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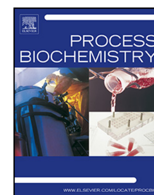

A two-stage process for succinate production using genetically engineered *Corynebacterium acetoacidophilum*

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ABSTRACT

Corynebacterium acetoacidophilum, which is related to *Corynebacterium glutamicum*, was genetically engineered to produce succinate for the first time. The results of genetic experiments in *C. acetoacidophilum* ATCC13870 showed that disrupting the lactate and acetate formation pathways, as well as overexpressing the anaplerotic enzyme pyruvate carboxylase, could increase succinate production and decrease the formation of by-products. Among the six engineered *C. acetoacidophilum* strains, Δ*ldhA*, Δ*ldhA*Δ*pta-ackA*, Δ*ldhA*Δ*pta-ackA*Δ*ctfA*, Δ*ldhA*Δ*aceE*, Δ*ldhA*/pXMJ19-*pyc*, and Δ*ldhA*Δ*aceE*/pXMJ19-*pyc*, merely deleting the L-lactate dehydrogenase gene *ldhA* and overexpressing the pyruvate carboxylase gene *pyc* yielded the optimum production of succinate under oxygen deprivation. A two-stage bioprocess was conducted in a 3-L fermenter to produce succinate using strain Δ*ldhA*/pXMJ19-*pyc*, in which cells were first grown aerobically, after which glucose and bicarbonate were directly added to the cultures for conversion under oxygen-deprived conditions. The succinate concentration reached 908 mM, with an overall yield of 1.34 mol (mol glucose)$^{-1}$ and volumetric productivity of 9.5 mM h^{-1}, suggesting a new potential approach for the industrial production of succinate.

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1. Introduction

Succinic acid (SA) is an intermediate in the citric acid cycle. It has been widely applied as a surfactant/detergent agent and ion chelator, as well as a food ingredient and health-related agent. Currently, bio-based SA is predicted to be one of a number of important platform chemicals for deriving high-value chemicals, such as 1,4-butanediol, gamma-butyrolactone, adipic acid, and tetrahydrofuran, as well as biodegradable polymers, including polybutylene succinate (PBS) and polybutylene succinic acid adipate. SA-producing microorganism and fermentation strategies have been improved so that they can replace petroleum-based SA in a cost-effective manner [1–7]. Naturally occurring bacteria, including *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, and *Basfia succiniciproducens* [8], and engineered strains, such as *Escherichia coli*, *Corynebacterium glutamicum*, and *Saccharomyces cerevisiae* [9], as well as fed-batch and continuous cultures with various bioreactors [10–14], have been studied for SA production (Table 1). In the last five years, large-scale fermentative production of SA has been implemented by four industrial consortia, including a low pH fermentation-based process using *S. cerevisiae* (by Reverdia-DSM/Roquette) or *Candida krusei* (by BioAmber), an electrodialysis-based process using *E. coli* (by BioAmber), an ammonia precipitation-based process using *E. coli* (by Myriant), and a magnesium-based process using *B. succiniciproducens* (by Succinity-BASF/Corbion Purac). All of these technologies have their own merits and challenges to become economically feasible [15].

Additionally, the industrially useful microorganism *C. glutamicum* is also regarded as a promising alternative for the production of SA, and alterations of metabolic pathways in wild-type *C. glutamicum* were studied to enhance SA synthesis by the means of genetic and metabolic engineering [16–21]. An engineered *C. glutamicum* R strain, Δ*ldhA*-pCRA717, which overexpressed the *pyc* gene encoding pyruvate carboxylase and which has a disruption in the *ldhA* gene that encodes L-lactate dehydrogenase, efficiently produced succinate at high-cell density (40–60 g dry cell weight (DCW) per liter) under oxygen deprivation conditions, and the succinate concentration and yield reached 1240 mM and 1.4 mol (mol glucose)$^{-1}$, respectively [16]. *C. glutamicum* BOL-3/pAN6-gap, a derivative of ATCC13032, was constructed with deletions in *ldhA* and three acetate-forming pathways genes (*cat*, *pqo*, *pta-ackA*), and chromosomal integrations of the *pyc* and *fdh* (encoding an NAD$^+$-dependent formate dehydrogenase) genes, as well as plasmid-borne overexpression of the gap gene (encoding glyceraldehyde 3-phosphate dehydrogenase). Using glucose, formate,

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Table 1
Some efficient engineered microorganisms for succinate production from glucose.

