

Citric acid production by *Yarrowia lipolytica* SWJ-1b using corn steep liquor as a source of organic nitrogen and vitamins

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ABSTRACT

In this study, *Yarrowia lipolytica* SWJ-1b was cultivated in the medium containing corn steep liquor (CSL) to replace yeast extract. In the medium containing 1.0 g/L of CSL, 27.5 g/L of citric acid (CA) was produced by *Y. lipolytica* SWJ-1b, with a 1.24-fold increase compare to the control medium containing yeast extract. It was showed that the addition of CSL could obviously influence the metabolism of *Y. lipolytica* SWJ-1b. The improved activity of the key intracellular enzymes indicated that the pyruvate carboxylation pathway was enhanced, which suggested carbon flux to CA in was redistributed in *Y. lipolytica*. During the fermentation in a 5 L fermenter, 52.3 g/L of CA was obtained from 60.0 g/L of glucose, with a productivity of 0.22 g/L/h. Only 5.5 g/L of isocitric acid was produced during the process, and 96.7% of glucose was consumed at the end of the fermentation. Results suggest that CSL could be used as an alternate source of organic nitrogen to replace the more expensive yeast extract in CA production by *Y. lipolytica* SWJ-1b. Furthermore, CSL could stimulate the rates of substrate uptake and improve CA yield. These results indicate the potential application of CSL for low-cost CA production on a commercial scale.

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1. Introduction

Citric acid (CA) is a key intermediate in the tricarboxylic acid (TCA) cycle, and the compound is abundant in fruits such as lemons or apples (Max et al., 2010). CA is one of the most commonly used organic acids in industries. Almost 70% of the total production of CA is used in the food industry (Darouneh et al., 2009). To date, the global demand for CA is mainly met by fermentative processes (Dhillona et al., 2013a). Different strains of filamentous fungi, which are mostly *Aspergillus niger* strains, are the main species used for the large-scale fermentative production of CA from molasses, sucrose, or glucose (Dhillona et al., 2013b; Kamzolova et al., 2011). However, the production of CA with the use of fungi is associated with the accumulation of significant amounts of solid and liquid waste. Thus,

this mode of production adds to environmental pollution and is also associated with increased costs. Furthermore, spores of *A. niger* can cause pulmonary aspergilloma; aspergillosis of operating staff have been frequently reported (Goldberg et al., 2006). Therefore, this species can be replaced by another production process with comparable economical results. Yeasts species of *Yarrowia lipolytica* are considered safe and can produce a wide spectrum of organic acids, including CA, from various carbon sources (Kamzolova et al., 2011). The bioprocess for CA production with *Y. lipolytica* has several advantages compared with the *Aspergillus* process, including a larger spectrum of substrates, lower sensitivity to low dissolved oxygen concentrations and heavy metals, genetic and mechanical stability, and less health hazards (Moeller et al., 2012).

Fig. 1 shows the proposed pathway of CA biosynthesis in *Y. lipolytica*. Generally, most of the CA from glucose is produced from pyruvate (PA) through pyruvate dehydrogenase (PDH) (Liu et al., 2013). When the dissolved oxygen in the medium is low, PYC will be functioned. PA is carboxylated into oxaloacetate (OAA) with the fixation of CO₂ under the function of PYC. After being transported to the mitochondria, OAA can be converted to CA (via the TCA cycle) and cell biomass, depending on the growth conditions (Jitrapakdee et al., 2008). Therefore, PYC has an influence on the synthesis of

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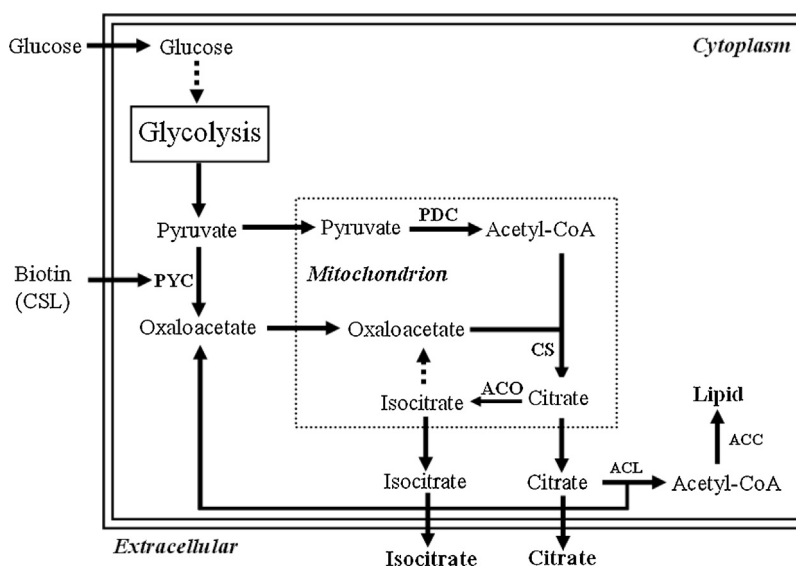


Fig. 1. The key enzymes involved in glucose metabolism and the CA synthesis in *Y. lipolytica*. PYC: pyruvate carboxylase; PDC: pyruvate dehydrogenase complex; CS: citrate synthase; ACO: aconitase; ACL: ATP-citrate lyase.

organic acid in the TCA cycle due to its central role in providing OAA to the TCA cycle. PYC is a biotin-dependent tetrameric enzyme (Jitrapakdee et al., 2008), which indicates that biotin is needed during the biosynthesis of CA.

