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ISOLATION OF *Pseudomonas* STRAIN EM5 WITH AN EFFICIENT NITRATE-DEGRADING ACTIVITY AND THE OPTIMUM CONDITIONS FOR NITRATE BIODEGRADATION USING IMMOBILIZED CELLS.

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ABSTRACT

Thailand is an agricultural country like so many other countries in the world. However, the excessive use of chemical fertilizers is causing groundwater pollution due to the effects of synthetic nitrogen fertilizer. Consequently, we initiated a study series to find a microorganism having good nitrate (NO_3) degrading ability, and to find the optimum condition for nitrate biodegradation. Several bacterial cultures were examined for their ability to utilize and convert nitrate into ammonia (NH_3), nitrogen dioxide (NO_2) and nitrogen (N_2) gas. We found only six isolates were capable of completely reducing NO_3 to molecular nitrogen, especially the isolate EM5. The isolate EM5 was identified as *Pseudomonas* sp. Several important parameters in the biological treatment process were tested in parallel with the controls, i.e., controlled pH conditions, selected amount of cell mass, and initial nitrate concentrations. The results showed that the denitrification property was significantly improved using immobilized cells compared to that of free cells, where one g of immobilized cell could totally remove 200 ml/L nitrate within two hours and was able to degrade up to 1,000 ml/L nitrate within four hours. It is expected to use the isolate EM5 in many applications to reduce the amount of nitrate in solution, such as immobilized cell reactor to reduce the amount of nitrate in drinking water.

Keywords: Microorganism, drinking water, nitrate, biological fertilizer, and degradation.

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INTRODUCTION

The nitrate-contaminated ground and surface water is an important environmental problem in many parts of the world (Rivett et al., 2008). The agricultural areas that use large quantities of fertilizers cause the contamination of water (Scanlon et al., 2007). In fact, plants can not use all the fertilizers that added into the soil by farmers. Thus, more than half of the fertilizers are lost where it is set down into soil and water, while some are circulated in the air. It has been shown that only 33% of the fertilizer is utilized for the plant growth in the plantation of grain crops. The remaining 67% are lost in various ways, i.e., erosion of topsoil, evaporated erosion, nitrogen release of plant processes, and processes reducing nitrate in the soil (Raun and Johnson, 1999). The excessive use of fertilizers can pollute the environment, and cause harmful serious effects to human health (Camargo and Alonso, 2005). The release of wastewater sludge onto the land is also causing this problem (Wakida and Lerner, 2005).

During the past 40 years, some scientists have reported that the rate of nitrogen fertilizer in Asia has increased by 17 times (Dobermann and Cassman, 2004). In most cases, nitrate-contaminated water is found in the areas that use large quantities of nitrogen fertilizers. Nitrogen contamination in water can cause a phenomenon called "algal bloom", which arises due to the presence of large quantities of nitrogen, phosphorus and potassium in water (Camargo and Alonso, 2006). This phenomenon is found to occur in the winter or rainy season due to leaching of the fertilizers into water which result in increased growth of the aquatic plants, especially algae, but the concurrent depletion of oxygen in the water results in death of the aquatic animals. Moreover, the people who live near the agricultural areas, or consume the water from this area are prone to the risk of health problems such as "blue baby syndrome" and "methemoglobinemia" in infants (Camargo and Alonso, 2005; Ghafari et al., 2008; Sadeq et al., 2008), which is arising due to the contamination of nitrate. Therefore, it is highly necessary to reduce the amount of nitrate in ground and surface water.

