

DENITRIFICATION EFFECT OF BACTERIA *THIOBACILLUS DENITRIFICANS*

DENITRIFIKACE ČINNOSTÍ BAKTERIÍ *THIOBACILLUS DENITRIFICANS*

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Abstract:

Thiobacillus denitrificans belongs to obligate chemolithoautotrophic and facultative anaerobic microorganisms. From an environmental perspective, *Thiobacillus denitrificans* can remediate natural groundwater and engineered water treatment systems by removing excess nitrate with coupling the oxidation of inorganic sulfur compounds to the reduction of oxidized nitrogen compounds (such as nitrate, nitrite). Work is focused on the chosen effects on the rate of autotrophic denitrification activity of bacteria *Thiobacillus denitrificans*. It is proven that sunshine does not affect denitrification activity. Sulfur particles with diameter less than 5 mm, high concentration of bacteria *Thiobacillus denitrificans* and temperature in the range from 21 to 37 °C have the positive effects on denitrification process. On the other hand, acidity of solution (pH less than 5,5) has negative effect on denitrification process, in the case where the limestone is not present.

Keywords:

Denitrification, pH, *Thiobacillus denitrificans*, waste water.

Introduction:

Thiobacillus denitrificans is located in the β -subclass of the *Proteobacteria* and belongs to obligate chemolithoautotrophic and facultative anaerobic microorganisms. Bacterial cells are short rod-shaped and they may be motile through a polar flagellum. *Thiobacillus denitrificans* is capable of effective reductive fixation of carbon dioxide by the Calvin cycle under both aerobic and anaerobic conditions. It uses oxidation of reduced inorganic sulfur compounds for respiratory reduction of oxygen or, under anaerobic conditions, of nitrate (or nitrite) to dinitrogen gas. It means, that *Thiobacillus denitrificans* belongs to important group of autotrophic bacteria occurring in nature linking the biogeochemical cycles of nitrogen and sulfur. It is found in soil, mud, marine and freshwater sediments, domestic sewage, industrial waste-treatment lagoons and digestion tanks. Denitrification capacity of *Thiobacillus denitrificans* could be used in the future as an alternative method for the removal of nitrates and nitrites from contaminated water. These bacteria also may be involved in the processes associated with the removal of unwanted sulfides such as acid gases and water. This is important from the environmental point of view. *Thiobacillus denitrificans* is not toxic and it is not among to pathogenic microorganisms, so we can consider their use in the long term (Beller and col., 2006; Kelly, Wood, 2000; Kelly, Wood, 2000; Shao, Zhang, Fang, 2010).

Material and methods:

Four series of batch experiments (A - D) were prepared to verify the selected effect on the course and speed of denitrification by *Thiobacillus denitrificans*. The experiments were carried out in glass bottles of one liter (total 12 pieces). Bottles differed in substance contained and experimental conditions, both between sets and between experiments. Specific contents of bottles is shown in table (Tab. 1).

Four bottles were prepared at series A of batch experiments. Bottles were labeled L7, L8, L9 and L10, and they were used for study of influence of solar radiation to speed and course of the denitrification by *Thiobacillus denitrificans*. All bottles contained 50 g sulfur pearls (with size from 0,5 to 5 mm) with bacteria *Thiobacillus denitrificans* and 1 l of a solution of sodium nitrate at concentration of 50 mg/l nitrate. In the bottles labeled L7 and L8 was added 50 g of limestone rubble. Sulfur pearls with bacteria *Thiobacillus denitrificans* and limestone rubble were purchased from the company Aqua Medic. All experiments in this series were carried out under laboratory conditions for free access of

air. Bottles labeled L7 and L9 were also left at the same conditions throughout the measurement in the dark.

Tab. 1: Summary of experiments and conditions

Bottle	Sulfur with <i>T. denitrificans</i> [g]	Sulfur [g]	CaCO ₃ [g]	NO ₃ ⁻ [mg/l]	H ₂ O [ml]	Solution active bacteria in water	Conditions
		Fraction size d [mm]					
L2	50	-	-	100	-	-	Light, 22° C
L3	50	-	-	-	1000	-	Light, 22° C
L7	50	-	50	50	-	-	Darkness, 22° C
L8	50	-	50	50	-	-	Light, 22° C
L9	50	-	-	50	-	-	Darkness, 22° C
L10	50	-	-	50	-	-	Light, 22° C
L18	50	-	50	50	-	-	Light, 7° C
L20	50	-	50	50	-	-	Light, 11° C
L21	50	-	50	50	-	-	Light, 37° C
L22	-	50	50	50	-	10	Light, 22° C
		10 < d					
L23	-	50	50	50	-	10	Light, 22° C
		5 < d < 10					
L24	-	50	50	50	-	10	Light, 22° C
		d < 5					

