Denitrification Performance of a Culture of Thermophilic Aerobic Bacteria *NBIMCC 3729*

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Original scientific paper
Received: July 17, 2002
Accepted: January 15, 2003

In the present paper the reduction of nitrate to nitrogen under thermophilic conditions was studied. A culture developed from the thermophilic aerobic bacteria *Geobacillus NBIMCC 3729* was used under non-aerated conditions. Instead of oxygen nitrates were used as an electron acceptor during the microorganisms' growth. The study was accomplished under batch conditions with model aqueous solutions of nitrates and peptone and meat extract as a complex carbon source. The temperature range of the biochemical reduction process, was determined. A two-step consecutive reduction process was observed and the presence of nitrite in the broth as an intermediate product was found out. It is confirmed that the first step in nitrate reduction is strictly associated with the microbial growth. A clear effect of substrate inhibition of the denitrification process was revealed at higher nitrate concentrations, resulting mostly as considerable extension of the lag-phase in the microbial growth.

Key words:
Thermophilic bacteria, denitrification, nitrite accumulation

Introduction – high temperature industrial waste waters treatment

The process of biological treatment of wastewater polluted by organic substances is connected with the design of a classic, conventional units.[1,2]

A new trend in the field of industrial wastewater treatment is the separate purification of specific industrial effluents, within one company.[3,4] In many cases the effluent wastewater is hot and with high organic pollutants concentration.[5] Until now, the direct biological treatment of such hot water flows was considered as impossible. Therefore, in order to achieve successful operation of the biological treatment process, cooling of the wastewater has been accomplished. The latter has led not only to energy losses but also to a decrease in the biological pollutant assimilation.[6]

Better results of the process of mass exchange are obtained at increased temperature.[5] The hygienic conditions of work with thermophilic microorganisms are also of importance – at a temperature higher than 60 °C the possibility of pathogenic bacteria proliferation is usually insignificant.[6,7]

For this purpose the use of thermophilic microorganisms for biological treatment of high temperature wastewater streams is necessary. There are known many classifications of bacteria towards their temperature optimum.[8,9] Usually, the thermophilic conditions of life are considered to be in the range from 55 to 70 °C.[10]

"Denitrification" is used to mark the reduction of oxidized nitrogen compounds to gaseous nitrogen. As a result of the reduction, different intermediate and end products are produced: nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), or molecular nitrogen (N_2). In the biological step of a wastewater treatment plant, the denitrification process to molecular nitrogen runs usually according to the following net reaction.[8]

$$4NO_3^- + 2H_2O \rightarrow 2N_2 + 5O_2 + 4OH^-$$

High nitrate concentrations are generated during the biological purification of the wastewater in the nitrification step, which can be accomplished only in presence of ammonia or other nitrogen compounds. Wastewaters, rich in ammonium cations, are usually formed in refineries, food, wine and tobacco industries and during the process of active sludge conditioning.[1]

As a second group, wastewater from companies, producing articles with nitrate contents or using nitric acid in the process of production, can be mentioned. Wastewater from such companies contains nitrate in high concentrations. Examples for such industries are factories for nitrate fertilizers and explosives production, as well as factories for electronics and galvanic cells. The nitrogen compounds, similarly to the carbon and phosphorus

compounds cause eutrophication in the water ponds. The continuous growth and death of the duckweeds and the other water plants worsen the water quality. One of the most distributed effects is the oxygen deficiency, which can lead to fish death.[11]

The health risk to the human beings is not only due to the nitrate ions, but also to their reduced form nitrite ions, which can be formed during biochemical processes in the human body.[11]

Depending on the conditions under which nitrate dissolves, the formation of dinitrogen oxide (N_2O) is possible.[12] This is one of the gases causing the greenhouse effect. Besides, N_2O can be oxidized in the stratosphere to form acid rain of nitric acid.

On the other hand there are many developments focused on the denitrification.[13–15] They use mesophilic strains in their studies. An excellent review on this subject is given by *Mateju et al.*[16] Furthermore, there were studied different specialized strains, performing the consecutive reduction process of nitrates to nitrogen by electrochemical techniques,[17] as well as the kinetics of bio-reduction and the accumulation of different intermediate products.[12,18]

There are efforts to replace oxygen by nitrate as an electron acceptor and thus to carry out biodegradation of organic matter.[19] This option is particularly important at treatment of hot streams, when oxygen solubility is sharply decreased and aerobic processes are not appropriate.

