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Efficient production of L-lactic acid from hydrolysate of *Jerusalem artichoke* with immobilized cells of *Lactococcus lactis* in fibrous bed bioreactors

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ABSTRACT

Hydrolysate of *Jerusalem artichoke* was applied for the production of L-lactic acid by immobilized *Lactococcus lactis* cells in a fibrous bed bioreactor system. Preliminary experiments had indicated that the high quality hydrolysate, which was derived from the 40 min acid treatment at 95 °C and pH 1.8, was sufficient to support the cell growth and synthesis of L-lactic acid. With the addition of 5 g/l yeast extract, the fermentative performance of free cell system was evidently improved. After the basal settlement of hydrolysate based fermentation, the batch mode and the fed-batch mode fermentation were carried out in the free cell system and the fibrous bed bioreactor system, respectively. In all cases the immobilized cells presented the superior ability to produce L-lactic acid. The comparison of batch mode and fed-batch mode also indicated that the growth-limiting feeding strategy could reduce the lag phase of fermentation process and enhance the production of L-lactic acid. The achieved maximum concentration of L-lactic acid was 142 g/l in the fed-batch mode. Subsequent repeated-batch fermentation of the fibrous bed bioreactor system had further exhibited the persistence and stability of this system for the high production of L-lactic acid in a long term. Our work suggested the great potential of the fibrous bed bioreactor system and hydrolysate of *J. artichoke* in the economical production of L-lactic acid at industrial scale.

1. Introduction

The fermentative production of L-lactic acid using renewable resources has attracted many interests in recent years. Many efforts have been made to improve the titer of L-lactic acid in microbial fermentation using food-based feedstock [1,2]. Recently, fermentation of non-food biomass has also gained considerable attention due to the forthcoming scarcity of fossil fuels and the increased lack of food and feed supplies over the world. Among them, the cheap and ubiquitous non-grain raw material *Jerusalem artichoke* is a good candidate and has been intensively studied. Hydrolysate of *J. artichoke* has been widely used to replace refined sugar, and been utilized for the submerged fermentation of economic product such as ethanol [3], single cell protein [4] and organic acid such as lactic acid [5] and butyric acid [6]. The dried tuber of *J. artichoke* contains approximately 60–70% insulin which can be hydrolyzed to fructose and glucose by inulinase or acid. Enzymatic hydrolysis of inulin is widely used for the production of fructose. However, the slow reaction rate and high cost of the feedstock pretreatment, enzyme production and enzyme recovery have limited the utilization of enzymatic hydrolysis. From the economic viewpoint, the hydrolysate derived from enzymatic hydrolysis may not be applicable for the production of low-priced lactic acid. On the contrary, acid-hydrolysis is a low-cost and simple quick operation which is more appropriate for some cost-effective industrial processes.

Conventional batch fermentation usually utilizes free cells for metabolite production, which has several limitations such as low productivity, product inhibition and batch-to-batch variation, leading to the high cost of fermentation. Thus, various novel fermentative techniques have been developed for efficiency improvement. In the previous study [7,8], some new bioreactors including membrane bioreactor and fluidized-bed bioreactor were introduced to improve L-lactic acid production. Especially, the fibrous bed bioreactor (FBB) with immobilized cells in the fibrous matrix has been successfully applied to produce several important organic acids, such as lactic acid [9], acetic acid [10], propionic acid [11] and butyric acid [12,13], with significantly improved productivity and titer of final product. One type of lactic acid bioproduction was depended on the fermentation of homolactic acid bacteria, which usually synthesize a mixture of L(+)- and D(−)-lactic acids. However, optically pure lactic acid is required for polylactic acid

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(PLA) manufacture and many other applications. Comparing to other strains, *Lactococcus lactis* ATCC19435 is an efficient L-lactic acid producing strain and normally no D-isomer could be detected during the fermentation process [14].

In this work, the influence of pH and temperature on the hydrolysis of *J. artichoke* was investigated. The addition of yeast extract was also investigated to consummate the hydrolysate-based fermentation medium. The batch, fed-batch and repeated-batch fermentative modes were studied respectively to evaluate the feasibility and robustness of the FBB system for the production of L-lactic acid from hydrolysate of *J. artichoke*.

