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L-lactic acid production from soybean straw hydrolysate using alginate-immobilised *Lactobacillus casei*

Juan Wang[1], Zhong Xu[2], Qunhui Wang[1]*, Wenyu Zhang[3], Yongzhen Ji[4], Wei Yin[4]

[1]Department of Environmental Engineering, University of Science and Technology Beijing, Beijing, 100083, (CHINA)

[2]Department of Environmental Engineering, Harbin University of Commerce, Harbin, Heilongjiang, 150076, (CHINA)

[3]Beijing Municipal Research Institute of Environmental Protection, Beijing, 100037, (CHINA)

[4]Department of Applied Chemistry, Harbin Institute of Technology, Harbin, Heilongjiang, 150001, (CHINA)

E-mail : wangqh59@sina.com

ABSTRACT

The effect of temperature, inoculum size, pH, initial reducing sugar concentration and Ca-alginate bead size on L-lactic acid production using immobilised *Lactobacillus casei* was investigated to promote the production of L-lactic acid from soybean straw enzymatic hydrolysate. The optimal conditions were as follows: temperature, 30°C; inoculum size, 10%; and pH, 5.5. L-lactic acid yield increased gradually as the reducing sugar concentration increased. The size of the Ca-alginate beads had no significant effect on lactic acid production, and the immobilised cell could be continuously used for more than 10 times. Immobilised cells were found to perform better than free cells in lactic acid production. The results indicated the feasibility of producing L-lactic acid using soybean straw enzymatic hydrolysate and provided a novel utilisation of soybean straw resources.

KEYWORDS

L-lactic acid;
Soybean straw;
Enzymatic hydrolysate;
Immobilisation;
Lactobacillus casei.

INTRODUCTION

Lactic acid can be classified into three types according to its optical activity: L-, D- and DL-lactic acid. Given that the human body only has L-lactic acid dehydrogenation enzyme, only L-lactic acid can be completely metabolised by the human body without producing any poisonous and side-effect products. Excessive absorption of D-lactic acid or DL-lactic acid can cause disorder even to an extent that leads to poisoning. Therefore, the preparation and application of L-lactic acid can be considered essential for the health of the human body. L-lactic acid, which is an organic acid, is also widely applied in many industries such as chemical industry, food and medicine, etc.[1,2]. Polymerised L-lactic acid that is synthesised from L-lactic acid is non-toxic, biodegradable and has broad applications in the market. Additionally, it may be considered as one of the most important high molecular materials in the future because of its environmentally friendly nature[3,4]. Lactic acid is traditionally fermented from sugar or starch[5-7]. Several studies have reported on lactic acid production from waste material, such as kitchen waste, agricultural residues and industrial by-products[8-11]. Utilisation of these wastes as raw material not only reduces the cost of L-lactic acid production, but can also

address the environmental problems caused by these wastes.

Soybean is one of the major crops in China. Approximately 17 million tons of soybeans are produced annually, and 26 million tons of soybean straw is generated simultaneously. Soybean straw generally is not reasonably utilised, and two-thirds of the straw is burned up. Apart from wasting resources, burning soybean straw also causes air pollution. Soybean straw can be hydrolysed into soluble sugar, which can then be biofermented to produce L-lactic acid. This phenomenon shows a promising method for reclaiming soybean straw, which also protects the environment.

Immobilised cell technology is used in numerous industries to protect the cell from unfavourable environments and realise continuous production. In food and fermentation industry, sodium alginate is widely used to immobilise enzymes and microorganism cells[12-14]. In this study, sodium alginate was used to immobilise *Lactobacillus casei* for L-lactic acid production. The enzymatic hydrolysate of soybean straw was used as the substrate, and parameters that affect L-lactic acid production such as temperature, inoculum size, pH, initial reducing sugar concentration and Ca-alginate bead size were investigated to obtain the optimal conditions for L-lactic acid production.

EXPERIMENTAL

Materials

The soybean straw was supplied by the Environmental Engineering Laboratory of Harbin Institute of Technology. The cellulase (15000 filter paper unites (FPU)/g) was provided by Wuxi Enzyme Factory. *L. casei* 1.6 was obtained from China Committee for Culture Collection of Microorganisms.

Culture medium

The bacteria were maintained on de Man, Rogosa and Sharpe (MRS) medium[15], and were subcultured every four weeks. The enrichment medium consisted of: glucose 3%, yeast extract 1.5%, peptone 1%, K_2HPO_4 0.5%, $CaCO_3$ 3%, water 1 l. The pH of the enrichment medium was adjusted to 6.0. The fermentation medium was composed of: peptone 5.0 g, yeast extracts 5.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 0.5 g, NaCl 0.1g, $CaCO_3$ 20.0 g, soybean straw enzymatic hydrolysate 1.0 l.

