Appl Biochem Biotechnol (2010) 160:244–254
DOI 10.1007/s12010-009-8649-1

Succinic Acid Production by *Actinobacillus succinogenes* Using Spent Brewer's Yeast Hydrolysate as a Nitrogen Source

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Received: 10 October 2008 / Accepted: 14 April 2009 /
Published online: 6 May 2009

Abstract To develop a cost-effective fermentation medium, spent brewer's yeast hydrolysate was evaluated as a nitrogen source for succinic acid production by *Actinobacillus succinogenes* NJ113 in glucose-containing media. Autolysis and enzymatic hydrolysis were used to hydrolyze the spent brewer's yeast cells to release the nutrients. The results showed that enzymatic hydrolysis was a more effective method due to the higher succinic acid yield and cell growth. However, the incomplete glucose consumption indicated existence of nutrient limitation. Vitamins were subsequently identified as the main limiting factors for succinic acid production using enzymatically hydrolyzed spent brewer's yeast as a nitrogen source. After the addition of vitamins, cell growth and succinic acid concentration both improved. As a result, 15 g/L yeast extract could be successfully replaced with the enzymatic hydrolysate of spent brewer's yeast with vitamins supplementation, resulting in a production of 46.8 g/L succinic acid from 68 g/L glucose.

Keywords Succinic acid · *Actinobacillus succinogenes* NJ113 · Brewer's yeast hydrolysate · Nitrogen source

Introduction

Succinic acid is valued as one of the key platform chemicals in the preparation of biodegradable polymers such as polybutylene succinate and polyamides or as a raw

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material for fine chemicals of the C4 family, which include 1,4-butanediol, tetrahydrofuran, *N*-methyl pyrrolidinone, 2-pyrrolidinone, and gamma-butyrolactone [1, 2]. Currently, succinic acid is produced commercially by chemical processes from maleic anhydride derived from petroleum, which limits the use of succinic acid for a wide range of applications due to the high conversion cost [3]. However, recent analysis showed that succinic acid production by fermentation from renewable resources and a greenhouse gas, CO_2, could be more cost-effective than the petroleum-based processes [4].

The fermentative production of succinic acid has been investigated with a wide variety of bacteria, but the most intensively researched are *Mannheimia succiniciproducens* [5, 6], *Actinobacillus succinogenes* [7–10], *Anaerobiospirillum succiniciproducens* [11–13], and recombinant *Escherichia coli* [14, 15] due to their ability to produce a comparatively large amount of succinic acid. The majority of studies on succinic acid production from glucose or lignocellulosic biomass have used media containing complex growth supplements to achieve high product concentration, such as yeast extract (YE), peptone, or tryptone [6, 12, 16, 17]. However, the cost of these supplements is not realistic for an industrial succinic acid fermentation process.

In China, spent brewer's yeast, a byproduct of the beer industry, is generally sold primarily as inexpensive animal feed after inactivation by heat [18]. However, because of the high level of protein, vitamin B complex, and minerals remaining, spent brewer's yeast cells might be used as a nutrient source for the growth of fastidious microorganisms or related product formation. Yeast autolysate has been used for ethanol formation by recombinant *E. coli* [19]. Hydrolysates of spent yeast cells have been used for lactic acid production by *Lactobacillus rhamnosus* [20, 21].

The aim of this study was to develop a cheap and suitable medium for succinic acid production with *A. succinogenes* NJ113 based on the utilization of spent brewer's yeast cells.

Materials and Methods

Chemicals and Gas

YE was obtained from Oxoid; other chemicals used were reagent grade and were obtained from either Sinochem (Shanghai, People's Republic of China) or Fluka Chemical (Buchs, Switzerland). CO_2 and N_2 were obtained from Nanjing Special Gases Factory (Nanjing, People's Republic of China). Spent brewer's yeast was obtained from a Jinlin beer factory (Nanjing, People's Republic of China).

