Simultaneous Saccharification and Fermentation of Bagasse Sulfite Pulp to Lactic Acid by *Bacillus coagulans* CC17

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Lignocellulosic biomass is an economical and renewable feedstock for microbial production of bulk chemicals such as lactic acid. In many cases, simultaneous saccharification and fermentation (SSF) can achieve lower cost and higher productivity than the classical double step fermentation. Thus, in the present study, bagasse sulfite pulp was directly employed to produce lactic acid by SSF, using thermophilic *Bacillus coagulans* strain CC17. The effects of various factors, including $CaCO_3$ addition time and the initial buffered pH, on lactate production were investigated. It was found that *Bacillus coagulans* strain CC17 could perform well at conditions that are also optimal for fungal cellulase. The addition of $CaCO_3$ as the buffering reagent is critical for the production of lactic acid and maintaining pH. Maximum production of lactic acid was obtained by adding $CaCO_3$ after 3 h fermentation. When pH 7 was used as the initial pH, strain CC17 produced about 20.68 g/L lactic acid from 20 g/L cellulose content of BSP with 15 FPU of Cellulast 1.5L/g cellulose and 15 CBU of Novozyme 188 /g cellulose. The results showed that this strain has potential to be used for direct lactic acid fermentation from lignocellulosic biomass via SSF.

INTRODUCTION

Lactic acid, one of the most important industrial chemicals, is widely used in food, pharmaceutical, leather, cosmetic, textile, and other industries (Datta and Henry 2006; Gao *et al*. 2011). Food and food-related applications account for approximately 85% of the demand for lactic acid, whereas the nonfood industrial applications account for only 15% of the demand (John *et al*. 2009). Recently, lactic acids has drawn wide attention for the production of biodegradable and biocompatible polylactate polymers, and as an environment-friendly alternative to conventional non-biodegradable plastics made from petrochemicals. This interest is driving a current market expansion for lactic acid (Huang *et al*. 2005; Marques *et al*. 2008; Abdel-Rahman *et al*. 2011).

There are two natural pure isomers of lactic acid, D- and L-lactic acid. Since elevated levels of the D-isomer are harmful to humans, L-lactic acid is the preferred isomer in the food and pharmaceutical industries (Huang *et al*. 2005; Gao *et al*. 2012). Lactic acid production can be achieved either by chemical synthesis routes or fermentative production routes. Presently 90% of all lactic acid produced worldwide comes from the fermentative production route (Zhang *et al*. 2007). The fermentative production route can be used to obtain pure L-lactic acid by choosing an appropriate

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strain, whereas the chemical synthesis production route always results in a racemic mixture of DL-lactic acid (Hofvendahl and Hahn-Hägerdal 2000; John *et al.* 2006). The widely used substrates for lactic acid production are food crops such as potato starch, corn starch, and wheat starch. However, the use of low-cost non-food materials for lactic acid production appears to be more attractive because they do not have any impact on the human food chain. They also contribute to economic, social, and security benefits at the national and local levels (Abdel-Rahman *et al.* 2011; Singh *et al.* 2008).

Lignocellulosic resources are generally considered to represent an attractive and inexpensive raw material for the production of lactic acid, as they are renewable, widely distributed, abundant, and cheap (John *et al.* 2007; Wee *et al.* 2006). The process using lignocellulosic materials includes four main processes: pretreatment, enzymatic hydrolysis, fermentation, and separation (Linde *et al.* 2007). However, the process using cellulosic-based material is complicated. First, cellulose is a persistent polymer, and its degradation requires expensive and complicated pretreatments and multi-enzymatic reactions (Okano *et al.* 2010; Budhavaram and Fan 2009; Zhang *et al.* 2013). Second, the efficiency of the enzymatic hydrolysis is not high due to the strong inhibition by glucose and cellobiose, and thus large amounts of enzymes are required, which are very expensive (Ou *et al.* 2011). Third, the hydrolysate of cellulosic material contains five-carbon sugars and six-carbon sugars, both of which need to be efficiently converted to lactic acid (Budhavaram and Fan 2009). Thus, developing an efficient process for hydrolysis of lignocellulose into sugars and fermentation of all available sugars to lactic acid could potentially decrease the cost of lactic acid production process (Suriyachai *et al.* 2013).

