0273-2289/06/129–132/844–853/$30.00

Optimization of L-(+)-Lactic Acid Production Using Pelletized Filamentous *Rhizopus oryzae* NRRL 395

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Abstract

Lactic acid is used as a food additive for flavor and preservation and a precursor 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 improve these problems. In this study, factors that affect lactic acid production in pelletized flask cultures using *R. oryzae* NRRL 395 were investigated in detail. Completely randomized designs were used to determine the influence of culture temperature, time, concentration of glucose, and inoculum size. Lactic acid fermentation using clump and pellet morphologies were performed in a 5 L 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 92 g/L. The data presented in the article can provide useful information on optimizing lactic acid production using alternative source materials.

Index Entries: *Rhizopus oryzae*; lactic acid; pellet morphology.

Introduction

Lactic acid ($CH_3CHOHCOOH$) is a colorless compound that is important in both international and domestic markets, and in several industries. Lactic acid is currently widely used as an acidulant, flavoring, and preservative in food industries. There is also increased interest in its

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application in the production of poly-lactic acid, which can be used as biodegradable plastic. Both the polymers and copolymers derived from lactic acid are especially attractive for biomedical application because of their biocompatibility, body absorption, and blood compatibility. The increased use of lactic acid in exciting applications and potential for use in biodegradable plastics has made producing lactic acid an attractive investment.

Rhizopus oryzae NRRL 395 is known to be a strain producing optically pure L-(+)-lactic acid *(1–5)*. However, there are some disadvantages in the fungal fermentative organic acids production. The fungi tend to form cotton-like mycelia in reactors, therefore the reactor is difficult to control under a homogeneous condition. Mass transfer and oxygen transfer through cotton-like mycelium are much more difficult than other forms of morphology. In addition, cotton-like morphology makes fungal biomass reuse impossible. All above factors ultimately lead to a low efficiency and yield of organic acid fermentation process *(6)*. Growing fungi in pellet form can significantly improve the mass transfer condition and benefits for lactic acid production.

In this article, the lactic acid production using palletized *R. oryzae* has been studied. The optimal conditions for the production of lactic acid with pellets were determined in flask culture. Enhanced production of lactic acid was conducted in a stirred fermentor using fed-batch culture.

Methods and Materials

Microorganism and Spore Culture Method

The fungus *R. oryzae* NRRL 395 (ATCC 9363) was obtained from the American type culture collection (Manassas, VA). The fungus was first grown on potato-dextrose agar (Difco, Sparks, MI) slants at 30°C for 7 d. 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 24 g/L potato dextrose broth (PDB) (Difco, Sparks, MI) with 6 g/L $CaCO_3$. In terms of achieving pellet form, spore solution was inoculated into a 125-mL Erlenmeyer flask containing 50-mL seed medium with a spore concentration of 1×10^6 spore/mL and cultured at 27°C with 170 rpm shake speed for one day. The broth was used as the pellet seed for the following experiments. The diameter of seed pellet was 1.13 ± 0.22 mm (Fig. 1). As for the culture of clump seed used in the section of lactic acid production in stirred fermentor, culture conditions were same as the culture of pellet seed, except that the culture was in the incubator without shaking.

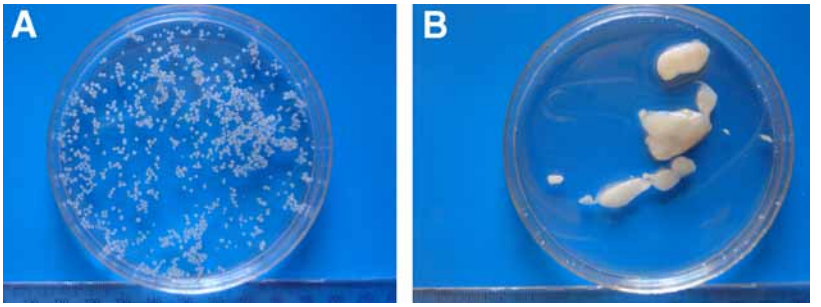

Fig. 1. The morphology difference of pellet and clump from seed culture. **(A)** is the seed pellets; **(B)** is the seed clumps.

