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PHENANTHRENE BIODEGRADATION EFFICIENCY OF BACILLUS SP. P4A ISOLATED FROM HYDROCARBON CONTAMINATED SOIL

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Abstract: In this study, phenanthrene-degrading bacteria was successfully isolated from hydrocarbon contaminated soil. The bacteria were identified based on their morphological, biochemical activities and molecular identification using 16S rRNA. The phenanthrene – degrading capabilities was cultivated in an enrichment culture containing phenanthrene as a sole source of carbon and energy. The phenanthrene degradation was analysed using Gas Chromatography-Flame Ionization Detector (GC-FID) and the result obtained indicated that the Bacillus sp. P4a was capable of degrading to about 83.01% in the enrichment culture. The Bacillus sp. P4a has been shown to degrade phenanthrene via salicylate pathway which produced salicylic acid and catechol compound after 14 and 28 days of incubation. The result revealed that this bacterial isolate can be highly recommended for bioremediation of hydrocarbon contaminants due to their capability to degrade phenanthrene.

Keywords: Phenanthrene, Bacillus sp. P4a, Biodegradation, Bioremediation, Contaminated soil

INTRODUCTION

In recent decades, the contamination of soil by pollutant such as polycyclic aromatic hydrocarbon (PAH) has caused serious concerns. PAHs have a widespread occurrence in various ecosystems which enable them to persist for a long time in the environment (El-Shahawi et al., 2010). Due to its toxic, mutagenic and carcinogenic properties, prolonged exposure to a high concentration of PAHs can cause acute and chronic health problems (Kim et al., 2013). Thus, bioremediation is a technique created to remediate the PAH contamination to reduce the negative impacts of the PAH. It is widely used to remove PAHs from the environment since it is cost effective and environmentally friendly compared to other conventional methods.

OBJECTIVE

The objective of the present study is to determine the biodegradation capability of the bacterial isolate using a shake flask system. It is also to determine the involvement of intermediates during the phenanthrene degradation process in the liquid system.
LITERATURE REVIEW

The presence of PAH pollutant creates harm to the environment and human because of their toxic and carcinogenic characteristics (Laws, 2013). There were 67 PAH compound showed mutagenic properties to human (Durant et al., 1996). Some of the PAH exposure may disturb our respiratory and gastrointestinal tract and also the skin (Ramesh et al., 2004). It also interferes with other life environment such as aquatic organisms through pollution in the food chain. It is harmful to human due to the consumption of this contaminated seafood.

Phenanthrene is a low molecular weight PAH which consists of three fused benzene rings with phenyl and anthracene compound attached together. It is toxic pollutant and occurs as a major component of PAH compounds in the environment (Slezakova et al., 2013). Therefore, biodegradation of this compound need to be done in order to bioremediate the contaminated soils.

Several bacterial strains have been shown to degrade phenanthrene such as Arthrobacter sp., Acidovorax sp., Brevibacterium sp., Burkholderia sp., Comamonas sp., Mycobacterium sp., Pseudomonas sp. and Sphingomonas sp. (Seo et al., 2009). These bacteria have the capability to degrade phenanthrene and utilize it as a nutrient source (Seo et al., 2009). Some of the bacteria from genus Mycobacterium have been demonstrated to metabolize phenanthrene using both enzymes dioxygenase and monooxygenase to react on the aromatic compound (Seo et al., 2012).

METHODODOLOGY

Sample Collection

The soil samples obtained near a palm oil mill factory were collected using clean stainless steel spatula and stored in sterile plastic bags before being transferred to the laboratory. The samples were stored at 4°C before analysis.

Enrichment and Isolation of Phenanthrene-degrading bacteria

The phenanthrene-degrading bacteria were enriched in flasks containing 150 ml of Minimal Salt Medium (MSM), 15 g of soil sample and 5 ml of phenanthrene (330 mg/l). The MSM contained (g/L): 1.0 g (NH₄)₂SO₄, 0.1 g CaCl₂·2H₂O, 0.8 g K₂HPO₄, 0.2 g KH₂PO₄, 0.012g FeSO₄·7H₂O, 0.003 g MnSO₄·7H₂O, 0.003 g ZnSO₄·7H₂O in 1 Liter of distilled water (modified from Coral & Karagoz, 2005). The flasks were incubated at 30°C with 150 rpm. Then, 5 ml aliquots were transferred to a new MSM containing 5 ml of phenanthrene. The flasks were shaken for 14 days. Bacteria were isolated by the serial where 0.1 ml from the series of dilution was spread onto the nutrient agar (NA) plates and incubated at 30°C for 48 hours.

Identification of the selected phenanthrene-degrading bacterial isolate

The identification and characterization of selected bacterial isolates were based on macroscopic and microscopic observations. The morphology of the bacterial isolates was observed using the Gram staining technique. The physiological characterization of the bacteria was performed using standard biochemical tests of the BBL Crystal Identification Kit (Jackson et al., 2004). The bacterial isolates were further characterized at the molecular level of DNA sequencing analysis of 16S rRNA.
Phenanthrene degradation analysis by locally isolated bacteria

The analysis of phenanthrene concentration in the culture medium was conducted at the regular interval every 4 days. Duplicated bacterial cultures were centrifuged at 5000 rpm for 10 minutes and at 4°C. The layer of the obtained supernatant was mixed with 100 ml of hexane in 250 ml separation funnel and vigorously shake. There were two layers or phases formed in the separation funnel. The extraction was repeated three times and the hexane phase (top layer) was separated and pooled in a beaker. Then, it was air dried in a fume hood. When the analysis was to be carried out, the samples were then dissolved with an equal volume of hexane (100 ml) and the phenanthrene was quantified by a Clarus 500 Gas Chromatography-Flame Ionization Detector (GC-FID).

