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Isolation and Characterization of *Pseudomonas* Strain Cabable of Degrading Hydrocarbons

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A facultative anaerobic bacterium *Pseudomonas nitroreducens* (D-3) isolated in the presence of naphthalene and pyrrolidine as the sole carbon and energy source from soil sample obtained from cashew nut food processing industry. On the basis of phenotypic and phylogenetic characteristics, it was identified as a member of the genus *Pseudomonas* with 100% sequence similarity to *P. nitroreducens*. The degradation efficiencies of naphthalene and pyrrolidine were examined by GC-MS revealed that the isolate was able to remove above 90% after 144 h at 37 °C. Under anaerobic conditions, degradation of pyrrolidine was coupled with a respiratory nitrate reduction. The strain was also able to degrade phenanthrene, petrol, diesel, diesel oil, kerosene, anthracene, pyridine, xylene and toluene. The pathway of naphthalene degradation was explored through analyzing the intermediary metabolites. Results revealed that the carboxylation process of napthalene produced 1, 2, 3, 4-tetrahydro-1-carboxynapthalene; decarboxylation leads to the formation of catechol, and carbon backbone rearrangements lead to ß-oxidation reaction. This study demonstrated that under anaerobic condition, naphthalene, pyrrolidine, phenanthrene and other hydrocarbons were effectively biotransformed by indigenous denitrifying bacteria.

Key words: Naphthalene, Pyrrolidine, Bio-degradation, *Pseudomonas nitroreducens*, Intermediary metabolites, GC-MS.

The persistence of a pollutant in the environment is influenced by the chemical nature of the contaminant, quantity of contaminant and the interaction between chemical, geological, physical and biological characteristics of the contaminated site. Polycyclic aromatic hydrocarbons (PAHs), perhaps the first recognized environmental carcinogens. They do not degrade easily under natural conditions because the cleavage of an aromatic ring is very difficult because of stability imparted by the high resonance energy of electrons. Though they are the chief pollutants of air and soil, acts as the ultimate repository of these chemicals. Pyrrolidine is commonly present in the environment but at low levels and that in fact, a common organic pollutant released by several industries causing negative impacts on the environment (Yucheng and Yongming 2008). This toxic chemical is widely used as a corrosion inhibitor, rubber additive, solvent, chemical intermediate, drug, herbicide, and photographic developer (Mjos 1978). Degradation of this commonly used chemical is very difficult process because it releases many toxic cancer causing pollutants to the environment. Whereas, novel microorganisms were identified to degrade potent carcinogen, N-nitrosopyrrolidine (Zielenska *et al*. 1990), in the presence of nitric oxides

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(Bamforth and Singleton 2005), therefore intensive attention has been paid in the identification of pyrrolidine degrading microorganisms by different routes. These hazardous chemicals released into the environment could be removed through many processes, such as photo-oxidation, chemical oxidation, bioaccumulation, biodegradation and adsorption. Among the different process, biodegradation is considered as an economic and versatile treatment process. Several biodegradation methods have been developed to remove these toxic chemicals from soil, water and air respectively (Alexander 1994; Romantschuk *et al*. 2000). Among the different methods, usage of microorganisms to clear the hazardous chemicals from the contaminated sites was attractive because this living organisms use the pollutants as the sole source of carbon and energy (Calmels *et al*. 1988). In the last 30 years many researchers identified a number of different microorganisms able to degrade hydrocarbons, especially n-alkanes, kerosene and crude oil. The hydrocarbon degrading microorganisms belong mainly to *Pseudomonas* sp., *Alcaligenes* sp., *Mycobacterium* sp. and *Rhodococcus* sp. genera (Weissenfels *et al*. 1990; Pagnout *et al*. 2007; Martinkova *et al*. 2009; Naas *et al*. 2009). Denitrifying bacteria; *Pseudomonas* species play an important role in the removal of hydrocarbons in the absence of molecular oxygen. *Pseudomonas* is a Gram-negative facultative anaerobic bacterium that performs denitrification, on some occasions. In the waste water systems, *Pseudomonas* plays a key role in the nitrogen cycle and effectively removes pollutants from waste water through assimilatory or dissimilatory process. Denitrification occurs under strict anaerobic conditions or under conditions of reduced oxygen tension. Some denitrification processes may occur in aerobic environments having anoxic micro habitats. The growth of *Pseudomonas*, on the other hand, occurs under both aerobic and anaerobic phases. The present work focuses on the characterization of bacterial strain *Pseudomonas nitroreducens* D-3 isolated from cashew (*Anacardium occidentale*) nut food processing industry soil sample polluted by organic contaminants and toxic elements. Cashew nut food processing industry soil mainly contains the phenolic constituent's such as benzoate, 3-hydroxybenzoate, sanacardic acid, cardol, cardanol and other hydrocarbons like naphthalene. Hot oil bath, expeller and kiln solvent extraction processes were used in this process. Their capability of *P. nitroreducens* D-3 growth in the presence of different organic contaminants and the effect of toxic elements was investigated. The isolated bacterium, *P. nitroreducens* D-3 was identified on the basis of its phylogenetic and phenotypic characteristics, and characterized with respect to the anaerobic degradation of naphthalene and pyrrolidine. For the first time, naphthalene and pyrrolidine degrading bacteria was compared to their capability to grow in the presence of different toxic compounds in order to obtain information on bacterial strain adaptation in the environment polluted by multiple contaminants.