In such cases the use of thermophilic microorganisms for denitrification purposes introduces new methods of industrial wastewater treatment at high temperature, when the oxygen in the medium is only chemically bound in the form of nitrate. The trend of the use of anaerobic conditions is well understood, mainly in the case of wastewater, rich in volatile compounds which could threaten human health.

It would be interesting to combine the advantages of the thermophilic properties of microorganisms with their dentrification potential. The goal of the present paper is to study the possibility for denitrification by thermophilic bacteria at higher temperatures without aeration and to use the nitrate as an electron-acceptor.

Aim of the research

The aim of this research was to study the possibilities for denitrification of model media with culture of thermophilic facultative aerobic bacteria from the genus *Geobacillus* and to establish the process optimal conditions.

Materials and methods

Used microorganisms

The research was accomplished, using a culture developed from the thermophilic bacteria *NBIMCC 3729* (genus *Geobacillus*, cf. ref.[20]), which was kindly supplied from the Bulgarian 'National Bank of Industrial Microorganisms and Cellular Cultures' (*NBIMCC*).

To evaluate and assess the results of our study, parallel results obtained from second thermophilic consortium was used, i.e. *Geobacillus thermodenitrificans* DSM 465/466.

For a reference, own data on the denitrification performance of the mesophilic strain *Pseudomonas denitrificans*, will be presented too.

Medium

The cultural medium is used in the experiments consisted of 3 g meat extract and 5 g peptone in 1000 ml distilled water. The pH value was adjusted within 7 and 8, using sulfuric acid and sodium hydroxide solutions.

Apparatus and experimental conditions

Inoculation

Usually the inoculum preparation preceded the fermentation, usually with 10 to 15 hours, during which the inoculum was kept in refrigerator at 4 °C. An hour prior to the start of the fermentation, the inoculum was put in a rotary shaker at 60 °C. The research was usually carried out with amount of inoculum of 10 to 15 % of the total fermentation volume.

Anaerobic cultivation of the inoculum was accomplished in flasks on an Elphan water bath shaker, type 357, with a thermostated water bath at 60 °C, at 120 rpm. Periodically 8.4 mg l^{-1} KNO_3 were added to the culture. Flasks of 100 ml were used and filled to 60 or 65 ml with the respective medium. In order to avoid an air penetration, the flasks were equipped with rubber flaps with a tight tubing, the end of which was put into a water-filled vessel. Thus, pressure equalization between the gas phase in the flask and the atmosphere pressure outside was ensured, without air penetration in the flask.

It was shown, that it was not necessary to purge the cultivated medium with nitrogen or helium[14] to eliminate dissolved oxygen before the start of anaerobic cultivation. It was established in our work, that the oxygen dissolved in the medium and present in the gas-phase was consumed so fast and right at the beginning of the reaction, that practically it had no significant influence on the process. More-

over, parallel experiments held with and without such a preliminary purging with nitrogen or argon, showed no difference in bacterial performance.

Cultivation under batch conditions

In order to determine the optimal conditions of the denitrification process with thermophilic facultative aerobic bacteria *NBIMCC 3729* batch experiments were carried out.

The reactor of 500 ml volume was supplied with a water jacket and connected to MLW U-15 thermostat. The reactor lid was equipped with several outlets, used to measure the temperature, to inject the inoculum and to remove the vapour and the gaseous denitrification products from the system. The gas-outlet was equipped with rubber hose, the top end of which was put into a water filled tube. Thus, pressure equalization between the gas phase inside the reactor and the atmosphere pressure outside was ensured, without air penetration into the reactor, and respectively without risks of infection. The samples were taken from the bottom of the reactor. No measures for sterile operation were taken.

The concentration of the nitrate-bound nitrogen (NO_3–N), of the nitrite-bound nitrogen (NO_2–N), and the light absorbance as an indicator for the cell growth, were the monitored process parameters.

