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Influence of Nitrogen Source on the Catabolism of Naphthalene by a *Pseudomonas aeruginosa* TU

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A B S T R A C T

A *Pseudomonas aeruginosa* TU isolated from diesel-contaminated site degraded 66% of an initial 10 mM naphthalene load after 48 h of incubation in a minimal-salts medium containing NH_4Cl as nitrogen source whereas, the same bacterium, when incubated in the minimal-salts medium containing KNO_3,degraded 10 and 20 mM initial naphthalene load within in 24 and 36 h respectively. The naphthalene enrichment medium containing NH_4^+ as nitrogen source slowed bacterial growth and compromised cell viability over 72 h while, naphthalene-minimal salts medium when supplemented by NO_3^-showed uniform and efficient cell growth. The bacterial degradation of naphthalene in the minimal-salts medium containing KNO_3 resulted in transient accumulation of salicylate (0.11mM) and catechol (102 µM) after 12 and 24 h respectively whereas, in the medium containing NH_4Cl, the naphthalene biodegradation caused decrease in the pH of culture medium from 7 to 3.8 and accumulation of 0.63 mM salicylate and 24 µM catechol as dead end products. Further, the cell-free extracts of the bacterium grown on naphthalene in the medium containing KNO_3showed high activities of naphthalene-degrading enzyme as compared to that of the cell-free extracts of bacterium grown on naphthalene and NH_4Cl.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are of natural and anthropogenic origin, are the compounds of human health and environmental concern due to their toxic, mutagenic and carcinogenic properties (Menzei *et al.,* 1992). Due to their calcitrant and highly persistent nature they are often found to bioaccumulate in aquatic organisms (Lotufo, 1998). Even though, PAHs are highly hydrophobic and persistent chemicals, reports of their degradation by variety of microorganisms are available like, Cerneglia (1993), Sutherland *et al.,* (1995), Manohar and Karegoudar (1999), Daane *et al.,* (2001), Bamforth and Singleton (2005). Human exposure to PAHs may occur due to incomplete combustion of fossil fuel, accidental discharge into aquatic and terrestrial environments during transport use and disposal of petroleum products, coal gastification and liquification process and through industrial effluents (Laflame and Hites, 1978, Paterson and Kodukala, 1981, Jacob *et al.,* 1986). Naphthalene, being the simplest homologue in the polycyclic series, has received considerable attention because, the knowledge on bacterial degradation of naphthalene has been valuable for understanding the fate of naphthalene in the environment and also to understand the pathways used for the degradation of more complex PAHs (Sutherland *et al.,* 1995, Mahohar and Karegoudar, 1995).

Naphthalene a possible carcinogen (IARC, US-EPA, Gervais *et al.,* 2010) is one of the important industrial and house hold chemical. Two-third of the global production of naphthalene was consumed in the production of phthalic anhydride, which is in turn used as starting material for synthesis of various industrial chemicals and also as plasticizer, pesticides and cleaner formulations. It is used in the manufacture of synthetic resins, celluloid, lampblack, smokeless powder, naphthalene sulfonates, polyethylene naphthalene, wetting agents and dispersants in paints and coatings, and in the manufacture of solvents used in lubricants and in motor fuels (Mason 1995, Lacson, 2000 and O'neil *et al.,* 2001). It is also used in the production of naphthalene ball (moth ball), toilet deodorant, leather tanning agents and carbaryl (ATSDR toxic substance portal, 2005). Naphthalene exposure to humans may occur due to automobile exhaust, transport and disposal of petroleum products, industrial effluents, refuse burning coal gasification, house-hold products, use of pesticides and insecticides and industrial activity.

Naphthalene induces oxidative stress and damages DNA in macrophages (Bagchi *et al.,* (1988) and acute haemolytic anaemia (Santucci and Shah, 2000). Exposure to naphthalene may prove fatal especially in the patients with glucose-6-phosphate dehydrogenase deficiency (Chugh *et al.,* 1977, Bradberry and Vale, 2014) and in normal human subjects, naphthalene poisoning cause prolonged haemolytic anaemia and methaemoglobinaemia (Kundra *et al.,* 2015) and haemoglobinurea (Chauhan *et al.,* 2014). Gastrointestinal effects, renal effects, respiratory effects, neurological effects, hepatic and ocular effects are some of the systemic effects of naphthalene exposure (US Environmental Protection Agency, 1988, 1995; Ekambaram *et al.,* 2017). Strawinski and Stone (1943), Klausmeier and Strawinski (1957), Arhana and Brown (1981), Grund *et al.,* (1992), Fuenmayor *et al.,* (1998), Manohar and Karegoudar (1995), Tomas-Gollado *et al.,* (2014), (Ghosal *et al.,* 2016), Nimatuzahroh *et al.,* (2017) reported salicylate accumulation during naphthalene degradation by different microorganisms however, Annweiler *et al.,* (2000) and Lin *et al.,* (2010) reported naphthalene degradation by *Bacillus sp*. occurs by different pathway. In this communication, the influence of nitrogen source on the pattern of naphthalene degradation by a *Pseudomonas aeruginosa* and the enzymes of naphthalene degradation has been investigated and reported.

