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Production of Lactic Acid from Raw Sweet Potato Powders by *Rhizopus Oryzae* Immobilized in Sodium Alginate Capsules

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Abstract *Rhizopus oryzae* immobilized in calcium alginate was applied in lactic acid fermentation with unhydrolyzed raw sweet potato powders as the sole carbon source. The effects of sodium alginate concentration, calcium chloride concentration, and the immobilized bead diameter on lactic acid production were investigated. Increase in sodium alginate concentration during the gelation process would harden the immobilized capsule, which led to a decrease in lactic acid production. The increase in calcium chloride would increase the thickness of the immobilized capsule, which would increase the mass transfer resistance. Nevertheless, while the calcium chloride was lower than 15%, it would not have obvious effects on lactic acid production. A larger bead could have more space for cell growth, which led to the maximum lactic acid production observed at the 5-mm bead diameter. Moreover, results of repeated-batch operation suggested that immobilized cells could have higher stability in lactic acid production than free cells. The total cumulative lactic acid in immobilized-cell operation could increase by 55% as compared with free-cell operation after 216 h (seven repeated-batches), and no loss of amylolytic activity was observed. The results indicated that immobilized *R. oryzae* by Ca-alginate could be suitable for lactic acid production from unhydrolyzed raw potato powders.

Keyword *Rhizopus oryzae* · Repeated-batch · Immobilization conditions · Bead size · Calcium chloride

Introduction

Lactic acid production from renewable crop biomass via fermentation was a known fermentation process, and this biological process had been widely used in most current lactic acid manufacturing procedures. The use of lactic acid, other than being an acidulent and preservative in the food industry, has recently been focused on the polymerization of

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lactic acid to form biodegradable materials polylactic acid and has received a great deal of attention. Henceforth, many lactic-acid-producing microbial species have been isolated and characterized for their efficient lactic-acid-producing potentials. In recent years, a fungus called *Rhizopus oryzae* has attracted much research interest, for it is one of the useful organisms which can produce the most optical pure L(+)-lactic acid, and is capable of simultaneous saccharification and fermentation (SSF) from untreated agricultural crops [1, 2]. The SSF ability of *R. oryzae* can achieve much more effective bioconversion of carbohydrate materials to lactic acid by mediating the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation of the derived glucose into a single step. Such SSF ability of *R. oryzae* can make it economically attractive in the scale-up production of lactic acid [3–5].

The SSF ability of *R. oryzae* has made commercial lactic acid production more feasible. However, commercial lactic acid production through the cultivation of *R. oryzae* was significantly restricted by the filamentous morphology of *R. oryzae* formed in bioreactors. In submerged cultures, *R. oryzae* could grow in any of the following morphological forms, including entangled clumps, small pellets, and flocs, depending on a variety of factors, such as inoculated spore concentration, agitation speed, and addition of flocculation agents [6, 7]. In the case of fermentation of filamentous morphology, mass transfer limitations were commonly a serious problem in lactic acid production, and growth of the mycelium on impellers or electrodes would further hamper the optimal control and the subsequent product recovery process. Therefore, confining or entrapping cell growth inside a capsule (or a matrix) through some immobilization processes might be a good way to avoid random mycelium growth.

Cell immobilization was one of the most promising methods in high cell density culture and had been widely used in many biological applications. Immobilized cells exhibited many advantages over suspended cell cultivation, such as the relative ease of product separation, reuse of biocatalysts, high volumetric productivity, improved process control, and reduced susceptibility of cells to contamination [8, 9]. Among various immobilization methods, entrapment in calcium alginate capsule had commonly been used in the immobilization of lactic acid bacteria (LAB). Several studies on lactic acid fermentation using Ca-alginate-entrapped LAB had been reported [10]. However, most researches focused on lactic acid production using pre-hydrolyzed starchy materials or using glucose by immobilized cells. The effects of immobilization parameters on lactic acid production from unhydrolyzed raw potato powders have not been reported.

