CO-PRODUCTION OF LACTIC ACID AND CHITIN USING A PELLETIZED FILAMENTOUS *RHIZOPUS ORYZAE* CULTURE FROM CULL POTATOES

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of YAN LIU find it satisfactory and recommend that it be accepted.

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Chair

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CO-PRODUCTION OF LACTIC ACID AND CHITIN USING A PELLETIZED FILAMENTOUS *RHIZOPUS ORYZAE* CULTURE FROM CULL POTATOES

Abstract

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Chair: Dr. Shulin Chen

This dissertation research explored feasibility of co-production of lactic acid and chitin from cull potatoes. Cull potatoes are underutilized agricultural products that provide only minimal financial return to the growers. However, fresh cull potato, with its rich composition in starch and nutrients, can be an ideal raw material for lactic acid production. Lactic acid is currently widely employed as an acidulant, flavoring, and preservative in the food industry. It can also be used to synthesize polylactic acid, a feedstock chemical for biodegradable plastics. A considerable amount of fungal biomass can be concurrently produced during the synthesis of lactic acid using some fungal strain. Chitin, the primary composition of the fungal cellular wall, can be extracted from the fungal biomass. Chitin and its derivatives are useful products that are utilized in wastewater treatments, cosmetics manufacturing and biomedical applications. The biomedical applications, in particular, are of great consequences as chitin has been proven to be constructive in promoting tissue growth, accelerating wound-healing and regenerating bone.

Rhizopus oryzae, a filamentous fungus used in this study, usually develops a cotton-like morphology that is associated with low yield and inadequate productivity of lactic acid. This is uneconomical for commercial fermentation purposes. Growing fungi in pellet form can circumvent these problems.

The overall goal of this study was to develop an innovative process for lactic acid and chitin co-production using pelletized *Rhizopus oryzae* NRRL 395 to improve both fermentation yield and productivity with cull potatoes as nutrient source. The specific objectives were to: (1) refine the pellet formation process to produce desired size and

number of pellet nuclei, (2) develop a mathematical model that describes the pellet formation as a function of culturing factors, and (3) optimize the pellet growth, lactic acid and chitin production by controlling operational parameters.

The major results obtained from this research were: (1) biodegradable polymer additive and spore storage time were determined to be the important factors in pellet formation. Multiple logistic regression models provided good pellet formation prediction, (2) high biomass and chitin yield were obtained by culture fungus on potato hydrolysate, (3) pelletized morphology significantly improved the lactic acid production in terms of productivity, yield, and lactate concentration in the broth, and (4) enhanced co-production of lactic acid and chitin were achieved by repeated-batch and fed-batch with mixed alkaline to adjust pH using cull potato and glucose as raw materials.

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Dedication

This dissertation is dedicated to my husband and my daughter.

CHAPTER ONE

INTRODUCTION

1. Background

Organic acids, such as lactic, citric and fumaric acid, are widely used as food additives for flavor and preservation Organic acids also serve as important building blocks for various chemicals such as biodegradable plastics, synthetic textile and synthetic resins (Magnuson, et al., 2004). Currently, a large percentage of these organic acids are derived from petroleum-based sources. As the demand of petroleum fuel continues to rise, there exists a significant market opportunity for the development of biological organic acid production. However, biological production of organic acids must be cost effective in order to be competitive in the market place. Reduced production costs and associated commercial advantages rely heavily on improvements in reactor operation to achieve close to the theoretical yield. Evidently, strain improvement and medium optimization should be completed in the early stages of process development (Doran, 1995). From an engineering perspective, there are three approaches to achieve the goal of cost minimization: (1) utilizing inexpensive substrates, (2) producing multiple products from a biological process, and (3) improving the process effectiveness through optimization of fermentation conditions. Therefore, this study made use of an inexpensive starch substrate from cull potatoes to develop a novel process to produce valuable lactic acid and chitin in tandem.

2. Cull potato as raw material

Potatoes are sorted for quality and size during harvesting and processing. For every acre of potatoes harvested, 10 to 15 percent of the crop is graded as culls. This includes undersized, bruised, damaged and deformed tubers. The inferior quality of culls is reflected in the low market price. Potato growers generally receive less than $10/ton for the culls, which cost about $65/ton to grow. However, fresh cull potato is rich in starch and nutrients, and can be an ideal feedstock chemical for producing lactic acid and chitin via fungal fermentation. The production of valuable chemicals from cull potatoes also assists the farmers financially in cultivating a more sustainable and economical potato industry.

3. Lactic acid

Lactic acid ($CH_3CHOHCOOH$, 2-hydroxypropanoic acid) is a colorless chemical that is important in several industries for both domestic and international markets.. Lactic acid is widely used as an acidulant, flavoring or preservative in food industry. There is also an increasing interest in its application in the synthesis of polylactic acid, which can be used for biodegradable plastic manufacturing. Polymers and co-polymers derived from lactic acid are especially appealing for biomedical application because of their biocompatibility, body absorption and blood compatibility. The additional uses of lactic acid in existing applications and potentials in biodegradable plastics have transformed lactic acid production into a lucrative investment.

4. Chitin

Chitin, a polysaccharide, is speculated to be the second most abundant biopolymer in the biosphere. The structure of chitin is a linear polysaccharide made up of unbranched β-(1,4)-2-acetamido-2-deoxy-D-glucopyranosyl residues which is also called N-acetyl- D-glucosamine (Fig. 1). The structural characters make chitin a very constructive biopolymer that can be used as coagulating agents in wastewater treatments, plant seed coating agents in agricultural industry, and biomaterials (e.g. absorbable sutures) in biomedical industry (Yusof et al., 2001). Chitin is widely distributed in the animal and plant kingdom, such as the exoskeletons of crustaceans, mollusks, algae, and fungi (Muzzarelli, 1977). Traditionally, chitin is commercially produced from marine invertebrates, which has some uneconomic liabilities such as non-reusable sources and limitations for biomedical usage due to the side effects from the residual invertebrate proteins and trace minerals (Khor, 2001). Chitin produced from fungi has no such shortcomings. Moreover, the fungal chitin content can be high, up to 90% by weight (Carlile, 2001). Therefore, chitin production from fermentation of fungal sources has garnered growing attention in the industrial sector (Khor, 2001).

5. The fungal strain – *Rhizopus oryzae* NRRL 395 (ATCC 9363)

Presently, over half of the lactic acid is produced chemically from coal, petroleum and natural gas, and the rest is produced from the bioconversion of carbohydrates and plant biomass (Tsao et al., 1999). Lactic acid from biological source can be produced by both bacteria and fungal fermentation. Generally, bacterial fermentation produces higher yields (Litchfield, 1996). However, fungi, such as *Rhizopus oryzae*, have been proved to

be a good lactic acid producer and have several advantages over bacteria fermentation. These advantages include: (1) fungi are more tolerant to a low pH environment, (2) the fungal biomass is relatively easy to separate from broth, and thus, facilitates the downstream process, and (3) *Rhizopus oryzae* has low nutrition requirements, which reduces the fermentation cost and simplifies downstream product separation (Tsao et al., 1999; Magnuson et al., 2004). In addition, *Rhizopus oryzae* can produce lactic acid with very high optical purities (Hang, 1989; Oda et al., 2002), which is preferable for food applications and biopolymer manufacturing. However, there is an inherent disadvantage with fungal organic acid production: fungi tend to form cotton-like mycelia in reactors, which makes it difficult to control the reactor in a homogeneous condition. Furthermore, the cotton-like mycelium limited the mass transfer of oxygen and nutrients into the microorganisms and the release of produced organic acids into the bulk solutions. All the above factors ultimately lead to low yields and productivities of organic acids in fungal fermentation.

Rhizopus oryzae can be formed into a "Pellet" morphology. Compared with cotton-like mycelia, pellet mycelia have a larger specific surface area which alleviates the mass transfer limitations. This type of morphology has a beneficial effect on broth rheology, which in turn affects mass and heat transfer in the reactor. Consequently, efficiency of mixing, aeration and cooling systems are enhanced (Olsvik et al., 1994). Another advantage of fungal pellet fermentation is that the pellet makes it possible to perform repeated batch cultures that further boost productivity (Yin et al., 1998).

Because the yield and productivity of organic acids are considerably influenced by fungi morphology, morphology control becomes a critical parameter in process

optimization. Two main approaches have been developed to handle fungi so that they will form the desired morphology – pellets: the first approach is through cell immobilization and the second approach is fermentation condition control. Vaidyanathan et al. (2000) reported that a polymer-type support medium (PVP) has been used to immobilize *Rhizopus oryzae* cells, resulting in a lactic acid yield of 71% and productivity of 1.8 times higher than that of free filamentous mycelia. For fermentation condition control, there are many factors that influence the fungal pellet formation, such as nutrients, pH of medium, agitation, aeration, inoculum level and substrate concentration (Metz et al., 1977). It has been reported that pellet-form mycelia of *Rhizopus oryzae* were obtained using xylose as the carbon source, or by controlling the initial pH and spore concentration (Yang et al., 1995; Du et al., 1998). Nevertheless, these literature methods are complex and difficult to implement.

6. Growth *Rhizopus oryzae* in the form of pellets

The alteration in fungal morphology is mainly influenced by medium compositions, inoculum, pH, medium shear, additives (polymers, surfactants, and chelators), culture temperature and medium viscosity (Metz et al., 1977; Nielsen et al., 1996; Papagianni, 2004; Znidarsic et al., 1998). For individual strains, each factor has a different effect on the growth morphologies; some strains such as *Rhizopus* sp. need strong agitation to form pellets, while strains such as *Penicillium chrysogenum* require high pH to form pellets (Metz et al., 1977). Thus, the study on fungal pellet formation is limited at the level of the individual strain. Zhou et al. (2000) investigated the effects of different metal ions (Mg^{2+}, Zn^{2+}, and Fe^{2+}) and pH on the pellet formation of *Rhizopus*

oryzae ATCC 20344 with glucose as the carbon source and urea as the nitrogen source. Byrne studied the effects of glucose concentration, peptone concentration, pH, and some additives on the pellet formation of *Rhizopus oryzae* ATCC 10260 (Byrne et al., 1989a; Byrne et al., 1989b). However, a comprehensive investigation of the effects of medium composition, inoculum and other factors, such as shaking speed, polymer additives, and temperature on the pellet formation and growth has not been reported to date.

Due to the fact that numerous factors affect pellet formation and growth, a complete predictability must include the probability of pellet formation, as well as pellet formation parameters when the pellet formation occurs in a specific environment. The approach of logistic regression model provides a useful way to describe the probability of occurrence of an event and has been applied progressively more in economics, environmental science, microbiology, the analysis of epidemiological data and clinical decision making (Neter et al., etc.1996; Skierve, 1992; Lopez-Malo, 2000). The response of the logistic model is usually binary and is defined to have the two possible outcomes -- the occurrence of an event and the absence of an event. We could define the response variable Y to have the two possible outcomes: forming pellets and not forming pellets (coded 1 and 0, respectively). Logistic regression is a powerful tool in probabilistic pellet formation modeling and it assists in exploring the effects of environmental conditions on spore germination.

7. Fermentation

Maximizing volumetric productivity is always a key criterion in fermentation process, because it can be used to minimize reactor size and, thus, decreasing both

operating and capital costs. Volumetric productivity depends on the concentration of microorganism cell and its specific rate of production (Doran, 1995). To achieve high volumetric rates, the reactor must therefore allow maximum catalytic activity at the highest cell concentration. Hence, one can obtain an optimal volumetric productivity by investigating the effects of cell concentration on fermentation.

The growth and metabolism of *Rhizopus oryzae* has been reported to be limited by nitrogen (Foster et al., 1939; Rhodes et al., 1959; Rhodes et al., 1962). A high concentration of nitrogen in the broth leads to a faster fungal growth than organic acid production. Therefore, in terms of improving the fermentation performance, the lactic acid fermentation processes could be divided into three steps: (1) pelletized seed culture, (2) fungal biomass cultivation with nitrogen, and (3) lactic acid production with limited nitrogen (Kenealy et al., 1986; Zhou et al., 2002; Romano et al., 1967). According to the characteristics of potato hydrolysate containing 100 g/L glucose and 10 g/L crude protein, the above three-step process is quite suitable to simultaneously produce lactic acid and chitin using potato hydrolysate and glucose. The potato hydrolysate can be first used as a nutrient source for fungal biomass growth and chitin accumulation, and then the fungal biomass can be transferred to a sole carbon source (glucose) broth to produce lactic acid.

8. Research hypothesis

Rhizopus oryzae NRRL395 is a well-known strain for producing optically pure L (+)-lactic acid (Hang, 1989; Oda et al., 2002). As a filamentous fungus, however, *R. oryzae* usually develops a cotton-like morphology that results in low yield and inadequate

productivity. For that reason, this is uneconomical for the commercial fermentation processes. The major challenge is to develop a process that transforms the morphology into pellets for purpose of improving both fermentation yield and productivity. There are many factors that influence the formation of fungal pellets, including agitation, medium nutrients, pH, oxygen tension, polymer additives, viscosity of the medium, surface-active agents, and inoculum (Metz, 1977; Papagianni, 2004). The author believes that there is an optimum operating condition in terms of pellet size, pellet concentration (numbers of pellets per unit volume), and pellet formation process, which maximizes fermentation efficiency. Accordingly, the research hypothesis is that the new techniques of pellet formation and Three-Steps process will allow fungal fermentation to become not only competitive with bacteria fermentation, in terms of lactic acid yield and productivity, but will be more economically sound by generating another product, chitin.

9. Research goal and objectives

The overall goal of this project was to develop a novel process for lactic acid and chitin co-production using pelletized *Rhizopus oryzae* NRRL 395 to improve both fermentation yield and productivity with underutilized cull potatoes and glucose as the nutrient source. The process included potato hydrolysis, fungal pellet formation, and co-production of lactic acid and chitin (Fig. 2). The specific objectives of this study were to: (1) refine the pellet formation process to produce desired size and number of pellet nuclei, (2) develop a mathematical model that describes the pellet formation as a function of culturing factors, and (3) optimize the pellet growth, lactic acid and chitin production by controlling operational parameters.

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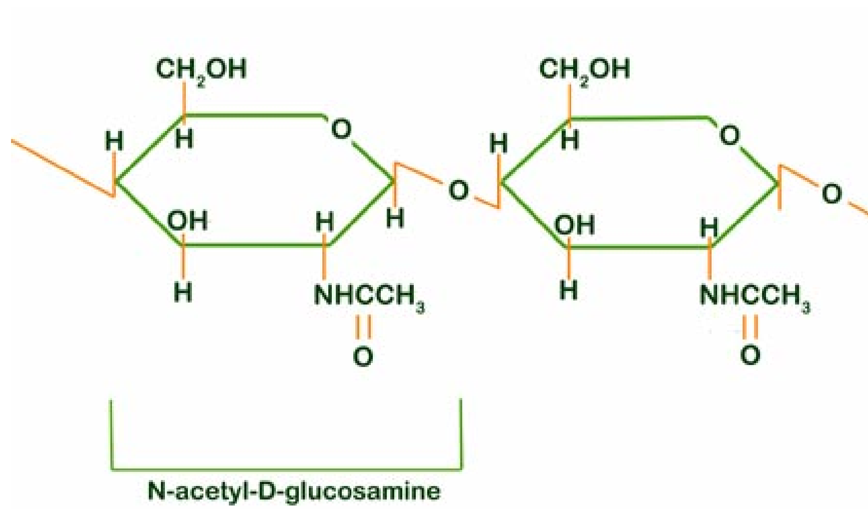

Fig. 1. Chemical structure of chitin

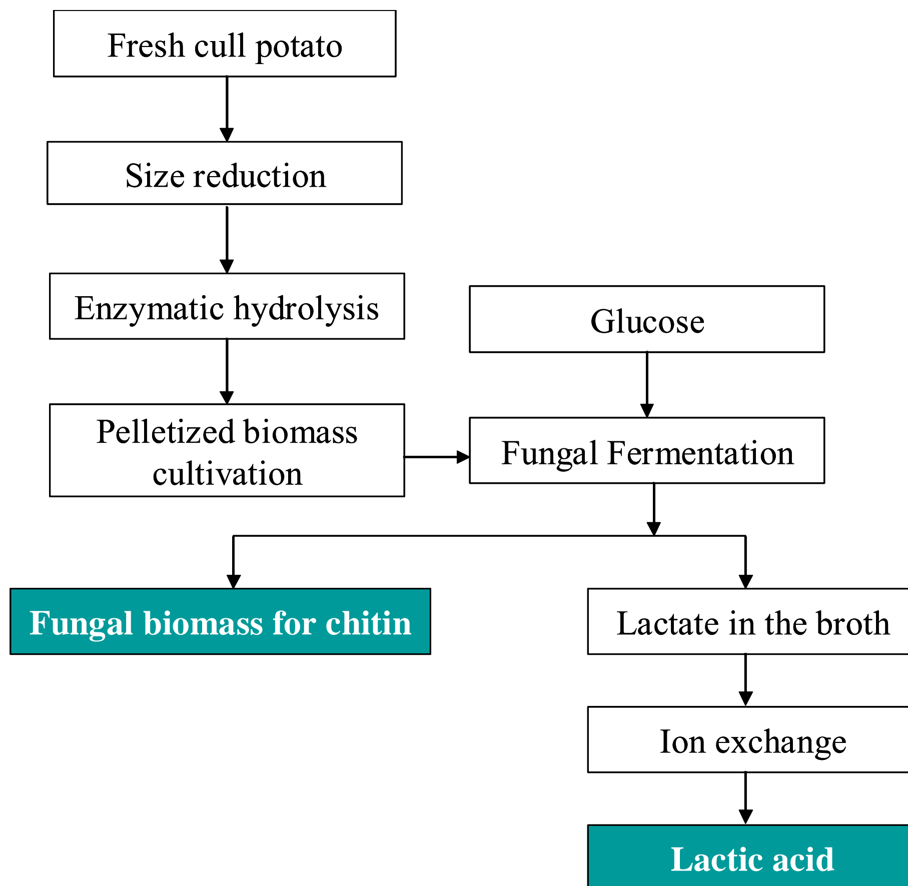

Fig. 2. Schematic flowchart of the process of co-production of lactic acid and chitin

CHAPTER TWO

OPTIMIZATION OF L (+)-LACTIC ACID PRODUCTION PROCESS FROM CULL POTATO BY *RHIZOPUS ORYZAE*

1. Abstract

Cull potato is currently an under utilized biomass within the potato processing states of the US. L (+)-Lactic acid production by three *Rhizopus* strains and one homofermentative, facultative anaerobe, *Lactobacillus amylophilus,* was investigated using potatoes as the sole nutrient supply in the culture medium. *Rhizopus oryzae* NRRL 395 was chosen as the strain for further studies because it showed the highest lactate yield. The fermentation condition for seed culture was studied for three treatment structures using a completely randomized design. Optimal conditions for seed culture were determined to be 2% potato medium, 10^4 spores/ml concentration, and 24 hours of fermentation. Plackett-Burman and central composite designs were used to screen and optimize factors for the lactic acid production. Substrate (potato) concentration, fermentation temperature, and shaking speed were found to be the most significant factors affecting both the yield and concentration of lactate. Optimal values for substrate concentration, fermentation temperature, and shaking speed were 10%, 27ºC, and 170 rpm, respectively. The lactate concentration under these optimal conditions was predicted to be 35.5 g/L by the model, which was verified by the experimental data (33.3g/L). The results indicate that cull potato can be a good feedstock for *Rhizopus oryzae* NRRL 395 in the production of lactic acid.

Key words: *Rhizopus oryzae*, lactic acid, cull potato, Plakett-Burman design, central composite design

2. Introduction

Potatoes are a major crop for the Pacific Northwest with 612,000 acres planted yearly in Washington, Oregon and Idaho, culminating in harvest averages of 251 million hundredweight (Araji et al., 2001). Among those potatoes harvested, about 10-15% are graded as cull potatoes, which include undersized, bruised, damaged, deformed, discolored, diseased, hollow-hearted, and low total solid tubers. Generally, these cull potatoes are composted, used as organic fertilizer, or fed to livestock, and, as a result of the low price for such uses, the growers pay the cost of growing these culls out of income from the marketable grades.

Use of cull potatoes' starch, protein, vitamin, and salt nutrients as feedstock for producing lactic acid, provides an alternative for cull potato utilization (Table 1). Lactic acid is a colorless compound that is currently widely used as an acidulant, a flavor additive, and a preservative in food industries. It can also be used in the production of polylactic acid, a precursor to biodegradable plastic. Presently, the increased use of lactic acid within the plastics field is making the production of lactic acid a potentially attractive investment.

Microbiological production of lactic acid from potato starch has been widely reported (Anuradha et al., 1999). Various bacteria such as *Lactobacillus*, *Lactococcus,* and *Streptococcus* and the fungi *Rhizopus* are commonly used as lactic acid producers (Litchfield, 1996; Tsao, 1999; Hofvendahl et al., 2000). Generally, though, the starch

must first be hydrolyzed to glucose before fermentation by the organisms can occur; thus increasing the complexity and cost of the process. Linko et al. (1996) used barley starch as raw material to produce lactic acid by simultaneous saccharification and fermentation (SSF). In their work, they found that using α-amylase, glucoamylase for starch saccharification and *L. Casei* for fermentation and achieved a yield of 87-98%. Lactic acid fermentation using bacterial culture with other waste, such as whey, was studied. Extra glucose and other nutrient source were required (Arasaratnam, et al., 1996). It has been reported that amylase-producing bacteria such as *Lactobacillus amylovorus*, *Lactobacillus amylophilus,* and the fungi *Rhizopus oryzae* can directly ferment starch into lactic acid, however extra nutrients such as a nitrogen source (ammonium, yeast extract) and salts (KH_2PO_4, $MgSO_4$, $FeSO_4$), are usually needed as supplements to the medium (Tay et al., 2002).

