Denitrification of Sodium Nitrate by Means of Mixed Culture of Microorganisms: (Part II.)

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This paper describes the reduction of nitrate (denitrification) from sodium nitrate in a synthetic medium by means of a mixed culture of microorganisms. Mixed culture was applied in experiments of oxidation of ammonia from ammonium sulphate (Part I). It was adapted to degrade nitrate in substrate with and without carbon source. The degradation of nitrate mass concentration of 0.20 g L^{-1} and 0.60 g L^{-1} was carried out in 0.5 L of synthetic medium in aerated reactor after adding one of carbon source: glucose, 3-Na-citrate, Na-acetate or methanol ($\zeta_{C/N}$=6:1). The initial pH value was 5.5 or 7.5 and during 18 or 24 h of degradation the temperature in reactor was 23 ºC. In both cases mixed culture of microorganisms reduced nitrate up to 99 % and accumulation of nitrite was low (3.88 mg L^{-1}). The exception was the sample at pH value 7.5 with Na-acetate added as the source of carbon (up to 11 mg L^{-1}).

Keywords:
Aerobic degradation, sodium nitrate, mixed culture of microorganisms, carbon source

Introduction

Nitrogen is one of the essential elements that can be found in living beings, mostly in proteins and nucleic acids. In general, humans and animals need ammonia nitrogen while plants prefer nitrate nitrogen. However, microorganisms utilize all kinds of nitrogen compounds and close carbon cycle in the nature.[1-5]

Reduction of nitrate to element nitrogen (N_2) takes place in the absence of oxygen in several steps which are catalyzed by bacterial enzymes as follows:[6,7] nitrate reductase, Cu-nitrite reductase, cd_1-nitrite reductase, NO-reductase and N_2O-reductase. The scheme of nitrate reduction is shown in Fig 1.

There are three different degradation pathways of nitrate: reductive assimilation, reductive dissimilation and denitrification[8]. Microorganisms that are capable to reduce nitrate to elemental nitrogen (N_2) belong to different bacterial genus such as *Thiosphaera*, *Paracoccus, Achromobacter, Alcaligenes, Bacillus, Flavobacterium, Corynebacterium, Micrococcus, Moraxella, Pseudomonas, Propionibacterium, Nitrobacter* and others.[2,4,9,12] The most denitrifying bacteria grow in the pH range of 6-8 and many of them demand presence of oxygen.[13] The influence of oxygen to denitrifying bacteria is not in total explained as well as if one or more mechanisms are involved. Many denitrifying bacteria are heterotrophic capable to utilize different carbon sources as electron donors.[14-17]

The aim of this work was to research the reduction of nitrate-*N* (denitrification) from sodium nitrate in synthetic medium in presence of oxygen by means of mixed culture of microorganisms.

Material and methods

Microorganisms

Mixed culture of microorganisms, used in this work, was composed of two different microbial populations: a) bacteria isolated from activated sludge from chemical industry, and b) yeast isolated from black liquor from cellulose industry. These two cultures were randomly selected because of their diversity and availability. Each sample was inoculated to the nutrient medium, bacterial and yeast one, and after incubation all grown colonies were collected and inoculated to the artificial medium (Table 1) with sodium nitrate as a source of nitrate. After 18 h or 24 h of aeration grown biomass was centrifuged and inoculated to the higher concentration of nitrate. At the end of that process the remaining cultures were isolated and identified.[18–20]

