

Isolation and Characterization of Surface and Subsurface Bacteria in Seawater of Mantanani Island, Kota Belud, Sabah by Direct and Enrichment Techniques

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Abstract. The distribution of hydrocarbon-utilizing bacterial may vary between surface and subsurface of the seawater. One of the identified contributors is the Total Petroleum Hydrocarbon. The isolation and characterization of bacteria using Direct and Enrichment techniques helps in identifying dominant bacterial populations in seawater of Mantanani Island, Kota Belud, Sabah, potential of further investigation as hydrocarbon degrader. Crude oil (5% v/v) was added as the carbon source for bacteria in Enrichment technique. For surface seawater, the highest population of bacteria identified for both Direct and Enrichment technique were 2.60×10^7 CFU/mL and 3.84×10^6 CFU/mL respectively. Meanwhile, for subsurface seawater, the highest population of bacteria identified for both Direct and Enrichment technique were 5.21×10^6 CFU/mL and 8.99×10^7 CFU/mL respectively. Dominant species in surface seawater were characterized as *Marinobacter hydrocarbonoclasticus*–RMSF–C1 and RMSF–C2 and *Alcanivorax borkumensis*–RMSF–C3, RMSF–C4 and RMSF–C5. As for subsurface seawater, dominant species were characterized as *Pseudomonas luteola*–SSBR–W1, *Burkholderia cepacia*–SSBR–C1, *Rhizobium radiobacter*–SSBR–C3 and *Leuconostoc-cremoisi*–SSBR–C4.

1. Introduction

Microorganisms' population is numerous in the open ocean. There are many different types of environmental-relevant microorganisms (ERM) mostly aerobes that are known to be able to degrade oil. The most important (based on frequency of isolation) genera of hydrocarbon utilisers in aquatic environment are of *Pseudomonas*, *Achromobacter*, *Micrococcus*, *Nocardia*, *Vibrio*, *Acinetobacter*, *Brevibacterium*, *Corynebacterium*, *Candida*, *Rhodutula* and *Sporobolomyces*. The most commonly isolated hydrocarbon-degraders are Gram Negative rods such as *Pseudomonas* and *Alcaligenes* [1]. Further studies continue to expand the list of microbial species that have shown capable of degrading various components of petroleum [2]. However, it is the isolates, single species or consortia that can degrade both aliphatic and aromatic hydrocarbons that are highly desirable.

Mantanani Island, Kota Belud, Sabah was chosen as the study site because of the potential to be a resort and one of the requirements to become a sea resort is free from any contamination and harmful-free marine species as Mantanani Island also known for dugong's populations. Therefore, the purpose of this study is to identify and determine the amount of oil and grease contamination and also the bacterial population in the surrounding sea of Mantanani Island. The aim of this study is to evaluate the physical (temperature and pH), chemical (Total Oil and Gas and Total Petroleum



Hydrocarbon) and biological aspects (bacterial populations) and examining the relationship between TOG, TPH and populations of bacteria found and latter identify the dominant bacterial populations of surface and subsurface waters of selected stations.

2. Materials and Method

2.1. Study Area

This study was done entirely at Mantanani Island, Kota Belud, Sabah with the coordinates (6°43'51.38" N, 116°21'13.56" E) and Environmental Microbiology Laboratory, Faculty of Science and Natural Resources, Universiti Malaysia Sabah. Seawater samples were collected at four sampling stations which were 1) Housing area of *Orang Ubian* (6°41.980" N, 116°21.251" E), 2) the end of the island next to the station 1 (6°42.298" N, 116°22.131" E), 3) rear area of the island and no settlement (6°43.206" N, 116°22.227" E), and 4) the Naval base area (6°42.533" N, 116°20.490" E).

2.2. Culture Medium

Culture media used in this study were Ramsay agar and broth. The preparation was according to Ramsay medium as described by Ramsay *et al.* (1983). The composition of Ramsay broth is of the same with Ramsay agar, only that 0.5gL⁻¹ MgSO₄.H₂O and 20.0gL⁻¹ glucose were added to the broth after being autoclaved at 121°C for 15 minutes. The glucose and MgSO₄.H₂O stocks were filter-sterilized under laminar floor before added to the culture media.

