ISOLATION AND CHARACTERIZATION OF CARBAZOLE AND DIBENZOFURAN DEGRADING BACTERIA FROM SOIL IN MANGROVE ENVIRONMENT

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Isolation and Characterization of Carbazole and Dibenzofurans Degrading Bacteria from Soil in Mangrove Environment

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A final project report submitted in partial fulfillment of the Final Year Project II (STF 3015) course

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Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak
2013
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I thank you all for your patience and attention.
Declaration

I declared that this project entitled “Isolation and Characterization of Carbazole and Dibenzofurans Degrading Bacteria from Soil in Mangrove Environment” is the result of my own research except as cited in the references. This project has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature: .......................................................

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<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>μl</td>
<td>Micro Liter</td>
</tr>
<tr>
<td>AGE</td>
<td>Agarose Gel Electrophoresis</td>
</tr>
<tr>
<td>bp</td>
<td>Basepair</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CAR</td>
<td>Carbazole</td>
</tr>
<tr>
<td>DBF</td>
<td>Dibenzofurans</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithioheitol</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>NAH</td>
<td>N-heterocyclic aromatic</td>
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<tr>
<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbons</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>SIM</td>
<td>Sulphide Indole Motility</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
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Isolation and Characterization of Carbazole and Dibenzofuran Degrading Bacteria from Soil in Mangrove Environment

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ABSTRACT
Heterocyclic hydrocarbons are toxic and mutagenic components of petroleum and creosote contamination that are frequently found in the groundwater, seawater, sediments, and soil sites. A study was carried out to isolate and characterize heterocyclic hydrocarbon degrading bacteria from soil samples collected at mangrove environment at Asajaya, Sarawak. This environment has physicochemical characteristics of high salinity, tidal range, strong winds, high temperature, crude oil and muddy soil. Crude oil often contains various heterocyclic compounds that are toxic for most life forms. Despite its toxicity, some bacteria species have the capability to mineralize these heterocyclic hydrocarbons compound efficiently. Two bacteria strains FNS01 and FNS02 was isolated and grown with enrichment of carbazole (CAR) and dibenzofuran (DBF) as the sole source of carbon. Characterization of these two isolated bacteria include motility test with the use of semi-solid medium, catalase test, salt tolerance test, hydrogen sulfide test, oxidase test, gram staining, and also different polycyclic aromatic hydrocarbons (PAHs) substrate test such as carbazole (CAR), biphenyl (BPH), fluorene (FLO), dibenzofuran (DBF), and dibenzothiophene (DBT) were studied. Total DNA was extracted and 16S rRNA amplified by PCR method and subsequently differentiates the species with restriction fragment length polymorphism (RFLP). Isolation of these strains might be useful in the bioremediation of environments contaminated by heterocyclic hydrocarbon.

Key words: heterocyclic hydrocarbon, 16S ribosomal RNA, heterocyclic hydrocarbon degrading bacteria, bioremediation

ABSTRAK
Hidrokarbon Heterosiklik adalah toksik dan mutagen komponen dalam petroleum dan kontaminasi kreasot yang kerap ditemui dalam air bawah tanah, air laut, sedimen, dan tapak tanah. Satu kajian telah dilakukan untuk mengasingkan dan mencirikan bakteria yang menjalankan proses degradasi kepada heterosiklik hidrokarbon daripada sampel air yang diambil dari persekitaran paya bakau di Asajaya, Sarawak. Persekitaran ini mempunyai ciri-ciri fizikokimia iaitu kemasinan yang tinggi, pelbagai pasang surut, angin yang kuat, suhu yang tinggi, minyak mentah dan tanah berlumpur. Minyak mentah sering mengandungi pelbagai sebatian heterosiklik yang menjadi toksik untuk kebanyakan kehidupan. Walaupun ia dalam keadaan yang bertoksik, beberapa spesies bakteria mempunyai keupayaan untuk untuk mengdegradasikan hidrokarbon heterosiklik kompaun dengan lebih berkesan. Dua bakteria strain iaitu FNS01 dan FNS02 telah diasingkan dan berkembang biak dengan penambahan karbazol (CAR) dan dibenzofuran (DBF) sebagai satu-satunya sumber karbon. Untuk pengelasan jenis bakteria ini termasuk ujian motiliti dengan menggunakan agar separuh pejal, ujian katalase, ujian toleransi garam, ujian hidrogen sulfida, ujian oksidase, pewarnaan Gram, dan juga ujian terhadap berbeza substrat polisiklik aromatik hidrokarbon (PAH) seperti karbazol (CAR), bifenil (BPH), fluorene (FLO), dibenzofuran (DBF), dan dibenzothiophene (DBT). Total DNA akan dikeluarkan dan penggandaan 16S rRNA dengan kaedah PCR dan kemudianya membezakan spesies dengan sekatan serpihan panjang polimorfisme (RFLP). Pengasingan jenis-jenis bakteria ini mungkin berguna dalam biopemulihan persekitaran yang tercemar disebabkan oleh hidrokarbon heterosiklik.

