

Isolation and Characterization of Hydrocarbon Tolerant Microorganisms from Marine Environment

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Abstract: Industrial activities have contributed to the releases of toxic organic compound into environment and have become a major public concern. This particularly of those industries that are located along coastal areas, which are the gateways for water transport. A study of the isolation and characterization of hydrocarbon tolerant microorganisms from marine samples collected at the jetty site of Tanjung Lumpur, Kuantan, Pahang, was conducted. There were very few studies have been done related to marine hydrocarbon tolerant microorganisms in Kuantan. Hence, this research was done to investigate the presence of microbial community that can thrive in the environment with oil-spillage. Enrichment culture technique by using MSM broth supplemented with 1% engine oil was utilized to isolate the desired microorganisms. Biochemical and molecular approaches were applied to identify and characterize the isolates. Six isolates were identified as genera *Vibrio*, *Halomonas*, *Pseudoaltromonas*, *Idiomarina*, *Staphylococcus* and Halophilic bacterium. In addition, phylogenetic study helps further in understand the relationship among the isolated bacteria.

Keywords: Halotolerant, Hydrocarbon tolerant, Bioremediation

1. Introduction

Microscopic organism or also known as microorganisms can be classified into bacteria, archaea, protists, microscopic plants, and animal such as plankton and planarian. Microbial communities play a significant role in the ecosystems, which they involve directly or indirectly in the nutrient cycling. Microorganisms are often at the end of the food chain where they act as the decomposers of dead living matters. They can exist either as unicellular or multicellular organisms and inhabit in various parts of the biosphere including soil, air, ocean floor, rocks, hot springs and even inside the body of other living organisms. With special adaptability, some microorganisms are able to survive extreme conditions such as high salinity water bodies, extreme temperature and pH, as well as polluted environment. Such microorganisms are referred as the extremophiles [1].

The occurrence of oil spills incidents highlight the need for cost effective and environmentally responsible ways to mitigate the issues. Enormous public concern has been raised by the incidents of oil spills in marine environment. A speedy and effective resolution of the problem is demanded. Some methods have been

introduced and practiced, such as the use of booms, skimmers and adsorbent, as the immediate response to remove the oil. However, these methods are usually costly and not very effective. The oil spills may still remain and contaminate the seawater. Therefore, there is a continuing search for alternative and additional methods.

Studies have shown that some microorganisms have the ability to thrive in the hydrocarbon contaminated environment. In fact, it is believed that all aerobic organisms can metabolize hydrocarbons [5]. It has been found that these microorganisms utilized hydrocarbon as their carbon/energy sources. Hydrocarbon-degrading bacteria or also known as the hydrocarbonoclastics were found to inhabit both marine and terrestrial environments. In marine environment, biodegradation of hydrocarbon is largely carried out by diverse bacterial populations, which are ubiquitously distributed in the oceans. The most commonly reported genera of hydrocarbon degrading microbes include *Alcanivorax* [16] and *Cycloclasticus* [7].

The ability of halophiles/halotolerants to oxidize hydrocarbons in the presence of salt is useful for the

biological treatment of saline ecosystems contaminated with petroleum products. Successful bioremediation of oil spills has been observed in marine, Arctic, and Antarctic environments [18]. In contrast to the assumption of an inverse relationship between oil biodegradation and high salinity, a new halo- and thermotolerant *Streptomyces albaxialis* was discovered with the ability to degrade crude oil and petroleum products even in a highly saline environment [14].

The technology of adopting halophiles in removing hydrocarbon has attracted many researchers to discover the valuable potential of these microorganisms in industrial application especially in bioremediation. Nevertheless, studies show that hydrocarbonoclastic activity by microorganisms is dependent on salinity of the environment where the degradation of hydrocarbon is usually impaired in a different range of salinity. This is believed to involve a unique cellular enzymatic machinery by halophiles that allows them to thrive in extreme saline environments. Due to the survival of microorganisms in hypersaline conditions requires specialized cellular and enzymatic adaptations, further understanding of hydrocarbon tolerant/degrading microorganism need to be explored. This work was conducted to study the diversity of halophiles in Kuantan seawater, and to test the hypothesis that some common marine bacteria would survive in hydrocarbon supplemented culture. Consequently, we have isolated, identified and characterized the indigenous microorganism from Tanjung Lumpur jetty, Kuantan, Pahang. A phylogenetic study was also conducted in order to examine the relationship of those isolates.

