Original Research Article

Rapid biodegradation of phenanthrene by a novel strain *Pseudomonas denitrificans* Fdl

ABSTRACT

Aims: The aim of the study was to find, isolate, identify and characterize a phenanthrene degrader strain, and examine its ability to degrade phenanthrene.
Place and Duration of Study: Faculty of Natural Sciences, Novosibirsk State University, between September 2013 and June 2014.
Methodology: Soil was sampled from the oil contaminated area in the Purov district of the Yamal-Nenetsk Autonomous region in Russia (63.00729 NL, 76.89418 EL), and the enriched culture of oil-degraders was spread on plates with polyaromatic hydrocarbons (PAH) to isolate PAH degraders. The isolated strain was characterized morphologically, biochemically, physiologically and genetically (16S rRNA gene nucleotide sequence).
Results: By using crude oil-contaminated soil to obtain a culture enriched with oil-degraders followed by plate cultivation on phenanthrene-amended agar a bacterial strain denoted Fdl was isolated. Its cells were Gram-negative motile rods 0.4-0.5 µm wide and 1.5-2.5 µm long with phenotypic traits common for the *Pseudomonas* genus. The 16S rRNA gene fragment nucleotide sequence showed 99% similarity with *Pseudomonas denitrificans*. The isolated *Pseudomonas denitrificans Fdl* strain was deposited into the GenBank under access number KM 436103. Incubation of the Fdl strain cells in the medium with phenanthrene as a sole carbon source resulted in phenanthrene concentration decrease, accumulation of corresponding metabolites and bacterial proliferation, which confirmed the strain's ability to utilize phenanthrene. Over 40 hours of incubation the phenanthrene concentration decreased from 100 to 1 ppm, proving the novel strain to be an effective phenanthrene degrader. Addition of Tween-20 non-ionic surfactant into the incubation medium accelerated phenanthrene degradation and cell proliferation as compared to phenanthrene degradation without a surfactant.
Conclusion: The isolated strain *Pseudomonas denitrificans* Fdl is capable of efficient phenanthrene degradation, especially in the presence of detergent, and hence can be a good candidate for biological preparations to be tested for bioremediation and sewage sludge treatment.

Keywords: oil-contaminated soil, phenanthrene, phenanthrene degrading bacteria, Pseudomonas denitrificans, novel strain

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are very common organic pollutants that are toxic and often carcinogenic [1]. Sixteen PAH, including phenanthrene, are included into the priority list of pollutants by the US Environmental Protection Agency [2]. The search for effective and cheap ways to clean PAH polluted territories has been increasingly actual issue. Bioremediation, i.e. using microbes, capable to destruct effectively various organic pollutants and preferably isolated from the contaminated site [3], is one of the most effective methods to remove PAH from environment.
Biodegradation of PAH has been quite intensively studied over the last years, and various bacterial strains, capable to degrade PAH in a broad range of environmental conditions, e.g. acidic and alkaline pH, low and high salinity, low and high temperatures, were isolated and characterized in detail [4-6]. Biodegradation of PAH is limited by their extremely low water solubility and hence low availability for bacterial degradation. To increase PAH bioavailability and intensify their biodegradation the addition of surfactants has been investigated [7]. However, surfactants are also known to negatively affect PAH biodegradation, as they can be more preferable carbon substrates for microbial utilization [8], exert toxic effect on microbial cells [9], inhibit biodegradation by blocking substrate access into the enzyme active

center by forming micelle [10], etc. Thus it is important to seek microorganisms, capable to degrade PAH effectively in the presence of surfactants. Phenanthrene, a polycyclic aromatic hydrocarbon with three benzene rings, is a dangerous organic pollutant, and due to its wide environmental occurrence and low water solubility (1.29 g/l) it is often used as a model substrate in microbial degradation studies, including isolation of novel PAH-degrading strains and estimating their potential for PAH biodegradation. The metabolic pathways of low molecular weight PAH degradation, and in particular, phenanthrene, were studied by many researchers, the established common intermediate being 1-hydroxy-2-naphthoic acid, which is further metabolized via one of the two pathways [11].

The aim of our study was to find, isolate, identify and characterize a novel phenanthrene degrader strain, and examine its ability to degrade phenanthrene in a liquid medium in the presence of non-ionic detergent.