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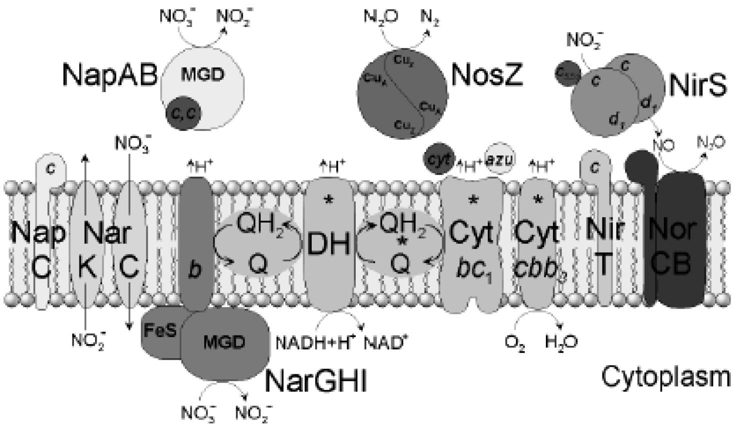
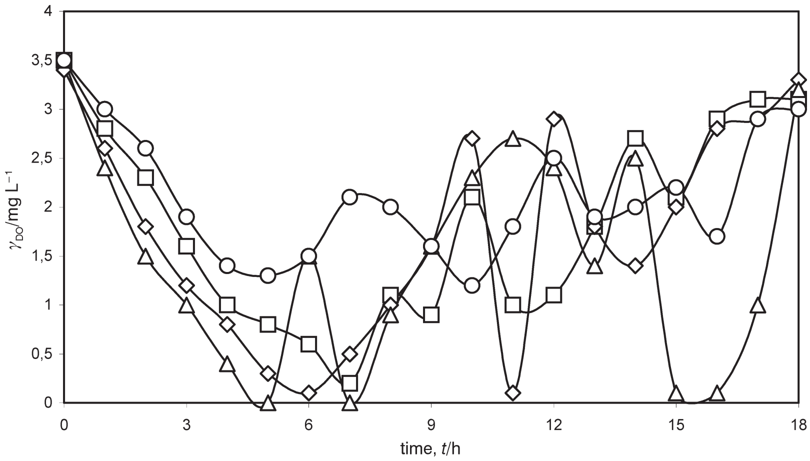
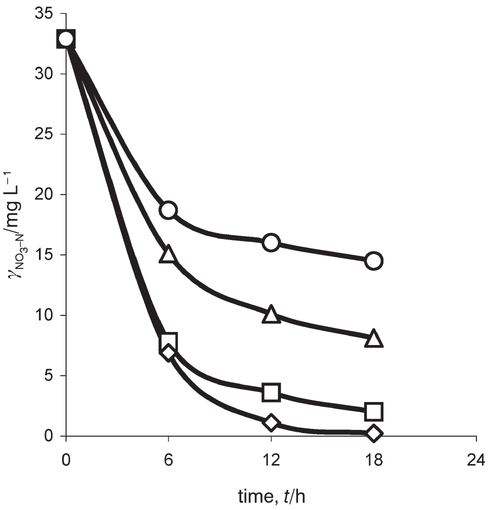


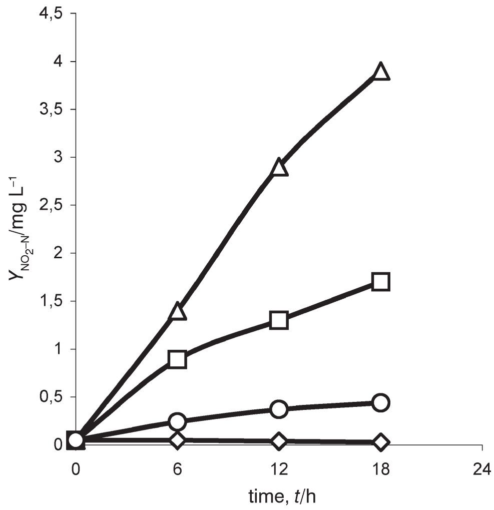
F i g . 1 – *The scheme of bacterial enzymes which catalyze the reduction of nitrate to element nitrogen (N_2)*



F i g . 2 – *The changing of dissolved oxygen (DO) during the reduction of nitrate from sodium nitrate concentration of 0.20 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◇-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 5.5 by means of mixed culture of microorganisms during 18 h at 23 °C*



F i g . 3 – *The reduction of nitrate from sodium nitrate concentration of 0.20 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◇-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 5.5 by means of mixed culture of microorganisms during 18 h at 23 °C*



F i g . 4 – *The accumulation of nitrite during the reduction of nitrate from sodium nitrate concentration of 0.20 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◇-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 5.5 by means of mixed culture of microorganisms during 18 h at 23 °C*

