Year: 2014; Volume: 1; Issue: 1
Article ID: BT14 07; Pages: 1-19

BioMed Research

The Open Access Publisher

www.bmrjournals.com

BMR Biotechnology

Research Article

Enhance the Biodegradation of Anthracene by Mutation from Bacillus species

Neelofur. M*, Shyam Prasad. V and Mahesh. M

Azyme Biosciences Pvt. Ltd., Bangalore, India

Correspondence should be addressed to Neelofur. M.

Received 17 May 2014; Accepted 27 May 2014; Published 09 June 2014

Abstract

Anthracene, a tricyclic aromatic hydrocarbon causes many problems associated with health and environmental impact. It results from incomplete combustion of fuels present in automobiles and found in high concentrations in the polycyclic aromatic hydrocarbon (PAH)-contaminated sediments, surface soils & waste sites. Anthracene serves as a signature compound to detect PAH contamination, since its chemical structure is found in carcinogenic PAHs. Therefore, the present study aimed to isolate bacteria capable of utilizing anthracene as the sole source of carbon and energy. Nine organisms was isolated among the 40 soil samples collected from (industrial area), Bangalore, based on the screening test the organism was selected and identified by 16s RNA sequencing. The organism subjected for optimise at different concentrations of anthracene, pH, temperatures, effect of carbon source and nitrogen source. After optimistaion, the more degradation was showing concentration at 10mg, at pH 5 and temperature at 35^{o}C but effect of nitrogen source, sodium nitrate was showing 60times more degradation and along with that effect of carbon source was increasing nearly 15 times. After UV mutation, the degradation was increased nearly 20 times. The cytochrome oxidase activity was determined which is involved in the degradation of anthracene and the degradation was analysed by high performance liquid chromatography (HPLC).

Keywords: Anthracene, PAH, RNA sequencing, MSM media, SDS- Native PAGE, cytochrome oxidase, NADPH

Introduction

Polycyclic aromatic hydrocarbons (PAHs) comprise a class of compounds consisting of three or more benzene rings fused in angular or cluster formation, composed solely of carbon and hydrogen atoms (Launen *et al.*, 1995). PAHs and heterocyclic compounds are considered environmentally significant because of their potential toxicity to the higher organisms & resistance to microbial attack (Kanaly & Harayama, 2000). PAH are formed as a result of the incomplete combustion of fossil fuels & are therefore present in a variety of products such as tar, coal, soot, petroleum, cutting oils & tobacco smoke (International Agency for Research on Cancer, 1983). Some PAHs are highly carcinogenic, genotoxic & cytotoxic (Boldrin *et al*, 1993; Cerniglia, 1993). Many PAHs including anthracene, phenanthrene, acenaphthene, acenaphthylene, fluoranthene, pyrene, benzo[a]pyrene (BaP) & benzo[a]anthracene (BAA) are in the United States Environmental Protection Agency Priority Pollutant List (Cerniglia, 1981). PAH & their derivatives have been recognized as the major culprits causing human lung cancer, anaemia, asthma, splenomegaly, bladder cancer & breast cancer etc (Doll & Peto, 1981; Okana-Mensah *et al.*, 2005; Booker & White, 2005; Hazra *et al.*, 2004; Miller *et al.*, 2005).

Anthracene together with other PAHs is a persistent and toxic soil contaminant (Hyotylainen *et. al.*, 1999, Juhasz and Naidu, 2000, Lotufo, 1997). Among the various PAH emitted from the fuel combustion anthracene, a tricyclic aromatic hydrocarbon causes many problems associated with health and environmental impact. It is released due to incomplete combustion of fuels present in automobiles. It is found in high concentrations in the PAH contaminated sediments, surface soils & waste sites. This hydrophobic contaminant is widely distributed in the environment, occurring as natural constituent of the fossil fuels & their anthropogenic pyrolysis products (Cerniglia, 1992; Kanaly & Harayama, 2000).

Anthracene exhibits toxicity to fish, algae and shows bioaccumulation in the food chain (Sutherland *et al.*, 1990; Sutherland *et al.*, 1992). Anthracene serves as a signature compound to detect PAH contamination, since its chemical structure is found in carcinogenic PAHs. It has also been used as a model PAHs to determine the factors that affect the bioavailability, biodegradation potential, & the rate of microbial degradation of the PAHs in the environment (Bouchez *et al.*, 1995; Cerniglia, 1992; Kanaly & Harayama, 2000; Sutherland *et al.*, 1995). Pollution by PAHs is usually found on the sites of gas factories and wood preservation plants. Bioremediation is an economically and environmentally attractive solution for cleaning those sites (Kastner and Mahro, 1996).

Microbial degradation plays a major role in the removal of PAHs from contaminated sediment and surface soils. The ability to degrade PAHs is shown by various groups of organisms including bacteria, fungi and algae. (Cerniglia, 1992; Cerniglia, 1993; Hammel, 1995; Sutherland et al., 1995; Muncnerova & Augustin, 1994; Feitkenhauer et al., 1996; Kanaly and Harayama, 2000; Van Hamme *et al.*, 2003).

By observing these problems, an approach should be taken to isolate microbes, which can degrade anthracene and its by products which may be harmful to human and animal health. With the aim of remediation & removal of anthracene from the environment, the present study is focused on the isolation & characterization of bacteria capable of degrading the anthracene.

Materials and Methods

Isolation of microorganism:

The bacteria consortium was isolated from a mixture of forty different samples which located near different industries are automobile, shoe, thermal, polymer, rubber and printing, the industrial area of Bangalore, India. The microorganism was isolated by using serial dilution method by using Mineral salt medium (MSM) along with 100ppm anthracene dissolved in 0.4ml of acetone and 0.6ml of distilled water.

Mineral salt medium:

The carbon free Mineral salt medium (MSM) contained KH_2PO_4-3.0g, Na_2HPO_4-6.0g, $MgSO_4$. $7H_2O$-0.1g, NH_4Cl-2.0g, NaCl-5.0g and Distilled water-1L. The final pH of the medium was adjusted to 7.4 and the medium was autoclaved at 121^{0}C for 15min. The stock solution of PAH (anthracene) was prepared in acetone and stored.

Growth curve:

Nine organisms were obtained after serial dilution subjected for growth curve and degradation by using MSM media along with 100ppm anthracene. The result was analysed by spectrophotometric method.

Effect of temperature:

Incubation was done at different temperatures 20°C 30°C, 40°C, and 50°C by using MSM media along with that 100ppm anthracene was added and incubated at room temperature for 72 hr. The degradation was measured by using spectrophotometeric method.

Effect of pH:

Increment the pH from 4, 5, 6, 7, 8 and 9 and incubate at room temperature for 72hr by using MSM media along with that 100ppm anthracene was added. The degradation was measured by using a spectrophotometeric method.

Effect of carbon and nitrogen sources:

Different carbon sources were taken are cellulose, starch, sucrose, fructose and dextrose by using MSM media along with that 100ppm anthracene was added and incubated for 72hr and the degradation was measured by spectrophotometric method.

Different nitrogen sources are peptone, tryptone, sodium nitrite, sodium nitrate and potassium nitrate by using MSM media along with that 100ppm anthracene was added and incubated for 72hr and the degradation was measured by spectrophotometrically at 340nm.

Effect of chemical mutation:

The isolated organisms were exposed to UV-mutation for 5min, 10min, 15min and 20min by using MSM media along with that 100ppm anthracene was added and incubated for 72hr. The growth was measured and estimated by spectrophotometric method.

Protein estimation:

Protein concentrations were measured by the method of Lowry *et al*

Enzyme estimation:

The cultured samples were taken and estimated the reductase activity and kept incubation for 72hr and the enzyme was estimated by spectrophotometric method.

Purification

The samples were purified by using some methods are salt precipitation, dialysis, ion-exchange chromatography, SDS-PAGE and Native PAGE.

