Isolation and Identification of Biphenyl Degrading Marine Bacteria from Seawater of Coast Of Sarawak

Toh Geh Heng
(22560)

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Department of Molecular Biology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
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LIST OF ABBREVIATIONS

PCBs	-	Polychlorinated biphenyls
16S rRNA	-	16 single ribosomal riboxynucleotide
BDDH	-	biphenyl-2,3-dihydrodiol-2,3-dehydrogenase
BDO	-	biphenyl-2,3-dioxygenase
BphH	-	2-hydroxypenta-2,4-dienoate hydratase
BphI	-	acylating acetaldehyde dehydrogenase
BphJ	-	4-hydroxy-2-oxovalerate aldolase
BLAST	-	Basic Local Alignment Search Tool
bp	-	base pair
DBDO	-	2,3-dihydroxybiphenyl-1,2-dioxygenase
g	-	gram
HOPDA	-	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate
HOPDAH	-	2-hydroxy-penta-2,4-dienoate by 2 –hydroxy-6-oxo-6-phenyl- hexa-2,4-dienoate hydrolase
ml	-	milliliter
mM	-	milimolar
ONR7a	-	artificial seawater mineral salt medium
SDS	-	sodium dodecyl sulphate
PCB_s	-	Polychlorinated biphenyls
°C	-	degree celcius
rpm	-	rotation per minute
DMSO	-	Dimethyl Sulphoxide
NaOH	-	Sodium Hydroxide
CTAB	-	cetyltrimethylammonium bromide
µm	-	micrometer
mbar	-	milibar

S.I.M	-	Sulfide-Indole-Motility
TAE buffer	-	Tris-Acetate-EDTA buffer
TE buffer	-	Tris-EDTA buffer
NaCl	-	Sodium chloride
$MgCl_2$	-	Magnesium chloride
dNTP	-	Deoxyribonucleotide triphosphate
PCR	-	Polymerase Chain Reaction
K	-	Potassium
Mg	-	Magnesium

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Isolation and identification of biphenyl degrading marine bacteria from seawater of coast of Sarawak

Toh Geh Heng

Resource Biotechnology Program
Department of Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

Biphenyl and polychlorinated biphenyl are widespread environmental pollutants that are degraded by biphenyl degrading bacteria in the bioremediation process into harmless product. The objectives of these studies are to isolate and characterize biphenyl degrading marine bacteria strain by morphological and molecular work. The samples of bacterial strain were collected from Muara Tebas and later the bacteria strain which designated BPH08 was grown in enrichment culture. Screening of the bacteria was successfully done by observing the growth of the strain on ONR7a agar plate containing biphenyl as sole carbon source. In addition, the strain BPH08 also undergoing morphological testing and the characteristics of bacteria was confirmed applying gram stain, salt tolerance test, motility test and growth in different PAH substrate test and showed that the bacteria was gram negative, could grow in wide range of salt concentration where the optimum growth could be achieved at 4% NaCl of ONR7a agar. It was also could grow in each of ONR7a agar containing different substrate supplemented with either biphenyl, dibenzofuran, fluorene, carbazole, or dibenzothiopene, was non-motile and tested for catalase and oxidase positive. Later the strain BPHO8 was selected to undergo molecular characterization and successfully amplifying the 16S rRNA gene sequence (1500bp) from the extracted total DNA of the bacteria using PCR. The PCR product was purified and the nearly complete 16S rRNA gene sequence (1255bp) was effectively sequenced. From the BLAST search, it showed that the species closest to the strain BPH08 was *Thalassospira profundimaris* strain with maximum identity of 98%.

