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# Direct Fermentation of Potato Starch in Wastewater to Lactic Acid by *Rhizopus oryzae*

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**Abstract** The fungal species of *Rhizopus oryzae* 2062 has the capacity to carry out a single stage fermentation process for lactic acid production from potato starch wastewater. Starch hydrolysis, reducing sugar accumulation, biomass formation, and lactic acid production were affected with variations in pH, temperature, and starch source and concentration. A growth condition with starch concentration approximately 20 g/L at pH 6.0 and 30°C was favourable for starch fermentation, resulting in a lactic acid yield of 78.3%~85.5% associated with 1.5~2.0 g/L fungal biomass produced in 36 h of fermentation.

*Keywords*: fermentation, lactic acid, *Rhizopus oryzae*, reducing sugars, starch hydrolysis, fungal biomass

## INTRODUCTION

Lactic acid, a naturally occurring multifunctional organic acid, has numerous applications in the food, pharmaceutical, leather, and textile industries and also as a chemical feedstock. One of its most promising applications is its use for biodegradable and biocompatible polylactate polymers such as poly-lactic acid (PLA), an environmentally friendly alternative to biodegradable plastics [1-3]. Owing to the unique property of PLA, lactic acid has the potential to be a substitute for plastics manufactured from petroleum derivatives and becomes a very large-volume commodity chemical intermediate. Lactic acid can be produced commercially either through chemical synthesis or biotechnological production. The biotechnological process can yield either D-(-)- or L-(+)-lactic acids, or a mixture in different proportions of the two isomers, depending on the microorganism, substrate, and growth conditions used, whereas the chemical production only results in a mixture of the two isomers [4-6]. Another significant advantage of biotechnological production over chemical synthesis is that it can use cheap raw materials, such as whey, molasses, starch waste, beet- and cane-sugar, and other carbohydrate-rich materials [6-8].

Conventional biotechnological production of lactic acid from starch materials, for instance, requires pretreatment by gelatinisation and liquefaction. This pretreatment is carried out at high temperatures of 90~130°C for 15 min followed by enzymic saccharification to glucose and subsequent conversion of glucose to lactic acid by fermentation [2,6]. This two-step process involving makes it economically unattractive.

Many representative bacteria, including *Lactobacillus* and *Lactococcus* species, have been used to effectively produce lactic acid from raw starch materials [2,9-11]. Lactic acid-producing fungi, such as *Rhizopus oryzae*, have recently received an interest in lactic acid production. The major advantage of the fungal process over bacterial fermentation is the low costs, due to (1) the use of raw and/or waste materials, (2) no need of specific nutrients, (3) little pH maintenance required, as the most fungi can be tolerant to low pH environments, and (4) easy and un-expensive separation of filamentous or pellet fungal biomass from the fermentation broth [12,13]. Two other important aspects for using the fungal lactic acid producer are they can take the role of carrying out consecutive enzymatic hydrolysis and fermentation process as both the enzyme and lactic acid producer, and they may secrete L-lactic acid as the only fermentation product. These benefits of direct fermentation allow the decrease in the inhibition caused by glucose accumulation during starch hydrolysis and the increase in the saccharification rate, which consequently increases the productivity and reduces the reactor volume and capital costs [3,4,12,14-17]. Many factors, such as pH, temperature, substrates, and product concentration of glucose and lactic acid, can affect the fermentation process [11,18]. A disadvantage of this direct fermentation is the difference in cultivation conditions, such as pH and temperature, for saccharification and fermentation. In many cases, the low pH, *e.g.* lower than 5, and the high temperature, *e.g.* > 40°C, may

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be favourable for enzymatic hydrolysis, whereas the low pH can surely inhibit the lactic acid production, and the high temperature may adversely affect the fungal cell growth [19-21]. Therefore, the determination of optimal process conditions that enhance the fermentation performance is of importance for an industrial process of the lactic acid production. However, little information of this kinetic process using fungous microorganisms for lactic acid production is available in the literature.