Materials and Methods

Chemicals

Naphthalene, salicylic acid and all the inorganic chemicals used for the preparation of medium were purchased from SD Fine chemicals, India. Organic solvents used in this study were obtained from Spectrochem India. 1,2-dihydroxynaphthalene and catechol were procured from Sigma-Aldrich and methanol from J. T. Baker.

Media, microorganism and growth condition

The mineral-salts medium used in the present investigation contained g L^{-1} of following constituents 0.38, K_2HPO_4; 0.2, $MgSO4.7H_2O$; 0.1, $FeSO_4$. $2H_2O$; 0.05 and 1, NH_4Cl or KNO_3as ammonical or nitrate nitrogen source. The pH of the medium was adjusted to 7. The medium was supplemented by 10 to 20 mM naphthalene (dissolved in minimal acetone) as specific and sole carbon source.

A naphthalene-degrading bacterium was isolated from a diesel-contaminated site (Arasikere, Hassan District, Karnataka, India) by enrichment culture technique in a mineral-salts medium gL^{-1} contained 6.3, K_2HPO_4; 1.8,KH_2PO_4; 1, NH_4NO_3; 0.1 $MgSO_4.7H_2O$; 0.1, $CaCl_2$; 0.1, $FeSO_4$; 0.1 $MnSO_4$, 0.006, $NaMoO_4$. The pH was adjusted to 7. A- 50 ml of the medium was dispensed in 250 ml conical flask and sterilized by autoclaving at 121^{o}C for 15 min. The medium was supplemented by naphthalene (5m M) as sole carbon source and aseptically inoculated by 5 ml of bacteria-soil suspension. The culture was incubated at 30±2^{o}C in a rotary shaker shaking at 160 rpm for two months. After the appearance of turbidity and colour in the culture medium, the naphthalene degrading ability of the bacterium was stabilized by performing several subcultures over a period of another four months. Further, the naphthalene degrading capacity of the bacterium was increased by gradual increase in the naphthalene levels over a period of four months.

The bacterial culture was isolated by serial dilution followed by standard spread-plate method on mineral-salts agar medium, which was supplemented by naphthalene in the form of vapours. The pure bacterial colonies were picked and aseptically inoculated on naphthalene-mineral-salts agar plates and the culture plates were incubated for 24 to 48 h in bacteriological incubator at 30±2^{o}C. Based on Gram staining, biochemical investigations and 16 S r-RNA partial gene sequencing, the isolated naphthalene-degrading bacterial culture was identified as *Pseudomonas aeruginosa.*

For studying naphthalene degradation, the bacterium was sub-cultured in 250 ml Erlenmeyer's flasks containing 50 ml of minimal-salts medium containing either NH_4Cl as ammonical (NH_4^+) or KNO_3(NO_3^-) as nitrate nitrogen source. The medium was supplemented by naphthalene (10 -20 mM) and the flasks were incubated on a rotary shaker shaking at 160 rpm at 30±2^{o}C. Growth of the bacterium at different time incubations was determined by standard plate-count method on naphthalene-minimal salts agar and also by reading the turbidity of the culture medium at 660 nm.

Analytical methods

Identification of bacterium

The genomic DNA was isolated from the naphthalene-utilizing bacterium and the fragment of 16S r-RNA gene was amplified by 27 F (AGAGGTTGATCMTGGCTCAG) and 1492 R (cggttaccttgt tac gactt) primers in a gradient PCR using template DNA (40 ng), primers (0.5 µM), dNTPs (500 µM) in Taq buffer containing $MgCl_2$ (1.5 mM) and 1 unit of Taq polymerase. The DNA was amplified for 35 cycles (DNA denatured at 94^{o}C for 30 sec. followed by annealing at 50^{o}C for 30 sec., amplification at 72^{o}C for 60 sec.) and the PCR product purified (1500 bp) purified. The amplified DNA was sequenced (sequencing primers, 27F AGAGGTTGATCMTGGCTC AG and 1492R CGGTTACCTTGT TAC GACTT) using BDT v3.1 cycle sequencing kit in genetic analyser (3730xl, Applied Biosystems) and consensus sequences of 16 *S* rDNA gene was generated using aligner 6. The 16*S* rDNA sequence was subjected to NCBI gene bank database using BLAST. Based on maximum sequence identity score, the first ten sequences were selected and aligned using Clustal W. The distance matrix generated and the phylogenetic tree was constructed (Kimura 1980, Kumar *et al.,* 2016, Felsenstein, 1985).

