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Biodegradation of carbazole by the seven *Pseudomonas* sp. strains and their denitrification potential

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ABSTRACT

Carbazole, one representative of non-alkaline nitrogen heterocyclic compounds, is widespread in the natural environment and harmful to human health. In this research, the seven bacterial strains using carbazole as their sole carbon, nitrogen and energy source were isolated from activated sludge of a coking wastewater treatment plant. All strains efficiently degraded 500 mg/L of carbazole in the medium within 36 h. Based on the DNA sequence and phylogenetic tree analysis, the seven strains were identified as the genera *Pseudomonas* with different evolutionary pathways. PCR analysis revealed that the seven isolates carried the *car* gene. Moreover, all of these strains could utilize and transform ammonium and nitrate efficiently, and the six strains except BC043 strain coded the nitrite reductase gene (*nirS*) and the nitrous oxide reductase (*nosZ*), that indicated their denitrification ability. All these strains may be useful in the bioremediation of environments contaminated by carbazole.

1. Introduction

Nitrogen heterocyclic compounds (NHCs), which are often generated from coking, petrochemical, and other related industries, are representative xenobiotics. Carbazole, quinoline, and pyridine are the typical NHCs in these industrial wastewaters. They are well-known to be toxic, teratogenic, and mutagenic pollutant [1,2].

Carbazole is a recalcitrant non-alkaline NHC. It is very difficult to be degraded by most of the microorganisms in environments. However, a few bacteria have evolved the necessary metabolic pathways and acquired the ability to degrade carbazole after a prolonged exposure to this pollutant [3]. These bacteria include *Pseudomonas resinovorans* CA10 [1], *Nocardioides aromaticivorans* IC177 [4], and *Sphingomonas* sp. KA1 [5]. For the first time, Ouchiyama et al. reported the degradation pathways and biodegradation products of carbazole by *P. resinovorans* CA10. This bacterial strain CA10 degraded carbazole to anthranilate and 2-hydroxypenta-2,4-dienoate through angular dioxygenation, *meta*-cleavage, and hydrolysis [1]. The enzyme carbazole 1,9-dioxygenase (CARDO), which participates in the angular dioxygenation and cleaves one of the two carbon–nitrogen bonds, is composed of terminal oxygenase (CarAa), ferredoxin (CarAc), and ferredoxin reductase (CarAd) [6]. CarAa is a unique type of oxygenase that shares low homology with other known dioxygenases based on the amino acid sequence homology and phylogenetic analysis [7,8].

It was also reported that during the biodegradation of carbazole, ammonium was produced as an inorganic product by the bacterial degrader [4,9], like the other degrader of NHCs [10]. However, little attention has been devoted to determine the further transformation of the NH_4^+–N by the same microbial degrader of carbazole. If the NH_4^+–N accumulated in the water, high concentration ammonium could not reach the discharge standard, even would cause serious environmental problems. Adding a new unit or modifying the treatment process to remove ammonia–nitrogen would increase the investment and operation cost. The bacterium with multiple functions could be regarded as an eligible solver.

In this study, the seven carbazole-degrading bacteria were isolated and phylogenetically analyzed. The biodegradation of carbazole by the seven strains was investigated, and the representative functional gene *carAa* was amplified from them. Their nitrification and denitrification potential was investigated using ammonium and nitrate brines. Furthermore, genes involved in the denitrifying process were detected, e.g. the nitrite reductase gene (*nirS*) and the nitrous oxide reductase gene (*nosZ*), that indicate the denitrification potential of these carbazole-degrading bacteria.

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2. Materials and methods

2.1. Chemicals

Carbazole was obtained from Sigma–Aldrich, Inc., USA. Dimethyl sulfoxide (DMSO) was purchased from Amresco, USA. NH_3–N, NO_2^-–N, and NO_3^-–N were received from the China Research Center of Certified Reference Materials. Tryptone and yeast extract were obtained from Oxoid Ltd., UK. Solvents for HPLC and GC/MS analysis were chromatographic grade. All other chemicals used in this study were analytical grade.

2.2. Media

Two kinds of basic media were used in the experiments. The Luria–Bertani (LB) medium [11] was used for bacteria enrichment and maintenance. The mineral salt medium (MSM), which does not contain N source as described by Bai et al. [12], was used as the basic ingredient for the bacterial degradation and transformation. The carbazole was dissolved in DMSO (30 g/L) and added to the MSM as the sole degradable carbon, nitrogen, and energy source in the biodegradation experiments. The MSM + NH_4Cl + glucose medium was used to determine the nitrification potential; and the MSM + KNO_3 + glucose medium was used to evaluate the denitrification potential of these carbazole-degrading strains. All media were sterilized at 121 °C for 20 min before use.

