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Diversity of Dibenzofuran-Utilizing Bacteria Isolated by Direct-Plating and Enrichment Methods

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Abstract

The effect of enrichment bias on the diversity of Dibenzofuran (DBF)-degrading bacteria recovered from soil was evaluated by direct plating, plating after in-soil adaptation, and plating after batch culture enrichment. Among colonies appeared on Bushnell Haas agar with DBF as the sole carbon source, 119 colonies (49, 38, and 32 from direct plating, plating after in-soil adaptation, and plating after batch culture enrichment, respectively) were arbitrarily selected based on the appearance of the colonies. Total DNA were then extracted from the rest of the colonies and analyzed for their diversity using Ribosomal Intergenic Spacer Analysis (RISA). Number of DNA bands obtained from direct plating was higher than the ones obtained after in-soil enrichment and batch culture enrichment. The RISA bands obtained from direct plating were also found to be distributed more evenly than the ones obtained after in-soil enrichment and batch culture enrichment. Dominant bands were observed on RISA from samples obtained after in-soil enrichment and batch culture enrichment. Out of 119, only 9 isolates were consistently able to grow on Bushnell-Haas broth with DBF as the sole carbon source as indicated by broth turbidity. All of the isolates were obtained from soil samples which were enriched in a batch culture. Some of the isolates were able to degrade more then 80 % DBF in the minimal medium.

Keywords : diversity, dibenzofuran-utilizing bacteria, direct-plating, enrichment methods

Introduction

Dibenzofuran (DBF) is a constituent of coal tar and its derivatives (Gaines, 1986; Sliwka *et al*., 2009), such as creosote which is used for wood preservatives. It is a common component of environmental pollutants, and has been identified in the air, fuel gas, fly ash from municipal incinerators, diesel exhaust, gas particulates, ground water, surface waters, sediments, and cigarette smoke (Watanabe and Hirayama, 1992; Padma *et al*., 1999). DBF possesses strong mutagenic and toxic activities (Culp *et al*., 1998; Goldstein *et al*., 1998). Chlorinated derivatives of DBF are even more toxic (Safe, 1986; Pohl *et al*., 1997; Isosaari *et al*., 2000).

The search for microorganisms able to grow on DBF, a model compound for highly toxic environmental pollutants like polychlorinated DBF, as a sole source of carbon and energy has led to the isolation of several bacterial strains belonging to the genera *Brevibacterium*, *Staphylococcus*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Microbacterium*, *Terrabacter*, *Janibacter*, and *Sphingomonas* (Strubel *et al*., 1989; Fortnagel *et al*., 1990; Monna *et al*., 1993; Wilkes *et al*., 1996; Schmid *et al*., 1997; Becher e*t al*., 2000; Iida *et al*., 2001; Habe *et al*., 2002; Yamazoe *et al*., 2004; Gai *et al*., 2007). All of the bacterial strains were isolated from either contaminated or

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uncontaminated soils after an enrichment process.

Batch culture enrichment is the most widely used method for isolation of bacteria expressing specific phenotypes. On their study to evaluate the potential for bias due to the use of enrichment cultures, Dunbar *et al*. (1997) reported that approximately 98 % of the enrichment isolates have a single pattern of catabolic gene, while isolates obtained by direct plating exhibit more diverse patterns of catabolic gene. The present paper describes the abundance and diversity of isolates, which able to grow on DBF as the sole source of carbon and energy, obtained from uncontaminated soil by direct plating, plating after in-soil adaptation, and plating after batch culture enrichment.

Materials and Methods

Soil samples

Soil samples were collected from a depth of 0-20 cm from three sites, 1 km away between each site, at mangrove forest located in the regency of Indramayu, Western Java, Indonesia (6° 21' 38" S, 108° 23' 25" E) which were considered not to be contaminated by polycyclic aromatic hydrocarbons. The soil samples were analyzed for their hydrocarbons and organic matter contents. Organic carbon content in the soil samples was analyzed by the Walkley–Black Procedure (Nelson and Sommers, 1982). Hydrocarbons in the soil samples were extracted according to previously described method (Dibble and Bartha, 1979), and the DBF content in the extract was determined using gas chromatography as described below.

