

The conversion of nitrate in water to diatomic nitrogen gas by immobilized *Pseudomonas stutzeri* on vermiculite

Badrie Fazeli

M.Sc. of Microbiology, University of Isfahan, Iran, badri.fazeli@gmail.com

Giti Emtiazi *

Professor of Microbiology, University of Isfahan, Iran, emtiaz@yahoo.com

Abstract

Denitrification is a reduction of nitrate by heterotrophic and autotrophic bacteria that may ultimately produce molecular nitrogen (N₂) through a series of intermediate nitrogen compounds. Vermiculite is a hydrous phyllosilicate mineral (Mg, Fe⁺², Fe⁺³)₃[(Al, Si)₄O₁₀](OH)₂·4H₂O with several layers for bacterial immobilization. The goal of this study was removal of nitrate from water with vermiculite and bacterial biofilm. In this process, 2×10⁷ of bacteria were trapped in one gram vermiculite with resting cells and growing cells and nitrate removal were compared by free cells. The data showed that the un-immobilized cell in resting state could only remove 45 mg nitrate in 5 days, however nitrate removal by resting cell on vermiculite was 180 mg in 10 days, while in free resting cells this removal was poor. The maximum removal of 450 mg nitrate occurred after 25 days with feeding of biofilm. The intermediate product of nitrate removal was nitrite and in all data, the end product was nitrogen.

Key words: Denitrification, Cell immobilization, *Pseudomonas stutzeri*, Vermiculite, Resting cell

Highlights

- Immobilization of *P. stutzeri* in resting cell status, improved nitrate removal efficiency versus un-immobilized bacteria.
- Feeding the immobilized bacteria, improved nitrate removal efficiency.
- Vermiculite introduced as a beneficial support media for immobilization of bacteria.

*Corresponding author, Iran National Science Foundation (INSF)

Introduction

Nitrate pollution becomes a serious problem in most of the countries around the world. Several technologies have been developed for removing nitrate from water, for example, Reverse Osmosis (RO), Ion Exchange (IE) and Electro Dialysis (ED). All of the called methods are so expensive and only separate nitrate from water, so that they may produce wastewater with high concentration of nitrate. Biological denitrification is a safe method for conversion of nitrate to N_2 .

The denitrification process is performed primarily by different bacteria such as, *Thiobacillus denitrificans*, *Paracoccus denitrificans* and various *Pseudomonads*. Generally, several species of bacteria are able to completely reduce nitrate to molecular nitrogen. Denitrification is a stable character for *Pseudomonas stutzeri*; it is one of the most active denitrifying, heterotrophic bacteria (1, 2). Previous studies indicate that *P. stutzeri* can carry out this process in the presence of high oxygen levels and can conduct denitrification simultaneously (3). Denitrification is widely used for quite ground water pollution with nitrate causing from extended usage of fertilizers in agriculture or used to eliminate nitrogen from waste-water (4, 5). For denitrification process, suspended or attached growth systems may be used (6).

The immobilization of viable cells has been explained by physical restriction of viable microbial cells to a particular defined area of space to limit their free movement. Cell immobilization can increase the yield of cell per bioreactor volume and improvement of process quality traditionally continues fermentations. Cell immobilization can

provide a direct contact between nutrients and the immobilized cells and increase resistance to environmental pressures (7, 8).

The easiest technique for cell immobilization is to transport cells from the bulk phase to the surface of support, followed by the sticking of cells by electrostatic and hydrophobic interactions, and consequent colonization of the support surface (9, 10). Vermiculite was used as support media for cell immobilization in this study.

Vermiculite is the mineralogical name generally applied to a layered silicate mineral which is composed of two silica tetrahedral sheets coupled symmetrically to another magnesium octahedral sheet in a tetrahedral octahedral tetrahedral layer lattice. Silicate layers were separated by a layer of water in this mineral. It is relatively inexpensive and is widely obtainable. The multilayer structure of vermiculite prepares effective aeration and space for microbial proliferation (11).

Material and Method

Vermiculite structure detection: To detect vermiculite structure, first thin slides of vermiculite were prepared, then they were observed with optic microscope. In the second step, fixed vermiculite was in resin and rubbed the surface and took image by Atomic Force Microscopy (AFM) to show nanolayers.