Effects of Glucose Concentration, Reaction Time, and Temperature on Lactic Acid Production in Flask Culture

The experiment was carried out by a completely randomized design, with three replicates of 18 culture combinations. Three glucose concentrations (60, 100, and 120 g/L) and three culture durations (2, 3, and 4 d) were studied at two different temperatures (27°C and 30°C). The shake speed was 170 rpm. Calcium carbonate as the neutralizer was added into the flasks in order to maintain the pH value of approx 6.0 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 0.45 g 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 different amount of solid glucose into PDB medium (PDB contains 20 g/L glucose). All media were autoclaved at 121°C for 15 min before 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 24 g/L PDB, 100 g/L glucose and 60 g/L $CaCO_3$. The culture temperature was 27°C. And the culture duration was 4 d. Other culture conditions were same as described in 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 Scienific, NJ) equipped with a pH controller was used to carry out lactic acid fermentation. For batch culture, 2 L of deionized (DI) water, 48 g PDB and 200 g glucose were added into fermentor and autoclaved at 121°C for 15 min. The aeration rate and agitation speed were 1 vvm (volume/volume/minute) and 200 rpm, respectively. The pH was adjusted at 7.0 ± 0.1 using 20% calcium hydroxide. 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 fermentor and cultured at 27°C for 3 d. For fed-batch fermentation, culture conditions were the same as the batch fermentation except that the inoculum size was 0.45 g dry biomass/L, and extra 150 mL medium with 80 g glucose and 12 g PDB powder was fed into the fermentation on d 2.5 and 3.5.

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 using the statistical analysis system program 8 (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. Because calcium carbonate was added to neutralize the pH, the lactate concentration in the broth was reported instead of the lactic acid concentration. The lactate in broth was analyzed using a Dionex DX-500 system (Sunnyvale, CA) including an AS11-HC (4 mm 10–32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler *(5)*. Glucose concentration was measured using the modified dinitrosalicylic acid method *(7)*. Dry biomass was determined by washing the pellet mycelia with 6 *N* HCl and then washing to pH 6.0 with DI water. The washed biomass was dried at 100°C over night before weight analysis. The diameter of seed pellets was determined using an Olympus microphotograph (Tokyo, Japan).

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 concentrations at 27°C and 30°C was shown in Figs. 2–4. At the culture duration of 2 d, lower glucose concentrations had higher lactate yields. 60 g/L glucose produced 33 g/L lactate (corresponding lactate yield of 55%) at both culture temperature of 27°C and 30°C; and 100 g/L and 120 g/L glucose at 30°C produced more lactate than that at 27°C (Figs. 2 and 3). Then, lactate yields from both 100 and 120 g/L glucose increased following the increase of culture duration, whereas the yields from 60 g/L glucose kept stable at approx 55% (Figs. 2 and 3). After 3 d of culture duration, the statistical analysis of pair wise comparison

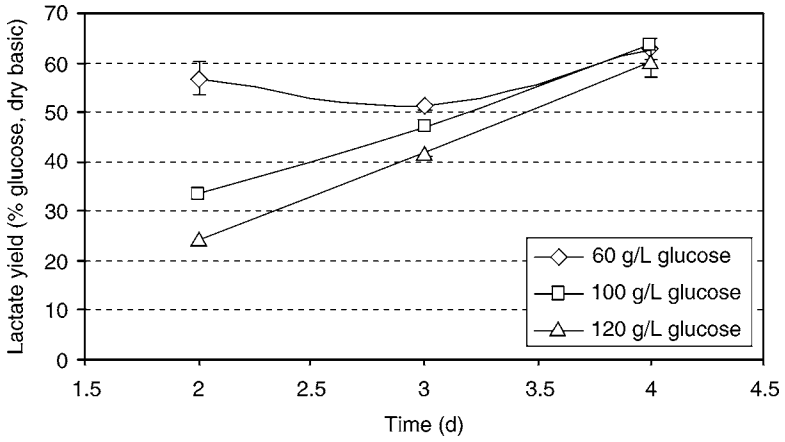

Fig. 2. Effects of glucose concentration on lactic acid yield. Fermentation was performed at 27°C with 0.45 g/L dry biomass inoculum size. Data was the average of triplicates with standard deviations ($n = 3$).

