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Carob Pod: A New Substrate for Citric Acid Production by *Aspergillus niger*

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ABSTRACT

The production of citric acid from carob pod extract by *A. niger* in surface fermentation was investigated. A maximum citric acid concentration (85.5 g/L), citric acid productivity (4.07 g/L/d), specific citric acid production rate (0.18 g/g/d), and specific sugar uptake rate (0.358 g/g/d) was achieved at an initial sugar concentration of 200 g/L, pH of 6.5, and a temperature of 30°C. Other kinetic parameters, namely, citric acid yield, biomass yield, specific biomass production rate, and fermentation efficiency were maximum at pH 6.5, temperature 30°C, and initial sugar concentration 100 g/L. The external addition of methanol into the carob pod extract at a concentration up to 4% (v/v) improved the production of citric acid.

Index Entries: Carob pod extract; kinetics of citric acid production; *Aspergillus niger;* surface fermentation.

INTRODUCTION

The carob pod is the fruit of the carob tree *(Ceratonia siliqua),* which is chiefly cultivated in the Mediterranean countries, and in many areas of North America. The annual production is about 340,000–400,000 metric t. Greece is a primary producer, with an annual harvest of 21,000 t *(1).* From the utilization viewpoint, two parts can be distinguished in the pod: the kibble, or locust bean; and the seeds, or locust kernel gum, a galactomannan that is highly valued in the food, textile, and cosmetic industries. The carob kibble contains the following (expressed as g/100g of kibble), 10–15 moisture; 40–50 total sugars (glucose, fructose, sucrose, and maltose); 3–4 protein; 1–2 pectin (7 cellulose; 5 hemicellulose; 20 phenolic compounds; 0.5–1.0 fat; and 2–3 ash *(1)*. Before the twentieth century, the carob pods were exclusively used as animal fodder, and also for human consumption. In more recent years, most carob pods were still being used in animal feeds; several applications of the kibble are in use. It is used in the preparation of antiarrheic and antiemetic products, pastry baking, and as cocoa substitution. *(2)* Because of the high concentration of sugars in the carob kibble, it is important to develop new and more attractive uses of these sugars.

Citric acid, a tricarboxylic acid, is used in the pharmaceutical, food, and beverage industries as an acidifying and flavor-enhancing agent, and is chiefly produced from beet and cane molasses by surface and submerged fermentation using *A. niger (3–6)*. In past years, a considerable interest has been shown in using agricultural products and their wastes, such as date, maize, citrus and kiwifruit peel, apple and grape pomace, and pineapple, mandarin orange, and brewery wastes for citric acid production by *A. niger (7–15)*. Carob trees have many distinct advantages over traditional crops, such as high carbohydrate yield, good growth in poor soil under favorable dry-farming conditions, and high tolerance to various plant diseases *(16)*. The value of carob pods is $135/t. Although the kernels represent only approx 10% of the weight of the pod, they contribute more than 60% of the pod market price *(16)*. For this reason, the carob kibble can be used as a cheap carbohydrate source for citric acid production. The production of citric acid from carob pod extract by *A. niger* has not been investigated.

The aim of this investigation was to examine the potential of carob pod as a source of citric acid production by *A. niger,* as well as to study the effects of various fermentation parameters, such as initial sugar concentration, pH, temperature, and methanol concentration, on kinetic parameters of carob-pod-extract fermentation.

MATERIALS AND METHODS

Microorganism

A. niger ATCC 9142 (American Type Culture Collection, Rockville, MD) was used throughout this investigation. It was maintained on potato dextrose agar slants at 4°C, and subcultured in intervals from 1–2 mo.

Inoculum

The cultures were incubated on potato dextrose agar slants at 30°C for 5 d. The spores obtained were suspended in 5 mL sterile distilled water, to prepare the inoculum.

Fermentation Medium

Carob pods (cultivar Tylliria) were obtained from the local market. After removing the seeds, kibble was chopped into small particles 0.3–0.6 cm in diameter. Forty-five g of particles were mixed with 180 mL distilled water (solid/liquid ratio 1:4), and the mixture was shaken on a rotary shaker/incubator (Lab-line Orbit-Environ shaker, Lab-Line Instruments, Melrose Park, IL), at 250 rpm for 2 h at 70°C, in order to extract the sugars from the kibble. The extract was centrifuged at 4000*g* for 15 min, and the supernatant (production medium) was used for the production of citric acid by *A. niger,* using surface fermentation.