2.3. Bacteria cultivation and isolation

The seven carbazole-degrading bacteria that utilized carbazole as their sole sources of carbon, nitrogen, and energy were isolated from the activated sludge of the coking wastewater treatment plant of Shougang Group, Beijing, China. The strains were identified by 16S rRNA sequence analysis. Genomic DNA was extracted from each pure culture using a TIANamp Bacteria DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.). The bacterial 16S rRNA gene sequence was amplified using the TaKaRa ExTaq hot-start polymerase (TaKaRa Bio., Japan) with the bacterial universal primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492r (5′-TAC GGT TAC CTT GTT ACG ACT-3′) [13]. The PCR thermal program was at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min, and then kept at 4 °C. The sequences were analyzed by the ABI 3730*xl* DNA analyzer (Applied Biosystems, USA).

The sequences obtained from the seven strains were searched in the National Center for Biotechnology Information (NCBI) database by using the BLAST program. Selected sequences of the same genera were extracted from the GenBank database. Sequence analysis was performed by Bioedit software, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA 4.0 [14].

2.4. Biodegradation of carbazole

A series of 250-ml Erlenmeyer flasks were used in the experiments. Each flask carried 100 ml of the MSM with a specific concentration of carbazole and the same initial amount of inoculated bacterium (OD_{602} = 0.1). In addition to above test flasks, the dead-cell control and negative control experiments were carried out at the same time. The dead-cell control flasks were inoculated with different species of heat-killed (autoclaved at 121 °C for 20 min) cells corresponding to the test flasks. The negative control flask had no inoculum. All flasks were sealed with sealfilm, shaken at 30 °C, 180 rpm, and sampled periodically. The samples were fully vortexed, centrifuged and extracted with isometric ethyl acetate for the carbazole analysis. All experiments including the negative controls were carried out in triplets.

2.5. PCR amplification and detection of dioxygenase in carbazole catabolism

In order to investigate the catabolic potential of the seven *Pseudomonas sp.* strains for carbazole, the total DNA extracted from each strain was analyzed for the presence of carbazole 1,9-dioxygenases (CARDO). The PCR amplification primer was designed from *Pseudomonas* sp. CA10 and *Pseudomonas* sp. OM1 as follows: sense: 5′-GCG AGC CGA AGA CAC TAA-3′, antisense: 5′-GCG TAG AAA TCC ACC ATA GC-3′. The PCR thermal program was set at 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min, and then kept at 4 °C. The TaKaRa Taq hot-start polymerase (TaKaRa Bio., Japan) was used for the PCR reaction. The sequence was analyzed by the same method as that used for 16S rRNA sequence analysis.

2.6. Nitrification and denitrification potential

A series of 500-ml Erlenmeyer flasks were used. Each flask was filled with 200 ml of the MSM + NH_4Cl + glucose or MSM + KNO_3 + glucose medium. The initial C/N ratio of the medium was kept at 25:1. All flasks were sealed with sealfilm, shaken at 30 °C, 180 rpm, and sampled periodically. The NH_4^+–N, NO_3^-–N, and NO_2^-–N were measured for the samples from the MSM + NH_4Cl + glucose medium; the NO_3^-–N and NO_2^-–N were measured for the samples from the MSM + KNO_3 + glucose medium.

For functional gene detection, two gene fragments encoding cytochrome cd1-containing nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*), which participate in denitrification, were amplified from the total DNA of the seven carbazole-degrading strains using the following primers [15]:

nirS-F: 5′-CAC GG**Y** GT**B** CTG CGC AAG GGC GC-3′
nirS-R: 5′-CGC CAC GCG CGG **Y**TC **S**GG GTG GTA-3′
nosZ-F: 5′-CG**Y** TGT TC**M** TCG ACA GCC AG-3′
nosZ-R: 5′-CAT GTG CAG **N**GC **R**TG GCA GAA-3′

Bold-face letters denote degenerate positions. B, G + T + C; M, A + C; N, A + C + G + T; R, A + G; S, G + C; Y, C + T.

The PCR thermal program used was described in our previous study [16]. The TaKaRa Taq hot-start polymerase (TaKaRa Bio., Japan) was used for the PCR reaction. The PCR products were separated by 1% agarose gel electrophoresis and stained using SYBR Safe DNA gel stain (Molecular Probes, USA).

2.7. Plasmid isolation

In order to determine the location of the genes encoding carbazole degradation and the genes participating in denitrification, plasmid DNA was isolated from each strain by the modified alkaline lysis method [11]. Positive control test was synchronized with the whole plasmid extraction.

2.8. Analytical methods

Bacterial growth was monitored by OD_{602} using a UV–Vis spectrophotometer (Shimadzu UV-2401PC).

