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Microbial Degradation of Aromatic hydrocarbon: Naphthalene through *Nocardiopsis alba* RD3

Ruby Doley[1], Manoj Barthakur[1*] and Bijoy S. Goswami[2]

[1]Department of Botany, B.Borooah College, Guwahati, India
[2]Department of Chemistry, B.Borooah College, Guwahati, India
**Corresponding author*

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A B S T R A C T

Microorganisms play a vital role incleaning up the environment by degrading the pollutants like monoaromatics andpolycyclic aromatic hydrocarbons(PAHs) which are toxic andare among the most prevalent and persistent pollutants inthe environment. Soil contaminated with petroleum generally contains a mixtureof polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatics. Aromatic compoundsderived from industrial activities often have functional groups such as alkyls, halogens andnitro groups.Naphthalene is a polycyclic aromatic hydrocarbon (PAH) which is persistent in the environment and is toxigenic, carcinogenic and mutagenic that has motivated scientists in putting efforts to remove it from the environment. Incomplete combustion of fossil fuels releases them into the environment thereby polluting it. Bioremediation is one of the natural processes that help to remove xenobiotic compounds from the environment with the help of microorganisms. The aim of our study is to isolate some potential microbial agents to degrade naphthalene into non-hazardous or less hazardous derivatives.A bacterial strainwas isolated from oil contaminated soil of Guwahati Refinery, labeled as RD3, and later identified as *Nocardiopsis alba*,was found tolerant to naphthalene in culture media and wasable to degrade the naphthalene into its derivatives.The productsthat were detected in naphthalene culture broth were identified mainly as acetic acid andphthalic acid. However, the ring fission products,2-dodecan-1-yl-succinic anhydride and octadec-9-enoic acid were also detected as transformation products, which confirmed the degradation of naphthalene by the bacterial strain.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) contain two or more benzene rings in their structure, which are hydrophobic and have lower solubility than the mono aromatics. PAHs are generally contaminating the environment as a result of oil refinery discharge, accidental oil spill or weathering of creosote-impregnated pylons (Coates, 2004). Naphthalene is an organic compound with chemical formula$C_{10}H_8$. It is the simplest polycyclic aromatic hydrocarbon, and is a white crystalline solid with a characteristic odour that is detectable at concentrations as low as 0.08 ppm by mass. As an aromatic hydrocarbon, naphthalene structure consists of a fused pair of benzene rings. It is best known as the main ingredient of traditional mothballs. Naphthalene is the most abundant single component of coal tar. Although the composition of coal tar varies with the coal from which it is produced, typical coal tar is about 10% naphthalene by weight. In industrial practice, distillation of coal tar yields oil containing about 50% naphthalene,

along with twelve other aromatic compounds. Naphthalene is one of the 16 PAHs classified as priority pollutant by US environmental Protection agency (USEPA, 1994, 2004). Exposure to large amounts of naphthalene may damage or destroy red blood cells, most commonly in people with an underlying G6PD (glucose-6-phosphate dehydrogenase) deficiency.

It is well known that microorganisms can degrade environmental pollutants. The continuous search for ways of cleaning up the environment has revealed a diverse range of microorganisms that can utilize these compounds as substrates and thereby convert them into less toxic products. It is well known that microorganisms play the central role in nutrient cycling and at one time, their vast enzymatic capacity was considered to be infallible (Alexander, 1999). Use of microorganisms to degrade environmental pollutants which is called bioremediation is one of the important ways to remove environmental contamination. Bioremediation is generally considered a safe and less expensive method for the removal of hazardous contaminants and production of non-toxic by-products (Ward *et al.,* 2003). Bioremediation process involves detoxification and mineralization, where the waste is converted into inorganic compounds such as carbon dioxide, water and methane (Varsha *et al.,* 2011). A number of authors have reported the degradation of naphthalene through application of different microbial agents. Mittal and Singh (2009) measured the microbial degradation value of polycyclic aromatic hydrocarbon like Naphthalene through Gas Chromatography. Kafilzadeh *et al.,* (2011) isolated 5 bacterial species belong to *Staphylococcus* sp. *Corynebacterium* sp. *Pseudomonas* sp. *Bacillus* sp. And *Micrococcus* sp. and reported that all these strains were significantly able to degrade naphthalene. Pawar *et al.,* (2013) characterized Gram positive and Gram negative bacteria belonging to genus *Micrococcus* sp, *Bacillus* sp, *Staphylococcus* sp and *Pseudomonas* sp isolated from marine and petroleum soil samples which showed degradation of naphthalene. A review on Microorganism as a tool for bioremediation technology using designed and developed laboratory Bioreactors for cleaning environment was conducted by Singh *et al.,* (2014).

