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THE REDUCTION OF NITRATE IONS BY BACTERIA *DESULFOMICROBIUM* SP. CRR3 UNDER THE DIFFERENT CULTIVATION CONDITIONS

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Optimization of cultivation conditions of sulphate-reducing bacteria in the medium with the addition of nitrate ions will help to develop the effective schemes that can be used in the cleaning up of contaminated soil and wastewater, that contain organic and inorganic substances. Our research focuses on the study of bacterial nitrate-reductive activity of *Desulfomicrobium* sp. CrR3 under the influence of different cultivation conditions. The aim was to study the usage of nitrate ions by sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3 under the influence of temperature, pH, the different adding donor of electrons and various concentrations of yeast extract. The effect of temperature (5, 15, 25, 35 and 45 °C), pH (5, 6, 7, 8 and 9), yeast extract (at the concentrations of 0.1, 0.25, 0.5 and 1 g/l) and different electron donor (sodium pyruvate, fumaric acid, glucose, ethanol and glycerol) on the reduction of nitrate ions by bacteria *Desulfomicrobium* sp. CrR3 was studied taking into consideration the change of growth, concentration of nitrate, nitrite and ammonium ions during 8 days cultivation. The optimum temperature for growth and reduction of nitrate ion by bacteria *Desulfomicrobium* sp. CrR3 was 25 and 35 °C and the optimum pH was 6-8. Yeast extract at a concentration of 0.5 and 1 g/l ensured the normal functioning of bacteria in the medium with nitrate ions. It has been found as a result of two-factor ANOVA analysis that the temperature and pH affected the growth and efficiency of reduction of nitrate ions with the presence of bacteria *Desulfomicrobium* sp. CrR3. The most effective electron donor for the reduction of nitrate ion by bacteria *Desulfomicrobium* sp. CrR3 was sodium lactate, sodium pyruvate and glucose.

Keywords: sulphate-reducing bacteria, nitrate ion, cultivation conditions.

Irrational usage of mineral nitrate-containing fertilizers leads to contamination of groundwater which is the source of drinking water [5, 12]. In addition, the presence of high concentration of oxanions of nitrogen facilitates the process of eutrophication of water [9].

In many countries, including our country, methods of wastewater treatment from nitrogen compounds are being developed. Physical and chemical technology of water purifying from nitrate ions are long term and ineffective [12] Bacterial–mediated amonification of nitrate ions and denitrification are considered to be an alternative to physical and physical-chemical methods of water cleaning contaminated with nitrogen compounds such as nitrates [6, 22]. The main parameters that limit the reduction of nitrate ions to ammonium ions is concentration of NO_3^-, the presence of organic compounds, temperature, pH, etc.

As a result of nitrate reduction carried out by bacteria *Pseudomonas, Klebsiella, Paracoccus* [14] etc the concentration of nitrate ions in groundwater decreases, which eliminates their negative impact on the environment and human health.

Sulphate-reducing bacteria have high biotechnological potential for cleaning soil and wastewater from various toxic substances (sulphates, nitrate ions, chromium and others) [6, 8, 15, 20]. Sholyak and others showed the efficiency of bacteria *Desulfomicrobium* sp. CrR3 for the treatment of wastewater from compounds of Cr (VI) [20].

It has been established that sulphate-reducing bacteria *Desulfovibrio* sp. and *Desulfomicrobium* sp. used nitrate ions as electron acceptors with the concentration of nitrate ions decreased, which eliminates their negative impact on the environment [6, 13, 21].

The aim of work was to investigate the usage of nitrate ion by sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3 under the influence of temperature, pH, organic substances and different concentrations of yeast extract.

Materials and methods

In this work sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3 excluded from wastewaters are used [21].

The bacteria were cultivated in a modified medium Posgate C of the following compounds (Ph 7.6) (g/l): potassium dehydrate − 0.5; calcium chloride hexahydrate − 0.06; magnesium chloride hexahydrate – 0.055; sodium lactate − 6; yeast extract − 1; sodium citrate dehydrate − 0.3; at the temperature of 30 °C, under anaerobic conditions. The tubes were completely filled with medium and closed using rubber stoppers [16]. Sterile water solution of KNO_3 was added.

