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THE EFFECT OF HEAVY METAL IONS ON THE ACTIVITY OF DENITRIFIERS FROM *BACILLUS* GENUS

The effect of such heavy metal ions as zinc, cadmium, mercury and lead on the proliferation and the activity of *Bacillus* bacteria in the denitrification process is reviewed. The concentrations at which these metals change and inhibit the nitrate reduction are presented. Toxicity of cations is arranged in descending order: $Pb^{2+} < Zn^{2+} < Cd^{2+} < Hg^{2+}$.

1. INTRODUCTION

The studies on biological purification of wastes have shown that *Bacillus* bacteria – isolated in our laboratory, could be used for simultaneous removal of nitrates and organic compounds from high-nitrate raw industrial wastes [1]. These microorganisms oxidise reduced carbon compounds and transfer the electrons to nitrates which are reduced to nitrogen. Under standard conditions, denitrification follows the kinetic model of irreversible subsequent reactions with stable intermediate product according to the following equation [3, 4]:

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2.$$

Agricultural and industrial wastes comprise in most cases some amounts of heavy metals [5]. Studies done so far [5]–[8] have shown that these metals are toxic to all living organisms and, of course, to denitrifying bacteria, too [9]. It is necessary therefore to find the concentrations affecting denitrification process and the mechanism of their action. We investigated the effect of Zn^{2+}, Cd^{2+}, Hg^{2+} and Pb^{2+} on the culture growth and nitrate reduction.

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2. MATERIALS AND METHODS

Bacillus bacteria were isolated and identified as described previously [1], [2]. Cells were grown in tightly closed bottles (capacity – 20 cm^3), filled with 10 cm^3 of medium which contained (in g/dm^3): sodium lactate as the sole carbon source (the C/N ratio is 1.8), KNO_3, 10; NH_4Cl, 0.25; $MgSO_4 \cdot 7H_2O$, 0.5; $Fe(NO_3)_3 \cdot 9H_2O$, 0.42; $Na_2HPO_4 \cdot 12H_2O$, 2.5; trace elements: $CaCl_2$, 0.05; $MnSO_4 \cdot 4H_2O$, $3.47 \cdot 10^{-2}$; H_3BO_3, $8.6 \cdot 10^{-3}$; $CuSO_4 \cdot 5H_2O$, $1.2 \cdot 10^{-2}$; $Co(NO_3)_2 \cdot 6H_2O$, $2.8 \cdot 10^{-3}$; $Zn(NO_3)_2 \cdot 6H_2O$, $1 \cdot 10^{-3}$; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, $1.8 \cdot 10^{-3}$; $NaHSeO_3$, $0.88 \cdot 10^{-5}$; $Ni(NO_3)_2 \cdot 6H_2O$, $1.8 \cdot 10^{-4}$. This culture medium was inoculated with 0.4 cm^3 of inoculum every 24 hours and it was held in thermostat at 37°C. The following heavy metals: zinc, cadmium, mercury and lead in the form of nitrate salts were added to medium before sterilization. The concentration of nitrate was maintened by the reduction of potassium nitrate. After autoclaving, the pH of medium was adjusted with Na_2CO_3 to the value of 8.0.

Nitrate concentration was determined using ion selective electrode. Nitrite concentration was measured colorimetrically at 520 nm, with sulphanilamid and N-1naftiletylendiammonium chloride [10] – using spectrophotometer Carl Zeiss, Jena. Optical density (OD) of the culture was measured at 550 nm by means of spectrometer "Specol" (Carl Zeiss, Jena).

3. RESULTS AND DISCUSSION

Figures 1a, b, c show the influence of zinc ions on the denitrification process. It appears that the biomass increase in the medium with 0.04 g Zn^{2+}/dm^3 is approximately the same as in the case of the blank test (fig. 1a). Only small changes in the curves of optical density were observed. Zinc concentrations in the range from 0.07 to 0.25 g/dm^3 cause decrease of maximum optical density which indicates the inhibition of proliferation. Consequently, it attenuates denitrification process. The amount of 0.04 g Zn^{2+}/dm^3 in the medium delays nitrate and nitrite reduction, but this reduction is complete (about 90% of NO_3^- and 100% of NO_2^-) and of similar rate in comparison to blanc sample. Progressive concentration of zinc ions, 0.25 g Zn^{2+}/dm^3, brings about inhibition of nitrate reduction (only 30%) as well as nitrite reduction (fig. 1b, 1c). At 0.3 g Zn^{2+}/dm^3, the denitrification process does not occur and the decrease of OD (to 0 in 12 hours) results in lethal effect of zinc ions.