Naphthalene from the culture medium were extracted and analysed by UV-Vis spectrophotometer. The culture medium growing on naphthalene was harvested at different incubation periods and extracted twice with equal volumes of diethyl ether. The ether fraction was concentrated and separated into neutral, acid and phenol fraction (Manohar *et al.,* 1999). The ether from different fractions was evaporated under vacuum (Buchi evaporator) and the residue dissolved in methanol. The neutral extract was separated by TLC (silica gel-G), naphthalene recovered and dissolved in cyclohexane. The naphthalene in the cyclohexane fractions were estimated at 275 nm in a spectrophotometer. The salicylate accumulated in the culture medium at different incubation periods was estimated by $FeCl_3$ method (Manohar and Karegoudar, 1995). Briefly, a 0.1 ml of freshly prepared aqueous $FeCl_3$ solution (5% w/v) was added to 5 ml of clarified culture medium (centrifuged for 10 min at 10000 rpm) and the absorbance of purple colour formed was read at 550 nm in a spectrophotometer (Elico SL-159 uv-vis spectrophotometer). The catechol formed by the bacterial transformation of naphthalene was estimated by the method as described by Barnum (1977).

Metabolite characterization

The *Pseudomonas aeruginosa* cultures were grown under naphthalene enrichment in minimal-salts medium containing either NH_4Cl or KNO_3 as nitrogen source and the culture was harvested at different time intervals. The bacterium from the culture medium was separated by centrifugation (10000 rpm, 10 min and 4^{o}C), the pH of the used medium was adjusted to 2 and extracted twice by two volumes of diethyl ether. Ether extract was concentrated under vacuum to about 20 ml, dried over anhydrous sodium sulphate and filtered. Ether was evaporated and the residue obtained was dissolved in minimum volume of HPLC grade methanol. The metabolites in the extract was analyzed by HPLC (Prominence, Shimadzu) fitted with PDA detector in isocratic mode. A 10 µl extract was injected and the metabolites were separated through a chromatographic column (Shim-pack hypersilCLC-8 (M) ODS, 4.6 x 150mm, 5µ particle size) in isocratic mode using solvent system a) methanol (containing 0.05% H_3PO_4) and b) 0.05% aqueous H_3PO_4 (9a:1b) at solvent flow rate of 1ml/ min. The metabolites were also detected by TLC and isolated by preparative TLC using a) benzene-methanol – acetic acid (45:8:8), b) benzene-dioxane– acetic acid (90:25:5) and c) benzene-acetic acid- water (125:75:3).

Enzyme assays

Preparation of cell-free extracts

Freshly growing, mid-logarithmic phase cells of *Pseudomonas aeruginosa* from naphthalene-minimal salts medium containing either NH_4Cl or KNO_3as nitrogen source were harvested by centrifugation for 10 min (10000 rpm, 4^{o}C) and washed by ice-cold Tris-Cl buffer (50 mM, pH 7.4). The bacterial cells suspension (200mg/ ml buffer) were sonicated (2 sec pulse followed by 5 sec. rest) at 0-4^{o}C until the absorption decreases by 95% in a sonicator (Vibra cell, Sonics and materials, USA). The unbroken cells and cell debris removed by centrifugation for 15 min. at 15000 rpm and 4^{o}C. The supernatant obtained was kept in ice and used as enzyme source. The protein content of the enzyme was estimated by Lowry's method (Lowry *et al.,* 1951).