2.3. Sampling

The seawater samples were taken at depth of 10 cm and 25 – 60 cm from surface, for surface and subsurface seawater respectively. For oil and grease determination, seawater samples were taken using the blue cap bottle. Meanwhile, for the determination of enrichment technique, 2 mL of seawater samples was injected into the universal bottles. Samples were stored in a cool box containing ice cubes all the way to the laboratory for analysis. Preservation of the samples was done by adding sulphuric acid until the pH is less than 2 and cooled at 4°C. Acidification works to stop the biological activity in the sample and also converting the fatty acids from marine salt water into free acid which is not water soluble, increases the total amount of oil and grease.

2.4. Temperature and pH analysis

Temperature and pH of surface and subsurface waters at selected stations were tested during sampling using pH meter. Prior to use, pH meter should be calibrated to avoid errors.

2.5. Direct and Enrichment techniques

Direct technique is a technique in which the seawater samples are taken directly for bacterial isolation. Meanwhile, Enrichment technique is a technique in which the bacteria are supplied with fuel as a food source to increase the amount of bacteria present in the original sample. This technique need to be used as total bacterial populations in seawater are few, so through enrichment technique the number of bacteria were doubled and the isolation process is easily conducted. Seawater was injected into Ramsay broth using a syringe. Bacteria were allowed to grow in Ramsay broth and incubated for 24 hours at 30°C. After 24 hours, the bacterial isolation was performed on samples.

2.6. Isolation of bacteria

Isolation of bacteria was done for the purpose of breeding bacteria that were found in the samples. Two methods that have been used are the pour plate and serial dilution method. These methods are according to the Standard Method for the Examination of Water and Wastewater (1998). Serial dilution of the sample was performed at the series of 10¹, 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷, 10⁸ and 10⁹. However, bacterial analysis was only performed at the series of 10³, 10⁴ and 10⁵ as for series before 10³ and after 10⁶ would not give accurate results. Each selected series of dilution were duplicated. The

samples were then placed into the incubator for 48 hours at 30°C and 40°C. Bacterial colonies formed after 48 hours were calculated using Colony Forming Unit (CFU/mL).

2.7. Biochemical tests

Biochemical tests were carried out to identify key chemical compounds found in isolated colonies. The tests were also done to study the reaction of each bacterial colony towards the addition of chemicals. A total of four biochemical tests were carried out prior to characterization of bacteria which were oxidase, catalase, spores and motility.

2.8. Characterization of bacteria

Characterization of bacteria was done using Gram staining process for identifying populations of bacteria found in samples of surface and subsurface waters. Pure culture was used for Gram staining. The preparation of a pure culture was by getting a single culture for each colony formed and incubated for 24 hours at 30°C.

2.9. Gram staining

A loopful of the pure culture were transferred to the surface of a clean glass slide using wire loop and added few drops of distilled water, spread over a small area and heat fixed until dried. Then, the smear stained with crystal violet for 2 minutes, washed briefly with distilled water. Next, the slide was treated with iodine or lugol to fix the stain for 2 minutes, washed briefly with distilled water. After that, destained the slides with drops of alcohol (95%) and finally counterstained with safranin for 10 seconds, washed briefly with distilled water. All slides of bacteria were then examined under the oil immersion lens.

2.10. Analytical Profile Identification (API)

Identification of the species of bacteria present in the sample surface and subsurface waters was done by using the API test. API tools that were used in this study were API 20NE, API 50CHL, and API 50CHB / E. API test results were recorded after 24 hours and 48 hours using results table sheets provided with API box. The results data obtained then placed in the computer software APIweb as input data to identify the species of bacteria and the percentage accuracy of the bacterial species. Percentage of bacterial species identification accuracy APIweb was acceptable in between 88% to 99%.