The main methods used to remove nitrate from water include physical, chemical and biological denitrification. The physical and chemical aspects used for nitrate removal are reverse osmosis, catalysis, electro-dialysis and ion exchange (Karanasios, 2010). However, the use of this process is limited due to high capital and energy costs (Shrimali and Singh, 2001). The biological treatment can provide a simple and cost effective solution to the treatment of water, while providing high quality, environmentally acceptable effluents (Mudder, 1987; Foglar, 2005). Free microorganisms usually used for denitrification are limited in terms of cell slow growth and sensitivity to environmental factors (Zheng, 2009). To solve this issue, an immobilization of microorganisms on the matrix for degradation of contaminants is considered to be an effective technique (Zhao, 2006). Different methods can be used to immobilize cells and these include cross-linking, physical entrapment, covalent coupling, and the natural process of adhesion (Freeman and Lilly, 1998). The most common immobilization matrix used nowadays is alginate and it has been used in various applications (Prasad and Mishra, 1995; Yoo et al., 1996; Abdel-Naby et al., 2000; Klinkerberg et al., 2001; Yan et al., 2001; Carvalho et al., 2003; Idris et al., 2003). The mild conditions for immobilization and its simplicity are some of the reasons that calcium alginate is chosen as the immobilization matrix (Yoo et al., 1996; Klinkerberg et al., 2001; Yan et al., 2001; Carvalho et al., 2003; Idris et al., 2003).

This study was aimed to isolate microorganisms that had the potential to reduce nitrate (NO_3) from biological fertilizer.

MATERIALS AND METHODS

Chemicals and reagents

The synthetic medium consisting of 420 mg/L KH_2PO_4, 375 mg/L K_2HPO_4, 244 mg/L $(NH_4)_2SO_4$, 40 mg/L $CaCl_2.2H_2O$, 30 mg/L NaCl, 30 mg/L $MgSO_4.7H_2O$, 3 mg/L $FeCl_3.6H_2O$, and 1 g/L glucose, which was modified from the formulation described by Chen et al. (2007), was used throughout the study. All the media were sterilized by autoclaving (at 121°C, 1.1 atm, for 15 minutes). Hydrochloric acid

(HCl) and sodium hydroxide (NaOH) were used for the pH adjustment. All chemicals used were of analytical grade and most of them were purchased from Fluka (Switzerland) or Sigma (USA).

Isolation of the microorganism from the biological fertilizer

Attempts were made to recover microorganisms from the biological fertilizer. The compositions of the biological fertilizer were Chinese cabbage, cabbage, morning glory and molasses. The biological fertilizer was allowed to ferment for a month before used as samples for isolation attempt. The biological fertilizer was homogenized with 90 ml of a 0.85% (w/v) sterile saline solution (SSS). Serial 10-fold dilutions were prepared using 9 ml of a 0.85% (w/v) SSS and 0.1 ml of each dilution was plated on Luria Broth (LB) agar and incubated at 37 ^{o}C for 18 h. Colonies appeared on LB agar, which showed as representatives of all different morphologies were chosen at random, purified by sub-culturing and maintained in LB medium.

The isolate that designated as EM5, which was subsequently identified as *Pseudomonas* sp., was immobilized onto calcium alginate as an alternative method to remove nitrate in synthetic wastewater. Various factors and their influences on biological denitrification were investigated, i.e., pH, amount of cell mass, and initial nitrate concentration. The immobilized cells that exhibited more nitrate removal at various conditions when compared to free cells were chosen as a candidate strain.

Ammonification test

Microorganisms were cultured in peptone broth medium. The production of ammonia was detected by adding Nessler's solution, a clear solution that had been undergone colorimetric reactions in the presence of different concentrations of the substance, as described by Sims and Collins (1960), and Rana and Mastrorilli (1998).

Nitrification test

Microorganisms were cultured in ammonium sulfate broth and nitrite broth medium. The production of ammonia, nitrite and nitrate were detected by adding Nessler's solution, Trommsdorf's solution, and diphenylamine, respectively, to show that the nitrite had been oxidized to nitrate as described by Sims and Collins (1960).

Nitrate reduction and denitrification test

Microorganisms were cultured in nitrate broth medium, which had Durham tube to collect gas. The production of ammonia, nitrite, and nitrate were detected by adding Nessler's solution, Trommsdorf's solution, and diphenylamine, respectively, as described by Sims and Collins (1960).

Non-symbiotic nitrogen fixation

Microorganisms were cultured in nitrogen free broth, and the microorganisms that contained a nitrogen fixation process could grow on the top layer or surface of the medium.