Key words: Biphenyl, biphenyl degrading bacteria, 16Sr RNA, PCR

ABSTRAK

Biphenyl dan polychlorinated biphenyl merupakan bahan pencemar yang merebak dalam alam sekitar yang dapat dilupuskan oleh bakteria pengurai biphenyl dalam proses bioremediasi dan menukarkannya ke produk yang selamat. Objektif dalm kajian ini adalah untuk mengasingkan dan menyaringkan bakteria tersebut melalui kerja-kerja morfologi dan molekular. Sampel bakteria dikumpul dari Muara Tebas, ditetapkan sebagai strain BPH08 dan ditumbuh dalam kultur kaya dengan mineral. Penyaringan bakteria berjaya dilakukan dengan memerhatikan pertumbuhan strain bakteria tersebut atas agar ONR7a yang mengandungi bifenil sebagai sumber tenaga dan karbon utama. Tambahan pula, strain bakteria BPH08 tersebut akan menghadapi ujian morfologi dan mengesahkan ciri-ciri bakteria tersebut dengan menggunakan 'gram staining', 'ujian penerimaan garam', 'ujian pergerakan' dan ujian pertumbuhan dalam pelbagai bahan kimia PAH . Keputusan menunjukkan bahawa bakteria tersebut ialah gram negatif, dapat tumbuh dalam kepekatan garam yang mempunyai jarak lingkungan yang luas di mana kadar pertumbuhan yang optima dicapai pada 4% NaCl dalam agar ONR7a, membuktikan bakteria dapat tumbuh di atas setiap agar ONR7a yang mempunyai bahan kimia yang berlainan seperti bifenil, dibenzofuran, florine, carbazole atau dibenzothiopene, tiada pergerakan dan diuji positif untuk ujian catalase and oxidase. Selepas itu, strain bakteria BPH08 tersebut dipilih untuk melalui proses identifikasi molekular dan berjaya mengamplifikasikan 16S rRNA urutan gen (1500bp) yang merupakan sebahagian dari pengeluaran keseluruhan DNA dalam bakteria dengan menggunakan PCR. Produk PCR tersebut ditulenkan dan 16S rRNA urutan gen (1255bp) yang hampir lengkap berjaya melalui proses pengurutan nucleotide. Daripada pencarian melalui BLAST, menunjukkan bahawa spesies yang paling dekat dengan strain bakteria BPH08 yang spesifik ialah Thalassospira profundimaris dengan identiti maksimum 98%.

Kata kunci: Bifenil, bakteria pengurai biphenyl, 16S rRNA, PCR

1.0 INTRODUCTION

1.1 Background of research

Biodegradation of dioxin and other compound such as biphenyl, dibenzofuran and dibenzo-p-dioxin has become subject of major concern in environmental microbiology in connection with bioremediation of polluted environments. Bioremediation of waste materials that contain hydrocarbons depend on the ability of microorganism to enhance their biomass growing on the substance and degrade them to non-toxic product such as H_20 and CO_2 (Toledo *et al.*, 2006). Pollutant such as polychlorinated biphenyl (PCBs) can be treated by microorganism but in large quantity of those substances will kill the microorganism due to the toxicity of PCBs. Therefore the search of useful microorganism and development of genetically engineered microorganism have been performed and progressed in consequence of remarkable advance of microbiology and genetic engineering for the last 10 years and over (Na *et al.*, 1998). Abundant of microorganism which capable of degrading biphenyl and dibenzofuran and their chlorinated analogues have been isolated and characterized from their physiology, biochemistry and genetic (Armengaud and Timmis, 1997; Furukawa, 2000; Wittich, 1998). The identification of key organisms that exert their function in pollutant degradation processes is significant to the development of optimal *in situ* bioremediation strategies (Viggiani *et al.*, 2004; Abed *et al.*, 2002).