Corn steep liquor (CSL) is a major by-product of corn steeping. CSL is a complex substance, which is high in amino acids, vitamins, and polypeptides, which are excellent sources of organic nitrogen. In addition, CSL typically contains approximately 1.0 mg/kg biotin, which can provide sufficient biotin and make a significant contribution to the nutritional requirement (Xi et al., 2013). Otherwise, the fermentation cost is a critical factor for the industrial production of CA, and the nitrogen source is a necessary ingredient in the medium. Yeast extract is an expensive component of the CA fermentation medium (Liu et al., 2014; Rywińska et al., 2010). To meet the demand for low-cost CA, the use of yeast extract should be avoided. As an economic alternative source of organic nitrogen and vitamins, CSL may replace the more expensive peptone or yeast extract in rich media (Xi et al., 2013). To date, CSL has been used in the production of various organic acids or other products (Khan et al., 2014; Xi et al., 2013).

However, the effects of using CSL for CA production by *Y. lipolytica* have not been investigated in detail. In this study, the submerged fermentation of *Y. lipolytica* SWJ-1b is used as a model to evaluate the potential of CSL as an efficient substrate for CA production. The effect of CSL on cell growth, CA production, and the yield of the by-product isocitric acid ICA were investigated. A higher concentration of CA was obtained when CSL was used in the place of yeast extract. The intracellular metabolic flux of *Y. lipolytica* was also improved. Therefore, CSL can be used as an economic alternative nitrogen source in CA production.

2. Materials and methods

2.1. Materials and microorganism

All chemicals were reagent grade. CSL was kindly supplied by the Baimai Green Biological Energy Co., Ltd. (Huaian, China). To produce consistent results, CSL from the same batch was used throughout the study. The CSL contained approximately 55.63% water, 8.58% reducing sugar, 6.51% amino acids, 12.24% lactic acid, 10.78% mineral elements, 0.11% vitamin B, and 0.01% biotin. The pH of CSL was 4.9.

The *Y. lipolytica* SWJ-1b used in this study was graciously provided by Professor Chi Zhenming of the Ocean University of China. This strain was isolated from the gut of the marine fish at the Bohai sea. The cells of *Y. lipolytica* SWJ-1b were kept at 4 °C on yeast peptone dextrose (YPD) agar slant contained 10.0 g/L yeast extract, 20.0 g/L peptone, 20.0 g/L glucose, and 20.0 g/L agar. Transfers were made on fresh agar slants monthly to maintain viability.

2.2. Growth conditions

The inoculums of *Y. lipolytica* SWJ-1b were prepared by transferring cells grown on a slant to 5.0 mL of YPD broth. The seed culture was incubated for 24 h at 28 °C and agitated at 180 rpm. The yeast was cultivated in a submerged culture with agitation in a shaker. The standard CA-producing medium consisted of 0.25 g/L of $(\text{NH}_4)_2\text{SO}_4$, 1.7 g/L of KH_2PO_4 , 12.0 g/L of Na_2HPO_4 , 1.25 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.006 g/L of vitamin B₁, and 0.25 g/L of yeast extract or CSL was added to the broth as the source of organic nitrogen and microelements. The media pH was adjusted to 6.0 and sterilized by autoclaving at 121 °C for 20 min. The cells of *Y. lipolytica* SWJ-1b were grown in YPD broth at 28 °C and agitated at 180 rpm for 24 h before 1.0 mL of the cultures ($\text{OD}_{600\text{ nm}} = 30$) was inoculated into 50.0 mL of the CA production medium and grown at 28 °C with shaking at 180 rpm. During cultivation, pH in the medium was maintained at 6.0 by adding 2.0 M KOH. Samples were withdrawn for analyses at regular intervals. All assays were conducted in triplicate and did not vary by more than 5%.

CA production was conducted in a 5 L fermentor (Major Science, Hong Kong) equipped with an alkali pump, an oxygen sensor, a stirrer, a heating element, and a temperature sensor. Seed cultures were prepared as described above. Briefly, 60.0 mL of the seed culture ($\text{OD}_{600} = 30$) was transferred into 3.0 L of the medium containing 60.0 g/L of glucose, 1.0 g/L of CSL, and 7.5 g/L Na_2HPO_4 . (The media pH was adjusted to 6.0, and the media were sterilized by autoclaving at 121 °C for 20 min). Fermentation was performed under an agitation speed of 250 rpm, aeration rate of 3.0 L/min/L medium, temperature of 28 °C, and fermentation period of 288 h. Finally, 20.0 mL of the culture was collected in 24 h intervals for determination.

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