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NITRATE REDUCTASE ACTIVITY OF SULPHATE-REDUCING BACTERIA *Desulfomicrobium* sp. CrR3 AT DIFFERENT CONDITIONS OF THE CULTIVATION

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The aim of the research was to study nitrate reductase activity of sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3 under different conditions of cultivation. To determine nitrate reductase activity, sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3 were cultivated in a modified medium of Postgate C. Nitrate reductase activity was determined under different conditions such as t° of 15–45 °C and pH of 5–9. The highest nitrate reductase activity of *Desulfomicrobium* sp. CrR3 was at t° of 25–35 °C and pH of 7–8. Lowering or raising the temperature and the pH caused the inhibition of the nitrate reductase. The highest nitrate reductase activity was found in the soluble fraction of cells (12 μmol nitrite·min^{-1}·mg protein^{-1}). Activity was lower in the cells'debris (5 μmol nitrite·min^{-1}·mg protein^{-1}), while it was absent in the culture fluid. It was established that nitrate reductase of bacteria *Desulfomicrobium* sp. CrR3 is constitutive enzyme. The value of K_m for nitrate for studied enzyme of bacteria *Desulfomicrobium* sp.CrR3 is 1.2 mM, V_{max} is 15.7 μM nitrite·min^{-1}·mg protein^{-1}), indicating high affinity of the enzyme with the substrate. Thus, the cultivation conditions significantly affect the nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3.

Key words: sulphate-reducing bacteria, nitrate reductase activity, conditions of cultivation.

Sulphate-reducing bacteria are unique group of microorganisms that are able to use various inorganic compounds including sulphate and nitrate as terminal electron acceptors during the obtaining energy. This exact feature is of considerable interest in studying mechanisms of nitrate reduction activity of sulphate-reducing bacteria.

Nitrate reduction is widely spread among prokaryotes and described in few sulphate-reducing bacteria, particularly in the genera of *Desulfovibrio* [1, 2], *Desulfomonas* [1] and others.

High concentrations of nitrates inhibit sulfate reduction [2, 3].

Nitrate reductase (EC 1.6.6.2; NR) is a key enzyme of nitrate reduction with molibdopterynguaninedinucleotide that is a part of the active site [4]. It belongs to dymethylsulphoxydreductase and is found both in eukaryotic and in prokaryotic organisms and plays an important role in converting nitrogen compounds [1].

Depending on its location and function in cells of microorganisms cytoplasmic assimilatory nitrate reductase (NAS), periplasmic dissimilatory nitrate reductase (NAP) and dissimilatory membrane nitrate reductase (NAR) are described [4].

For the first time the presence of nitrate reductase in sulphate-reducing bacteria *Desulfovibrio desulfuricans* ATCC 27774 was shown by Bursakov and others. [5].

Some features of reduction of sulphates into the representatives of the genus of *Desulfomicrobium* at the presence of nitrates are described in the works of Sholyak and Dorosh [2, 3].

The aim of this work was to investigate nitrate reductase activity of sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3 under the different culture conditions.

Materials and Methods

The object of the study was sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3, resistant to the compounds of hexavalent chromium [6].

The bacteria were grown in the medium of Postgate C at 30 °C in test tubes of 25 ml under anaerobic conditions. The test tubes were filled with the medium completely and closed with rubber plugs [7].

To determine nitrate reductase activity of bacteria they were cultivated in the modified medium of Postgate C of the following composition (g / l), potassium dehydrogen — 0.5; calcium chloride hexahydrate — 0.06; magnesium chloride hexahydrate — 0,055; sodium lactate — 6; yeast extract — 1; sodium citrate dehydrate — 0.3; pH — 7.6. Nitrate was added in the form of aqueous solution of KNO_3 in the concentration of 10 mM.

Nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 was determined in cell-free extracts of bacteria grown in the modified medium of Postgate C under the argon atmosphere. For obtaining the cell-free extracts twice washed cells of *Desulfomicrobium* sp. CrR3 were resuspended in 0.05 M K^+-phosphate buffer (pH 7.0) (10^{-5} M of ethylendiamintetraacetate was added to it to bind heavy metal ions and 10^{-5} M of phenylmethyl-sulphonylftoride was added for inhibition of proteases, which function at pH higher than 7.0) and destroyed by ultrasound (22 kHz) 5 times /30 s at 4 °C with the help of the ultrasonic desintegrator UZDN-2T. The resulting disintegrate was centrifuged (4000 g, 30 min, 4 °C), the precipitate was separated, and the supernatant was used for further research as cell-free extract [8].

Nitrate reductase activity was determined spectrophotometrically at a wavelength of 540 nm at a temperature of 30 °C. The reaction mixture for the determination of nitrate reductase activity contained potassium phosphate buffer (25 mM, pH 7.3), 10 mM of K^+-nitrate, 0.05 mM EDTA and freshly prepared 2 mM NADH. The reaction was started by adding cell-free extract. 5 min later the reaction was stopped by adding 58 mM solution of sulphanilamide 3 M HCl and 0.77 mM of NEDA reagent (N-(1-naphthyl) — ethylendiamindehydrochloride) [9]. Protein content in each sample was determined by the Lowry method [10].

Nitrate reductase activity was determined at different temperatures (15, 20, 25, 30, 35 and 45 °C) and pH (5, 6, 7, 8 and 9), using acetate, K^+-phosphate and Tris-HCl buffers [11].

To investigate the ability of nitrate reductase NADH, NADPH and FADN as electron donors were used, being contributed to the reaction mixture at a concentration of 2 mM.

Kinetic parameters of nitrate reductase activity (K_m, V_{max}) were determined in an incubation mixture under standard conditions described above. K_m and V_{max} were calculated by linearization data in the coordinates (1/V) and (1/S) [12]. From the resulting Lineweaver-Burke equation basic kinetic parameters of nitrate reductase of sulphate –reducing bacteria *Desulfomicrobium* sp. CrR3 were received.

In the studies phenylmethyl-sulphonyl-ftoride and N-(1-naphthyl) — ethylen-diamindehydrochloride (Acros Organics, Belgium) were used. All other reagents were of domestic production (Scope seven, Symbioses) of "cc" and "cda" qualification.

Statistical analysis of the results was performed using Origin 6.1, and Microsoft Excel. The research results are presented as the average adjusted standard error ($M \pm m$). Differences of averages are considered to be probable at a significance level of $P \leq 0{,}05$ [13].

Results and Discussion

Nitrate reductase activity was studied in the process of cultivation of bacteria *Desulfomicrobium* sp. CrR3 in the medium of sulphates and nitrates as electron acceptors only.

While growing bacteria *Desulfomicrobium* sp. CrR3 in the environment of nitrates (10 mM) as a terminal electron acceptor the maximum of nitrate reductase activity was observed during the first days of cultivation. It was 12 μmol nitrite/min·mg protein. After the first day of the cultivation nitrate reductase activity decreased, which is obvious due to the decrease in the concentration of substrate in the medium (Fig. 1).

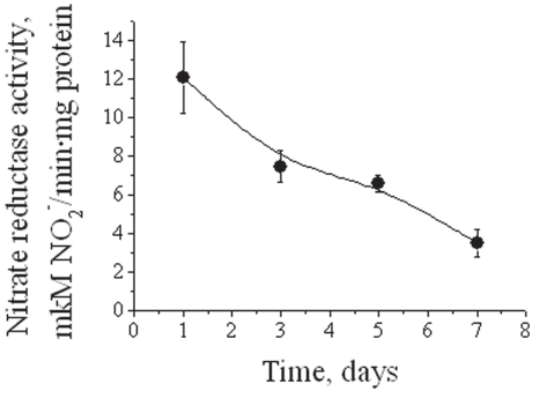

Fig. 1. **Nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 at the presence of 10 mM of nitrate in the medium**

The activity of nitrate reductase was found in the medium of sulphates as the sole electron acceptors. This allows us to assume that the nitrate reductase of bacteria *Desulfomicrobium* sp. CrR3 is constitutive enzyme (Fig. 2). Tarasova and others established the presence of the constitutive nitrate reductase in sulphate-reducing bacteria *D. vulgaris*1388 [14].

