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Process development of succinic acid production by *Escherichia coli* NZN111 using acetate as an aerobic carbon source

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ABSTRACT

Escherichia coli strain NZN111 could convert glucose to succinic acid efficiently in anaerobic conditions after the induction of gluconeogenic carbon sources in aerobic conditions. Acetate shows a strong effect on both yield and productivity of succinic acid. In this study, the fed-batch process of succinic acid production by NZN111 using acetate in a chemically defined medium in the aerobic stage was investigated and developed. Increasing cell density could increase succinic acid with a productivity of 3.97 g/(L h) in the first 8 h of the anaerobic phase with an overall yield of 1.42 mol/mol glucose in a 5 L fermentor. However, there was strong repression from succinic acid in the later anaerobic stage. When succinic acid exceeded 30 g/L, the glucose consumption rate began to drop sharply along with the succinic acid production rate. Supplementation with glucose from 30 to 70 g/L in the anaerobic stage showed little effect on succinic acid production. Acetic acid and pyruvic acid accumulated had no effect on succinic acid formation because of their low concentration. With acetate as the sole carbon source for aerobic cultivation in the following scale-up, 60.09 g/L of succinic acid was produced with a yield of 1.37 mol/mol in a 50 L bioreactor.

1. Introduction

Succinic acid, a C4 dicarboxylic acid produced as an intermediate of the tricarboxylic acid (TCA) cycle, can be useful in many industries such as food, pharmaceuticals, polymers, cosmetics, and detergent production [1,2]. It has been reported there is a $15 billion market of bulk chemicals that can be produced from succinic acid as an intermediary feedstock [1]. Succinic acid has also been identified as one of the top 12 building block chemicals that could be produced from renewable materials [3]. In addition, CO_2 can be bound to phosphoenolpyruvate (PEP) or pyruvate (PYR) to form succinic acid in the anaplerotic reactions. The incorporation of CO_2 in succinic acid formation provides further incentive for succinate biorefinery [4].

Many bacteria such as *Mannheimia succiniciproducens*, *Actinobacillus succinogenes*, and *Anaerobiospirillum succiniciproducens* can accumulate succinic acid as a major fermentation product [5–7]. However, these bacteria have more nutritional requirements including complex components and are difficult to be modified. Recently, *Escherichia coli*, with a less complicated genome and more effective genetic manipulation tools than other strains, has been studied by several groups for succinic acid over production [8–12]. Due to the deletion of byproduct pathway and elevation of anaplerotic metabolism and glyoxylate shunt, high yield and productivity (1.63 mol/mol, 1.3 g/(L h)) were achieved using *E coli* AFP111 when applying different feeding strategies at growth stage [11]. Overexpressing phosphoenolpyruvate carboxylase (PPC) and using lactose as inducer has also resulted in high yield and productivity (1.67 mol/mol, 1.7 g/(L h)), with a final concentration of 99.7 g/L [12].

E. coli NZN111was a mutant of W1485 and disrupted of ldhA and pflB genes, which result in NZN111 not grow anaerobically on glucose because of the failure to regenerate NAD^+ from NADH [13]. However, NZN111 has others enzymes capable of balance the redox, including fumarate reductase (FRD), malate dehydrogenase (MDH), and malic enzyme (ME). The reason NZN111 fails to metabolize glucose anaerobically is that these enzymes not be fully induced. In a two-stage fermentation, the activities of the anaplerotic pathway and the glyoxylate shunt in NZN111 can be enhanced significantly using acetate or glycogenic carbon sources for aerobic cultivation, and glucose can be efficiently fermented to succinic acid as the dominant metabolic product in the following anaerobic stage [14,15]. It has also been shown that when *E. coli* is cultured on acetate, gene expression relating to glycolysis and PTS is down-regulated, while gene expression relating to the glyoxylate shunt, the reductive branch of the TCA cycle, acetyl-CoA formation, and gluconeogenesis is up-regulated [16].

Acetate has the advantage of low cost, is readily available, and has been used as a carbon source for hydrogen and L-histidine production, among other things. Considering that the *E coli* strains cultivated on acetate have totally different gene expression, acetate

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may be an ideal metabolic regulator of NZN111 for industrial production of succinic acid. However, little research on fed-batch strategies under aerobic conditions has been conducted using acetate as a carbon source in succinic acid production. In this study, the two stage fermentation for succinic acid production was investigated and optimized in a fermentor. Different feeding strategies in the aerobic stage were compared. Additionally, high cell density cultivation of NZN111 was also investigated to increase succinic acid productivity. The scale-up was conducted in a 50 L bioreactor. Inhibition of succinic acid formation was remarkable during the anaerobic stage of fermentation; the possible contributory effects of glucose, acetic acid, pyruvic acid, and succinic acid concentrations were examined.

