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Denitrification of wastewater with immobilized cells of *Pseudomonas denitrificans*

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Nitrate contamination is one of the major problems in wastewater. The aim of the present investigation is to study the denitrification process with immobilized on different synthetic supports cells of *Pseudomonas denitrificans* (NBIMCC 1625). The bacterium from the agar slants was inoculated into YPD liquid medium. The activated culture was transferred to 100ml of a medium with potassium aspartate or methanol as carbon source. Preliminary study had been carried out with free cells in shake flasks, containing nitrate as KNO_3 and carbon source methanol, C/N ratio was 6. Complete nitrate removal was achieved for 3 hours. In order to determine the kinetic of process the influence of initial nitrate concentration on nitrate removal were examined. By applying linear fit to rate of denitrification vs. initial nitrate concentration Km and Vmax were found to be 63mg NO_3-N/l and 0,671mg NO_3-N/min/g cells respectively. The further investigation of the denitrification process was performed with immobilized on different synthetic supports cells. Denitrification in the continuous-flow column reactor was carried out. After 3 hours the steady state was reached and output concentration of 30mgNO_3-N/l. The result of this study demonstrated that nitrate concentrations up to 45mg NO_3/l can be removed from wastewater. In the continuous column process with immobilized cells at HRT=1h high denitrification rate was achieved.

***Key words**: denitrification, Pseudomonas denitrificans, magnetic support.*

INTRODUCTION

The use of fertilizers and other nitrogen components in various industries, contribute to nitrogen pollution. Ion exchange, adsorption and membrane processes have been developed for nitrogen compounds, like nitrate and nitrite removal. Each of them has advantages and disadvantages, from which biological method is found to be the most commonly used and effective method. The process of biological denitrification has been well studied in last years. There are a large number of bacteria, which can transform nitrogen compounds into harmless nitrogen gas with accompanying carbon removal. The denitrification could be achieved either with pure cultures of *Pseudomonas denitrificans*, *Ps. Stutzery* and other strains or by mixed cultures from wastewater treatment plants [1, 2]. The application of cell immobilization techniques to wastewater treatment process has recently gained much attention, because biological denitrification with free suspended cells is usually slow process [3]. The treatment of wastewater in different types continuous-flow column reactors using immobilized cells is attracting increasing interest and a variety of carriers and immobilization methods have been developed [4]. The advantages of immobilized cells application are in their longer operation stability and in their multiple uses in continuous bioreactors. Several natural materials [5] and synthetic polymers [6, 7] have been applied as support for cell immobilization. Among the various immobilization methods that are available, the immobilization by spontaneous biomass adhesion onto porous support (biofilm formation) has been chosen for its ease of use, low cost and operational stability.

EXPERIMENTAL

Materials and methods

Pure culture of *Pseudomonas denitrificans* was used in this study. A strain of *P. denitrificans* (*NBIMCC* 1625), provided from the Bulgarian National Bank of Industrial Microorganisms and Cell Cultures, was used. In order to prepare the inoculum, the strain was cultured in a medium containing: peptone, 5g/l; meat extract, 3g/l; glucose, 10 g/l, and was incubated for 24 h at 30 °C in a rotary shaker at low agitation speed, 50 rpm. The culture medium for biomass growing and biofilm formation had the following composition (in g/l): potassium aspartate 15; yeast extract 14; KNO_3 8; $MnSO_4$ 0,0025;

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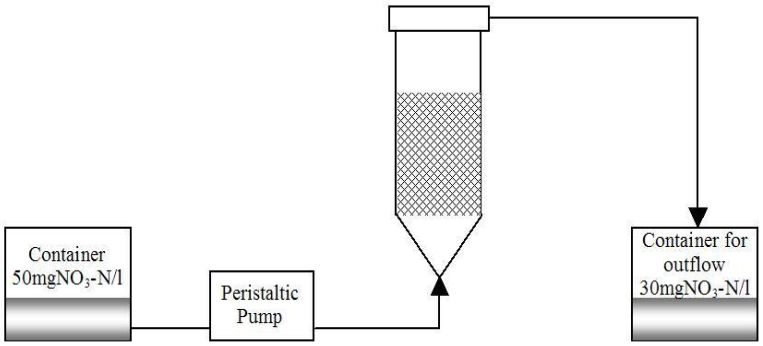

Fig. 1. Fixed bed column bioreactor for denitrification.