Using raw starch material as a single nutrient supply for lactic acid production has been tested for the fungi *Rhizopus oryzae*. For example, *Rhizopus oryzae* NRRL 395 has proven capable of producing lactic acid from several types of raw starch such as corn, barley, cassava, oat, and rice (Yang, 1989; Oda et al., 2002). In particular, Oda et al. (2002) screened a strain of *Rhizopus oryzae* that can grow using potato pulp as its single nutrient supply. However, compared with other raw materials, its lactic acid yield was relatively low.

To enhance the microbial production of lactic acid, optimization of culture conditions via statistically-based experimental design is needed. Lactic acid production was traditionally optimized by one-at-a-time methods, i.e., varying one factor while keeping all others constant. Although the strategy is simple and easy, it involves a

relatively large number of experiments and the interaction among factors is often ignored. In contrast, statistically-based experimental design is a more efficient approach that can deal with a large number of variables simultaneously as well as estimate the interaction among different variables.

The aim of this work was to apply statistically-based experimental designs to optimize lactic acid production from cull potatoes and provide basic information for developing a cost-effective process for high yield of lactic acid. The specific objectives of this study were to: (a) screen a strain which can directly produce lactic acid with cull potato; (b) determine the best seed culture condition for lactic acid fermentation; (c) study the influence of shaking speeds on lactic acid production; (d) investigate the impact of the major variables in lactic acid fermentation; and (e) optimize the significant factors.

3. Methods and Materials

Microorganisms

The fungi *Rhizopus oryzae* NRRL 395 (ATCC 9363), *Rhizopus oryzae* NRRL 1472 (ATCC 9374), and *Rhizopus oryzae* NRRL 1526 (ATCC 10260), and the bacterium *Lactobacillus amylophilus* NRRL B-4437 (ATCC 49845) were used. The fungi were first grown on potato-dextrose agar (PDA) (Difco, 213400) slants at 30°C for 7 days. For experimentation, the fungal spores in the slant were suspended with sterilized water and maintained in water at $4^{o}C$. For storage, the spores and the bacterium were placed in 20% glycerol solution at –80°C.

Screening of strain capable of producing lactic acid from cull potatoes

Four microorganisms (three fungi and one bacterium) were investigated for lactic acid production capabilities from cull potatoes. Three fungi spore suspensions, each of 0.5 mL (1×10^{8}spores/mL), were inoculated into 250 ml Erlenmeyer flasks containing 100 mL of potato-dextrose broth (PDB, Sigma P-6685). The cultures were performed at 30°C for 48 hours on an orbital shaker (Classic Series C24 Incubator shaker, New Brunswick Scientific, Edison, NJ, U.S.A.) at 175 rpm. Subsequently, 2.5 ml of the above seed culture broths were inoculated into 125 ml flasks containing 25 ml of the "production" medium. The "production" medium contained 2% potato (dry matter) that was crushed to mash and directly added into the medium. The shaker speed and culture temperature were 175 rpm and 30°C, respectively. For the inoculum preparation of the bacteria *Lactobacillus amylophilus*, the cells were grown in *Lactobacilli*-MRS-broth (Difco 288110) until the absorbance of cell density at 600 nm reached 0.1 at 10X dilution. Then the bacteria broth was inoculated into production medium without shaking. All the experiments were performed in triplicate and pH was maintained around 7 by additions of sterilized calcium carbonate. The fermentation time was seven days. Three flasks of each strain were taken out of the shaker each day to analyze the concentration of lactate, and one of them was used to analyze the concentration of reducing sugar.

Experimental design and data analyses

Completely randomized design of seed culture

A statistical design was used to determine the optimal conditions for amylolytic enzyme production within *R. oryzae*. A Completely Randomized Design (CRD) with two replications of 24 treatment combinations was used to analyze three-way treatments (potato concentration, spore concentration, and fermentation time). Three potato concentrations (2, 4, and 6% dry matter), two spore concentrations (10^4 and 10^5 spore/mL), and four treatment durations (15 to 24 hours) were studied. Samples were taken every three hours. The amount of enzyme activity in the broth was used as the criteria to judge the optimal seed culture.

After centrifugation, the liquid broth was diluted 20 times in order to analyze the total amylolytic enzyme activity (Yu et al., 1990). A unit of amylolytic enzyme activity is the amount of enzyme that liberates 1 mg of glucose per minute under the assay conditions. Glucose concentration was measured using the modified dinitrosalicylic acid (DNS) method (Miller, 1958). The General Lineal Model (GLM) for the Analysis of Variance (ANOVA) of enzyme activity was tested using the Statistical Analysis System (SAS 8.0) program.

Completely randomized design of shaking speed

A design was completed to determine the optimal shaking speed of flask culture for lactic acid production with *R. oryzae*. A completely randomized design (CRD) with 9 treatment combinations was used to analyze two-way treatments (shaking speed and fermentation time). Three shaking speeds (60, 110, and 170 rpm) and three fermentation

time levels (2, 3, and 4 days) were studied. The influence of shaking speed and fermentation time on lactic acid production was also tested using the Statistical Analysis System 8.0 (SAS Institute Inc. NC) program.

Plackett-Burman Design

Once the strain capable of producing lactic acid from cull potatoes was identified in the strain screening experiments described above, the optimization was initiated by identifying variables that significantly influence lactic acid production. The variables to be evaluated included medium components, temperature, and incubation time (Table 2). The Plackett-Burman (PB) design has proven very effective in recognizing the most important factors from a list of candidate factors and as shown in Tables 2 and 3, a 12-run Plackett-Burman design was used in this work (Haaland, 1989). The low levels of the variables were chosen according to previous reports (Hamamci et al., 1994). Statistical analyses were used to identify the significance of the variables (Wen et al., 2001). Here, only confidence levels above 80% (p value<0.2) were accepted as significant variables.

Central Composite Design

After the significant variables were identified, a 11-run central composite design (CCD) was used to optimize the levels of these variables (Haaland, 1989). The design matrix and the lactate concentration are shown in Table 4. The data was analyzed for optimal values by multiple regression using Matlab 6.1 (Mathworks, Inc. MA).

Verification experiment

The final step in the optimization process was to verify the predicted values through experimental comparison. Eight flask samples were cultured at the optimal conditions. The predicted lactic acid concentration was then compared to the resulting experimental data.

Analytical methods

A high-performance anion-exchange chromatography apparatus was used for the analyses. The lactate was analyzed using a Dionex DX-500 system (Sunnyvale, CA, USA) including an AS11-HC (4mm 10-32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler. The eluent conditions for lactate analysis are listed in Table 5.

Total reducing sugar in liquid was measured by modified Dinitrosalicylic Acid Reagent (DNS) method (Miller, 1958), which 3 ml dilute sample was added to 3 ml reagent that contained 1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, and 1% sodium hydroxide, and then 1 ml Rochelle salt (40% Potassium sodium tartrate solution) was added after the sample was heated in 100°C water bath for 15 minute. The color of this reaction was measured by spectrophotometer at 575 nm. Standard curve was obtained by measuring standard glucose from Sigma.

Carbon contents in both liquid and solid samples were measured by automated combustion techniques. The LECO CNS-2000 was used to measure the total carbon of solid samples, and Shimadzu TOC 500 analyzer was used to measure the total carbon of

liquid samples. Starch analysis was carried out using Salomonsson's method (Salomonsson et al., 1984)

Dissolved oxygen was measured by Fisher portable dissolved oxygen meter (Fisher Scientific, NJ)

4. Results and discussion

Screening strain capable of producing lactic acid from cull potatoes

Fig. 1 shows the lactate concentrations produced over time by the four species. The strain *R. oryzae* NRRL 395 produced the highest lactate among these four strains. The lactate concentrations for strains, *Rhizopus oryzae* NRRL 1472 , *Rhizopus oryzae* NRRL 1526, and the bacterium *Lactobacillus amylophilus* NRRL B-4437 were 0.1 g/L, 0.07g/L and 0.3g/L, respectively. For the culture of *R. oryzae* NRRL 395, lactate concentration increased sharply to 6.39 g/L within the first two days, and kept stable at around 6.5 g/L for the following 5 days. The glucose produced during hydrolysis first increased to its highest level and then leveled off following the increase of reaction time (Fig. 2). The lactate yield produced by *R. oryzae* NRRL 395 was 32% by weight. Meanwhile, the data of reducing sugars from other three strains, combined with their lactic acid profile (Fig. 1 and 2), further elucidate that although they could produce amylolytic enzymes, their lactic acid production ability was very low. As *R. oryzae* NRRL 395 could utilize starch more efficiently for lactic acid production than the other species, therefore, this fungus was used in the following optimization studies.

Optimization of seed culture conditions for lactic acid production by *R. oryzae* NRRL 395

The *R. oryzae* NRRL 395 strain can directly utilize starch material to produce lactic acid by excreting amylolytic enzymes with Fig. 3 showing the amylolytic enzyme activity produced over time by strain *R. oryzae* NRRL 395. The enzyme activity of each sample increased with the fermentation time. Enzyme activity in the 2% potato medium had the highest amount of enzyme units at 24 hours of fermentation, with every sample peaking at this time (Fig. 3). Overall, 10^4 spores/ml concentration exhibited higher enzyme activity than 10^5 spores/mL concentration and ANOVA results illustrated significant interactions between the medium concentration, spore concentration, and time ($p<0.05$). The spore concentration and time also had a significant interaction ($p<0.05$), though, this might have been solely a result of time, which had a p-value less than $p<0.001$; making it far more significant than the rest of the factors which showed insignificant effects ($p>0.05$) as shown in Table 6.

Influence of shaking speed

Figs. 4 and 5 show that the highest values of lactate and dissolved oxygen were obtained using a shaking speed of 170 rpm and a fermentation time of four days. The lactate concentration and relative dissolved oxygen rate in broth increased with higher rate of shaking speed. At the fourth day, the lactate concentrations were 32, 26, and 14 g/L and relative dissolved oxygen rates were 60, 29, and 6% at 170, 110, and 60 rpm, respectively. The Tukey-Kramer Multiple-Comparison test confirmed significant

differences between lactate concentration and dissolved oxygen at these three different shaking speed levels (p-value<0.05). From analysis of the metabolism pathway of *R. oryzae*, it is known that lactic acid is only produced during the anaerobic process (Longacre et al., 1997). However, the experimental data showed that the lactic acid production was also associated with the dissolved oxygen in the broth. One of the reasons might be that the lactic acid production rate was determined by biomass growth rate and total biomass produced in the broth, while the amount of biomass, itself, was directly associated with the dissolved oxygen in the broth. Moreover, the fungus formed cotton-like mycelia which decreased the oxygen and nutrients mass transfer and eventually influenced the lactic acid production.

Identification of significant variables by the Plackett-Burman design

Lactate concentrations of the different treatments varied widely as demonstrated in Table 7, although Runs 5, 3, and 8, in particular, produced high concentrations of lactate (16.89, 14.95, and 14.51 g/L). By referring to Tables 2 and 3, it is clear that all of these runs were at a high level of potato concentration and a low level of temperature. Runs 6 and 9 were also conducted at a high level of potato concentration. However, the yields of these two runs were lower than the others. These lower yields can be explained by poor fungus growth within the runs since the concentration of the reducing sugar in the broths (8.57 g/L for Run 6 and 12.63 g/L for Run 9) was high and the cell dry weight (26.4 g/L for Run 6 and 12 g/L for Run 9) was low in comparison with the other runs (Table 7).

While using yield as a response, Runs 2, 1, 4, 12, 11, and 7 produced good results (53.05, 49.76, 47.04, 46.01, 37.85, and 37.66%, respectively). These were all runs at a

low level of potato concentration (Tables 2 and 3). It can be concluded that potato concentration had a positive effect (3.65) on lactic acid concentration in the broth and a negative effect (-22.202) on yield. In addition, temperature had a negative effect on both lactic acid concentration (-5.56) and yield (-15.045). All other factors had positive effects. Table 8 shows the statistical results of the different variables. The important factors affecting lactic acid yield and broth concentration were potato concentration and fermentation temperature, as their p-values were less than 0.2. All salt factors had differing positive effects on both lactate concentration and yield but the positive effects were not significant (p-value>0.2). These results indicated that potato could be used as the sole nutrient supply for lactic acid production by *R. oryzae* NRRL 395.

Optimization by central composite design

According to the PB design, potato concentration had positive effects on lactate concentration; therefore, the range of potato concentration was set at 5-11%. The potato concentration could not be higher because of the corresponding increase in viscosity. Since the fermentation temperature had negative effects on the yield and concentration of lactic acid production, a lower temperature range (22-32°C) was chosen. Due to the undesirable economics of longer fermentation time, the best setting for fermentation time was 4 days despite the fact that increased length had a positive effect on lactic acid production. Since all of the salts in the PB design had insignificant positive effects on lactic acid production, only potato was added into the medium as a nutrient for optimization design. The results of the central composite design are shown in Table 4. The lactate concentration increased with the increase of potato concentration, and the

concentration was peaked at temperature of around 27ºC and potato concentration of around 10% (Fig. 6). The model used to characterize lactate concentration as a function of potato concentration and fermentation temperature was a second order (quadratic) polynomial model. It is of the form:

Lactate (g/L) = $-164.16 + 2.08C + 12.57T + 4.01\times10^{-2}C^2 - 0.23T^2 + 1.15\times10^{-2}C\times T$

where C is the potato concentration (%) and T is the fermentation temperature (°C).

Verification

The optimal conditions obtained from the model on the experimental range were potato concentration of 10% (dry basis) and temperature of 27°C. A verification test was carried out under these optimal conditions. According to the above model, the predicted lactate concentration in broth was 35.5 g/L and the experimental mean value was 33.3 g/L with the standard deviation equaling 2.5 g/L for the eight samples. There was no significant difference between the predicted value and the mean value of experimental data (p-value=0.68).

5. Conclusion

This paper was focused on lactic acid production using cull potatoes as the sole nutrients. The fungal strain, *Rhizopus oryzae* NRRL 395, could directly produce lactic acid without adding amylolytic enzymes to hydrolyze the potato starch, which would decrease the total cost although the lactate concentration was low compared with bacterial fermentation with addition of amylolytic enzymes. *Rhizopus oryzae* NRRL 395 could produce amylase, however, it was not suitable for using as an amylase producing

strain in technical amylase production. Therefore, the activity of amylolytic enzyme was low compared with other amylase producing strains. Improving amylolytic enzyme activity could increase the lactic acid yield because extra amylase could quickly hydrolyze the potato starch to glucose for the strain to grow. High shaking speed was benefit for the production of lactic acid due to the aerobic condition of fungal fermentation. The cake-like morphology hindered the mass transfer and then decreased the product yield. Pellet-form biomass could overcome this problem especially in industry application. Future work should focus on pellet morphology study in order to increase the yield and productivity.

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Table 1. Characteristics of fresh cull potato [a]

	Fresh cull potato
Dry Matter, %	18.50 ± 0.12
Starch, % dry matter	81.27 ± 1.46
C, % dry matter	10.85 ± 0.83
N, % dry matter	0.41 ± 0.01

a: all data are the average of three replications with standard deviations of the means (n=3).

Table 2. Variables in Plackett-Burman design

No.	Variables	Unit	Low level(-)	High level (+)
A	$(NH_4)_2SO_4$	g/L	2	4
B	KH_2SO_4	g/L	0.65	1.3
C	$MgSO_4 \cdot 7H_2O$	g/L	0.25	0.5
D	$ZnSO_4 \cdot 7H_2O$	g/L	0.025	0.05
E	Potato concentration(dry basis)	g/L	15.3	45.9
F	Temperature	°C	30	37
G	Time	day	2	4

Table 3. Worksheet for the 12- run, 7-factor Plackett-Burman design (PB0712)

Run	Variables										
	A	B	C	D	E	F	G	H	I	J	K
1	+	-	+	-	-	-	+	+	+	-	+
2	+	+	-	+	-	-	-	+	+	+	-
3	-	+	+	-	+	-	-	-	+	+	+
4	+	-	+	+	-	+	-	-	-	+	+
5	+	+	-	+	+	-	+	-	-	-	+
6	+	+	+	-	+	+	-	+	-	-	-
7	-	+	+	+	-	+	+	-	+	-	-
8	-	-	+	+	+	-	+	+	-	+	-
9	-	-	-	+	+	+	-	+	+	-	+
10	+	-	-	-	+	+	+	-	+	+	-
11	-	+	-	-	-	+	+	+	-	+	+
12	-	-	-	-	-	-	-	-	-	-	-

Table 4. 11-Run Optimization Experiment [a]

Run	Factors		Lactate concentration (g/L)
	Potato concentration (%)	Temperature(°C)	
1	6 (-1)	22 (-1)	19.46 ± 1.22
2	10(+1)	22 (-1)	30.83 ± 2.07
3	6 (-1)	32 (+1)	17.54 ± 1.32
4	10(+1)	32(+1)	29.37 ± 1.13
5	8 (0)	20(-1.41)	10.24 ± 2.56
6	8 (0)	34(+1.41)	14.45 ± 2.81
7	5(-1.41)	27 (0)	15.09 ± 0.96
8	11(+1.41)	27 (0)	34.00 ± 3.55
9	8 (0)	27 (0)	25.74 ± 2.50
10	8 (0)	27 (0)	28.32 ± 2.50
11	8 (0)	27 (0)	26.11 ± 2.50

a: all data are the average of three replications with standard deviations of the means (n=3).

Table 5. Eluent conditions for lactate analysis[a]

Time (min)	A (%)	B (%)	C (%)	Comments
Init	0.00	40.00	60.00	TTL1
0.00	0.00	40.00	60.00	
0.01	0.00	40.00	60.00	Inject
2.00	0.00	10.00	10.00	
18.00	0.00	99.00	1.00	
18.01	1.00	0.00	99.00	
28.00	30.00	0.00	70.00	
37.00	80.00	0.00	20.00	
38.50	80.00	0.00	20.00	
42.50	1.00	0.00	99.00	
42.51	0.00	99.00	60.00	
43.50	0.00	40.00	60.00	
50.00	0.00	40.00	60.00	

a: Eluent A: 100 mM NaOH; Eluent B: 1mM NaOH; Eluent C: water;
Eluent flow rate: 1.5 mL/min; Pressure limit: 2000~3000psi; SRS: 100mA.

Table 6. ANOVA table for seed culture.

Source	DF	Mean square	F-Value	P-Value
Potato	2	122.16	0.87	0.431
Spore	1	21.33	0.15	0.700
Potato×Spore	2	157.26	1.12	0.342
Time	3	5490.9	39.16	<.0001
Potato×Time	6	270.16	1.93	0.117
Spore×Time	3	593.89	4.24	0.016
Potato×Spore×Time	6	479.35	3.42	0.014

Table 7. Responses of 12-run PB design [a]

Run	Lactate (g/L)	Lactic acid yield (%)	Cell dry weight (g/L)	Reducing sugar (glucose g/L)
1	7.60	49.76	38	0.80
2	8.11	53.05	45.6	0.83
3	14.95	32.61	41.2	0.86
4	7.19	47.07	32	0.83
5	16.89	36.84	51.2	0.83
6	5.76	12.56	26.4	8.57
7	5.76	37.66	59.2	0.83
8	14.51	31.66	52.8	0.83
9	2.72	5.92	12	12.63
10	8.53	18.60	47.6	0.95
11	5.78	37.85	58.4	0.82
12	7.03	46.01	45.2	0.80

a: Data are presented as the mean of two replicates

Table 8. Effects of variables in PB design on lactic acid yield and concentration, and associated statistical tests

Variables	Lactic acid Yield (%)			Lactate concentration (g/L)		
	Effects	t-value	P-value	Effects	t-value	P-value
$(NH_4)_2SO_4$	4.362	1.134	0.339	0.55	0.283	0.795
KH_2SO_4	1.925	0.500	0.651	1.61	0.825	0.470
$MgSO_4 \cdot 7H_2O$	2.175	0.565	0.611	1.12	0.574	0.606
$ZnSO_4 \cdot 7H_2O$	2.468	0.642	0.567	0.92	0.471	0.670
Potato concentration	-22.202	-5.771	0.01	3.65	1.869	0.158
Temperature	-15.045	-3.911	0.03	-5.56	-2.846	0.065
Time	2.525	0.656	0.558	2.22	1.137	0.338

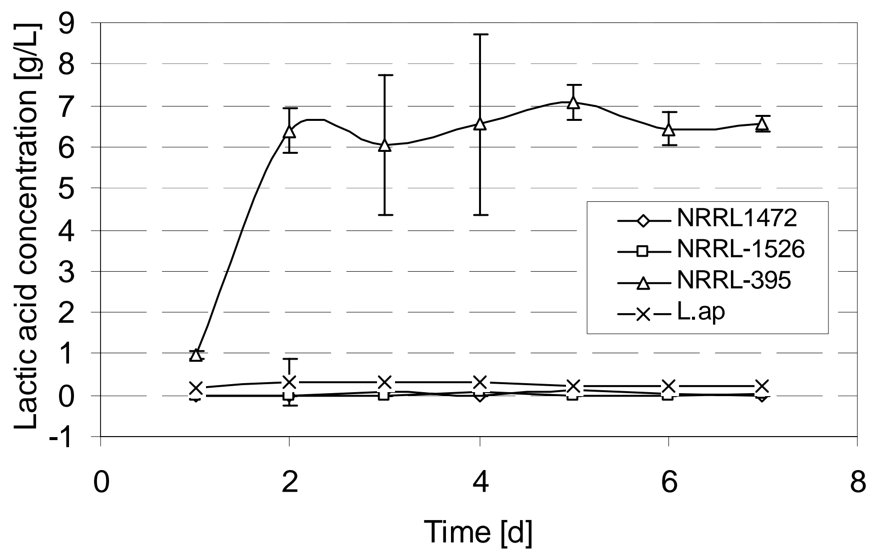

Fig. 1. Lactic acid production from cull potato by four species[a]
a: all data are the average of three replications with standard deviations of the means (n=3).