The carbazole concentrations were analyzed by a high performance liquid chromatography (HPLC) system (Shimadzu $LC10AD_{VP}$, $SPD10A_{VP}$ UV–Vis Detector; Rheodyne 7725i manual injector; Diamonsil C18 reverse-phase column, 250 mm × 4.6 mm, 5 μm). A methanol and water solution (9:1) was used as a mobile

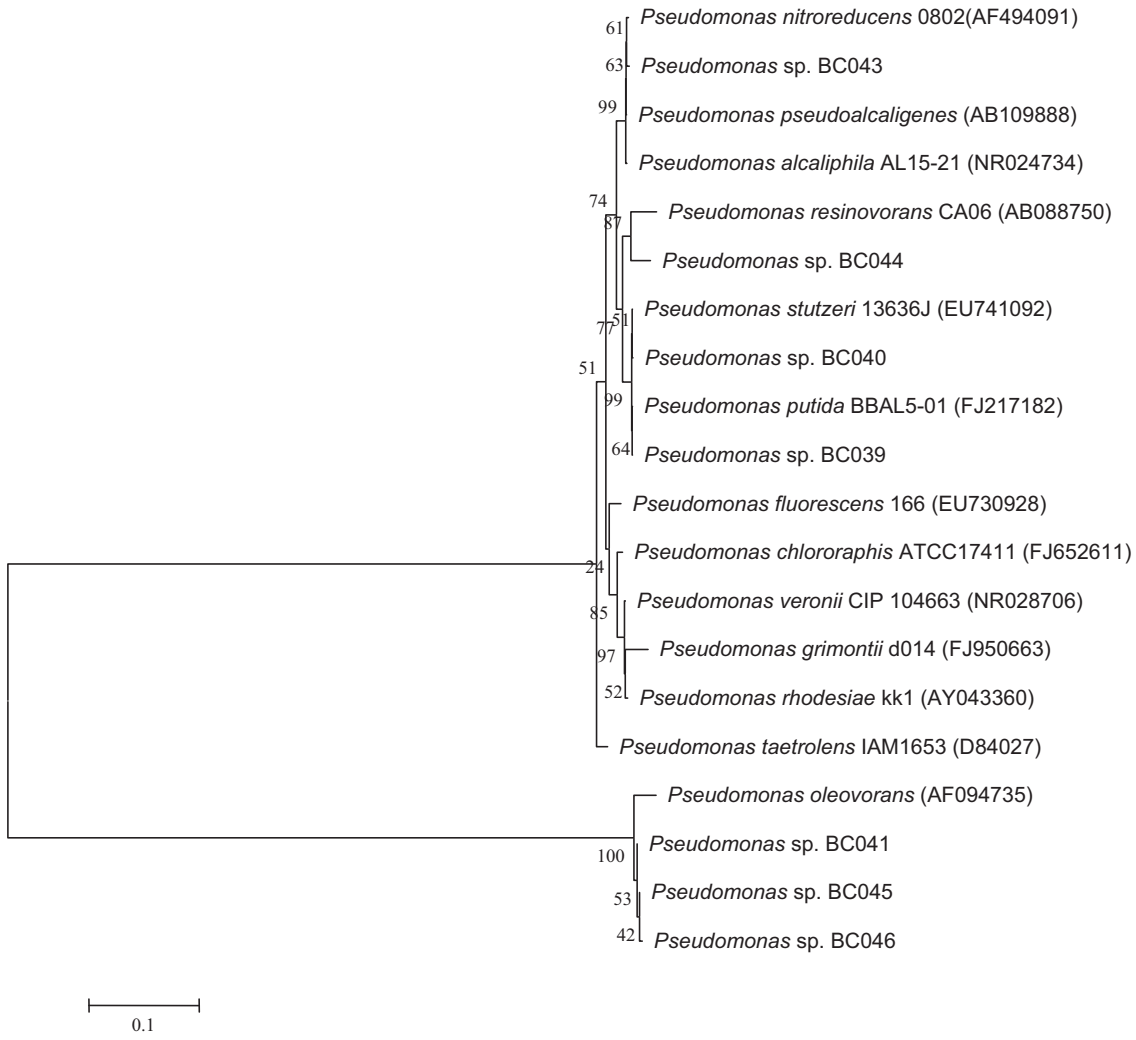

Fig. 1. Phylogenetic tree of *Pseudomonas* sp. BC039, BC040, BC041, BC043, BC044, BC045, BC046, and the members of representative bacteria of *Proteobacteria*. The numbers on the branch nodes demonstrate the percentages of bootstrap support for the clades based on 1000 bootstrap resamplings. The scale bar indicates the average numbers of nucleotide substitutions per site. The numbers in the brackets are each strain's GenBank accession in the NCBI.

phase in isocratic mode at the flow rate of 1.0 ml/min. Carbazole was detected at 254 nm wavelength.