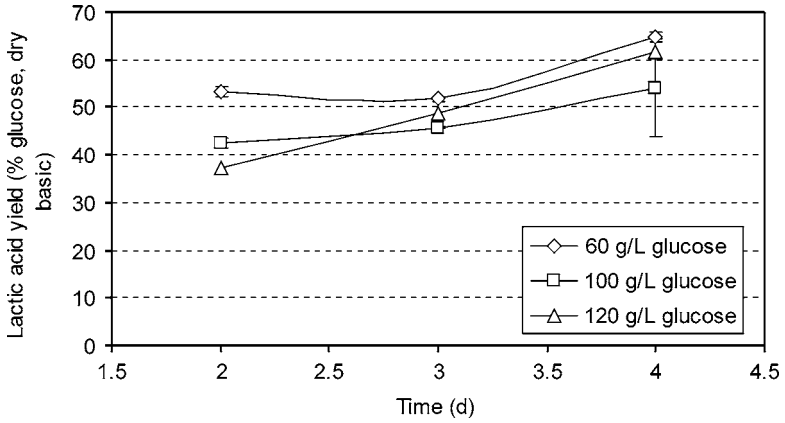

Fig. 3. Effects of glucose concentration on lactic acid yield. Fermentation was performed at 30°C with 0.45 g/L dry biomass inoculum size. Data was the average of triplicates with standard deviations ($n = 3$).

showed that there were no significant ($p > 0.05$) differences on lactate yields between each other of the three glucose concentrations, at each individual culture durations of 3 and 4 d (Figs. 2 and 3), in addition there were also no significant ($p > 0.05$) difference between culture temperatures of 27°C and 30°C at the culture duration of more than 3 d (Figs. 2 and 3). The highest lactate yield of 60% has been reached at 4 d of culture time, no matter what glucose concentration and culture temperature were. These results elucidated that 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 durations of 2 and 3 d. Glucose concentration had no significant

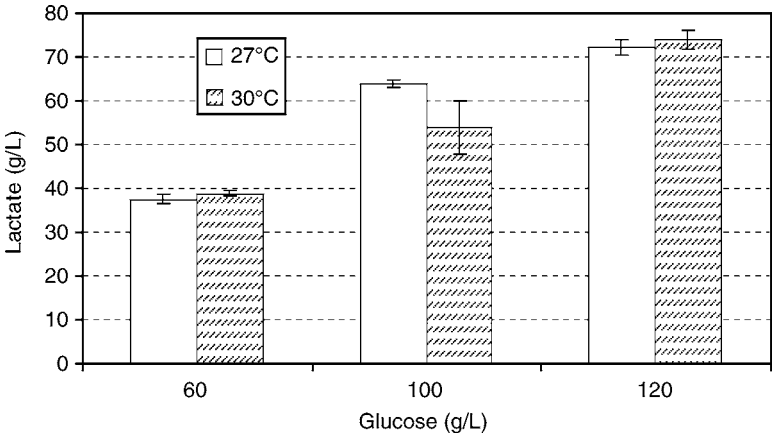

Fig. 4. Effect of temperature on lactate production. Fermentation was cultured for 4 d with 0.45 g/L dry biomass inoculum size. Data was the average of triplicates with standard deviations ($n = 3$).

influence on lactate yield at long culture durations, meanwhile, the higher glucose concentration produced more lactate at the same yield. Thus, the highest lactate concentration of 72 g/L was obtained at 4 d of culture duration with 120 g/L glucose concentration in the medium (Fig. 4). Therefore, 120 g/L glucose concentration and 4 d of fermentation at either temperature of 27°C and 30°C were chosen for the experiments in the following sections.

Effects of Inoculum Size on Lactic Acid Production in Flask Culture

Figure 5 and Table 1 showed that the lactate concentration varied with inoculum size. The inoculum size of 0.68 g/L dry biomass produced significantly less lactate than other inoculum sizes. The lowest lactate concentration of 37.3 g/L was obtained from the inoculum size of 0.68 g/L at 4 d of culture duration (Fig. 5). Meanwhile, the other three inoculum reached average lactate concentration of 78.5 g/L in the same culture duration. The Tukey-Kramer Multiple Comparison test confirmed that there was no significant ($p > 0.05$) difference on lactate concentration between each other 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 and 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 production.