In this study we used naphthalene, which is an aromatic polycyclic hydrocarbon and considered among the 16 PAHs classified as priority pollutants by USEPA. A bacterial strain was isolated from oil contaminated soil of Guwahati Refinery, Assam and identified as *Nocardiopsis alba*. Here, the process of degradation of naphthalene by our isolate has been studied and also the transformation products were identified by GC-MS.

Materials and Methods

Isolation and identification of bacterial strain

The oil contaminated soil sample was collected from Guwahati Oil Refinery, Assam, India. The soil sample was collected in sterile polythene bag and brought to the laboratory for microbial isolation. The stock solution was prepared by mixing 1 gm of soil in 100 ml sterile distilled water and serially diluted. Dilutions below 10^{-6} were inoculated into nutrient broth (pH 7.4) and incubated at 37°C for 7 days (Singh and Pandey, 2013). Characterization and identification of the bacteria was done by biochemical tests and16s rDNA sequence analysis through homology sequence and phylogenetic analysis.

DNA was extracted from the culture and its quality was evaluated on 1.0% agarose gel.

Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. 16srDNA sequencing was carried out by Eurofin Genomic, Bangalore, India.

The sequence obtained was compared with the nucleotide sequences of NCBI by using BLAST algorithm. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs. Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

Determination of growth at different concentration of naphthalene

Biodegradation ability of the isolated strain was studied using 100 ppm concentration of naphthalene in 100 ml nutrient broth and incubated at 37^0C. Within interval of 24 hours, 5 ml sample were collected periodically from the flask and the OD measured at 600 nm in UV spectrophotometer.

Determination of naphthalene degradation of isolate

Naphthalene degrading ability of the isolate was monitored by spectrophotometric method as used by Pawer *et al.,* (2013). 1 ml suspension of the isolate was inoculated in 100 ml nutrient broth containing 80 ppm and 100 ppm of naphthalene as the sole source of carbon.

Culture broth was incubated at 37^0C and naphthalene degradation ability of the isolate was monitored periodically at 24 hours intervals through UV spectrophotometer by taking the absorbance at 365 nm.

Sample preparation for FT-IR and GC-MS Analysis

To examine the naphthalene degradation, the bacterial strain was inoculated in nutrient broth supplemented with 80 ppm and 100 ppm naphthalene respectively and incubated at 37^0C. After 9 days of incubation the culture broths were centrifuged separately at 15000rpm. The supernatant were extracted in 50 ml n-hexane each and filtered through Whatman No. 42 filter paper. The filtrate was dried under vacuum. The residues thus obtained were dissolved in 3 ml n-hexane and used for FT-IR and GC-MS analysis. FT-IR spectra were recorded using KBr pellets in order to confirm the intermediate metabolites.

Results and Discussion

Isolation of bacteria

A total of 13 bacterial strains were isolated from the soil sample, however the strain designated as RD3 was found best for tolerance of naphthalene in culture broth (data not shown) and thus it was considered for further experiments.

