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THE EFFECT OF PHOSPHATE CONCENTRATION ON THE GROWTH OF BACTERIA IN A DENITRIFICATION PROCESS

Results of investigations of the effect of initial concentration of phosphates on biomass growth in the process of denitrification are presented. Kinetic parameters of the biomass growth were determined using empirically found dependence of protein growth on time at an optimum concentration of parent compounds which limit the denitrification process. The biomass growth was described by a hyperbolic Monod's function. The assumed kinetic model of the biomass growth enables us to determine maximum rate constant of the growth and Michaelis constant at a known initial phosphate concentration in a medium.

1. INTRODUCTION

Recently, concentrations of noxious and toxic nitrates and nitrites in surface, ground and drinking waters have been increased, which results from a disturbance in the cycle of natural transformations of nitrogen compounds (PHILIPAT and PATTE [9]). The noxious compound, coming mainly from industrial and municipal wastes as well as from the use of chemical fertilizers in agriculture, pose a serious threat to human health, hence there arises an urgent need to neutralize them by sewage treatment and water purification.

The most widely applied and efficient method of removing inorganic oxo-compounds of nitrogen from waters and wastes is denitrification. In this process, anaerobic bacteria use the oxygen of nitrates and reduce them to free nitrogen, the necessary energy being supplied from organic compounds (electron donors) dissolved in water

When kinetics is taken into account, this is a multistage redox process in which nitrates are terminal acceptors of electrons during the oxidation of organic compounds [4], [5]. This process is catalyzed by enzymes from reductase group, occurring in the cells of denitrificators. In our study, bacteria from the *Pseudomonas* genus have been applied since

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their biomass after denitrification is a valuable source of amino acids, peptides and some enzymes. Moreover, it has already been shown that they can be used as a component of fodder and fish feeding stuff [2]. The optimum growth of the biomass of denitrification microorganisms is determined by the rate of catalysis of parent substance by enzymes (the parent substances occurring at an optimum of carbon to nitrogen ratio), the content of inorganic assimilable compounds of phosphorus which are indispensable, e.g., to the synthesis of ATP and proteins and some other factors.

Our earlier investigations of the kinetic model of denitrification have proved that this process proceeds in accordance with the kinetic model of a system of irreversible consecutive first order reactions with a stable nitrite intermediate:

$$NO_3^- \xrightarrow[t_1]{k_1} NO_2^- \xrightarrow[t_2]{k_2} N_2 .$$

For this model, a kinetic equation for both stages of the reaction was derived, enabling calculation of appropriate rate constants (k_1, k_2) and induction periods (t_1, t_2) [5]. The effect of concentrations of nitrates and nitrites [6], temperature as well as pH of the medium [7] on the kinetic parameters of denitrification were also investigated. Besides, we have determined the influence of nitrate concentration on the growth of the biomass of denitrification bacteria [3].

The present paper deals with the effect of initial concentration of phosphates on the growth of the biomass during the denitrification. The process of the biomass growth is described by a hyperbolic Monod function [1], which in spite of its formal similarity to the Michaelis–Menten function cannot be used for describing single enzymatic reactions. The latter function, on the grounds of empirically established dependence of the biomass increase on optimum concentration of limiting parent compounds (C/N), can be used for determining kinetic parameters of enzymatic catalyst growth. The catalyst concentration was calculated by measurement of the protein amount.

2. EXPERIMENTAL

2.1. MATERIAL AND METHODS

Denitrification was performed in an isolated culture of *Pseudomonas* sp. under optimum conditions (pH 7.0, 37°C) at a mass ratio of organic carbon to inorganic nitrogen equal to 1.4 g/g, according to the method described earlier [4]–[6]. As a nutrient the following salt solutions (g/dm^3) have been applied: sodium lactate – 6.5, KNO_3 – 10.0, $CaCl_2$ – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.5, NH_4Cl – 0.25, $Fe(NO_3)_3$ – 0.25, as well as the following microelements (g/dm^3): $CuSO_4 \cdot 5H_2O$ – $1.2 \cdot 10^{-2}$, $Ni(NO_3)_2$ – $1.5 \cdot 10^{-4}$, $NaHSeO_3$ – $1.0 \cdot 10^{-5}$, $Zn(NO_3)_2 \cdot 6H_2O$ – $1.0 \cdot 10^{-3}$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ – $1.82 \cdot 10^{-2}$.

