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A Simultaneous Sucrose Bioconversion into Ethanol and Levan by *Zymomonas mobilis*

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INTRODUCTION

The interest of biotechnologists in the bacteria *Zymomonas mobilis* is mainly owing to the fact that these bacteria are more efficient than yeasts in converting glucose into ethanol. Very.high ethanol productivities have been achieved in bioreactors with immobilized cells, cell recycle, and flocculating strains (*1–3*).

Recently, it was demonstrated that *Z. mobilis* can be successfully used to obtain a number of other products of commercial interest: fructose (*4, 5*), sorbitol and gluconic acid (*6*), and fructose polysaccharide—levan (*7, 8*). Levan is used as a blood plasma substitute (*9*), a prolongator of the effect of medicinal drugs (*10*), a fructose source (*11*), and a specific immunomodulator. Several methods of levan production have been described for various bacteria: *Pseudomonas* (*12*), *Bacillus* (*13*), *Acetobacter* (*14*), and *Gluconobacter* (*15*).

In the present work, we studied the possibility of simultaneously obtaining ethanol and levan from sucrose, using *Z. mobilis 113*. Our aim was to create a laboratory system that would ensure an efficient conversion of sucrose into levan, ethanol, and CO_2, with an increased levan/ethanol ratio and a minimum production of other end products.

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METHODS

Microorganism and Maintenance

The culture of *Z. mobilis 113* (*16*) was maintained on a liquid medium and reseeded in each after 2 wk. The maintenance medium contained (g/L): KH_2PO_4, 2.5; $(NH_4)_2SO_4$, 1.6; $MgSO_4 \cdot 7H_2O$, 1.0; yeast extract (Olaine, USSR), 7.0; and sucrose, 100.0, at an initial pH 5.5. Cultivation was carried out at 30°C for 24 h. The culture was stored at 4°C. Population grown from one colony soon became heterogenous, with respect to the efficiency of levan production. The high levan-producing activity of the culture was maintained by a regular (every 2 wk) selection of the most productive clone plated on the abovementioned medium with 2% agar (Difco, Detroit, MI) and 4% sucrose). The colonies were grown at 30°C for 48–72 h. Colonies producing the biggest amounts of polysaccharide slime were selected and seeded on the liquid medium for maintenance.

Inoculate was grown at 25°C in flasks in a liquid medium of an analogous composition; except for the optimized variant, the cultivation temperature and the initial concentration of sucrose in the medium were 30°C and 50 g/L, respectively.

Medium and Cultivation Conditions

The cultivation medium contained (g/L): salts and yeast extract as above; sucrose, 150 or 300 as required; initial pH, 5.5. The cultivation temperature was 25°C. Bacteria were cultivated in a batch or continuous regime in flasks or a computer-coupled laboratory fermentor with a working vol of 5 L (MBR, Wetzikon, Switzerland), equipped with a quadrupole mass spectrometer (QMG-311 (Balzers, Liechtenstein). During the fermentation, temperature, pH, rH, pO_2, velocity of stirrer revolution, pressure in the apparatus, and flows of inlet and outlet gases were measured on-line (MBR, Switzerland). Anaerobic conditions in the fermentor were ensured by flushing the cultivation medium with nitrogen at the rate of 0.78 L/min. Velocity of stirrer revolution was 80 rpm. Continuous cultivation was carried out at a fixed dilution rate ($D = 0.05\ h^{-1}$) and varying medium pH values (4.0, 4.8, and 5.3).

The concentration of ethanol and dissolved gases was determined by the quadrupole mass spectrometer with membrane sensor (silicone membrane), whereas the CO_2 concentration in the outlet gases was determined by the gas analyzer Infralyt 2200 (VEB Junkalor, Dessau, DDR).

Analytical Methods

After centrifugation of the cells for 15 min (at 6000 rpm) to separate them from the cultivation medium, concentrations of levan, ethanol, glucose, fructose, sucrose, and sorbitol in the samples were determined off-line. Sugar and sorbitol concentrations were determined by the HPLC

method on a Shimadzu LC-4A, using a Zorbax NH_2 column (250×4.6 mm) and a refractometric detector. Elution was carried out using a solvent system, acetonitrile: water in the ratio 75:25, at the rate of 1 mL/min. Ethanol content was measured by the gas chromatography method (*17*). Levan was precipitated by 75% ethanol, and its concentration was determined by the content of fructose in polysaccharide hydrolysate, as described in Viikari (*8*).