1.2 Problem statement

Environmental pollutant of anthropogenic origin cause serious damage when introduced at certain concentration to the environment which impairs the biological functioning of ecosystem or pose risks to living organism (Scullion, 2006). Almost more than 1.7 million tons of PCB_s were formed worldwide, and a significant amount of these compounds have been discharged into the environment (Seeger and Pieper, 2009). Polychlorinated biphenyl and its derivatives are among the organic pollutant that have long be known to be a source of concern due to its high persistence, carcinogenic, mutagenic and tetraogenic potential. BisphenolA and benzophenone are biphenyl compounds that exhibited estrogenic activity in bioassays. These compounds are widely used to manufacture polyacrylates and phenol resins in which their residues are released as pollutant into rivers and seas. Frequently large number of small-scale spill accidents occurs. Oil pollution has become a worldwide problem, since it not only gives adverse effects on the natural environment and ecosystem but also causes serious damage on fisheries (Peterson *et al.*, 2003; Yamamoto *et al.*, 2003). The pollution caused by these xenobiotic compounds can be removed by biphenyl degrading bacteria used in bioremediation (Asturias and Timmis, 1993). The application of microorganisms 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 ideal to study bioremediation since a deep understanding of microbial ecology is essential to gain maximum benefits from this bioremediation process (Widada *et al.*, 2002).

The aim of this study is to isolate biphenyl degrading marine bacteria designated BPH08 by analyzing its growing ability on ONR7a agar containing biphenyl as sole carbon source. The study also includes morphological and physiological characteristics of the strain BPH08

applying Gram staining, salinity test, motility test, growth in different PAH (Polycyclic Aromatic Hydrocarbon) substrate test, catalase test and oxidase test. Besides that, amplification of 16S rRNA homolog sequence from the strain BPH08 will be used to search and analyze for closely related species of biphenyl degrading marine bacteria through BLAST search. The isolation and characterization of biphenyl degrading marine bacteria can provide rich information involve bioremediation of biphenyl in marine environment that will help in genetic engineering of the bacteria strain that can remediate range of contaminants which cannot be degraded by the pure bacteria strain. The information on the biphenyl degrading marine bacteria will also assist in designing and implement a successful bioremediation program that will be needed to manipulate ideal environmental parameters to allow microbial growth and degradation to proceed at faster rate especially microbes that are needed in *ex situ* bioremediation.

1.3 Objectives

The objectives of this study are:

- to isolate biphenyl degrading marine bacteria by observing its growing ability on agar plate containing biphenyl
- to study and analyze the physical and morphological characteristic of the bacteria strain BPH08
- to identify the genus and species of the bacteria strain BPH08 from BLAST program after sequencing of 16S rRNA

2.0 LITERATURE REVIEW

2.1 Biphenyl and Polychlorinated biphenyl

2.1.1 Biphenyl

Biphenyl (also called diphenyl), an aromatic hydrocarbon, is a white solid crystal compound at room temperature with a peculiar, strong odour similar to that of geraniums (BUA, 1990). Biphenyl is atypical in that the phenyl-phenyl linkage has a rotational degree of freedom not present in most polycyclic aromatic compounds (Dewey *et al.*, 2001). It has been used as an intermediate in the synthesis of many compounds such as emulsifier, optical brighteners and crop production product. It also has variety of specific capabilities that enable it to being used as a heat transfer medium in heating fluids, as a dyestuff carrier for textiles and copying paper, as a solvent in pharmaceutical production and especially in the preservation of citrus fruits to prevent damage from fungus during shipment and storage. Biphenyl has also been operated as a model compound to study bioavailability of soil sorbed chemicals and also used in polychlorinated biphenyls (PCBs) degradation studies (Wu *et al.*, 2003; Pieper, 2005). Biphenyl has low solubility in water and high solubility in organic solvent suggesting the use of two-phase partitioning bioreactor for microbial biphenyl degradation. In air, biphenyl is being degrade and convert to other chemicals and can attach to solid material in water. Microorganisms that survive in water and soil degrade biphenyl to other chemicals. The tendency of biphenyl to accumulate in the food chain is moderate. People will be affected by biphenyl when it get through the body when people breathe air which is contaminated with biphenyl or consume food or water contaminated with biphenyl. It can be penetrated into the body through skin contact. Biphenyl which inhaled over long period can cause liver and

nervous system damage. Biphenyl has high acute toxicity to aquatic life. The biphenyl industry has completed chronic aquatic toxicity studies in response to an EPA request for testing (Boehncke *et al.*, 1999).

