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Evaluation of Carbazole Degradation by *Enterobacter* sp. Isolated from Hydrocarbon Contaminated Soil

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Abstract

A gram-negative bacterium designated A8 was isolated from hydrocarbon contaminated soil collected from petrol pump of Ahmedabad, India. Its ability to degrade carbazole, nitrogen containing polycyclic aromatic hydrocarbon was checked in basal salt media containing 3 mM of carbazole. Growing cells of the isolate could degrade 83% of carbazole in 240 hours. On the basis of different biochemical and physiological tests, isolate A8 was identified as *Enterobacter* sp. To the best of my knowledge this is the first report on degradation of carbazole by *Enterobacter* sp.

Key Words: Bioremediation, Carbazole, *Enterobacter* sp., Bioavailability assay, *N*-Heterocyclic aromatics

Introduction

Nitrogen containing xenobiotic compounds is an important class of contaminants that are wide spread in the environment. They are frequently used as dyes, pesticides, explosives, petroleum products and are also involved in the production of pharmaceuticals [1, 2]. These N-containing xenobiotic compounds and their conversion products are both mutagenic and carcinogenic. Moreover, they also readily undergo radical chemistry to generate more genotoxic compounds [3, 4]. Most of these compounds are on the United States Environmental Protection Agency (EPA) priority list. The excessive use of these compounds and their harmful effect on human health and environment has generated serious concern for proper disposal. Investigation of microbial degradation is a promising method to elucidate the pathway and to use them for bioremediation. The N-containing synthetic compounds have been broadly classified into nitro aromatics, the nitrite esters and compounds containing nitrogen-ring heterocycles. Study of the degradation of these compounds by various microorganisms has led to the identification and characterization of the biochemical pathways and some of the genes and enzymes involved in the degradation [5-8]. Wealth of information is available for the degradation of nitro aromatics and nitrite esters but the information regarding degradation of heterocyclic compounds is very limited. This study reports the isolation of microorganism involved in the degradation of carbazole (CAR), a N-heterocyclic aromatic hydrocarbon. CAR is found in creosote, crude oil, shale oil and used as a feedstock for the manufacture of dyes plastics and medicines. It is the most abundant nitrogen containing compound in many petroleum samples and is therefore chosen as a model compound to investigate remediation strategies [9, 10]. A number of microbes have been reported for the degradation of CAR like *Sphingomonas* sp. [8], *Xanthomonas* sp. [11], *Gordonia* sp. [12], *Klebsiella* sp. [13], *Burkholderia* sp. [14], *Arthrobacter* sp. [15], *Novosphingobium* sp. [16] etc. The CAR degradation pathway and the genes (*car* genes) for *Pseudomonas resinovorans* CA10 have been well characterized [6, 7]. CAR degrading *car* genes are clustered in the form of an operon *carAaAaBaBbCAc*ORF7*Ad.* Carbazole 1,9a-dioxygenase (CARDO) is a multicomponent enzyme composed of terminal oxygenase (CarAa), ferredoxin (CarAc) and ferredoxin reductase (CarAd) units, which converts CAR to 2'-aminobiphenyl-2, 3-diol. The predicted CAR-degradation intermediate 2'-aminobiphenyl-2, 3-diol is then converted in to 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2, 4-dienoic acid by the action of meta-cleavage enzyme collectively encoded by *carBa* and *carBb* genes. Gene *carC* encodes the hydrolase enzyme that is supposed to convert 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2, 4-dienoic acid to anthranilic acid (Figure 1). Anthranilic acid is then converted in to catechol and further metabolized by β- ketoadipate pathway.