The influence of cadmium ions (fig.2a, b, c) is similar to that of zinc, but the changes in OD and nitrate reduction are observed at its concentration of 0.01 g/dm^3. At 0.06 g Cd^{2+}/dm^3 we observe conspicious inhibition of these processes. It is interesting that the optical density, even at cadmium concentration of 0.5 g/dm^3, has been constant at least for four days. These facts suggest that the influence of cadmium ions is rather bacteriostatic than bacteriocidal.

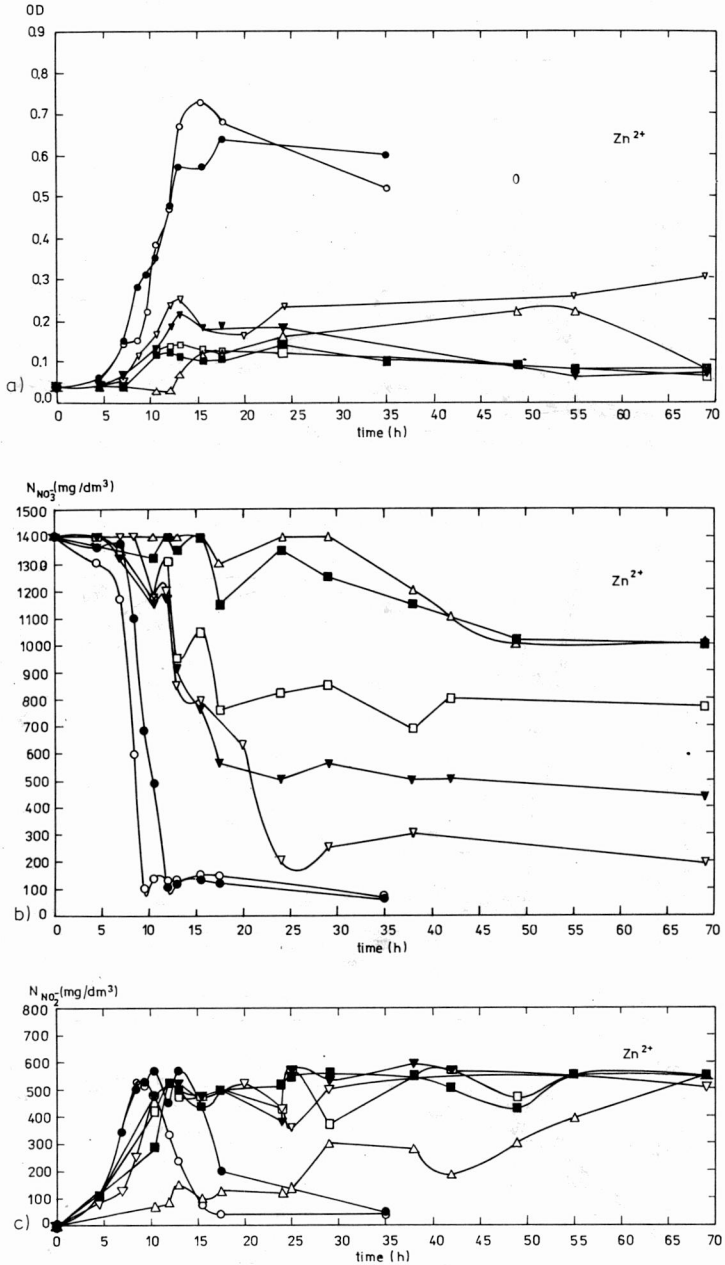

Fig. 1. The effect of Zn^{2+} ($Zn(NO_3)_2 \cdot 6H_2O$) concentration on the increase in the culture optical density (fig. 1a) and biological degree of nitrate (fig. 1b) and nitrite (fig. 1c) reduction.
(Reaction temperature = 37°C, pH of medium = 8.0)
Concentration of Zn^{2+} added (g Zn^{2+}/dm^3): ○ – 0.0 (blanc sample), ● – 0.04, ▽ – 0.07, ▼ – 0.1, □ – 0.15, ■ – 0.2, △ – 0.25

