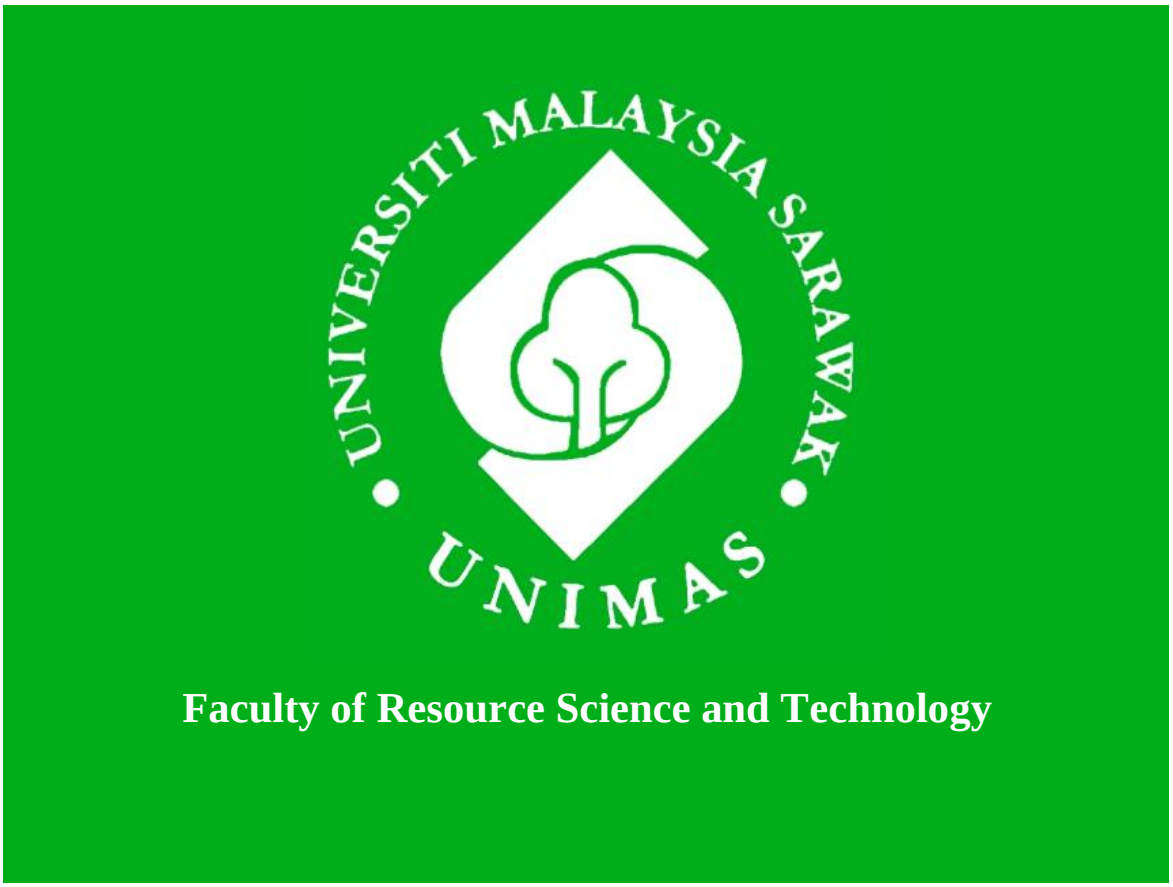

ISOLATION AND CHARACTERIZATION OF CARBAZOLE-DEGRADING BACTERIA FROM MANGROVE ENVIRONMENT

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Isolation and Characterization of Carbazole-Degrading Bacteria from Mangrove Environment

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Declaration

I declare that this thesis entitled "Isolation and Characterization of Carbazole-Degrading Bacteria from Mangrove Environment" is the result of my own work except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : …….

Name : …….....

Date : …….