2. MATERIAL AND METHODS

2.1 Chemicals and media composition

Phenanthrene and other chemicals used in the study were of analytical grade. The liquid minimal basal salts medium (MBS) contained the following (g/L): $(NH_4)_2SO_4$, 1.0; g KH_2PO_4, 5.0; g $MgSO_4$· 7H2O, 0.1; $Fe(NH_4)_2(SO_4)_2$, 0.005; and 1 ml of micronutrients' solution, containing 23 mg $MnCl_2$·$2H_2O$, 31 mg H_3BO_3, 36 mg $CoCl_2$·$6H_2O$, 10 mg $CuCl_2$·$2H_2O$, 20 mg $NiCl_2$·$6H_2O$, 50 mg $ZnCl_2$, 30 mg Na_2MoO_4·$2H_2O$. The medium pH was adjusted to 7.0. The solid MBS was prepared by adding 30 g of bacteriological agar into 1 liter of liquid MBS. The standard agarised Luria broth (LB) medium was used for colony-forming units (CFU) counting.

2.2 Isolation of a phenanthrene degrader strain and enrichment culture conditions

Soil was sampled from the long-term oil contaminated area the Purov district of the Yamal-Nenetsk Autonomous region in Russia (63.00729 NL, 76.89418 EL). The enrichment culture approach was employed to isolate the oil-degrading bacterial strains. Initially the enriched culture of oil-degraders was obtained. Then the culture was spread on plates with PAH to isolate PAH degraders. To obtain a culture enriched with oil-degraders 1 g of soil was put into the 250 ml Erlenmeyer flask with 100 ml of sterile liquid MBS medium. The sterilized crude oil at the rate of 2.5 % (v/v) was added into the medium as a sole carbon source. Flask cultivations were carried out in a rotary shaker at 150 rpm for 1 week at 35 °C. After that a 1 ml aliquot of the enriched medium was transferred into the new flask with similar medium for the next cultivation step. The process was repeated 3 times. After that a 100 µl aliquot of the enriched medium was spread on MBS agar plates. The phenanthrene solution in diethyl ester (0.1 % w/v) was pulverized over plates in a sterile box, the solvent being allowed to evaporate under regular laminar flux. The plates were cultivated for 1 week at 35 °C. The presence of a ring around a colony was interpreted as positive phenanthrene degradation. The colonies were sampled with a sterile loop and transferred onto LB agar plates to obtain a pure culture.

2.3 Morphological and biochemical characterization of a strain

Cell morphology was studied by phase-contrasting microscopy using Axiscop 40 microscope (Carl Zeiss, Germany). Biochemical and physiological traits, as well as pathogenicity, were determined by standard techniques [12, 13]. The antibiotic testing was studied using the antibiotics-impregnated paper discs produced by the Research Centre of Pharmacotherapy (Saint-Petersburg, Russia), the amount of antibiotics per disc being as following (µg): rifampicin, 5; penicillin, oxacillin, ampicillin and gentamicin, 10 each; oleandomycin, erythromycin and lincomycin, 15 each; streptomycin, neomycin, kanamycin, monomycin, tetracycline, levomycetin and ristomycin, 30 each; karbenicyllin, 100; and polymyxin,100 unites.

2.4 Molecular identification of a bacterial strain

The isolated strain was identified by analyzing its 16S rRNA gene nucleotide sequence. A 1350 bp gene fragment was amplified using the lyzate of the strain colonies. The oligonucleotides 16S-8-f-b 5'-AGRGTTTGATCCTGGCTCA-3' and 16S-1350-r-B 5'–ACGGGCGGTGTGTACAAG-3' were used as primers to identify bacteria by their 16S rRNA gene [14].

Sequencing of the obtained PCR-amplicons was performed with the same oligonucleotides and BigDye v.3.1 reagent under standard conditions. The reaction products were analysed electrophoretically using ABI Sequencing Analyzer 3500. The obtained nucleotide sequences were analysed using ABI Sequence Scanner and Sequencher v.4.1.4 software and compared to the 16S rRNA gene sequences deposited in GenBank using BLASTN software.