The simultaneous saccharification and fermentation (SSF) process combines the enzymatic hydrolysis step and the fermentation step (Lee *et al.* 2013). It has been widely used in ethanol production and gradually applied in the production of lactic acid. It requires less investment and a lower level of enzyme loading because the fermentation step helps reduce end product inhibition from the cellobiose and glucose formed during enzymatic hydrolysis (Kang *et al.* 2012; Watanabe *et al.* 2012; Karki *et al.* 2011). It also can accelerate processing time, save equipment investment, reduce the production cost, and increase productivity. The simultaneous saccharification and co-fermentation (SSCF) process based on SSF can both ferment five-carbon and six-carbon sugars (Sreenath *et al.* 2001). Temperature and pH play an important role in affecting the activity and cell growth. The main disadvantage of SSF and SSCF lies in different temperature optima for saccharification (46 to 50 ^{o}C) and fermentation (37 to 43 ^{o}C) (Hari Krishna *et al.* 2001; Zhang *et al.* 2013). To achieve great efficiency in both SSF and SSCF, it is desirable to establish conditions optimal for two biocatalysts: cellulases and the microorganism (Patel *et al.* 2006; Wooley *et al.* 1999). Recently, *Bacillus coagulans* strains have attracted much attention owing to their strong ability to ferment both hexose and pentose sugars to optically pure L-lactic acid using the pentose phosphate pathway with a theoretical yield of 100% (Patel *et al.* 2006; Ye *et al.* 2013; Ouyang *et al.* 2012). In addition, *B. coagulans* is moderately thermophilic and capable of fermenting at 50 to 55 ^{o}C, which reduces the risk of microbial contamination, specialized equipment requirements, and high energy consumption (Abdel-Banat *et al.* 2010; Ouyang *et al.* 2013b).

The present study deals with the utilization of bagasse sulfite pulp (BSP) as an alternative substrate for lactic acid production, using the *B. coagulans* strain CC-17. It is confirmed to grow and ferment well at 50 ^{o}C. Recent studies showed that sulfite

pretreatment could enable robust performance during the hydrolysis process (Ouyang *et al*. 2013a).

Compared to other cellulosic material, paper sludge has unique advantages, such as there is no need for costly pretreatment, negative feedstock cost, and potential integration into the current infrastructure (Budhavaram and Fan 2009; Fan *et al*. 2003; Lynd *et al*. 2001). The aim of this study is to investigate the influences of $CaCO_3$, citric acid, initial pH, and cellulose concentration on SSF.

EXPERIMENTAL

Materials

Bagasse sulfite pulp (BSP) was kindly provided by Jiangmen Sugar Cane Chemical Co., Ltd (Jiangmen, China). The material was washed 10-fold with distilled water (w/w) for the removal of inhibitors and sulfite groups, filtered, stored in sealed plastic bags at 4 ^{o}C upon arrival, and used unaltered (Ouyang *et al*. 2013a). The BSP was found to consist of 73.30% cellulose, 15.45% hemicellulose, and 6.15% lignin.

The *B. coagulans* strain CC-17, obtained from Nanjing Forestry University, was used in this work. The cultures were stored at -80 ^{o}C, and a fresh batch was prepared for each experiment.

Cellulase was purchased from Sigma Aldrich, produced by Novozymes. Yeast extract was purchased from Sigma Aldrich. Others were purchased from Sinopharm Chemical Reagent Co., Ltd.

Methods

Seed cultivation conditions

The pre-culture medium consisted of (g/L): glucose 20; corn syrup powder 2.5; yeast extract 1; NH_4Cl 1; $MgSO_4$ 0.2; $CaCO_3$ 10, and distilled water. The initial pH was 7.2. The medium for SSF was comprised of the following components (g/L): cellulose 20, corn steep powder 0.892, yeast extract 1.978, $FeSO_4 \cdot 7H_2O$ 0.03, $MnSO_4 \cdot H_2O$ 0.03, and $CaCO_3$ 10.

SSF experiments

A 250 mL flask containing 80 mL pre-culture medium was incubated on a rotary shaker at 50 ^{o}C, 150 rpm for 12 h. SSF reaction mixtures contained substrate, cellulase, 10% (v/v) culture inoculums and SSF basal medium to make up the volume to 100 mL. The pH was adjusted to 4.8 with 0.05 M citrate buffer. Reactions were carried out on a rotary shaker at 50 ^{o}C, 150 rpm. The enzyme loading is 15 FPIU/g of cellulose for all the experiments.