Preparation of soybean straw hydrolysate

Dry soybean straws were smashed and pretreated by 10% ammonia liquor for 24 h and filtered. The filter residue was dried at 80 ÚC to a constant weight and used as the substrate for enzymatic hydrolysis.

Hydrolysis of soybean straw was performed in a 250 ml Erlenmeyer flask containing 0.05 mol/l citric acid-sodium citrate buffer solution (pH 4.8) and 5% (w/v) of the pretreated soybean straw. After sterilizing, cellulase with 50 FPU/ (g straw) was added into the flask, and the hydrolysis was carried out at 50ÚC for 36 h. The main products of the hydrolysate were: glucose 15.58 g/l, xylose 6.87 g/l, and cellobiose 4.01 g/l, with a total reducing sugar production was 0.242 g/(g straw).

Preparation of cells immobilized with sodium alginate

L. casei cells were separated from the culture broth by centrifugation. After washing them with sterile water thrice, the cells were resuspended with phosphate buffer solution (10^{10} cells/ml). The bacterial suspension was added to sodium alginate solution and stirred for 5 min. The mixed solution obtained was then placed in a syringe and allowed to drop into a sterile 2% $CaCl_2$ solution that was stirred continuously. Alginate drops solidified upon contact with $CaCl_2$, forming beads and thus entrapping bacteria cells. The beads were allowed to harden for 1 h at 37 °C and then washed with sterile saline solution. Inoculate 15% of immobilized cells to the enrichment medium, and cultured at 30 °C for 24 h. After enrichment culture, the number of *L. casei* cells per 1 g Ca-alginate beads increased from 1.07×10^9 to 6.21×10^{12}.

Lactic acid fermentation of enzymatic hydrolysate

L-lactic acid fermentation was carried out in 250 ml Erlenmeyer flasks containing 100 ml sterilised fermentation medium and certain quantity of immobilized cells. Flush the flasks with nitrogen and seal them with rubber stoppers to maintain anaerobic condition. The fermentation flasks were placed in an incubator shaker with an agitation rate 140 r/min.

Analytical methods

Reducing sugar concentration was determined by

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dinitrosalicylic acid (DNS) method[16] with glucose as standard. Lactic acid was determined using a C_{18} column (4.6 mm 250 mm) and an ultraviolet absorption detector. Sulphuric acid (0.01 mol/l) at 0.7 ml/min was used as mobile phase, and detection was performed at 210 nm. The injection volume was 10 ml in both columns used for analysis. Column was maintained at room temperature (22±3 °C).

Sugar utilisation efficiency was calculated by dividing the reducing sugar consumed during fermentation by the initial reducing sugar concentration. And lactic acid conversion efficiency was calculated by dividing the produced L-lactic acid concentration by the sugar concentration consumed during fermentation.

RESULTS AND DISCUSSION

Effect of sodium alginate concentration

The concentration of sodium alginate can affect the sugar consumption and succeeding L-lactic acid production because it can alter the cross-linked structure of the Ca-alginate beads and consequently affect the diffusion of nutrients. In this study, four different sodium alginate concentrations were compared to determine the optimal concentration for L-lactic acid fermentation.

As shown in TABLE 1, the 2.5% sodium alginate concentration led to the maximum lactic acid production of 6.77 g/l and the largest conversion efficiency of 79.5%. At exceedingly low sodium alginate concentration, the produced beads were too soft and easily broken, thus causing leakage of the bacteria from the beads. Conversely at high sodium alginate concentrations, the resulting beads were too hard, and a more densely cross-linked structure was built, which resulted in thinner walls and diffusion problems. Consequently, the nutrients and substrates were restricted from easily diffusing to the *L. casei* cells. The 2.5% sodium alginate concentration produced higher amounts of lactic acid because of its less cross-linked structure that facilitated the diffusion of nutrients. This result is consistent with those obtained by Idris and Suzana[12], in whose experiment, liquid pineapple waste was fermented to produce lactic acid using immobilized *Lactobacillus delbrueckii* and the optimal sodium alginate concentration was 2.0%.