Bacterial identification by 16S rDNA analysis

Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. PCR amplification of the 16S rDNA gene produced fragments of approximately 1500 base pairs in size (Fig. 1). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA gene sequence was used to carry out BLAST with the database of NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs

Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4. Identification of the strains by 16S rDNA gene sequence analysis revealed that the strain shows 100% similarity with *Nocardiopsis alba.* Therefore the isolated bacterial strain has been identified as *Nocardiopsis alba* strain.

Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4. Identification of the strains by 16S rDNA gene sequence analysis revealed that the strain shows 100% similarity with *Nocardiopsis alba.* Therefore the isolated bacterial strain has been identified as *Nocardiopsis alba* strain.

Naphthalene degradation by *Nocardiopsis alba* strain

The bacterial strain *Nocardiopsis alba* RD3 grows in the culture broth utilizing naphthalene as the sole source of carbon and energy. Bacterial growth after the lag phase (Fig. 1) increases exponentially indicating that naphthalene has been utilized for cell growth. Residual naphthalene in the culture broth decrease simultaneously from 72 hrs onwards along with the cell growth (Fig. 2). Thus there is a correlation between the cell growth of the bacteria and degradation of naphthalene. The bacteria utilize naphthalene as carbon source rapidly in the exponential growth phase and thereby the concentration is depleted in the culture broth.

FTIR spectrum of hexane extract of culture broth supplemented with 60ppm (Fig. 3) naphthalene exhibited the characteristic peaks at 2984 cm^{-1} and 2834 cm^{-1} indicating the presence of $–CH_3$ and $–CH_2$ in aliphatic antisym and sym, peak at 2724 cm^{-1} suggested the presence of carboxylic acid O-H stretching, whereas the peaks at 1452 cm^{-1} and 1373 cm^{-1} showing –CH bending and peak at 1145 cm^{-1} suggested the presence of alkylated benzene in the extract. Similarly, 100 ppm (Fig. 4) naphthalene culture broth also showed characteristic peaks of carboxylic acid and C-O groups at around 2727 cm^{-1} and 1379 cm^{-1} respectively. The absence of such peaks at these regions in the control hexane extract supplemented with 60 and 100 ppm naphthalene (without inoculation of bacteria) reveals the degradation of naphthalene by the bacterial strain (Figs. 3 and 4).

