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A Biosensor for L-Proline Determination By Use of Immobilized Microbial Cells

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ABSTRACT

A biosensor to quantify L-proline within 10^{-5}–10^{-3} mole/L concentration is described. Immobilized *Pseudomonas sp.* cells grown in a medium containing L-proline as the only source of carbon and nitrogen were used to create the biosensor. The cells oxidized L-proline specifically consuming O_2 and did not react with other amino acids and sugars. The change in oxygen concentration was detected with a Clark oxygen membrane electrode. The cells were immobilized by entrapment in polyvinyl alcohol (PVA) cryogel. The resultant biocatalyst had a high mechanical strength and retained its L-proline-oxidizing ability for at least two months.

Index Entries: *Pseudomonas sp.;* L-proline-oxidizing activity; proline quantitation; immobilized cells.

INTRODUCTION

In recent years, remarkable progress has been made in designing analytical systems based on immobilized enzymes and cells. Such systems are used for the rapid and highly specific analysis of many organic compounds, including some amino acids (*1,2*). Compared to traditional

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methods, various biosensitive facilities have some advantages, such as assay rate, high selectivity, and simplicity of the method. Nevertheless, there have been no such systems to quantify proline. This may be because of the lack of stable enzyme or cell systems that could interact with high enough specificity to this amino acid, thus forming or utilizing an easily detectable compound.

Proline is an important compound. Its deficiency in humans seriously affects the mechanical properties of connective tissues, since it is needed to form a correct structure of collagen (*3*). Proline levels are one of the parameters that determine the qualities of grape wine and honey (*4*). It is evident from the above statement that it is important to design biosensitive facilities for proline quantitation in various biological and technological fluids, including the fermentation broth of the microbiological synthesis of L-proline.

The proline metabolism in microorganisms and animals, as discussed in the literature to date, seems obscure. The enzymes responsible for degradation of proline are termed differently in various works, different properties are ascribed to them (*5–7*), and although the proline oxidase is referred to in many of these studies none have distinctly described its isolation and properties.

The authors have found a *Pseudomonas sp.* strain capable of growth in a medium containing L-proline as the only carbon and nitrogen source. The cells grown under these conditions use proline as a respiration substrate, with a very high rate of oxygen consumption. Some properties of these cells have been investigated, the technique of their immobilization elaborated, and their application in an L-proline assaying biosensor described.

MATERIALS AND METHODS

Growth Medium

Pseudomonas sp. were grown in a synthetic medium containing 0.15% L-proline as the only source of carbon and nitrogen, 0.2% $K_2HPO_4 \cdot 3H_2O$, 0.05% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, pH 7.2–7.5.

Growth of Microorganisms

To produce the biomass, the cells were grown on a shaker (105 rpm) at 25°C in 1 L Erlenmeyer flasks containing 250 mL of the above liquid synthetic medium. The washoff of the cells grown on the slant agar (2%) was used as inoculum, with the content of the medium being the same. The inoculum volume was 0.4% of the fermentation medium volume. The grown cells were separated from the culture liquid by centrifuging at $6000 \times g$. They were washed twice by potassium-phosphate buffer (pH 8.0), and were then resuspended in the same buffer (1 g cells/1 mL buffer).

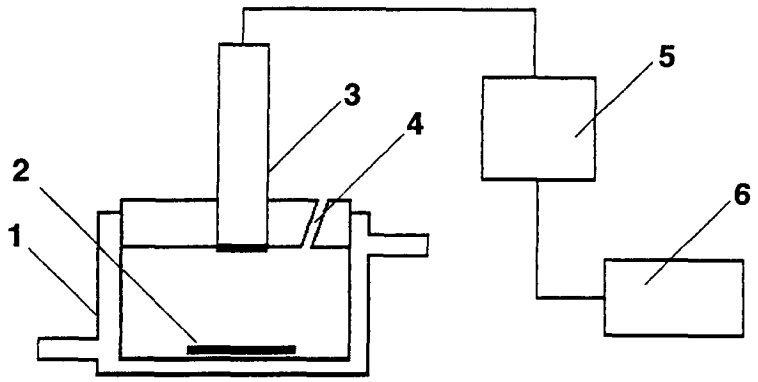

Fig. 1. The block diagrom of the setup: (1) amperometric unit: (2) magnetic stirrer; (3) oxygen electrode; (4) hole for sample injection; (5) electronic block for signal recording; and (6) plotter.