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List of Abbreviations

16S rRNA	16S ribosomal RNA
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
MR-VP	Methyl Red- Voges Proskauer
PAHs	Polycyclic Aromatic Hydrocarbons
TAE	Tris Acetate EDTA
CARDO	Carbazole 1,9a-dioxygenase
CO_2	Carbon dioxide
H_2S	Hydrogen sulfide
µL	Microliter
rpm	Rotation per minute
˚C	Degree Celsius
PCR	Polymerase Chain Reaction
$MgSO_4$	Magnesium sulfate

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ABSTRACT

Carbazole is an aromatic heterocyclic organic compound usually found in river sediment that can possess to toxic activities that will lend serious environmental problems. This study was conducted to isolate and characterize carbazole-degrading bacteria from mangrove environment. The samples were collected from Bako National Park and the presence of carbazole-degrading bacteria was confirmed through enrichment culture of the samples. Screening of the bacteria was successfully done by observing the growth of the strains on ONR7a agar containing carbazole as sole carbon source. Four different strains of carbazole degrading bacteria that are able to grow on carbazole source were isolated. Isolate strains were characterized using phenotypic and molecular techniques. From the BLAST search, four different strains of carbazole degrading bacteria were identified as *Sphingomonas* sp., *Flavobacterium* sp., *Gaetbulibacter* sp., and *Thalassospira* sp. with maximum identity of 100%, 97%, 99%, and 99% respectively. All strains appeared as a non-motile Gram-negative rod shaped bacterium.

Key words: Carbazole, Carbazole-degrading bacteria, 16S rRNA.

ABSTRAK

Karbazol merupakan sebatian aromatik heterosiklik organik yang biasanya boleh ditemui dalam sedimen sungai yang memiliki aktiviti toksik dan akan meminjamkan masalah alam sekitar yang serius. Kajian ini telah dijalankan untuk mengasingkan dan menyaringkan bakteria pengurai karbazol dari persekitaran bakau. Sampel-sampel telah diambil dari Taman Negara Bako dan kehadiran bakteria pengurai karbazol telah disahkan melalui pengkulturan sampel-sampel berkenaan. Penyaringan bakteria berjaya dilakukan dengan memerhatikan pertumbuhan strain atas ONR7a agar yang mengandungi karbazol sebagai sumber tenaga dan karbon utama. Terdapat empat bakteria strain yang berlainan boleh bertumbuh dan mengurai karbazol telah diasingkan. Identiti bagi isolat strain-strain telah dikenal pasti melalui kajian fenotipik and kajian molekular. Daripada pencarian melalui BLAST, empat strain bakteria pengurai karbazol telah dikenal pasti sebagai Sphingomonas sp., Flavobacterium sp., Gaetbulibacter sp. dan Thalassospira sp. dengan identiti maksimum 100%, 97%, 99%, 99% masing-masing. Semua strain bakteria pengurai karbazol menunjukkan bukan-mobil dan Gram-negatif bakteria yang berbentuk rod.

Kata kunci: Karbazol, bakteria pengurai karbazol, 16S rRNA.

1.0 Introduction

Carbazole, an environmental pollutant, is an aromatic heterocyclic organic compound or a nitrogen heterocycle normally used in drugs, dyes, insecticides and polymers (Collin & Hoke, 1986). Carbazole usually can found in river sediment (West *et al.*1986). Mangrove environment is a bridge between terrestrial and marine environment which have large unique microbial diversity. Mangrove environment is abundant with carbazole-degrading bacteria in mangrove sediments which important to bioremediation the polluted environments (Liu *et al*, 2010). According to Reynolds (2002), these microorganisms often use contaminants as a food source, thereby completely eliminating toxic compounds and transform them into basic elements.

A study by Guo *et al.* (2008) found that carbazole can possess to toxic activities that will lend serious environmental problems. Therefore, the global pollution of environment by carbazole has been concern to environmental microbiologists. It is reported that microorganism such as *Pseudomonas, Ralstonia,* and *Sphingomonas* revealed the metabolic pathway for initial carbazole methabolism (Ouchiyama *et al.* 1993, Gieg *et al.* 1996, Sato *et al.* 1997, Sheperd and Lloyd-Jones 1998, Ouchiyama *et al.* 1998, Kasuga *et al.* 2001, Nojiri *et al.* 2001).

