ISOLATION AND CHARACTERISTIC OF BIPHENYL DEGRADING BACTERIA FROM MANGROVE ENVIRONMENT

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Isolation and characteristic of biphenyl degrading bacteria from mangrove environment.

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Declaration

I hereby declare that no portion of the work referred in this project has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

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<tbody>
<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>cm</td>
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</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>ml</td>
<td>Milliliter</td>
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<tr>
<td>μl</td>
<td>Micro Liter</td>
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<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>16 single ribosomal ribonucleotide</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local Alignment Search Tool</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
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<td>CTAB</td>
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<tr>
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<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>ONR7a</td>
<td>Artificial seawater mineral salt medium</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbons</td>
</tr>
<tr>
<td>PCBs</td>
<td>Polychlorinated Biphenyl</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PCI</td>
<td>Phenol/Chloroform/Isoamyl alcohol</td>
</tr>
<tr>
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<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rotation per minute</td>
</tr>
<tr>
<td>S.I.M</td>
<td>Sulfide-Indole-Motility</td>
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<tr>
<td>TAE</td>
<td>Tris-Acetate-EDTA buffer</td>
</tr>
<tr>
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<td>Tris- EDTA</td>
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<tr>
<td>UV</td>
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Isolation and Characteristic of Biphenyl Degrading Bacteria from Mangrove Environment

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Abstract

Polychlorinated biphenyls (PCBs) and biphenyl often used in the chemical industry as dielectric fluid and plasticizer. It is known to be recalcitrant due to the presence of chlorine in the molecules. The aim of the study is to isolate and characterize the biphenyl degrading bacteria by using the molecular and biochemical test. The bacteria strain was isolated from the mangrove environment in Bako National Park. In this study, the isolation of biphenyl degrading bacteria was attempted using the ONR7a media which containing biphenyl as the sole carbon sources for the selection. Four different strains were successfully isolated which named as S1, S2, S3 and S5. Further characteristic of the enzymes and the degradation pathway of putative degrader were investigated by undergo the oxidase test, catalase test, motility test, MRVP test and also the gram staining. From the gram staining we can conclude that all four strains that obtain are gram negative bacteria. All four strains are non-motile bacteria which have a positive result in catalase and oxidase test. In the molecular characterization and successfully amplifying the 16S rRNA gene sequences (1500bp) from the extracted DNA of the bacteria by using the PCR method. From the best search it showed that the bacteria identified for S1 is Marinobacter sp.strain Trimyema-2 with 1423bp was successfully sequenced. Next, for the S2 bacteria strain, the result showed that 1347bp was successfully sequences of Salipiger sp strain BH85027. Then, the strain S3 was known as Thioclava sp.strain VS-126 with 1370bp was successfully sequences from this bacteria strain. Lastly, for the sample S5, the sequences of the bacterial strain showed highest homology toward Mycoplana sp. Strain G110. Further study on the enzymes of all the four strain which was isolated could produce a valuable commodity for biochemical industry.