Cell Immobilization

The immobilized biocatalyst was obtained by entrapment of *Pseudomonas sp.* cells in PVA cryogel beads about 2 mm in diameter by a method reported elsewhere (*8,18*). The content of the biomass in the granules was 10% by weight (wet weight of cells).

Measurements of Proline-Oxidizing Activity

The proline-oxidizing activity was measured amperometrically by recording the oxygen consumption in the reaction medium with a Clark membrane electrode. The block-diagram of the setup is shown in Fig. 1. The measurements were made of 30°C in a thermostated unit of 5 mL volume containing free or immobilized cells in 50 m*M* potassium-phosphate buffer, pH 8.0. The reaction was initiated by injecting 50 μL of proline solution (the final proline concentration in the working volume was 0.01–1.0 mmole/L). Initiation of the reaction by proline allowed us to take into account the endogenic respiration of free or immobilized cells.

One unit (U) of the specific activity of free or immobilized cells was expressed as oxygen quantity (μ*M*) consumed by 1 mg of cells (w/w) during 1 min.

Quantitation of ATP Concentration in Cells

ATP in free cells was quantified by the bioluminescence method with the aid of immobilized firefly luciferase, using dimethylsulfoxide (DMSO) as the ATP-extracting agent (*9*). ATP concentration in the immobilized cells was determined without preseparation of the cells from the carrier (*10*). ATP concentration in the extract was calculated according to the following formula:

$$(ATP)_{extr} = I \cdot V_1 (ATP)_{st} \cdot V_{st} / V \cdot I_{st} \cdot V_2, \quad (1)$$

where I is the luminescence intensity of the sample (mV); I_{st} is the standard luminescence intensity (mV); V_1 is the volume of the reaction mixture before addition of ATP standard (mL), and V_2 is the volume after addition of ATP standard (mL); V is the extract volume (mL); V_{st} is the volume of ATP standard (mL); and $(ATP)_{st}$ is the ATP concentration in the standard solution (μM/L).

Reagents and Apparatus

Reagents: L-proline, L-lysine, L-ornithine, L-glycine, L-arginine, D, L-threonine (all supplied by REANAL, Hungary), L-tryptophan, L-serine (supplied by REACHIM, Russia). Other reagents were of the "Chemical pure" grade (Russia).

Centrifuges K-70D (Germany) and Beckman G2-21 (USA) were used. The cells were disrupted by one of the following methods: UZDN-2T ultrasonic disintegrator (Russia), freezing and thawing, freezing and subsequent grinding with quartz sand, or in an X-press. Amperometric measurements were carried out with a LP-7 polarograph (Checkoslovakia). The luminescence intensity was measured by a 1250 Luminometer (LKB, Sweden).

RESULTS AND DISCUSSION

There are some contradictory data in the literature concerning the metabolism of L-proline as the carbon or nitrogen source for microorganisms. Classification of the enzymes involving proline conversion is also confusing. In one fundamental monograph (*5*), the process of proline oxidation is described by the following scheme:

$$\text{L-proline} + O_2 \xrightarrow{\text{prolineoxidase}} \Delta'\text{-pyrroline-5-carboxylate} \quad (2)$$

Dagly and Nickolson (*6*) suggested another scheme that also involved cytochrome C. Other authors (*7*) refer to proline dehydrogenase as the enzyme responsible for proline oxidation, and exclude molecular oxygen from the reaction. The *Enzyme Nomenclature* (*11*) contains none of the above names. It indicates that the enzyme responsible for L-proline oxidation is Δ'-pyrroline-5-carboxylate reductase. Adams and Frank (*12*) reviewed proline metabolism, and presented the data on a proline-oxidizing enzyme-proline oxidase.

