Journal of Industrial Microbiology, 11 (1992) 23–28
© 1992 Society for Industrial Microbiology 0169-4146/92/$05.00
Published by Elsevier

SIM 00449

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

Ali Demirci and Anthony L. Pometto III

Department of Food Science and Human Nutrition, Center for Crops Utilization Research, Iowa State University, Ames, IA, USA

(Received 6 April 1992; revision received 13 July 1992; accepted 18 July 1992)

Key words: Bacterium; Lactic acid; *Lactobacillus delbrueckii*; Mutagenesis; Fermentation

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 ≤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, Detroid, MI) stored at 4 °C. Cultures were transferred to fresh MRS broth every 2–3 weeks.

Correspondence to: A.L. Pometto III, Department of Food Science and Human Nutrition, Center for Crops Utilization Research, Iowa State University, Ames, IA 50011, USA.

* Journal Paper No. J-14087 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Projects No. 2889 and 0178.

L. delbrueckii ATCC 9649 wild-type is capable of growing in the presence of $\leq 6\%$ lactic acid, but not in 8%.

Culture broth analysis

Glucose consumption and 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 × 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA) using 0.012 N H_2SO_4 as a mobile phase at a flow rate of 0.8 ml/min with a 20-μl 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 × *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 mutant 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 $MgSO_4 \cdot 7H_2O$, 0.03 g $MnSO_4 \cdot H_2O$, 1.0 g sodium acetate, 0.5 g K_2HPO_4, and 0.5 g KH_2PO_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. *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 NH_4OH. Agitation was done by a magnetic bar on a plate stirrer. Inoculum was prepared from freeze dried *L. delbrueckii* DP3 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 $MgSO_4 \cdot 7H_2O$, 0.03 g $MnSO_4 \cdot H_2O$, 1.0 g sodium acetate, 0.5 g K_2HPO_4, and 0.5 g KH_2PO_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

for 24 h. Next, a series of flask fermentations was done using MRS medium in which pH was manually controlled at pH 6 with 5 N NaOH, and lactic acid concentrations were measured after incubation for 24 h.

Stirred batch fermentation. A 7.5-liter Microferm fermenter (New Brunswick Scientific Co., New Brunswick, NJ), with a working volume of 3 liters was used. The media consisted of filter-sterilized MRS, 1% YMO or 3% yeast extract, mineral salts, and 0.05% oleic acid (3% YMO) with 12% glucose. The 3% YMO medium required an additional 9% glucose, which was added when the residual glucose concentration decreased to ≤3%. The pH was maintained at 6 by addition of filter-sterilized 6 N NH_4OH. The fermentation temperature was 45 °C, and the agitation rate was 100 rpm. The fermentation was followed by measuring cell growth, glucose consumption, and lactic acid production. Biomass was determined by measuring absorbance at 620 nm, which was then converted to dry cell mass (g/l) via a standard curve prepared with log-phase cells. All fermentation data represent at least duplicate fermentation runs.

RESULTS AND DISCUSSION

Chemical mutagenesis and mutant selection

As determined by the kill curve, 0.9 ml of EMS per 20 ml of cell suspension killed 99% of the original microorganisms after 15 min of incubation; this was deemed the optimum procedure. Bacterial growth was detected only in the 8% lactic acid selection medium; even after repeated mutations, no growth was present in the tubes containing 12% or 16% lactic acid. Transfer of cultures from the 8% lactic acid selection medium to MRS medium and back to lactic acid again for a total of four transfers into 8% lactic acid selection medium was essential for selecting stable mutants. Mutant DP4 was treated with the EMS three times, as was DP2 and DP3. However, mutant DP4 was unable to grow in the third 8% lactic acid transfer culture tube after the third mutagenesis treatment, and its physiological characteristics were different from mutants DP2 and DP3 (Table 1). This emphasizes the importance of the bacterial growth in serial transfers after mutagenesis.

L. delbrueckii cells treated with MMS to a 99% kill did not grow in any of the lactic acid selection media. The main difference between the two chemical mutagens, MMS and EMS, is that 7-methyl guanosine produced during MMS treatments is not as stable as 7-ethyl guanosine, which is produced by the EMS treatment of DNA [10].

Another factor that could affect mutant selection and survival is the growth stages of the cells to be mutated. Hince and Neale [3] and Matijasavic and Zeiger [6] reported that mutagenesis was more effective if it were applied to cells in a stationary phase. We observed that, when log-phase cells were treated with EMS, no cell growth in any of the selection media was detected. Therefore, mutagenesis was routinely performed with cells in early stationary phase.