Key word: Biphenyl, biphenyl degrading bacteria, 16S rRNA, PCR

Abstrak

Polychlorinated biphenyl (PCBs) dan Biphenyl sering digunakan dalam industri kimia sebagai benda dielektrik dan pemiplastikan. Ia dikenali sebagai sesuatu bahan yang sukar untuk diuraikan kerana kehadiran klorin dalam molekul tersebut. Tujuan utama kajian ini adalah untuk memencangkan dan mencirikan bakteria terpoliklorin yang dapat diuraikan dengan menggunakan ujian molekul dan biokimia. Bakteria tersebut telah diambil dari perkesikitanan kawasan bakau di Taman Negara Bako. Dalam kajian ini, pengasingan bakteria terpoliklorin yang dapat diuraikan telah menggunakan media ONR7a yang mengandungi biphenyl sebagai sumber karbon tunggal bagi pemilihan. Empat strain yang berbeza telah berjaya disasingkan yang dinamakan S1, S2, S3 and S5. Ciri-ciri enzim dan laluan degradasi bahan pengurai telah dikenal pasti dengan menjalankan ujian oksidasi, ujian katalasi, ujian molatili dan juga ujian 'MRVP'. Daripada ujian pevarnaan gram, kita boleh membuat kesimpulan bahawa keempat-empat strain ini telah mendapat gram negatif bakteria. Dalam pencirian molekul yang telah berjaya dengan menggunakan kaedah penegeksstekanan DNA dengan memperluaskan 16S gen yujukan (1500bp) dari DNA bacteria dengan menggunakan kaedah PCR. Dengan menggunakn pencarian 'BLAST', ia menunjukkan bahawa bakteria yang dikenali pasti untuk S1 ialah S1 Marinobacter sp.strain Trimyema-2 dengan 1423bp telah berjaya dijukukan. Seterusnya, bagi strain bakteria S2, hasilnya menunjukkan bahawa 1347bp berjaya urutan terikan sp Salipiger BH85027. Kemudian, S3 dikenali sebagai Thioclava sp.strain VS-126 dengan 1370bp berjaya urutan dari strain bakteria ini. Akhir sekali, bagi sampel S5, Mycoplana sp. Tapis G110. Kajian lanjut mengenai enzim semua strain empat yang telah diasingkan boleh menghasilkan komoditi yang bernilai bagi industri biokimia.

Kata kunci: biphenyl, bacteria pengurai biphenyl, 16S rRNA, PCR
1.0 Introduction

1.1 Background of study

Environment preservation is one of the aims of the sustainable development. Environment pollution has increased in many regions due to industrialization. Biodegradation is one of the methods that used to break down the organic matter into nutrients that can be used by other organism. The natural forces of biodegradation can reduce waste and clean up some types of environment contamination.

The ecology of hydrocarbon degradation by microbial population in the natural environment is reviewed, phasing the physical, chemical, and biological factors that contribute to the biodegradation of individual hydrocarbons. The rates of biodegradation depend greatly on the composition, state, and concentration of the hydrocarbons with dispersion and emulsification enhancing rates in aquatics systems and absorption by soil particulars being the key feature of terrestrial ecosystems.

There are studies of micro-organisms that are able to decompose biphenyl. In general, these organisms work in one of two ways (Chauhan KR. et al., 2000). It is either they use the biphenyl as a carbon source or destruction takes place through reductive de-chlorination, with the replacement of chlorine with hydrogen on the biphenyl skeleton. Second, microbial de-chlorination tends to be rather slow-acting on biphenyl as a soil contaminant in comparison to other methods (Simon T. et al., 2007).

The main removal process for biphenyl in soil appears to be biodegradation. The following organisms have been shown to degrade biphenyl: Saccharomyces cerevisiae with the production of benzoic acid, Streptomyces sp., Achromobacter, Pseudomonasputida, Oscillatoria sp., gram negative bacteria, Acaligenes sp. (Kato et al., 2005). Bacteria generally oxidize biphenyl via cytochrome P-450 to 2,3-dihydroxybiphenyl.
(Kato et al., 2005). Fungi metabolize biphenyl to 4-hydroxy- or 2-hydroxybiphenyl and 4,4'-dihydroxybiphenyl (Kato et al., 2005). According to Kato et al. (2005), 9.1% of the biphenyl was degraded by activated sludge in 2 days.

Biphenyl is often used in chemical industry, the manufactures of textiles and dyes. It is also used to produce PCBs. It is known to be recalcitrant due to immobile in soils and the water bodies. In this study, the isolation of biphenyl degrading bacteria was attempted using minimal media containing biphenyl as the sole carbon sources for selection. Further characteristic of the strain and the degradation pathway of putative degraders were investigated using PCR method.