2.1.2 Polychlorinated biphenyl (PCB)

Polychlorinated biphenyls (PCBs) form a mixture of congeners consists of two phenyl rings with different number and location of chlorine atoms in molecule, which determines their physicochemical properties, environmental behavior and toxicity (McFarland and Clarke, 1989). PCBs were widely used in industry because of their good chemical and physical properties including low vapor pressures, low water solubility, excellent dielectric properties, and stability to oxidation, flame resistance and relative inertness (Kimbrough and Jensen, 1989). The proliferation of such products in routine household and commercial use, without any regulation of waste procedures, introduced vast amounts of PCBs into the environment. Polychlorinated biphenyl accumulates in biological tissue and related with decreased wildlife population, especially bird (bioaccumulation). Fish consumption advisories have been great issued due to high concentration of PCBs in sport fishes. The effects of PCBs on wildlife have included increased reproductive failure in fish-eating birds and elevated mink kit mortality (Allan, 1991; Giesy *et al.*, 1997). Polychlorinated biphenyl is suspected of having adverse effects on the human reproductive, endocrine, neural and immune system. (Swanson *et al.*, 1995). Most environmental contamination by polychlorinated biphenyl is a complex highly chlorinated mixture for example Aroclors 1242, 1245 and 1260. PCB is generally concentrated on sediment and soil surfaces, both organic and inorganic due to their low aqueous solubility and low volatility. Incorporation of PCBs into phytoplankton produces inhibitory effects on photosynthesis and cell motility. In addition to direct toxic effects on algae, accumulation

PCBs is readily introduced into aquatic food chain (Rohrer *et al.*, 1982). Exploitation of biological destruction systems applying microorganisms and recently also plants is due to the high production cost and public disagreement to current physical remediation technologies (Macek *et al.*, 2002) to clean-up PCB-contaminated sites.

2.2 Biphenyl Degrading Bacteria

2.2.1 Types of bacteria

Biphenyl-utilizing bacteria that produce biphenyl-catabolic enzymes are capable of catabolizing PCBs into chlorobenzoic acid via oxidative route. Biphenyl-degrading bacteria, including the genus *Pseudomonas* and *Rhodococcus* sp. strain RHA1 (RHA1) can grow on biphenyl with their oxidizing ability of PCBs via a biphenyl catabolic pathway (Masai *et al.*, 1995). Studies on different biphenyl- and PCB-degrading bacteria, whether they are gram-negative or gram-positive strains have created the biochemical and genetic bases for PCB bioremediation (Abramowicz, 1990; Brenner *et al.*, 1994; Furukawa, 1994). Masai *et al.*, (1995) have emphasized and concentrate his research regarding biphenyl degradation on Gram-negative bacteria, in particular members of the genus *Pseudomonas*. According to Mohn *et al.*, (1997), they have identified psychrotolerant PCB degrading organisms while according to Maeda *et al.*, (1998), they have characterized alkalitrophic PCB degrading bacteria. Various species of aerobic bacteria have been discovered which oxidatively attack PCBs. They belong predominantly to the genera *Pseudomonas, Alcaligenes, Achromobacter, Arthrobacter, Moraxella* and *Rhodococcus* (Furukawa, 1982, 1994; Abramowicz, 1990; Bedard, 1990).