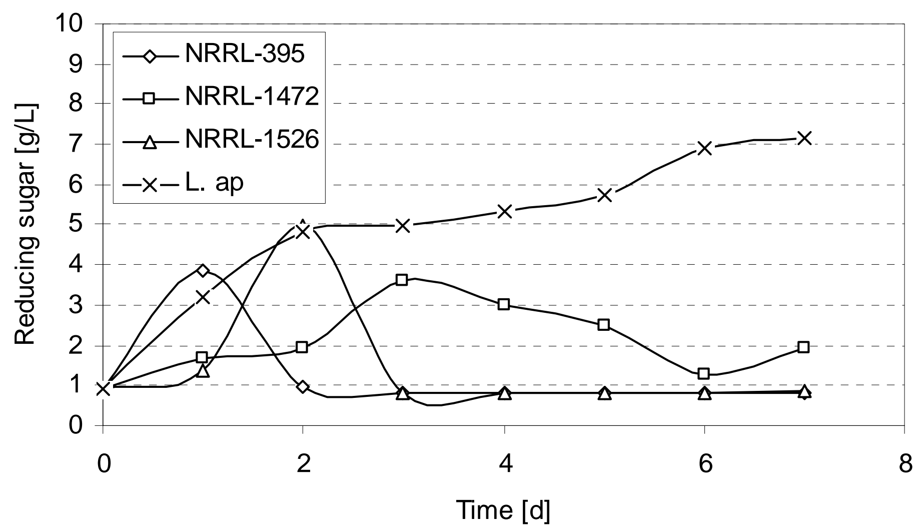

Fig. 2. The changes of reducing sugar during culturing four species on cull potato

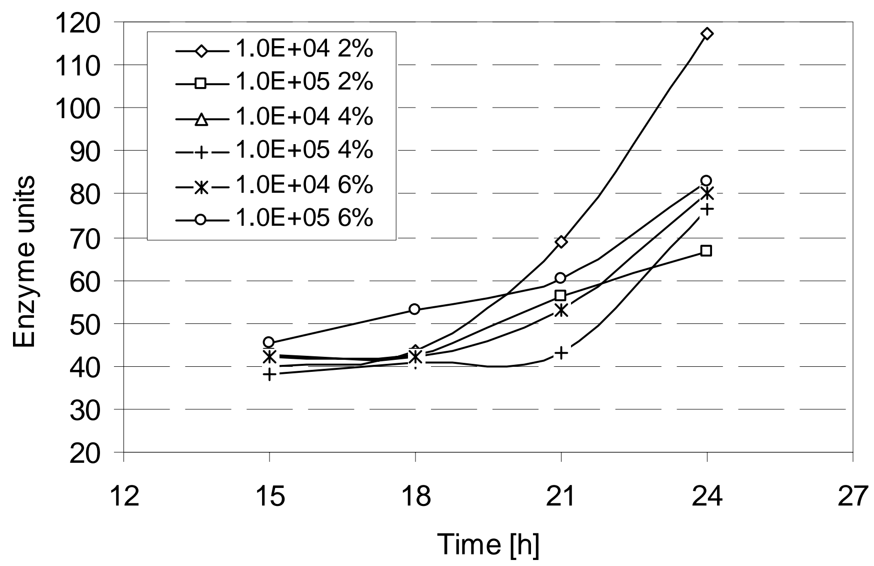

Fig. 3. Amylolytic enzyme activity of different seed culture treatments [a]
a: Data are presented as the mean of two replicates

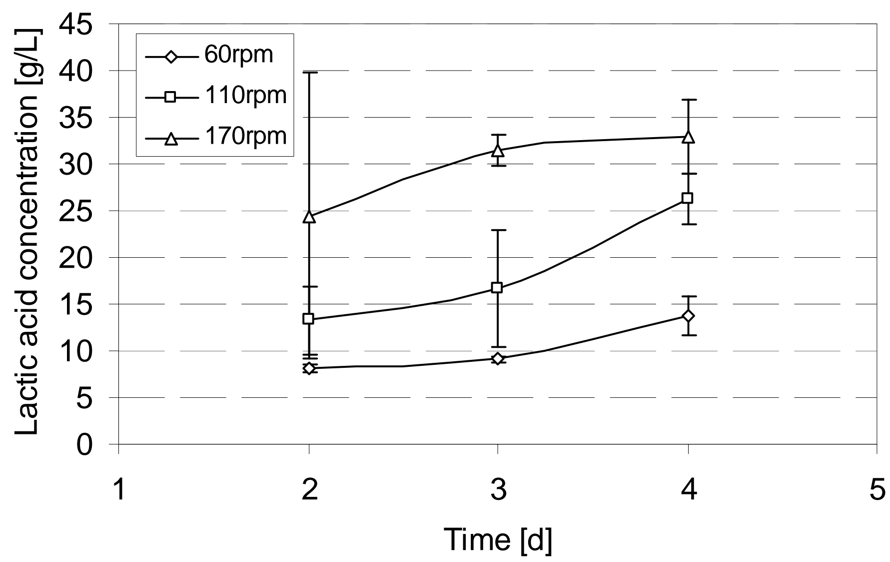

Fig. 4. Influence of shaking speed on lactic acid fermentation [a]
a: all data are the average of three replications with standard deviations of the means (n=3).

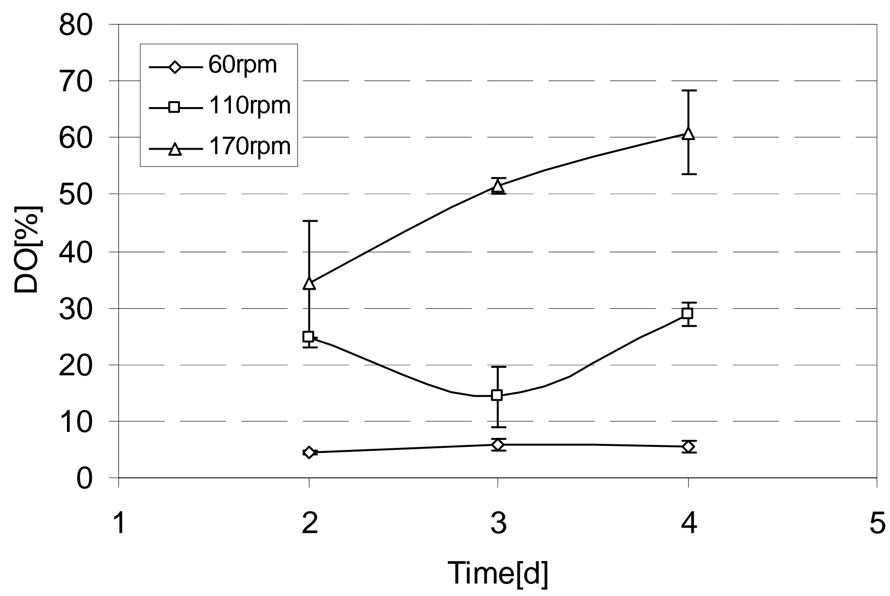

Fig. 5. Influence of shaking speed on dissolved oxygen [a]
a: all data are the average of three replications with standard deviations of the means (n=3).

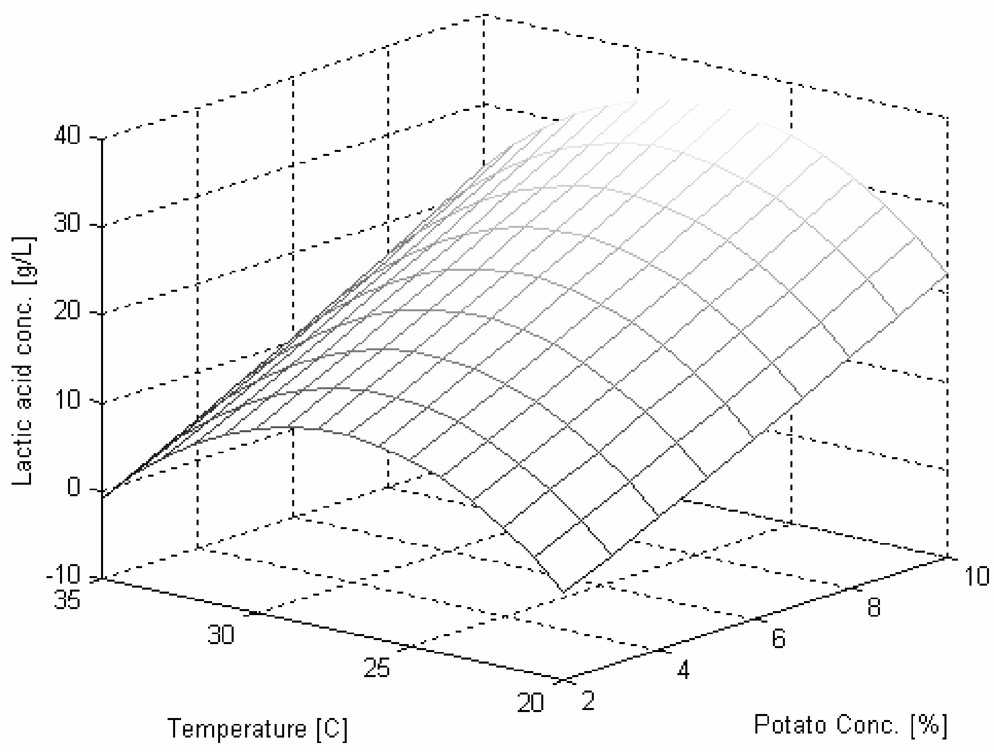

Fig. 6. Three-dimension plot of lactic acid production using *R. oryzae*

CHAPTER THREE

STUDY OF PELLET FORMATION OF FILAMENTOUS FUNGI *RHIZOPUS ORYZAE* USING A MULTIPLE LOGISTIC REGRESSION MODEL

1. Abstract

Fungal pellet formation is an important topic of fermentation research. It has been reported that many factors such as agitation, medium nutrients, pH, polymer additives, and inoculum size influence the formation of fungal pellets. However, a comprehensive investigation on the effects of all of these factors on fungal pellet formation has not been reported. This paper used a completely randomized design (CRD) on a filamentous fungus, *Rhizopus oryzae* NRRL 395, in order to discover the effects of the above factors on fungal pellet formation. In addition, other factors, such as addition of biodegradable polymers and spore storage time that have never been reportedly studied were examined and their effects on pellet formation were investigated. A multiple logistic regression model was established to predict the probability of pellet formation using the above factors and their interactions as predictor variables. Model building and diagnostics was obtained using the Statistical Analysis System (SAS 8.0) program. The model developed in this study can be used to predict the pellet formation of other *R. oryzae* strains as well.

Keyword: biodegradable polymer, pellet formation, logistic regression model, *Rhizopus oryzae,* storage time.

2. Introduction

Filamentous fungal fermentation is widely used to commercially produce useful products such as organic acids, enzymes, antibiotics, and cholesterol lowering drugs (Statins) (Cao et al., 1996; Casas Lopez et al., 2004, Chahal, 1985; Hang, 1989; Papagianni, 2004; Schuurmans et al., 1956; Steel et al., 1954). Fungi can be grown in submerged cultures by several different morphological forms: suspended mycelia, clumps, or pellets (Mets and Kossen, 1977). Many studies have discussed the advantages and disadvantages of growth morphologies in terms of producing different products (Calam, 1976; Konig et al., 1982; Martin et al., 1952). In bioreactors, the filamentous or clump mycelia can increase the viscosity of the medium, wrap around impellers, and block nutrient transport; all which can lead to a decrease in production efficiency and bioreactor performance. Fungal growth in pellet form then becomes the desirable morphology for industrial fermentation processors because it not only reduces the medium viscosity or possibility of wrapping around impellers, but also allows for fungal biomass reuse, improves culture rheology through enhanced mass and oxygen transfer, and lowers energy consumption for aeration and agitation (Van Sjijdam et al., 1980).

There are many factors that influence the formation of fungal pellets, such as agitation, medium nutrients, pH, oxygen tension, polymer additives, viscosity of the medium, surface-active agents, and inoculum size (Mets et al., 1977; Nielsen et al., 1996; Papagianni, 2004; Znidarsic et al., 1998). For individual strains, each factor has different importance to the growth morphologies. Some strains such as *Rhizopus* sp. needs strong agitation to form pellets, while others such as *Penicillium chrysogenum* require high pH to form pellets (Mets et al., 1977). Thus, the study of fungal pellet formation is often

limited to the level of an individual strain. In regard to the *Rhizopus oryzae* strain, Zhou et al. (2000) investigated the effects of different metal ions (Mg^{2+}, Zn^{2+}, and Fe^{2+}) and pH on the pellet formation of *R. oryzae* ATCC 20344 while Byrne et al. (1989a; 1989b) studied the effects of glucose concentration, peptone concentration, pH, and some additives on the pellet formation of *R. oryzae* ATCC 10260. Despite these individual studies on specific pellet forming factors, a comprehensive investigation of the effects of medium composition and inoculation on pellet formation and growth of *Rhizopus oryzae* has not been reported to date.

Due to the fact that numerous factors affecting pellet formation and growth, a complete predictability must include the probability of pellet formation as well as growth factors when the pellet formation occurs for a specific environment. It is the hypothesis of the author that a logistic regression model which is generally used to describe the probability of occurrence of an event in the fields of economics and environmental and microbiology (Neter et al., 1996; Skierve, 1992; Lopez-Malo, 2000), can, in fact, provide a possible mathematical mechanism to model the probabilistic pellet formation and help to explore the effects of culture conditions on spore germination. In the logistic model the response Y is usually binary and is defined to have two possible outcomes: occurrence and without occurrence. In this particular case, the response variable Y was defined as the pellet formation which has two possible outcomes: forming pellets or not forming pellets (coded 1 and 0, respectively), and the effects of the factors on the pellet formation can be evaluated using the logistic regression model.

The objective of this work included: (1) investigating the influence of fermentation factors such as pH, inoculum size, substrate concentration, biodegradable

polymer additives, storage time, shaking speed, and temperature on pellet formation using *Rhizopus oryzae* NRRL 395 as a model strain; (2) modeling pellet formation as a function of the above factors by logistic regression; and (3) verifying the model and predicting the pellet formation of other *R. oryzae* strains.

3. Material and methods

Microorganism

R. oryzae NRRL 395 (ATCC 9363), *R. oryzae* 20344 (ATCC), and *R. oryzae* 10260 (ATCC) were obtained from the American Type Culture Collection (Manassas, VA). The strains were first cultured on potato dextrose agar (Difco) slants, and further propagated on potato dextrose agar in 500 ml Erlenmeyer flasks to form spores. The culture temperature was 25°C. The spores were washed from the agar with sterile distilled water. The spore solution was stored at 4°C. Different storage durations of spore solution were used for the experiment. The spore concentration of the suspension was 1×10^{8} spores/ml.

Effects of biodegradable polymer additive on pellet formation

A biodegradable polymer, rice, with 4 kinds of sizes and 3 concentrations, was added to a 50 mL Potato Dextrose Broth (PDB) solution and autoclaved at 121°C for 15 minutes. A completely randomized design (CRD) with 12 combinations was carried out to study the effects of biodegradable polymer addition on pellet formation. Four sizes and three concentrations were tested (Table 1). The cultures were performed at 27°C for 48 hours using an orbital shaker bath (Lab-line Shaker, Model: 3540, Melrose Park, ILL,

U.S.A.) at 170 rpm. The culture temperature was fixed at 27°C. The spore storage time was 80 days.

Effects of inoculum, pH, and carbon source concentration on pellet formation

A three-way completely randomized design (CRD) with 48 treatment combinations was used to study the formation of the pellets. Factors such as spore inoculum ($3x10^9$, $2x10^9$, $1x10^9$, $0.2x10^9$, $0.1x10^9$ and $0.01x10^9$ spore/L), pH control (with or without $CaCO_3$), and carbon source concentrations (6, 12, 24 and 48 g/L PDB) were used to study the formation of the pellets (Table 2). The cultures were performed at 27°C for 48 hours using an orbital shaker (Classic Series C24 Incubator shaker, New Brunswick Scientific, Edison, NJ, U.S.A.) at 170 rpm. The spore storage time was 320 days.

Effects of shaking speed

Three levels of shaking speed (115, 170, 250 and 350 rpm) were investigated to test the formation of the pellets using an orbital shaker (Classic Series C24 Incubator shaker, New Brunswick Scientific, Edison, NJ, U.S.A.)(Table 3). The spore inoculum, $CaCO_3$ concentration, and PDB concentration were fixed at $1x10^9$ spore/L, 6 g/L, and 24 g/L, respectively. The culture temperature was 27°C and the spore storage time was 75 days.

Effects of temperature

Four levels of culture temperatures (22, 27, 33, and 38°C) were investigated to test the formation of the pellets (Table 4). The spore inoculum, $CaCO_3$ concentration, and PDB concentration were fixed at $1x10^9$ spore/L, 6 g/L and 24 g/L, respectively. The shaking speed was fixed at 170 rpm using an orbital shaker (Classic Series C24 Incubator shaker, New Brunswick Scientific, Edison, NJ, U.S.A.). The spore storage time was 372 days.

Statistical analysis

The effects of various factors on pellet formation were analyzed with a General Linear Model (GLM) using the Statistical Analysis System program 9.0 (SAS institute Inc., NC). ANOVA tables of different responses (pellet number and pellet yield) were used to evaluate the factors.

Logistic regression model

A multiple logistic regression model was used to predict the probability of occurrence of pellet formation. The multiple logistic response functions were expressed as equations (1) and (2).

$$E\{Y_i\} = \frac{\exp(\beta X_i)}{1+\exp(\beta X_i)} \qquad (1)$$

$$\beta X_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + ... + \beta_{21} X_{28} \qquad (2)$$

where Y_i are independent Bernoulli random variables with expected values $E\{Y_i\}$; the X_i are different predictor variables and interaction effects, and the coefficients (β_i) are the

parameters to be estimated by fitting the logistic model to the experimental data. The predictor variables could be quantitative or qualitative (Neter et al., 1996). Factors such as carbon source, inoculum size, temperature, shaking speed, addition of biodegradable polymers, neutralizer, spore storage time, and their interactions on pellet formation were the predictor variables (Table 5). Logistic regression was accomplished using the Statistical Analysis System program 9.0 (SAS institute Inc., NC). Backward stepwise selection was employed to fit the logistic regression equation. The significance of the coefficients was evaluated with individual coefficients being eliminated from the whole model if the p-value was greater than 0.2.

Model verification

After fitting the logistic regression equation, predictions of pellet formation were verified. Pellet formation experiments were carried out at different cultural conditions. Meanwhile, the predicted probabilities of forming pellets were calculated using the developed model. Three *R. oryzae* strains: *R oryzae* 9363, *R. oryzae* 20344 and *R. oryzae* 10260 were tested (Table 10).

Analytical methods

The morphology of the cultures was determined by examining submerged cultures dispersed in Petri dishes. An Olympus microphotograph (Tokyo, Japan) was used to observe the pellet morphology and measure the size of the pellets. The uniform pellet with diameter less than 5 mm was represented by the value of 1 while other non-pellet forms such as clump and non-uniform pellet/clump were represented by the value of 0

(Fig.1).The pH value was measured with a Fisher portable pH meter (Fisher Scientific, U.S.A.). Dry biomass was determined by washing the pellet mycelia with 6N HCL to neutralize excess $CaCO_3$ attached in the pellets, and then washing to pH 6 with DI water. The washed biomass was dried at 100ºC overnight before weight analysis.

4. Results

Effect of biodegradable polymer addition on pellet formation

All experimental treatments with rice as a biodegradable polymer at three different concentrations (4, 10, and 20 g/L) and four particle sizes formed pellets with diameters of 1.1~1.7 mm. There were no significant differences in pellet size between each of the 12 combinations (Fig. 2). Analysis of variance (AVOVA) further indicated that the interaction of rice concentration and rice particle size had no significant influence on both biomass yield and pellet number (Table 6 and 7), which indicates that mean values can be directly used to compare the pellet formation from different experimental combinations. The results show that different rice sizes had no significant influences on pellet number and biomass yield (Fig.3), while rice concentration had significant impacts on biomass yield although it did not influence the pellet number. The biomass yield reached the highest value of 16% at 4 g/L rice concentration (Fig. 4).

