Environment Protection Engineering

Vol. 18 1992 No. 1–2

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UPTAKE OF PHOSPHATES AND PHOSPHORITES BY DENITRIFYING BACTERIA *BACILLUS LICHENIFORMIS*

The effect of inorganic phosphate concentration on the growth of denitrifying bacteria *Bacillus licheniformis* and on the course of denitrification was studied. It was shown that up to 20 mg/dm^3 denitrification is limited by phosphorus content. The optimum P/N and P/C (g/g) ratios were found to be 0.14 and 0.07, respectively. Induction time and rate constants of denitrification at different phosphorus concentrations were calculated. Additionally the growth media were enriched with the "Courriba" phosphorites from Algiers as a sole P source. Although this mineral comprises predominantly insoluble $Ca_3(PO_4)_2$ and has very stable crystalline structure, denitrifiers proved to have enzymatic system which enables them to release phosphorus from apatite lattice. Concentration of phosphorite phosphorus exceeding that of phosphate phosphorus over 100 times was required to obtain comparable kinetic parameters of denitrification, suggesting that probably only phosphate anions from the surface of the mineral particles are enzymatically available.

1. INTRODUCTION

Phosphorus compounds are essential in the metabolism of living organisms. Stable, with multiple negative charges, phosphates seem to fulfil various biochemical functions. They are the components of DNA, RNA, most of the coenzymes as well as intermediate metabolites [1]. They are eventually the main reservoirs of energy ("high-energy"molecules such as ATP or pyrophosphate with a large free energy of hydrolysis) [2].

The growth of soil bacteria and plants is greathy influenced by the amount of the available phosphorus. The principal form of phosphorus taken up by many microorganisms seems to be orthophosphate and its uptake, in many cases, is stimulated by the presence of nitrate [3]. It has been shown that at high external

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phosphorus concentration, orthophosphate uptake is rapid and excess phosphorus is stored in the cell as polyphosphates, organized in rigid aggregates. When external phosphorus is limited, cells with the high level of stored phosphorus utilize these internal resources [4]. In most of the soils, the amount of the soluble phosphorus compounds is insufficient for the plants and fertilization is required.

Production of phosphorus fertilizers is based on water-insoluble apatites and phosphorites. Their poor solubilities are due to the stable crystalline structure of this group of minerals and an acid or thermal treatment is required for the breakage of the apatite structure [5].

Denitrifiers represent a widespread group of soil bacteria [6]. It has been shown that most of P_i (inorganic phosphorus) incorporated into cells during denitrification can be found in ATP and GTP [7], and ATP formation was proved to be coupled with reduction of nitrate to nitrite [8].

Phosphorus constitutes about 1.5–2.5% of dry matter of bacterial cell and can be released to the soil by autolysis or the action of other microorganisms.

The purpose of our studies was to show the effect of phosphorus concentration on denitrifying bacteria *Bacillus licheniformis* and to find out if phosphorites, without any initial treatment, can be dissolved and assimilated by these microorganisms.

2. MATERIALS AND METHODS

Microorganisms. A heterotrophic, facultatively anaerobic bacterium *Bacillus licheniformis*, originally isolated from the soil [9], has been used to study denitrification. The process was carried out in air-tight reactors and monitored at 37°C. The inoculum was taken out from the bacterial culture after 18 h corresponding to the end of logarithmic growth phase.

Growth media. When phosphorite was applied, the growth medium was lacking sodium phosphate and calcium chloride [10]. The initial value of pH was adjusted to 8.5.

Phosphorite. "Courriba" phosphorite from Algiers was homogenized and used in the growth medium. Atomic absorption analysis of phosphorite and standard method for phosphate determination [11] gave the following results (in weight %): Ca – 32, Fe – 1.6, Zn – 0.5, Cr – 0.2, Mg – 3, Sr – 0.35, traces of Ni, Cd, Cu and Pb, $P(H_2PO_4^-)$ – 0.11, $P(HPO_4^{2-})$ – 1.19, $P(PO_4^{3-})$ – 13.8. The concentration of phosphorus applied ranged from 2.9 to 22 g/dm^3. At the highest phosphorus concentration three series of experiments were carried out. First series – with untreated phosphorite containing primary, secondary and tertiary phosphates, second series – with primary phosphates and phosphates washed out, and third series – with primary and secondary phosphates washed out.

