LACTIC ACID PRODUCTION BY IMMOBILIZED *RHIZOPUS ORYZAE* IN A ROTATING FIBROUS BED BIOREACTOR

DISSERTATION

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By

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ABSTRACT

Lactic acid has long been widely used in food and pharmaceutical industries. Due to the evolution of new applications in solvent replacement, biodegradable polymer, and oxygenated chemicals, the estimated worldwide market of lactic acid, especially the L(+)-isomer, tends to increase rapidly. Lactic acid bacteria have been extensively used in industrial production of lactic acid because of their high growth rate and product yield. However, the major limitations including costly substrates and complicated product recovery make bacterial fermentation economically unattractive. In contrast, filamentous fungi, *Rhizopus oryzae*, can produce the optically pure L(+)-lactic acid from complex carbohydrates present in agricultural residues and plant biomass without prior treatment; therefore, can overcome the problems in bacterial fermentation.

However, cultivation of filamentous fungi in a stirred tank bioreactor is usually troublesome because of the diversity and change in morphology during fermentation which in turn affects lactic acid production. Therefore, in this research, fungal morphology was controlled by immobilization. Contrary to free cell culture in the stirred tank bioreactor, the fermentation carried out in a Rotating Fibrous Bed bioreactor (RFBB) resulted in good control of morphology, and improved oxygen transfer and lactic acid production from glucose. The improved oxygen transfer obtained in the RFBB not

only increased lactic acid production rate, but also limited undesirable ethanol production and allowed the bioreactor to be operated for long-term production.

To minimize the production cost, the feasibility of using low-value substrates derived from agricultural residues and plant biomass was studied. It was found that *R. oryzae* was capable of utilizing both starchy materials present in agricultural residues and pentose sugars which were abundant in plant biomass. Lactic acid yields obtained from these substrates were comparable to the yield from glucose. However, the production rate obtained from fermentation of pentose and insoluble starch was lower than that obtained from fermentations of glucose and soluble starch because of the complicated pentose metabolism and poor oxygen transfer in the cultivation with insoluble starch.

Process engineering techniques were also used to improve lactic acid production in the RFBB. Previous study reported the critical demand of oxygen for lactic acid production. In this research, it was found that increasing oxygen transfer rate led to the increase in lactic acid productivity. Although high oxygen transfer rate was maintained, ethanol production and the estimation of the critical biofilm thickness indicated an anoxic condition in the overgrown immobilized fungal cells on the rotating fibrous matrix. In this study, growth of immobilized cells was controlled by shaving-off the mycelia with rotational shear rate and by limiting the concentration of the nitrogen source in the medium.

In order to achieve controlled growth and immobilization of productive cells for stable long-term operation, spore germination and cell immobilization at the initial phase was studied. The effect of rotational speed on spore immobilization on different fibrous matrices was investigated. The mechanisms of spore immobilization on different fibrous matrices were elucidated.

The knowledge gained in these process engineering techniques is important to the development of the RFBB and its scale-up for lactic acid production from sugars.

Dedicated to my mother

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CHAPTER 1

INTRODUCTION

1.1 Background

The applications of lactic acid have long been discovered. Currently, the estimated worldwide market is approximately 50,000 tons per year with a high potential to increase due to the evolution of new applications in solvent replacement (lactic acid ester), biodegradable polymer (polylactic acid), and oxygenated chemicals (www.lactic.com, www.purac.com). Four common applications and functions of lactic acid are summarized in Table 1.1.

Lactic acid is commercially produced by either chemical synthesis or fermentation. In chemical synthesis, lactic acid is generally produced by the hydrolysis of lactonitrile formed in the reaction of acetaldehyde with hydrogen cyanide (Benninga, 1990). To make the production competitive to the chemical synthesis, the optimization of fermentation condition and the minimization of the bioprocess cost by using low-value substrate and a simple process are necessary. At the beginning, *Lactobacilli* have been extensively used in lactic acid fermentation because they can synthesize the optical isomers of lactic acid at a high production rate (Hofvendahl and Hahn-Hagerdal, 2000; Litchfield, 1996; Vick Roy, 1985). However, in pharmaceutical and food industries,

only L(+)-lactic acid is preferable because the D(-)-form is not metabolized by human body. In addition, pure L(+)-lactic acid is strictly required for biodegradable polylactic acid production (Tsai and Moon, 1998). Therefore, *Lactobacilli* are not the favorable L(+)-lactic acid producer although some mutants can produce L(+)-lactic acid but they are not commonly available. Moreover, *Lactobacilli* require complex media for growth. This leads to the complicated and costly final product recovery and purification (Hsieh and Yang, 1999; Silva and Yang, 1995).

To overcome the problems in costly substrates and complicated downstream processing, recently, the interest in fungal fermentation with *Rhizopus oryzae* to produce the optically pure L(+)-lactic acid from glucose, pentose sugars, and starch directly in a simple medium has been increasing (Ho, 1996; Tay, 2000; Woiciechowski et al., 1999; Yu and Hang, 1989; Zhou et al., 1999). Currently, a remarkable amount of agricultural residues from food processing and forestry cause several problems in industrial waste treatments relating to economical and environmental concerns. Due to the ability to secrete hydrolytic enzymes, *R. oryzae* is capable of utilizing starchy products and pentose sugars present in agricultural residues and plant biomass. Therefore, simultaneous saccharification and fermentation by *R. oryzae* to produce pure L(+)-lactic acid becomes a promising alternative because of a significant reduction in production cost.

Unfortunately, filamentous fungal fermentation is a complex process as compared to bacterial and yeast fermentations. Fungal morphology affects broth rheology which leads to numerous problems in gas dispersion, mass and heat transfer, and mixing in a conventional stirred tank bioreactor. The diversity and change in morphology during the

fermentation are difficult to control and often cause severe problems in operation. Many studies indicate that hydrodynamic properties including broth rheology and oxygen transfer as well as fungal morphology and metabolism, and product formation are closely related. As usually found in the stirred tank bioreactor, the freely dispersed mycelia cause the problem in mixing resulting in the pseudoplastic fermentation broth. This results in oxygen transfer limitation which causes the decrease in fungal growth and metabolism since filamentous fungi are highly aerobes (Sankpal et al., 2001; Cui et al., 1998). High agitation is normally used to provide rigorous mixing; and therefore, improve oxygen transfer. However, high agitation rate requires high power input and can cause mycelial damage which eventually results in fungal cell death. Besides growing fungal cells in the freely dispersed mycelia, pellet is another form of morphology which is usually found in submerged culture. It is indicated that oxygen transfer in the fermentation medium is improved in the fermentation with pellet morphology; however, in long-term cultivation, large pellets with high density often experience the problems in oxygen starvation occurring at the pellet core which is the result of oxygen diffusion limitation.

Therefore, controlling fungal morphology is required to obtain high production rate and good process performance (Domingues et al., 2000; Cui et al., 1998; Bai et al., 2003). Various cell immobilization methods to control the fungal morphology and to achieve high cell density and high production rate have been studied because immobilization of whole cells provides the way for the entrapment of multi-step and cooperative enzyme system present in the intact cell, repetitive use, and improved stability (Dong et al., 1996; Elibol and Ozer, 2000; Hang et al., 1989; Kosakai et al.,

1997; Lin et al., 1998; Nielsen, 1992; Park et al., 1998; Singh et al., 1992; Sun et al., 1998 and 1999; Tamada et al., 1992; Tay and Yang, 2002). This includes flocculation with polyelectrolyte, covalent binding to glycidyl ester copolymer, entrapment in gel, and adsorption onto the support (Angelova et al. 1998; Ates et al., 2002; Elibol and Ozer, 2000; Lusta et al., 2000; Sankpal et al., 2001; Sankpal and Kulkarni, 2002; Sun and Bai, 1999).

Similarly, high lactic acid production rate can be obtained from immobilized cells compared to the free cell cultures, partially due to reduced cell growth and increased specific cell productivity. With the immobilized cells, a high biomass density is achieved and can be repeatedly used for lactic acid production over a long period. In addition, power consumption in mixing and aeration is greatly reduced because low broth viscosity is maintained under cell-free condition. Eventually, this facilitates product recovery (Tay, 2000).

In this work, lactic acid fermentation by free cells of *R. oryzae* in the stirred tank bioreactor was compared with the fermentation by immobilized cells in a Rotating Fibrous Bed Bioreactor (RFBB). The feasibility of using low-value substrate derived from agricultural residues and plant biomass was also determined. Repeated-batch and fed-batch lactic acid fermentations in the RFBB were studied to observe long-term stability of the bioreactor. To improve lactic acid production, the influences of process parameters such as oxygen transfer and shear rate as well as the medium compositions were determined in order to control immobilized cell growth and minimize the problems in oxygen diffusion limitation which is usually found in the typical immobilized cell

bioreactor. Spore germination and immobilization were also studied to better understand the immobilization process in the RFBB. With the knowledge obtained from this work, this can help improve bioreactor design and process scale-up of the RFBB.

1.2 Research Objectives and Scope of the Study

The goal of this work was to develop L(+)-lactic acid fermentation by immobilized cells of *R. oryzae*. The production cost can be reduced by means of using low-value substrate and improved process performance. High production rate can be obtained from good process performance by controlling fungal morphology; therefore, the correlation between fungal morphology and lactic acid production as well as control of bioreactor should be well determined. In order to accomplish controlling fungal morphology and improving process performance along with high lactic acid production rate, different methods were applied in this study. To achieve the goal of this work, 4 specific objectives were studied.

Objective 1

Generally, immobilization provides a great advantage in morphological control, leading to high process efficiency. Therefore, in this study, fungal morphological control and lactic acid production were compared in 2 different types of bioreactors including the stirred tank bioreactor and the RFBB. Immobilized cell bioreactor usually suffers from

poor long-term stability due to reactor bed clogging, membrane fouling, cell deactivation or contamination. It was expected that the RFBB can circumvent these problems due to its geometry. Therefore in this work, the repeated batch fermentations were performed to observe the stability of the RFBB during long-term fermentation (Chapter 3).

Objective 2

One way to reduce the production cost is to use low-value substrates derived from agricultural residues and plant biomass. In this work, the fermentation kinetics in submerged fermentations of glucose, corn starch (both soluble and insoluble), xylose, and corn fiber hydrolysate were studied. Long-term lactic acid fermentation of mixed carbon sources in the RFBB was also observed. The preference of *R. oryzae* for carbon consumption was determined (Chapter 4).

Objective 3

Oxygen is one of the important factors in lactic acid fermentation. Under low oxygen concentration, *R. oryzae* shunts the pathway to ethanol production which results in low lactic acid production and eventually cell death. In order to better understand and control oxygen diffusion in the immobilized cells in the RFBB, oxygen diffusion model was used to estimate the critical thickness of the immobilized fungal cells attached on the fibrous matrix. The thickness of the immobilized fungal cells depended on the dissolved

oxygen level maintained in the fermentation medium. To prevent the overgrown immobilized cells on the matrix resulting in oxygen starvation, this could be done by shaving off the outer mycelia or manipulating the medium compositions to minimize the further growth. In this work, the shaving mechanism occurred in the RFBB was studied and the effects of nitrogen source on further cell growth appeared in the production phase and lactic acid production were determined (Chapter 5).

Objective 4

Both fungal growth and lactic acid production in the RFBB are affected by immobilization process. Immobilization in the RFBB included both entrapment of spores in the void spaces of the fibrous matrix and adsorption of spores on the surface of the fiber. The germination process of *R. oryzae* was studied and the immobilization mechanism in the RFBB was determined in this work (Chapter 6).

An overview of research objectives and the scope of this work with the specific objectives are summarized in Figure 1.1. The conclusions and recommendations are given in Chapter 7.

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www.lactic.com

www.purac.com

Applications	Main Functions
Food	• Flavoring • Antimicrobial • Calcium source • pH regulation
Industrial	• Complexing agent and sequestrant • Solvent • Cleaning agent • Raw material for chemical synthesis
Cosmetic	• Humectant and moisturizer • Skin rejuvenating • pH control • Antimicrobial
Pharmaceutical	• Sodium carrier • Mineral supplementation • Raw material for drug synthesis

Table 1.1 Lactic acid applications and functions (www.lactic.com and www.purac.com)

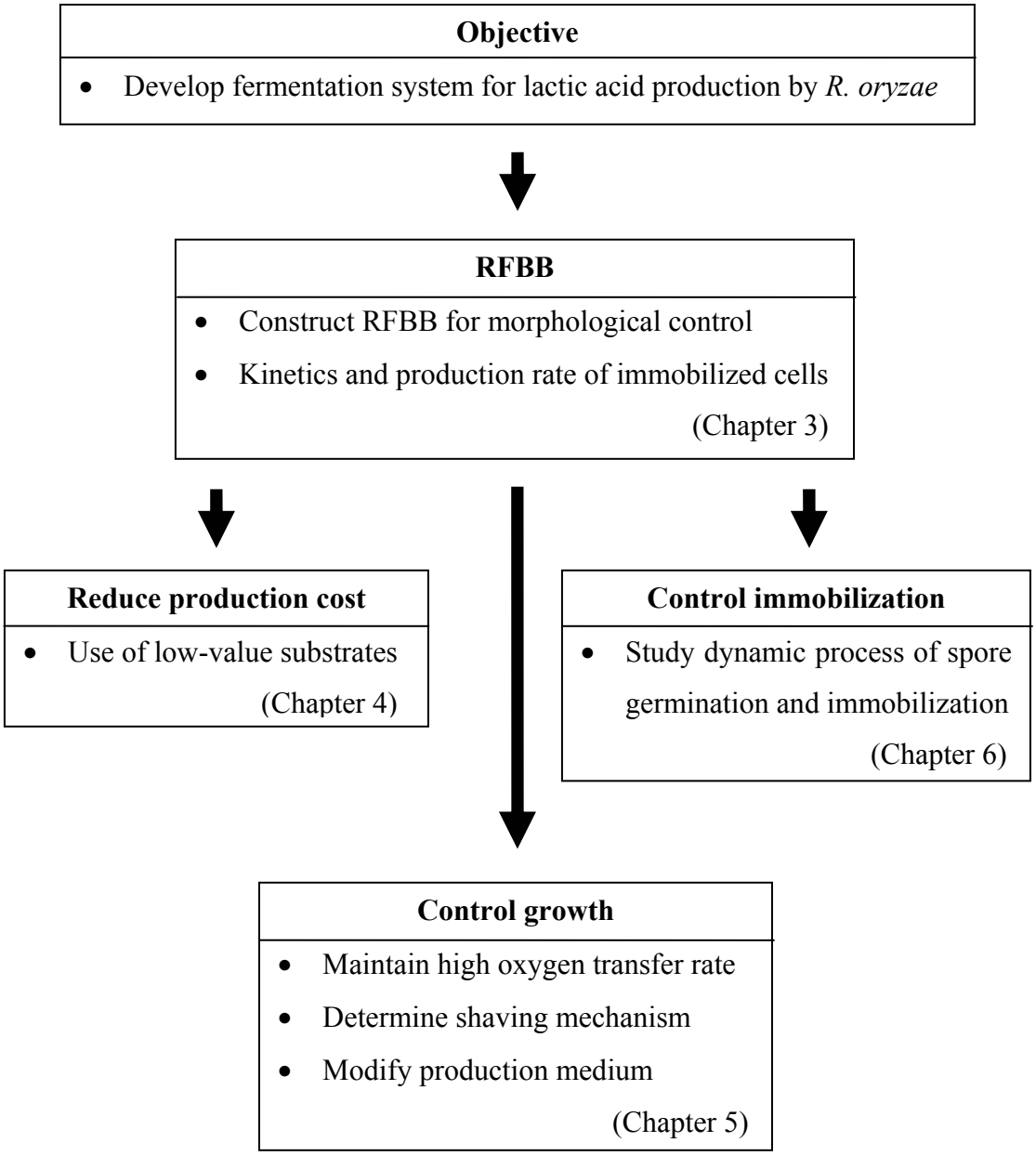

Figure 1.1 Scopes of this study

CHAPTER 2

LITERATURE REVIEW

2.1 Significance of Filamentous Fungal Fermentation

Filamentous fungi have been widely used in industry for several years because many strains have been classified as GRAS (generally regarded as safe). In addition, they can rapidly grow on relatively inexpensive substrates and secrete a remarkable amount of heterogeneous proteins. Organic acids such as itaconic acid, gluconic acid, citric acid, and lactic acid can be produced by filamentous fungi. Also, filamentous fungi can be used in the production of antibiotics and drugs, vitamins, and steroids. In agriculture, plant growth hormone, gibberellins, and growth promoter substance in cattle, zearalenone, are produced by *Furasium* species. Because of the high ability to excrete enzymes, half of the industrial enzymes used worldwide are produced by filamentous fungal fermentation including amylases, laccase, invertase, pectinases, lipases, acid proteases, and cellulases. In oriental countries, people have long been using filamentous fungi to produce the fermented food, e.g. ang-kak, hamanatto, miso, ontjom, shoyu, tempeh, sake, and soy sauce (Bennett, 1985; Bhargava et al., 2003).

Recently, recombinant filamentous fungal system has been extensively used for therapeutic protein expression because of the naturally high level of intracellular protein

synthesis and secretion. Besides, many studies also indicate that filamentous fungi are considered as the mitotically stable multicopy transformants and well-characterized strong promoters, both inducible and constitutive, are available. In addition, posttranslational modification patterns, e.g., glycosylation and disulfide bond formation, are similar to those of human proteins as that filamentous fungi can overcome the limitation in unicellular host systems (Bennett, 1985; Bocking et al., 1999; Covert and Cullen, 1992; Davies, 1992; Domingues et al., 2000; Gwynne, 1992; Martin and Demain, 1978).

Currently, a large amount of agricultural residues from food processing and forestry cause several problems in industrial waste treatments relating to economic and environmental concern. The use of natural products, particularly agricultural residues, as the carbon/energy source or the solid support/substrate offers the advantage of combining the utilization of low-value and problematic residues and the production of costly chemicals. Due to the ability to secrete a large amount of hydrolytic enzymes, filamentous fungi are capable of utilizing the cellulosic materials, starchy products, and pentose sugars present in agricultural residues and plant biomass. Therefore, direct saccharification and fermentation by filamentous fungi is a promising process, leading to many applications (Asther et al., 2002).

However, filamentous fungal culture is highly complex because of diversity and change in the morphology during fermentation. It is well known that fungal morphology affects broth rheology and causes severe problems in gas dispersion, mass and heat transfer, and mixing in industrial bioreactors. This results in the difficulty in bioreactor

control and design. Hydrodynamic conditions in the bioreactors, oxygen transfer, broth rheology, fungal morphology and metabolism, and product synthesis have been found closely related. (Bai et al., 2003; Cui et al., 1998a and b; Domingues et al., 2000; Sankpal et al., 2001a and b). Many researchers have studied various filamentous fungal strains to investigate changes in morphology during fermentation. Some of them have attempted to develop the mathematical models in order to understand and control fungal growth and morphology.

2.2 Fungal Morphology in Submerged Fermentation

Fungal morphology plays an important role in the metabolism during the fermentation process. Different morphology is required for an optimal product yield in different process (Park et al., 2002; Pazouki and Panda, 2000). For instance, in xylanase production by *Aspergillus awamori* as well as in exo-biopolymer production by *Paecilomyces japonica*, pellet morphology provides a higher production rate (Siedenberg et al., 1998; Sinha et al., 2001a and b). Whereas the filamentous morphology is preferable in lactic acid production by *Rhizopus arrhizus* and β-carotene synthesis by *Blakeslea trispora* (Jeong et al., 2001; Martak et al., 2003). High production rate of swainsonine by *Metarhizium anisopliae* in stirred tank bioreactor is obtained by mixed hyphal and pellet morphology (Tamerler and Keshavarz, 1999).

In submerged cultivation, filamentous fungi exhibit distinctive morphological forms depending on the culture environment. Fungal morphology is generally classified

into 2 different groups: dispersed mycelia and pellets (Figure 2.1). Dispersed mycelia can be further divided into freely dispersed mycelia and mycelial clumps. Freely dispersed mycelia compose of mycelia with up to 3 hyphal overlaps which result in hyphal loops whereas mycelial clumps contain more than 3 loops. Mycelia with a few overlaps are called entanglements (Riley et al., 2000). Freely dispersed mycelia can grow when dissolved oxygen and substrate concentration are sufficient in the culture medium. However, the strong mechanical forces can deactivate loose mycelia at some level of magnitude.

Cui et al., 1998b observed *A. awamori* CBS 115.52 growth and constructed the mathematical model. In their study, they claimed that the mechanical forces could deactivate cells or decrease fungal specific growth rate. They described that the deactivation was proportional to the specific energy dissipation rate with an exponent of 0.25 as expressed in eq (2.1).

$$\mu_f = \mu(1 - k_d \varepsilon^{0.25}) \qquad (2.1)$$

Where μ_f is the deactivated specific growth rate of loose mycelia, μ is the specific growth rate without mechanical damage, k_d is the inactivation coefficient of hyphae due to the mechanical damage, and ε is the specific energy dissipation rate. The results showed that such an inactivation mainly occurred on loose mycelia because they were more susceptible to mechanical force. Besides, the size of vacuoles formed in fungal hyphae, which is increasing with the fermentation time, leads to the reduction of hyphal activity per mass and the subsequent decrease in specific growth rate (Papagianni et al., 2002). The outer hyphae in mycelial clumps can be also shaved off by the hydrodynamic

forces resulting in small pieces of freely dispersed mycelia and the reduction of clump roughness. Li et al., 2000 suggested that shaving off by the hydrodynamic forces was the major mean by which mycelial clumps broke apart and the fragments found during agitation were primarily originated from mycelial clumps.

Based on the pellet structure, it can be divided into 3 distinctive zones (Figure 2.1). The outer zone or the hairy part, growing to the outside environment can be shaved off by hydrodynamic forces creating loose mycelia (Cui et al., 1997). The severity of pellet shaving is also the function of hydrodynamic forces. When substrate and oxygen are sufficient, the breakage of pellets occurs less often. In the penetrated zone, sufficient oxygen penetrates into this zone and the fungal cells in this region are active. The depth of this zone depends on the pellet density, growth rate and bulk dissolved oxygen concentration in the culture medium. In the starvation zone, oxygen and nutrients do not sufficiently penetrate into this zone; thus, dissolved oxygen is limited and autolysis occurs as the result of oxygen starvation (Cui et al., 1998a; Martin and Demain, 1978). Therefore, pellet growth is mostly controlled by oxygen transfer before the substrate is depleted in the bulk. Cui et al., 1997 reported that the shaving intensity was proportional to the specific energy dissipation rate.

$$R_{sha} = K_{sha} \cdot \varepsilon \cdot R_{PG} \tag{2.2}$$

Where R_{sha} is the shaving rate of hyphae from pellet surface (kg/m^3·s), R_{PG} is the pellet growth rate (kg/m^3·s), K_{sha} is the shaving coefficient (kg/W), and ε is the specific energy dissipation rate (W/kg).

In filamentous fungal fermentation, the rheological properties appear to be related to mycelial morphology. Growing filamentous fungi in freely dispersed morphology leads to a highly viscous and pseudoplastic fermentation broth. This results in the difficulty in mixing, which in turn affects mass and heat transfer, causing the decrease in productivity and the increase in the production of undesirable metabolites. Thus, rigorous mixing is required for adequate gas dispersion and homogeneity (Li et al., 2002). As the result, high power input needs to be provided. Although stronger mechanical force can result in a higher dissolved oxygen tension and more branching of hyphae which leads to the higher productivity, the excess mechanical intensity can damage the loose mycelia and create dense pellets (Amanullah et al., 1999; Cui et al., 1997; Cui et al., 1998a; Elibol and Ozer, 2000a and b; Gerlach et al., 1998; Jin and van Leeuwen, 1999; Ozbek and Gayik, 2001; Palma et al., 1996; Papagianni et al., 1999a and b; Piccoli-Valle et al., 2003; Sankpal and Kulkarni, 2002; Siedenberg et al., 1997; Woiciechowski et al., 1999; Yuguo et al., 1999). In Riley et al., 2000, they reported the influence of fungal concentration and morphology including clump roughness and compactness on the properties of fermentation broth. The correlations found with freely dispersed morphology constructed in their study and other related studies are summarized in Table 2.1, where C is compactness, C_m is fungal concentration, dry basis (g/L), K is consistency index (Pa·s^n), L_e^* is dimensionless length, L_{hgu} is hyphal growth unit (μm), R is roughness, and τ_y is yield stress (Pa).

On the other hand, when growing filamentous fungi in pellet morphology, the fermentation broth has Newtonian-like behavior with low viscosity. However, increase in

the pellet size during the fermentation creates the problems in nutrient transport into the pellet cores; therefore, reducing fungal activity at the pellet center. This results in the decreased production rate. Change in the pellet size during the fermentation is a function of growth rate, oxygen penetration depth in the pellets, shaving intensity, autolysis rate, and rate of change in pellet density. Cui et al., 1998b developed a descriptive mathematical model to describe pellet growth affected by hydrodynamic forces and oxygen penetration as shown in eq (2.3).

$$4\pi R_p \rho_p \frac{dR_p}{dt} = \frac{4\pi}{3}\left[\mu(1-K_{sha})(R_P^3 - R_{OP}^3)\rho_p - K_{aut} R_{OP}^3 \rho_p - \frac{d\rho_p}{dt} R_p^3\right] \qquad (2.3)$$

Where R_p is pellet radius (m), ρ_p is the pellet density (kg/m^3), R_{OP} is the radius where dissolved oxygen in the pellet is 0 (m), K_{sha} is the shaving coefficient (kg/W), and K_{aut} is the autolysis coefficient (s^{-1}). The left-handed side term represents rate of fungal cell accumulation in a pellet. At the right side of the equation, the first term in the bracket is rate of pellet growth minus shaving. The growth only occurs in the region where dissolved oxygen is available. Due to the hydrodynamic forces, shaving occurs at the outer part of the pellet. The second term refers to rate of autolysis during dissolved oxygen or nutrients starvation. The last term expresses fungal accumulation rate due to change in pellet density (Cui et al., 1998b; Riley et al., 2000).

Many studies indicate that environmental conditions and the genotype of fungal strain influence the growth pattern of filamentous fungi by affecting the morphology, growth rate, and product formation (Haq et al., 2002a and b; Li et al., 2000; Lucatero et al., 2003). This is because the change in fungal morphology during fermentation affects

nutrient consumption as well as oxygen uptake rate (Cho et al., 2002; Favela-Torres et al., 1998; Pazouki and Panda, 2000). Several researchers have attempted to control and maintain the optimal fungal morphology during the fermentation. Ethanol pulse feed, for example, was added into the continuous culture of *Mucor fragilis* in the production of γ-linolenic acid production. The results showed that ethanol could help control and maintain the small pellet culture at steady state throughout the fermentation. Small pellets consisted entirely of metabolically active cell with minimal transport limitation (da Silva et al., 2003). With the appropriate morphological control, high γ-linolenic acid production rate could be acquired. Also homogeneous culture could be obtained with small pellets without cell autolysis (usually occurred at the center of large pellets, mainly due to inefficient nutrient diffusion through the pellet).

2.2.1 Substrate

It is clear that nutrient uptake governs growth rate. Nutrient supplementation during the fermentation controls fungal fragmentation and vacuolation participating in morphological alteration (Haq et al., 2002b; Jennings, 1995). Sinha et al., 2001a and b observed the effect of substrate concentration on fungal morphology during exo-biopolymer production by *P. japonica* in a batch bioreactor. They reported that pellet morphology varied significantly with sucrose concentration. Pellet roughness and hairiness increased as sucrose concentration increased from 20 to 60 g/L. However,

insignificant difference in the circularity index showed that sucrose concentration had no effect on the shape of the pellets.

Papagianni et al., 1999b observed phytase production by *A. niger* in submerged fermentation using semi-synthetic fermentation medium at different concentrations of medium composition. They also observed the morphological change due to the modifications of the standard semi-synthetic medium by supplementing with 20 g/L wheat bran. The results indicated that wheat bran enhanced both biomass and phytase production and affected fungal morphology. Only the pellets were found in the fermentation with the standard semi-synthetic medium whereas a mixture of fine pellets and free mycelial trees (pulp) were formed in the presence of wheat bran.

Not only carbon sources are important in cell growth, nitrogen sources also involve in the biosynthetic pathways of the organism. Du et al., 2003 studied the effect of nitrogen sources on fungal morphology and antibiotic production. They indicated that in the medium containing different nitrogen compounds, the morphology of *Rhizopus chinesis* 12 varied significantly. They observed the differences in hyphal length and degree of branching. When peptone was present in the medium, dispersed mycelia were found whereas compact smooth pellets were found in the fermentation using $(NH_4)_2SO_4$ as the nitrogen source. Entangled filamentous growth was present when the nitrogen compound in the medium was yeast extract. The highest antibiotic production accompanied by pellet growth was achieved in the medium containing corn steep liquor (CSL). They described that the formation of more pellets was probably due to the high concentration of organic nitrogen source in CSL which accelerated spore germination and

cell growth. Furthermore, macromolecules or particles in CSL could gather new mycelial fragments or spores together to grow into pellets whereas pellet formation in the other medium was only due to spore aggregation or hyphal entanglement. In their study, they found that fluffy loose pellets offered the benefits of natural cell immobilization and led to low broth viscosity and simple product isolation. In addition, improved mass transfer could be found in small fluffy and loose pellets than in large and compact ones. They also believed that the inclusion of CSL certainly reduced the chance of spores to aggregate and form dense pellets.

Besides, various inorganic salts have influenced fungal morphology. Chen and Liu, 1996 determined the effects of K_2HPO_4 and $MgSO_4$ on the cultivation of *A. japonicus* TIT-90076 to produce β-fructofuranosidase. They observed fungal morphology and found that the morphology changed from filamentous form to pellet when growing fungi in the medium containing sucrose and yeast extract with an addition of K_2HPO_4 and $MgSO_4$. However, they reported that there was no major difference in cell growth and enzyme production. No clearly explanation on the morphological alteration by K_2HPO_4 and $MgSO_4$ has given in their paper.

Gerlach et al., 1998 also observed the effect of phosphate concentration on *A. awamori* morphology in an airlift tower loop reactor. The results indicated that with 0.3 g/L initial KH_2PO_4, loose pellets of 6 mm diameter with hairy surface and low density at the center were formed. At 1.05 g/L initial KH_2PO_4, dense pellets of 4 mm diameter with uniform pellet density were found. Hollow pellets of 7 mm diameter with dense surface were observed in the culture containing 2.1 g/L initial KH_2PO_4.

Haq et al., 2002b observed the effect of different copper sources ($CuSO_4$, $CuCl_2$, $Cu(NO_3)_2$, and $CuCO_3$) and their concentrations on citric acid production by *A. niger* GCB-47 and GCMC-7 in a stirred tank fermentor. They reported different morphology (viscous, gelatinous, gummy mass, last mycelia, fluffy mycelia, fine pellets, small pellets, intermediate pellets, large pellets, mixed pellets, broken pellets and laxy pellets) formed in the presence of different copper sources at various concentration. Among those, they indicated that 2.0×10^{-5} M $CuSO_4$ gave maximum citric acid production, probably due to $CuSO_4$ gave more Cu^{2+} ions. The presence of Cu^{2+} in the culture medium was helpful for fungal growth and induced the pellet like morphology of mycelia because it was believed that Cu^{2+} was related to the increase of mycelial branching. As the result, the presence of shorter and highly branched hyphae perhaps favored the formation of pellet which was preferable for citric acid production by *A. niger*.

As previously discussed, nutritional source and concentration strongly influence fungal morphology and production rate. Many studies have been conducted to determine the optimal nutritional source and concentration for a particular production. Fungal morphology accordingly varies from one process to another different process depending on the process parameters. Table 2.2 summarizes the difference in optimal fungal morphology in various filamentous fungal fermentations using different substrates to obtain maximum production performance.

2.2.2 pH

Fungal growth and metabolism inevitably lead to the change in hydrogen ion balance; and therefore, pH of the culture medium (Bradoo et al., 1997; Cheilas et al., 2000; Elibol and Ozer, 2000a; Gulati et al., 1999; Martak et al., 2003; Nagal et al., 1999). The pH of the culture medium is one of several parameters affecting fungal morphology. Change in cell size and shape as well as the metabolic rate are the results from the response of cell to pH change (O'Donnell et al., 2001; Sankpal et al., 1999 and 2001a and b). Tamerler et al., 1998 observed the effect of pH on swainsonine production and fungal morphology at different stages during the fermentation of *M. anisopliae*. They claimed that when there was no pH control, pH of the culture dropped from 6.5 to 3.8 within the first 72 hours and swainsonine concentration reached 43.3 mg/L after 168 hours. At the same time, fungal morphology gradually changed from free to entangled hyphae and eventually to pellets. When pH was kept constant either at the initial stage or throughout the fermentation, the maximum swainsonine level was only 8.4 mg/L corresponding with an increase in the formation of pellets. They also reported that a late pH control applied after 72 hours resulted in the maximum concentration of swainsonine at 45.5 mg/L.

Kim et al., 2003 optimized the culture conditions for exo-polysaccharide (EPS) production by *Cordyceps militaris* C738 in submerged culture. They claimed that pH strongly affected fungal morphology and EPS production. They compared morphological properties of the cultures with and without pH control by characterizing pellet roughness

and compactness and reported that the larger and more compact pellets formed at pH 6.0 were favorable for EPS production (0.91 g/g cell/day).

Martak et al., 2003 studied lactic acid production by *R. arrhizus* in a stirred tank reactor with a periodical bleed and feed operation (PBF). They indicated that using PBF cycles, fermentation was prolonged to 240 hours with an average lactic acid productivity of 2.31 $kg/m^3 \cdot h$ and a total yield of 67.3% at pH 5.30. They claimed that the most effective production required filamentous morphology because nutrient diffusion became less limited than that occurred in the dense pellets.

The intracellular pH controls the internal concentrations of various species of CO_2. This implies that CO_2 diffusion is influenced by pH of the culture medium. Many studies indicate that when CO_2 concentration increases, the degree of hyphal swelling increases corresponding with the morphological change and product synthesis. McIntyre and McNeil, 1997a, b, and 1998 used computerized image analysis to quantify the change in morphology of *A. niger* when CO_2 level increased. They found that short stubby hyphae, which was favorable for citric acid production, drastically reduced when CO_2 level was raised. In particular, the hyphal growth unit (HGU) increased with the exposure to CO_2 level of 5% or higher. They claimed that at the high level of CO_2 (12% and 15%), HGU values increased more than twice compared to the values obtained from the standard batch process. This result at high CO_2 level represented mycelial particles with fewer branches per unit length which caused the reduction of citric acid production.

2.2.3 Shear rate

Shear force in filamentous fungal fermentation markedly influences fungal morphology particularly under submerged condition. Strong mechanical force results in a high dissolved oxygen tension and high degree of hyphal branching. The pellet formed under high shear rate may become dense and strong with high tensile strength (Amanullah et al., 2002; Jeong et al., 2001). Amanullah et al., 1999 examined the effect of agitation intensity on mycelial morphology in chemostat cultures of recombinant *A. oryzae*. They reported that the increase in agitation speed to 550 rpm resulted in a rapid increase in the freely dispersed fraction to 42%. In their study, it was likely that the fragmentation of aggregates at the higher speed increased the proportion of biomass in the freely dispersed form. The interaction between the fungal cells and the vortices formed by mixing is the principle mechanism of mycelial damage due to excessive shear forces (Piccoli-Valle, 2003). Many works reveal that mixing by mechanical force causes the dramatic changes in morphology and productivity. Fungal mycelia undergo the complete transformation when the mechanical force changes (Papagianni et al., 1999a).

Palma et al., 1996 observed the influence of agitation rate on xylanase activity from *Penicillium janthinellum*. They indicated that agitation was to maintain the homogeneity to avoid the formation of large and metabolically inactive pellets. However, high shear force disrupted filamentous tissue and had a remarkable influence on xylanase production. They revealed that xylanase activity reduced when agitation speed was

increased from 100 to 400 min^{-1}. This could be due to hyphal disruption caused by high agitation speed.

Siedenberg et al., 1997 studied xylanase production by *A. awamori* and the effect of stirrer speed. They found that when increasing stirrer speed, the pellet size decreased. In order to evaluate the influence of stirrer speed on the fungal morphology, the fungal mass was taken and fractionated by test sieves in physiological salt solution. They reported the frequency distributions of the humid cell mass which passed the sieves at different mesh sizes. The results showed that at 200 rpm, pellet size of 1 mm was the largest fraction (80%). The remainder 20% consisted of small pellets and some filamentous mycelia which were perhaps originated from the pellet shear off. At 400 and 600 rpm, most of cell mass was in the fractions of 0.2 and 0-0.1 mm mesh sizes. At 400 rpm, 70% of cell mass was in 0.2 mm mesh size and 20% was in 0-0.1 mm fraction. At 600 rpm, 41% of cell mass was in 0.2 mm fraction and 18% was in 0-0.1 mm fraction whereas a total of 30% consisted of clumps of 0.45-0.8 mm.

Hellendoorn et al., 1998 studied intrinsic kinetic parameters of the pellet forming fungus *A. awamori* in a stirred tank reactor aerated with pure oxygen and stirred at 500 and 1000 rpm to determine the effect of shear stress on morphology and oxygen penetration depth. Microscopic observation of the pellets revealed that when cultivating at 1000 rpm, the pellets with denser structure and smaller diameter were found. From oxygen concentration profiles measured in these natural pellets, it was observed that fungal pellets grown at higher stirring rate became denser which resulted in the decrease in oxygen penetration depth. From the results in their study, they concluded that mass

transfer properties of pellets were strongly influenced by the morphology and undoubtedly by the mixing intensity.

Tamerler and Keshavarz, 1999 determined the optimized agitation rate for swainsonine production from *M. anisopliae* in the stirred tank. They claimed that the optimal agitation rate was 400 rpm with a mixed hyphal and pelleted morphology. The results showed that at low mixing, entangled morphology corresponding with lower carbon source consumption and lower growth rate was observed due to inadequate mixing. Whereas higher mixing led to better carbon source consumption and higher shear rate caused hyphal fragmentation and a subsequent formation of broken hyphae and pellets. Furthermore, hyphal fragmentation resulted in a morphological growth cycle: fragmentation, regrowth, and refragmentation and ended up with dense pellet formation.

Gerlach et al., 1998 observed the influence of reactor systems on the morphology of *A. awamori*. They reported that in shake flask culture without baffles, the pellet volume fraction dominated. But with 2 or 4 baffles, the clump volume fraction dominated. Moreover, with 2 baffles, the clump volume fraction was about 95% whereas with 4 baffles, it was initially 80% but reduced to 30% with the increased fermentation time.

As the fermentation proceeds, increased vacuolation weakens the filaments and makes them more susceptible to damage. Vacuolation is a time related process; therefore, the distribution of vacuoles in fungal hyphae is proportional to the age of the compartments. Papagianni et al., 1999a studied hyphal vacuolation and fragmentation in batch and fed-batch culture of *A. niger* and the relationship to citric acid production. They

reported the difference in time profile for mean diameter of vacuoles, d_v, at different agitation speed. In their study, the mean perimeter of clumps at 600 rpm represented a larger range of clump sizes with time compared to those obtained at 200 and 400 rpm. They explained that at 600 rpm the regrowth and formation of new clumps from the fragmented filaments occurred corresponding with the increase of the specific growth rate and the higher specific production rate compared to those of the other 2 speeds. From their study, increased agitation controlled the morphology by altering the specific growth rate and branching initially, following by fragmentation and regrowth. It is also believed that change in cell wall compositions and certain cell wall structures participate in the mechanism of resistance to shear force.

Many works report the degree of morphological damage by shear force in term of the specific energy dissipation rate. Three damaging mechanisms may occur, i.e. the interaction between fungal cell and vortices generated by the turbulent intensity and convective flow, the impact between fungal cell and impellers or baffles in the mechanically stirred tank and the flow collision among fungal cells. However, as discussed previously, several studies suggest that the most plausible mechanism of fungal cell damage is from the interaction between fungal cell and vortices. Cui et al., 1997 claimed that in submerged culture of *A. awamori* CBS115.52, the size and structure of pellets and the hyphal length as well as the pellet size were proportional to the specific energy dissipation rate according to the power function with an exponent of -0.25±0.08 and -0.16±0.03, respectively. They claimed that mechanical force caused hyphal chip-off from the pellet outer zone, contributing to free filamentous mycelia and reseeding the

growth instead of pellet break up. Other studies by Cui et al., 1998a and b reported that when the dissolved oxygen concentration was closed to the saturation concentration, *A. awamori* formed denser pellets and free filamentous mycelial fraction was almost zero at the power input of 1 W/kg. In their study, they also found that the porosity of pellets was inversely related to the specific energy dissipation rate. This was caused by the increased tensile strength of pellets increased with the increased specific energy dissipation rate. Hyphal fragmentation and morphology change are related to hyphal tensile strength. Li et al., 2002 claimed that hyphal fragmentation occurred when local shearing force exceeded hyphal tensile strength. They constructed the correlation of hyphal fragmentation rate constant (k_{frag}) with hydrodynamic variables as shown in eq (2.4).

$$k_{frag} \propto \left(\frac{P}{V_{impeller} t_c} \right)^{0.20} \left(\frac{\nu}{\sigma_h^2} \right)^{0.25} \tag{2.4}$$

Where t_c is circulation time (s), ν is kinematic viscosity (m^2/s), and σ_h is actual hyphal tensile strength (N/m^2). The first term in the equation represents EDCF defined as the product of energy dissipation in the dispersion zone and inverse circulation time $\left(P / kD_i^3\right)\left(1/t_c\right)$ where k is a geometric constant accounted for impeller type and dimensions. EDCF accounts for shearing level experienced by cells and the frequency to enter the impeller discharge zone. The second term accounts for hyphal tensile strength and broth rheology (Li et al., 2002).