2. Methodology

2.1 Isolation of Microorganism

Water and sediment samples were collected on the 8th January 2017 at Tanjung Lumpur jetty, Kuantan, Pahang.

Sea water sample

The sea water collected was filtered by using a vacuum filter with 0.45 µm filter membrane. The filter membrane containing trapped particles was placed onto nutrient agar and MSM + 1% engine oil plate. Samples were incubated at 37 °C for 24 hours.

Sediment sample

One gram of the soil sediment sample was diluted into 100 ml of minimal salt broth with 1ml of sterilized engine oil. The mixture was incubated overnight at 37 °C on the shaker for 3 days. The broth was spread onto both nutrient agar and minimal salt agar + 1% engine oil. All of the samples were repeatedly sub-cultured until pure cultures obtained. The pure cultures were maintained at 4 C.

2.2 Morphological Observation

Morphology of the single colonies was observed by using the dissecting microscope (Stemi DV4, ZEISS). The characteristics observed were optical density, shape, color, margin, elevation and texture.

2.3 Biochemical Characterization

Gram staining procedure was carried out to further classify them based on their Gram reaction. Biochemical test (Mc Conkey agar test, Oxidase test, Catalase test) was conducted according to the manufacturer's instructions (OXOID).

2.4 Molecular Characterization

DNA Extraction

Genomic DNA extraction was performed by using GF-1 Bacterial Nucleic Acid Extraction Kit (Vivantis).

DNA Amplification

The Polymerase Chain Reaction (PCR) was employed for 16s rRNA gene amplification. A PCR mix was prepared as presented in Table 1. Primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (3'-TACGGYTACCTTGTTACGACTT-5') specifically amplifying 16s rRNA sequences were used. DNA amplification was performed in the Mastercycler (Eppendorf). The machine was set up according to the standard PCR profile (Table 1).

Table 1: PCR profile

Process	Temperature (C ^o)	Duration (min)	No. of cycles
Initial denaturation	94	4	
Denaturation	94	2	25
Annealing	55	1	
Extension	72	1	
Final extension	72	10	
Hold	4	-	

Purification and DNA Sequencing

The PCR products obtained were sent for sequencing (with purification) at 1st BASE Laboratories Sdn. Bhd. The sequences obtained were analyzed by using multiple bioinformatics tools for identification of the bacteria species.

2.5 Computational Analysis of DNA Sequences

The sequences obtained were analyzed for sequence similarity by using the Basic Local Alignment Search Tool (BLAST) which is available on NCBI sequence database (www.ncbi.nlm.nih.gov). The sequences of the samples were aligned and compared with existing sequences from the database. Three most similar sequences were retrieved for every sample. All sequences were aligned and trimmed so that the length is similar for phylogenetic tree construction. This was done by using MEGA 6 software. Phylogenetic trees which gather the evolutionary relationship between the selected sequences were then constructed (Neighbor-Joining, Maximum Parsimony, and Maximum Likelihood).

3. Results and Discussion

Eight isolates obtained could be grouped based on their morphological characteristics. Most of the isolates showed circular form. Only two isolates were different: A3 with irregular and B2-I(a) with punctiform colony forms. The colonies in sample A3, B1-II and C1 presented with undulate margin. However, the rest of the samples exhibited entire margin. The texture of the isolates was mostly smooth. Slimy texture of colonies was observed on A3 and C1. Meanwhile, B2-I(a) has slightly rough or uneven texture. Going further on the observation of the morphological characteristics of the bacteria, colors of the colonies were also analyzed. The isolates were mostly whitish and creamy in color. Yellow colonies were presented in B2-I(a) sample.

