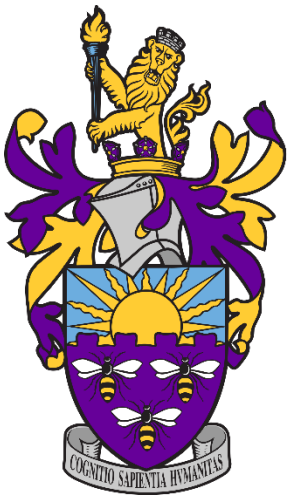
Towards a Fundamental Understanding of Solid State Bioprocessing



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Abstract

Solid state fermentation and solid state bioprocessing have been recognised for their unique features. However, there are several unsolved challenges which have prevented their widespread adoptions. In order to address these challenges, an investigation has been conducted, aiming at both identifying and addressing the problems. Two common applications of solid state bioprocessing, namely reducing toxins in raw materials and producing generic microbial feedstocks, were studied and are reported in Part A of the thesis (Chapter 3 and Chapter 4).

The findings reported in Chapter 3 show that by applying a two stage solid state bioprocess, the major toxin in rapeseed meal can be reduced to a level suitable for animal consumption. The first stage, pre-incubation, greatly assisted toxin reduction in the second stage (fermentation), through the addition of water into the substrate. Although the exact mechanisms were not elucidated, it was clear that water is a critical factor in the process. In Chapter 4, a three stage solid state bioprocess was used to produce a generic microbial feedstock from mixed rapeseed meal and sugarcane bagasse. The main findings from this study were that such processes are highly variable, and that the performance of solid state fermentation is highly influenced by the microscopic environment of the microorganism. This led to the conclusion that a more fundamental understanding of the system is required.

To obtain a better fundamental understanding, the effect of water on fungi cultivated on solid substrates (water bioavailability), was investigated in Part B of the thesis which includes theoretical and experimental investigations. The following theory has been developed from the findings.

“Fungal growth can only consume water from the surrounding local micro-environment. As germination and growth occur, they result in a modest depletion of water from the immediate vicinity. This creates a driving force for water to migrate from the bulk substrate (usually through diffusion), or to be absorbed from the gas phase, to replenish the water that has been consumed. The driving force within the substrate increases with fungal water consumption, while water absorption from the gas phase is driven both by the gas phase relative humidity and presence/absence of water at the substrate surface. Meanwhile, the resistance to mass transfer is greatly affected by both chemical and physical properties of the substrate. Fungal growth can continue, if, and only if water within their immediate vicinity can be replenished.”

Experiments showed that water bioavailability cannot be fully represented by terms such as “water content” and “water activity”, but is affected by the presence of water in the immediate vicinity of the fungus. This is under the influence of substrate chemical properties and consequently affects germination and early growth. It is also affected by mobility of water through the substrate which impacts on growth rate in the absence of gas phase water. Further, if there is water in the gas phase, it can fully compensate reduced water availability in the substrate, and even support growth with no substrate water.

Experiments also showed that solid state fermentation may have distinct advantages compared to submerged fermentation. The physical structure of solid substrates allows oxygen and fungal hyphae to penetrate by providing surfaces for oxygen transfer, without forced aeration and without disturbing the natural growth pattern of the fungus. Water can be variously supplied either from the substrate or from the gas phase, and therefore, compared to submerged fermentation, gives greater process flexibility, which may enable water to be used more effectively and the needs for downstream extraction to be reduced.

The project presented in this thesis is only the starting point for understanding the effects of bioavailability of water in solid substrates. Hopefully, it can be a pioneering work for gaining a better fundamental understanding of solid state bioprocessing systems, and by gaining this better understanding, can bring a new era to this ancient technology.

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Acknowledgement

This thesis is dedicated to

My parents, Li Lihua and Cao Zhigang, for their unreserved support and love.

谨以此文献给我的父母，李丽华和曹志刚，以感谢他们毫无保留的支持和关爱

And to

Professor Colin Webb

For his wisdom, guidance, and spoiling me like a child. For the wonderful time we had, the fantastic journey we went through. For the philosophical world we had explored in C77, and for the fact he had been always right.

And also to myself

May you always believe in yourself, and remember the persistence, the courage, and the victory of self-excelling.

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Chapter 1.

Introduction

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Chapter 1 Introduction

Back in ancient times, ever since their first appearance, humans have been processing biological materials, and the emergence of agriculture had marked the beginning of civilisation. Not long after they had learned how to grow their very first crops, they soon learned how to use some of these crops to make their very first wine [1]. Hence, fermentation has been an integral part of our lives for millions of years. It was originally used to produce food and beverages, the earliest evidence of fermentation can be traced back to 9000 years ago, on the banks of Yellow River in China. Even without having any scientific evidence, people in the past still learned the benefits of alcoholic drinks, for their antiseptic and antioxidant properties that prolonged life and increased productivity. In fact, in all ancient civilisations around the globe, similar techniques had been developed to make alcohols from sugary materials [2]. Soon after the discovery of producing alcohol, fermentation technology was extended to a much wider range of applications: mead, bread, cheese, mouldy tofu etc. Figure 1-1 shows a 3000 years old Chinese wine vessel.

Figure 1-1 A ritual wine vessel discovered in Henan province, China, approx. 1300-1050 BCE. [3]

Even without being fully understood, the technology of fermentation had been passed from generation to generation for thousands of years. The mechanisms of fermentation only became clear in the 19th century, when Louis Pasteur was able to demonstrate that fermentation is in fact a microbial metabolic process occurring in the absence of air, where living yeasts convert sugar into alcohols [4]. Along with alcohol, Pasteur also found that there were significant amounts of other metabolites, such as glycerine, succinic acid, and amyl alcohols, being produced during the fermentation. Ever since then, fermentation has attracted a lot of attention, for exploring its applications beyond traditional food production.

Before the First World War, the only chemical that could be fermented and produced at a large scale was alcohol. Industrial fermentation, for non-food applications, started exactly 100 years ago, at the very place where this thesis was written, the University of Manchester. Chaim Weizmann perfected the acetone–butanol–ethanol (ABE) fermentation as the first example of large scale

industrial chemical production using fermentation. Shortly after the war, driven by the need for post-war reconstruction, aseptic and aerobic fermentation processes were also developed to produce organic acids, e.g. citric acid. Using a submerged culture adapted from the citrus fruit industry, citric acid was successfully produced at an industrial scale. Soon after, this aseptic and aerobic process was further adapted to produce penicillin, which had saved millions of lives during the Second World War. Curiously, both citric acid and penicillin production began with solid substrates, it was only later that industrial processes favoured submerged fermentation. The large scaled production of penicillin established the foundation of modern industrial fermentation. Ever since, fermentation has been widely used for the production of chemicals, such as antibiotics, vitamins, amino acids, gibberellins, and enzymes [5].

In the 1980s, the rise of biotechnology brought the latest breakthrough to fermentation technology. Genetic engineering enables the expression of, amongst others, human and mammalian genes in microorganisms and thereby fermentation can facilitate a large scale production of human proteins, e.g. insulin. Nowadays, fermentation, this ancient technology that arose thousands of years ago, has become a vital element in our modern industry [6]. Table 1-1 is a chronology showing key fermentation development.

Table 1-1 chronology of key fermentation development, adapted from [5]and [6]

	First appearance in time	Fermentation product	Place
Ancient times (BC)	7000	Wine	China
	5400	Wine	Persia
	3400	Beer	Egypt
	3000	Sura	India
	2700	Wine	Babylon
	2000	Mead and wine	Greece and Rome
Modern times (AD)	1700	Vinegar	
	1850	Lactic acid	France
	1930	Organic acids	Italy
	1940	Penicillin	United Kingdom
	1960	Amino acids	Japan
	1970	Enzymes	World-wide
	1980	Insulin	United Kingdom

1.1 Solid State Bioprocessing and Fermentation

Ever since penicillin was first successfully produced from a submerged culture, industrial fermentation substrates have generally been in a liquid form. In fact, the term ‘fermentation technology’ is, to many, synonymous with the “use of submerged liquid culture of selected strains of microorganisms, plant or animal cells, for the manufacture of some useful product or products, or to gain insights into the physiology of these cell types” [7]. However, just as microorganisms can

thrive in water abundant environments and also under desiccated conditions, fermentation can take place in both submerged and solid culture.

The first scientifically reported fermentation carried out on a solid substrate was in 1935, for citric acid production. However, it was quickly overtaken by the success of submerged fermentation for penicillin production and didn't attract any scientific interest for the next 40 years. In the 1970s, inspired by traditional Japanese food fermentations, Hesseltine [8] described a process producing microbial metabolites using moist solid substrate. In this process, not only were the metabolites produced successfully, but compared to submerged fermentation, they were also produced at a much higher yield, and at a cheaper price. Hesseltine defined this process as "solid state fermentation". Clearly, it is obvious that such 'solid state fermentation' requires much less water than the equivalent submerged fermentation. It therefore, not only requires less space, but the products will be much more concentrated than when produced in submerged culture. In addition, solid state fermentation requires much less substrate preparation. Substrates used for submerged fermentation must be soluble in water, and therefore, almost none of them are in their naturally occurring form. As a consequence, the preparation of the substrate becomes such a heavy process that it is normally separated from the fermentation. To produce or buy those refined substrates, the cost can be very considerable for an industrial scale production. In comparison, substrates used for solid state fermentation require much simpler or even no treatment, mimicking the natural environment.

With more understanding of the process, nowadays, it is more common to process the raw materials through simple pre-treatments to create more favourable conditions for microbial growth. This simple upstream processing is normally integrated into the overall production process. It is also true that, compared to submerged culture, products of solid state fermentation, whether the biomass or

metabolites, cannot be easily separated. Therefore, it is also common to include a downstream section in the process.

As with submerged bioprocesses, because of the inclusion of upstream and downstream processing alongside solid state fermentation, it is important to distinguish the process as a whole, from the fermentation alone. Therefore, the term ‘solid state bioprocessing’ would seem to be more appropriate to describe a process integrating solid state fermentation with upstream and/or downstream processing. This terminology has been adopted throughout this thesis except where referring only to the microbial growth and production on a solid substrate, for which the term “solid state fermentation” has been used.

By definition, [9] “Solid-state fermentation (SSF) is defined as the fermentation involving solids in absence (or near absence) of free water; however, substrate must possess enough moisture to support growth and metabolism of micro-organism”. ‘Solid state bioprocessing’ on the other hand, refers to a series of operations divided into three stages, namely upstream, fermentation, and downstream (Figure 1-2). The solid state fermentation is central to the solid state bioprocess, which can therefore be defined as “the preparation and utilisation of solid substrates, for the growth of microorganisms, in the absence or near absence of free water, and the extraction and purification of desired products”.

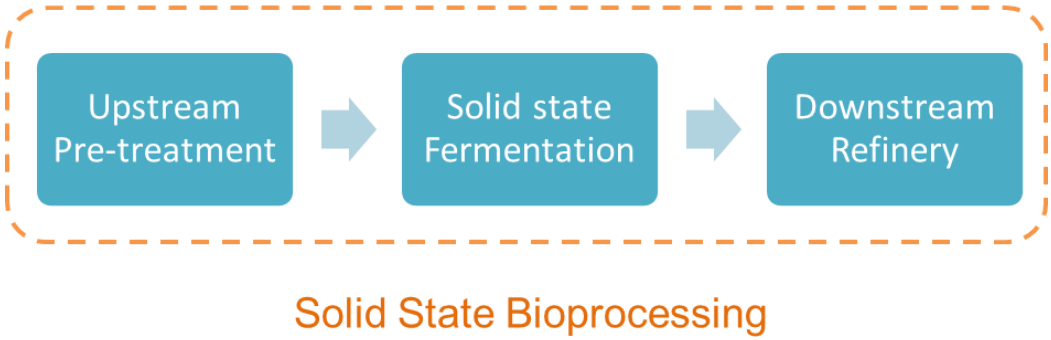

Figure 1-2 A typical 'solid state bioprocess' consisting of upstream, fermentation and downstream stages

1.2 Background to Solid State Fermentation

The key step in solid state bioprocessing is solid state fermentation. In this section, existing knowledge published within the domain of solid state fermentation is introduced. Solid state fermentation is first interpreted microscopically, and then factors affecting its performance are reviewed. Following that commonly used raw materials are discussed and summarised.

1.1.1 Microscopic Interpretation of Solid State Fermentation

From a microscopic point of view, there are three phases involved in the solid state fermentation: solid, liquid and gas [10]. Among them, the solid phase, which is essentially the solid substrate, takes the biggest part. It acts as the physical support to the microorganisms and in most cases, it also acts as the principal nutrient supply. The liquid phase is believed to be the place where all microbial activity takes place [10]. The gas phase has three functions in solid state fermentation. For aerobic systems, it carries oxygen, carbon dioxide and water. It also transports spores throughout the fermenter. Thirdly, due to the poor heat

conductivity of the solid phase and the very limited amount of liquid, most metabolic heat generated during fermentation is removed by the gas phase. As a consequence, fermentation temperature and consequently fermentation performance is in fact heavily affected by gas ventilation.

During solid state fermentation, the three phases do not exist alone, but have significant impacts on each other. For example, on one hand, the chemical composition, the availability and mobility of the liquid phase are affected by the physical and chemical properties of the solid phase. On the other hand, the solid phase is also affected by the liquid phase. For example, the solid phase can be hydrolysed by enzymes carried in the liquid phase and consequently change its physical structure [10]. In terms of the gas phase, it has an impact on the liquid phase by supplying/removing water from it. Similar to the liquid phase, the permeability and mobility of the gas phase is also affected by the physical properties of the solid phase, e.g. porosity.

The three intricately arranged phases delicately act together creating a complex and sensitive environment for microorganisms. It is the main reason why solid state fermentation is so much less controllable, yet offers more means to influence the fermentation performance, than submerged fermentation. With that being understood, factors affecting the performance of solid state fermentation are reviewed and summarised in the following section.

1.1.2 Effect of Microorganisms on Solid State Fermentation

The selection of microorganisms has perhaps the most significant impact on the performance of a solid state fermentation. This is not only because the selection of microorganism decides the final products of the fermentation, but also because the fermentation performance varies with microbial morphology and growth

pattern [11]. For example, some filamentous fungi, such as *Rhizopus oryzae*, can form a thick layer of hyphae and reduce both oxygen and heat transfer between the environment and the substrate. As a result, within the substrate, the depletion of oxygen and accumulation of metabolic heat makes the environment hostile for microbial growth and consequently impairs the performance of the fermentation. Therefore, optimal microorganism selection will depend on the type of solid substrate, growth requirements and targeted final product.

For solid state bioprocessing, both bacteria and fungi, in either a single or mixed culture, have been applied. Of the wide range of possible microorganisms, three filamentous fungi have been used in this project. *Rhizopus oryzae* is an edible fungus used for food production. In the first stage of growth, it can form a thick white layer of hyphae, which looks like a sheet of cotton (Figure 1-3). And during sporulation, it forms black spores. It is a lactic acid producer, which is perhaps why it can grow in acidic environments, as low as pH 3.5 [11].

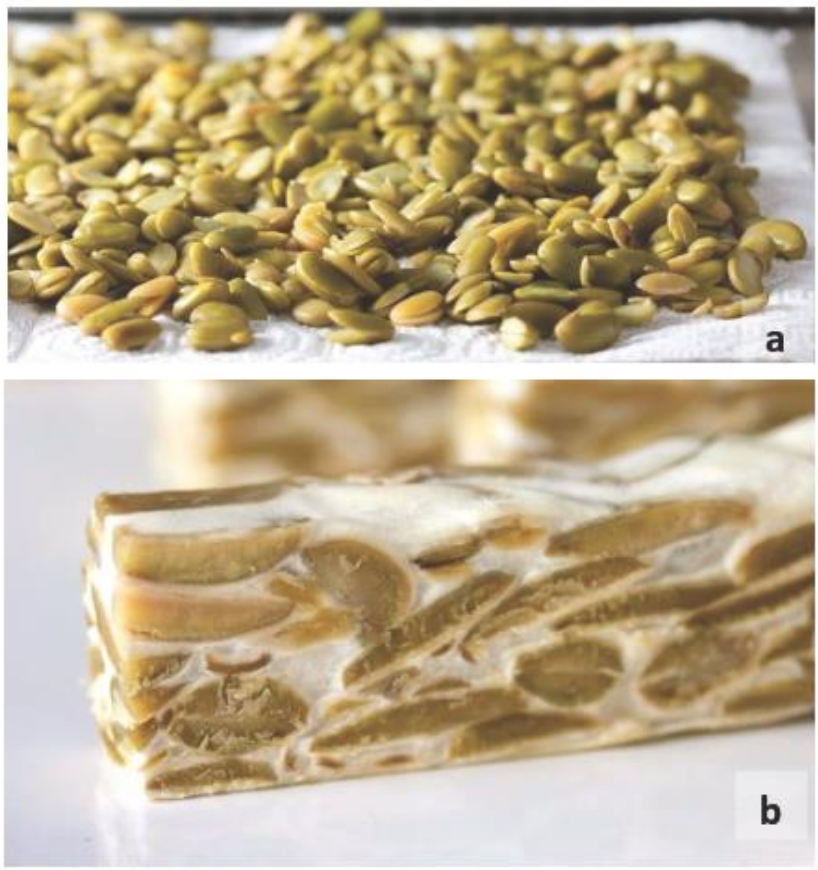

Figure 1-3 Soybeans before (a) and after (b) the growth of *R. oryzae* in the production of Tempeh, showing that the mycelia fills the inter-particle spaces leading to oxygen transfer limitations [11]

Aspergillus oryzae (Figure 1-4) and *Aspergillus awamori* are other two strains of filamentous fungi used in this project. Both can form white hyphae which are shorter and much less dense than those of *R. oryzae*. During the sporulation stage, *A. oryzae* produces green spores while *A. awamori* produces black ones. Like *R. oryzae*, *A. oryzae* is widely used in the food industry. It is also used as an industrial enzyme producer for protease and amylase production [12]. For *A. awamori*, because it can produce mycotoxin, it is not used directly in the food industry but is a source of hydrolytic enzymes, which include amylases, xylanases, pectinases and cellulases [13, 14].

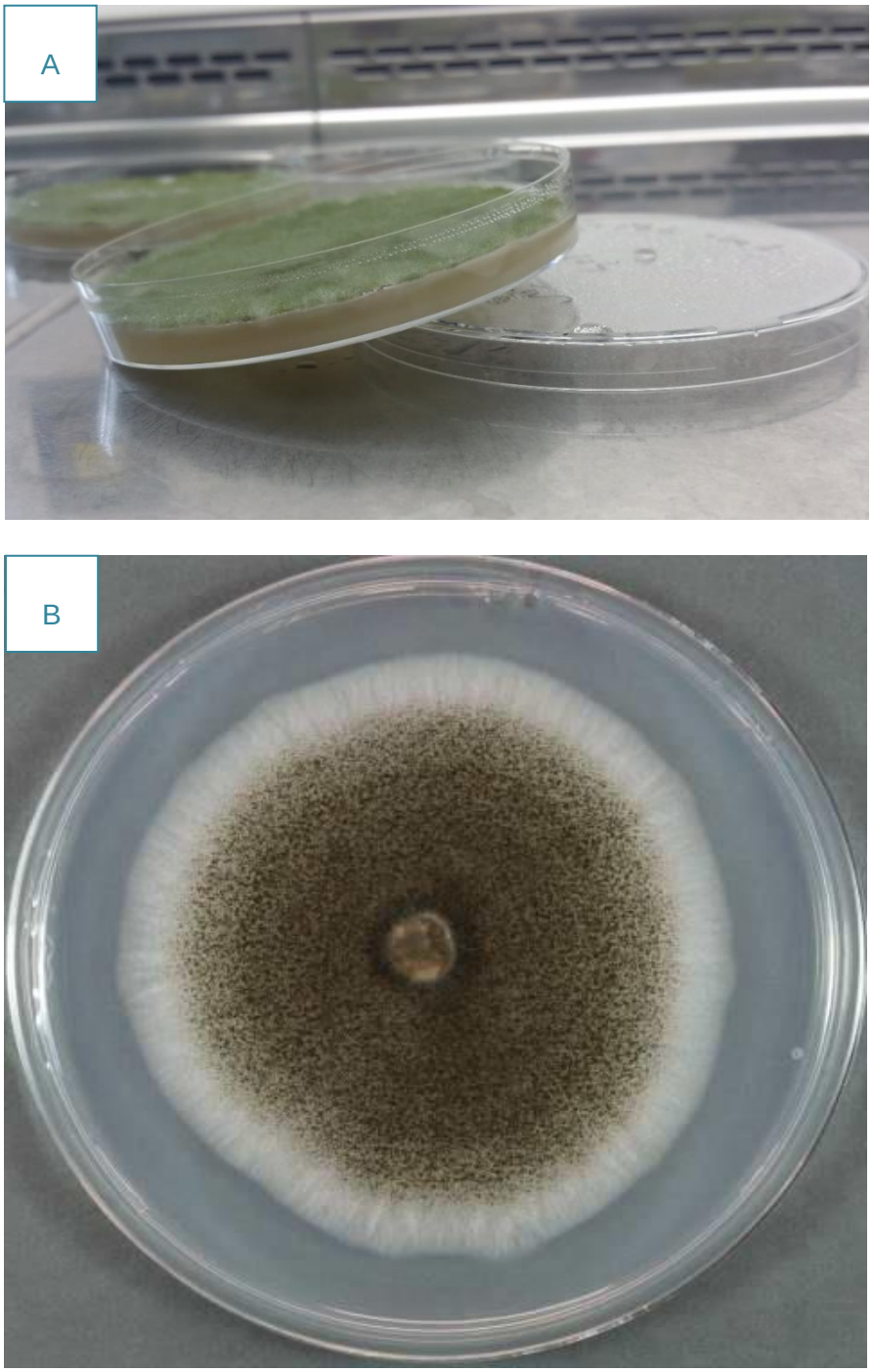

Figure 1-4 Solid substrate cultivated with (a) *A. oryzae* and (b) *A. awamori* [15]

1.1.3 Effect of Substrate Properties on Solid State Fermentation

As mentioned in Section 1.1.1, in a solid state fermentation system, the solid phase affects the performance of the fermentation through its physical properties (e.g. surface area, porosity, ability to remain intact, packing density, bed height,

etc.) and chemical properties (e.g. chemicals and their structures, amount of hydrophilic groups, homogeneity, etc). The liquid phase affects the fermentation through pH, solutes, liquid phase mobility and water availability.

It is commonly believed that among all the physical properties of the substrate, surface area has the most significant impact on the performance of solid state fermentation. On a solid substrate, microorganisms cannot use the inner cores of solid particles. This is because microorganism can only utilise the nutrients dissolved in the liquid phase, and since the hydrolysis of solid substrate only takes place at the surface of the solid substrate, the dissolved nutrients only exist on the surface of the particles. This is also because oxygen and water permeability in the solid phase is very poor, and therefore, the microorganisms cannot penetrate deeply into the solid particles. As a result, in a solid state fermentation systems, microorganisms can only attach on the surface of the solid particles, and therefore, the surface area affects the available area for microorganisms to grow. The total surface area of the substrate is decided by the porosity of the substrate, which in most of the cases, can be controlled by adjusting the particle size.

In addition to affecting the surface area, porosity also affects the availability and mobility of the liquid phase. On one hand, in the fixed substrate volume, reducing pore size and increasing their quantity can increase the total volume of void space, and consequently increase the amount of liquid phase available for the microorganisms. But on the other hand, the mobility of the liquid phase decreases with higher capillary force. Since the nutrients contained in the solid phase must be dissolved into the liquid phase, therefore, the mobility of the liquid phase affects the nutrients and water supply to the microorganism [16]. Similar to liquid phase, the gas phase is also affected by porosity of the solid substrate. Lowering the void space between particles and the connectivity between pores reduces the quantity and the mobility of the gas phase.

While the physical properties affect microbial growth, in many cases, microorganism can also affect the physical structure of the solid substrate [17]. As fermentation proceeds, the porosity of the substrate generally decreases. On one hand, this is because the pores are filled by germinated and extended hyphae, spores or by the reproduced bacterial cells [18]. On the other hand, the substrate is hydrolysed by extracellular enzymes excreted by the microorganisms, and consequently gradually dissolved into the liquid phase, resulting in reduced porosity and eventually, collapse of the physical structure [19].

As discussed above, the consequence of changing physical structure, e.g. reduced porosity, reduces available surface for microorganisms to grow on and makes ventilation more and more difficult [20]. Therefore, as fermentation proceeds, the metabolic heat accumulates but heat removal through the gas phase becomes more and more challenging, especially for those substrates whose physical structures are vulnerable to microorganisms and hydrolytic enzymes. For example, comparing to gel substrate (for which microorganisms cannot hydrolyse the structural biopolymers), starchy materials, such as wheat bran, accumulates more heat under the same fermentation condition, simply because the substrate collapses much more readily [21, 22]. On the bright side, the mass transfer within the substrate improves with the liquidation of the substrate, which means a better nutrients supply to microorganisms.

Similar to submerged fermentation, in a solid state fermentation system, the substrate acts as a nutrient reservoir for microorganisms and therefore, affects the viability of the cells. The essential nutrients that must be supplied by the solid substrates to the microorganisms are carbon, nitrogen, phosphorus and traces of inorganic elements. They are not only used for maintaining cellular activities, such as generating energy and building cellular organic compounds, but also for synthesis of extracellular metabolites (Figure 1-5) [23]. Most of the extracellular

metabolites are excreted in response to substrate conditions around the cells [24]. For example, extracellular enzymes are produced to hydrolyse macromolecular nutrients into small molecules that can be transported through cell membrane, and antibiotics are produced by fungi to protect food supply from bacteria. This means, those extracellular enzymes can only be triggered by environmental cues in the substrates. Therefore, the chemical composition of the substrate has a decisive impact on the microbial production and consequently, the outcome of the fermentation.

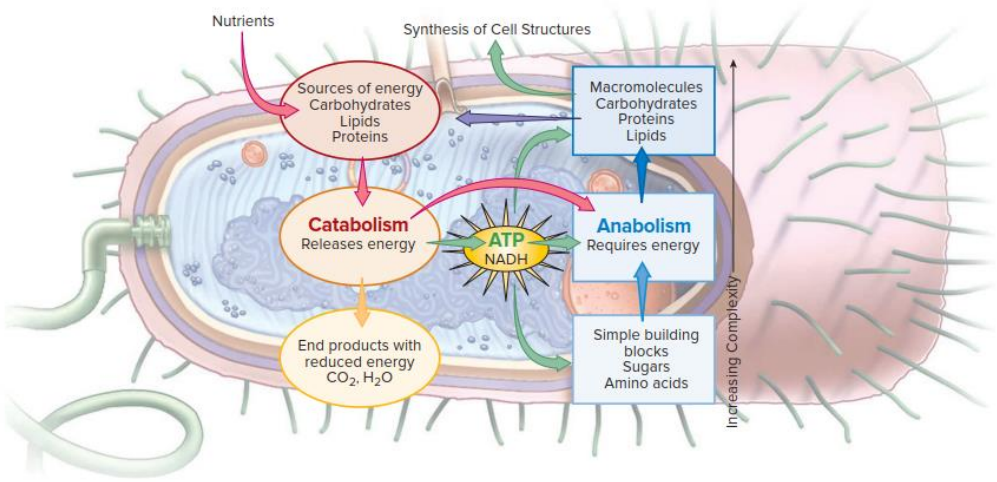

Figure 1-5 A model of cellular metabolisms, showing the conversion of nutrients in the substrate into cellular energy, structures and extracellular metabolites [23]

1.1.4 Common Raw Materials for Solid State Fermentation

Unlike submerged fermentation which requires the nutrients to be dissolved in the liquid phase, perhaps the only requirement for solid substrate is a solid material containing accessible moisture and nutrients for the microorganism. Indeed, in a natural environment, it is commonly seen that microorganisms grow on various solid surfaces, which means, even without any refinement, many natural materials

are suitable for solid state fermentation. This enables solid state fermentation to have a wide range of raw materials: from artificial hydrogels to animal manure, from lignocellulosic materials coming from tropical trees to nutritious industrial wastes. Most raw materials selected for the purpose of solid state fermentation research can be divided into two categories: the well-defined artificial substrate, e.g. hydrogels, or cheap and natural raw materials. Discussed below are three raw materials that are commonly used in solid state fermentation and which were used in the project reported in this thesis: rapeseed meal (Figure 1-6), sugarcane bagasse (Figure 1-14), and potato dextrose agar (PDA, Figure 1-15).

Figure 1-6 Rapeseed meal

1.1.5 Rapeseed Meal

Rapeseed (*Brassica napus*) is the seed of the rapeseed plant. It contains oil up to 40% of its dry weight, and is commonly cultivated as an oilseed. Industrially, rapeseed has been extensively used as a raw material for biodiesel

production [25]. Rapeseed meal (RSM) is the defatted residue of rapeseed after oil extraction and has a very high nutrient value: it contains 40% (w/w) protein which consists of a wide range of amino acids [26]. Rapeseed meal also contains approximately 20% neutral detergent fibre, which is the second largest component of the meal. Second to soybean meal, rapeseed meal is one of the largest protein meal resources, and its price is 40% cheaper than soybean meal. These features give rapeseed meal a great commercial potential particularly as an animal feedstock. However, its current utilisation is limited due to the presence of toxins. The most toxic components of rapeseed meal are glucosinolates and their hydrolysed derivatives. Other anti-nutritional factors include phytic acids and phenolic compounds [27].

Glucosinolates are present in all parts of the plants of the Brassicaceae family, though they are most located in the seeds [28]. Chemically, glucosinolates are alkyl aldoxime-O-sulphate esters (Figure 1-7) [29]. Due to the presence of sulphate and glucoside groups, glucosinolates are hydrophilic, this means, they remain in the meal after oil extraction.

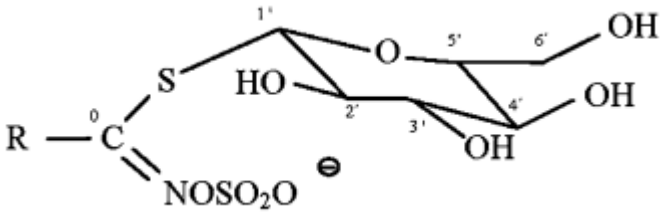

Figure 1-7 General structure of glucosinolates, R represents a side chain [30]

Glucosinolates vary in their side chains and consequently in their structure. In total, there are more than 120 glucosinolates have been identified [31]. In rapeseed meal, there are 3 major glucosinolates: progoitrin (epi-progoitrin), gluconapin and

glucobrassicanapin. Concentrations of the toxins vary with growing conditions and location. Typical values are listed in Table 1-2 for rapeseed meal.

Table 1-2 Major glucosinolates in rapeseed meal from Canada and EU [26]

Glucosinolates	Semi-systematic name	Canadian rapeseed meal (μmol/g)	European rapeseed meal (μmol/g)
Progoitrin	2-OH-3-butenyl-	98. 5	109.4
Gluconapin	3-butenyl-	32.2	33.3
Glucobrassicanapin	4-pentenyl-	8.9	8.2
Other		14.2	5.2
Total		153.8	156.1

Glucosinolates can be hydrolysed enzymically, for example by myrosinase, an enzyme contained in plant cells and some microorganisms. Myrosinase is also known as β –thioglucosidase, or thioglucoside-glycohydrolase (Figure 1-8). In an intact plant cell, myrosinase is stored separately from glucosinolates [28]. When the cell is damaged, for example during oil extraction, the myrosinase will be released, and with the presence of water, it will initiate the hydrolysis of glucosinolates. This will result in the production of isothiocyanates, oxazolidinethiones (5-vinyl-2-oxazolidinethione and 5-vinyl-1,3 oxazolidine-2-thione), thiocyanates, nitriles, epithionitriles and other indol-3-ylmethyl derivatives.

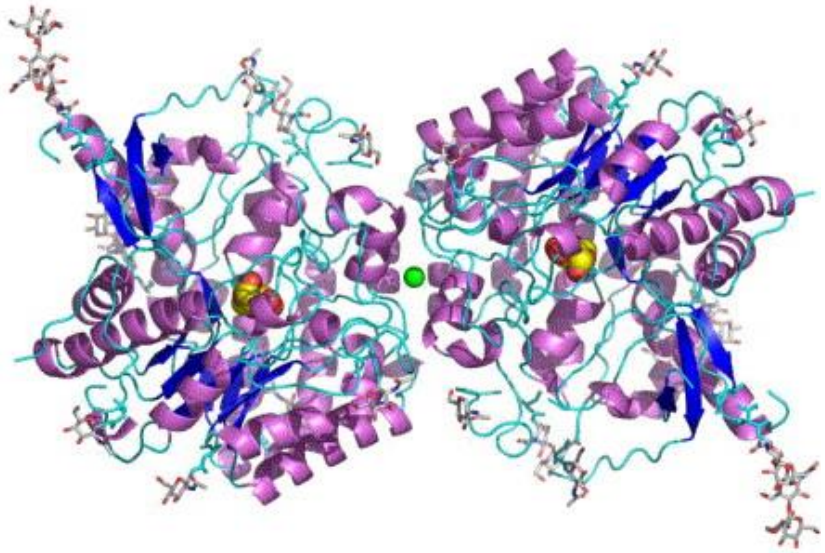

Figure 1-8 Chemical structure of myrosinase in 3D [32]

The actual outcome of the hydrolysis depends on which glucosinolates are present. It also varies with the pH of the microenvironment and whether ferrous ions and epithiospecific proteins (ESP) are present. If ESP is present, as it is in rapeseed meal, the glucosinolates will be predominantly hydrolysed into epithionitriles, whereas isothiocynate would be produced if ESP is absent (Figure 1-9).

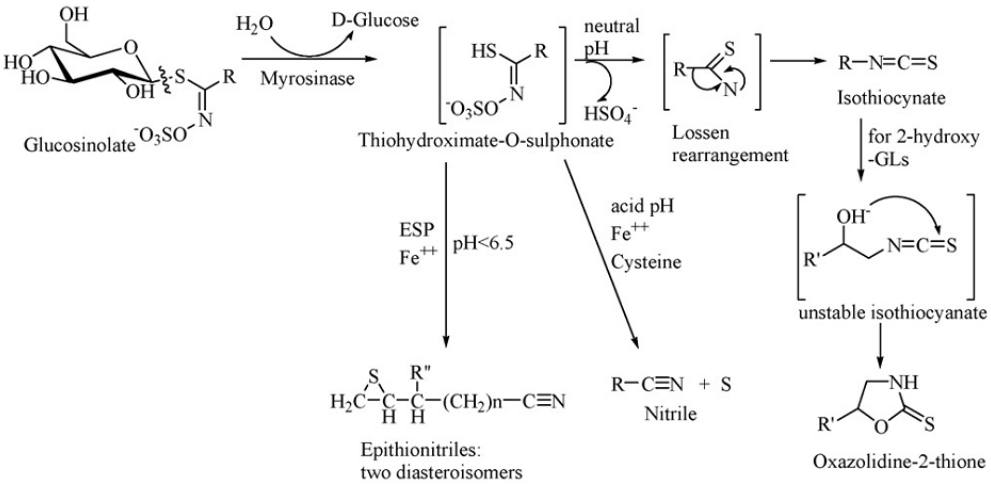

Figure 1-9 Outline of glucosinolate hydrolysis, R represents different side chains [33]

The toxicity of glucosinolates is closely related to the hydrolysis products, particularly thiocyanates, oxazolidinethiones and nitriles. These toxins can cause the following adverse effects in animals [28]:

- Damaged thyroid function and gland enlargement
- Necrosis of the gastrointestinal mucosa
- Reduced feed intake and inhibited growth
- Liver and kidney damage and enzyme leakage
- DNA damage
- Reduced fertility

Thiocyanates and oxazolidinethiones can reduce iodine availability to animals. Inorganic iodine enters animal thyroids through sodium-iodide symporters primarily as iodide. Thyroid peroxidase oxidizes the iodide to atomic iodine or iodinium. Thiocyanates and oxazolidinethione interfere with iodine intake by competing with the sodium-iodide symporter and binding the iodine to thyroglobulin, in other words, trapping iodine in the thyroid. Consequently, thiocyanates and oxazolidinethione damage thyroid function. As a result, animals display various abnormal behaviours which include reduced and/or impaired feed intake, growth, fertility or reproduction [28]. Secondly, nitriles can irritate the gastrointestinal mucosa, which leads to local necroses, hepatotoxicity and nephrotoxicity. Some researchers reported that nitriles are more toxic to animals than other degradation products [29]. In a 0.1% nitrile mixture feeding trials on rats, major lesions of kidneys along with severe growth inhibition were observed after 109 days [34].

Some glucosinolates and their hydrolysis products have also shown evidence of mutagenicity, genotoxicity and carcinogenicity. For example, glucosinolates can cause DNA damage, chromatid exchanges and clastogenic effects in *E. coli* and

mammalian cells [35]. However, certain types of glucosinolates and isothiocyanates have also been reported as having an anti-carcinogenic effect. For example, Phenyl isothiocyanates were reported to inhibit cancers from developing on various sites of rats. Similarly, indole-3-carbinol can prevent the growth of breast, stomach, colon, lung and liver cancers [36].

The individual impact of each glucosinolate and isothiocyanate on animals is not very clear [31]. Different animals respond very differently to glucosinolates, both in the nature of the negative effects and in the glucosinolate tolerance . The effects of various amounts of glucosinolates on different animals are presented in Table 1-3.

Table 1-3 Impacts of glucosinolate intake on different animals (adapted from references [28] and [33])

Animal	Glucosinolate intake (μmol/g diet)	Effect
Pigs	0.16–1.0	No adverse effect
	1.3	Reduced gain during finishing period
	1.3–2.79	Reduced feed intake and growth
	7.0	Severe growth depression
	9–10	Induced liver and thyroid hypertrophy
	10.0	Induced iodine deficiency, hypothyroidism, reduced bone and serum zinc content and alkaline phosphatase activity
Fish	1.4	No adverse effect
	2.2	Reduced growth by 15%
	19.3	Severe growth depression and thyroid disturbance
Poultry	0.9-4	No adverse effect
	7.7-11.6	Severe growth depression
	34.0	Severe growth depression, liver enlargement and haemorrhage
Rats	0.5	No adverse effect

Table 1-3 continues

Animal	Glucosinolate intake (μmol/g diet)	Effect
	3.3-4.4	Reduced intake and growth
	6.6	Poor weight gain, increased thyroid weight and changed thyroid morphology
	7.7	Depressed intake and growth
Calves	1.2–2.4	No adverse effect on thyroid and liver function of calves
Cows	11.0	Induced iodine deficiency
	11.7–24.3	Depressed feed intake and milk production
	⩾23.0	Reduced intake and milk production
	31.0	Thyroid disturbance and depressed fertility
Steers	10.0–15.0	No adverse effect on growth and feed conversion
Sheep	1.2–1.6	Reduced plasma levels of estradiol provoked reproductive disturbance
	1.2–2.2	Weight loss during lactation in ewes
	⩾4.22	Induced iodine deficiency and increased thyroid weight
	15.0	Reduced growth in lambs
	33.0	Growth depression in lambs
Rabbits	7.9	No apparent adverse effect on growth and health
	17.9–25.3	Severe growth depression and increased mortality

There have been numerous researches dedicated to reducing the toxicity of glucosinolates. The reduction of glucosinolates can be achieved by either decomposition through physical treatments, or changing their properties through chemical or microbial reaction. Physical treatments include: microwave irradiation, micronization and extrusion. Chemical treatments involve reactions with metals or alkali, and microbial reactions are achieved through bioconversion.

Heat treatment is reported to be an effective method for removing glucosinolates up to 95% [27]. This is because at high temperature, glucosinolates can be

degraded into isothiocyanates, oxazolidine-2-thione, nitriles and other products. The outcomes vary with different glucosinolates. Although such heat treatments are efficient, they are also energy intense and, more importantly, as discussed previously, the degradation products, such as isothiocyanates and nitriles, are also toxic to animals [32, 37].

Glucosinolates can also be hydrolysed under the influence of alkali, acid and metal ions. Typical products after chemical treatments are isothiocyanates, ammonia, thioglucose and with the right side chain, amino acids (Figure 1-10 and Figure 1-11) [38].

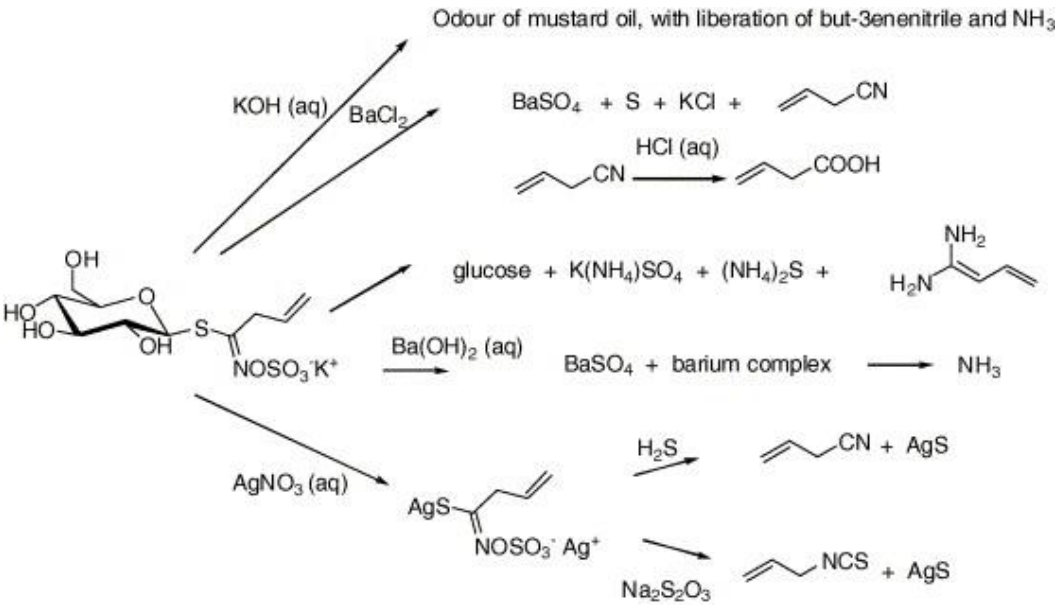

Figure 1-10 2-propenylglucosinolate degradation under the influence of alkali and metal ions [32]

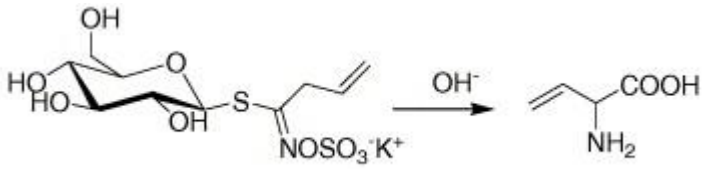

Figure 1-11 2-propenylglucosinolate degradation and amino acid formation under alkali condition [32]

Under acid conditions, the degradation of glucosinolates produces carboxylic acids, bisulphate, glucose and ammonium ions. The reaction is shown in Figure 1-11.

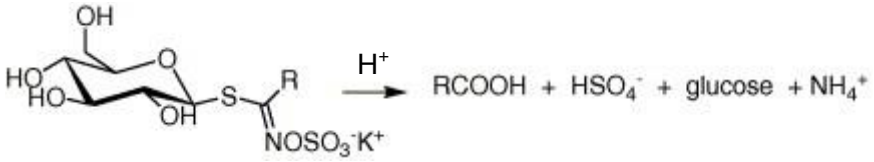

Figure 1-12 Degradation of glucosinolates under acid conditions, R represents side chain, adapted from [32]

With the presence of metabolic ions, isothiocyanates can be further developed into cyanide, nitriles or other chemicals. The mechanism is still not very clear, a possible explanation is that metabolic ions act as a bridge for electron transfer [39].

As with physical treatments, toxic products are generated during degradation of the glucosinolates and so the problem is not addressed. Due to the chemical complexity and inconsistency of the natural material, the process is also not completely controllable. Furthermore, in order to create a suitable environment for the reaction, it requires a large quantity of acid or alkali. This will bring negative impacts on both environmental and economic performances.

Microbial treatment is where glucosinolates and their hydrolysis products are bio-converted enzymatically and then used by microorganisms as their nutrient resources. This process occurs naturally inside mammalian intestines, where

certain strains of bacteria (*Bifidobacterium sp., B. Adolescentis* and *B. Longum*) have been shown to have the ability to digest glucosinolates and form particular isothiocyanates. Microbial digestions of glucosinolates can also take place beyond the mammalian intestine, when selected microorganisms are inoculated into, for example, rapeseed meal. For such systems, solid state bioprocessing is particularly favourable because of the advantages it has over submerged fermentation as mentioned above, especially when no product extraction is required. Most of the selected microorganisms in studies related to such microbial treatments are in the genera *Rhizopus* or *Aspergillus*. For example, in the research conducted by Chiang and colleagues, after 30 days of SSB, isothiocyanates in rapeseed meal had decreased from 119.6 μmol/g to 14.7 μmol/g. In the subsequent feeding trials on birds, no adverse effects were observed [40]. Another research revealed that 43% of glucosinolates had been eliminated by SSB using *Rhizopus Oligosporus* and *Aspergillus sp* after 10 days cultivation [33, 41].

Apart from glucosinolates, phytic acid and phenolic compounds are also considered to be anti-nutritional factors in rapeseed meal. The concentration of phytic acid and of phenolic compounds, namely tannins and sinapine in the case of rapeseed meal, and their effects on animals are presented in Table 1-4.

Table 1-4 Sinapine, tannin and phytic acid contents in rapeseed meal and their effects on animals [42]

Composition	Amount in canola meal (%, w/w)	Effects on animals
Sinapine	0.6 - 1.8	Bitter flavour, fishy eggs
Tannins	1.5 - 3.0	Impaired digestion of protein
Phytic acid	3.0 - 6.0	Binds minerals and affects its absorption

Rapeseed meal is rich in phenolic compounds, especially in sinapine (Figure 1-13) and tannings [43]. They are a group of polyphenolic chemicals with molecular weight ranging from 500 to 3000 Da. [42, 44].

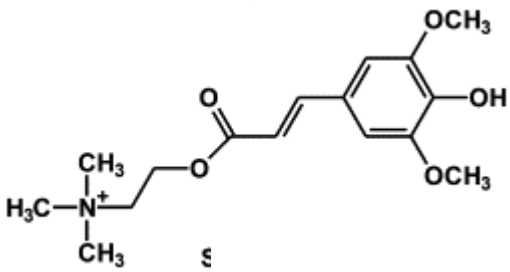

Figure 1-13 Chemical Structure of Sinapine [44]

Phenolic compounds are generated from glucose and phenylalanine. They act as UV protectors and as a supply of choline in plants. In general, the impacts of phenolic compounds are much less significant than glucosinolates [42]. They contribute to the bitter flavour (sour, astringent, bitter and/or phenol-like) of rapeseed meal, which may render it less desirable [45]. Similar to glucosinolates, sinapine can also cause off-flavoured eggs in susceptible birds [42]. Tannins can inhibit animal protein hydrolysis by interfering with digestive enzymes. They can combine with proteins through hydrogen bonds between the hydroxyl groups of phenol and carbonyl groups of peptides, especially under acid conditions. As the precipitation of proteins can also take place in saliva, tannins can cause puckering and drying sensations to the tongue.

Several researches have been dedicated to reducing the phenolic content in rapeseed meal and brassica vegetables. Heat treatment can reduce sinapine in rapeseed meal by 17%. Chemical treatment is achieved by administering ammonia, which can reduce sinapine by up to 74% [46]. Apart from heat and chemical treatment, tannins can be removed by water, methanol or ammonia. The combination of 10% ammonia in 95% methanol-hexane is reported to be more

effective than the individual chemical [47]. Solid state bioprocessing has also been reported to be effective for reducing phenolic compounds, the concentration can be reduced to below 5% of its original level [48]. Again, comparing to heat and chemical treatment, microbial conversion has distinctly better environmental and economic performance.

Phytic Acid (inositol hexakisphosphate) acts as a reservoir of phosphate and inositol in the seeds [49]. In animals, it can combine with metabolic ions, especially zinc, and form complex phytates. Therefore phytic acid can reduce ion availability to animals [42]. Negative impacts of phytic acid can be reduced by metabolic ion supplement, and microbial consumption. Many researchers have demonstrated the reduction of phytic acid through solid state bioprocesses. A few examples are listed in Table 1-5.

Table 1-5 Effects of solid state bioprocessing on phytic acid reduction with different fungi and culture time

Microorganism Used	Fermentation Time	Phytic Acid Reduction
Aspergillus niger [50]	120 h	Up to 74.7%
Aspergillus ficuum [51]	42 h	Around 87.5%
Rhizopus oligosporus [41]	240 h	Up to 42.4%

Summarising the previous section, rapeseed meal is rich in protein, cheap in price and sufficient in supply to be a successful animal feed. However, the presence of toxins and anti-nutritional factors greatly limit its utility. Many researchers have been focusing on enhancing the nutrient value of rapeseed meal, but a satisfactory result has not yet been reported. Solid state bioprocessing shows promise in terms of process efficiency, economics and environmental performance. However, none

of the previous reports have confirmed removal of all the anti-nutritional factors in a single process.

1.1.6 Sugarcane Bagasse

Sugarcane is the world's largest sugar crop. Between 2014 and 2016,1.84 billion tons were produced worldwide [52]. Sugarcane bagasse (SCB, Figure 1-14) is the cellulosic residue after sugar extraction. It contains 40% - 50% cellulose, 25% - 35% hemicellulose, and usually less than 30% lignin [53].

Cellulose is a polysaccharide consisting of a linear chain of several hundred to many thousands of D-glucose units, and consequently, the high proportion of cellulose makes sugarcane bagasse rich in carbon and makes it a potential carbon source for microorganisms [54]. However, cellulose chains form numerous intra- and inter-molecular hydrogen bonds, which account for the formation of rigid, insoluble microfibers. As a consequence, the nutrients are not accessible for microorganisms which are not able to excrete cellulosic enzymes. For those that can hydrolyse these long chain macromolecules, it would take them a long time to generate sufficient enzymes, especially at the beginning of the fermentation, resulting in a long fermentation time. Therefore, for lignocellulosic materials like sugarcane bagasse, pre-treatment can greatly enhance the accessibility of nutrients in the raw material, and consequently the fermentation efficiency. Pre-treatments of sugarcane bagasse can be achieved biologically such as by adding fungi, biochemically by adding enzymes, physically such as through hammer milling, chemically by adding acids or ammonia, or physico-chemically, such as by steam explosion [55]. These pre-treatments mainly aim at changing the physical structure of the sugarcane bagasse at a molecular level. As a result, the

ordered nature of cellulose, within its lignin matrix, is opened up, leading to increased total surface area.

Figure 1-14 Sugarcane bagasse [56]

Both rapeseed meal and sugarcane are by-products from agriculture and they are normally considered as wastes. Despite their differences in chemical composition, solid substrates made from agricultural wastes share some general features. The most significant one is their chemical and physical heterogeneity. The heterogeneity is not only due to the variation between crops, but it is also due to differences between the various parts of the plants/seeds. For example, a mature rapeseed is composed of approximately 18% (w/w) seed coat (hull) and 82% embryo [57]. The major compositions of the two components are very different: in the hull is fibre, and in the embryo are protein and oil for an intact seed [58]. In addition, the chemical composition of rapeseed also varies with the variety, maturity and growing conditions [58]. As a consequence, the chemical composition of defatted rapeseed meal also varies with growing conditions,

harvest timing, upstream oil extraction efficiency, and even from one particle to another. For example, in a solid substrate composed of rapeseed meal, there are particles shredded from the hull, the main composition of which is fibre. There are also particles from the embryo, of which the main component is protein. As a consequence, from a microscopic point of view, the available nutrients between different particles can be very different.