As described earlier, the shear rate strongly affected filamentous fungal fermentation in many ways. With the appropriate morphological control under the optimal shear rate, high production rate can be acquired.

2.2.4 Dissolved oxygen

Undoubtedly, oxygen transfer is the most important phenomenon to maintain the aerobic fungal metabolism because oxygen transfer rate is often the limiting factor for optimal growth and productivity (Thibault et al., 2000). The effect of dissolved oxygen concentration on fungal morphology has been observed in many studies but inconsistent results have been reported. In some papers, it was indicated that the morphology of free filamentous mycelia hardly changed with the large variation of oxygen concentration whereas some of them reported a remarkable difference in the mycelial appearance found when supplying the bioreactor with oxygen instead of air. Several studies determined the effects of oxygen concentration, shear rate, and broth rheology and found that these 3 parameters were closely related to morphology alteration with high complexity (Cui et al., 1998a and b; Dominguez et al., 2001; Palma et al., 1996; Piccoli-Valle et al., 2001 and 2003; Shojaosadati and Babaeipour, 2002). In submerged culture, oxygen transfer process involves the transfer from the air bubble into the solution, dissolved oxygen transfer through the culture broth to the surface of the fungal cell and the diffusion into the cell (Yuguo et al., 1999; Lu et al., 1998). The oxygen transfer rate from the gas phase into the culture broth is indicated by the volumetric oxygen transfer coefficient ($k_L a$). Many experimental results show that $k_L a$ is influenced by the air flow rate or superficial air velocity, agitation, air pressure, temperature, vessel geometry, fluid characteristics (density, viscosity, surface tension), the presence of antifoam agents, concentration and physical properties of immobilized materials (density, particle size) (Auria et al., 1995;

Elibol and Ozer, 2000b; Jin and van Leeuwen, 1999; Ozbek and Gayik, 2001; Sankpal et al., 1999).

Yuguo et al., 1999 studied citric acid production by *A. niger* in an external loop airlift bioreactor. They reported that $k_L a$ was influenced by superficial air velocity and the relationship between $k_L a$ and superficial air velocity was linear.

Ozbek and Gayik, 2001 observed the effects of agitation, aeration, and viscosity on $k_L a$ by static gassing out method. $k_L a$ was calculated from the experimental data using eq (2.5).

$$\frac{d[V_L \cdot C_L]}{dt} = k_L a\left(C^* - C_L\right)V_L \qquad (2.5)$$

Where $k_L a$ is the volumetric mass transfer coefficient (min^{-1}), $\left(C^* - C_L\right)$ is the driving force causing mass transfer, C^* and C_L represent oxygen concentration in liquid phase at saturation and at a particular time, respectively. In the stirred tank bioreactor, $k_L a$ in distilled water increased from 0.132 to 5.274 min^{-1} when increasing impeller speed from 100 to 500 rpm. In addition, the result indicated that a critical impeller speed, N_c, in the experiment was 300 rpm. They claimed that only above 300 rpm, a linear function of impeller speed and $k_L a$ occurred. They also estimated the relationship between $k_L a$ and the power number $\left(N^3 D^2\right)$ above 300 rpm as seen in eq (2.6).

$$k_L a \propto \left(N^3 D^2\right)^{0.42} \qquad (2.6)$$

In their study, the effect of aeration rate on $k_L a$ was also determined at 300 rpm, 37°C and pH 7.0. It was found that $k_L a$ increased from 1.728 to 5.35 min^{-1} when the

aeration was raised from 0.15 to 0.9 L/min. The exponential relationship between $k_L a$ and superficial gas velocity (v_s) in their study is as follows.

$$k_L a \propto (v_s)^{0.62} \tag{2.7}$$

Another factor affecting oxygen transfer is the fluid viscosity. They prepared glycerol solution at 10, 20, 30, 40, 50, and 100% in distilled water at pH 7.0 which gave the viscosity of 0.935-566.04 cP. They observed the effect of viscosity on $k_L a$ at 0.3 L/min aeration, 300 rpm and 37°C. The results showed that $k_L a$ decreased from 2.65 to 0.083 min^{-1} when viscosity increased from 0.935 to 566.04 cP. They reported the exponential relationship between $k_L a$ and viscosity (μ) in eq (2.8).

$$k_L a \propto (\mu)^{-0.30} \tag{2.8}$$

Bai et al., 2001 studied the effect of viscosity of the simulated broth on $k_L a$ in a split cylinder airlift bioreactor and reported that $k_L a$ decreased by 20% when the viscosity increased from 1.38×10^{-3} to 3.43×10^{-3} Pa·s. From previous studies, not only the process parameters such as air flow rate and agitation intensity have the strong influence on oxygen transfer but bioreactor configuration also affects $k_L a$.

The major problem in oxygen transfer from the gas phase into the liquid phase is because of the low oxygen solubility in the liquid medium. In the fermentation with the presence of freely dispersed mycelia, the highly viscous culture medium causes the limitation of gas-liquid oxygen transfer and the homogeneity in the bioreactor (Cui et al., 1998a and b). On the other hand, when growing the fungal cells in the pellet form, oxygen depletion in the pellet occurs even at the high bulk oxygen tension. Many works

have been proposed to improve the oxygen transfer rate in submerged fermentation. The simple technique to improve oxygen transfer is to increase agitation intensity and air flow rate. However, turbulence and shear rate, which are often associated with high agitation intensity and air flow rate cause the damage on the fragile cells like filamentous fungi.

Oxygen vector is applied in many fungal fermentation processes to improve oxygen transfer. It is believed that oxygen vector forms the new interfacial area between gas and liquid; hence, enhances oxygen transfer in bulk liquid. Some studies revealed that oxygen vector enhanced the production rate by altering fungal morphology. Jianlong, 2000 has used n-dodecane as the oxygen vector to improve oxygen transfer in citric acid production by *A. niger*. At 5% (v/v) level of n-dodecane in the fermentation medium, $k_L a$ increased as twice as that of the control experiment. Besides, n-dodecane promoted citric acid production and stimulated mycelial growth which was the favorable morphological form in citric acid production by *A. niger*.

Lai et al., 2002 examined the effect of oxygen carriers (n-dodecane, n-tetracane, and n-hexadecane) on fungal morphology and lovastatin production by *Aspergillus terreus* in both shake flask and fermentor cultures. They indicated that with an addition of 2.5% (w/v) n-dodecane into the shake flask culture, lovastatin production increased 1.4 fold corresponding to the morphological change resulting in the formation of small, uniform, compact pellets. In contrast, in the fermentor, the addition of 2.5% (w/v) dodecane resulted in low lovastatin production because an uncontrolled high dissolved oxygen level (>60%) caused unfavorable morphological change and the formation of star-like pellets.

Ates et al., 2002 have attempted to enhance citric acid production by immobilized and freely suspended *A. niger* using silicone oil as the oxygen vector. They found that citric acid production increased twice when 2% (v/v) silicone oil was present in the free cell culture medium. Whereas in immobilized culture, 15% (v/v) silicone oil was added to obtain 1.6 times higher production rate. They claimed that an extra resistance to oxygen transfer into the immobilized structure resulted in the difficulty in oxygen transfer so that higher concentration of silicone oil was required to reach the high production rate.

In many studies, high dissolved oxygen can be maintained by a mixture of air and oxygen, pure oxygen or an increase in partial pressure of oxygen in the fermentor. It is understood that high pressure applied into the fermentor helps increase the oxygen solubility, and eventually enhances $k_L a$ value (Sankpal and Kulkarni, 2002).

Immobilization in inert support provides many advantages on morphological control and high product yield compared to free cell cultivation. However, a rigorous study on mass transfer limitation is essential for immobilization process. Araujo et al., 1999 studied the respiration rate of immobilized *Cephalosporium acremonium* in calcium alginate gel beads. They estimated the respiration rate considering Monod kinetics and the effective diffusivity of oxygen into the gel beads. They determined the effectiveness factor, η, applied to the respiration rate at cell concentration C_x as expressed in eq (2.9).

$$\eta = \frac{v_{obs}}{R_{O_2} \cdot C_x} = \frac{v_{obs}}{\left[\frac{R_{\max} \cdot C_{O_2}}{k_{O_2} + C_{O_2}} \cdot C_x \right]} \tag{2.9}$$

Where v_{obs} and $R_{O_2} \cdot C_x$ refer to the respiration rate with and without oxygen diffusion limitation, respectively. In order to determine the immobilized cell respiration rate, v_{obs}, they assumed that (1) the gel beads are spherical (mean radius, r_p); (2) fungal cells are homogeneously distributed in a spherical shell of constant thickness, $\Delta R = \left(R_p - R_{cr}\right)$; (3) the culture medium is well mixed; (4) the resistance to oxygen transfer through the external film around the gel bead is negligible; (5) radial oxygen flow follows Fick's law; (6) oxygen concentration becomes zero below a critical radius, R_{cr} (dead core model); (7) the effective intraparticle diffusivity of oxygen, $D_{e_{O_2}}$ is constant during the process. The reaction-diffusion model expressed in eq (2.10) is used to describe oxygen profile between R_{cr} and R_p inside the gel beads, where C_x^{biop} is biomass concentration in gel bead.

$$\frac{\partial C_{O_2}}{\partial t} = D_{e_{O_2}} \cdot \left(\frac{\partial^2 C_{O_2}}{\partial r^2} + \frac{2}{r} \frac{\partial C_{O_2}}{\partial r} \right) - \frac{R_{\max} \cdot C_{O_2}}{K_{O_2} + C_{O_2}} \cdot C_x^{biop} \qquad (2.10)$$

By the assumption of quansi-steady state, the global oxygen consumption rate of biomass in gel bead was equal to oxygen diffusion rate flow through the surface of immobilized gel bead as seen in eq (2.11).

$$v_{obs} = \frac{A_p}{V_p} \cdot D_{e_{O_2}} \cdot \left(\left. \frac{dC_{O_2}}{dr} \right|_{r=R_p} \right) = \left. \frac{dC_{O_2}}{dt} \right|_{r=R_p} \qquad (2.11)$$

In their study, they determined the values of v_{obs} at several oxygen and fungal concentrations and took the derivatives of the curves obtained from the experimental data by spline interpolation. As the result, they were able to determine η using eq (2.9).

Cruz et al., 2001 developed the dead core model for immobilized *C. acremonium* in calcium alginate gel beads in a fed batch cephalosporin C production. They also indicated that despite the extremely low sugar concentrations, the reaction rate inside the pellet was limited by dissolved oxygen concentration. Besides they reported that fungal growth occurred only in the outer layers of the gel beads.

Pashova et al., 1999 observed immobilized *A. niger* in 3% calcium alginate beads. The specific oxygen uptake rate was determined during the fermentation. They found that for immobilized system, the specific oxygen uptake rate changed with the cultivation time. They indicated that the effectiveness factor, η, increased during the incubation time from 0.54 to 0.93. This was because immobilized mycelia were concentrated at the surface layer of the gel bead and no fungal hypha was found in the center of the gel bead. The dense growth on the outer surface led to the formation of a fur-like mycelial coat which provided a large surface area for oxygen transfer.

2.3 Reaction Systems Used in Submerged Filamentous Fungal Fermentation

As discussed earlier, filamentous fungal fermentation is a complicated system which requires a particular reactor design to overcome the problems in dramatic changes in hydrodynamic property and fungal morphology. Many researchers have been

attempting to develop the reaction system for the filamentous fungal fermentation in order to control and maintain the culture under the optimal operating conditions. In submerged culture, based on the appearance of fungal cells in the bioreactor, fermentation process can be divided into two categories, i.e. free cell and immobilized cell.

In free cell fermentation, fungal morphology can be either the dispersed mycelia or the pellets, depending on the process conditions. Usually in the typical stirred tank bioreactor, the problems in mixing and oxygen transfer occur due to high broth viscosity when freely dispersed mycelia are present. Although gas-liquid oxygen transfer is improved with the pellet morphology, oxygen starvation may occur in a large pellet because of the limitation of oxygen diffusion into the center of the pellet (Bai et al., 2003). The production rate is often low due to inappropriate morphology formed under improper operating conditions. In addition, other disadvantages of the mechanically stirred tank bioreactor include relatively high power requirement and heat generation rate, particularly in the large-scale bioreactor. Many researchers have attempted to improve the performance of the mechanically stirred tank bioreactor in various ways. Those methods involve manipulating the feed rate and mode, using fed batch or continuous processes to prolong the fermentation or using the sophisticated control system to control the process parameters in order to maintain reactor stability during the fermentation period. However, none of them can successfully work for filamentous fungal fermentation (Amanullah et al., 1999; Elibol and Ozer, 2002; Simpson et al., 1995).

Usually, the pellet is the morphological structure present in the fluidized bed bioreactors. Many studies show that the profound shear damage, which is often found in the mechanically stirred tank, can be reduced when using the fluidized bed bioreactor. The reactor performance strictly depends on the pellet size and the distribution of the pellet. Pellet aggregation often results from poor fluidization with slugging of the bed, although pulsing flow is applied into the bioreactor (Moreira et al., 1998).

Air-lift bioreactor is one alternative which has been studied and developed for free cell fermentation. In air-lift bioreactor, a region of gassed liquid is in contact to a region of ungassed liquid. The difference in hydrostatic pressure between the 2 liquid regions results in the circulation of liquid phase. As the results, this type of bioreactor provides many beneficial outcomes over the mechanically stirred tank bioreactor including the relatively lower energy requirement, higher mass, momentum and heat transfer rate, and lower shear damage (Aguilar et al., 2001; Ashokkumar et al., 2001; Bai et al., 2001; Gerlach et al., 1998; Jin and van Leeuwen, 1999; Jin et al., 1999 a and b; O'Donnell et al., 2001; Park et al., 1998; Sankpal et al., 2001a and b; Siedenberg et al., 1997; Srivastava and Kandu, 1999; Su and He, 1997; Sun et al., 1998 and 1999a and b; Yin et al., 1998; Yuguo et al., 1999).

In contrast to the typical free cell fermentation, immobilization provides the advantages of avoiding washout at a high dilution rate when operating the bioreactor continuously. Moreover, it offers higher cell concentration, easier downstream processing because cell separation is much easier, and much better morphological control (Elibol and Ozer, 2000a, Tay and Yang, 2002). Because immobilized cell is more stable than free

biomass, it is possible to operate the bioreactor in continuous or semi continuous processes as observed in many studies. With the better morphological control, higher fungal growth and metabolism lead to higher productivity (Araujo et al., 1999; Ates et al., 2002; Cruz et al., 2001; Junter et al., 2002; Lusta et al., 2000; Pashova et al., 1999; Sankpal et al., 1999).

Various immobilization techniques have been applied in submerged filamentous fungal fermentation. These include flocculation with polyelectrolyte, covalent binding to glycidyl ester copolymer, entrapment in gel, and adsorption onto support (Aguilar et al., 2001; Alekseiva et al., 1998; Angelova et al., 1998; Ashokkumar et al., 2001; Cruz et al., 2001; Jin et al., 1999b; Junter et al., 2002; Kosakai et al., 1997; Lusta et al., 2000; Sankpal and Kulkarni, 2002; Sankpal et al., 2001a and b; Srivastava and Kandu, 1999; Su et al., 1997; Sun et al., 1999a; Tay and Yang, 2002). For instance, L-lactic acid production by mycelial flocs of *R. oryzae* is accomplished in the jar fermentor with the optimal addition of supports and flocculants. In acid proteinase production, *Humicola lutea* 120-5 is immobilized in crosslinked polyvinyl alcohol (PVA) mixed with polyethylene glycol (PEG). To control the porosity of the matrix and to improve fungal activity, inert water soluble compound is also added into crosslinkable PVA by mixing with PEG. In polymethylgalacturonase production, a significant increase in production rate is observed when using *A. niger* 26 immobilized in calcium alginate beads. A study on gluconic acid production using immobilized *A. niger* on the porous cellulose support in a recirculation bioreactor indicates that for efficient bioconversion, an optimal fungal biomass on the porous cellulose support is necessary to be controlled; otherwise, an

overgrown mycelia will affect the productivity adversely. According to several advantages of immobilization, this technique has been applied in many bioreactors used in submerged culture including disk fermentor, magnetic drum contactor, reciprocating jet bioreactor, tower fermentor, hollow fiber bioreactor, and rotating bed bioreactor. However, without the optimal immobilization technique for a particular process, mass transfer limitation might cause the adverse effect to the production rate. Therefore, one should be aware of the mass transfer into the immobilized particle.

2.4 L(+)-Lactic Acid Production

L(+)-lactic acid, $CH_3CHOHCOOH$, a natural organic acid primarily found in milk is currently produced by many companies around the world. Due to the relatively high production cost, it and its salts and esters are only used in limited application. To date, the production of polylactic acid (PLA), a new biodegradable polymer with promising applications in food packaging, non-woven products, and disposable products becomes attracted by many producers. Therefore, with the evolution of new applications of L(+)-lactic acid, the production with lower cost needs to be developed. The typical route of L(+)-lactic acid fermentation by lactic acid bacteria is described in Figure 2.2.

Recently, there has been an increased interest to produce optically pure L(+)-lactic acid from the renewable agricultural residue by *Rhizopus* species. In the production by *Rhizopus*, the pretreatment of raw material by enzymatic hydrolysis followed by the extraction of residual sugars can be omitted because the ability of these fungal species to

secrete the hydrolytic enzymes. In addition, due to the production of optically pure L(+)-lactic acid, the separation and purification of L(+)-lactic acid from D(-)-lactic acid is not necessary. Among the different *Rhizopus* strains, *R. oryzae* has been widely used since it shows the synthesis of L(+)-lactic acid at the higher level compared with the other strains in these species (Oda et al., 2003; Yin et al., 1997).

2.4.1 Metabolic pathway of *R. oryzae*

Wright et al., 1996 and Longacre et al., 1997 used a flux analysis of glucose metabolism and developed a metabolic model of *R. oryzae* from the metabolic control theory (MCT). The generalized metabolic pathway of *R. oryzae* is illustrated in Figure 2.3. As shown in Figure 2.3, *R. oryzae* can secrete amylases to digest starch. Glucose from starch hydrolysis enters the Embden Mayerhof Parnar (EMP) pathway. The final product of the EMP pathway is pyruvate. In addition, *R. oryzae* can utilize xylose via the pentose phosphate (HMP) pathway to obtain pyruvate. In the presence of glucose and high dissolved oxygen concentration, Pyruvate is converted to lactate by an NAD^+-dependent lactate dehydrogenase (LDH) (Pritchard, 1973). During glucose depletion or sporulation, the activity of LDH rapidly decreases and the oxidation of lactate to Pyruvate by NAD^+-independent LDH is usually formed. The metabolism of Pyruvate in *R. oryzae* also involves the tricarboxylic acid (TCA) cycle occurring in the mitochondrion and the separated cytosolic pathway for fumaric acid formation involving Pyruvate carboxylase, malate dehydrogenase, and fumarase. In addition, *R. oryzae* possesses alcohol

dehydrogenase (ADH) to produce ethanol in a short period under low oxygen tension although *R. oryzae* cannot grow well during oxygen starvation. Therefore, depending on the fermentation condition, ethanol, CO_2, and fumarate can be produced as major byproducts in addition to lactate.

2.4.2 L(+)-lactic acid fermentation by *R. oryzae*

Currently, many researchers have attempted to develop L(+)-lactic acid production by *R. oryzae* due to its advantages of the simultaneous saccharification of agricultural residues and fermentation. Submerged fermentations including free cell and immobilized cell production have been extensively studied. In free cell fermentation, many studies report that filamentous form is the preferable morphology, accompanied with the high production rate. From the literatures, it was found that the stirred-tank bioreactor equipped with the sophisticated control and the air-lift bioreactor have been widely used in the fermentation (Table 2.3). In immobilized cell bioreactors, many immobilization techniques have been applied in various fermentation systems as discussed earlier. In L(+)-lactic acid fermentation by *R. oryzae*, the most favorable immobilization technique is cell entrapment in many types of supports such as calcium alginate gels, mineral supports, polymerix supports, or a fibrous matrix (Table 2.3). L(+)-lactic acid is recognized as an inhibitory product. As observed in many studies on the long-term fermentation, when the concentration of L(+)-lactic acid is high in the later period of fermentation, *R. oryzae* activity is declined corresponding with the lower lactic

acid production rate. Extractive fermentation provides the in situ removal of L(+)-lactic acid from the fermentation broth, reducing the product inhibitory effect. As the result, the problem in the activity decline and lower production rate in the later period of the long-term fermentation can be overcome (Sun et al., 1999a and b).

Recently, solid state fermentation (SSF) becomes attracted by many researchers; however, relatively few studies on SSF to produce L(+)-lactic acid have been conducted. Some previous studies reported the use of ground corn and corn cob as well as cereals as the solid substrates (Table 2.3). The results obtained form those studies show a great potential of SSF to be used in L(+)-lactic acid production. However, the information given in those studies does not provide the enough detail on mass and heat transfer control as well as the morphology related to growth and production rate which is usually required to develop the large-scale SSF process.

2.5 Conclusion

To economically produce a particular product involves the uses of bioreactors. It is well known that reactor performance has a profound effect on the productivity; therefore, understanding the nature of the fermentation system of such a particular product is helpful for bioreactor selection and design. For filamentous fungal fermentation including L(+)-lactic acid production by *R. oryzae*, morphology plays a significant role relating to product formation and secretion as well as the characteristic of the culture broth, mixing, heat and mass transfer. As discussed previously, fungal

morphology is closely related to the changes in environmental conditions, such as temperature, pH, shear force, oxygen, and nutrition. Therefore, to develop the high performance bioreactor to accommodate the fungal growth, one should be able to understand and control the fungal morphology. Many techniques have been developed for controlling the fungal morphology in order to reach high production rate. In the previous study of our research group, the RFBB has been developed for controlling *R. oryzae* morphology. The results showed that the RFBB provided many advantages in morphological control and a high potential for scale-up to the industrial fermentor. However, relatively little information about the morphology affecting mass transfer and L(+)-lactic acid in the RFBB is available. Therefore, in this study, *R. oryzae* morphological and mass transfer control will be further determined in the RFBB.

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Correlation	Organism	Rheological measurement technique	Fermentation system	Source
$K = C_m^{2.8} \times R^{0.7} \times C^{1.2} \times const^{a}$	*Penicillium chrysogenum*	Disk turbine rheometer	Batch	Tucker and Thomas, 1993
$K = C_m^{2.3} \times R^{-0.96} \times C^{0.79} \times 6.6 \times 10^{-5}$	*P. chrysogenum*	Disk turbine rheometer	Batch	Tucker, 1994
$K = -0.56 + 0.0018 \times R \times C_m^{1.7}$	*Aspergillus niger*	On-line impeller rheometer	Continuous and fed batch	Olsvik et al., 1993
$K = 0.38 + 4.8 \times 10^{-5} \times R \times C_m^{2.9}$	*A. niger*	On-line impeller rheometer	Batch	Olsvik and Kristiansen, 1994
$\tau_y = 4.2 \times 10^{-6} (C_m)^{2.6} (L_e^*)^{2.2}$, $\tau_y = 7.2 \times 10^{-3} (C_m)^{2.2} (L_{hgu})^{0.65}$, $\tau_y = 4.8 \times 10^{-7} (R)^{3.2} (C_m)^{2.5}$	*Streptomyces levoris and A. niger*	Rotating vane technique	Batch (fermentor) and shake flask culture	Mohseni and Allen, 1995

[a] Value of constant not given in the literature

Table 2.1 Correlations for rheological behavior prediction of various filamentous fungi, measured by different rheometric techniques (Riley et al, 2000)

Organism	Substrate	Product	Production rate	Fermentation condition	Morphology	Reference
A. niger	Molasses based medium containing 2.0×10^{-5} M $CuSO_4{\cdot}5H_2O$	Citric acid	92.0 g/L	Batch, fermentor, 6 days	Mixed pellets	Haq et al., 2002a
A. awamori	Synthetic medium containing 1.05 g/L KH_2PO_4	Cell biomass	~4.5 g/L	Airlift tower loop reator, ~60 days	Dense pellets with uniform density	Gerlach et al., 1998
A. japonicus	Sucrose and yeast extract with K_2HPO_4 and $MgSO_4{\cdot}7H_2O$	β-fructofuranosidase	Not affected but enhanced by the addition of surfactants and polymeric additives	Shake flask culture, 96 hours	Pellets	Chen and Liu, 1996
R. chinesis 12	Fermentation medium containing CSL	Antibiotics	6328.0 U/mL	Shake flask culture, 48 hours	Fluffy loose pellets	Du et al., 2003
A. niger	Semi-synthetic medium containing wheat bran	Phytase	8090 U/L	Shake flask culture, 144 hours	Pulp and pellets	Papagian-ni et al., 1999b

Continued

Table 2.2 Preferred fungal morphology found in different fermentation processes

Table 2.2 continued

Organism	Substrate	Product	Production rate	Fermentation condition	Morphology	Reference
P. japonica	Fermentation medium containing 40 g/L sucrose	Exo-biopolymer	25 g/L	Stirred tank fermentor, 12-14 days	Compact pellets with least hairiness and roughness	Sinha et al., 2001b
R. arrhizus NRRL2286	Fermentation medium containing 1 g/L glucose and 0.5 g/L corn oil	Lipase	325 μmol/L·min	Shake flask culture, 40-60 hours	Immobilized cells on polyurethane foam (natural attachment)	Elibol and Ozer, 2000a
Rhizopus oryzae	Fermentation medium with 90 g/L initial glucose and > 10 mg/L inorganic salts	L-lactic acid	60 g/L	Shake flask culture, batch, 18 hours	Immobilized cells on polyurethane foam cubes (natural attachment)	Sun et al., 1998

Continued

Table 2.2 continued

Organism	Substrate	Product	Production rate	Fermentation condition	Morphology	Reference
Acremonium chrysogenum	Minimal fermentation medium with methionine	Cephalosporin C	Not affected	Shake flask culture	Filamentous (prevented yeast-like morphology by prolonging the exponential growth phase)	Karaffa et al., 1997
A. niger	Fermentation medium containing a mixture of 1.5% w/w olive oil and 0.5% w/w sucrose	Lipase	29.6 triolein U/mL	Shake flask culture, 8 days	Mycelia	Macris et al., 1996
Aspergillus oryzae	Pulsed addition of limiting carbon	Glucoamylase	~80 units/mL	Stirred tank fermentor, fed batch, ~100 hours	Small fungal mycelia	Bhargava et al., 2003

Continued

Table 2.2 continued

Organism	Substrate	Product	Production rate	Fermentation condition	Morphology	Reference
A. niger 419	Sugarcane molasses-based medium with 0.5 g $CaCl_2$/L	Citric acid	0.37 g/h	Jar fermentor, 15 days	Loose pellet	Pera and Callieri, 1999
A. awamori	Fermentation medium containing oat xylan	Xylanase	N/A	Shake flask culture, 160 hours	Filamentous mycelia and small pellet (0.1-0.2 mm diameter)	Siedenberg et al., 1998
Penicillium griseoroseum	Minimal medium supplemented with sugar cane juice	Pectin lyase	~0.109 IU/mL	Fermentor, repeated batch, 53 hours (1^{st} cycle), 20 hours (2^{nd} and 3^{rd} cycles)	Pellets	Piccoli-Valle et al., 2001
Penicillium restrictum	Babassu oil cake supplemented with 2% olive oil	Lipase	30.3 U/g initial dry weight	SSF	Mycelia grown on solid babassu oil cake	Gombert et al., 1999

Continued

Table 2.2 continued

Organism	Substrate	Product	Production rate	Fermentation condition	Morphology	Reference
A. niger	Fermentation medium containing high glucose concentration (18%)	Gluconic acid	~175 g/L	Shake flask culture or modified batch recirculation fermentor	Immobi-lized cell on a highly porous cellulose support	Sankpal and Kulkarni, 2002

Fermentation conditions	Substrate	Yield	Productivity	Final product concentration	Operation period	Reference
Free cells in air-lift bioreactor	Corn starch, 120 g/L	85%	-	102 g/L	-	Yin et al., 1997
Free cells (mutants with low ADH) in shake flask under low O_2	Glucose, 100 g/L	-	-	40 g/L	Batch, 70 h	Skory et al., 1998
Free cells (pellet) in air-lift bioreactor	Corn starch, 90 g/L	75.8% (cycle 1-6)	2.02 g/L·h (cycle 1-6)	91 g/L (cycle 1-6) 76 g/L (cycle 7-9)	9 repeated batches, 14 days	Yin et al., 1998
Free cells in bubble column bioreactor	Glucose, 94 g/L	88%	2.58 g/L·h	83 g/L	Batch, 32 h	Zhou et al., 1999
Free cells in shake flask	Steam-exploded wood hydrolysate (reducing sugars), 75 g/L	-	-	-	Batch	Woiceichowski et al., 1999
Free cells in stirred tank bioreactor with a periodical bleed and feed (PBF)	Glucose, 100 g/L	67.3%	2.31 kg/m^3·h	~80 g/L	14 PBF cycles, 10 days	Martak et al., 2003

Continued

Table 2.3 Previous studies on L(+)-lactic acid production by *R. oryzae*

Table 2.3 continued

Fermentation conditions	Substrate	Yield	Productivity	Final product concentration	Operation period	Reference
Free cells with simultaneous saccharification and fermentation in shake flask	Potato wastewater	45%	-	21 g/L	Batch: saccharification, 20 h and fermentation, 28 h	Huang et al., 2003
Free cells (pellet) in stirred tank bioreactor	Glucose, 100 g/L	74.2%	-	74.92 g/L	6 repeated batches	Bai et al., 2003
Immobilized cells in calcium alginate gels in stirred tank bioreactor	Glucose, 130 g/L	72 %	-	62 g/L	5 repeated batches	Hang et al., 1989
Mycelial flocs entrapped in mineral supports in shake flask	Glucose, 120 g/L	80%	-	103.6 g/L	Batch, 60 h	Kosakai et al., 1997
Immobilized cells in polyurethane foam cubes in shake flask	Glucose, 50 g/L	-	-	~30 g/L	12 repeated batches	Sun et al., 1998

Continued

Table 2.3 continued

Fermentation conditions	Substrate	Yield	Productivity	Final product concentration	Operation period	Reference
Mycelial cotton-like flocs entrapped on mineral supports in air-lift bioreactor	Glucose, 120 g/L	87%	-	104.6 g/L	Batch	Park et al, 1998
Immobilized cells in polyurethane foam cubes in air-lift bioreactor	Glucose, 61 g/L	-	1.0-6.2 g/L·h (DR = 0.04-0.2 h^{-1})	40-60 g/L	Continuous	Sun et al., 1999a
Immobilized cells on a fibrous matrix in RFBB	Starch	~100%	1.65 g/L·h	127 g/L	Fed-batch	Tay and Yang, 2002
SSF in shake flask	Cassava, 10 g/100 mL distilled water	74%	-	~450 g/kg substrate	Batch, 96 h	Yu and Hang, 1989
SSF in shake flask	Ground corn, 15 g/100 mL distilled water	>44%	-	354 g/kg corn	Batch, 96 h	Hang, 1989
SSF in shake flask	Corncob, 5 g/100 mL distilled water	-	-	299.4 g/kg corncobs	Batch, 48 h	Ruengruglikit and Hang, 2003

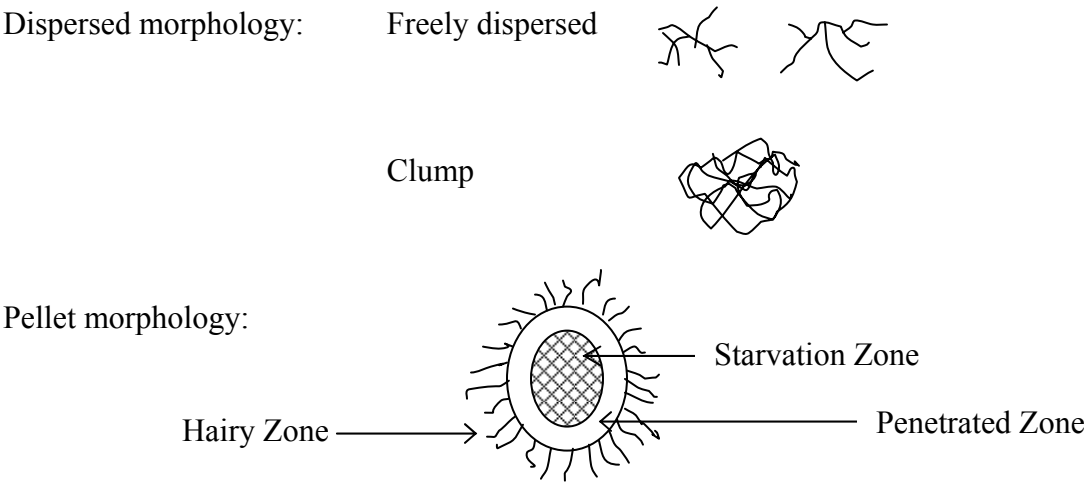

Figure 2.1 Fungal morphology in submerged culture

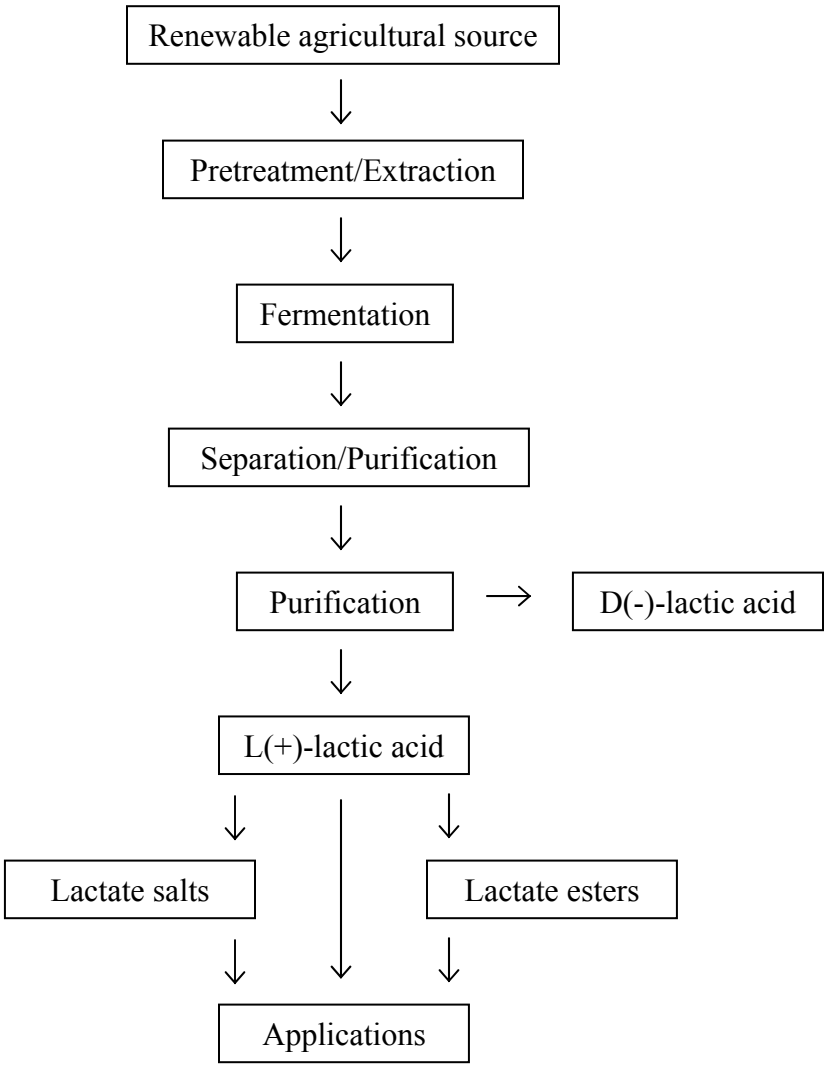

Figure 2.2 Typical route of L(+)-lactic acid fermentation by lactic acid bacteria (www.lactic.com)

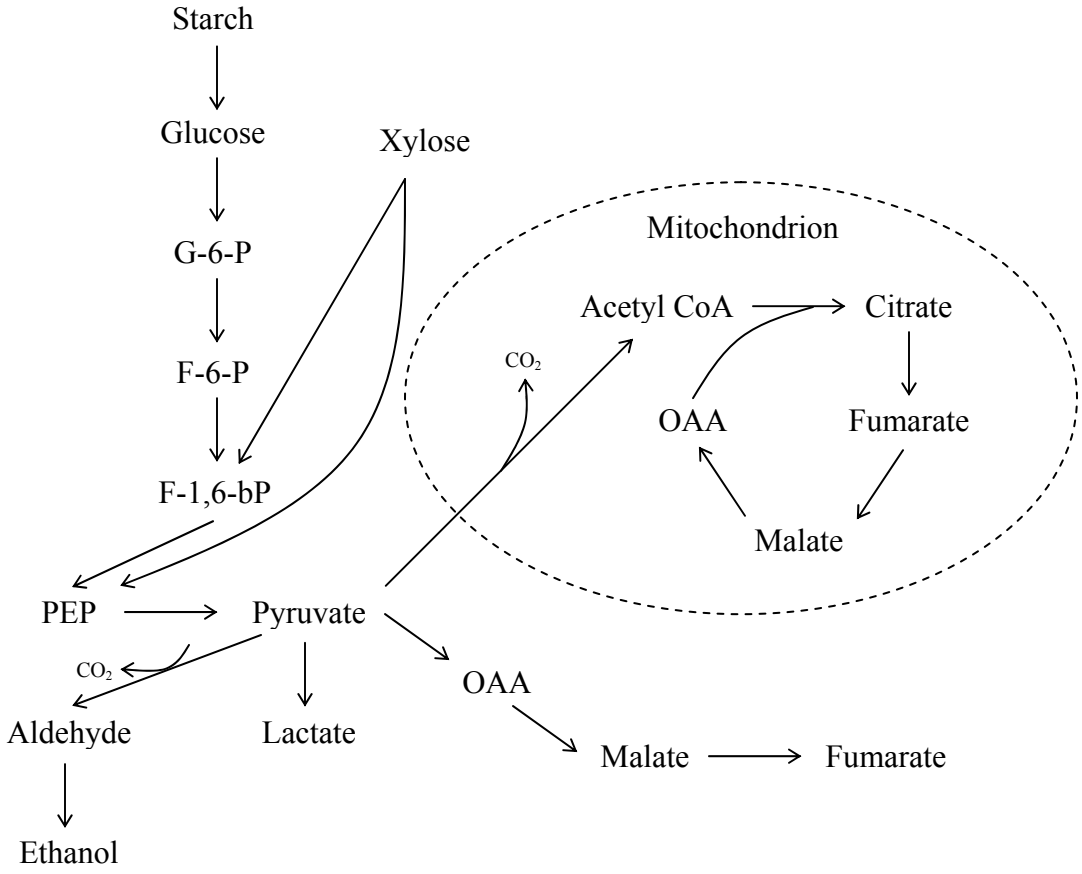

Figure 2.3 Proposed metabolic pathway of *R. oryzae*, where G-6-P is Glucose-6-phosphate, F-6-P is Fructose-6-phosphate, F-1,6-P is Fructose-1,6-bisphosphate, PEP is Phosphoenol pyruvate, and OAA is Oxaloacetate

CHAPTER 3

CONTROLLING FILAMENTOUS FUNGAL MORPHOLOGY BY IMMOBILIZATION ON A ROTATING FIBROUS MATRIX TO ENHANCE OXYGEN TRANSFER AND L(+)-LACTIC ACID PRODUCTION BY *RHIZOPUS ORYZAE*

Summary

Filamentous fungi are widely used in industrial fermentations. However, the filamentous morphology is usually difficult to control and often cause problems in conventional submerged fermentations. The fungal morphology has profound effects on mass transfer, cell growth, and metabolite production. Controlling the filamentous morphology by immobilization on a rotating fibrous matrix was studied for its effects on oxygen transfer and lactic acid production in aerobic fermentation by *R. oryzae*. Compared to the conventional stirred tank bioreactor, the fermentation carried out in the rotating fibrous bed bioreactor (RFBB) resulted in a good control of the filamentous morphology, and improved oxygen transfer and lactic acid production from glucose. A high lactic acid concentration of 137 g/L with a high yield of 0.83 g/g and reactor

productivity of 2.1 g/L·h was obtained with the RFBB in repeated batch fermentations. The process was stable and can be used for an extended operation period.

3.1 Introduction

Lactic acid is commercially produced by either chemical synthesis or fermentation. In chemical synthesis, lactic acid is generally produced by the hydrolysis of lactonitrile formed in the reaction of acetaldehyde with hydrogen cyanide (Benninga, 1990). *Lactobacillus sp*. have been commonly used in lactic acid fermentation due to their high volumetric productivity and yield (Hofvendahl and Hahn-Hagerdal, 2000; Litchfield, 1996; Vick Roy, 1985). However, the racemic mixtures of L(+)- and D(-)-lactic acids produced by most *Lactobacillus sp.* are difficult to use in the manufacture of biodegradable polylactic acid (Tsai and Moon, 1998). Although some mutants can produce pure L(+)-lactic acid, they are not commonly available. Furthermore, *Lactobacillus* requires complex media for growth which makes the final product recovery and purification difficult and costly (Hsieh et al., 1999; Ho, 1996; Silva and Yang, 1995). Recently, there have been increasing interests in fungal fermentation with *R. oryzae* to produce optically pure L(+)-lactic acid from glucose, pentose sugars, and starch directly in a simple medium (Ho, 1996; Soccol et al., 1994; Tay, 2000; Woiciechowski et al., 1999; Yu and Hang, 1989; Zhou et al., 1999). However, it is cumbersome to control the filamentous morphology in conventional submerged fermentation which greatly hampers reactor operation and limits the fungal fermentation process due to lowered product yield and production rate (Kosakai et al., 1997; Park et al., 1998; Yang et al., 1995). Table 3.1 compares homolactic *Lactobacillus sp.* and *R. oryzae* in their use for lactic acid production.

