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Economical succinic acid production from cane molasses by *Actinobacillus succinogenes*

Yu-Peng Liu, Pu Zheng, Zhi-Hao Sun *, Ye Ni, Jin-Jun Dong, Lei-Lei Zhu

Laboratory of Biocatalysis, School of Biotechnology, Southern Yangtze University, The Key Laboratory of Industrial Biotechnology, Ministry of Education, Wuxi 214122, PR China

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Abstract

In this work, production of succinic acid by *Actinobacillus succinogenes* CGMCC1593 using cane molasses as a low cost carbon source was developed. In anaerobic bottles fermentation, succinic acid concentration of $50.6 \pm 0.9\ g\ l^{-1}$ was attained at 60 h using an optimum medium containing molasses pretreated with sulfuric acid, resulting in a succinic acid yield of $79.5 \pm 1.1\%$ and sugar utilization of $97.1 \pm 0.6\%$. When batch fermentation was carried out in a 5-l stirred bioreactor with pretreated molasses, $46.4\ g\ l^{-1}$ of succinic acid was attained at 48 h and faster cells growth was also observed. Fed batch fermentation was performed to minimize the substrate (sugar) inhibition effect, giving $55.2\ g\ l^{-1}$ of succinic acid and $1.15\ g\ l^{-1}\ h^{-1}$ of productivity at 48 h. The present study suggests that the inexpensive cane molasses could be utilized for the economical and efficient production of succinic acid by *A. succinogenes*.

Keywords: Succinic acid; Cane molasses; Fermentation; *Actinobacillus succinogenes*

1. Introduction

Succinic acid is a C4-dicarboxylic acid produced in the metabolic pathway of several anaerobic and facultative microorganisms, which can be used as a precursor of numerous products, including commodity chemicals (and may replace maleic anhydride), pharmaceuticals, feed additives, green solvents, and biodegradable polymers (Willke and Vorlop, 2004). At present, succinic acid is mainly produced by the chemical process from *n*-butane through maleic anhydride, and the major raw material cost in the chemical process is $\$1.03\ kg^{-1}$ succinic acid (Song and Lee, 2006). The production of succinic acid from petrochemical feedstocks is expensive and suffers serious pollution problems. Recently, there has been an increasing effort on the production of succinic acid by microbial fermentation using renewable carbohydrates (Zeikus et al., 1999).

One of the key aspects in the fermentation process is the development of a cost-effective culture medium to obtain maximum product yield. Production of succinic acid has been reported from glucose (Guettler et al., 1996; Gallmetzer et al., 2002; Urbance et al., 2004; Vemuri et al., 2002), whey (Lee et al., 2000) and wood hydrolysate (Kim et al., 2004) using batch or continuous culture. Cane molasses is a byproduct of the sugar industry, which consists of water, approximately 50% (w/w) total sugars (sucrose, glucose, fructose), suspended colloids, heavy metals, vitamins and nitrogenous compounds, etc. (Roukas, 1998; Kotzamanidis et al., 2002). It is a relatively inexpensive raw material and has already been used for the production of a number of industrial important chemicals, such as ethanol (Ergun and Mutlu, 2000), sorbitol (Cazetta et al., 2005), lactic acid (Kotzamanidis et al., 2002; Shukla et al., 2004; Wee et al., 2004), citric acid (Wang et al., 2000; Haq et al., 2004), polysaccharide (Roukas, 1998). To our knowledge, cane molas-

* Corresponding author. Fax: +86 510 85918252.
E-mail address: sunw@public1.wx.js.cn (Z.-H. Sun).

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ses has only been used in one recent study to produce succinic acid by *Escherichia coli* (Agarwal et al., 2006). However, the yield and productivity of succinic acid were not satisfying for the industrial application.

In this article, we reported, for the first time, the economical production of succinic by *Actinobacillus succinogenes* from pretreated cane molasses. The effects of initial sugar concentration and complex nitrogen sources on cell growth and succinic acid production from molasses were also examined.

2. Methods

2.1. Chemicals and gas

All the chemicals used were of reagent grade and were obtained from either Sinochem Co. (Shanghai, PRC) or Fluka Chemical Co. (Buchs, Switzerland). Gas was obtained from Xinnan Gases Co. (Wuxi, PRC) and scrubbed free of oxygen by passage over a gas purifier.