Two bottles were prepared at series B of batch experiments and were labeled L2 and L3. These experiments were used for study of pH effect to speed and course of the denitrification. Bottles contained 50 g of sulfur pearls with *Thiobacillus denitrificans*. In the bottle labeled L2 was added 1 l of a solution of sodium nitrate at concentration 100 mg/l nitrate and in the bottle labeled L3 was added 1 l of drinking water. Both experiments at series B were carried out under laboratory conditions for free access of air.

At series C of batch experiments, three bottles were prepared. These bottles were labeled L18, L20 and L21 and were used for study of influence of temperature to course and speed of the denitrification by *Thiobacillus denitrificans*. All the bottles contained 50 g of sulfur pearls with bacteria, 50 g of limestone rubble and 1 l of solution of sodium nitrate at concentration 50 mg/l nitrate. Experiments were carried out under laboratory conditions for free access of air, but every bottle in the series C was kept at different temperature - L18 at 7 °C, L20 at 11 °C and L21 at 37 °C.

Three bottles were prepared at series D of batch experiments. They were labeled L22, L23 and L24. These experiments were used for study of influence of particle size of sulfur to course and speed of the denitrification. All the bottles at series D contained 50 g of limestone rubble, 50 g of sulfur with the different particle size, 10 ml of solution of bacteria *Thiobacillus denitrificans* in water and 1 l of solution of sodium nitrate at concentration 50 mg/l nitrate. In the bottle L22 was added sulfur having particle size larger than 10 mm, in the bottle L23 from 5 to 10 mm and in the bottle L24 smaller than 5 mm. Experiments were carried out under laboratory conditions for free access of air.

In all experiments the values of nitrate and nitrite concentration, pH, redox, temperature and dissolved oxygen concentration were measured. All assays were performed according to appropriate standards. It was also observed the concentration of bacteria in all experiments by culturing of determining the number of microorganisms. The results were expressed using CFU per 100 ml of sample (Colony forming unit). It was used medium containing L-asparagine and nitrates (Asparagine Nitrate Medium from HiMedia) as a culture medium.

Results and discussion:

Effect of pH process of denitrification:

During the autotrophic denitrification by bacteria *Thiobacillus denitrificans*, sulfur is oxidized to sulfuric acid, which acidifies the environment. Denitrification processes are stopped when pH drops below 5,5. Therefore it is necessary to add limestone (or otherwise maintain the pH in the neutral range) to the reaction. The limestone reacts with sulfuric acid and in this reaction are formed calcium sulfate and carbon dioxide. Carbon dioxide can be utilized as the carbon source by *Thiobacillus denitrificans*. The pH remains in the neutral range (Liu, Koenig, 2002; Moon and col., 2004).

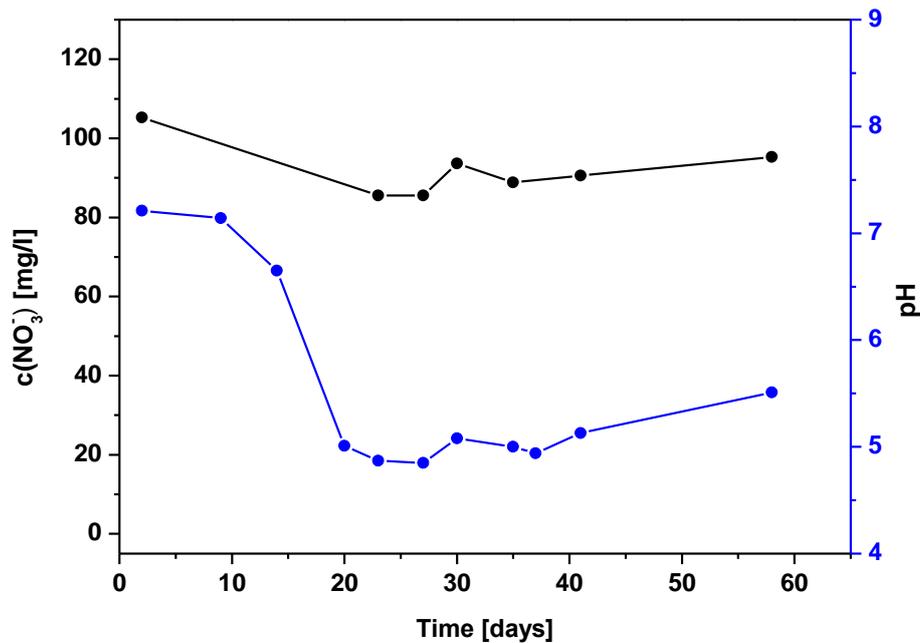


Fig. 1: Degradation of nitrate in the bottle L2 (in the absence of limestone)