2.5 Phenanthrene degradation

Phenanthrene biodegradation was studied in 100 ml of liquid MBS medium in 250 ml Erlenmeyer flasks with phenanthrene added at the rate of 100 mg/L. When studying the effect of a detergent on phenanthrene degradation, Tween-20 was added into the flasks to the 0.5% concentration (v/v). The medium was inoculated with 1 ml of suspension, containing 107 CFU/ml, obtained from colonies grown on the phenanthrene-containing agar and washed 3 times by sterile liquid MBS. Then the flasks were incubated at 35 ℃ in a rotary shaker at 150 rpm. When no detergent was added, the aliquots were sampled after 8, 16, 24, 32, 40, 48 hours of incubation. When the detergent was added, the aliquots were sampled after 2, 4, 6, 8, 16, 24, 48 hours of incubation. The pH of the aliquots was adjusted to 2.3 using concentrated hydrochloric acid (HCl), and then extracted 3 times by 30 ml of dichloromethane. The extracts were dried over anhydrous sodium sulphate and then evaporated in vacuum. The dried residue was solved in acetonitrile for further analysis.

2.6 Measurement of phenanthrene and 1-hydroxy-2-naphthoic acid concentration.

Concentrations of phenanthrene and 1-hydroxy-2-naphthoic acid in the obtained acetonitrile solutions were analysed by reversed-phase HPLC. HPLC was performed on a Milichrome A-02 chromatograph (Econova, Ltd., Russia), equipped with a ProntoSIL 120-5C18 AQ column (2 x 75 mm, B&W Separation Technologies Pvt. Ltd.) under the following conditions: solvent A, 0,2 M $LiClO_4$ - 0.05 M $HClO_4$; solvent B, acetonitrile; linear gradient of B in A from 0 to 100% in 40 min, flow rate of 100 µl/min, column temperature of 400 C, detection at 210, 220, 230, 240, 250, 260, 280 and 300 nm; the sample volume 3 µl. The presence of 1-hydroxy-2-naphthoic acid (HNA) was determined by comparing the retention time and spectrum of the analysed sample with the respective characteristics of the reference standard. The HNA and phenanthrene concentrations were determined by measuring the areas of the respective peaks, using the calibration curve with reference compounds.

2.7 Cell growth during biodegradation

Bacterial cell proliferation in course of biodegradation was estimated by counting the strain colonies grown over 48 hours on the LB agar. When no detergent was added, the aliquots were sampled after 8, 16, 24, 32, 40, 48 hours of cultivation. When the detergent was added, the aliquots were sampled after 2, 4, 6, 8, 16, 24, 48 hours of cultivation. The aliquots thus sampled were titrated in 0.9 % solution of NaCl in water and transferred onto LB agar plates to count CFU after 48 hours incubation at 35 ℃.

3. RESULTS AND DISCUSSION

3.1 Isolation and characterization of a novel strain of the phenanthrene-degrading bacterium

By using crude oil-contaminated soil to obtain a culture enriched with oil-degraders followed by plate cultivation on phenanthrene-amended agar we isolated a bacterial strain denoted Fdl. To identify it we

studied its morphological, physiological and biochemical properties and performed 16S rRNA genes sequencing.

3.2 Morphological and physiological characteristics of the isolated strain

The cells of the isolated strain were found to be Gram-negative motile rods, mostly solitary, but sometimes paired cells 0.4-0.5 µm wide and 1.5-2.5 µm long cells without endospores. On the LB agar the strain produced transparent yellowish glance round-shaped colonies with an even or slightly curving edge; on the solid medium containing fish peptone the strain produces yellow-orange pigment. The strain was found to be aerobic with the optimum growth temperature of 28-30 ºC, the moderate and weak growth rate being observed at 37 ºC and 10 ºC, respectively.

3.3 Biochemical characteristics of the strain

Standard biochemical characterization of the strain showed its negative reaction to indol, hydrogen sulphide, casein hydrolysis, as well as in Voges-Proskauer test reaction and in incubation with the methyl red dye. The strain was found to be able to reduce nitrates into nitrites and to utilize citrate as a carbon source. Under laboratory conditions the strain was not shown to secrete such enzymes as amylase, lipase, gelatinase, licitinase, urease, DNAse, RNAse, while positive reaction was displayed in oxidase and catalase tests (Tab.1). The isolated strain was found to be represented by a bacterium with a respiratory, rather than fermentation, metabolism as it does not hydrolyze sucrose, glucose, lactose, arabinose, ramnose, displaying weak acid-formation on media containing maltose and xylose (Tab.2).