Preparation of Spent Brewer's Yeast Hydrolysate

Autolysis

Spent brewer's yeast was mixed with 200 mL water to achieve a concentration of 10% (*w*/*v*) and 6.0 g NaCl was added as an autolysis accelerator. The initial pH was adjusted to 6.5. The yeast suspensions were then placed in 500 mL glass vessels immersed in a temperature-controlled water bath with shaker. The autolysis was carried out at 55 °C for 72 h with a speed of 120 rpm. After autolysis, the suspension was centrifuged for 15 min at 4 °C and 8,000 rpm, and the supernatant was designated as autolysate of brewer's yeast (ABY).

Enzymatic Hydrolysis

Spent brewer's yeast was mixed with 200 mL water to achieve a concentration of 10% (*w*/*v*). The yeast suspension's pH was adjusted to 8.0, and Alcalase (Novozymes) was added at 4.0 g/kg dry yeast cell. The yeast suspension was placed in a 500-mL glass flask immersed in a temperature-controlled water bath and treated at 60 °C for 12 h at a speed of 200 rpm. The suspension was then centrifuged for 15 min at 4 °C and 8,000 rpm, and the supernatant was designated as enzymatic hydrolysate of brewer's yeast (EBY).

Microorganism and Growth Conditions

Microorganisms

A. succinogenes NJ113 (China General Microbiological Culture Collection Center, CGMCC no. 1716) was used in all experiments.

Culture Media

The medium for inoculum preparation contained the following (in grams per liter): 10 glucose, 5.0 YE, 10.0 $NaHCO_3$, 8.5 $NaH_2PO_4{\cdot}H_2O$, and 15.5 K_2HPO_4.

The production medium contained the following (in grams per liter): salt solutions (including 3.0 KH_2PO_4, 0.2 $MgCl_2{\cdot}6H_2O$, 0.2 $CaCl_2$, and 1.0 NaCl) and 70 glucose. Glucose and nitrogen sources (YE, ABY, and EBY) were autoclaved separately.

In the "Identification of growth-limiting factors in succinic acid production" section, trace elements and vitamins and their concentrations were added according to McKinlay [22].

Cultivation Conditions

Inocula were prepared in 100 mL sealed anaerobic bottles with polytetrafluorethylene open top caps containing 50 mL medium with CO_2 as the gas phase. Vials were inoculated with a syringe from the headspace to ensure anaerobic conditions. The cultures were grown in a rotary shaker at 37 °C and 180 rpm for 12 h.

For anaerobic bottle fermentation, the cultures were performed using 100 mL anaerobic bottles containing 50 mL medium with CO_2 as the gas phase and 80 g/L $MgCO_3$ was added to maintain the pH. The cultures were grown in a rotary shaker at 37 °C and 180 rpm for 48 h.

Batch fermentation was carried out in a 3-L fermentor (Bioflo 110, USA) with an initial broth volume of 1.5 L, and nitrogen was bubbled through the medium for 30 min to remove oxygen before inoculation. All fermentations were performed at 37 °C with the agitation speed of 200 rpm and CO_2 flow rate of 0.5 L/min. The pH was set at 6.8 and maintained by addition of a concentrated base solution (10 N Na_2CO_3).

Analytical Methods

Dry cell weight (DCW) was computed from a curve relating optical density at 660 nm (OD_{660}) to dry weight. An OD_{660} of 1.0 represented 520 mg dry weight per liter.

Glucose was analyzed by a SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, People's Republic of China).

Fermentation products were analyzed by high-performance liquid chromatography (Chromeleon server monitor, UVD 170U detector, P680 pump, Dionex, USA) equipped with an ion exchange column (prevail organic acid 5u, 250×4.6 mm; Grace, USA) and 25 mM KH_2PO_4 (adjusted to pH of 2.5 by H_3PO_4) was used as a mobile phase with a flow rate of 1 mL/min.

Total nitrogen (TN) was assayed by according to Kjeltee [23], and amino nitrogen (AN) was assayed by formol titration [23]. Amino acids were analyzed using an amino acid analyzer (Amino Acid Analyzer A200, Aminonova, Germany).