Several cases of proline oxidase activity in cellular homogenates have been described. The experimental confirmation of oxygen consumption in the process of proline oxidation by a crude extract from microorganisms was reported by Deninger and Brill (*13*). Terming this enzyme proline:

Table 1
Oxygen Consumption by *Pseudomonas sp.* Cells in the Presence of Various Compounds

Substrate, concentration 20 mmole/L	Relative activity, %
L-Proline	100
L-Arginine	8
L-Lysine	5
L-Tryptophan	3
L-Serine	0
Glycine	0
L-Threonine	0
L-Phenylalanine	0
L-Ornithine	0
Glucose	0
Sucrose	0

oxygen oxidoreductase, they gave it the nomenclature number—1.4.3.1, but this number in the *Enzyme Nomenclature* (*11*) belongs to oxidase of L-amino acids. Manzel and Roth (*14*) reported the extraction of a protein with the molecular weight of 132 kDa that shows both proline oxidase and proline-5-carboxylate dehydrogenase activities. The above information testifies that the problem of finding a definite enzyme responsible for proline oxidation in the presence of oxygen remains open.

To obtain strains containing proline-utilizing systems, microorganisms from different collections and cultures obtained from soil were investigated. Over sixty strains belonging to *Escherichia, Pseudomonas, Serratia,* and *Micrococcus* species, as well as some unidentified cultures have been examined. The selection was first performed on Petri dishes with the agarized synthetic proline-containing medium. The cells selected at the first step were further grown during fermentation in a liquid synthetic proline-containing medium. The proline-oxidizing ability of the cells obtained was determined in an amperometric unit with an oxygen electrode, then the same activity of the cells disrupted by different methods was also tested. NAD, NADH, NADF, FAD, cytochrome C, ascorbate, magnesium, and copper ions were added into the measuring unit containing the disrupted cells to check their role in the manifestation of the proline-oxidizing activity.

A total of fifteen strains among the microorganisms studied had the ability of growing in a medium containing proline as a single source of carbon and nitrogen, but only the *Pseudomonas sp.* cells had a specific proline oxidizing activity. Table 1 presents the data defining the specificity of this strain, and shows that the cells do not use glucose, sucrose, or

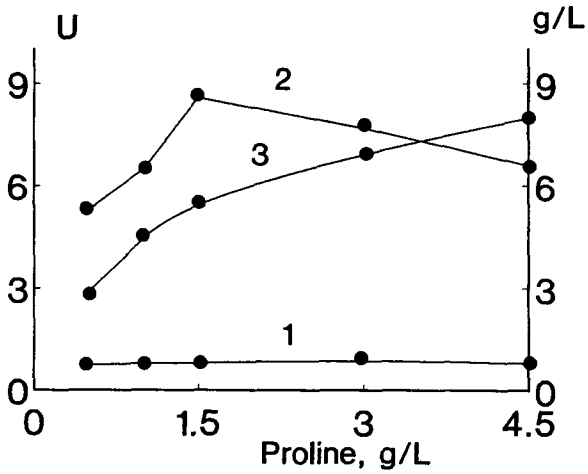

Fig. 2. Dependences of endogen respiration of cells (1) proline-oxidizing activity (2) biomass yield and, (3) on initial L-proline concentration in the growth medium.

some amino acids as respiration substrates. In principle, this organism can be used for L-proline quantitation. Since the proline-oxidizing possibility was induced only in the cells grown in proline-containing medium, the activity of the cells and the level of their endogenic respiration could depend on proline concentration in the growth medium.

Figure 2 shows the dependences of endogen respiration of the cells, their proline-oxidizing activity and biomass yield, on the initial proline concentration (0.5–4.5 g/L) in the growth medium. Curve 1 shows that the endogene respiration of cells remained practically the same, the maximum activity of the cells grown in the growth medium with 1.5 g/L of proline is shown in curve 2, and biomass yield are positively correlated with proline concentration variation in curve 3. Further experiments utilized a biomass cultured in such a medium up to the end of the logarithmic phase of growth.

