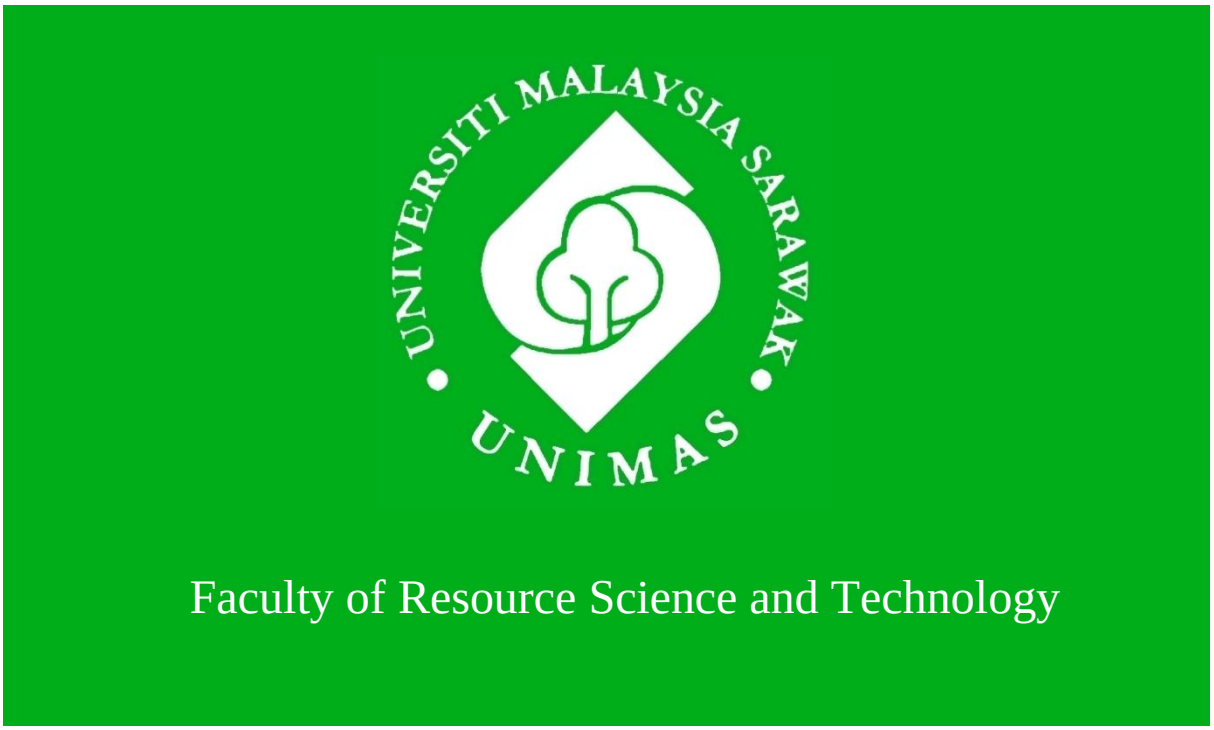

ISOLATION AND IDENTIFICATION OF DIBENZOFURAN DEGRADING MARINE BACTERIA FROM SEAWATER OF COAST OF SARAWAK

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Bachelor of Science with Honours
(Resource Biotechnology)
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Isolation and Identification of Dibenzofuran Degrading Marine Bacteria from Seawater of Coast of Sarawak

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DECLARATION

I hereby declare that this Final Year Project (FYP) entitled "Isolation and identification of dibenzofuran degrading marine bacteria from seawater of coast of Sarawak**"** is a bonafide and genuine research carried out by me under the guidance and supervision of Dr. Azham bin Zulkharnain, Department of Molecular Biology, Faculty of Resource Science and Technology (FRST), University Malaysia Sarawak (UNIMAS). I also declare that this Final Year Project has not been submitted in any form of another degree or diploma at any university. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given. FRST shall have the rights to preserve, use and disseminate this FYP report in print or electronic format for academic/research purpose.

