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Study on PAH degrading bacteria isolated from dioxin contaminated soil in Vietnam

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Abstract. Bacterial community in dioxin contaminated soil and mud samples from Danang airport was enumerated and studied for their potential degradation capability against three-ring compound carbazol. The cell number was determined on nutrient rich medium and on mineral medium supplemented with carbazol. It has been showed that number of cells presented in mud samples was significantly higher than in soil samples, in the range of 1 magnitude order. About 50% of the total cell number in each sample grown on the medium with carbazol. Analyzing 12 representative strains selectively isolated on the carbazol containing medium by using the ARDRA method with two endonucleases *Hae*III and *Msp*I showed that bacterial community at the studied areas was highly diversified. However non from these isolates could degrade carbazol significantly. Via enrichment and isolation steps using carbazol as the only energy and carbon sources, two carbazol degrading isolates R03 and R05 were obtained. Phylogenetic analyses based on 16S rDNA sequences showed that these strains were most affiliated to *Bacillus* and *Archromobacter* species, the most closely related species were *Achromobacter xylosoxydans* (99% homology) and *Bacillus subtilis* (98% homology). These strains were therefore designated the names *Bacillus* sp. R03 and *Achromobacter* sp. R05.

Keywords: Dioxin contamination, carbazol degrading bacteria, 16S rDNA, ARDRA.

1. Introduction

Dioxin is a group of highly toxic chemicals that are hardly degraded under natural condition [1]. Due to the high toxicity of dioxins to living organisms, model compounds such as carbazol, phenanthrene are usually used instead for most biodegradation studies [2]. It has been shown that microorganisms, especially bacteria, play an important role in biodegradation of poly-aromatic hydrocarbons (PAHs), including dioxins [3]. The oxidation of such aromatic ring-containing compounds is catalyzed by an enzyme called dioxygenase [2]. The enzyme has been detected in a variety of bacterial species, most of that belong to the genera *Pseudomonas*, *Sphingomonas*, *Rhodococcus*, *Terrabacter* [4, 5], and more new PAH-degrading species isolated from contaminated areas have been reported in recent studies [6, 7].

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During the Vietnam War, a large amount of agent orange was sprayed on forest areas in the middle region of Vietnam [8, 9]. It is shown that dioxins, including 2,3,7,8-TCDD, make a significant part of the agent orange used in the War [8]. As a military airport, Da Nang airport was one of the places where this toxic chemical was stored before the use, and therefore is heavily contaminated until today [10, 11]. Over 30 years exposed to dioxin, Da Nang airport becomes a unique natural enrichment of degrading microbes that worth to be explored. In this study, we investigated bacterial strains isolated from soil and mud samples at Danang airport, especially those strains having capability to degrade carbazol, which is used as a model compound instead of dioxins in the laboratory.

2. Materials and methods

2.1. Sampling technique

Two areas at Da Nang airport were selected for taking samples, the former agent orange storage area and the water collecting lotus pond. Soil samples (at positions S43, S74, S78 in the former agent orange storage area) and mud samples (at positions B31, B52, B55 in the lotus pond) were collected. These positions were different in contamination levels. At each position, 5 samples along the depth from surface to 80 cm below were taken, separated into closed vessels and stored at 4 °C until analyzing in the laboratory. In this study, samples from the first 5 cm surface were used for counting and isolation of aerobically respiring bacteria.

2.2. Couting, isolation and preservation of bacteria

The number of bacterial cells were determined by colony counting on agar plates. In this study, two kinds of media were applied. Total cell count was determined on rich nutrient agar medium (containing peptone 10 g, meat extract 3 g, NaCl 5 g, H_2O 1 L, pH 7.0) and the number of potential degrading cells was determined on carbazol free mineral (CFM) medium (containing K_2HPO_4 2.2 g, KH_2PO_4 0.8 g, NH_4NO_3 3 g, $MgSO_4.7H_2O$ 0.5 g, H_2O 1 L, pH 7.0), supplemented with 1 ml/L trace element solution [12]. Carbazol was added into the medium from a stock solution in DMSO at the concentration of 100 ppm. Counting experiments were carried out in dublicate and the middle values were taken. Baterial strains with capability to grow on mineral medium with carbazol were selected and transfered to agar slants with carbazol and preserved at −80 °C in 10% glycerol solution supplemented with carbazol at the concentration of 100 ppm.