Nitrate determination

The production of nitrate was measured by the brucine-sulfanilic method as described by Jenkins and Medsker (1964). The culture broth (150 μl) was centrifuged and a 100 μl of the supernatant was transferred into a new tube. A 50 μl of brucine-sulfanilic acid solution was added to the tube, which was left to stand for five minutes at room temperature followed by adding 500 μl of sulfuric acid (80%) to the mixture. The mixture was left to stand for 10 minutes in the dark. The absorbance at 410 nm was measured after the addition of distilled water (500 μl), and the mixture was subsequently left to stand for 30 minutes in a cool, dark place.

Determination of the optimal conditions of microorganism to degrade nitrate Study on the optimal condition for nitrate biodegradation by EM5 microorganism

The study on the optimal condition for nitrate biodegradation of the isolate EM5 was designed to find the most powerful condition of this isolate. Several parameters, i.e., pH, different cell mass, and different initial nitrate concentration were used. Two types of sample of the isolate EM5 were initially prepared, i.e., cell free sample and immobilized cell sample. The samples were inoculated into synthetic nitrate medium at various conditions, and the samples were subsequently collected to determine the nitrate concentration.

The determination of optimal conditions

for nitrate biodegradation by microorganism was carried out. Firstly, the effect of pH that varied from 6 – 8 using the 0.5 g wet cell prepared at pH7 in 200 mg/L of nitrate was studied. Bacterial cells were cultured in Luria-Bertani (LB) media until it reached the log-phase; the cells were subsequently separated by centrifugation at 5,000 g for 15 minutes. The effect of the amount of various cell masses, i.e., 0.10, 0.25, 0.50, 1.00, and 1.50, was determined the suitable condition for biodegradation of synthetic wastewater by using 100 mL synthetic media containing 200 mg/L of nitrate and pH7. Finally, various nitrate concentrations, i.e., 200, 400, 500, 600, 700, 800, and 1,000 mg/L with one g wet cells at pH7, were used to determine the suitable condition for the biodegradation of synthetic wastewater. In all experiments, the incubator shaker was set at 150 rpm at 37 °C to control the amount of oxygen and temperature for six hours. The sample was then precipitated by the centrifugation and the supernatant was kept for the future use. The suspended and immobilized cells were compared in a series of three experiments for determining pH, amount of cell mass, and initial nitrate concentration.

Immobilized microorganism with calcium alginate gel

Sodium alginate (3% (w/v)) and sodium carbonate (3% (w/v)) were freshly prepared and added into the bacterial cell suspensions, which were cultivated at 37 °C and 150 rpm using LB medium for 18 hours. The cells were separated by centrifugation at 5,000 rpm for 15 minutes, where the supernatant was removed. The mixture was transferred into calcium chloride (4% (w/v)) and incubated at 4 °C for one hour. The immobilized cells were washed with synthetic medium and incubated at 37 °C for two hours.

Statistical analysis

Each experiment was performed in a triplicate manner to make sure that the data were reproducible, while the values reported in the figures were the average of the measurements. The data were analyzed by the statistical tests using Microsoft Excel software. Statistical analysis consisted of summary statistics, including means, standard deviation and standard errors, where two way analysis of variance was done, and then comparison was conducted at the 0.05 level.

RESULTS

Screening the microorganism for nitrate degradation

Six isolates, i.e., EM1, EM2, EM3, EM4, EM5, and EM6, which could grow in the nitrogen free broth, were recovered from the biological fertilizer. Results of the nitrogen cycle activity tests including ammonification test, nitrification test, nitrate reduction test, denitrification test, and non-symbiotic nitrogen fixation test showed that all six isolates failed to convert nitrate to nitrite and ammonia in the nitrate reduction test. It was very interesting that while all of these isolates were cultured, they produced gas in the tube. Thus, it was speculated that they might reduce nitrate into nitrogen gas in the denitrification pathway (data not shown).