In this paper, the production of lactic acid from unhydrolyzed raw sweet potato powders by immobilized *R. oryzae* in Ca-alginate capsules was described. The effects of alginate capsule size, alginate concentration, and calcium chloride concentration on lactic acid production would be investigated. Finally, the comparison of lactic acid production from free cells and Ca-alginate immobilized cells in a repeated-batch operation would be presented to demonstrate the advantages of using immobilized cells in lactic acid production.

Materials and Methods

Microorganism and Medium

The freeze-dried *R. oryzae* BCRC 33071, purchased from the Bioresource, Collection, and Research Center (BCRC), Taiwan, was mixed with sterilized water and spread on PDA.

After incubation at 30 °C for 5–7 days, plenty of black spores were found to have grown on hypha. Water sterilized with Trition X-100 (0.1% *w*/*v*) was employed to prepare the spore suspension at a concentration of 5×10^6 counts/ml for further inoculation. Potato-dextrose broth was used as the seed medium. In batch lactic acid production, the fermentation medium (per liter) comprised 90 g of raw sweet potato powders, 2 g of NH_4NO_3, 0.25 g of $MgSO_4$ $7H_2O$, 0.25 g of $ZnSO_4$ 7 H_2O, 0.3 g of K_2HPO_4, and 0.3 g of KH_2PO_4. Calcium carbonate was used as the pH neutralizer to prevent from the pH collapse, which was added in the flask at 60 g/l. The raw sweet potato (species: Taiwan potato #57) was purchased from a local supermarket. Before further pulverization, the raw sweet potato was dried in an air-heated oven at 60 °C for over 24 h and the majority of dry sweet potato was milled to potato powders of particle size less than 1 mm by a homogenizer. The starch content in this sweet potato powders was 57% (gram of starch/gram of dry potato powders) based on data obtained from the website of Council of Agriculture Execute Yuan, Taiwan.

Immobilization Process

After 12 h of seed culture, the whole broth was then separated by centrifugation for 5 min at 3,000 rpm to remove the supernatants. The collected cells were suspended in a sterilized sodium alginate solution (concentration 0.5%, 1%, and 3%), and the obtained dispersion was extruded through a syringe into a gently stirred aqueous solution of calcium chloride (5%, 10%, 15%, and 20%) for forming the alginate capsules. The diameter of immobilized beads (1, 3, 4, and 5 mm) was controlled by the needle size of the syringe applied. The immobilized beads were washed with distilled water several times to remove the free cells and the alginate solution outside the capsules before being further applied in the experimental process.

Batch Fermentation Conditions with Ca-alginate Beads

Submerged fermentations of sweet potato powders were carried out in 250-mL Erlenmeyer flasks containing 50 mL of fermentation medium. The culture flasks were placed in an incubator shaker with an agitation rate of 150 rpm and 30 °C for 48 h.

1. The effects of sodium alginate concentration on lactic acid fermentation were examined using concentration such as 0.5%, 1%, and 3% (*w*/*v*), using inoculated bead diameter of 5 mm and 15% of calcium chloride.
2. The effects of bead diameter were studied at various bead diameters of 1, 3, 4, and 5 mm. The immobilization beads were prepared using 0.5% of sodium alginate and 15% of calcium chloride.
3. The effects of calcium chloride concentration on fermentations were investigated using concentrations such as 5%, 10%, 15%, and 20% (*w*/*v*) using inoculated bead diameter of 5 mm and sodium alginate of 0.5%.

Repeated-Batch Process

Immobilized beads of 5-mm diameter (prepared using sodium alginate of 0.5% and calcium chloride of 15%) and free cells were employed respectively in a repeated-batch operation for the stability comparison of lactic acid production. During this repeated-batch process, the initial 48 h of cultivation followed completely the same process described in the batch

operation. After 48 h, the whole broth was removed through a sterilized paper filter, but the biomass was retained inside the flask. Another 50 ml of fresh fermentation medium (using sweet potato powders at 90 g/l) with an extra 1 g of calcium carbonate were used for the next fermentation cycle. The culturing period in each cycle of repeated fed-batch process was 24 h, except for 48 h of cultivation for the first batch stage. Lactic acid concentration, residual starch concentration, and amylolytic enzyme activity were analyzed according to the analytical methods described below. The yield in this study was calculated according to the lactic acid production per gram of starch introduced (the starch content was 57% per gram of dry potato powders).

