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Series fermentation production of ornithine and succinic acid from cane molasses by *Corynebacterium glutamicum*

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ABSTRACT

The production of ornithine and succinic acid by *Corynebacterium glutamicum* CGMCC1006-Δ*ldhA* media was performed using cane molasses as a low-cost material under aerobic and anaerobic conditions, respectively. The use of a series fermentation process would reduce both production costs and excess bacteria. In this work, the ornithine concentration reached 14.93 g L^{-1} within 72 h when using the optimal medium containing molasses pretreated with sulphuric acid and coupled with polyacrylamide, whereas a succinic acid concentration of 13.11 g L^{-1} was attained after 12 h using a medium containing molasses pretreated with sulphuric acid. In fed-batch fermentation, the succinic acid concentration reached 35.13 g L^{-1} within 54 h when using molasses. The present study suggests that the use of molasses as a raw material in series fermentation for the production of ornithine and succinic acid by *C. glutamicum* can significantly reduce the production costs and the amount of excess biomass.

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1. Introduction

In the fermentation industry, reducing the raw material costs and improving the efficiency of resource use are the most common and most important ways to improve economic efficiency. The cost of raw materials can typically be reduced by substituting fine raw materials with cheaper coarse materials. The literature related to fermentation using *Corynebacterium glutamicum* as the production strain includes reports of the following applications: the use of glucose as a raw material for the production of a variety of amino acids and organic acids [1–5]; the use of fructose, lactose, sucrose, molasses and other raw materials for the production of lysine [6–8]; and the use of cellulose hydrolysate, formic acid, arabinose and other raw materials for the production of succinic acid [9,10]. The use of cheaper raw materials can further increase the efficiency of the production process. Sugarcane molasses is a byproduct of the sugar industry that contains 50–55% (w/w) sugar (primarily glucose, fructose, and sucrose), heavy metal ions, suspended solids, vitamins, and nitrogen compounds. Molasses is a relatively inexpensive material and is primarily used for the production of industrial chemicals, such as citric acid, lactic acid, alcohol, and polysaccharide [11–13]. To date, reports of studies on the use of molasses for the production of succinic acid are limited to production processes that use *Escherichia coli* and *Actinobacillus succinogenes* [14–17]. In addition, the series fermentation production of ornithine and succinic acid using molasses as a raw material has not yet been investigated using *C. glutamicum* as the fermentation production strain.

The use of *C. glutamicum* as the production strain for the individual fermentation of ornithine or succinic acid has been reported [18,19]. The aerobic fermentation production of ornithine will eventually produce a large amount of excess biomass. The production of succinic acid requires the accumulation of a large amount of bacteria that will be used in the acid production process under anaerobic conditions, and this requirement also leads to a large amount of excess biomass. To improve resource utilization and reduce waste generation, we propose a series fermentation process for the production of ornithine and succinic acid using *C. glutamicum*. During the proposed process, the excess biomass from the fermentation production of ornithine is collected and used directly for the production of succinic acid after its treatment. Compared with the individual fermentations of ornithine and succinic acid, this series process eliminates the need to accumulate bacteria for succinic acid production; therefore, this process reduces the total amount of excess biomass and the production costs.

To further improve the efficiency and reduce the production costs of the series process, this study provides the first demonstration of the use of molasses as a carbon source during the series

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fermentation production of ornithine and succinic acid by *C. glutamicum*. In this study, the differences in the utilization of different carbon sources by this strain were investigated using different sugars that are present in molasses as carbon sources. Based on the carbon utilization level of this strain during different fermentation stages, the impact of the molasses pretreatment method on the fermentation was studied.

2. Materials and method

2.1. Organism

C. glutamicum CGMCC1006-Δ*ldhA* (*C.g*1006-Δ*ldh*) was produced and preserved in our laboratory. The origin of the bacteria *C.g*1006 whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under accession JOLA00000000. The version described in this manuscript is version JOLA01000000. The *ldhA* gene in the *C.g*1006 strain was deleted. The successful deletion of *ldhA* was proven by PCR using the primers *ldhA*fow (5-GGCAAGCTTGGGATCGAAAATGAAAG-3) and *ldhA*rev (5-GGAATTCCAACTAGGCGCCAAAGATTTAGAAG-3). The sequence analysis showed that 150 bp were deleted. *C.g*1006-Δ*ldh* can produce ornithine under aerobic conditions and produce succinic acid under anaerobic conditions, both with high yield.

2.2. Pretreatment of sugarcane molasses

The sugarcane molasses was provided by Nanhua Sugar Group Co., Ltd. (Guangxi, China) and contains 35% (w/w) sucrose, 5% (w/w) glucose, 10% (w/w) fructose, and 3% (w/w) other carbohydrates. The molasses was diluted with water at a 1:1 ratio to prepare a molasses solution. The acid treatment entailed the addition of sulphuric acid to adjust the pH to 2.0 and subsequent incubation in a thermal bath at 100 °C for 30 min [15]. All of the other treatment methods used an acid-treated molasses solution as the material [11,20,21]. A 15% lime solution was added for the lime treatment, and the material was then incubated in a thermal bath at 65 °C for 30 min. The potassium ferrocyanide treatment entailed the addition of 0.3% potassium ferrocyanide and subsequent incubation in a thermal bath at 98 °C for 60 min. Five percent activated carbon was added to the solution for the activated carbon treatment, and the solution was then stirred at room temperature for 60 min. The flocculation treatment was performed by the addition of 1% polyacrylamide and subsequent agitation for 30 min at 200 rpm and room temperature. After the above-mentioned treatments, the pH level was adjusted to 6.5 by adding 10 M NaOH. The solution was then centrifuged for 15 min at 8000 rpm and room temperature, and the supernatant was reserved for the fermentation experiments.