Table 1 – *The composition of synthetic medium*

Ingredient	Mass concentration γ/g L^{-1}
$NaNO_3$	0.20 or 0.60
K_2HPO_4	0.85
$MgSO_4x7H_2O$	0.2
distilled water/dm^3	1.0
pH value*	5.5 or 7.5

*pH value was adjusted by adding sodium hydroxide solution concentration c = 2 mol L^{-1}, or by diluted sulfate acid φ = 20 %

The usual identification tests as well as Analytical Profile Index test (API) were applied for identification of microorganisms isolated from the mixed culture. The genera of bacteria[21,23] and yeast[22] were identified observing the morphological and culture characteristics of organisms and biochemical reactions. In total, five genera of bacteria (*Curtobacterium* sp., *Bacillus* sp., *Microccocus* sp., *Klebsiella* sp. and *Arthrobacter* sp.) and one genus of yeast (*Geotrichum* sp.) were isolated from adapted mixed culture of microorganisms.

Synthetic medium

Adaptation of mixed culture of microorganisms and aerobic degradation of nitrate was carried out in synthetic medium consisting of ingredients as it is shown in Table 1. The carbon source was one of these: glucose, 3-Na-citrate, Na-acetate or methanol. Initial pH values were 5.5 or 7.5 due to the composition of mixed culture. Yeast prefers lower and bacteria higher pH values.

Methods

Mixed culture of microorganisms, taken from the previous experiments of ammonia sulphate degradation,[17] was inoculated to the Petri dishes and adapted to degrade nitrate from sodium nitrate concentration from 0.020 to 1.5 g L^{-1}. Each of the carbon source: glucose, 3-Na-citrate, Na-Acetate and methanol, was added separately to the synthetic medium. The mass ratio between carbon and nitrate in synthetic medium was $\zeta_{C/N}$ = 6:1. The capability of the mixed culture in degradation of nitrate in synthetic medium was carried out in 1 L of aerated reactor with 0.5 L of synthetic medium (Table 1). When concentration of nitrate was 0.20 g L^{-1} (32.9 mg L^{-1} NO_3-N) the degradation time was 18 h and at 0.60 g L^{-1} (98.8 mg L^{-1} NO_3-N) it was 24 h, respectively. The initial pH value was 5.5 or 7.5 and during the experiment it was not corrected. The concentration of inoculum of wet biomass was from 0.96 to 1.2 g L^{-1} while the initial concentration of oxygen was up to 3.5 mg L^{-1}. The temperature of the medium in the reactor was maintained at 23 °C.

Analytical procedures

In synthetic medium the changing of nitrate and accumulation of nitrite were measured spectrophotometrically according the APHA standards.[18] The biomass of microorganisms was measured as wet biomass to speed up obtaining of the results. The concentration of dissolved oxygen was measured every 30 min with oxygen electrode (WTW) submerged into the medium.

Results and discussion

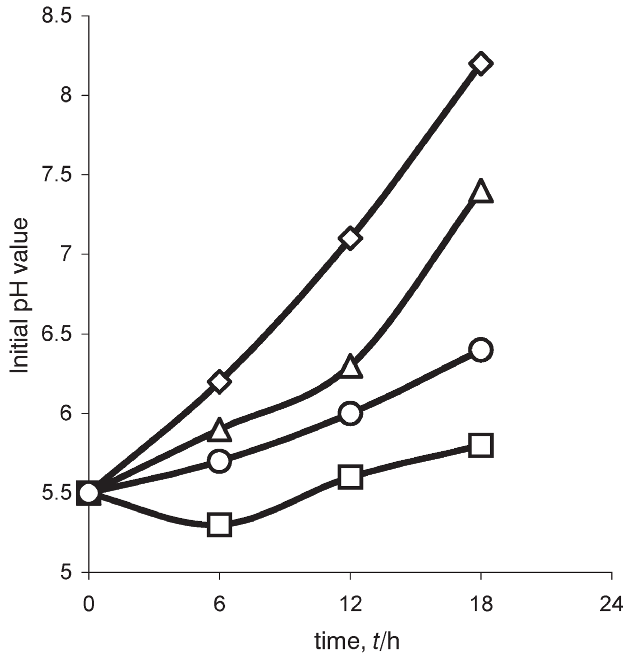
The changing of the initial concentration of dissolved oxygen during the nitrate reduction is shown in Fig. 2. The concentration of oxygen in the beginning was 3.5 mg L^{-1} and was going down. After 2.5 h the concentration reached zero and after a while it started to go up. The fluctuation in the concentration followed and at the end of reduction it reached almost the same value as it was in the beginning: According to the literature[2,13,17] the initial mass concentration of oxygen is in the range from 0.25 to 2.5 mg L^{-1}. During the reduction mixed culture of microorganisms uses two electron acceptors: nitrate and oxygen (mixotrophy).[14]

