DESIGN ASPECTS OF SOLID STATE FERMENTATION

A thesis submitted to the

UNIVERSITY OF MANCHESTER

for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Engineering and Physical Sciences

by

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University of Manchester

2014

The Microbe

The microbe is so very small

You cannot make him out at all

But many sanguine people hope

To see him through a microscope

His jointed tongue that lies beneath

A hundred curious rows of teeth

His seven tufted tails with lots of lovely pink and purple spots

On each of which a pattern stands

Composed of forty separate bands

His eyebrows of a tender green

All these have never yet been seen

But Scientist who ought to know

Assure us that they must be so

Oh, let us never, never doubt

What nobody is sure about

Hilaire Belloc

1870 – 1953

DESIGN ASPECTS OF SOLID STATE FERMENTATION

ABSTRACT

Solid state fermentation (SSF) refers to the microbial fermentation, which takes place in the absence or near absence of free water, thus being close to the natural environment to which the selected microorganisms, especially fungi, are naturally adapted. The current status of SSF research globally was discussed in terms of articles publication. This was followed by discussion of the advantages of SSF and the reason for interest in SSF as a notable bioprocessing technology to be investigated and compared to submerged fermentation (SmF) for the production of various added-value products. SSF also proved to be a potential technology to treat solid waste produced from food and agricultural industry and to provide environmental benefits with solid waste treatment. A summary was made of the attempts at using modern SSF technology for future biorefineries for the production of chemicals. Many works were carried out in the Satake Centre for Grain Process Engineering (SCGPE), University of Manchester, to prove the strategy of using SSF for the production of hydrolysate rich in nutrients for sequel microbial fermentation with or without adding any commercial nutrients.

The research findings presented in this thesis are based on a series of SSF experiments carried out on systems varying in complexity from simple petri dishes to our own design of bioreactor systems. They were conducted to assess a solution for biomass estimation, enzymes production, and successful mass and heat transfer. A proper technique for inoculum transfer prior to the start of the fermentation process was developed. In SSF, estimation of biomass presents difficulties as generally the fungal mycelium penetrates deep and remains attached with the solid substrate particles. Although many promising methods are available, the evaluation of microbial growth in SSF may sometimes become laborious, impractical and inaccurate. Essentially, this remains another critical issue for monitoring growth. In these studies, measurement of colour changes during SSF are presented as one of the potential techniques that can be used to describe growth, complementary to monitoring metabolic activity measurement, such as CER, OUR and heat evolution, which is directly related to growth. For the growth of *Aspergillus awamori* and *Aspergillus oryzae* on wheat bran, soybean hulls and rapeseed meal, it was confirmed that colour production was directly proportional to fungal growth. This colourimetric technique was also proved to be a feasible approach for fungal biomass estimation in SmF. This new approach is an important complementation to the existing techniques especially for basic studies. The key finding is that the colourimetric technique demonstrated and provided information of higher quality than that obtained by visual observation or spores counting.

The effect of aeration arrangements on moisture content, oxygen (O_2), mass and heat transfer during SSF was investigated. *A. awamori* and *A. oryzae* were cultivated on wheat bran in newly designed four tray solid state bioreactor (SSB) systems. The new tray SSB systems were: (1) single circular tray SSB, (2) multi-stacked circular tray SSB, (3) Single rectangular tray SSB and (4) multi-square tray SSB. The purpose was to study the effect, on heat and water transfer, of operating variables, fermentation on the perforated base tray and internal moist air circulation under natural and forced aeration. Temperature, O_2 and carbon dioxide were measured continuously on-line. Enzyme activity, moisture content and biomass were also measured. The results suggest that the air arrangements examined have a remarkable effect on the quantity of biomass produced using measurement of spores and enzymes production. The strategy presented in these studies allowed quantitative evaluation of the effect of forced internal moist air circulation on the removal of metabolic heat. With the proposed strategy, it was possible to maintain the bed temperatures at the optimum level for growth. However, the effect on moisture content was very different for the two fungi. It was found that the ability of *A. oryzae* to retain moisture was much higher than that of *A. awamori*. This is possibly due to the higher levels of chitin in *A. oryzae*. Greater spores and enzymes (glucoamylase, xylanase and cellulase) production was observed for *A. awamori* in multi-stacked circular tray and multi-square tray SSB systems compared to the conventional petri dishes and the other two systems. *A. oryzae* was excellent in producing protease in the same bioreactors.

A direct technique of establishing a correlation between fungal growth and CER, OUR, heat evolved was proven successful in this work. The information obtained from CER and OUR led to the estimation of respiratory quotient (RQ). RQ describes the state of the fungal population in the tray SSB and gives an indication of fungal metabolic behaviour. RQ values < 1 were obtained from 38 experiments using four tray SSB systems for the two fungi. A kinetic model based on CO_2 evolution instead of biomass concentration was examined in order to simplify the required experiments for kinetic model development. A Gompertz model was used to fit the integrated CO_2 data and predict the quantity of CO_2 evolution in all experiments. A correlation was found between the heat evolution and CER. The performances of tray SSB systems can be improved by constructing them as multi-trays. The multi-tray systems improved the mass transfer considerably compared with single tray systems. In addition, the multi-tray systems allowed precise measurement of the gradients of CO_2, enzymes, spores and fungal biomass. In addition, the air arrangements using moistened air were successful in maintaining moisture content, adequate O_2 supply and control of temperature, and hence, increased the productivity of both fungi. Overall *A. awamori* and *A. oryzae* have their own ability and performance to degrade and utilise the complex compositions contained in the solid substrate and fermentation conditions may lead to possible comparisons. In addition, multi-stacked circular tray and multi-square tray SSB systems demonstrated an excellent system for further investigations of mass transfer and possibly for large scale operation, though considerable optimisation work remains to be done, for example the height/diameter ratio and total number of trays should be optimised.

MUSAALBAKRI ABDUL MANAN   **PhD THESIS**   **2014**

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at University of Manchester or other institutions of learning.

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ACKNOWLEDGMENTS

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ
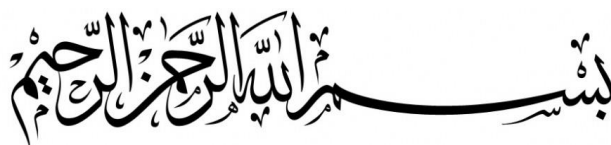
All praise to Allah S.W.T. who has showed me with kindness and affection during the course of my study that I cannot adequately thank for. His endless grace and love have provided me with the strengthen to finish the study.

Prof Colin Webb:

You probably do not know this, but even before I met you, I looked up to you. You are a smart, savvy, wise scientist who built a successful career based on your beliefs about science and desire to help others. What's not to envy, right? And then I met you, and my admiration only grew. You are sassy and funny (but sarcastic sometimes), and you always find time to answer my questions. You have helped me become a better researcher, and you have been a fantastic Guru to me as I have working on my project. I was delighted and deeply honoured that you agreed to accept me from beginning. THANK YOU VERY MUCH, Prof Webb. I know I will always continue to learn from you.

To the Malaysian Agricultural Research and Development Institute (MARDI) and Government of Malaysia for providing me an opportunity to further study and financial support during my study.

Finally, a special appreciation and deepest gratitude is extended to my wife, **Masita Mohd Ali**, for their love and support to encourage me to continue my endeavour, and to make this step in my life possible. Without love and support no work can be done.

CHAPTER 1

INTRODUCTION

"SSF is referring to the microbial fermentation, which takes place in the absence or near absence of free water, thus being close to the natural environment to which the selected microorganisms, especially fungi, are naturally adapted"
Colin Webb – Musaalbakri Abdul Manan
University of Manchester / Malaysian Agricultural Research and Development Institute
2014

1.1   BACKGROUND

There is a strong need to produce ingredients for new products (food and non-food, chemicals) from food and agro-industrial residues in a more sustainable way than is currently realized or possible. Sustainable production is defined here as a production that is efficient and effective in the use of raw materials and energy, that generates a minimum of waste or low-value streams, and that leads to new and existing products and/or materials with the required functionality, safety and integrity. Solid state fermentation (SSF) and related technologies offer alternative production routes for such biotechnology-based products.

SSF has been used in the world for a long time. This technology is commonly known in the East, for traditional manufacture of fermented foods, and in the west, for mould-ripened cheese. It can be defined as a system, in which the growth of selected microorganism(s) occurs on solid materials with low moisture contents and has been identified as a potentially important methodology and technique in biotechnology. Nowadays, SSF is an economically viable, practically acceptable technology for large-scale bioconversion and biodegradation processes. Development of sustainable SSF and bioprocess technology is an emerging, multidisciplinary field with possible application to the production of enzymes, chemicals, bioethanol and pharmaceuticals.

SSF offers many advantages over conventional submerged fermentation (SmF) such as, simple and inexpensive substrates, elimination of the need for solubilisation of nutrient from within solid substrates, elimination of the need for rigorous control of many parameters during fermentation, product yields are mostly higher, lower energy requirements, produce less waste water, no foam generation and relatively easy recovery of end products. SSF provides flexibility in terms of the raw materials to be used and their capability to produce various value-added products. Thus, SSF hold the highest potential for biorefinery.

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1.2   PROBLEM STATEMENT

Previous studies suggest that traditional methods for SSF are all very similar and often involve low technology. As a consequence, it is difficult to measure growth and difficult to control the environment in such systems. Therefore, it is necessary to significantly improve the ability to measure and control the physical environment in SSF systems in order to be able to develop new and effective processes. To confirm the particular end product, it is necessary to use analytical methods to evaluate the fermentation process. In contrast to conventional fermentation processes (e.g. SmF), SSF does not allow for an accurate biomass estimation. Attempts to estimate biomass have been made using many indirect techniques. For example, in the case of bacteria, ATP or DNA can be extracted and measured or, in the case of fungi, enzyme activity or glucosamine. However, none of these methods give satisfactory/conclusive results. The main disadvantage of most indirect techniques is that they are off-line, usually apply under a limited range of conditions and are labour intensive. By contrast, on-line techniques which measure oxygen consumption rate, carbon dioxide evolution rate and heat evolution rate are the most likely to meet with success.

One of the most important roles and functions of the microorganisms in SSF is the synthesis of enzymes, in particular extracellular enzymes. These enzymes generally hydrolyse complex compounds such as proteins, carbohydrates, polysaccharides, fats and so forth into smaller molecules, which in turn can be taken up by any cell. In the latter case, the fungus species play an additional role as they contribute towards texture, colour, aroma and taste and thus are involved in the final product formation. In this study, colour changes observed during the fermentation process are used as a new indirect method to estimate growth in SSF.

In SSF, the main factors affecting microbial activities are substrate heterogeneity, moisture gradients, temperature, and oxygen. In any aerobic bioprocess within any type of bioreactor, there are five basic factors that may limit the performance of the fermentation process: moisture content, oxygen transfer, carbon dioxide transfer, heat transfer and the bioreactor design itself. During the fermentation process, heat is generated which can consequently directly affect the metabolic activities of the microorganism causing, amongst others, moisture loss. Moreover, if temperature control and aeration fail, the selectivity of the biosynthesis process towards product formation could be lost, potentially even causing the process to fail completely. A good strategy is therefore needed to remove heat effectively from the system. Moisture content, heat transfer and carbon dioxide transfer requirements are strongly linked to oxygen transfer rates in aerobic processes. Direct aeration is the principal operation that results in oxygen transfer, heat removal and carbon dioxide

removal. A complication in SSF is that such effects are increased by high biomass concentrations in combination with the filamentous fungi.

Recently, SSF processes and the design of bioreactors along with biological aspects have received significant attention from researchers. However, results from previous and recent bioreactor designs are inconclusive regarding their effectiveness. This is surprising because SSF is one of the oldest biotechnological processes known. In order to gain a greater understanding, greater efforts are required not only in the taxonomic and engineering fields, but particularly in the microbial physiology and enzymology fields. The aim of this study was, therefore, to investigate the biological constraints of SSF on bioreactor design, which are, arguably, the major challenges within SSF processes. Many of these constraints are under investigation, as efforts to develop processes for renewable resource utilization consider the possible use of SSF, may not be possible to overcome. Even though the present study is on a small scale, it is hoped that the work with bioreactor systems will provide greater general understanding when looked at in conjunction with other studies. This could have several potential benefits including contributing to future designs and applications, and help to establish SSF as a viable alternative to SmF processes.

1.3   THESIS STRUCTURE

Chapter 2 reviews the history and development of SSF and general considerations about the technology. Due to the numerous benefits it offers, there has been a significant focus within current research and development on SSF. Modern SSF has shown much promise in the development of several bioprocesses and products. Published literature has provided significantly information on both fundamental and applied aspects of SSF. In addition, this section discusses several factors that influence SSF, as well as bioreactor design and mass transfer in SSF. At the end of the chapter, the benefits of new approaches to SSF and their applications are introduced. For example, by applying SSF in bioprocessing based biorefinery development, food and agro-industrial residues could be used to produce various valued added products. This could have positive benefits for producing lower levels of environmental waste.

Chapter 3 states the objectives of the research project, while Chapter 4 explains the outline of the experimental structure used to plan the research. Chapter 5 describes the materials and methods used throughout the project. Chapter 6 presents the results of studies on the physical characteristics of SSF. In Chapter 7 experiments on the different techniques for biomass estimation are described

and discussed. Several well-known indirect techniques to investigate biomass growth in simple petri dish and bioreactor cultures were studied. These were used to make biomass estimations, in order to carry out a critical informative comparison. Such techniques have the benefit of being practical, reliable and not too time-consuming in estimating fungal growth.

In Chapter 8, the hypothesis that biomass estimation can be based on observations of colour changes in SSF, is presented and tested. An investigation between colour developments within various parameters was carried out with both SSF and SmF. Besides using wheat bran as a subject material, detailed experiments with rapeseed meal and soy bean hull were also carried out. The two filamentous fungi *A. awamori* and *A. oryzae* were used in order to provide a comparative study.

Chapter 9 provides an analysis of the production of four enzymes, using simple single tray SSF systems, which consisted of either conventional petri dishes or trays with similar diameter but with an open perforated base. The enzymes produced were glucoamylase, protease, xylanase and cellulase. Chapter 10 describes and discusses four different tray-bioreactor designs. Experiments carried out in these tray bioreactors performed under sterile conditions, equipped with an on-line temperature recorder and gas analyser, for continuous oxygen and carbon dioxide measurement. An aeration strategy with varying flow-rates was applied to control the temperature and moisture content of the solid substrate. Wheat bran with *A. awamori* or *A. oryzae* were chosen as model systems for the study and the tray bioreactors are discussed as a suitable alternative for the effective production of the four enzymes refer to above.

The thesis concludes with Chapter 11, which includes suggestions and implications for future work and development. Finally, a reference list and appendices are provided at the end of the thesis.

The following presentations have been made as part of this work:

Conference proceedings:

1.   "Solid State Bioreactor Development – Integrated Enzyme Production and Fermentation"
       Post Graduate Student Conference 2011 School of Chemical Engineering and Analytical Science, University of Manchester – Poster Presentation

2.   "Colour Development in Solid State Fermentation"
       Post Graduate Student Conference 2012 School of Chemical Engineering and Analytical Science University of Manchester. 15 Jun 2012 – Oral Presentation

3.   "Colour Development during Growth in Solid State Fermentation as a Potential Method for use in Estimating, Quantitatively, Fungal Biomass Development"
European Federation on Biotechnology: Bioprocess Engineering Course 2012 Supetar Island Brac, Croatia. 2 – 8 September, 2012 – Oral and Poster Presentation

4.   "Colour Development as a Potential Method for Estimating Fungal Growth in Solid State Fermentation"
IChemE Biochemical Engineering Special Interest Group – Young Researchers Meeting, Manchester. 28 September 2012 – Oral and Poster Presentation

5.   "Evaluation of a strategy for temperature and moisture control in solid state tray bioreactors"
Chemical Engineering Day UK, Imperial College London. 25 – 26 March 2013 – Poster Presentation

CHAPTER 2

SOLID STATE FERMENTATION:
A REVIEW OF THE LITERATURE

"God did not create filamentous fungi to grow in a fermenter"
Prof A.P.J. Trinci
University of Manchester

2.1   INTRODUCTION

Solid state fermentation (SSF) has been practiced for centuries in the Orient and Asian region in both large and small scale applications in food processing and production of traditional fermented foods. Products such as tempe, soy sauce, tapai, koji, red fermented rice, annatto, miso, brewing of the Japanese rice wine (sake) among others are based on SSF. Some of these products have been in existence for over a thousand years, and their production was probably based on trial and error methods. Bread making is one of the oldest techniques known to man, and archaeological discoveries indicate that ancient Egyptians were making bread using a fermentation process even as early as 2600 B.C. (Krishna, 2005).

Fermented foodstuffs serve as an important component in the daily diet of a large majority of the families in that region as a source of protein and vitamins. However, the low level of traditional technology in the preparation of these products, the various sanitary requirements, the lack of built-in safeguards against undesirable microbial growth and toxins and other closely related constraints pose challenging problems to scientific and technological workers. There is therefore a perceived need for more basic understanding, in-depth studies and scientific as well as technological research on the various aspects of SSF.

The process of SSF has been practiced since ancient times and most of the processes are still practiced without any major modifications. The techniques applied are very simple, principally needing only raw materials, simple pre-treatment of the substrate to be fermented and microorganisms as an inoculum. The fermentation process proceeds at room temperature.

TABLE 2.1 shows the development of SSF from the past to the present and future. SSF technology has contributed many products for humans since the beginning of human civilisation (Chen, 2013).

TABLE 2.1: Development of SSF products. Adapted from Chen, 2013

Time	Products
2000 B.C.	Bread making by Egyptians
	Cheese making using *Penicilium roquefortii*
1000 B.C.	Sauce, koji
550 B.C.	Kojic acid
7th century	Kojic acid was introduced to Japan
Sixteenth century	Fermented tea
Eighteenth century	Vinegar from pomace, gallic acid in tanning, printing
1860 – 1900	Sewage treatment
1900 – 1920	Production of fungal enzymes, microbial enzymes, kojic acid
	Development of pneumatic drum-type fermenter
1920 – 1940	Production of gluconic acid, citric acid
	Development of drum-type fermenter
1940 – 1950	Tremendous development in fermentation industry
	Production of penicillin
1950 – 1960	Steroid transformation
1960 – 1980	Protein enriched food and production of mycotoxin
1990 – 2000	Development of fundamental aspects of SSF, bioprocess and products developments;
	Bioprocess:
	Bioremediation, biodegradation of hazardous materials, biological detoxification of agro-industrial wastes, biotransformation of crops and crop residues for nutritional enrichment, biopulping, etc.
	Products:
	Bioactive compounds: Aflatoxin, ochratoxin, bacterial endotoxins, gibberellic acid, zearalenone, ergot alkalois, penicillin, cephalosporin, cephamycin C, tetracycline, chlorotetracycline, oxytetracycline, iturin, actinorhodin, methylenomycinn, surfactin, monorden, cyclosporine A, ustiloxins, antifungal volatiles, destrucxins A & B, clavulonic acid, mycophenolic acid
	Enzymes:
	Various enzymes (cellulase, β-glucosidase, CMCase, laccase, xylanase, polygalacturonase, ligninase, β-xylosidase, a-arabinofuronosidase, Li-peroxidase, Mn-peroxidase, aryl-alcoholoxidase, catalase, phenol oxidase, proteases (acidic, neutral and alkaline), lipases, α-galactosidase, β-galactosidase, α-amylase, β-amylase, glucoamylase, glutaminase, inulinase, phytase, tannase, feruloyl para-coumaroyl esterase, etc.)
	Organic acids: Citric acid, fumaric acid, lactic acid, oxalic acid, gallic acid
	Other products:
	L-glutamic acid, pigments, carotenoid, xanthan gum, succinoglycan, ethanol, aroma compounds, vitamins B-12 and B-6, riboflavin, thiamine, nicotinic acid, nicotinamide, gamma-linolenic acid, biosurfactants, biopesticides/bioherbicides
2000 - present	SSF as a novel process to generate feedstock for biorefinery processes
Future	Move toward biorefineries for the production of industrial biochemicals

In recent years, the application and development of SSF technology has greatly expanded especially in Western countries because of its perceived advantages in the production of various secondary metabolites and novel foods (Oostra *et al.*, 2000). Detailed studies carried out in laboratories have helped to understand and to confirm the chemical and microbiological changes during fermentation. Some of the originally small scale, traditional SSF processes have become

large-scale industries; it should therefore be worthwhile to study those factors that are conducive for large scale production.

In the future, SSF may become more important as a technology to process alternative, bio-based feedstocks that will replace the declining petroleum resources. There will be efforts and ever-increasing pressure to move towards biorefineries for the production of industrial biochemicals (Mitchell *et al.*, 2011). The large scale cultivation of microorganisms will be an integral part of such biorefineries. This can be achieved since SSF technology offers the advantage and potential to minimise the addition of water and thus optimise process economics in biorefineries. On top of that, in order to meet the requirements of SSF as a potential technology, it is necessary to produce effective large-scale SSF bioreactors with optimised performance.

2.2   DEFINITION OF SSF

Solid state fermentation (SSF) has been defined in many ways. Many researchers in the field have introduced their own ways to define SSF. For example, Pandey *et al.* (2000) defined SSF as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can also be used as carbon and energy source. Mitchell *et al.* (2000) described SSF as any process in which substrates in a solid particulate state are utilised, while Viniegra-Gonzalez (1997) defined SSF as a microbial process occurring mostly on the surface of solid materials that have the property to absorb or contain water, with or without soluble nutrients. In 2006, Rahardjo *et al.*, in their review article, came out with a definition that SSF is the growth of microorganisms on moistened solid substrate, in which enough moisture is present to maintain microbial growth and metabolism, but where there is no free-moving water and air is the continuous phase. Rosales *et al.* (2007) gave a simple definition of SSF where the growth of microorganisms is on solid or semisolid substrates or support.

In the latest definition, Mitchell *et al.* (2011) defined SSF as a process that involves the growth of microorganisms on moist particles of solid materials in beds in which the spaces between the particles are filled with a continuous gas phase. Whatever the definition, we can understand that SSF is referring to the microbial fermentation, which takes place in the absence or near absence of free water, thus being close to the natural environment to which the selected microorganisms, especially fungi, are naturally adapted.

2.3   SSF – CURRENT STATE AND PERSPECTIVES

Over the last four decades, various terms have been used as synonyms of SSF. The most popular term is "solid state fermentation" itself, but terms such as "solid substrate fermentation", "solid state bioprocessing", "solid substrate cultivation", "solid substrate process", "solid state digestion", "solid state cultivation", "solid state culture", "surface cultivation" and "surface culture" have also been used to describe the same process. The extent to which each of these various terms has been used in publications since 1971 is shown in FIGURE 2.1.
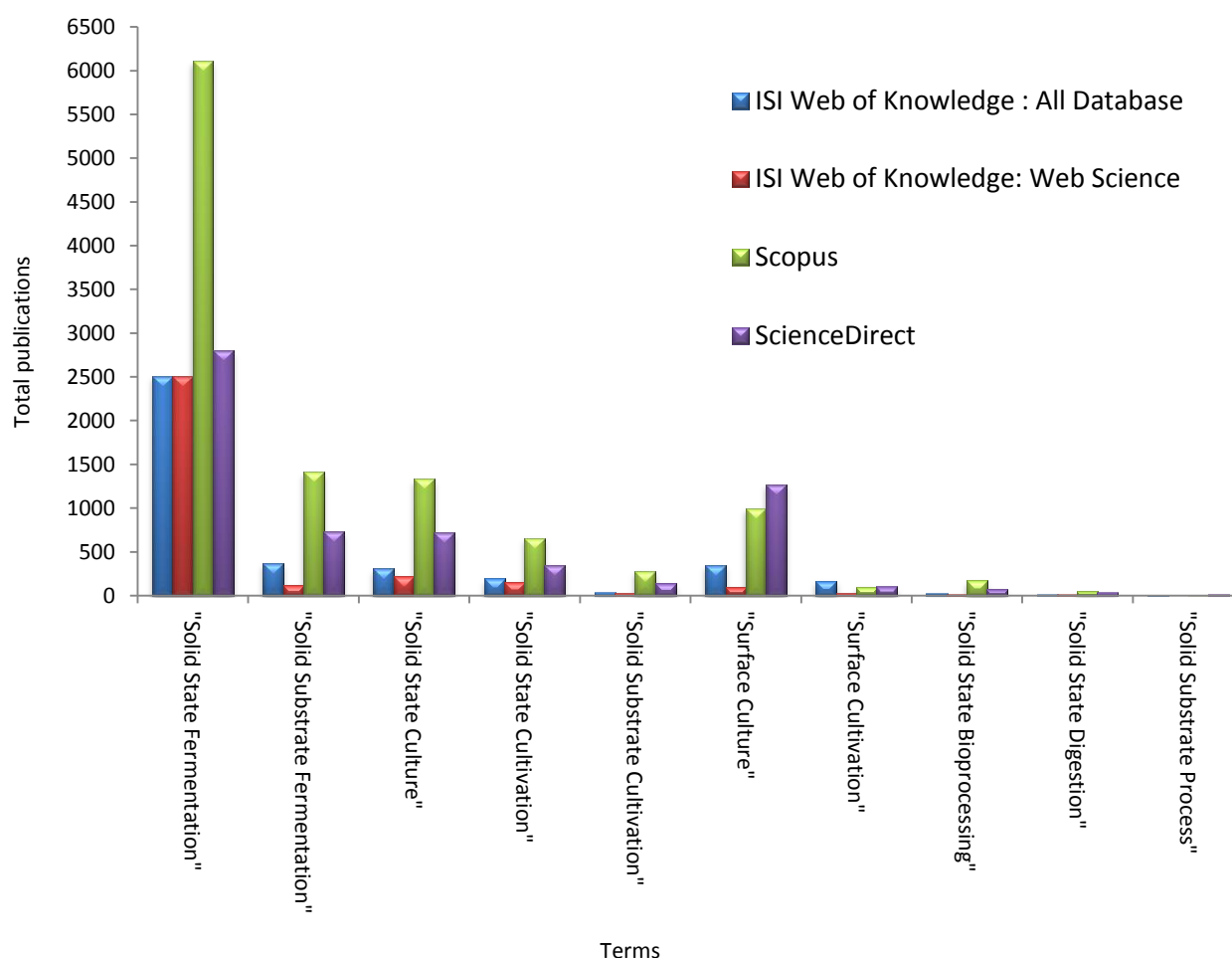
FIGURE 2.1:   **Number of publications using various terms equivalent to SSF during the period 1971-2012. (Data were obtained using the online databases mentioned in the legend)**

FIGURE 2.1 clearly shows that the term "solid state fermentation" is the most commonly used term followed by "solid substrate fermentation". During recent years, SSF has received a fresh attention from researchers and industries all over the world. This is due to several major advantages that it

offers over SmF, particularly in the area of solid waste treatment. Apart from the production of food and feed, SSF shows a tremendous potential in applications to produce high value-low volume products such as enzymes, biologically active secondary metabolites and chemicals. For the past five years, a new term has been developed by researchers to explain the process: "particulate bioprocessing". As an example from Botella *et al.* (2009), a novel process strategy has been developed for biorefineries (particulate bioprocessing) based on SSF. In this case, they described particulate bioprocessing as confined to those systems that involve the growth of microorganisms on moist solid materials in a particulate state.

For the last two decades, the interest in the topic of "solid state fermentation" has increased significantly. FIGURE 2.2 shows the publications by decade since the 1970s indicating a huge growth in SSF research all over the world. Tracing the history since 1971 reveals significant developments in SSF. These trend data were obtained using the term 'solid state fermentation' in four databases, namely ISI Web of Knowledge (publication in all languages), ISI Web of Science, Scopus and ScienceDirect. During the period 1981 – 2012, Scopus database itself, recorded more than 5,000 publications appearing in various journals, proceedings, books and patents.
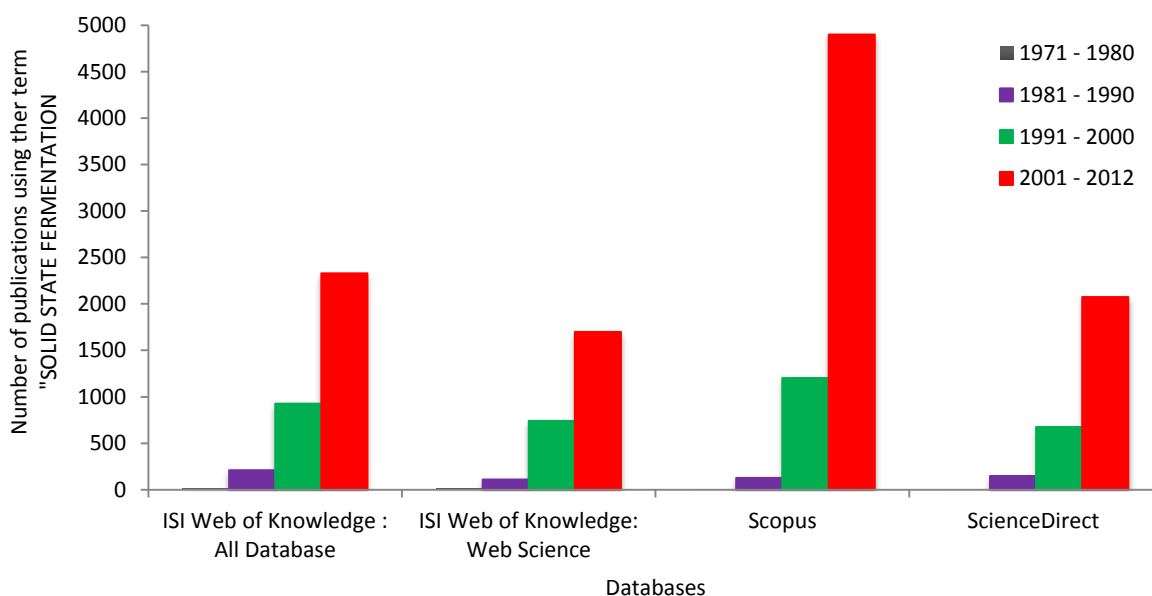
FIGURE 2.2: **The number of publications containing the term "Solid State Fermentation" by decade. (Data were obtained using an online search of the different databases mentioned above)**

Examining publications during the past decades reveals the principal authors in the field of SSF. Names such as Pandey, A., Soccol, C.R., Szakacs, G., Freire, D.M.G., Gomes, E., Mitchell, D.A. and

Rinzema, A. appear to be the most active scientists involved in SSF research. India proved to be the most active country based on the number of publications followed by Brazil and China. This is likely to be because both food and agro-industry wastes are produced in huge amounts in these countries. Considerable research activity is also evident in the USA, Mexico, Spain, France, Japan, the United Kingdom and the Netherlands, as shown in FIGURE 2.3.
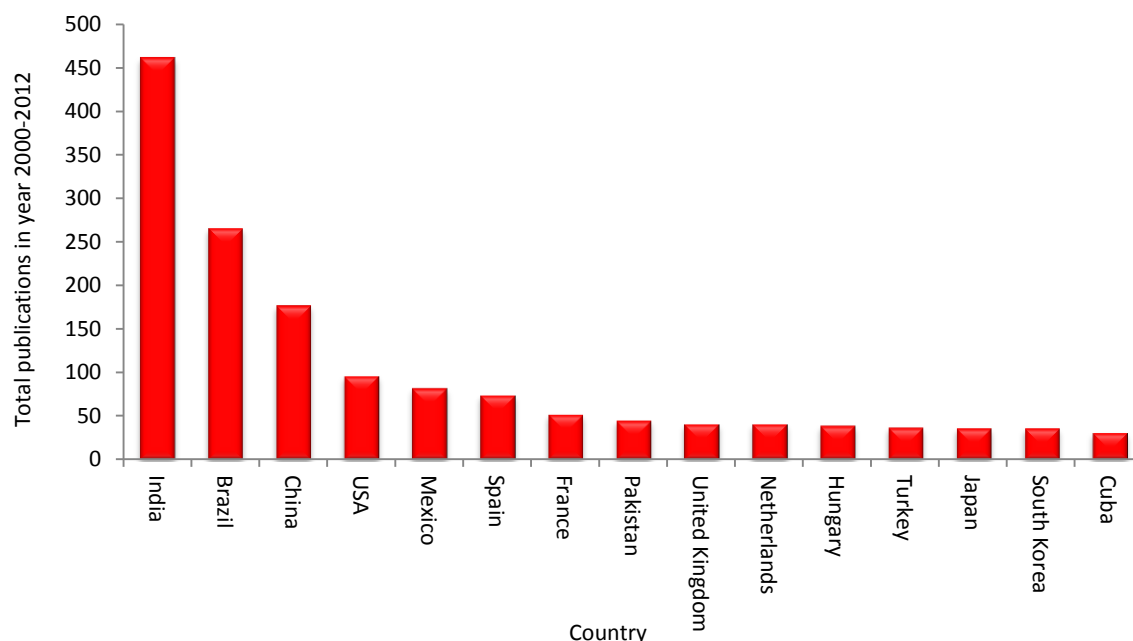
FIGURE 2.3: **Number of publications with term "Solid State Fermentation" by country in the period 2000 – 2012 (Data were obtained using the online database *ISI Web of Knowledge: All Database*)**

Industrial practice in SSF for secondary metabolites production has been led by companies such as Biocon Ltd. Biocon India developed technology based on SSF as a low cost, low energy option for the production of specialty enzymes. The company started as early as 1990 on an 8 year research and development programme to create a novel bioreactor capable of conducting SSF with comparable levels of automation and specialisation as those associated with SmF (Suryanarayan, 2003). The founder, Kiran Mazumdar Shaw started the biotechnology company at the age of 25 in Bangalore, India. In 2009, Biocon Ltd. was named by Forbes in the "Best under a billion" company list and Shaw herself as worth about £560 million (Stylist, 2011).

Cristobal *et al*. (2008) mentioned that the great success of SSF is not only related to the notable increase in research in this area, but also to significant industrial developments especially in enzyme production. The last two decades witnessed an unprecedented increase in interest in SSF. There has been a concerted effort to understand the SSF issues involved and to apply them to a wide range of

new products. The majority of publications are about the use of microbial fermentation and the possibility of utilisation using different solid wastes as raw material. Through SSF, solid wastes either from food or agro-industry can be used as commercially desirable substrates (Barrios-Gonzalez *et al.*, 1993).

Many research works have so far focused on the general applicability of SSF for the production of enzymes, metabolites and spores. Food and agro-industry provide much different solid waste as valuable solid substrates, which have been combined with many different microorganisms and resulted in a wide range of fermentation processes. For example, enzymes production by SSF is a growing field due to the simplicity of the processes, high productivity, and generation of concentrated products (Castilho *et al.*, 2000). Another important factor that influences the development of SSF is that both food and agro-industrial waste are rich in carbohydrates and other nutrients so that they can serve as a substrate for the production of bulk chemicals and enzymes (Cauto and Sanroman, 2006). In addition, SSF is a good alternative to help in solving pollution problems, rather than waste disposal into the land and causing environmental harm. Research studies on microbial growth in SSF are limited compared to those in SmF. The majority of published research focusses on the optimisation of environmental conditions to achieve maximum production and substrate utilisation rates (Papagianni *et al.*, 1999). With the advances of biotechnology and bioprocess nowadays, for example in the area of enzyme and fermentation technology, many new avenues have opened for their utilisation in SSF.

Growth and product formation kinetics, bioreactor design and process control in SSF are becoming popular subjects in research. With the increased interest in SSF, progress is being made nowadays with the goal of developing industrially applicable SSF systems. Research on kinetic studies has become one of the most popular and critical subjects to be explored, and with developments in computer and software technology, such studies have become easier. The prediction of microbial growth and product formation could be achieved with simulations and parameter estimation if we had a better understanding of how microorganisms grow and produce the desired products in SSF. Accurate modelling and determination of process variables in SSF such as moisture, temperature (Fanaeia and Vaziri, 2009; Dalsenter *et al.*, 2005; Hamidi-Esfahani *et al.*, 2004), pH (Nagel *et al.*, 1999), biomass determination by oxygen consumption rate, carbon dioxide production rate, ATP and glucosamine (Smits *et al.*, 1996), fungal growth and enzyme production (Smits *et al.*, 1999; Hamidi-Esfahani *et al.*, 2007) related to kinetic studies and modelling for optimisation of SSF have been investigated extensively but understanding still lags behind that for SmF.

Even though many articles have been published reporting on the kinetic modelling of SSF, there is not yet a comprehensive set of models and work is yet to be carried out in several areas related to SSF. Extensive studies are needed for the optimisation of kinetics and modelling in SSF to verify that this type of process can provide higher productivity. On the other hand, it is also important to carry out studies related to degradation or modification of substrates and end products.

Bioreactor design for SSF has been studied extensively (Durand, 2003) and is another important area for development. Developments during the past 15 years suggest that there is now considerable interest in studying various aspects of SSF bioreactor design. Bioreactors such as packed-beds (Fanaeia and Vaziri, 2009; Khanahmadi *et al.*, 2006; Cauto and Sanroman, 2005; Robinson and Nigam, 2003; Lu *et al.*, 1997; Pandey and Radhakrishnan, 1994), multi-layer packed-beds (Shojaosadati and Babaeipour, 2002; Lu *et al.*, 1998), rotating drums (Cauto and Sanroman, 2005; Robinson and Nigam, 2003) column bioreactor (Pandey *et al.*, 1996), magnetic drum contactors (Saha *et al.*, 1999), magnetic rotating biological contactors (Saha and Takahashi, 1997), fixed-beds (Cavalcanti *et al.*, 2005), immersion bioreactor (Cauto and Sanroman, 2005), tray systems (Bhanja *et al.*, 2007; Cauto and Sanroman, 2005; Robinson and Nigam, 2003), horizontal stirred tanks (Berovic and Ostroversnik, 1997) and other bioreactors (Takashi *et al.*, 2009) have been reported.

Many articles on growth and product formation kinetics, bioreactor design, and process control in SSF have been published in recent years. However, the information is very varied and it is difficult to establish a firm understanding of underlying principles. At laboratory scale, SSF appears to be superior to SmF but when up-scaled to industrial scale it appears to face a few problems including engineering problems such as build-up of temperature, difficulty of pH control, limited oxygen transfer, slow heat removal, heterogeneous substrates and moisture gradients (Holker *et al.*, 2004). It seems the advantages of SSF can be realised only at a laboratory scale. Nevertheless, better understanding of biochemical engineering aspects, particularly on bioprocess engineering and design of bioreactors, has made it possible to scale-up some SSF processes and some designs have been developed for commercialisation (Ramana Murthy *et al.*, 1993).

During the period 1980 – 2012, more than 6,600 publications have appeared in journals, conference proceedings, books and patents. Many review articles have also been presented discussing particular features of SSF. About 24 books have been published during the period 1981 – 2013, which are dedicated specifically to SSF. These are listed in TABLE 2.2.

TABLE 2.2: Books on SSF published during the period 1981 – 2013

No	Title	Author	Year publication
1	Solid state fermentation for foods and beverages (Fermented foods and beverages series)	Chen, J. and Zhu, Y.	2013
2	Effect of carbohydrates and amino acids on fermentative production of alpha amylase: Solid state fermentation utilizing agricultural wastes	Bose D. and Gangopadhyay, H.	2013
3	Lipase production by *Aspergillus niger* under solid state fermentation	Fahim, H.	2013
4	Solid-sate fermentation of agro by-products and production of lipase	Tripathi, S.S.	2013
5	Modern solid state fermentation: Theory and practice	Chen, H.-Z	2013
6	Chitinase production from *Actinomycetes* by solid state fermentation: Chitinase production by *Actinomycetes*	Choomponla, K.and Upadhyay, R.S.	2012
7	Bacterial cellulase production under solid State fermentation: *Eichhornia crassipes* (Water Hyacinth) as substrate	Prabhu, G.N. and Kurup, R.S.C.	2012
8	Alkaline protease production under solid state fermentation: *Bacillus* sp. C45 and properties of its enzyme	Gidamo, G. H.	2012
9	Solid-state fermentation of palm kernel cake: Locally-isolated strains	Farida Asras, M. F., Mohamad, R. and Ariff, A.	2012
10	Cellulases by *Aspergillus niger* in solid state fermentation: An experimental approach for biotechnological production of cellulases by *Aspergillus niger* in solid state fermentation	Chandra, M.S., Viswanath, B. and Reddy, B.R.	2012
11	Utilization of agro-wastes in fermentative production of alpha amylase: Production of fungal alpha amylase	Bose, D.	2012
12	Fermentative production of fungal alpha amylase and amyloglucosidase: Solid state fermentation for production of alpha amylase and amyloglucosidase enzymes utilizing agricultural wastes	Bose, D.	2012
13	Xylanase production using solid state fermentation	Mahatman, K.K., Garg, N. and Kumar, A.	2011
14	*Trichoderma* sp. a potent producer of xylanase enzyme: Xylanase production in solid state fermentation	Irfan, M.	2011
15	Current developments in solid-state fermentation	Pandey, A., Soccol, C.R. and Larroche, C.	2010
16	Solid-state fermentation bioreactors: Fundamentals of design and operation	Mitchell, D.A., Krieger, N. and Berovic, M.	2010
17	Advances in solid state fermentation	Roussos, S., Lonsane, B.K., Raimbault, M.and Viniegra-Gonzalez, G.	2010
18	Solid state fermentation	Pandey, A.	2009
19	Microbial biotechnology exploiting solid state fermentation	Nigam, P.S.	2003
20	Solid state fermentation of dye-adsorbed agricultural residues	Robinson, T.	2002
21	Solid-state fermentation in biotechnology: Fundamentals and applications	Pandey, A., Soccol, C.R., Rodriguex-Leon, J.A. and Nigam, P.S	2001
22	A novel spouted bed bioreactor for solid state fermentation	Silva, E.M.	1997
23	Solid state fermentation	Pandey, A.	1994
24	Solid state fermentation of ligno-cellulosic	Matteau, P.P.	1981

The biotransformation and biological upgrading of food and agro-industry waste for improved nutritional qualities can be achieved through SSF technology. This has been the most important area where the potential of SSF has been recognised offering economically feasible technology. Various high value biotechnological products such as enzymes, primary and secondary metabolites, antibiotics and chemicals could be produced through SSF because it uses cheap solid substrates, such as those mentioned above, which are available locally and are rich in carbohydrates and other

nutrients. In the case of wheat bran, research has been developed for its utilisation as SSF substrate for added value products. As examples, the production of the antifungal antibiotic iturin (Takashi *et al.*, 2009; Ohno *et al.*, 1992), nigerloxin (Chakradhar *et al.*, 2009), meroparamycin (El-Nagar *et al.*, 2009), GABA and phytic acid (Nagaoka, 2005), lovastatin (Valera *et al.*, 2005), gallic acid (Seth and Chand, 2000), L (+) lactic acid (Naveena *et al.*, 2005) and enzymes have been reported.

Many published articles refer to the advantages of SSF in enzyme production. These advantages include higher enzyme titers (de Barros Soares *et al.*, 2003; Tellez-Jurado *et al.*, 2006 ; Patil and Dayanand, 2006; Sandhya *et al.*, 2005; Fenice *et al.*, 2003; Romero-Gomez *et al.*, 2000), higher productivity levels (Nagel *et al.*, 2001; Aquilar *et al.*, 2001b; Balasubramaniem *et al.*, 2001; Solis-Pereira *et al.*, 1993), stability of excreted enzymes (Wolski *et al.*, 2009; Mateos Diaz *et al.*, 2006; Diaz-Godinez *et al.*, 2001), a low level of catabolic repression (Azeredo *et al.*, 2007; Diaz-Godinez *et al.*, 2001; Maldonado and Strasser de Saad, 1998; Minjares-Carranco *et al.*, 1997; Solis-Pereira *et al.*, 1993) and short fermentation time (Elinbaum *et al.*, 2002; Taragano and Pilosof, 1999; Maldonado and Strasser de Saad, 1998; Solis-Pereira *et al.*, 1993).

Production of enzymes can be stimulated by high sugar concentration in SSF but in SmF such high concentrations have an inhibitory effect because of catabolic repression (Diaz-Godinez *et al.*, 2001; Aquilar *et al.*, 2001a; Aquilar *et al.*, 2001b; Maldonado and Strasser de Saad, 1998, Ramesh and Lonsane, 1991). SSF appears to be more robust than SmF with regard to catabolic repression and can therefore be more productive with a wider variety of substrate mixtures (Aquilar *et al.*, 2001a; Aquilar *et al.*, 2001b). According to Castilho *et al.* (2000), SSF has many advantages over SmF for lipase production by *P. candidium* when it is grown on wheat bran substrate. The SSF process is very attractive from an economic point of view. Studies on economic analysis for lipase production showed that total capital investment needed for SmF was 78% higher than that needed for the SSF process (Castilho *et al.*, 2000).

Thus, there has been much development of SSF in various biotechnology applications and in product development. The last two decades have changed the perception of SSF as "low-technology" and it is becoming a promising technology for the production of added value, "low-volume and high-cost", products. The majority of publications indicate that filamentous fungi are the most suitable organisms for growing under SSF conditions.

2.4   PERCEIVED ADVANTAGES OF SSF

Claims of many advantages of SSF have been made compared to SmF in the literature. The question is; are these advantages real? Is it true that SSF is so much better than SmF? Maybe some claims are true, but many are still doubtful and need further proof. Indeed, SSF has centuries of history, but it is only in the last two decades that there has been a concerted effort to understand bioprocessing aspects involved in SSF and to apply them to a wide range of new products (Pandey *et al.*, 2008). Although significant advances have been achieved in understanding the controls of process performance, much research is still required.

Studies on SSF and some of their results have provided a substantial contribution to the improvement of the existing and widely used technology. To a certain extent, some of the research findings have widened the scope of research activities towards a better understanding of existing SSF systems. The various advantages that have been identified through the literature can be described based on different criteria, namely (1) biological advantages; (2) processing advantages; (3) environmental advantages and (4) economic advantage. TABLE 2.3 shows the main advantages of SSF listed and summarised within these four categories.

SSF has emerged as a viable technology for the bioremediation and biodegradation of hazardous compounds as well as the recycling of food and agro-industry wastes and biomass conservation. Moreover, SSF is a promising technology and is reliable for the development of various biotechnological products. Arguably, these advantages may in some circumstances outweigh the disadvantages of SSF that are discussed in the next section.

2.5   DISADVANTAGES OF SSF

Even though SSF offers certain unique advantages, it also suffers from some problematic disadvantages. Scientific studies that have been carried out in laboratories have helped to understand the chemical and microbiological changes that occur during the fermentation process. As we know from traditional technology for processing fermented food to produce valuable biotechnology products, SSF has a tremendous potential for industrial exploitation. In some cases, it is true that SSF has problems with scaling up but the process has been shown advantageous in competition with SmF.

TABLE 2.3: The perceived advantages of SSF

Type of advantage	Remarks
Biological	Biological advantages that have been demonstrated in enzymes production: ▪ Products produced in high volume ▪ Higher productivity level of the products ▪ Higher stability of products ▪ Absence of catabolic repression ▪ Tolerance to high substrate concentration Other biological advantages: ▪ Natural, complex raw materials often provide a complete medium ▪ Absence of rigorous control of fermentation process ▪ Easier aeration ▪ Low water demand helps to minimise contamination ▪ Practically involves fungi-producing spores. Spores can be used as inoculum, can be preserved for a long time and can be used repeatedly ▪ Absence of production of foam
Processing	Processing advantage that have been demonstrated in enzymes production: ▪ Bioreactors are usually in small volume and are compact ▪ The volume of the substrate loading is much higher Other processing advantages: ▪ The growth substrates are from natural resources proportionally simple and unrefined ▪ Pre-treatment and treatment of the natural resources can be very simple ▪ Downstream processing might be simple since products are concentrated ▪ Extraction of the products requires much less solvent (if necessary) ▪ The process does not involve anti-foam chemicals
Environmental	▪ Minimise the generation of pollutants or harmful waste during products manufacture ▪ Produce less liquid waste ▪ Solving waste problem as biological detoxification
Economic	▪ Substrates usually natural materials - Natural unusable carbon source which are extremely cheap, variable and abundant agro-industry and food waste ▪ Modified bioreactors are simple, cheap and user-friendly ▪ Low recovery cost in downstream processing ▪ SSF process is very attractive from an economic point of view and proved to be economically feasible

Research is needed to increase the capability of SSF for the development of biotechnological products. Being the aim of this study, one of the targets of this research activity is to explore any factors contributing to the disadvantages of SSF. Since such factors are not fully understood and not well reported, the fermentation strategy is based more or less on biomass and enzymes production combined with empirical studies. Some of the biological disadvantages in SSF have been defined and are summarised in TABLE 2.4. In this regard, having an understanding of growth characteristics is necessary. Moreover, such an understanding is important for rational design and process control in

SSF. It should be worthwhile to study those factors conducive for understanding and improvement of the SSF process.

TABLE 2.4: The disadvantages of SSF

No	Solid state fermentation disadvantage	Idea to overcome
1	Engineering problems due to the build-up of temperature, pH control, oxygen transfer, mass and heat transfer, substrate and moisture gradients	Measure build-up of temperature, oxygen and carbon dioxide gradients on-line and use these to control the system
2	Uneven distribution of the cell mass, nutrients, temperature, pH, moisture content	
3	Steady aeration throughout the substrate is difficult	Controlled by forced aeration. At the same time, this can control the temperature
4	Heat derived from metabolism and growth of the microorganism raises the temperature of the solid substrate and causes either loss of moisture or generation of watery substrate	Building temperature gradients capable of removing metabolic heat
5	Biomass measurement for microbial growth	Development of on-line measurements and use these to control the system
6	Growth and kinetics studies still difficult; even though information is variable, it is still relatively limited and scattered	Mathematical models for effective prediction and optimisation of data

2.6   THE DIFFERENCE BETWEEN SSF AND SmF

In contrast to SSF, the typical SmF is 100% liquid with, possibly, some suspended solids. The moisture content of SSF, on the other hand, is usually maintained in the range of 12 - 70%, and typically around 60% (Chen, 2013). SmF is by far the most common operation employed in the fermentation industry (Hata *et al.*, 1997). Most research in SmF is aimed at determining the production economics of the process including productivity and product yields (Castilho *et al.*, 2000) and maximising these parameters.

The use of filamentous fungi for the production of commercially important products has increased rapidly over the past half-century and the production of enzymes in SmF has long been established (Papagianni *et al.*, 1999). SmF currently produces commercial enzymes and several of the potential applications have been commercially exploited, primarily due to shortage and high cost of enzymes (Viniegra-Gonzalez *et al.*, 2003). Advantages of SmF include good control of environmental

parameters, relatively low labour costs, reduced space requirements and low scale-up requirements when compared with SSF (Singhania *et al.*, 2010).

Even though modern SmF offers many advantages, it suffers from some major disadvantages. de Barros Soares *et al.* (2003) reported that SmF for transglutaminase production showed some constraints such as a long fermentation process, excessive foam production that prevented optimum oxygen mass transfer and use of expensive culture media. Some of these difficulties arise when fermentation involves the use of filamentous fungi.

Professor A.P.J. Trinci (Pandey *et al.*, 2008) used the now frequently quoted phrase "God did not create filamentous fungi to grow in a fermenter". He claimed that SmF is an artificial condition for filamentous fungi because they live in nature in a solid state (Pandey *et al.*, 2008). Most microorganisms, especially filamentous fungi and many actinomycetes, primarily live and grow in nature in SSF conditions (Carlile and Watkinson, 1994). More than 98% of isolates from marine environment have been obtained from the underwater surfaces of solid substrates and less than 1% of all known fungi have been found in marine habitats (Kelecom, 2002). The morphology factor of the microorganisms plays an important role since it can affect many aspects of the process. TABLE 2.5 emphasises the major differences in characteristics between SSF and SmF (Chen, 2013).

2.7   FACTORS THAT INFLUENCE SSF

SSF processes are clearly different from SmF. In most cases, it is soluble substrate supported on a solid insoluble matrix in an environment of low moisture content. The advantage of SSF comes from its simplicity and its closeness to the natural habitat of many microorganisms. Through modern biotechnology, there are new initiatives to improve and enhance the productivity of SSF. Each microorganisms, solid substrate, and bioreactor system plays a major role in the success of SSF. The performance of the SSF process can be influenced by various factors. Broadly, the factors that influence the performance of SSF can be divided into three major categories, namely:

     i.   Biological factors

    ii.   Physico-chemical factors

   iii.   Mechanical factors

TABLE 2.5: Detailed comparison of SSF and SmF. Adapted from Chen (2013); Nigam and Pandey (2009); Wang, L. and Yang, S.-T. (2007)

SSF	SmF
There is no free water, and the water content of substrate is in the range 12 - 70%	Water is the main component of the culture
Microorganisms absorb nutrients from the wet solid substrates; a nutrient concentration gradient exists	Microorganisms absorb nutrients from the liquid culture; there is no nutrient concentration gradient
The culture system consists of three phases (gas, liquid and solid) and gas is the continuous phase	The culture system mainly consists of liquid; the liquid is the continuous phase
Inoculation size is large, more than 10%	Inoculation size is small, less than 10%
The required oxygen is from the gas phase; the process needs low energy consumption	The required oxygen is from dissolved oxygen; there is a larger amount of dissolved oxygen
Microorganisms attach and penetrate into the solid substrate	Microorganisms uniformly distribute in the culture system
At the end of fermentation, the medium is a wet state substrate, and the concentrations of products are high	At the end of the fermentation, the medium is liquid and the concentrations of products are low
High production rate and high product yield	Low production rate and low product yield
Mixing is difficult or impossible, some microorganisms are sensitive to mixing or agitation and the growth of microorganisms is restricted by nutrient diffusion	Mixing is easy, and the growth of microorganisms is not restricted by nutrient diffusion
Removal of metabolic heat is difficult	Temperature control is easy
Heterogeneity	Homogeneity
The fermentation parameters are hard to detect and control on-line	The fermentation parameters can be detected and controlled on-line
Extraction process is simple and controllable; little waste water	Extraction process is usually complex; there is a large amount of waste water
Low water activity	High water activity
Simple fermentation bioreactor	High-tech design fermentation bioreactor
Natural enrichment or artificial breeding systems	Pure strains
Energy consumption and equipment investment are high	Energy consumption and equipment investment are low
Low raw material cost	High raw material cost

2.7.1   Biological Factors

2.7.1.1   The type of microorganism

The most important criterion in SSF is the selection of a suitable microorganism, which has the ability to degrade the solid substrate. SSF processes are due mainly to the fermentation activity of fungi alone, bacteria alone, a mixture of fungi and yeasts or fungi followed by a mixture of bacteria and yeast. The selection of microorganism is usually dependent on the type of solid substrate, growth requirements and targeted final product (Krishna, 2005). These general criteria will affect the fermentation design and downstream processing. Filamentous fungi continue to dominate as an important microorganism in SSF due to their mycelia mode of growth as well as their neutral physiological capabilities (Mitchell *et al.*, 2011). The use of a single microorganism, especially in industrial SSF processes, has the advantage of improved rate of substrate utilisation and controlled

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product formation (Nigam and Pandey, 2009). Ensiling and composting are among the processes involving several microorganisms that exhibit symbiotic behaviour; in other words, mutual growth of microbial communities and thus mixed culture processes, as these exist in most natural habitats (Nigam and Pandey, 2009).

2.7.1.2   Inoculum

Inoculum can be described as a preparation containing high numbers of viable microorganisms, which may be added to bring about desirable changes in the solid substrate (Wolzapfel, 1997). The age of the inoculum, the medium used for its cultivation, and therefore its physiological state are of the utmost importance in many fermentation processes. According to Sheperd and Carels (1983), if the inoculum used for the production of secondary metabolites is not in the correct physiological state, a considerable decrease in production will occur. This is because the early hours of fermentation determine the future direction of the culture. Sekiguchi and Gaucher (1977) observed that with *Penicillium urticae* the type of inoculum used greatly influences the level of secondary metabolites produced. Smith and Calam (1980) reported that different yields were obtained in penicillin and griseofulvin fermentation using different types of inoculum. From their study, it was shown that biochemical factors, such as the level of enzyme activity and efficiency, were at least as important as morphology in determining yield, being carried forward from the inoculum to the production stage. For example, most fungi produce spores. Spores inocula are easy to prepare and can be stored for longer periods than vegetative cells. The chances for contamination are higher if low levels of inoculum density were used. According to Nigam and Singh (1994), by increasing the inoculum quantity, the time required for substrate utilisation can be shortened and this can also aid the inoculated fungus to displace any other microbes that may be present. This makes processes involving fungi more flexible since the synchronisation of inoculum production with the rest of the process is not that crucial. Sporulation is generally not desirable during the fermentation itself (Mitchell *et al*., 2002).

2.7.1.3   Substrates

Carbon sources supplied in the medium are of great importance to fungi since they provide the carbon source needed for the biosynthesis of cellular constituents. This includes carbohydrates, proteins, lipids, nucleic acids, and their oxidation provides energy for the cell (Gadd, 1988). The solid substrate is a major element in SSF. In addition to providing nutrients such as carbon and nitrogen, the solid substrate also performs the role of the physical structure that supports the growth of

microorganisms (Cauto, 2008). Another important factor in the selection of substrate is the water holding capacity that maintains moisture content of the fermented substrate (Nigam and Pandey, 2009). Generally, most of the solid substrates used in SSF are based on food and agro-industry crops and residues (Cauto and Sanroman, 2006). They are usually unprocessed and come with different particle sizes (Krishna, 2005). The quality and nutrients composition of the solid substrate might be different from one batch to another. This can lead to problems with heterogeneity and overall productivity of the fermentation process. Generally, solid substrates used in SSF can be classified into five main groups, namely:

(a)   Starchy substrates

Starchy substrates that have been used in SSF include rice, barley, oats, cassava, wheat bran, cassava meal, corn meal, okara, sweet potato residues, and banana peel. Starchy substrates, being rich in carbohydrates (important carbon source in many microbial fermentation processes), are hydrolysed to produce simple sugars that can be consumed by microorganisms.

(b)   Substrates with protein

Food and agro-industry by-products such as oil cakes are an ideal source of proteinaceous nutrients. Their use as a solid substrate is highly favoured in SSF. Pumpkin oil cake (63.52%) (Pericin *et al.*, 2008), soybean oil cake (51.8%) (Borucki Castro *et al.*, 2007), sesame oil cake (48.2%) (Yamauci *et al.*, 2006), groundnut oil cake (45.6%) (Batal *et al.*, 2005), safflower oil cake (44.0%) (Sivaramakrishnan and Gangadharan, 2009), rapeseed meal oil cake (42.8%) (Bell, 1984), cottonseed oil cake (41.0%) (Ramachandran *et al.*, 2005), mustard oil cake (38.5%), sesame oil cake (35.6%), sunflower oil cake (34.1%) and canola oil cake (33.9%) (Ramachandran *et al.*, 2007), linseed oil cake (32 - 36%) (Rani and Ghosh, 2011), coconut oil cake (25.2%) (Ghosh *et al.*, 2013), copra oil cake (23.11%) and palm kernel oil cake (20.4%) (Dairo and Fasuyi, 2008) and olive oil cake (4.77%) (Vlysside *et al.*, 2004) are the most abundant agriculture by-products. Oil cakes, being rich in proteins (important nitrogen source in many microbial fermentation) and supported by other nutrients such as carbohydrates and minerals, offer a wide range of alternative substrates in SSF for the production of various enzymes, a wide spectrum of secondary metabolites, biomass, organic acids and biofertilizer among other uses.

(c)   Cellulosic or ligno-cellulosic substrates

Most agricultural residues contain high levels of cellulose or ligno-cellulose, which have the potential to be used as solid substrates in SSF. These include sugarcane bagasse, soybean hulls, wheat bran, rice hulls, rice stover, corn cob, barley husk, sugar beet pulp, wheat straw, barley straw and wood. In this case, cellulolytic fungi such as *T. reseei*, *T. longibrachiatum*, *T. viride*, *A. niger*, *C. cellulolyticum*,

Rhizopus sp., and ligninolytic fungi such as white-rot fungi are able to degrade complex cellulose and lignocellulose to produce simple sugars.

(d)   Substrates with soluble sugars

Solid substrates containing significant amount of soluble sugars may be obtained from fruit processing such as molasses, grape pomace, apple pomace, kiwi pomace, lemon peel, lemon pulp, peach pomace, pineapple waste, sweet sorghum, fodder and sugar beets, sugar beet pulp, carob pods, and coffee pulp.

(e)   Defined media and inert carrier

There are various inert carriers that can be used to simulate the conditions of typical SSF. These include vermiculite, perlite, clay granules, pozolano particles (volcanic material), hemp, amberlite, polyurethane foam (PUF) and polystyrene. The inert carrier is filled with chemically defined liquid media. The advantages of SSF on inert support in comparison to SSF on natural solid substrates (Barrios-Gonzale and Mejia, 2008; Weber *et al*., 2002; Ooijkaas *et al*., 2000; Zhu *et al*., 1994; Auria *et al*., 1990; Aidoo *et al*., 1982), include: (1) enhancing the homogeneous aerobic conditions, (2) improving process control and monitoring, (3) the inert carrier has less physical structure changes or can even be constant during fermentation, (4) improved control of heat and mass transfer, (5) higher evaporation rates and thus better control of temperature, (6) good control of water activity, (7) shrinkage and channelling are avoidable, (8) less complicated and easy product recovery, (9) easy to extract extracellular products with fewer impurities, (10) the inert carrier allows precise modification of production liquid media, (11) easy and possible process modelling and process control because the production media are known and can be analysed, (12) biomass can be measured directly, (13) suitable to grow any microorganism based on defined media and (14) the inert carrier can be reused. Some natural solid substrates such as sugarcane bagasse and rice hulls can be used as inert carrier due to their low nutrients but high porosity and ability to provide a very good support in terms of controlling mass and heat transfer (Chen and He, 2012).

2.7.2   Physico-chemical Factors

2.7.2.1   Moisture content

The water requirements of microorganisms for microbial activity can be expressed quantitatively in the form of water activity (a_w) of the environment or substrate. The a_w gives an indication of the amount of free water in the substrate and determines the type of microorganisms that can grow in

SSF. The required a_w value for SSF varies depending on the microorganism, but it is usually recommended that the a_w be enough to permit growth of mycelium through the solid substrate particles without disintegrating the particles. According to Nigam and Singh (1994), microorganisms capable of carrying out their microbial activities at lower a_w values are suitable for SSF process. Bacteria mainly grow at higher a_w values of about 0.9, while yeasts grow at values of 0.8 and filamentous fungi are adaptable to lower a_w values ranging between 0.6 and 0.7 (Nigam and Singh, 1994; Beuchat, 1981). However, according to Ruijiter *et al.* (2004), the fungus *A. oryzae* accumulates high concentrations of polyols at water activities between 0.96 – 0.97 during SSF, which seems to be unusual for this type of processing. As the fermentation starts at low moisture content, the culture dries out. Consequently, the fungus grows poorly and growth does not occur before the fermentation is completed. To overcome this problem, an appropriate amount of water is occasionally added throughout the fermentation period (Lotong and Suwaranit, 1990).

Another approach is to apply saturated air. Saturated air is usually applied to the system as an alternative to maintain a_w and moisture content of the fermented substrate. It is also a common practice used to avoid substrate drying. This approach is suitable when the SSF is carried out in designated bioreactors. At high moisture content, solid substrate particles tend to stick together and thus reduce the surface to volume ratio of solid material. According to Mitchell *et al.* (2002), high moisture levels can cause agglomeration of medium particles in SSF and lead to oxygen transfer limitations. As a result, a great decrease is observed in the production of microbial metabolites. Hence, it is important to provide and monitor the moisture content at an optimum level.

2.7.2.2   pH

In SSF, pH is very difficult to measure and control. This is because of the nature of solid substrate, very low water content (lack of free water), heterogeneity in the conditions of the systems, and due to the lack (or absence) of suitable on-line pH measurement methods (Durand *et al.*, 1997). To the best our knowledge, there is no reliable electrode that can measure pH in the solid medium. Usually it is desirable to use microorganisms which will grow over a wide range of pH and which have broad pH minima. Individual groups of microorganisms react in different ways to the pH value of the fermentation environment. Bacteria generally prefer pH values near neutrality, fungus and yeast slightly acid pH values, and actinomycetes above neutrality. Villegas *et al.* (1993) suggested using of a potentiometric electrode or a standard pH electrode after suspending the fermented substrate in water.

2.7.2.3   Temperature

The problem regarding temperature arises during the SSF process due to the heat generated from microbial activity and accumulated in the system (Nigam and Pandey, 2009). Temperature due to heat and mass transfer effects presents difficulties in handling the SSF process (Krishna, 2005). The heat needs to be removed from the system to avoid overheating and thereby disturbing the growth of microorganisms and the formation of products (Pandey *et al.*, 2001). Therefore, in SSF, most studies on solid state bioreactor designs are focused on maximising heat removal (Figueroa-Montero *et al.*, 2011; Ashley *et al.*, 1999). The problem becomes crucial in large-scale systems where heat evolution leads to huge moisture losses and, under these circumstances, disturbing fungal growth (Khanahmadi *et al.*, 2006).   Another problem is that heat creates condensation such that a large amount of water is returned back to the fermented solid. This will create heterogeneity in the solid substrate. Because of this, it is difficult to maintain the temperature at an ideal range. To overcome this, air is usually blown into the system, to force out the heat generated via a gas outlet (Sato *et al.*, 1984). The flow rate of the air blown into the system needs to be taken into account to avoid the loss of moisture content from the fermented substrate (Shojaosadati *et al.*, 2007). A cooling system can also be installed into the system to solve this problem.

2.7.2.4   Gaseous environment

The gases of interest are oxygen and carbon dioxide. Oxygen must diffuse from the inter-particle space to the biomass. Adequate supply of oxygen is required to maintain aerobic conditions. Carbon dioxide must diffuse from the biomass to the inter-particle space and must be removed from the system. This requirement can be achieved by aeration or mixing of the fermenting solids. Oxygen limitation might occur at deep areas of the substrate. These can be solved by turning the fermenting substrate through mixing processes (Lonsane *et al.*, 1985).

2.7.2.5   Aeration

Microorganisms normally vary in their oxygen requirements. Oxygen or air is sparged into the medium. Aeration plays two important roles in SSF: (i) meeting the oxygen demand in aerobic fermentation and (ii) heat and mass transport in a heterogeneous system. Aeration provides and maintains high oxygen levels and low carbon dioxide levels in the inter-particle solid substrates. The points to take into account with the aeration are the flow rate and air quality. Dry air at high flow rate will have an effect on the moisture of fermented substrate even though it has an advantage in

terms of heat removal. Aeration rate was shown to have a positive effect on microbial growth and product formation (Assamoi *et al.*, 2008; Gutarra *et al.*, 2005; Zhang *et al.*, 2003). Alternatively, using saturated air is a common strategy to avoid substrate drying by maintaining moisture levels. In addition, the rate of aeration by saturated air controls the temperature and the moisture gradients of the solid medium (Saucedo-Castaneda *et al.*, 1992).

2.7.2.6   Particle size

The particle size properties of solid substrates will lead to the shape, accessible area, surface area and porosity of the solid substrates (Richard *et al.*, 2004). Processes like chopping, grinding and cutting create a condition for microorganisms to be active at the initial stages of growth and increase the degradation and hydrolysis rate since the solid substrate is insoluble (Ramana Murthy *et al.*, 1993). The most important physical factor is the particle size that affects the surface area to volume ratio of the solid substrate (Krishna, 2005). Smaller particle size would provide a larger surface area per volume and allow full contact of microorganisms with the nutrients but the diffusion of oxygen would be affected (Nigam and Pandey, 2009). Larger particle size provides small area per volume ratio and gives excellent diffusion of oxygen but contact with nutrients is affected (Nandakumar *et al.*, 1994). A suitable particle size should satisfy both mycelial growth and the demand for oxygen and nutrients (Nandakumar *et al.*, 1996). Particle size also affects the size of inter-particle voids and porosity (Mitchell *et al.*, 2002). Any change in porosity of the solid substrate bed changes the apparent density of solid substrate and diffusion of gases into the bed. A large pore size is suitable for an adequate oxygen supply (Pandey, 1991). If porosity is limited, the effective diffusivity of gases is less. Particle size and properties may change during fermentation. These do not only affect the growth of microorganisms, but also affect the monitoring of heat conductivity, substrate consumption, products concentration and water content (Rahardjo *et al.*, 2005).

2.7.3   Mechanical Factors

2.7.3.1   Agitation/Mixing

Agitation or mixing plays the same role as aeration. In addition, agitation is a possible alternative to solve heterogeneity problems in SSF and might improve homogeneity and disrupt gradients (Lonsane *et al.*, 1985; Xu and Hang 1988). Another benefit of agitation is that air flow is more evenly distributed which improves the conditions for microbial growth within the entire fermented bed (Suryanarayan, 2003). However, agitation affects mycelium formation as shear forces due to

agitation can destroy the mycelium. Continuous agitation also may create problems related to cell damage especially when filamentous fungi are used (Mitchell *et al.*, 2011). A slower agitation speed might be necessary and some bioreactors perform this by using intermittent agitation to avoid serious damage to the mycelium (Gasiorek, 2008). Agitation within an SSF process is preferred with bacteria or yeast as their cells are usually not in tight contact with the solid substrate surface. Overall, this agitation or mixing procedure is not always advisable.

2.7.3.2   Particular design of bioreactors

In the fermentation process, the bioreactor provides the suitable conditions for growth and activity for the microorganisms involved, which allow the microbiological activity. SSF can be considered to be a "closed system". At time t = 0, the sterilised solid substrate in the bioreactor is inoculated with the microorganism and incubation is allowed to proceed under optimal physiological conditions. In the course of the entire fermentation, nothing is added into the bioreactor except oxygen (in the form of air). The composition of the culture medium, the biomass concentration and the metabolites concentrations generally change constantly as a result of the metabolism of the cells. Despite the heterogeneity of the solid substrate in the bioreactor, there are several parameters such as transport of oxygen and metabolic heat which involves aeration/agitation, moisture content, temperature and the type of microorganism and solid substrate used, which are relevant to the particular design of the suitable bioreactor for each particular fermentation process. A detailed discussion will be provided in the next section on bioreactors for SSF.

2.8   ESTIMATION OF GROWTH IN SSF

It is difficult to estimate biomass in SSF directly because it is very difficult to separate the microorganisms and the solid particles. This is more the case for fermentations involving filamentous fungi because fungal mycelium penetrates deeply into the substrate and becomes inextricably entangled within the solids. For this reason, it is better to use indirect methodologies for biomass estimation and having information about the cell biomass in such systems is very important. Chattaway *et al*. (1992) state "in the case of biomass estimation, elemental balances are useful either for obtaining on-line estimates or for calculating biomass off-line, when direct measurement is difficult, during fermentations on solid-containing media".

Although there are a large number of studies on biomass estimation in SSF, the available methods do not perform well. There are operational and sample preparation difficulties around these existing

methods. For example, dry weigh measurements (including both cells and the solid particles), are used to estimate cell biomass concentration, resulting in accurate data. A range of indirect methods can be used to estimate biomass, which can be classified into six categories:

1. Measuring cell components not present in the substrate
2. Measuring biomass component present in both substrate and biomass
3. Measuring other secondary metabolites
4. Measuring metabolic activity
5. Measuring images from direct microscopic observation
6. Measuring biomass from the substrate matrix

2.8.1   Measuring Cell Components not present in the Substrate

Cell components (which are products of microbial activity) can be measured to estimate the growth of biomass. To do this, it is necessary to identify and measure components of the cell or mycelium, which are not normally present in the solid substrate. Cell components which can be measured include: glucosamine (Swift, 1973; Sparringa and Owens, 1999), chitin or ergosterol (Feng *et al*., 2005), total sugar, DNA assay (nucleic acids) (Solomon *et al*., 1983; Hashimoto *et al*., 1982), ATP (Thierry and Chicheportiche, 1988), proteins (Abd-Aziz *et al*., 2008; Raimbault and Alazard, 1980), enzymes and others secondary metabolites. The most popular methods involve glucosamine estimation and profile production of enzymes (Ramesh *et al*., 1996). However, identifying cell components especially glucosamine, DNA and ergosterol is difficult and time-consuming due to tedious extraction procedures. For example, sample preparation for glucosamine and ergosterol measurement can take more than 24 h to complete. Measuring enzymatic activity or other secondary metabolites could be a reliable method for biomass estimation (Raimbault, 1997; Mitchell, 1992). A more practical logistic model developed by Ramesh *et al*., (1996) describes α-amylase and protease production, which are two non-growth associated enzymes produced by *B. licheniformis* M27. However, it is not easy to perform sample preparation for the analytical assay.

2.8.2   Measuring Biomass Components Present in Both Substrate and Biomass

Protein content is related to cell biomass and is easy to measure when microorganisms are grown alongside non-protein solid substrates. Significant amounts of protein are usually found in solid materials. The microorganism will therefore hydrolyse the substrate protein and produce biomass

protein. Therefore, it can be difficult to tell the difference between protein from the cells and solid substrate protein. Measurements can wrongly be taken of total protein from both cells and solids. This biomass measurement technique is only reliable if solid substrate has no protein. Recent works by Oto *et al*. (2012, 2013) resulted in a non-destructive and real-time estimation method for monitoring microbial contamination. The method used a UV-Vis reflectance spectrum to measure ATP and/or a plate count on the surface of pork meat.

2.8.3   Measuring Other Secondary Metabolites

Microbial secondary metabolites are useful high value products because they undergo a large number of biological activities. Secondary metabolites produced by a SSF system such as penicillin, tetracyclin, cephalosporin, iturin A, cephamycin C, ergot, alkaloids, monacolin K, lovastatin and many others could be used to indicate growth in SSF. After microbial growth has occurred, secondary metabolites are synthesized in a fermentation medium (Krishna, 2005). Pandey *et al*., (2001) argue that secondary metabolites build up near the end of fermentation, known as the idiophase that follows the active growth phase called trophophase. Secondary metabolites produced in the idophase are independent of synthesised cell material and normal microorganism growth.

2.8.4   Measuring Metabolic Activity

During fermentation processes, carbon is aerobically transformed into biomass, primary or secondary metabolites, carbon dioxide and water (Cordova-Lopez *et al*., 1996). Growth happens because of the interactions of microorganisms and their environments. Besides product formation and nutrients consumed, other factors, which affect growth are: environmental conditions (oxygen and carbon dioxide level, temperature, pressure and pH) (Carrizalez *et al*., 1981). Continuously on-line monitoring of microbial growth could be performed by indirect methods measuring:

- Oxygen consumed (Sato *et al.*, 1983);
- Carbon dioxide produced (Saucedo-Castaneda *et al.*, 1994; Han, 1987; Carrizalez *et al.*, 1981);
- Metabolic heat (Marison and von Stokar, 1986; Ratkowsky *et al.*, 1983; Bayer and Fuehrer 1982) and
- Pressure levels during fermentation (Auria and Revah, 1994)

The advantage of using this method is that there are no tedious and time consuming sampling processes. It is assumed that total carbon dioxide produced is proportional to the cumulative cell concentration (Terebiznik and Pilosof, 1999). The metabolic state of culture is estimated from the respiratory quotient (RQ). The RQ value is found by using carbon evolution and oxygen consumption rates. This can give indication of how well the substrate is aerated as high RQ values indicate fermentative metabolism (Mitchell *et al.*, 2002). This could be applied to other biological systems where carbon dioxide is a growth product. Heat is also produced by microbial activity and so could be used to measure biomass. Another reported method shows that growth and pressure drop at the same rate across an aerated SSF fermentation bed (Auria and Revah, 1994). Once technology becomes more advanced over time, on-line microbial metabolism could be suitable for determining growth in aerobic SSF, by measuring rates of oxygen uptake, carbon dioxide evolve, heat evolution and pressure changes. Facilities could be expensive but useful for gaining information on microbial growth. These techniques are suited for use on-line and so are most likely to meet success in the future.

2.8.5   Measuring Images through Direct Microscopic Observation

Another method is image analysis through microscopic observation. This can be done using Scanning Electron Microscope or Confocal Microscopy. Growth patterns taken from digital image analysis (Yingyi *et al.*, 2012; Feng *et al.*, 2007; Couri *et al.*, 2012) are interpreted by computer software, which calculates total length or volume mycelium growing in the SSF system (Raimbault, 1997). Osma *et al.*, (2011) developed software using a Matlab platform to estimate fungal culture's occupied area and volume. This allows high-definition images from scanning electron and environmental scanning electron microscope to be analysed. To monitor the biomass growth of *A. niger*, Dutra *et al.*, (2008) measured the hyphae area in an image from stereomicroscope Carl Zeiss STEMI 2000-CS and found a correlation with lipase activity.

Another technique uses confocal scanning laser microscopy to measure concentration of penetrative biomass during growth (Nopharatana *et al.*, 2003a; 2003b). However, this technique is only suitable if artificial solid substrate is used. Miri *et al.*, (2003) studied fungal hyphae's morphology and structure during SSF. They used manual image analysis after staining the sample with a fluorescent contrast agent. Afterwards, they used a fluorescence microscope to see the fibres. This method involves expensive equipment and specific software needs to be developed. With these above methods, there are disadvantages. Some need special processes with tedious procedures, the amount of sample needed varies and operator fatigue can decrease accuracy level. In all cases, the cells attach to the

solid particles. Cells are scattered and not evenly distributed. Cell damages during sample processing are also high possibility. Despite clear developments in image analysis, predictive, easy to apply models have not been developed. Routine measurement of biomass using this method is not practical, despite great potential. Using haemocytometer under microscope to count spores or cells is usually a last resort.

2.8.6   Measuring Biomass from the Solid Matrix

In recent times, direct evaluation of biomass using a membrane filter has become possible. This is because the whole fungal mycelium can be easily recovered by peeling it off the membrane and weighing it immediately after drying (Ooijkaas *et al*., 1996). However, this technique is only suited to small scale bioreactor or lab studies. Another disadvantage is that it is difficult for fungal mycelium to penetrate especially large and deep solid particles. Terebiznik and Pilosof (1999) grew *A. oryzae* NRRL 3458 in wheat bran and measured dry matter weight loss in terms of mycelia growth. Okazaki *et al*., (1999) used the mathematical model for estimating fungal growth in SSF. They estimated biomass growth from dry matter weight loss by incorporating with carbon dioxide evolution into a relationship. Wang *et al*., (2010) adapted the logistic model as originally proposed by Okazaki *et al*, (1999). They successfully applied a dry weight reduction ratio for *A. oryzae* fermentation using rapeseed meal.

2.8.7   Other Techniques

Ramana Murthy *et al*., (1993) monitored biomass in SSF using light reflectance. They observed colour changes due to the growth of the fungus during SSF. They used CIE (Commission International de l'Eclairage) system based on the principles of additive colour mixing. The measurements taken were looked at against glucosamine concentration. The authors concluded that colour and glucosamine were closely linked. This technique for biomass estimation in SSF could be developed but no follow-up work has been done.

There is potential for using Biomass Monitor to measure radio-frequency dielectric properties (Aber Instruments Ltd, Science Park, Cefn Llan, Aberystwyth, Dyfed SY23 3DA, UK). The online method involves taking measurements of the dielectric permittivity at low radio-frequencies, by using biomass probe (the Bugmeter) which has four terminal sensors. Computerised system measures the capacitance at 0.3 MHz (in picofarads), which indicates of biomass. This technique was shown to

measure biomass in SmF without presence of solid particles either using yeast, bacteria or even fungus (Harris *et al.*, 1987).

Articles produced by Penaloza *et al.*, (1991) and Davey *et al.*, (1991); showed that the microbial biomass during SSF for tempe production could be estimated by the monitor measuring in capacitance (fungus *R. oligosporus* was used). See FIGURE 2.4(a) (Penaloza *et al.*, 1991) and FIGURE 2.4(b) (Davey *et al.*, 1991) which show the time course of the capacitance, during tempe fermentation. It was found that this technique produced linearity during the growth phase, between the dielectric permittivity and the hyphae length (as determined microscopically).

According to Darvey *et al*, 1991, the biomass monitor provides a reliable, one of a kind, easy to reproduce and on-line measurement of biomass in SSF. Botella (2007) also suggested that this technique has potential to monitor on-line fungal growth in SSF. As with light reflectance technique, there is no work reported after year 1991. Therefore, this technique shows potential for further development.
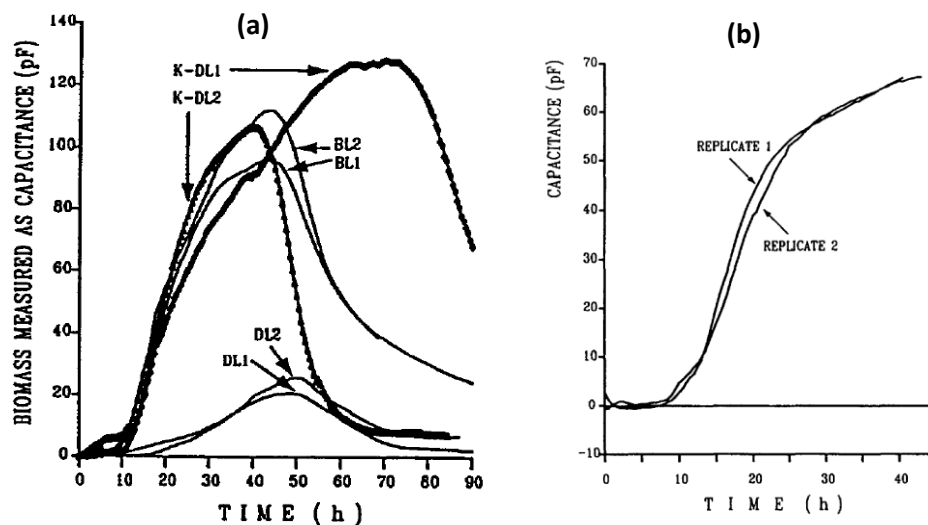
FIGUERE 2.4:   A graph showing the time course profile of biomass capacitance using a biomass monitor in tempe fermentation. Adapted from (a) Penaloza *et al.* (1991) and (b) Davey *et al.*, (1994)

There are disadvantages to most of the categories listed in TABLE 2.6. A common problem for indirect methods of biomass estimation is that the relationship between biomass and the factor measured can change over time. No methods currently involve calibration, which could better measure the relationship with biomass. It is necessary to have a direct measurement of the biomass through calibration methods**.**

TABLE 2.6: Summary of the categories of methods used to estimate biomass in SSF

Category	Comments
Measuring cell components that are not present within the solid substrate	▪ Most of the cell components are not in constant proportion of the cell mass during all stages of development ▪ Tedious extraction process, time-consuming ▪ Expensive reagents/chemicals, strong chemicals, buffer preparation
Measuring biomass components probably present in both solid substrate and biomass	▪ ▪ Difficult to differentiate biomass components coming from either cells or solids – interference with a substrate rich in protein ▪ Not accurate definition of biomass concentration ▪ Involves tedious extraction process, time-consuming ▪ Expensive reagents/chemicals/kits, buffer preparation
Measuring other secondary metabolites	▪ Most of the cell components are not in a constant proportion of the cell mass during all stages of development ▪ Not always related to growth ▪ Tedious extraction process, time-consuming, ▪ Expensive reagents/chemicals, strong chemicals, buffer preparation
Measuring metabolic activity	▪ The oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and metabolic heat evolution rate techniques are the easiest to operate on-line ▪ No regular sampling process ▪ Shows a potential for further development ▪ The metabolic production is proportional to biomass concentration but may vary with time during the fermentation ▪ Always related to growth ▪ Requires expensive equipment and dedicated software ▪ Requires frequent calibration
Measuring images through direct microscopic observation	▪ Labour-intensive, time-consuming, cells adhering to solid surface causes damage problems, requires considerable time ▪ Microscope has a higher resolution than the conventional light microscope ▪ Suitable if using an artificial solid media
Measuring biomass from the solid matrix	▪ Adapted for fungi only ▪ Suited only to a few cases, especially practical in lab scale studies ▪ Complete separation is difficult

2.9   BIOREACTORS FOR SSF

SSF bioreactor design greatly depends on the solid substrate. According to Zhong (2011), the bioreactor is the core of biological process. There are four major roles of the bioreactor which are:

1. To contain the substrate
2. To contain the process microorganism
3. To protect the process microorganism against contamination
4. To control environmental conditions to optimise growth and product formation

In SSF or SmF processes, the bioreactor provides suitable environment for microorganism growth and biological activity (Krishna, 2005). Bioreactors must be able to hold the media and be sealed well which prevents harmful environment substances entering in to bioreactor. Various types of bioreactor system have been developed and used in SSF processes. The most important benefit has been greater understanding of the biological system of the microorganism and biological processes in SSF (Mitchel *et al.*, 2002).

Research has found that temperature, pH, oxygen, carbon dioxide and heat removal need to be controlled and optimised (Zhong, 2011). According to Pandey *et al.*, (2008), SSF bioreactor design should carefully consider design and operating variables, especially aeration transfer, temperature control (including cooling system), effective diffusion of oxygen in gas phase and coefficient for heat transfer through bioreactor wall.

In SSF, oxygen transfer can often be inefficient in some designs. The problems are complicated, affecting the temperature and the water content of the solid medium, which both need to be, controlled tightly (Rodriguez *et al.*, 2005). Effective bioreactors have high biological reaction rates including growth performance, substrate consumption, product formation and by-products synthesis rates (Zhong, 2011). It is very important to understand which criteria provide the best bioreactor performance, so that bioreactors can be designed effectively.

TABLE 2.7 shows how the SSF bioreactors can meet many fermentation objectives and the limitations that prevent them from solving all problems. In SSF, matching a specific bioreactor to a certain bioprocesses needs a balanced consideration of many factors. These include oxygen transfer, mixing/agitation, aeration, temperature control, moisture gradients, operational stability and reliability, scale-up and cost. Selected bioreactor must have features and mode of operation that will

optimise results. Importantly, every selected bioreactor operation will increase the biological processes and factors involved.

TABLE 2.7: Strengths and weaknesses of small-scale SSF bioreactors

Strengths
▪ Operates under aseptic conditions
▪ Inoculation process in an aseptic manner post-sterilisation
▪ Ease to sample aseptically during fermentation
▪ Low cost bioreactors
▪ Low operating costs
▪ Operating is simple and inexpensive to conduct
▪ Bioreactors take up little space
▪ Require little infrastructure
▪ Easy for environment control – temperature, carbon dioxide
▪ Sensitivity of fungus to mechanical mixing/agitation and therefore fungus is not disturbed during fermentation process
▪ Ease of solid loading and unloading for transfer systems
▪ Potential for large numbers of parallel fermentations
▪ Ease of cleaning after fermentation
▪ Easy to scale by increasing the number of identical bioreactors
Limitations
▪ Limited maximum oxygen transfer rate
▪ Limited maximum heat transfer rate
▪ Limited maximum carbon dioxide removal
▪ Limited maximum water loss
▪ Limited maximum agitation rate
▪ Limited maximum water distribution into solid substrates
▪ Heterogeneity of the solid substrate
▪ Limited control of pH

Mitchell *et al.*, (2011) argue that one of the main issues of bioreactor design and operation is the need to remove enough metabolic heat waste. This is important to prevent temperature within the fermented bed from getting too high as this affects microbial growth and product formation. Heat removal must be effective. It depends on control and support systems and how these fit into the production system. Finally, other process needs and setbacks should also be considered.

2.9.1   Classification of Bioreactors for SSF

The main reason for designing SSF bioreactors is to address major problems such as transport of oxygen, removal of metabolic heat, moisture gradients and the heterogeneity of the solid substrate. According to Mitchell *et al.*, (2001), good bioreactor performance is controlled by two factors:

1. The interactions between the microorganism and its local environment.
2. Influence of the design and operating strategies on conditions in the microorganism's local environment.

Important parameters in bioreactor design include temperature, oxygen concentration, moisture gradients as well as mixing/agitation, aeration and heat transfer. Usually two strategies are used to produce excellent SSF processes, resulting in effective oxygen transfer, efficient heat removal, excellent water distribution and good substrate mixing with minimal mycelia damage. The two basic strategies of aeration and mixing are as follows (Mitchell *et al*., 2006a):

1. The air supply circulates around the fermented solid substrate particles.
2. The air goes through the inter-particles of fermented solid substrate particles.

