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Isolation of NH_4^+-Tolerant Mutants of *Actinobacillus succinogenes* for Succinic Acid Production by Continuous Selection

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***Actinobacillus succinogenes*, a representative succinic-acid-producing microorganism, is seriously inhibited by ammonium ions, thereby hampering the industrial use of *A. succinogenes* with ammonium-ion-based materials as the pH controller. Therefore, this study isolated an ammonium-ion-tolerant mutant of *A. succinogenes* using a continuous-culture technique in which all the environmental factors, besides the stress (ammonium ions), were kept constant. Instead of operating the mutant-generating system as a nutrient-limited chemostat, it was used as a nutrient-unlimited system, allowing the cells to be continuously cultured at the maximum specific growth rate. The mutants were isolated on agar plates containing the acid–base indicator bromothymol blue and a high level of ammonium ions that would normally kill the parent strain by 100%. When cultured in anaerobic bottles with an ammonium ion concentration of 354 mmol/l, the mutant YZ0819 produced 40.21 g/l of succinic acid with a yield of 80.4%, whereas the parent strain NJ113 was unable to grow. When using NH_4OH to buffer the culture pH in a 3.0 l stirred bioreactor, YZ0819 produced 35.15 g/l of succinic acid with a yield of 70.3%, which was 155% higher than that produced by NJ113. In addition, the morphology of YZ0819 changed in the fermentation broth, as the cells were aggregated from the beginning to the end of the fermentation. Therefore, these results indicate that YZ0819 can efficiently produce succinic acid when using NH_4OH as the pH controller, and the formation of aggregates can be useful for transferring the cells from a cultivation medium for various industrial applications.**

Keywords: Succinic acid, ammonium ion tolerant, continuous-culture technique, continuous selection, *Actinobacillus succinogenes*

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Succinic acid is an important C4 building block that has already been widely recognized as a potential platform chemical for the production of various value-added derivatives, such as butanediol, tetrahydrofuran, γ-butyrolactone, *N*-methyl pyrrolidinone, 2-pyrrolidinine, adipic acid, and diethyl succinate [9, 12, 16–18]. Furthermore, its use also extends to the synthesis of biodegradable polymers, such as polybutyrate succinate (PBS), polyamides [21], and various green solvents [14]. Currently, the commercial production of succinic acid is mostly chemically synthesized from butane derived from petroleum. However because of declining oil reserves, rising prices, and concerns over the environmental impact of oil-based industries, attention has recently shifted to the production of succinic acid by microorganisms as an alternative to petroleum-based processes [15, 22].

The fermentation process for numerous organic acids, including succinic acid, requires the pH to be maintained at an approximately neutral level, which is achieved by the addition of a base, such as a Ca-base, Na-base, or Mg-base, for succinic acid production. However, the downstream processing of the fermentation procedure is complicated, including the consumption of large amounts of reagents and the production of many by-products, which results in high costs [R. Datta *et al*. 1992, US patent 5168055; D. A. Glassner *et al*. 1992, US patent 5143834; K. A. Berglund *et al*. 1991, US patent 5034105].

The rumen bacterium *Actinobacillus succinogenes,* a representative succinic-acid-producing microorganism, has a unique ability to produce a relatively large amount of succinic acid from a wide range of substrates while remaining resistant to high concentrations of succinic acid [10, 15]. Among the various neutralization reagents mentioned above, $MgCO_3$ is the best for enhancing succinic acid production by *A. succinogenes*, especially when the fermentation proceeds with a high initial glucose concentration [4, 6]. However, because of the high cost of magnesium

salt and complicated downstream processing, magnesium salt is not suitable for the industrial production of succinic acid using *A. succinogenes*.

However, the above-mentioned problems would all be resolved if an ammonium-ion-based material (*e.g.*, ammonium hydroxide) could be used to maintain a neutral pH during the fermentation process. As diammonium succinate reacts with sulfate ion, combining diammonium succinate with ammonium bisulfate and/or sulfuric acid at a sufficiently low pH produces succinic acid and ammonium sulfate. Ammonium sulfate also thermally cracks into ammonia and ammonium bisulfate, while the succinic acid can be purified using a methanol dissolution step. Therefore, this process involves minimal use of additional reagents, produces virtually no waste by-products, and permits internal recycling of the base and acid values [K. A. Berglund *et al*. 1999, US patent 5958744].