Figure 3.1 shows a generalized catabolic pathway found in *R. oryzae*. *R. oryzae* usually converts glucose to pyruvic acid via the EMP pathway. *R. oryzae* can also use pentose phosphate pathway (HMP) in pentose sugar catabolism. In addition, *R. oryzae* is amylolytic; therefore, it can convert starch to glucose. Oxygen is a critical factor affecting lactic acid production from pyruvate (Skory et al., 1998). In the presence of glucose and sufficient dissolved oxygen concentration, pyruvic acid is converted to lactic acid by an NAD^+-dependent lactate dehydrogenase (LDH) (Pritchard, 1973; Skory et al., 1998). During glucose depletion or sporulation, the activity of LDH rapidly decreases and the oxidation of lactic acid to pyruvic acid is usually found. The metabolism of pyruvic acid in *R. oryzae* also involves the tricarboxylic acid (TCA) cycle occurring in mitochondria and the separated cytosolic pathway for fumaric acid formation involving pyruvate carboxylase, malate dehydrogenase, and fumarase. Although *R. oryzae* does not grow anaerobically, it possesses alcohol dehydrogenase (ADH) which allows the fungus to grow in a short period in the absence of oxygen. Depending on the fermentation conditions, ethanol, CO_2 and fumaric acid can be also produced as major byproducts in addition to lactic acid by *R. oryzae*.

Control of morphology and broth rheology is important to the fungal fermentation. The highly branched fungal mycelia may cause complex (viscous) broth rheology and difficulty in mixing and aeration in the conventional agitated bioreactor (Bai et al., 2003; Kosakai et al., 1997; Martak et al., 2003; Park et al., 1998; Skory et al., 1998; Yang et al., 1995; Yin et al., 1997 and 1998; Zhou et al., 1999). Various cell immobilization methods to control the fungal morphology and to achieve high cell

density and high production rate have been studied (Dong et al., 1996; Hang et al., 1989; Kosakai et al., 1997; Lin et al., 1998; Park et al., 1998; Sun et al., 1998 and 1999; Tamada et al., 1992; Tay and Yang, 2002). In general, higher lactic acid yield and productivity were achieved with immobilized cells than those from free mycelial cells, partially due to reduced cell growth and increased specific cell productivity. With the immobilized cells, a high fungal biomass density can be also achieved which can be repeatedly used for lactic acid production over a long period. Power consumption in mixing and aeration can be also greatly reduced because the fermentation broth is maintained at a low viscosity (similar to water) under cell-free condition which in turn facilitates product recovery from the relatively pure and simple medium by solvent extraction (Tay, 2000).

In this work, fungal spores and mycelia have been immobilized in a rotating fibrous matrix in a stirred tank bioreactor, and the reactor was used to produce L(+)-lactic acid from glucose for an extended period of more than 10 days, demonstrating the feasibility and advantages of the new fungal bioreactor. The effects of cell immobilization on fungal morphology, oxygen transfer, and lactic acid production by *R. oryzae* were determined. The dynamic method of gassing out was used to evaluate oxygen transfer and uptake during the fermentation. The effects of aeration rate on the fungal fermentation and RFBB performance were also determined and the results are discussed in this study.

3.2 Materials and Methods

3.2.1 Culture, inoculum preparation, and medium compositions

R. oryzae NRRL 395, a filamentous fungus producing L(+)-lactic acid obtained from the Northern Regional Research Center, Peroria, IL, was used in this study. The method to maintain fungal viability is described in Appendix A1. The spore suspension made from the 7-day culture on the potato dextrose agar plate was adjusted to 10^6/mL by dilution with sterile water. 10^6/mL *R. oryzae* spore suspension (10 mL) was used to inoculate the bioreactor (Appendix A2).

Unless otherwise noted, a growth medium contained 50 g/L glucose and a production medium contained 70 g/L glucose as a substrate. The other compositions required in each medium are shown in Appendix A3.

3.2.2 Rotating Fibrous Bed Bioreactor

A Rotating Fibrous Bed bioreactor (RFBB) was modified from a 5-L bioreactor (Biostat B, B. Braun) by affixing a perforated stainless steel cylinder mounted with a 100% cotton cloth to the agitation shaft. The bioreactor setup is described in Appendix B. The cylindrical matrix of 9 cm diameter and 15 cm height was used in this study. After sterilization, the bioreactor was set up the controlled parameters and inoculated with 10 mL spore suspension (10^6/mL). Unless otherwise mentioned, the bioreactor was

controlled at 30°C and pH 6.0 (during the production phase), agitated at 50 rpm, and aerated with filter-sterilized air at 1.0 vvm.

To study the fermentation kinetics, the growth medium was replaced with the production medium after spore germination and cell immobilization on the fibrous matrix had occurred (approximately after 48 h). The fermentation batch finished when the substrate was depleted or L(+)-lactic acid concentration was not increasing.

To study the long-term stability of the bioreactor, repeated batch fermentations were conducted by replacing the medium at the end of each batch when glucose was almost depleted and lactic acid production stopped. A total of 9 batches were conducted consecutively with each batch taking about 24 h. Repeated batch fermentations with high-concentration glucose media were also conducted to evaluate the maximum lactic acid concentration that can be produced by the fungal cells. About 2 L of the medium in the bioreactor were replaced with a concentrated medium whenever glucose was almost depleted, until the fermentation finally stopped due to inhibition of the cells by high concentration of lactic acid.

3.2.3 Determination of volumetric oxygen transfer coefficient

Before the end of each fermentation batch, the oxygen uptake rate (OUR) and the dynamics of oxygen transfer in the bioreactor were studied using the dynamic method of gassing out (Appendix C).

3.2.4 Analytical methods

3.2.4.1 Cell biomass

Growth of *R. oryzae* in the RFBB was determined at the end of fermentation. The detail is described in Appendix D1.

3.2.4.2 Substrate and product concentration

The fermentation sample was centrifuged to remove the suspended solid. The supernatant was analyzed for glucose and L(+)-lactic acid using a glucose/lactate analyzer (YSI 2700). HPLC was also used to analyze the organic compounds (glucose, lactic acid, fumaric acid, and ethanol) present in the supernatant. The HPLC system (Shimadzu Scientific Instruments) was equipped with a RID-10A reflective index detector and an organic analysis column (Aminex HPX-87H, Biorad). The details are given in Appendix D2 and D3.

3.3 Results and Discussion

3.3.1 Effect of immobilization on fungal morphology

After spore germination, cells generally grew into large mycelial clumps that were clinged to surfaces of agitation shaft, probes, and bioreactor wall in the conventional stirred tank bioreactor (Figure 3.2a), which made the bioreactor difficult to operate and control. However, when cotton cloth was present for cell attachment and immobilization in the RFBB, fungal mycelia were only attached on the cotton cloth and no cells were found in the fermentation broth or any other surfaces (Figure 3.2b). Consequently, the fermentation broth was clear and the bioreactor was easy to operate and control. It is clear that cotton cloth provided a preferential surface for cell attachment and the shear acting upon the rotating fungal biofilm helped maintaining its compactness. The different fungal morphologies found under these 2 different fermentation conditions also resulted in significant difference in lactic acid production by the cells. The fermentation kinetics obtained in these 2 bioreactor systems is shown in Figures 3.3a and 3.3b, respectively.

3.3.2 Fermentation kinetics

Figure 3.3a shows typical fermentation kinetics with fungal cells in a conventional stirred tank bioreactor. During the growth phase, the carbon source (glucose) was mainly used for growth and synthesis of cell biomass, and there were some

ethanol and a smaller amount of lactic acid produced. In the production phase, cell growth was greatly reduced due to nitrogen limitation and glucose was mainly used to produce lactic acid with ethanol as a fermentation byproduct. Similar fermentation kinetics was observed with the RFBB; however, faster and more lactic acid production was obtained with less ethanol produced in the production phase (Figure 3.3b). It is well known that cell growth and lactic acid production by *R. oryzae* require oxygen (Skory et al., 1998; Tay and Yang, 2002). More ethanol would be produced when there was oxygen limitation because the activity of lactate dehydrogenase (LDH) declined and alcohol dehydrogenase (ADH) was induced, resulting in the oxidation of lactic acid to pyruvic acid which then entered the ethanol fermentative pathway. The higher lactic acid production rate and yield obtained in the RFBB were attributed to the better oxygen transfer as indicated by the higher oxygen uptake rate (*OUR*) and volumetric oxygen transfer coefficient (k_La) which were determined from the dynamic gassing out method.

Cell growth could not be easily measured for immobilized cell fermentation in the RFBB. The immobilized fungal cells attached on the matrix were approximately 0.5 cm thick from the surface of the cotton cloth at the end of the growth phase, and increased to approximately 1 cm at the end of the production phase. The nutrients present in yeast extract were essential to spore germination, but not critical to maintain fungal activity. It is also possible that the residual nutrients left in the fermentation broth from the growth phase were sufficient to support continued cell growth in the production phase. The cell density at the end of the production phase was estimated by harvesting the cells in the bioreactor and measuring the total cell dry weight. More cell biomass was found in the

RFBB than in the free cell fermentation, indicating faster growth in the RFBB due to improved aeration (higher C_L and k_La values). Table 3.2 summarizes and compares the experimental results from the RFBB and the conventional stirred tank bioreactor.

It is clear that the RFBB gave better oxygen transfer and higher cell growth and lactic acid production, all of which can be attributed to the better controlled fungal morphology caused by cell immobilization on the rotating fibrous matrix. It should be noted that the values of k_La and C^* during the fermentation were estimated from the plot of $\left(\frac{dC_L}{dt} + OUR\right)$ versus C_L using the data obtained in the dynamic gassing out experiments conducted right after the fermentation was completed. The estimated C^* value of 0.17 mM was significantly lower than the solubility of oxygen in water (approximately 0.24 mM for air at 1 atm) at 30°C (Bailey and Ollis, 1986). The lower C^* value could be partially due to the electrolytes present in the medium and CO_2 production during the fermentation.

3.3.3 Long-term lactic acid production in the RFBB

The feasibility and stability of the fungal culture immobilized in the RFBB for long-term production of lactic acid from glucose were evaluated in repeated batch fermentations operated at 50 rpm, 1.0 vvm, pH 6.0 and 30°C. As shown in Figure 3.4a, except for the initial batch for cell growth, all subsequent 9 batches gave consistent lactic acid production during the 10-day period studied. There was no ethanol or other

byproducts found in the fermentation at the increased aeration rate of 1 vvm. The lactic acid yield and volumetric productivity from each batch fermentation are shown in Figure 3.4b, along with the *OUR* and k_La values estimated from the dynamic gassing out experiments performed at the end of each batch. As can be seen in Figure 3.4b, the lactic acid yield increased steadily from 0.53 to 0.74 g/g, while the reactor productivity remained relatively unchanged at 1.6 ± 0.1 g/L·h. In the mean time, the oxygen transfer coefficient k_La remained almost constant at approximately 0.44 ± 0.03 min^{-1}, C_L generally increased from approximately 0.11 mM in the first batch to approximately 0.15 mM in the 9th batch, while *OUR* fluctuated from batch to batch around 0.01 mM/min. The increased product yield could have been due to the increased dissolved oxygen concentration in the medium. However, it should be noted that the oxygen transfer experiments had relatively large experimental errors due to the instability of the dissolved oxygen probe during the long study period.

The increasing lactic acid yield during the repeated batch fermentations also could have been partially due to the reduced cell growth. Although there was continued cell growth as evidenced by the increasing thickness of the immobilized fungal cells on the rotating fibrous matrix, cell growth reduced significantly in the successive batch fermentations. The thickness of the immobilized fungal cells on the rotating fibrous matrix increased from 0.5 cm to 1 cm during the first production batch, but only increased to approximately 2 cm at the end of the 9th batch. The reduced cell growth might have allowed more glucose for lactic acid production.

Since oxygen transfer is critical to lactic acid production, further study of the fermentation was conducted with the RFBB operated at a higher aeration rate of 2.0 vvm. The fermentation was also operated at repeated batch mode with partial medium replacement at the end of each production batch. In order to evaluate the maximum lactic acid concentration that can be produced by the fermentation, high-concentration glucose media were used in the last 3 batches. As shown in Figure 3.5a, except for the initial batch for cell growth, lactic acid production was stable during the 11-day period studied and there was no ethanol or other byproducts formed in the fermentation. The highest lactic acid concentration reached in the last batch fermentation was 137.3 g/L, which is the highest ever reported in the literature (Tay and Yang, 2002). Figure 3.5b shows the lactic acid yield and volumetric productivity from each of the 8 production batches, along with the estimated OUR and k_La values from the dynamic gassing out experiments. It is clear that with the increased aeration rate of 2 vvm, the dissolved oxygen concentration (C_L) in the bioreactor increased to approximately 0.15 mM due to improved oxygen transfer with a high $k_L\,a$ of 0.67 ± 0.02 min^{-1}. Consequently, the fermentation gave a consistently higher lactic acid yield of ~0.83 ± 0.06 g/g. The reactor productivity was also higher, reaching approximately 2.1 g/L· h in the last four batches even though the lactic acid concentration was high. *OUR* was significantly lower in the last two batches, perhaps due to inhibition by lactic acid and/or glucose at high concentrations.

3.3.4 Effects of aeration on fermentation

It should be noted that both lactic acid production rate and yield in the RFBB can be further improved by improving oxygen transfer. Unlike homolactic acid bacteria, *R. oryzae* requires oxygen for growth and lactic acid production. The dissolved oxygen concentration was found to be a critical factor affecting lactic acid production from glucose (Tay and Yang, 2002). A high dissolved oxygen concentration was desirable for lactic acid production. At low dissolved oxygen concentrations, the fermentation was not only slower but also produced less lactic acid with more ethanol as the byproduct due to increased activity of alcohol dehydrogenase (Skory et al., 1998). As can be seen in Table 3.3, the reactor volumetric productivity and lactic acid yield from glucose increased with increasing the aeration rate due to increased dissolved oxygen concentration C_L resulted from more efficient oxygen transfer as indicated by the higher k_La value in the reactor. Also, the RFBB had better oxygen transfer and lactic acid production than the free cell fermentation at the same aeration rate. High dissolved oxygen concentration was necessary for efficient oxygen diffusion into the relatively thick immobilized fungal cells in the RFBB or the large mycelial clumps seen in the stirred tank bioreactor.

As already discussed earlier, during the repeated batch fermentations the fungal cells continued to grow and the thickness of immobilized cells on the rotating fibrous matrix continued to increase to approximately 2 cm. However, the lactic acid production rate did not increase with increasing fungal cell biomass. Diffusion limitation and oxygen starvation must have occurred to the inner fungal cells that were away from the outer

surface of the immobilized cells, resulting in lower specific productivity for those cells. Diffusion limitation can be alleviated by increasing the dissolved oxygen concentration in the medium which can be achieved by either increasing the oxygen solubility in the medium or increasing the oxygen transfer rate. The former can be achieved by applying a higher air pressure or using pure oxygen to increase the partial pressure of oxygen in the air; the latter can be done by increasing agitation and aeration rates. Lactic acid yield could be increased to more than 0.9 g/g when the dissolved oxygen concentration was increased by using oxygen-enriched air (Tay and Yang, 2002).

It is well known that filamentous fungi could have different morphologies under different fermentation conditions, particularly due to differences in oxygen tension and mechanical force or shear (Cui et al., 1998a,b). The filamentous fungal morphology often causes difficulties in agitation and aeration because of its complicated effects on the rheological property of the fermentation broth and bioreactor hydrodynamics. This morphological problem has been alleviated by controlling fungal growth into small pellets (Zhou et al., 1999) or immobilization in porous materials by entrapment (Hang et al., 1989; Yin et al., 1998) or on solid surfaces by attachment. However, long-term performance of these fungal fermentation systems have not been well studied (Sun et al., 1998; Yin et al., 1998), and further improvements in lactic acid yield and production rate are needed in order to compete with the commonly used bacterial fermentation process. With the RFBB, *R. oryzae* gave relatively stable, high production rate and yield for an extended period. However, the reactor productivity would be limited by the available surface area for cell attachment and oxygen transfer by diffusion into the immobilized

fungal cells on the rotating fibrous matrix. Further studies to increase the surface area in the RFBB and to control the thickness of immobilized cells are needed before the reactor can be scaled up for commercial applications.

3.4 Conclusion

This study demonstrated the feasibility and advantage of using the RFBB to control the filamentous fungal morphology in lactic acid production by *R. oryzae*. The cotton cloth used in the RFBB provided a preferential matrix for immobilizing fungal spores and mycelia, resulting in a cell-free broth that was better for fermentation operation and control. The improved oxygen transfer in the RFBB not only increased the fermentation rate and lactic acid production, but also eliminated undesirable byproduct ethanol and allowed the bioreactor to be used for long-term production. Since the dissolved oxygen concentration is a critical factor affecting lactic acid production, methods to enhance oxygen transfer into the immobilized cells on the rotating fibrous matrix should be further investigated.

3.5 References

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	Lactobacillus sp.	*R. oryzae*
Substrates	can't use starch and pentoses	can use starch and pentoses
Medium	require complex growth nutrients	simple medium composition
Growth conditions	anaerobic, pH > 4.5	aerobic, pH > 3
Products	usually mixtures of L(+) and D(-)-lactic acids	pure L(+)-lactic acid, plus other byproducts (e.g., ethanol, fumarate, CO_2)
Product yield from glucose	0.85 - 0.95 g/g	usually less than ~0.85 g/g
Product concentration	up to 150 g/L	up to ~130 g/L
Productivity	can be as high as 60 g/L·h	usually lower than 6 g/L·h
Reactor operation	easy	difficult due to the filamentous cell morphology

Table 3.1 Comparison between bacterial and fungal lactic acid fermentations

		Free cell	RFBB
Product yield (g/g)	Lactate	0.597	0.686
	Ethanol	0.133	0.074
Productivity (g/L·h)	Lactate	0.82	1.58
	Ethanol	0.20	0.14
Total cell dry weight (g)		10.97	18.62
Oxygen uptake rate (mM/min)		0.0086	0.0100
C^* (mM)		0.17	0.17
C_L (mM)		0.09	0.13
$k_L a$ (min^{-1})		0.13	0.36

Note: fermentation conditions: 50 rpm, 0.5 vvm, 30°C and pH 6.

Table 3.2 Comparison of free cell fermentation in the conventional stirred tank bioreactor and immobilized cell fermentation in the RFBB

Morphology	Aeration rate (vvm)	k_La (min^{-1})	C_L (mM)	Yield (g/g)	Productivity (g/L·h)
Free cells	0.5	0.13	0.09	0.60	0.82
Immobilized cells in RFBB	0.5	0.36	0.13	0.69	1.58
	1.0	0.44±0.03	0.14±0.01	0.72±0.06	1.60±0.10
	2.0	0.67±0.02	0.15±0.01	0.83±0.06	2.14±0.05

Table 3.3 Effects of aeration on lactic acid fermentations by free cells and immobilized cells in the RFBB

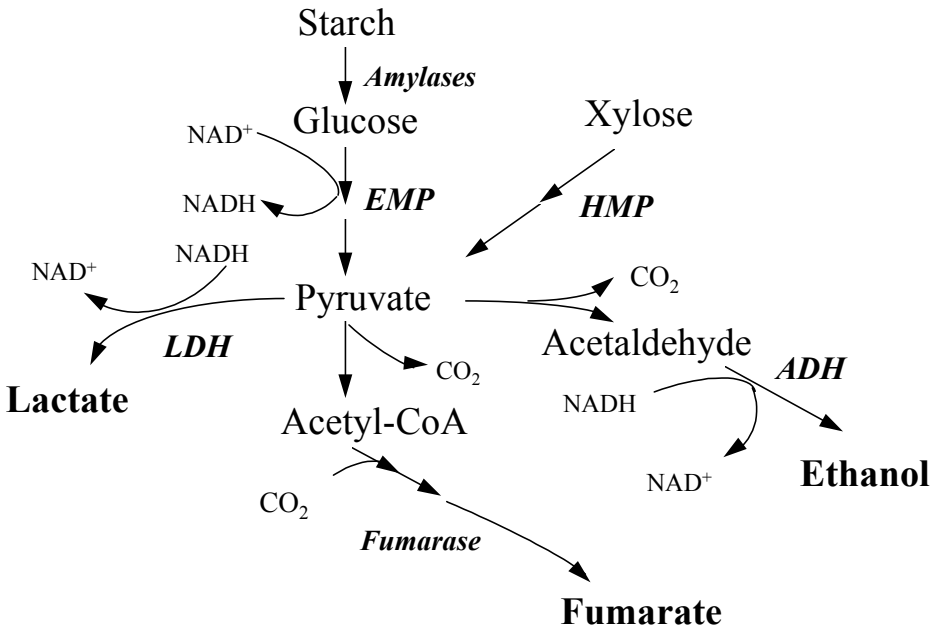

Figure 3.1 Pathways for production of lactic acid, fumaric acid, and ethanol in *R. oryzae* (LDH: lactate dehydrogenase; ADH: alcohol dehydrogenase)

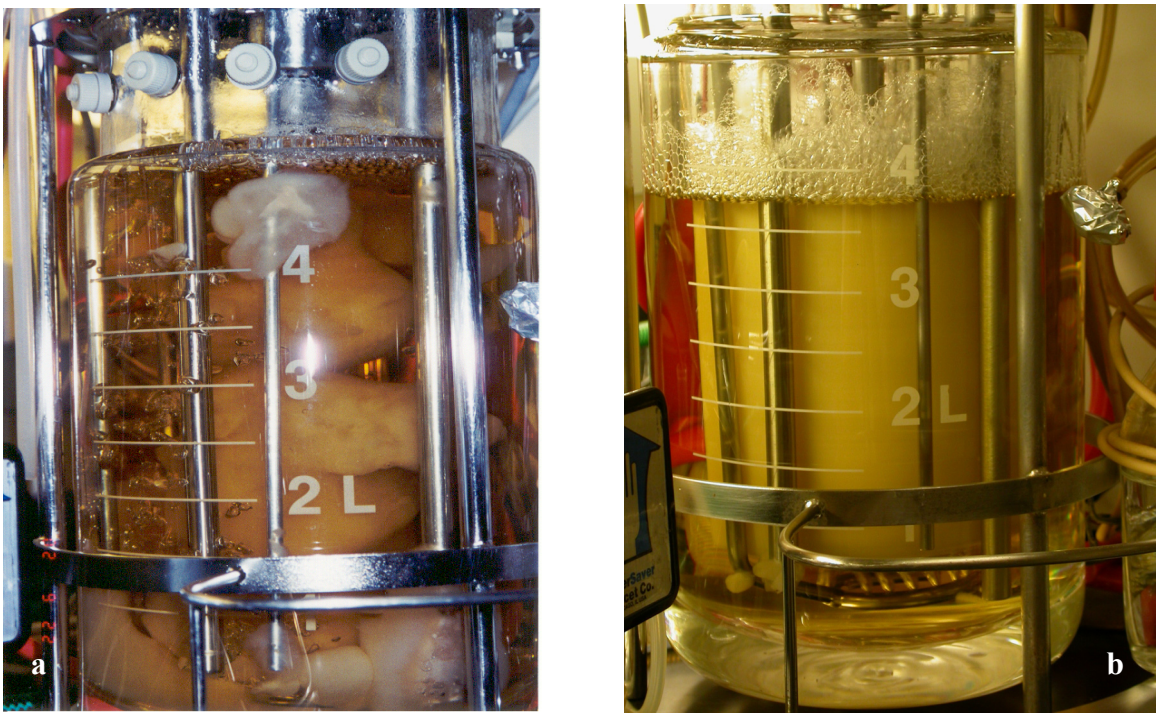

Figure 3.2 Fungal morphology seen in the bioreactor without cotton cloth, where fungal mycelia forming large clumps attached everywhere in the bioreactor (a), and in the RFBB, where fungal mycelia forming a sheet layer attached only on the cotton cloth in the bioreactor (b)

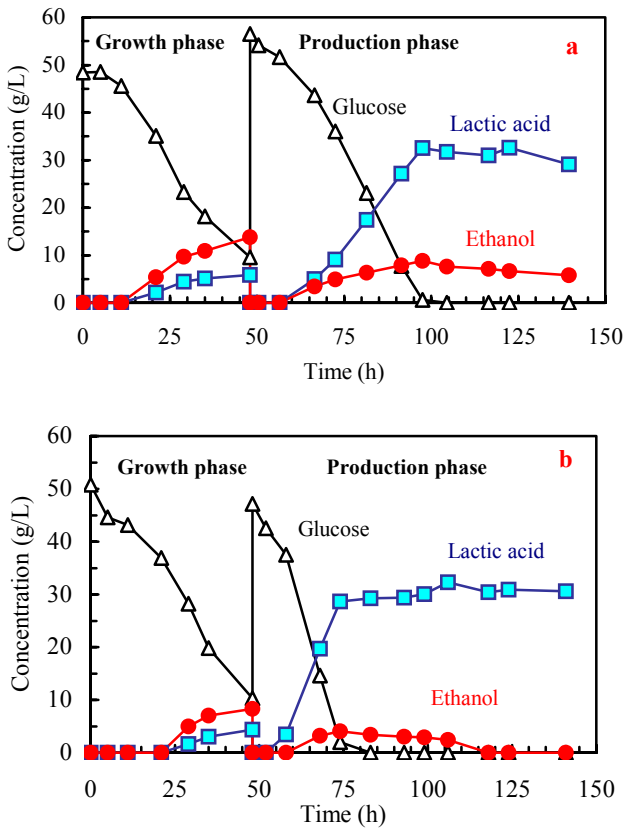

Figure 3.3 Fermentation kinetics during the growth phase and production phase by free cells in a stirred tank bioreactor (a) and by immobilized cells in the RFBB (b) at 50 rpm, 0.5 vvm, 30^{o}C, and pH 6.0

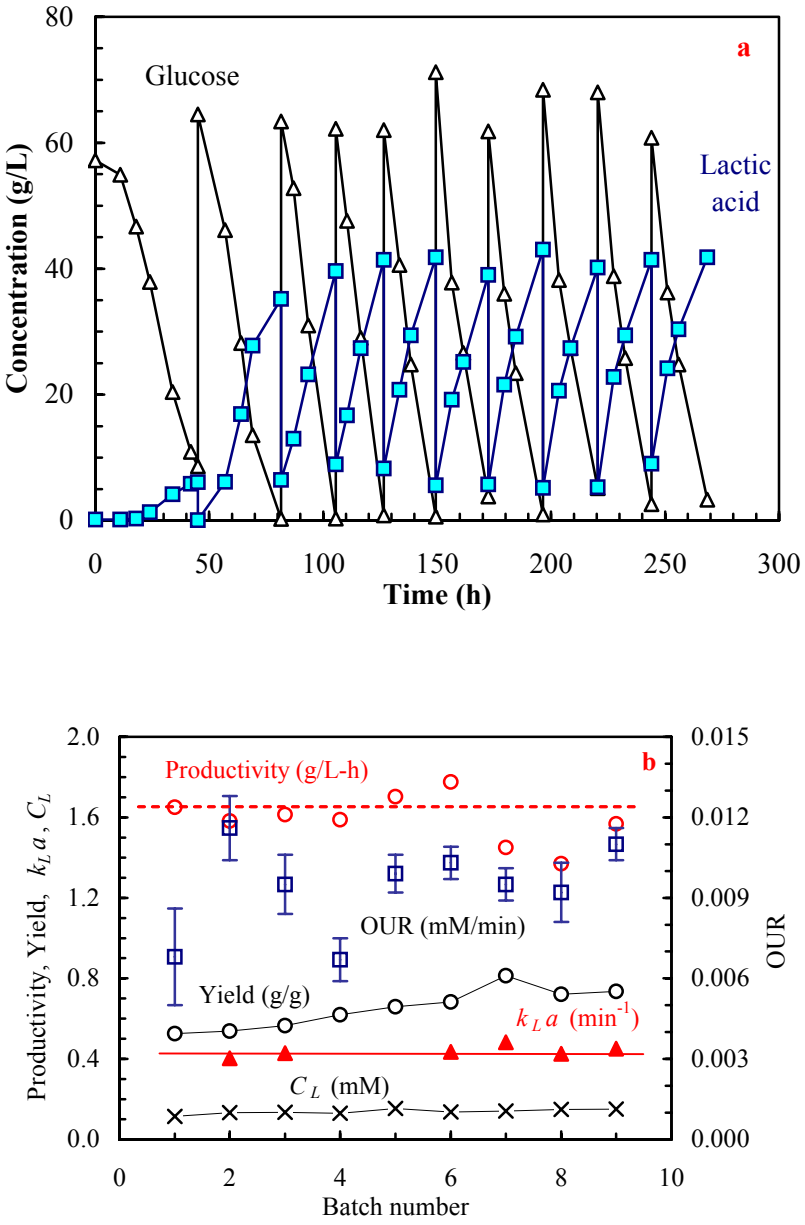

Figure 3.4 Kinetics of repeated batch fermentations in the RFBB at 50 rpm, 1.0 vvm, 30°C, and pH 6.0. The first batch was for cell growth in the growth medium, and all subsequent batches were conducted with the production medium; (a) glucose and lactate concentration profiles, (b) yield, productivity, and oxygen transfer data

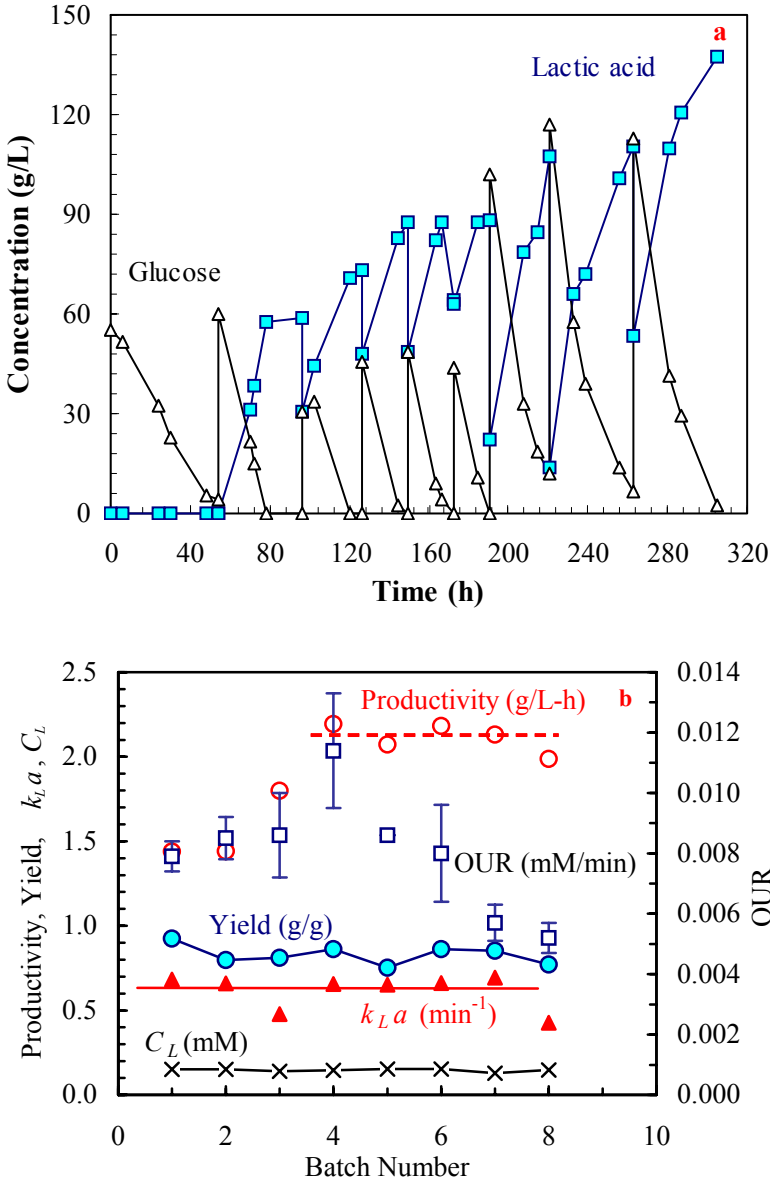

Figure 3.5 Kinetics of repeated batch fermentations in the RFBB at 50 rpm, 2.0 vvm, 30°C, and pH 6.0; the first batch was for cell growth in the growth medium, and all subsequent batches were conducted with the production medium; about half of the fermentation broth was replaced with fresh media containing high concentrations of glucose at the end of each batch; (a) glucose and lactate concentration profiles, (b) yield, productivity, and oxygen transfer data

CHAPTER 4

PREFERENCE OF CARBON ASSIMILATION IN LACTIC ACID FERMENTATION BY *RHIZOPUS ORYZAE* IN A ROTATING FIBROUS BED BIOREACTOR

Summary

Fermentation kinetics of lactic acid production from glucose, corn starch (both soluble and insoluble), xylose, and corn fiber hydrolysate (CFH) by *R. oryzae* NRRL 395 immobilized in a rotating fibrous bed bioreactor (RFBB) at pH 6.0 and 30°C were studied. Fermentations of glucose, corn starch (both soluble and insoluble), and xylose gave the similar lactic acid yield. However, the productivity of the fermentations of insoluble starch and xylose was lower than those of glucose and soluble starch. This indicated the slow saccharification process and poor mass transfer in the fermentation of insoluble starch. The low reactor productivity of the fermentation of xylose as the main carbon source was caused from the complicated xylose metabolism via the consecutive oxido-reductive, Pentose Phosphate (HMP), and Embden-Mayerhof-Parnas (EMP) pathways. The feasibility of using agricultural residues and plant biomass was also determined. In the fermentation of CFH, lactic acid production and reactor productivity

were low. The fermentation took longer time compared to those using other carbon sources and most CFH were utilized for biomass production. Long-term production of lactic acid from soluble starch showed the possibility of using the agricultural residues which usually contained plenty of starchy materials. In addition, the long-term study on mixed carbon fermentation (glucose/xylose) showed the preference of *R. oryzae* for glucose to produce lactic acid whereas xylose was preferably converted to biomass and fumaric acid.

4.1 Introduction

Lactic acid and its derivatives have long been widely used in food, pharmaceutical, and textile industries (Yun et al., 2003). Recently, there has been an increased interest in optically pure L(+)-lactic acid production by the fermentation route because of the increasing demand of polylactic acid in order to substitute for synthetic plastics such as polyethylene derived from the petroleum feedstocks (Datta et al., 1995; Huang et al., 2003; Skory, 2003; Yin et al., 1997; Zhu et al., 2002). Many bacteria including *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, *Clostridium*, and *Weissella*, have been reported to produce lactic acid. In the biological route, lactic acid is primarily produced via bacterial fermentation of simple sugars. Bacterial strains, such as *Lactobacillus* and *Lactococcus*, have been extensively used in the industrial production of lactic acid because of their high growth rate and product yield. However, the major limitations of bacterial fermentation include the high cost of substrate pretreatment and hydrolysis, supplementation of specific nutrients, pH control during the fermentation, and lactic acid recovery and purification (Huang et al., 2003; Skory, 2004). Therefore, bacterial fermentation of starchy materials consisting of the consecutive enzymatic hydrolysis and fermentation is economically unattractive. On the other hand, *R. oryzae* is amylolytic; and therefore, can produce the optically pure L(+)-lactic acid from starchy substrates without prior saccharification (Ruengruglikit and Hang, 2003; Tay and Yang, 2002; Yin et al., 1997).

However, it is well known that the cultivation of filamentous fungi in a typical stirred tank bioreactor is usually troublesome due to the diversity and change in morphology during fermentation. These problems in turn affect the production rate and bioreactor productivity (McCabe et al., 2001). To minimize the problems in dramatic change in hydrodynamic property and fungal morphology, immobilization of whole cells can provide the way for the entrapment of multi-step and cooperative enzyme system present in the intact cell, repetitive use, and improved stability (Elibol and Ozer, 2000; Nielsen, 1992; Singh et al., 1992; Tay and Yang, 2002).

It has been reported that a rotating fibrous bed bioreactor (RFBB) was successfully used to control fungal morphology in L(+)-lactic acid production by *R. oryzae*. The improved mixing and mass transfer, obtained from the perfectly immobilized fungal biomass on the provided surface and the cell free medium environment, resulted in the increase in lactic acid production rate compared to the fermentation using the stirred tank bioreactor (Thongchul and Yang, 2003).

As previously mentioned, one of the major barriers to lactic acid fermentation is the high substrate cost which is approximately 30-40% of the production cost (Huang et al., 2003). Unlike lactic acid bacteria, *R. oryzae* has the ability to secrete the enzymes to digest the complex carbohydrates such as the starchy products available in agricultural residues as well as hemicellulosic compounds which are rich in plant biomass. Therefore, agricultural residues and plant biomass provide the useful resources in lactic acid production by *R. oryzae* (Woiciechowski et al., 1999; Zhu et al., 2002). Most of the filamentous fungi including *R. oryzae* secrete amylases; therefore, the starchy products in

the agricultural residues can be directly converted to lactic acid without the prior hydrolysis. Plant biomass consists of 3 main components: cellulose, hemicellulose, and lignin. Cellulose is a linear β-D-glucan, associated with an amorphous matrix of lignin and hemicellulose. Hemicellulose is a heteropolysaccharide, containing neutral sugars, uronic acid, and acetyl groups (Woiciechowski et al., 1999). The hydrolysis of hemicellulose gives xylose, glucose, mannose, arabinose, galactose, and traces of other sugars depending on the type of plant biomass used. Therefore, besides the agricultural residues, plant biomass hydrolysate offers an attractive possibility to be used as the alternative substrate in lactic acid fermentation. In this study, lactic acid fermentation of different carbon sources was observed in the RFBB. The preference of *R. oryzae* in carbon assimilation process was determined to evaluate the feasibility of agricultural residues and plant biomass as the substrate to produce lactic acid in both single batch fermentation and long-term production.

4.2 Materials and Methods

4.2.1 Culture, inoculum preparation, and medium compositions

R. oryzae NRRL 395, a filamentous fungus producing L(+)-lactic acid obtained from the Northern Regional Research Center, Peroria, IL, was used in this study. The method to maintain fungal viability is described in Appendix A1. The spore suspension made from the 7-day culture on the potato dextrose agar plate was adjusted to 10^6/mL by

dilution with sterile water. 10^6/mL *R. oryzae* spore suspension (10 mL) was used to inoculate the bioreactor (Appendix A2).

The substrates used in this study included glucose, xylose, different forms of corn starch (soluble and insoluble), and corn fiber hydrolysate (CFH). The other compositions required in each medium are shown in Appendix A3.

4.2.2 Bioreactor setup

A Rotating Fibrous Bed bioreactor (RFBB) was modified from a 5-L bioreactor (Biostat B, B. Braun) by affixing a perforated stainless steel cylinder mounted with a 100% cotton cloth to the agitation shaft. The bioreactor setup is described in Appendix B. The cylindrical matrix of 9 cm diameter and 15 cm height was used in this study. After sterilization, the bioreactor was set up the controlled parameters and inoculated with 10 mL spore suspension (10^6/mL). Unless otherwise mentioned, the bioreactor was controlled at 30°C and pH 6.0 (during the production phase), agitated at 50 rpm, and aerated with filter-sterilized air at 1.0 vvm.

4.2.3 Lactic acid fermentation

To study the fermentation kinetics, first the RFBB containing the growth medium was inoculated and the growth phase took approximately 48 hours for spore germination and cell immobilization on the fibrous matrix. After 48 hours, the growth medium was

replaced with the production medium. The fermentation batch finished when the substrate was depleted or lactic acid concentration was not increasing.

To evaluate the potential of *R. oryzae* to produce lactic acid from agricultural residues and plant biomass, the fermentations of different substrates, i.e., glucose, xylose, the different forms of corn starch, and CFH were observed in the RFBB. The fermentation kinetics, lactic acid yield, and reactor productivity were compared.

To study the long-term stability of the RFBB in lactic acid fermentation from different substrates, repeated batch and semi fed-batch fermentations were conducted by replacing the medium at the end of each batch when the substrate was almost depleted and lactic acid production stopped.

4.2.4 Acid hydrolysis of corn fiber

CFH was prepared by hydrolyzing dried corn fiber with 0.25 M HCl at 121°C for 45 min. Insoluble materials were then removed by filtration and the remaining hydrolysate was stored at 4°C until used. The composition of the CFH was analyzed with high performance liquid chromatography (HPLC) (Zhu et al., 2002).

4.2.5 Oxygen transfer determination

Oxygen is one of the important factors in filamentous fungal fermentation. It involves in fungal growth and metabolism. Insufficient oxygen transfer can limit lactic

acid production and eventually cause cell death (Tay and Yang, 2002; Thongchul and Yang, 2003). Before the end of each fermentation batch, the oxygen uptake rate (OUR) and the dynamics of oxygen transfer in the bioreactor were studied using the dynamic method of gassing out (Appendix C).

4.2.6 Analytical methods

4.2.6.1 Cell biomass

Growth of *R. oryzae* in the RFBB was determined at the end of fermentation. The detail is described in Appendix D1.