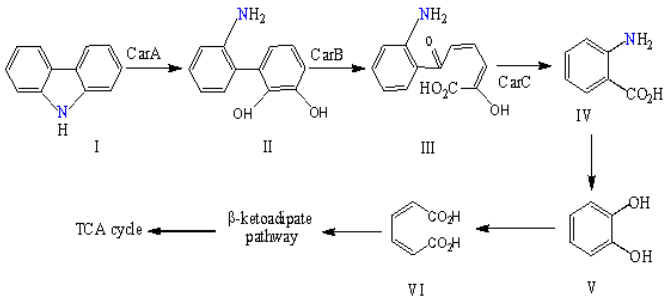

Figure 1: Degradation pathway of carbazole by *Pseudomonas* sp. strain CA10. CarA, carbazole 1,9a-dioxygenase; CarB, 2'-aminobiphenyl-2,3-diol-1,2-dioxygenase; CarC, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid hydrolase; I, Carbazole; II, 2'-aminobiphenyl-2,3-diol; III, 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid (*meta*-cleavage compound); IV, Anthranilic acid; V, Catechol; VI, *cis*,*cis*-muconic acid.

Materials and Methods

Enrichment, isolation and characterization of CAR degrading bacteria

The basal salt medium (BSM) containing final concentration of 3 mM CAR (Acros Organics, USA) was used for the isolation or cultivation of CAR degrading strains. The components of BSM (per litre) were 2.44 g of KH_2PO_4; 5.57 g of Na_2HPO_4; 2 g of Na_2SO_4; 2 g of KCl; 0.2 g of $MgSO_4$; 0.001 g of $FeCl_3.6H_2O$; 0.02 g of $MnCl_2.4H_2O$; 0.003 g of $CaCl_2.2H_2O$; 5 g of glucose and 6.4 ml of glycerol. For CAR degradation studies, CAR (dissolved in acetone) was the only nitrogen source. Cultures were incubated at 30°C with shaking at 180 rpm. Soil samples were collected from different petrol pumps located in Ahmedabad (Gujarat, India). One gram of soil sample was inoculated in 100 ml of BSM with CAR. After 4 days of incubation, 5% of enriched culture was transferred to fresh BSM and incubated under same conditions. This procedure was repeated four times. Later, samples were diluted serially and plated on solid Luria Bertani (LB) media to obtain isolated colonies. The LB media contained (per litre of distilled water): 10 g of tryptone; 5 g of yeast extract and 10 g of NaCl. Isolates were inoculated in BSM containing CAR (both as carbon and nitrogen source) and incubated at 30°C for seven days. Quantity of CAR degraded by isolates was analyzed by calculating initial and final concentration. CAR degradation for all isolates was visualized as a clearing halo on M9 minimal plates supplemented with 3 mM of CAR [17]. Selected isolate was identified, following different physiological and biochemical tests, using Bergey's manual of systemic bacteriology.

Bioavailability assay

The isolates were subjected to bioavailability assay to check their CAR utilization as both carbon and nitrogen source [18]. For bacterial growth test, four different types of BSM were used viz. M-I, M-II, M-III and M-IV. In M-I CAR (3 mM) was used as the only source of nitrogen with additional glucose (5 g/liter) and glycerol (6.4 ml/liter) as carbon sources; media M-II was taken as a positive control with NH_4Cl (2 g/liter) as an alternative nitrogen source instead of CAR. M-III was the test media containing CAR both as carbon and nitrogen source and lastly M-IV which was not supplemented with any nitrogen source (containing only carbon source) and used as a negative control media. All isolates were firstly inoculated in LB and after overnight incubation at 30°C, 2% of fresh washed cells were inoculated in 100 ml Erlenmeyer flasks containing 20 ml different basal salt media separately. After 3 days incubation at 30°C and 180 rpm growth was monitored spectrophotometrically by measuring the optical density at 600 nm (OD_{600}) in various test conditions. Isolate which would show good growth in M-I compared to M-III, believe to utilize CAR as nitrogen source only. Our aim is to search for a potential microorganism which would be able to break aromatic backbone of CAR by using it as carbon and nitrogen source.

High performance liquid chromatography

Supernatant from bacterial culture was obtained after centrifugation at 3,000 g for 15 minutes. Supernatant was acidified to pH 2 to 3 with HCl and extracted with ethyl acetate (1:1 v/v). The organic phase was separated from aqueous phase after centrifugation and then evaporated. The residue was resuspended in acetone for quantification.