Growth was calculated with the help of the device for determining the concentration of substances in solution-largest absorption of monochromatic using photoelectrocolorimeter (λ=340 nm).

Nitrate and nitrite ions were determined using n-naphthylethylendiamindichloride (λ=540 nm) [3]. The concentration of ammonium ions was determined using phenol reagent (λ=640 nm) [7].

The influence of temperature (15 °C, 25 °C, 35 °C and 45 °C), pH (5, 6, 7, 8 and 9), yeast extract (at concentrations of 0.1, 0.25, 0.5 and 1 g/l) and electron donor (sodium pyruvate, fumaric acid, glucose, ethanol and glycerol) addition on the reduction of nitrate ions using by bacteria *Desulfomicrobium* sp. CrR3 were examined. We measured changes in growth, concentration of nitrate-ions, nitrite ions and ammonium ions during the 8 days of cultivation.

Statistical analysis of the results was performed using Origin 6.1, Microsoft Excel and STATGRAPHICS Plus 5.0 programs. Using the experimental data, the basic statistical parameters (M–mean, m–standard error, M±m) have been calculated. The difference was reliable when $P \leq 0.05$ [1].

Results and discussion

The paper used bacteria *Desulfomicrobium* sp. CrR3 isolated from wastewater of city Lviv. These microorganisms are perspective objects for its using in the bioremediation processes. Beside sulfates and organic compounds, *Desulfomicrobium* sp. CrR3 able to use other compounds (nitrates, fumarate, pyruvate, etc.) in the metabolic processes. An important feature of the studied strain is its ability to reduction of the compound of hexavalent chromium, nitrite, which are toxic for living organisms.

Bacteria *Desulfomicrobium* sp. CrR3 used nitrate as an electron acceptor under anaerobic conditions, reducing nitrate ions to the ammonium ions [2, 7]. Bacteria *Desulfomicrobium* sp. CrR3 the most efficiently reduced nitrate ions at 25 and 35 °C (Fig. 1, A), with the content of nitrate ions in the medium decreased by 80 and 94 % after 3 days of cultivation, respectively, growth was about 2.5 g/l (Fig. 1, A). At temperature of 5 °C bacterial growth *Desulfomicrobium* sp. CrR3 significantly suppressed ($P<0.05$). However, it has been observed high efficiency of nitrate reduction at the temperature 45 °C ($P<0.05$). Similar results were obtained by Sulaiman Al-Zuhar and others that explored sulphate-reduction in sulphate-reducing microorganisms. All available substrates in the medium at high and low temperature were utilized for bacterial survival, but only a small amount of compounds used for growth [18, 19].

At the temperature of 25 °C the nitrite accumulated at a concentration of 3 mM in a medium of cultivation (Fig. 1, B). The concentration of ammonium ions was lower by 80−90 % at the temperature 5, 15 and 45 °C compared with the content of ammonium ions during bacteria cultivation at 25 and 35 °C (Fig. 1, B).