Phosphates (Na_2HPO_4) were added in different concentrations, but the results presented in this paper were obtained for phosphate concentrations of 0.625, 2.5 and 10.0 g/dm^3. Two series of experiments were carried out in parallel, one differing from the other in the additional amount of 4 g/dm^3 of EDTA (a complexing agent).

Concentrations of nitrates and nitrites were determined spectrophotometrically [11], using a VSU-2P spectrophotometer. Changes in biomass concentration as a function of time were determined by measurement of the protein concentration according to Lowry's method [8].

Kinetic parameters of the denitrification were found from the following equations [5]:

$$[NO_3^-] = [NO_3^-]_0 \, e^{-k_1(t-t_1)},$$

$$[NO_2^-] = \frac{[NO_3^-]_0 \, k_1 \, e^{k_1 t_1 - k_2 t}}{k_2 - k_1} \left[e^{(k_2-k_1)t} - e^{(k_2-k_1)t_2}\right]$$

where:

k_1 – rate constant of the first stage of reduction,

k_2 – rate constant of the second stage of reduction,

t_1 – induction period of the first stage of reduction,

t_2 – induction period of the second stage of reduction,

$[NO_3^-]_0$ – initial concentration of nitrates,

$[NO_2^-]_0$ – initial concentration of nitrites.

The above two-parameter equations were solved numerically using the least-square method [5]. The agreement between experimental data and theoretical rate equation was expressed by the correlation coefficient (r), which was calculated from n pairs of results in the first stage of reduction [10].

The rate of biomass growth was found from the following equation [1]:

$$\frac{dx}{dt} = \mu(t)\, x(t)$$

where:

$\mu(t$ – a time-dependent apparent rate constant of biomass growth,

$x(t)$ – protein concentration at time t.

The apparent rate constant (μ) is truly constant when the concentrations of parent substances are unlimited. If any of the components of the culture limits the growth, then the apparent rate of the process ($\mu(t)$) changes according to a hyperbolic function, which can be described by the following equation:

$$\mu = \frac{\mu_m \, s(t)}{k_m + s(t)}$$

where:

$s(t)$ – concentration of a parent substance which limits the process at time t,

k_m – Michaelis constant,

μ_m – maximum biomass growth rate constant.

The parent substance inhibiting the growth of bacteria in the culture tested is nitrate, the concentration of which is given by the following equation [6]:

$$s(t) = s_0 \, e^{-k_1(t-t_1)}$$

where:

s_0 – initial concentration of nitrates,

k_1 – rate constant of nitrate reduction,

t_1 – induction period of the first stage of denitrification.

Thus, the kinetic equation of the biomass growth has the form:

$$\frac{dx}{dt} = \frac{\mu_m s_0 \, e^{-k_1(t-t_1)}}{k_m + s_0 \, e^{-k_1(t-t_1)}} x(t) .$$

The solution of the above equation gives the concentration of biomass x at time t [3]:

$$x(t) = x_0 \left[\frac{e^{k_1(t-t_1)} (k_m + s_0)}{k_m e^{-k_1(t-t_1)} + s_0} \right] \frac{\mu_m}{k_1}$$

where:

$x(t)$ – biomass concentration at time t,

x_0 – initial concentration of biomass.

The values of kinetic parameters ($\mu_m \cdot k_m$) and $x(t)$ describing a biomass growth at a given time can then be calculated. The estimated mean error (S_y) is calculated from the equation:

$$S_y = \frac{\sum_{i=1}^{n} x_{t_i} - x_{d_i}}{n}$$

where:

x_t – theoretical value of protein concentration at time t,

x_d – experimental value of protein concentration at time t,

n – number of measurements.

The mean percentage error is described by the equation:

$$S = \frac{2S_y}{x_{max} - x} 100\%$$

where:

x_{max} – maximum protein concentration.