Analyses

pH

During the fermentation, the pH-value was measured off-line with a pH-meter Radelkis, OP-211/1 (Hungary), supplied with a glass probe. When necessary, pH was corrected using 1 mol l^{-1} NaOH.

Total solids (TS)

The *TS* content was determined in 20 ml samples of cultural medium, filtered through 0.45 μm effective pore size membrane filters (Gelman GN-6). The filters were previously dried during 24 hours at 105 °C to constant mass. The residues were washed twice with portions of 20 ml distilled water, so that only an insignificant amount of substrate or methabolits were left. The filter's weight was an indication about the *TS* concentrations. Then the *TS* values (related to the biomass concentration) were plotted *vs.* the light absorbance values in a calibration line.

Biomass concentration

The biomass concentration was estimated through the light absorbance *A*. The latter was measured photometrically with a Spekol 11 – Carl Zeiss (Jena, Germany) at λ = 543 nm. The samples were diluted with distilled water, so that the measurements were shifted into a range of A_{543} = 0.05 – 0.60.

Nitrate nitrogen (NO_3–N).

Nitrate mass concentrations were determined by UV-spectrophotometry[21] (Unicam/Helios β) at 220 nm.

Before each photometric determination of nitrogen compounds the samples were centrifuged for 15 min at 8000 rpm to remove the insoluble particles (cells, other medium components). Then 1 ml 1 mol l^{-1} HCl was added to the sample and the mixture was diluted to 50 ml. Then the light absorbance was read against re-distilled water. In order to avoid the interference of the organic matter the sample absorbance was measured at 275 nm, too. The corrected UV- light absorbance of nitrate in the sample was calculated by the equation

$$A_{corr} = A_{220} - 2 \cdot A_{275}.$$

The actual nitrate concentration in the samples were obtained from calibration curve, constructed by the same method. In our paper the data are given as nitrate bound nitrogen ($\gamma_{NO3–N}$).

Nitrite nitrogen (NO_2-N)

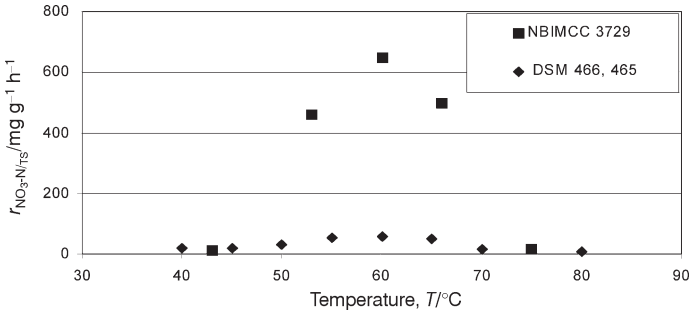
The concentration of nitrite-bound nitrogen was colorimetrically determined,[22] following the coupling of diazonium salt of sulphanilic acid (formed with nitrite) with α-naphtylamine at pH 2.0 – 2.5. The light absorbance of the resulting purple red azo-dye was determined at λ = 520 nm. The experimental data are given as nitrite-bound nitrogen ($\gamma_{NO2–N}$). The samples were pre-treated as above.

Results and discussion

The temperature effect on the denitrification activity of culture NBIMCC 3729

Our first task was to explore the temperature stability of thermophilic bacteria *NBIMCC* 3729 culture and to determine the temperature optimum of the denitrification activity. The experiments were carried out at different temperatures and according other conditions. The nitrate concentration was chosen in a low range $\gamma_{NO2–N}$ = 45 mg l^{-1}, so that the substrate inhibition effects be excluded. In the Fig. 1 the temperature optimum of the two microbes, i.e. *NBIMCC* 3729 and *Geobacillus thermodenitrificans DSM 465/466* are shown.

