Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Process engineering of cellulosic *n*-butanol production from corn-based biomass using *Clostridium cellulovorans*

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ARTICLE INFO

Keywords:
Clostridium cellulovorans
Butanol fermentation
Consolidated bioprocessing
Corn residues

ABSTRACT

The cellulolytic *Clostridium cellulovorans* has been engineered to produce *n*-butanol from low-value lignocellulosic biomass by consolidated bioprocessing (CBP). The objective of this study was to establish a robust cellulosic biobutanol production process using a metabolically engineered *C. cellulovorans*. First, various methods for the pretreatment of four different corn-based residues, including corn cob, corn husk, corn fiber, and corn bran, were investigated. The results showed that better cell growth and a higher concentration of *n*-butanol were produced from corn cob that was pretreated with sodium hydroxide. Second, the effects of different carbon sources (glucose, cellulose and corn cob), basal media and culture pH values on butanol production were evaluated in the fermentations performed in 2-L bioreactors to identify the optimal CBP conditions. Finally, the engineered *C. cellulovorans* produced butanol with final concentration > 3 g/L, yield > 0.14 g/g, and selectivity > 3 g/g from pretreated corn cob at pH 6.5 in CBP. This study showed that the fermentation process engineering of *C. cellulovorans* enabled a high butanol production directly from agricultural residues.

1. Introduction

n-Butanol is a potential substitute for gasoline, a raw material used to generate bio-jet fuel and biodiesel, and an important industrial chemical [1]. The butanol produced from conventional acetone-butanol-ethanol (ABE) fermentation is uneconomical in fuel market, mainly attributed to the high expense of starchy feedstock [2].

Compared to the starch-based fermentation, the production cost of butanol from lignocellulosic biomass, such as agricultural residues of corn, rice, wheat and soybean, grass, and wood, can be significantly reduced [3,4]. The lignocellulose is typically composed of cellulose, hemicellulose, and lignin. The pectinolytic enzymes and lignin degrading enzymes can loosen the cell wall and allow better access to cellulose and hemicellulose. The fermentable sugars, such as glucose, xylose, arabinose, galactose, mannose and rhamnose, can be converted from cellulose and hemicellulose by cellulase and hemicellulase, respectively [5,6].

Tremendous progress has been made to produce *n*-butanol from cellulosic biomass. For instance, the cellulosic hydrolysate has been fermented by solventogenic strains [7–11], but the use of cellulose hydrolysis has significantly increased the operation cost. Alternatively, butanol can be produced directly from biomass by consolidated bioprocessing (CBP) that combines cellulase and hemicellulase production, cellulose and hemicellulose hydrolysis, and hexose and pentose sugars fermentation. For example, a miniature cellulosome has been synthesized in solventogenic clostridia [12–14] for cellulosic butanol production in CBP, but the expression of heterologous cellulosome is unstable. The co-fermentations of cellulolytic and solventogenic strains, such as *C. thermocellum* & *C. acetobutylicum* and *C. cellulovorans* & *C. beijerinckii*, have been used to generate cellulosic butanol [15–17], but it is difficult to engineer the co-fermentation of clostridia due to the complicated cellular interaction.

Alternatively, the cellulolytic clostridia that express highly active cellulase and hemicellulase, such as *C. thermocellum* and *C. cellulovorans* [18], could be metabolically engineered to produce *n*-butanol. For instance, the heterologous bifunctional acetaldehyde/alcohol dehydrogenase (*adhE2*) catalyzing butyryl-CoA to butanol has been introduced into acidogenic *C. cellulovorans* (Fig. 1), producing 1.6 g/L of *n*-butanol from cellulose [19]. The cellulosome in *C. cellulovorans* has been well investigated [20–23], but the production of *n*-butanol from agricultural residues by CBP has not been fully explored.

The objective of this study was to develop a robust CBP that

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http://dx.doi.org/10.1016/j.procbio.2017.07.009
Received 14 May 2017; Received in revised form 22 June 2017; Accepted 13 July 2017

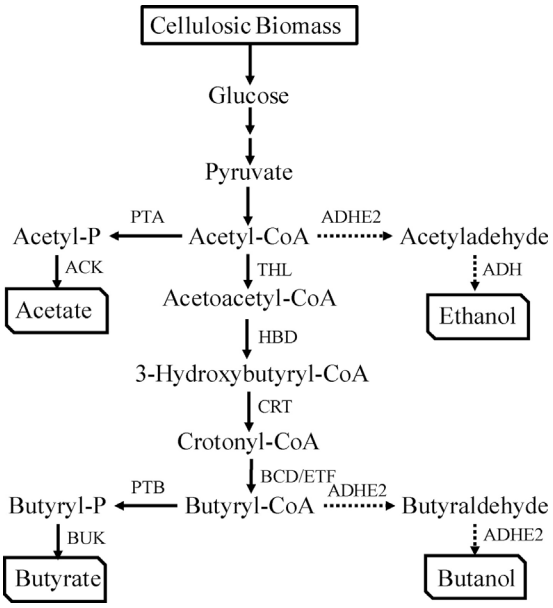

Fig. 1. Metabolic pathway in *C. cellulovorans-adhE2*. Abbreviations: THL, thiolase; HBD, beta-hydroxbutyryl-CoA dehydrogenase; CRT, 3-hydroxybutyryl-CoA dehydratase; BCD, butyryl-CoA dehydrogenase; ETF, electron transfer flavoprotein; PTA, phosphotransacetylase; ACK, acetate kinase; PTB, phosphotransbutyrylase; BUK, butyrate kinase; ADH, alcohol dehydrogenase; ADHE2, bifunctional acetaldehyde/alcohol dehydrogenase.

contains biomass pretreatment and cellulosic *n*-butanol production using the metabolically engineered *C. cellulovorans-adhE2*. Chemical pretreatment of four different corn-based biomass was investigated. The *n*-butanol fermentation process was optimized by evaluating the effects of carbon sources, basal media and culture pH. A higher level of butanol was produced from corn cob in CBP by applying the optimized conditions, which could offer an economical bioprocess for cellulosic *n*-butanol production.

