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ARTICLE TYPE

Continuous L-lactic acid production from defatted rice bran hydrolysate using corn stover bagasse immobilized carrier

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In this paper, L-lactic acid (LLA) was produced using defatted rice bran hydrolysate. The agricultural waste corn stover bagasse (CSB), in conditions of good mechanical and chemical stabilities, was used as the immobilized carriers. As a result, a maximal concentration of 88 g/L of LLA, with average yield of 0.95 g/g and productivity of 5.20 g/L·h were achieved in single-stage immobilized repeated-batch fermentation. Additionally, single-stage immobilized continuous fermentation was carried out, a maximal LLA concentration of 84 g/L was obtained while the average yield and productivity were 0.97 g/g and 5.73 g/L·h, respectively. To balance the increases of the productivity and concentration and yield of LLA, two-stage immobilized continuous fermentation was further performed. The LLA productivities of 6.20 g/L·h in first stage and 2.18 g/L·h in second stage were achieved, respectively. The whole immobilized fermentation process was demonstrated potential in L-lactic acid biorefinery and industrialization.

I. Introduction

With the deep worries of unsustainable development of human beings, recently, L-lactic acid (LLA) attracted headline-grabbing. LLA has versatile applications in food, pharmaceutical, cosmetic, textile, leather, and other chemical industries [1-4]. More importantly, LA is the precursor of PLA, a kind of renewable and biodegradable plastic. Hence the demand of LLA has been increasing continuously for years [5, 6]. Compared with other route of LLA production, fermentation has advantages in mild production conditions, low energy consumption [7], excellent optical purity of the product and safety [8]. However, unfortunately, suffering from the high costs of the food and starchy feedstock, the industrialization and large-scale applications of most case of LLA fermentation process were limited [9].

The utilization of agricultural residuals for LLA production was attractive due to the low prices of feedstock [10]. Among different types of agricultural residuals, the lignocellulosic biomass was more or less difficult to use because of the toxic of the hydrolysate and the uneconomical competitiveness of pretreatment [8]. As compared, defatted rice bran, the residue from brown rice, is one of the most abundant agricultural by-products in the South and East Asia [11]. After squeezing the rice bran oil, the residual powder of defatted rice bran contains a plenty of starch to make it feasible in LLA fermentation in an easy way according to our previous study [12].

Cell immobilization is a technique to improve the cell density and to keep the catalytic activity in repeated-use by physical or chemical means to position or limit the free cells in a specific region of space. Thus, with the increase of the cell concentration during immobilized fermentation, a higher productivity of LLA could be generated. In addition, cell immobilization has the ability of improving the fermentation system stability, decreasing the requirement to nutrients, and reducing the difficulties of LLA recovery to make the production more efficiency [13].

Traditional immobilized carrier materials are sodium alginate, polyvinyl alcohol [14, 15]. However, the immobilized carriers above have shortcomings of higher costs, damage to cells in the cumbersome immobilization process and the carrier softening. To overcome the disadvantages of the traditional immobilized carriers, various natural materials, such as fruit pieces [16], lignocellulosic materials [17], have been successfully applied as immobilized carriers in LLA fermentation.

Corn is an abundant agricultural crop [18] with extended planting area and good adaptation of margin land. The corn stover bagasse (CSB), residue of corn with commercial value, has been recognized as an excellent immobilized carrier according to previous research [19], which was not yet applied and studied in immobilized LLA fermentation. As the case in China, the main growing area of corn stover is coincident with the rice growing areas. Therefore, under the concept of biorefinery, the defatted rice bran and the CSB, two types of agricultural residuals, would be applied together in a certain immobilized fermentation process of LLA that retain the superiority of each [20].

The purposes of this research were to obtain efficient and inexpensive LLA with high LLA yield and productivity. The defatted rice bran was used as carbon source and the CSB was directly used as immobilized carrier. Both the single-stage immobilized repeated-batch and continuous LLA fermentation were performed. Besides, aiming to further get the increase of the productivity and yield of LLA, the two-stage immobilized continuous fermentation was also carried out.