Strain	Succinate (mM)	Productivity (mM h^{-1})	Yield(mol mol^{-1})	Process	Growth medium (C, N)	Reference
E.coli AFP111/pTrc99A-*pyc*	839	11	1.78	Dual phase[a]	Glucose, yeast extract, tryptone, $(NH_4)_2SO_4$	[39]
E. coli SBS550 MG/pHL314	350	4	1.60	Dual phase[a]-	Glucose, yeast extract, tryptone	[40]
E. coli KJ122	700	7.4	1.40	Anaerobic batch process	Glucose, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$	[41]
E. coli KJ134	606	6.3	1.53	Anaerobic batch process	Glucose, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$	[41]
C. glutamicum R Δ*ldhA*-pCRA717	1240	27	1.40	Growth-arrested bioprocess[b]	Glucose, yeast extract, casamino acid, $(NH_4)_2SO_4$, urea	[16]
C. glutamicum BOL-3/pAN6-*gap*	1134	21	1.60	Growth-arrested bioprocess[b]	Glucose, $(NH_4)_2SO_4$	[19]
C. glutamicum SA5	926	9.4	1.32	Growth-arrested bioprocess[b]	Glucose, yeast extract, tryptone	[38]
S. cerevisiae AH22ura3 Δ*sdh1*Δ*sdh2*Δ*idh1*Δ*idp1*	30.7	0.18	0.110	Aerobic batch process	Glucose, NH_4Cl, $NH_4H_2PO_4$	[42]
S. cerevisiae 8D (pRS426T-ICL-C)	7.63	–	0.0763	Aerobic batch process	Glucose, $(NH_4)_2SO_4$	[43]

[a] Dual phase process, *E.coli* growed aerobically and then turned to anaerobic succinate production in which cells still growed.
[b] Growth-arrested process, *C. glutamiucm* cells were cultured aerobically, then harvested and resuspened in mineral medium with high cell concentration to produce succinate under oxygen deprivation with cells growth-arrested.

and bicarbonate as substrates, this strain accumulated 1134 mM succinate in 53 h, with a succinate yield of 1.67 mol (mol glucose)$^{-1}$ [19]. The above two strains are currently the highest laboratory-scale SA producers yet described.

In addition, based on the characteristics of aerobic and oxygen deprived *C. glutamicum* processes, a bioprocess has been proposed in which growth-arrested cells of *C. glutamicum* were used as biological catalysts for converting sugars into value-added products [22]. This bioprocess, which used growth-arrested cells of *C. glutamicum* under oxygen deprivation, is promising for biorefinery research and development [23]. Oxygen-deprived cells of *C. glutamicum* could convert glucose to organic acids, mainly including L-lactate, succinate, and small amounts of acetate [24]. *C. glutamicum* R cells harvested after culturing were inoculated into a mineral salts medium under oxygen deprivation, and the production of succinate and lactate reached 11.7 g (L h)$^{-1}$ and 42.9 g (L h)$^{-1}$, respectively [25]. Another strain, *Corynebacterium crenatum* CICC 20219, was also shown to produce 43.6 g L^{-1} succinate and 32 g L^{-1} lactate using this bioprocess [26]. In our preliminary study, a *C. glutamicum*-related species, *Corynebacterium acetoacidophilum* ATCC 13870, was found to accumulate organic acids from glucose when cultured in a mineral medium [27], and it converted glucose to organic acids under oxygen deprivation, which was the same as that in *C. glutamicum* R and ATCC13032, although only about one-half of the close relatives of *C. glutamicum* tested were reported to have this ability [28].

C. acetoacidophilum ATCC 13870 was first isolated by Shiio et al. [29], and it produced L-glutamic acid in a medium containing acetate or sodium acetate as the sole carbon source. In our unpublished work, *C. acetoacidophilum* ATCC 13870 was shown to accumulate the highest amount of succinate of 14 glutamate-producing strains (including *C. acetoacidophilum* ATCC 13870 and, *C. glutamicum* ATCC 13032, ATCC13761, S9114, F343, and T613-41, *Corynebacterium melassecola* ATCC 17965 and 17966, *Microbacterium ammoniaphilum* ATCC 15354, and *Brevibacterium flavum* ATCC 14067 and BfV, *Brevibacterium tianjinese* T6-13, *Brevibacterium lactofermentum* B11 and B12) using a growth-arrested process (shown in Supplementary material). However, genetic manipulation of *C. acetoacidophilum* has seldom been reported, although a new T7 promoter-based expression system for *C. acetoacidophilum* was recently developed [30]. Here, several genetically engineered strains, including those with disruptions in lactate or acetate-producing pathways, and overexpressed pyruvate carboxylase, were constructed and their production of succinate using the growth-arrested process was characterized. A high SA-producer, as well as a two-stage process, in which sugar and carbonate are directly converted to SA without cell harvest after culture, was attained.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

All bacterial strains and plasmids used or constructed in the course of this work are listed in Table 2. In this study, LB medium contained 10 g L^{-1} peptone, 5 g L^{-1} yeast extract, and 10 g L^{-1} NaCl. K medium contained 25 g L^{-1} glucose, 10 g L^{-1} corn steep liquid, 2.5 g L^{-1} urea, 1.5 g L^{-1} K_2HPO_4, 0.6 g L^{-1} $MgSO_4 \cdot 7H_2O$, 5 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 0.2 mg L^{-1} biotin, and 0.2 mg L^{-1} thiamine. BT medium contained 0.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 6 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 4.2 mg L^{-1} $MnSO_4 \cdot H_2O$, 0.2 mg L^{-1} biotin, and 0.2 mg L^{-1} thiamine [16]. All the media were adjusted to pH 7 with NaOH.

2.2. Construction of deletion vectors

C. acetoacidophilum mutants with deletions in *ldhA*, *pta-ackA*, *ctfA*, and *aceE* were constructed via a two-step homologous recom-

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