MATERIALS AND METHODS

Chemicals and enzymes

The genomic DNA isolation kit and pGEM-T vector were purchased from Promega (Madison, WI, USA). The high fidelity *pfx* polymerase was obtained from Invitrogen. The miniprep and DNA gel extraction kits were purchased from Qiagen (Mannheim, Germany). PCR master mix with a novel *Top* DNA polymerase was purchased from Bioneer. API 20E test kits were acquired from Bio-Merieux SA, Marcy I'Etoile, France. All other chemicals were obtained from Himedia (Mumbai) and Sigma-Aldrich (St. Louis, MO, USA).

Sample collection

The soil samples were collected from cashew nut food processing industry, located at Marthandam, India. Soil samples were collected in sterile plastic bottles from the surface layer (5–15 cm), transported aseptically in a sterile plastic container to the laboratory and stored in refrigerator for isolation of bacteria. Ten different samples were used in this study for the cultivation of bacteria.

Isolation of naphthalene and pyrrolidine degrading microorganism

Strict anaerobic techniques were used in preparing the medium and handling cultures. Basal medium containing 100 mg; naphthalene, 3 mM; pyrrolidine 2 g; K_2HPO_4, 1.2 g; KH_2PO_4, 0.5 g; KCl, 0.25 g; $MgSO_4W_7H_2O$, and 0.05 g; $CaCl_2.2H_2O$ in 1 L of distilled water supplemented with 1 mL trace

element solution consisted of (g per litter): 2.32, $ZnSO_4$ $7H_2O$; 1.78, $MnSO_4$ $4H_2O$; 0.56, H_3BO_3; 1.0, $CuSO_4$ $5H_2O$; 0.39, Na_2MoO_3 $2H_2O$; 0.66, KI; 1.0, EDTA; 0.4 $FeSO_4$ $7H_2O$; 0.004, $NiCl_2$ $6H_2O$ was used for the isolation of bacteria. The pH of the medium was adjusted to 7.0 using 1N NaOH. A 25 mL medium was transferred into a 100 mL screw cap bottles and tightly closed with gas impermeable butyl rubber septum-type stoppers. Aluminium crimp seals were used to seal the bottles and completely airtight when closed. The bottles were then sterilized at 121 °C in an autoclave for 15 min. Then, the sterile media of each bottle were reduced by the addition of 0.5 mL of $Na_2S_9H_2O$ (4% w/v). An oxygen free mixture of argon was purged into the screw cap bottles for 15 min to create anaerobic conditions. The soil samples were suspended in sterile water and centrifuged at 12,000 rpm for 20 min to remove the heavy particulates. After proper dilution, the supernatant was serially diluted with 0.85% saline solution and transferred into the screw cap bottles. All the bottles were incubated at 37 °C in the dark. After 7 d of incubation with three cycles of enrichment culture; 1 mL of the aliquot was first suspended in a series of 10-fold dilutions of sterile saline water (0.85% NaCl) from 10^{-1} to 10^{-6}. An aliquot of 0.1 mL of each dilution was taken and spread evenly over the surface of the basal agar medium containing 100 mg; naphthalene and 3 mM; pyrrolidine. The bacterial colonies formed on the plate were transferred to new plates and incubated for further purification. The purified strains were incubated with basal liquid medium containing (50 mg) naphthalene and (3 mM) pyrrolidine individually to test the degradation activity. The selected strains were numbered as strain (D-1 to D-21). One strain having better growth and degradation activity was selected and named as strain (D-3); for further characterization the strain was preserved in 20% glycerol (v/v) at -80 °C.

