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Production of L-Lactic Acid from Raw Cassava Starch by *Rhizopus oryzae* NRRL 395

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ABSTRACT

Lactic acid, which is commonly used in food, chemical and pharmaceutical industries, has recently received much attention for the production of biodegradable plastic. L-lactic acid production by *Rhizopus oryzae* NRRL 395 from raw cassava starch as a sole carbon source was studied. The optimum production medium was as follows (g/l) : raw cassava starch, 120; $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; $ZnSO_4.7H_2O$, 0.04; pH 6.0. The maximum L-lactic acid production in shake flasks was 68.32 g/l on day 5 with the shaking speed of 200 rpm at 30°C. L – lactic acid yield and productivity were 0.59 g/g substrate and 0.57 g/lh, respectively. In a jar fermentor, the maximum L-lactic acid production was 54.62 g/l on day 4 of cultivation at the agitation speed of 400 rpm and the aeration rate of 1.5 vvm. The L – lactic acid yield was 0.49 g/g substrate with the productivity of 0.56 g/lh.

Key words: L-lactic acid, *Rhizopus oryzae*, cassava starch

INTRODUCTION

Lactic acid is an important organic acid widely used not only in the food industry as a preservative, a food acidulant and flavour agent but also in chemical industry as solvent. More-over, various lactic acid salts are also used in formulation of pharmaceutical products (Kascak *et al*., 1996 ; Vick Roy, 1985). Recently, lactic acid is used as a starting material of polylactic acid (PLA), a polymer used in the manufacture of new biodegradable plastics (Vert *et al*., 1992). Lactic acid has been produced by lactic acid bacteria (Bibal *et al*., 1991 ; VickRoy *et al*., 1982) and the fungus *Rhizopus oryzae* (Soccol *et al*., 1995 ; Yu and Hang, 1989). *Rhizopus oryzae* can produce large amounts of L-lactic acid and utilize both various sugars and starch as carbon sources (Yin *et al*., 1997 : Soccol *et al*., 1995 ; Yu and Hang, 1989). Lactic acid production using *Rhizopus oryzae* seems to be a viable alternative because it can grow on minimal liquid medium and on solid medium (Soccol *et al*., 1994).

Starch has been considered for use as a raw material for various fermentation because of its abundance and low price. However, when high concentration of starch is used in medium, an increase in viscosity of the medium due to gelatinization by heat will reduce the microbial growth. Recently, Yahiro *et al*. (1997) reported that itaconic acid could be produced from the medium consisting of 140 g/l corn starch hydrolyzed by nitric acid and higher than 60 g/l of itaconic acid was produced from *Aspergillus terreus*. The lactic acid production from *Rhizopus oryzae* using corn starch partially hydrolyzed with α-amylase or diluted hydrochloric acid as the sole carbon source was studied by Yin *et al*. (1997). The highest lactic acid concentration of 82 g/l was obtained from the

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medium containing 120 g/l corn starch (hydrolyzed with α-amylase) on day 4 of cultivation in a jar fermentor.

In this study, the optimum medium for the production of L-lactic acid in shake flasks was investigated when raw cassava starch pretreated with hydrochloric acid was used as a sole carbon source. Moreover, the influence of aeration rate and agitation speed on the L-lactic acid production in a jar fermentor was also determined.

MATERIALS AND METHODS

Microorganism and inoculum

Rhizopus oryzae NRRL 395 was obtained from the Northern Regional Research Center, Peoria, Illinois, and it was used throughout this study. The fungus was maintained on PDA slant and transferred to fresh slants every 4 months.

An inoculum was a spore suspension obtained by suspending spores from seven-day-old culture grown on PDA in 250 ml Erlenmeyer flask at 30°C with sterile distilled water containing 0.05% Tween 80 and filtering through a pad of sterile cotton wool. Spore concentration was determined using haemocytometer. Both flask culture and fermentor were inoculated with the inoculum so that the final concentration was 2-5 $\times 10^5$ spores/ml.