The biomass concentration was estimated by the optical density (540 nm) of a cell suspension prepared by centrifugation of the cells from the cultivation medium and a subsequent resuspension in distilled water.

For the determination of oligosaccharides and low molecular weight levan, the cultivation medium, after levan precipitation and ethanol evaporation, was hydrolyzed by 0.1*M* HCl for 1 h at 100°C and the concentration of reducing substances was determined (*18*). The concentration of oligosaccharides and low molecular weight levan was calculated as the difference between the concentrations of the total reducing substances in the hydrolysate and the sum of sugars (sucrose, fructose, and glucose), determined by the HPLC method in the cultivation medium without hydrolysis.

RESULTS

The Growth of *Z. mobilis 113* on Sucrose

In a batch culture, *Z. mobilis 113*, fermented 150 g/L of sucrose for 30 h at 25°C, produced 22 g/L of levan and 58 g/L of ethanol (Fig. 1A). The process of levan biosynthesis is seen to be characterized by a significant lag phase that lasted about 10 h from the beginning of fermentation. Unlike levan production, sucrose assimilation and ethanol synthesis started immediately after inoculation of the culture. Polysaccharide biosynthesis started after a full utilization of the inoculate-introduced levan (Fig. 1A). The time of complete inoculate-introduced levan utilization coincided with sorbitol formation and the increase of fructose concentration in the medium (Fig. 1B). After 30 h of fermentation, levan yield from the added substrate reached 0.147 g/g, with a productivity of 0.73 g/L/h.

The Effect of Glucose on Levan Synthesis by *Z. mobilis 113*

One of the reasons for a prolonged lag-phase of levan formation can be related to the presence of glucose in the medium resulting from partial sucrose hydrolysis during sterilization. Glucose is known to cause the inhibition of sucrose conversion to levan (*19*).

We have studied the effect of glucose concentration (2–10%) on levan production. Glucose was added to the *Z. mobilis 113* culture during the