The aim of this study was to identify how the cultivation conditions in terms of pH, temperature, and substrate affect the saccharification and fermentation kinetics in the lactic acid production from potato starch in wastewater by the fungal species of *R. oryzae* 2062. The saccharification and fermentation performance was characterized by starch hydrolysis, accumulation of reducing sugars, and production of lactic acid and fungal biomass.

## MATERIALS AND METHODS

### Microorganisms

The strain of *Rhizopus oryzae* 2062 was purchased from Culture Collection, Food Science Australia, Australia. This strain was propagated and stored on potato dextrose agar (PDA) slants at 4°C.

### Culture and Fermentation Medium

The composition of the pre-culture medium (g/L) was: soluble starch, 10; peptone, 5.0; yeast extract, 5.0; $KH_2PO_4$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2. This medium was autoclaved at 121°C for 20 min.

The potato wastewater was collected from a starch process waste stream, Freer Foods Pty Ltd. (Brisbane, Australia) mainly containing 15~30 g/L of potato starch, 0.5~1.2 g/L of reducing sugars, and 0.58~0.87 g/L of total Kjeldahl-N. If not stated otherwise, this starch wastewater was used as a production medium throughout the investigation.

### Saccharification and Fermentation Experiment

Spores were grown on the PDA slants at 30°C for 7 days. They were collected using a platinum loop and were suspended in sterilized distilled water. A 250-mL flask containing 100 mL of the pre-culture medium was inoculated with a final concentration of $10^5$ spores/mL and was incubated in a rotary shaker (150 rpm) at 30°C for 12 h. This overnight culture, used as the seed culture, was used to initiate growth in the potato starch medium that was used in this study.

Saccharification and fermentation were conducted in 250-mL Erlenmeyer flasks containing 100 mL final volume of the potato starch wastewater that was inoculated with 5 mL of seed culture. Unless stated otherwise, the agitation rate and incubation temperature were 150 rpm and 30°C, respectively.

### Sample Preparation

During fermentation, 100 mL samples were collected from one flask at certain time intervals for further analysis. Samples were filtered by a 106 μm sieve for the biomass measurement. The filtrated biomass was washed twice using 4 N HCl to remove the rest of the calcium carbonate, and the biomass concentration was determined by weighing the mass after drying at 60°C for 36 h. The filtered liquid was used for measuring starch and reducing sugar. Part of the filtered liquid was centrifuged at 6000 rpm for 10 min, and the supernatant was further filtered by a 0.45 μm Millipore and then was diluted two or three times for lactic acid analysis by HPLC. The samples added with 6.0% $CaCO_3$ were distinguishingly treated because of the calcium lactate precipitation under this situation. The treatment difference from other samples was that the collected acidic supernatant was also for lactic acid analysis by HPLC after the biomass was washed twice using 4 N HCl and the total lactic acid in these samples was the summation of lactic acid in liquid and solid phase.

### Analytical Method

The estimation of starch was carried out using the iodine colorimetric method as described by Tomas *et al.* [22]. Reducing sugars were estimated by the dinitrosalicylic acid method using glucose as the standard [23]. In the present study, the reducing sugars were described as the sum of the formation due to saccharification and consumption due to fermentation. Lactic acid was measured by HPLC. A Bio-rad HPX-87H column and a refractive index detector were used with 0.008 N $H_2SO_4$ as the eluant at 65°C.

The results reported were the arithmetic mean values of the tests in triplicates. Appropriate tests of significance, analysis derivations, and confidence difference at a 5% level were used in the data evaluations.

## RESULTS

### Effect of Growth pH

In order to determine the impact of growth pH on the starch saccharification and fermentation into lactic acid by *R. oryzae* 2062, the growth pH was controlled at 4.0, 5.0, 6.0, and 7.0 by adding 4 N NaOH solution at 4 h intervals during the course of cultivation. The variations in starch and reducing sugar concentrations of the *R. oryzae* 2062 cultures were measured at 4 and 8 h (Table 1).

