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ISOLATION AND CHARACTERIZATION OF A NOVEL DENITRIFYING BACTERIUM WITH HIGH NITRATE REMOVAL: *PSEUDOMONAS STUTZERI*

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ABSTRACT

The aim of this study was to isolate and characterize a high efficiency denitrifier bacterium for reducing nitrate in wastewater. Six denitrifier bacteria with nitrate removal activities were isolated from a petrochemical industry effluent with high salinity and high nitrogen concentrations without treatment. The isolated bacteria were tested for nitrate reomoval activity. One of the bacterium displayed the highest reduction of nitrate. The strain was preliminarily identified using biochemical tests and further identified based on similarity of PCR-16S rRNA using universal primers. Biochemical and molecular experiments showed that the best bacterium with high nitrate removal potential was *Pseudomonas stutzeri*, a member of the α subclass of the class *Proteobacteria.* The extent of nitrate removal efficiency was 99% at 200 mg/L NO_3 and the nitrite content of the effluent was in the prescribed limit. The experiments showed the ability of *Pseudomonas stutzeri* to rapidly remove nitrate under anoxic conditions. The strain showed to be potentially good candidate for biodenitrification of high nitrate solutions.

Key words: *Pseudomonas stutzeri*; Denitrification; Polymerase Chain Reaction, Isolation; Characterization

INTRODUCTION

Biological denitrification is a process carried out by numerous genera of bacteria. The denitrification enables transformation of oxidized nitrogen compounds by a wide spectrum of bacteria into harmless nitrogen gas with the accompanying carbon removal. The process uses nitrate as terminal electron acceptors, converting them to nitrogen, nitric oxide and nitrous oxide gases (Foglar *et al.,* 2005).

Heterotrophic denitrifying bacteria are one important functional group involved in the nitrogen cycle. They live most of their lives as heterotrophic, aerobic bacteria but have the ability to respire anaerobically using nitrogen oxides as electron acceptors, which are reduced to nitrous oxide and dinitrogen. The bacteria are one of the most diverse functional groups with members from almost all phylogenetic bacterial groups, and hence contain much genetic and metabolic diversity.

Denitrifying bacteria have been isolated from diverse environments (agricultural soils, deep sea sediments, wastewater treatment plants) and belong to diverse bacterial genera (Zumft, 1997). *Pseudomonas* species are generally presumed to be the predominant microorganisms through which denitrification is achieved (Janda *et al*., 1998). However, other studies have shown that various species, including

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Achromobacter, Agrobacterium, Alcaligenes, Bacillus, Chromobacterium, Flavobacterium and *Hyphomicrobium*, are responsible for denitrification (Otlanabo, 1993).

The *Pseudomonas* genus belongs to the gamma subgroup of *Proteobacteria*. *Pseudomonas stutzeri* belongs to the group of nonfluorescent *Pseudomonas* (Lalucat *et al*., 2006). It seems that *Pseudomonas* species are responsible for the denitrification that occurs in such incredibly diverse microbial consortia as exist in sewage treatment plants. It has been detected through specific DNA sequences (*nirS*, *nosZ*, *nifH*) extracted directly from environmental samples (Anzai *et al.,* 2000). The information derived from 16S rDNA sequences facilitates not only the identification of bacteria in environmental samples, but also a more complete understanding of microbial phylogeny (Cheneby *et al*., 2000).

The taxonomic status and biology of this species, isolated from a large diversity of terrestrial and marine environments, have been recently reviewed (Lalucat *et al.,* 2006). Some strains of *Pseudomonas* have attracted particular attention because of specific metabolic properties, such as denitrification, degradation of aromatic compounds, and synthesis of polyhydroxyalkanoates (Lewis *et al*., 2000). PCR has been developed for all the functional genes in the denitrification pathway, allowing researchers an unprecedented ability to amplify and analyse these genes in cultured isolates and environmental samples (Braker *et al*., 2003). Genetic analysis of denitrification usually proceeds from the nitrite reductase (nirK or nirS) and nitrous oxide reductase gene (nosZ) (Goregues *et al.*, 2005). Although the bacteria responsible for biological nitrogen removal in the petrochemical industry effluent are expected to have high efficiencies, no reports have appeared in the last decades on the screening of the bacteria existing in the petrochemical industry effluent. In this study, the isolated denitrifying bacteria with high denitrficaton potential were identified and characterized, using their morphological and biochemical properties, and 16S rRNA analyses. This bacterium was capable to grow in salt-containing media.

