**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$_4$OH 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$_4$OH 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-pyrrolidine, 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 diaminonium succinate reacts with sulfate ion, combining diaminonium 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 hamper 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$_4$OH 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$_4$OH 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), Na$_2$HPO$_4$·2H$_2$O (9.6), and KH$_2$PO$_4$·3H$_2$O (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$_2$HPO$_4$ (0.31), NaH$_2$PO$_4$ (1.6), K$_2$HPO$_4$ (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$_4$HCO$_3$ 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$_4$HCO$_3$ (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$_4$OH 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$_2$CO$_3$ solution. The pH of the supplemented medium was also controlled at 8.5 by a 25% Na$_2$CO$_3$ solution before sterilizing. To prevent foaming, 0.0015% (v/v) silicone antifoam 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$_4$Cl (20 g/l) was added to the agar plate medium, and the pH of the medium was adjusted to 7.0 with 25% Na$_2$CO$_3$. 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.; Chromelon 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$_2$PO$_4$ (adjusted to pH 2.5 by H$_3$PO$_4$), flow