When an NH_4^+-based material is used to maintain a neutral pH during fermentation, the accumulated concentration of ammonia is naturally high. However, *A. succinogenes* is inhibited by ammonium ions, which hampers the industrialization of *A. succinogenes* when using an ammonium-ion-based material as the pH controller [11]. Notwithstanding, if a mutant could be selected that can both tolerate a high concentration of ammonium ions and produce succinic acid efficiently with NH_4OH as the pH controller, this would dramatically reduce the cost of using *A. succinogenes* industrially to produce succinic acid, and greatly simplify the downstream processing. Moreover, isolating inorganic nitrogen-utilizing mutants would eliminate the dependence on expensive yeast extracts in the medium. Accordingly, this study designed a mutant-generating system based on a continuous-culture technique, along with a rapid screening method. Thereafter, the mutant performances were investigated in batch fermentations, with high concentrations of ammonium ions and NH_4OH as the pH controller.

Materials and Methods

Microorganism

Actinobacillus succinogenes NJ113 was isolated from rumen by the current authors and stored at the China General Microbiological Culture Collection Center, CGMCC No. 1716.

Media

The inoculum medium consisted of (g/l of distilled water) glucose (10), yeast extract (5.0), corn steep liquor (15), $NaHCO_3$ (10), $NaH_2PO_4 \cdot 2H_2O$ (9.6), and $K_2HPO_4 \cdot 3H_2O$ (15.5). The inoculum was prepared in 100-ml sealed anaerobic bottles containing 50 ml of the medium with CO_2 as the gas phase. The cultures were grown on a rotary shaker at 37°C and 180 rpm for 8 h. The glucose was autoclaved separately and added aseptically (similarly hereinafter). The batch medium consisted of (g/l of distilled water) glucose (50), sodium acetate (1.36), NaCl 1, $CaCl_2$ (0.2), $MgCl_2$ (0.2), Na_2HPO_4 (0.31), NaH_2PO_4 (1.6), K_2HPO_4 (3), yeast extract (5), and corn steep liquor (10). The continuous medium was the same as the batch medium, except the glucose was shifted to 20 g/l and NH_4HCO_3 was gradually added according to the required ammonium ion concentration (4, 8, 12, 16, and 20 g/l). The screening agar plate medium was based on the inoculum medium, with addition of 20 g/l powdered agar and 0.1 g/l bromothymol blue.

All the chemicals were of reagent grade and bought from either Sinochem (Shanghai, P. R. China) or Fluka Chemical (Buchs, Switzerland). The N_2 and CO_2 were obtained from Nanjing Special Gases Factory (Nanjing, P. R. China).

Batch Cultivation

The small batch cultures were held in 100-ml sealed anaerobic bottles containing 30 ml of medium. The ammonium ions were provided using NH_4HCO_3 (0–36 g/l). The pH decline was curbed by the presence of 35 g/l $MgCO_3$ that was added to the bottles prior to autoclaving. Sterile CO_2 was pumped into the bottom of the bottles before inoculation. The cultures were grown on a rotary shaker at 37°C and 180 rpm for 36 h. The larger batch cultures were held in a 3.0-l fermentor (Bioflo 110, U.S.A.) with an initial broth volume of 1.5 l, and N_2 was bubbled through the medium for 30 min to remove any oxygen before the inoculation. All the fermentations were performed at 37°C with an agitation speed of 200 rpm and CO_2 flow rate of 0.5 l/min. The pH was automatically controlled at 6.8 with the addition of 14 mol/l NH_4OH using a pump, or moderated by the presence of 35 g/l $MgCO_3$ that was added to the fermentor prior to autoclaving.

Continuous Cultivation

The fermentation was performed at 37°C with a CO_2 flow rate of 0.5 l/min. The pH of the vessel was set at 6.8 and controlled with the addition of a 25% Na_2CO_3 solution. The pH of the supplemented medium was also controlled at 8.5 by a 25% Na_2CO_3 solution before sterilizing. To prevent foaming, 0.0015% (v/v) silicone anti-foam was added to the complex medium.