II. Experimental section

A. Microorganism and culture

Lactobacillus rhamnosus LA-04-01 stored in the our lab was used throughout this work. The semi-synthetic medium consisted of 20 g/L of glucose, 15 g/L of yeast extract, 10 g/L of soya peptone, 5 g/L of sodium acetate, 2 g/L of ammonium citrate, 10 g/L of NaCl, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.05 g/L of $MnSO_4 \cdot 7H_2O$, 20 g/L of agar. It was grown on the semisynthetic medium at 42 ^oC for 36 h and the stock cultures were maintained at 4 ^oC. The medium for cell growth contained 40 g/L of glucose, 10 g/L of yeast extract, 10 g/L of soya peptone, 0.5 g/L of sodium acetate, 0.2 g/L of ammonium citrate, 0.01 g/L of NaCl, 0.2 g/L of KH_2PO_4, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 0.05 g/L of $MnSO_4 \cdot 7H_2O$ and 40 g/L of $CaCO_3$. The initial concentration of carbon source and yeast extract in the medium for LLA production were 120 g/L and 15 g/L, respectively, and the rest compositions were the same as those of the medium for cell growth.

B. Defatted rice bran hydrolysis

Hydrolysis of the defatted rice bran was routinely obeying the method of Wang [12]. An appropriate amount of defatted rice bran was formulated into a 200-250 g/L suspension of defatted rice bran with water. The pH of the defatted rice bran suspension was adjusted to 6.0 with 20 % sodium hydroxide solution. Then, an appropriate amount of amylase (Liquozyme ®Supra/2.2X) was added in the defatted rice bran suspension at the ratio of 1 mL/kg (v/w) in a high-pressure steam sterilization pot, the temperature was kept at 108 ^oC for 5 min and 95 ^oC for 60 min to complete the liquefaction of defatted rice bran. Till the temperature of the defatted rice bran suspension was cooled to 60 ^oC, the pH of the medium was adjusted to 4.2. An appropriate amount of glucoamylase (Dextrozyme DX/1.5X) was then added in the medium at the ratio of 1 mL/kg (v/w) at 60 ^oC for 24 h of hydrolysis. After hydrolysis, the defatted rice bran hydrolysate supernatant was obtained by centrifugation, and used as the substrate for the fermentation.

C. Pretreatment of corn stover bagasse as carrier

According to the method of Yu [21], the CSB were collected and peeled off. Then the spongy tissue of the stem was cut into suitable particle size, and placed in hot water to soak repeatedly 4-8 times until the sugar in the stem were fully released. After that, the CSB were dried at 60 ^oC . The particle size and the addition of carriers were adjusted to meet the different requirements of the experiments for further study.

D. Cell immobilization fermentation

The initial sugar concentration of the defatted rice bran hydrolysate supernatant medium was adjusted to 120 g/L. 250 mL flasks contained 100 mL medium of the defatted rice bran hydrolysate supernatant and 5 g of calcium carbonate in flask fermentation, while 1L fomentor contained 250 mL medium of the defatted rice bran hydrolysate supernatant and 7.5 g of calcium carbonate. According to the specific requirements of the experiments, different amounts of CSB carriers were added into the fermentor, and the pH of the medium was adjusted to 6.0. Then, the fermentor with medium inside were kept at 121 ^oC for 20 min of sterilization. The medium was cultivated at 42 ^oC, 180 rpm with the inoculum of 20 % (v/v). For repeated-batch fermentation, batch fermentation was carried out firstly in a 1 L fermentor, and after the end of batch fermentation, the suspend liquor was pumped out by a peristaltic pump (Baoding Chuangrui Precision Pump Co., Ltd). Then, the steriled defatted rice bran hydrolysate was pumped into the fermentation system under certain liquid to solid ratio for another batch of LLA fermentation. Thus, the fermentation could operation repeatedly and the immobilized cells on the surface of the corn stover carrier could maintain its activity because of the fresh substrate pumped into the fermentor periodically. For the single-stage continuous fermentation, fresh defatted rice bran hydrolysate was pumped into the fermentor continuously under different dilution rate, and the fermentation broth was pumped out continuously under the same rate of dilution to maintain the fermentation stable. As for the two-stage immobilized continuous fermentation, two 1 L fermenors in series was performed. The fermentation broth with high concentration of residual sugar after the first stage of fermentation was pumped into the second one as the substrate. Then, the immobilized cells were further use the sugar in the fermentation broth to produce LLA. The entire dilution rate of each stage was the same to maintain the two-stage system stable.