To determine probable localization of nitrate reductase in the cells of *Desulfomicrobium* sp. CrR3 the enzymatic activity was determined in the culture fluid, soluble fraction and debris of cells (Fig. 3). The highest nitrate reductase activity was found in the soluble fraction (12 μM nitrite/min·mg protein). The activity in cell debris fraction was about 2 times less (5 μmol nitrite/min·mg protein), and in the supernatant nitrate reductase activity wasn't detected (Fig. 3).

The obtained results show that nitrate reductase of bacteria *Desulfomicrobium* sp. CrR3 is localized in the cytoplasm (nitrate assimilation) or periplasmic space, that is proved by other scientists' researches[1, 4, 5].

The dependence of nitrate reductase activity from temperature was measured in the range of 15–45 °C (Fig. 4, *A*).

The maximum nitrate reductase activity was detected at the temperature range of 25–35 °C, which is the optimum temperature for life of the mesophilic sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3 (Fig. 4, *A*).

Nitrate reductase activity of cells of Desulfomicrobium sp. CrR3 depends on the pH (Fig. 4, *B*). The curve of the dependence

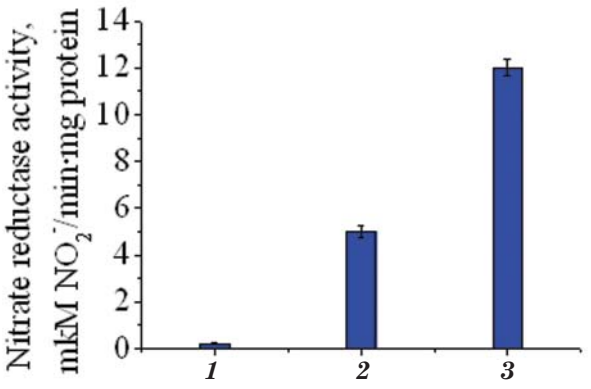

Fig. 3. **Nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 in different factions:**
1 — culture fluid;
2 — debris of cells;
3 — soluble fraction

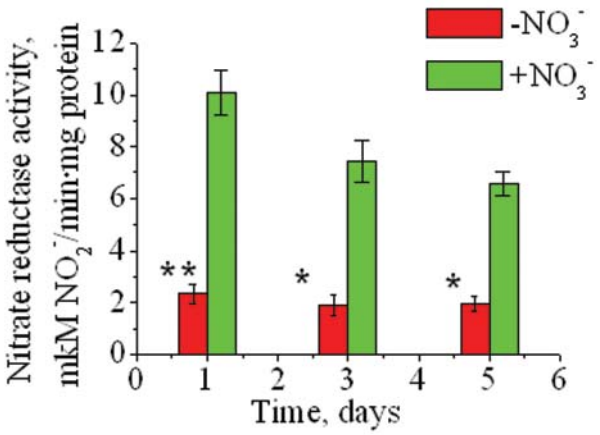

Fig. 2. **Nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 at the absence and presence of nitrates in the cultural medium**

Here in after: * $P < 0.05$; ** $P < 0.01$ relative to control activity.

Nitrate reductase activity at the presence of NO^{3-} in the medium served as a control activity