Sample analysis

The concentrations of sugars and organic acids in the samples were determined with a high performance liquid chromatography system (HPLC, Agilent technology 1200 series, Germany) equipped with a Bio-Rad Aminex HPX-87H column (300 $\times$ 7.8 mm) and a refractive index detector (Ouyang *et al*. 2013b). The mobile phase was 5 mmol/L sulfuric acid solution at a flow rate of 0.6 mL/min. The column temperature was maintained at 55 ^{o}C. Samples were filtered through 0.22-µm syringe filters to injection.

Calculations of conversion and lactic acid yields

Calculation of lactic acid yield was based on consumed sugars. The cellulose conversion, glucose yield and xylose yield in enzymatic hydrolysis process, and the lactate production rate are based on the equations below,

$$Cellulose \quad conversion = \frac{(C_{cel} \times 1.05 + C_{glu}) \times 0.05 \times 0.9}{G * W_1} \times 100\% \quad (1)$$

$$Xylose \quad Yield = \frac{C_{xyl} \times 0.88}{G * W_2} \times 100\% \quad (2)$$

$$Rate = \frac{(C_t - C_i)}{t} \times 100\% \quad (3)$$

where C_{cel}, C_{glu}, and C_{xyl} are the final volumetric concentration of cellobiose, glucose, and xylose, respectively, obtained from enzymatic hydrolysis (g/L), 0.05 is the total volume, 0.9 is the conversion of cellulose to glucose, 1.05 is the conversion of cellobiose to glucose, G is the dry weight of BSP (g), W_1 and W_2 are the cellulose and hemicellulose content of BSP, respectively, C_t is the lactic acid concentration at time (t) (g/L), C_i is the initial lactic acid concentration (g/L), and t is fermentation time (h). Equation 1 can also represent glucose yield when the value of C_{cel} is zero.

RESULTS AND DISCUSSION

Enzymatic Hydrolysis of BSP

Enzymatic hydrolysis of 20, 40, and 60 g/L cellulose content of BSP was carried out with two commercial enzyme preparations (15 FPU of Cellulast 1.5L/g cellulose and 15 CBU of Novozyme 188 /g cellulose) to evaluate the possibility of using BSP as the carbon source for lactic acid fermentation. The BSP hydrolysate was obtained after 48 h, 72 h, and 96 h, respectively, under 50 ^{o}C and 150 rpm (rotary shaker) with 0.05 M sodium citrate buffer (pH 4.8).

Table 1. Final Sugar Concentration and Overall Cellulose Conversion Obtained in Enzymatic Hydrolysis Experiments of Different Cellulose Content

Cellulose concentration (g/L)	Cellobiose (g/L)	Glucose (g/L)	Xylose (g/L)	Cellulose conversion (%)
20	0	18.03±0.01	3.48±0.01	81.16±0.02
40	0	34.87±0.02	6.72±0.04	78.41±0.09
60	0	51.79±0.54	10.04±0.09	77.69±0.81

Glucose concentration exhibited an upward trend during the whole enzymatic hydrolysis process, and the initial glucose accumulation rates increased from 1.42 to 3.58 g/(Lh) as the initial cellulose concentration was varied from 20 to 60 g/L in the first 6 h (Fig. 1). Cellobiose accumulated in the first 12 h and then quickly became hydrolyzed by β-glucosidase (data not shown). The final concentrations of xylose were 3.48, 6.72, and 10.04 g/L, respectively (Table 1).
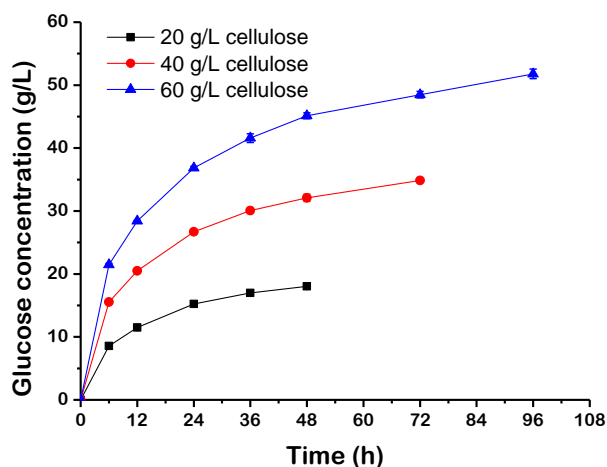
Fig. 1. Time-course of glucose accumulation in the process of 20, 40, and 60 g/L enzymatic hydrolysis