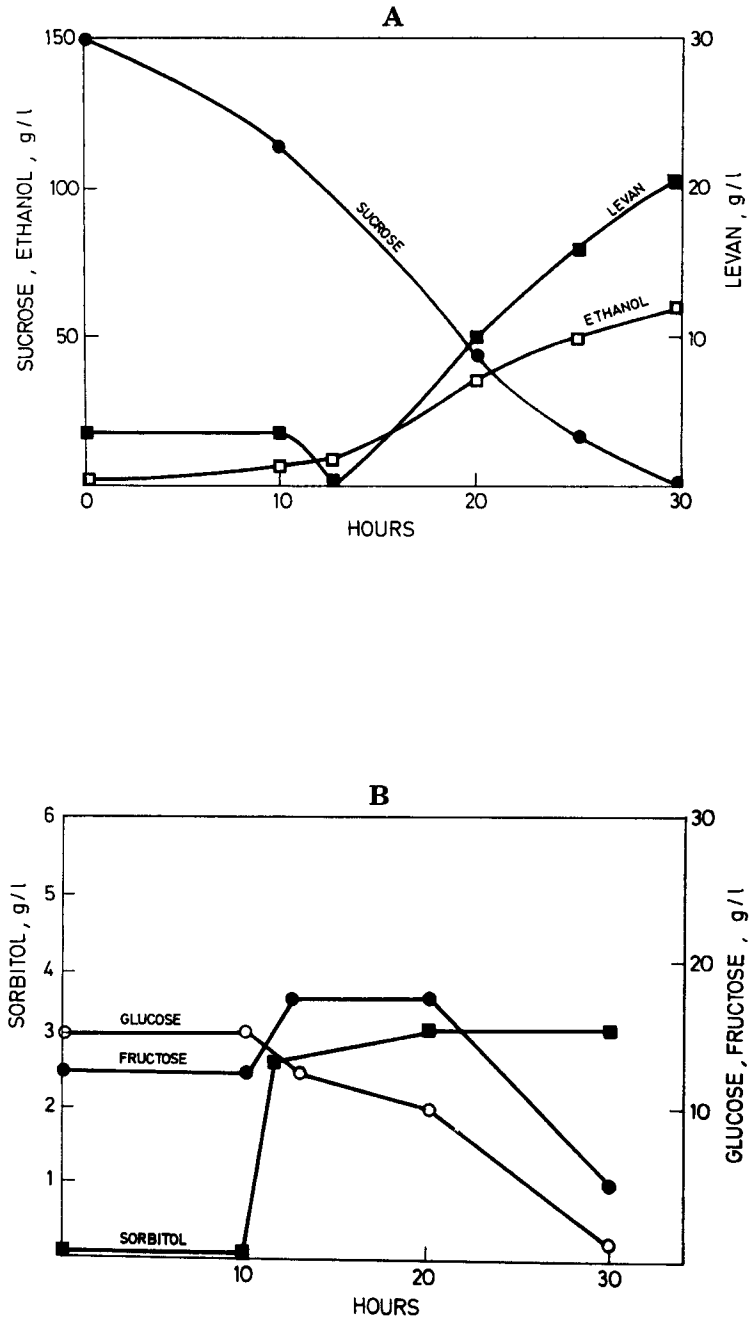

Fig. 1. Batch process of Z. *mobilis* growth on a 15% sucrose; (A) Time course of sucrose utilization, levan and ethanol production; (B) Time course of glucose, fructose, and sorbitol content.

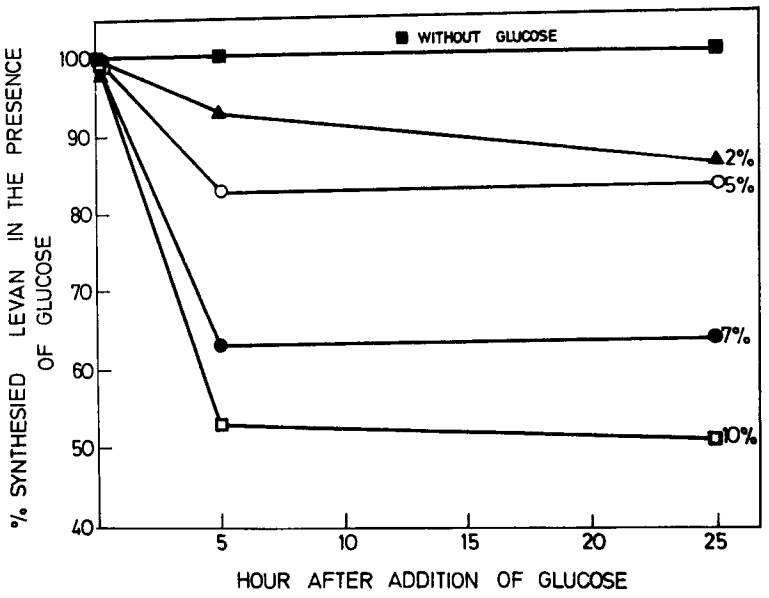

Fig. 2. The effect of glucose concentration on levan synthesis from sucrose in a batch culture of *Z. mobilis 113.*

exponential growth on 20% sucrose in flasks. The effect of levan biosynthesis inhibition by glucose is shown in Fig. 2. The addition of 10% glucose in the cultivation medium decreased levan concentration at the end of fermentation by half.

The effect of ethanol on levan synthesis by *Z. mobilis 113* was studied in batch culture grown on 20% sucrose in flasks (Fig. 3). Ethanol was added to the bacterial culture at the exponential growth phase at 2, 5, 7, and 10 wt% final conc. The addition of ethanol, in concentrations up to 5.0% into the medium, caused a small decrease of the levan-synthesizing activity of *Z. mobilis.* Yet, upon higher ethanol concentrations (7 and 10%), the synthesis of levan by the culture began to resume 5 h after the moment of ethanol addition. After adding ethanol in the final concentration, 100 g/L, there was no further decrease of medium pH and ethanol synthesis; yet, sucrose hydrolysis by *Z. mobilis 113* continued at a high rate. Sucrose utilization was accompanied by an accumulation of glucose (Fig. 4) and fructose (not shown) and a further synthesis of levan (Fig. 3).