E. Preparation of SEM sample of carriers

According to the literature [21], The CSB with the bacteria cells was firstly kept in 3.5 % glutaraldehyde solution and soaked for 6 h, then washed successively with 50 %, 60 %, 70 %, 90 %, 95 %, 100 % graded ethanol solution for dehydration, and then placed in a vacuum freezing dryer overnight to dry.

F. Analysis

1 mL of fermentation broth was collected and centrifuged at 8000 rpm for 5 min. After the appropriate dilution, the concentrations of LLA and glucose in the supernatant liquid were both detected by an SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, P.R. China) [22, 23].

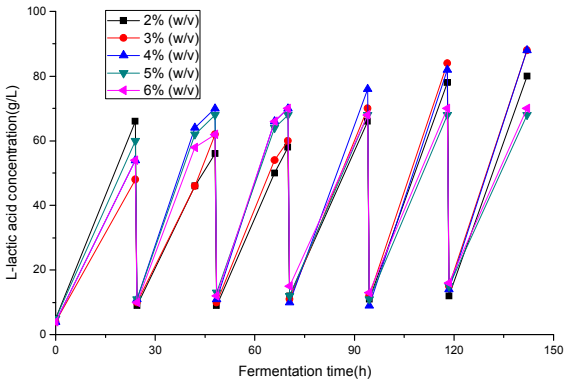
III. Results and discussion

A. The effect of carrier addition on fermentation

As the time of the immobilized repeated batch fermentation went on, the concentration and productivity of LLA increased. And the concentration and productivity of LLA gradually became stable after 5 batches of fermentation (Fig.1 (a) and Fig.1 (b)). The concentration and productivity of LLA did not increase with the increasing of the carrier loading until the carrier addition was above 4 % (w/v). On the contrary, the concentration and productivity of LLA were lower than those of 3-4 % (w/v). It might be caused by the low level of the liquid phase in the medium, leading to the non-uniform mixing and mass-transfer with the calcium carbonate promptly, resulted in lower pH, was harmful to the metabolism of the bacteria [13, 24, 25]. Thus, the optimized immobilized carrier loading was 3-4 % (w/v). After 4 batches of fermentation, the LLA productivity could be

maintained at about 3.00 g/L·h.

SEM of the cross-section and the vertical-section of the carriers with immobilization showed that, a large number of *Lactobacillus rhamnosus* cells were embedded in the cavities among the structure of the carrier. The structure of the carrier was under a rough surface, and the empty cavities would provide a stable mini-environment which was contributed to the metabolism of the bacteria (Fig.2) [19]. Therefore, cell



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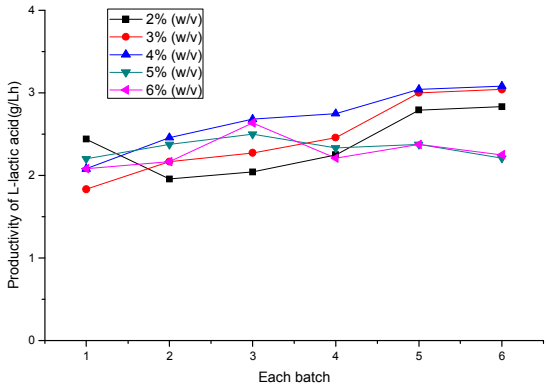
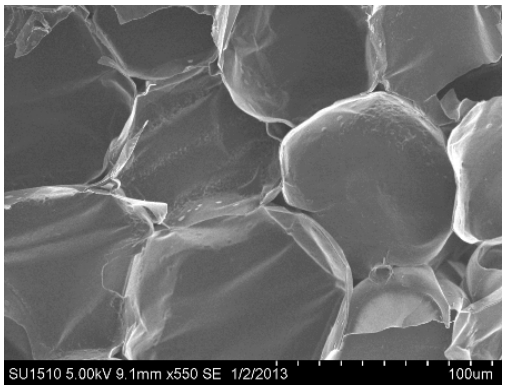


b

Fig.1. The effect of carrier addition on the immobilized fermentation: (a) The effect of carrier addition on the LLA concentration in immobilized fermentation(2% (w/v) : the 20 g carrier was added into the 1 L medium, the rest were the same); (b) The effect of carrier addition on the LLA productivity in immobilized fermentation(2% (w/v) : the 20 g carrier was added into the 1 L medium, the rest were the same)



