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Influence of Osmotic Stress on Fermentative Production of Succinic Acid by *Actinobacillus succinogenes*

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Abstract This study investigated the influence of osmotic stress on succinic acid production by *Actinobacillus succinogenes* NJ113. Both cell growth and succinic acid production were inhibited with the increase in osmotic stress of the medium. The use of three different osmoprotectants in the production of succinic acid was studied in order to decrease the inhibitory effects of osmotic stress during fermentation. Results indicated that proline offers optimal osmoprotection in the production of succinic acid by *A. succinogenes* NJ113. In tests of batch fermentation, the maximum cell concentration was observed to be 5.36 g DCW/L after the addition of 25 mmol/L proline to the fermentation medium. The cell concentration was 24% higher than that noted for the control. A total quantity of 56.2 g/L of succinic acid was produced, with a production rate of 1 g/L per hour, after 56 h of fermentation. The concentration and productivity of succinic acid was observed to be increased by 22.2% and 22%, respectively, as compared with the control. The specific activity levels of key enzymes in the metabolic network was noted to be higher following the addition of proline, particularly in the later stages of fermentation. This method of enhancing succinic acid production by the addition of an osmoprotectant may potentially provide an alternative approach for enhanced production of other organic acids.

Keywords Osmotic stress · Proline · *Actinobacillus succinogenes* · Succinic acid

Introduction

Succinic acid is extensively used in food, chemical, and pharmaceutical industries, and it has potential as a promising building-block chemical. In a report of the U.S. Department of Energy (USDOE), succinic acid was considered one among the top 12 sugar-derived chemicals that could be produced from both lignocelluloses and starch to serve as an

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economical driver in biorefineries [1]. Several microorganisms produce large amounts of succinic acid as their major metabolic byproducts [2, 3]. *Actinobacillus succinogenes*—which produces a relatively large amount of succinic acid under anaerobic conditions from a broad range of carbon sources—is a good candidate for application in the industrial production of succinic acid.

Based on reports from previous research studies on factors influencing the succinic acid fermentation process, both substrates and products have been identified to influence cell growth and succinic acid production by *A. succinogenes* [4, 5]. However, few studies have focused on the influence of metal ions. In most organic acid fermentation processes, alkaline materials are used to regulate pH within an optimal range. As a consequence, an increase in osmotic stress in the fermentation broth is usually observed if the addition of alkaline materials is continued with an aim to neutralize the organic acid formed during fermentation. This osmotic stress influences both cell growth and organic acid production during fermentation, particularly toward the end of fermentation [6]. Increased osmotic stress of the culture broth results in simultaneous cell shrinkage, water flux out of the cytoplasm, and reduced turgor pressure in most of the industrial strains. This results in the inhibition of a variety of physiological processes [7–11]. A variety of stress-adaptation mechanisms are acquired to protect these cells from such osmotic stress. These mechanisms include synthesis of compatible solutes and osmoprotectants [12, 13], redistribution of metabolic flux [14], and modulation of cell-surface properties [15, 16]. Osmoprotectants, such as trehalose, glycine, glycine betaine, and proline, are often used in the fermentation process to decrease the influence of osmotic stress. Andersson et al. reported on the addition of glycine betaine to the fermentation medium to reduce osmotic stress. They reported that the fermentation time was approximately 32 h lesser than the normal standard, with no added glycine betaine at 150 g/L glucose [17]. Xu et al. reported results following the addition of 1 g/L proline in the culture medium. In this case, cell growth was enhanced by 59% and pyruvate productivity by 14.3% [18]. Purvis et al. demonstrated that overproduction of trehalose in *Escherichia coli* could promote cell growth [19].

In the present study, the influence of osmotic stress on succinic acid production by *A. succinogenes* NJ113 was investigated. Further, an osmoprotectant was applied to decrease the osmotic stress during fermentation as well as to enhance the productivity and concentration of succinic acid. In addition, changes in specific activities of key enzymes in the metabolic network are discussed.