2.2. Organism

A. succinogenes CGMCC1593 was isolated by our laboratory and stored at China General Microbiological Culture Collection Center (CGMCC), which could produce a high concentration of succinic acid. A series of morphological, biochemical characteristics and sequence analysis of 16S rRNA gene (GenBank, No. DQ458786) have revealed that it belongs to *A. succinogenes* (Sun et al., 2006).

2.3. Pretreatment of molasses

Cane molasses was obtained from Jiangmen sugar-refinery (Guangdong, PRC), and it contained 35% (w/w) sucrose, 10% (w/w) converted sugars (glucose and fructose), 2.5% (w/w) other carbohydrates, 4.3% (w/w) crude protein, 0.06% (w/w) crude fat, 9.6% (w/w) ash, 4.6% (w/w) salt, 8.9% (w/w) metal ions such as calcium, potassium, sodium, iron, magnesium, copper, etc., and 25% (w/w) water. The crude molasses was diluted with distilled water to obtain 30% (w/v) total sugar concentration. For sulfuric acid treatment, the molasses solution was adjusted to pH 3.5 with 5 M H_2SO_4, and heated at 60 °C for 2 h. After centrifugation at 8000*g* for 15 min, the supernatant was adjusted to pH 6.5 with 10 M NaOH. Other pretreatment methods (potassium ferrocyanide, resin and activated carbon) described by Roukas (1998) and Kotzamanidis et al. (2002) were also studied. The industrial grade resins used for the pretreatment of molasses were obtained from Huazhen Tech. Co. (Shanghai, PRC).

2.4. Fermentation in anaerobic bottles

Fermentation in anaerobic bottles was carried out as described previously (Lee et al., 1999a). *A. succinogenes* CGMCC1593 was cultured in sealed 150-ml anaerobic bottles containing 50 ml AS medium. The AS medium contained per liter: 1.5 g $Na_2HPO_4 \cdot 12H_2O$, 1.0 g $NaH_2PO_4 \cdot 2H_2O$, 1.0 g NaCl, 0.2 g $MgCl_2$ and 0.2 g $CaCl_2$. For the seed medium, AS medium was supplemented with 5 g glucose and 5 g yeast extract. For the fermentation experiment, AS medium was supplemented with various concentrations of yeast extract and substrates such as sugars, cane molasses or artificial molasses (mixture of sucrose, glucose and fructose). The pH of the media was adjusted to 6.5 with concentrated NaOH. A solid $MgCO_3$ concentration of 25–60 g l^{-1} was also supplied in the medium to buffer the pH during fermentation. After the addition of $MgCO_3$, the initial pH of the fermentation medium would increase to 6.9–7.2. The medium was heat sterilized (15 min at 121 °C) in anaerobic bottles sealed with butyl rubber bungs with N_2 headspace. For the sterile medium, the N_2 headspace was replaced by CO_2, and $Na_2S \cdot 9H_2O$ (final concentration of 0.02%) was added before inoculation to ensure strict anaerobic condition (Lee et al., 2000). The seed medium was inoculated with 1 ml of stock culture maintained in cooked meat medium and incubated at 37 °C for 16 h. For the fermentation experiments, the medium was inoculated with 5% of seed culture and incubated at 37 °C for 40–60 h.

2.5. Fermentation in stirred bioreactors

Batch fermentation was carried out at 37 °C with 3.5 l medium in 5-l stirred bioreactors (BIOFLO 110, New Brunswick Scientific, Edison, NJ, USA). The fermentation medium was the same as the above and pH was strictly controlled at 6.7 with 3 M Na_2CO_3 during the fermentation process. External CO_2 gas sparging rate and agitation speed were controlled at 0.05 vvm and 200 rpm, respectively.

Fed-batch fermentation was carried out under the same conditions as batch fermentation. When the concentration of total sugar was lower than 10 g l^{-1}, a concentrated molasses solution containing 300 g l^{-1} total sugar was fed into the stirred bioreactor using a peristaltic pump to maintain the sugar concentration within 10–15 g l^{-1} during the course of fermentation.

2.6. Analytical methods

After the desired incubation period, the culture was diluted with 0.2 M HCl, and centrifuged at 8000*g* for 10 min. The cell pellets were washed for three times with distilled water to remove all media components, and the cell concentration was determined by measuring the absorbance at 660 nm using a spectrophotometer (U-3000, Hitachi, Japan).

Fermentation samples were centrifuged (8000*g*, 10 min) at 4 °C, and the concentrations of organic acids and sugars in supernatants were quantified using a HPLC system (Waters, Milford, MA) equipped with a refractive index detector (Waters, Milford, MA). Succinic acid, acetic acid