The results presented in Table 1 indicate that *R. oryzae* 2062 had a high enzymatic capability for saccharification of potato starch when the growth pH ranged from 4.0 to 7.0, and the starch hydrolysis and reducing sugar accumulation were influenced by the growth pH. A weaker starch saccharification occurred and the reducing sugar around 2.0 g/L was received in the growth pH range of

**Table 1.** Effect of pH on starch hydrolysis, reducing sugar accumulation, lactic acid production, and biomass

| Time (h) | Residual starch (%) | | | | Reducing sugars (g/L) | | | |
|---|---|---|---|---|---|---|---|---|
| | pH 4.0 | pH 5.0 | pH 6.0 | pH 7.0 | pH 4.0 | pH 5.0 | pH 6.0 | pH 7.0 |
| 4 | 98.2 | 98.9 | 99.1 | 99.5 | 2.26 | 2.03 | 1.20 | 2.19 |
| 8 | 28.4 | 8.86 | 4.55 | 3.61 | 8.78 | 8.86 | 9.02 | 9.06 |
| Time (h) | Lactic acid (g/L) | | | | Biomass (g/L) | | | |
| | pH 4.0 | pH 5.0 | pH 6.0 | pH 7.0 | pH 4.0 | pH 5.0 | pH 6.0 | pH 7.0 |
| 36 | 7.96 | 7.87 | 7.79 | 7.40 | 0.57 | 0.47 | 0.36 | 0.33 |
| 48 | 11.9 | 9.17 | 9.69 | 9.03 | 0.62 | 0.58 | 0.62 | 0.61 |

4.0 to 7.0 during the first 4 h. During the cultivation period from 4 to 8 h, *R. oryzae* 2062 demonstrated a high rate of starch hydrolysis from 3.5 to 4.8 g $L^{-1}$ $h^{-1}$ and reducing sugar accumulation from 1.6 to 1.8 g $L^{-1}$ $h^{-1}$. The rate of starch hydrolysis increased as the growth pH increased, and there was a slight difference in reducing sugar at pH 4.0~7.0 at 8 h. Consequently, a growth pH lower than 5.0 was unfavourable for starch saccharification in the culture.

The experimental results revealed that *R. oryzae* 2062 had a metabolic capability to produce lactic acid and fungal biomass using potato starch in a growth pH range between 4.0 and 7.0. From Table 1, it was interesting to note that the *R. oryzae* 2062 culture corresponded to an obvious decrease in the production of lactic acid and biomass with pH varied from 4.0 to 7.0. By observing the comparison of the production results of the 36 and 48 h fermentation presented in Table 1, the highest increase in lactic acid yields was given by 49% at the growth pH of 4.0. Unlike lactic acid production, *R. oryzae* 2062 culture demonstrated an unchangeable biomass production within the entire pH range of 4.0~7.0 except a slight decrease at pH 5.0 in the 48 h fermentation.

## Effect of Supplementation of $CaCO_3$

Lactic acid is known to be a strong inhibitor for both cell growth and production in lactic acid fermentation [4,18,21]. Calcium carbonate is one commonly used reagent in neutralizing lactic acid during fermentation. Its low solubility in water makes it possible to neutralize lactic acid and automatically maintain the pH at certain level. In this study, $CaCO_3$ powder was added to the media with dosages of 0.5, 1.0, 3.0, and 6.0% (w/v). A comparison experiment with no $CaCO_3$ addition was carried out by setting up an initial pH at 6.0 and during the course of fermentation, the growth pH was not adjusted. As indicated in Table 2, the addition of $CaCO_3$ significantly enhanced the microbial performances of not only enzymatic saccharification of starch, but also the metabolic fermentation of lactic acid. An obvious increase in terms of lactic acid production and fungal biomass formation was found with the $CaCO_3$ dosage varying