Materials and Methods

Microorganism

A. succinogenes NJ113 was isolated from rumen in the authors' laboratory and stored at the China General Microbiological Culture Collection Center, as CGMCC NO.1716.

Media Composition

The culture medium for seed culture comprised 10.0 g/L glucose, 5.0 g/L yeast extract, 10.0 g/L $NaHCO_3$, 8.5 g/L $NaH_2PO_4{\cdot}2H_2O$, and 15.5 g/L K_2HPO_4.

The medium for fermentation comprised 3.0 g/L KH_2PO_4, 0.2 g/L $MgCl_2{\cdot}6H_2O$, 0.2 g/L $CaCl_2$, 1.0 g/L NaCl, 5.0 g/L corn steep liquor, and 10.0 g/L yeast extract. Glucose was autoclaved separately and added with aseptic precautions.

Cultivation Conditions

Fermentation of the prepared media in anaerobic bottles was carried out as described in a previous study [20].

Batch fermentation was performed at 37 °C with an initial broth volume of 1.5 L in a 3-L fermentor (Bioflo 110; New Brunswick Scientific, Edison, NJ, USA). The culture medium used for fermentation was sparged with carbon dioxide (CO_2) for 30 min to remove oxygen prior to inoculation. All fermentation processes were carried out at an agitation speed of 200 rpm and CO_2 flow rate of 0.5 L/min. The pH was maintained at 6.8 by the addition of 3 mol/L Na_2CO_3. All experiments were performed in triplicate.

Analytical Methods

Fermentation samples were centrifuged at 9,400×*g* for 10 min at 4 °C in a microcentrifuge. The supernatant was filtered through a 0.22-micron syringe filter and stored at −7 °C until high-performance liquid chromatography (HPLC) analysis. Concentrations of organic content in the media were measured by HPLC (Chromeleon server monitor, UVD 170U detector, P680 pump; Dionex, USA), using equipment that had an ion-exchange column (PrevailTM 124 organic acid column; Grace, USA) [20]. Glucose concentrations were determined with the HPLC system described above; however, the system was modified by using a Series 3000 refractive index (RI) detector (Perkin-Elmer), a guard column, and an ion-exchange column (Aminex HPX87-P, BioRad), as described previously [21]. Dry cell weight (DCW) was computed from a curve correlating optical density at 660 nm (OD660) to dry weight. An OD_{660} of 1.0 represented 520 mg of dry weight per liter [20]. The succinic acid yield was defined as the amount of succinic acid produced following consumption of 1 g of glucose, and this yield was expressed as a percentage.

Enzyme Assay

For the measurement of intracellular enzymatic activity, cells were harvested by centrifugation at 9,400×*g* for 10 min at 4 °C and washed twice with cold Tris–HCl buffer (100 mmol/L; pH 7.5). These washed cells were suspended in the same buffer containing ethylene diamine tetraacetic acid (EDTA; 0.1 mmol/L) and sonicated on ice for 90 cycles (a working period of 3 s with a 10-s interval for each cycle) at a power output of 200 W with an ultrasonic disruptor (GA92-IID; ShangJia Biotechnology Co., WuXi, China). The cell debris was removed by centrifugation at 13,400×*g* for 20 min at a temperature of 4 °C, and the supernatant was used to measure enzymatic activity.

Enzymatic activity was measured in a temperature-controlled spectrophotometer (UV-2100, Unico, USA). Activities of phosphoenolpyruvate carboxykinase (PEPCK), pyruvate kinase (PK), malate dehydrogenase (MDH), and fumarate reductase (FR) were measured as described previously [22]. Enzymatic activity is expressed in units (U), and 1 U is defined as the amount of enzyme necessary to catalyze the conversion of 1 μmol of substrate per minute into specific products. Specific activity is expressed as units per milligram of protein.

Protein Assays

The protein content was determined by the Bradford method [23] and compared with bovine serum albumin (Sigma Chemical Co.) as the standard reference.