Bacterial cells and growth media: *Pseudomonas stutzeri* strain SEE-1 (Accession number in NCBI: HQ438282) was obtained from the microbial culture collection of microbiological laboratory of the University of Isfahan. The bacteria were grown in nutrient broth supplemented with 0.1% KNO_3 at 37°C for 24h with shaking (160 rpm) to prepare inoculum.

Nitrate reductase assay: 0.1 mL of supernatant was transferred to volumetric flask and distilled water was added to have 10 mL volume. Consequently, 0.2 mL of each nitrite determination reagents were added, mixtures were kept at room temperature for 15 min. The pink color was measured spectrophotometrically at 540 nm against the blank.

Immobilization of cells on vermiculite and nitrate removal by biofilm: Vermiculite particles (25/50 mesh; U.S. Standard Sieves) were washed with distilled water 3 times then autoclaved in erlenmeyer flasks. Sterilized vermiculites were dried in oven (60 °C) over night and dispensed (0.5% gram per liter of media) into flasks containing 400 mL nutrient broth supplemented with 0.1% KNO₃. The flask inoculated with 20 mL of bacteria with desired concentration (1.5×10^8 CFU/mL) and incubated at 37 °C for 48h with shaking (160 rpm). Then vermiculite was separated from media broth and washed 3 times with physiological saline (the media separated from vermiculite used for direct cell counting). Then 30 mL nitrate 0.1% solution was added to vermiculite and stored in room temperature for 24 h and then adding nitrates were repeated until inactivation of biofilm.

Nitrate removal by biofilm vermiculite by feeding: To feed inactive biofilm vermiculite, 400mL nutrient broth were added and supplemented with 0.1% KNO₃ to vermiculites and incubated at 37°C for 48h with shaking (160 rpm). Then, vermiculites were separated from media and washed 3 times with physiological saline. Then 30mL nitrate 0.1% solution was added to vermiculite and stored in room temperature for 24 h. We repeated

adding nitrate to vermiculite until inactivating and again fed to reactive vermiculites.

Nitrate removal by un-immobilized resting cell: Inoculated flask containing 400mL nutrient broth was supplemented with 0.1% KNO₃ with desired concentration (1.5×10^8 CFU/mL) of bacteria and incubated at 37 °C for 48h with shaking (160 rpm). Cells were harvested by centrifugation (4000g for 20 min) in sterile 50 mL centrifuge tubes. Cellular mass was desired to flask and added 30mL nitrate 0.1% solution to cellular mass and stored in room temperature for 24 h. and then adding nitrates was repeated until inactivation of cellular mass.

Detection of cell attached to vermiculite: A 1gram portion of the biofilm vermiculite was removed from the flask for bacterial population and purity determinations by suspending the vermiculite in 10 mL of sterile distilled water. The suspended samples were serially diluted and plated on nutrient agar plates.

Results

Vermiculite structure: Vermiculite particles were different in color; from gold to dark brown. Multi-layer structure of vermiculite can be seen by naked eyes but we cut the vermiculites and prepare a thin layer as possible and take images with optical microscope. (Fig.1)

Vermiculite structure as imaged by contact mode AFM: To take an image with contact mode AFM, we should prepare a flat surface of vermiculite. Because vermiculite is too soft; first fixed it in resin support and then rubbed the surface of it. As showed in fig. 2, vermiculite sub-layers are thinner than 20 nm.



Fig.1- Multi-layer structure of vermiculite in optical microscope. a) Magnification $\times 10$. b) Magnification $\times 4$.