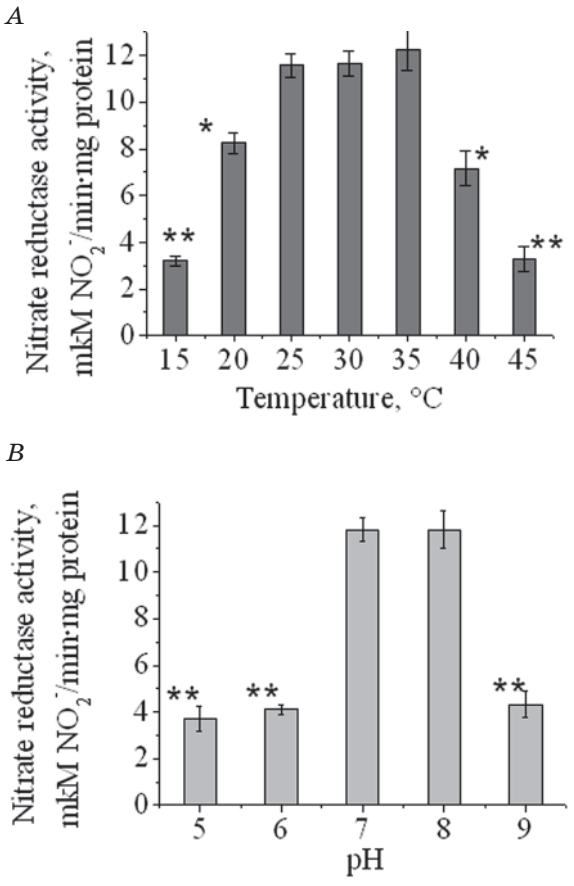

Fig. 4. **Nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 under the influence of temperature (*A*) and pH (*B*)**

Nitrate reductase activity under the optimal conditions such as temperature of 25 °C and pH 7 served as a control activity

of enzyme activity on pH is bell-shaped, and demonstrates the presence of at least one acid (H^+) and one alkaline (OH^-) piece in the active site of the enzyme [15]. Maximum activity of the enzyme was observed at pH of 7–8.

Thus, the maximum activity of nitrate reductase *Desulfomicrobium* sp. CrR3 showed at the temperature of 25–35 °C and pH 7.8 ($P \leq 0{,}05$).

The study of nitrate reductase activity of sulphate-reducing bacteria under the various culture conditions allows modeling the practical application of these bacteria in cleaning environment contaminated with nitrates and various organic substances that can be used as electron donors.

The results of research of the dependence of the activity of the enzyme on the concentration of the substrate (nitrate) are shown in Fig. 5. Basic kinetic properties of nitrate reductase reaction of bacteria *Desulfomicrobium* sp. CrR3, namely Michaelis-Menten constant and V_{max} were determined by linearization of data in coordinates of (1/V) and (1/S), where V is the activity of the enzyme, and S is the concentration of substrate (nitrate).

The growth of concentration of nitrate from 1 to 4 mM does not affect the nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 (phase "plateau"). Vmax for studied enzyme is 15.7 μM nitrite / min · mg protein.

Thus, we found out that nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 tends to saturate the enzyme with substrate.

The scores of nitrate reductase of microorganisms are described [16, 17], which allows us to compare the obtained data with results of other studies that is shown in the table.

The value of K_m for nitrate of studied enzyme of bacteria *Desulfomicrobium* sp. CrR3 is 1.2 mM, which substantially exceeds the nitrate K_m of *P. aeruginosa* (0.3 mM), *P. denitrificans* (0.3 mM), *T. lacustris* (0.23 mM) and *P. aerophilum* (0.14 mM), *Clostridium* sp. (0.5 mM) and *E. coli* (0.7 mM). Significantly higher K_m for nitrate reductase is described for bacteria *P. stutzeri* (3.8 mM) and *B. halodenitrificans* (2.7 mM), respectively (Table).

The electron donor for nitrate reductase can be both NADH and NADPH. It depends on the type of organism and the type of nitrate reductase. It was found that FADN can not be a donor of electrons for nitrate reductase in bacteria *Desulfomicrobium* sp. CrR3, but NADH and NADPH can (Fig. 6).

At the presence of NADPH in the reaction mixture the enzyme activity was 7 μM nitrite/ min·mg protein, whereas at the presence of NADH it was 12 μmol nitrite / min · mg protein ($P \leq 0{,}05$). Nitrate reductase of bacteria *Azotobacter indicum* can also accept the electrons from both NADH and NADPH [18].