Results and Discussion

Influence of the Osmotic Stress on Anaerobic Fermentation by *A. succinogenes* NJ113

The influence of the osmotic stress was investigated in anaerobic fermentation with NaCl as the osmo-regulator (Fig. 1). After the same duration of fermentation, the concentration of residual glucose increased rapidly with increase in concentrations of NaCl in the fermentation medium. The rate of consumption of glucose decreased from 1.98 to 0.23 g/L per hour, and succinic acid productivity decreased from 1.71 to 0.2 g/L per hour. At the conclusion of fermentation, a maximum concentration of 5.1 g/L was attained in a medium containing 0.1 mol/L of NaCl. However, the concentration of succinic acid was lower than that in a reaction where NaCl was not added. With increase in the concentration of NaCl from 0.1 to 0.3 mol/L, the concentration of succinic acid decreased by 42.62%, and levels of formic acid and acetic acid dropped by 29% and by 27.2%, respectively (Fig. 1). When the concentration of NaCl exceeded 0.5 mol/L, the inhibition of cell growth and formation of products was markedly enhanced. At an NaCl concentration of 0.7 mol/L in the fermentation medium, cell growth became nearly suspended, and fewer products were detected (Fig. 1). However, lesser inhibition was observed following the addition of NaCl at a later fermentation time (data not show). Liu et al. concluded that, in the production of pyruvate, the rate of cell growth and the concentration of pyruvate decreased with increase in NaCl and sorbitol concentrations [6]. Lee et al. reported that the maximum cell concentration of *A. succinicproducens* decreased when the concentration of NaCl was greater than 4 g/L [24]. Therefore, in the production of succinic acid by *A. succinogenes* NJ113, both cell growth and succinic acid production were inhibited in association with an increase in the medium's osmotic stress. Sodium ion concentrations at levels less than 0.3 mol/L inhibited the formation of succinic acid more strongly than that of formic acid and acetic acid. At a sodium ion concentration of more than 0.3 mol/L, there was no obvious difference in levels of inhibition.

Choice of Osmoprotectant

The resistance of *A. succinogenes* NJ113 to NaCl was evaluated in a fermentation medium that contained a high concentration of NaCl. Results indicate that the wild-type strain

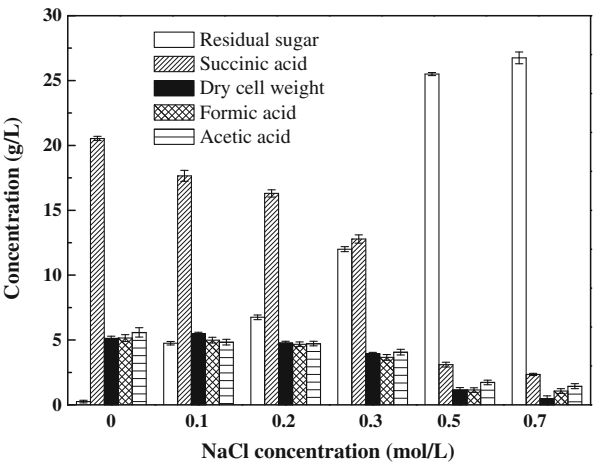

Fig. 1 Influence of the osmotic stress on anaerobic fermentation by *A. succinogenes* NJ113. Cells were grown in fermentation medium and concentrations of NaCl in anaerobic bottles for 12 h with an initial glucose concentration of 30 g/L