Medium and cultivation

Culture medium was as follows (g/l): carbon source, 120.0; $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.25; and $ZnSO_4.7H_2O$, 0.04. The initial pH was adjusted to 6.0. When raw cassava starch was used as a carbon source, it was partially hydrolyzed with 3 N HCL. The acid was added to the starch solution until pH 2.0 was reached and then the solution was autoclaved at 121°C for 30 min. The medium was sterilized at 121°C for 15 min.

In shake flasks, the fungus was grown in 250 ml Erlenmeyer flasks containing 50 ml of the medium. Four flasks were used in each experiment. The flasks were incubated for 5 days at 30°C on a rotary shaker with the shaking speed at 200 rpm. In order to prevent a decrease in pH, 3 g of sterilized calcium carbonate was added to each flask after 24 h of cultivation. Various concentrations of raw cassava starch, nitrogen sources, and K_2HPO_4 were investigated for L-lactic acid production. The optimum parameter from each experiment was used in the next experiment.

In a controlled fermentor, a 5-l jar fermentor (BIOSTAT-B, B.Braun, Germany) with instruments for the control of agitation speed, aeration rate, pH and temperature were used in this study. The fermentor was filled with 2.5 l of optimum medium and then autoclaved at 121°C for 40 min. The fermentation temperature was controlled at 30°C. The influence of aeration rate and agitation speed on lactic acid production was evaluated at 0.5, 1.0 and 1.5 vvm under 200, 300 and 400 rpm. After 24 h of cultivation, 150 g of sterilized calcium carbonate was added.

Samples were taken daily for analyses of pH, dry mycelial weight, total sugar, reducing sugar and lactic acid concentrations.

Analytical methods

pH value of culture broth was measured by a pH meter (Model HM-7E, TOA). Dry mycelial weight was measured by filtering the culture broth through filter paper, washing the mycelium with 3N HCl, following by distilled water and then drying at 90°C to constant weight. The supernatant fluid was used for total sugar, reducing sugar and lactic acid determinations. For the total sugar, the phenol – sulfuric acid method (Dubois, *et al*., 1956) was used. The DNS method (Bernfeld, 1955) was used for the reducing sugar measurement. The concentration of lactic acid was measured by the Barker – Summerson method (Barker, 1957).

RESULTS AND DISCUSSION

Effect of raw cassava starch on L- lactic acid production

Raw cassava starch concentrations of 50, 100, 120, 150 and 180 g/l were used as carbon source in the medium for flask cultivation. The results are shown in Figure 1. An increase of initial raw cassava starch concentration from 50-120 g/l, higher levels of L-lactic acid were produced. However, a further increase of the initial concentration over 120 g/l reduced the L-lactic acid production. The highest L-lactic acid concentration of 58.57 g/l was obtained in the medium containing 120 g/l of raw cassava starch on day 5 of cultivation. This result was lower than that reported by Yin *et al.* (1997) showing that L-lactic acid concentration of 95.10 g/l was produced by *R. oryzae* in the medium containing 120 g/l of corn starch (hydrolyzed with hydrochloric acid). This might be due to the difference in the compositions of cassava starch and corn starch.

Effect of nitrogen sources on L- lactic acid production

The effect of various nitrogen sources on L-lactic acid production was investigated using 2 g/l of ammonium sulfate, ammonium chloride, ammonium nitrate, yeast extract and corn steep liquor. The results are shown in Figure 2. The highest L-lactic acid concentration of 57.00 g/l was produced when ammonium sulfate was used as a nitrogen source. In case of ammonium chloride, 51.30 g/l of L-lactic acid was produced. When corn steep liquor was used as nitrogen source, L-lactic acid production was very low.