Caffeine was added to the mutagenesis MRS medium and to the 8% lactic acid selection medium. Caffeine is a mutation stabilizer which causes error-prone SOS rapid of the DNA in the cell [9]. Caffeine also slows down an error-prone, postreplicative DNA repair mechanism without changing the mutation frequency [11]. It was determined that the highest concentration of caffeine that *L. delbrueckii* could tolerate was 0.4%. Furthermore, to

TABLE 1

D(–)-lactic acid production (g/l) by wild-type and mutant strains of *Lactobacillus delbrueckii* grown in standing culture at 45 °C for 24 h without pH control

Strain	12% glucose/ MRS medium	12% glucose/ 1% YMO[a] medium
Wild	34	1.8
DP2	39	9.1
DP3	41	8.1
DP4[b]	38	5.6

[a] The 1% YMO is 1% yeast extract, mineral salts, and 0.05% oleic acid medium.

[b] Mutant DP4 was transferred into 8% lactic acid selection medium two times instead of four times.

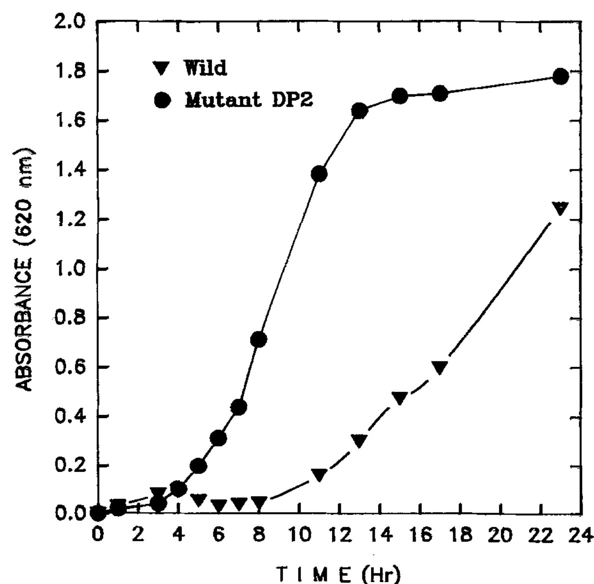

Fig. 1. Growth curve of wild-type *L. delbrueckii* and mutant DP2 in MRS medium stationary culture at 45 °C without pH control.

TABLE 2

Physiological differences between wild-type *Lactobacillus delbrueckii*, and mutants DP3 and DP3.19 in pH controlled fermentation in 12% glucose, 1% YMO medium at 45 °C[a]

Description	Wild-type	Mutant DP3	Mutant DP3.19
D(−)-lactic acid produced (g/l)	58	77	68
Glucose consumed (g/l)	73	95	75
Percent yield ($Y_{p/s}$)[b]	80	81	91
Max. growth rate $(dx/dt)_{max}$ (g/l/h)	0.09	0.12	0.03
Max. production rate $(dp/dt)_{max}$ (g/l/h)	0.72	1.72	1.17
Max. consumption rate $(ds/dt)_{max}$ (g/l/h)	0.96	1.99	1.29

[a] The total fermentation time was 100 h. All values are averages of two replicates. The 1% YMO is 1% yeast extract, mineral salts, and oleic acid medium.

[b] Yield is defined as the concentration of lactic acid produced divided by the amount of glucose consumed times 100 percent.

avoid the selection of crippled mutants, the most minimal medium possible was used for the 8% lactic acid selection medium.

Fermentation characteristics

Mutants DP2, DP3 and DP4 were evaluated initially by standing cultures in MRS-12% glucose medium without pH control. All mutant cultures became turbid more rapidly than wild-type cultures. Fig. 1 illustrates the shorter lag time and faster growth rate for one of the mutants (DP2) compared with wild-type. All mutants produced more lactic acid than the wild-type in MRS-12% glucose and 1% YMO medium (Table 1). In 1% YMO medium, mutants DP2 and DP3 produced about 4-times more lactic acid than the wild-type, whereas mutant DP4 produced only 3-times more lactic acid in 24 h. This difference between mutant DP4 and the other mutants illustrates the importance of mutant survival in the complete selection scheme.

Stressing the mutant

Continuous cultivation of mutant DP3 in a medium containing 8% L(+)-lactic acid was performed in an effort to stress further the mutant. Dilution rates were 0.007, 0.012, 0.024, and 0.048 h^{-1}. During this continuous fermentation mutant DP3.19 was the last survivor which was obtained at the dilution rate of 0.024 h^{-1} on the 13th day of the fermentation. This treatment resulted in poor growth and was harmful to the bacterium, resulting in an extended lag phase in MRS medium.

pH-controlled batch fermentations

In 1% YMO medium, pH-controlled batch-fermentation environment, the wild-type and the mutant strain DP3

TABLE 3

Physiological differences between wild-type *Lactobacillus delbrueckii*, and mutant DP3 and DP3.19 in pH-controlled fermentation in 12% glucose, 3% YMO medium at 45 °C[a]

