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Succinic acid production from corn stover by simultaneous saccharification and fermentation using *Actinobacillus succinogenes*

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ABSTRACT

Simultaneous saccharification and fermentation (SSF) technique was applied for succinic acid production by *Actinobacillus succinogenes* in a 5-l stirred bioreactor with corn stover as the raw material. The process parameters of SSF, including corn stover pretreatment condition, substrate concentration, enzyme loading and fermentation temperature were investigated. Results indicated that pretreating corn stover with diluted alkaline was beneficial for the succinic acid production, and succinic acid yield could be significantly increased when adding the cellulase supplemented with cellobiase. The maximal succinic acid concentration and yield could reach 47.4 g/l and 0.72 g/g-substrate, respectively. The corresponding operation conditions were summarized as follows: SSF operation at 38 °C for 48 h, diluted alkaline pretreated corn stover as substrate with concentration of 70 g/l, enzyme loading of 20 FPU cellulase and 10 U cellobiase per gram substrate. This result suggested an industrial potential of succinic acid production by using SSF and corn stover.

1. Introduction

Succinic acid, a C4-dicarboxylic acid, is widely used in chemical, food and pharmaceutical industries. It has been recognized as one of the most important platform chemicals in many reports (Song and Lee, 2006; Kamm and Kamm, 2007; James et al., 2007). Due to the increasing concerns on both the limited fossil resources and global greenhouse gas effect, fermentative production of succinic acid is recently attracting many attentions (Willke and Xorlop, 2004). However, from the view point of industrial application and commercialization, the bio-based succinic acid production still faces the strong competition from the existing petrochemical process (Werpy et al., 2006). The realization of bio-based succinic acid industrial production strongly depends on utilization of cheaper renewable resources, in addition to hard works of continuous strains improvement and purification simplification.

To date, the economically renewable resources used in succinic acid production reported are cheese whey (Samuelov et al., 1999; Lee et al., 2000,2003a; Wan et al., 2008), cane molasses (Agarwal et al., 2006; Liu et al., 2008), *Jerusalem artichoke* (Ren et al., 2008), wheat flour (Du et al., 2008), wood hydrolysate (Lee et al., 2003b; Kim et al., 2004; Hodge et al., 2009) and corn straw hydrolysate (Zheng et al., 2009). The cellulosic biomass, such as corn stover, is recently attracting more and more attention due to their natures of cheap, abundant and renewable. In China, the annual production amount of corn stover reaches 220 million tons, of which about 90% remains unused or is burnt resulting severe atmosphere pollution. In USA, more than 216 million tons (238 million tons) of corn stover (dry basis) is produced annually. They are mostly used for animal feeding and bedding besides a portion of them used for producing ethanol and other industrial products (Sokhansanj et al., 2002; Kadam and Mcmillan, 2003). Similar to the other cellulosic biomass, the digestion of corn stover is very difficult because of its complex crystal structure formed by cellulose, hemicellulose and lignin matrix (Ladisch et al., 1983).

Cellulose and hemicellulose in the corn stover must be hydrolyzed into monomer sugars before they are fermented to succinic acid by microorganisms. Fortunately, the succinic acid production strains, *Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes* and *Mannheimia succiniciproducens,* have the capability of fermenting both hexose and pentose (Guettler et al., 1999; Lee et al., 2002). Lee et al. (2003b) reported *A. succiniciproducens* grew on the medium containing wood hydrolysate and produced 24 g/l succinic acid (yield equal 88% w/w glucose). The wood hydrolysate was prepared with enzymatic hydrolysis of steam explosive oak wood chips with Celluclast (Novozymes Co.). Kim et al. (2004) used NaOH-treated wood hydrolysate as the carbon source to culture *M. succiniciproducens* MBEL55E, and have obtained 1.17 g/l/h and 3.19 g/l/h of succinic acid productivity in batch and continuous fermentation, respectively. Hodge et al. (2009) tested a metabolically engineered *Escherichia coli* to ferment the detoxified softwood

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dilute sulfuric acid hydrolyzates. As our previously reported (Zheng et al., 2009) corn stover hydrolysate was used as carbon source in batch fermentation by *A. succinogenes* CGMCC1593. A succinic acid concentration of 45.5 g/l was attained at the initial reducing sugar concentration in hydrolysate of 58 g/l.

All of the above reported cases are basically cellulosic biomass hydrolysis and fermentation separated processes (SHF), which involved with two steps, namely hydrolysates preparation process and succinic acid fermentation process. Whereas, another technique or process called simultaneous saccharification and fermentation (SSF) process has been developed (Takagi et al., 1977). It can simultaneously perform the cellulosic biomass enzymatic hydrolysis and fermentation at the same time and thus is considered as the most promising alternative way for the conversion of renewable raw materials into ethanol (Wingren et al., 2003; O¨hgren et al., 2007), lactic acid (Romanı et al., 2008; John et al., 2009) and hydrogen (Li and Chen, 2007). As SSF process is capable of treating cellulosic biomass and producing targeted product simultaneously in the same vessel, SSF has the advantages over SHF in aspects such as higher productivity, less glucose inhibition on both enzymatic hydrolysis and fermentation, as well as lower capital investment.

In this paper, succinic acid production was conducted in a SSF process with corn stover as the fermentation material and *A. succinogenes* CGMCC1593 as the production strain. The fermentation performance was evaluated, and the operation conditions such as corn stover pretreatment, substrate concentration, enzyme loading and fermentation temperature were also optimized.