Quantification of CAR was carried out by high-performance liquid chromatography (HPLC; Waters). CAR detection was performed at 233.7 nm with a photodiode array detector (PDA 2996; Waters). Separation was achieved with a reverse-phase column (C8, 3.3 µm; Waters RP 8; 150 x 4.6 mm) and the operating conditions were as follows: room temperature; mobile phase Acetonitrile: Water (80:20 v/v) and 0.5 ml/min flow rate.

Carbazole degradation by growing cells

The biodegradation of CAR was monitored in growing cell culture using 150 ml BSM supplemented with 3 mM CAR as sole source of carbon and nitrogen in 500 ml Erlenmeyer flasks. Cultures were incubated at 30ºC on incubator shaker at 180 rpm. The time course of CAR degradation was obtained by sampling at defined intervals and analyzing the various parameters like OD_{600} and quantification of CAR. In addition to test sample, *Pseudomonas resinovorans* CA10, a well known CAR degrading strain was taken as positive control and heat killed bacterium acted as a negative control. Test sample and controls studies were conducted in triplicates.

Results and Discussion

In this study, the biodegradation of CAR by microorganisms isolated from hydrocarbon contaminated soil was studied. The results on biodegradation are depicted as the

concentration of residual CAR with time. Control experiment with heat killed bacterium as an inoculum was used to evaluate the degradation of CAR. This control acted as a reference value and the results obtained were corrected in order to avoid the abiotic loss of CAR being attributed to bacterial degradation.

Enrichment and isolation of bacteria

The enrichment of soil samples for CAR degrading bacteria was carried out as mentioned above. Twelve different bacteria that can degrade CAR were isolated by enrichment culture using basal salt media supplemented with 3 mM of CAR as a nitrogen source and were designated consequently starting from A1 till A12. All the isolates were subjected to bioavailability assay (A1-A12). The growth of all the isolates was monitored in all the media by measuring absorbance at 600 nm. Based on bioavailability assay, it was inferred that all isolate could grow in M-I. M-II and M-III but isolate A8 showed better growth as compared to others (Figure 2).

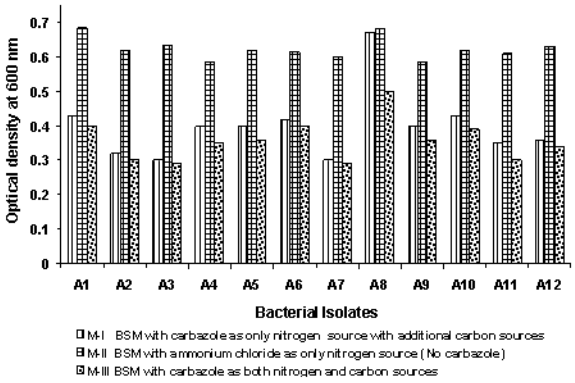

Figure 2: Spectrophotometric observations at 600 nm for all isolates in different basal salt media (BSM). No growth was observed in media M-IV, BSM without any nitrogen source (data not shown).

CAR degradation activity of all the isolates was also checked after seven days of incubation in BSM containing CAR both as carbon and nitrogen source. Among all isolates, isolate A8 was showing maximum CAR degradation (data not shown). Maximum growth and maximum utilization of CAR by the same isolate indicated that CAR is being utilized for the growth of the bacteria as both carbon and nitrogen source. Utilization of a pollutant (CAR in this case) as both carbon and nitrogen source is a prerequisite for a successful bioremediation process. No growth observed in M-IV (no nitrogen source) and growth in M-I (CAR as nitrogen source) suggested that nitrogen is required for the growth of the isolate and it could efficiently utilize nitrogen present in CAR.