Enzyme Activity Assay

α-amylase activity was assayed mainly according to the method described by Lee et al. [11]. One milliliter of supernatant (15 min centrifuged at 1,500×*g*) was added to 10 ml of 1% soluble starch solution (strength of acetate buffer at 0.1 M, pH 4.8). The mixture was incubated at 30 °C for 10 min. Then, 1 ml of 2 N HCl was added to stop the enzyme reaction. From this mixture, 0.5 ml was withdrawn and added to a 10-ml mixture comprising iodine solution of 0.005% and potassium iodine solution of 0.05%, and the absorbance of the mixture was measured at 580 nm [12]. One unit of amylase activity was defined as the amount which produced 10% reduction in the intensity of blue color of amylase–iodine complex under the conditions described above.

Glucoamylase activity was assayed using a reaction mixture containing 1-ml soluble starch of 1% dissolved in 0.1 M acetate buffer (pH 4.8) and 0.5 ml supernatant (15 min centrifuged at 1,500×*g*). After 30 min of reaction at 30 °C, 1 ml of the mixture was withdrawn and the amount of glucose liberated was determined by the glucose analyzer (YSI). One unit of glucoamylase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per minute under the conditions described above.

Analysis Methods

Lactic acid concentration was determined by HPLC under the following conditions: column, Vercopack inertsil 7 ODS-3 (250×4.68 mm); temperature, ambient; mobile phase, 50 mM $(NH_4)_2HPO_4$, 1% H_3PO_4 in H_2O (pH≈2.2); flow rate, 1.0 ml/min; and UV detector (Hitachi) at 210 nm. Glucose analyzer (YSI corporate) was adopted as the glucose concentration measurement. The improved iodine assay developed by Xiao et al. [12] was employed to estimate the residual starch concentration. All experiments were conducted in triplicate (except for the repeated-batch trial), and the results were expressed as mean ± standard deviation.

Results and Discussion

Effects of Alginate Concentration

The data shown in Fig. 1 suggested that the concentration of alginate applied during the immobilization process would have a serious impact on lactic acid production. An increase in alginate concentration of up to 3% would lead to decrease in lactic acid production. The alginate concentration of 0.5% had the maximum concentration of 50.2±2.5 g/l, which would rapidly reduce to 37.4±1.6 g/l at alginate concentration of 3%. However, Ca-alginate

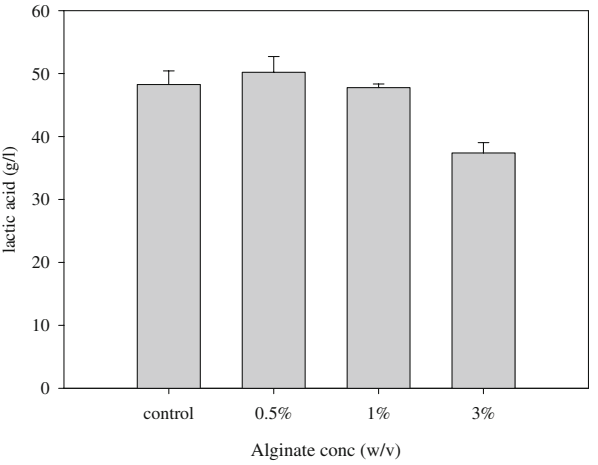

Fig. 1 Effects of alginate concentration (15% calcium chloride and 5-mm bead diameter) on lactic acid production as compared to the suspended free-cell culture (control)

immobilization at alginate concentration of 0.5% would not have much difference in lactic acid production as compared with the free-cell operation (control).