The NH_4^+–N concentrations were analyzed by the salicylate–hypochlorous acid method, NO_2^-–N by the N-1-naphthyl-ethylenediamine method, and NO_3^-–N by the UV-spectrophotometric determination [17,18].

3. Results and discussion

3.1. Identification of the carbazole-degrading strains

The sequences of the partial 16S rRNA gene fragments of the seven carbazole-degrading strains were uploaded to GenBank to find matching sequences. The database search result showed that the seven 16S rRNA sequences were nearly identical (up to 99%) to those of *Pseudomonas* sp. Phylogenetic tree analysis of the seven carbazole-degrading bacteria and the other similar *Pseudomonas* strains is shown in Fig. 1. Based on the 16S rRNA sequences and phylogenetic tree analysis, the seven strains were identified as the genera *Pseudomonas*, but represented different evolutionary pathways. BC039 and BC040 were closely related to *Pseudomonas stuzeri*; BC043 was similar to *Pseudomonas nitroreducens*; BC044 was identical to *P. resinovorans*; in addition, BC041, BC045, and BC046 were closely related to *Pseudomonas oleovorans*. Therefore, these gram-negative aerobic bacteria were identified as *Pseudomonas* sp. strains BC039, BC040, BC041, BC043, BC044, BC045, and BC046.

3.2. Carbazole degradation by the Pseudomonas sp. strains

All the seven *Pseudomonas* sp. strains utilized carbazole as their sole carbon, nitrogen and energy source (Fig. 2). All the strains degraded almost completely 500 mg/L of carbazole within 36 h and the removal efficiency of carbazole by BC039, BC040, BC041, BC043, BC044, BC045, and BC046 was 98.7%, 98.3%, 97.7%, 91.6%, 97.8%, 97.6%, and 97.3%, respectively (Fig. 2A). Therefore, carbazole removal efficiency by all strains except BC043 was above 97%, and BC039 strain showed the best result. The variation of the carbazole concentration of the negative control (Fig. 2A negative) and dead-cell controls (Fig. 2B) changed 6% and 12.8% in 48 h. These evidences indicated that the removal of carbazole was caused by biodegradation rather than by volatilization or adsorption.

During the initial period of carbazole biodegradation, within 8 h after the reaction, the degradation extent of the seven strains was not much different from each other. However, some discrepancy did exist in the degradation rate among these strains. During the

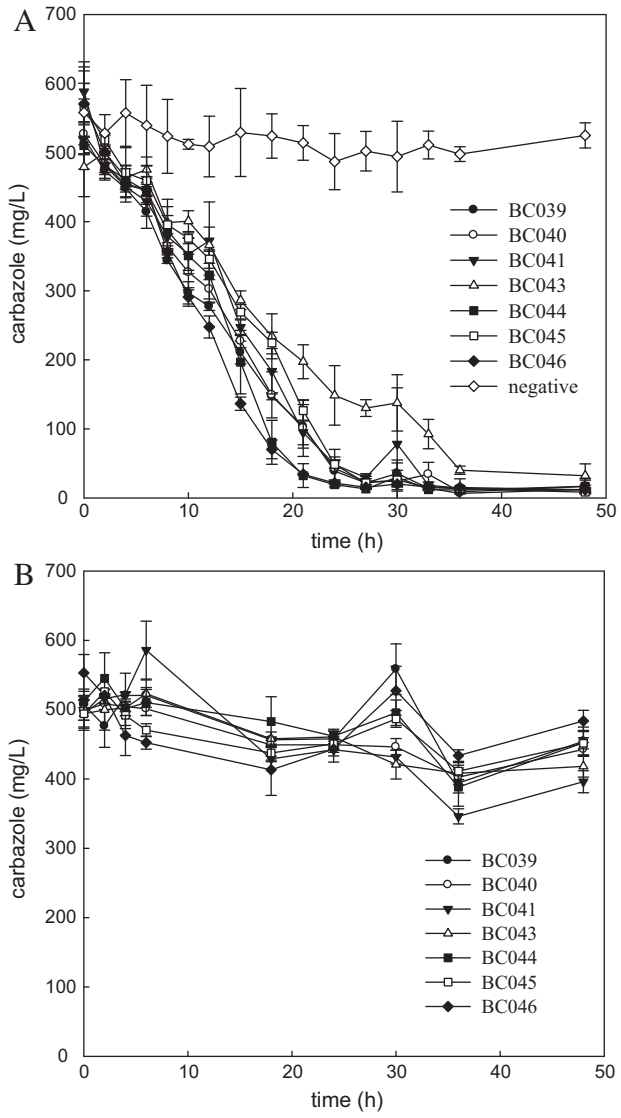

Fig. 2. Degradation of carbazole by the seven *Pseudomonas* sp. strains. (A) The degradation of carbazole 500 mg/L by the alive cells of the seven stains; (B) dead-cell controls of carbazole degradation.

rapid degradation process (0–21 h), the average degradation rate by the seven strains was 19.86, 20.27, 23.49, 13.43, 22.76, 21.02, and 25.53 mg/(L h), respectively. Among these bacteria, BC046 was the best carbazole degrader.