2.3. Determine growth of bacterial strains with carbazol

Bacterial strains were grown in liquid mineral medium containing carbazol (100 ppm) as the only carbon and energy sources. The growth was determined via mesuring total protein content over time with Bradford method. The experiment was carried out in dublicate. In addition, substrate (carbazol) consumption in cell cultures was determined via extraction with dichloromethane and measuring UV absorption.

2.4. DNA extraction, 16S rDNA amplification and sequencing

DNA of pure cultures was extracted following Marmur's method with some modifications [13]. Nearly full lenghth of 16S rDNA (1500 bp) was amplified via PCR by using the universal primers 27F (AGA GTT

TGA TCC TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACT T) [14]. The PCR products were purified with AccuPrep PCR Purification Kit (Bioneer, Korea) and subjected to sequencing with ABI Prism BigDye Terminator cycler sequencing Kit on automatic sequencer 3110 Avant Applied Biosystems. The obtained sequences were then aligned by CLUSTAL X program [15] with corresponding sequences available in the DDBJ/EMBL/GenBank databases. A phylogenetic tree was constructed by neighbour-joining method [16]. Topography of the constructed tree was evaluated by bootstrap analysis with 1000 replicates [17].

2.5. Analyzing diversity of the bacterial isolates by ARDRA method

Nearly full length sequenses of 16S rDNA (1500 bp) obtained via PCR were digested with restriction enzymes *Hae*III and *Msp*I (Fermentas). The digested DNA products were then separated by electrophoresis on 2% agarose gel at 100 V in 90 minutes. After the electrophoresis, the gel was stained in ethydium bromide solution (5 mg/ml) for 20 minutes before taking photographs under UV light on a GelDoc machine (BioRad). Differences in band pattern of strains indicated their genetic diversity.

3. Results and discussion

3.1. The number of bacterial cells in dioxin contaminated samples

It is shown that the studied dioxin contaminated soil and mud samples have relatively low total microbial cell content, laying in the range of 10^6 to 10^7 CFU·g^{-1}. This number is significantly lower than that of uncontaminated samples [18]. For both kinds of samples, cell content was in reverse ratio to the dioxin contamination level, indicating the effect of this toxic chemical on microbial processes there (Fig. 1).

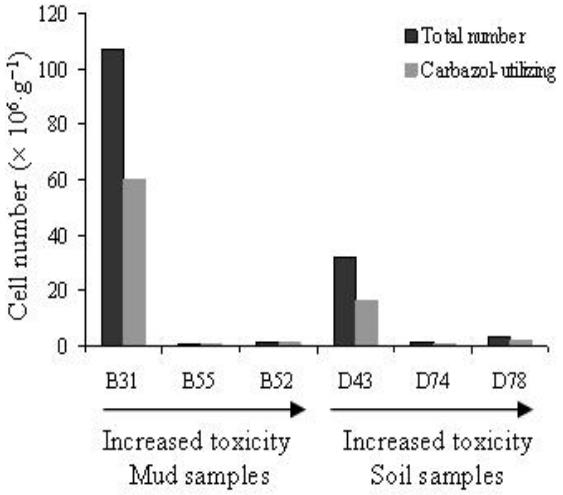

Fig. 1. Number of microbial cells in dioxin contaminated soil and mud samples. (■) Total cell number on rich nutrient medium and (■) carbazol-utilizing cell number on CFM medium supplemented with carbazol.

Apparetly, the mud samples have higher cell content than the soil samples, reflecting the higher content of organic carbon there. Besides that, the number of cell growing on CFM medium supplemented with carbazol was also determined in order to assess degradation potential of the native microbial communities. In all studied samples, the number of bacterial cells grown on CFM-carbazol agar medium was relatively high, making 50-64% of total cells count in the respective samples (Fig. 1). It is therefore expected that native microorganisms could be actively involved in degradation processes at the contaminated sites.

3.2. Isolation of carbazol-utilizing bacteria and study genetic diversity of the isolates

Based on colony characteristics and sample origin, 12 bacterial strains were isolated from

CFMM-carbazol agar plates. In addtion, two other strains R03 and R05 were isolated via enrichment and purification steps in medium containing carbazol as the only energy and carbon sources. These 14 strains were again purified on the same carbazol containing agar medium and stored at –80 °C in 10% glycerol solution with added carbazol.

