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Research Article

Isolation and characterization of anthracene degrading bacteria from soil contaminated with petroleum wastes

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ABSTRACT

Poly cyclic aromatic hydrocarbons are one of the most widespread organic pollutants. Among the polycyclic aromatic hydrocarbons (PAH), anthracene is highly carcinogenic. The vast amount of bacterial genomic data provides unparalleled opportunities for understanding the genetic and molecular bases of the degradation of organic pollutants In the present work, it is designed to isolate and identify anthracene degrading bacterial species from soil contaminated with wastes of petroleum industry. The efficiency of biodegradation capacity of isolated microorganism was measured by U.V spectroscopy. Soil samples contaminated with petroleum wastes were grown on MM9 medium. The cultures were then transferred onto the GSP agar medium for the presence of *Pseudomonas* species. The presence of yellow to red coloration indicated the presence of *Pseudomonas* species. Further, the isolates were identified on the basis of morphological, trophic and biochemical traits according to the Bergey's Manual of systemic bacteriology (1994). Chromosomal DNA is isolated from the isolated bacterial species. Spectrophotometric and electrophoretic analysis of the DNA has been done and compared with that *of Pseudomonas aeruginosa* ATCC 27853.DNA purity was estimated from $A_{280,}$ A_{260} and A_{230} values. Based on the percentage of cytosine to guanine content the isolated organism may be considered as *Pseudomonas stutzery*. Around 76% of anthracene was degraded by isolated pseudomonas species.

Keywords: Anthracene; PAHs; biodegradation; soil contaminated with petroleum wastes; *Pseudomonas*

INTRODUCTION

Interest in the microbial biodegradation of pollutants has increased in recent years. Bioremediation and biotransformation aroused to elimination of a wide range of pollutants and wastes from the environment (Kazuya Watanabe, 2001**).** The use of microorganisms to degrade aromatic pollutants is an interesting low-cost approach for the treatment of industrial sewage disposal, contaminated sediments, soil and groundwater. Typically, biodegradation is practiced by providing favorable environmental conditions in order to stimulate the removal of pollutants (Papazi A., 2008).

The poly cyclic aromatic hydrocarbons can be degraded by in environment include chemical oxidation, bioaccumulation and adsorption to soil particles, but the principal process for removal of PAH is thought to be microbial transformation and degradation (Heinonsalo J, 2000). Biological processes play a major role in the removal of contaminants. Polycyclic aromatic hydrocarbons are lipophilic and one of the most widespread organic pollutants.

PAHs have potentially deleterious effects on human health. Polycyclic aromatic hydrocarbons (PAHs) are toxic environmental pollutants that are known or suspected carcinogens or mutagens. (Jing-Liang Li, 2009). The microbial PAH degradation with special emphasis on both biological and physico-chemical factors influencing the biodegradation of poorly available PAHs is studied (Anders R. Johnsen, 2005). PAH are hydrophobic compounds with low solubility in water, they have a tendency to bind with organic matter or soil, limiting their availability to microorganisms. Despite these properties, many bacterial strains have been isolated for their ability to transform, degrade and utilize PAH as a source of carbon and energy (Leigh, M.B, 2006). Biodegradation of PAHs by fungi in contaminated soil containing cadmium and nickel ions were reported (Harrison Ifeanyichukwu Atagana, 2009). It is reported that Phenol and Benzoic Acid are degraded by *Pseudomonas aeruginosa* (Bared Razika, 2010). Biodegradation of beta-cyfluthrin is observed by *Pseudomonas stutzeri* strain (Nirmali saikia, 2005). Cyanide is a toxic nitrogen compound for almost every living organism since it binds irreversibly to haem-proteins. Pseudomonas pseudoalcaligenes is able to tolerate and

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degrade the cyanide present in the lixiviate from a gold mining industry located in Asturias, Spain (MIsabe Igeño, 2005).