Results and Discussion

Succinic Acid Production with Different Supplemental Nitrogen Sources

The effects of different nitrogen sources on the cell growth and succinic acid production by *A. succinogenes* NJ113 with an initial glucose concentration of 70 g/L were investigated in anaerobic bottle cultivation (Fig. 1). Each nitrogen source was added to give a TN concentration equivalent to 15 g/L YE. As shown in Fig. 1, the cell growth and succinic acid production by *A. succinogenes* NJ113 were markedly influenced by the type of nitrogen source. YE led to the best cell growth and highest succinic acid concentration with a variety of nitrogen sources. When $(NH_4)_2SO_4$ or NH_4Cl was supplemented as the sole nitrogen source, poor growth of *A. succinogenes* NJ113 was observed and succinic acid concentration fell to below 1.5 g/L, similar to that of the control. Among the used nitrogen sources, corn steep liquor (CSL) could be considered as a feasible and inexpensive alternative organic nitrogen source. Previous research showed that CSL can be used as a sole complex nutrient in succinic acid production from whey [24] and molasses [25, 26]. Unfortunately, when CSL was used as the sole nitrogen source in glucose-containing medium for *A. succinogenes* NJ113, only 9.5 g/L of succinic acid was obtained and a high residual glucose remained.

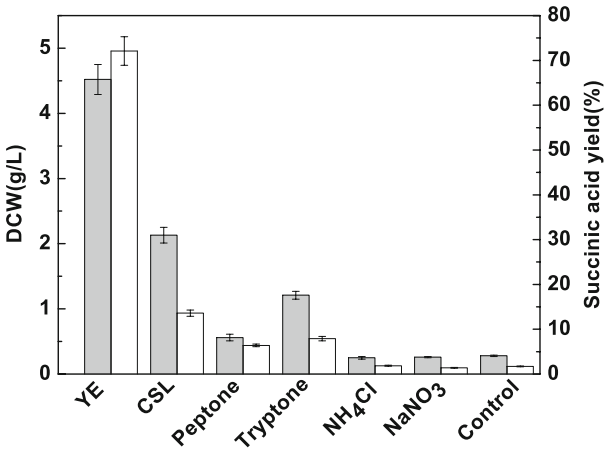


Fig. 1 Succinic acid production with different nitrogen sources supplement. *Shaded bars* DCW, *unshaded bars* succinic acid concentration

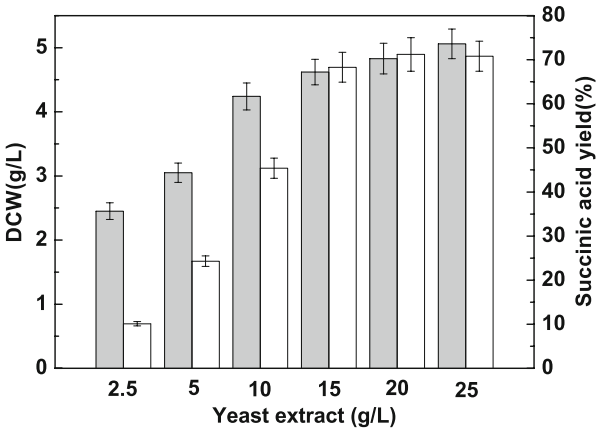


Fig. 2 Effect of YE on succinic acid production. *Shaded bars* DCW, *unshaded bars* succinic acid yield

Effect of YE Concentrations on Succinic Acid Production in Anaerobic Bottle Cultivation

YE is generally regarded as a suitable source of nitrogen and growth factors. As shown in Fig. 2, cell growth and succinic acid yield increased with the increase of YE concentration from 2.5 to 25 g/L. Addition of 15 g/L of YE was needed to completely utilize the 70 g/L of glucose with a succinic acid yield of 68.3%. It has been shown that *A. succinogenes* is a fastidious microorganism and YE supplied important growth factors for succinic acid production [17, 25]. From the above, we confirmed that YE was a good nutrient to ensure the growth of bacteria for succinic acid production, but its high cost is a limitation for its application in industrial processes. It is, therefore, desirable to find other more cost-effective nutrients to be used in industrial processes.