The biodegradation rate of nitrate by these six isolates is shown in Figure 1. The isolates EM2, EM3, and EM5 could reduce the nitrate in 100 mg/L initial nitrate concentration. The percentages of nitrate biodegradation rate in 24 hours were 94.56%, 96.62%, and 97.53%, respectively. When the initial nitrate concentration was increased to 200 mg/L, the three isolates (EM2, EM3, and EM5) still retained good nitrate biodegrading ability. The percentages of nitrate biodegradation rate in 24 hours were 89.74%, 89.56%, and 96.19%, respectively. The results showed that the isolate EM5 was significantly reduced nitrate at the level $p < 0.05$ with 200 mg/L initial nitrate concentration. This isolate was selected for use in the further experiments, since it was the most powerful bacteria that were able to biodegrade nitrate. It also had the ability to biodegrade nitrate into nitrogen gas as previously confirmed by denitrification test. However, the isolate EM5 could not convert nitrate into nitrite as previously confirmed by the nitrate reduction test. The isolate EM5 showed excellent denitrifying activities among all six isolates.

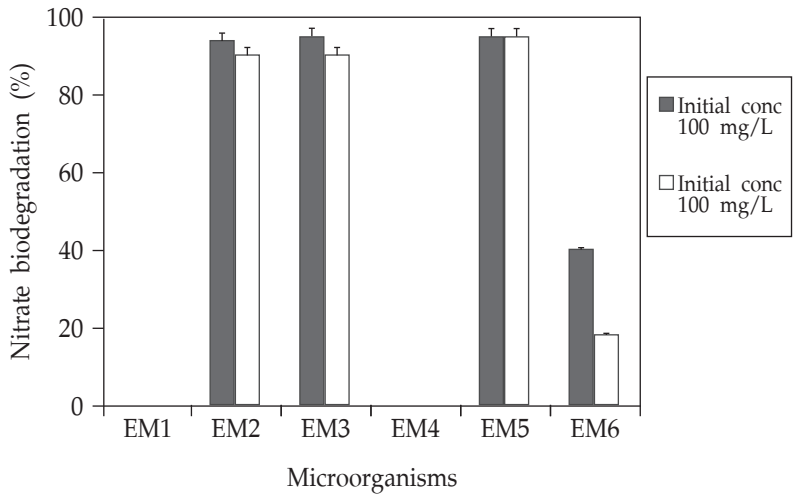

Figure 1. Showing a comparison of biodegradation rate of nitrate in 24 hours of all isolates for initial nitrate concentration of 100 mg/L and 200 mg/L.

Identification of the isolate EM5

The isolate EM5 was rod-shaped aerobic gram-negative motile bacteria. From the results obtained using King B Medium selective media, this bacterium was identified as *Pseudomonas* sp. The isolate EM5 was further examined using several characteristics, i.e., colony and cell appearance, gram staining, catalase and oxidase activities. It was shown that the bacterium was homofermentative, gram-negative rod, catalase- and oxidase-positive rods similar to that previously described by Harrigan and McCance (1966) and Sneath et al. (1986).

Effect of initial pH for nitrate biodegradation

The effect of various initial pH of the cell free and immobilized EM5 samples during the batch fermentation of synthetic wastewater is summarized and illustrated in Figure 2. The ability of the isolate EM5 to biodegrade nitrate at various pH in cell free sample showed that the nitrate concentration at zero hour was 200 mg/L, which was almost similar to the prepared synthetic nitrate as shown in Figure 2a. The nitrate concentrations in all pH levels were decreased as time increased. At the pH6 level, the nitrate concentrations in synthetic wastewater were 200, 65, 14, and 4 mg/L, at 1, 2, 3, and 4 h, respectively. Results showed that the nitrate concentration was almost removed at 4 h. On the other hand, the nitrate concentrations in synthetic wastewater at the pH7 level were 191, 3, 5, and 3 mg/L, at 1, 2, 3, and 4 h, respectively. This result showed that the nitrate concentration was almost completely removed at 2 h. The nitrate concentrations in synthetic wastewater at the pH 8 level were 200, 19, 18 and 5 mg/L, at 1, 2, 3, and 4 h, respectively. This result also showed that the nitrate concentration was almost completely removed at 4 h. When comparing the nitrate concentrations of all the experiments, the pH7 condition was presumably be the most suitable pH condition. It was possible that the higher initial pH brought too much stress on the organism metabolic abilities (Göksungur and Güvenç, 1997). The isolate EM5 showed the most powerful activity to biodegrade nitrate as the nitrate concentration in synthetic wastewater was almost completely removed in 2 h after inoculating with the isolate EM5.