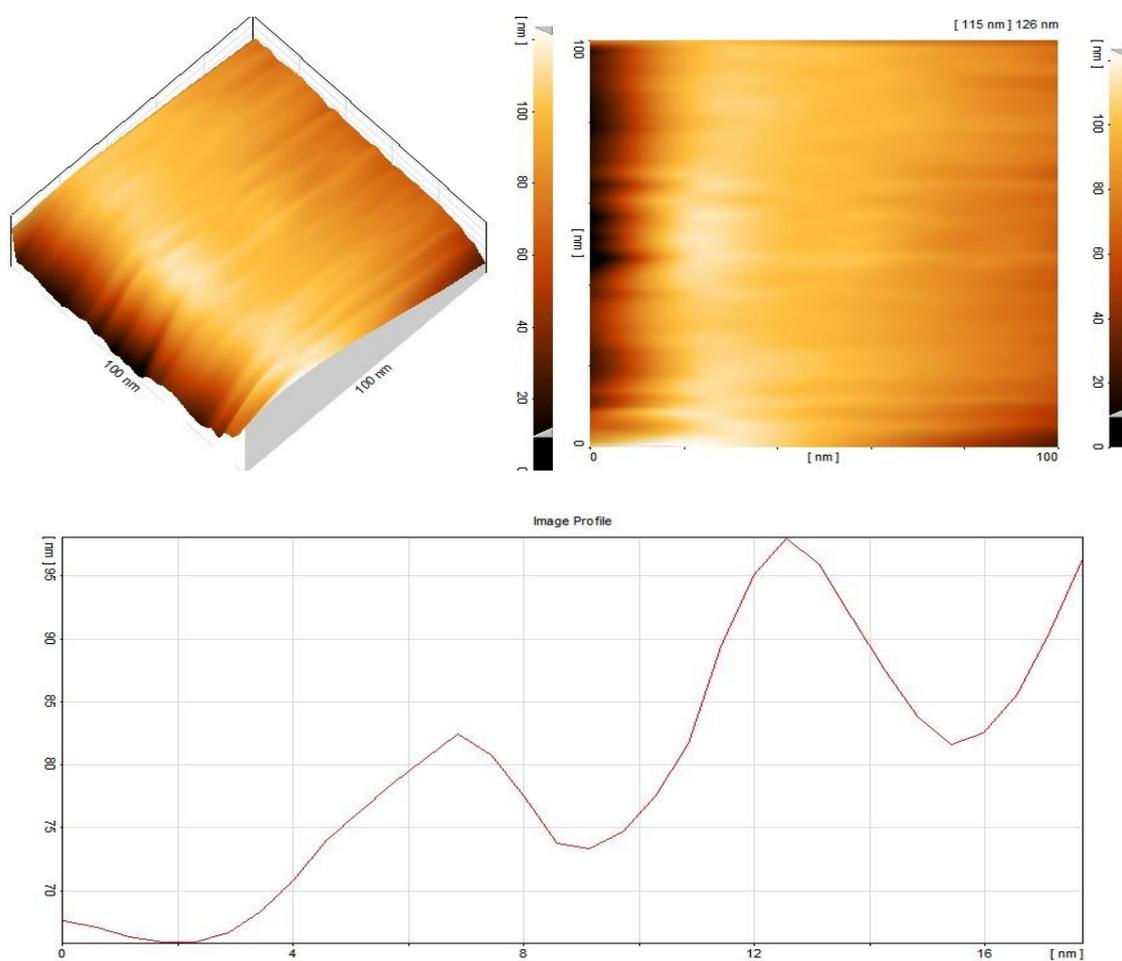


Fig 2- Contact mode AFM of vermiculite. Multilayer structure and size of sub-layers showed.

Cell entrapment in vermiculite: Plate count results showed that in supernatant of vermiculites we had 2×10^8 CFU/mL; however in media without vermiculite we had 6×10^8 CFU/mL bacteria. So we can say that 4×10^8 CFU/mL bacteria were entrapped in vermiculite. Plate count results of 1 gram of biofilm vermiculite showed that 2×10^7 CFU/mL bacteria were trapped in 1 gram vermiculite after 48h incubation.

Comparison of nitrate removal efficiency with different kind of cell status: Un-immobilized cellular mass were active only for five days and removed 45mg/L nitrate in 3 part addition. Immobilized bacteria were active for 10 days and removed 180 mg/L nitrate in 6 part addition. Immobilized bacteria which fed after

inactivation; stay active for 25 days by 3 time feeding and removed 450mg/L nitrate in 15 part addition. The results showed in fig. 3 and fig. 4.

Nitrate removal efficiency was measured based on nitrate and nitrite concentration. Nitrate concentration was measured by addition of Zn powder. Zn powder can reduce nitrate to nitrite and by measuring OD543 nm, calculate nitrate concentration. If the bacteria can reduce 30 mg nitrate to N_2 gas in 24 h, its efficiency is 100%. fig. 5, 6 and 7 showed nitrate and nitrite concentration during nitrate removal by immobilized bacteria without feeding, un-immobilized bacteria and immobilized bacteria with feeding, respectively.