In addition to heterogeneity, agricultural substrates act as both physical support and nutrient supply to microorganisms growing on them. In other words, the nutrients are in their solid form. As discussed above, since microorganisms can only utilise dissolved solutes in the liquid phase, the nutrients in the agricultural solid substrate are not directly accessible [10]. Therefore, for most agriculture substrates, the nutrients need to be, first, hydrolysed into soluble form, either by pre-treatment, or by microbial hydrolytic enzymes excreted during fermentation, and, second, must be dissolved in water, in order to become available to the microorganisms.

1.1.7 Potato Dextrose Agar

Comparing to agricultural substrates, as shown in Figure 1-15, PDA is much more homogeneous at a macroscopic level and even at the microscopic level. It is an artificial substrate made from potato infusion (10%, w/w), dextrose (51%) and agar (38%) [59]. The potato dextrose medium can, of course, also be used in liquid form (without the solidifying agar agent). In this form, it is referred to as potato dextrose broth (PDB). Both PDA and PDB have been used extensively in the project reported in this thesis.

The carbon and nitrogen ratio for PDA is 10:1 [60]. PDA is a type of hydrogel. Hydrogels are hydrophilic polymer networks, in which the polymers build up the

porous physical structure of the gel filled with liquid nutrient [61]. Apart from the degree of homogeneity, another difference between hydrogels and agriculture substrates is that the nutrients in the hydrogel are already in the liquid form and can be directly utilised by microorganisms. Therefore, as discussed above, the nutrient supply will be affected mainly by the mobility of the liquid phase, rather than by the chemical composition of solid particles.

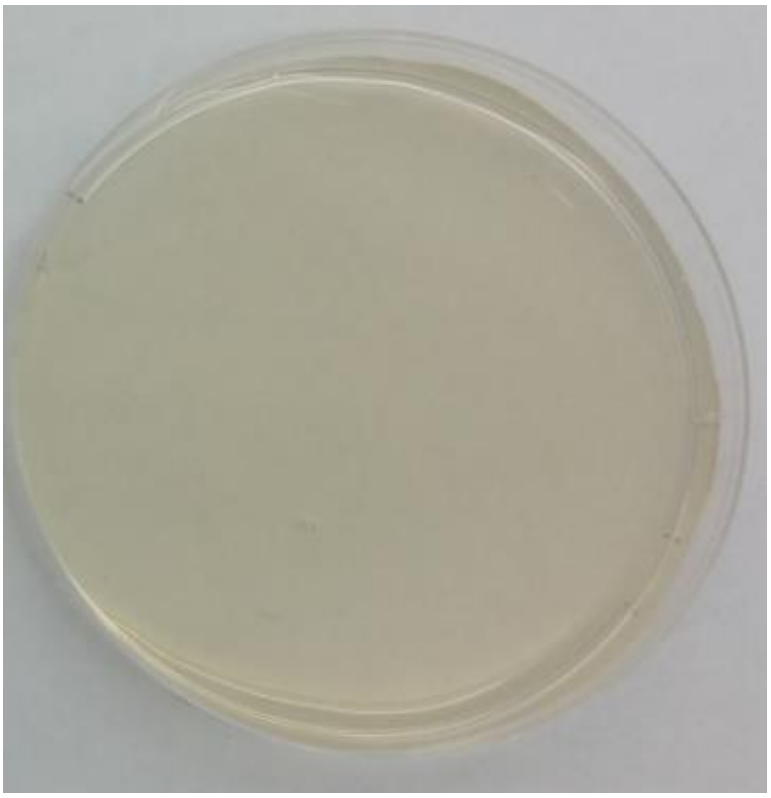

Figure 1-15 Hydrated potato dextrose agar (PDA)

1.3 Current Research and Challenges in Solid State Bioprocessing

Since the first report by Hesseltine [8], solid state fermentation and by implication, solid state bioprocessing, have attracted much attention from the scientific community. Some researchers have explored the possibility of using solid state bioprocessing to produce value added products from cheap raw materials (e.g. agricultural wastes), while others have focused on engineering aspects of the process, such as upstream processing, heat and mass transfer during fermentation, bioreactor design, mathematical modelling etc. Solid state fermentation and solid state bioprocessing have provided unique advances that could not have been achieved in submerged fermentations.

Despite this, in the meantime, there have been several unsolved challenges haunting almost every researcher working in the area: understanding the fundamental of the process, obtaining accurate and meaningful measurements of fermentation performance; achieving repeatability of experimental results; completing accurate mathematical descriptions [10, 62, 63]. As a result, the credibility of existing papers is impaired, the industrial scale up is problematic, and even providing meaningful interpretations of the experimental results has proven difficult.

However, most research has been applications oriented and therefore, very practical in nature [64-68]. The majority of the remaining research has focused on addressing the engineering aspects of the process, such as bioreactor design or mathematical description of mass and heat transfer during fermentation [63, 69-

73]. Only very few attempts have been made to understand the fundamentals of the process, such as that by Barrios-González [74] who reported the difference in physiology of microorganisms growing in liquid and solid cultures. Lopez – Perez [75] also reviewed the ecology of yeast growth on solid substrates. Apart from these rare cases, the majority of publications have not been aimed at gaining a fundamental understanding of the bioprocess and hence existing knowledge is not sufficient to address the challenges mentioned above. For example, even though the mechanisms of how microorganisms access nutrients in solid substrates has been well recognised by the research community, the importance of water and the liquid phase in solid state fermentation is very much underappreciated. Among the papers published in 2017 and 2018, in the field of solid state bioprocessing and solid state fermentation, more than 90% of them simply quantify the amount of water available to microorganisms in terms of water content, i.e. the ratio of the weight of water to the weight of substrate. However, water availability is a far more complex matter that cannot be sufficiently described by simple weight ratios. For example, whether water is available or not is affected not only by the total amount of water added to the substrate, but also by fermentation conditions, e.g., temperatures and humidity, and the chemical and physical properties of the substrate and consequently is affected by the heterogeneity of the substrate. At the moment, there is no information which can link the fermentation performance with the fundamental effects brought about by fermentation conditions on water dynamics. As a result, this prevents accurate descriptions of water availability in a solid state bioprocessing system, and consequently leads to poor repeatability of experiments and therefore results reported in the literature.

1.4 Project and Thesis Outline

Even though, in many publications, solid state fermentation and solid state bioprocessing have been praised for their unique features, there still lacks sufficient solutions to address the challenges mentioned above. This prevents the realisation of experimental findings at an industrial scale, or development of a generic solid state bioprocess which can be applied to produce different products from different substrates. Furthermore, due to the lack of systematic study to address the challenges mentioned above, it is not clear what the causes are that are responsible for them, or even if they truly exist. This is especially the case when, without even attempts to address these problems, many works published in this area still regard the experimental outcome as “effective”, “successful” or “attractive” [63, 68, 69, 76, 77].

The project reported in this thesis was aimed at both identifying and addressing problems occurring during solid state bioprocessing and in particular fermentation. This is a very ambitious aim, especially when solid state bioprocessing is such a complex system. So, to limit the scope of the topic, only static fermentations carried out on petri dishes, and with fixed air ventilation were investigated. To achieve the overall aim, an initial objective was to identify problems occurring during solid state bioprocessing and fermentation, by studying two common applications: reducing toxins in raw materials and producing generic microbial feedstocks. The two applications were carefully selected to gain a more comprehensive grasp of solid state bioprocessing: they are common topics which have been chosen by many researchers, and they have distinctly different natures. Through studies of these applications, it was concluded that there lacks sufficient

fundamental understanding of the system, especially in terms of water dynamics in the solid substrate. This prevented full advantage being taken of what solid state fermentation can provide, leading to a poor repeatability of the experimental results, and consequently preventing meaningful interpretation of the fermentation outcome.

After identifying the problems, the second part of the project was aimed at addressing them. In order to do so, two objectives were set: gaining a better fundamental understanding of the effects of water on solid state fermentation. This was achieved by conducting both literature based research and experimental work. In this way, information from related subjects (e.g. biochemistry, food science and soil science) was gathered and applied to solid state fermentation scenarios. Later, this information was further adapted and developed into the fundamental interpretations of water dynamics in the solid substrate.

In order to reflect the structure of the project, the thesis has also been organised in two parts. It starts with an introduction to general materials and experimental methods (Chapter 2). This is followed by Part A, which reports and summarises the findings from the two applications of solid state bioprocessing, namely reducing toxin content in RSM (Chapter 3) and producing a generic microbial feedstock from RSM and SCB (Chapter 4). These chapters highlight some of the problems of solid state bioprocessing, which are subsequently addressed in Part B. The literature based research into the importance of water to microbiological systems and the representation of water availability are reported in Chapter 5. The experimental work conducted to develop a fundamental interpretation of water bioavailability in solid substrates is then reported in Chapter 6. Finally, the conclusions and recommendations for further research bring together the two parts of the thesis in Chapter 7.

Gaining a fundamental understanding of solid state bioprocessing is indeed very challenging. This is not only because solid state bioprocessing is a very complex system, but also because there is so little information available within this domain that can be used directly. However, hopefully the project presented in this thesis offers some opportunity to gain insights into solid state bioprocessing, which can then help with solving existing challenges, interpreting the system with a fresh view, and developing this ancient technology to be fit for modern industry.

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Chapter 2.

General Experimental Equipment and Procedures

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Chapter 2

General Experimental Equipment and Procedures

Throughout this project, a wide variety of experiments have been conducted, many of which were progressive and developed as the project evolved. These are described in detail, at the relevant points in each of the experimental chapters. However, some of the materials, equipment and general procedures, particularly analytical, are used frequently and therefore described together. Hence, in this chapter, general materials and procedures that were applied throughout this project are described.

2.1 Materials

The principal raw materials used throughout the project were rapeseed meal (RSM) and sugarcane bagasse (RSM). Other chemicals, such as potato dextrose agar (PDA), potato dextrose broth (PDB) and polyethylene glycol (PEG) are described elsewhere. These were purchased from Sigma unless otherwise specified, except for PEG 1400, which was purchased from Alfa Aesar.

RSM was kindly provided by Archer Daniels Midland, UK and Mycozyme Ltd, UK, in vacuum-packed bags to maintain freshness. By observation, most of rapeseed meal had a particle size less than 0.5 mm, and was mixed with some other materials at a size larger than 0.5 mm. Therefore, for fermentation, particle size

was controlled by passing through a 0.5 mm sieve. After sieving, the <0.5 mm particles were stored at room temperature, in sealed containers. SCB was obtained from the School of Chemical Engineering and analytical science, the University of Manchester. The particle size of untreated SCB was between 0.5 mm and 0.85 mm. It was stored at room temperature and in a sealed container.

2.2 Preparation of Agar Gels and Czapek Medium

Gelled substrates used for this project were potato dextrose agar (PDA) and potato dextrose plus PEG agar (PDA + PEG). PDA was made by dissolving 3.9% (w/w) PDA powder into HPLC grade water then autoclaving at 121℃ for 20 min. The sterilised solution was then dispensed onto petri dishes, the exact amounts used in experiments are described in each chapter. The solution was allowed to cool in the petri dish and the dish was sealed with parafilm before storing at 4℃ prior to use.

PDA + PEG was made by dissolving 3.9% (w/w) PDA and 3.9% PEG into HPLC grade water then autoclaving at 121℃ for 20 min. Similar to the above, the solution was then dispensed onto petri dishes and allowed to cool before sealing with parafilm and storing at 4℃.

Czapek medium was made by dissolving 2.8% (w/w) glucose, 0.1% dipotassium phosphate, 0.001% iron (II) sulphate heptahydrate, 0.05% magnesium sulphate, 0.05% potassium chloride and 0.3% sodium nitrate into HPLC grade water. The solution was then autoclaved at 121℃ for 20 min before use.

2.3 Microorganisms

Several different fungi were used in the project and reason for their choice is given in the appropriate chapters. These were *Rhizopus oryzae*, *Aspergillus oryzae, Aspergillus awamori* and *Saccharomyces cerevisiae*. All had previously been used and readily available in the author's research laboratory at the School of Chemical Engineering and Analytical Science, the University of Manchester.

2.4 Inoculum Preparation

Two types of inoculum have been used for experiments involving the filamentous fungi reported in this thesis, spore suspensions and dry fungal spores. Spore suspensions were prepared freshly before inoculation. Prior to the experiment, fungi were cultivated on PDA dishes at 30℃ for 7 days. To harvest the spores, 10 mL sterilised water was added onto the fungal culture and the substrate surface was scratched with a disposable inoculation loop. The spore suspension was then carefully poured into a container. The harvesting procedure was performed twice. Spore concentration in the suspension was measured with a haemocytometer before use. One drop of the spore suspension was placed on the haemocytometer, and the spores were counted under the microscope using a standard procedure [11].

For dry spore inoculation, fungi were first inoculated onto a PDA dish and left in a 30°C incubator where humidity was 10% ± 2%, to allow the substrate to dry into a thin film after fungal cultivation (Figure 2-1). Spores were then taken directly from this master dried fungal culture using a disposable inoculation loop and inoculated onto the substrate surface. To estimate the number of spores in each such inoculation, the procedure was followed as described, but the loop was transferred into 1 mL sterile distilled water. A small sample of this suspension was counted on the haemocytometer, and for three repeats in two separate experiments, gave a count equivalent to approximately 1000 spores per inoculum.

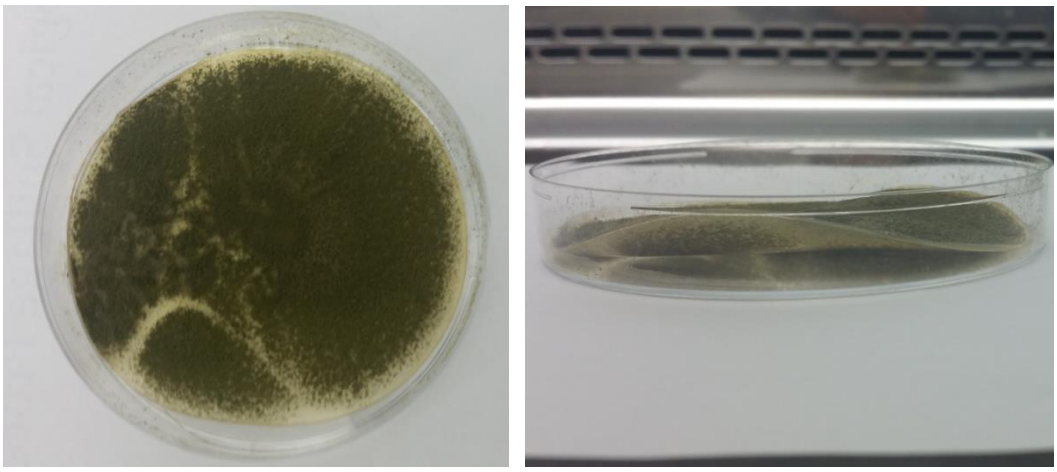

Figure 2-1 A dried *A.oryzae* culture cultivated on PDA and kept in 30 oven to constant weight. Photos were taken from front (left) and side (right)

For inoculation of *S. cerevisiae,* the yeast was cultivated on PDA substrate and incubated at 30℃ for 7 days. A liquid medium composed of 20 g/L glucose, 10 g/L yeast extract and 20 g/L peptone was also made by dissolving the chemicals into HPLC grade water. The solution was then autoclaved 121℃ for 20 min and allowed to cool to room temperature. Cells were scratched from the PDA dish using a disposable inoculation loop and transferred into the liquid medium, then shaken at 200 rpm at 30℃ for 48 h.

2.5 Measuring CO_2 production

As microbial activity occurs, CO_2 is being produced. Therefore, CO_2 concentration was used as an indicator for fungal growth on solid substrates.

To measure CO_2 concentration, an inoculated petri dish containing sterilised solid substrate was placed on a sponge in a sealed fermenter (Figure 2-2), without the dish lid. The fermenter was connected to a CO_2 gas analyser integrated with a built-in pump. The flow rate was set at 30 cc/min. The exhaust gas from the gas analyser was pumped back to the sealed fermenter, creating a complete gas circulation.

The fermenter was also connected to a humidity meter and an endoscopic camera, focused at the surface of the substrate, where fungi spores was inoculated. To obtain a visual record of fungal growth, a desk lamp was place at a fix point in the incubator as a light source, and videos were continuously produced by the endoscopic camera throughout fermentation.

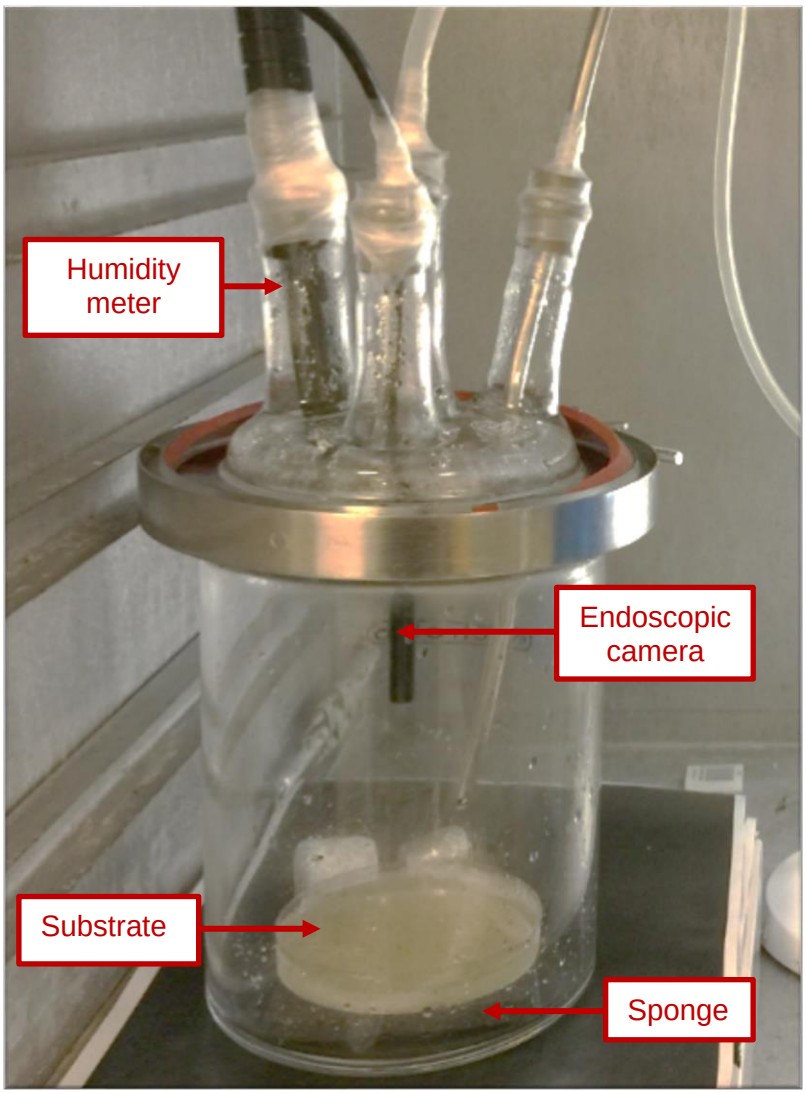

Figure 2-2 A sealed fermenter containing a sponge, substrate, endoscopic camera and humidity meter. The fermenter was connected to a gas analyser for monitoring CO2 production during fermentation

2.6 Analytical Methods

2.6.1 Extraction for Analysis of Solid State Fermentation Samples

In order to evaluate the performance of solid state fermentations, chemical analysis must be carried out using liquid extracts. To create these extracts, the following procedure was used.

After cultivation with the filamentous fungi, the fermented substrate was well mixed and ground using a pestle and mortar. 2 g of the ground meal was then taken and put into a 50 mL centrifuge tube with 20 g of distilled water added. The tube was sealed and vigorously shaken by hand before further shaking on an orbital shaker for 1 hour. Then all tubes were centrifuged at 10,000 rpm for 10 min. The liquid phase was decanted and stored at -18℃ as a liquid extract for analysis.

2.6.2 Dry Biomass Weight in Submerged Fermentation

To assess the performance of fermentations carried out in liquid culture, the dry biomass weight was measured. After fermentation, the liquid culture was transferred into a pre-weighed 50 mL centrifuge tube. The tube was then centrifuged at 10000 rpm for 10 min. The liquid phase was decanted, while the solid phase was kept in the tube. This was then placed in a 60℃ incubator until a constant weight was reached. This difference between this weight and the initial centrifuge tube weight indicated the dry biomass produced during the fermentation.

2.6.3 Fungal Growth at the Surface of Solid Substrates

In order to quantify fungal growth at the surface of solid substrates, photos were taken before and after solid state fermentations. They were then converted into grayscale using Photoshop. The fungal colony area in the photos taken after fermentation were identified and selected manually. The Chroma within that area were calculated against the same area of the photos which were taken before fermentation to give a value of Chroma for the substrate. In addition, a PDA dish inoculated with fungi was cultivated at 30℃ for 3 days. A photo was taken before and after cultivation. The Chroma of the area with fungi was calculated using the

same method to the above, to give a reference Chroma value for PDA. The fungal growth area was then calculated according to Eq. 2-1.

$$\text{Fungal growth area } [\%] = \frac{Chroma_{substrate}}{Chroma_{PDA}} \times 100\% \qquad \text{Eq. 2-1}$$

$Chroma_{substrate} = Chroma\ of\ fermented\ substrate$

$Chroma_{PDA} = Chroma\ of\ PDA\ \ after\ 3\ days\ fungal\ cultivation$

2.6.4 Total Reducing Sugars

Total reducing sugars concentration was measured using the DNS method based on the colour reaction between the reducing sugars and 3,5-dinitrosalycylic acid (DNS). The reaction yield was measured as absorbance of the sample at 540 nm using a spectrophotometer.

DNS reagent was prepared by dissolving 10.6 g/L 3,5-dinitrosalicyclic acid, 19.8 g/L sodium hydroxide, 8.3 g/L sodium sulphite anhydrous, 2.0 g/L phenol and 306.0 g/L potassium sodium tartrate into HPLC grade water. The solution was kept in a sealed bottle covered with foil to avoid light. The bottle was stored at 4℃ and consumed within 1 week.

A test tube containing 0.5 mL of liquid extract from the solid sample was made. The liquid extract was obtained as described in section 2.6.1. In addition, a series of dilutions of a glucose standard solution corresponding to concentrations ranging from 0 to 1.0 g/L was also prepared in test tubes in order to create a standard curve. The 0 g/L glucose standard, in effect, distilled water, was used as a blank.

All tubes were added with 0.5 mL DNS reagent and sealed with foil. The contents were vigorously shaken by hand, before incubating in a boiling water bath for 15 min. After that, the test tubes were placed inside a container with cool water. After cooling to room temperature, each tube was added with 4.5 mL distilled water and vigorously shaken by hand. For measuring UV absorbance, a cuvette was first filled with the blank sample and placed in a UV spectrophotometer (Shimadzu UV-VIS 2410PC, Japan). The absorbance of the blank sample at 540 nm was then set at 0 and the rest of the tubes were transferred into cuvettes. The relative absorbance of each was measured at 540 nm. The standard curve was produced using the absorbance of the glucose standards (Figure 2-3). The total reducing sugars in the liquid extract were calculated according to Eq. 2-2 using the slope of the standard curve for glucose (Figure 2-3).

$$Reducing\ Sugar\ \left[\frac{g}{L}\right] = \frac{A_{540} \times D}{m} \qquad \text{Eq. 2-2}$$

A_{540} $= absorbance\ of\ the\ sample\ at\ 540\ nm$

$m = slope\ of\ the\ glucose\ calibration\ curve$

$D = dilution\ factor$

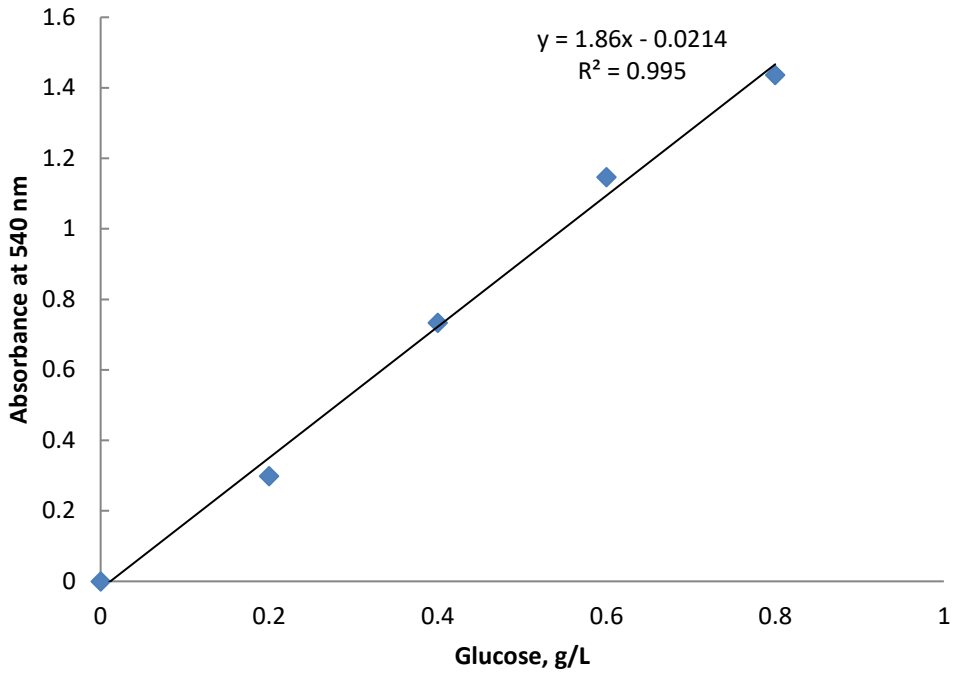

Figure 2-3 A standard calibration curve for absorbance at different glucose concentrations, used in the determination of reducing sugar concentrations

2.6.5 Free Amino Nitrogen

Free amino nitrogen (FAN) is the amount of individual amino acids and small peptides which can be utilised by microorganisms. For substrates that don't contain any FAN initially, such as RSM and SCB, it is produced by protease that has been excreted by fungi during fermentation. Therefore, it can be used as an indicator for assessing fermentation performance. To measure the FAN concentration a method was adapted from the standard ninhydrin colorimetric method [78].

Ninhydrin colour reagent was prepared by dissolving 49.7 g/L di-sodium hydrogen phosphate dehydrate, 60.0 g/L potassium dihydrogen orthophosphate, 5.0 g/L ninhydrin and 3.0 g/L fructose into distilled water. This was kept in an amber glass bottle at 4°C. In addition, a dilution reagent was made by dissolving 2.0 g

potassium iodate in 616 mL distilled water. Then 384 mL absolute ethanol was added into the solution. The reagent was also keep at 4°C.

A test tube containing 0.1 mL liquid extract of the solid sample and 0.9 mL distilled water was prepared. The liquid extract was obtained as described in Section 2.6.1. In addition, a series of dilutions of a glycine standard corresponding to concentrations ranging from 0 to 1.0 g/L glycine was also prepared in test tubes. The 0 g/L standard, in effect, distilled water, was used as a blank.

All test tubes were added with 1 mL of ninhydrin colour reagent, and sealed with foil. The contents were vigorously shaken by hand, before incubating in a boiling water bath for 15 min. After that, the test tubes were placed inside a container with cool water. After cooling to room temperature, each tube was added with 9 mL dilution reagent and vigorously shaken by hand. For measuring UV absorbance, a cuvette was first filled with the blank sample and placed in the UV spectrophotometer. The absorbance of this at 570 nm was set to 0. Then the rest of the tubes were also transferred into cuvettes, and the relative absorbance of each was measured at 570 nm.

FAN in the liquid extract was calculated according to Eq. 2-3 using the slope of the standard curve for glycine (Figure 2-4).

$$\text{Free amino acid} = A_{570} \times \text{Slope} \times \text{DF} \qquad \text{Eq. 2-3}$$

$A_{570} = absorbance\ of\ the\ sample\ at\ 540\ nm$

$m = slope\ of\ the\ glycine\ calibration\ curve$

$D = dilution\ factor$

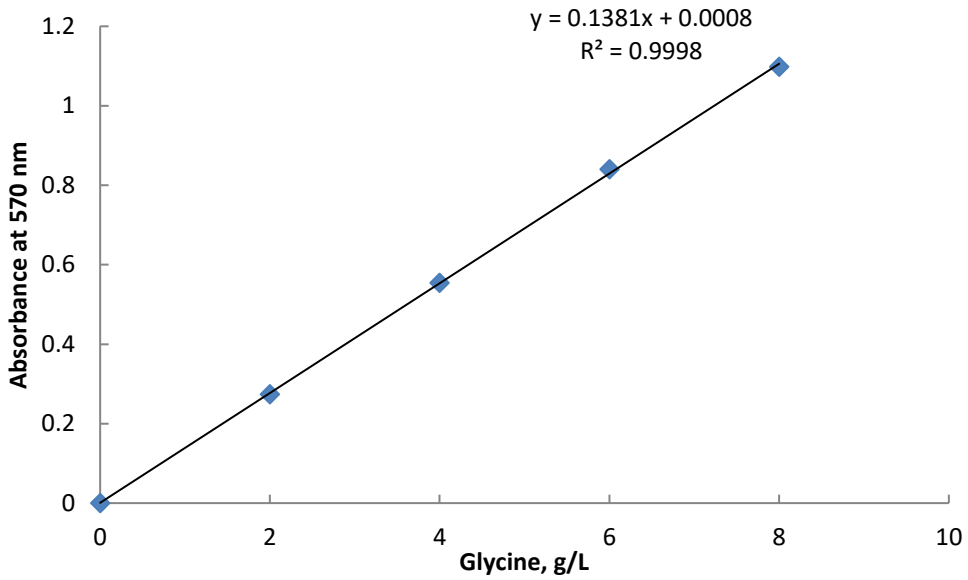

Figure 2-4 A standard calibration curve for absorbance at different glycine concentrations, used in the determination of FAN concentration

2.6.6 Protease Activity

Protease activity is defined as the amount of free amino nitrogen generated at 55℃ in 1 hour (U/mL). It was determined by measuring the production of FAN after incubating casein with the liquid extract obtained as described in Section 2.6.1. FAN concentration was measured following the procedure described in Section 2.6.5.

Casein solution was made by dissolving 2.5% (w/w) casein into distilled water and heating at 55℃ until dissolution. The supernatant was also incubated at 55℃ till temperature became constant. Then, 1 mL of the warm casein solution was transferred into a test tube, and added with 1 mL of the warm liquid extract. A tube containing 1 mL of distilled water and 1 mL of casein solution was made and used as the control. In addition, 1 mL of a commercial protease purchased from Sigma was also mixed with 1 mL casein solution in a test tube to provide a commercial reference.

All tubes were sealed and shaken vigorously by hand. Immediately after shaking, 1 mL of the mixed content was taken and added with 0.5 mL 10% trichloroacetic acid to stop the hydrolysis. Then all tubes were incubated at 55℃ for 1 hour, before adding another 0.5 mL trichloroacetic acid. The FAN concentration in all samples was determined as described in Section 2.6.5. The protease activity was calculated according to Eq. 2-4.

$$Protease\ activity\ \left[\frac{U}{ml}\right] = FAN_{1h} - FAN_0 \qquad \text{Eq. 2-4}$$

$FAN_{1h} = FAN\ production\ after\ 1\ h\ hydrolysis$

$FAN_0 = \ Initial\ FAN\ concentration\ before\ the\ hydrolysis$

2.6.7 Cellulase Activity

Cellulase activity was defined as the amount of reducing sugar generated at 55℃ in 1 hour (U/mL). It was determined by measuring the production of reducing sugar after incubating cellulose powder with the liquid extract obtained as described in Section 2.6.1. Reducing sugar concentration was measured following the procedure described in Section 2.6.4.

Cellulose suspension was made by mixing 10.0% (w/w) cellulose with distilled water and incubated at 55℃ till temperature become constant. Then, 1 mL of the warm cellulose suspension was transferred into a test tube, and added with 1 mL of the warm liquid extract. A tube containing 1 mL of distilled water and 1 mL of cellulose suspension was prepared and used as the control. In addition, 1 mL of a commercial carbohydrase, Viscozyme, purchased from Sigma was also mixed with 1 mL cellulose suspension in a test tube.

All tubes were sealed and shaken vigorously by hand. Immediately after shaking, 1 mL of the mixed content was taken and added with 0.5 mL 10% trichloroacetic acid to stop the hydrolysis. Then all tubes were incubated at 55℃ for 1 hour, and then added with another 0.5 mL trichloroacetic acid. The reducing sugar concentration in all samples was determined as described in Section 2.6.4. The cellulose activity was calculated according to Eq. 2-5.

$$Protease\ activity\ \left[\frac{U}{ml}\right] = RS_{1h} - RS_0 \qquad \text{Eq. 2-5}$$

$RS_{1h} = Reducing\ sugar\ production\ after\ 1\ h\ hydrolysis$

$RS_0 = Initial\ reducing\ sugar\ concentration\ before\ hydrolysis$

2.6.8 Ethanol Concentration

To measure the ethanol production from anaerobic fermentation, the liquid culture was transferred into a 50 mL centrifuge tube and then centrifuged at 10000 rpm for 10 min. The supernatant was poured into another container. A 2 mL sample was collected from the supernatant, filtered through a 0.45 µm syringe filter and dispensed into a HPLC vial. Analysis was performed on a HPLC (Dionex Ultimate 3000, USA), equipped with a Phenomenex Sphereclone C18, 250mm x 4.6mm, 5 µm column. Injection volume was 20 µL. The mobile phase was 0.001 N H_2SO_4, flow rate 0.7 mL/min, column temperature 45℃.

2.6.9 Glucose and PEG Concentration

To measure the residual glucose and PEG concentration after fermentation, the liquid culture or the liquid extract obtained as described in Section 2.6.1 was transferred into a 50 mL centrifuge tube and then centrifuged at 10000 rpm for 10

min. The supernatant was poured into another container. A 2 mL sample was collected from the supernatant, filtered through a 0.45 µm syringe filter and dispensed into a HPLC vial. Analysis was performed on a HPLC (Dionex Ultimate 3000, USA), equipped with a Phenomenex Sphereclone C18, 250mm x 4.6mm, 5 µm column and a evaporative light scattering detector (ELSD). Each run was set to complete at 30 min, the mobile phase was water for the first 5 minutes and acetonitrile for the rest of the analysis. Injection volume was 1 µL, column temperature was 30℃. The operation and also evaporation temperature for ELSD was 40℃, signal was acquired at 40 Hz. Results were compared with standard solutions.

2.6.10 Glucosinolates concentration

To measure the toxin levels, rapeseed meal, fermented or not fermented, was well mixed and ground using a pestle and mortar. 2 g of the ground meal was then taken and put into a 50 mL centrifuge tube with 20 g of boiling water. The tube was sealed and vigorously shaken by hand for 30 s. Then all tubes were centrifuged at 10,000 rpm for 10 min. A 2 mL sample was taken from the liquid phase, filtered through a 0.45 µm syringe filter and dispensed into a HPLC vial. Analysis was performed on a HPLC (Dionex Ultimate 3000, USA), equipped with a Phenomenex Sphereclone C18, 250mm x 4.6mm, 5 µm column and a UV detector. Injection volume was 20 µL. The mobile phase was 0.2 M ammonium sulphate at a flow rate of 0.8 mL/min. Column temperature of 30 ℃. The UV-Vis Absorbance detector was set at 229 nm.

Additionally, a rapeseed meal sample was sent to an outside analytical agency, Sciantec Analytical Services Ltd, to measure the glucosinolate concentration.

Then the same sample, with known value of glucosinolate concentration was used as a standard.

2.6.11 Dynamic Vapour Sorption (DVS)

Dynamic vapour sorption has been used to measure the water activity, the amount of water that can be absorbed from the gas phase and the absorption rate of solid samples. The DVS machine measures the weight change at 30℃ of a sample placed on a foil plate, in a sealed chamber under a wide range of relative humidities (0% - 97% saturation), against an empty foil plate under the same conditions. The DVS was set to move on from one humidity level to the next once the rate of mass change (dm/dt) becomes lower than 0.002%. A typical set of results from water absorption followed by desorption produced from the DVS is shown in Figure 2-5.

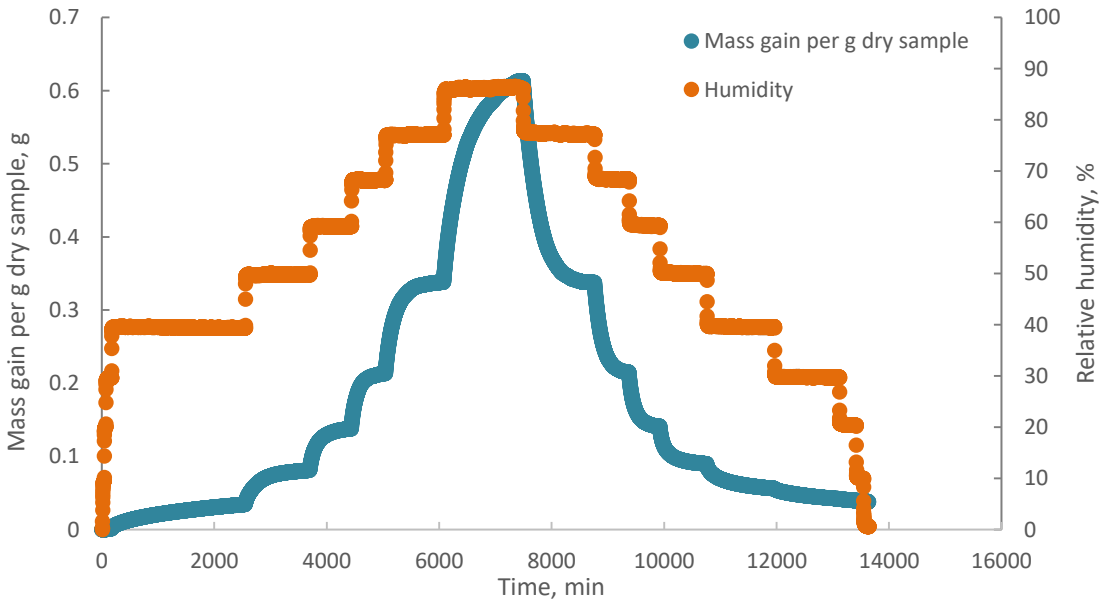

Figure 2-5 A typical set of DVS results for a dry PDA film, measured at 30℃ from 0% to 90% and back to 0% relative humidity, at 10% intervals

The water activity of a solid material changes with its water content. It is numerically equal to the relative humidity at equilibrium. Water content on the

other hand, is the ratio of the mass gain (water) against total weight (water + dry substrate) at the same equilibrium. A typical plot of water activity against water content is shown in Figure 2-6.

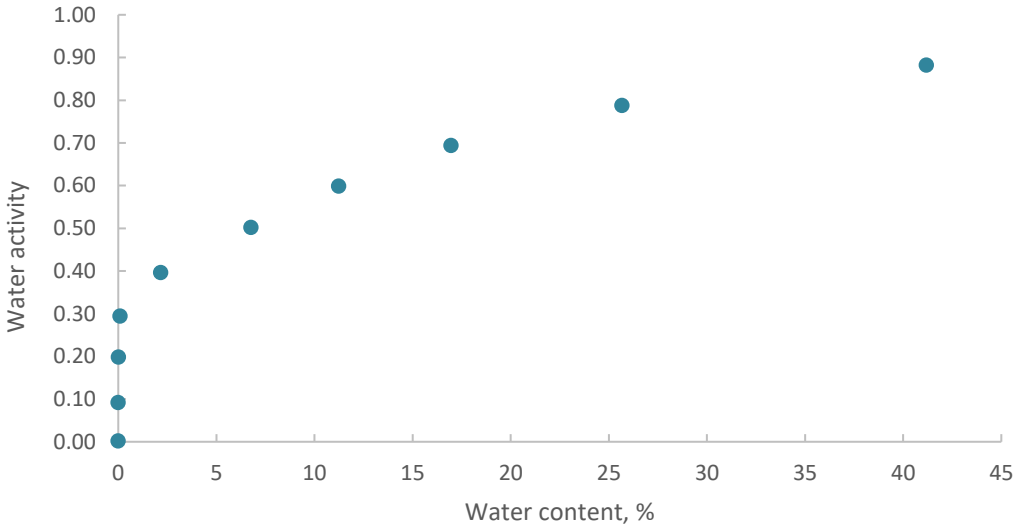

Figure 2-6 Relationship between water activity and water content for a dry PDA film, calculated from the DVS results shown in Figure 2-5

2.6.12 Nuclear Magnetic Resonance

To determine water mobility within the substrate, nuclear magnetic resonance (NMR) analysis was performed on a NMR 600. All samples were prepared with heavy water (D_2O) and placed in a NMR sample tube (outer diameter: 5mm, length: 7”, wall thickness: 0.43 mm). The tubes were then inserted into the NMR probe. The relaxation times (T2) were measured using the Carr-Purcell-Meiboom-Gill (CPMG) sequence. The parameters of CPMG were set as follows: corresponding resonance frequency (SF) for protons, 500 MHz; spectral width (SW), 20 ppm; echo time (TE), 600 ms; pulse widths at 90° (P1) and 180° (P2), 11 and 22 µs, respectively; waiting time (TW), 4 s, set 3. Data from 5000 echoes were acquired as 32 scan repetitions. The relaxation measurements were performed at the optimal operating temperature of 298 K. Data was processed by software: Topstia, version 3.5, and then analysed with software: Dynamic Centre.

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Part A:

Applications of Solid State Bioprocessing

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Chapter 3.

SSB Application I:

Reducing Toxins in Rapeseed Meal

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Chapter 3

SSB Application I: Reducing Toxins in Rapeseed Meal

As discussed in Chapter 1, rapeseed meal is high in protein. However, currently its utilisation is limited due to the presence of anti-nutritional factors. Reducing these would make it more suitable for animal consumption. In this chapter, the possibility of using solid state bioprocessing for toxin reduction is explored, followed by a critical analysis of its performance.

3.1 Experimental Programme

The research reported below was aimed at developing solid state bioprocesses for utilising rapeseed meal as animal feedstock, specifically to reduce anti-nutritional factors. Three objectives were established:

- Establish a feasible condition for microbial growth
- Test the effects of various treatments on fungal growth and enzyme production
- Test the effects of treatment plus fermentation as integrated solid state bioprocessing on the degree of fungal growth, enzyme production and toxin reduction

Working towards these objectives, the programme was developed into 3 stages. The first stage was creating a feasible environment for fungal growth. At this stage,

effects of key conditions on the fermentation performance had been investigated. These key conditions are sterilisation, water content, and microorganism species including indigenous microorganisms carried by the raw rapeseed meal. Based on these experimental results, the second stage was to develop a solid state process enhancing fungal growth and enzyme production. In this stage, a pre-incubation step was introduced prior to fermentation, where the raw rapeseed meal was mixed with water and incubated at 30℃. The effects of pre-incubation on fungal growth and enzyme production were studied and reported. The third stage involved investigating the effects of the integrated solid state bioprocessing on toxin reduction. A flow diagram summarising the programme is shown in Figure 3-1.

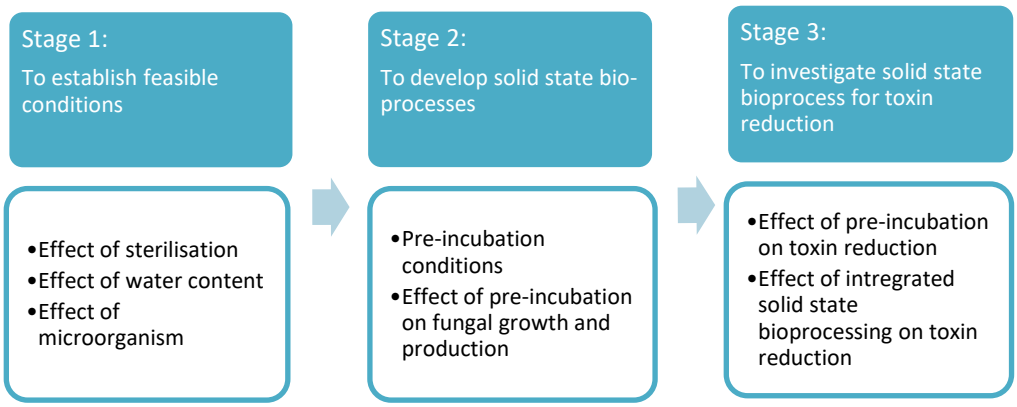

Figure 3-1 Summary flow diagram of experimental programmes for utilising rapeseed meal as animal feedstock through solid state bioprocessing

Based on previous research, rapeseed meal can successfully support fungal growth as a solid substrate [79, 80]. It was still, however, necessary to establish suitable conditions for the particular strains that were used for this research. Normally for bioprocesses, it is necessary to sterilise the substrate prior to inoculation. However, this is an expensive operation, which could render an

industrial scale process economically infeasible. It has been reported, for *R.oryzae,* that when a filamentous fungus grows on solid surface, it forms a thick layer of hyphae and consequently depletes oxygen supply for other microorganisms [11]. Therefore, it should be possible to perform solid state fermentation without the need for sterilisation, if the inoculated fungus can grow fast enough.

To test the possibility of bypassing sterilisation, an experiment was carried out to investigate the possibility of inoculating with *R.oryzae* onto moist untreated rapeseed meal. Based on results from this experiment, the effects of water and fungal strain on the rapeseed meal bioprocess were also tested. A further experiment was conducted, in which the naturally present microorganisms carried by untreated rapeseed meal, were encouraged to grow, consuming nutrients in rapeseed meal, and potentially reducing toxin levels.

3.2 Procedures and Results

3.2.1 Effect of Bypassing Sterilisation on Solid State Fermentation

As mentioned above, the first experiment of the programme was to test if solid state fermentation on rapeseed meal can be successfully performed without sterilisation. Based on the rapeseed meal provider's recommendation and on literature research [79], water content was set to 44% and 66% (w/w). After water was added, the wet rapeseed meal was mixed thoroughly then transferred onto petri dishes. The substrate weight in each dish was not controlled. The selected strain was *R. oryzae*, inoculum was 1 mL of spore suspension with a concentration of 10^6 per mL. All petri dishes were incubated at 30℃ for 72 h.

Photos were taken after fermentation and are shown in Figure 3-2. It can be seen in the photo on the left of the figure that some contamination existed in the group with 44% water content. In comparison, there was no obvious contamination in the other group. Contamination can be microorganisms brought by untreated rapeseed meal. One hypothesis to explain the different growth in the groups is that, when the water was insufficient, *R. oryzae* could not grow fast enough to form a hyphal barrier to oxygen and gave contamination a chance to grow. It is also possible that, because the untreated rapeseed meal contains very little moisture, the naturally occurring microorganisms are better adapted for dry environment.

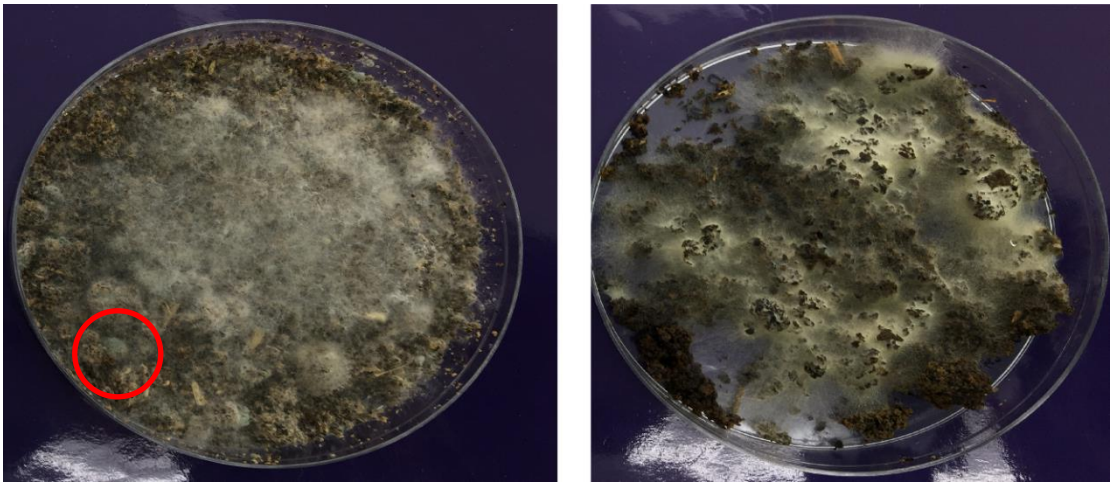

Figure 3-2 Rapeseed Meal inoculated with 10^6 spores of *R. oryzae* and fermented at 30°C for 72 h. Water content (w/w) was 44% (left) and 66% (right). The red circle indicates an area of contamination.

Due to the presence of contaminants, it was decided that sterilisation would be necessary. On the other hand, due to natural selection and evolution, those microorganisms naturally present, are likely to have a good ability to excrete relevant enzymes for utilising nutrients in rapeseed meal, possibly including the toxins such as glucosinolates. To investigate their potential, a further series of

experiments was designed and the results are reported in Section 3.2.3. Also in order to investigate the effect of water content on rapeseed meal solid state fermentation, the following experiment was designed and conducted.

3.2.2 Effect of Water Content on Solid State Fermentation

From the previous experiment, *R. oryzae* grew better in substrate containing 66% of water. In order to investigate the effect of water content further, 4 groups of experiments with rapeseed meal as solid substrate were set up.

Water content for group 1 and 2 were 44%, group 3 and 4 were 66% (w/w, based on dry rapeseed meal). To achieve a degree of sterilisation, two conditions were tested. Group 1 and group 3 were placed in boiling water for 30 min, group 2 and 4 were boiled for 60 min. After the treatment, the substrate was inoculated with *R. oryzae.* The concentration of spores in the spore suspension was 10^6 per mL and the inoculum was 1mL per 100 g wet rapeseed meal. The inoculated substrate was then transferred to petri dishes and incubated at 30℃ for 72 h. The weight of rapeseed meal in each dish was not controlled. Experimental details are shown in Table 3-1. After fermentation, the solids were ground till paste like. Free amino acids were extracted and analysed as described in Chapter 2.

Table 3-1 Experimental conditions for rapeseed meal solid state fermentation with different water contents and sterilisation times

Group	Water content (%, w/w)	Sterilization time (min)	Inoculum (mL)	Fermentation time (h)
1	44	30	1	72
2	44	60	1	72
3	66	30	1	72
4	66	60	1	72

Photo records of fungal growth on the surface of rapeseed meal substrate are shown in Figure 3-3. Free amino acid concentration before and after solid state fermentation is shown in Figure 3-4.

