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Biodegradation of anthracene by diazotrophic bacteria *Azotobacter vinelandii*

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ABSTRACT

The present study deals with the anthracene degradation ability of diazotrophic bacteria. Twelve diazotrophic isolates having anthracene degradation ability were isolated from pertroleum contaminated sites. Potential isolate was selected on the basis of growth rate and percent degradation. This isolate was identified as *Azotobactor vinelandii*. Growth of *Azotobactor vinelandii* in a mineral salt medium with 0.178g; 1mmol of anthracene as a sole carbon source resulted cell density at 96 h with O.D. value 0.27. At the end of 96 h. cell count were 1.0×10^{10} CFU mL^{-1} and 2.5 mg mL^{-1} of biomass. HPLC analysis of ethyl acetate extract at the end of 96 hours showed 40.74% degradation of anthracene. HPLC elution profile showed presence of anthracene degradation metabolites. GC-MS analysis of extract indicated degradation of anthracene with the formation of anthracene degradation products.

KEYWORDS

PAH;
Biodegradation;
Bioremediation;
Diazotrophs;
Anthracene.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHS) are common environmental pollutants, PAHs are a diverse group of compounds composed of two or more fused aromatic rings, which can have petrogenic, pyrogenic or biogenic origin[1]. Their presence in soils and sediments poses a significant risk to the environment, and they have cytotoxic, mutagenic and in some cases carcinogenic effects on human tissue[2-4]. The persistence of PAH in the environment is largely due to their low aqueous solubility and low availability[5].

Anthracene, a tricyclic PAH, is found in high amounts in PAH contaminated environments[6-8]. It is not itself genotoxic or carcinogenic[9], but it does represents a threat to the environment due to its toxicity to the aquatic life, particularly via photo induced toxicity[10]. Anthracene and phenanthrene are considered prototypic PAH and serve as signature compound to detect PAH contamination, since their chemical structures are found in carcinogenic PAH such as benzo[a] pyrene and benz[a] anthracene[11]. They have also been used as model PAH to determine factors that effect bioavailability, biodegradation potential and rate of microbial degradation of PAH in the environment[12-14]. Hence anthracene was used in the present studies.

A conventional physical remediation process like incineration land filling and chemical degradation have

been employed from time to time but these are expensive and inadequate for large scale application and does not successfully degrade many wastes[15]. Hence the recent focus is on bioremediation[16]. Bioremediation is defined as the process in which microorganisms are stimulated to rapidly degrade hazardous organic contaminant to environmentally safe level[17].

The biodegradation of hydrocarbons in the marine environment is influenced by the availability of nutrients such as nitrogen and phosphorus[18,19]. The addition of inorganic nitrogen and phosphorous stimulated the degradation of PAH in topsoil and aquifer sand[20,21]. To overcome the problem of nitrogen limitation, the present study was undertaken with diazotrophic bacteria *Azotobactor vinelandii*.

EXPERIMENTAL

Isolation and identification of diazotrophic anthracene degrading bacteria

Soil samples were collected from petroleum contaminated sites. One gram of soil sample transferred to a conical flask containing 50 mL of nitrogen free mineral medium [K_2HPO_4, 1.0g; $MgSO_4.7H_2O$, 0.2g; $FeSO_4.7H_2O$, 0.051g; $CaCl_2.2H_2O$, 0.1g; $Na_2MoO_4.2H_2O$, 0.001g; Glucose, 10g and distilled water 1000 mL, pH 7.00]. The flasks were incubated in a rotary shaker at 150 rpm and 30^{0}C for 96 hours. One mL of enriched culture was transferred to the flask containing nitrogen free mineral medium with 0.178g; 1mmol of anthracene as a sole carbon source and incubated on rotary shaker at 150 rpm and 30^{0}C for incubation period ranging from 5 days to 8 days. Pure anthracene degrading strains were obtained by plating dilution of enrichment broth on nitrogen free mineral agar plates containing 0.178g; 1mmol of anthracene.

Determination of anthracene degradation ability

The ability of isolated strains for degradation of anthracene, tested with a modification of Kiyohara's method[22]. Isolated colonies were transferred to the plates of nitrogen free mineral agar medium whose surface was previously sprayed with 1% anthracene solution in acetone : Hexane [1:1 $^v/_v$] after evaporating the organic solvent. After incubation 30^{0}C for 48 to 72 hrs, anthracene utilizing cultures were identified by the presence of clear halos around the colony indicating utilization of anthracene. The isolated anthracene degrading microorganisms were identified by using standard microbiological methods[23].