3.3. PCR amplification of functional gene for carbazole degradation

The *carAa* fragments were amplified from the total DNA of the seven *Pseudomonas* sp. strains and their electrophoresis patterns are shown in Fig. 3. All our seven bacteria held the *carAa* fragments. After sequencing, GenBank's BLAST program was used and the *carAa* fragments (917–926 bp) were more than 97% identical to the well-known carbazole-degrading stain, *P. resinovorans* CA10 [1]. However, except for BC044 strain, the genomes of the other six strains were significantly different from that of *P. resinovorans* as shown in Fig. 1.

In CA10 strain, the complete gene encoding CARDO located on a 199 kb IncP-7 circular plasmid pCAR1. The nucleotide sequence of the megaplasmid contained *tra* and *trh* genes that participate in the conjugative transfer, which indicated that the plasmid was

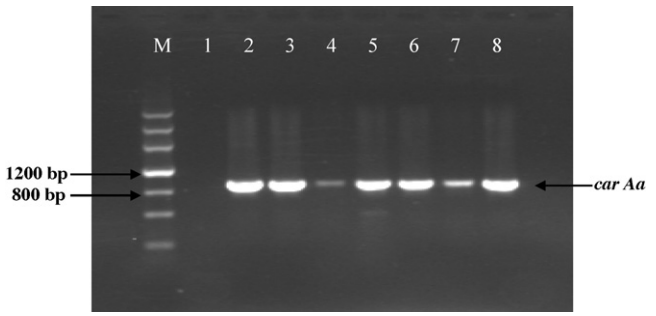

Fig. 3. PCR amplification of the *carAa* gene fragments from the seven carbazole-degrading strains. M, the molecular size markers, with the size of 200, 500, 800, 1200, 2000, 3000 and 4500 bp from bottom to top; lane 1, negative control of *carAa* gene fragment; lanes 2–8, *carAa* gene fragments of *Pseudomonas* sp. BC039, BC040, BC041, BC043, BC044, BC045, and BC046.

a self-transmissible mobile genetic element [19–21]. In our study, none of the seven strains harbored any plasmid. Therefore, the gene encoding carbazole degradation should locate on the genome of the seven bacteria. It was reported in several previous studies that strains from different geographic locations retained the same ability, because of the horizontal gene transfer, especially between the related hosts [21,22]. We speculated that the gene *carAa* or CARDO might be captured by a host bacterium through horizontal gene transfer and integrated into their own genome under a persistent pressure of carbazole pollution. A further and more detailed study would allow understanding of the ecological diversity and geographic distribution of these carbazole degraders as well as their potential capabilities.

The enzyme carbazole 1,9-dioxygenase (CARDO), which participates in the angular dioxygenation and cleaves one of the two carbon–nitrogen bonds of carbazole. Therefore, we concluded that the first step of carbazole biodegradation by the seven strains was angular dioxygenation, i.e. the bacteria degrade carbazole to 2′-aminobiphenyl-2,3-diol.

3.4. Nitrification of the Pseudomonas sp. strains

During the biodegradation of carbazole, the NH_4^+–N was generated by the seven bacteria (data were not shown). In the previous studies related to carbazole degradation, inorganic production of NH_4^+ was also detected in the solution [4,9]. However, NH_4^+–N is also a key pollutant and there is a strict standard on discharging it in wastewater. If these carbazole-degrading stains had the capabilities of nitrification and denitrification, the ammonium would be removed simultaneously. Until now, there has been no research to investigate the capabilities of nitrification and/or denitrification by any carbazole-degrading bacterium. The species of *P. stuzeri* and *P. nitroreducens* are recorded in the BERGEY'S MANUAL that possessed the ability of nitrogen transformation. Our seven strains are closely related to both species, therefore, they should possess denitrification capacity [23].

In order to determine whether the seven strains possess nitrification ability, the MSM + NH_4Cl + glucose medium was used for their metabolism. As shown in Fig. 4A, all the seven strains removed the NH_4^+–N completely within 30 h, when initial concentration of NH_4^+–N was 82.4 ± 4 mg/L and the initial OD_{602} of the each strain was 0.1.

During the removal of NH_4^+–N, more than 15 mg/L of NO_3^-–N was generated by all the strains except BC043 strain (Fig. 4B). A low concentration of NO_2^-–N (about 0.1 mg/L) was also detected in the solution (Fig. 4C). Within 10 h, the NO_3^-–N was almost completely removed; however the concentration of NO_2^-–N reached its highest (maximum) level.