The mutant-generating system was a 500-ml glass test tube with various tube fittings and an operating volume of 200 ml. The agitation was based on sterile CO_2 pumped into the bottom of the vessel. The overflow medium was forced out of the vessel by the air pressure. No samples were taken until three turnovers were completed. To ensure aseptic conditions, the operation of changing to a fresh medium was conducted in a 20% NaOH solution, using polyethylene gloves.

Screening Plate Cultivation

NH_4Cl (20 g/l) was added to the agar plate medium, and the pH of the medium was adjusted to 7.0 with 25% Na_2CO_3. The cultivation was performed at 37°C under anaerobic conditions (N_2:H_2:CO_2=8:1:1) in an anaerobic workstation (Bug box; RUSKINN, U.K.).

Analyses

The glucose was analyzed using an SBA-40C biosensor analyzer (Institute of Biology, Shandong Province Academy of Sciences, P. R. China), and the fermentation products were analyzed by high-performance liquid chromatography (Ultimate3000, Dionex, U.S.A.; Chromeleon server monitor, UVD 170U detector, P680 pump; Prevail organic acid column, Grace, U.S.A.). The analyses were carried out under the following conditions: sample volume of 20 μl, flow phase of 25 mM KH_2PO_4 (adjusted to pH 2.5 by H_3PO_4), flow

rate of 1 ml/min; column temperature of 25°C. The samples were centrifuged at 12,000 ×*g* for 10 min, diluted with the flow phase, and then filtered using a 0.22-µm nylon membrane filter. The dry weight was then determined as follows. Duplicate samples of 2×10 ml were collected in dried conical glass tubes, centrifuged at 12,000 ×*g* for 5 min, and the cells washed twice in deionized water (the cells came from the fermentation broth containing $MgCO_3$ diluted to a linear range with 1 mol/l HCl to make the $MgCO_3$ dissolvable). Thereafter, the tubes with pellets were dried for 24 h in 110°C and finally cooled in a desiccator prior to the final weighing. The ammonium ions were analyzed using a Nova Biomedical BioProfile Analyzer (Profile 300A; NOVA Biomedical, U.S.A.).

RESULTS

Design and Operation of Mutant-Generating System

The operation of the continuous culture is summarized in Fig. 1. A point of variation occurred after 434 h of fermentation: the cell density started increasing (2–3 times the initial density), and the consumption of glucose increased rapidly. At this time-point, the concentration of ammonium ions reached 253 mmol/l (5 times the initial concentration), while the dilution rate was maintained at D=0.37±0.01 h^{-1} (maximum specific growth rate was 0.49 ±0.02 h^{-1}). These shifts in the cell density and rate of glucose consumption were maintained through more than three turnovers, indicating that the shifts could be attributed to a culture change. At the same dilution rate, cultures fed with ammonium ions at 405 mmol/l began to be washed out.