McConkey agar is a selective and differential culture medium for bacteria designed to selectively isolate Gram-negative and differentiate them based on lactose fermentation. According to this test positive results were obtained for sample A3, B1-I, B2-I(a) and C1 (Table 2). Therefore, it can be said that the isolates that grow on McConkey agar are of Gram-negative. Based on the observation of the colonies that had grown on McConkey agar, they could be further categorized into the lactose fermenter and non-lactose fermenter. The lactose fermenter was presented by the pink colonies while the non-lactose fermenter Gram-negative bacteria showed no difference of color from the original colonies. From the result obtained, B1-I, B2-I(a) and C1 presented pink colonies and A3 colonies were colorless.

Oxidase test was performed by using the oxidase strips impregnated with tetramethyl-p-phenylenediamine reagent. The purpose of oxidase test is to identify bacteria that produce cytochrome c oxidase, the terminal enzyme of respiratory transport chain. With the presence of this enzyme in the cell, the reagent will be oxidized to indophenols, indicated by the violet blue color end product. Thus, oxidase positive bacteria should give violet blue stain to the oxidase strip within 5 seconds after they were placed onto it. Based on the result, all isolates gave positive reaction except for B2-I(a) and c1 in which no change of color was observed. Some of the commonly known oxidase positive bacteria are *Pseudomonas sp.*, *Vibrio cholera*, *Neisseria sp.*, *Campylobacter sp.*,

Helicobacter sp., *Haemophilus sp.*, and *Aeromonas sp.*

Catalase is an enzyme produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites, hydrogen peroxides (H_2O_2). This enzyme catalyses the breakdown of H_2O_2 into oxygen and water. Catalase test can be used to distinguish among Gram-positive cocci from other genera. Common catalase positive is among members of the genus *Staphylococcus*, while catalase negative is represented by members of the genera *Streptococcus* and *Enterococcus*. Molecular characterization on the other hand has used universal primers to direct the synthesis of a 1.5 kb DNA fragment. The PCR product all of the isolates gave clear visible bands (1500kb). The PCR products were purified prior sending for sequencing.

All sequences obtained from sequencing were compared with sequences from ncbi BLAST database. Six genera were identified. They were *Vibrio*, *Halomonas*, *Pseudoalteromonas*, *Staphylococcus*, *Idiomarina*, and the halophilic bacterium. Lists of possible species that correlated with the samples were studied by the constructing three types of phylogenetic trees (Maximum Likelihood (ML) (Figure 1), Neighbour-joining (NJ) (Figure 2) and Maximum Parsimony (MP) (Figure 3)). Based on the phylogenetic trees, it can be deduced that *Pseudoalteromonas* is highly related to *Idiomarina* because they are joined together under the same closest root. On the other hand, *Halomonas* is distantly related to *Pseudoalteromonas*, *Idiomarina* and *Vibrio*. Overall, the three phylogenetic trees, NJ, MP and ML exhibited consistent pattern. Thus, this consistent clade formation revealed a promising relationship among the isolates sequences.

According to Oren, A. (2002) [22], Most abundant halophilic bacteria in many subgroups is belongs to Halomonadaceae that is ubiquitously found in lakes and coastal areas. In addition, most of the bacterial genera found in this study have been detected in marine environments and associated with hydrocarbon degradation, including *Halomonas*, *Idiomarina*, *Halobacterium* [8] and *Pseudoalteromonas* [4].

Table 2:Summary of morphological, biochemical and molecular characterization tests.