Identification of microorganism

Biochemical tests were performed to identify isolate A8. Colonies of isolate A8 on LB agar medium are round with entire margins and convex elevation. It was further examined by gram staining, oxidase test, lactose fermentation, triple sugar iron agar reaction (TSI), indole production, methyl red test, citrate utilization and voges-proskauer test. On the basis of above biochemical tests, isolate A8 was determined as aerobic and facultatively anaerobic gram negative rod belonging to the *Klebsiella-Enterobacter* group. Method used to achieve the separation between *Klebsiella* and *Enterobacter*, is to employ motility or ornithine decarboxylase tests, or both [19, 20]. *Klebsiella* is nonmotile, and more than 98% of the strains are ornithine decarboxylase-negative while 5% of *Enterobacter* species may be ornithine decarboxylase-negative, so it is required to perform both ornithine decarboxylase and motility tests, to get accurate result [19]. The biological characteristics, as well as the results of biochemical and physiological tests for the isolate A8 are shown in Table 1. By referring to Bergey's Manual of Determinative Bacteriology isolate A8 was identified as *Enterobacter* sp.

Table 1: Physiological and biochemical characteristics of isolate A8

Physiological and biochemical characteristics	Isolate A8
Gram stain	–
Morphology	Small Rod
Pigments	–
Motility	+
Spore formation	–
Oxidase	–
Catalase	+
Nitrate reduction	+
Indole test	–
Methyl red test	–

Voges-Proskauer's test	+
Citrate utilization	+
Ornithine utilization	+
H_2S production	–
TSI test	A/A, g
Glucose fermentation	+
Lactose fermentation	+

Note: +, positive; –, negative; A, acid; g, gas.

Carbazole degradation by growing cells of Enterobacter sp.

The degradation of CAR by growing cells of *Enterobacter* sp. was studied for 240 hours. In the culture with 3 mM of CAR, OD_{600} increased till third incubation day and then decreased further when incubated for longer period of time. Maximum growth occurred after about 60 hours of cultivation. During the first 72 hours of incubation 50% of the CAR gets degraded by growing cells of *Enterobacter* sp. which finally resulted in 83% in 240 hours. A well established strain *Pseudomonas resinovorans* CA10 was taken as a positive control. This strain decreased the concentration of CAR to 56% in 240 hours. From the plot of time course of growth and CAR degradation (Figure 3), it can be concluded that isolate could degrade maximum concentration of CAR during exponential phase of their growth. In all the strains including positive control, no significant degradation of CAR was observed once the cell entered the stationary phase of growth.

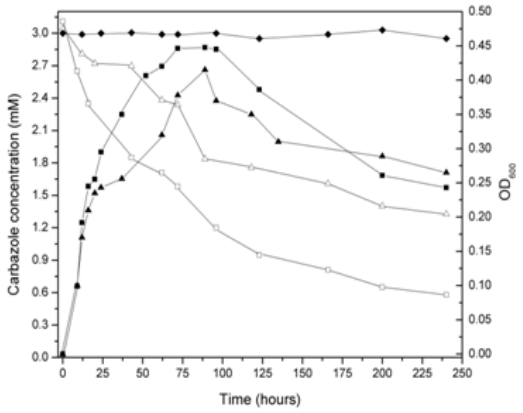

Figure 3: Degradation of carbazole by growing cells of A8 (□), positive control *Pseudomonas* sp. CA10 (∆) and heat killed negative control (♦). Time course of growth for A8 (■) and CA10 (▲). The values are means of three independent replicates. SD was within the acceptable range.

Conclusion

A CAR degrading gram-negative, motile, rod shaped bacterium *Enterobacter* sp. was isolated from hydrocarbon contaminated soil by an enrichment culture technique. Growing cells of the isolate could degrade 83% of CAR in 240 hours. Various *Enterobacter* sp. have been reported to be involved in metal detoxification, bioaccumulation [21] and bioremediation of organophosphate pesticide [22]. To the best of my knowledge this is the first report on degradation of CAR by *Enterobacter* sp. Utilization of CAR as both carbon and nitrogen source suggested that the isolate could completely degrade CAR and thus could be useful for the bioremediation of CAR from contaminated sites. Further work is underway to elucidate the complete pathway.

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