Mangrove wetlands diverse group microorganism in the sediments in which aromatic degrading bacteria are reported to be at high levels. (Macek et al., 2000). This indigenous community had considerable potential to degrade oils especially where the site was oil contaminated (El-Tarabily et al., 2002). However less is known concerning in the biodiversity and degradation abilities of PAH-degrading bacteria from different mangrove sediments. Therefore this study is an attempt to discover the bacteria from the mangrove environment have the ability to degrade the biphenyl component.
1.2 Problem statement

Polychlorinated biphenyls have been used not only as dielectric fluids in capacitors and transformers, but also as flame retardants, plasticizers and ink solvent. Commercial mixtures typically consist of 40-70 congeners. More than 1.7 million tons of PCBs were produces worldwide and important amounts of this compound have been released into the environment (Seeger et al., 2009). According to Mayes et al., (1998), PCB congeners have been reported to cause cancer. It is also causes of serious effects on endocrine, immune, nervous and reproductive systems (Faroon et al., 2001).

Oil pollution has becomes a worldwide problem, since it not only gives adverse effect on the natural environment and ecosystem but also causes serious damages on fisheries. The application of microorganism for degradation of pollutants is now an ideal technology for cleans up or restoration of polluted sites as it can be self-sustaining and inexpensive. The molecular biology methods are ideals to study bioremediation since a deep understanding of microbial ecology is essential to gain maximum benefits from this bioremediation process.

Biodegradation of PAHs is catalyzed by multicomponent enzymes from microbes. A key enzymes for attacking the aromatic ring structure of PAHs under aerobic is the initial dioxygenase which is substrate specific (Cerniglia et al., 1992). The isolation of PAH-degrading bacteria often utilizes a limited number of PAHs as their sole carbon and energy sources (Bouchez et al., 1995).
1.3 Aim of the study

This study was designed to investigate the degradation of biphenyl by newly isolated bacteria. The main objective of this study is to isolate and characterize the biphenyl degrading organism from the enrichment in minimal media containing of biphenyl as selection substrate. The aims of this study were listed in figures 1:

Aim 1: To isolate the biphenyl degrading bacteria from the mangrove environment.
Enrichment in ONR7a media.

Aim 2: To characterize and study the physical and morphological characteristic of the bacteria strain that isolated.
Biochemical test of the strain that isolated.

Aim 3: To identify the species of bacteria strain isolated by using the BLAST program by comparison of its 16S rRNA.
16S RNA gene sequencing after bacteria isolates were obtained.

Figure 1: The summary of the aim in this study
2.0 Literature Review

2.1 Xenobiotic compounds

According to IUPAC Recommendation 1997, a xenobiotic (Greek, xenos 'foreign'; bios 'life') is a compound that is foreign to a living organism. Principal xenobiotic included drugs, carcinogens and various compounds that have been introduced into the environment by artificial means. The number of man-made or natural organic compounds has reached 18 million molecular species with more than 60,000 used in commerce (Hou et al., 2003).

High production of xenobiotic in the last few years was due to the combinatorial chemistry (IUPAC Recommendation 1999) used in the biochemistry industry as well as the increasing demands in the pharmaceutical and agricultural industries (Dolle et al., 2004).

Xenobiotic such as herbicides and pharmaceutical drugs are useful and effective due to their abilities to target specific enzymes or cell components of weeds and pathogens.

Despite the known benefits to humans, there are several drawbacks in using xenobiotic as some of them are toxic and carcinogenic. For example, polychlorinated biphenyl (PCB) used in coolants, plasticizers for paints and also pesticides was found to disrupt thyroid functioning activity in humans. In addition, children exposed to PCB were shown to have increased mental retardation and neurodevelopment impairment (Jugan et al., 2010).

Apart from being physically removed, xenobiotic can also be biodegraded by bacterial enzymes. Some xenobiotic can be degraded into water and carbon dioxide and whilst others can be metabolized by bacteria as the carbon sources for their growths (Poelarends et al., 2000). Bacterial degradation of xenobiotic can occur under aerobic or anaerobic conditions, depending on the bacterial species (Yu and Welander 1995) and the specificity of each species depends on the enzymes it possess. Despite the extensive study of microbial degradative pathways, only less than 1000 compounds have been
characterized to date in comparison to the number of xenobiotic produced year by year (Ellis et al., 2006). Organisms that can degrade the structurally related natural compound often are able to degrade the substituted xenobiotic compounds although the process occurs at a slower rate.