Analytical procedure:

Residual amount of anthracene was determined by high performance liquid chromatography (HPLC) analysis for quantitative estimation of PAH degradation. High performance Liquid chromatography (HPLC) equipped with diode-array detector consists of reverse phase column (Waters) and dual piston reciprocal pump system. The injection volume was set at 10micro litre and the injector consists of 6-port binary valve system and the isocratic eluent was pumped at a rate of 1 ml/ min. The PAH concentrations were determined monitoring the absorbance at 254 nm for anthracene. The isocratic eluent consisted on 70%acetonitrile: 30% water (v/v) and pH was set for 3.6. The pH was setted and the mobile phase was filtered by vaccum filtration and kept for ultrasonication for 15min.The standard was dissolved in 10mg/25ml of mobile phase and filtered that standard by using membrane filter and the sample was directly taken and filtered by using membrane filter and injected into the injector.

Results and Discussion

Soil samples were contaminated with different industrial effluents causes serious threat to atmosphere. Bioremediation plays an important role to reduce the contamination of soils and employs microorganisms capable of degrading toxic contaminants. The polycyclic hydrocarbons (PAHs) anthracene represents one of the most widespread concerns because of its toxicity to biological systems (Doyle, 2008). Anthracene solubility is very low in aqueous medium. It is insoluble in water and slightly soluble inorganic solvents such as benzene, chloroform, methanol and hexane and soluble in organic solvents such as acetone, 1, 4-dichlorobutane, 1-butanol and 1-octanol. Anthracene is ubiquitous in the aquatic environment. It has been detected in industrial effluents, in run off waters, in surface water and sediments, in groundwater, and in drinking water. The industrial effluents that are most likely to contain polynuclear aromatic compounds including anthracene are wastewaters from the synthetic fuel industry.

Anthracene degrading bacterial isolates were obtained using anthracene as the sole source of carbon along with that MSM media and energy by using different techniques. The most degrading bacteria soil 1 was isolated.

This isolate was identified and characterized by 16sRNA sequencing and "Bergey''s manual of systemic bacteriology" (Vol I and II; Palleroni, 1984).

Growth curve:

Among the 9 bacterial isolates, the pure culture of one isolate from soil 1 was found to degrade initial concentration of anthracene most effectively when

incubated for 72hr as seen in Fig1 and that degradation abiltiy of the isolate decreases with a rise in incubation time and the organism was identified and confirmed by 16sRNA sequencing as *Protease sp.* The results of the present study confirmed the matter that many of bacterial strains , especially gram- negative bacteria were found to degrade poly aromatic hydrocarbons(PAHs) (Cerniglia, 1992, Kiyohara, Nagao and Yana, 1982, Sutherland, Rafii Khan and Cerniglia, 1995).

Table.1.Estimated the degradation by spectrophotometrically at 600nm

Incubation period	Thermal / OD	polymer	soil1	Shoe	printing	Auto mobile	Rubber	Rubber waste	soil2
12	0.024	0.022	0.066	0.064	0.019	0.025	0.024	0.022	0.017
24	0.028	0.024	0.013	0.071	0.048	0.046	0.048	0.042	0.048
36	0.086	0.028	0.024	0.076	0.086	0.082	0.084	0.086	0.082
48	0.126	0.116	0.125	0.126	0.124	0.122	0.124	0.126	0.125
60	0.146	0.126	0.146	0.148	0.145	0.148	0.148	0.146	0.146
72	0.182	0.148	0.185	0.182	0.198	0.184	0.182	0.184	0.186
84	0.215	0.211	0.214	0.212	0.204	0.214	0.212	0.214	0.218
96	0.272	0.264	0.254	0.252	0.256	0.258	0.264	0.254	0.268
108	0.279	0.274	0.275	0.273	0.272	0.278	0.275	0.274	0.276
120	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28	0.28

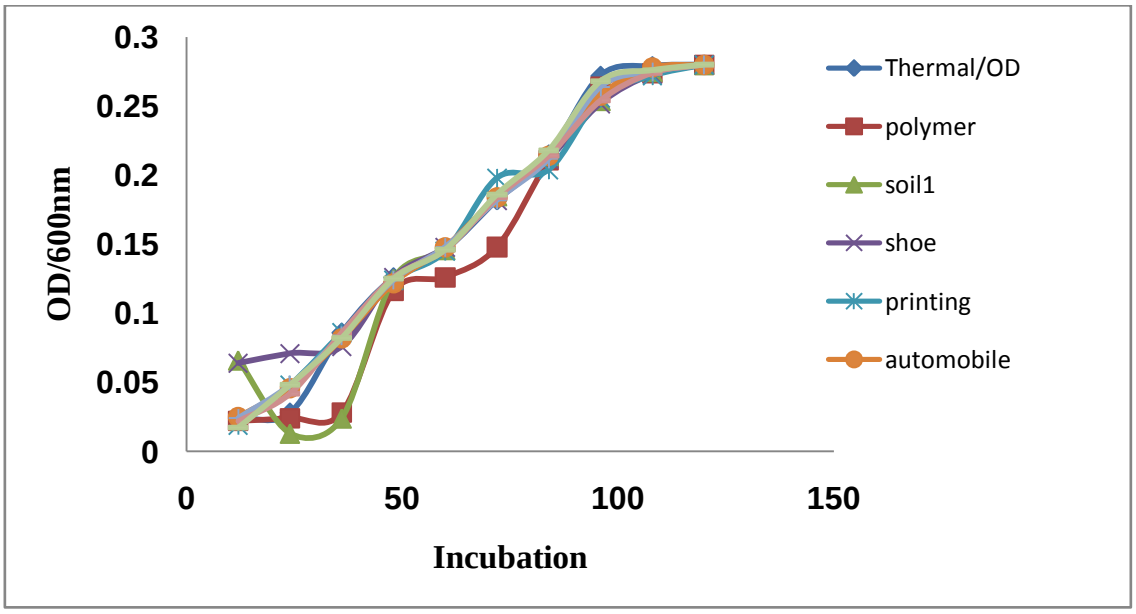


Fig.1. Growth curve for all the 9 samples base on their degradation. Here on x-axis taken as incubation period and on y-axis taken as OD values.

Effect of temperature:

The anthracene was degraded and showing the best degradation at 35^{0}C. Among the parameters controlling hydrocarbon biodegradation, temperature is generally considered as one of the most important area. Microbial metabolism increases as temperature increases (Leahy and Colwell, 1990), usually doubling for each 10^{0}C increase in temperature. Our study confirms that bioremediation of hydrocarbons in soil is feasible at 35^{0}C. Meanwhile, a temperature increases in the range from 20^{0}C-60^{0}C can accelerate the hydrocarbon biodegradation. These results according are in accordance with previous observations of Walworth and Reynolds (1995) that reported a positive result on temperature of hydrocarbon biodegradation.

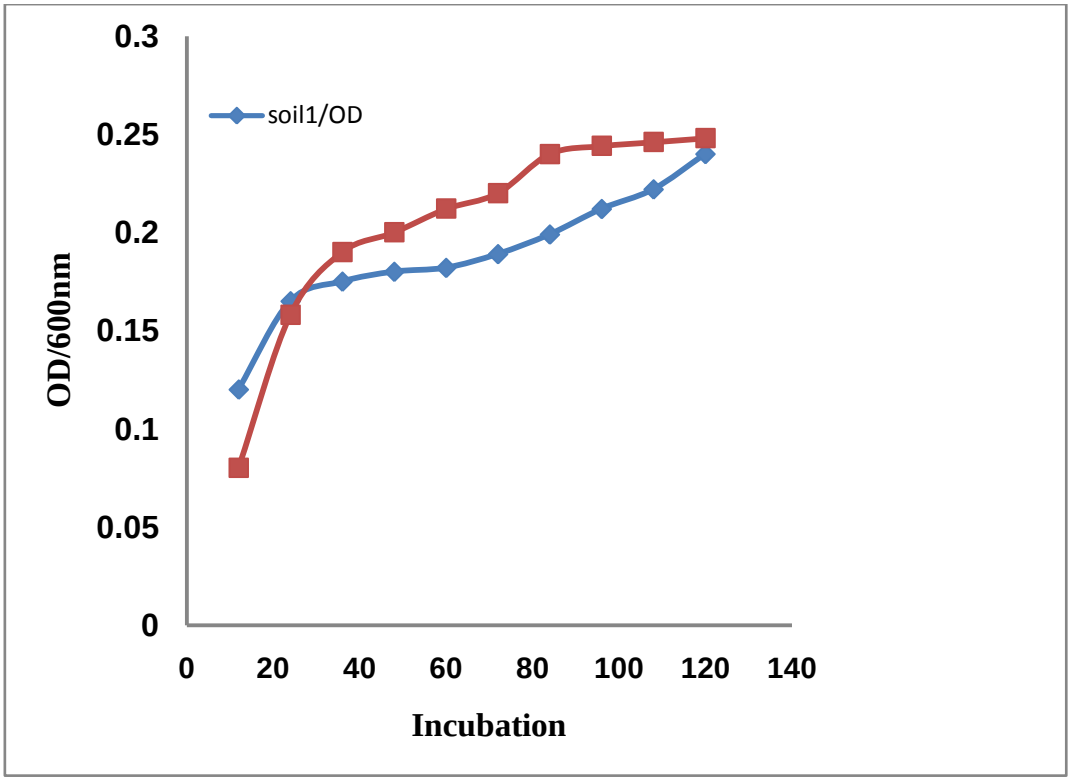


Fig.2 Estimated the degradation by different temperatures

Effect of pH:

The anthracene was degraded and showing the best degradation at pH 5. There was a gradual increase in rate of reduction of the compound till pH 5 (82.6%), after which it declined till it reaches to the point when there was no more reduction at pH 9. Degradation at pH 8 was the result of auto oxidation as no bacterial growth was observed at this pH level. pH plays a vital role in biodegradation and gives an insight to degradation process of anthracne as it biodegraded at pH ranges of 4 to 9 at different rates. The high sensitivity of Protese sp for pH and optimum pH for the degradation of anthracene by *Protease* was reported to be pH 5 (Zeyer et al., 1985; Rabia, 1998) that was similar to our current findings.

Estimated the degradation by different pH:

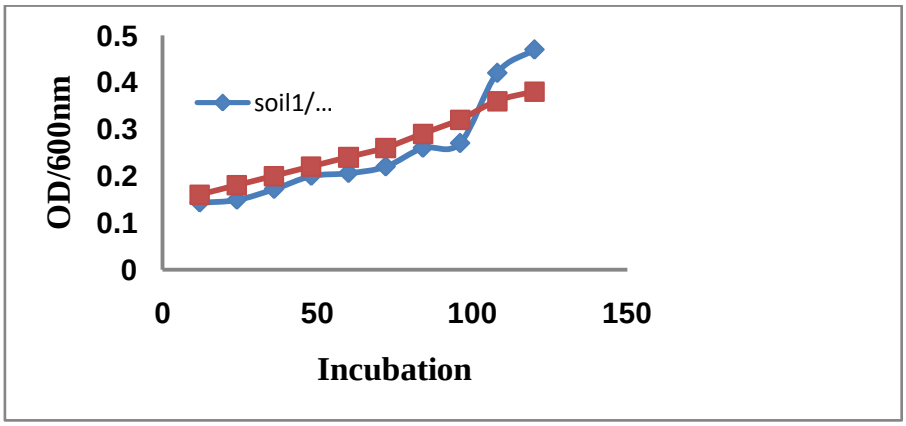


Fig 3. Growth curve of degradation of different pH

Effect of carbon and nitrogen sources:

The effect of carbon source i.e., sucrose showing the best degradation at 60mg and the nitrogen source i.e, sodium nitrite showing the max degradation of anthracene.

In laboratory conditions, the carbon source was added to support the higher growth of bacteria. Therefore, addition of carbon sources enhanced the anthracne biodegradation due to cometabolism. The presence of additional substrates could initiate cometabolism of the desired compounds (Felsot and Dzanto, 1995). Bacteria can degrade anthracene through co metabolism (Sette, de Oliveira, Manfio, 2005; Chirnside, Ritter, Radosevich, 2011; Ferrey , Koskinen, Blanchett , Burne, 1994).

Cells grown on nitrogen source have increased anthracene degradation reduction rate significantly. Many investigations showed a positive effect of nitrogen amendment on anthracene biodegradation by indigenous populations in soils (Entry, 1999; Abdelhafid, Houot, Barriuso, 2000).