Table 1. Biochemical characteristics of the isolated strain Fdl B-1299

Trait	Reaction	Trait	Reaction
Amilase	**– ***	***FP reaction***	**–**
Lecitinase	**–**	***MR reaction***	**–**
Lipase	**–**	***Oxidase***	**+**
Casein hydrolysis	**–**	***Catalase***	**+**
Gelatinase	**–**	***RNAse***	**–**
Indol produiction	**–**	***DNAse***	**–**
H_2S production	**–**	***Lysinedecarboxylase***	**–**
Urease	**–**	***Ornithinedecarboxylase***	**–**
Citrate utilization	**+**	***Argininedecarboxylase***	**±**
Nitrate reduction	**+**	***Phenylalaninnedesaminase***	**–**

** Symbols used: "+" means a positive reaction, and "-" means a negative one.*

Table 2. Carbohydrates utilization by the isolated strain on the OF medium

Carbohydrate	Gas emission	Acid production
Sucrose	-	-
Mannitol	-	-
Glucose	-	+
Lactose	-	-

Arabinose	-	-
Ramnose	-	-
Maltose	-	+
Xylose	-	±

Notably, the strain showed negative results as related to all four pathogenicity tests, i.e. hemolythic, plasmocoagulating, fibrinolythic and gelatinolythic activities. The absence of pathogenic properties in the isolated strain intended for bioremediation is very important as it shows its ecological safety. The strain was also found to be resistant to ampicillin, benzylpeicillin, carbenicillin, oxaxillin, rifampicin, levomycetin and lincomycin, (Tab.3).

Table 3. Antibiotic activity (zones of the reduced growth of the isolated strain around the antibiotic containing discs, mm)

Antibiotic	Reduced growth, mm	Antibiotic	Reduced growth, mm
polymyxin	**14**	***tetracycline***	**10**
amikacin	**25**	***ciprofloxacin***	**26**
vankomicyn	**15**	***azlocilline***	**21**
gentamycin	**15**	***streptomycin***	**18**
kanamycin	**20**	***imipenem***	**14**
ampicillin, benzylpenicillin, carbenicillin, oxaxillin, kanamycin, rifampicin, levomycetin, lincomycin	**0**	***neomycin***	**11**
		cefotaxime	**12**

The isolated strain showed phenotypic traits common for the representatives of the *Pseudomonas* genus. Further identification of the isolated Fd1 strain revealed its close relationship with *P. denitrificans* species (Tab.4). Comparison of the 16S rRNA gene sequence of the novel strain with the respective sequences stored in the international data base GenBank showed that the novel sequence had 99% similarity with the sequences of the following bacteria: *Pseudomonas nitroreducens* (NR 113601), *P. denitrificans* (NR 102805) and *P. multiresinivorans* (NR 119225). These information, together with the results of the phenotypic and genotypic analysis of the isolated strain Fdl led us to identify it as representing *Pseudomonas denitrificans* species. The 16S rRNA gene fragment nucleotide sequence of the *Pseudomonas denitrificans Fdl* strain was deposited into the GenBank under access number KM 436103.

Table 4. Morphological, physiological and biochemical characteristics of the bacterial genus *Pseudomonas*, its typical representative strain *P. denitrificans* and the novel strain *Fd1* B-1299

Trait	Genus *Pseudomonas*	*P. denitrificans* (reference strain KC1439 - ATCC 138667; [15])	Strain Fd1 B-1299
Cell shape	rods	rods	rods
Cell dimensions, width x length, μm	(0.5-1.0) ×1.5-4.0	(0.5-1) × (1.5-4.0)	(0.4-0.5) × (1.5-2.5)
Cell motility	+ *	+	+
Gram reaction	-	-	-

Respiratory, not fermenting metabolism	+	+	+
Strictly aerobic	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Growth at +4 ℃ and lower	+		+
Growth at 25℃	+	+	+
35℃		+	+
up to 42 ℃		[±]	±
pH 5.4	-	-	+
eskulin	-	-	n.d.
Lisindecarboxylase	-	-	-
Ornitinedecarboxylase	-	-	-
Argininedecarboxylase	[±]	[±]	+
Glucose oxidation	[±]	[±]	+
Xylose oxidation	[±]	[±]	±
Sucrose oxidation	-	-	-
Maltose oxidation	[±]	+	+
Citrate utilization	+	+	+
Nitrate reduction into nitrite	[±]	[±]	+
Indol production	-	-	-
Hydrogen sulphide production	-	-	-
Gelatine	±	-	-
Hemolysis	[±]	[±]	- (rabbit blood)
G+C	58-70 mol %		n.d.
NaCl, 0 %	[±]	+	+
6 %	[±]	[±]	-
Water solublepigment	+	[±]	+