2.11. Total Oil and Grease (TOG) and Total Petroleum Hydrocarbon (TPH)

Total oil and grease (TOG) were determined using gravimetric partition (APHA, 1998). Total petroleum hydrocarbon (TPH) was determined using the gravimetric method (silica gel treated n-hexane extractable materials @ SGT-HEM) similar to USEPA method 1664.

3. Results

3.1. Temperature and pH

As shown in Figure 1a and 1b, the temperatures of surface and subsurface of seawater are of similar ranges between 27.6°C to 29.8°C for Station 2, 3 and 4 where the lowest temperature of 27.8°C and 26.6°C was recorded at Station 1. Meanwhile, for pH value of surface and subsurface of seawater, Station 1, 3 and 4 also shows similar ranges from 8.01 to 8.31 where the lowest pH value of 7.23 and 7.74 was recorded at Station 2. Optimum temperature for marine water is from 10°C to 40°C in order for sustaining marine organisms in that particular environment. Based on the in-situ analysis of temperature in the study area, the obtained results are from 26.6°C to 29.8°C. This shows that the study area exhibits an optimum temperature.

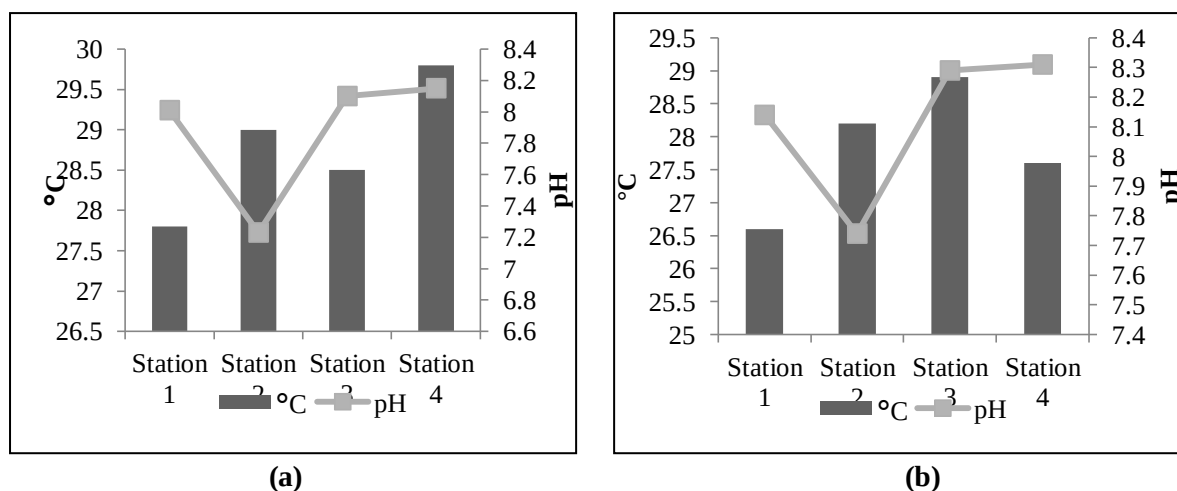


Figure 1 Temperature (°C) and pH of (a) Surface (b) Subsurface seawater in each sampling stations

Microbial growth rates in seawater are often associated with the surrounding temperature or optimum temperature. At temperature of about 20°C to 45°C, the microorganisms categorized as mesophilic. The optimum growth temperature is the temperature that is ideal for bacterial growth and rapid growth at this temperature [3]. The seawater temperature is also closely related to the concentration of petroleum hydrocarbons [4]. Evaporation rate of petroleum hydrocarbons from seawater into the air is influenced by water temperatures. In addition, water temperatures are correlated with the rate of degradation and microbial decomposition of petroleum hydrocarbons in water.

The range values of pH suitable for optimum marine conditions are from 7.0 to 8.5 meanwhile average range value of pH is form 7.9 to 8.3 [5]. Result shows that the range of pH values at study area are from 7.23 to 8.31, which is optimum and suitable for almost all aquatic organisms. A change of pH in the water column has an impact on aquatic life as aquatic organisms are very sensitive to changes in pH. Most microbes multiply rapidly in a neutral environment with a pH of 6.5 to 7.5, but based on the pH value recorded shows towards more alkaline environment. Thus, microbes in the environment can be categorized as Alkalophil. Alkalophil are of bacteria which tend to proliferate in the pH range of 8.5 to 9.0 [6].