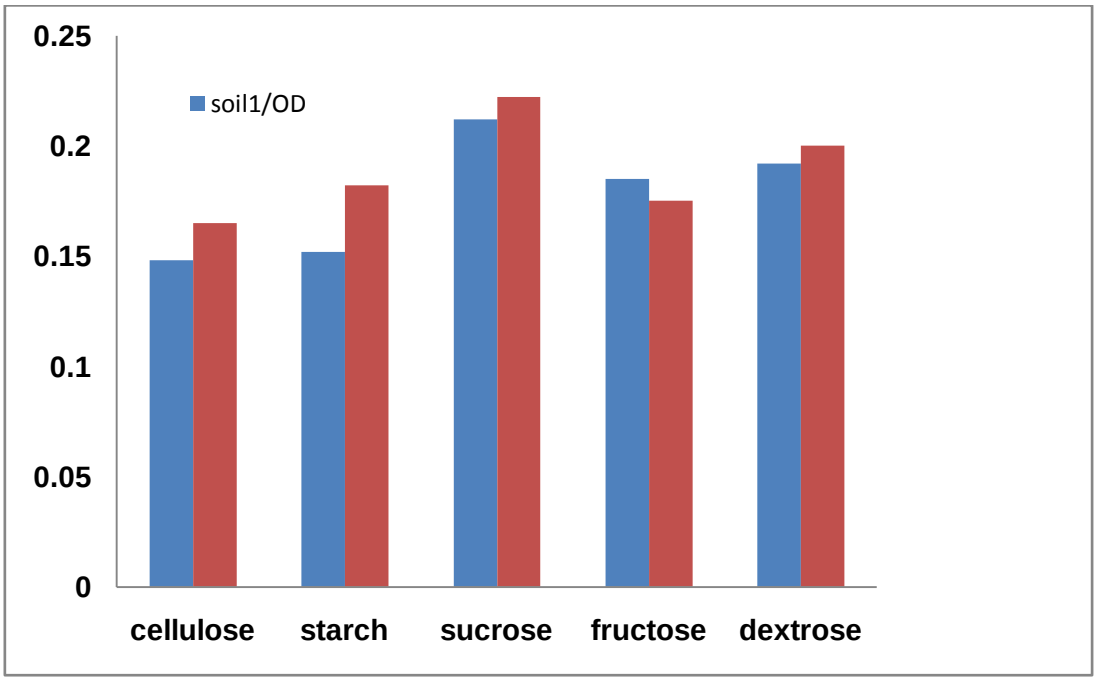


Fig.4. Estimated the degradation by different carbon sources

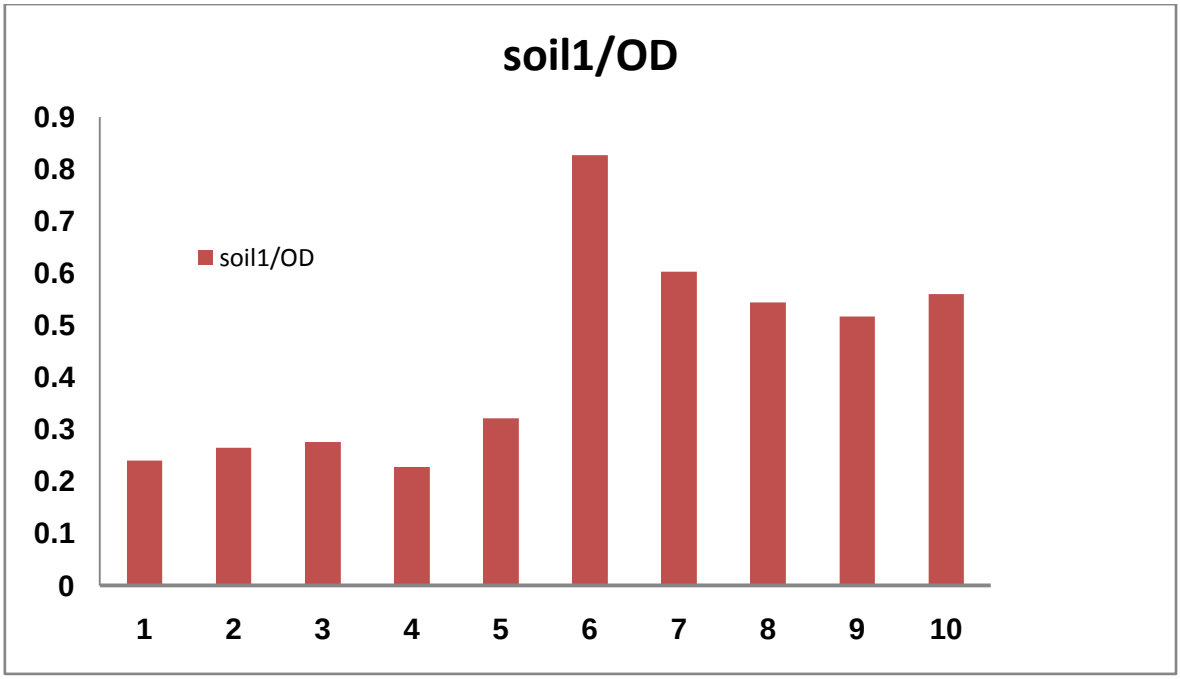


Fig .5. Estimated the degradation by different concentrations of sucrose

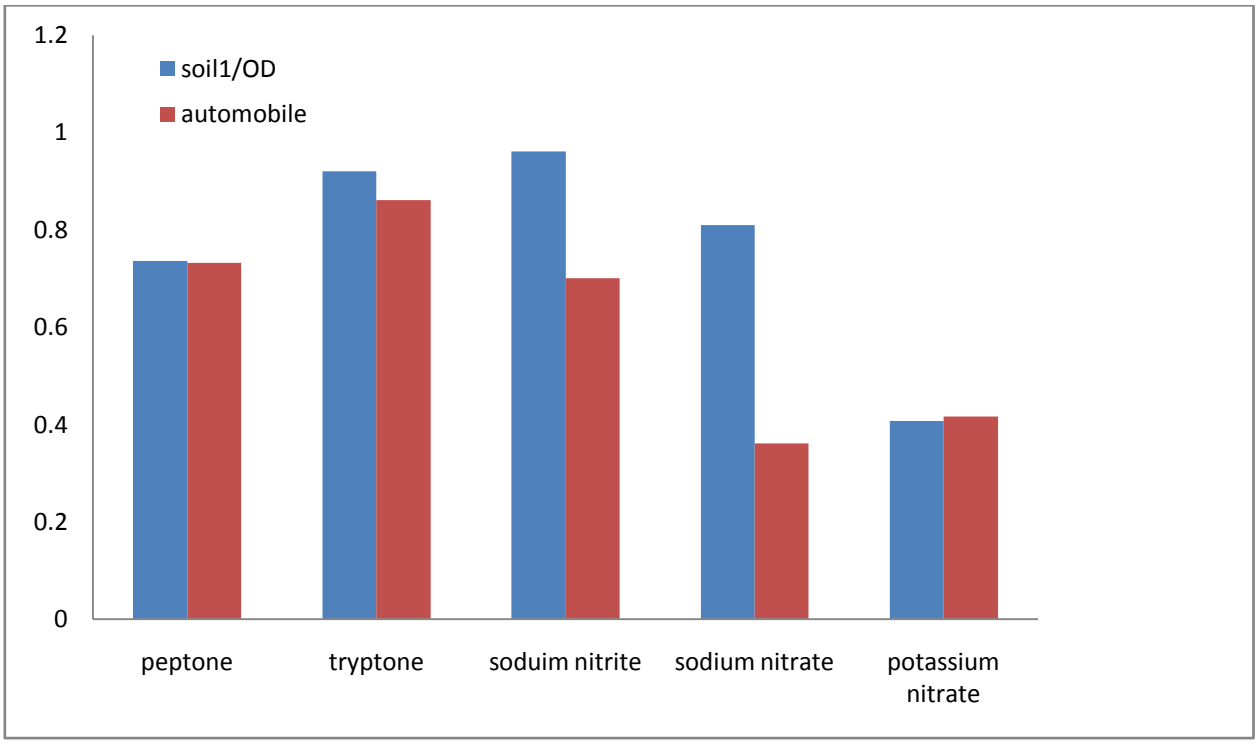


Fig .6. Estimated the degradation by different nitrogen sources

Effect of chemical mutation:

Isolation and maintenance of strains that utilize a anthracene as a sole carbon source can often be difficult, as many strains grown on minimal medium cannot degrade the anthracne or have lost the ability to degrade it (Cullington *et.al*, 1991). The selective nature of the isolation media originally used to obtain the wild-type degradative strain from soil was investigated and mutation using UV light was performed to enhance the degradation ability.

The pure culture of microbes on perti dish containing MSM when subjected to 15 minutes of UV light showed best growth. Hence it could be concluded that exposure to UV light for 15 minutes could improve the degradation ability significantly. Therefore the bacterial isolate when exposed to UV light to 15 minutes and on addition of 100 ppm of anthracene showed improved degradation ability as shown in Fig 7.

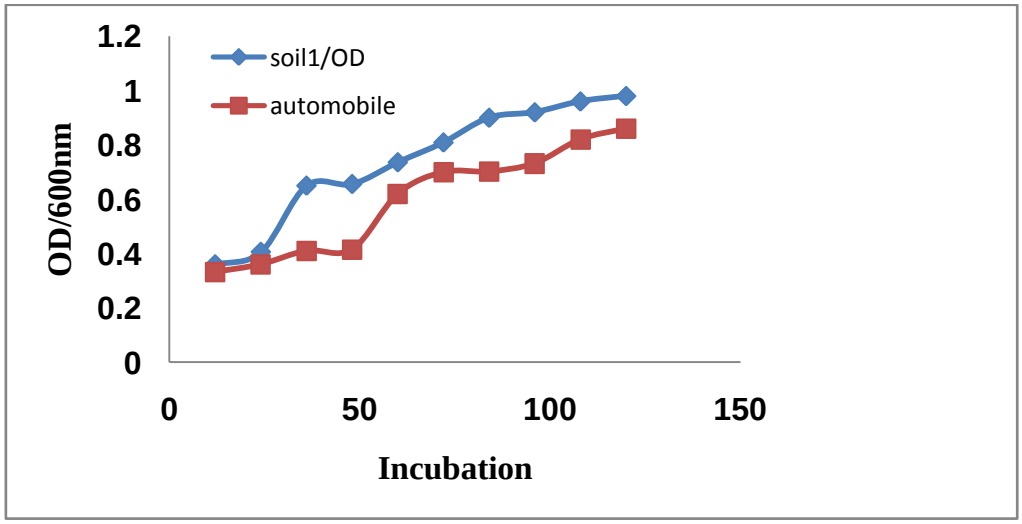


Fig.7 Growthcurve of mutated samples the degradation of anthracene. Incubation period of samples against OD values.

Enzyme estimation:

The possibility that cytochrome oxidase was an intermediate product was investigated by measuring specific enzyme activities. The crude cell extracts catalyzed the oxidation of NADH or NADPH in the presence of cytochrome oxidase, suggesting that the substrate was being reduced. This activity was detected only in cells grown in minimal medium containing anthracene as a sole source of carbon.

Cytochrome -oxidase seems to play an important role in oxidative stress, although it is able to create an electrochemical gradient for energetic requirements (Castresana 2011).