The effect of medium pH on levan synthesis in *Z. mobilis 113* was studied in a continuous culture at a constant medium dilution rate (D)—0.05 h^{-1}—and a sucrose concentration in the feeding medium—300 g/L. The concentrations of end-products and carbon yields are shown in Tables 1 and 2. The main products of sucrose conversion were levan, ethanol that

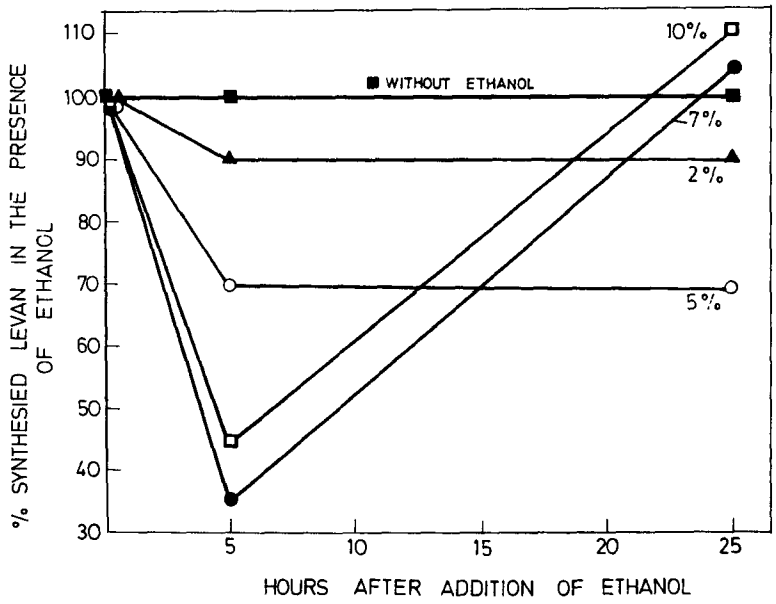

Fig. 3. The effect of ethanol concentration on levan synthesis from sucrose in a batch culture of *Z. mobilis 113* cultivation.

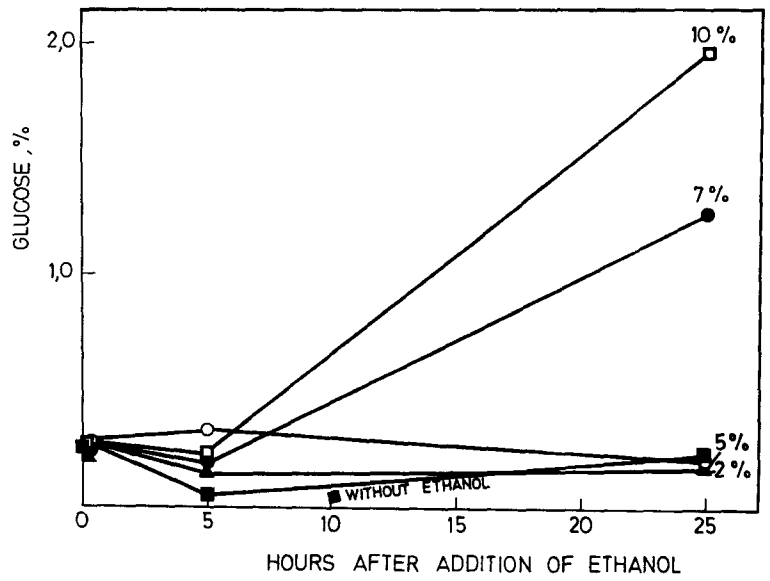

Fig. 4. The effect of ethanol concentration on glucose concentration after sucrose hydrolysis in the medium during a batch process of *Z. mobilis 113.*

Table 1
The Effect of Medium pH on Product Synthesis in a Continuous *Z. mobilis 113* Culture at $D = 0.05\ h^{-1}$ and a Sucrose Concentration in the Feeding Medium of 300 g/L

	Product concentration at different medium pH, g/L		
Products	4.0	4.8	5.3
Levan	54.1	64.2	41.0
Ethanol	75.0	49.2	58.0
CO_2	71.7	47.0	56.0
Biomass	5.1	5.2	4.8
Fructose + glucose	19.0	62.6	29.0
Residual sucrose	41.0	3.4	55.0
Sorbitol	3.0	9.1	4.2
Acetate	0.6	1.0	1.2
Lactate	2.2	2.0	2.0
Oligosaccharides + low molecular weight levan	24.0	60.0	45.0

Table 2
Carbon Balance in the Continuous Process of Sucrose Bioconversion into Levan and Ethanol by *Z. mobilis 113*[a]

Medium pH value	Carbon yield of the main end product					Carbon recovery
	Y^c_{lev}	Y^c_{eth}	$Y^c_{CO_2}$	Y^c_{biom}	C_{lev}/C_{eth}	
4.0	0.23	0.38	0.19	0.02	0.61	1.01
4.8	0.24	0.22	0.11	0.02	1.09	1.04
5.3	0.19	0.31	0.16	0.02	0.61	1.01

[a] $D = 0.05\ h^{-1}$; $S_o = 300$ g/L.

comprised 46–61% of the utilized sucrose carbon and CO_2. The minor end-products were oligosaccharides, glucose, fructose, sorbitol, and acetic and lactic acids. Taking the carbon content in the biomass as 50%, the total carbon recovery was close to 1.0 (Table 2). Among the three pH values we studied (4.0, 4.8, and 5.3), pH 4.8 was the most suitable for sucrose conversion into levan. At pH 4.8, we obtained the highest levan yield (Y^c_{lev})—0.24—and levan/ethanol ratio (C_{lev}/C_{eth})—1.09.