Chemotaxis has been reported to play an important role in enhancing biodegradation as it increases bioavailability of pollutants to bacteria (Parales, R.E, 2000). Microorganisms inoculated into PAH-contaminated soil environments must find and mobilize PAH before degradation and hence motility and chemotaxis are thought to be desired properties (Turnbull, 2011).

Anthracene is a solid polycyclic aromatic hydrocarbon consisting of three fused benzene rings. It is a component of coal-tar (Dean-Ross.*et.al*. 2001). It has been recently included in the Substances of Very High Concern list (SVHC) by the European Chemicals Agency (ECHA). Anthracene, as many other PAHs, is generated during combustion processes. Exposure to humans happens mainly through tobacco smoke and ingestion of food contaminated with combustion products. Anthracene is absorbed following oral and dermal exposure. Targets for anthracene toxicity are the skin, hematopoietic system, lymphoid system, and gastrointestinal tract. Adverse dermatologic effects have been observed in humans and animals in conjunction with acute and sub chronic exposure to anthracene. In humans, anthracene may cause acute dermatitis with symptoms of burning, itching, and edema. Prolonged dermal exposure produces pigmentation, cornification of skin surface layers, and telangiectasis.

In the present work, it is proposed to isolate and identify anthracene degrading bacterial species from soil contaminated with wastes of petroleum industry. The efficiency of biodegradation capacity of isolated microorganism is proposed to be measured by u.v spectroscopy.

MATERIALS AND METHODS

Materials required

Soil sample contaminated with petroleum waste, MM9 medium, GSP agar medium, ethyl acetate, hexane, ethyl ether, NaCl, CTAB, phenol, chloroform, ethidium bromide, EDTA, Tris and boric acid.

Soil sampling

For the isolation of anthracene degrading bacteria, soil samples were collected from petroleum refinery in cherlapally, Hyderabad. All soil samples were stored at 4^0C for further study.

Isolation of and identification of anthracene-degrading bacteria

Bacterial strains were isolated from soil samples by selective enrichment culture in modified M9 minimal medium (MM9). Soil samples (5g) were added to 10ml sterile distilled water. Anthracene was dissolved in ethyl ether and added to sterilized flasks, forming a thin film of anthracene on the flask bottom after evaporation of the ethyl ether, and then MM9 Medium was added. Flasks were capped and then sterilized at 121°C for 15 minutes. Soil suspensions were centrifuged at 2000 rpm for 10 minutes. Supernants from these suspensions were collected and 500 micro liter of each aliquot was then inoculated in to 100 ml MM9 medium. Flasks were incubated at 30°C in agitated conditions at 150 rpm using orbital shaker. Cultures were maintained in this medium for seven days. The cultures were plated on to glutamate starch phenol-red (GSP) agar for selection of pseudomonas genus. After the incubation at 30°C for 24 hours, colonies that showed a red precipitation were selected for identification and further characterization. The isolates were identified on the basis of the Bergey's Manual of systemic bacteriology (1994). Bacteria were maintained on MM9 agar slants at 4°C for further studies. The arginine deiminase system ("dihydrolase") catalyzes the conversion of arginine to citrulline and of citrulline to ornithine. It was used to identify the isolated species.

Physiological and Biochemical Tests

Test for nitrate reduction, casein hydrolysis, starch hydrolysis, gelatin hydrolysis, lipase production, L-lysine -decorboxylase production, indol production, hydrogen sulphite reduction, Utilization of citrate as carbon source and Pseudomonadaceae specific pigments production (pyocyanine, and fluoresceine) were performed (Aysel Uğu1, 2012).

The test for arginine-deiminase system

Peptone-starch-dextrose broth supplemented with 0.1% arginine hydrochloride was used to test for arginine deiminase and ornithine carbamoyl transferase activities. 50 pl of a cell suspension was added to 150 pl of each test medium, and the preparations were incubated at 37°C for 2 hrs. An inoculum from above culture was transferred aseptically to a sterile tube of ornithine decorboxylase broth. The inoculated tube was incubated at 35-37 C for 24 hours and the preliminary results were determined. The microbe must first use the glucose present to cause the p*H* to drop. This is indicated by a change from purple to yellow. Once the medium has been acidified, the enzyme ornithine decorboxylase is activated. The culture is incubated *an additional 24 hours* at 35-37 C to allow the microbe to now use the ornithine. The final results are then obtained by observing the tube at 48 hours. Change back to purple from yellow indicates a positive test for ornithine decorboxylase. Failure to turn yellow at 24 hours or to revert back to purple at 48 hours indicates a negative result (Jorge Lalucat, 2006).

