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PRODUCTION OF L(+)-LACTIC ACID FROM GLUCOSE AND STARCH BY FERMENTATIONS WITH IMMOBILIZED CELLS OF RHIZOPUS ORYZAE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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*****

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ABSTRACT

There has been a revival of interest in microbial production of lactic acid. The worldwide market for lactic acid is more than 240 million lbs/yr and the major routes for production are bacterial fermentation and petrochemical synthesis. The fungal fermentation process is still a very new technology, yet there is no industrial application for production of lactic acid via fungal fermentation. Lactic acid currently is being used widely in the food industry, in production of environmentally benign polymer poly lactic acid (PLA), and in production of propylene glycol and acrylic fibers. The production of PLA will increase if new economic production routes are developed, which will increase annual lactic acid consumption to billions of pounds.

The new methods to enhance lactic acid productivity and yield from fungal fermentation of starch and glucose were studied in this project. The immobilization of fungal cells on a fibrous matrix to increase mass transfer, reaction rate, and viable cell density were carried out. It was observed that by using immobilized cells with reduced or no growth activity; it was possible to enhance lactic acid yield since less carbon source was used for cell biomass formation. The lactic yield was increased from 50% to 80%,
and the productivity was increased from 0.6 g/L*h to 0.9 g/L*h by immobilization. Lactic acid yield and productivity were also affected by the pH and DO level. The increase in DO level from 20% to 90% resulted in an increase from 0.89 g/L*h to 2.62 g/L*h in the productivity and from 52.6% to 82.6% in the lactic acid yield. The change of pH from 4 to 6 resulted in a 60% increase in lactic acid productivity and 30% increase in lactic acid yield.

The amine based extractive fermentation to separate lactic acid in situ to reduce product inhibition and furthermore to enhance fermentation rate was also successfully demonstrated. It was observed that removing lactic acid continuously from the fermentation broth shifted the metabolic pathway towards lactic acid production; hence increasing lactic acid yield. The yield was increased from 60% to 92% by using extractive fermentation.
To Melissa
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CHAPTER 1

INTRODUCTION

1.1. Justification and Background

The industrial use of fungi is becoming very important with recent advances in biotechnology. The main use of fungi is associated with their ability to produce vast varieties of excreted enzymes and small molecules. Some of the major products from fungal fermentations include organic acids, enzymes, vitamins, antibiotics, and miscellaneous primary and secondary metabolites.

Plant biomass is the most abundant natural resource in the world. *Rhizopus oryzae* has the ability to convert cheap natural resources to value added products such as L (+)-lactic acid. If a proper fermentation design is achieved, it would lead to economic production of L(+)-lactic acid that is highly desirable in many industrial areas. Lactic acid bacteria usually produce a mixture of L(+) and D(-) -lactic acid which leads to very costly downstream processing. Moreover, *R. oryzae* produces L(+)-lactic acid from starch, xylose, and other plant biomass without requiring complex media formulation.
These important characteristics of *R. oryzae* not only reduce the cost of the fermentation process but also reduce the cost of the separation process. The simpler the medium composition is, the easier separation of the desired product will be. However, fungal fermentation with free cells generally involves very viscous fermentation medium caused by large amount of mycelia (Neway, 1989). This will lead to high power requirement for mixing and more importantly, cause a poor mass transfer rate. These problems can be avoided by immobilizing *R. oryzae* on a fibrous matrix to obtain cell free fermentation medium to produce pure L(+)lactic acid.

1.2. Plant biomass

The sun is the source of most renewable energy forms. Solar energy may be utilized through photosynthetic conversion to biomass. Biomass can be defined as all organic matter except fossil fuels or simply all-renewable organic matter. This includes all plant material whether grown on land or in water, all animal products and manure; all food processing and forestry by products, and urban wastes.

There is much interest in biomass conversion to heat and liquid or gaseous fuels and to value-added food products. Agricultural wastes mostly contain enough carbon sources. The agricultural industry generates a tremendous amount of waste every day. Agricultural biomass potential is shown in Table 1.1.
Successful use of these substrates by *R. oryzae* for the production of L(+)-lactic acid would result in economical benefits and reduce pollution problems. The bacterial fermentation for lactic acid production requires pretreatment of substrates for bacterial need, whereas *R. oryzae* can directly convert starch, xylose, arabinose to L(+)-lactic acid.

<table>
<thead>
<tr>
<th>Biomass source</th>
<th>106 tons/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn / soybean</td>
<td>120</td>
</tr>
<tr>
<td>Diverted crops</td>
<td>100</td>
</tr>
<tr>
<td>Agricultural residue</td>
<td>50</td>
</tr>
<tr>
<td>Forest biomass</td>
<td>200</td>
</tr>
<tr>
<td>Latent farmland</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>570</td>
</tr>
</tbody>
</table>

Table 1.1. Overall biomass potential in USA. (Lipinsky, 1985)

1.3. Demand for L(+)-lactic acid

There has been a continuous interest in lactic acid fermentation from biomass sources via fermentation. The market for lactic acid and its derivatives increases around 12% every year, which shows the need to investigate the new production routes. The microbial production route of lactic acid is currently bacterial fermentation that requires complex media composition and costly downstream processing. The worldwide demand for lactic acid is more than 240 million pounds per year. The major use of lactic acid is in food and food related areas, which account for approximately 85% of the demand (Datta et.al., 1995). Lactic acid occurs naturally in many food products. It has been in use as an acidulant, preservative and pH regulator for quite some time. Some of the important
applications of lactic acid in the food industry are detailed below. There are many properties of lactic acid, which make it a very versatile ingredient in the food industry. It has a pronounced preservative action, and it regulates the micro flora (www.purac.com; www.lactic.com). It has been found to be very effective against certain type of microorganisms. Sometimes a combination of lactic acid and acetic acid is used as it has a greater bactericidal activity. Because it occurs naturally in many foods, it does not introduce a foreign element into the food. The salts of lactic acid are very soluble, which creates the possibility of partially replacing the acid in buffering systems. Lactic acid is non-toxic and is deemed "Generally Recognized As Safe" (GRAS) as a general-purpose food additive in the USA. The same status is accorded in many other countries too. The calcium salt of lactic acid, calcium lactate, has a greater solubility than the corresponding salt of citric acid. In such products where turbidity caused by calcium salts is a problem, the use of lactic acid produces products that are clear. L(+) -lactic acid is the natural isomer found in biological systems, and hence its use as an acidulant does not introduce a foreign element into the body.

Lactic acid finds use as an acidulant in the confectionery industry. It is a better acidulant than citric acid since the sugar inversion is less when used for hardboiled candies. It does not have the initial burst of flavor and tanginess of citric acid. Lactic acid imparts a mellower and lasting sourness, which enhances the flavor much more. The use of buffered lactic acid in continuous production lines for high boiled sweets is a new
application. Liquid buffered lactic acid can be easily measured into molten syrups, even at high temperatures it can be used in depositing lines. Sugar inversion is minimal (Purac 1993).

Lactic acid is a natural beer acid, and hence is used for pH adjustments during the mashing process and in worth cooking. Lactic acid improves the microbial stability and also enhances the flavor. Lactic acid, due to its mild nature is the acidulant of choice in delicately flavored soft drinks and fruit juices. It does not mask or over power the natural flavor. Its flavor enhancing property makes the beverage more palatable and leaves a lingering taste. Lactic acid is preferred over citric acid for these reasons.

Green olives, gherkins and others are often packed in a solution of salt, lactic acid, and water. The lactic acid acts as a preservative and improves the clarity of the brine and flavor. A mixture of acetic acid and lactic acid in pickled products such as gherkins, and silver skin onion imparts a milder taste and flavor, and improves microbial stability. Calcium lactate is used as a firming salt, which has been used for canned fruits and vegetables (www.purac.com and www.lactic.com)

Direct acidification with lactic acid in dairy products, such as cottage cheese, is preferred to fermentation as the risks of failure and contamination can be avoided. The processing time also can be saved. Lactic acid is used as an acidulant in dairy products
like cheese and yogurt powder. For direct acidification of certain breads, lactic acid is the natural sour dough acid. The general appearance of a loaf of bread is greatly improved by the use of bacterial lactic acid, which results in a larger loaf per weight of bread with improved bloom, and color of crust. Lactic acid is directly added to certain types of fermented crispy dough biscuits. Lactic acid added to dough increases the shelf life due to its retarding action on molds and rope. The sodium and calcium stearoyl lactylates are used as emulsifiers in the baking industry as they provide substantial quality improvement of baked products besides reducing shortening levels (Purac, 1995).

Lactic acid is widely used in meat products as an antimicrobial agent. Decontamination of beef, poultry and pork carcasses in slaughterhouse operations is practiced to reduce *Salmonella* infection. In sausages, sodium lactate is used to reduce water activity and achieve higher shelf life. Lactic acid is widely used in the food industry as an additive for multipurpose. Many salts of lactic acid are used in the pharmaceutical industry for their therapeutic qualities. Lactic acid is also used as an intermediate compound for producing many drugs. L(−)-lactic acid can also be used in the manufacturing of a new biodegradable polymer, polylactic acid (PLA), which could play very important role in solving the environmental pollution problem due to non-degradable plastics.
The potential market for lactic acid and its derivatives in USA was prospected to be approximately 5.5-7.5 billion lbs/year; with a sale volume of 3.1-4.4 billion dollars (Datta et al., 1995).

1.4. Advantages of using immobilized *R. oryzae*

Lactic acid fermentation is generally carried out by homolactic acid bacteria. Bacterial fermentation can lead to the production of L(+)-lactic acid, D(-)-lactic acid or a racemic mixture of lactic acid. For developing environmentally friendly polymer, it is essential to produce pure L(+)-lactic acid. Moreover, the homolactic acid bacteria cannot ferment starch and pentoses, which are rich in plant biomass. The other disadvantage of bacterial fermentation is the requirement of a complex medium for lactic acid fermentation, which will make final downstream process very costly. In contrary, molds, such as *R. oryzae* can digest starch and pentoses and produce optically pure L(+)-lactic acid at a relatively low pH value (>3), but productivity and yield are lower for fungal fermentation than the bacterial fermentation. Table 1.2 compares the advantages and disadvantages of bacterial and fungal fermentation for lactic acid production.
### Table 1.2. Comparison between bacterial and fungal lactic acid fermentations.

<table>
<thead>
<tr>
<th></th>
<th>Bacteria</th>
<th>Molds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrate</strong></td>
<td>Most cannot utilize starch and pentoses</td>
<td>Can utilize starch and pentoses</td>
</tr>
<tr>
<td><strong>Medium composition</strong></td>
<td>Require complex growth nutrients</td>
<td>Simple medium composition</td>
</tr>
<tr>
<td><strong>Growth conditions</strong></td>
<td>Anaerobic, pH&gt;4.5</td>
<td>Aerobic, pH&gt;3</td>
</tr>
<tr>
<td><strong>Products</strong></td>
<td>Mixture of L(+) and D(-) lactic acid</td>
<td>Pure L (+) lactic acid and other by products (ethanol, fumarate, CO₂)</td>
</tr>
<tr>
<td><strong>Yield from glucose</strong></td>
<td>80–95 % (w/w)</td>
<td>~75 % (w/w)</td>
</tr>
<tr>
<td><strong>Product concentration</strong></td>
<td>up to 140 g/L</td>
<td>up to 120 g/L</td>
</tr>
<tr>
<td><strong>Productivity</strong></td>
<td>~60 g/L·h</td>
<td>~6 g/L·h</td>
</tr>
</tbody>
</table>
1.5. Research objectives and scopes of studies

The goal of this research was to develop an immobilized fungal fermentation process to economically produce L(+) -lactic acid from glucose and starch. To achieve this goal, the following specific objectives were pursued in this study.

1- To study the fermentation kinetics using starch and glucose as the substrates, and to find the effects of medium composition and the optimal fermentation conditions for reducing cell biomass and increasing lactic acid production. It is vital to establish optimum conditions for the production of L(+)-lactic acid before running other tests. These studies were carried out in shake-flasks and the results are reported in Chapter 3.

2- To develop and test a novel fibrous bed bioreactor for fermentation using immobilized cells of R. oryzae to attain high productivity, high lactic acid yield and stable long-term production. The optimum medium composition found in the objective 1 was applied to the bioreactor. The bioreactor studies were carried out in 5-liter fermenters equipped with pH and DO control units. Repeated batch and fed-batch fermentations were studied to evaluate pH and DO effects, and to observe long-term stability of immobilized cells in the bioreactor. The results are given in Chapter 4.

3- To investigate mass transfer rate in the immobilized fibrous bed bioreactor. The oxygen transfer rate is crucial for aerobic fermentation processes. The conversion of starch and glucose to L(+)-lactic acid by R. oryzae requires oxygen and a high
oxygen transfer rate in the fermenter is critical to lactic acid production. It is thus essential to understand the limiting transfer conditions. The oxygen transfer rate was greatly affected by bioreactor operating conditions and the results are also reported in Chapter 4.

4- To develop a solvent extractive fermentation for enhanced lactic acid production. The type of solvent and extraction conditions appropriate for fungal fermentation were first evaluated. In an extractive fermentation, the desired product is continuously removed from the fermentation medium in situ to prevent the accumulation of inhibiting product. The removal of product can eliminate product inhibition and improve reactor productivity. For heterofermentation, such as lactic acid production by R. oryzae, extractive fermentation also may increase product yield by shifting the metabolic reaction towards lactic acid production. The goal was to develop a solvent extractive fermentation process for enhanced lactic acid production. Compared with the conventional fermentation process, the extractive fermentation process was expected to have many advantages, including higher productivity, selectivity for lactic acid, highly concentrated cell-free product, and a continuous process with continuous feeding and product removal.

The results for lactic acid extractive fermentation are presented in Chapter 5. An overview of research objectives and the scope of the studies associated with each specific objective are summarized in Figure 1.1. The conclusions and recommendations for future work are given in Chapter 6.
Enhanced production of L(+)\textendash;Lactic acid from starch and glucose using immobilized \textit{R. oryzae} in a fibrous bed bioreactor

Fermentation kinetics and medium optimization
- Free cells vs. immobilized cells
- Nitrogen and carbon sources
- Substrate and end product
- Long-term stability
- Optimization of medium composition
Results are presented in Chapter 3.

Fibrous bed bioreactor
- Free cells vs. immobilized cells
- pH and DO effects
- Fed-batch fermentation
- Long-term stability
- Mass transfer ($k_{L}a$) study
Results are reported in Chapter 4.

Extractive fermentation
- Solvent toxicity and selection
- Process demonstration and long-term stability
Results are presented in Chapter 5.

Figure 1.1 Overview of research objectives and the scope of the present studies.
1.6. References


Purac (1993). Company profile. IL,USA


www.lactic.com

www.purac.com
CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

This chapter briefly reviews the background of fungal fermentation, lactic acid production, extractive fermentation, cell immobilization, and techniques to study mass transfer in bioreactor.

2.2. Fungal fermentation

Fungi play such a dominant role in human society that it could be argued that they are the most important biotechnologically useful organisms (Kurtzman, 1983; Smith and Berry, 1975). It is very difficult to argue against this proposition when we consider the importance of filamentous fungi and yeasts.

Traditional technologies that employ fungi include the production of biochemicals (such as citric acid) and antibiotics (e.g. penicillin) and the production and flavoring of foods (Everleigh, 1981; Arora and Elendar 1992). Table 2.1 shows some metabolites of industrial significance produced by fungi.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cephalosporium acremonium</em></td>
<td>Antibiotics</td>
</tr>
<tr>
<td><em>Emericiclospora</em></td>
<td><em>Cephalosporin C</em></td>
</tr>
<tr>
<td><em>Fusidium coccineum</em></td>
<td><em>Penicillin N</em></td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td><em>Fusidic acid</em></td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td><em>Varidin</em></td>
</tr>
<tr>
<td><em>Penicillium patulum</em></td>
<td><em>Penicillins G and V</em></td>
</tr>
<tr>
<td><em>Tolyposcidium inflatum</em></td>
<td><em>Griseofulvin</em></td>
</tr>
<tr>
<td><em>Asahya gossypii</em></td>
<td><em>Cyclosporin</em></td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td><em>Metabolites Other Than Antibiotics</em></td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td><em>Riboflavin</em></td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td><em>Citric acid</em></td>
</tr>
<tr>
<td><em>Ceratoctysis virescens</em></td>
<td><em>Kojic acid</em></td>
</tr>
<tr>
<td><em>Claviceps purpurea</em></td>
<td><em>Itaconic acid</em></td>
</tr>
<tr>
<td><em>Fusarium moniliforme</em></td>
<td><em>Flavors and fragrances</em></td>
</tr>
<tr>
<td><em>Penicillium stoloniferum</em></td>
<td><em>Alkaloids</em></td>
</tr>
<tr>
<td><em>Phycomyces biahsleaneus</em></td>
<td><em>Gibberellins</em></td>
</tr>
<tr>
<td><em>Rhizopus sp.</em></td>
<td><em>Mycophenolic acid</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td><em>-Carotene</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td><em>Fumaric acid</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td><em>Enzymes</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td><em>Glucosylase</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td><em>Lipase</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> niger</td>
<td><em>Peptidase</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> niger</td>
<td><em>Pentosanase</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> niger</td>
<td><em>-Glucanase</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> oryzae</td>
<td><em>Glucose oxidase</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> oryzae</td>
<td><em>Lactase</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> oryzae</td>
<td><em>-Amylase</em></td>
</tr>
<tr>
<td><em>Fusarium</em> spp.</td>
<td><em>Protease</em></td>
</tr>
<tr>
<td><em>Mucor</em> sp.</td>
<td><em>Penicillin acylase</em></td>
</tr>
<tr>
<td><em>Penicillium</em> sp.</td>
<td><em>Rennet</em></td>
</tr>
<tr>
<td><em>Trichoderma reesei</em></td>
<td><em>Dextranase</em></td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td><em>Cellulase</em></td>
</tr>
<tr>
<td><em>Aureobasiidium pullulans</em></td>
<td><em>Miscellaneous Compounds</em></td>
</tr>
<tr>
<td><em>Fusarium</em> sp.</td>
<td><em>Poliyols</em></td>
</tr>
<tr>
<td><em>Mucor</em> oryzae*</td>
<td><em>Pullulan</em></td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td><em>Microbial protein</em></td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td><em>Microbial insecticides</em></td>
</tr>
<tr>
<td><em>Penicillium roquefortii</em></td>
<td><em>Insulin</em></td>
</tr>
<tr>
<td><em>Rhizopus nigricans</em></td>
<td><em>Double-stranded RNA</em></td>
</tr>
<tr>
<td><em>Saccharomyces</em> sp.</td>
<td><em>Cheese manufacture</em></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td><em>Steroid conversions</em></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td><em>Ethanol</em></td>
</tr>
<tr>
<td></td>
<td><em>Interferons</em></td>
</tr>
<tr>
<td></td>
<td><em>Vaccines</em></td>
</tr>
</tbody>
</table>

Table 2.1. Some examples of fungi and fungal products (Aurora et. al 1992 and Everleigh, 1981)
The industrial production of biochemicals by fungi generally involves growing selected high-yielding species in a liquid medium contained in tanks or huge vessels. Alternatively, an approach, which is now increasingly used, is to grow the fungus on a solid substrate, such as wood chippings or straw, a process called solid state fermentation (Ward, 1989; Weyman, 1990).

2.2.1. Factors affecting the growth of mycelial cell

As production of biomass and/or synthetic byproducts is integrally related to the morphology of the mold, the determination and manipulation of these factors is critical in optimizing the fermentation. It is generally considered that the extremes of complete filamentous growth and large, hard pellet formation are detrimental for optimal cell and product formation.

2.2.1.1. General morphology

The mycelial structure of mold consists of a network of highly branched structures called hyphae. When the filamentous form of mold is present the hyphae are usually thin and growth of the mold occurs as more of an outward extension of the hyphae.

Under the influence of specific factors to be discussed below, the hyphae and mycelial structure can become swollen and very dense. In extreme cases, the mold will
develop into small, hard pellets. Generally, three types of pellets have been observed: large loosely formed pellets with more compact centers, uniform pellets with a smooth, hard outer surface, and small hollow pellets with smooth outer surfaces.

2.2.1.2. Factors influencing the morphology of filamentous fungi

There is a close relation between morphology of fungi and productivity of fungal fermentation. There are numerous studies of morphology effect of *Aspergillus niger* and citric acid production because citric acid fermentation is very mature compared to fungal lactic acid fermentation. The effect of different factors on morphology of molds will be discussed below.

a) Strain

The formation of pellets is somewhat influenced by the specific strain being investigated. The following influences on pellet morphology should be taken as general rules of thumb and extension to other mold strains should be confirmed by experimental investigations.

b) Agitation

Agitation plays an important role in the developing morphology of the mold. Numerous investigations into these phenomena have demonstrated that at low agitation intensity the mold tends to coagulate and form pellets. At higher rates of agitation, the
mold structures are swirling around at a faster rate reducing the influence of coagulating forces. In addition, at high agitation rates, the pellets that do begin to form are physically broken apart by the impeller motion and by contact with other reactor parts (Elmayergi et. al., 1973)

It should be noted that the viscosity of the fermentation broth is strongly influenced by the degree of pellet formation. When the mold is growing in filamentous form, the broth consists of a vast network of highly branched mycelia, which will tend to increase the broth viscosity, as the broth can become very soupy and dense. On the other hand, when pellet formation occurs the viscosity of the broth is reduced as the filamentous structure of the mold is eliminated. This effect strongly influences power requirements and oxygen diffusion rate for the fermentation.

c) Growth nutrients and Medium formulation

The type of nitrogen source and the total concentration of nitrogen in the medium strongly influence pellet formation. The use of ammonium salts and derivatives usually causes the mold to grow into hard pellets and even the use of corn steep liquor has been demonstrated to cause *P. chyrsoaenum* to form loose, fluffy pellets. In addition, at high concentrations of total nitrogen in the medium the mold tends to form small, hard pellets (Pirt and Callow, 1959).
As far as *A. niger* is concerned, experiments have shown that the amount of phosphorus present as well as trace amounts of manganese, zinc, and iron result in pellet formation and productivity increase (Roukas and Kotzekidou, 1987; Shu and Johnson, 1948; Clark et. al., 1966). In addition, some chelating agents, like EDTA and ferrocyanide, have shown some tendency to influence pelletized growth (Choudhary and Pirt, 1965).