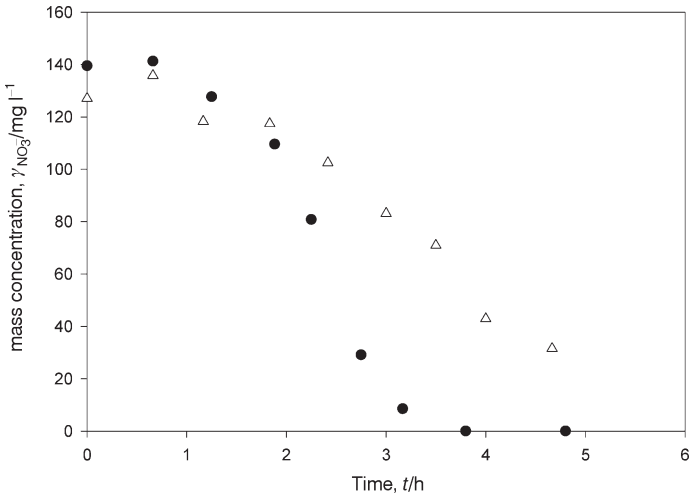
According to our data, the temperature range and the temperature optimum (60 °C) of denitrification activity for both bacteria species are equal.



F i g . 1 – *Temperature optimum of the nitrate ions reduction. Temperature range of activity r_{max} – comparison between NBIMCC 3729 and G. thermodenitrificans DSM 466/465 during the nitrates removal process*

The experimental data show a rate of nitrate removal by the culture *NBIMCC* 3729 about eleven times higher in comparison to *Geobacillus thermodenitrificans DSM 465/466*. At temperatures higher than 73 °C and lower than 43 °C cell growth was not observed.

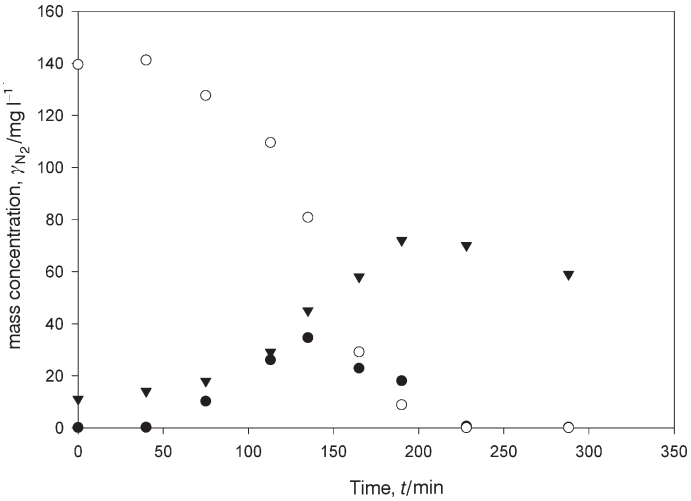
Fig. 2 shows comparative data for nitrate reduction of the culture *NBIMCC 3729* and its own data obtained with the mesophilic strain *Pseudomonas denitrificans*. It is evident, that the denitrification rate is considerably higher when the thermophilic culture is used.



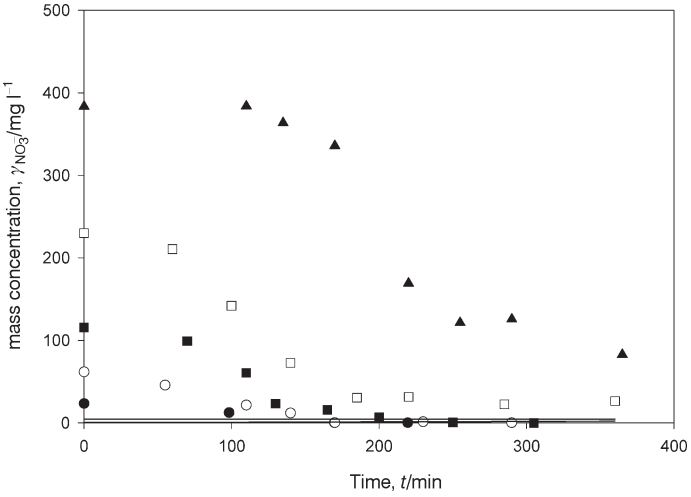
F i g . 2 – *Denitrification performance of two microbial cultures : the thermophilic culture NBIMCC 3729 (circles) and the mesophilic Pseudomonas denitrificans (triangles)*

Effect of nitrate concentration on the denitrification activity of culture NBIMCC 3729. Substrate inhibition

Batch cultivations with wide variety of initial nitrate nitrogen mass concentrations (20 – 380 mg l^{-1}), were performed. Some of the obtained data are shown in Fig. 3 and Fig. 4.