The cell suspension retained its proline-oxidizing ability over 12 d. The endogene respiration of the cell suspension at 4°C decreased over time, and around the 4–5 d, activity dropped to value close to zero. If the cells were incubated in a growth medium without proline being actively aerated, endogene respiration was lost after 2 d. A decrease of the intracellular level of ATP was observed during the same time period. The ATP concentration at the end of the logarithmic growth phase was $2.0\text{–}5.0 \cdot 10^{-11}$ mole/mg of wet weight of cells. During 4–5 d of storage at 4°C or 2 d at 28°C, the ATP decreased to $1.0\text{–}2.0 \cdot 10^{-12}$ mole/mg of cells. Since the ATP concentration characterizes cell metabolism (*10*), the decrease in its level can be considered the result of exhaustion of the endogene energetic substrates. The decrease of the level of endogene respiration of cells during storage in a medium not containing proline correlates positively with the change in their ATP concentration.

During a 12–14 d storage at 4°C or 5 d at 25°C, the cells retained their proline-oxidizing ability. Proline oxidation was detected immediately upon addition of proline to the medium. After 5 min incubation in a proline-containing medium, the ATP content of the cells is increased to $2.0–3.0 \cdot 10^{-11}$ mole/mg of wet weight of cells. When the cells lost their proline-oxidizing ability during storage, addition of proline to the incubation medium did not lead to the ATP level restoration in the cells.

Since these cells consume oxygen when oxidizing proline, the conditions for activity determination were optimized so that the oxygen concentration during the measurement was not limiting. To this end we investigated the influence of the microorganisms' concentration in the measuring unit against their specific proline-oxidizing activity. The cells had maximum specific activity when their concentration in the measuring device did not exceed 10 mg (wet weight) in 1 mL.

The literature concerning the proline metabolism in microbes is contradictory. An attempt to extract the proline-oxidizing enzyme from *Pseudomonas sp.* was unsuccessful. Then the sensitivity of the cells to KCN was checked. A 1.5 m*M* concentration of KCN suppressed the endogene respiration of the cells and their ability to oxidize proline. This effect occurred regardless of the order of KCN addition. Since the activity of proline oxidase (if it is present in these cells and is inhibited by KCN) does not manifest itself on the background of cellular respiration, the cells were disrupted by different methods, and attempts to deorganize the respiration chain of this bacterium were made. The cells were treated by ultrasound until the suspension was "clarified" (OD_{540} decreased by a factor of 10–12). The proline-oxidizing activity was then measured. After ultrasonic treatment the activity appeared to decrease by a factor of 5–6. The endogenic respiration was essentially suppressed. The addition of NAD, NADH, NADF, FAD, cytochrome C, magnesium, or copper ions into the medium did not restore the proline-oxidizing activity.

Interesting data were obtained after centrifuging the sonicated suspension (20 min, 10,000 rpm). There was no endogene respiration in the supernatant, and the proline-oxidizing activity was 60% of the ultrasonic treated suspension. The obtained sediment was resuspended in a potassium-phosphate buffer in a volume equal to that of the supernatant. In this new suspension there was endogene respiration equal to that of the sonicated suspension, while the proline-oxidizing activity was 40% of the analogous activity of the suspension treated by ultrasound. The results are presented in Table 2. Attempts to extract proline-oxidase from such a supernatant failed. Any fractionation of the protein mixture led to a complete loss of the proline-oxidizing activity. Continuous ultrasonic treatment of the cell suspension for more than 5 min also induced the loss of its proline-oxidizing activity. Other methods of disruption, such as X-pressing, led to similar results. These results indicate that the proline-oxidizing system of *Pseudomonas sp.* is a complicated, spatially organized