Nitrate assay. Nitrate concentration was measured using ion-selected electrode "Detector".

Nitrite assay. Nitrite concentration was measured colorimetrically [11] by means of Specord VSU2P, Carl Zeiss, Jena.

Protein assay. Protein concentration was measured in the growth media solution by Lowry method [12].

Phosphate assay. Primary, secondary and tertiary phosphates were separated based on their different solubilities and their concentrations were measured as ammonium phosphomolibdate [11].

Kinetic calculations. Under standard conditions, denitrification brought about by *Bacillus licheniformis* bacteria follows the kinetic model of irreversible subsequent reactions with stable intermediate:

$$NO_3^- \underset{t_i}{\overset{k_1}{\rightarrow}} NO_2^- \overset{k_2}{\rightarrow} N_2.$$

Kinetic parameters of the system studied were calculated based on kinetic model of denitrification proposed before [9]. The following equations have been used:

$$[NO_3^-] = [NO_3^-]_0 e^{-k_1 t + k_1 t_1},$$

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

$[NO_3^-]_0$ – initial concentration of nitrates,
$[NO_2^-]_0$ – initial concentration of nitrites,
t – time,
t_1, t_2 – induction time of both stages of the reaction,
k_1, k_2 – rate constants of both stages of the reaction.

3. RESULTS

In the first series of experiments, Na_2HPO_4 was used as the phosphorus source in the growth media. The effect of phosphate concentration on the time of denitrification is shown in figures 1, 2.

The increase in phosphorus concentration caused an increase in the rate constants of both nitrate (k_1) and nitrite (k_2) reduction, and simultaneous decrease of the induction times of denitrification (t_1) (table 1).

The maximum rate constants (0.2 h^{-1}) for both reaction stages and the shortest induction time of denitrification (5 h) were obtained when 200 mg P/dm^3 were applied in the growth medium. This value of rate constants for NO_3^- and NO_2^- reduction is about 7 times and 10 times higher than the values obtained for the lowest phosphorus concentration applied (2 mg P/dm^3). At concentration of phosphorus equal to 200 mg/dm^3, total reduction of NO_3^- and NO_2^- lasted 5 times and 8 times shorter, i.e. 13.5 h and 18 h, than at phosphorus concentration of 2 mg/dm^3

when the respective values of NO_3^- and NO_2^- reduction were 60 h and 144 h (figures 1–2). Further increment in the phosphorus concentration (up to 2 g P/dm^3) had no effect on the kinetics of the reaction.

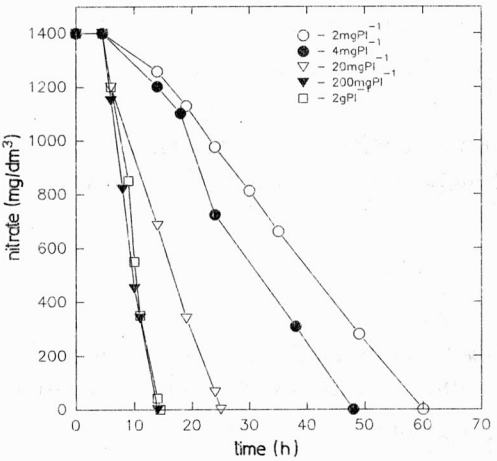

Fig. 1. The effect of phosphate phosphorus concentration on nitrate reduction

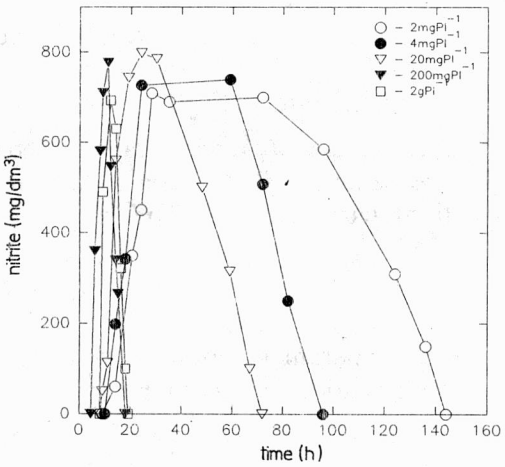

Fig. 2. The effect of phosphate phosphorus concentration on nitrite reduction

Table 1

The effect of phosphate phosphorus concentration on kinetic parameters of denitrification