The influence of medium constituents on mold morphology appears to be very particular to the strain utilized. Some species have shown susceptibility to form pellets in the presence of fatty acids and polymeric compounds, whereas, the presence of nonionic surface-active agents shows some ability to prevent formation of pellets (Takabashi et al., 1960). As these effects are specific to the strain, experimental results should be relied upon in order to determine influences on pellet growth due to the medium composition (Kumar and Ethiraj, 1976; Manonmani and Sreekantiah, 1988; Millis et al. 1963; Kobayashi and Suzuki, 1972).

c) pH

At low pH values pellet formation is not observed, however, as the pH increases the tendency of growth in the form of pellets is preferred. This effect is thought to be due to changes in cell wall structure. At low pH, the cells become more resistant to their
environment by thickening of the cell wall. Consequently, the hyphae will be more resistant to being broken apart and filamentous growth is observed.

*A. niger* is most notably observed with this effect in the production of citric acid, where pH is related to the product formation. The specific value of pH where these effects are observed depends on the mold strain being investigated. Of course, under extreme pH conditions the mold may be exterminated (Roukas and Harvey, 1988; Kubicek and Rohr, 1986; Srivastava and De, 1980). No detailed information is available on effect of pH on lactic acid production by *R. oryzae*.

e) Oxygen Supply

Increasing the availability of oxygen will result in an increase in cell growth and reproduction; however, there is a profound impact on the morphology of the mold when pure oxygen or oxygen-enriched air is utilized. At low oxygen tension, some strains will grow in filamentous form or as small, fluffy pellets; on the other hand, formation of hard pellets has been observed when the oxygen tension is increased. This effect seems to be due to increased hyphae branching and the presence of swollen hyphae at high oxygen tension levels.

The formation of pellets can have a significant effect on the rate of oxygen utilization, as diffusion into the pellets becomes a major concern. This is the primary
reason for the avoidance of large pellet formation for improving product formation. The decreased availability of oxygen at the center of the pellets due to intraparticle diffusion results in a lower rate of product formation.

The diffusion of nutrients to the pellet surface and diffusion into the particle itself play a significant role in pellet morphology. Once a pellet is formed, oxygen and other essential nutrients can become depleted at the center of the pellet. Under severe conditions, autolysis has been known to occur resulting in a hollowing of the pellet. Since fungi are primarily aerobic organisms, industrial production processes invariably involve aerating or oxygenating the liquid growth medium. However, since the solubility of oxygen in water is relatively low, oxygen transfer and uptake is often the rate-limiting step during large-scale industrial production. Maximum oxygen transfer at the lowest possible cost is therefore necessary, a process which generally involves the use of a stirred tank reactor or fermenter (Kristiansen and Chamberlain, 1983).

f) Growth Rate

The rate of growth of the mold will also affect the morphology of the mold. When the growth rate is increased by growth medium enhancement or some other means, a more filamentous form of the mold predominates. This results from an increase in branching frequency and growth of thinner hyphae. As the growth rate decreases resulting from reduction of the nutrient supply, the mold will branch less frequently and
begin to enlarge existing hyphae. Eventually a state is reached wherein pellets begin to form as hyphae become swollen and branching is virtually eliminated.

This type of occurrence of pellets will usually occur at the end of batch fermentations when nutrients become depleted or in continuous reactors when changes in the nutrient composition are made. One investigator suggested that this type of pellet formation may be a survival instinct similar to that of some bacteria which form endospores in harsh or unproductive environments, and the formation of large pellets usually result in diffusional limitations that produce a reduction in biomass and product formation (Righelato et. al., 1968). Current research efforts focus on immobilizing the mold so as to reduce these diffusional problems thereby improving fermentation performance.

Industrial fermentations involving fungi require, firstly, a substrate; this is usually a sugar, such as sucrose, glucose or lactose, although it can also be alcohol or some waste product such as distillery or confectionery effluents. The use of waste products obviously provides a potentially more economical feedstock than does the use of pure substrates. Secondly, a source of nitrogen is required, and finally minor nutrients and vitamins.
2.2.2. Immobilized cells and biofilm reactor system

During the early 1970s, considerable research effort was devoted to the use of immobilized enzymes. In this approach, substrates are allowed to interact with an enzyme that has been immobilized on a surface or in the form of an inert pellet. The great advantage of this system is that enzymes remain stable while being active for a relatively long period. At the same time as this technology was being developed, methods were introduced to immobilize whole cells of microorganisms, as well as of plants and animals.

Although there is relatively few industrial processes in operation that involve immobilized fungi, there is a general consensus that this approach will find more extensive commercial application in the future (Anderson, 1983). The main advantage of using whole cells compared with using immobilized enzymes is that it avoids the need to extract the enzyme used in the process in question. The process can also be operated at high cell densities and high substrate flow rates, while cell washout from the system is negligible.

A number of different approaches to fungal cell immobilization have been considered, including adsorption and entrapment techniques as well as immobilization achieved by natural adhesion and film growth.
Most of the methods of cell immobilization, which employ adsorption and entrapment techniques, are based on approaches developed for immobilizing enzymes. In the adsorption method, cells are directly linked to water insoluble carriers, an approach that relies on electrostatic interactions between the microbial cell surface and the carrier material. As this process is mild, the cells remain both viable and active. Both filamentous fungi and yeasts can be immobilized on ion exchange resins, fritted glass and zirconium ceramic. However, the most widely used approach is to entrap the cells in inert gels such as polyacrylamide and calcium alginate (Hang et al. 1989).

2.3. Lactic acid fermentation

Lactic acid fermentation has received a lot of interest during the last decade; this is basically because of significance of lactic acid in many industries. The production of lactic acid or 2-hydroxypropionic acid has a long history. In 1780, Carl Wilhelm Scheele, a Swedish chemist, first isolated lactic acid from sour milk. First Lafar in 1893 and independent of him Leichmann isolated a pure culture of lactic acid bacteria and applied these successfully in the distillery (Benninga, 1990).

Lactic acid was firstly assumed to be a dibasic by Liebig. The structure of lactic acid is shown in Figure 2.1. Engelhardt, after a study of crystal properties, the solubilities and some other physical characteristics of a number of lactate salts, he concluded that lactic acid from different sources such as meat (which he named as
Sarcolactic acid and souring fermentation has different properties. Sarcolactic acid, the only lactic acid natural to humans and mammals is called L(+) or according to latest convention S(+) lactic acid. The other optical isomer or enantiomer is then called the D(-) or R(-)-lactic acid.

![Structure of isomers of lactic acid.](image)

The letter L means levorotatory since it rotates plane polarized light counterclockwise and the letter D stands for dextrorotatory for rotating the plane polarized light clockwise. Their optical activities are exactly opposite; the precise value depends on concentration, temperature and wavelength of light. The properties of enantiomers are shown in Table 2.2.
Table 2.2. Some important properties of lactic acid isomers

There have been extensive researches involving lactic acid fermentation and majority of these studies have been reviewed. Litchfield (1994) in an elegant paper reviewed from the history of lactic acid to the immobilization techniques, from bacterial fermentation to the recovery methods. He also mentioned the wide range of use of lactic acid, which is shown in Table 2.3.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Food application</th>
<th>Code of Federal Regulation reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-(+)- lactic acid</td>
<td>Antimicrobial agent, curing and pickling, flavoring, enhancer, adjuvant, pH control, solvent and vehicle</td>
<td>21 CFR 184.1061</td>
</tr>
<tr>
<td>D-(−)-lactic acid (10326-41-7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL- lactic acid (598-82-3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium lactate (814-80-2)</td>
<td>Flavoring enhancer, firming agent, leavening agent, nutrient supplement, stabilizer, thickener</td>
<td>21 CFR 184.1207</td>
</tr>
<tr>
<td>Ferrous lactate (5905-52-2)</td>
<td>Nutrient supplements and in infant formula</td>
<td>21 CFR 184.1311</td>
</tr>
<tr>
<td>Potassium lactate (996-31-6)</td>
<td>Flavor enhancer, flavoring agent, humectant, pH control</td>
<td>21 CFR 184.1639</td>
</tr>
<tr>
<td>Sodium lactate (72-17-3)</td>
<td>Flavor enhancer, flavoring agent, humectant, pH control</td>
<td>21 CFR 184.1768</td>
</tr>
<tr>
<td>Calcium stearoyl-2-lactylate</td>
<td>Dough conditioner in bakery products, whipping agent in egg products, conditioning agent in dehydrated potatoes</td>
<td>21 CFR 172.844</td>
</tr>
<tr>
<td>Sodium stearoyl lactylate (25-383-997)</td>
<td>Dough conditioner, emulsifier, processing aid in baked products; emulsifier, stabilizer processing aid in milk or cream substitutes, snack dips, imitation cheeses, dehydrated potatoes</td>
<td>21 CFR 172.846</td>
</tr>
<tr>
<td>Lactylated esters of fatty Acids</td>
<td>Emulsifiers, plasticizers, surface active agents in foods</td>
<td>21 CFR 172.848</td>
</tr>
<tr>
<td>Lactylated fatty acid esters of glycerol and propylene</td>
<td>Emulsifiers, plasticizers, surface active agents in foods glycol</td>
<td>21 CFR 172.850</td>
</tr>
<tr>
<td>Glycero-lacto esters of fatty Acids</td>
<td>Emulsifiers, plasticizers, surface active agents in foods</td>
<td>21 CFR 172.852</td>
</tr>
</tbody>
</table>

Table 2.3. Food applications of lactic acids and lactic acid derivatives (Litchfield, 1994)
Lactic acids and its derivatives also have been used extensively in non-food areas. The non-food application of lactic acid is tabulated in Table 2.4.

<table>
<thead>
<tr>
<th>Industry</th>
<th>Compound</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electronics</td>
<td>Ethyl-lactate</td>
<td>Precision metal cleaning (Hill and Carter, 1993)</td>
</tr>
<tr>
<td>Cosmetics and Toiletries</td>
<td>Lactic acids, calcium and sodium lactate, acyl-lactate</td>
<td>Humectant, emulsifier, skin care (Smith 1993).</td>
</tr>
<tr>
<td>Plastics</td>
<td>Polylactic acid (PLA)</td>
<td>Medical garments, food service plasticware and many potential applications (Datta et al., 1995)</td>
</tr>
</tbody>
</table>

Table 2.4 Non-food applications of lactic acids and lactic acid derivatives.

The predominant industrial techniques for lactic acid production include batch fermentation of cane sugar, whey or hydrolyzed starch by lactic acid bacteria, often genus of *Lactobacillus*, and chemical synthesis involving the hydrolysis of lactonitrile formed by the reaction acetaldehyde with hydrogen cyanide. However, there are no any industrial applications of filamentous fungi for lactic acid production. The major use of fungi commercially is to produce citric acid using *Aspergillus niger* (Grewal and Kalra, 1995).

PLA was first discovered by Pelouze in 1845, when water evaporated from lactic acid solution at temperature of 130°C, a syrupy liquid was formed with a composition
containing less water than lactic acid itself (Benninga, 1990). Figure 2.2 shows the reaction for the formation of PLA.

\[
\begin{align*}
\text{HO-} &- \text{C-H} + \text{HO-} &- \text{C-H} \quad \text{heat, 130°C} \\
\text{CH}_3 & & \text{CH}_3 \\
\text{COOH} & & \text{COOH} \\
\end{align*}
\]

\[
\text{L(+) - lactic acid} \quad \text{Polylactic acid (PLA)}
\]

Figure 2.2. Formation of polylactic acid PLA upon heating.

The Dow Chemical Company and Cargill have formed a company, Cargill Dow Polymers, to manufacture and market polylactic acid (PLA) polymers. PLA polymers are a new polymer family derived from renewable agricultural resources, such as corn or sugar beets. These compounds are also commonly known as "bioplastics". Developers will market the fact that this plastic is made from renewable resources rather than depletable oil resources. They will displace oil hydrocarbon-based polymers, such as polyethylene, polystyrene, and polypropylene. They are expected to compete with oil-based plastics on a cost/performance basis.
PLA resins are composed of chains of lactic acid, a natural food ingredient that can be produced by converting starch into sugar and then fermenting it to yield lactic acid. Water is then removed to form lactide, which is converted into PLA resins using a solvent-free polymerization. This material biodegrades into carbon dioxide and water when composted in municipal or industrial facilities.

2.4. Lactic acid metabolism in R. oryzae

A possible metabolic pathway for production of L(+)-lactic acid by R. oryzae has been suggested by Marguiles and Vishniac (1961); Kanie et al. (1974) found that the major route of L(+)-lactic acid production by R. oryzae from glucose was the EMP pathway. R. oryzae also used pentose phosphate pathway (HMP) for catabolism of pentose such as xylose. R. oryzae also utilizes starch directly, because they produce required amylases for digestion of starch.

Pritchard (1971) found that two distinct lactate dehydrogenases were present in cultures of a lactic acid-producing strain of R. oryzae. During rapid vegetative growth, when lactic acid is being produced, the mycelium contains an NAD⁺-dependent lactate dehydrogenase which catalyses the reduction of pyruvate to lactate but not the reverse. Following the depletion of the glucose in the medium and the onset of sporulation, the activity of this enzyme decreases rapidly to an undetectable level and it is by an NAD⁺
independent dehydrogenase which catalyses the oxidation of L(+)-lactic acid to pyruvic acid. This enzyme shows no activity with D(-)-lactic acid.

Some of the researchers described a number of mitochondrial and cytosolic enzymes in *Rhizopus* metabolism (Wegener and Romano, 1964; Osmani and Scrutton, 1985; Kenealy et al., 1986; Peleg et al., 1989; Yu and Hang, 1991). The presence of pyruvate carboxylase, NAD-malate dehydrogenase, and fumarase in the cytosol led Osmani and Scrutton (1985) to propose a scheme of pyruvate metabolism in *Rhizopus*, which involves a normal tricarboxylic acid pathway in the mitochondrion and a separate cytosolic pathway for fumarate formation.

Wright et. al. (1996) and Longacre et. al. (1997) developed a model using TFLUX, a specific radioactivity curve-matching program. TFLUX was used for a steady state system where pool sizes and flux remain constant over the course of experiment. From the information gathered they concluded that there were two separately regulated pools of pyruvate in *R. oryzae*: a cytosolic pool channeled into ethanol, lactate, oxaloacetate, malate and fumarate synthesis, and the second pyruvate pool channeled into the tricarboxylic acid cycle. As can be seen in figure 2.3, ethanol, CO₂ and fumarate are three byproducts, in addition to lactic acid produced in the fermentation.
Figure 2.3. Metabolic pathways for lactic acid fermentation by R. oryzae.
2.5. Lactic acid production by *R. oryzae*

There are only a few studies involving L(+)lactic acid production by *R. oryzae*, which involved solid-state and submerged fermentation using different immobilization technique. In general, cell immobilization is achieved either by cell entrapment within a confined column through the use of a polymeric matrix or membrane, or by cell attachment via adsorption or covalent bond to a fixed matrix. In the lactic acid production by *R. oryzae*, entrapment of cells has been the major method for cell immobilization.

Lockwood et al. (1936) observed that free cells of *R. oryzae* usually requires fermentation time of 17 to 21 days for maximum production of lactic acid. They also studied the fermentation kinetics of cassava, corn, oats, and rice to lactic acid. They found that the lactic acid production and carbohydrate consumption were influenced by the fermentation temperature, type of substrate and presence of neutralizing agents. Hang et al. (1989) produced L(+)lactic acid from glucose in a chemically defined medium using immobilized *R. oryzae* in calcium alginate beads. They reported that immobilized cells have a higher yield of L(+)lactic acid than the free cells. They also stated that immobilized cells were more stable in repeated batch fermentations as compared to free cells. There are quite a few solid state fermentation applications to produce L(+)lactic acid. Soccol et al (1994) produced L(+)lactic acid by *Rhizopus oryzae* NRRL 395 in a solid medium on sugar cane impregnated with a nutrient solution containing glucose and CaCO₃. A comparative study was undertaken in submerged and solid-state cultures. They obtained a productivity of 1.38 g/L·h in liquid medium and 1.43 g/L·h in solid medium. In other studies the growth capacity of 19 *Rhizopus* strain was studied on solid state
medium of cassava starch. (Soccol et al., 1994b).

Yang et al., (1995) used R oryzae NRRL 395 for production of optically pure L(+)lactic acid. They first grew cell on chemically defined medium containing xylose to form pellets with a size of about 1 mm, and then used the pellets for production of L(+)lactic acid. They used poly vinyl pyridine (PVP) resin absorption column, calcium carbonate or sodium hydroxide for removal of lactic acid to reduce the effect of product inhibition. They obtained higher yields and productivity compared to other studies. The yields and productivity obtained were 62-74 % and 2.86-6.1 g/L-h, respectively. They also found that the maximum productivity was reached at pH 6 and temperature 33 °C. At room temperature the volumetric productivity was less than 1 g/L-h, whereas at 33 °C the productivity was about 1.8 g/L-h. Most other investigators, such as Hang (1989) and Soccol (1994), used 30°C as a fermentation temperature. The pH also has important effect on lactic acid production. The volumetric productivity when the pH was controlled at 6 was higher than those were when the pH was controlled at 5 and when there was no pH control (no numerical values of volumetric productivity was given).

Dong et al. (1996) immobilized R. oryzae BTC115 in polyurethane foam cubes for L(+)lactic acid in flasks. The immobilizing capacity reached 67.5 DCW/L-cube (DCW: dry cell weight). They found that the productivity of L(+)lactic acid could be increased by three fold by using immobilized cells, which could be steadily used in
repeated fermentation for more than 10 batches. The yield and productivity obtained were 72% and 19.8 g/L*h-cube respectively. In one of recent studies, L(+)-lactic acid production was investigated using 8 *Rhizopus* strains and various culture conditions. *Rhizopus oryzae* NRRL 395 showed the highest yield of L(+)-lactic acid among various *Rhizopus* strains. The optimum production medium contained the following: corn starch, 120 g/L; ammonium sulfate, 1.35 g/L; small amounts of mineral salts (potassium phosphate, magnesium sulfate and zinc sulfate). The data obtained for the flask culture were successfully reproduced using a 3L-airlift bioreactor under the optimum conditions. The yield (based on initial carbon source concentration) and final concentration of L(+)-lactic acid were 85% and 102 g/L, respectively, in the 3-L airlift bioreactor (Yin et al., 1997).

Woiciechowski and et al. (1999) carried out the fermentation of hemicellulosic hydrolysate from *Pinus taeda* chips, using the fungal culture *R. oryzae*, to produce L(+)-lactic acid and to optimize and enhance the biological conversion of reducing sugar into L-(+)-lactic acid using the experimental factorial design to evaluate the culture conditions. The first factorial design based on surface response with five factors (agitation level, substrate concentration, CaCO$_3$ concentration, C/N and C/P ratios) at low levels and one medium point was performed to optimize culture conditions. The second study tested two factors (substrate concentration and C/N ratio) at three levels. The statistical analysis of the data obtained from the factorial study showed that a C/N
ratio of 35 and substrate concentration of 90 g/L were the best conditions to produce L-(+)-lactic acid with *R. oryzae* on *P. taeda* hydrolysate, but in this case the statistical projection was not correct and the real optimized conditions were C/N ratio of 55 and substrate concentration of 75 g/L of reducing sugar.

Zhou et al. (1999) had attempted to optimize the conditions for lactic acid production using *Rhizopus oryzae* ATCC 52311. The effect of nutrients on L(+)-lactic acid production from glucose was investigated by using the shake-flask experiments. They found that a maximum lactic acid production rate of 2.58 g/L*h was obtained with an initial glucose concentration of 94 g/L. They also observed the final lactic acid concentration of 83 g/L was achieved after 32 h of fermentation with a yield of 0.88 g lactic acid/g glucose consumed.

The results from some fungal lactic acid fermentations are summarized in Table 2.5.
<table>
<thead>
<tr>
<th>Fermentation conditions</th>
<th>Substrate</th>
<th>Lactic acid yield</th>
<th>Productivity (g/L·h)</th>
<th>Product concentration (g/L)</th>
<th>Operation period</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immob. cells in calcium alginate</td>
<td>glucose</td>
<td>72 %</td>
<td>2.5</td>
<td>62 g/L</td>
<td>9 repeated batches</td>
<td>Hang et al., (1989)</td>
</tr>
<tr>
<td>Solid state fermentation</td>
<td>Starch in grains</td>
<td>23–74</td>
<td>-4.5 g/kg/h</td>
<td>450 g/kg subs.</td>
<td>batch</td>
<td>Yu and Hang, (1989)</td>
</tr>
<tr>
<td>Small cell pellets on xylose</td>
<td>glucose</td>
<td>62–74</td>
<td>2.9–6.2</td>
<td>60 g/L</td>
<td>42 repeated batches in 22 days</td>
<td>Yang et al., (1995)</td>
</tr>
<tr>
<td>Immob. cell in fluidized bed</td>
<td>glucose</td>
<td>65</td>
<td>1.6</td>
<td>73 g/L</td>
<td>batch</td>
<td>Hamamci &amp; Ryu, (1994)</td>
</tr>
<tr>
<td>Immob. cells in polymer matrix</td>
<td>glucose</td>
<td>65</td>
<td>-</td>
<td>-</td>
<td>batch</td>
<td>Tamada et al., (1992)</td>
</tr>
<tr>
<td>Solid state with sugar cane bagasse</td>
<td>glucose</td>
<td>77</td>
<td>1.4</td>
<td>137 g/L</td>
<td>batch</td>
<td>Soccol et al., (1994)</td>
</tr>
<tr>
<td>Immob. cells polyurethane foam cubes</td>
<td>glucose</td>
<td>75</td>
<td>-5</td>
<td>40 g/L</td>
<td>10 repeated batches</td>
<td>Dong et al. (1996)</td>
</tr>
<tr>
<td>Free cells</td>
<td>glucose</td>
<td>50</td>
<td>-</td>
<td>79 g/L</td>
<td>batch, 32hr.</td>
<td>Kristofikova et al. (1991)</td>
</tr>
<tr>
<td>Floes in jar fermenter</td>
<td>glucose</td>
<td>86</td>
<td>1.7</td>
<td>103 g/L</td>
<td>batch</td>
<td>Kosakai et al., (1997)</td>
</tr>
<tr>
<td>Free cells</td>
<td>glucose</td>
<td>72</td>
<td>-</td>
<td>93 g/L</td>
<td>Batch, 500 gallon fermenter</td>
<td>Snell &amp; Lowery, (1964)</td>
</tr>
<tr>
<td>Free cells</td>
<td>glucose</td>
<td>88</td>
<td>2.58</td>
<td>83</td>
<td>Batch, shake-flask</td>
<td>Zhou et al. (1999)</td>
</tr>
</tbody>
</table>

Table 2.5. Summary of lactic acid fermentations by *R. oryzae*
2.6. Extractive fermentation

Nowadays extraction has taken much of attention for recovery purpose of dilute organic acid from fermentation broth. An amine based solvent extraction technique was developed as a recovery process for separation of citric acid from the fermentation broth (Wennerstern, 1983). Unlike other conventional solvents, amine solvents have very high distribution coefficients, which range from 2 to 40 for organic acids because the higher affinity of amine solvents to react with organic acids to form complexes (Ricker et al, 1979). Due to this high affinity the organic acid can be recovered with less solvent, and distillation cost can thus be greatly reduced. In addition, amine solvent can be easily regenerated by back extraction with an alkaline solution (NaOH) solution because $K_d$ of an amine solvent for organic acids approaches zero at pH 7. Consequently, an energy efficient extraction process can be developed for recovering carboxylic acids from a dilute aqueous solution.