Growth media and conditions for isolation and cultivation

Carbon free Bushnell-Haas minimal medium supplemented with DBF as the sole carbon source was used for enrichment, isolation and cultivation of DBF degrading bacteria. The medium contains (per L): $MgSO_4$ (0.2 g), $CaCl_2$ (0.02 g), KH_2PO_4 (1 g), K_2HPO_4 (1 g), $FeCl_2$ (0.05 g), and NH_4NO_3 (1 g) (Atlas, 1993). DBF was added at a concentration of 1 g/l Bacto agar (12 g/l) was added to prepare agar plates. For enrichment medium, solid DBF was added to the medium just after autoclaving (Fukuda *et al*., 2002). For isolation medium of DBF degrading bacteria, DBF was added as solid crystals in the lid of the agar plates (Aly *et al*., 2008). For preparing medium for the determination of DBF degrading capability, DBF was added as dissolved form in dimethyl sulfoxide (DMSO) (0.1 g/ml) (Gai *et al*., 2007). Incubation was performed in sealed screw-capped tubes or plates to avoid evaporation. Nutrient agar (Oxoid Ltd., Basingstoke, UK) was used for maintenance of isolates.

In-soil enrichment

Soil (25 g) was well mixed with 25 mg of pulverized DBF and incubated for 15 days at room temperature. After incubation, 1 g of soil sample was taken and mixed with 9 ml of sterile distilled water and shaken for 20 min at 250 rpm on a rotary shaker and then settled for 5 min. Ten fold serial dilutions were then prepared for bacterial isolation.

Enrichment culture

Enrichment cultures were established by transferring 0.1 ml of soil slurry (prepared by mixing 1 g of soil with 9 ml of sterile distilled water, shaken for 20 min at 250 rpm on a rotary shaker, and then settled for 5 min) into 10 ml carbon free Bushnell-Haas broth supplemented with DBF and incubated for 15 days at room temperature. Ten fold serial dilutions of the enrichment culture were then prepared for bacterial isolation.

Direct plating

Soil (1 g) was mixed with 9 ml of sterile distilled water and shaken for 20 min at 250 rpm on a rotary shaker and then settled for 5 min. Ten fold serial dilutions were then prepared for bacterial isolation.

Bacterial isolation

Aliquots from serial dilution were spread on carbon free Bushnell Haas agar

plates and incubated for 3 days at room temperature with DBF crystals on the lid. The plates were placed upside down so the bacteria could utilize the vapor of DBF. Several colonies were arbitrarily selected among colonies appear on the plates, based on their appearance, picked up and transferred to nutrient agar.

DNA extraction of total isolates and its Ribosomal Intergenic Spacer Analysis (RISA)

Cells were collected from all of colonies appear on the DBF-coated carbon free Bushnell Haas agar plates by adding 0.85 % NaCl. The obtained mixtures of cells were then centrifuged at 12,000 rpm for 2 min to collect the biomass. Isolation of the total genomic DNAs were carried out as described by Maniatis *et al.* (1982). The bacterial IGS (Intergenic Spacer) located between the small- and large-subunit rRNA genes were amplified using puReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instruction. Sequences of 1406F (TGYACACACCGCCCGT) (universal rRNA small subunit) and 23SR (GGGTTBCCCCATTCRG) (bacterial 23S rRNA large subunit) were used as primers (Borneman and Triplett, 1997). PCR was carried out in ICycler Thermal Cycler (BioRad, CA, USA). After all necessary reagents were combined, the mixture was heated at 95°C for 5 min, followed by 30 cycles of PCR consisting of 94°C for 60 s, 50°C for 90 s, and 72°C for 120 s, followed by elongation at 72°C for 10 min. The PCR products were analyzed by 1% agarose (w/v) gel electrophoresis at 80 V for 1 h in 1X Tris-Acetic Acid-EDTA (TAE) buffer and made visible by ethidium bromide staining and UV transillumination.