2. Materials and methods

2.1. Microorganism and culture medium

L. lactis ATCC19435 was purchased and preserved as freeze-dried tube in our laboratory. As previously described, the strain was statically incubated in serum bottles containing MRS medium [15] under anaerobic conditions at 30 °C for 12 h. Unless otherwise noted, the fermentation medium contained a certain concentration of hydrolysate and other components including 5.0 g/l yeast extract, 0.25 g/l KH_2PO_4, 0.25 g/l K_2HPO_4, 6 g/l CH_3COONa, 0.51 g/l EDTA, 0.5 g/l $MgSO_4{\cdot}7H_2O$ and 0.07 g/l $MnSO_4$.

2.2. Pretreatment of Jerusalem artichoke

J. artichoke tubers were harvested from experimental plot on October (Jiangsu, PRC, Public taste Seasoning Co, Ltd.), with 25% of dry matter content. The tubers were sliced and dried at 60 °C for 12 h, and ground to fine powder. 100 g of such powder was mixed with 500 ml diluted sulfuric acid to give a final pH of 1.6, 1.8 and 2.0, respectively. Each pH has duplicates for temperature comparison, one was heated at 85 °C and the other was heated at 95 °C for 60 min. Samples were taken at 15-min intervals and the content of reducing sugars was analyzed by HPLC. A concentrated syrup for fed-batch study was prepared from the vacuum evaporation of hydrolyzed extracts.

2.3. Fermentative production of L-lactic acid

Both free-cell and immobilized-cell fermentations were carried out. Free-cell fermentation was conducted in a 5-l B. Braun fermentor (B. Braun Biotech, Germany) containing 2.5 l production medium. The 2.5 l fermentation medium was inoculated with 100 ml seeds from a serum bottle and cultivated at 30 °C and 100 rpm with flat blade disc turbine, and the pH in the bioreactor was controlled at 6.0 by 5 M NaOH. The anaerobic condition was maintained by initially sparging the medium with nitrogen and once for 30 min each day afterwards.

The immobilized-cell fermentation system consisted of a 5-l fermentor connected with a 0.5-l fibrous-bed bioreactor by a recirculation loop for pH and temperature control. The FBB with a working volume of 480 ml was made of a glass column packed with spiral wound cotton towel. The immobilized-cell fermentation was operated at 30 °C, and pH was controlled at 6.0 by addition of 5 M NaOH. The details of FBB construction was described elsewhere [10–13]. Cell immobilization was carried out by circulating the culture broth through the fibrous bed at a pumping rate of 25 ml/min to allow cells be immobilized onto the fibrous matrix. Batch fermentation was carried out to produce L-lactic acid with the concentrated hydrolysate medium supplemented with 5 g/l yeast extract. After the batch fermentation, the free-cell and immobilized-cell fed-batch fermentations were carried out with the pulse feeding of concentrated hydrolysate. The feeding process was not stopped until the concentration of lactic acid became steady in the broth. Samples were taken at regular intervals for the analysis of biomass and the concentrations of substrate and product. Finally, several repeated batch fermentation was done by removing the broth in the FBB when the concentration of the reducing sugars in the broth was almost consumed, and pumping fresh hydrolysate based medium into the fermentor. During this long-duration process, samples were taken at regular intervals for the rapid analysis of biomass and the concentrations of reducing sugars to determine the timing of running next batch. The concentration of L-lactic acid in the broth was then analyzed to determine the productivity.

2.4. Analytical methods

The cell density of the free cells in the bioreactor was determined by measuring the absorbance of the cell suspension at a wavelength of 660 nm (OD_{660}) with a spectrophotometer (Ultrospec 3300 pro, Amersham Bioscience). Dry weight of cell biomass in the broth was determined by centrifuging the fermentation broth at 10,000 × *g* for 10 min, washing the sediment with distilled water twice, and drying at 70 °C until constantly weighed. For immobilized cell fermentation, the immobilized cells were collected by washing the cells off the fibers twice with 0.2 M phosphate buffer (pH 6.0) after the end of the fermentation, then the OD660 of the collected solution was determined, finally the immobilized biomass in the FBB was calculated according to the predetermined coefficient (1 unit of OD660 was equivalent to 0.763 g/l for *L. lactis* cells) [16].