Effect of inoculum, neutralizer, and carbon source concentration

Table 2 presents the effects of carbon source, inoculum, and neutralizer on fungal morphology. The data show that fungal morphology varied with different combinations of factors. The final pH values of the experimental runs with addition of neutralizer

maintained around 5.59~6.87, while the runs without the neutralizer had lower pH values in the range of 2.44~2.75. In terms of pellet formation, seven runs (No. 7, 8, 13, 14, 19, 20, 21) with neutralizer were not able to form pellets, and another eleven runs (No. 25, 26, 31, 32, 33, 37, 38, 39, 43, 44, 45) without neutralizer did not form pellets as well. The results demonstrate that it is relatively difficult to conclude the effects of neutralizer itself on pellet formation.

Meanwhile, for most of the different PDB concentrations, the data also showed that the biomass yield increased following an increase in inoculum size (Table 2), and as for the inoculum, the lower the PDB concentration in the broth was, the higher the biomass yield obtained at each individual inoculum size (Table 2).

Effects of shaking speed on pellet formation

The effects of shaking speed on pellet formation were investigated (Table 3). The pellets from various shaking speeds were significantly different. The data demonstrated that low shaking speeds (from 115 rpm to 180 rpm) were beneficial to pellet formation, with pellet size increasing following a decrease in the shaking speed. The average pellet diameter from the 115 rpm shaking speed was 4.5 mm while the mean from 180 rpm was 1.35 mm.

Effects of temperature on pellet formation

The effects of temperature show that there were no significant differences on pellet biomass yield between 22~33°C, while the biomass yield at 38°C was significantly lower. This might be caused by the high cell maintenance required under high

temperature conditions. Additionally, temperature had an effect on the timing of pellet formation with a higher temperature leading to faster pellet formation (Table 4).

The logistic regression model

In order to better understand the combinatory effects of the above factors and their interactions on pellet formation, a multiple regression model was established by fitting the experimental data in Equation (1) using the Logistic Proc package of Statistical Analysis System program 9.0 (SAS institute Inc., NC). In this particular case, a P-value of 0.2 was used as the criterion to determine significant variables ($P<0.2$) and non-significant variables ($P>0.2$), and the determination was carried out by a backward stepwise procedure. Only significant variables were integrated into Equation (1) which was used to build the model. The statistic analysis elucidated that temperature was a non-significant variable and that most of the interaction terms (except the interaction between PDB concentration and inoculum spore concentration) were non-significant variables. Thus, the significant variables of pH control, PDB concentration, spore concentration, shaking speed, polymer addition, culture time, and the interaction of PDB and spore were included in the model. The coefficients of these variables in the model are presented in Table 8. The statistical analysis also demonstrated that the variables included in the model were statistically significant ($P<0.05$). In addition, the goodness of fit of the model was tested by the log likelihood ratio and Chi-square tests. The Hosmer-Lemeshow Chi square test indicated a very good fit ($P=0.97$) with empirical data. The significant results indicated that the model is valid as a predictor of outcome variables (formation of pellets or no formation of pellets).

The probabilities of pellet formation calculated using the logistic regression model at fixed temperature (27°C) and shaking speed (170 rpm) are presented in Figs. 5, 6, 7. Overall, the results generated from the model were: 1) higher PDB concentrations and higher spore concentrations had higher possibilities to form pellets; 2) the addition of biodegradable polymer had positive effects on pellet formation; 3) higher pellet formation probabilities were obtained at longer storage durations; and 4) pH control slightly benefited pellet formation.

The calculated critical spore concentrations at a probability of 0.95 on pellet formation at fixed PDB concentration (24 g/L) and temperature (27°C) are shown in Table 9. The data present that less spore was needed to form pellets at longer spore storage time no matter if the pH of cultures were controlled or not. For all of the tests with or without pH control, the strain required more spores to form pellets following an increase of shaking speed. The spore concentrations for test with pH control and without pH control increased 19.5 times and 11.2 times, respectively, once the shaking speed was changed from 170 rpm to 300 rpm. The data also show that the spore concentration can be decreased with addition of polymer. Compared to the test without polymer addition, the spore concentration decreased 7.2 times with an addition of 20 g/L polymer.

Table 10 presents the calculated polymer concentrations needed to form pellets at different shaking speeds, spore concentrations, and pH control. The polymer concentrations tested to fit the model were less than 20 g/L. Some calculated polymer concentrations were beyond this range, which were counted as invalid tests and the concentrations less than zero indicated that it is not necessary to add polymer at those conditions. Less polymer concentrations were predicted using a probability of 0.5 in

which there was a 50% chance of pellet formation. A greater margin of safety was obtained if a probability of 0.9 or 0.95 was used, which reflected in more polymer concentration. The data also demonstrated that shaking speed significantly affected pellet formation. Fewer polymers were required at lower shaking speed. The data present as well as that increasing spore storage time and adding $CaCO_3$ to control pH can compromise the polymer addition in terms of pellet formation.

Model verification

Model verification was carried out at different conditions using three filamentous *R. oryzae* strains: *R. oryzae* ATCC 9363, *R. oryzae* ATCC 20344, and *R. oryzae* ATCC 10260. The model prediction was consistent with all experimental results. Pellets were obtained with calculated probability of higher than 0.95, while clumps were observed at calculated probability of near to zero (Table 11). The data indicated that the established model was a good pellet formation prediction model for strains of *R. oryzae*.

5. Discussion

It has been reported that polymer additives such as anionic polymers of carbopol-934 (carboxypolymethylene) and Reten (polyacrylate), can decrease the agglutination of spores and produce a much more dispersed growth, which increased pellet number with an accompanying decrease of pellet size and density (Metz, 1977). Cereals, such as rice used in this research, which show a positive effect on fungal pellet formation, might have a similar function to influence the agglutination of spores before germination. Meanwhile, an advantage of biodegradable polymer additives is that the polymers can be

directly used by the strain as nutrients without separation problem. Other biodegradable polymers such as starch, wheat flour, corn, and millet etc. have similar positive effects on pellet formation (data not shown). A patent associated with this experimental result has been filed.

In addition to the development of biodegradable polymers for fungal pellet formation, the effects of spore storage duration on the pellet formation has been discovered as well. No literature was found in regard to the effects of spore storage duration on pellet formation. This study's results, though, conclude that the spore storage time is one of the main factors influencing pellet formation with short spore storage duration (new prepared spores) having a negative effect on pellet formation. Following the increase of storage duration the probability of forming pellets was correspondingly increased, and deactivation was not detected for the spores used in the research which was stored at 4°C for 1.5 years. The reason why new spores have a lower probability of pellet formation might be: (1) that their propensity for active hyphae in the spore solution directly leads to the formation of filamentous mycelia; and in relation (2) long storage duration leads to the hyphae having been nearly completely decayed and spores being left only in solution.

Inoculum size (spore concentration) is another important factor for pellet formation. Generally, the interaction of hyphae is considered to be the main factor for clump formation. In the early stage of growth, the higher the inoculum size is, the more interaction the hyphae have and the higher possibility clump biomass is formed. Thus, it has been concluded by other researchers that low inoculum concentrations are beneficial for pellet production (Foster, 1949). However, the maximum inoculum size varied from

strain to strain (Mets and Kossen, 1977). Most studies on pellet formation of *Rhizopus* strains were conducted at relatively low concentrations (less than 10^7 spores/L) (Byrne et al., 1989a; Byrne et al, 1989b; Znidarisic et al., 1998; Zndiarsic et al., 2000). The effects of inoculum on *R. oryzae* pellet formation conducted in this study, though, demonstrated that high inoculum spore concentration (up to $3x10^9$ spores/L) has a high probability to form pellets.

The pH of medium is also a very important factor for various fungi to form pellets. The influence of pH on pellet formation is mainly through a change in the surface properties of the fungi (Mets et al., 1977). It has been reported that different strains have different response to pH value (Mets et al., 1977). For this particular strain of *R. oryzae*, the results show that there were no significant differences on pellet formation with a pH range of 2.5 to 7, which means that this strain is relatively tolerant to pH compared to some other fungal strains such as *Aspergillus niger* and *Penicillium chrysogenum* (Galbraith et al., 1969; Pirt et al., 1959; Steel et al., 1954). However, neutralizer is still needed to prevent pH from dropping into the low pH range of 2-3 which is not favorable for biomass accumulation (Znidarsic et al., 1998). In this study, the results show that total amount of biomass from a medium with calcium carbonate was significantly higher than those without it. In addition, during fungal pellet formation, calcium carbonate not only is a neutralizer to keep pH stable, but also supplies Ca^{2+} ions. Calcium ions were usually recognized to induce mycelial aggregation during fungal growth, which will add some degree of benefit to the pellet formation (Jackson et al., 1993).

PDB as a good nutrient source has been widely used for fungal and yeast cultures. In this study, PDB was used as the sole nutrient source. It contains mainly glucose, some

vitamins, and a little nitrogen. The results indicated that PDB strongly interacted with other factors such as inoculum spore concentration to influence the pellet formation, which is in agreement with Liao et al. (2005) who also reported that PDB had a large impact on pellet growth such as pellet size, total biomass, and total amount of pellets.

Agitation as the main factor for dispersion of spore agglomerates was regarded as another important factor for pellet formation. Strong agitation can prevent the formation of pellets (Mets, 1977) and the experiments performed within this study showed similar results (Table 6). Additionally, the effects of temperature on pellet formation concluded that temperature was not an important factor for pellet formation although high growth rate was observed at high temperature.

6. Conclusion

Growth of fungi in pellet form is influenced by many factors and thus the prediction of pellet formation is difficult. Modeling the probability of pellet formation is a valuable approach to microbial modeling that was not explored prior to this study. A multiple logistic regression model provides an alternative way to predict fungal pellet formation. In the present study, a multiple logistic regression model was used for deciding which variables could serve as predictor variables for the occurrence of pellets of *R. oryzae* NRRL 395. The predictor variables include selected factors such as PDB concentration, inoculum spore concentration and their interaction, pH control, addition of biodegradable polymer, storage time and shaking speed. The established model can be used to predict pellet formation of other *R. oryzae* strains as well.

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Table 1. The experimental design of effects of biodegradable polymer additive on pellet formation

Size [a]	Rice (g/L)	PDB (mL)	CaCO3 (g)	Spore conc.(x10^8 spore/mL)
Original rice (3mm x 5mm)	20, 10, 4	50	0.3	0.5
Original rice~1.4mm	10, 4	50	0.3	0.5
1.4~0.6mm	20, 10, 4	50	0.3	0.5
<0.6mm	20, 10, 4	50	0.3	0.5

a: Rice was milled and particle size was separated by #12 U.S. standard screen and #30 U.S. standard screen.

Table 2. The effects of inoculum, PDB concentration, and pH on pellet formation [a,b]

Run	$CaCO_3$ (g)	PDB (g)	log(Inoculum)	diameter(mm)	pellet	Run	$CaCO_3$ (g)	PDB (g)	log(Inoculum)	diameter(mm)	pellet
1	0.3	0.3	7.0	0.94±0.10	1	25	0	0.3	7.0	-	0
2	0.3	0.3	8.0	1.13±0.26	1	26	0	0.3	8.0	-	0
3	0.3	0.3	8.3	1.47±0.23	1	27	0	0.3	8.3	0.70±0.13	1
4	0.3	0.3	9.0	0.93±0.07	1	28	0	0.3	9.0	0.45±0.07	1
5	0.3	0.3	9.3	0.69±0.12	1	29	0	0.3	9.3	0.35±0.12	1
6	0.3	0.3	9.5	0.66±0.13	1	30	0	0.3	9.5	0.34±0.06	1
7	0.3	0.6	7.0	-	0	31	0	0.6	7.0	-	0
8	0.3	0.6	8.0	-	0	32	0	0.6	8.0	-	0
9	0.3	0.6	8.3	1.75±0.67	1	33	0	0.6	8.3	-	0
10	0.3	0.6	9.0	0.96±0.19	1	34	0	0.6	9.0	0.92±0.18	1
11	0.3	0.6	9.3	0.73±0.14	1	35	0	0.6	9.3	0.86±0.14	1
12	0.3	0.6	9.5	0.86±0.19	1	36	0	0.6	9.5	0.67±0.12	1
13	0.3	1.2	7.0	-	0	37	0	1.2	7.0	-	0
14	0.3	1.2	8.0	-	0	38	0	1.2	8.0	-	0
15	0.3	1.2	8.3	1.79±0.69	1	39	0	1.2	8.3	-	0
16	0.3	1.2	9.0	1.90±0.56	1	40	0	1.2	0.5	1.06±0.20	1
17	0.3	1.2	9.3	1.02±0.21	1	41	0	1.2	1.0	1.04±0.09	1
18	0.3	1.2	9.5	1.00±0.23	1	42	0	1.2	1.5	1.20±0.29	1
19	0.3	2.4	7.0	-	0	43	0	2.4	0.0	-	0
20	0.3	2.4	8.0	-	0	44	0	2.4	0.1	-	0
21	0.3	2.4	8.3	-	0	45	0	2.4	0.1	-	0
22	0.3	2.4	9.0	2.29±1.10	1	46	0	2.4	0.5	1.16±0.22	1
23	0.3	2.4	9.3	1.69±0.31	1	47	0	2.4	1.0	0.98±0.28	1
24	0.3	2.4	9.5	1.29±0.19	1	48	0	2.4	1.5	0.68±0.18	1

a: All runs were carried out without polymer additives and cultured at 27°C, 170rpm for 2days. The spore storage time was 320day.
b: Data with "±" are the average of triplicates with standard deviations (n=3).

Table 3. The effect of shaking speed on pellet formation [a,b]

Shaking speed (rpm)	Pellet	diameter(mm)
115	yes	4.51±0.44
180	yes	1.35±0.32
250	No	-
350	No	-

a: All runs were carried out with polymer additives (20g/L) and cultured at 27°Cfor 2days. The spore inoculum, $CaCO_3$ concentration and PDB concentration were fixed at 1×10^9 spore/L, 6g/L and 24g/L respectively. The spore storage time was 75days.
b: Data with "±" are the average of triplicates with standard deviations (n=3).

Table 4. The effect of temperature on pellet formation [a,b]

Temperature (°C)	Pellet	Yield (%)
22	yes	11.8±0.5
27	yes	12.0±0.3
33	yes	12.3±0.4
38	yes	8.1±0.4

a: All runs were carried out without biodegradable polymer and shaking at 170rpm for 2days. The spore inoculum, CaCO3 concentration and PDB concentration were fixed at $1x10^9$ spore/L, 6g/L and 24g/L respectively. The spore storage time was 372days.
b: Data with "±" are the average of triplicates with standard deviations (n=3).

Table 5. Logistic model predictor variables and coefficients

Coefficient	Predictor variables
β_0	Constant
β_1	pH
β_2	P (PDB concentration g/L)
β_3	I (Log(Inoculum size spore/L)
β_4	T (Temperature °C)
β_5	S (Shaking speed, rpm)
β_6	Pm (addition of biodegradable polymer)
β_7	Time(storage time)
$\beta_8 \sim \beta_{28}$	Pair interaction coefficients

Table 6. Analysis of Variance of pellet number per unit volume broth [a]

Source Term	Degree of freedom	Sum of Squares	Mean Square	F-Ratio	P Level
A: Particle size	3	6049.52	2016.51	0.95	0.45
B: Polymer conc. (g/L)	2	9497.64	4748.82	2.24	0.16
AB: interaction	6	14644.56	2440.76	1.15	0.40
Total (Adjusted)	21	42825.45			
Total	22				

a: Term significant at alpha = 0.05

Table 7. Analysis of Variance of biomass yield [a]

Source Term	Degree of freedom	Sum of Squares	Mean Square	F-Ratio	P Level
A: Particle size	3	0.44	0.15	0.09	0.97
B: Polymer conc. (g/L)	2	37.60	18.80	11.11	0.00
AB: interaction	6	2.27	0.38	0.22	0.96
Total (Adjusted)	21	93.36			
Total	22				

a: Term significant at alpha = 0.05

Table 8. Analysis of maximum likelihood estimates of reduced model[a]

Parameter	Estimate	Standard error	Wald Chi-Square	P>Chi Square
Intercept (β_0)	179.5	79.8749	5.0516	0.0246
pH (β_1)	6.4594	2.9779	4.7050	0.0301
PDB (β_2)	-28.9887	10.9882	6.9600	0.0083
spore (β_3)	-16.9952	7.5509	5.0658	0.0244
PDB*spore(β_{15})	3.4649	1.3157	6.9357	0.0084
Shaking speed (β_5)	-0.5332	0.2631	4.1060	0.0427
Polymer (β_6)	2.8114	1.0136	7.6935	0.0055
Time (β_7)	0.1478	0.0531	7.7631	0.0053

a: Effects met the 0.20 significance level for entry into the model.

Table 9. Predicted critical log(inoculum size) for a probability of 0.95 on pellet formation[a]

Storage time (day)	polymer	CaCO3 control pH			Without pH control		
		170 rpm	220 rpm	300 rpm	170 rpm	220 rpm	300 rpm
30	0	9.1	9.5	10.3	9.1	9.6	10.2
	4	8.9	9.3	10.2	9.0	9.4	10.0
	10	8.6	9.0	9.9	8.7	9.1	9.8
	20	8.2	8.6	9.5	8.3	8.7	9.3
180	0	8.7	9.1	10.0	8.8	9.2	9.9
	4	8.5	9.0	9.8	8.6	9.0	9.7
	10	8.3	8.7	9.6	8.4	8.8	9.4
	20	7.9	8.3	9.2	8.0	8.4	9.0
365	0	8.3	8.7	9.6	8.4	8.8	9.4
	4	8.1	8.5	9.4	8.2	8.6	9.3
	10	7.9	8.3	9.2	8.0	8.4	9.0
	20	7.5	7.9	8.7	7.6	8.0	8.6

a: The PDB concentration and culture temperature were fixed at 24g/L and 27°C, respectively.

Table 10. Predicted critical polymer concentration for different probabilities (p) of pellet formation [a]

Storage time (day)	rpm	Log(spore)	With control pH			Without pH control		
			0.95	0.9	0.5	0.95	0.9	0.5
30	170	7	48.3	48.0	47.2	50.6	50.3	49.6
30	170	7.5	36.5	36.3	35.5	38.8	38.6	37.8
30	170	8	24.8	24.5	23.7	27.1	26.8	26.0
30	170	8.5	13.0	12.7	11.9	15.3	15.0	14.3
30	170	9	1.2	1.0	0.2	3.5	3.3	2.5
30	220	7	57.8	57.5	56.7	60.1	59.8	59.0
30	220	7.5	46.0	45.7	45.0	48.3	48.0	47.3
30	220	8	34.2	34.0	33.2	36.5	36.3	35.5
30	220	8.5	22.5	22.2	21.4	24.8	24.5	23.7
30	220	9	10.7	10.4	9.7	13.0	12.7	12.0
30	300	7	73.0	72.7	71.9	75.3	75.0	74.2
30	300	7.5	61.2	60.9	60.1	63.5	63.2	62.4
30	300	8	49.4	49.2	48.4	51.7	51.5	50.7
30	300	8.5	37.7	37.4	36.6	40.0	39.7	38.9
30	300	9	25.9	25.6	24.8	28.2	27.9	27.1
180	170	7	40.4	40.1	39.4	42.7	42.4	41.7
180	170	7.5	28.6	28.4	27.6	30.9	30.7	29.9
180	170	8	16.9	16.6	15.8	19.2	18.9	18.1
180	170	8.5	5.1	4.8	4.1	7.4	7.1	6.4
180	170	9	-6.7	-6.9	-7.7	-4.4	-4.6	-5.4
180	220	7	49.9	49.6	48.8	52.2	51.9	51.1
180	220	7.5	38.1	37.9	37.1	40.4	40.2	39.4
180	220	8	26.4	26.1	25.3	28.7	28.4	27.6

Storage time (day)	rpm	Log(spore)	CaCO3 control pH			Without pH control		
			0.95	0.9	0.5	0.95	0.9	0.5
180	220	8.5	14.6	14.3	13.5	16.9	16.6	15.8
180	220	9	2.8	2.6	1.8	5.1	4.9	4.1
180	300	7	65.1	64.8	64.0	67.4	67.1	66.3
180	300	7.5	53.3	53.0	52.3	55.6	55.3	54.6
180	300	8	41.5	41.3	40.5	43.8	43.6	42.8
180	300	8.5	29.8	29.5	28.7	32.1	31.8	31.0
180	300	9	18.0	17.7	17.0	20.3	20.0	19.3
365	170	7	30.7	30.4	29.6	33.0	32.7	31.9
365	170	7.5	18.9	18.7	17.9	21.2	21.0	20.2
365	170	8	7.2	6.9	6.1	9.4	9.2	8.4
365	170	8.5	-4.6	-4.9	-5.7	-2.3	-2.6	-3.4
365	170	9	-16.4	-16.6	-17.4	-14.1	-14.3	-15.1
365	220	7	40.2	39.9	39.1	42.5	42.2	41.4
365	220	7.5	28.4	28.1	27.4	30.7	30.4	29.7
365	220	8	16.6	16.4	15.6	18.9	18.7	17.9
365	220	8.5	4.9	4.6	3.8	7.2	6.9	6.1
365	220	9	-6.9	-7.2	-7.9	-4.6	-4.9	-5.6
365	300	7	55.3	55.1	54.3	57.6	57.4	56.6
365	300	7.5	43.6	43.3	42.5	45.9	45.6	44.8
365	300	8	31.8	31.5	30.8	34.1	33.8	33.1
365	300	8.5	20.0	19.8	19.0	22.3	22.1	21.3
365	300	9	8.3	8.0	7.2	10.6	10.3	9.5

a: The PDB concentration and culture temperature were fixed at 24g/L and 27C, respectively.

