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Simultaneous detoxification, saccharification and co-fermentation of oil palm empty fruit bunch hydrolysate for L-lactic acid production by *Bacillus coagulans* JI12

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ABSTRACT

Bacillus coagulans JI12 was used to produce L-lactic acid from both cellulose and hemicellulose sugars of oil palm empty fruit bunch hydrolysate at 50 °C without sterilization prior to fermentation. In fermentation of mixed glucose and xylose (10 g/L:100 g/L or 50 g/L:53 g/L), both sugars were simultaneously converted to L-lactic acid. *B. coagulans* JI12 was tolerant against up to 4 g/L of furfural and 20 g/L of acetate and able to metabolize furfural to 2-furoic acid. After acid hydrolysis, both the hemicellulosic and cellulosic fractions of oil palm empty fruit bunch were fermented to lactic acid in a simultaneous detoxification, saccharification and co-fermentation process supplemented with 25 FPU Cellic® CTec2 cellulase per g cellulose, yielding 80.6 g/L of lactic acid with a productivity of 3.4 g/L/h. Neither pre-detoxification nor separation of fermentable sugars from lignin was required. These results indicate that *B. coagulans* JI12 is a promising strain for L-lactic acid production from lignocellulosic biomass.

1. Introduction

Most studies on lactic acid production from lignocellulosic materials have been focused on using only the cellulosic fraction because the fermentation of hemicellulosic hydrolysates is more complicated due to the presence of pentose sugars such as xylose and L-arabinose that are not easily metabolized by most microorganisms [1] and the inhibitors generated during the hydrolysis of hemicellulose [2]. Some Lactobacilli have been reported to be able to ferment hemicellulosic sugars from the acid hydrolysates of agricultural wastes to produce lactic acid [3,4]. However, the lactic acid yield is usually low due to the co-production of acetic acid when C5 sugars are metabolized through the phosphoketolase pathway [5].

Thermotolerant *Bacillus coagulans* strains have been reported to be able to ferment pentose sugars completely to L-lactic acid via the pentose phosphate pathway [5,6]. For efficient and cost-effective conversion of lignocellulose to lactic acid, the complete utilization of both cellulose and hemicellulose sugars is essential. The thermo- and acid-tolerant properties of *B. coagulans* have been shown to lower the cellulase requirement in simultaneous saccharification and fermentation (SSF) of cellulose to lactic acid compared to the conventional mesophilic lactic acid bacteria [7]. The homofermentative and thermotolerant features of *B. coagulans* strains make them ideal biocatalysts for lactic acid production from lignocellulosic biomass. Lactic acid bacteria including *B. coagulans* strains have been reported to produce lactic acid either from hemicellulosic hydrolysates in batch fermentations [8–10] or from cellulosic hydrolysates in SSF [7,11]. If both the hemicellulosic and cellulosic fractions of lignocellulosic biomass can be utilized as the carbon sources in a co-fermentation process without separation, the commercial competitiveness of using lignocellulosic biomass as feedstock would be greatly enhanced. Such processes have been reported for ethanol production [12,13], but not yet for lactic acid production. A most similar report is the simultaneous saccharification and co-fermentation of crystalline cellulose and sugar cane bagasse hemicellulose hydrolysate by *Bacillus* sp. strain 36D1 [14], in which the hemicellulose hydrolysate was separated from the

Abbreviations: EFB, empty fruit bunch; SSF, simultaneous saccharification and fermentation; SDSCF, simultaneous detoxification, saccharification and co-fermentation; HMF, hydroxymethyl furfural.

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cellulose/lignin residue and detoxified by overliming before mixing with crystalline cellulose and using for fermentation.

Detoxification is often required before the lignocellulosic hydrolysates can be used for fermentation, since the generation of toxic byproducts such as furan derivatives and aliphatic acids during the pretreatment of lignocellulosic biomass would greatly inhibit the growth of lactic acid bacteria [15,16]. The conventional detoxification methods include overliming, sulfite addition, activated charcoal treatment, ion exchange, evaporation, enzymatic detoxification and biodetoxification, etc. [17]. Detoxification complicates the process increasing the production cost. Therefore, avoiding the detoxification by selecting a microbial strain that is resistant to high concentration of inhibitors and capable of detoxifying them would help simplify the process and reduce the production cost.