Within strategy two, three mixing types can be used on the fermented beds: static (unmixed), intermittently mixing (agitated), or continuous mixing (agitated). For both strategies, dry air or saturated air can be used. According to Mitchell *et al*., (2006a), SSF bioreactor designs can be put into four groups (FIGURE 2.5), which can be separated by aeration and mixing type:

- Group 1: Unforced aeration, without mixing/agitation (static)
- Group 2: Forced aeration, without mixing (static)
- Group 3: Unforced aeration, with continuous or intermittent mixing/agitation
- Group 4: Forced aeration, with continuous or intermittent mixing/agitation
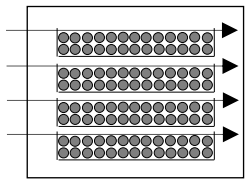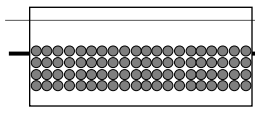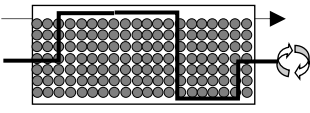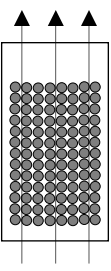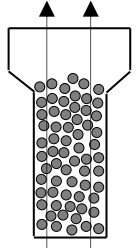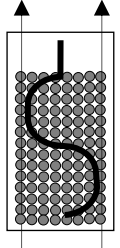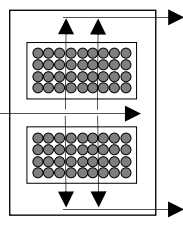
Aeration strategy	Agitation strategy	
	No mixing (or very infrequent)	Continuous mixing or frequent intermittent mixing
No forced aeration (air passes around bed)	Group 1 ▪ Tray Bioreactor Tray chamber	Group 3 ▪ Rotating drum bioreactors ▪ Stirred drum bioreactors Rotating drum Stirred drum
Forced aeration (air blown forcefully through the bed)	Group 2 ▪ Packed bed bioreactors ▪ Intermittently stirred packed-beds Packed bed	Group 4 ▪ Stirred-bed bioreactors ▪ Rocking drum bioreactors ▪ Gas-solid fluidised bioreactors Gas-solid fluidised bed Stirred bed Rocking drum

FIGURE 2.5: **Classification of SSF bioreactors according to agitation and aeration strategies used (Redrawn from Mitchell *et al*., 2006a)**

Mitchel *et al*., (2011) state there are three main objectives of designing bioreactors:

1. To provide adequate heat removal

2. To maintain adequate water activities

3. To provide high oxygen concentrations within the inter-particles spaces

FIGURE 2.6 from Mitchell *et al.*, (2011) shows a typical SSF bioreactor and bioreactor phases.
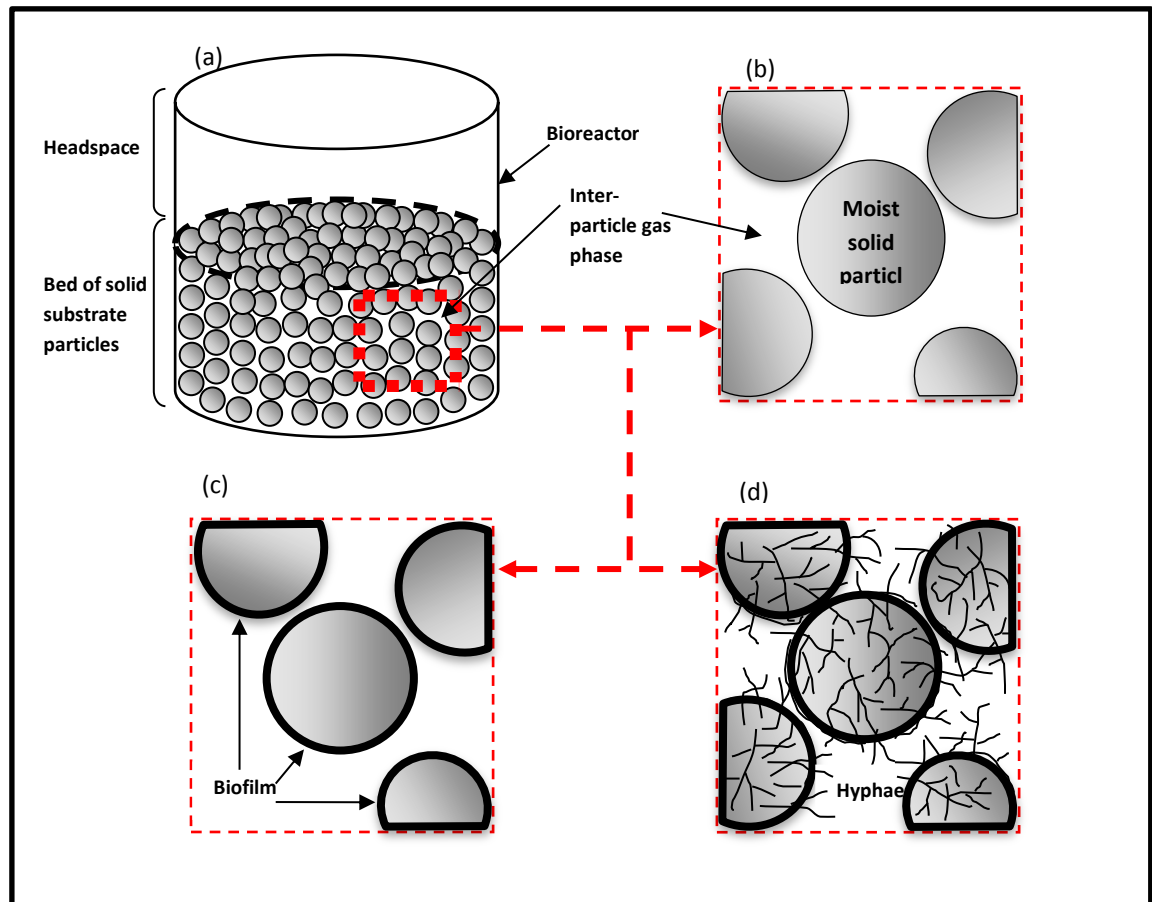
FIGURE 2.6: The phases within a SSF system (redrawn from Mitchell *et al*., 2011)

FIGURE 2.6 gives an overview of SSF phases as follows:

FIGURE 2.6(a):   Overview of the bioreactor system at the macro-scale, sometimes referred to as bioreactor vessel. At this scale, differences between solid substrate particles (fermented bed), headspace, and the wall of the body structure of the bioreactor are identified.

Generally, from a macroscopic viewpoint, there are three main subsystems within SSF bioreactors:

1. A bioprocess takes place in the body structure of the bioreactor.
2. The fermented bed, which combines moist solid substrate particles and inter-solid substrate particles, which are filled with the oxygen.

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3. The area on and above the surface of the fermented bed (known as the headspace), which has high oxygen concentration.

FIGURE 2.6(b):   Micro-scale overview of un-inoculated substrate. This can be known as solid phase. At this scale, moist solid substrate particles hold important nutrients and growth factors. Space between solid substrate particles known as void fraction, is filled with water and gas. Furthermore, the gradients of nutrients, oxygen and water will affect microbial growth.

FIGURE 2.6(C):   Overview of bacterial or yeast cultures growing and sticking to surfaces of the solid substrate particles, shown by the thick black layer at the particle surface.

FIGURE 2.6(d):   Overview of filamentous fungi growing by sticking to the surface and hyphae. Therefore able to deeply penetrate into solid substrate particles to consume nutrients, oxygen, excreting metabolites and enzymes.

At a small scale level, petri dishes and Erlenmeyer flasks can be used as laboratory scale bioreactors for SSF. Conditions are entirely aseptic, making this an advantage of using laboratory scale bioreactors. However, the disadvantage is that agitation and aeration cannot be carried out. Many industrial bioreactors have been designed. However, they have problems of sterilisation, substrate loading, heat generation, heat removal and handling difficulties.

2.9.1.1   Group 1

Tray bioreactors

Historically, tray bioreactors have been used widely in traditional SSF. Using trays is the oldest system and they are very simple in design and used in static conditions, unmixed beds with no forced aeration on the solid substrate. The fermentation is done in stationary trays with no mechanical agitation. The bottom of the tray is perforated with mesh to hold the solid substrate to allow a normal aeration. This system type only holds limited amount of solid substrate to be fermented. This is because only thin layers must be used, to avoid overheating from happening and to maintain aerobic conditions (Robinson and Nigam, 2003). Alcantara and da Silva (2012) and Vaseghi *et al*., (2013) found that substrate thickness; surface area and chamber temperature gave the positive effect on enzymes activity and could improve metabolic heat and gas transfer (Xie *et al*., 2013).

Thickness of solid substrates bed can be varied. Usually trays are place in the incubating room, where temperature and humidity are controlled for optimal growth. Trays are arranged one above the other with suitable gaps between them. Tray design has not changed majorly (Nigam and Pandey, 2009). Chen *et al*., (2005) studied the effect of two dynamic changes of air (including air pressure pulsation and internal air circulating) in a tray bioreactor and observed changes in the temperature gradient. In their results, internal air circulation was beneficial, accelerating heat transfer between the substrate surface and the outside air. Furthermore, Ruiz *et al*., (2012) and Assamoi *et al*., (2008) designed column-tray bioreactors with forced aeration. This allowed better control of environmental conditions in the bed due to manipulation of temperature and flow rate of the air. FIGURE 2.7 shows general schematic multiple tray fermenters.
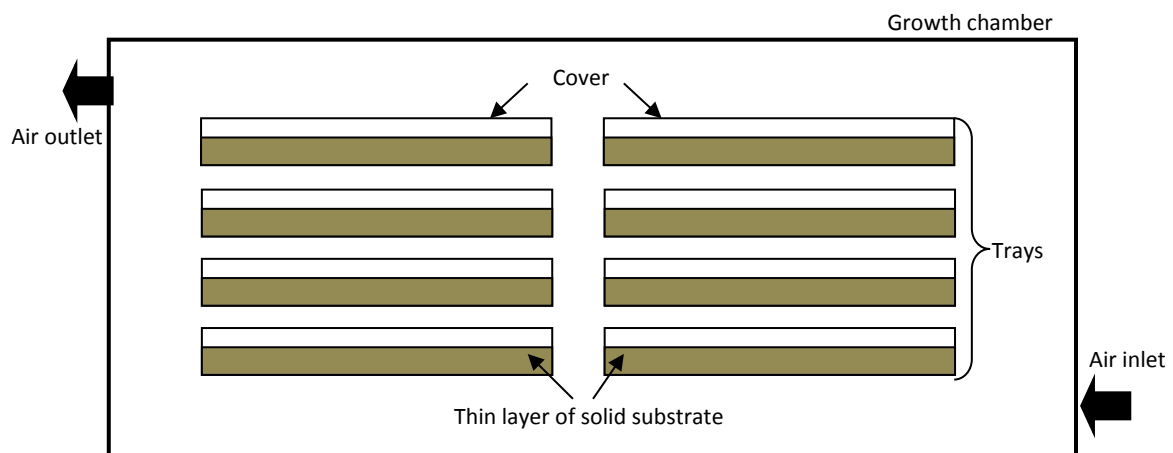
FIGURE 2.7: Scheme of tray bioreactor (redrawn from Cauto and Sanroman, 2006)

2.9.1.2   Group 2

Packed-bed bioreactors:

Packed-beds bioreactors are built from glass or plastic columns and are made up of unmixed beds with perforated base. Forced aeration is applied to the bottom of the column (Nigam and Pandey, 2009). These systems are useful for product developments with efficient process controls, particularly for heat removal (Robinson and Nigam, 2003). Forced aeration using moistened air improves the moisture gradients of fermented bed and temperature control, compared with forced dry air (Assamoi *et al*., 2008). However, according to Gutierres-Rojas *et al*., (1996), not all the heat generated during fermentation process is eliminated. To achieve this, they suggested an injection of cool-dry air and replacing moisture at different points in the packed-bed. Salum *et al*., (2010) cultivated *B. cepacia* LTEB11 on a mixture of sugarcane bagasse and sunflower seed meal. They found that the fermentation solid can be used to catalyse the ethanolysis of soybean oil to produce biodiesel in a fixed packed-bed bioreactor (a co-solvent-free system). This strategy does not need expensive processing steps, for examples enzyme recuperation and immobilization and co-solvent separation. It is very effective and has potential to reduce the costs with enzyme-catalysed biodiesel synthesis. FIGURE 2.8 shows a general schematic packed-bed fermenter.
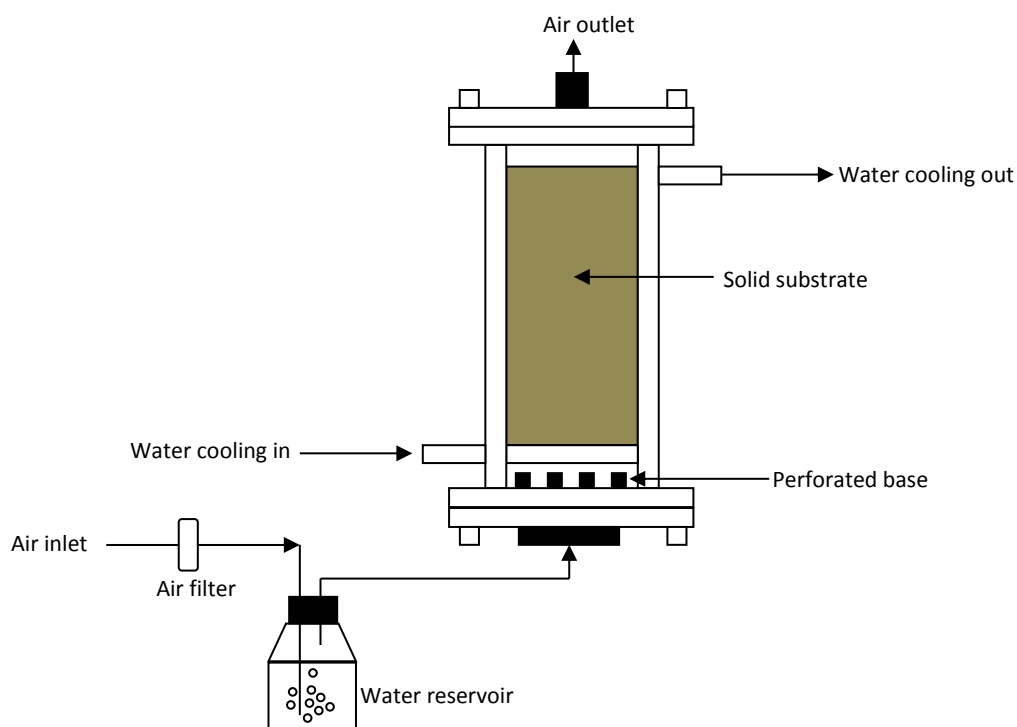
FIGURE 2.8: Scheme of a packed-bed bioreactor (redrawn from Cauto and Sanroman, 2006)

2.9.1.3   Group 3

***Rotating drum bioreactors*:**

Rotating drums bioreactors mix intermittently without forced aeration, operating on continuous or semi-continuous mode. A rotating drum bioreactor is a horizontal cylinder. The drum is semi-filled with a bed of substrate. The fermented bed cannot be too high and this creates good oxygen and carbon dioxide transfer (Mitchell and Berovic, 2010). Lonsane *et al.*, (1992) found even at small scale that temperature control in drum bioreactors is quite difficult. Solid substrate is mixed differently for different microorganisms. Mixing could be continuous, intermittent or mixed. This prevents metabolic heat generated by microbial activity accumulating. Temperature control also depends on the mixing effect on the solid substrate (Wang *et al.*, 2010). Air also could be blown through the headspace. Also in forced aeration, conditioned air is passed through the bed. Metabolic heat is removed from the bed, transfers into headspace by diffusion or through the conductive wall to the environment (Ali *et al.*, 2011).

During intermittent mixing, growth of the microorganisms is found to be more even and less damaging to fungal mycelium (Mitchell and Berovic, 2010). Continuous mixing could increase damage to fungal mycelium, affecting microorganism growth. Stuart and Mitchell (2003) observed the operating variables of the rotating drum bioreactor when growing *A. oryzae*. They found that the growth rate decreased when rotational speed increased because of shear forces. Ali *et al*., (2011) suggest two possible ways to address these problems: (i) intermittently mixing and aeration into the headspace. Both mixing and aeration could control the temperature, moisture gradients, uniformity and oxygen concentrations. (ii) Equipped with baffles on the inner wall. FIGURE 2.9 shows a general schematic rotating drum bioreactor.
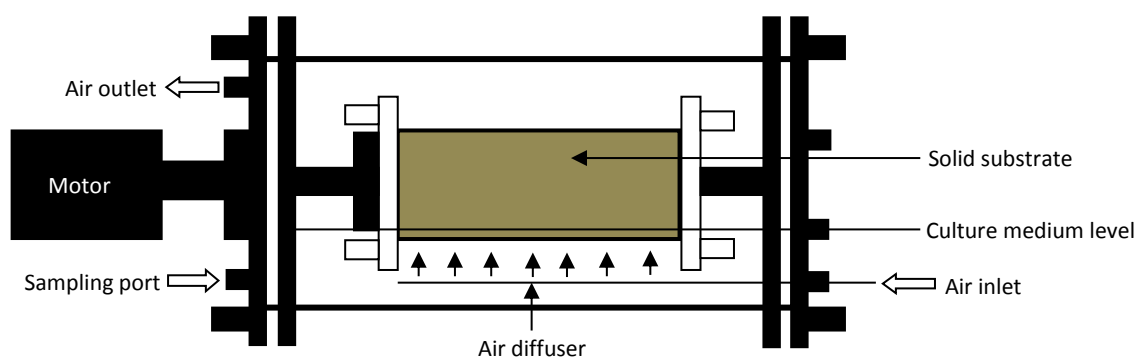
FIGURE 2.9: Scheme of a rotating drum bioreactor (redrawn from Cauto and Sanroman, 2006)

2.9.1.4   Group 4

(i) Fluidised-bed bioreactors:

Typically, fluidized-bed bioreactors are constructed from a vertical chamber with a perforated base plate. Forced aeration is applied at the bottom chamber at sufficient speed to fluidize the solid substrate particles and cause mixing. Also, the bioreactor has an agitator (clump breaker), breaking up agglomerates that can form and settle to the bottom (Tanaka *et al.*, 1986). The bed expands and so enough headspace is needed (Mitchell and Berovic, 2010). The mixture of solid particles and gas will behave like a liquid. This fluidized-bed bioreactor provides a good mixing behavior of gas, solid and liquids (Wang and Yang, 2007). Evaporating water can cool the biomass (Moebus and Teuber, 1982). The substrate properties of gas-solid fluidized-bed bioreactors influence bioreactor effectiveness. For example, a sticky substrate will form large agglomerates (clumps), which cannot be fluidized (Ali *et al.*, 2011; Foong *et al.*, 2009b).

In order to fluidize all, the solid substrate should have the same particles size. With size differences, some small size particles might fluidize and the large particles size might not (Jang and Yang, 2008). There is no problem with controlling the temperature and cooling the substrate bed, because high flow rates for fluidization should provide a large enough convective cooling capacity (Foong *et al.*, 2009a) and good rate of heat and mass transfer (Wang and Yang, 2007). However, mixing steps in fluidized bed could damage the penetrative mycelium of the fungus because fungi are affected by hard force (Raghavarao *et al.*, 2003). The design scheme of the fluidized-bed bioreactor can be seen in FIGURE 2.10.
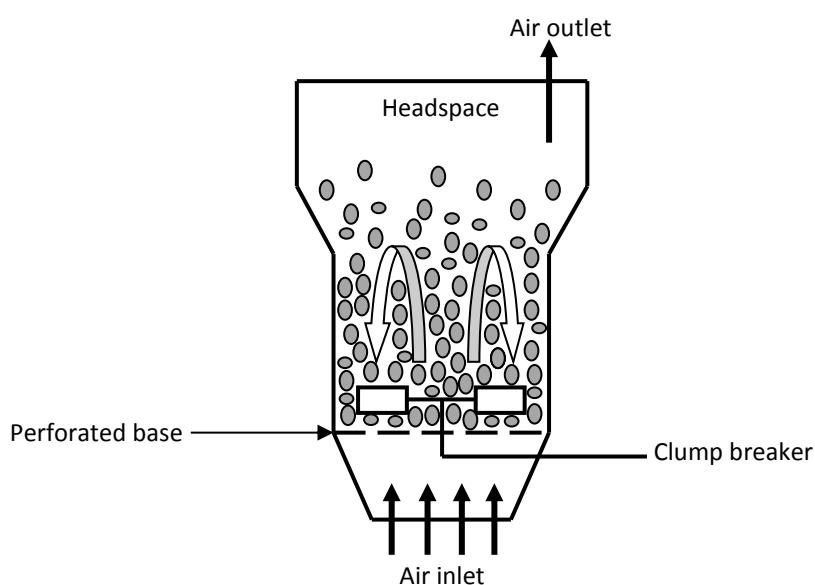
FIGURE 2.10: Scheme of a gas-solid fluidised-bed bioreactor (redrawn from Mitchell *et al.*, 2006b)

(ii) Spouted-bed bioreactors:

In spouted-bed bioreactors, air is only blown upwards through the central axis of the solid bed. As a result, only part of the bed is fluidized and the bed can be expanded. Due to the vigorous contact between gas and solids, solids slip down the bottom of bioreactor due to its sloped sides and solids cycle continuously (Mitchell *et al*., 2006, Olazar *et al*., 2003). The advantage of the spouted-bed bioreactor is that it prevents particles agglomeration caused by high speed impacts in the spouted-bed's core region (Wang and Yang, 2007, Viswanathan *et al*., 2009). This is suitable for handling solids, which have sticky nature, irregular texture or size distribution that cannot be treated in fluidized-bed bioreactors (Olazar *et al*., 2003). Also spouted-bed bioreactors are able to handle large coarse solid particles, different densities and shapes (San Jose *et al*., 2006), which are related to solid substrate for the SSF process. Furthermore, spouted-bed bioreactors have other advantages for mass and heat transfer. This is because exceptional mixing of solid substrates in the bioreactor creates high mass and heat transfer rates (Viswanathan *et al*., 2009; Szafran and Kmiec, 2004; Larachi *et al*., 2003; Olazar *et al*., 2003).

The spouted bed bioreactor solves the problems of more common SSF carried out in tray and packed-bed bioreactors. Spouted bed bioreactor show improved fermentation performance, including higher product titers, yields, and productivity (Silva and Yang, 1998). They studied the spouted-bed bioreactor with intermittent spouting with air, which achieved high production levels both of total protein and enzymes. The result was similar to packed-bed bioreactor, but spouted-bed had uniformity and had no solids-handling problems. However, it was found that continual spouting was found to be unfavourable to this SSF, possibly because of shear impact damage to fungal mycelium during spouting. The design scheme of the spouted-bed bioreactor is illustrated in FIGURE 2.11.
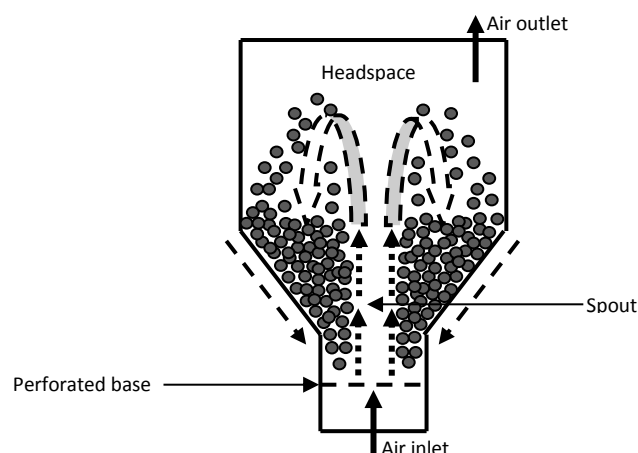
FIGURE 2.11: Scheme of a spouted-bed bioreactor (redrawn from Mitchell *et al*., 2006b)

TABLE 2.8 lists common features and problems with SSF bioreactors. Various researchers have sorted the SSF bioreactors into classifications shown in TABLE 2.9. Currently, there are about eighteen different SSF bioreactors types, which are given different names by different authors. All designs are based on applying simple aeration and mixing strategies to the system, both for small scale and large scale. Particularly, packed-bed, tray, rotating drum and fluidized-bed bioreactors gained great attention from researchers. Choosing the most suitable bioreactor system depends on which solid substrates and microorganisms are used in the SSF (Mitchell *et al*., 2000).

Importantly, research and development are interested in improving performance and productivity, by improving existing SSF bioreactor systems and processes (Mitchell *et al*., 2000). The results of the research studies increase possibilities for improving quality of SSF bioreactor systems. Results are because of manipulation of the substrates or the microorganisms used. Bioreactor designs are more progressive, due to investments in design development. This is possible because of advantages of SSF, which have been discovered. Also because economic factors such as having choice of using wider ranges of raw materials (Nigam and Pandey, 2009).

For the past 15 years, a lot of research has focused on bioreactor engineering to improve mixing, aeration, heat removal, moisture gradients and mass transfers, either in small or large scale SSF bioreactor. Mostly the research based on trial and error, or adopted from somewhere else. Some of the findings showed that a "new" system design and technology can be developed by manipulating the bioreactor systems and process (Wang and Yang, 2007).

Despite progressive research, there are still many problems to be solved. These include problems with oxygen transfer, removing of metabolic heat and water activity distribution through the solid substrate particles. These issues were the most serious problems for SSF and are summarised in TABLE 2.9.

The table shows the interaction between different factors in the SSF bioreactor. According to Mitchell *et al*., (2006a), ideal balance can be created by: combining the most desired operating conditions within the SSF process with desired conditions in SSF bioreactors, needed for: (1) minimizing deviations from the optimum temperature, (2) minimizing damage to microorganism (3) minimizing deviations of the bed water activity from the optimum value and (4) maximizing the supply of oxygen to the solid substrate particles.

TABLE 2.8: **Features and problems of commonly used SSF bioreactors. Adapted from Nigam and Pandey (2009), Wang and Yang (2007) and Mitchell *et al*. (2000)**

Bioreactor type	Features/Problems
Bench scale: Petri dish Erlenmeyer flask	- Very simple, low cost, small quantity of substrate, less contamination, - Allows many experiments, passive aeration, easy regulation of temperature and airflow rate - Optimisation of the process and experimental variables in a short time
Tray bioreactor	- Simple structure, easy to operate - Non-aeration, non-mixed - Heat accumulation - Temperature and moisture gradients generated - Bed caking - Required larger area - Labour intensive
Packed-bed bioreactor	- Forced-aeration, non-mixed - Axial temperature and gas concentration gradients exist - Non-uniform growth - Difficulties to harvest the final product - Difficult to scale-up - Bed caking - Labour intensive
Zymotis bioreactor	- Packed-bed bioreactors with internal cooling plates - The spacing between the internal cooling plates and the temperature of the cooling water - Cooling water temperature is varied during fermentation in response to bed temperature - Overcomes the problem of heat removal - Substrate must be maintained static - Leads to reasonably stable bioreactor performance - High productivities at large scale – easy scaling up
Rotating-drum bioreactor	- Continuously or intermittently mixed/agitation with forced-aeration - Improved mass and heat transfer - Considered to be better and more uniform - Operating on continuous or semi-continuous mode - Shear effect may cause damage to microorganisms - Slumping flow may cause a little mixing - Complicated reactor construction - Difficult operation for large scale fermentation
Fluidised-bed bioreactor	- Good moisture and temperature controlling due to good mixing - High mass and heat transfer rate - Removal of metabolic heat is efficient - Excellent growth of aerobic microorganisms due to efficient aeration - No bed caking - High shear damage to microorganisms - Difficult to fluidize large, coarse and sticky particles - High cost
Spouted-bed bioreactor	- Continuously or intermittently mixed - High mass and heat transfer rate - Lower power requirements than fluidised-bed systems - Good in handling large, coarse, non-uniformly sized and sticky particles - Need further investigation on characterisation and scale-up

TABLE 2.9: Research and development in design of fermenters for SSF

Microorganism	Substrate	Products	Remarks	Reference
Tray bioreactor				
Rhizopus oryzae	Sugarcane bagasse	Lipase	▪ The temperature and humidity of the cabinet, depth of solid bed, particle size, initial moisture content and supplementary substrate effect on lipase production.	Vaseghi *et al.*, 2013
Beauveria bassiana Bb-202	Rice	Conidia production	▪ Cutting solid substrate into many pieces would increase the surface area of substrate. ▪ Metabolic heat and gas transfer in the centre of substrate could be improved. ▪ Conidia yields were significantly increased.	Xie *et al.*, 2013
Aspergillus niger CCT0916	Cashew apple dry bagasse	Polygalacturonase	▪ Temperature and substrate thickness influence the process and enzymes production.	Alcantara and da Silva, 2012
Mixed-culture fungi: *Aspergillus niger* *Trichoderma reesei*	▪ Rice straw ▪ Cauliflower waste ▪ Kinnow pulp ▪ Pea-pod wastes ▪ Wheat bran	▪ Cellulase ▪ β-glucosidase ▪ Endoglucanase ▪ Xylanase	▪ Mixed-culture better than single culture. ▪ Provision of aeration during static tray SSF and control conditions enhanced enzyme activities.	Dhillon *et al.*, 2011
Aspergillus niger C28B25	Perlite as an inner support impregnated with Pontecorvo medium	Studied on temperature and water content	▪ Improvement of heat removal in SSF tray bioreactors by forced air convection without drying the fermentation bed.	Figueroa-Montero *et al.*, 2011
Trichoderma virens	Rice grains	Heat and mass transfer study	▪ The bed height, relative humidity and moisture content important factors for temperature maintenance. ▪ The bed height improved moisture content. ▪ An intermittent mixing process is essential.	Jou and Lo, 2011
Bacillus thuringiensis CM-1 *Penicillum decumbens* JUA10	Steam-exploded straw	▪ Bio-pesticide ▪ Cellulase	▪ The strategy benefit of internal air circulation was to accelerate the heat transfer between the substrate surface and the outside air.	Chen *et al.*, 2005
Penicillium simplicissimum	Babassu cake	Lipase	▪ By adding nutrient supplement, lipase production was optimum without aeration.	Gutarra *et al*, 2005
Penicillium decumbens JUA10	Steam-exploded wheat straw and wheat bran	▪ Cellulase ▪ β-glucosidase	▪ Operated with periodic pressure oscillation coupled with forced aeration through the medium. ▪ Gave efficient control of temperature	Zhang *et al.*, 2003
Column-tray bioreactor				
Aspergillus niger Aa-20	Lemon peel pomace	Pectinase	▪ The forced aeration allows better control of environmental conditions in the bed due to the ability to manipulate the temperature and flow rate of the process air.	Ruiz *et al.*, 2012

Packed-bed bioreactor				
Kluyveromyces marxianus var. marxianus	▪ Pressmud	Inulinase	▪ Higher inulinase production under optimised conditions.	Dilipkumar *et al*., 2013
Metarhizium anisopliae	▪ Wheat bran ▪ Rice bran ▪ Hemp impregnated with a nutrients medium	Insecticides (against malaria mosquitoes)	▪ Aerated static packed beds are the most suitable bioreactor for large production of spores. ▪ *M. anisopliae* cannot withstand mixing (Scrapped drum bioreactor). ▪ Evaporative cooling system for temperature control was successful.	Van Breukelen *et al*., 2011
Kluyveromyces marxianus NRRL Y-7571	Sugarcane bagasse	Inulinase	▪ Batch and fed-batch modes. ▪ Different strategies for feeding the inlet (saturated and unsaturated air) as an alternative to remove metabolic heat.	Astolfi *et al*., 2011
Burkholderia cepacia LTEB11	Mixture of sugarcane bagasse and sunflower seed meal	Biodiesel	▪ The system has potential to decrease the cost of enzyme-catalysed trans-esterification reactions.	Salum *et al*., 2010
Aspergillus niger	Wheat bran	Modelling of temperature gradients	▪ Provides useful tools for investigating the heat transfer dynamics and performance under different operating conditions.	Fanaei and Vaziri, 2009
Aspergillus niger	Sugarcane bagasse	Citric acid	▪ Aeration and packing density helps in heat and mass transfer. ▪ Excellent in removal of metabolic heat. ▪ Higher product yield. ▪ 90% recovery of the product. ▪ Potential for scaling up and commercialisation.	Kumar and Jain, 2008
▪ *Phlebiopsis gigantean* ▪ *Streptomyces sp.* K61	Silica gel impregnated with a nutrients medium	Spores bio-fungicides	▪ Suitable for different types of microorganisms such as fungi and bacteria. ▪ Suitable for many kinds of solid materials. ▪ The temperature of substrate was controlled by changing ambient temperature.	Virtanen *et al*., 2008
Aspergillus niger	Wheat bran	Temperature and moisture control	▪ Use of cooling water in the wall of the solid state bioreactor is a suitable strategy for reduction of bed temperature and moisture gradient in order to obtain uniform fungal growth with high biomass production.	Shojaosadati *et al*., 2007
Aspergillus niger	Wheat bran	Study on moisture content control	▪ Developed a method based on simple measurements of the temperature of the inlet and outlet gas for estimating moisture content of the bed in a forcefully aerated system. ▪ Cheap tool for making decisions about when to add water to the bed. ▪ How much water to add during each mixing and water-addition event.	Khanahmadi *et al*., 2006
Penicillium simplicissimum	Babassu cake	Lipase	▪ The aeration rate was shown to have a positive effect on lipase production but had a negative effect on temperature. ▪ High lipase production (30%) compared to tray system.	Gutarra *et al*., 2005
Penicillium simplicissimum	Babassu cake Sugar cane molasses	Lipase	▪ The effect of air flow rate and temperature on lipase production was assessed.	Cavalcanti, *et al.*, 2005

			▪ Aeration shown to have a positive effect on lipase activity.	
Aspergillus oryzae	Wheat grain Wheat flour pellets	α-amylase	▪ The effects of bed porosity, bran and specific surface area on the oxygen uptake rate and α-amylase production during growth.	Rahardjo *et al.*, 2005
Coniothyrium minitans IVT1	Hemp impregnated with glucose and yeast extract	Bio-control agent	▪ Effective for temperature control by increasing the aeration rate. ▪ Temperature control resulting in the highest yield at the lowest costs for production conidia.	Jones *et al.*, 2004
Coniothyrium minitans IVT1	Hemp impregnated with glucose and yeast extract	Bio-control agent	▪ The combination system with inert support impregnated with enriched medium excellent in temperature control, respiration, growth and sporulation of the bio-control fungus.	Weber *et al.*, 2002
Aspergillus niger	Starchy substrate	Strategies for overheating problems	▪ Frequent mixing decreases the maximum temperature, but air reversal does not.	Ashley *et al.*, 1999
Aspergillus niger	Wheat bran	Validation of a model describing two-dimensional heat transfer	▪ Developed a two-dimensional heat transfer that can describe heat transfer.	Sangsurasak and Mitchell, 1998
Aspergillus niger	Kumara (starch-containing root crop)	Citric acid	▪ Improved aeration for high production.	Lu *et al.*, 1998
Aspergillus niger	Amberlite IRA-900	Citric acid	▪ Different mechanisms of heat removal (conductive, convective and evaporative). ▪ The unsteady-heat balances indicated that conductive heat transfer was the least efficient mechanism (8.65%) when compared with convective (26.65%) and evaporative (64.7%) mechanisms.	Gutierrez-Rojas *et al.*, 1996
Aspergillus niger	Wheat bran	Amyloglucosidase	▪ The moisture content of fermented substrate was found to affect oxygen transfer rates.	Gowthaman *et al.*, 1995
Aspergillus niger CFTRI 1105	Wheat bran	Amyloglucosidase	▪ Employed with forced aeration. ▪ Gas concentration and temperature gradients decreased with increasing air flow rate.	Gowthaman *et al.*, 1993
Aspergillus niger	Wheat bran	Glucoamylase	▪ Space loading is an important factor in this bioreactor. ▪ A good compromise between space loading and aeration rate.	Pandey and Radhakrishnan, 1992
Multi-layer packed-bed bioreactor				
Aspergillus niger	Wheat bran	Investigation of the "cycling" and "continuous plug-flow" operating strategies	▪ The performance of bioreactor can be improved by constructing it as multi-layer beds and operating it in a continuous plug-flow mode ▪ This strategy results in a lower heat generation rate.	Mitchell *et al.*, 2010
Penicillium canescens 10-10c	Soya oil cake	Xylanase	▪ Forced aeration induces more sporulation of strain and reduces xylanase production. ▪ Forced moistened air improves production compared to production obtained with forced dry air.	Assamoi *et al.*, 2008

Aspergillus niger	Apple pomace	Citric acid	▪ Higher aeration rate gave negative effect on citric acid production due to shear stress and harmful effect on the filamentous fungi.	Shojaosadati and Babaeipour, 2002
Aspergillus niger	Kumara (starch-containing root crop)	Citric acid	▪ Improved the mass transfer considerably compared with a single-layer packed-bed with the same substrate loading. ▪ Allowed precise measurement of the gradients for carbon dioxide, citric acid, starch and fungal biomass.	Lu *et al.*, 1998
Rotating drum bioreactor				
Aspergillus niger NRRL 567	Apple pomace	Citric acid	▪ Bio-production trend with intermittent agitation. ▪ Higher citric acid production was achieved with apple pomace supplemented with methanol.	Dhillon *et al.*, 2013
Aspergillus oryzae	Wheat grains	Biomass	▪ Sufficient aeration was provided to control the temperatures near the optimum value for growth during the first 45 – 50 hours. ▪ Forced aeration through a single pipe did not provide homogenous cooling in the substrate bed. ▪ Biomass yield decreased with increasing size of the fermenter due to large temperature gradients. ▪ Air flow improvement required to avoid the need for frequent mixing.	Schutyser *et al.*, 2004
Aspergillus oryzae CBS 570.64	Wheat grains	Biomass	▪ Mixing in this fermenter was needed to first disrupt the mycelium network formed between the substrate particles, rather than to homogenise excessive temperature or moisture gradients. ▪ The main heat removal mechanism during SSF with forced aeration is evaporative cooling (i.e. heat removal via evaporation of water).	Schutyser *et al.*, 2003a
Aspergillus oryzae	Wheat grains	Biomass	▪ Water was added to the bed via spray nozzles to overcome desiccation of the bed during evaporative cooling. ▪ A short spraying period, followed by a period of mixing without water addition can be applied.	Schutyser *et al.*, 2003b
Aspergillus oryzae	Wheat bran	Evaluating heat and mass transfer	▪ Rotational speed, aeration characteristics, substrates loading and surrounding temperature were varied to explore the performance of wheat bran fermentations.	Stuart and Mitchell, 2003
Thermoascus aurantiacus	Wheat straw	Enzymes	▪ The strategy with an intermittent agitation effectively increased enzyme production.	Kalogeris *et al.*, 2003
Aspergillus oryzae	Wheat bran	Establishment of axial temperature profiles during fermentation	▪ Axial temperature gradients can arise; lack of provision for axial mixing. ▪ Suggested to use angled lifters within the bioreactor to promote axial mixing.	Mitchell *et al.*, 2002
▪ Simulations of grain mixing in three different rotating drum designs for SSF.			▪ Valuable detailed knowledge about particle transport processes help to understand and optimise related heat and mass transfers process in SSF.	Schutyser *et al.*, 2002
▪ Discrete particle simulations predicting mixing behaviour of solid substrate particles in a rotating drum fermenter.			▪ Success in the mixing progress as a function of the degree of filling and size of the drum.	Schutyser *et al.*, 2001

			• Potential tool for design and scale-up of mixed SSF.	
Scrapped-drum bioreactor				
Aspergillus oryzae	Wheat grain	Biomass	• Continuous mixing provides promising possibilities for simultaneous control of temperature and moisture content.	Nagel *et al.*, 2001a
Aspergillus oryzae	Wheat grain	Biomass	• Developed model for on-line moisture content control during fermentation.	Nagel *et al.*, 2001b
Coniothyrium minitans IVT 1	Oats	Bio-pesticides	• Convective cooling is effective, but evaporative water losses result in unacceptable shrinkage of the substrate and in a critically low water activity. • A non-mixed system is unfit for large scale spore production. • A mixed bioreactor offers better scale-up possibilities as mixing improves conductive cooling.	Oostra *et al.*, 2000
Horizontal drum bioreactor				
Aspergillus nifer F3	Citrus peel	• Pectinase • Xylanase	• Air flow intensity was found do not improve pectinase and xylanase production.	Rodriguez-Fernandez *et al.*, 2011
Cylindrical bioreactor				
Aspergillus niger	Amberlite IRA-900	Citric acid	• Evaluation of unsteady-state heat accumulation as well as conductive, convective and evaporative contributions to metabolic heat removal.	Gutierrez-Rojas *et al.*, 1996
Column bioreactor				
Mortierella alpina	Rice bran	PUFA	• The cultures could grow better in the bioreactor with aeration. • Moisture content and temperature at upper, middle and bottom at optimum. • The final product of SSF could be dried and consumed directly without further extraction treatment.	Jang and Yang, 2009
Aspergillusniger NRRL 3	Buckwheat seeds	Spore	• Fixed bed glass column with a jacket for circulation of water to control temperature. • Supplied continuous aeration from the bottom of bioreactor.	Ramachandran *et al.*, 2008
Beauveria bassiana	Mixture: potatoes and sugarcane bagasse	Spore	• A column bioreactor with forced aeration with saturated air produced highest spore levels due to the maintenance of temperature, moisture content and aerobic conditions in the bioreactor.	Santa *et al.*, 2005
Penicillium chrysogenum ATCC 48271	Sugarcane bagasse impregnated with a nutrients medium	Penicillin	• Criteria for the interpretation of respirations kinetics (cumulative carbon dioxide produced and carbon dioxide production rate) were established.	Dominguez *et al.*, 2000
Aspergillus niger	Wheat bran	Glucoamylase	• A column bioreactor produced about 50% higher enzyme than flask experiments.	Pandey *et al.*, 1996
Metarhizium anisopliae	Mixture: rice bran and rice husks	Spores	• Heat removal with forced aeration is inefficient and water losses occur from the fermented bed. • Evaporative cooling was employed as an efficient means of temperature control.	Dorta and Arcas, 1998

Fluidised-bed bioreactor				
Fungal strain TW1	Palm kernel cake	Mannose	• The moisture gradients of fermented bed were under controlled when high air velocity into the bioreactor was employed.	Foong *et al*., 2009a
Palm kernel cake	Palm kernel cake	Palm kernel cake (PKC)	• Excellent heat transfer between palm kernel cake and air allows SSF of PKC without accumulation of metabolic heat in the fermenter. • Increase of adsorbed water in PKC was proportional to air relative humidity and inversely proportional to superficial air velocity.	Foong *et al*., 2009b
Gel beads	5 types of gel beads differing in diameter and density	Hydrodynamic behaviour of gel beads	• The drag force between gel beads and flowing liquid is smaller than that for conventional solids.	Van Zessen *et al*., 2005
Saccharomyces cerevisiae	Semisolid potato	Baker's yeast	• Air-fluidised bed fermenter with aeration. • The growth of baker's yeast was easily controllable in this highly aerated system.	Hong *et al*., 1988
Saccharomyces cerevisiae	Wheat bran powder	• Amylase • Protease	• Air-solid fluidised bed fermenter with agitators. • High enzymes production compared to SmF due to high flow rate of air access into the system.	Tanaka *et al*., 1985
Saccharomyces cerevisiae	Inert carrier with glucose	Ethanol	• The design of the system was improved in order to obtain fermentation rates comparable with SmF in liquid fermentation for ethanol production.	Moebus and Teuber, 1982
Horizontal stirred tank bioreactor				
Aspergillus niger	• Apple pomace • Soya flour • Wheat bran	• Polygalacturonase • Pectinesterase	• Performed in situ sterilisation of solid substrate with periodical mixing	Berovic and Ostroversnik, 1997
Vertical cylindrical shaped bioreactor (Fed-batch SSF)				
Gibberella fujikoroi	Wheat bran	Gibberellic acid	• The efficiency of this reactor during fed-batch SSF process by taking as a model the production of secondary metabolites. • Performed in full aseptic conditions. • The ability to take samples and to add products during the fermentation without problems.	Bandelier *et al*., 1997
Gas-solid spouted-bed bioreactor				
Aspergillus oryzae	Rice	• α –amylase • β -amylase • Glucoamylase	• The system with intermittent spouting with air achieved. • High production levels in both total protein and enzymes. • No uniformity and solving solids-handling problems.	Silva and Yang, 1998

Zymotis bioreactor				
Aspergillus niger	Starchy substrate		▪ Packed-bed bioreactors with internal cooling plates. ▪ To overcome the problem of heat removal. ▪ Substrate bed must be maintained static. ▪ Minimise the degree to which growth is restricted by heat transfer limitations.	Mitchell and von Meien, 2000
Trichoderma harzianum	Mixture: sugarcane bagasse and wheat bran	Cellulase	▪ A large quantity of metabolic heat can be easily removed by the novel cooling system employed. ▪ Higher enzyme production compared to other bioreactors.	Roussos *et al*., 1993
Trichoderma harzianum	Mixture: sugarcane bagasse and cassava flour	Spore production	▪ Advantages of producing large scale conidiospore	Roussos *et al*., 1991
Hexahedral modular bioreactor				
Aspergillus niger	Mixture: rice meal and rice husks		▪ Keeps the homogeneity of the fermented bed at optimal levels. ▪ The geometry allows cooling the bioreactor without the need for an external cooling system. ▪ The admission of air provides oxygen and cooling to the system. ▪ Constructed with the principle of minimum heat resistance.	Colembergue da Cunha *et al*., 2009
Novel deep-bed cubical bioreactor				
Mixed culture: ▪ *Trichoderma reseei* ATCC 26921 ▪ *Aspergillus oryzae* ATCC 12892	Soybean hulls	▪ Cellulase ▪ β-glucosidase ▪ Endoglucanase ▪ Xylanase	▪ Bioreactor prototype that allows for effective temperature control. ▪ Internal aeration to achieve improved temperature control.	Brijwani *et al*., 2011
SSF reactor (SSFR)				
Bacillus subtillis RB14-CS	Okara (waste form tofu industries)	Antibiotic iturin A	▪ SSFR has powerful agitation ability due to the fact that the agitation rotor and the culture floor of the reactor turn mutually in the opposite direction for thorough mixing and SSFR is also equipped with temperature control and airflow unit.	Takashi *et al*., 2009
Continuous counter-current reactor				
Aspergillus niger	Sugarcane bagasse	Glucoamylase	▪ No mycelium damage or sporulation was observed. ▪ Successful for continuous production of glucoamylase under no sterile conditions. ▪ The system able to reduce fermentation time. ▪ Aeration was accomplished by natural convection.	Varzakas *et al.,* 2008

2.10   MASS TRANSFER PHENOMENA IN SSF

Performance of SSF depends greatly upon issues such as mass transfer phenomena and bioreaction rates, as well as effective bioreactors system design and operation. 'Micro-scale' and 'macro-scale' are used to describe mass transfer within the SSF bioreactor system. FIGURE 2.12 shows the relationship between micro-scale and macro-scale phenomena.

According to Mitchell *et al*, (2011): (1) 'micro-scale' involves microorganism growth which is affected by interaction between microorganisms and the local environment inside the solid substrate particles; (2) 'macro-scale' refers to effectiveness of SSF bioreactor system design and operation strategy and how this may affect conditions in local environment of microorganism. Generally mass transfer phenomena in SSF are not easy to measure, because different phenomena take place at the same time. Also, the relative importance of phenomena changes with bioreactor type, scale and other factors (Garcia-Ochoa and Gomez, 2009).

2.10.1   Micro-scale Phenomena

Micro-scale phenomena are of great interest because they are a major disadvantage of SSF. FIGURE 2.13 shows the schematic of some micro-scale phenomena that happen during SSF (as illustrated by Holker and Lenz (2005)). SSF has three major phases: which are the solid, gas and liquid phase. These phases make the process quite complicated and varied. Following this thought, microorganisms have a limited access to nutrients contained in the solid substrate, oxygen, water, enzymes and also limited carbon dioxide and heat removal. This will lead to poor growth, reduced bioreaction rates, low productivity and poor performance of the bioreactor system due to these limitations. In this situation, where SSF involves the use of fungi, mass transfers in micro-scale phenomena can be classified into four categories:

1. Intra-particle mass transfer
2. Inter-particle mass transfer
3. Heat transfer and
4. Water transfer
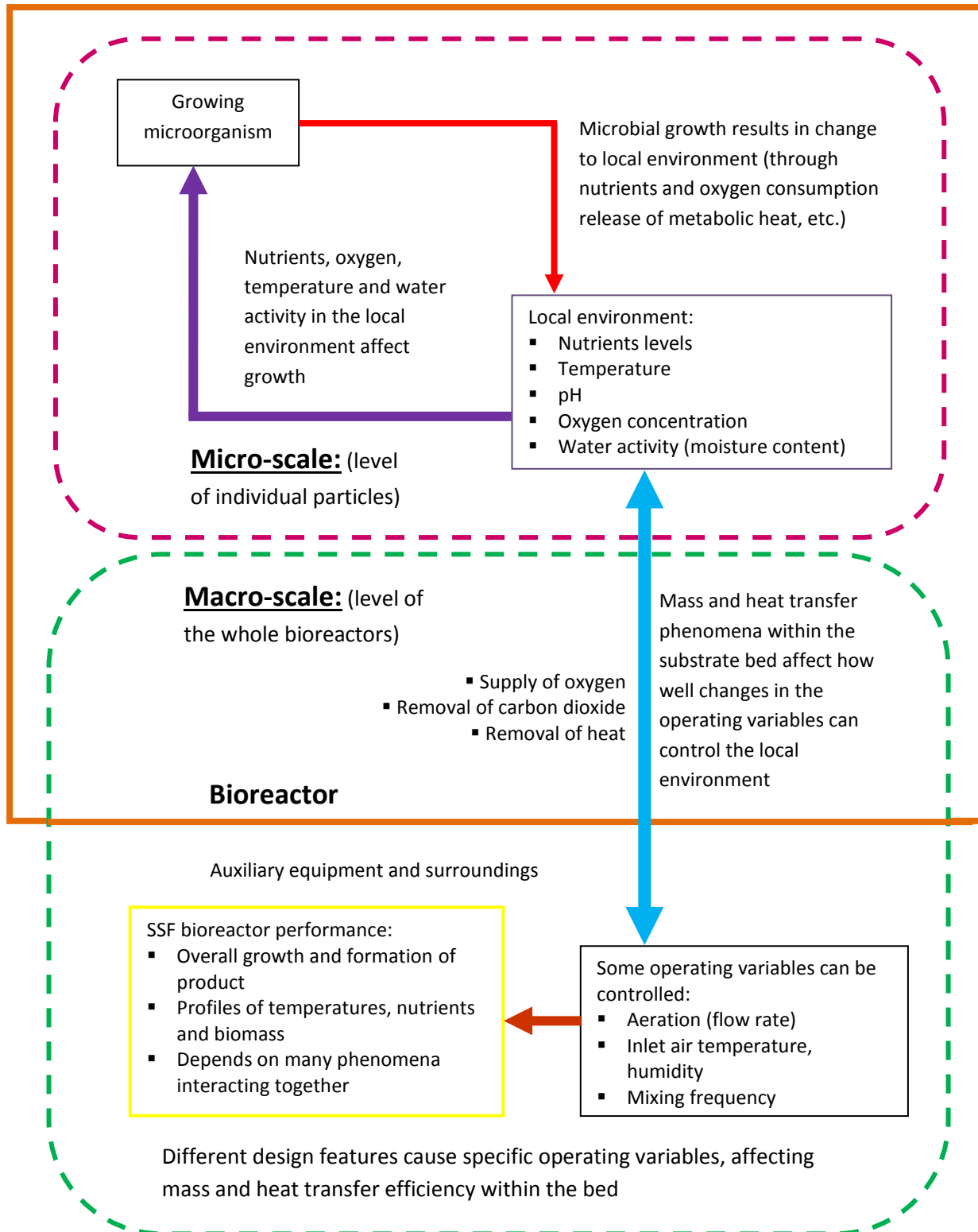
FIGURE 2.12: Summary of the micro-scale and macro-scale interactions between different factors which can affect SSF systems performance (Adapted from Mitchell *et al.*, 2011)

2.10.1.1   Inter-particle mass transfer

In SSF systems, the liquid film on the substrate surface limits oxygen transfer. Oxygen mostly comes from the gas phase and also from that dissolved in water due to growth and metabolism of the culture (Garcia-Ochoa and Gomez, 2009). However, compared to SmF, in SSF, gas transfer is much higher due to high interfacial area-to-liquid volume ratios of solid substrate particles (Hamidi-Esfahania *et al.*, 2004; Gowthaman *et al.*, 1993). During the fermentation process, the microorganism consumes oxygen, decreasing oxygen concentrations. Levels become less and less along the penetration depth, possibly reaching zero at a certain depth. Penetration depth of the mycelium can be increased by increasing oxygen transfer.

Gas volume within solid substrate particles inside bioreactors represents the void fraction. The void space depends on the solid substrate particle characteristics and also moisture content. Moisture content should be optimum; not too high or too low. If the moisture level becomes too high, void space becomes filled with water, causing oxygen concentration to decrease greatly. Conversely, if the moisture content is too low, the growth of the microorganism will be negatively affected, even though the space between solid particles becomes filled with sufficient oxygen.

In SSF, inter-particle mass transfer involves oxygen transfer to the growing fungus from the void fraction within the solid substrate particles. Internal oxygen concentration plays a very important role. Oxygen first passes through the actively respiring biomass in the solid substrate particle surface and subsequently diffuses through the liquid phase of the substrate (Richard *et al.*, 2004). Aeration and mixing are a good method to cause inter-particle oxygen transfer under the correct conditions of parameters such as void fraction and moisture content. Both continuous or intermittent mixing and aeration will resupply oxygen required for respiration. In addition, these processes function to prevent the exhaustion of oxygen in localised regions of the solid substrate particles. At the same time, they represent a good way to release the entrapped metabolic heat and carbon dioxide.
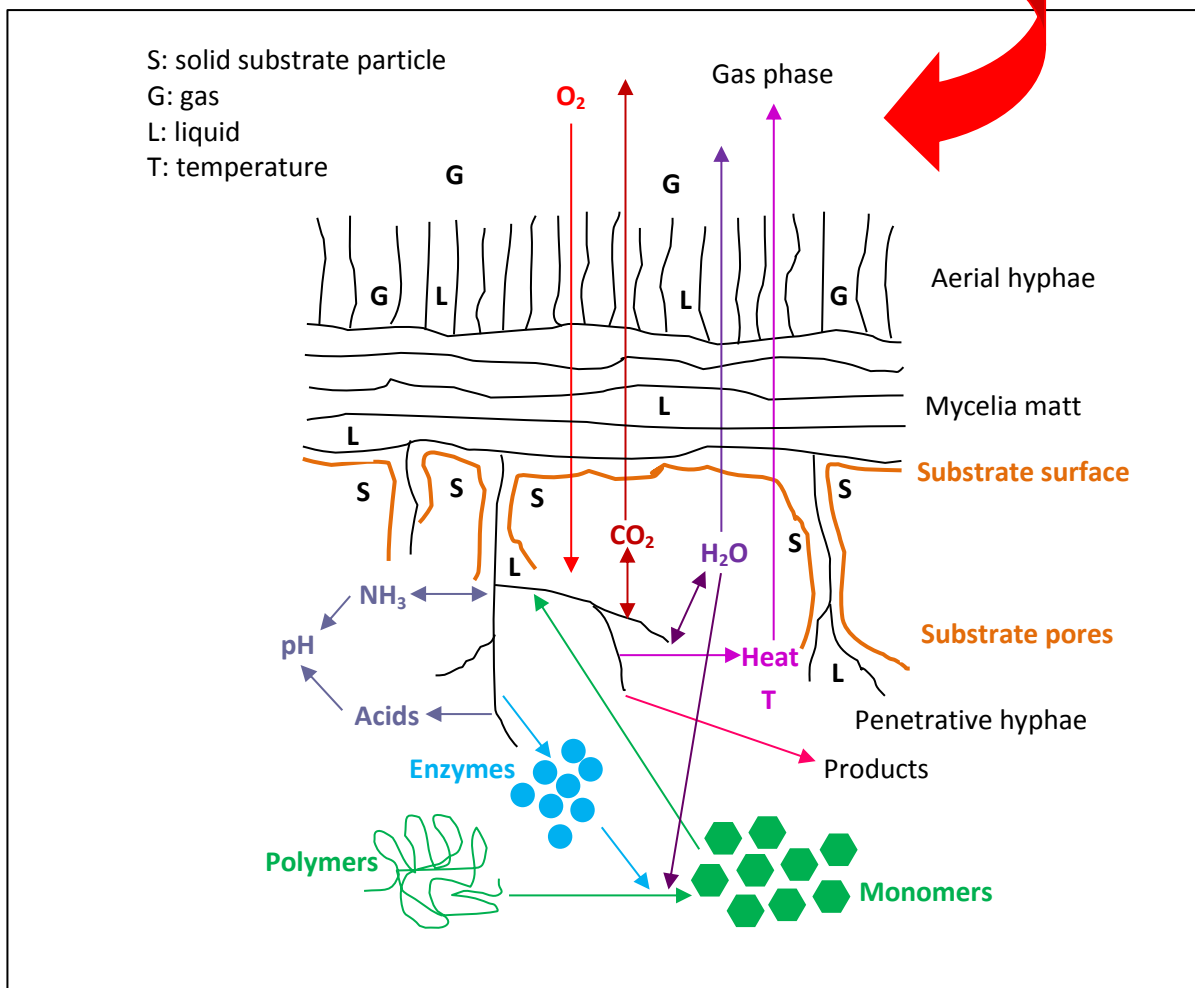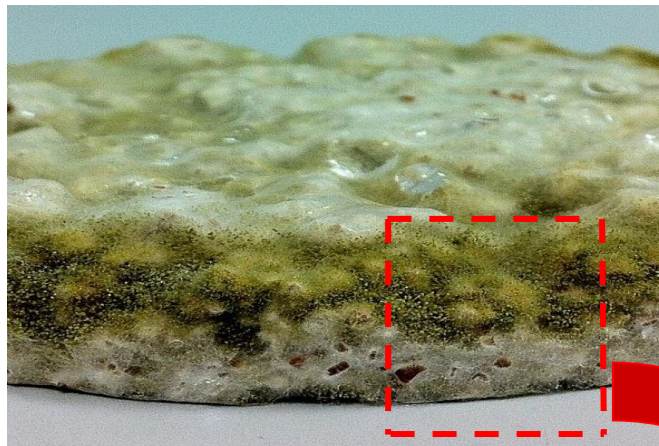
FIGURE 2.13: Schematic of the micro-scale processes that occur during SSF involving fungi (Redrawn from Holker and Lenz, 2005). Above: Fermented wheat bran with *A. oryzae* from the current study

2.10.1.2   Intra-particle mass transfer

Intra-particle mass transfer concerns transfer of nutrients and enzymes inside solid substrate particles. Here, the main issue is the diffusion of oxygen needed in metabolic respiration. Oxygen is consumed and carbon dioxide, water, heat and other products are produced. Additionally, the substrate containing the biomass produces enzymes, other polymers and secondary metabolites. The growing microorganisms secrete further enzymes, which help in the biodegradation of solid substrate particles. According to Mitchell *et al*. (1990), there are three steps of intra-particle mass transfer: (1) enzymes are released from the mycelium, (2) enzyme diffusion and (3) the process of degrading the substrate. The degradation process happening inside the solid substrate and the pore space between solid substrate particles improves nutrients and enzyme diffusion. Using small particles decreases the diffusion of solutes including enzymes, nutrients and oxygen (Mitchell *et al*., 2011).

Porosity of solid substrate particles changes during the course of SSF. Due to this, the rate of diffusion of oxygen, nutrients and enzymes may vary. Diffusivity processes limit the growth rate, especially within the solid substrate particles (Khanahmadi *et al*., 2006). Another consideration is that solid substrate particles should have high enough moisture content. This will increase the chance of efficient diffusion processes for oxygen, nutrients and enzymes. According to Mitchell *et al*. (2011), high water content can help to improve diffusion coefficients. With respect to this, the physical morphology of the solid substrate particles changes during SSF, especially porosity and particle size, which influences how much of the surface area is accessible to oxygen, nutrients, enzymes and the microorganism itself (Nandakumar *et al*., 1996).

2.10.1.3   Heat transfer

During SSF, a high amount of metabolic heat is produced. The amount of heat depends on the metabolic activity levels of the microorganism. Heat builds up in the fermentation medium because the solid substrate is a good thermal conductor (Ramana Murthy *et al*., 1993). The transfer of heat into or out of the SSF system is closely linked with the microorganism's metabolic activity, as well as the aeration of the fermentation system. During SSF, heat transfer is needed for temperature control. High temperature affects growth and product formation negatively. Low temperatures are unfavourable for growth and biochemical reactions. High moisture content in the SSF system makes it difficult to achieve good heat transfer. As fermentation progresses, oxygen diffuses and undergoes bio-reactions, which release heat. At the same time, solid substrate particles shrink, reducing

porosity, which further reduces heat transfer. Under these circumstances, heat builds up in the system. Mixing and aeration are good strategies because they lead to high rates of both heat removal and oxygen supply. Mixing or aeration with saturated air is an efficient solution, which helps with temperature control. Mitchell *et al*. (2011) suggest a strategy that promotes convective heat and mass transfer in the substrate bed through bulk flow of gas through the inter-particle spaces. As a result, controlling the bed temperature is more effective compared to strategies that restrict heat and mass transfer within the bed (reducing conduction and diffusion).

2.10.1.4   Water transfer

During SSF, there are four factors, which aid status and water balance inside the system (Nagel *et al*., 2001). These include water level in the hydrolysis step, metabolic water production, uptake of intracellular water during biomass production and metabolic-heat production causing water evaporation (FIGURE 2.14). It is important to keep water content at an optimal level to avoid poor growth in SSF.
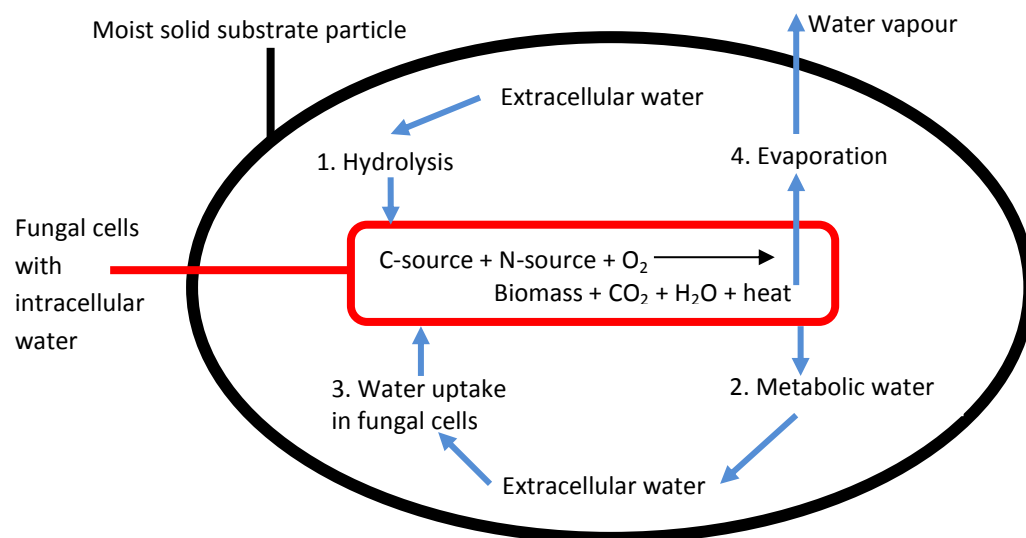
FIGURE 2.14:   A schematic overview of the four different contributions in water balance during SSF (Redrawn from Nagel *et al*., 2001)

Gervais *et al.* (1988) describe water activity as a 'water potential', with osmotic and matric functions. The first function is due to dissolved solutes and the second one is due to capillary forces. Monitoring water activity or moisture content and solute concentration can help to keep the water potential at the right level for SSF. However, water activity decreases with fermentation time. This is due to the

evaporation of water on removing heat of the fermented substrate. At the same time (in almost all cases), heat builds up in the system and causes condensation and therefore water, as can be seen in FIGURE 2.15. This water returns back into the fermentation bed, however this water collects unevenly in the solid substrate and causes irregularly formed areas. In areas of greater water content, the growth of microorganisms becomes disrupted. In practice, a solution for this problem involves controlling temperature by supplying air into the bioreactor which influences water activity in SSF. Usually, cooling air is used and a high amount of air is able to overcome the problem.

FIGURE 2.15: **Water formation in the bioreactor system (a) on the lid, (b) on the wall and (c) inside the bioreactor. Pictures are from the current study**

12.10.2   Macro-scale Phenomena

Macro-scale phenomena describe how effective the design and operation strategy of the SSF bioreactor system are and how they affect conditions in the microorganism's local environment (such as provision for aeration, mixing or agitation and heat removal). According to Mitchell *et al*. (2000), macro-scale phenomena refer to several situations and issues faced by SSF bioreactor systems:

1. The movement of air into and out of the bioreactor can affect how well bulk transport, especially of oxygen, carbon dioxide, water and heat, occurs. Usually, it occurs in the headspace or inter-particle spaces depending on the bioreactor type.

2. If the bioreactor is operated with forced aeration, this can influence how effective bulk transport is, especially inter-particle transport. Dry forced air can cause large moisture losses and drying of the solid substrate.

3. If the bioreactor is operated with agitation or mixing, two important criteria need to be taken into account. First, the solid substrate particles must cope with shear stress and

must not coagulate after agitation or mixing. Second, the microorganisms should be able to cope with shear stress, otherwise mixing or agitation could damage them.

4. Bulk transport could happen as a result of natural conduction, convection, and diffusion.

5. Bulk heat transport through conduction across bioreactor walls to the surrounding can occur.

6. Convective cooling across the bioreactor walls to the surrounding can also occur.

7. Physical properties of the solid substrate such as density, porosity (void space) and stickiness change during fermentation. Also, oxygen can only move by diffusion once it is transferred from the gas phase into the liquid phase within the particle (Mitchell *et al.*, 2011).

2.11 SSF BIOPROCESSING – BASED BIOREFINERY DEVELOPMENT

A wide number of substrates are used for SSF. These include waste products from the agricultural and food industry. There is a need to decrease food and agro-industry processing waste because of environmental and economic problems (Reade *et al.*, 1972). Instead, a better use of raw materials and more efficient processing of food and agro-industry solid materials into added value products are needed (Stabniko *et al.*, 2010). Nowadays, scientists are using SSF to develop new process strategies for biorefineries.

Currently, commercially viable SSF processes involve solid substrates for producing products ranging from biofuels, bio-ethanol, bio-methanol, bio-gas and bio-degradable plastics to commodity, platform and specialty chemicals like succinic acid and pharmaceutical products. This is achieved by microbial bioconversions or enzymatic bio-transformations (Koutinas *et al.*, 2007). Using food and agro-industry solid materials provide almost complete nutrient source (Reed and Nagodawithana, 2001). This is an advantage because during fermentation, solid materials can be used with/without supplementation.

Processes using microorganisms (for use in food and agro-industry waste processing) are very efficient and can be controlled (Cauto and Sanroman, 2006). An example includes using the fungus *A. niger* in bioconversion of apple pomace into a multi-enzyme bio-feed (Zhong-Tao *et al.*, 2009). Another example is making use of agro-industry waste (coconut husks, apple pomace, orange and lemon peels) as these materials can be used as an inert carrier in SSF and allows good microorganism growth (Orzua *et al.*, 2009). As found by many researchers globally, solid materials from food and agro-industry waste could be used as feedstocks for fermentation processes. They can be applied to

biological activities such as producing various enzymes, animal feed, food ingredients, primary and secondary metabolites or nutraceutical and pharmaceutical products.

Emerging technologies have high potential because of applications in producing non-food materials. Examples include bio-ethanol and bio-fuels produced by food (Mahro and Timm, 2007) and agro-industry waste (Kim and Dale, 2004; Fatma and Fadel, 2010), biopulping (Giles *et al.*, 2011; Wall *et al.*, 1993), biological control (bio-insecticides) (Desgranges *et al.*, 1993), biodegradable plastic (poly (3-hydroxybutyrate): PHB) (Xu *et al.*, 2010; Calabia., 2008), and the production of functional chemicals (Tokiwa and Calabia, 2008; Koutinas *et al.*, 2007). It is desirable to use food and agro-industry waste solids as a renewable resource for sustainable chemical and non-chemical production through microbial bioconversions. In recent times, SSF has developed phases created by modern strategies and major compounds used in different industrial fields.
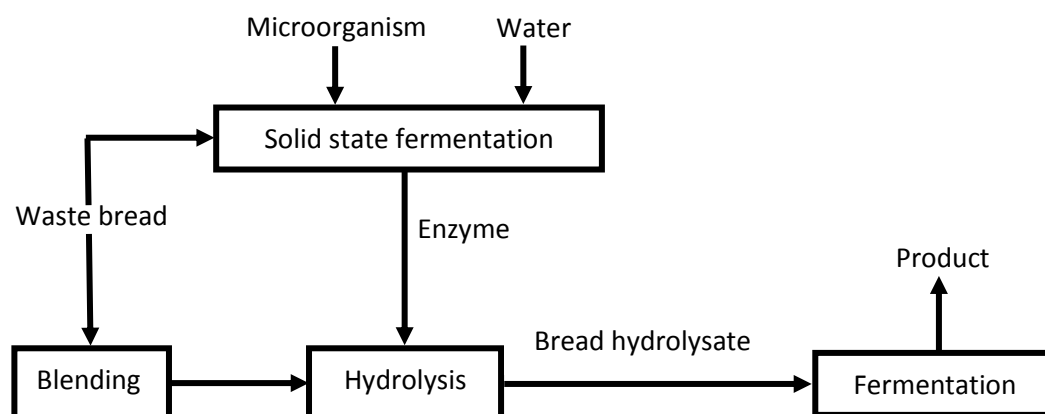
FIGURE 2.16:   **Novel bioprocess for the utilisation of waste bread based on multi-enzyme-producing SSF. Adapted from Melikoglu (2008)**

Biomass from food and agro-industry (through microbial bioconversion either for the production more added value products) has helped in creating more viable biorefineries based on SSF. Generating a generic fermentation feedstock through fungal SSF provides a possibility of using residual solid waste from food and agro-industry. In the Satake Centre for Grain Process Engineering (SCGPE) in the University of Manchester, successful research demonstrated that two filamentous fungi, one producing amylolytic enzymes (*A. awamori*) and the other producing proteolytic enzymes (*A. oryzae*), were both able to grow on various waste solids. Research focusing on the production of hydrolytic enzymes from small portion of waste bread through SSF, and subsequent use of the enzymes to hydrolyse the remaining portion for the production of a nutrient rich hydrolysate, was carried out by Melikoglu (2008) (FIGURE 2.16). As a result of the process, the nutrient rich

hydrolysate taken from both fermented substrates (containing carbon and nitrogen sources), was then afterwards used to support growing a range of microorganisms including yeasts and bacteria.

Within their continuous studies, Du *et al*. (2008) also applied a SSF of *A. awamori* and *A. oryzae* on wheat bran to produce amylolytic-rich and proteolytic-rich solutions for efficient hydrolysis of wheat starch and protein. This produced glucose-rich and nitrogen–rich streams. In the following year (Dorado *et al*., 2009), a wheat bran fraction was used as the only solid medium in two SSF processes of *A. awamori* and *A. oryzae* that produced enzyme complexes rich in amylolytic and proteolytic enzymes respectively.

Recently, research work by Leung *et al*. (2012) reported how a glucoamylase and protease rich multi-enzyme solution was formed from waste bread using the fungi *A. awamori* and *A. oryzae*. The SSF based biorefining presented by researchers in the University of Manchester can be divided into three important steps as shown in FIGURE 2.17: (1) SSF of a selected fungus was carried out on solid waste material to obtain enzymes-rich fungal solids, (2) the fermented solids were subsequently added to a media suspension to produce a nutrient-rich hydrolysate with optimal C/N source and (3) another subsequent bacterial fermentation was carried out using the waste solids hydrolysate for the production of chemicals or targeted products.
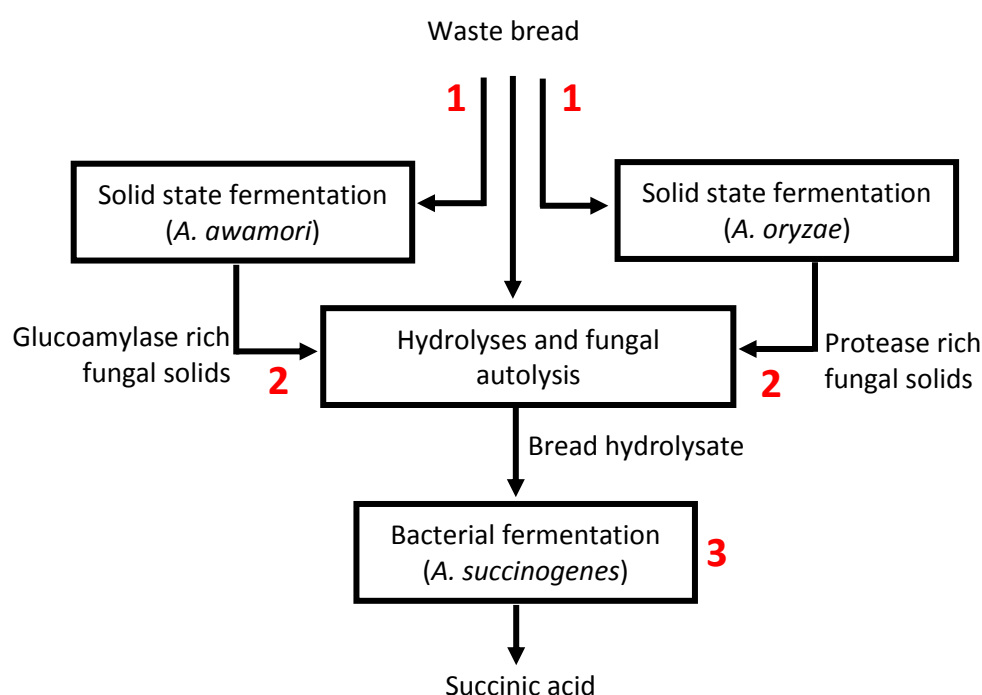
FIGURE 2.17:   **Novel bioprocess based on the production of amylolytic and proteolyic enzymes via SSF followed by subsequent submerged fermentation. Adapted from Leung *et al*. (2012)**

Prior to that, Koutinas *et al*. (2010) designed an integrated biorefinery that produced high quality wheat flour and upgraded the by-product stream into a nutrient-enrichment animal feed and added value chemicals through microbial bioconversions. FIGURE 2.18 shows this process based on the bio-production of succinic acid. One of the processes developed in FIGURE 2.18 used wheat bran in fungal SSF for enzyme production. This was then used in hydrolysis reactions to produce nutrient rich hydrolysates from wheat macromolecules (e.g. starch, protein). The nutrient rich hydrolysate is then converted into the desired products by proper fermentation as shown in FIGURE 2.18.
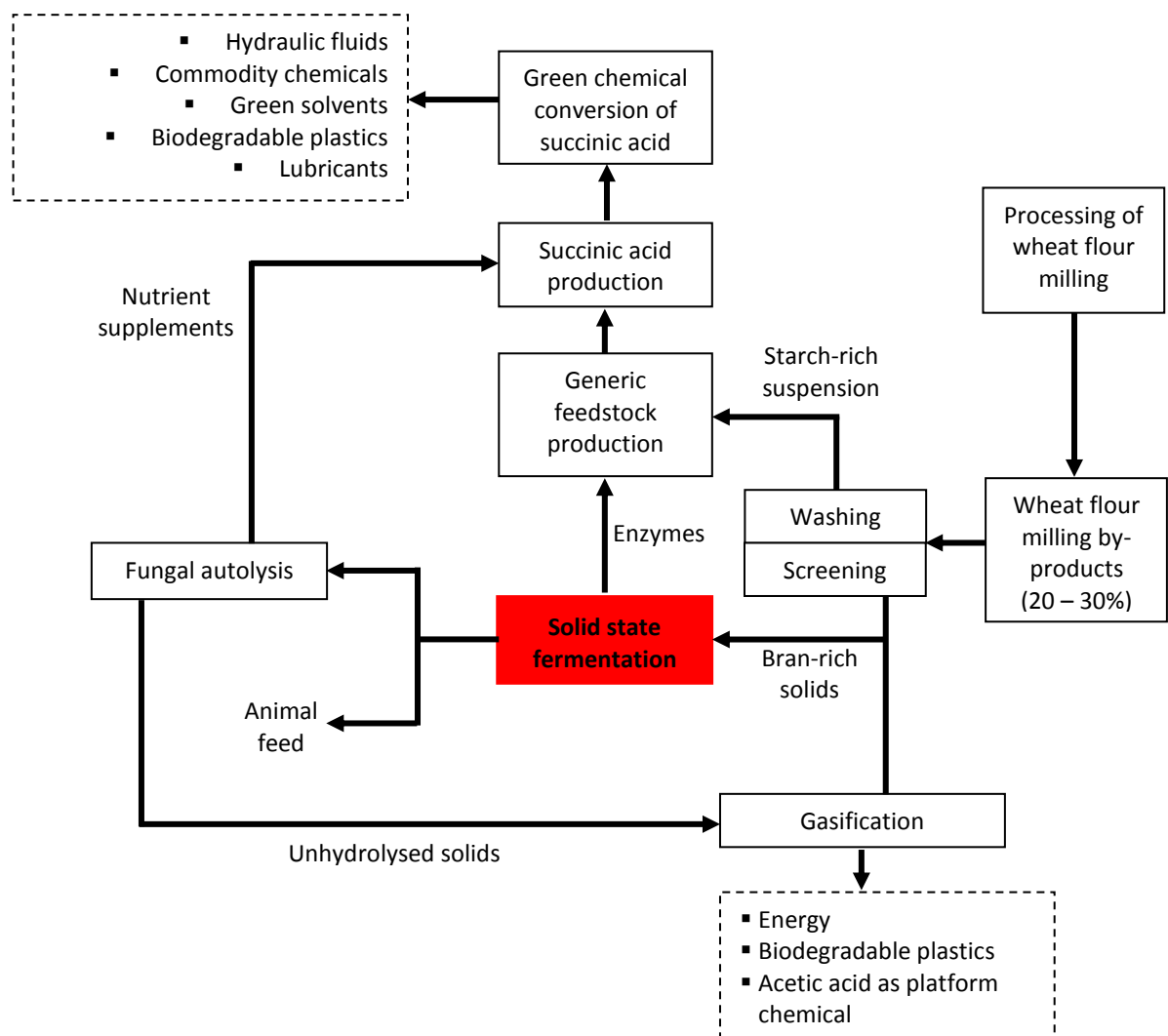
FIGURE 2.18:   Schematic diagram of SSF rules in a possible biorefinery utilising wheat flour milling by-products to produce added value products. Adapted from Koutinas *et al*. (2010)

Another work by Botella (2007) explained how the nutrient rich hydrolysate was produced via SSF of wheat grains using *A. awamori* and followed next by SmF by *W. eutropha* to produce the biodegradable plastic PHB (Polyhydroxybutyrate) and by *S. cerevisiae* for ethanol production.

Salakkam (2012) worked with nutrient rich hyrolysate derived from rapeseed meal via SSF by *A. oryzae* followed by the hydrolysis of fermented solids to produce hydrolysate, which later was used for PHB production through SmF by *C. necator*.

Added value technology through microbial bioconversions technology will have a powerful impact on the food and agro-industry waste processing and creating exciting opportunities for addressing environment issues and economic concerns (Iyayi and Aderolu, 2004). SSF with the use of microorganisms could lower operating costs by eliminating the need to purchase unnecessarily purified commercial enzymatic products (Koutinas *et al*., 2010). In addition, nutrient rich hydrolysates with high glucose (Botella, 2007) and free amino nitrogen (FAN) (Salakkam, 2012 and Botella, 2007) levels were shown to have a great potential to replace more expensive synthetic media.

A potential integrated biorefinery based on food and agricultural industry waste solids can be summarised in the schematic diagram in FIGURE 2.19. At the end of SSF, hydrolysates are mixed with water and could be directly used for subsequent fermentation. However, nutrient complete generic fermentation can be achieved with another step, which is continuing with simultaneous hydrolyses and fungal autolysis. In this step, enzyme activity, such as glucoamylase and protease activities, continues to degrade the solids from the fermented substrates to produce glucose and free amino nitrogen.

Research showed that hydrolysates could either be used separately or in a mixture. Nutrient supplements might need to be added if separate feedstocks are used. With the respect to this, if carbon rich hydrolysates from one microorganism are used, nutrient with nitrogen source should be added. Likewise, if hydrolysates rich in nitrogen from a second microorganism are used, nutrient with carbon source might be added. As a result, the study proved the performance of microorganisms was extremely efficient when both generic fermentation feedstocks were used together in a mixture. This is due to achieving the optimal nutrient media with carbon and nitrogen sources (C/N ratio) to support growth of microorganisms for selected fermentations. This leads to the production of chemicals and of targeted added value products.

It is predicted that SSF technology will appeal to countries, which have a high level of agro-industry wastes, as these can be used as inexpensive raw materials. On top of that, advanced biorefining strategies have been restructured in order to reduce environmental impact, improve overall economics and meet market and societal needs (Koutinas *et al*., 2007). The promising results taken

from this research led to an extension of the study, aiming for greater understanding of SSF technology and the process to produce targeted added value products. It can be concluded that the biomass from renewable resources, such as food and agro-industry waste, has practical benefits and should be further taken advantage of within future biorefining strategies.
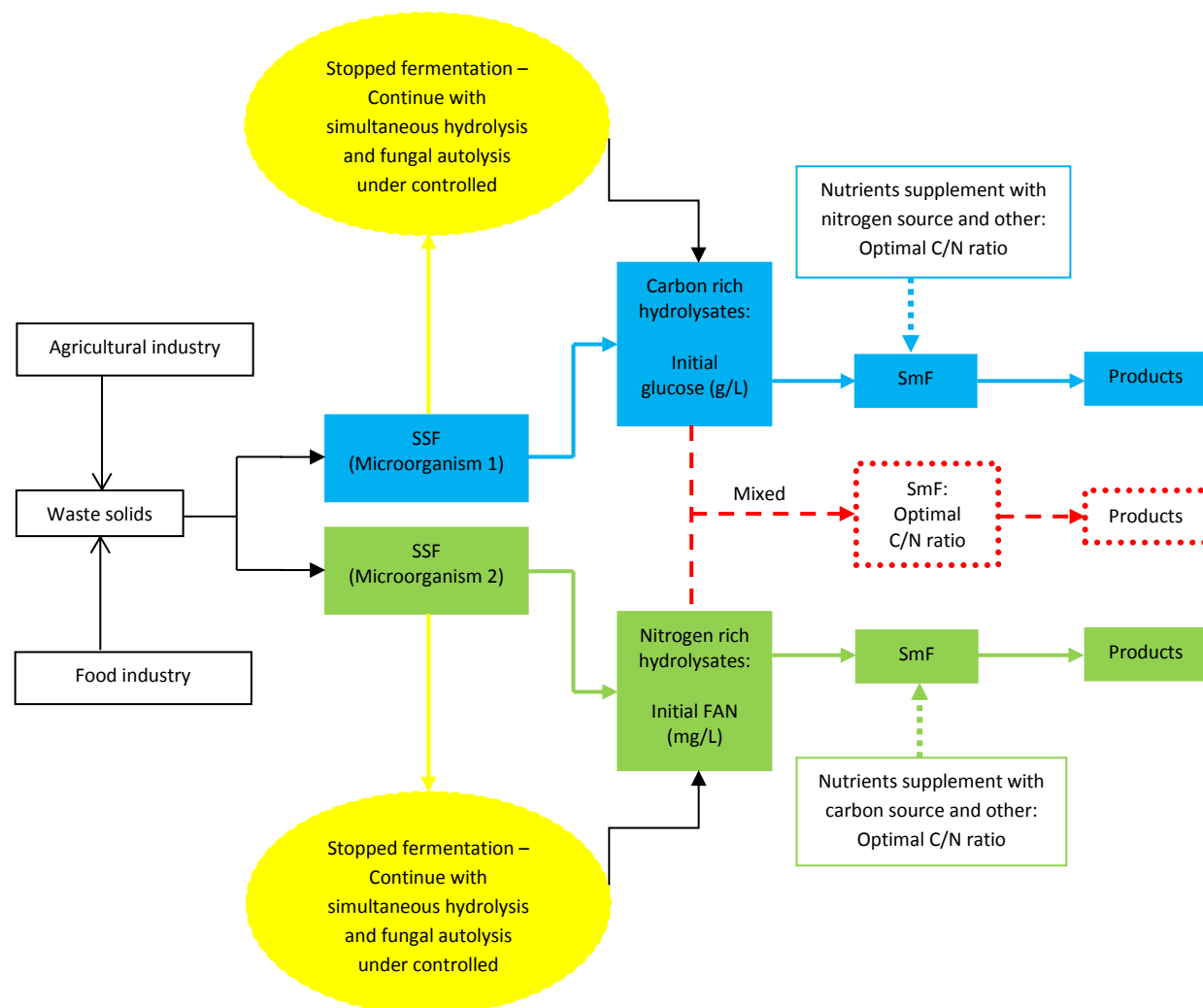
FIGURE 2.19: **Summary of a novel process strategy for biorefineries based on SSF to produce generic fermentation feedstocks**

2.12   CONCLUDING REMARKS

The decade of 1980 – 1990 (TABLE 2.1), witnessed a remarkable growth in research activities on SSF technology for the production of various primary and secondary metabolites and the development of various types of fermenters as well as forming new theories on kinetics and modelling. Since 1990, the entire focus has changed, placing more emphasis on the development

of bioprocess via SSF. This phenomenon includes research on SSF as a tool in biorefinery development for bioethanol and biofuel production. The research on SSF and some of the results have provided a weighty contribution to improving existing and widely used technology. To a certain extent, the types of research activities have increased due to some of the recent research findings. This has created a better understanding of the existing SSF process and provided future development opportunities.

However, it is not possible to carry out a detailed analysis of SSF because the history, research development and scientific investigation of SSF are varied and inconsistent, despite being explored and reviewed time and again by many scientists. SSF bioreactor systems for the production of enzymes and secondary metabolites were previously an important area of development of the SSF process technology. The popularity of SSF technology increased as a result of several advantages and despite its drawbacks. Current research on biorefineries based on SSF has the aim of using generic fermentation feedstock to produce a range of potential added value end products. This later application shows great potential especially in biochemical industries. Such application will lead to a promising future for SSF technology as an integral part of such biorefineries.

Many microorganisms including bacteria and fungi were found to be capable of producing many miscellaneous compounds. The most important is the ability of these microorganisms to degrade waste solids directly. For this purpose, SSF technology has been applied successfully and is gaining momentum rapidly. Another area where SSF technology can be fully exploited is in the manufacture of SSF bioreactor systems with features to suit the specific microorganism used. SSF bioreactors may lead to an industrial feasibility. This in turn presents some bioengineering challenges since a variety of bioreactors need to be designed and implemented for SSF metabolic production. The opportunity to produce new products via manipulation of culture conditions, fermentation process development, media development, bioreactor design coupled with efficient downstream processing and product recovery will be a unique challenge for new areas of SSF technology.

CHAPTER 3

PROJECT OBJECTIVES AND PLANS

"The release of atomic energy has not created a new problem. It has merely made more urgent the necessity of solving an existing one"
Albert Einstein

Despite the fact that there is already much information available in the literature concerning solid state fermentation (SSF), it is always interesting to explore fresh ideas that may provide a better understanding. Literature studies clearly show that SSF offers a great and promising technology for future research and development in industry. Many benefits can be obtained through SSF technology and it can potentially be as advantageous as submerged fermentation (SmF) technology in bioprocessing. Lacks and disadvantages seen with SSF need to be overcome in order to establish SSF technology as a possible solution to treat and process food and agro-industry waste for the generation of added-value products. The growth of modern SSF as a tool in the development of biorefinery processes is necessary to realise more advances especially in bioprocess engineering and bioreactor system design in terms of both theoretical and practical features of this system.

In order to establish SSF in future bioprocess development, certain aspects and challenges need to be considered during research and process development. In this study, SSF has been chosen with the aim to produce new informative data that can be useful to support the technology as an alternative for SmF for the production of certain biotechnological products. In order to exploit the advantages of the different aspects of SSF, which have been discussed in Chapter 2, the development and establishment of a novel process strategy for the production of enzymes through SSF using wheat bran as a model solid substrate was attempted in this research. In selected experiments, soybean hulls and rapeseed meal were also used for the purpose of comparison with wheat bran. At the same time, some of the disadvantages of SSF have been investigated for future bioprocessing of SSF. *Aspergillus awamori* and *Aspergillus oryzae* were chosen as model microorganisms because they are broadly used to produce enzymes and have a long history of safe use in the food industry. In addition, hydrolytic enzymes: glucoamylase, cellulase, xylanase and protease were chosen as model enzyme products for this study.

Before developing specific objectives for the project, it is necessary to critically explore the various advantages and disadvantages of SSF by reviewing the literature. The scope of this study was focused on the development of a process aimed at establishing high performance SSF. From the literature, it is evident that SSF aspects, including proper technique for monitoring growth, inoculum transfer and

bioreactor design, in addition to a strategy for controlling the moisture gradient, oxygen demand, mass and heat transfer and system operation, are all important aspects that need attention in the development of a successful SSF technology. Therefore, the development of future biorefineries for SSF systems must include consideration of all the aspects mentioned above during process design and implementation.

From the above discussion, the experimental work can be divided into the following categories:

- Investigation of various physical variables of the solid substrate, fungi and process that may influence the fermentation outcome.
- Investigation of a new technique for biomass determination using colourimetric measurement as a potentially efficient and reliable technique to describe fungal growth.
- Investigation of enzyme production in lab-scale tray systems, known as closed and opened systems.
- Testing the performance of a novel tray SSF bioreactor system.

Rationally, the optimisation of SSF is dependent on the determination of essential fermentation parameters that are related to fungal growth and enzyme production. In addition to biological parameters, the SSF process is also dependent on physical parameters such as substrate density, porosity, tortuosity and water retention value. The fungus itself with different morphology and different physical characteristics reacts differently during the process of SSF. Such data should also be acquired because optimisation is strictly dependent on both biological ad physical fermentation parameters.

Preparation of the solid substrate and subsequent inoculation are very important because the initial conditions strongly affect the entire SSF process. Usually, it is very difficult to correct the inoculation transfer and there is always an uneven distribution of spores on the solid substrate. Thus, standard procedures for inoculum transfer were developed to obtain satisfactorily homogenous transfer of the spores into the solid substrate particles prior the fermentation process. In addition, a study was carried out in petri dishes to investigate the possibility of incubating *A. awamori* using wheat bran as the sole nutrient. These simple preliminary experiments have a great impact on the whole process, as described in Chapter 4.

Biomass estimation is a very important variable in the control of any fermentation process. However, in the case of filamentous fungi, the mycelium penetrates the solid substrate and is difficult to

separate from it. At the beginning of this study, fungal biomass estimation in the course of SSF using five identified methods was carried out. The first method was capacitance measurement using a biomass monitor, which uses an on-line probe to apply high frequency electrical field to the culture. This allows the measurement of the capacitance of the biomass. The second method was an off-line technique that measures organic matter loss. The third involved dry weight reduction measurement. In addition, other well-known techniques for biomass estimation, including measuring metabolic activity such as oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and temperature evolved during fermentation, were also carried out. Finally, measurement of colour changes of the fermented substrate observed during fermentation was developed as a new technique for describing fungal growth in SSF. The rapid response of the fungi to colour changes allowed the elaboration of certain control mechanisms without resorting to sophisticated analytical techniques. The colourimetric technique is proposed and extensively explored in Chapter 7 and 8 for estimating fungal growth. This technique was also introduced and studied in SmF with solid substrate particles.