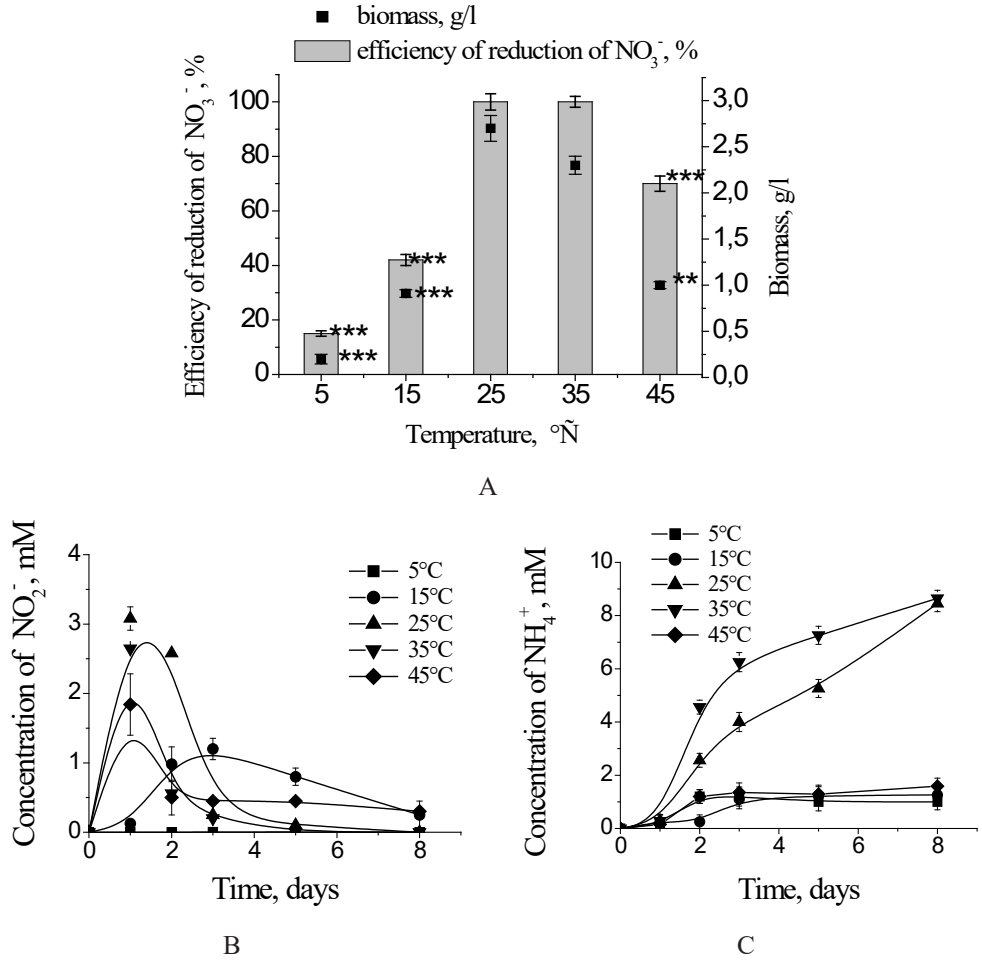


Fig. 1. Efficiency of nitrate reduction, growth of bacteria (A), the formation of nitrite ion (B) and ammonium ions (C) by bacteria *Desulfomicrobium* sp. CrR3 under the influence of different temperatures cultivation. Note: *** P<0.001; ** P<0.01 compared with control. Optimal conditions for the cultivation of sulfate-reducing bacteria (temperature – 25 °C, pH 7, concentration of yeast extract 1 g/l) in the medium served as a control

During bacteria *Desulfomicrobium* sp. CrR3 cultivation in the medium containing nitrate ions (10 mM) at pH 6, 7 and 8 for two days the content of nitrate ions decreased by 95 % (Fig. 2, A), while growth was almost 3 g/l (P≤0.05) (Fig. 2, A). It has been observed slight inhibition of efficiency of nitrate reduction by 15 and 10 %, at pH 5 and 9, respectively.

The growth of bacteria under these conditions also declined and amounted to about 1 g/l (P≤0.05). The maximum concentration of nitrites (3 mM) revealed on the first day of cultivation at pH 7−8 (Fig. 2, B). The concentration of ammonium ions peaked at the eighth day of cultiva-

tion and calculated 8 mM at pH 7−8 (Fig. 2, B). At pH 5 the ammonium ions were not observed during 8 days of cultivation.