Levan overproduction also was improved in *Z. mobilis 113* batch culture by setting the pH at 4.8 after the pH fell to this point. With a controlled pH value, the bacteria produced 35 g/L levan from 150 g/L sucrose. The levan yield and productivity were 0.24 g/g and 0.90 g/L/h, respectively.

DISCUSSION

During the batch process of *Z. mobilis 113* growth on 15% sucrose, 65.6% of substrate carbon was transferred to levan and ethanol. The ratio C_{lev}/C_{eth} was 0.30 (Fig. 1A).

It is possible to increase the yield and productivity of levan synthesis, first shortening the lag-phase (Fig. 1A). Obviously, there are three reasons for an increased lag-phase of a batch culture of *Z. mobilis* on sucrose. The first—*Z. mobilis*—are anaerobic bacteria, and the lag-phase is connected with the elimination of the dissolved oxygen from the medium (*17,20,21*). In our experiments, we carried out deoxygenation of the medium by flushing nitrogen through the nutrient medium prior to the inoculation of the culture. The second reason could be caused by the inhibition of levansucrase by glucose (*19*), which can be formed during the sterilization of sucrose-containing medium. It was established that glucose in the concentration of 20 g/L, in the exponentially growing culture, inhibited levan synthesis by about 15% (Fig. 2). The weakening of the standard medium sterilization regime (121°C; 120 kPa; 10 min) to 111°C, a 51 kPa overpressure for 10 min, and a rapid cooling reduced the initial glucose concentration from 16.0 to 5.4 g/L in the last experiment.

The third reason may be connected with the fact that sucrose grown *Z. mobilis 113* form a polysaccharide capsule that disturbs culture growth. The time course of levan concentration showed that the synthesis of polysaccharides started only after reutilization of inoculate-introduced levan from the medium (Fig. 1B). The capsule forming inoculum can be eliminated either by growing the inoculum on a medium with a low initial sucrose concentration (less than 50 g/L) and a growth temperature that is not optimal for levan synthesis (30°C), or by growing on glucose. We made use of the first option.

There are reference data that ethanol in the concentration of 1.6*M* inhibits the levan-sucrase activity (*19*), and when its content exceeds 100.0 g/L (*22*), any metabolic activity and cell growth cease. We observed an unexpected effect. Ethanol in concentrations exceeding 70 g/L, 5 h after its addition, began to restore and even stimulated levan production (Fig. 3). Earlier, the uncoupling effect of ethanol on the growth of *Z. mobilis* was reported (*23*). The results of our investigation indicated that sucrose hydrolysis, accumulation of monosaccharides, and levan synthesis are not inhibited at high ethanol concentrations. Accumulation or addition of ethanol at concentrations >70 g/L may be even favorable for increasing the levan yield.

The C_{lev}/C_{eth} ratio at the end of fermentation greatly depends on medium pH (Table 2). Between 46 and 61% of the carbon of the added sucrose were transferred into levan and ethanol. The rest of the carbon was transferred into CO_2, biomass, mono- and oligosaccharides, sorbitol, and acetic and lactic acids, and a part remained nonutilized. Taking into account these end products, the carbon balance was close to 100% (Table 2).

Two methods for simultaneously obtaining levan and ethanol from sucrose with Z. *mobilis* could be used. We have demonstrated a batch process with a controlled pH (=4.8) of the conversion of 15% sucrose, with a transfer of 66.7% of substrate carbon into levan and ethanol. In the batch process, the levan end concentration was 35 g/L, levan yield was 0.24 g/g, ethanol yield was 0.31 g/g, levan productivity 0.90 g/L/h, and ethanol productivity 1.1 g/L/h.