Date:……. Signature:

……

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
rRNA	Ribosomal ribonucleic acid
Sec	Second
Min	Minute
g	Gram
μg	Microgram
cm	Centimeter
RPM	Revolutions per minutes
Taq polymerase	*Thermus aquaticus* DNA polymerase
TAE	Tris-EDTA buffer
%	Percent
^{o}C	Degree Celcius
μl	Microliter
V	Volts
NCBI	National Center for Biotechnology Information
ml	Mililiter
mM	MiliMolar
M	Molar or molarity (moles of solutes per liter solution)
bp	Base pair
kb	Kilo base pair
SDS	Sodium Dodecyl Sulfate
PCI	Phenol-Chloroform-Isoamyl alcohol
CIA	Chloroform-Isoamyl alcohol

UV	Ultraviolet
PCR	Polymerase Chain Reaction
-OH	Hydroxyl group
DBF	Dibenzofuran
DMSO	Dimethyl Sulphoxide
HOHPDA	2-hydroxy-6-oxy-6-(2-hydroxyphenyl)-hexa-2,4-dienoate
NaOH	Sodium Hydroxide
ONR7a	Artificial seawater mineral salts medium
PES	Polyethersulfone
THB	2,2',3-trihydroxybiphenyl
v/v	Volume per volume
w/v	Weight per volume
CTAB	Cetyltrimethyl Ammonium Bromide

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

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ABSTRACT

A dibenzofuran-utilizing marine bacterium, designated strain DBF01 was isolated from seawater in Miri, Sarawak. This strain grew aerobically with DBF as the principal sole carbon source and energy. In this study, Artificial seawater mineral salts medium (ONR7a) was used as the basal medium for isolating and culturing microorganisms. They produced a soluble opaque colored with ONR7a suspension culture and the isolate formed smooth, circular creamy-white, small-sized colonies on the surface of the ONR7a agar. Gram staining analysis showed that the isolate was a Gram-negative, short straight rod-shaped cell. Strain DBF01 required Na^+ for growth and exhibited optimal growth at 3% NaCl. They were capable of degrading various different recalcitrant aromatic compounds including biphenyl, carbazole dibenzothiophene and fluorene. Strain DBF01 was obligately marine bacterium, aerobic, motile, oxidase and catalase positive. DNA extraction was carried out by PCI method and 16S rRNA gene sequence was amplified by universal primers (PA/PH), purified and sequenced. The 16S rRNA-based phylogenetics analysis has revealed that strain DBF01 belonged to γ-subclass of *Proteobacteria.* The species closest to strain DBF01 was *Pseudomonas pachastrellae* (99% similarity in 16S rRNA gene sequence). PTG4-14 is designated as the type strain (EU603457.1)

Key words: ONR7a, bioremediation, 16S rRNA, *Pseudomonas pachastrellae,* dibenzofuran

ABSTRAK

Bakteria masin, ditetapkan sebagai strain DBF01 yang mempunyai keupayaan untuk mengurai dibenzofuran telah berjaya diasingkan dari air laut yang diambil dari Miri, Sarawak. Strain ini dapat tumbuh secara aerobik dengan DBF sebagai sumber tenaga dan karbon utama. Kajian menunjukkan bahawa media garam mineral air laut buatan (ONR7a) digunakan sebagai perantara basal untuk tujuan pengasingan dan pengkulturan bakteria. Bakteria ini telah menyebabkan kultur suspensi ONR7a bertukar warna kepada putih kabur dan bakteria ini membentuk koloni yang putih-krim, halus, bulat dan bersaiz kecil pada permukaan agar ONR7a. Analisa daripada pewarnaan Gram menunjukkan bahawa bakteria ini adalah Gram-negatif dan berbentuk rod. Strain DBF01 memerlukan ion Na^+ untuk membiak dan menunjukkan pertumbuhan optimum pada NaCl 3%. Strain ini mampu mengurai pelbagai sebatian aromatik yang berbeza termasuk bifenil, carbazole, dibenzothiophene dan florine. Ekstraksi DNA dilakukan dengan menggunakan kaedah PCI dan urutan gen 16s rRNA diamplifikasi dengan primer universal, (PA/PH), dipurifikasi dan dihantar untuk sequencing. Analisis filogenetik 16S rRNA menunjukkan bahawa strain DBF01 dirujuk kepada Proteobakteria kelas-γ. Spesis yang paling hampir dengan strain DBF01 adalah Pseudomonas pachastrellae (99% kemiripan dalam urutan gen 16S rRNA). PTG4-14 ditetapkan sebagai strain bakteria ini (EU603457.1)