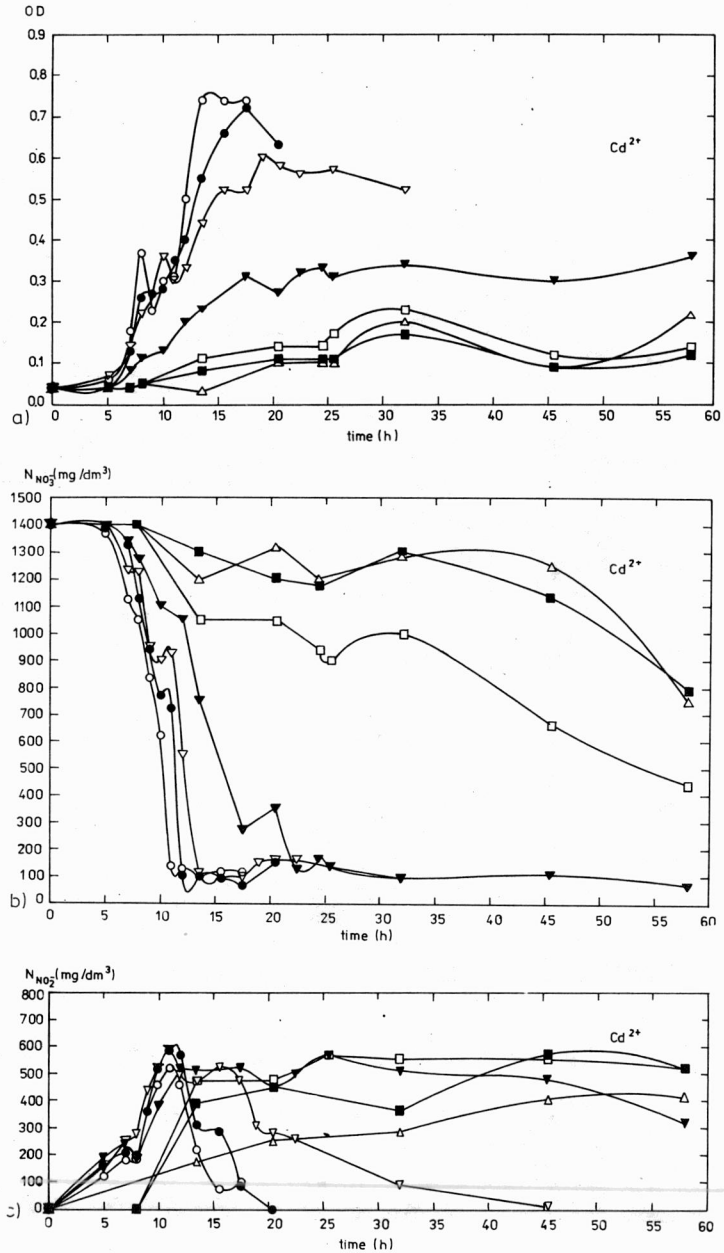

Fig. 2. The effect of Cd^{2+} ($Cd(NO_3)_2 \cdot 4H_2O$) concentration on the increase in culture optical density (fig. 2a) and biological degree of nitrate (fig. 2b) and nitrite (fig. 2c) reduction.
(Reaction temperature = 37°C, pH of medium = 8.0)
Concentration of Cd^{2+} added (g Cd^{2+}/dm^3): ○ – 0.0 (blanc sample), ● – 0.01, ▽ – 0.02, ▼ – 0.04, □ – 0.06, ■ – 0.08, △ – 0.1