Here we report the efficient production of optically pure L-lactic acid from both the hemicellulosic and cellulosic fractions of oil palm empty fruit bunch (EFB) by *B. coagulans* strain JI12 in a simultaneous detoxification, saccharification and co-fermentation (SDSCF) process.

2. Materials and methods

2.1. Fermentation of mixed glucose and xylose

For preparing seed culture, *B. coagulans* JI12 (ATCC PTA-13254) was grown overnight in 100 mL mineral salts medium [5] containing 5% (w/v) xylose or glucose and 3% (w/v) $CaCO_3$ in 250 mL conical flasks at 50 °C and 200 rpm. Cells from the culture were collected by centrifugation (4000 rpm, 10 min) and resuspended in 50 mL fresh fermentation medium. The cell suspension was then used as the inoculums for the subsequent fermentation experiments.

Mixed sugar fermentations were carried out in a 2 L bioreactor (Biostat® B plus, Statorius Stedium Biotech, Germany) containing 1 L of fermentation medium at 50 °C, 100 rpm. The fermentation medium was composed of 2 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 2 g/L NaCl, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $MnSO_4{\cdot}7H_2O$, 0.01 g/L $FeSO_4{\cdot}7H_2O$, 10 g/L yeast extract and mixed sugars (xylose:glucose = 100:10 or 53:50, g/L). NaOH (15 M) was automatically added to neutralize the lactic acid produced during the fermentation to maintain the pH at 6.0. All fermentations were conducted openly (without sterilization of the medium).

2.2. Growth and lactic acid fermentation in the presence of furfural or acetate

Cells of *B. coagulans* JI12 were grown at 50 °C and 200 rpm in 100 mL mineral salts medium containing 83 ± 2 g/L of xylose, 10 g/L of yeast extract and furfural or acetate at predetermined concentrations. $CaCO_3$ (5%, w/v) was added to neutralize the lactic acid produced during fermentation. Xylose consumption and lactic acid production was analyzed by HPLC and cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}).

2.3. Acid hydrolysis of oil palm empty fruit bunch (EFB)

Lignocellulosic hydrolysates were prepared by acid-catalyzed hydrolysis of empty fruit bunch (EFB) of oil palm trees. EFB was kindly provided by Wilmar International Limited, Singapore. It was dried and grounded as previously described [18]. Dried powder of EFB (150 g) was added into 450 mL water containing 2% (w/v) of H_2SO_4 and 0.8% (w/v) of H_3PO_4. Acid-catalyzed hydrolysis of EFB was carried out in a 1 L Parr reactor (Fike, Blue Springs, MO, USA) at 130 °C for various durations.

For batch fermentation, the hemicellulosic hydrolysate was separated from the solid cellulose–lignin complex by filtration after hydrolysis. The filtrate from multiple hydrolysis runs was combined and over-limed as described previously [19]. The hydrolysate was then centrifuged to remove the resultant gypsum.

For simultaneous detoxification, saccharification and co-fermentation (SDSCF), 300 mL water was used after hydrolysis at 130 °C for 30 min to wash and collect all the solid residue and liquid fractions from the Parr reactor, followed by addition of solid $Ca(OH)_2$ into the mixture (consisting of both hemicellulose hydrolysate and solid cellulose–lignin complex) to adjust its pH at 5.5.

2.4. Fermentations of EFB acid hydrolysates

For batch fermentations, into 550 mL of EFB hydrolysate in a 2 L fermenter was added 1% of yeast extract and 0.2% of $(NH_4)_2SO_4$ followed by inoculation of the seed culture. The seed culture was prepared in 60 mL of mineral salts medium containing 5% of xylose, 1% of yeast extract and 3% of $CaCO_3$ in 250 mL conical flasks at 50 °C and 200 rpm overnight. After centrifugation at 4000 rpm, 4 °C for 10 min, the cells were collected and re-suspended in 50 mL of EFB hydrolysate.

