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Biodegradation of Carbazole by Newly Isolated *Acinetobacter* spp.

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Abstract In this study, two bacterial isolates designated Alp6 and Alp7 were isolated from soil collected from dye industries and screened for their ability to degrade carbazole. Growing cells of the isolates Alp6 and Alp7 could degrade 99.9% and 98.5% of carbazole, respectively in 216 h. The specific activity for degradation by the resting cells of Alp6 was found to be 7.96 μmol/min/g dry cell weight, while for Alp7 it was 5.82 μmol/min/g dry cell weight. Phylogenetic analysis based on 16S rDNA gene sequences showed that isolates Alp6 and Alp7 belonged to the genus *Acinetobacter*. To the best of our knowledge, this is the first report on the *Acinetobacter* spp. showing utilization of carbazole as carbon and nitrogen source.

Keywords *Acinetobacter* sp. · Biodegradation · Carbazole · 16S rDNA

N-Heterocyclic aromatic hydrocarbons (NHAs) belong to the class of organic molecules which are most widely distributed toxic pollutant of air, surface soil and aquatic systems. Major sources of environmental NHAs contamination are incomplete combustion of fossil fuels, coal liquefaction, volcanic eruptions, forest fires, uncontrolled hazardous industrial and oily sludge waste dump (Grosser et al. 1991). They are highly recalcitrant and resistant to nucleophilic attack because of their strong molecular bonds and presence of π-electrons surrounding benzene rings (Johnsen et al. 2005). Degradation of various NHAs like quinoline, pyridine and phenanthridine has been well studied and the large numbers of microorganisms involved in the degradation of the same have been reported (Padoley et al. 2008; Willumsen et al. 2001). However, limited information is available regarding the degradation of carbazole (CAR). CAR and its derivatives are used as a feedstock in dyes, plastics and pharmaceutical industries and naturally found in crude oil, shale oil and creosote (Mushrush et al. 1999; Singh et al. 2010). They are known to be mutagenic and carcinogenic (Benedik et al. 1998). Moreover, due to its hydrophobic nature, CAR tends to be associated with sediment in aquatic system and thus result in toxicity to benthic aquatic life with long lasting effects (Ololade 2010). In the present study, CAR is chosen as a model compound to investigate strategies for bioremediation of toxic *N*-heterocyclic aromatic hydrocarbons. Selection of CAR as a model compound for biodegradation studies is motivated by its broad distribution, toxicity and high persistence in contaminated sites. Number of microbes has been reported for the degradation of CAR like *Sphingomonas* sp. (Meiying et al. 2009), *Gordonia* sp. (Santos et al. 2006), *Klebsiella* sp. (Li et al. 2008), *Pseudomonas* sp. (Ouchiyama et al. 1993), *Enterobacter* sp. (Singh et al. 2011) etc. The CAR biodegradation pathway and the genes involved in the degradation of CAR (*car* genes) for *Pseudomonas resinovorans* CA10 have been well characterized (Sato et al. 1997). In this paper, we report two newly isolated *Acinetobacter* spp. utilizing CAR as carbon and nitrogen source. These isolates could be useful for the bioremediation of CAR from contaminated sites.

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Materials and Methods

The basal salt medium (BSM) containing final concentration of 3 mM CAR (Acros Organics, USA) was used for