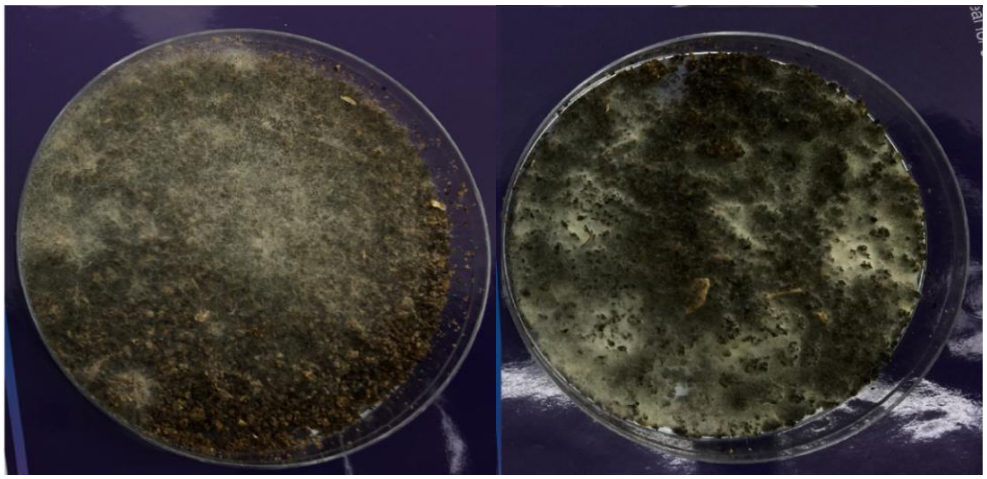

Figure 3-3 Rapeseed Meal with Different Water Content (44% (left) and 66% (right)) after heating in Boiling Water and 72 h Fermentation with R. oryzae

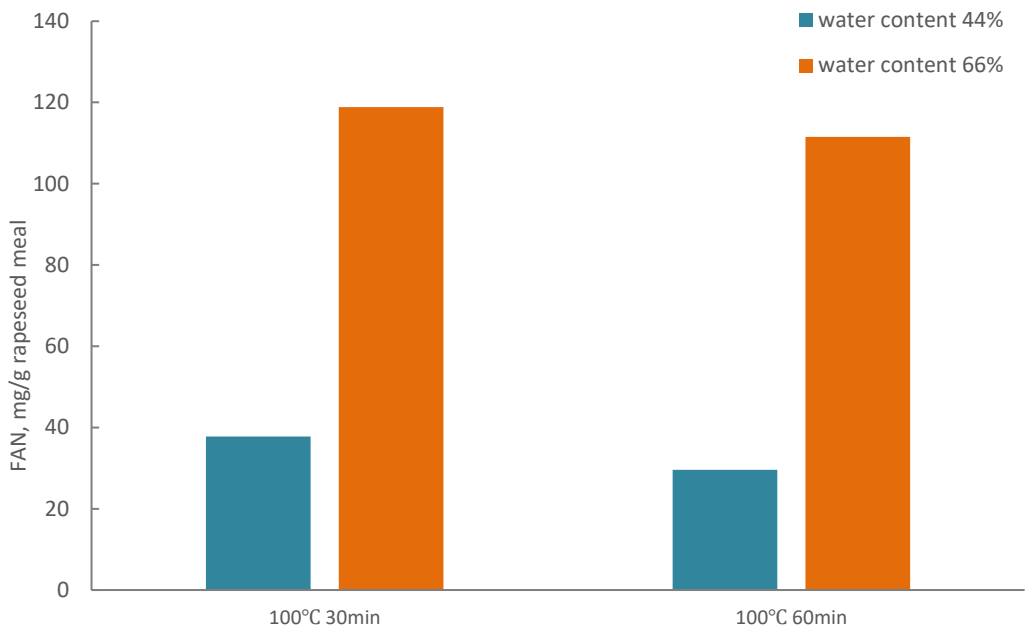

Figure 3-4 Free amino acid concentration of rapeseed meal with different water content, after heating in boiling water and 72 h fermentation with R. oryzae

Free amino acids are generated through the action of fungal proteases; higher free amino acids concentrations indicate a higher production of protease. In this experiment, concentration was much higher in the groups with 66% of water than with 44%. This is consistent with the photo record, where more fungi can be observed in the plate with 66% water. Therefore, water content of 66% was selected for future experiments. Boiling time, on the other hand, did not appear to have a significant impact on fungal growth, both 30 min and 60 min groups showed no indication of contamination and similar FAN production at the end of fermentation.

3.2.3 Utilising Naturally Occurring Microorganisms in Rapeseed Meal

From the experiment reported in Section 3.2.1, it appeared that untreated rapeseed meal naturally carries some indigenous microorganisms. Due to natural selection and evolution, such microorganisms are very likely to be able to excrete relevant enzyme for hydrolysing nutrients in rapeseed meal, including possibly its toxins such as glucosinolates, though it is not certain if they are safe or not. It was decided to allow the growth of these naturally carried microorganisms to see whether they would produce hydrolysing enzymes better than the lab strains. The detailed experimental procedure is described below.

As the naturally carried microorganisms appeared to grow better in lower water content, the water content in rapeseed meal was adjusted to 44% (w/w). The wet meal was mixed then pre-incubated, without inoculating, at 30℃, 50℃ and 70℃ for 30 min, and 60 min.. All petri dishes were then inoculated with *R. oryzae,* as in the previous experiment, but without autoclaving. Then the inoculated wet meal was distributed onto petri dishes. The weight in each dish was not controlled. All petri dishes were incubated at 30℃ for 72 h. A summary of experimental conditions is given in Table 3-2. Three repetitions were performed. After fermentation, the substrates in parallel dishes was well mixed together by a pester and mortar. The liquid extract was prepared as described in Chapter 3. FAN was measured before and after fermentation, and wet, untreated rapeseed meal was used as a control group. Fungal growth area was also calculated, using the photographic images and image analysis software. Fungal growth area and the production of FAN after pre-incubation and also after fermentation is shown in Figure 3-5.

Table 3-2 Summary of experimental conditions for rapeseed meal pre-incubated at different temperature and time before inoculation and fermentation

Group	Pre-incubation temperature (℃)	Pre-incubation time (min)	Fermentation (h)
1	30	30	72
2	30	60	72
3	50	30	72
4	50	60	72
5	70	30	72
6	70	60	72
Control	N/A	0	72

From Figure 3-5 it can be seen that pre-incubation at 30℃ for 60 min had a significant effect on the production of FAN after fermentation. This is also consistent with FAN concentration after pre-incubation alone. The highest production appeared in groups pre-incubated at 30℃ for 60 min.

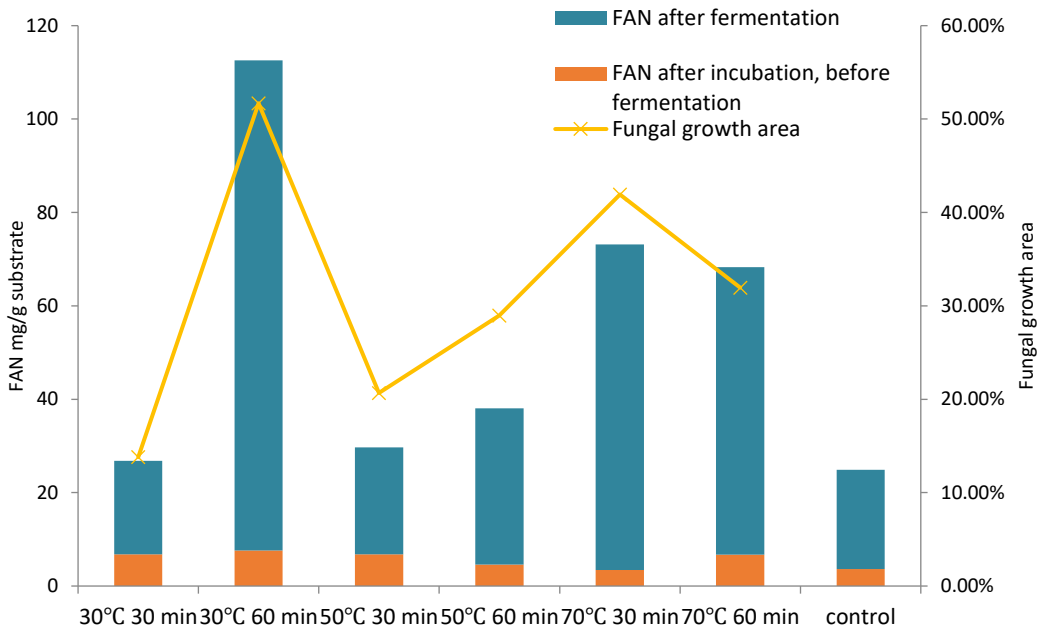

Figure 3-5 FAN productions after pre-incubation at 30°C, 50°C or 70°C for 30 or 60 min, and after 72 h *R.oryzae* fermentation at 30°C. The control was fermented rapeseed meal, but without pre-incubation

The trends between fungal growth and FAN production are consistent. It is clear that fungal growth and FAN production are enhanced by pre-incubation, either by incubating the wet rapeseed meal at a high temperature (70°C) for a short time (30min) or at a lower temperature (30°C) but for longer. The optimal temperature and duration of incubation appear to be 30°C and 1 hour. The increased fungal growth and production brought about by pre-incubation is unlikely to be a result of microbial activity, as the timescale is too short for indigenous microorganisms to have had a noticeable impact. However, there is clear benefit from pre-incubation, even at 30°C, on growth and FAN production. Further investigation, in relation to toxin reduction is reported in Section 3.2.5.

3.2.4 Comparison of *R. oryzae* and *A. oryzae* in Rapeseed Meal Solid State Bioprocessing

As mentioned in Chapter 1, rapeseed meal contains up to 40% protein. *R. oryzae* is not a particularly good protease producer, and since now the need for sterilisation has been established, it was decided to compare *R. oryzae* with *A. oryzae*, another food grade strain isolated from fermented soybean and known for its ability to produce proteases and carbohydrases. In order to do that, dry rapeseed meal was mixed with distilled water to a water content of 66% (w/w). The wet meal was sealed and then incubated at 30℃ for 5 hours, before being autoclaved at 121℃ for 20 min. The sterilised meal was distributed onto petri dishes, so each contained 15 g wet substrate. Then the dishes were divided into 4 groups. Groups 1 and 2 were inoculated with 2 mL and 1.5 mL, respectively, of *R.oryzae* spore suspension. Groups 3 and 4 were inoculated similarly, but with *A.oryzae*. The concentrations of spores in the suspensions were 3.4×10^5 per mL for *R.oryzae* and 3.75×10^5 per mL for *A.oryzae*. Plates were kept at 30℃ for 120 h.

To prevent rapeseed meal from drying out, relative humidity in the incubator was controlled within the range 68% to 93%. A dish containing sterilised, wet rapeseed meal, without any inoculation was used as control. A summary of conditions is given in Table 3-3. Three repetitions were performed. After fermentation, the substrates in parallel dishes was well mixed together by a pester and mortar. The liquid extract was prepared as described in Chapter 3.

Table 3-3 Summary of experimental conditions for rapeseed meal fermented with *R. oryzae* and *A. oryzae*

Group	Species	Inoculum	Incubation time and temperature
1	*R. oryzae*	2 mL, $3.4x10^5$ per mL	30℃ for 120 h
2	*R. oryzae*	1.5 mL $3.4x10^5$ per mL	30℃ for 120 h
3	*A. oryzae*	2 mL, $3.75x10^5$ per mL	30℃ for 120 h
4	*A. oryzae*	1.5 mL, $3.75x10^5$ per mL	30℃ for 120 h

FAN concentration was measured as previously. In order to extract hydrolysing enzyme, fermented substrate was ground using a mortar and pestle. Then, 2 g of the ground meal were taken and put into centrifuge tubes with 5 g of water added. The mixture was vigorously shaken by hand for 30 s then again on an orbital shaker for 1 hour. All tubes were then centrifuged at 10,000 rpm for 10 min to extract supernatant. Enzyme activity was measure as described in Chapter 2. The enzyme activity was then compared with those of water and Viscozyme. The concentration of FAN is shown in Figure 3-7. Protease and carbohydrase activities are shown in Figure 3-7.

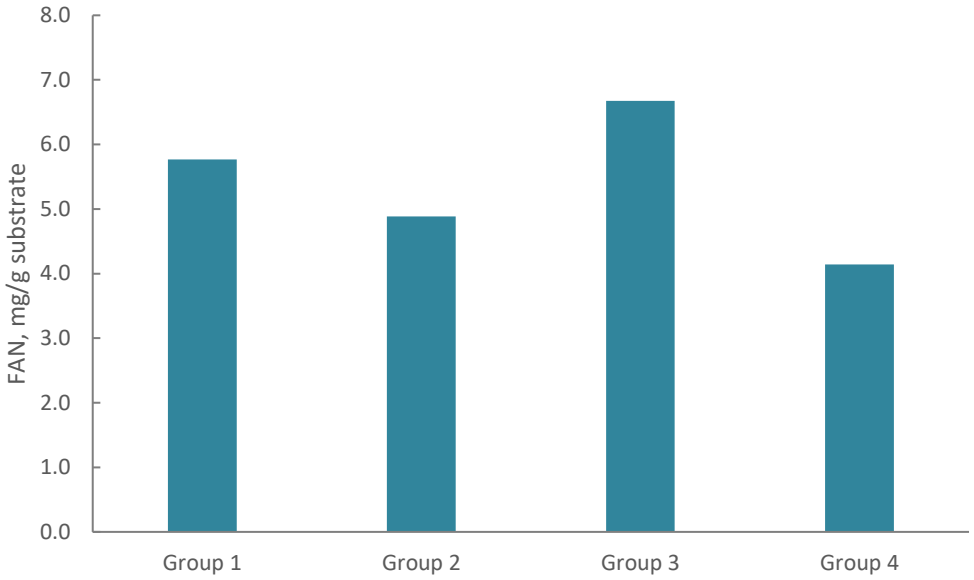

Figure 3-6 FAN concentration after fermentation of rapeseed meal inoculated with *R. oryzae* (group 1 and 2) and *A.oryzae* (group 3 and 4), inoculated with 2 mL (group 1 and 3) or 1.5 mL (group 2 and 4) of spore suspension

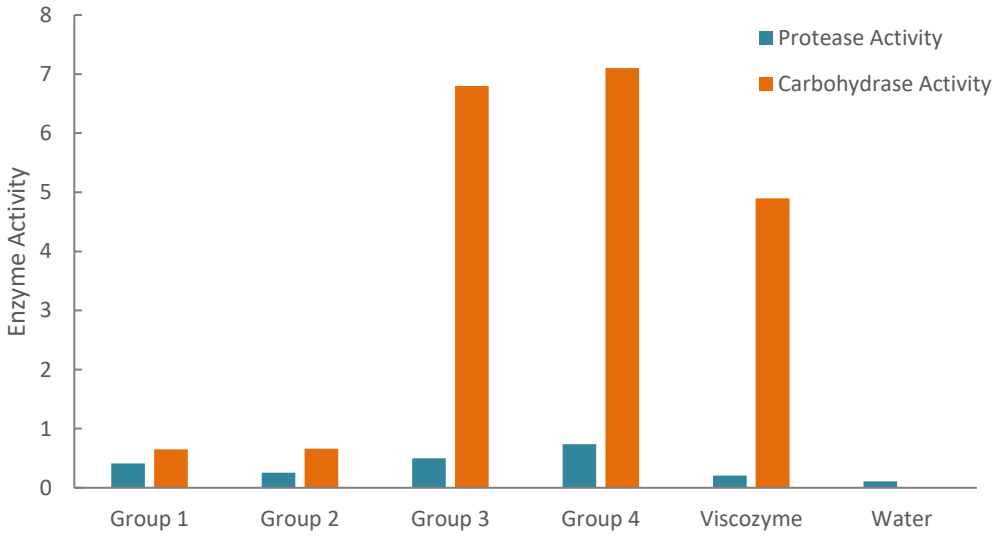

Figure 3-7 Enzyme activity produced from rapeseed meal fermented with *R. oryzae* (group 1 and 2) and *A.oryzae* (group 3 and 4), inoculated with 2 mL (group 1 and 3) or 1.5 mL (group 2 and 4) of spore suspension, compared to the enzyme activities of Viscozyme and water.

The results show that higher inoculum results in a better production of FAN, and that *A.oryzae* produces more FAN than *R.oryzae*. Similar to FAN, the production of hydrolytic enzymes was better in groups fermented by *A.oryzae*, especially for

carbohydrase, where an activity of 5 g enzyme extraction from 2 g of fermented rapeseed meal was better even than the commercial enzyme (Viscozyme). The effect of inoculum on enzyme production was not significant. The success of *A.oryzae* in growing on rapeseed meal and in producing both protease and carbohydrase enzymes suggested it would be highly suitable for solid state bioprocessing, and its potential should therefore be investigated further.

3.2.5 Impacts of Solid State Bioprocessing on Glucosinolate Reduction

Previously in Section 3.2.3, pre-incubation was shown to have a significant impact on enhancing fungal growth in subsequent fermentation, but the mechanism for this was unclear, as was the impact on toxin reduction. Therefore, effects of pre-incubation on glucosinolate reduction over a longer period were investigated. Glucose concentration after pre-incubation and after fermentation was also measured, in order to determine how pre-incubation assists fungal growth. The detailed experimental procedures are described below.

Rapeseed meal containing 66% water (w/w) was thoroughly mixed then incubated at 30℃ for 3, 6, 12, 15, 19, 24 hours, before autoclaving at 121℃ for 20 min. Then the sterilised meal was distributed onto petri dishes, each containing 15 g of substrate. There were 5 identical petri dishes in each group. These dishes were inoculated with 2 mL *R.oryzae* spore suspension ($2.5x10^5$ per mL). All dishes were incubated at 30℃ for 210 h. After fermentation, the substrates in parallel dishes was well mixed together by a pester and mortar. The liquid extract was prepared as described in Chapter 3.

Samples were taken after pre-incubation, before autoclave, except for the group that was incubated for 12 h before fermentation, due to access restrictions. Glucosinolate concentration in all samples was measured as described previously. Glucose concentration in the supernatant was also measured and shown in Figure 3-8. Raw rapeseed meal was used as a control.

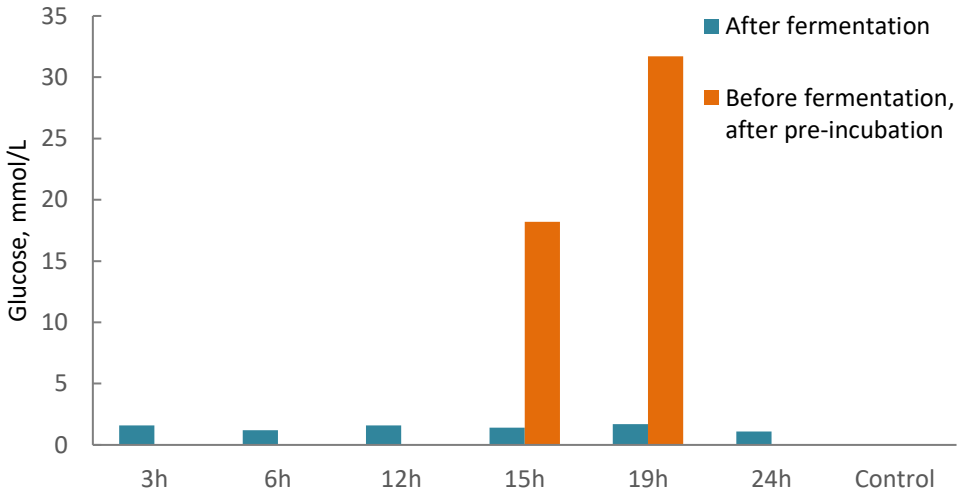

Figure 3-8 Glucose concentration (from rapeseed meal) after pre-incubation at 30°C for 3, 6, 12, 15, 19, 24 h, and after 210 h fermentation with *A.oryzae*. Data for 12 h pre-incubation before fermentation were not collected due to access restriction

The results show that significant quantities of glucose were detected only in the samples subjected to 15 and 19 h of pre-incubation. Therefore, the enhanced fungal growth observed with just 1 h of pre-incubation in previous experiments was not because of glucose production during the pre-incubation (see Section 3.2.3). The highest glucose concentration was observed after 19 h of pre-incubation, though none was detectable in the 24 h pre-incubation group. It is likely that the production of glucose was the result of indigenous microbial activity and that hydrolysing enzymes were excreted, resulting in glucose production from carbohydrates (and subsequent consumption). The accumulated glucose was soon consumed by the growing microorganisms, in samples with more than 19 h

of pre-incubation. After 210 h of fermentation, however, the glucose concentration for all groups was at a very low level (<2 mmol/L), indicating that demand was greater than supply.

In order to determine the effect of pre-incubation and fermentation on toxin reduction, glucosinolates concentrations before and after fermentation were measured and are shown in Figure 3-9 and Figure 3-10.

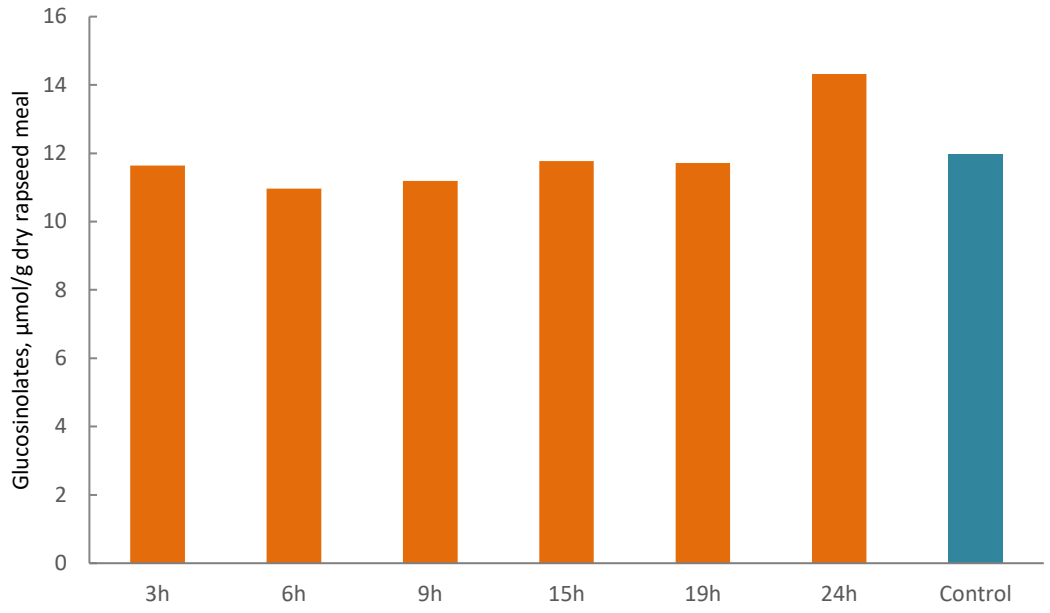

Figure 3-9 Glucosinolates concentration in rapeseed meal pre-incubated for 3, 6, 9, 15, 19 and 24 h at 30°C, control represents untreated rapeseed meal

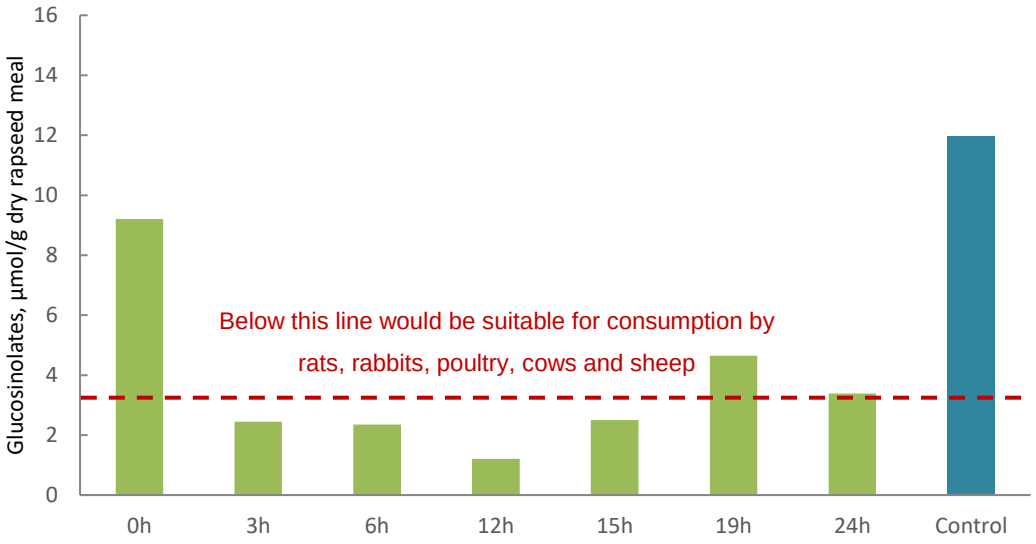

Figure 3-10 Glucosinolates concentration in rapeseed meal after 0, 3, 6, 9, 12, 15, 19 and 24 h pre-incubation plus 210 h fermentation with *A.oryzae* at 30°C, control represents untreated rapeseed meal

From Figure 3-9 and Figure 3-10 it can be seen that pre-incubation on its own did not reduce glucosinolates. The concentration of toxins remained at a similar level to that in untreated rapeseed meal. The slightly higher concentration after pre-incubation for 24 h was deemed to be within the range of normal variations between different rapeseed meal samples.

On the other hand, fermentation by *A.oryzae* did clearly reduce toxin levels, and this reduction was significantly enhanced by pre-incubation. Without pre-incubation, glucosinolates concentration in fermented samples was 9.2 µmol/g dry substrate, compared to 11.97 µmol/g in untreated rapeseed meal. However, if pre-incubation was applied before the fermentation, even just for 3 h, glucosinolates were reduced to a level which is safe for rats, rabbits, poultry, cows and sheep to consume. The lowest level of toxin appeared in the group incubated for 12 h, where glucosinolates concentration was reduced to just 1.21 µmol/g dry substrate.

3.3 Discussion and Concluding Remarks

Taking all of the results of the above experiments together, it seems clear that, the major toxin in rapeseed meal, glucosinolates, can be reduced by solid state bioprocessing, to a level suitable for animal consumption. The solid state bioprocessing involves two stages, pre-incubation and fermentation. During the first stage, within a few hours, a certain form of reaction takes place after the rapeseed meal has been wetted and placed in the incubator. It is not clear the exact mechanism, but it is clear that water has played a critical role in this process.

It is possible that with the help of water, toxins were exposed in a form that is more vulnerable to fungal bioconversion. The longer the pre-incubation, the better will be the toxin reduction during subsequent fermentation. Taking the results shown in Figure 3-5 into consideration, fungal growth can also be significantly enhanced even after just 1 hour of pre-incubation of rapeseed meal with water. Therefore, the reaction is more likely to be enzymatic or chemical, rather than microbial. Enzymatic reactions can be the result of naturally occurring enzymes present in the rapeseed, e.g. myrosinase, and having survived the process of oil extraction. When water was added prior to pre-treatment, these enzymes could be activated and the toxins hydrolysed into a form which is easier for microorganisms to convert or consume. As a consequence, both toxin reduction and fungal growth were enhanced by pre-treatment. Chemical reaction can also take place after water has been added (the pH of wet rapeseed meal was 5.6). Whichever form the reaction is, it is not sufficient on its own to reduce the glucosinolates, but does appear to reduce the barrier for fungi to consume the toxins in the second stage, solid state fermentation. This could be either transforming glucosinolates to a form that fungi can consume, or generating certain products that can assist fungal growth in later

fermentation, or perhaps eliminating substances toxic to the fungi. This also means that the original hypothesis, which suggested that the naturally occurring microorganisms carried by raw rapeseed meal had an ability to hydrolyse the toxins, was not quite correct.

It would be fascinating to know how water assists in the pre-incubation and why it has such a significant impact on fungal growth and toxin reduction. However, at the moment, we do not yet possess sufficient understanding of water, either in terms of its effects on solid state fermentation or on toxin reduction. As a result, this prevents meaningful interpretation of the results being made and hence, full advantage cannot be taken of what solid state bioprocessing could offer.

In addition, *A.oryzae* was shown to have an excellent ability to produce enzyme with wet rapeseed meal as substrate, the enzyme activity was surprisingly better than that of a concentrated commercial enzyme. It is not clear though, whether this is a result of using rapeseed meal as the substrate, or effects brought about by pre-incubation, or solid state fermentation, or the fungi themselves, but the result did reveal a potential of utilising rapeseed meal to produce hydrolytic enzymes. This was investigated further and is reported in Chapter 4.

Nonetheless, solid state bioprocessing provides a very promising prospect for reducing toxins in rapeseed meal, however, there are still problems that need to be addressed: there is insufficient understanding of the role of water in solid state bioprocessing; it is not clear why fungal growth can be so uneven on the same substrate; it is not clear how to reduce maldistribution or how it affects the overall fermentation performance.

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Chapter 4.

SSB Application II:

Producing a Microbial Feedstock

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Chapter 4

SSB Application II: Producing a Microbial Feedstock

Results in Chapter 3 show that after solid state bioprocessing using rapeseed meal as substrate, hydrolytic enzymes, both proteases and carbohydrases, were produced. The enzyme activity was considerable, especially for the carbohydrases, which was even better than a commercial enzyme (Viscozyme). Since the price of commercial carbohydrase is much higher than that of animal feed (£1000 per litre enzyme, in comparison with £170 – £300 per ton animal feed), enzyme production could clearly be an attractive proposition. Therefore it was decided to explore the potential of producing enzymes through solid state bioprocessing, and the results are presented in this chapter.

Furthermore, as discussed in Chapter 1, rapeseed meal contains a comprehensive range of nutrients for many microorganisms. However, most of these nutrients are not in a form that is directly accessible for industrially important strains, e.g. yeasts. This is because most are macromolecules, and not all microorganisms can produce the necessary hydrolytic enzymes to break them down. In 2010, Wang et al [81] demonstrated that through a combination of solid state fermentation and fungal autolysis, it was possible to hydrolyse such macromolecules into small, simple nutrients to create a generic microbial feedstock. According to the report, the first stage was producing *in situ* hydrolytic enzymes through solid state fermentation. The second stage was incubating the fermented substrate at a temperature higher than that used for fermentation, to accelerate further hydrolysis by *in situ* enzymes. Incubation at high temperature

also triggered fungal autolysis, a process releasing nutrients from the cells back into the feedstock [81]. However, the authors noticed that the resultant feedstock did not have sufficient carbon source, in comparison with the amount of nitrogen present, especially for yeasts. After a short period of cultivation using the feedstock, yeasts soon suffered from carbon deficiency while plenty of free amino acids were still left. Wang et al compensated the carbon deficiency by adding glucose as an additional carbon source.

This Chapter explores the potential of using mixed agriculture wastes, namely sugarcane bagasse and rapeseed meal, to create a balanced generic microbial feedstock. As mentioned in Chapter 1, sugarcane bagasse is cheap and rich in cellulose which makes it ideal to compensate the fact that rapeseed meal alone has insufficient carbon.

The first part of the work reported in this chapter was aimed at developing a solid state bioprocess for utilising sugarcane bagasse and rapeseed meal to produce a feedstock suitable for the growth of microorganisms. Based on these experimental results, the second part of this chapter is focused on increasing the repeatability and reliability of the solid state fermentation. General materials and experimental procedures are as listed in Chapter 2. The experimental programme related to the work reported in this chapter is reported below.

4.1 Experimental Programme

In the first part of this experimental programme, a three-stage solid state bioprocess, consisting of pre-treatment, solid state fermentation and fungal autolysis, was designed and tested, for using sugarcane bagasse and rapeseed

meal to produce a generic microbial feedstock. The feedstock was evaluated for its enzyme activity and its content of simple nutrients, namely reducing sugar and FAN. The accessibility of these nutrients was then tested by culturing *Saccharomyces cerevisiae* in the feedstock. However, results proved to be unreliable, as significantly different outcomes were obtained from parallel groups cultivated under the same conditions. This was especially the case for groups tested immediately after the solid state fermentation.

The second part of work was therefore aimed at increasing the repeatability and reliability of solid state fermentation. Using an orthogonal design approach, a set of experiments was conducted to test the effects of combinations of selected conditions on fermentation performance. The results are reported in this chapter.

4.1.1 Producing a Generic Microbial Feedstock from Rapeseed Meal and Sugarcane Bagasse

As mentioned above, a three-stage solid state bioprocess was designed and conducted to produce a generic microbial feedstock using rapeseed meal and sugarcane bagasse. The first stage was pre-treatment. In this stage, the sugarcane bagasse and rapeseed meal were adjusted and modified, in terms of their physical and chemical properties, to create a substrate suitable for fungal growth. To adjust the physical properties of the substrate, particle size of both sugarcane bagasse and rapeseed meal was controlled below 1.4 mm based on previous research [54]. For chemical aspects, based on the results shown in Chapter 3 that pre-incubation can enhance fungal growth on rapeseed meal, sugarcane bagasse and rapeseed meal was well mixed with water and then pre-incubated for 12 h.

The second stage was solid state fermentation to produce hydrolytic enzymes, and to convert macromolecules into simple nutrients. As reported in Chapter 3, *A. oryzae* is a good enzyme producer with rapeseed meal as substrate, but its ability to utilise cellulosic material (sugarcane bagasse) was not known. Therefore, *A. awamori,* a better cellulase producer, was selected and compared. The mixed substrate was inoculated with fungal suspension and was then incubated at 30℃ for 72 h.

The third stage was fungal autolysis. This involved incubating the fermented substrate at a temperature higher than that used for fermentation, to create an environment suitable for further enzymatic hydrolysis of the substrate and to trigger fungal autolysis to release nutrients from the fungal cells. The fermented meal was mixed with water and incubated at 55℃ for 72 h. As recommended by Wang et al [81], the ratio between fermented substrate and water added was controlled at 1:50 (w/w). In addition, to increase the concentration of nutrients in the feedstock and the enzyme activity, another group was autolysed with a much lower substrate/water ratio (1:3, w/w) under the same condition.

To evaluate the accessibility of nutrients in the microbial feedstock, a subsequent 'downstream' fermentation with *Saccharomyces cerevisiae* was conducted at 30℃ for 72 h in sealed flasks. A summary flow diagram of the experiments is shown in Figure 4-1.

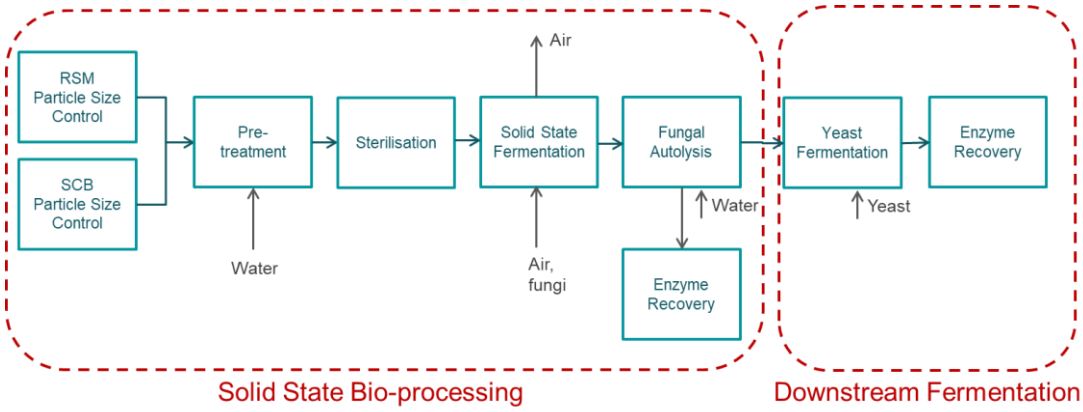

Figure 4-1 Process Flow Diagram of enzyme and generic microbial feedstock production, RSM: rapeseed meal; SCB: sugarcane bagasse

4.1.2 Improving Consistency of Solid State Fermentation Results

In order to improve the repeatability and reliability of solid state fermentation using rapeseed meal and sugarcane bagasse, a set of experiments was designed using orthogonal approach. As discussed in Chapter 1, experimental conditions affecting the performance of solid state fermentation include substrate composition, substrate particle size, water availability, pH, microorganism species, inoculum size, fermentation temperature, and oxygen availability. Fermentation conditions selected for the orthogonal experiment design were water content, pH, inoculum size and fermentation temperature. The selection was based on previous results and equipment availability. Other factors affecting solid state fermentation were controlled at the same levels for all groups.

4.2 Procedures and Results

4.2.1 Producing a Generic Microbial Feedstock from Rapeseed Meal and Sugarcane Bagasse

Rapeseed meal and sugarcane bagasse were both dried at room temperature and then sieved prior to the experiment as described in Chapter 2. The particle size for both materials was less than 1.4 mm. Due to the significant difference of density between sugarcane bagasse and rapeseed meal, the ratio of two raw materials was set at 2:1 (w/w). Of this ratio, the volume of rapeseed meal and sugarcane bagasse are at a similar level, which contributes to an even distribution of the two materials. Water content was adjusted to 65% (w/w). The two raw materials were then well mixed by hands for 5 minutes before pre-incubating at 30℃ for 12 h.

After pre-incubation, the mixed substrate was autoclaved at 121℃ for 20 min, then distributed onto petri dishes. Each dish contained 10 g of substrate and 5 identical dishes were made. Then they were divided into four groups. Spore suspensions of *A. oryzae* and *A. awamori* were prepared as described in Chapter 2 with 7.5 x 10^6 and 6.5 x 10^6 spores/mL, respectively. Groups 1 and 2 were inoculated with *A. awamori* and groups 3 and 4 were inoculated with *A. oryzae*. The inoculum for each dish was 1 mL of spore suspension. Fermentation was carried out in a 30℃ incubator for 72 hours and humidity was kept above 60%.

After fermentation, the fermented substrates in the same group were well mixed together using a pestle and mortar. A few grams of the mixed substrates were taken transferring into flasks and mixing with distilled water. The meal/water ratio

for groups 1 and 3 was 5 g meal with 15 g water (1:3, w/w), and for groups 2 and 4 was 2 g of meal and 100 g of water (1:50, w/w). The flasks were then sealed, and autolysis was carried out in 55℃ incubators for 72 h while stirring by magnetic stirrers. Groups 1 and 3 were designated as 'solid' autolysis and groups 2 and 4 as 'liquid' autolysis. A duplicate was made for each group.

After 72 h of autolysis, the resultant feedstock was inoculated with directly 10% (w/w) of *S. cerevisiae* without further sterilisation. The yeast was prepared as described in Chapter 2. The flasks were sealed and stirred with magnetic stirrers and the downstream fermentation was carried out at 30℃ for 7 days.

Samples were taken at the end of the solid state fermentation stage, the autolysis stage and after the downstream fermentation stage. Free amino nitrogen, reducing sugar content and enzyme activity in the liquid phase were measured as described in Chapter 2. Detailed experimental conditions are summarised in Table 4-1.

Table 4-1 Experimental conditions for producing generic microbial feedstock utilising rapeseed meal and sugarcane bagasse

Group	Pre-incubation		Solid state fermentation		Autolysis		Downstream fermentation	
	Temperature (℃)	Time (h)	Strain	Time (h)	Meal: water ratio	Time (h)	Strain	Time (h)
1	30	12	*A. awamori*	72	1:3	72	*S. cerevisiae*	72
2	50	12	*A. awamori*	72	1:50	72	*S. cerevisiae*	72
3	30	12	*A. oryzae*	72	1:3	72	*S. cerevisiae*	72
4	50	12	*A. oryzae*	72	1:50	72	*S. cerevisiae*	72

Reducing sugar and free amino nitrogen concentrations after each of the solid state fermentation, autolysis and downstream fermentation stages are shown in Figure 4-2 and Figure 4-3. From the results it can be seen that, after solid state

fermentation, the production of reducing sugar was higher than of amino acids, the difference between groups inoculated with different strains is very small.

For the next stage in the process, the concentration of both nutrients after autolysis was much lower than after solid state fermentation. The reduction was much more significant in free amino acids concentration than reducing sugar. This suggests that microbial activity was taking place during autolysis and consumed the nutrients. It is not clear whether the activity was of the inoculated strains, or of contaminating microorganisms. The difference between groups inoculated with different strains was, as with the fermentation stage, not significant. However, the water/substrate ratio applied for autolysis did have a significant impact on the level of sugar reduction: residual reducing sugar in liquid autolysed samples was much higher than in the solid autolysed samples. Though this was not the case for free amino nitrogen.

The final stage in the process was a downstream fermentation to test the suitability of nutrients produced from solid state bioprocessing for microbial growth. After this yeast fermentation, for groups 1 and 3, where solid autolysis was applied, reducing sugar was lower than in the previous stage. For groups 2 and 4, where liquid autolysis was applied, reducing sugar was higher. On the other hand, free amino nitrogen concentrations in all groups were higher than they had been directly after autolysis.

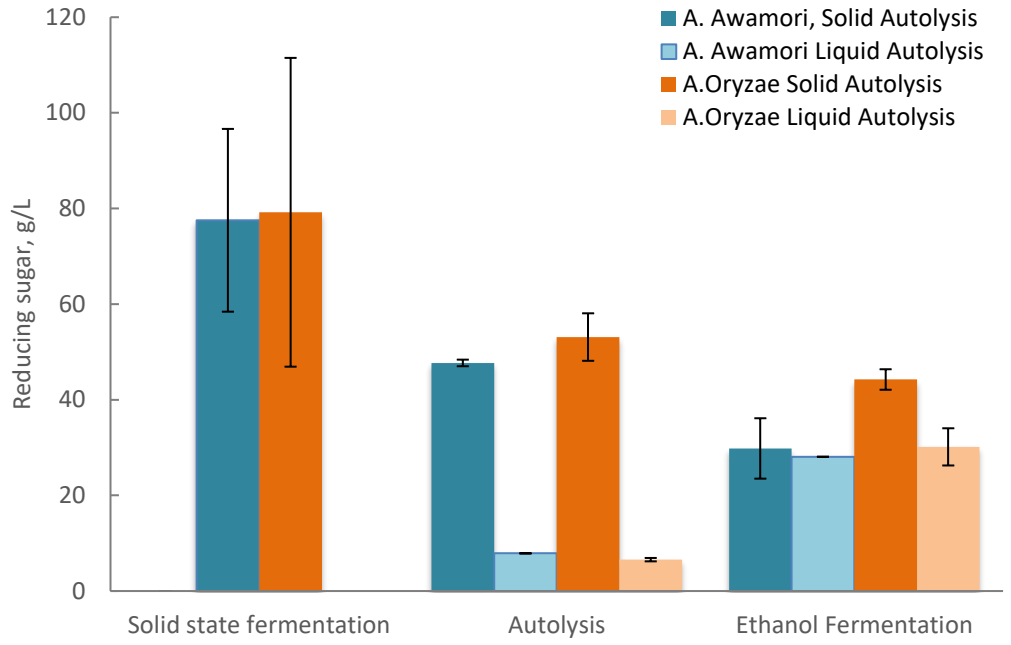

Figure 4-2 Reducing sugar concentration after solid state fermentation (with *A.oryzae* and *A. awamori* at 30℃ for 72 h), fungal autolysis (at 30℃ for 72 h) and yeast fermentation (with *S. cerevisiae* at 30℃ for 7 days), The bars represent average values of duplicate plates, and error bars represent actual data values

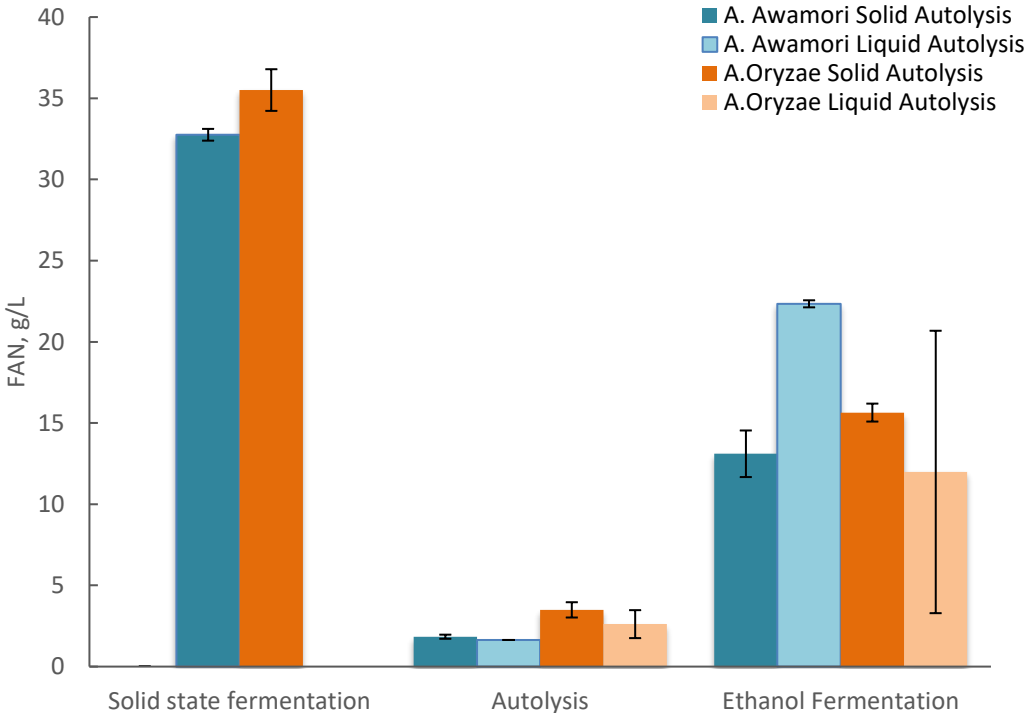

Figure 4-3 Free amino acid concentration after solid state fermentation (with *A.oryzae* and *A. awamori* at 30℃ for 72 h), fungal autolysis (at 30℃ for 72 h) and yeast fermentation (with *S. cerevisiae* at 30℃ for 7 days. The bars represent average values of duplicate plates, and error bars represent actual data values

As shown in Figure 4-4 and Figure 4-5, both cellulase and protease activities were lower after autolysis than after solid state fermentation. Consistent with previous observation (Chapter 3), enzyme levels, higher than those in a commercial preparation, were obtained, in this case for protease. *A. oryzae* was better at producing protease and *A. awamori* was better at producing cellulase. The water/substrate ratio applied in autolysis did not have a noticeable impact on protease activity, but did have an influence on cellulase activity: samples processed with liquid autolysis had higher cellulase activity than with solid autolysis.

For the downstream fermentation, both cellulase and protease activities were higher than directly after autolysis. Again for cellulase activity, the differences between fungal strains were less than between process conditions. No such difference was observed for protease.

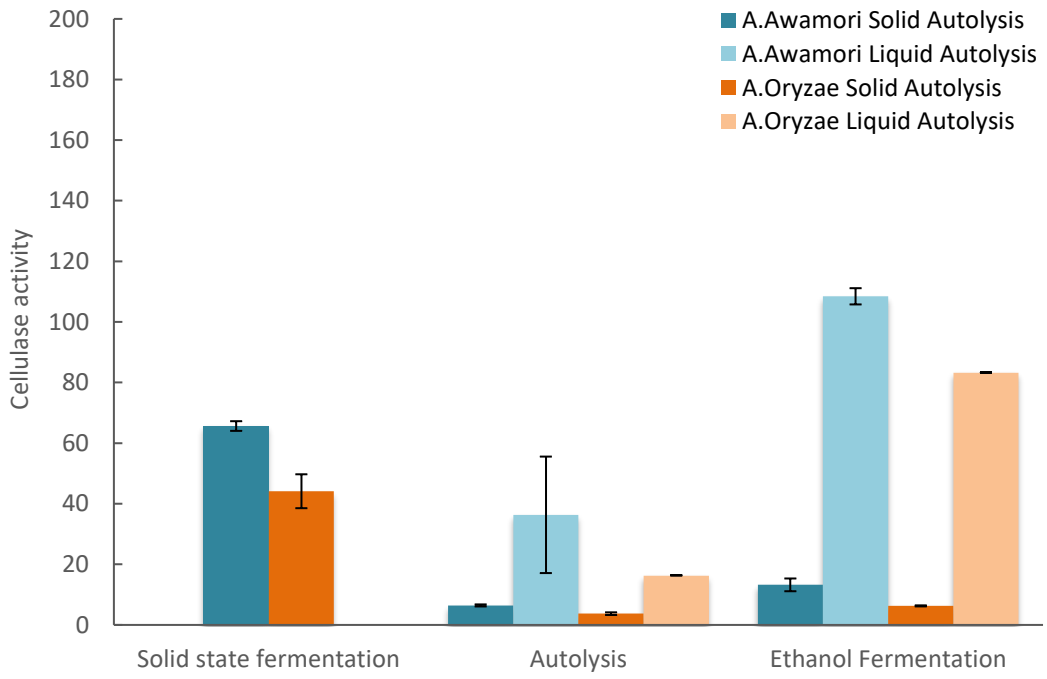

Figure 4-4 Cellulase activity, after solid state fermentation (with *A.oryzae* and *A. awamori* at 30°C for 72 h), fungal autolysis (at 30°C for 72 h) and yeast fermentation (with *S. cerevisiae* at 30°C for 7 days). The bars represent average values of duplicate plates, and error bars represent actual data values

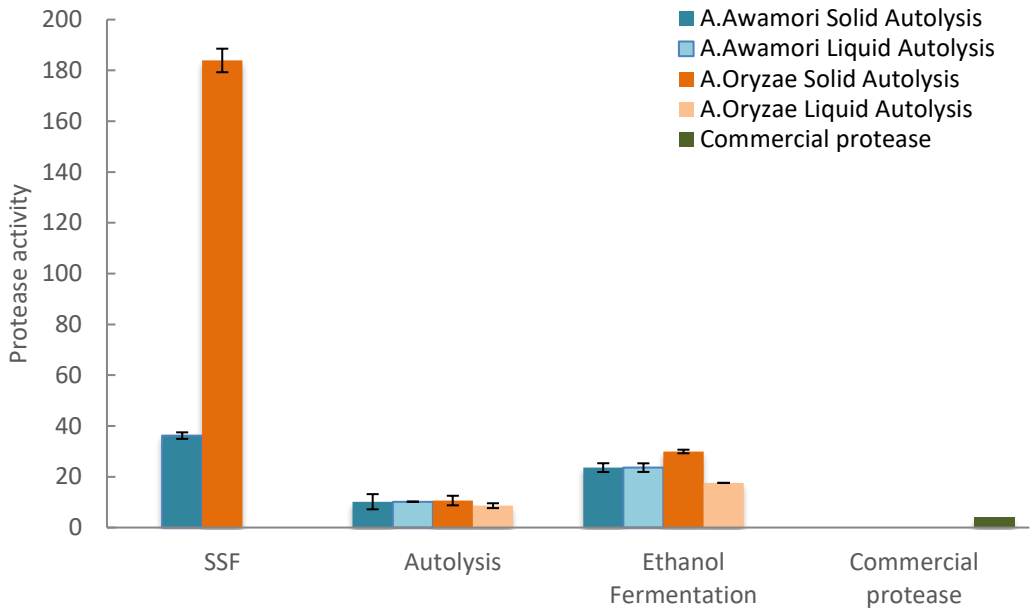

Figure 4-5 Protease activity after solid state fermentation (with *A.oryzae* and *A. awamori* at 30°C for 72 h), fungal autolysis (at 30°C for 72 h) and yeast fermentation (with *S. cerevisiae* at 30°C for 7 days), in comparison with a commercial protease. The bars represent average values of duplicate plates, and error bars represent actual data values

It is worth mentioning that some of the error bars in the above figures are very large, which represents significant differences between parallel experiments. This potentially undermines confidence in the reliability of the results and prevents conclusive interpretation. In summary, the outcome of this set of experiments was not as expected, particularly changes occurring during autolysis need further investigation. However, before further investigation, it is critically important to improve the reliability, repeatability and predictability of solid state fermentation. In order to do this, effects of pre-treatment, different pH, water content, inoculum size and fermentation temperature on solid state fermentation were studied using a set of experiments based on orthogonal design.