3.2. Concentration of Total Oil and Grease (TOG) and Total Petroleum Hydrocarbon (TPH)

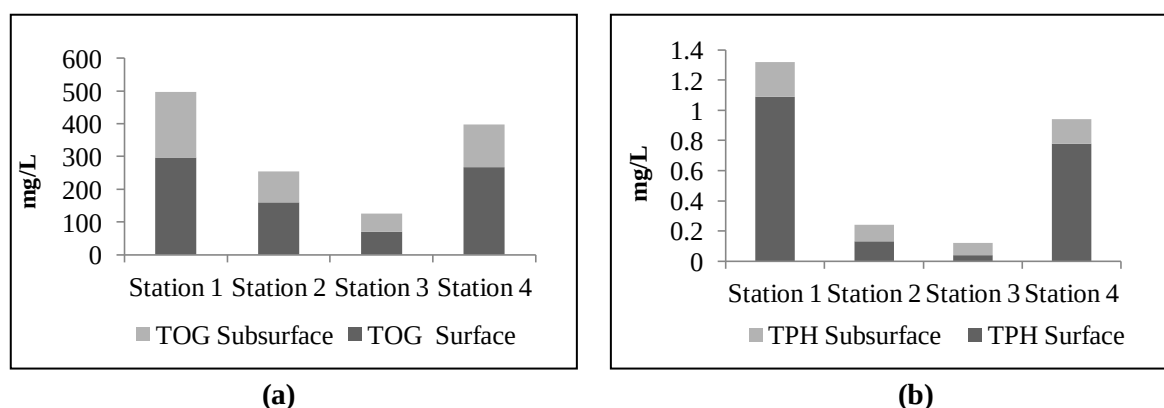


Figure 2 (a) TOG (b) TPH concentration of surface and sub surface seawater in each stations

Figure 2a shows TOG concentrations in surface and subsurface waters of selected stations. Highest reading recorded at Station 1 and the lowest is at Station 3, with a 4-fold difference. Meanwhile, highest TOG concentration for subsurface recorded at Station 1 and the lowest is at Station 3, similar to the surface seawater, only that the difference is a 3-fold.

Figure 2b shows the highest reading recorded at Station 1 and the lowest is at Station 3, with a 24-fold difference. Meanwhile, TPH concentrations for subsurface water range from 0.08 to 0.23 mg/L where the highest reading recorded at Station 1 and the lowest is at Station 3, similar to the surface seawater, only that the difference is almost 3-fold.

Based on the results, Station 1 recorded the highest concentration of TOG and TPH for both surface and subsurface seawater which may due to environmental factors that affect the concentrations of these compounds found in surface and subsurface waters. Through observation, Station 1 has a denser population distribution compared to the other three stations. Factors that may contribute to higher TOG and TPH concentrations are wastewater resulting from the daily activities of the villagers. Wastewater may contain a variety of contaminants such as food waste, cooking oil and many more. TOG also have poor solubility properties and tend to form a layer on the surface of the water [5]. TPH is one of the components in TOG. Higher concentration of TOG may contribute to the higher concentration of TPH. Among the major contributors to the high concentrations of TPH is from boating activity that uses gasoline as an energy source. Marine transportation is among the anthropogenic sources that introduced petroleum hydrocarbons into marine waters [7].

3.3. Bacterial population

Bacterial population for surface seawater using Direct technique has the range from 4.00×10^6 CFU/mL to 4.32×10^7 CFU/mL where the highest populations recorded at Station 4 with 4.32×10^7 CFU/mL and the lowest is at Station 3 with 4.00×10^6 CFU/mL, a 10-fold difference. Bacterial populations for Station 1 and Station 2 are 2.45×10^7 CFU/mL and 5.50×10^6 CFU/mL respectively. Meanwhile, bacterial population for subsurface seawater using Direct technique has the range from 4.00×10^5 CFU/mL to 1.57×10^7 CFU/mL where the highest reading recorded at Station 1 and the lowest is at Station 3, similar to the surface seawater, only that the difference is a 39-fold. Bacterial populations for Station 2 and Station 4 are 3.10×10^6 CFU/mL and 7.10×10^6 CFU/mL respectively.