4.2.6.2 Substrate and product concentration

The fermentation sample was centrifuged to remove the suspended solid. The supernatant was analyzed for glucose and L(+)-lactic acid using a glucose/lactate analyzer (YSI 2700). HPLC was also used to analyze the organic compounds (glucose, xylose, arabinose, lactic acid, fumaric acid, and ethanol) present in the supernatant. The HPLC system (Shimadzu Scientific Instruments) was equipped with a RID-10A reflective index detector and an organic analysis column (Aminex HPX-87H, Biorad). The details are given in Appendix D2 and D3.

To determine the starch concentration, the fermentation sample was first hydrolyzed with 37%w/v HCl (2 mL sample and 0.2 mL 37%w/v HCl, autoclaved at 121°C, 15 psig, 10 minutes). Then the hydrolyzed sample was adjusted to neutral pH, centrifuged, and diluted before measuring starch concentration as glucose equivalent by YSI or HPLC.

4.2.6.3 Enzyme assay

When starch was used as the substrate, the enzymatic starch hydrolysis by *R. oryzae* was determined by measuring the activity of α-amylase and glucoamylase. To measure α-amylase activity, the reaction mixture containing 0.125 mL cell-free fermentation sample and 0.125 mL 1% starch in 0.1 M sodium acetate (pH 6.0) was reacted at 40°C for 30 minutes. 0.5 mL 3,5-dinitrosalicylic acid (DNS) reagent was added to stop the reaction and the reaction mixture was incubated at 100 °C in water bath for 10 minutes (DNS reagent reacts with the reducing sugars released from starch break down by α-amylase). The control was prepared from the reaction mixture of DNS reagent with the sample with deactivated enzyme or with sample added after reaction. The reducing sugar was determined by comparing the absorbency at 540 nm (200 μL sample in microplate reader, SpectraMax 250) of the reaction mixture to a standard curve of glucose solution (1 mg/mL-10 mg/mL). The released reducing sugar was defined as the reducing sugar present in the sample subtracted with the control at the particular time.

One unit of α-amylase activity was defined as the release of 1 μmol reducing sugar from the soluble starch per minute.

The DNS reagent was prepared by dissolving 10 g 3,5-dinitrosalicylic acid in 200 mL 2 N NaOH while gradually adding 500 mL water over gentle heat. Then, 300 g potassium sodium tartrate was added and the water was added to mixture to adjust the total volume to one liter. The solution was stored at room temperature in a dark colored container.

Glucoamylase was determined using the assay described by Holm, 1986. The reaction mixture containing 125 μL sample and 250 μL PNPG solution (0.1%w/v p-nitrophenyl-α-D-glycopyranoside in 0.1 M sodium acetate buffer, pH 5.0, equilibrated at 30°C) was incubated at 37°C for an hour. The reaction was terminated by the addition of 375 μL 0.1 M borax, and the absorbency at 400 nm (200 μL sample) was measured using the spectrophotometer (microplate reader, SpectraMax 250). The concentration of glucoamylase was determined from the absorbency, after subtracting the background absorbency obtained from the same reaction mixture but the reaction was stopped prior to the addition of PNPG solution, and compared with the standard curve prepared from glucoamylase standard (0 mg/L-200 mg/L).

4.3 Results and Discussion

4.3.1 Fermentation of glucose and corn starch

Batch fermentations of glucose and corn starch (both soluble and insoluble forms) as the major carbon source by *R. oryzae* were studied. The fermentation batch consisted of growth and production phases. In the growth phase, yeast extract, the organic nitrogen source, was required for spore germination and growth initiation. After 48 h, the immobilized fungal biofilm in the RFBB was washed thoroughly with sterile water to remove the excess yeast extract and the growth medium was replaced by the production medium. To increase the carbon flux to lactic acid production in the production phase, the continuous fungal growth was minimized by using the inorganic nitrogen source (urea, in this study). As shown in Figure 4.1 to 4.3, both glucose and corn starch were readily fermented to produce lactic acid. Lactic acid concentration increased rapidly between approximately 53 h and 77 h in the fermentations of glucose and soluble starch whereas in the fermentation of insoluble starch, lactic acid concentration increased slower and the fermentation time took longer. At the end of the production phase, the fermentation reached a maximum lactic acid concentration of 32.2 g/L from 51.2 g/L glucose, 38.0 g/L from 61.0 g/L soluble starch, and 21.8 g/L from 38.3 g/L insoluble starch. It was found that fermentations of glucose and both soluble and insoluble starches gave the similar lactic acid yield (0.60-0.65 g/g). However, the reactor productivity was higher with soluble starch (1.37 g/L·h) compared to those with glucose (1.18 g/L·h) and insoluble

starch (0.30 g/L·h). In addition, more ethanol production was obtained along with more biomass growth (0.22 g/g) compared to the fermentations of glucose (0.09 g/g) and insoluble starch (0.10 g/g) (Table 4.1).

4.3.2 Fermentation of xylose

The feasibility of using pentose sugars as substrates for lactic acid fermentation was studied. The result in Figure 4.4 indicated that *R. oryzae* could utilize xylose to produce lactic acid at a little lower yield (0.59 g/g) compared to the fermentation of glucose. However, the reactor productivity was much lower (0.09 g/L·h). At the beginning of the production phase, the lag phase took approximately more than 24 h before lactic acid was produced while in the fermentations of glucose and corn starch, the lag phase was approximately 4-6 h. Yin et al., 1998 also reported the long cultivation time when xylose was used as the sole carbon source. In the production phase, the highest lactic acid concentration of 20.0 g/L was obtained from 50.4 g/L xylose after 400 h of the fermentation (Figure 4.4). Later, lactic acid production declined while xylose consumption was still continued along with the accumulation of fumaric acid in the fermentation. No ethanol production appeared in the fermentation of xylose as the sole carbon source. The highest biomass yield (0.30 g/g) was obtained from the fermentation of xylose compared to other carbon sources used in this study (Table 4.1).

4.3.3 Fermentation of CFH

To investigate the economical use of plant biomass for industrial fermentation, the feasibility of using CFH as the substrate was also studied. The HPLC analysis showed that the acid hydrolysate of corn fiber contained a mixture of fermentable sugars including glucose (27.4%), arabinose (11.9%), and xylose (22.2%). It also included some acetate (2.7%) and lactate (0.5%) (Zhu et al., 2002). In the fermentation of CFH, *R. oryzae* consumed glucose and xylose simultaneously at the beginning of the growth phase (less than 80 h) while arabinose was rarely metabolized (Figure 4.5). Later, glucose was rapidly metabolized until depletion then started to accumulate whereas xylose was slowly consumed which indicated the slightly preference of *R. oryzae* for glucose. The increase in glucose concentration while xylose concentration decreased indicated the metabolic pathway shift from pentose sugars to the favorable form of hexose sugars which provide the higher energy production for growth and metabolism (Stryer, 1995). Ethanol production was observed in both growth and production phases. A small amount of fumaric acid was also found. The high biomass production was obtained (0.29 g/g) whereas lactic acid yield (0.02 g/g) and reactor productivity (0.02 g/L·h) were low (Table 4.1).

4.3.4 Long-term fermentation of corn starch and glucose/xylose mixture

Agricultural residues and plant biomass contain plenty of starchy products and pentose sugars. Due to the large increase in the residues from agricultural industry and plant biomass supply in the world market, it is necessary to determine the method to convert the starchy materials and pentose sugars to the valuable products (Aristidou and Penttila, 2000; Dale, 1999; Gallagher et al., 2003; Lynd et al., 1999; Prathumpai et al., 2003; Wooley et al., 1999). In this study, the repeated batch fermentation of soluble starch was conducted to determine the reactor stability for a long-term production of lactic acid. As shown in Figure 4.6, except for the initial growth phase, all subsequent 6 batches gave the consistent lactic acid production during the 500-h period studied (approximately 50-69 g/L for each batch). A small amount of fumaric acid started to accumulate after 2 production batches. The higher ethanol production was observed in the growth phase compared to that in the production phase. After the 4^{th} production batch, spore formation was clearly found at the surface of the immobilized fungal biofilm indicating that *R. oryzae* entered the dormant state due to unfavorable chemical and/or physical conditions. This caused no synthesis of new cellular materials and much lower metabolic activity (Nielsen, 1992). The cumulative lactic acid yield of 0.65 g/g was obtained. The reactor productivity gave the highest value at the first production batch (1.36 g/L·h) whereas at the rest of the production batches, the reactor productivity became lower (0.73-1.02 g/L·h). At the end of fermentation, the total accumulated biomass was 62.03 g (Table 4.2).

The fed-batch fermentation of glucose/xylose mixture was studied to determine the preference of *R. oryzae* for the assimilation of hexose and pentose sugars in the long-term lactic acid production. It was clear that the biomass growth was very slow when xylose was used as the sole carbon source; therefore, glucose was fed with the yeast extract in the growth phase to enhance spore germination and initial cell growth (Figure 4.7). Later, in the production phase, both glucose and xylose were used in the fermentation. The fermentation was carried out for 12 production batches over 1000 h. It was clear that *R. oryzae* could readily consume glucose whereas it metabolized xylose very slowly. In the 1st production batch, both glucose and xylose (1:1) were fed into the fermentation but only glucose was rapidly metabolized and became depleted. In the 2nd and 3rd batches, high concentration of glucose was fed. In these batches, glucose was still rapidly consumed whereas xylose was slowly metabolized. The increase in lactic acid at the end of each batch was found from the 1st batch to the 3rd batch. At the end of the 3rd batch, the maximum lactic acid concentration of 139.33 g/L was obtained.

Both glucose and xylose (1:1) were fed into the 4th batch. Later, in the 5th and 6th batches, only glucose was fed. Similar to the fermentation in the first 3 batches, glucose was still rapidly consumed; however, xylose metabolism became even slower than the previous batches (Figure 4.7). Besides, lactic acid production was decreased as indicated from the slightly decrease in final concentration at the end of each batch. At the 7th batch, both glucose and xylose (1:1) was fed and later only glucose was fed in the 8th, 9th, and 10th batches. During these batches, lactic acid production declined and fumaric acid production increased. Later, in the 11th and 12th batches, it was found that *R. oryzae*

stopped producing the metabolites (lactic acid and fumaric acid). This was probably because *R. oryzae* lost its metabolic activity and entered the dormant state. At the end of the fermentation, the total amount of the accumulated biomass was 145.75 g. The total lactic acid cumulative yield was 0.43 g lactic acid/g sugars and the average reactor productivity was 0.52 g/L·h.

Further cell growth was observed in the fermentation after the growth phase. From Figure 4.7b, during the first 4 batches, lactic acid yield and reactor productivity were higher than the rest of the production batches. This was probably because during this period, lactic acid production was by the fungal cells produced from glucose in the growth phase and they were maintained by the addition of glucose during the production phase. Fungal cells produced from glucose might have the high metabolic activity; therefore, they consumed sugars and produced lactic acid at the high production rate. Later, fungal growth was produced by glucose/xylose mixture. This new growth was accumulated on the previous fungal biofilm produced from glucose; thus embedded the fungal cells which had high metabolic activity. It was claimed that growth from xylose showed the lower metabolic activity compared to that from glucose which generated more NADH involving in the oxidation of pyruvic acid to lactic acid. Therefore, lactic acid and productivity dropped in the later production batches (Pritchard, 1973).

4.3.5 Oxygen transfer in the fermentations of different carbon sources

From Table 4.1, the oxygen transfer from the gas phase to the fermentation broth was similar in the fermentation of glucose, soluble starch, and xylose (k_La ~ 0.44-0.49 min^{-1}). However, k_La in the fermentation of insoluble starch was only 0.37 min^{-1} because insoluble starch was not readily soluble in the fermentation broth; therefore, it formed the suspension which in turn increased the broth viscosity and resulted in poor mass transfer.

Oxygen uptake rate in the RFBB was related to the diffusion of oxygen into the fungal biofilm immobilized on the fibrous matrix. It was found that the oxygen uptake rate in the fermentation of soluble starch was the lower than those in the fermentations of glucose and insoluble starch. This result was consistent with the production of ethanol in both growth and production phases, which indicated that the diffusion limitation in the fermentation of soluble starch was severe, compared to those of other carbon sources (Skory, 2003).

The oxygen uptake rate in the fermentation of xylose was less than that in the fermentation of glucose and insoluble starch. This was probably due to the slow metabolic activity in the fermentation of xylose.

4.3.6 Effect of carbon source

Besides starch, xylose is the most abundant substrate in plant biomass. However, the bioconversion of xylose to chemicals has been limited by the preference of

microorganisms for glucose as the carbon and energy sources (El Kanouni et al., 1998). *R. oryzae* showed the preference for glucose in lactic acid fermentation. In the fermentation of glucose/xylose mixture, glucose was readily consumed whereas xylose was slowly metabolized. This was probably because glucose and xylose were metabolized via different metabolic routes. *R. oryzae* converts glucose to pyruvic acid by the Embden-Meyerhof-Parnas (EMP) pathway (Figure 4.8). Pyruvic acid is then converted to lactic acid, fumaric acid, ethanol, cell biomass, and energy. On the other hand, the first step of xylose catabolism by *R. oryzae* is its conversion to xylulose via the oxido-reductive pathway. Xylose is first reduced to xylitol in the presence of NAD(P)-linked xylose reductase which is then reoxidized by NAD(P)-linked xylitol dehydrogenase to give xylulose. Later, the steps after the formation of xylulose phosphate appear to use a combination of Pentose Phosphate (HMP) and EMP pathways to the key intermediate pyruvic acid whereas only EMP pathway is used in glucose catabolism (Figure 4.8). Finally, 3 moles xylose are converted into 5 moles pyruvic acid with the net synthesis of 5 moles ATP and 5 moles reduced pyridine nucleotide but 1 mole glucose is converted via the EMP pathway into 2 moles pyruvic acid, 2 moles ATP, and 2 moles NADH. Figure 4.8 clearly shows that the metabolic pathways in xylose (pentose) conversion are more complicated than the glucose (hexose) conversion. This may be the plausible explanation for the slow conversion rate of xylose compared with glucose (Nielsen, 1992; Singh et al., 1992; Stryer, 1995).

Unlike in bacterial fermentation which usually cannot directly use starch as the substrate, *R. oryzae* is amylolytic; therefore, it can readily metabolize starch to chemicals.

Compared to insoluble starch which has a larger molecule, soluble starch has a smaller molecule; thus, in the same initial concentration used in the fermentation, soluble starch contains more non-reducing ends, the sites for amylase binding. This results in the higher enzymatic hydrolysis rate which in turn provides the higher glucose production rate for the catabolism process. Moreover, soluble starch is soluble in the fermentation medium; therefore, *R. oryzae* can readily digest the starch molecule dissolved in the fermentation broth. According to the results of the enzyme expression level, it shows the similar expression level in both fermentations; however, the expression was slightly slower in the fermentation of insoluble starch due to the low availability of non-reducing ends to induce amylase secretion at the beginning of the fermentation (Figure 4.9). Therefore, the fermentation of soluble starch gave the higher reactor productivity compared to that of insoluble starch.

Compared to the fermentation of glucose/xylose mixture, which showed reasonably higher lactic acid yield and reactor productivity, CFH, containing mainly glucose, xylose, and arabinose, was rarely metabolized to lactic acid and the reactor productivity was very low. This is probably because during the hydrolysis of corn fiber, some toxic compounds such as furan derivatives (2-furaldehyde and 5-hydroxymethyl-2-furaldehyde) and organic acids (formic acid, acetic acid, levulinic acid) are also formed and they may cause the inhibitory effects on the fermentation by *R. oryzae* (Woiciechowski et al., 1999). Unlike the butyric acid fermentation from the direct acid hydrolysis of corn fiber by *Clostridium tyrobutyricum* reported by Zhu et al., 2002, in the fermentation of CHF by *R. oryzae*, it is necessary to minimize the inhibitory effects using

various pretreatments such as steam stripping, neutralization of the hydrolysate with alkali or activated charcoal, ion exclusion chromatography, solvent extraction, enzymatic detoxification and molecular sieving.

Fumaric acid production was found in all of the fermentations using xylose as the substrate. Fumaric acid is a product of the mitochondrial oxidation of pyruvate, but this type of high accumulation in *R. oryzae* is more likely a product of cytosolic enzymes. This cytosolic pathway also competes for available pyruvate to form oxaloacetic acid (OAA) with the enzyme pyruvate carboxylase. Malate dehydrogenase then catalyzes OAA to malic acid and eventually to fumaric acid by a cytosolic fumarase (Skory, 2004). It is claimed that pentose sugars including xylose are preferably metabolized via the HMP pathway to obtain the nucleotides and amino acid building blocks for cell biomass synthesis. OAA plays the central role in the construction of these building blocks. However, besides involving in the metabolism to the nucleotides and the amino acids, the excess OAA can be converted to fumaric acid (Figure 4.8). Therefore, the fermentation of xylose-based substrate resulted in fumaric acid production as the by product (Nielsen, 1992; Singh et al., 1992; Stryer, 1995).

4.4 Conclusion

The carbon sources influencing lactic acid fermentation by *R. oryzae* were studied. Besides glucose, soluble corn starch was readily metabolized by *R. oryzae* to produce lactic acid at the high reactor productivity. Fermentation of insoluble starch gave

the low reactor productivity due to the slow starch hydrolysis rate and poor mass transfer (Bai et al., 2003). *R. oryzae* used the oxido-reductive pathway to metabolize xylose followed by the combination of HMP and EMP pathways to obtain lactic acid. Due to the complicated metabolic routes, the fermentation of xylose-based substrate gave low reactor productivity. In the fermentation of CFH, *R. oryzae* rarely metabolized CFH to lactic acid indicating that CFH might contain some inhibitory compounds; therefore, further treatments of CFH after acid hydrolysis was required for lactic acid fermentation.

The long-term fermentation of soluble starch showed the stable performance of the RFBB in lactic acid fermentation by *R. oryzae*. For the fed-batch fermentation of glucose/xylose mixture, the optimal feeding mode is required to maintain cellular activity and enhance lactic acid production.

4.5 References

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Parameters	Carbon sources				
	Glucose	Soluble starch	Insoluble starch	Xylose	CFH
Lactate					
Production yield (g/g)	0.65±0.10	0.63±0.08	0.60±0.01	0.59±0.02	0.02
Productivity (g/L·h)	1.18±0.31	1.37±0.01	0.30±0.12	0.09±0.03	0.02
Ethanol production	No	Both in growth and production phase	In production phase	No	In production phase
Fumarate production	No	No	No	In production phase	Both in growth and production phase
Biomass					
Total mass (g)	15.89±3.53	24.56±4.06	21.82±6.62	26.94±5.81	54.26
Overall yield (g/g)	0.09±0.05	0.22±0.02	0.10±0.03	0.30±0.05	0.29
OUR (mM/min)	0.0088±0.0011	0.0078±0.0006	0.0089±0.0022	0.0076±0.0013	n/a
k_La (min^{-1})	0.44±0.02	0.44±0.02	0.37±0.11	0.46±0.04	n/a

Table 4.1 Effect of carbon sources on lactic acid production by *R. oryzae*

Carbon source	Soluble starch	Glucose/xylose mixture
Lactate yield (g/g)		
Cumulative	0.65	0.43
Average	0.69±0.11	0.42±0.28
Lactate productivity (g/L·h)	1.00±0.21	0.52±0.44
Total biomass production (g)	62.03	145.75

Table 4.2 Long-term lactic acid fermentations of soluble corn starch and glucose/xylose mixture in the RFBB at 50 rpm, 2 vvm air, and 30°C

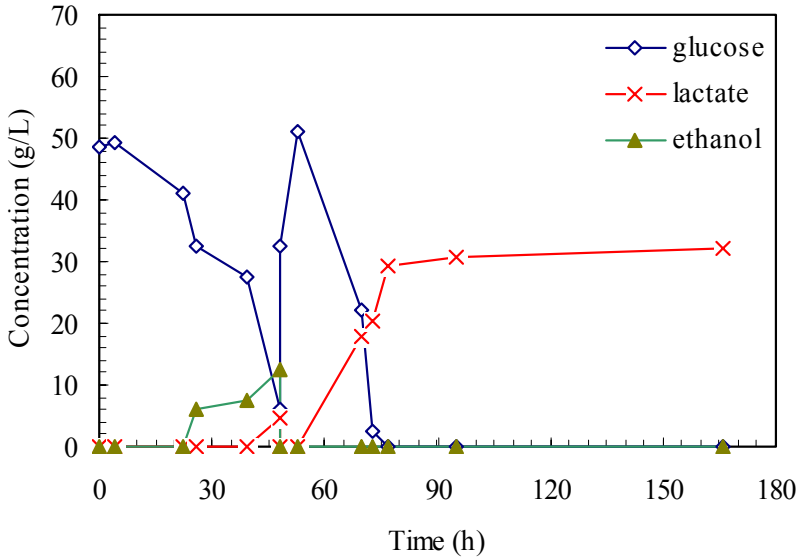


Figure 4.1 Fermentation kinetics of lactic acid production from glucose by *R. oryzae*

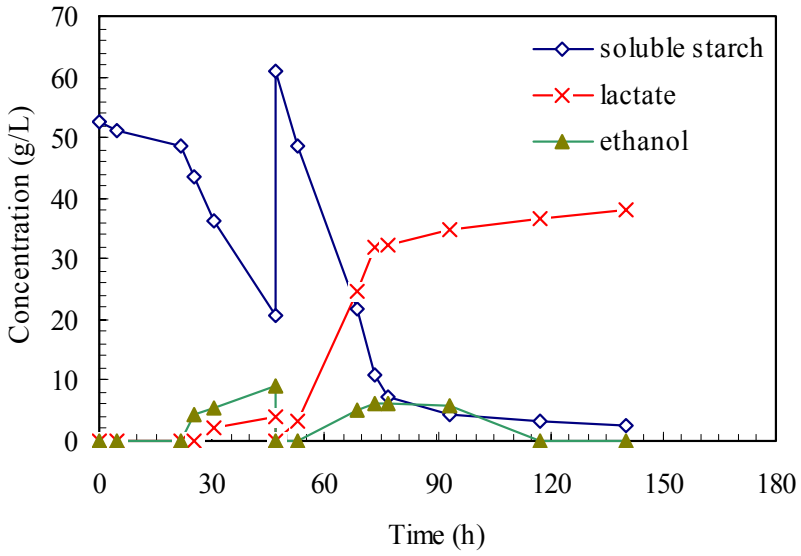


Figure 4.2 Fermentation kinetics of lactic acid production from soluble starch by *R. oryzae*

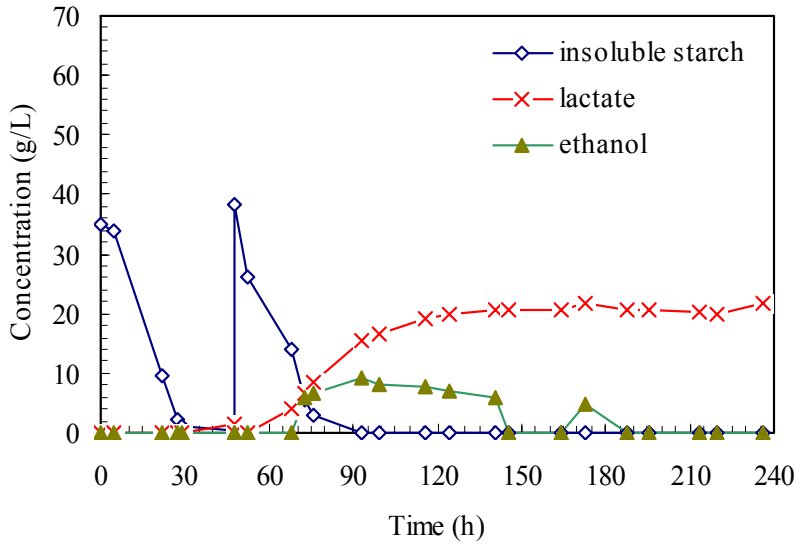


Figure 4.3 Fermentation kinetics of lactic acid production from insoluble starch by *R. oryzae*

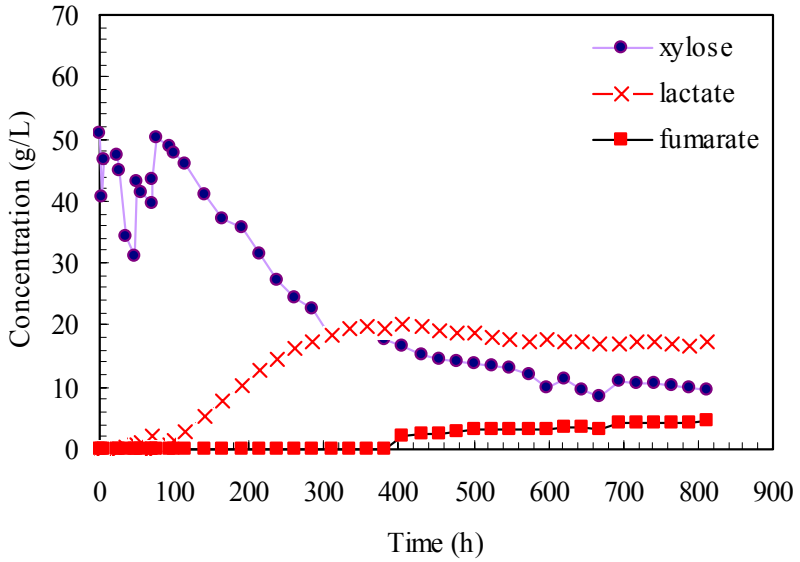


Figure 4.4 Fermentation kinetics of lactic acid production from xylose by *R. oryzae*

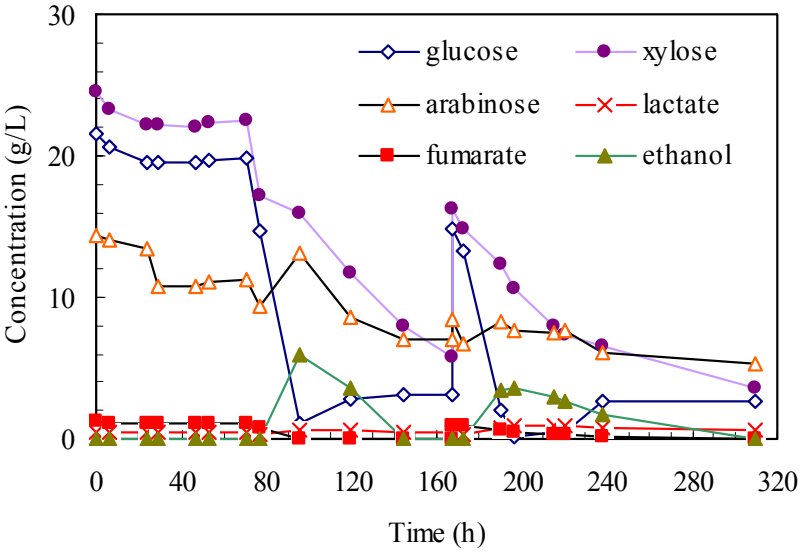


Figure 4.5 Fermentation kinetics of lactic acid production from CFH by *R. oryzae*

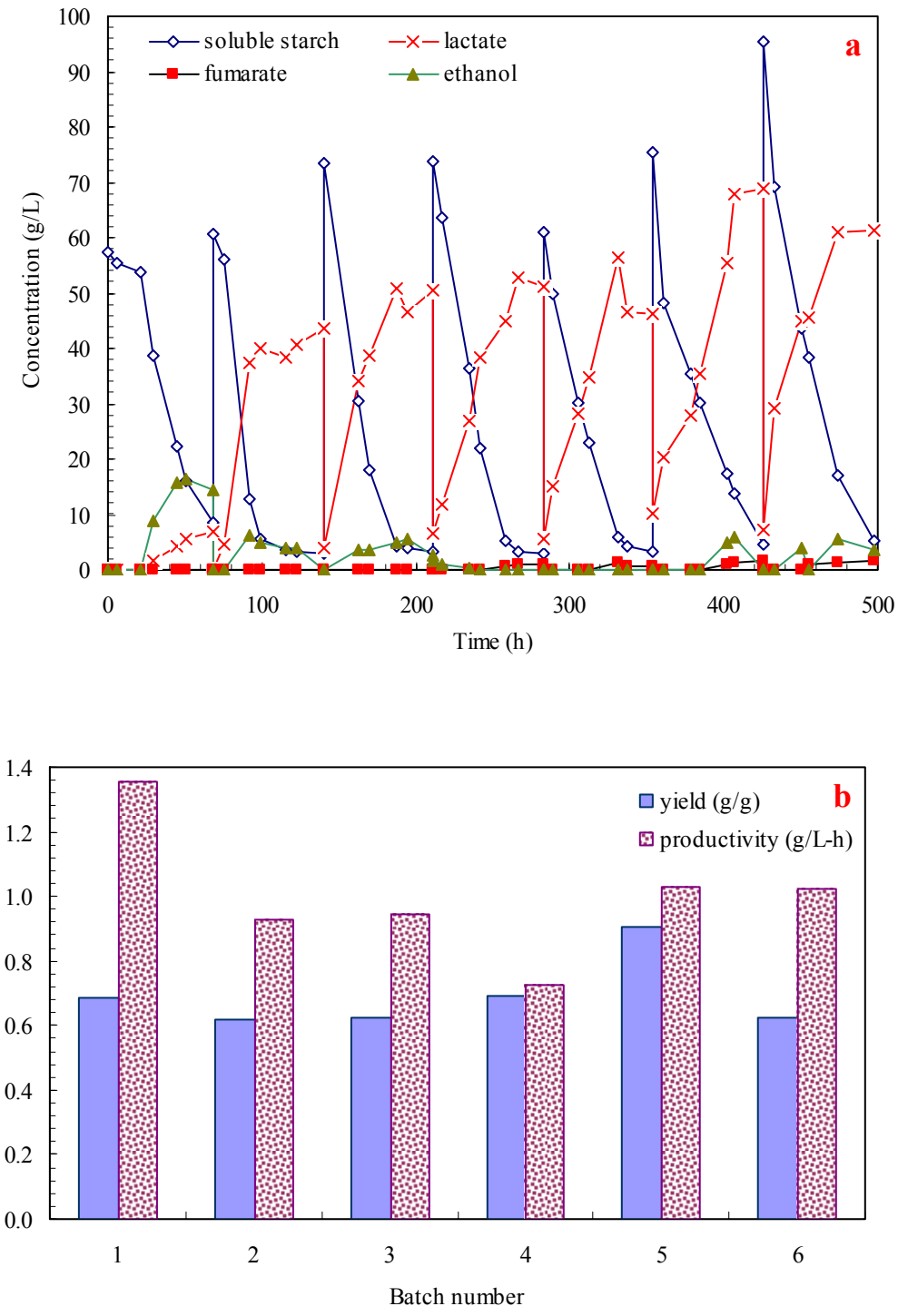
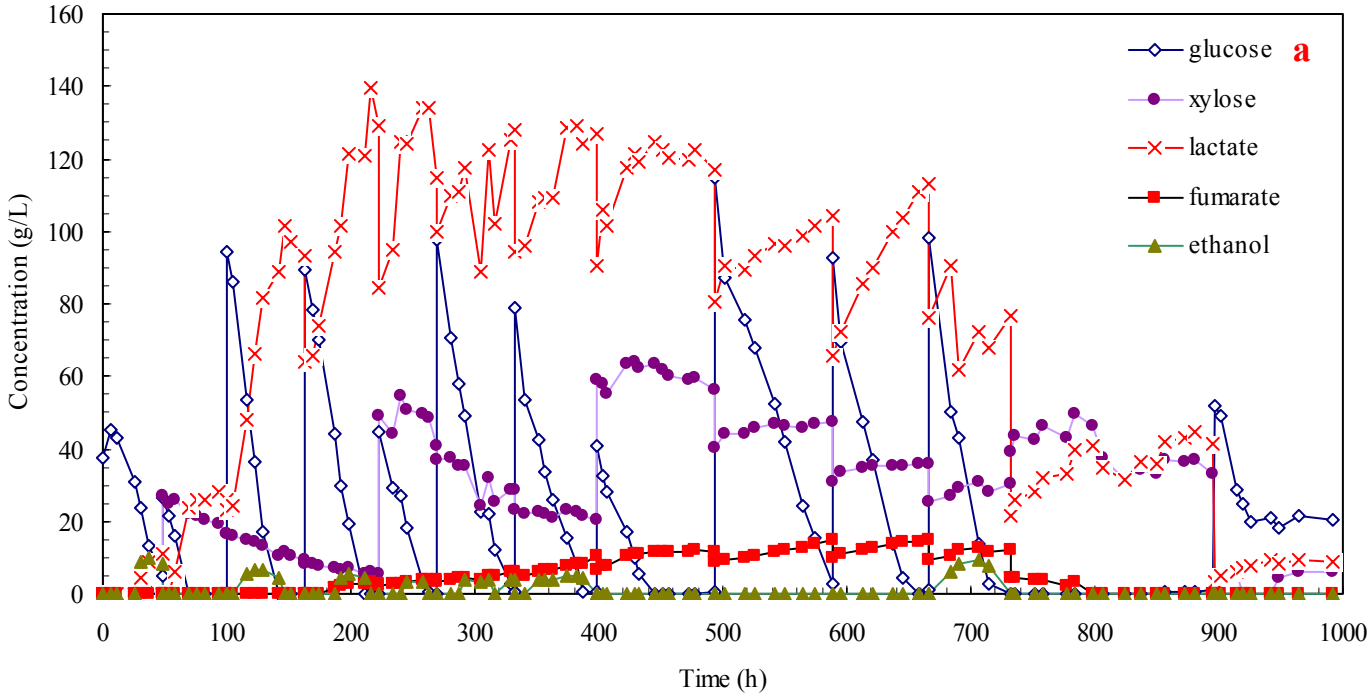


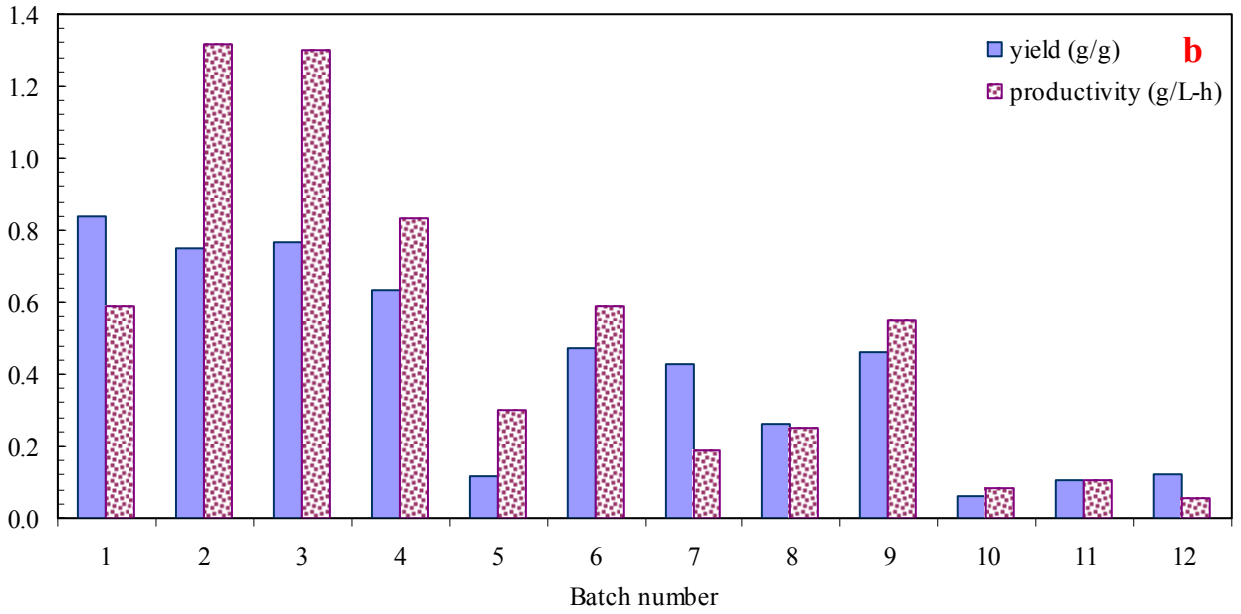
Figure 4.6 Kinetics of repeated batch fermentation of soluble corn starch, (a) fermentation profiles, (b) lactate yield and reactor productivity



Continued

Figure 4.7 Kinetics of fed batch fermentation of glucose/xylose mixture, (a) fermentation profiles, (b) lactate yield and reactor productivity

Figure 4.7 continued



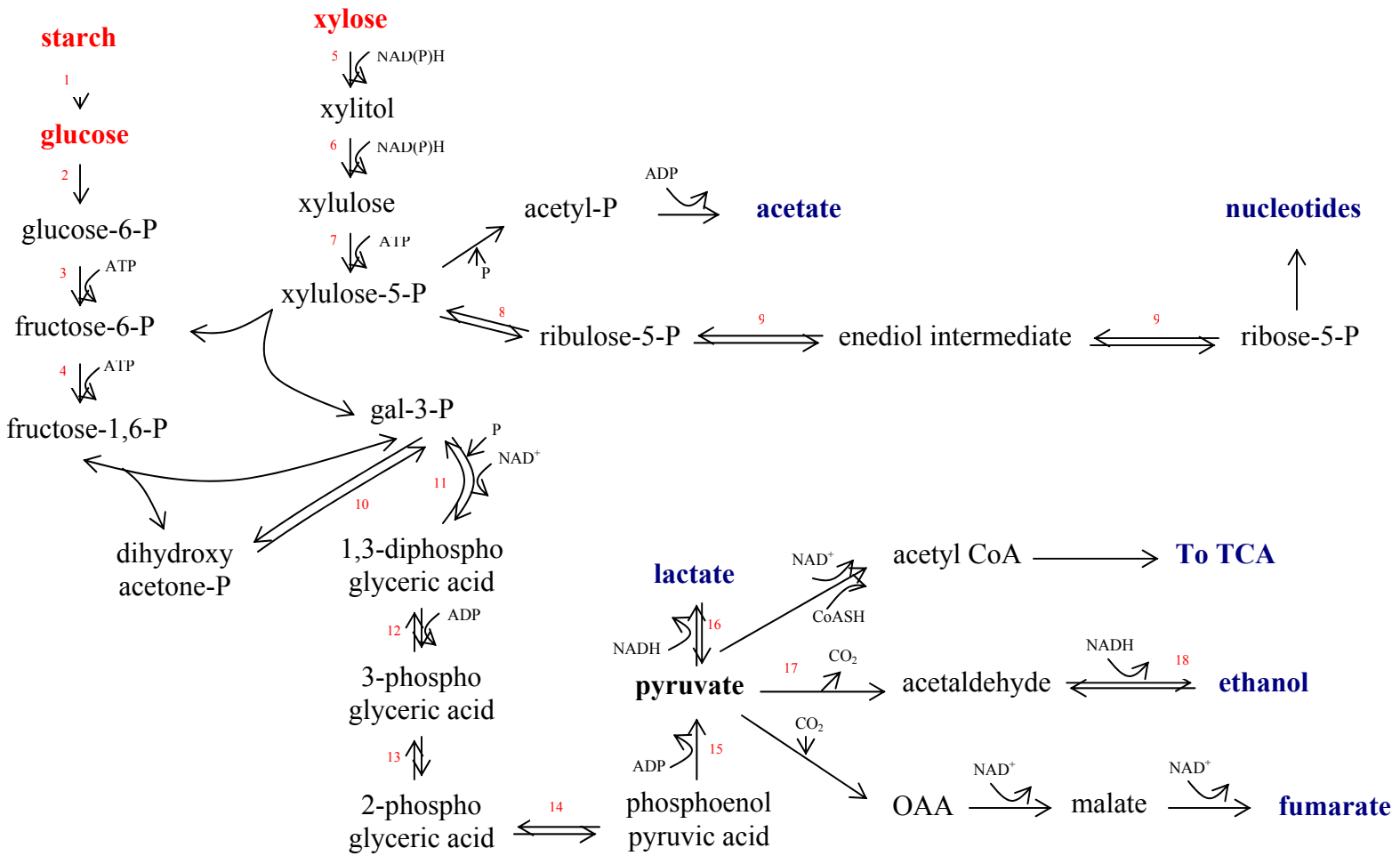


Figure 4.8 Metabolic pathway of *R. oryzae*; the number indicates the enzymes involving in the pathway ([1]amylase, [2]hexokinase, [3]phosphohexoisomerase, [4]phosphofructokinase, [5]xylose reductase, [6]xylitol dehydrogenase, [7]xylulose kinase, [8]phosphopentose epimerase, [9]phosphopentose isomerase, [10]triose phosphate isomerase, [11]gal-3-P dehydrogenase, [12]phophoglycero kinase, [13]phosphoglyceromutase, [14]enolase, [15]pyruvate kinase, [16]lactate dehydrogenase, [17]pyruvate decarboxylase, [18]alcoholdehydrogenase)

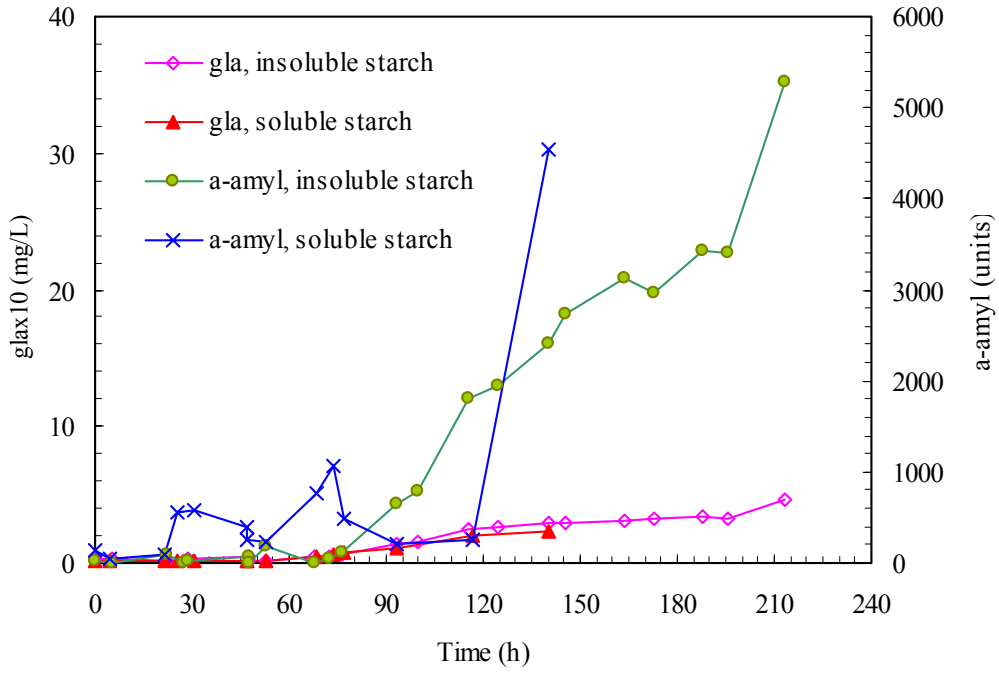


Figure 4.9 Amylase expression level of *R. oryzae* in lactic acid production from different types of starch where gla is glucoamylase and a-amyl is α-amylase

CHAPTER 5

CONTROLLING *RHIZOPUS ORYZAE* GROWTH ON THE ROTATING FIBROUS MATRIX IN A ROTATING FIBROUS BED BIOREACTOR

Summary

Oxygen is one of the factors controlling lactic acid fermentation by *R. oryzae*. Lack of oxygen results in low lactic acid production because of the pathway shunted to ethanol production and eventually leads to loss in fungal activity and cell death. It was observed that maintaining high dissoloved oxygen (DO) in the RFBB did not improve lactic acid production but the increase in oxygen transfer rate led to the increase in lactic acid productivity. In this study, ethanol production was found even at high oxygen transfer rate in the fermentation medium. This indicated an anoxic condition occurring in the fungal cells immobilized on the rotating fibrous matrix. This result was consistent with the estimation from oxygen diffusion model indicating the occurrence of oxygen starvation in the overgrown biofilm. Controlling biofilm thickness to prevent oxygen diffusion limitation could be achieved by shaving off the fungal hyphae at the outermost region of the biofilm. The hyphal shaving off in the RFBB is caused from the shear stress due to the rotational speed of the matrix. Fungal tensile strength plays roles in controlling

shaving mechanism. Shaving occurs only when the shear stress is higher than the fungal tensile strength and the shaving rate is proportional to the specific energy dissipation which is in turn affected by the rotational speed of the matrix. However, the rotational speeds tested in this study did not show the influence on controlling biofilm growth and improving lactic acid production. Besides the optimization of process parameters including mixing and aeration, biofilm growth and metabolic pathway of *R. oryzae* could be controlled by manipulating the medium composition. It was found that biomass production was limited and lactic acid production increased in the absence of urea in the production phase. However, lack of nitrogen source could cause the cease in fungal activity and product synthesis in long-term cultivation. Therefore, periodical addition of urea in the production phase is necessary to limit the overgrown fungal biofilm, maintain fungal viability and activity, and delay sporulation which eventually promotes lactic acid production.