MATERIALS AND METHODS

Bacterial isolation

Ten grab samples of raw wastewater were collected from a petrochemical industry in Iran, with high-nitrate concentration (1 g N/L). The samples were stored at 4°C before experiments. The wastewater was analyzed according to the standard methods (APHA, 2005). For bacterial isolation, serial dilutions within the range of 10^{-4}-10^{-7} were inculcated on the denitrification medium (disodium succinate hexahydrate=10 g; $K_2HPO_4.2H_2O$ = 1 g; $NaNO_3$ = 1 g; KCl = 0.2 g; $MgSO_4.7H_2O$ = 0.2 g; and $FeSO_4.7H_2O$ = 1 mg, in 1000 mL of deionized water; pH=7.2). The inoculated media were incubated with constant shaking (120 rpm) at 25 °C. The inoculation mixture with serial dilution was then spread on the plate of the same medium containing 1.5% (w/v) agar and incubated at 25 °C until the visible colonies were formed.

Different kinds of colonies could be distinguished on the plates. For comparison of isolated bacteria and selection of best denitrifier, batch tests were conducted at vials with 120 mL volume at anoxic condition. The initial NO3–N concentration was 200 mg/L. Nitrate removal rate in the vials, under the batch tests was checked with different isolated bacteria.

Denitrifying bacteria identification

Initial identification schemes were performed with biochemical tests as suggested by the Bergeys Manual of Systematic Bacteriology (Krieg and Holt, 1984). Biochemical tests were consisted of pigment, catalysis, mobility, starch analysis oxides, Oxidation/Fermentaion(O/F) and maltose tests. Specific identification was performed using 16S rRNA sequencing. For performing the molecular diagnostic technique, the following extraction and amplification protocols were conducted.

DNA extraction

The bacteria were grown in (LB) medium at 25°C for 18 h. After growing, they were suspended by mixing with a vortex mixer in suspension buffer, (TE) buffer (10 mM Tris, 1 mM EDTA) in pH in 8.0. Genomic DNA was obtained from pure cultures by lysozyme-proteinase K-sodium dodecyl sulfate (SDS) treatment

followed by phenol-chloroform extraction and subsequent ethanol precipitation (Braker *et al.,* 2003). The purity and concentration of the DNA preparations were determined spectrometrically. Electrophoresis of the extracted DNA was carried out on 0.8 % agarose gels at 3.0 Vcm-1 in TAE buffer.

16S rRNA amplification

The DNA amplifications were performed with general methods (Sambrook *et al*., 2001). Polymerase Chain Reaction (PCR) was used to amplify denitrification genes encoding nitrous oxide reductase (nosZ) from isolated denitrifier strains. Total bacterial *16S* rRNA genes were amplified by PCR using the universal primer pair(5′-AGAGTTTGATCCTGGCTCAG) and(5′AAGGAGGTGATCCAGCCGCA). The *16S* rRNA genes from nosZ were amplified under similar conditions using the primer pair (5′-GCGAGGAAATGAAGCTG) and(5′-AAGGTGATCGACGAGGTC) PCR amplifications from pure cultures samples were performed in a total volume of 50 µL containing 5 µL of 10× PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris-HCl [pH=8.4], 0.1% Triton X-100), 200 µM each deoxyribonucleoside triphosphate, 1.0 U of *Taq* polymerase (5 U/µL; Pharmacia Biotech), 25 pmol of both primers (5 pmol/µLeach), and DNA (10 to 100 ng).

The reaction mixtures were covered with mineral oil and placed in a thermocycler (Perkin Elmer). The PCR was run with initial denaturation of the DNAs at 94°C for 3 min followed by 30 cycles of 30 s at 94°C (denaturation), 1 min at 46°C (annaling), and 1 min at 73°C (extention). The reaction was completed after 10 min at 75°C. The DNA fragments were analyzed by electrophoresis on a 1% (w/v) agarose gel followed by a 15_{min} staining with ethidium bromide (0.5 mg/L).

The amplified genes were purified from the agarose gel using a gel extraction kit according to the manufacturer's guide (Viogen). The 16S rRNA sequences were compared with all accessible sequences in databases using the BLAST server at NCBI (National Centre of Biotechnology Information). The sequences were aligned with those belonging to representative organisms of the L-subclass of *Proteobacteria*. The strains were assigned to a genus based on the obtained 16S rRNA gene sequence similarities.

