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Biodegradation of Carbazole By a Promising Gram-Negative Bacterium

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***Abstract*—**In the present work we report a gram negative bacterial isolate, from soil of a dye industry, with promising biorefining and bioremediation potential. This isolate (GBS.5) could utilize carbazole (nitrogen containing polycyclic aromatic hydrocarbon) as the sole source of nitrogen and carbon and utilize almost 98% of 3mM carbazole in 100 hours. The specific activity of our GBS.5 isolate for carbazole degradation at 30°C and pH 7.0 was found to be 11.36 µmol/min/g dry cell weight as compared to 10.4 µmol/min/g dry cell weight, the highest reported specific activity till date. The presence of *car* genes (the genes involved in denitrogenation of carbazole) was confirmed through PCR amplification.

***Keywords*—**Biodenitrogenation, Biorefining, Carbazole degradation, Crude oil.

I. Introduction

DIESEL is the major transportation fuel worldwide and thus much emphasis is laid on improving its quality. Nitrogen, sulfur and aromatic compounds are the major impurities present in diesel. Presence of nitrogen containing compounds promote tank corrosion and oil degradation during storage. It also leads to the poisoning of refining catalyst by getting adsorbed to the active sites [1]. Carbazole, a nitrogen containing polycyclic aromatic compound that is difficult to remove using conventional physico-chemical methods, is considered as the model compound in most of the denitrogenation studies. It is also used as feedstocks for the production of dyes, medicines and plastics [2] and found in crude oil, tar sand sources, shale oil [3], [4] and wood preserving wastes [5]. Carbazole and its derivatives being carcinogenic and mutagenic [6]-[8], readily undergo radical chemistry to generate the more genotoxic hydroxynitrocarbazoles. Thus microbes capable of degrading carbazole serve a dual purpose of biorefining and bioremediation. A number of microbes have been reported for the degradation of carbazole like *Sphingomonas* sp. [9], [10], *Pseudomonas* sp. [11], *Xanthomonas* sp. [12], *Gordonia* sp. [13], *Klebsiella* sp. [14], *Burkholderia* sp. [15], *Arthrobacter* sp. [16], *Novosphingobium* sp. [17] etc. The carbazole biodegradation pathway and the genes (*car* genes) for *Pseudomonas resinovorans* CA10 have been well characterized [11], [18]-[20]. In the present work we have isolated a microorganism which could utilize carbazole much more efficiently as compared to already reported microorganisms. The presence of car genes has also been confirmed through PCR studies.

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II. Material and Methods

A. Chemicals

Carbazole was purchased from Acros Organics. Organic solvents used (acetonitrile, acetone, ethyl acetate) were of HPLC grade from Qualigens. Other materials were of analytical grade from CDH, Qualigens and Himedia. PCR chemicals were purchased from Sigma-Aldrich.

B. Enrichment and Isolation of carbazole degrading bacteria

Various samples rich in polyaromatic hydrocarbons were collected from oil refineries, activated sludge and dye industries from different parts of Indian subcontinent. Microbes isolated from these samples were cultivated in basal salt medium (BSM pH 7.0) with 3 mM carbazole. Basic components of BSM (g l^{-1}) were KH_2PO_4, 2.44; Na_2HPO_4, 5.57; Na_2SO_4, 2; KCl, 2; $MgSO_4$, 0.2; $FeCl_3.6H_2O$, 0.001; $MnCl_2.4H_2O$, 0.02 and $CaCl_2.2H_2O$, 0.003. Carbazole was used as the sole carbon and nitrogen source.

C. Analytical Methods

HPLC analysis of the samples were done using a Waters model 515 HPLC pump equipped with a model 2996 photodiode array detector. A reversed phase column (RP-8, 4.6 x 150 mm long with 3.3 µm diameter packing material) was used and extracts were analyzed using acetonitrile and water (80:20 v/v) as mobile phase, at a flow rate of 0.5 ml per min. The effluents were monitored at 233 nm.

D. Carbazole degradation by growing cells

The biodegradation of carbazole was monitored in growing cell culture using 150 ml BSM supplemented with 3 mM carbazole in 500 ml Erlenmeyer flasks. Cultures were incubated at 30ºC on incubator shaker at 180 rpm. The time course of carbazole utilization was obtained by sampling at defined intervals and analyzing the various parameters like OD_{600} and quantification of carbazole. In addition to test sample, *Pseudomonas resinovorans* CA10, a well known

carbazole degrading strain was taken as positive control and media without any inocula acted as a negative control. Test sample and controls studies were conducted in triplicates.

E. Resting cell experiment

Cells in BSM supplemented with 3 mM of carbazole were grown on an incubator shaker at 30ºC and 180 rpm. The cells from the late logarithmic phase were harvested (9000 rpm, 10 min, 4°C), washed twice with 50 mM potassium phosphate buffer (pH 7.0) and finally resuspended in equal volume of potassium phosphate buffer. In this cell suspension 3 mM carbazole was added and the reaction was allowed to proceed at 30°C and 180 rpm for 320 minutes. The samples were collected at regular intervals and analyzed for carbazole.

