©Copyright 1991 by The Humana Press Inc.
All rights of any nature whatsoever reserved.
0273-2289/91/3002—0203$02.80

Continuous Potable Alcohol Production by Immobilized *Saccharomyces cerevisiae* on Mineral Kissiris

A. A. KOUTINAS,*[1] C. GOURDOUPIS,[1] C. PSARIANOS,[1] A. KALIAFAS,[2] AND M. KANELLAKI[1]

[1]*Department of Chemistry, University of Patras, Patras, Greece; and* [2]*Department of Biology, University of Patras, Patras, Greece*

Received September 25, 1990; Accepted December 1, 1990

ABSTRACT

A biocatalyst prepared by the immobilization of *Saccharomyces cerevisiae* on the surface of the mineral kissiris was used in the present study for continuous potable-alcohol production. An ethanol productivity (calculated on the basis of liquid volume) of 10.5 g/L/h was obtained at a 0.7/h dilution rate, 121 g/L sucrose content, and 29.6% conversion employing molasse as feed material. Glucose, raisin extracts, and molasse were successively used as feed materials without stopping the operation of the reactor for 6 mo. The ethanol productivity and yield remained constant during the operational-stability study of the reactor, carried out for 44 d. Biomass productivity, yield, and free-cell concentration in glucose, raisin extracts, and molasse were examined. Finally, a system with two continuous reactors joined successively was also studied in the present investigation.

Index Entries: Kissiris, alcohol production; continuous fermentation; *Saccharomyces cerevisiae*.

INTRODUCTION

Continuous alcohol production can be obtained by the use of biocatalysts prepared after the immobilization of *Saccharomyces cerevisiae* or *Zymomonas mobilis* cells on a solid support. During the last 10 years, con-

*Author to whom all correspondence and reprint requests should be addressed.

siderable attention has been given to industrial development of the continuous alcohol-production process. This is because continuous alcohol production, as compared with batch and fed-batch processes, leads to the attainment of (i) high conversion of substrate to ethanol, (ii) higher ethanol productivity, (iii) elimination of the centrifuge separator for cell recycle, and (iv) reduction of biological and organic load in the wastewater of plants.

Several investigators in the recent past have reported continuous ethanol production mainly via immobilized *Z. mobilis* on organic supports (*1–5*) and on inorganic supports, such as vermiculite (*6*). The investigations mentioned above were carried out with the aim of producing fuel-grade ethanol. Recently, the use of supported γ-alumina biocatalyst, prepared by the immobilization of *Zymomonas mobilis* cells on γ-alumina pellets, for continuous potable alcohol production using raisin extracts was proposed (*7*).

Although a number of immobilization supports have been used for continuous alcohol production, alcohol is not yet produced by immobilized cells in industry because of the prerequisites for cost-effective immobilization:

1. High viability of immobilized cells;
2. Availability of a suitable low-cost support material; and
3. Abundance and accessibility of the material.

Recently, the abundant mineral kissiris was proposed for the immobilization of *Saccharomyces cerevisiae* in order to satisfy the above prerequisites (*8*) as well as for the promotion of molasses alcoholic fermentation (*9*).

The support employed in the present investigation is the mineral well-known in Greece as kissiris, elaphropetra, or Thiraiki gi. It is a volcanic rock usually formed by the foam thickening of volcanic lava and characterized as a natural glass foam with porosity and relatively high specific surface area. In Greece it occurs in the Aegean islands, Sandorini, Milos, and Nissiros, in a layer 30–50 m thick. Kissiris contains 70% SiO_2, 13% Al_2O_3, and other inorganic oxides (*8*). It is a cheap mineral material with a price not exceeding $40/tn. In contrast to γ-alumina pellets proposed for continuous potable-alcohol production (*7*), kissiris is a porous raw mineral; γ-alumina is prepared and converted to a porous solid after specific treatment, which increases its production cost.

Although the bacteria *Z. mobilis* ferments raisin extracts faster and is more alcohol-resistant than *Saccharomyces cerevisiae* (*10*), the latter microorganism is uniquely employed in the potable-alcohol production industry using raisin, molasse, or other related raw material. The attainment of immobilization of *Saccharomyces cerevisiae* on the surface of porous mineral kissiris (*8*) led to the study of this kissiris-supported biocatalyst in the continuous potable-alcohol production using molasse or raisin extract.