Kata Kunci: hidrokarbon heterosiklik., 16S ribosomal RNA, bakteria yang menjalankan proses degradasi kepada heterosiklik hidrokarbon, biopemulihan
1.0 INTRODUCTION

1.0 Background of Study

Heterocyclic aromatic compounds can be defined as a cyclic compound that has atoms of at least two different elements as members of its ring. It is also known to possess toxic and mutagenic activities (Gai et al., 2007). Bacteria that can degrade NHA, such as carbazole have also been isolated (Shotbolt-Brown et al., 1996). Other examples of these heterocyclic hydrocarbons are carbazole, biphenyl, fluorene, dibenzofuran, and dibenzothiophene which their degradation products have been detected in groundwater, seawater, sediments, and soil sites contaminated with leaks of petroleum and industrial wastes.

A bioremediation goal is to transform organic pollutants into harmless metabolites or mineralize the pollutants into carbon dioxide and water using such microorganisms (Seo et al., 2009). Environments that are contaminated with these compounds may elicit serious health threats as these heterocyclic compounds are mutagenic and carcinogenic (Gai et al., 2007). Thus, marine degrading bacteria play a primary role in the removal of many types of chemical pollutants from the contaminated water and soil environment. The contribution of these microorganisms and biodegradation of PAHs can be a useful approach for eliminating these pollutants (Hop and Omori, n.d). Some example of microorganism such as Ralstonia sp. is the first reported species that is capable of degrade carbazole (Schneider et al., 2000). Other is Pseudomonas resinovorans strain CA10 that exhibits the potential to enhance degradation of carbazole in soil (Widada et al., 2002).
Previous study has shown the bacterial degradation pathway of carbazole is initiated by the angular dioxygenase catalyzed by CAR 1,9a-dioxygenase (CARDO), which is encoded by the carAa, carAc, and carAd genes. Some example of bacterial species such as *Pseudomonas resinovorans* CA10 is shown to degrade CAR to 2'-aminobiphenyl-2,3-diol via this CARDO. This CARDO is composed of terminal oxygenase, ferredoxin, and ferredoxin reductase used for bioremediation in marine environments since it can transforms dioxin compounds and polycyclic aromatic hydrocarbons (PAHs) (Maeda *et al*., 2010). Consequently, present study indicates that the contaminated water and soil samples from mangrove water could contain a diverse population of N-heterocyclic aromatic (NHA) degrading bacteria since this environment has various species composition and special characteristic.

### 1.1 Problem Statement

Currently, an exploration, production, refining, transport and storage of petroleum and petroleum products, some accidental spill could occur. In addition, industrial effluents and poor waste management recently has shown contributed to heavy metal contamination in the sediments. As a result, contamination of certain soil area and seawater might cause a serious health threats and for our ecosystem. In order to solve this issue, bioremediation has been focused by researchers since it might aid in restoration of these contaminated sites. For implementation of effective bioremediation programs in marine environments, it requires the use of natural microorganisms like heterocyclic hydrocarbon degrading bacteria. Therefore, this
study aims to study degrading bacteria that are useful in the bioremediation that will lead to a safe environment in an effort to reduce water pollution.

1.2 **Objectives**

The purposes of this study are to:

a. Isolate heterocyclic hydrocarbon from bacteria strains at mangrove environment.

b. Study the characterization of heterocyclic hydrocarbon degrading bacteria isolated from water and soil samples.

c. Assess degradation ability of isolated bacteria using different substrates.
2.0 LITERATURE REVIEW

2.1 Bioremediation

Bioremediation is a process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition. Microbial degradation is a natural mechanism to clean up the hydrocarbon pollutants (and crude oil) from the environment (Cristol, 1983). The recognition of biodegraded derived aromatic hydrocarbons in marine sediments. Several microorganisms are capable to degrade crude oil including Arthrobacter, Burkholderia, Mycobacterium, and Pseudomonas (Hill et al., 1999).

Research has been conducted to understand bioremediation for environmental pollutants such as N-heterocyclic aromatic (NHA) compounds that are among the most prevalent and persistent environmental pollutants. Bioremediation can be successful with the presence of microorganisms with appropriate metabolic capabilities dependent on nutrients, oxygen, and pH (Im et al., 2004). Biodegradation of hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present.

