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An active PAH-degrading microbial consortium developed from dioxin-contaminated sediments via enrichment technique

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Abstract. Dioxin contaminated environments could serve as natural sources of microorganisms with special degradation capabilities. Via enrichment procedures carried out on a dioxin contaminated sediment sample from lotus pond at Danang airport, a microbial community assigned as DN553 with high capability of carbazol degradation was established. Analyses of community structure by using denaturing gradient gel electrophoresis (DGGE) of 16S rDNA fragments indicated that *Achromobacter* and *Alcaligenes* species dominated in this enrichment culture. In addition to carbazol, the enrichment culture was also able to utilize other PAH compounds such as naphthalene and phenanthrene as the only carbon and energy sources. In the presence of different PAH as growth substrates, the community structure changed accordingly, however the *Achromobacter* and *Acaligenes* groups still remained. Thus, the enrichment cultures DN553 could be a potential microbial source for the treatments of PAH contamination.

Keywords. Enrichment culture, PAH degradation, DGGE, 16S rDNA, *Achromobacter*, *Alcaligenes*.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) make a class of organic compounds that consist of two or more fused benzene and pentacyclic rings that are arranged in various structural configurations. They are highly recalcitrant molecules that can persist in the environment due to their high hydrophobicity and low water solubility [1]. PAHs are ubiquitous in the natural environment and originate from two main sources, natural (biogenic and geochemical) and anthropogenic [2], of which the latter is the major cause of environmental pollution. PAHs naturally occur in fossil fuels such as coal and petroleum, but are also formed during the incomplete combustion of organic materials [3,4]. PAHs are highly lipid soluble and thus readily absorbed to the gastrointestinal tract of mammals including human and cause serious health problems [5]. Many PAHs show toxic, mutagenic and carcinogenic properties [6, 7], therefore are of environmental concern. Bioremediation is an approach that has been used to clean up land and waters from PAH contamination. Although a number of PAH-degrading microbial pure strains have been

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isolated in different laboratories and applied for the remediation processes, the use of communities in this field now becomes more and more attractive to researchers.

Danang airport is known as a hot spot of dioxin contamination since the time of the Vietnam War. Over more than 40 years exposed to this toxic chemical [8,9], the place has became a unique natural enrichment of PAH-degrading microbes. By using sediment samples taken from this area for enrichment, in this study we successfully produced a stable bacterial consortium that actively degraded PAH compounds under laboratory conditions.

2. Materials and methods

2.1. Sampling

Sediment samples were collected from heavily dioxin contaminated pond at Danang airport and stored at 4°C until use in the laboratory. For the enrichment experiments, samples at 10 cm surface were used.

2.2. Establish PAH-degrading communities via enrichment

Enrichment experiments were carried out in carbon-free mineral (CFM) medium (containing per liter K_2HPO_4 2.2 g, NH_4NO_3 3 g, $MgSO_4.7H_2O$ 0.5 g, pH 7.0), supplemented with 1 ml/L trace element solution and 1 ml/L vitamin mixture solution [10]. After sterilization, carbazol was added from a stock solution in DMSO at the concentration of 100 ppm as the only carbon and energy sources. Sediment samples were used at the ratio of 10% (vol/vol) as inoculums. The enrichments were performed in erlenmeyer flasks under shaking condition at 100 rpm at 28°C and transferred every two weeks.

2.3. Determine growth of bacterial communities with PAH compounds

In addition to carbazol, two other PAH compounds, naphthalene and phenanthrene (Fig. 1), were used in the degradation experiments.

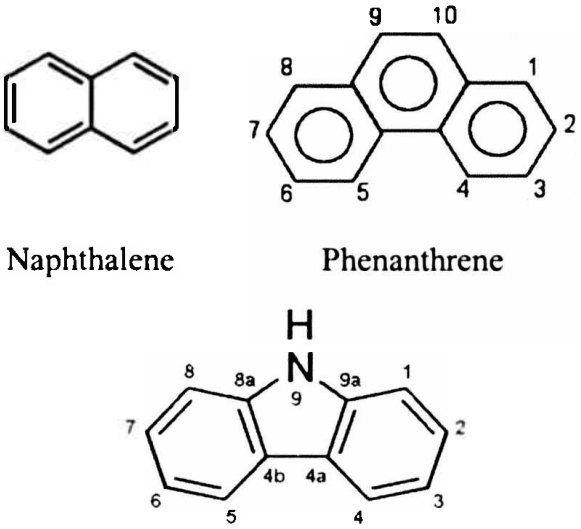

Fig. 1. Structure of PAH compounds used in the degradation experiment.