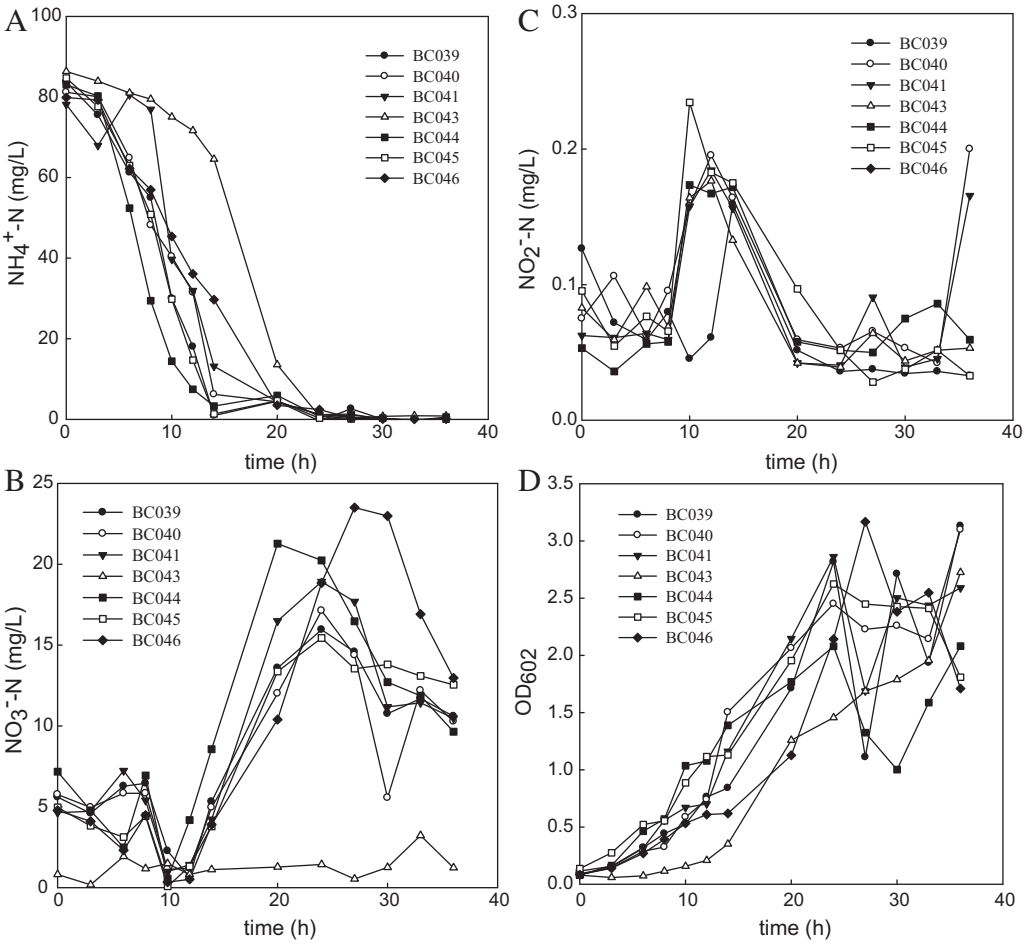

Fig. 4. Utilization and transformation of NH_4^+–N by the seven *Pseudomonas* sp. strains. (A) The removal of NH_4^+–N; (B) the yield of NO_3^-–N; (C) the yield of NO_2^-–N; (D) the growth of the seven strains.

The seven carbazole-degrading strains grew rapidly, as the concentration of the NH_4^+–N decreased significantly (Fig. 4D). This indicated that the seven strains grew on NH_4^+–N and glucose as their N and C sources. According to the data shown in Fig. 4A–D, more than 18.2% of NH_4^+–N was transformed into NO_2^-–N and NO_3^-–N.

3.5. Denitrification of the Pseudomonas sp. strains

Whether the seven strains could transform the NO_2^-–N and NO_3^-–N into harmless gaseous nitrogen was the key to nitrogen removal. In order to determine the denitrification potential of the seven strains, MSM + NO_3^-–N + glucose was used as their metabolic medium. The NO_3^-–N was reduced completely within 72 h by all the seven strains (Fig. 5) when the initial concentration of NO_3^- was 98.0 ± 2.5 mg/L and the initial OD_{602} of each strain was 0.1.