The aim of the present study was to investigate the possibility of cost-effective ethanol production by continuous potable-ethanol production by immobilized *Saccharomyces cerevisiae* on kissiris, using glucose, molasse, and raisin extracts to feed an upflow reactor system.

MATERIALS AND METHODS

Baker's yeast from ZAAE Co., Pireous, Greece was employed in the present study. Pressed wet weight cells (20 g) were used directly in the continuous fermentation process.

The continuous culture media in the case of glucose solutions contained 113 and 150 g/L glucose, 0.4% yeast extract; 0.1% $(NH_4)_2SO_4$; 0.1% KH_2PO_4; and 0.5 $MgSO_4 \cdot 7H_2O$ in distilled water. These complete media were sterilized at 130°C. The reactor temperature was controlled at 30°C by placing the reactor in a constant-temperature water bath. The initial pH was adjusted to 5.6.

Greek sugar-beet molasse was used. The rarified products were prepared by the addition of tap water to obtain the appropriate °Be density. Also, these solutions contained 121, 116.8, and 115 g/L sucrose. The pH was adjusted to 4.7 by the addition of sulfuric acid, and 0.5 g/L KH_2PO_4 was added as nutrient. All liquids were sterilized at 130°C.

Raisin extracts were prepared as follows: Raisins (variety trechumena) and tap water were placed in a conical flask and extracted at 72°C for 4 h. The desired invert-sugar content was obtained by the addition of tap water. The initial pH was adjusted to 3.2 by the addition of sulfuric acid. Extracts were used as continuous media without nutrient addition or sterilization. For immobilization, mineral kissiris was used (*8*).

Biomass was determined using the absorbance experimental procedure (*3,5*). Wet free-cell concentrations are given in g wet wt/L and were determined using standard curves. Dry cell concentrations were estimated at 30% of wet weight. Residual sugar estimations were made with the anthrone test (*11*).

The ethanol concentrations were analyzed using a gas chromatograph (Varian 1400), with Porapac S as column material, nitrogen as carrier gas (40 mL/min), 210°C injector temperature, column temperature programming 120–152°C, and 220°C detector temperature.

Pilot Plant

The experimental apparatus, both with a continuous reactor and with two successively connected continuous reactors, is shown in Fig. 1. The reactors were glass tower reactors (1060 mL total working vol and 700 mL liquid vol when a glass tower reactor was used and twofold total working and liquid vol were employed).

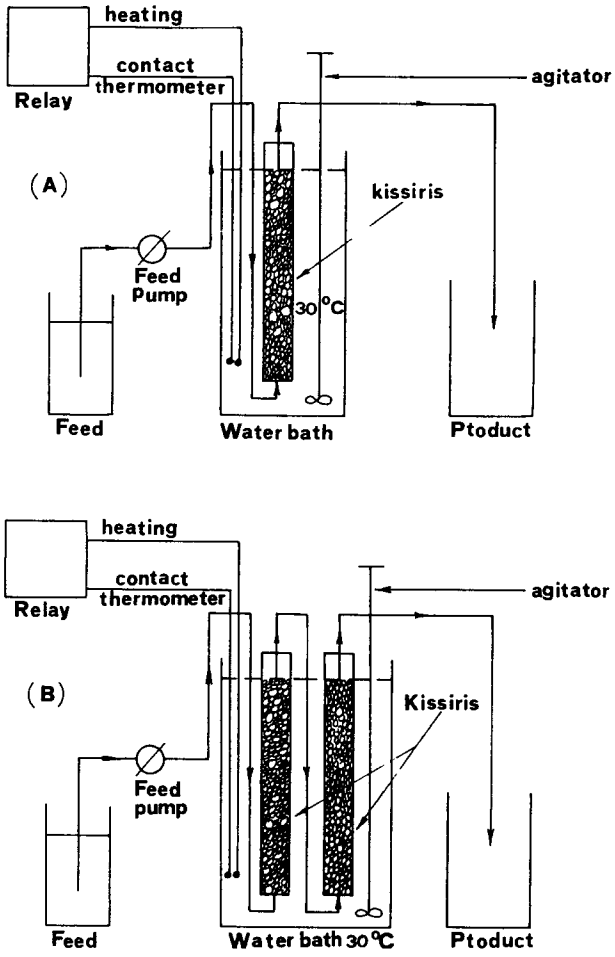

Fig. 1. Pilot plant operations for continuous ethanol production by immobilized *S. cerevisiae* on mineral kissiris. (A) Using one reactor, (b) two reactors connected successively.