2.2 Biphenyl and Polychlorinated biphenyl

2.2.1 Biphenyl

Biphenyl can be also called as diphenyl. It is a colorless solid and occurs naturally in trace amount. Biphenyl is mainly used in chemical industries. It is used as a heat transfer agent and a starting material to make polychlorinated biphenyl (Duinker et al., 1998). Biphenyl dissolves poorly when it is mixed with water. Biphenyl breaks down to other chemicals settles as dry deposits to water and land. Biphenyl normally attaches to solid material in water. Microorganisms living in water and in soil breaks down biphenyl to other chemical. There are lots of effects of biphenyl on human health and the environment (Baker et al., 1990). The effect is depends on the exposure of the individual occurs. The exposure for a short period of time has experiences nausea, vomiting, irritation of the eyes and respiratory tract. The inhalation of small amount of biphenyl over long period of time has caused damage to the liver and nervous system of exposed workers (Doucette et al., 1988). Laboratory studies show that exposure to large amount of biphenyl by ingestion damages the kidney and blood and reduces growth and life expectancy. Biphenyl is highly toxic to aquatic life. The biphenyl industry has completed chronic aquatic toxicity studies in response to an EPA request of testing (Baker et al., 1990).
2.2.2 Polychlorinated Biphenyl (PCBs)

Polychlorinated biphenyls (PCBs) are priority pollutants that were used worldwide for a variety of applications for more than 50 years. PCB molecules are composed of a biphenyl backbone substituted with 1 to 10 chlorines (Thomas et al., 1992). These PCBs persist in the sediments, accumulate in biota, and biomagnified in the food chain. Multiple adverse health effects have been attributed to them, and they are suspected human carcinogens (Thomas et al., 1992). Microbial reductive PCB de-chlorination provides a natural means of detoxifying PCBs in aquatic sediments because it reduces their persistence and increases their biodegradability and metabolism by other prokaryotes and by higher organisms.

Polychlorinated biphenyls (PCBs) are toxic, persistent pollutants of worldwide concern whose cleanup using conventional methods like incineration or relocation to specialized landfills is often prohibitively expensive (Masuda et al., 1997). An alternative strategy for in situ PCB removal is biodegradation by microorganisms capable of metabolizing PCBs. Biodegradability is related to the amount of chlorination of a specific PCB. The higher the chlorine content of a PCB, the less the biodegradability (Masuda et al., 1997). The lack of degradability of PCB compounds results in bioaccumulation of PCBs in the environment.

PCBs is so harmful because it has the characteristic which is so hazardous to the environment. The high thermal and chemical resistances of PCBs showed that it is not readily break down when exposed to heat and chemical treatment (Masuda et al., 1994) It is a group of compounds that are generally both biohazards and stable are the polycyclic aromatic hydrocarbons (PAHs). Although some PAHs are toxic, carcinogenic, or teratogenic, a variety of bacteria can degrade certain PAHs completely to CO₂ and
metabolic intermediates, en route gaining energy and carbon for cell growth (Wood et al., 1999).

2.3 Biphenyl degrading bacteria

The microbial degradation of PCBs is regarded as one of the most effective procedures for removing them from the environment. Many PCB-degrading bacteria have been isolated which able to degrade biphenyl. The gram – negative bacteria have the ability to degrade biphenyl including genera *Pseudomonas, Alcaligenes, Achromobacter, Burkholderia, Acinetobacter, Comamonas, Sphingomonas* and *Ralstonia* (Masai et al., 1995). The strain of *Pseudomonas* is biphenyl-utilizing polychlorinated biphenyls (PCB)-degrading bacteria which belong to different phylogenetic groups, which indicates that the same geographical location does not ensure the same ancestor of degradative enzymes. The genus *Pseudomonas* and *Rhodococcus* sp. strain RHA1 (RHA1) grow on biphenyl by oxidizing PCBs via a biphenyl catabolic pathway (Masai et al., 1995). According to Masai et al (1995), until the recent research on biphenyl degradation has focused on gram negative bacteria in particular members of the genus *Pseudomonas*.