Kata kunci: ONR7a, biodegradasi, 16S rRNA, Pseudomonas pachastrellae, dibenzofuran

1.0 INTRODUCTION

In the past decades, rapid expansion of the industries to fuel the growth of the global economy coupled with the increasing knowledge of chemical synthesis, inevitably resulted in the pollution of the natural environments. Environmental pollutants are compounds that are toxic to living organisms released into the ecosystem, usually as a consequence of human activities. Dibenzofurans are toxic heterocyclic organic substances, chemically hazardous and highly recalcitrant (Oh *et al.,* 1999; Pollitt, 1999) to biological degradation. This problem has gained major concerned by public since they are harmful to human health. This has led to intensifying efforts to find effective detoxification processes.

Much effort has been made in developing physico-chemical and biological methods for degradation of these compounds (Hong *et al.,* 1999; Kubota *et al.,* 2005). From ecological and economical point of view, bioremediation techniques are more effective and cost beneficial than other remedial action methods to be applied for relatively low contaminated area (Hiraishi, 2003; Kubota *et al.,* 2004). Decontamination using microorganisms promise an improved substitute for ineffective and costly physico-chemical remediation methods (Diaz, 2004; Paul *et al.,* 2005). This is due to physiochemical methods are caused irreversible damage and not feasible to remedy large areas of contaminated terrestrial as well as marine environments (Hiraishi, 2003).

Bioremediation is defined as the natural process by which microorganisms are stimulated to rapidly degrade hazardous organic contaminants by mean of their enzyme to environmentally safe levels in soils, subsurface materials, water, sludge, and residues. The microorganisms use the contaminants as a food source and convert the contaminants into biomass and harmless by-products of metabolism reducing the amount of hazardous compounds in the environment.

Bacteria has become versatile tools for biotechnology applications nowadays as their adaptability to almost any type of environments allowed them to be utilized in many situations especially in environmental management.

As cited by EPA, dibenzofuran is one of the compounds on the Hazardous Substance Lists (U.S. EPA, 1994). According to New Jersey Department of Health and Senior Services (1998), dibenzofuran is brownish sand-like powder, which derived from *Coal Tar* and mainly used in the production of insecticides and to make other chemicals.

In this study, seawater samples were collected from coastal ecosystem in Miri, Sarawak. Upon returning to the laboratory, the samples were then subjected to filtration process as liquid component was drained off while marine bacteria retained on the filter membrane. With reference to the view of Dyksterhouse *et al.,* (1995), an artificial seawater mineral salts medium (ONR7a), was used as basal medium for isolating and culturing marine bacteria. The media was prepared according to standard formulation and isolation process proceeded through enrichment method using dibenzofuran compound as sole carbon and energy sources. Isolated strains were then subjected to identification and molecular characterization in order to generate data pertaining particular bacterial strain.

This research aimed mainly to isolate bacteria that best degrade the dibenzofuran in marine environment. The project also intended to analyze physical, morphological characteristics and biochemical properties of strain DBF01. This strain was subjected to characterization by comparing against 16S rRNA sequences held in NCBI GenBank through BLAST algorithm. By understanding the characteristics of the suspected bacteria, this will be essential in manipulating them in order to improve the bioremediation of poorly water-soluble pollutants.

2.0 LITERATURE REVIEW

2.1 Dibenzofuran

Chemical pollutants such as dibenzofuran are a great danger to people as humans are unable to detect them by the naked eye or smell although they are prevalent in the marine environments. Animal testing of dibenzofuran and polychlorinated dibenzofurans (PCDFs) have been performed and the result has gained a public concern for several decades because of their strong toxicity (Schecter *et al.,* 1987; Safe, 1990). Due to relatively lipophilic and chemically stable, these hazardous compounds tend to accumulate in the body fat (Schecter *et al.,* 1987 and Safe, 1990).

Dibenzofuran (CAS Registration Number: 132-64-9) are mostly derived from the coal tar (NJ Department of Health and Senior Services, 1998). These compounds are accidentally formed as unwanted by-products during combustion of domestic and industrial waste (Czucwa and Hites, 1984 and 1986) as well as during the synthesis of herbicides or pesticides in which later released into the environment as recalcitrant contaminants. Dibenzofuran and its substituted analogues occurred as stress chemicals, so called phytoalexins in several woody plants (Gottstein and Gross, 1992).