Description	Wild-type	Mutant DP3		Mutant DP3.19
		Initial culture	Freeze dried[b]	
D(−)-lactic acid produced (g/l)	67	117	117	89
Glucose consumed (g/l)	88	152	160	89
Percent yield ($Y_{p/s}$)[c]	75	76	73	99
Max. growth rate $(dx/dt)_{max}$ (g/l/h)	0.15	0.34	0.17	0.05
Max. production rate $(dp/dt)_{max}$ (g/l/h)	1.35	6.46	5.57	2.25
Max. consumption rate $(ds/dt)_{max}$ (g/l/h)	1.86	7.82	7.16	1.70

[a] The total fermentation time was 100 h. All values are averages of two replicates. The 3% YMO is 3% yeast extract, mineral salts, and 0.05% oleic acie medium.

[b] Freeze dried DP3 which was stored at −20 °C for 22 months.

[c] Yield is defined as the concentration of lactic acid produced divided by the amount of glucose consumed times 100 percent.

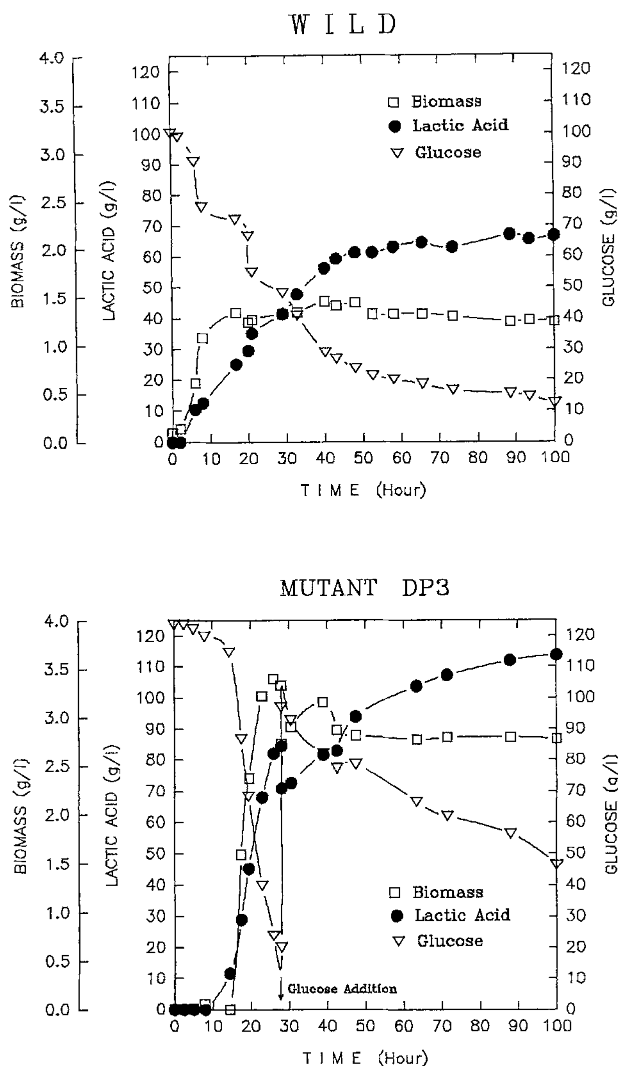

Fig. 2. Wild-type and mutant DP3 *L. delbrueckii*; cell mass production, D(–)-lactic acid production, and glucose consumption in pH-controlled, stirred-tank fermentation in 3% YMO medium.

produced 58 g/l and 77 g/l D(–)-lactic acid in 100 h, respectively (Table 2). The data for the wild-type matched a previous report for lactic acid production under similar conditions [7]. The percent yield was about 80% for both the wild-type and mutant DP3. The maximum growth rate of mutant DP3 was about 40% faster than that of the wild-type. All mutants tested, except DP3.19, demonstrated shorter lag times and faster growth rates than the wild-type. The maximum lactic acid production rate for mutant DP3 was more than 2-times faster than that of the wild-type. This greater maximum production rate decreased the batch fermentation time, and increased volumetric productivity. Mutant DP3.19 showed higher lactic acid concentration, yield, and production rate then wild-type.