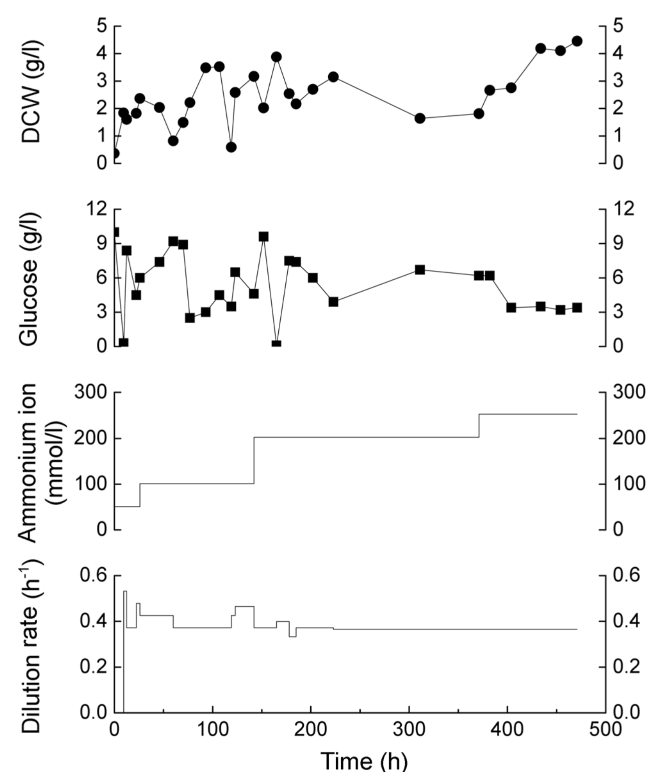

Fig. 1. Process of continuous culture operation.
The fermentations were initiated batchwise [4% (v/v) inoculum of *A. succinogenes* NJ113] and continued to the exponential phase. Continuous cultures were then established by pumping in a fresh medium, starting at a rate of 133 ml/h. The fresh feed medium contained ammonium ions from 50.6 to 253 mmol/l (ammonium ions were supplemented using NH_4HCO_3). As the ammonium ion concentration gradually increased, the dilution rate was adjusted accordingly to prevent the biomass from being washed out of the fermentor vessel.

Selection of Ammonium-Ion-Tolerant Mutants

As the high cell density and low residual glucose suggested the appearance of ammonium-ion-tolerant mutants, samples were obtained under sterile conditions and spread on agar plates containing a high level of ammonium ions, which normally kills 100% of the wild-type strain, and bromothymol blue, which turns yellow around colonies producing organic acid. The mutant colonies circled with yellow were picked and cultured in an NH_4^+ medium to check their viability. Table 1 compares the ammonium ion tolerance of four mutants with that of the wild-type strain.

The growth of the wild-type strain NJ113 in a batch culture was seriously inhibited and no products were detected by HPLC. In contrast, the newly isolated mutants YZ0206, YZ0819, YZ03, and YZ05 were able to grow in the medium with 253 mmol/l NH_4^+ (Table 1). Furthermore, the succinic acid yield of the mutants reached 70%, which the wild-type strain NJ113 was only able to achieve in a medium without NH_4^+. Mutant YZ0819 exhibited the highest fermentation viability among the four mutants, and was used in the following experiments.

Performance of Mutant YZ0819 with High Ammonium Ion Concentration in Anaerobic Bottle Cultivation

As bacteria do not generally adjust the velocity of their catabolism to the anabolic situation, it can be expected that the maximum cell yield will only be obtained if the growth

Table 1. Comparison of fermentation results for mutants and parent strain NJ113[a].

Strains	DCW (g/l)	Succinic acid (g/l)	Succinic acid yield[b] (%)
NJ113	0.164±0.001	-	-
YZ0206	4.550±0.002	31.71±0.14	70.52±0.04
YZ0819	4.578±0.001	33.01±0.18	73.44±0.08
YZ03	2.288±0.003	28.11±0.12	62.53±0.02
YZ05	3.005±0.001	29.24±0.16	65.05±0.03

[a]The fermentations were performed in anaerobic bottles containing 253 mmol/l NH_4^+ and all data are expressed as the means of duplicate tests (±standard deviation). No products of NJ113 were detected by HPLC.
[b]The succinic acid yield was defined as the percentage of the succinic acid concentration to the total glucose (45 g/l).