The study of the ability to biodegrade nitrate at various pH conditions in immobilized cells of the isolate EM5 (Figure 2b) showed that the nitrate concentration at zero h was 200 mg/L, which was almost similar to that of the prepared synthetic nitrate. The nitrate concentrations at all pH conditions were decreased as time increased. At pH6, The nitrate concentration in synthetic wastewater at the pH 6 condition were 101, 10, 8, and 1 mg/L at

1, 2, 3, and 4 h, respectively. This result showed that the nitrate concentration was almost completely removed at 2 h. The nitrate concentrations in synthetic wastewater at the pH 7 conditions were 81, 9, 8, and 3 mg/L at 1, 2, 3, and 4 h, respectively. This result showed that the nitrate concentration was almost completely removed at 2 h. The nitrate concentrations in synthetic wastewater at the pH8 conditions were 83, 9, 8, and 3 mg/L at 1, 2, 3, and 4 h, respectively. This result showed that the nitrate concentration was almost completely removed at 2 h. When comparing the nitrate concentrations in immobilization experiments, it was found that all pH conditions were suitable for nitrate biodegradation, since the immobilized technique would protect the cells from the unsuitable pH.

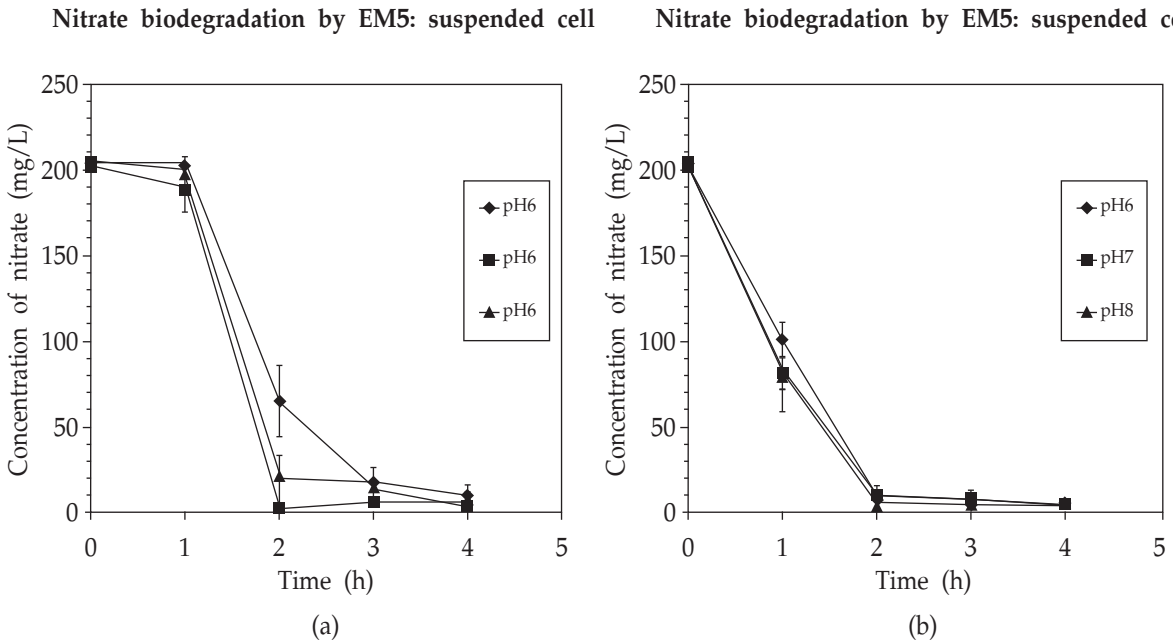

Figure 2. Showing nitrate biodegradation by the isolate EM5 in various pH conditions, i.e., (a) suspended cells, and (b) immobilized cells.