The objectives of this project are:

1. To isolate carbazole-degrading bacteria from mangrove environment.
2. To examine the physico- chemical properties of isolate through morphological and biochemical characterization.
3. To identify isolate by its 16S rRNA gene sequences.

2.0 Literature Review

2.1 Mangrove Environment

Mangrove environment is a bridge between terrestrial and marine environment which provided important permanent and temporary habitats for a large number and range of marine and terrestrial organisms such as microorganism, flora and fauna. The soil from the mangrove usually made up from sand, silt and clay in different combination. There is large unique microbial diversity found in the mangrove soil. These microbial play different roles of activities in the mangrove environment. Smith *et al.* (1991) found that mangrove supports abundant life through a food chain that starts with the trees and the micro-biota in the coastal areas of tropical countries. Mangrove environment is abundant with Polycyclic Aromatic Hydrocarbon-degrading bacteria in mangrove sediments which important to bioremediation the PAH polluted environments (Liu *et al.,* 2010).

2.2 Bioremediation

Bioremediation is a process which uses the enzymes from microorganisms to degrade environmentally harmful contaminants and turn them into non-toxic compound. According to Reynolds (2002), these microorganisms often use contaminants as a food source, thereby completely eliminating toxic compounds and transform them into basic elements such as carbon dioxide and water, a process known as mineralization. However, some microorganisms are partial breakdown of the original contaminant to a less complex form or changing the toxic compound to a different chemical structure that may affect the toxicity and mobility of the original agent. Bioremediation is a relative slow process, requiring few weeks to months to effect cleanup, but this process can be very cost-effective if done properly.

Bioremediation basically can be classified as in situ which involves treating the contaminated material at the site and ex situ involves the removal of the contaminated material to be treated elsewhere (Microbiology Reader Bioscreen C, 2005). Soil bioremediation might be performed under either aerobic or anaerobic conditions, and involve optimization of the metabolic pathways of bacteria for degradation of hydrocarbons and aromatic compounds.

2.3 Carbazole

According to Santodonato and Howard (1981), Carbazole ($C_{12}H_9N$) or also known as 9-azafluorene is an aromatic heterocyclic organic compound or a nitrogen heterocyclic detected in diverse environments like atmospheric sample. Besides that, it also can be found in river sediment (West *et al.*1986). Carbazole is a chemical that normally used in to drugs, dyes, insecticides and polymers (Collin & Hoke, 1986). A study by Guo *et al.* (2008) found that carbazole can possess to toxic activities that will lend serious environmental problems.

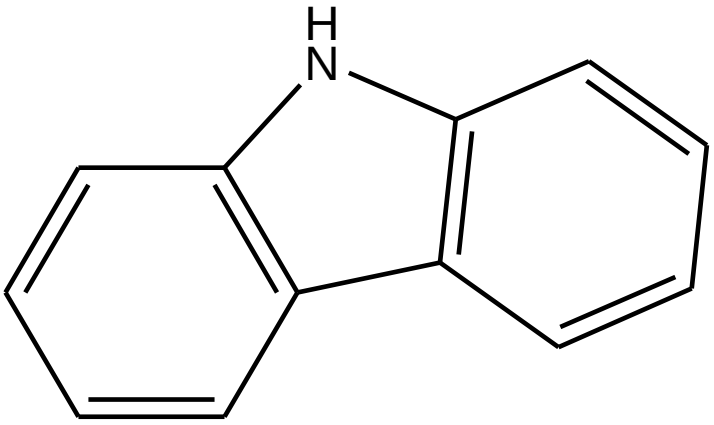

Figure 1: Chemical structure of carbazole.

2.4 Carbazole Degrading Bacteria

Carbazole-degrading bacteria are a type of microorganisms which play important role in removal of carbazole chemical pollutant from the environment. There microorganism usually able to utilize carbazole as the sole sourse of carbon, nitrogen, and energy (Guo *et al*., 2008). It is reported that microorganism such as *Pseudomonas*, *Ralstonia*, and *Sphingomonas* revealed the metabolic pathway for initial carbazole methabolism (Ouchiyama *et al.* 1993, Gieg *et al.* 1996, Sato *et al.* 1997, Sheperd and Lloyd-Jones 1998, Ouchiyama *et al.* 1998, Kasuga *et al.* 2001, Nojiri *et al.* 2001).