Table 2
The Effect of Ultrasonic Disintegration on the Endogene Respiration and on Proline-Oxidizing Activity of *Pseudomonas sp.* Cells

Stage of treatment	ABS_{540}	Endogene respiration $\mu M\ O_2 \cdot mg^{-1} \cdot min^{-1}$	Activity $\mu M\ O_2 \cdot mg^{-1} \cdot min^{-1}$
Initial cell suspension	1.9	0.8	9.6
Homogenate	0.2	0.2	2.0
Supernatant	—	0	1.2
The precipitate resuspended in the buffer	—	0.2	0.8

The measurements were made out in a 50 m*M* potassium-phosphate buffer, pH 8.0, at 30°C and 1 mmole/L proline concentration in the measuring unit. The cell concentration was 10 mg/mL.

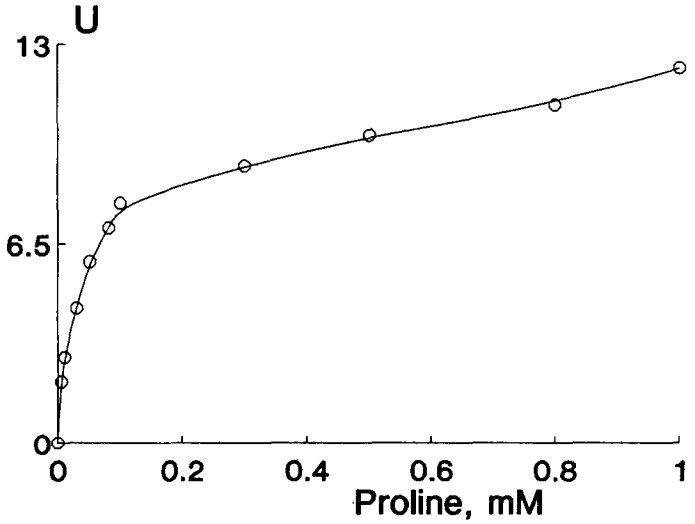

Fig. 3. The dependence of respiratory activity of native cells, namely L-proline concentration in the measuring unit.

multienzyme complex that catalyzes the series conversion of proline and the oxygen acceptance.

Taking into consideration the high substrate specificity of the *Pseudomonas sp.* strain (see Table 1), it seems possible to use whole cells as a sensitive basis for a biosensor.

First, it was important to investigate the relationship between the oxygen consumption rate and the L-proline concentration in the medium. Only if such a dependence exists can one think of applying the cells as a biosensor for determination of L-proline (Fig. 3).

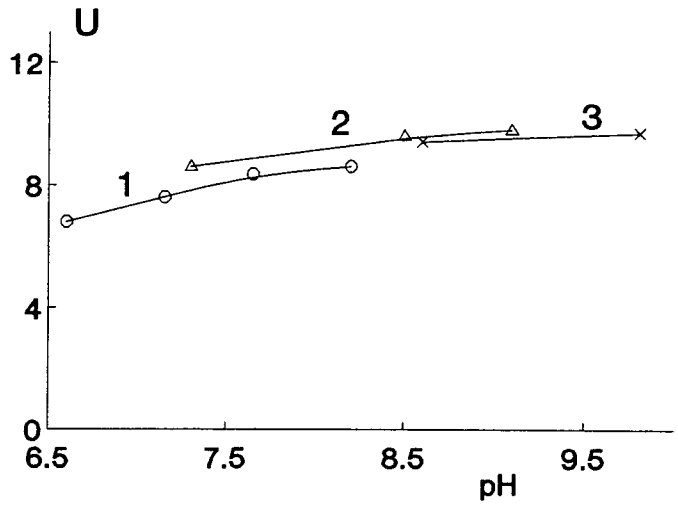

Fig. 4. The pH dependence of the proline-oxidizing activity of native *Pseudomonas sp.* cells (proline concentration 1 mmole/L): (1) 50 mmole/L potassium-phosphate buffer; (2) 50 mmole/L Tris-HCl buffer; and (3) 50 mmole/L glycine-KOH buffer.