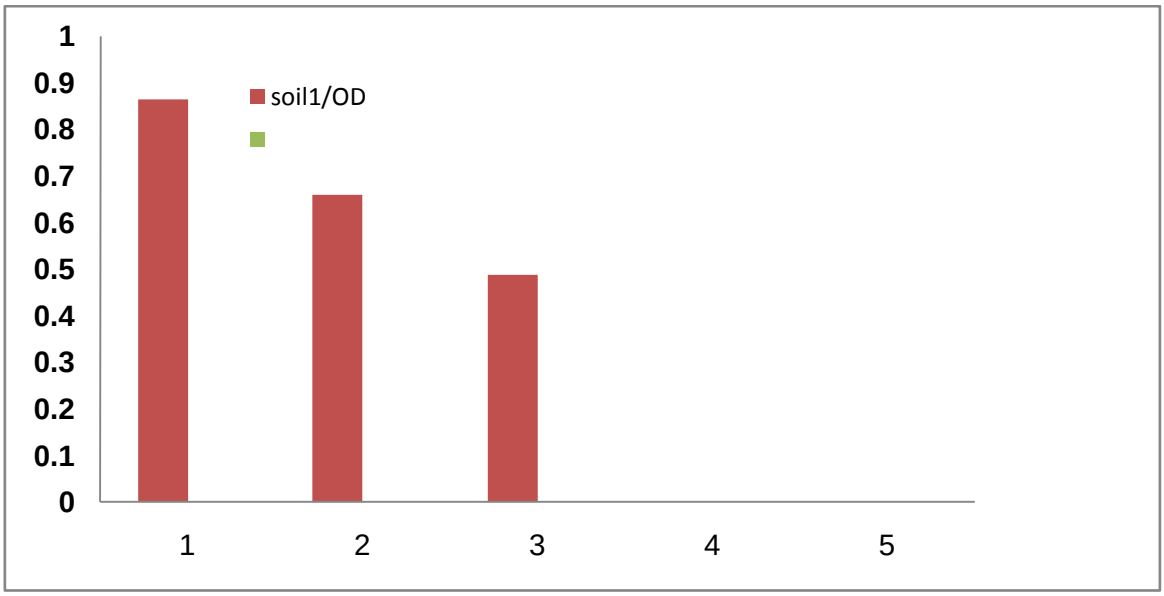


Fig.8 Enzyme estimation of mutated samples

Effect of Substrate concentration:

The anthracene was degraded and showing the best degradation at 20mg.

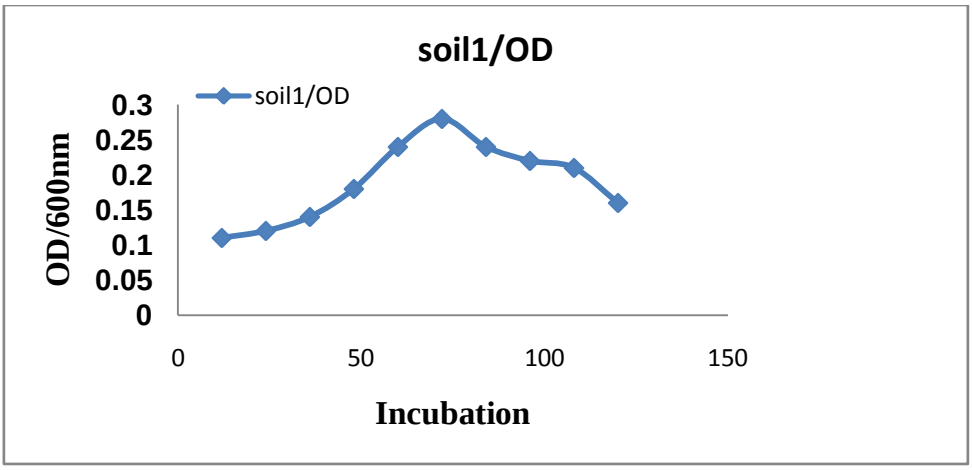


Fig.9 Effect of substrate concentration

Effect of Activators:

By using $MgCl_2$ as a activator, the anthracene was degraded and the best result at 50mg. Increasing concentration of the activator, magnesium chloride, increased enzyme activity and maximum activity was observed with 1.0 ml of activator.

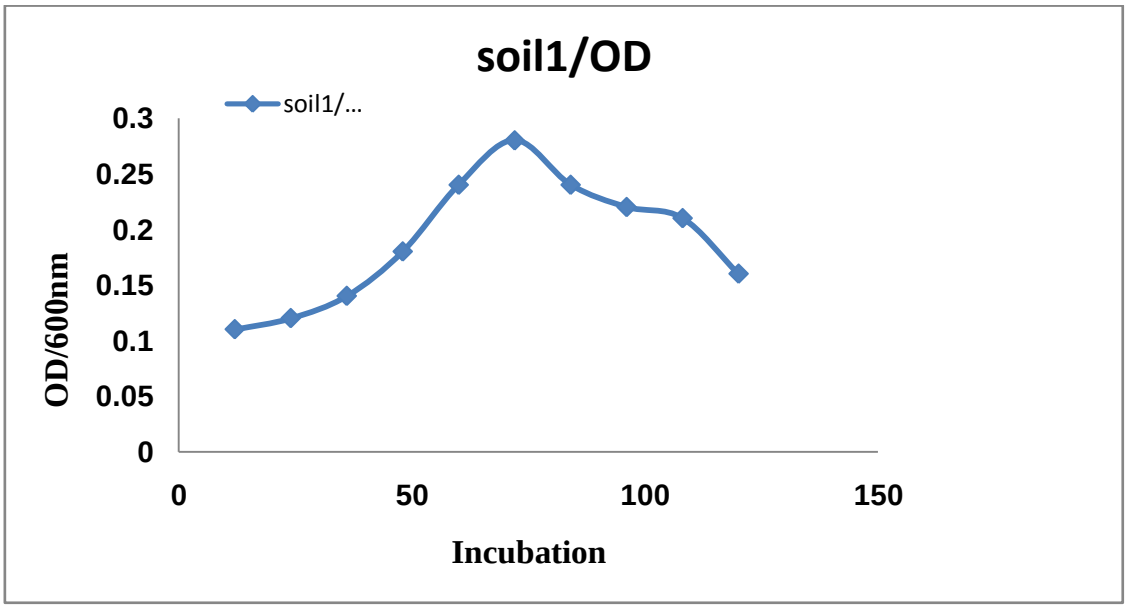


Fig 10. Effect of activators

Effect of Inhibitors:

By using EDTA, the anthracne was degraded and showing the best result at 10mg. Increasing concentration of the inhibitor, EDTA, decreased the enzyme activity.

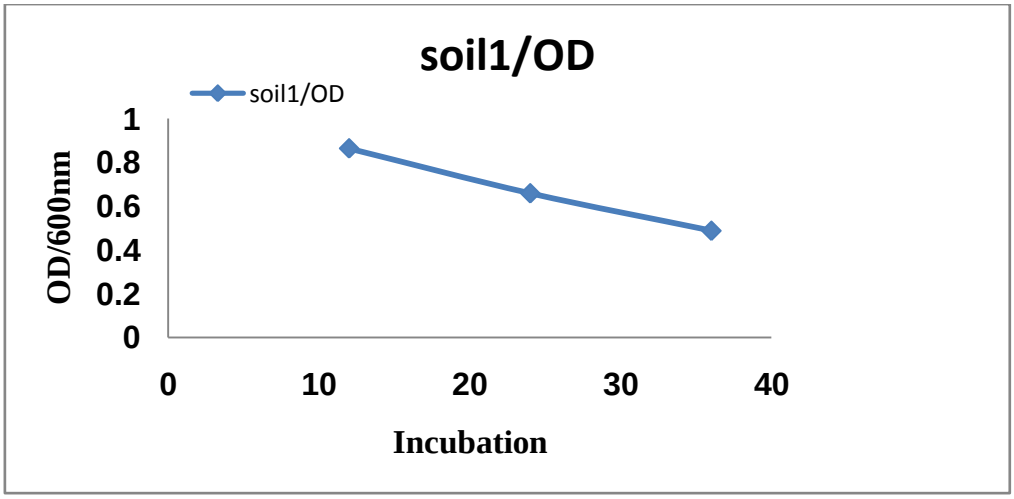


Fig.11 Effect of inhibitors

By observing these results, we confirmed that anthracene was degrading by bacteria. Identification and characterization of bacteria was carried out on the basis of morphology, staining reactions and biochemical activities (Table 2) and HPLC analysis. Therefore, the anthracene degrading bacteria was identified as rod shaped, Gram-positive in all nine samples.