The effect of initial ammonium sulfate concentration was also determined. As shown in Figure 3, the optimum concentration of ammonium sulfate providing L-lactic concentration of 60.80 g/l on day 5 of cultivation was 3.0 g/l. It could be concluded that ammonium sulfate was the most effective nitrogen source for L-lactic acid production in *R. oryzae*. This is in agreement with Soccol *et al*. (1994) and Kosakai *et al.* (1997). However, Yin *et al*. (1997) showed that ammonium sulfate at the concentration of 1.35 g/l resulted in the maximum L-lactic acid production by *R.oryzae* when corn starch was used as a sole carbon source.

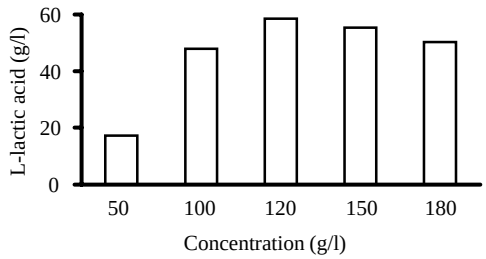

Figure 1 Effect of raw cassava starch concentration on L-lactic acid production.

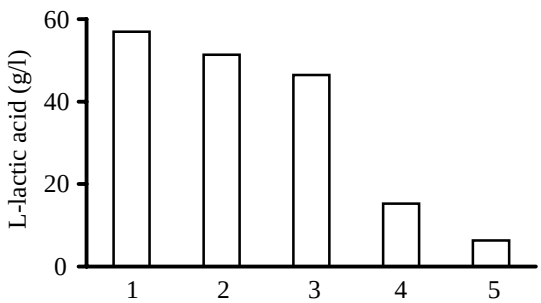

Figure 2 Effect of various nitrogen sources on L-lactic acid production.
(1, $(NH_4)_2 SO_4$; 2, NH_4Cl: 3, NH_4NO_3; 4, yeast extract; 5, corn steep liquor)

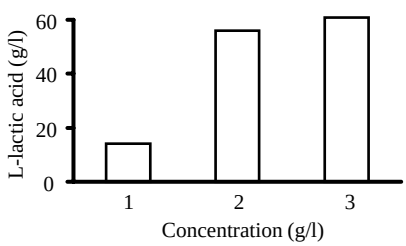

Figure 3 Effect of $(NH_4)_2 SO_4$ concentration on L-lactic acid production.

Effect of KH_2PO_4 concentration on L-lactic acid production

KH_2PO_4 concentrations of 0, 0.3, 0.6 and 1.0 g/l were investigated. The results are shown in Figure 4. The maximum L-lactic acid with the concentration of 63.33 g/l was obtained with 1.0 g/l KH_2PO_4. As the concentrations of L-lactic acid were reduced to 58.80 g/l and 59.47 g/l in the medium containing 0.3 g/l and 0.6 g/l KH_2PO_4, respectively.

In conclusion, an optimized medium for L-lactic acid production by *R.oryzae* NRRL 395 contained (g/l) : raw cassava starch, 120.0; $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; $ZnSO_4.7H_2O$, 0.04 and pH 6.0. Figure 5 shows time courses of growth and L-lactic acid production of *R.oryzae* cultivated in the optimized medium using shake flasks. The production of L-lactic acid appeared related to the mycelial growth and reached the maximum concentration of 68.32 g/l on day 5 of cultivation. Then it gradually decreased. L – lactic acid yield (based on an initial cassava starch concentration) and productivity were 57% and 0.57 g/lh, respectively.

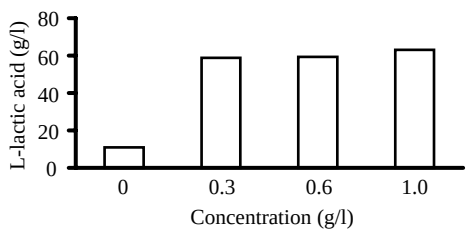

Figure 4 Effect of KH_2PO_4 concentration on L-Lactic acid production.