Naphthalene-1,2-dioxygenase assay was performed by monitoring the decrease in the absorption at 340 nm due to the oxidation of NADH (Dua and Meera, 1981). The assay mixture in a total volume of 3.11 ml contained enzyme (0.1 mg protein), NADH (1.5 µM) in phosphate buffer (50 mM, pH 6.5). The E_{340} nm (6.22 x 10^3MolL-1). The mixture was incubated for 3 min to allow background oxidation of NADH, which otherwise would interfere with assay results. The reaction was initiated by the addition of naphthalene (1 µM, dissolved in 0.1 ml 2-methoxyethanol). The activity of 1,2-dihydroxynaphthalene dioxygenase was followed by change in the absorption at 331 nm as suggested by (Kuhm *et al.,* 1991). The assay mixture (1 ml) contained 50 µl of acetic acid- sodium hydroxide buffer (50 µM, pH 5.5) and suitably diluted cell-free extract (100µg of protein). The reaction was initiated by forcing 1,2-dihydroxynaphthalene (10 µl, dissolved in tetrahydrofuran) through a 25µl syringe. The molar reaction coefficient (ε) of 2.60 mM^{-1} cm^{-1} used to calculate enzyme activity. Salicylaldehyde dehydrogenase (Shamsuzzaman and Barnsley, 1974) was determined by the increase in the absorption at 340 nm. Enzyme assay mixture in a final volume of 3 ml contained buffer (2.75 ml, tetrasodiumpyrophosphate – HCl, 20 mM, pH 8.5), cell-free extract (0.5 mg protein), NAD (0.1 ml,150 mM). The reaction was initiated by the addition of salicylaldehyde (3 mM aqueous). Salicylate hydroxylase (Shamsuzzaman and Barnsley, 1974) assay was estimated at 340 nm by measuring the oxidation of NADH in a reaction mix containing EDTA (1 mM), NADH (147 µM) and sodium salicylate (133 µM) in phosphate buffer (20 mM, pH 7).Catechol-1,2-dioxygenase (Hegeman, 1966) activity was measured by following the increase in the absorption at 260 nm due to the formation of *cis, cis*-muconic acid in the assay mixture (3 ml) containing catechol (1 µM), EDTA (10 µM) and suitably diluted enzyme (1 mg protein) and phosphatebuffer (200 µM, pH 7). Catechol-2,3-dioxygenase (Fiest and Hegeman, 1969), assay mix3 ml comprising of 0.5 ml of catechol (10 mM) and 0.1 ml cell-free extract (0.1 mg protein) in phosphate buffer (100 µM, pH 7.5). The enzyme reaction was monitored by the increase in absorbance at 375 nm due to the formation of α-hydroxymuconicsemialdehyde. The gentisate-1,2-dioxygenase enzyme assay was carried out by the method as described by Crowford *et al.,* (1975).Protocatechuate-3,4-dioxygenase and protocatechuate-4,5-dioxygenase enzymes were assayed by the method McDonald *et al.,* (1954). One unit of enzyme activity was defined as µM substrate converted min^{-1} mg^{-1}protein.

Results and Discussion

A bacterium capable of utilizing naphthalene as sole carbon source was isolated from the soil samples obtained from diesel-contaminated site by enrichment culture technique. The bacterium was cultivated in the mineral-salts medium supplemented by naphthalene as sole carbon source. Based on Gram staining, physicochemical and biochemical studies, 16*S* r-DNA partial gene sequence homology and molecular phylogenetic analysis, the bacterium was identified as *Pseudomonas aeruginosa* (Fig. 1) and designated as *Pseudomonas aeruginosa* TU

The bacterium was shifted to grow in a minimal-salts medium containing NH_4Cl or KNO_3 as nitrogen source and supplemented by naphthalene alone as specific and only carbon source. The bacterium was acclimatized to grow on increasing levels of naphthalene over a period of one year by performing several subcultures. The growth behaviour of *Pseudomonas aeruginosa* on naphthalene in the minimal-medium containing NH_4Cl or KNO_3 as nitrogen source was studied and the results are presented in Figure 2.

Growth study

It is evident from Figure 2 that the growth of the bacterium increased with the increase in the incubation time. In the minimal-salts medium containing ammonical 'NH_4' nitrogen source and enriched with naphthalene (10 mM) as the specific and sole carbon source, the initial bacterial-cell population of 9 x 10^5 colony-forming units (CFU) ml^{-1} reached to 3 x 10^7 CFU ml^{-1},5 x 10^7 CFU $ml^{-1}$and 8 x 10^7 CFU ml^{-1} at the end of 24, 36 and 48 h of incubation. Further, even though there was an appearance of turbidity, substrate utilization and metabolism, the viable cell population decreased to 4^4 CFU ml^{-1} at 60 h and ceases to exist after 96 h of incubation. The utilization of naphthalene by the bacterium has resulted in the decrease in the pH of the culture medium amended with NH_4^+as nitrogen source, the initial pH 7 of the culture medium decrease to pH 5.4 and 4.3 at the end of 24 and 36 h and reached to pH 3.8 after 48 h of incubation (Fig. 2A). The growth of the bacterium in the minimal-salts medium that received nitrate (NO_3^-) as nitrogen source and supplemented by naphthalene (10 and 20 mM) is depicted in Figure 2B. The initial cell population of 1.6 x 10^6 CFU ml^{-1}in the minimal-salts medium containingKNO_3 as nitrate nitrogen source and supplemented by naphthalene (10 mM) as sole carbon source increased to 7 x 10^9 CFU $ml^{-1}$and 8 x 10^9 CFU ml^{-1} after 24 and 36 h of incubation. It was also observed that, with the increase in the initial naphthalene to 20 mM, the initial cell population of 10^6 CFU ml^{-1} in the culture medium increased to 9 x 10^9 to 1 x 10^{10} CFU ml^{-1} after 36 and 48 h of incubation. Naphthalene utilization by the bacterium showed a slight increase in the pH of the culture medium from pH 7 to 7.4, 7.8 and 8.2 at the end of 24, 36 and 48 h of incubation (Fig. 2B). The culture medium which received NH_4Cl turned fluorescent yellow where as the one that received KNO_3 as nitrogen source turned dark brown. Maximum growth of the bacterium observed between 36 to 40 h of incubation of bacterium in the minimal-salts medium containing NH_4NO_3 as nitrogen source and naphthalene as sole carbon source (Fig. 2C). Naphthalene biodegradation in NH_4NO_3 containing medium followed the NH_4Cl type pattern of naphthalene degradation, wherein, the naphthalene utilization by the bacteria lead to the decrease in the pH of the culture medium from an initial pH 7 to 5.2 and the maximum viable cell population from an initial 10^6CFU ml^{-1} increased to 10^8 CFU ml^{-1} after 36 to 48 h. Further the cell viability decreased.