The genetic diversity of the isolates was investigated through ARDRA analyses by using two restriction enzymes *Msp*I and *Hae*III. These enzymes have been shown with high resolution in a number of studies on bacterial diversity [19]. After amplification steps, the 1500 bp PCR products of 16S rDNA were treated with these two enzymes in single digetions, then separated by electrophoresis on 2% agarose gels.

After treatment with the enzyme *Msp*I, the isolates could be organized into 6 different genetic groups (Fig. 2), including a bigest group G1 with 7 strains (R02, R03, R06, R07, R09, R11, R12), a smaller group G2 with three strains (R05, R08, R10) and remaining four groups (G3, G4, G5, G6) each contained only a single strain (B11, B45, B50 and R04 respectively).

Treatment with the second enzyme *Hae*III allowed to rearrange the strains of two first groups G1 and G2 created by the enzyme *Msp*I. The 7 strains in G1 group were now splited into 3 different groups, namely G1a (R02, R03), G1b (R06, R12) and G1c (R07, R09, R11). Similarly, three strains of the group G2 were splited into G2a with only one strain R05 and G2b with two strains R08 and R10.

Thus, the ARDRA analyses using combination of two enzymes *Msp*I and *Hae*III leading to arrangement of the 14 isolates into 9 different genetic groups as shown in the table below.

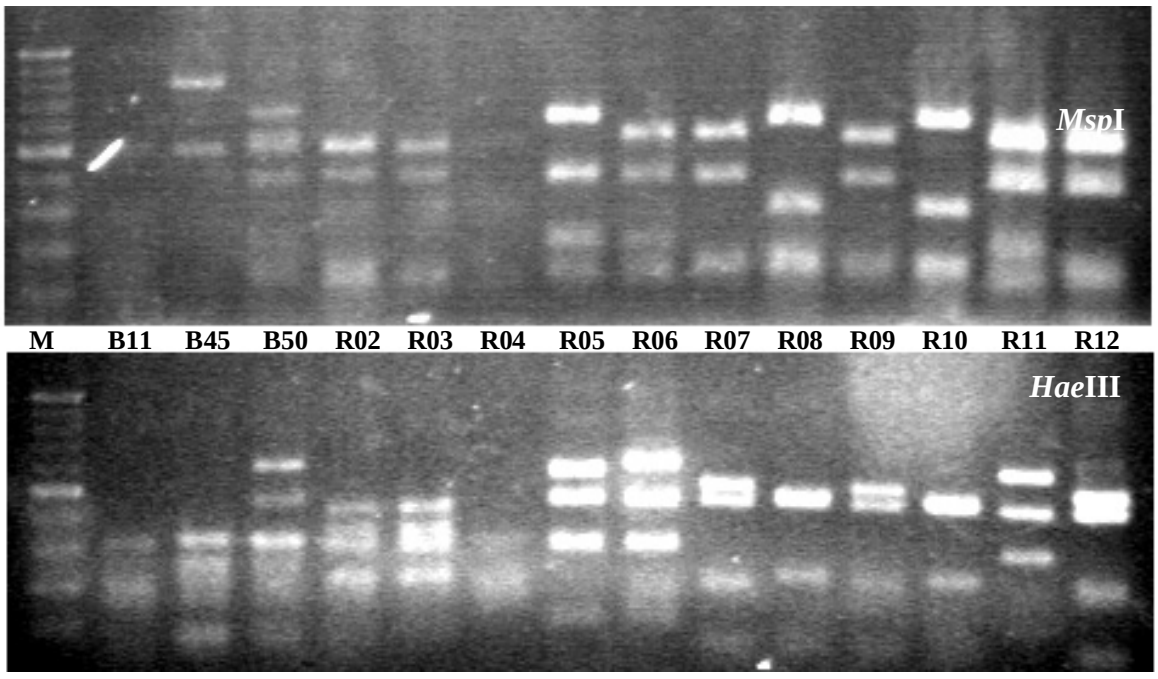

Fig. 2. Restriction profiles of 16S rDNA fragment of bacterial isolates obtained in ARDRA analyses using two endonucleases *Hae*III and *Msp*I.

Table 1. Arrangement of bacterial isolates based on the ARDRA analyses with *Msp*I and *Hae*III.