Cellulast 1.5L is a complex enzyme system that contains highly active xylanase. Thus, there was no need to add additional xylanase during enzymatic hydrolysis process. And the cellulose conversion of 20, 40, 60 g/L BSP reached 81.13%, 78.41%, and 77.69%, respectively (Table 1). The cellulose content rose 40 g/L along with a 3.44% decrease of cellulose conversion. The glucose and xylose yields (81.16% and 72.27%) were comparable to those reported by Silva and his coworkers for sugarcane bagasse. The glucose and xylose yields were 49.3% and 36.7% for WDM-treated bagasse, and 78.7% and 72.1% for BM-treated bagasse (Silva *et al.* 2010). Hence, it was found that BSP has higher enzymatic hydrolysis efficiency compared with bagasse, which makes it a highly favored raw material compared with other cellulosic materials. In this investigation BSP was used as carbon source with the initial concentration of 20 g/L since this gave its highest cellulose conversion. However, it appears that additional basic study is needed to more fully understand what happens under low concentration conditions.

Monosaccharide Fermentation

B. coagulans strains have been well known for their strong ability for using both hexose and pentose sugars as the carbon sources to produce optically pure L-lactic acid (*ee* 99.6%). (Patel *et al.* 2006). In order to evaluate its ability to convert hexose and pentose to lactic acid, preliminary fermentation of 20 g/L reagent-grade glucose, xylose and cellobiose were carried out in shake-flasks. The initial pH and temperature were adjusted to 4.8 (0.05 M citrate buffer) and 50 $^{\circ}$C with the addition of 10 g/L $CaCO_3$ as the neutralizing agent. The parameters measured in this experiment were sugar consumption and lactic acid production.

Glucose was rapidly consumed in 12 h, while xylose consumption exhibited a delay at the beginning of fermentation. This phenomenon was also evident in Wang's study. It may be due to an initial lack of related enzymes. Therefore there is a lag time before the enzymes required for assimilation appear when cells are exposed to xylose (Wang *et al.* 2010). All sugars were consumed within less than 24 h, and all retained a minor amount of unused sugars. After 48 h of fermentation the lactic acid yield of glucose, xylose, and cellobiose fermented were 86.64%, 70.85%, and 84.07%, respectively (Fig. 2). These results implied that this strain has a good fermentation

performance under enzymatic hydrolysis condition. Our observations indicated that this strain could directly metabolize cellobiose so that it may have the potential to reduce the cost of SSF by minimizing the amount of fungal cellulases, a significant cost component in the use of biomass as a renewable resource (Marques *et al*. 2008). Its ability to completely assimilate xylose with a high lactic acid production favored its use over other lactic acid bacteria (Ouyang *et al*. 2012). All these findings demonstrated that the *B. coagulans* strain CC-17 has huge potential in SSF and especially in SSCF.
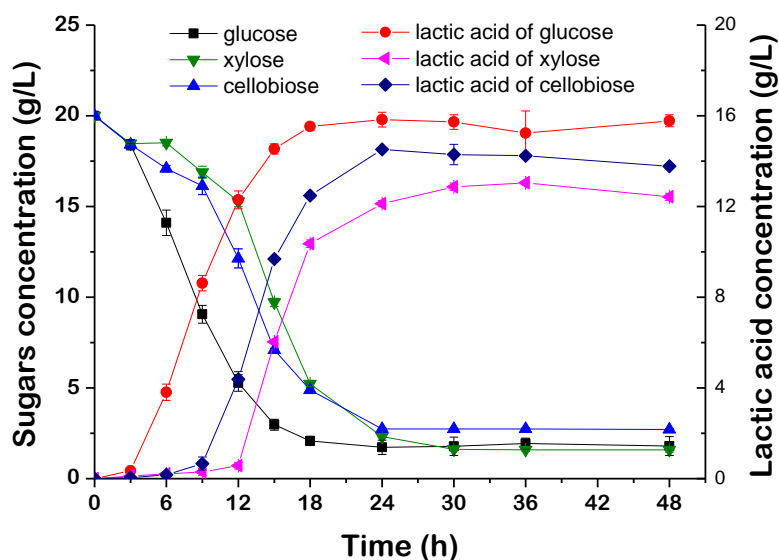