**Table 2.** Effect of the addition of $CaCO_3$ on starch hydrolysis, reducing sugar accumulation, lactic acid production, and biomass formation

| Dosage (w/v, %) | Residual starch (%) | | Reducing sugars (g/L) | |
|---|---|---|---|---|
| | 4 h | 8 h | 4 h | 8 h |
| 0 | 99.8 | 28.4 | 1.04 | 8.55 |
| 0.5 | 90.2 | 76.2 | 1.30 | 1.33 |
| 1.0 | 81.1 | 64.5 | 1.21 | 1.49 |
| 3.0 | 78.4 | 62.4 | 1.14 | 1.56 |
| 6.0 | 77.8 | 61.7 | 1.01 | 1.60 |
| Dosage (w/v, %) | Lactic acid (g/L) | | Biomass (g/L) | |
| 0 | 8.25 | | 0.64 | |
| 0.5 | 13.4 | | 1.47 | |
| 1.0 | 19.0 | | 1.51 | |
| 3.0 | 15.0 | | 1.88 | |
| 6.0 | 15.1 | | 1.99 | |

from 0 to 1.0%. There was a dramatic reduction in reducing sugar accumulation in the cultivated media due to the addition of $CaCO_3$, and the variation in reducing sugar quantity was little with dosing of $CaCO_3$ ranging from 0.5 to 6.0%. The data of starch hydrolysis and reducing sugar accumulation in the experiments with and without the addition of $CaCO_3$ revealed that $CaCO_3$ increased the starch hydrolysis in the initial 4 h of incubation and decreased the accumulation of reducing sugar during the 8 h incubation time. These results proved the different role of $CaCO_3$ within the different incubation period. The discovery that there was little impact on starch hydrolysis and reducing sugar accumulation by the addition of more than 1.0% of $CaCO_3$ was found from the experimental data. The addition of $CaCO_3$ ranging from 0.5 to 6.0% resulted in a constant increase in biomass quantity. However, an over dosage of $CaCO_3$ (more than 1.0%) resulted in a slight decrease in the lactic acid yield. Therefore, the supplementation of 1.0% (w/v) of $CaCO_3$ may be sufficient for maintaining a growth pH to achieve the optimum fungal cell growth and lactic acid production using potato starch.

## Effect of Temperature

The impact of the cultivation temperature on the fermentation process was investigated by controlling the growth temperatures at 22°C, 30°C, 35°C, and 40°C. The results, by measuring the concentration of the residual starch and reducing sugar at 4 h and 8 h of cultivation, indicated that there was a slight increase in residual starch and reducing sugar accumulation as the cultivation temperature increased from 22 to 30°C, and a further increase in the temperature from 30 to 40°C resulted in

**Table 3.** Effect of temperature on starch hydrolysis, reducing sugar accumulation, lactic acid production, and biomass formation

| Time (h) | Residual starch (%) | | | Reducing sugars (g/L) | | |
|---|---|---|---|---|---|---|
| | 22°C | 30°C | 38°C | 22°C | 30°C | 38°C |
| 4 | 80.0 | 81.1 | 80.3 | 0.93 | 1.28 | 1.30 |
| 8 | 62.6 | 64.5 | 63.5 | 0.98 | 1.49 | 1.55 |

| Time (h) | Lactic acid (g/L) | | | | Biomass (g/L) | | | |
|---|---|---|---|---|---|---|---|---|
| | 22°C | 30°C | 35°C | 38°C | 22°C | 30°C | 35°C | 38°C |
| 36 | 5.79 | 9.58 | 8.96 | 6.99 | 0.99 | 0.93 | 0.66 | 1.00 |
| 48 | 9.01 | 13.9 | 12.6 | 11.7 | 1.13 | 1.20 | 0.99 | 1.03 |

**Table 4.** Effect of potato starch and soluble starch on saccharification and fermentation by *R. oryzae* 2062

| Time (h) | Residual starch (%) | | Reducing sugars (g/L) | |
|---|---|---|---|---|
| | Potato starch | Soluble starch | Potato starch | Soluble starch |
| 4 | 88.2 | 68.4 | 1.57 | 4.37 |
| 8 | 37.3 | 16.7 | 2.66 | 4.77 |

| Time (h) | Lactic acid (g/L) | | Biomass (g/L) | |
|---|---|---|---|---|
| | Potato starch | Soluble starch | Potato starch | Soluble starch |
| 36 | 7.85 | 9.34 | 2.64 | 3.52 |
| 48 | 10.3 | 15.7 | 2.38 | 3.44 |