Succinic Acid Production from Spent Brewer's Yeast Hydrolysate in Anaerobic Bottle Cultivation

Spent brewer's yeast contains a lot of protein, lipid, RNA, vitamins, and minerals and, therefore, could be used as an alternative inexpensive nitrogen source. Autolysis and enzymatic hydrolysis are the two methods used for breaking down the cells to release nutrients [27, 28]. Autolysis is an economic disruption method but it has some disadvantages: low yield and the high content of residual salt in the autolysate [27]. As shown in Table 1, compared with enzymatic hydrolysate, the TN content of the hydrolysate by autolysis was comparable, but the AN content was less than half. Therefore, the AN/TN ratio of EBY was twice as high as that of ABY. Furthermore, the AN/TN ratio of EBY were

Table 1 Specifications for nitrogen sources.

Nitrogen source	TN(g/100 mL)	AN (g/100 mL)	Total amino acids (g/100 mL)	AN/TN
ABY	0.50±0.08	0.18±0.02	0.74±0.25	0.36±0.04
EBY	0.54±0.09	0.41±0.08	4.10±0.43	0.75±0.06
YE[a]	0.54	0.27	2.49±0.36	0.49

[a] Comparing with EBY at the same TN, the AN content, TN content, and AN/TN ratio were achieved from the supplier

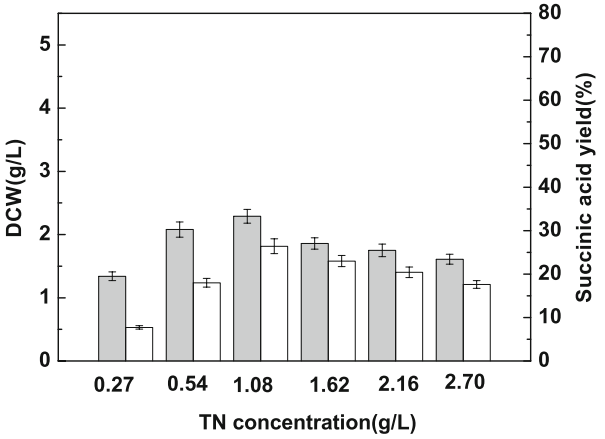


Fig. 3 Succinic acid production from ABY. *Shaded bars* DCW, *unshaded bars* succinic acid yield

also higher than that of YE. The analysis of amino acids content revealed that much of the nitrogen existed in the form of free amino acids in the EBY.

The effects of ABY and EBY on succinic acid production were also investigated (Figs. 3 and 4). In the medium containing ABY, maximum succinic acid yield was only 26.4% at the initial TN concentration of 1.08 g/L. The succinic acid yield and biomass decreased with increasing TN concentration from 1.08 to 2.7 g/L. The lower performance of ABY could be attributed to the fact that *A. succinogenes* NJ113 had difficulty utilizing the unhydrolyzed protein. When EBY was used as nitrogen source, the highest succinic acid yield of 58.3% was achieved at a TN content of 2.16 g/L, equal to 20 g/L of YE, which was approximately 10% lower than that with YE.

Therefore, enzymatic hydrolysis was a more effective method for treating the spent brewer's yeast cells and the enzymatic hydrolysate could be used as an inexpensive nitrogen source for succinic acid production. However, incomplete glucose consumption with the EBY indicated the existence of nutrient limitation.

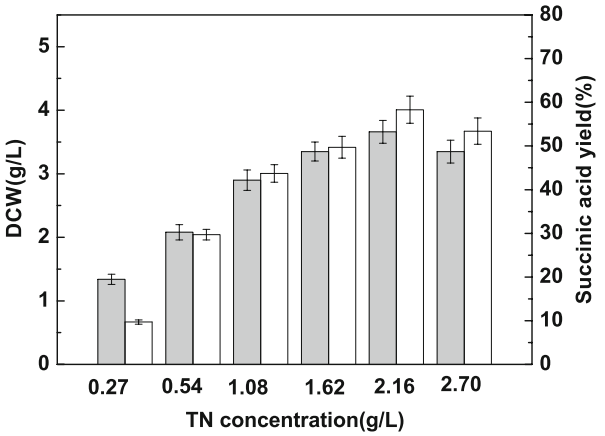


Fig. 4 Succinic acid production from EBY. *Shaded bars* DCW, *unshaded bars* succinic acid yield

Table 2 Effect of growth-limiting factor supplementation on succinic acid production.