DNA extraction of selected isolates and its rep-PCR

Selected isolates were grown in Luria-Bertani broth for 24 h at room temperature. Cells were then collected by centrifugation at 12,000 rpm for 2 min. Isolation of the genomic DNAs were carried out as described by Maniatis *et al.* (1982). For genome finger printing, repetitive DNA elements were amplified using puReTaq™ Ready-To-Go™ PCR Beads (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instruction. BOX A1R primer (5'-CTACGGCAAGGCGACGCTGACGCTGA CG-3') was used as primer (Genersch and Otten, 2003). PCR was carried out in ICycler Thermal Cycler (BioRad, CA, USA) with a condition as follows: After the initial activation step (95°C, 4 min), 30 cycles at 92°C for 1 min, at 50°C for 1 min 30 s, and at 68°C for 8 min were run followed by a final elongation step at 68°C for 10 min. The PCR products were analyzed by 1 % agarose (w/v) gel electrophoresis at 80 V for 1 h in 1X Tris-Acetic Acid-EDTA (TAE) buffer. The DNA bands were stained with ethidium bromide and visualized by UV light.

Determination of DBF consumption by selected isolates

For determination of DBF consumption, DBF concentrations in the spent medium were determined. Cells of selected isolates were inoculated into 3 ml of carbon free Bushnell-Haas broth supplemented with DBF to reach a final cell concentration of 10^7 CFU/ml and then incubated with shaking at room temperature for 7 days. DBF in the spent medium were extracted with the same volume of ethyl acetate containing 100 ppm phenantrene as an internal control. The organic phase was then passed through a column of Na_2SO_4. Without derivatization, the resulted filtrate was subjected to gas chromatography which was carried out on a Shimadzu model GC-14B gas chromatograph (Kyoto, Japan) with a flame ionization detector at 310°C equipped with a 25-m by 0.2 mm (inside diameter) HP-1 (100 % dimethyl polysiloxane fused-silica column) (Hewlett-Packard, DE, USA). Nitrogen was used as the carrier gas (150 kPa) with a flow rate of

25 ml/min. The column temperature was programmed from 80 to 300°C at 10°C. min^{-1} (Hammer *et al*., 1998; Fortnagel *et al*., 1990). The reported data are mean values for three replicate batch cultures.

Results

Soils from the floor of mangrove forest used in this study were characterized by a low (1.2 % at the site 1) to moderate organic matter contents (2,8 % and 3.8 % at the site 2 and site 3, respectively). No DBF was detected in all of the soil samples. Isolation of DBF degrading bacteria from the soil samples was carried out by three different ways, i.e. direct plating, after in-soil enrichment, and after batch culture enrichment. The use of the methods was intended to elucidate the best methods for the isolation of bacteria with the highest DBF degradation capability as well as the best method for studying diversity of bacteria with DBF degradation capability.

Among colonies appeared on the carbon free Bushnell Haas agar plates supplemented with DBF as the sole carbon source, 119 were arbitrarily selected based on the appearance of the colony, picked up, and transferred to nutrient agar. The number of isolates selected from each sites and methods of isolation were shown in Table 1. The selected colonies were then purified according to standard procedures.

The rest of the colonies were scrapped, collected, and their total DNA were extracted and analyzed for their diversity using RISA. The result of RISA after DNA electrophoresis and visualization were shown in Figure 1.

Figure 1 shows that the numbers of DNA bands from RISA of total DNAs isolated from all colonies obtained by direct plating were higher than the ones obtained after in-soil enrichment and batch culture enrichment. The RISA bands of total DNAs obtained by direct plating were also found to be distributed more evenly than the ones obtained after in-soil enrichment and batch culture enrichment. Dominant bands were observed on RISA of total DNAs isolated from all colonies obtained after in-soil enrichment and batch culture enrichment.