2.5 Carbazole Degrading Pathway

So far, different species of carbazole degraders all appear to follow a same carbazole degrading pathway. The carbazole degrading bacteris will degrade the carbazole to 2'-aminobiphenyl-2, 3-diol by carbazole 1,9a-dioxygenase (CARDO). The products is futher converted by CarB and CarC proteins to 2-aminobenzoate (anthranilic acid) and 2-hydroxypenta-2, 4-dienoate. Then, these two compounds will be converted to TCA cycle intermediate which entered the tricarboxylic acid cycle to generate energy for the bacteria.

2.6 The 16S rRNA Gene

Traditionally, the identification of bacteria is based on the phenotypic characteristics of bacteria which are not accurate as identification based on genotypic methods. As Jill E. Clarridge III (2004) mentions, the 16S rRNA gene approximately 1,550 bp long sequence has emerged as a preferred genetic technique to distinction among bacterial strain which allow

bacterial identification that is more robust, reproducible, and accurate than that obtained by phenotypic testing in clinical microbiology laboratories.

2.7 Polymerase Chain Reaction

Polymerase Chain Reaction or PCR is a process to analyze specific short sequence of DNA fragment from minute quantities of source DNA material, even when that source DNA is of relatively poor quality (Erlich, 1989). Generally, there are three steps involved in PCR which are denaturation, annealing and extension. These three steps are repeated can generate millions of copies only in a few hours.

3.0 Materials and Methods

3.1 Sample collection and filtration

The mangrove water and soil samples were collected from the mangrove environment of Bako National Park. Then, all samples were sent to the laboratory for isolation of carbazole-degrading bacteria. The soil samples were stored at 4℃ in fridge, while the water samples store in cool room.

The mangrove water collected from Bako National Park was sent to the laboratory for filtration. Vacuum filtration was operated by using vacuum [MILIPORE EXPRESS^{TM} PLUS (Milipore, USA)] and filter medium with pore size of 0.22 µm. The filter medium was then cut out and transferred into a falcon tube. Later it was washed with mangrove water and removed the filter medium before vortexing using vortex mixer. Two layers would be formed. The supernatant that contained the bacterial strain was extracted out into ONR7a medium.

3.2 Preparation of ONR7a suspension and agar

3.2.1 ONR7a suspension

ONR7a which is known as artificial seawater mineral salt medium was prepared according to the standard formulation. All the chemical compounds must be weighted using digital balance before transferred into 1 L conical flask.

Table 1: The composition of artificial seawater mineral salt medium (ONR7a).

Chemical compound	Weight (g)
NaCl	22.79
Na_2SO_4	3.98
KCl	0.72
NaBr	0.083
$NaHCO_3$	0.031
H_3BO_3	0.027
NH_4Cl	0.27
$Na2HPO_4$	0.04715
TAPSO	1.3
$MgCl_2 \cdot 6H_2O$	11.18
$CaCl_2$	1.102
$SrCl_2 \cdot 6H_2O$	0.0024
$FeSO_4 \cdot 7H_2O$	0.002

Next, 1L of distilled water was added and the solution was stirred using magnetic stirrer on the hot plate. The conical flask was wrapped with aluminum foil and sent to autoclave at 121 ℃/15atm for 90 minutes.

3.2.2 ONR7a agar

ONR7a agar was prepared with the same chemical compound of ONR7a suspension with additional of Bacto Agar (Difco) and later autoclave at 121 ℃/15atm for 90 minutes. ONR7a agar solution was poured into agar plate in vertical flow hood. The plates were kept in fridge at 4 ℃ for further use.