2. Materials and methods

2.1. Strain and culture media

A mutant strain of *C. cellulovorans* ATCC 743B with overexpressed heterologous *adhE2* gene, which produced *n*-butanol from cellulose [24], was used in this study. The seed culture was anaerobically maintained in the modified DSMZ 520 medium supplemented with 30 μg/mL of thiamphenicol (Tm, Alfa Aesar, Ward Hill, MA) and 20 g/L of glucose at 37 °C. Both modified DSMZ 520 and ATCC 1345 media were used, as reported previously [25,26], and evaluated by comparing the cell growth and *n*-butanol production. Different carbon sources, including glucose, cellulose (microcrystalline, Alfa Aesar), corn cob (Northern Tool, Burnsville, MI), corn husk (collected from fresh corn and dried at 100 °C), corn fiber (Cargill, Wayzata, MN), and corn bran (Honeyville, Brigham City, UT), were tested. All chemical reagents were purchased from Thermo Fisher Scientific (Waltham, MA), unless otherwise specified.

2.2. Pretreatment (delignification) of corn residues

The size of corn cob was 0.29–0.38 mm, the corn bran was purchased in the form of fine powder, and the fiber and husk were ground with a Cuisinart DBM-8 Supreme Grind Automatic Burr Mill. The pretreatment of biomass was carried out in 200-mL screwed cap media bottles to evaluate the delignification efficiency. Each bottle containing 10 g of biomass and 100 mL of 0.5% H_2SO_4, 0.2 M $Ca(OH)_2$ or 0.4 M NaOH, respectively, was autoclaved at 121 °C for 2 h. During pretreatment, the lignin structure of the biomass was disrupted and the hemicellulose substrates were extracted and solubilized. After cooling down, the treated biomass was neutralized by washing with tap water and filtering through Whatman Grade 1 qualitative filter paper, and dried at 80 °C for 2 days. The pretreated biomass samples that mainly contain cellulose were stored in sealed plastic bags at 4 °C until used.

2.3. Cellulosic butanol fermentation

The cellulosic butanol fermentations of *C. cellulovorans-adhE2* were performed in 2-L stirred-tank bioreactors (FS-01-A; Major science, Saratoga, CA). The bioreactors containing basal medium and carbon source, i.e. delignified biomass, cellulose or glucose (control), were autoclaved at 121 °C for 60 min and purged with nitrogen at 10 mL/min for 3 h to reach anaerobiosis. Fresh seed culture with optical density at 600 nm (OD_{600}) of 1.0 was used to inoculate the fermentation medium to reach seeding density of OD_{600} of ~0.05. All fermentations were operated at temperature 37 °C, agitation 100 rpm, and various pHs (6.5, 7.0 and 7.5) controlled with 5 M NaOH. The bioreactors were sampled at regular intervals (once or twice a day) to monitor cell growth and titrate the substrate and products. The preparation of the seed cultures for the bioreactors and the mini butanol fermentations were conducted in static 100-mL serum bottles by manually adjusting the pH to 7.0 twice a day using 5 M NaOH. All fermentations were carried out in duplicate and the data were presented as the average of replicates with standard deviation.

2.4. Analytical methods

In this study, the composition of cellulose, hemicellulose and lignin in the corn cob was analyzed using the previously developed RI method [27]. The cell density was estimated by measuring the OD_{600} of cell suspension using a spectrophotometer (Biomate; Thermo Fisher Scientific, Waltham, MA). The concentrations of fermentation products, including butanol, butyrate, acetate and ethanol, were analyzed using high performance liquid chromatography system (HPLC, Shimadzu, Columbia, MD) equipped with Rezex RHM-Monosaccharide H^+ column (Phenomenex, Torrance, CA) and a refractive index detector (Shimadzu RID-10A) [28].

3. Results and discussion

3.1. Effect of basal media

Two basal media were tested for *n*-butanol production by *C. cellulovorans-adhE2* in bioreactors at pH 7.0, including ATCC 1345 and DSMZ 520. As shown in Fig. 2, faster cell growth and higher cell density could be reached in DSMZ 520 medium, with a maximum OD_{600} of 1.11 in ATCC 1345 (Fig. 2A) and 2.02 in DSMZ 520 (Fig. 2B) after 50 h in fermentation, respectively. Both media produced similar levels of *n*-butanol, butyrate and acetate with final concentrations of 1.6–1.8 g/L, 0–0.73 g/L, and 1.26–1.85 g/L, respectively. However, ATCC 1345 produced a significantly lower concentration of ethanol than DSMZ 520, 0.92 g/L vs. 3.32 g/L. In addition, the fermentation time using DSMZ 520 was much shorter than of ATCC 1345, 53 h vs. 66 h. These fermentation data showed that DSMZ basal medium was more efficient in butanol production. In previous studies, the basal medium ATCC 1345 was originally used to grow *C. cellulovorans* for cellulosome study [29] and the DSMZ 520 medium was originally designed to cultivate *C. cellulolyticum* [30]. As compared to ATCC 1345, the DSMZ 520 medium contained the added 4 g/L of tryptone and an increased yeast extract from 1 g/L to 2 g/L, which improved the cell growth and shortened the fermentation time of *C. cellulovorans-adhE2* [31].

3.2. Effect of pretreatment of corn-based biomass

Various pretreatment strategies were developed to delignify the