Fig. 2. Time-course of sugar consumption and lactic acid accumulation in monosaccharide fermentation

SSF Fermentation

Effect of CaCO₃ addition on lactic acid production in SSF

CaCO$_3$ is a commonly used reagent to neutralize lactic acid during fermentation. Its low solubility in water makes it possible to neutralize lactic acid and maintain the pH at a certain level automatically (Huang *et al*. 2005). In this paper, CaCO$_3$ was added at different times with a dosage of 10 g/L. An experiment of no CaCO$_3$ addition was carried out by setting up an initial pH at 4.8 using 0.05 M sodium citrate buffer. The pH during fermentation was measured by pH test paper.

As shown in Fig. 3, a very low concentration of about 2.53 g/L lactic acid was produced during the experiment in which there was no CaCO$_3$ addition; the low results were attributed to the drop of pH to near 4. Glucose and xylose concentrations showed upward trends during SSF, which indicated that it has little effect on enzymatic hydrolysis. Little assimilation of sugars and low lactic acid concentration showed that the strain neither grew well nor worked well at pH close to 4 (Budhavaram and Fan 2009). It was confirmed that the presence of CaCO$_3$ as a buffering reagent is necessary for SSF of lactic acid in the absence of active pH control. The CaCO$_3$ was added at 0 h, 3 h, and 6 h fermentation in this study under the same conditions of 150 rpm, 50 °C for 48 h. The pH could be maintained at around 5.5 by the addition of CaCO$_3$. Many other references had reported its high efficiency to maintain pH during fermentation (Huang *et al*. 2005; Marques *et al*. 2008). The maximum lactic acid concentration of 18.26 g/L was produced for the experiment with the addition of CaCO$_3$ at 3 h fermentation in about 30 h, which

was 4% higher than 0 h addition and 10.27% higher than 6 h addition. Therefore, 3 h may be the optimum $CaCO_3$ addition time for maintaining a growth pH to achieve a high lactic acid production using BSP as the substrate. It was found that citric acid concentration decreased after adding $CaCO_3$ along with sugar consumption during fermentation except for the experiment in which no $CaCO_3$ was added. The consumption of citric acid may have an impact on SSF, and further experiments were carried out to study this aspect.
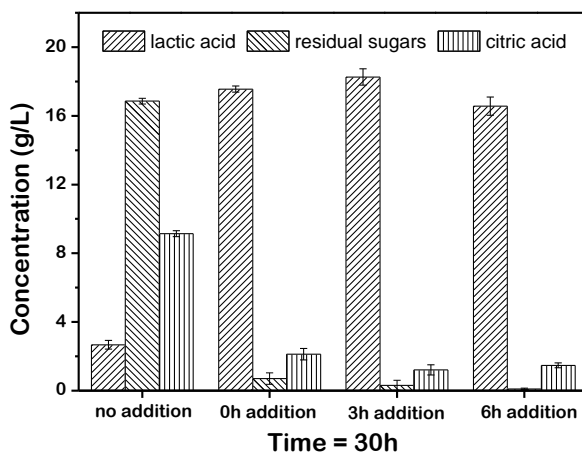

Fig. 3. Lactic acid concentration, residual sugars concentration, and citric acid concentration at 30 h fermentation of different $CaCO_3$ addition time experiments

Effect of buffer on lactic acid production in SSF

The investigation of the influence of sodium citrate consumption in the SSF process was conducted in flask fermentation using distilled water and 0.05 M citrate buffer to make up the final volume to 100 mL. Experiments were carried out at the same conditions of 50 $^{\circ}$C and 150 rpm on a rotary shaker. The pH of the experiment with distilled water was adjusted to 4.8 by using 72% H_2SO_4 to match the same pH as with the citrate buffer.

An experiment of no pH control was carried out with the initial pH measured as 5.67. The lactic acid was produced at a rate of 1.13 g/(Lh) along with the consumption of citric acid. After 12 h of fermentation, lactic acid was produced at a slower rate 0.362 g/(Lh) during 12 h to 24 h and then reached the maximum concentration of 17.96 g/L in 24 hours (Fig. 4). Citric acid concentration decreased from 8.78 to 1.04 g/L.