Results of the aerobic reduction of nitrate in synthetic medium are shown in figures 3-14. During 18 h (Fig. 3 and 6) of aerobic reduction it was noticed that there was decrease of nitrate of initial concentration of 0.20 g L^{-1}. In samples with different carbon sources there were a different reduction as well. Minimal reduction was in sample when methanol was added and maximal reduction was in sample with 3-Na-citrate as it is shown in Fig. 3.

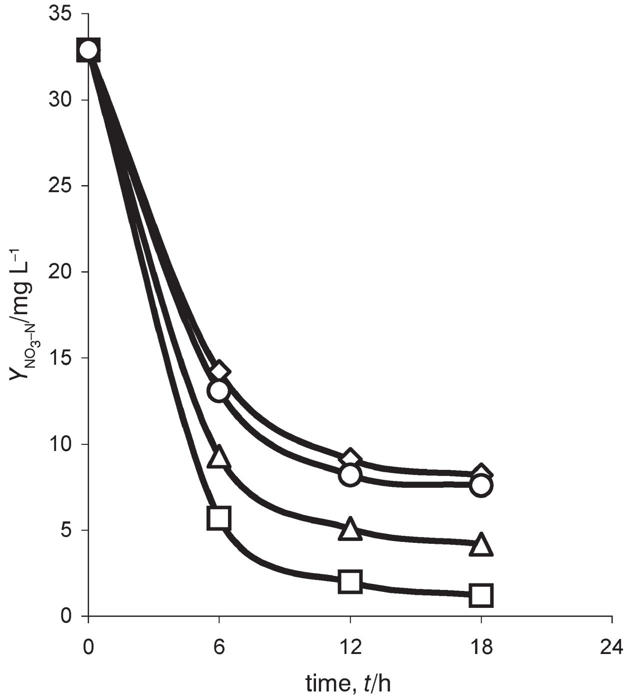
In those cases the initial pH value was 5.5. The results of reduction were something better when initial pH value was 7.5 (Fig. 6). Only in sample when 3-Na-citrate was added the reduction was as the same as with methanol. At higher mass concentration of nitrate (0.60 g L^{-1}) and initial pH value of 5.5 almost all nitrate was reduced (Fig 9). When initial pH was 7.5, nitrate was reduced when Na-acetate and glucose was added (Fig. 12).

According to literature[9,10,13,15] adaptation to methanol, as a carbon source, needs a longer period of time and the degradation of C_1 component does not take place throughout the three carboxylic acids cycle.

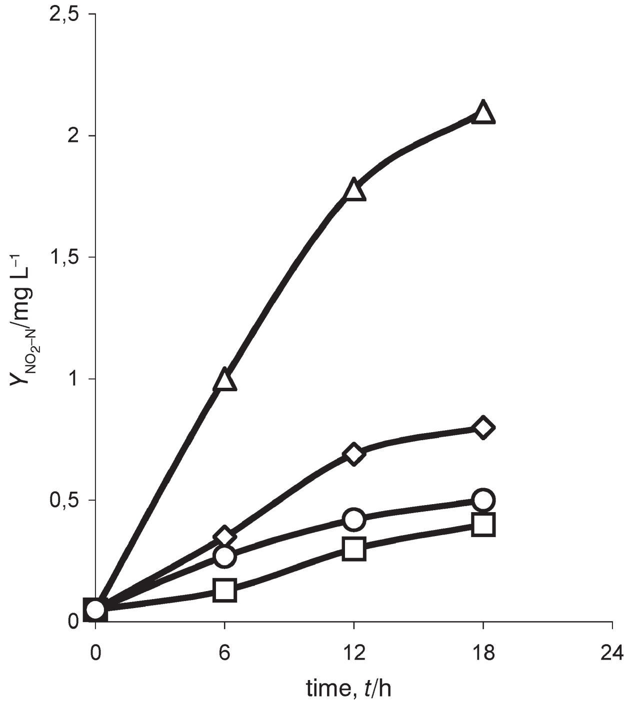
As the reduction progress nitrite accumulates successively (Fig. 4, 7, 10 and 13). At initial pH