P g/dm^3	P/C mg/dm^3	P/N g^{-1}	t_1 h	k_1 h^{-1}	k_2 h^{-1}	Final protein content g/dm^3
0.002	0.7	0.0014	8	0.03	0.02	0.48
0.004	1.4	0.0028	7	0.04	0.03	0.85
0.020	6.8	0.014	5	0.1	0.04	0.90
0.200	68	0.14	5	0.2	0.2	1.34
2.000	680	1.4	5	0.2	0.2	1.30

The exponential bacterial growth up to 19–24 h was observed with all phosphorus concentrations applied (figure 3).

The final protein concentration, i.e. 1.3 g/dm^3, in the growth medium was highest at the dose of 200 mg P/dm^3. Further increase of phosphorus concentration did not result in the increment of the final protein concentration (figure 3). In the case of lower phosphorus concentrations applied, the final protein concentrations decreased by 66% for 20 mg P/dm^3, by 60% for 4 mg P/dm^3 and by 34% for 2 mg P/dm^3.

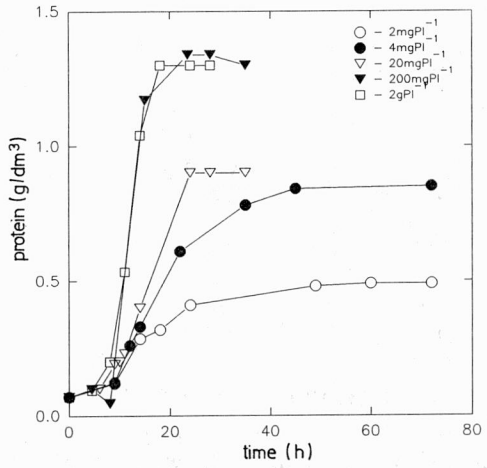

Fig. 3. The effect of phosphate phosphorus concentration on bacterial growth

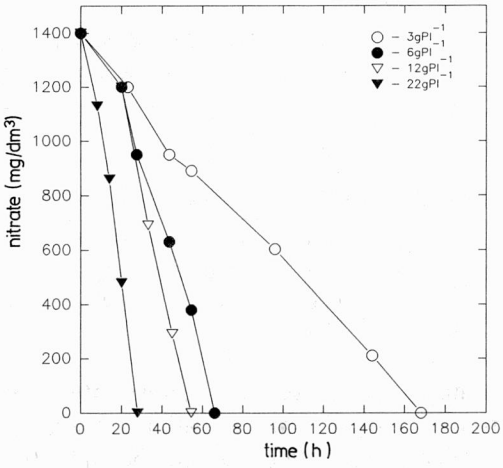

Fig. 4. The effect of phosphate phosphorus concentration on nitrate reduction

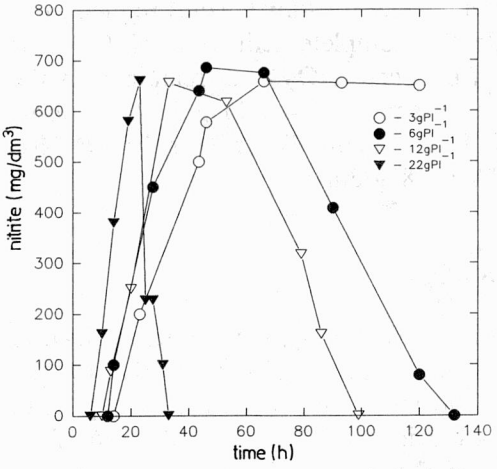

Fig. 5. The effect of phosphorite phosphorus concentration on nitrite reduction

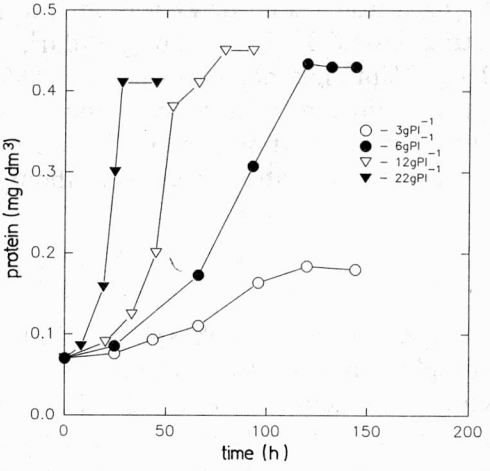

Fig. 6. The effect of phosphorite phosphorus concentration on bacterial growth

In the second series of experiment, natural phosphorites were used as the only phosphorus source in the growth medium. The effect of phosphorite phosphorus concentration on the denitrification is shown in figures 4 and 5. The kinetic data for this set of experiments are given in table 2. With the increasing amount of phosphorites applied the rate constant of nitrate reduction increased 9 times (from 0.01 h^{-1} for 2.9 g P/dm^3 to 0.09 h^{-1} for 22 g P/dm^3). The rate constant of nitrite reduction increased over 16 times (from 0.006 h^{-1} for 2.9 g P/dm^3 to 0.1 h^{-1} for 22 g P/dm^3).

Table 2

The effect of phosphorite phosphorus concentration on kinetic parameters of denitrification

P g/dm³	P/C g/g	P/N g/g	t_1 h	k_1 h^{-1}	k_2 h^{-1}	Final protein content g/dm³
2.9	1.0	2.1	15	0.01	0.006	0.18