Study of Fermentation Parameters

Initial Sugar Concentration

The carob pod extract, containing 12% total sugars, was diluted with distilled water, or concentrated at 50°C under vacuum, in order to contain 10, 15, 20, and 25% initial sugars. The pH of the extract was adjusted to 6.5 with 1 *N* NaOH, and the solution was sterilized at 121°C for 15 min. A set of 500-mL conical flasks, containing 100 mL production medium were inoculated with 1 mL of the inoculum, to give a final concentration of approx 1.0×10^6 spores/mL. The flasks were incubated at 30°C as surface fermentation for 21 d.

Initial pH

A series of conical flasks containing 100 mL production medium (20% initial sugars) at different initial pH (3.5, 4.5, 5.5, and 6.5) were incubated at 30°C for 21 d.

Temperature

A set of conical flasks containing 100 mL production medium (20% initial sugars, pH 6.5) were incubated at different temperatures (25, 30, 35, and 40°C) for 21 d.

Methanol Concentration

A series of conical flasks containing 100 mL sterilized carob pod extract (20% initial sugars, pH 6.5), at different methanol concentrations (1, 2, 3, 4, and 5%, v/v), were incubated at 30°C for 21 d. Methanol was added on the third day of fermentation *(17).*

Analytical Techniques

At appropriate time intervals, fermentation flasks were removed, and the contents analyzed. Citric acid was determined by the method of

Saffran and Denstedt *(18)*, using a Zeiss PMQII spectrophotometer. Mycelial dry wt was determined by filtering, washing with distilled water, and drying at 105°C to constant weight. Residual sugars (glucose, fructose, sucrose, and maltose) were determined as glucose by the method of Dubois and coworkers *(19)*. pH was measured using a knick 646 pH meter equipped with a glass electrode.

Each experiment was repeated 3× and the results were reported as averages of three repetitions. Statistical evaluation of the data was carried out through analysis of variance, using the randomized block design. Comparison of the means was assessed using the LSD-test.

RESULTS AND DISCUSSION

Citric Acid Production via Surface Fermentation

The production of citric acid from carob pod extract (initial sugars 20%, pH 6.5) by *A. niger* in surface fermentation is shown in Fig. 1. The concentration of citric acid increased with the increase of fermentation time. The maximum citric acid concentration (85.5 g/L) was obtained after 21 d of fermentation, and then declined on 24 d. The decline in concentration of citric acid may have been caused by a decay in the enzyme system responsible for the production of citric acid upon exhaustion of the fermentable sugars *(20)*. Morikawa and coworkers *(21)* and Papagianni and coworkers *(22)* found that a maximum citric acid concentration (67.6 and 125 g/L) was obtained when *A. niger* was grown in synthetic medium in surface and submerged culture, respectively. Roukas and Alichanidis *(3)* and Berovic and coworkers *(23)* found that maximal citric acid concentrations of 65 and 100 g/L were obtained when *A. niger* was grown in beet molasses in surface culture and stirred-tank fermentor, respectively. Al-Obaidi and Berry *(7)* reported that a high concentration of citric acid (85 g/L) was obtained when *A. niger* was grown in date syrup using submerged culture. There are some possible reasons for these differences, including the strain of organism used, the chemical composition of the substrate, the fermentation system, and, generally, the conditions under which the fermentation takes place.

The biomass dry wt followed a pattern similar to citric acid concentration, with maximum biomass concentration observed at the same time as the maximum concentration of citric acid was observed (Fig. 1). The highest mycelial dry wt (22.5 g/L) was obtained after 21 d of fermentation, and then remained constant.

