

**EFFECT OF THE SUPPORT ON THE IMMOBILIZATION OF *PSEUDOMONAS DENITRIFICANS* CELLS**

Tsvetomila I. Parvanova-Mancheva, Venko Beschkov, Evgenia Vasileva

Institute of Chemical Engineering, Bulgarian Academy of Sciences, str. "Acad. G. Bonchev" bl. 103,  
1113 Sofia, Bulgaria**Abstract**

*Biological means for biodegradation of pollutants in wastewater have relatively low cost compared to conventional physical and chemical processes. The use of immobilized cells is considered as more effective than for free cells. Reason for this is easier operation of solid-liquid separation, better operation stability, higher biodegradation rates, protection from toxic substances, increased plasmid stability. Many cells have the ability to adhere to other organisms (aggregate) or to solid surfaces. In this article different carriers for immobilization of cell of the strain Pseudomonas denitrificans are compared. They are granulated activated carbon, pyrolytic graphite fabric, graphite and polymer particles. The granulated activated carbon was the most suitable one.*

**Key words:** *immobilized cells, Pseudomonas denitrificans, wastewater, denitrification*

**1. INTRODUCTION**

Immobilized cells are being used or investigated for many different applications: wastewater treatment, biomedical applications, biosensors, etc. Immobilized cell systems have the potential to degrade toxic chemicals faster than conventional wastewater treatment systems, because of the high cell densities of specialized microorganisms that are used. The immobilization of cells can be a natural process or can be induced by chemicals. Some microorganisms have a natural tendency to adhere to surfaces and to become immobilized. The immobilization methods are many and variety. Figure 1 shows schematically several ways of immobilization techniques. Cell immobilization by biomass entrapment within various carriers is one of the progressive approaches for the creation of immobilized biocatalysts. It has been suggested that the earliest purposeful use of immobilized cells in the beginning of the 19th century took advantage of this type of microbial immobilization with a biological film in a trickle-filter system for producing acetic acid (Brodelius, P., et al.), (Chibata, I., 2013), (Chibata, I., 2013).

Some useful papers on microbial immobilization have appeared as early as the 70s (Buchholz, K.(ed.), 1979), (Venkatasubramanian, K. (ed.), 1979). The immobilization of microorganisms can be defined as technique for limiting the free migration of cells.

Cell mobility can be restricted by aggregating the cells or by retaining them into, or attaching them to, a solid support. Various terms have been used to describe different types of immobilization, but these terms do not seem to be accepted universally. The types of immobilization are two: (1) attachment, where the microorganisms adhere to surface or other organisms by self-adhesion or chemical bonding; and (2) entrapment, where the organisms are captured in the pores of fibrous or porous materials or are physically retained within or by a solid or porous matrix such as a stabilized gel or a membrane (Scott, Ch. D. et al. 1987).

The ideal carriers for immobilizing microbial cells should fulfil as many as possible the next criteria: maximum surface area to volume ratio, high biomass loading capacity, simple non-toxic' immobilization procedure, adequate access to nutrient media, optimum diffusion distance from flowing media to centre of the support, mechanical stability, easy separation of media from cells and carriers. Activated carbon and polymer particles possess most of these characteristics.