2. Materials and methods

2.1. Strain

E. coli strain NZN111 [F^+ λ^- *rpoS*396(Am) *rph*-1 Δ*pflB*::Cam Δ*ldhA*::Kan] [15] was used exclusively in this study, which was kindly provided by Prof. D.P. Clark, Southern Illinois University. The strain was kept as a stock in 25% (w/w) glycerol at −20 °C.

2.2. Media

The medium for primary seed cultures of NZN111 was LB containing tryptone (Oxoid, UK) 10 g/L, yeast extract (Oxoid, UK) 5.0 g/L, and NaCl 10 g/L. In fed-batch 1 (FB-1) and fed-batch 2 (FB-2) experiments, the salt medium (SM1) for secondary seed cultures contained (per liter): glucose 5.0 g, $Na_2HPO_4{\cdot}12H_2O$ 15.12 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, 1 M $MgSO_4$ 2.0 ml, 1.0 M $CaCl_2$ 0.1 ml, 1% (w/v) vitamin B1 0.2 ml, and trace elements solution 0.1 ml. The stock solution of trace elements contained the following (per liter) in 3.0 M HCl: $FeSO_4{\cdot}7H_2O$ 80 g, $AlCl_3{\cdot}6H_2O$ 10 g, $ZnSO_4{\cdot}7H_2O$ 2.0 g, $CuCl_2{\cdot}2H_2O$ 1.0 g, $NaMoO_4{\cdot}2H_2O$ 2.0 g, $MnSO_4{\cdot}H_2O$ 10 g, $CoCl_2$ 4.0 g, and H_3BO_4 0.5 g. The salt medium (SM2) for secondary seed cultures in fed-batch 3 (FB-3) experiments was identical to SM1 with the following exceptions: 5.2 g/L of sodium acetate was added and glucose was omitted. Instead of NH_4Cl and sodium acetate, 5 g/L of ammonium acetate was used in the salt medium (SM3) for second seed culture in fed-batch 4 (FB-4). The media for batch cultivation in the FB-1 experiment was as follows (per liter): glucose 15 g, NH_4Cl 10 g, $Na_2HPO_4{\cdot}12H_2O$ 3.78 g, KH_2PO_4 0.75 g. The concentrations of $MgSO_4$, $CaCl_2$, vitamin B1 and trace elements were identical to SM1. In the FB-2 experiment, NH_4Cl was changed to 5.0 g/L in batch cultivation. In FB-3 and FB-4, ammonium acetate 5.0 g/L was used as the initial carbon and nitrogen sources for batch cultivation. The media coded SMN, SMNG, SMNS, SMNA and SMNP were used to evaluate the inhibition effects of glucose and metabolites on succinic acid production in anaerobic fermentation in flasks. SMN was SM1 supplemented with 15 g/L of glucose and 10 g/L of $NaHCO_3$, but without addition of NH_4Cl. The initial glucose concentration of SMNS, SMNA and SMNP was the same as SMN. SMNG was prepared with 15, 30, 50, or 70 g/L of glucose in SMN. SMNS was prepared by adding 7.5, 10, 20, 30, 50, or 70 g/L of succinic acid. SMNA and SMNP medium were prepared by adding 3.66 g/L of acetic acid or 2.44 g/L of pyruvic acid to SMN, respectively. The initial pH of the SMNG, SMNS, SMNA and SMNP was adjusted to 7.0.

2.3. Cultivations

The primary seed culture was prepared by the transfer of 1 ml of NZN111 glycerol stock to 30 ml of LB medium in a 250-ml flask, which was then aerobically incubated for 9 h at 37 °C and 220 rpm. The secondary seed culture was prepared by inoculating 500-ml flasks containing 100 ml of SM1 or SM2 medium with 2 ml of primary seed culture and cultivating at 37 °C and 220 rpm for 5 h or 15 h, respectively. Two flasks of the secondary seed culture were then combined to inoculate 3.0 L of medium in a 5 L fermentor. For scale-up in the 50 L fermentor, the primary seed culture was prepared by the transfer of 3 ml of the glycerol stock to 100 ml of LB medium in a 500-ml flask and aerobically incubated for 9 h at 37 °C and 220 rpm. The secondary seed cultivation was conducted in a 5 L fermentor with 2.5 L SM3 medium inoculated with 100 ml of primary seed culture at 37 °C for 10 h, in which DO was maintained above 30%.