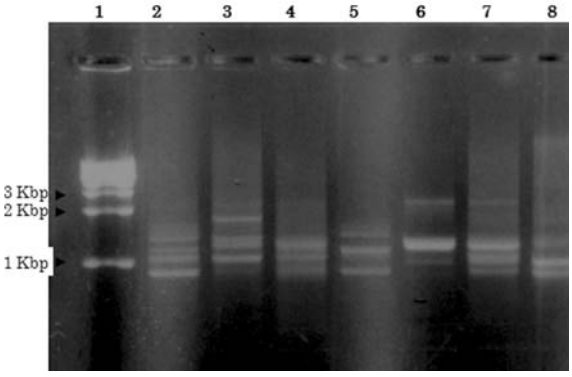

Figure 1. RISA from total bacterial isolates obtained after direct plating, plating after in-soil enrichment, and plating after batch culture enrichment. The intergenic spacer region was PCR amplified and resolved on 1 % agarose gel. Lanes: 1, molecular weight markers; 2, 3, and4, RISA from total isolated bacteria obtained after direct plating of soil sample from site 1, 2, and 3 respectively; 5, 6, and7, RISA from total isolated bacteria obtained from plates after in-soil enrichment of soil sample from site 1, 2, and 3 respectively; 8, 9, and 10, RISA from total isolated bacteria obtained from plates after batch culture enrichment of soil sample from site 1, 2, and 3 respectively.

The selected 119 isolates were then examined for their ability to grow on carbon free Bushnell-Haas minimal medium supplemented with DBF as the sole carbon source. Out of 119, only 9 isolates showed to consistently able to grow on Bushnell-

Table 1. Number of isolates selected from each sites and methods of isolation

Method of isolation	Number of isolate selected from		
	Sampling site 1	Sampling site 2	Sampling site 3
Direct plating	18	17	14
In-soil enrichment	14	11	13
Batch culture enrichment	14	10	8

Haas minimal medium with DBF as the sole carbon source as indicated by broth turbidity yellow color formation. All of the isolates were obtained from soil samples which were enriched in a batch culture. The nine isolates were then analyzed for their genetic similarity by PCR analysis using BOX A1R primer. The resulted amplified DNAs were separated by electrophoresis on 1.5 % agarose gel, stained with ethidium bromide, visualized by UV light, and showed in Figure 2.

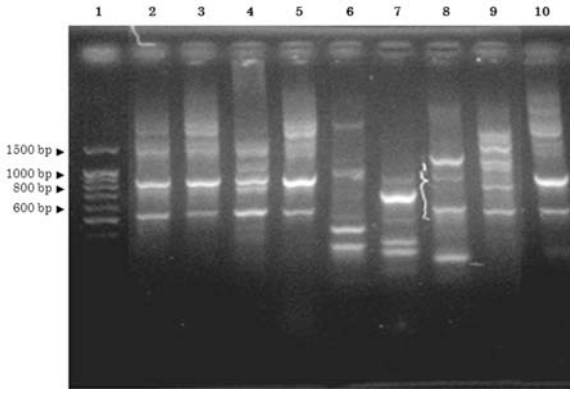

Figure 2. Repetitive-PCR patterns of DBF-degrading bacteria after resolved on 1 % agarose gel. Lanes: 1, molecular weight markers; 2 to 10, repetitive-PCR of isolates A, B. C, D, E, F, G, H, and I, respectively

Figure 2 shows that while six other isolates have quite different patterns of rep-PCR, isolates A, B, and D have very similar ones. It indicates that isolates A, B, and D have close relationship, which means that only seven different isolates have been obtained. The ability of the 9 isolates to degrade DBF was then examined by growing them in carbon free Bushnell-Haas minimal medium supplemented with DBF as the sole carbon source at room temperature for 7days with reciprocal shaking at a speed of 180-200 rpm. Un-inoculated carbon free Bushnell-Haas minimal medium supplemented with DBF incubated under the same conditions was prepared to assess the decrease of DBF concentration due to non-biological factors. The remaining DBF in the spent medium was determined using gas chromatography analysis. The results of the determination of remaining DBF concentration in the spent medium presented in Figure 3.