Naphthalene utilization

Studies on the naphthalene utilization by the bacterium in the minimal-salts medium containing ammonical or nitrate nitrogen were conducted and the results are presented in Figure 3. The naphthalene-degrading *Pseudomonas aeruginosa* when incubated with naphthalene in the minimal-medium containing NH_4Cl as nitrogen source, could degraded a maximum of 66% of the initial 10 mM naphthalene load after 120 h. The results indicate that the 48, 54 and 58% of an initial 10 mM naphthalene in the culture medium was degraded by the bacterium within 24, 36 and 48 h of incubation and there after the degradation of naphthalene slowed down drastically. Contrary to this, the same bacterium showed the complete degradation of initial 10 and 20 mM naphthalene concentrations at the end of 24 and 36 h respectively in the culture medium that received KNO_3 as nitrogen source (Fig. 3).

Estimation of salicylate and catechol

Naphthalene utilization by the bacterium has resulted in the accumulation of salicylate (0.58 mM) and catechol (24 µM) after 48 and 36 h respectively as the end products of naphthalene degradation in the culture medium, which received NH_4Cl as nitrogen source whereas, the culture medium that received KNO_3 as nitrogen source, showed 102, 74 and 60µM of catechol at the end of 24, 36 and 48 h respectively and a transient accumulation of about 1.1 mM salicylate at 6 to 8 h which disappeared after 14 h (Fig. 4).

Metabolite characterization

The results on the metabolite characterization by TLC are presented in Table 1. The ether extracts of the spent medium obtained from naphthalene grown culture (36 h), which received NH_4Cl as nitrogen source showed the presence of salicylic acid and catechol whereas, only catechol as an intermediary metabolite was found in the extracts of the culture medium that contained KNO_3 as nitrogen source (Table 1).

The results on the metabolite characterization by HPLC are presented in Figure 5.

The HPLC elution profile of the metabolites showed the presence of 1,2-dihydroxynaphthalene, salicylic acid and catechol in the extracts of *Pseudomonas aeruginosa* culture medium amended with NH_4Cl as nitrogen source and naphthalene as carbon source whereas, catechol and detectable amount of salicylate was observed in the culture filtrate of the cells grown for 12 h in the naphthalene minimal-salts medium containing KNO_3 as nitrogen source, however, at the end of 24 h both salicylate and naphthalene disappeared and new metabolites at retention time (t_R) 1.7, 2.6, 2.9 and 4.34 appeared.

Enzyme investigations

The results on the enzyme investigations in the crude cell-free extracts of *Pseudomonas aeruginosa* TU grown in naphthalene-minimal salts medium containing ammonical or nitrate nitrogen source are shown in Table 2.

It is evident from the results presented in Table 2, that the cell-free extracts of the naphthalene grown cells showed the activities of all the above enzymes tested except for that of gentisate dioxygenase and protocatechuate dioxygenases. The results suggest that the cell-free extracts of the naphthalene-grown cells of *P. aeruginosa* TU in the presence of KNO_3as nitrogen source, exhibited higher activities of all the naphthalene-degrading enzymes as it is compared to that of the enzyme activities in the cell-free extract grown in NH_4Cl containing medium.