5.8	2.0	4.2	17	0.04	0.03	0.43
11.6	4.0	8.4	18	0.06	0.03	0.45
21.8	7.6	15.7	7	0.09	0.1	0.41

Removal of the primary phosphates and both primary and secondary phosphates from the phosphorites had no effect on the kinetics of denitrification process.

The slight differences in the induction time (from 25 h to 18 h) were observed when phosphorus concentration ranging from 2.9 g/dm³ to 11.6 g/dm³ was used in the growth medium. The significant decrease of the induction time occurred when 22 g P/dm³ was applied (7 h). Time required for the complete nitrate reduction has changed from 28 h (for 22 g P/dm³) to 16.9 h (for 2.9 g P/dm³). Total reduction of nitrite lasted 33 h at 11.6 g P/dm³, while the complete reduction of NO_2^- at 2.9 g P/dm³ has not occurred and 0.65 g of nitrogen (NO_2^-) has remained in the system. Final protein concentrations were very similar (0.4–0.45 g/dm³) at phosphorus concentration ranging from 5.8 g to 11.6 g (figure 6). At 2.9 g P/dm³ final protein concentration decreased about 53% (0.18 g/dm³).

4. DISCUSSION

Optimum dose of phosphorus (in the form of Na_2HPO_4) for the denitrification brought about by *Bacillus licheniformis* was found to be 0.2 g/dm³. In this case, the shortest induction time, the maximum rate constants and maximum final protein concentration in both stages of the process are evidenced. The concentration of 0.2 g P/dm³ seems to represent already an excess of phosphorus, since further increment of its concentration has no effect on reaction course. It corresponds to P/N ratio equal to 0.14 g/g. This value is consistent with the results obtained by RHEE and GOTHAM [13] for algae. They reported that optimum ratio of phosphorus to nitrogen varied from 0.08 to 0.3. Our experiments have shown that optimum P/C ratio is equal to 70 mg/g. This result is in the range of values obtained by VODSTEIN [14] according to whom it is from 0.02 to 0.1 g/g. Lower phosphorus concentration seems to limit the bacterial growth which results in extended total reduction time for both steps of denitrification.