A general problem encountered in the cell immobilization process was the increase in mass transfer resistance, including nutrients and dissolved oxygen, owing to the thickness of capsule membrane. However, this did not seem to be a problem in this study when using raw potato powders as the substrate for lactic acid production by Ca-alginate immobilization at a low alginate concentration of 0.5%. As shown in Fig. 1, there is not much difference between the free-cell culture (control) and the immobilized-cell culture (at alginate concentration of 0.5%). In the bioconversion of undissolvable potato powders to lactic acid by encapsulated *R. oryzae*, the powders will be first hydrolyzed into soluble carbohydrates by amylolytic enzymes (saccharification step) in the broth, and then transferred into the alginate capsule for the subsequent fermentation steps. A similar lactic acid fermentation level between operation involving free cells (control) and immobilized cells (sodium alginate of 0.5%) implied that the increase in capsule thickness at sodium alginate of 0.5% would cause decrease in lactic acid production.

However, the increase in sodium alginate concentration would harden the beads during the gelation process, which would possibly impede further the transfer of nutrients [13]. Therefore, lactic acid production would rapidly reduce to 37.4±1.6 g/l at alginate concentration of 3% as compared with 50.2±2.5 g/l at alginate concentration of 0.5%. Even so, a low alginate concentration would lead to formation of a less dense bead with a low cross-linking lattice. It might be dubious whether such a soft bead could resist the high shear force inside the scale-up fermenter or not, if such immobilization process was applied in the fermenter.

In brief, the results indicated that lactic acid production by immobilized alginate beads might depend on the availability of nutrients. Under the premise of keeping immobilized beads intact, lower alginate concentration would attain higher lactic acid production.

Effects of Bead Size

In the trials with various alginate bead sizes, Fig. 2 clearly indicated that increase in alginate bead size would lead to increase in lactic acid production to the maximum of 53.6± 2.4 g/l of lactic acid at bead diameter of 5 mm.

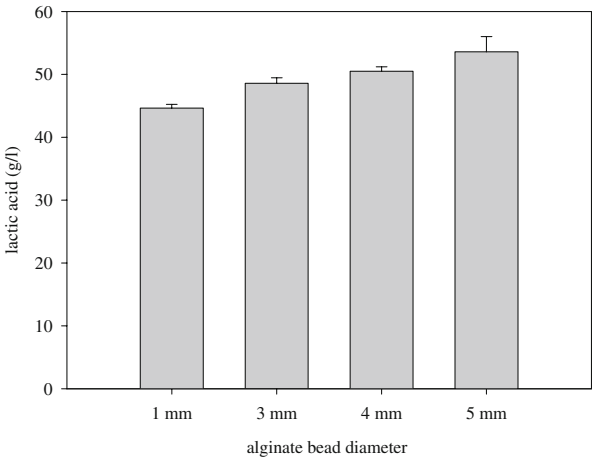

Fig. 2 Effects of capsule size on lactic acid production (prepared by 0.5% alginate concentration and 15% calcium chloride)

The increase in bead size from 1 to 5 mm would increase lactic acid production, which was against the conclusion obtained by Idris and Suzana [14], who indicated that a smaller alginate bead diameter produced higher lactic acid concentration by immobilized *Lactobacillus delbrueckii*. The reason was the high surface volume ratio in the small bead, which might reduce the mass transfer limitation. However, the limitation space inside a small bead might inhibit cell growth, which would not attain the high cell density culture [10]. In this study, an ample space inside the alginate bead for the growth of *R. oryzae* seemed to be crucial to lactic acid production, when using sweet potato powders as the substrate. Examining the bead diameter effects revealed that a 5-mm alginate bead achieved the maximum of 53.6±2.4 g/l lactic acid production, as compared with 44.6±0.6 g/l in the batch with 1-mm bead inoculated.

