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Rudiments of a nitrate assimilating bacterium in bioremediation

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ABSTRACT

The microbial pathway of nitrate assimilation may have bioremediation potential in cases where the need for nitrate removal is paired with a desire to reuse the captured nitrogen in biomass form. In order to assess the nitrate bioremediation potential of *Methylobacterium fujisawaense*, both freely suspended cells and those immobilized on calcium alginate beads were assessed in aerobic test tube and batch reactor settings for their ability to reduce NO_3-N. Nitrate removal results were as high as 100% for freely suspended cells after 96 h and 95% for immobilized cells also after 96 h. A statistical analysis of the first order kinetic/exponential decay trend found in both free cell and immobilized cell systems found no significant difference in overall nitrate removal rates between the two systems. These findings suggest that the organism is capable of a significant assimilation of NO_3-N. The assimilating ability of *M. fujisawaense* is also not greatly altered by immobilization, a characteristic that adds to the list of its potential benefits in a bioremediation setting.

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1. Introduction

A study that collected data from 1993 to 2003 found that 90% of the 190 streams sampled within US had nitrate concentrations that exceeded their natural, expected levels (Dubrovsky et al., 2010). Although physicochemical methods, ion exchange utilizing zeolites for example, are effective at the removal of nitrate and other soluble nitrogen forms from water, factors such as their considerable cost and difficulties in maintenance, regeneration, and disposal have so far kept these processes from becoming dominantly used in the water treatment sector (Ergas and Reuss, 2001; Shrimali and Singh, 2001; Liu, 2007). Nitrate assimilation by bacteria can remove nitrate from soil or water by transforming it into biomass; nitrate in the cell is reduced to nitrite, then the ammonium ion, and finally becomes integrated into organic forms of nitrogen needed for the cell (Metcalf and Eddy, 1991; Moreno-Vivian and Flores, 2007). In contrast to denitrification, the nitrate becomes part of the biomass, which is degradable by heterotrophic organisms, and so the nitrogen is considered reactive and available for use by living organisms (Galloway et al., 2003). Polymeric carriers have been used to immobilize microbes in the bioremediation of nitrate contaminated water via denitrification (Song et al., 2005; Tenokuchi et al., 2006; Hill and Khan, 2008; Rezaee et al., 2008; Siripattanakul et al., 2010). Polymeric carriers have practical benefits over a bioremediation system with freely suspended cells including the following: the prevention of cell washout from the system; easier product separation at the end of the process; a lower risk of contamination of system components by the biological agent used; and higher levels of stability in the biological processes occurring in the bioreactor (Konsoula and Liakopoulou-Kyriakides, 2006; Pramanik and Khan, 2009). The objectives of this study were to: (a) Determine the degree to, and the rate of, which the nitrate assimilating bacterium, *M. fujisawaense*, can deplete NO_3-N, and (b) explore the effect of immobilization on the nitrate-assimilating ability of *M. fujisawaense*.

2. Materials and methods

2.1. Growth curve

M. fujisawaense ATCC® No. 35065™ was acquired from the American Type Culture Collection (ATCC) in Manassas, VA. Broth medium ATCC® 1354 was prepared and contained the following per liter of deionized water: 1 g of $MgSO_4$, 0.2 g $CaCl_2$, 2 mL chelated iron solution, 1 g KNO_3, 0.5 mL trace element solution, 0.272 g KH_2PO_4, and 0.717 g Na_2HPO_4. The chelated iron solution contained the following per 100 mL of deionized water: 0.1 g ferric (III) ammonium citrate, 0.2 g EDTA, sodium salt and 0.3 mL

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concentrated HCl. The trace element solution contained the following per 1 L of deionized water: 500 mg EDTA, 200 mg $FeSO_4 \cdot 7H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, 3 mg $MnCl_2 \cdot 4H_2O$, 30 mg H_3BO_3, 20 mg $CoCl_2 \cdot 6H_2O$, 1 mg $CaCl_2 \cdot 2H_2O$, 2 mg $NiCl_2 \cdot 6H_2O$, and 3 mg $Na_2MoO_4 \cdot 2H_2O$. The medium was autoclaved at 121 °C for 15 min and 1 mL of filter-sterilized methanol was added as a carbon source. 30 mL of ATCC® 1354 broth media was inoculated with one colony of *M. fujisawaense* and incubated on an 18 rpm rocker at 25 °C. Spectrophotometer readings (600 nm) on a Thermo Scientific Biomate 3™ and dilution series plates were then taken and made three times daily until a consistent decline or stagnation in spectrophotometer values occurred. Two more growth curves were generated for *M. fujisawaense* but the 80 mL cultures were agitated at 200 rpm to ensure non-limiting oxygen levels and incubated at an average temperature of 28 °C. Spectrophotometer readings and dilution plates were also taken and made using the same schedule as the first growth curve.