The two-stage cultivation was also applied for succinic acid production [9,10]. During the aerobic stage, DO was regulated by varying the agitation speed from 250 to 1000 rpm and the air flow from 3 to 10 L/min. The two-stage cultivation process was divided into three or four phases. Phase I was an aerobic batch cultivation using acetate or glucose. Phase II was a fed-batch cultivation using ammonium acetate or glucose, added after the initial carbon source was exhausted. During Phase II, 214 g/L ammonium acetate or 400 g/L of glucose was fed continuously to maintain the required specific growth rate. The phase III was a transformation process under anaerobic condition and achieved by flushing the fermentor headspace with CO_2 for 5 min. Then the gas exit in the fermentor was shut off and the headspace was switched to a CO_2 gas bag (GSB). The agitation rate was maintained at 500 rpm. Once the anaerobic stage was started, different amounts of glucose were added to the reactor. When the glucose concentration dropped below 6 g/L, glucose was added again. The phase IV represented a stage in which the production rate of succinic acid was decreased significantly. During the whole process of two-stage succinic acid fermentation, the temperature was maintained at 37 °C. The pH was controlled at 7.0 in the aerobic stage and at 6.3 in the anaerobic stage by automatic addition of 8 M NaOH. All of the fermentations were terminated when glucose was exhausted or the glucose consumption rate was lower than 0.2 g/(L h).The feeding method was based on the following formula:

$$V_S = \frac{XV\exp(\mu t)}{Y_{x/s}S_F}$$

V_s, the volume of ammonium acetate or glucose needed to be fed in 2 h. S_F, the concentration of ammonium acetate or glucose used as feed medium. μ (h^{-1}), required specific growth rate of *E. coli*. $Y_{x/s}$, yield of cell based on glucose or NH_4^+.

2.4. Analytical methods

The biomass was determined by measuring the optical density of the culture sample at 600 nm (OD_{600}), after the appropriate dilution. One unit of OD_{600} was equivalent to 0.41 g (dry cell weight)/L. The concentration of glucose was assayed with a commercial analysis kit (Institute of Biological Products, Shanghai, China) containing glucose oxidase. Acetic acid, pyruvic acid, ethanol and succinic acid concentrations were determined by HPLC with an aminex HPX-87H ion exclusion column (Bio-Rad, USA). Chromatography was performed on an LC Solutions system (Shimadzu Corporation, Kyoto Japan) with a UV/vis detector (Shimadzu Corporation, SPD-20A) and refractive index detector (Shimadzu Corporation, RID-10A). The mobile phase was 5 mM H_2SO_4 solution at a flow rate of 0.6 ml/min. The HPLC column was operated at 65 °C.

3. Results

3.1. Effects of different feeding strategies using glucose or acetic acid as a carbon source for aerobic cultivation of NZN111

3.1.1. Fed-batch fermentation for succinic acid production by using glucose as a carbon source (FB-1)

E. coli NZN111 has a set of enzymes capable of regenerating NAD from NADH including fumarate reductase (FRD), malate dehydrogenase (MDH), and malic enzyme (ME). It can be inferred that NZN111 could metabolize glucose anaerobically through the reductive tricarboxylic acid (TCA) pathway. However, these pathways are regulated by the intermediates in carbon metabolism. The induction of acetate and other glycogenic carbon sources was investigated and reported, which indicated a new process for succinic acid production by NZN111 [14,15]. In FB-1, the initial 15 g/L of glucose was exhausted at 6 h. Later, 400 g/L of glucose was fed in to the medium to control the specific growth rate at 0.12 h^{-1}. Once the cell density reached 11.77 g DCW/L, the anaerobic stage was started. In the anaerobic stage, a total of 80 g of glucose was added into the fermentor with a maximum glucose concentration below 30 g/L in the 5 L fermentor. In the first 11 h of the anaerobic stage, 9.42 g/L of succinic acid was produced and 7.51 g/L of pyruvic acid was formed simultaneously. The maximum specific succinic acid production rate was 115.43 mg/(g DCW h). Pyruvic acid was produced quickly in the first few hours, and the maximum volumetric pyruvic acid production rate reached 0.67 g/(L h). As shown in Fig. 1, the final concentration and yield of succinic acid were 16.32 g/L and 0.58 mol/mol, respectively.

3.1.2. Fed-batch fermentation using glucose and acetic acid in the aerobic stage (FB-2)

Acetate has been found to significantly induce and increase the activities of several enzymes such as ICL, MDH, PCK, and ME when *E. coli* strains are cultivated using acetate as the sole carbon source in aerobic conditions. However, a complex component, namely yeast extract (YE), was used in the medium in our former study [14]. To improve the process economics, YE was deleted in this study. The feeding of ammonium acetate was controlled to sustain the specific growth rate at 0.06 h^{-1}. In FB-2, 60% (v/v) of acetic acid was used