Biochemical and physiological test for identification

To prepare the inoculum for identification experiments, one glycerol stock vial was used to inoculate 100 mL bottle containing 25 mL medium. The activation culture was grown at the 37 °C for 72 h under anaerobic condition. Biochemical and physiological properties of the strain was analyzed using routine methods. API 20E test kits were used to characterize phenotypically. The API test strips were prepared according to the instructions of the kit supplier.

Molecular identification

Strain D-3 DNA was extracted using kit method also manually by boiling a loop full of culture in sterilized distilled water for ten minutes and centrifugation at 13,000 rpm for 10 min. The supernatant containing the DNA was used to amplify 16S ribosomal DNA fragments by PCR (Bio-Rad I cycler) using 27F (5' AGA GTT TGA TCG TGG CTC AG 3') and 1492R (3' GGT TAC CTT GTT ACG ACT T 5') primers. The conditions for thermal cycling were as follows: initial denaturation of the target DNA at 95 °C for 10 min followed by 30 cycles of amplification, denaturation at 95 °C for 2 min, primer annealing at 58 °C for 1 min and primer extension at 72 °C for 2 min. At the end of the cycle, the reaction mixture was held at 72 °C for 10 min and cooled at 4 °C. Amplified DNA was visualized at 100 V and 400 mA for 25 min on agarose gel (1% (w/v) in TAE buffer 1x, 0.1 ¼l Ethidium Bromide solution). Concentration of DNA was determined by using a double beam spectrophotometer (Lambda 20, PerkinElmer; Norwalk, CT, USA). The amplified PCR products were purified by PCR Purification Kit (Qiagen, Crawley, UK). The PCR product was ligated into the pGEM-T cloning vector by following the instructions given by the manufacturer (Promega, Madison, WI, USA). Plasmids were transformed into *Escherichia coli* DH5± competent cells. Recombinant transformants were selected by blue/white colony screening. Individual white colonies were grown at the 37 °C overnight with rotary shaking in 25 mL of LB medium containing ampicillin (50 µg mL^{-1}). Plasmid DNA was extracted and purified by QIA Purification Kit. After plasmid preparation, 2 µL (out of 50 µL) of each sample was amplified by PCR (Bio-Rad I cycler) using M13-F (5' GTT TTC CCA GTC ACG AC 3') and M13-R (3' CAG GAA ACA GCT ATG AC 5') primers to check for the presence of insert DNA. Only plasmids containing the expected 1,500 bp inserts were sequenced. The obtained sequences were subjected to BLAST at http://www.ncbinlm-nih.gov/ search in NCBI database for phylogenetic relationship.

Analysis of fatty acids methyl ester

Phospholipid ester-linked fatty acids of strain D-3 were analyzed in the form of fatty acid methyl ester (FAME) using MIDI system (Microbial

Insights, Newark, DE). Extraction of fatty acids has been done according to the instruction provided by the manufacturer with some modification. Briefly, cells harvested following the 24 h of growth on TSA (Tryptic Soy Broth Agar) were heated to 100 °C with NaOH-methanol to saponify cellular lipids and the released fatty acids were methylated by heating with HCl-methanol at 80 °C. FAMEs were solvent-extracted, and analyzed by gas chromatography with flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). FAMEs were identified by comparing their retention time and mass spectra with authentic standards provided by the MIDI database. To examine the fatty acids shifted in response to carbazole, cells grown on TSB were collected and washed twice in potassium phosphate buffer (pH 7.0). After 24 h of incubation at 37 °C, changes in the composition of FAMEs were analyzed as described above.