**Table 5.** Effect of initial starch concentration on starch hydrolysis and reducing sugar accumulation in 8 h of cultivation and lactic acid production in 36 hrs of fermentation by *R oryzae* 2062

| Starch concentration (g/L) | Starch Hydrolysis (%) | Reducing sugar accumulation (g/L) | Starch concentration (g/L) | Lactic acid production (g/L) |
|---|---|---|---|---|
| 15.5 | 86.4 | 1.84 | 12.5 | 5.02 |
| 20.5 | 83.6 | 2.27 | 18.6 | 18.02 |
| 30.6 | 68.2 | 2.87 | 27.0 | 11.79 |
| 40.0 | 56.7 | 2.92 | 37.5 | 5.34 |
| | | | 62.7 | 5.46 |

a slight improvement for the saccharification and fermentation performance (Table 3). The lactic acid production and biomass growth were obviously affected by the cultivation temperature. From Table 3, the peak lactic acid concentration was found at 30°C, while the lowest biomass concentration was produced at 35°C. Consequently, 30°C appeared to be the optimum cultivation temperature for both saccharification and fermentation by *R. oryzae* 2062.

## Effect of Substrates

In order to identify the saccharification and fermentation capacity of *R. oryzae* 2062 and to use different starch materials, the fungal culture was cultivated in the potato starch wastewater with a starch concentration of 21.7 g/L and also in soluble starch medium which consisted of soluble starch 20.0 g/L, $(NH_4)SO_4$ 2.0 g/L, $KH_2PO_4$ 0.6 g/L, $MgSO_4 \cdot 7H_2O$ 0.25 g/L, and $ZnSO_4 \cdot 7H_2O$ 0.04 g/L. As expected, this *Rhizopus* fungus performed at a high saccharification rate in terms of starch hydrolysis and reducing sugar accumulation in the soluble starch medium (Table 4). This is because the fungal microorganism can easily access the starch in the soluble state rather than insoluble state. During the 8 h of cultivation, *R. oryzae* 2062 had a starch hydrolysis rate of 2.26 g $L^{-1}$ $h^{-1}$ in the soluble starch medium and a rate of 1.74 g $L^{-1}$ $h^{-1}$ in the potato starch wastewater. The reducing sugar in the cultivated media in 8 h of fermentation remained a volumetric concentration of 4.77 g/L in soluble starch medium, while 2.66 g/L reducing sugar was measured in the potato starch wastewater medium. The results of the lactic acid and fungal biomass production in potato starch wastewater and soluble starch media are shown in Table 4. It was interesting to note that a relatively high production of lactic acid and fungal biomass was found in the soluble starch medium. These phenomena may be explained by a fast starch hydrolysis that resulted in producing a high quantity of reducing sugars, in particular the glucose, which lead to a high production of lactic acid and a fast cell growth termed as biomass production.

## Effect of Starch Concentration

The effect of the starch concentration varying from 12.5 to 62.8 g/L in the potato starch wastewater of the fermentation process was investigated, and the results were shown in Table 1. From the results presented in Table 5, it was found that the starch saccharification capacity of *R. oryzae* 2062 was limited when the starch concentration increased from 15 to 40 g/L under the cultivation conditions. Therefore, during the 8 h saccharification, the reducing sugar concentration only increased from 1.5 to 3.1 g/L with the initial starch concentration varying from 15.5 to 40.0 g/L. The lactic acid concentra-