Fig.1 gDNA 16s PCR amplicon

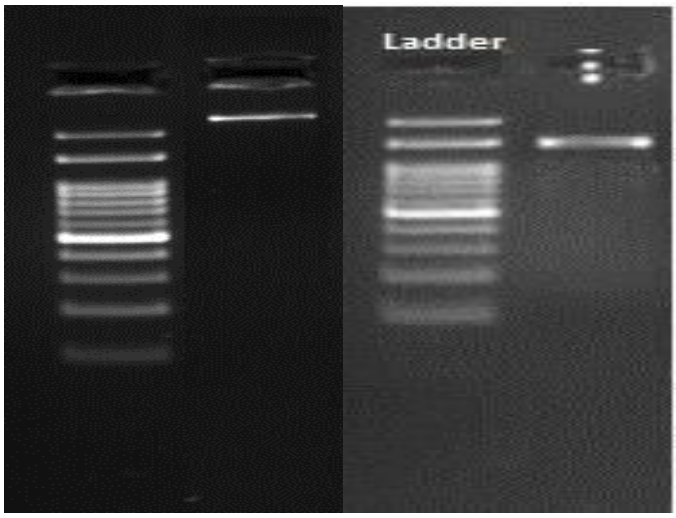


Fig.2 Phylogenetic tree of *Nocardiopsis alba*

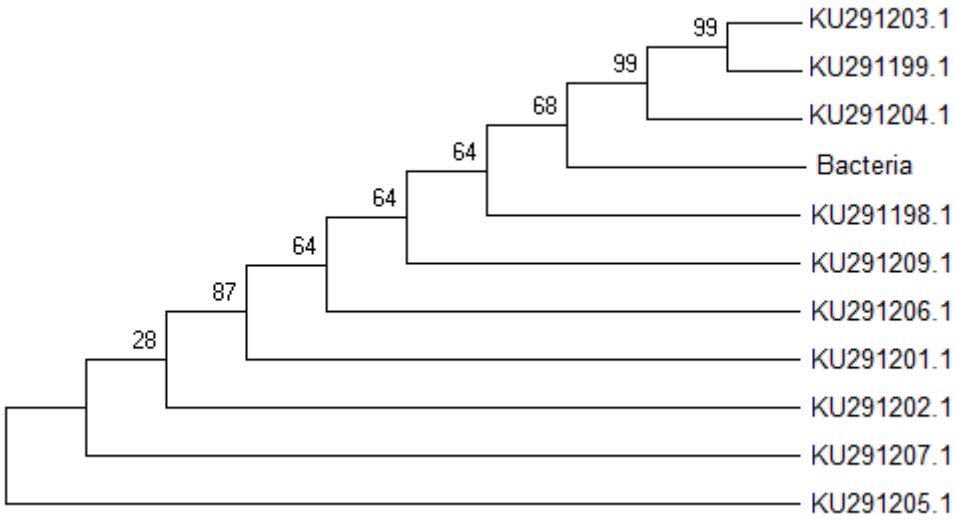


Fig.3 Growth of *Nocardiopsis alba* (in 100 ppm naphthalene broth) against time of incubation

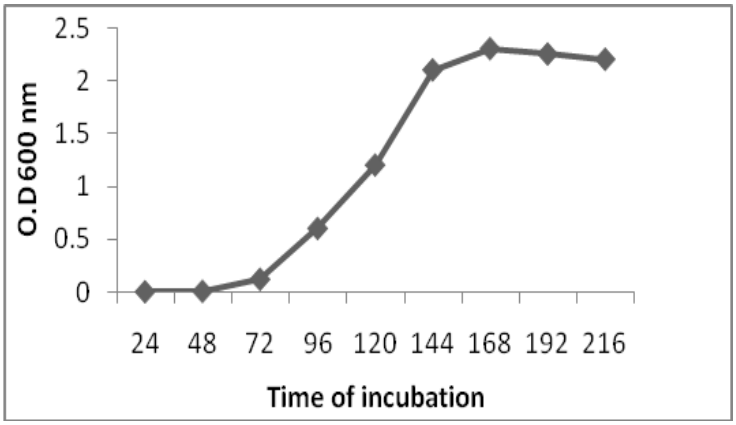


Fig.4 Growth of *Nocardiopsis alba* (in 100 ppm naphthalene broth) against time of incubation