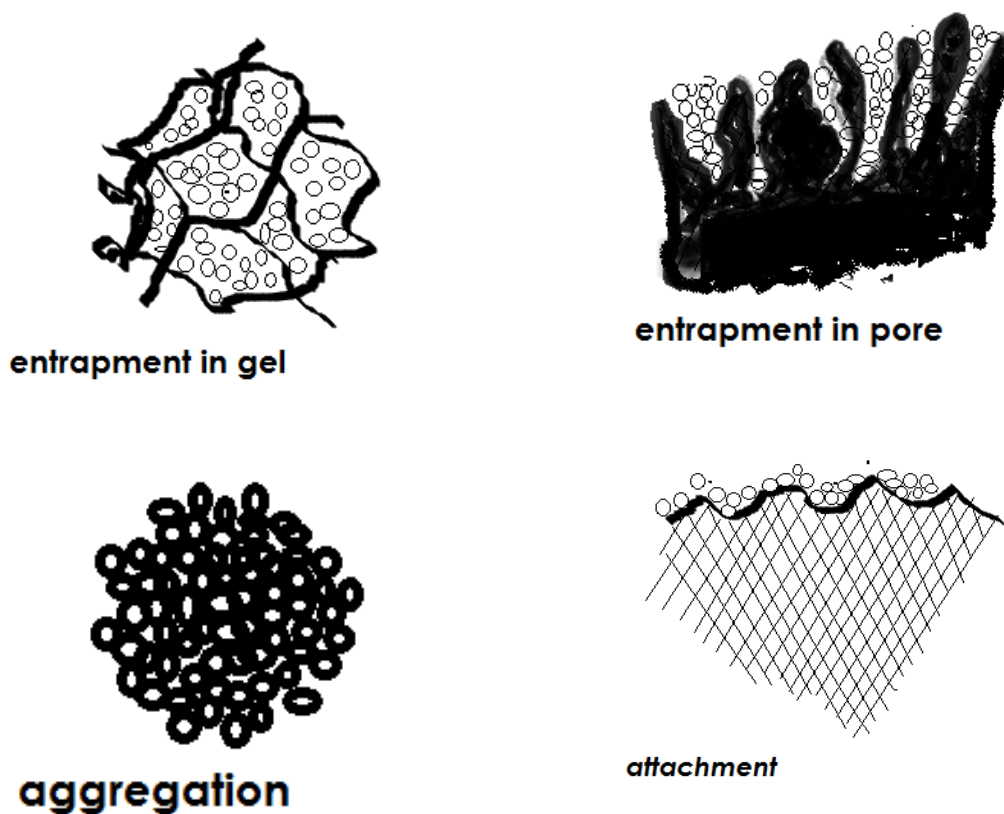


Fig. 1. Methods of immobilization

## 2. MATERIALS AND METHODS

### *Strain cultivation*

The strain of *Pseudomonas denitrificans* (NBIMCC 1625), delivered from the Bulgarian National Bank of Industrial Microorganisms and Cell Cultures is used. The microbial cells are inoculated in a medium containing: NaCl, 10 g/l; peptone, 10 g/l; yeast extract, 1 g/l. The culture is incubated at 30°C for 24 h on a rotary shaker at low speed 50 RPM.

### *Media*

The working medium for the microorganism is comprised of two solutions: (i) A phosphate buffer at pH=7, containing also: MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 g/l; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2 g/l; NaCl, 5 g/l; and (ii) potassium nitrate solution – 10 g/l.

The investigations of nitrate degradation and immobilization are accomplished in flasks of 250 ml in a rotary shaker at 100rpm and at 30°C. The activated carbon, type Fujikasui (Japan), has a specific surface area of 680 m<sup>2</sup>/g.

### *Immobilization*

The carriers used for immobilization of cells *Pseudomonas denitrificans* is granular activated carbon, pyrolytic graphite fabric, graphite particles and polymer particles.

### *Polymer carrier*

Immobilization was carried out using the method described in (Lalov, I.G. et al. 2001). 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 0.1M phosphate buffer at pH=7.5. The microbial cells were harvested from the inoculum culture by centrifuging at 5000  $x$   $g$  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<sup>3</sup> with a specific area about 30 cm<sup>-1</sup>.

### *AGC, pyrolytic graphite fabric and graphite particles carriers*

The immobilization of microbial cells by active granular carbon is used. The preliminary cultivated bacterial suspension was mixed with the washed and dried to constant weight activated carbon (AGC). Then the mixture was transferred to shaking flasks at 30°C for at least 48 hours. This same procedure was used for the immobilization on the other two bearers.