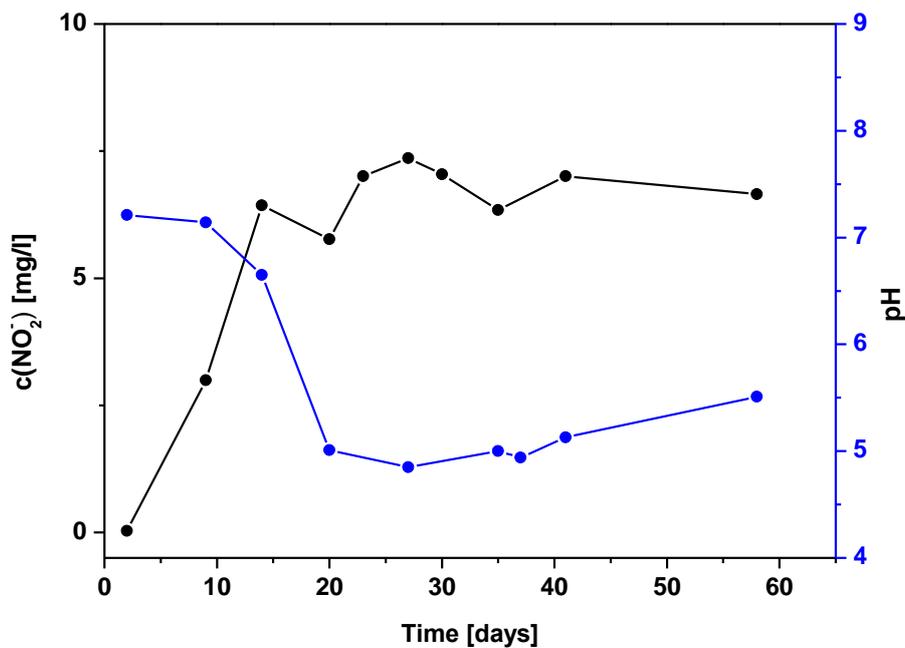


Fig. 2: Formation of nitrite in the bottle L2 (in the absence of limestone)

Experiments labeled L2, L3, L9 and L10 were observed to verify the effect of pH to the course and speed of denitrification processes. These bottles contained only sulfur pearls with bacteria and solution of sodium nitrate. In the figure 1 (Fig. 1) and 2 (Fig. 2), there is graphically illustrate the course of denitrification in the bottle L2 - figure 1 using values depending nitrate concentration and pH versus time and figure 2 using values depending nitrite concentration and pH versus time.

These experiments demonstrated, that bacteria need to adapt 10 - 15 days. After this phase, there are significant changes in the monitored parameters - reduction of nitrate concentration (Fig. 1) and the corresponding increase of nitrite concentration (Fig. 2). After decrease of pH below 5,5, denitrification processes were stopped, because of the absence of limestone to neutralize the sulfuric acid.

In the bottle L3 was a low initial value of the nitrate concentration (approximately 5 mg/l). There was complete elimination of nitrates and nitrites even before the pH drops below 5,5. Comparable amounts of nitrate was reduced also in bottles L2, L9 and L10. However, since there was a much higher initial nitrate concentration, this decrease is classified only as a reduction in nitrate concentration.

Effect of solar radiation to process of denitrification

Experiments labeled L7, L8, L9 and L10 were observed to verify the effect of solar radiation to the course and speed of denitrification. These bottles contained sulfur pearls with bacteria, solution of sodium nitrate and limestone - limestone was added only in the bottles labeled L7 and L8. Bottles L7 and L9 were left throughout the experiment in the dark. Figure 3 (Fig. 3) shows graphically the progress of the denitrification in the bottles L7 and L8, which is expressed in dependence of the values of nitrate and nitrite concentration to time.

Thiobacillus denitrificans does not belong to the group of photosynthetic organisms, thus the assumption was verified that the absence of solar radiation has no effect to the course of denitrification (Beller a col., 2006; Kelly, Wood, 2000; Kelly, Wood, 2000; Shao, Zhang, Fang, 2010). This is shown on figure 3 (Fig. 3) - denitrification runs identically in the bottles L7 and L8. Denitrification processes run also identically in the bottles labeled L9 and L10, but there was not complete elimination of nitrates and nitrites, because these bottles not contained a limestone. This corresponds to a decrease in pH below 5,5, thereby to stop the denitrification processes.