F i g . 3 – *Time profiles of nitrate and nitrite mass concentrations (as nitrogen) at batch fermentation with NBIMCC 3729 at initial nitrates concentration 140 mg l^{-1}. (●) – nitrite, mg l^{-1}; (○) – nitrate, mg l^{-1}; (▼) – light absorbance of the broth at 543 nm wavelength (%)*



F i g . 4 – *Kinetics of nitrate reduction process at different initial mass concentrations of NO_3^-–N (mg l^{-1}): (●) – 23; (○) – 61; (γ) – 116; (□) – 230; (▲) – 383*

Representative profiles of the process quantities ($\gamma_{NO3\text{-}N}$, $\gamma_{NO2\text{-}N}$ and A_{435}) during one typical denitrification experiment are shown in Fig 3. The profiles can be interpreted as follows: the first phase is the *lag-phase*, where the measured quantities remain constant, then the second phase is the *exponential phase*, which, after short *steady phase,* turns into the *phase of cell decay.*

After the *lag-phase*, when the measured quantities remain constant, the *exponential phase* of microbial growth follows, associated with the reduction of main part of nitrates (70 to 85 % in dependency of the initial $\gamma_{NO2\text{–}N}$ concentration, see Fig. 4). During the fast removal of nitrate, a rapid accumulation of nitrites were found.

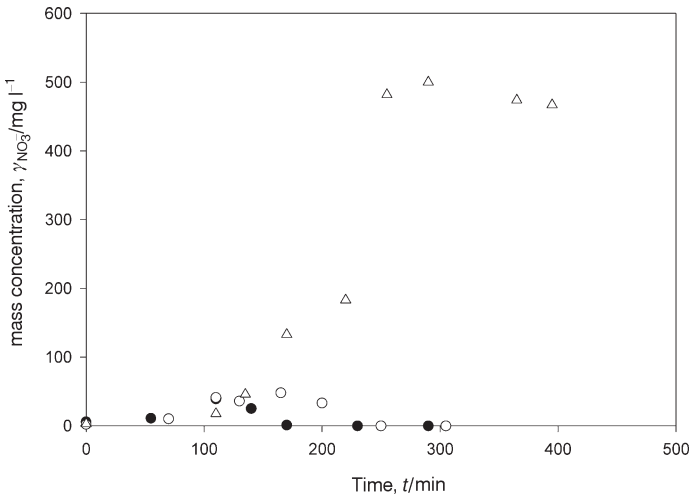
The experimental data showed, that nitrite were accumulated until nitrate is exhausted. Obviously the culture prefers to perform the oxidation of the carbon source using nitrate as the electron acceptor. In the late *exponential phase*, or at the beginning of the *steady phase*, after exhaustion of nitrate, a reduction of nitrite starts. A typical maximum in the time profile of the nitrite concentration is observed when both consecutive reduction processes run with comparable rates.

The specific growth rate varies between μ = 0,36 and 0,75 h^{-1}, depending on the initial $\gamma_{NO3–N}$ concentration. Lineweaver-Burk plot was used to determine the kinetic constants, according to the growth model of Monod. The estimated model quantities are:

$$\mu_{max} = 0{,}75\ h^{-1} \qquad K_s = 27{,}4\ mg\ l^{-1}$$

Fig. 4 shows the influence of the initial ($\gamma_{NO3–N}$) mass concentration on the nitrate reduction. At lower initial mass concentrations (e.g. 23 mg l^{-1}), the reduction is very fast, whilst the higher concentrations slow down the process. In this case a long *lag-phase* is observed, which could be due to a substrate inhibition effect.

The time profile of the accumulation/removal process of $\gamma_{NO2–N}$ is shown in Fig 5. The nitrite concentration in the fermentation medium shows maximum, which appears in the late *exponential phase* of microbial growth and nitrate reduction process, followed by a reduction to gaseous products, performed in the *stationary* and *cell decay phase*. During the first half of exponential growth phase a fast removal of nitrate and accumulation of nitrite were found, unless the initial $\gamma_{NO3–N}$ concentration was above the limiting level of 230 mg l^{-1}.