2.2. Nitrate utilization

Since most bacterial nitrate and nitrite reductase assimilation enzymes have been found to contain molybdenum and iron (e.g. Lin and Stewart, 1998; Moreno-Vivian and Flores, 2007), a test to affirm the necessity of inclusion of the bacterium's trace element solution containing these elements was conducted. A 10 mg L^{-1} NO_3-N stock solution was autoclaved at 121 °C for 15 min. 30 mL volumes were then aseptically transferred into eight sterilized 50 mL Kimax™ culture tubes. Two tubes containing 30 mL NO_3-N solution and no further additions along with two tubes containing NO_3-N solution plus 30 μL of filter sterilized methanol and 15 μL of 10× sterile (ATCC 1354) trace element solution served as controls. A control of a combination of nitrate and trace element solution of equal concentrations as the aforementioned controls but without the addition of methanol was run at a later date under the same conditions of temperature and sterilization as the controls described here. Two tubes had the addition of 30 μL of filter sterilized methanol and were then inoculated with 1 mL of log phase *M. fujisawaense* broth culture. The final two tubes received the additions of 30 μL of filter sterilized methanol, 15 μL of sterile 10× trace element solution and a 1 mL inoculation of the same log phase *M. fujisawaense* culture. The quantities of methanol and trace element solution added were chosen to result in 1 g L^{-1} and 5 g L^{-1} concentrations, respectively. This methanol concentration was equal to that found in the growth medium while the trace element concentration was ten times the concentration of the growth medium to ensure that nitrate would be the limiting nutrient. The tubes were then placed on 18 rpm rockers and incubated at 25 °C. Three, 1 mL samples were removed daily from each tube, filtered using an Aladn™ brand 0.22 μm polysulfone syringe filter, and diluted in deionized water by a factor of ten in order to determine the nitrate concentration using a Dionex ICS-2000 system. 100 μL aliquots of the *M. fujisawaense* containing tubes were taken at each sample time to make dilution series plates and establish a measure of cell concentration throughout the experiment.

2.3. Free and immobilized cells

To compare the nitrate utilization between freely suspended and immobilized *M. fujisawaense* cells, batch experiments in duplicate were performed. 10 g L^{-1} NO_3-N solution was prepared and sterilized as previously described and had resulting concentrations of 1 g L^{-1} methanol, 5 g L^{-1} trace element solution, and 10 mg L^{-1} NO_3-N. Bacterial cells for both the free cell and immobilized cell treatments were grown in ATCC 1354 liquid growth media for 72 h before being harvested via centrifugation at 4000 rpm for 25 min in a Hettich Rotina 35 R centrifuge. The calcium alginate bead method used followed the methods of Konsoula and Liakopoulou-Kyriakides (2006) with the modification of Pramanik and Khan (2009) of hardening the beads overnight in $CaCl_2$ solution and a further modification of use of 2% w/v $CaCl_2$ solution in place of the 3.5% w/v $CaCl_2$ solution of Konsoula and Liakopoulou-Kyriakides (2006). The treatments were prepared as follows: after hardening overnight in 2% $CaCl_2$ solution, the beads inoculated with 0.2% (w/v) *M. fujisawaense* were rinsed with sterile, deionized water; 5 mL of the inoculated beads were placed in 20 mL of the prepared simulated nitrate-contaminated water in sterile, 50 mL Kimax™ culture tubes. For the free cell treatment, the same nitrate solution was inoculated with an equal mass of *M. fujisawaense* as the beads (30 mg), and 20 mL of the inoculated solution were placed in sterile 50 mL Kimax™ culture tubes. Both treatments were run in duplicate. To serve as a control, 5 mL of blank beads were placed in 20 mL of the nitrate solution, also in 50 mL sterilized Kimax™ culture tubes. The blank preparation was also run in duplicate. All treatment and control tubes were placed on 18 rpm tube rockers and were incubated at 25 °C. One mL samples were removed daily from each tube and centrifuged at 18,000 rpm for 15 min in order to remove the suspended bacteria. The resulting supernatant was diluted in deionized water by a factor of ten in order to measure the nitrate concentration using the Dionex ICS-2000 system. 100 μL aliquots of the treatment tubes inoculated with free or immobilized *M. fujisawaense* were taken at each sample time to make dilution series plates to establish both a measure of cell concentration in the free cell treatment and a measure of cell release from beads in the immobilized treatment.

2.4. Aeration

To remedy any oxygen limiting conditions, air stones connected to Tetra™ brand air pumps were used for aeration in the experimental setup instead of the tube rockers. A larger volume experiment was also undertaken to give a larger yield of potential sample volume, allowing for the analysis of nitrate, pH, temperature, and to simulate a bench-scale aerated batch reactor as found in other studies (e.g. Song et al., 2005; Tenokuchi et al., 2006; Hill and Khan, 2008; Rezaee et al., 2008). To grow the bacterial cells, two 250 mL Erlenmeyer flasks were filled with 100 mL each of ATCC® 1354 liquid media and inoculated with *M. fujisawaense*. The flasks were shaken at 200 rpm and 25 °C for 72 h to allow for sufficient cell concentration and to attain cells in their log phase of growth. The cells were then harvested by centrifuging 1 mL volumes of the culture in microcentrifuge tubes at 20,800 rpm for 25 min. 0.5 g (wet weight) of the resulting bacterial pellets were added to 250 mL of 2% alginic acid solution, and beads were made according to Konsoula and Liakopoulou-Kyriakides (2006) method with the modification of Pramanik and Khan (2009) and one further modification: in place of hand-syringing, a VWR™ peristaltic micropump was used to add the 2% alginic acid solution drop-wise to the 2% $CaCl_2$ solution, allowing for greater consistency in size of the beads (average diameter 3 mm). The beads were then allowed to harden overnight in the 2% $CaCl_2$ solution. $CaCl_2$ solution was then drained from the beads, and the beads rinsed with deionized water. 750 mL of sterilized 10 mg L^{-1} NO_3-N solution, 1 g L^{-1} methanol, and 5 g L^{-1} trace element solution was then added to the beads to give a total volume of 1 L. 750 mL of the same sterilized nitrate solution was inoculated with 0.5 g (wet weight) of the bacterial pellets in a separate reactor to serve as a free cell comparison to the immobilized cells in the first reactor. Glass bottle reactors were aerated constantly through the air stones, and samples were taken from each reactor daily for nitrate, temperature, and pH analysis. Nitrate analysis was done by filtering the samples taken with

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