Table 11. Verification of model using three *R. oryzae* strains [a]

Factors	*R. oryzae* (9363)	*R. oryzae* (9363)	*R. oryzae* (10260)	*R. oryzae* (10260)	*R. oryzae* (20344)	*R. oryzae* (20344)
$CaCO_3$ g	0	0.3	0.3	0.3	0.3	0.3
PDB g	24	24	20	20	20	24
log(Inoculum)	7	9	7	9	7	9
Temp(°C)	27	27	27	27	27	27
Shaker (rpm)	170	170	170	170	170	170
polymer (g/L)	0	20	0	20	0	0
storage time (day)	3	3	2	2	2	90
number of runs	5	10	3	3	3	3
number of runs forming pellets	0	10	0	3	0	3
predicted probability	0	1	1.3×10^{-6}	1	1.3×10^{-6}	1

a: Culture conditions: All experiments were cultured for two day.

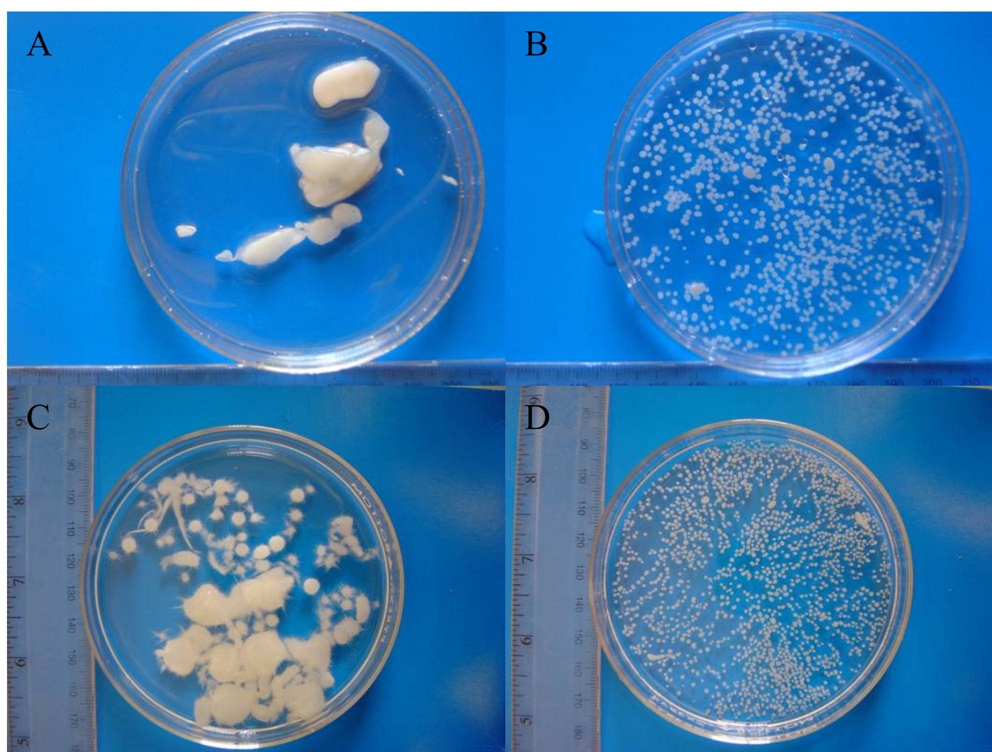


Fig. 1. Morphology of fungal biomass

A: Clump: Temperature=27°C, PDB concentration=12g/L, inoculum spore concentration=1x10^8 spore/L, $CaCO_3$ concentration=6g/L, without biodegradable polymer, spore storage time=320day and shaking speed=170rpm.
B: Pellets: Temperature=27°C, PDB concentration=48g/L, inoculum spore concentration=3x10^9 spore/L, $CaCO_3$ concentration=6g/L, without biodegradable polymer, spore storage time=320day and shaking speed=170rpm.
C: Clump: Temperature=27°C, PDB concentration=24g/L, inoculum spore concentration=1x10^7 spore/L, without $CaCO_3$, without biodegradable polymer, spore storage time=320day and shaking speed=170rpm.
D:Pellets: Temperature=27°C, PDB concentration=48g/L, inoculum spore concentration=3x10^9 spore/L, without $CaCO_3$, without biodegradable polymer, spore storage time=320day and shaking speed=170rpm.

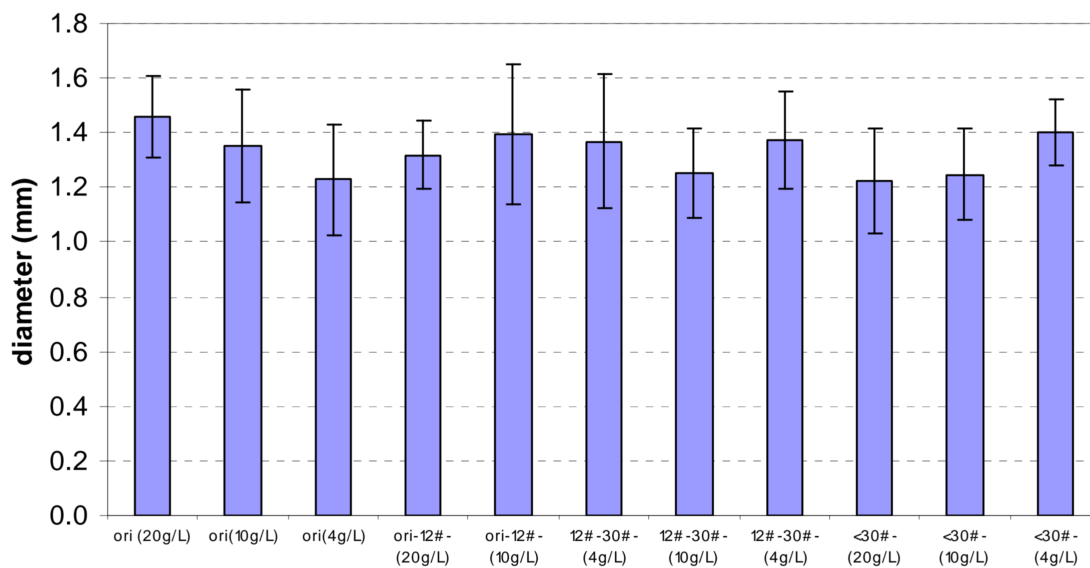


Fig. 2. The effects of polymer on pellet diameter[a]

a: data are presented as the mean of ten replicates with standard deviation.

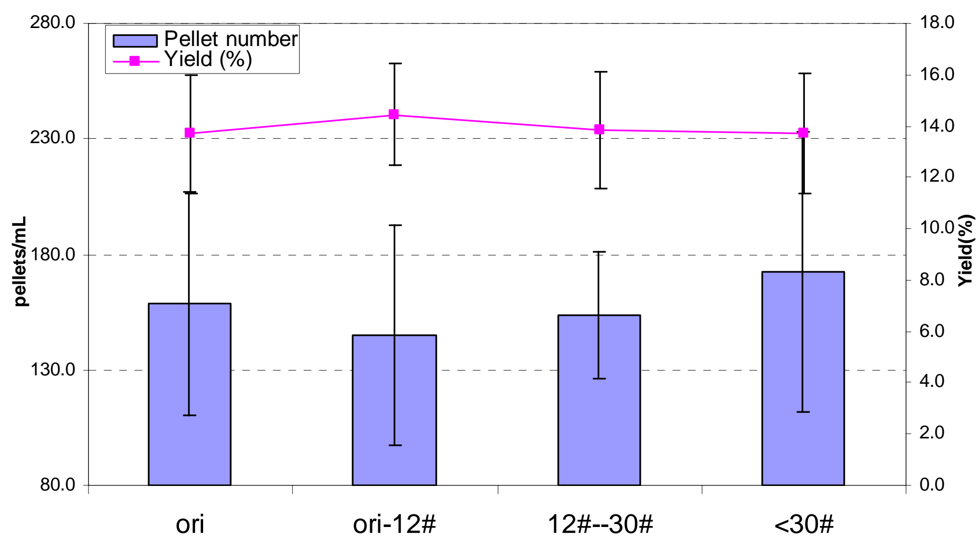


Fig. 3. The effects of polymer size on pellet number and yield [a]

a: data are presented as the mean of three replicates with standard deviation.

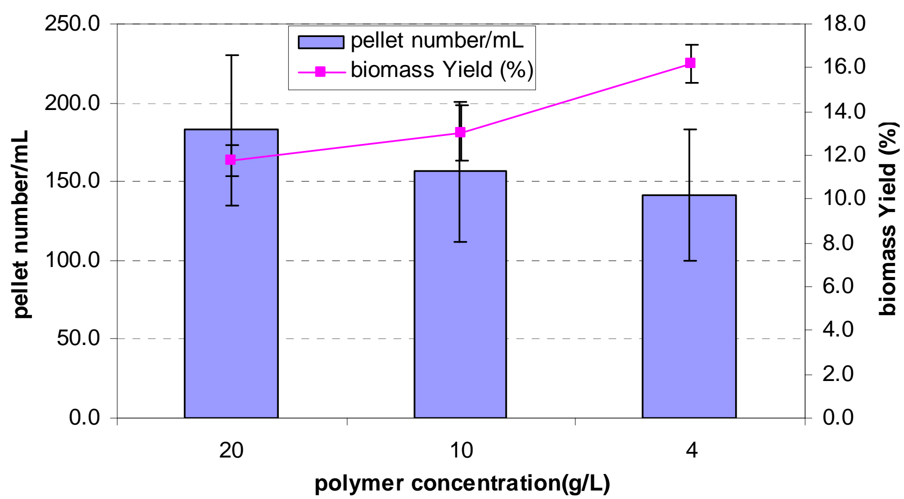
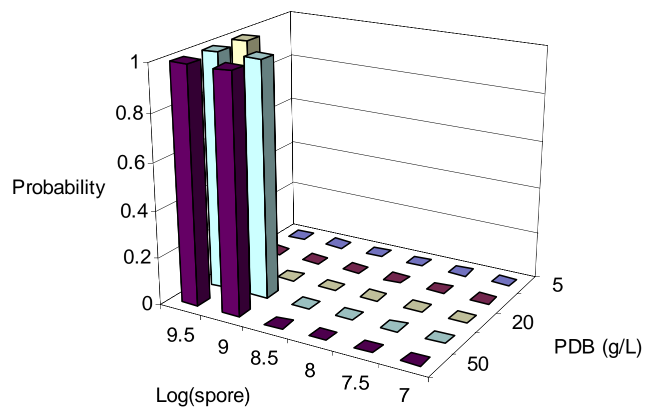
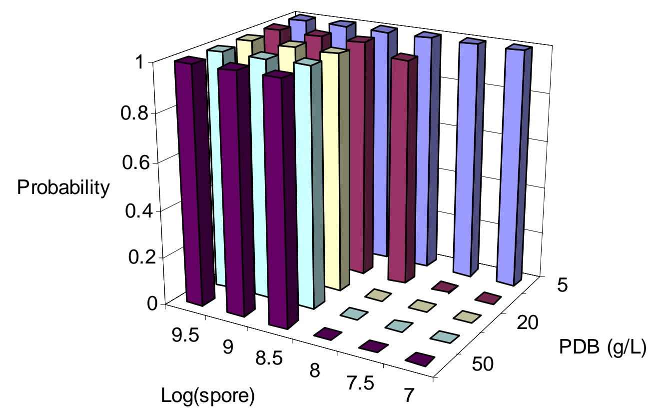


Fig. 4. The effects of polymer concentration on pellet number and yield[a]

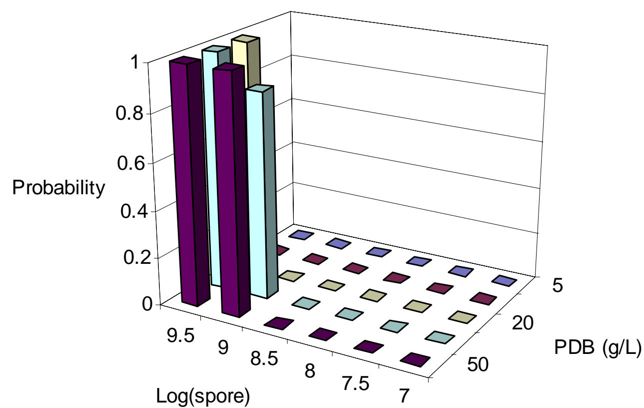
a: data are presented as the mean of three replicates with standard deviation.



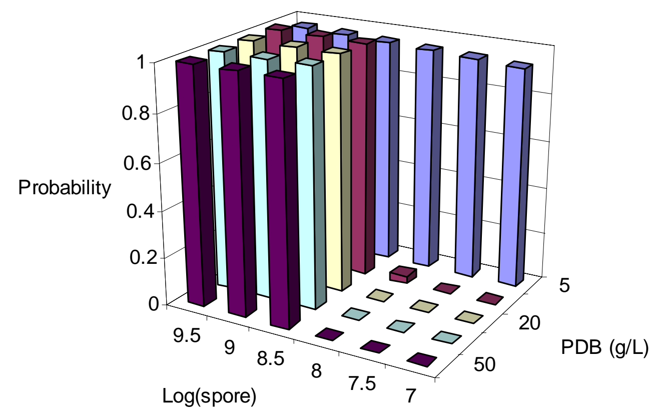
a). With pH control & without polymer



b). With pH control & with polymer

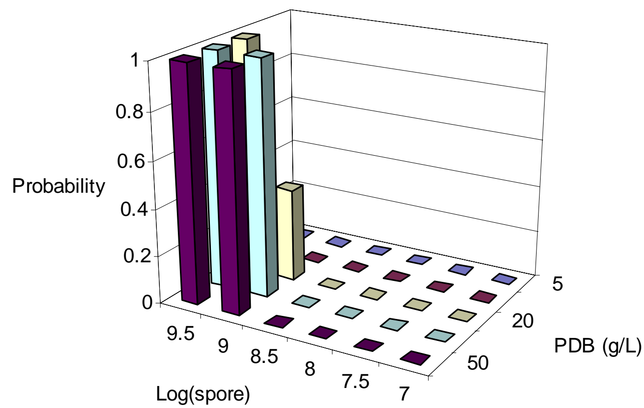


c). Without pH control & without polymer

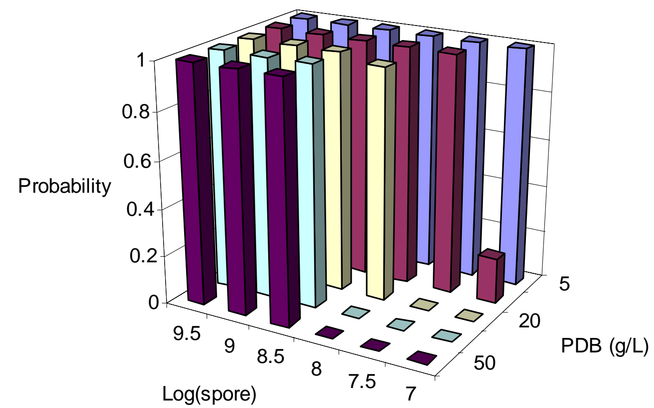


d). Without pH control & with polymer

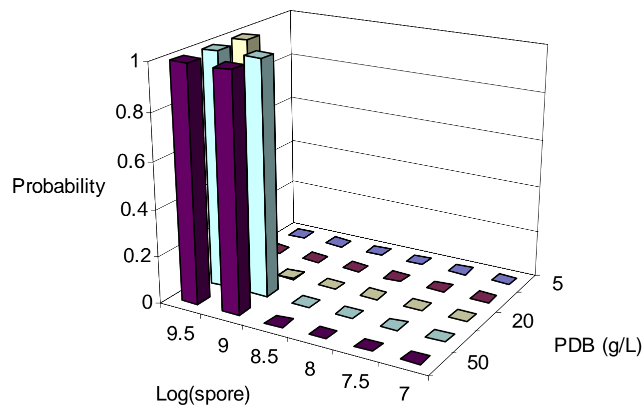
Fig. 5. Probabilities of pellet formation at the storage duration of 1 day[a]
a: Culture temperature was 27 °C, and shake speed was 170 rpm



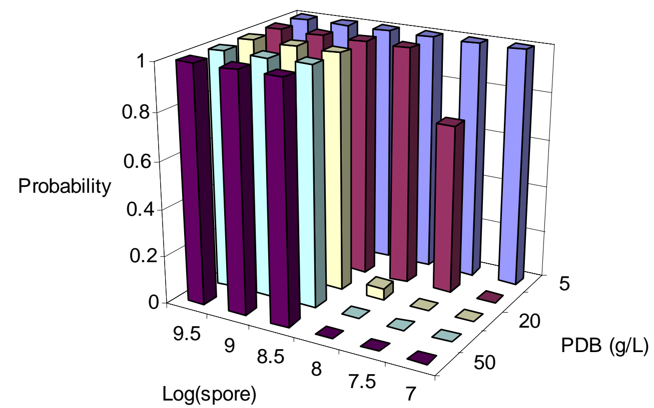
a). With pH control & without polymer



b). With pH control & with polymer



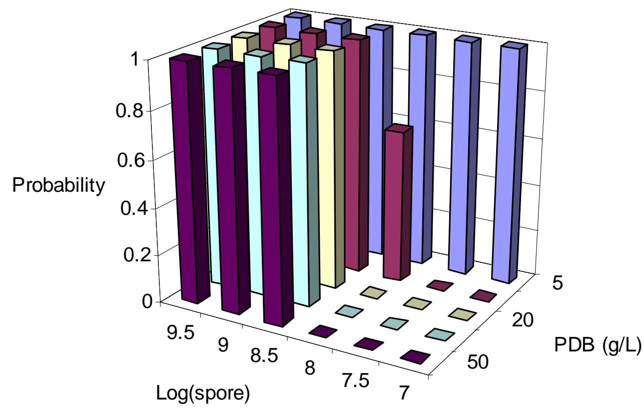
c). Without pH control & without polymer



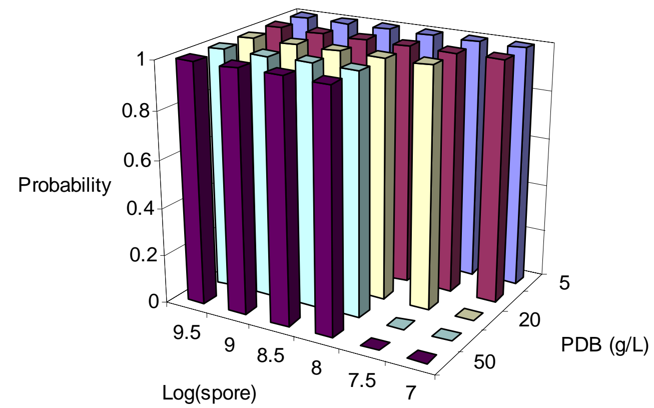
d). Without pH control & with polymer

Fig. 6. Probabilities of pellet formation at the storage duration of 90 day [a]

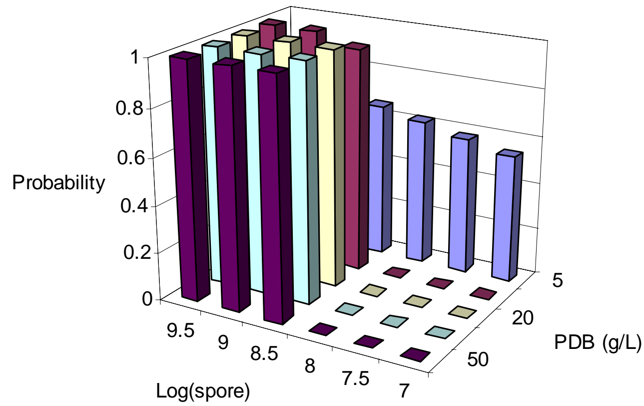
a: Culture temperature was 27 °C, and shake speed was 170 rpm



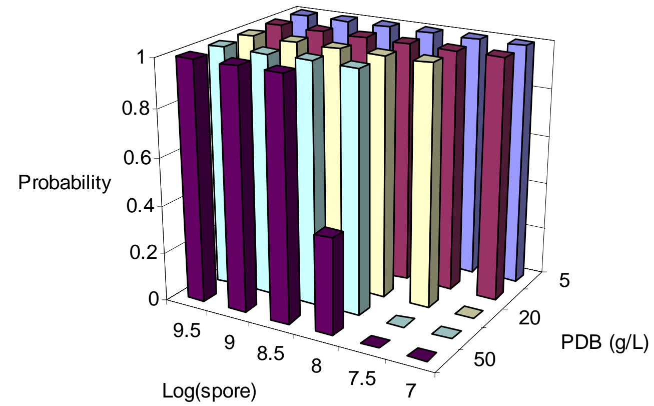
a). With pH control & without polymer



b). With pH control & with polymer



c). Without pH control & without polymer



d). Without pH control & with polymer

Fig. 7. Probabilities of pellet formation at the storage duration of 365 day [a]
a: Culture temperature was 27 °C, and shake speed was 170 rpm

CHAPTER FOUR

OPTIMIZATION OF L-(+)-LACTIC ACID PRODUCTION USING PELLETIZED FILAMENTOUS *RHIZOPUS ORYZAE* NRRL 395

1. Abstract

Lactic acid is used as a food additive for flavor and preservation and in the development of poly-lactic acid, a product used to make biodegradable plastics and textiles. *Rhizopus oryzae* NRRL 395 is known to be a strain that produces optically pure L-(+)-lactic acid. The morphology of *Rhizopus* cultures is complex, forming filamentous, clumps and pellet mycelia. Different morphology growth has significant effects on lactic acid production. In bioreactors, the filamentous or clump mycelia increase the viscosity of the medium, wrap around impellers and block the nutrient transportation, leading to a decrease in production efficiency and bioreactor performance. Growing fungi in pellet form can significantly solve these problems. In this study, factors that affect lactic acid production in pelletized flask cultures using *Rhizopus oryzae* NRRL395 were investigated in detail. Completely randomized designs (CRD) were used to determine the influence of culture temperature, time and concentration of glucose and inoculum size. Lactic acid fermentation using clump and pellet morphologies was performed in a 5L fermentor at the optimal values obtained from flask culture. Finally, fed-batch culture was used to enhance the lactate concentration in broth. The final lactate concentration of fed-batch culture reached 92g/L. The data presented in the paper can provide useful information on optimizing lactic acid production using alternative source materials.

