Enhanced production of D(-)-lactic acid by mutants of *Lactobacillus delbrueckii* ATCC 9649*

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SUMMARY

Chemical mutagenesis with ethyl methanesulfonate (EMS) was used to develop strains of *Lactobacillus delbrueckii* (ATCC 9649) that tolerated increased lactic acid concentrations while continuously producing the acid. Three mutants (DP2, DP3 and DP4) were compared with wild-type *L. delbrueckii* by standing fermentations with different glucose concentrations. All three mutants produced higher levels of lactic acid than the wild-type. In pH-controlled (pH 6.0) stirred-tank-batch fermentations, mutant DP3 in 12% glucose, 1% yeast extract/mineral salt/oleic acid medium produced lactic acid at a rate that was more than 2-times faster than the wild-type. Mutant DP3 also produced 77 g/l lactic acid compared with 58 g/l for the wild-type. Overall, compared with wild-type, the mutants DP2 and DP3 exhibited faster specific growth rates, shorter lag phases, greater lactic acid yields, tolerated higher lactic acid concentrations, and produced as much as 12% lactic acid in 12% glucose, 3% yeast extract/mineral salt/oleic acid medium which required an additional 9% glucose when the residual glucose concentration decreased to \( \leq 3\% \). Mutant DP3 was stable for over 1.5 years (stored freeze dried). The strain development procedure was very successful; mutants with enhanced lactic acid-producing capacity were obtained each time the procedure was employed.

INTRODUCTION

Lactic acid is the smallest natural molecule to exhibit optical activity. It exists in two isomeric forms, D(-)-lactic acid and L(+)-lactic acid [12]. Lactic acid is an organic acid that can be produced chemically from coal, petroleum and natural gas, and biologically from the bioconversion of carbohydrates, agricultural and industrial wastes and plant biomass [2,12]. In the food industry, lactic acid is a bacterial fermentation product essential for the manufacture of cheeses, pickles, yogurt, cultured sausages, and buttermilk. As a food additive, it is used as an acidulant, a flavor agent, and a preservative. It is generally recognized as safe for human consumption [4]. Lactic acid can also be used as a feedstock for the production of plastics [5,12], other organic acids, propylene glycol, ethanol, and acetaldehyde [4].

Lactic acid production via fermentation provides only about 50% of the world supply [7] because product inhibition and recovery limit economics of the conventional fermentation process. To improve production, novel fermentation techniques have been applied. These include immobilized-cell bioreactors [14], hollow-fiber reactors [8], cell-recycled reactors [7], and extractive fermentations [13]. To overcome feedback inhibition problems, strain development via chemical and irradiation mutagenesis or molecular genetic approaches need to be employed.

In this paper, we describe a reliable chemical mutagenesis procedure that can be used to develop enhanced strains of *Lactobacillus*. Three mutants were selected, and their physiological differences were examined. In all instances, the mutants demonstrated faster growth rates and higher product yields than the wild-type in various medium modifications.

MATERIALS AND METHODS

Microorganism

*Lactobacillus delbrueckii* (ATCC 9646), a homofermentative D(-)-lactic acid producer, was obtained from the American Type Culture Collection (Rockville, MD), and maintained in *Lactobacillus* MRS medium (Difco Laboratories, Detroit, MI) stored at 4 °C. Cultures were transferred to fresh MRS broth every 2–3 weeks.
**L. delbrueckii** ATCC 9649 wild-type is capable of growing in the presence of ≤6% lactic acid, but not in 8%.

**Culture broth analysis**

Glucose consumption and \( \text{D(\(-\))-lactic acid production were analyzed by using a YSI model 2000 glucose/L(\(+\)-lactic acid analyzer (Yellow Springs Instruments, Yellow Springs, OH) and a Water's high-performance liquid chromatograph (HPLC) (Milford, MA) equipped with Waters model 401 refractive index detector, respectively. The separation of lactic acid, glucose, and other broth constituents was achieved on a Bio-Rad Aminex HPX-87H column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using 0.012 N \( \text{H}_2\text{SO}_4 \) as a mobile phase at a flow rate of 0.8 ml/min with a 20-\( \mu \)l injection loop. Bacterial growth was followed by measuring the absorbance at 620 nm on a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, NY).