Biochemical tests Results:

Biochemical test	Result
Indole	-ve
Methyl red and voges proskauer test	+ve and –ve
Citrate utilisation	+ve
Casein hydrolysis	+ve
Starch hydrolysis	+ve
Gelatin test	+ve
Hydrogen sulphite test	-ve
Catalase test	+ve
Urease test	-ve
Oxidase test	+ve

Purification results:

The purified enzyme was protein profiled by SDS PAGE for determination of molecular weight of the enzyme. The result thus obtained was a protein band of a molecular weight of approximately 45 kDa.

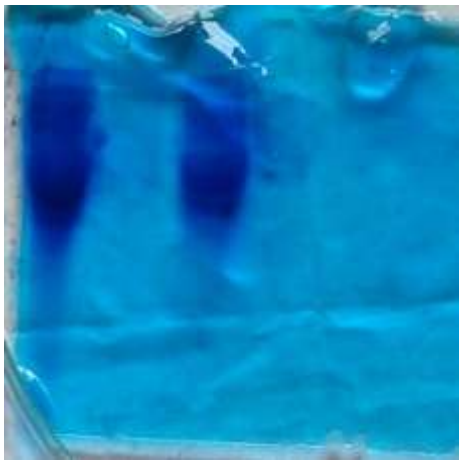


Fig. 14. Result of SDS-PAGE

Fig .15. Result of Native-PAGE

The Fig.14 showing the two running samples are first one is the marker compared with sample containing enzyme and fig.15 showing the results of native PAGE containing the little content of enzyme compared with marker.

HPLC Analysis Result:

25 µl aliquots of culture supernatants were analyzed with C18 column. The samples were: Soil sample 1 with anthracene concentration of 100ppm;UV mutated sample with 100ppm of anthracene.

HPLC analysis allowed us to monitor the disappearance of anthrcane and the formation and/or destruction of reaction products during the treatment of soil samples on exposure to UV light. In all cases, retention time curves (3.037) are characterized by a relatively rapid and regular decrease in the anthracene concentration. There were 2 peaks observed when anthracene was injected which was used as standard. On comparison of degradation ability it was found that the isolate containing 100ppm of anthracene on mutation showed an increase of 99% degradation(graph 3) and the isolate containing 100ppm of anthracene showed an increase of 78%(graph 2).

Calculation

Calculation of sample = Retention time of standard – Retention time of sample/Retention time of standard*100

Calculation of sample = 5.137-1.054/5.137*100 = 79.482% of anthracene degraded in sample-1.

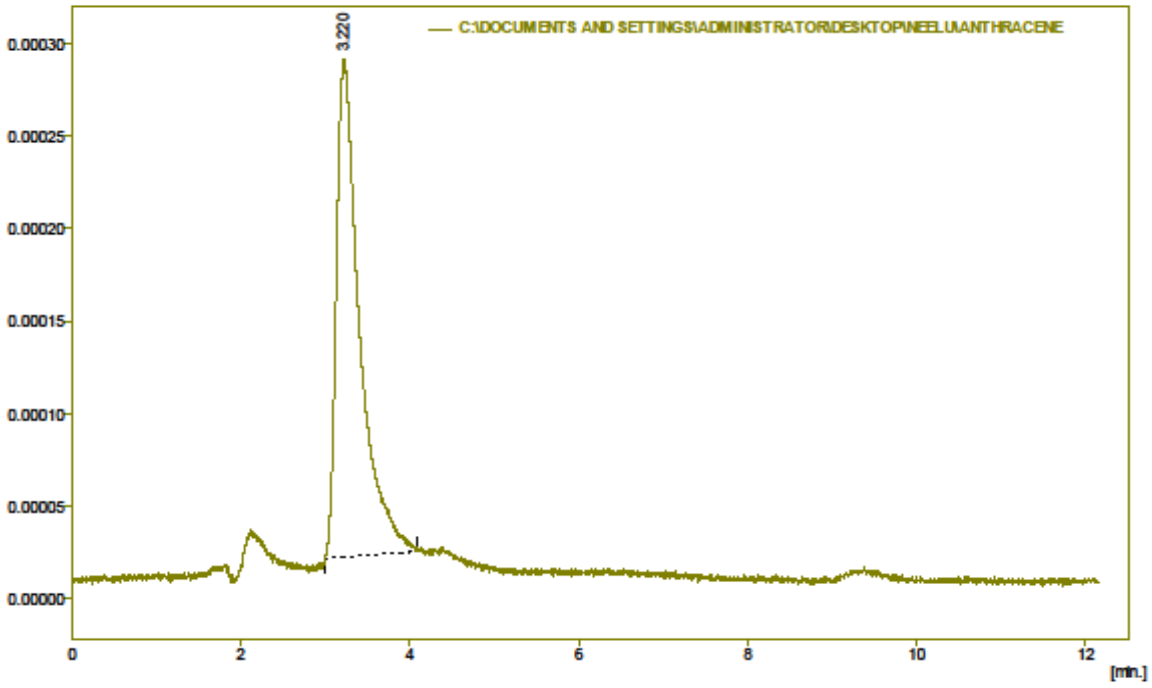
Result of Anthracene (Standard):

4/24/2014 10:15 AM Chromatogram C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\NEELU\ANTHRACENE.PRM Page 1 of 1

AZYME BIOSCIENCES PVT LTD

Column	:	Detection	:
Mobile Phase	:	Temperature	:
Flow Rate	:	Pressure	:
Note	:		

Autostop	: None	External Start	: Start - Restart, Down
Detector 1	: Signal 1	Range 1	: Bipolar, 1250 mV, 10 Samp. per Sec.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\NEELU\ANTHRACENE)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	3.220	5.137	100.0
	Total	5.137	100.0

Graph 1

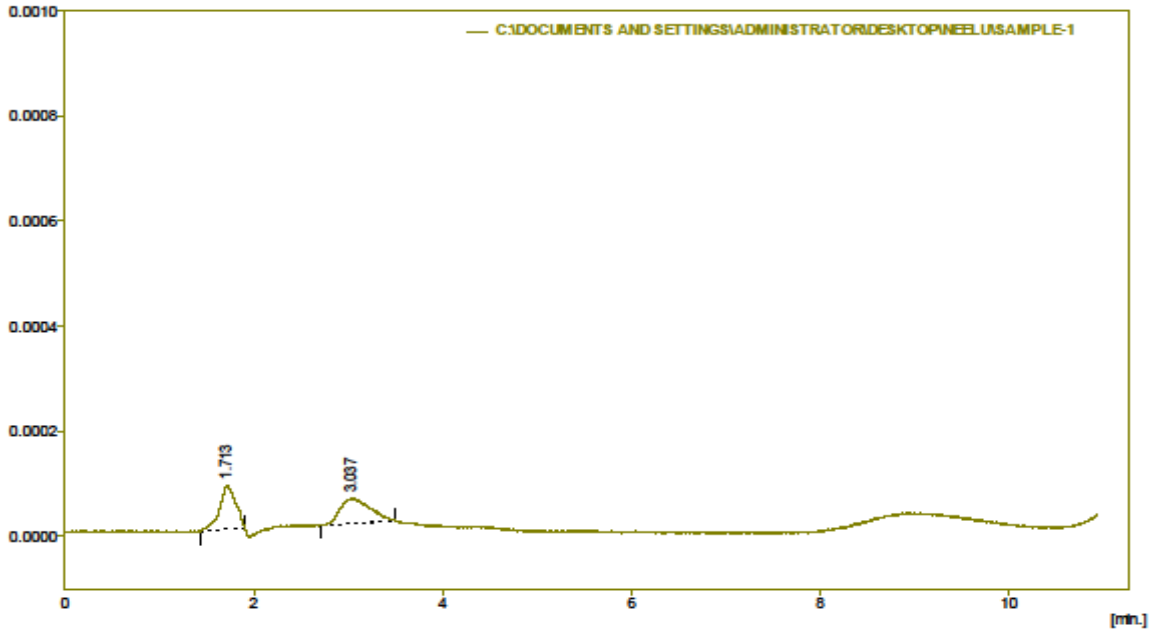
Result of sample 1

4/24/2014 10:17 AM Chromatogram C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\NEELU\SAMPLE-1.PRM Page 1 of 1

AZYME BIOSCIENCES PVT LTD

Column	:	Detection	:
Mobile Phase	:	Temperature	:
Flow Rate	:	Pressure	:
Note	:		