SSF has been successfully applied for the production of various enzymes. It has to be verified though that it can be considered as a suitable technology for the production a wide range of enzymes. A thorough literature review revealed that the production of enzymes has received the most extensive attention in worldwide research. As reviewed in Chapter 9, glucoamylase, protease, xylanase and cellulase are studied in two simple tray SSF systems known as closed and opened tray systems. Like most SSF processes, one of the major problems that hinder such an achievement is controlling moisture content and mass and heat transfer. This study provides an idea of how important such parameters as moisture content and mass and heat transfer influence the performance of fungi during the SSF process. Such information enables the design of a better air flow arrangement for closed tray solid state bioreactor (SSB) systems (Chapter 10).

Studies on bioreactor performance identify and discuss current bottlenecks related to the performance of biochemical production processes, in particular with the bioreaction component. Further, they assist in formulating opinions and weighing out advantages and disadvantages of SSF. The studies also explore how developments in modern measurement techniques can be exploited to improve process knowledge. Among the processes available, temperature, oxygen supply and moisture content of the substrate are the most important for the control of SSF. However, there is always an uneven distribution of these physical and chemical conditions in the solid substrate fermented bed. Emphasis is placed on the question of how the transport properties of bioreactors impact process performance. Experiments with selected tray SSB with improved aeration and heat

and mass transfer capabilities on the response of *A. awamori* and *A. oryzae* to environmental conditions are reported out in Chapter 10.

Four tray SSB systems were designed and used for further exploration in this study. Growth of *A. awamori* and *A. oryzae* and enzyme activities (glucoamylase, protease, xylanase and cellulase) were used as example parameters to test the contribution of mycelium to respiration and production of enzymes in real tray systems using wheat bran as a substrate. The optimisation of the fermentation process stage is dependent on the determination of essential fermentation parameters that are related to fungal growth and product formation. At this stage, the study was carried out to investigate the effect of aeration during the fermentation process and the ability to monitor temperature changes (mass and heat transfer), oxygen uptake, carbon dioxide evolution and enzyme production during the growth of *A. awamori* and *A. oryzae*. The evolved heat reduces the moisture content of the solid substrate by evaporation. The mechanisms of mycelium penetration and oxygen transfer into the solid substrate are one of the most significant problems in the analysis of SSF.

Several strategies for air flow arrangement into the system were examined in terms of growth performance. Thus, a control system for SSF in which the temperature and moisture content in the fermented solid substrate were automatically controlled, was developed. Since the heat evolved, OUR and CER are often strongly dependent on fungal growth, an understanding of growth characteristics is essential. Moreover, such understanding is important for rational design of tray SSB systems and process control in SSF. As mentioned earlier, this can be accomplished by finding a method to measure fungal growth throughout the fermentation process. Heat evolved, CER and OUR have motivated extensive studies of biomass estimation. OUR and CER lead to determine respiratory quotient (RQ) that can be correlated with changes in the metabolic activity of fungal growth. In order to analyse the kinetic behaviour of the fungal culture, the raw carbon dioxide evolved, which represents an accumulation term, was integrated with respect to time and modelled using a logistic-like equation.

According to the above-mentioned discussion, specific objectives were formulated in order to establish the process of SSF for future bioprocess development. Hence, the goals of this research were:

1. To obtain a full understanding of the aspects of SSF in order to properly design suitable tray solid state bioreactor systems.
2. To enhance the operational efficiency of solid state bioprocess systems.

Leading to the following objectives:

a. To evaluate inoculation techniques for better homogeneity of transfer to solid substrate particles.

b. To evaluate biological and physical characteristics that influence the SSF process.

c. To evaluate and critically compare techniques for biomass estimation from different perspectives including ease, reliability and how informative the technique is.

d. To select or develop a new technique for biomass estimation based on the outcome of objective (C).

e. To study enzyme production in simple SSF systems.

f. To design a solid state bioreactor system utilising the outcome to the above objectives.

CHAPTER 4

EXPERIMENTAL DESIGN AND RESEARCH PLAN

"Biotechnology is all about life and life-related reactions, so it is necessary to start with some basic information about the types of cellular systems encountered in biotechnology"
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University of Manchester

4.1   INOCULUM TRANSFER PROCEDURES

A standard procedure was developed in these studies to prepare inoculum transfers for the inoculation process into solid substrates. This was done to make sure the spore inoculum distributed homogenously into all solid particles before the fermentation was started.

Firstly, 12.0 g wheat bran, 12.0 soybean hulls, and 20.0 g rapeseed meal were weighed and placed into separate 250 mL flasks before being sterilised at 121 °C for 15 min. The substrates were allowed to cool at room temperature before inoculating with *A. awamori* and *A. oryzae* spores and being moistened with an amount of sterile distilled water to obtain the initial moisture content needed for each experiment. About 1.2×10^6 spores/g substrate were inoculated into the flask and mixed well with a sterile spatula under aseptic conditions to uniformly distribute the spores within the substrate. After mixing well, the content was distributed into petri dishes, tray and tray bioreactor before being incubated at 30 °C for 72 h. At the end of the fermentation period, a sample was taken out for sample analysis.

In order to make sure the spores homogenously transfer into every solid substrate, three strategies for inoculum transfer were performed in this study:

- Technique 1: the spore suspension was transferred into the centre of the solid substrate over the solid surface.
- Technique 2: the spore suspension was distributed on the surface of solid substrate randomly.
- Technique 3: Following the standard procedure described above with homogenous distribution of the spore suspension in the flask before being distributed into petri dishes.

The rationale of the study:

- The first cultivation on a solid substrates solid state fermentation (SSF) system used an inoculum that serves as a starter microorganism to make the fermentation successful.
- Inoculum transfer process in SSF represents one of the important subjects that need to be focused on to make sure the process is successful. Compared to submerged fermentation (SmF) where inoculum preparation is very easy in terms of creating homogeneity within the substrate preparation. The homogenous conditions between inoculum and substrate in SSF sometimes become very difficult to achieve and absolutely will affect the process.

This work was carried out using wheat bran as a model solid substrate while *A. awamori* was used as a model microorganism. Briefly, 12.0 g wheat bran was weighed and put into the 250.0 mL flask before being sterilised at 121 °C for 15 min. For technique 1 and 2, sterile uninoculated moistened wheat bran was distributed into petri dishes. By using a sterile pipette, a spore suspension was transferred onto the solid surface according to strategies 1 and 2 described above. While for technique 3, inoculum transfer was performed inside the flask and mixed homogenously by stirring with a sterile spatula. After mixing well, the content was distributed onto petri dishes. All petri dishes were incubated at 30 °C for 72 h. At defined time intervals (48 h), one petri dish for every technique was taken out for organic matter loss analysis (details are explained in Chapter 7).

FIGURE 4.1 shows an image of growth for comparison of three inoculum transfer techniques. Through visual observation, inoculum transfer upon the cultivation of SSF showed different patterns of fungal growth. For more details on the degree of fungal growth, data from organic matter loss are shown in FIGURE 4.2. Fungal growth occurred where almost 75% substrates were consumed (measured at day 16) when inoculum transfer was homogenously transferred into solid substrate using technique 3. With fermentation using technique 2, almost 50% of solid substrate was consumed and at the end of fermentation, there was solid substrate not utilised. technique 1 promoted poor growth (33%) and growth was observed in the centre and occurred a limited distance from the original inoculation.

The mycelium of fungal growth gives the filamentous fungi the ability to penetrate into solid substrates; however, this process can be influenced by many factors. These include the physical characteristics of the solid substrate, such as particle size and moisture content, which lead to the porosity of the fermented bed.
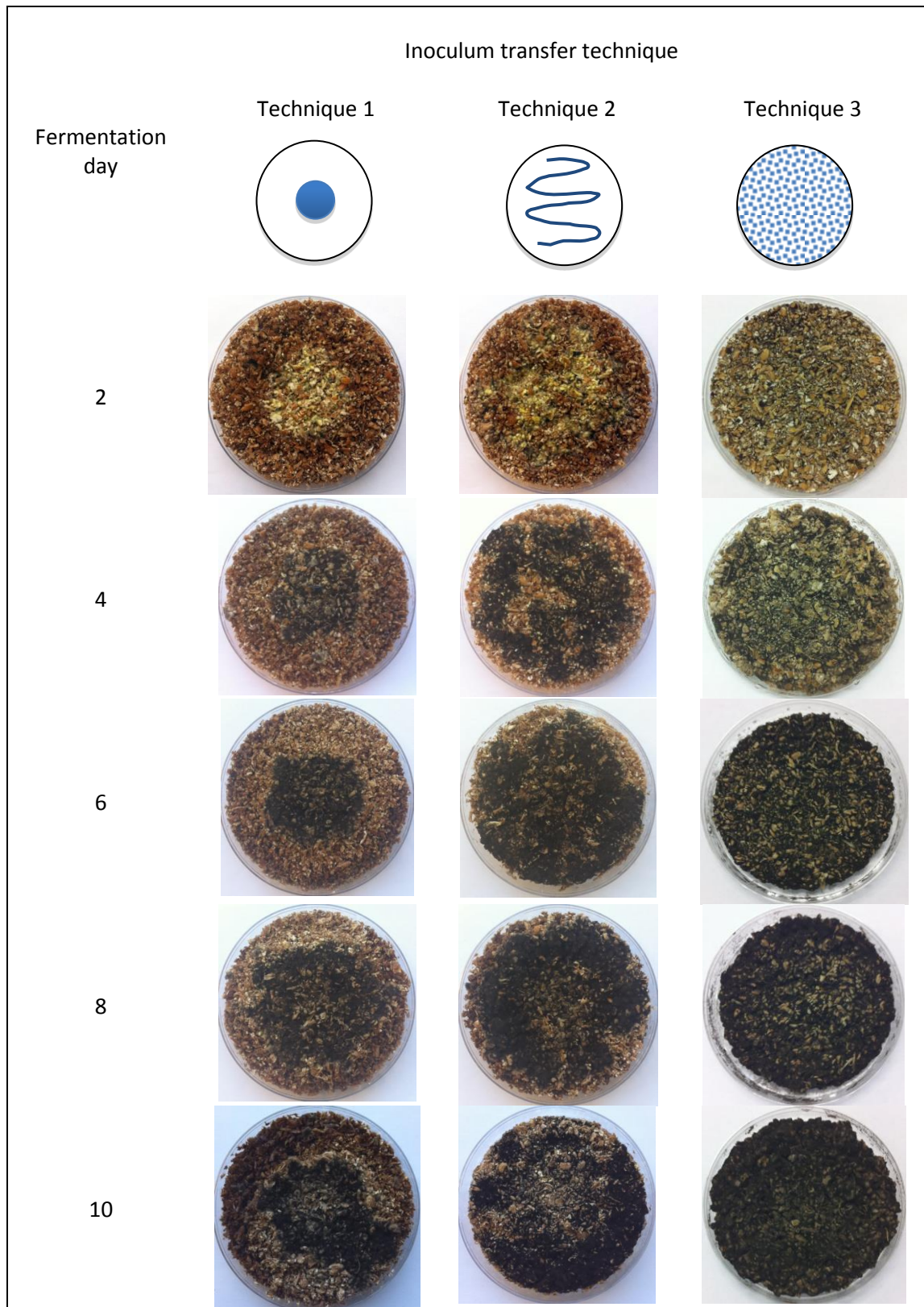
FIGURE 4.1: **Image of the fermented substrate during fermentation process with three different inoculum transfer techniques**
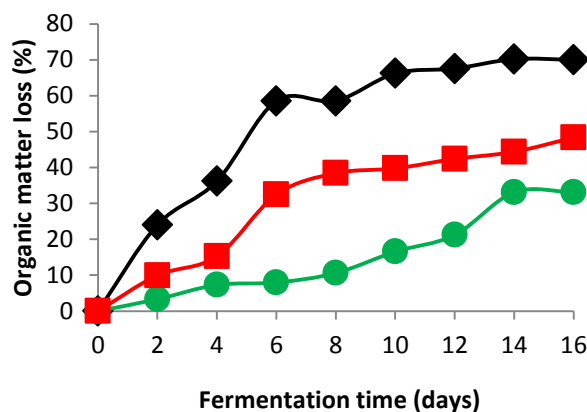
FIGURE 4.2:   Comparison of growth profiles between inoculum transfer strategies into solid substrate based on organic matter loss. (●): technique 1; (■): technique 2 and (◆): technique 3

In SSF, the inoculum process definitely will involve labour intensive due to the necessity to make homogenous conditions especially in large scale. However, this will gives a major advantage for the colonization of the substrate and the utilisation of the available nutrients as shown in FIGURE 4.3. Processing steps in inoculum transfer must be taken into account to obtain satisfying of fungal growth. Generally, this standard procedure applies in small scale laboratory work. In large scale, small reactor with mixing device can be built upon the inoculation process under sterile conditions. Technique 3 will be standard procedures for inoculum transfers for the whole study reported in this thesis.
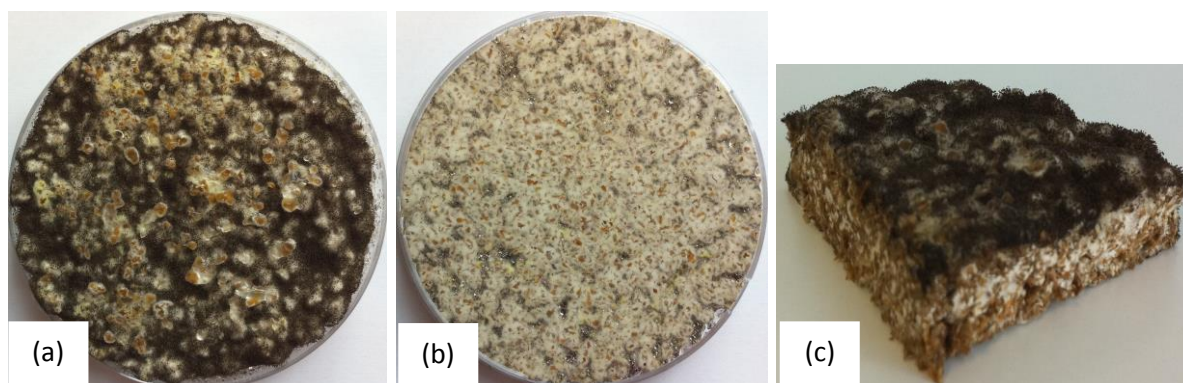
FIGURE 4.3:   Images of fermented wheat bran for technique 3 of inoculum transfer. (a) image taken from the top of the surface of fermented solid; (b) image taken from the bottom of the petri dish and (C) cross section pieces of solid fermented cake

4.2   PETRI DISH EXPERIMENTS

All experiments were carried out in commercial petri dishes, 1.5 cm high and 9.0 cm in diameter. This simple SSF system, which has similarities with tray bioreactors, was filled with the inoculated solid substrate. All the petri dishes experiments were placed in a growth incubator with temperature strictly under control at 30 °C. Several preliminary and micro-scale effects experiments such colour studies, organic matter loss, weight reduction ratio, biomass monitor were carried out using petri dishes. A standard procedure for inoculum transfer was performed as described in Section 4.1 before loading onto petri dishes. At defined time intervals, the fermented substrate on petri dishes was taken out for further analysis.

4.3   BIOMASS MONITORING

4.3.1   Organic Matter Loss

The technique for organic matter loss as described previously by Kumar *et al*. (2003) was followed. Biomass was estimated on the basis of loss of organic matter or dry solid during the course of fermentation. It was assumed that organic matter loss is due to carbon dioxide formation by fungal metabolic activities during the fermentation process. This technique involves a drying process at 80 °C using an oven. The study was performed in petri dishes.

First, a known amount of uninoculated solid substrate was dried and the weight was recorded as the initial dry weight before fermentation. Second, a known amount of solid substrate (should be the same as the uninoculated substrate) moistened with a known amount of water to achieve 65% initial moisture content and inoculated with 1.2×10^{6} spores/g substrate before transfer onto a petri dish. The petri dishes were incubated at 30 °C for 12 days. At defined time intervals, fermented substrate was harvested and dried in an oven overnight to a constant weight, which was recorded as the final dry weight of solid substrate after fermentation. Organic matter loss was then calculated as the weight difference and expressed as a percentage of the initial dry weight of the samples.

$$Organic\ matter\ loss\ (\%) = \frac{W_i - W_f}{W_i} \times 100 \qquad\qquad (4.1)$$

Where;

W_i :   The initial dry weight of solid substrate before fermentation

W_f :   The final dry weight of solid substrate after fermentation

4.3.2   Dry Weight Reduction Ratio

Dry weight reduction ratio is defined as the ratio between dry weight loss to the initial dry weight after subtracting average water evaporation measured in the accompanying non-fermented substrate according to Equation 7.3 (Wang *et al*., 2010). The experiments were carried out in petri dishes. A number of petri dishes were prepared and fermentation was carried out as described in Chapter 4 (Section 4.2).

$$r = \frac{W_i\,(1-65\%)-\,W_t(1-m_t)}{W_i(1-65\%)} - \frac{W_{bi}-W_{bt}}{W_{bi}\,(1-65\%)} \qquad (4.2)$$

Where;

r : The dry weight reduction ratio (%)

W_i : The initial weight of wet solid substrate (g)

W_t : The instantaneous weight of fermented substrate (g)

65% : The initial moisture content of wet solid substrate in both fermented and non-
                 fermented substrate

m_t : The instantaneous moisture content of fermenting substrate

W_{bi} : The initial solid weight of non-fermented substrate (g)

W_{bt} : The instantaneous solid weight of non-fermented substrate (g)

4.3.3   Biomass Monitor

According to Harris *et al*., (1986), capacitance is strongly affected by the presence of viable cells with an intact, electrically insulating, outer membrane envelope. If high concentrations of cells are present in the medium, then the capacitance of the suspension, usually measured in pF will increase (Harris *et al*., 1986). FIGURE 4.4 shows simplified illustrations of a cell with an intact membrane that is a characteristic of viable cells only and the biomass monitor's electric field charging the membrane (http://www.aber-instruments.co.uk/attachments/60).

Macromolecule

Impermeable lipid bi-layer
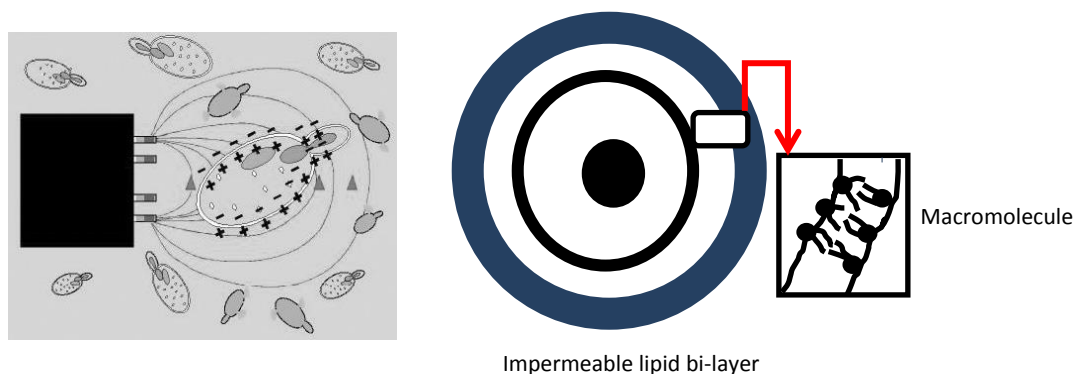
FIGURE 4.4: **Simplified illustrations of a cell with an intact membrane (viable cells only), the biomass monitor's electric field charges the membrane and the principle of using capacitance to measure live biomass. Adapted from** http://www.aber-instruments.co.uk/attachments/60

When a radio-frequency alternating current is applied across a suspension of cells, an electrical polarisation process is set into motion which results in a charge separation across the insulating cell membrane. The cells with their induced dipoles can be compared with tiny capacitors. The charge on the membrane is similar to that found on an electrical capacitor and this principle is used to detect and measure presence of viable spores (Davey *et al*., 1991; Penaloza *et a*l., 1991).

The Procedures

12.0 g wheat bran was weighed and placed into a 250 mL flask before sterilisation at 121 °C for 15 min. The substrates were allowed to cool at room temperature before inoculating with *A. awamori* spores (1.2×10^6 spores/g substrate) and moistening with 40.0 mL of sterile distilled water to obtain 65% initial moisture content. A standard procedure for inoculum transfer was performed as described in Section 4.1 before loading onto petri dishes. All petri dishes were incubated at 30 °C for 16 days. At defined time intervals (2 days), one plate was taken out for capacitance analysis.

The monitor measures capacitance at different frequencies using a probe with four sensors. First, the fermented sample was harvested in every 48 h. One (1) piece of 4 x 4 cm of mycelial blocks of *A. awamori* was transferred into 20.0 mL beaker. 20.0 mL of potassium chloride (KCl; MW: 74.55; Sigma-Aldrich) solution (10.0 g/L) was added into the beaker. By using a spatula, the fermented substrate was homogenously broke and mixed with the salt solution which increases the conductivity during the measurements. The probe (FIGURE 4.5a) is submerged inside a beaker with continuous stirring on a stirring plate to avoid cell precipitation. The probe is then left until the capacitance reading is stable. This system works by measuring the electrical capacitance (pF) of the spore's

suspension at low radio frequencies (0.3 MHz). The reading is recorded manually since this system not equipped with the computer for on-line measurement. As an alternative, sodium chloride (NaCl; MW: 58.44; Sigma-Aldrich) with the same concentration can be used instead of KCl.

Besides SSF, SmF was performed using 500.0 mL shake flask containing 12.0 g whole-wheat flour with a working volume of 150.0 mL distilled water without adding any nutrients. The medium was autoclaved at 121 °C, 15 min. A spore suspension with concentration of 1×10^6 spores/mL was used as inoculum and the flasks were incubated at 30 °C with shaking at 250 rpm. Fermentation was carried out for 12 days. At defined time intervals (24 h), 10.0 mL samples were collected for capacitance measurement (FIGURE 4.5b) following the steps described above. In this experiment, there was no need to add a KCl or NaCl solution since the samples from SmF were already in liquid form. The system was calibrated using baker's yeast suspension with known concentrations prior measuring the capacitance in the fermented substrate solution.
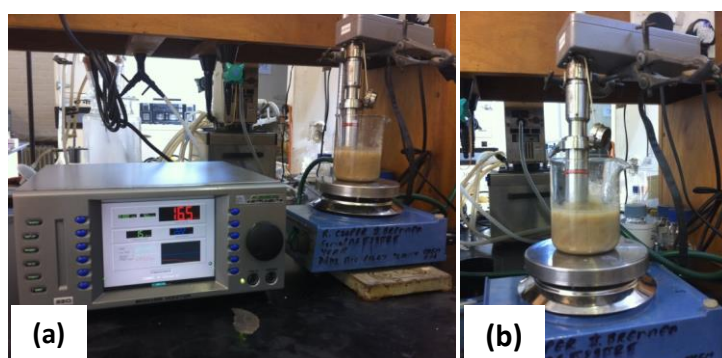
FIGURE 4.5: Biomass monitor

4.3.4 Metabolic Measurements

The consumption of oxygen (O_2) and the evolution of carbon dioxide (CO_2) and heat are directly associated with cellular metabolism and as such could be used as indicators of fungal growth in SSF.

4.3.4.1 Oxygen uptake rate and carbon dioxide evolution rate

Continuous monitoring of O_2 and CO_2 concentrations in the exhaust gas outlet of four tray solid state bioreactors (SSB) were carried out in this study. The tray SSBs used for this study are described in Chapter 4 (Section 4.4). Measurement of O_2 and CO_2 concentrations were carried out using a gas

analyser as described in Chapter 4 (Section 4.4.5). O_2 uptake rate (OUR) and CO_2 evolution rate (CER) (Sukatch and Dziengel, 1987) were calculated using following equations.

Oxygen uptake rate:

$$OUR = \frac{F_1}{V_m \times V_0} \left(X_{O_2(in)} - \frac{1 - \left(X_{O_2(in)} + X_{CO_2(in)} \right)}{1 - \left(X_{O_2(out)} + X_{CO_2(out)} \right)} \times X_{O_2(out)} \right) \tag{4.3}$$

Carbon dioxide evolution rate:

$$CER = \frac{F_1}{V_m \times V_0} \left(X_{CO_2(out)} \times \frac{1 - \left(X_{O_2(in)} + X_{CO_2(in)} \right)}{1 - \left(X_{O_2(out)} + X_{CO_2(out)} \right)} - X_{CO_2(in)} \right) \tag{4.4}$$

Where;

OUR	: Oxygen uptake rate (mole/L.h)
CER	: Carbon dioxide evolution rate (mole/L.h)
F_1	: Air flow rate of inlet gas (L/h) at 1 atm and 30 °C
V_m	: Molar volume of gases = 24.88 L/mole at 1 atm and 30 °C
V_0	: Working volume, solid phase (L)
$X_{O_2(in)}$	: Molar fraction of oxygen at gas inlet
$X_{O_2(out)}$	: Molar fraction of oxygen at gas outlet
$X_{CO_2(in)}$	: Molar fraction of CO_2 at gas inlet
$X_{CO_2(out)}$	: Molar fraction of CO_2 at gas outlet

This equation is based upon the inert gas balance. It is assumed that CO_2 is the only gaseous product of the fermentation (Sukatch and Dziengel, 1987).

4.3.4.2   Heat evolution

A thermocouple K was used to measure the temperature of fermented bed in the four tray SSBs as described in Chapter 4 (Section 4.4.5).

4.3.5   Colourimetric Technique

The experiments were carried out in petri dishes using *A. awamori* and *A. oryzae* for fermentation. Wheat bran, soybean hulls and rapeseed meal were used as model solid substrates. The procedures for substrate preparations and inoculum transfer were described in Chapter 4 (Section 4.2). After the end of the incubation period, the sample was harvested for analysis. About 2.0 g (wet weight) of the fermented samples was extracted with 40 mL distilled water and shaken for 1 h on a rotary shaker (Infors AG-CH4103, Switzerland) at 250 rpm and 30 °C. To make sure of obtaining a very clean extraction solution, first the suspensions were centrifuged using a laboratory centrifuge (Eppendorf Centrifuge 5804, Germany) at 10,000 rpm for 10 min before being recovered by filtration through a filter paper (Whatman No. 1). The solution must be very clean and free from spores and other substances to avoid interference during UV spectrophotometric analysis.

Colour estimation was carried out by measuring optical density at its absorbance maximum expressed as the concentration of colour. The analysis of colour production was done by measuring absorbance maximum of colour extract by spectrum analysis using a Shimadzu UV-VIS 2410PC (Japan) series scanning spectrophotometer.

4.4   TRAY STUDIES

This tray system is another example of laboratory scale tray bioreactor system. Generally, this simple stainless steel perforated base tray test is similar to a petri dish experiment with dimensions: 3.5 cm high and 10.0 cm in diameter (FIGURE 4.6).

FIGURE 4.6: A stainless steel perforated base tray

This system was known as an "opened system" because of the bottom of the tray is made up of a sieve plate or a wire mesh with different apertures to help airflow. The top mouth of the trays is covered with a lid. The trays need to be kept in a sterile environment to prevent contamination. After sterilising, the solid substrate was loaded in every tray with 2.0 cm bed substrate height. A standard procedure for substrate preparation and inoculum transfer was performed as described in Section 4.1 before loading onto trays. All the trays are placed in growth incubator with temperature strictly under control at 30 °C. A suitable space is left between the two trays. In laboratory scale tray bioreactors (petri dishes and stainless steel trays), maintenance of completely aseptic conditions is possible to a certain extent. Low costs and simplicity in construction are two of the main advantages of using this system. However, aeration and agitation are not possible. A standard procedure for solid substrate and inoculum transfer was used as described in detail in Section 4.1.

4.5   TRAY SOLID STATE BIOREACTOR STUDIES

In general, for each SSF process, the most suitable bioreactor system must be designed to give the most suitable environment for growth and to allow metabolic activity to occur. Besides that, the material used to build bioreactors, also play another important role for successful fermentation process. TABLE 4.1 shows the standards of materials used in the construction of bioreactors for SSF that must be taken into account to obtain a proper design. Metabolic activity occurs most efficiently within optimum ranges of environmental parameters, and in SSF processes these conditions must be maintained on a micro-scale so that each cell is equally provided for as already described in Chapter 2.

TABLE 4.1: Standards of materials used in bioreactor design (Adapted from Smith, 2009)

1	All materials coming into contact with the solid substrates entering the bioreactor or the actual microorganism culture must be corrosion resistant to prevent trace metal contamination of the process
2	The materials must be non-toxic so that slight dissolution of the material or compounds does not inhibit culture growth
3	The materials of the bioreactor must withstand repeated sterilisation with high-pressure steam
4	The bioreactor system, entry ports and end plates must be easily machinable and sufficiently rigid not to be deformed or broken under mechanical stress
5	Visual inspection of the medium culture is advantageous, transparent materials should be used wherever possible
6	Cleaning should be easier and therefore this reduces the possibility of contamination and spoilage

The handling of solid substrate in SSF is also particularly important. In contrast to SmF, solid substrate cannot be transferred into a bioreactor system by pumping which can be used with liquids. Therefore, the chance of contamination of solid substrate is very high in solid state bioreactor. In this study, the solid substrate was sterilised in a different Erlenmeyer flask and inoculums can be introduced into sterilised solid substrate before loading onto each tray and then transfer into the bioreactor. All of the work was carried out in a laminar cabinet under sterile conditions. In this study, four different designs of the tray solid state bioreactor (SSB) were implemented. These are:

- Single circular tray SSB
- Multi-stacked circular tray SSB
- Single rectangular tray SSB
- Multi-square tray SSB

4.5.1   Single Circular Tray Solid State Bioreactor

For this bioreactor, the bioreactor chamber is shown in FIGURE 4.7 includes one circular perforated tray with 10.0 cm diameter. The bioreactor was designed to be capable of accommodating a removable tray. The solid substrate sits on the top of perforated tray.
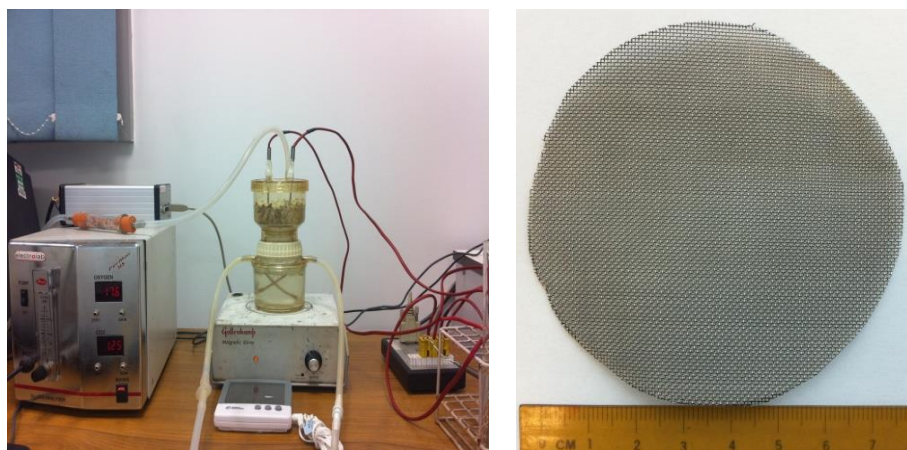
FIGURE 4.7: Single circular tray SSB and circular mesh tray

The internal structure of a bioreactor and the dimensions are illustrated in the scheme shown in FIGURE 4.8. The bioreactor was made of polycarbonate material and compatible with autoclaving. After sterilising, a standard procedure for substrate preparation and inoculum transfer was

performed as described in Section 4.1 before loading into bioreactor with 2.0 cm bed substrate height. In this work, a strategy for the operation of tray bioreactors used for SSF involves a static bed aerated with air moistened by passing through a reservoir of water at the bottom of the bioreactor. The moistened air may be used as a cooling system to remove the heat generated from the microbial activity while at the same time supplying moist air and oxygen in order to improve fungal growth. The moistened air sparges into the bioreactor system according to the strategy explained in detail in Chapter 10. The entire bioreactor is placed in a temperature controlled room at 30 °C. No mechanical agitation used in this bioreactor. A schematic diagram of the experimental set-up in this system is shown in FIGURE 4.9.
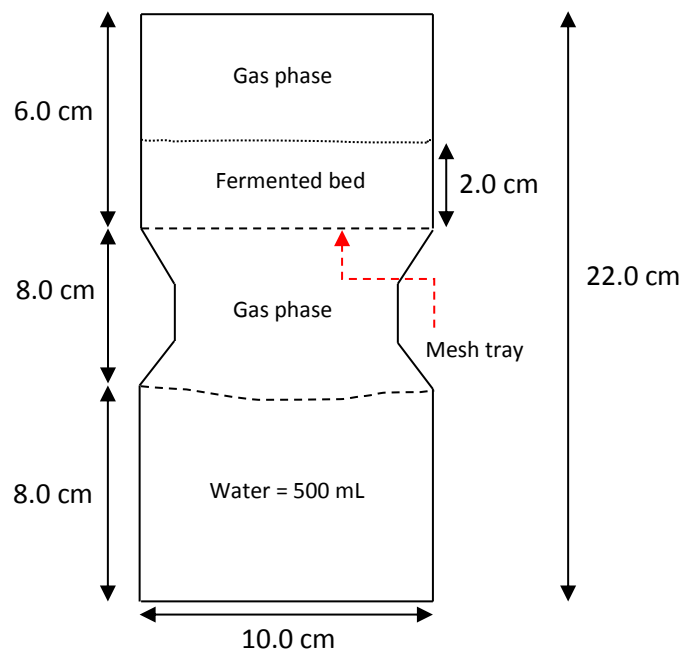
FIGURE 4.8: **A perspective view of a single circular tray SSB from a side view, with emphasis on the location of the tray**
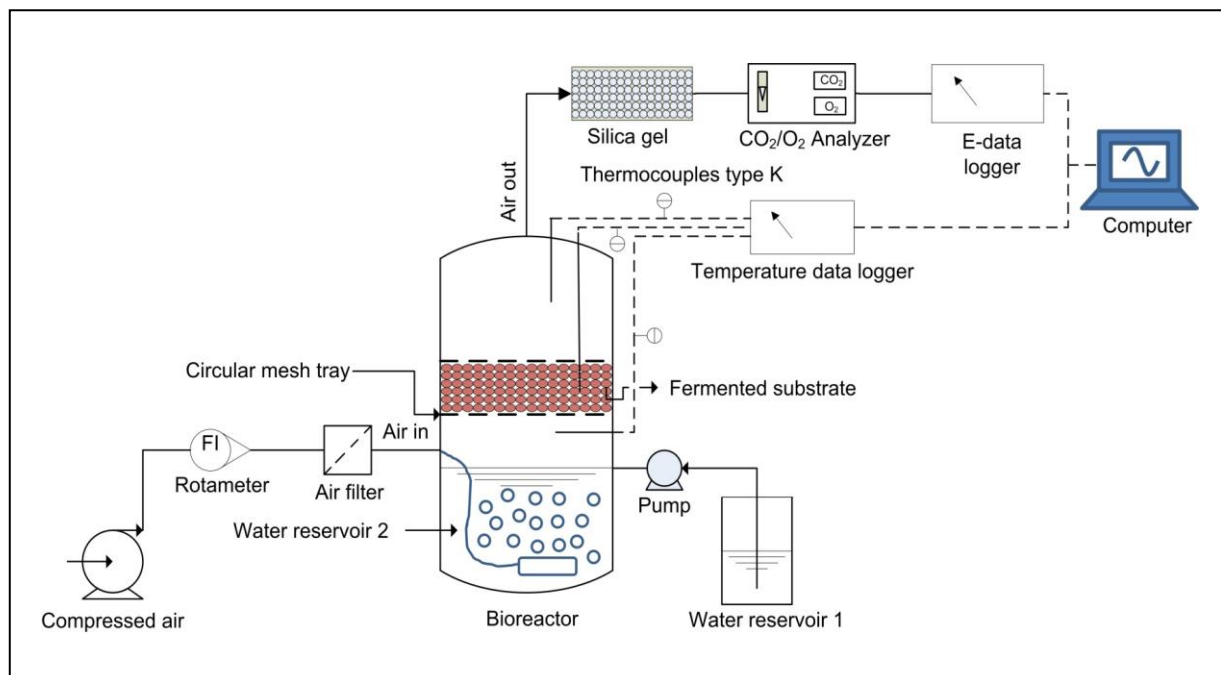
FIGURE 4.9:   A schematic diagram of experimental set-up of the single circular tray SSB

4.5.2   Multi-stacked Circular Tray Solid State Bioreactor
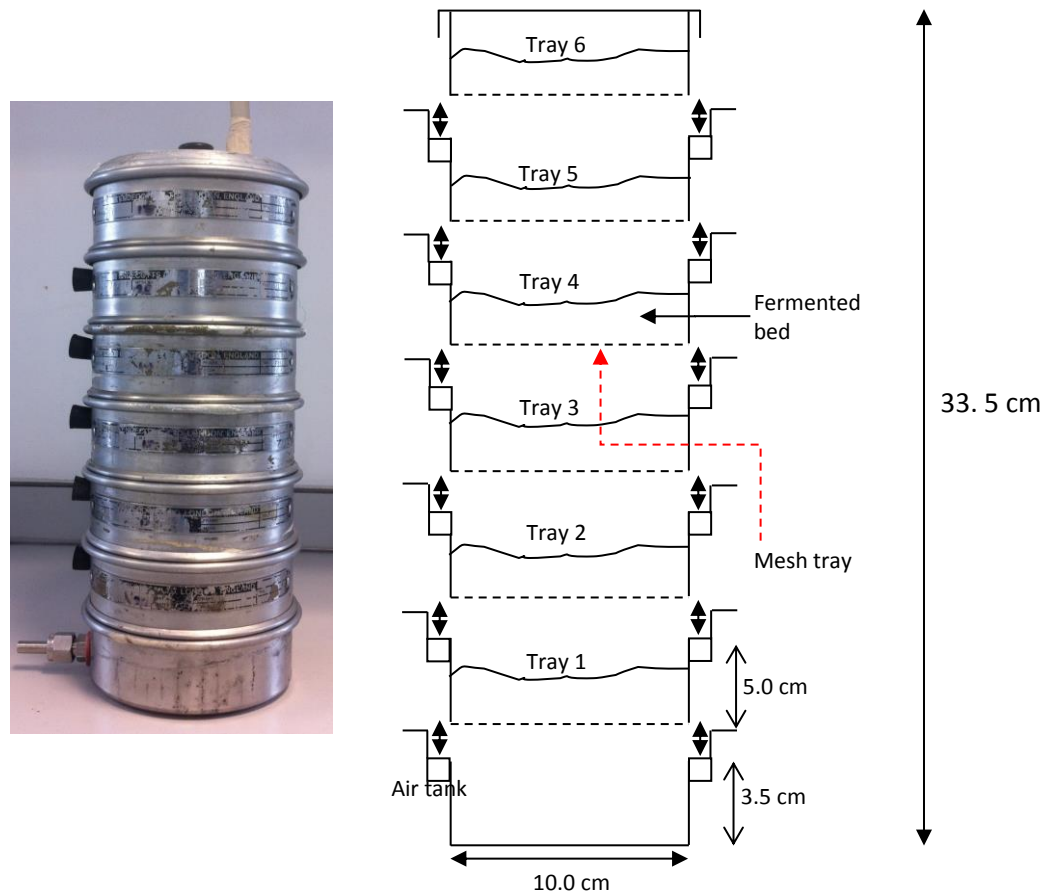
FIGURE 4.10:   Left: A multi-stacked circular tray SSB. Right: A perspective view of a bioreactor according to a first embodiment of the multi-stacked trays, with emphasis on the location of the seals, which are represented by the symbol " □ ", removed and a split tray installed
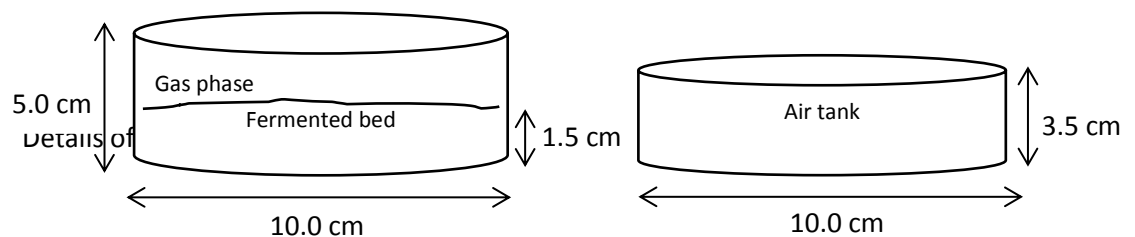
FIGURE 4.11:   Left: A perspective view of an individual perforated base circular tray. Right: The tray at the bottom represents an air distributor at the bottom of the bioreactor

An image of a multi-stacked circular SSB and a schematic diagram of a bioreactor system are shown in FIGURE 4.10. The bioreactor was constructed of multiple individual circular trays that can be fitted on top of one another. As shown in FIGURE 4.10, the bioreactor contains six perforated base trays with 10.0 cm diameter and 5.0 cm height (FIGURE 4.11a) which were tightly stacked one over another and brought the total height to 33.5 cm (FIGURE 4.10). The closed tray at the bottom is as an air distributor with the same diameter and 3.5 cm height (FIGURE 4.11b) and will be referred to as the base tray. The air passes into this base tray before forcing though to another tray by continuously aeration (from bottom to the top). The mounted trays are sealed in such a manner that prevents leakage from the outside to the inside environment and vice versa.

The bioreactor was constructed from stainless steel. A perforated base tray with different aperture sizes (500, 600, 710, 850, 1000 and 1400 µm) was used for uniform distribution of air, as well as to support and prevent the solid particles from flowing through. After sterilising, a standard procedure for substrate preparation and inoculum transfer was performed as described in Section 4.1 before loading onto every tray with 2.0 cm bed substrate height. During fermentation, the moistened air sparges into the bioreactor system (from bottom to top of the bioreactor) according to the strategy explained in detail in Chapter 10. A schematic diagram of the experimental set-up consisting of the multi-stacked circular tray SSB with aeration, control and an on-line monitoring system is shown in FIGURE 4.12.

FIGURE 4.12:   A schematic diagram of the experimental set up of the multi-stacked circular tray SSB

4.5.3   Single Rectangular Tray Solid State Bioreactor

This constructed tray bioreactor is a single rectangular solid state tray, with equal top and bottom height of headspace. The bioreactor itself serves to contain only one tray, while the enclosure assembly serves to provide an enclosed sterile environment for the interior of the bioreactor chamber. The outer casing of the bioreactor is insulated. The enclosure assembly has a chamber lid attached to the main body of bioreactor. The dimensions of a rectangular box in one of the structures are 30.0 cm length, 22.0 cm width and 10.0 cm height. In one structure, the dimensions of the tray are of 22.0 cm length, 13.0 cm width and 1.5 cm height (FIGURE 4.13). The bioreactor was constructed from polycarbonate material. The tray was constructed from Perspex material with a perforated base (150 µm aperture size) that permits operation, sterilisation and high conduction. The enclosure of the bioreactor is of durable transparent material, thereby enabling viewing of the bioreactor chamber contained therein.
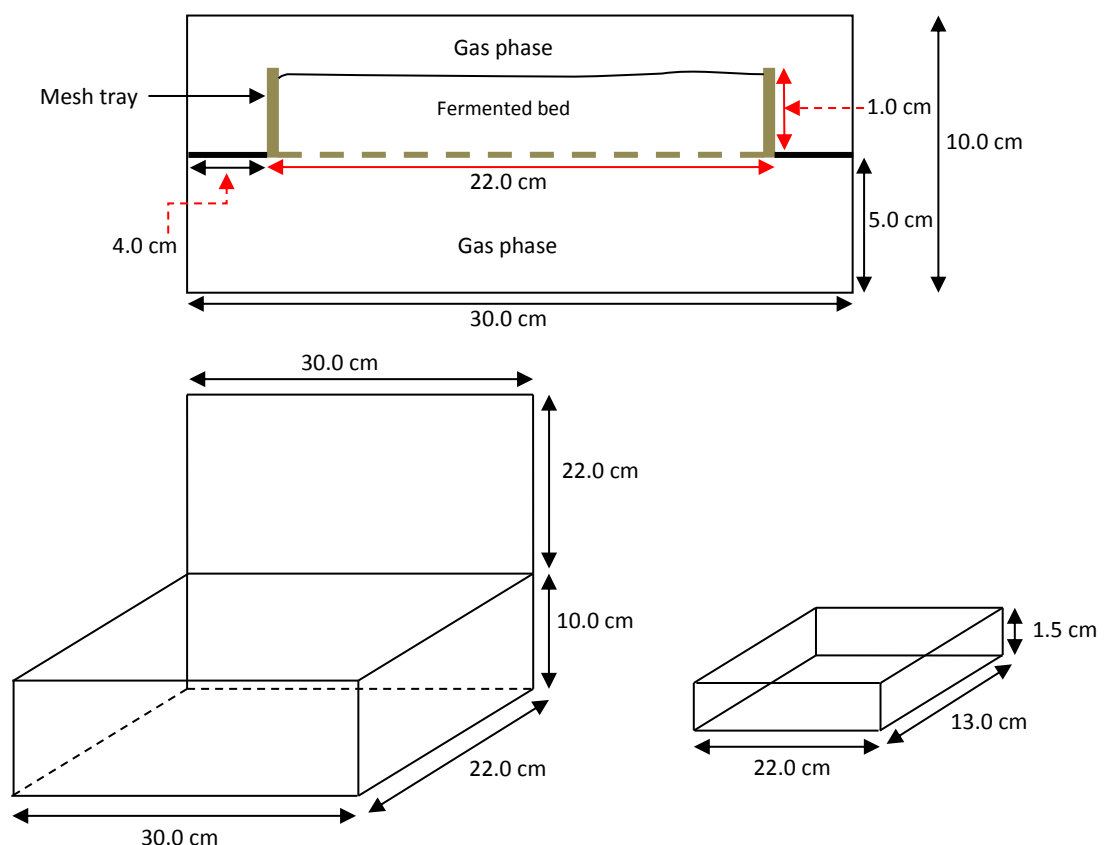
FIGURE 4.13:   A perspective view of a single rectangular tray SSB from the side, with emphasis on the location of the tray

Images of a single rectangular tray SSB are shown in FIGURE 4.14. FIGURE 4.15 shows an image of SSB have ports for air inlets, thermocouples and air outlet to monitor temperature and O_2/CO_2 gas during SSF.
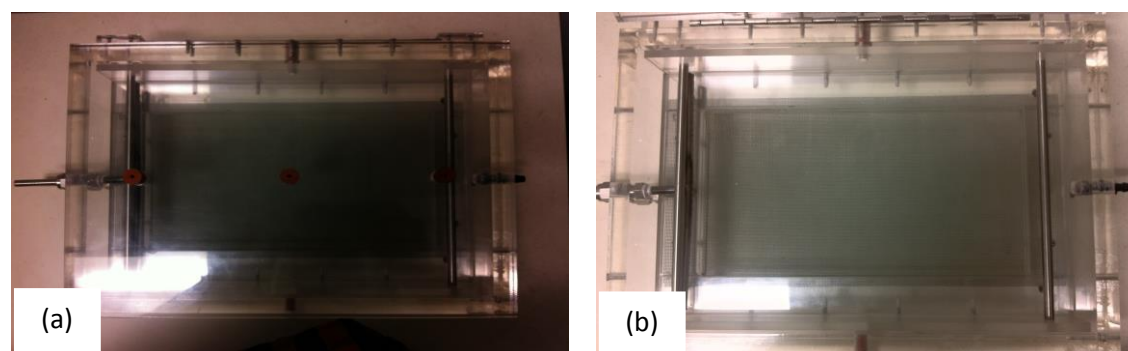
FIGURE 4.14: **(a) view from the top with the lid and (b) view of the inside of a single rectangular tray SSB with emphasis on the location of the tray**
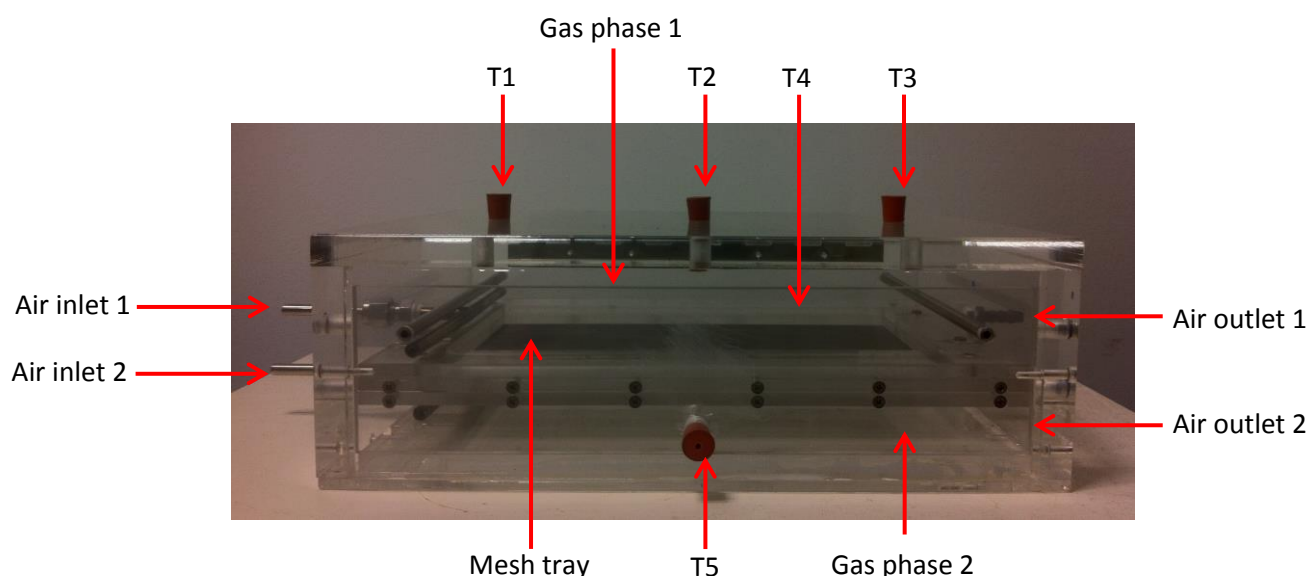
FIGURE 4.15: **A side view with temperature position. T1, T2, T3, T4 and T5 represent the temperatures at area 1, 2, 3, the gas phase over the fermented bed and the gas below the tray respectively**

The bioreactor chamber assembly is configured as a module that can be placed in a growth incubator. The bioreactor was autoclaved for 15 min at 121 °C, prior the operation. After cooling to room temperature, it was filled with sterile inoculated substrate in a laminar flow cabinet under sterile conditions. A standard procedure for inoculum transfer was performed as described in Section 4.1. The bed height at the beginning of the fermentation was approximately 1.5 cm with 60.0 g wheat bran. Moistened air sparges into the bioreactor system according to the strategy explained in

detail in Chapter 10. A schematic diagram of the experimental set-up consisting of the single rectangular tray SSB with aeration, control and on-line monitoring system is shown in FIGURE 4.15.
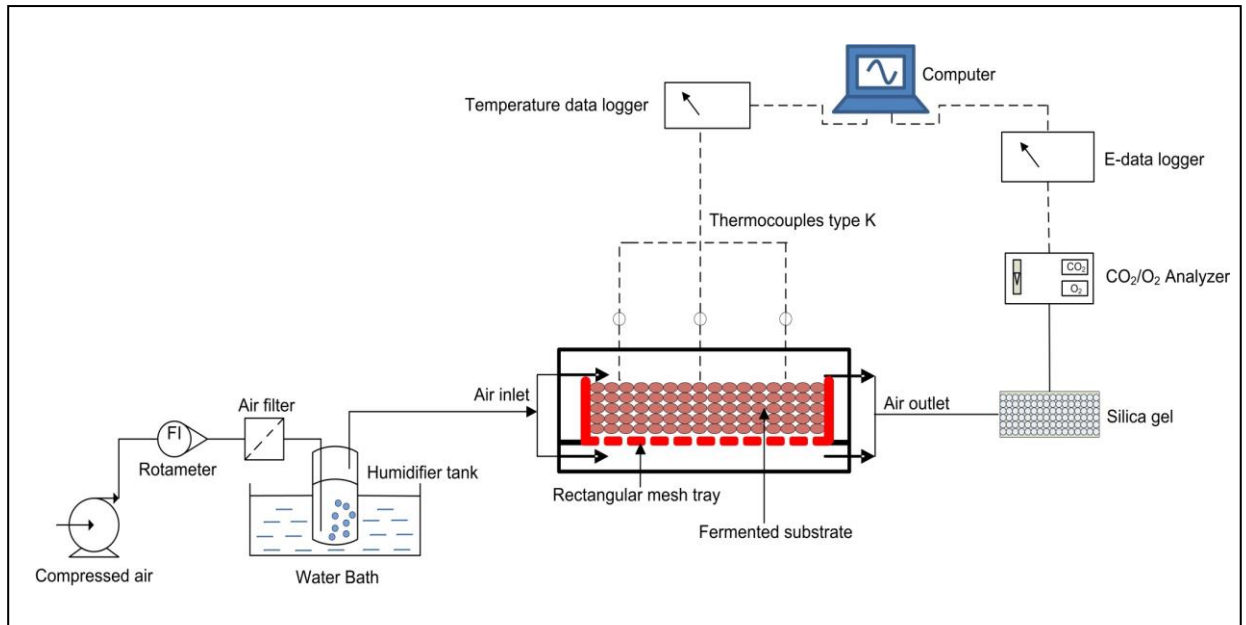
FIGURE 4.15:   A schematic diagram of the experimental set-up of a single rectangular tray SSB

4.5.4   Multi-square Tray Solid State Bioreactor

The bioreactor chamber depicted in FIGURE 4.16 consists of eight identical square perforated base trays arranged one above another. The enclosure assembly has a door is attached to the main body of the bioreactor.
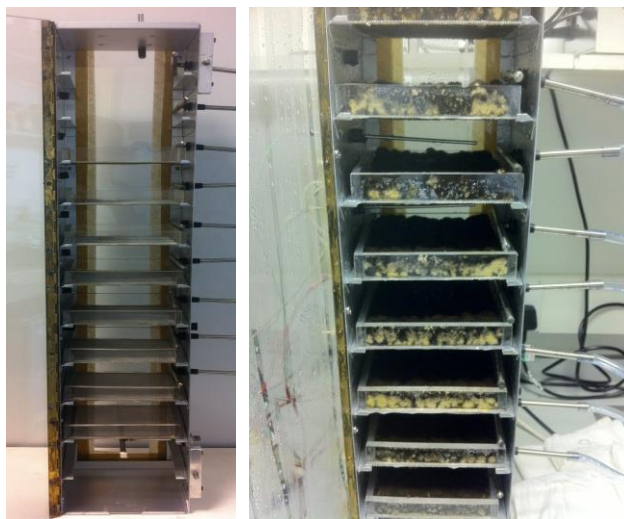
FIGURE 4.16: A multi-square tray SSB

The dimensions of a rectangular bioreactor in one structure are 14.0 cm length, 14.0 cm width and 73.5 cm height (FIGURE 4.17).
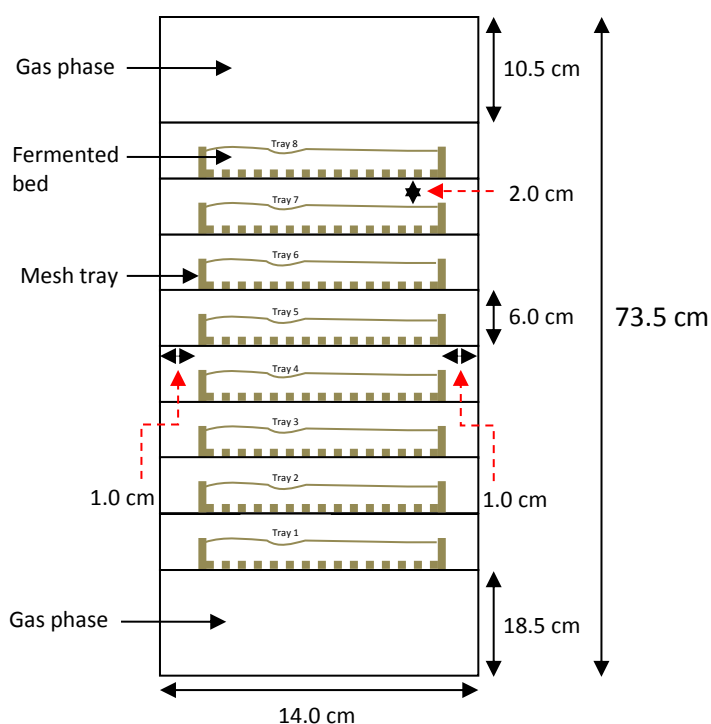
FIGURE 4.17: A schematic of a multi-square rectangular tray SSB viewed from the side, with emphasis on the location of the tray
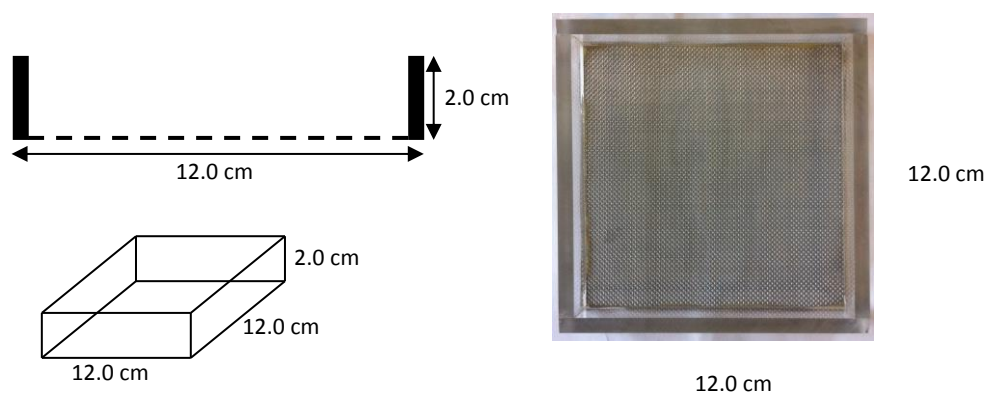
FIGURE 4.18: A square mass tray

In one structure, the dimensions of the tray are of 12.0 cm length, 12.0 cm width and 2.0 cm height (FIGURE 4.18). The main body of the bioreactor made of aluminium while the wall at the back and the door are made of Perspex material. The tray was constructed from Perspex material and equipped with a perforated based with aperture size of 150 μm that permits operation, sterilization and high conduction. The enclosure of the bioreactor is of a durable transparent material, thereby enabling viewing of the bioreactor chamber contained therein).

The bioreactor chamber assembly is configured as a module that can be placed in an autoclave. The bioreactor was autoclaved for 15 min at 121 °C prior the operation. After cooling to room temperature, it was filled with sterile inoculated substrate in a laminar flow cabinet under sterile conditions. Every individual tray can hold up to about 20.0 g of solid substrate with 1.5 cm bed height. The tray medium enters the bioreactor chamber through the door that can later be perfectly sealed to avoid any access from environment into the system and vice versa. Assuming that each tray was filled in the same way from previously prepared and inoculated solid substrate, all trays are therefore under equal conditions. The moistened air sparges into bioreactor system according to the strategy explained in detail in Chapter 10. A schematic diagram of the experimental set-up consisting of the multi-square tray SSB with aeration, control and on-line monitoring system is shown in FIGURE 4.19.

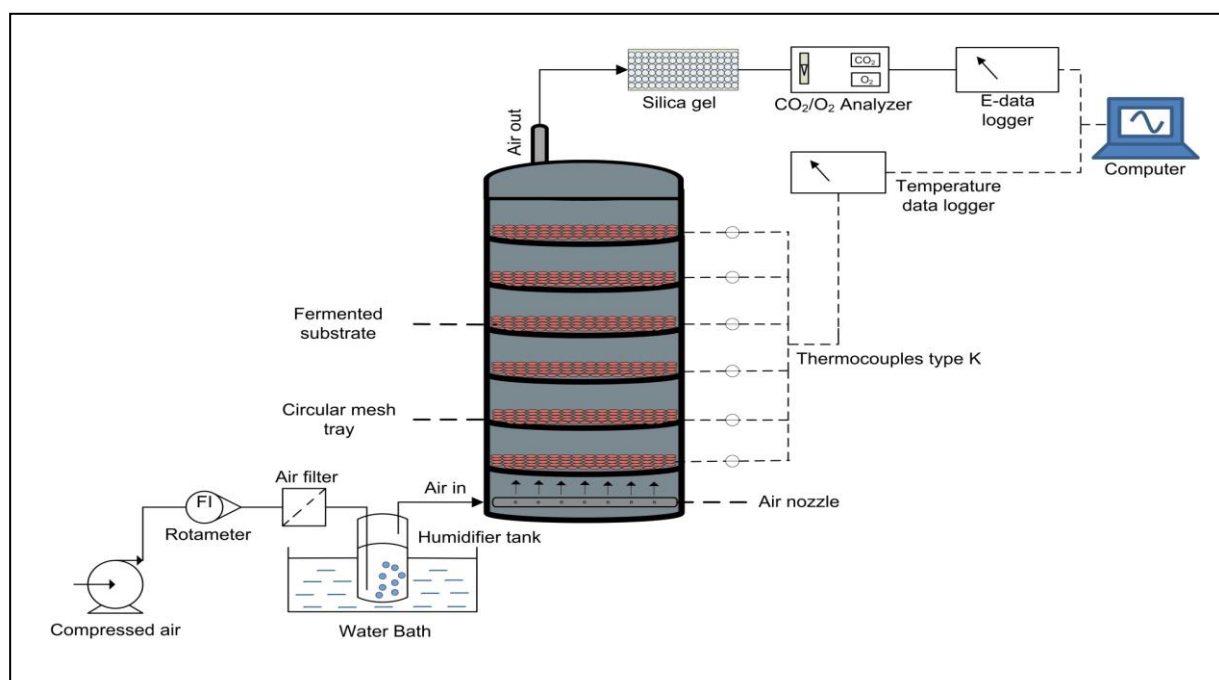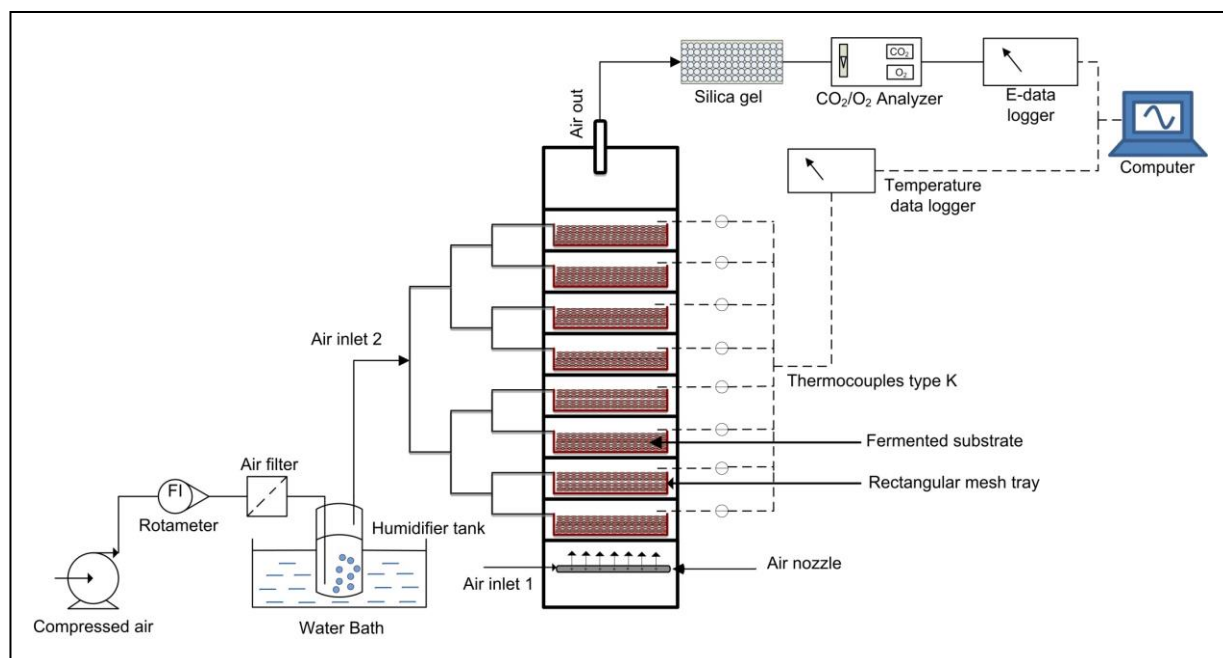**FIGURE 4.19:   A schematic diagram of the experimental set-up of a multi-square tray SSB**

4.5.5 Bioreactor Set Up

The bioreactor and solid substrate were autoclaved at 121 °C for 15 min, prior to operation. In this work, only wheat bran was used as a model substrate with *A. awamori* and *A. oryzae*. The sterilised substrate was inoculated with 1.2 x 10^6 spores/g substrate in another Erlenmeyer flask before loading in the sterile tray before transfer into the bioreactor as described details in Section 4.1. All work was carried out in a laminar flow cabinet under sterile conditions. The bioreactor was then placed in an incubator growth room for 72 h at 30 °C. Sterile dry or moistened air as a fluidising medium was introduced at the bottom or on the surface of the tray from the compressor depending on the air strategies applied to every experiment and explained in detail in Chapter 10.

For moistened air, dry air from the compressor (Air compressor JUN-AIR, USA) was passed through a humidifier chamber before entering the system. The flow rate of the air into the bioreactor system needs to be accurately measured, normally by using a rotameter. Forced aeration at different flow rates is applied to increase accessibility to oxygen. The air was sterilised by passing it through a 0.45 µm cellulose acetate membrane filter and then into humidifier chamber with sterilised distilled water which is monitored in a water bath at 30 °C. In the lower part of the bioreactor, there is another compartment which plays the role of an air distributor before passing through to every tray. The bioreactor is equipped with thermocouples and O_2/CO_2 dioxide analyser. Details operating conditions are described details are shown in Table 2.

Table 4.2: Operating conditions of the solid state tray bioreactor

Parameter	Value
Average wheat bran particle size	494.19 µm
Bulk density of wheat bran	0.37 g/mL
Particle density of wheat bran	1.19 g/mL
Porosity	67.29%
Initial moisture content	65%
Incubator room temperature	30 ± 3 °C
Initial bed temperature	28 ± 3 °C
Inlet air temperature	30 ± 2 °C
Bed temperature sampling time interval	Every 60 min
O_2/CO_2 sampling time interval	Every 60 min
Fermentation period	72 h
Air flow rate	Various

Temperature

Every bioreactor is equipped with a thermocouple type K (Pico Lab Technology, UK) placed at a different point on the surface of the fermented substrate to continuously record the bed temperature over time. All of the on-line data are monitored on screen and recorded using a computer.

Respiratory gases

The system used for quantifying CO_2 and O_2 is able to continuously measure exhaust gas compositions online. An air outlet is situated in the headspace of the highest tray. The exit gas may need to be conditioned (moisture removed) before going into the gas analyser. Therefore, the humid exhaust air from the bioreactor was firstly dried by passing through silica gel tubes before entering an O_2/CO_2 analyser (FerMac 368 Electrolab, UK). This analyser was connected to the Electrolab eLogger data logging and it translates the concentration of O_2 and CO_2 to be recorded using a computer system.

CHAPTER 5

RESEARCH AND ANALYTICAL TECHNIQUES

"Biotechnology is one of the most applied technologies 'microbial-biotechnology' in modern biology"
Prof Ashok Pandey
University of Allahabad, India

5.1   MICROORGANISMS

Aspergillus awamori and *Aspergillus oryzae* obtained from the School of Chemical Engineering and Analytical Science, Faculty of Engineering and Physical Science, University of Manchester are used throughout this study. Fungal spores in universal bottle were stored at 4 °C in agar slopes of solid sporulation medium containing 5% (w/v) whole wheat flour and 2% (w/v) agar (Sigma-Aldrich) as a stock culture and they were sub-cultured in the time interval of every two months. This strain produces compact colonies with huge spores and accumulates large quantities of black and green pigments during growth in the form of mycelium for *A. awamori* and *A. oryzae*, respectively (FIGURE 5.1).
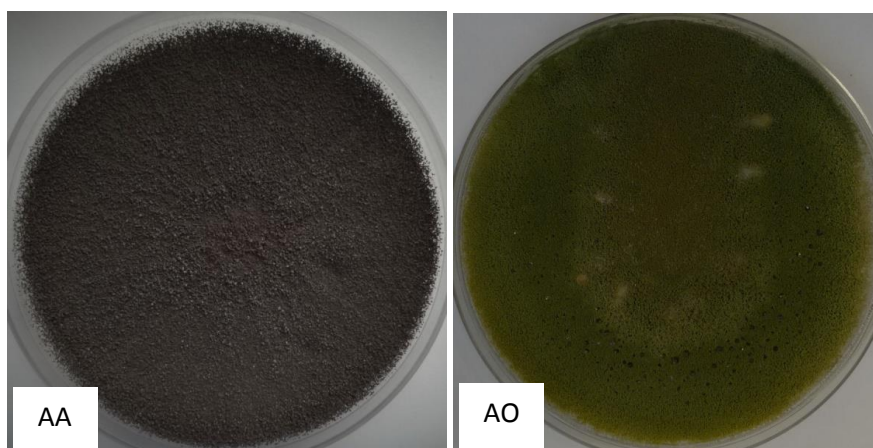
FIGURE 5.1:   *A. awamori* **(AA) and** *A. oryzae* **(AO) on whole wheat flour agar plate. Growth of colonies after 7 days of incubation at 30 °C**

5.2   PREPARATION OF STANDARD INOCULA

5.2.1   Monospore Isolation Technique

One of the important aspects of the development of the fermentation process is the ability and suitability of the microbial strain to be employed. Great care has to be taken to ensure that the strain is pure and produced the desired product at an optimal level. The strain has to be preserved using the right technique to maintain its viability, stability and activity for as long as possible. At first, the strain was improved for its ability by monospore isolation method. The isolation of monospore from the strains *A. awamori* and *A. oryzae* was carried out using the spread plate technique. Through this technique, it is possible to avoid development of heterokaryons which consist of several unstable monokaryons. As a result of the monospore isolation step, a stable pure monokaryon is isolated; this has a better ability to produce targeted products.

First, a small amount of the spore suspension (0.5 mL) obtained from the Morton Laboratory, University of Manchester, was spread on the surface of a 100.0 mL solid sporulation medium [5% (w/v) whole wheat flour and 2% (w/v) agar] in 500.0 mL Erlenmeyer flasks, and the inoculated flasks were incubated at 30 °C for 7 days. Several glass beads (4-mm diameter) and 50.0 mL of sterile 0.1% (v/v) Tween 80 solution (Merck) were added to the flasks to form a stock spore suspension after gentle shaking.

Monospore isolation was performed by serially diluting the fungal spore from 10^1 to 10^9. A 10.0 µL of each serially diluted spore suspension was plated onto a solid medium of 5% (w/v) whole wheat flour and 2% (w/v) agar and incubated at 30 °C for 7 days. The clearly observed single colony was isolated from the plates and sub-cultured by sixteen-streak dilution for three passages. The progeny obtained were sub-cultured on a solid medium of 5% (w/v) whole wheat flour and 2% (w/v) agar and the single colonies or monospores obtained were then transferred to agar slants for spore formation and incubated for another 7 days at 30 °C.

5.2.2   Fungal Inocula Preparation

The spores were washed by lightly scrapping with wire loop in 10.0 mL of sterile 0.1% (v/v) Tween 80. 0.5 mL of the spore suspension was further transferred onto the surface of 100.0 mL of the same sporulation medium in 500.0 mL Erlenmeyer flask and incubated for another 7 days at 30 °C. After

the incubation period, 50.0 mL of sterile 0.1% (v/v) Tween 80 solution and several sterile glass beads (4-mm diameter) were added to the flask. The spores were suspended by shaking the flask gently and collected in one bottle as a spore suspension.

The concentration of the spore suspension was measured by haemocytometer. Spores suspension should be diluted enough so that the spores do not overlap with each other on the grid and should be uniformly distributed as it is assumed that the total volume in the chamber represents a random sample. To prepare the haemocytometer, the mirror-like polished surface is carefully cleaned with lens paper and 75% (v/v) ethanol (Fisher Scientific). The spore suspension is introduced onto the surface using a Pasteur pipette (about 200.0 µL). The cover slip is then placed over the counting surface prior to adding the spore's suspension. The haemocytometer is then placed on the microscope stage and the counting grid is brought into focus at lower power (10X) and then followed with high power at 40X. The spores are counted in selected squares so that the total count is 100 spores or so (number of spores needed for a statistically significant count). The main divisions separate the grid into 9 large squares. Each square has a surface area of 1.0 mm^2. The depth of the chamber is 0.1 mm. Each square of the haemocytometer with the cover slip in place represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1.0 cm^3 = 1.0 mL, the subsequent spore concentration per mL can be determined (FIGURE 5.2).
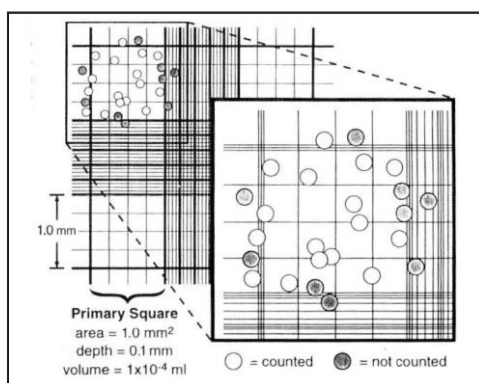
FIGURE 5.2:   **A square under a microscope on how to make a proper spore count with a haemocytometer**

If there are less than 50 or more than 200 spores per large square, the procedure is repeated by adjusting using and appropriate dilution factor. The subsequent spore concentration per mL and the total number of spores is determined using the following equation.

$$Number\ of\ spores/mL\ =\ [average\ number\ of\ spores/grid]\ \times dilution\ factor\ \times\ 10^4 \qquad (5.1)$$

Total spores number will represent spores per mL multiply the original volume of the spore stock suspension from which the spores sample was harvested. The volume of suspension needed for the inoculation of the solid was calculated for each experiment to reach a concentration of around 1.0 x 10^6 spores/g solid substrate.

5.3   SOLID SUBSTRATE

Wheat bran was obtained from Cargill Wheat Processing Plant, Manchester, UK. Soybean hulls and rapeseed meal were obtained from Brocklebank Oilseed Processing Division, Cargill Wheat Processing Plant, Liverpool, UK. All the substrates were kept in airtight container and stored in cold room for future use.

Wheat bran, soybean hulls and rapeseed meal, low cost residues of the milling industry, are an interesting solid substrate for solid state fermentation (SSF). These materials may be seen as a model of cheap and abundant agricultural waste and they have potential in making the entire SSF process feasible.

The composition of wheat bran, soybean hulls and rapeseed meal used in this study are reported in TABLE 5.1. Rapeseed meal is composed of a large proportion of protein (38.9%) followed by wheat bran (at 15.4%) and soybean hulls (at 14.2%). However, wheat bran is particularly rich in starch (23.4%). Soybean hulls are considered to have a much less nutritional and can be classified as lignocellulosic material. Carbohydrates are a major component of soybean hulls (50.7%) represents a structural component constituent in cell walls, including cellulose (36.4%) and hemicellulose (12.5%).

TABLE 5.1: Composition of solid substrate

Component	Wheat bran[1] (%)	Soybean hulls[2] (%)	Rapeseed meal[3] (%)
Moisture	10.3	10.1	10.6
Ash	5.9	4.2	7.5
Crude fibre	8.7	32.3	14.97
Cellulose	10.6	36.4	-
Hemicellulose	29.7	12.5	-
Protein	15.1	14.2	38.9
Total nitrogen	2.4	2.3	6.2
Lignin	2.42	0.75	8.9
Starch	23.3	1.8	4.9
Oil	-	-	2.5
Phosphorus	1.0	0.20	0.8

Adapted from: (1): Lorenzo et al., (2012); (2): Deisy, (2008) and (3): Wang et al., (2010)

5.4   MOISTURE CONTENT

The moisture content of the samples was determined by weight loss after heating to a constant weight at 95°C. Samples were placed in pre-dried and known weight metallic dishes which were immediately weighed. After being dried in the oven for 24 h the samples were cooled for 30 min in desiccators before being finally weighed. Moisture content was calculated as follows:

$$MC = \left[\frac{W_i - W_f}{W_i}\right] \times 100 \qquad\qquad (5.2)$$

Where,

MC :   Moisture content (%)

W_i :   Total weight before drying (g)

W_f :   Total weight after drying (g)

5.5   SPORE COUNTS

About 2.0 g (wet weight) of fermented substrate of *A. awamori* and *A. oryzae* were used to harvest the spores in a 250 mL flask containing 40 mL 0.1% (v/v) Tween 80. The flasks were kept shaking in orbital shaker at 30 °C, agitated at 100 rpm for 30 min. Then the spore suspensions were filtered using a stainless steel sieve with aperture size 45 µm to separate the solid particles. The procedure of spores count was carried out by using a haemocytometer as described in Section 5.2.2 above.

5.6   SAMPLE EXTRACTION

During the fermentation process, a sample was taken at various time intervals for chemical analysis. All the fermented samples (2.0 g on a wet basis) were extracted with distilled water (40.0 mL) and shaken for 30 min on a rotary shaker (Infors A – CH 4103 Switzerland) at 250 rpm and 30 °C. Then the solid suspensions were centrifuged at 10,000 rpm for 10 min (4 °C). The clear supernatant was used for the measurement of pH, total reducing sugar (TRS), free amino nitrogen (FAN), colour and enzyme activity.

A normal observation in SSF is un-even growth on fermented substrate. This situation will usually result in unsatisfied outcome. Therefore, a standard procedure was developed in this study to obtain a satisfactory sampling process. To overcome this issue and to satisfy the need for a homogenous sampling process, an amount of fermented mass was harvested, crashed and vigorously mix using spatula. If the experiments were conducted in petri dishes, the whole fermented mass was blended using a food-processing blender.

5.7   GLUCOSAMINE

The method was adapted from Swift (1973) with slight modification to suit the sample study. The method is based on the fact that glucosamine is a monomer component of chitin, present as acetylglucosamine. Chitin is an insoluble polymer present in the mycelium (Aidoo *et al.*, 1981). The process consists of the polymerisation of chitin; followed by the liberated glucosamine. The principle difficulty with this method is the lengthy analytical procedure.

5.0 mL of 2.0 M hydrochloric acid (HCl; MW: 36.46; FLUKA) was added to 0.1 g of fermented substrate and boiled for 2 h. In this step, it is essential that the tube is stirred vigorously at regular time intervals to ensure complete homogeneity of the fermented mass. To a 1.0 mL of the aliquot, two drops of phenolphthalein ($C_{20}H_{14}O_4$; MW: 318.32; Fisher Scientific) in 0.5% (v/v) ethanol were added. 1.0 M sodium hydroxide (NaOH; MW: 40; Fluka) was added drop wise until the aliquot turned pink. Neutralisation was carried out using 1% (w/v) monopotassium phosphate (KH_2PO_4; MW: 136.09; Fluka) until the mixture became colourless.

The volume was adjusted with distilled water to give a final volume of 5.0 mL. To 3.0 mL aliquot, 1.0 mL of reagent A (1.0 mL of acetylacetone ($C_5H_8O_2$; MW: 100.13; Fluka) - and 50.0 mL of 0.5 M sodium carbonate (Na_2CO_3; MW: 105.99; Fluka) were added and the mixture boiled for 20 min. The mixture was cooled for about 5 min and 6.0 mL absolute ethanol was added followed by the addition of 1.0 mL Ehrlich's reagent; Fluka) (This is a poisonous chemical and should be treated accordingly; reaction of Ehrlich's reagent releases a poisonous gas and work must be performed in fume hood).

The mixture was mixed well and all the tubes placed in a water bath at 65 °C for 15 min. The mixture was then cooled down and absorbance at 530 nm was read against a reagent blank (UA_{540}: unit absorbance at 540 nm). The absorbance of the colour formed should be measured within 60 min of preparation. The concentration of glucosamine from fermented substrate was quantified according to a standard curve of D-(+)-glucosamine hydrochloride ($C_6H_{13}NO_5$.HCl; MW: 215.63; Sigma-Aldrich)

(FIGURE 5.3) which was linear for glucosamine concentration ranging from 0 to 0.2 mg/mL. Glucosamine standard solution was prepared by following assay procedures described above and the glucosamine concentration throughout this study was expressed in units of milligrams per gram material measures on a dry basis (mg/g [db]).
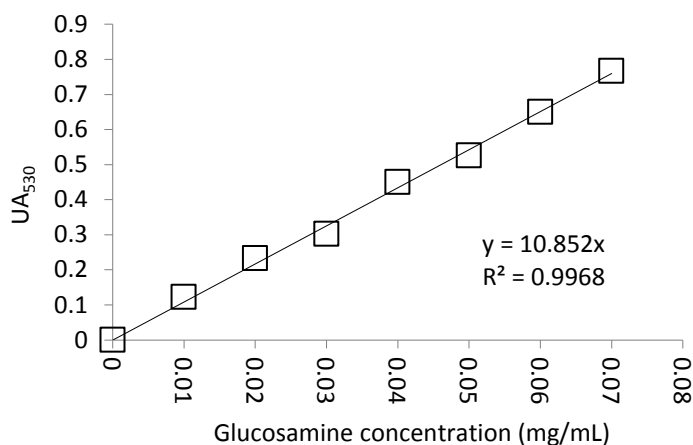
FIGURE 5.3: A standard curve of D-(+)-glucosamine hydrochloride

5.8   TOTAL REDUCING SUGARS

Total reducing sugars (TRS) concentration in the supernatant was measured using the method proposed by Miller (1959). The principle involves the determination of total reducing sugars based on the colour reaction between the reducing sugars and 3,5-dinitrosalycilic acid method (DNS method, Miller, 1959). The reaction yield can be measured as absorbance of the sample at 540 nm using a Shimadzu UV-VIS 2410PC series scanning spectrophotometer (Japan).

Preparation of DNS reagent:
The procedure for sample preparation, analysis and calculation of total reducing sugars is given detail below:

▪ The chemicals in TABLE 5.2 are weight accurately. If necessary, a heater and stir plate are used for constant stirring to aid dissolution. The solution should not be boiled. In this step, it is essential that the liquid be stirred vigorously to ensure complete dissolution of the chemicals. The reagent should be dissolved and mixed well. The volume is then adjusted to 1.0 L with distilled water,

taking into account the original weight of chemical ingredients. Once prepared, the reagent is then kept in an amber bottle at room temperature.

- Preparation of standard solution:

Standard solution – 0.1% (w/v) maltose ($C_{12}H_{22}O_{11}.H_2O$; MW: 360.3; Fisher Scientific)

g maltose is weighed accurately and dissolved in 100.0 mL deionised water in a volumetric flak. A series of dilutions of the maltose solution is made for concentrations ranging from 0 to 1.0 mg/mL.

TABLE 5.2: List of ingredients of DNS reagent

Distilled water:	500.0 mL
3,5-dinitrosalicyclic acid: ($C_7H_4N_2O_7$; MW: 228.1; Sigma-Aldrich)	10.6 g
Sodium hydroxide: (NaOH; MW: 40; Fluka)	19.8 g
Sodium sulphite anhydrous: ($NaSO_3$; MW: 126.04; Fluka)	8.3 g
Phenol: (C_6H_5OH; MW: 94.11; Fluka)	2.0 g
potassium sodium tartrate: ($KNaC_4H_4O_6.4H_2O$; MW: 282.1; Sigma-Aldrich)	306.0 g

Procedures:

0.5 mL broth supernatant from the extraction process is added to test tubes. A dilution is made if necessary. In a separate test tube, 0.5 mL of deionised water is added as a blank. 0.5 mL of DNS reagent is added to each of the test tubes. The tubes were mixed on a vortex mixer and incubated in a boiling water bath for 10 – 15 min. Under running tap water or in a cold water bath, the mixtures are cooled. 4.5 mL deionised water was added to each of the test tubes and the mixtures were mixed on a vortex mixer. The absorbance of each sample was measured at 540 nm against the reagent blank using a Shimadzu UV-VIS 2410PC (Japan) series scanning spectrophotometer and the value was expressed as unit absorbance 540 nm (UA_{540}).

Standard curve:

A series of dilutions of maltose solution corresponding to concentrations ranging from 0 to 1.0 mg/mL was made in test tubes. In a separate test tube, 0.5 mL deionised water was used as a blank. For the rest of procedure, the steps explained above (under procedure) are followed. The TRS in culture filtrate were quantified according to the standard curve of maltose (FIGURE 5.4), which was

linear for maltose concentrations ranging from 0 to 1.0 mg/mL. The TRS concentration was expressed throughout this study in units of milligrams per gram material measured on a dry basis (mg/g [db]).

$$[Total\ reducing\ sugars](mg/mL)\ =\ \frac{UA_{540}}{m} \times D \tag{5.3}$$

Where;

UA_{540} :   Unit absorbance of sample at 540 nm

D :   Dilution factor of broth supernatant (if applicable)

m :   Slope of calibration curve
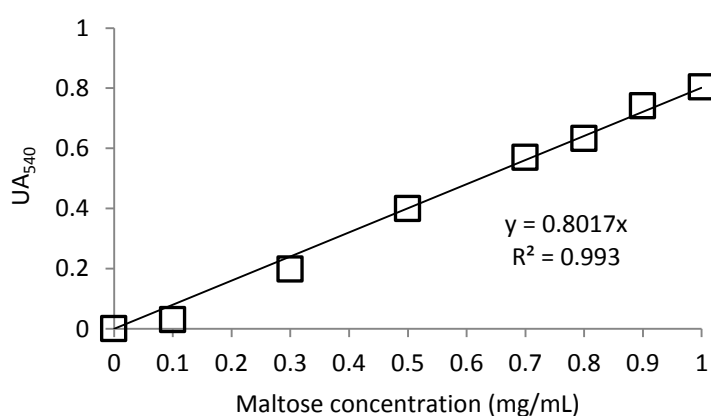
FIGURE 5.4: A standard calibration curve for measuring maltose (TRS) concentration

5.9   GLUCOSE

Glucose concentration in the supernatant was measured using Glucose Analyser (GL6 Analox Instruments Ltd. London, UK). The measurement was based on the reaction between β-D-Glucose from the sample mediated by an enzyme from the enzyme reagent solution (glucose oxidase) to form D-gluconic acid and hydrogen peroxide. The analyser employs an oxygen electrode to measure the change of the rate of oxygen consumption when the sample is injected in the presence of an enzyme reagent solution.

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{D-Gluconic acid} + H_2O_2$$
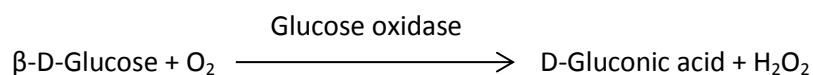
Samples were prepared by centrifugation of the the supernatant at 10,000 rpm for 5 min in order to obtain a very clean supernatant prior to concentration determination using the glucose analyser. In order to measure the concentration of glucose, an appropriately diluted sample (5 µL) was injected

into the reaction chamber of the analyser. The machine was calibrated using 5 g/L standard glucose solution prepared in the laboratory. Lastly, the glucose concentration was expressed throughout this study in units of grams per gram material measured on a dry basis (g/g [db]).

5.10   FREE AMINO NITROGEN

Free amino nitrogen (FAN) is believed to be a good index for potential microbial growth and fermentation efficiency. Adequate levels of FAN in fermentation ensure efficient microbial growth and hence a desirable fermentation performance. FAN concentration was measured using the ninhydrin colourimetric method as outlined by the European Brewery Convention (Lie, 1973) with modifications made by Wang (1999). The method based on the colour reaction between ninhydrin and amino acids at pH 6.7, which gives an estimate of amino acids, ammonia and in addition the terminal alpha-amino nitrogen groups of peptides and proteins.

Ninhydrin colour reagent:

49.71 g di-sodium hydrogen phosphate dihydrate ($HNa_2O_4P \cdot 2H_2O$; MW: 177.99; Fluka); 60.0 g potassium dihydrogen orthophosphate (KH_2PO_4; MW: 136.09; Sigma-Aldrich); 5.0 g ninhydrin spectrophotometric grade ($C_9H_6O_4$; MW: 178.14; Fluka) and 3.0 g fructose ($C_6H_{12}O_6$; MW: 180.16; Fisher Scientific) were weighed and dissolved in 750.0 mL distilled water. The volume was adjusted to exactly 1.0 L with distilled water, taking account of the original weight of chemical ingredients, and the solution was mixed well in a volumetric flask. The pH was adjusted to values between 6.6 – 6.8 by the addition of acid or alkali. This reagent was kept in an amber glass bottle at 4 °C in a refrigerator.

Dilution reagent:

2.0 g of potassium iodate (KIO_3; MW: 214; Fluka) was accurately weighed and dissolved in 600.0 mL distilled water. The volume was adjusted to exactly 1.0 L in volumetric flask by the addition of 398.0 mL absolute ethanol (CH_3CH_2OH; MW: 46.04; Fisher Scientific). This reagent was then kept at 4 °C in a refrigerator.

Glycine stock solution:

0.005 mg of glycine ($C_2H_5NO_2$; MW: 75.07; Fluka) was weighed accurately and dissolved in distilled water in a 100.0 mL volumetric flask to give an accurate concentration of 50.0 mg/L. The solution was kept at 4 °C in a refrigerator.

Glycine standard solution:

A series of dilutions was made for glycine concentration ranging from 0 to 50.0 mg/L.

Procedures for FAN analysis:

Sample preparation:

Whenever necessary, the samples were diluted with distilled water. Usually a dilution factor between 10 – 50 is sufficient. 1.0 mL of diluted sample was introduced into 2 test tubes (to provide duplicate measurements) and then procedure of FAN analysis was followed.

Blank:

A volume of 1.0 mL of distilled water was introduced into 2 test tubes (duplicate) and then the procedure of FAN analysis was followed.

Standard preparation:

A volume of 1.0 mL of glycine standard solution was introduced into 2 tests tubes (duplicate) and then the procedure of FAN analysis was followed.