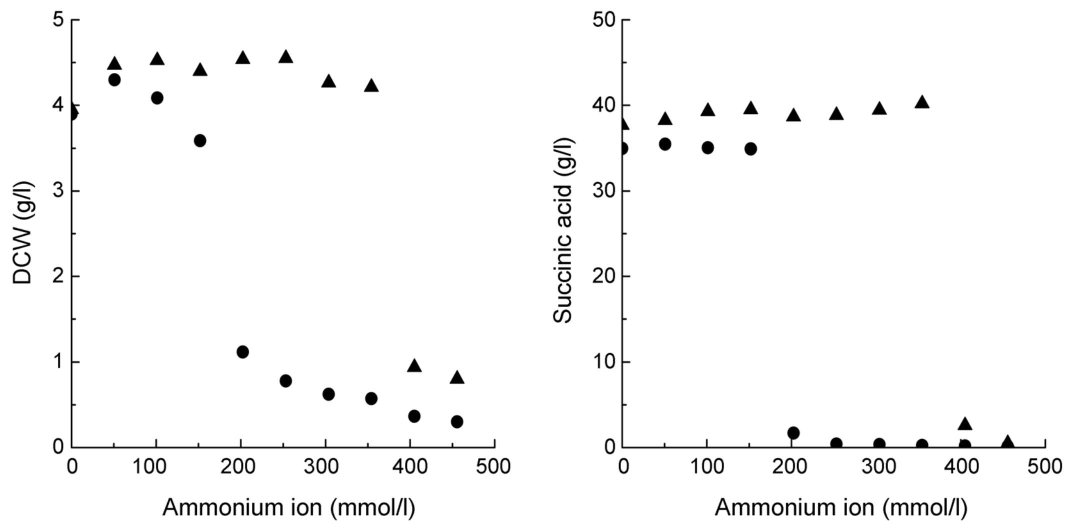

Fig. 2. Comparison of ammonium ion tolerance between YZ0819 (▲) and NJ113 (●) in anaerobic bottle cultivation.

is limited by the energy supply [19]. Thus, with ammonium ions present in the medium, faster growth and greater cell yields were measured (Fig. 2). However, since the uptake of ammonium ions and extrusion of protons increased the energy requirement of the cells [3], the cell growth was inhibited when the ammonium ion concentration exceeded a certain amount. As such, the cell growth and succinic-acid-producing activity decreased sharply when the concentration of ammonium ions in the medium reached 354−405 mmol/l for mutant YZ0819 and 151−202 mmol/l for the wild-type strain NJ113. Thus, when performing fermentation in anaerobic bottles with 354 mmol/l NH_4^+ (the highest medium concentration of NH_4^+ in which mutant YZ0819 was able to grow and produce succinic acid normally), YZ0819 produced up to 40.21 g/l of succinic acid with a yield of 80.4% (Fig. 2).

Batch Fermentations with NH_4OH as pH Controller

When using $MgCO_3$ to buffer the culture pH, the parent strain NJ113 produced 36.8 g/l of succinic acid in 34 h with an initial glucose concentration of 50 g/l (Fig. 3C). As shown in Fig. 3, when using NH_4OH as the pH controller, NJ113 was severely inhibited and the concentration of succinic acid was only 13.76 g/l (Fig. 3A). Although the concentration of ammonium ions in the fermentor (437 mmol/l after 15 h and 718 mmol/l at the end of the fermentation; Fig. 3B) exceeded the tolerance range of YZ0819 for ammonium ions (Fig. 2), YZ0819 still produced 35.15 g/l of succinic acid, 155% more than that produced by NJ113. The succinic acid yield and productivity of YZ0819 was 70.3% and 1.03 g/l/h, respectively (Fig. 3B). Moreover, the rate of glucose consumption was much quicker than that when using $MgCO_3$ as the neutralizer.

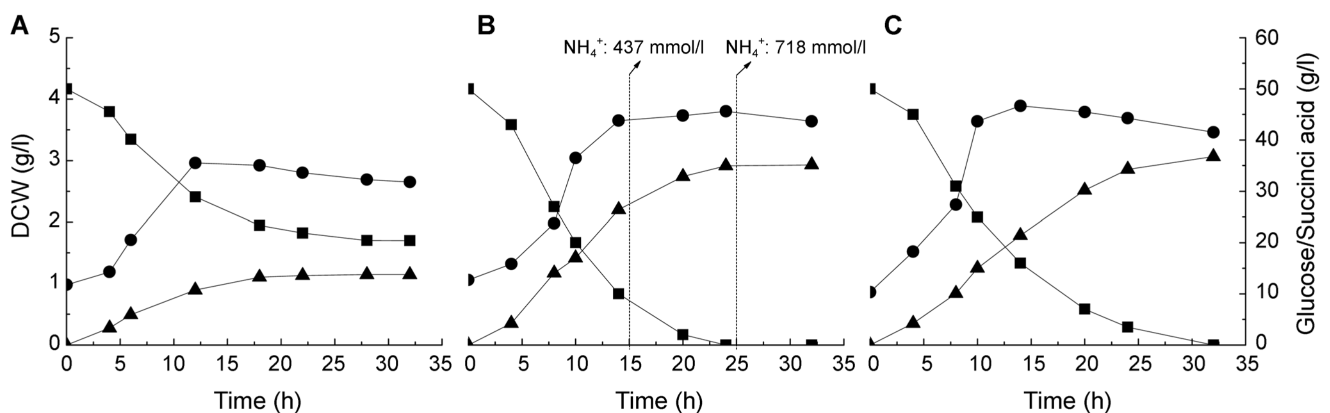

Fig. 3. Comparison of fermentation results for YZ0819 and NJ113 with NH_4OH or $MgCO_3$ as pH controller: fermentation by NJ113 with NH_4OH (**A**) or with $MgCO_3$ (**C**) as neutralizer, and fermentation by YZ0819 with NH_4OH as neutralizer (**B**) [dry cell weight (●), glucose (■), and succinic acid (▲)].