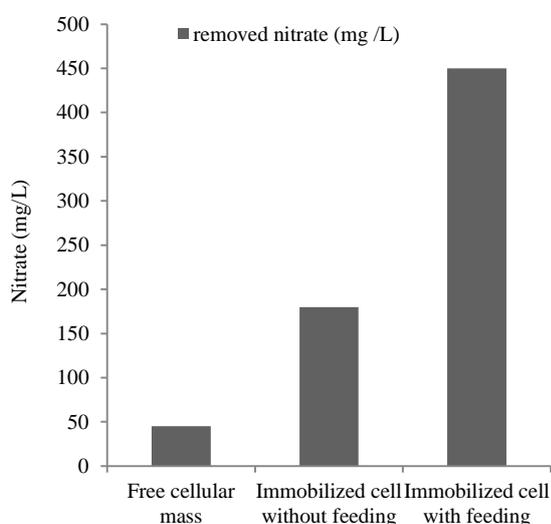


Fig 3- Total nitrate removal (mg/L) in different situation of immobilization and un-immobilization of *P. stutzeri* (4×10^8 CFU/mL)

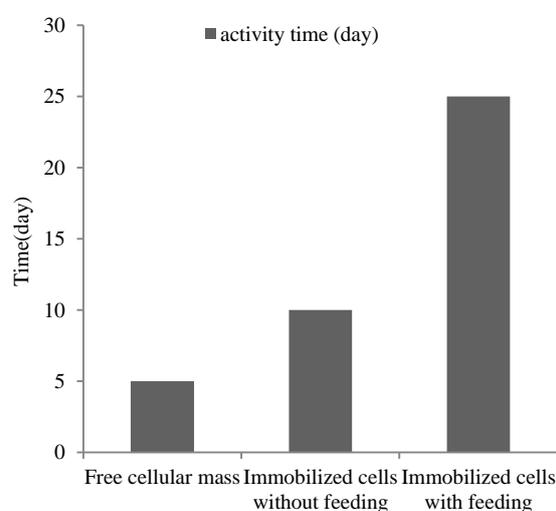


Fig 4- Time of denitrification activity (day) in different situation of immobilization and un-immobilization of *P. stutzeri* (4×10^8 CFU/mL).

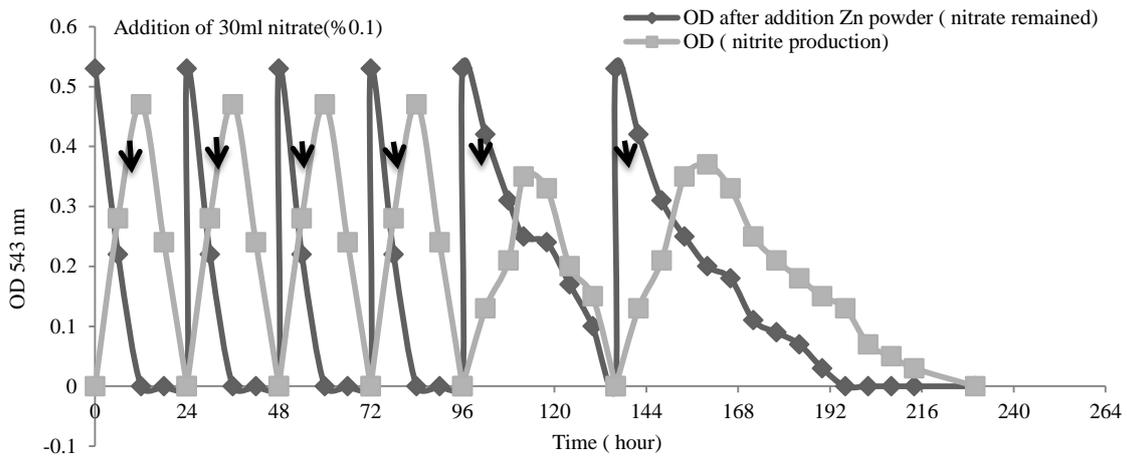


Fig 5- Nitrate and nitrite concentration (OD 543nm) in continuous usage of immobilized *P. stutzeri* (4×10^8 CFU/mL) on vermiculite without feeding.