FAN analysis:

0.5 mL of colour reagent was added to all test tubes prepared as 1 (sample), 2 (blank) and 3 (standard). The test tubes were sealed to prevent evaporation and place it in a boiling water bath for exactly 16 min. He tubes were cooled to room temperature under running tap water. To each tube, 2.5 mL of dilution reagent was added and the tubes mixed thoroughly on a vortex mixer. Absorbance was read for each sample at 570 nm against the reagent blank and the value was expressed as unit absorbance 570 nm (UA_{570}). The colour formed should be measured within 30 min. The FAN concentration in culture filtrate was quantified according to the standard curve of FAN (FIGURE 5.5) which was linear for glycine concentration ranging from 0 to 50.0 mg/L. The concentration was expressed throughout this work in units of milligrams per gram material measured on a dry basis (mg/g [db]).

Expression of results:

The concentrations of FAN in the samples were calculated by:

$$FAN \left(\frac{mg}{mL}\right) = \frac{UA_{570}}{m} \times 2 \times D \qquad (5.4)$$

Where;

Abs_{570}:   Unit absorbance of sample at 570 nm

m: The slope from glycine standard solution

2:      Amount of free amino nitrogen in the glycine standard solution (mg/L)

D: Dilution factor of the sample
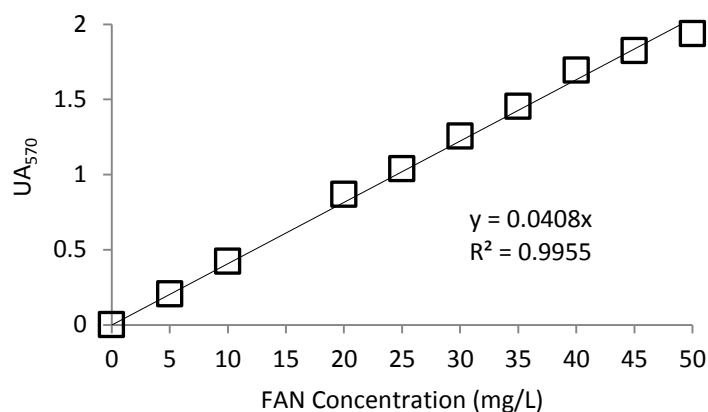
FIGURE 5.5: A standard curve for measuring FAN concentration

5.11   pH

The pH of supernatant obtained after the extraction process was directly measured using pH meter (HANNA Instruments HI 221, UK).

5.12   ENZYMES

5.12.1   Glucoamylase Activity

Glucoamylase hydrolyses of 1,4-linked α-D-glucose residues successively from non-reducing ends of polysaccharides chains and then produces glucose. When the next bond in sequence is 1,4 linked, the enzyme is also able to hydrolyse the α-1,6-glucosidic and α-1,3-glucosidic linkages. Glucoamylase was assayed using the method as described by Ariff and Webb (1996) using maltose as a substrate. The procedures of glucoamylase determination were conducted as follows:

60.0 mM maltose ($C_{12}H_{22}O_{11}.H_2O$; MW: 360.3; Fisher Scientific) was prepared in 0.1 M sodium acetate buffer pH 4.4. 18 mL of substrate was incubated in a test tube at 40 °C for 10 min. A 2.0 mL

sample of diluted supernatant was added and the time was taken as 0 min of reaction. At time intervals (10, 20 and 30 min), 0.5 mL of sample was collected for the measurement of glucose concentration and therefore the degree of substrate hydrolysis. The enzyme reaction in the sample was stopped by adding 0.5 mL of 0.05 M Tris/HCL buffer (Fluka) at pH 9 and then kept in the ice batch until the glucose concentration measurement. Glucose concentration in the reaction mixture was measured by using Glucose Analyser (as described above in Section 5.9). Samples were centrifuged the supernatant at 10,000 rpm for 5 min in order to obtain a very clean supernatant prior to determination of glucose concentration.

Glucoamylase activity was determined by measuring the initial rates of glucose production and expresses as μmole of glucose liberated per minute per mL broth supernatant (μmole min^{-1} mL^{-1}) or unit per mL (U/mL). The method for the calculation of glucoamylase activity is described below. Glucoamylase activity is expressed throughout this study in units of U/g material measured on a dry basis.

Kinetic data and calculation of glucoamylase activity:

Glucose Analyser reading (g/L) = A

Correlation for samples dilution (g/L) = (A x 2) = B

g of glucose in 20.0 mL of reaction mixture=(B x 20) = C

Blank discount (mg glucose/20 mL) = (C – Blank) = D

μmole glucose in 20 mL = (D x 5.5) = E

5.5: The numerator in the equation is derived from the factor for converting the glucose equivalents generated in the assay to 1 μmole of glucose (1/0.18016)

$$Glucoamylase\ activity\ (U/mL)\ =\ \frac{E \times dilution\ factor}{time \times sample\ volume}$$ (5.5)

5.12.2   Protease activity

Protease is involved in digesting long protein chains into short fragments, splitting the peptide bonds that link amino acid residues. During growth on solid substrates, filamentous fungi produce proteases in order to utilise the proteins found in the environment. Protease activity was evaluated by the formation of FAN, which was analysed following the procedure described in Section 5.10.

The amount of proteases for the production of one milligram FAN in one minute under controlled conditions was defined as one unit of activity (U/mL). The procedures of protease determination were conducted as follows:

2.5% substrate solution of casein in 0.2 M phosphate buffer, pH 7.0 was prepared. The suspension was heated at temperature 60 − 70 °C until dissolution. The solution should not be boiled. The reaction tube containing 1.0 mL substrate was incubated at 55 °C, 10 min to maintain a constant temperature in the substrate solution. At time zero, 1.0 mL enzyme supernatant was added into the tube and immediately 0.5 mL of the reaction mixture was transferred into a micro-centrifuge tube containing 0.5 mL 10% trichloroacetic acid ($C_2HCl_3O_2$; 163.39; Sigma-Aldrich) to stop the reaction. Incubation was continued for 30 min with intermittent mixing on a vortex mixer.

At time 30 min, another 0.5 mL of reaction mixture was transferred into a micro-centrifuge containing 0.5 mL 10% TCA to stop the reaction. The micro-centrifuge tubes were centrifuged at 10,000 rpm for 5 min (Mini-centrifuge Hermle Z 160M, Germany). The supernatant was used for FAN determination following the procedure described above (Section 5.10). The amount of proteases for the production of one milligram FAN in one min under controlled conditions was defined as one unit of activity (U/mL). The protease activity throughout this study was expressed in units U/g material measured on a dry basis.

$$Protease\ activity\ (U/mL)\ =\ \frac{(FAN_{30} - FAN_0)}{time\ of\ the\ assay\ (30\ min) \times concentration\ of\ solid\ used\ in\ the\ assay} \quad (5.6)$$

5.12.3   Xylanase activity

Determination of xylanase activity was conducted according to the method developed by Bailey *et al*. (1992). The assay is based on the release of reducing sugars from 1% (w/v) xylan (Sigma-Aldrich) solution prepared in 0.05 M citrate buffer pH 5.4 by 3,5-dinitrosalycylic acid method (DNS method, Miller, 1959) at 50 °C by using xylose to generate a standard curve. The procedures of xylanase determination were conducted as follows:

1% (w/v) substrate suspension of xylan in 0.05 M citrate buffer, pH 5.4 was prepared. The suspension was heated at temperature 60 − 70 °C until dissolution. The solution should not be boiled. 10.0 mL of the substrate solution was incubated at 50 °C, 10 min to maintain a constant temperature in the substrate solution. 5.0 mL of appropriate diluted enzyme supernatant was added. At time intervals

(0, 2, 4, 6, 8 and 10 min), 0.5 mL of reaction mixture was collected for the measurement of xylose and therefore the degree of substrate hydrolysis. The enzyme reaction in the sample was stopped immediately by adding 1.0 mL of DNS reagent. The solutions were mixed well and the tubes were placed in a boiling water bath for 10 – 15 min. These were then cooled under running tap water. 9.0 mL of deionised water was added to each of the test tubes and the tubes were mixed on a vortex mixer.

The absorbance for each sample was measured at 540 nm against the reagent blank using spectrophotometer. A blank solution was also prepared using 1.0 mL distilled water, 0.5 mL of diluted enzymatic extract solution, and 1.0 mL DNS by following procedures explained above. Using the xylose ($C_5H_{10}O_5$; MW: 150.13; Sigma-Aldrich) standard curve (0 – 1.0 mg/mL) the amount of xylose released for each samples was determined.

The xylanase activity was expressed throughout this work in units of U/g material measured on dry basis. One unit of xylanase activity was defined as the amount of enzyme producing 1 µmole xylose equivalents per minute under assay conditions.

Standard curve:

A working stock solution of D(+)-xylose ($C_5H_{10}O_5$; MW: 150.13; Sigma-Aldrich) at 1,000.0 µg/mL was made up. A series of dilutions are made from the working stock within the range 1 to 1,000.0 µg/mL. In a separate test tube, 0.5 mL of deionised water was used as a blank. Further steps were carried out following the procedure explained above. The TRS were quantified according to standard curve of xylose, which was linear for xylose concentration ranging from 1 to 1,000.0 µg/mL (FIGURE 5.6).
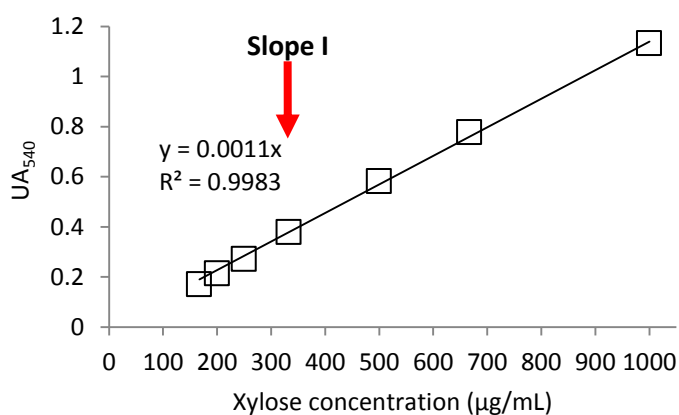
FIGURE 5.6: A standard curve for measuring xylose concentration

Enzyme activity was constructed by plotting time (min) against unit absorbance value (at 540 nm) obtained from enzyme analysis for every each sample and the slope was obtained from the curve as shown in FIGURE 5.7.
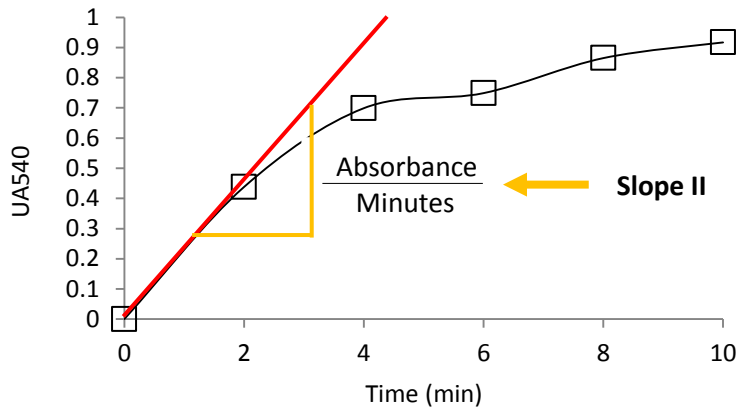
FIGURE 5.7: A graph of xylanase enzyme reaction

$$Xylanase\ activity\ (U/mL) = \frac{Slope\ II}{Slope\ I} \times \frac{1\ \mu mole}{150.13\ \mu g} \times \frac{total\ volume\ of\ reaction\ mixture\ (mL)}{Enzyme\ solution\ volume\ in\ assay\ (mL)} \qquad (5.7)$$

Slope I = $\dfrac{UA_{540}}{Xylose\ concentration\ (\frac{\mu g}{mL})}$ (From xylose standard curve) (FIGURE 5.6)

Slope II = $\dfrac{UA_{540}}{time\ (min)}$ (FIGURE 5.7)

Total volume reaction = 15.0 mL

Enzyme volume = 5.0 mL

5.12.4   Cellulase activity

Filter paper cellulase activity was measured according to IUPAC recommendations (Adney and Baker, 2008) employing filter paper Whatman No. 1 as a substrate (Ghose, 1994). The procedures were designed to measure cellulase activity in terms of "filter paper units" (FPU) per millilitre (mL) of original (undiluted) enzyme solution. Quantitative results of the enzymes preparations must be compared on the basis of significant and equal conversion.

The value 2.0 mg of total reducing sugars as glucose from 50.0 mg of filter paper in 60 min reaction time was designated as the intercept for calculating filter paper cellulase units (FPU) by IUPAC. The

total reducing sugars was then determined by the DNS method (Miller, 1959). The cellulase activity was expressed throughout this study in units of FPU/g material measured on a dry basis.

Procedures:

The substrate is a 50.0 mg Whatman No. 1 filter paper strip (1.0 x 6.0 cm). A rolled filter paper strip was placed into each glass test tube. 1.0 mL of 0.05 M sodium citrate buffer, pH 4.8 was added to every test tube. Filter paper should be submerged and saturated inside the buffer. The tubes were incubated at 50 °C in a water bath for about 10 min to maintain a constant temperature in the substrate solution.

At the same time, a blank is prepared which contains 1.5 mL sodium citrate buffer. 0.5 mL enzyme solution diluted appropriately was added into the substrate-buffer solution. At least 2 "enzyme dilutions" from the original enzyme solution were prepared. The tubes were mixed well and placed in a water bath at 50 °C for exactly 60 min. At the end of the incubation period, each tube was removed from the water bath and 3.0 mL DNS reagent was added immediately to stop the enzyme reaction. The tubes were mixed on a vortex mixer.

In addition to the assay tubes, blank and control tubes were prepared:

- Reagent blank: 1.5 mL citrate buffer
- Enzyme control: 1.0 mL citrate buffer + 0.5 mL enzyme dilution (a separate control was prepared for each dilution tested)
- Substrate control: 50.0 mg filter paper strip + 1.5 citrate buffer

Preparation of glucose standard solution:

A working stock solution of anhydrous glucose ($C_6H_{12}O_6$.; MW: 180.16; Fisher Scientific) (10.0 mg/mL) was made up. A series of dilutions were made up for glucose concentration ranging from 0 to 10.0 mg/mL. Glucose standard tubes should be prepared by adding 0.5 mL of each glucose dilution to 1.0 mL sodium citrate buffer. Reagent blank, enzyme control, substrate control and glucose standards were incubated along with the enzyme assay tubes at 50 °C and the procedure was completed by adding 3.0 mL of DNS reagents. The tubes were mixed on a vortex mixer.

Colour development:

All tubes were boiled for exactly 15 min in boiling water. After boiling, the tubes were cooled under running tap water or in a cold water bath. Tubes were kept until all the pulps have settled. 0.2 mL of

colour-developed reaction mixture was pipetted and 2.5 mL distilled water was added in a spectrophotometer cuvette.

The absorbance for each sample was measured at 540 nm against the reagent blank using spectrophotometer. Using the glucose standard curve (FIGURE 5.8), the amount of glucose released for each of the samples was determined after subtraction of values obtained for the enzyme control and substrate control. The concentration of enzyme, which would have released exactly 2.0 mg of glucose, was estimated by means of a plot of glucose liberated against the enzyme concentration (enzyme dilution).

Enzyme concentrations for all samples = 1/dilution

= 1/2 and 1/5 (= 0.5 and 0.2)

Enzyme concentration for original supernatant = 1

The term "enzyme concentration" refers to the proportion of the original enzyme solution present in each enzyme dilution (i.e., the number of mL of the original solution present in each mL of the dilution):

$$\frac{2\ mg\ glucose/0.18016\ mg\ glucose\ \mu mole^{-1}}{0.5\ mL\ enzyme\ \times 60\ min} = 0.37\ \mu mole\ min^{-1}mL^{-1} \tag{5.8}$$

0.37: The numerator in the equation is derived from the factor for converting the 2.0 mg of "glucose equivalent" generated in the assay to mmoles of glucose (2.0/0.18016). The denominator is the volume of the enzyme being tested that is used in the assay (0.5 mL) and from the incubation time (60 minutes) required for generation of the reducing equivalents.
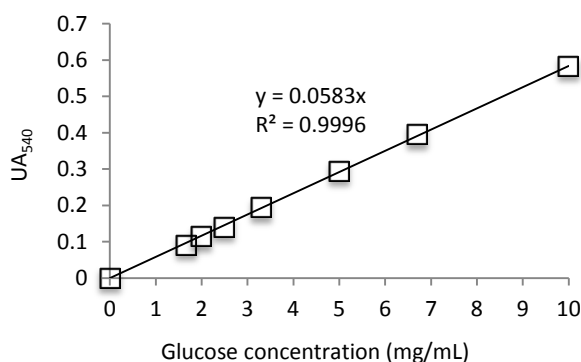
FIGURE 5.8: A standard curve for glucose measurement

Determination of the concentration of enzyme that would have released exactly 2.0 mg of glucose was carried out by plotting the concentration of glucose liberated against enzyme concentration as shown in FIGURE 5.9.
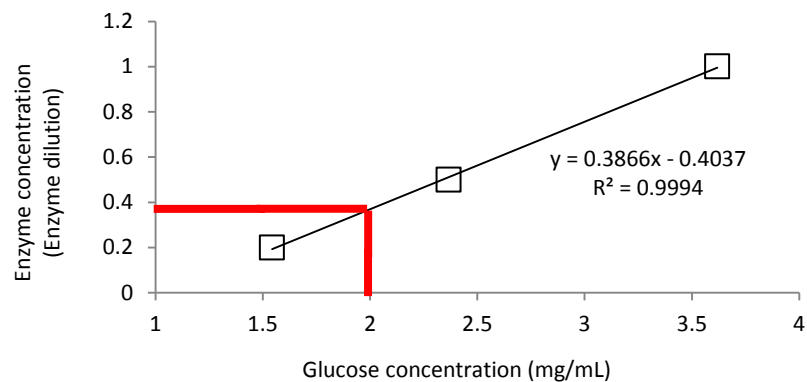
FIGURE 5.9: Calculation of FPU from a plot of enzyme dilution versus glucose concentration

Cellulase activity can be determined using the following equation:

$$Cellulase\ activity\ (FPU/mL) = \frac{0.37\ \mu mole\ min^{-1} mL^{-1}}{Enzyme\ concentration\ to\ release\ 2.0\ mg\ glucose} \tag{5.9}$$

CHAPTER 6

STUDIES OF PHYSICAL CHARACTERISATION OF
SOLID STATE FERMENTATION

"Science never gives up searching for truth, since it never claims to have achieved it"
John Charles Polanyi
University of Manchester

6.1   INTRODUCTION

The objective of this study is to analyse the viability of wheat bran, soybean hulls and rapeseed meal for solid state fermentation (SSF) processes by exploring their physical properties. Such properties include bulk density, particle density, porosity, water retention value (WRV) and tortuosity and the percentage of pores filled with water. Indeed, in SSF, the availability of surface area plays a critical role for microbial attachment and mass transfer (Anto *et al.*, 2006) and is dependent upon the particle size of the support. Particle size affects the rigidity and porosity of the solid substrate, which further influences mass transference and heat transference, as well as microbial growth and metabolite production (Nandakumar *et al.*, 1996; 1994).

The ability of solid substrate to retain water is also addressed in this chapter (WRV). This is   highly relevant to the study as it provides a basis to understand other important solid particle properties for SSF, such as how much water can be stored in the solid substrate, how fast water and heat will be transferred through the solid particle, how easily the mycelium of fungus can penetrate through the solid particle, and the potential of total water needed to be supplied both at the beginning and throughout the entire process of SSF to support the growth of microorganisms. In addition, experiments were carried out in order to determine the ability of the fungus itself to retain water within its own cells. Different fungi have different cell morphologies, which results in differing abilities to retain water. This factor will influence the ability of a fungus to fully utilise the water content provided in the system and also its ability to maintain the moisture content at an optimum level during the SSF process.

Metabolic heat is produced during fermentation as a result of metabolic activity. It has been found that this metabolic heat generation and the slow heat transfer inside the bioreactor cause a temperature rise which is difficult to regulate (Khanahmadi *et al.*, 2004). Therefore, the fermented bed and bioreactor temperature need to be controlled as closely as possible to remain at an optimum temperature level. However, this process poses some difficulties due to the nature of SSF.

The forced addition of air is usually necessary to allow evaporative cooling of the fermented substrate. However, this method of cooling, although effective, results in a loss of water from the system. In some cases, this water is replaced as a by-product of metabolism. The water activity can be maintained by humidified air whereby air saturated with water is blown into the bioreactor. Therefore, fermented bed temperature and bioreactor control strategies require inlet air-humidity manipulation achieved by adjusting the temperature, humidity, and flow rate of the inlet air. Varying the proportion of dry to wet air is the preferred method of controlling evaporation as opposed to alternating between wet and dry air. Preliminary experiments for aeration studies are analysed within this chapter in order to study the effect of temperature on the humidity of the air entering the bioreactor. The air entering the bioreactor will play a major role in expelling the excess heat whilst simultaneously controlling the moisture content of the fermented substrate. Consequently, the amount of water carried by the air must be tightly regulated during the fermentation process.

6.2   MATERIALS AND METHODS

6.2.1   Evaluating Particle Size

The method proposed by Baker and Herrman (2002) was used to evaluate particle size of wheat bran, soybean hulls and rapeseed meal. Samples were sieved using a SATAKE PLSB-Series 2000 Simon Laboratory Sifter (UK). A series of known test sieves values of 45, 53, 120, 180, 212, 500, 850, 1180, 1400, 1700 and 2000 µm aperture size were used in order to evaluate particle size distribution in the original solid substrate. Each sieve separates solid substrate particles according to size. In this method, the first step in particle size analysis is to obtain a representative sample. A 150.0 g sample was measured by using a full stack of sieves to avoid accumulation of more than 20.0 g over any one sieve. After the 150.0 g sample had been weighed, the following stages in the separating process were followed:

- The sieve stack was arranged with the following order: the greatest size at the top and the finest at the bottom.
- The sample was placed onto the top sieve and the sieve stack was placed onto the shaker.
- The shaker was allowed to run for about 5 min on laboratory sifter to ensure complete separation.
- The sides of each sieve were gently tapped with a brush before removing the sieve from the stack.
- The sieve stack was removed from the shaker.

- Each sieve was placed with the retained solid particles on a balance to weigh the sieve and retained solid particles together.

- Solid particles were removed and the sieves were thoroughly cleaned.

- The empty sieves were weighed and the weights recorded. The difference between the weight of the sieve with and without material was calculated to determine the weight of material. The weight values were entered in the appropriate columns of a spreadsheet. The average particle size of material retained on each sieve is calculated as the geometric mean of the diameter openings in the two adjacent sieves in the stack. Equation 6.1 shows this calculation.

$$d_i = (d_u \times d_0)^{0.5} \tag{6.1}$$

As it is not practical to count each particle individually and calculate an average, the average particle size can be calculated on the basis of weight. This can be done with Equation 6.2:

$$d_{gw} = log^{-1}\left(\frac{\sum(W_i \times \log d_i)}{\sum W_i}\right) \tag{6.2}$$

The standard deviation can be calculated as follows:

$$S_{gw} = log^{-1}\left(\frac{\sum W_i(\log d_i - \log d_{gw})^2}{\sum W_i}\right)^{0.5} \tag{6.3}$$

Where

        d_{gw} = the average particle size

        S_{gw} = standard deviation (dimensionless)

        d_i = diameter of ith sieve in the stack

        d_u = diameter opening through which particles will pass (sieve preceding the ith)

        d_0 = diameter opening through which particles will not pass (i[th] sieve)

        W_i = weight of retained solid particles in every sieve (i[th] sieve) (g)

The number of particles per gram and amount of surface area can be calculated from d_{gw} and S_{gw} obtained from Equations 6.2 and 6.3 respectively.

From this value, the particles per gram and surface area can be calculated as follows:

$$particles/gram = \left(\frac{1}{\rho_p \beta_v}\right) \exp\left(4.5\, ln^2 S_{gw} - 3 \ln d_{gw}\right) \tag{6.4}$$

$$SA = \left(\frac{\beta_s}{\rho_p \beta_v}\right) \exp\left(0.5\, ln^2 S_{gw} - \ln d_{gw}\right) \tag{6.5}$$

Where;

SA = surface area (cm^2/g)

β_s = shape factor for calculating surface area of particles = 6

β_v = shape factor for calculating volume of particles

ρ_p = particle density of solid substrate (g/cm^3)

For these calculations, the shape factors β_s and β_v are assumed to be 6 and 1 respectively. The particle density (ρ_p) can be obtained within the next section. Since the specific weight is expressed in g/cm^3, it is necessary to convert the average particle size (d_{gw}) to cm. This can be done by multiplying the value by 0.0001.

Volumetric specific surface area (VSA) (cm^{-1}) can then be obtained from Equation 6.5 by multiplying it by particle density (ρ_p) of solid substrate (g/cm^3), that is:

$$Volumetric\ specific\ surface\ area\ (VSA) = Surface\ area\ (SA) \times \rho_p \tag{6.6}$$

According to Ishizawa *et al*. (2007), volumetric specific surface area is attributed to the creation of surface openings or internal pore spaces and by the removal of cell wall components, which enhances the direct physical contact between the enzymes and the substrate.

6.2.2   Properties of Solid Substrate

Density is measured as mass per unit volume (mass divided by volume). Solid substrate density depends on the chemical composition and structure of the minerals in the solid substrate. Density of any materials (solid substrate) can be divided into two categories: (1) bulk density and (2) particle density. Bulk density refers to the volume of the solid portion of the solid substrate particles along with the spaces where the air and water exist. Bulk density differs from particle density as particle density is only concerned with solid substrate particles and the pore spaces occupied within the solid substrate. Bulk density is used along with particle density to calculate porosity. Porosity (expressed in

percentage) refers to pore space occupied by air and water within a solid substrate. This knowledge about the properties of a solid substrate makes it possible to have a better understanding of how the solid substrate functions within specific conditions. It also allows for a more accurate interpretation of solid substrate measurements to be carried out. Percentage of pores filled with water and bed tortuosity can be defined as a function of moisture content.

Procedures:

Bulk density

Solid substrates (wheat bran, rapeseed meal and soybean hulls) with different moisture contents were poured into a measuring cylinder of known volume (30.0 mL) and weighed to determine the bulk density (Abalone *et al*., 2004). Bulk density of solid particles was calculated using Equation 6.7.

$$Bulk\ density\ (\rho_b) = \frac{mass\ of\ dry\ solid\ substrate\ (g)}{total\ volume\ of\ solid\ substrate\ and\ air\ (mL)} \tag{6.7}$$

Particle density

Particle density at various moisture contents was determined using a standard soil particle density protocol (Globe, 2002).

- The weight of an empty 100.0 mL volumetric flask without a cap was measured.
- Approximately 12.0 g of solid substrate was weighed and mixed homogenously with a suitable amount of water to obtain an initial moisture content of 0, 11, 35, 50, 60, 65, 70, 75 and 80%.
- The solid substrate was placed in the volumetric flask using a funnel.
- The weight of the volumetric flask containing the solid substrate was measured at different moisture contents.
- About 50 mL distilled water was added to the solid substrate in the volumetric flask.
- The solid substrate/water mixture was brought to a gentle boil by placing the volumetric flask on a hot plate. The flask was gently swirled for 10 seconds once every minute to keep the solid substrate/water mixture from foaming over. The boiling process was continued for 10 min to remove air bubbles.
- The volumetric flask was removed from the heating plate and the mixture was allowed to cool.
- Once the volumetric flask has cooled, the flask was capped and let to sit for 24 h.
- After 24 h, the cap was removed and the flask filled with distilled water, so that the bottom of the meniscus is at the 100 mL line.
- The 100 mL solid substrate/water mixture was weighed in the volumetric flask.

- The weight values were recorded in the appropriate columns of the spreadsheet for further data analysis.

$$Particle\ density\ [\rho_p] = \frac{mass\ of\ dry\ solid\ substrate\ (g)}{Volume\ of\ solid\ substrate\ particles\ only\ (mL)} \qquad (6.8)$$

Porosity can also be expressed as percentage of pores filled with air. The amount of pore space, or porosity, is expected to decrease by increasing the moisture content of the solid substrate. Porosity (ε) can be calculated with the following equation:

$$\varepsilon = 1 - \left(\frac{\rho_b}{\rho_p}\right) \times 100 \qquad (6.9)$$

Bed tortuosity (τ), which accounts for elongation of the diffusion path due to the presence of solid substrate particles, is expected to increase with bulk density (Khanahmadi *et al.*, 2005). Bed tortuosity (dimensionless) can be calculated using the following equation:

$$\tau = \frac{1}{(0.2+\varepsilon)^2} \qquad (6.10)$$

By knowing the bulk density, porosity, water density and the ratio of water mass to solid sample mass, the percentage of pores filled with water (ε_w) can be measured as followed:

$$\varepsilon_w = \left(\frac{g\ water/g\ substrate}{\varepsilon}\right) \times \left(\frac{\rho_b}{density\ of\ water}\right) \times 100 \qquad (6.11)$$

6.2.3   Water Retention Value

Water retention value (WRV) is an empirical measurement of the capacity of a test solid substrate to hold water. WRV was calculated as the ratio of weight of water retained by wet solid particles after centrifugation under specified conditions to the weight of the same solid sample after oven drying.

This was carried out by using a specially modified centrifuge-holding tube, as illustrated in FIGURE 6.1. The method used to determine *WRV* has been taken from Scandinavian pulp, paper and board SCAN-C 62:00 (2000) with slight modifications appropriate for the apparatus available.
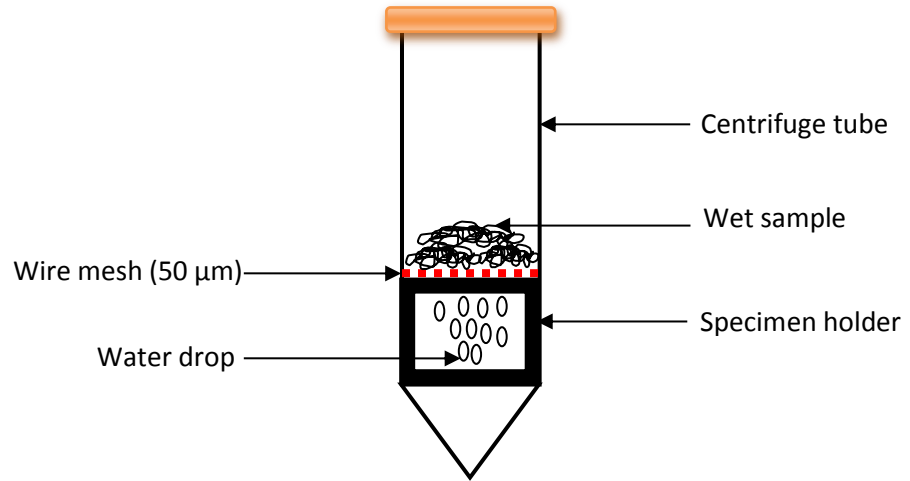
FIGURE 6.1: Modified centrifuge-holding tube to hold to the rim of the centrifuge rotor

Procedure:

- 5.0 g of solid substrate at moisture content 0% after drying at 80 °C overnight was immersed in distilled water for about 1 hour at room temperature.

- Sample was removed from the water and allowed to drain for a few minutes. Some amount of the wet sample was then transferred into the specially modified centrifuge tube (FIGURE 6.1) and centrifuged at 7,500 rpm for 15 min. During centrifugation, the unit hangs on the rim of the centrifuge rotor, leaving enough space underneath to accommodate drained water.

- After centrifugation, samples were weighed (W_{ww}) and next dried in an oven at 80 °C for overnight. The samples were weighed as quickly as possible after centrifugation in order to obtain a wet weight prior to any evaporation occurring.

- After drying, samples were put into a desiccator to cool and then weighed (W_{dw}).

- The WRV was expressed in grams per gram, according to the following equation:

$$WRV = \frac{W_{ww} - W_{dw}}{W_{dw}} \qquad (6.12)$$

Where;

W_{ww} = wet weight of sample after centrifugation (g)

W_{dw} = dry weight of sample after drying (g)

In addition to measuring WRV of solid substrates, the same procedure was also performed to measure WRV of fungus cell material. The procedure for WRV for fungi was carried out according to the following steps:

- First, matured culture fungus (7 days old) was autoclaved at 121 °C for 5 min to kill the fungus and after that melted agar was removed. Fungal mycelium mat was obtained and washed under running tap water.
- Sample (fungal mycelium mat) was then dried in an oven at 80 °C for 24 h until the moisture content was almost zero.
- Dried fungal mycelium mat was crushed using a mortar to obtain pieces of fungal mycelium
- About 2.0 g of fungal pieces was weighed and placed in 250 mL flask.
- 40.0 mL of distilled water was added and the flask was placed on a shaker at 250 rpm and 30 °C for about 24 h.
- The sample was then transferred into a normal centrifuge tube and centrifuged at 10,000 rpm for 10 min. The supernatant was discarded.
- The wet sample was transferred into a new centrifuge tube equipped with a wire mesh (50 μm) on top of the specimen holder (FIGURE 6.1) and centrifugation was performed according the procedure explained above.
- WRV was calculated, in units of gram per gram, according to Equation 6.12.

6.2.4   Aeration Studies

Significantly, several studies reported that saturated air can be applied as a strategy for providing O_2 to the bioreactor, whilst also playing an important role in controlling temperature and maintaining the moisture content of the fermented substrate. In order to study the effect of the air entering the bioreactor, an experiment was carried out to measure the amount of water carried by the air.

To measure the amount of water carried by the air, a tube with silica gel was connected to the system (FIGURE 6.2) and the accumulation of water was measured every two hours by the difference in the weight of silica tube. To ensure that all the water carried by the air was retained in the silica, a second and a third tube with silica gel were connected to the first one. These were also weighed every two hours, observing that the weight was constant during all experiments.
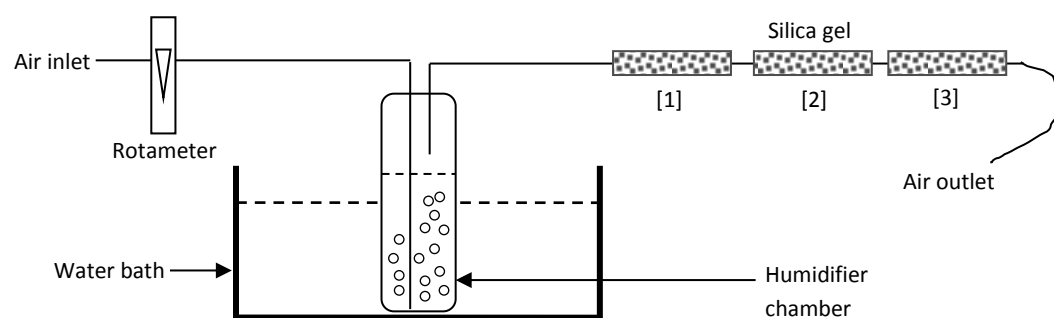
FIGURE 6.2: **Experimental system used to study the amount of water carried by air after passing through a humidifier chamber**

A series of experiments was carried out to study the amount of water carried by air. This was done by passing the air through a humidifier chamber as explained below:

i.   Experiment 1: At different temperatures of water bath – 20, 30, 40 and 50 °C

  ▪ Size of humidifier chamber: 250 mL

  ▪ Working volume of water: 150 mL

  ▪ Flow rate: 1 L/min

ii.   Experiment 2: At different flow rates – 1, 2, 3, 5 and 7 L/min

  ▪ Size of humidifier chamber: 250 mL

  ▪ Working volume of water: 150 mL

  ▪ Temperature of water bath: 30 °C

iii.   Experiment 3: At different working volumes of water in the humidifier chamber – 50, 75, 100, 125, 150, 175 and 200 mL

  ▪ Size of humidifier chamber: 250 mL

  ▪ Flow rate: 1 L/min

  ▪ Temperature of water bath: 30 °C

  ▪ Observation has been made after 3 h

iv.   Experiment 4: A series of humidifier chambers in the water bath – 1, 2, 3, 4 and 5 humidifier chambers

  ▪ Size of humidifier chamber: 250 mL

  ▪ Working volume of water: 150 mL

  ▪ Temperature of water bath: 30 °C

  ▪ Air flow rate: 5 L/min

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6.3   RESULTS AND DISCUSSION

Particle size is one of the most important physical parameters in SSF. Particle size distribution will affect the surface area to volume ratio of the particles that are initially accessible to the microorganism and the packing density within the surface mass. The space between particles is occupied by a continuous gas phase (Botella *et al*., 2009) and the size of the substrate particles determines the pore space that is occupied by air. This space will aid gaseous exchange as well as heat and mass transfer between particles. As the rate of O_2 transfer into the pore space affects growth, the substrate should contain particles of suitable size to enhance mass transfer (Singhania *et al*., 2009).

In addition, the chemical composition of solid substrate will determine its ability to retain sufficient water supplies to support growth. The results presented here provide a basic idea of the limitations and difficulties that are faced in the development of SSF. Furthermore, understanding some of these physical properties will help in developing the design of research strategies and experiments and in defining experimental parameters such as setting a suitable initial moisture content prior to the start of the SSF process.

6.3.1   Evaluating Particle Size

FIGURE 6.3 shows percentage distribution of solid particles over the sieve and cumulative data from particle size distribution. In summary, particle size analysis of wheat bran revealed cumulative undersize of 29.05%, 25.75% and 17.49% at diameter of 500, 212 and 850 µm respectively. For soybean hulls, about 35.65% of particles are 500 µm in size followed by 21.43% and 19.06% the particles 212 and 850 µm in size respectively. For rapeseed meal, about 36.95% of particles are 500 µm in size followed by 34.35% and 9.16% of particles 212 and 200 µm in size.

Particle analysis provided volumetric specific surface area measurements, for wheat bran (301.99 cm^{-1}) > rapeseed meal (214.0 cm^{-1}) > soybean hulls (173.40 cm^{-1}). The higher volumetric specific surface area in wheat bran can be correlated with its starch content. However, by considering the particles per gram value of wheat bran compared to rapeseed meal and soybean hulls, which contained only husk, wheat bran presents as a promising substrate in SSF. Soybean hulls might be useful as an inert carrier. Mixed with other substrates, soybean hulls can be used to create inter-particle spaces, thus increasing the surface area for better air circulation and nutrient diffusion.

However, this is not the only factor, which will determine its suitability because factors such as the type of microorganism and moisture content will also have an important impact on SSF.
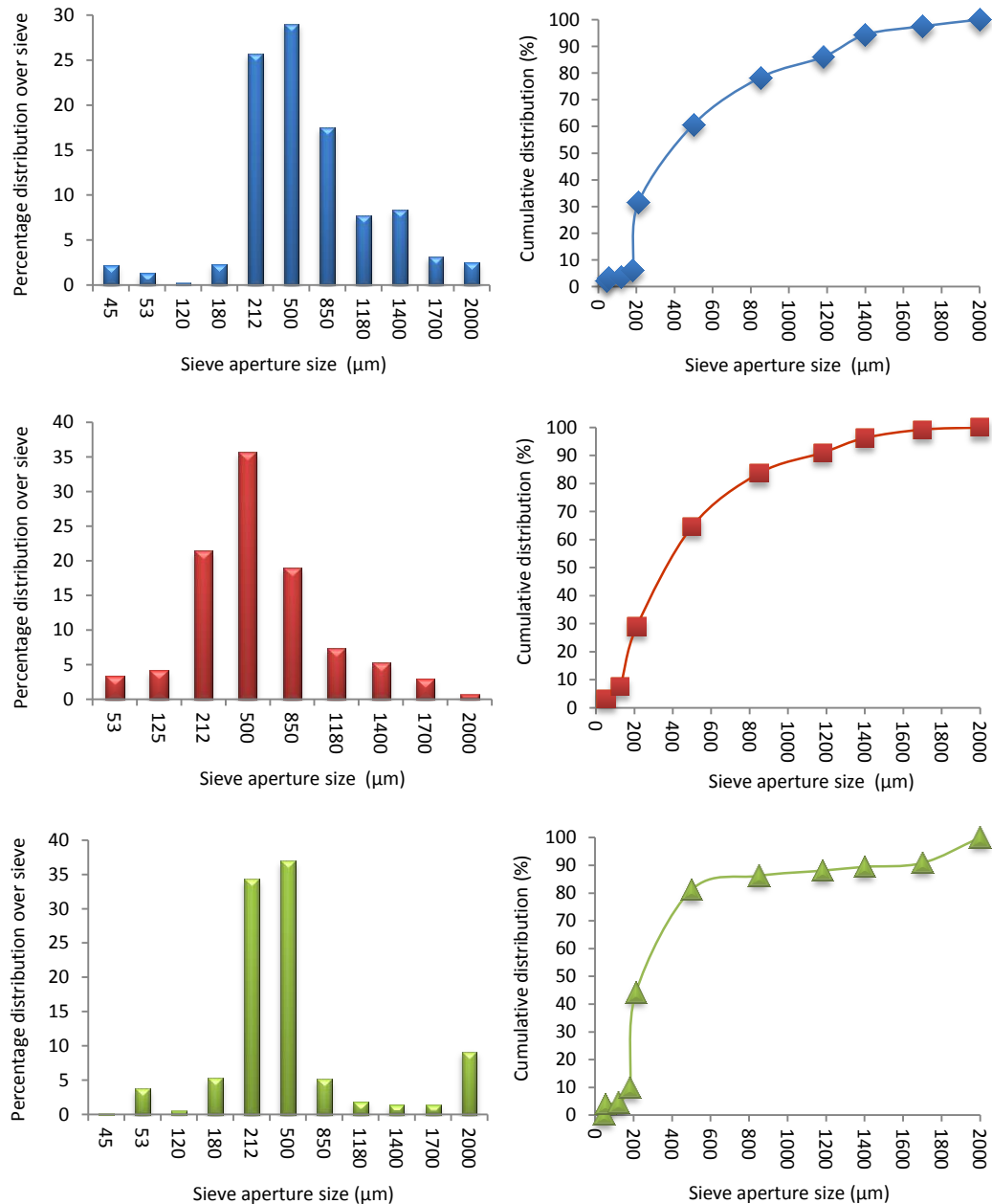
FIGURE 6.3: **Left: Bar chart of percentage solid particle distribution over sieve. Right: Cumulative size distribution of solid particles. (◆): wheat bran; (■): soybean hulls and (▲): rapeseed meal**

6.3.2   Properties of Solid Substrate

In this study, bulk density, particle density, porosity, tortuosity and percentage of pores filled with water for wheat bran, soybean hulls and rapeseed meal were experimentally measured at different moisture contents.

According to FIGURE 6.4, bulk density decreased from 0.26 to 0.24 g/mL when moisture content increased from 11 to 35% in wheat bran, then it increased from 0.24 to 0.94 g/mL when moisture content further increased from 35 to 80%. The same trend was observed in soybean hulls where the bulk density decreased from 0.33 to 0.23 g/mL when moisture content increased from 11 to 35%, then increased from 2.3 to 1.01 g/mL when moisture content changed from 35 to 80%. FIGURE 6.4 also shows that the bulk density of rapeseed meal decreased from 0.5 to 0.39 g/mL when moisture content changed from 11 to 30%. The bulk density then started to increase from 0.39 to 1.04 g/mL when moisture content increased from 30 to 70%. Different trends in bulk density due to increase in moisture content can contribute to different responses of solid materials to moisture content.

Particle density decreased from 1.29 to 1.07, from 1.49 to 1.08 and from 1.29 to 1.11 g/mL for wheat bran, soybean hulls and rapeseed meal respectively, showing a linear relationship, when moisture content changed from 0 to 80 % (FIGURE 6.4). If the increase in moisture content results in greater decrease in solids volume than mass, particle density will have a negative relationship with the moisture content.

By gaining information on both the bulk density and particle density of the solid substrate, it is possible to measure the pore space (porosity) that is occupied by air (oxygen) (Equation 6.9). Furthermore, by knowing the solid bulk density, particle density and the density of water, the ratio of the volume of water to the volume of solid substrate may be calculated along with the percentage of the pore space filled with water (Equation 6.11).

Figure 6.5 shows the correlation between porosity (pores filled with air/O_2) and the percentage of pores filled with water. In all three solid substrates tested, porosity decreased from 82.45 to 6.26%, showing a non-linear relationship when moisture content increased from 0 to 80%. By contrast, the opposite trend was observed for the percentage of pores filled with water, which increased exponentially when moisture content increased from 0 to 80%.
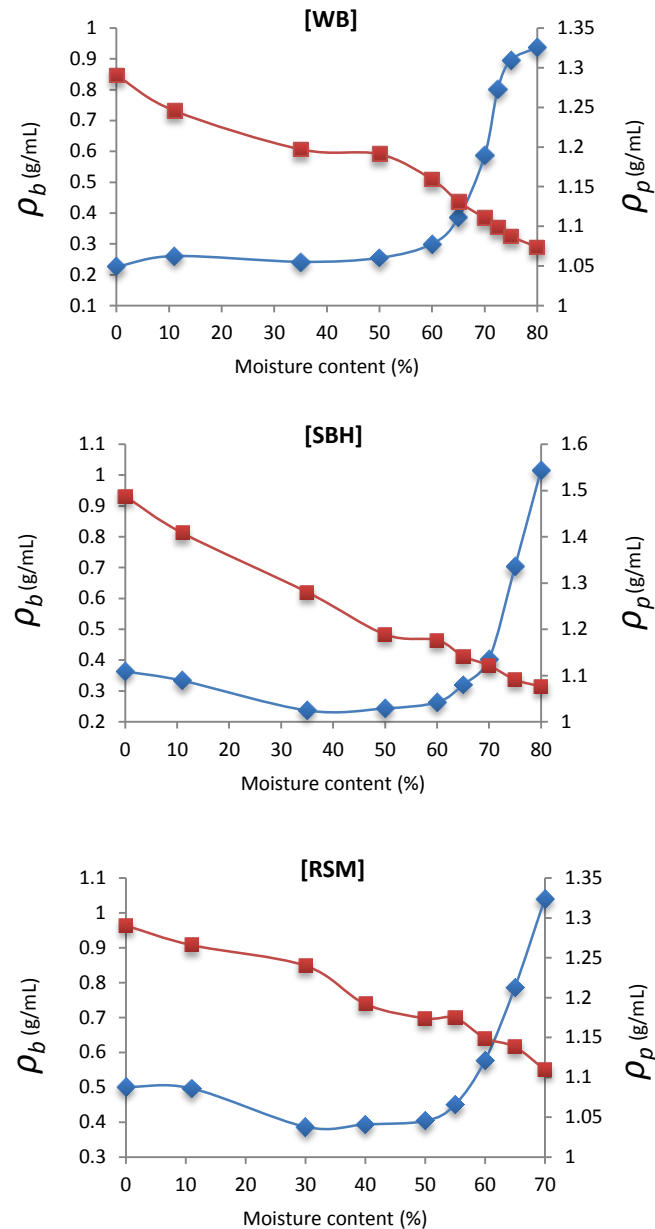
FIGURE: 6.4:   **Bulk density (◆) and particle density (■) at different moisture contents. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal**

By using the information in FIGURE 6.5, a preliminary decision about the most suitable moisture content for initial SSF experiments can be made. For example, with wheat bran and soybean hulls, a suitable moisture content might be set at 65% while this would be 55% for rapeseed meal. For wheat bran and soybean hulls, the percentage of pores filled with air and the percentage of pores filled with water were 65.95% and 91.64%, and 79.97% and 74.11% respectively, at moisture content of 65%. For rapeseed meal, the percentage of pores filled with air and the percentage pores filled with water were 61.69% and 72.22% respectively, at moisture content of 55%. The percentage of pores filled

with water for wheat bran was slightly higher due the high starch component, which has a high ability to absorb water.
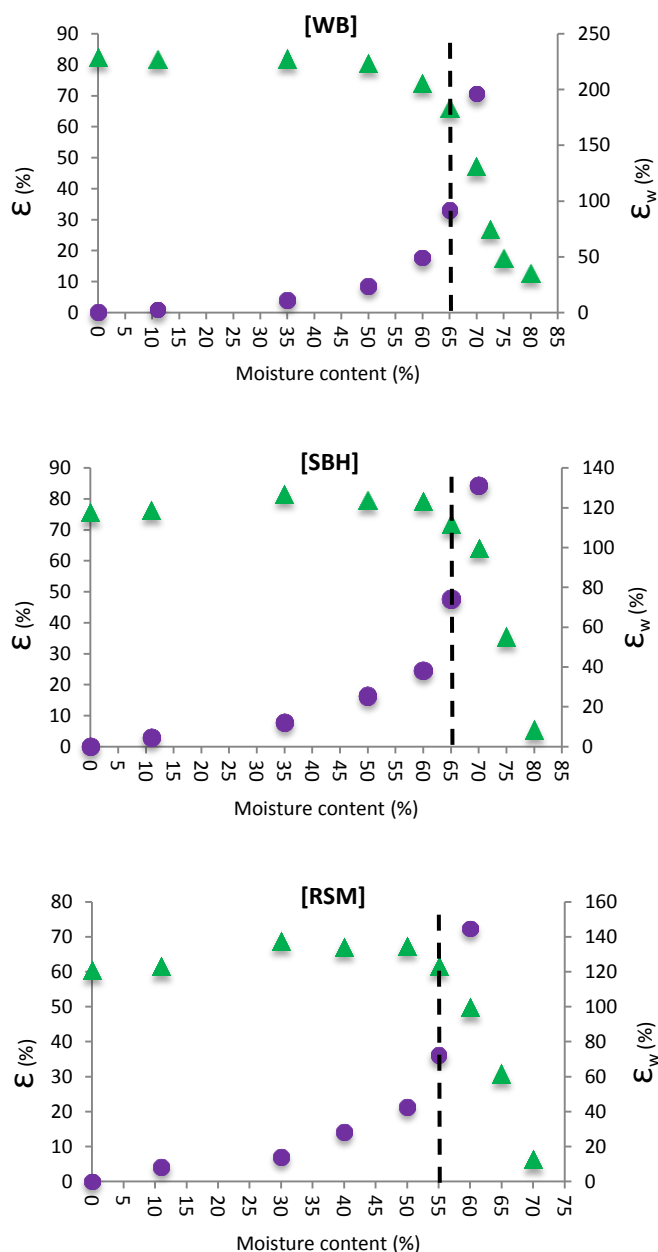
FIGURE 6.5:   Relationship between the percentage of pores filled with water (●) and those filled with air (porosity) (▲) as a function of moisture content. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal

Bed porosity ensures O_2 availability between the moist solid substrate particles. At high moisture content, water flooded the system at one point, not allowing air in or out and leading the bulk density to increase, particle density to decrease and specific volume to decrease. High moisture content also resulted in porosity decreasing to almost zero. This could provide a reason for the fungal

growth being restricted to the surface of the solid particle as difficulty with penetration results in poor mycelium growth. At low moisture content (below 30%), even with high O_2 content, conditions are not favourable to support a greater fungal growth due to lack of water.

According to Valera *et al*. (2005), there are two effects of particle size on SSF at any given moisture content. The first effect is the increase in surface area for the growth of the microorganism with decreased particle size. The second is the reduction in pore space and hence gas phase O_2 transfer with decreased particle size.

Arasaratnam *et al.* (2001) found that paddy husk mixed with substrates, such as rice bran, corn flakes, soya flour and soy meal powder, during SSF increased glucoamylase production. This has been attributed to the efficient air circulation and nutrient diffusion caused by the inter-particle space created by paddy husk, along with the increase in the surface area for the spores to germinate and mycelia to grow, providing them with easy access to nutrients.

This phenomenon was also observed by Rahardjo *et al*. (2005) when using various model solid substrates with different porosities for the production of α-amylase in SSF of *A. oryzae*. They reported that model substrates with high porosity exhibited better enzyme production compared to those with less porosity.

The pore spaces between particles are occupied with a continuous gas phase. Gas phase in SSF is strongly affected by the size of particles, the shape of particles and the tortuosity of a network of gas-filled pores (porosity) (Brijwani and Vadlani, 2011; Moldrup *et al*., 2001). According to Moldrup *et al*. (2001), a tortuosity phenomenon of pore spaces influences the transport of water solutes and gases within the solid substrate.

FIGURE 6.6 shows the relationship between porosity and tortuosity for wheat bran, soybean hulls and rapeseed meal at different moisture contents. At high porosity (low moisture content), the low value of tortuosity indicates that the transport of water solutes and gases is facilitated. In addition, within this study, tortuosity and porosity were considered together because they were simultaneously altered when solid substrate particles were subjected to a particular moisture content (specified in this study).
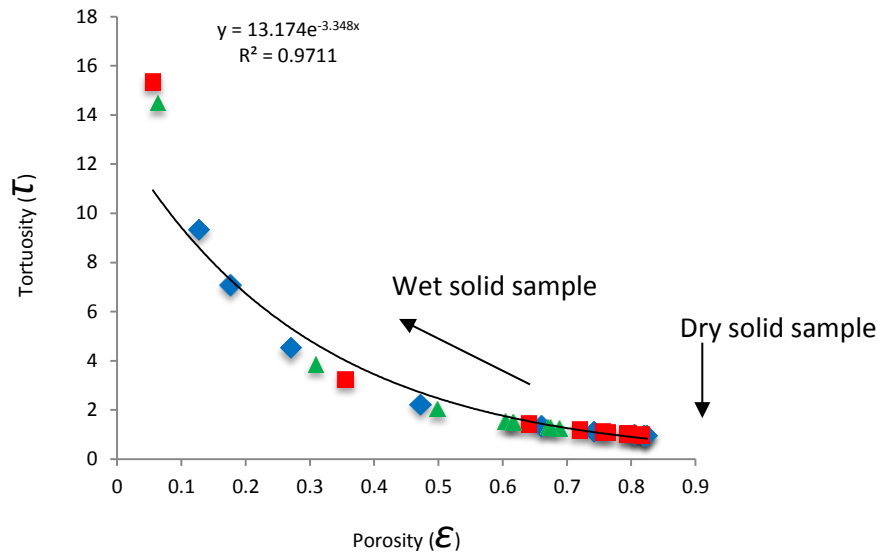
FIGURE 6.6: Correlation between porosity ($\mathcal{E}$) and tortuosity (τ) for three different solid substrates measured at different moisture contents. (◆): wheat bran; (■): soybean hulls and (▲): rapeseed meal

Furthermore, at low porosity (high moisture content), the value of tortuosity was higher indicating that the transport of water and gases was difficult. This is due to the decrease of inter-particle spaces and substrate agglomeration in most of the cases, which may interfere with microbial respiration and mass transfer. By contrast, high water content provides less respiration efficiency due to decreased inter-particle space. As a result, this condition will create limited surface area for microbial penetration and attachment. From this study, the values of tortuosity (dimensionless) for wheat bran, soybean hulls and rapeseed meal are 1.35, 1.18 and 1.50 respectively. These values are based on moisture content of 65% for wheat bran and soybean hulls and 55% for rapeseed meal.

6.3.3   Water Retention Value

WRV is used to determine the amount of water absorbed under specified conditions. It is also often used to quantify fibre swelling behaviour and it represents the water absorbency that is a key quality in solid substrate particles (SCAN-C, 2000). The pore characteristics, such as volume and size distribution, are key parameters for water capacity and absorbency.

FIGURE 6.7 shows values of WRV measured for wheat bran, soybean hulls and rapeseed meal. An average WRV for wheat bran was 2.63 g/g (range between 2.53 and 1.78 g/g), with an average coefficient of variation between replicates of 1.12%. Soybean hulls had an average WRV of 1.69 g/g

(range between 1.60 and 1.79 g/g) with an average coefficient of variation between replicates of 0.82%. Rapeseed meal had an average WRV of 1.36 g/g (range between 1.30 and 1.43 g/g) with an average coefficient of variation between replicates of 0.63%. The ability of wheat bran to retain water in the solid substrate is 56% higher than that of soybean hulls and rapeseed meal. This may be explained by the high starch content in wheat bran (23.3%) as starch absorbs more water compared to the other two solid substrates. Nevertheless, these polysaccharide components are an important factor in terms of the physicochemical properties of solid substrates in the SSF process as they contribute a nutritional value. In addition, starch content can be related to the available surface area of the wetted solid particles.
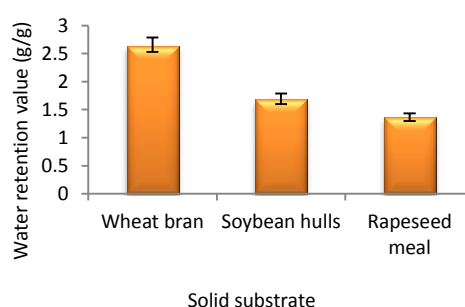
FIGURE 6.7: Water retention values for wheat bran, soybean hulls and rapeseed meal

The WRV for *A. awamori* and *A. oryzae* was determined (FIGURE 6.8) and each fungus was tested using four replicates. The average WRV for *A. awamori* was 1.67 g/g (range between 1.53 and 1.79 g/g) and it had an average coefficient of variation between replicates of 1.37%. The fungus *A. oryzae* showed an average WRV of 2.90 g/g (range between 2.79 and 3.01 g/g) and had an average coefficient of variation between replicates of 1.16%. Briefly, these results show that the ability of *A. oryzae* to retain water in the cells was higher (73% higher) than that of *A. awamori*.
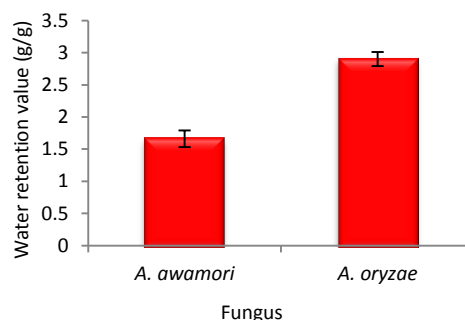
FIGURE 6.8:   Water retention values obtained for fungal mycellium mat of *A. awamori* and *A. oryzae*

To support this finding, another experiment was carried by measuring the decrease of moisture content of fungal mycellium mat of *A. awamori* and *A. oryzae* at 80 °C (at different times). A mature fungal mycellium mat (after 7 days) was submerged in water for 24 h at 30 °C. The mat was removed, pat dried with a lint free cloth and weighed. This gives an initial moisture content to the fungal mycellium mat. Subsequently, the fungal mat was placed in an oven and heated at 80 °C. At regular time intervals, the fungal mat was weighed as quickly as possible and then immediately placed back into the oven. This allows measuring the moisture content at those particular times. The process was repeated until the weight of fungal mycellium mat reached a constant value indicating that all the water from fungal mycellium mat was removed.

The rate of removal of the moisture content from fungal mycellium mat is illustrated in FIGURE 6.9. The initial removal of moisture content (AB) occured as the fungal mat and the water within it experienced a slight temperature increase. During this period, a heating process accoured whereby the fungal mycellium mat was still able to absorb heat from the environment. Following the initial stages of drying, significant reductions in moisture content occurred at a constant rate. The constant rate drying period (BC) matched the temperature of the oven. At this stage, the constant rate during the drying period continued until the moisture content was reduced to a critical moisture content (after 120 min). The diffusion rate drying period (CD) then followed. At this stage, the critical moisture content was further defined due to the abrupt change in the rate of moisture removal.
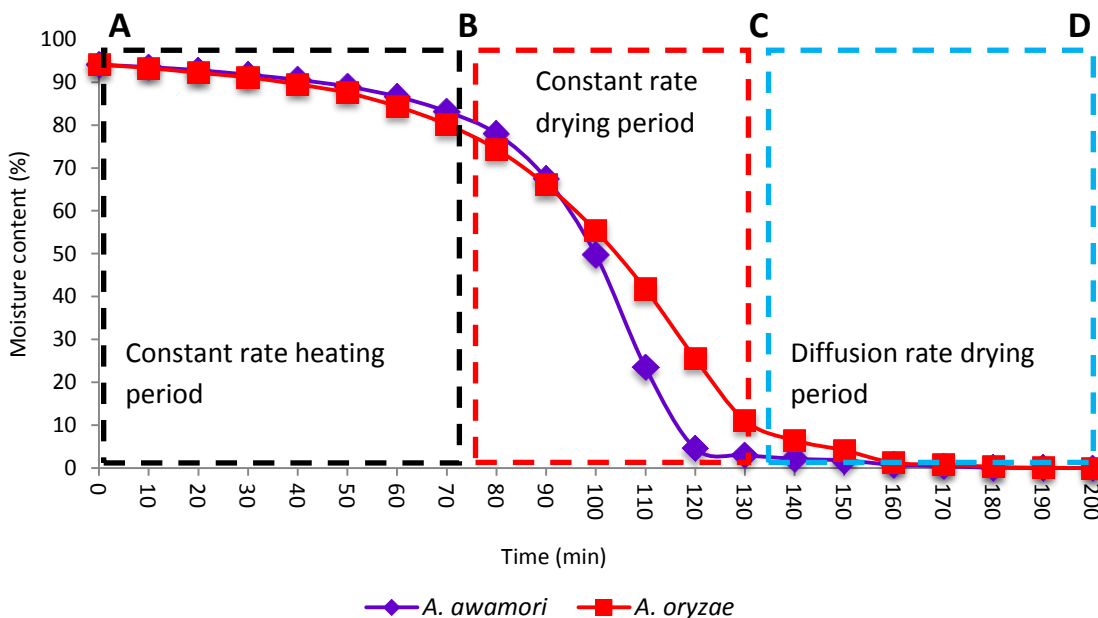
FIGURE 6.9: Profiles of moisture content removal for the fungal mat of *A. awamori* and *A. oryzae*

FIGURE 6.10 clearly shows that a constant rate drying period for both fungi occurs from 60 to 120 min. Moisture content of the fungal mycellium mat of *A. awamori* drastically decreased from 86.63% to 4.6%, while moisture content of *A. oryzae* fungal mycellium mat was still high at 25.6% at 120 min and only reached 1.22% after 160 min. Here, it can be seen that *A. oryzae* is able to retain water content about 5 times higher than *A. awamori*. In addition, moisture content loss from *A. awamori* is 46% higher than that from *A. oryzae*.
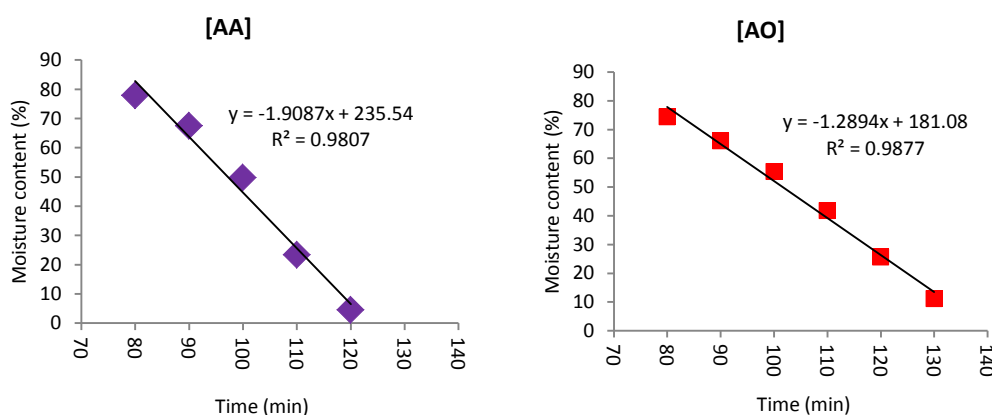
FIGURE 6.10:   Constant rate drying period for the two fungi. (◆): *A. awamori* and (■) *A. oryzae*

Interactions of microorganisms at the interfaces with their environment are known to be controlled by physicochemical properties of their cellular surface (Chau *et al*., 2009). Fungal mycellium mat from *A. awamori* is soft, fragile and non-elastic compared to fungal mycellium mat from *A. oryzae*, which is hard, elastic and not easy to break. The filamentous fungal mycellium mat of *A. awamori* is difficult to handle and can be easily damaged. This is possibly due to the higher levels of chitin in *A. oryzae.* In this case, glucosamine was measured based on the fact that glucosamine is a monomer component of chitin, present as acetylglucosamine (Aidoo *et al*., 1981). Glucosamine concentration was carried out according to the methods described in Chapter 5 (Section 5.7). TABLE 6.1 shows the concentration of glucosamine in fermented wheat bran, soybean hulls, rapeseed meal and fungal mat obtained from solid agar medium. It was observed glucosamine concentration in *A. oryzae* samples were higher between 7 – 16.83% compared to *A. awamori*. According to Peter (2005), variations in the concentrations of glucosamine (amounts of chitin) may depend on physiological parameters in natural environments as well as on the fermentation conditions in biotechnological processing or in cultures of fungi. In addition, chitin serves as a fibrous strengthening element responsible for cell wall rigidity.

TABLE 6.1: Glucosamine concentration

Fungus	Glucosamine concentration (mg/g) [db]			
	Fermented wheat bran	Fermented soybean hulls	Fermented rapeseed meal	Fresh Fungal mycelium from solid agar
A. awamori	120.35	85.69	93.02	100.46
A. oryzae	132.57	92.14	104.92	120.79

Examined under Environmental Scanning Electron Microscope (ESEM), fungal mycelium mat from *A. oryzae*, whether from a wet sample or after being treated with gold, showed a tightly fused and rigid structure. The abundant fungal mycelium in *A. oryzae*, which is fused and tightly bound, has several advantages as it is more hydrophilic and can hold water for moisture content maintenance. This can be observed in micro-images of fungal mycelium mat of *A. oryzae* when treated with gold (FIGURE 6.11 – AO1 and AO2: at magnification 1000X and 8000X, respectively) with some fibre-like structure but mainly a smooth surface. Micro-images of fungal mycelium mat of *A. awamori* treated with gold (FIGURE 6.11 – AA1 and AA2: at magnification 600X and 8000X respectively) showed micro-structures of a porous body (mycelium) with abundant, large pores and interconnected mycelium.
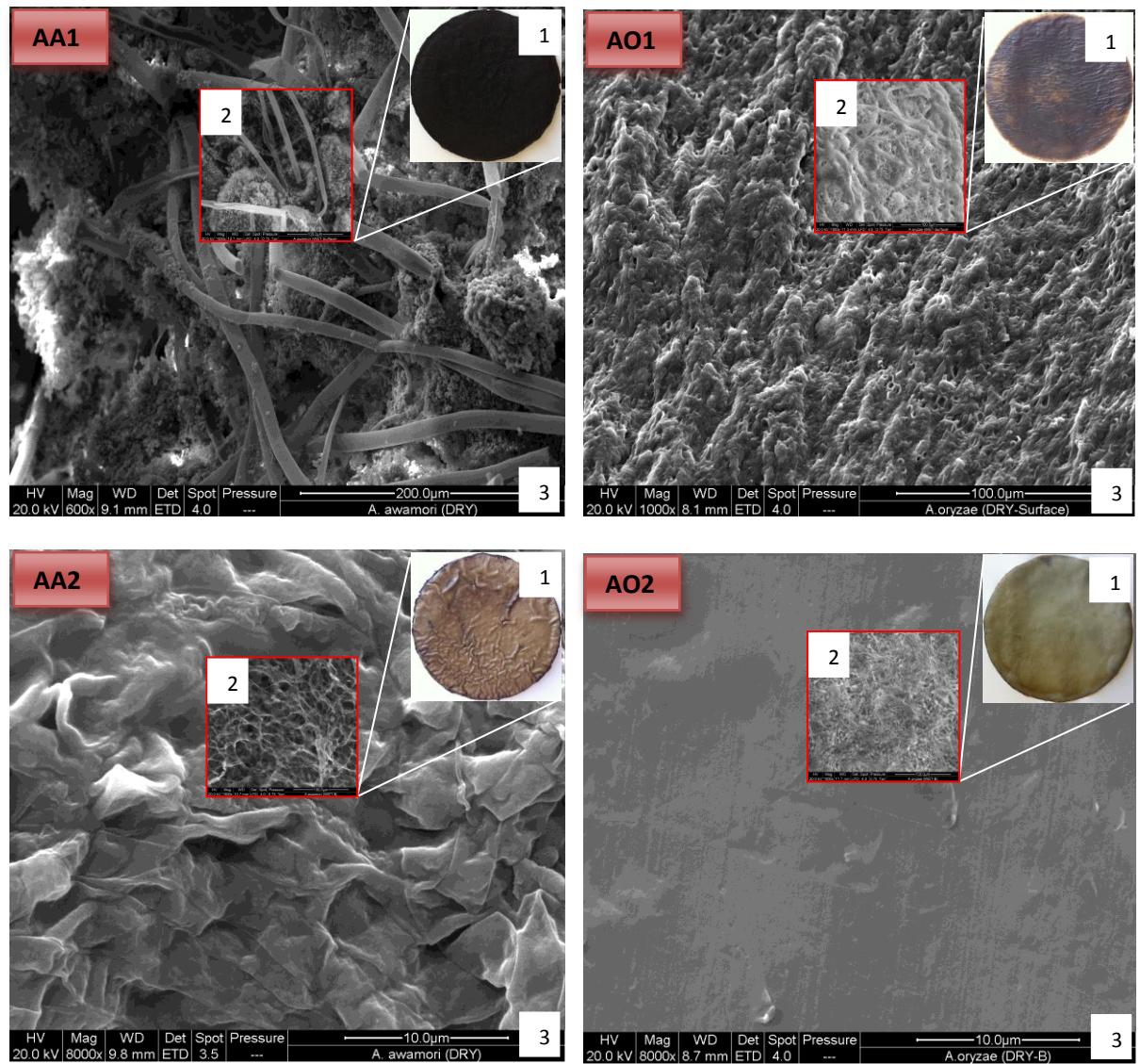
FIGURE 6.11: **ESEM images of fungal mat surface (AA1 and AO1) and the lower surface (AA2 and AO2) of *A. awamori* (AA) and *A. oryzae* (AO). Image 1 from live sample, image 2 from wet sample and image 3 from sample treated with gold**

500.0 mL of distilled water was poured into a 1.0 L beaker and the fungal mycelium mat was placed onto the surface of the water carefully (FIGURE 6.12). The first weight of water with fungal mycellium mat was recorded. Both beakers (containing fungal mat from the two fungi) and a control beaker (without fungal mat) were placed into an incubator growth chamber with an exact temperature of 30 °C. At specific time intervals, the weight of each beaker was recorded to measure the amount of water which evaporated from the beaker.
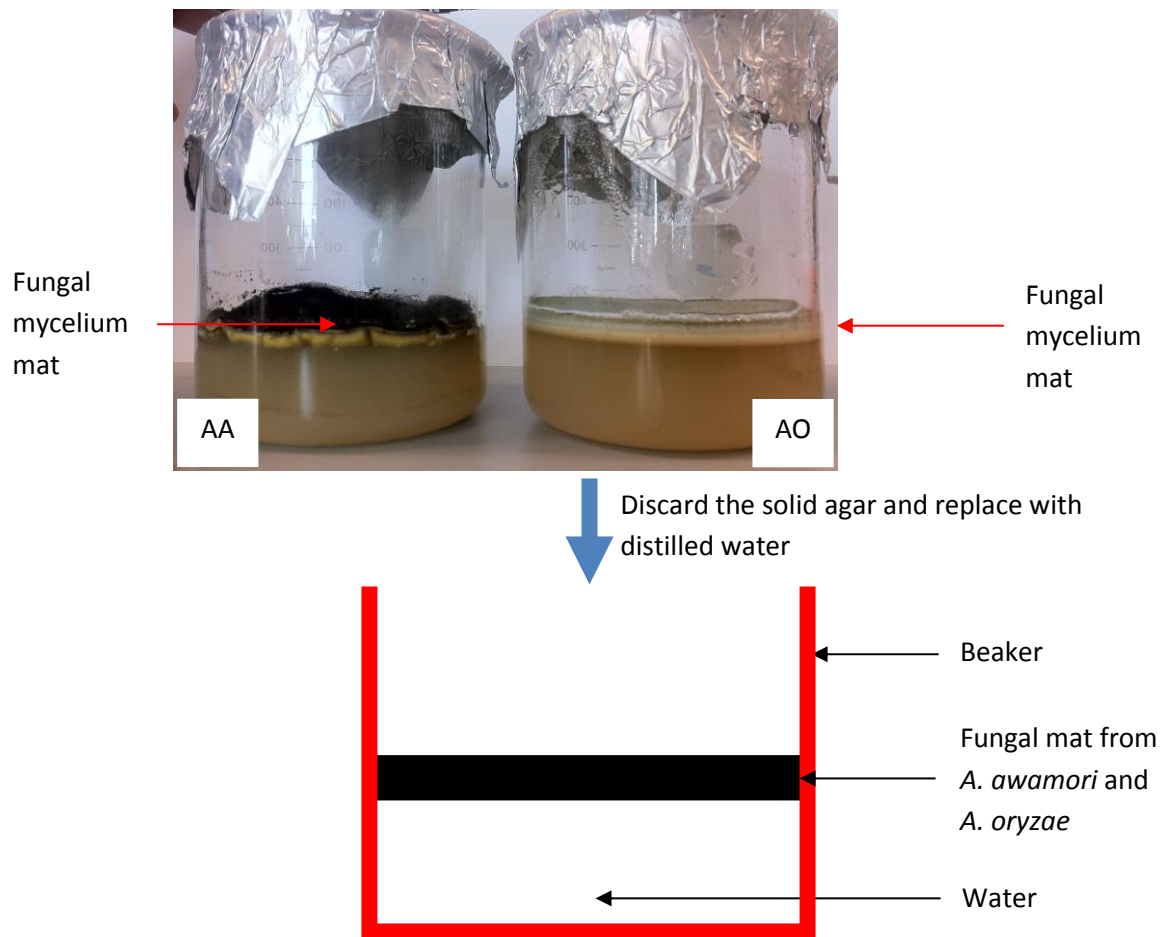
FIGURE 6.12: **Above: Growing *A. awamori* (AA) and *A. oryzae* (AO) on whole wheat flour agar for 7 days to obtain fungal mat. Below: The experiment used to study the evaporation process**

FIGURE 6.13 shows total amount of water that evaporated from each beaker after 7 days. Here, it is observed that water evaporated from *A. awamori* at a rate 8% higher than from *A. oryzae*. This result shows that fungal mycellium mat from *A. oryzae* has better ability to prevent water from evaporating. This may also indicate that A. *oryzae* was able to retain water in its cells. *A. oryzae* can therefore retain moisture content during the fermentation process better than *A. awamori*. This can be seen in the final moisture content at the end of the fermentation process, which is discussed further in Chapter 10. From this observation, it can be concluded that the filamentous fungal mat of *A. oryzae* is more hydrophilic than A*. awamori*. This study has also found that, in addition to the ability of solid substrates to absorb and retain water, fungal mycelium mat also plays important role in retaining water within the cells. As already explored in Chapter 2, water plays an essential role during the fermentation process.
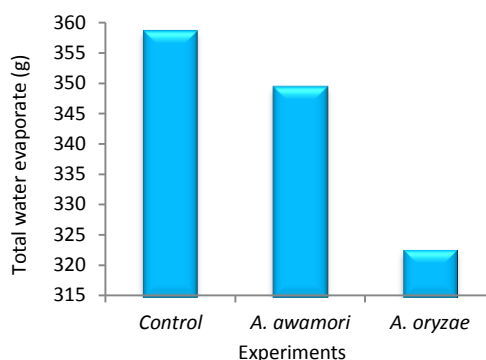
FIGURE 6.13: Total amount of water evaporated from fungal mat of *A. awamori* and *A. oryzae*

6.3.4   Aeration Studies

In order to study the quality of saturated air entering the SSF bioreactor, the first parameter examined was the water content in the air at the air inlet (bioreactor) at different temperatures. The humidifier chamber was placed in a water bath that was set at a constant temperature. Dry air at constant flow rate (1 L/min) was sparged into the humidifier chamber to 'create' saturated air. The temperature of the water bath was changed during this study to observe the water content of the air after passing through the humidifier chamber at 20, 30, 40, 50 and 60 °C. The system used for this study was the same as the one used in SSF using the bioreactor system described in FIGURES 4.12, 4.15 and 4.19 (Chapter 4).

FIGURE 6.14 shows that the amount of water carried by air is highly temperature dependent and increases at higher temperatures. This was observed by measuring the total water, which accumulated in the silica gel (measurements were carried out every two hours). Humidity ratio and relative humidity of saturated air were determined using a psychrometric chart. The humidity ratios are plotted against temperature in FIGURE 6.15, illustrating a linear relationship. The humidity ratios (grams of water per kilogram of dry air) increased proportionally as temperature increased. This means that increasing the temperature also increases the amount of vapour present, and so it increases the saturated vapour pressure. Briefly, when temperature increases, the energy of the particles present also increases. Therefore, more water molecules have enough energy to escape from the surface of the water. This tends to increase the saturated vapour pressure (humidity of the air).
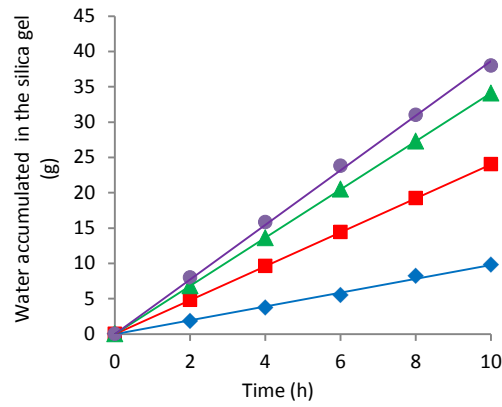
FIGURE 6.14: **Profiles of water accumulated in a silica gel after passing through a humidifier chamber at a constant flow rate (1 L/min) and different temperatures. Temperature of the water bath: (◆): 20 °C; (■): 30 °C; (▲): 40 °C and (●): 50 °C**

These values were compared with the psychrometric values, also represented in FIGURE 6.15. It can be concluded from this study that air passing through the humidifier did not reach saturation. However, this system was able to create moist air, as air entering the bioreactor held some degree of water vapour.
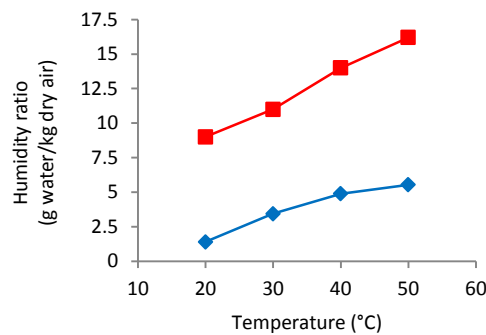
FIGURE 6.15: **Humidity ratios obtained from psychrometric charts (■) and this study (■) at different temperatures**

By measuring the slope of each temperature profile (FIGURE 6.14) and using values of flow rate and density of dry air, humidity ratios at different temperatures can be calculated according to Equation 6.13.

$$HR = \frac{(m/FR)}{\rho_{air}}$$
(6.13)

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Where;

HR = humidity ratio (g water/kg dry air)

m = slope from FIGURE 6.14 (g water accumulated/min)

FR = flow rate (L/min)

ρ_{air} = density of dry air (kg/L)

FIGURE 6.16 shows relative humidity of saturated air is strongly temperature dependent and decreases at higher temperatures (values obtained from psychrometric chart). This is because relative humidity is the ratio of the actual moisture content in the air to the moisture capacity of dry air at the same temperature, and moisture capacity increases with temperature.
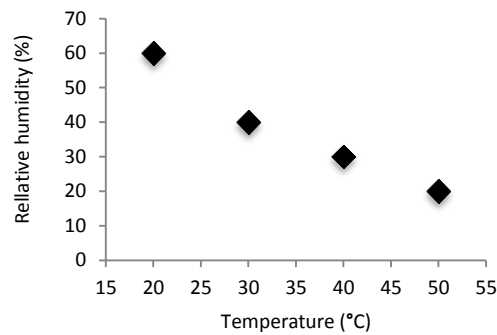
FIGURE 6.16:   Relative humidity at different temperatures obtained from psychrometric chart

In addition to obtaining values for the amounts of water carried by air, it is also possible to measure the amount of O_2 concentration in air-moistened water. The O_2 concentration of any liquid is defined by Henry's law as being proportional to the partial pressure (or percent) of O_2 in the gas above it. Solubility of O_2 in water can be calculated according to Equation 6.14.

$$C_L = \frac{P_G}{H'}$$
(6.14)

Where;

C_L = O_2 concentration in air-saturated water (mg/L)

P_G = partial pressure of O_2 in the gaseous phase (bar)

H' = Henry's constant. At 25 °C: H' = 0.026 (bar.L/mg O_2)

Partial pressure of a gas (O_2) in a humid gas mixture (air) can be calculated according to Equation 6.15.

$$P_G = (P_B + P_a - P_W)\left(\frac{A}{100}\right)$$ (6.15)

Where;

P_G = partial pressure of gas (O_2) in a humid gas mixture (air) (bar)

P_B = barometric pressure (bar)

P_a = partial pressure of dry air (bar)

P_W = partial pressure of water vapour (bar)

A = the portion of gas in the dry air gas mixture, in % volume (for O_2 in air: A = 21)

Theoretically, the effect of temperature on the solubility of O_2 in water was determined using the following equation:

$$C_L = 14.16 - 0.39437\,T + 0.0077141\,T^2 - 0.0000646\,T^3$$ (6.16)

Where C_L the amount of dissolved O_2 (mg/L) in water which has been moistened with O_2 at temperature T °C and pressure 1.0 atm. Using Equation 6.16, at 30 °C, theoretically it was found that 100% saturation requires 7.02 mg O_2/L water. O_2 solubility is strongly temperature dependent and decreases at higher temperatures (FIGURE 6.17). O_2 is poorly soluble in water, and its concentration is difficult to control. According to Standbury and Whitaker, (1984), O_2 is approximately 6000 times less soluble in water than glucose. Thus, it is not possible to initially provide a microbial culture with sufficient O_2 to complete respiration of the glucose (or any carbon source). O_2 must be supplied during growth at a rate sufficient to satisfy the demand by the microorganism. Therefore, it is suggested that the temperature of the humidifier chamber should not exceed 30 °C.
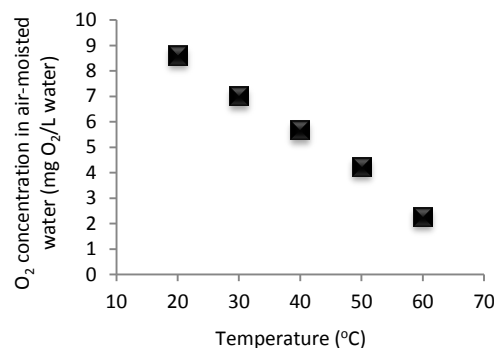
FIGURE 6.17:   Calculated O_2 concentration in moistened air at different temperatures using Equation 6.16

FIGURE 6.18 shows the amount water carried by dry air, accumulated in the silica gel with time at different air flow rates. This study (using a humidifier chamber in a water bath) was carried out at a constant temperature 30 °C. In a similar trend to the results obtained at different temperatures, a linear relationship was observed between amount of water carried by the dry air and air flow rate. When the air flow rate increases, the amount of water also increases.
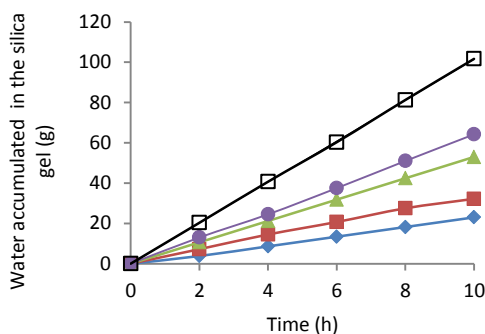
FIGURE 6.18: **Profiles of water accumulation in a silica gel after passing through a humidifier chamber at constant temperature (30 °C) and different flow rates. Flow rates: (◆): 1 L/min; (■): 2 L/min; (▲): 3 L/min; (●): 5 L/min and (□): 7 L/min**

By measuring the slope from each flow rate profile (FIGURE 6.18) and using the flow rate and density of dry air, humidity ratios at different flow rates can be calculated according to Equation 6.13. Figure 6.19 shows an almost constant correlation between the humidity ratio of the air and the flow rate, with a value of around 2.17 g water/kg dry air.
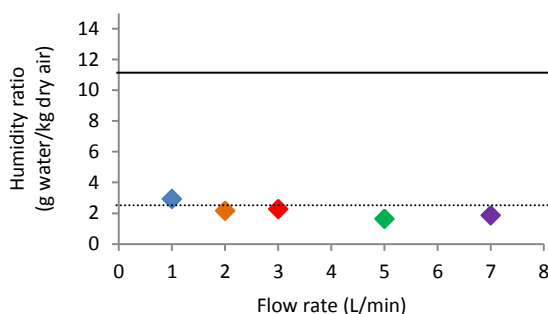
FIGURE 6.19: **Humidity ratios at different air flow rates and constant temperature (30 °C). Solid line represent saturated humidity ratio at 30 °C (———————)**

The following experiment was carried out with different amounts of working volume of water in the humidifier chamber range from 50 – 200 mL. The capacity of humidifier tank was 250 mL. The gas

phase from the water surface inside the humidifier chamber therefore had a range between 200 to 50 mL. FIGURE 6.20 shows the total amount of water accumulated in silica gel after 3 h of passing through the humidifier chamber. The humidity ratio was measured according to the Equation 6.13. The amount of water carried by air was strongly dependent on water volume and decreased at higher volumes of water in the humidifier chamber (FIGURE 6.20). At a low working volume of water in the humidifier chamber (for example at 50 mL water), bubbles are created vigorously and water molecules are more likely to escape from the surface of the liquid due to higher kinetic energy. The sparingly soluble O_2 is transferred from a rising gas bubble into a solid phase and ultimately to the site of O_2 reaction in cell particles. At a working volume of 50 mL, the humidity ratio was 4.2 g water per kg of dry air, which is 1.6 times higher than that corresponding to the highest working volume (200 mL). From this experiment, it can be concluded that at lower working volumes, the volume of gas inside the humidifier chamber tends to be larger, so the saturated vapour pressure will be higher.
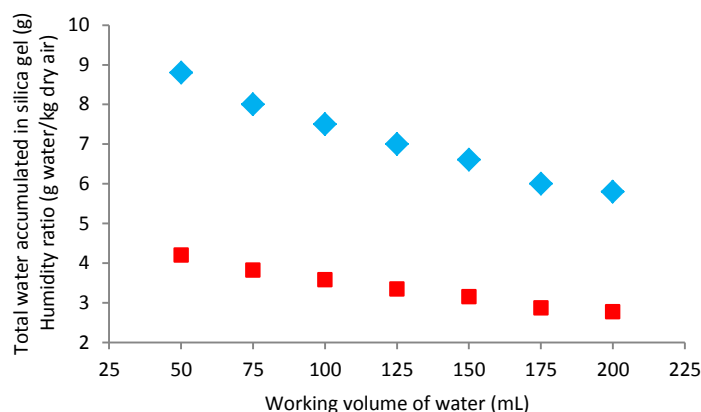
FIGURE 6.20: (◆): **Total water accumulated in a silica gel after 3 h passing through a humidifier chamber with different working volumes of water at constant temperature (30 °C) and flow rate (1 L/min). (■): Humidity ratio**

Another experiment was carried out by sparging dry air at a flow rate of 5 L/min into a series of humidifier chambers, each with a working volume of 200.0 mL of distilled water. This experiment used a variety of combinations of humidifier chambers: one, two, three, four and five humidifier chambers and also a water bath at 30 °C. From this study, it was observed that the amount of water accumulated in the silica gel increases when the number of humidifier chambers increases. The air and water mixture becomes humidified in the first humidifier chamber forming vapour. This vapour is transported into the next humidification chamber to become further humidified. The second chamber (then followed by a third, a fourth and a fifth chamber) becomes a condensing chamber where the hot and humidified vapour becomes condensed, transferring its water vapour into cooling

water through the water surface. After passing through humidifier chamber number five, the total water accumulated in the silica gel increased to almost six times higher than when a single humidifier chamber was used. In this study, it was assumed that air saturation improved because of the insulated chamber system made up of a series of chambers. Even though the effect of temperature and flow rate was not studied, from the results observed in FIGURE 6.21, it can also be expected that both accumulation and evaporation would increase as the number of humidifiers increases.
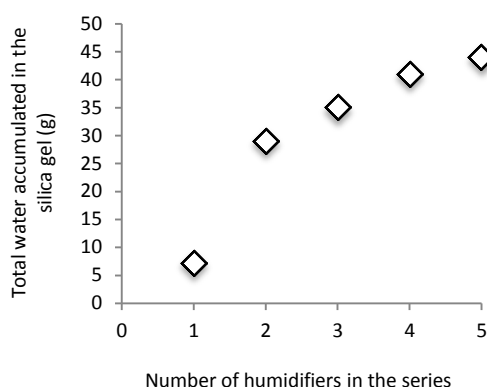
FIGURE 6.21: **Total water accumulated in a silica gel after 3 hours passing through a series of humidifier chambers at a constant temperature (30 °C) and flow rate (5 L/min)**

6.4   SUMMARY AND CONCLUSIONS

The main objective of this chapter was to study solid substrate particles and their physical characteristics in relation to SSF, analysing how of these characteristics vary with different solid substrate particles and moisture contents. The importance of physical properties, such as bulk density, particle density, specific volume, porosity, particle size, surface area, volumetric specific surface area, WRV and tortuosity, was identified in this study. These are listed in TABLE 6.2.

In summary, it can be concluded that, in addition to nutritional value, there are many important characteristics that a good solid substrate should have. Many factors are involved in a successful fermentation process. These include physical factors discussed and listed in TABLE 6.2. In addition to the solid substrate used, the fungus itself has physical properties that influence the SSF process. For example in this study, the ability of fungus cells to absorb and retain water was explored. This property will also determine the moisture content during SSF and show how cells of the fungus itself play an important role in maintaining moisture content. Further details are provided in Chapter 10, exploring how fermented *A. awamori* and *A. oryzae* have different final moisture contents in SSF.