$(NH_4)_6Mo_7O_{24}.4H_20$ 0,0025; $FeCl_3.6H_2O$ 0,006; pH 6,75. After cultivation with weak shaking for 24h at 30^0C, cells were harvested by centrifugation (15min, 4000g), washed twice in saline solution and stored at 4^0C. The accessibility to biofilm formation on four different carriers was tested by cultivation for one week. The culture medium was replaced every second day. The first two support materials were a copolymer of acrylonitrile and acrylamide formed as granules with an average diameter of 2mm [8], without and with incorporated Fe_3O_4 nanoparticles. The second two carriers were polyurethane foam (PUF) cut into cubes with approximately 3mm x 3mm x 3mm cubic sizes, also without and included magnetite. Magnetite nanoparticles were incorporated in support matrix by coprecipitation of ferric and ferrous salts with alkaline solution [9], then washed and dried overnight at 50^0C. The study of denitrification activity of free and immobilized biomass was carried out with batch fermentation in shake flasks, containing nitrate as KNO_3 and carbon source methanol, C/N ratio was 6. The continuous-flow reactor for denitrification was set up as an upflow fixed bed, as shown in Fig. 1.The reactor was run in continuous mode at 25°C, and with a flow volume speed of 100 ml/h. It consisted of a container, column reactor with ID=50mm and 100ml total volume, peristaltic pump and container for outflow. The column was packed with 25g support and solution with $50mgNO_3$-N/l was pumped thru the column. Nitrate concentrations were determined through UV-spectrophotometry [7]. Before each spectrophotometric determination of nitrate, the samples were centrifuged for 15 min at 4000 rpm to remove cells from the supernatant. Then 0,1 ml 1N HCl was added to 5 ml of diluted sample. The light absorbance of samples was read against redistilled water at 220 nm on a UV–vis-spectrophotometer (Perkin-Elmer, Germany). In order to avoid the interference of the organic matter, the absorbance of samples was also measured at 275 nm. The corrected UV-light absorbance of nitrate in the sample A was calculated by the equation: $A=A_{220}-2.A_{275}$ and calibration curve was used to determine the concentration of nitrate. The concentration of nitrites in the centrifuged samples was determined spectrophotometrically from the amount of the diazonium salt of sulphanilic acid (formed from the nitrites present) by coupling with α-naphthylamine at pH = 2.0–2.5. The analyses of nitrites were carried out as follows. The assay involved two reagents. The Griess 1 reagent consisted of 0.6 g sulphanilic acid dissolved in 100 ml of distilled water. The Griess 2 reagent contained 0.6 g α-naphthylamine dissolved in distilled water. The solution was mixed with 25ml of glacial acetic acid, and diluted to 100ml with distilled water. Five milliliters of diluted sample was mixed with 1 ml of each of Griess 1 and Griess 2 reagents. The light absorbance of this solution was measured after 40 min using Spekol spectrophotometer at 543 nm against distilled water. When necessary, the samples were diluted prior to the addition of the Griess reagents. The nitrite concentrations were calculated using a calibration curve composed by the same method for concentrations from 0.05 to 1 mg/l. All chemicals were of analytical grade.

RESULTS & DISCUSSION

Batch cultivations with potassium aspartate and methanol as carbon source were performed. The potassium aspartate was replaced with equivalent quantity of methanol in culture medium. The experimental data showed that there are not significant differences between growth kinetics. Results of a typical s-shaped growth curves are presented in Fig.2. The experimental data showed that biomass concentrations after 24h cultivation was 9,6g/l wet biomass, when potassium aspartate

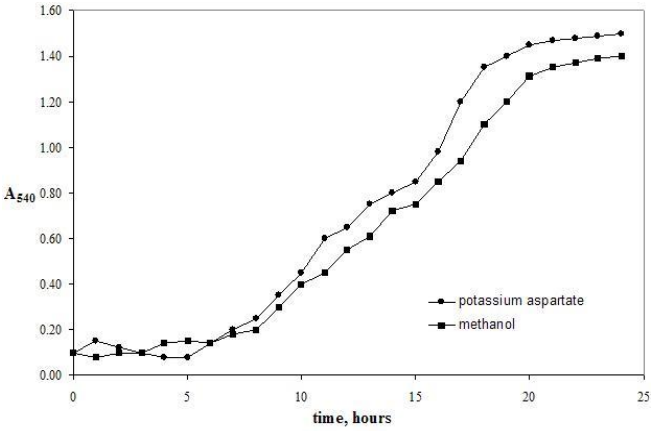

Fig. 2. Kinetics of biomass growth on different carbon sources.