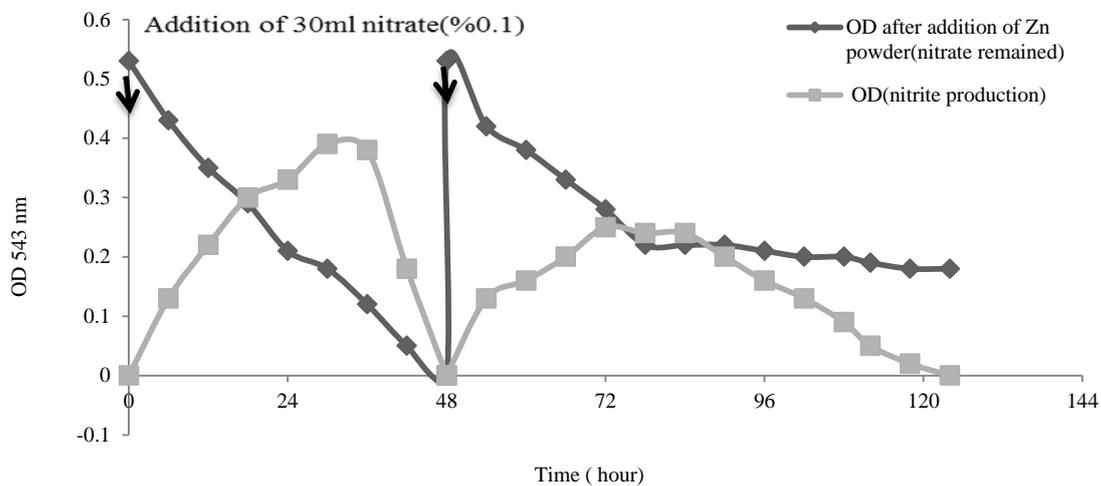


Fig 6- Nitrate and nitrite concentration (OD 543nm) in continuous usage of un-immobilized *P. stutzeri* (4×10^8 CFU/mL).

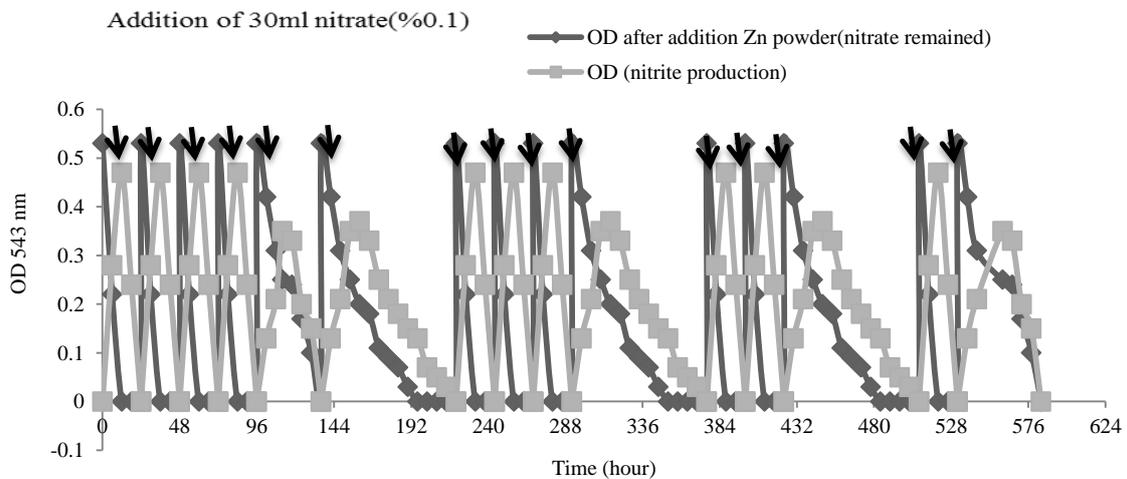


Fig 7- Nitrate and nitrite concentration (OD 543nm) in continuous usage of immobilized *P. stutzeri* (4×10^8 CFU/mL) on vermiculite with feeding.