They are also occurred in marine sediments and marine organisms (Koistinen *et al.,* 1997; Yamashita *et al.,* 2000). Many independent scientific studies affirm that DBF has a negative impact on human health and must be treated with seriousness.

Ecosystem contaminated with dibenzofuran may elicit serious health risk and consequences particularly to human (Resnick and Gibson, 1996) due to their carcinogenic and mutagenic

properties (Ouchiyama *et al.*, 1993; Jensen *et al.*, 2003). It can cause potential adverse effects such as eyes irritation, rashes and burns to skin, and mucous membranes (Cooper, 1997).

Dibenzofuran is a white or brownish and sand-like powder in physical appearance at room temperature (NJ Department of Health and Senior Services, 1998). Dibenzofuran is slightly soluble in water due to the large portion of aromatic hydrocarbon and yet soluble in alcohol, acetone, acetic acid and hot benzene. Due to poor solubility in water and hydrophobic nature, it is strongly adsorb to particulate material, and accumulate in marine sediments. Similar observation is reported by Dyksterhouse *et al.*, (1995) for polycyclic aromatic hydrocarbon (PAH).

Despite their refractory in nature, dibenzofuran compounds are degraded by microbial activity which occurring naturally in marine environments. Degradation of dibenzofuran has been studied most thoroughly and described (Fukuda *et al.*, 2002; Fortnagel *et al.*, 1989; Kubota *et al.*, 2004).

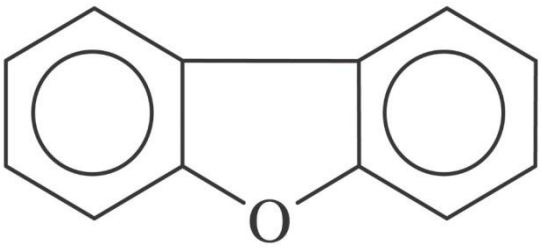

Figure 1. Molecular structure of dibenzofuran. Picture taken from http://upload.wikimedia.org/wikipedia/commons/thumb/9/93/Dibenzofuran-numbering-2D-skeletal.png/800px-Dibenzofuran-numbering-2D-skeletal.png

The above figure is a molecular structure of a heterocyclic organic substance known as dibenzofuran**.** It is composed of a fusion of 2 benzene rings to one ring in the middle called furan ring. Additionally, it is also an aromatic ether which having the chemical formula $C_{12}H_8O$.

2.2 Dibenzofuran degrading bacteria

The study of microbes has intrigue scientists since the 1600's as causative agents of diseases and emerging diseases. But we know today that microbes play important roles in the daily lives or can be used to accomplish beneficial tasks. Microbes assist human in many areas including food manufacturing (wine, bread, and cheese), medical industries (antibiotics) and bio-remediation.

Studies on biological methods using microorganisms capable of dioxin degradation in natural environments started to appear in 1970's (Kearny *et al.,* 1972; Matsumura and Benezet, 1973; Ward and Matsumura, 1978). Since then, an increase number in isolation and characterization of microorganisms capable of degrading dioxin and related aromatics have been done (Hiraishi, 2003). Remarkable and significant catalytic ability of certain species of marine bacteria to metabolized dibenzofuran has created an explosion of interest in the utilization of marine bacteria in DBF abatement.

Jannibacter terrae strain XJ-1 capable of growing on DBF and metabolize it as sole carbon and energy source (Jin *et al.,* 2005). Strain XJ-1 was isolated from East Lake in Wuhan, China and the strain grown aerobically on DBF. Within 5 days, the strain was almost completely degraded 100 mg/ml of DBF.