TABLE 6.2: Physical properties of wheat bran, soybean hulls and rapeseed meal particles

Parameter	Values		
	Wheat bran	Soybean hulls	Rapeseed meal
ρ_b of sample at moisture content 0%	0.22 g/mL	0.36 g/mL	0.50 g/mL
ρ_b of sample at moisture content 11%	0.26g/mL	0.33 g/mL	0.50 g/mL
ρ_b of sample at moisture content 65%	0.39 g/mL	0.32 g/mL	0.79 g/mL
ρ_p of sample at moisture content 0%	1.29 g/mL	1.49 g/mL	1.29 g/mL
ρ_p of sample at moisture content 11%	1.25 g/mL	1.41 g/mL	1.27 g/mL
ρ_p of sample at moisture content 65%	1.13 g/mL	1.14 g/mL	1.13 g/mL
ε of sample at moisture content 0%	82.45 %	75.57 %	60.42 %
ε of sample at moisture content 11%	81.96 %	76.32 %	61.51 %
d_{gw} at moisture content 11%	494.20 µm (0.0494 cm)	474.26 µm (0.0474 cm)	391.61 µm (0.0391 cm)
S_{gw} at moisture content 11%	1.04	2.21	2.31
Particles/gram at moisture content 11%	6.95×10^3 particles/gram	1.12×10^5 particles/gram	3.01×10^5 particles/gram
SA at moisture content 11%	241.51 cm^2/gram	122.98 cm^2/gram	168.54 cm^2/gram
VSA at moisture content 11%	301.99 cm^{-1}	173.40 cm^{-1}	214.0 cm^{-1}
WRV	263.55 %	164.56 %	132.36 %
τ (dimensionless)	1.35 (MC: 65% ε: 65.95%)	1.18 (MC: 65% ε: 79.97%)	1.50 (MC: 65% ε: 61.69%)

If the air entering the SSF bioreactor is not saturated with water, this will cause evaporation and drying out the fermented bed. As a result, the water activity will decrease to an unacceptable level, unfavourable for fungal growth and products formation. In order to minimise evaporation, saturated air should be sparged at the air inlet, which prevents air humidity from being an operating variable and provides more consistent inlet air humidity. It is important to keep in mind that the use of saturated air does not prevent evaporation from occurring within the fermented bed. However, it does minimise evaporation when compared to the use of unsaturated air.

In this study, it was observed that the humidifier system developed did not achieve 100% saturation, but at one point it successfully created moistened air – enough to supply some water into the fermented bed. Although the amount of water vapour in the air is low, it plays a major role in SSF processes. Therefore, it is an important consideration in air-cooling applications. It is impossible to prevent evaporation from occurring in a SSF system even if the air supplied to the fermented bed is saturated.

CHAPTER 7

ESTIMATING GROWTH IN SOLID STATE FERMENTATION

"Without doubt, the single most vital yet most problematical value sought during fermentation is biomass estimation"
Carleysmith and Fox (1994)

7.1   INTRODUCTION

Biomass is a fundamental parameter in the characterisation of microbial growth. In solid state fermentation (SSF), estimation of biomass, which is essential for kinetic studies, poses difficulties as generally the microbial cells remain attached with the substrate and it is difficult to separate them. In the case of fungal cultures, it is more difficult because fungal mycelia penetrate and tightly bind to the solid substrate particles. There are several indirect techniques to estimate biomass as discussed in Chapter 2; however, almost every method has its own drawbacks. Because direct estimation of biomass is difficult, the following global stoichiometric equation is used.

$$Carbon\ source +\ O_2 + H_2O + P + N_2\ \rightarrow biomass\ +\ CO_2 + metabolites + heat \qquad (7.1)$$

Measuring any one of the above components allows for determining the evolution of others if all the coefficients are maintained constant. In this chapter, four common techniques in addition to one new technique were experimented with to estimate biomass in SSF. The data obtained form every technique will be discussed and compared in terms of ease, comparable, meaningful to describe fungal growth. For colour techniques, biomass estimation was also carried out in SmF with the presence of solid particles to expand the technique.

The four common techniques were: organic matter loss, dry weight reduction ratio, biomass monitor and metabolic measurements (oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and heat evolution). The new proposed technique in this study is colour change during SSF as an indicator of fungal growth.

The main objective of this chapter is to evaluate relatively simple techniques to identify fungal growth phases in SSF processes. The techniques used to estimate biomass in the present study are classified in this chapter according to strategy or concept as opposed to discussing individual techniques, to observe in selecting an approach to a new problem or situation.

7.2   MATERIALS AND METHODS

7.2.1   Microorganisms, Inoculum and SSF

The detailed process of microorganism, inoculum and SSF preparation is described in Chapter 4 and 5. Briefly, the progeny obtained from monospore isolation of *A. awamori* and *A. oryzae* described in Section 5.2.2 was used. Fungal spores in universal bottle were stored at 4 °C in agar slopes of solid sporulation medium containing 5% (w/v) whole wheat flour and 2% (w/v) agar as a stock culture. Standard procedures for inoculum preparation and transfer for solid substrate SSF described in Section 4.1 were followed.

7.2.2   Biomass Measurement

7.2.2.1   Organic matter loss, dry weight reduction ratio and biomass monitor

Sample preparation for organic matter loss, dry weight reduction ratio and biomass monitor were carried out according to the method described in Chapter 4 (Section 4.3.1, 4.3.2 and 4.2.3, respectively). The experiments were carried out in petri dishes. In case for biomass monitor technique, biomass estimation also was carried out using a sample from SmF in the presence of solid substrate particles. For a standard calibration of biomass monitor, *S. cereviasie* was used at different concentrations growing in SmF.

7.2.2.2   Metabolic measurements

The data obtained from 4 tray SSB systems (Chapter 10) which performed with *A. awamori* and *A. oryzae* on wheat bran, moisted air at flow rate 2 L/min were used to elaborate fungal growth based on O_2 uptake rate (OUR), CO_2 evolution rate (CER) and heat evolution. Details bioreactor operational and measurement of oxygen, carbon dioxide concentration at gas outlet and temperature were described in Chapter 4 (Section 4.5.5).

7.2.2.3   Colourimetric technique

Sample preparation and procedures involved for colour extraction from fermented solid substrate were carried out according to the method described in details in Chapter 4 (Section 4.3.5)

In this study also, an attempt was made to extract the colour from fermented substrate. Fermented substrate of *A. awamori* on wheat bran, soybean hulls and rapeseed meal were used in this study. SSF was carried out at different initial moisture contents and inoculum sizes. For this purpose, three types of solvent (water, perchloric acid and enzyme solution) and different treatments were evaluated for each procedures separately. Different extraction procedures employing different approaches described in details in TABLE 7.1:

TABLE 7.1: Extraction procedures for colour from fermented mass

Procedure	Description
Procedure A	Extraction with distilled water, with shaking at 250 rpm, 1 h, and 30 °C
Procedure B	Extraction with distilled water, samples boiled for 20 min, shaken at 250 rpm, 1 h, 30 °C
Procedure C	Extraction with 1 M perchloric acid, shaken at 250 rpm, 1 h, 30 °C
Procedure D	Extraction with 1 M perchloric acid, samples boiled for 20 min, shaken at 250 rpm, 1 h, 30 °C
Procedure E	Extraction with distilled water, samples autoclaved at 121 °C, 15 min
Procedure F	Treatment with glucanase enzyme (5XL Biocatalysts) (Enzyme activity – 3000 U/mL, 50 °C, 1 h)

Extraction was performed to select the best and practical procedure to extract colour in fermented substrate. After a suitable dilution, the absorbance of colour was then determined by UV-Vis specrophotometer at wavelength 300 nm. This was transformed in unit absorbance (in UA_{300}/g) relative to substrate dry mass, multiplying the absorbance by dilution factors and dividing by the substrate dry mass on fermentation medium. This method permits direct comparison between different processes, since it reflects the amount of coloured matter produced by SSF.

7.3   RESULTS AND DISCUSSION

Many efforts have been made to find a reliable and sensitive method that is convenient to use at every stage during SSF for biomass estimation in a way that better describes the growth of microorganisms. It is often desirable to know the current biomass or the quantity that will be present in the future if a product is subjected to particular treatments (Scotti *et al*., 2001). Thus, by measuring fungal growth once using a desirable method, one could tell how a future population will develop under the same conditions (even though in SSF this scenario is impossible due to the nature of the growth environment).

From the literature, it is clearly demonstrated that studying microorganisms in controlled, well-phased cultures is of great importance. Above all, studying the early developments of the culture is particularly important because it is this phase, together with the physiological state of the microorganism, that determines the later stages of development (Desgranges *etal*., 1991). Measuring growth through a direct or an indirect technique is also important to provide a better understanding of process control and also to give an idea regarding the productivity of a cell culture (Borzani, 2000). Normally, estimation of organic matter loss, dry weight, spores count and glucosamine content are made by taking samples intermittently and carryig out laboratory analysis.

Biomass estimation is of fundamental importance in many microbial fermentation processes. However, this estimation is very difficult to make when solid substrate particles are present and bind to the cells. This chapter examines and evaluates five common techniques that have been used to estimate biomass in SSF and one new technique proposed based on colour changes of fermented substrate during fermentation. Preliminary SSF using petri dishes and four tray SSB systems was carried out in order to study the growth of *A. awamori* and *A. oryzae*.

7.3.1   Organic Matter Loss

Because of the difficulties associated with direct measurement of fungal biomass in an SSF system, the organic matter loss technique described in this chapter may improve on studies of biomass estimation. This is because it may identify the fungal biomass growth phases and also it easily describes fungal growth curve profile.

FIGURE 7.1 shows the profile of organic matter loss for *A. awamori* and *A. oryzae* preparations on three solid substrates under study. The loss of organic matter followed a typical growth curve

indicative of its relationship with biomass formation. Both fungi caused variable loss in organic matter during SSF which depended on the solid substrate. Different fungi were consistent in their rate of consumption during the 12 day fermentation. For *A. awamori*, the loss of organic matter with 65% moisture content was obtained 74.1%, 59.1% and 54.2% for wheat bran, soybean hull and rapeseed meal, respectively. For *A. oryzae*, rapeseed meal, wheat bran and soybean hull recorded 73.2%, 64.1% and 50.2% loss of organic matter respectively after 12 days of fermentation.
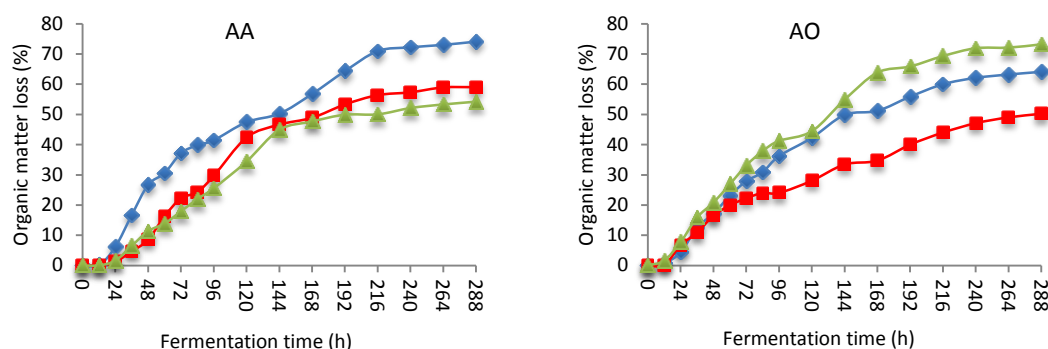
FIGURE 7.1: Growth profile of *A. awamori* [AA] and *A. oryzae* [AO] based on organic matter loss. (◆) wheat bran; (■) soybean hulls and (▲) rapeseed meal

This study was carried out in a petri dishes and in this kind of system, it is assumed that there is a limitation in oxygen supply to the cells due to the close contact with the solid substrate, which is demanding for substrate utilisation. According to Rahardjo *et al*., (2001) mainly 70% oxygen is taken up by the aerial hypahae of *A. oryzae* in SSF. The nature system of SSF contribute another reason a lack of oxygen supply to the penetrative hyphae during growth on a solid substrate (Oostra *et al*., 2001). The variation in organic matter loss in the present work can be attirubuted to the difference in efficiency of the strains and the variation in cell components and metabolites during different phases of fungal growth.

7.3.2   Dry Weight Reduction Ratio

In the process of estimating weight reduction ratio, the instantaneous moisture content for both non-fermented and fermented substrate need to be recorded. The moisture content in fermented wheat bran has the same pattern with non-fermented wheat bran. The moisture content in both media started to decrease only after 200 h of incubation time. The moisture content in fermented wheat bran was slightly higher in non-fermented wheat bran since there is more heat generated from microbial activity. The fermentation heat can only dissipate at the lid surface and internal vapor

condensation occurs because of the closed system. Water was consumed when enzyme hydrolysed substrate components to supply nutrients for fungal growth. Although water consumption should continue to increase alongside the development of fungal growth and hydrolytic enzyme activity in the fermenting solids, gradual recovery suggested increase in metabolic water production sustained by the accumulation of fungal biomass. This can be seen in FIGURE 7.2 where after 200 h of fermentation time, moisture content started to decrease and fungal activity became slow and fungal growth reached a stationary phase (FIGURE 7.3).
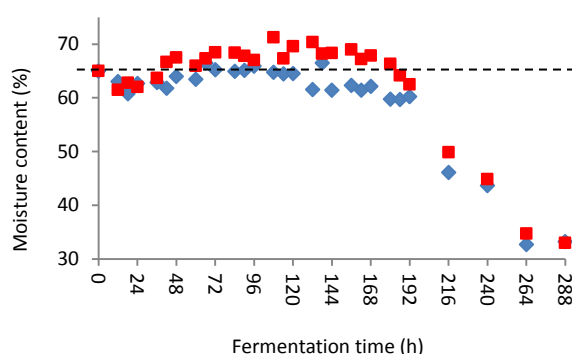
FIGURE 7.2: **Moisture profile in non-fermented (◆) and fermented wheat bran (■). Dashed line refers to initial moisture content 65%**

FIGURE 7.3 shows that subsequent exponential growth of *A. awamori* on wheat bran was signalled by the increase in dry weight reduction ratio. Fungal growth has a very long log phase between 48 and 200 h, a growth deceleration phase between 200 and 275 h and a stationary phase beyond 275 h. Fungal growth started to decelerate at about 200 h and entered into a stationary phase at about 240 h.
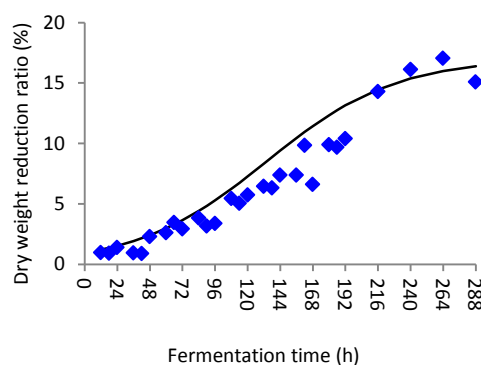
FIGURE 7.3: **Growth profile of *A. awamori* represented by dry weight reduction ratio (%). (◆) from experimental data (Equation 4.2, Chapter 4) and (——————) the solid line obtained by fitting with Equation 7.5**

The basic equations for growth:

$$dX = \frac{\mu X}{dt} \qquad (7.2)$$

$$\mu = \frac{1}{X} \times \frac{dX}{dt} \qquad (7.3)$$

A widely used relationship for μ is the following expression proposed by the equation:

$$\frac{dX}{dt} = \mu = \mu_{max} \left(1 - \frac{X}{X_{max}}\right) \qquad (7.4)$$

Integrating with X = X (0) at t = 0, this gives:

$$X(t) = \frac{X_{max}}{1 + [(X/X_0) - 1]\, e^{-\mu_{max}t}} \qquad (7.5)$$

Where;

X_0 = the intial of microbial biomass concentration (percentage dry weight reduction)

X_{max} = the maximum of microbial concentration (percentage dry weight reduction)

t = time (h)

μ = the specific growth rate (h^{-1})

μ_{max} = the maximum specific growth rate (h^{-1})

In order to consider theoritically the mode of growth of *A. awamori*, a log curve was introduced. By assuming a linear relationship between dry weight reduction ratio and fungal cell concentration, the integrated form of the log model (Equation 7.5), as proposed by Wang *et al.* (2010), satisfactory described the profile of dry weight reduction ratio. As a result, FIGURE 7.3 showed an accurate fit of the log model to dry weight reduction ratio experimental data. The exponential growth phase generated a specific growth rate (μ) of 0.02 h^{-1} and a maximum dry weight reduction ratio of 17.03% with a regression coefficient (R^2) of 95.35%. It was ascertained that the mathematical model was accurate in the case of wheat bran. Hashemi *et al.*, (2011) investigated a feasible approach for modelling the phases of the bacterial growth curve and production of α-amylase by *Bacillus* sp. in a SSF process based on variation in dry weight. However, according to Terebiznik and Pilosof (1999), even though this technique is able to estimate biomass in SSF, this is particularly true in the case of fermentation performed without forced aeration.

7.3.3   Biomass Monitor

7.3.3.1   Capacitance measurement using biomass monitor in SmF

At first, SmF was carried out using *S. cereviasiae*. Correlation was made between dry cell weight and capacitance measurement as shown in FIGURE 7.4. The result obtained showed there is a strong correlation between the two parameters tested.
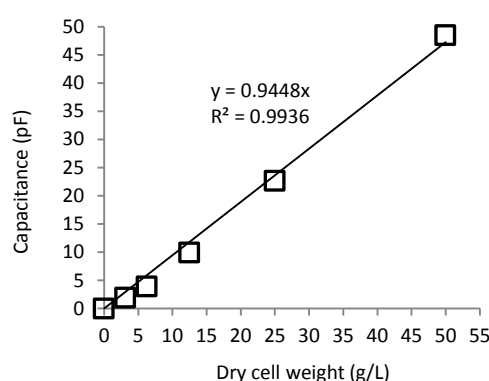
FIGURE 7.4: Correlation between dry cell weight and capacitance of *S. cereviasiae*

Fehrenbach *et al*., (1992) reviewed that a linear correlation was found between the on-line capacitance measurement and the off-line measurement (optical density, packed mycelial volume, biomass concentration and colony forming units). Biomass monitor was proved to be a suitable method for biomass estimation and this was reviewed in Chapter 2 (Section 2.9.7) adapted from previous experiments (Kell *et al*., 1990; Harris *et al*., 1987).

Details of SmF are described in Chapter 4 (Section 4.2.2). Briefly, SmF was performed using shake flasks containing 15.0 g whole hard wheat flour with a working volume of 150.0 mL distilled water without adding any nutrients. The media were autoclaved at 121 °C for 15 min. Spore suspension with concentration of 1 x 10^6 spores/mL was used as inoculum and the flasks were kept shaking at 30 °C, 250 rpm. Fermentation was carried out for 12 days. At defined time intervals, samples were collected for online measurement of capacitance, biomass, dry cell weight and glucoamylase activity in order to describe the growth profile of *A. awamori* in SmF.

At beginning, capacitance measurement of biomass was conducted at different frequencies ranging between 500 and 1,000 kHz. The purpose of this analysis is to determine the suitable frequency for

biomass in fungal mycelium form. The method is based on measurements of the dielectric permittivity at radiofrequencies, using a four-terminal instrument. In all cases, excellent linearity was observed during the growth phase as shown in FIGURE 7.5. In this study, the results have shown that it is possible to measure biomass accumulation by exploiting the radiofrequency dielectric properties of mycelium suspensions. The great frequency for fungus in SmF was found suitable at 600 Hz.
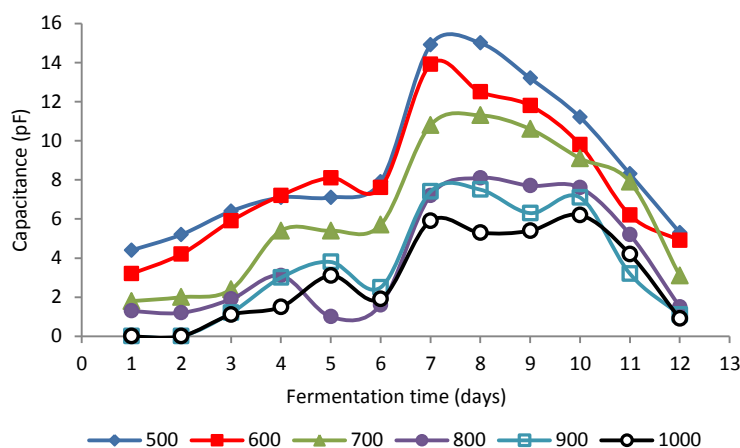
FIGURE 7.5:   **Capacitance measurement of the accretion of *A. awamori* biomass during submerged fermentation in whole hard wheat flour at different frequencies (kHz)**

The results of off-line analyses of dry cell weight and glucoamylase activity as compared with results of capacitance measurement (at 600 kHz) for *A. awamori* fermentation are shown in FIGURE 7.6. Dry cell weight and glucoamylase activity increased approximately linearly with fermentation time. The online and off-line results of SmF for *A. awamori* might be interpreted that there is an excellent linear relationship between the capacitance change in pF and the dry cell weight and glucoamylase activity (U/mL) to describe the growth profile during the process.

FIGURE 7.6 shows that subsequent exponential growth of *A. awamori* on wheat bran was signalled by the increase in dry cell weight, glucoamylase activity and capacitance. Fungal growth has a log phase between 2 to 4 days (dry cell weight and glucoamylase activity) and between 2 to 6 days (capacitance). No stationary observed in glucoamylase activity and fungal growth started to decelerate after day 5. Clear stationary phase observed in dry cell weight profile (5 to 9 days) and a growth deceleration phase beyond 9 days. For capacitance, fungal growth started to decelerate at about 8 days and entered into a death phase at about 11 days.
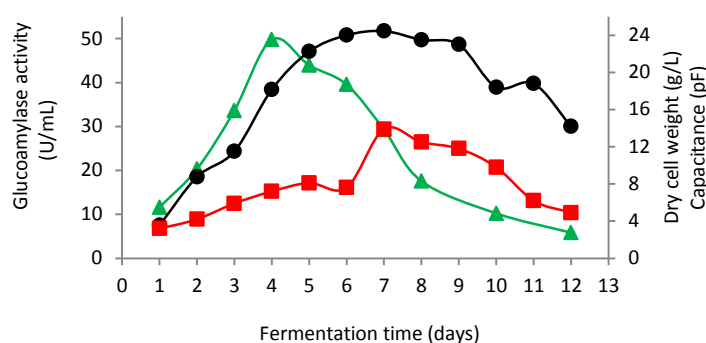
FIGURE 7.6: Profiles of capacitance measurement, dry cell weight and glucoamylase activity during growth of *A. awamori* in submerged fermentation. Symbols represent experiments using the substrate whole hard wheat flour: (■): capacitance; (●) dry cell weight and (▲) glucoamylase activity

Capacitance provides a unique, reliable, reproducible and above all online measurement of both homogenous (uni-cellular) and heterogeous (mycelial) cultures in bioreactors (Fehrenbach *et al*., 1992; Harris and Kell, 1985). Indeed, capacitance measurement was reported to possibly be the most precise method for studying on biomass formation during process optimization in SmF (Sonnleitner *et al*., 1992; Kell *et al*., 1990). Kell *et al*., (1994) reviewed that this patented device has been very successfully exploited in media and in systems of substantial conductivity at laboratory and industrial scales including bacterial and yeast fermentations, bacterial biofilms, immobilised cells, filamentous organisms in liquid and SSF, in both human blood and animal cell cultures in isotonic media.

7.3.3.2   Capacitance for biomass measurement in SSF

To prove this, SSF of *A. awamori* using wheat bran as a substrate was carried out by applying three initial moisture ratios with measurement of capacitance. The biomass monitor measured values for capacitance; however, the results obtained fluctuated. Some measurements returned a negative reading for capacitance even though visual inspection indicated significant fungal growth on the substrate. On the other hand, some measuremnts returned a positive reading for capacitance for poor grow on the substrate. Therefore, the results of capacitance measurement did not show any pattern to reflect fungal growth in SSF. FIGURE 7.7 shows an example of the results from the experiments of capacitance measurement. The results showed that the biomass monitor may not work for biomass measurement in SSF. Biomass monitor may be suitable in SmF but not in SSF in this case.
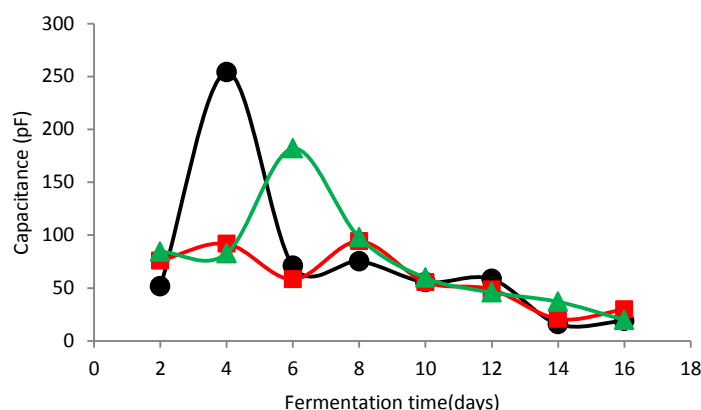
FIGURE 7.7: **Time course of online capacitance measurement of biomass during growth of _A. awamori_ on wheat bran in SSF. Symbols represent experiments with different initial moisture ratios: (●): 45%; (■): 60% and (▲): 75%**

The capacitance is still not proven as a way to evaluate the biomass concentration and needs more studies for its utility to be established. One of the determinants that should be studied further in the process of capacitance measurement in SSF is sample preparation. The excellent results seen with the biomass monitor in SmF, where the cells in liquid form, are promising. Liquid conditions allow the development of conductivity and permittivity to generate homogenously dielectric characteristics and connectivity between cells and the probe (Kell _et al_., 1990). The conditions are totally different in solid substrate. Some of the factors which affect the performance of the biomass monitor in SSF might be: (1) there is no homogeneity in substrate since the amount of liquid is very limited and (2) the presence of solid particles may be lead to disadvantageous conditions to the probe. Therefore, the biomass monitor does not seem reliable to evaluate biomass and growth in SSF.

7.3.4   Metabolic Measurements

It is possible to estimate biomass development in SSF by considering OUR, CER and heat evolved, which are easily measurable parameters. According to Mitchell _et al_. (2004; 2006), OUR and and CER offer the advantage of a fast response time and are directly linked to the metabolism of the microorganism. This is also true for the heat evolved during fermentation as a result of microbial metabolism (Harris and Kell, 1985). This can refer to the global stoichiometric Equation 7.1. FIGURE 7.8 and 7.9 show profiles of metabolic measurements (heat evolved and OUR, CER respectively) from a four-tray SSB.

Refering to FIGURE 7.8, during the first 28 h of SSF, the bed temperature profile increased rapidly to a range between 32 – 44 °C, which indicates that both fungi are extensively growing. A few hours later, a rapid bed temperature decrease was recorded after 30 h of fermentation. From the temperature curve (FIGURE 7.8), it can be asserted that the fungal growth consisted of 3 general stages. During the first 6 hours, it was assumed that both fungi take time to adjust upon finding themselves in a new environment. Initially, the number of cells does not increase. The first stage of fungal growth is a lag phase that lasted approximately 12 h when spore germination took place. At this stage cells may need to synthesise new enzymes and start the degradation of complex substances in the solid substrate. The second stage was an acceleration phase that lasted approximately 20 hours followed by an exponential growth phase. The exponential growth phase switched smoothly, after 30 hours of fermentation, to a decelerating phase. This also can be seen in the profiles of OUR and CER as shown FIGURE 7.9.
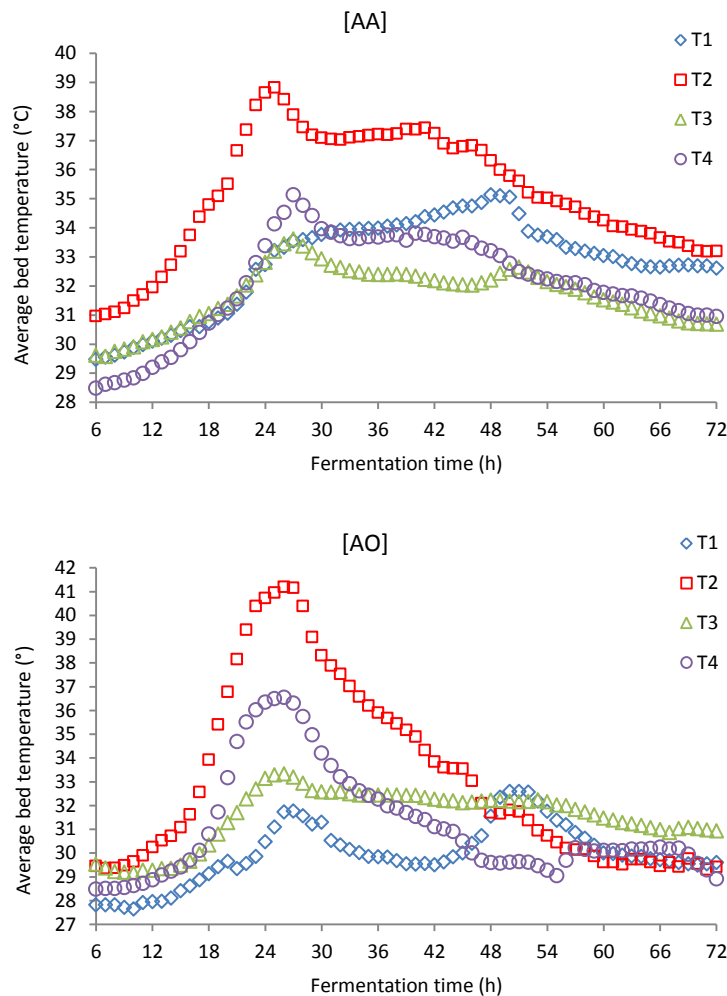
FIGURE 7.8: Indirect measurement of microbial growth rate of *A. awamori* [AA] and *A. oryzae* [AO] in four tray SSBs with respect to heat evolved, with moistened air at flow rate 2 L/min. (T1): single circular tray SSB; (T2): multi-stacked circular tray SSB; (T3): single rectangular tray SSB and (T4): multi-square tray SSB
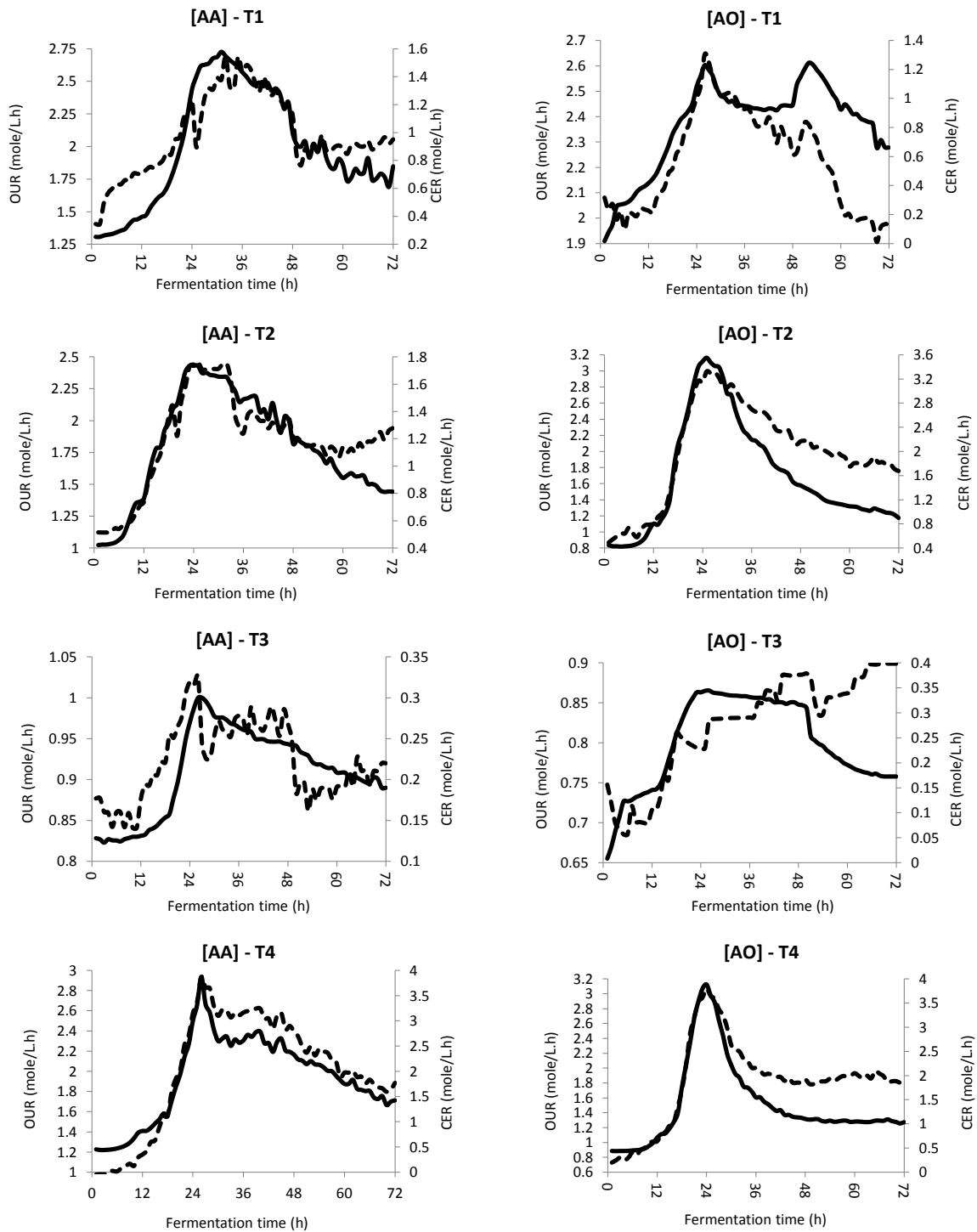
FIGURE 7.9: Indirect measurement of microbial growth rate of *A. awamori* [AA] and *A. oryzae* [AO] from four tray SSBs with respect to OUR (▬ ▬ ▬ ▬ ▬ ▬ ▬ ▬ ▬ ▬ ▬) and CER (▬▬▬▬▬▬▬▬▬▬) with moistened air at flow rate 2 L/min. (T1): single circular tray SSB; (T2): multi-stacked circular tray SSB; (T3): single rectangular tray SSB and (T4): multi-square tray SSB

Clearly reflected in OUR, CER and heat evolved curves, the stationary phase, for both fungi in all tray SSB systems tested, was very short and lasted for 1 – 2 h of fermentation time. The curves were not

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of a sigmoidal shape and reached the maximum value of O_2 consumption, CO_2 generation and heat evolution approximately between 24 – 30 h of fermentation. After this peak, the gradual decrease of heat and O_2 and CO_2 concentrations indicated the beginning of the last phase which corresponds to slower fungal growth. This is a very slow progress phase because the active spores still consume the O_2 (plus other nutrients) and produce CO_2 and heat. Ikasari and Mitchell (1998), using the *R. oligosporus* SSF process with rice bran as the substrate, observed similar results, reported as increase in OUR and heat evolved around 24 h and a significance decrease was reported at 72 h fermentation. In this case, it was considered that the high content of readily available carbon source in wheat bran (starch in this case) was the reason for the comparatively high OUR, CER and heat evolved indicating high fungal growth during the first 24 – 30 h of SSF. Also, in SSF, fungi cannot grow continuously as the amount of nutrients available is finite and waste products will accumulate. These conditions might be the reason for the deceleration of fungal growth after achieving maximum activity. Growth still continued at a slow rate and did not stop eventually because of the presence of nutrients. At the same time, inhibitory metabolites built up due to waste accumulation in the system. The temperature often reaches values which severely limit growth or even kill the microorganims (Mitchell *et al*., 2002). High temperature might affect spore germination and growth, and product formation. This can be observed in temperature profiles illustrated in FIGURE 7.8 where it was increased above 30 °C in all bioreactor system tested. This is might be the reason for the observation that the growth curves did not have a sigmoidal shape.

The main advantages and disadvantages of most of these techniques are discussed above and summarised in TABLE 2.7 (Chapter 2, Section 2.9.7). Some of these techniques are offline and usually apply under a limited range of conditions while being labour intensive. Of all the techniques discussed in this chapter, the metabolic measurement (OUR and CER) techniques are the easiest to operate online. If both values are used, it is possible to determine the respiratory quotient of the microorganism, which can provide information about the metabolic state (discussed in Chapter 10). Kabanova *et al*., (2012) developed a method for the investigation of bacterial growth, growth of colonies in solid-state matrices using isothermal microcalorimetry. They showed that microcalorimetry measurements was a very powerful instrument in studying quantitative detailed peculiarities of SSF and it is possible to calculate heats evolved during the growth with numbers of bacteria in the sample.

According to Kennedy *et al*. (1992), these techniques suffer from the disadvantage that substrate consumption and product formation rates will not always vary linearly with cell concentration, especially during the different stages of the fermentation. For example, during the stationary phase

the substrate consumption and product formation can occur at a rate that is different from that during exponential growth. Therefore, the measurement is not an accurate estimate of biomass. This suggests that the limitations of using this technique as a cell concentration measurement method must be carefully considered.

7.3.5   Colourimetric Technique

In this work, colourimetric technique is proposed as a tool with which to characterize and monitor fungal growth in SSF. The methodology proposed in this work was based on the colour solution extracted from fermented substrate determined by UV-vis specrophotometer at suitable wavelength. Two independent experiments (different initial moisture content and inoculume size) involving of two culture of fungi were analysed to observe the spectra features and to assess how the parameters obtained from the spectra vary as function of time.

The experiments were performed in three sets using three solid substrates (wheat bran, soybean hulls and rapeseed meal) with two filamentous fungi, *A. awamori* and *A. oryzae*. It is a general observation that the colour of the fermenting solid substrate changes during the course of the SSF due to the growth of the fungus. During the course of the investigation of fungal growth on solid substrate, it was found that the colour of the solid substrate became darker with progressive growth as shown in FIGURE 7.10 and 7.11.
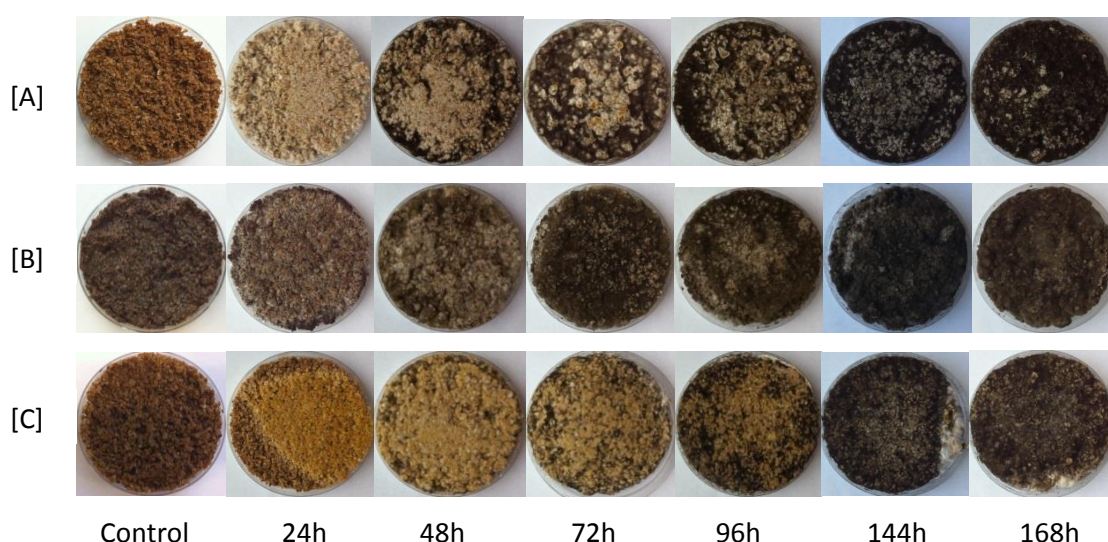
FIGURE 7.10:   Visual observation of the colour changes during the course of the SSF of *A. awamori* on three different solid substrates. [A]: wheat bran; [B]: rapeseed meal and [C]: soybean hulls
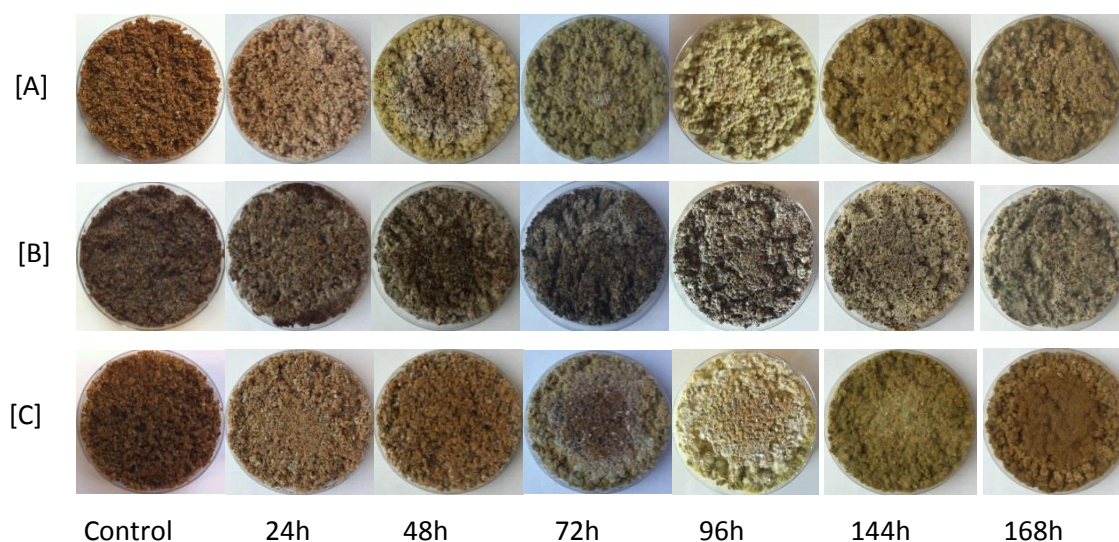
FIGURE 7.11:   **Visual observation of the colour changes during the course of the SSF of** *A. oryzae* **on three different solid substrates. [A]: wheat bran; [B]: rapeseed meal and [C]: soybean hulls**

For example, FIGURE 7.12 shows the colour changes with time when the fermented mass was extracted using distilled water. If this colour changes could be quantitatively related to fungal growth, it might become a new technique for the estimation of fungal growth in SSF, which is known to be very challenging. This colour change may be due to the formation of several pigments by the growing fungus. The colour change that occurs with the growth of biomass cannot be attributed to a single factor and a quantitative measurement of the change in different fungi is needed.
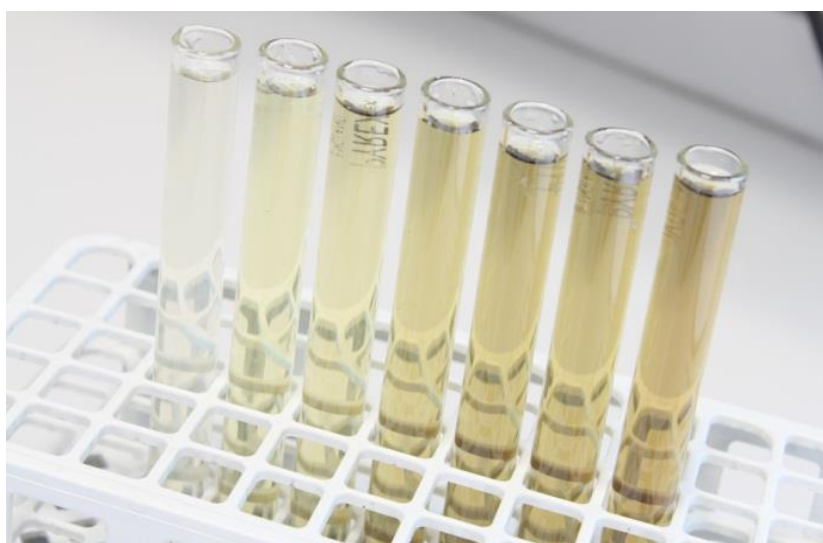
FIGURE 7.12:   **Water extraction of fermented mass during the course of SSF with** *A. awamori* **on wheat bran**

7.3.5.1   Preliminary petri dish experiments

At first, the colour was extracted from fungi (spores and mycellium), non-fermented substrate and fermented substrate using distilled water with shaking at 250 rpm for 1 h. Details of the standard procedures for colour extraction are explained in Chapter 8. This colourimetric technique explores the growth behaviour of *A. awamori* and *A. oryzae* from the perspective of multiwavelength UV-vis spectrophotemetric. The sample information is obtained from the spectrophotometric analysis of colour solution measured over a broad range of wavelengths (200 – 800 nm). The data were then analysed to observe the variability in spectrum features and to assess how the parameters obtained from the spectra vary as function of fermentation time.

FIGURE 7.13 shows the typical normalised optical density spectra of colour solution measured for *A. awamori* and *A. oryzae* growing on wheat bran, soybean hulls and rapeseed meal at different growing stages. Experimentally, it is demonstrated that the spectra signatures of the both fungi evolves as a function of time. The colour density increased with time which indicates the progressive growth of both studied fungi in the course of SSF. Optical density showed a maximum peak between 260 – 300 nm wavelength for all samples extracted at different incubation times. The optical density spectra obtained also showed identical features for each colour solution extracted from each substrate. From the theoretical point of view, it is demonstrated that the spectra signatures could be adequately represented an interpretation model based on spectrophometric theory. The parameters from the interpretation spectra reflect changes in cells fungus and chemical composition known (for example colour in this case) to take place in the fungal during growth.
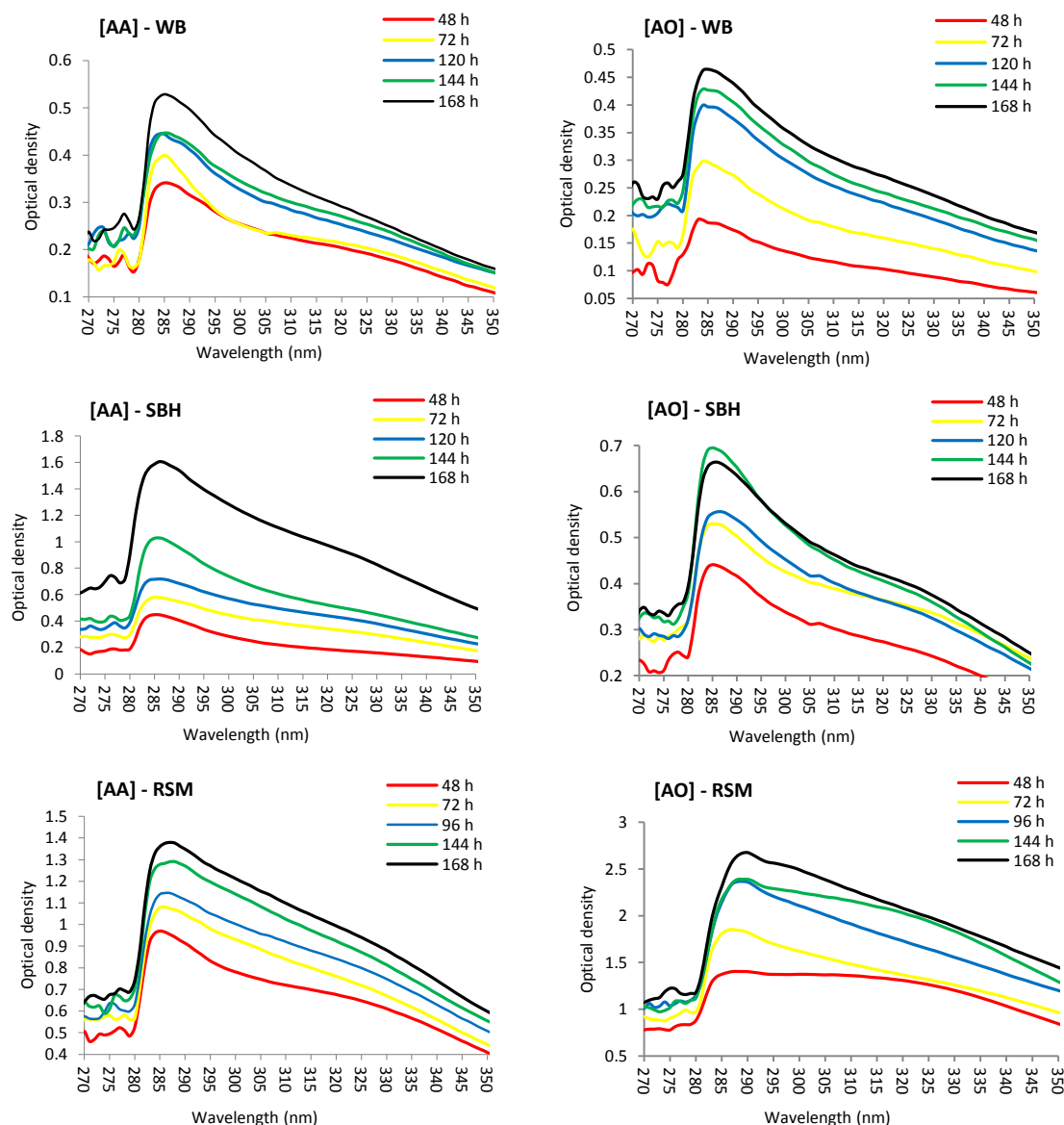
FIGURE 7.13: **Comparison of typical normalised optical density spectra measured from colour solution extracted from fermented mass as function of time for [AA]:** *A. awamori* **and [AO]:** *A. oryzae***. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal**

FIGURE 7.14 shows typical normalised optical density spectra measured for non-fermented substrate, fermented substrate and the fungi (spores and mycelium). The spectra for non-fermented substrate were different from those for fermented mass and those for fungi. These spectra clearly showed that there was a new component produced as a result of microbial activity of fungi during fermentation on the solid substrate. Notice the peak around 300 nm, which is generally (but not necessarily), attributed to the maximum absorption of new component from fermented substrate. This new component might be responsible for the colour characteristic reflected in the spectra.

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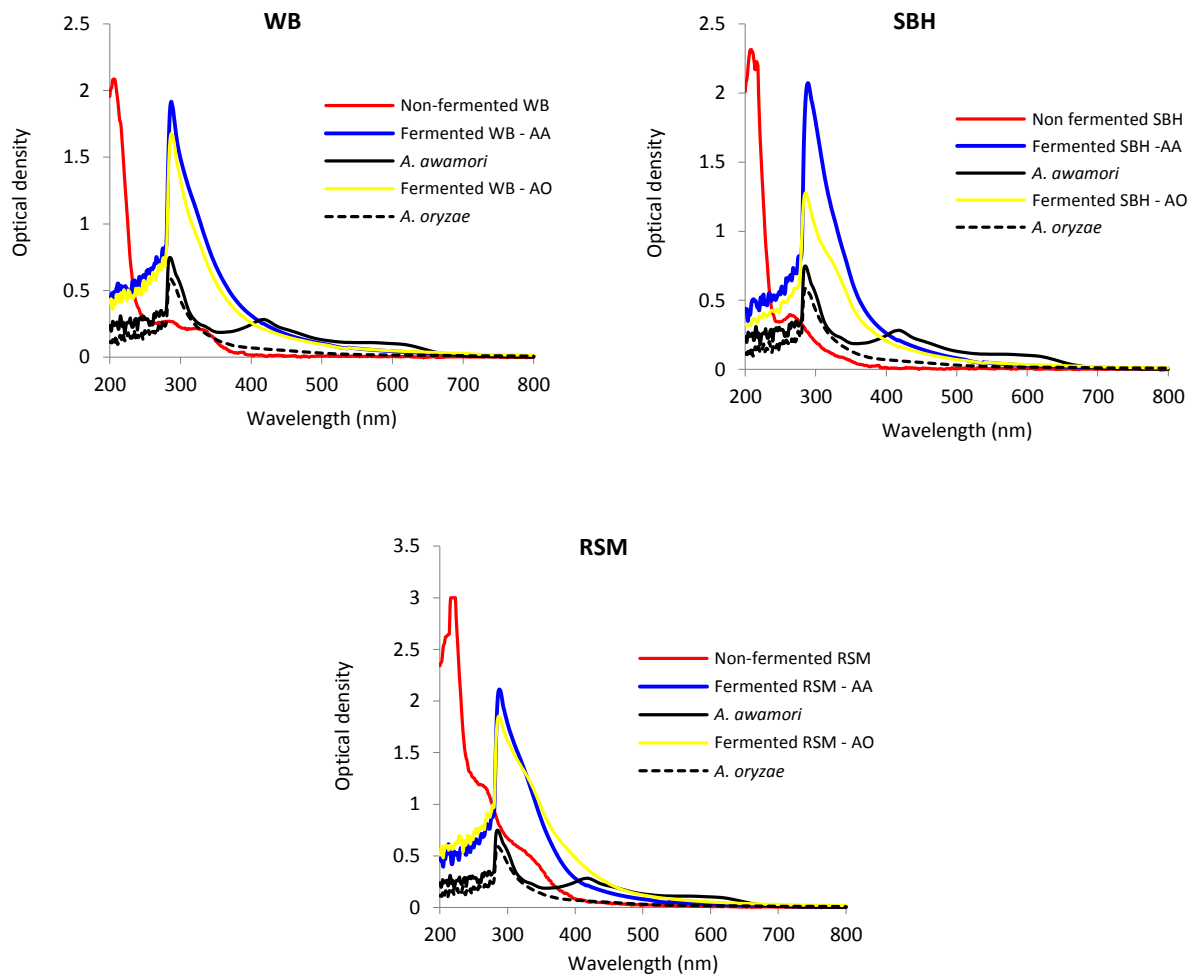
FIGURE 7.14: Comparison of typical normalised optical density spectra measured from colour solution extracted from non-fermented substrate, fermented substrate with *A. awamori* [AA], fermented substrate with *A. oryzae* [AO]; fungus *A. awamori* and *A. oryzae* (7 days old, grown on whole wheat flour agar. [WB]: wheat bran; [SBH]: soybean and [RSM]: rapeseed meal

Colour solutions extracted from fermented substrates at different times of fermentation were used to measure the abosorbance at different wavelengths; 260, 280 and 300 nm. FIGURE 7.15 shows the profiles of colour measurements at three different wavelenghts. This was carried out to decide the wavelength which is more representative of the colour change. In FIGURE 7.15, the colour concentration gradually increased at the same rate when measured at 260, 280 and 300 nm.

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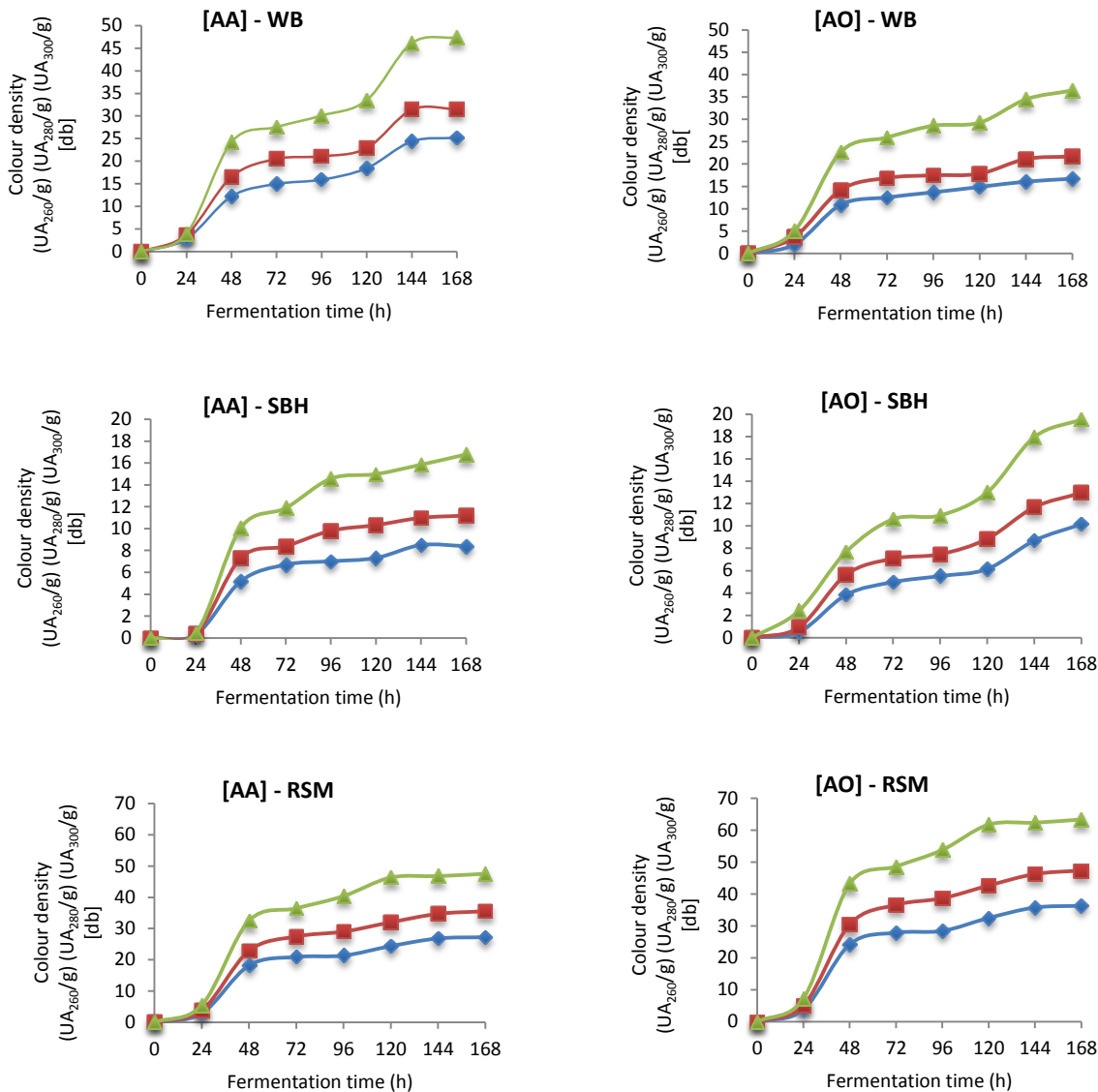
FIGURE 7.15: Growth profiles of *A awamori* [AA] and *A. oryzae* [AO] on three different solid substrates based on colour estimation at different wavelengths, expressed as unit absorbance (UA/g) [dry substrate]. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal. (◆): 260 nm; (■): 280 nm and (▲): 300 nm

The growth curve for a microorganisms population is usually divided into four phases: lag phase, exponential phase or log phase, stationary phase and death phase. Notice that the measured optical density at 260, 280 and 300 nm, follows the expected behaviour growth curve. However, death phase was not clearly observed in this work. This is might be because of the SSF was carried out only 7 days and need to prolong following the nature of SSF. Clearly, the changes in the colour of fermented substrate during SSF can be related to the changes of the cells population. The cells population developed on fermented substrate could be grouped in changes in number of cells (spores), cell size (hyphae elongation), shape (morphology), chemical composition (colour in this

work) and changes in the internal structure (can be observed under scanning microscope). In principle, all these differences may be detected through multiwavelength spectrophotometric measurements.

7.3.5.2   Effect of extraction techniques on the quality of colour from *A. awamori*

In order to study the utility of the colourimetric technique proposed in this study, the pigment responsible for the colour needs to be efficiently extracted from the fermented substrate. Also, it is important to define optimal conditions of extraction for further colourimetric technique development. As mentioned earlier, colour extraction procedures were performed using different solvents and treatments to obtain the colour solutions. The selection of a suitable of initial moisture content and inoculum size were considered as the most important parameters in the optimisation of SSF. Both parameters selected in this exercise were observed to influence physical and morphological properties of the fermented substrate.

7.3.5.2.1   *Effect of intial moisture content*

FIGURE 7.16 show images of the fungus *A. awamori* growing on wheat bran, soybean hulls and rapeseed meal respectively after 72 h of fermentation. Visual observation showed that different initial moisture contents led to different morphology of fungal growth, which most likely represents different degrees of growth. Through visual observation, an initial decision can be made concerning which initial moisture content is favourable for the growth of *A. awamori*; based on colour appearance on the fermented surface, identification of good and poor growth can be made.
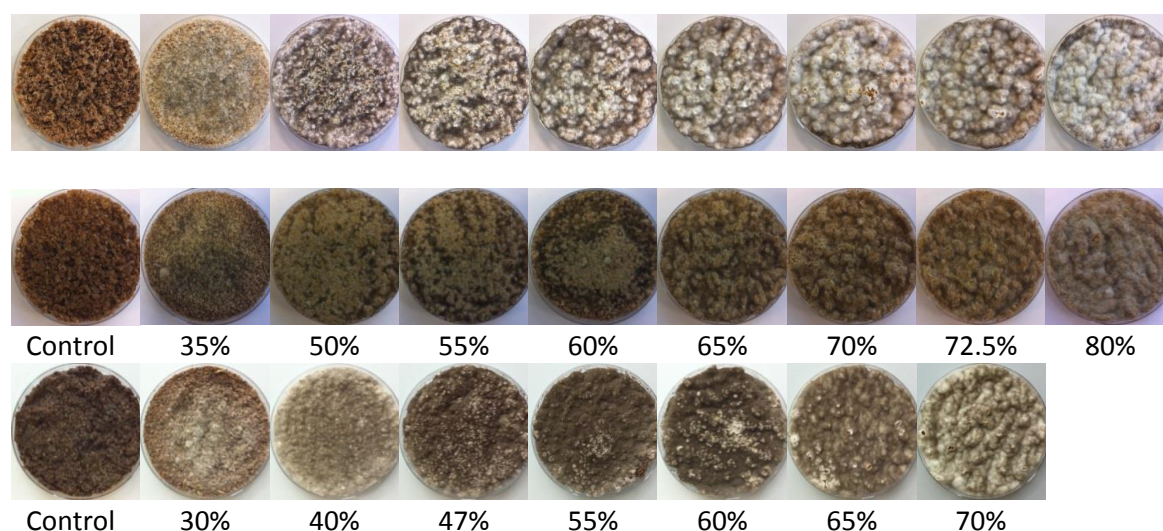
First line: Fermented solid of wheat bran
Second line: Fermented solid of soybean hulls
Third line: Fermented solid of rapeseed meal

FIGURE 7.17:   Visual observation of the course of the solid state fermentation of *A. awamori* on different initial moisture contents. Image taken from the top of the surface of fermented solid. Control experiment with initial moisture content of 65%

The effect of extraction techniques on the colour density values of fermented *A. awamori* at different initial moisture contents is shown in FIGURE 7.18 (wheat bran, soybean hulls and rapeseed meal, respectively). High colour concentration was observed in initial mositure content ranging between 35 – 65%. For colour production measurement, the extraction processes for the three substrates under study which resulted in high absorbance reading at 300 nm are are listed in TABLE 7.2.

TABLE 7.2:   Performance of colour extraction with different extraction procedure regards to high concentration of colour extracted

Fermented solid substrate	Colour concentration	
	Initial moisture content	Inoculum size
Wheat bran	F > D > C > E > B > A	F > D > C > E > B > A
Soybean hulls	E > B > D > A > C > F	F > E > C > D > B > A
Rapeseed meal	C > F > E > D > A > B	C > F > E > D > A > B
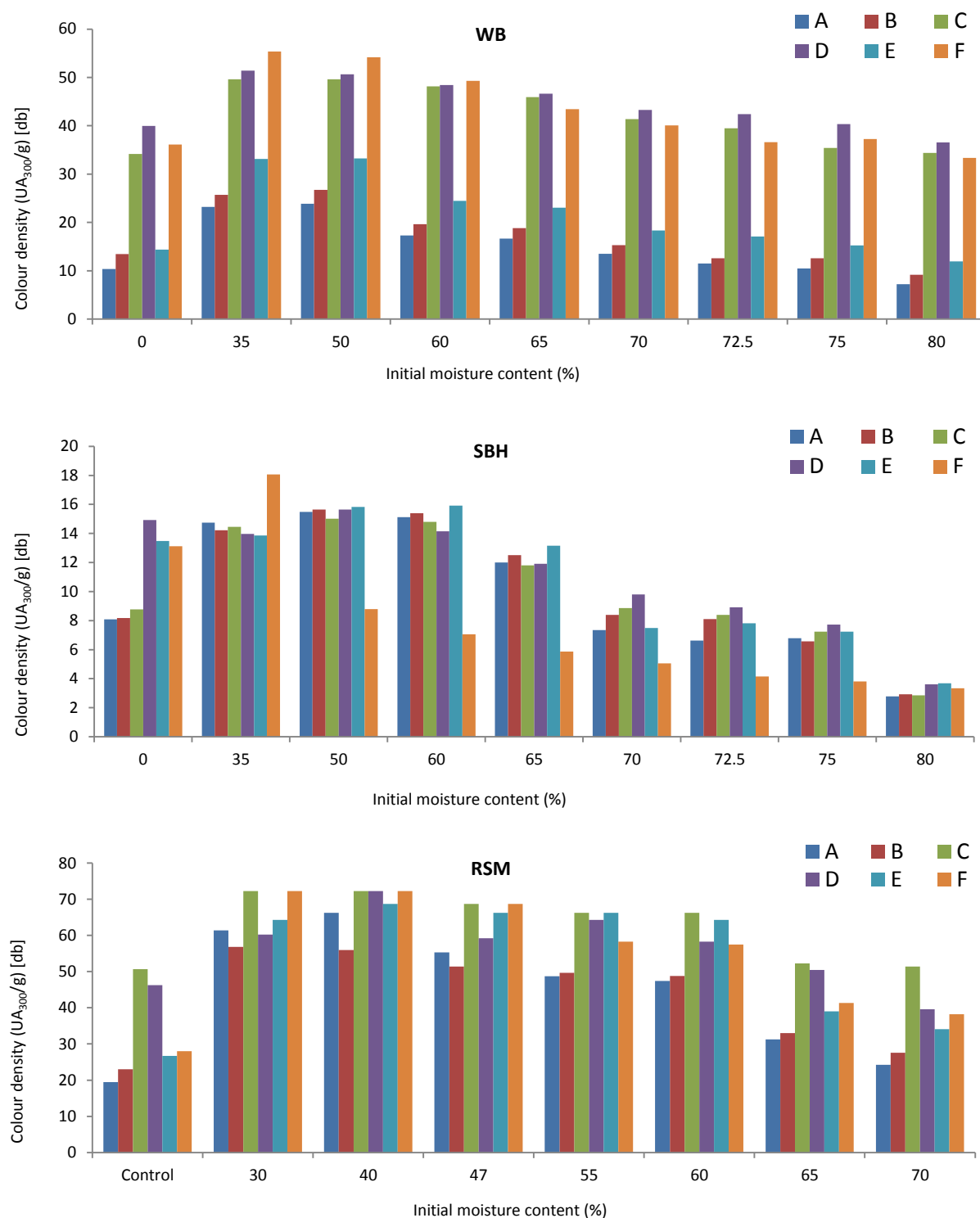
FIGURE 7.18: **Effect of different extraction techniques for extracting pigment from *A. awamori* fermented wheat bran [WB], soybean hulls [SBH] and rapeseed meal [RSM] at different initial moisture contents.** *[Procedure A]: distilled water, 250 rpm, 1h, 30 °C; [Procedure B]: A + boiling 20 min; [Procedure C]: 1M perchloric acid, 250 rpm, 1h, 30 °C; [Procedure D]: C + boiling 20 min; [Procedure E]: A + Autoclaving, 121 °C, 15 min and [Procedure F]: enzyme treatment*

7.3.5.2.2   Effect of inoculum size

FIGURE 7.20 shows image of the fungus *A. awamori* growing on wheat bran, soybean hulls and rapeseed meal respectively after 72 h of fermentation at different inoculum sizes. Visual observation shows that growth was considerable at all inoculum sizes tested and it is difficult to decide which of the inoculum sizes corresponds with the best growth.

| 3.6 x 10^4 spores/g | 1.67 x 10^5 spores/g | 4.4 x 10^5 spores/g | 1.2 x 10^6 spores/g | 1.33 x 10^6 spores/g | 2.39 x 10^6 spores/g | 4.57 x 10^6 spores/g |

First line: Fermented solid of wheat bran
Second line: Fermented solid of soybean hulls
Third line: Fermented solid of rapeseed meal
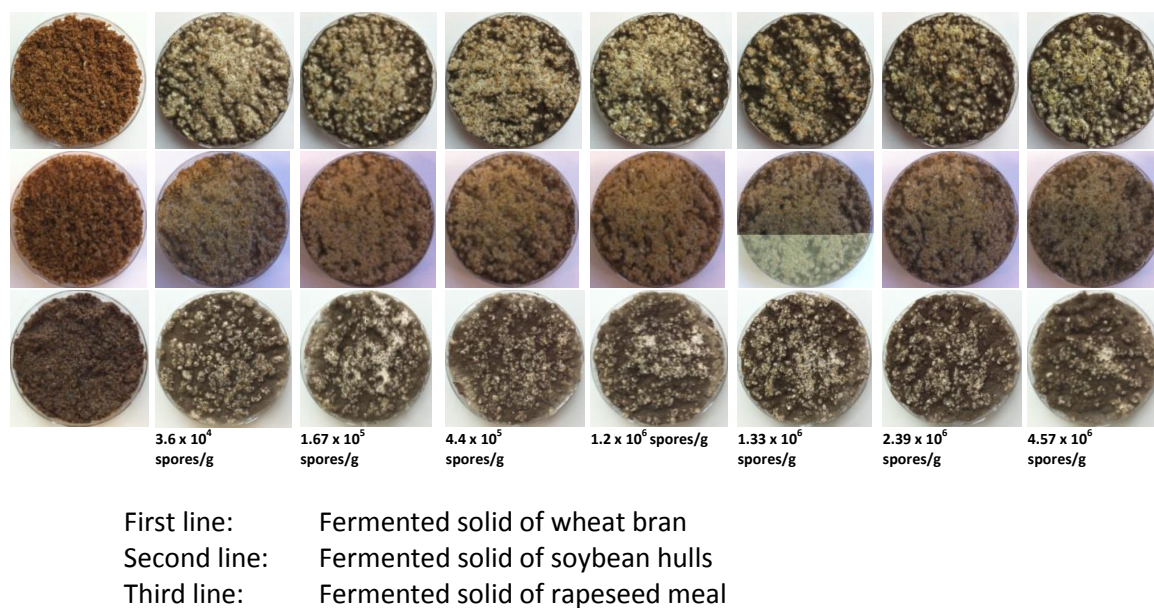
FIGURE 7.19:   Visual observation of the course of the SSF of *A. awamori* on wheat bran, soybean hulls and rapeseed meal at different inoculum sizes and an intial moisture content of 65%. Image taken from the top of the surface of fermented solid. Control experiment with initial moisture content of 65%

The effect of extraction techniques on the colour density values of fermented *A. awamori* is shown in FIGURE 7.20 (wheat bran, soybean hulls and rapeseed meal, respectively). For colour production measurement, the extraction processes for the three substrates under study which resulted in high absorbance reading at 300 nm are are listed in TABLE 7.2.
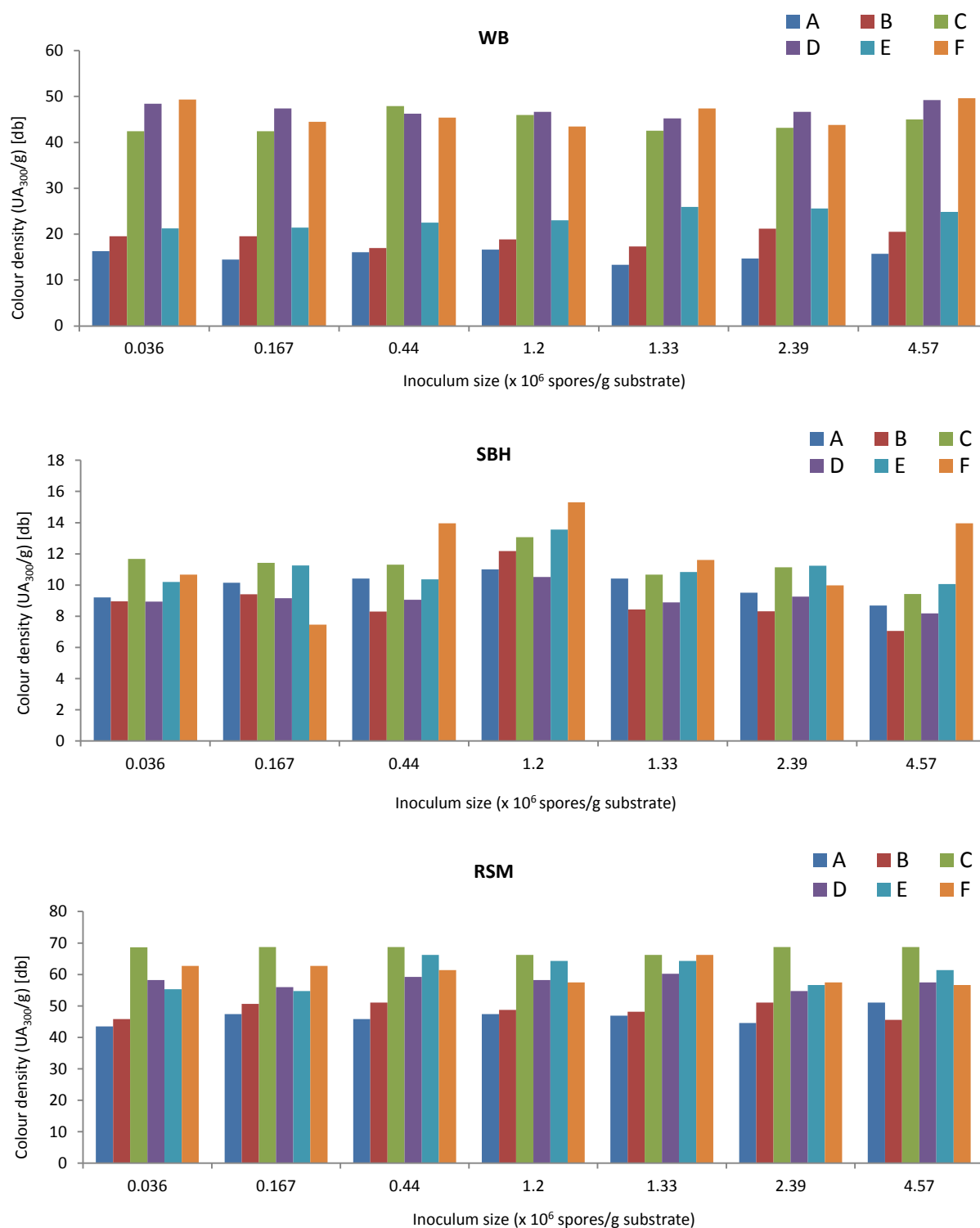
FIGURE 7.20: Effect of different extraction techniques for extracting pigment from *A. awamori* fermented wheat bran [WB], soybean hulls [SBH] and rapeseed meal [RSM] at different inoculum sizes. *[Procedure A]: distilled water, 250 rpm, 1h, 30 °C; [Procedure B]: A + boiling 20 min; [Procedure C]: 1M perchloric acid, 250 rpm, 1h, 30 °C; [Procedure D]: C + boiling 20 min; [Procedure E]: A + autoclaving, 121 °C, 15 min and [Procedure F]: enzyme treatment*

Notice that the colour concentration per extraction procedure follows a similar behavior under parameter studied in wheat bran and rapeseed meal (TABLE 7.2). In this case, procedure F and C produced high colour concentration in wheat bran and rapeseed meal, respectively. However this was not observed in fermented soybean hulls. The colour concentration profile obtained from initial moisture content studies shows different with obtained from inoculum size. Procedure E and F produced high colour concentration from sample with different initial moisture content and inoculum size, respectively. Procedure F involves the use of glucanase enzyme and it believed that hydrolysis was occurred at high rate and as a result produced high colour concentration, not only from cells fungus, but also from solid substrate itself.

The results of this study revealed that the procedures used had a significant effect on the concentration of the colour that was extracted. The extraction method using procedure F (treatment with enzyme) provided a maximum concentration of colour in wheat bran followed by procedure E (extraction with distilled water, samples autoclaved at 121 $^{\circ}$C, 15 min) in soybean hulls and procedure C (1 M perchloric acid, boiled 20 min then shaken at 250 rpm for 1 h at 30 °C) in rapeseed meal. The colour concentration demonstrated different levels of solubility in every procedure.

The main objective this work was to find a very easy, inexpensive and fast process that does not require expensive or strong chemicals during the extraction process. By using strong chemicals with excessive procedures, for example perchloric acid in this study, it is possible that colour from the solid substrate will also be extracted leading to colour contamination. On top of that, the used of enzyme not practical since it is more expensive and not suggested in this study. However, extraction using procedure A; distilled water with shaking at 250 rpm for 1 h at 30 °C, produced a significant amount of colour and was easy, non-toxic and inexpensive. In addition, water can be categorised as a mild solvent. Solid substrate also carries a certain colour and by using water, the possibility of extracting the colour of solid substrate in very mild conditions can be minimised. As a result of this work, extraction using procedure A will be used for further studies. Further studies will also use absorbance at 300 nm to measure colour density of extracted solutions from fermented solid substrate.

7.4   SUMMARY AND CONCLUSIONS

In this study, there was no significantly different pattern in fungus growth development demonstrated using organic matter loss and dry weight reduction ratio. However, this study suggests that it is possible to estimate biomass development in SSF by considering organic matter loss and dry weight reduction ratio. This observation is particularly true in the case of fermentations performed in a very small scale experiment such as in a petri dish or a flask but not in large scale fermentation that involves large scale bioreactor systems.

Measuring metabolic activities by estimation of OUR, CER and heat evolved are considered to be the most accurate for the estimation of growth of microorganisms in SSF. These activities can be measured continuously online. With the increasing computing power nowadays, it may become appropriate to include a gas analyser and a temperature device in the bioreactor system, allowing a more direct evaluation of the metabolic measurement. This will help to avoid futile attempts to manipulate the SSF operating variables in order to improve SSF process that is limited by biomass estimation. The results obtained from metabolic measurement also showed that measuring the heat evolved during fermentation can be another promising technique to describe growth. It was also found that there is a strong correlation between the heat evolved with OUR/CER during SSF; when heat evolved increases, OUR/CER also increases. A detailed explanation of this observation can be found in Chapter 10.

The increase in optical density and the subsequent scattering of light by microbial colour provides a good means to estimate fungal growth. This method is cost-effective, rapid, quantitative and works well for colour in the logarithmic phase of growth. In this study, it is demonstarted that the colour changes observed during the growth of fungal SSF can be used to obtain quantitative information of their growth behavior. Such predictive microbiolgy is based on observation. The hyphothesis is that fungal growth is an intrinsic characteristic of the fungus and will occur reproducibly in the same environment. Effectively, extraction procedure A (distilled water, shaking at 250 rpm for 1 h at 30 °C) was efficient enough to desorb all the colour fixed on the fungal cells and the spectrophometric technique at wavelength 300 nm was sensitive enough to estimate colour concentrations. Another reasons measurement at 300 nm were selected because at this wavelength changes in the colour concentration are reflected. Further motivation for these studies were carried out to explore details of the phenomenon of colour change during SSF in Chapter 8.

CHAPTER 8

COLOUR DEVELOPMENT AS A POTENTIAL TECHNIQUE FOR ESTIMATING FUNGAL GROWTH

"The colourimetric technique demonstrated and gave information better than just what we can get by visual observation or spore counting"
Colin Webb - Musaalbakri Abdul Manan
University of Manchester / Malaysian Agricultural Research and Development Institute
2014

8.1   INTRODUCTION

Achieving a positive result in determining biomass remains a major challenge in SSF. Fungi are well characterised microorganisms and are widely used in solid state fermentation (SSF) due to their ability to colonise and penetrate into the solid substrate. The compressed structure of the mycelia and the solid substrate does not allow a complete recovery of the biomass, which may not be insurmountable. In contrast to submerged fermentation (SmF), SSF does not allow accurate biomass estimation. However, SmF faces the same problem when the fermentation media contain solid particles. Although significant advances have been made with the availability of various techniques; however, progress has been very unsatisfactory.

In order for a particular strategy to be useful, it is necessary to have analytical methods for the analysis and evaluation of fungal growth. Such quantification is possible by measuring the colour produced in SSF and SmF using colourimetric technique. In this study, fungal fermentation of *A. awamori* and *A. oryzae* are carried out on complex heterogeneous solid media; wheat bran, soybean hull and rapeseed meal, which are constituted of various soluble and insoluble solid particles.

As an extension of Chapter 7, in the present work, experiments were carried out to quantify the colour of fermentation medium as a means for estimating growth. The main aim of this study is to provide better insight into monitoring biomass growth in *A. awamori* and *A. oryzae* SSF using colourimetric technique. Other objectives of this study are: (1) to investigate colour production from different fungal components: spores, mycelium and a combination of both; (2) to establish an accurate estimation model using a spectrophotometer at a wavelength ranging from 200 to 800 nm; (3) to investigate colour production changes with three different solid substrates used in SSF; (5) to investigate correlation between colourimetric measurement and several available biomass estimation techniques and (6) to investigate colour production in SmF.

8.2   MATERIALS AND METHODS

8.2.1   Microorganisms and Inoculum Preparation

Fungal progeny obtained with monospore isolation of *A. awamori* and *A. oryzae*, as described in Chapter 5, were used throughout this study. Fungal spores in a universal bottle were stored at 4 °C in agar slopes of solid sporulation medium containing 5% (w/v) whole wheat flour and 2% (w/v) agar as a stock culture. For preparing a spore suspension, well sporulated slants of both fungi were used and prepared according to the methods described in Chapter 5.

8.2.2   Fermentation Medium and Media Preparation

8.2.2.1   Solid agar medium

All three selected substrates (wheat bran, soybean hulls and rapeseed meal) and whole wheat flour were used as a solid agar medium for growing *A. awamori* and *A. oryzae*. All experiments were done in 9.0 cm petri dishes. Solid sporulation media containing 5% (w/v) of each substrate and 2% (w/v) agar in distilled water were prepared and sterilised at 121 °C for 15 min. In addition, potato dextrose agar (Sigma-Aldrich, UK) and nutrient agar (Sigma-Aldrich, UK) were prepared according to the manufacturer's instructions. After autoclaving, the agar media were immediately distributed onto petri dishes and allowed to cool down and solidify at room temperature before inoculating with 100 µL *A. awamori* and *A. oryzae* spores. All petri dishes were incubated at 30 °C for 7 days. At the end of incubation period, the colour was extracted from spores, mycelium and a combination of the spores, mycelium and agar with distilled water.

8.2.2.2   Solid state fermentation

All three selected substrates (wheat bran, soybean hulls and rapeseed meal) were used without any treatment as a solid medium for growing *A. awamori* and *A. oryzae*. All experiments were carried out in 9.0 cm petri dishes. Details of substrate preparation and inoculum transfer are described in Chapter 4 (Section 4.3.5). At the end of the fermentation period (72 h), samples were taken out for colour analysis.

8.2.2.3   Submerged fermentation

The medium consisting of the same substrate (5% w/v) as described above were prepared without adding any nutrients. The flasks were sterilised at 121 °C for 15 min. 1.2×10^6 spores/mL were used to inoculate the media in 500 mL flasks containing 100 mL working volume of distilled water. The flasks were incubated in an orbital incubator shaker (Gallenkamp, Germany) at 30 °C operating at 250 rpm. Sampling was carried out at defined time intervals for colour analysis.

In order to study whether the colour is developed by the fungus itself or as a result of microbial activity another set of experiments in SmF using synthetic medium (non-proteinaceous) was carried out. Inorganic nitrogen was used to avoid colour development in the original medium and therefore the final medium was colourless. In this approach, both fungi (*A. awamori* and *A. oryzae*) grow in submerged medium where protein must be absent from the medium.

The medium proposed by Ariff (1993) was used in this study. This medium consists of glucose (50.0 g/L) as a carbon source and $(NH_4)_2SO_4$ (5.0 g/L) as nitrogen source. Other salt components of the medium are: $CaCl_2.2H_2O$ (0.1 g/L), $FeSO_4.7H_2O$ (0.1 g/L), $MgSO_4.7H_2O$ (0.5 g/L) and KH_2PO_4 (0.2 g/L). The glucose solution and the salts solution need to be prepared and autoclave separately. After cooling, the two solutions were mixed together.

SmF was conducted in 500 mL flasks with 200 mL working volume. 1.2×10^6 spores/mL inoculum size was inoculated into each flask and flasks were shaken at 250 rpm at 30 °C. Sampling was done at defined time intervals for biomass determination using dry cell weight. Colour measurement using a spectrophotometer (Shimadzu UV-Vis 2410PC, Japan) was measured using samples from the supernatants and cell free extracts from the fungal mycelium using water as described in Section 8.3.1.3. The overall colour measurement was obtained by adding the two values for the supernatant and cell free extract.

8.3   ANALYTICAL METHODS

8.3.1   Colour Extraction

8.3.1.1   Samples from solid agar media

At the end of the incubation period, the colour was extracted from spores, mycelium and a combination the spores, mycelium and agar. 10 mL of sterile distilled water and several sterile glass beads (4 mm diameter) were added onto a petri dish. The spores were suspended by shaking the petri dish gently and collected in 250 mL flask as a spores suspension. This step was repeated until all the spores were harvested and the final volume of the spores suspension was made up to 40 mL. The petri dish with the washed spores from the previous step was used to obtain mycelial material. The surface was gently scratched with a spatula to collect about 2.0 g (wet weight) of mycelium in 250 mL flask containing 40 mL distilled water.

One (1) piece of 4 x 4 mm of mycelial blocks of *A. awamori* and *A. oryzae* were used to extract the colour in 250 mL flask containing 40 mL distilled water. All the flasks were kept shaking on ain an orbital rotary shaker (Infors AG-CH4103, Switzerland) at 30 °C and 250 rpm for 30 min. The suspensions were then centrifuged (Eppendorf Centrifuge 5804, Germany) at 10,000 rpm for 10 min at 4 °C. This procedure was repeated until a clear solution is obtained.

8.3.1.2   Samples from SSF

At the end of the incubation period, samples were harvested for analysis. Approximately 2.0 g (on a wet basis) from all the fermented samples was extracted with 40 mL distilled water and shaken for 1 h on a rotary shaker (Infors AG-CH4103, Switzerland) at 250 rpm and 30 °C. To make sure of obtaining very clean extraction solution free from spores, the suspensions were centrifuged using a laboratory centrifuge (Eppendorf Centrifuge 5804, Germany) at 10,000 rpm for 5 min before recovery by filtration through a filter paper (Whatman No. 1). Centrifugation was repeated when needed under similar conditions to achieve complete separation of spores from the colour solution. The solution must be very clean from spores or contaminating substances to avoid interfering with UV spectrophotometric analysis.

8.3.1.3   Samples from SmF

Samples collected during the fermentation were centrifuged at 10,000 rpm for 5 min using laboratory centrifuge (Eppendorf Centrifuge 5804, Germany). The colour was present in the fractions, filtrate and mycelium. In order to measure colour in the mycelium, extraction of the pigment was carried out using distilled water. The method of extraction used was as follows: 10 mL of distilled was added to 1 g wet cells in 250 mL flask and this was shaken at 250 rpm for 1 h at 30 °C. The mixture was then filtered through a filter paper (Whatman No. 1) and the filtrate was used for colour determination.

8.3.2   Spectrophotometric Analysis

To identify the most suitable wavelength for absorbance measurement of the filtrate obtained using the extraction procedure, samples were scan over the wavelength range 200 - 800 nm and the wavelength corresponding to absorption maxima were determined. Water was used as a blank. Whenever necessary, the filtrate samples were diluted with distilled water prior to absorbance measurement. Colour production was calculated by multiplying the absorbance units by the dilution factor. The value was expressed as UA_{300} g^{-1} of dried fermented substrate (dry basis) [db]. The readings of absorbance were used for the calculation of optical density using the formula below.

$$Unit\ absorbance\ (UA_{300}) = \frac{Abs_{300} \times dilution\ factor}{Sample\ weight \times (1-moisture\ content/100)} \qquad (8.1)$$

Colour yield was expressed as unit absorbance (UA_{300}) (measured at absorbance 300 nm) using its absorbance maximum per gram dry fermented substrate as described in Equation 8.1. In the case of samples from SmF, total extra-cellular and intra-cellular colour was measured, which is the sum of the absorbance of extra-cellular colour (the absorbance of the liquid culture medium after filtration) and intra-cellular colour (the absorbance of a water extract of the mycelium after filtration).

It is important in this study that all the samples are analysed using a scanning spectrophotometer within the wavelength range of 200 – 800 nm to observe a typical normalised UV absorption spectrum. The spectrum of colour absorbance was obtained using a Shimadzu UV-Vis 2410PC series scanning spectrophotometer (Japan) taking into consideration the dilution factor of samples.

8.3.3   Biomass Estimation

Biomass estimation techniques; spores concentration (Section 5.5), glucosamine concentration (Section 5.7) and protease activity (Section 5.12.2), were carried out according to the methods described in Chapter 5. Organic matter loss determination procedures were conducted according to the methods described in Chapter 7 (Section 7.2.2.1).

In the case of SmF, dry cell weight was determined using filtration and oven drying method. A known volume of culture sample (10 – 20 mL) withdrawn from the fermentation was filtered through a pre-weighed Whatman filter paper No. 1 by using vacuum pump. After a drying period of more than 24 hours in an oven at 80 °C, until a constant weight was achieved, the filter paper and cells were re-weighed and the dry cell weight (DCW) was calculated by difference by using the following equation.

$$DCW\ (g/L) = \frac{Weight\ of\ dry\ filter\ paper + dry\ cells\ (g) - weight\ of\ dry\ filter\ paper\ (g)}{sample\ volume\ (L)} \qquad (8.2)$$

8.4   RESULTS AND DISCUSSION

Based on the idea of estimation of the growth of the fungus *M. purpureus*, the analysis of the colour produced by micro-organisms is usually done by measuring the absorbance of colour solutions in the range near 400, 470 and 500 nm for yellow, orange and red pigments respectively (Juzlova *et al*., 1994; Johns and Stuart, 1991; Lin *et al*., 1992, Wong and Koehler, 1981).

In order to cover the objective of this work to determine whether the proposed hypothesis could be applied for quantifying fungal growth, different sets of experiments were conducted. Each one of the sets of experiments and fermentations were planned to address one or several of the objectives described above. Preliminary experiments in petri dishes were carried out in order to confirm the theory of using colour changes in fungal SSF as an approach for biomass estimation.

All the parameters examined in these studies, which are the weight of fungi, spores concentration, organic matter loss, glucosamine concentration, protease activity and dry cell weight, are among the indirect techniques used for biomass estimation. The theory was tested by examining quantitatively the relationship between the above-mentioned parameters and colour changes that occur during fermentation.