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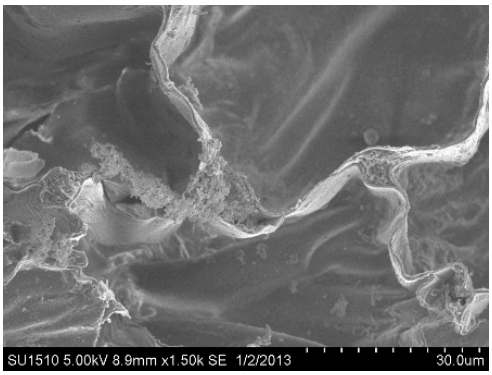

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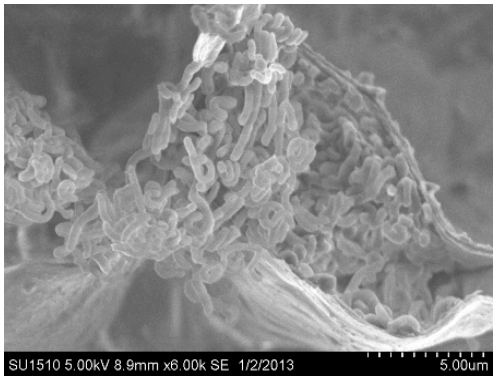

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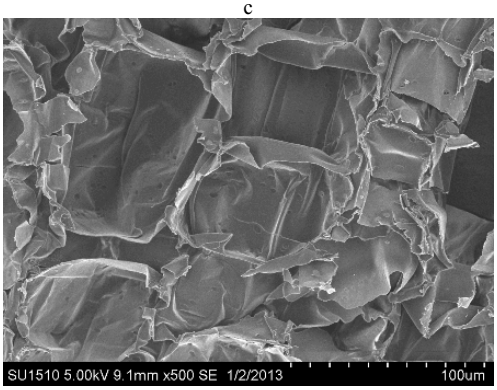

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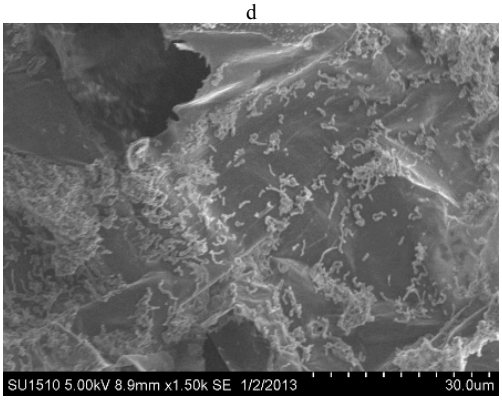


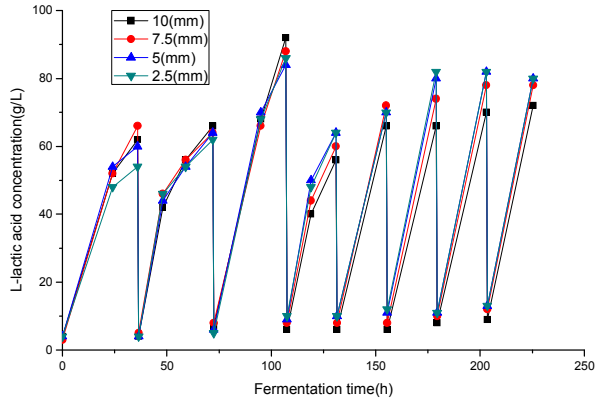
e

Fig.2. The carrier with *Lactobacillus rhamnosus* embedded inside and the original carrier under the SEM: (a) Transverse section of the original carrier showed plant stem cell cavities; (b) Transverse section of the carrier showed a large number of *Lactobacillus rhamnosus* entrapped in the plant stem cell cavities; (c) the center enlargement of Fig.(b); (d) Longitudinal section of the original carrier showed plant stem cell cavities; (e) Longitudinal section of the carrier indicated a large number of *Lactobacillus rhamnosus* entrapped in the plant stem cell cavities

immobilization using CSB, a kind of lignocellulosic biomass, had a significant effect for cell enrichment, and good reproducibility of the batch fermentation (Fig.1(a) and Fig.1(b)). That was, the natural CSB had excellent biocompatibility for cell immobilization.