Table.1 R_f values and λ_{max} of metabolites isolated from cultures filtrates of naphthalene grown cells

Compound	A		B		C		λ_{max}	
	a	b	a	b	a	b	a	b
Salicylic acid	0.82	0.81^N	0.71	0.71^N	0.72	0.72^N	234, 296	234, 296
Catechol	0.7	0.7^N	0.63	0.63^N	0.69	0.69^N	276	276^N
Salicylic acid	0.81	ND^K	0.71	ND^K	0.72	ND^K	234, 296	ND^K
Catechol	0.71	0.7^K	0.63	0.64^K	0.69	0.69^K	276	276^K

Table.2 Specific activities of naphthalene metabolizing enzymes in the cell-free extracts of *Pseudomonas aeruginosa* TU grown in minimal-salts medium containing NH_4Cl or KNO_3 as nitrogen source and supplemented by naphthalene as sole carbon source

Growth substrate	Specific activities of enzymes in crude cell-free extracts						
	NDO	1,2-DHNDO	SALDDH	SALH	C1,2O	C2,3O	GDO
Naphthalene + NH_4Cl	0.38	3.2	1.2	0.18	0.03	0.12	ND
Naphthalene +KNO_3	0.82	4.9	2.2	0.6	0.05	0.42	ND
Glucose	ND	ND	0.1	ND	0.04	ND	ND

NDO- naphthalene dioxygenase, 1,2-DHNDO- 1,2-Dihydroxynaphthalene dioxygenase, SALDDH-salicylaldehyde dehydrogenase, SALH-salicylate hydroxylase, C1,2O-catechol-1,2-dioxygenase, C2,3O-catechol-2,3-dioxygenase, GDO-gentisate dioxygenase. ND-not detected

Figure.1 Molecular phylogenitic analysis of 16 *S* r-RNA partial gene-sequence

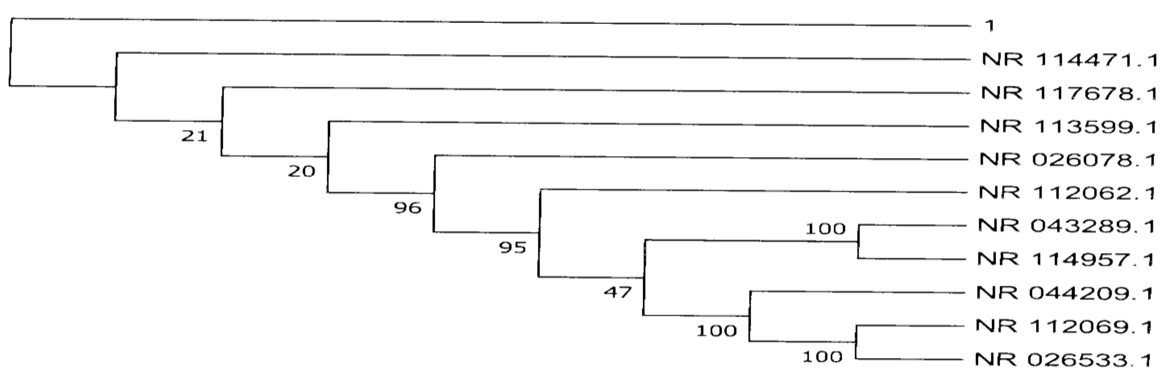


Figure.2 Growth of *Pseudomonas aeruginosa* TU on naphthalene in the minimal-salts medium containing NH_4Cl (A) and KNO_3 (B) and the change in pH of the culture medium.

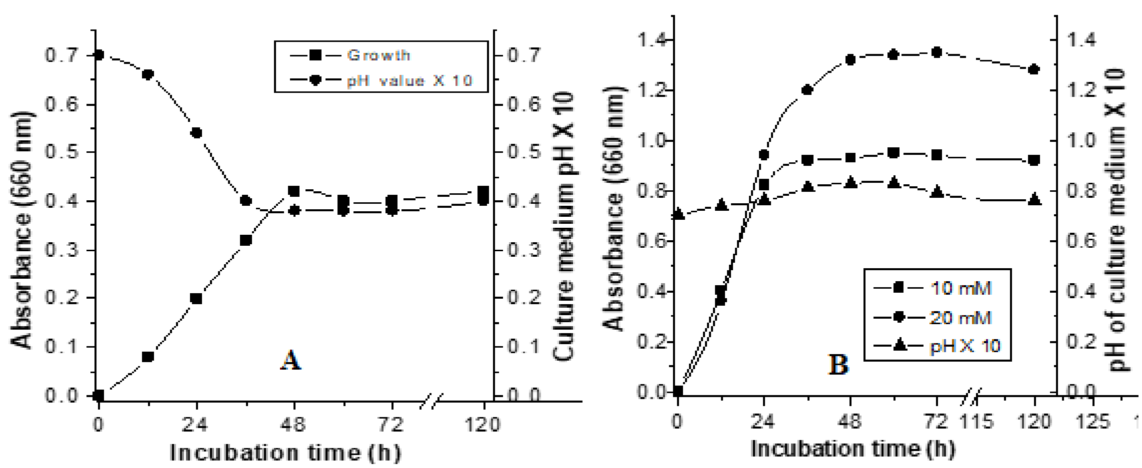


Figure.3 Time dependent utilization of naphthalene by the bacterium in the minimal-salts medium containing NH_4Cl and KNO_3 as nitrogen source