PAH compounds were added from stock solutions in DMSO to the CFM medium at the concentration of 500 ppm (for carbazol and phenanthrene) and 4000 ppm (for naphthalene) as the sole carbon and energy sources. Liquid enrichment cultures previously grown with carbazol were inoculated in the medium at 10% (vol/vol) and shake at 28°C. 1 ml samples were taken every 2 days for analyzing total protein content by using Bradford method [11]. The experiment was carried out in duplicate.

For analyzing carbazol content in the medium after incubation with microbial cells, dichloromethane was added to the liquid culture to dissolve the remaining carbazol completely and compare UV light absorption of the samples with that of the control without microbes.

2.4. Analyzing community structure of the enrichment cultures

Total DNA of bacterial communities in the enrichment cultures were extracted by using the method described by Zhou et al. [12] with some modifications. 550 bp fragments of 16S rDNA from the samples were amplified via PCR with primer pair 907R and GM5F-GC [13]. These fragments were then subjected to denaturing gradient gel electrophoresis (DGGE) on polyacrylamide gel 6% with denaturing range from 30 – 60% urea/formamid for 15 hours at 100 V and 60 °C. After the electrophoresis, the gel was stained in ethidium bromide solution (5 mg/mL) in 30 min, washed in water for 5 min and photographed under UV light.

Representative bands from the DGGE gel were excised and DNA was eluted in 50 µl water overnight at 4°C. The DNA was then used as template for PCR with primer pair 907R and GM5F [13]. 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 compared with the sequences available on the database GeneBank by using Blast Search tool.

3. Results and discussion

3.1. Enrichment of PAH-degrading bacteria from the dioxin contaminated sediment

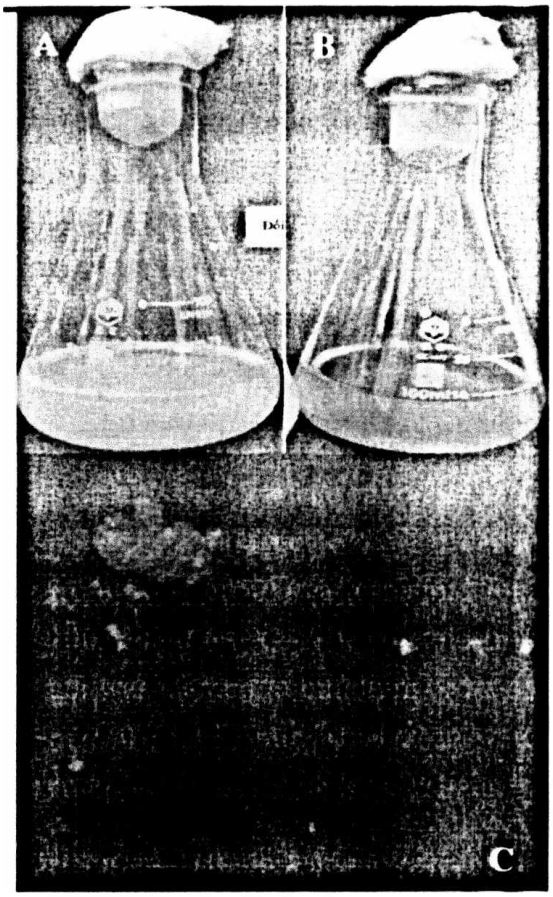

Fig. 2. Enrichment of PAH-degrading microbes using carbazol as the only energy and carbon sources. A, B – liquid cultures after 5 day incubation (A – control without bacteria; B – enrichment culture DN553); C – Microscopy image of cells in the enrichment culture DN553 after staining with DAPI.

Dioxin contaminated sediment sample DN55 was collected from lotus pond at Danang airport and used as the initial source of PAH-degrading microbes for the enrichment experiment. The sample was inoculated in bottles containing mineral medium supplemented with vitamins, trace elements, and carbazol as the only carbon and energy sources. The culture was incubated at 28°C in the dark and was transferred every 2 weeks for three times. The decomposition of the substrate in the bottles could be observed by eyes through the changes of color and the stage of the culture liquid. As the result of the microbial

metabolic activity, the white suspension of carbazol in the liquid medium (Fig. 2A) became homogenous and changed colors due to generated intermediates (Fig. 2B). After three transferring steps, an active enrichment culture DN553 was obtained.

The time the enrichment culture DN553 required to reach the homogenous stage of medium containing carbazol was shortened obviously from the first transferring step to the last one (from 2 weeks to 4 days). Moreover, the growth of microbes in the enrichment culture at every transferring step could also be proven based on the observation of cell density in the liquid culture. Here, to distinguish the cells and substrate crystals, the culture liquid was stained with DAPI and observed under fluorescent microscopy (Fig. 2C). It turned out that a significant pat of microbial cells in the enrichment culture DN553 grew in close contact with the substrate crystals.