Autostop	: None	External Start	: Start - Restart, Down
Detector 1	: Signal 1	Range 1	: Bipolar, 1250 mV, 10 Samp. per Sec.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\NEELU\SAMPLE-1)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	1.713	0.988	48.4
2	3.037	1.054	51.6
	Total	2.042	100.0

Graph 2

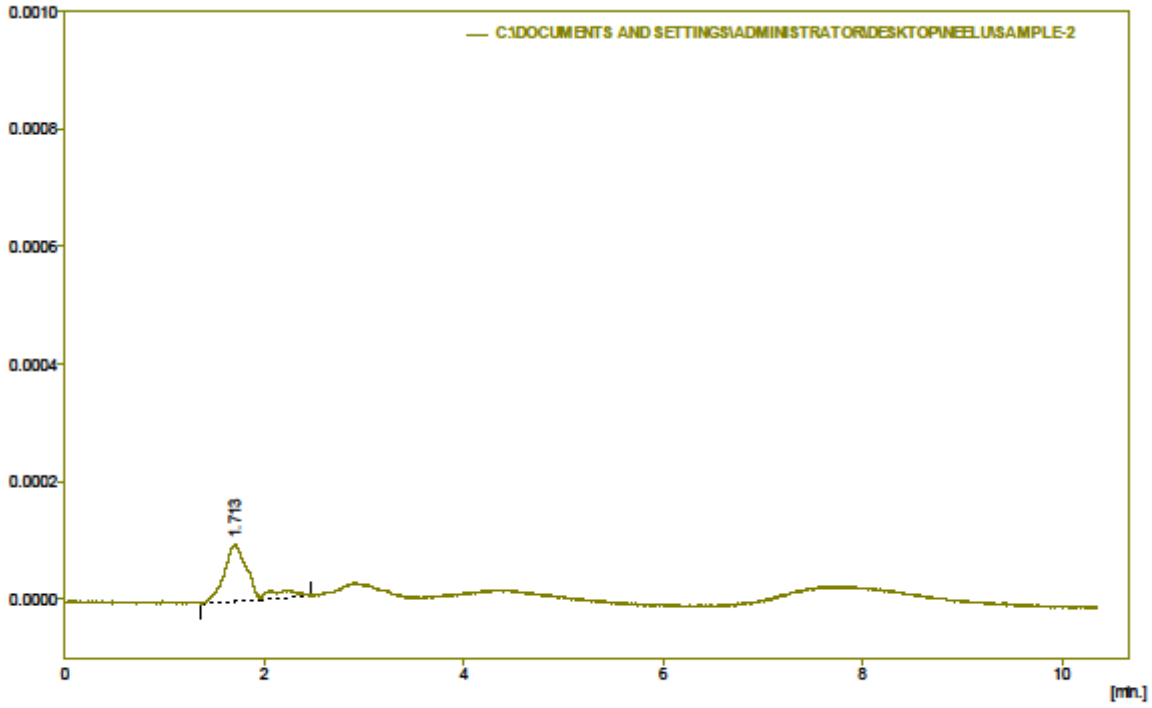
Result of sample-2

4/24/2014 10:19 AM Chromatogram C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\NEELU\SAMPLE-2.PRM Page 1 of 1

AZYME BIOSCIENCES PVT LTD

Column	:	Detection	:
Mobile Phase	:	Temperature	:
Flow Rate	:	Pressure	:
Note	:		

Autostop	: None	External Start	: Start - Restart, Down
Detector 1	: Signal 1	Range 1	: Bipolar, 1250 mV, 10 Samp. per Sec.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\NEELU\SAMPLE-2)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	1.713	1.744	100.0
	Total	1.744	100.0

Graph 3

Chromatogram showing retention times of the above mentioned samples:

4/24/2014 10:20 AM Chromatogram C:\Documents and Settings\Administrator\Desktop\neelu\sample-2.PRM Page 1 of 2

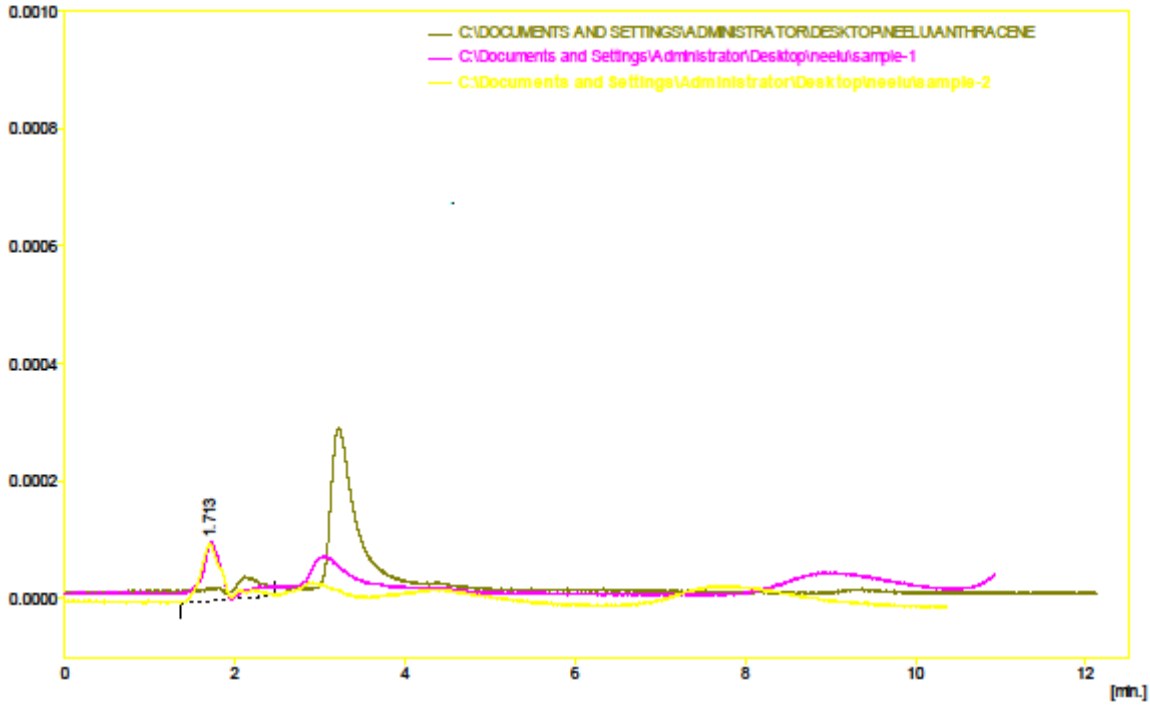
AZYME BIOSCIENCES PVT LTD

Column :
Mobile Phase :
Flow Rate :
Note :

Detection :
Temperature :
Pressure :

Autostop : None
Detector 1 : Signal 1

External Start : Start - Restart, Down
Range 1 : Bipolar, 1250 mV, 10 Samp. per Sec.



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\ADMINISTRATOR\DESKTOP\NEELU\ANTHRACENE)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	3.220	5.137	100.0
	Total	5.137	100.0

Result Table (Uncal - C:\Documents and Settings\Administrator\Desktop\neelu\sample-1)

	Reten. Time [min]	Area [mV.s]	Area [%]
1	1.713	0.988	48.4
2	3.037	1.054	51.6
	Total	2.042	100.0

Conclusion:

The present study reports the isolation, characterization and identification of a *Bacillus sp*, which is capable of utilizing anthracene as a source of carbon. Results from the present study suggest that the isolated *Bacillus sp* is able to grow in minimal salt medium in the presence of anthracene (100ppm). An enhancement in degradation ability with an exposure time of 15 minutes under UV light was also observed. The most potent degrader on mutation was found to be the one containing 100ppm anthracene which be used for bioremediation of anthracene - contaminated soil. These isolated strains of bacteria are highly adapted to the existing environmental conditions and thus could be effectively utilized for bioremediation and metabolic detoxification of cytochrome oxidase.

Therefore, it is concluded that the bacteria was a efficient degrader for anthracene and can be employed for the removal and containment of PAHs from contaminated ecosystems thereby leading to reduction of health risks associated with exposure to anthracene & other PAHs.