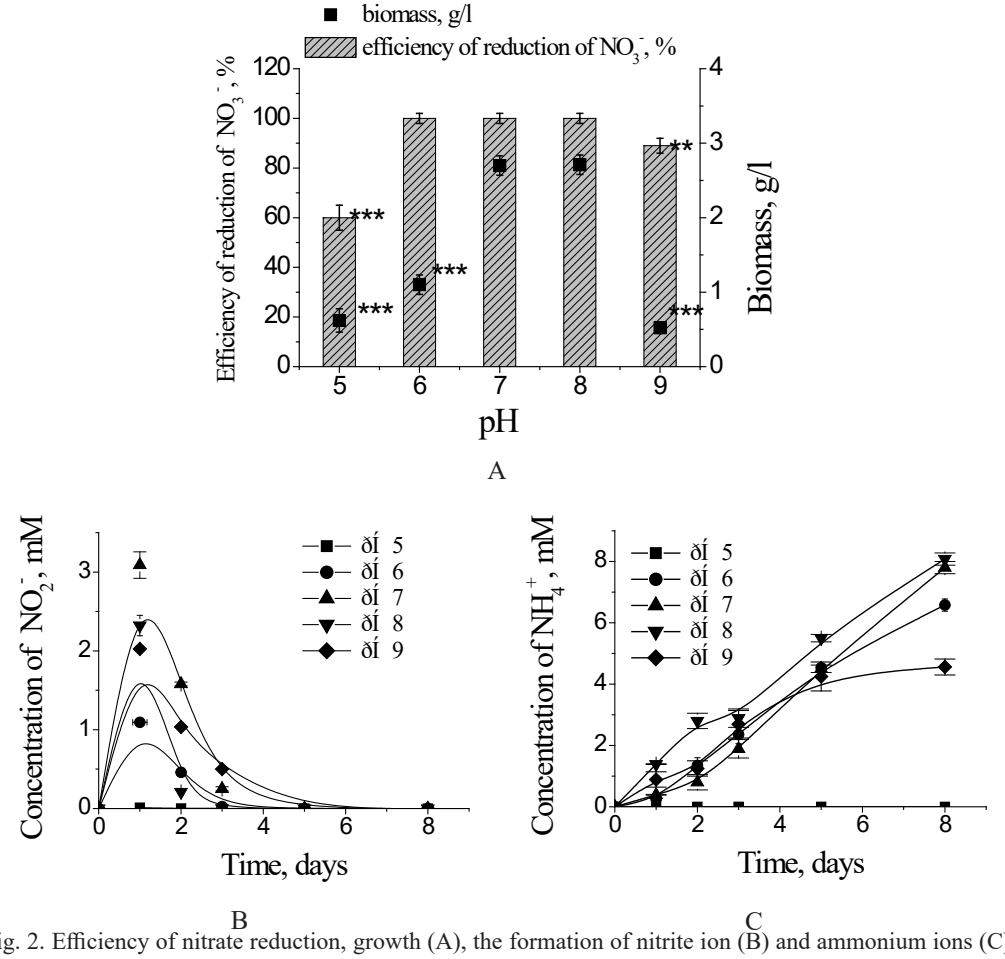


Fig. 2. Efficiency of nitrate reduction, growth (A), the formation of nitrite ion (B) and ammonium ions (C) by bacteria *Desulfomicrobium* sp. CrR3 under the influence of different pH of medium cultivation. Note: *** P<0.001; ** P<0.01 compared with control. Optimal conditions for the cultivation of sulfate-reducing bacteria (temperature – 25 °C, pH 7, concentration of yeast extract 1 g/l) in the medium served as a control

The yeast extract is one of the components in the medium of Posgate C for the cultivation of sulphate-reducing bacteria. Saez-Navarette and others have found out that the optimal concentration of yeast extract for sulphate reduction by the sulphate-reducing bacteria *Desulfobacterium autotrophicum* was 0.5 g/l at a temperature of 38 °C [17].

The influence of yeast extract on the growth and reduction of nitrate are important in the process of cultivation of sulfate-reducing bacteria. For reduce the cost of media of Posgate C for cultivation bacteria *Desulfomicrobium* sp. CrR3 we have studied the influence of yeast extract on the growth and reduction of nitrate ions of microorganisms studied.

Bacteria *Desulfomicrobium* sp. CrR3 accumulated maximum growth (2.7 g/l) in a modified medium of Posgate C contained of yeast extract of 1 g/l (Fig. 3 A). The lowering of concen-

tration of yeast extract to 0.5 g/l it has been observed a slight decrease of growth (P≤0.05) (Fig. 3, A), but the effectiveness of nitrate reduction was about 100 % (P≤0.05). At the lowering of concentrations of yeast extract in the medium to 0.1 and 0.25 g/l growth decreased to 1,5 −1,75 g/l, respectively (P≤0.05). In the absence of yeast extract, bacterial growth was 0.8 g/l, the efficiency of nitrate reduction was 40 % (P≤0.05).