FTIR results for show binding of bacteria to vermiculite: Fourier transform infra-red spectra for most bacteria have four recognizable regions. Region I (3000-2800 cm^{-1}) represents cell membrane fatty acids, with three detectable peaks (2960, 2925 and 2860 cm^{-1}). Region II (1700-1500 cm^{-1}) shows amide I (1650 cm^{-1}) and amide II (1550 cm^{-1}) bands of proteins and peptides. Region III (1500-1200 cm^{-1}) corresponds to fatty acids as well as proteins and phosphate-carrying molecules. Three major peaks at 1455 cm^{-1} , 1400 cm^{-1} and 1240

cm^{-1} depict changes to lipids and proteins; carbohydrates and nucleic acids or phospholipids, respectively. Bands at 1080 cm^{-1} are also related to nucleic acids. Region IV (1200-900 cm^{-1}) shows absorption bands typical of polysaccharides or carbohydrates of microbial cell walls with an absorption peak between 1100-950 cm^{-1} (12).

As showed in fig. 9, FTIR spectra of vermiculite changed after binding bacteria. Specific peaks which confirm bacterial binding to vermiculite focused in fig. 10.

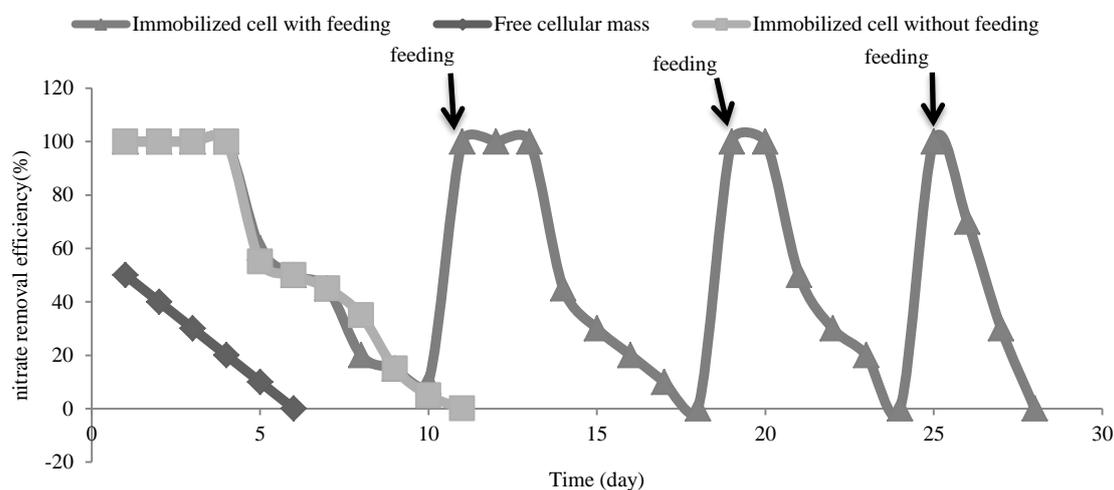


Fig 8- Comparison of nitrate removal efficiency in different situation of immobilization and un-immobilization of *P. stutzeri* (4×10^8 CFU/mL).