the isolation and 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 $FeCl_3{\cdot}6H_2O$; 0.02 g of $MnCl_2{\cdot}4H_2O$; 0.003 g of $CaCl_2{\cdot}2H_2O$. Higher amount of Na_2HPO_4 with respect to KH_2PO_4 were used to provide good buffering capacity to BSM to maintain pH around 7.0 ± 0.1 (Santos et al. 2006) throughout the cultivation. CAR degrading strains were isolated by enrichment from various soil samples, collected from dye industries (located in Gujarat, India). One gram of soil samples were inoculated in 500 mL sterilized Erlenmeyer flask containing 100 mL of BSM supplemented with CAR both as carbon and nitrogen source (dissolved in acetone). 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 and incubated aerobically at 30°C in rotary incubator shaker with 180 rpm for 7 days. Quantity of CAR utilized by isolates was analyzed by calculating initial and final concentration. Selected isolates were subjected to bio-availability assay (Kilbane et al. 2000) to check their CAR utilization as both carbon and nitrogen source. Selected isolates showing maximum CAR degradation, were identified following different morphological, physiological and biochemical tests, using Bergey's manual of systemic bacteriology. PCR amplification of the approximately 1.5 kb 16S rDNA fragment was performed commercially. The reaction mixture contained 2.5 μL of 10× Taq buffer, 1 μL of deoxynucleoside triphosphate mix (2.5 mM each), 1 μL of forward and reverse primers (100 ng each), 0.5 μL of 5 U/μL Taq DNA polymerase with 25 mM $MgCl_2$ and 1 μL of template DNA (20 ng) in a 25 μL reaction volume. The cycles involved initial denaturation at 94°C for 5 min followed by 35 amplification cycles (94°C for 1 min, 55°C for 45 s and 72°C for 1 min 30 s) and a final extension for 10 min at 72°C. Sequenced PCR products were then aligned with the nucleotide sequences available in NCBI GenBank and RDP database. CLUSTAL W and BLAST programs were used for multiple alignment and comparison of 16S rDNA gene sequences from different nucleotide databases, respectively. Pairwise evolutionary distance matrices (based on 16S rDNA sequence homology) were computed using Kimura-2 parameter. Phylogenetic trees were constructed applying neighbour-joining, maximum-likelihood and maximum-parsimony methods using bootstrap values based on 1,000 resamplings.

The biodegradation of CAR was monitored in growing cell culture of selected isolates. Isolates were firstly inoculated in LB and after overnight incubation at 30°C, 2% of fresh washed cells were inoculated in 500 mL sterilized Erlenmeyer flasks containing 150 mL BSM supplemented with 3 mM CAR as sole source of carbon and nitrogen. Cultures were incubated at 30°C on rotary incubator shaker with 180 rpm. The time course of CAR degradation was obtained by sampling at defined intervals and analyzing the various parameters like optical density at 600 nm (OD_{600}) and quantification of CAR. In addition to test sample, *P. resinovorans* CA10, a well known CAR degrading strain was taken as positive control and heat killed bacterium acted as a negative control. Test samples and controls studies were conducted in triplicates.

Specific activity for CAR degradation was calculated by resting cells of selected isolates. Isolates were cultivated in 100 mL BSM supplemented with 3 mM of CAR and incubated under same incubation condition described above. The cells from the late logarithmic phase were harvested by centrifugation at 9,000 rpm for 10 min at 4°C, washed twice with 50 mM potassium phosphate buffer (pH 7.0) and finally cells pellet (approximately 0.35 g dry cells/L) were resuspended into same volume of potassium phosphate buffer. In this cell suspension nearly 1.6 mM CAR was added and the reaction was allowed to proceed at 30°C and 180 rpm for 390 min. Samples were collected at regular intervals and analyzed for residual CAR concentration. In addition to test sample, *P. resinovorans* CA10 was taken as positive control and heat killed bacterium acted as a negative control. Test samples and controls studies were conducted in triplicates.

Supernatant from aliquoted bacterial cultures was obtained after centrifugation at 3,000 rpm for 15 min. Residual CAR was then quantified after extraction with ethyl acetate using high-performance liquid chromatography (HPLC; Waters). Separation was achieved with a reverse-phase column (C8, 3.3 μm; Waters RP 8; 150 × 4.6 mm) using Acetonitrile:Water (80:20 v/v) as a mobile phase at 0.5 mL/min flow rate. CAR detection was performed at 233.7 with a photodiode array detector (PDA 2996; Waters).