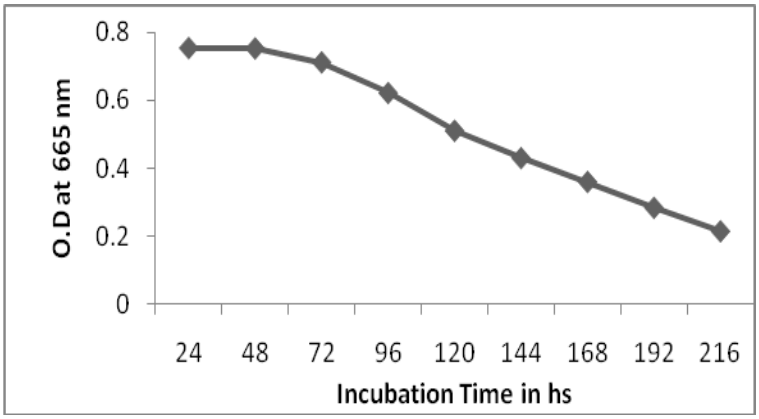
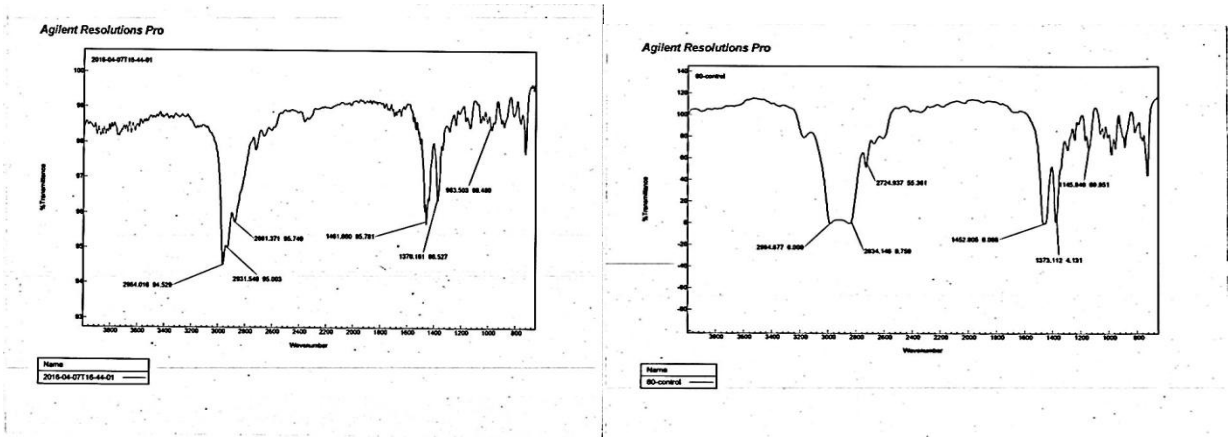
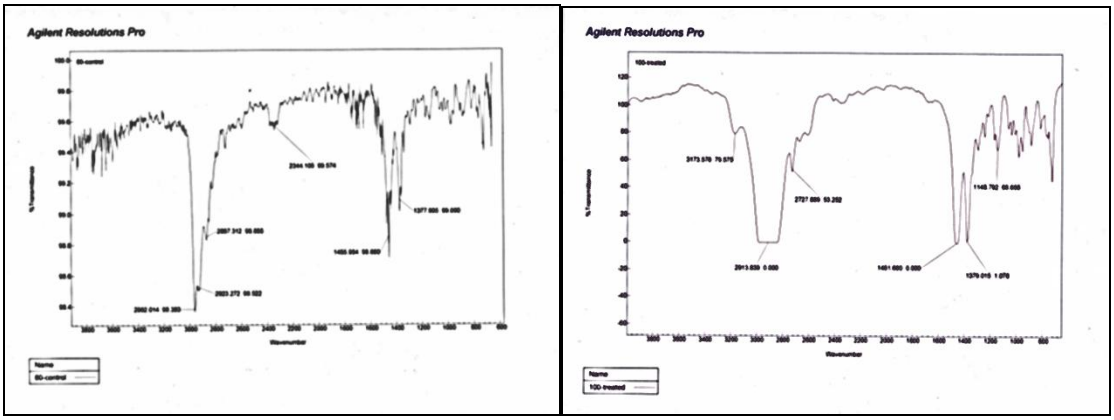