In the continuous process at $D=0.05\ h^{-1}$ and pH 4.8, 64.2 g/L levan was produced. Levan yield was 0.22 g/g, ethanol's 0.17 g/g; levan productivity was 3.2 g/L/h, ethanol's 2.5 g/L/h. The results with Z. *mobilis 113* are much better than the known literature data for *Bacillus sp.* yielding 28.2 g/L levan, with the productivity of 0.94 g/L/h (*13*).

SUMMARY

Two biotechnological systems were developed for sucrose conversion into levan and ethanol with *Zymomonas mobilis*, ensuring a 66.7% transfer of substrate carbon in a batch and 61% carbon transfer in a continuous culture. The effect of glucose, ethanol, and medium pH on sucrose conversion by Z. *mobilis* was studied. The addition of ethanol to the fermentation medium, in the final conc. of 100 g/L, uncoupled levan synthesis from ethanol fermentation. For a continuous culture, the most efficient conversion of substrate carbon into levan was reached at pH 4.8, giving 64.2 g/L levan, with the levan yield of 0.22 g/g and the productivity of 3.2 g/L/h.

REFERENCES

1. Rogers, P. L., Lee, K. J., Skotnicki, M. L., and Tribe, D. E. (1982), *Adv. Biochem. Eng.* **23,** 37.
2. Jain, V. K., Toran-Diaz, J., Baratti, J. (1985), *Biotechnol. Bioeng.* **27,** 273.
3. Lee, J. H., Skotnicki, M. L., Pagan, P. J., and Rogers, P. L. (1983), *Proc. Pac. Chem. Eng. Congr.* **4,** 132.
4. Doelle, H. W. and Greenfield, P. F. (1985), *Appl. Microbiol. Biotechnol.* **22,** 411.
5. Bringer-Meyer, S., Scollar, M., and Sahm, H. (1985), *Appl. Microbiol. Biotechnol.* **23,** 134.
6. Chun, U. H. and Rogers, P. L. (1988), *Appl. Microbiol. Biotechnol.* **29,** 19.
7. Dawes, E. D., Ribbons, D. W., and Rees, D. A. (1966), *Biochem. J.* **98,** 804.
8. Viikari, L. (1984), *Appl. Microbiol. Biotechnol.* **19,** 252.
9. Schechter, J. and Hestrin, S. (1963), *J. Lab. Clin. Med.* **61,** 962.
10. Tkatchenko, A. A., Loityanskaya, M. S., Rabinovich, I. M., and Vavilin, G. I. (1971), *Physiologically and Optically Active Polymeric Substances* (in Russian), Zinatne, Riga, USSR, pp. 206–211.
11. Behrens, U. and Ringpfeil, M. (1965), *GDR Patent 24763, 30/72537.*
12. Behrens, U. and Ringpfeil, M. (1964), *Mikrobielle Polysaccharide,* Academie-Verlag, Berlin.

13. Elisashvili, V. I. (1984), *Prikl. Biokh. Mikrobiol.* (in Russian) **20,** 101.
14. Feingold, D. S., Avigad, G., and Hestrin, S. (1956), *Biochem. J.* **64,** 351.
15. Loityanskaya, M. S. and Elisashvili, V. I. (1981), *Bulletin* (in Russian) **32,** 116.
16. Beker, M. J. Klintsare, A. A., Shvinka, J. E., and Pankova, L. M. (1984), *Bulletin* (in Russian) **32,** 77.
17. Pankova, L. M., Shvinka, J. E., Beker, M. J., and Slava, E. E. (1985), *Mikrobiologiya* (in Russian) **54,** 141.
18. Plevako, J. A. and Bakushinskaya, O. A. (1964), *Microbial Chemical Control of Yeast Production* (in Russian), Pistchevaya Promyshlennostj, Moscow, 267 pp.
19. Lyness, E. W. and Doelle, H. W. (1983), *Biotechnol. Lett.* **5,** 345.
20. Pankova, L. M., Shvinka, J. E., and Beker, M. E. (1988), *Appl. Microbiol. Biotechnol.* **28,** 583.
21. Shvinka, J. E. Pankova, L. M., Mezbarde, I. N., and Licis, L. J., (1989), *Appl. Microbiol Biotechnol.* vol. 31, p. 240.
22. Osman, Y. A. and Ingram, L. O. (1985), *J. Bacteriol.* **164,** 173.
23. Raynina, E. I., Batchurina, G. P., Makhlis, T. A., and Sinitsin, A. P. (1986), *Biotekhnologiya* (in Russian) **4,** 65.