Result and Discussion

As a result of several rounds of enrichment cultivation, fifteen different bacterial isolates having CAR degrading ability were isolated and designated consequently starting from Alp1 till Alp15. CAR degradation activity of all the isolates was checked after 7 days of incubation in BSM containing CAR both as carbon and nitrogen source. Among all isolates, the isolates Alp6 and Alp7 showed maximum CAR degradation. Bioavailability assay results

Table 1 Morphological and biochemical characteristics of newly isolated *Acinetobacter* spp. Alp6 and Alp7

Morphological and biochemical characteristic	Alp6	Alp7
Gram stain	–	–
Morphology	Rods	Rods
Pigments	–	+
Motility	–	–
Spore	–	–
Oxidase	–	–
Catalase activity	+	+
Nitrate reduction	–	+
Indole test	–	–
Methyl red test	–	–
Voges–Proskauer's test	+	–
Citrate utilization	–	–
TSI test	K/K	K/K
H_2S (on TSI)	–	–
Gas (on TSI)	–	–
Glucose fermentation	–	–
Lactose fermentation	–	–
Growth on MacConkey agar	+	+
Assimilation of		
Glucose	+	+
Lactose	–	–
Adonitol	–	–
Arabinose	–	–

+ Positive, – negative, K alkaline

suggested their (Alp6 and Alp7) CAR degradation ability by utilizing CAR as both carbon and nitrogen source.

Different biochemical tests were performed to identify these isolates (Table 1). Both isolates were non motile gram negative rods and forms round with convexly elevated colonies on LB agar medium. 16S rDNA sequencing was also done to identify these isolates. Comparison of the 16S rDNA gene sequence, conducted using the GenBank and RDP database, indicated that Alp6 showed the highest level of sequence similarity with *Acinetobacter radioresistens* strain F71005 (99%), followed by *A. baumannii* strain GTCR407 (98%) and *Acinetobacter baylyi* strain 723 (96%) while isolate Alp7 showed maximum sequence similarity with *Acinetobacter radioresistens* strain F71005 (98%) followed by *A. lwoffii* strain ISP4 (98%) and *Acinetobacter baumannii* strain GTCR407 (98%). Phylogenetic trees of the isolates was constructed with closely related microorganisms showing maximum sequence similarity and other validly published representatives of the *Acinetobacter* species (Dijkshoorn et al. 2007), according to three different methods viz. maximum-parsimony, maximum-likelihood and neighbour-joining algorithms, using MEGA package (Version 5; Tamura et al. 2011). Phylogenetic tree shows that the isolate Alp6 and Alp7 formed a monophyletic clade with *A. radioresistens* (GenBank accession no. HQ90872) and *A. baumannii* (GenBank accession no. GU319977) with a high bootstrap value (Fig. 1). Similar phylogenetic tree patterns were obtained using all the three methods. 16S rDNA sequences of isolates Alp6 and Alp7 are submitted under GenBank accession number JF828047 and JF828048 respectively. Alp6 and Alp7 showed sequence similarity of 98% with each other. The levels of 16S rDNA gene sequence similarity and the results of the phylogenetic analysis indicate that isolates Alp6 and Alp7 are member of the genus *Acinetobacter*.

The degradation of CAR by growing cells of *Acinetobacter* spp. was studied for 216 h in BSM supplemented with CAR as both carbon and nitrogen source. In the culture with 3 mM of CAR, growth of Alp6 increased till third day and that of Alp7 till fourth day. During the first 72 h of incubation 84% of the CAR gets degraded by growing cells of *Acinetobacter* sp. Alp6, decreasing its amount from 3 to 0.48 mM, which finally resulted in 99.9% degradation in 216 h. Growing cells of *Acinetobacter* sp. Alp7 also showed significant decrease in CAR concentration from 3 to 1.20 mM in 72 h (60%) and after 216 h incubation it was turned into 98% CAR degradation. There was no considerable change in pH during the cultivation. A well established strain *P. resinovorans* CA10 was taken as a positive control and it showed 54% CAR degradation in 216 h. No decrease in CAR concentration was seen in heat killed bacterium. From the plot of time course of growth and CAR utilization (Fig. 2), it can be concluded that isolate could utilize maximum concentration of CAR during exponential phase of their growth. In all the isolates including positive control, no significant degradation of CAR was observed once the cell entered the stationary phase of growth. Time course of CAR degradation by resting cell suspensions in BSM with approximately 1.6 mM CAR was performed at 30°C in potassium phosphate buffer (pH 7.0). Resting cells of *Acinetobacter* spp. Alp6 and Alp7 harvested from late log phase were able to degrade 61% and 51% CAR, respectively in 390 min. The specific activity for CAR degradation by the resting cells of Alp6 was found to be 7.96 µmol/min/g dry cell weight, while for Alp7 it was 5.82 µmol/min/g dry cell weight (Fig. 3). Comparison of CAR degradation activity with the reported activity is not possible as media composition is different in each study. However, the results for biodegradation assay are very promising and comparable with the best results expected for potential bioremediating strains.