The concentrations of glucose and fructose were analyzed by Agilent 1100 HPLC system consisted of a refractive index detector (Agilent 1100, G1362A) and an Aminex HPX-87H column (250 mm × 4.6 mm, 5 μm; BioRad, USA), with the mobile phase of 5 mM H_2SO_4 and a flow rate of 0.6 ml/min at 45 °C. Reducing sugars were was detected with 3,5-dinitrosalicylic acid timely to control the limit of residual sugars in the repeated-batch fermentations. The concentrations of D- and L-lactate were enzymatically measured using the test combination "D-lactic acid/L-lactic acid" kit (Cat. No. 1112 821) from Boehringer Mannheim (Mannheim, Germany). The method is based on the measurement of NADH derived lactic acid oxidation at the UV absorbance of 365 nm, in the presence of D- or L-lactate dehydrogenase (LDH), respectively [17].

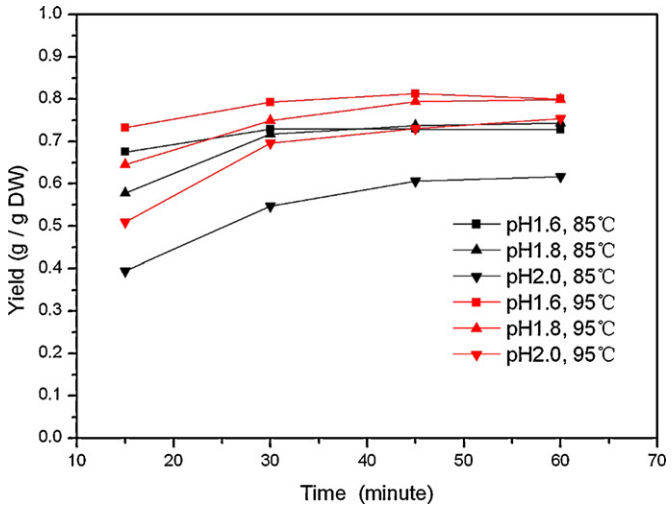

Fig. 1. Effect of pH and temperature on the hydrolysis of *Jerusalem artichoke*. The hydrolytic yield is calculated by the determination of reducing sugars in the samples.

3. Results

3.1. Acid hydrolysis of J. artichoke tuber

Since *L. lactis* could not directly utilize inulin, hydrolysis of *J. artichoke* by acid treatment was necessary to transform inulin into available fructose and glucose. Because fructose is thermostable below 100 °C, the hydrolysis of *J. artichoke* is usually taken at 85 °C or 95 °C, depending on the type of *J. artichokes* [18]. Moreover, the pH of acid also contributes a lot to the hydrolysis process. To determine the optimal condition for the hydrolysis, three pH values (1.6, 1.8 and 2.0) were tested at 85 °C or 95 °C, respectively. As shown in Fig. 1, higher temperature and lower pH could enhance the efficiency of hydrolysis obviously. Considering about the cost and efficacy, the optimal hydrolysis was carried out at pH 1.8 and 95 °C for 45 min, which could convert 79% of dry weight to reducing sugars with an approximate 5:1 ratio of fructose and glucose.

3.2. The effect of yeast extract on lactic acid production

To test the nutrient efficiency of hydrolysate, especially for fermentation purpose, the addition of yeast extract was conducted in the 1 l shaking flask fermentation. The final concentration of reducing sugars in each flask was diluted to 80 g/l, the concentrations of yeast extract in the initial media were ranged from 0 to 20 g/l. Although the high levels of nutrients in the hydrolysate were sufficient to support the cell growth and production of L-lactic acid, the addition of 5 g/l yeast extract could notably enhance the titer of lactic acid (Fig. 2). However, further increase of yeast extract concentration could only lead to unconspicuous improvement of L-lactic acid production. As a result, the fermentation medium