The pH decreased during fermentation (Fig. 1). This was caused by the citric acid production during fermentation of sugars. The lowest value of pH was accompanied by the greatest concentration of citric acid, then

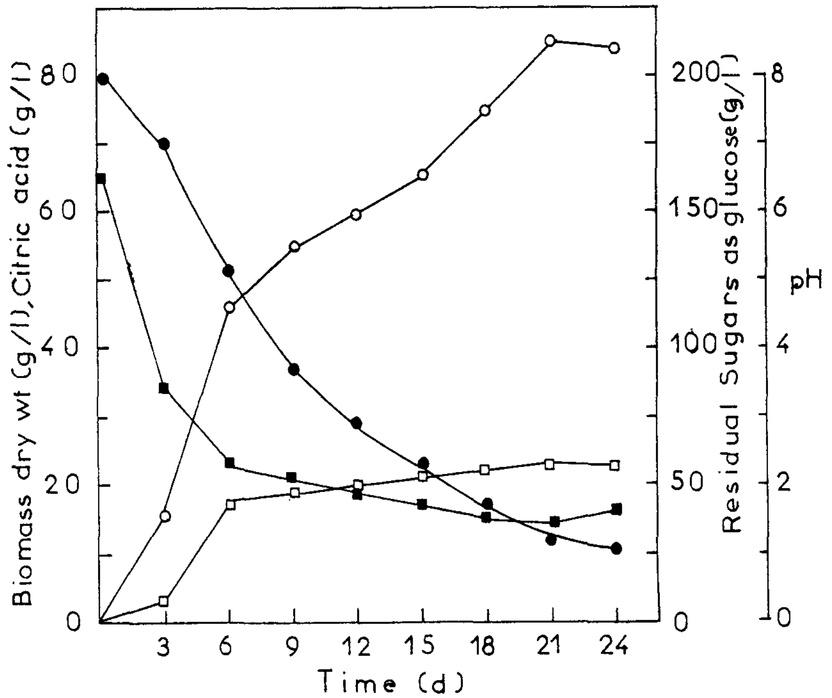

Fig. 1. Fermentation kinetics of *A. niger* during citric acid production from carob pod extract in surface fermentation. ○, citric acid; □, biomass dry wt; ●, residual sugars as glucose; ■, pH. Each point is the mean of three repetitions.

the pH value increased slightly because of oxidation of citric acid by the fungus *(24)*.

The concentration of residual sugars decreased during the fermentation, coinciding with an increase in biomass and citric acid production (Fig. 1). The concentration of residual sugars decreased rapidly during the first 6 d of fermentation, and kept decreasing at a slow rate, until it reached a minimum sugar level (30 g/L) after 21 d of incubation, because of the rapid increase of biomass and citric acid concentration observed at the same time. When the maximum concentration of citric acid was achieved, 50.5% of sugars consumed were converted to citric acid; the total amount of utilized sugars was 84.7%.

Effect of Initial Sugars

The effect of initial sugar concentration on kinetic parameters of carob pod extract fermentation is shown in Table 1. The citric acid concentration increased with the increase of initial sugar concentration, up to 200 g/L but decreased beyond this value. The highest concentration of citric acid (85.5 g/L) was obtained in culture grown at initial sugar concentration

Table 1
Kinetic Parameters of Carob-Pod-Extract Fermentation by *A. niger* at Different Sugar Concentrations

Kinetic parameters	Initial sugar concentration (g/L) 100	150	200	250
Citric acid concentration, p(g/L)	65.5[a]	70.0[b]	85.5[c]	76.5[d]
Citric acid productivity, R(g/L/d)	3.11[a]	3.33[b]	4.07[c]	3.64[d]
Citric acid yield, Yp/s (g citric acid/ g sugar utilized)	0.73[a]	0.56[b]	0.50[c]	0.41[d]
Specific citric acid production rate, q_b (g citric acid g biomass/dry wt/d)	0.164[a]	0.158[a]	0.180[b]	0.140[c]
Biomass dry wt, X(g/L)	19.0[a]	21.0[b]	22.5[c]	26.0[d]
Biomass yield, $Y_{x/s}$ (g biomass dry wt/ g sugar utilized)	0.212[a]	0.168[b]	0.132[c]	0.140[d]
Specific biomass production rate, q_x (g biomass dry wt/g sugar utilized/d)	0.010[a]	0.008[b]	0.006[c]	0.006[c]
Specific sugar uptake rate, q_a (g sugar/ g biomass dry wt/d)	0.224[a]	0.283[b]	0.358[c]	0.338[d]
Fermentation efficiency, f_e (g sugar utilized/100 g initial sugar)	89.5[a]	83.3[b]	84.7[b]	74.0[c]