3.2. PAH-degradation by the enrichment culture DN553

Growth of microbes in the enrichment culture DN553 on PAH sources were determined via measuring the total content of cell protein. Growth curves of this culture with naphthalene, phenanthrene or carbazol based on the synthesis of protein over time (Fig. 3A) showed that the microbes in this sample indeed utilized the PAH compounds as the carbon and energy sources for their growth.

Among the three PAHs, napthalene seemed to be the best substrate for the microbes and was degraded most easily, then phenanthrene and carbazol. This result was in consistence with previous studies about the fate of these compounds under biodegradation processes [1, 14]. The growth curves had log phase in the first 4 days of inoculation, afterward the growth speed slowed down and the total amount of protein did not increase significantly in the days after.

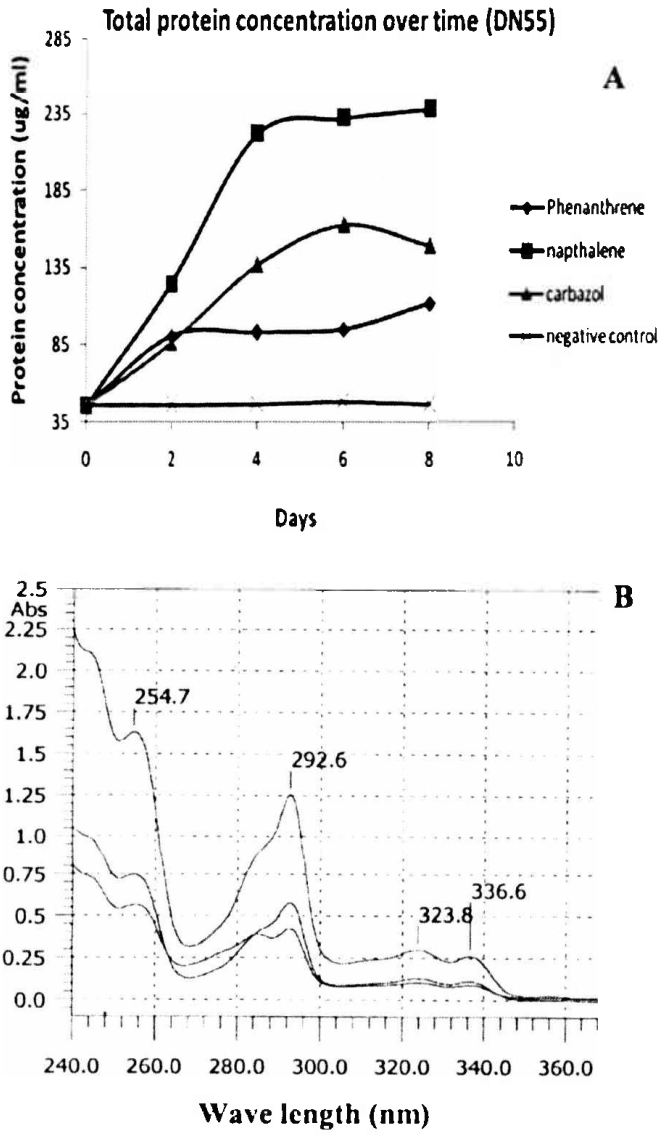

Fig. 3. Growth of the enrichment culture DN553 in mineral medium with different PAH compounds as the only carbon and energy source. A – Growth curves based on measuring total protein content over time; B – carbazol utilization after 5 day incubation.

Analysis of carbazol content in the culturing medium after 5 days of incubation (Fig. 3B) showed that more than 50% of the added substrate was disappeared. Although microbes in the enrichment sample DN553 ceased to synthesize

protein after 6 day incubation with carbazol (Fig. 3A), they were still metabolically active and continued to utilize the substrate as energy source. It therefore could be expected that more carbazol would disappeared at longer incubation periods. This degradation capacity is comparable with that shown in some microbial consortia have been reported [15,16]. As the trend of using mix cultures instead of pure cultures in bioremediation due to high degradation capability and adaptation ability [17], this enrichment culture could serve as microbial source for cleanup processes of PAH pollution.

3.3. Community structure of the enrichment culture DN553 as revealed by PCR-DGGE analysis of 16S rDNA

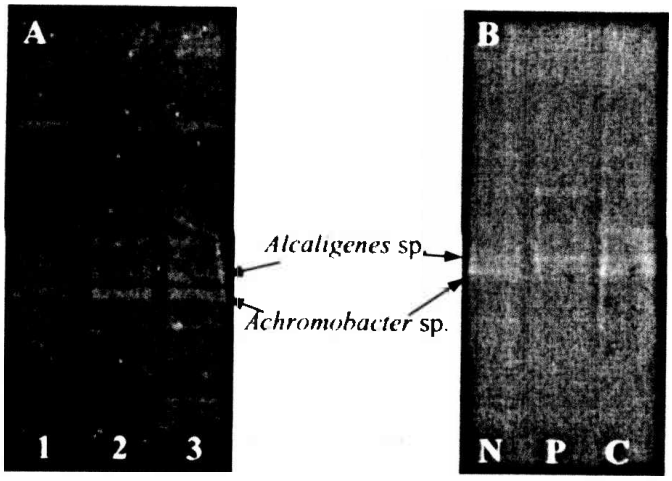

Fig. 4. Analyzing community structure of the enrichment culture DN55 by DGGE of 16S rDNA fragments. A – enrichment cultures DN55 through 3 transferring steps 1, 2 and 3; B – the enrichment culture DN553 cultivated with naphthalene (N), phenanthrene (P) or carbazol (C) as growth substrates.