The results obtained from these studies are described in the sections below. Typical normalised UV absorption spectra were obtained using a scanning spectrophotometer for each colour solution. These demonstrated that samples used in this work have very similar patterns. Multiple linear regressions were constructed in order to examine correlations between the concentration of colour produced and the indirect parameters studied. It is important to prove that the measurement of colour changes can provide a clear correlation to growth of fungi in SSF. The information obtained can also be applied to estimate cell concentration in SmF in the presence of solid particles.

8.4.1   Effect of Different Solid Agar Media on Growth, Sporulation and Colour Production of *A. awamori* and *A. oryzae*

This experiment was carried out to demonstrate the effect of different solid agar media on the growth of solid culture of *A. awamori* and *A. oryzae* and to examine whether spores and mycelial growth on plates correlates with colour production. Examining the colour density and morphology of fungal growth on different solid agar media on plates would provide preliminary information consisting of spectral profiles obtained by scanning UV-Vis spectrophotometry at wavelengths between 200 - 800 nm.

It is a necessity to confirm whether the production of colour from either fungal species is from the spores, from the mycelium or from a combination of spores and mycelial material. In addition, correlation between spores concentration and colour production and that between colour produced by fungi and cell weight of fungi growing on solid agar medium were examined.

Growth morphology and absorbance of colour extracted in distilled water for *A. awamori* and *A. oryzae* are in FIGURE 8.1 and FIGURE 8.2, respectively. Visual observation indicated different growth morphology on different agar media. The UV absorption spectra showed that colour could be produced either by the spores themselves or by the mycelium. In addition, there were some significant optical density spectral patterns as shown in the two figures.

Maximum absorbance was observed to occur in the region 270 – 300 nm for all samples tested indicating maximum absorbance for the pigment largely responsible for the colour. Variations in the wavelength corresponding to absorption maxima and in the size of peaks in the spectra might be because of contaminants present in the colour solution. For that reason, centrifugation and filtration need to be carried out adequately in order to obtain a clear and clean colour solution since UV absorption is very sensitive to impurities.

Growth and sporulation response was markedly affected by the solid medium used. Both fungi grew well on whole wheat flour agar as their sole source of carbon and energy. In both cases of *A. awamori* and *A. oryzae*, sporulation is highly efficient when fungi are grown on whole wheat flour agar and colour production was also highest, followed by wheat bran agar, rapeseed meal agar and soybean hulls agar respectively. Both fungi showed poor growth on nutrient agar since nutrient agar was made to be more suitable for growing bacteria.

FIGURE 8.1:
Different morphologies of *A. awamori* colonies cultivated using six different solid agar media after 7 days. Spectra from scanning spectrophotometer (200 – 800 nm wavelengths) extracted from
[▬ ▬ ▬ ▬]: spores;
[▬▬▬▬▬]: mycelium and
[▬▬▬▬▬]: fungus together with agar medium
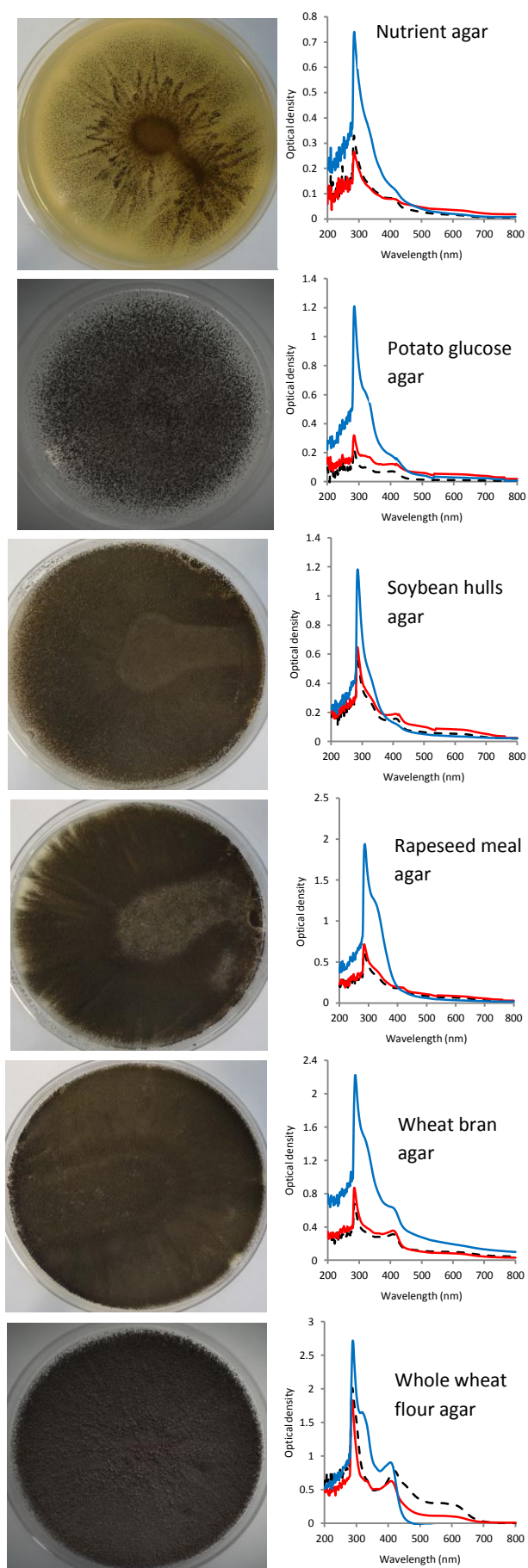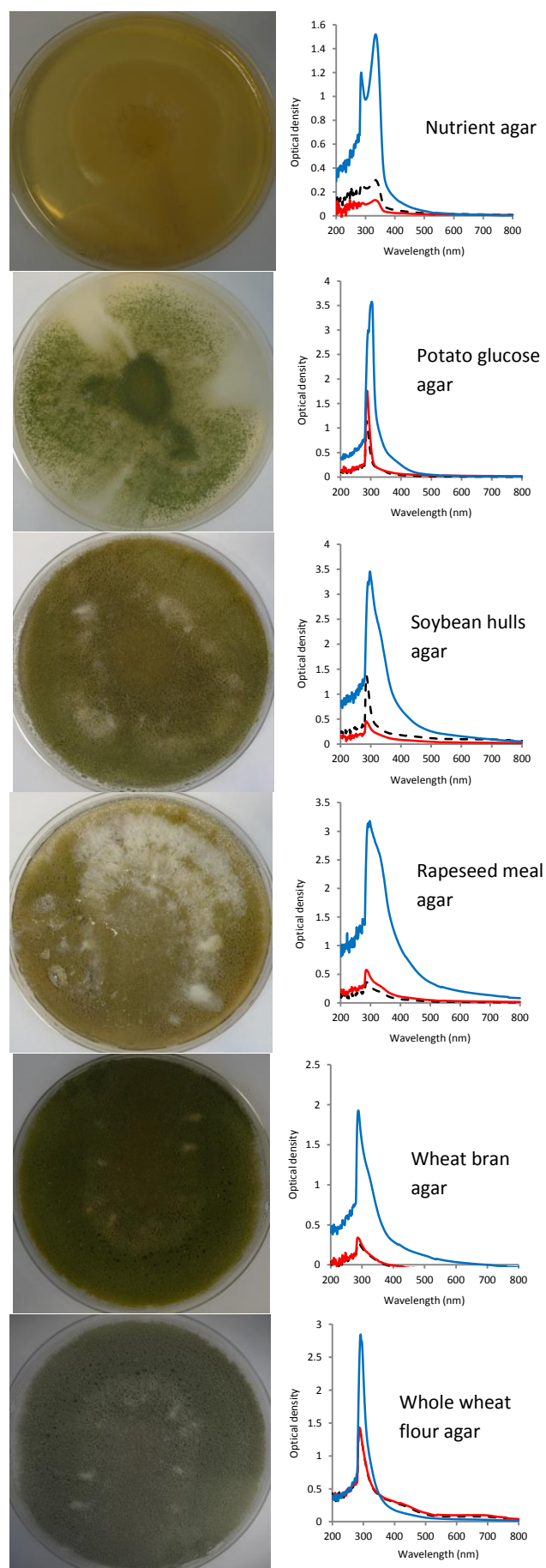
FIGURE 8.2:
Different morphologies of *A. oryzae* colonies cultivated using six different solid agar media after 7 days. Spectra from scanning spectrophotometer (200 – 800 nm wavelengths) extracted from
[― ― ― ―]: spores;
[―――――]: mycelium and
[―――――]: fungus together with agar medium

FIGURE 8.3 shows the relationship between colour density (UA_{300}/g fungus) and weight of fungus (g/petri dish) for the period of 0 to 7 days. The colour density proportionally increased as weight/cell mass of fungus increased. The linear relationship of weight of fungus growing on different solid agar media with colour density was observed throughout the incubation period. Correlation by linear regression between fungal weight and UA_{300} for *A. awamori* and *A. oryzae* are represented in FIGURE 8.3. In all solid agars tested, correlation coefficients were greater than 0.98 and 0.95 for *A. awamori* and *A. oryzae* respectively, indicating a strong positive correlation between the colour produced and weight of fungi.
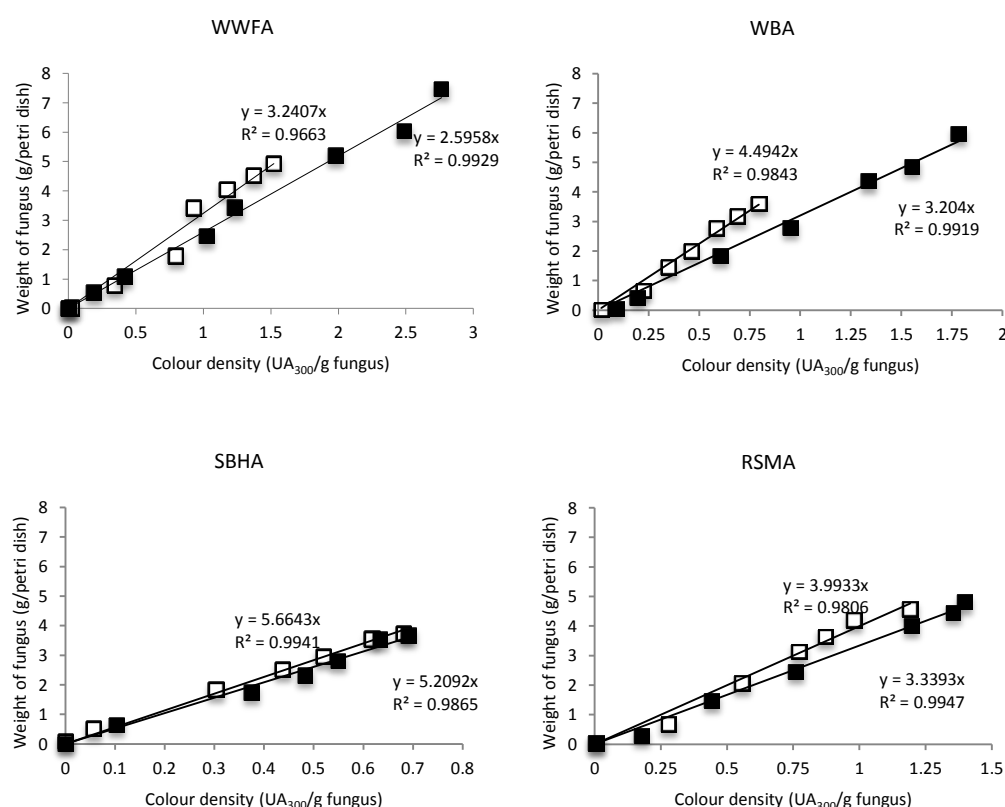
FIGURE 8.3: **Correlation between weight of fungi and colour of both fungi growing on different solid agar media. [■]: *A. awamori*; [□]: *A. oryzae*. [WWFA]: whole wheat flour agar; [WBA]: wheat bran agar; [SBHA]: soybean hulls agar and [RSMA]: rapeseed meal agar**

The results from this study revealed that the effect of different carbon sources on sporulation and cell production during the cultivation of *A. awamori* and *A. oryzae* on solid media is substrate dependent. This can be shown in FIGURE 8.4 where the data from both fungi are combined for different agar media. On the other hand, the weight of fungi and colour concentration is constant

whatever the age of cultivation and culture conditions may be. Weight of fungi increased with different solid media, which may be related to increasing C/N ratio in the solid media (not determined in this case) up to certain levels, depending on the strain, where a drastic reduction in sporulation occurred. From the correlation between the colour produced and the weight of fungi, it was observed that the preferred medium for growth may necessarily be suitable to enhance spore production. It is very obvious that a specific medium formulation is required for the enhancement of growth and sporulation of the specific *A. awamori* and *A. oryzae* strains and that the weight of fungi in these cases correlated strongly with colour production.
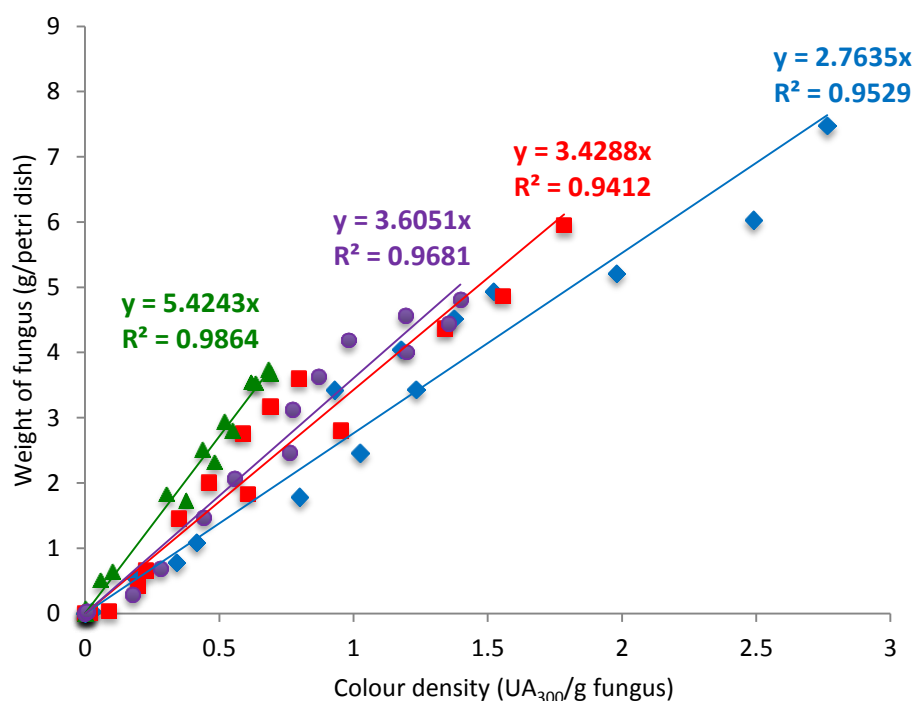
FIGURE 8.4: Correlation between colour and weight of fungus from a combination of data for *A. awamori* and *A. oryzae* on different solid agar media tested. (◆): whole wheat flour agar; (■) wheat bran agar; (●) rapeseed meal agar and (▲): soybean hulls agar

8.4.2   Correlation between Spores Concentration and Colour Density

In view of the considerations mentioned above, attention was focused on the development of a standard curve showing a strong relationship between the concentration of spores and colour density. FIGURE 8.5 shows the correlation between the concentration of spores harvested at different fermentation times from 24 to 168 hours with colour density measured at wavelength 300 nm.

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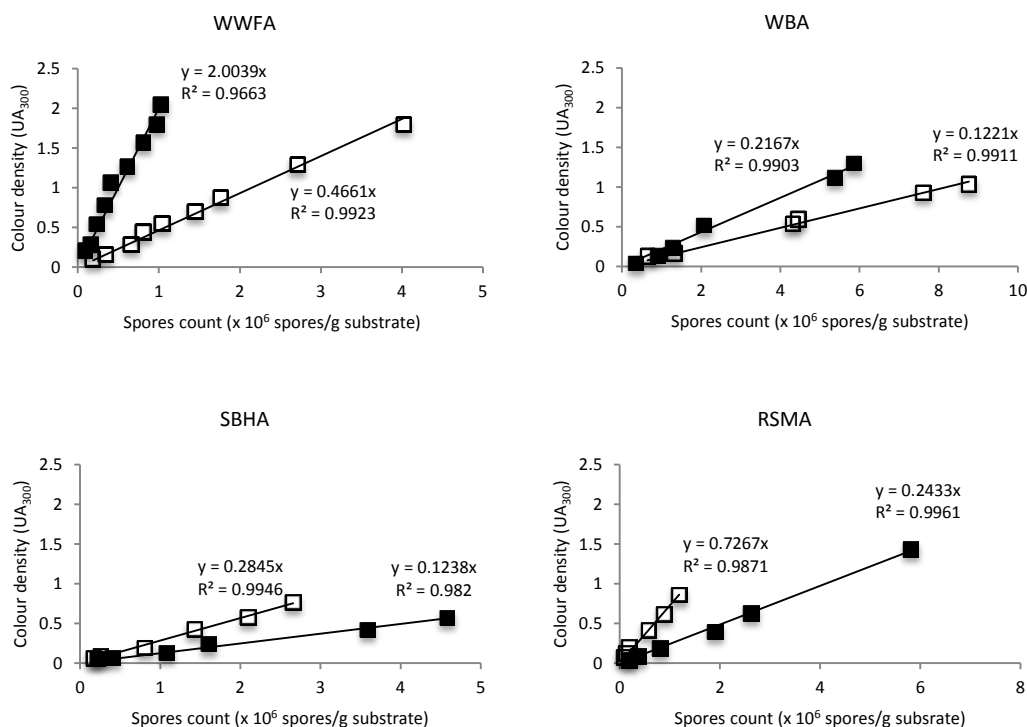
FIGURE 8.5: Correlation between spore concentration and colour of [■]: *A. awamori* and [□]: *A. oryzae* growing on different substrate solid agars. [WFA]: whole wheat flour agar; [WBA]: wheat bran agar; [SBHA]: soybean hulls agar and [RSMA]: rapeseed meal agar

FIGURE 8.5 shows correlation coefficients for both fungi growing on different media. The correlation coefficient was higher than 0.96 and 0.98 for *A. awamori* and *A. oryzae* respectively. Nevertheless, comparison of the four regression equations indicated that the amount of colour produced per 10^6 spores/g substrate was different for the four different solid media. The origin of the straight lines was the same but the slopes were different. From this finding, two questions needed to be answered: (i) is it possible to construct a calibration curve based on the concentration of spores during fungal SSF? (ii) is it possible to use the calibration curve to estimate spores production in fungal SSF?

The linear relationship between spores concentration and absorbance at 300 nm observed for both fungi on the solid substrates tested was strong and positive where colour density increased proportionally with spores concentration. This result showed it is possible to develop a standard curve based on spores concentration. Therefore, it would be possible to predict spores production from SSF using colour extracted from fermented mass. Moreover, these results show that spores concentration can be different depending on the fungal strain and the solid substrate.

Different fungi growing on different substrates will produce different colour density. Another explanation may be that the ability of fungi to sporulate is related to a sophisticated system of positive and negative gene regulation processes, which are affected by environmental and nutritional factors (Suh and Sin, 2000). Temporal and spatial regulation of gene expression, cell specialisation, and intercellular communication may affect the sporulation in the fungal system (Suh and Sin, 2000; Adams *et al.*, 1996). In other words, the correlation between colour density and spores concentration is very satisfactory; however, it does not permit comparison between processes.

8.4.3   Correlation between Organic Matter Loss and Colour Density

Dry matter weight of fermented substrate gradually decreased as fermentation progressed, and this was reflected by an increasing percentage of organic matter loss. Organic matter loss is presumably due to loss of CO_2 (Kumar *et al.*, 2003). This observation can be related to colour production indicating the gradual utilisation of nutrients in the substrate for biomass and products synthesis. A correlation between organic matter loss and colour density obtained is presented in FIGURE 8.6. Colour density increased proportionally with organic matter loss for both fungi. For all substrates tested, the correlation coefficients were greater than 0.98 and 0.96 for *A. awamori* and *A. oryzae*, respectively.

During the fermentation process, nutrients are consumed by the fungus and it starts producing spores and mycelium. The type of substrate definitely influences the performance of the fungus. FIGURE 8.7 shows that the growths of both fungi were affected by the type of solid medium. Organic matter loss and colour production appear to depend on the medium composition of solid substrate. The figure also shows that both fungi seem to produce the same degree of colour concentration regardless of the solid substrate used. The complexity of fungi in terms of chemical composition, morphology and internal structure suggests that development and colour changes can be indicators that growth happens.

WB

y = 2.3194x
R² = 0.9803

y = 2.6403x
R² = 0.9689

Organic matter loss (%)

Colour density (UA$_{300}$/g) [db]

SBH

y = 4.3276x
R² = 0.9914

y = 3.2144x
R² = 0.9989

Organic matter loss (%)

Colour density (UA$_{300}$/g) [db]

RSM

y = 1.1989x
R² = 0.9853

y = 1.2727x
R² = 0.9921

Organic matter loss (%)

Colour density (UA$_{300}$/g) [db]
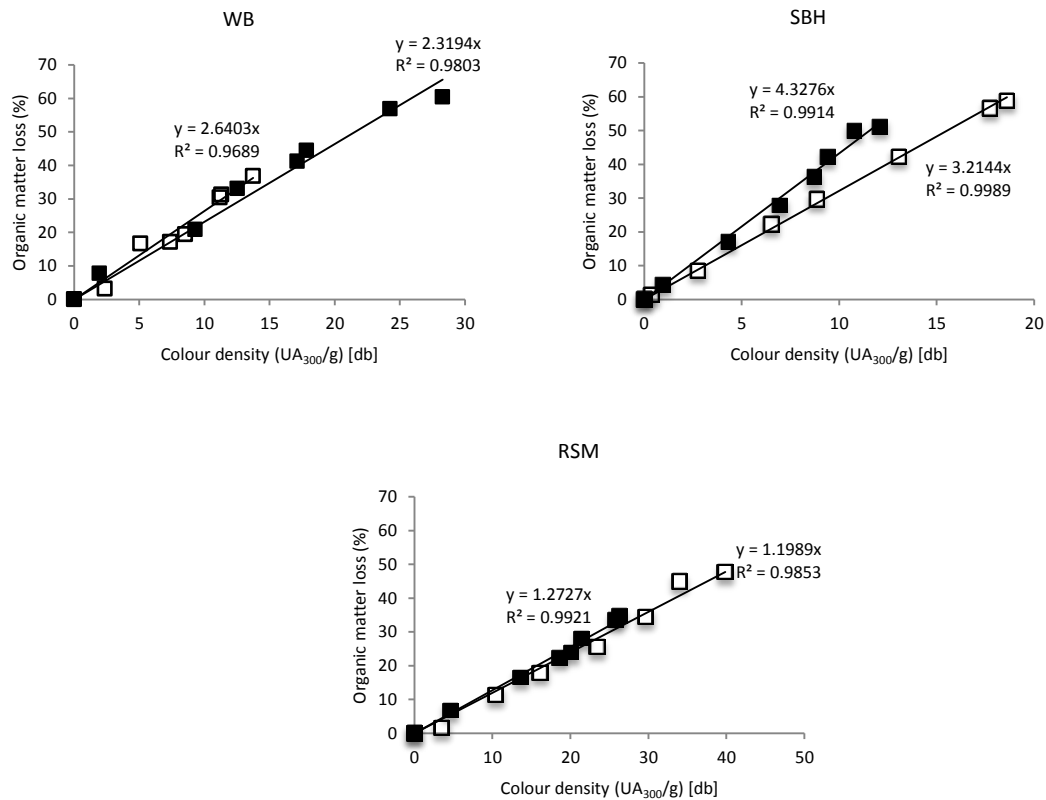
FIGURE 8.6:   **Correlation between organic matter loss and colour density at wavelength 300 nm. [■]:** *A. awamori* **and [□]:** *A. oryzae***. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal**

The changes in the fungal population can be grouped in changes in the number of cells/spores, morphology, chemical composition (for example colour in this case) and changes in the internal structure (which can be observed under a microscope). From a theoretical point of view, it is demonstrated that colour changes as a function of time in fungal SSF can be explained by changes in chemical composition either in the solid material or the fungus itself. Organic matter loss represents one of the indirect techniques used to describe growth of fungi and here correlation clearly indicates that when organic matter loss increases, so does colour production.
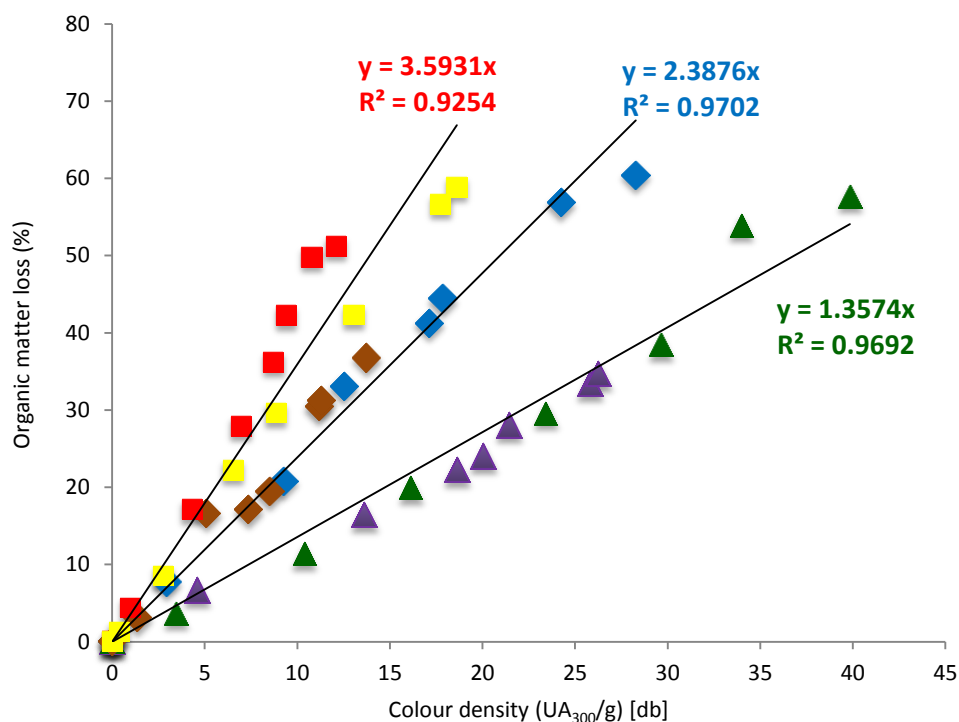
FIGURE 8.7: **Correlation between colour density and organic matter loss from a combination data for *A. awamori* and *A. oryzae* at different initial moisture contents and inoculum sizes for three substrates tested. (◆) *A. awamori* – wheat bran; (◆): *A. oryzae* – wheat bran; (■): *A. awamori* – soybean hulls; (▢): *A. oryzae* – soybean hulls; (▲): *A. awamori* – rapeseed meal and (▲): *A. oryzae* – rapeseed meal**

8.4.4   Correlation between Glucosamine Concentration and Colour Density

Glucosamine concentration and colour density in the present study showed a varying pattern between solid substrates tested with regards to time. This may be due to the difference in production of metabolites during growth phases. *A. awamori* degraded wheat bran at a faster rate than *A. oryzae*. However, *A. oryzae* degraded rapeseed meal at a faster rate than *A. awamori*. The rate of degradation can be measured by glucosamine concentration and colour density. In this work, a linear correlation was found between the colour produced and glucosamine concentration in fermented material of A*. awamori* and *A. oryzae* (FIGURE 8.8). A positive effect of initial moisture content and inoculum size was seen on the concentration of glucosamine and also on the concentration of colour produced.
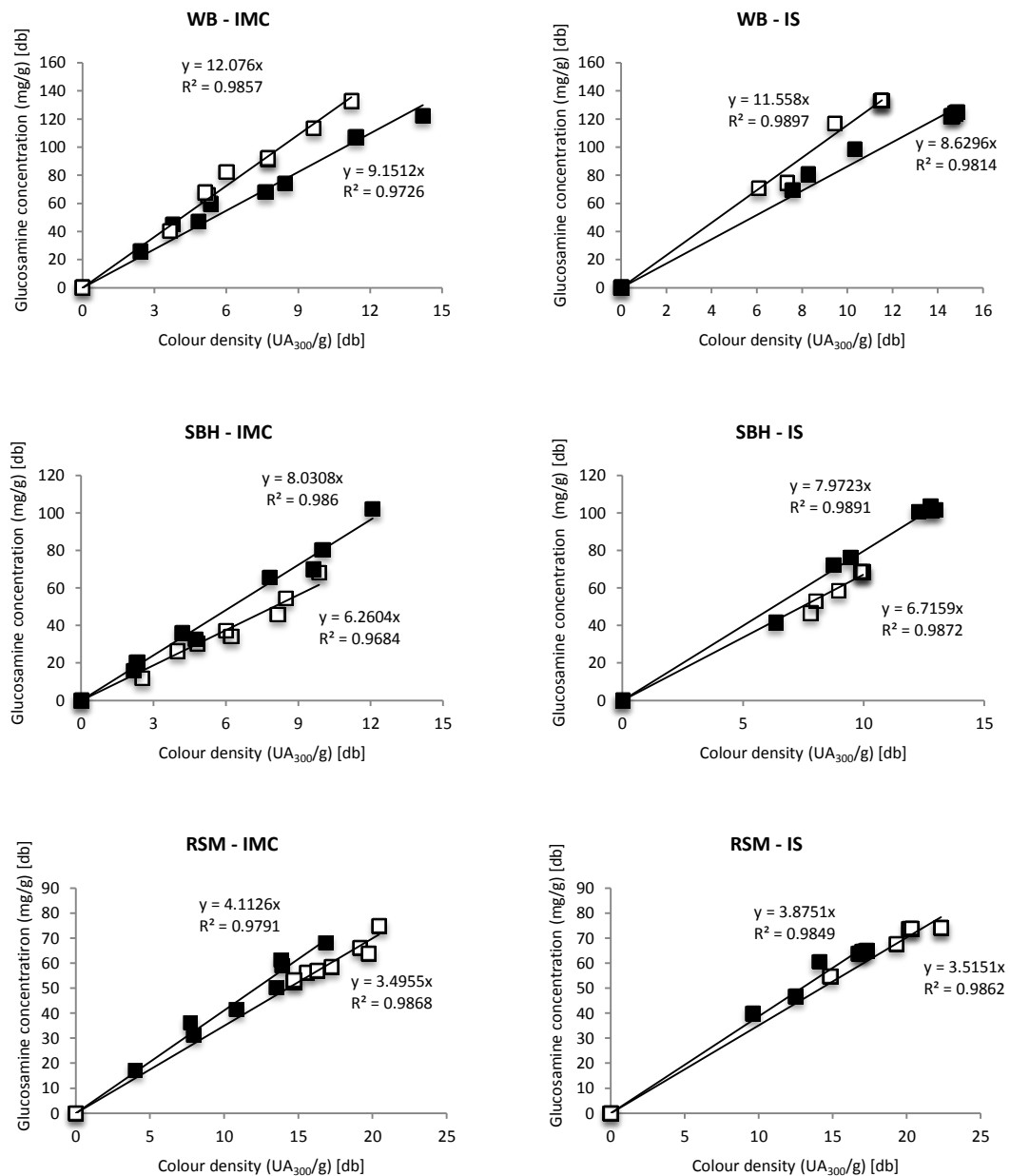
FIGURE 8.8: **Correlation between colour produced and concentration of glucosamine tested at different initial moisture content [IMC] and inoculum size [IS] for both [■]: *A. awamori* and [□]: *A. oryzae* on different substrates. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal**

This result provides a useful tool for investigating the use of the correlation of glucosamine concentration with colour changes in the growing fungi for biomass estimation. In essence, high fungal biomass can be reflected by high colour density and high glucosamine concentration. Each of the two fungi produced a different colour density but almost the same concentration of glucosamine. In addition, the same fungus produced different levels of colour density and glucosamine when grown on different substrates. It should borne in mind that the colour produced cannot be attributed

a single substance; it might involve one or more substances and therefore it cannot be attributed to a single wavelength.

Referring to the complexity of pigment mixtures produced by *Monascus* species, it is reported in most studies that pigment composition is determined spectrophotometrically using absorption maxima exhibited around 400 nm, 470 nm and 500 nm corresponding to yellow, orange and red compounds respectively (Juzlova *et al.*, 1996). In the case of *Monascus* species, intensive research has been carried out to identify the compounds responsible for the colour. The pigment produced by this microorganism has been used successfully to describe growth in either SmF or SSF.

To examine, in detail, the correlation between colour density and glucosamine concentration, data obtained from both fungi and both parameters were combined for each solid substrate tested as shown in FIGURE 8.9. A good correlation was found between colour density and glucosamine concentration using the combined data. The correlation coefficients obtained for wheat bran, soybean hulls and rapeseed meal were 0.82, 0.94 and 0.93 respectively. However, comparison of the three regression equations indicated that the amount of glucosamine produced per unit absorbance was different for the three solid substrates. Indeed, the origin of the straight lines was the same and the slopes were different for the solid substrates. This shows that the correlation obtained for the effects of inoculum sizes and initial moisture contents was similar for both fungi.

Results presented in this section show that the colourimetric technique could be considered as a reliable indirect method for estimating fungal biomass in the case of both fungal species. FIGURE 8.9 shows that glucosamine concentration appears to depend on the solid substrate used. In other words, glucosamine concentration depends on the medium composition of the solid substrate. Indeed, the level of glucosamine was affected by the nature of the solid substrate (carbon and nitrogen source), but not by the type of microorganism used or the treatment conducted (the effect of inoculum size and initial moisture content in this study).

This study also demonstrates the strong and positive correlation between colour density and glucosamine concentration. Therefore, it is possible to make a good estimation of the level of glucosamine using the colourimetric method developed in this study. By using the linear regression equation obtained in SSF for each fungus and solid substrate, quantities of colour measured could be easily converted into milligram glucosamine per gram of dry solid substrate (on a dry basis) (mg/g [db]).
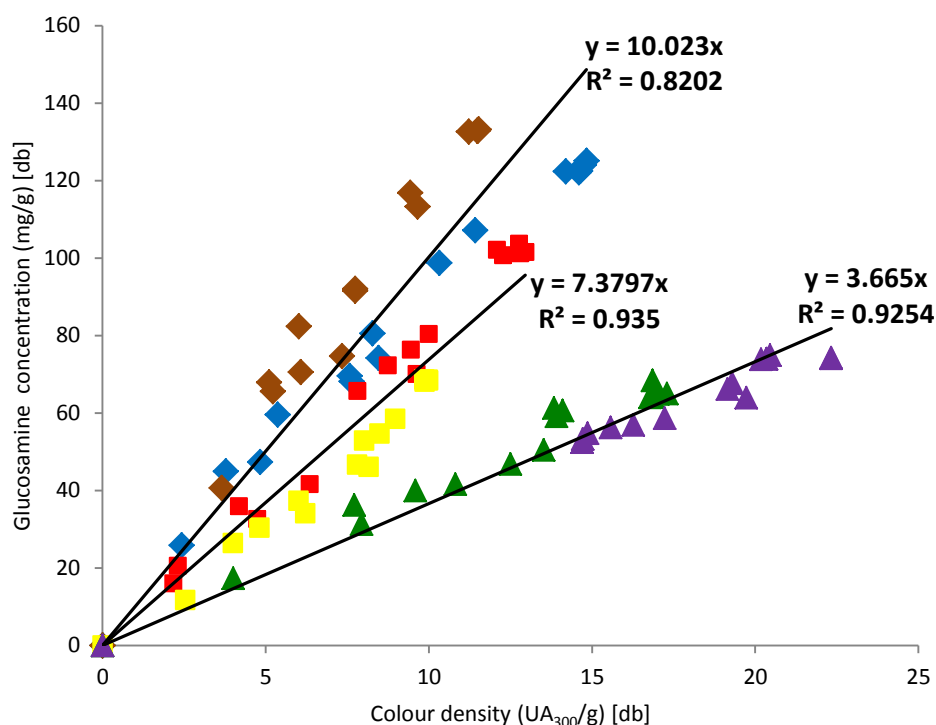
FIGURE 8.9:   Correlation between colour density and glucosamine concentration using a combination of data for *A. awamori* and *A. oryzae* from different tests of initial moisture content and inoculum size for the three substrates tested. (◆): *A. awamori* – wheat bran; (◆): *A. oryzae* – wheat bran; (■): *A. awamori* – soybean hulls; (■): *A. oryzae* – soybean hulls; (▲): *A. awamori* – rapeseed meal and (▲): *A. oryzae* – rapeseed meal

8.4.5   Correlation between Enzyme Activities and Colour Density from SSF

In this study, the colour extract was also used as a supernatant crude enzyme extract for further analysis by determining the activity of enzymes such as glucoamylase, protease, xylanase and cellulase in order to investigate whether a correlation exists between the activity of enzymes within the extract and the intensity of its colour. It is worth noting from visual observation that different colour density of supernatant crude enzyme extract will give different activity of enzymes. The darker will results high enzymes activity. This phenomenon also observed by Ariff (1993) that high production of glucoamylase was accompanied by high production of yellow pigments. Therefore, in the SSF experiments used in this work, the activity of protease is used as a reference to verify how efficient the applied technique to quantify the biomass growth during the process was.

FIGURE 8.10 shows correlation between protease activities and colour density extracted from fermented *A. awamori* and *A. oryzae* on wheat bran, soybean hulls and rapeseed meal. Experiments

were carried out at different initial moisture contents and inoculum sizes. Cell free extracts were obtained from fermented fungal biomass after 72 h of fermentation. The results showed a positive and significant correlation between colours produced by fungal SSF and protease activity. In contrary with results presented above, it was observed that colour density exponentially increases with the protease activity. The production of enzymes and pigments would be independent on solid substrates and fungi.
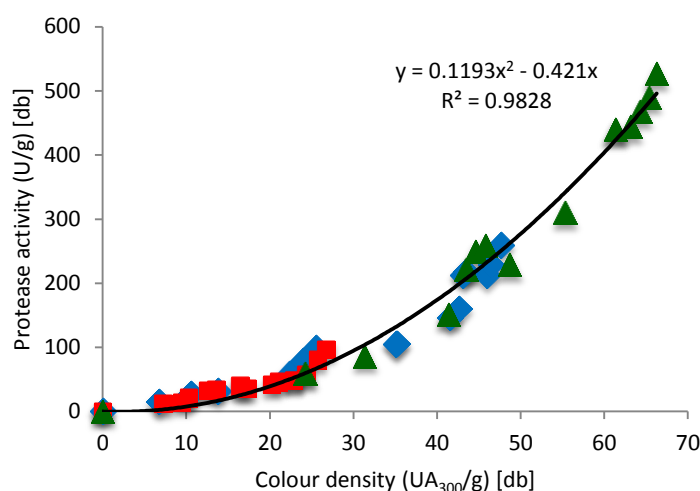
FIGURE 8.10: Correlation between colour produced and protease activity at different initial moisture contents and inoculum sizes for three different substrates. (◆): wheat bran; (■): soybean hulls and (▲): rapeseed meal

A. awamori and *A. oryzae* showed a satisfactory growth and produced high protease activity and high colour concentration on rapeseed meal followed by wheat bran and soybean hulls. This is expected since the composition of rapeseed meal is known to be rich in protein content (Chapter 5, Table 5.1). It should borne in mind that the colour produced cannot be attributed a single substance; it might involve one or more substances and therefore it cannot be attributed to a single wavelength. Dutra *et al.*, (2008) reported that the digital image process technique could be used to monitor biomass growth in a SSF process. They observed that the image processing have a linear relationship with glucosamine and lipase activity, and to correlate biomass growth.

8.4.6   Colour Development in SmF using Solid Substrates (I)

Fungal SmF was carried out on complex heterogeneous media such as whole-wheat flour, wheat bran, soybean hulls and rapeseed meal, which are constituted of various soluble and insoluble solid particles. The estimation of the course of fungal growth during such fermentation, conducted using

either SmF or SSF, is hindered by the presence of insoluble particles. In such systems, the adherence of the fungus to solid particles hinders the partition of the solid mass to its components. Therefore, direct estimation of fungal growth using the dry weight method is not possible due. Another reason might be due to the mixture of the content of cells and high molecular weight compounds, which cannot be filtered out.

To implement a fermentation process based on such solid particles, the determination of cell concentration throughout the fermentation process is essential. In order to achieve this, the problem caused by the presence of various heterogeneous and insoluble solid particles should be overcome. Since the use of a direct analytical method such as dry cell weight is impractical, the use of an indirect estimation method is the only alternative. Arguably, monitoring colour changes occurring in SmF can be used as an alternative indirect method for measuring fungal growth during fermentation. The production of colour in both SSF and SmF would be directly or indirectly affected by the cultivation environment and cultivation methods.

This study was conducted with the objective of examining colour development in SmF using *A. awamori* and *A. oryzae* with four different heterogeneous fermentation media. For the purpose of comparison, data are also obtained with SSF to determine the colour profiles developed in these two systems. Colour solutions from the liquid broth and colour extracts from cell/fungal mycelium are measured using a spectrophotometer to obtain the UV absorption spectra at a wavelength range of $200 - 800$ nm.

FIGURE 8.11 depicts the UV absorption spectra for the two fungi, *A. awamori* and *A. oryzae*, in different substrates measured at different fermentation times. Colour density increased with time indicating growth of fungi in the tested media. In all cases, the spectra had a maximum absorption in the region $260 - 300$ nm. The UV absorption spectra at their absorbance maxima were used to express the concentration of colour produced. Both *A. awamori* and *A. oryzae* growing on substrates tested in SSF and SmF showed identical patterns of optical density spectra obtained using a scanning spectrophotometer. In SmF using whole wheat flour with both fungi also showed almost the same spectral pattern.
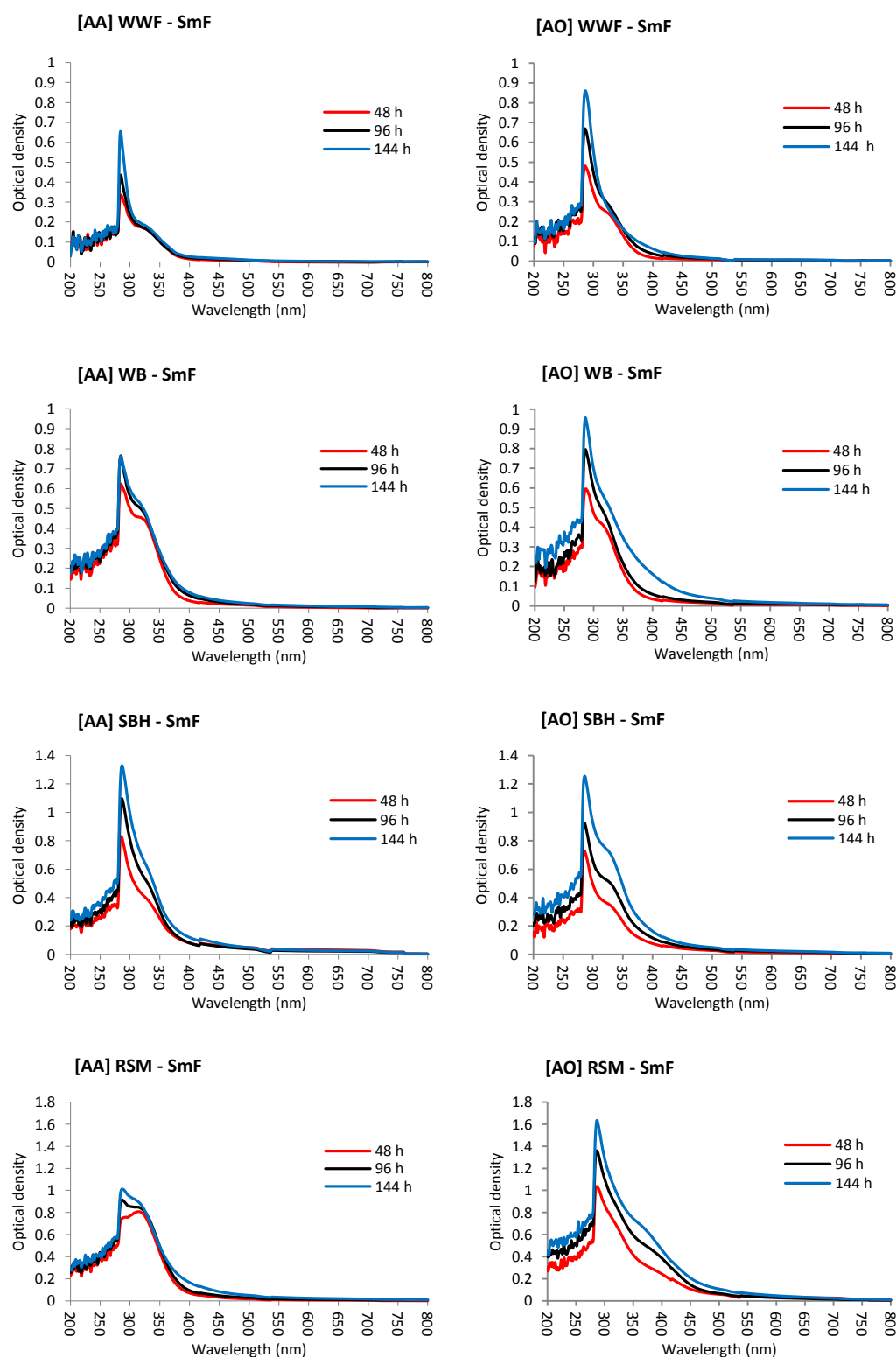
FIGURE 8.11: Typical normalised optical density spectra using a scanning spectrophotometer (200 – 800 nm wavelengths) obtained with SmF at different fermentation times for [AA]: *A. awamori* and [AO]: *A. oryzae*. [WWF]: whole wheat flour; [WB]: wheat bran; [SBH} soybean hulls and [RSM]: rapeseed meal

The absorbance maxima of colour extracts from SSF and SmF were compared. The screening process showed that the maximum absorbance wavelength for SSF was between 280 – 300 nm. On the other hand, this was between 284 – 307 nm for SmF. For SmF with *A. awamori* on rapeseed meal, the maximum absorbance wavelength was between 287 – 317 nm. However, these small differences are reasonable.

This study revealed that the colour produced from microbial activity either in SSF or SmF might correspond to the same pigments for both fungi. This was suggested based on the identical spectra obtained using UV-Vis spectrophotometry. In both fermentation processes, colour is associated with fungal growth. Naturally, this equivalence should not apply to crude colour extracts, which may contain several substances with different absorbance patterns. The fact that the colour was produced as a mixture of compounds probably affects the analysis especially when a simple analytical method is used such as measuring colour absorbance.

8.4.7   Colour Development in SmF using a Synthetic Medium (II)

Finally to acquire an idea about the production of colour in fungi, another study was carried out to investigate the colour production in a synthetic medium. This synthetic medium contained zero protein concentration except for an inorganic nitrogen source. This medium was colourless and constituted a very clear solution. In previous experiments using SmF, there was an amount of organic protein present in solid substrates. Through metabolic activity, fungi consumed this protein and synthesised other proteins and compounds (such as proteins responsible for the colour itself, enzymes and other secondary products).

Explanation the production of colour during the fermentation process by fungi may require further study. As described above, colour solutions from the liquid broth and colour extract solutions from cell/fungal mycelium are measured using a scanning spectrophotometer to obtain optical density spectra within a wavelength range between 200 to 800 nm. The UV absorption spectra at their absorbance maxima are used to express the concentration of pigment produced by fungal growth.

FIGURE 8.12 shows the typical normalised UV absorption spectra for both fungi growing in a synthetic medium measured at different fermentation times. In line with observations with SmF and SSF using solid substrates, colour density increased with time indicating growth of fungi. *A. awamori* and *A. oryzae* growing in the synthetic medium showed a similar pattern of UV absorption spectra. In all cases, the spectra had a maximum absorption in the region 260 – 300 nm, which corresponds to the colour. A comparison was made with spectra from SSF and SmF using solid substrates, as explained above, and the spectra were very comparable for all samples with a maximum absorbance wavelength between 260 – 300 nm.
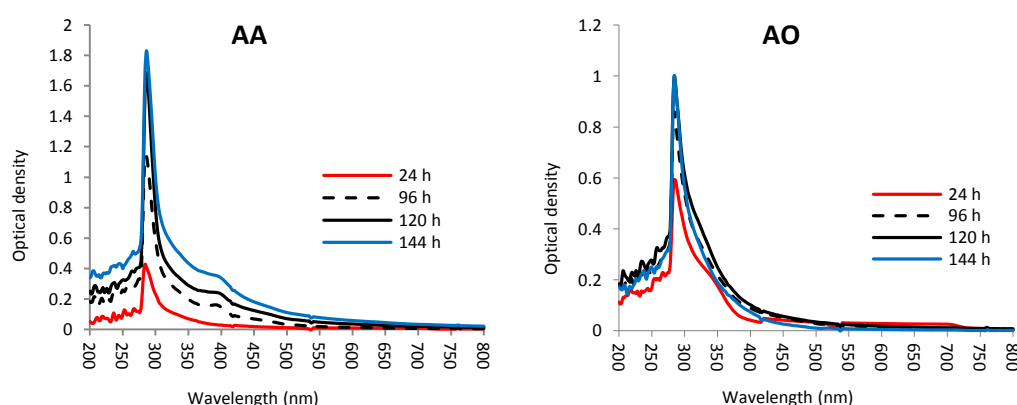
FIGURE 8.12: **Optical density spectra measured at wavelength range 200 – 800 nm for [AA]:** ***A. awamori* and [AO]:** *A. oryzae* **in SmF using a synthetic medium at different fermentation times**

This study suggests that the colour is produced from microbial activity during the fermentation process is linked to the cellular component dynamics. FIGURE 8.13 shows the correlation between dry cell weight and absorbance at 300 nm for both fungi: *A. awamori* and *A. oryzae*. Colour density proportionally increases with dry cell weight for both fungi with very good linearity and a correlation coefficient of 0.96. Dry cell weight is well known technique for biomass estimation in SmF. In this work, comparison of colour density was carried out for media having the same composition. The results show that the amount of pigment can be the same for different dry cell weights in different fungal strains. As mentioned earlier in case of SSF, the amount of pigment produced was different depending on the solid substrate used. This was assumed to be true for SmF in different synthetic media with different compositions.
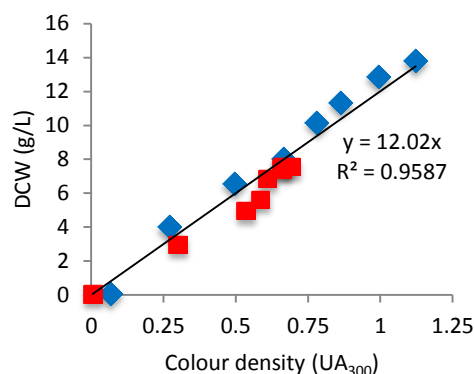
FIGURE 8.13: Correlation between colour density and dry cell weight in a synthetic medium inoculated with (◆):*A. awamori* and (■): *A. oryzae.* DCW: dry cell weight

By using the linear regression equation obtained for SmF data using the same medium with different fungi, quantities of dry cell weight could be easily measured and expressed as grams/litre (g/L). Under these considerations, this study showed that biomass estimation can be carried using the UV absorption spectra since the change in colour density and dry cell weight are shown to be closely related. Poilpre *et al*., (2008) reported the on-line estimation of biomass concentration during transient growth on yeast chemostat culture using light reflectance was shown to be accurate for rapid biomass changes.

This investigation showed that the colour was produced by fungus itself either in the spores or the mycelium as a result of microbial activity. This shows that when nutrients are consumed by the fungus, it starts producing spores and mycelium and at the same time it also produces colour, protein, and other primary and secondary products, which are related to the growth of the fungus. The approach of colourimetric estimation seems to be very similar to measuring optical density (usually at absorbance 550 – 600 nm), which has been widely used in SmF for biomass determination. In the case of SmF, the solid biomass of bacteria or yeast is very easily separated by centrifugation for dry cell weight estimation.

According to Alupoaie and Garcia-rubio (2004), multi-wavelength transmission spectra of microorganisms and cells suspension consists of combined absorption and scattering phenomena resulting from the interaction of light with microorganisms or cells typically suspended in a non-absorbing media. In their work, they are studied the growth behaviour of *E.coli* using UV-Vis spectroscopy and demonstrated that the spectroscopy method have high potential for real-time analysis and characterisation of microbial populations. Callahan *et al*., (2003) demonstrated that the combined scattering and absorption properties of suspended particles in SmF could be obtained as a

function of wavelength by measuring the complete UV–Vis spectrum. Recently, Oto *et al.*, (2012) demonstrated a non-destructive estimation of ATP contents and plate count on pork meat surface by UV-vis spectroscopy spectra analysis that can be used for sanitation management.

From a theoretical point of view, it is clearly demonstrated that the colour produced increased with fermentation time in fungal SmF and SSF and this can be explained by changes in the chemical composition (colour in this case) of the fungus itself and by microbial activity as a results of solid substrate degradation by fungi to acquire nutrients for growth in the case of SSF. To solve the long-standing problems of biomass estimation, this approach could be useful as an indirect estimation of fungal biomass in SSF and may become an important complementary technique to the existing indirect methods. Additionally, the use of colourimetric technique seems promising for direct comparison between SSF and SmF, especially for basic laboratory based research.

8.5   SUMMARY AND CONCLUSIONS

The theory of measuring colour changes in SSF by UV-Vis spectroscopy demonstrates that this colourimetric technique provides valuable information complementary to visual observation and spore counting to describe the growth of fungal mycelium on SSF. Colour from cell free extracts absorbs light in the region 260 – 300 nm that largely corresponds to pigments in the supernatant. Colour density measured for fungal SSF in cell free extracts can be determined quickly and accurately using absorbance measurement at the chosen wavelength of 300 nm.

The advantages of this method include the fact that the procedure is cheap, fast, objective, simple to carry out, non-destructive and no special or expensive reagents are required. Making cell free extracts from fermented fungal biomass was carried only using distilled water and was performed in less than 2 h. Water was efficient in the recovery of colour. Cell free extracts can also be used for multiple analyses including total reducing sugars, free amino nitrogen, glucose concentration, pH and enzymes activities.

The results of this study indicated that the colourimetric technique could be used to monitor biomass growth in a SSF process and to correlate biomass growth and weight fungus, spores concentration, organic matter loss, glucosamine and enzyme activity. Another finding from this study is that colourimetric technique can be also implemented as a real time continuous monitoring system for fungal culture growth in SmF with presence solid particles. The typical normalised optical density spectra obtained using a scanning spectrophotometer show a local absorbance maximum near

300 nm, a region where colour absorbance rapidly increases, which is a similar observation to spectra obtained for SSF extracts.

In general, colour-based methods seem to be the most promising approach for biomass estimation in SSF. It was suggested that the colour developed during fermentation progress is closely related to either or both of the growth rate and the actual biomass of the fungus. Understanding the variations in biomass components and their interactions can enable development of new techniques for biomass estimation in SSF. Apart from being a potential technique to describe growth in fungal SSF, colour production measurement in fungi provides a very elegant technique to monitor the way fungi use their metabolism to survive and adapt to different environments. Moreover, after verification of the absence of interference from solid particles, the colour will also be useful to estimate growth of fungus in SmF. Overall, the estimation of colour density through UV-Vis spectrophotometry from SSF and SmF would be a favourable indirect method to describe the growth of fungi. No method is ideally suited to all situations and hence the most appropriate method to a particular fermentation application must be chosen in each case based on the simplicity of the procedure, its cost and its accuracy.

CHAPTER 9

ENZYMES PRODUCTION STUDIES IN SINGLE TRAY SYSTEM

"Fermentation is not a simple and unique phenomenon, but a very complex one, as is often the case with phenomena closely correlated with life, which gives rise a variety of products, all of which are necessary"
Louis Pasteur

9.1   INTRODUCTION

Solid state fermentation (SSF) processes play an important role in the production of various microbial enzymes. The production of enzymes by SSF is associated with the history of the development of SSF and enzymes have been known to be produced by SSF for many years (Pandey, 1992). Enzymes have been established to be useful due to their wide range of applications. Enzymes have tremendous applications in a number of industrial processes such as the food industry, fermentation, the textile industry and the production of paper and also in chemical feedstocks. Therefore, there is an increasing demand for their production (Rodriguez-Fernandez *et al.*, 2011). Although, all commercial production of enzymes as well as almost all research works on microbial enzymes employs submerged fermentation (SmF) as the technique of fermentation, SSF has also been successfully used to cultivate many microorganisms using various solid substrates.

SSF offers several potential advantages for the production of enzymes. It has been well established that enzyme titres produced in SSF are many fold more concentrated than those produced by SmF. George *et al.* (1997) reported that the total protease activity present in one gram wheat bran (SSF) was equivalent to that present in 100.0 mL broth (SmF). In addition, SSF can be of special interest in those processes where the crude fermented product may be used directly as a source for enzymes (George *et al.*, 1997). The enzymes in SSF crude fermented product are concentrated; thus, they can be used directly in such agro-biotechnology applications as silage or feed additives, ligno-cellulosic hydrolysis and natural fibre processing (Pandey *et al.*, 1999).

The selection of an excellent microorganism strain for the production of enzymes is very important. The microorganism selected, especially the fungus in this case, must have relatively stable characteristics and the ability to grow rapidly and vigorously (Ito *et al.*, 2011). The most important characteristic to look for in the selection of the microorganism is its ability to degrade the complex solid substrates. This will lead to the production of higher yields of the desired enzymes. The selection of a particular strain of microorganism, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved (Raimbault, 1998). This will be dependent

upon a number of factors, mainly including the nature of substrate and the environmental conditions.

Due to the lack of free water in SSF, smaller fermenters are required for this process, and therefore, less effort is required for downstream processing (van Breukelen *et al*., 2011). Tray systems are the simplest of all types of fermenters used in SSF. As has been mentioned in Chapter 2, there are several other types of designs of fermenters that have been applied by researchers for various purposes. The bed height of a tray reactor is limited due to heat and mass transfer limitations and heat transfer gradients (Rajagopalan and Modak, 1995). By contrast, rotary reactors are deemed to have complex operational systems, low fill volumes (typically less than 30%) and low shear in the substrate bed (Yang, 2007).

In this chapter, studies were carried out in a simple tray system, which was used as a closed system or an opened system. For the closed system, normal petri dishes were used. For the opened system, a circular stainless steel tray was used. The bottom of the opened tray is perforated (with different aperture sizes) in such a way that it holds the substrate and allows aeration. This system was designed to address the current problems in SSF and to ensure consistent water content in the fermented solid substrate during fermentation. The study tested fermentation with *Aspergillus awamori* and *Aspergillus oryzae* and monitored the efficiency of multi-enzyme production (glucoamylase, protease, xylanase and cellulose).

9.2   MATERIALS AND METHODS

9.2.1   Microorganisms, Preparation of the Inoculum and the Substrates

The fungi *A. awamori* and *A. oryzae* were used to study enzyme production. Procedures for preparation of fungi and inoculum were described in detail in Chapter 5. The solid substrates: wheat bran, soybean hulls and rapeseed meal, were used. After autoclaving, inoculum transfer was carried out aseptically in a laminar cabinet according to the standard operational procedures described in Chapter 4.

9.2.2   The Tray System

Two types of tray systems, known as a closed or opened system, were used in this study. A closed system consists of normal petri dishes with 9 cm diameter and 1.5 cm depth covered with a lid. The opened system consists of circular stainless steel trays with 10 cm diameter and 3.5 cm depth (FIGURE 4.6, Chapter 4). The top of the tray is covered with a lid and the bottom of the tray is equipped with a perforated base to allow for aeration of the bottom surface of the bed and, to some extent, the inner layers within the bed.

As an opened system, the aperture size of perforated base was 45, 53, 106, 180, 300, 425, 500, 600, 710, 850, 1000, 1400, 2000 or 2800 μm. These trays are autoclave compatible. 12.0 g of wheat bran and soybean hulls and 15.0 g of rapeseed meal were used with both systems. These amounts were used to bring the height of the substrate bed in the tray to 1.0 cm. After the inoculation process (as described in Chapter 5), all the trays were placed in a growth incubator with temperature strictly under control at 30 °C. The initial moisture content of the substrate was 65%. At the end of the incubation period (72 h), the performance of the two systems was evaluated in terms of the production of four enzymes, which are glucoamylase, protease, xylanase and cellulase.

9.2.3   Moisture Content and Spores Count

Moisture content of the fermented samples at the end of 72 h of the fermentation period was determined by weight loss after heating at 95 °C to a constant weight. The procedures are described in detail in Chapter 5. The procedure for counting spores in every tray is described in Chapter 5.

9.2.4   Preparation of Enzymes Supernatant

After the 72 h fermentation period, samples were taken for enzyme analysis. A standard operational procedure for enzyme extraction developed in this study, as explained in detail in Chapter 5, was followed. In addition, the supernatant was also used for determination of pH, total reducing sugars (TRS), free amino nitrogen (FAN) and colour. All the procedures for the above-mentioned analyses can be found in Chapter 5. Enzymes assays for glucoamylase, protease, xylanase and cellulase were prepared according to the procedures described in detail in Chapter 5.

9.3   RESULTS AND DISCUSSION

Food and agro-industry solid waste residues are generally considered the best substrates for SSF processes and enzyme production in SSF in terms of nutritional value (Stoilova and Krastanov, 2008). These substrates, which are derived from various sources, vary in their nature, structure and composition. The quality of substrates differs due to their composition and the ratios of different ingredients. These include starch, protein, lignin, cellulose and hemicellulose. Therefore, cultures from different strains of microorganisms biodegrade substrates in varied fashions and produce different enzymes, such as glucoamylase, protease, xylanase and cellulase. In the end product of fermentation, enzymes should be present in a heterogeneous mixture as a result of the metabolic activity of the employed microorganism (Wang *et al.*, 2010).

Preliminary results show that there are no significance differences in the values obtained for moisture content, pH, TRS, spore concentration, colour, FAN and enzyme production in the opened system with different aperture sizes (perforated base aperture size 45, 53, 106, 180, 300, 425, 500, 600, 710, 850, 1000, 1400, 2000 and 2800 µm). Thus, in this chapter, the values obtained using the opened system will be presented as an average for the different measurements corresponding to aperture sizes ranging from 45 to 2800 µm. The data are compared with those from the closed system to investigate the performance differences between the two systems.

9.3.1   Moisture Content

FIGURE 9.1 shows the final moisture content for the two fungi in both systems for wheat bran, soybean hulls and rapeseed meal. In the closed system, moisture content increased from the initial moisture content (65%) after 72 h of fermentation time. However, in the opened system, drastic moisture content reduction was observed reaching an average moisture content of 15.56% and 33.27% after 72h of fermentation for *A. awamori* and *A. oryzae* respectively. Hence, the closed system was found to be more capable of retaining moisture.

According to Nagel *et al*. (2000), water balance in SSF is influenced by four main contributors, which are (1) water needed for hydrolysis, (2) metabolic water production, (3) uptake of intracellular water during biomass production and (4) water evaporation as a consequence of metabolic heat production (refer to FIGURE 2.14, Chapter 2). These four contributions may explain the incremental change of moisture content in the closed system.

In addition, in the opened system, it was observed that the moisture content of fermented substrate with *A. oryzae* was higher compared to that of fermented substrate with A. *awamori*. This is because *A. oryzae* was found to have higher ability to retain water compared to *A. awamori* as described in Chapter 6 (Section 6.3.3). Other experiments were carried out to observe moisture content profile between non-fermented and fermented wheat bran in petri dishes (closed system) and result was presented in FIGURE 7.2 (Chapter 7, Section 7.3.2). Instantaneous moisture content in fermented substrate slightly higher compared to non-fermented substrate. During growth and respiration, amount of water was generated. The growth morphology of fungi by producing hyphae and mycelium covers the substrate will helps to retain moisture in the system and so the rate of evaporation may be lower in fermented substrate. It was assumed that the same level of water evaporation would occur in the fermenting substrate as in the non-fermented substrate.
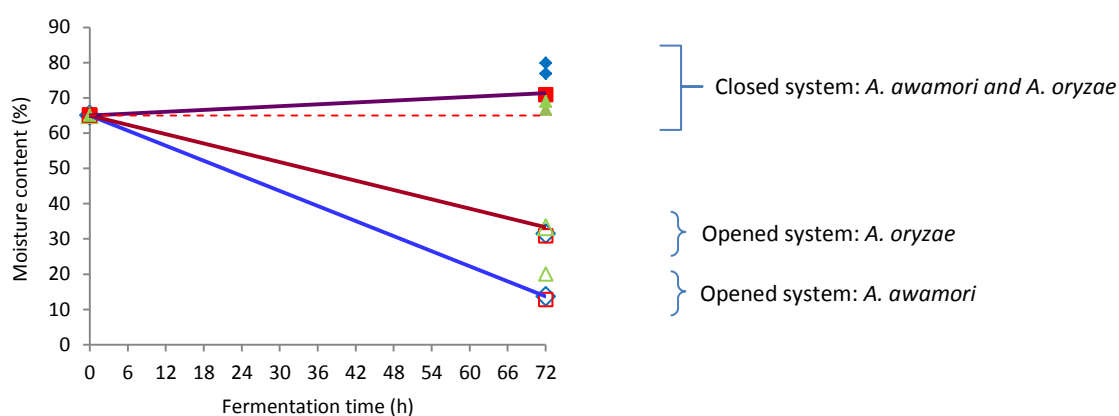
FIGURE 9.1:   **Comparison of final moisture content between *A. awamori* and *A. oryzae* in closed and open systems for all three substrates tested. (   ) represents moisture content at 65%. (◆ ◇): wheat bran; (■ □): soybean hulls and (▲ △): rapeseed meal**

9.3.2   pH Profile in SSF

FIGURE 9.2 shows pH values for the two fungi in both systems obtained by measuring the acidity of the solution supernatant extracted from fermented substrate after 72 h of fermentation. The pH value from *A. awamori* fermented mass was acidic, between 4.6 and 5.2; while the pH of *A. oryzae* fermented mass ranged between 6.7 and 8.0 in all substrates tested either in the closed system or the opened system.

According to Karr-Lilienthal *et al*. (2005), xylan contains a low proportion (approximately 4%) of side chains of single units of D-glucopyranosyluronic acid residues attached to the main chain by 1→2 linkage. Upon hydrolysis, xylan yields a mixture of acidic sugars that are considered to be constituents of wheat bran, rapeseed meal and soybean hulls: polysaccharide V, xylose, a minor component of glucoronic acid, higher oligosaccharides and traces of L-rhamnose. This might be one of the reasons that explain the acidic pH value of *A. awamori* fermented mass, pH 4.53 – 5.17, in all substrates tested. This explanation may also be supported by xylanase activity from *A. awamori* fermented mass, which was higher compared to *A. oryzae* fermented mass, where pH ranged between 6.7 and 8.0 in both systems.
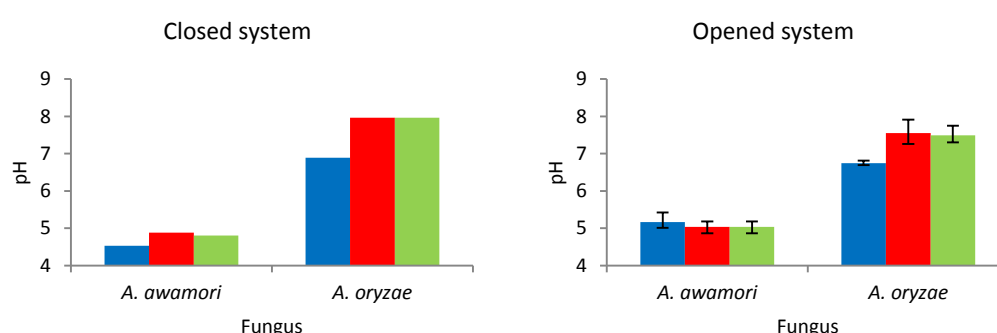
FIGURE 9.2: **Comparison of pH profiles in both systems between *A. awamori* and *A. oryzae* after 72 h fermentation time. (◼): wheat bran; (◼): soybean hulls and (◼): rapeseed meal. Data points show the average from opened tray system with 14 different mesh aperture sizes**

9.3.3 Biomass Estimation

As shown in FIGURE 9.3, use of the colourimetric technique was feasible for indirect determination of fungal biomass and accurate comparison with spores concentration. The colour density and spores concentration had similar trends in the two systems for both fungi with all three solid substrates tested. It was observed from this work that the open system was excellent in spores production for *A. awamori* and *A oryzae*. Colour density measured at wavelength 300 nm increased proportionally with spores concentration.

In this study, use of the colourimetric technique for biomass estimation was proved to be a feasible approach for indirect measurement of fungal biomass in SSF. Although the technique may not be suitable for SSF in industrial or practical processes, this new approach is still an important complementation to existing techniques, especially for basic research.
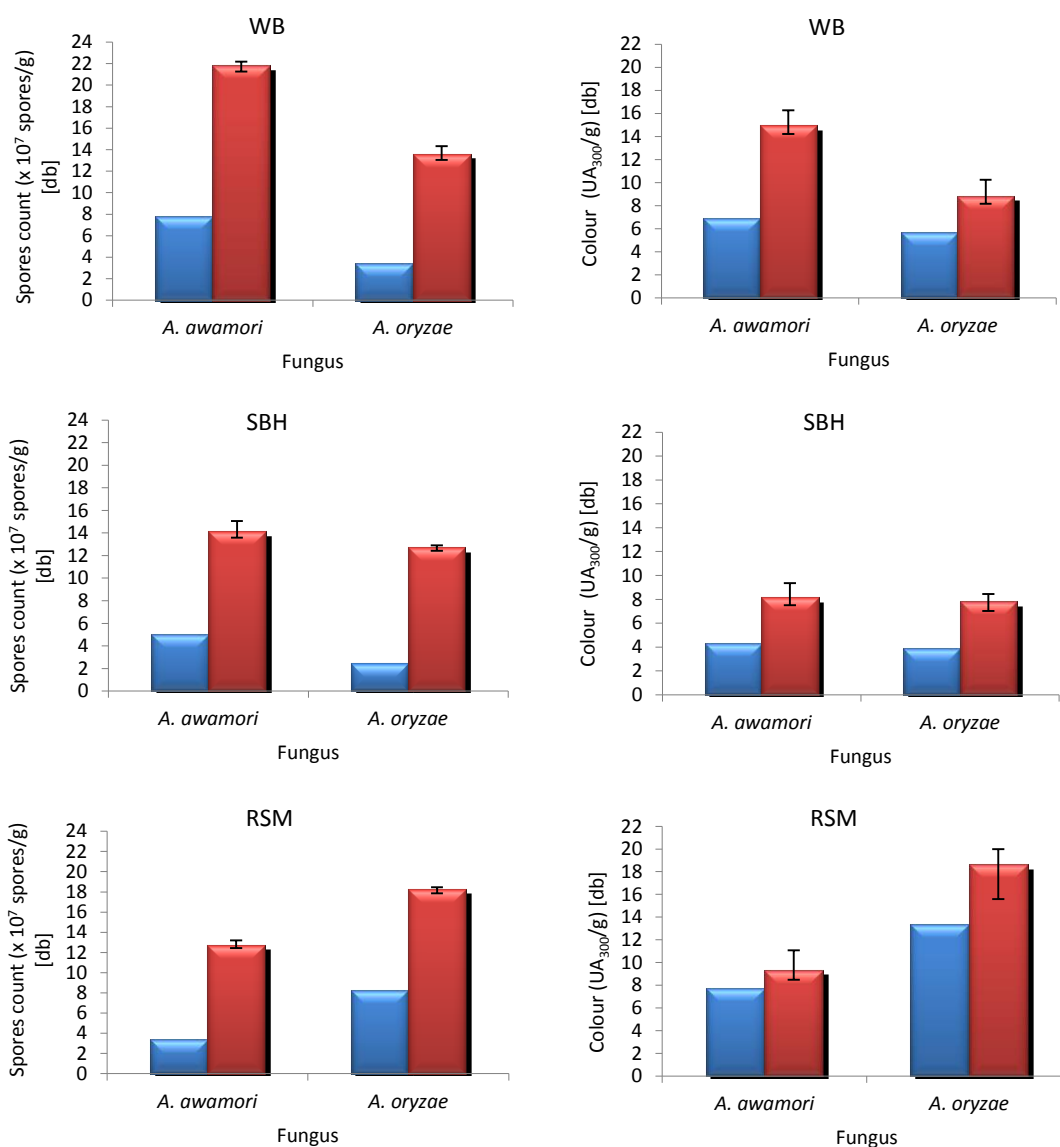
FIGURE 9.3: Comparison of spores count and colour produced in closed (■) and opened system (■) by the fungal cultures of *A. awamori* and *A. oryzae* on different solid substrates. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal. Data points show the average of measurements for the opened tray system with 14 different mesh aperture sizes

9.3.4 Total Reducing Sugars and Free Amino Nitrogen

Total reducing sugars (TRS) and free amino nitrogen (FAN) were released from all three solid substrate (FIGURE 9.4). TRS concentrations of 43.20, 21.66 and 4.35 mg/g [db] were obtained after 72 h of fermentation time in the closed system with *A. awamori* for wheat bran, soybean hulls and rapeseed meal respectively. *A. awamori* in the opened system produced an average TRS

concentration of 38.73, 20.75 and 14.92 mg/g [db] on wheat bran, soybean hulls and rapeseed meal respectively.

Wheat bran fermentation with *A. awamori* was observed to produce the highest levels of TRS followed by soybean hulls and rapeseed meal in both systems, which can be explained by several reasons. The first reason may be the high starch concentration in wheat bran (23.3%), which can lead to increased glucoamylase activity and starch hydrolysis. The second reason might be the elevated xylanase activity and increased hemicellulose material hydrolysis. In addition to xylanase, cellulase can also release reducing sugars from lignocellulose, and the liberated reducing sugars are essentially from the hydrolysis of lignocellulose material.

TRS concentrations of 14.22, 14.73 and 9.43 mg/g [db] were obtained after 72 h of fermentation time in the closed system with *A. oryzae* for wheat bran, soybean hulls and rapeseed meal respectively. *A. oryzae* in the opened system produced an average TRS concentration of 21.41, 15.77 and 13.97 mg/g [db] on rapeseed meal, soybean hulls and wheat bran respectively. There was no significant difference in TRS production on solid substrates between the two systems. However, the production of TRS in the opened system was slightly higher than that in the closed system on rapeseed meal.

FAN concentrations of 713.99, 475.42 and 244.43 mg/g [db] were obtained after 72 h of fermentation time in the closed system with *A. awamori* for rapeseed meal, wheat bran and soybean hulls respectively. *A. awamori* in the opened system showed a slight increase in FAN production; an average FAN concentration of 756.27, 737.52 and 370.12 mg/g [db] was obtained for rapeseed meal, soybean hulls and wheat bran respectively.

FAN concentrations of 1797.74, 895.72 and 315.02 mg/g [db] were obtained after 72 h of fermentation time in the closed system with *A.oryzae* for rapeseed meal, soybean hulls and wheat bran respectively. *A. oryzae* in the opened system produced an average FAN concentration of 1864.48, 867.41 and 650.86 mg/g [db] on rapeseed meal, wheat bran and soybean hulls respectively. There was no significant difference in FAN production on rapeseed meal between the two systems. The opened system showed slightly higher FAN production. By contrast, the opened system with wheat bran showed almost 2.5 times higher FAN production compared to the closed system. In contrast to rapeseed meal and wheat bran, the production of FAN in the opened system was about 1.5 times lower on soybean hulls.

FIGURE 9.4:   Comparison of TRS and FAN produced in the closed (🟦) and opened system (🟥) by the fungal culture of *A. awamori* and *A. oryzae* on different solid substrates. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal. Data points show the average of measurements for the opened tray system with 14 different mesh aperture sizes

As expected, the production of FAN was elevated in rapeseed meal substrate with *A. oryzae* in both the closed system and the opened system, followed by wheat bran and soybean hulls (FIGURE 9.4). As reported earlier, *A. oryzae* favoured to grow on rapeseed media. Rapeseed meal is a nitrogen-rich substrate as it consists of approximately 38.9% (w/w) protein. High protease activity sustained continuous FAN production. However, *A. awamori* was also reasonably efficient in producing FAN in the opened system on wheat bran and rapeseed meal compared to soybean hulls. Usually reducing sugars will continue to be produced by the fungus as a result of hydrolysis and, at the same time, these sugars will be consumed by the fungus continuously for growth maintenance. This

phenomenon can lead to the production of FAN as a result of protein hydrolysis. This provides a suitable condition for fungal growth in the SSF process.

It is worth noting that *A. awamori* and *A. oryzae* have their own capabilities to utilize the carbohydrate, protein and ligno-cellulosic fraction from every solid substrate. In a biorefinery, usually the above-mentioned fraction is in the holding the attention. Considering the proportion of these fractions in solid substrate, its utilisation is also of great relevance. Fungal utilisation leads to different sugar monomers and FAN. In the present work, enormous quantities arising in the fungal utilisation and its specific properties, all the solid substrate can be considered as a feedstock for future biorefineries.

9.3.5   Enzymes Production in SSF using the Tray System

Many researchers have reviewed the production of enzymes in various modified solid state tray systems. Nahid *et al*. (2012) reviewed glucoamylase production and Mitra *et al*. (1996) reviewed the production of proteolytic enzymes. Babu and Satranayanam (1996) examined bacterial enzyme production, while Gupta and Kar (2008) reviewed the production of cellulolytic enzymes. In addition, Dhillon *et al*. (2011) reported the production of cellulase and xylanase enzymes in tray SSF that employed mixed-cultured fungi.

9.3.5.1   Glucoamylase

Activity levels for glucoamylase that was produced in the two systems are shown in FIGURE 9.5. Glucoamylase activity levels of 299.47, 88.74 and 75.96 U/g [db] were obtained after 72 h of fermentation time in the closed system with *A. awamori* for wheat bran, soybean hulls and rapeseed meal respectively. *A. awamori* in the opened system produced an average glucoamylase activity of 336.08, 90.92 and 32.82 U/g [db] obtained for wheat bran, soybean hulls and rapeseed meal respectively. Glucoamylase activity levels were higher when SSF was carried out in the opened system with wheat bran and soybean hulls. This was attributed to the efficient air circulation and nutrient diffusion caused by the perforated base resulting in an increase in O_2 and mass transfer. However, almost a two-fold glucoamylase activity decrease was recorded for rapeseed meal culture in the opened system compared to the closed system.

In addition, glucoamylase activity levels of 121.62, 85.96 and 79.06 U/g [db] were obtained after 72 h of fermentation time in the closed system with *A. oryzae* for wheat bran, soybean hulls and rapeseed

meal respectively. *A. oryzae* in the opened system produced an average glucoamylase activity of 81.30, 76.94 and 57.30 U/g [db] obtained for wheat bran, soybean hulls and rapeseed meal, respectively. This is a contrast to the result obtained with *A. awamori*. Glucoamylase activity decreased in the opened system almost 1.5 times in *A. oryzae* culture on rapeseed meal compared to the closed system.

FIGURE 9.5: **Activity of glucoamylase produced in the closed (■) and the opened system (■) by the fungal culture of *A. awamori* and *A. oryzae* on different solid substrates. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal. Data points show the average of measurements for the opened tray system with 14 different mesh aperture sizes**

A. awamori produced high glucoamylase levels in the opened and the closed system respectively. *A. awamori* favoured growth on wheat bran and produced high glucoamylase activity on this substrate. Higher enzyme production on wheat bran can be correlated with its high starch content (23.3%). However, *A. oryzae* does not favoured on wheat bran. *A. awamori* with wheat bran media resulted in double the enzyme production by *A. oryzae* culture.

Ellaiah *et al*. (2002) reported that wheat bran gives higher enzyme yield compared to rice bran and other agricultural waste. Hata *et al*. (1997) compared glucoamylase levels produced in SSF and SmF systems using *A. oryzae* and showed these systems exhibited different characteristics. In their work,

they reported that glucoamylase produced by SSF could digest raw starch but that produced by SmF could not. Pandey (1990) reported that glucoamylase production in trays occurred optimally after 36 h of fermentation in comparison to the typically required 96 h periods in flask fermentation processes.

9.3.5.2   Protease

Protease activity levels measured in samples in the studied systems are shown in FIGURE 9.6. Protease activity levels of 93.08, 42.05 and 40.04 U/g [db] were obtained after 72 h of fermentation time in the closed system with *A. awamori* for rapeseed meal, soybean hulls and wheat bran respectively. However, *A. awamori* in the opened system produced an average protease activity of 165.03, 114.92 and 70.32 U/g [db] obtained from rapeseed meal, soybean hulls and wheat bran, respectively. Protease activity increased about two-fold when SSF was carried out in the opened system compared to the closed system.

Meanwhile, protease activity levels of 1017.70, 972.35 and 964.71 U/g [db] were obtained after 72 h of fermentation time in the closed system with *A. oryzae* for rapeseed meal, soybean hulls and wheat bran, respectively. In contrast to the closed system, *A. oryzae* in the opened system produced an average protease activity of 2029.51, 919.50 and 1327.76 U/g [db] obtained from rapeseed meal, soybean hulls and wheat bran, respectively. In line with observations with *A. awamori*, protease activity increased almost two-fold in the opened system compared to the closed system.

These results proved that *A. oryzae* is an excellent fungus for protease production with rapeseed meal as a solid substrate. This is expected since rapeseed meal content is higher in protein content (38.9%) than wheat bran (15.1%) and soybean hulls (14.2%). In this case, the opened system proved to be more favourable for protease production for both fungi. Improvement of mass transfer in the opened system might be a reason explaining this behaviour. Another can be low temperature and moisture content of the fermented solid that may be beneficial and may cause efficient degradation of substrate and uptake of nutrients. The decrease in moisture content in the opened system, especially with *A. oryzae* culture, did not reach a critical point that can inhibit growth. Protease produced by *A. oryzae* was efficient in breaking down the peptide bonds of proteins contained in rapeseed meal, and hence, forming more FAN (FIGURE 9.4).
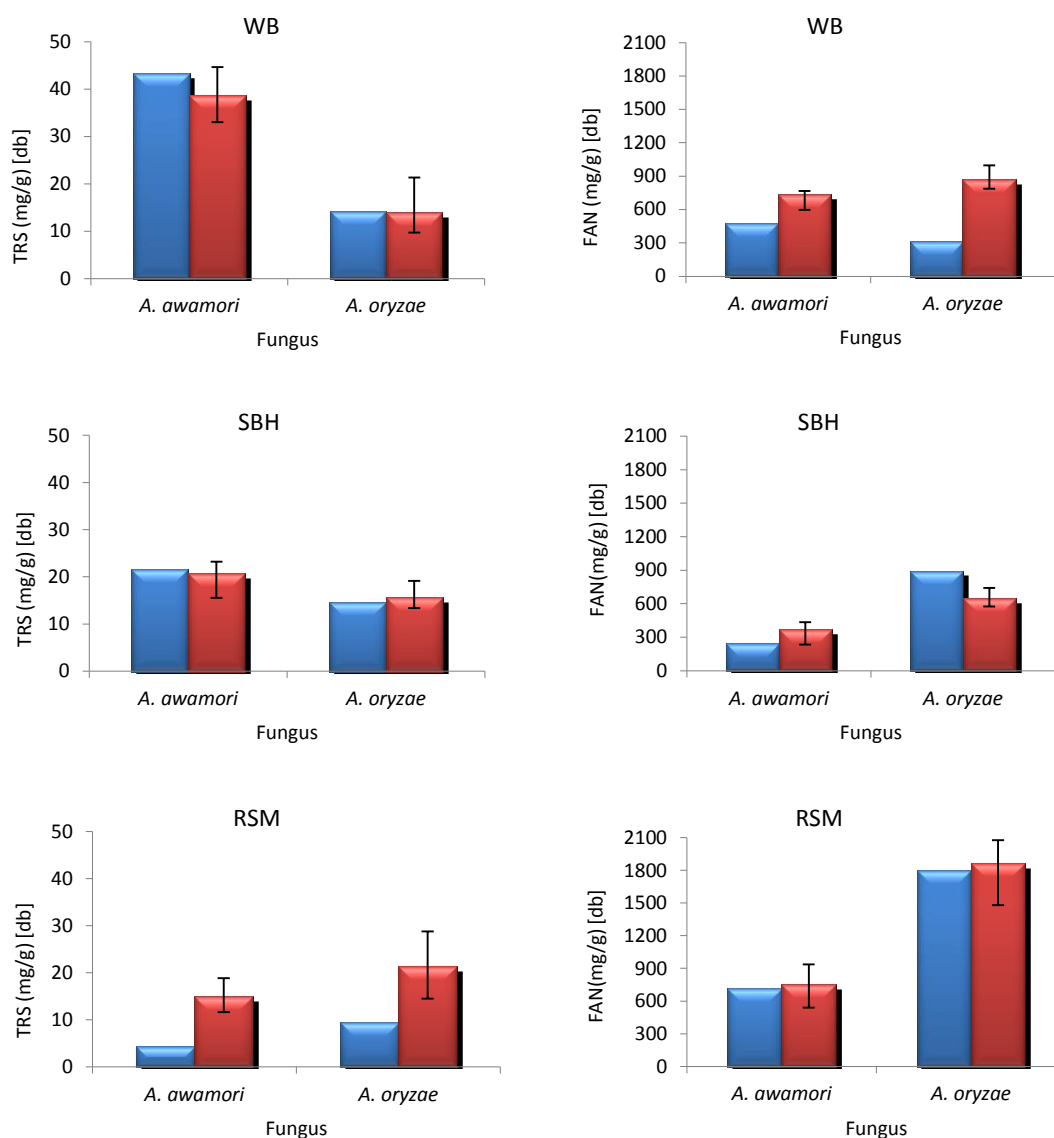
FIGURE 9.6: **Activity of protease produced in the closed (■) and opened system (■) by the fungal culture of *A. awamori* and *A. oryzae* on different solid substrates. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal. Data points show the average of measurements for the opened tray system with 14 different mesh aperture sizes**

George *et al*. (1997) reported that one gram of wheat bran was found to produce about 250,000 U/mL protease activity in SSF using *B. amyloliquefaciens* by subjecting the microorganism to natural selection and high positive organic nitrogen pressure. Meanwhile, Belmessikh *et al*. (2013) reported that protease activity was nine times higher in SSF (21,309 U/g) compared to SmF (2343.5 U/g) using *A. oryzae* on tomato pomace.

9.3.5.3   Xylanase

Xylan is a polysaccharide found in the hemicellulose fraction of lignocellulose. Hemicellulose of agricultural substrate can be used as an appropriate substrate for xylanase production in SSF (Chai *et al*., 2006). Xylanase hydrolyses xylan to xylose or xylooligosccharide. Activity levels for xylanase extracted in both studied systems with *A. awamori* and *A. oryzae* are shown in FIGURE 9.7.

Maximum xylanase activity levels of 728.24, 586.17 and 450.63 U/g [db] were obtained after 72 h of fermentation time in the closed system with *A. awamori* for wheat bran, soybean hulls and rapeseed

meal respectively. However, *A. awamori* in the opened system produced an average xylanase activity of 581.42, 389.65 and 349.49 U/g [db] obtained for wheat bran, soybean hulls and rapeseed meal, respectively. There were significant differences between the closed and opened system for all reported activities. *A. awamori* was observed to be superior in producing xylanase in the closed system compared to the opened system. This observation can be related to the increase in moisture content in the closed system, which may favour xylanase production. It was reported that the optimum moisture content for xylanase production by *T. longibrachiatum* (Ridder *et al*., 1999) and *A. tereus* (Gervais and Molin, 2003) on wheat bran were 55 and 75% respectively. Production of xylanase decreased in the opened system, which might be related to loss of moisture content and subsequent inhibition of *A. awamori* growth.

Maximum xylanase activity levels of 103.94, 52.71 and 17.14 U/g [db] were obtained after 72 h of fermentation time in the closed system with *A. oryzae* for wheat bran, soybean hulls and rapeseed meal, respectively. However, *A. oryzae* in the opened system showed an increase in xylanase activity to an average of 219.71, 138.71 and 117.87 U/g [db] observed for soybean hulls, wheat bran and rapeseed meal, respectively. In contrast to the results obtained for *A. awamori* above, the production of xylanase was higher in the opened system compared to the closed system.