3. RESULTS AND DISCUSSION

The effect of initial concentration of orthophosphates on kinetic parameters of denitrification was described on the basis of changes in concentrations of inorganic nitrogen compounds as a function of reaction time. From the theoretical model [6], the rate constants (k_1, k_2) of both stages of the reaction induction periods (t_1, t_2), maximum concentration of an intermediate product ($NO_{3\,max}^-$) and the time (t_{max}), at which the concentration of

nitrites reaches its maximum value, have been calculated. The results are presented in table 1.

Table 1

Kinetic parameters of denitrification proceeding under optimum conditions (temperature 37 °C, pH=7, $[N_{NO_3^-}]_0$=1.4 g/dm^3) at different concentrations of $[PO_4^{3-}]_0$

Concentration of $[P_{PO_4^{3-}}]_0$ (g/dm^3)	k_1 (h^{-1})	t_1 (h)	k_2 (h^{-1})	t_2 (h)	t_{max} (h)	$[N_{NO_2^-}]_{max}$ (g/dm^3)	r	S_y
lactate medium								
0.055	0.234	5.4	0.359	5.5	8.9	0.400	0.91	0.144
0.220	0.202	4.2	0.414	4.7	8.5	0.310	0.90	0.093
0.880	0.441	4.7	0.470	4.8	8.5	0.407	0.90	0.170
lactate medium with EDTA								
0.055	0.330	5.9	0.514	6.3	9.4	0.340	0.91	0.138
0.220	0.283	3.9	0.476	4.7	8.6	0.290	0.95	0.096
0.880	0.475	4.4	0.500	4.4	6.5	0.500	0.92	0.175

k_1 – rate constant of the first stage of denitrification.

t_1 – induction period of the first stage of denitrification.

k_2 – rate constant of the second stage of denitrification.

t_2 – induction period of the second stage of denitrification.

t_{max} – time of maximum nitrite concentration $[N_{NO_2^-}]_{max}$ in the denitrification process.

r – correlation coefficient.

S_y – mean estimation measurement error.

The kinetic parameters obtained were used for calculating the concentrations of $[NO_3^-]$and $[NO_2^-]$ which were compared with the experimental data. A plot illustrating this correlation is given in fig. 1, whereas the correlation between the theoretical and experimental data on the biomass increase is shown in fig. 2.

Rate constants and induction periods as functions of initial concentration of orthophosphates are presented in figs. 3 and 4.

As follows from the data presented, rate constants of both stages of denitrification as well as induction periods depend on the initial concentration of orthophosphates. An increase in their concentration usually results in an initial decrease in the rate constants k, but then leads to their increase, i.e. acceleration of the denitrification. The character of these changes is the same in EDTA-containing as well as in EDTA-free media. (The addition of EDTA results in an increase of nitrate solubility.) However, rate constants of the

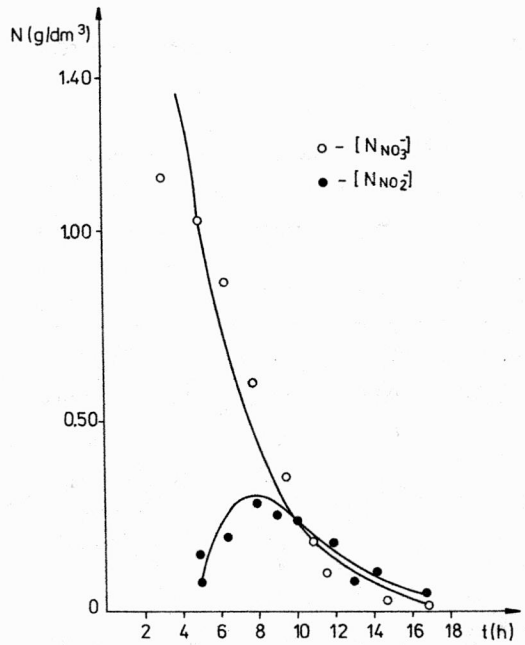

Fig. 1. Correlation between experimental data (circles) and theoretical ones (solid line) obtained on the basis of the assumed model of denitrification (temperature 37°C, pH 7.0, C/N 1.4)