The composition of bacterial species in the enrichment sample DN55 at every transferring step was characterized via analyzing the diversity of 16S rDNA sequences by denaturing gradient gel electrophoresis (DGGE) (Fig. 4A). It could be noted that the number of electrophoresis bands in the sample tended to increase through the transferring steps. The DGGE bands marked by arrows were kept through all transferring steps and reached strong intensity at the third transfer. These bands represented major groups that have been enriched in the consortium.

The most significant DGGE bands (indicated with arrows) were excised, again amplified with the primers GM5F and 907R, then subjected to sequencing. The obtained results showed that these bands had the highest homology to *Achromobacter* and *Alcaligenes* species, two groups of β-proteobacteria. In a number studies, the group β-proteobacteria has been shown with different species having capability of PAH degradation [18], among those *Alcaligenes* species are frequently reported, but not *Achromobacter*. This bacterial group could be a special characteristic of the investigated environment.

On the other hand, the bands disappeared through transferring steps represented groups being excluded from the community because they could not adapt to the conditions in the enrichment experiment. Most notably was the appearance of many new bands through transferring steps, meaning that many groups of bacteria were enriched together with the dominant groups at the same time. These additional groups could be supported by the high variety of intermediates generated during biodegradation process of such complex substrates like PAHs.

Community structure in the enrichment culture DN553 cultivated with one of the three PAH compounds were also analyzed using DGGE technique (Fig. 4B). The results showed that the community structure of this sample changed a little when it was cultivated with one

of the three different PAH substrates. Especially, two bands presenting the dominant groups *Achromobacter* and *Alcaligenes* remained almost the same under all three cultivation conditions. It is possible that these bacterial groups were able to utilize all the three PAH compounds tested and their dioxygenases had broad range of substrates. Such kind of microbes would be quite useful for field treatment of PAH pollution.

4. Conclusion

A PAH-degrading microbial consortium DN553 was obtained from dioxin contaminated sediment sample via enrichment technique. This culture was able to utilize carbazol, naphthalene and phenanthrene as the only carbon and energy sources.

Denaturing gradient gel electrophoresis of 16S rDNA fragments showed that the enrichment culture DN553 consisted of several bacterial groups, among them *Achromobacter* and *Alcaligenes* species were identified as the most abundant.

When grown on different PAH substrates, this culture showed flexible changing in their community structures, however the *Achromobacter* and *Alcaligenes* groups remained almost unchanged. Thus, the enrichment culture DN553 could be a potential microbial source for treatments of PAH contamination.

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Thiết lập quần thể vi sinh vật phân hủy tích cực PAH từ mẫu trầm tích nhiễm dioxin thông qua phương pháp làm giầu

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Môi trường nhiễm dioxin có thể là nguồn vi sinh vật quí giá với nhiều khả năng phân huỷ sinh học đặc biệt. Mẫu quần thể vi sinh vật DN553 có khả năng phân giải tích cực carbazol được thiết lập thông qua phương pháp làm giàu từ nguồn vi sinh vật trong mẫu trầm tích nhiễm dioxin thu tại hồ sen thuộc sân bay Đà Nẵng. Phân tích cấu trúc quần thể bằng phương pháp điện di biến tính đoạn gen 16S rADN cho thấy các nhóm vi khuẩn *Achromobacter* và *Alcaligenes* chiếm số đông trong mẫu quần thể này. Ngoài carbazol, mẫu vi sinh vật này còn có khả năng sử dụng một số hợp chất carcbuahydro thơm đa nhân khác như naphthalene hay phenanthrene làm nguồn carbon và năng lượng duy nhất. Trong môi trường có mặt các PAH khác nhau làm cơ chất, cấu trúc quần thể bị thay đổi, tuy nhiên hai nhóm *Achromobacter* và *Alcaligenes* vẫn được giữ nguyên. Như vậy mẫu quần thể DN553 có thể được sử dụng làm nguồn vi sinh vật hữu hiệu trong xử lý ô nhiễm PAH.

Từ khoá. Mẫu quần thể, phân huỷ PAH, DGGE, 16S rADN, *Achromobacter*, *Alcaligenes*.