B. The effect of carrier particle size on fermentation

As it was shown in Fig.3 (a) and Fig.3 (b), the different particle size of the CSB carrier had slight impact on the concentration and productivity of LLA. However, with the increasement of the carrier particle size, the increasing range of the LLA yield was lower. Therefore, the efficient surface areas of different carrier particle sizes were almost the same. As Fig.3 (b) indicated, the carrier particle size of 5 mm was the optimized choice. After 4 batches of fermentation, the average LLA productivity was stable and maintained at about 2.90 g/L·h, and the highest productivity of LLA was currently measured at 2.96 g/L·h. Besides, since CSB have characteristic of porous, the immobilized carrier had a wide range of applications, and it could be pre-treated by various methods to control and change the particle size and the porosity of the immobilization carriers, which would significantly improve the capacities of immobilization carriers, including high cell density and high activity.



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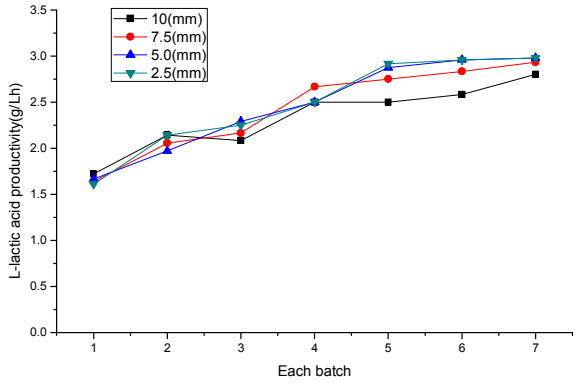

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Fig.3. The effect of carrier particle sizes on the immobilized fermentation: (a) The effect of carrier particle sizes on the LLA concentration in immobilized fermentation; (b) The effect of carrier particle sizes on the LLA productivity in immobilized fermentation

C. The immobilized repeated-batch fermentation

The CSB carriers had an excellent immobilization property in LLA fermentation with good reproducibility. In this section, the fermentation was carried out for 20 batches, of which the initial four batches were at the stage of stable growth phase of the immobilization fermentation system (Fig.4). From the fifth batch, the LLA production from fermentation was gradually stabilized with a total of about 16 batches of repeated-batch fermentation. As a result, the LLA concentration was remained at about 84 g/L in each batch. The productivity of the LLA was remained at about 5.2 g/L·h, which was almost twice as much as that of fermentation in flasks. The yield of LLA was basically about 0.95 g/g at the same time. Due to the electrostatic interaction between cells and carrier, the immobilized cell system had very strong impact of resistance and abrasion resistance. It could protect cells and reduce the activity loss of cells in long terms of fermentation [26, 27]. The structure of the CSB carrier played an important role in maintaining the stability of the intracellular pH, preventing acidification, and protecting microorganisms from the impact of high shear forces [28]. Consequently, the CSB fully met the needs of LLA fermentation with cell immobilization.

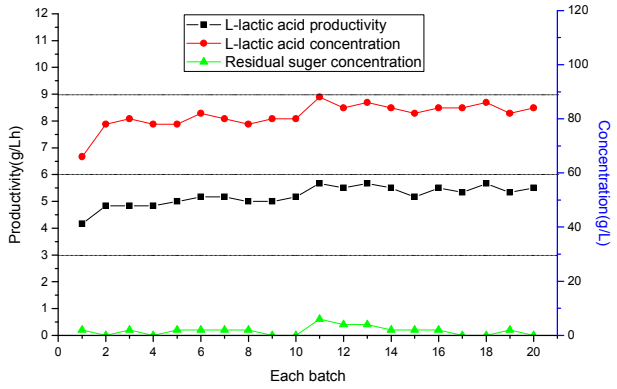

Fig.4. The trend of LLA concentration, sugar concentration and LLA productivity in each batch of the immobilized repeated-batch fermentation