Several bacteria capable of degrading dibenzofuran and some of their respective halogenated analogues are summarized in **Table 1.**

Table 1. Lists of dibenzofuran-degrading bacteria capabale of utilizing DBF as sole carbon source

Dibenzofuran-degrading bacteria	Reference
Sphingomonas sp. strain RW1	Wilkes *et al.*,1996; Keim *et al.,* 1999 and Hong *et al.,* 2002
Sphingomonas sp. strain HL7	Fukuda *et al.,* 2002
Klebsiella sp.	
Rhodococcus opacus strain SAO101	Kimura and Urushigawa, 2001
Terrabacter sp. DBF63	Habe *et al.,* 2001
Pseudomonas sp. strain CA10	Sato *et al.,* 1997 and Nojiri *et al.,* 1999
Pseudomonas aeruginosa	Ishiguro *et al.,* 2000
Xanthomonas maltophilia	
Terrabacter sp. strain DPO360	Schmid *et al.,* 1997
Bulkholderia sp. strain JB1	Parsons *et al.,* 1998
Porphyrobacter sanguineus	Hiraishi *et al.,* 2002
Comamonas sp.	Wang *et al.,*2004
Pseudomonas sp., strain HH69	Fortnagel *et al.,* 1989 and 1990
Brevibacterium sp., strain DPO1361	Strubel *et al.,* 1989 and 1990

2.3 Dibenzofuran degradation pathway

Angular dioxygenation, lateral dioxygenation and lateral oxygenation are three pathways for initial steps in biodegradation of DBF (Xu *et al.,* 2006). By refering to **Appendix A**, the angular attack occurs at carbon atoms 4 and 4a of DBF. Molecular oxygen is incorporated into the angular position of DBF and hydroxylation occurs at carbon 4a which breaks open the ether bridge. The unstable phenolic hemiacetal products are formed and cleaved spontaneously to yield 2,3,20-trihydroxybiphenyl (THBP) by removing phenolate group (Fortnagel *et al.,* 1990 and Harms *et al.,* 1995). Subsequent *meta*-ring cleavage of THBP is catalyzed by extradiol dioxygenases to yield 2-hydroxy-6-(2-hydrophenyl)- 6-oxo-2,4-hexadienoic acid (HOHPDA), which is then hydrolyzed to 3-(chroman-4-on-2-yl)-pyruvate and salicylic acid.

Salicylic acid is then converted to catechol or gentisic acid. These compounds are further degraded and routed into the tricarboxylic acid (TCA) cycle.

This pathway is similar to the carbazole-degradation process carried out by *P. resinovorans* CA10 (Benedik *et al.,* 1998). The initial angular dioxygenase plays important role to determine substrate range in aromatic compounds biodegradation including dibenzo-*p*-dioxins and other dibenzofurans (Nojiri *et al.,* 1999).

By contrast, lateral dioxygenation, mainly performed by naphthalene- or biphenyl-utilizing bacteria, occurs at the carbon 1,2, the 2,3 and the 3,4 positions to produce DBF-dihydrodiols (Becher *et al.,* 2000 and Yamazoe *et al.,* 2004). Ring fission of 1,2- dihydroxydibenzofuran results in the production of 2- hydroxy-4-(30-oxo-30H-benzofuran-20-ylidene)but-2-enoic acid, which is degraded to salicylic acid. Salicylic acid is then metabolized to catechol or gentisic acid followed by incorporation into the TCA cycle, similar to the angular dioxygenation pathway.

The lateral dioxygenation pathway is also used in the co-metabolic degradation of DBF by biphenyl-utilizing bacteria. There are only a few reports of bacteria that transform DBF by both angular and lateral dioxygenation for example *Janibacter* sp., strain YY-1 (Stope *et al.,* 2002; Fuse *et al.,* 2003 and Yamazoe *et al.,* 2004).

2.4 Genes responsible for dibenzofuran degradation

Wittich (1998) has reported that the genes encoding for catalytic enzyme essential for DBF degradation in *Sphingomonas* sp. strain RW1 are not clustered but there are spread over the chromosome. Near to dioxygenase, the present of several genes in the form of cluster are found which code for an enzyme for degradation of hydroxylated DBF.

In previous research by Kasuga *et al*., (2002), they had successfully isolated a gram positive *Terrabacter* sp. strain DBF63 and reported based on their ability to utilize dibenzofuran via initial dioxygenation by a novel angular dioxygenase enzyme. In addition, 2 genes known as *dbfA1* and *dbfA2* genes respectively are responsible for DBF degradation by encoding the large and small subunits of the dibenzofuran 4,4a-dioxygenase.