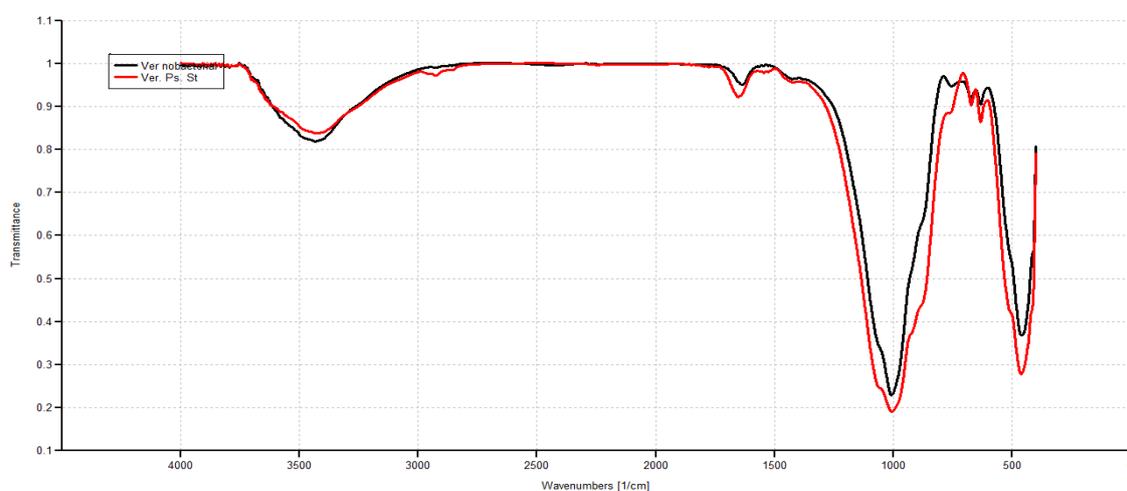


Fig 9- FTIR spectra of vermiculite versus vermiculite binding to *P. stutzeri*.

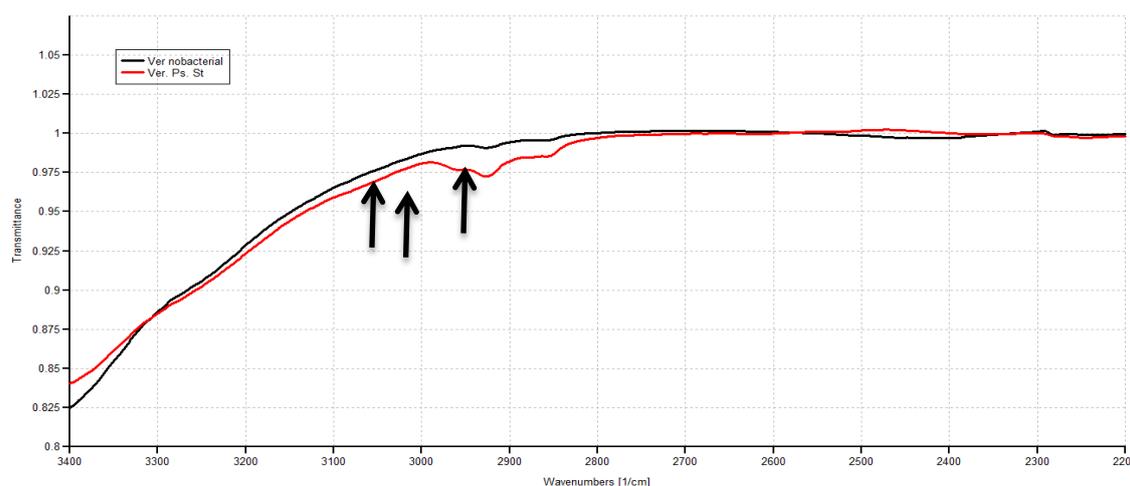


Fig10- Special peaks appear after binding bacteria to vermiculite. Three peak (2860,2920 and 2960 cm^{-1}) appear in red curve after binding bacteria to vermiculite have been shown by this image.

Discussion and Conclusion

Polypropylene and polyoxymethylene were used for immobilizing *Pseudomonas stutzeri* and then brought in to a fluidized bed bioreactor to omit nitrates from synthetic wastewater using methanol as carbon source (13). In another research, *P. stutzeri* immobilized on microbial cellulose for denitrification (14). This is a first report on immobilization of *P. stutzeri* on vermiculite and also using resting cell for nitrate removal.

Sterile exactly vermiculite used as media support for directly fermentation bacterial cultures to prepare bacterial inoculants. Because of the unique characteristics of vermiculite, direct fermentation of bacteria on nutrient-supplemented vermiculite provided a reliable process for producing bacterial inoculants (15). This is the first usage of vermiculite as a nanofilter media support. As the result showed in fig. 8; immobilization of bacteria in vermiculite can increase nitrate removal efficiency. Feeding of immobilized bacteria improved nitrate removal efficiency and activity time. So that, vermiculite was a perfect media support for bacterial immobilization. It was

shown that vermiculite has nanolayers by AFM image. FTIR spectra showed binding of bacteria to vermiculite. It can be used to design a nanofilter to continue nitrate removal. Production of this nanofilter is very easy, inexpensive and there is no need to prepare specific conditions like carbon source and anaerobic bioreactor so that it's available to use it in industry and agriculture. It's suggested to use cheaper nutrient material for bacterial growth or using extended method for bacteria immobilization on vermiculite to improve nanofilter stability and bacterial activity. However there are other environment factors like temperature pH, aeration, ammonium concentration, toxic compounds, sodium azide, heavy metals that might affect on denitrification by immobilized cells.