Experiments with distilled water and no pH control exhibited nearly the same variation trend, and could get the maximum lactic acid concentration of 17.76 and 17.84 g/L in 48 h. The glucose and xylose concentration changed at a similar rate for all experiments.

The results showed that the consumption of citric acid did not increase the production of lactic acid and had little effect on enzymatic hydrolysis. So it was decided to carry out SSF experiments without citrate buffer. And adjustment of the initial pH was found to have little influence of lactic acid production. Experiments need to be carried out to evaluate the influence of different initial pH.
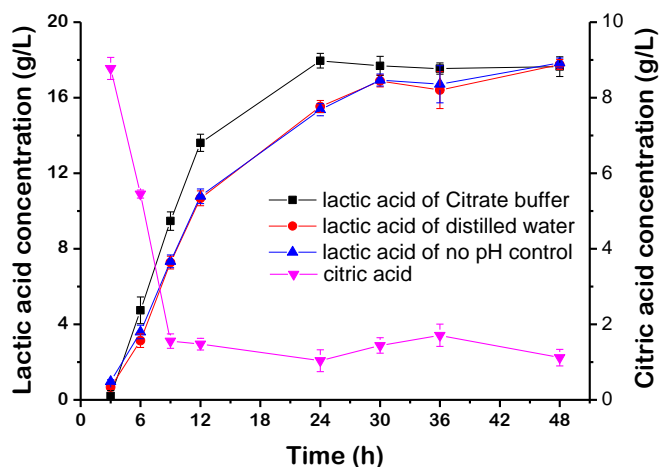
Fig. 4. Time-course of lactic acid production and citric acid consumption in SSF using citrate buffer, distilled water and no pH control broth

Effect of initial pH on lactic acid production in SSF

To investigate the impact of initial pH on SSF, the initial pH was adjusted to 4.0, 5.0, 6.0, and 7.0 by adding 72% H_2SO_4 or 50% NaOH. All experiments started with an initial cellulose concentration of 20 g/L under the conditions of 50 ^{o}C and 150 rpm for 48 h. A dosage of 10 g/L calcium carbonate was added at 3 h fermentation. And the pH was measured by universal indicator paper during SSF. The results showed that after 9 h fermentation, the pH was stabilized around 5.5 for all experiments. Lactic acid production corresponding to an initial pH of 7 was at a higher rate than others during 0 h and 12 h about 0.92 g/(Lh) and then at a slightly lower rate close to others after. From Fig. 5 it can be seen that four experiments showed similar sugar consumption trends. It is possible that the initial pH had little effect on the enzymatic hydrolysis process due to the fast degradation of substrate at low concentration. Xylose was found to be totally consumed when the initial pH was 7, but other initial pH experiments in this study showed accumulations after 48 h fermentation. This result means that the strain converted sugars to lactic acid more efficiently at pH 7, which is near its optimal pH value of 7.2 based on previous studies (Ouyang *et al*. 2013b). The highest final concentration of about 20.68 g/L lactic acid was produced at pH 7 within 48 h fermentation. The maximum lactic acid was based on the total concentration of glucose and xylose is 0.77 g/g.

To date, production of lactic acid from various renewable low-cost lignocellulosic materials has gained much attention. Cui *et al* used 30 g/L NaOH-treated corn stover for lactic acid SSF and obtained 21.0 g/L lactic acid (Cui *et al*. 2011). Rice bran was used as substrate in Tanaka's study, where 28 g/L lactic acid was obtained from 100 g/L substrate (Tanaka *et al*. 2006). The use of cellulosic biosludge as substrate for lactic acid SSF was investigated, and a product yield of 37.8 g lactic acid per 100 g biosludges was obtained after 48 h of fermentation (Romaní *et al*. 2008). In comparison with these renewable substrates, a higher lactic acid yield was obtained from BSP, demonstrating its feasibility as a raw material for L-lactic acid production. Most that reported lactic acid production from renewable biomass used *Lactobacillus* strains. Recently, several *Bacillus* sp. strains had been isolated for lactic acid SSF. Compared with other *Bacillus* sp. strains, a relatively better result was obtained in this study. Budhavaram *et al*. obtained 21.1 g/L lactic acid after 140 h fermentation using strain 36D1 in batch fermentation. Additionally,