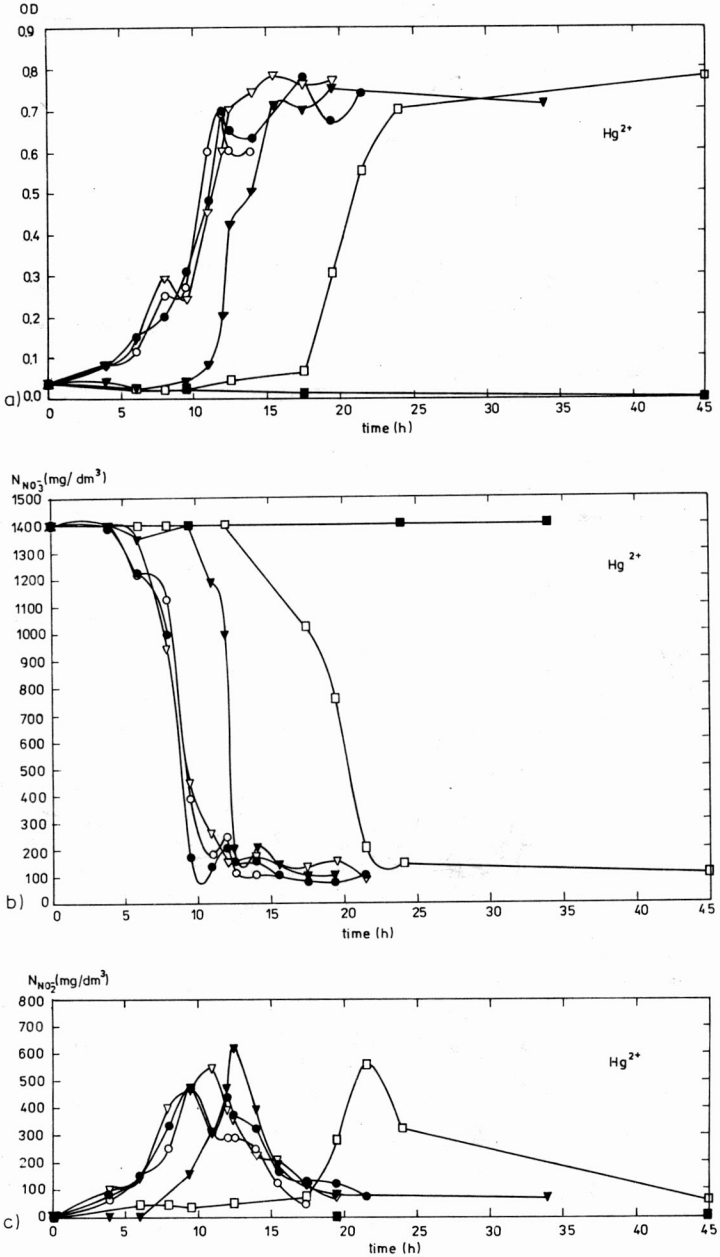

Fig. 3. The effect of Hg^{2+} ($Hg(NO_3)_2 \cdot 0.5\ H_2O$) concentration on the increase in culture optical density (fig. 3a) and biological degree of nitrate (fig. 3b) and nitrite (fig. 3c) reduction.
(Reaction temperature = 37°C, pH of medium = 8.0)
Concentration of Hg^{2+} added (g Hg^{2+}/dm^3): ○ – 0.0 (blanc sample), ● – 0.00025, ▽ – 0.0005, ▼ – 0.00075, □ -- 0.001, ■ – 0.0015

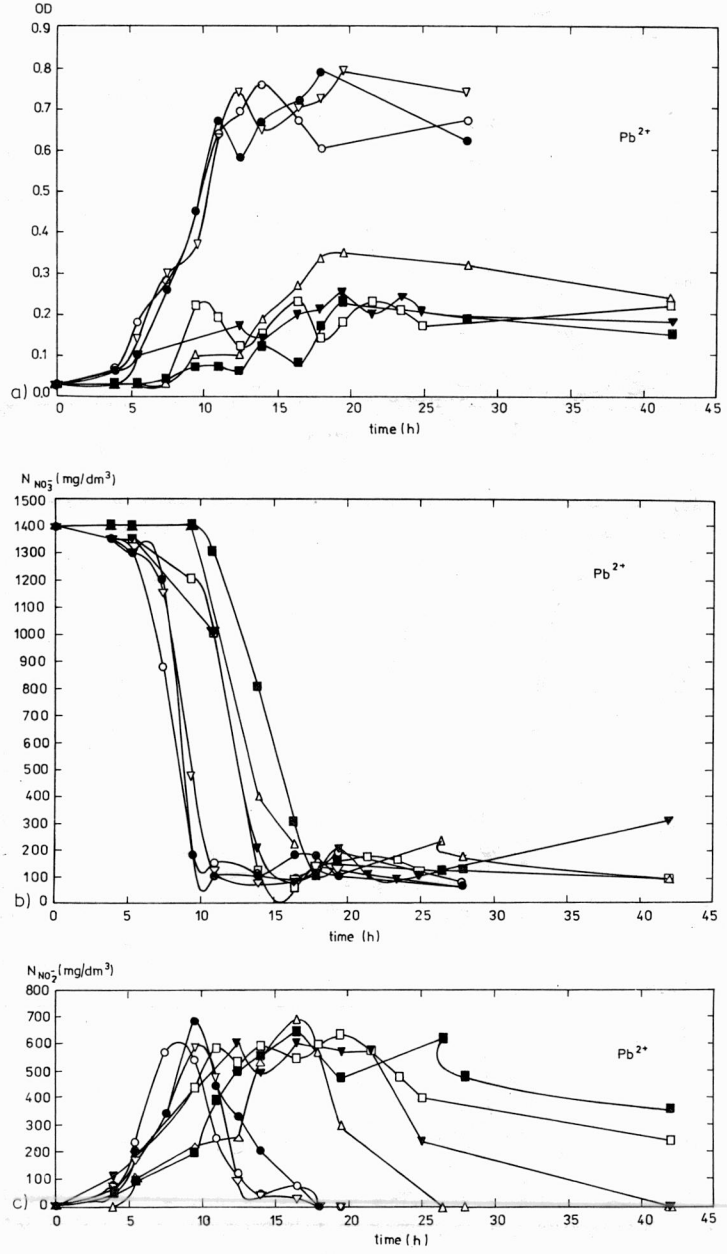

Fig. 4. The effect of Pb^{2+} ($Pb(NO_3)_2$) concentration on the increase in culture optical density (fig. 4a) and biological degree of nitrate (fig. 4b) and nitrite (fig. 4c) reduction.
(Reaction temperature = 37°C, pH of medium = 8.0)
Concentration of Pb^{2+} added (g Pb^{2+}/dm³): ○ – 0.0 (blanc sample), ● – 1, ▽ – 2, ▼ – 3, □ – 4, ■ – 7, △ – 10