Pieces of mineral kissiris (500 g) were placed in each of the reactors and used as the immobilization support for continuous operation. The continuous-fermentation medium was pumped in an upflow stream with the aid of a high-accuracy peristaltic pump (Cole Parmer Instrument Co., Chicago, IL).

Experimental Procedure

In the case in which an upflow reactor was used, the experiments were performed in the order of Tables 1–3, using successively glucose, molasse, and raisin extract. Cells were immobilized as follows: The glass tower fermenter was charged with 700 mL of culture media containing 113 g/L glucose and 20 g/L pressed baker's yeast *Saccharomyces cerevisiae*. The pH was adjusted to 4.7 and the mixed culture was allowed to ferment

Table 1
Results of Continuous Ethanol Production by Immobilized
S. cerevisiae on Mineral Kissiris, Using Glucose and One Reactor System

Dilution rate (h^{-1})	Test Duration (h)	Feed conc (g/L)	Residual sugar (g/L)	Ethanol conc (g/L)	Ethanol productivity (g/L/h)	conversion (%)	Ethanol Yield factor (g/g)
0.08	48	113	-	39	3.1	-	-
0.10	48	113	23.7	37	3.7	79.1	0.41
0.25	24	113	79.8	14	3.4	29.4	0.41
0.53	24	113	90.3	10	5.1	20.1	0.42
1.00	24	113	102.2	6	5.5	9.6	0.51
0.11	48	150	91.9	25	2.7	38.7	0.43
0.25	24	150	111.2	15	3.8	25.8	0.39
0.54	24	150	130.8	8	4.1	12.8	0.42

Table 2
Results of Continuous Ethanol Production by Immobilized
S. *cerevisiae* on Mineral Kissiris, Using Sugar Beet Molasse and One Reactor System

Dilution rate (h^{-1})	Test Duration (h)	Feed conc (g/L)	Residual sugar (g/L)	Ethanol conc (g/L)	Ethanol productivity (g/L/h)	conversion (%)	Ethanol Yield factor (g/g)
0.05	48	121	60.1	25	1.3	50.3	0.41
0.08	48	121	-	20	1.7	-	-
0.25	24	121	-	19	4.6	-	-
0.70	24	121	85.2	15	10.5	29.6	0.42
1.00	24	121	-	5	4.7	-	-

Table 3
Results of Continuous Ethanol Production by Immobilized
S. cerevisiae on Mineral Kissiris, Using Raisin Extracts and One Reactor System

Dilution rate (h^{-1})	Test duration (h)	Feed conc (g/L)	Residual sugar (g/L)	Ethanol conc (g/L)	Ethanol productivity (g/L/h)	conversion (%)	Ethanol Yield factor
0.13	48	117.5	69.0	20	2.5	41.3	0.41
0.27	24	117.5	76.3	18	4.9	35.1	0.43
0.55	24	117.5	95.5	9	5.1	18.7	0.42
0.13	48	171.5	100.2	28	3.6	41.6	0.40
0.26	24	171.5	137.4	14	3.5	19.9	0.40
0.52	24	171.5		7	3.8		-

without feeding. After 4 h, feed having a pH of 5.6 and 113 g/L of glucose was pumped at a dilution rate of 0.08/h. The fermenter was operated continuously for 240 h for biomass attachment. Subsequently, samples were collected in a dilution-rate range of 0.08–1.00/h. The column was pumped with synthetic media containing glucose solution for 25 d. The experiments were made in the order presented in Table 1. After that, we started to feed molasse, and the reactor was operated in that way for 32 d. Samples were collected in a dilution-rate range of 0.09–1.00/h. The results were obtained in the order presented in Table 2. Then the reactor was operated for 50 d with raisin extracts in a dilution-rate range of 0.13–0.52/h. In this case the experiments were made in the order presented in Table 3. For the operational-stability study, the reactor was fed for 22 d with raisin extract and for 22 d with molasse solution, using a dilution rate of 0.26/h. The results are shown in Fig. 2. All measurements were made at the steady state. Samples were collected at each dilution rate and analyzed for residual sugar biomass and ethanol.

For the two-reactor system, the operation for biomass attachment was repeated and the reactors fed with synthetic medium containing glucose for 50 d and with molasse for 15 d. Finally, the experiments were made in the order presented in Table 4.