D. The single-stage immobilized continuous fermentation

A total 600 h of the single-stage immobilized continuous fermentation in 1 L fermenter was then performed, the dilution rates of 0.025 /h, 0.050 /h, 0.075 /h, 0.100 /h and 0.125 /h were chosen to examine the immobilized continuous fermentation, respectively. As Fig.5 showed, in general, the LLA concentration of 84 g/L and the productivity of 2.10 g/L·h in fermentation came with the dilution rate of 0.025 /h. With the increasement of the dilution rate, the concentration of LLA was decreased markedly, while the productivity of LLA significantly increased. When the dilution rate rose to 0.125 /h, the LLA concentration fell to about 44 g/L with a productivity of 5.80 g/L·h. The significantly decrease of the yield of LLA could be due to the high concentration of the residual sugar in medium. In spite of the inhibitory effect of the initial sugar, the inhibition of LLA from the fermentation also had a dramatic impact on the production of LLA. In addition, the concentration of LLA increased in the fermentation broth, the LLA productivity decreased significantly. While, compared to the fermentation under high dilution rate,

higher concentration of LLA was obtained at lower dilution rate. Therefore, to maximize the productivity of LLA and make full use of the residual carbon source in the medium, the multi-stage immobilized continuous fermentation was a better choice. The progressive consumption of the carbon source resulted in the low residual sugar concentration to improve the yield of LLA.

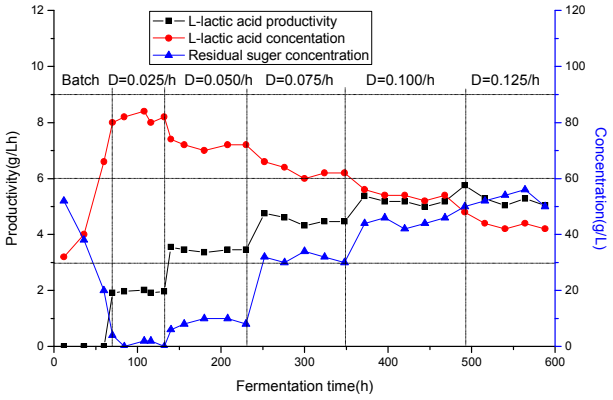

Fig.5. The trend of LLA concentration, sugar concentration and LLA productivity in single-stage immobilized continuous fermentation under the different dilution rates

E. The two-stage immobilized continuous fermentation

Although a higher productivity of LLA was achieved using the single-stage immobilized continuous fermentation under a higher dilution rate, however, the final concentration and yield of LLA were relatively low with a high concentration of residual sugar. In order to maximize the final concentration of LLA and minimize the residual carbon source concentration, two 1 L fermentors under series connection were performed according to the previous study [28] to break the dilution limitations. The optimized parameters above were used in the multiple stage of immobilized fermentation process.

A total 700 h of the two-stage immobilized continuous fermentation was performed under the dilution rates of 0.050 /h, 0.075 /h, 0.100 /h and 0.125 /h, respectively. Since the concentration of residual sugar in the first stage was almost 0 g/L under the dilution rate of 0.025 /h. Substantially, the basic need of the LLA fermentation of the second stage was limited. So the dilution rate of fermentation liquid was chosen from 0.050 /h. As apparent from Table 1, Fig. 6(a) and Fig. 6(b), in the first stage of fermentation, the dilution rate increasing would result in the decrease of LLA production remarkably, while the productivity of LLA increased significantly, which was the phenomenon of the single-stage immobilized continuous fermentation. In general, the LLA concentration of 78 g/L with a productivity of 3.90 g/L·h and a yield of 0.93 g/g was achieved respectively under the dilution rate of 0.050 /h. But when the dilution rate reached 0.100 /h, the LLA concentration was fell from 78 g/L to 62 g/L with an upward movement of LLA productivity to 6.20 g/L·h, which was 1.71 times of that in our previous study [12].