** Symbols used: "+" denotes a positive reaction, "-" denotes a negative reaction; [±]means variable trait, while "±" means weak trait and "n.d." means no datum.*

3.4 Phenanthrene degradation by the isolated strain

Incubation of the Fdl strain cells in the medium with phenanthrene as a sole carbon source was found to result in phenanthrene concentration decrease, accumulation of corresponding metabolites in the medium and bacterial cell proliferation, all together confirming the ability of the strain to utilize phenanthrene as a carbon source. The kinetic curve of phenanthrene biodegradation was characterized by three portions, coinciding with the main phases of cell proliferation and growth (Fig.1). After 4 hours of incubation the lag-phase ended, and the exponential growth started and continued up to 24 hours of incubation with the maximal degradation rate being observed between 8 and 16 hours. After 32 hours of incubation, when almost all phenanthrene in the medium was utilized, the number of living cells started to decrease correspondingly. Over 40 hours of incubation the phenanthrene concentration decreased from 100 ppm down to 1 ppm, proving the novel Fdl strain to be an effective phenanthrene degrader.

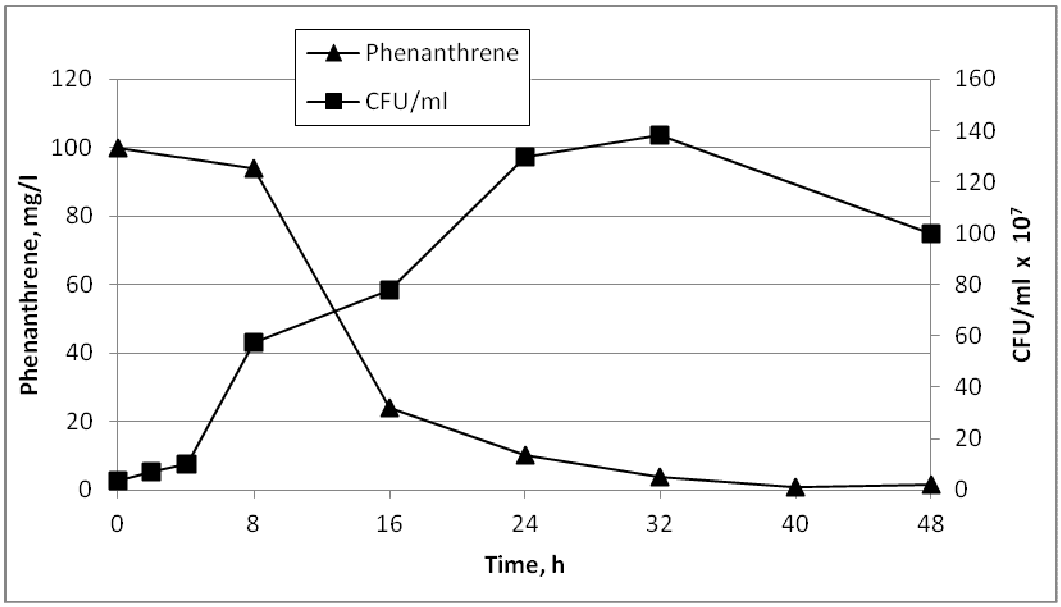

Fig. 1. Phenanthrene degradation and cell proliferation

3.5 Identification and accumulation of 1-hydroxy-2-naphthoic acid

The HPLC analysis of phenanthrene degradation products in the incubation medium was found to contain significant amount of HNA known to be the key metabolite in phenanthrene degradation. Kinetic studies of the HNA accumulation (Fig.2) showed increase in HNA concentration during the first 24 hours of incubation. After 32 hours of incubation when phenanthrene concentration decreased below the HNA concentration, the latter started to decrease as well. The further degradation of HNA, as was reported earlier, goes via salicylic or phthalic acid production. However, HPLC analysis found no such compounds in the incubation medium, which may result from their extremely short life time in the medium. The fact that the strain was found to be able to proliferate on/in the medium containing salicylic acid as the sole carbon source and not on/in the medium with the phthalic acid as the sole carbon source (the data are not shown) can serve as indirect evidence in favour of the salicylic acid production pathway.