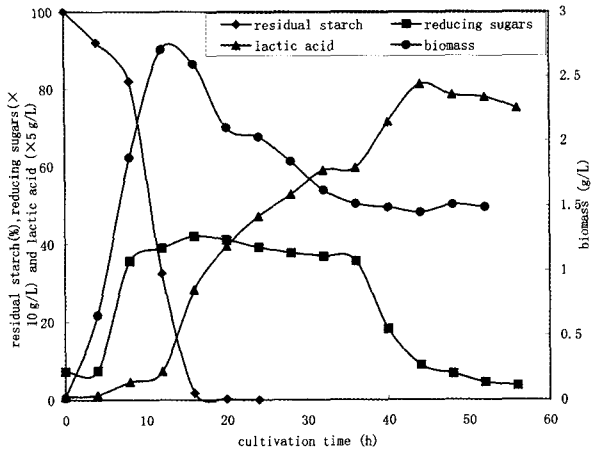


**Fig. 1.** Kinetic profiles of starch hydrolysis, reducing sugars accumulation, lactic acid production and biomass formation by *R. oryzae* 2062 during the direct fermentation course. This figure showed the simultaneous change of starch, reducing sugars, lactic acid and biomass in the cultivation medium at different cultivation time.

tion decreased approximately 50% with the increase in initial starch concentration from 18.6 to 27.0 g/L. Cultivation of *R. oryzae* 2062 in potato starch wastewater with the initial starch concentration ranging between 37.5 and 62.8 g/L only resulted in approximately 5.5 g/L lactic acid production. This might be due to low accessibility of amylolytic enzymes to starch substrates and product inhibition during the later stages of fermentation where there is a high concentration of substrates as stated by Yin *et al.* [4]. However, in terms of the lactic acid yield, a very low yield of 36.0% was produced at the initial starch concentration of 12.5 g/L and the maximum yield for *R. oryzae* 2062 was 85.5% corresponding to the starch concentration of 18.6 g/L. At the maximum yield of 85.5%, yield of the by-products maltose, ethanol, and succinate in the fermentation medium was very low with 0, 0.59 and 0.27% respectively, and these data demonstrated an almost complete conversion of potato starch to lactic acid.

### Kinetic Characteristics during the Course of Direct Fermentation

The investigation of kinetic characteristics in the fermentation process was conducted in shake flask fermentation using potato starch wastewater containing 20.5 g/L of potato starch. Cultivation conditions were set up at an initial pH of 6.0 with supplementation of 1% (w/v) $CaCO_3$ at 30°C. Samples were taken at 4 h intervals during the course of 56 h of fermentation. Fig. 1 presents the fermentation kinetic profiles of (a) starch saccharification, (b) reducing sugar accumulation, (c) fungal biomass formation, and (d) lactic acid production during the 56 h of cultivation.

During the cultivation, the potato starch was saccharified into glucose by enzymes, such as amylase and glucoamylase, which were generated by the *Rhizopus* fungus, and the glucose was metabolized by the fungous microorganism and converted into the lactic acid. There was a lag phase for the saccharification and fermentation during the 8 h of cultivation. During the initial 12 h of cultivation, the saccharification rate was greater than the fermentation rate, resulting in an accumulation of reducing sugars. The highest saccharification rate was observed between the 4th and the 12th h corresponding to a starch hydrolysis rate of 2.01 g $L^{-1}$ $h^{-1}$. Starch was undetectable by the iodine colorimetric method after 16 h. During the exponential saccharification phase of 4~12 h, the reducing sugars accumulated at a high rate of approximately 0.40 g $L^{-1}$ $h^{-1}$. The reducing sugars remained at a high constant level of approximately 4 g/L between the 12th and the 36th h in the *R. oryzae* 2062 culture. After the exponential fermentation phase, the reducing sugar concentration was lower than 1.0 g/L.

