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EXPERIMENTAL ARTICLES

Streptomyces sp. Z-11-6, a Novel Producer of Extracellular L-Glutamate Oxidase

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Abstract—Glutamate oxidase activity was studied in 1254 *Streptomyces* strains isolated from the zonal soils of various regions of Russia and other countries. Seven strains proved to be producers of extracellular L-glutamate oxidase. The most active producer strain was identified, and the conditions of enzyme biosynthesis were optimized. A multistep mutagenesis–selection procedure allowed a genetically stable strain, *Streptomyces* sp. Z-11-6, to be obtained, whose glutamate oxidase activity was 40 times higher than that of the original natural isolate.

Key words: L-Glutamate oxidase, optimization of biosynthesis, *Streptomyces* sp. Z-11-6.

Glutamate oxidase (L-glutamate: O_2 oxidoreductase (deaminase), EC 1.4.3.11) can be used as an analytic reagent and as a basis for developing biosensors for the determination of L-glutamate, L-glutamine, ammonia, and creatinine; these biosensors can be used in analytical chemistry (for quantitative and qualitative assays of enzymatic processes) and in the food industry (to assess the quality of foodstuffs [1, 2]). Of particular importance is the use of the enzyme in clinical biochemistry for the determination of glutamate–pyruvate transaminase and glutamate–oxalacetate transaminase in biological fluids, which makes early diagnostics of heart and liver diseases possible [3]. According to the available data, the main producers of glutamate oxidase are streptomycetes [4–9], which are convenient agents for the industrial production of L-glutamate oxidase. Production of the enzyme has begun in several countries. However, in Russia, L-glutamate oxidase is not industrially produced. The search for new producers of L-glutamate oxidase and the development of efficient procedures to obtain it remain topical. Microorganisms excreting L-glutamate oxidase into the medium are the most promising producers of the enzyme.

The aim of the present work was to search for new producers of extracellular L-glutamate oxidase.

MATERIALS AND METHODS

Soil sampling. Samples of zonal soils were collected in various geographical regions of Russia and other countries by the random sampling method. To promote the isolation of *Streptomyces* representatives, air-dry soil samples (1 g) were mixed with 0.1 g $CaCO_3$ and incubated at 28°C for 7–9 days at a humidity corresponding to 60% of the soil moisture-holding capacity. Before microbiological analysis, the soil was humidified to a pasty state, ground, and agitated for 15–20 min in shaken flasks to desorb spores and mycelium from soil particles. Dilutions of soil suspensions were plated onto starch–casein agar with 0.2% glutamate. The medium was supplemented with 10–50 μg/ml nystatin and 50–100 μg/ml cycloheximide (actidione) to inhibit fungal growth and with 1 μg/ml penicillin to inhibit bacterial growth. Inoculated Petri dishes were incubated at 28°C for 14 days. All grown colonies were tested for glutamate oxidase activity by the method described below.

Screening for producers of extracellular glutamate oxidase. To reveal producers of extracellular glutamate oxidase, grown colonies were poured with modified F upper minimal agar [10], which contained (per 100 ml) 0.8 g Difco agar, 0.8 g NaCl, 250 units horse radish peroxidase, 0.1 mmol 4-aminoantipyrine, 1.75 mmol phenol, 1 mmol L-glutamate, and 0.1 M K–phosphate buffer (pH 7.0) and incubated at 28°C for 24 h. Glutamate oxidase activity was assessed visually from the red staining of the medium developing around the colonies as a result of the chromogenic peroxidase reaction. Active clones were transferred to a liquid medium containing 3% glucose, 0.6% corn extract, 0.6% ammonium sulfate, 3% calcium carbonate, 0.3% calcium chloride, 0.1% magnesium chloride, and 0.1% potassium chloride (pH 7.0). The inoculum was grown overnight and introduced in an amount of 5 vol % into 100 ml of the medium in 750-ml shake flasks. Cultivation was performed at 26°C on a shaker (190 rpm). Grown mycelium was separated from the culture liquid by filtration through a dense cloth, after which glutamate oxidase activity was determined in the filtrate by the method described below.