Effects of $CaCl_2$ Concentration

As shown in Fig. 3, $CaCl_2$ concentration of less than 15% had no apparent effects on lactic acid production. However, using $CaCl_2$ concentration of 20% during the gelation process

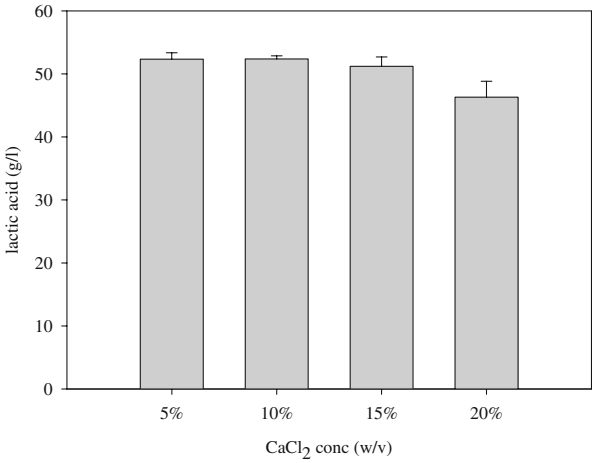

Fig. 3 Effects of $CaCl_2$ concentration on lactic acid production with 0.5% sodium alginate and 5-mm bead diameter

would decrease lactic acid production to 46.3±2.5 g/l as compared with 52.3±1.0 g/l obtained in the batch with $CaCl_2$ concentration of 5%.

During the gelation process of calcium alginate formation, a divalent metal ion-Ca^{2+} connected two alginate chains to form an ordered structure. Since $CaCl_2$ acted like a connecter, a higher $CaCl_2$ concentration would increase capsule thickness, which would enlarge the mass transfer resistance and reduce lactic acid production [13]. Therefore, as shown in Fig. 3, $CaCl_2$ concentration of 15% would not obviously affect lactic acid production, while $CaCl_2$ concentration of 20% would lead to decrease in lactic acid production. It was presumed that $CaCl_2$ concentration of up to 15% would not apparently cause mass transfer problems. In other words, limitation of nutrients would not be observed at $CaCl_2$ concentration of less than 15%, while $CaCl_2$ concentration of 20% would create a more condensed bead membrane, which might decrease the nutrient transfer and oxygen diffusion.

Repeated-Batch by Immobilization Cells and Free Cells

A repeated-batch fermentation with raw potato powders as the single carbon source was carried out as well to demonstrate the stability of Ca-alginate immobilized cells and free cells. As shown in Fig. 4, both free-cell and immobilized cell operations would have similar lactic acid production at the end of the first batch (after the initial 48 h). However, Ca-alginate immobilized cells could achieve more stable lactic acid production as compared with free cells in the subsequent cycles. A decline in lactic acid concentration was observed in the repeated-batch operation with free cells. Moreover, the lactic acid production ability of immobilized cells could be maintained for over 216 h in the repeated-batch without any reduction observed. A stable lactic acid production in a repeated-batch operation by immobilized cells was also observed by Dong et al., who revealed that immobilized cells in polyurethane foam cubes could be steadily used in repetitive fermentations for more than ten batches [15]. As shown in Fig. 5, all cumulative lactic acid curves maintained an almost straight line in the operation with immobilized cells. Conversely, the lactic acid accumulative rate seemed to reduce gradually with the increase in repeated cycles in the operation with free cells. The total cumulative lactic acid increase was 55% (*w*/*w*) in the operation when immobilized cells were used compared with that when using free cells. The corresponding yields were 47% and 73% (total lactic acid produced/total starch introduced)