Supplement	DCW (g/L)	Succinic acid (g/L)	Residual glucose (g/L)
None	3.60±0.23	39.2±1.4	15.3±1.4
Vitamins	4.05±0.26	47.6±1.3	1.6±0.5
Trace elements	3.51±0.16	39.6±0.8	13.8±1.4
Trace elements and vitamins	4.13±0.23	48.3±1.0	0.8±0.2

Identification of Growth-Limiting Factors in Succinic Acid Production

Two potential categories of limiting components, namely, vitamins and trace elements, were considered for growth of *A. succinogenes* NJ113 and succinic acid production. As shown in Table 2, when trace elements were added to the medium, there was no significant effect on cell growth or succinic acid concentration. However, when a mixture of vitamins and trace

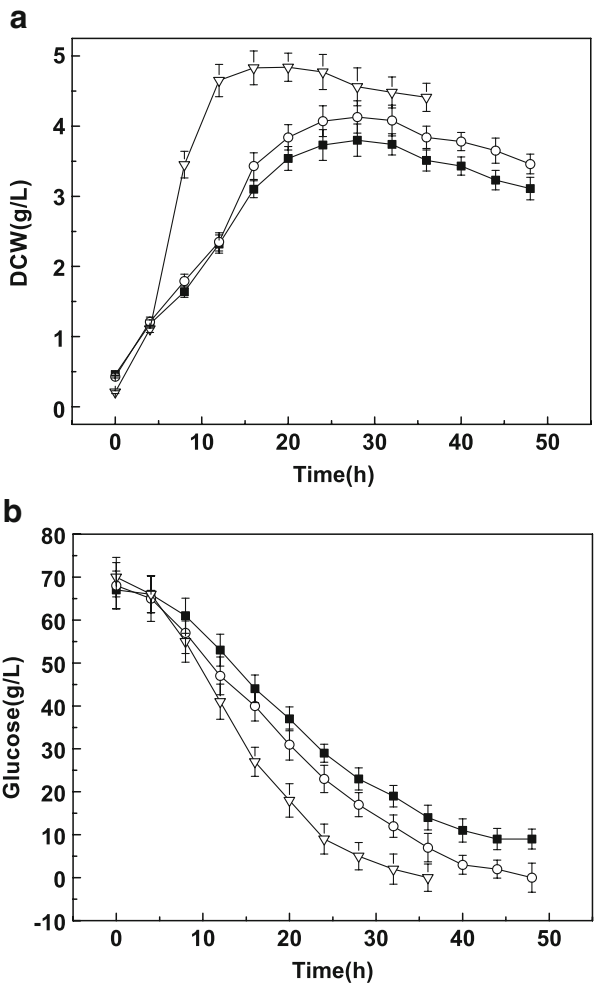
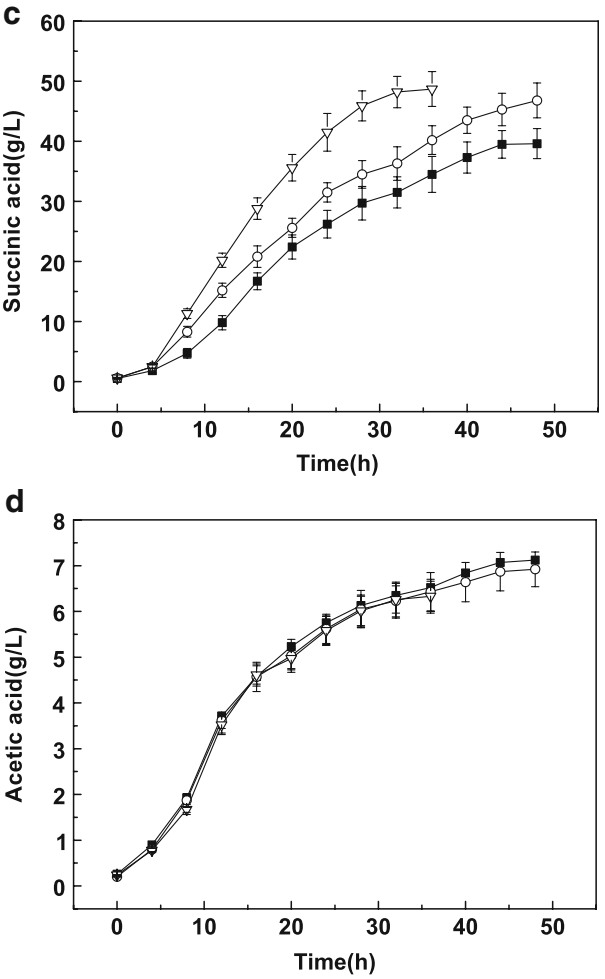