For SDSCF, the seed culture was prepared following the same procedures except that the cells were grown in 80 mL of mineral salts medium containing 5% of xylose, 5% of glucose, 1% of yeast extract and 3% of $CaCO_3$. SDSF was initiated by adding the inoculums and 25 FPU of Cellic® CTec2 cellulase (Novozymes, Bagsværd, Denmark) per gram of cellulose.

For both batch fermentations and SDSCF, $Ca(OH)_2$ (35%, w/v) was automatically added to neutralize the lactic acid produced to maintain the pH at 6.0 for batch fermentation and at 5.5 for SDSF. Samples were taken at predetermined time intervals for HPLC analysis to monitor the inhibitors, sugars and lactic acid.

2.5. Investigation of furfural detoxification by B. coagulans JI12

B. coagulans JI12 was inoculated to 50 mL L-Broth supplemented with 1 g/L furfural to investigate the biodetoxification of furfural. The reaction was started with 10% inoculation of overnight grown seed culture. The experiment was conducted in triplicates. Samples were taken at predetermined time points for HPLC analysis. Furoic acid and furfuryl alcohol were used as external and internal standards for identification of the furfural metabolites. At the end of the reaction, the culture broth was collected, centrifuged to remove the cells, filtrated and subjected to LC–MS analysis on a Waters Quattro Micro API LC–MS/MS equipped with a C18 column and a Waters 2996 Photodiode Array Detector (Waters, Milford, MA, USA).

2.6. Analytical methods

Xylose, glucose, L-arabinose, lactic acid, acetic acid, furfural and its metabolite were analyzed by a Shimadzu HPLC system (LC-10AT, refractive index detector SPD-10A, Shimadzu, Kyoto, Japan) equipped with a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad, Herculse, CA, USA) at 50 °C. The mobile phase was 12 mM H_2SO_4 at 0.65 mL/min. The optical purity of lactic acid was checked on the same HPLC equipped with a Mitubishi MCI(R) GEL CRS15W column (50 mm × 4.6 mm, Mitubishi Chemical, Tokyo, Japan) at 30 °C using 2 mM $CuSO_4$ as the mobile phase at 0.4 mL/min.

3. Results and discussion

3.1. Fermentations of mixed xylose and glucose

Glucose and xylose are the two major sugar components in lignocellulosic biomass. Efficient utilization of these two sugars is a

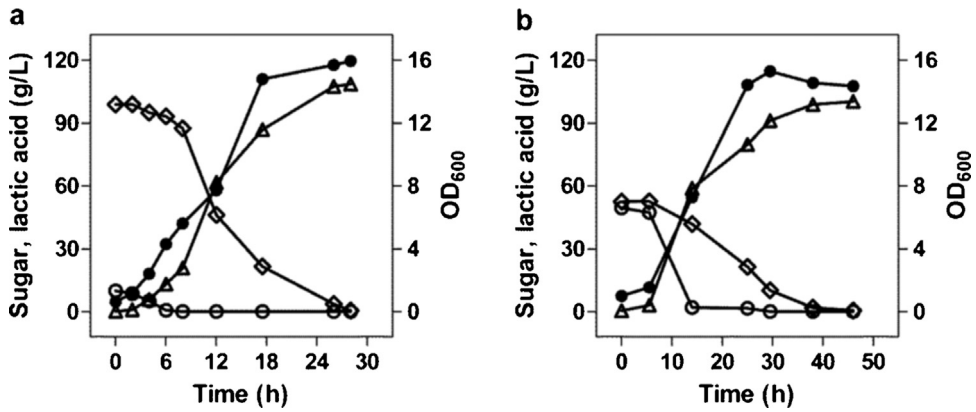

Fig. 1. Time courses of xylose (◊) and glucose (○) consumptions, L-lactic acid production (△) and cell growth (OD_{600}, ●) in mixed sugar fermentations by *Bacillus coagulans* JI12. Fermentations were conducted in mineral salt medium with 1% yeast extract (w/v) as nitrogen source at pH 6.0 and 50 °C with (a) 10 g/L glucose and 100 g/L xylose, or (b) 50 g/L glucose and 53 g/L xylose as carbon sources. NaOH (15 M) was used as the neutralizing agent.