Significant differences were observed in the produced xylanase activity between *A. awamori* and *A. oryzae* in both systems. In the closed system with *A. awamori* on wheat bran, xylanase production was 7 times higher compared to *A. oryzae* in the same system. Production of xylanase seemed very poor with *A. oryzae* in both systems with all three substrates tested. These findings show that *A. awamori* is efficient in producing xylanase and in actively degrading the bonds of hemicellulose contained in wheat bran, soybean hulls and rapeseed meal and forming more reducing sugars. This is another reason TRS was higher in *A. awamori* culture. High activities were obtained for wheat bran followed by soybean hulls, both of which contained a high proportion of hemicellulose (29.7% and 12.5%, respectively) (TABLE 5.1, Chapter 5) and therefore induced xylanase production. Rapeseed meal, however, has lower hemicellulose content. Hemicellulose of agricultural substrate can be used as an appropriate substrate for xylanase production in SSF (Chai *et al*., 2006). However, *A. oryzae* is observed to be very poor in the production of xylanase.
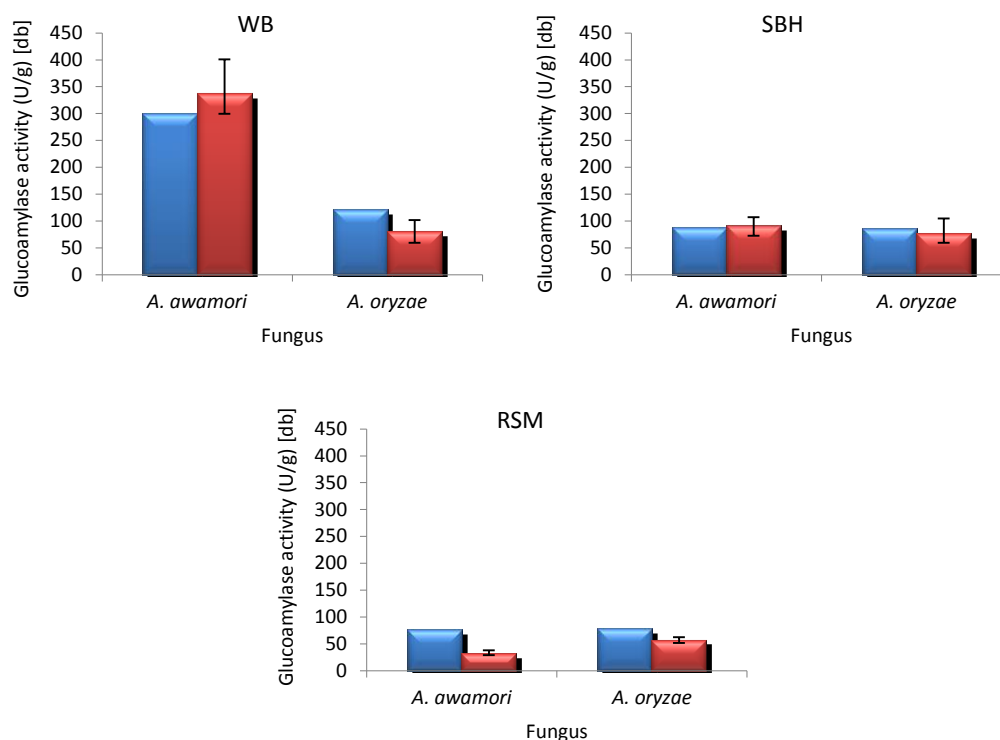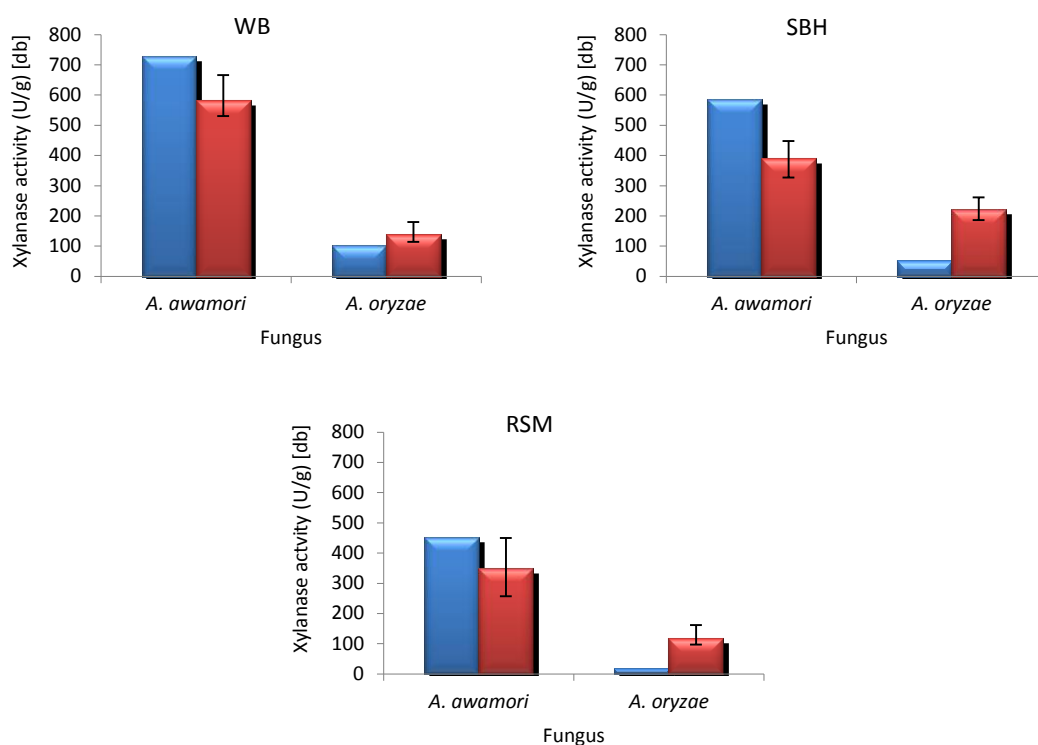
FIGURE 9.7: Activity of xylanase produced in the closed (■) and opened system (■) by the fungal culture of *A. awamori* and *A. oryzae* on different solid substrates. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal. Data points show the average of measurements for the opened tray system with 14 different mesh aperture sizes

Observations of this work indicate the production of xylanase is higher in a closed system. In the closed system, it was assumed that heat accumulation occurred and therefore incubation temperature increased and subsequently the production of xylanase. This phenomenon was also observed in glucoamylase production (FIGURE 9.5). Cai *et al*. (2006) reported production of thermostable xylanase in an SSF system using *A. niger* A3 and this enzyme was more thermostable (55 °C) than that produced in SmF. Archana and Satyanarayana (2003) also reported production of cellulase-free xylanase at levels 22 fold higher in SSF compared to SmF using thermophile *B. licheniformis* A99. In addition, the SSF process was also observed to induce production of higher levels of xylanase by thermostable *Melanocarpus albomyces* due to the close contact between the mycelium and the solid substrate compared to SmF (Jain, 1995). According to Pandey *et al*. (1999), although xylanase is produced by fungi, yeast and bacteria, filamentous fungi are preferred for commercial production as the levels of enzyme produced by fungal cultures are higher than those obtained from yeast or bacteria.

9.3.5.4   Cellulase

Cellulases are among the most important enzymes that are employed in the processing of ligno-cellulosic materials for the production of feed, fuel and chemical feedstocks (Pandey *et al.*, 1999). Activity levels of cellulase involved in both studied systems were measured using an enzyme extract that was prepared after fermentation with *A. awamori* and *A. oryzae* (FIGURE 9.8).

Maximum filter paper activity levels of 17.28, 16.99 and 16.08 FPU/g [db] were obtained after 72 h of fermentation time in the closed system with *A. awamori* for wheat bran, rapeseed meal and soybean hulls, respectively. However, *A. awamori* in the opened system produced an average filter paper activity level of 14.03, 10.19 and 8.19 FPU/g [db] obtained for soybean hulls, rapeseed meal and wheat bran, respectively. There was no significant difference between solid substrates especially in the closed system.

Maximum filter paper activity levels of 15.18, 13.88 and 11.67 were obtained in the closed system with *A. oryzae* for soybean hulls, wheat bran and rapeseed meal, respectively. In the opened system, an average filter paper activity level of 12.57, 11.48 and 3.32 FPU/g [db] was obtained for soybean hulls, rapeseed meal and wheat bran, respectively.

A. awamori was observed to be superior in producing cellulase on wheat bran and soybean hulls in the closed and opened system. *A. oryzae* was observed to be excellent in producing cellulase on soybean hulls in both systems compared to the other two substrates. Cellulose content in soybean hulls (36.4%) was obviously higher than that in wheat bran (10.6%), and as expected, cellulose is ideal for efficient growth of fungal cultures and cellulase production. The results of static tray SSF highlighted the importance of using the closed or the opened system. Overall, cellulase production was significantly higher in closed system SSF as compared to the opened system for both fungi. Production of cellulase decreased in the open system for both fungi, which might be due to the drastic loss of moisture content in this system.
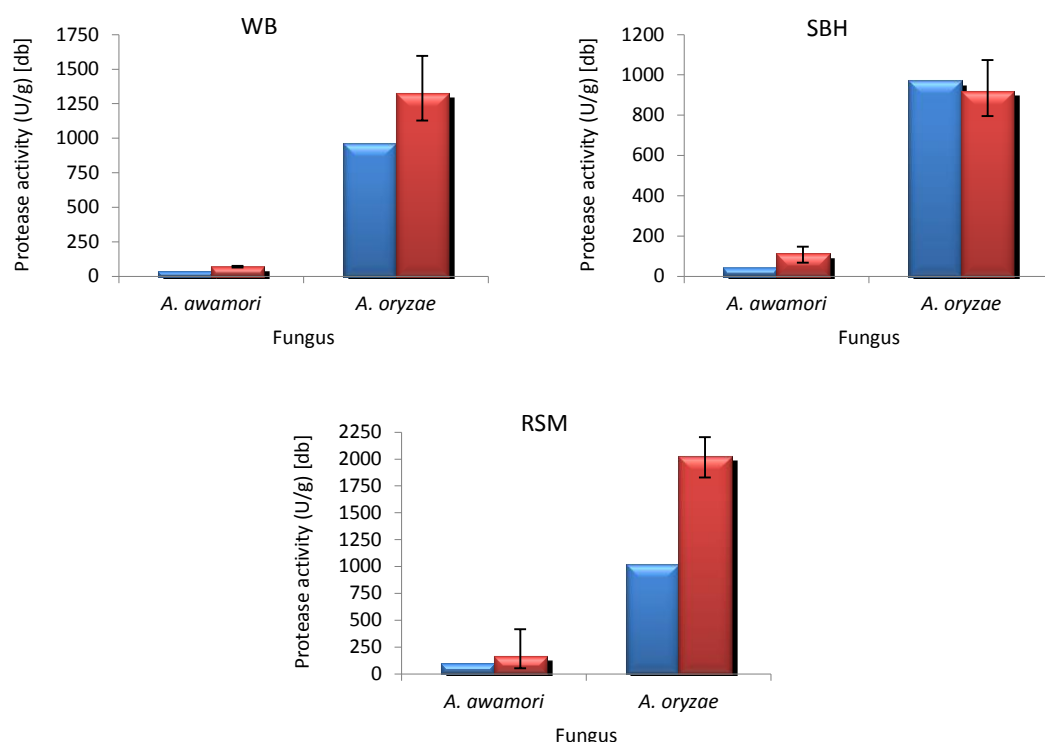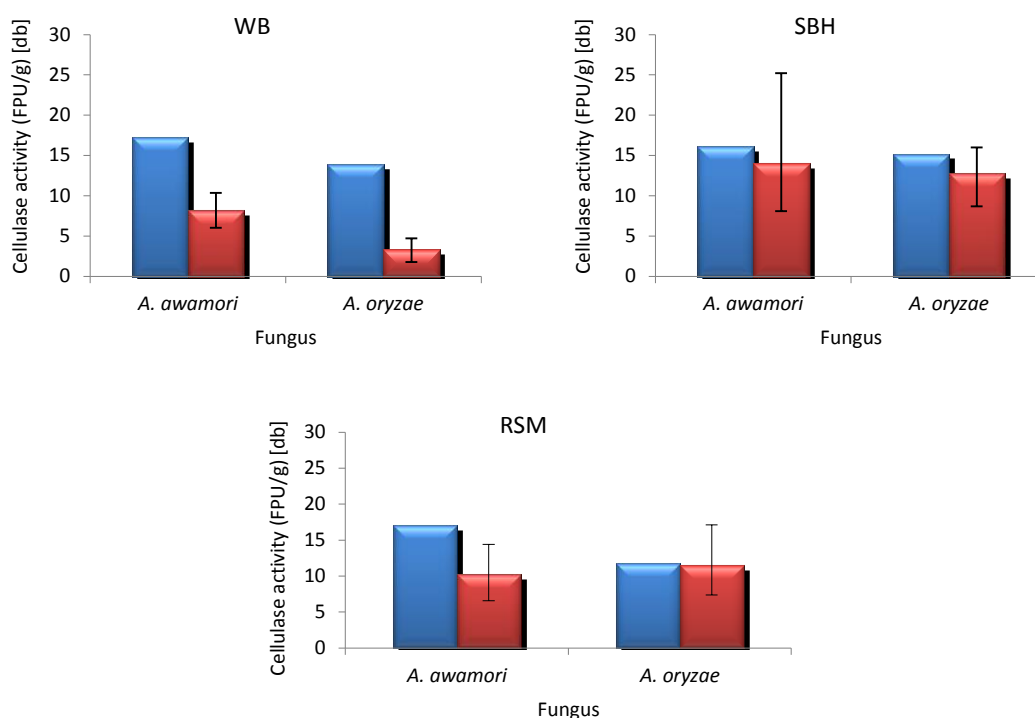
FIGURE 9.8: **Activity of cellulase produced in the closed (■) and opened system (■) by the fungal culture of *A. awamori* and *A. oryzae* on different solid substrates. [WB]: wheat bran; [SBH]: soybean hulls and [RSM]: rapeseed meal. Data points show the average of measurements for the opened tray system with 14 different mesh aperture sizes**

Brijwani *et al.* (2010) reported that SSF of soybean hulls supplemented with wheat bran produced a maximum filter paper activity of 10.7 FPU/g [db] after a 96 h incubation period in a static tray. According to Amorea Faracoa (2012), only fungi naturally produce the needed titers of cellulases required for the complete saccharification of pretreated lignocellulose. Cellulolytic fungi (*T. reesei*, *Aspergillus spp.*, *Fusarium spp.*, *Rhizopus spp.*) including thermophilic fungi, such as *T. emersonii* and *T. aurantiacus*, produce a high concentration of cellulase enzymes to digest lignocellulose efficiently, assimilate all ligno-cellulosic sugars and convert these sugars to ethanol, showing that they naturally possess all pathways for the conversion of lignocellulose to bioethanol (Viikari *et al*., 2012). The efficiencies of the degradation of cellulose were dependent on the nature and concentration of the compounds added in the SSF system. The production of fermentative sugars as a result from the hydrolysis of biomass component (hemicellulose and cellulose) is largest economic and technological barrier for the production of cellulosic biofuels (Viikari *et al*., 2012).

9.3.6   Mass Transfer Phenomenon in the Tray System

In this work, it was observed that *A. awamori* and *A. oryzae* react differently to the design applied in the tray system. There were also differences between of *A. awamori* and *A. oryzae* in the degradation of the solid substrates tested. These differences led to variations in growth performance and production of enzymes. *A. awamori* favoured the opened system for glucoamylase production, while *A. oryzae* favoured the closed system. Glucoamylase production increased when access to O_2 was improved in the opened system using a perforated tray compared to the closed system. Xylanase and cellulase production was observed to be superior in the closed system with *A. awamori*, and protease production was excellent in the opened system with *A. oryzae*.

From visual observation (in the case of *A. awamori*), the morphology growth in the closed and opened systems shows some difference as shown in FIGURE 9.9. The same phenomenon was observed for *A. oryzae*. In this work, two types of mycelia were observed in the vegetative growth of both fungi. First, the growth penetrating the surface of the solid substrate leads to the formation of penetrated mycelium. Second, the aerial growth leads to the formation of aerial mycelia. However, it is difficult to differentiate between these mycelia on the surface. According to Sudo *et al.* (1995), after germination, the penetrated mycelium grows first, and then aerial mycelium develops above it. The sporangiophores that produce the conidiospores are formed later within the aerial mycelia. Additionally, Sudo *et al*. (1995) studied differences between these two types of mycelium in terms of the production of α-amylase in rice koji. They observed that α-amylase was found to be produced highly by the penetrated mycelium rather than the aerial mycelium.

FIGURE 9(a) shows the mycelium penetrates deeply into the inner solid substrate particles of wheat bran. The deep penetrating clean white mycelium and binding together the solid substrate, covered almost every part of the fermented bed and produced a compact fermented cake. FIGURE 9(b) shows the large aerial mycelium on the surface of the fermented bed, showing sporangiophores, which would produce large spores. The fermented cake observed is quite loose and porous from the middle of the fermented cake to the bottom surface of the perforated base. The production of mycelium in the open system was lower compared to the closed system. It was assumed that, in the area exposed to the environment, the concentration of O_2 is very high yet moisture content is low. This will lead to lower diffusivity of glucose and other nutrients into solid particles of solid substrates of low moisture content. These results mean that the production of glucose and other nutrients is restricted at low moisture content and any glucose and other nutrients available are consumed rapidly before they diffuse along the particles (this phenomenon is described in Chapter 2 (Section 2.11.1.2)). As a result,

some starvation may occur on solid substrates of low moisture content, which may affect growth performance. Therefore, it was concluded that moisture content in the fermented substrate is one of the most important factors for the production of enzymes in SSF.
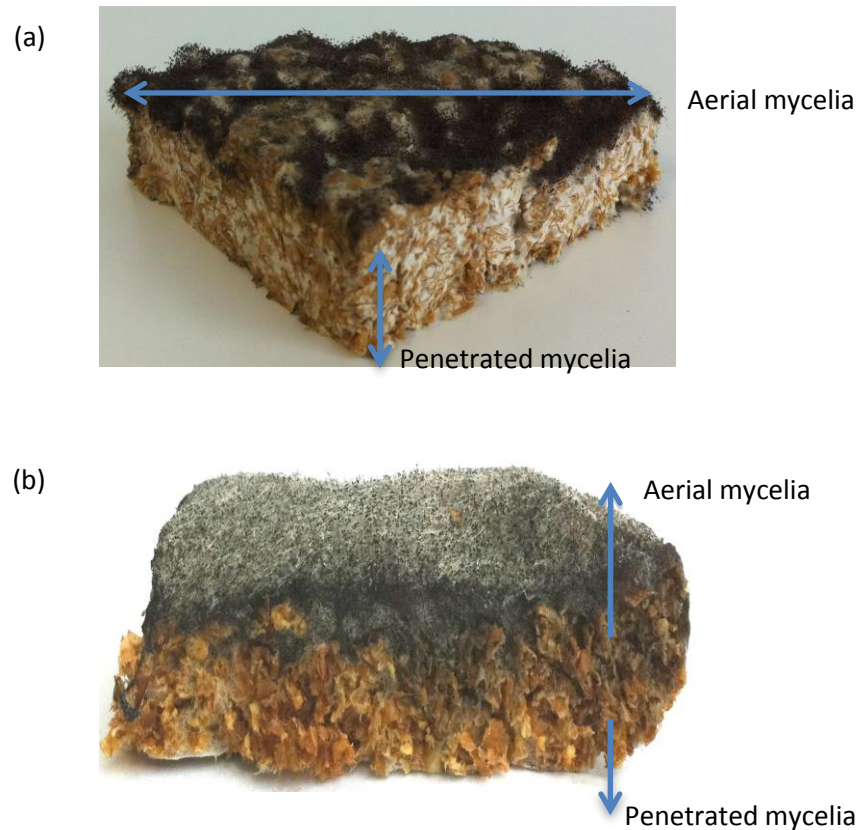
FIGURE 9.9: **Images of the fermented cake of *A. awamori* on wheat bran in (a) the closed system and (b) the opened system**

FIGURE 9.10(a) shows the closed non-aerated system showing the advantages of tray SSF; it allows for the strict control of water content in the substrate during culture by allowing internal vapour condensation. O_2 concentration in the system might be low, but this disadvantage might be overcome where fungi are totally dependent on water content to support growth. Internal vapour condensation might become detrimental because it can create non-homogenous conditions. Fungi grew more vigorously in the cultures with higher water content. It was observed from the study in the lab that moisture content in the closed non-aerated system could be maintained for up to 200 h of fermentation time. Consequently, the increase in moisture content of the fermented bed could be cause decreased porosity, change in particle structure and lower O_2 transfer in the solid substrate bed. The moisture content in the closed system was assumed to be reasonable between 65 – 73%. The moisture content profiles of fermented wheat bran and non-fermented wheat bran can be found in the FIGURE 7.2 (Chapter 7).
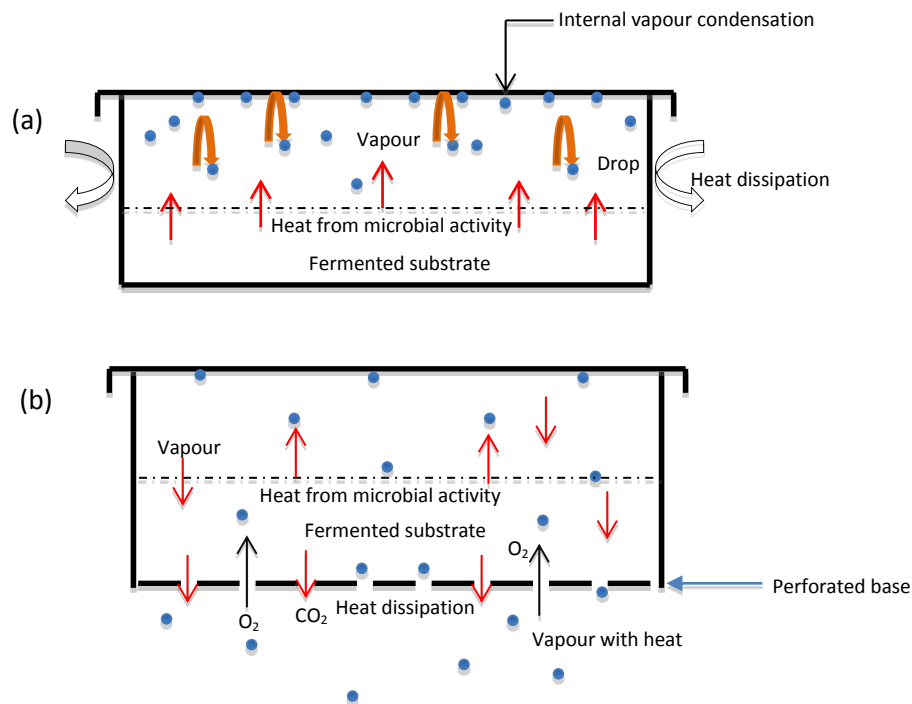
FIGURE 9.10: Schematic cross-sectional diagrams of the (a) closed system and (b) opened system. Briefly, phenomenon mass transfer during fermentation process

As in the opened system (FIGURE 9.10(b)), the contribution of water evaporation as a consequence of metabolic heat production might not happen because water evaporation occurs at the perforated base. No water accumulation is observed. These results indicated that the vapour generated from microbial activity could efficiently dissipate out of the tray through the perforated base. Nevertheless, the total amount of evaporated water was estimated to be higher in the opened system. Water evaporation depends on the amount of metabolic heat evolved, so it can be suggested that for the opened system, the heat evolved reduces the moisture content of the solid substrate by evaporation in the case of both fungi.

One of the important roles and functions of the fungi in SSF is the synthesis of enzymes, in particular extracellular enzymes. These enzymes generally hydrolyse complex compounds such as starch, proteins, and polysaccharides into smaller molecules, which in turn can be taken up by any cell. As a consequence, the reduction of moisture content leads to reduce diffusion of enzymes and nutrients in the fermented substrate, lower degree of swelling and increased water tension on the microorganism.

9.4   SUMMARY AND CONCLUSIONS

As an overall conclusion, a perforated tray system was developed in this study that permits direct access to O_2. Testing the tray system with different perforated mesh aperture sizes in this study did not yield different results in growth performance of *A. awamori* and *A. oryzae*. Reducing sugars can be obtained from hydrolysis of starch, hemicellulose and cellulose components of all solid substrates tested. In addition, free amino nitrogen (FAN) is produced as a result of protein hydrolysis. Reducing sugars and FAN available due to degradation of substrates are consumed rapidly in SSF. The opened system is observed to be favourable for spores production by both fungi.

A. awamori and *A. oryzae* can be very versatile in producing various enzymes with different substrates with different starch, protein, hemicellulose and cellulose contents. These studies indicate that *A. awamori* is more suitable for the efficient production of multiple enzymes in the closed system including xylanase and cellulase, while the production of glucoamylase and protease is superior in the opened system. *A. oryzae* is more suitable for the efficient production of protease and cellulase in the closed system, while the production of protease is more favourable the opened system. *A. awamori* efficiently consumed starch (which known as simple nutrient to degrade) in wheat bran medium and produced very high glucoamylase activity, and after that, the fungus efficiently produced other enzymes to degrade other complex nutrients such as protein, hemicellulose and cellulose.

Accumulation of high amylolytic and proteolytic enzymes observed in this study is desired as this will result in a successful hydrolysis process that follows the process of SSF and targets the production of a medium rich in fermentative sugars and nitrogen. Through a strategy for a biorefinery based on SSF, this can lead to the production of bioethanol, biofuels, biodegradable plastic and valuable chemicals (as discussed in Chapter 2, Section 2.12).

From this work, the following conclusions can be made with regards to the systems used:

■ The closed system:

1. O_2 concentration is a limiting factor that influences fungal growth.
2.   The ability to maintain water is high but it creates heterogeneous conditions.

■ The opened system:

1. O_2 concentration might not be a problem, but the drastic loss of moisture content of about 80% (especially in the culture system with *A. awamori*) after 72 h of fermentation is a limiting factor that influences fungal growth.

2. The chance to lose water through evaporation is very high through the uncontrolled perforated base tray.

It is possible that the opened system exhibits such a high reproducibility of enzyme production because it allows efficient mass transfer and heat dissipation, although it is also observed to be more efficient in evaporation of water from the substrate. SSF in the closed system faces difficulty in controlling these parameters because it is a closed environment. On top of that, it is difficult to achieve reproducible results under appropriate culture conditions because both *A. awamori* and *A. oryzae* respond differently with varied growth performances. This can be observed to be reflected in enzyme production. However, the results obtained in this work provide an idea of how to make an air arrangement into the closed solid state tray bioreactor system (Chapter 10). The important idea is that it is possible to achieve uniform and exact regulation of temperature and water content during the fermentation process without introducing unfavourable factors such as substrate mixing.

CHAPTER 10

BIOREACTOR STUDIES

"Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world"
Louis Pasteur

10.1   INTRODUCTION

Despite the early promise of solid state fermentation (SSF) technology, researchers have faced challenges in SSF processes that serve a predominantly bioreactor design. Bioreactors for SSF can be designed to maintain physiological parameters at desired levels and enhance mass transport rates. An appropriate condition development by providing the necessary guides allows metabolic activity and biochemical reactions in a bioreactor. In order to determine design criteria for SSF bioreactor, it is necessary to understand how complex the systems/processes are within the physical context of solid particles and microorganisms.

The requirements for tray solid state bioreactor (SSB) will vary depending on the dimensions, complexity and physiologically environment involvement. Moreover, such bioreactor has to be able to operate under aseptic conditions. This would allow for higher efficiency in the SSF process, as well as a high degree of microbial performance. Normally in SSF, as the fermentation progresses, oxygen (O_2) must diffuse to the region of the bioreaction. The bioreaction itself liberates heat, which is not easily dissipated due to the poor thermal conductivity of the substrate.

In this work, the growth performance and ability of *Aspergillus awamori* and *Aspergillus oryzae* to grow in four tray solid state bioreactor (SSB) systems was explored. It involved the study of the influence of the initial moisture content and air arrangement on growth and enzyme production.

In addition to that, the purpose was to study the effect, on heat and water transfer, of operating variables, fermentation on the perforated mesh tray and internal moist air circulation under natural and forced aeration. The dry or moistened air may be used as a cooling system to remove the heat generated from the microbial activity while at the same time supplying water and O_2 in order to improve fungal growth. In this chapter, simultaneous measurements of bioreactor/bed temperature, oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and instantaneous moisture content for *A. awamori* and *A. oryzae*, a fungi growing actively on wheat bran, were carried out. Also, certain strategies on applicability of air flow rate with various arrangements using dry and moist air for

control of the bioreactor/bed temperature and moisture in tray SSB were proposed and compared for operation in the fungi used.

10.2 MATERIALS AND METHODS

10.2.1 Microorganisms, Inoculum and Substrate Preparation

The fungi *A. awamori* and *A. oryzae* were used to study enzyme production. Procedures for preparation of fungi and inoculum were described in detail in Chapter 5. The solid substrate wheat bran was used as a model substrate. After autoclaving, inoculum transfer was carried out aseptically in a laminar cabinet according to the standard operational procedures described in details in Chapter 4.

10.2.2 The Tray Solid State Bioreactor

The experiments were carried using four tray solid state bioreactors (SSB) where *A. awamori* and *A. oryzae* were cultivated on wheat bran. Temperature, O_2 and carbon dioxide (CO_2) were measured continuously on-line (Chapter 4, Section 4.5.5). Enzyme activity, moisture content and biomass (spores) were also measured. All the procedures were similar to those described previously in Chapter 5. The bioreactor set up and tray SSBs used throughout this study are described in Chapter 4 (Section 4.5) as follows:

1. Single circular tray SSB (FIGURE 4.9, Section 4.5.1)
2. Multi-stacked circular tray SSB (FIGURE 4.12, Section 4.5.2)
3. Single rectangular tray SSB (FIGURE 4.15, Section 4.5.3)
4. Multi-square tray SSB (FIGURE 4.19, Section 4.5.4)
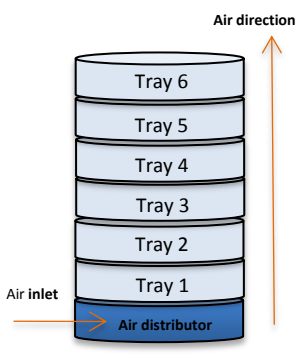
10.2.2.1   Initial moisture content arrangement

Multi-stacked tray SSB

In this work, first, moist air is sparges into the bottom of the tray, which is described as air distributor. The air then is forced from bottom to the top of the tray. In this study, experiments were carried out on an arrangement of different initial moisture contents in every tray instead of air arrangement. The airflow rate was 2 L/min. Four different initial moisture content arrangements in every tray are described in TABLE 10.1.

TABLE 10.1: Arrangement of initial moisture content in multi-stacked tray SSB

Experiment	Description: Initial moisture content (IMC) arrangement						
Exp 1	**Tray**	**1**	**2**	**3**	**4**	**5**	**6**
	IMC	50	55	60	65	70	75
Exp 2	**Tray**	**1**	**2**	**3**	**4**	**5**	**6**
	IMC	75	70	65	60	55	50
Exp 3	**Tray**	**1**	**2**	**3**	**4**	**5**	**6**
	IMC	65	65	65	65	65	65
Exp 4	**Tray**	**1**	**2**	**3**	**4**	**5**	**6**
	IMC	15	15	15	15	15	15

10.2.2.2   Air arrangement

As fermentation systems were further developed, improvements in aeration have been set up by arrangement consideration in two directions. In the first arrangement, internal dry or moist air circulation under natural and forced aeration was developed from the bottom to the top of a series of perforated mesh tray (FIGURE 10.1a).

In the second approach, air distribution is achieved by blowing the air from the side of the tray and the air moves directly onto the surface of fermented substrate. In addition, the air also is blown below the fermented substrate from the bottom of perforated mesh tray. In this system, there is only one tray located inside the closed bioreactor (FIGURE 10.1b).

(a)

Perforated
mesh tray
contains
solid
substrate

Bioreactor

(b)

Bioreactor

Perforated mesh tray contains solid substrate
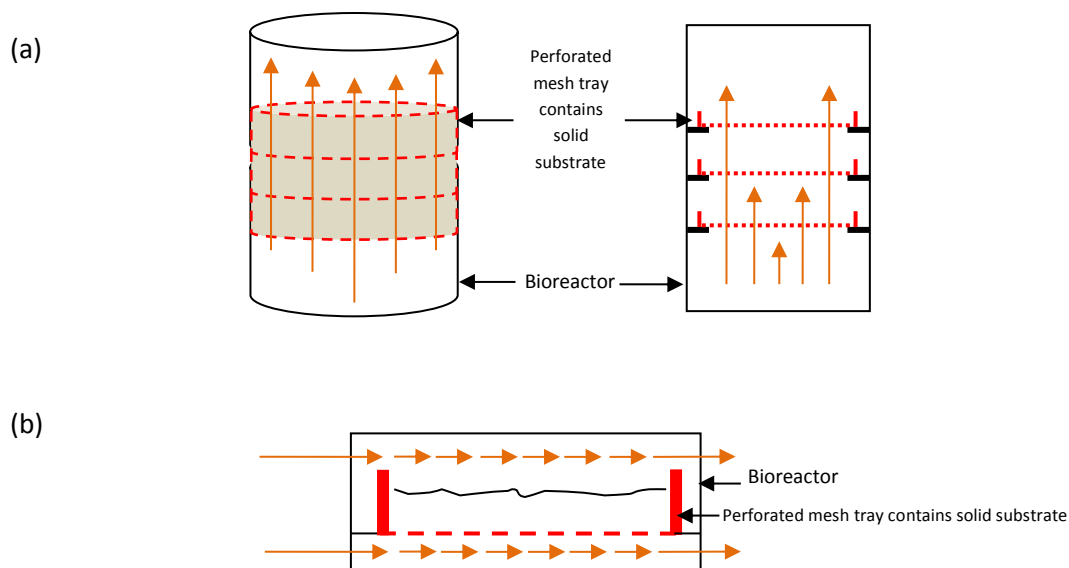
FIGURE 10.1: **The phenomena of aeration with uniform flow across the whole cross section of the fermented bed. (a) Forced air from the bottom to the top of bioreactor and (b) Air blown from the side onto the surface of fermented substrate and below the perforated mesh tray (⟶ : air flow direction)**

FIGURE 10.2a shows micro-scale view of air diffusion through solid particles in the first arrangement (FIGURE 10.1a) and how the air is forced through the solid particles. FIGURE 10.2b shows micro-scale view of air distribution and diffusion into solid particle and uptake by fungus without force aeration from along the fermented bed surface in the second arrangement (FIGURE 10.1b).

(a)

Solid substrate particle

(b)

Solid substrate particle
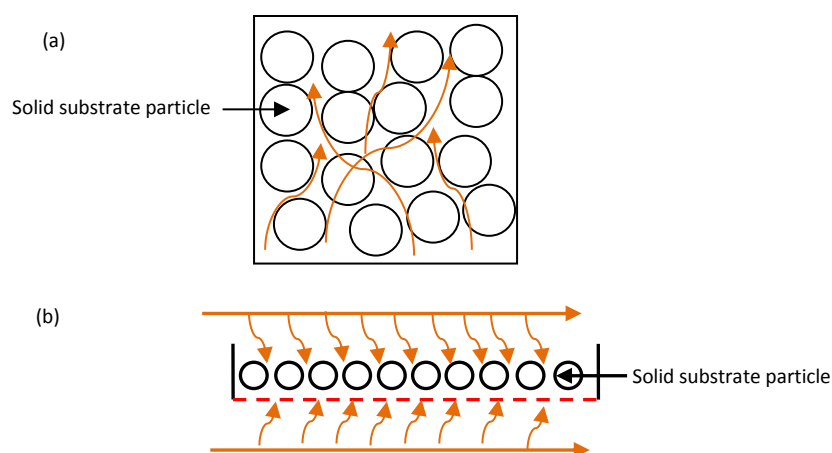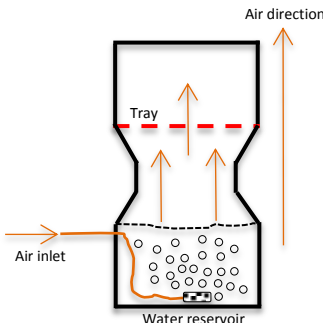
FIGURE 10.2: **Micro-scale view of aeration pattern in both systems showing how the O_2 and mass transfer through the bed of particles (⟶ : air flow direction)**

1.   Single circular tray SSB

TABLE 10.2 describes in detail the air arrangement carried out in single circular tray SSB. In this work, a strategy for the operation of tray bioreactors used for SSF involves a static bed aerated with air moistened by passing through a reservoir of water at the bottom of the bioreactor.

TABLE 10.2:   Air arrangement in single circular tray SSB

Experiment	Air arrangement	Description	
1	Control 1	No water, no air	
2	Control 2	With water, but no air	
3	Control 3	Dry air at flow rate 2 L/min is sparged from the bottom the bioreactor	
4	Air flow rate = 1 L/min	Air moistened by passing through a reservoir of water at the bottom of the bioreactor	
5	Air flow rate = 2 L/min		
6	Air flow rate = 4 L/min		
7	Air flow rate = 6 L/min		

2.   Single rectangular tray SSB

In this work, dry air and moistened air were used. Air flow rate was set constant at 2 L/min for each experiment. Air arrangement carried out in this system is described in TABLE 10.3. The perforated mesh tray was placed in the center of the bioreactor with the same amount of gas phase on the surface and from the bottom of the tray.

TABLE 10.3:   Air arrangement in single rectangular tray SSB

Experiment	Description	Air direction
Exp 1	Dry air is blown onto the surface of fermented substrate only	Air inlet → Tray → Air outlet
Exp 2	Moistened air is blown onto the surface of fermented substrate only	
Exp 3	Dry air is blown from below the surface of perforated mesh tray	Tray → Air outlet; Air inlet →
Exp 4	Moistened air is blown from below the surface of perforated mesh tray	
Exp 5	Dry air is blown onto the surface of fermented substrate and below the surface of perforated mesh tray	Air inlet → Tray → Air outlet; Air inlet → → Air outlet
Exp 6	Moistened air is blown onto the surface of fermented substrate and below the surface of perforated mesh tray	

3.   Multi-square tray SSB

This system consists of 8 trays placed inside the bioreactor one above the other with identical space between them. Two air flow rate were chosen as a moistened air is sparged in two arrangements. The air arrangement is described in TABLE 10.4.

TABLE 10.4:   Two air arrangements in multi-square tray SSB

Experiment	
Exp 1	Exp 2
Moistened air at flow rate 8 L/min pass into air distributor at the bottom of the bioreactor before being forced through the perforated mesh tray from bottom to the top	Moistened air at flow rate 1 L/min is passes onto the surface of each fermented substrate
Air direction; Tray 8, Tray 7, Tray 6, Tray 5, Tray 4, Tray 3, Tray 2, Tray 1, Air distributor; Air inlet →	Air direction; Tray 8, Tray 7, Tray 6, Tray 5, Tray 4, Tray 3, Tray 2, Tray 1, Air distributor; Air inlet →

10.3   RESULTS AND DISCUSSION

In this chapter, studies were developed for both moisture content and mass transfer to study the effect of operating variables on mass and internal air circulation under natural and forced aeration in various arrangements. In addition to investigating enzyme production that is related to fungal growth, final moisture content, temperature evolved, oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) during fermentation were investigated. Analysis of O_2 as well as CO_2 in the exhaust gas is becoming generally accepted and is likely to be applied in this study as a standard measuring technique in bioprocessing because the analysis does not interfere with the sterile barrier.

This experiment was carried out as a comparison to previous experiments (Chapter 9) where the system was designed to generate moistened air where it was provided into the four trays SSB from a different perspective. The moistened air provided can also be used as a cooling system to remove the heat generated from microbial activity and at the same time as strategy for supplying water and O_2 with the purpose of improving fungal growth. Additionally, certain initial moisture contents and air arrangements were proposed and compared for applicability of air for control of the fermented bed temperature (plus the whole system generally) and moisture for operation in the four trays SSB. The results obtained will be discussed and reviewed for the two fungi reacting with every study proposed.

10.3.1   Profile Final Moisture Content

10.3.1.1   Single circular tray SSB

The profiles of final moisture content for *A. awamori* and *A. oryzae* in single circular tray SSB are shown in FIGURE 10.3. In control experiments with uninoculated wheat bran, final moisture content in Control 1, Control 2, moistened air at flow rate 1, 2 and 4 L/min exhibited an increment of final moisture content from the initial level (65%) after 72 h. The final moisture content reached about 66.65 – 70.97%. Furthermore, at air flow rate 6 L/min, the final moisture content was about 64.41%, showing a slight decrease. Although, not 100% saturated, moistened air used favours to supply water and at the same time uninoculated wheat bran (at initial moisture content 65%) has high ability to retain water even at high flow rates. However, when dry air at 2 L/min was sparged into the system, the moisture content hugely decreased to about 44.41% after 72 h. This is expected to happen because dry air carries huge amounts of water from the solid particles of wheat bran to environment.

According to Dorta *et al*. (1994), operational conditions accompanied with the selection of the solid substrate and the initial moisture content could prevent losses of water from SSF systems, thus improving the performance of the process.
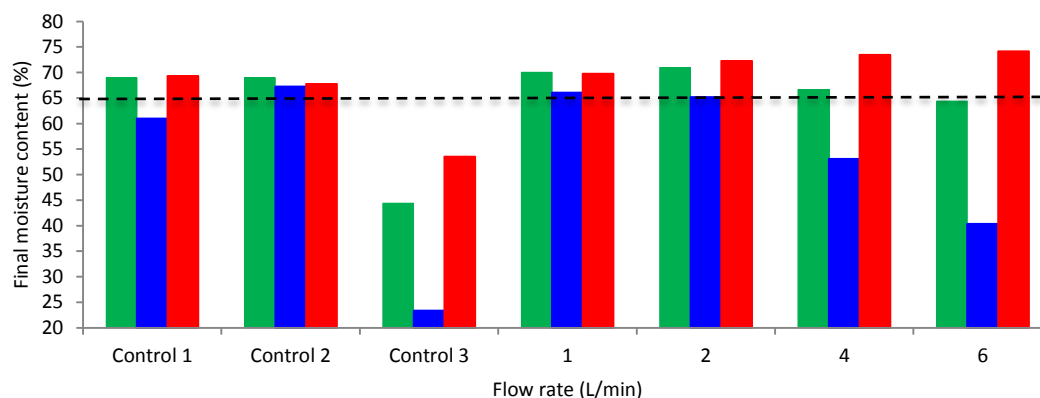
FIGURE 10.3: **Profile of final moisture content at 72 h has labelled for control experiment (uninoculated wheat bran: ■), fermented wheat bran with *A. awamori* (■) and *A. oryzae* (■) growing in single circular tray SSB with different air flow rates. Control 1: no water – no air; Control 2: with water – no air and Control 3: no water with dry air at 2 L/min. Dash line represents initial moisture content at 65%**

In this system, the final moisture content after 72 h fermentation for both fungi shows interesting differences. First, in the case of fermented *A. awamori*, the final moisture content in Control 1 decreased to about 60.99%. It was assumed that the moisture decrease was caused by evaporation process. In Control 2, moisture content was recorded to increase slightly (67.25%). Evaporated water from the water provided below the tray might give an advantage by supplying water vapour to the fermented substrate. At moistened air flow rates 1 and 2 L/min, the moisture content was around 66.03 and 65.20%, respectively. It was believed that the moistened air created in this system succeeded to maintain the moisture content of fermented substrate. However, by increasing the air flow rate to 4 and 6 L/min, the moisture content hugely decreased to 53.11 and 40.36%, respectively. This is because at high flow rates, pressure will be created inside the bioreactor and will carry some amount of water to the gas outlet.

In Control 3 (dry air at 2 L/min), a greater loss of substrate moisture and consequent drying of the solids were observed. The final moisture content was recorded at 23.39% and this is one of the problems that we tried to solve in this study. Dry air at 2 L/min might be advantageous to remove heat and to discard carbon dioxide evolved from the system. However, the dry air and high flow rate

due to forced aeration will cause serious problems in the SSF system, and as a consequence, the water loss at such high rates in this system with *A. awamori*.

Second, in the case of *A. oryzae*, the final moisture content increased for all flow rates applied in this study except in the experiment: Control 3 (dry air at flow rate 2 L/min). However, the final moisture content in Control 3 was still at a reasonable value, which is about 52%, compared to control uninoculated wheat bran (44.41%). The final moisture content in Control 1, Control 2, flow rate 1, 2, 4 and 6 L/min was recorded at about 69.31, 67.76, 69.76, 72.26, 73.43 and 74.16%, respectively. The moist air created in this system with different air flow rates succeeded to provide water to the fermented substrate. It has to be taken into account that in addition to water produced from metabolic activities, some water displaced via condensation during the fermentation process also contributes to the final moisture content of the fermented substrate.

The results obtained in this study motivated us to study the water retention ability in both fungi. As presented earlier in Chapter 6 (Section 6.3.3) , *A. oryzae* proved to have high ability to retain water in its cells compared to *A. awamori*. Thus, a combination of the ability of wheat bran and *A. oryzae* to retain water resulted in high final moisture content in fermented *A. o*ryzae.

10.3.1.2   Multi-stacked circular tray SSB

The profiles of final moisture content in every tray of the multi-stacked circular tray SSB are shown in FIGURE 10.4. In Exp 1 (initial moisture content arrangement from low (50%) to high (75%)) with *A. awamori*, trays at positions 1 and 2 lost about 17.72 and 2.13% moisture content, respectively. However, trays at positions 3, 4, 5 and 6 showed an increment in moisture content of about 4.37, 8.48, 7.41 and 3.83%, respectively. In the case of *A. oryzae*, trays at positions 1, 2, 3, 4, 5 and 6 showed an increment in moisture content of about 11.44%, 15.66%, 17.68%, 12.36% and 5.73%, respectively.

In Exp 2 (initial moisture content arrangement from high (75%) to low (50%)), *A. awamori* recorded loss in moisture content of about 16.88 and 8.6% in trays at positions 1 and 2, respectively. However, there were increments of about 9.67, 6.10, 5.76 and 9.88% in final moisture content for trays at positions 3, 4, 5 and 6, respectively. In the case of *A. oryzae*, the final moisture content in trays 1, 2, 3, 4, 5 and 6 increased by about 0.11, 4.41, 11.77, 17.15, 14.75 and 21.50%, respectively.
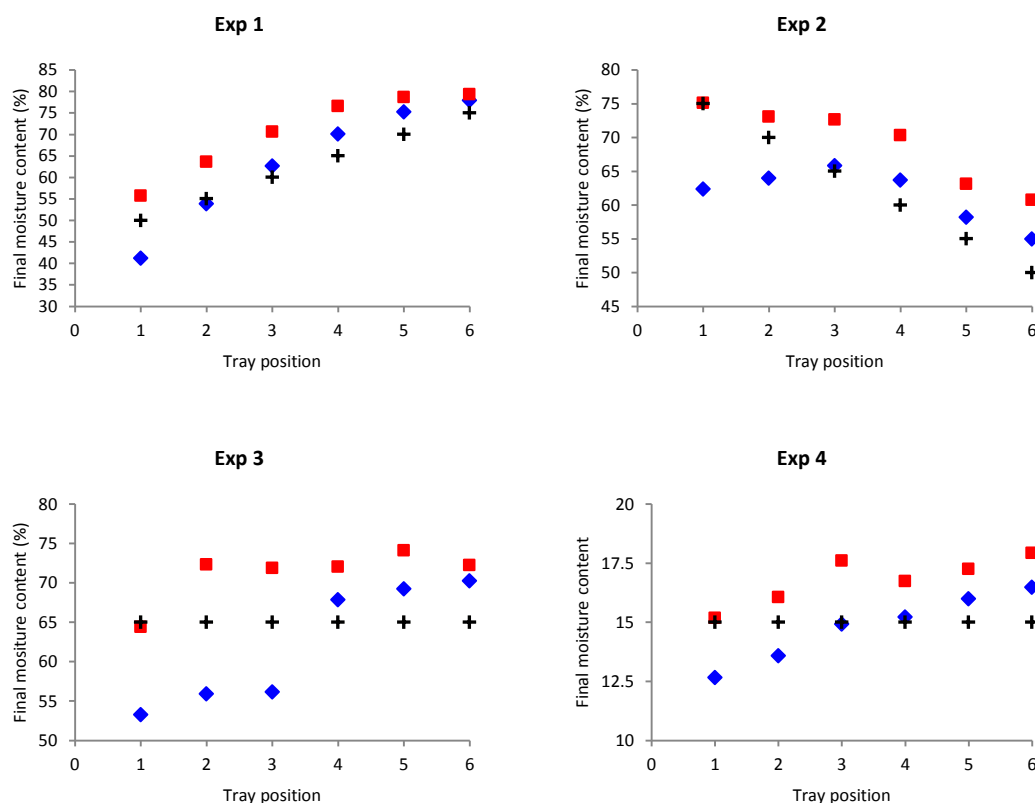
FIGURE 10.4: Final moisture content at 72 h fermentation period for different initial moisture content arrangement in multi-stacked circular tray SSB. (+): initial moisture content 65%; (◆): *A. awamori* and (■): *A. oryzae*

In Exp 3 (initial moisture content was set up at 65% in every tray), the final moisture content in fermented solid with *A. awamori* decreased by about 18.08, 13.99 and 13.68% in trays at positions 1, 2 and 3, respectively. However, it increased in trays at positions 4, 5 and 6 by about 4.35, 6.49 and 8.06, respectively. In the case of *A. oryzae*, only the tray at position 1 showed a decrease in final moisture content of about 0.96%. Final moisture content in trays at positions 2, 3, 4, 5 and 6 showed increments of about 11.26, 10.56, 10.76, 13.97 and 11.10%, respectively.

Finally, in Exp 4 (initial moisture content was set up at 15% in every tray), the final moisture content in the first three trays from the bottom decreased by about 15.61, 9.53 and 0.53% (tray 1, 2 and 3, respectively) for fermented *A. awamori*. However, increments were observed in trays 4, 5 and 6 of about 1.40, 6.57 and 9.85%, respectively. As observed in the first three experiments above, the final moisture content in every tray increased for A. *oryzae*. The increments were recorded at about 1.19, 7.06, 17.30, 11.5, 14.96 and 19.51 for tray in position 1, 2, 3, 4, 5 and 6, respectively.

It can be noticed that the tray at position 1 suffered higher loss of moisture especially with *A. awamori* culture due to access to moistened air being very large. As observed in aeration studies (Chapter 6), the strategy to create humidified air through a humidifier chamber was not observed to create 100% saturation. If the air is 100% saturated, it cannot carry away any moisture from the substrate. The air forced into the system is only moistened and is still able to carry some amount of water from the fermented substrate. This is the reason why the tray at position 1 (and sometimes at positions 2 and 3) was observed to lose moisture content at a high level.

In this system, it was assumed that the trays were tightly stacked above each other. There is no access for air from inside or outside. The moistened air forced inside the bioreactor passes through tray-by-tray and the heat (from microbial activity) is moved along the tray from bottom to the top. It is found that in the present cross flow arrangement in the system, the fermented substrate is effectively used in heat and moisture content exchange. The metabolic heat evolved from every tray will give advantage to other trays at the levels above it. The heat will goes up from the surface of the fermented bed in the tray below and become trapped at the fermented substrate in the above trays. The perforated mesh tray will allow mass transfer between the trays. Here, condensation process will occur and will generate water and directly provide moisture into the fermented substrate.

Both heat and moisture contents are transferred between the trays when they flow through the fermented substrate. Thus, heat and moisture content could be recovered from the forced moistened air provided; the excess heat and moisture could be transferred to the other trays in order to cool and dehumidify the next fermented substrate in the tray at the top. This process continuously occurs parallel with continuous air supply into the system. Here, a synergistic relationship is applied where each tray will provide an advantage to the other trays. The metabolic heat generated from microbial activity was observed to be advantageous for maintaining moisture content, especially in the trays above the tray at position 1.

10.3.1.3   Single rectangular tray SSB

In this study, for moisture content determination, samples at 5 different places were used as labelled in FIGURE 10.5 for both fungi. The effect of air arrangements on the average final moisture content at 72 h is presented in the FIGURE 10.6.
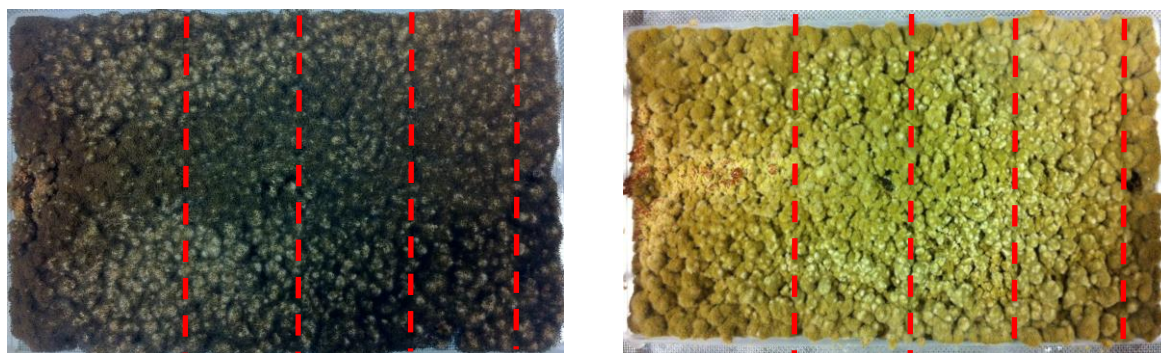
FIGURE 10.5:   Sampling area in fermented *A. awamori* (left) and *A. oryzae* (right)

The final moisture content obtained in this system was variable and different from that observed in the previous two systems described above. It was clearly observed that both fungi show almost the same pattern. Experiments with dry air (Exp 1, 3 and 5) resulted in a decrease in the final moisture content; however, experiments with moistened air (Exp 2, 4 and 6) recorded high moisture content for both fungi. In the case *A. awamori*, the final moisture content decreased when dry air at 2 L/min was used with values of 13.50, 9.20 and 14.36% for Exp 1, 3 and 5, respectively. The same observation was recorded for the final moisture content of fermented *A. oryzae* with values of 47.29, 4.34 and 5.33% for Exp 1, 3 and 5, respectively. However, when moistened air was used, fermented *A. awamori* recorded a final moisture content with values of 63.5, 61.75 and 66.12% for Exp 2, 4 and 6, respectively. No huge differences were observed in the final moisture content of fermented *A. oryzae*. However, the final moisture content of fermented *A. oryzae* decreased in Exp 2 and 4 with values of 55.96 and 64.93, respectively. An increment only occurred in Exp 6 with a value of 66.22%.

It was believed that in this system, the movement of air along the surface of fermented substrate either from the top or bottom of the tray seems to be a key factor in water loss from fermented substrate. When dry air moves along the surface, large amounts of water are carried away from the surface easily. This system was prepared in a way that large surface area is exposed to the gas space. It is clearly shown that water content is affected by variations in the ratio of surface area to volume (Mitchell *et al*., 2010); it is found that in each of the four systems tested, when this ratio is high, the amount of water loss is high and vice versa. However, this is only true with dry air. Dry air is not suitable for this system and will cause dryness of the fermented sample easily where the amount of water loss very high. The idea of sparging moistened air along the fermented surface provided the benefit of maintaining the moisture content even in *A. awamori* culture. The final moisture content was still at optimal level after 72 h of fermentation period for both fungi.
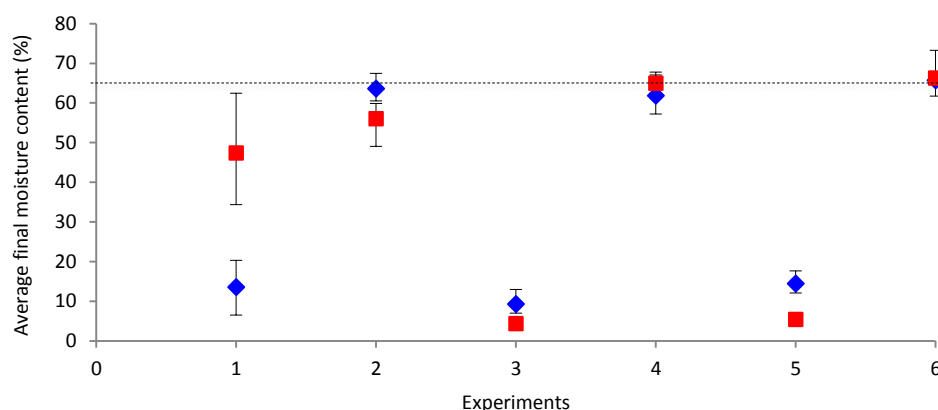
FIGURE 10.6: The average final moisture content at 72 h fermentation time for *A. awamori* (◆) and *A. oryzae* (■) growing in a single rectangular tray SSB with six different air arrangements using dry air (Exp 1, 3 and 5) and moistened air (Exp 2, 4 and 6). Dash line represents the initial moisture content 65%

10.3.1.4   Multi-square tray SSB

This system is completely sealed with no access for air from the inside or from the outside. However, large gas spaces between the trays were included in this system. Exp 2[AA] and Exp 2[AO] with air sparged directly onto the surface of the fermented substrate provided advantages to the above trays. For example substrate in the tray at position 2 has access to air from: (i) the surface itself and (ii) the bottom of the perforated tray on which the air is sparged the tray in position 1 and moves into the next tray above. The tray at position 3 has the same advantage as the tray at position 2. It seems that the trays at positions 2, 3, 4, 5, 6, 7 and 8 were aerated from both the surface and the bottom. Only the tray at position 1 was aerated from the surface.

FIGURE 10.7 shows profiles of the average final moisture contents from multi-square tray SSB. Fermented *A. awamori* in Exp 1[AA] (the air from the bottom to the top) experienced high loss of moisture content for every tray (ranging between 51.95 to 59.82%). However, with Exp 2[AA], the moisture content loss was not as critical as Exp 1[AA]. The same was observed in a single rectangular system and the final moisture content was still at optimal levels with a range between 59.81 to 62.22% in every tray. As expected, the final moisture content for fermented A. *oryzae* was above the initial level of 65%. Exp 1[AO] recorded final moisture content within the range 66.04 to 67.84%, while in Exp 2[AO], the final moisture content was between 66.71 to 69.89% for all the eight trays. Water loss from this system was not as critical as that observed in the previous system, especially for SSF with *A. awamori* culture.
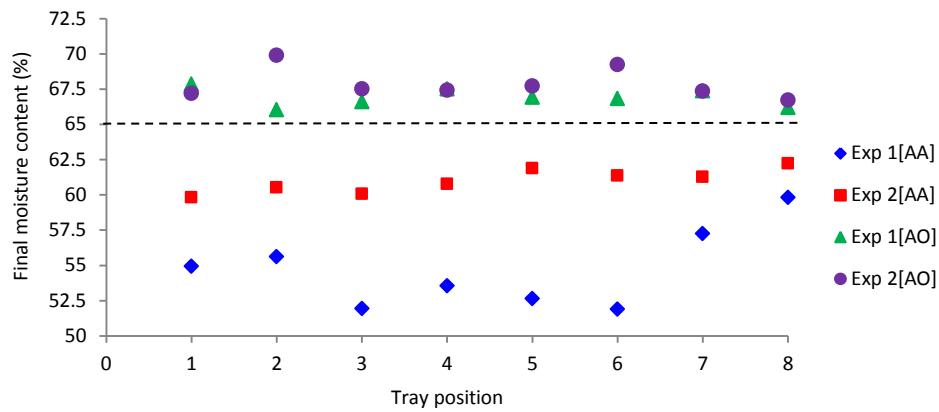
FIGURE 10.7: **Final moisture content at 72 h fermentation time for *A. awamori* [AA] and *A. oryzae* [AO] growing in multi-square tray SSB with two different air arrangements. Dash line represents initial moisture content 65%**

There are some key factors affecting the moisture content in SSF and it is important to know their influence on the processes. It could be summarised that *A. awamori* and *A. oryzae* were very versatile and also sensitive fungi in terms of air arrangement in bioreactor systems and moisture contents in the fermented substrate. Dry air negatively affected the final moisture content for both fungi. The use of moistened air as an alternative to provide adequate O_2, which is suitable for the moisture content control. The air flow through the fermented substrate was also observed to play an important role in carrying moisture from the fermented substrate. When the air was forced from the bottom to the top, it had a negative effect on *A. awamori* culture but a positive effect on *A. oryzae* culture. When the air was blown through the surface of fermented substrate, it had a negative effect on moisture content for both fungi. However, this is not the only factor to determine high productivity of the culture.

10.3.2　The Gompertz Curve as a Growth Curve

Benjamin Gompertz (1825) first proposed the Gompertz model (Winsor, 1932). The Gompertz model is a sigmoid function, as the logistic curve and has been used it as a growth curve, both for biological economic phenomena (Winsor, 1932). Skadias and Skadias (2008) came out with a derivation based on systems theory to consider some of the mathematical properties of this curve and to indicate to some extent its usefulness and its limitations as a growth curve. In differential equation form, the model is based on the equation:

$$(\ln x)' = -b \ln x \qquad\qquad (10.1)$$

Where;

x = the function of time

b = a positive constant expressing the rate of growth of the system

Without loss of generality, the function of x can be assumed bounded (0 < x < 1, with x = 1 corresponding to the entire population), so that x is the probability density function of the growth process. Direct integration of equation 10.1 gives as solution of the Gompertz function (Skadias and Skadias, 2008):

$$x = \exp(\ln(x_o)\exp(-bt)) \tag{10.2}$$

The integrated Gompertz model, as reported by Saucedo *et al*., (1990), Meraz *et al*., (1992) and Christen *et al*., (1997), was used to analyse the kinetic data. In this logistics-like model the product carbon dioxide ($[CO_2]$) is a function of time (t) according to the following equation.

$$[CO_2] = [CO_{2_{max}}]\exp(-b\exp[-kt]) \tag{10.3}$$

Where;

$[CO_{2_{max}}]$ = the maximum CO$_2$ concentration (at t -----------> ∞) (mole)

b = a constant related to the initial conditions (when $t = 0$, then $[CO2] =$ $[CO_{2_0}] = [CO_{2_{max}}]\, exp\,(-b)$ (dimensionless)

k = the specific CO$_2$ evolution rate (h^{-1})

t = fermentation time (h)

The constants $[CO_{2_{max}}]$, b and k were estimated from the data using a non-linear regression programme. The value of k (h^{-1}) refers to the time it takes for the value of $[CO_2]$ to reach $[CO_{2_{max}}]$. Larger values of k cause $[CO_2]$ to reach $[CO_{2_{max}}]$ in a shorter time. The value of b (dimensionless) will determine the shape of the sigmoidal curve. Larger values of b cause the initial exponential growth phase to be slower and the deceleration phase to reach asymptote faster. Smaller values of b cause a rapid exponential growth phase and slower deceleration before the asymptote of $[CO_2]$ is reached (Winsor, 1932). Here, asymptote refers to a line that continually approaches a given curve but does not meet it any finite distance.

10.3.2.1   Single circular tray SSB

The growth of *A. awamori* and *A. oryzae* on wheat bran as solid support was studied for different air flow rates. CO_2 was chosen as growth indicator and its evolution is presented in FIGURE 10.8. FIGURE 10.8 shows profiles of accumulated CO_2 evolution for every air arrangement. The respiratory activity measured as CO_2 evolution was very low for all air arrangement. When air flow rate increased up to 6 L/min, the respiratory activity and the growth were poorer compared to control experiments without air supply (Control 1 and 2). In the case of *A. awamori* (FIGURE 10.8[AA]), the experiment with Control 1 recorded the highest CO_2 evolution followed by the experiment with moistened air at flow rate 2 and 1 L/min, then Control 2, and Control 3 (dry air at 2 L/min). In the case of *A. oryzae* (FIGURE 10.8[AO]), Control 1 evolved the highest of CO_2 followed by Control 2, moistened air at flow rate 4 L/min, Control 3 (dry air at flow rate 2 L/min) and moistened air at flow rate 2 and 1 L/min.

The experiment with moistened air at flow rate 6 L/min evolved the lowest CO_2 for both fungi. The CO_2 evolved decreased when the air flow rate was increased to 6 L/min. This is probably due to the high flow rate; O_2 was washed out from the system and caused O_2 limitation in the system. Another reason is because of the loss of high water content in *A. awamori* culture and accumulation of high water content in *A. oryzae*. Too dry and too wet fermentation conditions are not suitable for fungal growth. It is important to consider that high retention of water in *A. oryzae* can also affect O_2 diffusion. For *A. awamori*, improvement in CO_2 evolution was found at flow rate of 1 and 2 L/min (moistened air) as opposed to 4 L/min for *A. oryzae*.

From FIGURE 10.8, it can be observed that the experimental data obtained from the system using wheat bran were reproducible for every air arrangement experiment for CO_2 evolution for both fungi. These raw data were then integrated and the Gompertz model was applied. Fitted parameters and correlation coefficients (R^2) are reported in TABLE 10.5. The $[CO_{2max}]$ values confirmed the influence of air arrangement. For *A. awamori*, it can be seen that $[CO_{2max}]$ evolution decreased linearly when the flow rate of moistened air was increased from 0 to 6 L/min. Likewise, CO_2 evolution was higher at 4 L/min for *A. oryzae*. Control experiments without air supply showed higher CO_2 evolution for both fungi. It is likely that the presence of different CO_2 content in the headspace depends on the concentration of O_2 in the solid medium which is available to be used by fungi in the respiration process. By providing air flow, some amount of O_2 might be moved out from the solid medium due to the pressure build-up because of the flow especially at higher air flow rate.
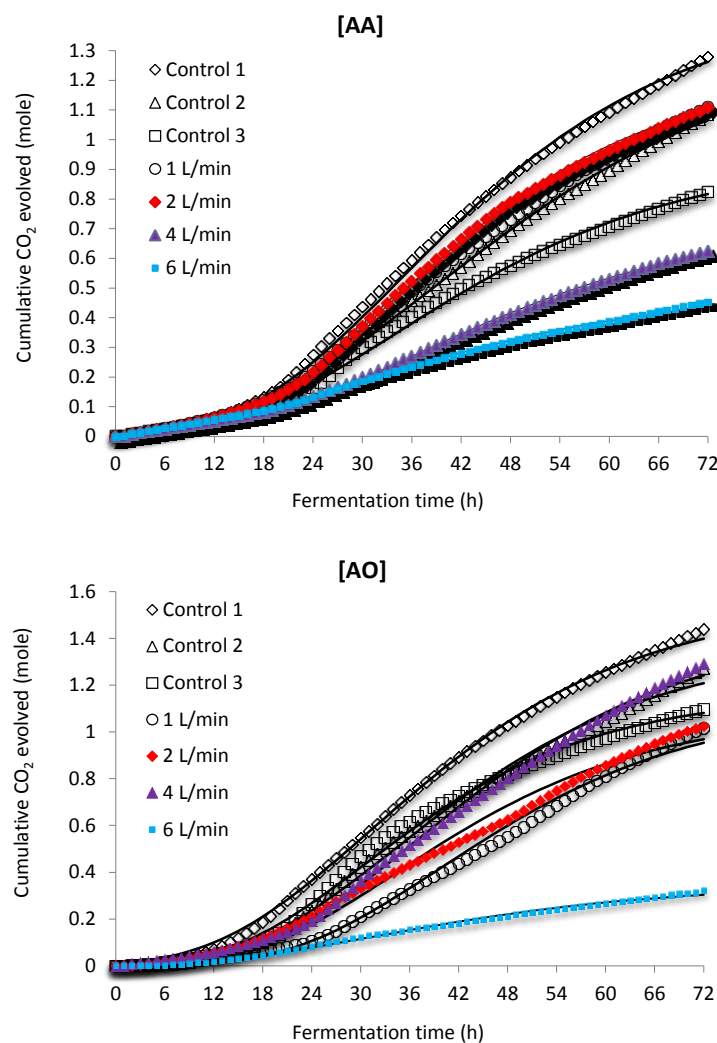
FIGURE 10.8:   Variation of cumulative CO₂ evolved with time at different air flow rate during the growth of *A. awamori* [AA] and *A. oryzae* [AO] on wheat bran in single circular tray SSB. Symbols represent experimental data. The Gompertz model is shown as a solid line

The evolution rate (k) values were comparable, all being between $0.44 - 0.54$ and $0.49 - 0.61$ for *A. awamori* and *A. oryzae*, respectively, though clear tendency can be seen in relation to the air supply. Values increased when moistened air flow rate was increased from 0 to 2 L/min, however values decreased when the flow rate was increased from 4 to 6 L/min. However, there was no clear relationship between values of this parameter and the amount of CO_2 evolved characterised by $[CO_{2_{max}}]$. The t_{max} values calculated were close to those observed experimentally. These values were between 30.2 to 38.4 h and 30.4 to 39.7 h and did not seem to be influenced by the air arrangement. Fitting the model to all the experimental points gave R^2 values above 0.998 and 0.991 for *A. awamori* and *A. oryzae*, respectively. Hence, it may be concluded that the experimental set-up and the analytical technique used for CO_2 measurement in this study was sufficient.

TABLE 10.5:   Kinetics constants for different air flow rate according to Gompertz model

Experiment (Flow rate – L/min)	*A. awamori*					*A. oryzae*				
	CO_{2max} (mole)	k (h^{-1})	b	t_{max} (h)	R^2 coefficient	CO_{2max} (mole)	k (h^{-1})	b	t_{max} (h)	R^2 coefficient
Control 1	1.46	0.052	6.14	34.9	0.999	1.57	0.053	5.21	31.2	0.999
Control 2	1.35	0.043	5.22	38.4	0.999	1.38	0.054	6.44	34.5	0.997
Control 3	0.95	0.050	5.53	34.2	0.998	1.17	0.061	6.37	30.4	0.996
1	1.25	0.052	6.18	35.0	0.999	1.13	0.055	8.87	39.7	0.995
2	1.25	0.054	6.20	33.8	0.999	1.09	0.057	7.14	34.5	0.991
4	0.75	0.046	5.36	36.5	0.999	1.45	0.053	7.02	36.8	0.998
6	0.51	0.044	3.77	30.2	0.999	0.35	0.049	4.86	32.3	0.996

10.3.2.2   Multi-stacked tray SSB

In this system, moistened air supply into the system was constant at flow rate of 2 L/min and air was sparged from the bottom to the top of the multi-stacked tray bioreactor. However, different initial moisture contents were set up at every tray according to the experimental design explained in TABLE 10.1. FIGURE 10.9 shows the fitting of Gompertz model to these data for both fungi during SSF with wheat bran. It is quite clearly visible that with the different initial moisture contents in every tray, CO_2 evolved during SSF was variable for *A. awamori* in different experiments. With *A. oryzae*, CO_2 evolution was comparable with Exp 1[AO], 2[AO] and 3[AO] but very low in Exp 4[AO] (15% moisture content in every tray). It seems that in response to different initial moisture content clear differences occur in parameters of growth characteristics of *A. awamori* and *A. oryzae*. The application of the Gompertz model to the raw experimental data confirmed that maximum CO_2 evolution can be obtained. As the initial moisture content was reduced to 15%, $[CO_{2max}]$ decreased. In all cases, the fitting of the model to the experimental data was excellent (R^2 > 0.999 and R2 > 0.994 for *A. awamori* and *A. oryzae,* respectively).

In TABLE 10.6, modelling parameters of the integrated CO_2 evolution data are presented. The $[CO_{2max}]$ values confirmed the influence of the initial moisture content arrangement. For example, it can be seen that CO_2 evolution was lower on wheat bran when the initial moisture content was set up at 15% for both fungi (Exp 4[AA] and Exp 4[AO]). In experiment Exp 1[AA] with *A. awamori* (initial moisture content from low to high) greatly enhanced evolution of CO_2 was observed. However, Exp 3[AO] with *A. oryzae* (initial moisture content at 65% in every tray) observed among the highest evolution of CO_2. The evolution rate (k) values were variable, all being in the range 0.038 – 0.063 hr^{-1} and 0.043 – 0.073 hr^{-1} for *A. awamori* and *A. oryzae*, respectively, though clear tendency can be seen in relation to the initial moisture content arrangement. There is still no apparent relationship between this parameter and the amount of CO_2 evolved characterised by [CO_{2max}]. The t_{max} values

calculated were close to those obtained experimentally. These values were in the range 30.5 – 43.2 h and 24.9 – 30.7 h for *A. awamori* and *A. oryzae*, respectively, and seem to be very strongly influenced by the studies carried out. The same observation was recorded with *b* values.

FIGURE 10.9: Variation of cumulative CO_2 evolved with time at different moisture content arrangement at air flow rate 2 L/min during the growth of *A. awamori* [AA] and *A. oryzae* [AO] on wheat bran in multi-stacked tray SSB. Symbols represent experimental data. The Gompertz model is shown as a solid line

Interestingly, it is noticeable that the two fungi react differently to the same arrangement of growth characteristics. As reflected by the intensity of CO_2 evolved, it seems that initial moisture content has a direct influence on metabolic activities and thus on the rate and amount of CO_2 evolved. Moisture content was significant as a source of variation for biomass production (measured in this case by CO_2 evolution). This was confirmed by the distribution of each kinetic constant, presented for the four

most productive experiments (TABLE 10.6). In these cases, *A. awamori* with Exp 1[AA] showed a high overall growth performance on wheat bran based on CO_2 evolution.

TABLE 10.6: Kinetics constants for carbon dioxide evolved by *A. awamori* and *A. oryzae* with different initial moisture content arrangements in multi-stacked tray SSB

Experiment (Flow rate – L/min)	*A. awamori*					*A. oryzae*				
	$[CO_{2max}]$ (mole)	k (h^{-1})	b	t_{max} (h)	R^2 coefficient	$[CO_{2max}]$ (mole)	k (h^{-1})	b	t_{max} (h)	R^2 coefficient
Exp 1	4.31	0.048	7.92	43.2	0.999	1.83	0.069	5.57	24.9	0.994
Exp 2	2.55	0.063	6.83	30.5	0.999	1.78	0.063	5.08	25.8	0.998
Exp 3	1.59	0.048	4.48	31.3	0.999	1.98	0.073	7.67	27.9	0.997
Exp 4	1.03	0.038	4.24	37.6	0.999	1.11	0.043	3.74	30.7	0.997

10.3.2.3   Single rectangular tray SSB

The Gompertz model gave excellent fitting of growth kinetics (FIGURE 10.10) for all data obtained from experiments with air arrangements in a single rectangular tray SSB (TABLE 10.3). A reasonable fit ($R^2 > 0.99$) to the accumulated CO_2 evolution profile was achieved by using the Gompertz model (Equation 10.3) in each fermentation. Therefore, the proposed Gompertz model provided a suitable simulation of CO_2 evolution for *A. awamori* and *A. oryzae* fermentation using SSF.

It is noticeable that in this system, the evolution of CO_2 was lower compared to the other three systems. However, CO_2 was greatly evolved in Exp 1[AO] (TABLE 10.3 - dry air 2 L/min onto the surface only) with *A. oryzae*. This system has a large surface area of fermented substrate with the same thickness as in the other system. In this system, the air was blown from the edge of the tray and the air was moved along the surface of the fermented substrate. It was believed that the concentration of O_2 was higher at the edge of the tray and became lower when it reached the opposite edge of the tray. Here, it was assumed that the concentration of O_2 might not be adequately supplied. Consequently, the apparent microbial respiration occurring in this system was lower and variable. The kinetics of microbial growth also showed that the apparent CO_2 concentrations under different conditions did not always increase with the fermentation time.
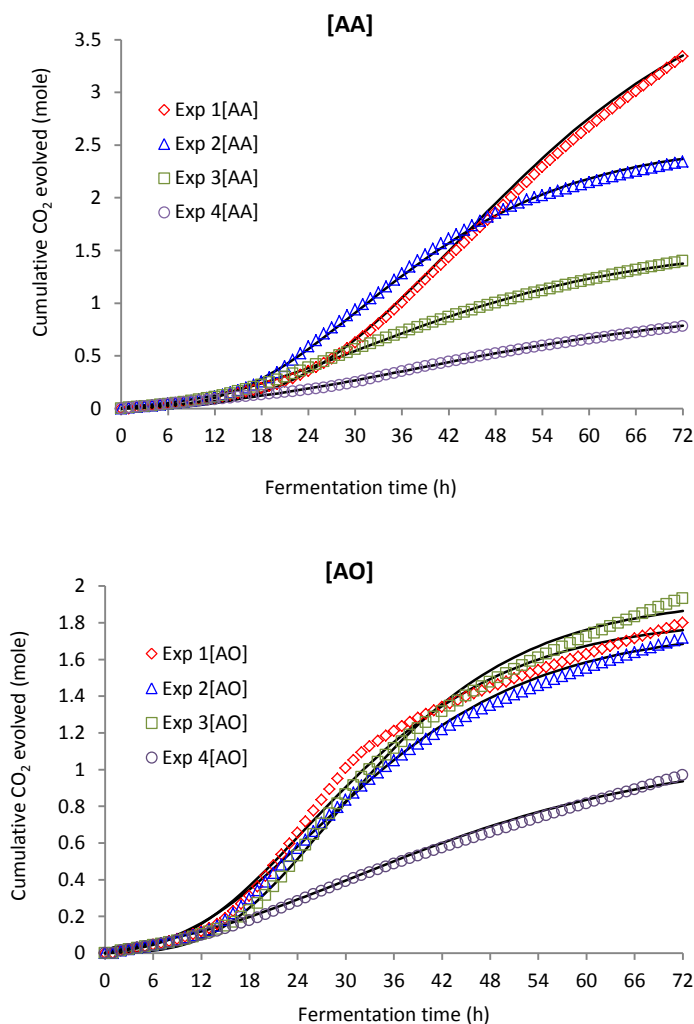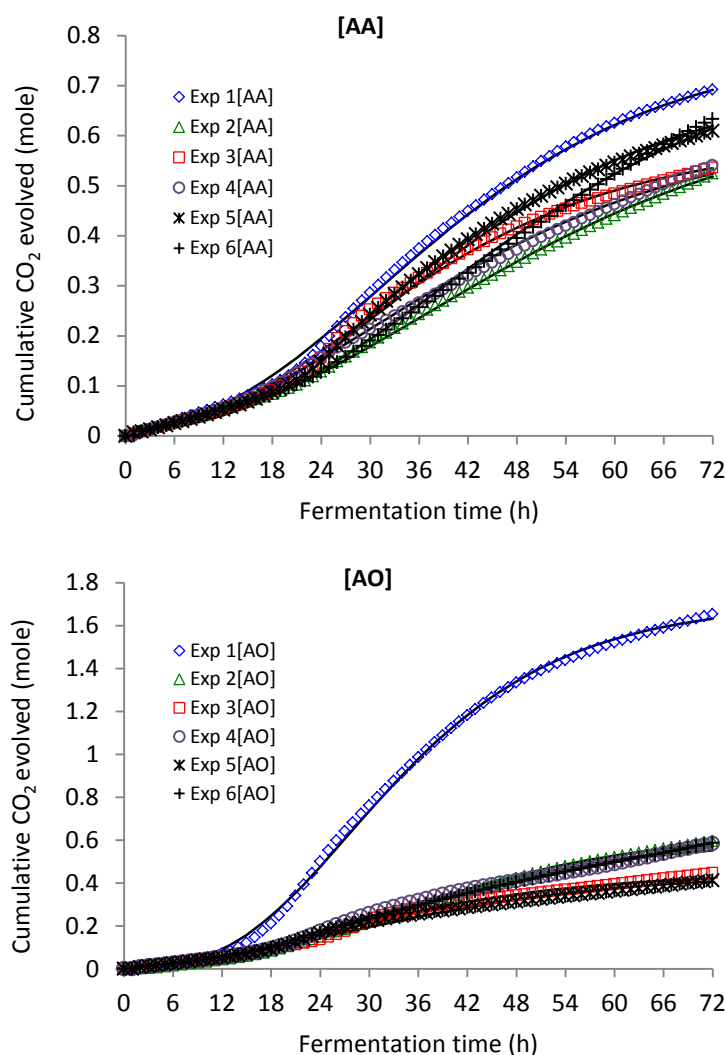
FIGURE 10.10: **Variation of cumulative CO$_2$ evolved with time at different air arrangement at flow rate 2 L/min during the growth of *A. awamori* [AA] and *A. oryzae* [AO] on wheat bran in single rectangular tray SSB. Symbols represent experimental data. The Gompertz model is shown as a solid line**

The CO$_2$ evolution throughout the time of cultivation in SSF was determined within six air arrangements (TABLE 10.3) and the results are presented in TABLE 10.7. The experimental evidence points out that air arrangement significantly influences the rate of CO$_2$ evolution. The maximum CO$_2$ evolved ($[CO_{2_{max}}]$) was variable between *A. awamori* and *A. oryzae*. The highest $[CO_{2_{max}}]$ was determined in Exp 1[AO] with dry air blown onto the surface of fermented substrate. The evolution rate (k) was also variable in all experiments for both fungi. The addition of air supply (either with dry or moistened air) onto the surface and below the bed (Exp 6) did not promote the growth of either of the two fungi. The experimental evidence suggests that differences between the two fungi are responsible for variations in the yields of enzymes (as discussed in Chapter 9) as the amount of $[CO_{2_{max}}]$ was observed to be higher for *A. awamori* than *A. oryzae*.

TABLE 10.7:   **Kinetics constants for different air arrangements according to Gompertz model. The blue highlighted rows represent experiments with dry air and the non-highlighted rows are those with moistened air**

Experiment (Flow rate – L/min)	A. awamori					A. oryzae				
	CO_{2max} (mole)	k (h^{-1})	b	t_{max} (h)	R^2 coefficient	CO_{2max} (mole)	k (h^{-1})	b	t_{max} (h)	R^2 coefficient
Exp 1	0.79	0.049	4.53	30.9	0.999	1.71	0.069	6.69	27.6	0.999
Exp 2	0.67	0.039	4.26	37.1	0.999	0.65	0.055	5.66	31.5	0.997
Exp 3	0.59	0.053	4.45	28.2	0.998	0.45	0.058	4.19	24.7	0.995
Exp 4	0.62	0.044	3.99	31.4	0.999	0.62	0.050	4.20	28.7	0.996
Exp 5	0.72	0.048	4.78	32.6	0.998	0.45	0.051	3.88	26.6	0.992
Exp 6	0.79	0.041	4.82	38.4	0.999	0.66	0.048	4.67	32.1	0.997

10.3.2.4   Multi-square tray SSB

As expected, all the experimental data obtained from two different air arrangements in the multi-square tray SSB (TABLE 10.4) were fitted into Gompertz model to observe the growth of *A. awamori* and *A. oryzae* during SSF on wheat bran. FIGURE 10.11 shows the use of the Gompertz model for the description of fungal growth. In this system, it is clear that air arrangement has a direct influence on the metabolic activity and CO_2 evolved. The strategy by supplying air onto the surface of fermented substrate (Exp 2) with moistened air at flow rate 1 L/min greatly enhanced the evolution of CO_2 compared with Exp 1 (moistened air sparged from below to the top).

For Exp 2 (for both *A. awamori* and *A. oryzae*), the evolution of CO_2 was higher probably due to high concentration of O_2 at the surface of fermented substrate. With low flow rate at 1 L/min, the accumulation of O_2 in the headspace (between the trays) was adequate to supply enough O_2 for fungi. Compared with Exp 1, the concentration of O_2 might be variable in different locations of the trays. The bottom tray might have a high concentration of O_2 compared to the above trays. Another reason for these differences may be that some amount of O_2 might be moved out from the system due to high pressure build caused by the high flow rate 8 L/min.
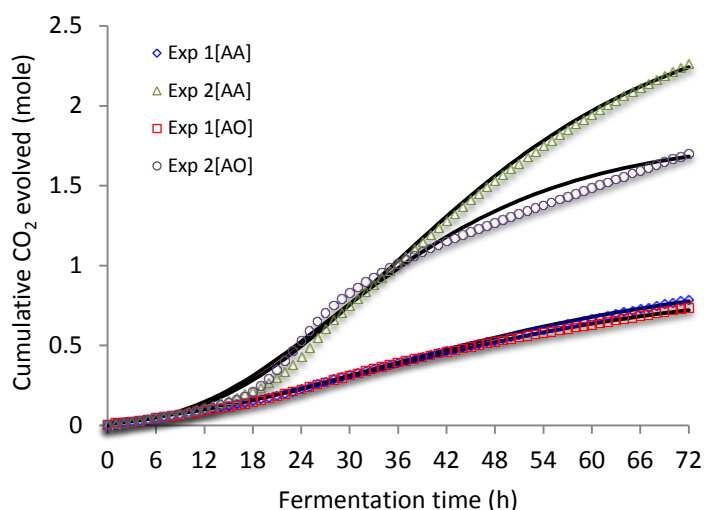
FIGURE 10.11: **Variation of cumulative CO$_2$ evolved with time at different air flow rate and air arrangement during the growth of *A. awamori* [AO] and *A. oryzae* [AO] on wheat bran in multi-square tray SSB. Symbols represent experimental data. The Gompertz model is shown as a solid line**

The kinetics constants are presented in TABLE 10.8. The model was found to be adequate to describe the integrated data from CO$_2$ evolution as could be seen from the R^2 coefficients (R^2 > 0.997). The arrangement of trays with air sparged onto the surface of fermented substrate (Exp 2) greatly enhanced $[CO_{2_{max}}]$ (more than 2 fold) compared to Exp 1 for both fungi. This was expected since in this system there are eight trays with the same amount of solid substrate.

TABLE 10.8: **Kinetics constants for different air flow rate according to Gompertz model**

Experiment (Flow rate – L/min)	*A. awamori*					*A. oryzae*				
	CO$_{2max}$ (mole)	k (h^{-1})	b	t$_{max}$ (h)	R^2 coefficient	CO$_{2max}$ (mole)	k (h^{-1})	b	t$_{max}$ (h)	R^2 coefficient
Exp 1	0.98	0.038	3.59	33.6	0.999	0.89	0.039	3.48	31.9	0.999
Exp 2	2.65	0.048	5.31	34.8	0.999	1.81	0.059	5.10	27.6	0.997

The evolution rate (k) values were 2.65 and 1.81 h^{-1} for *A. awamori* and *A. oryzae*, respectively (Exp 2). Yet, there is no clear relationship between this parameter and the amount of CO$_2$ produced characterised by $[CO_{2_{max}}]$ as observed from the previous three systems (FIGURE 10.12). The t$_{max}$ parameters values coincide with those obtained for the maximum evolution of CO$_2$ and in most cases correspond to those obtained experimentally.
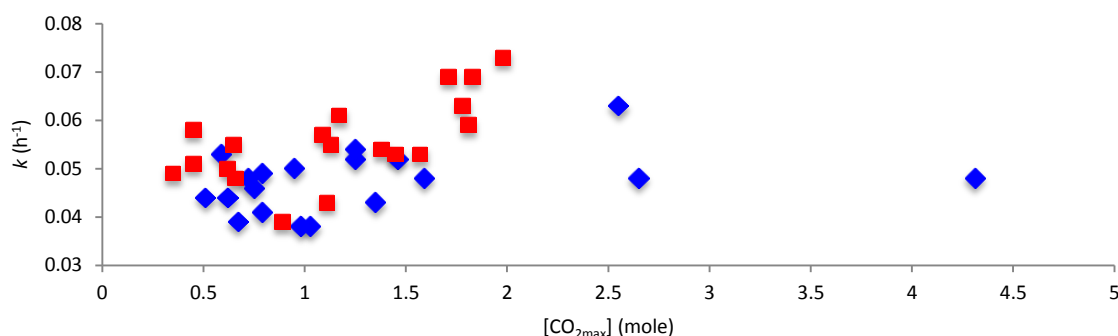
FIGURE 10.12: Experimental correlation between $[CO_{2_{max}}]$ with the evolution rate (k) using data of *A. awamori* (◆) and *A. oryzae* (■) from all experiment tested using wheat bran

10.3.2.5   Fitting models

Fitting growth models to accumulated CO_2 evolution raw data is meaningful and easier to handle for further analysis. It is recognised that in all the cases in this study, it was possible to use Gompertz model to describe fungal growth in SSF based on CO_2 evolution. Data of CO_2 evolution as a result of metabolic activity during SSF with *A. awamori* and *A. oryzae* on wheat bran were easy to handle with this model. It was observed in this study that the concentration of CO_2 increases during SSF over time following a sigmoidal curve that describes fungal growth. Also, the variation patterns of sigmoidal curve were produced in response to different conditions (for example, different air arrangements and initial moisture contents).

FIGURE 10.13 shows the correlation between experimental and predicted data for the cumulative CO_2 evolved by the behaviour of *A. awamori* and *A. oryzae* during SSF on wheat bran at various air and initial moisture content arrangements in four tray SSB systems calculated with the Gompertz model. An excellent agreement between experimental and predicted values of *A. awamori* and *A. oryzae* were obtained with $R^2 > 0.99$. Thus, this model allowed an excellent prediction of the effects of various air and initial moisture content arrangements on CO_2 evolution during SSF. From all experiments carried out in this study for CO_2 evolution, the parameters showed that the total amount of $[CO_{2_{max}}]$ was dependent on the type of fungus, initial moisture content and air arrangement. Ultimately, given the assumptions that need to be considered, the Gompertz model would show potential and can be capable of describing what happens under different culture conditions.

For this study, Gompertz model was selected because it can reflect the sigmoidal nature of growth. In this study, for all tray SSBs and for both fungi, growth followed a typical pattern with four distinct phases: a lag phase, an acceleration phase, a log (exponential) phase and a deceleration phase. However, there was no clear stationary phase and no accelerated death phase observed. In conclusion, it can be summarised that the Gompertz model is a model of choice for the description of the growth curves of *A. awamori* and *A. oryzae*. It is noteworthy that the potential of models to describe growth curves is not the only criterion for its assessment. Some other criteria, such as prediction of growth curve and estimation of some biological parameter, may be used for the selection of appropriate model.
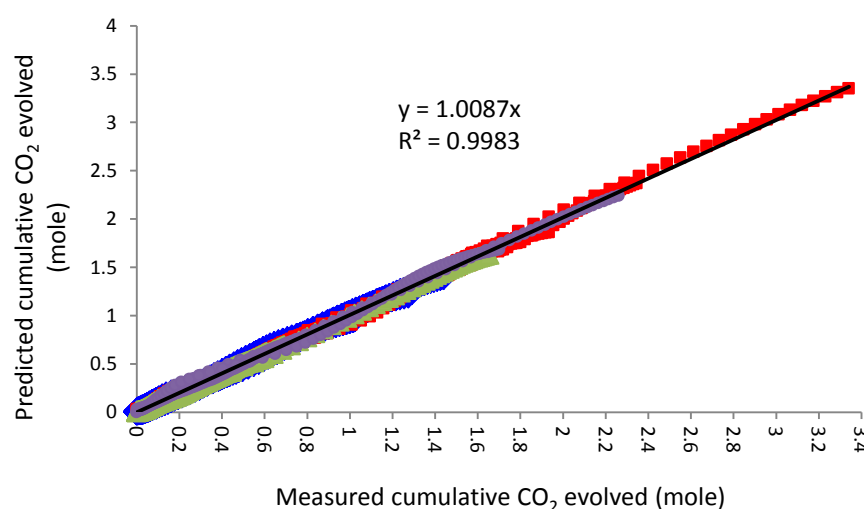
FIGURE 10.13: **Correlation between measured and predicted data of *A. awamori* and *A. oryzae* from all experiment conducted using wheat bran in (◆): single circular tray SSB; (■): multi-stacked circular tray SSB; (▲): single rectangular tray SSB and (●): multi-square tray SSB**

According to *Mitchell et al.* (2004), introducing a relevant approach to modelling the product formation kinetics in SSF is valuable owing to the difficulty in monitoring the different variables involved in the fermentation systems. Christen *et al*. (1997) used the Gompertz model to represent the growth of *Ceratocystis fimbriata* in SSF. They stated that the curve gives good fit for the integrated data of CO_2 and volatiles production. Soares *et al*. (2000) also applied the Gompertz model to the growth of the *C. fimbriata* to describe the total volatile compounds during SSF on coffee husks. Erkmen (2008) used the Gompertz model to represent the growth in *Listeria monocytogenes*, aerobic bacteria and lactic acid bacteria, during ripening and storage. They reported that the model provides parameters for different production methods in Gompertz model against microorganisms. Other authors used the Gompertz model growth curve to describe the time course of fermentation under

different conditions for lipopeptide production by *Bacillus amyloliquefaciens* in SSF (Zhu *et al.*, 2013). Braissant *et al.,* (2013) suggested the use of the Gompertz model as a growth model and potential application to microcalorimetric data. They promoted further use of microcalorimetry in microbiology and biology and the use of such model that has become available to isothermal microcalorimetry end user. According to Winsor (1932), the Gompertz and log model possess similar properties that make them useful for the empirical representation of growth phenomena.