Carbon source utilization

Various aromatic compounds were added as carbon sources to the basal liquid media (composition explained in isolation of naphthalene and pyrrolidine degrading microorganism section). Strain D-3 was grown in 100 mL medium containing naphthalene (100 mg) and pyrrolidine (3 mM) or other carbon sources (0.1%); and incubated at 37 °C at 200 rpm for 144 h in an orbital shaker under anaerobic condition. The compounds were: petrol, diesel, diesel oil, kerosene, phenanthrene, anthracene, benzene, pyridine, xylene, toluene, salicylic acid and phenol. Growth was measured after 144 h by the increase of OD 600_{nm} of the culture. Flasks without hydrocarbons served as controls. Each test ûask was prepared in triplicate.

Effect of environmental condition on degradation of naphthalene and pyrrolidine

Various conditions for degradation of naphthalene and pyrrolidine were as follows: Incubation temperatures of 20, 30, 37 and 45 °C and autoclaved medium initial pH of 5, 6, 7, 8 and 9 using 1 M HCl or 1 M NaOH and incubated for 144 h under anaerobic condition. Flasks without hydrocarbons served as controls. Each test ûask was prepared in triplicate. After incubation the cell density was measured at OD_{600nm}.

Analytical methods for determination of naphthalene and pyrrolidine

The concentration of pyrrolidine was analyzed spectrophotometrically as described by Stevens and Skov (1965) and Knapp *et al*. (1982). Naphthalene degradation efficiencies were detected on 7th d by GC. The concentration of naphthalene (1 mg mL^{-1}) in the samples was analyzed using gas chromatography (GC) equipped with a flame ionization detector (FID). The chromatographic separation was performed on a CP-Sil 8 CB (30 m, i.d. 0.32 mm and 0.25 µm film thicknesses) column (Agilent Technologies, CA). The initial column temperature was 52 °C and detector and injector temperatures were 280 and 260 °C, respectively. The injection sling was 10 ¼L (manually). The degradation of naphthalene and pyrrolidine as a whole was expressed as the percentage of naphthalene and pyrrolidine degraded in relation to the amount of the remaining fractions in the appropriate control samples (external standard). The degradation efficiency (DE) was determined based on the decrease in the total concentration of hydrocarbons, by using the following equation:

$$DE\ (\%) = 100 - (A_s \times 100/A_{ac})$$

where A_s is the total area of peaks in each sample, A_{ac} is the total area of peaks in the appropriate control, DE (%) is the efficiency of degradation.

RESULTS AND DISCUSSION

Isolation of naphthalene and pyrrolidine degrading microorganism

The present investigation showed that ten samples were used for the isolation of naphthalene and pyrrolidine degrading microorganisms. Twenty one suspected *Pseudomonas* strains were isolated and purified based on their capability to grow on agar medium. All the strains were selected by growing on agar plates with naphthalene and pyrrolidine as sole carbon source. Initially the isolates were selected based on cell morphology and cell growth. An isolate was named as strain (D-3), grew well on naphthalene and pyrrolidine containing medium over other strains. This strain was subjected to microbial analysis and biochemical characterization, which was identified as a facultative anaerobic, motile and appears as a light cream in colour on agar medium. Biochemical identifications presented in the API 20E micro tests (Himedia, Mumbai) were determined. Table 1 shows

the biochemical, morphological and physiological characterization of strain (D-3). The strain could grow on dextrose, fructose, maltose, lactose, sucrose, galactose and glycerol. However, it did not grow on mannitol, xylose, mannose, cellobiose, rhamnose, xylitol, arabinose, inositol, sorbitol and arabbitol. Utilization of various carbon sources indicated a wide pattern of carbon source assimilation. These biochemical tests revealed that this isolate is a mesophilic microorganism which is unable to produce hydrogen sulphide while capable of reducing nitrite. The optimal pH and temperature for growth was 7.0-8.0 and 30-37 ^{o}C, respectively. It displayed negative results for gelatinase enzyme production test. From these analyses, the strain was identified as *Pseudomonas* and designated as *Pseudomonas* sp. (D-3). The strain was classified into the genus within the ^{3}-subdivision of the *Proteobacteria*, because the phenotypic characteristics were closely matched those of *Pseudomonas*, i.e., Gram-negative, rod shape, motile, facultative anaerobic, usually oxidase positive, use nitrate as alternative electron acceptor, and optimum growth in the neutral pH ranges (Palleroni 1984). The 16S rRNA sequences of *Pseudomonas* sp. (D-3). (Comprising 1450 nucleotides) were determined, and deposited in GenBank database under the accession number JX534215. Phylogenetic tree was constructed on MEGA-5.05 aligned sequences of the 16S rRNA gene (Figure 1). An analysis with the NCBI BLAST