Nitrate biodegradation using different amount of cell masses

This experiment was aimed to study the suitable amount of EM5 cells to biodegrade 200 mg/L nitrate. Various wet cell volumes, i.e., 0.10, 0.25, 0.50, 1.00, and 1.50 g were used to biodegrade the initial nitrate concentrations of 200 mg/L in 100 ml at the pH7 conditions. The degradation performance was increased when the amount of cells was increased, as shown in Figure 3a. The suitable cell mass of EM5 suspended cells to biodegrade 200 mg/L nitrate was 1.5 g as this could totally degrade nitrate for 4 h. The ability of the isolate EM5 at different amount of cell masses in immobilized cells to biodegrade nitrate showed that the nitrate concentration was almost completely removed at 2 h at all cell masses, 0.10, 0.25, 0.50, 1.00, and 1.50 g, respectively, as shown in Figure 3b. It was found that the more the cell mass used, the more degradation rate was. This observation was in agreement with those of many other studies (Chen et al., 2008; Chen et al., 2010), which showed that with an increase of cell mass, there was also an increase in biodegradation rate. In our experiment, we found that on adding more cell mass, the biodegradation rate occurred within 2 h, and more times and cost were required to prepare more cells. Thus, the suitable cell mass in immobilized cell method of our study was concluded at 0.10 g per 100 ml of 200 mg/L nitrate.

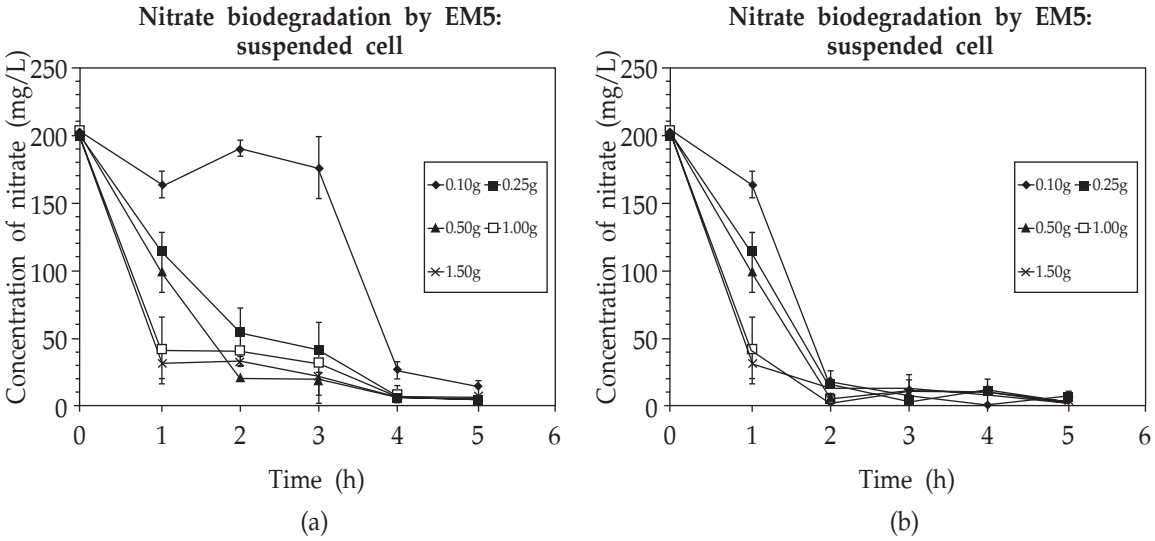

Figure 3. Showing nitrate biodegradation by the isolate EM5 in various amounts of cell masses, i.e., (a) suspended cells, and (b) immobilized cells.