5.1 Introduction

The industrial importance of filamentous fungi has been continuously increasing due to the wide-range applications in the production of antibiotics, organic acids, proteins, and food. In addition, during the last 2 decades, filamentous fungi have been extensively used as eukaryotic hosts for foreign gene expression because their saprophytic life results in an ability to secrete a large amount of proteins and they also offer the similar posttranslational modifications of proteins to the human proteins. Above of all, many fungal species are generally regarded as safe by Food and Drug Administration (Amanullah et al., 1999).

Despite the widespread industrial use, relatively little is known about the influence of engineering variables including mass transfer on the fungal morphology in submerged cultivation (Jin and van Leeuwen; 1999; Pazouki and Panda, 2000; Riley et al., 2000). In conventional filamentous fungal fermentation, high agitation is necessary for providing adequate mixing and oxygen transfer to avoid the high apparent broth viscosity and the non-Newtonian fluid behavior. However, mycelial damage at high shear rate results in the limitation of using high agitation; and therefore, causes oxygen transfer limitation and lowers the production rate (Amanullah et al., 1999).

To date, many studies on immobilized filamentous fungi in the inert supports have been widely determined because immobilization provides many advantages such as the possible high product yield in long-term fermentation and simple downstream processing. However, less knowledge of the novel development regarding scale-up is

available, probably due to oxygen diffusion limitation corresponding to the inappropriate fungal morphology in the immobilized process (Araujo et al., 1999; Cronenberg et al., 1993; Huang and Bungay, 1973; Oostra et al., 2001; Revsbech and Ward, 1983). The viability and activity of the immobilized fungal cells can be predicted by evaluating the diffusion effect of oxygen through the supports. Recently, oxygen diffusion in the immobilized fungi present in many reactor geometries ranging from the stirred tank bioreactor to the air-lift bioreactor have been rigorously studied and many mathematical models explaining oxygen diffusion in the pellet have been developed using the dead core model, the morphologically structured model, and the reaction-diffusion model (Agger, 1998; Cruz et al., 2001; Cui et al., 1998; Hellendoorn et al., 1998; Kobayashi et al., 1973; Oostra et al., 2001; Sun et al., 1999).

As mentioned previously, to minimize the limitations usually found in the stirred tank bioreactor, many novel bioreactors have been developed for controlling fungal morphology. This includes a rotating fibrous bed bioreactor (RFBB) which provides a large surface area for fungal immobilization on the fibrous matrix. Tay and Yang, 2002 reported that fungal immobilization has been achieved in the RFBB; therefore, provided the better oxygen transfer and eventually resulted in increased lactic acid production. However, oxygen diffusion limitation in the overgrown biofilm could lower lactic acid productivity obtained from this bioreactor. Since no relevant study on oxygen diffusion in the immobilized fungal biofilm has been conducted and little is known about controlling fungal biofilm, oxygen diffusion in the fungal biofilm immobilized on the fibrous matrix in the RFBB was determined in this study. The dissolved oxygen in the fermentation

medium creates oxygen concentration gradient inside the fungal biofilm during migration. Oxygen starvation resulted from oxygen diffusion limitation causes cell deactivation and autolysis. Controlling biofilm growth is required since excess growth beyond the critical level can lead to oxygen limitation. An oxygen diffusion model was developed to estimate the critical biofilm thickness under certain dissolved oxygen concentration. Besides, the attempts to control biofilm growth in the RFBB by either optimization of the rotational speed or medium composition were described.

5.2 Materials and Methods

5.2.1 Culture, inoculum preparation, and medium compositions

R. oryzae NRRL 395, a filamentous fungus producing L(+)-lactic acid obtained from the Northern Regional Research Center, Peroria, IL, was used in this study. The method to maintain fungal viability is described in Appendix A1. The spore suspension made from the 7-day culture on the potato dextrose agar plate was adjusted to 10^6/mL by dilution with sterile water. 10^6/mL *R. oryzae* spore suspension (10 mL) was used to inoculate the bioreactor (Appendix A2).

To study the effect of dissolved oxygen and rotational speed, a growth medium contained 50 g/L glucose and a production medium contained 70 g/L glucose as a substrate. To determine the effect of urea in the production phase, 3 levels of urea was

studied, i.e. 0 g/L, 0.15 g/L, and 0.30 g/L. The other compositions required in each medium are shown in Appendix A3.

5.2.2 Bioreactor setup

A Rotating Fibrous Bed bioreactor (RFBB) was modified from a 5-L bioreactor (Biostat B, B. Braun) by affixing a perforated stainless steel cylinder mounted with a 100% cotton cloth to the agitation shaft. The bioreactor setup is described in Appendix B. The cylindrical matrix of 6 cm diameter and 10 cm height was used in the study of the effects of dissolved oxygen and rotational speed whereas the matrix of 9 cm diameter and 15 cm height was used in the study of the effect of urea in the production phase. After sterilization, the bioreactor was set up the controlled parameters and inoculated with 10 mL spore suspension (10^6/mL). Unless otherwise mentioned, the bioreactor was controlled at 30°C and pH 6.0 (during the production phase), agitated at 60 rpm, and aerated with filter-sterilized air at 1.0 vvm.

5.2.3 Fermentation study

To study the fermentation kinetics, first the RFBB containing the growth medium was inoculated and the growth phase took approximately 48 hours for spore germination and cell immobilization on the fibrous matrix. After 48 hours, the growth medium was

replaced with the production medium. The fermentation batch finished when glucose was depleted or lactic acid concentration was not increasing.

5.2.4 Oxygen transfer determination

5.2.4.1 Volumetric oxygen transfer coefficient

Oxygen is one of the important factors in filamentous fungal fermentation. It involves in fungal growth and metabolism. Insufficient oxygen transfer can limit lactic acid production and eventually cause cell death (Tay and Yang, 2002; Thongchul and Yang, 2003). Before the end of each fermentation batch, the oxygen uptake rate (OUR) and the dynamics of oxygen transfer in the bioreactor were studied using the dynamic method of gassing out (Appendix C).

5.2.4.2 Effective diffusivity

The effective diffusivity (D_e) was determined by the equipment developed from the biological oxygen monitor as shown in Figure 5.1 (Hellendoom et al., 1998). The equipment consisted of 2 consecutive compartments. Deactivated *R. oryzae* biofilm harvested from the fermentation in the RFBB was inserted into the slot at the middle of the vessel; thus, separated the 2 compartments containing well-mixed water. Before the measurement began, dissolved oxygen probes were calibrated with nitrogen gas and air.

Since the liquid in both compartments was well-mixed, it was assumed that no concentration gradient occurred at the interface of the outermost surface of the biofilm and the bulk liquid. Additionally, there was no convective force present. D_e was dependent on the geometric structure of the biofilm indicating by density or porosity of the biofilm (Cussler, 1999; Geankoplis, 1993).

The diffusivity measurement was begun by aerating one of the compartments (#1) and biofilm with air until saturation (approximately 30 min) whereas the other compartment (#2) was separately flushed with nitrogen gas to obtain the oxygen-free condition for approximately 30 min. Later, nitrogen flushing in #2 was stopped and oxygen transfer was allowed from #1 passing through the biofilm to #2. DO levels from both compartments were recorded. Figure 5.2 shows the dissolved oxygen concentration profile in both compartments.

D_e was determined by the dynamic method of gassing out (Appendix C). The rate of increase in oxygen concentration in #2 was owing to oxygen diffusion through the biofilm. The oxygen concentration at the outermost surface of the biofilm was equal to bulk oxygen concentration; therefore, the rate of change in oxygen concentration in #2 was equal to the oxygen diffusion rate through the biofilm.

$$V_{imb} \cdot \frac{dC_{L,2}}{dt} = \frac{D_e \cdot A \cdot \left(C_{L,1} - C_{L,2}\right)}{\delta} \tag{5.1}$$

Where A is the surface area of the biofilm. $C_{L,1}$ and $C_{L,2}$ are bulk oxygen concentrations in compartment #1 and #2, respectively. V_{imb} is the wet biofilm volume. $\frac{dC_{L,2}}{dt}$ was

determined from the slope of the plot of $C_{L,2}$ versus time. $\frac{dC_{L,2}}{dt}$ and $C_{L,1}$-$C_{L,2}$ were calculated and the plot of $\frac{dC_{L,2}}{dt}$ and $C_{L,1}$-$C_{L,2}$ was used to determine D_e from the slope of the plot $\left(\frac{D_e \cdot A}{\delta \cdot V_{imb}}\right)$ (Figure 5.3).

Table 5.1 shows the effective diffusivity of oxygen and glucose in 2 different geometric structures of the biofilm (one with low density and high porosity and the other one with high density and low porosity). It was found that both oxygen and glucose diffusion through the biofilm having low density and high porosity was faster than that through the biofilm having high density and low porosity. Glucose diffusion in the biofilm was faster than oxygen diffusion indicating that oxygen was the limiting substrate in lactic acid fermentation by *R. oryzae* immobilized in the RFBB.

5.2.5 Tensile strength

R. oryzae biofilm harvested from the fermentation was tested for biomechanical strength using a tensiometer (MicroInstron). The biofilm was cut into small pieces (1.0cm × 2.0cm). The sample was placed vertically between 2 clamps of the tensiometer and subjected to a force applied with a constant speed until rupture. Maximum load and extension were recognized as the maximum force and stress withheld before rupture. Tensile strength (σ) was determined as the maximum breaking strength per cross-sectional area. Cross-sectional area was determined from the product of the area

subjected to testing between the jaws (distance between 2 clamps) and the width of the sample being tested (1.0 cm).

Tensile strength of the single mycelia can be empirically estimated from the model proposed by Li et al., 2002.

$$\sigma_{pseudo} = \rho(\varepsilon\nu)^{1/2} \ln\left(\frac{15^{1/2}(\varepsilon\nu)^{1/2}}{q_{frag}}\right) \quad (5.2)$$

Where ρ is the density fermentation medium. ε is the specific energy dissipation rate. ν is the kinematic viscosity and q_{frag} is the average specific fragmentation rate.

5.2.6 Analytical methods

5.2.6.1 Cell biomass

Growth of *R. oryzae* in the RFBB was determined at the end of fermentation. The detail is described in Appendix D1.

5.2.6.2 Substrate and product concentration

The fermentation sample was centrifuged to remove the suspended solid. The supernatant was analyzed for glucose and L(+)-lactic acid using a glucose/lactate analyzer (YSI 2700). HPLC was also used to analyze the organic compounds (glucose, lactic acid, fumaric acid, and ethanol) present in the supernatant. The HPLC system

(Shimadzu Scientific Instruments) was equipped with a RID-10A reflective index detector and an organic analysis column (Aminex HPX-87H, Biorad). The details are given in Appendix D2 and D3.

5.2.6.3 Scanning electron microscopy

Fungal growth and activity were examined at the end of the fermentation in the study on the effect of urea on lactic acid fermentation by scanning electron microscopy. The method of sample preparation is described in Appendix D4.

5.3 Results and Discussion

5.3.1 Effect of dissolved oxygen

The effect of DO level on lactic acid fermentation was observed in the RFBB using glucose as the substrate at pH6.0, 30°C, and 60 rpm. The DO level was maintained constant by either controlling the aeration rate or using the oxygen-enriched air to achieve high DO level at 100% air saturation. The result shows that similar fermentation kinetics was observed in the fermentations with DO from 40% to 100% air saturation (Figure 5.4). However, there was a decrease in ethanol production at high DO levels (80 and 100%). At 100% air saturation, lactic acid concentration reached 33.7 g/L in 102 h, while only 3.5 g/L ethanol was produced in the production phase. Additionally, ethanol

production in the growth phase was less than those at the lower DO level. Previous results showed that lactic acid production only occurred in the presence of oxygen; and therefore, indicated the critical demand of oxygen for lactic acid production (Elibol and Ozer, 2000; Skory et al., 1998; Skory, 2003; Tay and Yang, 2002; Thongchul and Yang, 2003). In this study, it was found that higher lactic acid productivity was obtained at higher DO level but the high DO level did not greatly improve lactic acid yield (Table 5.2). Generally, oxygen plays a role as an electron acceptor in the respiratory system which directly affects cell growth (Elibol and Ozer, 2000; Skory, 2003). However, the result indicates that the influence of oxygen on cell growth was not clearly observed under the DO levels studied (Table 5.2). In Table 5.2, k_La values at 80 and 100% air saturation were higher than those and 40 and 60% due to the higher aeration rate of oxygen-enriched air and this, in turn, led to the higher lactic acid productivity. According to the results, the DO level might not be an intrinsic parameter which determined cell growth and lactic acid production. On the other hand, it was found that the change of oxygen transfer rate affected lactic acid productivity (Table 5.2). This indicates that DO level could not be directly used to correlate the production rate of lactic acid fermentation but the effect of oxygen could be explained by its transfer rate (Elibol and Ozer, 2000).

5.3.2 Oxygen diffusion in *R. oryzae* biofilm

Although high oxygen transfer rate was achieved at high DO level in this study; lactic acid production from glucose was shunted to ethanol production pathway. This

indicated an anoxic condition occurring in *R. oryzae* biofilm immobilized on the fibrous matrix which was related to the enhancement of *pdc* transcription and pyruvate decarboxylase activity involving in ethanol fermentation pathway (Skory, 2003).

Oxygen diffusion was determined in order to better understand and prevent oxygen limitation in the immobilized biofilm (Figure 5.5). It was assumed that the effect of immobilization on growth was restricted to a change in microenvironments as an effect of oxygen concentration profile and to the absence of the internal shear stress. Oxygen diffusion in the biofilm was described by Fick's law. Fungal mycelia were homogeneously distributed on the fibrous matrix and fungal growth was described with zero order kinetics with respect to oxygen concentration. This assumption was supported by the small value of Monod constant for oxygen (0.024 mol/m^3), compared to bulk oxygen concentration used in this study. The experimentally determined effective diffusivity (D_e) of oxygen was constant during the process. The oxygen consumption rate or *OUR* (v) was determined by the dynamic method of gassing out described in Appendix C. At pseudo steady state, oxygen consumption rate depending on oxygen diffusion in the biofilm can be described as follows (Appendix E1):

$$D_e \frac{d}{dr}\left(r \frac{dC}{dr} \right) = \frac{vr}{2} \tag{5.3}$$

Where r is the radial distance from the center of the cylindrical fibrous matrix. Because of well-mixed fermentation medium, there was little external mass transfer limitation; and therefore, the oxygen concentration at the surface of the biofilm could be assumed to be equal to bulk oxygen concentration. Due to the homogeneously distributed biofilm

growth, the growth on both sides of the cylindrical fibrous matrix (inside and outside) was assumed to be symmetrical. Thus, eq (5.3) can be derived (Appendix E1) and the oxygen concentration profile in the biofilm can be described by eq (5.4) (Figure 5.5).

$$C_L - C = \frac{vR^2}{4D_e}\ln\frac{r}{R_o} + \frac{v}{8D_e}\left(R_o{}^2 - r^2\right) \qquad (5.4)$$

Where C_L is bulk oxygen concentration. C is the oxygen concentration in the biofilm. R_o is the radial distance from the center of the cylindrical matrix to the outermost surface of the immobilized biofilm. r is any radial distance from the center of the matrix to any region in the biofilm.

When the biofilm growth reached the critical thickness, the oxygen starvation occurred as indicated from the absence of oxygen at the center of the biofilm (surface of the cylindrical matrix). The critical thickness of the biofilm under various bulk DO levels (C_L) was numerically determined by substituting $C = 0$ at $r = R$ into eq (5.4). It was clear that the anoxic condition occurred at the internal region of the biofilm in all fermentations at different DO levels observed in this study (Figure 5.6). This indicated that the internal region of the biofilm would likely have reduced oxygen availability compared to that at the surface, regardless of the level of aeration. The result obtained from the model estimation was consistent with ethanol production appeared in all fermentations. This indicated the excess biofilm growth and inappropriate control of immobilization on the fibrous matrix (Skory, 2003).

5.3.3 Effect of rotational speed

Many studies reported the effect of agitation on fungal morphology in submerged cultures. Filamentous fungal fermentation in the stirred tank bioreactor usually experiences the high apparent viscosity and the non-Newtonian broth behavior which lead to the use of high agitation speed to provide adequate mixing and oxygen transfer in order to improve the production rate. An increase in biomass production at high agitation speed is also possible due to the fragmentation of hyphae resulting in a morphological growth cycle; fragmentation, regrowth, and re-fragmentation which eventually leads to high biomass production. However, mycelial damage at high power input from rigorous agitation can limit the acceptable range of agitation speed. This is probably caused from the rigorous shaving resulting in the higher shaving rate than the reproduction rate of the biomass. Therefore, the high rate of cell damage could lower growth and product formation (Amanullah et al., 2002; Li et al., 2000; Tamerler and Keshavarz, 1999).

Likewise in the stirred tank bioreactor, the rotational speed of the fibrous matrix in the RFBB possibly influenced lactic acid fermentation by *R. oryzae*. Due to the RFBB geometry, the higher rotational speed than 100 rpm could cause damages to the bioreactor and controlling system. Therefore, in this study, the effect of rotational speed was determined at the levels of 40, 60, 80, and 100 rpm. Figure 5.7 shows the kinetics of the fermentation of glucose at different rotational speeds at the DO level of 80% air saturation. It was found that the rotational speed tested in this study did not greatly affect lactic acid yield; however, the increase in lactic acid productivity was observed at high

rotational speed (Table 5.3). It was also found that rotational speeds tested in this study did not influence fungal growth as indicated from the similar biomass yield and productivity. In addition, ethanol production was observed along with lactic acid production in all fermentations at different rotational speeds indicating the problem in oxygen limitation in the biofilm although oxygen transfer in the fermentation medium was improved at high rotational speed (Table 5.3 and Figure 5.7). Therefore, the range of rotational speed used in this study could not be successfully used for controlling fungal growth to prevent diffusion limitation and to enhance lactic acid production.

5.3.4 Controlling biofilm thickness

The previous results imply that oxygen diffusion limitation in the biofilm was mainly caused from the inappropriate control of growth and immobilization in the RFBB. Generally, agitation has an influence on significant changes in fungal morphology and reactor productivity in the stirred tank bioreactor (Cui et al., 1997; Papagianni et al., 1999). Fungal mycelia suffer from a complete transformation when the agitation level increases. It is claimed that changes in cell wall composition and structure have been involved in the resistant mechanism to shear forces. Many studies reported the reduction of pellet size, hyphal length, number of tips, and hyphal growth units under intensive agitation in the stirred tank bioreactor. Intensive agitation controls the morphology by actual breakage of fungal mycelia, where a cycle of fragmentation and regrowth of mycelia predominates at high agitation speed (Cui et al., 1997; Papagianni et al., 1999).

In the RFBB, fungal morphology could possibly be controlled in the similar way of that in the stirred tank bioreactor. Controlling biofilm thickness for preventing diffusion limitation could be achieved by shaving off the fungal mycelia at the surface of the biofilm when the tensile strength of the mycelia was less than the local shear stress. The shear stress in the RFBB was mainly due to the rotational speed of the cylindrical matrix. It was assumed that the biofilm exhibited the fluid-like behavior. The fermentation medium had constant density. The fermentation medium was considered to have the Newtonian fluid behavior because fungal growth appeared only on the cylindrical matrix, preventing the presence of filamentous mycelia in the fermentation medium. This, in turn, prevented the problem in high viscosity (non-Newtonian like fluid). The shear stress at the surface of the rotating matrix can be described as follows (Appendix E2).

$$\tau_{r\theta}\big|_{r=R} = \frac{2\mu\omega R_T^2}{\left(R_T^2 - R^2\right)} \tag{5.5}$$

Where $\tau_{r\theta}$ is the shear stress acting on the rotating matrix as a function of rotational speed (ω), radius of the cylindrical matrix (R), and radius of bioreactor vessel (R_T). μ is the fermentation medium viscosity.

Rate of increase in the amount of fungal cells immobilized on the rotating matrix in the RFBB mainly depends on fungal growth rate and rate of biofilm shaving off due to shear stress (eq (5.6)).

Rate of biofilm accumulation = Growth rate – Shaving rate (5.6)

The fungal cell biomass accumulated on the cylindrical matrix can be described by eq (5.7).

$$\frac{dM}{dt} = \rho_b \frac{dV}{dt} = 2\pi\rho_b h \frac{dr}{dt} \tag{5.7}$$

Where M is the amount of fungal biomass immobilized in the matrix. ρ_b is the immobilized fungal cell density. h is the height of the cylindrical matrix. r is the radial distance from the center of the matrix.

It is claimed that the extension rate of a hypha is proportional to its length. Due to a linear extension rate of hyphae and the formation of new tips, mycelial growth is effectively equivalent to that of unicells; and therefore, the exponential growth rate and Monod's expression for the specific growth rate (μ_G) were used to describe biofilm growth rate (R_G).

$$R_G = \mu_G M = \pi\mu_G \rho_b h r^2 \tag{5.8}$$

In the RFBB, the hyphal shaved off starts from the surface of the fungal cells immobilized on the matrix due to the mechanical forces. The shaving process controlled the increase in biofilm thickness during growth. It is claimed that shaving occurs at the outermost zone of the biofilm only when the local shear stress (τ) is higher than fungal tensile strength (σ) and the shaving rate (R_{sha}) is proportional to the specific energy dissipation rate (ε) (Cui et al., 1997; Lejeune and Baron, 1998; Li et al., 2002).

$$R_{sha} = \begin{cases} 0 & , \tau < \sigma \\ K_{sha}\varepsilon R_G & , \tau \geq \sigma \end{cases} \tag{5.9}$$

Where K_{sha} is the shaving coefficient empirically determined to best fit the model. Cui et al., 1998 reported the K_{sha} value of 0.06 kg/W in the cultivation of *Aspergillus niger* in

the stirred tank bioreactor. The specific energy dissipation rate in the RFBB depended on the torque (T) required to rotate the cylindrical matrix at certain rotational speed.

$$\varepsilon = \frac{P}{\rho V} = \frac{T}{\rho V \omega} \tag{5.10}$$

$$T = \frac{4\pi\mu\omega h R^2 R_T^2}{R_T^2 - R^2} \tag{5.11}$$

Where ρ and V are the density and volume of fermentation medium. P is the power input required to rotate the cylindrical matrix.

Accordingly, shaving mechanism in the RFBB is related to fungal tensile strength at the surface of the biofilm and the rotational speed of the matrix. Fungal tensile strength is exceptionally difficult to measure or estimate and only a few studies have attempted to do so (Ayazi Shamlou et al., 1994; Johansen et al., 1998; Lejeune and Baron 1998; Li et al., 2002; Nielsen and Krabben, 1995; van Suijdam and Metz, 1981). It is claimed that during the cultivation, fungal cell wall composition has changed dramatically during growth and autolysis (Lahoz et al., 1986). This, in turn, may lead to changes in tensile strength. Therefore, it is cumbersome to measure and estimate the tensile strength of the immobilized fungal biofilm during the cultivation in the RFBB. In this study, the tensile strength of the biofilm at the end of the fermentation could be measured and the result provides the interesting information. It was found that the measured biofilm tensile strength with the thickness of 0.7-1.0 cm (0.09-0.15 MPa) was 10^7 times higher than that of the single mycelium (0.01-0.03 Pa) estimated by the tensile strength model proposed by Li et al., 2002. This difference could be explained by immobilization created the tight entangled network of fungal hyphae; therefore, increased the strength of the biofilm. This

could possibly affect the shaving mechanism at the outermost region of the biofilm immobilized on the matrix as well.

The shear stress as the function of the rotational speed is shown in Figure 5.8. Compared to the estimated tensile strength of the single mycelia (approximately 0.02 Pa), it was found that shaving mechanism in the RFBB started at the rotational speed higher than 600 rpm. According to the shaving mechanism, at lower rotational speed than 600 rpm, the change in biofilm thickness is mainly due to fungal growth rate. Therefore, the low level of rotational speed in the RFBB could not be successfully used to control biofilm thickness to prevent diffusion limitation.

5.3.5 Effect of medium composition

The type and concentration of nitrogen source strongly influence fungal morphology and growth. Similarly to other filamentous fungi, *R. oryzae* requires organic nitrogen source such as yeast extract for spore germination and initial cell growth during the growth phase. However, excess organic nitrogen source in the production phase decreases lactic acid production. Tay, 2000 reported that urea at the level of 0.3 g/L was most suitably used in the production phase of lactic acid fermentation by *R. oryzae* in the shaken flask culture compared to other nitrogen source. However, subsequent growth and ethanol production, which in turn resulted in low lactic acid yield, were found in the fermentation using 0.3 g/L urea in the RFBB (Figure 5.9). This was possibly because when the excess urea is present, ammonium ions in urea counteract the inhibition of

phosphofructokinase by ATP; therefore, stimulate glycolysis which results in a large pyruvate pool. Besides ammonium ions stimulate growth by decreasing the intracellular level of NADH, depressing glucose-6-phosphate dehydrogenase; and therefore, enhancing the activity of Pentose Phosphate pathway (Elibol and Ozer, 2000; Papagianni et al., 1999; Pritchard, 1973; Seidel et al., 2002; Soto-Cruz et al., 1999; Woiciechowski et al., 1999). Therefore, to increase lactic acid production in the RFBB, growth pattern and metabolic pathway of *R. oryzae* require to be manipulated besides the optimization of other environmental parameters such as mixing and aeration. In this study, lactic acid fermentation using different concentrations of urea in the production phase was observed. It was found that less biomass was produced in the fermentation without urea whereas the highest biomass production was found in the fermentation using 0.3 g/L urea (Table 5.4). This result was consistent with the lowest ethanol production in the fermentation without urea indicating that less glucose was converted to biomass (Figure 5.9). Consequently, oxygen diffusion limitation in the fermentation without urea was less severe because there was less subsequent growth of the biofilm in the production phase. Therefore, this led to higher lactic acid yield (Table 5.4). However, nitrogen is still necessary to maintain *R. oryzae* viability and activity during fermentation. Lack of nitrogen could cause the cease in cell growth and product synthesis in long-term cultivation (Papagianni et al., 1999). Figure 5.10 shows that the fungal cells immobilized on the fibrous matrix at the end of the fermentation without urea (approximately 94 h cultivation) had a smaller hyphal diameter compared to those observed in the fermentation with urea. Besides, more hyphal shrinkage as the indication of more inactive or damaged cells was found when

there was no urea present in the production phase. Therefore, periodical addition of urea is required to limit overgrowth of fungal biofilm, maintain fungal activity, and delay sporulation which, in turn, promotes lactic acid production.

5.4 Conclusion

Lactic acid production in the RFBB was mainly influenced by the *R. oryzae* biofilm immobilized on the rotating matrix. Although high oxygen transfer rate in the fermentation medium was achieved by high aeration rate or high rotational speed, oxygen limitation in the biofilm was still found. This, in turn, caused low lactic acid production rate. It was found that high DO level did not improve lactic acid production but oxygen transfer rate in the fermentation medium played a role in increasing lactic acid production rate. According to the shaving mechanism, high rotational speed not only affected oxygen transfer but also caused high fragmentation rate which could affect lactic acid production by fungal growth manipulation. It was found that subsequent biofilm growth in the production phase could cause oxygen limitation in the biofilm; thus, shunted the pathway to ethanol production. This resulted in less glucose converted to lactic acid, instead glucose was consumed for biomass and ethanol production. The subsequent biofilm growth in the production phase could be minimized by manipulating the addition of nitrogen source. This can be done by periodically supplying nitrogen to the fermentation to minimize subsequent growth, maintain fungal activity, delay sporulation, and eventually prevent cell death.

5.5 References

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Density (g/cm^3)	Porosity	Medium	Effective diffusivity (m^2/s)	
			Oxygen	Glucose
0.0257	0.9154	Water	$(8.41\pm1.65)\times10^{-9}$	$(1.60\pm0.46)\times10^{-8}$
0.1584	0.8178	Water	$(1.93\pm0.36)\times10^{-10}$	$(7.98\pm1.06)\times10^{-10}$

Table 5.1 Effective diffusivity of oxygen and glucose in different geometric structures of *R. oryzae* biofilm

DO (% air saturation)	40	60	80	100
Aeration rate (vvm)	0.1	0.2	1.0	2.0
Gas composition	100% air	100% air	100% air	17% O_2 83% air
C_L (mM)	0.10±0.00	0.14±0.01	0.21±0.00	0.22±0.00
$OUR \times 10^3$(mM/min)	2.3±0.0	2.9±0.1	3.8±0.0	3.7±1.3
k_La (min^{-1})	0.10±0.00	0.12±0.02	0.43±0.02	0.61±0.11
Lactate				
Yield (g/g)	0.53±0.06	0.49±0.05	0.51±0.04	0.53±0.05
Productivity (g/L·h)	0.58±0.13	0.55±0.08	0.88±0.10	0.95±0.11
Biomass				
Yield (g/g)	0.05±0.000	0.05±0.003	0.05±0.003	0.05±0.001
Productivity (g/L·h)	0.04±0.001	0.04±0.003	0.04±0.002	0.04±0.001
Thickness (mm)	5	6	7	7

Table 5.2 Effect of dissolved oxygen on oxygen transfer, lactic acid production, and immobilized *R. oryzae* growth on the rotating cylindrical matrix (6cm × 10cm) in the RFBB operated at 30°C, pH 6.0, and 60 rpm

Rotational speed (rpm)	40	60	80	100
C_L (mM)	0.21±0.00	0.21±0.00	0.20±0.00	0.18±0.00
$OUR \times 10^3$(mM/min)	2.3±0.0	3.8±0.0	4.6±0.0	3.5±0.0
k_La (min^{-1})	0.42±0.01	0.43±0.02	0.47±0.02	0.50±0.05
Lactate				
Yield (g/g)	0.55±0.06	0.51±0.04	0.58±0.07	0.55±0.10
Productivity (g/L·h)	0.90±0.14	0.88±0.10	0.77±0.05	0.81±0.16
Biomass				
Yield (g/g)	0.04±0.009	0.05±0.003	0.05±0.004	0.05±0.007
Productivity (g/L·h)	0.03±0.006	0.04±0.003	0.04±0.004	0.04±0.005
Thickness (mm)	5	6	6	5

Table 5.3 Effect of rotational speed on oxygen transfer, lactic acid production, and immobilized *R. oryzae* growth on the rotating cylindrical matrix (6cm × 10cm) in the RFBB operated at 30°C, pH 6.0, and 80% air saturation

Urea concentration	No urea	0.15 g/L urea	0.30 g/L urea
Lactate			
Yield (g/g glucose)	0.66±0.11	0.64±0.12	0.57±0.16
Productivity (g/L·h)	1.38±0.19	1.35±0.25	1.15±0.29
Biomass			
Total dry weight (g)	25.21	27.29	29.88
Yield (g/g glucose)	0.06	0.07	0.07
Productivity (g/L·h)	0.06	0.07	0.08

Table 5.4 Effect of urea in the production phase on lactic acid fermentation by immobilized *R. oryzae* on the rotating cylindrical matrix (9cm × 15cm) in the RFBB operated at 30°C, pH 6.0, 60 rpm, and 80% air saturation

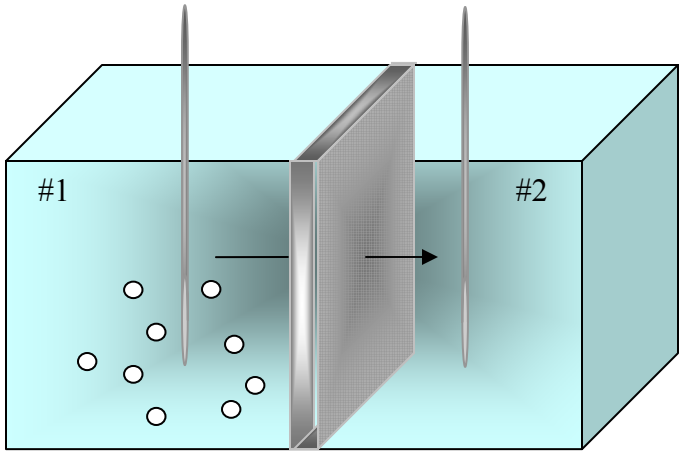

Figure 5.1 An experimental setup for determining the effective diffusivity in *R. oryzae* biofilm

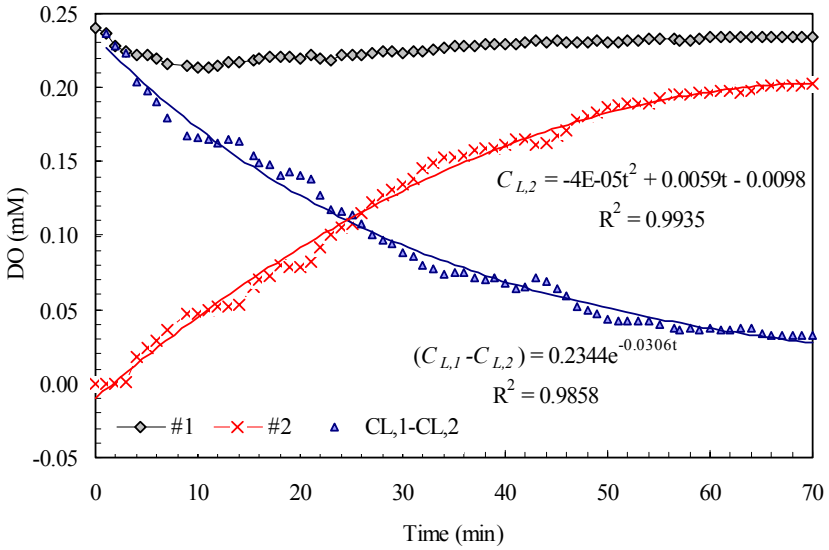

Figure 5.2 Dissolved oxygen concentration profiles in both compartments, where #1 is dissolved oxygen concentration in compartment#1 and #2 is the dissolved oxygen concentration in compartment#2

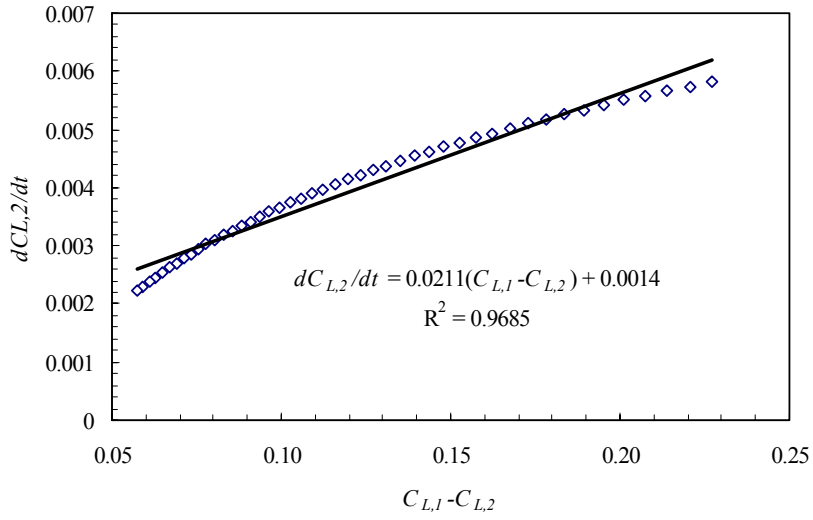

Figure 5.3 An example of the plot used to determine the effective diffusivity in fungal biofilm

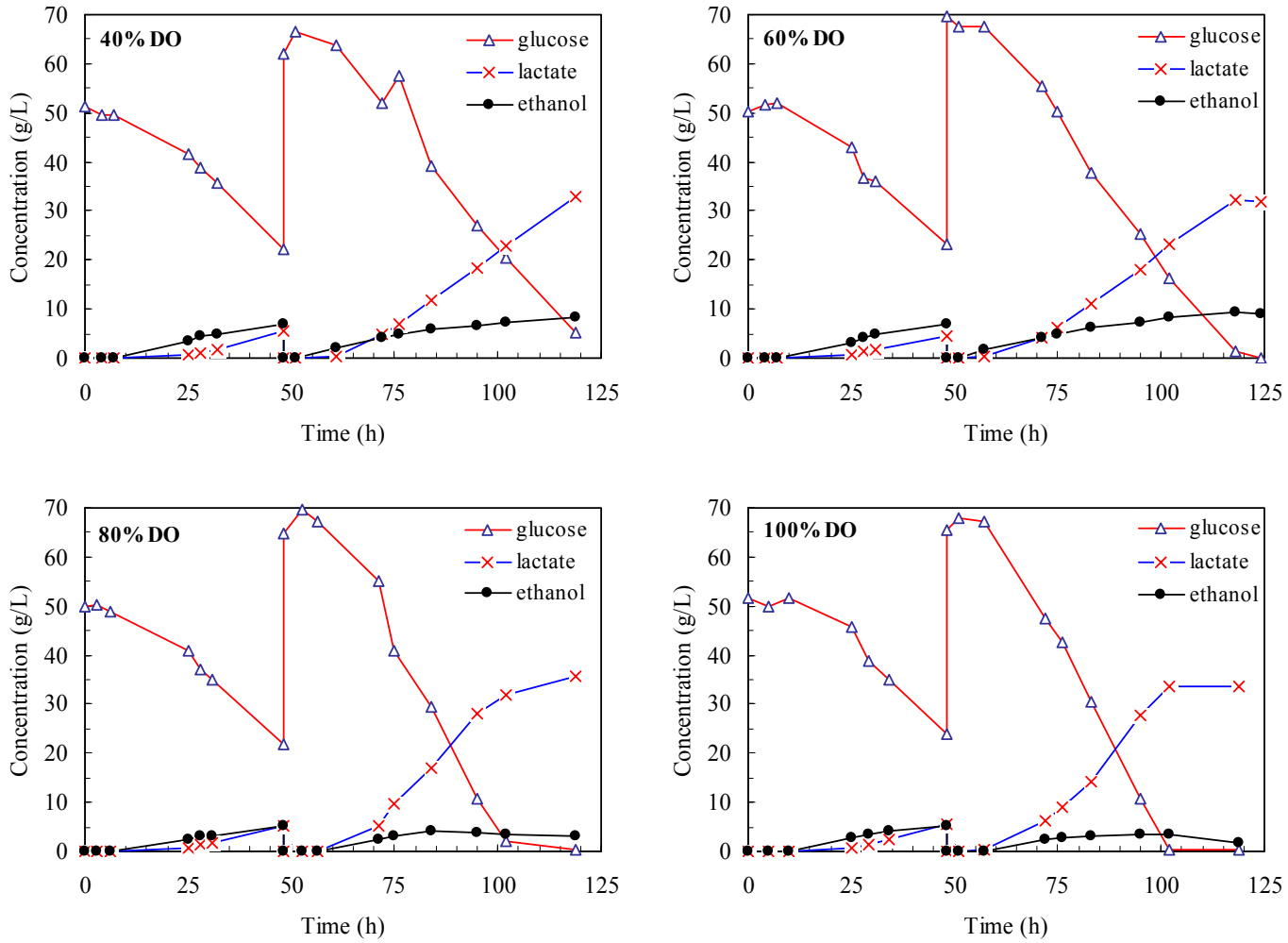

Figure 5.4 Fermentation kinetics of *R. oryzae* in the RFBB at 60 rpm with different dissolved oxygen concentrations