Study of growth and biodegradation of anthracene

Isolates were cultured in conical flask containing 50 mL of nitrogen free medium with 0.178g; 1mmol anthracene as a sole carbon source. The anthracene had been added to the bottom of the flasks as a solution in hexane which was left to evaporate before the addition of the medium and sterilization. All flasks were inoculated with 1ml of inoculum which was in exponential growth phase and having O.D. 0.3 at 610 nm.

Initial viable cell population CFU mL^{-1} was determined by standard plate count method. Flasks were incubated in a shaker at 150 rpm and 30^{0}C. Sampling was made at an interval of 24 h. for estimation of growth. Growth studies were made by measuring optical density of the culture at 610 nm and cell count CFU mL^{-1} estimated by direct plate count method.

When cultures attained its early stationary phase 96 hours of incubation, it is acidified to pH 2.5 and extracted with ethyl acetate. Further treated with 5% $NaHCO_3$, 5% NaOH and finally solvent layer separated, allowed to evaporate. The residue dissolved in methanol. O.D. of the appropriate diluted sample determined by using UV/visible spectrophotometer at 254 nm. The percentage of degradation was calculated from the standard curve.

HPLC

The residue dissolved in methanol at suitable dilution was subjected reversed phase HPLC. Using Younglin Isocratric liquid cromatography system. Model no. Acme 9000 equipped with rhedyne injector with 20 mL fixed loop, UV/Visible detector model SP 930d. The C-18 column varian (250 × 4.6 mm, partical size 5m) was used as stationary phase.

GCMS analysis

Analysis of residue dissolved in methanol also performed on gas chromatograph – Perkin Elimer Autosystem with Turbomass, column PE – 5 MS (30 meters × 0.250 microns thickness × 250 micron dimeter), carrier – Helium, injector temperature 250, oven temp : 100 for 5 min, Run time 40 minutes, El

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source temp : 250, injectionvolume -0.2 microlitre.

RESULTS AND DISCUSSION

Isolation and identification of diazotrophic anthracene degrading bacteria

Colonies that grow on anthracene (0.178g; 1mmol) containing nitrogen free mineral agar and that showed clearing of anthracene (halo) were isolated in pure culture and characterized. Among twelve isolates three isolates were rapid grower and are gram negative, cysts forming bacteria belonging to the genus *Azotobacter*. Isolates identified as *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Azotobacter beijerinckii*.

Study of growth and biodegradation of anthracene

Isolates from the genus Azotobacter were subjected for growth study. Amount of growth measured in terms of O.D. of culture at 610 nm at the interval of 24 hours upto 96 hours. *Azotobacter vinelandii* showed maximum cell density with O.D. value 0.27 at the end 96 hours (Figure 1). The cell count was 1.0×10^{10} CFU mL^{-1} and biomass was 2.5 mg mL^{-1}.

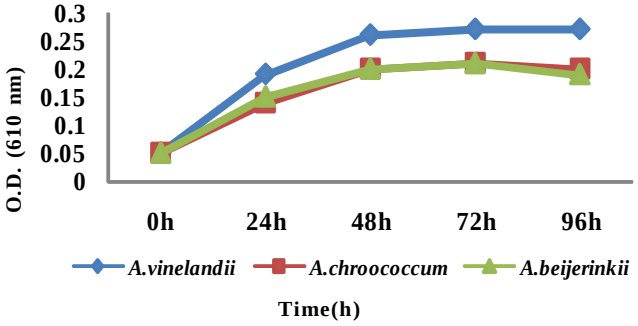

Figure 1 : Bacteria growth in the presence of anthracene as sole carbon source

Growth rate during exponential growth phase at 48 hr. was 0.31 h^{-1}. Residual concentration of anthracene determined by UV/visible spectrophotometer by monitoring O.D. at 254 nm, indicated maximum 40% degradation of anthracene by *Azotobactor vinelandii* hence used for further study.

HPLC: HPLC analysis indicated 41.74 percent degradation of anthracene at the end of 96 hours of incubation. HPLC elution profile revealed the presence of several metabolites. Two metabolites having retention times of 8.23 and 4.26 identified as peak I, II respectively (Figure 2).