Table 1. Biochemical tests for characterization of *Pseudomonas nitroreducens* (D-3)

Test	D-3
Lactose	+
Xylose	-
Maltose	+
Fructose	+
Dextrose	+
Galactose	+
Raffinose	-
Trehalose	-
Melibiose	-
Sucrose	+
L-Arabinose	-
Mannose	-
Inulin	-
Sodium Gluconate	-
Glycerol	+
Salicin	-
Dulcitol	-
Inositol	-
Sorbitol	-
Mannitol	-
Adonitol	-
Arabitol	-
Erytritol	-
á-Methyl-D-Glucoside	-
Rhamnose	-
Cellobiose	-
Melezitose	-
á-Methyl-D-Mannoside	-
Xylitol	-
ONPG	-
Esculin hydrolysis	-
D-Arabinose	-
Citrate utilization	+
Malonate utilization	-
Sorbose	-
Indol	-
Methyl red	-
Voges Proskauer's	-
Lysine utilization	-
Ornithine	-
Urease	-
Phenylalanine Deaminase	-
Nitrate reduction	+
H_2S Production	-

+ : Positive (more than 90%)
-: Negative (more than 90%)

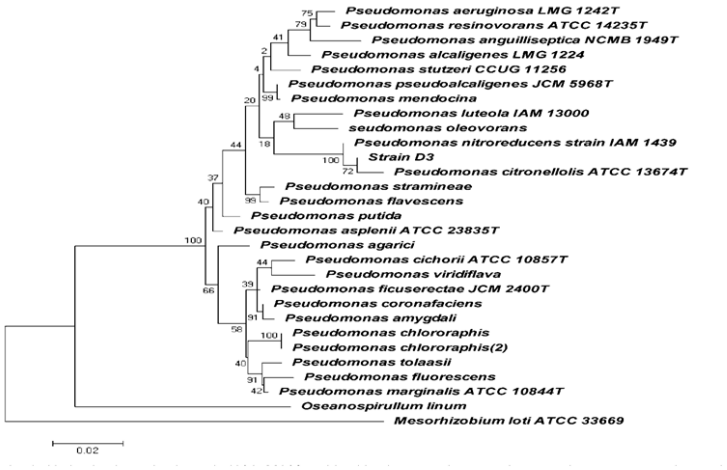
Table 2. Utilization of different carbon substrates by *Pseudomonas nitroreducens* (D-3) and cell growth (mean ± SD)

Carbon source	After 144 h ($OD600_{nm}$)
Naphthalene	1.34±0.1
Pyrrolidine	1.34±0.12
Petrol	1.9±0.10
Diesel	1.89±0.09
Diesel oil	2.3±0.14
Kerosine	1.78±0.21
Phenanthrene	0.98±0.19
Anthracene	1.1±0.15
Benzene	0.045±0.001
Pyridine	0.89±0.1
Xylene	1.15±0.2
Toluene	1.78±0.22
Salicylic acid	0.114±0.01
Phenol	0.02±0.011

Growth was followed by measuring the increase of $OD600_{nm}$ for 144 h.
At 0 h the $OD600_{nm}$ 0.1