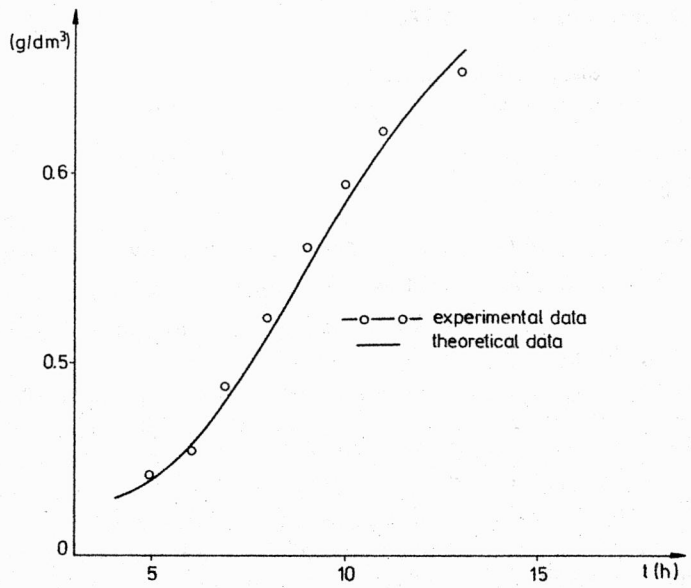

Fig. 2. Correlation between experimental (circles) and theoretical data (solid line) obtained on the basis of the assumed model of biomass growth (g/dm^3)

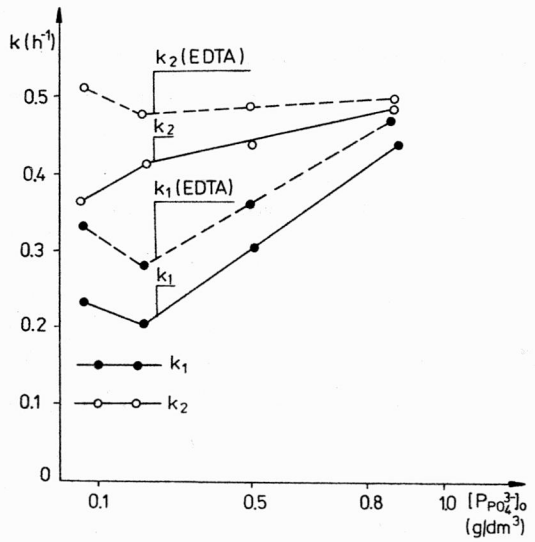

Fig. 3. Dependence of rate constants of nitrate reduction on initial phosphate concentration. Solid line: EDTA-free medium, dashed line: EDTA-containing medium

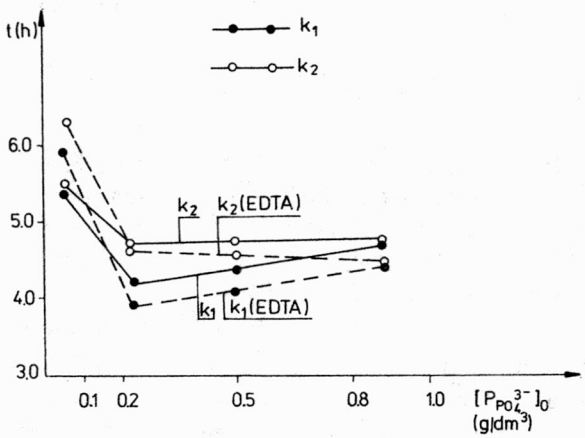

Fig. 4. Dependence of induction period on initial phosphate concentration. Solid line: EDTA-free medium, dashed line: EDTA-containing medium

second stage (k_2) show another dependence upon initial concentration of orthophosphates (fig. 3, table 1, column 4). In this case, an increase in the concentration of $[P_{PO_4^{3-}}]_0$ from 0.055 to 0.22 g/dm^3 causes a rise in the rate constant k_2 only in the experiments without EDTA.