ARDRA groups	Enzymes used to distinguish the groups	Bacterial isolates
G1a	*Msp*I, *Hae*III	R02, R03
G1b	*Msp*I, *Hae*III	R06, R12
G1c	*Msp*I, *Hae*III	R07, R09, R11
G2a	*Msp*I, *Hae*III	R05
G2b	*Msp*I, *Hae*III	R08, R10
G3	*Msp*I	B11
G4	*Msp*I	B45
G5	*Msp*I	B50
G6	*Msp*I	R04

The results of ARDRA analyses indicated that bacteria in the dioxin contaminated areas at Da Nang airport were highly diverse. Such a high diversity of prokaryotic microbes has been shown in other studies performed at polyaromatic hydrocarbon (PAH) contaminated environments [18, 20]. This diversity might be due to the complexity of degradation pathways of PAH, including dioxins, which creates a vast number of intermediates serving as substrates for microbial growth.

3.3. Carbazol utilization by the bacterial isolates

The capability of the isolates to utilize carbazol was examined via a growth experiment in liquid mineral medium containing carbazol as the only energy and carbon sources. Growth of the bacterial isolates in this medium could be observed via colour changing of the medium. In addition, the increase of cell number in the culture liquid as observed under microscope was also a useful parameter to recognize the growth. Based on these two parameters, non of the 12 isolates obtained directly on mineral-carbazol-agar medium could degrade carbazol significantly. On the other hand, two isolates R03 and R05 obtained via enrichment procedure showed out as promising carbazol degrading candidates. These two strains could change colour and stage of the culture liquid from colorless with white precipitation to yellow and brownish colour without precipitation after just 5 days shaking at 28 °C (Fig. 3A).

Growth of the two selected isolates R03 and R05 with carbazol was determined quantitatively via changing of total cell protein over time (Fig. 3B). The obtained results showed that these strains grown exponentially after two days shaking and their growth lasted for a week before going into a stationary phase. In addition, the amount of carbazol was quantified after 5 day cultivation with these bacterial strains. The results showed that in the presence of strains R03 or R05, about 70% of added carbazol was disappeared as compared with control without bacteria (Fig. 3C, 3D). Such a high degradation capacity was also observed in some PAH degrading microorganisms that have been isolated at the same contaminated sites [21].