The obtained results concerning the properties of nitrate reductase of bacteria *Desulfomicrobium* sp. CrR3 allow better understand the reduction of nitrate by sulphate-reducing bacteria, and find the optimal conditions (temperature, pH, substrate concentration, etc.) for effective nitrate reduction of these bacteria.

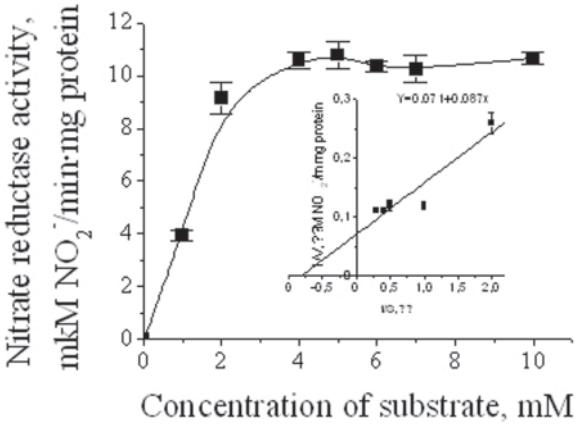

Fig. 5. **Dependence of nitrate reductase activity on substrate concentration in direct coordinates and Lineweaver-Burke coordinates**

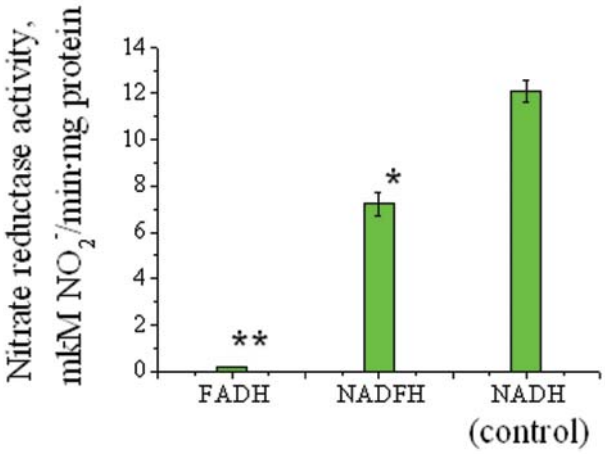

Fig. 6. **Nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 under the influence of various electron donors**

Features of nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 compared with those of other species of microorganisms

Species of microorganisms	Features of nitrate reductase in microorganisms		
	Optimum, pH	Optimum temperature, °C	K_m, mM
Pseudomonas sp.SH7	6.5–7.5	35	0.3
Pseudomonas stutzeri	7.2	ns	3.8
Pseudomonas isachenkovii	6.8–7	70–80	ns
Bacillus halodenitrificans	8–8.2	ns	2.7
Paracoccus denitrificans	7.5	ns	0,3
Thiothrix lacustris	7.2–7.3	63–65	0.23
Clostridium sp.	ns	ns	0.5
Pyrobaculum aerophilum	6.5	95	0.14
Escherichia coli	7–8	31	0.7
Desulfovibrio desulfuricans ATCC 27774	7.5–8.5	30	5.4
Desulfomicrobium sp. CrR3	7–8	25–35	1.2

Note: "ns" — not studied.

It was found that the nitrate reductase of the bacteria *Desulfomicrobium* sp. CrR3 was located in the cytoplasm (nitrate assimilation) or periplasmatic space.

The optimal conditions for nitrate reductase activity of the bacteria *Desulfomicrobium* sp. CrR3 are the following: the temperature of 25–35 °C and the pH of 7–7.5. Lowering or raising the temperature or the pH results in decreasing activity of the enzyme.

Electron donor for nitrate reductase activity of bacteria *Desulfomicrobium* sp. CrR3 can be both NADH and NADPH.

The value of K_m for nitrate for investigated enzyme of bacteria *Desulfomicrobium* sp.CrR3 is 1.2 mM, V_{max} is 15.7 mM nitrite / min · mg protein. This demonstrates the high affinity of the enzyme with the substrate.

