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A complete industrial system for economical succinic acid production by *Actinobacillus succinogenes*

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ABSTRACT

An industrial fermentation system using lignocellulosic hydrolysate, waste yeast hydrolysate, and mixed alkali to achieve high-yield, economical succinic acid production by *Actinobacillus succinogenes* was developed. Lignocellulosic hydrolysate and waste yeast hydrolysate were used efficiently as carbon sources and nitrogen source instead of the expensive glucose and yeast extract. Moreover, as a novel method for regulating pH mixed alkalis ($Mg(OH)_2$ and NaOH) were first used to replace the expensive $MgCO_3$ for succinic acid production. Using the three aforementioned substitutions, the total fermentation cost decreased by 55.9%, and 56.4 g/L succinic acid with yield of 0.73 g/g was obtained, which are almost the same production level as fermentation with glucose, yeast extract and $MgCO_3$. Therefore, the cheap carbon and nitrogen sources, as well as the mixed alkaline neutralize could be efficiently used instead of expensive composition for industrial succinic acid production.

1. Introduction

The development of biorefineries has recently attracted increasing attention as a means of providing sustainable alternative solutions to convert renewable agricultural materials into numerous economically viable products (fuels, chemicals, and other target molecules) and offer competitive performance compared to traditional petrochemical refineries (Ji et al., 2009; Dorado et al., 2009; Lynd and Wyman, 1999). Many chemicals that could only be produced by chemical processes in the past could potentially be generated biologically from annually renewable resources (Ragauskas et al., 2006).

Microbial production of succinic acid is a good example. Interest in this bioprocess has been increasing recently due to that succinic acid could serve as a precursor to various commodity chemicals used in industries like adipic acid, 1,4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts, gamma-butyrolactone, poly-butyrate succinate, polyamides, and various green solvents (McKinlay et al., 2007; Zheng et al., 2009). Succinic acid is traditionally manufactured from petrochemicals through expensive processes. The biologic production of succinic acid from renewable resources and the greenhouse gas CO_2 would alleviate the dependence on specialty chemical production from petroleum. However, bio-based succinic acid has not yet become competitive with petrochemical-based production, mainly because of high production costs (McKinlay et al., 2007; Song and Lee, 2006). There is therefore a need to develop a cost-effective process for succinic acid production from renewable resources.

Among succinic acid producers such as *Actinobacillus succinogenes* (Guettler et al., 1999), *Anaerobiospirillum succiniciproducens* (Lee et al., 2010), *Mannheimia succiniciproducens* (Lee et al., 2002), and recombinant *Escherichia coli* (Jantama et al., 2008), *A. succinogenes* not only could efficiently produce succinate (Mckinlay et al., 2007), but also could use a wide range of carbon sources, including lactose, xylose, arabinose, cellobiose, and other reduced sugars, to produce succinic acid as the major end product (Guettler et al., 1999; Van der Werf et al., 1997). The cost of carbon sources has been reduced by many renewable resources, such as straw (Zheng et al., 2009), corn fiber (Chen et al., 2010a), crop stalk wastes (Li et al., 2010c), whey (Wan et al., 2008), wheat (Dorado et al., 2009), and cane molasses (Liu et al., 2008b), all of which could be hydrolyzed into mixed sugars. The process of succinic acid production from lignocellulosic materials is economically attractive. In addition to carbon sources, nitrogen sources are also used to reduce the cost of succinic acid fermentation. Our previous research have shown that *A. succinogenes* NJ113 could use an enzymatic hydrolysate of spent yeast cells as a nitrogen source for succinic acid production in glucose-based and corn fiber hydrolysate-based media (Jiang et al., 2010; Chen et al., 2010a).

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Succinic acid is an acid product. Large amounts of alkaline neutralizer are required to maintain the pH during succinic acid fermentation. The cost of alkaline neutralizer accounts for a significant portion of raw material costs. Majority of studies on succinic acid production have used $MgCO_3$ as alkaline neutralizer to achieve high product concentrations. Nevertheless, the cost of $MgCO_3$ supplementation is not practical for industrial succinic acid fermentation. In a previous study, our laboratory also investigated the replacement of $MgCO_3$ with Na_2CO_3, $NaHCO_3$, or $NH_3{\cdot}H_2O$ as alkaline neutralizer (Li et al., 2010a, 2010b; Ye et al., 2010). However, this could only happen under low initial carbon source concentrations. There have been no reports regarding the use of mixed alkalis as alkaline neutralizer for regulating pH for succinic acid production.

In this study, mixed alkalis were first used as a novel method for regulating pH for succinic acid production. A complete industrial system using lignocellulose hydrolysate as the carbon source, waste yeast hydrolysate as the nitrogen source, and mixed alkali as the alkaline neutralizer to achieve high-yield, economical succinic acid production by *A. succinogenes* was developed.

2. Methods

2.1. Preparation of corn stover hydrolysate

Corn stover hydrolysate was from BBCA Group Corp. (Anhui, China). The corn stover was pretreated with 1% (w/v) diluted aqueous sulfuric acid solution, and then with 12% (w/v) 20 FPU g^{-1} cellulase with 0.05 M Tris–HCl as pH buffer. Corn stover hydrolysate components consisted of 326.80 g/L total sugar (of which glucose was 180.12 g/L, xylose was 92.20 g/L, and arabinose was 44.61 g/L), 1.35 g/L furfural, and 0.59 g/L hydroxymethylfurfural (HMF). Hydrolysate was diluted into different concentrations as the substrate for succinic acid fermentation.