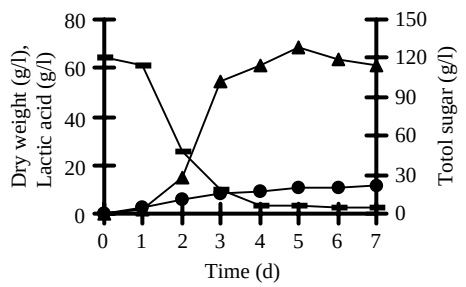

Figure 5 Time courses of L-lactic acid fermentation by *R.oryzae* in shake flasks. (▲, L-lactic acid concentration; ●, dry weight; –, total sugar)

Effect of aeration rate and agitation speed on L-lactic acid production in a jar fermentor

Three aeration rates of 0.5, 1.0 and 1.5 vvm and three agitation speeds of 200, 300 and 400 rpm were combined giving nine conditions for L-lactic production. The results are summarized in Table 1. An increase in aeration rate increased specific growth rate (μ) at all agitation conditions studied. The maximum specific growth rate of 0.06 h^{-1} was obtained at the agitation rate of 400 rpm with 1.5 vvm. At the agitation rate of 400 rpm, L- lactic acid production, L – lactic acid yield ($Y_{P/S}$) and productivity were increased when the aeration rate was raised from 0.5 to 1.5 vvm. L – lactic acid production was highest (54.62 g/l) at the aeration rate of 1.5 vvm on day 4 of cultivation. At agitation rate of 200 rpm, L – lactic acid production was increased by an increase in aeration rate of 0.5 to 1.0 vvm (36.33 and 41.68 g/l with 0.5 and 1.0 vvm, respectively) while an increase to 1.5 vvm only 42.41 g/l of L – lactic acid was obtained. It was found that L – lactic acid yield and productivity were independent of the aeration rate tested. For the aeration rate of 300 rpm, L- lactic acid production, L – lactic acid yield and productivity were not different when the aeration rate was increased from 0.5 to 1.5 vvm. Comparing to various conditions, it was elucidated that the maximum L-lactic acid concentration was 54.62 g/l and L – lactic acid yield of 0.49 g/g substrate with a corresponding the productivity of 0.56 g/lh were obtained on day 4 of cultivation when the fermentation was carried out at the agitation speed of 400 rpm and the aeration rate of 1.5 vvm (Figure 6).

From these results, the values of specific growth rate increased with increasing the aeration rate demonstrating that growth was dependent on

Table 1 Kinetic parameters of L-lactic acid fermentation by R.oryzae in a jar fermentor.

Agitation (rpm)	Aeration (vvm)	μ (h^{-1})	L-lactic acid (g/l)	$Y_{p/s}$ (g/g)	Productivity (g/lh)
200	0.5	0.026	36.33	0.37	0.38
200	1.0	0.030	41.68	0.36	0.43
200	1.5	0.035	42.41	0.38	0.44
300	0.5	0.036	40.35	0.40	0.42
300	1.0	0.042	41.94	0.41	0.44
300	1.5	0.046	42.32	0.41	0.44
400	0.5	0.048	44.88	0.44	0.46
400	1.0	0.054	49.80	0.46	0.51
400	1.5	0.060	54.62	0.49	0.56

μ = specific growth rate; Productivity = g L – lactic acid produced per liter per hour
$Y_{p/s}$ = L-lactic acid yield (g L – lactic acid produced per g total sugar consumed)