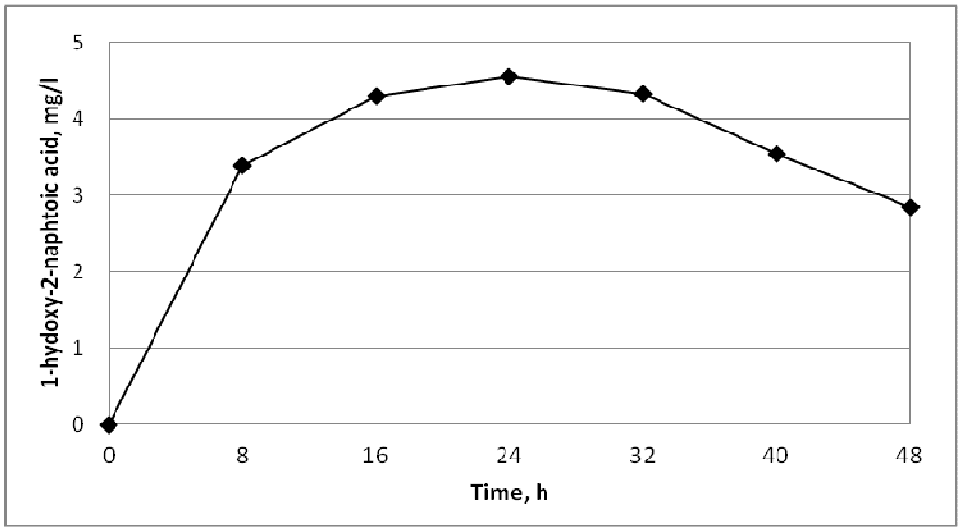

Fig. 2. Accumulation of 1-hydroxy-2-naphtoic acid in course of phenanthrene degradation

3.6 The effect of Tween-20 surfactant on phenanthrene degradation

Addition of Tween-20 non-ionic surfactant into the incubation medium accelerated phenanthrene degradation and cell proliferation as compared to phenanthrene degradation without a surfactant (Fig.3). When Tween 20 was added, the phenanthrene concentration was found to decrease already after 2 hours of incubation, decreasing to 1 ppm over 24 hours, with the maximal degradation rate being recorded at 2-8 hours interval. Since Tween 20 is known as a good carbon source to be utilized by bacterial cells, its effect on phenanthrene degradation may be due both to a facilitated assimilation/uptake of phenanthrene by bacterial cells and/or increased cell biomass. Judging by the CFU counts, we may conclude that after 4 hours of incubation cell proliferation is supported mostly by Tween 20 as a growth substrate, with phenanthrene contributing not more than 10 %. However, as phenanthrene degradation was observed to start already after 2 hours of incubation, i.e. before the end of the lag-phase, the increased phenanthrene uptake by cells may be suggested to contribute into the accelerated degradation as well.

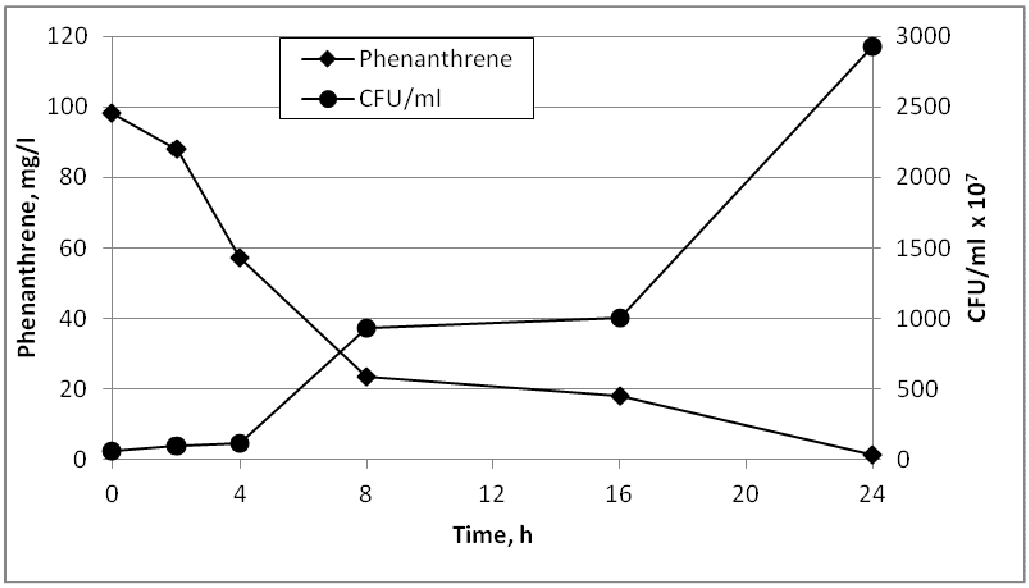

Fig. 3. Phenanthrene degradation and bacterial cell proliferation in the medium with Tween 20 added