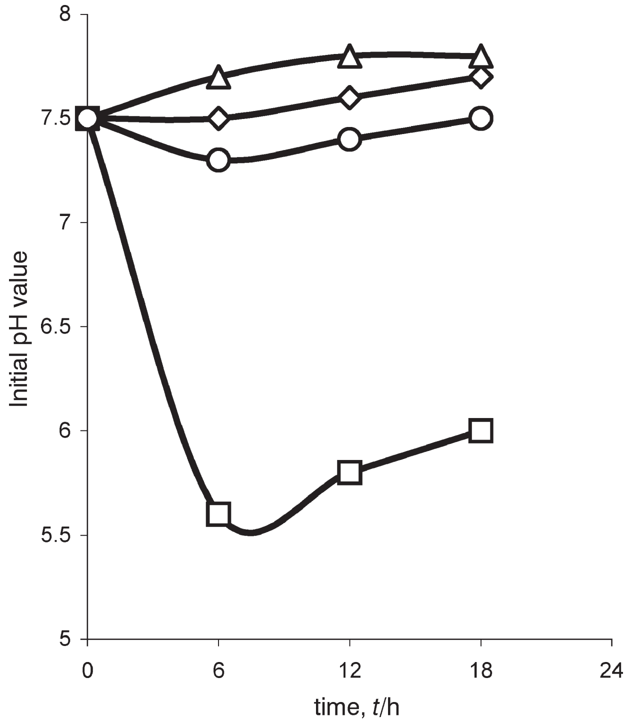
F i g . 5 – *The changing of initial pH value 5.5 during the reduction of nitrate from sodium nitrate concentration of 0.20 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◊-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium by means of mixed culture of microorganisms during 18 h at 23 °C*



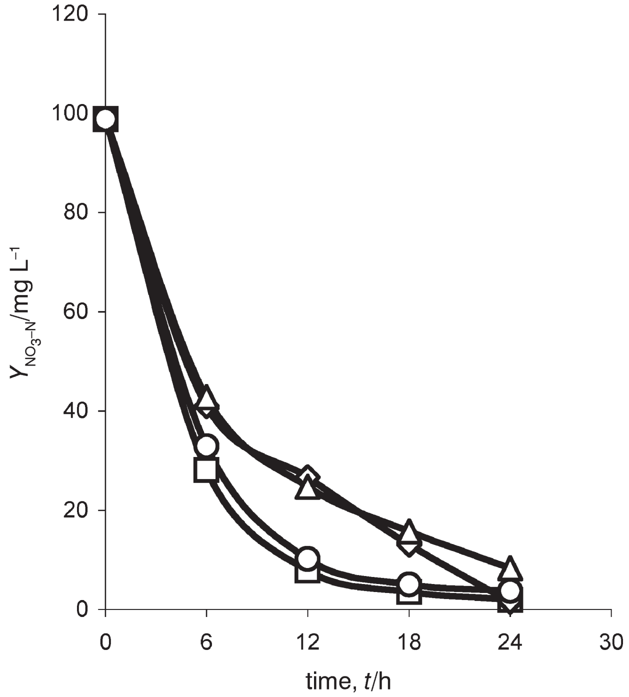
F i g . 6 – *The reduction of nitrate from sodium nitrate mass concentration of 0.20 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◊-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 7.5 by means of mixed culture of microorganisms during 18 h at 23 °C*