ceased to grow when the concentration of NaCl was more than 0.7 mol/L. Osmoprotectants are small molecules that help cells maintain membrane integrity and protein stability and, thus, survive during extreme osmotic stress. They can be synthesized intracellularly by microorganisms or be utilized from the growth medium [25, 26]. Osmoprotectants, such as trehalose, glycine, glycine betaine, and proline, are often used during fermentation to minimize the influence of osmotic stress. To decrease the inhibitory effects of osmotic stress during fermentation by *A. succinogenes* NJ113, the effects of trehalose, glycine betaine, and proline were investigated in succinic acid fermentation. A quantity of 0.3 mol/L of NaCl was added into the fermentation medium to generate an osmotic stress. After 14 h of fermentation (Table 1), it was observed that with the increase in concentrations of osmoprotectants, glucose consumption increased at different levels. Trehalose was noted to affect succinic acid formation, with a yield of 63%. However, under the same experimental conditions but without the addition of trehalose, the yield of succinic acid was observed to be 70%. Following the addition of glycine betaine or proline, the yield of succinic acid reached similar levels to that of the control; proline increased glucose consumption significantly, and the rate of glucose consumption increased from 1.57 to 2.02 g/L per hour. These results indicate that trehalose, glycine betaine, and proline undergo intracellular transport against high osmotic stress. Proline was observed to be the most efficient of these three osmoprotectants during succinic acid production by *A. succinogenes* NJ113. Therefore, it was selected for further study.

Comparison of Fermentation Results with or Without Proline Addition

Batch fermentation was performed in a 3-L stirred bioreactor to investigate cell growth and succinic acid formation. Methods (a) and (b) depict the processing of succinic acid production from an initial glucose concentration of 85 g/L, with or without added proline, respectively (Fig. 2). In method (a), cell growth occurred rapidly and reached a maximum concentration of 4.65 g/L at 8 h. Concentrations of succinic acid, formic acid, and acetic acid continuously increased until no further glucose consumption was noted after approximately 48 h into the reaction. The maximum glucose consumption was 72 g/L, and a total amount of 46 g/L of succinic acid was produced with a productivity of 0.82 g/L and 68% yield per hour, based on the glucose consumption. In method (b), the cell growth was better in comparison with the control, and a maximum concentration of 5.77 g/L was observed at 8 h; this was 24% higher than that in the control. In addition, the cell growth of *A. succinogenes* NJ113 during the entire fermentation process was observed to be much better. When the fermentation reaction was terminated, 84.5 g/L glucose was found to have been consumed in 56 h of fermentation. The concentration and yield of succinic acid were 56.2 g/L and 67%, respectively. The concentration of succinic acid increased by 22.2%, and the yield of succinic acid was close to that observed for method (a).

An evaluation of the first 24 h (Table 2) of the early fermentation process revealed that glucose consumption in method (a) was 55.75 g/L, the concentration of succinic acid was 35.9 g/L, and productivity was 1.39 g/L per hour. Following the addition of proline, glucose consumption increased by 5.8%, and concentration and productivity of succinic acid increased by 13.2% and 12.7%, respectively. With continued fermentation, the concentration of organic acid increased gradually. To neutralize pH, Na_2CO_3 was added to the medium continuously, and it promoted the concentration of sodium ions; thus, it significantly influenced cell growth and the production of succinic acid. At 24 h, the concentration of sodium ions in the fermentation medium reached 0.9 and 1 mol/L in methods (a) and (b), respectively. The fermentation period from 24 to 56 h was analyzed.