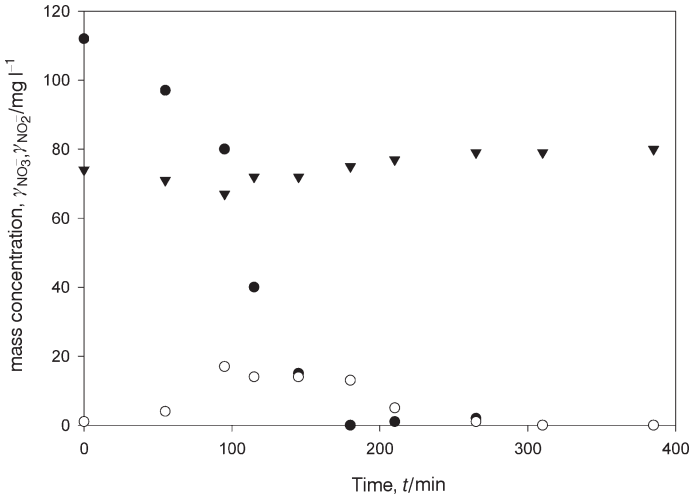
F i g . 5 – *Kinetics of nitrite reduction process at different initial mass concentrations of* NO^-_3 *– N, mg* l^{-1}*: (●) –23.3; (○) – 61.64; (Δ) – 383*

At very high initial nitrate concentrations (and nitrite ones, respectively) almost no nitrite degradation was observed, cf. Fig. 5.

One can seek the reason of this rate limitation of nitrite reduction in the disturbed mass balance between the electron donors and the electron acceptors, and carbon source limitation. According to the data, presented by *Mateju et al.*[16] for the successful and complete reduction of nitrate, a stoichiometric balance of the electron donors (e.g. the carbon source) and the electron acceptor (e.g. nitrate) is required.

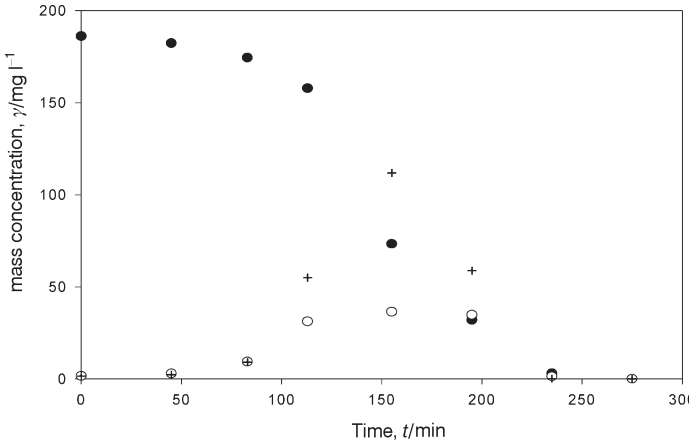
Although the use of the stoichiometric balance proposed by *Mateju et al.*[16] is not sufficiently adequate, we can estimate that the ratio of the concentrations of the carbon source and the nitrates should be greater than 0.475. In our experiments we have been always on the safe side, i.e. carbon source limitations could not be the explanation.

It is obvious also, that the maximum of nitrite concentration coincides with the minimum in pH values, cf. Fig. 6. This fact was observed in all of our experiments. It could be associated with the accumulation of nitrous acid.



F i g . 6 – *Comparison of nitrite accumulation and removal with pH evolution. (●) – nitrate, mg* l^{-1}*; (○) – nitrite, mg* l^{-1}*; (▼) –pH × 10*