F. PCR amplification of car genes

Bacterial colony was lysed in 50 µl of nuclease free hyclone water by heating at 95ºC for 10 min. It was then centrifuged at 3000 rpm for 5 min. Aliquot of 2 µl was taken and used as a template. PCR was performed in a DNA Engine PTC-200, Peltier thermocycler (BioRad). The reaction mixture contained 1 mM of each dNTP, 2.5 pmole of each primer, 2.5 U Taq Polymerase (Sigma) and 2 µl of template in a 20 µl reaction volume. The cycles involved initial denaturation at 94ºC for 10 min followed by 30 amplification cycles (94ºC for 30 sec, 58ºC for 30 sec and 72ºC for 60 sec) and a final extension for 10 min at 72ºC. The primers used in this work are listed in table 1. To amplify the DNA fragment carrying the *car* gene cluster of the isolated strain, primers were designed based on the conserved regions of the known sequences of *car* operon (*car*Aa, *car*Ac, *car*Ba, *car*Bb and *car*C)

TABLE I
LIST OF PRIMERS USED IN THIS STUDY

Name of genes	Primer sequences	Expected size
carAa F	5'gtaccacgcgtggacctatc3'	551bp
carAa R	5'acggattgaccttgagaacgc3'	
carAc F	5'tggcgatcgcttgtacgcc3'	210bp
carAc R	5'tcctccggcgacataaacttc3'	
carBa F	5'atccagtagaccgcctgattc3'	181bp
carBa R	5'tgcatctgcagaaccggatg 3'	
carBb F	5'cgatgggtgacatggacattc3'	575bp
carBb R	5'gaattccattcctccgattcc3'	
carC F	5'ttggttgaaagtatgtgctgc3'	282bp
carC R	5'cctctttgccgtctaccacg3'	

Primers were designed based on the conserved sequences of the *car* genes from various known sequences. F denotes the forward primer and R denotes the reverse primer for every corresponding primer. The expected size of the amplified fragment, as calculated from the sequence of *Pseudomonas resinovorans* CA10 is also listed.

III. Results and Discussion

A. Enrichment and isolation of bacteria

130 different bacterial strains were isolated from various samples. Carbazole degradation ability of all the isolated strains was monitored by its ability to grow in BSM with carbazole as the only carbon and nitrogen source. After several rounds of enrichment cultivation, 20 isolates showed high growth in BSM supplemented with carbazole. These isolates when streaked on BSM plates with carbazole also showed clear halo zones indicating the utilization of carbazole [21]. Quantification of Carbazole for these 20 isolates was further carried out indicating more than 450 ppm of carbazole utilization by 4 isolates. No additional peak was detected in the organic layer indicating that carbazole is either converted to CO_2 or its metabolites are not soluble in organic layer. Among these four strains, one gram negative rod designated GBS.5 was selected for further studies.

B. Carbazole removal by growing cells

Carbazole is a carcinogen and a mutagen which is predominant in petroleum, dye contaminated soil. Moreover, being an environmental contaminant it could also contaminate underground water and is a potential health hazard. Fig 1 shows the time course of carbazole utilization by GBS.5 compared with CA10. The strain could completely degrade 3 mM of carbazole in minimal media after 100 hrs. Fig 2 is showing the time course of carbazole degradation by resting cells of GBS.5 and CA 10. The specific enzyme activity obtained by the resting cells (Fig 3) was 11.36 µmol/min/g dry cell weight. To the best of my knowledge, in the literature maximum specific enzyme activity obtained was 10.4 µmol/min/g dry cell weight by *Pseudomonas* sp XLDN4-9 [22].

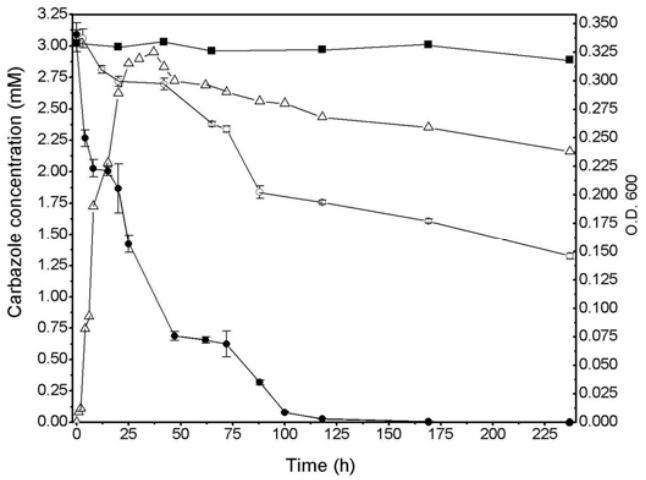

Fig. 1 Utilization of carbazole by GBS.5 and *Pseudomonas resinovorans* CA10. Time course of growth (Δ) for GBS.5, utilization of carbazole by positive control CA10 (○), by our isolate GBS.5 (●) and carbazole concentration in negative control without inoculation (■).The values are means ± standard deviations of at three replicates.