Determination of glutamate oxidase activity was carried out on the basis of the chromogenic peroxidase

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Table 1. Glutamate oxidase activity of the most active clones obtained after the third treatment of the wild strain *Streptomyces* sp. Z-11 (initial glutamate oxidase activity, 0.04 units/ml) with nitrous acid

The number of the culture transfer	Glutamate oxidase activity of mutant strains, units/ml				
	Z-11-1	Z-11-6	Z-11-9	Z-11-7	Z-11-24
1	0.130	0.130	0.139	0.130	0.139
2	0.143	0.191	0.150	0.160	0.150
3	0.193	0.432	0.146	0.156	0.291
4	0.191	0.610	0.120	0.158	0.270
5	0.104	0.601	0.110	0.132	0.220

Table 2. Glutamate oxidase activity of the clones derived from *Streptomyces* sp. Z-11-6 by successive treatments with nitrous acid

	The wild strain *Streptomyces* sp. Z-11	The mutant strain *Streptomyces* sp. Z-11-6	The number of the mutagenic treatment			
			1	2	3	4
Glutamate oxidase activity, units/ml	0.04	0.601	0.643	0.760	0.910	1.650

reaction. The reaction mixture (1 ml) contained 2.5 units of horseradish peroxidase, 1.0 μmol 4-aminoantipyrine, 17.5 μmol phenol, 10 μmol L-glutamate, and 0.1 M K–phosphate buffer (pH 7.0). The reaction mixture was incubated at 37°C for 2 min; then, the reaction was initiated by adding an aliquot of culture filtrate. Glutamate oxidase activity was determined from the changes in the optical density of the reaction mixture measured at 500 nm on a Hitachi 200-20 spectrophotometer (Japan). The activity was calculated by the formula

$$A_{GO} = \frac{\Delta V_{tot}}{\varepsilon_M T V_{enz}},$$

where A_{GO} is the glutamate oxidase activity, Δ is the change in the optical density of the reaction mixture in 1 min, V_{tot} is the total volume of the reaction mixture, V_{enz} is the volume of the sample, T is time in minutes, and ε_M is the molar extinction coefficient (equal to 6) of the chromogenic compound quinonimine formed as a result of the reaction. The amount of the enzyme catalyzing the formation of 1 μmol of hydrogen peroxide per minute at 37°C was taken as the activity unit.

Natural and induced mutability. Natural mutability and mutability induced by a mutagen were studied by commonly accepted methods [10]. Nitrous acid was used as the mutagen at a concentration resulting in an 0.01–0.1% spore survival rate. To decrease the proportion of auxotrophs, mutagen-treated cultures were grown overnight in minimal medium [10]. Active producer strains were screened for and further cultivated on medium containing 2% oat meal, 1.8% agar, and 0.2% glutamate.

Analytical grade reagents purchased from Reakhim (Russia) were used in this work.

RESULTS AND DISCUSSION

As a result of screening 1254 streptomycete strains, 7 strains (0.56%) proved to be producers of extracellular glutamate oxidase. The occurrence frequency of the producers of extracellular glutamate oxidase was also reported to be low in one of the first works devoted to the description of this enzyme (2 producers were revealed among 500 streptomycete cultures tested) [7]. For further work, we chose strain *Streptomyces* sp. Z-11, which was the most active producer of extracellular L-glutamate oxidase (0.01 unit/ml). Species identification of this strain, carried on with the use of the manual by Gauze *et al.* [11] and based on morphological, cultural, physiological, and biochemical properties, allowed strain Z-11 to be assigned to *Streptomyces canofumeus* Krassilnikov 1970 (type strain, INMI 516 = VKM 70).

According to data available in the literature, optimization of the cultivation medium is an efficient means to increase the biosynthetic activity of a culture [12]. Therefore, we carried out the optimization of the cultivation conditions of strain Z-11 (medium composition, pH, temperature, cultivation time). As a result, the enzyme yield increased to 0.04 unit/ml.

A classical method for the enhancement of the enzyme yield consists in the treatment of the culture with a mutagen and the subsequent selection of active clones [12]. Strain *Streptomyces* sp. Z-11 was treated with nitrous acid as a mutagen. Out of 1386 clones tested, only 24 exhibited glutamate oxidase activity. The five most active clones were subjected to further treatments with nitrous acid (Table 1). To check the genetic stability of the clones, they were subjected to culture transfers with subsequent assays for glutamate oxidase activity. The genetic stability of the clones was rather low; an exception was strain *Streptomyces* sp. Z-11-6, which was subjected to four additional treatments with nitrous acid (Table 2).

As a result of the multistep mutagenesis–selection procedure, we obtained a genetically stable mutant strain *Streptomyces* sp. Z-11-6 whose glutamate oxidase activity was 40-fold higher than that of the original wild strain and constituted 1.6–1.8 units/ml culture liquid. After 4 months of storage at 4°C, strain Z-11-6 retained 98% of its productivity.