The effect of pH is very dramatic on extractive fermentation. There has been successful application of amine based extraction for recovering citric acid from its fermentation broth by using Alamine 336 (a tertiary amine) in hexane as the extractant and hot water back extraction for solvent regeneration. However, a similar process did not work with lactic acid fermentation, because the fermentation broth had a relatively high pH value and need to be acidified with a strong inorganic acid before extraction.
could be carried out (Scholler et al, 1993). In case of acidifying, extraction efficiency was found to dramatically drop to a conventionally low level in this case. This dramatic drop of extraction efficiency was caused by the interference of the inorganic acid. Although there are several reports on the mechanism of anion interference, the mechanism is not yet fully understood. Thus, the example of industrial application of amine based extraction technique for the time being is just limited to citric acid recovery.

There are several studies aimed at reducing the recovery cost of a fermentation product by integrating the fermenter with the downstream recovery process. The general purpose is to remove the fermentation product continuously from the fermenter, which can avoid product inhibition and improve productivity of the fermentation process. Meanwhile, the product is separated, purified, and concentrated. Such processes have been reported; including: using adsorption (Bradley, 1987) and solvent extraction of the product (Jenemann, 1933; Finn, 1966), using vacuum or inert gas like CO$_2$ to remove the volatile product from the fermentation broth (Dale et al, 1985), dialysis (Steiber and Gerhardt, 1981), ion exchange (Srivastava, et al., 1992) and electrodialysis (Kyung and Gerhardt, 1984). Among all these integrated processes, amine based solvent extractive fermentation is the most attractive one because extraction is a simple, mature, and energy efficient separation technology commonly used in industry. Although the vacuum extractive process can remove volatile products such as ethanol and acetic acid from their fermentation broth, the equipment size and energy cost for creating the vacuum could be
very high. Further concentration is also needed because the product is usually at a very low concentration level. Electrodialysis needs more expensive equipment and operation is complicated by competition from other salt ions and membrane fouling. Ion exchange is an effective process, but it usually has a low adsorption capacity to organic acid and requires frequent regeneration of the adsorbents. A comparison of these different types of integrated fermentation processes is summarized in Table 2.6.

<table>
<thead>
<tr>
<th>Types</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Applications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent extraction</td>
<td>Simple operation</td>
<td>- Low pH</td>
<td>Organic acids and alcohols</td>
<td>Daugulis, 1987; Yabannavar and Wang, 1991; Coelho et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Solvent toxicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ion exchange</td>
<td>Simple operation</td>
<td>- Low capacity</td>
<td>Organic acids</td>
<td>Seevaratnam et al., 1991; Srivastava et al. (1992)</td>
</tr>
<tr>
<td>adsorption</td>
<td></td>
<td>- Costly regeneration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrodialysis</td>
<td>Biocompatible</td>
<td>- Ion competition</td>
<td>Organic acid</td>
<td>Weier et al., 1992; Kyung and Gerhardt, 1984</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Fouling</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- High energy cost</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Difficult to scale up</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gas stripping</td>
<td>Simple operation</td>
<td>- Big equipment size</td>
<td>Alcohols and volatile organic</td>
<td>Dale et al. 1992</td>
</tr>
<tr>
<td>(or vacuum)</td>
<td></td>
<td>- High energy cost</td>
<td>acids</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Require higher volatility</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>products</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6. Comparison of some of integrated methods

Nerabska and Staniszewski (1998) studied the facilitated membrane extraction (FME) for continuous isolation of lactic acid from its dilute solutions. The technique is intended for continuous fermentation, with the pH of a broth regulated by extraction of the acid. Standard dialysis membranes and a conventional diffusion dialyzer was the equipment used for carrying out the separation. The concentration of lactic acid left in a
feed after extraction was $10^3$-$10^4$ mol/L. FME integrated with electrodialysis seems to offer an effective separation system for continuous fermentation.

It has been reported that for ethanol and acetone-butanol fermentations that volumetric productivity can be increased significantly by using in situ solvent extractive fermentation. As in ethanol extractive fermentation, the major obstacle in developing an organic acid extractive fermentation is the solvent toxicity problem or the biological effect of solvents on cells. However, it is more difficult to find biocompatible, a nontoxic solvent for organic acid extractive fermentation than for alcohol extractive fermentation because solvents that have better $K_d$ values and a higher extraction power for organic acids tend to be also more toxic to cells. The amine solvents with very high distribution coefficients for organic acids also turned out to be highly toxic to cells (Dave et al, 1979). Another problem in developing organic acid extractive fermentation is that fermentation usually favors a pH around 7, but extraction works efficiently only at a pH below pKₐ value 4.5 (King, 1992). However, few fermentation can work at pH 4.5 and amine-based extraction can not work at pH 7. Except a study for propionic acid extraction by Jin et.al. (1998), no practical organic acid extractive fermentation process has been demonstrated. Many studies focused on acid-tolerating fermentation, such as lactic acid fermentation, which was demonstrated with a small improvement in productivity (Yabanna and Wang, 1987; 1991). The potential new technique, amine based extraction, does not work well with fermentation broth because of the anion
interference. The in situ extractive fermentation is supposed to be able to avoid the anion interference in amine based extraction, however, it falls into the deadlock that there hardly exists an operation condition ideal for both fermentation and extraction. Further improvement in organic acid extractive fermentation depends on overcoming the solvent toxicity problem and the pH deadlock. It was suggested that amine based extraction had better be treated as a chemical reaction rather than an extraction process (Scholler et al., 1993; Lazarova and Peeva, 1994). A distribution coefficient is not specific enough to describe a complexation reaction, which might happen in both aqueous and organic phases. Jin (1997) introduced an ionic form complexation reaction in the aqueous phase instead of a molecular form neutralization reaction to explain anion interference in amine-based extraction process.

It is known that the extraction process can work with a low product concentration in the fermentation, in which the solvent regeneration is a must. Non-dispersion phase contact in both extraction and back-extraction would be preferred rather than direct contact extraction process. For this purpose, a new phase contact technique called membrane-based extraction was recommended to conduct both extraction and back extraction. Membrane-based phase contact is a relatively new technique using the static phase interfaces that are immobilized by the micropores of the membrane as the mass transfer surface instead of the dynamic phase interfaces in mixing and settling. The high volumetric specific surface (area/volume) of the hollow fiber membrane cartridge can
give a higher mass transfer efficiency in this type of phase contact than in the conventional phase contact. Some of the advantages of membrane-based phase contact include non-dispersion, no flooding, and no flow rate limitation. But most importantly, this configuration protects the cells from the contact with solvent phase, which could get rid of the problems such as clogging and aggregation of cells caused by phase contact.

We believe that lactic acid production can be enhanced if extraction system is being implemented to fungal fermentation system because *R. oryzae* can grow and produce lactic acid at a lower pH as compared to bacteria.

2.6.1. Organic acid extractive fermentation

Solvent extractive fermentation was first suggested by a patent assigned to du Pont, which is based on isopropyl ether (Jenemann, 1933). Schopmeyer (1954) and Vick Roy (1985) described the purification of lactic acid after cell removal. In the patent assigned to Cargill more than 70% lactic acid was extractively recovered from aqueous solutions by basic amine tricaprylyl amine (Eyal et al., 1998). Lactic acid production from cellulotic biomass by cellulase and *Lactobacillus delbrueckii* was studied in a fermenter-extractor employing a microporous hollow fiber membrane (MHF) (Chen and Lee, 1997). This bioreactor system was operated under a fed-batch mode with continuous removal of lactic acid by an in situ extraction. They found that a mixture of 20%
Alamine 336, 40% oleyl alcohol and 40% kerosene was the most effective in the extraction of lactic acid.

Roychoudhury, et al. (1996) in an elegant review with 80 references discussed lactic acid production methods, with emphasis on the process parameters for enhancement of the lactic acid production in extractive bioconversion processes. In general, organic acid fermentation has not been as successful as alcohol fermentation when in situ solvent extraction was applied. Most of the in situ organic acid extractive fermentations reported were with homolactic acid fermentation. No organic acid extractive fermentation experiment was reported until 1986, although the first success with ethanol was in 1981. Yabannavar and Wang (1987, 1991) first presented the extractive fermentation with lactic acid. The extractant was 15% Alamine 336 in oleyl alcohol, which was found to be the best in terms of extraction power and biocompatibility. Immobilized Lactobacillus delbrueckii was used. However, only slight improvement in productivity was reached. One reason for this slow progress with organic acid extractive fermentation is probably the difficulty in finding a suitable solvent that meets the same criteria as the solvent for alcohol fermentation. The solvent effect in organic acid fermentation seems more complicated and more species-dependent than in alcohol fermentation (Daugulis, 1985). Due to the severe toxic effect of organic acid extraction solvents, efforts were made to better immobilize cells to protect cells from toxicity (Yabannavar and Wang, 1991; Lewis and Yang, 1992; Wang et al, 1994). A group
of extractants, mostly secondary and tertiary amines, have very high distribution coefficients for organic acids (Ricker and King, 1988), but in general their toxicity to cells is also very high (Dave et al, 1979). These extractants affect cell growth in a more complicated way, which has not been fully understood. Investigators found that paraffin oil was not toxic to *Lactobacillus delbrueckii*, while all of the amines tested were highly toxic (Seevaratnam et al, 1991; Herrfurth and Hartmeier, 1990; Mattiasson et al, 1990). However, the low capability of the paraffin solvent cannot improve productivity very much. Another main problem is that extraction requires low pH to extract acids in undissociated form, but most of the fermentations can only work at pH 6 to 7. Therefore, an optimum pH for organic acid extractive fermentation must be carefully chosen (Wang et al, 1991; King, 1992; Hano et al, 1993). For long time only lactic acid fermentation that can tolerate low pH has been demonstrated experimentally for extractive fermentation, but there was no solvent regeneration loop and no long-term test studied (Scholler et al, 1993; Yannabavar and Wang, 1991; Honda et al, 1995; Ye et al, 1996). Propionic acid fermentation by *Propionibacterium acidipropionici*, which has an optimum pH between 6.0 and 7.0, was also studied for *in situ* product recovery (Lewis and Yang, 1992). The extractant used was 50% Alamine 336 in 2-octanol, which was only partially compatible to the microorganism. The toxicity was greatly reduced by immobilizing the bacteria in a spiral wound fibrous matrix. Acetic acid was recovered *in situ* in a fluidized bed mixing culture bioreactor using solvent extraction (Busche, 1991). The yield unfortunately was very poor. Membrane based processes were also developed
by using simulated fermentation broths or acid solutions (Wang et al, 1991; Basu and Sirkar, 1992). A membrane based extractive fermentation system for propionic acid was demonstrated by Jin and Yang (1997). They reported five times increase in productivity and 20 % increase in yield as compared to conventional batch fermentation. A summary of previous research on organic acid extractive fermentation is given in Table 2.7.

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>Extractant</th>
<th>Process configuration</th>
<th>Performance improvement</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid</td>
<td>50% TOPO in oleyl alcohol and pure oleyl alcohol</td>
<td>In situ two stage extraction</td>
<td>slight increase in productivity</td>
<td>Honda et.al., 1991, 1995</td>
</tr>
<tr>
<td></td>
<td>50% TOPO in oleyl alcohol</td>
<td>In situ multistage</td>
<td>increase in productivity</td>
<td>Ye et. al. 1996</td>
</tr>
<tr>
<td></td>
<td>15% alamine 336 in oleyl alcohol</td>
<td>In situ</td>
<td>50 % increase in productivity</td>
<td>Yabannavar and Wang, 1991</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>50% Alamine 336 in 2-octanol</td>
<td>Ex situ</td>
<td>100 % increase in productivity</td>
<td>Lewis and Yang, 1991</td>
</tr>
<tr>
<td></td>
<td>50% TOPO in oleyl alcohol</td>
<td>In situ</td>
<td>300 % increase in productivity</td>
<td>YE et.al., 1996</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Mixing culture, fluidized bed</td>
<td>Poor yield</td>
<td></td>
<td>Busche, 1991</td>
</tr>
<tr>
<td>Butyric acid</td>
<td></td>
<td></td>
<td>125% increase in productivity</td>
<td>Hatzinikoalou and Wang, 1992</td>
</tr>
</tbody>
</table>

Table 2.7 Summary of the previous research on organic acid extractive fermentation.
2.6.2. Solvent Selection Criteria

There are a few important criteria to consider before starting to the extractive fermentation. These critical parameters are discussed in the following sections.

2.6.2.1. Solvent toxicity

Problems caused by solvents generally can be categorized into two categories. The first one is the emulsification caused by phase contact and related clogging and aggregation of biomass. The second problem is the biological effect of the water-soluble part of the solvent. The first problem can be avoided by proper phase contact technique such as using hollow fiber membrane. The second one is more chemical and biological in nature, which is actually what most of the toxicity research focused on.

Solvent toxicity was first studied by Dave (1979) when hazardous properties of solvents were of interest. Some of the commonly used solvents, such as aliphatic amines and tri-n-butyl-phosphate, were found to inhibit the growth of green algae, *Chlorella emersonii*, and fish. Most amines were found to be toxic to algae and fish as well as cellulose-degrading bacteria when the nitration was even at a low saturation level (Dave, 1979). The quaternary amine compound, Aliquat 336, was the most toxic. However, one of the 13 solvents reported to be nontoxic to *Methanobac* was Aliquat 336 (Playne, 1983). Based on the experimental results, Playne reported that generally the extractant chemicals were not toxic unless present at a level in excess of that expected to be
required to saturate the aqueous phase. As reviewed by Roffier (1991), 2-octanol was found not to strongly inhibit cell growth in one study, but in another study all alcohols tested was reported to inhibit the growth of the same bacterium (Roffier, 1991). Biocompatible solvents found so far mostly include kerosene, cyclooctane, cyclohexane, dodecane, benzyl benzoate, diethylphthalate, dibutylphthalate, dodecanol, and oleyl alcohol (Roffier, 1991). The conclusions about solvent toxicity seem sometimes inconsistent and contradictory which sometimes lead to many debates and controversy.

In general, the mechanism of solvent toxicity to a microorganism has not been well studied and all previous experiments may not have been carried out under well-controlled conditions. Brink and Tramper (1985) found that solvent toxicity is related to the solvent properties, such as the polarity and the molecular size. The toxicity has also been studied as a function of solvent saturation level in media (Dave, 1979; Yabannavar and Wang, 1991; Lewis and Yang, 1992). Until today no data are available about the solvent toxicity to *R. oryzae*.

2.6.2.2 Solvent polarity and molecular weight

There are many different parameters, such as the dipole moment, dielectric constant, and polarizibility that can be used to indicate the polarity of a compound. A weak, negative correlation was established between the retained activity of the cell and polarity parameter that is expressed as the solubility parameter
$\delta = \rho \left( \frac{\Delta H_v - RT}{MW} \right)^{0.5}$

where $\Delta H_v$ is the enthalpy of vaporization, $R$ is the gas constant, $T$ is the temperature, $MW$ is the molecular weight, and $\rho$ is the density (Brink and Tramper, 1985). This correlation indicates that a low polarity tends to cause less toxicity, but not remarkably. However, a significant relation existed when the retention of cell activity was considered as a function of both the solubility parameter and the molecular weight.

2.6.2.3. Saturation level

The presence or absence of a separate organic solvent phase could be a critical factor in affecting the toxicity to cells (Brink and Tramper, 1985). Often rapid loss of catalytic activity occurred when a separate immiscible solvent phase existed. When the fermentation broth was saturated with a secondary or tertiary amine, the cell growth was completely stopped (Roffler, 1991). The effect of Alamine 336 saturation level on cell growth was also studied (Yabannavar and Wang, 1991; Lewis and Yang, 1992; Solichien et al, 1995).

2.6.2.4. Cell immobilization

Immobilization has been reported to give cells more tolerance to solvent. Further study concluded that immobilization can protect cells against the immiscible phase, but
the water soluble portion of the solvent would still be toxic to the cells (Yabannavar and

2.6.3. Solvent screening procedures

Solvent screening methods have been systematically developed for alcohol
extractive fermentation. Kollerup and Daugulis (1985; 1986) in their study; used
UNIFAC/UNIQUAC models to predict liquid-liquid equilibrium conditions, which was
then combined with other available data such as physico-chemical properties, price, and
toxicity data, if available, to rank over 1,500 extractants in terms of their predicted
performance on single or aggregate criteria. The criteria used were biocompatibility,
favorable distribution coefficient for the product, non-biodegradability, phase stability,
low mutual aqueous solubility, chemical and thermal stability, non-hazardous nature, low
price, and availability in bulk quantities. 70 selected solvents were then investigated
experimentally. Non-toxic solvents found include:

(a) double bond unsaturated aliphatic alcohols (C>12),
(b) saturated branched chain aliphatic alcohols (C>14),
(c) double bond aliphatic acids (C>12),
(d) aliphatic and aromatic mono, di, or tri esters (C>12)
(e) aliphatic noncyclic ketones and aldehydes (C>12), and
(f) mixture of (a) to (e) (Kollerup and Daugulis, 1985).
Among those solvents, 20 of them, including oleyl alcohol and dodecanol were identified as completely biocompatible with yeast cells. This work was also expanded to test the validity of applying yeast biocompatibility data to algae, for a purpose of finding some general parameters to predict toxicity to microorganisms (Frenz et al, 1989).

2.6.4. Amine solvents

Although secondary and tertiary amines known to be very toxic to most of the microorganisms, they are receiving a lot of research attention because they have very high extraction power for organic acids. Conventional extractants have distribution coefficients \((K_d)\) around 1.0; however, amine solvents have a \(K_d\) value of higher than 10 for some organic acids. Understanding amine extraction is thus very important in developing a practical extractive fermentation process.

The distribution coefficient \((K_d)\) of an organic acid can be defined as the concentration of the acid in the organic phase divided by that in the aqueous phase.

\[ K_d = \frac{C_{org}}{C_{aq}} \]

The distribution coefficient is constant for extraction if there is no reaction involved. Unlike the physical extraction which occurs without any chemical reaction, \(K_d\) in amine extraction is not a constant and it is greatly function of many parameters such as
pH value in the aqueous phase, concentration of amine in the solvent phase, nature of amines and diluents, and of course the organic acid concentration in aqueous phase.

Amines as extractants for organic acids were first studied by Wardell and King (1978) and followed by Ricker and King (1979), Wennerstern (1983), and Kertes and King (1986). Amine-based extraction for the recovery of organic acids was also reviewed by King (1992). There are four different kinds of amines that can be used as a extractant: primary, secondary, tertiary, and quarternary. Most of the amines that have been studied are secondary or tertiary amines because primary amines are not good as a solvent due to their high solubility in water. Quarternary amines did not show very high distribution coefficients for organic acids and were found to be able to extract both dissociated and undissociated acids (Yang et al, 1991). Tertiary amines, especially alamine 336 (Henkel), have received most of the research attention. Secondary amines, such as Adogen 283 (Ashland), are subject to amide formation when distillation is used to regenerate the solvent, although secondary amines provide even higher $K_a$. Different organic acids, amines, and diluents continue to be examined for their $K_a$ values (Achour et al, 1994; Bizek et al, 1992; Malmary et al, 1993; Prochazka et al, 1994). Some significant parameters affecting the amine based solvent extraction are summarized in the following sections.
2.6.4.1 pH effect

The $K_d$ value depends on pH value in the aqueous phase (Yang et al, 1991; King, 1992). It has been pointed out that $K_d$ nearly goes to zero when the pH in the aqueous phase is greater than 7, whatever the $K_d$ is at low pH. This phenomenon is due to the fact that the tertiary amine can only extract the undissociated organic acid. The pH effect was described by the following model based on dissociation chemistry (Yang et al, 1991):

$$K_d = \frac{K_1 + \frac{K_2}{[H^+]}}{1 + \frac{K_3}{[H^+]}}$$

where $K_a$ is the equilibrium or dissociation constant of the acid, and $K_1$, and $K_2$ are two intrinsic distribution coefficients at extremely low and high pH values respectively. Reisinger et al, (1995) and Tung et al, (1994). also investigated that some liquid extractants and solid sorbents could extract acid at a pH above the pK_a value. It was possible to extract organic acids at a pH higher than the pK if more basic extractants or sorbents were used for extraction.

2.6.4.2 Effects of physical and chemical properties of amine and diluent

Different diluents can lead quite different $K_d$ values even if other conditions are kept the same, which basically means that amine based extraction depends strongly on the nature of diluent. Many diluents, such as chloroform, 2-octanol, heptanone and
hexane have been studied. It seems that a more polar diluent tends to yield a greater $K_d$ value (Ricker and King, 1979). The diluent was assumed to influence the stability of the amine-acid complex by providing solvation. In general, secondary amines have higher $K_d$ values than tertiary amines because secondary amines tend to form the complex more easily.