Dilution rates wer calculated by dividing the flowrate of liquid by the liquid volume of the fermenter. The ethanol yield factor was the ratio of g ethanol/g utilized sugar in the fermentation. Furthermore, the ethanol productivity was expressed in g ethanol/L produced in 1 h and calculated on the basis of liquid volume by multiplying the dilution rate by ethanol concentration. Biomass productivity was g dry wt/L produced in 1 h. The biomass yield factor was the ratio of g dry wt/g of utilized sugar. Conversion percentage was calculated using the equation

$$(\text{Feed conc.} - \text{residual sugar})\ 100/\text{feed conc.}$$

RESULTS

Baker's yeast *Saccharomyes cerevisiae* and pieces of kissiris were mixed in the synthetic medium containing glucose with a pH 4.7 to obtain the immobilization and so the kissiris-supported biocatalyst. This operation was done in a glass column and, after 4 h, it was pumped for a relatively long period of time at a low dilution rate to achieve better biomass attachment. Then the system was operated at a different glucose concentration. In the two glucose concentrations used, the one-reactor system was fed at different dilution rates, lower than 1/h. Then the system was operated using molasses containing 121 g/L sucrose. The reactor was also pumped with various dilution rates lower than 1/h. After that, raisin extracts containing 117.5 and 171.5 g/L invert sugar were supplied at dilution rates in

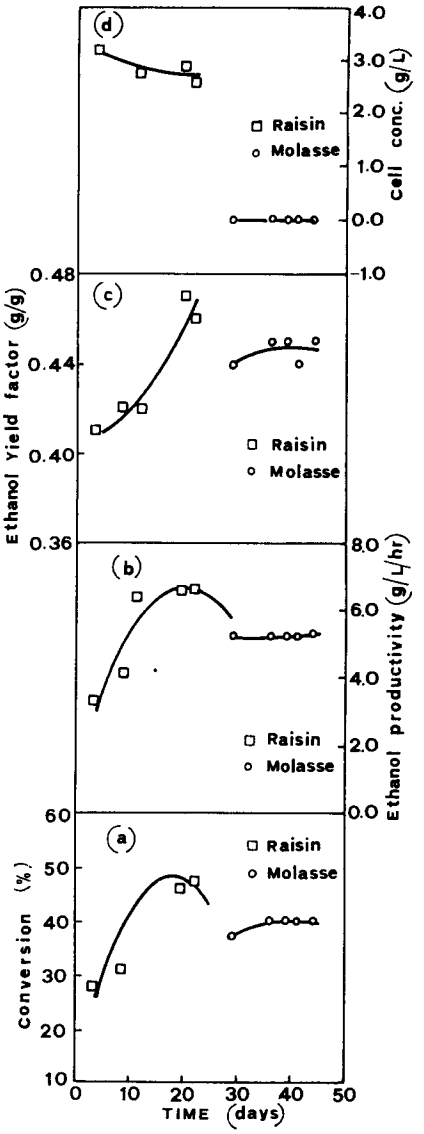

Fig. 2. Operational stability of the one-reactor system with mineral kissiris. The dilution rate was 0.26/h; feed concentration was 117 g/L sugar.

the range of 0.13–0.55/h. The results are summarized in Tables 1, 2, and 3 for glucose, molasse, and raisin extract, respectively.

In the case of glucose, ethanol productivities increase as the dilution rate increases at the values studied. Conversions were higher at the lower feed concentrations used. The ethanol productivity and conversion drop as the feed concentration increases from 113 to 150 g/L. The ethanol yield factor in most of the experiments was at an acceptable level. In the continuous alcoholic fermentation of molasse, the ethanol productivity increased

Table 4
Results of Continuous Ethanol Production by Immobilized
S. cerevisiae on Mineral Kissiris, Using Two Successively Connected Reactors

Substrate	Dilution rate (h^{-1})	Test duration (h)	Feed conc (g/L)	Residual sugar (g/L)	Ethanol conc (g/L)	Ethanol productivity (g/L/h)	conversion (%)	Ethanol Yield factor (g/g)
Glucose	0.07	48	113	6.0	43	2.9	94.7	0.40
	0.06	48	113	8.0	49	2.9	92.9	0.46
	0.13	24	113	48.5	32	4.1	57.1	0.50
	0.25	24	113	67.5	22	5.4	40.3	0.48
Glucose	0.06	48	150	47.0	51	3.2	68.7	0.49
	0.13	24	150	80.7	34	4.3	46.2	0.49
	0.26	24	150	121.2	14	3.5	19.2	0.48
Molasse	0.06	48	115	6.7	49	3.0	94.2	0.45
	0.13	24	115	19.8	43	5.6	82.8	0.45
	0.26	24	115	53.8	29	7.4	53.2	0.46

at dilution rates close to 0.7/h at which the higher ethanol productivity was obtained. At dilution rates higher than 0.7/h, the ethanol productivity fell. Likewise, in continuous ethanol fermentation with raisin extract, the ethanol productivity increases as the dilution rate approaches 0.55/h at a feed concentration of 117.5 g/L and it remains constant at 171.5 g/L.