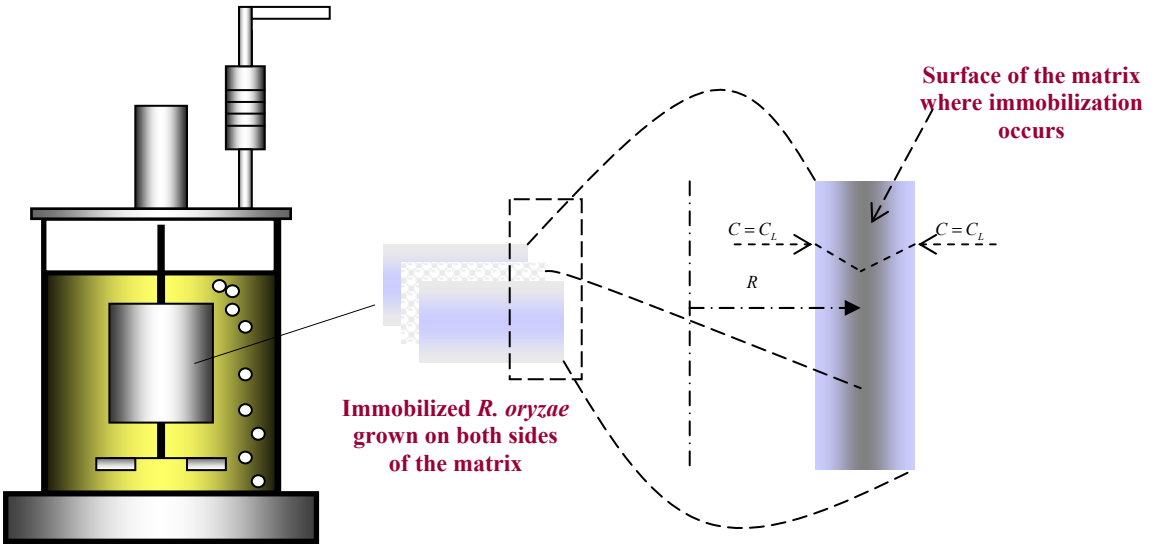

Figure 5.5 *R. oryzae* immobilized on the cylindrical matrix (6cm×10cm) in the RFBB in the study of the effects of dissolved oxygen concentrations and rotational speeds

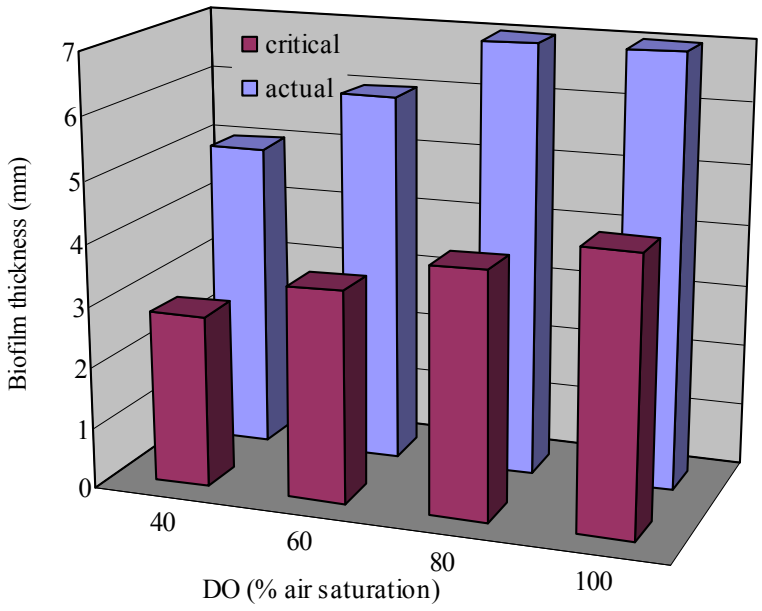

Figure 5.6 Comparison of the actual biofilm thickness to the predicted critical values in lactic acid fermentation from glucose at different DO levels

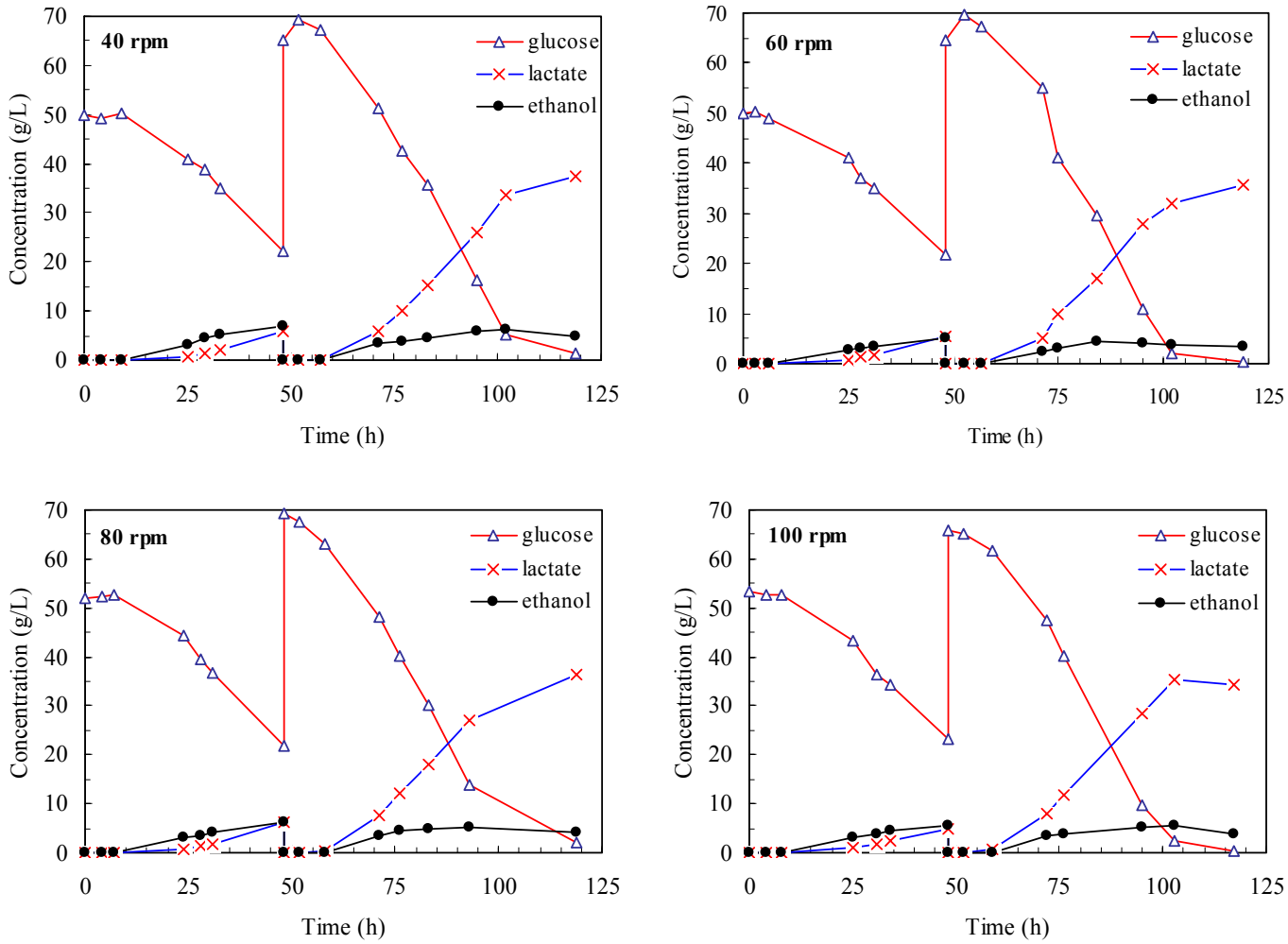

Figure 5.7 Fermentation kinetics of *R. oryzae* in the RFBB at 80% air saturation with different rotational speeds

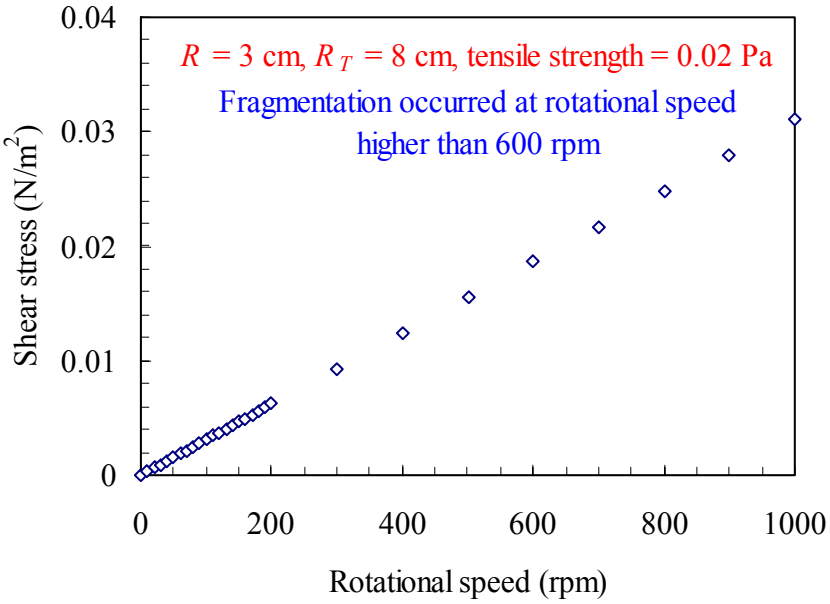

Figure 5.8 Shear stress acting on the fibrous matrix where immobilization occurred as a function of the rotational speed

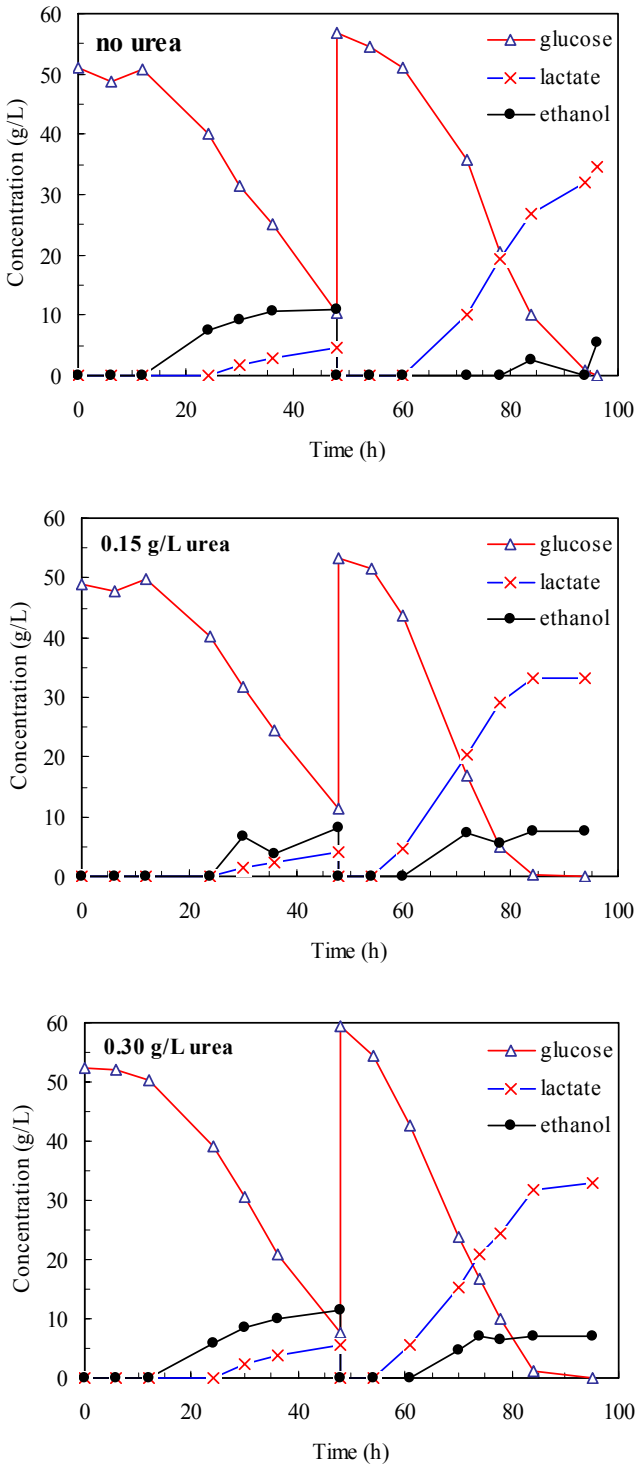

Figure 5.9 Fermentation kinetics of *R. oryzae* in the RFBB with different urea concentrations in the production phase

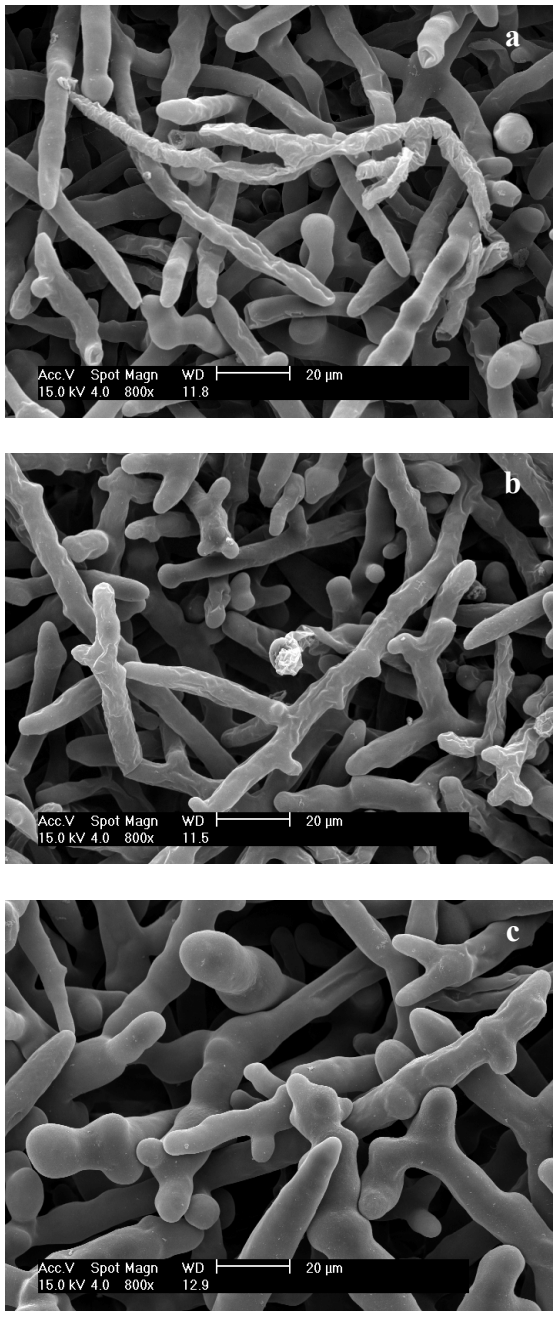

Figure 5.10 *R. oryzae* immobilized on 100% cotton (non-woven) fibrous matrix in the fermentation using different urea concentrations during the production phase, (a) no urea, (b) 0.15 g/L urea, and (c) 0.30 g/L urea

CHAPTER 6

SPORE GERMINATION AND CELL IMMOBILIZATION IN A ROTATING FIBROUS BED BIOREACTOR FOR CONTROLLING MORPHOLOGY AND LACTIC ACID PRODUCTION BY *RHIZOPUS ORYZAE*

Summary

The kinetics of germination and immobilization of *R. oryzae* spores in a rotating fibrous bed bioreactor (RFBB) were studied. In general, spore germination consisted of 2 distinguishable phases: spore swelling and growth polarization (germ tube extension). The effects of agitation or rotational speed of the fibrous bed on spore germination and immobilization were studied at 20 and 60 rpm. The results showed that the rotational speed did not significantly affect the spore germination process, but influenced spore immobilization in various types of fibrous matrices (non-woven and woven cotton and polyethylene terephthalate (PET) cloth) studied. Among them, 100% cotton fibrous matrices provided the best support for spore immobilization in the RFBB. Immediately after inoculation, a large number of spores were immobilized by entrapment in the void spaces of cotton matrices, which was followed by adsorption of spores and then germinated spores until all spores had been immobilized in less than ~9 h, resulting in a

cell-free broth in the subsequent fermentation period. In contrast, spore immobilization on PET matrices was mainly by adsorption and was relatively slow until most spores had been germinated in the medium. By that time, a significant amount of germinated spores had grown into cell clumps and in many cases they remained in the suspension instead of being immobilized on the matrix. In general, the higher rotational speed promoted spore immobilization because more spores could be intercepted and entrapped in the fibrous matrix, although it reduced the strength of spore adsorption on the fiber surface. Mathematical models for spore germination and immobilization (adsorption) were also developed and are presented in this chapter.

6.1 Introduction

Recently, L(+)-lactic acid production by *R. oryzae* has been extensively studied because of its advantages in producing an optically pure L(+)-lactic acid from the renewable sources. However, it is well known that the cultivation of filamentous fungi in a conventional stirred tank bioreactor is usually troublesome due to the diversity and change in cell morphology during fermentation. This causes the problems in mixing and mass transfer due to high medium viscosity. In addition, undesirable growth on wall and sensors hampers bioreactor control and adversely affected the fermentation and reactor productivity (McCabe et al., 2001)

Cell immobilization has been widely used as a method to overcome the problems caused by dramatic changes in fungal morphology and hydrodynamic property of the fermentation broth. Compared to the conventional free cell culture, immobilization provides the advantage of avoiding cell washout when operating a continuous bioreactor at a high dilution rate. It also offers higher cell concentration, easier downstream processing and better morphological control, which in turn increase the bioreactor productivity (Elibol and Ozer, 2000; Nielsen, 1992; Singh et al., 1992; Tay and Yang, 2002).

Many immobilization techniques have been used to control fungal morphology, including flocculation with polyelectrolyte, covalent binding to glycidyl ester copolymer, entrapment in gel, and adsorption onto solid supports (Angelova et al., 1998; Ates et al., 2002; Elibol and Ozer, 2000; Lusta et al., 2000; Sankpal et al., 2001; Sankpal and

Kulkarni, 2002; Sun et al., 1999). Recently, a rotating fibrous bed bioreactor (RFBB) has been developed and successfully used to control fungal morphology in L(+)-lactic acid production by *R. oryzae* (Tay and Yang, 2002). In the RFBB, fungal growth appeared only on the rotating fibrous matrix provided; thus, resulting in a cell-free fermentation broth that was better for mixing and aeration. As a result of the improved oxygen transfer, the RFBB increased L(+)-lactic acid production as compared to the conventional fungal fermentation in the stirred tank fermentor (Tay and Yang, 2002; Thongchul and Yang, 2003). The RFBB was also easy to start up and operate because of its simple way for cell immobilization as compared to other methods. However, inappropriate spore immobilization and cell growth in the fibrous matrix may cause operational and diffusion problems, which in turn could lower bioreactor productivity (d'Enfert, 1997). It is thus important to understand how fungal spores and cells were or can be better immobilized on the fibrous matrix in order to further optimize the process.

Fungal growth on the fibrous matrix inside the RFBB begins with spore germination and immobilization. Spore germination may vary with fungal strains and is affected by many environmental factors, including temperature, pH, water activity, aeration, and carbon and nitrogen sources (d'Enfort, 1997; Medwid and Grant, 1984; Osherov and May, 2001). Spore immobilization in the RFBB may be influenced by the rotational speed of the fibrous bed and the chemical and physical properties of the fibrous matrix, including surface hydrophobicity and roughness of the fibers and the weaving structures of the matrix. Therefore, the main objectives of this study were to understand the mechanism of and to determine the optimal conditions for initial spore germination,

immobilization and subsequent cell growth on the fibrous matrix. Various types of fibrous matrices, including non-woven and woven cotton and polyethylene terephthalate (PET) cloth, were studied as supports for *R. oryzae* spore immobilization. The effects of the rotational speed of the matrix on spore germination and immobilization were also investigated. Based on the experimental results, plausible immobilization mechanisms and mathematical models were proposed and are presented in this chapter.

6.2 Materials and Methods

6.2.1 Microorganism, inoculum preparation, and medium composition

R. oryzae NRRL 395, a filamentous fungus producing L(+)-lactic acid, was obtained from the Northern Regional Research Center, Peroria, IL. The stock culture was maintained on potato dextrose agar (PDA) plates and subcultured every month to maintain cell viability. The growth medium containing 50 g/L glucose and 5 g/L yeast extract was used in spore germination and initial cell growth study. The spore suspension was prepared from 7-day culture on the PDA plate and adjusted to 10^7/mL by dilution with sterile water. The spore suspension was then used as the inoculum to the RFBB in spore germination and immobilization studies.

6.2.2 Bioreactor setup

The rotating fibrous bed bioreactor (RFBB) used was modified from a 5-L bioreactor (Biostat B, B. Braun). Briefly, a perforated stainless steel cylinder (9 cm in diameter and 15 cm in height) affixed with a fibrous matrix for spore immobilization was mounted onto the agitation shaft of the bioreactor. Detailed description of the RFBB setup is given in Appendix B. The reactor with 2 L of the growth medium was autoclaved at 121°C, 15 psig for 60 min. After cooling, a sufficient amount of the spore suspension was inoculated into the bioreactor to an initial spore concentration of ~10^6/mL. Unless otherwise noted, the RFBB was controlled at 30°C, aerated with 1 vvm sterile air, and the fibrous bed operated at a low rotational speed of 20 rpm or a higher speed of 60 rpm.

6.2.3 Spore germination and immobilization study

Five different fibrous matrices, including 100% cotton cloth (non-woven), cotton towel (woven), 100% PET (non-woven and woven), and 65% PET 35% cotton (woven) were studied. After inoculation, medium samples were taken immediately and every 1 h thereafter until no free spore remained in the medium. The spore morphology and concentrations in the liquid samples were examined under a light microscope and counted using a haemacytometer. Three types of spores were identified and counted separately – dormant, swelling, and germinated spores. Swelling spores were differentiated from the dormant spores by their increased diameter. Spore germination was defined as the

extension of a germ tube to a length equal to one-half of the diameter of the spore. Freely dispersed mycelia containing no more than 3 hyphal clumps were also counted as the germinated spores. For example, a freely dispersed mycelial clump containing 3 hyphae that had just been developed from the entanglement of 3 germinated spores was counted as 3 germinated spores, whereas a mycelial clump containing more than 3 hyphal loops was treated as self-immobilization and not counted as free spores. The concentration of immobilized spores, including both immobilized in the fibrous matrix and self-immobilization, was determined by subtracting the total spore concentration remained in the medium from the initial spore concentration.

6.2.4 Scanning electron microscopy

The spore morphology and localization in the fibrous matrices at different stages of spore germination were examined by scanning electron microscopy, which is described in Appendix D4.

6.3 Results and Discussion

6.3.1 Germination process

Figure 6.1 shows *R. oryzae* sporangiospores from different stages of germination. Germination process consisted of 2 phases. In phase I (spore enlargement), the spore

gradually swelled, resulting in an increase in both diameter (approximately from 5 μm to 12 μm) and biomass (Figure 6.1a and 6.1b). In this phase, there were rapid increases in the number of mitochondria and in the content of the active cell material (the size of the endoplasmic reticulum which is the location of the ribosome). New wall layers were also formed and laid down uniformly over the entire surface of the spore. In phase II, spore germination occurred by the initiation of growth polarity and the cease of wall formation followed by the outgrowth of hyphal element (germ tube) from the swelled spore (Figure 6.1c). The germinated spores tended to clump together, especially at the lower rotational speed (20 rpm) (Figure 6.1d). From the microscopic observation, an increase in the spore size was apparent after ~3 h. The presence of the germinated spores was found usually after ~4 h (Figures 6.2-6.6). Figure 6.7 shows the kinetics of spore germination at two different rotational speeds. It appeared that spore germination was slightly faster at 60 rpm than at 20 rpm, probably due to the higher mixing and mass transfer rates preventing spore aggregation and substrate limitation, which could occur inside spore aggregates and thus lower the germination rate (Bosch et al., 1995; Medwid and Grant, 1984; Nielsen, 1992; Nielsen and Carlsen, 1996).

6.3.2 Immobilization in the RFBB

R. oryzae spore immobilization occurred differently when using different fibrous matrices and rotational speeds. Figure 6.2a shows almost half of the total spores were irreversibly immobilized in the 100% cotton (non-woven) matrix at 60 rpm in the first

hour. Later, spores were slowly immobilized as indicated from the slow decrease in the total spore concentration remained in the medium during the period between 1 h and 6 h. After half of the spores were already germinated (approximately after 6 h), spores immobilization occurred rapidly again. Spore immobilization in the 100% cotton (non-woven) matrix at 20 rpm was almost similar to that at 60 rpm but the process was slightly slower (Figure 6.2b). Besides, some spores were not immobilized in the matrix but became aggregated and developed to small pellets later. Spore immobilization in the cotton towel matrix occurred very rapidly before the germination process started (Figure 6.3). Within the first hour, almost 70% and 80% of spores were immobilized in the matrix at 20 rpm and 60 rpm, respectively. Perfect spore immobilization in the cotton towel was observed at both rotational speeds as indicated by no cell biomass growth outside the fibrous matrix.

Figure 6.4 shows that before 6 h, spores were rarely immobilized in the 100% PET (non-woven) matrix at both rotational speeds. The fluctuation in spore concentration in the medium during this period showed that the spores were reversibly immobilized in the matrix. Later, spores were rapidly immobilized in the matrix. Pellet formation appeared in the later period ($t > 7$ h) at 20 rpm, whereas perfect immobilization in the matrix was observed at 60 rpm. Spore immobilization dynamics in the 100% PET (woven) matrix appeared similarly to that in the non-woven type (Figure 6.5); however, the spore adhesion strength was much weaker, which resulted in pellet formation and cell detachment from the fibrous matrix later. At 60 rpm, spores were rarely immobilized in the matrix but they germinated and self-immobilized in the medium producing pellets,

whereas at 20 rpm, more spores were immobilized in the matrix and less pellets were formed in the medium. The attachment to the matrix was also weak at 20 rpm; therefore, after 10 h, growth detachment from the matrix was found. The dynamics of spore immobilization in the 65% PET and 35% cotton matrix was similar to those in the PET matrices, at both rotational speeds (Figure 6.6).

6.3.3 Germination kinetics

Germination of *R. oryzae* sporangiospores was reported as a function of the spore population. A simple statistical model proposed by Bosch et al., 1995 was used to describe the germination kinetics as follows:

$$p(t) = K\frac{(t/\lambda)^c}{1+(t/\lambda)^c} \tag{6.1}$$

Where $p(t)$ is the fraction of the spores that germinate before time $t > 0$, $K(0 \le K \le 1)$ is the maximum fraction of germination; thus, by eq (6.1), $\lim_{t\to\infty} p(t) = K$. λ is the time required to reach 50% of the maximum germination, or $p(\lambda) = K/2$. The λ value is usually considered as an estimate of the germination rate. $c(c > 0)$ is the synchronization factor. In a perfectly synchronized process ($c \to \infty$), all spores are likely to have the same age and the cellular properties; therefore, they enter the germination process and the vegetative growth cycle at the same time. This indicates that $p(t) = 0$ for $t < \lambda$ and $p(t) = K$ for $t \ge \lambda$ ($p(t)$ becomes a step function).

It is indicated that phase II of the germination process begins when at least 1% of spores presents the germ tube. The lag phase of the germination process (t_l) is then determined by substituting $\beta = 0.01$ into eq (6.2) (Bosch et al., 1995; Medwid and Grant, 1984).

$$t_\beta = \lambda\left[\frac{\beta}{K-\beta}\right]^{1/c} \tag{6.2}$$

In the presence of glucose and yeast extract at both rotational speeds, *R. oryzae* spores completely germinated $(K = 1)$. At 60 rpm, spores started to germinate after approximately 2.91 h, whereas at 20 rpm, the lag phase (swelling process) was longer (approximately 3.49 h). The higher rotational speed increased the germination rate as indicated by the smaller value of λ (λ was 5.10 ± 0.39 h at 60 rpm and 5.43 ± 0.20 at 20 rpm). However, compared to other environmental factors such as temperature, pH, and nutrients, the rotational speed did not strongly influence *R. oryzae* spore germination in the RFBB (d'Enfert, 1997; Gabler et al., 2001; Medwid and Grant, 1984; Osherov and May, 2001).

6.3.4 Immobilization mechanisms

In the immobilization process, spores are retained partly by surface attachment (adsorption) and partly by self-aggregation in the pores and in the zones with the very low flow or dead end area in the matrix (entrapment) (Bazaraa et al., 1998; Grivel et al., 1999). According to surface attachment, it is indicated that surface properties of the

matrix determine the speed and the strength of spore adsorption. Three major surface properties include hydrophobicity, surface charge, and surface roughness (Meyer, 2003; Gotz, 2002; Flemming et al., 1996; Videla, 2002; Morton et al., 1998; Pasmore et al., 2001; Hibiya et al., 2000).

Spore can attach to both hydrophobic and hydrophilic surfaces but the attachment to hydrophobic surface is stronger. This is because although spore has more hydrophilic area on the surface of its membrane, these sites are highly attractive to water; and therefore, water-spore surface interaction is reversible and in rapid equilibrium. On the other hand, the hydrophobic sites exclude water through their interaction with the surface of the matrix; thus, this interaction is usually significantly stronger (Guimaraes et al., 2002).

Charged surface can readily provide sites for cell attachment through electrostatic attraction and ionic bond. Spore surface is generally made up of phospholipids, lipopolysaccharides, polysaccharides, and proteins. All of these macromolecules are charged in the physiological pH range which makes the membrane surface strongly attractive to the sites having the opposite charge.

Surface roughness affects spore attachment in 2 major ways. The first effect is the disruption of fluid flow. Rough surface creates the area of low shear where the force which may remove the attached spores is significantly reduced. Secondly, the increased roughness increases surface area, providing more available sites for spore attachment because rough surface contains contours and valleys, providing the location where spore can adsorb.

It is claimed that non-germinated spores reversibly adsorb on the surface of the matrix whereas germinated conidia irreversibly adsorb. This is because germinated conidia have a marked increase in the adhesive property. The adhesion mechanism usually consists of 2 steps: initial adhesion which results from a pre-existing glycoprotein layer or from a component of the cell wall and tighter adhesion which results from metabolic activation and protein synthesis. The tighter adhesion exists during the swelling process when the new cell wall components are constructed; therefore, resulting in the irreversible adhesion of germinated spores. Moreover, in the area of low shear disruption such as in the pores and the dead end zones, spore entrapment also occurs irreversibly (Osherov and May, 2001).

6.3.5 Immobilization kinetics

Spore immobilization either by the adsorption on the surface of the fiber in the matrix or the entrapment in the dead end zones or the void spaces in the matrix usually follows the first order kinetics as follows:

$$N + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} N - S \tag{6.3}$$

Where N is the total population of the spores including dormant, swelling, and germinated spores. S is the immobilization site. $N - S$ represents the spore-site interaction referring to the spore immobilization in the matrix. k_1 is the adsorption rate

constant and k_2 is the desorption rate constant. Thus, spore immobilization rate can be expressed as follows:

$$-\frac{dN}{dt} = k_1[S][N] - k_2[N-S] \tag{6.4}$$

Although the adsorption process is reversible, for germinated spores, the desorption rate is very slow as compared to the adsorption rate. Moreover, the spore entrapment occurs irreversibly. Therefore, the immobilization rate of the germinated and entrapped spores can be described by the following equation:

$$-\frac{dN}{dt} = k_1[S][N] = k[N] \tag{6.5}$$

By integration, eq (6.5) becomes:

$$N = N_0 e^{-kt} \tag{6.6}$$

Where $k = k_1[S]$ is the irreversible spore immobilization rate constant. N_0 is the initial spore concentration. From eq (6.6), by plotting the semi log plot of $\frac{N}{N_0}$ versus t, k can be determined from the slope of the plot.

Figures 6.8-6.10 compare spore immobilization kinetics at 2 different rotational speeds in different fibrous matrices. Spores were slowly immobilized at the first 6 h in most of matrices except for the cotton towel, in which spores were immobilized immediately after inoculation. After 6 h, a dramatic decrease in spore concentration in the medium indicated that spores were rapidly immobilized in the matrices and at the same time, some spores formed self aggregates which later developed to pellets or mycelial clumps in the medium.

According to the germination kinetics, *R. oryzae* spore started to germinate after approximately 3 h (2.91 h at 60 rpm and 3.49 h at 20 rpm); therefore, in the first 3 h, irreversible spore immobilization in the matrix was mainly by entrapment of non-germinated spores. Later immobilization occurred by either entrapment or adsorption of germinated spore on the fibrous surface. Besides *R. oryzae* germinated conidia might form self aggregates (pellet/clump) instead of immobilization in the matrix when the inappropriate rotational speed and the fibrous matrices were used in the immobilization process.

Table 6.1 shows the spore immobilization rate constant (k) at slow and fast immobilization periods. During the first 3 h, spore immobilization in the 100% cotton (non-woven) fiber at 60 rpm occurred mainly by the entrapment in the dead end zones (Figure 6.11a). Due to the limitation in the availability of dead end area and the less of germinated spores, the immobilization rate partly by entrapment and adsorption of germinated spores before 6 h was slow as indicated by the low k. After 6 h, the immobilization rate increased rapidly because of the increasing amount of the germinated spores. During this period, germinated spores were either entrapped in the void space or adsorbed on the surface of the fiber (Figure 6.11b). The similar immobilization process was also observed at 20 rpm; however, the rate during the slow immobilization period was slower because the lower rotational speed reduced the chance of spore attachment on the surface of the individual fibers in the matrix or entrapment in the dead end areas or the void spaces. Since the slow rotational speed caused the slow immobilization rate in the matrix, instead of being immobilized in the matrix provided, some of germinated

spores in the medium formed self aggregates as indicated by the presence of the pellets after 7 h in the medium (Table 6.1).

Spore immobilization in the cotton towel matrix mostly occurred at the first 3 h mainly by entrapment in the dead end areas or the void spaces in the fibrous matrix (Figure 6.3). This was probably because the cotton towel provided more available sites for spore immobilization compared to the other matrices. This led to the perfect spore immobilization in the matrix and no pellet or clump formation in the medium at both rotational speeds although the immobilization rate at 20 rpm was slower (Table 6.1).

The immobilization in the 100% PET (non-woven) matrix by entrapment rarely occurred in the first 6 h as indicated by the lower k compared to those in the cotton matrices. After spore germination started, it was found that the immobilization rate became faster indicating that the immobilization occurred mainly by adsorption of germinated spores on the surface of the fibrous matrix. Spore entrapment in the void space in the fibrous matrix rarely occurred (Figure 6.12). At 20 rpm, the similar spore immobilization process was obtained with the slower rate during the fast immobilization period. Besides, pellet formation was found at 20 rpm due to the slower immobilization rate in the matrix caused from the slower mixing.

Spore immobilization rate in the 100% PET (woven) matrix was similar to those in 100% PET (non-woven); however, the strength of immobilization was weaker. Due to the tight network structure and the smooth surface of the individual fiber of the matrix, it did not facilitate either entrapment or adsorption of germinated spore in the 100% PET

(woven) type (Figure 6.13). This resulted in the formation of pellet and the detachment of germinated conidia and subsequent growth later at the high rotational speed.

The immobilization process in 65% PET 35% cotton matrix showed the similarity to that found in the 100% cotton (non-woven) matrix. However, spore entrapment rarely occurred during the first 6 h (Table 6.1). In addition, the adsorption of germinated conidia in the matrix was slow so that self immobilization was observed (pellet formation) at both rotational speeds. This clearly indicated that besides, 100% PET (woven) matrix, 65% PET 35% cotton was not appropriate for *R. oryzae* spore immobilization.

6.3.6 Effects of fibrous matrix and rotational speed on immobilization

The results in this study show that spore immobilization in the cotton matrices (100% cotton fiber and cotton towel) was better than those in the PET (both non-woven and woven types) and the 65% PET 35% cotton matrices as indicated by the higher immobilization rate constant (k) during the slow immobilization rate period because the surface of the cotton matrices was rougher and the structure of the matrix consisted of the dead end zones and the well-fitted void spaces to the spores (Figure 6.13a and 6.13b). This could facilitate the adsorption on the surface of the fiber and the entrapment in the void spaces which, in turn, resulted in the better immobilization (Figure 6.11). On the other hand, the PET matrices constructed of the very smooth individual fibers (Figure 6.13c and 6.13d). This limited the chance of both non-germinated spores and germinated

conidia to adsorb on the surface of the individual fiber and eventually limited spore immobilization due to the high chance of shear disruption (Figure 6.12).

Compared to the 100% cotton (non-woven) matrix, cotton towel (woven) had the smaller void spaces between the individual fibers and the fibrous structure was denser and more uniformly (Figure 6.13a and 6.13b). Therefore, the individual spore (either non-germinated or germinated) could be easily entrapped in those void spaces which resulted in the high immobilization rate and the perfect immobilization in the matrix.

In case of PET matrices, the woven type provided the worse sites for spore immobilization compared to the non-woven type. This is because the woven PET had a very dense and uniform structure which was difficult for spores to get through this structure (Figure 6.13d). Therefore, immobilization could occur mostly at the outer surface of the matrix where the high shear disruption occurred. This resulted in the weak immobilization strength and cell detachment from the matrix. On the other hand, the strength of spore adsorption at the surface of the non-woven type fiber was stronger because of the fluffier structure of the matrix network (Figure 6.13c). The 65% PET and 35% cotton (woven) and the 100% PET (woven) matrices had the similar structure; therefore, the immobilization behavior and strength were very similar to each other (Figure 6.13e). However, the 65% PET 35% cotton fibrous matrix partly contained cotton fibers which had the rough surface; thus, reducing the shear disruption. This led to the slightly higher immobilization rate compared to that of the PET (woven) matrix.

Due to the higher flow through the network of the matrix, this provided the better opportunity for spore to get in and attach in the matrix and resulted in the higher spore

immobilization rate in all 5 of the fibrous matrices at the higher rotational speed. Additionally, the high rotational speed helped prevent germinated spores forming aggregates and developed to the pellet in the medium. This, in turn, led to the perfect immobilization in the fibrous matrix. However, the higher chance of shear disruption limited spore adsorption at the outer surface of the matrix especially when using the matrix such as the PET matrices that had the smooth fiber surface and lacked the contours and valleys to lower the shear force (Table 6.1).

6.4 Conclusion

Immobilization of *R. oryzae* spores appeared differently in different fibrous matrices. In the cotton matrices, spores were irreversibly entrapped in the void spaces or the dead end zones and became swelled and germinated to produce the vegetative conidia (Figure 6.11). The higher rotational speed promoted the spore entrapment resulting in the rapid immobilization process. In the 100% PET and 65% PET 35% cotton matrices, non-germinated spores were reversibly adsorbed on the surface of the fiber in the matrix. After the germination started, germinated spores were irreversibly attached on the surface of the fiber in the matrix due to the tighter adhesion, whereas immobilization by entrapment rarely occurred in these matrices (Figure 6.12). In these cases, the lower rotational speed resulting in a lower shear force disruption promoted the stronger spore adsorption at the outer surface of the matrix. The results in this study showed that the rotational speed, the surface property of the fiber, and the structure of the matrix (non-

woven or woven) were important factors determining the immobilization process in the RFBB. Generally, at the low rotational speed, a higher chance of self-immobilization in the medium occurred. The structure of the matrix played a role in irreversible spore entrapment. The matrix void spaces, approximately having the similar size of the swelled spore, were required for perfect immobilization in the matrix. Also, they helped prevent pellet or clump formation in the medium. Rough surface of the cotton fibers reduced shear damage due to its shear disruption effect and more surface areas for spore adsorption. Therefore, rough surface was more favorable to the immobilization process as well.