2.6.4.3. Effect of initial acid concentration

It was observed that $K_d$ went down when the acid concentration in the aqueous phase increases (Ricker and King, 1979). In some cases $K_d$ can become a constant when the acid concentration is high enough.

2.6.4.4. Effect of temperature

It was observed that the most of the amine extractions were not temperature sensitive in terms of the reaction rate. The equilibrium can be reached quickly at ambient temperature (Kertes and King, 1986). It is not necessary to work at high temperatures. In fact most of the amine-based extraction can be carried out at room temperature. However, $K_d$ has a strong dependence on temperature and the solvent can be stripped by hot water (Wennerstern, 1983).
2.6.4.5. Anion interference

The first example of amine as an extractant was in the metal recovery industry as a liquid ion exchanger (House, 1983). Both secondary and tertiary amines were used to extract a metal anionic complex from ore. It was also mentioned that amines can extract both organic acids and inorganic acids. H₂SO₄ was also reported to be extracted by an amine (Tung and King, 1994). The ion exchange mechanism in extraction for organic acids was first discussed when liquid membrane extraction did not work with the lactic acid fermentation broth (Scholler et al, 1993). Anion interference actually makes amine extraction in practice more difficult than it appears.

2.6.5. Operation of membrane based extraction

The interface of the phases is immobilized in the pores of the membrane. The position of the interface is a function of the pressure difference of the two phases, the nature of the two phases, and the nature of membrane material. When the membrane is hydrophobic, the pressure in the aqueous phase must be maintained higher than that in the organic phase to keep the interface inside the pores. Otherwise, the pressure in the organic phase needs to be higher than that in the aqueous phase when the membrane is hydrophilic. There is a pressure value for any given system that is called the breakthrough pressure. When the pressure difference of the two phases exceeds the breakthrough pressure, the interface will lose stability and one of the two phases will go
through the membrane pores into the other phase. Therefore, the pressure difference of the two phases is a critical parameter in membrane-based extraction. To maintain proper pressure difference between two phases throughout the whole module, so a co-current flow mode can be suggested. The membrane material used in most commercial hollow fiber modules for extraction is polypropylene because of its high resistance to various organic solvents, strong acids, and strong bases.

2.6.6. Breakthrough Pressure

Some data on measurement of breakthrough pressure for organic-water systems are available in literature. The breakthrough pressure for a membrane depends on the pore-size distribution, the interfacial tension between the two liquids, the contact angle of the aqueous (nonwetting) phase on the membrane wetted by the organic (wetting phase), and the size and shape of the openings in the membrane. A membrane with a wide pore-size distribution would have some pores much larger than the average pore size, and fluid would break through these large pores first. In order to force a nonwetting fluid through the pores of a membrane the applied pressure should be at least equal to the pressure drop across the interface $P_A - P_B$ given by the Young-Laplace equation:

$$P_A - P_B = 2\gamma \cos \theta (1/r_1 + 1/r_2)$$
where $P_A$ is the pressure in phase $A$, $P_B$ is the pressure in phase $B$, $\gamma$ is the interfacial tension, $\theta$ is the angle at which the interface meets the capillary wall (contact angle) as measured in phase $B$ and $r_1$, and $r_2$ are the two radii of curvature for the surface. The contact angle of a fluid with a solid is a measure of the wettability of the solid by the fluid. The interfacial tension is a liquid-liquid property while the contact angle is a liquid-liquid-solid property. Prasad and Sirkar (1987) made measurements of the breakthrough pressure for the non-wetting fluid (water), but they did not measure interfacial tension and contact angles. Lee et al. (1984) measured permeability of water through hydrophilic membranes at increasing levels of oil fouling. They used the capillary equation to estimate the pressure required to force oil droplets into the membrane:

$$P_A - P_s = 2\gamma \cos \theta / r$$

where $r$ is the capillary radius. Increased membrane fouling (and reduced water permeability) was observed for operation above the calculated capillary pressure.

Kim and Harriot (1987) have modeled the breakthrough pressure for the non-wetting fluid in the pores of hydrophobic membrane by combining the model by Pucell (1949) with the model of Crisp and Thorpe (1948) and obtained the following equation:
\[ \Delta P_{\text{eff}} = 2\gamma \cos \theta_{\text{eff}} / r \]

where \( \theta_{\text{eff}} \) accounts for the pore and was evaluated from measurement of the contact angle \( \theta \) on the membrane surface.
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CHAPTER 3

FERMENTATION KINETICS AND STABILITY STUDIES

Summary

The growth kinetics of *Rhizopus oryzae* NRRL 395 was studied under immobilized and free cell fermentation conditions. The effects of different carbon sources and nitrogen sources on cell growth were investigated. It was observed that the best nitrogen source for the growth phase was yeast extract; whereas there was no spore germination or growth when other nitrogen sources, including urea, ammonium nitrate, and ammonium sulfate were used.

The effects of various parameters such as carbon source, nitrogen source, and mineral concentration on L(+)-lactic acid production by *R. oryzae* were studied. The full factorial and response surface analysis experimental designs at five levels with four parameters were constructed to optimize the medium composition for the production phase. The parameters studied were urea, ZnSO₄, KH₂PO₄, and MgSO₄. The productivity, yield, and biomass were observed at the end of each experiment. It was
observed that urea was the most important factor since there was a significant difference (p< 0.05) between levels.

The effects of immobilization and end-product concentration on lactic acid productivity and yield were also investigated. The effect of initial L(+) lactic acid concentrations on the fermentation was also studied. The long-term stability of immobilized cells was evaluated. It was seen that immobilized cells of *R. oryzae* were capable of producing L(+) lactic acid for 10 consecutive batches. The SEM pictures of immobilized cells were taken at different stages to observe morphological differences.

Key words: *Rhizopus oryzae*, L(+) lactic acid fermentation, optimization, and long-term stability
3.1. Introduction

Lactic acid fermentation has received a lot of interest because of its many industrial applications (Soccol et al. 1994a,b; Tamada 1992; Yang 1995; Hamamci 1994) and potential use as the feedstock for the production of PLA, a biodegradable polymer. According to Argonne National Laboratory (www.anl.gov), the production of lactic acid may rise to billions of pounds if a more successful way of producing good quality PLA can be found. Producing better quality PLA relies on producing inexpensive and quality lactic acid. The majority (85%) of lactic acid is produced being used by the food industry in various applications. The two main routes for lactic acid production are chemical synthesis and fermentation. Fermentation has many advantages over chemical synthesis in terms of product quality and renewable resources. Lactic acid fermentation traditionally has been carried out via bacterial fermentation. Nowadays industry uses many strains of *Lactobacillus delbureckii* to produce lactic acid. Recently, there has been new interest in L(+)-(+)-lactic acid fermentation using the filamentous fungus *Rhizopus oryzae*. However, more research is needed to understand the kinetics and stability of fermentation process by *R. oryzae*.

In this work, the kinetics and stability of L(+)-(+)-lactic acid fermentation by *R. oryzae* were investigated in details. The effects of nitrogen source, carbon source, minerals, and end-products on the fermentation were studied in shake-flasks. Moreover, the effect of cell immobilization on stability of *R. oryzae* fermentation was thoroughly
investigated. The advantages of using immobilized cell over free cell were also studied in details.

3.2. Materials and Methods

3.2.1. Culture

*Rhizopus oryzae* NRRL 395, obtained from the Northern Regional Research Center, Peroria, IL, was used in this study. *R. oryzae* is a filamentous fungus, which produces L(+)-lactic acid. The fungus was maintained on potato dextrose agar slants (200 g/L potatoes (infusion form), 20 g/L Bacto dextrose, 15 g/L Bacto agar). Stock cultures were stored in the refrigerator and transferred to a new potato dextrose agar (PDA) slant once a month to maintain the viability of the cultures. The sporangiospores were produced from PDA petri plates by shaving and extracting the spores with sterile water or from medium, which contained 50g/L glucose, and 5g/L yeast extract. The stock culture was filtered through a sterilized cheese cloth. The number of spores in the stock culture was adjusted to 10^6 spore/mL by dilution. The spore count was carried out by hemacytomoter and plate counting methods.

In hemacytomoter method 0.1 µL spore solution was placed on hemacytometer slide and spores in 5 diagonal squares were counted under a light microscope (Figure 3.1) The number obtained from those 5 squares was multiplied by 2.5x10^4. Then the proper dilutions were made to obtain the desired spore count of 10^6 spores/mL.
Total number of spores = (2.5 \times 10^4) \times N_d

Where \( N_d \) is the number of spores obtained from 5 diagonal squares.

In the plate count method, the stock culture was diluted to certain levels and then plated on the PDA agar. The number of colonies were counted after 36-48 hours.

3.2.2. Materials

Glucose and corn starch (DE 24) were obtained from Cargill, L(+)-lactic acid and fumaric acid were obtained from Sigma (St. Louis, MO, USA). Urea (Mallinckrodt), sodium hydroxide (Mallinckrodt), \( \text{KH}_2\text{PO}_4 \) (Sigma), \( \text{MgSO}_4\cdot7\text{H}_2\text{O} \) (J.T. Baker), \( \text{ZnSO}_4\cdot7\text{H}_2\text{O} \) (Matheson Coleman & Bell), 200 proof ethanol (McCormick), \( (\text{NH}_4)_2\text{SO}_4 \) and \( \text{NH}_4\text{NO}_3 \) (Fisher) were obtained from the stores at The Ohio State University. Yeast extract was obtained from USB (United States Biochemical Corporation). All reagents used were in analytical grade except regular corn starch (ARGO) was in consumer grade.

3.2.3. Media

Growth medium

The growth medium consisted of 50 g/L carbon (glucose or starch) and 5 g/L yeast extract. 100 mL of the medium in a 250-mL erlenmeyer flask was sterilized at 121°C for 30 min in an autoclave (AMSCO). Yeast extract was separated from the carbon-containing portion to prevent any undesirable reaction during autoclaving. No \( \text{CaCO}_3 \) was added because the control of pH was not necessary during the growth phase since the main purpose was to produce cell biomass during the growth phase. The pH
changed from 6.5 to 3.5 during the growth phase. In case of immobilized cell fermentation, 2 g of cotton pieces (with dimension of 2 cm x 2 cm x 0.4 cm) were added as the matrix for cell immobilization.

Production medium

Unless otherwise noted, the medium for L(+)-lactic acid production consisted of 70 g/L carbon source (starch or glucose), 0.6 g/L KH₂PO₄, 0.25 g/L MgSO₄, 0.088 g/L ZnSO₄, 0.3 g/L nitrogen source (urea, (NH₄)₂SO₄ or NH₄NO₃) and 50 g/L CaCO₃, which was added to maintain the medium pH at ~6. 100 mL of the medium in a 250-mL erlenmeyer flask was autoclaved at 121°C for 30 min. Urea and ZnSO₄ were separated from the remaining medium to prevent unwanted reaction during autoclaving.

3.2.4. Methods

Free cells

One mL of spore suspension obtained by the method described in section 3.2.1 was used in the growth medium. The flask was then placed in a shaker-incubator for cell growth at 30°C and 210 rpm for 2 to 4 days.

Immobilized cells

The same procedure described in section 3.2.3 was used, except that 2 g of 100% cotton fiber (2 cm x 2 cm x 0.4 cm) was placed in each flask and autoclaved before
inoculation. Mycelia grew and firmly attached to the fiber, with virtually no suspended cells in the medium.

3.2.5. Production of L(+)‐lactic acid

In order to produce L(+)‐lactic acid, mycelia were first grown in the growth medium for approximately 2‐4 days. Afterward, the growth medium was poured out from the flask and a sterilized production medium was added. The flask containing free cells or immobilized cells for the production phase was then placed in a shaker‐incubator at 30°C and 210 rpm. Production of L(+)‐lactic acid finished when all carbon sources were utilized. Samples were taken from each flask at proper time intervals (4‐8 hours). The procedures are shown in Figure 3.2.

To evaluate long‐term stability repeated batch fermentations were carried out by replacing with new production medium at the end of each fermentation

3.2.6. Assay procedures

3.2.6.1. Cell biomass

Growth of fungus is usually extensive and difficult to measure without destroying cells. Biomass of R. oryzae was measured at the end of each fermentation run. Cells were taken out of the flasks and washed with distilled water to remove any residue, then the cells were put into the oven for drying. Cells were dried in the oven at 105°C for overnight (at least 18 hours) and the dry weights were measured. The biomass yield (YXS)
was determined by the amount of dry cell produced at the end of the fermentation divided by the total substrate consumed.

\[ Y_{XS} = \frac{\text{g dry cell}}{\text{g substrate consumed}}. \]

3.2.6.2. Substrate and product concentration

The concentration of L(+) -lactic acid and glucose were measured by using either High Performance Liquid Chromatography (HPLC) or glucose-lactate analyzer (YSI-2000). Starch was first hydrolyzed to glucose by using concentrated HCl (37% w/v) and measured as glucose equivalent (Method described in Silva, 1997). Fumaric acid and ethanol were analyzed by the HPLC only. In this study, lactic acid yield \( (Y_{PS}) \) was reported in percent yield and determined by plotting the amount of lactic acid vs. the amount of glucose.

\[ Y_{PS} \text{ (%)} = \frac{\text{g lactic acid produced}}{\text{g glucose consumed}} \times 100 = \text{-slope} \times 100 \]

Productivity was determined from the slope of lactic acid produced (g/L) vs. time plot.

3.2.6.3. High Performance Liquid Chromatography (HPLC)

HPLC was used to determine the concentrations of glucose, ethanol, fumaric acid and lactic acid in the fermentation broth. Samples from the fermentation broth were vortexed, and then diluted with a known amount of double distilled water, again vortexed and then centrifuged for 4 minutes. 15 µL of this clear, diluted cell free sample were injected by autosampler (Shimadzu-SIL-10Ai) into an organic acid analysis column (Bio-Rad, Aminex-Model HPX-87H- Ion exclusion organic acid column; 300 mm x 7.8 mm).
maintained at 45°C in a column oven (Shimadzu- CTO- 10A VP). 0.01 N H$_2$SO$_4$ was used as the eluant at 0.6 mL/min flow rate. A RI detector (Shimadzu- RID 10A) was used at the range of 200 to detect the organic constituents in the stream. A standard containing 2 g/L of the each component was used periodically to determine the unknown sample concentrations. The peak height was selected as a basis of comparison. It is noted that HPLC would detect both L(+) and D(-)-lactic acids. A typical HPLC chromatogram is shown in Figure A.1 in the Appendix section.

3.2.6.4. Glucose and L(+) -lactic acid analyses by YSI-2700

Glucose and L(+) -lactic acid were also analyzed using YSI Model 2700 glucose-lactate analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). This is an analytical instrument that is not affected by turbidity and pH and is accurate within a range of 0.04 g/L.

Samples of the fermentation broth were centrifuged for 4 min and diluted 10 times by distilled water, then the diluted sample was presented to the glucose-lactate analyzer and glucose and L(+) -lactic acid results were recorded. Results from HPLC and YSI-2700 were compared and found to be the same (± 0.01%), indicating that only L(+) -lactic acid was produced in the fermentation.
Buffer used in the YSI analyzer was prepared according to the following composition:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$H$_2$EDTA</td>
<td>4.4g</td>
</tr>
<tr>
<td>Kanamycin sulfate</td>
<td>0.05g</td>
</tr>
<tr>
<td>Sodium Benzoate</td>
<td>7.3g</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>12.0g</td>
</tr>
<tr>
<td>Na$_2$PO$_4$</td>
<td>54.7g</td>
</tr>
<tr>
<td>NaCl</td>
<td>21.5g</td>
</tr>
</tbody>
</table>

The substances above were blended thoroughly, and then 12.7 g of the mixture was mixed with 900 mL of distilled water to form the buffer solution. The standard solution for YSI-2700, which contained 2.5 g/L of glucose and 0.5 g/L of L(+)-lactic acid, was obtained from YSI Inc. More information can be found in the operating manual for YSI 2700.

### 3.2.6.5. Scanning Electron Microscopy

The morphology of *R. oryzae* was studied at different stages of fermentation. The samples were taken from the growth phase and the production phase. In the case of long-term stability, the samples were also taken from different batches. The common procedure for scanning electron microscopy was followed. The steps for SEM are summarized as follows:

1. The samples were treated with 3% glutaraldehyde (EM grade) solution containing 1.7 g/L of sucrose and 0.2 M phosphate buffer of pH 7.0 for overnight.
2- The samples were then washed several times with phosphate buffer in 1 hour time period.

3- Then the samples were placed in different concentration of ethanol for drying.

The sequence and duration of sample treatments in each concentration were as follows:

- 50% of ethanol (by volume) for 30 min.
- 70% of ethanol for 30 min.
- 80% of ethanol for 30 min.
- 90% of ethanol for 30 min.
- 100% of ethanol for 1 hr.

4- After ethanol treatment, samples were brought to critical point drying by flushing CO₂ for at least 1 hour.

5- Then, samples were coated with palladium and gold by using the spotter coating machine in the presence of the medium containing argon gas.

6- Samples were examined by using Philips 30 XL brand SEM.

3.3. Results and Discussion

3.3.1. Growth kinetics

The YE was required for germination of spores. In a separate try without any YE in the medium, there was no growth of mycelia. However, YE was too rich for the production phase; including YE would decrease lactic acid yield and productivity. In the
growth phase, no neutralizer (CaCO₃) was added to the medium because the goal was to produce mycelia rather than L(+)-lactic acid. The pH changed from 6 to 3.5 during the growth phase due to L(+)-lactic acid production. Another important point is that the same type of carbon source was used for the growth phase and the production phase.

The effect of cell immobilization on the growth phase was investigated. Growth parameters were kept the same for both immobilized cell and free cell fermentations. The only difference was 2 g of cotton fiber (terry cloth) was added to each flask in the immobilized cell fermentation. The main advantage of immobilized cell over free cell was ease of separation and transfer of cells from one batch to the subsequent batch or to the production phase. Cells were attached firmly to the cotton fiber and the liquid medium was virtually cell free in immobilized cell fermentation.

Figure 3.3 shows the production of L(+)-lactic acid in immobilized and free cell fermentations utilizing glucose. The initial glucose concentrations were ~50 g/L for these fermentations. The final L(+)-lactic acid concentrations for free cell and immobilized cell fermentations were 11.5 and 14.2 g/L, respectively. A lag phase of ~15 hours was observed for both fermentations; there was no significant L(+)-lactic acid production during that time. The lag phase perhaps was attributed to the time required for spore germination and maybe physiological change to induce lactic acid production.

Similar results were observed for starch as the carbon source. Figure 3.4 shows
the L(+)-lactic acid production kinetics using corn starch. The initial corn starch concentrations were measured to be 53 g/L and 46 g/L for immobilized and free cell fermentations, respectively. The final L(+)-lactic acid concentrations of 14.7 and 17.7 were observed after 58 hrs of fermentation for free and immobilized cell, respectively. A lag phase of 18 hrs was observed for both fermentation types.

Lactic acid yield, productivity, and biomass yield are given in Figure 3.5. As can be seen in this figure, that the immobilized cells gave higher lactic acid yield, productivity, and biomass yield as compared to those for free cells. The yields from glucose were 29.9% and 23%; and were 26.5% and 29.6% from corn starch, for immobilized and free cell fermentations, respectively. The yields were relatively low compared to the production phase where yields of 75-85% were observed. The productivity values of 0.198 and 0.245 g/L*h were observed from glucose, whereas 0.23 and 0.26 g/L*h from corn starch, for free and immobilized cells, respectively. Although the lag phase for L(+)-lactic acid from glucose was slightly shorter than the fermentation with starch, the observed productivity values were slightly higher for fermentation utilizing starch for both immobilized and free cells.

One big challenge of fungal fermentation is the lack of a fast online measurement technique to monitor the biomass concentration. The current method of drying cells is destructive and it is impossible to precede fermentation after the biomass measurements. In this study, the final concentration of biomass was obtained after each fermentation
cycle. The observed biomass concentration values indicated that immobilized fermentation technique and starch gave higher biomass concentration as compared to free cells and glucose. The difference for starch and glucose can be due to chemical degradation of starch to glucose where every hydrolysis utilizes one mole of water.

3.3.2. Production kinetics

The aims of studying the production kinetics were (a) to investigate the effects of medium composition on L(+)-lactic acid fermentation, (b) to investigate the feasibility of L(+)-lactic acid production from glucose and starch, (c) to determine and demonstrate the feasibility of a fibrous matrix as an immobilization support for fungal cells, (d) to demonstrate the long-term stability of immobilized cells for L(+)-lactic acid production, and finally (e) to investigate the optimum conditions for improved L(+)-lactic acid yield and productivity.

3.3.2.1. Effects of free and immobilized cells on L(+)-lactic acid production

Figure 3.6 shows L(+)-lactic acid production in immobilized and free cell fermentations utilizing glucose as the substrate. It is observed that L(+)-lactic acid production in the immobilized cell fermentation was higher than that in the free cell fermentation. L(+)-lactic acid concentrations were 45 and 49 g/L at the end of 54 hrs of fermentation for free cells and the immobilized cells from 70 g/L of initial glucose respectively.
The lactic acid yields from glucose for immobilized and free cells were 76%, 72.6%, respectively. The volumetric productivity at the end of fermentation of 54 hrs was also higher for immobilized cells. The value of productivity for immobilized cell fermentation was 1.05 g/L*h, whereas for free cell fermentation was 0.94 g/L*h. Ho (1996) also observed similar findings between immobilized and free cells. The difference between these two fermentation techniques could be because of increased surface area of fungi due to immobilization. Consequently, the higher surface area will give a better mass transfer rate and nutrient to the cells. Free cells suspended in medium in the shake-flask may be subjected to high shear stress from high agitation rate (210 rpm). The fiber matrix may protect the cells from high hydrodynamic forces created in the shake-flask.

Similar results were obtained from starch (DE 24). As seen in Figure 3.7, L(+)-lactic acid concentrations were 49 and 54.3 g/L at the end of fermentation of 51 hrs. The immobilized cell fermentation gave a yield of 74.3%, whereas free cell fermentation yield was around 70.31%. The corn starch also gave a slightly lower yield and productivity of L(+)-lactic acid than glucose. This fact was observed for both immobilized and free cells fermentation techniques.