Nitrate biodegradation using different initial nitrate concentrations

The ability of the isolate EM5 to biodegrade nitrate at various initial nitrate concentrations in cell free system showed that at small amount of initial nitrate concentrations, the isolate EM5 was able to biodegrade quickly, as shown in Figure 4a. The isolate EM5 could biodegrade rather slowly when the initial nitrate concentration was increased, whereas it failed to biodegrade when an initial nitrate concentration was 1,000 mg/L. The ability to biodegrade nitrate at different initial nitrate concentrations in immobilized cells of the isolate EM5 (Figure 4b) showed that the nitrate concentration was almost completely removed by one g of cell mass at 2 h with the given nitrate concentrations, i.e., 200, 400, 500, 600, 700, and 800 mg/L, as shown in Figure 4b. Thus, one g of immobilized cells had the ability to degrade up to 1,000 mL/L nitrate within 4 h.

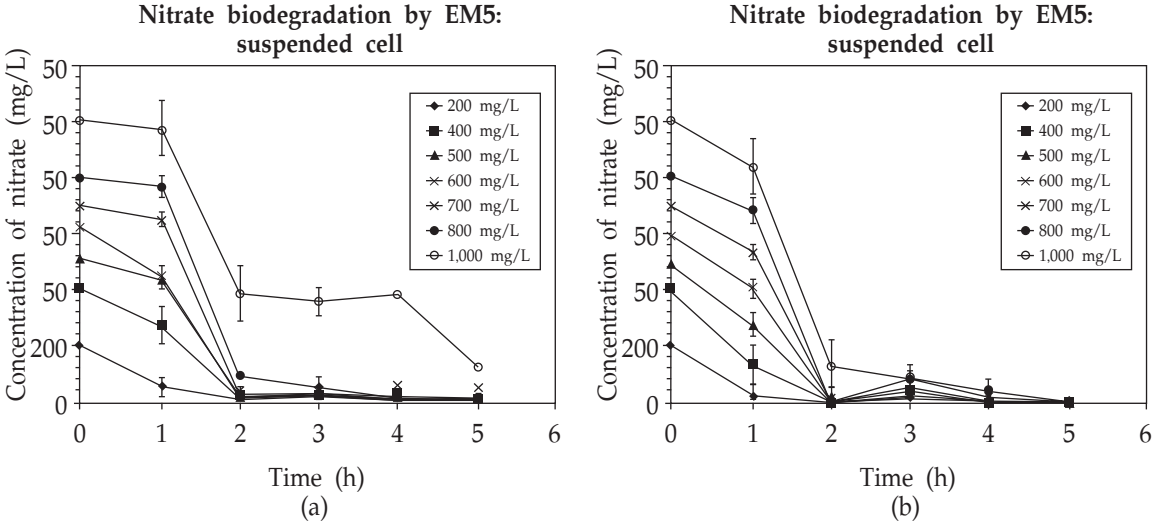

Figure 4. Showing nitrate biodegradation by the isolate EM5 in different initial nitrate concentrations, (a) suspended cells, and (b) immobilized cells.

DISCUSSION

The removal of nitrate from contaminated water results from industrial wastewater, and the use of fertilizers can have a severe impact on human health such as methemoglobinemia and cancer (Camargo and Alonso, 2005; Ghafari et al., 2008; Sadeq et al., 2008). Biological denitrification is an alternative technology and considered to be the most economical strategy to be used because it does not require post-treatment or produce any by-products (Mudder, 1987; Foglar, 2005). It is a cost-effective and environmentally friendly method of nitrate removal despite being a slower process in terms of lower denitrification rates (Ghafari, 2010).

The results of this study show that the selected microorganism that capable of degrading nitrate is the isolate EM5 as it can completely reduce nitrate to nitrogen gas. However, it cannot convert nitrate back into nitrite. The highest percentage of microbial reduction of nitrate was observed in the ability of the isolate EM5 as compared to other microorganisms. The isolate EM5 is identified as *Pseudomonas* sp., which is widely distributed in nature.

