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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