Figure 3 shows that isolates A, B, C, D, and F were able to degrade more then 80 % DBF in the minimal medium.

Discussion

Diversity of DBF utilizing bacteria

The patterns of RISA indicate that the genetic diversity of bacteria obtained by direct plating showed a high degree of variability

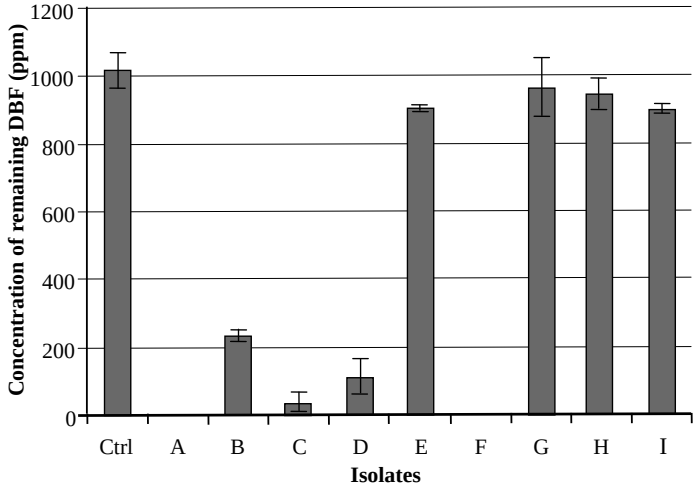

Figure 3. Remaining DBF concentration in the spent medium inoculated with isolates after 7 days incubation. Ctrl is un-inoculated carbon free Bushnell Haas minimal medium supplemented with DBF as the sole carbon source. Averages of triplicate experiments. Standard errors of the means indicated by error bars

compare to the ones obtained after in-soil and batch culture enrichments. It is in accordance with the finding of Dunbar *et al*., (1997) who studied the effect of enrichment on the diversity of 2,4-dichlorophenoxyacetate (2,4-D)-degrading bacteria. The results support their opinion that evaluation of diversity and distribution of catabolic pathways in nature can be highly distorted by the use of enrichment culture techniques.

In addition, the RISA bands from bacterial community obtained by the method of enrichment, both in-soil as well as in batch culture, look thicker which indicate the existence of dominant of strains/species. The patterns of RISA from bacterial community obtained by direct plating were almost evenly distributed. No domination by certain types of bacteria was observed. The existence of dominant of strains/species may also explain the low diversity of *Sphingomonas* strains in soils contaminated with high concentrations of polycyclic aromatic hydrocarbons (Leys *et al*., 2004).

The isolation of only five high ability DBF utilizing isolates from among 119 was in line with the results obtained by Futamata *et al*. (2004) who reported the isolation of 17 DBF degrading bacterial isolates from among 3455 isolates obtained from soil. All of those DBF utilizing isolates were obtained from polluted soils. Moreover, the selection of isolates through the formation of yellow color compound narrowed the range of isolated bacteria. Initial reactions of dibenzofuran degradation can be classified into angular and lateral dioxygenation and only the lateral dioxygenation produces 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-2,4-hexadienoic acid that is yellow (Seo *et al*., 2009).

In some ways, in-soil enrichment with DBF may imitate soil contamination. However, no DBF utilizing isolates was isolated from in-soil enrichment. The short incubation period of in-soil enrichment may be the cause of inability to obtain DBF utilizing isolates from the in-soil enrichment.

In conclusion, isolation of specific compound degrading-microbes from sites contaminated by that compound and their enrichment in the presence of the compound may distort the apparent diversity of those compound degrading-microbes. Isolation of microbes from contaminated sites followed by their enrichment is more appropriately used to obtain microbes with high capability.

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