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Microbial denitrification by immobilized bacteria *Pseudomonas denitrificans* stimulated by constant electric field

T. Parvanova-Mancheva, V. Beschkov*

Institute of Chemical Engineering, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

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ABSTRACT

A comparative study of microbial reduction of nitrate ions by immobilized *Pseudomonas denitrificans* cells in the presence and in the absence of a constant electric field was carried out. The experiments were carried out in a continuous stirred tank reactor at a broad range of dilution rates. The cathode potential was maintained constant around the standard potential value of the nitrate/nitrite redox couple. The residual outlet concentrations of nitrate, when the electric field was applied, were much lower than those obtained without the electric field, all other conditions being equal. It was demonstrated that the electric field had a significant effect on the nitrate reduction rate allowing the use of much higher inlet flow rates compared to the flow rate in the absence of an electric field.

1. Introduction

Nitrogen-containing ions such as nitrate and nitrite occur widely in a variety of process streams like those coming from the extensive use of fertilizers. They can have serious impact when released to the environment due to the possible health effects on many organisms, including humans. The key problem is the reduction of nitrate to nitrite, the latter being considerably more toxic than the nitrate itself [1]. There are a large number of mesophilic bacteria capable of utilizing nitrate instead of free oxygen as an electron acceptor under anoxic conditions, converting the nitrate to nitrogen [2–6]. The following consecutive reactions exhibit the nitrate-to-nitrite conversion, and the nitrite reduction to gaseous nitrogen:

$$(1)NO_3^- \rightarrow NO_2^- \rightarrow NO(g) \rightarrow N_2O(g) \rightarrow N_2(g).$$

Efforts have been made to use immobilized microbial cells for denitrification in wastewater treatment [7–13]. The advantages of immobilized cell usage are in their longer operational stability and in their multiple uses particularly in continuous bioreactors. The application of immobilized cells is favorable for the process of nitrification at temperatures far below the optimal temperature of 30–35 °C [7]. Garbayo et al. [8] stated that the cells of *Chlamydomonas reinhardtii* entrapped in calcium alginate gel showed high rates of nitrate uptake and higher viability compared to the freely suspended cells, explained by the protective effect of the polymer on the biological activity. Nitrite inhibition of the nitrate consumption by *C. reinhardtii* cells, entrapped in calcium alginate, was observed and explained by local accumulation of ammonia within the alginate beads [9]. Another drawback of the cell entrapment was the diffusion limitations within the gel particles [10,11]. The attachment of the cells onto a carrier surface should be preferable when the reaction product is an inhibitor [13].

Another wide area of research on microbial denitrification is dedicated to the bio-electrochemical reduction of nitrate [14–26]. There are several approaches in this sphere. The presumption of one of them is to facilitate the microbial activity by cathode production of hydrogen which is itself a strong nitrate reducer [15–26]. These studies referred to the galvanostatic performance at relatively high electric currents, i.e. up to 200 mA. In addition, a membrane bio-electrochemical reactor was applied for separation of biomass and for preventing release of microorganisms [22].

It was previously shown [14] that at potentiostatic mode and at very low electric currents (in microamperes) bacterial denitrification took place with satisfactory rates and efficiency. The bio-electrochemical process depended on the cathode potential, and its optimum value was close to the standard potential of the nitrate-to-nitrite reduction, i.e. 0.01 V (in reference to the saturated hydrogen electrode, S.H.E.).

A drawback of this approach is the delicate balance between free cell proliferation and nitrate reduction in a continuous flow. The disturbed balance (i.e. if the specific microbial growth rate is not high enough to maintain a constant cell density at the corre-

* Corresponding author. Tel.: +359 2 8702088; fax: +359 2 8707523.
E-mail address: bioreac@bas.bg (V. Beschkov).

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sponding dilution) may lead to washing out of the microbial cells at higher flow rates and irreversibly disrupt the bioreactor operation at shorter hydraulic retention times.

That is why it seems useful to test the capacity for electrically stimulated nitrate biodegradation by microbial cells immobilized on a solid support. Feleke and Sakakibara [20], Park et al. [21] and Prosnansky et al. [22] have studied the process of denitrification by cells, immobilized on a cathode. However, the microbial denitrification capacity was limited because of the low specific area of the electrode. Therefore, a larger interface area between the nitrate ions in the solution and the cells is desirable. Increased specific area of the cell carrier was achieved in a bio-electrochemical packed bed reactor with immobilized cells [23] at relatively high specific currents, i.e. 5 and 10 A/m^2.

In this paper we demonstrate the denitrification capacity of *Pseudomonas denitrificans* cells attached to a solid support, which operate in a continuous stirred tank bioreactor at constant electric field in potentiostatic mode, and at extremely weak electric currents.

2. Materials and methods

2.1. Strain culturing

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, 10 g/l; yeast extract, 1 g/l; NaCl, 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 comprised two solutions, sterilized separately:

(A) A phosphate buffer at pH = 7, containing: $MgSO_4 \cdot 7H_2O$, 0.2 g/l; $CaCl_2 \cdot 2H_2O$, 0.2 g/l; NaCl, 5 g/l; and
(B) A potassium nitrate solution – 10 g/l. It was added to the phosphate buffer after sterilization in a calculated amount to reach the desired initial nitrate concentrations in the culture media, ranging from 50 to 300 mg/l. No methanol or any other organic compounds as a carbon source were added to these solutions.