Values are reported at 21 d fermentation. Means with different letters in the same line are significantly different at the 5% level by the LSD-test.

of 200 g/L; in cultures grown at initial sugar concentration of 100, 150, and 250 g/L, the citric acid concentration was lower by 23.4, 18, and 10.5%, respectively. Jernejc and coworkers *(25)* and Honecker and coworkers *(26)*, who studied the effect of initial sugar concentration on citric acid production from synthetic medium by *A. niger*, found that maximum citric acid concentrations of 85 and 50 g/L were achieved when *A. niger* was grown at initial sugar concentrations of 140 and 160 g/L, respectively. There were significant differences (at the 5% level) in citric acid concentration, citric acid productivity, and citric acid yield between cultures grown at initial sugar concentrations of 100, 150, 200, and 250 g/L. The maximum citric acid productivity (4.07 g/L/d) was obtained in culture grown at an initial sugar concentration of 200 g/L. The citric acid yield decreased with the increase of sugar concentration from 100 to 250 g/L. The highest citric acid yield (0.73 g/g) was obtained with an initial sugar concentration of 100 g/L. As shown in Table 1, increasing the initial sugar concentration from 100 to 250 g/L significantly affected (at the 5% level) the biomass dry w, the biomass yield, and the specific sugar uptake rate. The highest values of biomass dry wt (26 g/L), biomass yield (0.212 g/g), and specific sugar uptake rate (0.358 g/g/d) were obtained in cultures grown at initial sugar concentration's of 250, 100 and 200 g/L, respectively. There were no significant differences (at the 5% level) between cultures grown at initial sugar concentration's of 100 and 150 g/L, 200 and 250 g/L, and 150 and 200 g/L in terms of specific citric acid production rate, specific biomass production rate, and fermentation efficiency, respectively. The cultures grown at initial sugar concentration's of 100 and 200 g/L resulted in a better specific biomass production rate, fermentation efficiency, and specific citric acid production rate, respectively, compared to the other sugar concentration. The fermentation efficiency remained almost unaffected by initial sugar concentrations of 150 and 200 g/L, but decreased significantly as the initial sugar concentration was increased from 200 to 250 g/L. The decreased efficiency encountered with the highest concentration treatment is probably a result of osmotic effects. It has been reported that, above a critical substrate concentration, the decreased water activity and the onset of plasmolysis combine to cause a decrease in the rates of fermentation and product concentration *(27)*. The culture grown at initial sugar concentrations of 100, 150, 200, and 250 g/L utilized 89.5, 83.3, 84.7, and 74% of the sugars, respectively.

Effect of Initial pH

One important factor that affects the performance of carob-pod-extract fermentation is the initial pH of substrate. The purpose of this experiment was to determine the optimum initial pH of carod pod extract that would result in the highest citric acid concentration. As shown in Table 2, the citric acid concentration, citric acid productivity, citric acid yield, and specific citric acid production rate were increased with the increase of initial pH from 3.5 to 6.5. The highest values of the above fermentation parameters were achieved at an initial pH of 6.5. These results agree with those of Roukas and Alichanidis *(28)*, who studied the effect of initial pH on citric acid production from beet molasses by surface fermentation. At pH 3.5, the kinetic parameters, except biomass dry wt, were significantly different (at the 5% level), in comparison to the other pH values. There were no significant differences (at the 5% level) in citric acid concentration, citric acid productivity, and specific citric acid production rate, between cultures grown at pH 4.5 and 5.5; the biomass yield, the specific biomass production rate, the specific sugar uptake rate, and the fermentation efficiency were not significantly different among pH 4.5, 5.5, and 6.5. The biomass dry wt remained constant over the pH range 3.5–6.5. The maximum biomass dry wt (22.7 g/L) and fermentation efficiency (84.8%) were obtained in cultures grown at pH 4.5. Generally, the results showed that the optimum pH for citric acid concentration, citric acid productivity, citric acid yield, and specific citric acid production rate was 6.5.