The calibration plot consists of two linear parts, 0.01–0.1 and 0.1–1.0 mmole/L of proline. Proline can be quantified in the above whole range: 0.01–1.0 mmol/L. To optimize the L-proline determination using *Pseudomonas sp.*, the dependence of the proline-oxidizing activity on the temperature and pH of the medium as well as on the nature of the buffer was examined. The cells were most active at 29–30°C. No sufficiently pronounced effect of pH or buffer content on the proline-oxidizing activity of cells were observed (Fig. 4).

Pseudomonas sp. can be used in an amperometric determination of L-proline. However, in a real analytical system responding to modern requirements, the use of a cell suspension will lead to difficulty in solving problems. As previously mentioned, during storage the cells gradually lose their proline-oxidizing ability. It is therefore necessary to grow biomass regularly. Moreover, the accuracy of the L-proline quantitation depends essentially on the number of cells inserted into the measuring unit, the homogeneity of the cell suspension being rather relative. It is also impossible to use free cells in flow-through analytical systems.

Obvious advantages include analyzers that use immobilized microorganisms as a biocatalyst. The detailed analysis of modern designs of such systems are presented in other reports (*15,16*). Various methods and carriers are utilized to immobilize the microbes used as biosensors (*17*). In cases when the product to be determined is formed or consumed in a reaction catalyzed by a single enzyme, as in the case of determining glucose or ethyl alcohol (*15*), immobilized cells are used instead of the corresponding immobilized enzymes (glucose and alcohol oxidases), which essentially

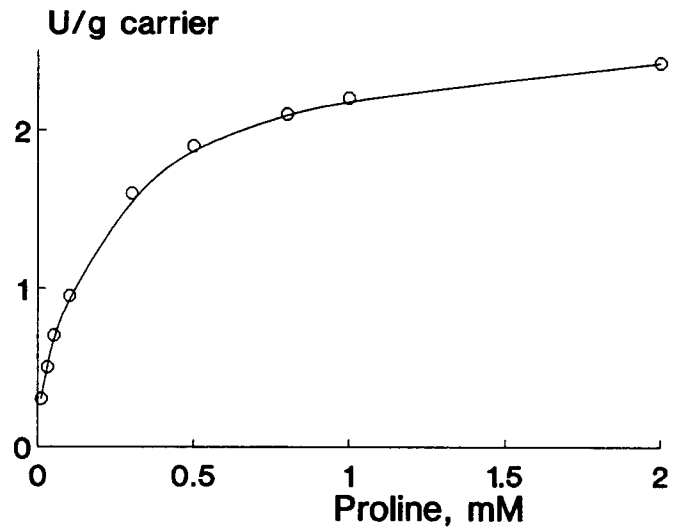

Fig. 5. Calibration curve for L-proline quantification by immobilized *Pseudomonas sp.* cells.

lowers the cost and simplifies the biocatalyst production. For this type of system there are various successfully used methods of immobilization, including the use of agents toxic for the cells or treatments killing the cells, but not affecting their specific enzymatic activity. To create biocatalysts based on native, metabolically active cells, the choice of the method of immobilization is a difficult problem. Entrapment of cells into κ-carrageenane and Ca-alginate gels is considered the "mildest" method today.

The results of these investigations have shown that *Pseudomonas sp.* bacteria entrapped in a gel matrice can utilize L-proline in the same way as do the free cells. However, when used repeatedly, the granules lost their mechanical strength because of the intense agitation in the measuring device.

It seems rather perspective to entrap cells into the so-called PVA cryogel. The latter is obtained by the procedure of freezing-thawing of aqueous PVA solution. The matrix formed is highly porous and has satisfactory mechanical strength. The successful immobilization of microbial cells in the PVA cryogel was reported elsewhere (*8,18,19*).