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Matrix	Speed (rpm)	Immobilization on the matrix		Slow immobilization		Fast immobilization	
		Perfect	Partial	k (h^{-1})[a]	Time (h)	k (h^{-1})[a]	Time (h)
100% cotton (non-woven)	60	•		0.1594	0-6	1.1864	6-9
	20		•	0.1290	0-8	1.7777	8-10
Cotton towel (woven)	60	•				0.7472	
	20	•				0.4271	
100% PET (non-woven)	60	•		0.0252	0-6	1.3123	6-9
	20		•	0.0417	0-6	1.1401	6-9
100% PET (woven)	60		•	0.0767	0-6	1.6720	6-9
	20		•	0.0369	0-7	0.7780	7-10
65% PET 35% cotton (woven)	60		•	0.0810	0-7	1.4311	7-9
	20		•	0.0432	0-7	1.7987	7-9

[a] First-order spore immobilization kinetics is expressed by $N = N_0 e^{-kt}$ where k is the immobilization rate constant

Table 6.1 Spore immobilization behavior and kinetics on the different rotating fibrous matrices in the RFBB

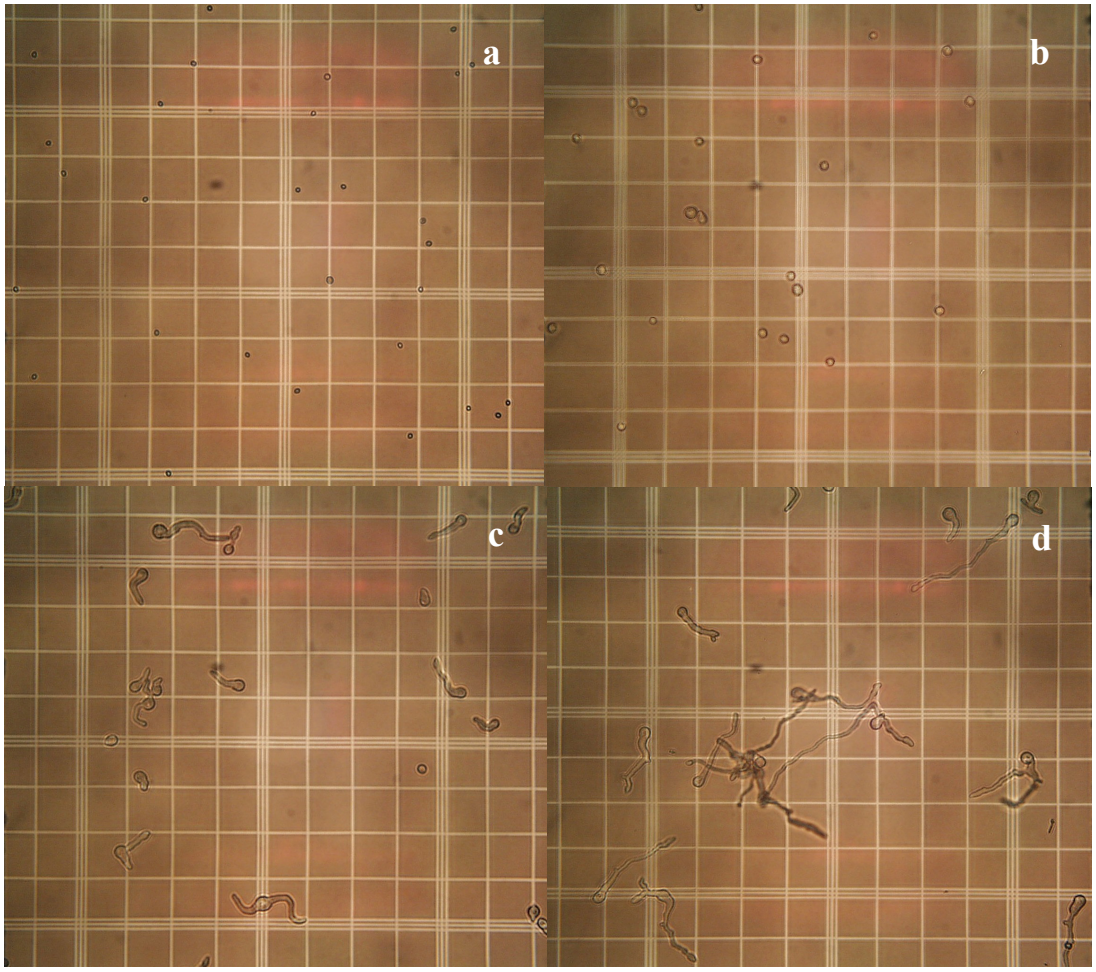

Figure 6.1 (a) Dormant sporangiospores, (b) Swelling sporangiospores, (c) Germinating sporangiospores, (d) Germinated spores forming an aggregate

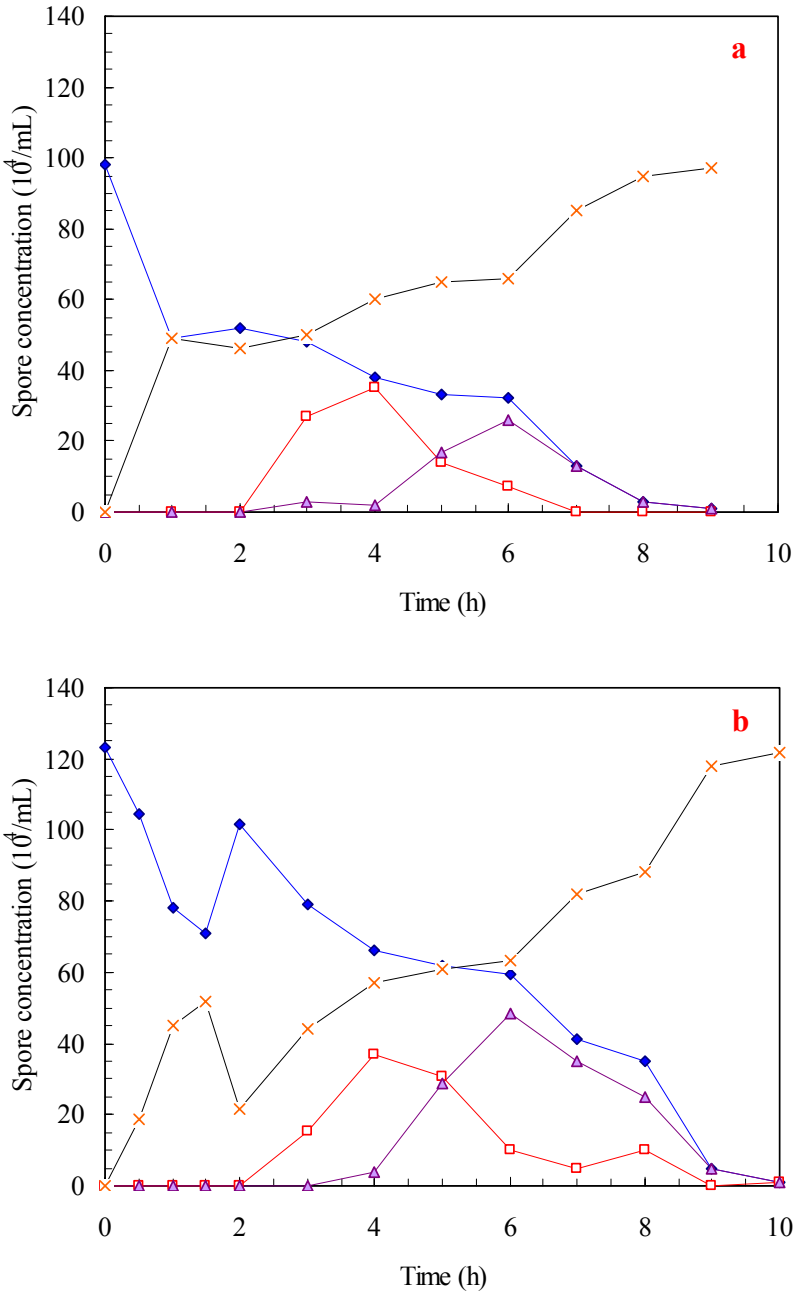

Figure 6.2 Dynamics of *R. oryzae* spore germination and immobilization in 100% cotton (non-woven) fiber at (a) 60 rpm, and (b) 20 rpm where ◆ is the total spore concentration, □ is the swelling spore concentration, △ is the germinated spore concentration, and × is the immobilized spore concentration

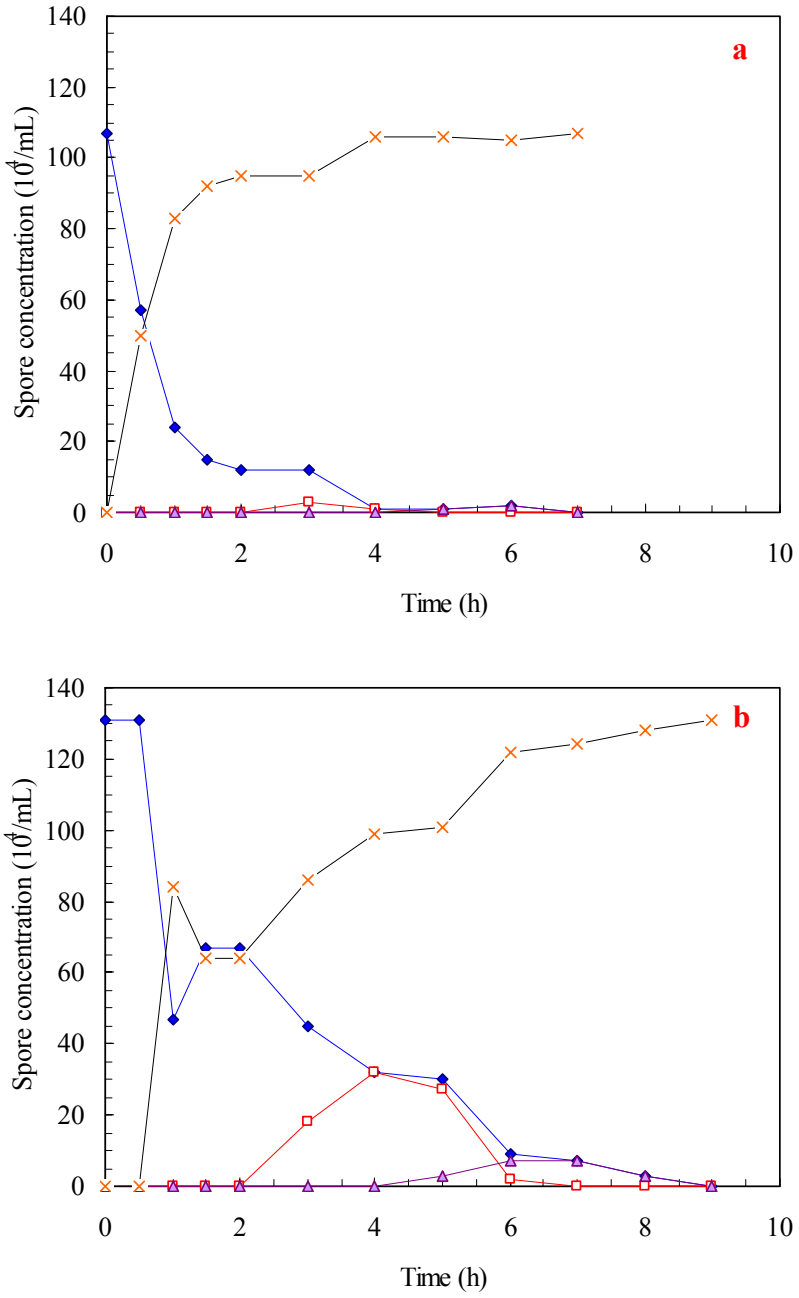

Figure 6.3 Dynamics of *R. oryzae* spore germination and immobilization in cotton towel fiber at (a) 60 rpm, and (b) 20 rpm where ◆ is the total spore concentration, □ is the swelling spore concentration, △ is the germinated spore concentration, and × is the immobilized spore concentration

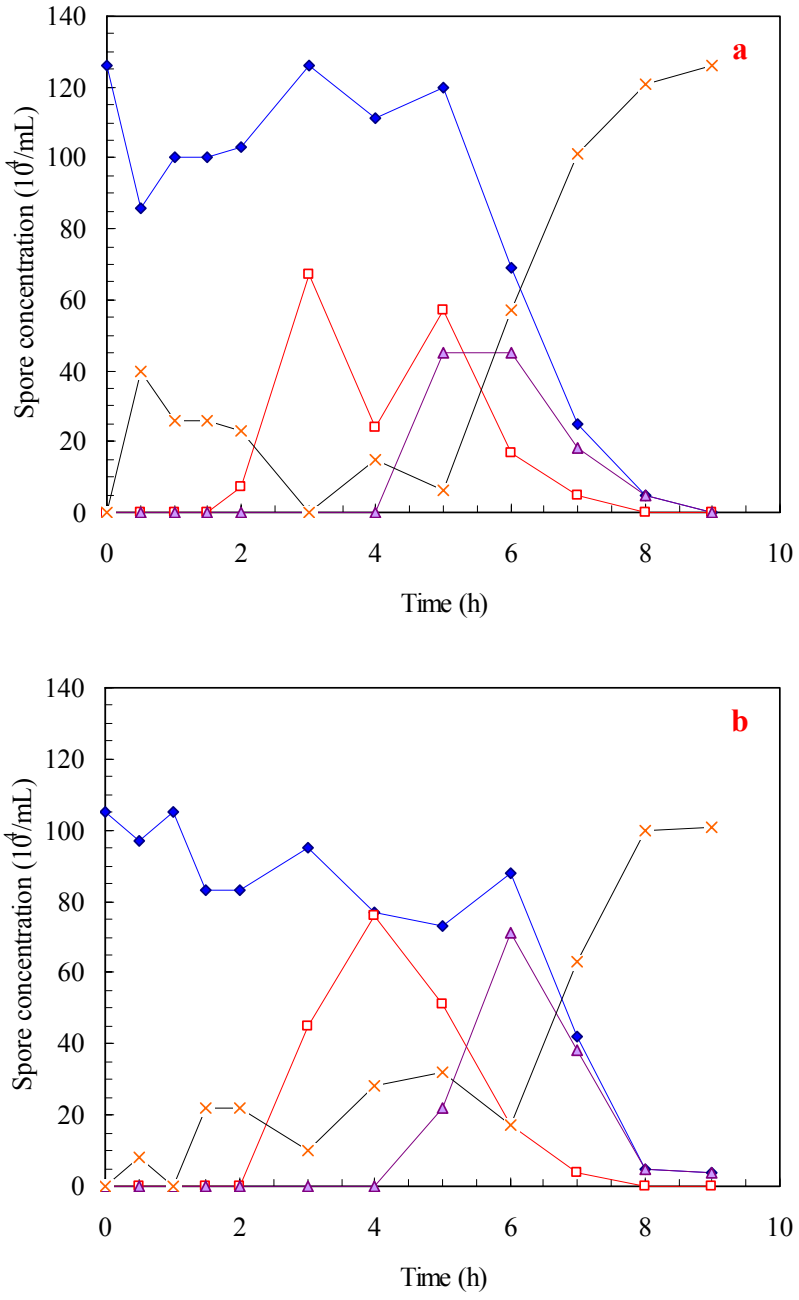

Figure 6.4 Dynamics of *R. oryzae* spore germination and immobilization in 100% PET (non-woven) fiber at (a) 60 rpm, and (b) 20 rpm where ◆ is the total spore concentration, □ is the swelling spore concentration, △ is the germinated spore concentration, and × is the immobilized spore concentration

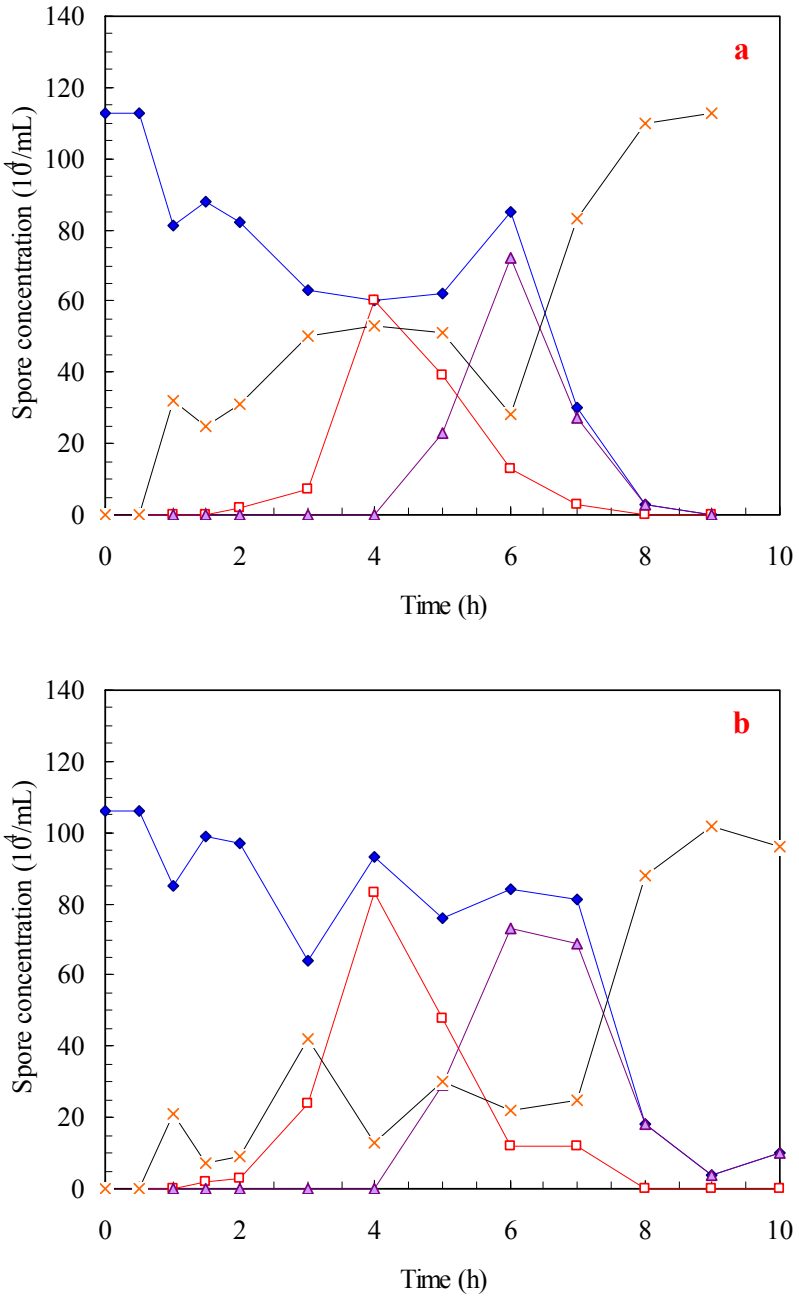

Figure 6.5 Dynamics of *R. oryzae* spore germination and immobilization in 100% PET (woven) fiber at (a) 60 rpm, and (b) 20 rpm where ◆ is the total spore concentration, □ is the swelling spore concentration, △ is the germinated spore concentration, and × is the immobilized spore concentration

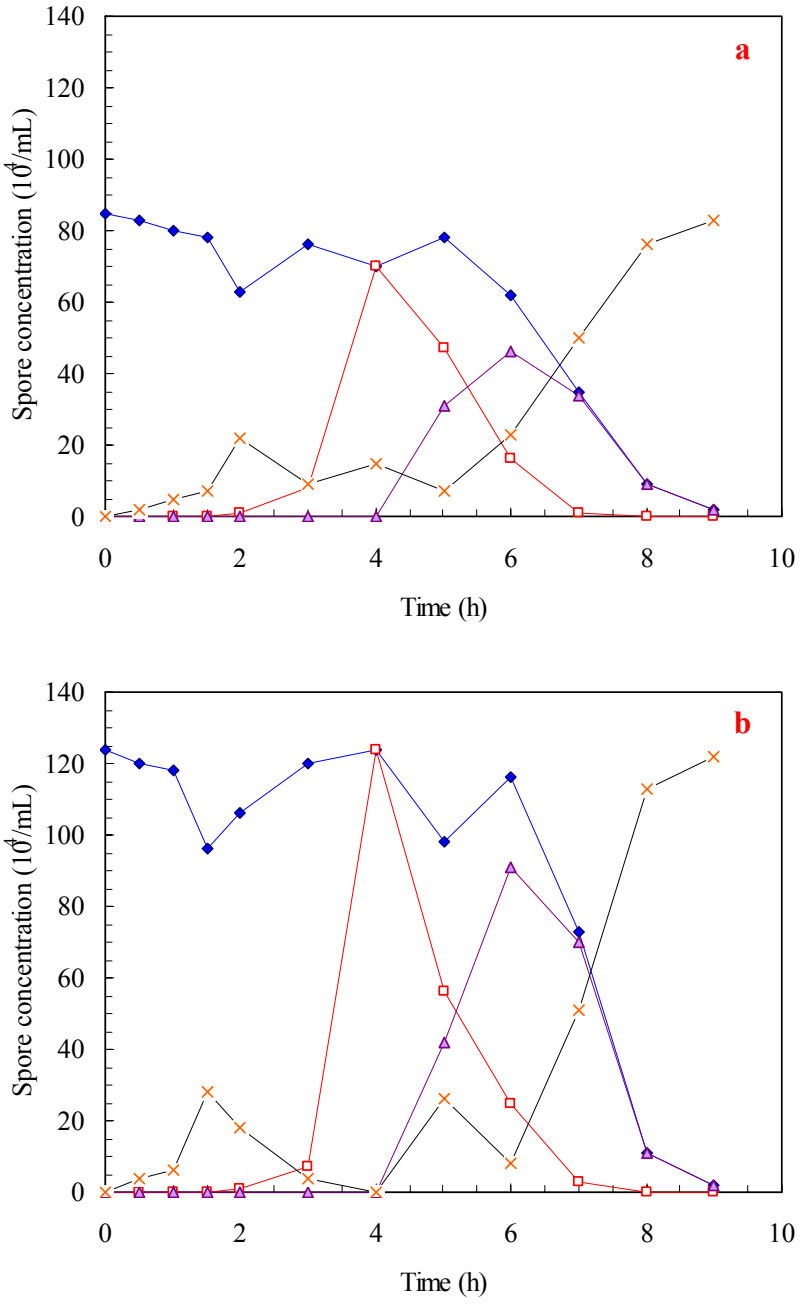

Figure 6.6 Dynamics of *R. oryzae* spore germination and immobilization in 65% PET 35% cotton (woven) fiber at (a) 60 rpm, and (b) 20 rpm where ◆ is the total spore concentration, □ is the swelling spore concentration, △ is the germinated spore concentration, and × is the immobilized spore concentration

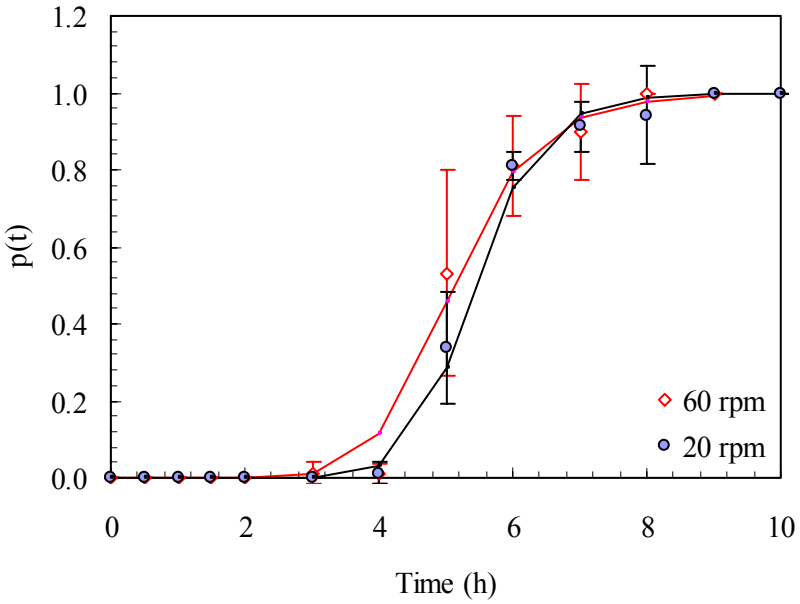

Figure 6.7 Germination kinetics of *R. oryzae* in the RFBB (50 g/L glucose, 5 g/L yeast extract, 30°C, and 1 vvm air)

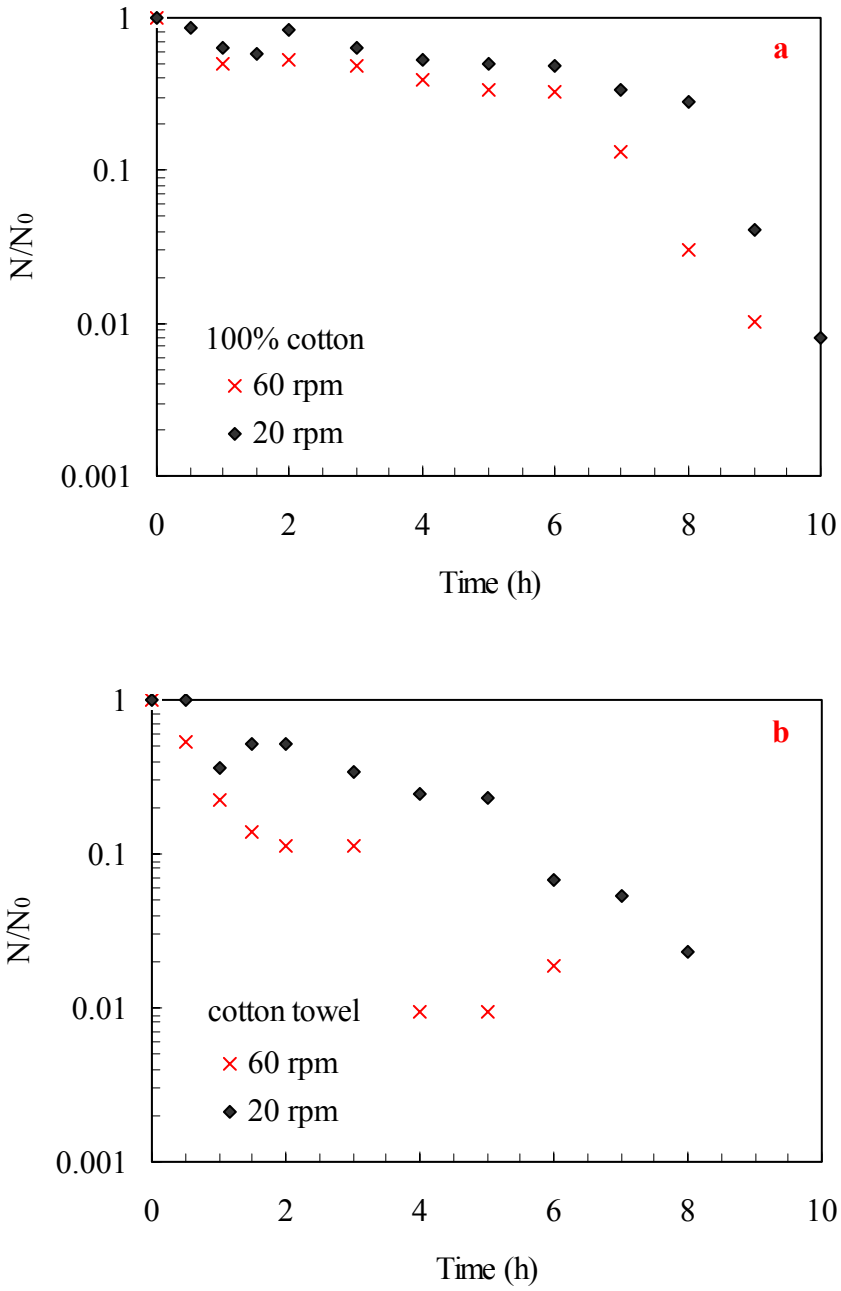

Figure 6.8 *R. oryzae* spore immobilization kinetics at 20 and 60 rpm in (a) 100% cotton fiber (non-woven), and (b) cotton towel (woven)

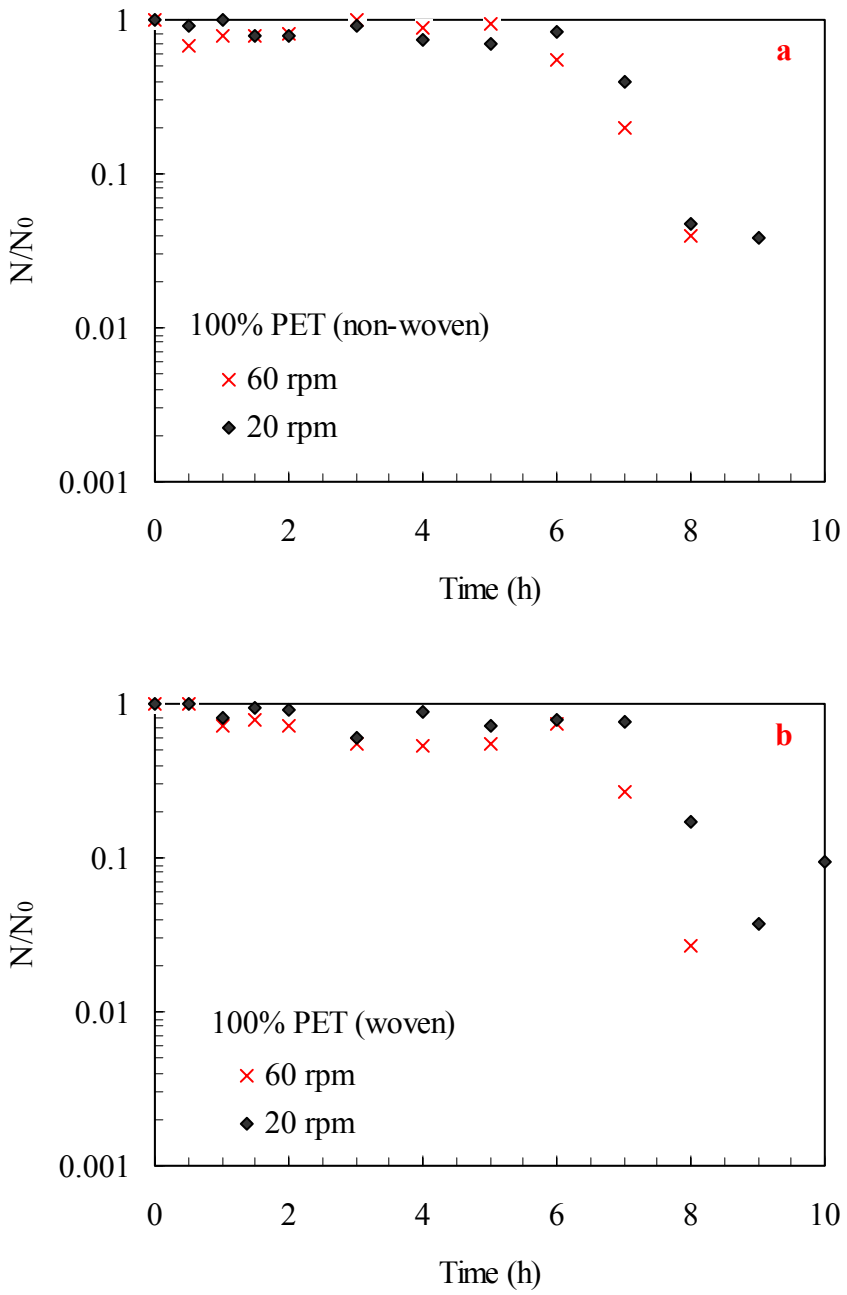

Figure 6.9 *R. oryzae* spore immobilization kinetics at 20 and 60 rpm in 100% PET fiber, (a) non-woven type, and (b) woven type

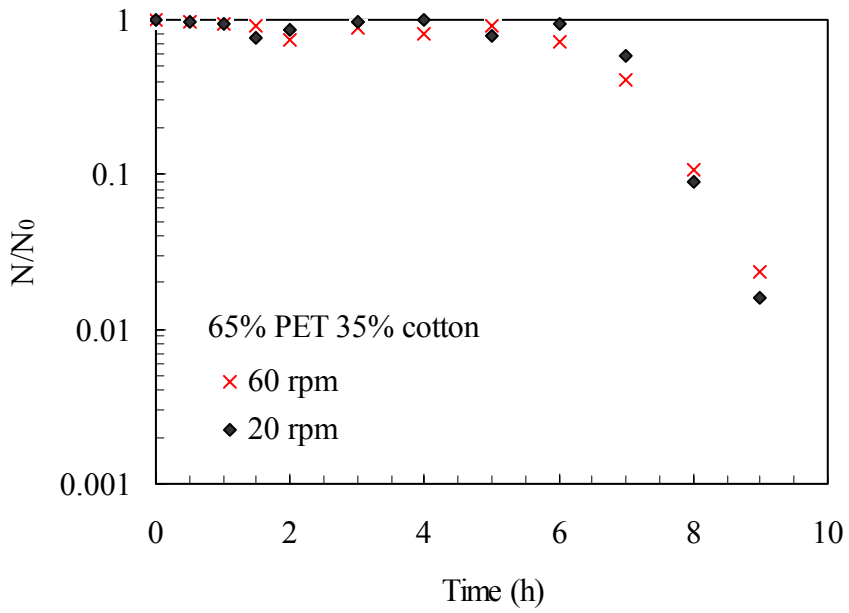

Figure 6.10 *R. oryzae* spore immobilization kinetics at 20 and 60 rpm in 65% PET 35% cotton (woven) fiber

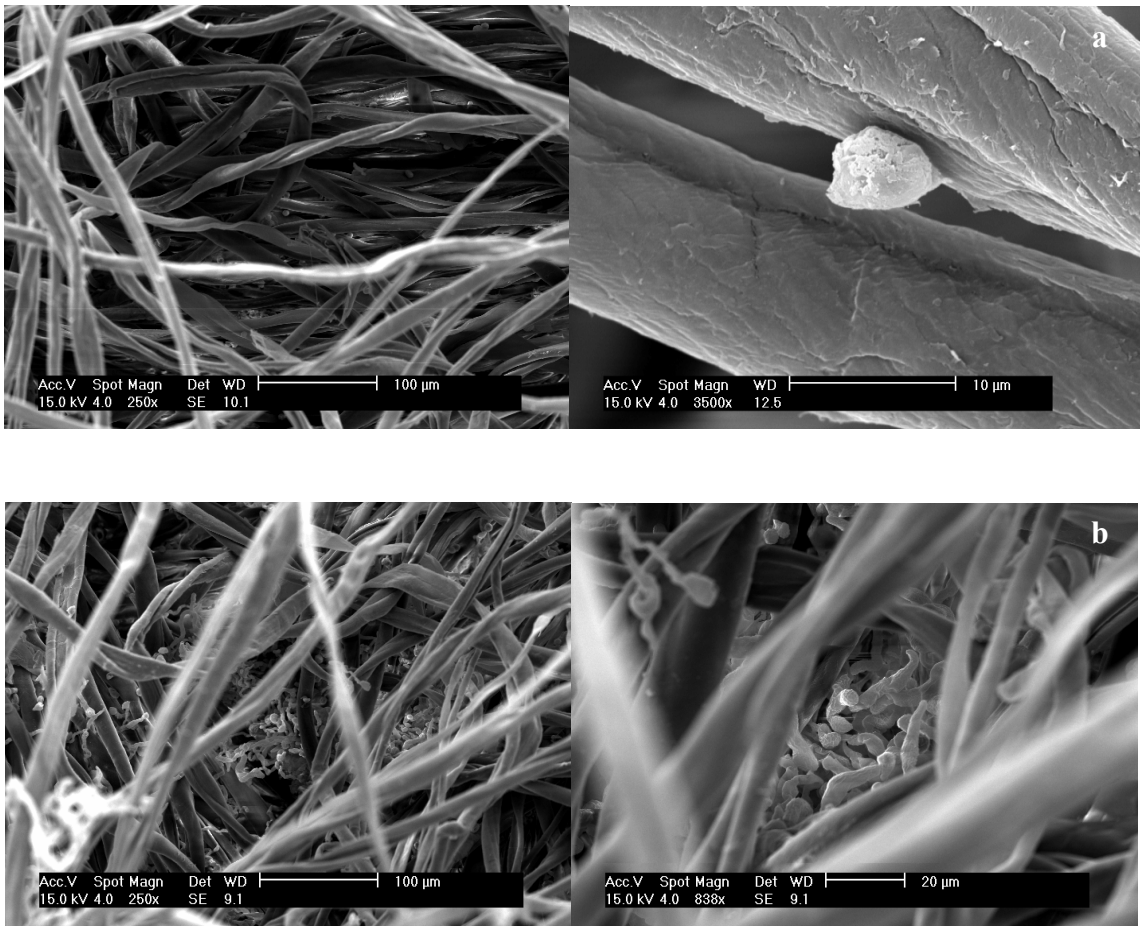

Figure 6.11 *R. oryzae* spore localization in 100% cotton (non-woven) fibrous matrix at the germination period of (a) 3 h, and (b) 6 h

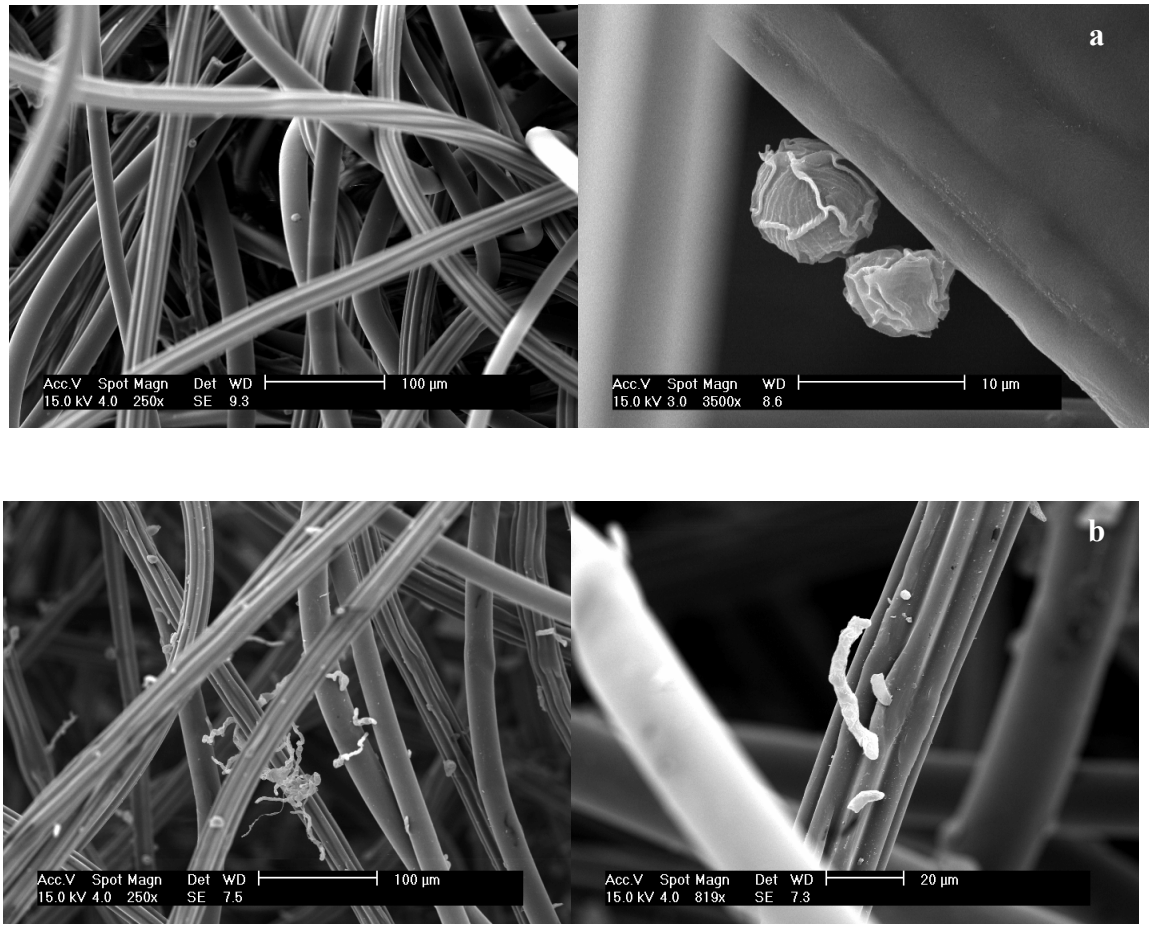

Figure 6.12 *R. oryzae* spore localization in 100% PET (non-woven) fibrous matrix at the germination period of (a) 3 h, and (b) 6 h

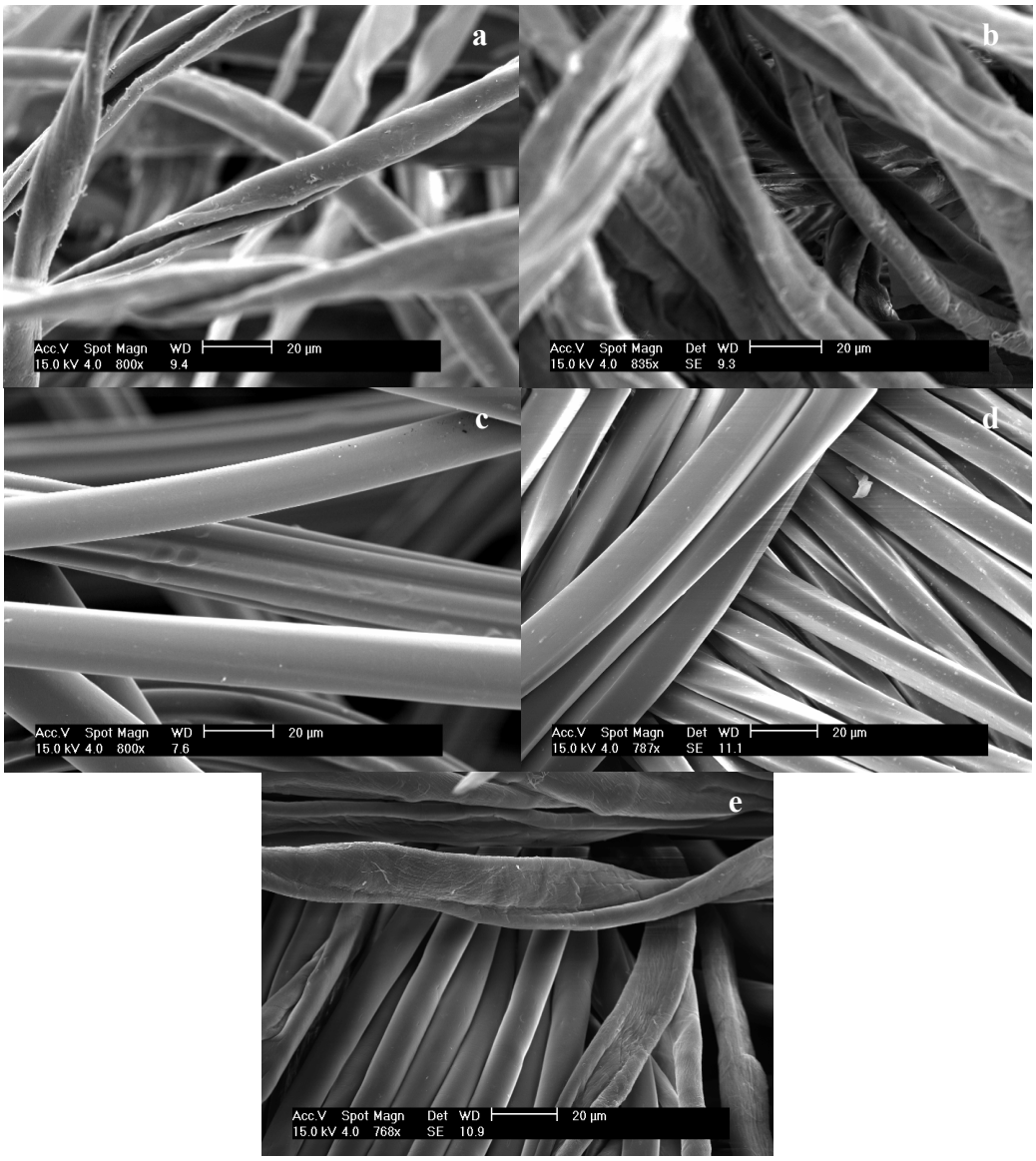

Figure 6.13 Structure of the fibrous matrices, (a) 100 % cotton (non-woven), (b) cotton towel (woven), (c) 100% PET (non-woven), (d) 100% PET (woven), and (e) 65% PET 35% cotton (woven)

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

This work demonstrated the advantages of using an immobilized cell bioreactor RFBB for controlling fungal morphology, the feasibility of using low-value substrates derived from agricultural residues and plant biomass for the production of lactic acid, and the control of fungal cells immobilized on a rotating fibrous matrix in the RFBB. The important results and conclusions obtained in this study are summarized below.