Table 1 Fermentation results by adding concentrations of osmoprotectants

	Osmoprotectants concentration (mol/L)	NaCl (mol/L)	Residual glucose (g/L)	Consumption rate of glucose (g/L/h)	Succinic acid (g/L)
Trehalose	0.001	0.3	7.75±0.20	1.59±0.04	14.30±0.30
		0	0±0.05	2.14±0.12	20.6±0.09
	0.005	0.3	7±0.18	1.64±0.01	14.96±0.21
		0	0±0.13	2.14±0.06	19.3±0.31
	0.01	0.3	5±0.24	1.79±0.03	16.50±0.10
		0	0±0.08	2.14±0.24	18.98±0.13
	0.03	0.3	3±0.30	1.93±0.07	17.55±0.08
		0	0±0.12	2.14±0.22	18.48±0.22
	0.05	0.3	2.75±0.12	1.95±0.02	17.61±0.06
		0	0±0.02	2.14±0.27	17.90±0.34
Glycine betaine	0.001	0.3	8±0.24	1.57±0.01	15.18±0.22
		0	0±0.05	2.14±0.23	20.59±0.14
	0.005	0.3	7.75±0.12	1.59±0.05	15.13±0.30
		0	0±0.14	2.14±0.11	20.23±0.33
	0.01	0.3	6.75±0.08	1.66±0.06	15.81±0.12
		0	0±0.05	2.14±0.15	20.19±0.11
	0.03	0.3	5±0.20	1.79±0.04	17.00±0.01
		0	0±0.14	2.14±0.32	21.17±0.03
	0.1	0.3	4.5±0.14	1.82±0.01	17.34±0.02
		0	0±0.09	2.14±0.12	20.69±0.23
Proline	0.001	0.3	7±0.21	1.64±0.04	16.10±0.15
		0	0±0.18	2.14±0.09	19.55±0.22
	0.005	0.3	6±0.06	1.71±0.08	16.80±0.21
		0	0±0.11	2.14±0.22	19.89±0.03
	0.01	0.3	4.75±0.30	1.80±0.01	17.68±0.11
		0	0±0.16	2.14±0.33	20.64±0.14
	0.025	0.3	1.75±0.24	2.02±0.05	19.78±0.23
		0	0±0.05	2.14±0.13	21.24±0.30
	0.05	0.3	2±0.15	2±0.01	19.60±0.04
		0	0±0.11	2.14±0.23	20.15±0.20
Control 1	0	0	0±0.04	2.14±0.01	20.87±0.16
Control 2	0	0.3	8±0.20	1.57±0.03	15.84±0.05

Cells were grown in fermentation medium and concentrations of NaCl in anaerobic bottles for 14 h with an initial glucose concentration of 30 g/L. Each value is the average of results in triplicate experiments and is reported as mean±standard deviation

The glucose consumption in method (a) amounted to 15.25 g/L, while that of the sample with added proline was 25.75 g/L, which indicated an increase of 68.9%. At the conclusion of fermentation, the concentration of sodium ions in the fermentation medium reached 1.4 and 1.18 mol/L in methods (a) and (b), respectively. By-products of this reaction, formic acid and acetic acid, were produced mainly in the early stage of fermentation. From the conclusion presented in "Influence of the osmotic stress on anaerobic fermentation by *A.*

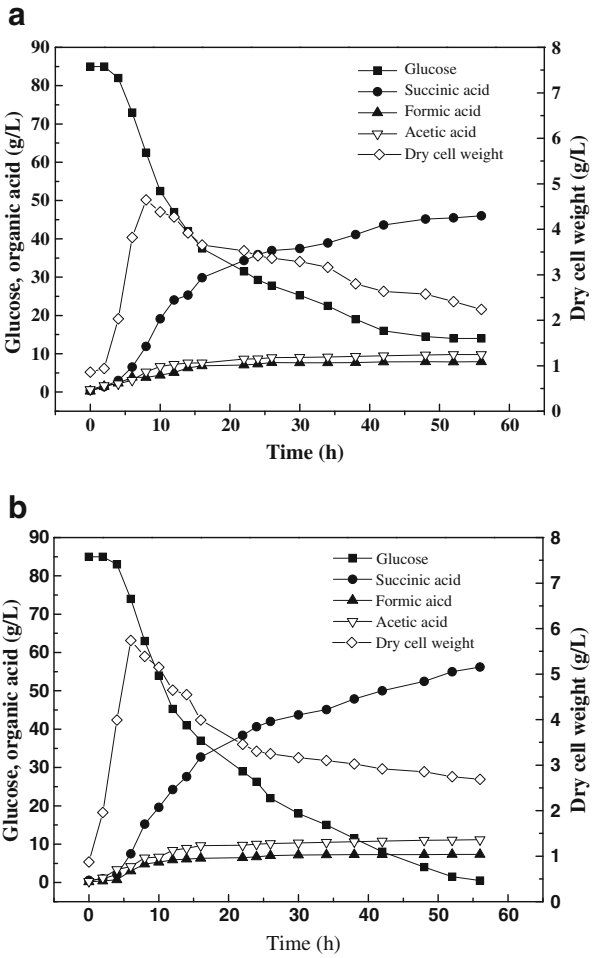

Fig. 2 Fermentation progress of cell growth and production of organic acids in batch fermentation from glucose by *A. succinogenes* NJ113 in a 3-L stirred bioreactor. Cells were grown with an initial glucose concentration of 85 g/L. The pH was constant at 6.8 and modulated with Na_2CO_3. Plotted data were the mean of two replicates, where SD was within 7%. Method (a): fermentation progress of succinic acid production without proline addition. Method (b): fermentation progress of succinic acid production with proline addition