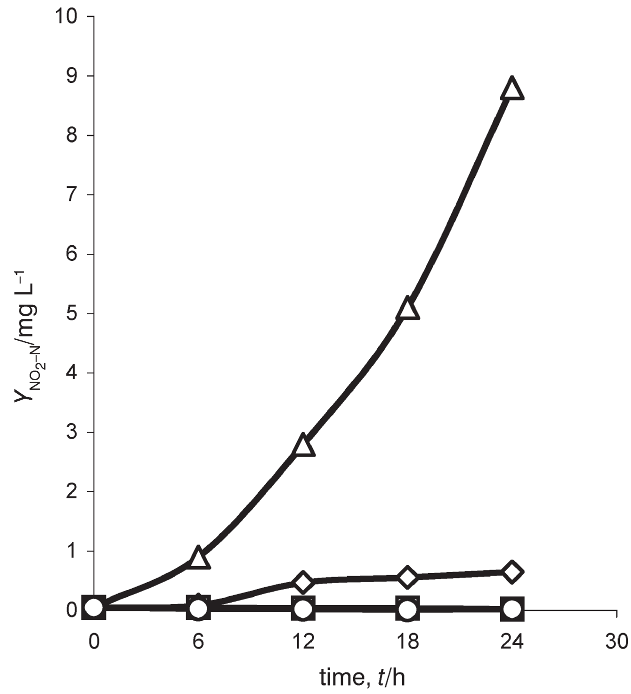
F i g . 7 – *The accumulation of nitrite during the reduction of nitrate from sodium nitrate mass concentration of 0.20 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◊-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 7.5 by means of mixed culture of microorganisms during 18 h at 23 °C*



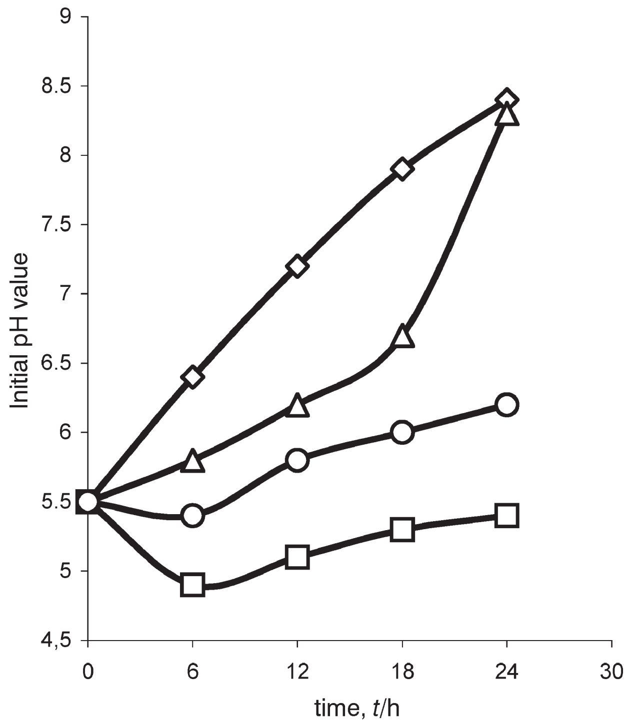
F i g . 8 – *The changing of initial pH value 7.5 during the reduction of nitrate from sodium nitrate concentration of 0.20 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◊-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium by means of mixed culture of microorganisms during 18 h at 23 °C*