2.2.2 Properties and behaviors of bacteria

Most aerobic PCB degraders are obligate aerobes, motile, gram negative rods which are enriched by growth on biphenyl and can be selected for their capability to grow on biphenyl or PCB agar plate by clearing of biphenyl around colonies on agar. There are some properties of biphenyl utilizing bacteria that improve the bioavailability of PCBs. Ability of bacteria of cell attachment and surface hydrophobicity, motility and chemotaxis processes will makes it to be near the solid substrates, and this may helps biphenyl-utilizing bacteria looking for new substrates when they are depleted in a specific toxic component area (Chavez *et al.*, 2005). Recently, Raman confocal microscopy was applied to discriminate between cultures of B. xenovorans LB400 that were exposed to different pollutants (Singer *et al.*, 2005). This bioassay could be used as a modern tool to study bioavailability and toxicity in PCB contaminated environments. Wu *et al.*, (2003) reported that two motile biphenyl degrading bacteria (*Pseudomonas putida* P106 and *Rhodococcus erythropolis* NY05) showed significant positive chemotactic response toward biphenyl. The accumulation of inorganic polyphosphate (polyP) in bacteria is a finely regulated process that depends on phosphate and energy sources availability as well as in the presence of K and Mg ions (Nesmeyanova, 2000). polyP accumulate in reaction to nutrient deficiency has been reported in the PCB-degrading bacteria *Pseudomonas sp.B4* and *B.xenovorans* LB400 accumulated great amount of large electron-dense granules when grown in biphenyl in all stages of growth and in glucose only when the cell entered the stationary phase (Cha'vez *et al.*, 2004).

2.3 Degradation pathway of biphenyl degrading bacteria

2.3.1 Biphenyl upper pathway

Biphenyl 2, 3-dioxygenases comes from a large family of Rieske non-heme iron oxygenases (Gibson and Parales, 2000). They consist of a terminal oxygenase that is composed of a large α- and a small β-subunit, a ferredoxin and a ferredoxin reductase. The degradation of biphenyl to its reaction products occur through sequence of reaction catalyzed by specific enzymes which is called biphenyl upper pathway. The reaction is initiated by multicomponent enzyme biphenyl-2, 3-dioxygenase (BDO) which attacked biphenyl that leads to formation of biphenyl-2, 3-dihydrodiol of cis stereochemistry (Haddock *et al.*, 1993). Dehydrogenation catalyzed by biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (BDDH) enzyme will form 2,3-dihydroxybiphenyl, which then continue to go through meta-cleavage attack by 2,3-dihydroxybiphenyl-1,2-dioxygenase (DBDO) enzyme to produce 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA). This substance is later changed to benzoate and 2-hydroxy-penta-2, 4-dienoate by 2 –hydroxy-6-oxo-6-phenyl-hexa-2, 4-dienoate hydrolase (HOPDAH). A specific microorganism that has particularly worth for its ability to attack a broad spectrum of PCB is the gram negative strain *Pseudomonas* sp.LB400 (Bopp, 1986; Bedard *et al.*, 1986; Bedard, 1990). This bacteria attacks mono to hexachlorinated congeners and show a degraditive preference for ortho-CB. Meer *et al.*, (1992) postulated that a number of catabolic genes related in the degradation of aromatic compounds share a common ancestry and form gene superfamilies. Study by using *Pseudomonas sp. LB400* has provided various information about these two pathways of biphenyl 'upper' pathway and 'lower aliphatic' pathway occuring in this specific organism which both at the level of DNA as well as protein sequence. The enzymes require for the biphenyl 'upper' pathway in *Pseudomonas sp. LB400*

are BDO which are encoded by *bphAEFG* (Erickson and Mondello, 1992) in which they are also referred to as *bphA.* BDDH enzyme is encoded by *bphB,* DBDO enzyme is encoded by *bphC,* and HOPDAH enzyme, encoded by *bphD.*

2.3.2 Lower biphenyl catabolic pathway

On the other hand, the enzymes that involved in the 'lower aliphatic' pathway are 2-hydroxypenta-2,4-dienoate hydratase, acetaldehyde dehydrogenase (acylating) and 4-hydroxy-2- oxovalerate aldolase (Hofer *et al.*, 1994). The lower biphenyl catabolic pathway oxidizes 2-hydroxypenta-2, 4-dienoate and converted to pyruvate and acetyl-CoA (Seeger *et al.*, 1997). 2-hydroxypenta-2, 4-dienoate is transformed by 2-hydroxypenta-2,4-dienoate hydratase (BphH), an acylating acetaldehyde dehydrogenase (BphI) and 4-hydroxy-2-oxovalerate aldolase (BphJ) into acetyl-CoA that later enter Krebs cycle.