Meanwhile, bacterial population for surface seawater using Enrichment technique has the range from 5.00×10^6 CFU/mL to 3.80×10^7 CFU/mL where the highest populations recorded at Station 1 with 3.80×10^7 CFU/mL and the lowest is at Station 3 with 0.05×10^8 CFU/mL, almost 8-fold difference. Bacterial populations for Station 2 and Station 4 are 9.00×10^6 CFU/mL and 2.00×10^7 CFU/mL respectively. As for bacterial population for subsurface seawater, the range is from 2.30×10^7 CFU/mL to 1.64×10^8 CFU/mL where Station recorded the highest population with 1.64×10^8 CFU/mL and Station 3 recorded the lowest population with only 2.30×10^7 CFU/mL, similar the surface seawater, only that the difference is a 7-fold. Bacterial populations for Station 2 and Station 4 are 3.20×10^7 CFU/mL and 1.50×10^8 CFU/mL respectively.

Based on the results, the bacterial population for surface and subsurface seawater has been identified and calculated using both Direct and Enrichment techniques. Bacterial populations were recorded high in surface waters using the Direct method, and using the Enrichment method showed high bacterial population in the subsurface.

3.4. Relationship of Total Petroleum Hydrocarbon (TPH) and Bacterial Population in Surface and Subsurface seawater

Bacterial population was recorded highest at Station 1, where TPH concentrations are also of the highest. As concentration of TPH decreases in Station 2 and 3, the bacterial population also declined and increased again at Station 4 when TPH concentrations increased. This suggests that the

concentration of TPH and bacterial populations through Direct technique and Enrichment technique are directly proportional.

High bacterial population recorded at Station 1 shows the ability of the bacteria to tolerate the high concentrations of TPH. The presence of bacteria in the water column is important because microbes are the major organisms identified in the water column that are able to help in the decomposition of waste including hydrocarbons and other metabolites.

Typically, marine environment contaminated with petroleum hydrocarbons have an extensive bacterial population, which of population capable to degrade petroleum as compared to that population from uncontaminated areas. Bacteria that present on the surface and subsurface layer are highly resistant to UV radiation and chemicals. Resistance to pollution and the ability to degrade indicates that the bacteria isolated from surface and subsurface seawater are important in the process of bioremediation.

3.5. Isolation and characterization of surface and subsurface bacteria

3.5.1. Isolation of surface and subsurface bacteria. Morphological, physiological and biochemical identification of the isolates from surface and subsurface seawater samples were investigated. A selected number of the investigated sequences are shown in Table 1 and 2. A total of eight dominant bacterial colonies were identified (Table 1). Determination purpose of the dominant bacteria is to determine the ability of the bacteria to tolerate with high concentration of TPH. If the bacteria can survive and grow in a particular concentration of TPH, thus it can also decompose or degrade hydrocarbons in that particular area. Based on Table 1, there are two types of configurations for the selected colonies which are round and spread whilst the sides are wavy and smooth. All selected strains had similar colony colour of cream and white. All strains exhibits similar cell morphology which is rod-shaped. Through Gram staining, strains RMSF-C1 until RMSF-C5 identified as Gram-negative while RMSF-C6 until RMSF-C8 are Gram positive.

Biochemical tests performed on each strain to determine the APIs test that will be used for each selected bacterial strains. Almost the entire bacterial strains showed negative results for Spores Tests, where most of the bacterial strains do not produce spores. Instead, endospores are being produced by bacterial cells to survive. Endospore is a hard layer that enables the strain to be resistant to radiation and chemicals [4]. Catalase test showed that the eight strains showed positive results. Catalase is an enzyme containing iron. Meanwhile, Oxidase test showed that only strains RMSF-C6 and RMSF-C8 showed positive results. This indicates that all other strains are not of anaerobic bacteria. Most marine bacteria are facultative anaerobe which breeds well in the presence of oxygen.