Microbe cells were bonded to the surface of the activated carbon particles *via* chemical adsorption. The *Pseudomonas* strain are able to produce exo-polysaccharides in the biofilm layer which function was to bind activated carbon particles and microbial cells (Beschkov, V., 2008). The volume of the microbial culture towards activated carbon volume was 2 %.

### *Analyses*

**Nitrate concentrations** were determined through UV-spectrophotometry (Goldman, E. and R. Jacobs, 1961).

Before each spectrophotometric determination of nitrate, the samples were centrifuged for 3 min at 14800 rpm to remove the cells and other insoluble components from the supernatant. Then 1ml 1N HCl was added to 1 ml of supernatant and the mixture was diluted to a constant volume of 50 ml. The light absorbance of samples was measured against the blank sample (redistilled water) at 220 nm on a UV/Vis-spectrophotometer.

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  $A_{corr}$  in the sample was calculated by the following equation:

$$A_{corr} = A_{220} - 2 \times A_{275}$$

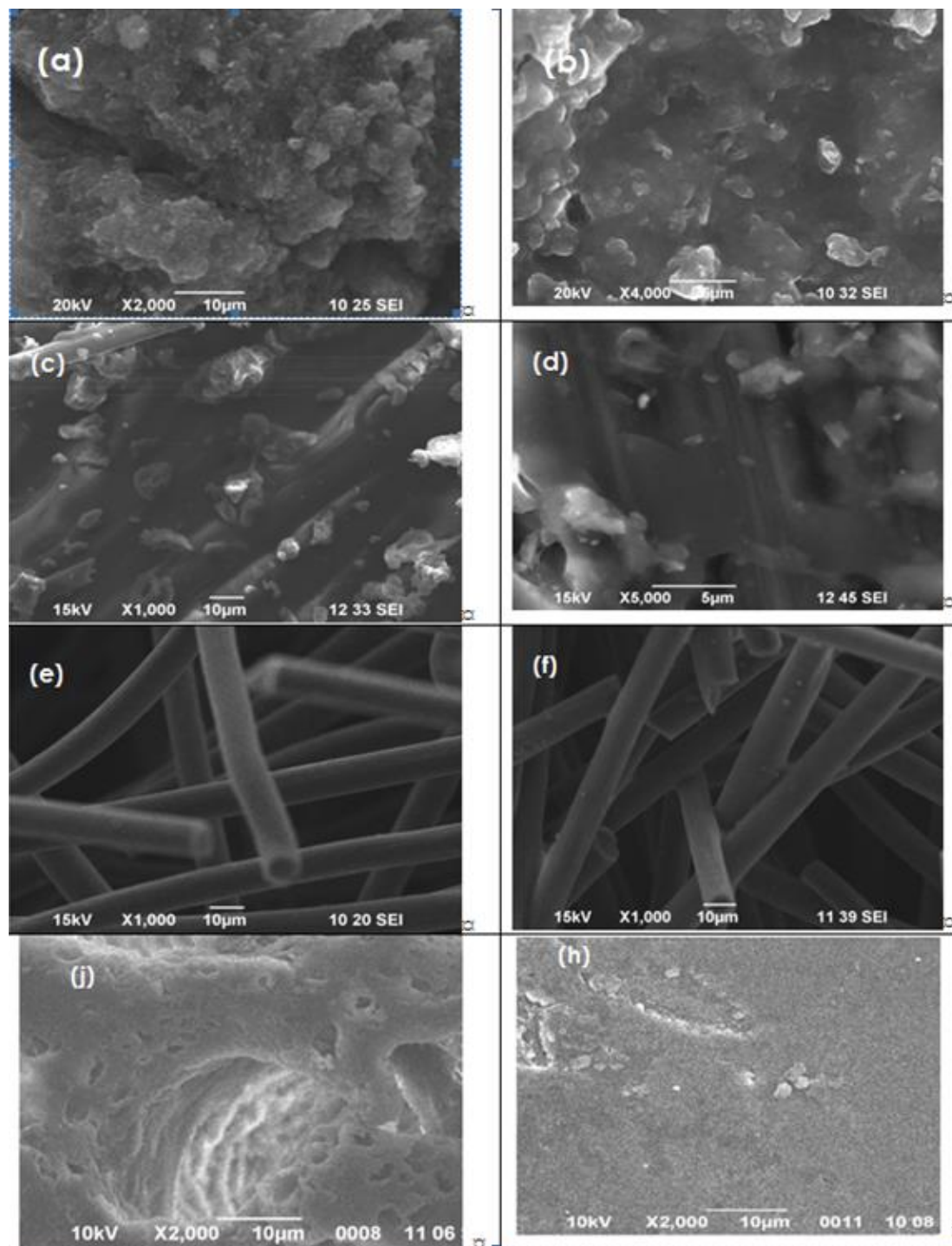
During denitrification process, **pH** value was measured periodically by a pH-meter, model CH-9100 Herisau.