NO_2^-–N ions were generated during the reduction of the NO_3^-–N. However, the modes of denitrification by the seven *Pseudomonas* sp. strains were very different. As shown in Fig. 5, within the first 24 h of the NO_3^-–N reduction, a slight accumulation of NO_2^-–N was observed, which indicated that six of the strains preferentially utilize NO_3^-–N before using NO_2^-–N as their N source. Only BC046 did not accumulate NO_2^-–N during denitrification and this clearly indicated that this particular strain can utilize NO_3^-–N and NO_2^-–N simultaneously. Moreover, in comparison with the other six strains, BC043 transformed the highest amount of NO_3^-–N (31%) into NO_2^-–N (Fig. 5A and B), and utilized less NO_3^-–N for biomass growth. As shown in Fig. 5C, the biomass of BC043 was 2–3 times lower than the other six strains. This phenomenon indicated that when a strain transformed more NO_3^-–N into NO_2^-–N, the biomass of the strain grew correspondingly less.

3.6. PCR amplification of functional genes for denitrification

In our tests for nitrification and denitrification potential, we found that these seven carbazole-degrading bacteria could utilize and transform ammonia and nitrate. Bacterial denitrification uses NO_3^- and NO_2^- as electron acceptors. Several functional genes are involved in the process, where NO_3^- and NO_2^- are reduced to gaseous products as NO, N_2O, and N_2. The *nirS* is cytochrome cd1 contains the nitrite reductase gene, which is known as a marker for detecting denitrifying bacteria in activated sludge [24,25]. The *nosZ* is a nitrous oxide reductase gene and it is generally unique to denitrifying bacteria [26].

The genes of *nirS* and *nosZ* (approximately 700 bp fragments) that are involved in microbial deniftrification were amplified from genome of the seven carbazole-degrading bacteria. Based on the electrophoresis (Fig. 6), the *nirS* and *nosZ* genes were found in six strains (BC039, BC040, BC041, BC044, BC045, and BC046) except BC043. The lengths of these gene fragments of the six strains were identical to other denitrifying bacteria [15,16,27]. We concluded that the six strains could convert NO_3^- and NO_2^- to N_2.

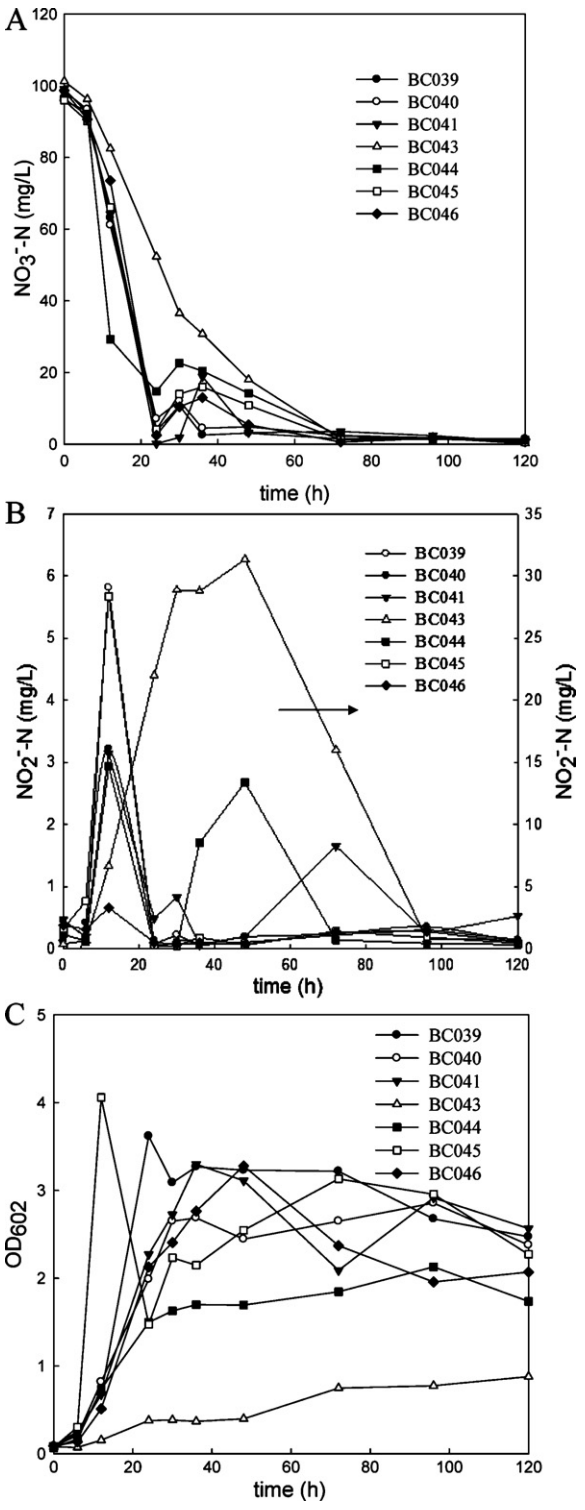

Fig. 5. Utilization and transformation of NO_3^- by the seven strains. (A) The reduction of NO_3^-–N; (B) the yield of NO_2^-–N, the right *Y* axis was for the concentration of NO_2^-–N generated by BC043, the left *Y* axis was for the other six strains; (C) the growth of the seven strains.