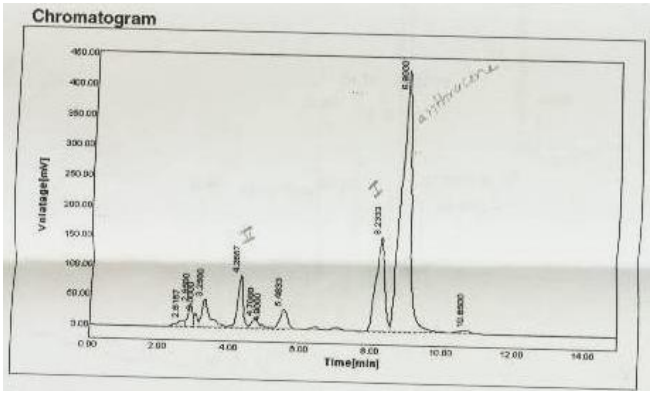

Figure 2 : HPLC elution profile of ethyl acetate extractable metabolites produced during the growth of azotobactor vinelandii in the presence of anthracene.

GC-MS: GC-MS analysis of extract revealed the presence of several degradation products. Metabolites having retention time 16.91, 19.78, 22.05, 26.38, 26.99 are probably – 9.10 anthraquinone, Phenol 2 – (l – Phenyl ethyl), Phthalic acid dibutyl ester, phenol 2, 4 Bis (1 – phenyl ethyl) and O – benzoyl benzene (Figure 3).

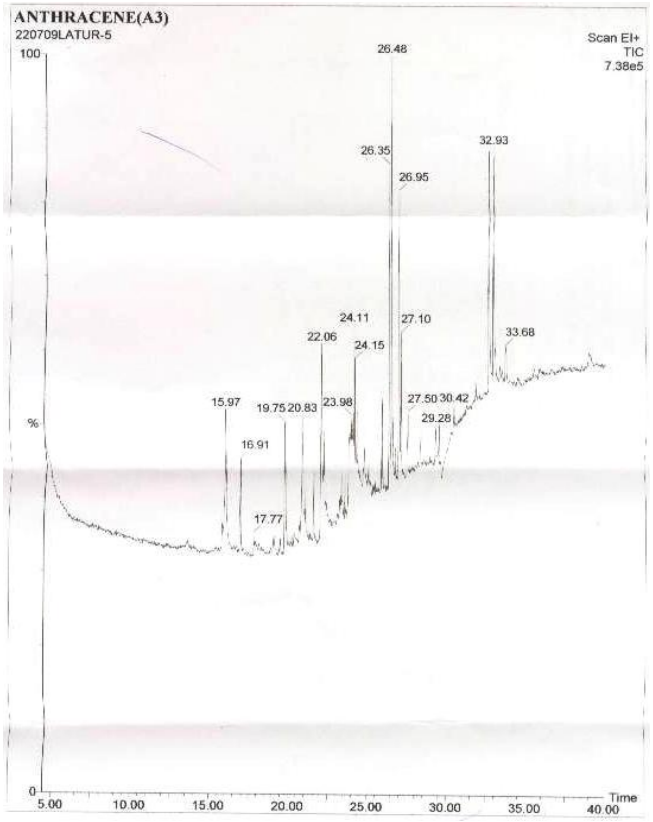

Figure 3 : GC-MS elution profile of ethyl acetate extractable metabolites produced during the growth of azotobactor vinelandii in the presence of anthracene.

Moody *et.al.*[24] reported that M. vanabaalenii PYR-1 can catalyze 9, 10 – hydroxylation of anthracene to produce anthracene – 9, 10 – dihydrodiol, which is coverted to 9, 10 – dihydroxy – anthracene, which is spontaneously tautomerises to 9, 10 – anthroquinone.

R.Van Herwijnen *et.al.*[25] reported accumulation of O-phthalic acid and protocatechuic acid as anthracene degradation products by *Mycobacterium sp*. and suggested existence of anthracene degradation pathway through O-phthalic acid.

The results of present study when compared with these reports indicated that *Azotobacter vinelandii* possess very high potential to degrade anthracene. This microorganism is of special interest in contributing nitrogen economy of both terrestrial and marine ecosystems polluted with PAH. Thus *Azotobacter vinelandii* could contribute substantially to the nitrogen requirement of PAH degrading bacteria in the PAH polluted sites.

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