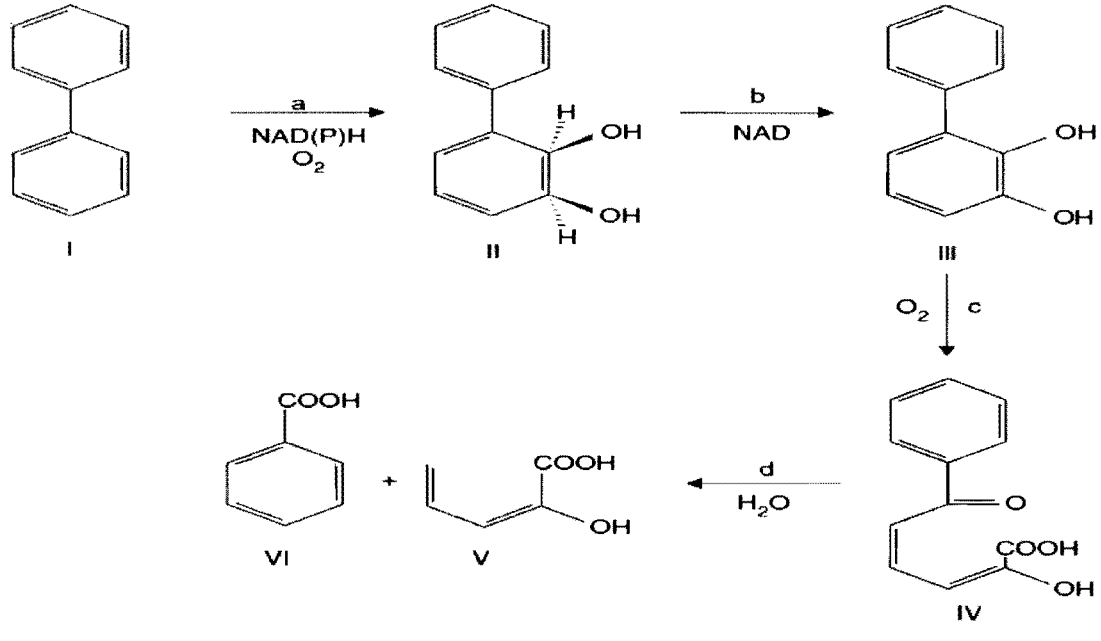

Figure 1. Catabolic pathway for the degradation of biphenyl. I, biphenyl; II, biphenyl-2,3-dihydrodiol; III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-phenylhexa-2,4-dienoic acid; V, 2-hydroxypenta-2,4-dienoate; VI, benzoate. (a) biphenyl dioxygenase (BphA); (b) biphenyl- 2,3-dihydrodiol dehydrogenase (BphB); (c) 2,3-dihydroxybiphenyl dioxygenase (Bph- C); (d) 2-hydroxy-6-phenylhexa-2,4-dienoic acid hydrolase (BphD); illustration modified from Haddock *et al.*, (1997).

2.4 Motility test for bacteria

S.I.M Medium is a semisolid agar used for identification and differentiation of members of the family Enterobacteriaceae by detecting indole formation, sulfide production, and motility. Green *et al.*, (1951) first described the application of S.I.M Medium that suggests that a reduced amount of medium will improve the incubation times for motility detection. In 1940, Sulkin and Willett demonstrated motility, hydrogen peroxide production and carbohydrate fermentation by members of Salmonella and Shigella groups. When the organisms are stabbed into the semisolid agar with a straight wire, the bacteria will migrate by flagella away from the stab line. As a result turbidity is produced throughout the medium. Triphenyltetrazolium chloride (TTC) is a soluble compound incorporated in the medium. When the substance is taken up by bacterial cell, the substance is reduced releasing acid formazan, a highly pigmented red, insoluble compound. SIM with TTC demonstrates motility by means of a diffuse pink color throughout the medium. Non motile organisms produce a straight pink line with no diffusion along the stab line.