prerequisite for cost-effective conversion of lignocellulose to value-added chemicals. To simulate the major sugar components in the hemicellulosic fraction and in the whole lignocellulose hydrolysate (including hemicelluloses and cellulosic fractions) and investigate the possible glucose repression in mixed sugar fermentations by *B. coagulans* JI12, mixtures of xylose and glucose at different ratios were tested as carbon sources. Xylose is the major component of hemicellulose sugars, accounting for 60–70% or above [20], while glucose is often present as a minor component. Therefore, a mixture of 100 g/L xylose and 10 g/L glucose was used to simulate the sugar compositions in hemicellulose hydrolysate (Fig. 1a). Xylose consumption started before the depletion of glucose (8 h) and all sugars were used up within 28 h, yielding 108.6 g/L lactic acid. The average productivity reached 3.9 g/L/h.

Since cellulose and hemicellulose account for 35–50% and 20–40% of the lignocellulosic biomass [21], respectively, another fermentation was conducted using a mixture of 50 g/L glucose and 53 g/L xylose (Fig. 1b) to simulate sugars in whole lignocellulose hydrolysate. All sugars were converted to 100.3 g/L lactic acid within 38 h, giving a yield of 98% and a productivity of 2.6 g/L/h. The consumptions of glucose and xylose started almost simultaneously, which is different from most other bacteria. For most microorganisms, xylose and glucose are usually not simultaneously metabolized and the xylose consumption begins only when there is almost no glucose left in the medium, so-called glucose repression [22,23]. Glucose repression is one of the major limitations in mixed sugar fermentations for cost-effective production of fuels and chemicals. In our experiments, although the average consumption rate (1.3 g/L/h) of xylose at higher glucose:xylose (1:1) ratio was lower than that (3.7 g/L/h) at lower glucose:xylose (1:10) ratio, the presence of glucose (up to 50 g/L) did not obviously prevent the xylose consumption by *B. coagulans* JI12, indicating that the expression of xylose-catabolizing genes in *B. coagulans* JI12 might be less repressed by glucose than those in other bacteria [24]. These results indicate that *B. coagulans* JI12 is a promising bacterial strain for co-fermentation of mixed sugars derived from lignocellulosic biomass.

3.2. Effect of furfural and acetic acid on xylose fermentation

The release of pentose and hexose sugars during pretreatment and enzymatic hydrolysis is often accompanied by the liberation of byproducts such as acetic acid and furans. Acetic acid is derived from the hydrolysis of the acetyl groups bound to the hemicellulosic monomers [25]. The acid may cause inhibitory effect on microbial growth as it can enter the cell membrane and decrease intercellular pH, consequently affecting the metabolisms of the microorganisms [15]. Furfural is generated by threefold dehydration of xylose and is one of the key toxic furan derivatives in acid pretreated lignocellulose hydrolysates, which causes detrimental effects that result in a decrease of specific growth rate, fermentation yield and productivity for both yeasts and bacteria [26].

For efficient fermentation of sugars in lignocelluloses hydrolysates, it would be ideal if the microorganisms used could also tolerate or even metabolize theses toxic compounds. In mineral salts medium containing 8% xylose, the tolerances of *B. coagulans* JI12 against furfural and acetate were tested in concentration ranges of 0–5 g/L and 0–30 g/L, respectively. As shown in Table 1, up to 4 g/L of furfural and 20 g/L of acetate were tolerated by *B. coagulans* JI12. In the co-presence of ≤4 g/L of furfural or 20 g/L of acetate, the fermentation underwent a longer lag phase but the lactic acid yield was not reduced, whereas 5 g/L of furfural and 30 g/L of acetate (sodium salt) were found to be completely inhibitory to *B. coagulans* JI12. At concentrations not exceeding 4 g/L, furfural did not influence the cell biomass, whereas the cell growth was inhibited by 76% at 5 g/L of furfural. Similarly, acetate at up to 15 g/L had no obvious influence on the cell biomass of *B. coagulans* JI12, whereas the cell growth was inhibited by 6% and 79% at 20 g/L and 30 g/L of acetate, respectively. Similar inhibition trends were observed for xylose consumption and lactic acid production.