Table 2
Kinetic Parameters of Carod-Pod-Extract Fermentation by *A. niger* at Different pH Values

Kinetic parameters	pH 3.5	pH 4.5	pH 5.5	pH 6.5
Citric acid concentration, P(g/L)	63.4[a]	77.5[b]	80.0[b]	85.5[c]
Citric acid productivity R(g/L/d)	3.01[a]	3.69[b]	3.80[b]	4.07[c]
Citric acid yield, $Y_{p/s}$ (g citric acid/ g sugar utilized)	0.42[a]	0.45[b]	0.48[c]	0.50[c]
Specific citric acid production rate, q_p (g citric acid/g biomass dry wt/d)	0.137[a]	0.162[b]	0.170[b]	0.180[c]
Biomass dry wt, x(g/L)	22.0[a]	22.7[a]	22.4[a]	22.5[a]
Biomass yield, $Y_{x/s}$ (g biomass dry wt/ g sugar utilized)	0.147[a]	0.133[b]	0.136[b]	0.132[b]
Specific biomass production rate, q_x (g biomass dry wt/g sugar utilized/d)	0.007[a]	0.006[b]	0.006[b]	0.006[b]
Specific sugar uptake rate, q_s(g sugar/ g biomass dry wt/d)	0.322[a]	0.355[b]	0.350[b]	0.358[b]
Fermentation efficiency, f_e (g sugar utilized/100 g initial sugar)	74.6[a]	84.8[b]	82.3[b]	84.7[b]

Values are reported at 21 d fermentation. Means with different letters in the same line are significantly different at the 5% level by the LSD-test.

Effect of Temperature

The effect of temperature on kinetic parameters of carob-pod-extract fermentation is shown in Table 3. There were no significant differences (at the 5% level) between cultures grown at 25 and 40°C in terms of citric acid concentration and citric acid productivity. No significant differences (at the 5% level) were noted in biomass yield, specific biomass production rate, and specific sugar uptake rate among cultures grown at 25 and 30°C. On the other hand, increasing the fermentation temperature from 25 to 40°C significantly affected (at the 5% level) specific citric acid production rate and biomass dry wt. The citric acid concentration, citric acid productivity, citric acid yield, specific citric acid production rate, and specific sugar uptake rate increased with the increase of fermentation temperature from 25 to 30°C, and decreased above 30°C. The maximal citric acid concentration (85.5 g/L), citric acid productivity (4.07 g/l/d), citric acid yield (0.5 g/g), specific citric acid production rate (0.18 g/g/d), and specific sugar uptake rate (0.358 g/g/d) were obtained in culture grown at 30°C. Rokosu and Anenih *(29)* studied the effect of temperature on citric acid production from molasses by *A. niger*, and found that the maximum citric acid concentration was obtained at a fermentation temperature of 28°C. Other

Table 3
Kinetic Parameters of Carob-Pod-Extract Fermentation by *A. niger* at Different Temperatures

	Temperature (°C)			
Kinetic parameters	25	30	35	40
Citric acid concentration, P (g/L)	67.7[a]	85.5[b]	76.6[c]	70.0[a]
Citric acid productivity, R (g/L/d)	3.22[a]	4.07[b]	3.64[c]	3.33[a]
Citric acid yield, Yp/s (g citric acid/ g sugar utilized)	0.45[a]	0.50[b]	0.46[a]	0.39[c]
Specific citric acid production rate, q_p (g citric acid/g biomass dry wt/d)	0.157[a]	0.180[b]	0.140[c]	0.111[d]
Biomass dry wt, x(g/L)	20.5[a]	22.5[b]	26.0[c]	30.0[d]
Biomass yield, Yx/s (g biomass dry wt/g sugar utilized)	0.138[a]	0.132[a]	0.157[b]	0.170[c]
Specific biomass production rate, q_x (g biomass dry wt/g sugar utilized/d)	0.006[a]	0.006[a]	0.007[b]	0.008[c]
Specific sugar uptake rate, q_s (g sugar/g biomass dry wt/d)	0.343[a]	0.358[a]	0.303[b]	0.279[c]
Fermentation efficiency, f_e (g sugar utilized/100 g initial sugar)	74.0[a]	84.7[bc]	82.7[c]	88.0[b]

Values are reported at 21 d fermentation. Means with different letters in the same line are significantly different at the 5% level by the LSD-test.

kinetic parameters, namely, biomass dry wt, biomass yield, specific biomass production rate, and fermentation efficiency, were maximum at 40°C. At 25°C, the fermentation efficiency was significantly different (at the 5% level), in comparison to the other temperature values. The cultures grown at fermentation temperatures 25, 30, 35, and 40°C utilized 74, 84.7, 82.7, and 88% of the sugars, respectively.