Therefore, the results showed that YZ0819 can efficiently produce succinic acid when using NH_4OH instead of $MgCO_3$ as the pH controller.

DISCUSSION

This study used a continuous-culture technique in which all the environmental factors, besides the stress, were kept constant. With this technique, the inhibitor concentration in the feed was increased manually from time to time, as maximizing the stress promotes the selection of tolerant mutants [8]. Since the specific growth rate was presumed to decrease when increasing the stress, the dilution rate was adjusted based on the cell density and concentration of the residual glucose. Furthermore, the mutant-generating system was not operated as a nutrient-limited chemostat (where the specific growth rate of a mutant is dependent only on the concentration of a single growth-rate-limiting nutrient) at a dilution rate below the maximum specific growth rate (μ_{max}), but rather as a nutrient unlimited at μ_{max}. The residual glucose in the vessel was maintained at a low level. Thus, with this nutrient-unlimited glucose-stat system, the mutations that resulted in an improved μ_{max} were assumed to be the most frequently occurring mutations that conferred a selective advantage [5].

As a result, a mutant with resistance to a high concentration of ammonium ions was obtained after a continuous culture under ammonium ion stress conditions (Fig. 2). It should also be noted that a significant change occurred in the fermentation broth. The cells of the mutant YZ0819 flocculated or aggregated, even under high-speed agitation conditions, from the beginning to the end of the fermentation, and settled quickly at the bottom of the fermentor when the agitation was stopped, whereas the parent strain NJ113 did not exhibit this phenomenon (Fig. 4). Further study found that the aggregation of YZ0819 also occurred in the medium without ammonium ions, and did not disappear even after 5 generations, indicating that the flocculated morphology was stable for future cultures and not only expressed in the presence of ammonium ions (data not shown). Ammonium ions, which are very similar to potassium ions both in charge and size, are transported *via* the high-affinity potassium transport system of *Escherichia coli* (Kdp), leading to a futile cycle of ammonium ions and ammonia molecules (plus protons) across the cytoplasmic membrane [3]. Thus, any transport system capable of transporting ammonium ions into the cell also induces a futile cycle, resulting in wasted energy due to the energy required for the ammonium transporting system and the need to extrude protons to maintain the pH homeostasis in the cell [2]. Consequently, since the permeability of the cell membrane for ammonia molecules is very high [7], if cells do not have enough energy or are unable to maintain their pH homeostasis while growing in a high ammonium ion medium, the strain will become inhibitive and even cause cell death.

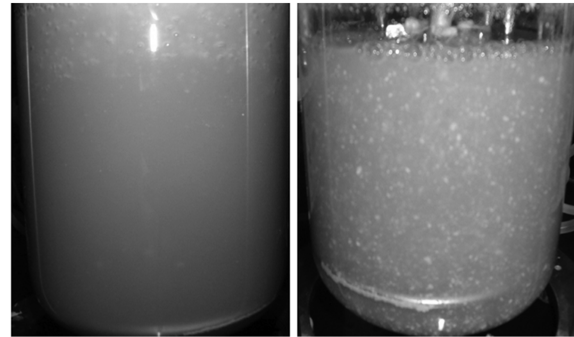

Fig. 4. Aggregation of mutant YZ0819 in fermentation broth (**left**, parent NJ113; **right**, mutant YZ0819).