Lida *et al.*, (2001) had cloned and characterized the novel genes which encode for extradiol dioxygenase enzyme involves in dibenzofuran degradation. *Rhodococcus* sp. strain YK2 was selected for the degradation analysis in their study. Five genes (*dfdB* and *bphC1* to C4) of YK2 were cloned and sequenced. Lida and his coworkers had analyzed the hybridization between the extradiol dioxygenase genes with genomic DNA of the actinomycetes and YK2 mutant strains. As a result, they demonstrated that the *dfdB* and *bphC4* were spread in several actinomycetes and *dfdB* product was thought to be involved in degradation process by YK2 strain.

In 2005, Lida *et al*., again had shown that two *Paenibacillus validus* JCM 9077 strains gained the ability to metabolize DBF into 2,2'3,-triohydroxybiphenyl and salicylic acid after acquiring *dbf* gene clusters. This result suggested that the isolated gene was responsible in DBF metabolism.

2.5 16S rRNA Analysis

The 16S rRNA gene is also designated as 16S rDNA, and the terms have been used interchangeably (Clarridge, 2004). According to Woese *et al.*, (1985 and 1987), determination of phylogenetic relationship of bacteria and all life-forms could be done by comparing a stable part of the genetic code, 16S rRNA gene, which is universal in bacteria

Some of the reasons to perform 16S rRNA analysis are to identify the causative agent of an infection in a clinical laboratory, confirming the identity of a microorganism in a research laboratory, and also accurate and precise method for environmental isolates identification which may impact the sterilization process of a medical product (Matzinger, 2004) or biodegradation process.

16S rRNA is a widely accepted method for genetic determination as genetic information in 16S rRNA is conserved throughout the microorganism's life cycle. The degree of conservation is assumed to result from the importance of the 16S rRNA as a critical component of cell function (Clarridge, 2004).

Currently, this method is the most reliable resource for bacterial identification since it is independent of stage of growth or even viability of the microorganisms (Matzinger, 2004). Hence, 16S rRNA gene was commonly used for taxonomic purposes for bacteria (Bottger, 1989; Palys *et al.*, 1997; Kolbert and Persing, 1999; Garrity and Holt, 2001; Tortoli, 2003; Harmsen and Karch, 2004).

"Percent difference" is the result of this comparison which identifies the specific base positions that are different (Matzinger, 2004). It is guaranteed to the species if the percent difference is less than 1% to the closest library database match while it is guaranteed to the genus level if the differences are greater than 1% and less than 10%. The 1500 base sequences would be compared to other library databases to determine if any closer match exists for percent difference of greater than 1%. Moreover, a part from species confirmation, results obtained from 16S rRNA analysis is also used to verify genetic consistency from one microorganism to another.

The 16S rRNA gene sequence is composed of both variable and conserved regions, roughly about 1500 bp in length. Interspecific polymorphisms of 16S rRNA gene are methodologically sufficient to provide distinguishing and statistically valid measurements as the gene is large enough (Clarridge, 2004).

Therefore, 16S rRNA gene sequences offering precise method of bacterial identification that is more robust, reproducible, and accurate than phenotypic testing. Also, non-culturable bacteria can be identified by 16S rRNA gene sequence analysis.

2.6 Basic Local Alignment Search Tool (BLAST)

BLAST is a tool used for the comparative analysis in order to detect the best local alignment together with possible closer match in between of query and the target sequence. Zaki *et al.* (2003) showed that the basic algorithm of BLAST is robust, simple, and can be applied in a wide context including for motif search, DNA and protein database search, gene identification search, the analysis of multiple region of similarity in long DNA sequences and for protein classification. In a simple term, it is a tool utilized to compare homologous sequence of sequence samples and sequences in GenBank for bacterial identification.

The idea is, in both directions, the alignment is extended to tally the cumulative score from a series of matches, mismatches and gaps then assembling them until a local alignment of maximal length is constructed, resulting in high-scoring segment pair (Buxevanis, 2005).

3.0 MATERIAL AND METHODS

3.1 Sampling

Seawater samples for marine bacteria were taken from Miri, Sarawak, at the upper surfaces and at depth of roughly 30 – 70 cm of by using pail or suitable containers. Seawater samples were then poured into 2 empty screw-capped gallons of 10 liters each pre-rinsed with seawater. All the samples were clearly labeled with sampling site and date as well as name of collector. Samples were not refrigerated during transportation. Seawater samples were stored in the cold room upon return to laboratory before undergone further processing.