It has been reported that the growth and fermentation of *B. coagulans* MXL-9 were completely inhibited by 30 g/L of sodium sulfate and the presence of 30 g/L of ammonium acetate extended the lag phase [27]. High concentrations of both sodium and acetate were inhibitory to cell growth of *Bacillus* species. These results are comparable to our observations with *B. coagulans* JI12. When furfural tolerance is concerned, *Bacillus spp.* IFA 119 showed 78% growth inhibition by 1.0 g/L furfural [16] and the growth of *Escherichia coli* LY01 was inhibited by 50% in the presence of 2.4 g/L furfural [8], while the growth of *B. coagulans* JI12 was not obviously inhibited by up to 4 g/L furfural, indicating its higher furfural tolerance.

3.3. Biodetoxification of furfural during batch fermentation of EFB hydrolysate

Fermentation was conducted using overnight grown cells of *B. coagulans* JI12 as inoculums and 35% $Ca(OH)_2$ as the neutralizing agent. The sugar compositions of EFB hydrolysate were 6.9 g/L of glucose, 53.9 g/L of xylose and 6.9 g/L of L-arabinose. The inhibitor concentrations were 18.1 g/L of acetic acid, 1.4 g/L of furfural and 0.3 g/L of 5-hydroxymethylfurfural (HMF).

Table 1
Cell growth and lactic acid production of *B. coagulans* JI12 in the presence of furfural or acetate.

Inhibitor	Maximal growth (OD_{600})	Time to maximal growth (h)	Lactic acid titer (g/L)	Fermentation time (h)
No inhibitor	8.0	23	82.3	30
2 g/L furfural	8.0	23	80.7	30
3 g/L furfural	7.9	30	83.2	30
4 g/L furfural	7.9	30	82.4	48
5 g/L furfural	1.9	48	2.1	48
10 g/L acetate	7.9	23	81.5	30
15 g/L acetate	8.1	23	81.0	30
20 g/L acetate	7.5	48	80.6	55
30 g/L acetate	1.7	55	1.0	55

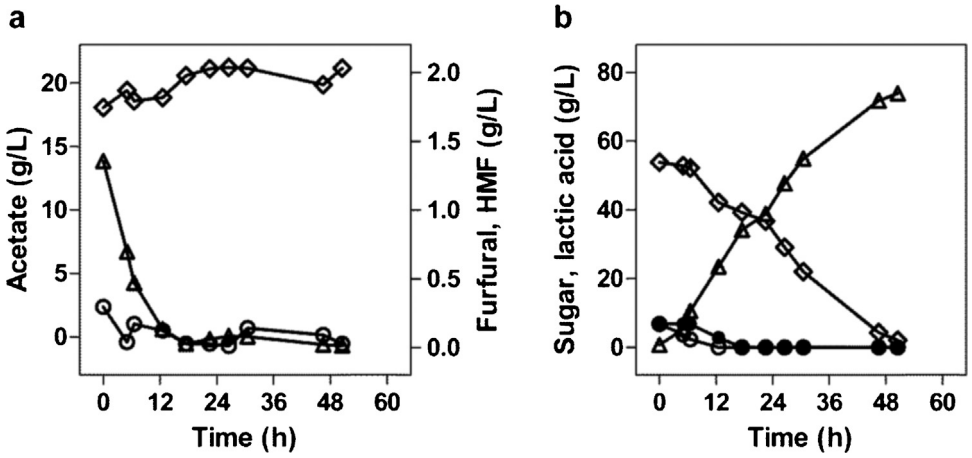

Fig. 2. Detoxification of furfural and fermentation of hemicellulosic hydrolysate of oil palm empty fruit bunch (EFB) to lactic acid by *B. coagulans* JI12 in batch fermentation. (a) Time courses of HMF (◯), furfural (△) and acetic acid (◊); (b) time courses of glucose (◯), xylose (◊) and arabinose (●) consumption and L-lactic acid production (△). Fermentation was conducted at pH 6.0 and 50 °C with supplement of 1% yeast extract (w/v) and 0.2% $(NH_4)_2SO_4$. 35% $Ca(OH)_2$ was used as the neutralizing agent.