4.2.2 Improving Consistency of Solid State Fermentation Results

To study the effects of fermentation conditions on the performance of solid state fermentation, four factors were selected: pH, water content, inoculum size and fermentation temperature, each factor was tested at 3 levels. Fermentation performance was evaluated using protease activity as an indicator. This was chosen because, compared with residual nutrients, enzymes are only generated by fungal activity while nutrient concentrations are a balance between production and consumption. In addition, generic microbial feedstocks are produced by enzymatic hydrolysis of the substrate, therefore, enzyme activity is a better indicator of fermentation performance.

Rapeseed meal and sugarcane bagasse were both dried at room temperature and sieved prior to the experiment. The particle size for both materials was controlled to be less than 1.4 mm. The rapeseed meal was then mixed with the sugarcane bagasse at a ratio of 3:2 (w/w). PBS buffered water was made at pH of 5, 5.7 and 7 and was mixed with the substrate to reach moisture contents of 75.0%, 90% and 95% (w/w). To study the effect of pre-incubation, the substrate was divided into two sets: the first was autoclaved directly after the buffer was mixed with the substrate (i.e. “Not pre-incubated”). The others were mixed with buffered water and “Pre-incubated” for 24 h before being autoclaved at 121℃ for 20 min.

After autoclaving, the substrate was distributed onto petri dishes. Each dish contained 10 g of substrate and was inoculated with *A. oryzae* spores. The spore suspension was prepared as described in Chapter 2 with 7.5 x 10^6 spores/mL. Inoculum size was set to 5%, 10% and 15%. Fermentation was carried out at 30℃, 35℃ and 37℃ for 96 hours; humidity was keep above 60%. All experiments were performed with duplicates. After the fermentation, enzymes were extracted and the activity measured as described in Chapter 2. A summary of the orthogonal

experiment design is given in Table 4-2. Impacts of fermentation conditions (water content, pH, fermentation temperature and inoculum size) on protease activity is shown in Figure 4-6 to Figure 4-9.

Table 4-2 Orthogonal experiment design for improving the reliability of solid state fermentation based on fermentation with *A.oryzae* for 96 h

Group	Water Content	Inoculum （v/w）	pH	Temperature
0	75%	5%	uncontrolled	30℃
1	75%	5%	5	30℃
2	80%	10%	5.7	35℃
3	80%	15%	7	37℃
4	95%	5%	5.7	37℃
5	90%	10%	7	30℃
6	95%	15%	5	35℃
7	90%	5%	7	35℃
8	95%	15%	5.7	30℃

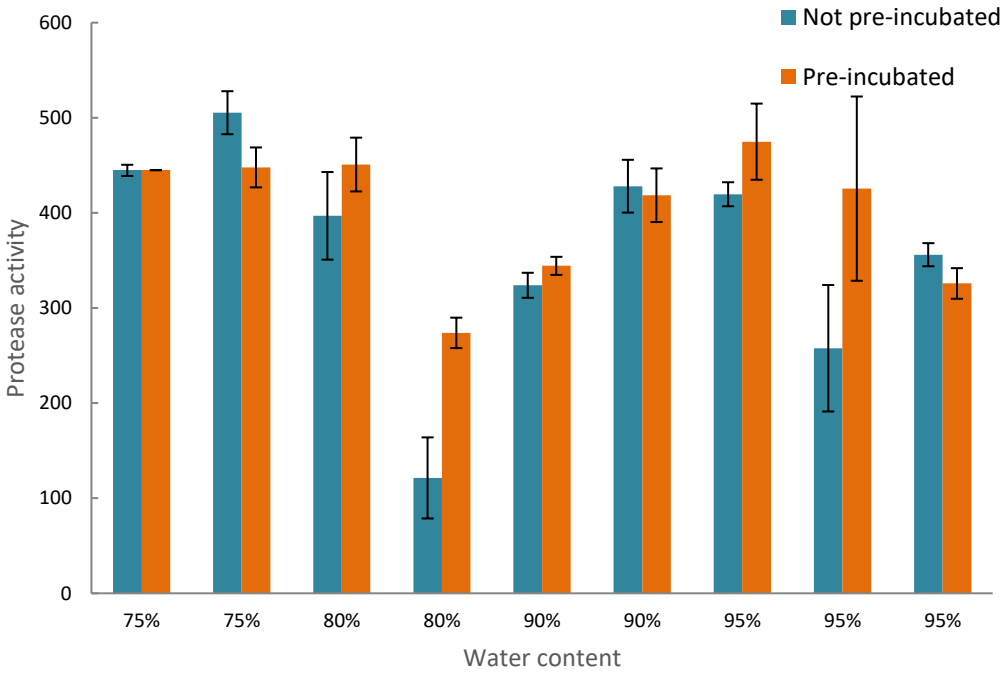

Figure 4-6 *Effect of water content on protease activity after 96 h fermentation with A.oryzae. The bars represent average values of duplicate plates, and error bars represent actual data values*

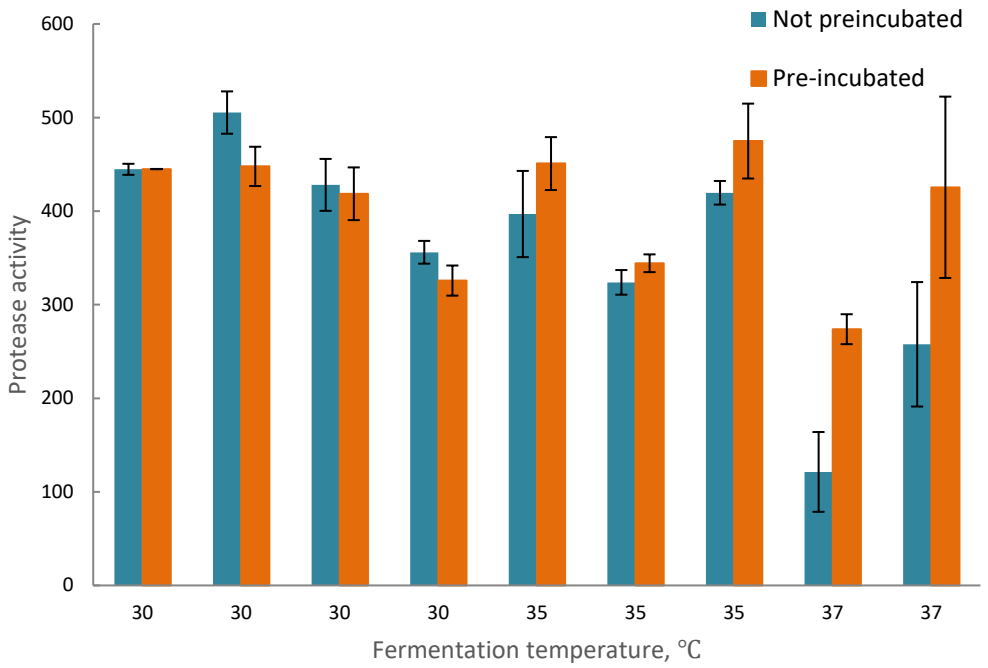

Figure 4-7 Effect of fermentation on protease activity after 96 h fermentation with *A.oryzae*. The bars represent average values of duplicate plates, and error bars represent actual data values

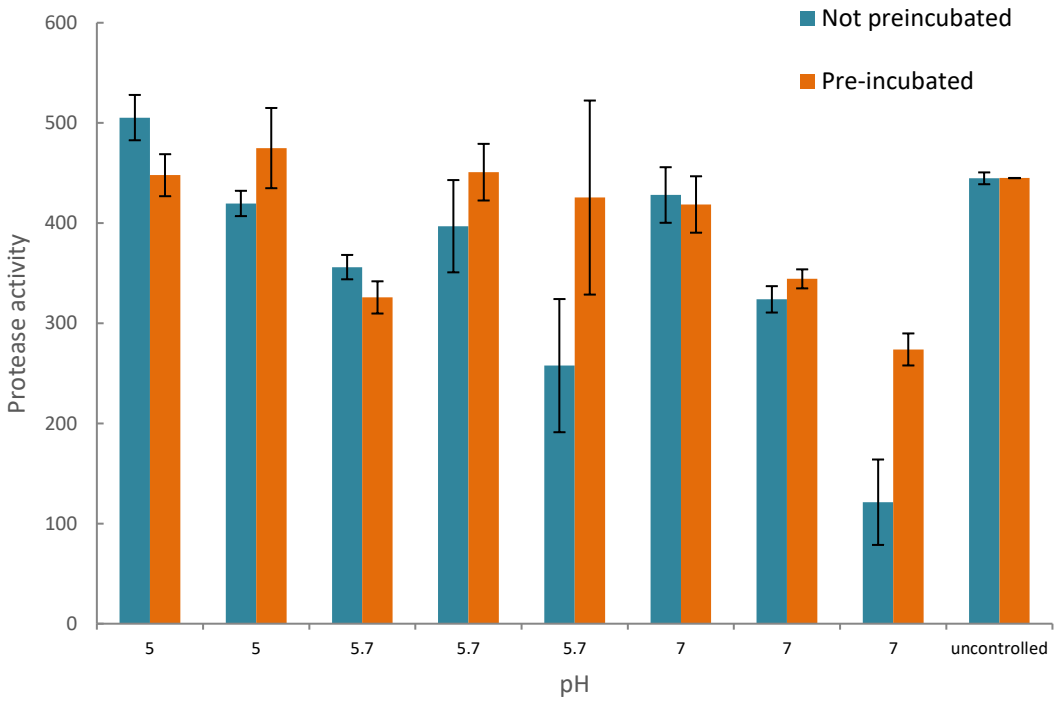

Figure 4-8 Effect of pH on protease activity after 96 h fermentation with *A.oryzae*. The bars represent average values of duplicate plates, and error bars represent actual data values

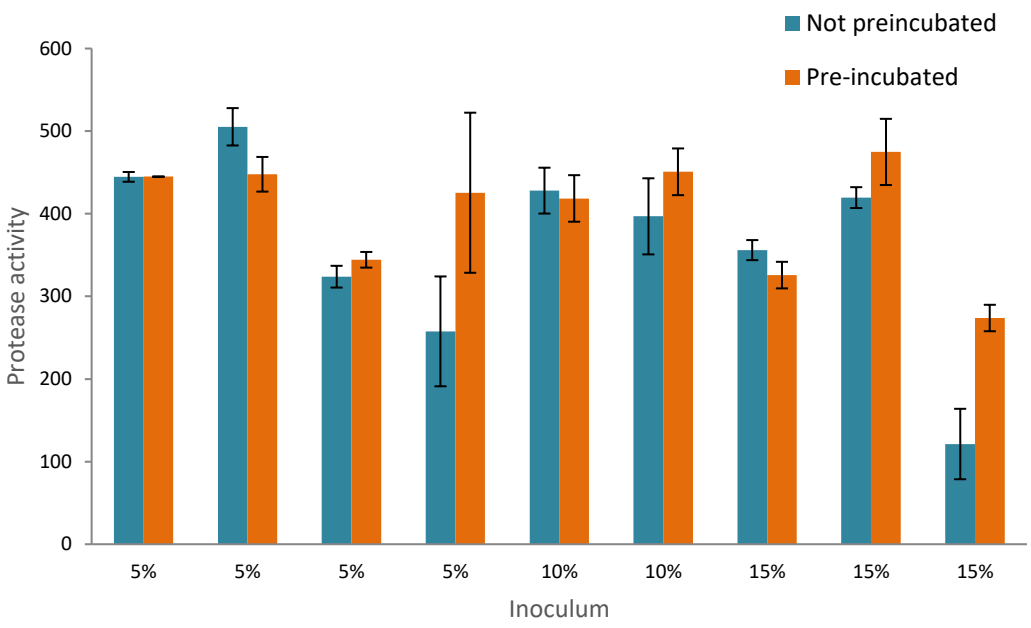

Figure 4-9 Effect of inoculum on protease activity after 96 h fermentation with *A.oryzae*. The bars represent average values of duplicate plates, and error bars represent actual data values

The results show, among all the fermentation conditions tested in this series of experiments, that the factor that can influence enzyme production in the most negative way is high fermentation temperature. Enzymes produced in all groups fermented at 37 ℃ had much lower activity than those produced at other temperatures. This adverse impact was slightly compensated by increasing water content in the substrate. On the other hand, the factor that can influence enzyme production in the most positive way is low pH. All groups with initial pH 5 resulted in higher enzyme activity than the others.

The results also show that the influence of water on enzyme production is highly dependent on the fermentation temperature. At lower temperatures, enzyme activity is higher in groups with lower water content, while at higher temperature, it is higher in groups with higher water content. Surprisingly, larger inocula did not give rise to a higher enzyme activity, which suggests that it does not compensate for the adverse impacts of fermentation temperature or lack of water. Adverse fermentation conditions not only resulted in lower enzyme activity, but were also

associated with much larger error bars. Indeed, whenever production was good, results were much less variable between parallel groups than when enzyme production was poor. In general, compared with samples fermented without pre-incubation, the pre-incubated groups had higher enzyme activity, especially under adverse conditions. Interestingly, these samples also showed much better reproducibility.

4.3 Discussion

4.3.1 Producing Generic Microbial Feedstock Utilising Rapeseed Meal and Sugarcane Bagasse

Both *A.oryzae* and *A.awamori* were able to produce small molecular nutrients (reducing sugars and free amino nitrogen) from the macromolecules in the combined substrate composed of rapeseed meal and sugarcane bagasse. Both fungi were able to produce both protease and cellulase after solid state fermentation, the difference between fungal strains in this set of experiments was not significant, compared to the differences between individual process stages or different process conditions.

In all experimental groups, both reducing sugar and free amino nitrogen concentrations were lower after autolysis than after solid state fermentation. This was not as expected from the original hypothesis, that autolysis can facilitate further enzyme hydrolysis of macromolecular nutrients into small ones. This could be because microbial activity was taking place during autolysis, which consumed the nutrients. The level of consumption was affected by the amount of water present during autolysis: more nitrogen source was consumed than carbon source

in situations where water was scarce. However, if water was plentiful, both nutrients were consumed readily.

Following autolysis and without further sterilisation, the process was continued to downstream yeast fermentation. After this final stage, the nutrient concentration was higher than it had been after autolysis except for those groups where solid autolysis had been applied. Again, this is contrary to the original hypothesis that yeast would consume the nutrients produced by the bioprocess. This suggests that, during yeast fermentation, further enzymatic hydrolysis of the substrate took place, for which production was greater than the microbial consumption. This could be the action of enzymes produced previously during solid state fermentation. It could also be because the spores produced during solid state fermentation germinated and grew during the downstream fermentation, though they did not grow very well in feedstocks produced via solid autolysis. The change in reducing sugar concentration suggests the latter: both reducing sugar consumption and cellulase activity were lower in solid autolysed groups than in liquid autolysed groups. This also suggests the enzymes produced in the first stage, solid state fermentation, may lose some of their activity. This was consistent with the observation that both cellulase and protease activities were lower after autolysis than after solid state fermentation.

The reduction in enzyme activity is possibly because enzymes are proteins and the proteases present may have hydrolysed them. This would, of course, result in the production of free amino acids but, as mentioned earlier, there was still microbial activity during the autolysis stage, so it is likely that the free amino acids were quickly consumed by those microorganisms, resulting in no net free amino nitrogen production. If the microorganisms active during autolysis were contaminations, they may have changed the pH of the substrate or excreted chemicals which result in the denaturation of cellulase and protease enzymes.

After autolysis, the process moves on to downstream yeast fermentation. After this final stage, the concentration of nutrients was higher than after autolysis except for those groups processed with solid autolysis. This could be due to the hydrolytic action of enzymes, which were produced in the solid state fermentation stage, or to microbial activity resulting from spores, also produced during solid state fermentation that germinated and grew. The change in reducing sugar concentration suggests the latter: i.e. if sufficient reducing sugar was present in the substrate (groups 1 and 3), microorganisms would consume the sugar first. However, if not sufficient (i.e. groups 2 and 4), then microorganisms would produce hydrolytic enzymes to hydrolyse the macromolecules in the substrates. This also suggests that enzymes produced during the first stage lose some of their activity.

4.3.2 Improving Consistency of Solid State Fermentation Results

The results presented in Figure 4-6 to Figure 4-9 show that, among the various fermentation conditions tested, high incubation temperature influenced enzyme production in the most negative way, and was only compensated by water content but not by inoculum size. This is because high temperatures are not only unsuitable for fungal growth but also accelerate water evaporation, resulting in less water being available for the fungi. Perhaps this is why many aerobic fermentations take place at lower temperatures than anaerobic fermentations.

The results also suggest that performance of solid state fermentation is unrelated to inoculum size but more closely related to the immediately microenvironment of the spores. In other words, if the fungi cannot grow well, it doesn't matter how many spores are inoculated into the substrate, they still cannot produce a lot of protease. This is because extracellular enzymes are largely produced in response

to diminishing nutrient availability within the vicinity, i.e. to produce more food, while unfavourable conditions, e.g. high temperatures, are more likely to result in spore production, i.e. to seek new environments. However, without sufficient hydrolysing enzymes, the fungus will also suffer from a shortage of nutrients, resulting in a worse environment for survival.

In contrast, the factor that influenced enzyme production most positively was low pH. All groups incubated on substrate mixed with pH 5 buffer resulted in higher enzyme activity. This is probably because *A.oryzae* is an acid producer and prefers an acidic environment. When buffer was applied, the pH was maintained at a certain level, even if the level was an unfavourable one. Hence, the group where pH was not uncontrolled resulted in higher enzyme production than those controlled at unfavourable levels.

In terms of reproducibility, the reason why better reproducibility appeared in the groups yielding higher enzyme production is possibly related with the heterogeneous nature of the solid substrate. Even though the substrate was well mixed and parallel dishes were fermented at the same temperature, the microscopic environments can be different from one to another within the same substrate, in terms of water availability, porosity, chemical properties etc. If the macroscopic fermentation conditions were set at a favourable level, after inoculation, most of the spores can germinate and grow well. Even in some places where the microscopic environment is not good, the fungi have the ability to adjust it by extending hyphae and excreting extracellular enzymes. However, if the macroscopic fermentation conditions are unfavourable, e.g. fermented at a temperature which is too high, or if the water content is too low, the microscopic environments where spores land become critical. For example, because water is distributed unevenly within the substrate, if spores land on a spot where water is

more available than in other places, they have a better chance of germinating, growing and producing extracellular enzymes.

By comparison, if spores land on a spot where water is scarce, or where pores are larger resulting in higher evaporation rates, they can soon die from water insufficiency after germination, before having had a chance to extend their hyphae. This is especially so at higher temperatures, where water evaporation is greater. It is possible that in one petri dish most spores happen to land in a favourable microscopic environment, but in another most land in unfavourable environments. This uncertainty, resulting from the heterogeneous nature of the solid substrate, increases the differences between parallel groups and lowers the reproducibility of the fermentation outcome. On the other hand, the heterogeneity of the solid substrate can be reduced by pre-incubation, where soaking the substrate in water can encourage more even distribution, not only in terms of water, but also in terms of soluble nutrients, hence reducing the differences between parallel groups.

However, virtually all solid state fermentation related research has focused on the effect of conditions on performance at a macroscopic level. There has been little interpretation/information available for the microscopic level. Therefore, a better fundamental understanding at that level is critically important, to improve the consistency of fermentation outcomes and for exploring the full potential of solid state bioprocessing.

4.4 Concluding Remarks

So far, two applications of solid state bioprocessing have been tested and reported. In summary, by adding water, pre-incubation has a distinct positive influence on fermentation performances, in terms of toxin reduction, fungal growth, enzyme production, and also on the consistency of the fermentation outcome. However, the detailed mechanisms are not straightforward, and there is very little information available in the literature. Therefore, a systematic study is required to obtain a more fundamental understanding of water in the solid state fermentation.

In addition, it is not clear yet, how the microscopic environment surrounding the fungi affects growth and fermentation outcome, especially under unfavourable conditions. This results in inconsistency of fermentation and lowers the reliability of the outcome.

To address these issues, investigations into the fundamentals of solid state bioprocessing have been conducted. The effect of water on fungi cultivated on solid substrates (water bioavailability) was selected and investigated, due to the absolute necessity of water, and its critical role during the pre-incubation stage. The investigation was conducted both at theoretical and experimental levels.

Chapter 5 reports the theoretical investigation for the effects of water on different aspects, namely small molecules, biomolecules, microorganisms and microbial communities, in solid state bioprocessing systems.

The chemical and physical properties of the substrate on water bioavailability and consequently fungal growth were studied. In addition, the effects of water present in the gas phase and the microscopic water distribution on the substrate surface

were also studied. Based on experimental findings, a theory of water bioavailability in the solid state fermentation system was developed and tested. The findings are reported in Chapter 6.

Chapter 7 brings the entire thesis together by discussing and concluding the major findings of the research. It also looks forward to possible further research that could be carried out in the light of the project outcomes.

Part B

Water Bioavailability in Solid State Systems

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Chapter 5.

Effects of Water on Solid State Fermentation:

A Theoretical Investigation

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Chapter 5

Effects of Water on Solid State Fermentation, a Theoretical Investigation

In Part A of this thesis, two applications of solid state bioprocessing have been reported and discussed. To provide a theoretical background for understanding and realising its importance in solid state bioprocessing systems, the effects of water on different aspects of solid state bioprocessing (small molecules, biomolecules, microorganisms and microbial communities) have been summarised from related disciplines and reported in this chapter.

In most living systems, water accounts for approximately 70% of cellular weight. It is the largest component in the cell and well recognised to be essential for active life. Reviews on solid state fermentation systems, always acknowledge the importance of water [10, 63, 73]. However, the fundamentals of the interactions between biomolecules and water are rarely mentioned. In order to begin understanding these fundamentals, as related to solid state fermentation, it is necessary to gather information from many disparate sources. Even then, there will still be significant gaps in understanding, but it is hoped that a foundation can be laid that will facilitate a more complete appreciation of the topic. In this section, water, its structure and expression, its impact on microbial biochemistry, cellular behaviour and on the microbial community are examined in detail.

5.1 The Structure of Water

Owing to the dipole structure and high dielectric constant, in bulk water, water molecules are separated and held apart by hydrogen bonds. Those bonds can be formed within picoseconds and repaired almost instantly if “broken”. Each water molecule can form up to four tetrahedrally directed hydrogen bonds and forms a hydrogen bonded network (Figure 5-1). These hydrogen bonds are not only formed between water molecules, but also with other polar and ionic groups on macromolecules [82]. The interaction between water and its solutes is called solvation. Since most cell based molecules are macromolecules, water has a unique significance to microorganisms.

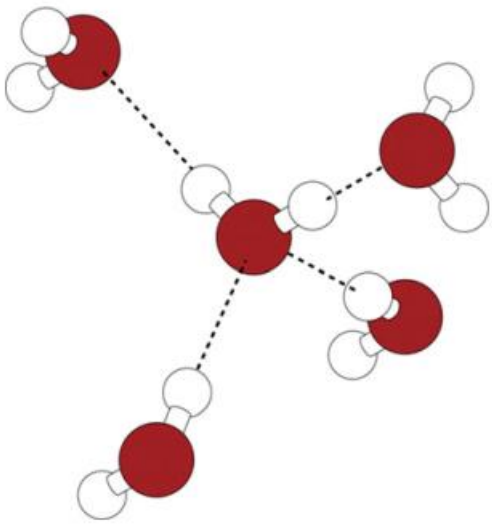

Figure 5-1 A typical hydrogen bonded water network [83]

Water affects the structure and solubility of macromolecules, and consequently the stability, reactivity, and eventually their function. It is believed, the hydrogen bonds are the main cause for the unique significance of water [84]. To understand how water affects macromolecules and cells, it is important to understand how it

affects ionic, hydrophilic and hydrophobic particles first. This is because, in an aqueous solution, these particles have different behaviours and also different impacts on macromolecules, and eventually these impacts becomes the driving force for macromolecules to form their structures and to perform their biological functions. For example the aggregation tendency of hydrophobic materials is in fact the driving force for proteins to fold. The effects of water on biologically important macromolecules is further discussed in Section 5.2.3 and 5.2.4.

5.2 Effects of Water at a Molecular Level

5.2.1 Effects of Water on Ionic and Small Hydrophilic Solutes

In aqueous solution, small ionic molecules do not exist as free entities. By columbic force, they are tightly bound to a layer of water molecules (solvation shell), on the oppositely charged end of the water dipole. Anions are bound to the hydrogen atoms on water molecules while cations are bound to the oxygen atom (Figure 5-2) [85].

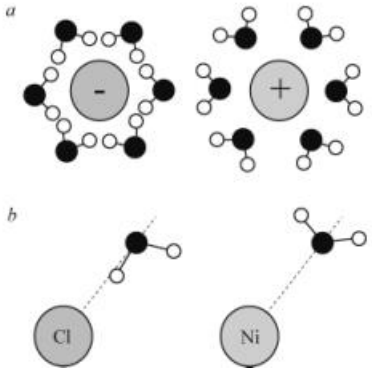

Figure 5-2 Solvation of cations and anions. (a) The conventional view. (b) Water orientations revealed by neutron scattering. *[85]*

Owing to the ion-water interactions, the hydration of the ion perturbs the water hydrogen bonded network and occasionally brings a redistribution of charge

amongst the water molecules. On one hand, highly charged ions are tightly bound to water, and therefore, decrease the availability of water molecules for the solvation of other particles, e.g. biomolecules, and hence lower their solubility. On the other hand, however, low charged ions are preferentially absorbed at hydrophobic surfaces of macromolecules and therefore increase their solubility [86].

At the current time, it is still not clear the exact mechanisms by which the ion solvation affects the solubility of macromolecules. It can be the result of a competition for water molecules between ion and macromolecule. It can also be the disturbance caused by ions, on the hydrogen bond network, and hence affects the bond between water and macromolecules. The mechanisms can furthermore be in the form of a direct interaction between ions and macromolecules.

It is possible that in a water abundant environment, e.g. submerged culture, the solubility of macromolecules is in fact more affected by the direct interactions between ions and macromolecules, than by other mechanisms [87-89]. However, in a water scarce environment, e.g. with solid substrates, the competition for water molecules between ions and macromolecules is also significant. Nonetheless, the presence of ions can affect the solubility of macromolecules both positively and negatively [86].

Similarly to charged ionic molecules, uncharged hydrophiles are also capable of forming strong hydrogen bonds, disturbing the water network and consequently affecting the macromolecules both positively and negatively. For example, urea is a small hydrophile but a strong protein denaturant. It can perturb the water network structure and encourage solvation of hydrophobic groups. It can also expel water from proteins, not only from the surrounding hydration shell, but also from any other region of the protein. This is because, comparing to water, urea

preferentially binds to the protein. Urea can also denaturalise protein by penetrating into the hydrophobic core and suppressing its native conformation, and consequently changing its functional structure [90]. In comparison, trimethylamine-N-oxide, another hydrophile, can act as a protein stabiliser against denaturation. Even though the mechanism has not been fully explained, recent studies have shown that trimethylamine-N-oxide has a strong interaction with water, which leads to a preferential exclusion from hydrophobic surfaces and apolar side chains, and such exclusion may play a significant role in protein stability enhancement [91].

5.2.2 Effects of Water on Small Hydrophobic Solutes

Nonpolar molecules, with neither charged groups, nor dipole moments, are insoluble in aqueous solutions. This is because, compelled by water, the hydrophobic surfaces face each and aggregate [85]. However, as with hydrophilic interactions, the detailed mechanism for hydrophobic interaction is not fully understood. Some researchers believe that instead of forming hydrogen bonds with nonpolar molecules, water molecules arrange themselves in an ordered fashion around the hydrophobic surface and form relatively rigid hydrogen bonds between each other as a cage around the nonpolar molecule (Figure 5-3) [83].

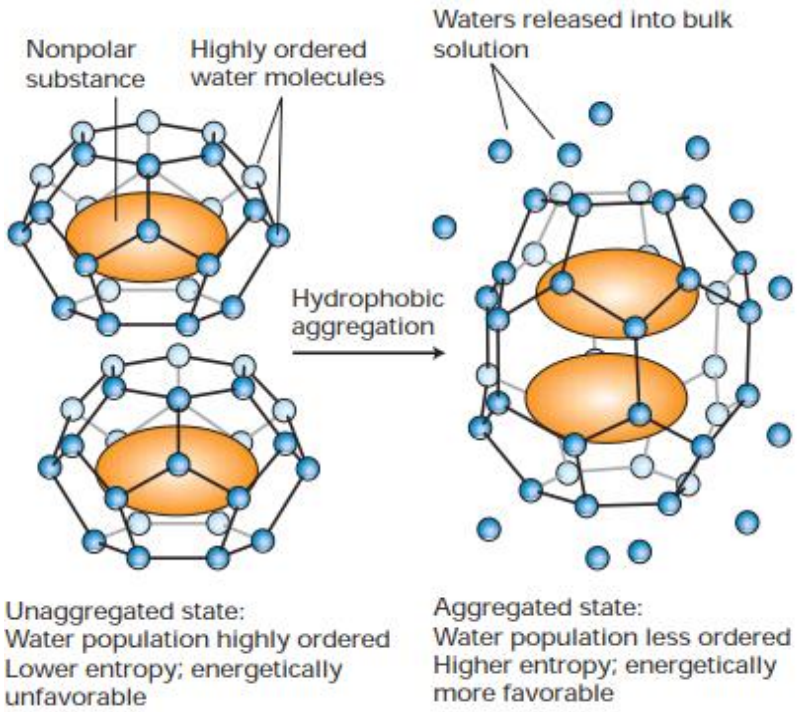

Figure 5-3 Schematic depiction of the hydrophobic effect. Cages of water molecules that form around nonpolar molecules in solution are more ordered than water molecules in the surrounding bulk liquid. Aggregation of nonpolar molecules reduces the number of water molecules involved in highly ordered cages, resulting in a higher-entropy, more energetically favourable state (right) compared with the unaggregated state (left) [83].

However, others share a different view that, apart from introducing a tangential orientation preference in the water molecules on the surface of hydrophobes, there is no evidence supporting any enhancement of the ordering of water [92, 93]. Recent studies [94-96] reported a thin water layer with reduced density around the hydrophobic surface. There were a few tangential oriented water molecules as a disordered hydration shell surrounding the hydrophobes, while the first neighbouring water structure in the hydration shell remain unchanged. Those researchers proposed that, instead of forming a highly ordered cage, the hydrophobic effect is brought about by a limited ability to open cavities within the water structure and thereby accommodate hydrophobes.

Things are even more complex for the solvation of macromolecules. It is not even entirely true that water is the driving force for macromolecules to aggregate. To begin with, if it is possible to fit small hydrophobes in the water network by opening small cavities, it would be very challenging to fit macromolecules. They would have to break the entire water network. Indeed, it has been argued that there is a qualitative difference between the solvation of macromolecules and small hydrophobic molecules. The difference is brought about by the structural heterogeneity of the macromolecules, whether apolar, polar, ionic, hydrophilic or hydrophobic groups, all are affected differently by water.

The effects of water on macromolecules may seem to be a subtle and delicate interplay between local surface, water molecules and water network. For example, the phenomenon known as dewetting transition, occurs when two different macromolecules come too close (Figure 5-4). If the approaching surfaces are hydrophobic, under capillary evaporation, water molecules are evaporated from the gap between the macromolecules. Therefore, the macromolecules are pulled together by surface tension. On the other hand, if surfaces are not sufficiently hydrophobic, say containing a few polar residues, water molecules will be squeezed out one by one. In this circumstance, instead of water completely evaporating from the gaps, it actually wets the approaching surface of the macromolecules. Hence, a subtle conformational change may have significant impact on the hydration of macromolecules and consequently their structure and function.

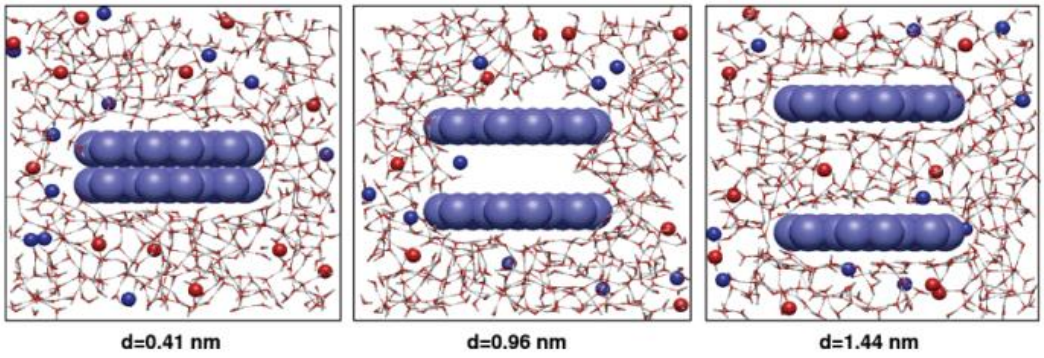

Figure 5-4 Effect of solutes on the dewetting transition of hydrophobes, using ionic solutes as an example. The ions are shown as dots in red (positive) and blue (negative). Hydrophobes are shown as blue plates. The distribution of highly charged ions around hydrophobic solutes at varying plate separations d . The ions are preferentially excluded at the surfaces and in the intervening water film, which retreats in a drying transition at around d 0.96 nm. Copyright 2007 American Chemical Society [85].

In general, ions and small hydrophiles affect the macromolecules in a very complex manner, very likely resulting in complex interplays. These interplays are highly dependent on local conditions and can, therefore, only be described individually through case by case study. The lack of current understanding casts a huge shadow of uncertainty to understand the more complex situation: how macromolecules are affected by water in a cellular environment. But fortunately, this current knowledge of the small molecules still enables us to interpret the behaviours of macromolecules in aqueous environment.

Nonetheless, the significant importance of water to macromolecules and to the biological system is certain: it causes amphiphilic lipids to hide their hydrophobic tails as they aggregate into bilayers (as in the creation of membranes); it causes proteins to bury their hydrophobic surfaces as they fold; it even empowers the proteins of their biological functionality, which are in fact exquisite interplays between the surrounding water and the various hydrophilic and hydrophobic groups.

In the following sections, two particular macromolecules with great biological importance, especially to solid state bioprocessing (proteins and nucleic acids),

are selected for detailed examination of how water affects them, in terms of structure and function.

5.2.3 Effects of Water on Protein Folding, Structure and Function

Proteins are the most common products produced from solid state bioprocesses. They are also of vital importance to cells. They participate in virtually every cellular process and perform a vast array of functions. Surprisingly, such active participants by themselves are passive, their structure and functionality is completely driven and empowered by water. To begin with, the polypeptide chains do not have any biological functionality without adapting into specific folded structures, and the folding process is, in fact, driven by water [97].

Using typical cytoplasmic globular proteins as an example, Sen and Voorheis [98] summarised a model which shows how water drives protein folding into functioning structures: Immediately after being extruded from the ribosomal exit tunnel, water molecules begin to form an ordered structure (water sheath) around the polypeptide chains. If the extruded amino acids are hydrophobic, driven by the hydrophobic effect discussed earlier, water molecules are expelled from the surfaces and the polypeptide chains are twisted and compressed together. If the number of extruded hydrophobic amino acids is more than 6 (e.g. on the protein backbone chain), the water structure around the chain will rotate 360 ℃ and consequently, insert with the rest of water sheath (Figure 5-5). Taking a closer look at the intersection, water molecules around it will be diffused away and the water sheath becomes thinner and thinner as it gets closer to the intersection, till it completely breaks at the point. As a result, the twisting motion of the polypeptide chain increases, and results in a stream of water molecules detaching from the adjacent water structure. Sen and Voorheis suggested that this change of water

structure is the cause of peptide chain bending or doubling up. With peptide chain folding, the movement of water molecules in the water sheath is dramatically reduced and water entropy is significantly increased. Many researchers believe that the increased water entropy is the principal driving force for protein folding processes [99, 100]. Some studies also suggest that apart from being driven by hydrophobic effects, protein chain folding is also driven by hydrogen bonding interactions in the polypeptide backbones [101]. Daniel et al [102] found that the dissection of side-chain and backbone interactions can sufficiently drive polypeptide chains to collapse (fold) by forming local and long-range contacts between specific sites.

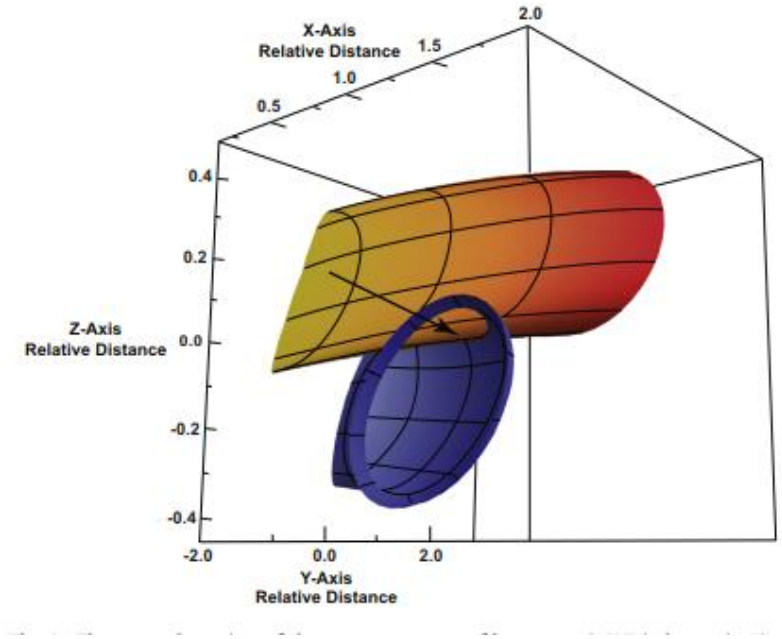

Figure 5-5 The central portion of a segment of lysozyme (1LYD), viewed at an oblique angle along the axis of the segment and its water sheath as it enters the bend and illustrating the intersection of the water sheath. The arrow marks the region where the segment of the sheath entering the bend intersects the segment of the sheath exiting the bend in the z dimension *[98]*.

Protein folding is not a 'blind' process in which the polypeptide chains miraculously find the optimal place. In fact, it occurs under the 'guidance' of water. Water can form folding intermediates (chaperonins) with the hydrophobic surfaces on the

protein chain. These chaperonins first bind the unfolded protein chains to their hydrophobic sites, and then trigger the conformational change that orientates the hydrophobic regions towards the interior. They also enable partially misfolded proteins to unravel and refold properly [84]. If, however, the backbones are not properly folded, or the hydrophobic sites are exposed to water, the protein loses its basic function and aggregates into β-sheet-like structures. These misfolded proteins are notoriously well known as the causes of such incurable human maladies, as Creutzfeldt-Jakob and Alzheimer's diseases.

After being folded, hydrogen bonds between polypeptide chains are formed to replace original hydrogen bonds with water molecules. Thus, the hydrophobic core is tightly packed and secondary structures are formed and ready to proceed to their final structure. At the final stage of protein folding, most water molecules are squeezed out, leaving a few to become an integral part of the protein backbone [84]. Later in this section it can be seen that water buried in the hydrophobic core has an important role in maintaining the stability of the protein.

The model proposed above provides current interpretations of how a globular protein is folded, but beyond this scope, lie so many other proteins. Just a relatively small number of amino acids (e.g. 150) can give rise to an enormous number (10^{68}) of possible conformations. It has been more than half a century since the structure of globular proteins was determined, and yet, there still remains a whole field of research attracting numerous researchers. We do not yet fully understand the detailed mechanism of protein folding. For example, there is little information about membrane proteins, and we do not know what the protein folding energy landscapes are in real life, or how to reverse protein mis-folding processes [103]. To make the problem even more complex, but closer to fermentation scenarios, the folding process is also under the influence of other

solutes especially when the latter are concentrated like in a cellular environment or a solid substrate. These are further discussed in the following sections.

Nonetheless, as may seem clear now, the protein structure is in fact a delicate manoeuvre of polypeptide chains driven by water. So it may not be surprising that the structure and stability of proteins are also essentially maintained by water molecules. To begin with, protein stability is highly dependent on the network of hydrogen bonds in the hydration shell [104]. The hydration shell around the protein surface contains three layers of water molecules: the first layer is tightly bound to the hydrophilic surfaces and on top of that, is the second layer, which is loosely bound to those regions. The third and most distant layer, covers the whole protein molecule and corresponds to those water molecules which are unbound, but also disturbed by the presence of the protein [98]. Figure 5-6 shows a typical hydration shell around a protein.

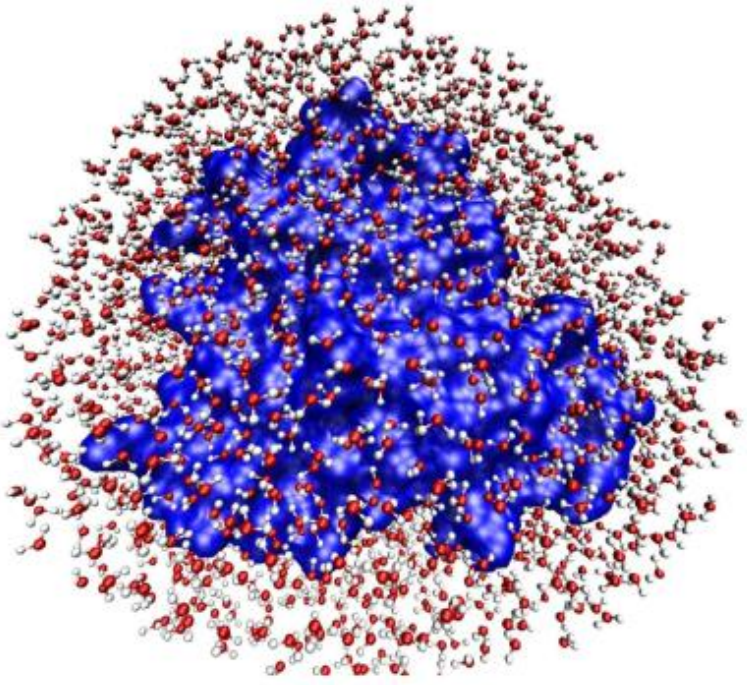

Figure 5-6 The hydration shell of myoglobin (blue surface) with 1911 water molecules (CPK model), the approximate number needed for optimal function (h = 2). The water molecules form a shell ≈ 5Å thick around the protein. Approximately 200 water molecules are distinguishable from the background with high-resolution X-ray crystallography *[105, 106]*

The effects of water on protein structure is a rather delicate balance. On the one hand, water molecules contribute to the stability of the protein. As mentioned above, even deep in the hydrophobic core there are water molecules tightly bounded which become an integral part of the protein structure. Those water molecules in the backbone stabilise protein structure by filling the internal cavities and interacting with polar atoms [107]. On the surface of the protein, the hydration shell can saturate the H-bond propensity of polar/charged groups and hence avoid their collapse and intramolecular rigidification [97] (Figure 5-7). Indeed, when hydrated, some proteins may even gain an increased thermal stability over the dry state. This suggests that, in solid state bioprocessing, when the substrate is low in moisture but high in temperature, such as during extraction and purification, proteins such as enzymes are more vulnerable to denaturation.

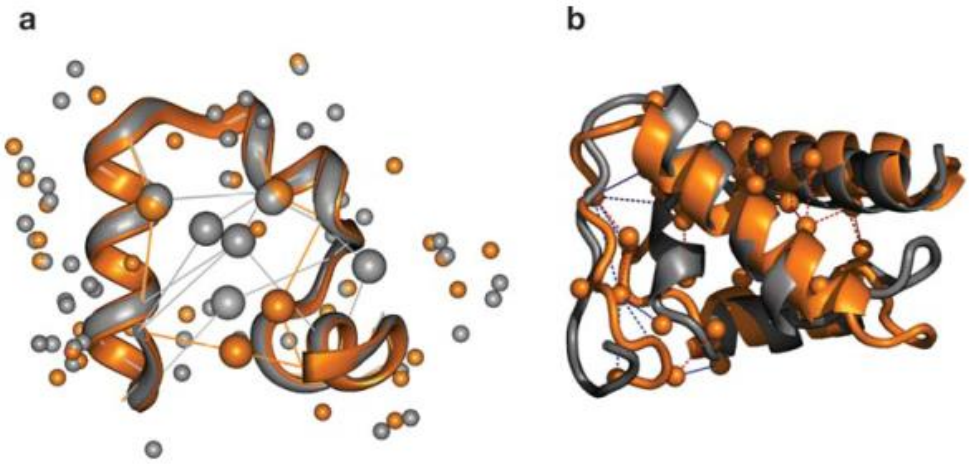

Figure 5-7 Water maintains protein three-dimensional structures. (a) High-resolution X-ray crystal structures of the villin headpiece subdomain at pH 5.1 (orange, 1.55 Å resolution, PDB code 1WY4) and pH 7.0 (grey, 0.95 Å resolution, PDB code 1WY3). Water molecules that mediate ternary contacts (thin lines) are shown as large spheres, indicating that they are an integral part of the structure and that some are conserved in both crystallization conditions. (b) The prediction of the structure of the protein is improved when using an optimized energy function that includes water-mediated interactions. The native and predicted structures are grey and orange, respectively. The virtual water molecules (defined by distance and the residue solvent accessibility) are shown as spheres *[104]*.

However on the other hand, surrounded by water molecules, proteins seem rather vulnerable: if the protein vibrates too fast, for example when heated, the weakly

bound structure becomes unfolded and loses it functionality; if the order of water in the hydration shell is increased, such as lowering temperature below the freezing point, the protein also unfolds, possibly due to the weakened hydrophobic effect and the entropic loss. Even under normal temperatures but with applied pressure, water can still be squeezed into the folded protein structure leading to denaturation [84, 108].

The delicate balance maintained by water is perhaps why proteins are so sensitive to thermal changes, and consequently, why most life forms, including microorganisms, can only thrive within a narrow range of temperatures. In fact, one author has even described water and protein as having a 'love-hate relationship' [109], though the love seems stronger: proteins are not only 'made' and maintained by water, their functionalities are also completely empowered by water. As mentioned above, there is a structured hydration shell at the surface of proteins, and the interaction between this and the protein is the very cause of the empowerment. The fast reorientational motion of water induces a slow motion to the side chains of the protein, and this propagates along the protein structure into the backbone, generating the longer time scale motion necessary for its function. In other words, the interfacial water rotational dynamics are the very source of the entropy that drives the protein dynamics [110].

In addition, water molecules can also form a network and actively participate in protein functions. The water network consists of short range interactions in the first hydration shell and long range interactions that connect several other hydration shells [111]. It is affected by the chemical composition of the protein surface, if for example, the hydrogen accepting/donating sites on the surface are removed, the shell loses its connectivity. Taking enzymatic reaction as an example, Dielmann-Gessner *et al.* [112] found that after mixing an enzyme with a substrate, the substrate bound to the enzyme but not immediately at the active site. Right

before the binding, there is a sharp gradient of water dynamics near the active site, which assists the substrate binding with the active site via charge-induced water retardation. Once the enzyme-substrate complex is formed, the hydration dynamics of the substrate is further retarded, and towards the active site, establishing a mild gradient of water dynamics (hydration funnel). The authors further concluded that water dynamics contributes to the net enzyme reactivity and impacts the catalysis process.

Apart from the water network, another important contribution of water to enzymatic reaction is water wires. Water wires are chains of water hydrogen bonds which can link to several amino acids. They can significantly enhance the mobility of protons and transport them rapidly by successive water molecules flipping their hydrogen bonds. This enables protons to hop over many water molecules (Figure 5-8). Such water wires are particularly effective to transport hydrogen ions from the surrounding solvent into protein active sites. The water wires and, in effect, the hydrogen bond networks also play an important role in protein binding at interfaces. The interfacial water between proteins can provide a dielectric shielding which results in highly directed electrostatic interactions between the hydrophilic groups of the proteins. In other words, the interfacial water network acts as a 'glue' between protein molecules and assists in the binding [84].

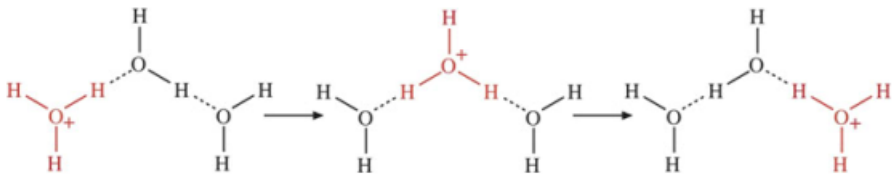

Figure 5-8 The Grotthuss mechanism for rapid proton transport in hydrogen-bonded chains of water molecules. The hydronium ion (red [grey]) shifts essentially by the rearrangement of electronic rather than nuclear configuration [84]

Apart from transporting protons to active sites and assisting local binding, the hydration network also participates in the allosteric conformational shifts of proteins. These shifts can lead to a large scale conformational change which is necessary for protein function [113]. For example, introduced by dynamic rearrangements of the hydrogen bond network, water molecules can open or close a hydrophobic pocket on the protein molecules by wetting (binding) or drying (detaching from) the hydrophilic crevice [114]. Beyond local hydrations, water may also be able to induce a remote allosteric change in conformation as a whole protein molecule. Fast IR spectroscopy shows that a change in water density in the vicinity of the active site of a protein propagates slowly through the water network over about 100 ns until it reaches the other side of the protein. It means, the conformational change is in fact governed by the rearrangement of the water network on the protein surface [115, 116]. Indeed, these changes in the hydration, either locally or remotely, are so important that one may argue water is, not only the maker of protein structure, but also the switch of their functions.

5.2.4 Effects of Water on Nucleic Acids

Compared to the intense research interests driving towards the relationship between water and proteins, the effects of water on nucleic acids seem rather underappreciated, even though water is of no less importance to nucleic acids than it is to protein. Water is vital to DNA structure and function: it assists the folding of nucleic acids and stabilises the double helix structure; it mediates the interactions between DNA and proteins or small molecules; it also forms an activation barrier for DNA binding and other essential biological processes. In fact, the effects of water on nucleic acids and proteins share great similarities. For example, water assists the folding of both protein and nucleic acids. RNA folding

is essentially a hydrophobic collapse driven by expulsion of water from hydrophobic regions. However, unlike polypeptides, the backbone of RNA is a uniformly polar chain consisting of sugar and phosphate groups [84] which can trap water more easily between the hydrophobic bases. As a result, as the compaction proceeds, DNA has greater potential for water-mediated interactions [117]. Around the phosphate backbone, water may also serve as a primary heat sink accepting excess vibrational energy from the macromolecule [118].

Similar to proteins, DNA is also surrounded by structured water molecules which form several hydration layers. This is known as 'the spine of hydration' (Figure 5-9). Back in 1953, when the double helix structure of DNA was being proposed by Watson and Crick, they predicted that the structure should be a delicate relationship between DNA structure and aqueous environments [119]. Indeed this is the case. Even though the solid-state crystal structures of the hydrated molecule at low temperatures was reported in 1983 [120], it was only very recently that scientists managed to observe the spine of hydration at ambient temperature, which is much closer to a cellular environment [121]. The hydration layer around DNA is built up by water bridges hydrogen-bonded to the DNA strands in a complicated manner. Compared to proteins, it has an analogously broad distribution of residence times and fast fluctuations in the hydrogen bond-network [118, 122].