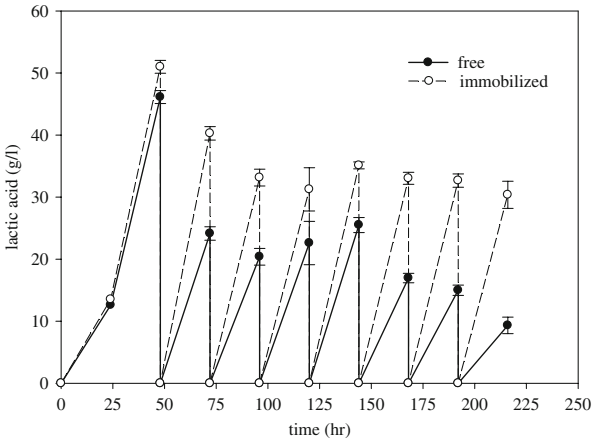

Fig. 4 Lactic acid production in a repeated-batch operation with free cells and alginate immobilized cells

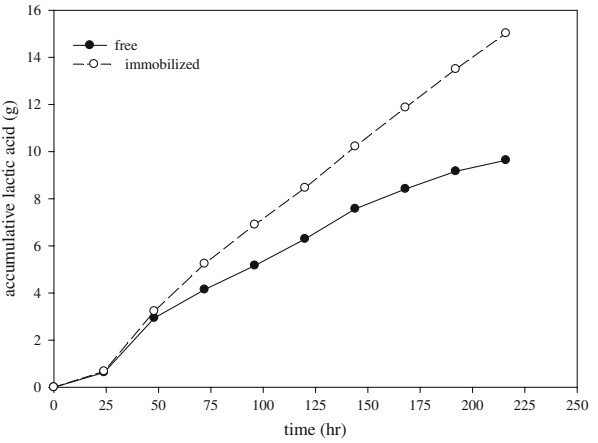

Fig. 5 The accumulative lactic acid in a repeated-batch operation with free cells and alginate immobilized cells

in the operation with free cells and immobilized cells, respectively. The analysis of amylolytic enzyme activity showed that there was not much different in amylase activities between the operation with immobilized cells and free cells (as shown in Fig. 6). However, the operation with immobilized cells had a higher glucoamylase activity than that with free cells. In addition, the glucoamylase activity in the operation with immobilized cells seemed to increase with the increase in repeated cycles. In contrast, a slight decline in the glucoamylase activity was observed in the operation with free cells. The decrease in glucoamylase activity might be the reason leading to the decline of lactic acid concentration in the repeated-batch with free cells. Overall, Ca-alginate immobilization could maintain lactic acid production for over 216 h, and showed no reduction in lactic acid concentration or amylolytic enzyme activity.

Conclusions

Cell immobilization was often adopted in the fermentation process to increase production efficiency. Nevertheless, lactic acid production by immobilized cells of *R. oryzae* using unhydrolyzed sweet potato powders was not investigated in the literature. The

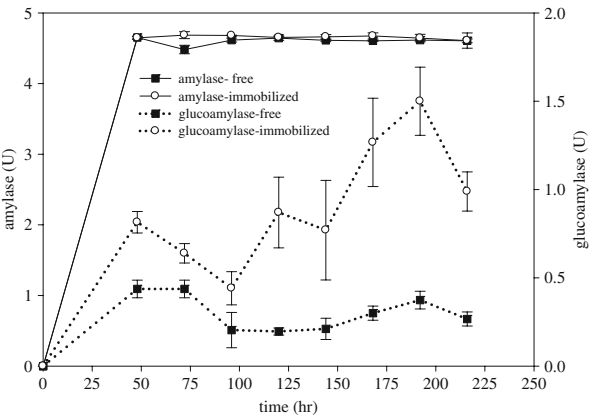

Fig. 6 The enzyme activities of amylase and glucoamylase in a repeated-batch operation with free cells and alginate immobilized cells

undissolvable characteristic of raw crops might be the reason why relevant studies in the literature for lactic acid production by the Ca-alginate immobilization of *R. oryzae* were rare. In this study, cell immobilization by calcium alginate (sodium alginate of 0.5% and calcium chloride of 15%) was suggested to be able to achieve stable lactic acid production for over 216 h with a total yield of 73%. The total cumulative lactic acid would increase by 55% in the operation with immobilized cells compared with that using free cells. Therefore, lactic acid production by the alginate immobilization process might be worth being applied in the scale-up production.

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