Our study shows that *Acinetobacter* species isolated form the soil of dye industry are good CAR degraders. Species of the *Acinetobacter* have been attracting increasing attention in environmental cleanup. It can degrade and

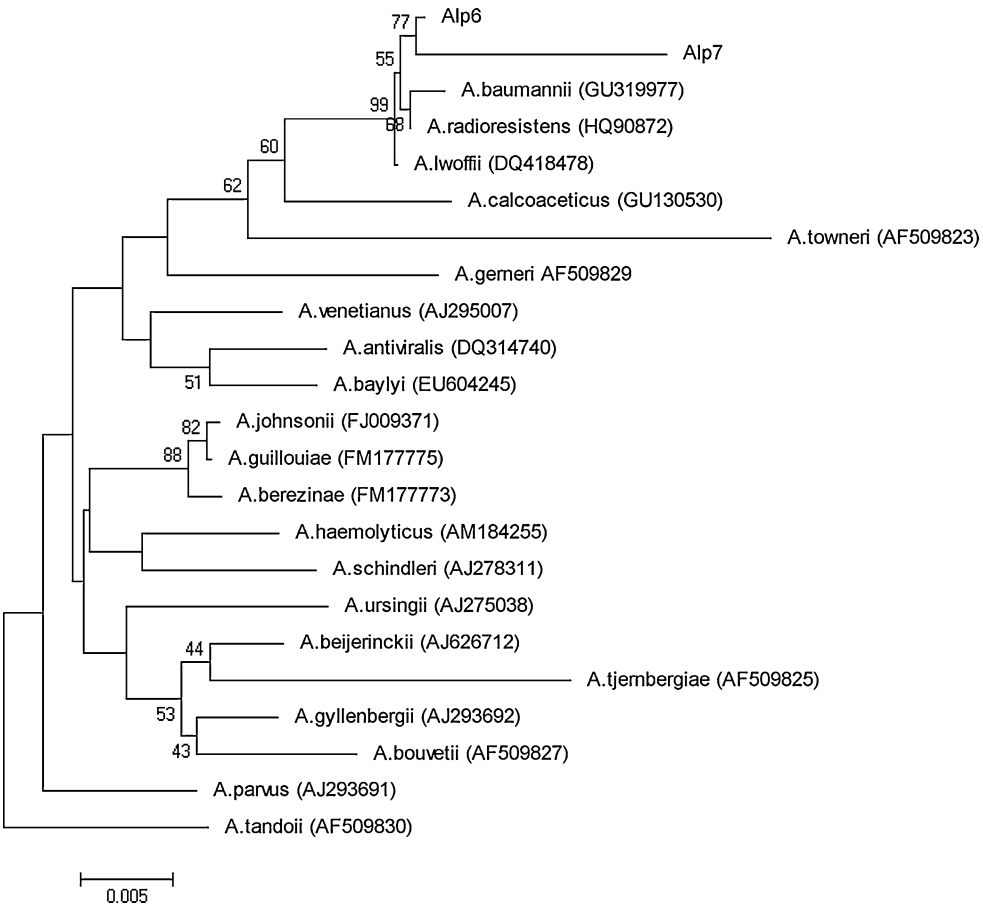

Fig. 1 Neighbour-joining tree based on 16S rDNA gene sequences, showing relationship of newly isolated *Acinetobacter* spp. Alp6 and Alp7 with closely related and validly published species from the genus *Acinetobacter*. The *numbers* indicate bootstrap values >40% (based on 1,000 resamplings). GenBank accession numbers are given in parentheses. *Bar*, 0.005 nucleotide substitutions/position