Table 2 Fermentation results of succinic acid production at initial glucose of 85 g/L

Method	Fermentation time (h)	Glucose consumption rate (g/L/h)	Succinic acid productivity (g/L/h)	Average rate of glucose consumption (g/h/L)	Average rate of succinic acid productivity (g/h/L)
a[a]	0–24	2.15	1.50	1.27	0.82
	24–56	0.47	0.32		
b[b]	0–24	2.46	1.69	1.52	1.00
	24–56	0.80	0.49		

Fermentation progress of cell growth and production of organic acids in batch fermentation from glucose by *A. succinogenes* NJ113 in a 3-L stirred bioreactor. Cells were grown with an initial glucose concentration of 85 g/L. The pH was constant at 6.8 and modulated with Na_2CO_3

[a] Fermentation progress of succinic acid production without added proline

[b] Fermentation progress of succinic acid production with added proline

Table 3 Influence with or without proline addition on key enzymes activities at different time

Fermentation time (h)	PEPCK (U/mg)		MDH (U/mg)		PR (U/mg)		PK (U/mg)	
	Method a[a]	Method b[b]	Method a	Method b	Method a	Method b	Method a	Method b
8	1,652±120	2,072±121	1,350±57	1,500±110	1,201±23	1,600±89	1,150±67	1,420±56
16	1,155±58	1,610±23	1,000±20	1,240±50	900±58	1,300±45	800±23	1,040±34
24	540±23	1,120±25	690±34	789±23	400±19	750±21	410±54	689±23
36	120±39	340±29	200±38	380±13	159±12	250±8	180±25	290±18

Cells were grown in fermentation medium with or without added proline for 56 h with an initial glucose concentration of 85 g/L. Each value is an average of three parallel replicates and is reported as mean±standard deviation

[a] Processing of succinic acid production without added proline

[b] Processing of succinic acid production with added proline

succinogenes NJ113", this can probably be attributed to low sodium ion concentration in the fermentation medium.

Proteins, such as enzymes in the metabolic network, evolve to function only within a specified normal range of water activity; outside this range, certain essential cellular functions become impaired. As a consequence, microbial production capacity cannot reach its theoretically predicted maximum level [18]. PEPCK and PK are two key enzymes in succinic acid formation and form byproducts such as formic acid, acetic acid, etc., in fermentation by *A. succinogenes*. MDH and FR were also investigated. From Table 3, these authors note that enzymes demonstrated highest activities at 8 h into the fermentation process. With progression of the process, these specific activities of enzymes declined gradually. All the key enzymes in the metabolic network were observed to exhibit higher specific activities after the addition of proline, particularly in the later stages of fermentation. Therefore, the higher specific activities of key enzymes observed in method (b) could explain why the glucose consumption rate and succinic acid productivity were higher than for method (a).

From these experimental results, proline appears to effectively regulate the cell growth environment, and, thus, is more conducive to bacterial growth and acid production.

Conclusion

The authors believe that this article presents the first report on the influence of osmotic stress in succinic acid production by *A. succinogenes*. Increased osmotic stress was observed to be an important factor that influenced cell growth and product formation during the fermentation process. Proline was found to play an important role in succinic acid production and could regulate the osmotic stress of the medium while maintaining specific activities of key enzymes at a high level. Concentrations of cells and succinic acid were increased by 24% and by 22.2%, respectively, following the addition of proline. This technique for enhancing succinic acid production by the addition of osmoprotectant may provide an alternative approach to enhance the production of other organic acids.

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