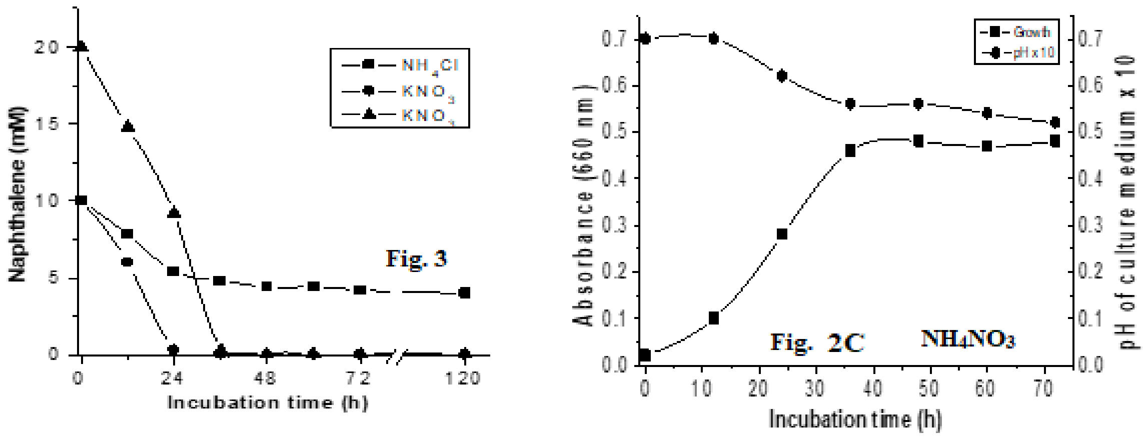


Figure.4 Biotransformation of salicylic acid and catechol from naphthalene by a *Pseudomonas aeruginosa* TU in the culture medium containing NH_4Cl and KNO_3 as nitrogen source

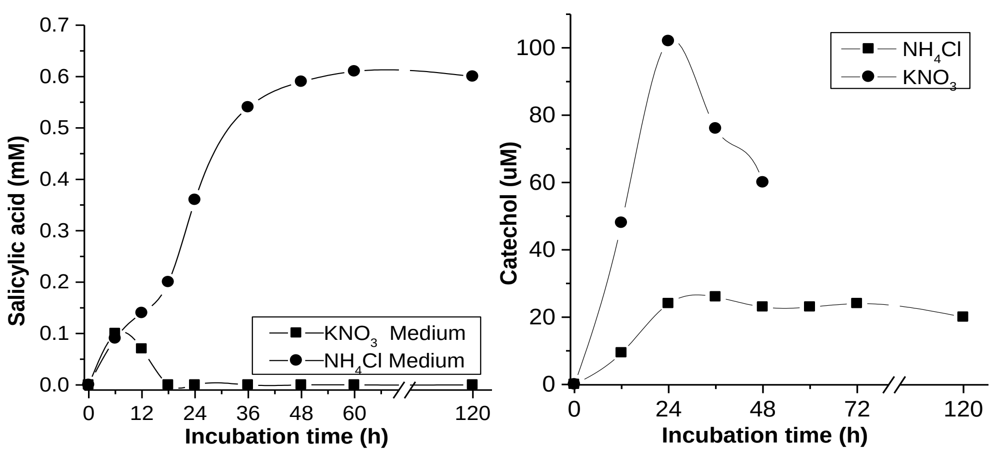
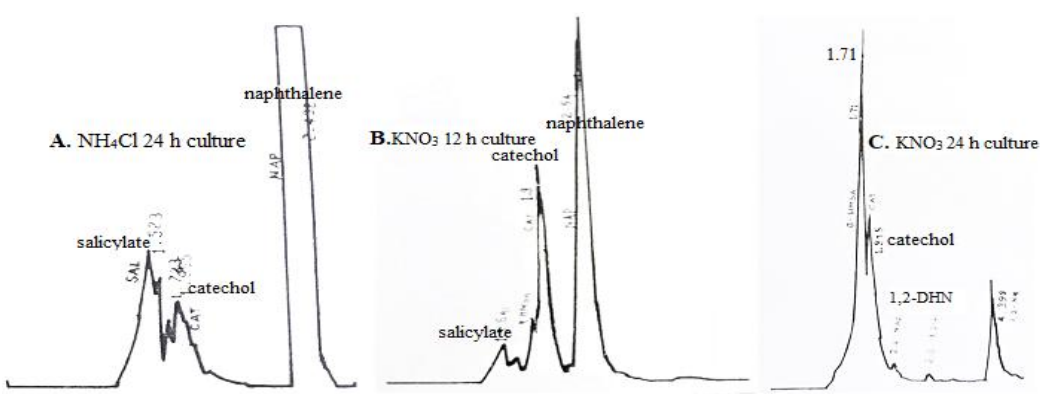