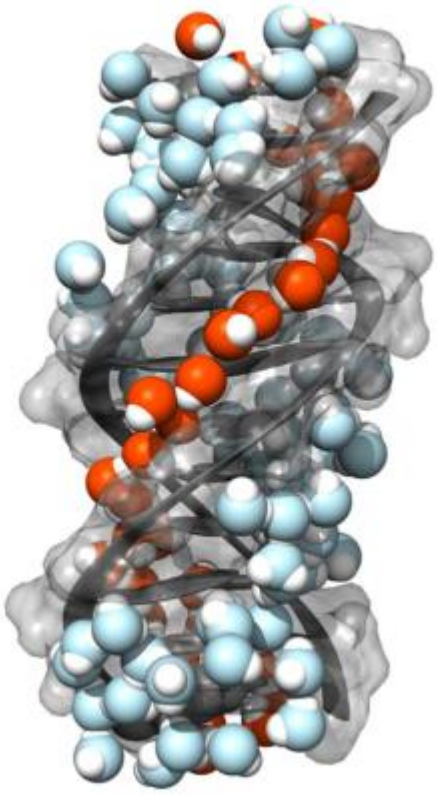

Figure 5-9 . Water structure in the minor groove (red) and major groove (blue) of the double-stranded DNA from a molecular dynamics simulation [121].

These hydration layers have a strong connection to DNA stability: the conformational state of double stranded DNA in solution is very sensitive to hydration. For example, without sufficient hydration, DNA transits from B form, the most biologically relevant form, to others. The stability of DNA in B form, in fact, depends on whether the water cluster in the primary hydration shell can be fully connected within the major grooves (where the DNA backbones are far apart). And, in a very similar manner, the stability of DNA in the A form is affected by the water cluster within the minor grooves [123, 124].

Apart from stabilising DNA structure, water also affects its function. For example, the specific binding of protein and DNA is always accompanied by the displacement of water from the DNA hydration shell. Such binding can be greatly suppressed by lowering water activity, which indicates the protein-DNA interaction

is in fact mediated by water molecules [125]. In addition, the distribution of water at the interface between protein and DNA shows sequence dependent variations, which control not only the number of water molecules released, but also the contribution of local entropy to the binding free energy. In other words, water at the interface serves as a hydration fingerprint of a given DNA sequence [126]. Generally speaking, water mediates the recognition and binding between DNA and peptides, proteins, or other molecules.

5.2.5 Macromolecules in Crowded Environments

So far, the effects of water on biomolecules in dilute solutions have been discussed. However, from a biological point of view, one may ask, should the cellular environment be considered as a dilute solution? Furthermore, do biomolecules in water scarce situations, e.g., in a solid state fermentation systems, behave the same as they are in a diluted solutions? For both questions, the answer is clearly no. To begin with, cellular environments are in fact extremely crowded, and have even been described as ‘a kind of very thick soup’ [127]. The concentration of macromolecules in the cellular environment is in the range of 80-400 mg/mL and the total volume occupied by macromolecules can take up to 40% of cellular space. Even though this may not feel like an overly crowded environment, the distribution of cellular content is highly heterogeneous. In fact, both cytoplasm and nucleoplasm contain various membrane-less organelles (tiny cellular structures that perform specific functions) where macromolecules are gathered at such high concentrations that, on average, the distance between each macromolecule is only around 1-2 nm (Figure 5-10) [127-129]. In such crowded environment, each macromolecule excludes others from its immediate vicinity, which gives rise to the excluded volume phenomenon [130]. In plain words,

macromolecules 'push' each other out of the way (Figure 5-11). Such phenomenon can be observed in both cellular and water scarce environments.

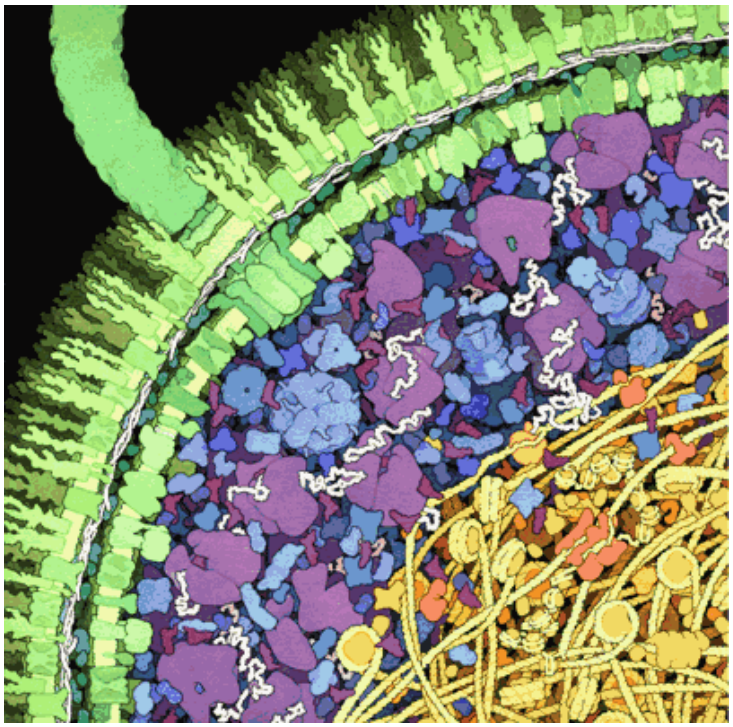

Figure 5-10 Schematic representation of part of an E. coli cell, including cell wall (green), cytoplasm area (blue and purple), and nucleoid region (yellow and orange). Water molecules are not shown [131].

To study effects of this phenomenon on macromolecular behaviour, scientists have applied inert crowding agents, such as PEG, PVA and Ficoll 70, to mimic a crowded cellular environment [127, 132]. What they found was, if the total volume occupied by cellular macromolecules becomes considerable, and if the size of crowding agents is similar to or larger than the macromolecules, then, the total entropy of the crowded solution is drastically decreased, and the free energy and thermodynamic activity of the solutes are increased [133]. As a result cellular solutes, both large and small molecules, at both high and low concentrations, are changed under the excluded volume effect, namely in their folding mechanisms,

structure, stability, and consequently in their functions, reactivities and interactions [127].

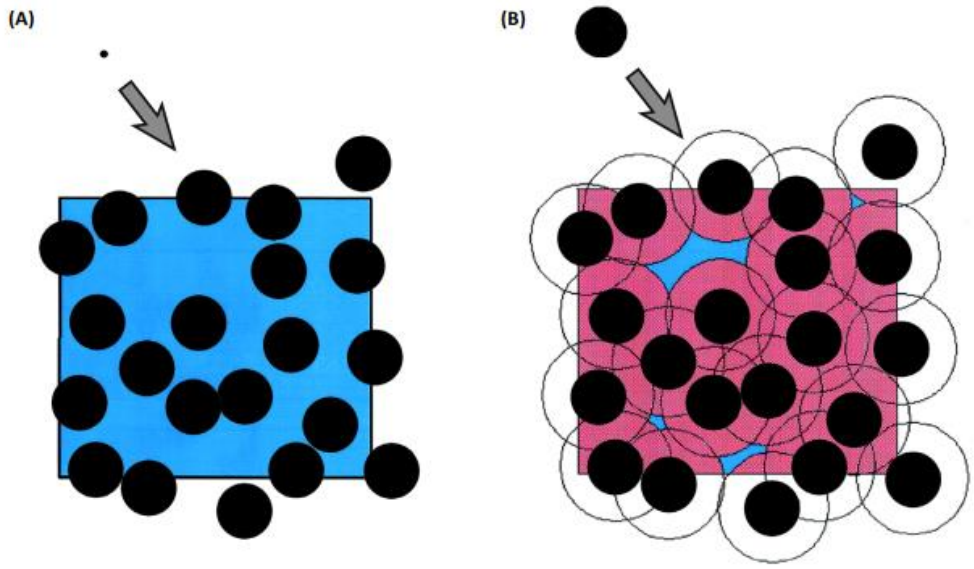

Figure 5-11 Schematic depiction of available volume (blue) into which a newly added molecule can move. In case (A) the small molecule is not impeded by existing macromolecules. In case (B) where the newly added molecule is comparable in size to the existing macromolecules there is an instantaneous exclusion effect (represented in pink) *[134]*.

To begin with, the crowded environment can increase the amount of secondary structure in the folded state. Research shows than the proportion of structural content of flavodoxin and VIsE was enhanced by 33% and 70%, respectively, in the presence of crowding agents [135]. In addition, Ai *et al.* [136] found that, in a moderately crowded environment, the folding rate of protein can be significantly accelerated. In fact, it increases exponentially with increasing concentration of the crowding agents; whereas the unfolding rate remains mostly unchanged [137]. These findings indicate that the crowded environment preferentially stabilises the compact states over the unfolded ones which experience increasing difficulty in refolding [138]. As a result, if the environment becomes too crowded, the refolding process of unfolded protein is almost completely lost due to protein aggregation [139]. Indeed, proteins seem to be much more stable in a crowded environment than in the dilute solution. To begin with, they are more stable against mechanical

forces. For example, Yuan *et al.* [140] reported that the average mechanical force required to unfold a single ubiquitin molecule was 210 pN, but in a crowded environment, this increased to 234 pN. In addition, the crowded environment can increase protein midpoint denaturation temperature, with a constant enthalpy change of the thermal denaturation process. In other words, it increases the thermal stability of protein (by 5℃ to 10℃), especially in the presence of a destabiliser (by 10℃ to 20℃) [141]. However, such effect is largely dependent on the protein and also on other conditions of the crowded environment. For example, at pH 4, crowding agent Ficall 70 can stabilise RNase A and α-LA, but not lysozyme [142].

In addition to mechanical and thermal stability, many researchers have shown that the crowded environment can affect the functionality of macromolecules, normally in a positive manner. For example, the activity of ATPase was increased significantly with the presence of crowding agent [143]. Similarly the catalytic activity of DNA ligases were completely inactive under conventional assay conditions, but it can be activated by increasing the concentration of macromolecules in the environment [144]. This is partly due to the crowded environment shifting the conformational equilibrium of proteins into a more compact state, and thereby modulating the conformational behaviour, which generally results in an increased activity [129]. This can also be because crowded environments can increase the affinity of proteins, especially enzymes, in binding with substrates and DNA. For example, the enzyme activity of DNA polymerase I was dramatically increased in a crowded environment due to the increased binding between polymerase and its DNA template-primer [145]. Similarly, crowding agents can also enable recA protein pairing and exchanging with DNA strands, which would be very difficult in dilute solutions [146].

On one hand, as a result of increased binding between macromolecules, the gene expression is influenced by crowded environments. Tan et al [147] reported that increasing concentration of crowding molecules can result in an increase of DNA expression rates, more significantly if the crowding agent has higher molecular weight than lower. However, on the other hand, due to impaired diffusion rate, the expression rate can also be reduced by crowding agents that are too concentrated. In fact, it appears that, in nature, cells are able to modulate the expression rate by coupling the crowding densities with both promoters and ribosomal binding sites, and consequently increasing the robustness of the system [147]. For example, in a highly crowded environment, in the presence of ammonium ions (which lower the stability of macromolecules), gene expression experiences less perturbation than in a less crowded one. This finding suggests that the gene expression is actively tuned with the crowding density of the environment.

Even though many researchers hav\e reported the positive effects of a crowded environment on macromolecules, it must be pointed out that this is not always the case. For example, crowding agents did not accelerate the substrate binding in the trypsin-catalysed hydrolysis of p-nitrophenyl acetate [148]. Similarly, Vöpel and Makhatadze [149] also found that three glycolytic enzymes, phosphoglycerate kinase, glyceraldehyde 3-phosphate dehydrogenase, and acylphosphatase remain unaffected in the presence of crowding agents. Furthermore, the hydrolysis rate of alkaline phosphatase was actually reduced by crowding agents, the effect increasing with the molecular weight of the crowding agents [150]. These findings are consistent with simulation results, which show that the reaction rate is always decreased with increasing volume fraction of the crowding agents [151]. One reason accounting for the negative effect brought by crowded environment is again the reduced molecular diffusion rates [132]. This reduction applies for both large and small molecules, but much more significantly for the

former, ranging from three- to tenfold comparing to a dilute solution. This means a macromolecule may travel ten times slower before reaching the binding site, and consequently results in a lower reaction rate [132]. Thus, taking the negative effect into account, the effect of crowded environment on macromolecules become a complex matter: on one hand, it reduces the reaction rate of macromolecules, while on the other hand, reduced diffusion is accompanied by increased thermodynamic activities, as discussed above. It needs to be taken into consideration that the electrostatic interactions between macromolecules was found to counteract the effect introduced by the crowded environment. Also, mixed macromolecules may behave very differently from pure solutions [152, 153]. Indeed, an actual intracellular environment is far more complex than simplified experiments conducted in a lab, and that is without considering the difference in cellular content brought about by different species of microorganisms. The overall effect of crowding on macromolecules is therefore rather multifaceted. Even though considerable effort has been put into interpreting the behaviour of macromolecules in a crowded environment, it is not yet possible to provide accurate predictions [154].

Nonetheless, it is certain that macromolecules behave differently in a crowded environment. Perhaps, some of the positive effects mentioned above provide some insights for explaining the mystery around solid state fermentation performance, such as why it is intuitively believed to be ‘more productive’ than a submerged one, especially when many of the crowding agents can also reduce the availability of water to microorganisms, either incidentally or associatively.

So far the biochemical effects of water have been examined. It is truly amazing that such a simple molecule shapes life on earth: it drives and guides macromolecules to fold, it empowers and facilitates their functions, it even influences the behaviour of macromolecules based on their concentration. It is

also surprising that so much is still unknown about this molecule that is so closely associated with life: we can't predict how macromolecules fold under the guidance of water, we don't know the detailed mechanisms of how water facilitates macromolecular functions, we have very limited experiments to back up theories and simulations, and we have little knowledge about how macromolecules behave in an actual cellular environment, let alone in different cells. And yet, without any conscious effort but with millions of years of evolution, molecules are adapted to take advantage of water, of its versatility, sensitivity and responsiveness, thus becoming the basis of life [84].

5.3 Effects of Water at a Cellular Level

Leaving the limited biochemical knowledge aside, it is time to examine the influence of water at a cellular level. At this level, the difference in water potential between a cell and its surroundings is the driving force for water inflow. It means, when placing a cell in a dilute solute with lower osmotic pressure than the cellular cytoplasm (i.e. a hypotonic solution), water flows from the environment into the cell. As a consequence, the cell will expand. In some cases, this expansion can reach 400% of the original volume, before it bursts (cytolysis, Figure 5-12) [155, 156].

If, on the contrary, the cell is placed in a solution with higher osmotic pressure than the cellular cytoplasm (a hypertonic solution), water migrates from the cell to its surroundings. As a result, the cell experiences a series of physiological and morphological changes. The immediate changes include loss of cellular water and hence cytoplasm volume, leading to a reduction in turgor pressure. This, in

turn, leads to cell membrane shrinkage, and hence to reduced cellular volume and loss of biomass [156-158]. If there is cell wall, the cell membrane can become separated from it (plasmolysis, Figure 5-12) [159].

Apart from these immediate changes, since turgor pressure is the driving force for cell wall expansion, cells grown in water stressed environments also experience reduced growth rate, and in the case of fungi, shorter but more frequently branched hyphae [160, 161]. At a molecular level, as discussed above, osmotic pressure can reduce the mobility of water and other molecules, as well as change the shapes of biomolecules. It can also impair their function, and ultimately cause their denaturation. As a result, the reaction, metabolic rate and total microbial activity are also reduced, which can lead to impaired cellular growth, delayed germination and even cell death and lysis. Reduced cellular water content also makes a crowded cellular environment even more compact. This is normally accompanied by changing the fraction of cytoplasmic volume occupied by macromolecules which further disturbs the intracellular dynamics as discussed above [162, 163].

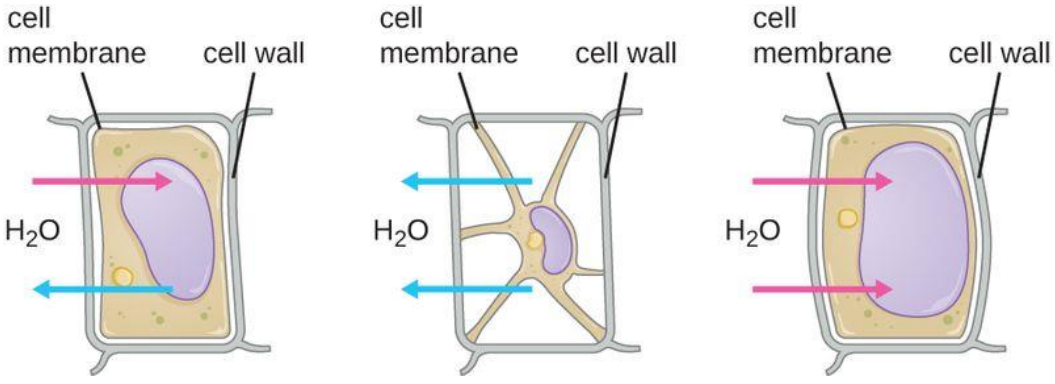

Figure 5-12 Schematic representation of cells in isotonic solution (left), hypertonic solution (centre) and hypotonic solution (right) [164]

Even though microorganisms seem to be completely under the control of water availability in their surroundings, this doesn't mean they are totally defenceless in unfavourable conditions. In fact, in a natural environment, the availability of water to microorganisms is rarely stable, and in response, microorganisms have developed a number of osmoregulation strategies actively controlling the influx and efflux of solutes from the cell, and consequently maintaining the necessary cellular homeostasis [165]. At the cellular membrane are located multiple osmosensing transporters and mechanosensitive channels, and by monitoring changes in cellular properties (e.g. turgor pressure or membrane strain), they can detect and respond to changes in osmotic pressure. In the case of increased osmotic pressure, microorganisms can alter the compositions of the cytoplasm by accumulating electrolytes or small organic solutes (known as osmoprotectants or compatible solutes), and consequently, increase cellular osmotic pressure. This can be done either through intracellular synthesis or selective uptake from the surrounding environments [137, 162]. For example, microorganisms start accumulating K^+ or its glutamates, as soon as the osmotic pressure is increased above a certain critical value. The total cytoplasmic concentration of potassium can be increased from 0.2 to 0.7 M, if the osmolality of the medium increases from 0.1 to 1.0 mol/kg [162]. Comparing to the accumulation of compatible solutes, accumulation of ions is faster and far less energy expensive. However, if the concentration of intracellular salt becomes too high, biomolecules, such as proteins and DNA, are still threatened by denaturation. Perhaps this is why in media with a high osmotic pressure, microorganisms replace the accumulation of ions with non-ionic, organic solutes. For bacteria, these compatible solutes are sugars (e.g. trehalose), amino acids (e.g. glycine betaine, proline, etc.), and various peptides [166]. For fungi, the compatible solutes are mainly polyols (e.g. glycerol, mannitol, arabinitol, and erythritol) [137]. Compared to ions, compatible solutes are much less toxic, better at withholding cellular water against diffusion,

and can even show the ability to restore the enzyme activity inhibited by high concentrations of ions [162, 167].

Examining at a molecular level, these cellular strategies in response to water stressed situations are achieved by expression and regulation of related genes. Taking fungi as an example, cellular response to osmotic pressure is governed by many signalling pathways, the central and the most famous one, is the high osmolarity glycerol (HOG) pathway (Figure 5-13). This pathway is composed of upstream osmosensors, and a downstream signalling pathway with a core of Hog1 MAP kinase (MAPK) cascade, which eventually leads to a complex change in the global cellular gene expression [163, 168, 169]. To begin with, cells have osmosensors (in the case of yeast, Sln1 and Sho1) located at the cellular membrane and also possibly within the cell. These osmosensors respond to changes in osmotic pressure, by sensing physiological changes in the membrane (e.g., stretching and curving), or by sensing macromolecular crowding and/or diffusion restrictions [163]. For example, Sln1 is activated upon the reduction of turgor pressure, and Sho1 is activated through assembly and regulation of a membrane-associated complex with the scaffold proteins. In other words, Sho1 is activated by a series of changes and interactions of transmembrane and response regulator proteins [163, 170]. The two sensing systems can function independently. However, in comparison, Sln1 is generally more sensitive to osmotic changes. In fact, in filamentous fungi, Sho1 appears to have a completely different role than osmosensing, such as sensing contact with the surface [171].

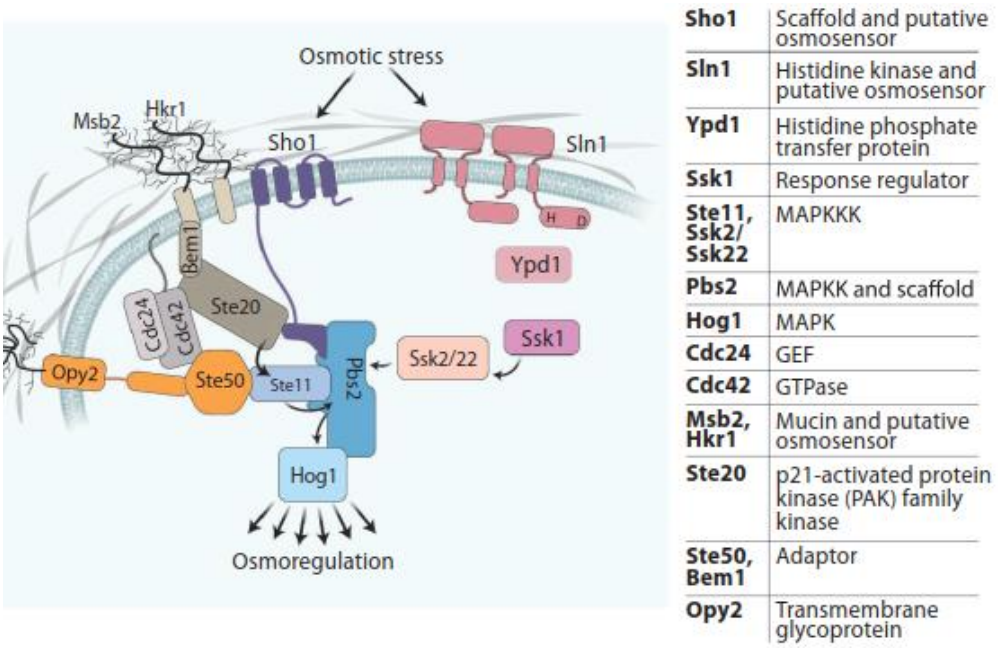

Figure 5-13 A summary of the current model of the HOG pathway in yeast. Osmotic stress releases Sln1-dependent inhibition of Ssk2/22 to activate the pathway. The Sho1 branch activation requires the membrane-embedded mucin proteoglycans Msb2 or Hkr1 to interact with Sho1 and Ste20 in a complex with the MAPK components. Opy2 is a transmembrane glycoprotein that serves as an anchor for Ste50. Cdc24 and Cdc42 are the cytosolic guanosine triphosphatase (GTPase) and guanine nucleotide exchange factor (GEF) that activate Ste20. Diagram adapted from [170].

Once a hyperosmotic shock is detected, sensor proteins pass the signal to the core protein (Hog1) in the osmoregulation pathway and consequently stimulate and activate it [172]. Immediately after stimulation, phosphorylated (activated) Hog1 translocates to the nucleus within seconds, and modulates transcription factor activity, which involves more than 300 genes [170]. Among these genes, a small fraction mediates the uptake and synthesis of cytosolic compatible solute (glycerol) and directly affects its concentration. Other genes affect the regulation of cytosolic proteins, such as reducing the release of cellular glycerol, and consequently participate in the osmoregulation process [163]. In this manner, the cell can rapidly respond to osmotic changes by accumulating intracellular compatible solutes, to avoid a sudden decrease in cell volume.

If the cell is exposed to a hyperosmotic environment for longer than about 20 min, the active Hog1 gives preferential expressions of genes responsive to, not only

osmotic pressure, but also diverse stresses including pH, temperature etc. This cluster of genes is known as the environmental stress response [173]. In the meantime, the expression of other genes involved in cell growth and proliferation is temperately stopped, ensuring proper stress adaptation before entering the cell cycle again [169]. This means exposure to one type of stress can trigger the protective adaptation of the cell and prepare it for other subsequent environmental stresses. Indeed, in a natural environment, many adverse conditions often appear together, such as heat and drought [174]. Pascual-Ahuir *et al.* [169] found that Hog1 is also associated with nutrient regulated protein kinases, so that hyperosmotic shock could result in a shift of metabolic balance towards using internal fatty acids instead of sugars. The authors believed that such a strategy is adapted to counteract the nutritional stress which is normally associated with high salinity. Through this general response to various environmental stresses, cells are more robust in hostile environments, and are able to maintain their normal microbial behaviour, at least to a certain extent.

The general stress response system may provide another insight to interpret certain phenomena occurred in solid state fermentation, which is more complex than it seems. For example, research shows that ‘stress-free’ environments may even have adverse impacts on cells, such that those exhibiting optimal activity have thinner cell walls, lack resilience to change and may also be more vulnerable to competitive microorganisms. Hallsworth [174] even suggested stress-free microbes “lack vitality”. This is perhaps why microorganisms seem to be more vulnerable to contaminations in a submerged culture, than in solid state substrates.

It is worth mentioning that the effects of a water stressed environment on individual cells within a microbial community can be highly varied [174]. This is partly because, the requirement for water availability varies by microbial species, and also varies with stage in the life cycle. It is also because, the microscopic

environment around each cell can be distinctly different from one to another, especially in heterogeneous environments, such as solid substrates. However, despite these individual differences, the effects of changing water availability on the whole microbial community do follow some general patterns. These are further discussed in the following section.

5.4 Effects of Water at a Microbial Community Level

Generally speaking, water availability has impacts on the total microbial activity and on the structure of the microbial community. To begin with, it is well known that the performance of submerged fermentation, such as cellular growth and production can be significantly inhibited by high concentrations of substrates or microbial metabolites. This substrate or product inhibition, has been studied for decades in fermentation, with researchers developing many empirical equations to explain and predict the phenomena. However, despite these efforts, the detailed scientific mechanisms of inhibition are still not completely clear. The effect could be brought about by the accumulation of chemicals and toxic metabolites, which interfere with normal cellular function, and/or the reduced availability of water affecting the function of biomolecules and cells as discussed above [175-177].

In a natural environment, reduced water availability also affects microbial community structure, and more significantly, microbial biofilms [178]. A biofilm is a highly hydrated porous matrix of microbial cells adhered to each other and/or to surfaces or interfaces. The largest component of this matrix is water. The major dry component on the other hand, is a wide range of biopolymers, e.g., polysaccharides and proteins, produced by the microorganisms, and to which the

cells are attached. Apart from providing physical support, the biofilm also serves many functions for the attached microbial community, such as absorbing organic compounds and supplying them to the microorganisms as nutrients. It can also bind and stabilise enzymes through interaction with polysaccharides, and facilitate the exchange of genetic information between cells [179]. Also, when the concentration of a certain molecule reaches a critical threshold, a cascade of events is triggered, including changes in gene expression, prompting the bacteria to be released from the biofilm and disperse [180]. In fact, the presence of a biofilm enables microorganisms to have a lifestyle completely different from the freely dispersed state. This can confer both advantages, and disadvantages, on the microbial community in the biofilm which can potentially be exploited [181].

Due to the presence of many hygroscopic components, biofilms retain water entropically rather than through specific water binding mechanisms [179]. This means that they can hold a lot of water but allow limited transport through the film, retaining it underneath. As a result, they can protect cells not only from desiccation, but also from external fluctuations in osmotic pressure, at least to a certain extent. At a cellular level, the effect of osmotic pressure varies with microorganisms. For example, Pemmaraju et al [182] reported that osmotic stress had a positive effect on the formation of *Candida albicans* biofilms, Rubinstein et al [183] on the other hand, found that an increase in osmotic pressure repressed the expression of related genes in *Bacillus subtilis*. At a microbial community level, the growth and spreading of the biofilm is closely related to the osmotic pressure. For example, Seminara et al [184] found that the secretion of biopolymers, e.g. exopolysaccharides, can generate osmotic pressure gradients in the extracellular space. These gradients drive surface motility and consequently contribute to the growth and spreading of the biofilm. In agreement with this finding, Yan et al [185] reported that by physically swelling, the osmotic

pressure difference between the biofilm and the external environment promotes the expansion of the biofilm, and consequently the microbial colony, on nutrient surfaces.

The natural tendency of microbial aggregation and adhesion to solid surfaces raises questions, if not doubts, for researchers working in modern fermentation, for example, are the benefits brought about by cells attaching to a porous, nutritious solid substrate still valid in an artificial fermentation environment? If so, how much are these benefits impaired by using tradition submerged fermentation techniques, which disperse the cells throughout the liquid culture? Are these advantages consciously taken into account when developing techniques for cell immobilisation?

Moving on to solid substrates, many studies have shown that microbial activity is closely related with water availability in the substrate. For example, many researchers have reported that increasing osmotic pressure in the substrate can reduce the microbial respiration rate and total biomass production [166, 186, 187]. In addition, it can also result in a change in cellular composition (carbon and nitrogen ratio) and substrate consumption, which is a result of microbial osmoregulation strategies as discussed above [188]. If, however, there is too much water in the substrate, the air ventilation and oxygen availability is likely to be impaired. As a result, anaerobic microorganisms will prosper and aerobic ones perish [166]. Indeed, the structure of microbial communities is heavily affected by water availability and other environmental conditions: those microorganisms that can adapt to or tolerate environmental changes will thrive, and those that cannot will not. Chowdbury et al [189] further proposed that the microbial community sacrifices a proportion of microorganisms, in order to ensure the survival of the rest.

5.5 Expressions of Water Availability in Solid Substrates

In the literature, the main term used to represent the availability of water in a solid state bioprocessing system is ‘water content’ (also referred to as ‘moisture content’), which is the ratio between the weight of water and the weight of substrate. Based on a random sampling of 35 of the relevant papers published during 2017 and 2018, 34 used the term ‘water content’ or ‘moisture content’. However, despite it being widely used, many researchers have questioned whether the term ‘water content’ can truly represent the bioavailability of water, as it lacks any recognition of the effects of the chemical properties of the substrate [54, 63, 190, 191].

Another term that can be used to describe the availability of water to microorganisms is ‘water activity’. This is defined as the ratio between the vapour pressure of water in the substrate and the vapour pressure of pure water, under the same conditions. Therefore, the water activity for pure water is 1, and by adding chemicals into solution, its value is lowered. The lower its value, the less water is available to cells.

Theoretically, all chemicals can change water activity by being dissolved into the liquid phase and the most commonly used water activity reducing agent are common salts. However, there is only a limited number of water activity reducing agents that are suitable for studying the bioavailability of water. This is because many chemicals interfere with macromolecules and metabolic processes, and consequently with microbial growth. For example, salts and heavy metals can

denature proteins, glucose can be utilised by microorganisms as nutrient source, and glycols can be accumulated in microbial cells as part of their osmotic stress response. One potentially suitable agent is polyethylene glycol (PEG, Figure 5-14). It is inert, thermally stable, non-toxic and most importantly, cannot be utilised by microorganisms [192]. It can bind water molecules via hydrogen bonds, and therefore, lowers water activity. However, PEG can also form hydrogen bonds with some gelling agents, such as agar and therefore, at high concentration (>10%), it prevents agar from solidifying.

For this project, PEG 1400 (the average molecular weight is around 1400) was selected as water activity reducing agent, principally for its low cost. At 298.5 K and 2.46% (w/w), its viscosity is 0.1216 Pa.s which is much higher than water (8.9×10^{-4} Pa.s). In addition, its volatility is also well below that of water which means it will not be lost by evaporation [193].

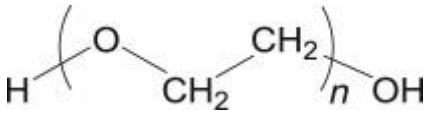

Figure 5-14 Chemical structure for polyethylene glycol (PEG)

Experimentally, water activity can be measured by dynamic vapour sorption (DVS). The DVS equipment measures the amount of water a particle absorbs, and the time it takes to reach equilibrium under different conditions such as temperature and humidity, by measuring weight change of the particle in a sealed chamber. At equilibrium, the water activity is numerically equal to the relative humidity at that temperature. Water activity is widely used in food science and the minimum water activity required for different microorganisms to thrive has been determined and is summarised in Table 5-1. The minimum requirement for most *Aspergillus*

species is in the range 0.75 to 0.82. In comparison, the minimum requirement of *R.oryzae* is 0.88, which is much higher than the *Aspergillus* fungi.

Table 5-1 Minimum water activity for microbial growth, adapted from [194] and [195]

Water Activity	Type of Microorganism	Name
0.97	Bacteria	*Clostridium botulinum E* *Pseudomonas fluorescens*
0.95	Bacteria	*Escherichia coli* *Clostridium perfringens* *Salmonella spp.* *Vibrio cholerae*
0.94	Bacteria	*Clostridium botulinum A, B* *Vibrio parahaemolyticus*
	Fungi	*Stachybotrys atra*
0.93	Bacteria	*Bacillus cereus*
	Fungi	*Rhizopus nigricans*
0.92	Bacteria	*Listeria monocytogenes*
0.91	Bacteria	*Bacillus subtilis*
0.90	Bacteria	*Staphylococcus aureus (anaerobic)*
	Fungi	*Trichothecium roseum*
	Yeast	*Saccharomyces cerevisiae*
0.88	Yeast	*Candida* *Rhizopus oryzae*
0.85	Fungi	*Aspergillus clavatus*
0.84	Fungi	*Byssochlamys nivea*
0.83	Fungi	*Penicillium expansum* *Penicillum islandicum* *Penicillum viridicatum*
	Yeast	*Debarymoces hansenii*
0.82	Fungi	*Aspergillus fumigatus* *Aspergillus parasiticus*
0.81	Fungi	*Penicillum cyclopium* *Penicillium patulum*
0.80	Fungi	*Penicillium citrinum*
	Yeast	*Saccharomyces bailii*
0.79	Fungi	*Penicillum martensii*
0.78	Fungi	*Aspergillus flavus*
0.77	Fungi	*Aspergillus niger* *Aspergillus ochraceous*
0.75	Fungi	*Aspergillus restrictus* *Aspergillus candidus*
0.71	Fungi	*Eurotium chevalieri*
0.70	Fungi	*Eurotium amstelodami*
0.62	Yeast	*Saccharomyces rouxii*
0.61	Fungi	*Monascus bisporus*
<0.60	No microbial proliferation	

5.6 Summary

So far, the effects of water on molecules and cells have been examined. At the molecular level, water shapes and stabilises biomolecules and assists their function; at cellular level, water affects cellular shape, composition, gene expression, metabolite production and growth. At the community level, it affects structure and total microbial activity. The effects of water on microorganisms is such a broad topic that within the limitation of a chapter, it can't be comprehensively summarised or explained in full detail. Despite this, aspects covered in this chapter provide a glimpse of how life is entirely governed by water from the molecular reaction to the cellular community. And yet, notwithstanding advances in biochemistry and microbiology, there lacks sufficient connection to translate existing knowledge into fermentation and industrial technology. For example, as discussed above, in natural liquids, individual cells are in fact closely connected and communicated with each other through soluble chemicals. In such manner, they can cooperatively respond to environmental changes. However, in a normal submerged culture, cells are dispersed, chemical cues are diluted, and their communication is interrupted.

This leads to a number of interesting possibilities. Is it possible to bring effects to the fermentation system by mimicking the natural environment? Is it possible to compare solid state fermentation with a submerged one where cellular communication is still preserved? Is it possible to take advantage of the general stress response system of microbes and make fermentation more robust against contamination? Is it possible to mimic the effect of macromolecular crowding on enzyme activity enhancement in a submerged culture? There are clearly opportunities to adapt existing knowledge to biotechnological applications.

Apart from adaption of existing knowledge, there is also currently a lack of fundamental understanding of the systems. For instance, in solid state fermentation, the most common expression of water quantity is through simple water content. However, water availability has effects from the molecular level to the microbial community level, and knowing the heterogeneous properties of the substrate, water content is clearly not enough to describe the amount of water required at a molecular level, cellular level or at a microbial community level. In addition, does the water stressed condition normally occurring in solid state fermentation bring about any differences, if not advantages, compared to a liquid culture?

To obtain a specific fundamental understanding of water in the context of the solid state fermentation system, an experimental investigation into the bioavailability of water to microorganisms growing on solid substrates was conducted and is reported in the following chapter (Chapter 6).

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Chapter 6.

Effects of Water on Solid State Fermentation:

An Experimental Investigation

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Chapter 6

Effects of Water on Solid State Fermentation, an Experimental Investigation

In the previous chapter, a theoretical investigation of the effects of water on molecules, cells and microbial community has been reported. In this chapter, experimental work was conducted and reported, in order to develop a specific fundamental understanding of the bioavailability of water in solid state fermentation, in terms of its dynamic relationship with the substrate and its effects on microbial growth. It starts with examining if existing representations, water content and water activity, are sufficient for representing water bioavailability in solid systems, in other words, examining if water bioavailability is actually closely related with the ratio of weights or with the chemical properties of the substrate.

6.1 Investigating Water Content and the Bioavailability of Water

In the field of solid state bioprocessing, the water available to microorganisms (bioavailability) is usually represented as percent water content. However, as many have questioned, does water bioavailability directly correlate with the ratio of the weights of water and substrates? To answer this question, some preliminary experiments were conducted and are reported below.

Rapeseed meal (RSM) and sugarcane bagasse (SCB) were dried in separate containers in a 60℃ oven. Potato dextrose broth (PDB) was dissolved in HPLC grade water and then the solution was well mixed with SCB. The ratio between the two components was 2.4 g PDB per 100 g wet substrate. The wet substrate was then divided into four groups, the water content in groups 1 and 2 was 65% (w/w), while in groups 3 and 4 it was 90%. For comparison, rapeseed seal was also mixed with PDB solution, the concentration of which was 2.4 g PDB per 100 g wet substrates, and the water content was 65% (w/w).

The wet substrates were then autoclaved at 121℃ for 20 min before dispensing onto petri dishes. Each petri dish contained 15 g of the wet substrate, and was inoculated with 1 mL of *A.oryzae* spore suspension, in the centre of the substrate. The spore count was 2.4×10^6 per mL. After inoculation, groups 1 and 3 were incubated in a sealed container where humidity was controlled at a low level by adding desiccants. Groups 2 and 4 were placed on a rack in another sealed container where the humidity was kept above 90% by adding water into the bottom of the container below the petri dishes. Rapeseed meal was also divided into two groups (5 and 6). Group 5 was incubated in the dry environment whereas Group 6 was incubated in the humid environment. For all groups, the fermentation was carried out at 30℃ for 72 h, and photos taken after the fermentation are shown below (Figure 6-1 and Figure 6-2). Experimental details are given in Table 6-1.

Table 6-1 Experimental conditions for solid state fermentation with different substrates, water content and fermentation humidity

Group	Water content (%, w/w)	Solid substrates	Inoculum (mL)	Fermentation humidity (%)	Fermentation time (h)
1	65	SCB+PDB	1	low	72
2	65	SCB+PDB	1	>90%	72
3	90	SCB+PDB	1	low	72
4	90	SCB+PDB	1	>90%	72
5	65	RSM+PDB	1	low	72
6	65	RSM+PDB	1	>90%	72

Figure 6-1 presents images of the substrates fermented in dry environments at 30℃ for 72 h. It is clear that, for the two substrates that had the same water content (65%, Group 1 and Group 5), fungal growth was much better on the substrate composed of rapeseed meal and PDB. PDB contains simply nutrients which are preferentially utilised prior to SCB and RSM by microorganisms. Therefore, nutrient supply to microorganisms is assumed to be very similar. In addition, the poor fungal growth on the SCB + PDB substrate was greatly enhanced by increasing the amount of water in the substrate (Group 3). This further suggests the difference in fungal growth between Group 1 and Group 5 were mainly brought about by the difference in water bioavailability. In other words, substrate properties have direct impacts on the bioavailability of water and these impacts cannot be fully represented by the ratio between the weight of water and substrates (water content).

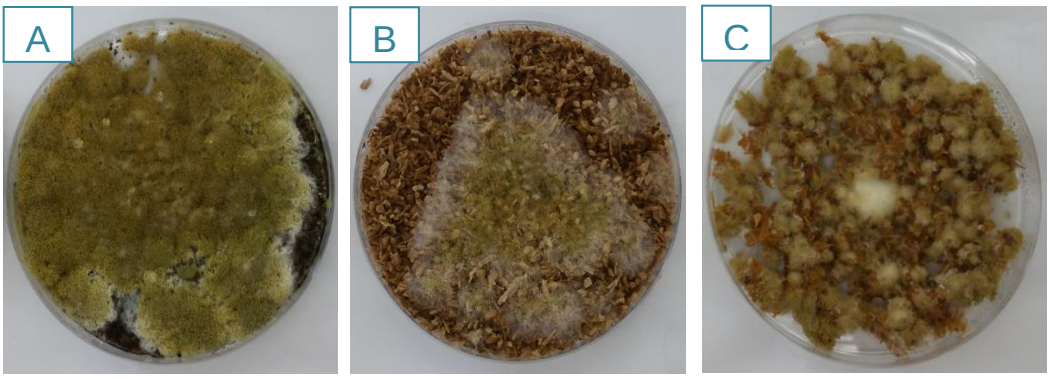

Figure 6-1 Different substrates fermented with *A.oryzae* in dry environments for 72 h, at 30℃. A: RSM+PDB with 60% (w/w) water content. B: SCB+PDB with 60% water content. C: SCB+PDB with 90% water content.

In addition to physical and chemical properties of the substrates, water bioavailability also seems to be affected by the presence of water in the gas phase i.e. humidity, but again, such impact is influenced by the properties of the substrate. Figure 6-2 shows a comparison of the effects of humidity on microbial growth on different substrates. On one hand, comparing to Group 1, incubating substrates in humid environments (Group 2) can significantly enhance fungal growth, despite both groups having started with the same water content. In fact, growth in Group 2 was as good as in Group 3, in which, the substrate water content was 90%. On the other hand, the enhancement of growth brought about by the humid environment did not appear in groups where RSM+PDB was used as substrate. Even though Group 5 and Group 6 were fermented in distinctly different humidities, the fungal growth in the two groups were almost identical.

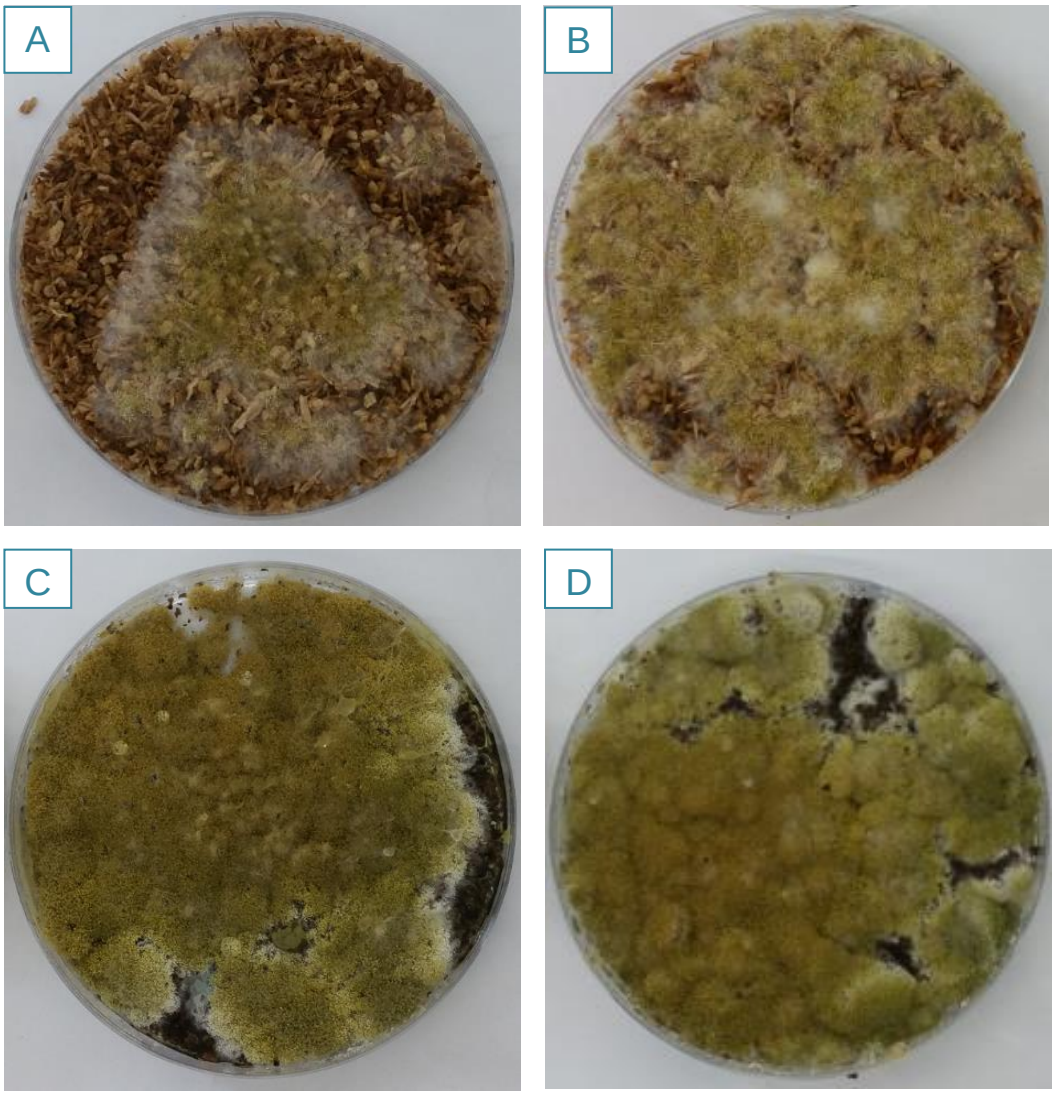

Figure 6-2 Different substrates containing 60% (w/w) water, fermented with *A.oryzae* in different humidity for 72 h, at 30℃. A: SCB+PDB, humidity was controlled at a low level. B: SCB+PDB, humidity was more than 90%. C: RSM+PDB, humidity was controlled at a low level. D: RSM+PDB, humidity was more than 90%.

At this stage, it was not quite clear why water requirements for rapeseed meal and sugarcane bagasse are so distinctly different, nor how water bioavailability is affected by substrate properties or humidity. It is nevertheless certain that water bioavailability cannot be effectively represented by substrate water content alone.

6.2 Investigating Water Activity and Water Bioavailability

As discussed in the previous chapter, apart from water content, water bioavailability can also be represented in terms of water activity. To examine if water activity can fully represent water bioavailability in a solid state fermentation system, a set of experiments was designed and conducted (Section 6.2.1). To begin with, it was necessary to test if PEG is a suitable water activity reducing agent, by testing if it can be utilised by *A.oryzae* or has any toxicity to the fungus. This was achieved by carrying out fermentations in Czapek media in which PEG either replaced the glucose as the sole carbon source or was supplemental to a complete medium to test its toxicity. Following validation of its suitability, the effects of PEG on fermentations carried out in submerged culture and on solid substrates were explored and are reported in the second part of this section (6.2.2).

6.2.1 PEG as a Water Activity Reducing Agent

Czapek medium, as described in Chapter 2, was used as the basis for submerged and solid fermentations. Modifications of the medium were made by replacing or supplementing glucose with PEG 1400. The variations are summarised in TableTable 6-2. In Group 1, glucose was replaced by 2.2% PEG, while in Group 2, the same amount of PEG was added to an otherwise complete medium. Group 3 was exactly as described in Chapter 2 and was used as a control. All media were autoclaved at 121℃ for 20 min before dispensing as 50 mL aliquots into 100 mL flasks. Each flask was inoculated with 1 mL *A.oryzae* spore suspension with a spore count of 2.6×10^6 per mL. In each group there were 4 flasks. After

inoculation, all flasks were incubated in a 30℃ incubator, and shaken at 180 rpm. A single flask was taken out at 48, 90, 120 and 192 h. The flask contents were transferred into a 50 mL centrifuge tube and centrifuged at 10000 rpm for 10 min, after which, the liquid phase was separated from the solid phase by decanting into another container and then sealed and frozen at -18℃ before further analysis. The solid phase was kept in the centrifuge tube and, as described in Chapter 2, was used to measure biomass dry weight. Concentrations of residual glucose and PEG in the liquid phase were also analysed as described in Chapter 2.

In addition to the above, 2 groups of solid media (4 and 5) containing the same nutrients as Group 1 and Group 2 were made by adding 1.5% agar. The media were well mixed and autoclaved at 121℃ for 20 min before dispensing onto petri dishes. Each dish contained 15 g of medium and was inoculated with dry spores using a sterile loop. After the inoculation, all dishes were incubated at 30℃ for 90 h in an incubator where humidity was controlled below 20%. The detailed recipe for all experimental groups is summarised in Table 6-2.

Table 6-2 Variations of Czapek media used for liquid and solid fermentations

Ingredients	Group 1 % (w/w)	Group 2 % (w/w)	Group 3* % (w/w)	Group 4 % (w/w)	Group 5 % (w/w)
Agar	0	0	0	1.5	1.5
PEG	2.2	2.2	0	2.2	2.2
Glucose	0	2.8	2.8	0	2.8
K_2HPO_4	0.1	0.1	0.1	0.1	0.1
$FeSO_4 \cdot 7H_2O$	0.001	0.001	0.001	0.001	0.001
$MgSO_4$	0.05	0.05	0.05	0.05	0.05
KCl	0.05	0.05	0.05	0.05	0.05
$NaNO_3$	0.3	0.3	0.3	0.3	0.3

* Unmodified Czapek medium

Resultant biomass dry weight for groups 1, 2, and 3 are shown in Figure 6-3. It is clear that biomass production in Group 1 was much lower than in the other two groups and unexpectedly, Group 2 had the highest biomass production. This was possibly because some of the PEG remained in the solid phase after centrifugation, and was counted as biomass. As discussed in the previous chapter, the viscosity of PEG is very high and therefore, it is not easily separated from the solid phase, especially as cells provide additional surface for attachment. It also cannot be removed by evaporation in an oven set at 60 ℃. As a result, the actual biomass production in Group 1 could be even lower than the value shown in Figure 6-3. Therefore, biomass dry weight is not ideal for evaluating submerged fermentation when PEG is present.