10.3.2.6   The relation between $[CO_{2max}]$ and spores production

FIGURE 10.14 shows the correlation between total accumulated carbon dioxide ($[CO_{2max}]$) and spores measured at 72 h fermentation time for both fungi using the data obtained from all experiments using four tray SSB systems. The total CO_2 produced correlates linearly to the spores produced. As observed in FIGURE 10.14, total spores and CO_2 produced are dependent on the type of fungus used and fermentation conditions. The multi-stacked tray bioreactor showed excellent spores production in *A. awamori* culture, followed by the multi-stacked circular tray SSB. Based on the total spores production in the multi-stacked tray SSB ([Exp 2[AA]) and the apparent weight of the solid substrate, an average value of 5.5 x 10^{12} spores/g [dry basis] was produced. This value is about four times higher than that obtained in the single rectangular tray SSB.

FIGURE 10.14:   Relationship between $[CO_{2max}]$ with average spores production obtained from every air arrangement using 4 tray systems with *A. awamori* [AA] and *A. oryzae* [AO] cultures. (◆): single circular tray SSB; (■): multi-stacked circular tray SSB; (▲): single rectangular tray SSB and (●): multi-square tray SSB

The tray systems developed in this study were declared as an "opened system" due to the large ratio of area per volume where adequate moistened air and O_2 are available in the system. However, the

system is still closed from access to the environment as discussed in tray studies in Chapter 9. This result makes it evident that the fermentation conditions including the bioreactor design and air arrangements created favourable conditions for growth and spores production. The same result was observed in *A. oryzae* culture. In addition, it is evident that *A. awamori* was superior in spores production to *A. oryzae*. This sporulation behaviour was also observed by Dorta and Arcas (1998) and Dorta *et a*l. (1996) in cultures of *Metarhizium anisopliae* using column bioreactors packed with different substrates with forced aeration. Van Breukelen *et al*. (2011) demonstrated the feasibility of an aerated packed bed bioreactor cultivated with the same fungus showing its potential for large scale production of *M. anisopliae* spores. The CO_2 evolution after 72 h of fermentation was due mainly to fungal respiration. Therefore, it represents the active growth of *A. awamori* and *A. oryzae* and spores respiration. These results were similar to others results presented by Lareo *et al*., (2006) for growth and sporulation of *Mucor bacilliformis* in SSF on an inert support. The CO_2 evolved during SSF expected to be regards with spores production, however, to be insignificant between fungus.

10.3.2.7   The relation between [$CO_{2_{max}}$] and enzymes production

FIGURE 10.15 shows correlation between total accumulated carbon dioxide ([$CO_{2_{max}}$]) and the production four enzymes (glucoamylase, protease, xylanase and cellulase) measured at 72 h fermentation time for both fungi using the data obtained from all experiments carried out in four tray SSBs. As observed with spores production, the total CO_2 produced correlates linearly with the enzymes produced. Enzyme production can be considered a fermentation product similar to spores production. The correlation between [$CO_{2_{max}}$] and these four enzymes is most likely dependent on the type of fungus used and the fermentation conditions. This demonstrates that CO_2 evolution and enzymes production refer to the quality and composition of nutrients and the current initial moisture content and air arrangement.

A. awamori and *A. oryzae* have the ability and performance to degrade and utilise complex compositions contained in wheat bran. The same results as those obtained for spores production were observed; the multi-square tray SSB (Exp 2[AA]) was proven to be an excellent system for the production of all enzymes tested during SSF of *A. awamori* on wheat bran. This was followed by the multi-stacked circular tray SSB by applying the initial moisture content arrangement. The same observation was seen with *A. oryzae* culture. Yet, *A. awamori* proved to be excellent in glucoamylase, xylanase and cellulase production while *A. oryzae* was excellent in protease production. Enzyme production also was higher compared to conventional tray systems using normal petri dishes previously described in Chapter 9.
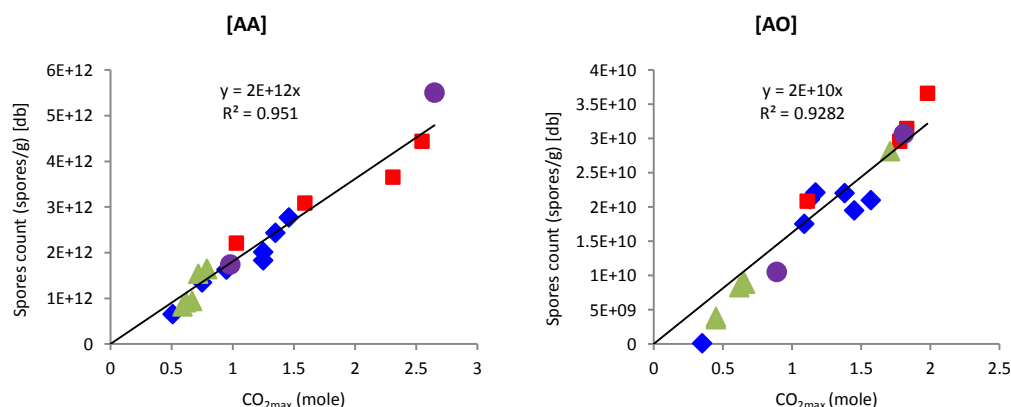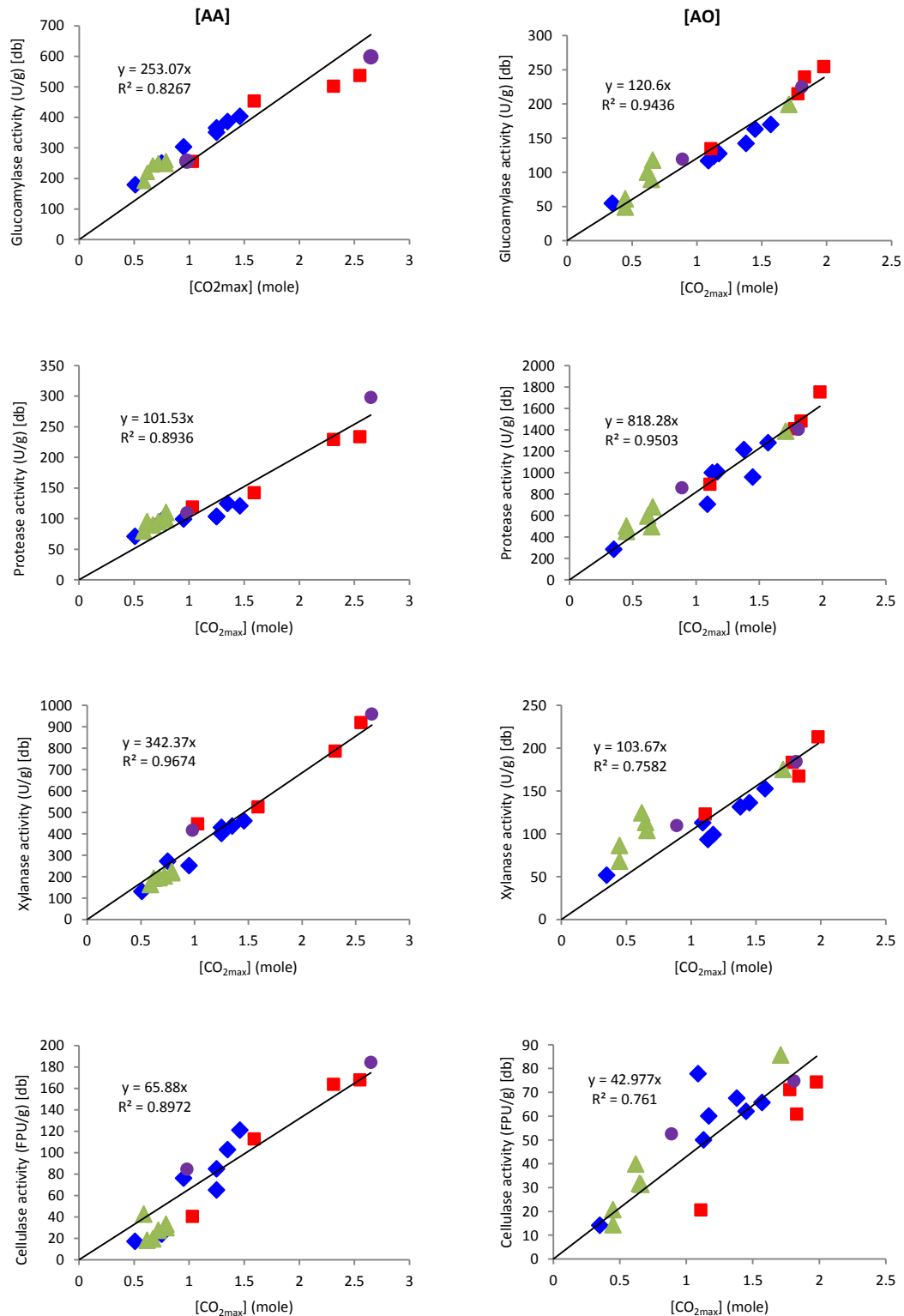
FIGURE 10.15: Relationship between $[CO_{2max}]$ with average enzymes production obtained from every air arrangement using 4 tray systems with *A. awamori* [AA] and *A. oryzae* [AO] cultures. (◆): single circular tray SSB; (■): multi-stacked circular tray SSB; (▲): single rectangular tray SSB and (●): multi-square tray SSB

10.3.3   Gas Balance - Respiratory Quotient

The respiratory quotient (RQ) can be calculated directly when the composition of the exhaust gas is known. All these values are interesting because they characterise physiology, specific performance of SSF bioreactor or total performance of fermentation. Since, those can be made available on-line, the RQ can be calculated on-line simply as:

$$RQ = \frac{CER}{OUR}$$ (10.4)

Where;

$RQ =$ Respiratory quotient (dimensionless)

$CER =$ Carbon dioxide evolution rate (mole L^{-1} h^{-1}) – Equation 7.4 (Chapter 7)

$OUR =$ Oxygen uptake rate (mole L^{-1} h^{-1}) – Equation 7.5 (Chapter 7)

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + Energy$$ (10.5)
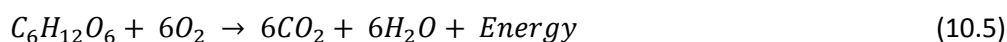
RQ characterises the state of the fungal population in the tray SSB and gives an indication of the fungal metabolic behaviour. According to Pandey *et al*. (2001), when RQ = 1, substrates are simple sugars or related substances where glucose oxidation is taking place. According to Equation 10.5, a theoretical RQ of oxidation of glucose for aerobic microorganisms is equal to 1. The equation shows that during glucose fermentation through aerobic fermentation, the rate of O_2 consumption in ideally six molecules and six molecules of CO_2 are produced per glucose molecule. It must be kept in mind, solid substrate waste used in SSF has varying composition of carbon and nitrogen. Some contain complex compositions such as hemicellulose, cellulose and lignin. In this situation, microorganisms need to utilise the entire complex compound and produce simple fermentative sugars before these can be easily consumed. In practical terms in SSF, it is impossible to obtain RQ = 1.

When RQ < 1, it is assumed that one or more non-constitutive or newly induced metabolites have possibly been produced. In other words, when O_2 was consumed by fungus, CO_2 was not the only product produced, other products such as energy (ATP), water and secondary metabolites were produced. Also O_2 is not only be used for the respiration process, it is also used for other tasks such as cell maintenance. Equation 10.6 summaries the SSF of organic material in solid substrates and is followed by Equation 10.7 for the oxidation of sugars (glucose).

$$Organic\ material + nO_2 \rightarrow Biomass + sugars + nCO_2 + nH_2O + Energy$$ (10.6)

$$Sugars\ (C_6H_{12}O_6) + nO_2 \rightarrow Biomass + nCO_2 + nH_2O + Energy \qquad (10.7)$$
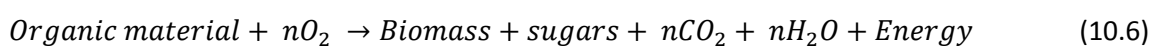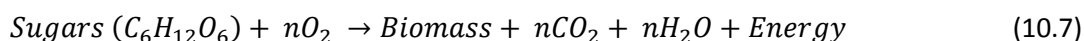
RQ > 1 indicates that O_2 uptake is low and fermentation of glucose is occurring. In practical terms, RQ values greater than one are taken as an indication that anaerobic fermentation is occurring. Usually in high aerated fermentation systems, it must be considered that the higher production of CO_2 relative to O_2 corresponds to the presence anaerobic zones in the fermenter (Pandey *et al*., 2001).

Further discussion of off gas analysis is provided in this chapter for every tray SSB with variable initial moisture contents and air arrangements. The rates of O_2 consumption and CO_2 evolution are presented along the calculated RQ values, giving further insights into the behaviour of the fermentation process. FIGURE 10.16 shows the profiles of RQ obtained using Equation 10.4 for both fungi in four tray SSBs with various air and initial moisture content arrangements. The RQ values are presented in FIGURE 10.17 by obtaining the slope from every experiment illustrated in FIGURE 10.16. It was observed in these studies that all RQ values obtained from tray SSBs with different initial moisture contents and air arrangements are below 1 (FIGURE 10.17).

Various values of RQ were obtained in this study as response to the reaction of fungi with the quantity of air provided into the system. However, it is difficult to correlate the effect of different flow rates of moistened air with the fungal behaviour in this study. For example, in the case of single circular tray SSB, RQ = 0.507 in Control 1 experiments (no water and no air) was the highest among the others in fermented *A. awamori*. Surprisingly, moistened air at different flow rates had apparently no remarkable effect on the RQ value.

The experiments with flow rate 4 L/min with adequate O_2 and water content exhibited low RQ compared to control experiments (Control 1 and Control 2), which had been initially assumed to promote high RQ values. However, in fermented *A. oryzae*, moistened air at flow rate 4 L/min recorded the highest RQ value (RQ = 0.789), which indicates that good growth occurred in this system. In experiments with flow rate 6 L/min, the RQ values averaged 0.357 and 0.159 for *A. awamori* and *A. oryzae*, respectively, which reflected the current metabolic activity in environments dominated by O_2 deficient substrates. It was believed that at high flow rate, most of the O_2 was washed out from the system. This can be supported by FIGURE 10.8 where the production of $[CO_{2_{max}}]$ was among the lowest evolved during SSF.
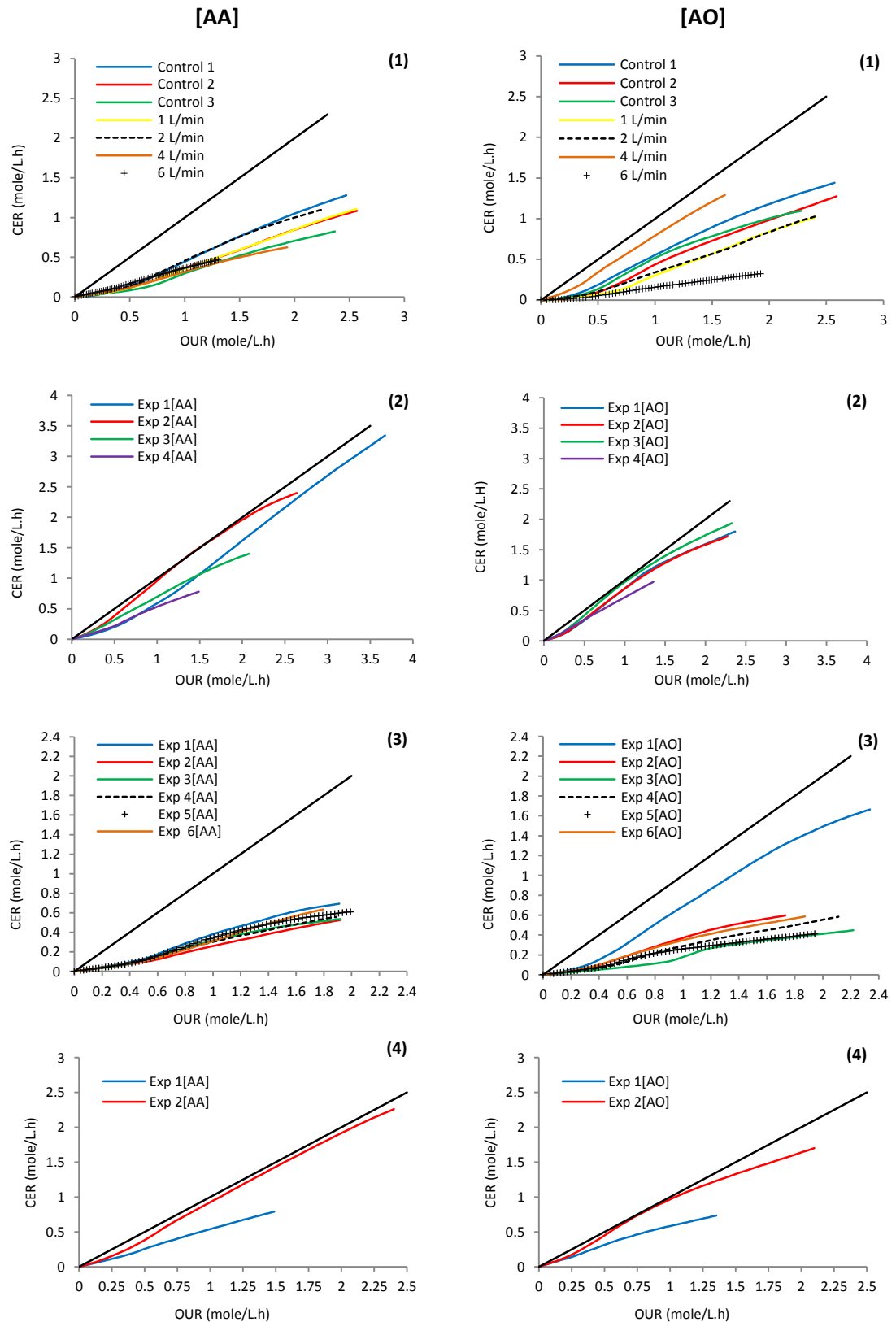
FIGURE 10.16: Profiles of RQ in four tray SSB systems with the fungi *A. awamori* [AA] and *A. oryzae* [AO] with different initial moisture contents and air arrangements. (1): single circular tray SSB; (2): multi-stacked circular tray SSB; (3): single rectangular tray SSB and (4): Multi-square tray SSB. The straight line represents RQ = 1

Interestingly, the RQ values were greater in the multi-stacked circular tray SSB with different initial moisture content arrangements. For *A. awamori* with Exp 1[AA], 2[AA] and 3[AA], the RQ values were 0.856, 0.954 and 0.694, respectively. RQ values for *A. oryzae* were 0.807, 0.807, 0.844 and 0.712 for Exp 1[AO], 2[AO], 3[AO] and 4[AO], respectively. Exp 4[AA] (initial moisture content at 15% in each tray) recorded the lowest RQ, being 0.520 for *A. awamori*. This low value of RQ can also be linked to the low water content and created unfavourable conditions for *A. awamori*.

The single rectangular tray bioreactor recorded the lowest RQ value compared the other bioreactors. In the case of *A. awamori*, the RQ values were variable between 0.296 and 0.374. Only in Exp 1[AO] (dry air onto fermented substrate) *A. oryzae* recorded higher RQ value (0.731). In others experiment, the RQ values were within the range 0.207 to 0.357. It was observed here that dry or moistened air does not give clear benefits for fungal growth. Based on features design of single rectangular tray SSB with air arrangement, the concentration of O_2 in the medium of respiring fungus, depends on the rate of O_2 transfer from the surface (gas phase) to the culture solid (on the rate at which is transported to the site of utilisation). It was suggested that the O_2 consumption rate by fungus is low and leads to the low CO_2 evolved.

In any case, a low RQ indicates that less CO_2 is evolved per unit of O_2 consumed. However, low RQ values cannot directly be linked to anaerobic conditions. With large are of fermented surface and the flow of air, it seems O_2 taken by fungus is less and more O_2 is carried into the gas outlet. This might be because O_2 has to diffuse from the gas phase to inside the fermented bed whereas the CO_2 is generated inside throughout and has to diffuse outside compared to the other air arrangement where moistened air was forced through the fermented substrate from bottom to the top.

Other factors, such as the effect of readily degradable nutrient compounds contained in the fermented substrate, seem to be key for determining the RQ value. Therefore, subsequent study should be considered to explore this in more details. These results can also be supported by poor spores and enzymes production by both fungi as described details in Section 10.3.2.6 and 10.3.2.7 (FIGURE 10.14 and 10.15, respectively).
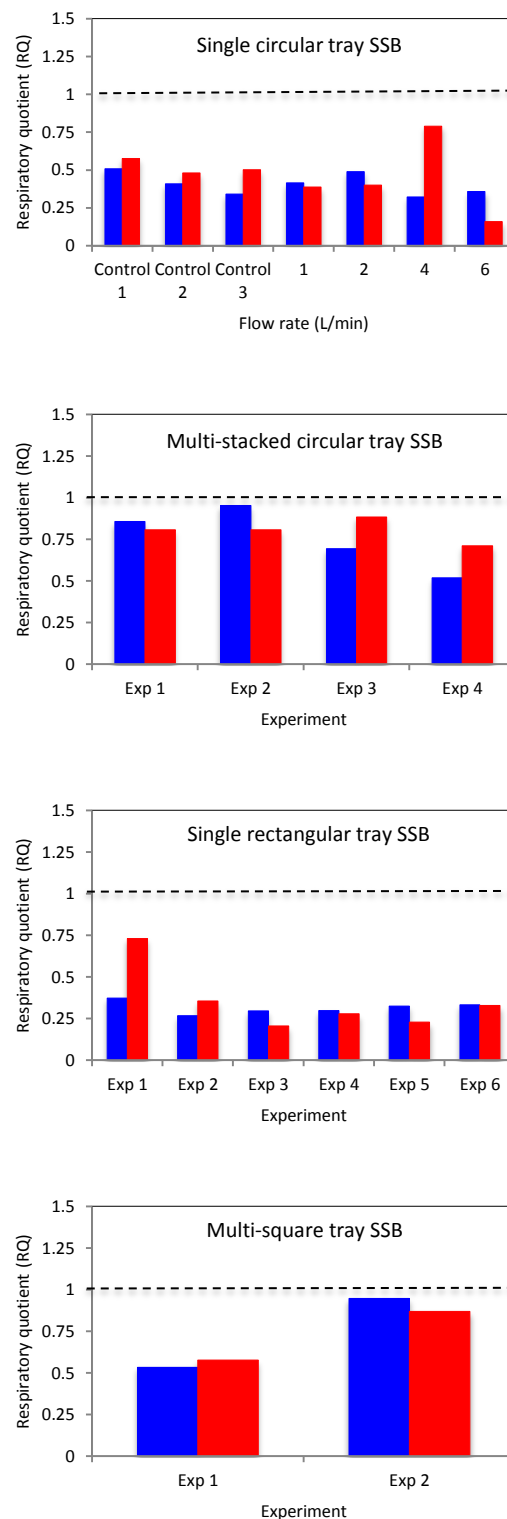
FIGURE 10.17: Respiratory quotient (RQ) during SSF of *A. awamori* (■) and *A. oryzae* (■) on wheat bran using four tray SSBs with different initial moisture content and air arrangement. The dash line represents RQ = 1

According to Gildyal *et al*. (1992), the mycelium growth of fungi is restricted by the diffusion of O_2 in the fermented solid substrate especially in a deep area. They argued that poor growth might occur

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deep because O_2 transfer inside the solid substrate is blocked due to the compact structure of solid particles. These situations were observed by Okazaki *et al.* (1980) where poor growth of the deeply submerged mycelium is observed in rice koji at high moisture contents, possibly because the pathways of O_2 inside the rice grain are blocked. Rajagopalan and Modak (1995) observed the growth of *A. niger* in a tray SSF system that was limited by heat and O_2 transfer. They suggested that by increasing the thickness of the mold biofilm around the solid particles, the porosity of the substrate bed and diffusivity of O_2 in the bed are reduced.

As expected, RQ values were higher in Exp 2 in the multi-square tray SSB for both fungi. As discussed before, the gas phase between the trays and the air arrangement by blowing the moistened air at 1 L/min onto fermented surface gives a higher advantage for fungal growth. It was assumed adequate O_2 concentration is available in the system. The concentration of O_2 is higher in the arrangement of trays in a bioreactor. This situation arises because of the perforations at the bottom plate of trays that facilitate mass transfer to the gas surroundings of the substrate bed, both at the top and the bottom. With the air arrangement in Exp 2, the RQ value for *A. awamori* and *A. oryzae* was 0.947 and 0.868, respectively. *A. awamori* showed RQ values around 1 and it seems degradation and utilisation processes of nutrient components during 72 h SSF provides adequate nutrients and fungal biomass. By contrast, in Exp 1, the RQ value was 0.533 and 0.578 for *A. awamori* and *A. oryzae*, respectively. According to Govind *et al*. (1997), RQ values are close to 1 until O_2 uptake reaches a maximum level and thereafter RQ becomes higher than 1 due to utilisation of metabolite products. Becerra and Gonzalez-Siso (1996) argued that changes in RQ values could be associated with the production of multiple products by *Streptomyces cattleya* during SSF. Barrios-Gonzalez *et al.* (1993) reported that an RQ of 0.7 indicates oil-supported metabolism for *Nocardia lactamdurans* during the production of efrotomycin in SSF.

10.3.3.1   The relation between respiratory quotient and carbon dioxide evolution

The experiments conducted in this study showed that the rate of O_2 consumption and CO_2 evolution in *A. awamori* and *A. oryzae* highly correlated. FIGURE 10.18 shows linear regression analysis of the relationship between $[CO_{2_{max}}]$ and RQ giving a straight line (R^2 > 0.862) for both fungi using data from 38 experiments. Changes in RQ value can be correlated with changes in the metabolic condition of the fungus. The higher RQ values of the metabolic activity may result from beneficial microbial respiration by O_2 consumption and CO_2 evolution rate. Thus, *A. awamori* and *A. oryzae* apparently adapt to the degree of complete oxidation (microbial respiration) and utilisation of the available nutrients provided in the system (water, O_2 etc.).

Undoubtedly, measuring the amount of CO_2 evolved or O_2 consumed during SSF will lead to obtaining RQ that is able to describe different patterns of fungal metabolic behaviour. RQ has proven to be an effective indicator for the control of metabolic conditions in SSF processes. When used as a guide for growth development, RQ levels associated with biomass accumulation phase should be maintained throughout the development of the fermentation conditions. If any of the subsequent RQ phases are allowed to occur during the growth, subsequent productivity can be compromised. It must be kept in mind that all of these considerations are based on the employment of solid substrates comprising simple sugars or related carbohydrates or polysaccharides. According to Pandey *et al*. (2001), when dealing with different solid substrates, the substrate properties and the overall metabolic pathway must be taken into account to obtain a proper value for the RQ. However, it would be convenient for a basic laboratory SSF bioreactor to have a system for gas analysis at the outlet of the bioreactor.
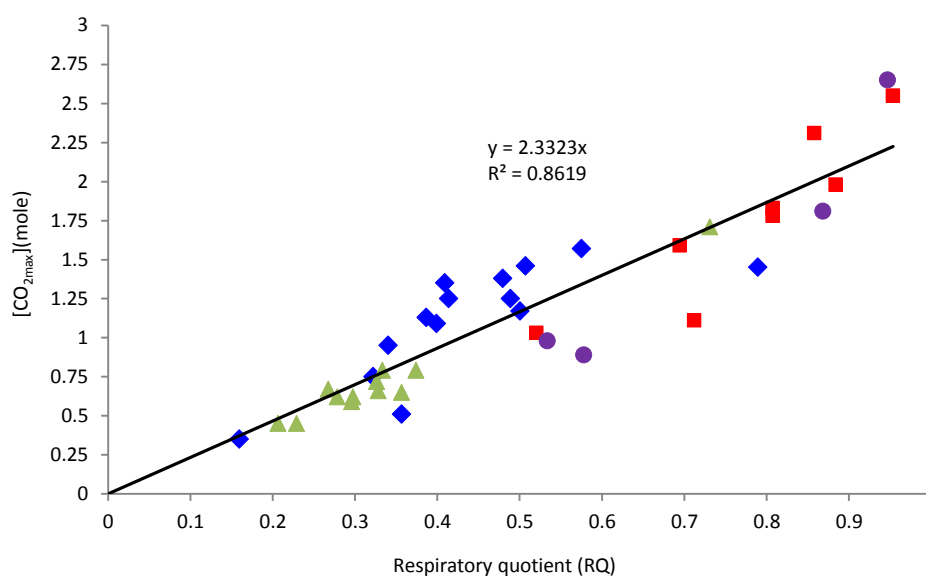
FIGURE 10.18: **Relationship between RQ with $[CO_{2max}]$ obtained from every air arrangement using 4 tray systems with *A. awamori* and *A. oryzae* cultures. (◆): single circular tray SSB; (■): multi-stacked circular tray SSB; (▲): single rectangular tray SSB and (●): multi-square tray SSB**

10.3.4   Heat Evolution during SSF

Temperature is the limiting design consideration for most SSB systems. A high amount of metabolic heat is produced during growth. The temperature of SSF cultures often increases to levels incompatible with growth. As the scale of SSB increases, the mechanism of heat transfer rapidly becomes insufficient. The forced addition of air is usually necessary to allow evaporative cooling of

the fermented substrate and also the system. Some form of moisture control is then required. Forced aeration with saturated (moistened in this study) air then can be introduced into the system.

10.3.4.1   Single circular tray SSB

The temperature profiles of the fermented bed were recorded during 72 h fermentation time shown in FIGURE 10.19 for *A. awamori* and *A. oryzae*. As illustrated in TABLE 10.2, air circulated from below the perforated mesh tray results in temperature and gas concentration gradients within the fermented bed. It was assumed that heat is driven off by forcing it through the top of the fermented bed. To observe the effectiveness of moistened air in this system, experiments without air were also carried out (Control 1 and 2).

In the case of *A. awamori*, during the first 18 h of fermentation time, the bed temperature stayed constant between 30 – 31 °C for all experiments except for flow rate 6 L/min. Only after 48 h, the temperature of the bed started to increase gradually and this showed that at this point the growth of fungi and metabolic activities were at a high level. Experiment Control 1 and that with moistened air at 2 L/min showed an increment temperature of almost 5 °C. Control 2, Control 3, moistened air at 1 L/min recorded an increment in the range 2 – 3 °C. Experiment with flow rate at 4 L/min showed temperature at a constant range 30 – 31 °C during 72 h of fermentation. The temperature of fermented bed was recorded below 30 °C until the end of fermentation for the higher flow rate at 6 L/min.

In the case of *A. oryzae*, experiment Control 1 recorded metabolic heat and achieved almost 40 °C. Control 1 experiment, where there is no air introduced into the system, showed heat accumulation in the system. However, in Control 2, when only air was blown below the tray, it still showed an effect on bed temperature. This result showed that maximum temperature reached about 34 °C and after that it was maintained between 31 – 32 °C until the end of fermentation. As predicted, Control 3 with dry air was greatly efficient in discarding metabolic heat and able to control temperature below 32 °C. Other experiments (moistened air at 1, 2 and 4 L/min), the temperature was recorded at optimal level and an increment in the range 1 – 4 °C was observed. This flow rate is assumed to give a good performance and as a result a better temperature control. In the experiment with flow rate 6 L/min, the temperature of fermented bed was recorded below 30 °C until the end of fermentation. For culture system with *A. oryzae*, the role of either dry air or moistened air succeeded in removing the heat form the system.

[AA]

[AO]
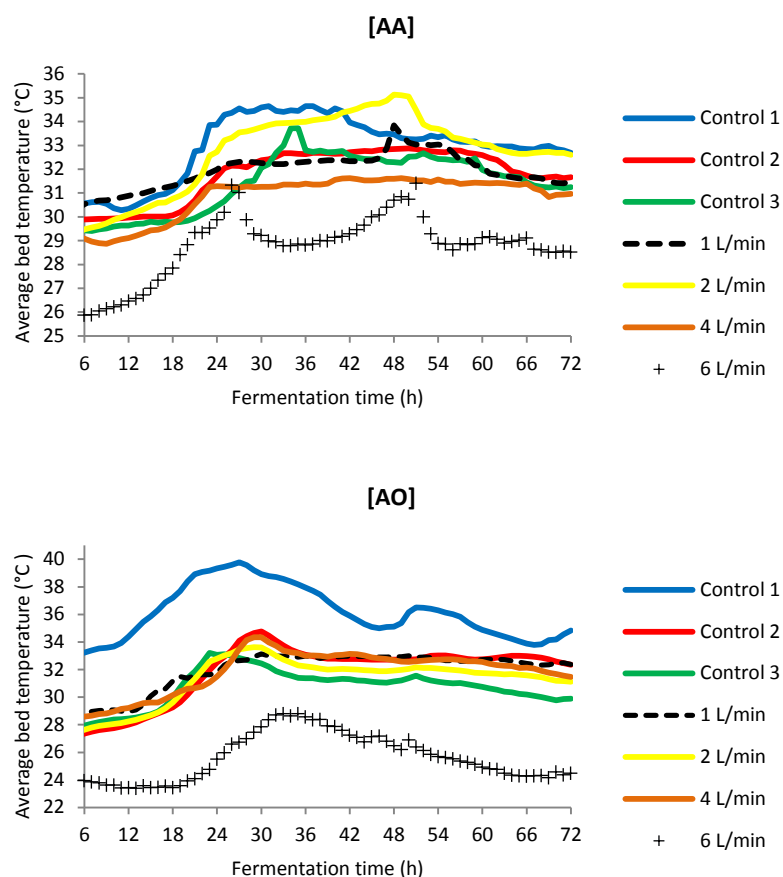
FIGURE 10.19: Effect of the different air flow rate on the heat evolution of *A. awamori* [AA] and *A. oryzae* [AO] grown on wheat bran performed in single circular tray SSB

In this strategy where the air flow rate was set up at a higher rate of 6 L/min into the water reservoir below the tray, air was blown forcefully through the tray and solid particles and heat removal was clearly observed. It is clearly observed from this system, heat accumulation is partially overcome by forced aeration and cooling strategies, but high temperatures can still be reached at the gas phase and near the air outlet with certain experiments. As an example from these studies, we found that during the time of peak heat production, the temperature can rise almost to 8 - 10 °C or more above the optimum temperature. A large amount of heat is generated during SSF, which is directly proportional to the metabolic activities of fungus (Shojaosadati *et al*., 2007). Heat accumulation is high, which can cause denaturation of the products formed (enzymes in these studies) (Rodriguez-Fernandez *et al*., 2012). According to Mitchell *et al*, (2003), in forcefully aerated bioreactors, the air is blown directly into fermented substrate resulting in improving contact between the air and the substrates particles. At the same time when the temperature decreased to 5 - 7 °C below optimum temperature, this also can have a negative effect on growth and microbial activity.

10.3.4.2   Multi-stacked circular tray SSB

As the positions of multi trays vary, so does their temperature. As a result; each tray is exposed to several temperature cycles during fermentation. The temperature of fermented bed in the tray at position 1 is predicted to be the lowest and the temperature increases gradually in the trays above. The temperature of fermented bed in the tray at position 6 is predicted to reach the highest. The moistened air blown into the bioreactor is maintained at 30 °C (the optimum temperature for *A. awamori* and *A. oryzae*), in an attempt to control bed temperatures.

The temperature of the moistened air increases with height and this increases the air water holding capacity, causing some evaporation to occur in the trays. This phenomenon can be proved by the final profile of moisture content (especially in *A. oryzae* culture). However, the average of fermented bed temperature was presented in this system from six temperature profiles. Growth in all trays is almost similar, with various growth profiles being quite close to the profile for the average of all six trays. This result is clearly observed in the culture of *A. oryzae* (Exp 1[AO], 2[AO] and 3[AO]).

FIGURE 10.20 shows the average temperature profiles for both fungi during SSF on wheat bran with different initial moisture content arrangements. During the period 6 – 12 h, various profiles of temperature were observed. After that period, temperature rises gradually as an indication of increase in metabolic activity. Exp 1[AA] recorded temperature peaks of between 32 – 35 °C, while Exp 2[AA] and 3[AA] recorded a maximum temperature of almost 39 °C. Exp 1[AA], 2[AA] and 3[AA] recorded temperature profiles above 30 °C until the end of the experiments. Exp 4[AA] recorded temperatures below 30 °C and only reached maximum (32 °C) at 32 h. This indicates that growth in Exp 4[AA] was very slow compared to other experiments because of lack of water in the fermented bed.

In Exp 1[AO], 2[AO] and 3[AO], the maximum temperatures reached were 41.57, 40.71 and 41.20 °C after 26 h. As the initial moisture content was low in Exp 4[AO] (15% moisture content) and because of slow growth as observed with *A. awamori* culture, the maximum temperature reached was only 33.42 °C.

[AA]

[AO]

FIGURE 10.20: Effect of the different initial moisture content arrangement on the heat evolution of *A. awamori* [AA] and A. *oryzae* [AO] grown on wheat bran performed in multi-stacked circular tray SSB

The growths of both fungi more or less corresponded with each other. The cultures in every tray all started growing slowly and entered a rapid growth phase together. As a result, fermented cultures in every single circular tray reached their peak generation at about the same time. It has to be kept in mind that the chosen initial moisture content in Exp 1 and Exp 2 was assumed to be optimal to support growth of both fungi. Only in Exp 4, the limited water content (15%) in the culture system was the reason the growth of both fungi was slow.

In this multi-stacked circular tray SSB, proper stacking and sealing of the attached trays to a lip at the bottom of the tray would ensure a good sealing against the supporting tray above each others. If proper sealing is not achieved, then the air will pass around outside the trays rather than being forced to flow upwards through them. The temperature and moisture gradients can lead the fermented substrate at different tray heights to have very different temperature profiles.

10.3.4.3   Single rectangular tray SSB

In order to investigate the effects of air arrangements on the fermented bed during the fermentation process, dry and moistened air arrangements were carried out as described in TABLE 10.3. Measurement of temperature of the fermented bed were done at the starting point (near air inlet), center and at the end (near air outlet). The average results of temperature at different points on the fermented bed of the single rectangular tray SSB under the conditions of 30 °C (temperature of incubator growth room) are shown in FIGURE 10.21 and 10.22 for experiments using dry air and moistened air, respectively.

The results of average temperature of fermented bed in the single rectangular tray SSB with dry air for both fungi (Exp 1, 3 and 5) are shown in FIGURE 10.21. According to FIGURE 10.21, during the 18 h of fermentation, the bed temperature profile was approximately constant, but a few hours later with an increase of growth rate, a rapid increase in the bed temperature to approximately 36 and 38 °C was recorded for *A. awamori* and *A. oryzae*, respectively. In the case of *A. awamori*, the maximum heat production levels reflected in the average temperature gradients for Exp 1[AA], 3[AA], and 5[AA] were 35.35, 33.77 and 35.76 °C, respectively. After 30 h, the temperature profile started to decrease constantly indicating that fungal growth in all three experiments became slower until the end of the fermentation time.

In the *A. oryzae* culture, the maximum temperature was 38.04, 36.05 and 36.01 for Exp 1[AO], 2[AO] and 5[AO], respectively. In contrast with *A. awamori*, for *A. oryzae* culture in Exp 3, fungal growth only started to decrease after 34 h. However, Exp 1[AA] and 3[AA] have almost the same pattern with *A. awamori* culture. In this air arrangement, the fermented bed suffered from loss of moisture content which was already discussed in Section 10.3.1.3. It was predicted here that high temperatures are generated and accumulate in this system and play one of the key roles in the loss of water from the fermented bed. Despite the non-uniform fungal growth in this system using all of the above-mentioned air arrangement, low biomass concentration was observed due to aeration and low fermented bed moisture.

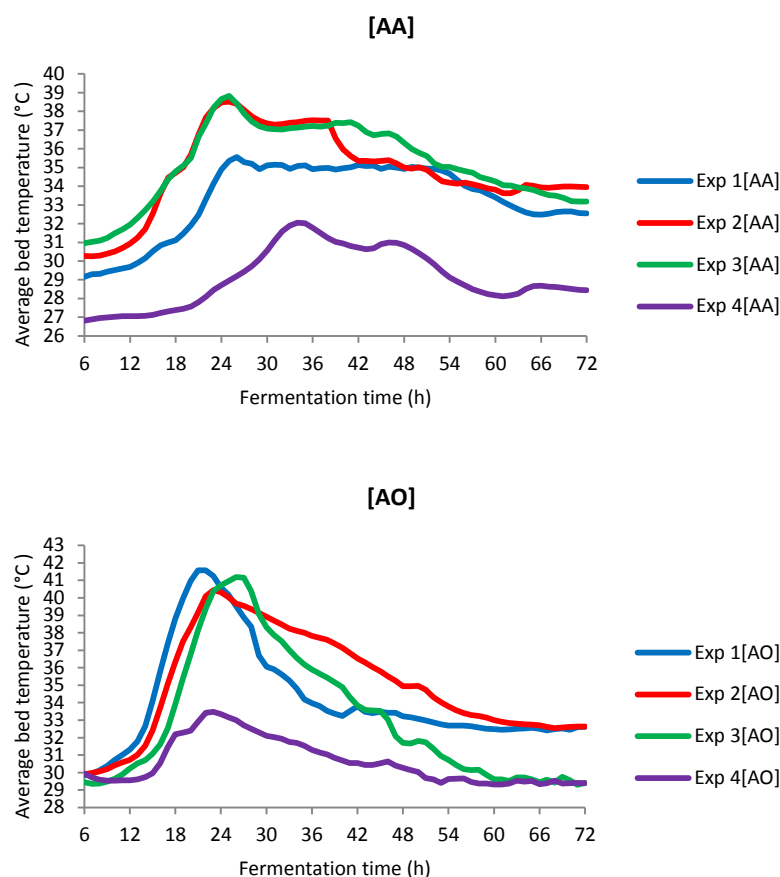**FIGURE 10.21:** Effect of dry air arrangement on the heat evolution of *A. awamori* [AA] and *A. oryzae* [AO] grown on wheat bran performed in single rectangular tray SSB. (◆): Exp 1; (■): Exp 3 and (▲): Exp 5

In order to reduce the fermented bed temperature gradient, the moistened air flow at 2 L/min was carried out. Uniform fungal growth was observed in the bioreactor, fungal growth and temperature (FIGURE 10.22) decelerated after 26 h except for Exp 6[AA], the growth only decelerated after 45 h. Fermented bed temperature for all experiments was near the optimum growth temperature which is below 35 °C.

The air arrangement with moistened air was able to control the temperature of the fermented bed and at the same time control moisture content as described in Section 10.3.1.3. It is likely that minimum evaporation of fermented bed moisture occurred during which aeration rate, control of temperature and O_2 consumption rates were at maximum. Despite the constant aeration with moistened air, low fermented bed temperature (near optimal growth temperature), high biomass concentrations (without apparent gradient) and also lower reductions in the fermented bed moisture were observed for both fungi.

According to the results obtained from this system, it can be concluded that the dry air used for the simultaneous control of the fermented bed temperature and moisture may not be an appropriate strategy. Use of moistened air in the system is a suitable strategy for reducing the fermented bed temperature and moisture gradient in order to obtain uniform fungal growth with high biomass.

[AA] - Moistened air
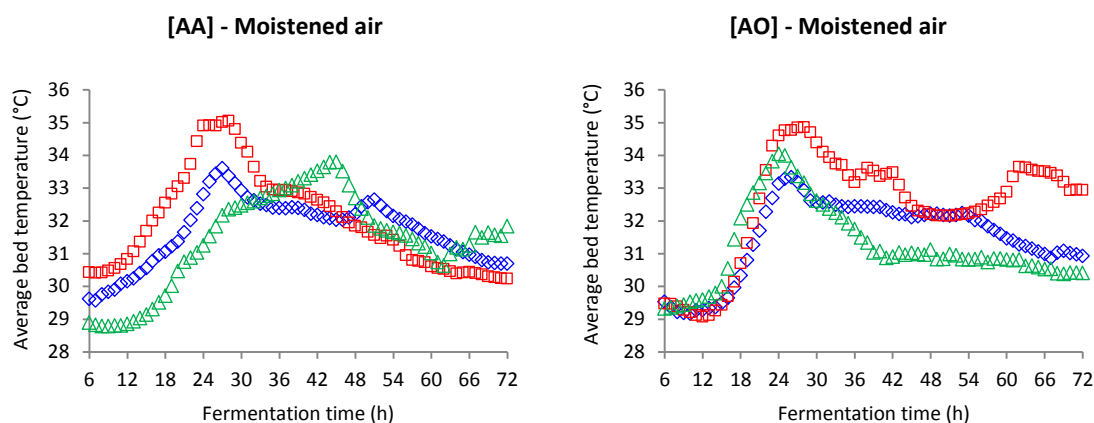
[AO] - Moistened air

FIGURE 10.22: **Effect of moistened air arrangement on the heat evolution of *A. awamori* [AA] and *A. oryzae* [AO] grown on wheat bran performed in single rectangular tray SSB. (◇): Exp 2; (□): Exp 4 and (△): Exp 6**

10.3.4.4   Multi-square tray SSB

Effects of moistened air with two arrangements in multi-square tray SSB are shown in FIGURE 10.23. Fungal growth for both fungi with Exp 2 provided a great respond based on temperature profiles during 72 h fermentation time. The temperature reached maximum at about 27 h with 37.03 and 36.55 °C for *A. awamori* and *A. oryzae,* respectively. All experiments show a rise in temperature after 18 h of fermentation reaching maximum between 27 and 30 h before it started to decrease until the end of fermentation. Exp 2 with moistened air at flow rate of 1 L/min tended to be suitable for growth conditions, and for both fungi, the temperature recorded was below 40 °C. Exp 1 with flow rate 8 L/min succeeded to move out the heat created from every tray from the bottom to the top due to the high flow rate.

Better growth performance was observed in Exp 2 due to adequate O_2 concentration. Further, in Exp 2, the air is not blown forcefully through the bed but rather circulated through the headspace above the bed and the substrate supported on the perforated mesh tray allows better air circulation around the trays. Perforation in a tray base allows air flow through the tray to assure uniform mass and heat transfer. The system was properly sealed with a gap of about 2.0 cm between the trays to allow better aeration and O_2 accumulation (FIGURE 4.17, Chapter 4). This system also proved to be very excellent in spores (Section 10.3.2.6) and enzymes production (Section 10.3.2.7) compared to the other 3 system (FIGURE 10.14 and 10.15, respectively).
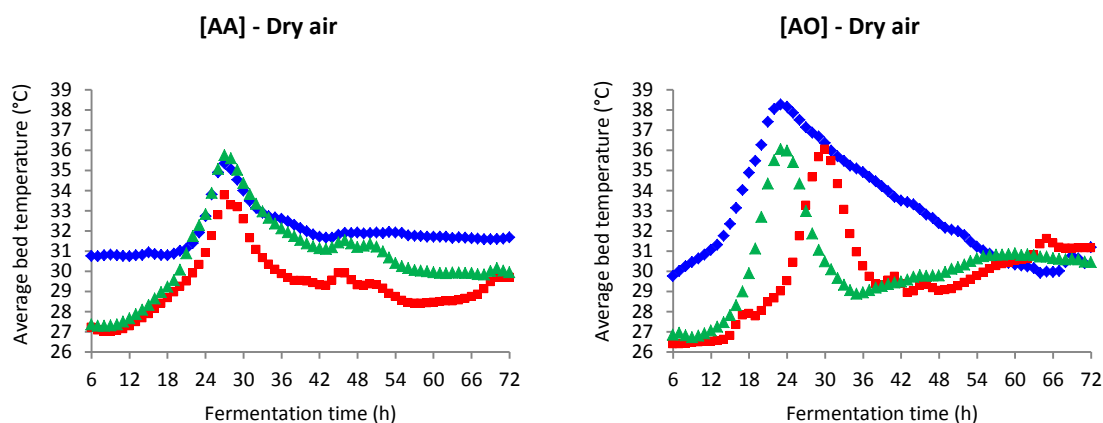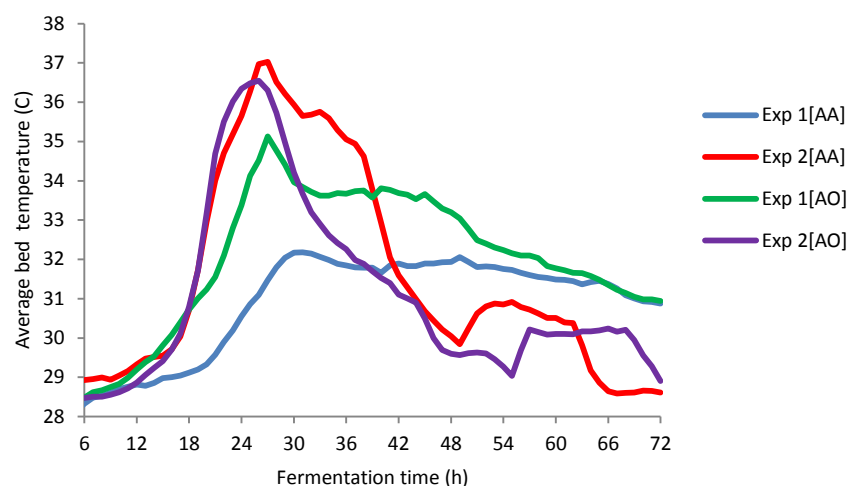
FIGURE 10.23: Effect of moistened air arrangement on the heat evolution of *A. awamori* [AA] and *A. oryzae* [AO] grown on wheat bran performed in multi-square tray SSB

In the tray system bioreactors studied here, it is impossible to maintain the bed temperatures at the optimum value for growth, and therefore the fungus will suffer variations in temperature during the fermentation process. It is worth mentioning that from these studies, huge amount of water loss and drying of the substrate were only observed in the single rectangular tray SSB when dry air was used. However, this was not observed in the other three tray SSBs. The information about the fungus itself in terms of dealing with fermentation conditions is important. For example in this study, *A. awamori* suffered from higher water loss compared to *A. oryzae*. However, the growth of *A. awamori* tended to be excellent in all experiments carried out. This was proven by spores and enzymes production.

The approach taken by creating moistened air flow in the system created a cooling system able to supply moisture into the fermentation substrate. At one stage, the substrate possessed enough moisture to support growth and fungal metabolic activities. In the majority of cases, the growth rate was expressed solely as a function of the current temperature according to the experimental results collected.

On top of that, the temperature can be expressed as a function of the CO_2 evolved in the system. The profile for both parameters recorded in this work can almost provide an adequate estimation of the whole growth curve including the lag, log, exponential growth, and stationary phases. However, this profile cannot provide complete representation that includes the death phase, instead it clearly shows the growth becomes slower after the exponential phase.

10.3.4.5   Heat evolution and correlation with CER during fungal growth

Through observation during these studies, one of the most remarkable findings is the strong correspondence between temperature rise and the concentration of CO_2 evolved observed during 72 h fermentation. A high degree of correlation was found to exist relating evolving CO_2 and metabolic heat as a results of utilising the carbon source. During the period of CO_2 evolution, the total amount of CO_2 evolved with time was found to correlate with the changes in temperature evolved during microbial activity.

Motivated by these observations, evaluation of data of carbon dioxide evolution rate (CER) and temperature changes during SSF for both fungi were carried out. Data from four tray SSB systems with different air arrangements were chosen as follows:

1.   Single circular tray SSB: Control 1 without air
2.   Multi-stacked circular tray SSB: Exp 2 with different initial moisture content
3.   Single rectangular tray SSB: Exp 5 with dry air at 2 L/min
4.   Multi-square tray SSB: Exp 2 with moistened air at 1 L/min

FIGURE 10.24 and 10.25 show the profiles of temperature changes and CER over time for A. *awamori* and A. *oryzae*, respectively.   Commencement of CO_2 evolution occurred during 6 − 24 h of fermentation for all the above-mentioned experiments. This also can be observed with temperature rise where the profile shares almost the same pattern as the CER profile. When the CO_2 concentration increases, temperature rises. Likewise, when the CO_2 concentration decreases, temperature also goes down. The relationship between temperature and CO_2 evolved is consistent with a feedback between CO_2 evolution rate (and might be also correspond with oxygen uptake rate, OUR).

(A)

(B)

(C)

(D)

FIGURE 10.24: Profile of temperature and CER of *A. awamori* culture from four different experiments using four tray SSB systems. **(A):** Control 1 without air [single circular tray SSB]; **(B):** Exp 2 with different initial moisture content [multi-stacked circular tray SSB]; **(C):** Exp 5 with dry air at 2 L/min [single rectangular tray SSB] and **(D):** Exp 2 with moistened air at 1 L/min [multi-square tray SSB]. (◇): temperature profile and (☐): CER profile

According to Hendry *et al*. (1993), heat and CO_2 are microbially produced indicating the existence of metabolic activity of microorganisms, consumption of O_2, evolution of CO_2 and utilisation of organic carbon. In the case of aerobic microorganisms, the catabolic heat evolution rate is regarded to be proportional to the OUR and CER. According to Cooney *et al*. (1968), if the transfer ratio of heat evolution rate is constant, the change of temperature can be calculated by monitoring to the O_2 consumption or CO_2 evolution.
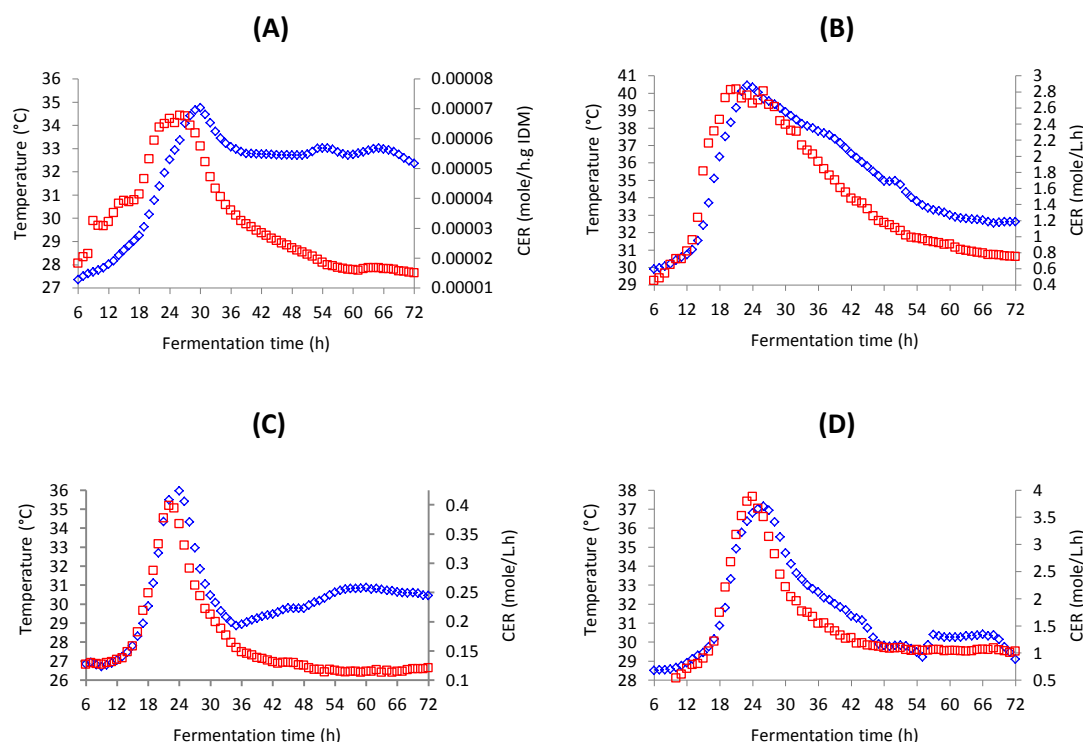
FIGURE 10.25: Profile of temperature and CER of *A. oryzae* culture from four different experiments using four tray SSB systems. (A): Control 1 without air [single circular tray SSB]; (B): Exp 2 with different initial moisture content [multi-stacked circular tray SSB]; (C): Exp 5 with dry air at 2 L/min [single rectangular tray SSB] and (D): Exp 2 with moistened air at 1 L/min [multi-square tray SSB]. (◇): temperature profile and (□): CER profile

The data obtained from all experiments was further evaluated to study the correlation between temperature changes with CER. The data for the rate of CO_2 evolution and temperature rise for 8 experiments (from both fungi) are shown in FIGURE 10.26. These data are plotted for two different fungi in the same experiments. In every tray SSB experiments, the temperature rise during SSF was found to correlate with the rate CO_2 evolution. A linear correlation exists between the temperature rise and CER regardless of the growth rate, initial moisture content, air arrangement, bioreactor design and type of fungus. It can be noticed that from FIGURE 10.26(B) and 10.26(D), a straight line with the slope of 2.352 and 2.224 °C/mole of CO_2 was about the same. This is might be due to moistened air used in the system even though at different flow rates and initial moisture contents. However, the features of the systems are almost the same involving of multiple fermented substrates. However, a strange linear correlation was observed in the study without air and such a tendency is not noted as can be seen in FIGURE 10.26(A). A straight line was also observed in the experiment with dry air at flow rate 2 L/min (FIGURE 10.26(C)). However, the trend is not apparent in the data with moistened air (FIGURE 10.26(B) and 10.26(D)). The correlation between temperature

rise and CO_2 evolution is very satisfactory; however, it does not permit comparison between processes.
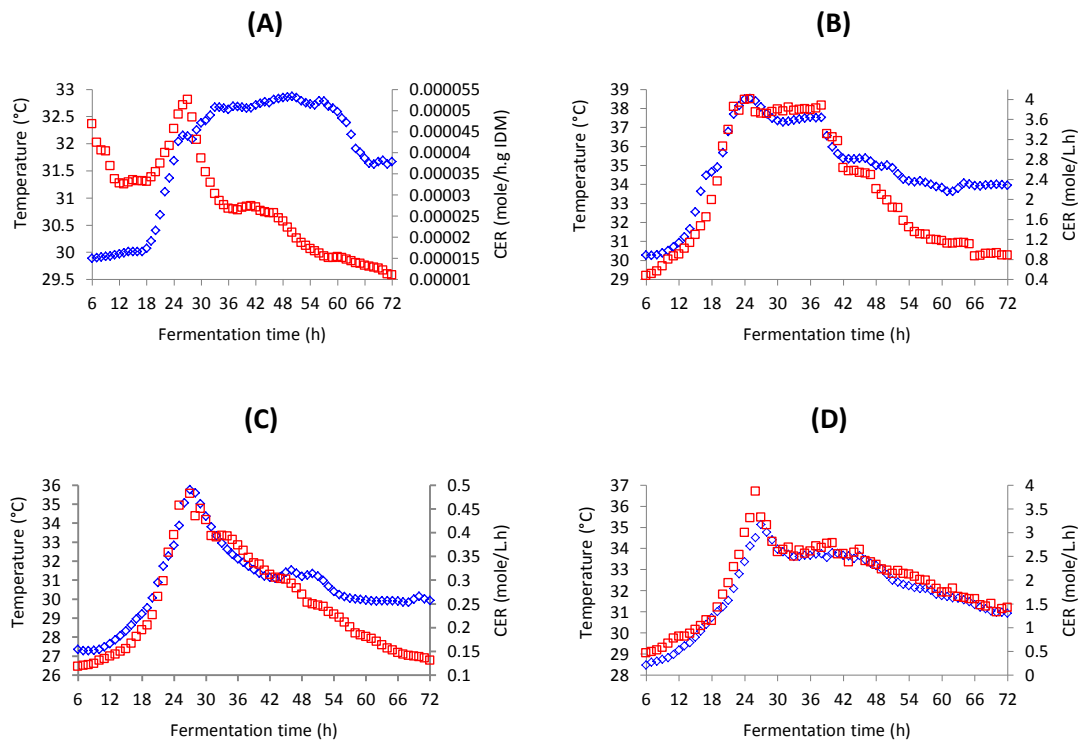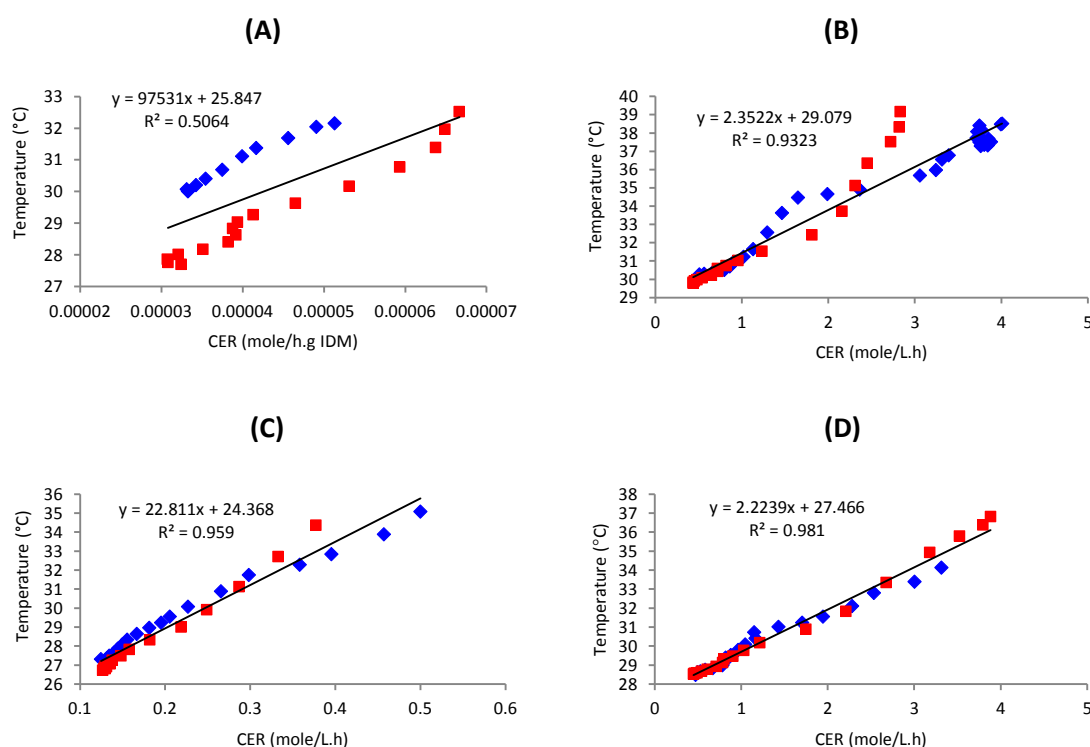

FIGURE 10.26: **Profile of temperature and CER of *A. awamori* and *A. oryzae* cultures from four different experiments using four tray SSB systems. (A): Control 1 without air [single circular tray SSB]; (B): Exp 2 with different initial moisture content [multi-stacked circular tray SSB]; (C): Exp 5 with dry air at 2 L/min [single rectangular tray SSB] and (D): Exp 2 with moistened air at 1 L/min [multi-square tray SSB]. (◆): *A. awamori* and (■): *A. oryzae***

The data shown in FIGURE 10.26 suggest a possible linear dependence between the temperature rise and CER on the type of fungus. It appears possible to draw separate lines for *A. awamori* and *A. oryzae* for every experiment that will correlate well with the respective data. Temperature rise and rate of CO_2 evolution reflect the metabolic activity of the fungus during the SSF process. According to Hendry *et al*. (1993), rising CO_2 levels correlated with rising temperatures because CO_2 is believed to trap natural heat caused by surface warming of the fermented substrate by microbial activity. They also added that CO_2 would enclose the surface and trap the heat causing the fermented bed surface to become insulated and causing temperature rise. In addition, the evolution of heat during microbial activity can provide useful data and converted into biologically meaningful data such as growth rate, lag phase, or maximum growth (Braissant *et al*., 2013). According to Braissant *et al*., (2013), the use

of this technique in the biological and biomedical field has been increasing such as detection and characterisation of pathogens, drug testing, parasitology and tissue engineering.

10.4   SUMMARY AND CONCLUSIONS

One of the remarkable findings from this study was that the ability of *A. oryzae* to retain moisture was much higher than that of *A. awamori.* This is possibly due to the higher levels of glucosamine (chitin monomer) in *A. oryzae.* However, as an overall observation, *A. awamori* demonstrated excellent growth and high productivity although lacking in retaining water content within its cells. With operational bioreactor design in multi trays as observed in multi-stacked circular and multi-square tray SSBs, combined with aeration arrangement using moistened air, *A. awamori* demonstrated great potential in spores and enzymes production compared to *A. oryzae*.

Continuous monitoring of O_2 and CO_2 concentrations in the exhaust gas of SSF bioreactors is very useful to assess the physiological state and respiration rate of culture. CO_2 evolved as a result of metabolic activity during *A. awamori* and *A. oryzae* SSF on wheat bran is easy to handle with a simple Gompertz model. Data obtained in four tray SSBs modelled using Gompertz model for *A. awamori* and *A. oryzae* in SSF at various initial moisture contents and air arrangement experiments were fitted in a satisfactory way. The Gompertz model is useful for the empirical representation of fungal growth. However, it does not describe the biomass decay after the stationary phase.

Measurements of O_2 and CO_2 are used to derive OUR and CER. The studies showed that OUR and CER during SSF of *A. awamori* and *A. oryzae* were generally highly correlated. In turn, the ratio CER over OUR can be used to calculate the RQ allowing an easy assessment of the performance of the bioreactor system. The RQ values were less than 1 for data obtained from 38 experiments using four tray SSB systems with two fungi. This indicates relatively high O_2 consumption during the current fungal SSF on wheat bran. The multi-stacked circular tray SSB with different initial moisture content arrangements exhibited greater RQ values. In addition, the multi-square tray SSB system with air blown onto the fermented surface also recorded high RQ values almost equal to 1. The RQ value varies due to the composition of available substrates, the current physiology of the fungi *A. awamori* and *A. oryzae*, fungal adjustment to the nutritional conditions and also physical conditions in the bioreactor system. Different initial moisture content and air arrangements, different systems and different fungi showed varying CO_2 evolution rates, spores and enzymes production, and RQ values during SSF, which are indicators of the fungal growth.

Heat removal is one of the major constraints in SSF processes. The strategy presented in this study allowed quantitative evaluation of the effect of the forced internal moist air circulation on the removal of metabolic heat. A combination of the control of aeration and moisture content of the fermented substrate is thus favourable in the SSF process using moist (humidified) air. The rate of aeration by moistened air is able to control the temperature and the moisture content of the fermented substrate. In addition, it will allow a sufficient O_2 concentration for fungal growth. Through the study with the proposed moisture and air arrangements, it was possible to maintain the bed temperatures and moisture at the optimum value for growth. Increasing the flow rate of moistened air into the bioreactor improved heat removal. A correlation was found between heat evolution and CER during SSF. The linearity constant for the correlation is independent of the design of the bioreactor system, but slightly dependent on type of air (dry or moistened air), the air and moisture arrangement and possibly dependent on the type of fungus.

As mentioned in the above reports, encouraging improvements in production can be achieved by using tray SSB systems developed in this study. The principal advantages of tray SSB systems over conventional petri dish fermentation or other tray type fermentation are: (i) higher productivity and (ii) ability to relieve repression under specific nutrient limitation (O_2 concentration and water activity in this case). In some cases, a simple operated closed system tray SSB with constant moistened airflow rate was already adequate to improve the productivity to the required levels. In certain cases, even the application of a very complicated aeration arrangement did not further improve the results of the simplest aeration mode. In conclusion, the selection of aeration arrangement and the quality of air into the system is determined by the expected productivity.

CHAPTER 11

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

"The ability of microorganisms, especially filamentous fungi, to convert biomass through SSF bioconversion will have a great impact on food and agro-industry in every aspect of life from food and medicine to fuel. This is a future that undoubtedly will present challenges, but one that I believe we should embrace."
Musaalbakri Abdul Manan
Malaysian Agricultural Research and Development Institute

11.1 CONCLUSIONS

The promise of industrial biotechnology has been around since Chaim Weizmann developed the acetone-butanol-ethanol fermentation here in the University of Manchester in 1917 and the prospects nowadays look brighter than ever. Today's biorefinery technologies would be almost unthinkable without biotechnology. This is a growing trend and biorefineries have also increased in importance in agriculture and food industry. A novel biorefinery process using solid state fermentation (SSF) technology has been developed in the Satake Centre for Grain Process Engineering (SCGPE), University of Manchester, as an alternative to conventional processing routes, leading to the production of added-value products from agriculture and food industry raw materials. SSF involves the growth of microorganisms on moist solid substrate in the absence of free flowing water. Future biorefineries based on SSF aim to exploit the vast complexity of the technology to modify biomass produced by agriculture and food industry for valuable by-products through microbial bioconversion.

For example, wheat is one of the main agricultural crops in the United Kingdom. The United Kingdom produces about 13.4 million tonnes of wheat per year, and this agricultural activity is estimated to produce about 1.61 million tonnes of wheat bran. Through many generations, wheat bran has been used as an animal feed and can be categorised as waste. Value addition of wheat bran through microbial fermentation technology using selected microorganisms can hopefully improve the nutritional content and make it more valuable. The value of wheat bran can be improved through fermentation technology either by using SSF or submerged fermentation (SmF). These two techniques are not entirely independent. SSF has been chosen since it appeared to be exceptional and more favourable over SmF in several aspects where it gave advantages in terms of biological, processing, environmental and economic aspects to produce food, enzymes, chemicals and bio-oils. SSF offers a more favourable environment for fungal growth, yielding higher productivity in a relatively low-cost process by using nutrient-rich agro-industrial residues as substrates. With the increasing interest in SSF nowadays, researchers are keen to discover as many new ways to explore

the usage of this technology as possible to develop new added-value materials from by-products. The technology know-how, protocol and manufacturing process will be obtained and optimised. The use of renewable resources will make an essential contribution towards sustainable development, while at the same time, the generation of pollutants or harmful wastes during product manufacture can be minimised.

This work presents the SSF process of 'waste' solids using *Aspergillus awamori* and *Aspergillus oryzae* for the production of an enzymatic consortium containing glucoamylase, protease, cellulase and xylanase. Prior to starting the work, this study showed the importance of considering the inoculum transfer technique in assessing the productivity of any culture. A proper transfer technique was developed to achieve favourable distribution and homogeneity of inoculum within the fermented solid substrate and this is discussed in detail in Chapter 4.

The growth of *A. awamori* and *A. oryzae*, two related species of fungi, in an aerated tray solid state bioreactor (SSB) of moist solid substrate is studied. The study deals with both the physical and biological aspects of the system, which may vary from process to process. Because the microorganisms mostly used for SSF are fungal species, the main focus of the discussion is also based on fungal species and this is discussed in Chapter 6.

Because of the difficulties associated with the direct estimation of biomass in SSF, changing colour of the substrate during the growth of fungi was used as an indirect method and its potential for use in estimating (quantitatively) fungal biomass development is investigated and discussed in detail in Chapter 8. Fermented mass was extracted using distilled water to obtain a cell free extract, which can be determined quickly and accurately using UV-Visible spectrophotometer measured absorbance at 300 nm. The results showed a significant correlation between colours produced from fungal SSF and the concentration of spores, weight of fungus cells, organic matter loss, and enzyme activity. We found that the colour density proportionally increased when the studied parameters mentioned above increased. The advantages of this method are that the procedure is simple to carry out, it is non-destructive and no special and expensive reagents are required and the process is very cheap.

The theory of measuring colour changes in SSF by UV-visible spectroscopy demonstrates that the colour method gives some valuable information of higher quality than just obtaining a visual observation or spore count to describe growth of fungal mycelium in SSF. The ideas obtained from this exercise might provide a quick and convenient method for quality control of fungal growth. Prior to that, a critical review and comparison of several known techniques for biomass estimation:

organic matter loss, weight reduction ratio, oxygen (O_2) uptake rate (OUR), carbon dioxide (CO_2) evolution rate (CER) and heat evolved, was carried out and discussed in detail in Chapter 7. In addition, other techniques to measure growth such as spores count and glucosamine measurement were also performed and can be found in Chapters 8, 9 and 10.

The robustness of *A. awamori* and *A. oryzae* as enzyme producers is exploited in fungal fermentation on agricultural solid waste. High level production of extracellular glucoamylase, protease, cellulase and xylanase has been achieved. Three different types of 'waste' solids (wheat bran, soybean hulls and rapeseed meal) have been used in studies of SSF. Optimisation of the fermentation conditions for enhanced enzymes production involves optimising moisture content and O_2 concentration. The enzymes could be produced in significant levels by controlling the moisture content and continuously supplying O_2 through the tray system known as "closed" and "opened" tray systems. Supplying O_2 itself without controlling the moisture content does not give a good condition to enhance the growth of microorganisms in the SSF process. This combination acts as an inner support, and optimising medium porosity enhances gas transfer under the appropriate water content; these aspects are presented in Chapter 9.

Four new tray SSB systems were fabricated and tested in Chapter 10. The overall goal of Chapter 10 is to have systems that reliably and reproducibly form, accumulate and deliver functional and improved fungal growth. *A. awamori* and *A. oryzae* grow in a wide spectrum of physical and chemical environments; their growth and other physiological activities are in fact a response to their physico-chemical environment. In this work, the growth performance and ability of *A. awamori* and *A. oryzae* to grow in four tray SSB systems was explored and described in detail. It involved the study of the influence of the initial moisture content and air arrangement (dry and moistened air) on fungal growth and spores/enzyme production. In general, designing a solid state tray bioreactor was focused on three main areas: (1) moisture content control, (2) O_2 transfer and (3) maximising heat removal and monitoring temperature.

In aerated tray SSBs, blowing and sparging air through the fermented solid substrate by various air arrangements removed metabolic heat that is released from microbial activity. The effectiveness of the aeration is the result of both the heat uptake capacity of the air itself and of the evaporation of moisture into the air. A side effect of the air arrangement (plus evaporation) is that the decreasing moisture level in the substrate can become a limiting factor to fungal growth. Evolution of heat during fermentation, however, is unwanted and needs to be removed from the system. Thus, one of the main objectives of setting up air arrangements in the bioreactor system is efficient heat removal.

Awareness about the effect of moisture content on the fermenting substrate was the beginning of a study on the response of fungi to changing moisture content (after 72 h of fermentation). Various air arrangements were designed into the system that allowed the dynamic response of the fungus to decreasing water content to be measured. The experimental set-up was based on initial moisture content and air arrangement by sparging dry or moistened air. For such a series of experiments, a good comparison of the effect of air arrangement on fungal growth behaviour was possible.

During the experiments in the tray SSB involving *A. awamori,* a shrinkage of the fermented bed occurred because the fungus tied the substrate particles together due to the loss of moisture content. This was observed when dry air was used. However, the loss of moisture content was still at optimum level when using moistened air except with experiments using dry air in single rectangular tray SSB where greater loss was observed and led to poor fungal growth for both fungi. *A. oryzae* culture did not have critical problems of moisture loss as observed with *A. awamori* culture in the other systems with the same fermentation conditions. The ability of *A. oryzae* to retain water in the cells was much greater compared to *A. awamori*. Contrary to expectations, the culture system with *A. awamori*, that experienced moisture loss, showed an increased fungal growth. *A. awamori* was found to be excellent in the production of glucoamylase, xylanase and cellulase. In addition to that, the system was proven to be very suitable for spores production especially in multi-stacked circular and multi-square tray SSB systems.

During this study, it was observed that the use of *A. awamori* was favourable to growth and producing spores but less favourable to mycelial formation; whereas, *A. oryzae* produced less spores and was favourable to formation of mycelial material during SSF. The higher mycelial formation led to high concentration of glucosamine in all samples isolated from *A. oryzae* cultures. This is might be the reason for *A. oryzae* being able to retain water greater than *A. awamori* through mycelial material. *A. oryzae* culture was observed to be very excellent in protease production in all experiments using the tray systems but very poor in glucoamylase, xylanase and cellulase production.

For a successful series of experiments, we need to obtain 100% saturated air so that it can supply adequate water to the culture system. However, although not saturated, moistened air created in these studies was still able to supply some amount of water to the culture system.

Monitoring the microbial activity, including monitoring O_2 consumption and carbon dioxide and heat evolved during SSF, can directly reveal the activity of the fungal culture in SSF. Microbial activity is the most important parameter in the operation of the bioreactor and it would be of great value to

monitor. In the operation of SSF process, it is important to be able to monitor these parameters. Thus, all four new tray SSBs are equipped with instrumentation (a O_2/CO_2 gas analyser and a thermocouple) to continuously monitor O_2 consumption and CO_2 and heat evolved, which can directly be used to monitor the fungal biomass.

Detailed kinetic studies are also described in Chapter 10. The integrated Gompertz model was used to describe the accumulated evolution of CO_2. The results from the models strongly suggest that the evolved and accumulated CO_2 can be used to describe fungal growth excellently. Another important parameter that can be determined by the gas balance method is the respiratory quotient (RQ). This is the ratio of the CO_2 evolution rate (CER) to the O_2 uptake rate (OUR). The use of CER and OUR confirms that correlated measurements of microbial activity are available, and the determination of RQ may propose an explanation for differences from expected levels.

At the same time, analysis of temperature profile during fermentation may also be used to obtain important knowledge about microbial activity and was also covered in detail in Chapter 10. The assessment was done by correlating respiration profile (CER) differences between the dry and moistened air from the four tray SSBs with temperature changes during SSF. It turned out that considerable differences between the dry and moistened air can exist with different systems, which can indicate the importance of using the type of fungi and fermentation conditions that can be compared to optimise the fermentation process. It was decided that using a temperature rise for the description of heat evolution would incorporate CER in the overall aerated tray SSB. The heat evolved, in further studies presented, was therefore in linear correlation with CER and both profiles could be used as the most suitable technique to estimate fungal growth.

The bioreactor developed in this study is optimised for spores and enzyme production technology based on SSF as a low-cost, low-energy option for the production of the above-mentioned specialty enzymes. In principle, all the bioreactors used in this chapter were capable and showed potential to provide the suitable physical stimulations to both fungi for monitoring growth, controlling water content, continuous supply of O_2, continuous removal of CO_2 and heat, and better temperature control. The best strategies are by supplying moistened air to control the moisture content and at the same time the concentration of O_2 must be high.

11.2 MAIN CONCLUSIONS

A significant number of interesting results were obtained from this study, and the main conclusions can be summarised as follows:

❖ The available knowledge on bioreactor design aspects for SSF processes shows that not enough details are available and mostly these are verifiable by observation or experience and experiment. The satisfying point is that there is continuous increase on availability of information on mechanisation and automation of SSF bioreactors. It is hoped that the enormous efforts being made all over the world will certainly lead to the development of suitable bioreactor systems for the SSF process.

❖ Physical studies:

▪ In addition to nutritional composition of solid substrate used for SSF medium, physical properties of solid substrate also represent another important idea to be focused on during SSF. Physical properties measured in this study include bulk density, particle density, porosity, tortuosity, water retention value, which will influence the behaviour and the productivity of microorganisms involved for substrate utilisation and product formation.

❖ Estimating growth in SSF:

▪ Organic matter loss and dry weight reduction were successfully proven for biomass estimation. However, these techniques were only satisfactory in laboratory study and did not permit estimation in large scale SSF.

▪ The online measurement of capacitance using the Biomass monitor technique seemed very promising but might not be approachable for SSF especially in large scale. However, it works excellently and should be explored further for fungal biomass estimation in large scale SmF involving solid substrate particles. The measurement procedures can be done on-line without involving tedious sampling processes, although they are more expensive. It was regretted that due to lack of complete equipment, we were unable to utilise this

technique in the current study. If properly calibrated and compensated, these devices may be useful to measure the capacitance in SSF and SmF.

■ Metabolic respiratory (OUR, CER) and temperature profiles during SSF are linearly related with biomass synthesis in an aerobic fermentation. They are considered to be the most accurate for determination of growth of the fungus in SSF. The technique is also suitable for large scale SSF due to its advantages such as overcoming the sampling process, avoiding damage of mycelium and the on-line and fast delivery nature of the technique.

■ OUR, CER and heat evolved are the most meaningful techniques to estimate biomass in SSF. The data obtained from OUR, CER and heat evolved allows the estimation of the kinetics of fungal growth. Supervision is a higher level automation, in which measurements are processed in real time to generate more useful information. The devices are expensive but very accurate.

❖ Colourimetric technique:

■ The results showed that colour changes during SSF would be a promising technique with which growth in SSF is indirectly estimated. The ideas obtained from this exercise might give a quick, convenient method for the quality control of fungal growth. The procedures for colour extraction are simple, easy, fast and cheap by using water only.

■ Through the colourimetric technique, the potential to extract large amounts of information regarding fungal growth from a single multi-wavelength measurement makes UV-visible spectrophotometry a powerful characterisation tool. Multi-wavelength UV-visible spectrophotometry provides the added benefits of being versatile, rapid, quantitative, cheap and relatively simple to operate. In addition, it is a reliable analytical tool that has immediate applications as a biosensor for the detection, identification and enumeration of microorganisms and cells.

❖ Simple tray SSF studies:

■ Studies on simple tray SSF using "closed" and "opened" systems reviewed various results on production of enzymes using wheat bran, soybean hulls and rapeseed meal. Every fungus with a different solid substrate reacts with a degree of metabolic activity. Glucoamylase, xylanase and cellulase were greatly produced with *A. awamori* culture,

while *A. oryzae* was excellent in protease production. Overall, *A. awamori* and *A. oryzae* are very versatile in terms of substrate utilisation for enzymes production.

❖ Bioreactor studies:

■ Four new tray SSB systems were designed in this study known as:

   1.   Single circular tray SSB

   2.   Multi-stacked circular tray SSB

   3.   Single rectangular tray SSB

   4.   Multi-square tray SSB

■ The strategy with different air arrangements using either dry or moistened air was able to supply an amount of water and promote effective O_2 and mass transfer to fermenting solid in the bioreactor. However, the effect on the final moisture content was very different for the two fungi. It was found that the ability of *A. oryzae* to retain moisture was much higher (about 5 times higher) than that of *A. awamori.* Another reason is possibly due to the higher concentration of glucosamine (monomer component of chitin) in fungal mycelium of *A. oryzae* with levels between 7 – 16.83% compared to *A. awamori*.

■ The kinetic behaviour of the fungal culture, using raw CO_2, which represents an accumulation term, was integrated with respect to time and fitted to a Gompertz model, a log-like equation. The model can be used to generate parameter values that may be used to verify the experimental data, and also to simulate and optimise the process. The data for maximum CO_2 concentration ($[CO_{2_{max}}]$) accumulated from 38 experiments were generally highly correlated with spores and enzymes (glucoamylase, protease, xylanase and cellulase) production.

■ The study showed that the rate of O_2 consumption and CO_2 evolution were generally highly correlated. Respiratory quotient (RQ) values < 1 were recorded during SSF for both fungi from 38 experiments. Different types of fungi, SSB systems and fermentation conditions showed varying RQ values during metabolic activity and indicated variation in fungal growth. Linear relationship between $[CO_{2_{max}}]$ and RQ was observed, which can be explained by varying fungal metabolism in fermentation conditions.

■ The strategy presented in this study allowed quantitative evaluation of the effect of the forced internal moist air circulation on the removal of metabolic heat. With the proposed strategy, it was possible to maintain the bed temperatures at the optimum value for

growth. Aeration arrangements with moistened air gave positive effect on temperature controlling during SSF. Overall, it was possible to maintain the temperature below 30 °C, and under that condition, productivity of fungal growth was increased. Strong correlation was observed between CER and heat evolution from each experiment. When temperature increases, CER also increases.

- The results reported in this thesis support the validity of the SSF process proposed in this project for the production of enzymes from wheat bran using solid state tray bioreactors. All the objectives have been met, and detailed knowledge about SSF has been obtained. The proposed bioreactor systems present some important findings especially for utilisation of solid wastes through SSF. The newly developed tray SSBs generate useful information about fermentation conditions and the physiological state of fungi in the stages of research and bioprocess development. At the same time, this provides a partial solution to various added-value (namely spores and enzymes in this thesis) production and proposes ways for potential income from a wastes resources.

11.3 SUGGESTIONS FOR FUTURE WORK

Although this research study has generated some major and important findings and advanced knowledge in development of SSF, there are some aspects of the research that might be useful to be identified and investigated in future studies. Full exploitation of SSF in bioprocess technology awaits further investigation to overcome the various limitations and hurdles. Suggestions for future work, which might be required for the improvement of the process, are as follows:

- Even though many articles were published on SSF research work, it is about time a global group of eminent academics and industry experts produced a book or manuscript to pave the way for new innovations in the exploitation of SSF for modern biotechnology. The book must address the whole spectrum of SSF from microbial fermentation to bioprocess engineering including fermentation kinetics and control of fermentation. The mathematical modelling and equations must be specifically designed to illustrate the applications of central concepts and current state of the art technologies. In this growing multidisciplinary field of biotechnology nowadays, there is a need for a clear, concise and handy reference book or manuscript from which students, scientists and engineers can benefit, thus ensuring a good balance between theory and practice of SSF. For this reason, the book should be considered as essential reading for researchers and

students concerned with the science and technology of SSF. So far, the current books that we have access to be a compilation of knowledge and experience from various sources.

- The development and establishment of standard procedures for inoculum transfer into solid substrate for homogenous distribution prior to the SSF process. Small reactors equipped with mixing devices or machinery to produce efficient homogenisation. This factor might determine the success of the SSF process.

- A higher number of experiments and improvements on the colour changes technique in SSF using various microorganisms and solid substrates are needed to validate the technique. Further studies should be carried out to obtain more data to support the colourimetric technique using different fungi and bacteria, and also using different types of solid substrate whether in single form or mixed-solid substrate from different solid wastes.

- In addition to SSF, the technique based on colour changes for biomass estimation should also be extensively studied in SmF using filamentous fungi with solid substrate particles.

- For bench scale bioreactors, it would be very desirable to produce standard modifications of tray solid state bioreactors with proper arrangements for aeration, cooling mechanism, aseptic conditions with sampling, charging and discharging parts. These proper controls will allow the study and characterisation of SSF as is already the case for SmF. There is a need to ensure that fermentation conditions are constant, and for example, gas and heat analysis have become very important knowledge. Process descriptors such as the RQ are very important since this parameter reveals knowledge about microbial activity. In addition, knowledge of CER and OUR and heat evolved provides a measure of production in the SSF process. This will overcome the problems in fungal biomass estimation, which are the main disadvantageous aspects in SSF.

- From this study, multi-stacked circular and multi-square tray SSB systems proved to be excellent for the production of spores and enzymes. The aeration strategies and arrangements applied in these studies exceeded the optimum requirements and allowed accurate measurements of gas composition, temperature, moisture content and fungal biomass gradients. Both systems may provide an excellent system for further investigations of mass transfer and possibly for large scale operation.

- With the present advanced technology, bioreactors should be designed with an on-line facility for monitoring of several parameters as well as studies that consider transport phenomena at micro-scale levels, primarily including heat and mass transfer in a system characterised by a gas-solid interface.

- In these studies, enzymes production was selected as the test parameter; thousands of other added-value products could be produced from the solid waste. SSF has proved to be a great success in the production of biologically active secondary metabolites. In optimised SSF, these products are produced in high concentrations. Therefore, it would be beneficial to explore other profitable products in future studies.

- It is about time now for research to be carried out in biosensor technology to design special electrodes for on-line pH measurement in solid substrate with the presence of little amount of water. Biosensors are compact and highly specific analytical instruments that comprise biological sensing components such as tissues, cells, enzymes or antibodies. To the best our knowledge, this type of instruments has not be evaluated in SSF so far.

- Recycling fermented substrate after fermentation for sequel fermentation can also be further investigated. This is because not all the solid substrate can be utilised during fermentation. After the extraction process to obtain the nutrient rich hydrolysate, fermented substrate should not be discarded and it is suggested to be used for a second SSF. At this time, the complex structures of solid substrate have changed much and together with the nutrient enriched fungus protein body, it can be easily consumed with a new and fresh inoculum.

- Another important challenge to be considered for further study is downstream processing. Separation of products from the solids is a technically and economically challenging process. Considerable research needs to be done in recovering the products from the fermented substrate with a minimum number of steps.

- Future biorefinery studies can be carried out to use one of the possible tray SSBs produced in this study for further research for the production of nutrient rich hydrolysate for sequel fermentation for the production of value chemicals. The productivity of the systems can be enhanced by modification and adding several control parameters for better control of SSF processes.

- SSF proved to be excellent technology for solid wastes treatment. It can be suggested that utilisation of food and agro-industry solid waste can be carried out for the production of various added-value products. Industrial bodies should consider this scenario as part of their strategy for tackling the food and agro-industry waste problem and for the environmentally friendly production of enzymes, secondary metabolites, chemicals and even bio-fuels. Therefore, this can overcome the issues of the solids wastes being thrown into the land, which can create another problem for the environment.

Advances in the understanding of microbiology and of the composition of targeted products and their raw materials (biomass), as well as the development of advanced SSF bioreactor, allow more consistent research and development on SSF. Thus, we arrive at the modern day bioprocessing and microbial fermentation processes. Despite many limitations, many industrial facilities worldwide successfully operate SSF processes, although some of them produce relatively small quantities of high added-value products that do not require large scale bioreactors. In other cases, the bioreactors do not operate optimally, or bioreactors that are not easily adaptable for different processes are used. We believe that in the near future flexible and optimum performance large scale SSF bioreactors will be designed, built and operated successfully, although more engineering research is needed. These bioreactors will use low-cost and reliable instruments, especially designed for SSF, and sophisticated control strategies that will include advanced control techniques such as expert and model based controlled systems. The ability of microorganisms, especially filamentous fungi, to convert biomass through SSF bioconversion will have a great impact on food and agro-industry in every aspect of life from food and medicine to fuel. This is a future that undoubtedly will present challenges, but one that I believe we should embrace.

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APPENDICES

The raw data and calculation can be found electronically in the CD appendix attached at the end of the thesis. The content of the CD is described below:

APPENDIX A: Raw data and calculations from Chapter 6

APPENDIX A: Raw data and calculations from Chapter 7

APPENDIX A: Raw data and calculations from Chapter 8

APPENDIX A: Raw data and calculations from Chapter 9

APPENDIX A: Raw data and calculations from Chapter 10