It was very interesting to find that the small mass or aggregation of cells helped the cells to be tolerant to the ammonium ions. As a kind of self-preservation mechanism, the amount of space between aggregated cells can affect the permeability of solutes into the cells from the surrounding solution [1]. Thus, the flocculation and agglutination of the *A. succinogenes* mutant may have interfered with the access of the ammonium ions to the cells, thereby ensuring that the cells were not injured. In many microorganisms, aggregation results from interactions of molecular components on the cell surface. In the case of *Saccharomyces cerevisiae*, flocculation and agglutination are a mating-type-specific cell–cell aggregation that occurs between mating partners through a protein–protein interaction of α-agglutinin [20]. Whether the ammonium-ion-tolerant mutant secreted a kind of agglutinin requires further study. Nonetheless, the cell aggregation by the mutant may be a useful aid when transferring the cells from a cultivation medium for various industrial applications.

Table 2. Comparison of fermentation results for YZ0819 and NJ113 with different concentrations of yeast extract[a].

Strains	0 g/l Yeast extract		2.5 g/l Yeast extract		5 g/l Yeast extract	
	DCW (g/l)	Succinic acid (g/l)	DCW (g/l)	Succinic acid (g/l)	DCW (g/l)	Succinic acid (g/l)
NJ113	0.264±0.003	1.89±0.51	3.574±0.001	30.18±0.43	4.087±0.001	35.03±0.58
YZ0819	0.278±0.004	2.43±0.67	3.317±0.001	33.01±0.48	4.524±0.002	39.29±0.48

[a]Medium containing 101 mmol/l NH_4^+ and 50 g/l glucose. All data are expressed as the means of duplicate tests (±standard deviation).

The inclusion of yeast extract (supplying organic nitrogen, glutamate, and other nutrient substances) in a medium can increase the rate of fermentation and succinic acid concentration [M. V. Guettler *et al*. 1996, US patent 5573931]. However, since yeast extract is expensive, finding an alternative nitrogen source with a similar effectiveness is clearly desirable. In this study, the mutant YZ0819 grew well, resulting in greater cell yields than with the parent strain NJ113 in a medium containing a high level of ammonium ions (Fig. 2). Unfortunately, the mutant YZ0819, like the parent strain NJ113, did not utilize ammonia-nitrogen as its sole nitrogen source. Thus, the *A. succinogenes* strains did not grow well in the medium without yeast extract (Table 2). Although *A. succinogenes* cell extracts have aspartate:glutamate transaminase activity, they have been found to be auxotrophic for glutamate [13]. This study also found that *A. succinogenes* NJ113 was unable to grow in a chemically defined medium without glutamate (data not shown). As cells utilize NH_3-nitrogen mainly *via* a process of glutamate synthesis [13], the cells were unable to grow in the medium containing NH_3-nitrogen without yeast extract.

The efficacy of a continuous-culture technique for selecting mutants tolerant to extreme environmental stress is already well documented. The dominant ammonium-ion-tolerant mutant YZ0819 was selected and shown to be effective for the production succinic acid when using NH_4OH as the pH controller. However, further study is required to determine the reasons for the flocculated morphology. Moreover, the higher the tolerance to ammonium ions, the higher the glucose consumption, allowing the mutant to be used in large-scale fermentation. The continuous-culture technique could also be used to screen inorganic nitrogen-utilizing mutants of *A. succinogenes* for succinic acid production, based on a metabolic flux analysis.