Figure 3.8 compares the yield, productivity, and biomass yields (g/g) in immobilized cell fermentation and free cell fermentation. The biomass yields were found to be slightly higher for immobilized cells and starch.
Although it looks like there is difference between all these treatments (free cells vs. immobilized cells or starch vs. glucose) it is hard to say that the difference is statistically significant. One way to analyze the effect of treatments is to carry out a series of experiments and run t-test. However the general trend of higher productivity and yield from glucose was observed for all experiments where the conditions may vary. Although one can say the data can be categorized into free cells vs. immobilized cells without any consideration to the substrate type. The interaction of parameters should be negligible or independent, although in this case is unknown.

3.3.2.2. Effects of substrate concentration

The effect of substrate concentration on L(+) -lactic acid fermentation was investigated. The purpose of this was to find the optimum substrate concentration for lactic acid fermentation and observe the maximum substrate concentration that *R. oryzae* can efficiently produce L(+) -lactic acid.

All experimental runs were carried out in shake-flasks except very high concentration of substrate (270 g/L) where it was impossible to maintain pH around 6 and prevent the precipitation of L(+) -lactic acid with CaCO₃. High concentration of Ca-lactate may cause precipitation during fermentation. Corn starch (DE 24) was used as the substrate for all run. The concentration of the substrate varied from 30 to 270 g/L. CaCO₃ was added as a neutralizer but the concentration was varied according to the projected maximum L(+) -lactic acid concentration at the end of each fermentation run. The
concentrations of CaCO₃ were 50-60 g/L for initial substrate concentration values of 30 and 70 g/L, whereas it was 140 g/L for 150 g/L of starch containing medium. The medium containing 270 g/L of starch did not need any CaCO₃ since it was carried out in a rotating fibrous bed bioreactor (RFBB) with pH control. Details of RFBB are given in Chapter 4.

Figure 3.9 shows the kinetics of L(+)-lactic acid production from 270 g/L of initial substrate concentration. The pH was maintained at 6 and the DO level was around 25% throughout the fermentation. The final L(+)-lactic acid concentration of 78 g/L was reached after 148 hrs of fermentation. The substrate consumption rate can be found from the slope of starch curve, which was found to be 1.6 g/L*h. However the L(+)-lactic acid production rate was found to be just 0.5 g/L*h. Therefore only ~1/3 of starch was converted into L(+)-lactic acid. The ethanol and fumaric acid concentrations were 8.1 and 2.4 g/L, respectively, at the end of fermentation. The total consumption rate of substrate for ethanol and fumaric acid was only about 0.055 g/L*h. This fact means that the rest of the substrates consumed should have been used for another by-product, CO₂, and biomass. The biomass yield was measured to be 0.09 g/g at the end of the fermentation. Therefore, it can be concluded that beginning with high initial concentration of substrate there is a high tendency for biomass formation. Similar results were obtained by Soccol et al. (1999); they found that the best substrate concentration was around 90 g/L of hemicellulosic hydrolysate. Zhou et al. (1999) also found that the optimum substrate concentration was around 94 g/L in a bubble column bioreactor.
Figures 3.10 and 3.11 show the fermentation kinetics with 150 g/L and 30 g/L of corn starch, respectively. The final L(+)-lactic acid concentration of 85.1 g/L was reached after 74 hrs of fermentation for 150 g/L of substrate concentration, whereas the final L(+)-lactic acid concentration of 22.92 g/L of L(+)-lactic acid was observed for 30 g/L of corn starch after 37 hrs of fermentation. In the light of all experimental runs, it can be easily seen that there are lower and higher limits of substrate concentration for L(+)-lactic acid production using immobilized L(+)-lactic acid in terms of final L(+)-lactic acid concentration obtained at the end of each fermentation run. Figure 3.12 shows the effects of various substrate concentrations on lactic acid production in terms of lactic acid yield, productivity, and biomass yield. It can be seen that as the substrate concentration increased, the yield decreased and biomass yield increased. The productivity was highest at the level of 150 g/L with the value of 1.15 g/L*h. On the other hand, better results were obtained at 70 g/L in which the lactic acid yield of 76% and productivity of 0.88 g/L*h were obtained. The results confirm the theory that a high initial substrate concentration promotes high cell growth and biomass formation.
3.3.2.3. Effects of end-products on lactic acid production

The effects of end products (L(+)-lactic acid, fumaric acid, and ethanol) were investigated. It is known that the accumulation of end products after a certain point can inhibit the product formation. In order to investigate the effect of product inhibition, the medium containing initially different levels of L(+)-lactic acid ranging from 10 to 60 g/L were used in the production phase. The other parameters such as substrate concentration, temperature, and agitation speed were kept the same. Corn starch was used as the substrate at 60 g/L. The mycelia grown on the fiber matrix were washed with 100 mL of sterile distilled water several times. The pre-grown cells were placed in the production medium containing different levels of L(+)-lactic acid. The results from these experiments are shown in Figure 3.15. The corn starch utilization was higher for 10 g/L while it was very low for 60 g/L L(+)-lactic acid concentration. The L(+)-lactic acid production was very low for 60 g/L containing flask. The final L(+)-lactic acid concentrations were 69 g/L and 47 g/L for 60 and 10 g/L initial L(+)-lactic acid containing flasks, respectively. As the initial L(+)-lactic acid concentration increased, the production rate for L(+)-lactic acid decreased dramatically. It can be concluded that the L(+)-lactic acid has an inhibitory effect on R. oryzae to produce L(+)-lactic acid.

In order to understand L(+)-lactic acid inhibition and its inhibition magnitude, the yield, productivity, and biomass values were plotted in Figure 3.16. As can be seen both L(+)-lactic acid yield and volumetric productivity decreased sharply as the initial L(+)-
lactic acid concentration increased. The yield value for a flask containing 10 g/L lactic concentration was 66%, whereas it was 22.5% for the flask containing 60 g/L initial L(+)-lactic acid. The difference in volumetric productivity for low and high initial L(+)-lactic acid concentration was also a proof of product inhibition. The productivity values for 10 and 60 g/L initial L(+)-lactic acid concentration were 1.2 and 0.31 g/L*h, respectively. The productivity was almost 4 times higher in 10 g/L initial L(+)-lactic acid concentration than the 60 g/L initial L(+)-lactic acid concentration. All these results strongly suggest that the initial L(+)-lactic acid concentration is a critical factor in L(+)-lactic acid fermentation. The studies on L(+)-lactic acid production pathway in R. oryzae also shows that L(+)-lactic acid concentration can be detrimental to cells, and therefore can affect the fermentation cycle (Yang et. al.,1995; Yu and Hang, 1991).

It can be concluded that the removal of L(+)-lactic acid from the fermentation broth in situ can decrease the profound effect of L(+)-lactic acid inhibition on L(+)-lactic acid fermentation, thus increase the yield and volumetric productivity of L(+)-lactic acid fermentation. In Chapter 5, the effect of L(+)-lactic acid removal in situ on lactic acid production in an extractive fermentation is discussed in details.

The effect of other by-products (ethanol and fumaric acid) on L(+)-lactic acid production was also investigated. The main reason to study this was to understand the effect of each component on L(+)-lactic acid metabolism and production flux. The flasks initially containing 15 g/L of ethanol and the mixture of 10 g/L ethanol and 5 g/L fumaric
acid were prepared the same way that was discussed earlier in the methods section. The effects of ethanol and ethanol-fumaric acid mixture are shown in Figures 3.12 and 3.13. The final L(+)-lactic acid concentration obtained from 15 g/L ethanol containing medium was around 73.3 g/L from 95 g/L of initial corn starch at the end of 110 hrs of fermentation. The ethanol concentration was 20.5 g/L at the end of fermentation, which means that there was only 5 g/L of ethanol production during the entire fermentation cycle. The concentration of ethanol without any initial ethanol was generally around 8-10 g/L for 60 hrs of fermentation. The yield and volumetric productivity values were higher for initial ethanol containing flask than non-containing ones. The L(+)-lactic acid yield was 86 % for ethanol containing experimental runs, whereas the general observed yield were ranged from 65-75 % for the experiments without ethanol additions. Similar results were observed for volumetric productivity values. The productivity of 1.1 g/L*h was observed for ethanol containing run while the observed values for productivities without any initial ethanol ranged from 0.75–0.95 g/L*h.

Since ethanol production was substantially low for 15 g/L ethanol containing flask, it can be concluded that there was a significant effect of ethanol on ethanol production and also a metabolic pathway shift towards L(+)-lactic acid production.
There are three fates of pyruvic acid in *R. oryzae*, as illustrated in Figure 2.3. One of the fates goes to L(+)‐lactic acid, whereas the other two go towards ethanol and fumaric acid. It was concluded that by adding ethanol and fumaric acid to the fermentation broth, it is possible to decrease fumaric acid and ethanol production and increase L(+)‐lactic acid accumulation. Therefore, it is possible to draw a conclusion that there is a metabolic shift towards L(+)‐lactic acid in ethanol containing fermentation runs. This leads to another assumption that there is an equilibrium state between all metabolites. By increasing or decreasing the initial concentration of these metabolites it is possible to alter this equilibrium state towards a desired end product, rather than undesirable end products.

### 3.3.3. Optimization of production medium

In this section, the optimization of the production medium was investigated. It is crucial to obtain the optimized concentrations of all medium components for L(+)‐lactic acid production with enhanced productivity and yield. Medium optimization is very vigorous and needs many tedious experimental runs. The number of experimental runs can be very high depending on the number of parameters and their levels. The optimization studies of fermentation media have received a lot of attention recently (Soccol et al., 1999; Zhou et al., 1999).
The primary objective in developing a fermentation medium is to ensure that the required nutrients are present in appropriate forms and at non-inhibitory levels. The common optimization strategy that is generally employed is the one-variable-at-a-time method in which one independent variable is optimized while the others are held constant. The optima for the remaining variables are determined using the same approach, provided the variables do not interact with one another. However, variables frequently do interact, preventing the use of this optimization approach from always yielding an optimum set of conditions. Therefore, in this study the response surface method was chosen to optimize and find the interactions of all selected variables.

The number of parameters generally determines the best way to design and construct the experiment for optimization purposes. Currently the response surface analysis to determine the optimum fermentation conditions is very common. In this study, the response surface analysis for four parameters at five different levels was performed. The selected variables were urea, $\text{KH}_2\text{PO}_4$, $\text{MgSO}_4$ and $\text{ZnSO}_4$ for the optimization study. The experiments were run at five different levels. Table 3.1(a) shows factors and the levels that have been investigated for optimization purpose. MINITAB statistical software program was used to generate all possibilities for four variables at five different levels using the response surface analysis option under designing of experiment (DOE). The possible 31 experiments are shown in Table 3.1(a). The yield, productivity, and biomass were measured at the end of each fermentation run and these values were used as the response values. The response surface and interaction of variables and effects of
variables on L(+)-lactic acid yield, productivity and biomass were evaluated by MINITAB statistical software program. The response surface wire frame plots were also generated to observe the interaction of all variables and their effects on L(+)-lactic acid productivity, yield and biomass concentration. These graphs are shown in Figures 3.17 - 3.33.

Effects of urea

All microorganisms require nitrogen to support the biosynthesis of nitrogenous metabolites, both primary and secondary. Both organic and inorganic sources of nitrogen can be used in the fermentation broth. In this study, an inexpensive inorganic source, urea, was used as the nitrogen source. The response surface graphs (wire frame) were generated using MINITAB. The estimated coefficient for productivity, yield and biomass are shown in Tables 3.3-3.4.

It can be seen that the most significant changes on yield, productivity and biomass were observed when the concentration of urea differs from one level to another. The effects of different levels of urea concentrations were significant at α = 0.05, since the p-value for the urea was 0.027 for productivity, 0 for yield, and 0 for biomass accumulation. The optimum level for urea concentration was around level -1 (0.3 g/L) for productivity and yield. The biomass concentration increased as the concentration of urea increased. But the productivity and yield values peaked at -1 level. From the graphs (Figure 3.35- 3.37) the interaction of urea with other variables and their effects on
productivity, yield, and biomass can be seen. In order to generate the graphs the concentration of two variables were held constant at the central levels while the other two variables were ranged from -2 to 2 level.

**Effects of minerals**

Growth and production media generally include phosphate, sulfate, potassium, magnesium, zinc and other trace minerals. Minerals play important roles in the intermediary metabolism, in the biosynthesis of certain enzymes, activators and cofactors for many enzymic systems and as biosynthetic regulators of many secondary metabolites. Although these minerals are minor constituents of growth and production phase their levels are important. The stimulatory and inhibitory levels are important and should be optimized. The effects of different minerals, such as $\text{KH}_2\text{PO}_4$, $\text{MgSO}_4$ and $\text{ZnSO}_4$, were investigated. The effects of all minerals and their interactions on L(+) lactic acid yield, productivity, and biomass are also given in Tables 3.2-3.3.

It can be seen that as the levels of minerals increased the productivity also increased, but the changes for the selected levels were not significant at $\alpha=0.05$ where the p values were greater than 0.05. But the yield decreased significantly for $\text{KH}_2\text{PO}_4$ as its level increased ($p<0.05$). Therefore, it is hard to conclude which value would be the optimum for lactic acid production because increasing mineral levels would increase productivity, but reduce the yield.
The effects of medium components on productivity (P), yield (Y), and biomass (B) can be given in the following quadratic polynomial models:

\[
P = 0.98 + 0.0108[\text{ZnSO}_4] + 0.0566[U] + 0.00417[\text{MgSO}_4] + 0.02917[\text{KH}_2\text{PO}_4] - 0.0075[\text{ZnSO}_4][U] + 0.0875[\text{ZnSO}_4][\text{MgSO}_4] + 0.00625[U][\text{MgSO}_4] - 0.0750[U][\text{KH}_2\text{PO}_4] + 0.00875[\text{KH}_2\text{PO}_4][\text{MgSO}_4] + 0.0154[\text{ZnSO}_4]^2 - 0.058[U]^2 + 0.0667[\text{MgSO}_4]^2 + 0.02292[\text{KH}_2\text{PO}_4]^2
\]  
(3.1)

\[
\]  
(3.2)

\[
B = 5.2 + 0.139[\text{ZnSO}_4] + 0.5221[U] + 0.1362[\text{MgSO}_4] + 0.2463[\text{KH}_2\text{PO}_4] - 0.0644[\text{ZnSO}_4][U] + 0.0394[\text{ZnSO}_4][\text{MgSO}_4] + 0.0194[\text{ZnSO}_4][\text{KH}_2\text{PO}_4] + 0.644[U][\text{MgSO}_4] - 0.0431[U][\text{KH}_2\text{PO}_4] - 0.0244[\text{KH}_2\text{PO}_4][\text{MgSO}_4] - 0.098[\text{ZnSO}_4]^2 - 0.1468[U]^2 - 0.1068[\text{MgSO}_4]^2 - 0.1193[\text{KH}_2\text{PO}_4]^2
\]  
(3.3)

From these equations the productivity, yield, and biomass can be determined for different concentration values. The above equations can be simplified by eliminating non-significant parameters. The significant parameters were urea and urea-urea interactions whereas the other parameters (\text{KH}_2\text{PO}_4, \text{MgSO}_4, \text{and ZnSO}_4) and their interactions at the studied levels were insignificant (p>0.05).
3.3.4. Long-term stability

One important advantage of using immobilized cells is its capability to use the same cells for long-term production without any substantial loss in productivity and yield. Immobilized cells can be recycled and used many times easily as compared to free cell fermentation, where cell separation and recycling can be a major problem.

In this study, the long-term stability of immobilized cells of *R. oryzae* was investigated in media containing glucose and corn starch. The glucose contained a medium composed of different nitrogen sources to evaluate the effectiveness of different nitrogen sources, such as NH$_4$(NO$_3$) and (NH$_4$)$_2$SO$_4$, besides urea. In the case of starch, urea was the only nitrogen source in the production medium. The procedure for long-term stability studies was the same as described in the method section. The only difference was after each batch run, the immobilized cells were washed several times with 100 mL of sterile distilled water and a fresh production medium, with 50 g/L of CaCO$_3$ was added. The samples were taken at proper time intervals and L(+)-lactic acid and glucose or glucose equivalent were monitored.

The results from repeated batch fermentation studies utilizing glucose as the substrate are shown in Figures 3.35-3.38. The yield and productivity values for all batches stay in the range of 70-80% and 0.75-0.9 g/L*h, respectively. In most of the batches, urea gave slightly better productivity and yield. The biomass concentration was almost doubled at the end of 8th batch and averaged 9 g/L, whereas for the first batches
the biomass concentrations were generally around 4.5-5.0 g/L. The productivity tended to decline after the 7th batch, which could be attributed to cell aging after long-term run. The culture-aging problem may be prevented by adding yeast extract to freshen the cells. The results obtained are comparable to those found in the literature. Hang et al. (1989) used immobilized cells of R. oryzae on calcium alginate and obtained lactic acid yield of 72% in 9 batches. Sun et al (1996) observed an average yield of 72% and a productivity of 1.32 g/L*h with the immobilization of cells on a polyurethane cube.

A similar experiment was carried out using corn starch as the carbon source. The medium composition was the same as mentioned in the method section. The sole source of nitrogen in this case was urea. The total carbon content was expressed in terms of glucose. The kinetics for all batches are shown in Figure 3.9. The individual batches are given in Figures A.2-A.5. The comparison of yield and productivity for all batches are shown in Figure 3.40.

The yield for L(+)-lactic acid started to increase after the 1st batch and reached its highest point of 84% in the 3rd batch, then started to decline again and reached the lowest point of 62% by the end of 10th batch. After the 4th and 5th batch, the yield slightly declined which could be due to the aging of cells or mass transfer difficulties caused by the thick layer of mycelial cells at the end of fermentation. The other reason for lowering yield can be the low levels of oxygen tension in shake flasks, which makes oxygen transfer very difficult to the cells. The long-term stability if conducted in bioreactor, which provided better control of DO level and other parameters may give different
results. In fact, study conducted with the bioreactor (RFBB) shows no significant decrease in productivity and yield after 6 batches (See Chapter 4).

3.3.5. Morphology of immobilized cells

The morphology of mycelia can affect production of L(+) -lactic acid. The morphology of immobilized cells was investigated by using Scanning electron microscopy (SEM). The immobilized cells from different stages were taken and the procedure for SEM was followed. In order to understand the morphology changes throughout fermentation, samples were taken from the growth phase, 1st batch, 10th and starved cells. The starvation time was 7 days at 30°C after the depletion of glucose in the fermentation broth. The small pieces (0.25 cm x 0.25 cm) of immobilized cells on cotton fiber were taken, followed by SEM sampling procedure, Philips XL-30 SEM. The samples of SEM pictures from different stages are shown in Figures 3.41-3.44.

Figure 3.41 shows the cells after the growth phase with the spot magnification of 800x. It can be seen that the cells look healthy and rod-shaped. Under the same magnification of 800x, the cells from the 1st batch (Figure 3.42) look bigger in size. In some spots there were few spores, which indicates the growing and extension of cells. Figure 3.43 shows the cells after 10 batches. Cell density and sizes sharply decreased after 10 batches. This may be an indication that cells were dying after long-term fermentation. Some crystals, salts, calcium carbonate, or calcium lactate were also present in and around the cells. The reason for that could be due to the decrease in cell
activity to utilize substrates for L(+)-lactic acid production, since the higher amount of L(+)-lactic acid produced, the higher the amount of calcium carbonate dissolved and neutralized. The other important difference between the 10th batch and the others (growth, 1st) was the extensive damage to the hyphae. It was observed that cells were tarnished and mechanically abused during the long-term fermentation. In the case of starved cells, the cells started to sporulate, and many bud like structures were observed (Figure 3.44)
3.4. Conclusions

- *R. oryzae* is able to utilize glucose and starch to produce L(+)-lactic acid. This could lead to the development of an economic fermentation process for L(+)-lactic acid production using agricultural wastes and other inexpensive plant biomass.

- Yield and productivity strongly depend on the concentration of urea, salts and substrate.

- Effects of immobilization on productivity and yield are very small in shake flask.

- The immobilized cells gave virtually cell-free fermentation broth, which facilitated the separation of cells from the broth.

- Immobilization of cells provided stable fermentation for at least 10 batches. This study shows that immobilized *R. oryzae* can be used repeatedly for lactic acid production.

- SEM pictures demonstrate that there are morphological changes at the different fermentation stages.

- Response surface analysis shows that urea plays a very significant role in volumetric productivity, yield, and biomass production.

- Response surface analysis shows that the optimum urea concentration for L(+)-lactic acid production is around 0.3 g/L, while the concentrations for other salts are around -1 level.
3.5. References


Table 3.1  (a) The levels of variables studied in response surface analysis, (b) The responses (yield productivity and biomass) obtained at different combinations of levels.

(a) Levels (g/L)

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(b) RunOrder  ZnSO4  Urea  MgSO4  KH2PO4  Product (g/L*h)  Yield (%)  Biomass g/L

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<th>Run</th>
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<th>MgSO4</th>
<th>KH2PO4</th>
<th>Product (g/L*h)</th>
<th>Yield (%)</th>
<th>Biomass g/L</th>
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## Estimated Regression Coefficients for Productivity

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<td>0.02852</td>
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<td>0.02852</td>
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<tr>
<td>MgSO₄*KH₂PO₄</td>
<td>0.00875</td>
<td>0.02852</td>
<td>0.307</td>
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S = 0.1141  R-Sq = 53.5%  R-Sq(adj) = 12.9%

### Analysis of Variance for Productivity

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### Unusual Observations for Product

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<th>St Resid</th>
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R denotes an observation with a large standardized residual

The analysis was done using coded units.

Table 3.2  Response Surface Regression. The analysis was done using coded units. Estimated coefficients for productivity

100
Estimated Regression Coefficients for Yield (%)

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<tr>
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<th>T</th>
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<td>0.196</td>
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<td>-0.708</td>
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\[ S = 4.176 \quad R^2 = 81.8\% \quad R^2(\text{adj}) = 66.0\% \]

Analysis of Variance for Yield (%)

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Unusual Observations for Yield (%)

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<th>StDev</th>
<th>Fit</th>
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\( R \) denotes an observation with a large standardized residual.