Isolation and purification of chromosomal DNA

Genomic DNA was isolated from 3 ml of overnight culture (28^oC in liquid LB, 250 rpm). After harvesting, cells were resuspended in 700 µl TEG pH 8.0 (Tris 25 mM, EDTA 10mM, Glucose 50mM). Cellular walls were bro-

Figure 1: Morphological characterization of Pseudomonas stutzery

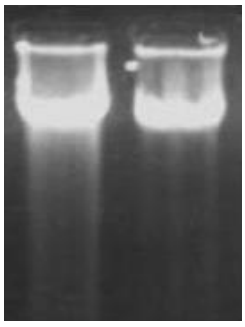

Figure 2: Agarose gel electrophoresis of genomic DNA

Lanes: 1.Isolated Organism; 2.*Pseudomonas aeruginosa*ATCC27853

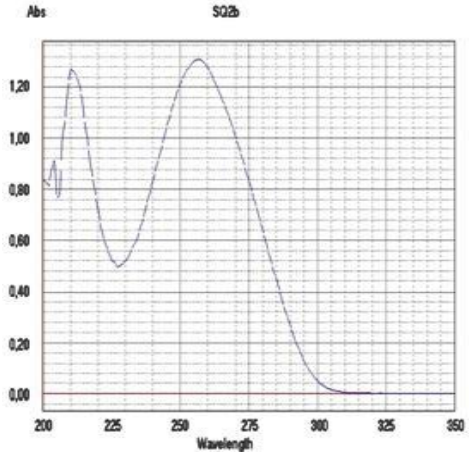

Figure 3: Wave scan spectrum of chromosomal DNA of Pseudomonas. aeruginosa ATCC 27853

ken using thermal shock between -70°C and +42°C for 20 min, repeated for 3 times. Cell lysis was performed with SDS 20%. Protein precipitation was performed with NaCl 5M and CTAB/NaCl 10%/ 0.7% (v/v) by incubation at 65°C for 15 minutes. The deproteinization was done with isoamylic alcohol/ chloroform 24:1 (v/v) and phenol/chloroform 1:1 (v/v).

Nucleic Acids Electrophoresis

Electrophoretic analysis of DNA was performed using horizontal submerse agarose gel 1 %(w/v) in TBE buffer (Tris 0.089M, boric acid 0.089M, EDTA 0.002M, pH 8.5). Electrophoresis was run at 2.5V/cm and DNA stained with ethidium bromide 0.5 µg/ml.

Spectrophotometric Analysis of DNA

Spectrophotometric analysis of chromosomal DNA was carried out with an UV-VIS ULTROSPEC 3000 (Pharmacia-LKB) spectrophotometer. Absorption spectra were obtained for wavelength ranging between 200 and 350 nm. DNA purity was estimated from A_{280}, A_{260} and A_{230} values. Contamination was considered to be minimum for A_{260}/A_{280} values ranging between 1.8 and 2.0 and, respectively, greater than 2.0 for A_{260}/A_{230}.

Determination of Molar Percentage of Guanine plus Cytosine

In order to determine the Tm values of chromosomal DNA, we used a UV-VIS ULTROSPEC 3000 (Pharmacia-LKB) equipped with a Peltier unit and SWIFT-Tm v1.05 program. DNA denaturation was performed in a temperature range between 20-100°C with a rate of 1°C/min and the DNA absorbance values at λ= 260nm have been continuously monitored. Molar percentage guanidine plus cytosine (mol % GC) was calculated using Owen's formula: % mol GC =2.08 T_m =106.4.