B. coagulans JI12 exhibited a strong ability toward metabolizing furfural. During the fermentation of EFB hydrolysate, furfural (1.4 g/L) was completely metabolized within 17.5 h (Fig. 2a). When furfural concentration was decreased to 0.7 g/L at 5 h, obvious increases in both rates of sugar consumption and lactic acid production were observed, indicating that furfural at >0.7 g/L inhibited cellular metabolisms in the hydrolysate. This lowered tolerance concentration to furfural in the hydrolysate compared to that in the mineral salts medium (Table 1) might be ascribed to the coexistence of other inhibitors such as acetic acid, HMF and phenolic compounds. The time courses of sugar consumption and lactic acid production in the fermentation of EFB acid hydrolysate are shown in Fig. 2b, giving 73.9 g/L of lactic acid within 50.5 h with a productivity of 1.5 g/L/h and a yield of 109%. The higher than predicted lactic acid yield might be caused by the conversion of remaining oligosaccharides in the hydrolysate to lactic acid as reported for other lactic acid bacteria [28].

Previous studies have shown that furfural might be converted to less toxic compounds such as furfuryl alcohol or furoic acid [29]. A number of organisms including fungi and bacteria have been reported to be able to metabolize furfural [30–32] and serve as potential biological detoxification agents for pretreatment of lignocellulosic biomass [33,34]. The conversion products of furfural by *B. coagulans* have not yet been identified so far.

To investigate the furfural metabolites by *B. coagulans* JI12, the strain was inoculated into LB broth supplemented with 1.08 g/L of furfural and incubated at 50 °C. Samples were taken every 2–3 h for HPLC analysis. It was found that *B. coagulans* JI12 converted all furfural to 0.97 g/L furoic acid within 9 h (Fig. 3), which was verified by LC–MS (Fig.A.1 in Appendix), indicating that each molecule of furfural was oxidized to yield one molecule of furoic acid by *B. coagulans* JI12.

3.4. SDSCF of acid pretreated EFB whole slurry

Owing to the ability of *B. coagulans* JI12 for fermentation of both glucose and xylose and its tolerance to high concentrations of furfural and acetate, fermentations were also conducted directly using the acid-pretreated whole EFB slurries including the hemicelluloses hydrolysate and cellulose–lignin complex without detoxifying the mixture by over-liming. The initial inhibitor concentrations were: 12.9 g/L of acetic acid, 1.4 g/L of furfural and 0.04 g/L of HMF. After inoculating *B. coagulans* JI12 and adding Cellic® CTec cellulase, the SDSF process was initiated at 50 °C and pH 5.5. The amount of

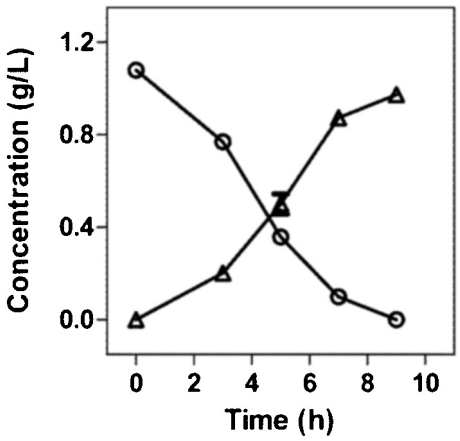

Fig. 3. Time courses of furfural detoxification by *Bacillus coagulans* JI12 in LB medium. Biodetoxification was initiated in 20 mL LB medium with 1.08 g/L furfural by inoculating 2 mL overnight cultured *B. coagulans* JI12. The culture was kept at 50 °C and 200 rpm. Symbols: residual furfural (◯), furoic acid (△).