The analysis was done using coded units.

Table 3.3  Response Surface Regression. The analysis was done using coded units. Estimated coefficients for yield.
Estimated Regression Coefficients for Biomass

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S = 0.3759  \quad R-Sq = 82.1%  \quad R-Sq(adj) = 66.5%

Analysis of Variance for Biomass

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Unusual Observations for Biomass

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* R denotes an observation with a large standardised residual

Table. 3.4  Response Surface Regression. The analysis was done using coded units. Estimated coefficients for biomass.
Figure 3.1  Schematic representation of hemacytometer.
1. Growth phase

Inoculated with spores and incubated at 30°C and 210 rpm.

Immobilized cells on cotton fiber

2. Production phase

Growth medium replaced with production medium and incubated at 30°C and 210 rpm.

L(+) lactic acid

Figure 3.2 Schematic representation of procedures for lactic acid fermentation using immobilized *R. oryzae* cells in shake-flasks.
Figure 3.3 Kinetics of *R. oryzae* in immobilized and free cell fermentations utilizing glucose at 30°C, 210 rpm, and initial pH 6 during the growth phase.
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Figure 3.18  Response surface analysis: Effect of urea and KH$_2$PO$_4$ on lactic acid productivity; MgSO$_4$ and ZnSO$_4$ concentrations were kept at central levels of 0.4 and 0.132 g/L, respectively.
Figure 3.19  Response surface analysis: Effect of urea and MgSO$_4$ on lactic acid productivity; KH$_2$PO$_4$ and ZnSO$_4$ concentrations were kept at central levels of 1.1 and 0.132 g/L, respectively.
Figure 3.20  Response surface analysis: Effect of ZnSO₄ and KH₂PO₄ on lactic acid productivity; urea and MgSO₄ concentrations were kept at central levels of 0.5 and 0.4 g/L, respectively.
Figure 3.21  Response surface analysis: Effect of ZnSO$_4$ and MgSO$_4$ on lactic acid productivity; urea and KH$_2$PO$_4$ concentrations were kept at central levels of 0.5 and 1.1 g/L, respectively.
Figure 3.22  Response surface analysis: Effect of ZnSO₄ and urea on lactic acid productivity; MgSO₄ and KH₂PO₄ concentrations were kept constant at central levels of 0.4 and 1.1 g/L, respectively.
Figure 3.23  Response surface analysis: Effect of MgSO$_4$ and KH$_2$PO$_4$ on lactic acid yield (%); urea and ZnSO$_4$ concentration were kept at central levels of 0.5 and 0.132 g/L, respectively.
Figure 3.24  Response surface analysis: Effect of urea and KH$_2$PO$_4$ on lactic acid yield (%); MgSO$_4$ and ZnSO$_4$ concentrations were kept at central levels of 0.4 and 0.132 g/L, respectively.
Figure 3.25  Response surface analysis: Effect of urea and MgSO₄ on lactic acid yield (%); KH₂PO₄ and ZnSO₄ concentrations were kept at central levels of 1.1 and 0.132 g/L, respectively.
Figure 3.26 Response surface analysis: Effect of ZnSO₄ and KH₂PO₄ on lactic acid yield (%); urea and MgSO₄ concentrations were kept at central levels of 0.5 and 0.4 g/L respectively.
Figure 3.27  Response surface analysis: Effect of ZnSO₄ and MgSO₄ on lactic acid yield (%); urea and KH₂PO₄ concentrations were kept at central levels of 0.5 and 1.1 g/L, respectively.
Figure 3.28  Response surface analysis: Effect of ZnSO₄ and urea on lactic acid yield (%); MgSO₄ and KH₂PO₄ concentrations were kept at central levels of 0.4 and 1.1 g/L, respectively.
Figure 3.29   Response surface analysis: Effect of MgSO$_4$ and KH$_2$PO$_4$ on the biomass (DW); ZnSO$_4$ and urea concentrations were kept at central levels of 0.132 and 0.5 g/L, respectively.
Figure 3.30  Response surface analysis: Effect of urea and KH$_2$PO$_4$ on the biomass (DCW); ZnSO$_4$ and MgSO$_4$ concentrations were kept at central levels of 0.132 and 0.4 g/L, respectively.
Figure 3.31  Response surface analysis: Effect of urea and MgSO₄ on the biomass (DCW); ZnSO₄ and KH₂PO₄ concentrations were kept at central levels of 0.132 and 1.1 g/L, respectively.
Figure 3.32  Response surface analysis: Effect of ZnSO₄ and KH₂PO₄ on the biomass (DCW); urea and MgSO₄ concentrations were kept at central levels of 0.5 and 0.4 g/L, respectively.
Figure 3.33  Response surface analysis: Effect of ZnSO$_4$ and MgSO$_4$ on biomass (DCW); urea and KH$_2$PO$_4$ concentrations were kept at central levels of 0.5 and 1.1 g/L, respectively.
Figure 3.34  Response surface analysis: Effect of ZnSO₄ and urea on the biomass (DCW); MgSO₄ and KH₂PO₄ concentrations were kept at central levels of 0.4 and 1.1 g/L, respectively.
Figure 3.35  Long-term stability of L(+)-lactic acid production from glucose by immobilized cells of *R. oryzae*; Repeated batch fermentation kinetics with 0.3 g/L urea as the nitrogen source at 30°C, 210 rpm, and pH ~6.
Figure 3.36 Long-term stability of L(+) lactic acid production from glucose by immobilized cells of *R. oryzae*; Repeated batch fermentation kinetics with 0.3 g/L NH₄NO₃ as the nitrogen source at 30°C, 210 rpm and pH ~6.
Figure 3.37  Long-term stability of L(+) -lactic acid production from glucose by immobilized cells of R. oryzae; Repeated batch kinetics fermentation with 0.3 g/L (NH₄)₂SO₄ as the nitrogen source at 30°C, 210 rpm, and pH ~6.
Figure 3.38 Comparisons of yield (a) and productivity (b) in repeated batch fermentation with different nitrogen sources.

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Figure 3.39 Long-term stability of L(+)-lactate acid production from starch (DE 24) using immobilized cells of L. casei at 30°C, 210 rpm, and pH 6.

Wear as the nitrogen source at 30°C, 210 rpm, and pH 6.
Figure 3.40  Comparisons of productivity and lactic acid yield in repeated batch fermentation utilizing corn starch (DE 24) as the substrate.
Figure 3.41  SEM pictures of *R. ozyae* after growth phase. The top picture shows the top view and the bottom picture shows the side view of the matrix cell. (Spot magnification 800x.)
Figure 3.42 SEM pictures of *R. oyzae* after 1st batch. (Spot magnification 800x.)
Figure 3.43  SEM pictures of *R. oryzae* after 10\textsuperscript{th} batch. (Spot magnification 800x.)
Figure 3.44  SEM pictures of *R. ozyae* after starvation. (Spot magnification 800x.)
CHAPTER 4

FIBROUS BED BIOREACTOR STUDIES

Summary

In Chapter 3 various optimization and stability studies were carried out in shake-flasks. In order to study some of the important parameters such as pH and dissolved oxygen (DO), experiments were carried out in a fibrous bed bioreactor.

In this Chapter, the effects of pH and DO on lactic acid productivity and yield were investigated. The effect of pH on lactic acid production was investigated by maintaining the pH at different levels. It was observed that pH 6 created a better physiological environment and gave higher productivity and yield than the lower pH values. On the other hand, lactic acid production was still significant at a low pH value of 4. The other important characteristic of immobilized R. oryzae was interesting; by maintaining the DO level above 90% it was possible to obtain superior yield and productivity. A high yield of 83% was achieved at DO of 90%. The productivity was almost 4 times higher than the one obtained at a low level of oxygen tension. The
productivity of 2.66 g/L*h was achieved at the end of fermentation. More interestingly even at a low pH of 4 it was possible to obtain a substantial amount of lactic acid. The yield of 61% and productivity of 0.8 g/L*h were reached at DO 90% and pH 4.

The long-term stability of using a rotating fibrous bed bioreactor was also studied. It was possible to run the fermentation for at least 140 hrs without any significant loss in yield and productivity. A high concentration of 129 g/L lactic acid was reached after 4 consecutive feedings in the fed-batch fermentation. Immobilization on the fibrous matrix resulted in a virtually cell free fermentation broth, which facilitated the operation and long-term stability of the bioreactor.

The possibility of using corn starch for lactic acid fermentation was another issue that has been investigated. Due to the difficulties of handling the regular starch, corn starch with dextrose equivalent (DE) of 24 was successfully used with comparable results to those using glucose as the substrate.

Key words: R. oryzae, lactic acid, fermentation, rotating fibrous bed, immobilization, long-term stability, and dissolved oxygen (DO)
4.1. Introduction

Lactic acid fermentation has received a lot of interest during the last decade; this is basically because of the significance of lactic acid in many industries. The production of lactic acid or 2-hydroxypropanoic acid has a long history. In 1780, Carl Wilhelm Scheele, a Swedish chemist, first isolated lactic acid from sour milk. First, Lafar in 1893 and independent of him, Leichmann isolated a pure culture of lactic acid bacteria and applied these successfully in the distillery (Benninga, 1990).

There are only a few studies involving L(+)-lactic acid production by \textit{R. oryzae}, which involved solid-state and submerged fermentation using different immobilization techniques. In general, cell immobilization is achieved either by cell entrapment within a confined column through the use of a polymeric matrix or membrane, or by cell attachment via adsorption or covalent bond to a fixed matrix. In the previous production of lactic acid by \textit{R. oryzae}, entrapment of cells was the major method for cell immobilization.

Lockwood et al. (1936) first observed that free cells of \textit{R. oryzae} usually require a fermentation time of 17 to 21 days for maximum production of lactic acid. They also studied the fermentation kinetics of cassava, corn, oats, and rice to lactic acid. They found that the lactic acid production and carbohydrate consumption were influenced by the fermentation temperature, type of substrate, and presence of neutralizing agents. Hang
et al. (1989) produced L(+)-lactic acid from glucose in a chemically defined medium using immobilized *R. oryzae* in calcium alginate beads. They reported that immobilized cells have a higher yield of L(+)-lactic acid than that of the free cells. They also stated that immobilized cells were more stable in repeated batch fermentations as compared to free cells. There are quite a few solid-state fermentation applications to produce L(+)lactic acid. Soccol et al (1994) produced L(+)-lactic acid by *R. oryzae* NRRL 395 in a solid medium on sugar cane impregnated with a nutrient solution containing glucose and CaCO$_3$. A comparative study was undertaken in submerged and solid-state cultures. They obtained a productivity of 1.38 g/L·h in liquid medium and 1.43 g/L·h in solid medium. In another study, the growth capacity of 19 *Rhizopus* strains were studied on a solid-state medium of cassava starch. (Soccol et al., 1994b).

Yang *et al.*, (1995) used *R. oryzae* NRRL 395 for production of optically pure L(+)-lactic acid. They first grew cells on chemically defined medium containing xylose to form pellets with a size of about 1 mm, and then used the pellets for the production of L(+)-lactic acid. They used a polyvinyl pyridine (PVP) resin absorption column, calcium carbonate or sodium hydroxide for the removal of lactic acid to reduce the effect of product inhibition. They obtained higher yields and productivity as compared to other studies. The yields and productivity obtained were 62-74% and 2.86-6.1 g/L·h respectively. They also found that the maximum productivity was reached at pH 6 and temperature 33°C. At room temperature, the volumetric productivity was less than 1 g/L·h, whereas at 33°C the productivity was about 1.8 g/L·h. Most other investigators
such as Hang (1989) and Soccol (1994) used 30°C as the fermentation temperature. The pH also has important effects on lactic acid production. The volumetric productivity when the pH was controlled at 6 was higher than those when the pH was controlled at 5 and when there was no pH control (no numerical values of volumetric productivity were given). Nowadays, the interest in lactic acid production using *R. oryzae* has increased, but there is no implementation of rotating fibrous bed bioreactor. Many studies focus on free cell fermentation. One of the major problems in free cell fermentation is its high viscosity resulting from pellets and hyphae formation. This definitely causes poor oxygen and mass transfer, which is critical in lactic acid fermentation by *R. oryzae*.

In this work the rotating fibrous bed was developed and tested for lactic acid fermentation. The effects of pH, DO and carbon source on fungal fermentation were studied. The objectives of this research were (a) to determine and demonstrate the effectiveness of using the fibrous matrix as a support surface for immobilizing fungal cells, (b) to investigate the optimum conditions for improved L(+)-lactic acid yield and productivity in RFBB, (c) to evaluate the long-term stability of immobilized mycelia for long-term production of L(+)-lactic acid, and finally (d) to evaluate the feasibility of L(+)-lactic acid production from glucose and starch. By immobilizing the mycelia on the surface of the fiber matrix, it is possible to better operate and control the reactor and facilitate the separation of the liquid medium from the mycelial cells. The advantages and performance of RFBB were also studied by comparing with conventional free cell fermentations. The results are reported in this Chapter.
4.2. Materials and Methods

4.2.1 Culture and Media

*Rhizopus oryzae* NRRL 395, obtained from the Northern Regional Research Center, Peroria, IL, was used in this study. *R. oryzae* is a fungus, which produces L(+) lactic acid. The fungus was maintained on potato dextrose agar slants (200 g/L potatoes (infusion form), 20 g/L Bacto dextrose, 15 g/L Bacto agar). Stock cultures were stored in the refrigerator and transferred to a new potato dextrose agar (PDA) slant once a month to maintain the viability of cells. The sporangiospores were produced from PDA petri plates by shaving and extracting the spores with sterile water or from the growth medium, containing 50 g/L glucose, and 5 g/L yeast extract. The spore suspension was filtered through a sterilized cheese cloth to remove mycelia. The number of spores in the suspension was adjusted to $10^6$ spore/ml by dilution. The spore count was carried out by hemacytometer and plate counting methods. The spore suspension was used to inoculate the fermenter containing the growth medium.

Unless otherwise stated, all the growth conditions used in this study were at 60 rpm, 30°C and the medium contained 5 g/L yeast extract (YE) and 50 g/L carbon source (glucose or starch). The main purpose of the growth phase of fermentation was to germinate the spore and to accumulate the mycelial biomass for the subsequent production phase. The fermentation was carried out in a 5 L bioreactor for both immobilized and free cell fermentation in which volume of growth medium was 5 L.
4.2.2. Rotating fibrous bed bioreactor (RFBB)

4.2.2.1. Bioreactor construction

Figure 4.1 shows the fibrous bed bioreactor, which was modified from a 5-liter fermenter by attaching a fibrous matrix to the agitation shaft. The fibrous bed (9 cm x 15 cm x 0.5 cm) was made of one sheet 100% cotton cloth affixed to the surface of the porous stainless steel cylinder mounted on the agitation shaft. The fermenters (BIOFLO II, New Brunswick and Biostat B, B. Braun) were equipped with automatic pH, agitation, and aeration (DO) control.

4.2.2.2. Reactor start-up and cell immobilization

Before use, the fibrous bed bioreactor was autoclaved twice at 121°C for 60 minutes with an overnight interval. The reactor containing 5 L of the growth medium was prepared as described in Chapter 3 and autoclaved at 121°C for 60 minutes. After cooling to room temperature, the reactor vessel was connected to the controller system. Unless otherwise stated, the reactor was operated at 30°C, 60 rpm and without pH control during the mycelial cell growth. The air was filtered and sterilized by passing through a filter (0.2 m, Micropore). 0.5 mL/L of antifoam A (Sigma) was added to the medium to prevent foaming during fermentation. 10 mL of spore suspension (10^6 spores/ml) was inoculated aseptically. All mycelial cells grew and attached to the surface of the cotton fiber within 2-days, and there was no visible mycelial cells in the liquid medium.
4.2.2.3. Fermentation studies

After the initial growth and immobilization phase, 5 L of production medium was prepared as described earlier in Chapter 3 in a 6-L flask, then autoclaved at 121°C for 60 minutes. The growth medium in the bioreactor was replaced with the production medium and the fermentation kinetics were studied with glucose and starch as the substrate under the repeated batch and fed-batch conditions. In repeated batch fermentations, the medium at the end of each batch was replaced with fresh medium to start a new batch. Each batch usually lasted for 2-5 days, depending on the conditions studied. In fed-batch fermentation, a new carbon source (either glucose or starch) was added to the medium when the carbon source was about to be depleted in the medium. The addition of carbon source was repeated several times to allow the production of lactic acid to reach a high concentration level (>120 g/L). Samples were taken at proper time intervals (4-8 hrs), and were analyzed by HPLC and YSI 2700 analyzer for glucose, L(+)-lactic acid, ethanol and fumaric acid. The yields and productivities were determined as stated in Chapter 3 and reported in Figures B.1-B12 (Appendix B). The details of the assay procedure have been given in Chapter 3.
4.2.3. Oxygen transfer experiment

The dynamic method was employed to determine the $k_L a$ (volumetric mass transfer coefficient) values of production medium in RFBB system. The estimation of $k_L a$ by this technique depends on monitoring the increase in dissolved oxygen concentration of a solution during aeration. According to Van't Riet (1979), the use of commercially available electrodes with a response time of 2 to 3 seconds should enable a $k_L a$ of up to 360 h$^{-1}$ or 0.1 s$^{-1}$ to be measured accurately (Stanburg and Whitaker, 1984). The bioreactor with immobilized cell was used for this study. The initial DO level was around 60-65%, the aeration stopped and the oxygen uptake rate (OUR) was monitored. The deoxygenated liquid was then aerated, and the increase in the dissolved oxygen concentration was monitored. The DO level was measured with a dissolved oxygen (DO) probe connected to a DO meter (Fisher Scientific).

4.2.3.1. Determination of $C^*$ and $C_L$

The dissolved oxygen concentrations in the broth were calculated from dissolved oxygen tension (DOT) values. The dissolved oxygen concentration in solution can be assumed to be proportional to DOT, with 100%-DOT in water equivalent to the solubility of oxygen in water. The water solubility of oxygen at 1 atm pure oxygen at 30°C is 1.16 mM (Bailey and Ollis, 1986). Since total pressure is usually not far from atmospheric pressure, proportionality of solubility and partial pressures (Henry's law) can be assumed without introducing appreciable errors. The solubility of oxygen at 1 atm air in water at
30°C can be determined from the volumetric percentage of oxygen in air, i.e. 21%, and then should be 0.2436 mM. The solubility ($C^*$) and concentration of dissolved oxygen ($C_L$) in fermentation broth were determined from the measured dissolved oxygen tension (DOT) values (in percentage) times the solubility of oxygen in pure water (0.2436 mM).

4.2.3.2. Determination of $k_La$ and oxygen uptake rate (OUR)

Oxygen transfer rate (OTR) has been used to indicate the aeration ability of a fermentation system for a long time in which various aeration requirements can be done. The OTR from air bubble to the liquid phase is generally described by the equation:

$$\frac{dC_L}{dt} = k_La(C^* - C_L) - ROX = OTR - OUR$$

where $C_L$ (mM) is the concentration of dissolved oxygen in the fermentation broth; $t$ is the time in h; $\frac{dC_L}{dt}$ is the change in oxygen concentration over a time and $ROX$ is the oxygen uptake rate (OUR). Practical difficulty exists in measuring both $k_L$ (mass transfer coefficient) and $a$ (specific surface area) separately in a fermentation; therefore, the two terms are generally combined in the term $k_La$ (h$^{-1}$), the volumetric transfer coefficient. The value of $k_La$ is a measure of the aeration efficiency and capacity of a fermenter under the test conditions. The larger the $k_La$ the higher the aeration capacity of the system. $k_La$ is thus frequently used as an indication of the oxygen transfer rate that can be achieved in a bioreactor. The determination of $k_La$ of a fermenter becomes essential in order to
establish the aeration efficiency of a system and to quantify the effects of operating
variables on the provision of oxygen.

A number of factors have been shown to affect the $k_l$ value in a fermentation
system. Such factors include aeration rate, the degree of agitation, the rheological
properties of the culture broth, and the presence of antifoam agents. The airflow rate in
the range of 0.5 to 1.5 vvm has a relatively small effect on the $k_l$ value in conventional
agitated systems.

OUR can be determined by using the data without aeration ($k_l = 0$), then the
equation 4.1 becomes:

$$\frac{dC_L}{dt} = -R_{O_2}X = OUR$$

(4.2)

The $C_L$ vs t can be plotted where the -slope equals to $R_{O_2}X$.

The $k_l$ can be evaluated by using the data with aeration. Upon rearranging the equation
4.1 the following equation can be obtained:

$$C_L = C^* - \frac{1}{k_l} \left[ R_{O_2}X + \frac{dC_L}{dt} \right]$$

(4.3)

$C_L$ vs. $\frac{dC_L}{dt}$ or $\left[ R_{O_2}X + \frac{dC_L}{dt} \right]$ can be plotted to find $k_l$ where the -slope equals to

$1/ k_l$.
4.3. Results and Discussion

4.3.1. Growth kinetics in RFBB and free cell bioreactor

The lactic yield and productivity were relatively low during the growth phase because YE was used in the growth medium. In the growth phase no neutralizer (NaOH) was added to the medium because the main goal was to produce mycelia rather than L(+)-lactic acid. The pH dropped from 6 to 3.5 during the growth phase due to L(+)-lactic acid production. The other important point is that the same type of carbon source was used for the growth phase and production phase.

The effect of immobilization on the growth phase was also investigated. Growth conditions in free cells and immobilized cells were the same. The only difference was the rotating fibrous bed that affixed to the agitator in the immobilized fermentation system. Figure 4.2 shows the mycelial growth inside the bioreactor. The main advantage of immobilized cell over free cell fermentation in a bioreactor is obvious. The immobilized cells provided a virtually cell free medium, which facilitated the control and operation of the bioreactor. The other benefit of immobilized cells or RFBB was the higher oxygen transfer rate due to cell free medium and low broth viscosity. Without the fibrous matrix, big clumps of mycelia were formed inside the bioreactor, which resulted in the formation of big air bubbles that deteriorated the transfer of oxygen from the gas phase to the liquid phase. Also mycelia were grown on the surfaces of the agitation shaft, pH and DO probes, and reactor vessel wall, hindering the reactor’s ability to control pH and DO.
The fermentation kinetics during the growth phase were studied with free cells and immobilized cells. Figure 4.3 shows the kinetics of lactic acid production and glucose consumption in free cells and RFBB. The initial glucose concentrations were around 53 g/L for free and immobilized cells fermentations. The final L(+)-lactic acid concentrations for free cell and immobilized cell fermentation were 11.4 and 17.8 g/L from glucose. The lag phase of 14 hrs was observed for both immobilized and free cell fermentation where there was no significant L(+)-lactic acid production during that time. The lag phase perhaps is due to spore germination or the bioamass concentration required to reach a certain level to produce a significant amount of L(+)-lactic acid.