Table 2

Changes in protein concentration in the denitrification process (temperature 37 °C, pH=7.0, $[N_{NO_3^-}]_0$=1.4) at different initial concentrations of orthophosphates

Measurement time (h)	Initial concentration (g/dm^3) of phosphates $[P_{PO_4^{3-}}]_0$					
	EDTA-free medium			EDTA-containing medium		
	0.055	0.220	0.880	0.055	0.220	0.880
3	–	0.230	–	–	–	–
4	0.280	0.220	0.235	0.270	0.230	0.150
5	0.270	0.290	0.220	0.260	0.310	0.190
6	0.326	0.350	0.320	0.275	0.378	0.280
7	0.400	0.405	0.420	0.398	0.460	0.443
8	0.480	0.520	0.520	0.555	0.590	0.620
9	0.550	0.640	0.660	0.727	0.740	0.798
10	0.620	0.750	0.790	0.920	0.952	0.960
11	0.700	0.951	0.903	1.153	1.134	1.110
12	0.766	1.100	1.117	1.274	1.231	1.240
13	0.837	1.212	1.180	1.290	1.301	1.270
14	0.887	1.240	1.200	1.300	1.310	
15	0.888	1.244				

The addition of the complexing agent (EDTA) affects the dependence, causing a decrease in the value of the rate constant k_2. This implies that as the concentration of orthophosphates increases, the rate constant decreases, which means that the reaction becomes slower. Moreover, in this range of orthophosphate concentration the induction periods (t_1 and t_2) are markedly reduced.

Further increase in $[P_{PO_4^{3-}}]$ concentration usually prolonges slightly these periods. Such a behaviour was observed both in EDTA-containing and EDTA-free media.

Having compared the kinetic parameters of the above systems, it is possible to come to a conclusion that an addition of a complexing agent (EDTA) to these systems accelerates both stages of denitrification and reduces their induction periods. The effect of initial concentration of phosphates on the rate of biomass growth was determined by measurement of an increase in protein concentration in time at different initial phosphate concentrations. The results obtained are compiled in table 2, and some exemplary kinetic curves illustrating increase in protein amount are presented in fig. 5.

Using the proposed kinetic model of biomass growth it was possible to find the maximum rate constants of protein concentration increase (μ_m) and Michaelis constants (K_m). The data obtained are shown in table 3.

Table 3

Dependence of maximum value (μ_m) of the biomass growth and Michaelis constants (K_m) on initial concentration of orthophosphates ($[P_{PO_4^{3-}}]_0$)

$[P_{PO_4^{3-}}]_0$ (g/dm^3)	k_1 (h^{-1})	t_1 (h)	X_0 (g/dm^3)	μ_m (h^{-1})	K_m (g/dm^3)	S (%)
lactate medium						
0.055	0.234	5.4	0.270	1.03	3.75	4.1
0.220	0.202	4.2	0.224	0.43	0.80	8.2
0.880	0.441	4.7	0.200	0.37	0.15	5.5
lactate medium with EDTA						
0.055	0.330	5.9	0.260	1.35	2.50	7.1
0.220	0.283	3.9	0.220	0.43	0.48	7.3
0.880	0.475	4.4	0.150	0.48	0.16	4.0

X_0 – initial protein concentration.

μ_m – maximum rate of biomass growth.

K_m – Michaelis constant.

S – mean percentage error.