References

- (1) Lalucat, J., Bennisar, A., Bosch, R., García-Valdés, E., Palleroni NJ. *Biology of Pseudomonas stutzeri*. *Microbiology and Molecular Biology: Pseudomonas Stutzeri Reviews* **2006**, 70 (2), pp. 510- 47.
- (2) Rezaee, A., Godini, H., Dehestani, S., Kaviani,

- S. Isolation and characterization of a novel denitrifying bacterium with high nitrate removal. *Iranian Journal of Environmental Health Science & Engineering* **2010**, 7 (4), pp 313-320.
- (3) Deng, B., Fu, L., Zhang, X., Zheng, J., Peng, L., Sun, J., et al. The denitrification characteristics of *Pseudomonas stutzeri* SC221-M and its application to water quality control in grass carp aquaculture. *PloS one* **2014**, 9 (12):e114886.
- (4) Sharma, SK., Sobti, RC. Nitrate removal from ground water: a review. *Journal of Chemistry* **2012**, 9 (4), pp 1667- 75.
- (5) Mohseni-Bandpi, A., Elliott, DJ., Zazouli, MA. Biological nitrate removal processes from drinking water supply-a review. *Journal of Environmental Health Science and Engineering* **2013**, 11 (1), pp 35-42.
- (6) Della Rocca, C., Belgiorno, V., Meriç, S. Overview of in-situ applicable nitrate removal processes. *Desalination* **2007**, 204 (1), pp 46-62.
- (7) Kilonzo, P., Bergougnou, M. Surface modifications for controlled and optimized cell immobilization by adsorption: applications in fibrous bed bioreactors containing recombinant cells. *Journal of Microbiology, Biochemistry and Technology Science*. **2012**, 8, pp18- 21.
- (8) Bayat, Z., Hassanshahian, M., Cappello, S. Immobilization of Microbes for Bioremediation of Crude Oil Polluted Environments: A Mini Review. *The Open Microbiology Journal* **2015** 9, pp 48-52.
- (9) Martins SCS., Martins CM., Fiúza L., Santaella ST. Immobilization of microbial cells: A promising tool for treatment of toxic pollutants in industrial wastewater. *African Journal of Biotechnology* **2013**,12, pp 4412-441 8.
- (10) Vijayakumar, S., Manoharan, C. Treatment of dye industry effluent using free and immobilized cyanobacteria. *Journal of Bioremediation & Biodegradation* **2012**, 3(10):1000165-1000171.
- (11) Marcos, C., Rodriguez, I. Vermiculites irradiated with ultraviolet radiation. *Applied Clay Science* **2015**, 109, pp 127- 35.
- (12) Booyens, J., Thantsha, MS. Fourier transform infra-red spectroscopy and flow cytometric assessment of the antibacterial mechanism of action of aqueous extract of garlic (*Allium sativum*) against selected probiotic *Bifidobacterium* strains. *BMC complementary and alternative medicine* **2014**, 14 (1), pp 1-8.
- (13) Naik, SS., Setty, YP. Biological denitrification of wastewater with immobilized cells of *Pseudomonas stutzeri* attached to polypropylene and polyoxymethylene. *International Journal of Biology, Ecology Environment Science*. **2012**, 1, pp 42- 5.
- (14) Rezaee, A., Godini, H., Dehestani, S., Yazdanbakhsh, AR., Mosavi, G., Kazemnejad, A. Biological denitrification by *Pseudomonas stutzeri* immobilized on microbial cellulose. *World Journal of Microbiology and Biotechnology* **2008**, 24 (11), pp 2397- 402.
- (15) Graham-Weiss, L., Bennett, ML., Paa, AS. Production of bacterial inoculants by direct fermentation on nutrient-supplemented vermiculite. *Applied and environmental microbiology* **1987**, 53 (9), pp 2138- 41.
- (16) Shahrokh, S., Hosseinkhani, B., Emtiazi, G. The impact of nano-silver on bacterial aerobic nitrate reductase. *Journal of Bioprocessing & Biotechniques* **2014**, 4, pp 162-168.