7.1.1 Comparing free cells with immobilized cell culture

- The fermentation carried out in the RFBB resulted in good control of the filamentous morphology, and improved oxygen transfer and lactic acid production from glucose.
- A high lactic acid concentration of 137 g/L with a high yield of 0.83 g/g and reactor productivity of 2.1 g/L·h was obtained with the RFBB in repeated batch fermentations of glucose.

- The improved oxygen transfer in the RFBB not only increased the fermentation rate and lactic acid production, but also eliminated undesirable ethanol production and allowed continuous operation of the bioreactor for long-term production.

7.1.2 Preference of carbon source for lactic acid production

- Fermentations of glucose, corn starch (both soluble and insoluble), and xylose gave similar lactic acid yield.
- Fermentations of insoluble starch and xylose gave lower productivity than those of glucose and soluble starch because of the slow saccharification step and poor oxygen transfer in the fermentation of insoluble starch and the complicated xylose metabolism via consecutive oxido-reductive, Pentose Phosphate (HMP), and Embden-Mayerhof-Parnas (EMP) pathways.
- Long-term lactic acid production from soluble starch showed the possibility of using agricultural residues containing starchy materials in fermentation by *R. oryzae*.
- Long-term fermentation of mixed carbon substrates (glucose/xylose) showed the preference of *R. oryzae* for glucose to produce lactic acid whereas xylose was preferably converted to biomass and fumaric acid.
- In the fermentation of corn fiber hydrolysate (CFH), *R. oryzae* rarely metabolized CFH to lactic acid, indicating that CFH might contain some compounds which inhibited lactic acid production.

7.1.3 Controlling *R. oryzae* growth

- Maintaining a high dissolved oxygen (DO) level did not improve lactic acid production although lactic acid productivity increased with increasing the oxygen transfer rate.
- Ethanol production was found even at high oxygen transfer rate, indicating an anoxic condition in the fungal cells immobilized on the rotating fibrous matrix.
- The estimation of the critical biofilm thickness from the oxygen diffusion model showed the occurrence of oxygen starvation inside the overgrown biofilm.
- Hyphal shaving off by shear rate could be used to control biofilm thickness; and therefore, prevent oxygen diffusion limitation.
- From the shaving mechanism and the proposed model, it was found that the rotational speed used in this study was too low to be effective in controlling biofilm growth; and thus, did not improve lactic acid production.
- Biomass production was reduced but lactic acid production increased when there was no urea in the production medium.

7.1.4 Spore germination and cell immobilization

- Spore germination consisted of 2 distinguishable phases, including spore swelling and growth polarization (germ tube extension).

- Compared to other environmental factors, the rotational speed in the RFBB did not greatly affect germination process.
- High rotational speed promoted spore immobilization by entrapment in the matrix but it also reduced the strength of spore adsorption on the surface of the fibers in the matrix.
- Rotational speed, surface property (roughness), and structure of the matrix (non-woven or woven) were important factors determining the immobilization process.
- Cotton matrices including non-woven and woven cotton towel were the preferential fibrous matrices for *R. oryzae* spore immobilization as compared to polyethylene terephthalate (PET) matrices.
- After inoculation, the entrapment of non-germinated spores occurred in the void space of cotton matrices where the germination occurred, whereas in PET matrices, spores were mostly germinated in the medium before they were adsorbed onto the PET fibers due to the increased surface adhesion force of the germinated spores.

7.2 Recommendations

This research used process engineering methods to optimize the bioprocess for lactic acid production; however, many problems still remain unsolved and need to be studied in more details in order to provide better understanding of the fermentation process. Two major areas are suggested for future study.

7.2.1 Process engineering methods

- So far the problem in oxygen diffusion in the immobilized cells on the rotating fibrous matrix is still unsolved, although oxygen transfer in the fermentation medium has been improved. The biofilm structure such as density and porosity plays an important role in diffusion; therefore, to enhance oxygen diffusion, the structural effect of the biofilm should be clearly determined and controlled.
- Due to the limitation in increasing rotational speed in the RFBB, the shaving mechanism was limited by the limited shear force. To limit the overgrown biofilm thickness, the other techniques to increase shear force acting on the surface of the biofilm should be identified.
- To better control the subsequent growth after spore immobilization, it is necessary to determine the dynamic growth after spore immobilization. The strength of cell attachment as well as cell detachment should be studied at various states of fermentation with various cell types, including active, inactive, damaged, and dead cells. Types of interaction (both physical and chemical) and conditions in cell immobilization at various states of fermentation should be clearly determined.

7.2.2 Metabolic/genetic engineering methods

- In the fermentation of CFH, besides the carbon sources, all other compounds such as nitrogen sources and inhibitory compounds should be clearly analyzed. In case

that inhibitory compounds are found, pretreatment may be required to remove these substances before use in the fermentation. In addition, the nitrogen source, which was not sufficiently present in the CFH, could be the key determining the metabolism of this substrate by *R. oryzae*.

- In long-term fermentation of mixed carbon substrates (glucose/xylose), an optimal feeding mode is necessary to maintain fungal activity and enhance lactic acid fermentation. Therefore, it is important to determine the expression level of key enzymes involving in glucose/xylose metabolism and lactic acid production during the fermentation. By combining the enzyme expression level and fermentation kinetics, the metabolism of *R. oryzae* and the optimal feeding strategy can be clearly determined.
- Besides carbohydrate metabolism, nitrogen flux analysis in *R. oryzae* should be studied to better understand the physiology of this fungal strain adapted in the RFBB. With this knowledge, fungal growth can be better controlled to further enhance lactic acid production.
- Use of genetically engineered *R. oryzae* to overexpress lactic acid production genes and limit cell growth and ethanol production from the substrate derived from plant biomass (hemicellulose-based substrate) is another alternative to improve lactic acid production. Various genetic engineering methods should be explored, including overexpression of *ldh* gene encoding lactate dehygenase required in the conversion of pyruvic acid to lactic acid, suppression of *pdc* gene encoding pyruvate carboxylase to prevent the conversion of pyruvate to acetyl

CoA and aldehyde, disruption of *adh* gene encoding alcohol dehydrogenase involving in the ethanol production, and effective regeneration of NADH required in lactic acid production.

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APPENDIX A

CULTURE, INOCULUM PREPARATION, AND MEDIUM COMPOSITIONS

A1 Culture

Rhizopus oryzae NRRL 395, a filamentous fungus producing L(+)-lactic acid was obtained from the Northern Regional Research Center, Peroria, IL. The stock culture was kept on potato dextrose agar (PDA) plate at 4°C. To maintain the viability, in every month the stock culture was transferred to the new PDA plate and incubated at 30°C for 7 days.

A2 Inoculum Preparation

The sporangiospores were collected from the 7-day culture on PDA plates by shaving and extracting the spores with sterile water. The spore concentration was determined by spore counting using a haemacytometer. The spore suspension was then adjusted to desired concentration by dilution with sterile water.

A3 Medium Compositions

Growth medium

The growth medium consisted of 50 g/L substrate and 5 g/L yeast extract. Yeast extract was autoclaved separately from the substrate to prevent any undesirable reaction due to heat sterilization. The control of pH was not necessary during the growth phase.

Production medium

Unless otherwise mentioned, the medium for enhancing lactic acid production consisted of 70 g/L substrate, 0.6 g/L KH_2PO_4, 0.25 g/L $MgSO_4$, 0.088 g/L $ZnSO_4$, and

0.3 g/L urea. $ZnSO_4$ and urea were autoclaved separately from the other compositions to prevent undesirable reaction.

APPENDIX B

BIOREACTOR SETUP

B1 Bioreactor Construction

A Rotating Fibrous Bed bioreactor (RFBB) was modified from a 5-L bioreactor (Biostat B, B. Braun) by affixing a perforated stainless steel cylinder mounted with a fibrous matrix (usually, 100% non-woven cotton fiber) to the agitation shaft (Figure B.1). Two different sizes of the cylindrical matrices were used in this research including the one with 6 cm diameter and 10 cm height and the other one with 9 cm diameter and 15 cm height.

B2 Bioreactor Start-up and Cell Immobilization

Before use, the bioreactor was sterilized twice at 121°C for 60 min with an overnight interval. The bioreactor containing growth medium was autoclaved at 121°C for 60 min. After cooling, the bioreactor was connected to the controller. The dissolved oxygen (DO) probe was calibrated with air and nitrogen. Unless otherwise noted, the bioreactor was operated at 30°C and agitated at 60 rpm, without pH control during the growth phase, and aerated with filter-sterilized air at 1.0 vvm (0.2μ, Micropore). The bioreactor was then inoculated with spore suspension at desired concentration and amount. Antifoam A (Sigma) was manually added to prevent foaming during fermentation. With the 10 mL of 10^6/mL spore suspension, usually cell growth and immobilization occurred within 2 days with no visible filamentous growth in the medium.

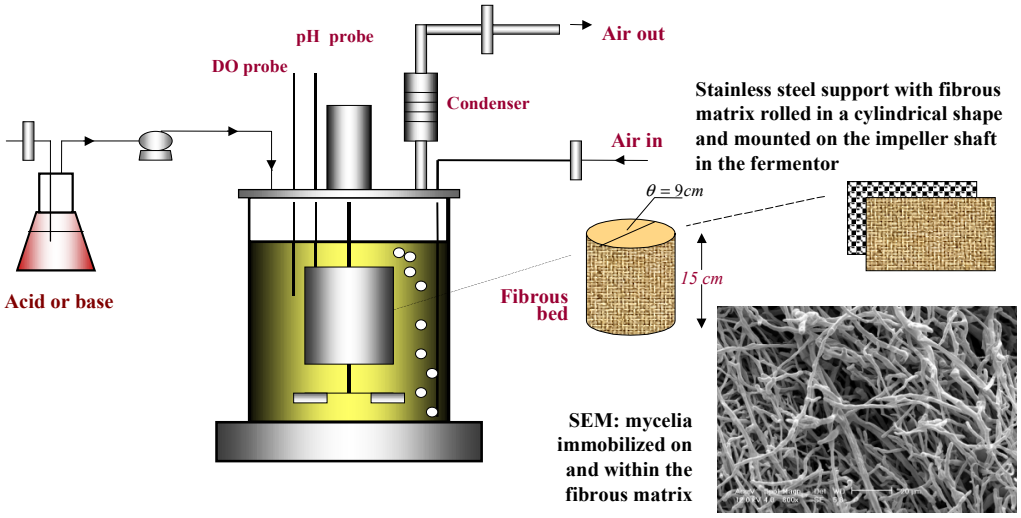


Figure B.1 A rotating fibrous bed bioreactor (RFBB) used in this study for filamentous fungal fermentation

APPENDIX C

OXYGEN TRANSFER DETERMINATION

Before the end of each fermentation batch, the oxygen uptake rate (OUR) and the dynamics of oxygen transfer in the bioreactor were studied using the dynamic method of gassing out. In the experiment, aeration was stopped to allow the dissolved oxygen concentration (C_L) in the medium to decrease, and aeration was then resumed. Figure C.1 shows a typical oxygen concentration profile during one experiment. The change in C_L with time, $\frac{dC_L}{dt}$ can be expressed by the following equation:

$$\frac{dC_L}{dt} = OTR - OUR \tag{C.1}$$

where OTR is the oxygen transfer rate in the medium due to aeration and can be expressed as:

$$OTR = k_L a \cdot (C^* - C_L) \tag{C.2}$$

where C^* is the solubility of oxygen in the medium (mM) and $k_L a$ is the volumetric oxygen transfer coefficient (min^{-1}). When there is no aeration, $OTR = 0$ and OUR (mM/min) can be determined from the slope of the plot of C_L versus time during the period without aeration. Both $k_L a$ and C^* can be then estimated from the plot of $\left(\frac{dC_L}{dt} + OUR\right)$ versus C_L during the period with aeration, with $k_L a$ equal to the negative slope and C^* equal to the Y-intercept divided by $k_L a$.

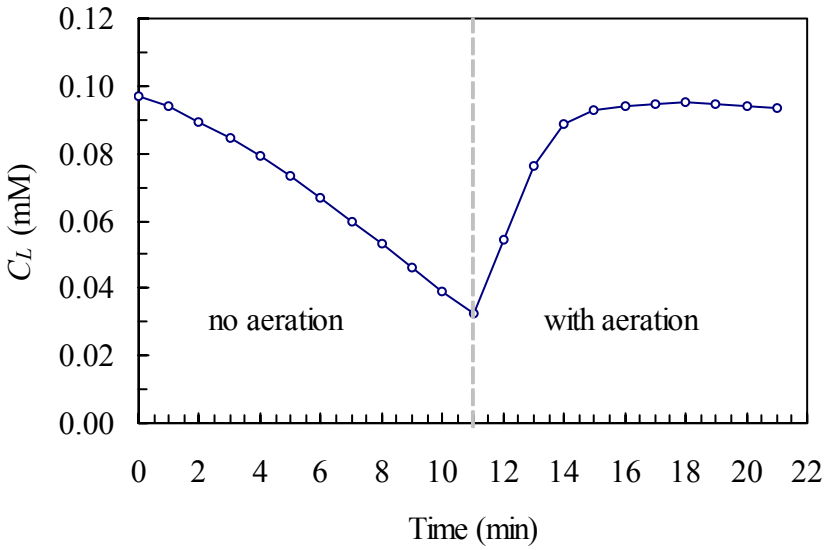


Figure C.1 A typical dissolved oxygen profile in the bioreactor obtained using the dynamic method of gassing out in the oxygen transfer experiment

APPENDIX D

ANALYTICAL METHODS

D1 Cell Biomass

Cell biomass of *Rhizopus oryzae* was measured at the end of fermentation. Biomass was harvested from the bioreactor and washed with water to remove the residues. Washed biomass was dried at 105°C until constant weight was obtained. Dry biomass weight was measured. The biomass yield, $Y_{x/s}$ was calculated from the total dry biomass weight divided by the total substrate consumed.

D2 Glucose Analyzer

Glucose and L(+)-lactic acid were analyzed by YSI 2700 glucose analyzer (Yellow Spring Instrument Co., Inc.). This analytical instrument is accurate within the range of 0-2.5 g/L glucose and 0-0.5 g/L lactic acid. Before measurement, fermentation broth was centrifuged and diluted with distilled water. The calibrator standard contained 0.5 g/L L(+)-lactic acid, 2.5 g/L glucose, 1.0 g/L benzoic acid, and 2.0 g/L NaEDTA. The buffer powder used in this equipment was prepared by mixing 4.4 g K_2H_2EDTA, 0.05 g kanamycin sulfate, 7.3 g sodium benzoate, 12.0 g NaH_2PO_4, 54.7 g Na_2PO_4, and 21.5 g NaCl together and grinded to powder-form. The buffer solution was prepared by dissolving 12.7 g buffer powder in 900 mL distilled water.

D3 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was used to analyze the organic compounds (glucose, xylose, arabinose, lactic acid, fumaric acid, and ethanol) present in the fermentation broth. Samples from the fermentation broth were centrifuged,

and diluted with distilled water. 15 μL diluted particle-free samples were injected by an autosampler (Shimadzu-SIL-10Ai) into an organic acid analysis column (Biorad, Aminex HPX-87H ion exclusion organic acid column; 300mm×7.8mm) maintained at 45°C in a column oven (Shimadzu-CTO-10A VP). 0.01 N H_2SO_4 was used as an eluant at 0.6 mL/min flow rate. An RI detector (Shimadzu-RID-10A) was set at the range of 200 to detect the organic compounds. A standard containing 2 g/L of each component was injected as a reference to determine the sample concentration. The peak height was used for the comparison basis. It is noted that HPLC can detect both L(+) and (D)-lactic acids.

D4 Scanning Electron Microscopy

Small pieces of samples (0.5cm × 0.5cm) were cut from the different parts of the matrices. The samples were fixed by immersing in 2.5% glutaldehyde solution overnight at 4°C. Later they were rinsed by distilled water and progressively dehydrated in various concentrations of ethanol (from 20% to 100%) for 20 min at each concentration. The samples were then immersed in various concentrations of hexamethyl disilazane (HMDS) (ethanol:HMDS; 3:1, 1:1, 1:3) for 15 min at each concentration and finally immersed in 100% HMDS for 15 min 3 times. Before examination, the samples were coated with palladium and gold using the spotter coating machine in the presence of the medium containing argon gas. After coating, the samples were examined using Philips 30 XL scanning electron microscope.

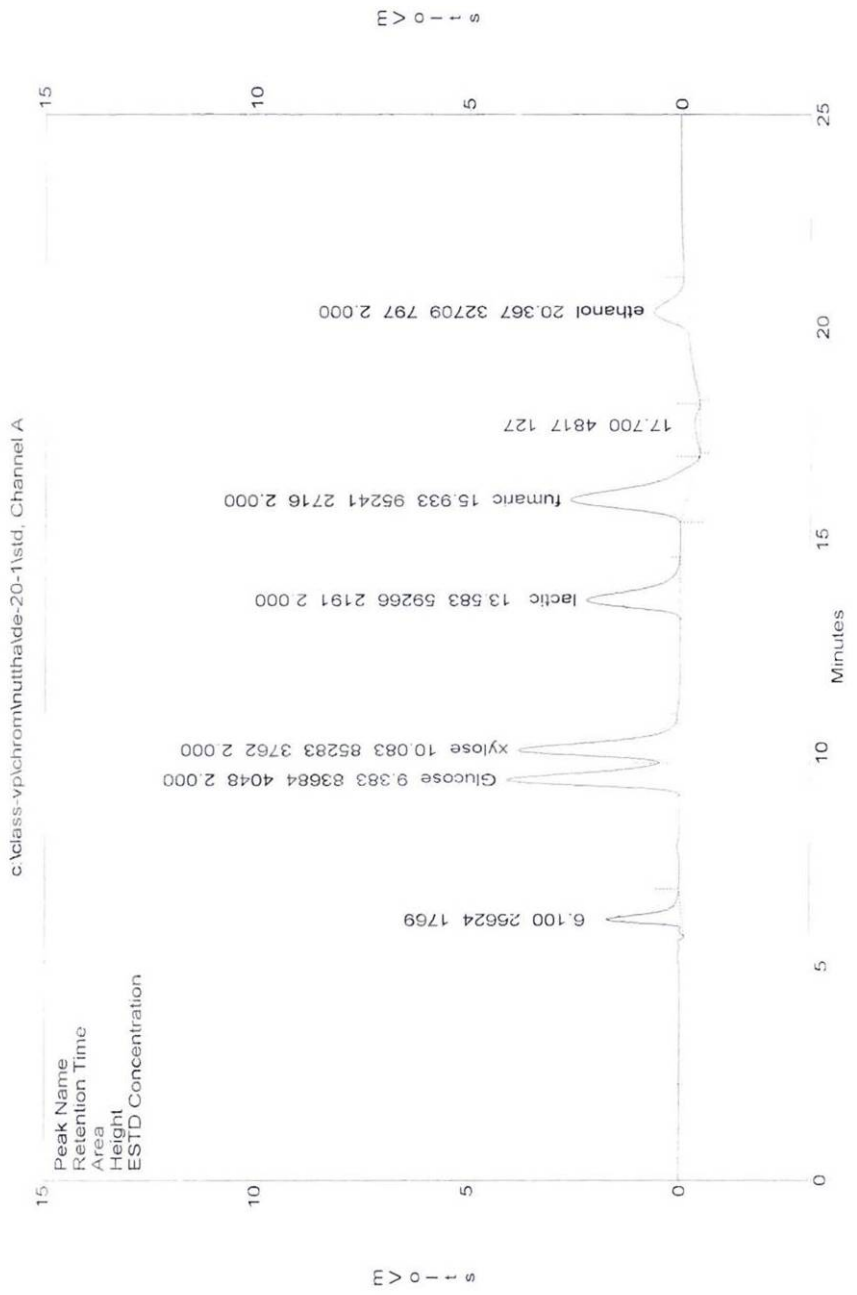


Figure D.1 The standard HPLC chromatogram of lactic acid fermentation from glucose and xylose by *R. oryzae*

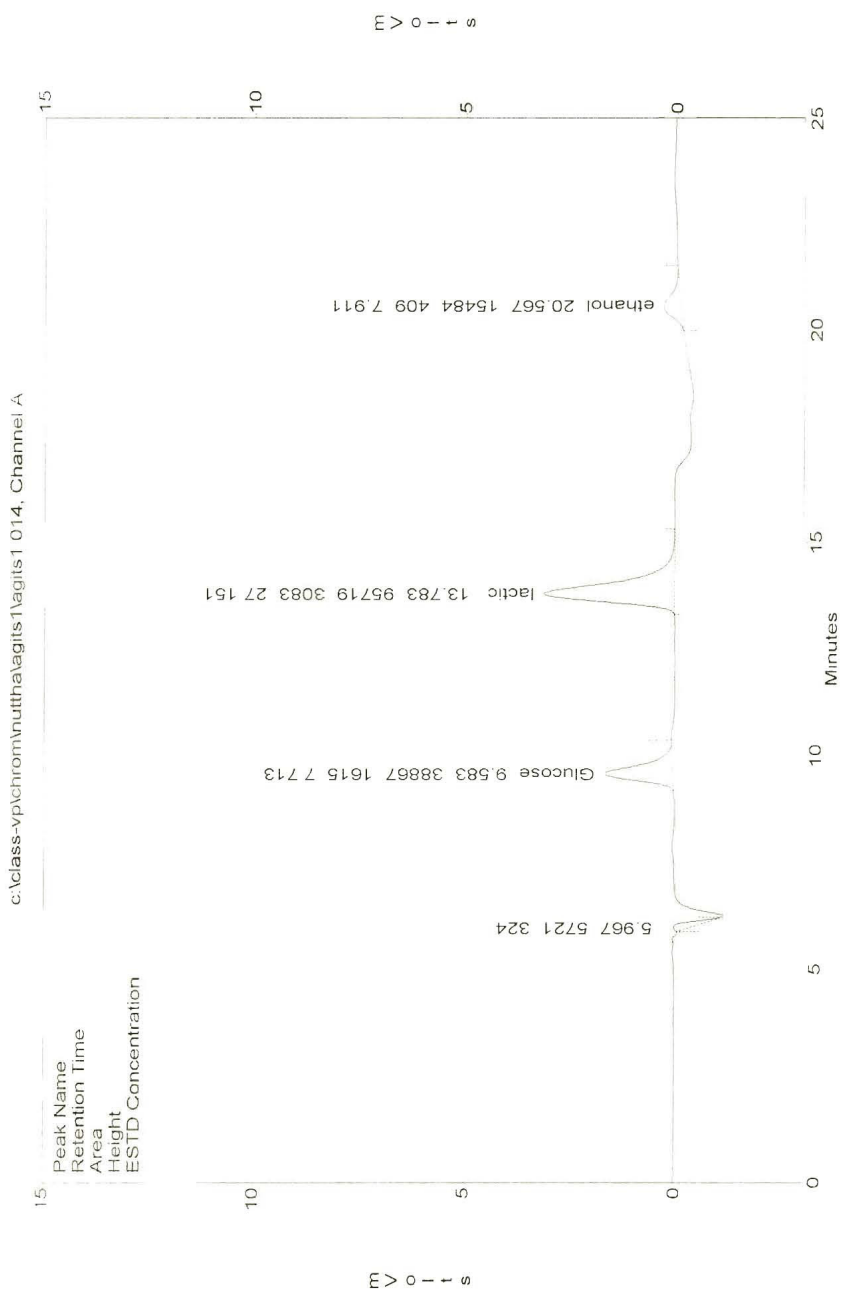


Figure D.2 The HPLC chromatogram of lactic acid fermentation from glucose by *R. oryzae*

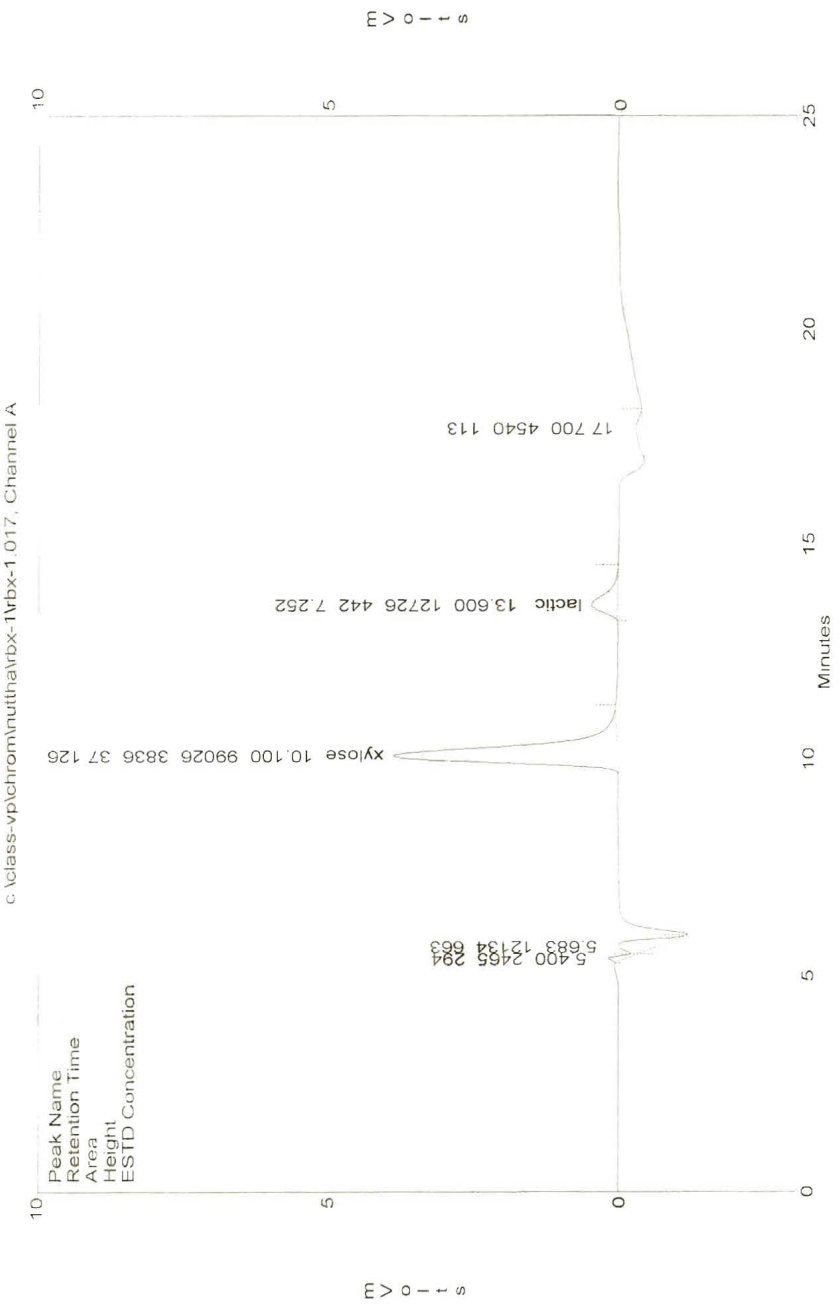
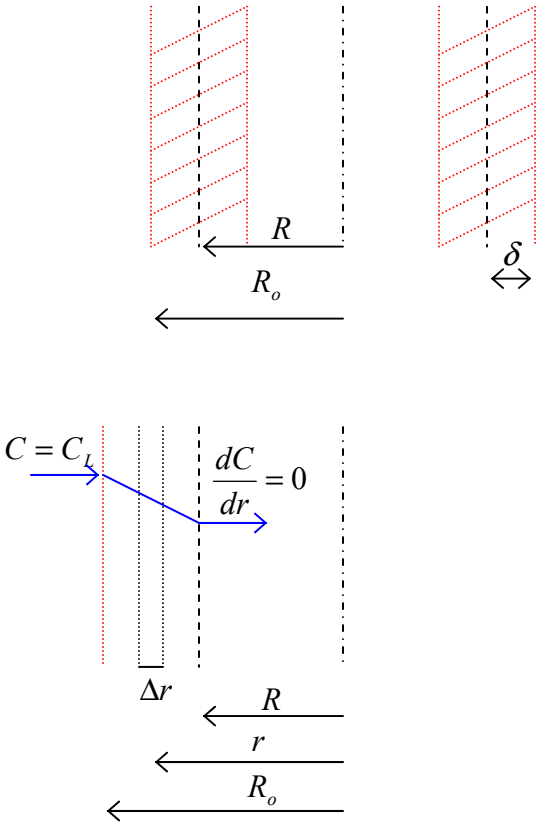


Figure D.3 The HPLC chromatogram of lactic acid fermentation from xylose by *R. oryzae*

APPENDIX E

MATHEMATICAL MODELS

E1 Oxygen Diffusion Model



R. oryzae biofilm (thickness 2δ) immobilized on both sides of the perforated stainless cylinder covered with cotton fiber.

Figure E.1 Schematic diagram shown oxygen concentration profile in the immobilized fungal cells attached on the rotating fibrous matrix

Nomenclature

C_L = bulk oxygen concentration

C = oxygen concentration in the biofilm

R_o = radial distance from the center of the cylindrical matrix to the surface of the immobilized biofilm

R = radius of the cylindrical matrix

D_e = effective diffusivity of oxygen

ν = oxygen uptake rate (OUR) experimentally determined (Appendix C)

Assumptions

1. The influence of immobilization on growth depends on oxygen concentration profile as indicated by the diffusivity data described in 5.2.4.2.
2. The effect of shear stress inside the biofilm is negligible.
3. Oxygen transfer is described by Fick's law.
4. Fungal biofilm is homogeneously distributed and symmetrically grows on both sides of the cylindrical matrix.
5. Fungal kinetics can be described with zero order kinetics (both oxygen and glucose)
6. The effective diffusivity is constant
7. Pseudo steady state

Considering the growth on the fibrous matrix outside the stainless cylinder and assuming the symmetric growth from both inside and outside the stainless cylinder. Oxygen balance of the immobilized biofilm on the cylindrical matrix (at $C_{o_2} > 0$) was given by

$$0 = \left(2\pi r H \cdot D_e \frac{dC}{dr} \right)\Bigg|_{r+\Delta r} - \left(2\pi r H \cdot D_e \frac{dC}{dr} \right)\Bigg|_{r} - 2\pi r H \Delta r \cdot \frac{v}{2} \qquad \text{(E.1)}$$

Divided eq (E.1) by $2\pi H \Delta r$,

$$D_e \cdot \frac{\left(r \frac{dC}{dr}\Big|_{r+\Delta r} - r \frac{dC}{dr}\Big|_{r} \right)}{\Delta r} = \frac{vr}{2} \qquad \text{(E.2)}$$

Take limit $\Delta r \to 0$, eq (E.2) became,

$$D_e \frac{d}{dr}\left(r \frac{dC}{dr} \right) = \frac{vr}{2}$$

$$\therefore \frac{d}{dr}\left(r \frac{dC}{dr} \right) = \frac{vr}{2D_e} \qquad \text{(E.3)}$$

Integrate eq (E.3),

$$\int d\left(r \frac{dC}{dr} \right) = \int \frac{vr}{2D_e} dr$$

$$r \frac{dC}{dr} = \frac{vr^2}{4D_e} + a$$

$$\frac{dC}{dr} = \frac{vr}{4D_e} + \frac{a}{r}; \qquad a = \text{constant} \qquad \text{(E.4)}$$

Integrate eq (E.4),

$$\int dC = \int \left(\frac{vr}{4D_e} + \frac{a}{r} \right) dr$$

$$C = \frac{vr^2}{8D_e} + a \ln r + b; \quad b = \text{constant} \qquad \text{(E.5)}$$

Boundary conditions:

B.C.1 at $r = R_o$, $C = C_L$

B.C.2 at $r = R$, $\frac{dC}{dr} = 0$

Substitute B.C.2 into eq (E.4),

$$\frac{dC}{dr} = 0 = \frac{vR}{4D_e} + \frac{a}{R}$$

$$\therefore a = -\frac{vR^2}{4D_e} \tag{E.6}$$

Substitute B.C.1 and eq (E.6) into eq (E.5),

$$C_L = \frac{vR_o^2}{8D_e} + \left(-\frac{vR^2}{4D_e} \right) \ln R_o + b$$

$$b = C_L - \frac{vR_o^2}{8D_e} + \frac{vR^2}{4D_e} \ln R_o \tag{E.7}$$

Substitute eq (E.6) and eq (E.7) into eq (E.5),

$$C_L - C = \frac{vR^2}{4D_e} \ln \frac{r}{R_o} + \frac{v}{8D_e} \left(R_o^2 - r^2 \right) \tag{E.8}$$

When the biofilm growth reaches the critical thickness ($\delta \to \delta_c$ or $R_0 \to R_c$), oxygen concentration (C) at the middle of the biofilm (at the surface of the cylindrical matrix, r = R) became zero. Therefore, the critical biofilm thickness is numerically determined from Eq (E.8) at known C_L and ν.

E2 Shear Stress Acting on the Rotating Matrix

Assumptions

1. Biofilm exhibits fluid-like behavior
2. Newtonian fluid behavior
3. Constant density (ρ) and viscosity (μ)
4. End effects are negligible
5. Steady state

6. $g_r = g_\theta = 0$ and $g_z = g$

Using the cylindrical coordinate:

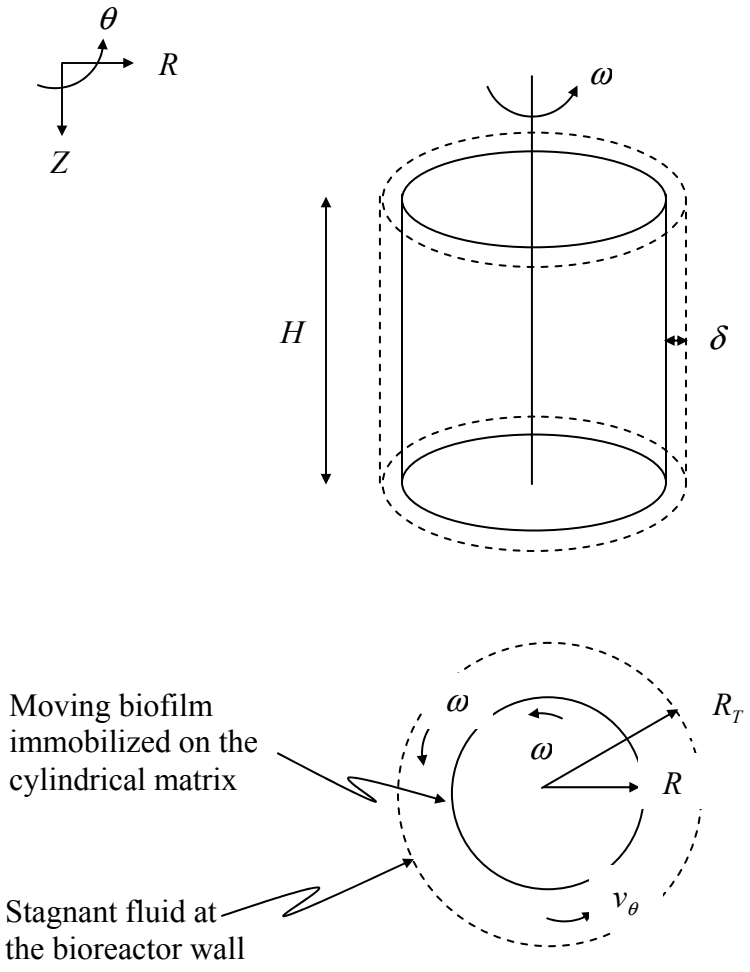


Figure E.2 Schematic diagram shown shear stress acting on the rotating fibrous matrix where fungal immobilization occurred

Nomenclature

R = radius of the cylindrical matrix providing the surface area for biofilm attachment

R_T = radius of bioreactor vessel

h = height of the matrix

p = Static pressure

μ = medium viscosity

ρ = medium density

Both velocities in the radial and axial directions (v_r and v_z) are zero. Also, $\frac{\partial \rho}{\partial t} = 0$ at steady state. There is no pressure gradient in the θ direction. The equation of continuity becomes:

$$\frac{\partial \rho}{\partial t} + \frac{1}{r}\frac{\partial (\rho r v_r)}{\partial r} + \frac{1}{r}\frac{\partial (\rho v_\theta)}{\partial \theta} + \frac{\partial (\rho v_z)}{\partial z} = 0 \qquad \text{(E.9)}$$

All terms in eq (E.9) are zero.

The equations of motion are as follows:

r-component: $$-\rho \frac{v_\theta^2}{r} = -\frac{\partial p}{\partial r} \qquad \text{(E.10)}$$

θ-component: $$0 = \frac{d}{dr}\left(\frac{1}{r}\frac{d(r v_\theta)}{dr}\right) \qquad \text{(E.11)}$$

z-component: $$0 = -\frac{\partial p}{\partial z} + \rho g_z \qquad \text{(E.12)}$$

Integrate eq (E.11)

$$\therefore v_\theta = C_1 r + \frac{C_2}{r} \qquad \text{(E.13)}$$

Where C_1 and C_2 are constant. Consider the cylindrical rotating matrix with the immobilized biofilm at the rotational speed ω in the stagnant fluid. Assume that medium present inside the rotating bed was moving at the same rotational speed. Therefore, at $r = R$, the velocity v_θ was ωR whereas at the wall of the bioreactor vessel, $v_\theta = 0$,

Boundary conditions:

B.C.1: at $r = R$, $v_\theta = \omega R$

B.C.2: at $r = R_T$, $v_\theta = 0$

Substitute B.C.s into eq (E.13) and solve for C_1 and C_2

$$C_1 = -\frac{\omega R^2}{R_T^2 - R^2} \qquad C_2 = \frac{\omega R^2 R_T^2}{R_T^2 - R^2}$$

Substitute C_1 and C_2 into eq (E.13)

$$v_\theta = -\frac{\omega R^2}{\left(R_T^2 - R^2\right)} r + \frac{1}{r}\frac{\omega R^2 R_T^2}{\left(R_T^2 - R^2\right)} = \frac{\omega R^2}{\left(R_T^2 - R^2\right)}\left(\frac{R_T^2}{r} - r\right) \tag{E.14}$$

Shear stress component for Newtonian fluids

$$\tau_{r\theta} = \tau_{\theta r} = -\mu\left(r\frac{\partial\left(v_\theta / r\right)}{\partial r}\right) \tag{E.15}$$

Substitute eq (E.14) into eq (E.15) and differentiate

$$\tau_{r\theta} = \frac{2\mu\omega R^2 R_T^2}{\left(R_T^2 - R^2\right)}\frac{1}{r^2} \tag{E.16}$$

$\therefore$ The velocity gradient and the shear stress distribution on the cylindrical rotating bed with the immobilized biofilm in the stagnant fluid are as follows:

Velocity gradient: $$v_\theta = \frac{\omega R^2}{\left(R_T^2 - R^2\right)}\left(\frac{R_T^2}{r} - r\right)$$

Shear stress: $$\tau_{r\theta} = \frac{2\mu\omega R^2 R_T^2}{\left(R_T^2 - R^2\right)}\frac{1}{r^2}$$