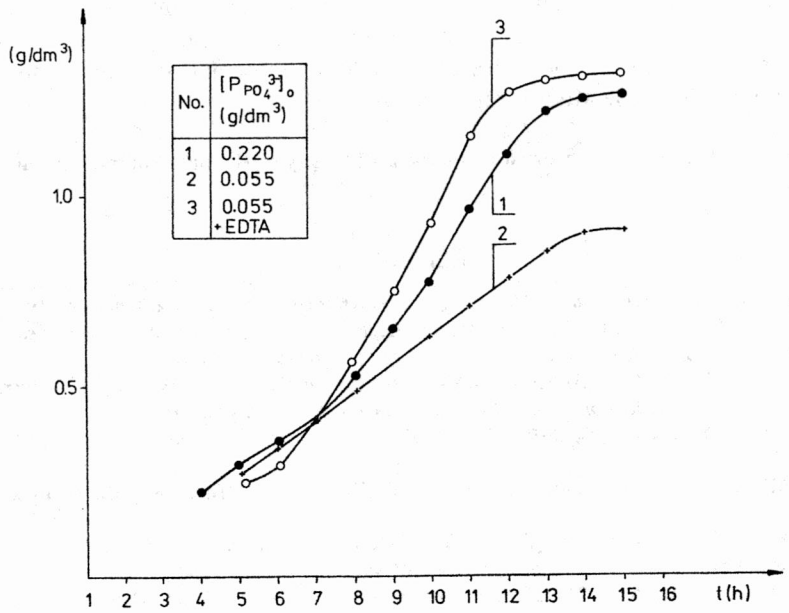

Fig. 5. Dependence of biomass concentration (g/dm^3) on the duration of denitrification and initial phosphate concentration

The data in table 3 proves that an increase in the initial phosphate concentration in a medium leads to a decrease in the maximum rate constant of biomass growth and Michaelis constant. A high value of this constant, characteristic of a medium containing 0.055 g of phosphate per dm^3, points to a low chemical affinity of enzyme to the reaction substrate in this system. In such a case, an addition of EDTA to the medium enhances the chemical affinity of the enzyme to the substrate. The most pronounced change being observed at 0.055 g/dm^3 concentration of $[P_{PO_4^{3-}}]$. This effect may be due to the complexing of Ca^{2+} and Mg^{2+} ions present in the nutrient by EDTA. The introduced phosphorus occurs then in the form of HPO_4^{2-} ions which are assimilated better by microorganisms.

In all the series investigated, an apparent rate of biomass increase (μ) was calculated from the equation given in the foregoing part of this paper, and it was concluded that the rate is not constant, but it decreases with duration of the process. When phosphates were added at the concentration of 0.88 g/dm^3, the initial rate calculated from the equation was roughly equal to the maximum rate, which is equal to 0.37 and 0.48 for EDTA-free and EDTA-containing media, respectively. As has already been shown, a relative rate of biomass growth decreases with the time of the reaction, but due to a maximum value of this rate in the beginning of this process, the reaction is not limited by the substrate [3].

Using a kinetic equation of denitrification [5] and the kinetic parameters obtained, a kinetic model of biomass growth of denitrificators was proposed. It enables determination of maximum constant of biomass increase (μ_m) and Michaelis constant (K_m) at the initial concentrations of phosphates in lactate medium.

It should be noted here that the mean percentage error (S), expressing a deviation of the theoretical values of protein concentration (estimated from the postulated model) from the experimental values, does not exceed 9%. As far as the microbiological processes are concerned, such an error provides a satisfactory conformation of the proposed kinetic model of biomass increase after denitrification.

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WPŁYW STĘŻENIA FOSFORANÓW NA PRZYROST BIOMASY BAKTERII PODCZAS DENITRYFIKACJI

Przedstawiono wyniki badań nad wpływem początkowego stężenia fosforanów na przyrost biomasy podczas denitryfikacji. Parametry kinetyczne przyrostu biomasy wyznaczono na podstawie empirycznie ustalonych zależności przyrostu białka w czasie przy optymalnym stężeniu substratów limitujących denitryfikację. Do opisu przyrostu biomasy wykorzystano funkcję hiperboliczną Monda. Zaproponowany model kinetyczny przyrostu biomasy denitryfikatora pozwala na wyznaczenie maksymalnej stałej wzrostu i stałej Michaelisa, gdy znane jest stężenie początkowe fosforanów w pożywce.

ВЛИЯНИЕ КОНЦЕНТРАЦИИ ФОСФАТОВ НА ПРИРОСТ БИОМАССЫ БАКТЕРИЙ ВО ВРЕМЯ ДЕНИТРИФИКАЦИИ

Представлены результаты исследований влияния начальной концентрации фосфатов на прирост биомассы бактерий во время денитрификации. Кинетические параметры прироста биомассы определены на основе эмпирически установленных зависимостей прироста белков во времени при оптимальной концентрации субстратов лимитирующих денитрификацию. Для описания прироста биомассы использована гиперболическая функция Монода. Предложенная кинетическая модель прироста биомассы денитрификатора позволяет определить максимальную постоянную роста и постоянную Михаелиса, когда известна начальная концентрация фосфатов в питательной среде.