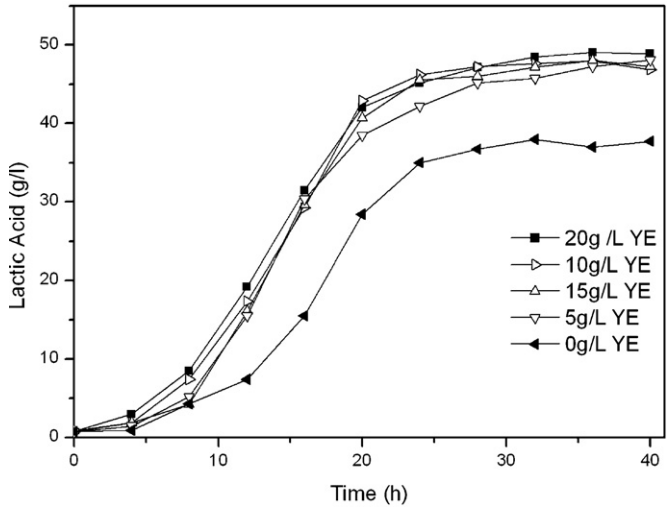

Fig. 2. Effect of yeast extract addition on the production of L-lactic acid with hyrolysate of *Jerusalem artichoke*. The final concentration of reducing sugars in each 1 l shaking flask was diluted to 80 g/l; the concentrations of yeast extract were ranged from 0.0 to 20.0 g/l with an increment of 5 g/l.

containing hydrolysate and 5 g/l yeast extract was used for the following fermentative trials.

3.3. Batch fermentation of free cells and immobilized cells

The batch fermentation of *L. lactis* using free cells and immobilized cells was compared (Fig. 3). The time-course results indicated that glucose and fructose were metabolized simultaneously. Because of the extra growth stage of free cells, the fermentation using free cells was lagged comparing to that of immobilized cells. At the end of fermentation, the highest concentration of lactic acid (92.56 g/l) was observed in the free cell fermentation (Fig. 3a), while the highest concentration of lactic acid was improved up to 120.5 g/l (Fig. 3b) in the FBB bioreactor. It was also observed that the productivity of lactic acid was improved from 0.5 g/(l h) in free-cell system to 1.0 g/(l h) in immobilized cell system, and this improvement was contributed by the high cell density of immobilized cells in the FBB because the profiles of cell density in the broth seemed to be not quite different between in free-cell and immobilized fermentations. Moreover, these immobilized cells in the fiber bed column improved the product yield from 0.68 g/g to 0.92 g/g, which might be contributed by the alleviated distribution of cellular energy for biomass synthesis in immobilized cell fermentation.

3.4. Fed-batch fermentation of free cells and immobilized cells

To eliminate the effect of possible substrate inhibition, the pulse fed-batch feeding was applied to improve product concentration. The concentration of fructose was set in the range of 10 g/l to 60 g/l by the supplementation of concentrated syrup (400 g/l reducing sugars) derived from hydrolysate of *J. artichoke*. The fermentative performance in both batch mode and fed-batch mode was very similar, except that the titer of L-lactic acid was obviously improved in fed-batch mode. In fed-batch mode, the maximum yield of 142 g/l lactic acid was obtained in the FBB system, which was 27.92% higher than that (103 g/l) in free-cell system (Fig. 4).

3.5. Repeated-batch fermentation in FBB

To evaluate the long-term performance of FBB system, repeated-batch fermentation was also studied. As shown in Fig. 5, no lag phase was presented in the next 8 continuous batches. The cells grew rapidly within the following 12 h after replaced with fresh broth, indicating the high vitality of immobilized cells. The yield of L-lactic acid from individual batch varied from 0.84 to 1.01 g/g, with an average yield of 0.92 g/g. The productivity was ranged from 0.71 to 2.85 g/(l h). Such discrepancies were mainly related to the initial concentration of reducing sugars. When the initial concentration of reducing sugars was below 100 g/l, the fermentation cycle was relatively short, and the cell density as well as the titer of L-lactic acid could only reach moderate value. After five batches, the immobilized cells were accustomed to the environment, and the resistance to the high concentrations of both substrate and product was improved. With the increase of initial fructose content, the fermentation process was prolonged, and the cell density was concomitantly improved as well as the production of L-lactic acid (Fig. 5). However, further increase of fructose concentration over 150 g/l could result in the decreased yield of L-lactic acid. For economical purpose, the optimal concentration of reducing sugars should be in the range of 100 g/l to 150 g/l. In summary, the repeated-batch fermentation has proved the endurance and stability of FBB system for long-term operation.