Mercury ions (Hg^{2+}) are the most toxic of all ions tested (fig. 3a, b, c). At the concentration of 0.001 g/dm^3 they result in the delay of logarithmic growth phase. Progressing amounts of Hg^{2+} lenghten the lag phase. The increase in OD and denitrification is not observed at the concentration of 0.0015 g Hg^{2+}/dm^3. Similarly to Zn^{2+}, mercury ions are lethal to the bacteria tested (the OD decrease was observed).

In our studies on the lead effects on denitrifiers, we have observed the curious phenomenon (fig. 4a, b, c): lead ions in amounts of 4 and 7 g/dm^3 markedly inhibited the OD increase, resulting in incomplete reduction of nitrate. However, after nearly 10 hours of lag phase at the concentration of 10 g Pb^{2+}/dm^3, the rapid increase in cells quantity was observed, and denitrification was complete (lack of NO_2^- ions after 50 hours). Assuming that all phosphate and sulphate anions precipitate with lead ions (this process could be catalysed by bacteria [6], [14], [15]) and the latter become nontoxic for bacteria, it is easy to explain why the concentration of 2 g Pb^{2+}/dm^3 (9.65 mM) is a threshold value beyond which lead cations inhibit both growth and denitrification processes. But it is difficult to explain why bacteria are active at 10 g Pb^{2+}/dm^3 again.

The interactions between heavy metals and microorganisms are diverse, but can be divided into two groups:

1. Interactions in which metals are essential for metabolism (e.g., micronutrient Zn^{2+}), or they disturb metabolism (by binding with proteins).

2. Interactions comprising defence mechanisms of organisms such as a) accumulation of metals, including accumulation at organism surfaces, and intracellular uptake of metals, b) biochemical transformations of metals which can involve solubilization or precipitation, valency changes during oxidative or reductive processes, and the interconversion of inorganic and organic metal compounds [12]–[15].

Our studies have been mostly focused on four heavy metal cations, which are most toxic for living organisms. We can arrange them according to growing toxicity to denitrifiers from *Bacillus* genus: $Pb^{2+} < Zn^{2+} < Cd^{2+} < Hg^{2+}$. Noticeable disturbances of denitrification process occur in media of the concentration of 0.5 mg Hg^{2+}/dm^3, 10 mg Cd^{2+}/dm^3, 40 mg Zn^{2+}/dm^3 and 2 g Pb^{2+}/dm^3. The mechanism of action of these metals is still unknown.

The results presented in this paper are of a great importance in the designing of biological treatment. The attention should be focused on heavy metal concentrations in the wastes in which the beneficial chemical reactions are catalyzed by bacteria (especially by *Bacillus*).

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WPŁYW JONÓW METALI CIĘŻKICH NA AKTYWNOŚĆ DENITRYFIKATORÓW Z RODZAJU *BACILLUS*

Przedstawiono wyniki badań nad wpływem jonów metali ciężkich, takich jak: cynk, kadm, rtęć i ołów na aktywność i proces namnażania bakterii z rodzaju *Bacillus*. Wyznaczono granice stężeń tych metali, które mają działanie inhibitujące na proces redukcji azotanów. Z badań wynika, że można je uszeregować w następujący sposób według wzrastającej toksyczności $Pb^{2+} < Zn^{2+} < Cd^{2+} < Hg^{2+}$.

ВЛИЯНИЕ ИОНОВ ТЯЖЕЛЫХ МЕТАЛЛОВ НА АКТИВНОСТЬ ДЕНИТРИФИКАТОРОВ ТИПА *BACILLUS*

Представлены результаты исследований влияния ионов тяжелых металлов, таких как: цинк, кадмий, ртуть и свинец на активность и процесс размножения бактерий типа *Bacillus*. Определены пределы концентраций металлов, которые характеризуются ингибитирующим действием на процесс редукции нитратов. Из исследований вытекает, что можно их систематизировать следующим образом по растущей токсичности: $Pb^{2+} < Zn^{2+} < Cd^{2+} < Hg^{2+}$.