Table 1. Morphological, physiological and biochemical identification of the isolated bacteria from surface seawater

Items	Isolates							
	RMSF-C1	RMSF-C2	RMSF-C3	RMSF-C4	RMSF-C5	RMSF-C6	RMSF-C7	RMSF-C8
Colony color	Cream	Cream	White	Cream	White	Cream	Cream	White
Colony surface	Smooth	Wavy	Smooth	Smooth	Smooth	Wavy	Smooth	Wavy
Shape of cells	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram staining	-	-	-	-	-	+	+	+
Endospores	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	-	+	-
Motility	+	+	-	-	-	-	-	-
Positive (+)		Negative (-)						

Table 2. Morphological, physiological and biochemical identification of the isolated bacteria from subsurface seawater

Items	Isolates					
	SSBR-C1	SSBR-C2	SSBR-C3	SSBR-C4	SSBR-C5	SSBR-W1
Colony color	Cream	Cream	Cream	Cream	Cream	White
Colony surface	Smooth	Wavy	Wavy	Irregular	Smooth	Smooth
Shape of cells	Rod	Rod	Rod	Rod	Rod	Rod
Gram staining	-	+	-	-	+	-
Endospores	-	-	-	-	-	+
Catalase	+	+	+	+	+	-
Oxidase	+	+	+	+	+	-
Motility	-	+	+	+	+	+
Positive (+)	Negative (-)					

However, the growth rate of marine bacteria is very slow compared to most soil bacteria [8]. Motility test only showed positive results for strain RMSF-C1 and RMSF-C2. Motility test using SIM aims to determine whether the bacteria are moving on its own or elsewhere during the growth occurs [9].

A total of six dominant bacterial colonies were identified (Table 2) from subsurface seawater. Based on Table 2, there are three types of configurations for the selected colonies which are round, spread and L-shaped, whilst the sides are smooth, wavy and irregular. All selected strains had similar colony colour of cream and white. All strains exhibits similar cell morphology which is rod-shaped. Through Gram staining, strains SSBR-CR1, SSBR-CR3, SSBR-CR4 and SSBR-W1 identified as Gram-negative while SSBR-CR2 and SSBR-CR5 are Gram positive.

Almost the entire bacterial strains showed negative results for Spores Tests, except for SSBR-W1 which show positive result by producing spore. Catalase test showed that the 5 strains showed positive results, only SSBR-W1 that show negative result which indicates that SSBR-W1 does not equipped with catalase, an enzyme containing iron. Meanwhile, oxidase test showed that only strains SSBR-W1 showed negative results. This indicates that all other strains are of anaerobic bacteria due to it exists in subsurface column of seawater. Motility test only showed negative results for strain SSBR-C1.

3.5.2. Characterization of surface and subsurface bacteria. API tests (API 20NE and API 50 CHL) are the test that showing results for characterization of surface and subsurface bacteria. API 20NE are used to identify the species of non-enteric bacteria of a Gram-positive with a rod-shaped. The API 50CHL are used to identify Gram positive bacteria with a rod-shaped and does not produce spores.

Table 3 shows the results of API test and percentage of identification of each bacterial species for surface seawater. Based on the API tests, there are three species of bacteria that have been identified in surface waters which are *Bacillus coahuilensis* sp, *Marinobacter hydrocarbonoclasticus* sp and *Alcanivorax borkumensis* sp. According to API Web, the percentage of identification of species for *Bacillus coahuilensis* sp for strain coded RMSF-C5, RMSF-C6 and RMSF-C8 are weak, while *Marinobacter hydrocarbonoclasticus* sp for strain coded RMSF-C1 and RMSF-C2 and *Alcanivorax borkumensis* sp for strain coded RMSF-C3, RMSF-C4 and RRMSF-C5 are good.