Similar results were observed for starch utilization. Figure 4.4 shows the L(+)-lactic acid production kinetics using corn starch. The initial corn starch concentrations were ~51 g/L for both immobilized and free cell fermentations. The final L(+)-lactic acid concentrations of 14.7 and 18 were observed after 58 hrs of fermentation for free and immobilized cells, respectively. A lag phase of 16 hrs was observed for both fermentation types using starch as the substrate.

The comparisons of yield, productivity, and biomass concentrations are given in Figure 4.5. Compared to free cells, the immobilized cells gave higher yield, productivity and biomass concentrations as. The yields (%)(g L(+)-lactic acid produced/ g carbon source consumed *100) from glucose were 34.4 and 22.8%, and were 35.1% and 34.4%
from corn starch, for immobilized and free cell fermentations, respectively. The yields were relatively low as compared to the production phase where yields of 75-85% were observed. The productivity values of 0.378 and 0.231 g/L*h were observed from glucose whereas these values were 0.31 and 0.25 g/L*h from corn starch for immobilized and free cells, respectively.

The biomass concentration was found by drying the mycelia for 18 hrs at 105°C. One of the big challenges of fungal fermentation certainly is the lack of fast online measurement techniques to monitor the biomass concentration. The current traditional method is destructive and is impossible to precede fermentation after the biomass measurements. Although in some studies, especially when free cell and pellet form were employed; the samples can be taken from the fermenter and biomass can be measured assuming that there is homogeneity in the fermenter. In this study the final concentration of biomass was obtained after each fermentation cycle. The observed biomass concentration values indicated that immobilized fermentation techniques and starch gave higher biomass concentration as compared to free cell and glucose. The difference for starch and glucose can be due to chemical degradation of starch to glucose where every hydrolysis utilizes one mole of water. The biomass concentrations (DCW)/L for immobilized fermentation were 4.8 and 4.0 g/L from starch and glucose containing growth medium. For free cells the fermentation values were 3.9 and 3.7 g/L for starch and glucose. Table 4.1 summarizes the results for free cells and immobilized cells during the growth phase.
4.3.2. Production kinetics in immobilized and free cells fermentations

Lactic acid production in immobilized and free cell fermentations were investigated and compared using glucose as the substrate under the same pH and DO conditions. For the free cell fermentations the cells were collected from the growth phase and washed by sterile distilled water. It is relatively difficult to transfer the production phase for free cell fermentation. The conditions studied were 30°C, 25% DO and pH 6. The agitation speed was kept at 100 rpm. The results for free cell and immobilized cell fermentations are shown in Figures 4.6 and 4.7, respectively.

It is clear that the L(+)-lactic acid production in the immobilized cell fermentation was higher than that in the free cell fermentation. The lactic acid yield for free cell fermentation was only 50.1 (0.501 g L(+)-lactic acid/ g glucose), comparing to ~70% for immobilized cell fermentation. Apparently, more substrate was spent towards biomass accumulations rather than lactic acid accumulation in free cell fermentation. Thus, it is safe to say that the immobilized cell fermentation provided better utilization of the substrate and efficient lactic acid production. The volumetric productivity at the end of fermentation (53 hrs) was also lower for free cell. The productivity for free cell fermentation was 0.59 g/L*h, whereas for immobilized cell fermentation was 0.80 g/L*h. The higher productivity in the immobilized cell fermentation could be attributed to improved reactor control and mass transfer by immobilized mycelia on the fibrous matrix.
4.3.3. Effects of pH

The sequence of fermentation was pH 6, pH 5 and pH 4. The aging of the cell during fermentation may also have affected the results obtained at different pH values. The DO level was controlled at 25%. Figures 4.7, 4.8, and 4.9 show lactic acid production in RFBB at pH of 6, 5, and 4, respectively. The pH 6 provided a better physiological environment for lactic acid production and thus resulted in higher lactic acid concentration than those with pH 5 and 4. The concentration of lactic acid was 41 g/L at pH 6 at the end of 51 hrs of fermentation. The lactic acid concentrations were 30 g/L and 39 g/L for pH 4 and 5, respectively. The lactic acid concentration was 22 g/L for the system where pH was not controlled by base addition. Figure 4.10 shows the kinetics of lactic acid production without pH control. The initial pH dropped from 6 to 3.9 during the 38 hrs of fermentation. The fermentations were carried out in repeated batches where the same immobilized cells were used for all fermentations.

Figure 4.11 shows the effects of pH on yield and productivity. In general, both yield and productivity are higher at a higher pH value. Also, the immobilized cell system gave better productivity as compared to those for free cell fermentation at the same pH value. Similar results were obtained in a study by Ho (1996), where he carried out the shake-flask experiments with and without neutralizer (CaCO₃). There is no previous research on the effect of pH on lactic acid production by R. oryzae.
4.3.4. Effect of Dissolved oxygen (DO)

Dissolved oxygen is an important criterion in submerged fermentation systems where the oxygen is limited by its poor solubility in water. Thus, the oxygen tension in the fermenter becomes a critical factor in the fermentation. In free mycelial fermentation, cells form pellets and hyphae and create a very viscous fermentation environment that reduces the mass transfer of oxygen to the fermentation broth. The immobilized cell system provides a cell-free liquid environment and can be a solution to the high viscosity problem.

The effect of DO was investigated in RFBB utilizing glucose as the substrate. The experiments were carried out at three different DO levels. The fermentation conditions were kept constant for the DO level. The DO was measured and controlled by the DO probe (Mettler-Toledo). The DO was adjusted by pumping air or air-oxygen mixture to the fermenter. The pH and temperature were maintained at 6 and 30°C, respectively. To maintain the DO level at a high value of 90%, the mixture of air-oxygen was used at the ratio of 5 to 1 by volume. This was adjusted by mixing 5 L/min of air to 1 L/min of pure oxygen by means of flow meters.

Figure 4.12 shows the fermentation kinetics at the DO level of 90%. The lactic acid concentration reached 49 g/L within 18 hrs. The productivity was 2.62 g/L*h whereas the yield was 83%. The other important observation was that the amount of ethanol produced was low (~3.4 g/L) compared to 10-12 g/L produced at the lower DO
levels. Figures 4.13 and 4.14 show the fermentation at the DO levels of 50 and 20%, respectively. The lactic acid concentration was 36.3 g/L at DO 50% and was 30.5 g/L at DO 20%. The ethanol production was higher at DO 20%, where the ethanol concentration reached 11.5 g/L at the end of fermentation. The results showed that as the DO level increased the lactic acid production increased, whereas the ethanol production decreased. The experiment was also carried out at the DO level of 90% and pH 4 to understand the interaction of DO and pH. As shown in Figure 4.15, even at a low pH of 4, it was possible to produce a substantial amount of lactic acid if the DO level was high. The concentration of lactic acid reached 41.1 g/L at 47 hrs of fermentation time.

The yield and productivity values for all three DO levels studied are given in Figure 4.16. It is clear that the DO level of 90% and pH 6 gave the highest productivity and yield. Similar results were found by Zhou et. al (1999) using a bubble column fermenter to maintain the high DO levels to obtain a lactic acid yield of 88% and productivity of 2.55 g/L*h. The repeated batch method was followed for all DO levels and the sequence for fermentation was 90%, 50% and 20%. Thus, there was also a possibility of the cell aging effect on lactic acid yield and productivity. The results obtained at all DO and pH levels studied are summarized in Table 4.2.
4.3.5. Fed-batch fermentation and long-term-stability in RFBB

In order to understand how the immobilized cells in RFBB would behave after a long fermentation time and how that would affect lactic acid production; a series of experiments were conducted using glucose and starch (DE 24) as the substrates.

Figure 4.17 shows the repeated fed-batch fermentation utilizing glucose in RFBB. The fermentation was carried out at 30°C and DO 25%. Fresh production medium was added as the substrate depleted. This was done by removing 1 L of the fermentation broth and replacing it with 1 L of fresh production medium. The initial glucose level was 42.8. The final lactic acid concentration reached was 123 g/L. Six fed-batches were carried out in 139.5 hrs. Figure 4.18 shows the fed-batch fermentation at DO level of 90%. The total fermentation time was 54 hrs and the final lactic acid concentration 129 g/L after 3 fed-batches.

The fed-batch experiment was also carried out using starch (DE 24) as the substrate. The water soluble, DE 24 starch was chosen to avoid difficulty encountered in the sterilization of suspension. Figure 4.19 shows the fermentation with regular corn starch (ARGO). In this experiment it was very difficult to start with high concentration of starch. However, the experiment shows the ability of \textit{R. oryzae} utilizing regular corn starch. The yield was around 60%, whereas the productivity was 0.26 g/L*h. In separate experiments, regular corn starch (ARGO) was sterilized in dry powder form and suspended in the fermentation broth. The initial starch concentration was 70 g/L and the
final lactic acid concentration was 45 g/L at the end of fermentation. The glucose concentration was usually around 25-30 g/L during the fermentation, suggesting that the breakdown from starch to glucose was not a rate determining or limiting step, thus there was always available glucose for lactic acid production and other metabolic activities of \textit{R. oryzae}. The results from starch and glucose were comparable indicating that the reaction from starch to glucose is not a limiting step. Figures 4.20 and 4.21 show the fed-batch fermentations using DE 24 at different DO levels. As expected, the higher DO level made it possible to give superior amounts of lactic acid production.

Figure 4.22 illustrates the overall productivity and yield comparison at different DO levels. The results showed that the overall productivity of 2.53 g/L*h was the highest value for using glucose as the substrate at DO 90%. The overall yield was 88.96% for glucose and 89% for corn starch at DO 90%. The productivity and yield from DO 25% was 1.1 g/L*h; 79% from glucose and 0.73 g/L*h; 81% from starch, respectively. These results are summarized in Table 4.3.

It can be concluded that the fed-batch fermentation is an efficient way to produce lactic acid as compared to the fermentation where a high initial substrate concentration was employed. It is also noted that the ability of immobilized \textit{R. oryzae} to produce lactic acid was not diminished after 6 fed-batches. For each experiment newly grown immobilized cells were used to carry out the fed-batch fermentation. Thus, the effect of cell aging was eliminated in fed-batch fermentations.
4.3.6. Evaluation of the volumetric mass transfer coefficient \((k_{L_a})\) in RFBB

The dynamic method was followed for the evaluation of \(k_{L_a}\). The production medium consisted of 70 g/L of DE 24 corn starch and other minerals indicated in Chapter 3. The experiment was carried out with the immobilized cells on the surface of RFBB. The experiment was done at different levels of agitation speed and aeration rate.

Figure 4.23 shows the typical dissolved oxygen tension profiles obtained with different levels of agitation and aeration rate. The medium was first aerated to high values of DO levels then the aeration was stopped and the OUR was monitored. Then, the aeration started again to monitor the DO changes and to evaluate \(k_{L_a}\) values. The \(k_{L_a}\) values were calculated by plotting the \(C_L\) vs \(dC_L/dt+OUR\) and the individual graphs for each fermentation condition are presented in Figure 4.24. The calculated \(k_{L_a}\) values at various fermentations conditions are presented in Table 4.4.

It was observed that a higher \(k_{L_a}\) was obtained at higher aeration rate and agitation rate. The agitation rate did not have as great an effect on \(k_{L_a}\) as the aeration did. Thus, the \(k_{L_a}\) was 0.33 min\(^{-1}\) at 100 rpm and 1 vvm, which was almost 5 times higher than that at 50 rpm and 0.5 vvm. The \(k_{L_a}\) value dropped from 0.33 min\(^{-1}\) to 0.239 when the agitation rate change from 100 rpm to 50 rpm while the aeration rate was 1 vvm. The difference between \(k_{L_a}\) values was not significant when compared to changing the aeration rate from 1 vvm to 0.5 vvm. The OUR was a function of \(C_L\), as the \(C_L\) decreased, the OUR, also decreased. The opposite was true for OTR, an increase in \(C_L\) caused a
decrease in OTR. The relationship between OTR-OUR and $C_L$ is presented in Figure 4.25.

4.4. Conclusions

- *R. oryzae* is able to utilize glucose and starch to produce L(+) lactic acid. This could lead to the development of an economic fermentation process for L(+) lactic acid production using agricultural wastes and other inexpensive plant biomass.

- Yield and productivity strongly depend on the level of DO and pH values.

- Effect of immobilization on productivity and yield was very high in RFBB

- The immobilized cells gave virtually cell free fermentation broth, which facilitated the separation of cells from the broth.

- Immobilization of cells provided stable fermentation for at least 6 batches with superior yield and productivity values. This study shows that immobilized *R. oryzae* can be successfully used in RFBB for a prolonged fermentation period.

- The $k_l a$ value was strongly affected by the aeration rate. The agitation did not show any significant effect on $k_l a$. 
4.5. References


<table>
<thead>
<tr>
<th>Substrate</th>
<th>Free cell fermentation</th>
<th>Immobilized cell fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Starch</td>
</tr>
<tr>
<td>Biomass yield (g/g)</td>
<td>0.0740</td>
<td>0.0763</td>
</tr>
<tr>
<td>Lactic acid yield (%)</td>
<td>22.80</td>
<td>28.70</td>
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<tr>
<td>Lactic acid productivity (g/L*h)</td>
<td>0.2310</td>
<td>0.2530</td>
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Table 4.1 Comparison of yields and productivity during the growth phase in free cell and immobilized cell fermentation at 30 °C, DO= 25 %, and pH 6
<table>
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<th>pH</th>
<th>6.0</th>
<th>5.0</th>
<th>4.0</th>
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<tr>
<td>DO (%)</td>
<td>20</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Yield (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>52.6</td>
<td>66.0</td>
<td>63.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>18.8</td>
<td>13.5</td>
<td>13.5</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>1.60</td>
<td>2.90</td>
<td>1.70</td>
</tr>
<tr>
<td>Productivity (g/L*h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.89</td>
<td>0.78</td>
<td>1.70</td>
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<tr>
<td>Ethanol</td>
<td>0.32</td>
<td>0.15</td>
<td>0.2</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>0.037</td>
<td>0.035</td>
<td>0.06</td>
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Table 4.2  Effects of pH and DO on yields and productivity for fermentation of glucose in the RFBB at 30 °C
### Table 4.3 Overall yields and productivities in fed-batch fermentations.

<table>
<thead>
<tr>
<th>DO</th>
<th>90 %</th>
<th>25 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Glucose</td>
<td>Starch</td>
</tr>
<tr>
<td>Product yield (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactic acid</td>
<td>89.4</td>
<td>90.4</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Fumaric acid</td>
<td>2.3</td>
<td>1.34</td>
</tr>
<tr>
<td>Productivity (g/L*h)</td>
<td>2.52</td>
<td>1.62</td>
</tr>
<tr>
<td>Final lactic acid concentration (g/L)</td>
<td>125</td>
<td>127</td>
</tr>
</tbody>
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Table 4.4 The $k_La$ values at various fermentation conditions.

<table>
<thead>
<tr>
<th>Fermentation conditions</th>
<th>$k_La$ (min$^{-1}$)</th>
<th>Steady state $C_L$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5vvm-50 rpm</td>
<td>0.0785</td>
<td>4.4</td>
</tr>
<tr>
<td>0.5vvm-100rpm</td>
<td>0.15</td>
<td>4.7</td>
</tr>
<tr>
<td>1vvm-50 rpm</td>
<td>0.239</td>
<td>6.1</td>
</tr>
<tr>
<td>1vvm-100 rpm</td>
<td>0.33</td>
<td>6.5</td>
</tr>
</tbody>
</table>
Figure 4.1 Schematic diagram of the rotating fibrous bed-bioreactor for the production of lactic acid by *R. oryzae*. 
Figure 4.2  Pictures of the fermenters with (a) and without (b) the fibrous matrix for cell immobilization during *R. oryzae* fermentation.
Figure 4.3  Kinetics of \textit{R. oryzae} grown on glucose in the growth medium in immobilized and free cell fermentations at 30°C and DO 25 %. The medium pH was not controlled.
Figure 4.4  Kinetics of *R. oryzae* grown on starch (DE 24) in the growth medium in immobilized and free cell fermentations at 30°C and DO 25 %. The medium pH was not controlled.
Figure 4.5 Comparisons of productivity, yield and biomass yield at different growth conditions.
Figure 4.6 Production of lactic acid from glucose with free cells in the bioreactor at 30°C, DO 25%, and pH 6.
Figure 4.7 Production of lactic acid from glucose in the rotating fibrous bed bioreactor (RFBB) at 30°C, DO 25% and pH 6.
Figure 4.8 Production of lactic acid from glucose in the rotating fibrous bed bioreactor (RFBB) at 30°C, DO 25%, and pH 5.
Figure 4.9   Production of lactic acid from glucose in the rotating fibrous bed bioreactor (RFBB) at 30°C, DO 25%, and pH 4.
Figure 4.10 Production of lactic acid from glucose in the rotating fibrous bed bioreactor (RFBB) at 30°C, DO 25%, and without pH control.
Figure 4.11 Effects of pH on productivity (g/L*h) and yield (%) of lactic acid in RFBB utilizing glucose as the substrate. (DO=25%)
Figure 4.12  Production of lactic acid from glucose in the rotating fibrous bed bioreactor (RFBB) at 30°C, DO 90%, and pH 6.
Figure 4.13 Production of lactic acid from glucose in the rotating fibrous bed bioreactor (RFBB) at 30°C, DO 50%, and pH 6.
Figure 4.14  Production of lactic acid from glucose in the rotating fibrous bed bioreactor (RFBB) at 30°C, DO 20%, and pH 6.
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Figure 4.17 Production of lactic acid from glucose by fed-batch fermentation in RFBB at 30°C, pH 6, and DO 25%.
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Figure 4.19  Production of lactic acid from regular corn starch in RFB at 30° C, pH 6, and DO 25%.
Figure 4.20 Production of lactic acid from corn starch (DE 24) by fed-batch fermentation in RFBB at 30°C, pH 6, and DO 25%.
Figure 4.21 Production of lactic acid from corn starch (DE 24) by fed-batch fermentation in RFBB at 30°C, pH 6, and DO 90%.
Figure 4.22 Overall yield and productivity comparison for fed-batches at different DO levels for glucose and corn starch (DE 24).
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CHAPTER 5

EXTRACTIVE FERMENTATION

Summary

In this section of study, lactic acid was continuously removed from the fermentation broth \textit{in situ} and collected in a base tank as Na-lactate by solvent extraction. The general expectation was by removing lactic acid from the fermenter \textit{in situ}, it would be possible to enhance reactor productivity by preventing lactic acid accumulation and thus product inhibition and to increase yield by shifting the metabolic pathway towards lactic acid production.

Lactic acid extraction from the fermentation medium using various solvents at various concentrations and flow rates were studied. Based on extraction coefficient and solvent toxicity, the best extractants for extraction were found to be Alamine 336 and Adogen 283, and the best diluent was oleyl alcohol. The extraction rate of 2.0 g/L was reached at the flow rate of 80 mL/min and 30% alamine 336-10% Adogen 283 concentration of extractants. The lactic acid yield was also greatly enhanced and an
overall yield of 92% (w/w) was achieved. This yield value was higher than the average values (65%) obtained from fermentation at the same pH value. Therefore, a conclusion can be drawn that by removing lactic acid from the medium it was possible to shift the metabolic pathway towards lactic acid in *R. oryzae*. The amount of ethanol produced in the extractive fermentation was negligible as compared to the fermentation process without extraction.

**Key words:** Lactic acid, solvent extraction, extractive fermentation, and *R. oryzae*
5.1. Introduction

Recently solvent extraction has received much attention for recovering dilute organic acid from fermentation broth. An amine-based solvent extraction technique was developed as a recovery process for the separation of citric acid from the fermentation broth (Wennerstem, 1983). Unlike other conventional solvents, amine solvents have very high distribution coefficients, which range from 2 to 40 for organic acids, because the higher affinity of amine solvents reacts with organic acids to form complexes (Ricker et al, 1979). Due to this higher affinity the organic acid can be recovered with less solvent, and the cost can thus be greatly reduced. In addition, amine solvents can be easily regenerated by back extraction with an alkaline solution (NaOH), because $K_d$ of an amine solvent for organic acids approaches zero at pH 7. Consequently, an energy efficient extraction process can be developed for recovering carboxylic acids from dilute aqueous solutions.

The effect of pH is very dramatic on extractive fermentation. There have been successful applications of amine-based extractions for recovering citric acid from its fermentation broth by using Alamine 336 (a tertiary amine) in hexane as the extractant and hot water back extraction for solvent regeneration. However, a similar process did not work with lactic acid fermentation because the fermentation broth had a relatively high pH value and needed to be acidified with a strong inorganic acid before extraction
could be carried out (Scholler et al, 1993). In the case of acidifying, the extraction efficiency was found to dramatically drop to a conventionally low level. This dramatic drop in extraction efficiency was caused by the interference of the inorganic acid. Although there are several reports on the mechanism of anion interference, the mechanism is not yet fully understood. Thus, industrial application of amine-based extraction techniques for the time being is limited to citric acid recovery.