The pH condition of culture medium can affect microbial diversity and activity, which can affect transport processes, nutrient solubility, and enzyme activity (Lin, 2010). The efficiency of denitrification at various ranges of culture medium pH values (pH 6-8) for both free and immobilized cells were analyzed. It was found that the efficiency of denitrification using free cells in 2 h was significantly increased from 67.25% (65 mg/L remained) to 98.38% (3 mg/L remained) when the pH of the culture medium was ranged from 6 to 7, as shown in Figure 2a. As the pH value increased to 8, the denitrification efficiency of free cells declined from 98.38% (3 mg/L remained) to 90.10% (19 mg/L). These results are consistent with most studies, where microorganisms favored growth at pH ranging from 6.0 to 8.0 (Lin, 2010). The finding of El-Naas et al. (2009), who studied the biodegradation of Phenol by *Pseudomonas putida*, showed that the highest biodegradation rate was at pH7 level. It is likely that such acidic or alkaline conditions affect the activity of organisms and hence denitrification. When the performance of nitrate biodegradation rate of the isolate EM5 in cell suspension and immobilized cells was compared, it showed that the nitrate biodegradation rate of the isolate EM5 in immobilized cells at various pH levels had high performance than those in cell suspension (Figure 2b). It was, therefore, demonstrated that the denitrification using immobilized cells was less sensitive to the medium pH changes. This result was in agreement with the study of Chen et al. (2010), who immobilized *Klebsiella oxytoca* to treat wastewater-containing cyanide, and found that *K. oxytoca* could biodegrade nitrate at pH7. They also found that the immobilized cells performed their activities more than that of the cell suspension. The immobilized carrier could protect cells, therefore it was good for keeping the stable pH value inside bacteria to avoid acidification of microorganism (Zheng, 2009).

Our result showed that the amount of immobilized cells of the isolate EM5 had a greater ability to degrade nitrate than that of suspended cells even at the same cell concentrations. It was in agreement with Kao et al. (2009), who studied the biodegradation of cadmium by using immobilized *Escherichia coli* cell in PVA gel, which showed that at the same concentration of cell, the immobilized cell showed greater ability to degrade cadmium than that of the suspended cell.

Studies on effect of initial nitrate concentrations on denitrification were also examined to assess the capacity of free and immobilized cells that might tolerate nitrate concentrations. Results showed that the isolate EM5 was enwrapped with calcium alginate and was protected from a direct contact with high volume and the concentration of nitrate. As shown in Figures 4a and 4b, there was no difference in denitrification between freely suspended cells and immobilized cells at an initial nitrate concentration ranging from 200-700 mg/L, since low initial nitrate concentration favored the removal of nitrate (Rezaee, 2008). However, as the initial concentration of nitrate increased from 700-1,000 mg/L, the denitrification by immobilized cells increased significantly. This indicates that a high nitrate concentration is resulted from a longer lag phase, and consequently resulted

in declining nitrate removal efficiency in free cells. Toxic effects may occur and inhibit the growth of microorganisms (Chen and Lin, 2007). It was shown that the inhibition of a high concentration of nitrate on immobilized bacteria was less than that of free cell. This was in agreement with many reports such as evidence found by Lijun et al. (2005), who studied LAS biodegradation by *Pseudomonas aeruginosa* and showed that the immobilized cell had a higher biodegradation rate than that of the suspended cell. Our results is also in agreement with the results obtained from the biodegradation of pyridine by *Paracoccus* sp. strain KT-5 immobilized on bamboo-based activated carbon, where the removal rate of pyridine by immobilized microorganisms increased at various initial nitrate concentrations compared to that of the free cell (Qiao et al., 2010).

Our batch experiments showed that immobilized cells had a high tolerance to environmental factors, which affected the process of nitrate removal. On the basis of the results obtained from the isolate EM5, *Pseudomonas* sp., which immobilized on calcium alginate had much potential as a technique for biologically removing nitrate from water.

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