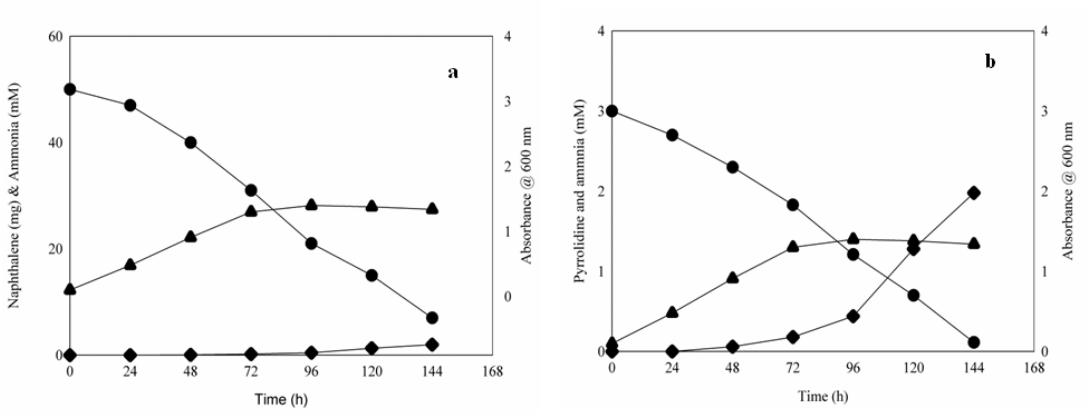
program, it was found that *Pseudomonas* sp. (D-3) is closely clustered with *P. nitroreducens* IAM 1439T, having 100% sequence similarity.

Cellular fatty acids detected from the strain were: straight saturated fatty acid, 16 (20.1%); unsaturated fatty acid, 18:1 7c/ω9t/ω12t (18.7%); cyclo fatty acids, 19:0 cyclo É8c (14%) and 17:0 cyclo (11%); hydroxylated fatty acids, 10:0 3-OH (7%), 12:0 2-OH (4%); 12:0 3-OH (6.3%); and other minor fatty acids. Different types of carbon source were tested as sole carbon substrate of *P. nitroreducens* (D-3). The strain was able to grow in the presence of naphthalene, phenanthrene, petrol, diesel, diesel oil, kerosene, anthracene, pyridine, xylene and toluene under anaerobic condition, but it could not grow on benzoates. The cell growth clearly revealed that the strain could degrade the hydrocarbons (Table



The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.45569599 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1234 positions in the final dataset. Evolutionary analyses were conducted in MEGA-5.05.

Fig. 1. Evolutionary relationships of *Pseudomonas nitroreducens* (D-3)



a, naphthalene degradation; b, pyrrolidine degradation.
Symbols: closed circle, residual naphthalene and pyrrolidine; closed triangle, OD600 and closed diamond, ammonia.

Fig. 2. Anaerobic degradation of naphthalene and pyrrolidine by *Pseudomonas nitroreducens* (D-3)

2). When *P. nitroreducens* (D-3) was cultivated with pyrrolidine and naphthalene the optimal pH and temperature was 7.0-8.0 and 30-37 °C, respectively.

Biodegradation of naphthalene and pyrrolidine

The degradation pattern of selected isolate on naphthalene and pyrrolidine was individually evaluated by GC-MS and spectrophotometrically. The results demonstrated that *P. nitroreducens* (D-3) degraded almost 90.1% naphthalene and 97.48% pyrrolidine within 7 d; this showed that naphthalene and pyrrolidine were an efficient carbon source (Figure 2 a and 2 b). Present results were slightly similar to Lin *et al*. (2010) who reported that the degradation of naphthalene rose to 99.1% within 4 d under optimum conditions. In previous results, Young *et al*. (2002) reported that nearly 99% of pyrrolidine was degraded by *Pseudomonas* sp., strain YG1 within 70 h . Many different species of bacteria, mostly from soil environments, have the ability to degrade naphthalene and other PAHs whereas, *Pseudomonas* genus was identified as the majority of the PAH degrading bacteria (Cerniglia 1993; Daane *et al*. 2001). However, investigations of contaminated soils have uncovered naphthalene-degrading bacteria that did not hybridize with NAH7-derived gene probes (Ahn *et al*. 1999; Lloyd-Jones *et al*. 1999), and indicate that there are still many unidentified bacteria with diverse PAH biodegradation pathways. The strain reported in the current study extends our knowledge of the