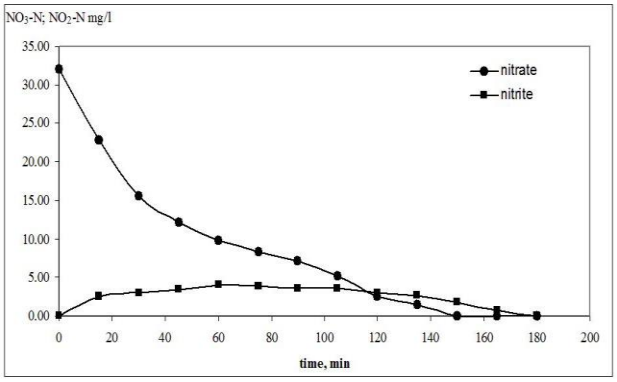

Fig. 4. Nitrate reduction by free cells in culture media containing 32,1mg NO_3-N/l and 2g/l dry biomass

was used vs. 8,8g/l wet biomass when methanol was used like carbon source. It was found that there is no difference between denitrification activities of biomass from two different carbon sources. In orderto determine the optimal conditions of the process nitrate reduction activity of free cells as a function of pH in denitrification media was tested. The denitrification profile obtained for the strain of *Pseudomonas denitrificans* is shown in Fig. 3. The Figure shows that the pH optimum of denitrification activity is between 7.25 and 7.5. At pH higher 9.0 nitrate reduction also was observed. The time profile of denitrification process with free cells is shown in Fig. 4. Complete nitrate removal was achieved after 150 minutes. Slight accumulation of nitrite was observed, during the experiment, but final nitrite concentration was low. In order to determine the kinetic of process the influence of initial nitrate concentration on nitrate removal was examined. The effect of different initial nitrate concentrations is shown in Fig. 5. The data from batch experiments were used to calculate the rate of nitrate reduction as a function of initials concentrations.

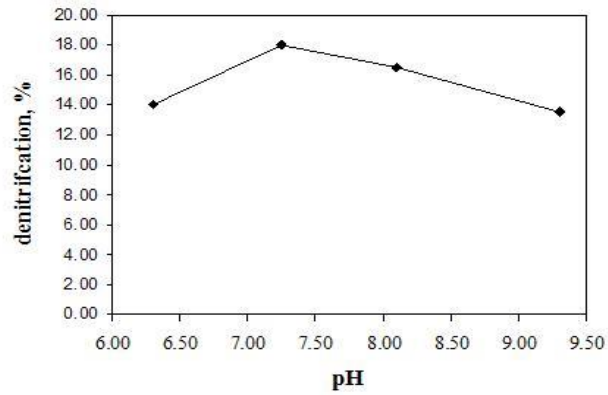

Fig. 3. pH optimum of the nitrate reduction by free cells.

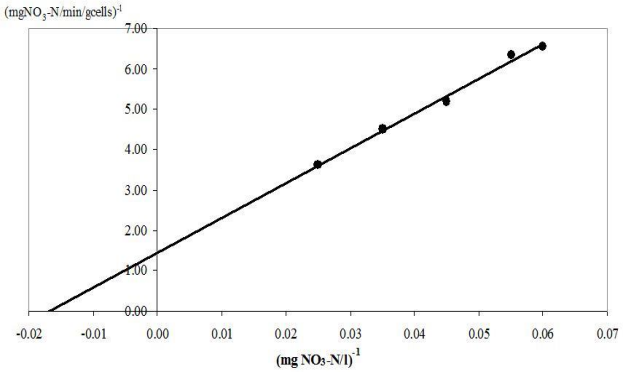

Fig. 5. Lineweaver-Burk plots (1/v versus 1/[S]) derived from initial rate of nitrate reduction at different nitrate concentration.

The rate of nitrate reduction was observed to depend on its concentration as predicted by the Michaelis–Menten equation. By applying linear fit to rate of denitrification vs. initial nitrate concentration Michaelis-Menten constants Km and Vmax were found to be 63mg NO_3-N/l and 0,671mg NO_3-N/min/g cells respectively. A comparison of the experimental results for the processes using immobilized on different supports cells in batch culture is shown in Fig. 6.

It is clearly seen from fig. 6 that nitrate removal increases when the polyurethane foam beads are used. The best results were obtained with magnetite containing polyurethane foam support. There are many investigations on bacterial biofilm application, but initiation of biofilm formation is poorly understood, and in particular, the contribution of chemical bond formation between bacterial cells and metal oxides (titanium dioxide and iron (II, III) oxide) has received much attention [10]. The Fe_3O_4 probably has a complex effect on the bacterial community, but did not show a straightforward toxic effect. It was found that nanoparticles of Fe_3O_4 changed the hydrolytic activity and bacterial community composition.