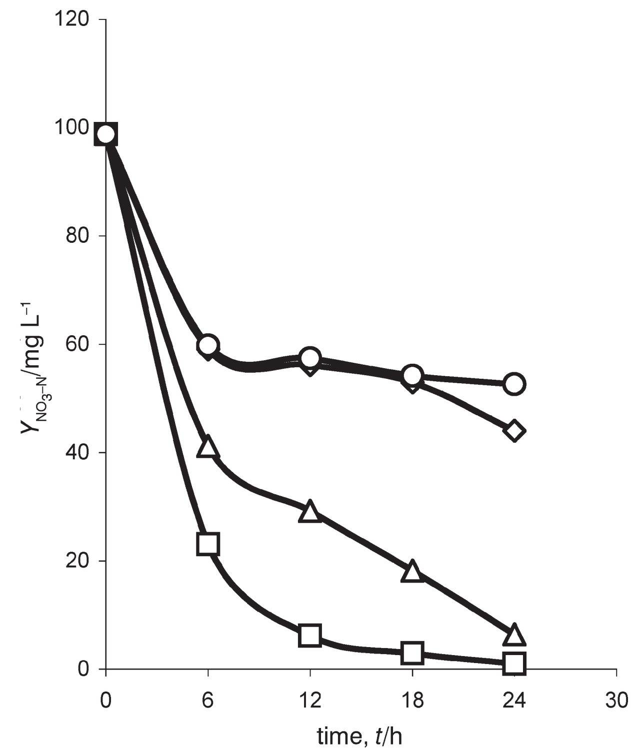
F i g . 9 – *The reduction of nitrate from sodium nitrate mass concentration of 0.60 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◊-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 5.5 by means of mixed culture of microorganisms during 24 h at 23 °C*



F i g . 1 0 – *The accumulation of nitrite during the reduction of nitrate from sodium nitrate mass concentration of 0.60 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◊-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 5.5 by means of mixed culture of microorganisms during 24 h at 23 °C*



F i g . 1 1 – *The changing of initial pH value 5.5 during the reduction of nitrate from sodium nitrate concentration of 0.60 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◊-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium by means of mixed culture of microorganisms during 24 h at 23 °C*



F i g . 1 2 – *The reduction of nitrate from sodium nitrate mass concentration of 0.60 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◊-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 7.5 by means of mixed culture of microorganisms during 24 h at 23 °C*

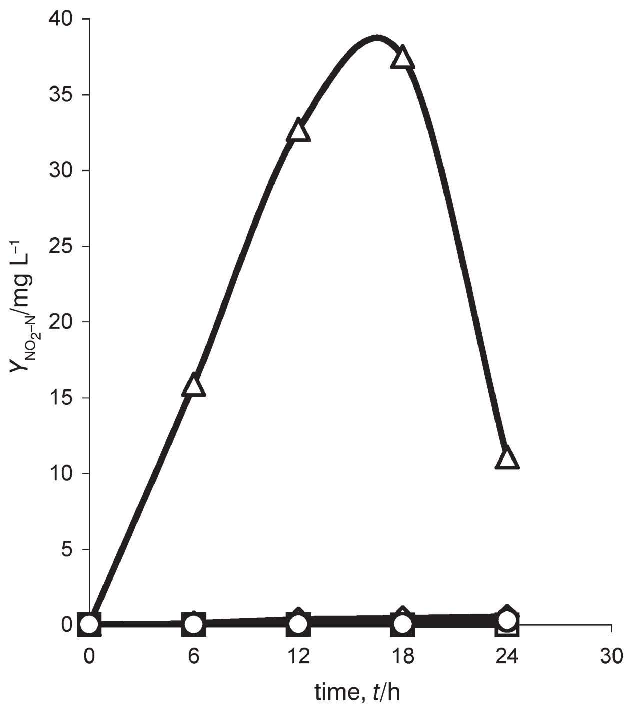


Fig. 13 – *The accumulation of nitrite during the reduction of nitrate from sodium nitrate concentration of 0.60 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◇-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium at the initial pH value 7.5 by means of mixed culture of microorganisms during 24 h at 23 °C*