In 3% YMO medium, mutant DP3 produced 117 g/l lactic acid compared with 67 g/l lactic acid by the wild-type strain (Table 3, Fig. 2). Furthermore, mutant DP3 had maximum growth and production rate that were 2- and 5-times higher, respectively, than those of the wild-type. These higher growth and lactic acid production rates in the presence of 3% YMO as compared to 1% YMO indicated the importance of yeast extract for *L. delbrueckii*. This observation was supported by Aeschlimann and von Stockar [1]; they reported that high yeast extract concentrations increase lactic acid concentration, and production rate for *L. helveticus*. Mutant DP3.19 produced 89 g/l lactic acid with a 99% yield, whereas the wild-type produced 67 g/l lactic acid with a 75% yield (Table 3). However, in either 1% or 3% YMO medium, the maximum growth rate of DP3.19 was 3-times slower than that of the wild-type (Tables 2 and 3). Therefore, continuous stressing with a high level of lactic acid altered the physical characteristics of DP3 negatively. Finally, after 22 months storage at −22 °C of freeze-dried cultures of mutant DP3 demonstrated an excellent stability by producing 117 g/l lactic acid at almost the same yield, and production rate (Table 3). However, the maximum growth rate returned to the level of the wild-type, but evidently this did not affect the lactic acid production rate. On the other hand, subculturing the mutant in MRS medium resulted in a loss of stability of lactic acid production of the mutants after about six transfers, probably, because of the DNA repair mechanism in the cell during repeated unstressed growth.

CONCLUSION

To our knowledge this is the first report of a successful strain development protocol to increase lactic acid production by *Lactobacillus* sp. Stable enhanced mutants were obtained rapidly. Mutants, like mutant DP3, could be used to reduce lactic acid production costs because of the faster production rates, and higher levels attained. The proposed mutagenesis and selection protocol developed might have applications to enhance lactic acid production by other lactic acid bacteria particularly those not as fastidious as *L. delbrueckii*.

ACKNOWLEDGEMENTS

This research was supported by the Center for Crops Utilization Research, by a scholarship to Ali Demirci from the University of Gaziantep, Turkey, by the Iowa Corn Promotion Board, and by the Iowa Agriculture and Home Economics Experiment Station.

REFERENCES

1 Aeschlimann, A. and U. von Stockar. 1990. The effect of yeast extract supplementation on the production of lactic acid from whey permeate by *Lactobacillus helveticus*. Appl. Microbiol. Biotechnol. 32: 398–402.

2 Cheng, P., R.E. Mueller, S. Jaeger, R. Bajbai and E.L. Iannotti. 1991. Lactic acid production from enzyme-thinned corn starch using *Lactobacillus amylovorus*. J. Ind. Microbiol. 7: 27–34.

3 Hince, T.A. and S. Neale. 1977. Physiological modification of alkylating agent induced mutagenesis. II. Influence of the numbers of chromosome replicating forks and gene copies on the frequency of mutations induced in *Escherichia coli*. Mutat. Res 43: 11–24.

4 Holten, C.H., A. Muller and D. Rehbinder. 1971. Properties and Chemistry of Lactic Acid and Derivatives. Verlag Chemie Gmbh, Weinheim/Bergstr.

5 Lipinsky, E.S. and R.G. Sinclair. 1986. Is lactic acid a commodity chemical? Chem. Eng. Prog. August: 26–32.

6 Matijasavic, Z. and E. Zeiger. 1988. DNA binding and mutagenicity of ethyl methanesulfonate in wild-type and uvrB cells of *Salmonella typhimurium*. Mutat. Res. 198: 1–8.

7 Ohleyer, E., H.W. Blanch and C.R. Wilke. 1985. Continuous production of lactic acid in a cell recycle reactor. Appl. Biochem. Biotechnol. 11: 317–331.

8 Roy, T.B.V., H.W. Blanch and C.R. Wilke. 1982. Lactic acid production by *Lactobacillus delbrueckii* in a hollow fiber fermenter. Biotechnol. Lett. 8: 483–488.

9 Shetty, K., D.L. Crawford and A.L. Pometto A.L. 1986. Production of L-phenylalanine from starch by analog-resistant mutants of *Bacillus polymyxa*. Appl. Environ. Microbiol. 52: 637–643.

10 Singer, B. 1974. The chemical effects of nucleic acid alkylation and their relation to mutagenesis and carcinogenesis. Prog. Nucleic Acid Res. Mol. Biol. 15: 219–284.

11 van Zeeland, A.A. 1978. Post-treatment with caffeine and the induction of gene mutations by ultraviolet irradiation and ethyl methanesulphonate in V-79 Chinese hamster cells in culture. Mutat. Res. 50: 145–151.

12 Vickroy, T.B. 1985. Lactic acid. In: Comprehensive Biotechnology: the Principles, Applications, and Regulations of Biotechnology in Industry, Agriculture and Medicine. Vol. 2. (M. Moo-Young, ed.), pp. 761–776, Pergamon Press, New York.

13 Yabannavar, V.M. and D.I.C. Wang. 1991. Analysis of mass transfer for immobilized cells in an extractive lactic acid fermentation. Biotechnol. Bioeng. 37: 544–550.

14 Zayed, G. and A.S. Zahran. 1991. Lactic acid production from salt whey using free and agar immobilized cells. Lett. Appl. Microbiol. 12: 241–243.