The results of the operational-stability study are presented in Fig. 2. An inspection of this figure reveals that the reactor operated for a relatively long period with a stable ethanol productivity and conversion. Raisin extracts seem to give about 25% higher ethanol productivity than molasse. The ethanol yield factor was at an acceptable level. Free-cell concentration in a wet-wt basis was low in the case of raisin extract and near zero when molasses was pumped into the reactor.

When the system using two successively connected reactors was employed, the reactors were charged and the cells were immobilized as in the one-reactor system described. At first the reactors were supplied with synthetic media containing glucose (113 and 150 g/L) at dilution rates in the range of 0.06–0.26/h. Then they were pumped with molasse containing 115 g/L sucrose in a dilution-rate range of 0.06–0.26/h. The results are shown in Table 4.

In the similar feed concentration of glucose and molasse containing sucrose, the ethanol productivity increases as the dilution rate is augmented. Conversion was higher when the lower concentration was used and the ethanol yields were acceptable in all dilution rates and feed concentrations supplied.

In the one-reactor system, the biomass productivity, yield, and free-cell concentration obtained were lower in glucose solutions than in raisin extract (Fig. 3). Also, in the two-reactor system, the product stream contained higher wet free cell, and higher biomass productivity and yield were obtained in the case of glucose solutions.

When the reactor's systems were pumped with molasse, the product stream contained no free cells and the fermentation was carried out only by the immobilized (Figs. 3 and 4).

DISCUSSION

Molasse is a raw material used in potable-alcohol production in most countries of the world. Raisin is also used in Greece and some other countries in the ethanol-production industry. The raw materials molasse and raisin gave, in most cases studied, higher ethanol productivities than the synthetic media containing glucose. Although raisin extracts were pumped into the reactor system without nutrient addition, the wet free-cell concentration in the product stream was greater than in the synthetic media containing glucose. In contrast to raisin extract, no free cells were mea-

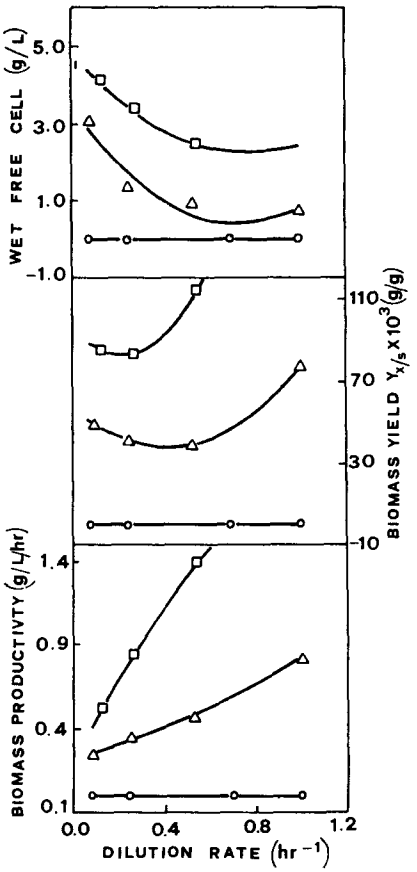

Fig. 3. Biomass productivity, yield (Yx/s) and free cells vs dilution rate using one reactor. △: glucose, 113 g/L; ○: molasse, 121 g/L sucrose; □: raisin extracts, 117.5 g/L invert sugar.

sured in molasse. This is an advantage for molasse, because it led to a diminution of the water pollution caused by the waste-water of the alcohol-production industry. Also, the free-cell concentration obtained in the product stream when raisin extracts were employed can be characterized as a low cell content in terms of water pollution.

The continuous alcohol produced using molasse without any free cells, by the kissiris-supported biocatalyst supports further the immobilization of *Saccharomyces cerevisiae* on kissiris as it was reported in a recent study (*8*).