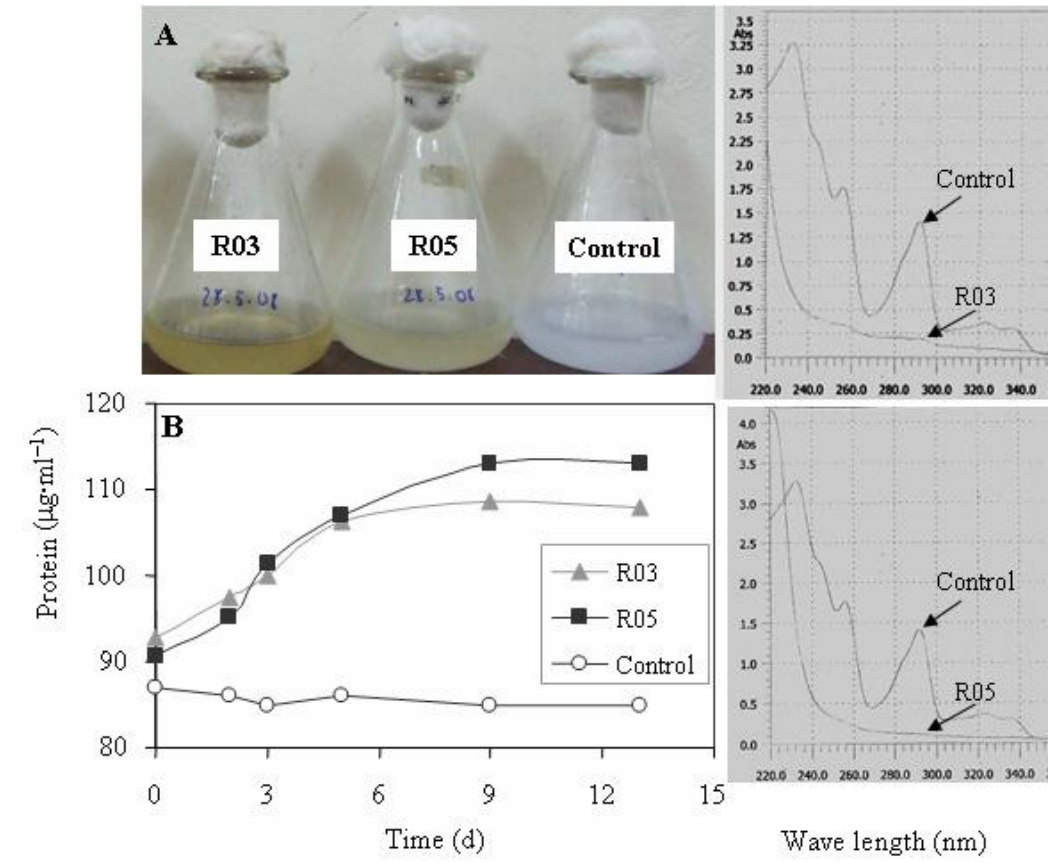

Fig. 3. Carbazol utilization by the bacterial isolates R03 and R05. A-Culture liquid after 5 day shaking; B-Growth curves based on the increasing total protein content over time; C and D-Analyses of carbazol content in liquid cultures of strains R03 and R05, respectively, after 5 days of incubation.

3.4. Physiological and phylogenetic analyses of carbazol-utilizing strains R03 and R05

Both strains R03 and R05 were isolated from sample DN55, a heavily dioxin contaminated area in the lotus pond. These strains belonged to typical mesophilic group, grown best at temperature in the range of 25 – 37 °C. Because pH of the environment where these strains originated from was slightly acidic (5.5 – 6.5), these strains grown better at pH 6.5. Next to carbazol, these two strains could also utilize other PAH compounds such as phenanthrene and naphthalene. Besides that, they also grown on other common organic substrates such as carbohydrates and organic acids. The growth was stimulated by yeast extract as well as vitamins and microelement mixtures. Growth in the absence of oxygen was not observed.

Strains R03 and R05 contained short rod-shaped cells of the size 1×2 – 3 µm and 1×3 – 4 µm respectively (Fig. 4 A,B). Comparison analyses of partial sequences of 16S rDNA showed that strain R03 belonged to the genus *Archromobacter* and strain R05 to the genus *Bacillus*, the most closely related organisms were *A. xylooxydans* (99% homology) and *B. subtilis* (98% homology) respectively (Fig. 4C). These strains therefore were designated as *Archromobacter* sp. R03 and *Bacillus* sp. R05.