The detached cells were monitored by **biomass concentration in the bulk**. It was controlled by optical density readings of the broth at  $\lambda=660$  nm using a Spekol spectrophotometer.

The **concentration of nitrite** in the centrifuged samples was determined spectrophotometrically by the amount of the diazonium salt of sulphanilic acid (formed from the nitrites present) by coupling with  $\alpha$ -naphthylamine at pH=2.0–2.5. The analyses of nitrites were carried out as follows. The assay is involved two reagents. The Griess 1 reagent consisted of 0.5 g sulphanilic acid dissolved in 150 ml of 12% wt. acetic acid upon heating. The Griess 2 reagent consisted of 0.25 g  $\alpha$ -naphthylamine dissolved in 20 ml distilled water. This solution was boiled for 5 min and then filtered through a hot filter. The filtrate was mixed with 150 ml of 12% wt. acetic acid. One milliliter of the centrifuged sample was mixed with 1 ml of each of Griess 1 and Griess 2 reagents. The mixture was diluted to 50 ml in a measuring flask. The light absorbance of this solution was measured after 40 minutes 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.

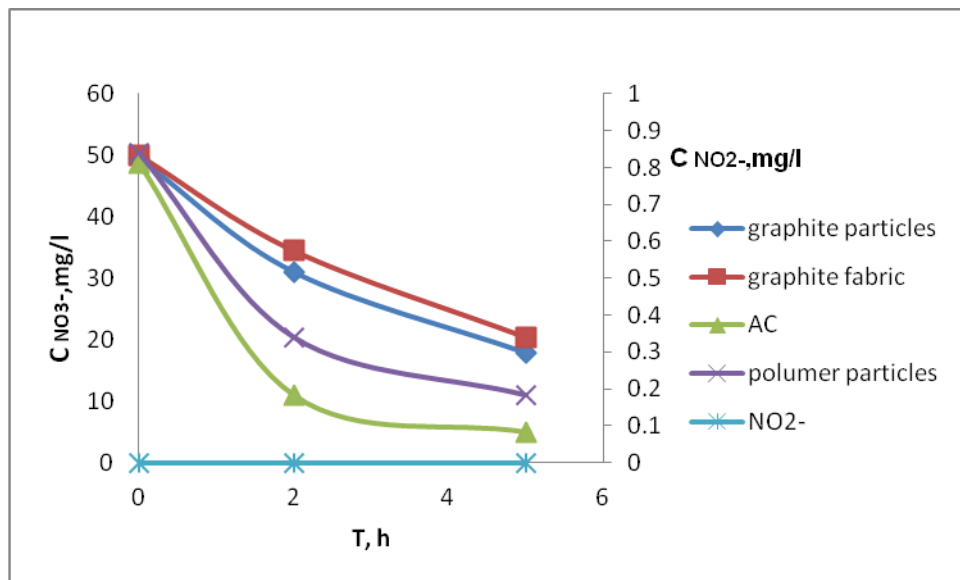
### 3. RESULTS

The images of the activated carbon, pyrolytic graphite fabric, graphite particles and polymer particles supports with and without the immobilized cells are made by scanning electron microscopy (Fig.2).