Table 3. Cultural properties of the producer strain *Streptomyces* sp. Z-11-6

Medium [12]	Aerial mycelium	Substrate mycelium	Soluble pigment
Mineral agar 1	Well-developed, light gray to gray	Yellowish gray, later gray	No
Glycerol–nitrate agar	Lacking	Yellow to grayish yellow	Slightly yellow
Organic agar	Scanty, white	Brown	Brown
Oat agar	Well-developed, gray	Grayish yellow, later gray	No
Peptone–yeast extract–iron agar (ISP 6)	Well-developed, light gray	Brown	Melanoid pigments
Glucose–asparagine agar	Poorly developed, light gray	Colorless to yellowish cream	No
Glucose–nitrate agar	Well-developed, light gray	Yellowish brown	No
Glycerol–asparagine agar (ISP 5)	Scanty, light gray	Grayish yellow	No
Starch–ammonia agar (ISP 4)	Well-developed, light gray	Yellowish gray–brown	No

An additional advantage of strain Z-11-6 is that during cultivation in liquid media containing yeast or corn extract, it produces much less melanoid pigments than the wild strain; this facilitates enzyme extraction and purification.

Morphological and Cultural Properties of the Mutant Strain Streptomyces sp. Z-11-6

Vegetative hyphae are 0.6–0.8 μm in diameter and form a well-developed mycelium that does not exhibit a tendency toward fragmentation. The hyphae of aerial mycelium are 0.9–1.0 μm in diameter; upon maturation, they carry spore chains in the form of well-developed, regular, stretched spirals having 3 to 8 coils. Spores are ellipsoids measuring 0.6–0.7 × 0.9–1.0 μm; they are nonmotile and have a smooth surface. No structures resembling sclerotia, sporangia, picnidia, or cinnemia were detected.

The strain is gram-positive and not acid-fast. The cell wall contains L-diaminopimelic acid (type 1 cell wall [13]).

Cultural properties of strain Z-11-6 are given in Table 3. After 7–14 days of growth on oat agar at 28°C, dense agar-ingrowing concentric colonies 1–3 mm in diameter with scalloped edges are formed. The colony surface is covered with hydrophobic gray velvety aerial mycelium.

Growth of strain Z-11-6 occurs within a temperature range of 20–37°C, with an optimum at 26–28°C. The strain is aerobic.

Physiological and Biochemical Properties of the Mutant Strain Streptomyces sp. Z-11-6

Utilization of carbon sources. The range of carbon sources utilized was determined on ISP 9 medium [11] with various carbon sources at 28°C. The results were recorded on the 7th, 14th, and 21st days of cultivation; medium without a carbon source served as a negative control, and medium with D-glucose was used as a positive control. *Streptomyces* sp. Z-11-6 actively utilized glucose, arabinose, sucrose, xylose, mannitol, fructose, galactose, maltose, rhamnose, starch, glycerol, and casein hydrolysate. Poorer growth was observed on raffinose. Gelatin liquefaction was weak. Inositol was not utilized.

Utilization of nitrogen sources. The range of nitrogen sources utilized was determined on medium containing (%) glucose, 1; $MgSO_4 \cdot 7H_2O$, 0.05; NaCl, 0.05; $FeSO_4 \cdot 7H_2O$ – 0.001, K_2HPO_4, 0.1; agar, 1.5 (pH 7.0). The following nitrogen sources (0.1%) were tested: L-arginine, L-cysteine, L-histidine, L-methionine, L-phenylalanine, L-valine, L-serine, L-threonine, L-proline, DL-diamino-*n*-butyric acid, potassium nitrate, ammonium sulfate, and ammonium nitrate. The results were recorded on the 15th day of cultivation; medium without a nitrogen source served as a negative control, and medium with L-proline was used as a positive control. Strain Z-11-6 showed good growth with ammonium sulfate, proline, threonine, serine, histidine, methionine, and valine; poorer growth occurred with potassium nitrate.

Strain *Streptomyces* Z-11-6 was deposited at the Culture Collection of the Microbiology Department of the Moscow State University Biological Faculty.

Compared with the previously described producers of extracellular L-glutamate oxidase, the mutant strain *Streptomyces* Z-11-6 obtained in this work has certain advantages. The previously known producer strains accumulated 0.2–0.6 units of enzyme activity per ml of culture liquid after 7–10 days of cultivation in a complex medium containing peptone, yeast extract, glucose, and starch [4–6], whereas our strain Z-11-6 accumulated 1.6–1.8 units of enzyme activity per ml of culture liquid after 2 days of cultivation in a simpler and cheaper medium. The simpler composition of the medium and the low amount of melanoid pigments produced by strain Z-11-6 facilitate the isolation and purification of the enzyme.

Thus, we managed to isolate a wild strain producing extracellular L-glutamate oxidase and to obtain on its basis a genetically stable mutant strain accumulating as much as 1.6–1.8 units of enzyme activity per ml of culture liquid.

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