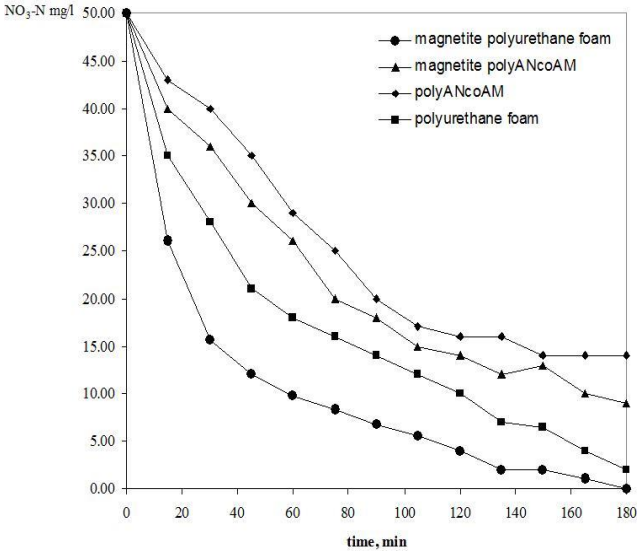

Fig. 6. Nitrate reduction by immobilized cells in culture media containing 50,0mg NO_3-N/l and 100g/l beads.

Generally, magnetite tends to cover cell surfaces, but no damage to the cell's integrity was reported in different studies dealing with this problem [11]. Overall, magnetite nanoparticles did not affect bacteria in culture media and model waste waters enough to indicate significant risk.

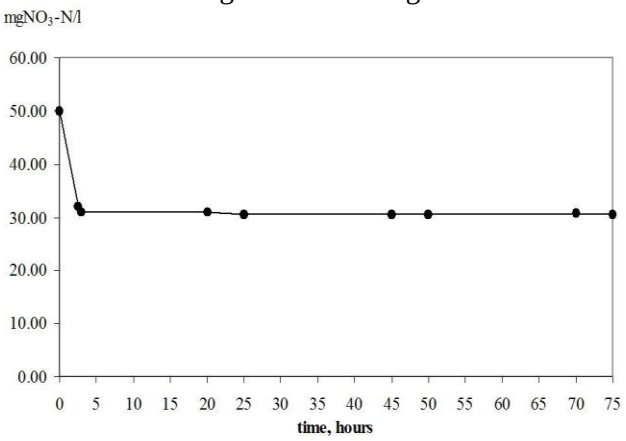

Fig. 7. Denitrification in continuous-flow column reactor.

The results from denitrification in continuous-flow column reactor are shown in Fig. 7. The results of column experiments show that after 3 hours the steady state was reached and output concentration of 30mgNO_3-N/l.

The result of this study demonstrated that nitrate concentrations up to 45mg NO_3/l can be removed from wastewater. In the continuous column process with immobilized cells of *Pseudomonas denitrificans* at HRT = 1 h high denitrification rate was achieved. Through the description of the Fig. 7, it can be concluded that flow rate of 0.1l/h should be chosen as the optimum hydraulic loading, and two columns should be used to reach desired nitrate reduction.

CONCLUSIONS

The microbial cells of gram-negative bacteria *Ps. denitrificans* were immobilized by adhesion onto four different kinds of polymer supports. Denitrification processes with free and immobilized cells have been investigated. Our findings, based on the experimental results, suggested that the use of immobilized cells in column bioreactor for water denitrification ensured a stable process over a long period of time without biomass wash out. An increase in the efficiency of the denitrification was observed in the presence of a magnetite (Fe_3O_4 nanoparticles) embedded in a matrix. The effect of the iron oxide nanoparticles on biofilm formation and denitrification should be studied better. The technique of using magnetic biomass carriers was shown better results. An accumulated biomass of microorganisms was achieved inside the reactor during a continuous process when applying this technique. Magnetite containing PUF was the most appropriate carrier of the particles evaluated and could be applied in Magnetic carrier technology. Our results confirm that stabilized magnetite nanoparticles interact with bacterial surfaces without causing damage sufficient to inhibit cell growth. Magnetite is also relatively cheap and available, why magnetic separation and reintroduction can be achieved to a relatively low cost in large-scale production.

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ДЕНИТРИФИКАЦИЯ НА ОТПАДНИ ВОДИ С ИМОБИЛИЗИРАНА БИОМАСА ОТ *Pseudomonas denitrificans*

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(Резюме)

Един от основните замърсители в отпадните води са нитратите. Целта на настоящата работа е да се изследва и оптимизира процес на денитрификация с имобилизирана върху различни носители биомаса *от Pseudomonas denitrificans* (NBIMCC 1625). От твърдата хранителна среда микроорганизмите бяха развити на течна среда съдържаща калиев аспартат или метанол като въглероден източник. При използване на метанол в процеса на денитрификация и съотношение на C/N = 6 със свободна биомаса беше достигнато пълно отстраняване на нитратите за 3h. Определени са и основните кинетични параметри Km = 63 mg NO_3-N/l и Vmax=0,671 mg NO_3-N/min/g клетки. Процеса на денитрификация беше изследван и с имобилизирана на различни синтетични носители биомаса. При използване на колонен реактор запълнен с имобилизирана биомаса в непрекъснат процес е достигната концентрация от 30 mg NO_3-N/l на изход от колоната и е установено че времепрестой от 1 час е достатъчен за достигане на висока степен на денитрификация.