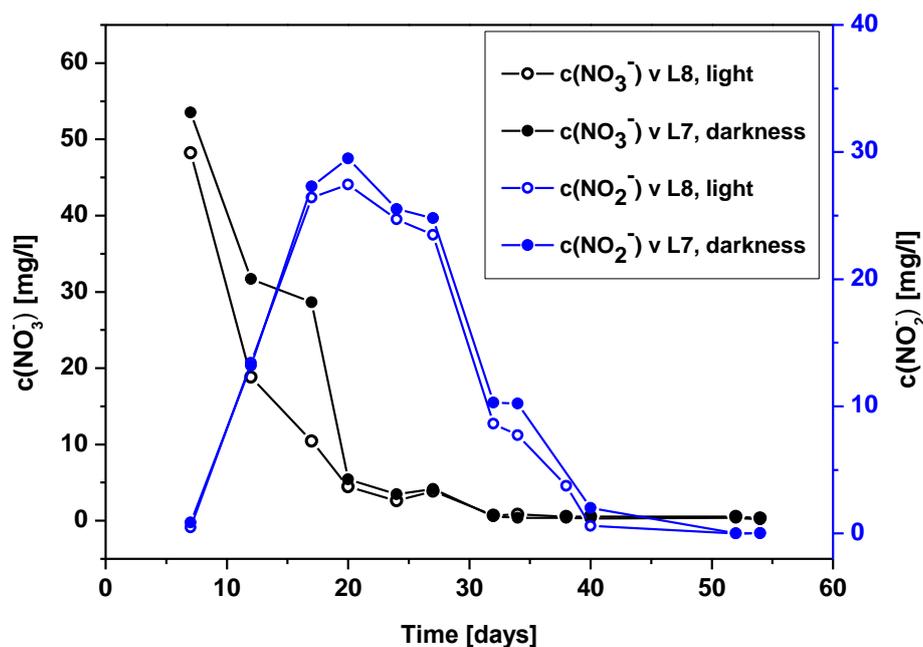


Fig. 3: The course of denitrification in the bottles L7 and L8.

Effect of temperature to process of denitrification

Experiments labeled L18, L20 and L21 were observed to verify the effect of temperature to the course and speed of denitrification. These bottles contained sulfur pearls with bacteria, limestone and solution of sodium nitrate. Each of the bottles was tempered at a different temperature.

Optimal temperature for autotrophic denitrification by *Thiobacillus denitrificans* is in the range of values from 33 to 35 °C (Oh and col., 2000). The experiments at three different temperatures have confirmed this fact - denitrification ran the fastest at 37 °C and the slowest at 7 °C.

Effect of particle size of sulfur to process of denitrification

Experiments labeled L22, L23 and L24 were observed to verify the effect of particle size of sulfur to the course and speed of denitrification. These bottles contained bacteria *Thiobacillus denitrificans*, limestone, solution of sodium nitrate and sulfur with different size of particles.

Figure 4 (Fig. 4) and figure 5 (Fig. 5) show graphically the progress of the denitrification in these bottles, which is expressed in dependence of the values of nitrate (Fig. 4) and nitrite (Fig. 5) concentration to time.

The experiments with three different sizes of sulfur particles showed the fastest elimination of nitrates and nitrites in the bottle labeled L24. This bottle contained sulfur with particle size smaller than 5 mm. The elimination of nitrates and nitrites in the bottle L23 was lower than elimination in the bottle L24. In the bottle labeled L22 with particle size larger than 10 mm, there was the lowest elimination. For autotrophic denitrification by *Thiobacillus denitrificans* is preferable to use smaller sulfur particles, which have larger reaction surface.

The experiments in this series were observed a shorter period than the previous series (only 26 days), because these bottles contained a solution of already active bacteria *Thiobacillus denitrificans*. The adaptation phase was conspicuously accelerate.