The fungous cell growth was very fast, which resulted in a sharp increase in fungal biomass production at a rate of 0.27 g $L^{-1}$ $h^{-1}$ within the 4th to the 12th h range. There was a short biomass stationary phase between the 12th and the 16th h, and it was followed by an obvious biomass deduction phase. This may be due to the rapid cell lysis that occurred in the *R. oryzae* 2062 culture under the growth conditions. Experiments indicated that there was an initial phase, where the saccharification rates were greater than the fermentation rates, wherein glucose was accumulated. Therefore, a lag period in the fermentation of lactic acid was introduced in the simulation. During this period, the glucose generated by the enzymatic reaction was used for maintaining the fungal cell growth. Unlike the biomass formation, the lactic acid production corresponded to a long lag phase, and a fast production phase was observed within the 12~32 h period, which was associated with a production rate of 0.52 g $L^{-1}$ $h^{-1}$. These results indicated that lactic acid formation took place followed by the exponential cell growth phase. The highest lactic acid yield of starch was 70.2% in 44 h, and the maximum productivity was 0.40 g $L^{-1}$ $h^{-1}$ in 20 h.

## DISCUSSION

In the present study, the biochemical kinetics of the saccharification and fermentation processes of lactic acid production from potato starch wastewater using *R. oryzae* 2062 was studied with respect to the variations in growth pH, temperature, starch amount and type. The results of the present investigation indicated that *R. oryzae* 2062 had the ability to perform a single stage fermentation process for lactic acid production using potato starch material. In the cultivation medium that contained approximately 20 g/L of potato starch, this amylolytic *Rhizopus* strain enzymatically hydrolysed more than 80% of the total starch at a saccharification rate of 1.5 to 2.0 g $L^{-1}$ $h^{-1}$ in 8 h of cultivation time, and a complete starch hydrolysis was achieved within 16 h. A fermentative formation of lactic acid took place simultaneously, but a high production rate from 0.57 to 0.98 g $L^{-1}$ $h^{-1}$ was observed during the period from 12 to 28 h. This fungous microorganism

had a high enzymatic and metabolic capability to not only carry out the direct fermentation process but also to utilize potato starch material as a sole source for lactic acid production at a wide pH and temperature range: 4.0~7.0 and 22~40°C, respectively. Although this direct fermentation performance was affected with respect to the variation in pH and temperature, a growth condition with a pH between 5.0~6.0 at 30°C was found to be favourable for both starch saccharification and lactic acid fermentation. Associated with 1.5 and 2.0 g/L fungal biomass that was produced, lactic acid yield from 78.3 to 85.5% was achieved in 36 h of fermentation.

Microbial fungus *Rhizopus* has been known for its capacity to secret amylose in producing lactic acid from starchy substrates such as potato starch without prior saccharification [15]. *R. oryzae* NRRL 395 was extensively researched and has been recognised as one of the most suitable lactic acid producers [4,12,15]. The lactic acid production from 60 to 80 g/L using *R. oryzae* NRRL 395 was obtained in a batch process lasting more than 3 days with a lactic acid yield between 65 and 78% [6,13,24,25]. The ability of *Rhizopus* to aerobically produce lactic acid under additional nutrient environments has been studied [4,12,16]. Strain *R. oryzae* ATCC 52311 can produce lactic acid that has a concentration of 83 g/L and with a yield of 88%, which is consumed in a culture containing glucose and ammonium sulphate and other inorganic salts [16]. *R. oryzae* was capable of saccharifying and fermenting corn and potato pulp to lactic acid, respectively [4,25]. Khalaf [3] reported that two prototrophic hybrids that mutated from *R. oryzae* Leu and *R. arrhizus* Cys2 produced lactic acid with a concentration of 75 g/L.