In the present work, the immobilized biocatalyst was prepared by a previously described method (*8,18*). The granules of about 2 mm diameter were incubated in a proline-containing culture medium for 1 d. During this period, the ATP concentration increased and became practically the same as in the cells before immobilization. The endogene respiration of the cells was restored at the same time. The biocatalyst granules obtained in that way were used in the biosensor to quantify the L-proline. Figure 5 shows a calibration curve for L-proline. The comparison of Figures 3 and 5 indicates similar dependence of the oxygen consumption rates, namely,

L-proline concentration for both free and immobilized cells. In addition, ranges of L-proline quantification in these cases were equal. The proline-oxidizing ability of the immobilized as well as the free cells depended slightly on the buffer pH. The experiments on determining the biocatalyst storage conditions have shown that the catalyst retains its activity best when the granules are maintained in a 50 m*M* potassium-phosphate buffer, pH 7.5, at 4°C, or in the same buffer containing low concentrations of proline with no more than 0.01 mole/L at 25–30°C with a forced aeration. Under such conditions the biocatalyst retained its activity and mechanical strength for over two months. Preliminary results have been obtained on the successful application of a biocatalyst for the quantitation of proline in flow-through analytical systems.

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REFERENCES

1. Danielsson, B. and Mosbach, K. (1988), *Methods in Enzymology* **134,** *Part D,* Academic, NY, p. 3.
2. Romette, J., Yang, J., Kusakabe, M., and Thomas, D. (1983), *Biotechnol. and Bioeng.* **25,** 2557.
3. Bohinski, R. C. (1986), *Modern Concepts in Biochemistry,* Allyn and Bacon, Newton, MA, p. 118.
4. Minguzzi, A. (1973), *"SETA"* **3,** No. 6, 371 (in Italian).
5. White, A., Mandler, P., Smith, L., and Mill, R. L. (1978), *Principles of Biochemistry,* McGraw-Hill, NY, p. 187.
6. Dagley, S. and Nicholson, D. (1970), *An Introduction to Metabolic Pathways,* Blackwell Scientific Publications, Oxford and Edinburg, p. 184.
7. Lehninger, A. L. (1985), *Principles of Biochemistry,* Worth Publishers, NY, p. 522.
8. Lozinsky, V. I., Vainerman, E. S., and Rogozhin, S. V. (1986), *Inventor's certificate of the USSR,* No. 1400071.
9. Ugarova, N. N., Brovko, A. U., Trdatian, I. U., and Rainina, E. I. (1987), *Prikladnaya biokhimia i mikrobiologia,* **23,** 14 (in Russian).
10. Rainina, E. I., Makhlis, T. A., and Bachurina, G. P. (1987), *Immobilized Cells in Biotechnology,* Puschino, p. 140 (in Russian).
11. *Enzyme Nomenclature: Recommendation (1972) of IUB* (1979), Moscow, p. 63 (in Russian).
12. Adams, E. and Frank, L. (1980), *Ann. Rev. Biochem.* **49,** 1005.
13. Deninger, S. and Brill, W. (1970), *J. of Bacteriol.* **103,** 144.
14. Manzel, R. and Roth, J. (1981), *J. Biol. Chem.* **256,** 9755.

15. Karube, J. (1987), *Biosensors. Fundamentals and Applications,* Oxford University Press, Oxford, p. 15.
16. Riedel, K. and Renneberg, R. (1989), *J. Chem. Technol. Biotechnol.* **44,** 85.
17. Woodward, J. (1988), *Immobilized Cells and Enzymes. A Practical Approach,* IRL Press Oxford, Washington DC, p. 130.
18. Lozinskuy, V. I., Faleev, N. G., Zubov, A. L., Ruvinov, S. B., Vainerman, E. S., Antonova, T. V., Belikov, V. M., and Rogozhin, S. V. (1989), *Biotechnol. Lett.* **11,** 43.
19. Lozinsky, V. I., Zubov, A. L., Vainerman, E. S., and Rogozhin, S. V. (1990), *Biotekhnologia,* **No. 5,,** 32 (in Russian).