Key words: *Rhizopus oryzae*, lactic acid, pellet morphology

2. Introduction

Lactic acid ($CH_3CHOHCOOH$) is a colorless compound that is important in both international and domestic markets to several industries. Lactic acid is currently widely used as an acidulant, flavorant and preservative in food industries. There is also increased interest in its application in the production of poly (lactic) acid, which can be used in the production of biodegradable plastic. In addition, both the polymers and co-polymers derived from lactic acid are attractive to biomedicine because of their biocompatibility, particularly in regard to body absorption and blood compatibility. These new and growing markets have generated active research interest in enhancing lactic acid production.

Rhizopus oryzae NRRL 395 is a strain that through fermentation produces optically pure L-(+)-lactic acid (Hang, 1989; Soccol, 1994, Oda *et al.*, 2002; Ying, 1997; Liu, et al., 2005). Use of a fungal fermentative process for organic acid or lactic acid production, though, has some disadvantages. These include the production of cotton-like mycelia and mycelia-associated mass transfer and oxygen transfer difficulties which generate reactor control concerns as well as the impossibility for fungal biomass reuse. All of the above factors ultimately lead to a low efficiency and yield of lactic acid in the fermentation process (Yin, 1998). Growing fungi in pellet form, however can significantly improve the mass transfer condition and reactor performance and consequently benefit the lactic acid production.

In this study, optimal conditions for the production of lactic acid with pellets were determined in flask culture, and two different morphologies, clump and pellet, were used to study the effects of morphology on lactic acid production using batch culture and a stirred tank fermentor. After flask scale optimization and batch culturing, enhanced production of lactic acid

was studied using fed-batch culture in a stirred tank fermentor.

3. Methods and Materials

Microorganism and spore culture method

The fungus *Rhizopus oryzae* NRRL 395 (ATCC 9363) was obtained from the American Type Culture Collection (Manassas, VA). The fungus was first grown on potato-dextrose agar (PDA) (Difco, 213400) slants at 30°C for 7 days. For experimentation, the fungal spores in the slant were suspended in sterilized water maintained at 4°C. For storage, the spores were placed in 20% glycerol solution at –80°C.

Seed culture

The composition of the seed medium was 24g/L potato dextrose broth (PDB) (Difco, 254920) with 6g/L $CaCO_3$. In terms of achieving pellet form, the spore solution was inoculated into a 125mL Erlenmeyer flask, containing 50mL seed medium with a spore concentration of $1x10^6$ spore/ml. The cultures were performed at 27°C for 48 hours on an orbital shaker bath (Lab-line Shaker, Model: 3540, Melrose Park, ILL, U.S.A.) at 170 rpm. The culture temperature was fixed at 27°C. The spore storage time was 180 days. The broth was used as the pellet seed for the ensuing experiments. The diameter of seed pellet was 1.13±0.22 mm (Fig. 1). As for the culture of clump seed used in the stirred fermentor comparison experiments, culture conditions except spore concentration ($1x10^4$/ml) were kept the same as the culture of pellet seed.

Effects of glucose concentration, reaction time, and temperature on lactic acid production in flask culture

The experiment was carried out according to a Completely Randomized Design (CRD) with three replicates of 18 culture combinations. Three glucose concentrations (60, 100, and 120 g/L) and three culture durations (2, 3, and 4 days) were studied at two different temperatures (27 and 30°C). The shake speed was 170 rpm. Calcium carbonate as the neutralizer was added into flasks in order to maintain the pH value at around 6 during the culture. The ratio of $CaCO_3$ to glucose was 1:2. The seed for all cultures was inoculated into the flasks at a fixed concentration of 0.45g dry biomass/L. The cultures were performed in 250 ml flasks containing 100 ml of culture medium. The culture medium with varied glucose concentration (60, 100, and 120 g/L) was obtained by adding a different amount of solid glucose into the PDB medium (PDB medium included 20g/L glucose). All media were autoclaved at 121ºC for 15min prior to inoculation.

Effects of inoculum size on lactic acid production in flask culture

Four seed concentrations (0.13, 0.23, 0.45 and 0.68 g dry biomass/L) were inoculated to the media which contained 24g/L PDB, 100 g/L of glucose and 60 g/L of $CaCO_3$. The cultures were performed at 27°C for 4 days using an orbital shaker (Classic Series C24 Incubator shaker, New Brunswick Scientific, Edison, NJ, U.S.A.) at 170 rpm. Other culture conditions were the same as described in the previous section.

Lactic acid production in stirred fermentor

A 7-L (5.6 L effective volume) stirred tank fermentor (Bioflo 110 Modular Benchtop Fermentor, New Brunswick Scientific) equipped with a pH controller was used to carry out lactic

acid fermentation. For batch culture, two liters of DI water, 48g PDB, and 200g glucose were added into the fermentor and autoclaved at 121ºC for 15min. The aeration rate and agitation speed were 1 VVM and 200rpm, respectively. The pH was adjusted to 7.0 ± 0.1 using 20% $Ca(OH)_2$. Two different seeds of clump and pellet were used to study the effects of morphology on lactic acid production. 0.23 g dry biomass/L was inoculated into the fermentor and cultured at 27ºC for 3 days. For fed-batch fermentation, culture conditions were the same as in the batch fermentation except that the inoculum size was 0.45 g dry biomass/L, and an extra 150mL medium with 80g glucose and 12 g PDB powder was fed into the fermentation on day 2.5 and 3.5 of a 5.5 day run.

Statistical analysis

The effects of glucose concentration, reaction time, temperature, and inoculum size on lactic acid production in flask culture were analyzed by General Lineal Model (GLM) using the Statistical Analysis System program 8.0 (SAS institute Inc., NC). Pair wise comparison and Tukey-Kramer multiple comparison were conducted to identify the difference of lactic acid production from different culture combinations.

Analytical methods

A high-performance anion-exchange chromatography apparatus was used for the analyses. The lactate in broth was analyzed using a Dionex DX-500 system (Sunnyvale, CA) including an AS11-HC (4mm 10-32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler (Liu et al., 2005). Glucose concentration was measured using the modified dinitrosalicylic acid (DNS) method (Miller, 1958). Dry biomass

was determined by washing the pellet mycelia with 6N HCl and then washing to pH 6 with DI water. The washed biomass was dried at 100°C overnight before weight analysis. The diameter of seed pellets was determined using an Olympus microphotograph (Tokyo, Japan).

4. Results and Discussion

Effects of substrate concentration, temperature and fermentation time for lactic acid production by pelletized *R. oryzae* NRRL 395 in flask culture

The production of lactic acid by pelletized *R. oryzae* with different medium glucose concentration at 27ºC and 30ºC is shown in Figs 2, 3 and 4. At the culture duration of 2 days, lower glucose concentrations had higher lactate yields with the lowest glucose concentration of 60g/L glucose, producing 33 g/L lactate or 55% yield at both culture temperatures of 27ºC and 30ºC. Although producing lower yields, the 100 g/L and 120 g/L glucose concentrations at 30ºC produced more lactate than at 27ºC (Figs. 2, 3). As culture duration increased past 2 days, the lactate yields from both the 100 g/L glucose and 120 g/L glucose concentrations increased while the yields from 60g/L glucose kept stable at about 55% (Figs. 2, 3). After 3 days of culture duration, the statistical analysis of pair wise comparison showed that there were no significant ($P>0.05$) differences on lactate yields between each of the three glucose concentrations (Figs. 2, 3). In addition there were also no significant ($P>0.05$) differences between culture temperatures of 27ºC and 30ºC at culture durations of more than 3 days (Figs. 2, 3). The highest lactate yield of 60% was reached at 4 days of culture time no matter what the glucose concentration and culture temperature were. Interpretation of the following data: (1) the influence of temperature on lactate yield was diminished once the culture duration was increased although it had significant ($P>0.05$) influences at low glucose concentration in the short culture duration of 2 and

3 days; (2) glucose concentration had no significant influence on lactate yield at long culture durations; (3) while the higher glucose concentration produced more lactate at the same yield, led a highest lactate concentration of 72 g/L obtained at 4 days of culture duration with 120 g/L glucose concentration in the medium (Fig. 4). As a result, the following optimized choices for glucose concentration, temperature, and reaction time were used in later studies: 120 g/L glucose concentration and 4 days of fermentation at either a temperature of 27ºC or 30ºC.

Effects of inoculum size on lactic acid production in flask culture

Fig. 5 and Table 1 show that the lactate concentration varied with inoculum size. The inoclum size of 0.68 g/L dry biomass produced significantly less lactate than the other inoculum sizes, achieving the studies' lowest level of 37.3 g/L at 4 days of culture duration (Fig. 5). Meanwhile, the other three inoculums reached average lactate concentration of 78.5 g/L during the same culture duration. The Tukey-Kramer Multiple Comparison test confirmed that there was no significant ($P>0.05$) difference in lactate concentration between each of the inoculum sizes of 0.13, 0.23, and 0.45 g/L dry biomass (Table 1). This means that inoculum size between 0.13~0.45 g/L dry biomass had no significant influences on lactic acid production. Therefore, inoculum sizes in the range of 0.13~0.45 g/L dry biomass were the optimal ones for flask culture of lactic acid.

Comparison of lactic acid production using different fungal morphologies in a stirred tank batch culture

Lactic acid fermentation comparing clump and pellet morphologies was performed in a 5L stirred fermentor (Fig. 6). The results demonstrated that there was a significant ($P<0.05$)

difference on lactic acid production between clump and pellet morphologies. The lactate concentration of clump fermentation reached 33 g/L in 2.5 days of culture duration while the pellet fermentation produced 60 g/L. The data indicated that the lactic acid production was significantly increased using pelletized fungal fermentation.

Enhancing lactic acid production by fed-batch culture in a stirred tank fermentor

In order to improve lactic acid production, a fed-batch culture was performed under the optimal conditions obtained from the previous flask culture work. Fig. 7 shows that lactate concentration from batch fermentation reached 50 g/L in 2 days. Two extra glucose solutions were then fed into the fermentor at 2.5 and 3.5 days, respectively. After the feedings the lactate concentration eventually reached 92 g/L in 5.5 days. The data also presented differences in production rate at different phases of the batch and fed-batch fermentations. The production rate after feeding was 0.53 $g \cdot L^{-1} \cdot h^{-1}$, which was much slower than 1.02 $g \cdot L^{-1} \cdot h^{-1}$ before feeding (Fig. 7). The low production rate was mainly caused by the formation of calcium lactate crystals. During the fermentation process, calcium lactate started crystallizing out once the lactate concentration reached 70 g/L, and calcium lactate crystals were then attached on the surface of pellets, influencing the mass/oxygen transfer though pellets and further reducing the production rate.

There is product inhibition and substrate limitation in fermentation processes and in this particular case, it was apparent that both substrate limitation and product inhibition played equally important roles on lactate production. Fed-batch fermentation can only minimize the substrate limitation, but it has nothing to do with production inhibition. In terms of further improving the fermentation performance, the product inhibition of calcium lactate crystals has to

be eliminated. Other alkali such as sodium hydroxide and ammonia which produce soluble lactate during the fermentation process could be good alternatives. However, high concentration of those cations could inhibit the lactic acid production. Thus, a mixture of alkali such as calcium hydroxide, sodium hydroxide, and ammonia could be used as a neutralizer to control pH in fed-batch culture, which will result in a low concentration of each cation and a high concentration of soluble lactate.

Comparison of different processes of lactic acid production using *R. oryzae*

Several cell immobilization methods have been developed to control fungal morphology to achieve higher content of fungal biomass and eliminate mass transfer limitations inside the fungal mycelia to increase the total amount of final product and its productivity (Table 2). In these studies, fungal mycelia were either entrapped in a polymeric matrix or attached to a support surface and the performances were apparently improved. The best results obtained in these studies are: 126 g/L for final lactate concentration in broth, 2.5 g/L/h for productivity and 90% for lactic acid yield (Tay et al., 2002). The fungal cell was immobilized on a cotton cloth and maintained at high DO levels of 90%. The immobilized cell in a fluidized bed reactor reached a higher productivity of 1.6 g/L/h (Hamamci et al., 1994). However, the systems with cell immobilization will add extra cost on lactic acid production. Thus, if the cell can directly form small pellets, the operation would be both more efficient and more economical. To that end, Yin et al. (1998) formed small pellets in an air-lift fermentor and generated lactic acid concentrations of 86 g/L (Table 2). Compared to the pellet fermentation of the air-lift system, our submerged pellet fermentation achieved a higher lactate concentration of 92 g/L. In addition, the average

productivity and yield of the submerged pellet fermentation were 0.7 g/L/h and 60%, respectively.

5. Conclusion

Pelletized morphology significantly increased lactic acid production, almost doubling the production rate produced by clump morphology under similar optimized conditions. The optimal fermentation conditions of pelletized flask culture with *Rhizopus oryzae* NRRL 395 were 120 g/L of glucose concentration, 0.13~0.45 g/L dry biomass inoculum size, 27-30ºC culture temperature and 3-4 days fermentation time. Under these conditions, the lactic acid productivity, yield and lactate concentration reached 1.1 g/L/hr, 63% and 76 g/L, respectively. Feb-batch culture can significantly increase the lactate concentration in the broth. The final lactate concentration reached 92 g/L after 5.5 days of fermentation. To enhance lactic acid concentration, productivity, and yield in broth, though, future work should focus on the fed-batch fermentation using mixed alkali to adjust pH.

6. References

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Table 1. Turkey-Kramer multiple-comparison test of lactate concentration[a]

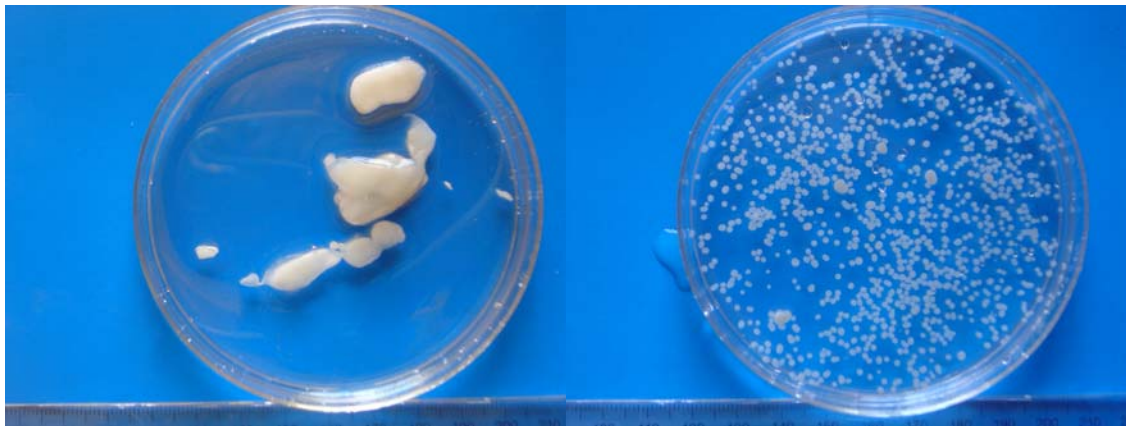
Group of Inoculum size (g/L dry biomass)	Mean of lactate concentration (g/L)	Different from groups
0.68	37.3	0.45, 0.23, 0.13
0.45	76.3	0.68
0.23	79.5	0.68
0.13	80.6	0.68

a: This report provides multiple comparison tests for all pair wise differences between the means at alpha equal to 0.05. Fermentation was performed for four days at 27ºC with 120g/L glucose concentration.

Table 2. Comparison of different processes of lactic acid production using *R. oryzae*[a]

Fermentation conditions[a]	Reactor	Lactate (g/L)	Yield (g/100g glucose)	Productivity (g/L/h)	Reference
Cells immobilized on cotton cloth	Rotating bed	126	90	2.5	Tay and Yang, 2002
pellets	Air-lift	86	-	1.07	Yin et al., 1998
Immobilized cells	Fluidized bed	73	65	1.6	Hamamci and Ryu,1994
Pellets	Flask	76~80	63~67	1.1	This study
Pellet (batch)	Stirred tank	60	66	1	This study
Pellets (Fed batch)	Stirred tank	92	60	0.7(average)	This study

a: Glucose was used as substrate for all fermentations.



(A) (B)

Fig. 1. The morphology difference of pellet and clump from seed culture

A is the seed pellets; B is the seed clumps.