Our results revealed that the pH of the culture media is one of the most important factors that significantly affect the lactic acid production. The experimental observations indicated that the pH of the cultivated medium decreased dramatically during the first 12 h, and maintained a very low pH afterwards (approximately 2.5), if no pH control was involved in the process (Fig. 2a). A low pH not only affects cell growth but also inhibits the biochemical reactions for lactic acid formation [26,27]. A pH control procedure was conducted in the present study through the addition of NaOH solution at 4 h intervals during the cultivation course. A controlled growth pH of around 6.0 may be desirable to obtain a promising microbial performance in terms of starch saccharification and lactic acid production. However, the pH in the cultivated medium dropped quickly after the pH adjustment as lactic acid formed (Fig. 2b). Therefore, the controlled growth pH in this study was an unstable pH level. On the other hand, a constant pH between 5.0 and 6.0 may be maintained by the addition of 1~3% $CaCO_3$ during the lactic acid fermentation (Fig. 2a). Present results, as stated above, revealed that the optimum pH for starch saccharification somehow may differ from that of the lactic acid fermentation. The growth pH also governed the metabolic reaction for lactic acid and biomass formation using the limited carbon source. Therefore, the optimum growth pH, which may enhance the saccharification or

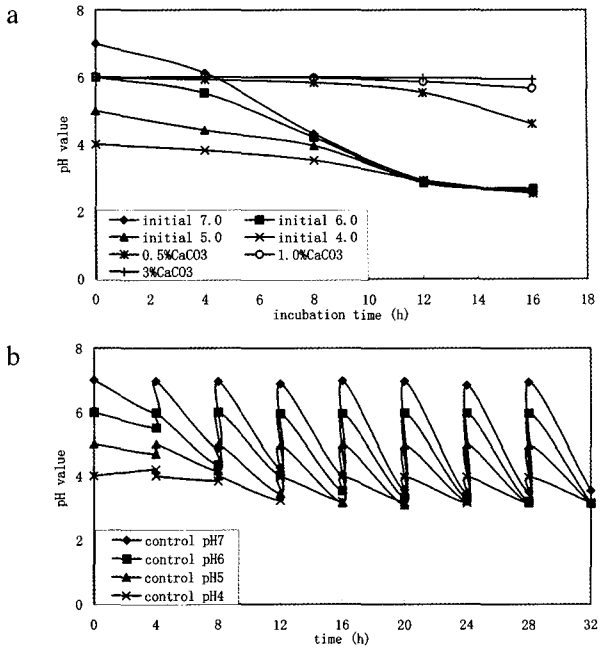


**Fig. 2.** Variation in pH in *R. oryzae* 2062 culture by (a) addition of $CaCO_3$ with no pH control and by (b) addition of NaOH solution during the direct fermentation course. Addition of 1%-3% $CaCO_3$ can maintain the system pH (a), meanwhile, the pH control by addition of NaOH solution at 4 h interval was unstable (b). The peaks of curves in Figure (b) mean "after pH adjustment" and the bottoms of curves mean "before pH adjustment".

fermentation, needs to be further investigated by an online pH control system. Lactic acid is known to be a strong inhibitor for cell growth, enzymatic hydrolysis, and microbial activity production in lactic acid fermentation [26,27]. Therefore, in order to prevent this self-inhibition, the addition of a neutralising agent is necessary. Sodium hydroxide has been often used to control the pH of fermentation broths. Present results confirmed that a neutralizing agent such as $CaCO_3$ must be present in the fermentation media to achieve a maximum production of lactic acid by *Rhizopus sp.* The addition of $CaCO_3$ could neutralize $H^+$ that are released from the lactic acid which may reduce the synthesis reaction forward toward lactic acid production [13,15,28]. A significant improvement in terms of lactic acid and biomass production was obtained by the addition of 1% $CaCO_3$ in the fermentation media. However, this process may bring into an extra cost for lactic acid purification and biomass recovery in a downstream process.

Current results may reveal that the cultivation conditions in terms of growth pH, temperature, carbon source, and substrate concentration affected not only the saccharification rate, but also biomass formation and lactic acid production. The quantity and the accumulation rate of glucose, termed as reducing sugars in this study, may take an important role in this direct fermentation process. A high reducing sugar accumulation rate may result in a

high lactic acid production. On the other hand, a very high concentration of glucose may also inhibit the lactic acid formation. Associated with the glucose, an inhibition from the lactic acid itself may be an important factor which affected the microbial accessibility and fungal cell growth, as well as the lactic acid formation. Therefore, these phenomena need to be addressed in further fermentation investigations.

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