BC043 strain appears distinct from the other six strains in both functional genes and nitrogen transformation. In the denitrification experiment, BC043 accumulated NO_2^- for a long time when the NO_3^- had been exhausted. It utilized NO_3^- in preference to NO_2^- for its growth. The nitrogen transformation pathway by BC043 strain is under further study.

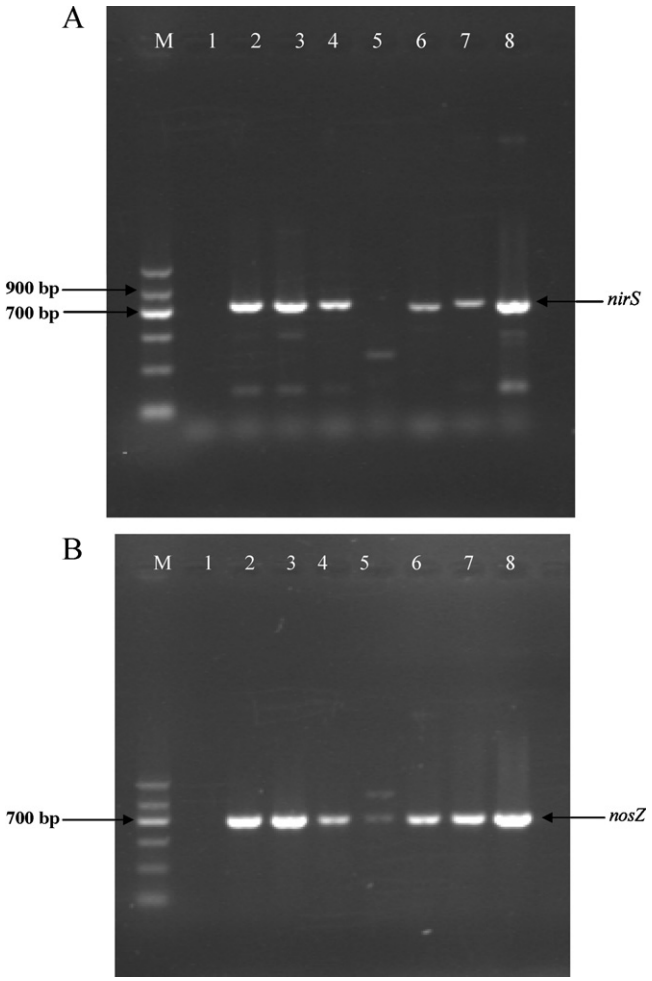

Fig. 6. PCR amplification of the specific fragments of *nirS* and *nosZ* gene from the seven carbazole-degrading strains. (A) M, the molecular size markers, with the size of 100, 300, 500, 700, 900 and 1200 bp from bottom to top; lane 1, negative control of *nirS* gene fragment; lanes 2–8, *nirS* gene fragments of *Pseudomonas* sp. BC039, BC040, BC041, BC043, BC044, BC045, and BC046; (B) M, the molecular size markers, with the size of 100, 300, 500, 700, 900 and 1200 bp from bottom to top; lane 1, negative control of *nosZ* gene fragment; lanes 2–8, *nosZ* gene fragments of *Pseudomonas* sp. BC039, BC040, BC041, BC043, BC044, BC045, and BC046.

4. Conclusion

The seven carbazole-degrading bacteria were isolated from the activated sludge of a coking wastewater treatment plant. All the bacteria were identified as *Pseudomonas* sp. strains (BC039, BC040, BC041, BC043, BC044, BC045, and BC046). All the strains degraded 500 mg/L carbazole efficiently within 36 h, with removal efficiencies above 97% except BC043 (91%). We concluded that the *carAa* genes were located on the genomes of the seven strains. The NH_4^+–N was produced during carbazole degradation. The seven degrading bacteria could utilize and transform NH_4^+–N, because they all possessed nitrification and denitrification ability. The genes *nirS* and *nosZ* were detected on the genome of all the strains except BC043.

The carbazole biodegradation, nitrification, and denitrification capabilities of these degrading bacteria should be useful in bioremediation for NHCs and ammonium polluted environments.

Nucleotide sequence accession numbers

The accession numbers of the isolates 16S rRNA gene of the seven strains (BC039, BC040, BC041, BC043, BC044, BC045, and

BC046) on the GenBank are HQ105008, HQ105009, HQ105010, HQ105011, HQ105012, HQ105013, and HQ105014, respectively.

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