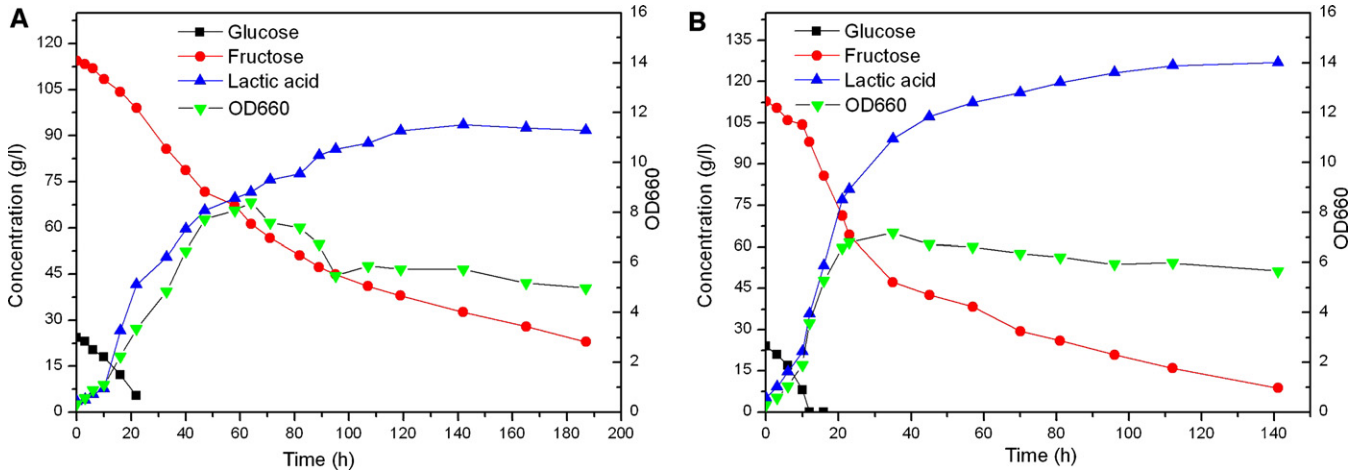

Fig. 3. Kinetics of batch fermentation of JA acid hydrolysate by free (a) and immobilized cells (b) of *L. lactis* in FBB at pH 6.0 and 30 °C. Free cell batch was started by inoculation of 100 ml cell suspension into fermentor placed with 2 l hydrolysate medium. For repeated batch, when the reducing sugars concentration in the broth decreased to zero, the broth in the fermentor was pumped out thoroughly and new fresh hydrolysate was added into the fermentor to start a new batch.

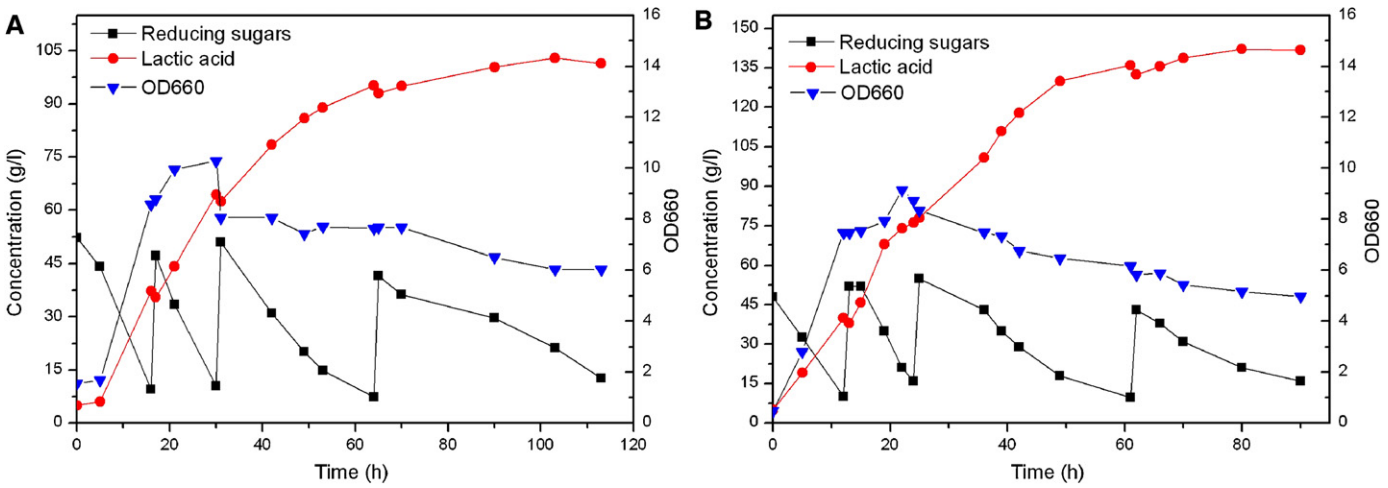

Fig. 4. Kinetics of fed-batch fermentation of JA acid hydrolysate by free (a) and immobilized cells (b) of *L. lactis* in FBB at pH 6.0 and 30 °C. The both fed-batch mode were started with 2 l of fresh JA acid hydrolysate, followed with pulse feeding concentrated hydrolysate whenever its concentrations of reducing sugars in the fermentation broth was close to 10 g/l.