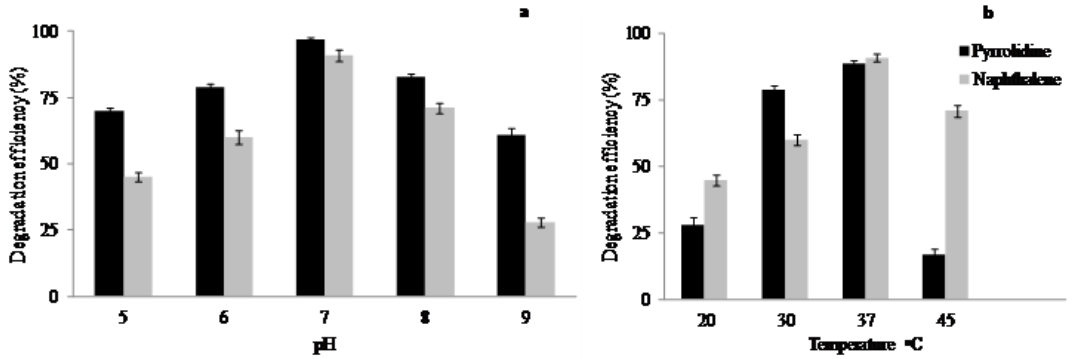


Fig. 3. Effect of different conditions on biodegradation of naphthalene and pyrrolidine by *Pseudomonas nitroreducens* (D-3). a, different pH; b, different temperature.

range of naphthalene degrading bacteria found in the soil environment. This work suggests that many undiscovered bacteria might enhance a key role in PAH degradation on contaminated industrial soil environment.

Anaerobic cultivation of *P. nitroreducens* (D-3) in the basal medium containing pyrrolidine is represented in Figure 2 b. Pyrrolidine degradation and ammonia production were directly proportional; 3 mM pyrrolidine was depleted within 144 h. During the anaerobic degradation of pyrrolidine, maximum cell growth was increased to about 1.4 OD_{600} indicating that the strain used pyrrolidine as a carbon and energy source for its growth. The amount of ammonia accumulated in the medium reached 1.98 mM, indicating that in the process of pyrrolidine metabolism, amine moieties were released. The nitrate used in the medium might have enhanced the degradation of pyrrolidine. Results indicated the degradation of naphthalene and pyrrolidine were pH dependent (Figure 3 a). The optimum pH for the degradation was 7.0 (after 144 hr >90% of pyrrolidine and naphthalene were degraded whereas the efficiency of degradation was slightly lower at pH less than 7.0 and significantly poor above 8.0 and 9.0). These results are in agreement with the report by Chen *et al*. (2006) who found that microorganisms favored growth at pH level ranging from 6 to 8. A series of degradation tests was carried out at various optimum temperatures from 20 to 45 °C (Figure 3 b). The strain showed good effect at the optimum temperature ranging from 37 °C for the degradation of pyrrolidine and naphthalene under anaerobic

condition. *Pseudomonas* sp. (HOB1) degraded 97% of naphthalene within 24 h (Pathak *et al*. 2009).

Identification of bi-products and metabolic pathways in degradation of naphthalene

As the identification of metabolic pathways is important for the investigation, in this study we identified the strain at the metabolic levels. It is understood that the degradation of naphthalene by biological process occurs through the formation of salicylate or benzaldahyde as an intermediate (Eaton *et al*. 1992; Grund *et al*. 1992). In order to evaluate the metabolic pathway degradation metabolites were analyzed by GC–MS. The degradation products identified were benzaldehyde, catechol and phenylacetic acid. The fragments detected in each of the identified metabolites are given below (*m/z*, relative intensity). Benzaldehyde (a) 106(M+, 100), 107[M+H]$^+$ (7.85), 105(45.70), 78(18.34), 77[M-CHO]$^+$ (99.88), 75(4.43), 74(9.04), 52(12.29), 51(47.21). Catechol (b) 110 (M+, 100), 92 (11.47), 81 (13.86), 64 (41.34), 63 (21.62), 53 (12.66). Phenylacetic acid (c) 136 (M+, 19.88), 92 [M-CO2]$^+$ (19.48), 91 [M-COOH]+ (100%), 89 (4.65), 85 (4.03), 77 [M-CH2COOH]$^+$ (1.98), 65 (18.66), 63 (6.87). Under anaerobic condition, naphthalene degraded into 2-methylnaphthalene derivatives by the alkylation reaction. After that oxidation process might be activated to form the carboxylic acid metabolites (Safinowski *et al*. 2006). It is clear that the addition of nitrate acts similarly to fumarate, therefore nitrate may activate 2-methylnaphthalene to form naphthyl-2-methylsuccinate and naphthyl-2-methylenesuccinic acid. Further, dehydration process undergoes to the formation of naphthyl-2-methylenesuccinate followed by benzylsuccinic acid, benzylsuccinyl-CoA, phenylitaconyl-CoA and benzyl-CoA intermediary metabolite formation. Microorganism belongs to *Pseudomonas* family comprises many aerobic naphthalene-degrading bacteria. McNally *et al*. (1998) identified *P. stutzeri*, *P. putida*, and *P. fluorescens* which degraded naphthalene, acenaphthene, anthracene, phenanthrene, and pyrene under anaerobic condition. Rockne and Strand (1998) reported anaerobic degradation rates of PAH were comparatively slower in nature, because the initial cleavage of the ring structure of PAH were the rate-limiting step. Carboxylation step is the initial ring breakdown reaction of the anaerobic naphthalene biodegradation pathways in a sulfate-reducing condition (Zhang and Young 1997). It is assumed that *P. nitroreducens* (D-3) might be used carboxylation pathway to degrade naphthalene (Figure 4). Carboxylation process of naphthalene produce 1, 2, 3, 4-tetrahydro-1-carboxynapthalene; and further decarboxylation leads to the formation of catechol, and carbon backbone rearrangement and ß-oxidation Yen and Serdar (1988) reported the metabolism of naphthalene by *P. putida* (Yen and Serdar 1988). But in the present study salicylic