3.2 Filtration

Initially, 20 liter of seawater samples taken from Miri division were pre-screened (50 μm) and subsequently filtered by using 0.22 μm pore sizes filter membrane (MILIPORE EXPRESS^{TM} PLUS, Milipore, USA) until the filters were completely clogged. 1000 ml of filtered seawater was kept and maintained in 1000 ml DURAN universal bottle for bacterial suspension preparation. After that, the filter papers were cut into sections as shown in **Figure 2** and stored at -20^{o}C until further processing.

3.3 Collection of Bacteria and Bacterial Suspension Preparation

At the completion of filtration, each of the filter membranes used during the filtration process was cut by using a sterile blade into 4 sections as shown below.

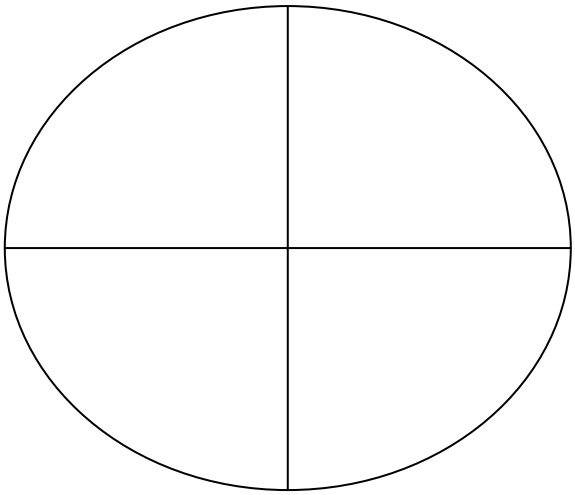

Figure 2. Overview of cut filtered membrane

Cut filter membranes were placed inside the sterilized falcon tube. Approximately 10 ml of 1000 ml filtered seawater was poured into falcon tube and capped. The cut filter membranes were then washed by vortexing the falcon tube several times using vortex machine. The bacterial suspension was poured into new falcon tube leaving the washed filter membranes. The suspension was left to stand for a period of time to allow denser particles (i.e sand particles) to settle at bottom.

3.4 ONR7a Suspension and agar preparation

Artificial seawater mineral salts medium (ONR7a) was used for culturing and isolating microorganisms (Bidwell and Spotte, 1985) in this study. ONR7a medium (per liter of distilled or deionized water) was prepared according to standard formulation. All the chemicals and reagents were aseptically weighed based on the determined weight (**Table 2**)

using digital balance and dissolved in 1000 ml of distilled water. The mixture was stirred by rotation of the magnetic stirrer until all the chemical compounds were dissolved on the hot plate. pH of solution was measured using pH meter (Thermo Scientific) and adjusted to 7.8 with addition of 1M NaOH. Then, it was sterilized by autoclaving.

Table 2 Composition of ONR7a

Compound	Weight
NaCl	22.79g
Na_2SO_4	3.98g
KCl	0.72g
NaBr	0.083g
$NaHCO_3$	0.031g
H_3BO_3	0.027g
NH_4Cl	0.27g
Na_2HPO_4	0.04715g
$MgCl_2.6H_2O$	11.18g
$CaCl_2$	1.102g
$SrCl_2.6H_2O$	0.0024g
$FeSO_4.7H_2O$	0.02g
Bacto agar	15.0g (add in for ONR7a agar preparation)

3.5 Bacterial enrichment media preparation

0.1% (w/v) of biphenyl was prepared (refer to **Section 3.6 Preparation of 0.1% biphenyl**). Four sterilized 500 ml DURAN conical flasks were prepared and each of the flasks were labeled with ONR7a (control), ONR7a (1), ONR7a (2) and filtered seawater. 3 of the conical flasks were filled with 200 ml of autoclaved ONR7a suspension. The remaining flask was filled with 200 ml of filtered seawater. 2 ml of prepared bacterial suspension was added into conical flasks labeled ONR7a (1), ONR7a (2) and filtered seawater. Carefully, 1 ml of 0.1% (w/v) previously prepared was pipette into each of conical flasks. All the conical flasks