Isolates	Gram	Shape	Mc Conkey	Oxidase	Catalase	Related Species	Accession No.	Max. Identity (%)
A3	+	Coccus	+	+	+	<i>Vibrio parahaemolyticus</i> SMHW4.4 <i>Vibrio sp.</i> JF2-1 <i>Vibrio sp.</i> S1194	KJ620890.1	96
							KT369808.1 KM273120.1	9594
B1.1	+	Bacillus	+	+	+	<i>Halomonas sp.</i> VSS1 <i>Halomonas sp.</i> O-1 <i>Halomonas sp.</i> NTN129	KR296930.1 AB894359.1 AB167043.1	969695
B1.11	+	Coccus	-	+	-	<i>Pseudoalteromonas sp.</i> Strain 22-19 <i>Pseudoalteromonas sp.</i> Strain 8-13 <i>Pseudoalteromonas shioyasakiensis</i>	KX816432.1 KX806622.1 LC131142.1	989795
B1.111	+	Coccus	-	+	+	<i>Idiomarina sp.</i> SN-3-2 <i>Idiomarina sp.</i> H-76 <i>Idiomarina Fontislapidosi</i>	JX119039.1	96
							KF021759.1	96
							KT673806.1	94
B2.1.a	+	Coccus	+	-	+	<i>Staphylococcus warneri</i> Strain JCR13 <i>Staphylococcus warneri</i> Strain Na58 <i>Psychrobacter pulmonis</i> T-15	KU714597.1	98
							HQ831387.1	98
							HQ202844.1	98
B2.1.b	+	Coccus	-	+	+	<i>Idiomarina baltica</i> 0S145 clone <i>Idiomarina sp.</i> SN-3-2 <i>Idiomarina sp.</i> H-76	KP150441.1	95
							JX119039.1	94
							KF021759.1	94
B2.11	+	Coccus	-	+	-	Halophilic bacterium LC2 <i>Staphylococcus sp.</i> Y6 <i>Staphylococcus arlettae</i>	JX406388.1	97
							KC502905.1	97
							KR047785.1	97
C1	+	Coccus	+	-	+	<i>Aeromonas so.</i> Strain JH155 <i>Vibrio xiamenensis</i> Strain 0264 <i>Halomonas sp.</i> X27	KX708618.1	100
							KP236383.1	100
							KM974780.1	100

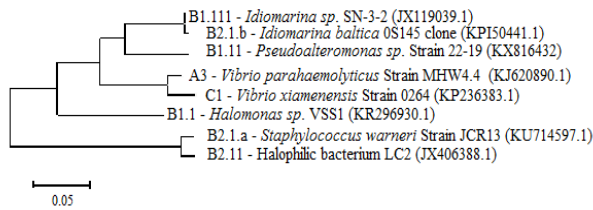


Figure 1: Maximum Likelihood

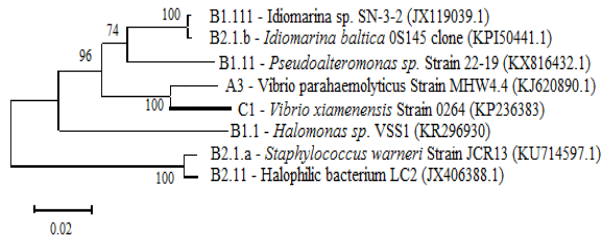


Figure 2: Neighbour-joining

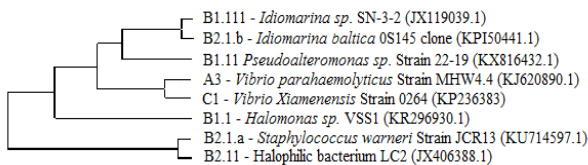


Figure 3: Maximum Parsimony

4. Summary

The isolated bacteria in this study were considered as presumptive hydrocarbon tolerant. They are capable of growing in the selective medium containing 1% engine oil. Six genera were identified and characterized as *Vibrio*, *Halomonas*, *Pseudoalteromonas*, *Idiomarina*, *Staphylococcus* and Halophilic bacterium.

Based on the phylogenetic tree, it can be deduced that *Pseudoalteromonas* is highly related to *Idiomarina*. On the other hand, *Halomonas* is distantly related to *Psuedomonas*, *Idiomarina* and *Vibrio*. Consequently, the study showed that most of the bacterial genera found in this study are common marine bacteria and are presumptive hydrocarbon degraders.

However, further study has to be done for advance understanding of the benefits and limitations of hydrocarbon tolerant microorganisms. Such knowledge may be highly useful in order to enhance the bioremediation process for the treatment of oil spillage in future.

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