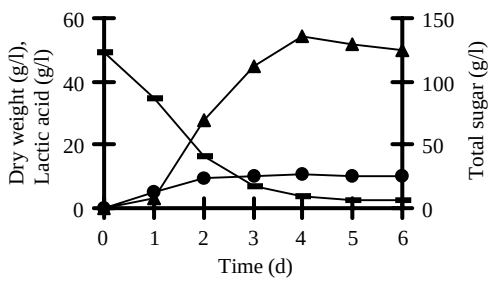

Figure 6 Time courses of L-lactic acid fermentation by *R.oryzae* in a jar fermentor at agitation speed of 400 rpm and aeration rate of 1.5 vvm.
(▲, L-lactic acid concentration; ●, dry weight; –, total sugar)

oxygen supply. In case of the agitation rate of 400 rpm, production, yield and productivity of L – lactic acid were higher than those obtained at the aeration rates of 200 and 300 rpm. This might be due to the medium homogeneity and higher transfer rates of substrate and oxygen resulting from more thorough mixing. In addition, an increase in aeration rate also increased oxygen supply in the medium which promoted not only mycelial growth but also L – lactic acid production. *Rhizopus oryzae* NRRL 395 produced high level of L-lactic acid in aerobic culture while ethanol was produced instead in oxygen limited culture (Soccol *et al*., 1995). Similar result reported by Skory *et al*. (1998) showed that ten-fold increase in alcohol dehydrogenase activity was obtained under oxygen limited condition compared to aerobic condition during L-lactic acid fermentation by *R. oryzae.*

Comparison of L-lactic acid production by *R. oryzae* NRRL 395 between shake flasks and a jar fermentor

The results are summarized in Table 2. For flask culture, the highest L-lactic acid concentration of 68.32 g/l was obtained on day 5 of cultivation with a productivity of 0.57 g/lh and L-lactic acid yield of 0.59 g/g substrate. In jar fermentor, the maximum L-lactic acid production was 54.62 g/l on day 4 of cultivation with a productivity of 0.56 g/lh and a product yield of 0.49 g/g substrate. The reason for L-lactic acid production in the jar fermentor was lower than in the shake flasks might

Table 2 Comparison of L-lactic acid production by *R. oryzae* in shake flasks and in the fermentor.

Fermentation type	Time (day)	μ (h^{-1})	L-lactic acid (g/l)	$Y_{p/s}$ (g/g)	Productivity (g/lh)
Shake flasks	5	0.038	68.32	0.59	0.57
Fermentor@	4	0.060	54.62	0.49	0.56

@ = agitation speed of 400 rpm and aeration rate of 1.5 vvm.

be due to a difficulty in morphological problems and wall growth of mycelium in the fermentor. In the early growth phase of *R. oryzae*, fluffy mycelia were formed. After 2-3 days of culture, the mycelia clung to baffles and impellers causing clumped mycelia form. This might be due to diffusional limitation of oxygen and substrate transfer to the inner aggregated mycelia resulting in low L-lactic acid production. *R. oryzae* was characterized by hyphal growth, formation of a big pellet – like cake, and twisting of hyphae in baffles and impellers of a jar fermentor (Kosakai *et al*., 1997; Yin *et al*., 1997) and Kosakai *et al*. (1997) also reported that L-lactic acid production in a jar fermentor by *R.oryzae* was enhanced by addition of mineral support and polyethylene oxide in the medium. It was found that the yield of lactic acid was 1.7-fold higher than that without the support and polyethylene oxide. This might be due to the formation of dispersed mycelia in the culture broth. Yin *et al*. (1998) reported that small mycelial pellets were effective for the production of lactic acid by *R.oryzae* in an air-lift bioreactor.

CONCLUSION

In this study, we found that *R.oryzae* NRRL 395 has the ability to produce L-lactic acid by using partially hydrolyzed raw cassava starch and ammonium sulfate as carbon and nitrogen sources, respectively. The production of L-lactic acid in the jar fermentor by *R.oryzae* is affected by both agitation and aeration. However, in the fermentor L-lactic acid production was lower than obtained in the shake flask culture. So, it is difficult to produce high yield of L-lactic acid in the stirred tank bioreactor. Further investigation will be required to use proper type of bioreactor and to optimize bioreactor operation for mass production of L-lactic acid by *R.oryzae*

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