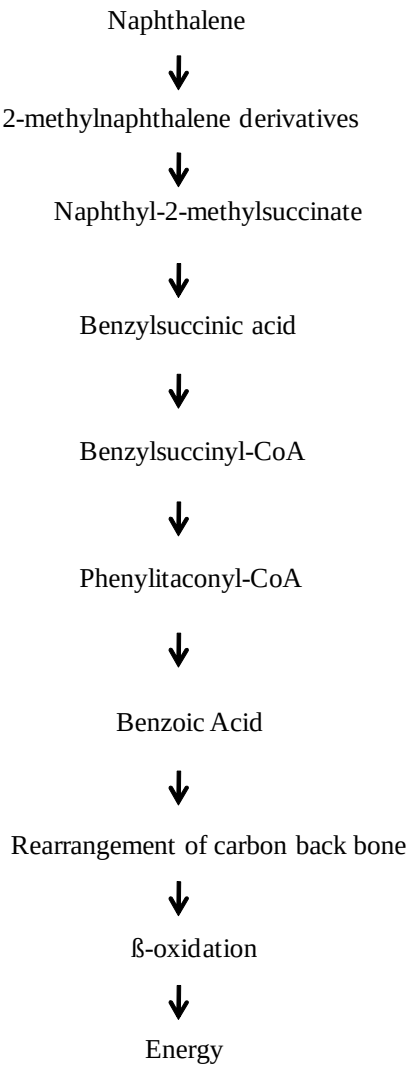


Fig. 4. Degradation pathway of naphthalene by *Pseudomonas nitroreducens* (D-3) under anaerobic condition

acid was not detected in the culture extract and growth on salicylic acid was also negative. Therefore, the main route for degradation of naphthalene in *P. nitroreducens* (D-3) was alkylation reaction or a naphthalene carboxylase enzyme. Many microorganisms have been identified from various sources of environment for their ability to degrade naphthalene under anaerobic conditions. This *P. nitroreducens* (D-3) can be exploited for the development of effective biodegradation for PAHs in the environment.

CONCLUSION

In conclusion, *P. nitroreducens* (D-3) capable of degrading pyrrolidine and naphthalene in *in vitro* condition was isolated. It was identified as a member of the genus *Pseudomonas* on the basis of its phenotypes and phylogenetic properties. The degradation result showed that the isolate could remove more than 90% of pyrrolidine and naphthalene in 144 h at 37 °C. Incorporation of nitrate in the basal medium stimulates the anaerobic degradation of pyrrolidine. *P. nitroreducens* (D-3) completely degraded pyrrolidine and naphthalene without the accumulation of hazardous metabolites such as N-nitrosopyrrolidine. A degradation pathway has been elucidated for the degradation of naphthalene under anaerobic conditions.

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