The presence of Tween 20 in the medium resulted in the accumulation of large amount of HNA, with a pattern similar to the one without Tween 20 (Fig.4). The maximal HNA concentration was registered after 16 hours of incubation. Despite the fact that it is a less preferable substrate for bacterial utilization, as compared with Tween 20, HNA did not accumulate in the medium in course of incubation.

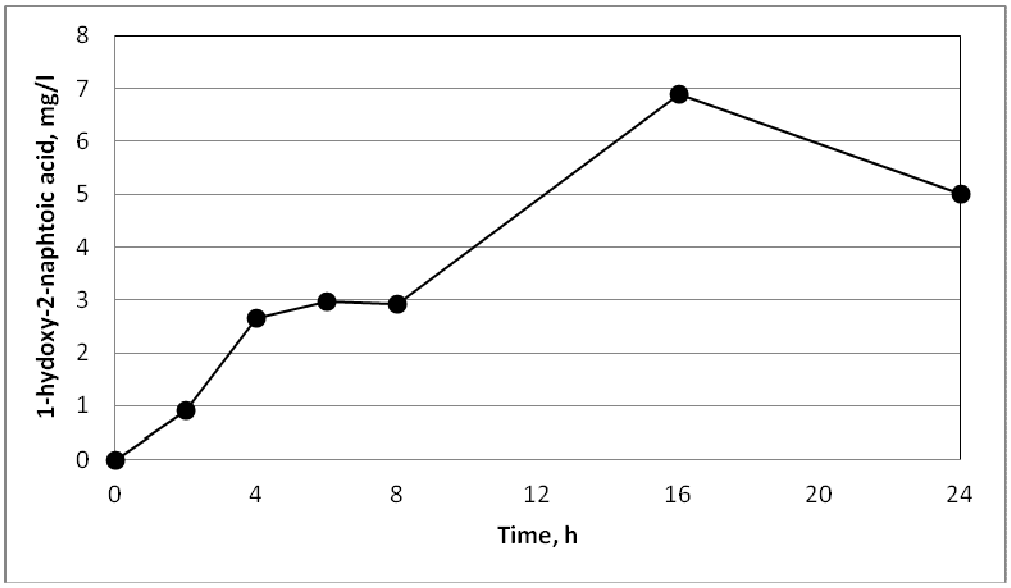

Fig. 4. The accumulation of 1-hydroxy-2-naphtoic acid in course of phenanthrene degradation in the medium with Tween 20 added

Many bacteria, representing diverse genera such as *Bacillus* [16], *Nocardia* [17], *Pseudomonas* [18-21], *Sphingomonas* [22], *Sphingobium* [23], *Mycobacterium* [24], *Sinorhizobium* [25], *Rhizobium* [26], *Novosphignobium* [27] and others [28, 29] were reported to degrade phenanthrene. However, their degradation rate in liquid medium with 100 ppm phenanthrene ranged 96-99 % over 77-360 hours, whereas the *Pseudomonas denitrificans* Fdl strain isolated in this study was shown to degrade phenanthrene over 48 hours without any additions present, and twice as rapidly if phenanthrene assimilation/uptake was facilitated by the detergent present in the medium. The closest to our strain was a *Pseudomonas* strain, reported to degrade 100 % of 60 mg/L phenanthrene within 60 hours [30].

Some phenanthrene-degrading *Pseudomonas* strains were found to produce biosurfactants, facilitating PAH bioavailability [31]. Although we did not examine its capability to produce biosurfactants, the isolated strain may be quite likely to do so, which might enhance the strain's prospects as a potential bioremediator.

4. CONCLUSION

The isolated strain *Pseudomonas denitrificans* Fdl is capable of efficient phenanthrene degradation, especially in the presence of detergent, and hence can be a good candidate for biological preparations to be tested for industrial bioremediation and sewage sludge treatment.

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