TABLE 1: Effect of sodium alginate concentration on L-lactic acid production and sugar consumption

Sodium alginate (%)	Initial sugar (g/l)	Residual sugar (g/l)	Lactic acid (g/l)	Sugar utilisation efficiency (%)	Lactic acid conversion efficiency (%)
1.5	10	3.75	3.06	62.5	49.0
2.0	10	3.02	4.25	69.8	60.9
2.5	10	1.48	6.77	85.2	79.5
3.0	10	2.96	4.13	70.4	58.7

Effect of temperature

Temperature can remarkably affect the growth, reproduction and metabolic activity of microorganisms. *L. casei* is classified as a mesophilic bacterium, with an optimum temperature of 37 °C. The effect of temperature on L-lactic acid production using immobilised *L. casei* was investigated at the various temperatures for 54 h and at a reducing sugar concentration of the soybean hydrolysate of 13.3 g/l.

As shown in Figure 1, the highest L-lactic acid production was obtained at 30 °C, with a yield of 11.8 g/l and a conversion efficiency of 99.0%. Increasing the

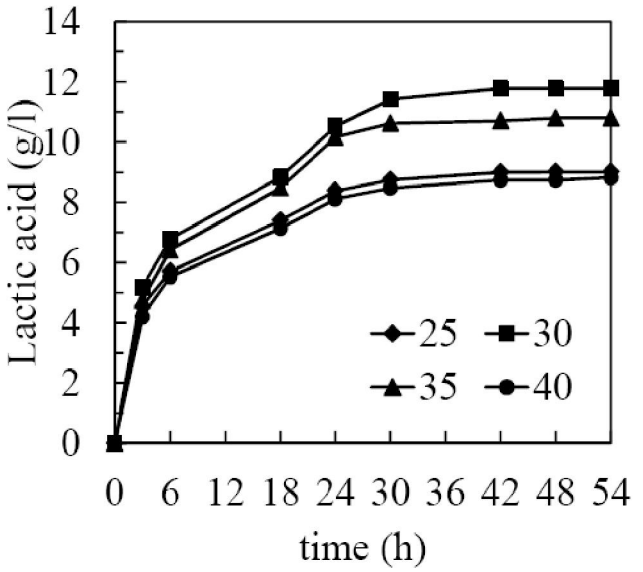

Figure 1 : Effect of temperature on lactic acid production with soybean hydrolysate

temperature beyond 35 °C or operating below 30 °C did not promote lactic acid production. These results differ from those reported by Qin *et al.*[17], who found that the optimal temperature for the strain *L. casei* G-03 to produce L-lactic acid was 41 °C. The difference may have been caused by the dissimilarity in substrates used in the experiments.

Effect of inoculum size

The effect of inoculum size was studied at various inoculum sizes, namely, 5%, 10%, 15%, and 20%. The initial reducing sugar concentration of the fermentation medium was set to 11.7 g/l. The flasks were incubated at 30 °C for 54 h.

The amount of cells inoculated into the fermentation system will affect the growth speed of the microbe. In a certain range, the time needed for the microorganisms to reach the logarithmic phase shortens as the inoculum size increases. Consequently, the growth speed is higher, and more microbial cells are involved in L-lactic acid production. An inoculum size of 5% generated a lower lactic acid yield, which was due to the lower growth speed of the cells (Figure 2). However, increasing the inoculum size to 10% or more did not promote lactic acid production. Therefore, the optimal inoculum size was considered to be 10%.

Effect of pH

The effect of pH on lactic acid production of immobilised *L. casei* with soybean hydrolysate is illustrated in Figure 4. The pH of the fermentation liquid was controlled with phosphate buffer solution, and the control experiment was conducted without any pH adjustment. The flasks were incubated at 30 °C, with an inoculum size of 10% and initial reducing sugar concentration of 12.4 g/l.

Lactic acid production of the control group was the lowest, which may be due to the low pH of the fermentation system resulting from the accumulation of generated lactic acid. The highest lactic acid production (9.8 g/l), with a conversion efficiency of 85.6% was obtained at pH 5.5. *L. casei* grew well in a pH range from 5.0 to 6.0, but the cell activity was restrained in highly acidic environment (pH 4.5).