When natural phosphorites were applied as the sole phosphorus source, much higher phosphorus concentrations had to be used. Even with the concentration of

phosphorus 100 times higher than the optimum for phosphates, the kinetic parameters of denitrification have not approached the optimum values. The reason for that lies in the fact that over 90% of phosphorite is in the form of insoluble $Ca_3(PO_4)_2$ and phosphorite represents a very stable crystalline structure. Release of phosphate anions from that structure requires initial enzymatic treatment which results in the increase of the induction time of denitrification. It seems possible that only phosphate anions from the surface of the mineral particles are available which can explain why such a great excess of phosphorite phosphorus is needed. There was a possibility that so high phosphorite phosphorus concentration is required because the bacteria are capable of releasing only the most readily soluble primary phosphates. It was not the case since the removal of primary and secondary phosphates from the mineral did not affect the bacterial growth and the kinetics of denitrification.

These results suggest that the denitrifying bacteria *Bacillus licheniformis* in the media with limited concentration of phosphorus have developed the mechanism of enzymatic solubilization of insoluble phosphates. Apart from many other functions in ecosystems, these microorganisms, similarly to sulphur bacteria [15], can be a phosphorus source for the metabolism in the soil.

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PRZYSWAJANIE FOSFORANÓW I FOSFORYTÓW PRZEZ BAKTERIE DENITRYFIKUJĄCE *BACILLUS LICHENIFORMIS*

W warunkach laboratoryjnych odtworzono proces denitryfikacji zachodzący przy udziale bakterii *Bacillus licheniformis* i na podstawie wyznaczonych parametrów kinetycznych ustalono wpływ stężenia fosforu nieorganicznego, pochodzącego z rozpuszczalnych fosforanów oraz z nierozpuszczalnych w wodzie fosforytów (Courriba – Algier), na maksymalną szybkość procesu.

Stwierdzono, że denitryfikacja zachodzi w warunkach optymalnych, gdy stosunek P/N oraz P/C (g/g) wynosi odpowiednio 0.14 i 0.07. Wykazano również, że badany minerał (fosforyt), składający się głównie z nierozpuszczalnego $Ca_3(PO_4)_2$ o stabilnej siatce krystalicznej, w przemianie mikrobiologicznej przechodzi z formy nierozpuszczalnej w rozpuszczalną. Osiągnięcie parametrów kinetycznych (stałe szybkości, okresy indukcyjne), porównywalnych z odpowiednimi wartościami otrzymanymi w wyniku hodowli bakterii w pożywce zawierającej rozpuszczalny fosforan wymaga w przypadku minerału fosforytowego 100-krotnego jego nadmiaru i wiąże się z przemianami zachodzącymi na powierzchni mikroorganizmy–minerał.

УСВАИВАНИЕ ФОСФАТОВ И ФОСФОРИТОВ ДЕНИТРИФИЦИРУЮЩИМИ БАКТЕРИЯМИ *BACILLUS LICHENIFORMIS*

В лабораторных условиях воспроизведен процесс денитрификации, происходящий при участии бактерий *Bacillus licheniformis* и на основе определенных кинетических параметров установлено влияние концентрации неорганического фосфора, происходящего из растворимых фосфатов, а также из нерастворимых в воде фосфоритов (Курриба – Алжир), на максимальную скорость процесса.

Было установлено, что денитрификация происходит в оптимальных условиях, когда отношение P/N, а также P/C (г/г) составляет соответственно 0,14, и 0,07. Было также обнаружено, что исследуемый минерал (фосфорит), состоящий главным образом из нерастворимого $Ca_3(PO_4)_2$ стабильной кристалической сетки, в микробиологическом процессе превращается из нерастворимой формы в растворимую. Достижение кинетических параметров (постоянные скорости, индукционные периоды), сравнимых с соответствующими значениями, полученными в культуре бактерий в среде, содержащей растворимый фосфат, в случае фосфоритового минерала требует 100-кратного его избытка и связано с процессами, происходящими на поверхности микроорганизмы–минерал.