Figure.5 HPLC elution profile of the metabolites extracted from the cultures of naphthalene grown cells of *Pseudomonas aeruginosa* TU in the medium containing NH_4Cl –A- and KNO_3- B and C



It is evident from the results presented in Figure 2 and 3 that the *Pseudomonas aeruginosa* TU showed better growth and naphthalene degradation in the medium containing KNO_3 as nitrogen source as it is compared to that of the medium amended with NH_4Cl as nitrogen source. In the minimal-medium containing NH_4Cl as nitrogen source, bacterium could degrade only 66% of an initial 10 mM naphthalene further, naphthalene degradation by bacterium was accompanied by the concomitant accumulation of salicylate (0.63 mM) in the culture medium, which may have decrease in the pH of the culture medium from 7 to 3.8. The accumulation of salicylate, catechol (Fig. 3 and 5) and other phenolic products in the culture medium during naphthalene degradation may have slowed bacterial growth, which may have resulted in loss in cell viability (Aranhaand Brown, 1981; Manohar and Karegoudar, 1995). Contrary to this, in the present investigation, the same bacterium degraded 10 and 20 mM initial naphthalene within 24 and 36 h of incubation in the minimal-medium containing KNO_3 as nitrogen source, interestingly, there was an increase in the pH of the culture medium from pH 7 to 8.2. Such observations were made by Aranha and Brown (1981) and reported slight change in pH of the culture medium from 7 to 7.3 after 48 h of growth. In the present investigation, we have made an observation that the minimal-salts medium amended with KNO_3 as nitrogen source supported better and uniform growth of the bacterium as compared to the growth in NH_4Cl containing medium. The bacterial growth in naphthalene minimal-medium containing ammonical nitrogen source slowed, cells occluded and lost viability within 4 days. Majority of the studies on naphthalene degradation by various bacterial species have been conducted using 'NH_4^+' as ammonical nitrogen source (Strawinski and Stone, 1943; Klausmeier and Strawinski, 1957; Shamsuzzaman and Barnsley, 1974; Dua and Meera, 1981; Grund *et al.,* 1992; Fuenmayor *et al.,* 1998; Manohar and Karegoudar, 1995; Tomas-Gollado *et al.,* 2014; Ghosal *et al.,* 2016; Nimatuzahroh *et al.,* 2017) and reported the production of salicylate in the medium but, none of the investigations reported decrease in pH of the culture medium. Aranha and Brown (1981) reported salicylate and the decrease in pH of the culture medium containing NH_4Cl as nitrogen source and suggested pH remains stable in the culture medium containing KNO_3. We have observed that the degradation of naphthalene by the *Pseudomonas aeruginosa* TU occurs via salicylate and catechol in both NH_4Cl and KNO_3 containing medium but, interestingly just detectable levels of salicylate accumulates transiently at the early growth phase (6 to 14 h) in KNO_3 containing medium and after 36 to 48 h the pH increases to 8.3 whereas, high levels of salicylate builds up in NH_4Cl containing culture medium and subsequently the pH decreases to 3.8 at the end of 48 h. The higher activities of the enzymes upstream to salicylate hydroxylase and low activities of salicylate hydroxylase and catechol oxygenases in the cell-free extracts of bacterium grown in the medium containing NH_4Cl supports the possible accumulation of salicylate in the culture medium as compared to the enzyme activities observed in the cell-free extracts of cells grown on naphthalene in mineral salts medium containing KNO_3. Since naphthalene, a possible carcinogen, is found naturally in the environment and also used as starting material in various industrial processes (Mason 1995, Lacson, 2000 and O'neil *et al.,* 2001), human exposure may occur by both natural and anthropogenic activities (Paterson and Kodukala, 1981; Jacob *et al.,* 1986) thus, its effective bioremediation is the only strategy to clean up the environment, which needs understanding of the optimal conditions for bioremediation process is prerequisite. The outcome of the present investigations may aid in developing bioremediation technology for toxic PAHs and their derivatives from industrial effluents and contaminated sites.

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