Comparison of Lactic Acid Production Using Different Fungal Morphologies in a Stirred Tank Batch Culture

Lactic acid fermentation with clump and pellet morphologies was performed in a 5-L stirred fermentor (Fig. 6). The results demonstrated that

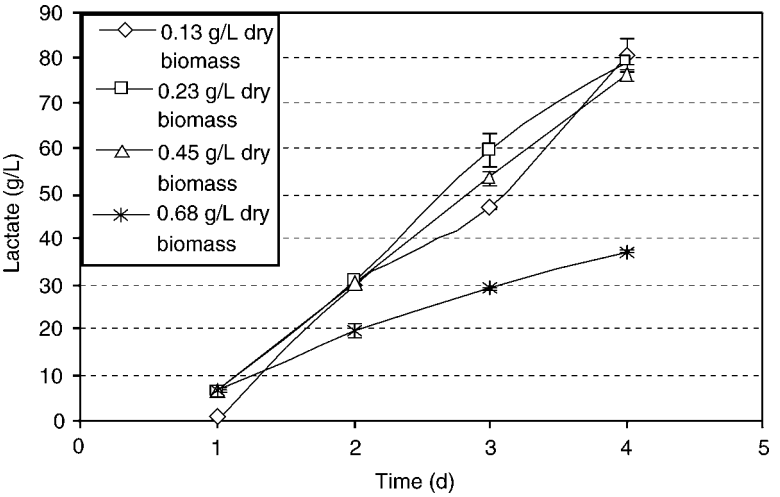

Fig. 5. Effects of inoculum size on lactic acid production. Fermentation was performed at 27°C with 120 g/L glucose in medium. Data was the average of triplicates with standard deviations ($n = 3$).

Table 1
Tukey-Kramer Multiple-Comparison Test of Lactate Concentration*

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

*This report provides multiple comparison tests for all pair wise differences between the means at $\alpha = 0.05$. Fermentation was performed for 4 d at 27°C with 120 g/L glucose concentration.

the there were significant ($p < 0.05$) difference on lactic acid production between clump and pellet morphologies. The lactate concentration of clump fermentation reached to 33 g/L in 2.5 d of culture duration whereas the pellet fermentation produced 60 g/L. The data indicated that the lactic acid production was significant 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 condition obtained from the previous section of the flask culture. Figure 7 showed that lactate concentration from the part of batch fermentation reached 50 g/L in 2 d, then two extra glucose solutions were fed into the fermentor at the culture durations of 2.5 and 3.5 d,

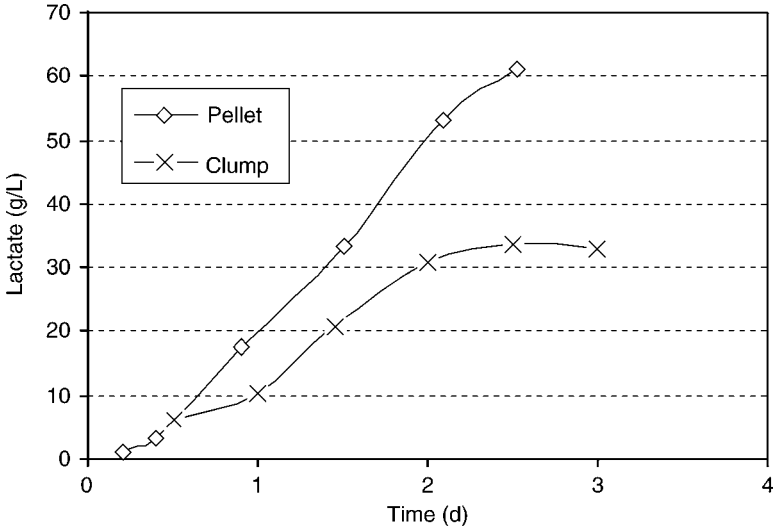

Fig. 6. Batch culture of lactic acid production using pellet and clump morphology in a 5-L stirred tank. The aeration rate and agitation speed were 1 vvm and 200 rpm, respectively. The pH was adjusted at 7.0 ± 0.1 with 20% $Ca(OH)_2$ and 0.23 g/L dry biomass seed was inoculated into fermentor and cultured at 27°C.