2.2 Mangrove environment

Mangrove can be defined as a habitat that consist of numerous halophytic (salt-tolerant) of plant species which there are more than 12 families and 50 species worldwide (Jennifer, 2012). In nature, mangrove soils normally are acidic which has high salt concentration and hence, the roots of mangrove plants are adapted to filter
salt water, and their leaves can excrete salt for their survival (Kathiresan and Bingham, 2001). Additionally, this environment also has various species that utilize the mangroves and physicochemical characteristics of high salinity, tidal range, strong winds, high temperature and muddy soil. For the reason that they are surrounded by loose sediments, greater diversity of microorganism such as bacteria can be found since they are able to adapt themselves in such adverse condition. Therefore this study is carried out to discover which bacteria strains from the mangrove environment have the ability to degrade the certain heterocyclic hydrocarbon compound.

2.3 Dibenzothiophene compound

One group of compounds that are generally both biohazards and stable are the polycyclic aromatic hydrocarbons (PAH). Dibenzothiophene compound (DBT) is one of PAHs compound which can persevere for up to 3 years after an oil spill along with its derivatives in particular. Meanwhile other more susceptible compounds might have been biodegraded by heterocyclic hydrocarbon degrading bacteria (Gai et al., 2007). DBT is a sulfur-containing PAH and numerous reports on biodesulfurization have been published since DBT is broadly used as a model for biodegradation and petroleum biodesulfurization (Gai et al., 2007). DBT degradation pathway has been studied in detail for a few bacterial strains including Pseudomonas, Sphingomonas, Rhodococcus, Mycobacterium, Terrabacter, Burkholderia, Paenibacillus, Gordonia and others (Cooper, 2009). DBT degradation pathways as shown in Figure 2.1 when DszC catalyzes the conversion of dibenzothiophene (DBT) to the sulfoxide dibenzothiophene 5-oxide (DBTO) and further to the sulfonedibenzothiophene 5,5-
dioxide (DBTO₂). DszA then further degrades DBTO₂ to 2-(2’-hydroxyphenyl) benzene sulfinate (HBPSi). Finally, DszB catalyzes the conversion of HBPSi to 2-hydroxybiphenyl (HBP) and sulfite (SO₃²⁻).

Figure 2.1: The metabolic pathway for the desulfurization of DBT to HBP and sulfite (Gai et al., 2007).

2.4 Biodegradation of carbazole and dibenzofurans

Carbazole is nitrogen containing polycyclic aromatic compound and its derivatives being carcinogenic and mutagenic (Singh et al., 2010). It is an example of heterocyclic hydrocarbon that derived from creosote, crude oil, and shale oil, which can be
mineralized by bacteria strain (Gai et al., 2007). *Ralstonia* sp is the first reported of species that able to degrade carbazole (Schneider et al., 2000). Carbazole degradation of some previously described *Pseudomonas* sp. is said to be shared other characteristics with *Ralstonia* sp. (Ouchiayama et al., 1993).

Bacterial catabolism of dibenzofurans starts at insertion of two oxygen atoms catalyzed by enzyme dioxygenases. The initial reactions of biodegradation pathway for dibenzofuran and carbazole are classified into angular and lateral dioxygenation which is then catalyzed by different enzymes (Figure 2.2). These enzymes can be found in the isolated Gram positive or Gram negative bacteria. For instance, some bacterial dioxygenases from *Pseudomonas* sp. CA10 able to catalyze mainly angular insertion of oxygen meanwhile the commonly known naphthalene dioxygenase from *Pseudomonas* sp. that only catalyzes by lateral dioxygenation (Seo et al., 2009). However some dioxygenase able to catalyze both reactions for instance cloned dioxygenase of *Norcardioides aromaticivorans* IC177 from previous studies. Metabolism of dibenzofuran via lateral dioxygenation has been reported in *Ralstonia* sp. strain SBUG 290 (Figure 2.3). It has been shown that during cometabolic processes, a complete degradation of dibenzofuran via lateral dioxygenation and meta cleavage of the aromatic structure is possible (Becher et al., 2000).
Figure 2.2: Simplified bacterial catabolic pathways of dibenzofuran (X = O) and carbazole (X = N) (Seo et al., 2009)

Figure 2.3: Dibenzofuran degradation via lateral dioxygenation of Ralstonia sp. strain SBUG 290 (Becher et al., 2000)
2.5 **Bacterial species identification**

Morphological test is one of the methods that can identify species of isolated bacteria by using gram staining to differentiate into two large groups which are Gram-positive and Gram-negative. Under 100x magnification of light microscope, morphology of the isolated bacteria can be identified depends on chemicals, physical properties of cell wall and presence of thick peptidoglycan. Earlier studies have showed that certain carbazole (CAR)-degrading bacteria such as *Pseudomonas resinovorans* CA10 and *Sphingomonas* sp. strain KA1 are Gram-negative bacteria and the *Nocardoides aromaticivorans* IC177 belongs to Gram-positive bacterium (Maeda et al., 2009).