Identification of phenanthrene metabolites

An analysis of phenanthrene metabolites (Mallick et al., 2007) in the culture medium was conducted at Day 0, Day 14 and Day 28 of incubation. The cells were removed by centrifugation at 5000 rpm for 10 minutes and 4°C. The supernatants obtained were membrane-filtered and acidified to pH 2. The supernatant were extracted with three volumes of 100 ml ethyl acetate. The organic extracts were pooled and concentrated to 10 ml using rotavapour unit (Buchi, Germany). The extracts were air dried and stored at room temperature until further analysis. The residue was then dissolved in 1 ml of methanol and analysed using Gas Chromatography-Mass Spectrometry (GC-MS Agilent 6890N/59731).

FINDINGS

Identification of phenanthrene-degrading bacteria

The morphology of the potential phenanthrene-degrading bacterial was observed using the Gram staining method. It was shown that the Isolate P4a is Gram-positive rod-shaped bacteria. It was produced a white colony possessed a dry surface, a filamentous form of the colony, flat elevation and filiform margin. The identification that based on a biochemical test and BBL Crystal Identification Kit showed that Isolate P4a was preliminarily identified as Bacillus sp. The molecular approach such as 16S rRNA gene analysis of Isolate P4a was carried out using genomic extraction, PCR and sequencing. The sequence showed 99% of the Bacillus sp. N30 identity respectively. Bacillus sp. was the most widely reported phenanthrene degraders isolated from the environment (Patel et al., 2013).

Degradation activity of phenanthrene by Bacillus sp. P4a

There was about 83.00% of phenanthrene degradation throughout the 28 days incubation. The degradation was analyzed using GC-FID to measure the decrease in the peak area of phenanthrene. Concomitant phenanthrene degradation with the growth of bacteria can be observed (Figure 1). The bacteria count increases from Log CFU/ml 4.10 at day 0 to Log CFU/ml 5.67 after 8 days of cultivation. Referring to Figure 1a, it shows that the bacteria grow in the log phases on the first day of cultivation up to 8 days after inoculation. This condition suggests that the bacteria have been previously exposed to PAH particularly during the isolation and screening processes. Once the bacteria have adapted to the new medium, they begin to reproduce and multiply their numbers for each increment of time. However, the bacterial growth steady has shown a decrease from Log CFU/mL 5.67 at day 8 to Log CFU/mL 3.27 at day 28 of cultivation This is supported by the finding of Zhao et al. (2017) where the bacterial reproduction and growth regularly reduced because the carbon and
nutrient sources have been used by the bacteria in the cultivation medium. By the end of the cultivation period, the bacterial growth entered the final growth phase which nutrients have been depleted and cell number decreases. The phenanthrene degradation activity is different from one species to another. Referring to Ling et al. (2011), Bacillus sp. could utilize various PAH such as phenanthrene as a sole source of carbon.

Figure 1: Profile of viable cell count and phenanthrene concentration (mg/L) in flask system containing Bacillus sp. P4a

Metabolites production in phenanthrene degradation by Bacillus sp. P4a

In the present study, there were three metabolites identified by GC-MS from the organic extracts of Bacillus sp. The P4a culture containing phenanthrene was incubated at Day 0, Day 14 and Day 28 (Table 1). The metabolites obtained correspond with 1-naphthalencarboxylic acid, salicylic acid and catechol, respectively (Figure 2).

Table 1
GC-MS data for metabolites of phenanthrene obtained from organic extract of Bacillus sp. P4a culture

<table>
<thead>
<tr>
<th>Incubation (Day)</th>
<th>Metabolites</th>
<th>Retention time (min)</th>
<th>m/z of fragment ions</th>
<th>% Relative Intensity</th>
<th>Possible structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A</td>
<td>10.7</td>
<td>184 (16, M*), 155 (100, 140 (4), 127 (70), 113 (3), 101 (6), 87 (5), 77 (12), 63 (19), 51 (11), 27 (2)</td>
<td>1-Napthalene carboxylic acid</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>B</td>
<td>9.73</td>
<td>138 (17,M*), 121 (100), 109 (2), 93 (23), 81 (3), 65 (25), 53 (5), 39 (82), 27 (2)</td>
<td>Salicylic acid</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>C</td>
<td>8.95</td>
<td>126 (2, M*), 124 (100), 107 (12), 95 (7), 78 (48), 62 (7), 51 (19), 39 (18), 27 (5)</td>
<td>Catechol</td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSION

*Bacillus* sp. P4a showed the capability in degrading phenanthrene which was 83.01% at 28 days of incubation. It has been shown to degrade phenanthrene via salicylate pathway which produced salicylic acid and catechol compound after 14 and 28 days of incubation. Thus *Bacillus* sp. P4a gave a great potential to be applied in bioremediation of environments polluted by phenanthrene. These potential bacterial culture can be highly recommended for bioremediation of hydrocarbon contaminants due to their capability to degrade phenanthrene.
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