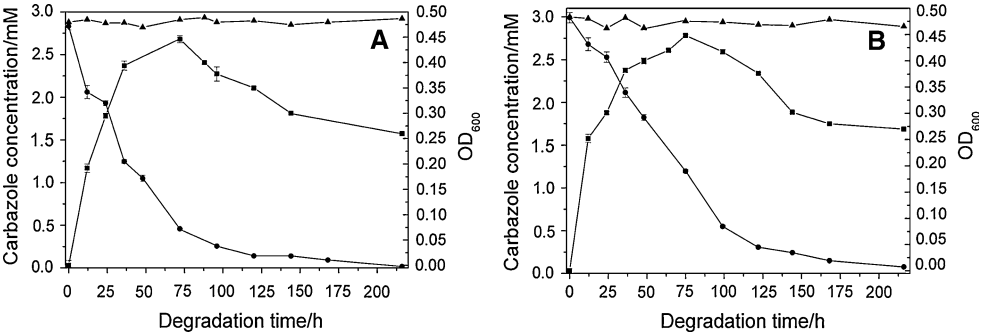

Fig. 2 Degradation of CAR by growing cells of *Acinetobacter* spp. Alp6 (**a**) and Alp7 (**b**). Time course of CAR degradation (*circle*), growth (*square*) and heat killed negative control (*triangle*). The values are means of three independent replicates. SD was within the acceptable range

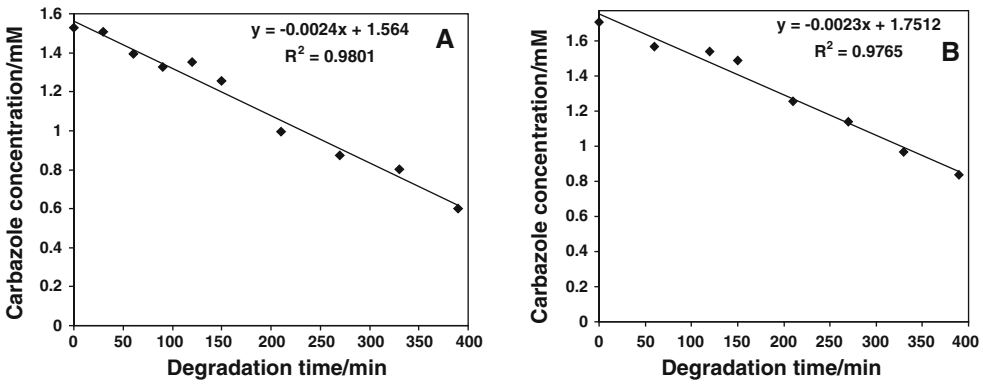

Fig. 3 Time course of CAR degradation (linear fit graph) by resting cells of *Acinetobacter* spp. Alp6 (**a**) and Alp7 (**b**). The values are means of three independent replicates. SD was within the acceptable range

remove wide range of organic and inorganic compounds. Strains of the genus *Acinetobacter* are reported to be involved in the degradation of pollutants like biphenyl, aniline, phenol, benzoate, acetonitrile (Abdel-El-Haleem 2003) and quaternary ammonium compounds (Al-Ahmad et al. 2000). It is also known to be involved in the removal of heavy metals or phosphate. To the best of our knowledge, this is the first report on degradation of CAR by *Acinetobacter* spp. Elucidation of the intermediates of CAR degradation is an important step in determining whether bacteria can be used for bioremediation. Current work is underway to elucidate the genetic/enzymatic basis for CAR degradation to ensure that these processes can be used for removal of CAR from contaminated sites.

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