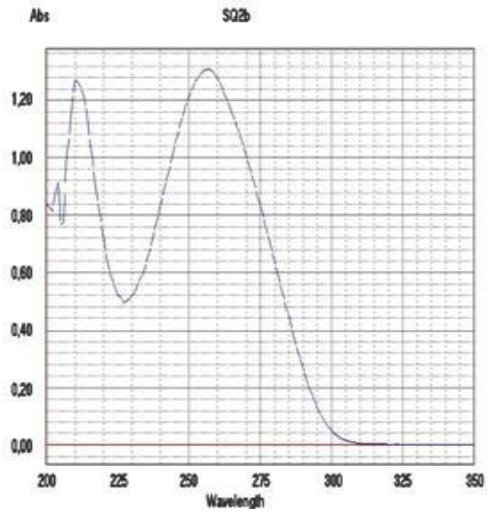

Figure 4: Wave scan spectrum of chromosomal DNA of isolated microorganism

Biodegradation testing

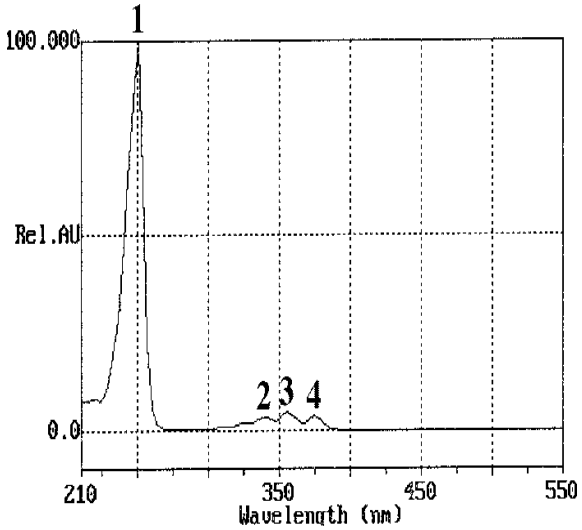

Figure 5: UV-Spectrum of anthracene in ethyl acetate

Anthracene degradation experiments were conducted in flasks containing 100 ml of MM9 medium. Anthracene was added to MM9 liquid media to final concentration of 500mg/ml. Inocula for all experiments were prepared by diluting the recovered cultures with desired media to give a Spectrophotometric reading of 0.05 at 600nm. Cell suspension (500micro liters) was inoculated in to a 100 ml MM9. The cultures were incubated on orbital shaker at 150rpm at 30°C for 7 days.

Anthracene extraction from culture medium

Two ml of hexane were added to 5ml MM9 medium in screw cap glass tubes that were then shaken several times. 1 ml of the upper phases (hexane) were transferred to clean tubes and evaporated on a rotary evaporator. The remaining pellets were dissolved in 2 ml of ethyl acetate and stored at 4°C.

U.V. Spectrophotometric analysis

The λ max of anthracene in ethyl acetate was determined to be 254 nm. It was used to determine the concentration of anthracene in ethyl acetate extract. The λ max was used to determine the concentration of anthracene in ethyl acetate extract.

RESULTS AND DISCUSSION

Soil contaminated with petroleum wastes causes serious threat to groundwater. Bioremediation offers a promising means to reclaim such contaminated soil. Bioremediation employs microorganisms capable of degrading toxic contaminants. Among the polycyclic aromatic hydrocarbons (PAHs) anthracene represents one of the most widespread concerns because of its toxicity to biological functions (DoyleE, 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. It has melting and boiling points of 218 and 354°C, respectively (RamsayJA, 2003). The productive metabolism of anthracene occurs through the formation of 1-hydroxy-2 naphthoic acid, which is further transformed to salicylic acid. Many of the micro organisms have the ability to degrade naphthalene, a two ring PAH. Therefore, it is quite evident to characterize and

Table 1: Spectrophotometric analysis of chromosomal DNA samples

Strain	A230	A260	A280	A260/ A230	A260/ A280	µg DNA/µl
Ps. aeruginosa ATCC 27853	0.45	1.15	0.55	2.55	2.1	11.5
Isolated organism	0.5	1.35	0.40	2.70	2.3	13.5

Table 2: Values of molar % G+C of the strains studied

Strain	Tm	Molar % G+C
Ps. aeruginosa ATCC 27853	79.7	59.4
Isolated organism	79.3	58.5

investigate the microorganisms that are able to utilize anthracene. (HadibarataT, 2012).