2. Methods

2.1. Raw materials

Corn stover (obtained from Shijiazhuang, Hebei Province, China) was chopped, air-dried and stored at room temperature. Before pretreatment it was milled into particles sizing about 2–10 mm. Based on the analysis procedures described by Goering and Van Soest (1970), the dry particles was determined to have 38.4% cellulose, 24.7% hemicellulose and 17.2% lignin.

2.2. Microorganisms and enzymes

A. succinogenes CGMCC1593 (Liu et al., 2008) was used for succinic acid fermentations. *Trichoderma reesei* CICC40359 purchased from China Center of Industrial Culture Collection (CICC) was used for producing cellulase by submerged fermentation. Cellobiase (Sigma C6105) from *Aspergillus niger* (Novozym 188) was purchased from Sigma–Aldrich Co., and its β-glucosidase activity was measured as 311 U/ml using the method described by Ghose (1987).

2.3. Pretreatment of corn stover

Corn stover particles (CS) were pretreated with different methods before fermentation, including diluted acid, diluted alkaline, alkaline peroxide and aqueous-ammonia soaking. Diluted acid or alkaline pretreatment was carried out by immersing CS in the solution of 0.75% H_2SO_4 (v/v) or 1% NaOH (v/v) with solid–liquid ratio of 1:15, and then they were placed in an autoclave at 121 °C for 1 h. Alkaline peroxide pretreatment was carried out by soaking CS in 2% (v/v) H_2O_2 solution with the solid–liquid ratio of 1:15. The resulting slurry was then adjusted to pH 11.5 using 4 M NaOH and kept at 30 °C for 16 h. For aqueous-ammonia soaking pretreatment, CS was immersed in 10% ammonia (v/v) with the solid–liquid ratio of 1:15. The solution was placed at 30 °C for 24 h. The corn stover subject to the pretreatments was then collected by filtration, followed by water washing until pH of the filtrate reached neutral, and then the collected particles were placed in an oven to be dried at 60 °C until reaching constant weight. The contents of cellulose, hemicellulose and lignin in the pretreated and dried corn stover (PCS) were analyzed. Finally, these PCS were used as the substrates in the subsequent enzymatic hydrolysis process.

The corn stover pretreated with steam explosion, provided by Institution of Process Engineering of Chinese academy of sciences, was prepared at 1.1 MPa for 5 min in the steam-exploded vessel with water addition ratio 1:1 and then a valve on the wall of vessel was suddenly opened to bring the reactor rapidly to atmospheric pressure. It contained 46.3% cellulose, 29.7% hemicellulose and 15.7% lignin.

2.4. Preparation of cellulase

The stock culture of *T. reesei* CICC40359 was grown on potato dextrose agar slants at 30 °C for 6 d, then the spores were washed with sterile water, and spores suspension was inoculated into a 500 ml shake flask, containing 100 ml sterilized fermentation medium. The fermentation medium composition was as follows: wheat bran 40 g/l, avicel 10 g/l, peptone 3 g/l, yeast extract 0.5 g/l, $(NH_4)_2SO_4$ 2.0 g/l, $CaCl_2{\cdot}2H_2O$ 0.3 g/l, $MgSO_4{\cdot}7H_2O$ 0.3 g/l, KH_2PO_4 4.0 g/l, Tween-80 0.2 g/l, pH 5.5. After incubating *T. reesei* CICC40359 at 28 °C and 180 rpm for 5 d, the fermentation broth was centrifuged at 3000 rpm for 10 min. The supernatant was collected as crude cellulase liquid (CCL) and stored at 4 °C. The filter paper activity (FPA) of obtained CCL was assayed around 7– FPU/ml based on Ghose (1987).

2.5. Enzymatic hydrolysis of corn stover pretreated with different methods

Enzymatic hydrolysis experiments were conducted in 150 ml shaking flask at 50 °C and 80 rpm for 36 h. The PCS was soaked in citrate buffer (0.05 M, pH 4.8) at concentration of 40 g/l (or 80 g/l), and CCL was suitably added make a total working volume of 50 ml and enzymatic activity around 20–25 FPU per gram PCS. For some cases, cellobiase of 10 U per gram PCS was further supplemented to strengthen the hydrolysis. All experiments were carried out in triplicates. Samples were taken from the reaction mixture periodically during incubation, and boiled for 10 min to terminate the reaction for sugar analysis. The enzymatic conversion was calculated as follows:

$$\text{Enzymatic conversion}(\%) = \frac{\text{Reducing sugar} \times 0.9}{\text{Carbohydrate in substrate}} \times 100$$

2.6. Succinic acid production using different PCS hydrolysates based medium

The PCS samples after enzymatic hydrolysis were centrifuged at 3000*g* for 10 min. The supernatants were collected and used as the carbon source for succinic acid fermentation. The following substances were supplemented into the fermentation medium, including corn steep liquor (CSL) 20 g/l, sodium acetate 1.0 g/l, $K_2HPO_4{\cdot}12H_2O$ 1.5 g/l, $NaH_2PO_4{\cdot}2H_2O$ 1.5 g/l, $MgCl_2$ 0.2 g/l, and $CaCl_2$ 0.2 g/l. 50 ml of the medium was placed in each 150 ml anaerobic bottle, and the initial pH was adjusted to 6.5. The medium was autoclaved at 120 °C for 20 min, and then *A. succinogenes* CGMCC1593 was inoculated (5%, v/v). Succinic acid production was performed under anaerobic condition (Liu et al., 2008). $MgCO_3$ powder (about 40 g/l) was added into the medium to buffer pH