2.2. Cell immobilization

Immobilization was carried out using the method described in [27]. The support material was a copolymer of acrylonitrile and acrylamide formed as porous granules with an average diameter of 2 mm. The polymer granules were activated for 4 h, using a 12.5% solution of formaldehyde in 0.1 M phosphate buffer at pH 7.5. The microbial cells were harvested from the inoculum culture by centrifuging at $5000 \times g$ for 20 min, washed in phosphate buffer at pH 7.5, and re-suspended in the same buffer to achieve a biomass content of 10 mg/ml. Then the microbial suspension was mixed with the activated polymer beads for 20 min under careful stirring. Thereafter the granules were thoroughly washed with distilled water until no free cells were detected in the flush. The overall volume of the beads was 55 cm^3 with a specific area of about 30 cm^{-1}.

Images of the polymer support with and without the immobilized cells were made using scanning electron microscopy (SEM) in a secondary electron imaging (SEI) mode. A JSM 6390 electron microscope (JEOL, Japan) was used for this purpose. The microphotographs are shown in Fig. 1. The porous surface of the cell free polymer is obvious (cf. Fig. 1a), whereas the surface covered with the immobilized cells seems to be smooth at the same magnification (Fig. 1b). This observation corresponds to the statement that a significant number of pseudomonads can produce exopolysaccharides in biofilms that are known as slime layers [28], thus filling the surface pores. The picture taken at higher magnification shows that the bacteria are predominantly attached to the pore edges and are not observed inside the pore (Fig. 1c).

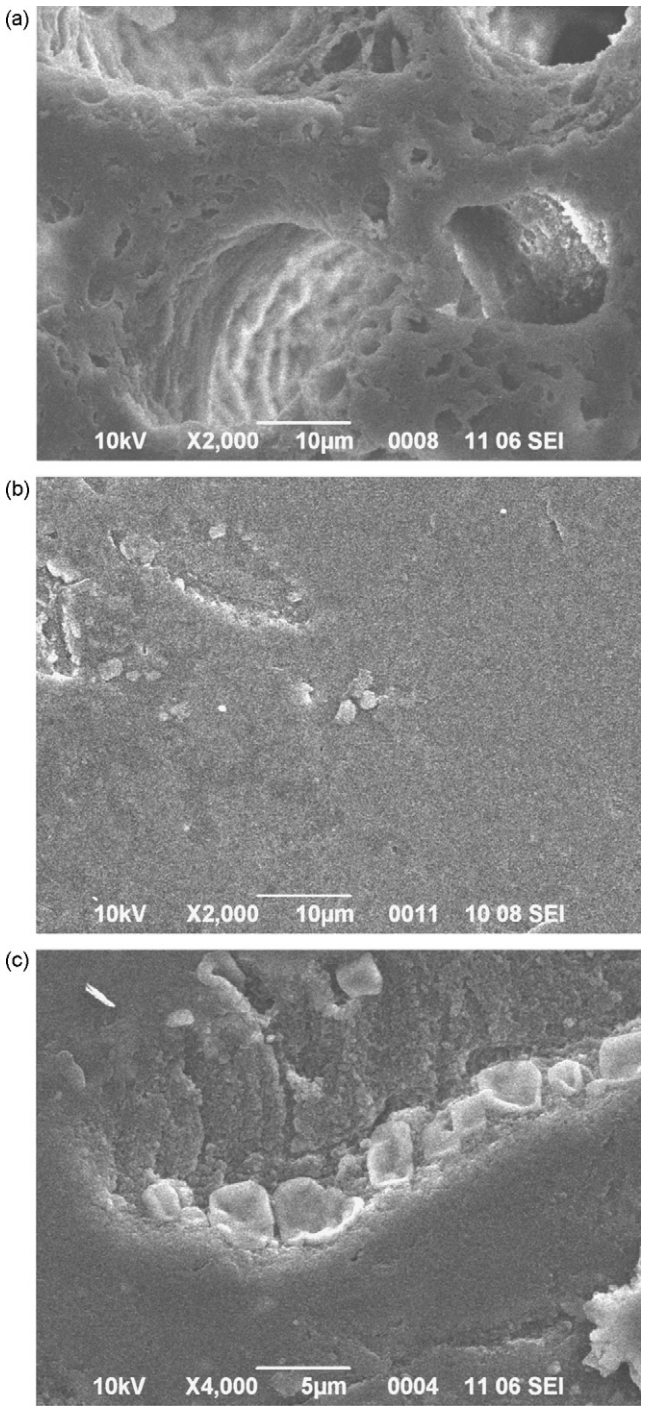

Fig. 1. SEM images of the polymer support surface without and with immobilized cells. (a) The polymer surface without bacteria (×2000 magnification); (b) the polymer surface with bacteria (×2000 magnification); (c) the polymer surface with bacteria (×4000 magnification).

2.3. Analyses

During the fermentation, the pH value was measured periodically (off-line) by a Radelkis pH-meter, model OP-211/1 (Budapest, Hungary) with a glass probe.