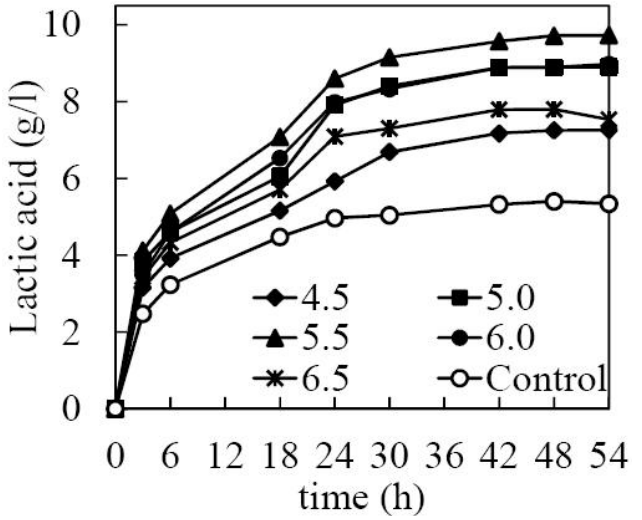

Figure 3 : Effect of pH on lactic acid production using soybean hydrolysate

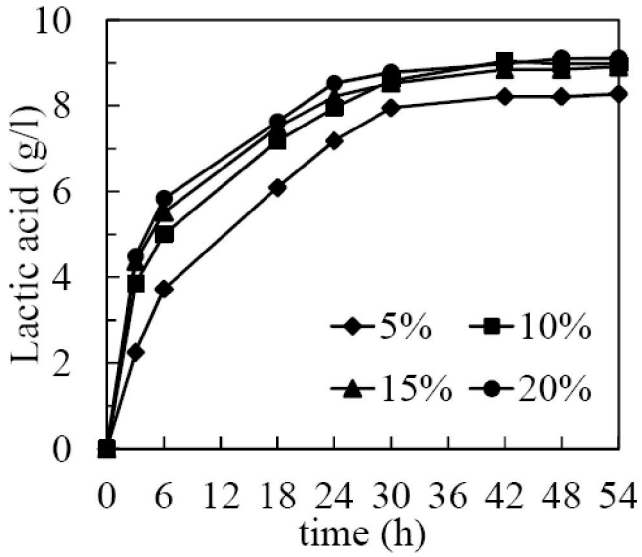

Figure 2 : Effect of inoculum size on lactic acid production with soybean hydrolysate

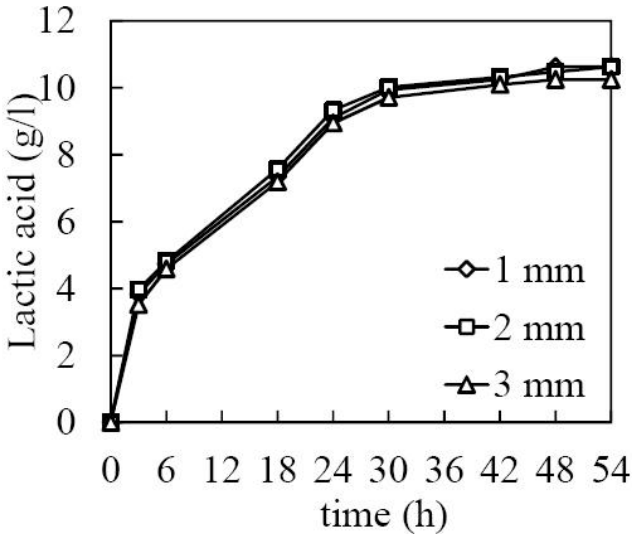

Figure 4 : Effect of bead diameter on lactic acid production with soybean hydrolysate

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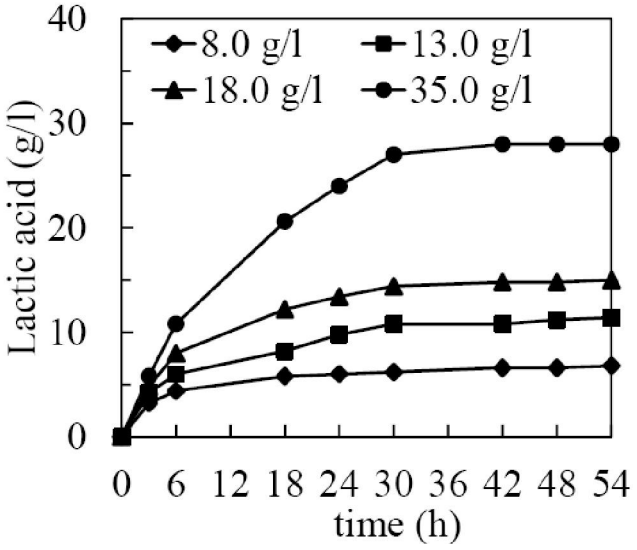

Figure 5 : Effect of initial reducing sugar concentration on lactic acid production with soybean hydrolysate

Effect of bead diameter

The lactic acid productions of three different Ca-alginate bead diameters were compared. Bead size ranging from 1 mm to 3 mm did not significantly affect lactic acid production (Figure 4). Abdel-Naby *et al.*[18] showed that more lactic acid was produced with smaller bead diameter because of the increase in specific surface. Additionally, smaller beads had better permeability, thus facilitating the diffusion of the substrate and the product-lactic acid. In this study, all bead sizes chosen were too small, and no difference in lactic acid production was observed. However, further increase in bead size may restrict the lactic acid yield.