**Chemical mutagenesis and killing curve determination**

A 1-ml inoculum from an overnight static culture in MRS medium was added to 100 ml of MRS medium in a 250-ml flask. The culture was incubated as a standing culture in a 45 °C water bath until it reached stationary phase; then, 20 ml of cell suspension (10^7 cells/ml) was harvested by centrifugation at 1000 \( \times \) g for 10 min. The supernatant was decanted, and the cell pellet was resuspended in 20 ml fresh MRS medium containing 0.4% (w/v) caffeine (Sigma Chemical Co., St. Louis, MO), which was the maximum level that the bacterium could tolerate. After the zero-time sample was taken, 0.4 ml of ethyl methanesulfonate (EMS) or methyl methanesulfone (MMS) (Sigma Chemical Co.) was added to the tube, mixed, and the tube was incubated at 45 °C. One-ml samples were taken after 5, 10, 15, and 30 min. The samples were serially diluted in sterile water, and survivors determined by using pour plates of MRS agar medium without caffeine in replicates of three. Viable counts were determined after incubation at 45 °C for 24–48 h. The same procedure was repeated by using 0.6, 0.8, 1.0, 1.2, 1.5 ml of EMS or MMS, and kill curves for the bacterium were constructed for both mutagens.

**Mutagenesis and mutant selection**

The mutation selection medium contained 5% (w/v) glucose; 1% (w/v) yeast extract (Difco); 8, 12 or 16% (w/v) L(\(+\))-lactic acid (Aldrich Chemical Co., Milwaukee, WI); 0.4% caffeine (w/v) in mineral salt solution (0.6 g \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 0.03 g \( \text{MnSO}_4 \cdot \text{H}_2\text{O} \), 1.0 g sodium acetate, 0.5 g \( \text{KH}_2\text{PO}_4 \), and 0.5 g \( \text{KH}_2\text{PO}_4 \) per liter of deionized water, pH adjusted to 6.5) and 0.05% (w/v) oleic acid (Sigma Chemical Co.). The glucose and mineral salts solutions were sterilized separately in an autoclave, cooled, and mixed; filter-sterilized oleic acid was added aseptically. \( \text{L. delbrueckii} \) ATCC 9649 was treated with EMS to 99% kill. The cells were removed by centrifugation, washed once in 0.1 M phosphate buffer (pH 7), and resuspended in 5 ml of phosphate buffer. One ml of cell suspensions was transferred to 9 ml of selection medium. From culture tubes with growth, 1 ml was transferred to 9 ml of fresh MRS medium without lactic acid, and incubated standing in a 45 °C water bath. Next, 1 ml was transferred to 9 ml of the mutant selection medium and incubated until visible growth was detected. The same sequential transfer procedure between MRS medium and selection medium was applied two more times for a total of four transfers in the lactic acid selection medium. Finally, the culture was transferred to fresh MRS medium. After overnight incubation, the culture was inoculated into 100 ml of MRS medium in a 250-ml flask, and the flask was incubated standing in a 45 °C water bath until stationary phase. This culture was mutated a second and third time as already described. A total of four serial transfers into 8% lactic acid selection medium followed by MRS medium was performed after each mutagenesis treatment, except for one mutant (DP4) that survived only two transfers. Mutants DP2, and DP3 were obtained by following the complete procedure described. The mutants were freeze dried in 20% skim milk powder solution and stored at -20 °C.

To further stress mutant DP3, continuous fermentation was performed by using filtered sterilized medium containing 1% yeast extract, 5% glucose, mineral salt solution, 0.05% oleic acid and 8% L-lactic acid. A custom fitted 1800-ml Fleaker beaker was used as the reactor vessel with a working volume of 500 ml, at 45 °C and pH maintained at 6 with 7 N \( \text{NH}_4\text{OH} \). Agitation was done by a magnetic bar on a plate stirrer. Inoculum was prepared from freeze dried \( \text{L. delbrueckii D} \) which was suspended in 100 ml MRS medium and incubated for 24 h. A 50 ml inoculum was added to reactor. After 48-h batch fermentation, continuous fermentation was started and 0.007, 0.014, 0.024, 0.048 h⁻¹ dilution rates were initiated on days 2, 5, 12, and 15, respectively. The mutant selected during this continuous fermentation was DP3.19.

**Comparison of wild-type to the mutants**

**Standing culture fermentation.** Growth rates and lactic acid production rates of the wild-type and mutants were compared by using MRS medium containing 12% glucose and 1% yeast extract, mineral salts (0.6 g \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 0.03 g \( \text{MnSO}_4 \cdot \text{H}_2\text{O} \), 1.0 g sodium acetate, 0.5 g \( \text{K}_2\text{HPO}_4 \), and 0.5 g \( \text{KH}_2\text{PO}_4 \) per liter of medium) and 0.05% oleic acid (1% YMO) with 12% glucose in standing culture without pH control at 45 °C. Absorbance at 620 nm and lactic acid concentration were determined after incubation