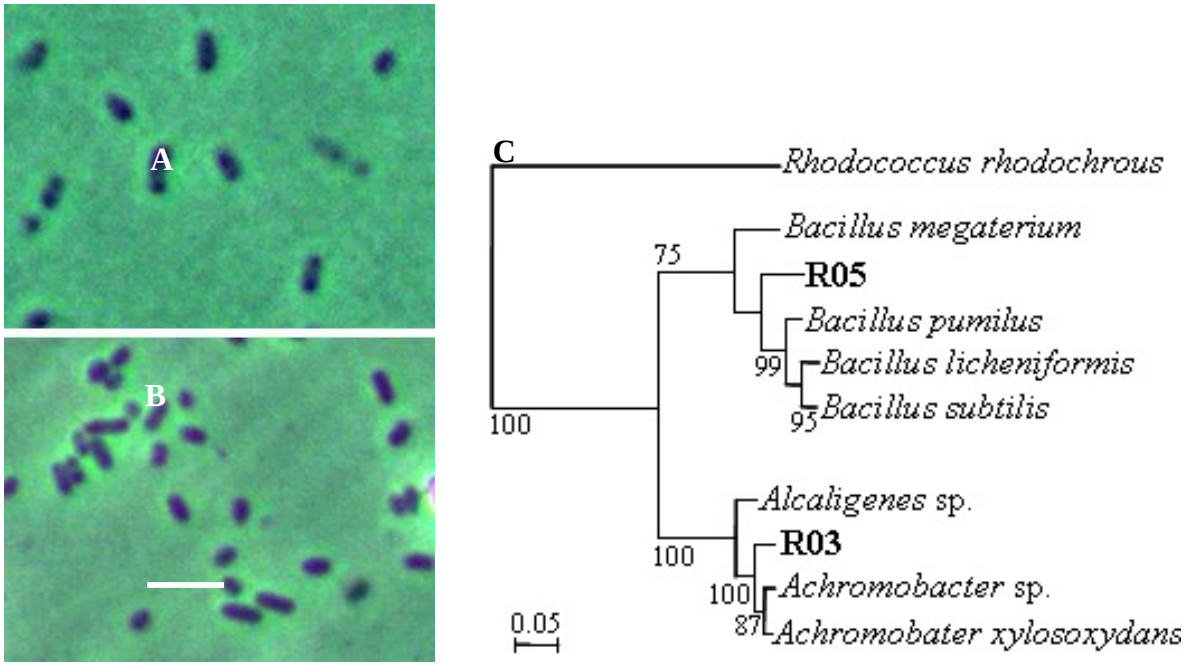

Fig. 4. Cell morphology and phylogenetic relationship of strains R03 and R05. A, B – Microscopic images of living cells of strains R03 and R05, respectively, previously grown on mineral medium supplemented with carbazol, scale bar = 5 μm; C – Phylogenetic tree of strains R03 and R05 with closely related genera based on the 16S rRNA gene sequences. The tree was constructed using the neighbour-joining method. Scale bar = 0.005 K_{nuc} in nucleotide sequences. The numbers on the branches are the confidence limits estimated by bootstrap analysis with 1,000 replicates (only values above 50% are presented).
Rhodococcus rhodochrous is selected as outgroup.

4. Conclusion

By using carbazol as a substituted substrate for studying degradation capacity of bacteria, it could be shown that potentially degrading organisms made a high proportion of the total cell count at dioxin contaminated sites at Danang airport.

12 representative strains of bacteria grown with carbazol were isolated and studied for their genetic diversity. ARDRA analyses of 16S rDNA with two restriction enzymes *Hae*III and *Pst*I brought about high diversity among the isolated strains. However, the ability to utilize carbazol as the only energy and carbon sources of these strains was not significant.

Via enrichment and isolation techniques carried out in mineral medium supplemented with carbazol, two strains R03 and R05 were obtained with high degrading capacity. Phylogenetic analyses of these two strains based on 16S rDNA sequences showed that they belonged to the genera *Achromobacter* and *Bacillus*, respectively. Accordingly, the names *Achromobacter* sp. R03 and *Bacillus* sp. R05 were designated for these bacterial strains.

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Nghiên cứu vi khuẩn phân hủy PAH phân lập từ đất nhiễm dioxin tại Việt Nam

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Quần thể vi khuẩn trong đất và bùn nhiễm dioxin tại sân bay Đà Nẵng được xác định về số lượng và nghiên cứu về khả năng phân huỷ đối với hợp chất có cấu trúc 3 vòng thơm carbazol. Số lượng tế bào được xác định trên môi trường thạch giàu dinh dưỡng và môi trường khoáng có bổ sung carbazol. Kết quả thu được cho thấy số lượng vi khuẩn trong các mẫu bùn cao hơn đáng kể so với các mẫu đất, khoảng 1 số mũ. Tại mỗi điểm nghiên cứu, số lượng vi khuẩn có khả năng sinh trưởng trên môi trường khoáng-carrbazol chiếm 50% tổng số tế bào đếm được trên môi trường giàu dinh dưỡng. Nghiên cứu 12 chủng vi khuẩn đại diện phân lập từ các đĩa thạch khoáng-carbazol bằng phương pháp ARDRA sử dụng 2 enzyme giới hạn *Hae*III và *Msp*I cho thấy tính da dạng cao về mặt di truyền của vi khuẩn tại vùng nhiễm dioxin. Tuy nhiên không có chủng nào trong số này thể hiện khả năng phân huỷ cao đối với carbazol. Thông qua kỹ thuật nuôi tích luỹ và phân lập trên môi trường khoáng-carbazol hai chủng khác có tên là R03 và R05 được phân lập và thể hiện khả năng sinh trưởng tốt với carbazol làm nguồn carbon và năng lượng duy nhất. Phân tích phả hệ dựa trên trình tự 16S rADN cho thấy hai chủng này thuộc các chi *Bacillus* và *Archromobacter*, các loài gần gũi nhất là *A. xylooxydant* (99% tương đồng) và *Bacillus subtilis* (98% tương đồng). Hai chủng R03 và R05 được đặt tên tương ứng là *Achromobacter* sp. R03 và *Bacillus* sp. R05.

Từ khoá. Nhiễm dioxin, carbazol, vi khuẩn phân huỷ PAH, 16S rADN, ARDRA.