Effect of Methanol

As shown in Fig. 2, the citric acid concentration and the citric acid yield increased with the increase of methanol concentration from 1 to 4% (v/v), and decreased as the methanol concentration was increased beyond 4%. The highest values of citric acid concentration (120 g/L) and the citric acid yield (70%) were obtained in the presence of methanol at a concentration of 4% (v/v). Hang and coworkers *(30)* and Roukas and Kotzekidou *(17)* reported that the addition of methanol at concentrations of 1–4% (v/v) resulted in a marked increase in the amount of citric acid formed by *A. niger* on spent grain liquor and brewery wastes, respectively. The observed increases in citric acid concentration show that methanol has a profound effect on the metabolism of sugars by *A. niger*. The mechanism

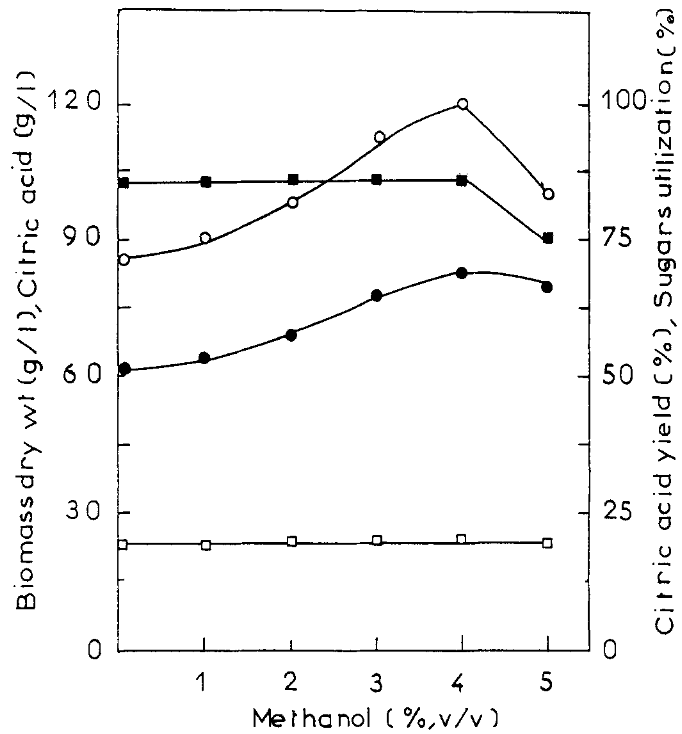

Fig. 2. Kinetic parameters of carod pod extract fermentation by *A. niger* at different methanol concentrations. ○, citric acid; □, biomass dry wt; ●, citric acid yield; ■, sugar utilization. Each point is the mean of three repetitions. Values are reported at 21 d fermentation.

by which methanol stimulates citric acid production from sugars is not clear. Maddox and coworkers *(31)* reported that the effect of methanol is at the cell-permeability level, allowing citrate to be excreted from the cell; the cell then responds by increasing its citrate production, via repression of 2-oxoglutarate dehydrogenase, in an attempt to maintain an adequate intracellular level of the metabolite. The biomass dry wt remained almost constant, with the increase of methanol concentration from 1 to 5%. The sugar utilization remained practically constant, with the increase of methanol amounting to 4%, and decreased beyond this value. This was caused by a rapid decrease of citric acid concentration, observed at the same time (Fig. 2). The maximum biomass dry wt (24 g/L) and sugar utilization (86.5%) were obtained at a methanol concentration of 4% (v/v).