2.5 16S ribosomal RNA analysis

Molecular method for evaluating phylogenetic relationship such as DNA-rRNA hybridizing and 16S rRNA oligonucleotide cataloging has its own advantages and limitation. But in general macromolecular sequences for evaluating phylogenetic tree is preferred because they permit quantitative inference of relationship. 16S rRNA is the major molecule of the small ribosomal subunit of prokaryotes. 16S rRNA, have proven and tested to be the most appropriate for creating distant relationships compare to all the macromolecules used for phylogenetic analysis due to their properties of high information content, conservative nature,

and universal distribution (Lane *et al.*, 1985). The comparison of rRNA sequences among organism can be powerful mechanism for analyzing phylogenetic and evolutionary relationships for bacteria, archaebacteria and eukaryotic organisms (Weisburg *et al.*, 1991). According to Weisburg *et al.*, (1991) a few PCR primers which can attach to 16S rRNA sequences can be operated for phylogenetic study between a few species of eubacteria. 16S rRNA different in their nucleotide sequence but contain region that are conserved perfectly, or nearly among all organism that are investigated so far. Certain of the 16S rRNA region that are conserved perfectly is in the mean time adjacent to less-conserved regions that are useful and beneficial for phylogenetic evaluations which provide broadly applicable initiation sites for primer elongation sequencing techniques. Synthesizing of oligodeoxynucleotides, 15-20 residues in length, which are complementary to be part the conserved sequences, are being tested as primers for dideoxynucleotide-terminated sequencing reactions with reverse transcriptase and 16S rRNA templates.16S rRNA has been used in discrimination of mRNA initiation sites, tRNA binding, and association of the two ribosomal subunits (Lane *et al.*, 1985). Phylogenetic microarray, or "phylochips" have been used to discriminate between diverse 16S rRNA genes exists within cultured or environmental microorganisms (Wilson *et al.*, 2002; Brodie *et al.*, 2006; Palmer *et al.*, 2006; Huyghe *et al.*, 2008). 16S rRNA sequences have been proven to become a widely used tool in bacterial systematic, bacterial typing and studies of the composition of microorganisms in environmental samples. Comparative sequence analysis of small subunits rRNA has been recognized as the gold standard for rebuilding phylogenetic relationships among prokaryotes for the aim of classification (Ludwig *et al.*, 2001).

2.6 Basic Local Alignment Search Tool (BLAST)

BLAST (Basic Local Alignment Search Tool) is a tool that is maintained by the National Center for Biotechnology Information (NCBI). BLAST is used to scan a nucleotide or amino acid sequence database for "hits." A BLAST hit contains one or more high-scoring segment pairs (HSPs). BLAST is operated to detect and analyzed the best local alignment, as well as other likely alignment between query sequence and the target sequence to identify previously characterized sequences and find phylogenetically related sequences such as the amino acid sequences of different proteins or the nucleotides of DNA sequences. The search initiated by aiming a small subset of letters from the query sequence.

The result of the search will be query in the sequence and any related sequence where conservation substitution had been introduced. Homologues sequence can be detected by searching series of ungapped sequence alignment and then assembling them to form a longer sequence alignment (Xu, 2004). Advantage of BLAST is an order of magnitude faster than existing sequence comparison tools of comparable sensitivity. The method will sense weak but biologically important sequence similarities, and is more than an order of magnitude which is faster than existing heuristic algorithms (Altschul *et al.*, 1990). The BLAST wrapper supports all five types of BLAST searches: BLASTn, BLASTp, BLASTx, tBLASTn, and tBLASTx.

3.0 MATERIALS AND METHODS

3.1 Sample collection and filtration

Sample of sea water that contains bacteria strain was collected at jetty of Muara Tebas of about 10 liter. Then it 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 into four sections before transferred into a falcon tube. Later it was washed with filtered sea 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 falcon tube and kept for next step.

3.2 Preparation of ONR7a suspension and agar

3.2.1 ONR7a suspension

ONR7a which was 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 flasks.