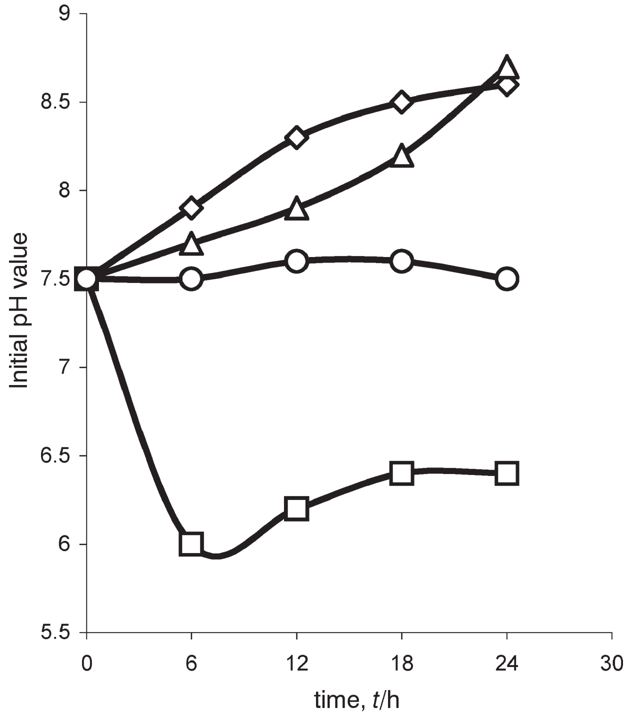


Fig. 14 – *The changing of initial pH value 7.5 during the reduction of nitrate from sodium nitrate concentration of 0.60 g L^{-1} with glucose (-□-), or 3-Na-citrate (-◇-), or Na-acetate (-Δ-) or methanol (-○-) in synthetic medium by means of mixed culture of microorganisms during 24 h at 23 °C*

value of 5.5 the concentration of nitrite was very low when 3-Na-citrate was added (Fig. 4). The results were somewhat better at higher concentration of nitrate (Fig. 10). This time the accumulation of nitrite didn't occur when glucose or methanol was added. When initial pH value was 7.5 the accumulation of nitrite didn't happen, except when Na-acetate was added (Fig. 13).

Although the concentration of accumulated nitrite, during the experiment at higher pH value 7.5 was very high (Fig. 13), it was reduced and remained at 11 mg L^{-1}.

According to results it seems that Na-acetate as carbon source contributed to nitrite accumulation.

Simultaneously, the initial concentration of biomass of mixed culture reduces from $\gamma = 1.2$ g L^{-1} to 0.85 g L^{-1} weighed as wet biomass. At the same time organic matter (measured as chemical oxygen demand, COD) diminished for 28 % and 82 % when methanol or 3-Na-citrate were added, respectively.

Knowing which of the microorganisms are involved in reduction of nitrate is one of preconditions for maintaining the degradation process, its stability as well as the balance in interactions of different groups of microorganisms.[9-14] Yeasts *Geotrichum* sp. demands lower pH value (5.5-6) for growth while bacteria (in this case genera *Curtobacterium*, *Bacillus*, *Micrococcus, Klebsiella and Arthrobacter*) need somewhat higher pH value (6-9). As the mixed culture of microorganisms consisted of five genera of bacteria and one yeast it was the reason why two different pH values were applied, which were not corrected during the experiments. However, the change in pH values was noticed in the range from 5.5 to 8.3 (Fig. 5, 8, 11 and 14).

Aerobic and anaerobic reductions of nitrate have been reported by many authors[2-17] and for explanation of nitrification-denitrification processes in their works they have employed different types of microorganisms. In the most cases they were monocultures of bacteria, such as *Nitrobacter* sp., *Clostridium* sp., *Pseudomonas* sp., *Paracoccus* sp., *Bacillus* sp., *Micrococcus* sp., *Vibrio* sp., *Chromobacterium* sp., *Flavobacterium* sp., *Corynebacterium* sp.; or fungi *Alternaria* sp., *Helminthosporium* sp; or yeasts[9,10] *Candida* sp., *Pachysolen* sp., *Sporobolomyces* sp., *Trichosporon* sp.; or an undetermined mixed culture of microorganisms in form of activated sludge. Denitrification processes have been carried out in synthetic medium enriched with different salts and growth factors. Acetate[8,9,16] was the most often used carbon source, or some particular wastewater, whereas pH value[10,13] was 6 up to extreme 11, while temperature[13,17] varied from 0 to 50 °C.

Conclusion

Mixed culture of microorganisms has shown reduction of nitrate mass concentration of 0.2 and 0.6 g L^{-1} with different carbon source: glucose, or 3-Na-citrate, or Na-acetate or methanol. The same culture is able to reduce nitrite too.

List of symbols

γ – mass concentration, mg L^{-1}, g L^{-1}
$\zeta_{C/N}$ – mass ratio, m_C/m_N
t – time, h
c – concentration, mol L^{-1}
φ – volume fraction, %

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