The percentage identification accuracy plays a very important role in determining the selection of bacterial strains. This is because only a percentage of accuracy approaching 100% will be accepted as a species of bacteria of the strain.

Table 3. Surface bacterial species identification using API and percentage of identification accuracy

Colony code	API	Identified species	Percentage of Identification (%)	Comment
RMSF-C1	20 NE	<i>Marinobacter hydrocarbonoclasticus sp</i>	96	Good
RMSF-C2	20 NE	<i>Marinobacter hydrocarbonoclasticus sp</i>	90	Good
RMSF-C3	20 NE	<i>Alcanivorax borkumensis sp</i>	90	Good
RMSF-C4	20 NE	<i>Alcanivorax borkumensis sp</i>	91	Good
RMSF-C5	20 NE	<i>Alcanivorax borkumensis sp</i>	89	Good
RMSF-C6	50 CHL	<i>Bacillus coahuilensis sp</i>	60	Weak
RMSF-C7	50 CHL	<i>Bacillus coahuilensis sp</i>	63	Weak
RMSF-C8	50 CHL	<i>Bacillus coahuilensis sp</i>	50	Weak

Meanwhile, Table 4 shows the results of API test and percentage of identification of each bacterial species for subsurface seawater. Based on the API tests, there are five species of bacteria that have been identified in subsurface waters which are *Burkholderia cepacia sp*, *Rhizobium radiobacter sp*, *Leuconostoc mesenteroides ssp cremois*, *Rhizobium radiobacter sp* and *Pseudomonas iuteola sp*.

According to API Web, the percentage of identification of all five species for *Burkholderia cepacia sp* for strain coded SSBR-C1, *Rhizobium radiobacter sp* for strain coded SSBR-C3, *Leuconostoc mesenteroides ssp cremois* for strain coded SSBR-C4, *Rhizobium radiobacter sp* for strain coded SSBR-C5 and *Pseudomonas iuteola sp* for strain coded SSBR-W1 are good.

Table 4. Subsurface bacterial species identification using API and percentage of identification accuracy

Colony code	API	Identified species	Percentage of Identification (%)	Comment
SSBR-C1	20 NE	<i>Burkholderia cepacia sp</i>	99	Good
SSBR -C3	20 NE	<i>Rhizobium radiobacter sp</i>	99	Good
SSBR -C4	50 CHL	<i>Leuconostoc mesenteroides ssp cremois</i>	78	Good
SSBR -C5	20 NE	<i>Rhizobium radiobacter sp</i>	97	Good
SSBR -W1	20 NE	<i>Pseudomonas iuteola sp</i>	99	Good

4. Conclusion

In this paper, it is examined that the highest bacterial population were obtained for surface and subsurface seawater using Direct technique and Enrichment technique. This suggests that the concentration of TPH and bacterial populations through direct technique and Enrichment technique are directly proportional. Morphological, physiological and biochemical identification of the dominant isolates from surface and subsurface seawater samples were investigated and results shows that the total of eight strains from surface seawater are showing almost similar morphological, physiological and biochemical characteristics with the total of six strains from surface seawater, although some are differ with another one. However, from API tests, the strains species for surface are of *Bacillus coahuilensis sp*, *Marinobacter hydrocarbonoclasticus sp* and *Alcanivorax borkumensis sp* while the strains species for subsurface are of *Burkholderia cepacia sp*, *Rhizobium radiobacter sp*, *Leuconostoc mesenteroides ssp cremois*, *Rhizobium radiobacter sp* and *Pseudomonas iuteola sp*. This shows that the differences in depth of water column for surface and subsurface do plays role in determining the species of bacteria identified. Ultimately, all of the results demonstrated by both surface and subsurface bacteria selected in our study may be applicable for further investigation on potential of

biodegrading petroleum hydrocarbon of certain concentrations. Microbial degradation is one of the cost-effective treatments for hydrocarbon polluted environment. Nonetheless, periodic monitoring of TOG, TPH and bacterial population at Mantanani Island, Kota Belud is crucial to ensure the marine environment well maintained.

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