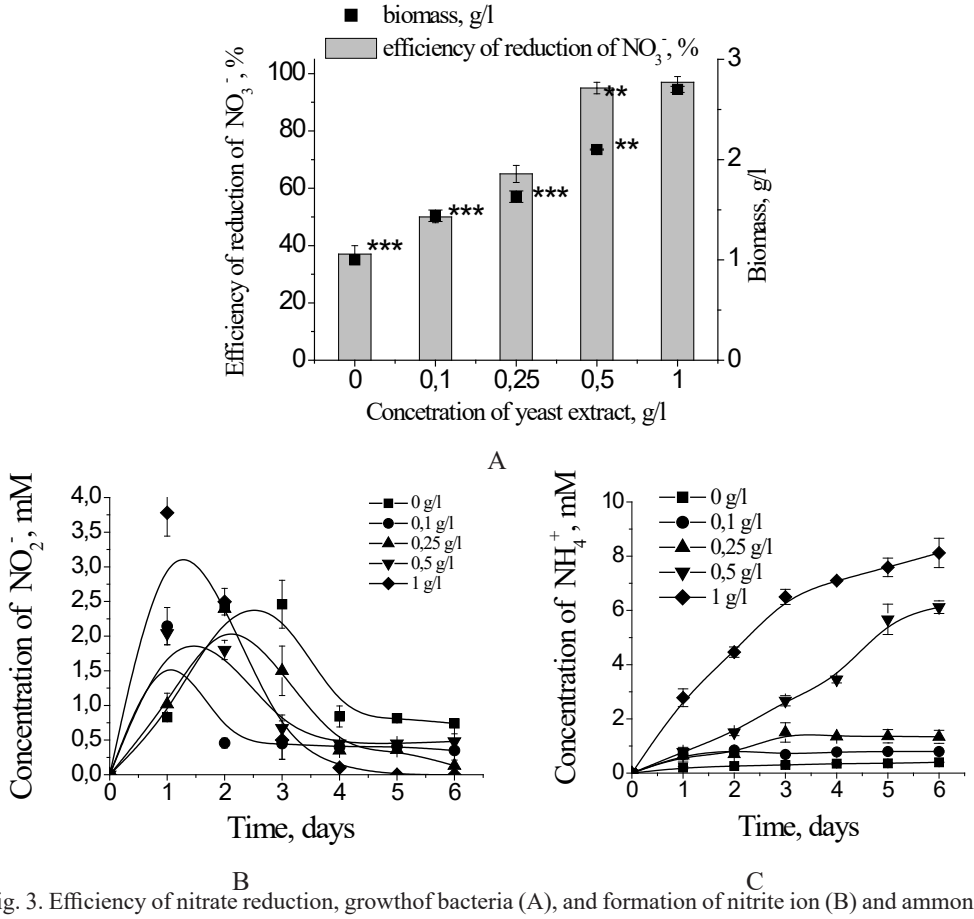


Fig. 3. Efficiency of nitrate reduction, growthof bacteria (A), and formation of nitrite ion (B) and ammonium ions (C) by bacteria *Desulfomicrobium* sp. CrR3 under the influence of different concentrations of yeast extract in a medium of cultivation. Note: *** P<0.001; ** P<0.01 compared with control. Optimal conditions for the cultivation of sulfate-reducing bacteria (temperature – 25 °C, pH 7, concentration of yeast extract 1 g/l) in the medium served as a control

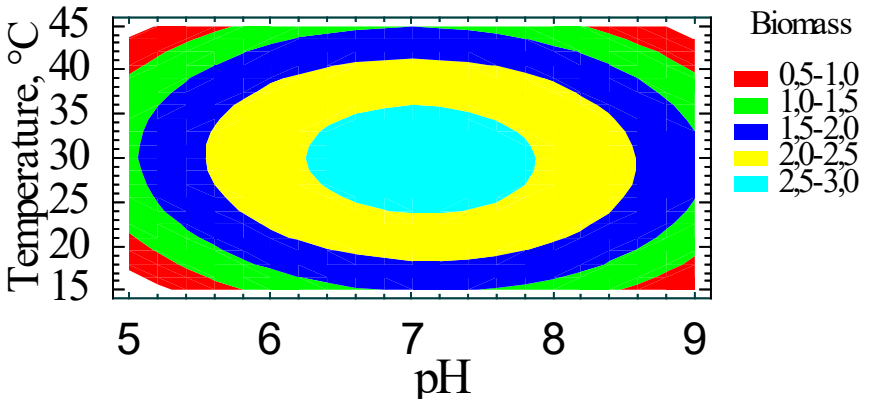
The intermediates of nitrate reduction by bacteria *Desulfomicrobium* sp. CrR3 were nitrite ions, which were accumulated in the provided medium cultivation at a concentration of 3 mM (Fig. 3, B), the final products are ammonium ions at a concentration of 8 mM (Fig. 3, B).