Denitrification activity

The bioreactor used for testing the reduction of nitrate consisted of a plexiglass cylinder [90 cm length and 0.8 cm diameter]. Inlet and outlet points were set at 2 cm from the bottom and top of the column, respectively. A headspace of around 30 cm^3 was allowed in the column. The synthetic wastewater contained: 0.1 g $MgSO_4.7H_2O$; 1 g KH_2PO_4; 2.5g K_2HPO_4; 0.17 g $CaCl_2.2H_2O$ and 5g NaCl.

The column was initially filled with 10 L of synthetic solution, and after complete removal of nitrate, the continuous process was started by running the effluent through the reactor and 1.5-3 L/h flow rates. The reactor was operated at anoxic conditions with the denitrifier. The KH_2PO_4 loading was chosen in such a way that P/N ratio was kept constant at 0.03 throughout the whole work. 1 M aqueous solutions of hydrochloric acid (HCl) and sodium hydroxide (NaOH) were used for pH adjustments. Effects of different carbon sources and initial concentrations of nitrate were evaluated.

Analytical methods

Samples were collected in vials at interval times. The samples were centrifuged for 20 min at 14000 rpm in an Eppendorf centrifuge. The obtained supernatant was used for nitrate, nitrite, CODand alkalinity analysis using Standard Methods (APHA, 2005). The pH was measured routinely throughout the trials.

RESULTS

Six denitrifiers were isolated from the petrochemical wastewater by culture method. Among these bacteria, *Pseudomonas. stutzeri* with 200 mg/L nitrate-N removal at less than 24 hours retention time was the best denitrifier bacterium. The composition of the effluent used for bacterial isolation is presented in Table 1. All the denitrifiers isolated were bacteria according to biochemicals and PCR analysis (Fig. 1). The *Pseudomonas. stutzeri* was selected among six

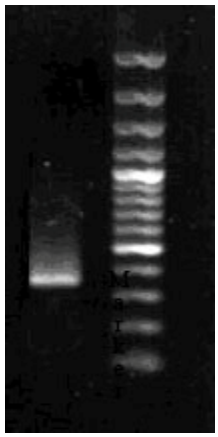

Fig. 1: PCR amplification of 16S rRNA of *P. stutzeri*

Table 1: Characteristics of the petrochemical effluent

Parameter	Average value
Temprature (^{o}C)	30
COD (mg/L)	216
BOD_5 (mg/L)	34
pH	8.8
TDS (mg/L)	16450
NO_3-N (mg/L)	60.44
NO_2-N (mg/L)	1.93
Ammonia-N (mg/L)	597.64

different denitrifying bacteria isolated from the petrochemical effluent, based on nitrate removal. Initial nitrate concentration role is shown in Fig 3. Complete removal of nitrate was observed for the *Pseudomonas stutzeri* until 24h of cultivation for 200 mg/L initial nitrate concentration. While this happened for the 400-800 mg/L initial nitrate concentration at the longer time (Fig. 3). *Pseudomonas stutzeri* showed a fast denitrification activity at 200-800 mg/L initial

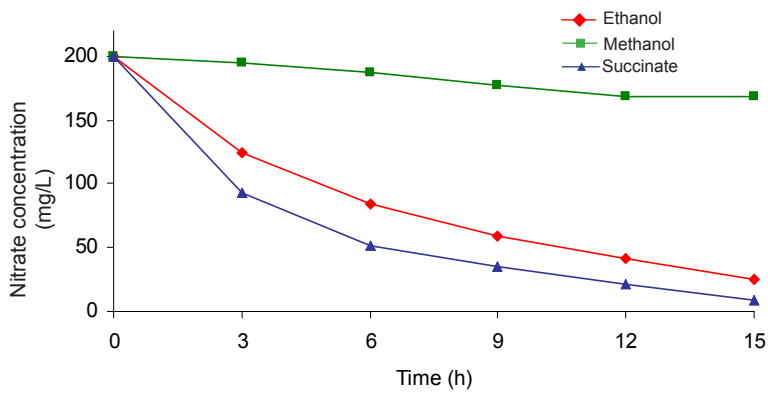

Fig. 2: Effect of carbon source (succinate, methanol and ethanol) on biological denitrification by the isolated *P. stutzeri*

nitrate concentration (Fig. 3). It was a short rod, motile, gram-negative, catalase-positive, and oxidase-positive bacterium. It was found capable of starch hydrolysis and acid production. It grew at temperatures between (4-42)^{o}C. A trend of faster growth in the higher temperature was observed.