4. Discussion

In this work, the hydrolysate of *J. artichoke* was used for L-lactic acid production by immobilized cells of *L. lactis*. As a fastidious organism, the growth of *L. lactis* normally requires complex organic substances such as vitamins, nucleotides and amino acids. Fortunately, the hydrolysate of *J. artichoke* after acid treatment contains high levels of vitamins, potassium, calcium, phosphorus, iron and zinc, as well as amino acids derived from hydrolyzed proteins [19]. Yeast extract is a most commonly used nitrogen source for lactic acid production, but the high cost usually limits its large scale application. Based on our results, with the optimized hydrolysis of *J. artichoke* concerning about the temperature and pH, the high quality hydrolysate of *J. artichoke* was sufficient to support the cell growth and production of lactic acid, and the addition of only 5 g/l yeast extract was enough to enhance the concentration of lactic acid to a stable level, whereas 10–30 g/l yeast extract was used for lactic acid fermentation in previous studies [20–23]. This outcome could obviously reduce the cost of lactic acid and beneficial to the subsequent large-scale application.

Comparing to the conventional free cell fermentation system, the FBB system has several advantages for efficient production of lactic acid, such as high cell density, high yield and productivity of final metabolite. The primary merit could be attributed to the high viable cell density maintained in the fibrous matrix. Cell immobilization usually includes physical entrapment and chemical adsorption. In physical entrapment phase, cells were loosely attached or entrapped in the fibrous matrix by simple filtration effect; while in chemical adsorption phase, the chemical forces could make a stronger attachment with fibrous matrix. In FBB system, after the end of fermentation, approximately 35–42 g/l of immobilized cell biomass was determined in the fibrous matrix, whereas only 7–10 g/l of cells were suspended in the fermentation broth. The scanning electron micrographs has exhibited that high density of *L. lactis* cells were immobilized on the fiber surface and large cell clumps were formed via cell adsorption (Fig. 6). These loosely entrapped cell clumps could be washed off during the medium recirculation, which redistributed the cells in the FBB to make the immobilized cells uniform.

Although Jerusalem artichoke has already been proposed as a possible substrate for ethanol production in fermentation process [24–27], there have been few studies on lactic acid production from *J. artichokes*. It was reported that *J. artichoke* was employed as substrate for lactic acid production in a simultaneous saccharification and fermentation process (SFF), and the highest lactic acid concentration of 120.5 g/l was obtained in 36 h of the fed-batch fermentation with high conversion efficiency of 0.945 g/g [5]. However, the high productivity of 3.34 g/(l h) was based on the inoculation volume of 35%, which is impractical for industrial-scale production. The same group [28] investigated the influence of citrate on the metabolism and physiology of the strain, significantly improved the lactic acid productivity and increased the final acid