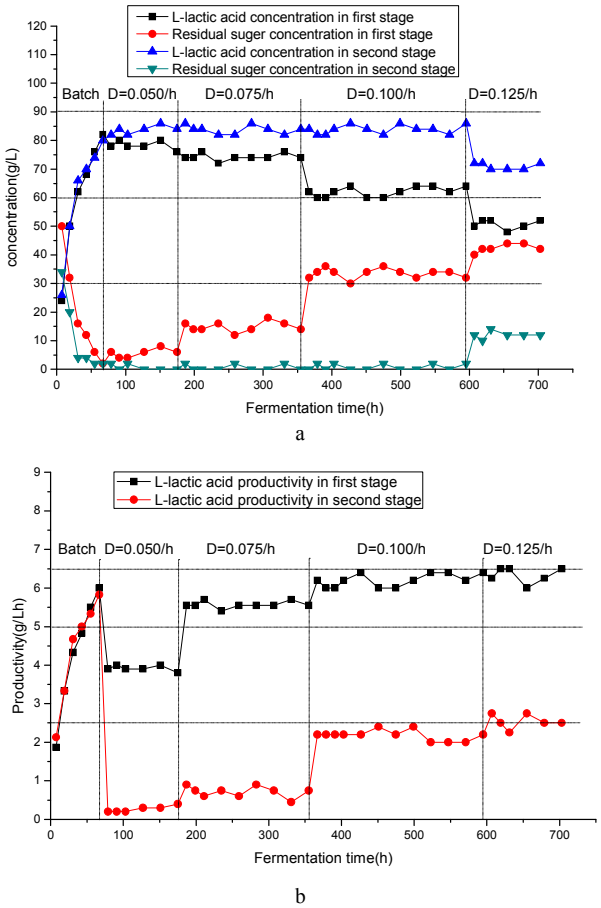

Fig.6. The trend of LLA production in two-stage immobilized continuous fermentation: (a) The trend of LLA concentrations and glucose concentrations in two-stage immobilized continuous fermentation; (b) The trend of LLA productivities in two-stage immobilized continuous fermentation under the different dilution rates

However, unlike the trend of LLA production of the first stage, the LLA concentration of the second stage leaped from 5.34 g/L to 23.83 g/L with the increase of dilution rate (Table.1). It could be due to the residual sugar concentration of medium outflowed from the first stage under a low dilution rate. As the dilution rate increased, the residual sugar concentration of medium outflowed from the first stage slightly increased. Therefore, a higher LLA concentration could be achieved under a higher dilution rate. In addition, the productivity of LLA produced from the second stage could be gradually increased with the incensement of LLA concentration in the second stage, on the contrary, the LLA yield decreased with the increase of the dilution rate.

According to Fig. 6(b) and Table 1, the optimized dilution rate of two-stage immobilized continuous fermentation was 0.100 /h. In this condition, the LLA concentration and yield reached their peaks, which were 86 g/L and 0.98 g/g, respectively, and the productivities of LLA were 6.20 g/L·h in first stage and 2.18 g/L·h in second stage, respectively. Meanwhile, the residual sugar concentration of medium outflowed from the second stage under the dilution rate of 0.100 /h was 0 g/L substantially (the maximal residual sugar concentration $\leqslant$ 2 g/L), which ensured the entire cell immobilization fermentation system could maintain at a higher dilution rate to achieve a higher processing throughput.

Table 1. Performance of the two-stage immobilized continuous fermentation under the different dilution rates

Dilution rate (/h)	The LLA production in first stage			The LLA production in second stage		
	Concentration(g/L)	Yield(g/g)	Productivity(g/L·h)	Concentration (g/L)	Yield (g/g)	Productivity (g/L·h)
0.050	78.33	0.93	3.92	5.34	0.98	0.27
0.075	74.22	0.83	5.57	9.56	0.98	0.72
0.100	62.00	0.64	6.20	23.83	0.98	2.38
0.125	50.67	0.54	6.33	20.33	0.83	2.54

Although as the dilution rate increased, the LLA productivity rose continuously, while, the yield and the concentration of LLA significantly decreased (less than 0.90 g/g and 80 g/L), the residual sugar concentration of fermentation broth from the second stage under a higher dilution rate was more than 10 g/L, which was not conducive to improving the subsequent separating operation of the LLA.

IV. Conclusions

Utilization of CSB as immobilization carrier material to make LLA production on defatted rice bran hydrolysate was studied in this paper. The LLA fermentation with cells immobilized onto CSB carriers could greatly simplify the process (including carriers preparation and cell immobilization) to improve the efficiency. As a conclusion, the optimized size of carrier was 5 mm, and the addition of carrier was preferable in the range of 3.0-4.0 % (w/v). Besides, the phenomenon of the larger particle size bringing the poor mass transfer effects was proved. During the two-stage immobilized continuous fermentation, a maximum LLA concentration of 86 g/L with a yield of 0.98 g/g was achieved at the dilution rate of 0.100 /h. In this condition, the high LLA productivities in first stage and second-stage of immobilized continuous fermentation was 6.20 g/L·h and 2.18 g/L·h was generated. The biorefinery process based on the defatted rice bran and CSB shows promising in industrial L-lactic acid production.

Acknowledgements

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Notes and references

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