CONCLUSIONS

The results showed some important aspects of citric acid production from carob pod extract by *A. niger*. The optimum conditions for carob-pod-extract fermentation were pH 6.5, temperature 30°C, and initial sugar concentration of 200 g/L. The addition of methanol at concentrations up to 4% (v/v) resulted in a marked increase in the citric acid concentration. The carob pod extract was an attractive medium for the production of citric acid by *A. niger.*

REFERENCES

1. Roukas, T. (1993), *Food Biotechnol.* **7,** 159–176.
2. Calixto, F. S. and Canellas, J. (1982), *J. Sci. Food Agric.* **33,** 1319–1323.
3. Roukas, T. and Alichanidis, E. (1991), *J. Ind. Microbiol.* **7,** 71–74.
4. Roukas, T. (1991), *J. Ind. Microbiol.* **7,** 221–226.
5. Hamissa, F. A. and Radwan, A. J. (1977), *Gen. Appl. Microbiol.* **23,** 325–329.
6. Kundu, S., Panda, J., Majumdar, S. K., Guha, B., and Bandyopadhyay, K. (1984), *Biotechnol. Bioeng.* **26,** 1114–1121.
7. Al-Obaidi, Z. and Berry, D. R. (1979), *Biotechnol. Lett.* **1,** 153–158.
8. Esuoso, K. O., Oderinde, R. A., and Okogun, J. I. (1991), *J. Ferment. Bioeng.* **71,** 200–202.
9. Rodriguez, J. A., Echevarria, J. Rodriguez, F. J., Sierra, N., Daniel, A., and Martinez, O. (1985), *Biotechnol. Lett.* **7,** 577–580.
10. Hang, Y. D., Luh, B. S., and Woodams, E. E. (1987), *J. Food Sci.* **52,** 226–227.
11. Hang, Y. D. and Woodams, E. E. (1984), *Biotechnol. Lett.* **6,** 763–764.
12. Hang, Y. D. and Woodams, E. E. (1985), *Biotechnol. Lett.* **7,** 253–254.
13. Tran, C. T. and Mitchell, D. A. (1995), *Biotechnol. Lett.* **17,** 1107–1110.
14. Kumagai, K., Usami, S., and Hattori, S. (1981), *Hakkokogaku* **59,** 461–464.
15. Roukas, T. and Kotzekidou, P. (1986), *J. Food Sci.* **51,** 225–226, 228.
16. Roukas, T. (1994), *Appl. Biochem. Biotechnol.* **44,** 49–64.
17. Roukas, T. and Kotzekidou, P. (1987), *Enzyme Microb. Technol.* **9,** 291–294.
18. Saffran, M. and Denstedt, J. (1948), *J. Biol. Chem.* **175,** 849–855.
19. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem.* **28,** 350–356.
20. Kristiansen, B. and Sinclair, C. (1979), *Biotechnol. Bioeng.* **21,** 297–315.
21. Morikawa, R. S., Kirimura, K., and Usami, S. (1995), *Appl. Microbiol. Biotechnol.* **42,** 839–843.
22. Papagianni, M., Mattey, M., and Kristiansen, B. (1994), *Biotechnol. Lett.* **16,** 929–934.
23. Berovic, M., Cimerman, A., Steiner, W., and Koloini, T. (1991), *Appl. Microbiol. Biotechnol.* **34,** 579–581.
24. Hang, Y. D., Splittstoesser, D. F., and Woodams, E. E. (1975), *Appl. Microbiol.* **30,** 879–880.
25. Jernejc, K., Cimerman, A., and Perdih, A. (1982), *Eur. J. Appl. Microbiol. Biotechnol.* **14,** 29–33.
26. Honecker, S., Bisping, B., Yang, Z., and Rehm, H. J. (1989), *Appl. Microbiol. Biotechnol.* **31,** 17–24.
27. Roukas, T., Lazarides, H. N., and Kotzekidou, P. (1991), *Milchwissenschaft* **46,** 438–441.
28. Roukas, T. and Alichanidis, E. (1988), *8th International Biotechnology Symposium, Abstract,* Paris, p. 218.
29. Rokosu, A. A. and Ahenih, C. A. (1980), *Enzyme Microb. Technol.* **2,** 61–62.
30. Hang, Y. D., Splittstoesser, D. F., Woodams, E. E., and Sherman, R. M. (1977), *J. Food Sci.* **42,** 383–384.
31. Maddox, I. S., Hossain, M., and Brooks, J. D. (1986), *Appl. Microbiol. Biotechnol.* **23,** 203–205.