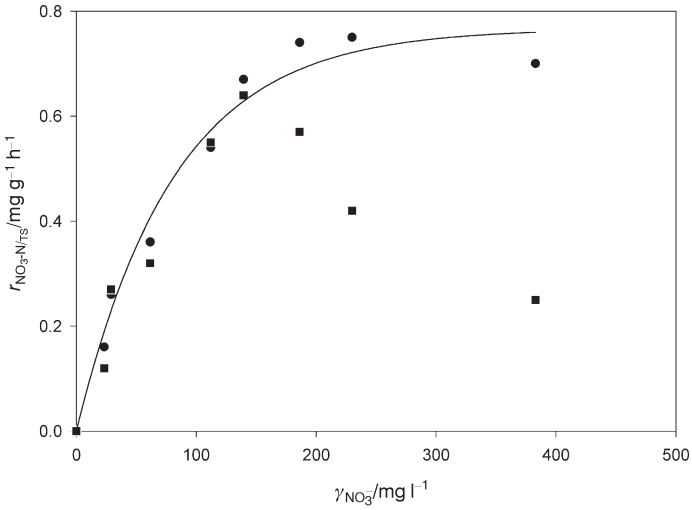
Almeida et al.[23] claim that the main inhibition effect on the denitrification performance of the strain *Pseudomonas fluorescens* is due to the presence of nitrous acid. We made calculations of the concentration of non-dissociated nitrous acid from our experiments based on its dissociation constant, the measured pH-values, and nitrite concentrations. The results are shown in Fig. 7. One can see, that in our case the maximum in nitrous acid concentration coincides with the end of nitrate reduction and its degradation follows the nitrite decay almost com-



F i g . 7 – *Time profile of nitrate (●), nitrite (○) mass concentrations (measured, mg l^{-1}) and nitrous acid one (+, calculated, μg l^{-1}). Initial nitrate nitrogen concentration – 186 mg l^{-1}*

pletely. On the other hand, the extension of the lag-phase occurs before the nitrite formation. Therefore, we cannot state that the considered strain nitrites and nitrous acid are the main inhibitors of the denitrification process, unless in the second step, provided the initial nitrate and subsequent nitrite and nitrous acid concentrations are extremely high, cf. Fig. 5.

The specific rates of nitrate and nitrite removal, observed during batch fermentations is shown in Fig. 8. A maximum rate value [mg g^{-1} h^{-1} NO_3–N in TS] is reached at an initial $\gamma_{NO3–N}$ mass concentration of 200 mg l^{-1}, which obviously is the limiting concentration under the mentioned conditions of the studied process.



F i g . 8 – *Reaction rate of nitrate (●) and nitrite (■) reduction, [mg g^{-1} h^{-1} NO_3–N in TS], depending on the initial nitrate-nitrogen mass concentration*

The review of the data for nitrite degradation presented on the same figure shows, that the second step is quite sensitive to the initial nitrate concentrations, through the concentration of accumulated nitrite. Whereas both nitrate and nitrite degradation rates are almost equal at initial concentrations of nitrate-bound nitrogen below 200 mg l^{-1}, beyond this value the nitrite degradation rates drops drastically. This fact is in agreement with our statement, that the accumulated nitrite ions as well as the free nitrous acid are inhibitors as substrates only for the second consecutive step of the net denitrification process.

Therefore, we can expect, that substrate inhibition at high initial nitrate concentrations is the main reason of process retardation.

Conclusions

The obtained results lead to the conclusion that the utilized culture *NBIMCC 3729* is stringently thermophilic, with temperature optimum of 60 °C. The studied culture is superior to selected mesophilic strain, e.g. *Pseudomonas denitrificans*. A comparison between *NBIMCC 3729* and *Geobacillus thermodenitrificans* DSM 466/465 revealed the higher effectiveness of *NBIMCC 3729* as a denitrification culture bacteria under thermophilic conditions. It successfully reduces nitrate ions, without nitrite accumulation, unless the initial nitrates concentration exceeds quite high values. It is interesting to repeat this study at genuine wastewater conditions at genuine $\gamma_{NO3–N}$ mass concentrations, i.e. wastewater from food industry, beverage production, etc. In future approaches the possibility should be tested to run the process as continuous and with immobilized cells.

ACKNOWLEDGEMENTS

This research was supported by the National Council for Scientific Research (Republic of Bulgaria) by grant TN-901/1999.

The culture Geobacillus thermodenitrificans DSM 465/466 was given to our disposal from the 'Deutsche Sammlung von Mikroorganismen und Zellkulturen' with the kind assistance of Prof. Dr. G. Antranikian, Technical University of Hamburg-Harburg, Germany.

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