Thus, the growth and the nitrate reduction by bacteria *Desulfomicrobium* sp. CrR3 changed different concentrations of yeast extract. The increase of the concentration of yeast extract up to 2 or more g/l where was uneconomical, but the decrease from 0.25 to 0 g/l depressed the nitrate reduction by bacteria *Desulfomicrobium* sp. CrR3 in the case of our experiments.

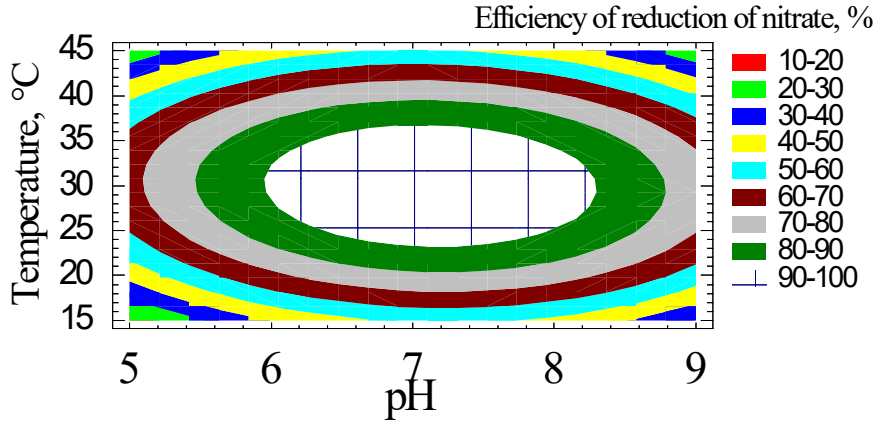
The control in all experiments served as the optimal conditions for sulfate-reducing bacteria: pH 7, temperature 25 °C, the concentration of yeast extract 1 g/l. At the absence of bacteria

Desulfomicrobium sp. CrR3 the content of nitrate ions did not change during 8 days of cultivation.

Using double-factor analysis it has been found, that the temperature and pH affected on the growth of bacteria *Desulfomicrobium* sp. CrR3 estimably (Fig. 4, A). The optimal temperature of what for maximum accumulation of growth of bacteria (2.5–3 g/l) was 25–35 °C and pH was 6.5–8.



A



B

Fig. 4. Effect of temperature and pH on the growth (A) and efficiency of nitrate reduction (B) by bacteria *Desulfomicrobium* sp. CrR3

It has been observed that if the temperature and pH effect on the nitrate reduction by bacteria *Desulfomicrobium* sp. CrR3 simultaneously, the temperature played significant role in the nitrate reduction (Fig. 4, B). The optimal conditions for nitrate reduction by bacteria *Desulfomicrobium* sp. CrR3 were the temperature 25 and 35 °C and pH 6 and 8 of cultivation medium.

It has been observed that bacteria *Desulfomicrobium* sp. CrR3 reduced nitrate ions completely in the medium containing lactate, glucose and fumaric acid as donor electrons. When as the donor of electrons sodium lactate was added the efficiency of nitrate-ion usage was 98.7 %. The growth of bacteria was 2–3.5 g/l in the presence of sodium glucose, sodium pyruvate and fumaric acid in the medium. Significantly less growth was accumulated in the medium with glycerine (1.2 g/l) and ethanol (1.5 g/l). It has been observed, that the accumulation of ammo-

nium ions in the concentration 4.2–8.5 mM as a final product of nitrate reduction by bacteria *Desulfomicrobium* sp.CrR3 in a medium cultivation. The most effective electron donors for the nitrate reduction by bacteria *Desulfomicrobium* sp. CrR3 was sodium lactate, sodium pyruvate and glucose (see Table).