Soil samples contaminated with petroleum wastes were grown on MM9 medium. The cultures were then transferred onto the GSP agar medium for the presence of Pseudomonas species. The presence of yellow to red coloration indicated the presence of *Pseudomonas* species. Further, the isolates were identified on the basis of morphological, trophic and biochemical traits according to the Bergey's Manual of systemic bacteriology (1994).

Microscopically, cells of isolated microorganism were gram-negative short rods, single cells, or in pairs star-shaped, dark-yellow, rough-surfaced colonies (Figure 1). Positive results were obtained for nitrate reduction, starch hydrolysis, gelatin hydrolysis, lipase production and utilization of citrate as carbon source performed. The arginine deiminase system ("dihydrolase") catalyzes the conversion of arginine to citrulline and of citrulline to ornithine. No yellow colour is observed after 24 hours incubation when inoculum was grown in ornithin decorboxylase medium. By the literature review it was revealed that All *P. stutzeri* strains give a negative test result for this reaction. Hence it can be analysed that the isolated microorganism may be *Pseudomonas stutzery.*

Spectrophotometric and electrophoretic analysis revealed that chromosomal DNA was highly concentrated (Figure 2).Wave scan spectrum of Chromosomal DNA of *Pseudomonas aeruginosa* and isolated micro organism were recorded (Figure 3 and Figure 4). In Spectrophotometric analysis of DNA, A_{260} readings should be between 0.1 and 0.5 to be considered valid. Readings outside these ranges may not accurately reflect the DNA concentration. If higher than 0.5, you may consider performing another measurement on a more dilute sample. If it is lower, different dilution of samples should be prepared for measurement. The latter may not be possible with a limited sample volume and the low measurement may be considered acceptable depending upon the procedure for which the DNA will be used. The concentration is given in µg/ml, which is equivalent to ng/µl. The purity of the DNA sample can be gauged by the A_{260}/A_{280} ratio. Pure DNA has an A_{260}/A_{280} ratio of 1.8-2.0. Readings below 1.6 indicate significant amounts of contamination, mainly protein. For the isolated bacterial strain the A_{260}/A_{280} value is found to be 2.3.

Pseudomonas aeruginosa strains found to contain 11.5 µg/ µl of DNA where as isolated strain contains 13.5 µg/µl (Table1). Molar Percentage of Guanine plus Cytosine were determined for isolated organism. Results obtained for the isolated strain shows 58.5 % GC content that are in ranging between 58-69 for *Pseudomonas stutzeri* and 58-69 for *Pseudomonas aeruginosa* ATCC 27853 (Table 2).

These results proves that the isolates micro organism belongs to the *Pseudomonas stutzeri* but further analysis, especially at a molecular level is necessary, for a more accurate taxonomic identification.

From the literature it was found that the λ max of anthracene in ethyl acetate was 254 nm by u.v. spectroscopy. Hence, it was used to determine the concentration of anthracene in ethyl acetate extracts. A single peak at 254 nm showd the maximum absorbance when ethyl extract of anthracene was taken as sample (Figure 5). Hence anthracene was confirmed in the sample. It was found that around 76% of anthracene was degraded by isolated pseudomonas species.

CONCLUSIONS

The results obtained in this work indicates that Pseudomonas species is involved in the degradation of poly cyclic aromatic hydrocarbons especially anthracene. The isolated microorganism may be *Pseudomonas stutzery.* Further molecular analysis including 16S r RNA sequencing has to be done to get more accurate results.

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