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НІТРАТРЕДУКТАЗНА АКТИВНІСТЬ СУЛЬФАТВІДНОВЛЮВАЛЬНИХ БАКТЕРІЙ *Desulfomicrobium* sp. CrR3 ЗА РІЗНИХ УМОВ КУЛЬТИВУВАННЯ

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Метою роботи було дослідити нітратредуктазну активність сульфатвідновлювальних бактерій *Desulfomicrobium* sp. CrR3 за різних умов культивування. Для визначення нітратредуктазної активності сульфатвідновлювальні бактерії *Desulfomicrobium* sp. CrR3 культивували у модифікованому середовищі Постгейта С. Застосовували різні умови культивування: t° 15–45 °С, pH 5–9. Найвищу нітратредуктазну активність бактерії *Desulfomicrobium* sp. CrR3 виявляли за t° 25 та 35 °С і pH 7–8. Зниження чи підвищення температури та pH призводило до зниження нітратредуктазної активності. Найвищу нітратредуктазну активність виявлено в розчинній фракції клітин (12 мкмоль нітриту·хв$^{-1}$·мг протеїну$^{-1}$), в уламках клітин вона була нижча (5 мкмоль нітриту·хв$^{-1}$·мг протеїну$^{-1}$), а в культуральній рідині – відсутня. Встановлено, що нітратредуктаза у бактерій *Desulfomicrobium* sp. CrR3 є конститутивним ензимом. Величина K_m за нітратом для досліджуваного ензиму бактерій *Desulfomicrobium* sp. CrR3 становить 1,2 мкмоль, V_{max} — 15,7 мкмоль нітриту·хв$^{-1}$·мг протеїну$^{-1}$, що свідчить про високу спорідненість ензиму до субстрату. Таким чином, умови культивування істотно впливають на нітратредуктазну активність бактерій *Desulfomicrobium* sp. CrR3.

Ключові слова: сульфатвідновлювальні бактерії, нітратредуктазна активність, умови культивування.

НИТРАТРЕДУКТАЗНАЯ АКТИВНОСТЬ СУЛЬФАТРЕДУЦИРУЮЩИХ БАКТЕРИЙ *Desulfomicrobium* sp. CrR3 ПРИ РАЗЛИЧНЫХ УСЛОВИЯХ КУЛЬТИВИРОВАНИЯ

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Целью работы было исследование нитратредуктазной активности сульфатредуцирующих бактерий *Desulfomicrobium* sp. CrR3 при различных условиях культивирования. Для определения нитратредуктазной активности сульфатредуцирующие бактерии *Desulfomicrobium* sp. CrR3 культивировали в модифицированной среде Постгейта С. Применяли различные условия культивирования: t° 15–45 °С, pH 5–9. Наивысшую нитратредуктазну активность бактерии *Desulfomicrobium* sp. CrR3 проявляли при t° 25 и 35 °С, pH 7–8. Снижение или повышение температуры и pH приводило к снижению нитратредуктазной активности. Наивысшая нитратредуктазная активность обнаружена в растворимой фракции клеток (12 мкмоль нитрита·мин$^{-1}$·мг протеина$^{-1}$), в обломках клеток она была ниже (5 мкмоль нитрита·мин$^{-1}$·мг протеина$^{-1}$), а в культуральной жидкости — отсутствовала. Установлено, что нитратредуктаза у бактерий *Desulfomicrobium* sp. CrR3 является конститутивным энзимом. Величина K_m по нитрату для исследуемого энзима бактерий *Desulfomicrobium* sp. CrR3 составляет 1,2 мкмоль, V_{max} — 15,7 мкмоль нитрита·мин$^{-1}$·мг протеина$^{-1}$, что свидетельствует о высоком сродстве энзима к субстрату. Таким образом, условия культивирования существенно влияют на нитратредуктазную активность бактерий *Desulfomicrobium* sp. CrR3.

Ключевые слова: сульфатредуцирующие бактерии, нитратредуктазная активность, условия культивирования.