There are several studies that have attempted to reduce recovery costs of fermentation products by integrating the fermenter with the downstream recovery process. The general purpose is to remove the fermentation product continuously from the fermenter, which would avoid product inhibition and improve productivity of the fermentation process. Meanwhile, the product is separated, purified, and concentrated. Such processes have been reported, including: using adsorption (Bradley, 1987) and solvent extraction of the product (Jenemann, 1933; Finn, 1966), using vacuum or an inert gas like CO$_2$ to remove the volatile product from the fermentation broth (Dale et al, 1985), dialysis (Steiber and Gerhardt, 1981), ion exchange (Srivastava, et al., 1992) and electrodialysis (Kyung and Gerhardt, 1984). Among all these integrated processes, amine based-solvent extractive fermentation is the most attractive and promising one because extraction is a simple, mature, and energy efficient separation technology commonly used in the industry. Although the vacuum extractive process can remove volatile products such as ethanol and acetic acid from their fermentation broth, the equipment size and energy cost for creating the vacuum could be very high. Further concentration is also
needed because the product is usually at a very low concentration level. Electrodialysis needs more expensive equipment and operation is complicated by competition from other salt ions and membrane fouling. Ion exchange is an effective process, but it usually has a low adsorption capacity of organic acid and requires frequent regeneration of the adsorbents.

Vick Roy (1985) described the purification of lactic acid after cell removal. In the patent assigned to Cargill, more than 70% lactic acid was extractively recovered from aqueous solutions by basic amine tricaprylyl amine (Eyal et al., 1998). Lactic acid production from cellulosic biomass by cellulase and *Lactobacillus delbrueckii* was studied in a fermenter-extractor employing a microporous hollow fiber membrane (MHF) (Chen and Lee, 1997). This bioreactor system was operated under a fed-batch mode with continuous removal of lactic acid by an *in situ* extraction. They found that a mixture of 20% Alamine 336, 40% oleyl alcohol, and 40% kerosene was most effective in the extraction of lactic acid.

In general, organic acid fermentation has not been as successful as alcohol fermentation when *in situ* solvent extraction was applied. Most of the *in situ* organic acid extractive fermentations reported were with homo-lactic acid fermentation. No organic acid extractive fermentation experiment was reported until 1986, although the first success with ethanol was in 1981. Yabannavar and Wang (1987, 1991) first presented the extractive fermentation with lactic acid. The extractant was 15% Alamine 336 in oleyl
alcohol, which was found to be the best in terms of extraction power and biocompatibility. Due to the severe toxic effect of organic acid extraction solvents, efforts were made to immobilize cells to protect cells from solvent toxicity (Yabanavar and Wang, 1991; Lewis and Yang, 1992; Wang et al, 1994). A group of extractants, mostly secondary and tertiary amines, have very high distribution coefficients for organic acids (Ricker and King), but in general their toxicity to cells is also very high (Dave et al, 1979). These extractants affect cell growth in a more complicated way, which has not yet been fully understood. Investigators found that paraffin oil was not toxic to *Lactobacillus delbrueckii*, while all of the amines tested were highly toxic (Seevaratnam et al, 1991; Herrfurth and Hartmeier, 1990; Mattiasson et al, 1990). However, the low capability of the paraffin solvent can not improve productivity very much. Another main problem found was that extraction requires a low pH to extract acids in undissociated form, but most of the fermentations can only work at pH 6 to 7. Therefore, an optimum pH for organic acid extractive fermentation must be carefully chosen (Wang et al, 1991; King, 1992; Hano et al, 1993). Only lactic acid fermentation that can tolerate a low pH has been demonstrated experimentally for extractive fermentation, but there was no solvent regeneration loop and no long-term test studied (Scholler et al., 1993; Yannabavar and Wang, 1991; Honda et al, 1995; Ye et al, 1996). Propionic acid fermentation by *Propionibacterium acidipropionici*, which has an optimum pH between 6.0 and 7.0, was also studied for *in situ* product recovery (Lewis and Yang, 1992; Hsu and Yang, 1991;). The extractant used was 50% Alamine 336 in 2-octanol, which was only partially compatible to the microorganism. The toxicity was greatly reduced by immobilizing the
bacteria in a spiral wound fibrous matrix. Acetic acid was recovered in situ in a fluidized bed mixing culture bioreactor using solvent extraction (Busche, 1991). The yield unfortunately was very poor. Membrane based processes were also developed by using simulated fermentation broths or acid solutions (Wang et al, 1991; Basu and Sirkar, 1992). As seen, there are numerous applications of solvent extractive fermentation, however, until today there was no application of on-line solvent extractive fermentation to lactic acid fermentation by *R. oryzae*.

5.2. Materials and Methods

5.2.1 Culture and Media

*Rhizopus oryzae* NRRL 395 was used in this study. Glucose and starch (DE 24) was used for the fermentation. The production medium also contained 0.3 g/L urea, 0.6 g/L KH$_2$PO$_4$, 0.25 g/L MgSO$_4$ and 0.088 g/L ZnSO$_4$. Details about the medium are stated in Chapter 3.

5.2.2 Fermentation process

The fermentation was carried out at 30°C and DO of 90%. The growth conditions were the same as stated in Chapter 4. The immobilized cells were washed and 4 L of the production medium was added. The pH in the fermenter was not controlled by adding the base.
5.2.3. Solvent selection

The amines, Adogen 283 and Alamine 336, were used as the extractants in this study. Adogen 283, Di-tridecyl amine, is a secondary amine and was provided by Witco. Alamine 336, tricaprylyl amine, is a tertiary amine and was provided by Henkel. Oleyl alcohol (Acros) and 2-octanol (Fisher) were used as diluents. Kerosene was obtained from Super America. Cyanex 923, is a mixture of octyl-phosphine oxide and hexyl-phosphine oxide and was provided by American Cyanamide Company. The solvent was washed with fermentation broth before use to prevent the migration of nutrients and water from the fermentation broth.

Solvent selection was done by testing each of the solvents for their extraction capacity ($K_d$), toxicity to the cell, and ability of back extraction by NaOH. To determine the distribution coefficient, $K_d$, 1 mL of lactic acid solution was mixed with 1 mL solvent. The mixture was vortexed for 5 min. and then centrifuged at 4000 rpm for 5 min. The aqueous phase was analyzed for lactic acid content.

The toxicity test was carried out by saturating the growth medium with the solvent. The growth procedure in Chapter 3 was followed. The total reduction in biomass yield was found at the end of the each testing.

The back extraction was also carried out for each solvent to test the regeneration.
of the solvent. 1 mL of base solution (6 N NaOH) was mixed with 1 mL of solvent for $K_d$ testing. The mixture was vortexed and centrifuged for 5 min. The lactic acid concentration in the base was found and the percent of lactic acid removed was determined. Table 5.1 shows the solvent screening results.

5.2.4. Hollow fiber membrane extractors

Each hollow fiber membrane extractor (HFME) (Liqui-Cel Extra Flow, 2.5 in x 8 in, Celgard) contained 800 polypropylene hollow fibers with a total membrane surface area of 1.4 m². The schematics and specifications of the hollow fiber membrane extractor are shown in Figure 5.1 and Table 5.2, respectively.

5.2.5. Operating conditions

The fermentation broth was circulated between the fermenter and the extractor shell side. The fermentation broth was maintained at 30°C. The pH was not controlled during the extractive fermentation. The total medium volume in the circulation tubing and the shell side of the extractor was ~4 L. The organic solvent was circulated through the tube side of the extractor and back-extractor. The volume of solvent was 500 mL. The base solution (~500 mL) was circulated between the shell side of the back-extractor and the base tank. The fermenter and two membrane extractors were connected with flexible tubing (Masterflex Norprene #16, Cole-Parmer), and peristaltic pumps (Masterflex, Cole-Parmer) were used. The flow rate of aqueous and solvent was maintained at 80 mL/min. The pH in the base tank was maintained at 11 by adding NaOH pellets. The pressure
difference across the membrane was adjusted by partially closing a valve at the outlet of the aqueous phase. The pressure difference was ~2 psi, with aqueous phase (shell side) being higher than the organic phase (tube side), to prevent solvent breakthrough. Figure 5.2 shows the diagram of the extractive fermentation system.

### 5.2.6. Sterilization

The fermenter pressure gauge and some of the tubing were sterilized by autoclaving at 121.1°C for 60 min. The extractors were sterilized by circulating the 75% ethanol solution through the shell side for 4 hours. The residual chemical in the extractor was removed by flushing with sterile water. Then the extractor was dried by filtered sterile (0.2 m, Micropore) air passing through the shell side. The outlet of the shell side was closed to force air through the pores of the membrane to the tube side of the extractor.

### 5.2.7. Analytical methods

Assay procedures described in Chapter 3 were followed for sample analysis.

### 5.3. Results and Discussion

The aim of this study was to demonstrate on-line extraction of lactic acid from the fermentation broth. According to the solvent screening data (Table 5.1), there were two extractants of interest Alamine 336 and Adogen 283. The diluent can be either 2-octanol
or oleyly alcohol. Although kerosene and Cyanex 923 were biocompatible, back extraction of lactic acid with NaOH solution was impossible for these solvents.

Figure 5.3 shows the extraction of lactic acid from glucose containing medium. The solvent mixture was composed of 30% alamine 336 in 2-octanol. The extraction rate was very high, about 1.7 g/L of lactic acid. But after 18 hrs of extraction there was no lactic acid production and lactic acid extraction. The total extraction rate was 0.88 g/L, which was very low compared to extraction with 30% Alamine and 5 % Adogen 283 in oleyly alcohol. The extraction was stopped because the pH in the fermenter increased to 5.8, which made the extraction inefficient. It is hard to explain why lactic acid production stopped and why the pH of the fermenter was continuously rising. One possible explanation can be the solvent toxicity. The other possible explanation could be the migration of OH ion from the base to the fermenter side.

Figure 5.4 shows the extraction of lactic acid from fermentation with glucose as the substrate. The Alamine content was varied during the extraction process and oleyl alcohol was used as the diluent. In order to observe at what level the Alamine would become toxic, the initial concentration was kept at 5% and gradually increased. For the first 20 hrs of extraction, the Alamine 336 content was kept at 5%. During this time lactic acid in the fermenter reached 24 g/L. However, lactic acid in the base tank was just 1.1 g/L. Then, the amine content was increased to 10% to increase the extraction rate. Finally the amine content was increased to 30% to further enhance lactic acid extraction. At the
end of 240 hrs of extraction, unfortunately lactic acid in the base tank was just about 82 g/L. The starting pH was 5.35 and final pH was 2.84. Then, the production medium was changed with fresh medium and Adogen 283, a secondary amine, was added at the level 5% into the mixture of Alamine-oleyl alcohol. The flow rate was 60 mL/min for both the solvent phase and aqueous phase. The lactic acid yield for the final stage was around 92%.

After understanding the extraction mechanism of lactic acid in fungal fermentation, one more experiment was conducted utilizing starch as the substrate. Figure 5.5 shows the on-line extraction kinetics from starch containing medium. The extraction was started when the levels of starch and lactic acid in the fermenter were 35.6 g/L and 30.7 g/L, respectively. The solvent was 30% Alamine 336 and 5% Adogen 283 in oleyl alcohol. To increase the extraction rate the solvent and aqueous flow rate were adjusted to 80 mL/L. As the starch was consumed, the fresh medium was added to maintain the level around 30-35 g/L of starch. The total lactic acid produced in fermentation during the fermentation was 135.77 g/L. The final lactic acid concentration in the base tank was 286 g/L at the end of 145 hrs of extractive fermentation. The liquid volume in the base tank increased from 500 ml to 1125 ml. Figure 5.6 shows the cumulative substrate consumption and lactic acid production throughout the course of extractive fermentation. For unknown reason, lactic acid production stopped after 145 hrs of fermentation. Excluding the period after 145 hrs, overall lactic acid yield was 92% (See Figure 5.7) and the overall fermenter productivity was around 0.73 g/L*h. The overall lactic acid yield
was significantly higher than the yield obtained in fermentation without extraction (See Chapter 4), suggesting that there was a metabolic shift towards lactic acid production. Another indicator of the pathway shifting was the low ethanol production during the extractive fermentation. The ethanol concentration was around 0.8 g/L, which was 5 times lower as compared to that found in the fermentation carried out at the same level of DO (See Chapter 4). The 92% yield is so far the highest yield reached in lactic acid fermentation by *R. oryzae*. The productivity for extractive fermentation was not better than that for fermentation without extraction due to the low extraction rate, only 2.0 g/L. A larger extraction and more powerful extraction system is necessary to improve the productivity of the extractive fermentation process.
5.4. Conclusions

- It is possible to employ on-line solvent extraction to lactic acid fermentation by *R. oryzae*.

- Kerosene and oleyl alcohol were the most biocompatible solvents to *R. oryzae*.

- The back-extraction with kerosene-Cyanex 923 system was insufficient.

- The extraction depends on the type and the content of amines used.

- The overall yield of 92% was achieved. The best value reached among all experiments.

- Alamine 336–oleyl alcohol system provided better results than the Alamine 336-2- octanol system.

- High lactic acid yield and low ethanol production indicated the metabolic shift towards lactic acid production.

- The extractive fermentation can be an alternative to metabolic engineering to enhance lactic acid productivity and yield.
5.5. References:


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<table>
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<tr>
<th>Solvent</th>
<th>$K_d$ (C$<em>{org}$/C$</em>{aq}$)</th>
<th>Toxicity (percent reduction in biomass)</th>
<th>Back extraction with NaOH</th>
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<tbody>
<tr>
<td>Alamine 336</td>
<td>3.5</td>
<td>14%</td>
<td>excellent</td>
</tr>
<tr>
<td>Adogen 283</td>
<td>8.5</td>
<td>63%</td>
<td>excellent</td>
</tr>
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<td>Cyanex 923</td>
<td>2.9</td>
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<td>poor</td>
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<tr>
<td>Kerosene</td>
<td>-</td>
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<td>very poor</td>
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<tr>
<td>Oleyl-alcohol</td>
<td>-</td>
<td>5.4%</td>
<td>good</td>
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Table 5.1 Solvent screening results for lactic acid and *R. oryzae*
### Fiber Characteristics

<table>
<thead>
<tr>
<th>Fiber Type</th>
<th>Celgard X-30 Microporous Polypropylene</th>
</tr>
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<tbody>
<tr>
<td>Fiber Internal Diameter</td>
<td>240 um</td>
</tr>
<tr>
<td>Fiber Wall Thickness</td>
<td>30 um</td>
</tr>
<tr>
<td>Effective Pore Size</td>
<td>0.03 um</td>
</tr>
<tr>
<td>Porosity</td>
<td>40%</td>
</tr>
<tr>
<td>Fiber Potting Material</td>
<td>Polyethylene</td>
</tr>
</tbody>
</table>

### Operating Characteristics

| Maximum Transmembrane Differential Pressure | 4.1 bar (60psi) |
| Maximum Operating Temperature              | 40°C (104°F)   |
| Maximum Operating Pressure                 | 4.1 bar (60psi) |

### Cartridge Characteristics

| Cartridge Length  | 2.5 X 8 |
| Effective Fiber Length | 20.3 cm (8in) |
| Effective Surface Area  | 15 cm (5.9in) |
| Effective Area/Volume   | 1.4m² (15.2ft²) |
| Priming Volumes:        | 29.3cm²/cm³ (74.4in²/in³) |
| Tubeside (incl. endcaps)| 145 mL |
| Shellsid               | 195 mL |

### Case Material

| Polypropylene |

Table 5.2 Specification of hollow fiber membrane extractor.
Figure 5.1  Schematic diagram of hollow fiber membrane.
Figure 5.2  Schematic diagram of solvent extractive fermentation for lactic acid
Figure 5.3  Kinetics of extractive fermentation with glucose as the substrate at 30°C and DO 90%. 30 % Alamine 336 in 2-octanol was used as solvent for extraction.
Figure 5.4  Kinetics of extractive fermentation with glucose as the substrate at 30°C and DO 90%. The solvent used in the extraction varied during process.
Figure 5.5  Kinetics of extractive fermentation with glucose as the substrate at 30°C and DO 90%. The solvent used in extraction consisted of 30 % Alamine 336, 5% Adogen 283, and 65% oleyly alcohol.
Figure 5.6  Cumulative substrate consumption and lactic acid production in extractive fermentation with starch as the substrate.
Figure 5.7  Estimation of overall yield from starch in extractive fermentation.
CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The major conclusion arising from this work is that it is possible and advantageous to conduct fungal fermentations in the Rotating fibrous-bed bioreactor (RFBB). Unlike in free cell fermentation, RFBB remained a cell free environment. This can be a solution to the high viscosity problem in filamentous fungal fermentations. Several other conclusions were made during the course of this investigation.

6.1.1 Shake-Flask Fermentation Conclusions

1. Within the range of 0.1 to 0.9 g/L urea, the concentration of 0.3 g/L gave best production and yield.

2. Long-term stability studies showed that it is possible to use immobilized *R. oryzae* for repeated batch fermentations. 10 repeated batches were carried out without significant decrease in yield and productivity.

3. Immobilized cell fermentation and starch resulted in higher biomass yields than the free cells and glucose during the growth phase.
4. Of the three nitrogen sources tested, urea, ammonium nitrate, and ammonium sulfate, urea yielded the highest level of lactic acid.

5. The substrate concentration of 70 g/L was the optimum level for lactic acid production among the tested substrate concentrations.

6.1.2 RFBB

1. It was possible to conduct a fungal fermentation in RFBB. RFBB facilitated the control and operation of fungal fermentation process.

2. *R. oryzae* is able to utilize glucose and starch to produce L(+) - lactic acid. This could lead to the development of an economic fermentation process for L(+) - lactic acid production using agricultural wastes and other inexpensive plant biomass.

3. Among the pH values tested, (pH 4, 5, and 6), pH 6 gave the highest yield and productivity.

4. DO level was critical in lactic acid fermentation. As the DO level increased, the lactic acid yield and productivity also increased, while the yield and productivity of the other by-products (ethanol and fumaric acid) decreased.

5. It was possible to produce substantial amounts of lactic acid at low pH of 4 when the DO level was 90%.

6. Between the substrates tested (starch and glucose), starch generally gave a higher yield than glucose, while glucose gave higher productivity.
6.1.3 Extractive fermentation

1. It was possible to employ on-line solvent extraction to lactic acid fermentation by \textit{R. oryzae}.

2. Among all experimental runs, extractive fermentation resulted in the highest lactic acid yield of 92%.

3. Alamine 336- oleyl alcohol mixture provided better results than the Alamine 336- 2-octanol system.

4. Extractive fermentation can be an alternative for metabolic engineering to enhance the product formation.

5. The ethanol and fumaric acid production was negligible compared to fermentation without extraction.

6.2. Recommendations

The RFBB can be further improved not only for lactic acid production but also for the other submerged fermentation systems, such as citric acid and penicillin production. In order to understand the mass transfer in RFBB, more studies should be done.

The removing of lactic acid from fermentation broth by solvent extraction should be improved to further enhance the reactor productivity. This depends on finding a better solvent, which can extract lactic acid faster and in the meantime should be biocompatible to \textit{R. oryzae}.


Galactic, www.lactic.com


Purac (1993). Company profile. IL, USA


Purac, [www.purac.com](http://www.purac.com)


APPENDIX A

LONG-TERM STABILITY STUDIES
Figure A.1. A typical HPLC chromatogram.
Figure A.2. Long-term stability utilizing glucose with different nitrogen sources. The production conditions were 30°C, 210 rpm, and pH 6. (a) Batch # 1 and (b) Batch # 2.
Figure A.3  Long-term stability utilizing glucose with different nitrogen sources. The production conditions were 30°C, 210 rpm, and pH 6. (a) Batch # 3 and (b) Batch # 4.
Figure A.4  Long-term stability utilizing glucose with different nitrogen sources. The production conditions were 30°C, 210 rpm, and pH 6. (a) Batch # 5 and (b) Batch # 6.
Figure A.5 Long-term stability utilizing glucose with different nitrogen sources. The production conditions were 30°C, 210 rpm, and pH 6. (a) Batch # 7 and (b) Batch # 8.
Figure A.6  Long-term stability utilizing corn starch (DE 24) at 30°C, 210 rpm, and pH 6. (a) Batch #1 and (b) Batch #2.
Figure A.7   Long-term stability utilizing corn starch (DE 24) at 30°C, 210 rpm, and pH 6. (a) Batch # 3 and (b) Batch # 4.
Figure A.8  Long-term stability utilizing corn starch (DE 24) at 30°C, 210 rpm, and pH 6; (a) Batch # 5 and (b) Batch # 6.
Figure A.9 Long-term stability utilizing corn starch (DE 24) at 30°C, 210 rpm, and pH 6. (a) Batch # 7 and (b) Batch # 8.
Figure A.10  Long-term stability utilizing corn starch (DE 24) at 30°C, 210 rpm, and pH 6; (a) Batch # 9 and (b) Batch # 10.
APPENDIX B

DETERMINATION OF YIELDS AND PRODUCTIVITIES
Figure B.1 Determination of yields and productivities from glucose for free cell fermentation in the bioreactor at 30°C, pH 6, and DO 25%.
Figure B.2 Determination of yields and productivities from glucose for immobilized cell fermentation in RFBB at 30°C, pH 6, and DO 25%.
Figure B.3 Determination of yields and productivities from glucose for immobilized cell fermentation in RFBB at 30°C, pH 5, and DO 25%.
Figure B.4  Determination of yields and productivities from glucose for immobilized cell fermentation in RFBB at 30°C, pH 4, and DO 25%.
Figure B.5  Determination of yields and productivities from glucose for immobilized cell fermentation in RFBB at 30°C, DO 25%, and without pH control.
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Figure B.7 Determination of yields and productivities from glucose for immobilized cell fermentation in RFBB at 30°C, pH 6, and DO 50%.
Figure B.8 Determination of yields and productivities from glucose for immobilized cell fermentation in RFBB at 30°C, pH 6, and DO 20%.
Figure B.9  Determination of yields and productivities from glucose for immobilized cell fermentation in RFBB at 30°C, pH 4, and DO 90%.
Figure B.10  Determination of overall yields and productivities from glucose for fed-batch fermentation in RFBB at 30°C, pH 6, and DO 25%.
Figure B.11  Determination of overall yields and productivities from starch for fed-batch fermentation in RFBB at 30°C, pH 6, and DO 25%.
Figure B.12  Determination of overall yields and productivities from glucose for fed-batch fermentation in RFBB at 30°C, pH 6, and DO 90%.
Figure B.13  Determination of overall yields and productivities from starch for fed-batch fermentation in RFBB at 30°C, pH 6, and DO 90%.