Fig. 5 Succinic acid production from YE (*diamonds*), EBY (*closed squares*), and vitamin-supplemented EBY (*closed triangles*). **a** DCW, **b** glucose, **c** succinic acid, **d** acetic acid

Fig. 5 (continued)



elements or only vitamins was added, cell growth and succinic acid concentration both improved. In these experiments, vitamins seemed the most likely candidate as growth-limiting factors in succinic acid production.

Lactic acid bacteria also need YE to supply specific minerals, vitamins, peptides, and some unknown nutrients to ensure their optimum growth for lactic acid fermentation [29]. Some low-cost industrial byproducts, such as soy protein hydrolysate [30, 31], fish wastes [32], and spent cell hydrolysate [20, 33], have been used for lactic acid production to replace commercial YE. They resulted in lower performance in lactic acid production compared with YE, except when growth factors, such as vitamins, were added [30]. It has also been reported that *A. succinogenes* is a fastidious organism, which requires vitamins for cell growth [34]. Although the amino acids content of EBY was higher than that of YE at the same TN content, it might lack sufficient vitamins and lead to nutrient limitation. Further research is necessary to optimize the components and concentrations of the vitamins and to analyze the effect of each vitamin on cell growth and succinic acid production.

Succinic Acid Production from EBY in Batch Cultivation

To confirm the performance of EBY, batch cultivation was carried out (Fig. 5). Cultivation with 15 g/L of YE was performed first as a reference. The 70 g/L of glucose was utilized in 36 h with 15 g/L of YE as the nitrogen source, and 48.7 g/L of succinic acid was obtained. The volumetric productivity and yield of succinic acid were 1.4 g/L h and 69.6%, respectively, taking into account the volumetric change due to pH control. When EBY was used as the nitrogen source, the 67 g/L of glucose was utilized to 9 g/L and succinic acid concentration reached 39.6 g/L with the yield and productivity of 0.83 g/L h and 59.1%, respectively, after 48 h culture. After the addition of vitamins, final succinic concentration and glucose consumption were both improved, but succinic acid productivity was still lower than that obtained with YE, which implied that some other components were limited. However, it seemed that there was no significant influence on the concentration of the byproduct of acetic acid. As a result, when EBY supplemented with vitamins was used as the nitrogen source, the fermentation came to an end in 48 h with a succinic acid concentration of 46.8 g/L and a yield of 68.8%, comparable with the fermentation using 15 g/L YE. Thus, EBY seems to be a promising alternative to YE as a source of nitrogen and growth factors.

Concluding Remarks

YE is most often used in fermentation studies as a supplement, but its high price hinders its use in large quantities. This study describes how spent brewer's yeast hydrolysate was used as a nitrogen source in succinic acid production by *A. succinogenes* NJ113 to replace commercial YE. Autolysate of spent brewer's yeast resulted in low performance in succinic acid production. However, enzymatic hydrolysis showed high performance in succinic acid production with a yield of 59.1%, which was approximately 10% lower than that with YE. Furthermore, the addition of vitamins to the enzymatic hydrolysate resulted in succinic acid yield of 68.8% from 68 g/L of glucose, which was comparable with 15 g/L YE. Therefore, spent brewer's yeast could be an alternative nitrogen source for the economic production of succinic acid by *A. succinogenes* NJ113.

Acknowledgements This work was supported by grant no. 2006AA02Z235 from "863" Program of China, grant no. 20606017 from National Natural Science Foundation of China, grant no. 2009CB724701 from "973" Program of China.

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