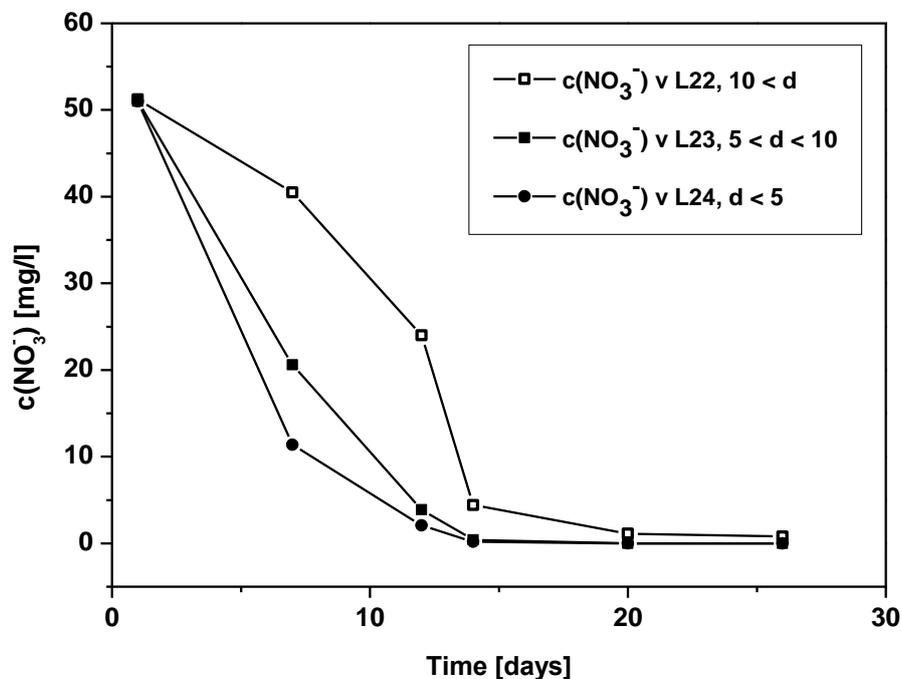


Fig. 4: Degradation of nitrate in the bottles L22, L23 and L24

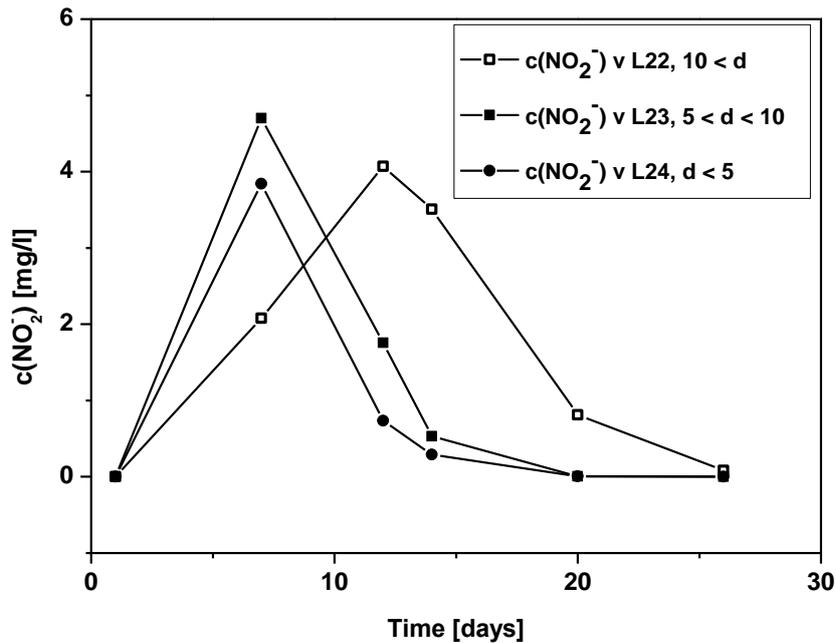


Fig. 5: Formation and reduction of nitrite in the bottles L22, L23 and L24

Conclusion:

In the experiments were tested various initial nitrate concentration (from 5 to 100 mg/l) for study of selected influences to the autotrophic denitrification activity of bacteria *Thiobacillus denitrificans*. These bacteria were able to remove the tested nitrate range, because in all cases, there were initiated denitrification processes. It was demonstrated, that solar radiation has no effect to the course and speed of autotrophic denitrification by *Thiobacillus denitrificans*. Further, it was shown that pH lower than 5,5 has a significant negative effect to these denitrification processes. Conversely, for bacteria is preferred to use of smaller particles of sulfur with larger reaction surface. From the practical point of view, there is very important influence of the temperature, since the necessity of increasing the temperature is costly. The experiments showed that the rate of denitrification was significantly changing at different temperatures - denitrification at 37 °C was approximately 1,5 - 2 times faster than at 11 °C.

Acknowledgement:

Author gratefully thanks for financial support to SGSFChT 2014006.

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