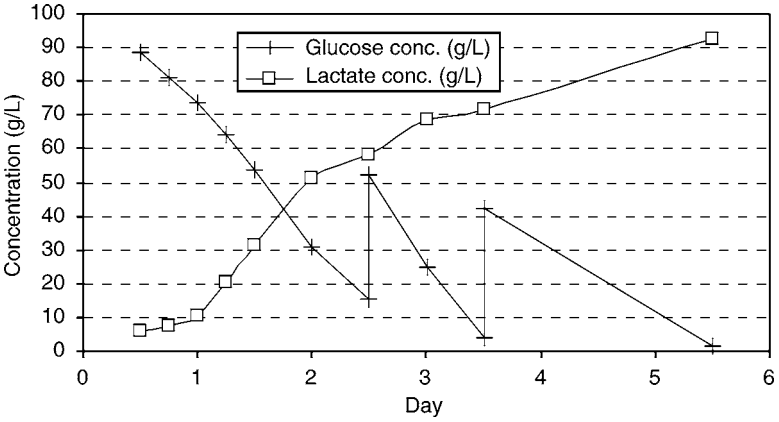

Fig. 7. Lactic acid production by fed-batch culture of *R. oryzae* NRRL 395 in a 5-L stirred tank fermentor with the aeration rate and agitation speed were 1 vvm and 200 rpm, respectively. The pH was adjusted at 7.0 ± 0.1 with 20% $Ca(OH)_2$ and 0.45 g/L dry biomass seed was inoculated into fermentor and cultured at 27°C for 5.5 d and 150-mL fed-medium with 80 g glucose and 12 g PDB powder was added at 2.5 and 3.5 d.

respectively. After feeding, the lactate concentration of fed-batch culture eventually reached 92 g/L in 5.5 d. The data also presented the difference of production rates on different phases of batch fermentation and fed-batch fermentation. The production rate after feeding was 0.53 g/L h, which was much slower than 1.02 g/L h of the rate before feeding (Fig. 7). The low production rate was mainly caused by the formation of calcium

Table 2
Comparison of Different Processes of Lactic Acid Production Using *R. oryzae*

Fermentation conditions*	Reactor	Lactate (g/L)	Yield (g/100g glucose)	Productivity (g/L h)	Reference
Cells immobilized on cotton cloth	Rotating bed	126	90	2.5	(8)
Pellets	Air-lift	86	–	1.07	(6)
Immobilized cells	Fluidized bed	73	65	1.6	(9)
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

*Glucose was used as substrate for all fermentations.

lactate crystalline and substrate limitation. During fermentation process, the product of calcium lactate started crystallizing out once the lactate concentration reached 70 g/L. The calcium lactate crystals were attached on the surface of pellets and influenced the mass/oxygen transfer though pellets, and further reduced the production rate.

It is well known that production of fermentation processes are mainly controlled by both product inhibition and substrate limitation. In this particular case, it was apparent that both the substrate limitation and product inhibition played equal 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 crystalline has to be eliminated. Other alkalis such as sodium hydroxide and ammonia, producing soluble lactate during fermentation process, could be good alternatives of calcium hydroxide. However, high concentration of those cations could inhibit the lactic acid production. Thus, mixture of alkalis such as calcium hydroxide, sodium hydroxide, and ammonia could be used as neutralizers to control pH in fed-batch culture, which will result in low concentration of each cation and 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 on a support surface. The performances were apparently improved. The best results obtained in these studies are: 126 g/L final lactate concentration in broth, 2.5 g/L h productivity, and 90% lactic acid yield from the immobilized

fungal biomass on a cotton cloth *(8)*. 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 still highlys efficient and much more economical. Yin *(6)* formed small pellets in an air-lift fermentor to produce lactic acid. The lactic acid concentration from the air-lift fermentor was 86 g/L (Table 2). Compared with the pellet fermentation of 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.

Conclusions

Pelletized morphology significantly increases lactic acid production. The optimal fermentation conditions of pelletized flask culture with *R. oryzae* NRRL 395 were 120 g/L of glucose concentration, 0.13–0.45 g/L dry biomass inoculum size, 27–30°C culture temperature and 3–4 d fermentation time. Under these conditions, the lactic acid productivity, yield, and lactate concentration reached 1.1 g/L h, 63%, and 76 g/L, respectively. Fed-batch culture can significantly increase lactate concentration in broth. The final lactate concentration reached 92 g/L after 5.5 d of fermentation. To enhance lactic acid concentration, productivity, and yield in broth, future work should focus on the fed-batch fermentation using mixed alkali to adjust pH.

Acknowledgments

This research was supported by the Washington State IMPACT Center (International Marketing Program for Agricultural Commodities and Trade) and the Agricultural Research Center of Washington State University. We are grateful to Craig Frear, Mike Richardson, and Dan Hardesty for editorial assistance.

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