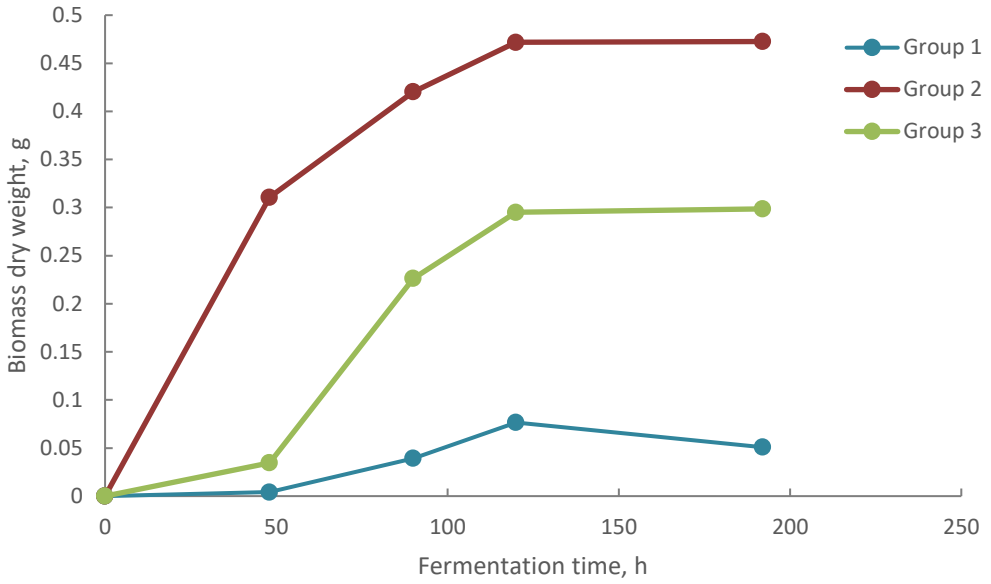

Figure 6-3 Biomass dry weight for groups 1, 2 and 3, after 0, 48, 90, 120 and 192 h fermentation at 30°C and shaken at 180 rpm

Figure 6-4 shows the residual glucose and PEG concentrations for group 1, 2 and 3. It is clear that, during 192 hours of fermentation, PEG concentration in all groups remained at roughly the same level, or in some cases, slightly increased, which was due to the water loss from evaporation. Combining the fact that very little biomass was produced in Group 1, where PEG was used as the sole carbon source, this means PEG cannot be readily utilised by *A.oryzae*. In addition, the fact that glucose consumption in groups 2 and 3 were almost identical means PEG is not toxic to *A.oryzae*. The apparent increase in biomass in Group 2 compared to Group 3 is clearly not linked to increased consumption of glucose or PEG and is therefore, mostly likely the result of poor separation as suggested above.

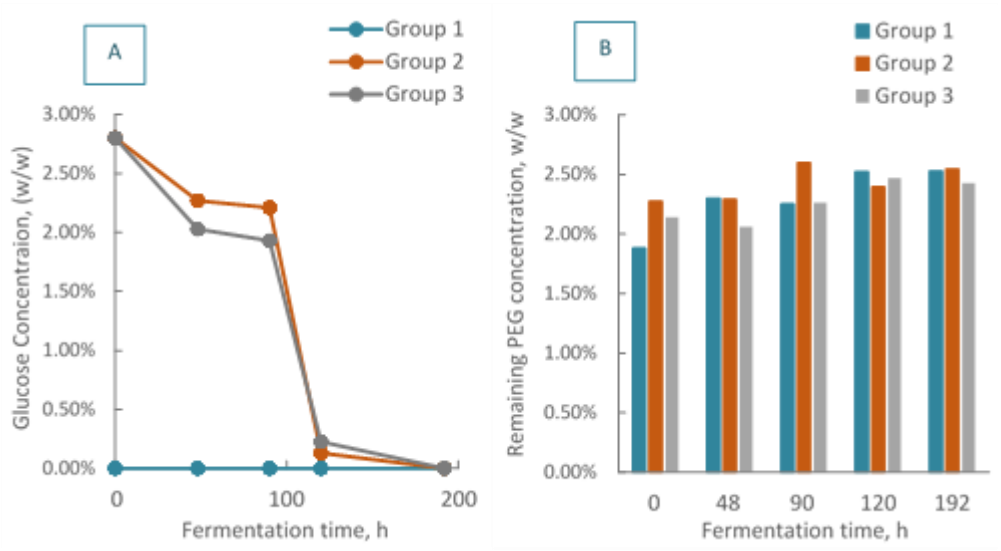

Figure 6-4 Residual glucose (A) and PEG (B) concentration of groups 1, 2 and 3, after 0, 48, 90, 120, and 192 h fermentation at 30℃ and shaken at 180 rpm

For solid state fermentation, photos of groups 4 and 5 after 90 h fermentation are shown in Figure 6-5. It is clear that, as with liquid cultures, fungi could not grow on solid media where PEG was used as sole carbon source. The results show not only that *A.oryzae* does not use PEG as carbon source either in liquid medium or on solid substrate, but also that PEG is not toxic to *A.oryzae*. It was therefore considered to be suitable for use as a water activity adjusting agent for subsequent experiments.

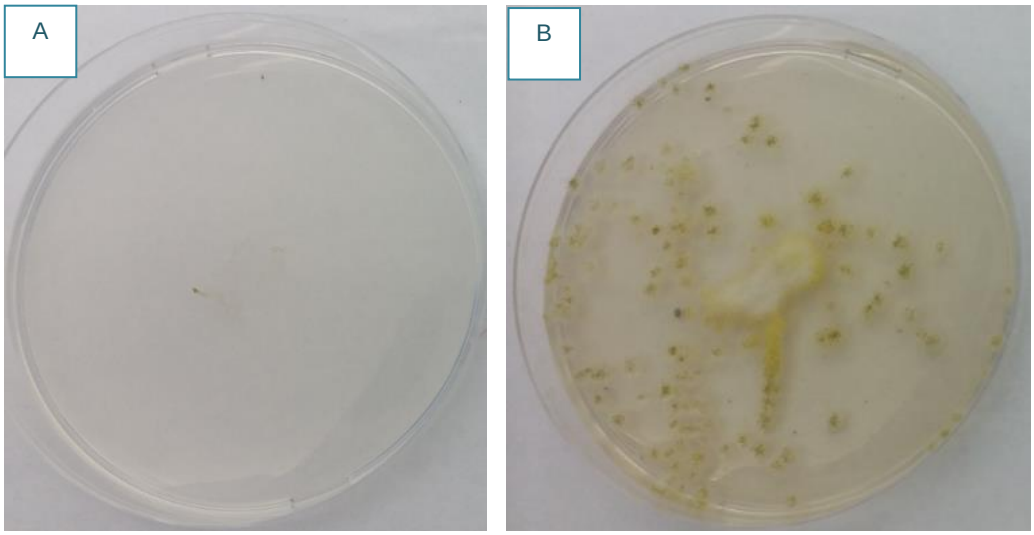

Figure 6-5 Photos of solid medium, Group 4 (A) and Group 5 (B), after 90 h incubation at 30 ℃

6.2.2 Effect of PEG on Microbial Growth in Liquid and Solid Substrates

After testing the suitability of PEG as a water activity reducing agent, the next step was to examine if water activity is a meaningful representation of the bioavailability of water in a solid substrate. The effects of reducing water activity on fermentations carried out in submerged culture and on solid substrates were therefore explored. The findings are reported and compared in this section.

For these experiments, liquid PDB culture (PDB concentration 3.9 %, w/w, in HPLC grade water) was divided into 3 groups. Group 1 did not contain any PEG whereas 30% and 50% (w/w) of PEG were added into Group 2 and Group 3 respectively. Water activity of the three cultures were measured using DVS, as described in Chapter 2. All liquid media were autoclaved at 121℃ for 20 min before dispensing into 100 mL flasks. There were 5 flasks in each group and each flask contained 50 mL of medium. Of these, 4 flasks were inoculated with 1 mL *A.oryzae* spore suspension (Spore count was 2.6×10^6 per mL) and 1 flask was left as a blank. All flasks were incubated at 30℃ and shaken at 180 rpm. At 48, 66, 144 and 192 h after inoculation, a single flask from each group was taken from the

incubator for analysis. In addition, under sterile conditions, a 10 mL sample was also taken from the blank flask of each group. For the flasks in which fermentations were carried out, photos were taken after 48, 144 and 192 h. The liquid content in each flask was then transferred into a 50 mL centrifuge tube and centrifuged at 10000 rpm for 10 min. The liquid phase was separated from the solid phase by decanting into another container and this was sealed and frozen at -18℃ before further analysis. Glucose concentration in all liquid samples was analysed. The detailed experimental procedures for submerged fermentation are summarised in Table 6-3.

Table 6-3 Experimental conditions for submerged fermentation with PDB and PDB+PEG media, inoculated with *A.oryzae* spore suspension

Group	PDB Concentration (%, w/w)	PEG concentration (%, w/w)	Shaking speed (rpm)	Fermentation temperature (℃)
1	3.9	0	180	30
2	3.9	30	180	30
3	3.9	50	180	30

In addition to the above, solid PDA and PDA+PEG substrates were also made. Two groups (Group 4 and Group 5) of pure PDA were prepared as described in Chapter 2, each petri dish contained 25 g PDA. Furthermore, four groups of PDA with PEG infused in them, were also prepared. For the infusion, PEG solution (PEG concentration 50%, w/w) was poured on top of 25 g of solidified PDA, which were then placed with the lid open to evaporate excess water in a sterile laminar flow cabinet at room temperature. The final PEG concentration was controlled by adjusting the amount of PEG solution poured onto the PDA and also the amount of time the dish was left for water evaporation. After evaporation, Group 6 and Group 7 contained 30% PEG and approximately 65% of water whereas Group 8 and Group 9 contained 50% PEG and the overall water content was around 45%.

Each group comprised of 2 dishes, one was inoculated with dry *A.oryzae* spores in the centre of the dish, and the other (kept as a blank) was sampled. Two samples were taken from the surface, one to measure water activity (as described in Chapter 2), the other for weighing, both before and after drying in a 60℃ oven to determine its water content. Groups 4, 6 and 8 were kept in a sealed container where humidity was maintained at low level by adding desiccants. Groups 5, 7 and 9 were placed on a rack in another sealed container where the humidity was maintained above 90% from water kept at the bottom of the container below the petri dishes. Fermentation for all groups was carried out at 30℃. At 48, 92, and 144 h, photos of each petri dish were taken. The detailed experimental procedures are summarised in Table 6-4.

Table 6-4 Experimental conditions for solid state fermentations inoculated with *A.oryzae* spores carried out on PDA dishes with different PEG concentrations and humidities

Group	PDA Concentration (%, w/w)	PEG concentration (%, w/w)	Fermentation humidity (%)	Fermentation temperature (℃)
4	3.9	0	low	30
5	3.9	0	>90%	30
6	3.9	30	low	30
7	3.9	30	>90%	30
8	3.9	50	low	30
9	3.9	50	>90%	30

Photos for submerged fermentations (groups 1, 2 and 3) carried out for 48, 144 and 192 h are shown in Figure 6-6. Water activity values for substrates used in groups 1, 2 and 3 are presented in Table 6-5. As shown in Figure 6-6, during 48 h fermentation, the fungus grew into many balls of hyphae when they were cultivated in media that did not contain any PEG (Group 1). These hyphal pellets can be clearly seen from all the flasks in Group 1. In comparison, even after 192 h

fermentation, there was very limited visible hyphae in both Group 2 and Group 3 where PEG was present, especially in Group 3 in which the largest amount of PEG had been applied. This is as expected, since more PEG results in a lower culture water activity (Table 6-5), and the lower the water activity, the less fungal growth is apparent.

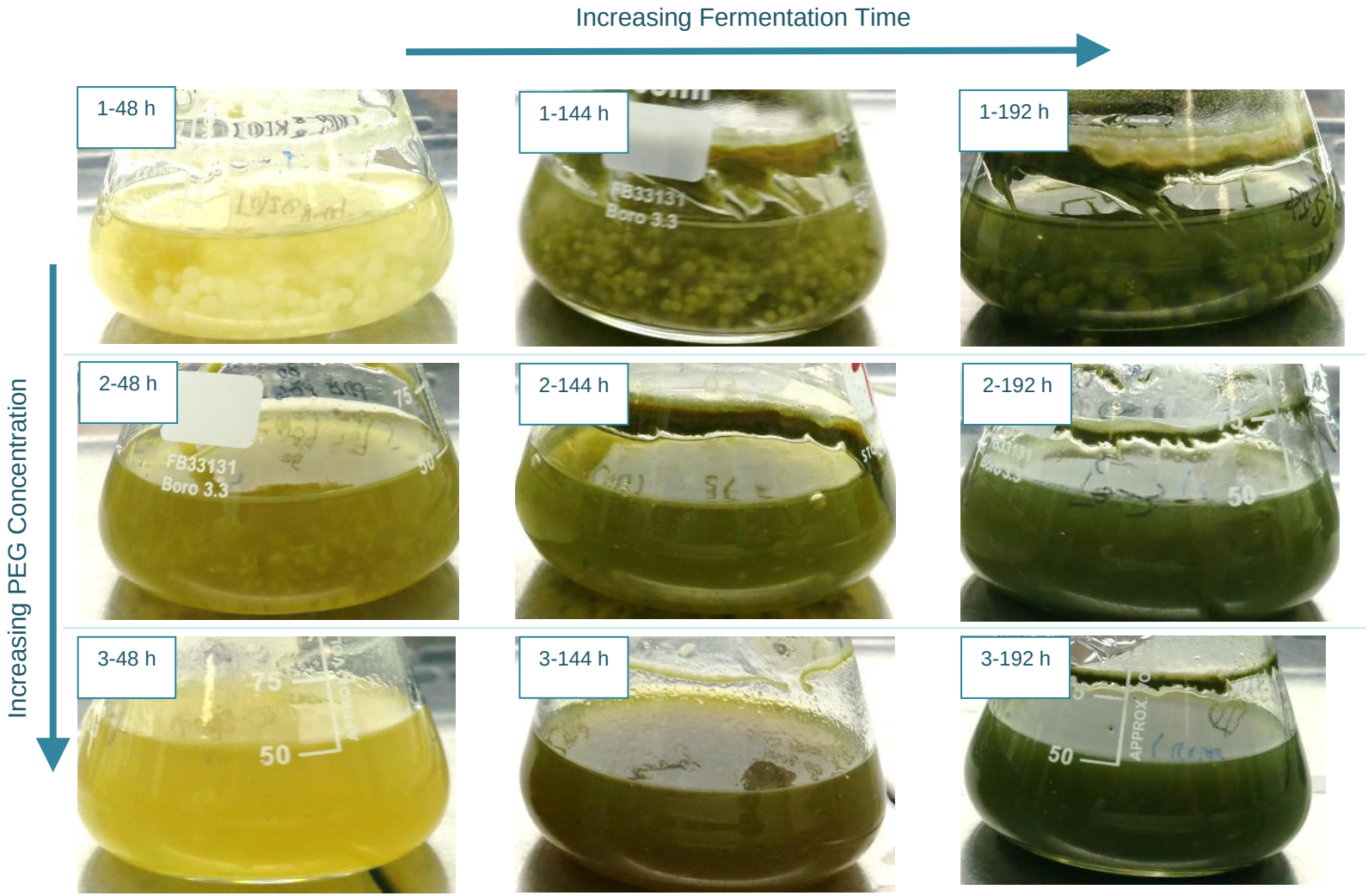

Figure 6-6 Submerged fermentations carried out in PDB media containing different levels of PEG, at 30°C for 48, 144 and 192 h. Group 1: PDB, Group 2: PDB + 30% PEG, Group 3: PDB + 50% PEG

Table 6-5 Water activity determined by DVS for PDB media mixed with different levels of PEG

Group	PDB Concentration (%, w/w)	PEG concentration (%, w/w)	Water Activity
1	3.9	0	~1
2	3.9	30	0.93
3	3.9	50	0.91

Fungal glucose consumption over 192 h fermentation is shown in Figure 6-7. Fungal glucose consumption is the difference in the glucose concentration between the blank samples and the inoculated ones. Similar to the photo records, the highest glucose consumption occurred in Group 1, in which the liquid culture did not contain any PEG. The difference in glucose consumption between groups became more distinct with time, such that by the end of 192 h fermentation glucose consumption in Group 1 was much higher than the other two. This is possibly because PEG concentration increases with time due to water loss through evaporation and lowers the water activity in groups 2 and 3 even further. As a result fermentations become more and more difficult in the cultures where PEG was present. Comparing Group 3 to Group 2, the former had slightly higher glucose consumption, which could be a result of fungal osmotic stress response. As discussed in the previous chapter, in cultures with low water activity, fungal cells produce and accumulate compatible solutes (e.g. glycerol) to balance the intracellular and extracellular osmotic pressure and to prevent the loss of cellular water. The lower the water activity of the solution, the greater is the requirement for compatible solutes and hence, more glucose is consumed as the carbon source and also as the energy source for the microorganisms.

In general, the effects of water activity on fungal growth and consumption in submerged fermentation is as expected and as reported in the literature [191].

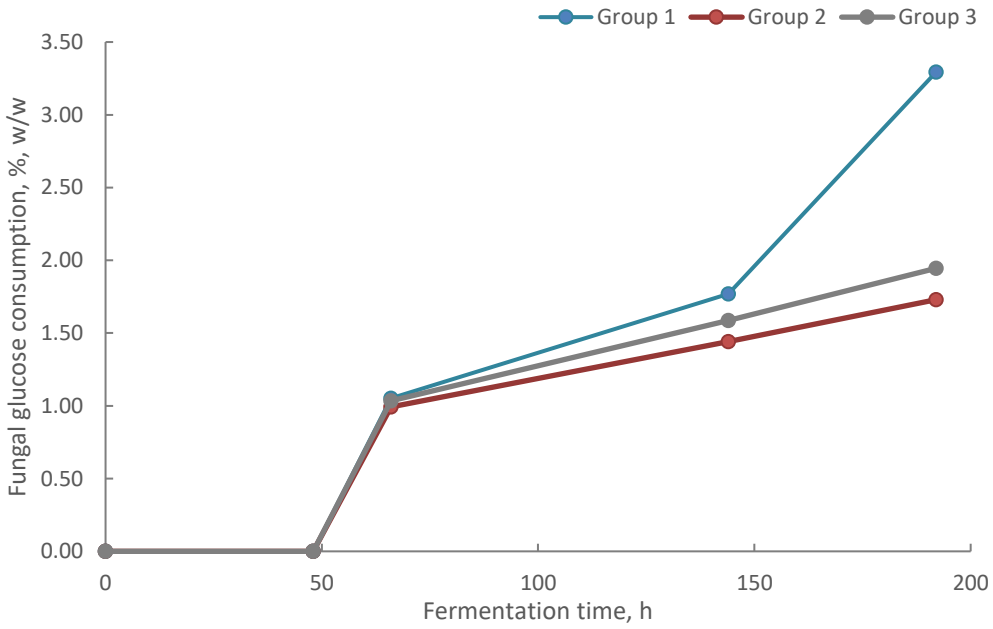

Figure 6-7 Glucose consumption by *A.oryzae* in groups 1, 2 and 3 after 48, 66, 144 and 196 h of fermentation at 30°C and shaken at 180 rpm

Water activity values for solid substrates in groups 4 – 9 are shown in Table 6-6. It appears that samples taken from the surface of PDA+PEG dishes have a much lower water content than the average for the whole dish. Consequently, the water activity at the surface was also much lower than expected. This means that water activity at the place where the fungus was inoculated and grew had a much lower value than the liquid cultures containing the same amounts of nutrients and PEG. This is because a highly concentrated PEG solution was poured onto the PDA, forming a thick PEG layer covering the PDA layer. During the evaporation process, water did not evaporate evenly from the two layers, but mostly evaporated from

the PEG layer. Therefore, water content and consequently water activity of samples taken from the surface were much lower than expected.

Table 6-6 Water activity measured by DVS and water content for PDA and PDA+PEG infused dishes

Group	PDA Concentration (%, w/w)	PEG concentration (%, w/w)	Water Content at the surface (%, w/w)	Water Activity at the surface
4	3.9	0	96%	~1
5	3.9	0	96%	~1
6	3.9	30	38%	0.85
7	3.9	30	38%	0.85
8	3.9	50	17%	0.77
9	3.9	50	17%	0.77

Photo records for fungi cultivated on solid substrates in groups 4 – 9 for 48, 90 and 144 h are shown in Figure 6-8 to Figure 6-10. It is clear that as with submerged fermentation, fungi grew better on the substrates that did not contain PEG (Group 4 and Group 5). For these groups, humidity did not appear to have any effect on fungal growth.

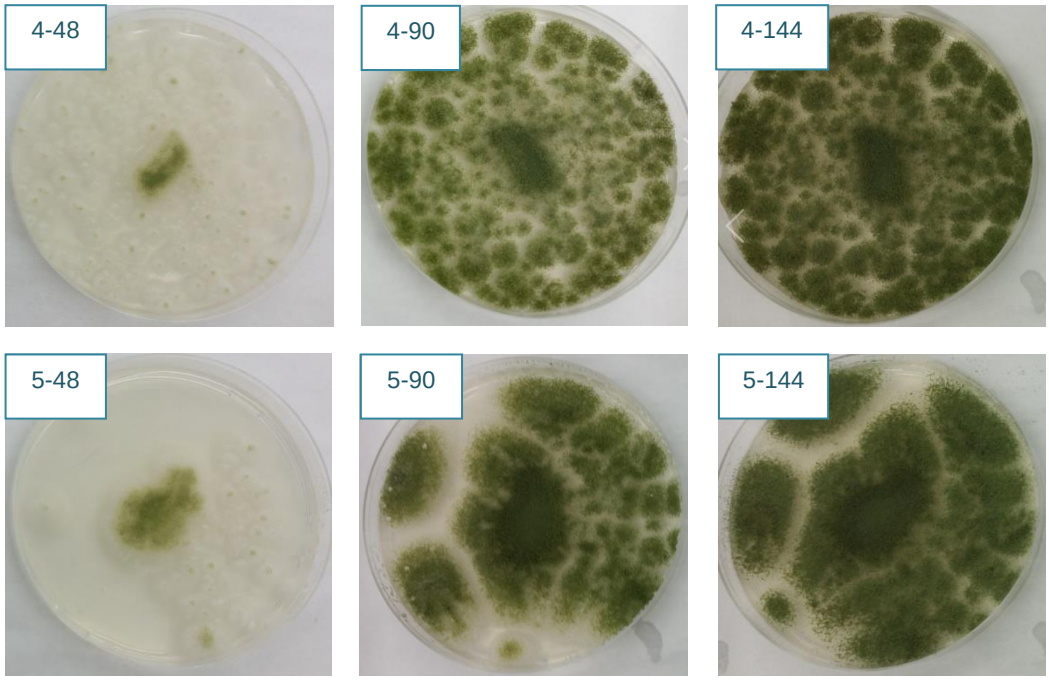

Figure 6-8 Solid state fermentations carried out on PDA substrates in dry humidity (Group 4, fermented with dessicant) and high humid (Group 5,humidity >90%) at 30°C. Dishes are shown for 48, 90, and 144 h after inoculation

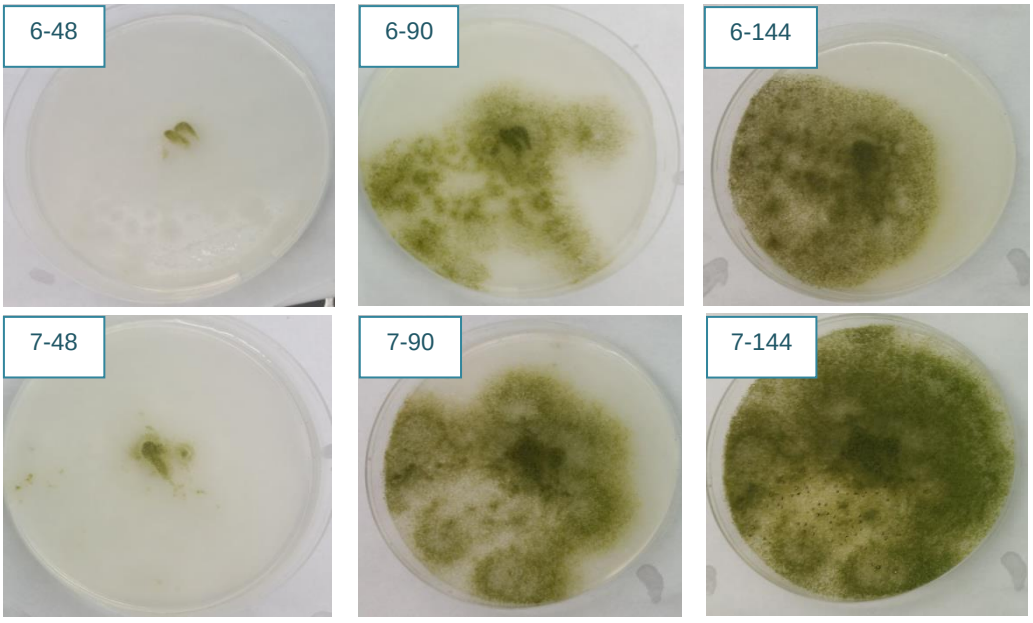

Figure 6-9 Solid state fermentation carried out on PDA substrate infused with 30% PEG, in dry humidity (Group 6,fermented with dessicant) and high humid (Group 7, humidity>90%), at 30°C. Dishes are shown for 48, 90, and 144 h after inoculation.

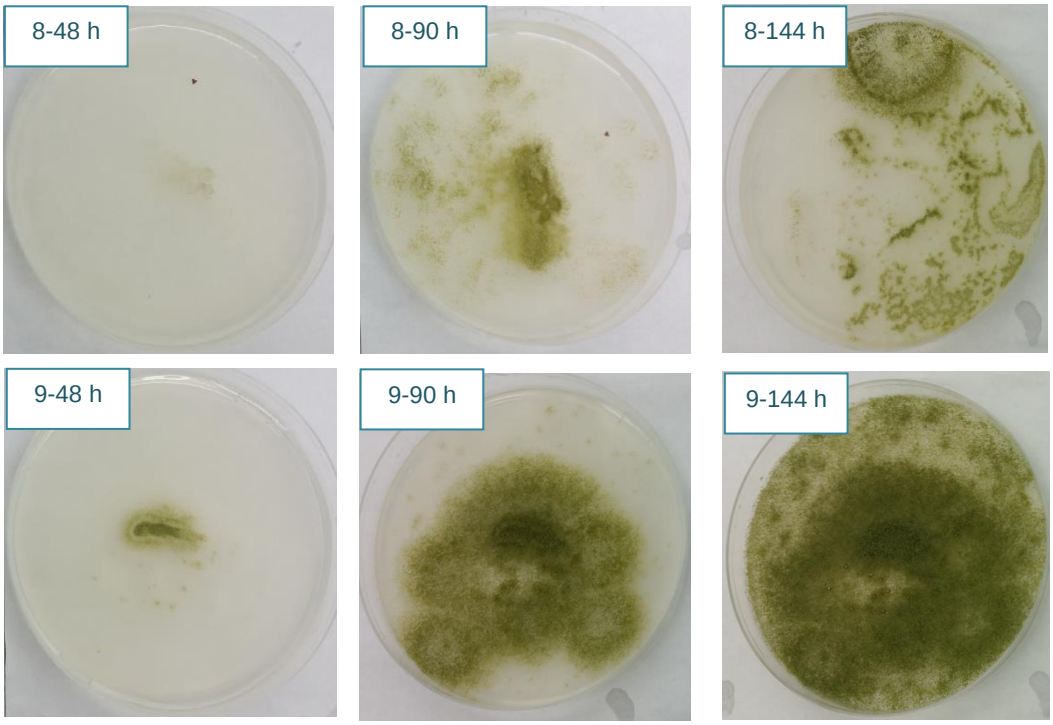

Figure 6-10 Solid state fermentation carried out on PDA substrate infused with 50% PEG, in dry humidity (Group 8, fermented with dessicant) and high humidity (Group 9, humidity>90%) environments, at 30°C. Dishes are shown for 48, 90, and 144 h after inoculation.

For fermentations that were carried out in dry environments, the presence of PEG in the substrate significantly inhibited fungal growth. The more PEG the substrate contained, the poorer was the growth. For example, as shown in Figure 6-10, even after 144 h fermentation, *A.oryzae* had barely grown on substrates in Group 8. This is as expected because in this group, water activity at the surface was only 0.77, which is near the minimum requirement for growth of this microorganism. Surprisingly, the growth inhibition caused by low water activity was significantly eased in the more humid environment. Both Group 7 and Group 9, in which the substrates were cultivated under humid conditions, had much better growth than the same substrates incubated in dry environments. In fact, despite the distinct difference in substrate water activity, Group 7 and Group 9 appear to have very similar overall growth.

The enhancing effects brought about by humidity on fungi growing on solid substrates with low water activity is very similar to the result that has been reported in Section 6.1, in which, water in the gas phase also significantly enhanced fungal growth on relatively dry sugarcane bagasse. The relationships between water activity and water content for rapeseed meal (RSM) and sugarcane bagasse (SCB) are shown in Figure 6-11.

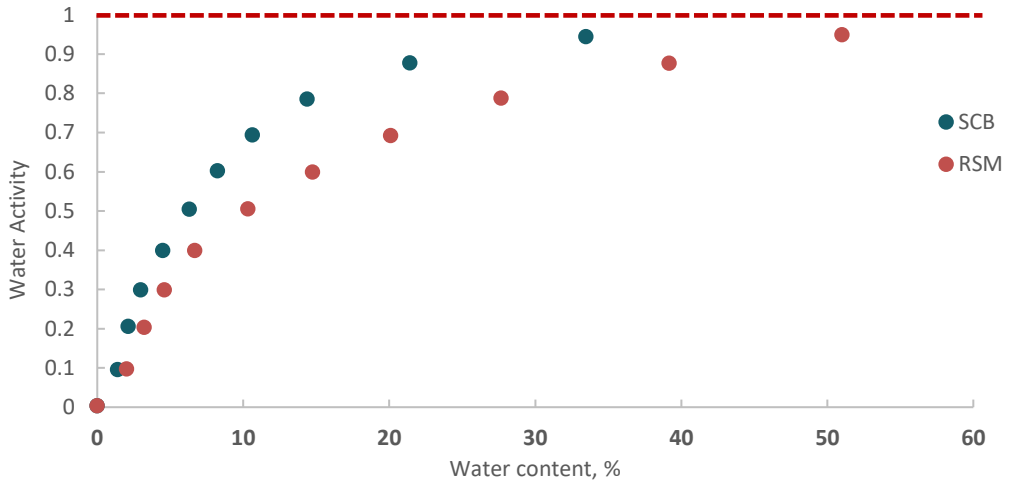

Figure 6-11 Water activity of RSM and SCB at different water contents, the dotted line shows the maximum possible value (i.e. =1)

It is surprising to see that sugarcane bagasse in fact needs less water content in the substrate to reach the same water activity as rapeseed meal. This means, if water activity were sufficient for describing the bioavailability of water, sugarcane bagasse would need less water for fermentation than rapeseed meal. However, as shown in Figure 6-1, this is not the case.

It is also interesting that the enhancement on fungal growth due to humid environment only affects those substrates low in water content or water activity. It seems that water in the gas phase can act as an alternative water source for

microorganisms, and therefore compensate the insufficient water supply in the solid substrate. At this stage, it is not yet clear how fungi access water from the gas phase. Nonetheless, It is clear that both water content and water activity are insufficient to describe the bioavailability of water in solid substrates. In other words, water bioavailability is not straightforwardly related to the ratio between the weights of water and substrate, nor the chemical properties of the substrate.

Microbial growth measurement for solid state fermentation systems has always been a challenge, because it is very difficult, if not impossible, to separate the biomass from the solid substrate. Clearly, a more accurate technique is needed for measuring fungal growth on solid substrates if the effect of water on growth is to be fully quantified. Neither visual observation nor determination of fungal growth area can accurately reflect the extent of fungal growth beyond the surface of the substrate. Photo quality is also heavily affected by environmental conditions, such as lighting, camera setting, camera positions etc. In addition, both substrates and fungi vary in colour and texture. As a consequence, while photographic records and visual observations are valuable and useful, they are also very limited in their applicability. Even though direct measures of biomass production in solid state fermentation systems cannot be achieved, quantifiable measures of metabolism do exist in submerged fermentation technology and therefore could be used as alternative indicators. In aerobic fermentations, CO_2 is produced from metabolic processes and therefore, can be used to indicate biomass production. This was tested for solid state fermentation systems as part of the experiment reported in Section 6.3.

6.3 How does Water in the Gas Phase Impact Growth on Solid Substrates

As seen in the previous section, water in the gas phase plays an important role in solid state fermentation systems. However, it is not clear how microorganisms access this water. There are three possibilities: either the microorganism can utilise the water directly from the air, or the water is absorbed into the solid substrate and then becomes available to the microorganism, or a combination of both the above. In order to understand the detailed interaction between water in the gas phase and microorganisms growing on solid substrates, the effect of using water in the gas phase as the sole supply of water was investigated and is reported below. The effects of substrate water absorption from the gas phase were also investigated and are reported later.

In an attempt to obtain more quantitative data, CO_2 production was monitored as an indicator for fungal growth in the following experiments. PDA dishes were prepared as described in Chapter 2, each dish containing 25 g substrate. These were inoculated with dry fungal spores in the centre of the dish and were placed on a wet sponge within a sealed sterilised fermenter to maintain humidity above 90%. The fermenter was then incubated at 30℃, and CO_2 production was monitored as described in Chapter 2 using a gas analyser. In order to test the feasibility and reliability of CO_2 production as an indicator for biomass production, photos were taken at 17, 34, and 65 h, using an endoscopic camera placed in the fermenter. In addition, an identical fermentation was carried out as a duplicate to test reproducibility.

In addition to the above, a PDA dish was dried in a 60℃ oven until its weight became constant. The resultant dried PDA film (shown in Figure 6-12) was then inoculated with dry fungal spores in the centre of the dish, and the whole dish was placed on a sponge in a sealed fermenter as described above. Fermentation was again carried out at 30℃ with humidity maintained above 90%.

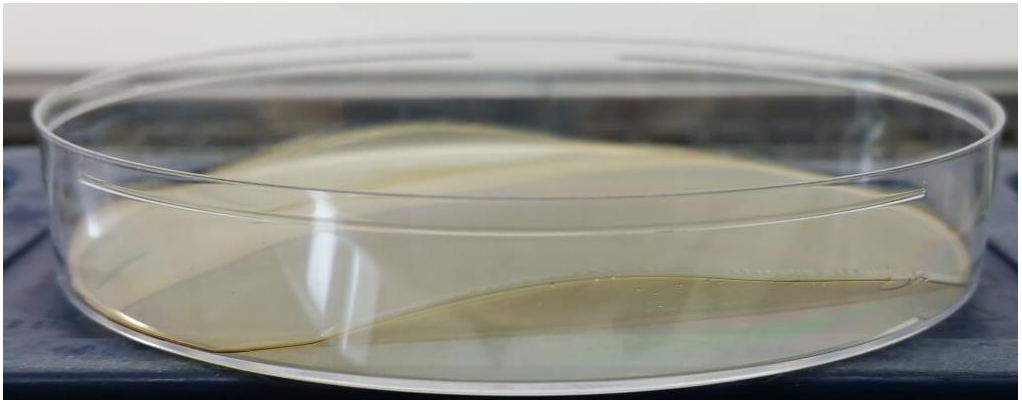

Figure 6-12 A PDA substrate after drying in a 60℃ oven to constant weight (PDA film)

Endoscope photos were continuously produced throughout the fermentations. Figure 6-13 shows example photos from dish 1, chosen to illustrate onset of growth (17 h), mid growth phase (34 h) and peak growth (65 h) for *A.oryzae* growing on hydrated PDA. Also included, for completeness, are photos for inoculated PDA film at the same times, indicating essentially no growth. The field of view of the endoscope is limited and hence only a fraction of the substrate surface can be seen. Corresponding CO_2 production from both substrates is shown in Figure 6-14.

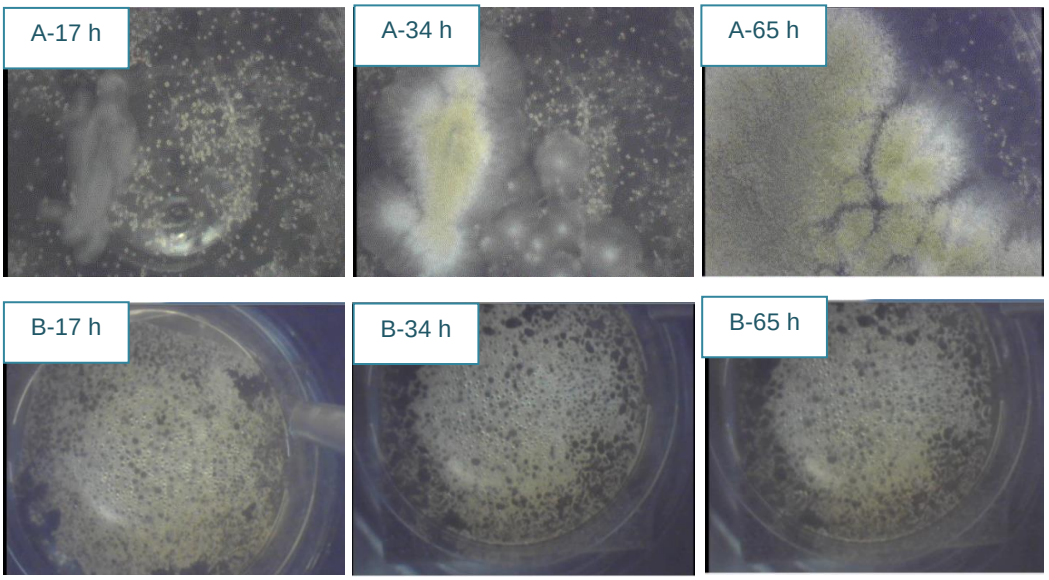

Figure 6-13 Fungal growth on a hydrated PDA substrate (A) and dry PDA film (B) for 17, 34 h and 65 h, fermentation temperature was 30°C, humidity was >90%

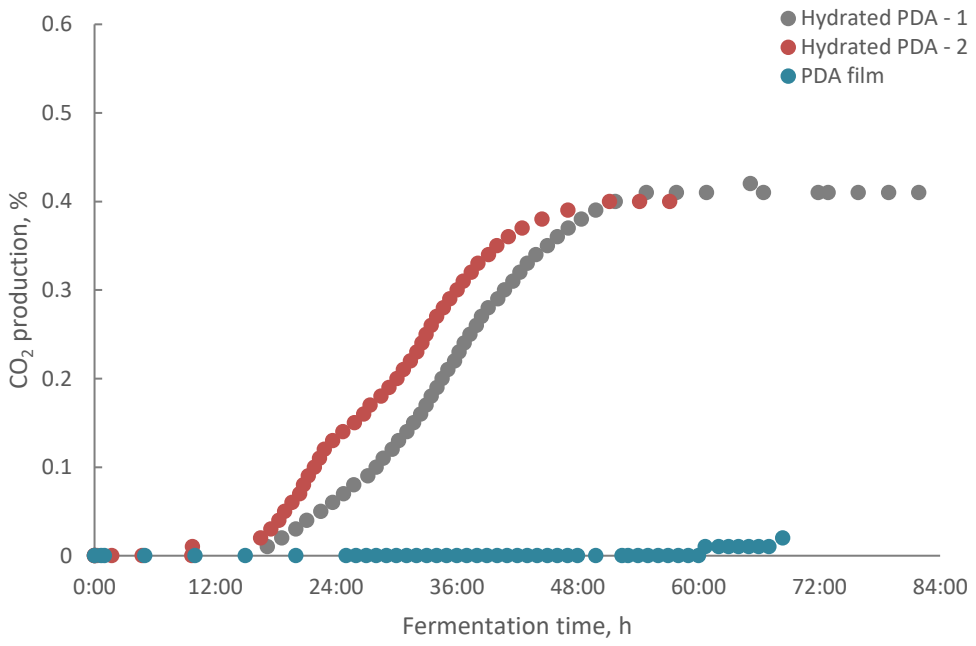

Figure 6-14 CO_2 production by *A.oryzae* on hydrated PDA and PDA film. Fermentations were carried out at 30°C, with humidity >90%

As shown in Figure 6-14, CO_2 production from hydrated PDA dishes showed good repeatability, considering that it is impossible to inoculate with identical numbers of viable spores. It is also clear that CO_2 production can successfully reflect biomass production. However, such reflection is not always as sensitive as visual observation: in the experiment, germination became visible prior to the time when CO_2 production first became detectable. This is because CO_2 concentration was measured by a gas analyser for which the minimum detectable change in gas concentration is 0.01%.

The gas analyser consumes a small amount of the sample gas and as gas recirculation continues, CO_2 is continually lost. Therefore, the value shown on the analyser should be slightly lower than the actual CO_2 concentration. This effect is shown in the results (Figure 6-15) of a simple experiment in which a standard gas (1.4% CO_2) was circulated within the closed system used for the fermentation experiments. In Figure 6-14, CO_2 concentration clearly reached a peak value, suggesting maximum metabolic activity. However, the fungal colony was still visibly expanding, though probably at a lower metabolic rate. The CO_2 concentration appeared to remain constant throughout the period until growth actually stopped. It subsequently reduced, presumably due to CO_2 consumption by the analyser (see Figure 6-16). Nonetheless, CO_2 production is still a good indicator for fungal growth especially for the early stages of the fermentation and was therefore used throughout the experiments reported in this chapter.

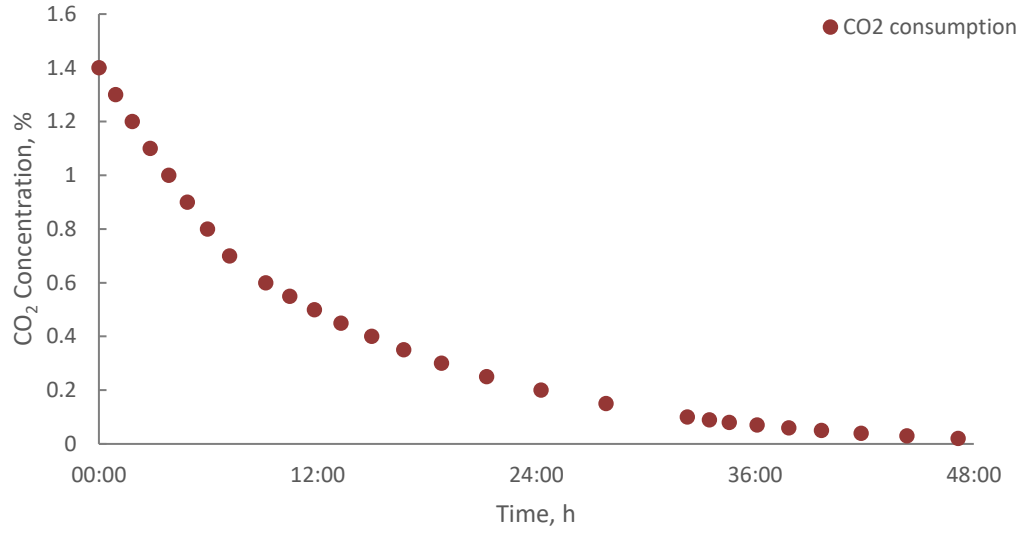

Figure 6-15 CO_2 consumption by gas analyser, measured by a standard gas (1.4% CO_2)

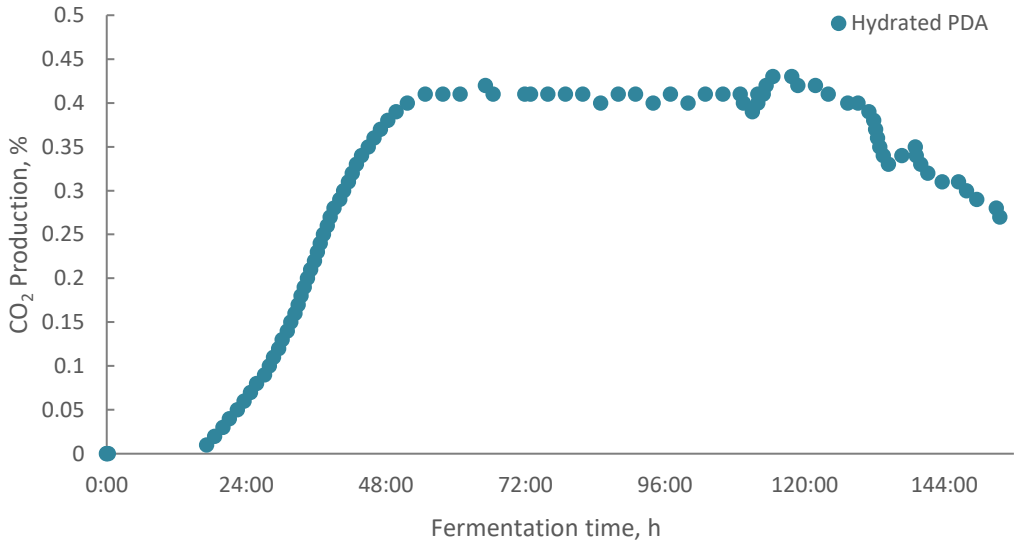

Figure 6-16 14 CO_2 production by *A.oryzae* on hydrated PDA over 156 h. Fermentations were carried out at 30℃, with humidity >90%

From Figure 6-14 it can also be seen, by comparing with the hydrated PDA, that CO_2 production was barely noticeable in the experiment where PDA film was used as the substrate. This suggests that fungal spores do not germinate when there is

only water in the gas phase. It is likely that water from the gas phase must be absorbed into the substrate first. As discussed in the previous chapter, water can be absorbed into the substrate chemically, such as through hydrogen bonding, and physically, such as by capillary action or diffusion. In other words, both chemical and physical substrate properties can affect water absorption and consequently water availability to microorganisms. The effects of substrate chemistry were studied next and are reported below in Section 6.4.

6.4 Effects of Substrate Chemistry on the Bioavailability of Water from the Gas Phase

To study the effect of substrate chemical properties on water absorption and water bioavailability, four groups of substrates were prepared. Group 1 and Group 2 were pure PDA, Group 3 and Group 4 were PDA + 4% PEG. The detailed preparation is described in Chapter 2. Each dish contained 25 g of substrate and was inoculated with dry *A.oryzae* spores in the centre of the dish. After inoculation, a single dish was placed on a sponge in a sealed fermenter. Groups 1, and 3 were incubated in dry environments where humidity was maintained at a low level by adding desiccants in the sealed fermenter. Groups 2, and 4 were incubated in humid environments where humidity was maintained above 90% by water added to the sponge beneath the dish prior to inoculation. Fermentations were carried out at 30℃ and CO_2 production was measured as described in Chapter 2. In addition, to quantify how much water the substrate can absorb from the gas phase and also how long it takes to reach equilibrium, samples of PDA and PDA + PEG

(4%) were dried to constant weight in a 60℃ oven, then analysed in the DVS to determine weight changes under different humidity conditions.

The DVS results indicating the amount of water that can be absorbed by the substrates are shown in Figure 6-17. At lower humidities (hence water activity) the pure PDA absorbed much more than the PDA + PEG but at 90%, weight gain was similar for both substrates.

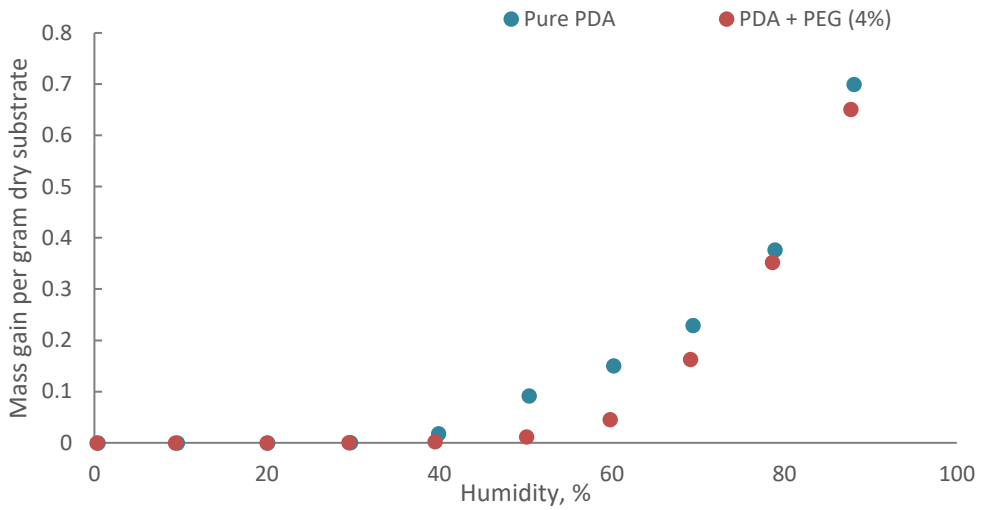

Figure 6-17 The amount of water absorbed by PDA, and PDA + PEG (4%), at different humidities

CO_2 production by *A.oryzae* fermented on the two substrates in dry and humid environments is shown in Figure 6-18 and Figure 6-19. The results confirm that if the fermentation is carried out in a dry environment, i.e. no water in the gas phase, fungal growth is dominantly affected by the presence of water in the substrate, which is consequently under the influence of the chemical properties of the substrate. The onset of exponential growth appeared much later in Group 3, in which PEG was incorporated into the substrate than in Group 1 where pure,

hydrated, PDA was used. The growth rate was also much lower in Group 3 and therefore, the overall CO_2 production was much lower.

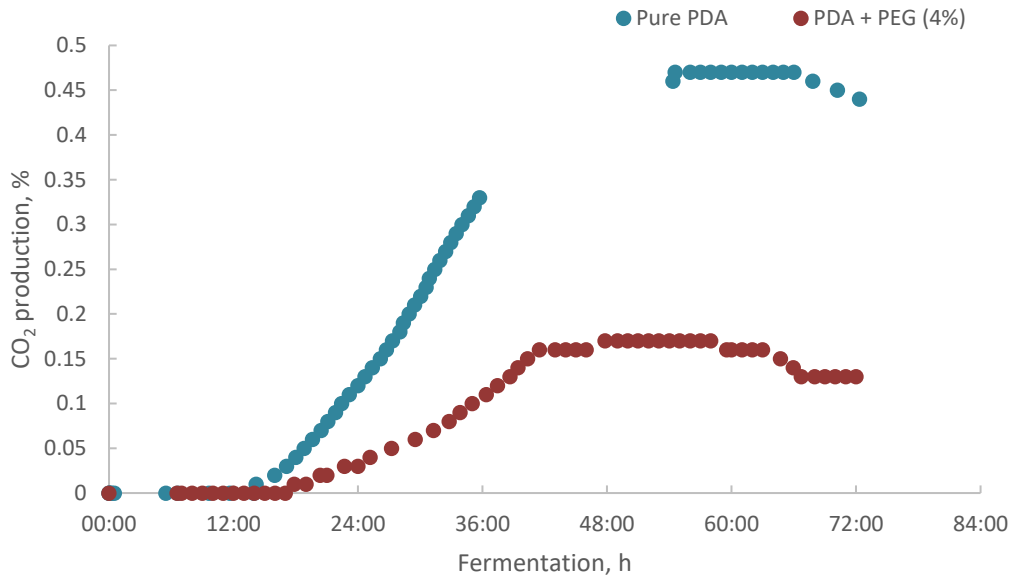

Figure 6-18 CO_2 production by *A.oryzae* growing on different substrates, PDA and PDA + PEG (4%), in dry humidity

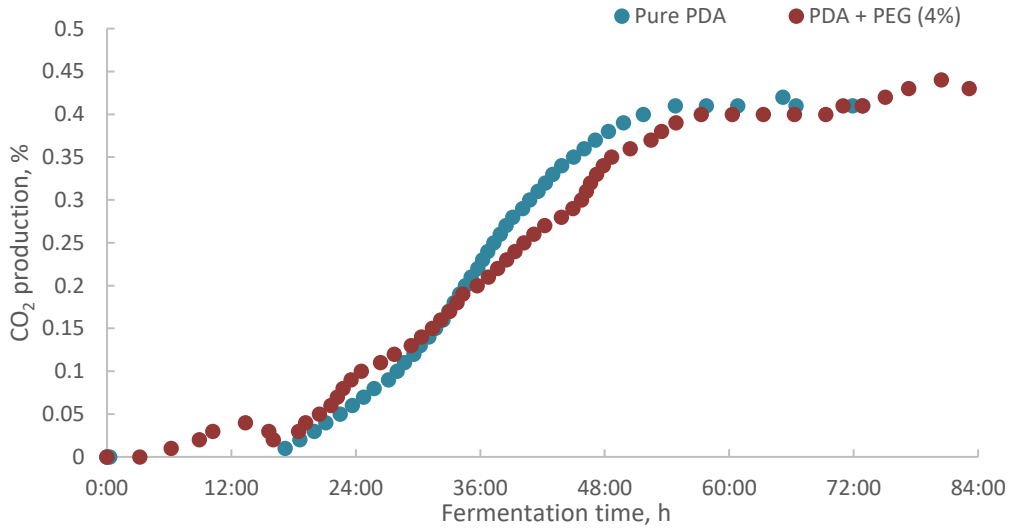

Figure 6-19 CO_2 production by *A.oryzae* growing on different substrates, PDA and PDA + PEG (4%), humidity >90%

If, on the other hand, the fermentation is carried out in a humid environment, water in the gas phase can be such a significant supply for microorganisms growing on the solid substrate that it can completely compensate for any reduction in water activity due to the addition of PEG. The CO_2 production from the two groups cultivated in the humid environment were very similar, both in terms of the length of the pre-exponential growth period, the exponential growth rate, and the maximum CO_2 concentration reached, despite their distinct difference in PEG concentration. This result provides quantitative evidence that water absorbed from the gas phase vitally affects fungal growth on the solid substrate. To explain this phenomenon, the following theory is proposed…

> "Fungal growth can only consume water from the surrounding local micro-environment. As germination and growth occur, they result in a modest depletion of water from the immediate vicinity. This creates a driving force for water to migrate from the bulk substrate (usually through diffusion), or to be absorbed from the gas phase, to replenish the water that has been consumed. The driving force within the substrate increases with fungal water consumption, while water absorption from the gas phase is driven both by the gas phase relative humidity and presence/absence of water at the substrate surface. Meanwhile, the resistance to mass transfer is greatly affected by both chemical and physical properties of the substrate. Fungal growth can continue, if, and only if water within their immediate vicinity can be replenished."