3.3 First and second stage enrichments of Carbazole-Degrading Bacteria

Approximately 0.5 g of the soil samples and 1 mL of mangrove water sample were suspended in 100 mL of artificial seawater mineral salt medium (ONR7a) containing 1mL of carbazole. All the samples were incubated with agitation for few weeks at room temperature until degradation can be confirmed through coloration of media. After two weeks of incubation, the second stage enrichment was carried out by transferring 100 µl of bacteria sample from first enrichment into 10 mL of same media supplemented with 100 µl of carbazole. The samples were reciprocally shaken at 120 rpm in room temperature for few days.

3.4 Isolation of Carbazole Degrading Bacteria

Bacteria culture from the second stage enrichment was inoculated onto ONR7a agar supplemented with carbazole on the lid of Petri dish and incubated for several days at room temperature. Besides ONR7a, the culture has been inoculated onto Marine agar (Difco, USA) without supply of carbazole and incubated for several days at room temperature. Subculture of bacteria on ONR7a and Marine agar were carried out in order to isolate the suspected carbazole degrading bacteria.

3.5 Morphological Test

3.5.1 Gram Staining

A single bacterial colony from overnight culture was obtained using a sterile inoculating loop. The colony was mixed with two drops of distilled water to form a smear on a microscope slide. Then, the smear was air dry and heat fix by passing through the flame of Bunsen burner for few seconds. Next, heat-fixed culture mounts on glass slide was stained with few drops of crystal violet (primary stain) for 1 minute. After that, the stain was washed off with distilled water gently. The smears were covered with gram's iodine solution for another 1 minute. Afterwards, the smears were washed with distilled water and 95% ethanol was added for 15 seconds to decolorize the smear. After the smear decolorized, the slide was washed with distilled water before added with safranin for 20-30 seconds. Lastly, the slide was washed with distilled water and the stained slide was observed under light microscope to determine the gram stain and shape of bacterial cells.

3.6 Biochemical Tests

3.6.1 Methyl Red and Voges-Proskauer Test

For Voges-Proskauer tests, the bacteria were inoculated into MR-VP broth and incubated at 37℃ for 24-48 hours. After incubation, 1mL of culture was aseptically pipette to a clean test tube. Then, 0.5 mL of Barritt's reagent A and 0.5 mL of Barritt's reagent B were added to the 1mL of culture. The positive result was indicated by a color change from pink to red within 20 minutes. The remaining broth from the cultures grown for the Voges-Proskauer test was used to perform the methyl red test. A few drops of methyl red solution were added to the

cultures. The positive result was indicated by a color change to red, while yellow color indicates a negative reaction.

3.6.2 Hydrogen Sulfide Test

The bacteria was streaked on the surface of the SIM agar with a loop and stabbed down through the middle of the SIM agar to the bottom of the slant with a needle. The culture was incubated at room temperature for overnight. The production of hydrogen sulfide can be determined by observing the formation of black precipitate in the agar. Contrarily, the absence of black precipitate in the agar indicates a negative reaction.

3.6.3 Motility Test

A motility test was implemented to test for flagullar bacterial movement. A semi-solid SIM medium was used to test. An inoculation needle was used to pick up a small amount of bacteria and stab directly in and out of the middle of the semi-solid medium. A positive for motility was recorded if the bacteria moved outward from the stabbed area.

3.6.4 Catalase Test

A catalase test was implemented to demonstrate catalase activity. A loop was used to pick up and spread a small amount of bacteria (18 to 24 hours old) onto a microscope slide. Two drops of 3% hydrogen peroxide was added on top of the bacteria. The catalase activity will be indicated if production of bubbles.

3.6.5 Oxidase Test

A toothpick was used pick up bacteria and scrape onto filter paper. Three drops of oxidase reagent namely N,N,N,N-tetramethyl-p-phenylenediamine was added to the bacteria. The positive result was indicated by a color change to purple, while negative reaction is observed when oxidase reagent remains colorless.

3.6.6 Citrate Test

A single bacterial colony was inoculated onto Simmon's citrate medium and incubated at room temperature for overnight. The positive result was indicated by a color change of the Simmon's citrate medium to blue, whereas negative reaction is obtained when the medium remains green.