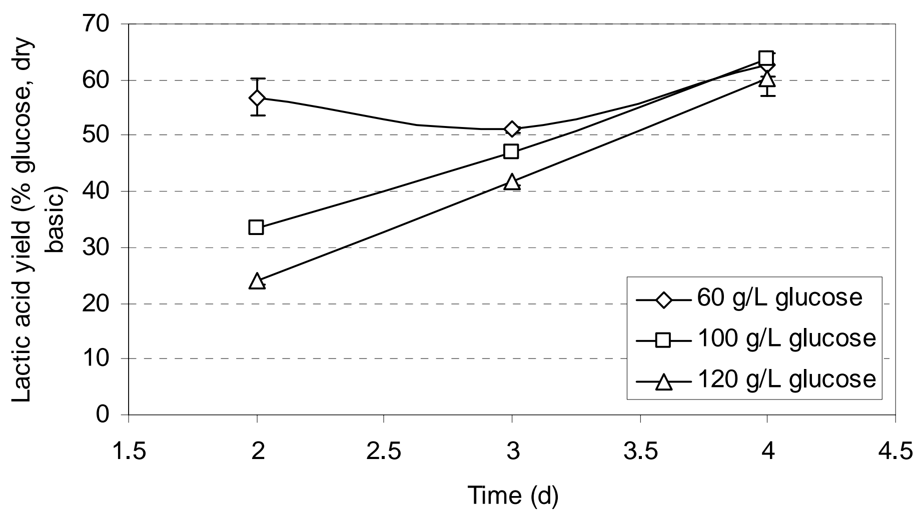


Fig. 2. Effects of glucose concentration on lactic acid yield

Fermentation was performed at 27ºC with 0.45g/L dry biomass inoculum size. Data was the average of triplicates with standard deviations (n=3)

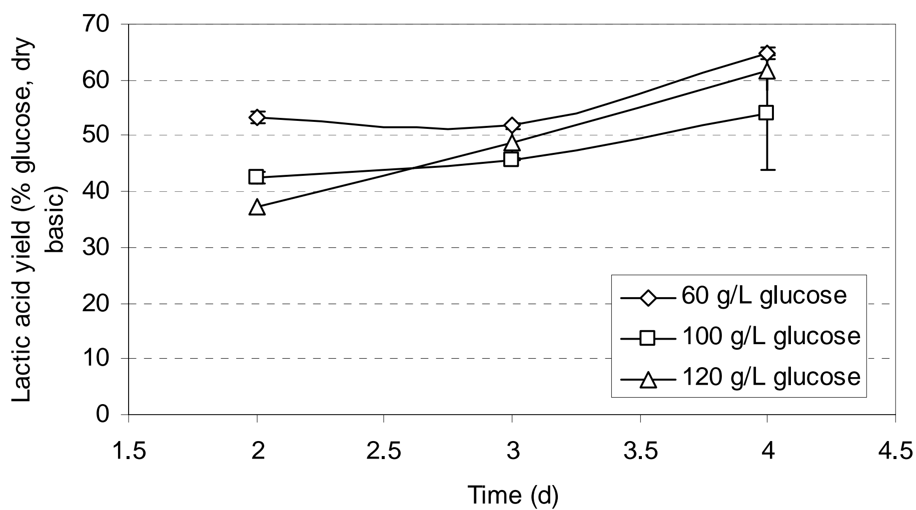


Fig. 3. Effects of glucose concentration on lactic acid yield

Fermentation was performed at 30ºC with 0.45g/L dry biomass inoculum size. Data was the average of triplicates with standard deviations (n=3)

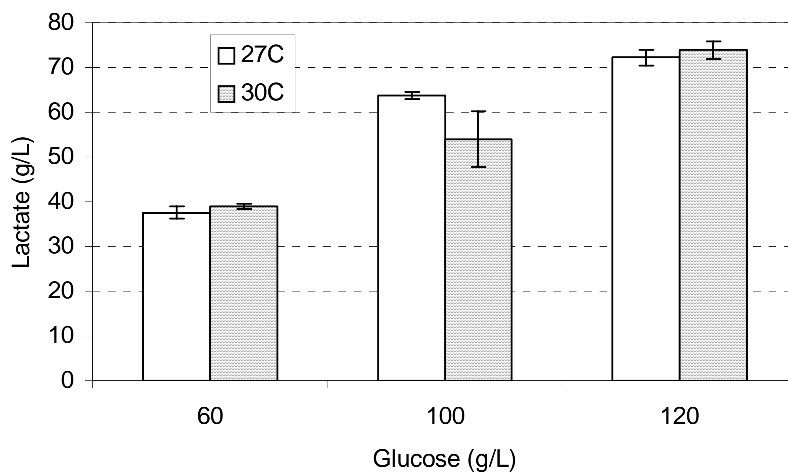


Fig. 4. Effects of temperature on lactate production

Fermentation was cultured for four days with 0.45g/L dry biomass inoculum size. Data was the average of triplicates with standard deviations (n=3)

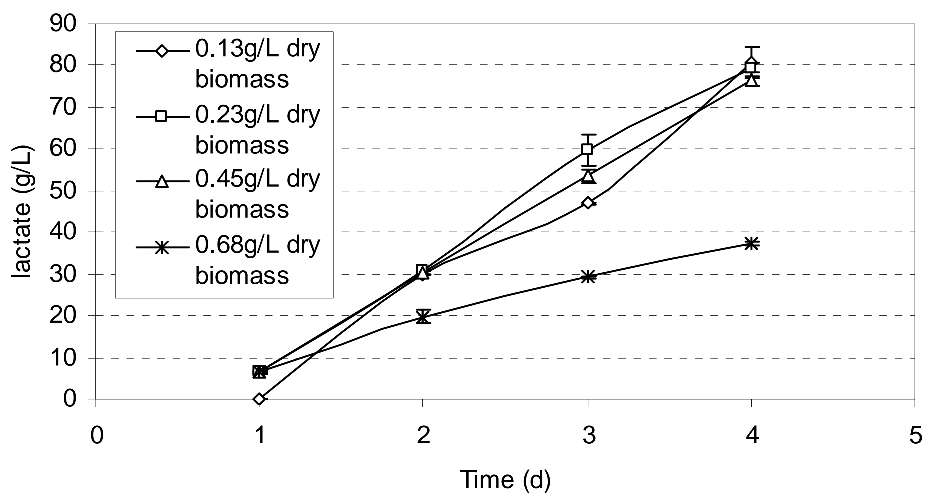


Fig. 5. Effects of inoculum size on lactic acid production

Fermentation was performed at 27ºC with 120g/L glucose in medium. Data was the average of triplicates with standard deviations (n=3)

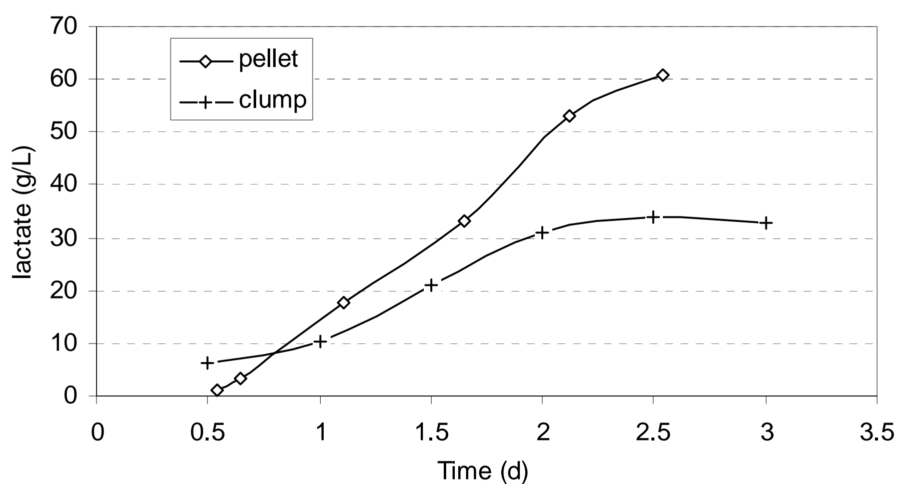


Fig. 6. Batch culture of lactic acid production using pellet and clump morphology in a 5L-stirred fermentor

The aeration rate and agitation speed were 1vvm and 200rpm, respectively. The pH was adjusted at 7.0±0.1 with 20% Ca $(OH)_2$. 0.23g/L dry biomass seed was inoculated into fermentor and cultured at 27ºC.

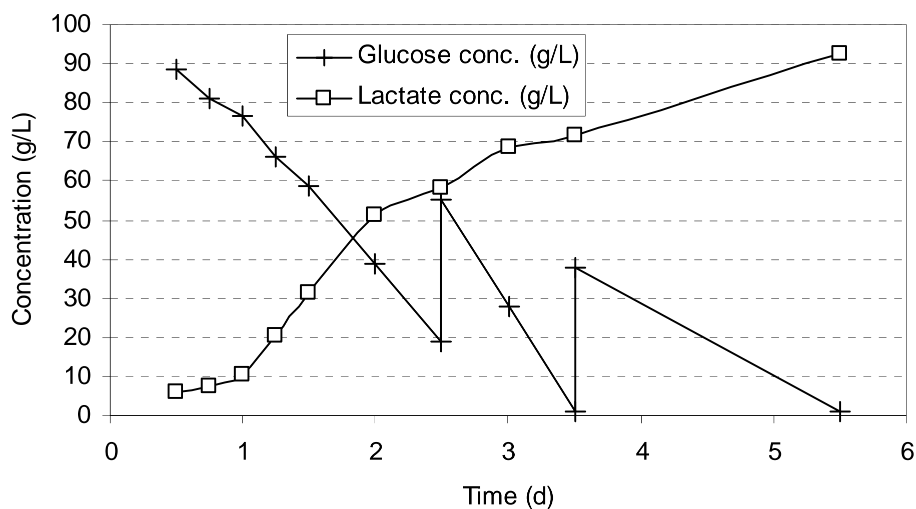


Fig. 7. Lactic acid production by fed-batch culture of *R oryzae* NRRL 395 in a 5L-stirred fermentor

The aeration rate and agitation speed were 1vvm and 200rpm, respectively. The pH was adjusted at 7.0±0.1 with 20% $Ca(OH)_2$. 0.45g/L dry biomass seed was inoculated into fermentor and cultured at 27°C for 5.5days. 150mL fed-medium with 80g glucose and 12 g PDB powder was added at day 2.5 and day 3.5.

CHAPTER FIVE

CO-PRODUCTION OF LACTIC ACID AND CHITIN USING A PELLETIZED FILAMENTOUS FUNGUS *RHIZOPUS ORYZAE* CULTURED ON CULL POTATOES AND GLUCOSE

1. Abstract

This paper developed a novel process for lactic acid and chitin co-production of the pelletized *Rhzious oryzae* NRRL 395 fermentation using underutilized cull potatoes and glucose as nutrient source. The biomass and chitin production was first optimized using different potato hydrolysate medium in cultivation process. Whole potato hydrolysate medium produced the highest pelletized biomass yield accompanying the highest chitin content in biomass. An enhanced lactic acid production then followed up using batch, repeated batch and fed batch culture with glucose as sole nutrient. The lactic acid productivity peaked at 2.8 and 3 g/L/h in repeated batch culture and batch culture, respectively. The highest lactic acid yield of 86% was obtained in fed batch and repeated batch culture. The fed batch culture had the highest lactate concentration of 138 g/L.

Key words: batch culture, biomass cultivation, chitin, fed batch culture, fungal pellet, lactic acid, repeated batch culture, *Rhizopous oryzae*

2. Introduction

Lactic acid ($CH_3CHOHCOOH$) is a colorless compound that is important in both international and domestic markets for several industries. Lactic acid is widely used as an

acidulant, flavoring and preservative in food industry. There is also an increased interest in its application in the production of polylactic acid, which can be used in the production of biodegradable plastics (Tsao et al., 1999). Lactic acid from biological sources can be produced by both bacteria and fungi fermentation. Generally, bacterial fermentation has a higher yield (Litchfield, 1996). However, fungi, such as *Rhizopus oryzae* have been proven to be a good lactic acid producer and have several advantages compared with bacteria fermentation. These include: (1) it is more tolerant to a low pH environment; (2) the fungal biomass is relatively easy to separate from broth, and thus, facilitates the downstream process, and (3) *Rhizopus* mold has lower nutrition requirements which reduced the fermentation cost and simplifies downstream product separation (Tsao et al., 1999; Magnuson et al., 2004).

When lactic acid is produced using the fungal strain *Rhizopus oryzae*, a considerable amount of fungal biomass, whose cellular wall is primarily composed of chitin, was concurrently produced. Chitin, as one of polysaccharides, is the second most abundant biopolymer after cellulose in the biosphere. The structure of chitin is a linear polysaccharide of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranose, where each individual residue is N-acetyl-D-glucosamine. The structure characteristics make chitin a very important biopolymer. It is used as a coagulating agent in water treatment, plant seed coating agents in agriculture and biomaterials (e.g., absorbable sutures) in biomedical industry (Yusof et al., 2001; Khor, 2001).

However, there is an inherent challenge in the fermentative organic acid production with filamentous fungi. The fungi tend to form cotton-like mycelia which limited the mass transfer of oxygen and nutrients onto the microorganisms and the release

of the produced organic acids into bulk solutions. These factors ultimately lead to a low yield and productivity of organic acids in fungi fermentation. Growing fungi in pellet form can alleviate these problems. The pellet formation of the fungus *Rhizopus oryzae* NRRL 395 has been explored in a previous study (Liu et al., 2005).

It has been reported that the growth and metabolism of *Rhizopus oryzae* is limited by nitrogen (Foster et al., 1939a; Rhodes et al., 1959; Rhodes et al., 1962). Too much nitrogen in the broth leads to a faster fungal growth than organic acid production. Therefore, in terms of improving fermentation performance, the lactic acid fermentation processes can be divided into three steps: 1) pelletized seed culture; 2) fungal biomass cultivation with nitrogen; and 3) lactic acid production with less nitrogen or no nitrogen (Kenealy et al., 1986; Zhou et al., 2002; Romano et al., 1967). The process of lactic acid production provides a possibility to simultaneously produce lactic acid and chitin using potato hydrolysate and glucose. For example potato hydrolysate contains 100 g/L glucose and 10 g/L crude protein, which represents not only a carbon source but also a good nitrogen source for microorganisms. This means that potato hydrolysate can be used as a nitrogen source to grow fungal biomass and for chitin accumulation. Then the fungal biomass is transferred to a sole carbon source (glucose) broth to produce lactic acid.

The overall goal of this study was to develop a novel process of lactic acid and chitin co-production using pelletized *Rhizopus oryzae* NRRL 395 to improve both fermentation yield and productivity on potato hydrolysate and glucose as nutrient source. The specific objectives were to: (1) determine the influence of nitrogen and carbon concentration on lactic acid production and chitin content in biomass, (2) investigate the influence of potato hydrolysate on lactic acid and biomass production in cultivation

process, and (3) enhance the lactic acid production by increasing the biomass concentration, depriving nitrogen source in the broth of batch, repeated batch, and fed-batch fermentations.

3. Methods and Materials

Material and preparation

Three kilograms of fresh cull potatoes were boiled and mashed before adding 0.3 % α-amylase (A7595-250ml, Sigma_Aldrich Corp., St. Louis, MO) to liquefy the starch at 75ºC for one hour. Then 0.1% glucoamylase (A7255-100G, Sigma-Aldrich Corp., St. Louis, MO) was added and reacted at 55ºC until the reducing sugar content of hydrolysate (detected by DNS method) no longer increased within two hours. After solid and liquid separation, the liquid hydrolysate was used to prepare a cultivation medium. The liquid hydrolysate contained 100 ±20 g/L of glucose and 9.4 ±1.0g/L of crude protein.

Microorganism and spore culture method

The fungus *Rhizopus oryzae* NRRL 395 was obtained from the American Type Culture Collection (Manassas, VA). The fungus was first grown on potato-dextrose agar (PDA) (Difco, 213400, Sparks, MD) slants at 30°C for 7 days. For experimentation, the fungal spores in the slant were suspended in sterilized water maintained at 4^oC. For storage, the spores were placed in a 20% glycerol solution at –80°C. Pelletized seed was cultured on the medium with 24g/L potato dextrose broth (PDB) (Difco, 254920, Sparks, MD) mixed with 6g/L $CaCO_3$. In terms of achieving pellet form, the spore solution was

inoculated in a 125ml Erlenmeyer flask, containing 50ml of seed medium with a spore concentration of $1x10^6$ spore/ml, and cultured at 27ºC on a orbital shaker bath (Lab-line Shaker, Model: 3540, Melrose Park, ILL, U.S.A.) set at 170 rpm for one day. The culture temperature was fixed at 27°C. The spore storage time was 90 days. The resulting broth was used as the pellet seed for the ensuing experiments. The diameter of seed pellet was 1.13±0.22 mm.

Biomass cultivation

A Completely Randomized Design (CDR) with three replicates of six cultures was used to study the effects of the potato hydrolystate content on fungal biomass and chitin during cultivation. Six potato hydrolysate contents (0, 10, 25, 50, 75, 100 %) were studied. For each individual potato hydrolysate content medium, additional glucose was added making the total glucose concentration 120g/L. Calcium carbonate was used to keep the pH at 5. The inoculum for all cultures was fixed at 0.12 g dry pellet biomass/L. The cultures were processed for 2 days in 250 ml flasks containing 100 ml medium at 27°C using an orbital shaker (Classic Series C24 Incubator shaker, New Brunswick Scientific, Edison, NJ, U.S.A.) at 170 rpm.

Lactic acid production

Effects of pellet biomass concentration on lactic acid production using glucose as a carbon source

Two replicates of five cultivated biomass concentrations (1.73, 4.09, 6.47, 13.90, and 17.30 g dry biomass/L) were inoculated onto the media which contained only 100

g/L of glucose. Calcium carbonate (35 g/L) was added as the neutralizer to maintain the pH around 5.5. The cultures were processed for 2 to 4 days in 250 ml flasks containing 100 ml medium at 27°C using an orbital shaker (Classic Series C24 Incubator shaker, New Brunswick Scientific, Edison, NJ) at 170 rpm.

Repeated batch fermentation

The initial culture conditions were the same as described in the previous section. The inoculum size was 13.7g/L pelletized fungal biomass. The cultures were processed in 1000 ml flasks containing 300 ml of the medium. The strategy of repeated batch culture was: using the same fungal biomass, separating the product solution and adding the fresh culture medium for each subsequent batch. Two replicates were carried out for this experiment. The initial glucose concentration for the first batch was 80 g/L, and the inoculum was 13.7 g/L pelletized biomass. At the end of each batch, the pellet fungal biomass was sterilely separated from the medium solution by a No. 40 standard screen; and then a 300 ml fresh medium solution with a sole carbon source of 100 g/L glucose was added to the flask to create the next batch of culture.

Fed batch culture

The pelletized fungal biomass was cultured using the same method as described in previous section. The biomass of 13.9 g/L was inoculated into 1 L fermentor (Bioflo 110 Modular Benchtop Fermentor, New Brunswick Scientific, NJ) containing 300 ml production medium that included 100g/L glucose and 35 g/L $CaCO_3$. The fermentation was carried out at 27°C with 200 rpm agitation speed and 1vvm aeration rate. An extra

100mL medium with the glucose concentration of 600g/L was fed into the fermentation at the culture duration of 30 hours. During the stage of fed-batch fermentation, sodium hydroxide solution (25% NaOH), instead of calcium carbonate, was used as the buffer to maintain the broth pH at 5.0.

Statistical analysis

A pair wise comparison using the Statistical Analysis System program 8.0 (SAS institute Inc. NC) was conducted to identify the effects of potato hydrolysate on fungal biomass and chitin content during cultivation. The effects of pellet biomass concentration and repeated batch on lactic acid production were analyzed using the pair wise comparison as well.

Analytical methods

The lactate in the broth was analyzed using a Dionex DX-500 system (Sunnyvale, CA) (Liu, 2005). Dry biomass was determined by washing the pellet mycelia with 6N HCl and then washing to a pH 6 with DI water. The washed biomass was dried at 100°C overnight before weighing. The diameter of seed pellets was determined using an Olympus microphotograph system (Tokyo, Japan).

N-Acetyl-D-glucosamine (NAG), as the major component of chitin, was used to express the total amount of chitin in the biomass. A modified analysis method of hydrochloric acid hydrolysis (Muzzarelli et al., 1985) was used. A 0.25g dry biomass mixed with 25 ml of 6 N HCl solution was hydrolyzed at 100°C for 4 hours. The hydrolysate was neutralized by 1 N NaOH to a pH of around 5, and the NAG

concentration in the hydrolysate was measured using a Dionex DX-500 system coupled with a CarboPac PA 10 guard (4x50 mm) and analytical (4x250 mm) columns and a ED40 detector (Sunnyvale, CA). The running eluent was 18 mmol NaOH, and the washing eluent was 200 mmol NaOH.

4. Results and Discussion

Effects of potato hydrolysate on fungal biomass cultivation and chitin accumulation

Different percentages of potato hydrolysate had significant ($P<0.05$) influence on fungal biomass cultivation (Fig. 1). The data show that both total biomass and chitin content decreased following a decrease in the percentage of potato hydrolysate (Fig. 1, Table 1). The cultivation on 100% potato hydrolysate had the highest fungal biomass at 11 g/L with respect to the highest chitin content of 0.25 g/g biomass (Table 1) and a lactate concentration of 30 g/L (Fig. 2). It has been reported that nitrogen had a positive effect on chitin accumulation during fungal growth (Arcidiacono et al., 1992.). Since the potato hydrolysate contained 9.4 g/L of protein, most of it being proteins and amino acids, which can be well utilized by the fungus to increase both biomass and chitin content. Thus, the higher the protein concentration was in the medium, the more biomass and chitin produced from the culture.

In addition, the lactate concentrations obtained from the cultures, produced from the different percentages of potato hydrolysate, demonstrate the effects of potato hydrolysate on lactate production during the cultivation. The lactate concentration first increased following a decrease in the percentage of potato hydrolysate. It reached the highest value of 69 g/L in three days culture at the 25% of potato hydolysate. Then the

lactate concentration leveled off following the further decrease of the percentage of potato hydrolysate. This indicates that protein influenced the lactate production by controlling the biomass growth. A large amount of protein in the medium was favorable to biomass growth but correspondingly it inhibited the lactate production, while less protein content leads to less biomass growth which also caused the reduction of lactate production. Thus, in terms of cultivation which is to produce more chitin with less or no lactate production, 100% potato hydrolysate was chosen for fungal biomass cultivation. At the 100% potato hydrolysate concentration, the cultivation produced 11 g/L of fungal biomass and 0.25g/g of chitin along with 6000 pellets/L with an average pellet diameter of 2.9 mm.

Lactic acid production

Effects of biomass concentration on lactic acid production

Fig. 3 shows that in the lactic acid production step, the lactate concentration varied with different biomass concentrations. The lactate production increased following the increase of initial biomass concentration. The lactate concentration reached 58 g/L at an initial biomass concentration of 17.3 g/L in 17 hours of culture, which is much higher than 35 g/L from initial biomass concentration of 1.7 g/L in 52 hours (Fig. 3). Meanwhile, the data for lactic acid productivity further demonstrate that the production rapidly increased from 0.8 g/L/h to 2 g/L/h with respect to the change of biomass concentration from 1.7 g/L to 3.9 g/L (Fig. 4). And then production further increased following the increase of biomass concentration (Fig. 4). It reached 3 g/L/hr when the biomass concentration was increased to 17.3 g/L. In addition, the data also shows that the

fungal biomass had no significant growth without a nitrogen source during the lactic acid production step (Table 2). These results demonstrate that the maintenance of fungal metabolism does not require a nitrogen source and the lactic acid production was mainly influenced by the total amount of fungal biomass in the broth. Thus, in terms of both lactate productivity and final lactic acid concentration, the data indicate that the more pelletized fungal biomass was inoculated in the culture, the higher lactate productivity and concentration could be reached. However, the high biomass concentration means a high pellet density, which could cause problems with mass and oxygen transfer and further make it difficult to maintain the high lactate productivity and increased lactic acid production. Thus, in this study, the pelletized fungal biomass of 13.9 g/L was selected as the operational inoculum for the next step in lactic acid production.