Fig.5 FT-IR of 60 ppm Naphthalene



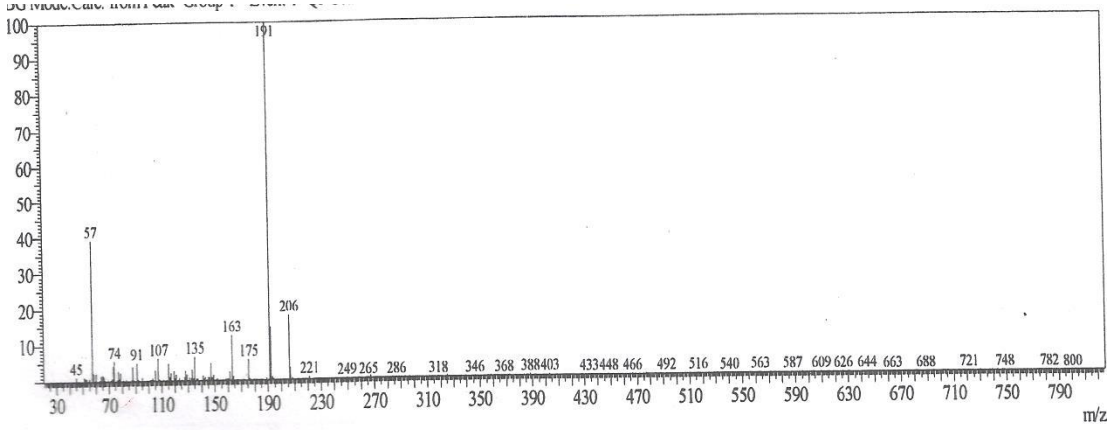
Control **Treated**

Fig.6 FT-IR of 100 ppm naphthalene degradation



Control **Treated**

Fig.7 GC-MS spectrum of degraded naphthalene of 100 ppm broth culture



Catabolism of alkyl polycyclic aromatic hydrocarbons (PAHs) in aerobic bacteria suggests the involvement of a diversity of enzymes (Mahajan *et al.,* 1994). These include oxidation of methyl group to alcohol, aldehyde, or carboxylic acid, decarboxylation, demethylation, and deoxygenating. However, PAHs and their alkyl derivatives can be transformed by various anaerobes through novel catabolic pathways (Annweiler *et al.*, 2002; Zhang and Young, 1997). In comparison with aerobic bacteria initiating dioxygenation, anaerobic bacteria usually insert carboxyl groups from carbon dioxide or succinic acids (Mahajan *et al.,* 1004). In our studies naphthalene transformation products in the extract of 60 ppm naphthalene broth culture were detected through GC-MS and identified as Cholesta-4,6-dien-3-ol, Oleyl alcohol, acetic acid,2-dodecan-1-yl-succinic anhydride and fumaric acid (Figs. 5–7).

However, GC-MS of the 100 ppm of naphthalene broth detected octadec-9-enoic acid and phthalic acid (Mrozik *et al.,* 2003) the other transformation products of naphthalene. Acetic acid and fumaric acid as detected in 60 ppm naphthalene broth were not detected in 100 ppm broth. Mallick *et al.*, (2007) reported that a novel *meta*-cleavage of 2-hydroxy-1-naphthoic acid to form *trans*-2, 3-dioxo-5- (2'-hydroxyphenyl)-pent-4-enoic acid in *Staphylococcus* sp. The ring fission product 4-(2-Hydroxyphenyl)-2-oxo-but-enoic (2-hydroxybenzal pyruvic acid) and *trans*-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid as reported by different authors were not detected in our studies with *Nocardiopsis alba*, however, another product octadec-9-enoic acid was identified. Phthalic acid, the transformation product of naphthalene detected in our study may arise from degradation of 2-carboxylcinnamic acid (Annweiler *et al.,* 2000). However, 2-carboxylcinnamic acid was not detected in our study; probably it has been instantly transformed to phthaic acid before extraction**.** Succinic acid the transformed product of naphthalene as reported by many authors (Rainer *et al.,* 2004) was not detected in our study, however 2-dodecan-1-yl-succinic anhydride has been identified which is probably derived from unstable succinic acid in due course. Acetic acid and fumaric acid are the other derivative of naphthalene detected in our study similar to other authors (Annweiler *et al*., 2002; Meckenstock *et al*., 2004).

The occurrence of phthalic acid, acetic acid, fumaric acid, octadec-9-enoic acid and 2-dodecan-1-yl-succinic anhydride strongly suggests that *Nocardiopsis alba* efficiently degrades naphthalene into its derivative products.

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