The pH range of growth under stationary conditions was between 6-9. In this pH range, the bacterium could completely remove 200 mg NO_3-/L of the medium without accumulation of nitrite. The isolate was found to be able to utilize succinate, ethanol and microbial cellulose as sole carbon sources. It could not utilize methanol as carbon source (Fig. 2).

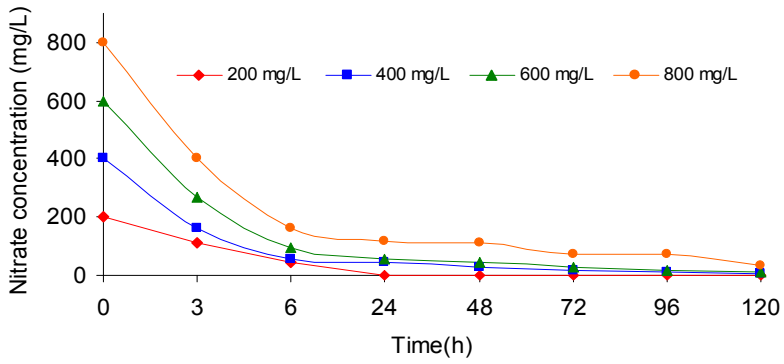

Fig. 3: Effect of initial nitrate concentration (200-800 mg/L) on biological denitrification by the isolated *P. stutzeri*

To describe the isolated bacterial diversity, a molecular approach based on the sequence variability of the 16S rRNA gene was used. The results of the above biochemical tests along with the phylogenic identification of the bacterium using 16S rDNA sequence analysis, proved that the genus of the bacterium was *Pseudomonus* and close to the *stutzeri* sp. In 16S rDNA sequence analysis, more than 98% similarity was observed between the sequences of the isolate strain and the sequences of *Pseudomonus* stutzeri in database.

DISCUSSION

Pseudomonus stutzeri was first described by Burri and Stutzer in 1895 with the definition designation as by *Pseudomonus stutzeri* (Lalucat *et al.,* 2006). The bacterium has been shown to be involved in nitrification and denitrification processes as well as in the degradation of environmental pollutants.

The strains of *Pseudomonus stutzeri* have been used successfully in many developed countries for treating of wastewater (Su *et al.,* 2001). *Pseudomonus stutzeri* strains have strong ability to remove nitrogen in wastewater in piggery wastewater. Su *et al.* (2001) reported that *Pseudomonus stutzeri* NS-2 strain, isolated from piggery wastewater systems, had excellent denitrifying capability under aerobic and anaerobic conditions, as revealed by nitrate disappearance and nitrogen gas production. *Pseudomonus. stutzeri* changes nitrate to dinitrogen (N_2) anaerobically (Carlson and Ingraham, 1983). Sikorski *et al*. (2005) recovered some isolates from soils and marine sediments and used 16S–23S rRNA to identify 8 genovars (genovar 11 to 18). Bennasar *et al*. (1998) revealed genetic diversity and the relationships among *Pseudomonus stutzeri* strains by rapid molecular typing methods. Sikorski *et al*. (2002) carried out random amplified polymorphic DNA (RAPD) PCR analysis in their study of *Pseudomonus stutzeri* isolates from marine sediments and soils in geographically restricted areas. The trend of nitrate removal of the *Pseudomonus stutzeri* was obtained using various carbon sources under stationary culture conditions. No nitrate removal was observed when methanol was used as a sole carbon source. Faster nitrate removal rates were observed with succinate and ethanol, in comparison to methanol (Fig. 2).

The use of succinate was tried in these experiments, since it resulted in a faster nitrate removal rate. The isolate *P. stutzeri* can be employed in purpose of denitrification of high nitrate containing wastewaters such as the wastewaters from the regeneration step of the ion exchange columns which produce nitrate concentrations up to 1000 mg/L (Felsfelstein, 1993). Other study has shown that *Pseudomonus stutzeri* isolated from the Ariake Sea tideland, Japan, had the capability to fully remove as high as 225.8 mg NO_3-N/L under stationary culture conditions without accumulation of nitrite as an intermediate (Hamedaani, 2004). According to literature, different industrial wastewaters contain

more than 200mg NO_3–N/L, and their biological denitrification usually takes a few days. The *P. stutzeri* is one of the most active denitrifying heterotrophic bacteria, and it has been considered as a model system for the denitrification process (Zumft, 1997; Lalucat, 2006).

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