Studies also show that a Gram-negative bacterium, strain LY402 has the ability to degrade biphenyl, belonging to the genus *Enterobacter*. The strain readily degraded certain highly chlorinated and recalcitrant polychlorinated biphenyls (PCBs) (Nicholson et al., 1994). Analysis of PCB degradation indicated that strain LY402 could effectively degrade PCB congeners with chlorine substitutions in both ortho- and para-positions. Consequently, this is the first report of *Enterobacteria* that can efficiently degrade both low and highly chlorinated PCBs under aerobic conditions (Nicholson et al., 1994).
2.4 Degradation pathway of biphenyl degrading bacteria

A number of bacteria have the ability to initiate the degradation of the compound by adding molecular oxygen to the ring. *Pseudomonas* sp. strain LB 400 is the particular noteworthy (Ivanov *et al.*, 1992). It encodes four enzymes and catabolize biphenyl to benzoate and 2- hydroxypenta-2-4 diene through four steps, often called as the biphenyl upper pathway. Many bacteria can oxidize polychlorinated biphenyl (PCBs), a group of man-made compound composed of biphenyl molecules containing from 1 to 10 chlorines, and persistent and toxic in biosphere (Jugan *et al.* 2010). It has been shown that PCBs follow the same catabolic pathway as biphenyl and use the same enzymes (Ellis *et al.*, 2006). Biphenyl dioxygenase plays a critical role in PCB degradation by catalyzing the first step.

The biodegradation of PAHs by bacteria has been observed under aerobic and anaerobic conditions. Anaerobic biodegradation proceeds very slowly and the biochemical mechanism of this process has not yet been determined in detail. (Dagher *et al.*, 1997). The pathway and mechanism for biphenyl degradation has been simplified in Appendix A.

2.5 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 natural mechanism to clean up the hydrocarbon pollutants and crude oil from the environment (Cristol *et al.*, 1983). Biodegraded derived aromatic hydrocarbons in marine sediments, demonstrate that multiple microorganisms are capable to degrade crude oil, including *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas*, (Hill *et al.*, 1999).

An important requirement for successful bioremediation is the presence of microorganisms with appropriate metabolic capabilities dependent on multiple factors such
as nutrients, oxygen, and pH (Im W. T. et al., 2004). Biodegradation of hydrocarbons is a complex process that depends on the nature and on the amount of the hydrocarbons present. Petroleum hydrocarbons can be divided into four classes: the saturates, the aromatics, the asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and the resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) (Im W. T. et al., 2004).

2.6 16S ribosomal RNA analysis

16S ribosomal RNA analysis comparison of the bacterial 16S rRNA gene sequences has emerged as a preferred genetic technique. 16S rRNA gene sequences analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains, can be routinely used for identification of mycobacteria, and can lead to the recognition of novel pathogen and non-cultured bacteria (Juhasz et al., 2000). The 16S rRNA genetic analysis is an essential element. Genetic information is conserved throughout the microorganism. It is independent of stage of growth or even viability and is currently the most reliable sources for bacterial identification.

The analysis uses the RNA type found in the ribosomes of all self-replicating cells. The genetic fingerprint or gene sequences are retrieved using a primer that targets a specific gene sequences which capture a 500 base sequences sample (Hwang et al., 2002). This data is compared to the same 500 base sequences in such genetic libraries as MicroSeq, Genbank or Ribosomal Database Project (RDP) (Hedlund et al., 1999). The comparison result in a “percent differences” from the library databases and identifies the specific base positions that are different. If the percent different to the closed library database match is under 1%, the sample is guaranteed to the species, if the differences are greater than 1% and less than 10%, it is guaranteed to the genus level. If greater than 1 1% differences is seen, the 500 base sequences would then be compared to other database libraries to determine if a closer match exists (Ho et al., 2000).