In the case of PDA, water is abundant within the substrate, and consequently sufficient to support fungal germination, and hence relatively early onset of exponential growth. In addition, the water mobility within the substrate is rapid and consequently the migration of water from the bulk substrate rehydrates the water depleted area in a timely manner. Combining with the fact that PDA has a relatively high water activity, there is only a small driving force for water to be absorbed from the gas phase. Therefore, diffusion within the substrate becomes virtually the sole means of supplying water for fungal growth. Hence, CO_2 production from hydrated PDA substrates cultivated in both humid and dry conditions are at the same level.

In the case where PEG is present, due to the additional hydrogen bonding between the PEG and water, the availability of water within the immediate vicinity of the spores is significantly reduced. Therefore, when cultivated in a dry environment, the presence of PEG delays the onset of exponential growth. Through the same hydrogen bonding mechanism, the presence of PEG reduces the mobility of water within the substrate and consequently lowers the rate at which water can be replenished. As a result, exponential growth rate is limited. However, by forming hydrogen bonds with water molecules from the gas phase, the presence of PEG also increases the driving force for water to be absorbed into the substrate, and therefore, when cultivated in a humid environment, water from the gas phase becomes an important supply for the microorganisms.

This theory of water mobility and water bioavailability was further studied and is reported in the following sections. A preliminary experiment to investigate the effect of water distribution on its availability was conducted and is reported in the next section (6.5). After that, the effect of mobility between the gas phase and the substrate was investigated and is reported in Section 6.6. Then, the effect of water mobility within the substrate was tested and reported (Section 6.7). Finally, in

Section 6.8, the theory is further tested by using it to explain the water requirement in the solid state fermentation applications, i.e., with rapeseed meal and sugarcane bagasse substrates.

In addition to the above explanation concerning the bioavailability of water, it is also possible that, once germinated, hyphae are able to access water directly from the gas phase, such that greater than normal growth becomes theoretically possible. This is because additional water drawn from the gas phase by aerial hyphae could supplement that in the immediate vicinity of the hyphae at the substrate surface. This has also been studied and is reported below in Section 6.5.

6.5 Substrate Water Distribution and Bioavailability, a Preliminary Study

In Section 6.3, it was suggested that fungal growth can only consume water from its surrounding local micro-environment. In order to test if the distribution of water within the substrate affects fungal growth, a preliminary experiment was conducted. Hydrated PDA dishes were prepared as described in Chapter 2, and then dried in a 60℃ oven to produce a dried PDA film. As demonstrated in Section 6.3, fungi cannot grow for at least 60 h on a dried PDA film laid flat and cultivated in a humid environment. For the current experiment, the dried PDA film was placed at an angle (tilted) on a moist sponge in a fermenter (Figure 6-20). The sponge was carefully separated from the dried film by the plastic wall of the dish. Dried fungal spores were inoculated onto the PDA film at the lowest point of the substrate (Point A shown in Figure 6-20), before sealing the fermenter. Humidity

was kept above 90% by water in the sponge. Fermentation was carried out at 30℃ and CO_2 production was monitored.

In this set up, water distribution within the substrate became very uneven because, driven by gravity, the water absorbed from the gas phase by the dried film should accumulate at the lowest point of the dish (Point A), where the fungal spores were inoculated. If water in the local micro-environment has no impact on fungal growth, the CO_2 production from the tilted PDA film should be similar to that from the flat PDA film. In addition, if after germination, hyphae cannot utilise water directly from the gas phase then, as with the flat PDA film, there will be little fungal growth beyond point A. CO_2 production from the tilted PDA film is compared with that from the flat PDA film in Figure 6-21. At the end of the fermentation, photos were taken and are shown in Figure 6-23. In addition, endoscope photos were being continuously produced throughout the fermentation, three of these were chosen and presented in Figure 6-22 to illustrate the beginning of fermentation (0 h), mid growth phase (48 h) and peak growth (78 h) for *A.oryzae* growing on the tilted PDA film. For comparison, photos for the fermentation carried out on the flat PDA film at 0 h, 48 h and 67 h are also shown in Figure 6-22.

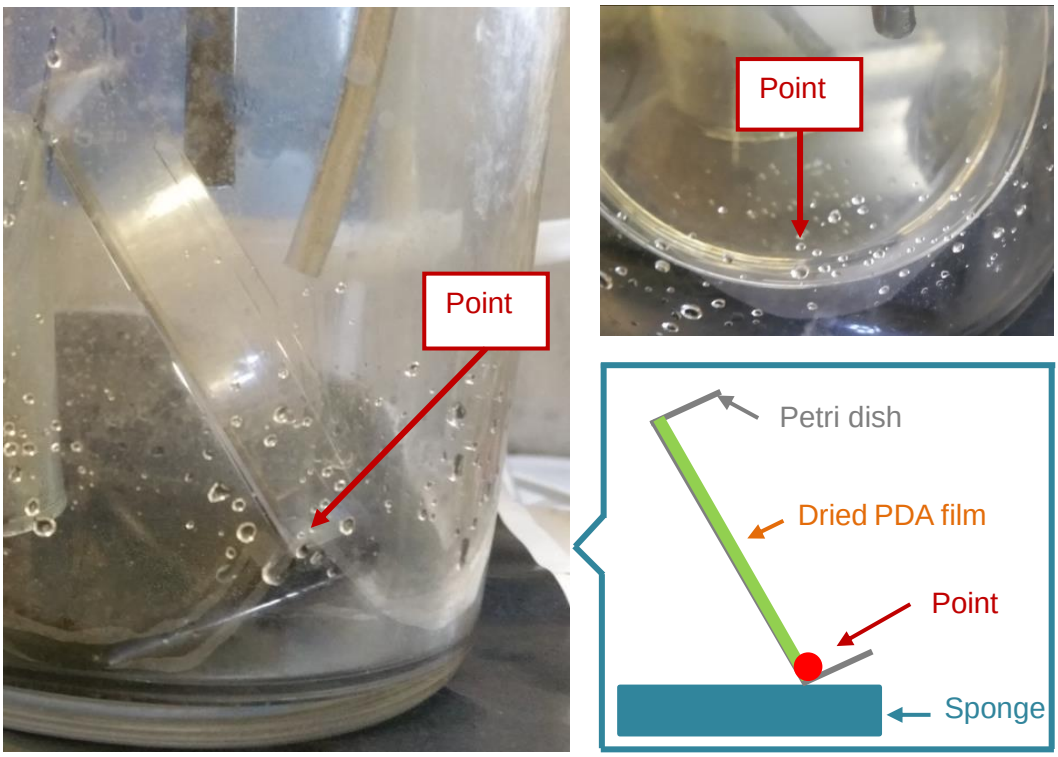

Figure 6-20 Photos for the tilted PDA film in the sealed fermenter, illustrating the inoculation point (Point A).

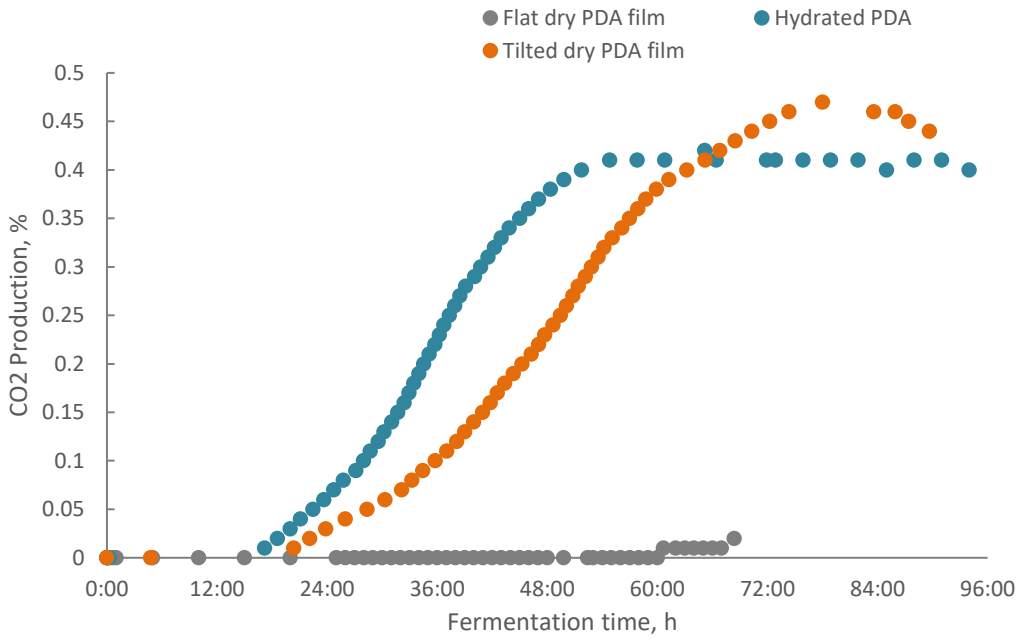

Figure 6-21 CO_2 production by *A.oryzae* on a tilted PDA film, in comparison with that produced from hydrated PDA and flat PDA film. Fermentations were carried out at 30°C, with humidity >90%

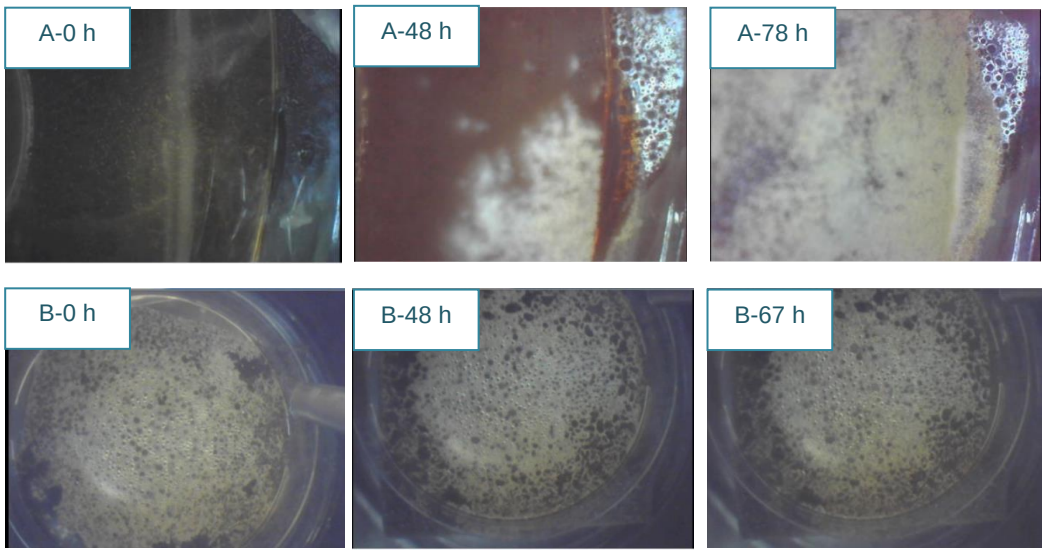

Figure 6-22 Endoscope photos of fungal growth on a tilted PDA film (A) and flat PDA film (B). Fermentation temperature was 30℃, humidity was >90%

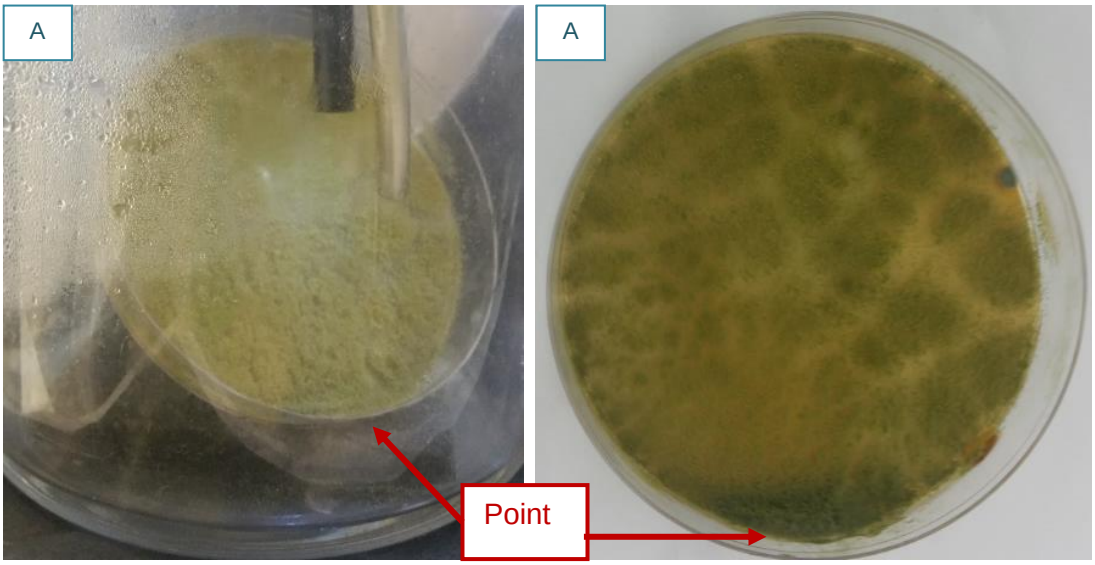

Figure 6-23 Tilted PDA film after 96 h fermentation at 30℃, 90% humidity, showing the inoculation point (Point A). A: within the fermenter, A2: after the dish was taken out

From Figure 6-21 to Figure 6-23 it can be seen that the fungi grew significantly better on the tilted PDA film, than on the flat one, both in terms of fungal colony area and also the CO_2 production. As shown in Figure 6-22, germination started from the bottom of the dish (Point A), where spores were inoculated, and where

water had accumulated by absorption from the gas phase. Combining with the CO_2 production results, the detectable germination time for the titled PDA film dish was around 20 h, which was slightly later than from the hydrated PDA (17 h). This is because, unlike on the hydrated PDA where water is immediately available, water absorption and accumulation at Point A takes time and hence results in a delayed germination.

After germination, along the PDA film, fungi grew from the bottom of the dish all the way to the top, giving a full coverage of the surface of the substrate. This indicates that, once germinated, hyphae do seem to have the ability to utilise water directly from the gas phase. This is supported by the fact that there was no visible growth nor any CO_2 production on the flat PDA dish until well after 60 h. In addition, the overall CO_2 production from the tilted PDA film was more than from the hydrated PDA substrate which indicates a greater fungal growth had occurred. As explained in the previous section, this could be because water drawn from the gas phase forms a thin water film around the hyphae within the immediate vicinity. The better fungal growth could also be due to the influence of better oxygen transfer between the gas phase and the cells through this thin water film, than through substrate alone.

As can be seen from Figure 6-23, fungi grew more around Point A. This is because the spores were inoculated there, and were therefore more abundant than on the rest of the dish where they were present only through transportation via air ventilation and only after this initial growth at Point A had resulted in the production of more spores. Towards the end of the experiment, a small colony of contaminating microorganisms was observed on the dish. It is possible that by growing on the substrate, the *A.oryzae* had altered the surrounding micro-

environment and make it more conducive for growth, even of other microorganisms.

The above provides convincing evidence that, for solid state fermentation, the bioavailability of water is determined by the accessibility of that water within the immediate vicinity of the microorganisms at a microscopic level, which is not necessarily reflected by the description of water at a macroscopic level, i.e., water content or water activity. If this is the case, water bioavailability is then defined by the amount of water within the micro-environment surrounding the microorganisms, and also the mobility of the water within the substrate or between the gas phase and the substrate. In other words, water bioavailability depends on whether there is sufficient water surrounding the microorganisms, and whether it can be supplied/replenished from beyond the immediate vicinity in a timely manner.

The bioavailability of water in the gas phase and in the substrate were studied separately and are reported in the following sections. Substrate water absorption rate, and consequently, the amount of water being absorbed within a fixed time, was altered by changing substrate physical structure. The effects of absorption rate are reported in Section 6.6, while the effect of water mobility within the substrate are reported in Section 6.7.

6.6 Effects of Substrate Physical Structure on the Bioavailability of Water in the Gas Phase

First, different drying techniques and their effects on substrate physical structure were studied. Then, substrates with identical/similar chemical composition, but

very different physical structures were made such that they would absorb similar amounts of water but at different rates. These substrates were used for fungal fermentation, to study the effects of water mobility between the gas phase/substrates on its bioavailability. The findings are reported below.

6.6.1 Effects of Drying Techniques on Substrate Physical Structure

Identical hydrated PDA dishes were prepared as described in Chapter 2, and then dried either in a 60℃ oven or in a freeze drier. In addition, the hydrated PDA was also cut into pieces and then soaked in ethanol for 24 h, before drying in a vacuum oven. After drying, photos were taken and are shown in Figure 6-24. Samples were also taken from the dried dishes and observed under scanning electron microscope (SEM), to obtain a better knowledge of the detailed physical structure (Figure 6-25).

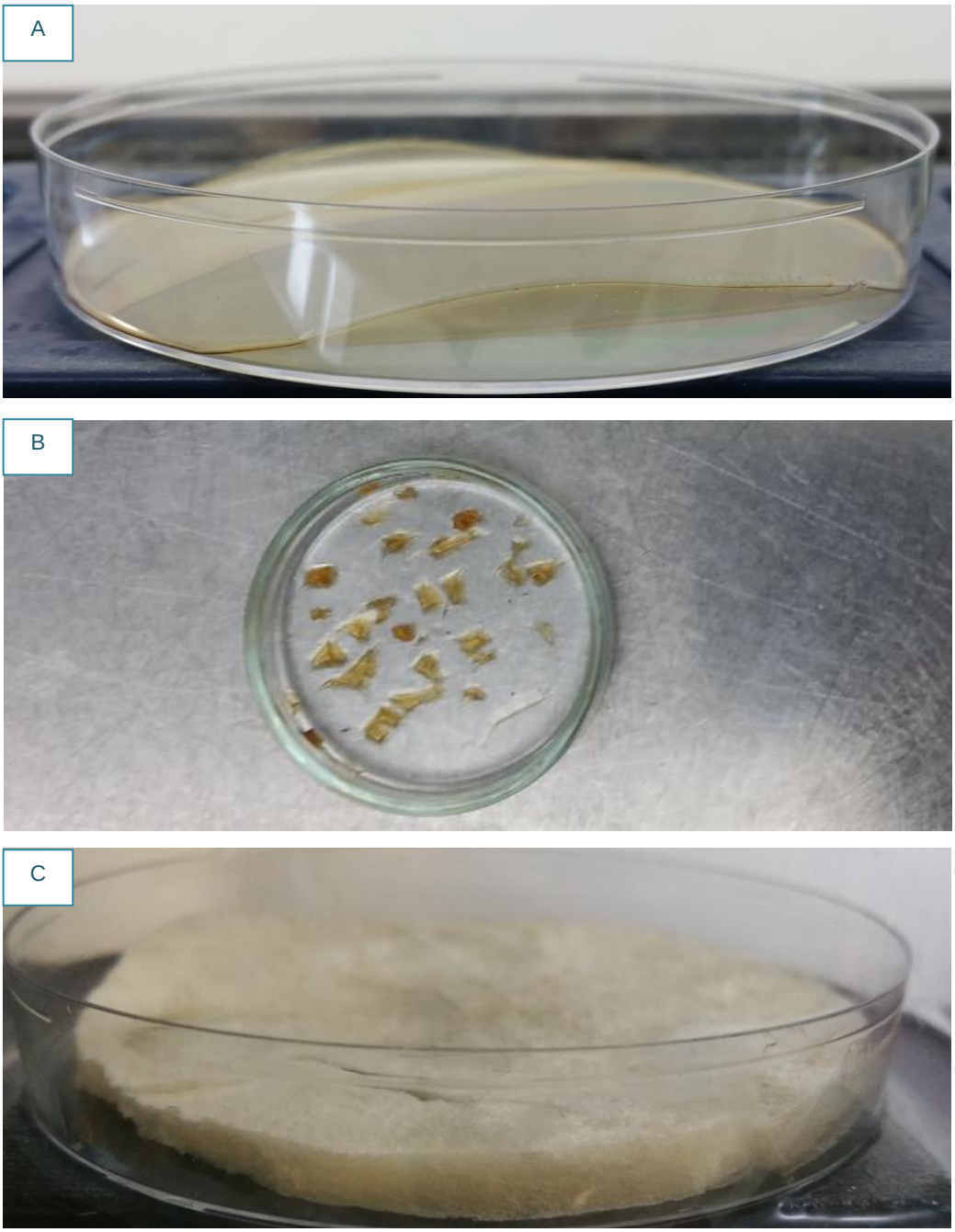

Figure 6-24 Hydrated PDA dishes after (A) drying in a 60°C oven. (B) Soaking in ethanol and then drying in a vacuum drier. (C) drying in a freeze drier

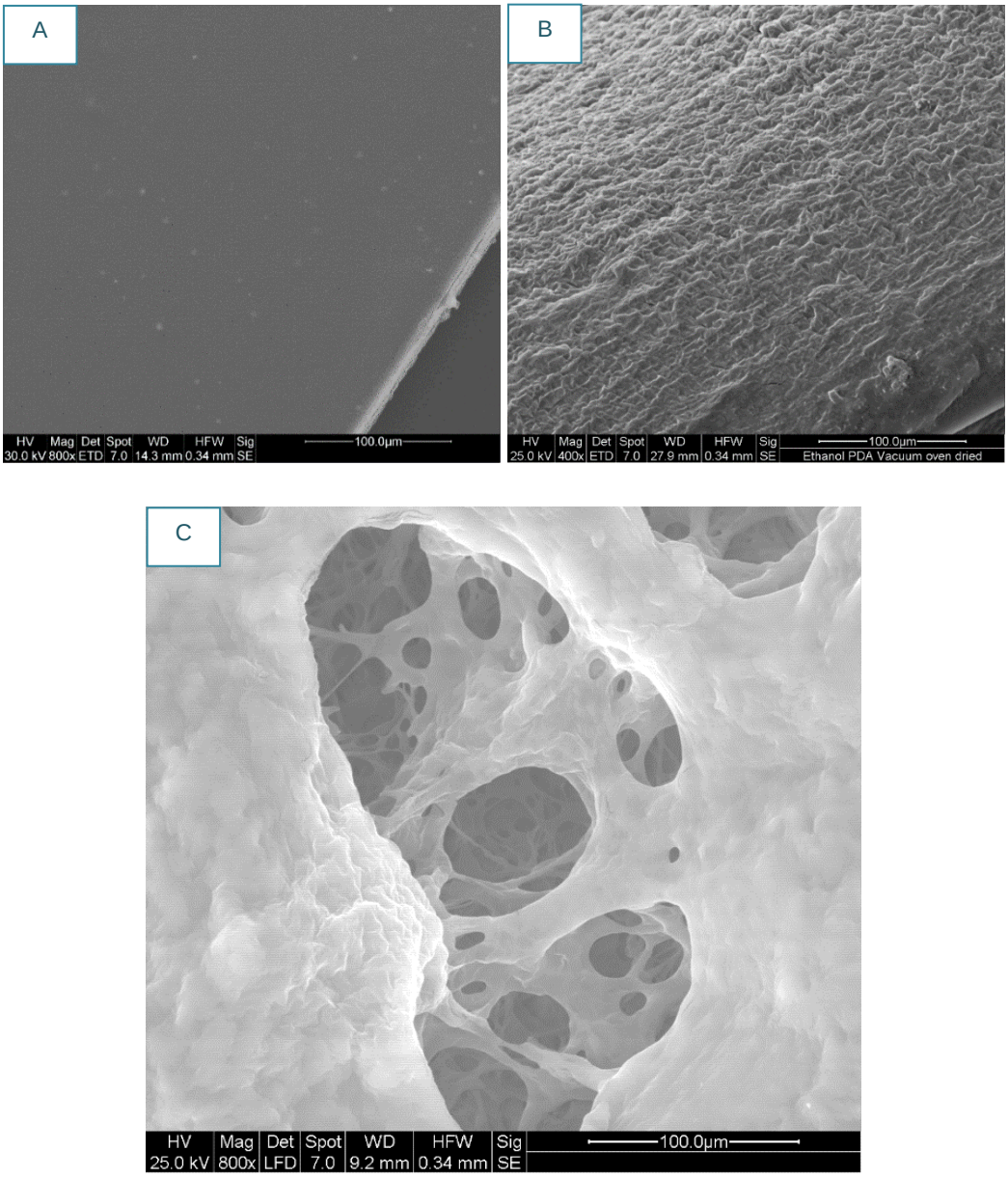

Figure 6-25 SEM photos for PDA substrate after (A) drying in 60℃ oven, showing the edge of the dry film, (B) soaking in ethanol then drying in a vacuum drier showing the surface, and (C) drying in a freeze drier showing internal structure

From the photos it can be seen that the differences between the different drying techniques are obvious and significant. After drying in the oven or vacuum oven, the hydrated PDA became a thin film. When observed under the SEM, neither showed any visible internal structure. By comparison, freeze drying seems to be

able to retain the mesh crosslink network of the hydrated PDA, the physical arrangement of which can be clearly seen under SEM. At the macroscopic level, the freeze dried substrate shrank moderately, but in general remained at a similar size to the hydrated one. This freeze dried PDA substrate is referred to as PDA foam in the following sections.

6.6.2 Effects of Substrate Physical Structure on Water Absorption and Bioavailability

Hydrated PDA dishes were prepared as described in Chapter 2 and dried either by a 60℃ oven (PDA film) or by a freeze drier (PDA foam). Dishes containing PDA and extra agar (PDA + agar) were also prepared by dissolving 3.9 % (w/w) PDA and 1.5 % agar power into HPLC grade water and then autoclaving at 121 for 20 min. Then 25 g sterilised substrate was transferred onto each petri dish. After solidification, PDA + agar dishes were also dried in a freeze drier (PDA + agar foam). PDA + agar foam was observed under SEM and compared with PDA foam (Figure 6-26).

Dried PDA foam and PDA + agar foam were then inoculated with *A.oryzae* and placed on a moist sponge in a sealed fermenter. Fermentation was carried out at 30℃ and 90% humidity, with the humidity level being maintained by water in the sponge. CO_2 production was monitored as described in Chapter 2 and the results are shown in Figure 6-27 and Figure 6-29. The CO_2 production data from hydrated PDA and PDA film, fermented under the same conditions were used as a comparison, with those from Figure 6-14.

Samples were also taken from un-inoculated PDA foam and PDA + agar foam to measure water absorbance in different humidity by DVS as described in Chapter 2. The results are shown in Figure 6-28 and are compared with those for PDA film.

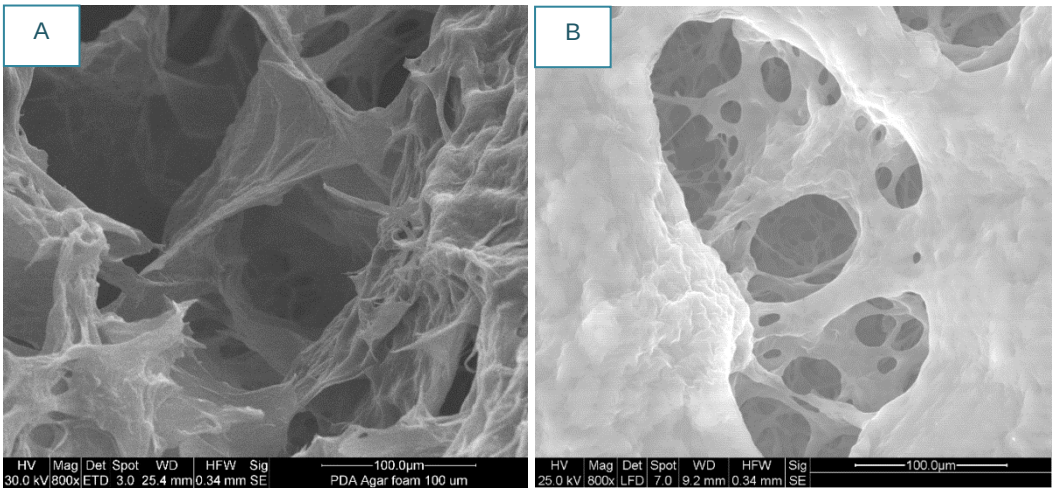

Figure 6-26 PDA + agar foam (A) and PDA foam (B) observed under SEM at 100 µm

From Figure 6-26 it can be seen, PDA + agar foam has a different physical structure to PDA foam. The extra agar contained in the PDA + agar foam appears to have produced more mesh crosslinks within the solid substrate, as would be expected. As a result, there are also more layers and consequently more surface area within the PDA + agar foam, than in the PDA foam.

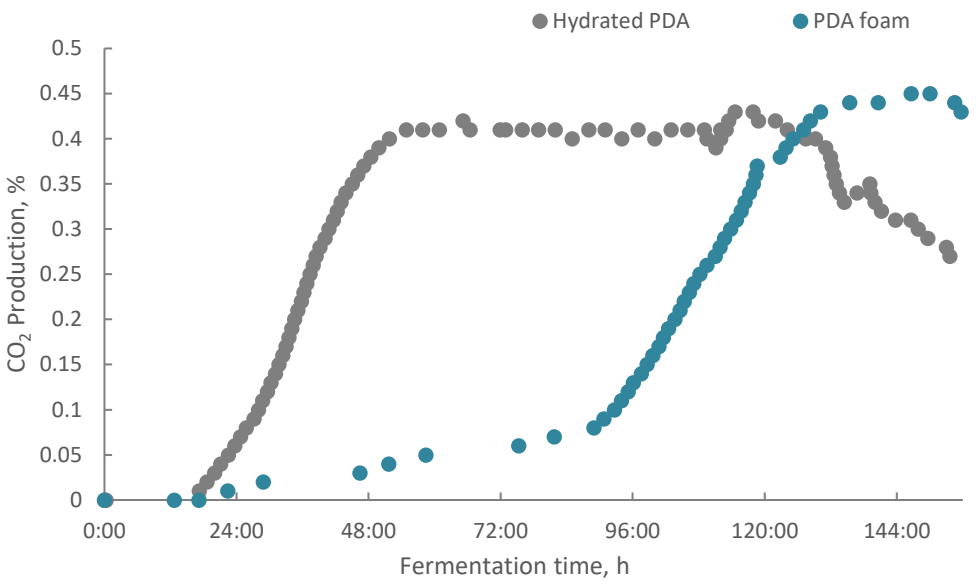

Figure 6-27 CO_2 production by *A.oryzae* on hydrated PDA, and PDA foam. Fermentations were carried out at 30℃, with humidity >90%

Figure 6-27 shows the CO_2 production from a hydrated PDA and a PDA foam. Even though at the beginning of the fermentation, PDA foam did not contain any water in the substrate, the CO_2 production was as good as from the hydrated PDA, but just with delayed germination and onset of exponential growth. This result shows that it is possible for water to support fungal growth when only present in the gas phase. In other words, it is possible to carry out fermentation without water in the substrate. Comparing to submerged fermentation where excess water has been applied, this finding enables water to be used more effectively, and in an industrial production, may reduce the needs for product extraction.

In addition, as expected, it is also likely that the rate of water migration from the gas phase to the substrate mainly affects fungal growth by affecting the onset of exponential growth. In other words, the length of the pre-exponential growth phase depends on whether there is enough water within the immediate vicinity around the fungus.

If this is the case, by altering the physical structure and consequently substrate water absorption rate, the fungal germination and pre-exponential growth should be affected. Therefore, dried PDA film, PDA foam and PDA + agar foam were placed in the DVS to measure the amount of water they can absorb, and also the absorption rate (Figure 6-28). Then, CO_2 production from these substrates was compared and is shown in Figure 6-29.

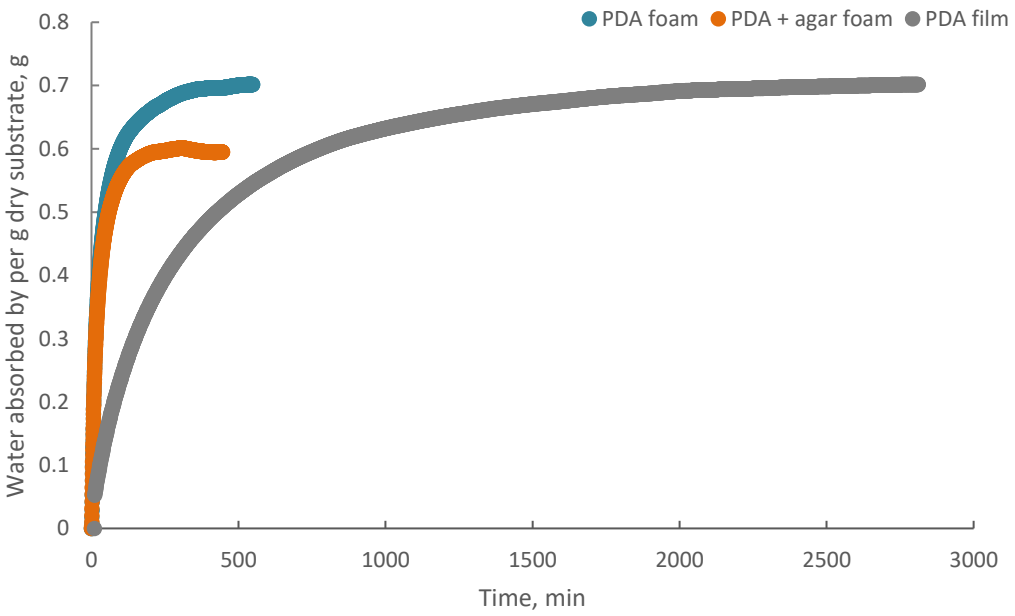

Figure 6-28 The amount of water that can be absorbed by PDA film, PDA foam and PDA + agar foam under 90% humidity, and the time required to reach equilibrium, measured by DVS

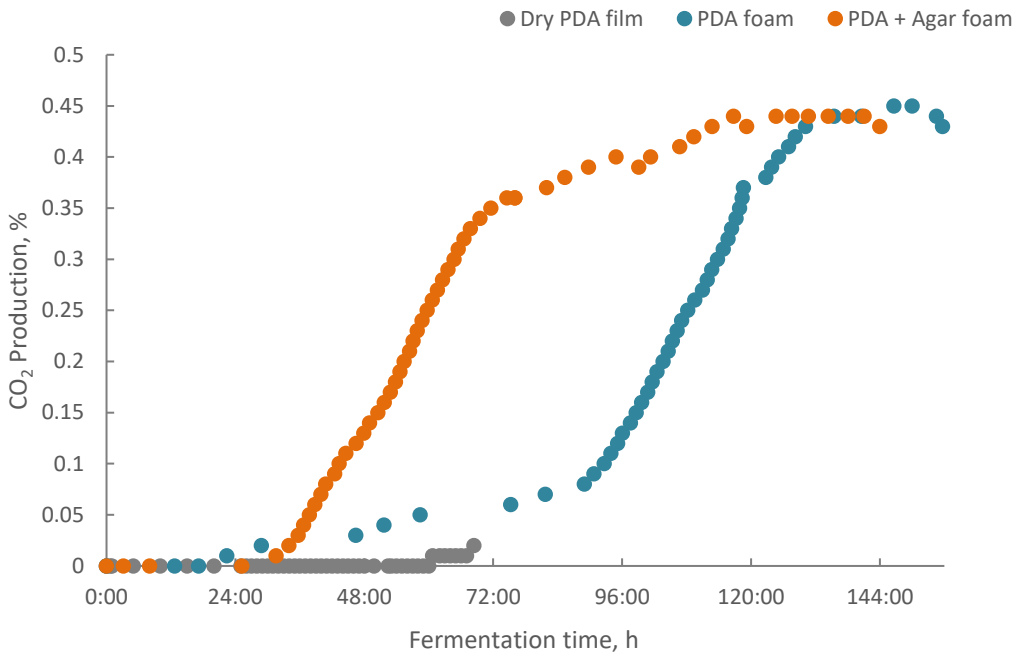

Figure 6-29 CO_2 production by *A.oryzae* on PDA film, PDA foam and PDA + agar foam. Fermentations were carried out at 30℃, with humidity >90%

As revealed by the DVS results (Figure 6-28), PDA foam and PDA film absorbed the same amount of water at 90% humidity. This is as expected as the two substrates have the same chemical composition. However, due to the presence of pores and mesh crosslink network, as shown in Figure 6-25, PDA foam had a much higher surface area within the substrate, than the PDA film did. Therefore PDA foam can absorb water much faster than PDA film. As a consequence, PDA foam can make water in the gas phase available to the microorganisms more rapidly, whereas for PDA film, the substrate couldn't accumulate enough water for any fungal growth during at least 60 h of incubation, or, the water absorption rate was so low that fungal growth rate and consequently the CO_2 production rate was lower than CO_2 consumption rate by the gas analyser. It is likely that the fungal spores would eventually germinate and grow, but it is clear that this would be much later than in the other cases.

Comparing PDA foam with PDA + agar foam, even though PDA foam absorbs more water than PDA + Agar foam at 90% humidity, the absorption rate of PDA + agar is faster than for simple PDA foam. Correspondingly, the former also supported earlier germination and exponential growth than the latter. This provides convincing evidence that for substrates with similar chemical compositions, germination and the onset of exponential growth are significantly affected by substrate water absorption rate. This is despite the water absorption rate of PDA + agar foam being only moderately faster than that of simple PDA foam. This further suggests that germination and growth are heavily influenced by substrate water absorption rate, and the relationship is not likely to be a linear one.

Therefore, for fungi growing on solid substrates in humid environments, the water bioavailability in the gas phase is not only affected by the substrate chemical composition (water activity), but also affected by substrate physical structure and consequently, water absorption rate. The exact mathematical relationship between substrate properties and the bioavailability of water in the gas phase would require considerable further experimentation, which could be done by using substrates with different water absorption rates and absorption abilities. However, this would not be a simple matter and therefore, a preliminary conclusion must be drawn from the studies reported here.

Nonetheless, the results presented in this section do support the theory that water in the gas phase can be supplied to microorganisms via substrate water absorption. In addition, they also suggest that water in the gas phase not only should be considered as an additional supply to that in the substrate, but in some cases, can replace water in the substrate and become the sole source of water without overly compromising fungal growth. In other words, under humid

conditions, it is possible to carry out solid state fermentation without water in the substrate.

6.7 Effects of Water Mobility in Substrate on its Bioavailability

The bioavailability of water contained in liquid cultures has been intensely investigated by researchers working in the related areas, e.g., food science and microbiology, and is reported in Chapter 5. It is affected by the chemical properties of the medium. However, solid substrates also have physical structures, which might further influence the bioavailability of water. Unfortunately, there is little information available for the effects brought about by substrate physical structure on water availability and consequently on microbial growth.

As proposed in Section 6.4, apart from being affected by water in the gas phase, microbial growth is also affected by whether the water consumed within the immediate vicinity can be replenished in a timely manner. Consequently, the bioavailability of water in solid substrates is affected by its mobility within the substrate. Physical structures should increase the resistance for water migration, due to the increased tortuosity, the existence of friction, capillary forces and/or hydrogen bonding between water and crosslinked networks. Therefore, the mobility of water within the substrate should be lower than that in simple liquid cultures. If water bioavailability is not affected by substrate water mobility, the presence of physical structure should have no impact on microbial growth. In other words, under the same conditions, fungal growth should be identical in liquid cultures and on solid substrates.

In order to gain a better understanding and to examine this hypothesis, an investigation was conducted, using solid and liquid cultures with the same chemical composition, to test if substrate physical structure has any effect on water bioavailability and microbial growth. The solid and liquid cultures were analysed by NMR to measure the mobility of water in the substrate. Based on these findings, a solid substrate with an alternative physical structure was produced, and its effects on growth were investigated and are reported in the second part of this section.

6.7.1 The Presence of Physical Structure and Water Bioavailability

In order to test if the presence of a physical structure in the substrate has any effect on water bioavailability, a PDB solution was made by dissolving 3.9% (w/w) PDB powder into HPLC grade water. A PDB + PEG solution was also made by dissolving 3.9% PDB and 3.9% PEG into HPLC grade water. The solutions were then autoclaved at 121℃ for 20 min. After sterilisation, the solutions were cooled to room temperature and then mixed thoroughly with 1.5% (w/w) agar powder before dispensing onto petri dishes. This was done in order to produce media with identical chemical compositions to those of solidified agar substrates (PDA and PDA + PEG), but without the presence of the physical structures associated with the gelled agar (agar must be heated in solution to above 90℃ to form a mesh crosslink network). Dishes containing PDB solution and agar powder are referred to as “PDB + agar powder”, and those containing PDB + PEG solution and agar powder as “PDB + agar powder + PEG” dishes.

Each dish contained 25 g of solution and was carefully placed on a moist sponge in a fermenter. Dried *A.oryzae* spores were carefully inoculated onto the surface

of the liquid substrates. In order to avoid any difference brought about by different evaporation rates, humidity was maintained above 90% by water contained in the sponge. The fermenter was then sealed and the cultivation was carried out at 30℃. After cultivation, photos were taken and are shown in Figure 6-30. In addition, CO_2 production was monitored and compared with the CO_2 production from solidified PDA and PDA + PEG. The results are shown in Figure 6-32.

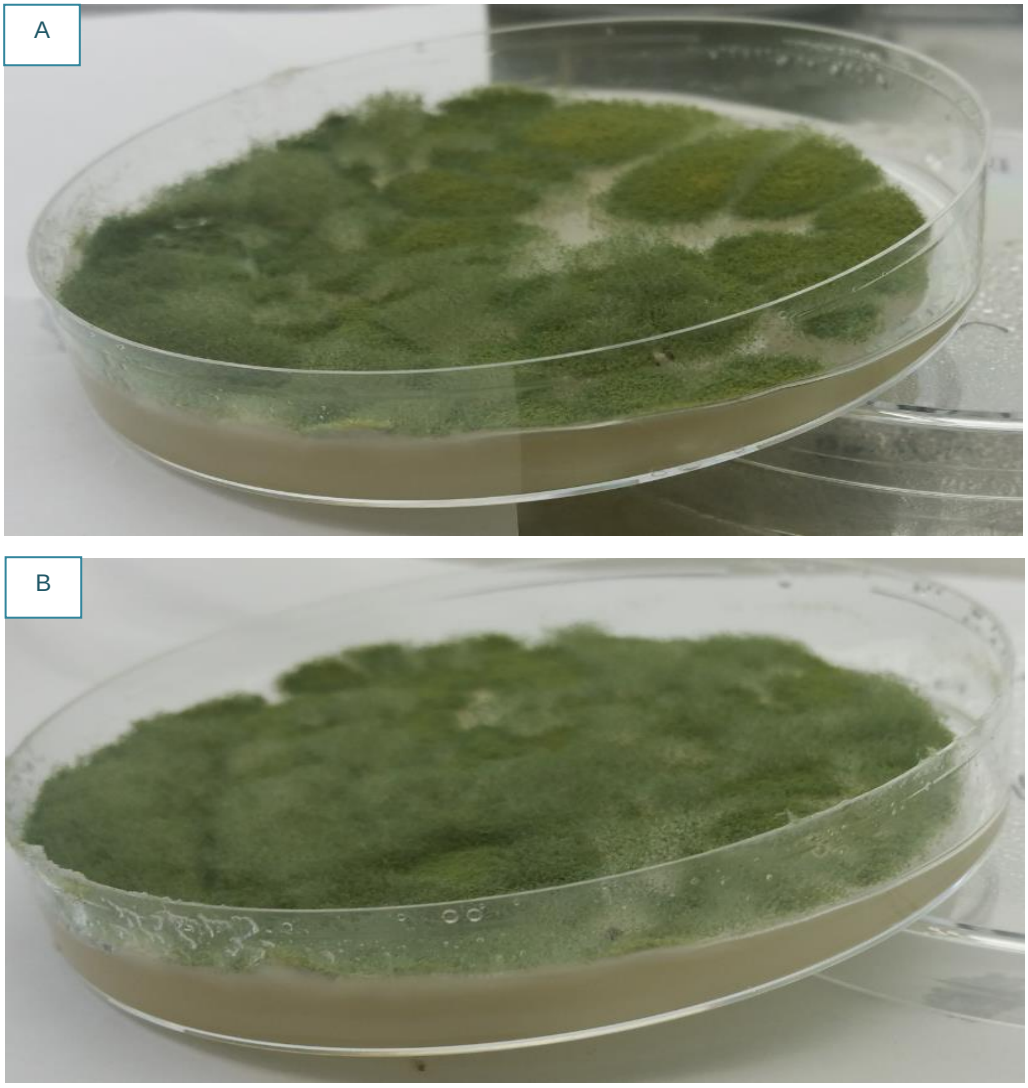

Figure 6-30 *A.oryzae* after 144 h cultivation on liquid "PDB + agar powder" medium (A) and liquid "PDB + agar powder + PEG" medium (B), at 30℃, humidity >90%

Due to the hydrophobic nature of *Aspergillus* spores, when inoculated at the surface of the liquid media, under static conditions, the spores stayed at the surface and germinated there [196, 197]. As shown in Figure 6-30, unlike in submerged fermentation where spores become suspended in the liquid and grow into hyphal pellets, the fungal hyphae formed a biofilm floating at the medium surface. In this case, the fungus was cultivated in an environment that is very similar to that of a homogenous solid substrate (e.g. solidified agar) in terms of nutrient composition, oxygen and heat transfer. As a consequence, fungi cultivated on "PDB + agar power" displayed very different growth pattern and spore production to PDB submerged fermentation that was carried out in shake flasks (shown in Figure 6-6, Section 6.2.2), but was similar to that cultivated on solid PDA substrate (shown in Figure 6-8, Section 6.2.2). To make the comparison clear, static cultivations of *A.oryzae* growing on liquid "PDB + agar powder" and solid PDA substrate, were compared with a cultivation in liquid PDB shaken at 180 rpm. These are all shown in Figure 6-31.

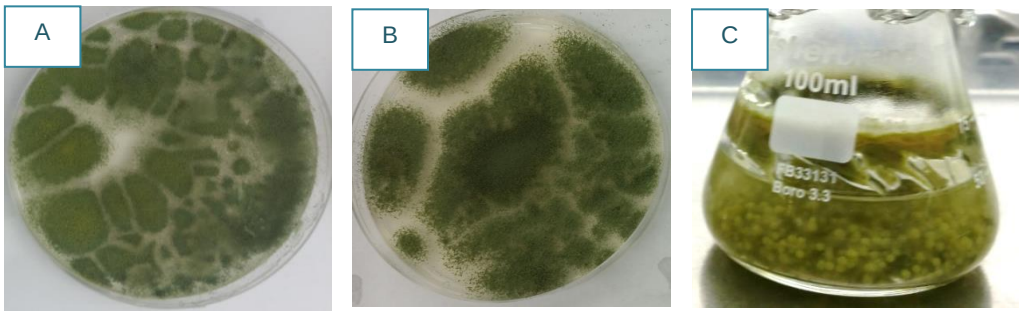

Figure 6-31 Photos showing *A.oryzae* growing in different conditions after 144 h cultivation at 30°C. (A): Liquid "PDB + agar powder". (B): solid PDA. (C): liquid PDB. For A and B, cultivation was static, for C, it was shaken at 180 rpm. Photos B and C are reproduced from Figure 6-6 and Figure 6-8

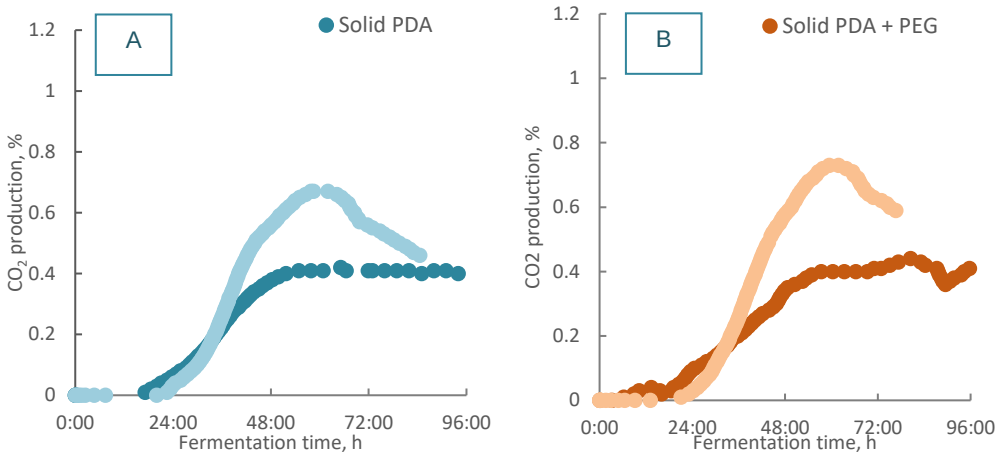

Figure 6-32 CO_2 produced from fermentations carried out at 30°C and 90% humidity with different substrates. A: Solidified PDA and liquid "PDB + agar powder". B: Solidified PDA + PEG and liquid "PDB + agar powder + PEG"

Despite the similarity shared between cultivation on PDA and "PDB + agar powder", as shown in Figure 6-32, the CO_2 production from the liquid cultures is still much higher than from the solid substrate. The difference is mainly reflected by the increasing rate of exponential growth, which was much higher in the liquid cultures, and consequently, the maximum CO_2 concentration was also higher. In addition, in the liquid cultures, CO_2 concentration reduced drastically after the peak, which indicates significantly reduced fungal activity. In comparison, on solid substrates, CO_2 production maintained at a plateau for a period of time before decreasing which suggests the fungal activity was also at the same level for that period.