Effect of initial reducing sugar concentration

The soybean straw hydrolysate solution was concensed to obtain different reducing sugar concentrations. The effect of initial reducing sugar concentration on lactic acid production was investigated at 30 °C and pH 5.5, with an inoculum size of 10%. The results are shown in Figure 5.

The reducing sugar was the main carbon source of *L. casei* cells in this study, and its concentration had a significant effect on lactic acid fermentation. A considerably low sugar concentration resulted in low lactic acid productivity, whereas excess sugar inhibited the growth and metabolism of the microorganisms. Higher lactic acid was produced as the initial reducing sugar concentration increased (Figure 5). At an initial sugar concentration of 35 g/l, 28.0 g/l of lactic acid was produced, and the conversion efficiency was 83.9%, which approached the conversion efficiency at initial sugar concentrations of 13 and 18 g/l. This result indicated that no inhibition of *L. casei* cells was observed in the tested sugar concentration range (8 g/l to 35 g/l). This characteristic may be attributed to the diffusion assistance through the beads that made the sugar concentration of the extracellular microenvironment lower than that of the outside medium. Consequently, immobilised cells can tolerate a higher substrate concentration than free cells.

TABLE 2 : Lactic acid production in different fermentation batches

Batch	1	2	3	4	5	6	7	8	9	10	11
Lactic acid (g/l)	8.7	8.9	9.2	9.3	9.3	9.4	9.4	9.6	9.5	9.3	9.1

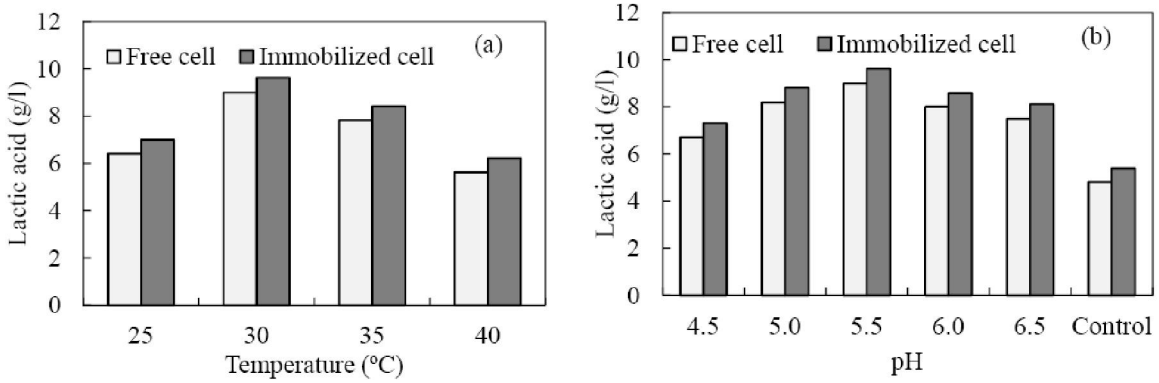

Figure 6 : Comparison of lactic acid production between free cells and immobilised cells at different temperatures (a) and different pH (b)

Lactic acid production in successive fermentation batches

To investigate the operational stability of the

immobilised *L. casei* cells, 11 successive fermentation batches were performed at a substrate concentration of 12.0 g/l and temperature of 30 °C.

TABLE 2 shows that no significant change in lactic acid production was observed during the 11 successive batches. The steady lactic acid production indicated that the Ca-alginate-entrapped *L. casei* cells retained their catalytic activity for an indefinite period of time. This result suggested that the Ca-alginate beads can be successfully reused for at least 20 d without yield reduction.

Comparison of performance between free and immobilised cells

The lactic acid production abilities of free and immobilised *L. casei* are shown in Figure 6. Lactic acid yields at different temperatures and pH were investigated.

Immobilised cells showed better fermentation performance compared with free cells at 25 °C to 40 °C or pH 4.5 to 6.5. Immobilisation of the cells had provided a more stable environment for the cells and can tolerate unfavourable environment better than free cells.

CONCLUSIONS

In the lactic acid fermentation process with soybean straw hydrolysates, the obtained optimal conditions were as follows: sodium alginate concentration, 2.5%; inoculum size, 10%; temperature, 30 °C; and pH, 5.5. The size of Ca-alginate beads had no distinctive effect on lactic acid fermentation. High concentration of the initial reducing sugar (up to 35 g/l) did not inhibit lactic acid production of the immobilised cells, and the beads can be reused for at least 10 times without significant decrease in performance. Comparison of the lactic acid yield with free cells and immobilised cells at different temperatures and pH showed that immobilised cells perform better than free cells.

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