Acknowledgments

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References

1. Arnold, W. N. and J. S. Lacy. 1977. Permeability of the cell envelope and osmotic behavior in *Saccharomyces cerevisiae*. *J. Bacteriol.* **131:** 564–571.
2. Booth, I. R. 1985. Regulation of cytoplasmic pH in bacteria. *Microbiol. Rev.* **49:** 359–378.
3. Buurman, E. T., M. J. Teixeira de Mattos, and O. M. Neijssel. 1991. Futile cycling of ammonium ions *via* the high affinity potassium uptake system (Kdp) of *Escherichia coil*. *Arch. Microbiol.* **155:** 391–395.
4. Du, C., S. K. C. Lin, A. Koutinas, R. H. Wanga, M. P. Dorado, and C. Webba. 2008. A wheat biorefining strategy based on solid-state fermentation for fermentative production of succinic acid. *Bioresour. Technol.* **99:** 8310–8315.
5. Duncan, R. S., J. M. Withers, M. G. Wiebe, G. D. Robson, and P. J. A. Trinci. 1998. Mutants with general growth rate advantages are the predominant morphological mutants to be isolated from the Quorn® production plant. *Mycol. Res.* **102:** 221–227.
6. Jiang, M., K. Chen, Z. Liu, P. Wei, H. Ying, and H. Chang. 2010. Succinic acid production by *Actinobacillus succinogenes* using spent Brewer's yeast hydrolysate as a nitrogen source. *Appl. Biochem. Biotechnol.* **160:** 244–254.
7. Kleiner, D. 1985. Energy expenditure for cyclic retention of NH_3 NH_4^+ during N_2 fixation by *Klebsiella pneumoniae*. *FEBS Lett.* **187:** 237–239.
8. Lane, P. G., S. G. Oliver, and P. R. Butler. 1999. Analysis of a continuous-culture technique for the selection of mutants tolerant to extreme environmental stress. *Biotechnol Bioeng.* **65:** 397–406.
9. Lee, P. C., W. G. Lee, S. Y. Lee, H. N. Chang, and Y. K. Chang. 2000. Fermentative production of succinic acid from glucose and corn steep liquor by *Anaerobiospirillum succiniciproducens*. *Biotechnol. Bioprocess. Eng.* **5:** 379–381.
10. Li, X., Z. Zheng, Z. Wei, S. Jiang, L. Pan, and S. Weng. 2009. Screening, breeding and metabolic modulating of a strain producing succinic acid with corn straw hydrolyte. *World J. Microbiol. Biotechnol.* **25:** 667–677.
11. Liu, Y., P. Zheng, Z. Sun, Y. Ni, J. Dong, and P. Wei. 2008. Strategies of pH control and glucose-fed batch fermentation for production of succinic acid by *Actinobacillus succinogenes* CGMCC1593. *J. Chem. Tech. Biotech.* **83:** 722–729.
12. McKinlay, J. B., C. Vieille, and J. G. Zeikus. 2007. Prospects for a bio-based succinate industry. *Appl. Microbiol. Biotechnol.* **76:** 727–740.
13. McKinlay, J. B., J. G. Zeikus, and C. Vieille. 2005. Insights into *Actinobacillus succinogenes* fermentative metabolism in a chemically defined growth medium. *Appl. Environ. Microbiol.* **71:** 6651–6656.
14. Rudner, M. S., S. Jeremic, K. A. Petterson, D. R. Kent, K. A. Brown, M. D. Drake, W. A. Goddard, and J. D. Roberts. 2005. Intramolecular hydrogen bonding in disubstituted ethanes. A comparison of $NH\cdots O^-$ and $OH\cdots O^-$ hydrogen bonding through conformational analysis of 4-amino-4-oxobutanoate (succinamate) and monohydrogen 1,4-butanoate (monohydrogen succinate) anions. *J. Phys. Chem. A* **109:** 9076–9082.
15. Song, H. and S. Y. Lee. 2006. Production of succinic acid by bacterial fermentation. *Enzyme Microb. Technol.* **39:** 352–361.
16. Sriram, V. and J. M. Dennis. 1999. Catalytic upgrading of fermentation-derived organic acids. *Biotechnol. Prog.* **15:** 845–854.
17. Stols, L. and M. I. Donnelly. 1997. Production of succinic acid through overexpression of NAD+ dependent malic enzyme in an *Escherichia coli* mutant. *Appl. Environ. Microbiol.* **63:** 695–701.

18. Van der Werf, M. J., M. V. Guettler, M. K. Jain, and J. G. Zeikus. 1997. Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentation by *Actinobacillus* sp. 130Z. *Arch. Microbiol.* **167:** 332–342.
19. Van Uden, N. 1969. Kinetics of nutrient limited growth. *Annu. Rev. Microbiol.* **23:** 473–486.
20. Wen, Z., U. Mitsuyoshi, and T. Atsuo. 2001. Genetically controlled self-aggregation of cell-surface-engineered yeast responding to glucose concentration. *Appl. Environ. Microbiol.* **67:** 2083–2087.
21. Willke, T. h. and K. D. Vorlop. 2004. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. *Appl. Microbiol. Biotechnol.* **66:** 131–142.
22. Zeikus, J. G., M. K. Jain, and P. Elankovan. 1999. Biotechnology of succinic acid production and markets for derived industrial products. *Appl. Microbiol. Biotechnol.* **51:** 545–552.