For identifying isolated bacteria, 16S rRNA gene sequencing is commonly used to obtain an accurate and precise bacteria species. 16S rRNA is the component of the subunit of prokaryotic ribosomes. This analysis is predominantly important for bacteria with unusual phenotypic profiles, rarely isolated bacteria, slow-growing bacteria, uncultivable bacteria and culture-negative infections (Janda and Abbott, 2007). The 16S rRNA has been used for housekeeping genetic marker since its presence in almost all cellular life forms and the function of 16S RNA gene has not changed over time so it is more accurate to make an inference of evolutionary relatedness. Moreover, it is relatively large enough about 1500 bp for informatics purposes (Janda and Abbott, 2007). The gene sequences are retrieved using primer that targets a specific gene sequences which capture a 500 base sequences samples. This data is compared to the same 500 base sequences in genetic libraries which are MicroSeq, Genbank or Ribosomal Database Project (RDP) which will suggest the strains is related to any genus (Hedlund et al., 1999).
3.0 MATERIALS AND METHODOLOGY

3.1 Samples collection

This study was conducted throughout mangrove environment at Asajaya, Sarawak. Soil samples were aseptically collect with a total of soil sample of 20 g. The samples were collected at three different sites. Then these samples were placed into sterile polythene bags respectively and stored at 4°C immediately after they were brought to the laboratory before analyzing it.

3.2 Bacteria enrichment media preparation

3.2.1 ONR7a agar and suspension preparation

The basal medium used was artificial seawater medium ONR7a. A standard formulation was used for preparing ONR7a medium. Digital balance was used to weight all the chemical compounds before transferring into 1L conical flask. ONR7a medium were consisted of as table below:
Table 3.1: Basal medium ONR7a mixture (Dyksterhouse et al., 1995)

<table>
<thead>
<tr>
<th>Chemical compounds</th>
<th>Weight</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>22.79g</td>
</tr>
<tr>
<td>Na\textsubscript{2}SO\textsubscript{4}</td>
<td>3.98g</td>
</tr>
<tr>
<td>TAPSO</td>
<td>1.3g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.72g</td>
</tr>
<tr>
<td>NH\textsubscript{4}SO\textsubscript{4}</td>
<td>0.27g</td>
</tr>
<tr>
<td>Na\textsubscript{2}HPO\textsubscript{4}∙7H\textsubscript{2}O</td>
<td>0.047g</td>
</tr>
<tr>
<td>KBr</td>
<td>0.083g</td>
</tr>
<tr>
<td>NaHCO\textsubscript{3}</td>
<td>0.031g</td>
</tr>
<tr>
<td>H\textsubscript{3}BO\textsubscript{3}</td>
<td>0.027g</td>
</tr>
<tr>
<td>MgCl\textsubscript{2}∙6H\textsubscript{2}O</td>
<td>11.18g</td>
</tr>
<tr>
<td>CaCl\textsubscript{2}∙2H\textsubscript{2}O</td>
<td>1.10g</td>
</tr>
<tr>
<td>SrCl\textsubscript{2}∙6H\textsubscript{2}O</td>
<td>0.0024g</td>
</tr>
<tr>
<td>FeCl\textsubscript{3}∙6H\textsubscript{2}O</td>
<td>0.002g</td>
</tr>
</tbody>
</table>

The solution was stirred using magnetic stirrer on the hot plate after adding 1L of distilled water. After that, the pH was adjusted to 7.8 by adding NaOH into the medium. Then, the medium was sterilized by autoclaving at 121°C/15 atm for 1 hour. After it is cooled, then it was poured into the conical flask and wrapped with aluminum foil and sent for shaking at 140 rpm on an orbital shaker.

Same chemical compound was used for preparing ONR7a agar. In addition, Bacto Agar (Difco, USA) was added to the agar followed by autoclaving at 121°C/15 atm for 1 hour. In vertical flow hood, the ONR7a agar solution was poured into agar plate and kept at 4°C in fridge for further use.