2.3. Aerobic fermentation production of ornithine

Pre-cultures of *C.g*1006-Δ*ldhA* were grown overnight on LB plates. After the cells were washed with CgXII medium (containing 2.5 g of urea, 1.5 g of $K_2HPO_4{\cdot}3H_2O$, 0.5 g of KH_2PO_4, 0.4 g of $MgSO_4{\cdot}7H_2O$, 0.02 g of $MnSO_4{\cdot}H_2O$/L, 0.02 g of $FeSO_4{\cdot}7H_2O$, 100 μg of biotin, and 200 μg of VB_1 per liter) [22], the cells were inoculated into 30 mL of CgXII medium containing 8% glucose to obtain an initial OD_{600} of 1 for the fermentation broth. The solution was poured into a 500-mL Erlenmeyer flask and cultured for 72 h with constant agitation at 200 rpm and 30 °C. Different carbon sources were substituted for the glucose in the CgXII medium, and the concentrations of the substituted carbon sources were the same as the original concentration of glucose.

2.4. Anaerobic fermentation production of succinic acid

The aerobic bacterial cells in the fermentation were collected via centrifugation at 5000 rpm for 5 min and washed with carbon-free BT medium (containing 9.3 g of $NaHCO_3$, 0.5 g of $K_2HPO_4{\cdot}3H_2O$, 0.5 g of KH_2PO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 6 mg of $FeSO_4{\cdot}7H_2O$, 6 mg of $MnSO_4{\cdot}H_2O$, 0.2 mg of biotin, and 200 μg of VB_1 per liter). The fermentation process in the anaerobic bottle followed the procedure that was previously reported by Okino et al. [19]. The cells were resuspended in 10 mL of BT medium containing 2% glucose and were placed in a 25-mL anaerobic bottle; the initial OD_{600} of each bottle was 40. Three percent calcium carbonate was added to each bottle to stabilize the pH. N_2 was injected to produce an anaerobic environment and the samples were cultured for 12 h with constant agitation at 200 rpm and 30 °C. Different carbon sources were substituted for the glucose in the BT medium and the concentration of these carbon sources was the same as the original concentration of glucose. For succinic acid production using a fed-batch culture, the washed cells were suspended in 100 mL of BT medium at a concentration of 10 g-dry cell L^{-1}, and the pH was maintained by supplementing with 15 N sodium hydroxide solution.

2.5. Analytical methods

The cell growth was estimated by measuring the OD_{600} using a spectrophotometer, where 1 OD_{600} unit equals 0.25 g of cell dry weight (CDW) per liter [23]. The fermentation sample was used to determine the total sugar, ornithine, and succinic acid contents after centrifugation for 3 min at 10,000 rpm and room temperature. The total sugar was determined using the phenol–sulphuric acid method [24]. The ornithine and succinic acid contents were determined through high-performance liquid chromatography (HPLC) [1,25]. The ornithine content was determined using a Hedera ODS-2 column (4.6 mm × 250 mm, 5 μm, Hanbon Sci. & Tech., Jiangsu, PRC). The mobile phase of 50 mM sodium acetate solution (pH 6.5) was added at a flow rate of 1 mL/min, and the column was operated at 40 °C. The contents of succinic acid and acetic acid were measured using a Sepax HP-C18 column (4.6 mm × 250 mm, 9 μm, Sepax Technologies, Inc.) with a mobile phase of 50 mM KH_2PO_4 solution at a flow rate of 1 mL/min, and the column was operated at 30 °C. The nitrate concentration was determined using an NO_2/NO_3 Assay Kit-C II (colorimetric) and a Griess Reagent Kit (Dojindo, Kumamoto, Japan).

3. Results and discussion

3.1. Ornithine production by C.g1006-ΔldhA using different carbon sources

The impact of the use of glucose, fructose, and sucrose as carbon sources for the production of glutamate and lysine by *C. glutamicum* was reported previously [6,8], but the impact of different carbon sources on the production of ornithine by *C. glutamicum* has not yet been investigated. Therefore, we compared the fermentation production of ornithine by *C.g*1006-Δ*ldhA* using glucose, fructose, sucrose and mixed sugar (specifically, a mixture of glucose, fructose, and sucrose). Under aerobic conditions, this fermentation process can produce approximately 15 g L^{-1} ornithine using 80 g L^{-1} glucose (Table 1).

The results indicate that *C.g*1006-Δ*ldhA* can effectively accumulate ornithine in the culture medium using glucose, fructose, or sucrose as the sole carbon source; however, the fructose yield was slightly reduced compared with that obtained with glucose, sucrose or the mixture. The main sugar components of cane molasses are glucose, fructose and sucrose, and a

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