Maas and his coworkers used *B. coagulans* DSM 2314 to ferment polymeric material and obtained a final lactic acid concentration of 40.7 g/L, which accounted for 43% (w/w) of the theoretical yield (Budhavaram *et al*. 2009; Maas *et al*. 2008). All these prove that *B. coagulans* CC-17 is a promising strain in lactic acid SSF from lignocellulosic materials.
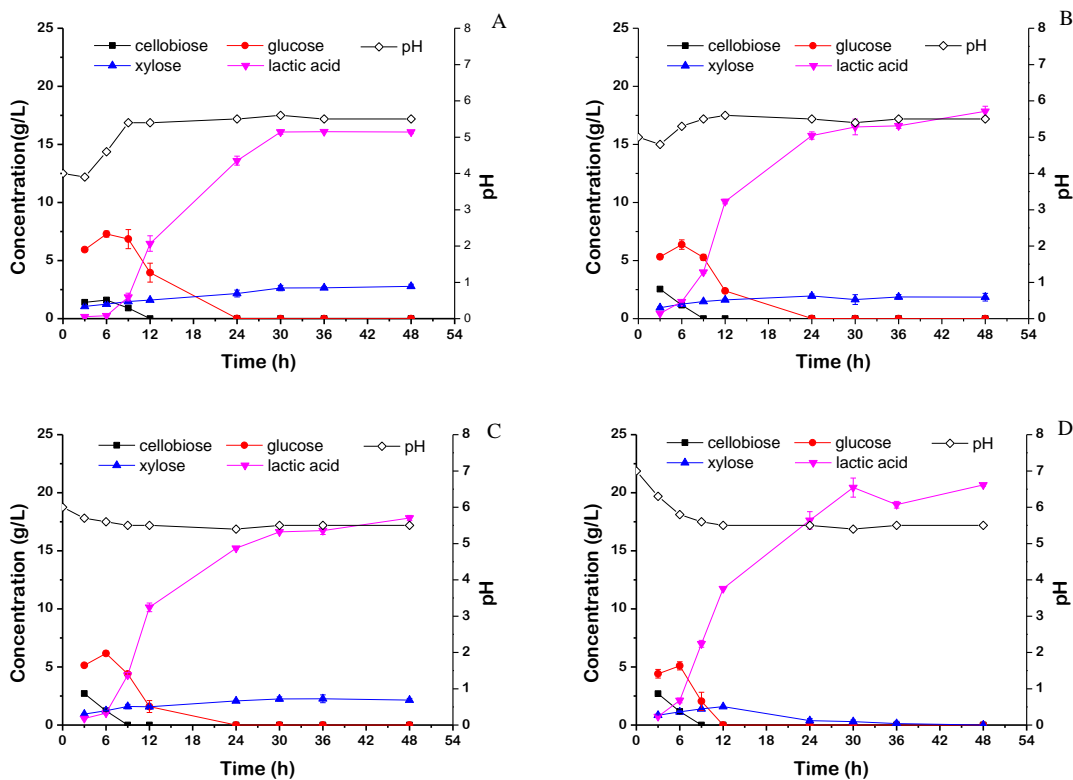

Fig. 5. Time-course of SSF process at different initial pH (A) pH 4, (B) pH 5, (C) pH 6, (D) pH 7

CONCLUSIONS

1. *Bacillus coagulans* strain CC-17 was used to convert the sugars in bagasse sulfite pulp to lactic acid and found to perform well at 50 $^{\circ}$C in SSF mode. Its ability to convert glucose, xylose, and cellobiose into lactic acid makes it a promising strain for lactic acid SSF.

2. BSP was directly used as the carbon source, and it resulted in a good enzymatic hydrolysis performance within the range of 20 to 60 g/L cellulose content.

3. Results of SSF experiments indicate that the addition of $CaCO_3$ as the buffering reagent is critical for the production of lactic acid and maintaining pH. Maximum production of lactic acid was obtained by adding $CaCO_3$ at 3 h fermentation. The consumption of citric acid had little impact on enzymatic hydrolysis and lactic acid production. And the initial pH 7 was found to get the maximum lactic acid production of 20.68 g/L.

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