In the case of continuous potable-alcohol production using the two successively joined reactors, the ethanol productivity was higher than was obtained by the one-reactor system. This has greater significance if we take into account that the ethanol concentration in the second reactor of the system with two reactors is larger than in the one-reactor system. The latter is in agreement with an observation made in a previous study con-

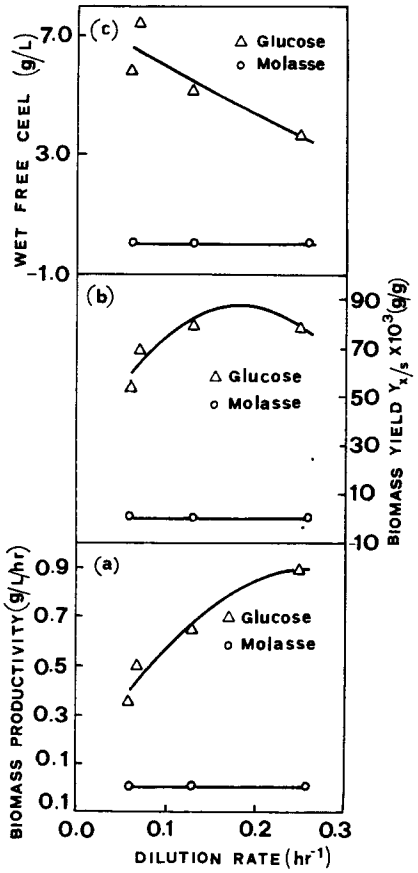

Fig. 4. Biomass productivity, yield (Yx/s) and free cells vs dilution rate, using two reactors connected successively.

cerning the fact that, in the presence of mineral kissiris, the fermentation is carried out at even higher alcohol concentrations than in its absence (*9*).

The operational-stability study led to the conclusion that the system can operate for a long period with constant ethanol productivity and yield. Also, the low cell concentration of the product stream contributed to a reduction of the biological and organic load of the wastewater and to a diminution of the water pollution.

The ethanol productivity obtained is fourfold higher than that achieved in the Greek potable-alcohol production industry. An inspection of Table 5 shows that the cost increase attributable to using kissiris as the immobilized support material in continuous potable-alcohol production using molasse is very low. The benefit obtained by the increase of productivity is evidently much higher, making kissiris an attractive support for potable-alcohol production. However, further research has to be done in order to increase ethanol productivity using kissiris as a support material.

Table 5
Cost Increase in Ethanol Production Due to Use of Kissiris as Immobilized Support Material in the Continuous Potable Alcohol Production Using Molasse

Time, using the same amount of kissiris	Cost of Support	
	$	$
(months)	m^3 reactor ·year	1000 l C_2H_5OH produced[b]
4	36[a]	0.70
8	18	0.35
12	12	0.23
16	9	0.17

[a]Calculated on the basis of $40/tn support.
[b]Calculated taking into account 0.3 tn support/m^3 reactor volume as well as a productivity in molasse 7 g ethanol/m^3/h.

ACKNOWLEDGMENTS

We acknowledge the financial support of this work by the Greek government (Organization EOMMEX).

REFERENCES

1. Margaritis, A. and Rowe, C. E. (1983), *Dev. Ind. Microbiol.* **24,** 329.
2. Jain, W. K., Toran Diaz, I., and Barattit, J. (1985), *Biotechnol. Bioeng.* **27,** 613.
3. Klein, J. and Kressdorf, B. (1983), *Biotechnol. Lett.* **5(8),** 497.
4. Krug, T. A. and Dangulis, A. S. (1983), *Biotechnol. Lett.* **5,** 159.
5. Bajpai, P. K. and Margaritis, A. (1986), *Biotechnol. Bioeng.* **28,** 824.
6. Bland, R. R., Chen, H. C., Jewell, W. J., Bellamy, W. D. and Zahl, R. R. (1982), *Biotechnol. Lett.* **4(5),** 323..
7. Koutinas, A. A. and Kanellaki, M. (1990), *J. Food Sci.* **55(2),** 525.
8. Kana, K., Kanellaki, M., Psarianos, C., and Koutinas, A. A. (1989), *J. Ferment. Bioeng.* **68(2),** 144.
9. Tsoutsas, T., Kanellaki, M., Psarianos, C., Kaliafas, A., and Koutinas, A. A. (1990), *J. Ferment Bioeng.* **69(2),** 93.
10. Koutinas, A. A., Kanellaki, M., Typas, M. A., and Drainas, C. (1986), *Biotechnol. Lett.* **8(7),** 517.
11. Scott, T. A. and Melvin, E. H. (1953), *Anal. Chem.* **25,** 1651.