References

1. Boldrin B, Tiehm A, Fritzsche C: Degradation of Phenanthrene, Fluorene, Fluoranthene and Pyrene by a Mycobacterium *sp.*, Applied environmental microbiology, 1993, 59: 1927-1930.
2. Booker CD, and White KL: Benzo[a] pyrene induced anemia and splenomegaly in NZB/WF-1 mice, Food Chemistry & Toxicology, 2005, 43: 1423- 1431.
3. Bouchez M, Blanchet D, Vandecastelle JP: Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism, Applied microbiology & biotechnology, 1995, 43: 156-164.
4. Brezna B, Kweon O, Stingley RL, Freeman JP, Khan AA, Polek B, Jones RC, Cerniglia CE: Molecular characterization of cytochrome P450 genes in the polycyclic aromatic hydrocarbon degrading *Mycobacterium vanbaalenii* PYR-1. Appl Microbiol Biotechnol, 2006, 71: 522-532.
5. Ceringlia CE: Aromatic Hydrocarbons: Metabolism by bacteria, fungi and algae. In Review in biochemical toxicology, Edited by E. Hodyson, J. R. Bend, and R. M. Philpot. Elsevier North-Holland Inc; New York, 1981, 3: 321-361.
6. Cerniglia CE: Biodegradation of polycyclic aromatic hydrocarbons, Current opinion in biotechnology,1993, 4: 331- 338.
7. Cerniglia CE, and Crow SA: Metabolism of aromatic hydrocarbons by *Yeasts*, Archives in microbiology, 1981, 129:9-13.
8. Cerniglia CE, Sutherland JB, Crow, SA: Fungal metabolism of aromatic hydrocarbons. In microbial degradation of natural products, ed. G. Winkelmann. VCH Verlagsegesellschaft, Weinheim, 1992.
9. Cerniglia CE, and Heitkamp A: Microbial degradation of PAHs in the aquatic environment. In metabolism of PAHs in the Aquatic environment, ed. University. Varansi. CRC Press, Boca Raton, FL, 1989, 41-68.
10. Cerniglia CE, Campbell WL, Freeman JP, Evans FE: Stereoselective fungal metabolism of methylated anthracenes, Applied environmental microbiology, 1990, 56:661- 668.
11. Cerniglia CE, White GL, Heflich RH: Fungal metabolism and detoxification of PAHs, Archives in microbiology, 1985, 50: 649-655.
12. Cerniglia CE, Campbell WL, Freeman JP, Evans FE: Identification of a naval metabolite in phenanthrene metabolism by the fungus Cunninghamella elegans, Applied environmental microbiology, 1989, 55: 2275-2279.
13. Cerniglia CE: Initial reactions in the oxidation of anthracene by Cunninghamella elegans, Journal of general microbiology, 1982, 128: 2055-2061
14. EPA, US 1995. QA/QC guidance for sampling and analysis ofsediments, water, and tissues for dredged material evalations,Office of Water, Washington, DC, p. 297
15. EPA, US 1998. IRIS summary: anthracene. Office ofResearch and Development, http://www.epa.gov/iris/subst/0434.htm#woe
16. Evans WC, Fernley HN, Griffiths E: Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads, Biochemistry journal, 1996, 95: 819-831
17. Field JA, Vledder RH, vanZeist JG, Rulkens WH: The tolerance of lignin peroxidase and manganese-

dependent peroxidase to miscible solvents and the in vitro oxidation of anthracene in solvent:water mixtures. Enzyme Microb Technol 1996; 18:300–8

18. Faust, R.A., 1997.Toxicity summary for anthracene. ChemicalHazard Evaluation and Communication Group, Biomedical andEnvironmental Informtion Analysis Section, Health and SafetyResearch Division, http://www.risk.1sd.ornl.gov/tox/profiles/anthrace.shtml

19. Faust, R.A., 1997. Toxicity summary for anthracene. ChemicalHazard Evaluation and Communication Group, Biomedical andEnvironmental Informtion Analysis Section, Health and SafetyResearch Division, http://www.risk.1sd.ornl.gov/tox/profiles/anthrace.shtml

20. Hammel KE, Kalyanaraman B, Kirk TK: Oxidation of polycyclicaromatic hydrocarbons and dibenzo[p]-dioxins by Phanerochaete chrysosporium ligninase. J Biol Chem 1986, 261: 16948–52.

21. Hammel KE, Green B, Gai WZ: Ring fission of anthracene by a eukaryote. Proc Natl Acad Sci USA 1991, 88:10605–8

22. Hilyard EJ, Jones-Meehan JM, Spargo BJ, Hill RT: Enrichment, isolation, and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from elizabeth river sediments. Appl Environ Microbiol, 2008, 74: 1176-1182.

23. International Agency for Research on Cancer (IARC), Anthracene. Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Polycyclic Aromatic Compounds, Part 1. Chemical, Environmental, and Experimental data, World Health Oranization, Lyon, France, 1983, 32: 105–121.

24. Juhasz A, Stanley G, Britz M: Microbial degradation and detoxification of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia* strain VUN 10,003. Lett Appl Microbiol, 2000, 30: 396-401.

25. Kastner M, Breuer- Jammali M, Mahro B: Enumeration and characterization of the soil microflora from hydrocarbon- contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons, Applied microbiology & biotechnology, 1994, 41: 267-273.

26. Kleespies M, Kroppenstedt RM, Rainey FA, Webb LE, Stackbrandt E: Mycobacterium hodleri, sp. nov., a new member of the fast- growing mycobacteria capable of degrading polycyclic aromatic hydrocarbons, International journal of systematic bacteriology, 1996, 46: 683- 687.

27. Kim S, Kweon O, Freeman JP, Jones RC, Adjei MD, Jhoo J, Edmondson RD, Cerniglia CE: Molecular cloning and expression of genes encoding a novel dioxygenase involved in low- and high-molecular-weight polycyclic aromatic hydrocarbon degradation in *Mycobacterium vanbaalenii* PYR-1. Appl Environ Microbiol, 2006, 72: 1045-1054.

28. Kim S, Kweon O, Jones RC, Edmondson RD, Cerniglia CE: Genomic analysis of polycyclic aromatic hydrocarbon degradation in *Mycobacterium vanbaalenii* PYR-1. Biodeg, 2008, 19: 859-881

29. Lee PH, Ong SK, Golchin J, Nelson GL: Use of solvents to enhance PAH biodegradation of coal tar-contaminated soils. Water Res 2001, 35:3941–9

30. Mackay D, Shiu WY: Aqueous solubility of polynuclear aromatic hydrocarbons. J Chem Eng Data 1977, 22: 399–402

31. Ogino H, Ishikawa H: Enzymes which are stable in the presence of organic solvents. J Biosci Bioeng 2001, 91:109–16

32. Schneider J, Grosser R, Jayasimhulu K, Xue W, and Warshawsky D: Degradation of pyrene, Benzo[a] anthracene and Benzo[a]pyrene by Mycobacterium sp. strain RJG II -135, isolated from a former coal gasification site, Applied Environmental Microbiology, 1996, 62: 13-19.

33. Sutherland JB, Rafii F, Khan AA, Cerniglia CE: Mechanisms of polycyclic aromatic hydrocarbon degradation. In L.Y.Young and C.E. Cerniglia (ed.), Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York, 1995, N.Y, 269-306.

34. Sutherland JB: Detoxification of polycyclic aromatic hydrocarbons by fungi, Journal of industrial microbiology, 1992, 9: 53-62.
35. Shuttleworth KL, Cerniglia CE: Environmental aspects of PAH biodegradation. *Appl.* Biochem. Biotechnol, 1995, 54: 291-302
36. Tiehm A, Fritzche C: Utilization of solubilized and crystalline mixtures of polycyclic aromatic hydrocarbons by a Mycobacterium sp., Applied microbiology biotechnology, 1995, 42: 964- 968
37. Zeng J, Lin X, Zhang J, Li X. Isolation of polycyclic aromatic hydrocarbons (PAHs)-degrading *Mycobacterium* spp. and the degradation in soil. J Hazard Mater, 2010, 183: 718-723.