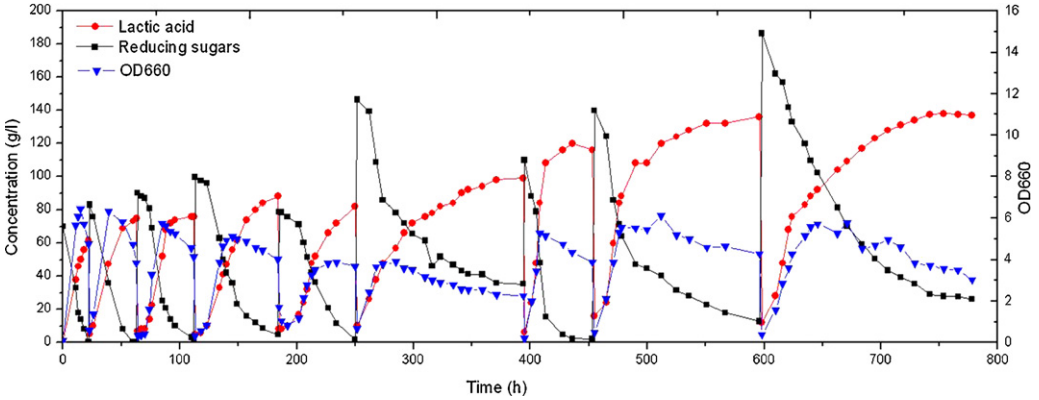

Fig. 5. Kinetics of repeated-batch fermentation of JA acid hydrolysate by *L. lactis* immobilized in FBB at pH 6.0 and 30 °C. In the repeated batch mode, the fermentation broth was replaced with sterile JA acid hydrolysate as the carbon source to start a new batch.

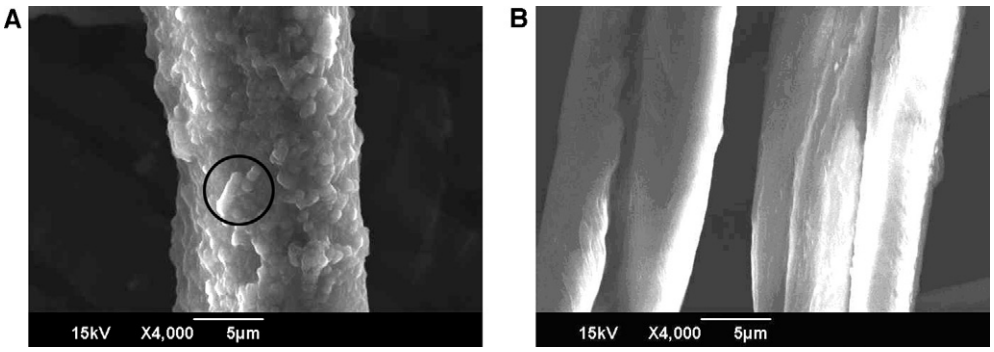

Fig. 6. Scanning electron micrographs of (a) immobilized *Lactococcus lactis* cells on the fiber surfaces; (b) control fiber without cell immobilization. The fiber samples were processed through a progressive dehydration with 20–100% (v/v) ethanol at 10% (v/v) increment, dried and coated with gold/palladium. The samples were scanned and photographed with a Philips XL 30 scanning electron microscope at 15 kV.

concentration to 140.5 g/l. However, to maximize the rate of saccharification, the fructose concentration in the culture has been kept at a low level, and 10 g/l of sodium citrate was added as supplement to maximize the lactic acid productivity, which may complicated the process of upscale production of lactic acid. In the present repeated fermentation, 120 g/l of lactic acid averagely in four batches (batch 6–9) was reached approximately in 132 h. This high lactic acid concentration was achieved by only using fed-batch fermentation operation in many literatures [17]. The FBB process using Jerusalem artichokes as substrate can endure high substrate concentration up to 180 g/l of reducing sugars, which owing to high cell density immobilized in the bioreactor and natural mutation during adapting in fermentation process. It is not unusual to have high fermentation productivity with an immobilized cell bioreactor. Conventional immobilized cell bioreactors, however, suffer from productivity loss when the cells are used continually or repeatedly in continuous or fed-batch fermentation, due largely to limited mass transfer and accumulation of dead cells. This reactor degeneration problem was overcome by continual cell renewal in the fibrous bed, which also provided an effective mechanism for adapting the culture to tolerate a high concentration of lactic acid.

Apparently, cell density and the concentration of substrate were two major factors to affect the production of lactic acid. With the application of efficient FBB system and development of fed-batch strategy, the fermentative performance was greatly improved. The achieved high cell density not only stimulated the synthesis of lactic acid, but also resisted the possible contamination. Moreover, the lag phase of cell growth was eliminated in the FBB system, which accelerated the fermentation process. The high conversion rate of substrates and the persistence of efficient continuous operation made the FBB system more potential and practical for the industrial trials.

5. Conclusion

In this study, the good performance of fibrous bed bioreactor system with immobilized *L. lactis* cells was achieved for lactic acid production from hydrolysate of *J. artichoke*. Using the fed-batch strategy, the maximum concentration of 142 g/l lactic acid was produced with supplementation of concentrated hydrolysate of *J. artichoke*. With the high efficiency and long-term persistence, the whole system developed in this work was really potential for the economical production of lactic acid on industrial scale.

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