Lactic acid production with repeated batch culture

Developing an efficient lactic acid production system is one of targets of this study. The pellet morphology makes it possible to perform repeated batch culture to enhance the fermentation efficiency. Meanwhile, repeated batch culture has the advantages of high productivity and a low possibility of contamination as well (Yin et al., 1998). In this system, fermentation broth was pumped out at the end of each batch, and then fresh medium is added to the fermentor to begin the next batch. This was repeated continuously until lactic acid production began to decrease significantly. Lactic acid will be separated from the broth and chitin will be extracted from fungal biomass. Therefore, high yield/productivity of lactic acid and chitin can be achieved.

Fig. 5 shows the fluctuations of lactate and glucose during fermentation over nine repeated batches. The lactate concentration increased following the increase of repeated batches at the first 3 batches (Fig. 5a). The average lactate concentration of batch 3-6 was 70 g/L. The statistical analysis of pair wise comparison demonstrate that there were no significant ($P>0.05$) differences in lactate concentration from batch 3 to batch 6. After the sixth batch, the lactate concentration rapidly decreased during the last three batches. The lactate generated from the ninth batch was only 40 g/L. The effects of repeated batches on the productivity and lactate yield are illustrated in Fig. 6 and 7. The lactate production during the first 7 batches was higher than 2.0 g/L/h. The trend of productivity during the repeated batch fermentation was: the lactate productivity slightly, but significantly ($P<0.05$), increased in the first 3 batches; it reached the highest productivity of 2.9 g/L/h at batch 4; and then the productivity decreased in subsequent batches; at batch 9 the productivity dropped to 1.2 g/L/h. The lactate yields during the repeated batch fermentation present a similar trend. The only difference is that the lactate yield kept increasing in the first 6 batches and then leveled off. The highest yield of 86 % was achieved at batch 6.

In addition, the pellet size and the biomass concentration from repeated batch cultures with a sole carbon source did not significantly ($P>0.05$) change during the entire fermentation course (Data not shown). It has been reported that the size and concentration of fungal pellets from cultures on the medium containing both nitrogen and carbon sources increased from one batch to the next. This leads to high density of large fungal pellets which makes mass and oxygen transfer more and more difficult once the repeated batch fermentation progressed, eventually influencing the fermentation performance (Yin

et al., 1997). The aeration and agitation have to be adjusted with respect to the fermentation process in order to maintain proper mass and oxygen transfer. Compared to that, the fungal biomass, with uniform pellet size and concentration during the entire course of lactic acid production, apparently had much better mass and oxygen transfer efficiency.

The results indicate that not only the pelletized fungus is able to utilize the glucose as a sole carbon source to produce lactic acid, but also the fermentation process with a sole carbon source is much easier and simpler to be operated. The data show that the first seven batches of fermentation had average values of 66 g/L of lactate concentration, 71% of lactate yield, and 2.4 g/L/h of productivity.

Lactic acid production with fed batch culture

High concentration of lactate in the broth is the other target of this study since high concentration of final product is favorable for the downstream separation and can significantly reduce the operation cost of the separation. However, repeated batch has the limitation on the increase of final lactate concentration although it can effectively improve productivity. A fed batch culture was used to carry out this task. The changes of lactate concentration and glucose concentration during the fed batch culture were shown in figure 8. The final lactate concentration reached at 138g/L in a reaction duration of 100 hours with an overall yield about 86%. A higher productivity of 2.8 g/L/h was observed in the culture time of first 28 hours, while the productivity was decreased to 0.9 g/L/h once the fresh medium was fed. The average productivity of whole process was 1.4g/L.h. Compared to the previous study of fed batch lactic acid production from low biomass

concentration (2g/L) (Liu et al., 2006), the productivity was doubled and the yield and concentration increased 50% and 43%, respectively. The results further indicated that the higher concentration of pelletized biomass was an important step in lactic acid production no matter what following fermentation process was adopted. In addition, since the lactate concentration from fed batch culture reached a very high level, and the solubility of calcium lactate is around 70 g/L, in order to avoid the formation of crystal calcium lactate, sodium hydroxide, instead of calcium carbonate, was used to neutralize the broth once lactate concentration reached 65 g/L.

5. Conclusion

This research developed a new three-step process for lactic acid and chitin co-production using pelletized *Rhizopus oryzae* NRRL 395 with potato hydrolysate and glucose as the nutrient source. Separation of the biomass cultivation and the lactic acid production can not only improve lactic acid production yield and productivity, but also enhance the chitin content. Cull potato hydrolysate used as a good nutrient source for biomass cultivation can significantly increase both biomass yield and chitin content. The biomass concentration and chitin content from culture on 100% potato hydrolysate reached 11 g/100 g glucose and 0.25 g/g biomass, respectively. Meanwhile, different fermentation method had different influences on lactic acid production. The comparison of three different cultures to produce lactic acid concluded that: (1) the lactic acid production was significantly enhanced by increasing the biomass concentration, removing the nitrogen source in the fermentation broth, and repeated or fed batch fermentations; (2) a high productivity of 3 g/L/h was achieved from the batch culture at

the high biomass concentration of 17.4g/L; (3) the repeated batch culture with a sole carbon source has been shown to be a favorable fermentation method in terms of enhancing the efficiency of lactic acid production, which the lactic acid productivity and yields from the first seven batches reached the averages of 2.4 g/L/hr and 71%, respectively. (4) the fed batch culture had the highest lactic acid yield and lactic acid concentration of 86% and 138 g/L, respectively, while its overall productivity was 1.4 g/L/h.

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Table 1. Chitin contents in biomass from cultures on different percentage of potato hydrolysate [a]

Potato hydrolysate content (%)	Chitin content, g/g biomass
100	0.25 ± 0.05
50	0.14 ± 0.03
25	0.10 ± 0.02
10	0.07 ± 0.03

a. data are the average of triplicates with standard deviations (n=3)

Table 2. Comparison of biomass during the lactic acid production [a]

	Initial biomass concentration inoculated for lactic acid production (g/L)	Biomass concentration after lactic acid production (g/L)
Culture 1	1.71	1.82
Culture 2	3.90	4.12
Culture 3	6.55	6.63
Culture 4	13.92	14.79
Culture 5	17.37	17.85

a. data are the mean value of two replicates

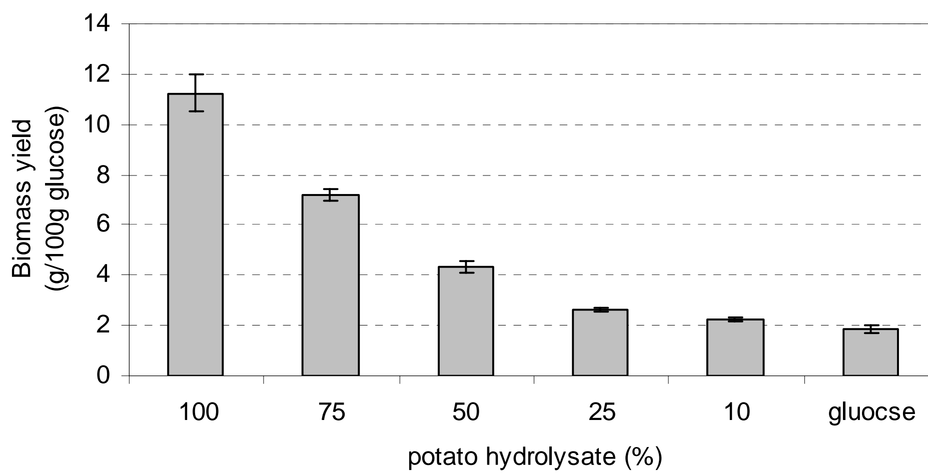


Fig. 1. Effects of potato hydrolysate on biomass production[a]

a: data are presented as the mean of three replicates with standard deviation.

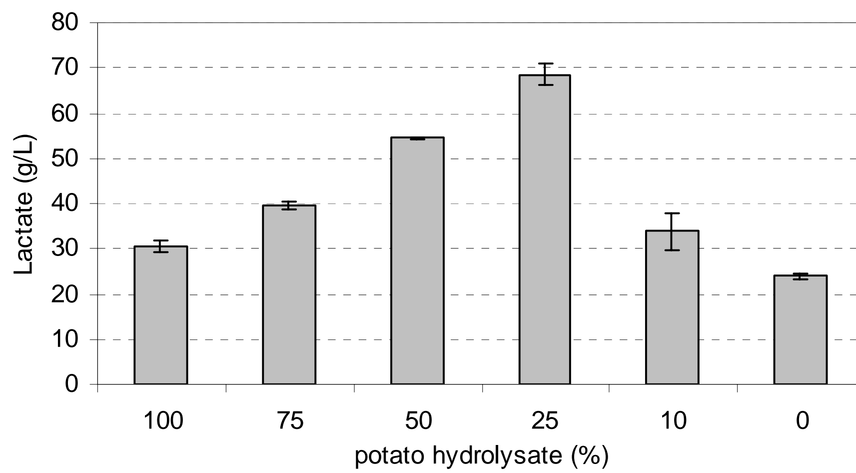


Fig. 2. Lactic acid production during the cultivation[a]

a: data are presented as the mean of three replicates with standard deviation.

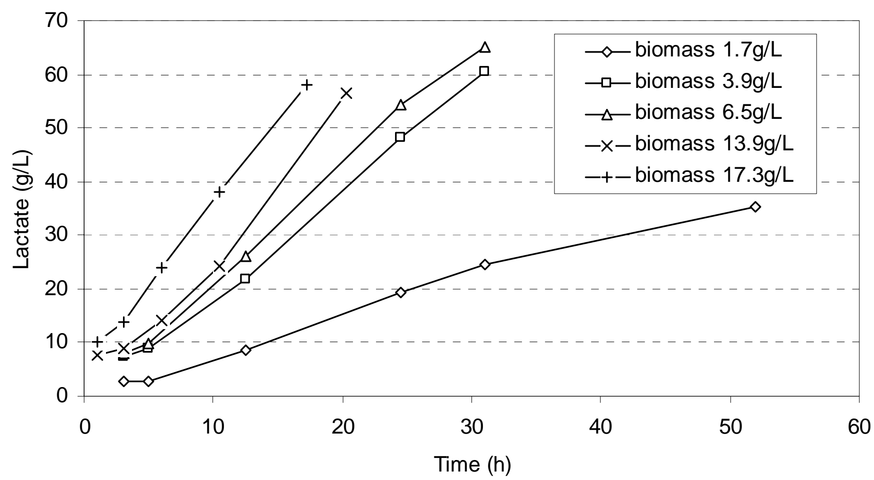


Fig. 3. Effects of fungal biomass on lactic acid production[a]

a: data are presented as the mean of two replicates

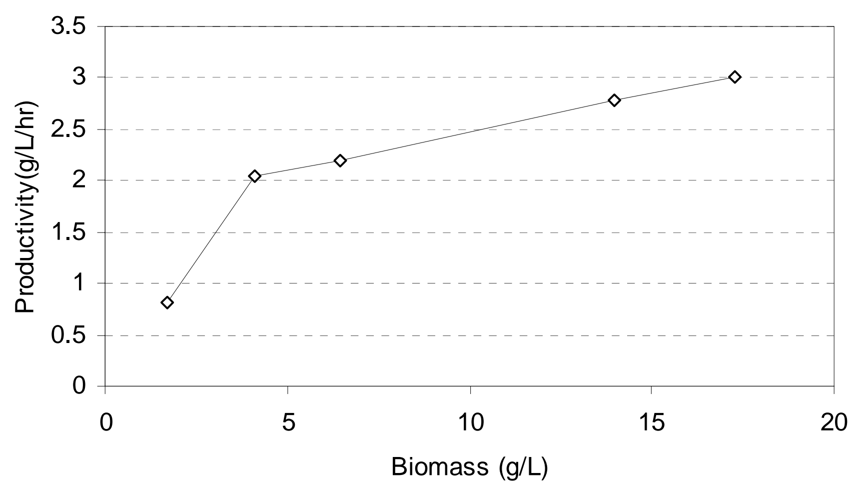
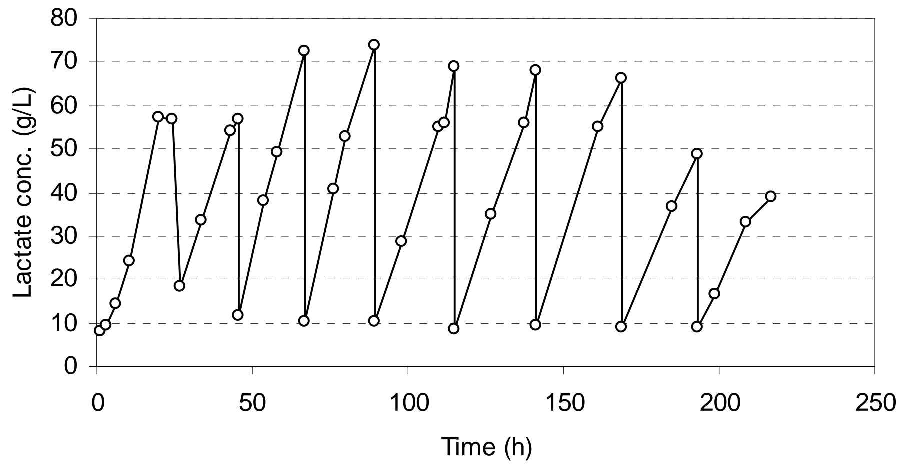
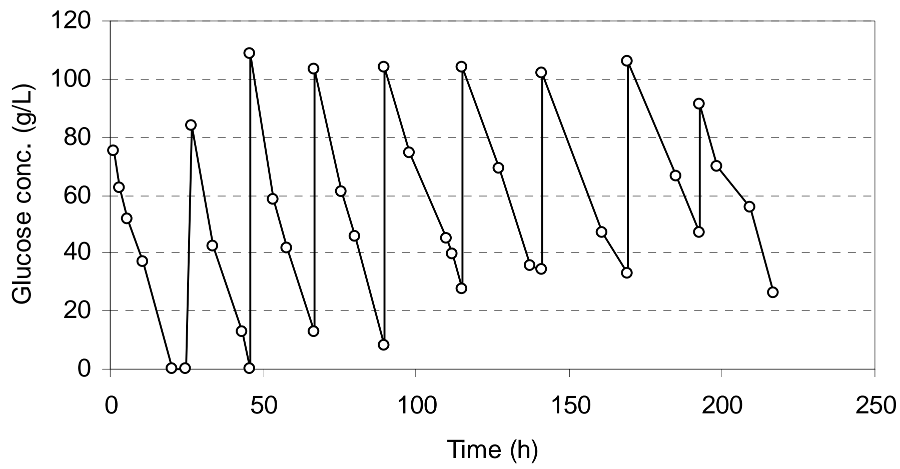


Fig. 4. Effects of fungal biomass on lactic acid productivity[a]

a: data are presented as the mean of two replicates



a. Lactate



b. Glucose

Fig. 5. Repeated batch fermentation of lactic acid production[a]

a: data are presented as the mean of two replicates

Fig. 6. Productivity of each batch during the repeated batch fermentation[a]

a: data are presented as the mean of two replicates

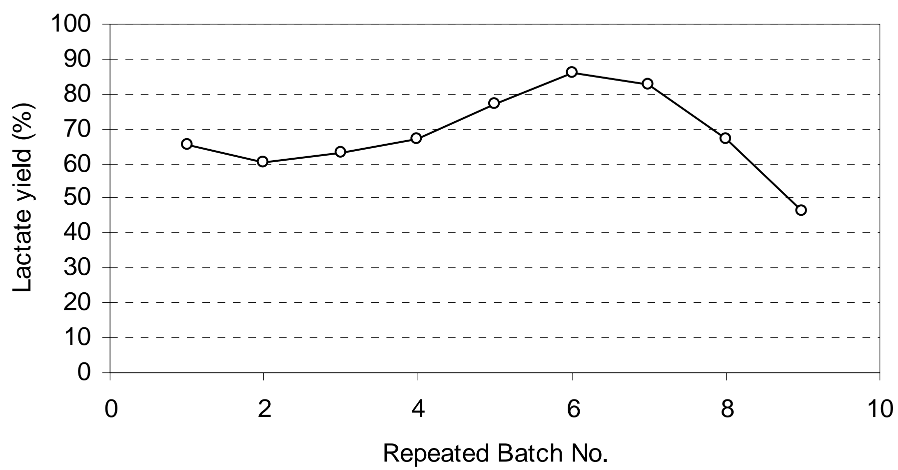


Fig. 7. Lactate yield of each batch during the repeated batch fermentation[a]

a: data are presented as the mean of two replicates

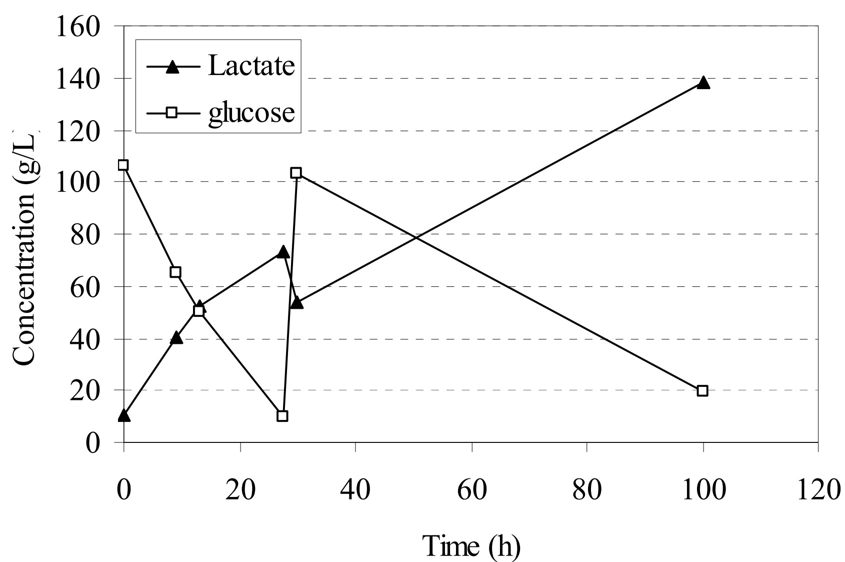


Fig. 8. Lactic acid production using fed batch culture of *R. oryzae* NRRL 395 in a 1L-stirred tank fermentor

The aeration rate and agitation speed were 1vvm and 200rpm, respectively. The pH was adjusted at 5.0±0.1 with CaCO3 and 25% NaOH. 13.9 g/L pelletized biomass seed was inoculated into fermentor and cultured at 27°C for 100 hours. 100mL fed-medium with 60g glucose was added at the 30th hour.

CHAPTER SIX

SUMMARY

In this study, a novel process was developed to convert underutilized cull potato into valuable lactic acid and chitin through pelletized fungal fermentation. There are three innovations in this study: co-production of lactic acid and chitin, controlled fungal pellet formation, and three-step fermentation to enhance lactic acid production. The main findings obtained from this research are summarized below:

1. Lactic acid production using cull potatoes as the sole nutrients.

The fungal strain, *Rhizopus oryzae* NRRL 395, was able to directly produce lactic acid without adding amylolytic enzymes to hydrolyze the potato starch. However, the lactate concentration was low (33 g/L in batch culture) compared to fermentation using potato hydrolysate (60~70g/L in batch culture), because the hydrolysis of potato starch during the fermentation was ineffective, which eventually influenced the lactic acid production. Adding extra amylolytic enzyme to the fermentation system improved lactic acid production. High shaking speed benefited the production of lactic acid due to the aerobic requirement of fungal biomass growth. The cotton-like morphology hindered mass transfer and decreased the product yield and productivity.

2. Fungal pellet formation

Biodegradable polymer additives, such as rice, were discovered to be able to greatly enhance pellet formation. The effect of spore storage time on pellet formation was another finding. Longer storage time had positive effect on pellet formation. Meanwhile, a multiple logistic regression model was established to predict the probability of forming pellet as a function of pertinent factors such as biodegradable polymer additive, spore storage time, inoculum size, substrate concentration, pH, and shaking speed. The experimental data indicated that the established model was a good pellet formation prediction model for strains of *Rhizopus oryzae*.

3. Co-production of lactic acid and chitin using pelletized *Rhizopus oryzae* cultured on cull potatoes and glucose

A novel three-step process of lactic acid and chitin co-production using pelletized *Rhizopus oryzae* NRRL 395 with potato hydrolysate and glucose as nutrient source was developed. Separation of the biomass cultivation and the lactic acid production not only improve lactic acid production yield and productivity, but also enhance the chitin content. Using cull potato hydrolysate as a good nutrient source for biomass cultivation can significantly increase both biomass yield and chitin content. Different fermentation methods had different influences on lactic acid production. High productivity was achieved from the batch culture at the high biomass concentration. The repeated batch culture was a favorable fermentation method in terms of enhancing the efficiency of lactic acid production. The fed batch culture produced high lactic acid concentration with relatively high yield.

4. Future work

As a result of the low solubility and the limited volume mass transfer in bioreactors, oxygen is one of the limiting factors for fermentations. The situation could be made worse with high biomass concentration, given that oxygen uptake rate is proportional to biomass concentration. Devising methods to evaluate oxygen mass transfer in this heterogeneous system and to increase the mass transfer efficiency will be good future research topics. Additionally, other factors that influence the production of lactic acid and chitin content in biomass, such as product inhibition and biomass decay, can be investigated as well.