The exponential growth and the peak CO2 production on the other hand, appeared around the same time (60 h) in liquid cultures, and slightly earlier in solid culture (56 h). The presence of PEG did not have any significant effect on fungal growth cultivated under humid conditions. This is because the fungus was growing on the surface of the substrates, and consequently had access to water in the gas

phase as discussed previously in this chapter. The difference in growth rate and maximum CO_2 production from both solid and liquid cultures confirms that the presence of a physical structure does have a clear and negative impact on fungal growth. However, it is not certain yet if water mobility within the solid substrate is lower than within the liquid culture, and therefore, it is not clear if the growth rate was limited by substrate water mobility. To obtain a better understanding, the four substrates, PDA, PDA + PEG, "PDB + agar powder", "PDB + agar powder + PEG" were analysed in a NMR 600, to determine the substrate water mobility. The detailed procedures are described in Chapter 2, and the results are shown in Figure 6-33.In addition, water mobility of PDB was used to test the repeatability of NMR results and is also shown in Figure 6-33.

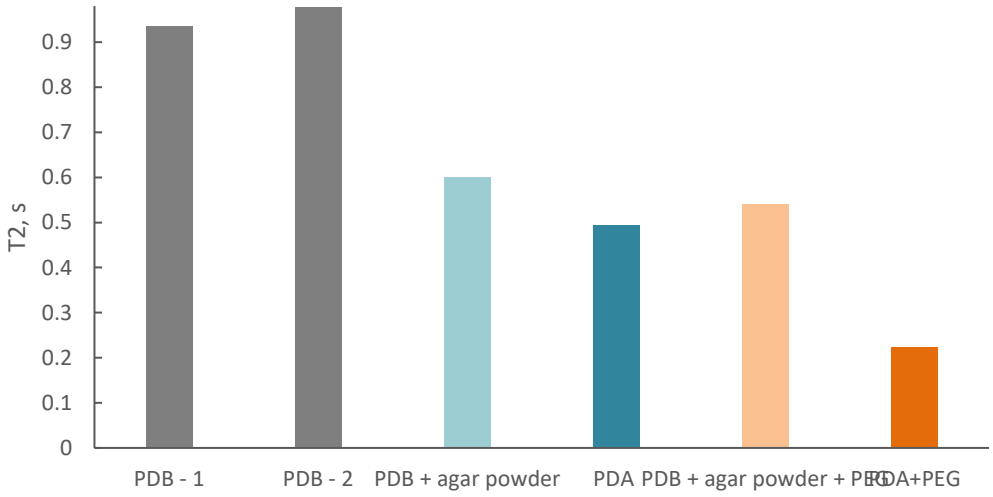

Figure 6-33 The effect of substrate properties on T2 relaxation times for water molecules measured by NMR 600. PDB -1 and PDB -2 are repeats, PDA and "PDB + agar powder" are equivalent to PDB 1 and 2 but with agar present, while PDA + PEG and "PDB + agar powder + PEG" are again similar compositions but with agar and PEG added

The value of the relaxation time, T2, represents the length of time required for atomic nuclei in water molecules to reorient in a static magnet field. Longer T2 times mean water molecules vibrate more intensely and are less bonded by other

molecules/forces. Therefore, the higher T2 value indicates better mobility within the substrate. As can be seen from Figure 6-33, the differences between the two measurements of T2 in PDB solution are negligible, which gives enough confidence in the reliability and reproducibility of the results, which were obtained using different samples and on different days. Comparing PDB with “PDB+ agar powder”, the presence of the agar powder in the liquid reduces water mobility, such that T2 value is much lower. This suggests some chemical bonding between the water and agar molecules. In addition, the presence of a physical structure (gelled PDA) clearly lowers water mobility within the substrate as shown in the figure. Despite having identical chemical compositions, the liquid “PDB + agar powder” has much longer relaxation time, than the gelled PDA. The effect of physical structure on water mobility reduction was also observed between the liquid “PDB + agar powder + PEG” and the gelled PDA + PEG, where the T2 value of the latter is much lower than the former.

Furthermore, as expected, the presence of PEG lowers water mobility via hydrogen bonding. Both liquid and solid substrates containing PEG have lower T2 values than those without. The effect of PEG on water mobility reduction is more significant within the solidified substrate than in the liquid; the difference between PDA + PEG and pure PDA is very large. The effect of PEG on water mobility fits the theory proposed in Section 6.4, in that, after water within the immediate vicinity has been depleted by fungal growth, for growth to continue, water must be replenished. When the fermentation is carried out in a dry environment, i.e. no water in the gas phase, the rate of water being replenished, and consequently water bioavailability is dominantly affected by the mobility of water within the substrate. In the case presented in Section 6.4, PDA and PDA + PEG have very similar gelled agar physical structure and therefore, the water mobility is mainly affected by the chemical properties. As a consequence, by forming hydrogen

bonds with water molecules, the presence of PEG reduces water mobility and consequently fungal growth. In the case being discussed here, both the chemical properties and the physical structures significantly affect water mobility.

Considering that the presence of a physical structure is the only difference between PDA and “PDB + agar powder”, and also between PDA + PEG and “PDB + agar powder + PEG”, combining this with CO_2 production (shown in Figure 6-32), these results suggest that the physical structure does indeed have a clear impact on water mobility. It would appear that the effect on fungal growth, mainly in terms of exponential growth rate, is also a consequence. If this is the case, then by altering water mobility in the solid substrate, which can be achieved by altering its physical structure, the CO_2 production should be affected accordingly. This was investigated and is reported below.

6.7.2 The Effects of Physical Structure on Water Bioavailability

In order to change substrate physical structure from agar gel, an inert melamine foam was used. Melamine foam (Figure 6-34), also known as ‘magic sponge’, is a microporous hydrophobic polymer (formaldehyde-melamine-sodium bisulphite copolymer). Unlike agar, due to its hydrophobicity, melamine foam does not form any hydrogen bond with water, but holds water by capillary action. Since the binding strength of capillary action is much weaker than that of hydrogen bonds, the mobility of water within the foam must be much higher than it is in gelled PDA. To observe its detailed physical structure, melamine foam was examined under SEM and a photo is presented in Figure 6-35.

Figure 6-34 A block of Melamine foam cut and fitted into a petri dish

The foam was cut and fitted into petri dish bases. The height of the foam was fixed to give a similar level to that of a hydrated PDA substrate. For disinfection, the melamine foam was soaked in 70% ethanol and dried in a 60℃ oven. PDB solution was made by dissolving 3.9% PDB into HPLC grade water and then autoclaved at 121℃ for 20 min. The sterilised solution was then added into the melamine foam in the petri dish (PDB sponge) and this was carefully massaged to distribute the solution evenly throughout the foam. Water mobility within PDB sponge was determined by NMR 600, as described in Chapter 2. The result is shown in Figurer 6-37.

The PDB sponge was then inoculated with dried *A.oryzae* spores in the centre of the substrate surface and carefully placed on a moist sponge in a fermenter. Humidity was maintained above 90% as in previous experiments, to avoid any difference brought about by water evaporation rates. The fermenter was then sealed and the cultivation was carried out at 30℃. CO_2 production was monitored and is shown in Figure 6-36. In addition, PDA solution, 3.9% (w/w), was autoclaved at 121℃ for 20 min before dispensing onto a petri dish. The dish

containing 25 g of PDA and was inoculated in the centre with *A.oryzae*. It was then placed in on a rack in a sealed container, with the humidity maintained above 90% by water beneath the dish. The dish was placed in the same incubator as the fermenter containing the PDB sponge, the cultivation was started and also stopped at the same time as that for the PDB sponge. After cultivation, the PDB sponge and the PDA were dried in a freeze drier, photos were taken (Figure 6-38) before taking samples from the dried substrates, which were observed under the SEM (Figure 6-39).

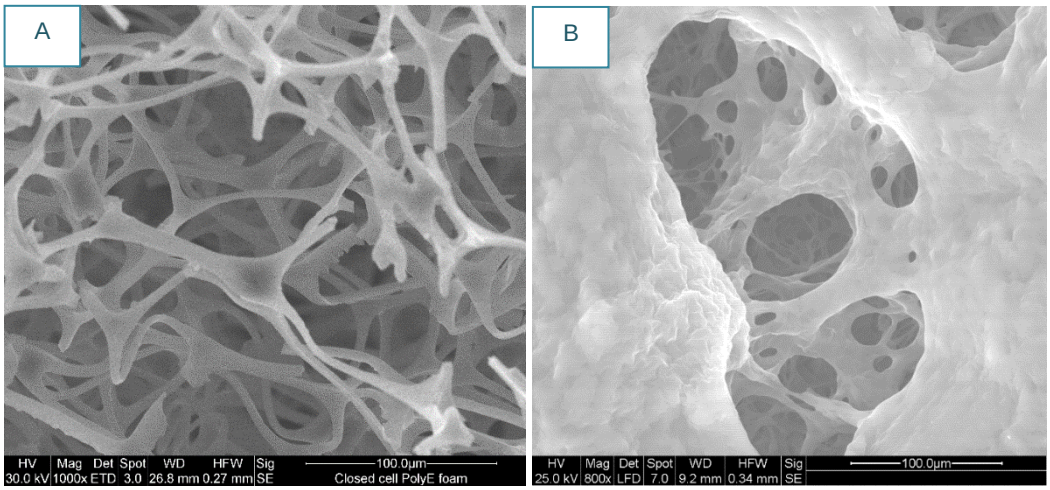

Figure 6-35 Untreated melamine foam (A) and PDA foam (B) observed under SEM

As shown in Figure 6-36, the CO_2 production was much higher from the PDB sponge, than all other dishes. Similar to previous experiments, the exponential phase appeared around the same time (20 h) for all dishes. The peak time for PDB sponge, appeared around the same time as for liquid cultures (60 h), which is slightly later than for the solid substrates (56 h). The growth rate, on the other hand, was highest in PDB sponge and therefore resulted in the highest maximum CO_2 concentration.

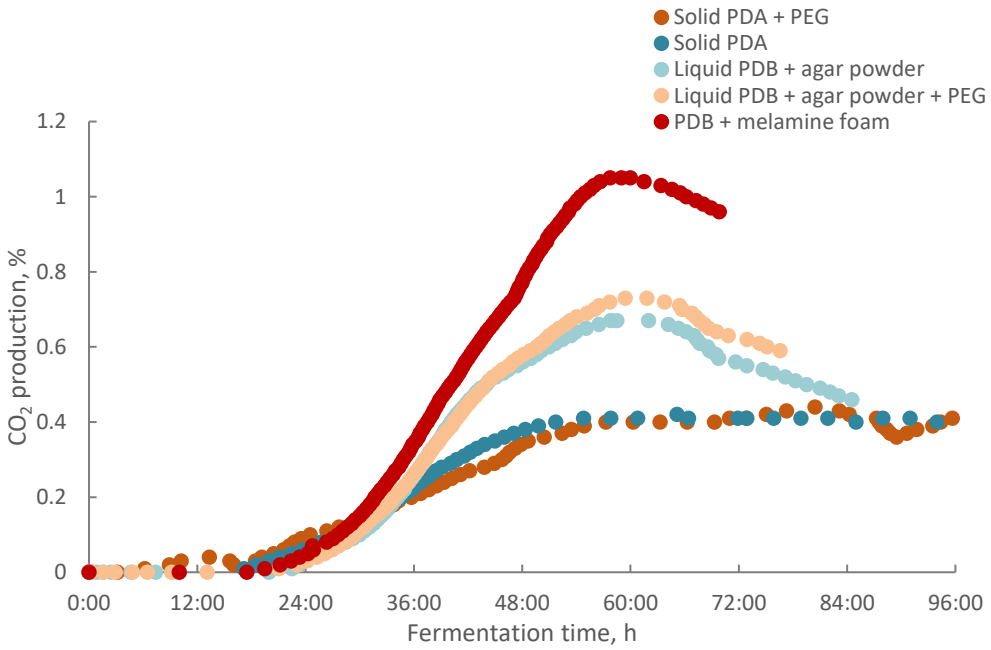

Figure 6-36 CO_2 produced during fermentations carried out at 30°C at 90% humidity with different substrates as identify in the legend

Considering the results shown throughout this section (6.7), it seems that when germination starts, there is abundant water surrounding the spores and therefore, the germination times for all dishes were similar. However, as fungal growth occurs, water was consumed but replenished at different rate in different substrates. Water mobility within the agar gels is the lowest among all groups, and therefore the growth rate on agar gels are also the lowest. The liquid cultures on the other hand, have a better water mobility, resulting in a higher growth rate. In the case of PDB sponge, comparing to simple liquid cultures, due to the lack of agar powder and therefore associated hydrogen bonds, water mobility is higher within PDB sponge. This has been confirmed by NMR analysis (Figure 6-37).

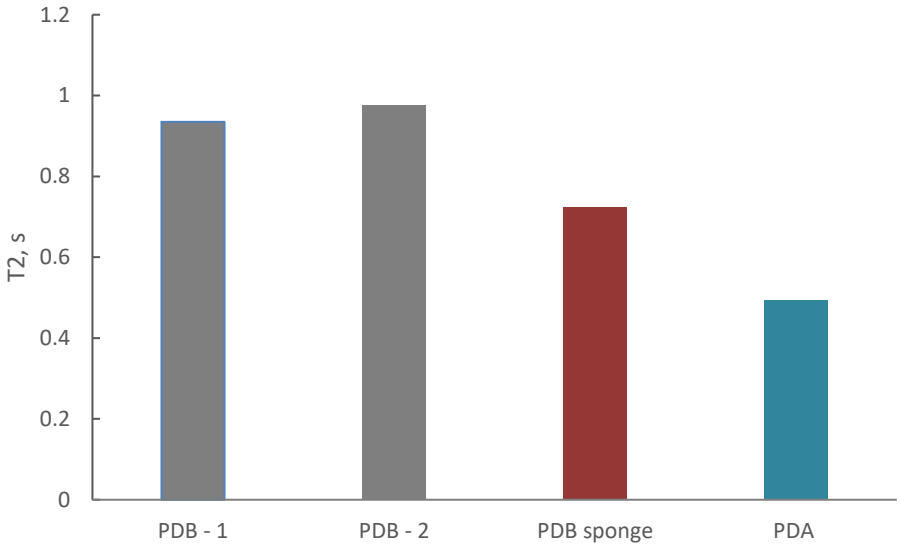

Figure 6-37 The. T2 relaxation times for water molecules within PDB sponge measured by NMR 600, compared with PDB and PDA. PDB -1 and PDB -2 are repeats

Comparing to gelled agar, as shown in Figure 6-35, the contiguity and openness of the pores in the PDB sponge is much greater than within the agar gel structure and therefore, the effect on water mobility is also lower.

Apart from water mobility, the connected pores also enhance fungal growth due to the following aspects. To begin with, as the nutrients are dissolved within the liquid phase, a better mobility can also bring about better nutrient supply. In addition, the well connected pores allow fungal hyphae to extend much deeper into the substrate than within the agar gels. As shown in Figure 6-38, fungal growth can be clearly seen on the bottom of the PDB sponge, but cannot be observed on the bottom of the PDA. This is further confirmed by SEM, as shown in Figure 6-39, fungal hyphae can only be seen at the surface of the PDA whereas on it can be seen throughout the PDB sponge.

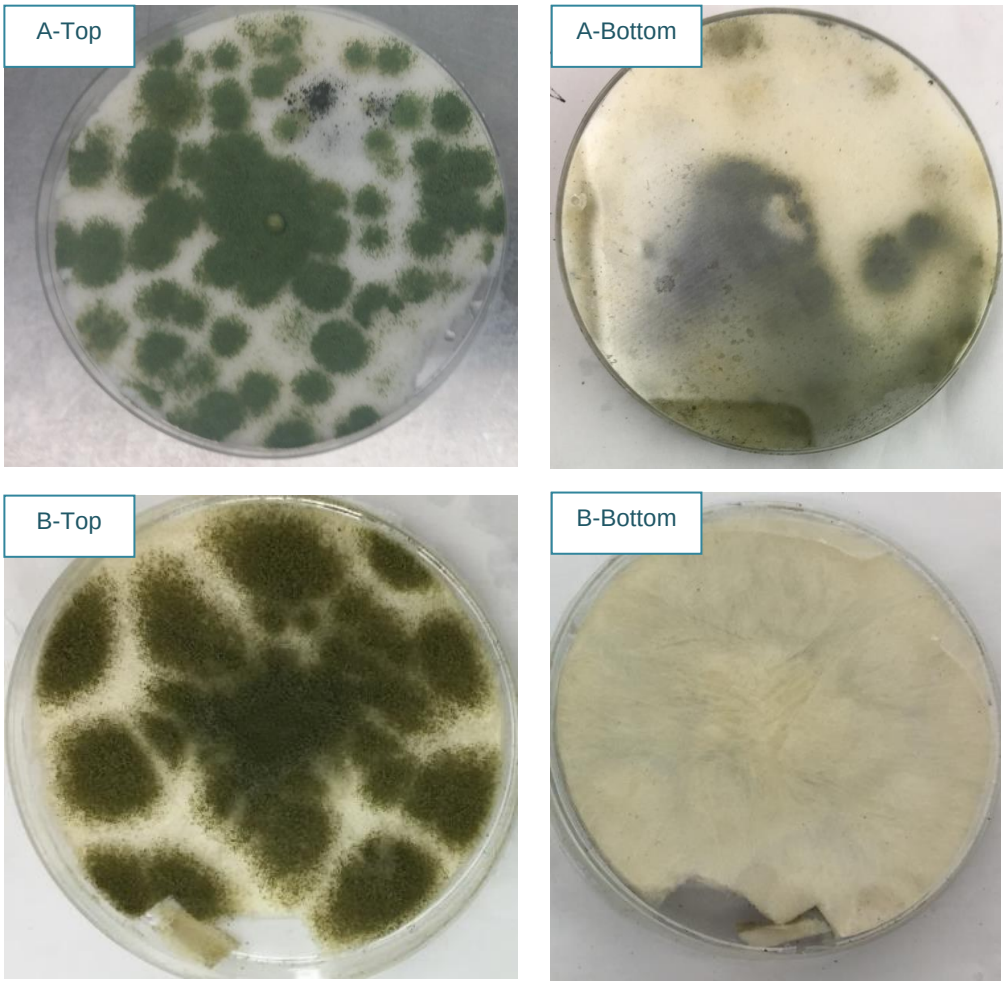

Figure 6-38 Photos showing *A.oryzae* growth on top and bottom of PDB sponge (A) and PDA (B). Samples were freeze dried before photos were taken

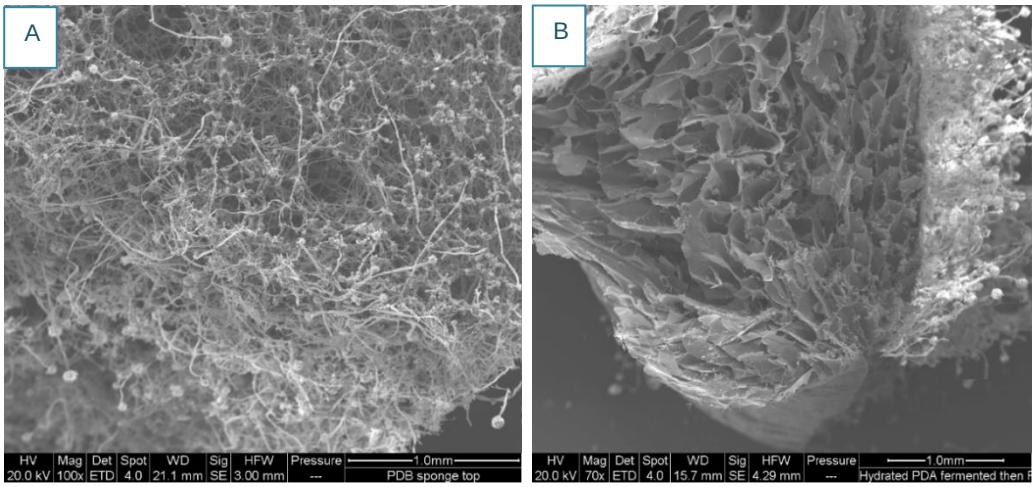

Figure 6-39 SEM photos showing thin cross sections of PDB sponge (A) and PDA (B) after cultivation with *A.oryzae*. Samples were taken from the centre of the dishes shown in Figure 6-38, following freeze drying.

Growth may have occurred throughout the PDB sponge because some of the pores were not entirely saturated with the liquid but remained filled with air, and consequently the availability of oxygen and also the surface area for oxygen transfer were considerably greater. Whereas for liquid cultures and for solid gels, oxygen transfer is only available through the surface and via diffusion within the substrate at a much lower rate. Once the surface is fully covered by hyphae, oxygen transfer becomes very difficult, if not impossible, and consequently limits growth. In addition, since fungal spores are only produced at the tips of aerial hyphae, they are very difficult to produce in a submerged culture. On a static liquid culture, most of the spores are separated from the liquid culture by the biofilm and remain in the gas phase, and therefore, do not germinate. However, in the PDB sponge, as can be seen from Figure 6-39, fungal spores were produced at the tips of hyphae extended within the substrate. In fact, aerial hyphae with spores can be observed throughout the sponge. This confirms the presence of a gas phase within the substrate, which also allows those spores within the substrate to germination and grow.

In summary, physical structure does have a significant impact on water mobility. On one hand, it can significantly reduce water mobility by forming hydrogen bonding with the crosslinked networks, and/or lowering the connectivity of the pores. On the other hand, in a well-connected porous substrate, physical structure assists fungal growth by assisting with spore migration, providing more surface area for oxygen transfer and consequently encouraging hyphal extension within the substrate. Better water mobility increases water bioavailability, nutrient supply and spore migration and consequently increases fungal growth rate.

So far, the theory proposed in Section 6.4, has been developed and tested. Fungal growth can only consume water from the surrounding local micro-environment.

For germination, it can only occur if there is sufficient water within the immediate vicinity, therefore, the length of the pre-exponential growth period mainly depends on water bioavailability in the substrate surrounding the fungus (sections 6.4, 6.5 and 6.6).

Once germinated, fungal hyphae are able to utilise water directly from the gas phase (6.5). As germination and growth occur, they result in a modest depletion of water from the immediate vicinity. This creates a driving force for water to migrate from the bulk substrate (usually through diffusion), or to be absorbed from the gas phase, to replenish the water that has been consumed. If the fermentation is carried out in a dry environment, i.e. no water in the gas phase, water bioavailability is mainly affected by the rate of water being replenished, which is the water mobility within the substrate. The effect of water mobility on fungal growth is mainly reflected in the fungal exponential growth rate (Section 6.7).

Better water mobility increases water bioavailability, nutrient supply and spore migration and consequently increases fungal growth rate (Section 6.7). Water mobility within the substrate is under the influence of both chemical properties and physical structure of the substrate. It is reduced if water forms hydrogen bonds with either dissolved chemicals or the crosslinked network within the substrate, and consequently lowers water bioavailability and fungal growth rate. Conversely, in open porous substrates without hydrogen bonds, the presence of a physical structure assists fungal growth by assisting with spore migration, providing more surface area for oxygen transfer and consequently permitting hyphal extension within the substrate.

On the other hand, if there is water in the gas phase, it can become an important water supply to microorganisms growing on solid substrates. The reduced availability of water within the substrate can be fully compensated by water in the

gas phase (Section 6.4). Indeed, it is even possible to carry out fermentation solely using water in the gas phase and without any in the substrate. In this case, substrate water absorption rate affects the length of the pre-exponential growth phase (Section 6.6).

So far, the theory appears to fit well and can be used to explain the phenomena that have been observed on simplified systems, involving homogenous solid substrates, agar gels. In the next section, the theory is further tested by applications using heterogeneous materials, namely, rapeseed meal and sugarcane bagasse.

6.8 Water Bioavailability in Natural Solid Substrates

Earlier in this chapter (Section 6.1), a question was asked: why water requirements for rapeseed meal (RSM) and sugarcane bagasse (SCB) are so different (water content 65% and 90%, respectively), and how water bioavailability is affected by substrate properties and humidity. In this Section, the theory of water bioavailability that has been studied throughout this Chapter was further examined, by using it to explain the water requirements for solid state fermentation carried out on RSM and SCB. This has been done from the two following aspects: first, when fermentation was carried out in dry environments, i.e. water in the gas phase is essentially absent, SCB containing the same water content (65%) as RSM supports much poorer fungal growth. According to the theory, the availability of water within the immediate vicinity and water mobility within the substrate are the factors affecting fungal growth in such environments. To examine if this is indeed

the case, water mobility within RSM and SCB was measured using NMR 600. The availability of water on the surface of the two substrates was also assessed by observing the moist substrates under environmental scanning electron microscope (ESEM). The ESEM has a specialized electron detector that allows a gaseous, humid environment in the specimen chamber, thus enabling the collection of electron micrographs of wet samples [198]. If the availability of water within the immediate vicinity of fungal spores does not have a direct impact on germination and growth, the amount of water present at the surface (where fungal spores are inoculated) of RSM particles with 65% water content should be similar to that of SCB with 65% water content. The findings are reported in the first part of this section.

Secondly, when fermentation was carried out in a humid environment, growth on SCB with 65% water was almost as good as with 90% in the dry environment. However, such enhancement did not appear in the dishes where rapeseed meal was used as the substrate. Since water in the gas phase is proposed as an alternative water supply and its bioavailability is affected by substrate water absorption rate, RSM and SCB were placed in the DVS to measure absorption rate in humid environments (90%). The findings are reported in the second part of this section.

6.8.1 Water Bioavailability within Natural Solid Substrates

Three moist samples were made using RSM and SCB. These were: RSM containing 65% (w/w) water (RSM-65), SCB containing 65% and 90% water (SCB-65, SCB-90). They were observed under ESEM separately. To obtain a clear picture of the behaviour of water on the particle surface, water was allowed to evaporate within the specimen chamber by changing chamber pressure. Time-

lapse photos were produced throughout the evaporation process at an interval of 20 s, to create videos comprising 110 to 180 frames. These videos are available as supplementary materials to this thesis. Sample photos are also shown in Figure 6-40. To determine water mobility within moist RSM and SCB, the two materials were soaked in D_2O for 12 hours before placing in the NMR 600 to measure relaxation time. The results are shown in Figure 6-41. In addition, to check the effect of soaking on water mobility, separate measurements were taken immediately after the D_2O was added into dried RSM and SCB.

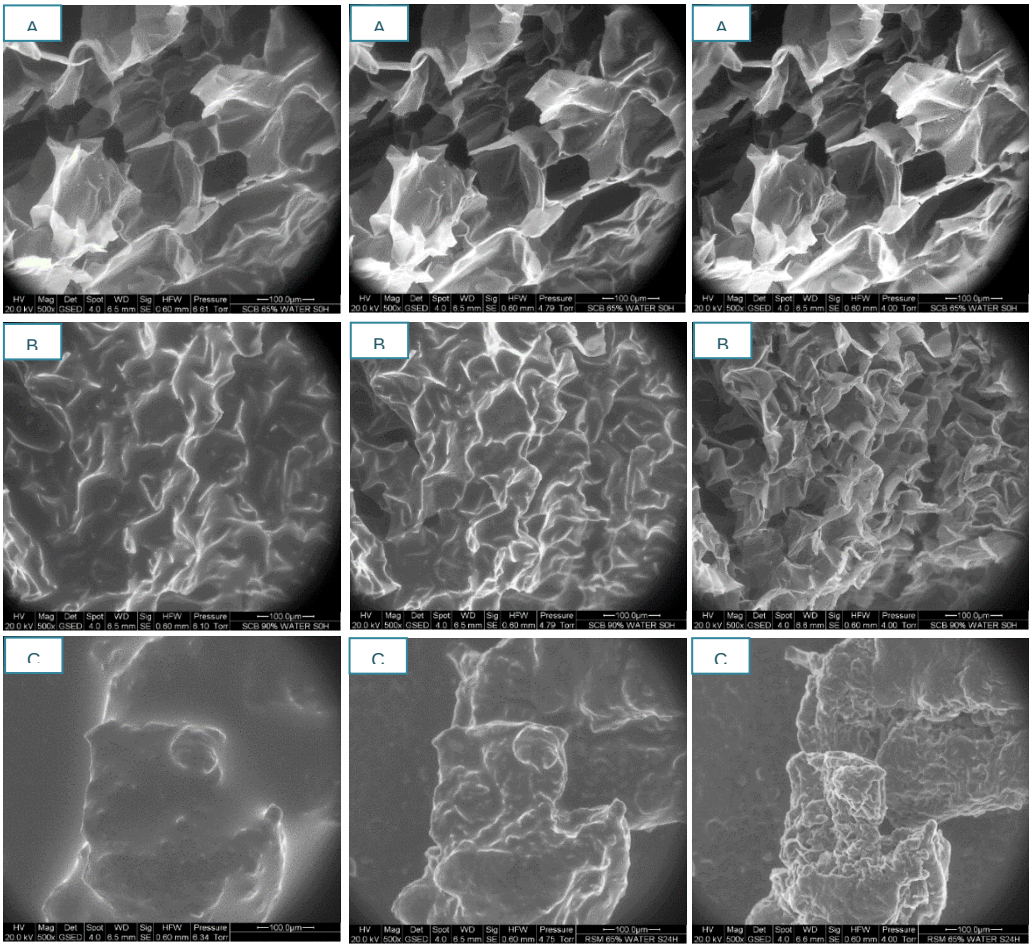

Figure 6-40 ESEM photos of SCB initially containing 65% water (A), SCB initially containing 90 % water (B) and RSM initially containing 65% water (C) with decreasing Chamber pressure (around 6.3 Tor, 4.7 Tor and 4.0 Tor), at 100 µm, showing water disappearance from the surface

From Figure 6-40 it can be seen, for the SCB sample containing 65% water that the particle surface remains relatively the same as chamber pressure decreases.

The detailed structure can be clearly seen throughout the evaporation process. By comparison, for the SCB sample containing 90% water, the topographical features only become clearly distinct when chamber pressure decreases. Comparing to its dry state (B3), the surface of SCB-90 (B1) seems much smoother, the angular terrain on the SCB particles appears soft, with groups of undulating bumps rather than the sharp edges in the dry state. This is because, the penetration depth of the electron beam is only around 1 – 5 µm so, if there is a layer of water covering the particle that is deeper than 5 µm, the detailed structure of the particles will be obscured.

As chamber pressure decreases, more water evaporates from the particle surface. The water layer becomes thinner and thinner, and consequently, the topographical features become clearer. Therefore, on one hand, the distinct difference between fresh SCB-90 (B1) and its dried state (B3) indicates that, with 90% water content, water forms a layer at the surface of the SCB particles. On the other hand, the fact that the detailed surface structure of SCB-65 can be clearly seen even before water evaporation, and throughout the evaporation process, suggests that no water film exists in this case. By contrast, a water film can be clearly observed on RSM with only 65% water content (RSM-65). The detailed surface structure, in this case, is only revealed towards the end of the evaporation process, and can only be seen clearly in C3 (Figure 6-40). This, and the other transitions can more easily be seen dynamically in the videos submitted as supplementary materials.

Combining the above understanding with the fermentation results shown at the beginning of this chapter, in Figure 6-1, the presence of a water film is closely correlated with the performance of solid state fermentations when carried out in dry environments. The fungal growth on RSM-65 and SCB-90 is much better than on SCB-65. This is because, with RSM-65 and SCB-90, the presence of a water

layer provides good accessibility of water within the immediate vicinity of the spores which were inoculated at the surface of the substrate. Whereas in the case of SCB-65, water is fully absorbed into the particles and hence availability within the immediate vicinity, is limited.

The reason that SCB requires much more water than RSM to form a water film on the surface of the particles is possibly related with hydrogen bonds formed between the SCB and water molecules. SCB is a cellulosic material which has a large amount of hydroxyl groups that can form hydrogen bonds with water molecules, whereas the main component of RSM is protein, which typically form fewer hydrogen bonds. This speculation is consistent with the relaxation times of water within the substrates (Figure 6-41).

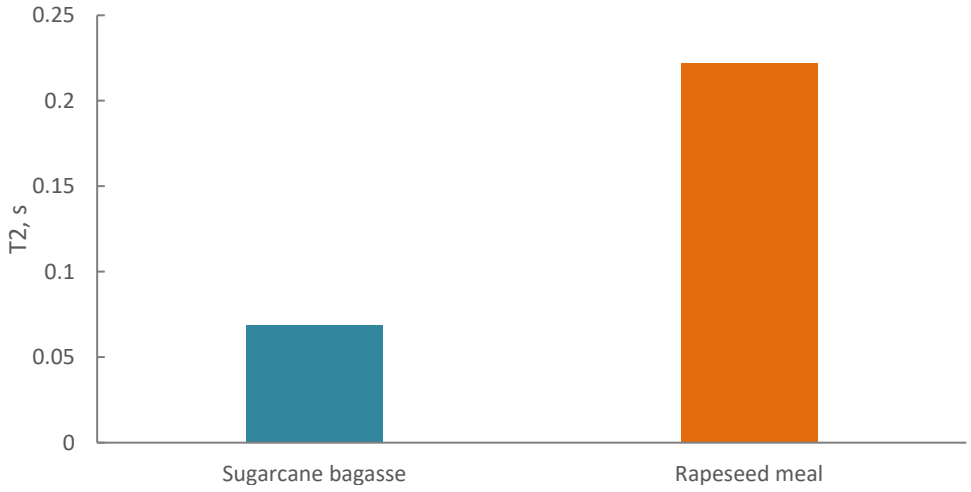

Figure 6-41 Relaxation times of water molecules within sugarcane bagasse and rapeseed meal samples that have been soaked for 12 h in D_2O, measured by NMR 600

As shown in Figure 6-41, the relaxation time of water in SCB that had been soaked in D_2O for 12 h is much lower than that of RSM, suggesting that the water molecules are much more tightly bonded in SCB, and hence the water mobility is much lower. It is worth mentioning that even though samples without the soaking

process were also made, the measurements for these were not successful. This was because of the formation of microbubbles and the maldistribution of water within the substrates, both of which are removed by the soaking process.

Nonetheless, the relaxation times of RSM and SCB that were soaked for 12 h provide sufficient information to interpret the water mobility within the two substrates. The results indicate that after the water within the immediate vicinity had been consumed by fungal growth, its replenishment is much more difficult for SCB than for RSM, when fermented in a dry environment.

The results shown above provide insights for understanding why without water in the gas phase, SCB requires much more water than RSM, for fermentation to carry out. Due to the presence of large amounts of hydroxyl groups on SCB, compared to RSM, water is easily absorbed into the particles. As a result of this, SCB requires much more water to form a water film that can stay at the surface, and become available to the microorganisms. In addition, once water within the immediate vicinity is depleted, the rate of replenishment is much lower in SCB than in RSM, which further limits fungal growth.

The findings can also be used to explain a question that was asked in Chapter 4: in a fermentation using mixed RSM and SCB as substrate, why can consistency of fermentation outcome be heavily affected by fermentation conditions, especially under unfavourable ones?

As discussed earlier this chapter, the distribution of water on the surface of the substrate, and the mobility of water within the substrate vary with the physical and chemical properties. Therefore, the evaporation rate, under the same conditions, should also be different. This is confirmed by DVS results for SCB and RSM as shown in Figure 6-42.

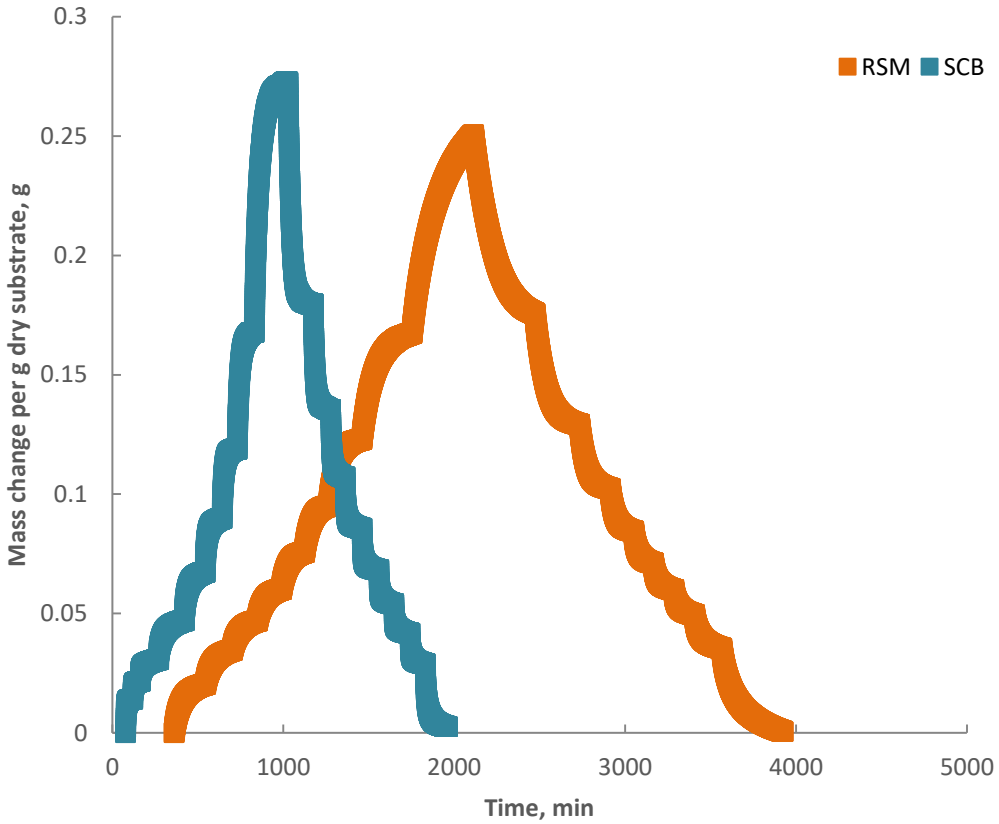

Figure 6-42 Absorption and desorption curves for RSM and SCB under varying humidity, measured by DVS

As shown in Figure 6-42, SCB can lose water by evaporation much faster than RSM. Therefore, when cultivated at a temperature that is higher than optimal, without water in the gas phase, the water loss from SCB will be much larger than for RSM. Consequently, the water bioavailability to fungal spores that land on the SCB is much lower than on RSM. The landing spots affect fungal growth and hence, the overall fermentation outcome. As the landing spots could not be artificially controlled, the fermentation outcomes presented in Chapter 4, showed poor consistency.

The pre-incubation effects on fermentation outcome (Chapter 3) can also be explained, as discussed earlier in this section, in that soaking can assist more

even distribution of water within the substrate, and consequently should affect water bioavailability to fungal spores. However, the effect of water bioavailability on fungal metabolite production needs another systematic study, which was not possible in this project.

6.8.2 Water Bioavailability from the Gas Phase for Natural Solid Substrates

To determine the bioavailability of water from the gas phase for the two natural solid substrates, water absorption rate of dried RSM and SCB in 90% humidity was measured using the DVS. The results are shown in Figure 6-43.

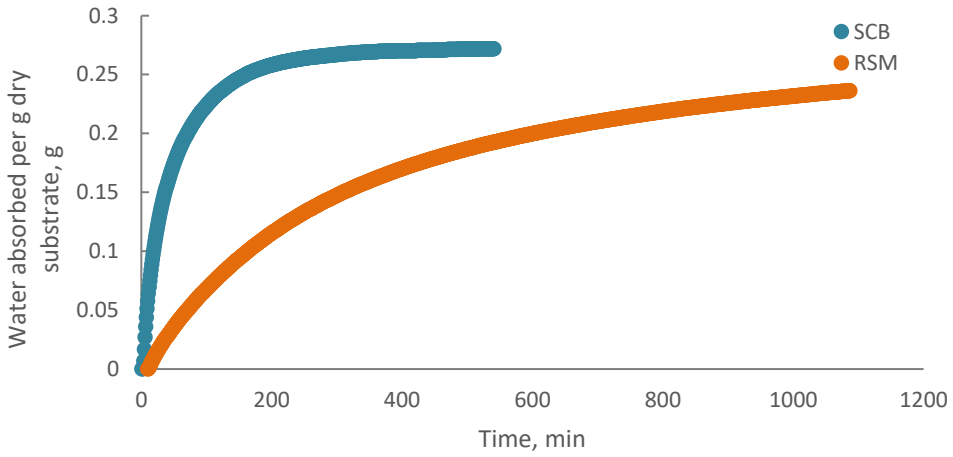

Figure 6-43 Water absorption by SCB and RSM in a 90% humidity environment, measured by DVS

As shown in Figure 6-43, the amount of water that can be absorbed by SCB, and also the absorption rate at 90% humidity are much higher than those for RSM. This suggests that the water can be rapidly absorbed into SCB and become available to microorganisms. This could also explain why earlier results, presented in Figure 6-2, showed that SCB with 65% water content, grew much better at 90%

humidity than under dry conditions. This further confirms that the theory that the gas phase is an important source of water for solid state fermentation, and therefore, has a direct impact on microbial growth. Furthermore, unlike in submerged fermentation where virtually all water comes from the liquid culture, water supply to solid state fermentation is more diverse. Sometimes, it is more efficient coming from the gas phase, than from the substrate, especially for substrates with 'low water activity' which can absorb large amounts of water from the gas phase. In other words, contrary to common belief that low water activity results in poor fermentation, solid substrates with lower water activity can support fungal growth just as well as those with high water activity, if sufficient water is present in the gas phase.

6.9 Concluding Remarks

In this Chapter, an experimental investigation of the bioavailability of water for fungi growing on solid substrates has been reported. The following theory was developed from the experimental findings:

> "Fungal growth can only consume water from the surrounding local micro-environment. As germination and growth occur, they result in a modest depletion of water from the immediate vicinity. This creates a driving force for water to migrate from the bulk substrate (usually through diffusion), or to be absorbed from the gas phase, to replenish the water that has been consumed. The driving force within the substrate increases with fungal water consumption, while water absorption from the gas phase is driven both by the gas phase relative

> humidity and presence/absence of water at the substrate surface. Meanwhile, the resistance to mass transfer is greatly affected by both chemical and physical properties of the substrate. Fungal growth can continue, if, and only if water within their immediate vicinity can be replenished."

In general, water bioavailability for fungi growing on solid substrates cannot be fully represented by either of the commonly used concepts of "water content" or "water activity", but is affected by three factors: the availability of water in the substrate, in the immediate vicinity of growth, the mobility of water within the substrate, and the availability of water from the gas phase.

The first factor is under the influence of substrate chemical properties. It affects germination time and the length of the pre-exponential growth period. This is because, to germinate, there must be sufficient water surrounding the spores. The mobility of water within the substrate is under the influence of both chemical and physical properties. It can be significantly lowered by the presence of hydrogen bonds. Lower water mobility means water is being replenished at a lower rate, Therefore, without water in the gas phase, growth is mainly affected by substrate water mobility and is reflected in the growth rate.

If there is water in the gas phase, on the other hand, it is an important water supply. This is not only because solid substrate can absorb water from the gas phase and make it available to the microorganisms, but also because fungi can utilise water directly from the gas phase after germination. Through these mechanisms, water in the gas phase is able to compensate reduced water availability in the substrate. As demonstrated in this chapter, fungal growth on substrates with low water activity can be as good as on those with high water activity. In fact, it is even possible to carry out fermentation with gas phase water as the sole source. In this

case, water bioavailability is affected by the amount of water that can be absorbed and the absorption rate, which is consequently affected by both physical and chemical properties of the substrate.

The results also show that solid state fermentation may have distinct advantages compared to submerged fermentation. For example, the physical structure of solid substrates allows oxygen and consequently fungal hyphae to penetrate into the substrate by providing surface for oxygen transfer, without artificial aeration and without disturbing the natural growth pattern of the fungi. In addition, water can be diversely supplied from the substrate and also from the gas phase. Therefore, comparing to submerged fermentation, this may enable water to be used more effectively and therefore, lower the needs for downstream extraction.

The detailed relationship between water bioavailability and substrate properties or gas phase water needs further systematic study, which alas was not possible here. Nonetheless, the author sincerely hopes that the outcomes reported in this chapter have contributed to gaining a better understanding of solid state fermentation and will assist with further exploration of its potential.

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Chapter 7.

Conclusion

And

Further Recommendations

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Chapter 7

Conclusions and Further Recommendations

7.1 Conclusion

As discussed in Chapter 1, solid state fermentation and, by implication, solid state bioprocessing have been recognised and praised for their unique features. However, there have been several unsolved challenges haunting almost every researcher working in the area: obtaining accurate and meaningful measurements of fermentation performance; achieving repeatability of experimental results; completing accurate mathematical descriptions; and understanding the fundamentals of the process.

In order to address these challenges, an investigation of solid state bioprocessing and fermentation has been conducted, aiming at both identifying the problems occurring during solid state bioprocessing and in particular fermentation, and addressing them. To identify the problems, two common applications of solid state bioprocessing, namely reducing toxins in raw materials and producing generic microbial feedstocks, were studied and are reported in Part A of the thesis (Chapter 3 and Chapter 4).

The findings reported in Chapter 3 show that by applying two stage solid state bioprocessing, pre-incubation and solid state fermentation, the major toxin in rapeseed meal, glucosinolates, can be greatly reduced to a level suitable for

animal consumption. During pre-incubation, just by adding water and incubating at 30℃, a certain form of reaction takes place within only a few hours, which greatly enhances the toxin reduction during solid state fermentation. It is not clear what the exact mechanism is, but it is clear that water plays a critical role in this process.

In Chapter 4, a solid state bioprocess consisting of pre-incubation, solid state fermentation, and fungal autolysis were applied to produce a generic microbial feedstock from a mixed substrate composed of rapeseed meal (RSM) and sugarcane bagasse (SCB). However, the experimental outcome greatly differed from what was expected: both cellulase and protease activities were reduced after fungal autolysis, compared to their values after solid state fermentation. It was not possible to obtain meaningful interpretations, due to poor consistency of the results of the solid state fermentation. Therefore, a set of experiments was planned using an orthogonal design approach to improve the consistency of the results. The outcome of these experiments suggested that performance of solid state fermentation is closely related to the microscopic environment surrounding the fungi, especially when conditions are unfavourable. However, yet again, the detailed mechanisms were not clear, and it was therefore concluded that a more fundamental understanding of solid state fermentation at a microscopic level is required.

To address the key problem identified in Part A, the effects of water on fungi cultivated on solid substrate, in other words, water bioavailability, were investigated in Part B. These investigations were conducted both theoretically and experimentally. Chapter 5 reports the theoretical investigation for the effects of water on different aspects, namely small molecules, biomolecules, microorganisms and microbial communities, in solid state bioprocessing systems. The outcome of extensive experimental investigations is reported in Chapter 6. By

combining the findings of both studies, and in particular the experimental observations, it was possible to synthesis and propose a theory to describe the dynamic relationship between water, fungi and solid substrate:

> "Fungal growth can only consume water from the surrounding local micro-environment. As germination and growth occur, they result in a modest depletion of water from the immediate vicinity. This creates a driving force for water to migrate from the bulk substrate (usually through diffusion), or to be absorbed from the gas phase, to replenish the water that has been consumed. The driving force within the substrate increases with fungal water consumption, while water absorption from the gas phase is driven both by the gas phase relative humidity and presence/absence of water at the substrate surface. Meanwhile, the resistance to mass transfer is greatly affected by both chemical and physical properties of the substrate. Fungal growth can continue, if, and only if water within their immediate vicinity can be replenished."

The experimental results reported in Chapter 6 show that the water bioavailability for fungi growing on solid substrates cannot be fully represented by the commonly used concepts of "water content" or "water activity", but is affected by three factors: the availability of water in the substrate surrounding the fungi, the water mobility within the substrate and the availability of water from the gas phase. The first factor is affected by substrate chemical properties and consequently affects germination and the length of the pre-exponential growth period. The mobility of water within the substrate is under the influence of both chemical and physical properties of the substrate. It can be significantly lowered by forming hydrogen bonds in the solid substrate. Lower water mobility means water is being

replenished at a lower rate, Therefore, without water in the gas phase, growth is mainly affected by substrate water mobility, as reflected by the growth rate.

If there is water in the gas phase, it can become an important water supply. Solid substrates can absorb water from the gas phase and make it available to the microorganisms, and fungi also can utilise water directly from the gas phase after germination. Therefore, water in the gas phase is able to compensate for reduced water availability in the substrate. It is even possible to carry out fermentations with gas phase water as the sole source. In this case, water bioavailability is affected by the amount of water that can be absorbed and the absorption rate, which is consequently affected by both physical and chemical properties of the substrate.

The results also show that solid state fermentation may have distinct advantages compared to submerged fermentation. For example, the physical structure of solid substrates allows oxygen and consequently fungal hyphae to penetrate into the substrate by providing surfaces for oxygen transfer, without artificial aeration and without disturbing the natural growth pattern of the fungus. In addition, water can be diversely supplied from the substrate and also from the gas phase. Therefore, compared to submerged fermentation, this may enable water to be used more effectively and therefore, lower the needs for downstream extraction.

The findings can be used to explain that, in a fermentation using mixed RSM and SCB as substrate (Chapter 3), why the consistency of fermentation outcomes are worse under high temperature, than under an optimal one. This is because, the evaporation rates of the two substrates are different. Therefore, the water bioavailability to the fungus on SCB is much lower than on RSM, and consequently, the microenvironment affects fungal growth and hence, overall fermentation

outcome. As the microenvironments cannot be artificially controlled, the fermentation outcomes are variable.

The absolute necessity and critical importance of water to microorganisms has always been recognised by researchers working with microorganisms. And yet, in the field of fermentation/bioprocessing, water seems not to have attracted particular interest. This almost feels like water has been taken for granted. Maybe this is permissible in the case of submerged fermentation, in which water is abundant, but it should certainly not be the case in solid state bioprocessing. As discussed in this thesis, interactions between water, substrate and fungi are far more complicated than has been recognised generally. Unsurprisingly, none of the existing concepts (water content or water activity) is sufficient to describe them.

It is possible that complexity is the reason why the study of mechanisms of solid state bioprocessing have been neglected: on one hand, solid state systems are too complex to understand fully. On the other hand, for thousands of years, fermentations on solid substrates have been carried out successfully based purely on empirical knowledge and there is therefore, a tendency to continue in the same manner. However, it is the author's belief that the fundamental duty of a researcher is not merely to use and to adapt, but also to explore, to discover and strive to understand.

Just as the understanding of microorganisms in submerged fermentation has enabled development of that industry, it should be possible through better understanding of solid state bioprocessing fundamentals, to achieve a similarly successful outcome, producing a wider range of products, at higher efficiency and larger scale but with lower cost.

7.2 Recommendations for Further Research

Further research should be aimed at determining the detailed relationship between water bioavailability and substrate properties/gas phase water. The effect of water mobility within the substrate on fungal growth can be studied by gradually changing the substrate chemical properties in a small area, adjacent to fungal spores. To study the effects of water distribution on fungal growth at the substrate surface, it requires a monitoring system that is able to observe fungal activity at the microscopic level. The effects can then be studied by changing the water retention time on the particle surface by changing the initial amount of water on the dry surface that will be absorbed into the substrate.

To study the effects of physical properties on water mobility within the substrate and from the gas phase on fungal growth, an experiment can be designed to vary the porosity of the substrate, while retaining identical/similar chemical properties.

In the present study, the microorganisms used were only filamentous fungi. Therefore, research could be carried further by studying yeast and bacteria. The bioavailability of water should have different effects on these due to the absent of hyphae.

After obtaining a detailed relationship between water bioavailability and fungal growth, the experiment can be further extended to investigate the relationship with production of microbial metabolites.

Clearly, the project presented in this thesis is only the starting point for understanding the effects of bioavailability of water in solid substrates. Hopefully,

it can be a pioneering work for gaining a better fundamental understanding of solid state bioprocessing, and by gaining this better understanding, can bring a new era to this ancient technology.

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