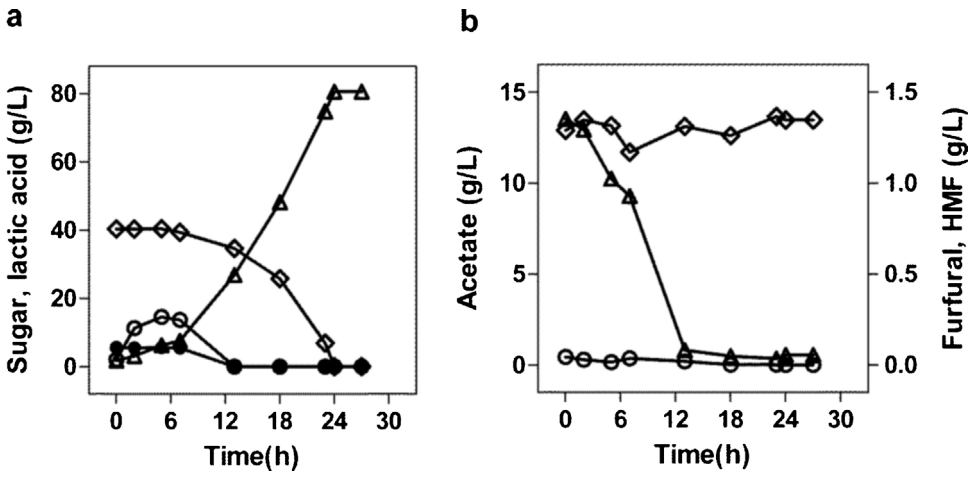

Fig. 4. Time courses of simultaneous detoxification, saccharification and co-fermentation of EFB hydrolysate by *Bacillus coagulans* JI12. Fermentation was conducted at pH 5.5 and 50 °C with supplement of 1% yeast extract (w/v), 0.2% $(NH_4)_2SO_4$ and 25 FPU Cellic® CTec2 cellulase/g cellulose. 35% $Ca(OH)_2$ was used as the neutralizing agent. Symbols: (a) glucose (◯), xylose.

cellulase was optimized to 25 FPU/g cellulose in a pre-test. The pH of 5.5 was chosen by compromising the pH optima of the cellulase (5.0–5.5) and the lactic acid fermentation (5.5–6.0).

As shown in Fig. 4, glucose was continuously released from the cellulosic fraction by the Cellic® CTec cellulase and accumulated to a peak at 5 h and then depleted at 13 h without further accumulation. L-Arabinose was also completed consumed within 13 h. The depletion of xylose was observed at 24 h. The conversion of both xylose and L-arabinose seemed to be inhibited within the first 7 h. Although there was no significant lag phase in lactic acid production, the production rate obviously increased after 7 h. The inhibition of sugar consumption and lactic acid production in the first 7 h might be caused by the higher furfural concentration (>1 g/L). When the furfural concentration was decreased to 0.9 g/L at 7 h and further decreased to 0.1 g/L at 13 h, the fermentation rate was obviously increased. At the end of the fermentation, 80.6 g/L of lactic acid was produced, giving an average productivity of 3.4 g/L/h and a yield of 0.49 g lactic acid per g EFB. In comparison, only 35 g/L lactic acid was produced with a maximal lactic acid productivity of 0.6 g/L h in the simultaneous saccharification and co-fermentation of crystalline cellulose and an over-limed sugar cane bagasse hemicellulose hydrolysate by *Bacillus* sp. 36D1 [14].

4. Conclusions

B. coagulans JI12 simultaneously converted glucose and xylose to L-lactic acid in mixed sugar fermentation. It also showed tolerance to 20 g/L of acetate and 4 g/L of furfural. Furfural was metabolized to furoic acid. In a simultaneous detoxification, saccharification and fermentation process, *B. coagulans* JI12 metabolized all furfural in the acid pretreated EFB whole slurry and produced over 80 g/L of lactic acid in the presence of cellulase. The lactic acid productivity reached 3.4 g/L/h. This integrated process made *B. coagulans* JI12 a promising strain for converting lignocellulosic sugars to L-lactic acid.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2013.12.005.

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