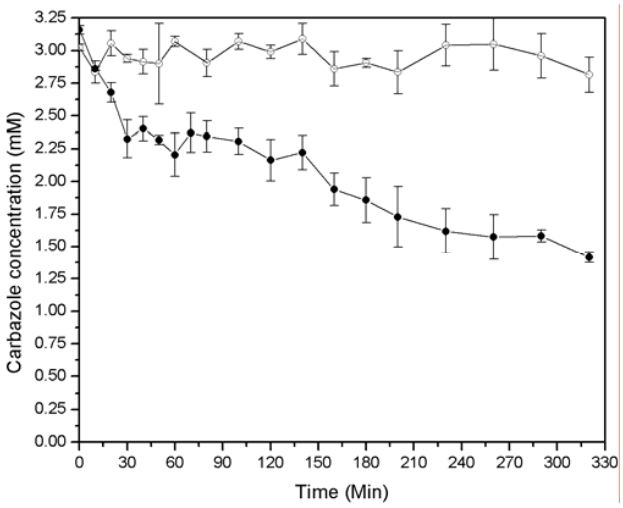

Fig. 2 Resting cell of GBS.5 and *Pseudomonas resinovorans* CA10 are exposed to 3mM carbazole for 320 min. Time course utilization of carbazole by resting cell of positive control CA10 (○) and GBS.5 (●).The values are means ± standard deviations of at three replicates.

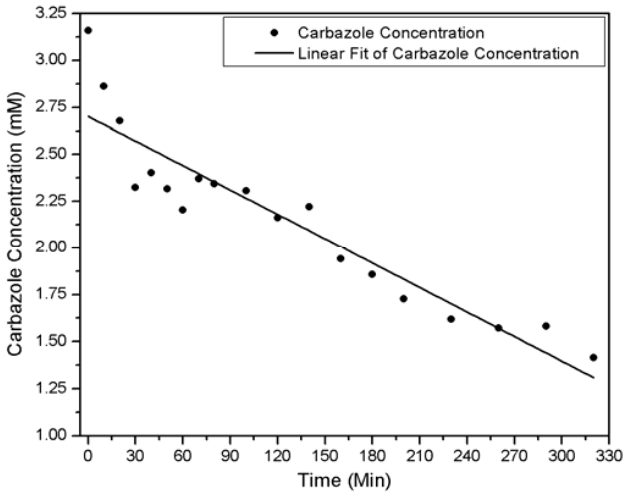

Fig. 3 Linear fit graph for carbazole utilization by GBS.5. The specific enzyme activity obtained by the resting cells was 11.36 µmol/min/g dry cell weight.

It is undoubted that fast degradation of carbazole is a key to a good bioremediation process. This strain degrade carbazole at a fast rate as compared to others reported in the literature thus these results imply that the bacterium could prove to be a good candidate for a bioremediation process. The strain was cultivated in BSM supplemented with 3mM carbazole as sole carbon and nitrogen source. Data are the mean result of the analysis of triplicate samples from three different experiments. The average standard deviation from all data points was 5% or less.

C. PCR amplification

In the literature, carbazole degradation is reported to be performed by a genes arranged in the form of an operon. Primers were designed based on the conserved sequence of the *car* gene reported in literature. The results obtained via PCR with GBS.5 using the *car* primers showed approximately the same size as obtained with CA10 as shown in Fig 4.

IV. Conclusion

A carbazole degrading bacterium designated GBS.5 was isolated by enrichment technique in our laboratory. Specific activity of carbazole degradation of the isolated strain (11.36 µmol/min/g dry cell weight) is higher than that of the best known microorganism till date (10.4 µmol/min/g dry cell weight). Thus the strain could be a good candidate for bioremediation and biorefining purposes. Furthermore, PCR amplification of the DNA using the primers for the conserved region of the *car* genes showed that GBS.5 produced amplicons of the same size as that of CA10 indicating that it contains the gene for *car* operon. However, further analysis of the DNA sequence is needed.

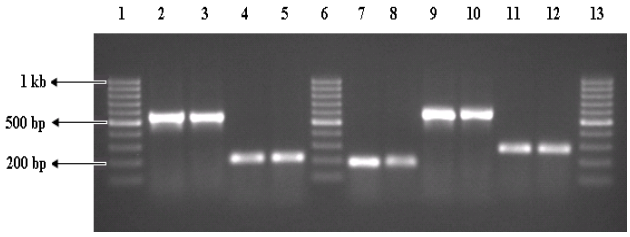

Fig. 4 PCR pattern obtained with different primers for GBS.5 and *Pseudomonas resinovorans* CA10. Lane 2, 4, 7, 9 and 11 shows amplification pattern observed in CA10 using primers 1, 2, 3, 4 and 5 respectively. Lane 3, 5, 8,10 and 12 shows amplification pattern observed in GBS.5 using primers 1, 2, 3, 4 and 5 respectively. Lane 1, 6 and 13 are 100 bp ladder. The amplicons of the same size were obtained both in CA10 and GBS.5. Electrophoretic separation was performed in 1 % agarose gel in a TAE buffer.

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