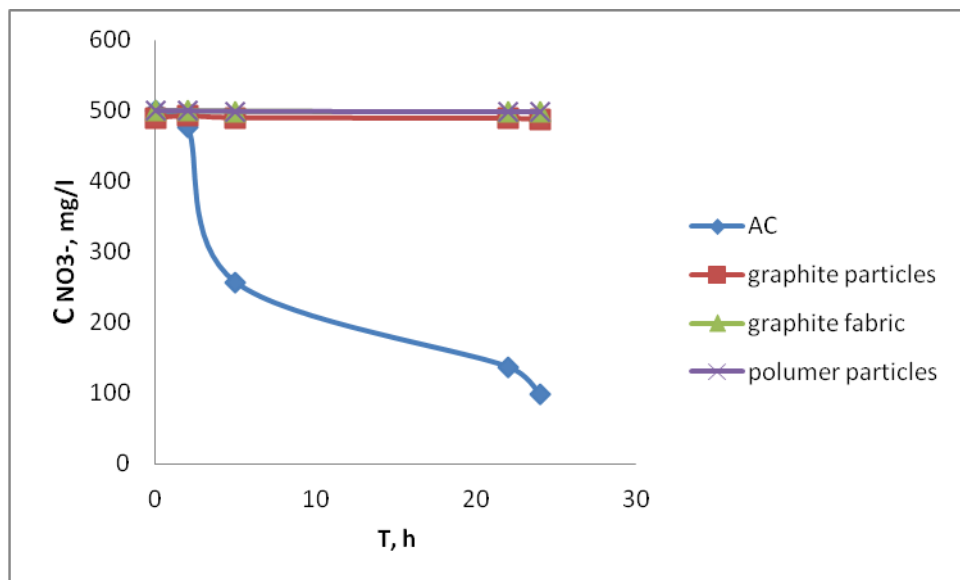


**Fig. 2.** Scanning electron microscopy images of the support surface without and with immobilized cells. (a) The granulated activated carbon without bacteria; (b) The granulated activated carbon with bacteria; (c) the graphite particles without bacteria; (d) the graphite particles with bacteria; (e) the pyrolytic graphite fabric without bacteria; (f) the pyrolytic graphite fabric with bacteria; (j) the polymer particles without bacteria (Parvanova-Mancheva et al., 2009); (h) the polymer particles with bacteria (Parvanova-Mancheva et al., 2009)

Figure 3 illustrates the speed of the nitrate reduction with a concentration of 30 mg/l by immobilized cells on different supports of *Pseudomonas denitrificans* at a temperature of 30°C. On the base of these results, it can be concluded that the reduction proceeds rapidly with cells immobilized on granulated activated carbon. Experiments with higher concentration of nitrate ions up to 500 mg/l showed that only the immobilized cells on the granulated activated carbon were denitrified. At the rest carriers, the process is inhibited by the substrate from the very beginning (Fig. 4). The nitrites were determined qualitatively.



**Fig. 3.** Nitrate reduction (30mg/l) by immobilized cells *Pseudomonas denitrificans* on different carriers



**Fig. 4.** Nitrate reduction (500mg/l) by immobilized cells *Pseudomonas denitrificans* on different carriers

#### 4. CONCLUSION

The granulated activated carbon is the most suitable one for immobilization and denitrification by the strain *Pseudomonas denitrificans* of the studied carriers.

The activated carbon protects the immobilized microorganisms by adsorbing nitrate ions and low quantities of the adsorbed ions released for biodegradation gradually. Therefore, this combination of physical adsorption and biological degradation of nitrate ions is applicable for the treatment of wastewaters, containing relatively high nitrate concentrations, which are toxic for microbial cells.

The immobilized cells could utilize most of the adsorbed nitrate ions. The microorganisms still grow although no nitrates are presented in the medium. The adsorbed nitrate ions diffused out of the activated carbon and were available to the microorganisms.

#### ACKNOWLEDGEMENTS

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