Effect of different electron donors on the nitrate reduction by bacteria *Desulfomicrobium* sp. CrR3

Electron donors	Growth, g/l	Efficiency of nitrate reduction, %	Concentration of NH_4^+, mM
Sodium lactate	2.8±0.6	98.7±2.2	7.6±0.6
Glucose	3.5±0.8	99.2±3.1	8.5±0.3
Fumaric acid	2.9±0.3	95.2±1.7	8.2±0.3,
Sodium pyruvate	2.3±0.7	85.0±2.1	8.3±0.1
Glycerin	1.2±0.2	60.0±1.8	5.3±0.3
Ethanol	1.5±0.4	70.0±1.5	4.2±0.2

Similar results were obtained by K. Sholyak and others [21] on the impact of various organic compounds to sulphate reduction by bacteria *Desulfomicrobium* sp. CrR3. Sodium lactate, sodium pyruvate, glycerin and ethanol are the main electron donors for sulphate-reducing bacteria of genera *Desulfomicrobium* [11]. Fructose, acetate, fumaric acid, methanol, pyruvate and malate do not provide the growth in the medium with sulphates by bacteria *Desulfomicrobium hypogeium* sp. Nov. [10].

Thus, the optimal conditions for nitrate reduction by bacteria *Desulfomicrobium* sp. CrR3 were the temperature of 25 and 35 °C, pH of 6–9, the concentration of yeast extract of 0.5–1 g/l, and the electron donors such as sodium lactate, fumaric acid and glucose. Optimization of cultivation conditions of sulphate-reducing bacteria *Desulfomicrobium* sp. CrR3 in the medium with nitrate ions was important in order to develop effective schemes aimed to treat wastewater and contaminated soil containing organic substances with nitrate ions.

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ВІДНОВЛЕННЯ НІТРАТ-ЙОНІВ БАКТЕРІЯМИ *DESULFOMICROBIUM* SP. CRR3 ЗА РІЗНИХ УМОВ КУЛЬТИВУВАННЯ

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Оптимізація умов культивування сульфатвідновлювальних бактерій у середовищі з нітратами дасть змогу розробити ефективні біоремедіаційні схеми, які можна застосовувати в очищенні забруднених стічних вод і ґрунтів, які містять органічні та неорганічні речовини. Наші дослідження спрямовані на вивчення нітратвідновлювальної активності бактерій *Desulfomicrobium* sp. CrR3 за впливу різних умов культивування. Метою роботи було дослідити використання нітрат-йона сульфатвідновлювальними бактеріями *Desulfomicrobium* sp. CrR3 за впливу температури, pH, різних концентрацій дріжджового екстракту і донорів електронів. Вплив температури (5, 15, 25, 35 та 45 °C), pH (5, 6, 7, 8 та 9), дріжджового екстракту (в концентрації 0,1, 0,25, 0,5 та 1 г/л) та донорів електронів (натрій пірувату, фумарової кислоти, глюкози, етанолу та гліцерину) на відновлення нітрат-йона у бактерій *Desulfomicrobium* sp. CrR3 досліджували за зміною біомаси бактерій, концентрації нітрат-йона, нітрит-йона та йонів амонію впродовж 8 діб культивування. Оптимальна температура та pH для росту бактерій і процесу нітратредукції у бактерій *Desulfomicrobium* sp. CrR3 становить 25−35 °C та pH 6−8. Дріжджовий екстракт у концентрації 0,5 та 1 г/л повністю забезпечує функціонування бактерій у середовищі з нітрат-йонами. За результатами двофакторного аналізу ANOVA встановлено, що температура та pH впливають на біомасу й ефективність відновлення нітрат-йона бактеріями *Desulfomicrobium* sp. CrR3. Найефективнішими донорами електронів для відновлення нітрат-йона бактеріями *Desulfomicrobium* sp. CrR3 були натрій лактат, натрій пірувat і глюкоза.

Ключові слова: сульфатвідновлювальні бактерії, нітратредукція, умови культивування.