2.2. Enzymatic hydrolysis of spent yeast cells

Spent yeast cells were obtained from the Jinlin beer factory (Nanjing, China). Enzymatic hydrolysis of the spent yeast cells was conducted as described (Jiang et al., 2010; Chen et al., 2010b). The supernatant was used as a yeast cell hydrolysate (YCH).

2.3. Microorganism and medium

A. succinogenes NJ113 (China General Microbiological Culture Collection Center, CGMCC No. 1716) was used for succinic acid fermentations following described procedures (Chen et al., 2010b). The seed culture medium contained the following (in g/L): 10.0 glucose, 5.0 yeast extract (YE), 10.0 $NaHCO_3$, 8.5 $NaH_2PO_4{\cdot}H_2O$, and 15.5 K_2HPO_4. The culture medium contained the following (in g/L): 3.0 KH_2PO_4, 0.2 $MgCl_2{\cdot}6H_2O$, 0.2 $CaCl_2$, 1.0 NaCl, 5.0 corn liquor steep, and 10.0 YE (or YCH).

Fermentation was done in anaerobic bottles as described (Chen et al., 2010b). Batch fermentation was done at 37 °C at an initial broth volume of 1.5 L in a 3 L fermentor (Bioflo 110, New Brunswick Scientific, Edison, NJ, USA). The culture medium was used for fermentation and was purged with nitrogen gas for 30 min to remove oxygen before the inoculation. Carbon sources (glucose, corn stover hydrolysate) and nitrogen sources (YE, YCH) were separately autoclaved. The pH of the medium was maintained with the addition of different alkaline neutralizers. When $CaCO_3$ or $MgCO_3$ was used as pH regulators, they were added initially to the medium. When $Ca(OH)_2$, NaOH, Na_2CO_3, $NaHCO_3$, $Mg(OH)_2$ or $NH_3{\cdot}H_2O$ was used as pH regulators, they were made into the proper concentration solutions by means of exogenous feeding during the course of fermentation. All fermentations were done at an agitation speed of 200 rpm and CO_2 flow rate of 0.5 L/min. All experiments were repeated three times.

2.4. Analytical methods

Glucose was analyzed using an SBA-40C biosensor analyzer (Shandong Province Academy of Sciences, China). The concentrations of organics were measured by HPLC (Chromeleon server monitor, UVD 170U detector, P680 pump, Dionex, USA) equipped with an ion-exchange column (Prevail™ organic acid column, Grace, USA) (Li et al., 2010a). The column was maintained at 48 °C and KH_2PO_4 (25 mmol/L; adjusted to pH 2.5 with H_3PO_4) was used as the mobile phase at a flow rate of 1 mL/min. Sugar concentrations were determined with the HPLC system using a Series 3000 refractive index (RI) detector (Perkin–Elmer), a guard column, and an ion-exchange column (Aminex HPX87-P, BioRad) as described previously (Andersson et al., 2007). Dry cell weight (DCW) was obtained as described (Chen et al., 2010a). The total nitrogen (TN) content in the samples was measured *via* the Kjeldahl method (AOAC, 1999).

3. Results and discussion

3.1. Utilization of corn stover hydrolysate as carbon source

Lignocellulosic materials represent the largest reservoir of potentially fermentable carbohydrates. These carbohydrates, obtained *via* either acid-promoted hydrolysis or by enzymatic hydrolysis, are intrinsically a mixture of pentoses, such as, xylose and arabinose, and hexoses, such as, glucose and mannose, among others (Stephanopoulos, 2007). Generally, complete hydrolysis of corn stover ideally generates a solution primarily containing glucose and xylose at an approximate ratio of 2:1 (w/w) (Yan et al., 2009). Our previous research showed that *A. succinogenes* NJ113 could simultaneously uptake glucose, fructose, xylose, arabinose, and lactose to produce succinic acid (Chen et al., 2010b). Therefore, effective co-fermentation of mixed sugars from lignocellulosic hydrolysates may be achieved with *A. succinogenes* NJ113.

As shown in Fig. 1, succinic acid production from corn stover hydrolysate, with total sugar concentrations from 50 to 110 g/L, was investigated by anaerobic bottle cultivation. A total sugar utilization of approximately 100% was achieved at total sugar concentrations from 50 to 100 g/L. However, total sugar utilization

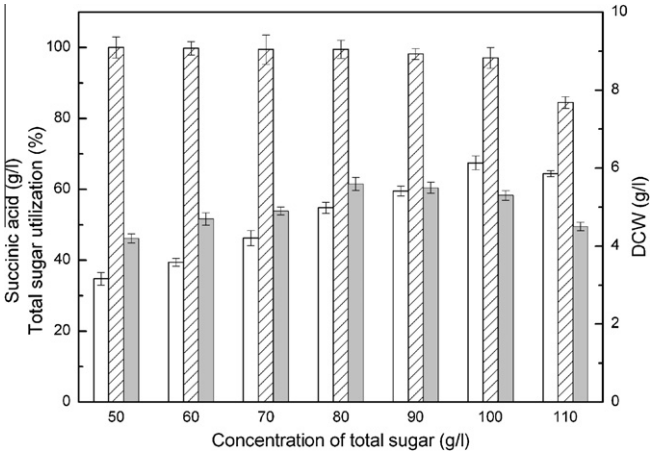

Fig. 1. Succinic acid production with corn stover hydrolysate at total sugar concentrations from 50 to 110 g/L. Unshaded bars, succinic acid; sparsely shaded bars, total sugar utilization; light gray bars, dry cell weight.