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Phylogenetic Study of Presumptive Oil-degrading Microbes Isolated from The North-western Tip of Pahang

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Abstract: Many construction areas are often contaminated with petroleum compounds. The aim of this work were to isolate and characterize indigenous bacteria isolated at a moderate temperature site as well as to study the pattern of phylogenetic tree among bacterial communities associated with oil degradation. No profound studies have yet been done in the construction site at Tanah Rata. Hence, this research was carried out to find existing status of microbial community from a few selected spots. Enrichment culture technique by using MSM broth has been used to isolate the desired microorganisms. Isolation and characterization tests using phenotypic and genotypic approaches (based on genes encoding 16S rRNA) had led to the discovery of 18 isolates. The 16S rRNA was used due to its functional constant, universally distributed and moderately well discovered across broad phylogenetic distances. The successfully identified genera were *Pseudomonas, Bacillus, Exiguobacterium, Stenotrophomonas, Acinetobacter, Serratia* and *Gamma Proteobacterium.*

Keywords: Oil-degrading microbes, 16S rRNA, phynotypic and phylogenetic.

1. Introduction

Microorganisms are critical for nutrient recycling in ecosystems as they act as decomposers. Nowadays, microbes are also exploited in biotechnology, as a bioremediation tool. Bioremediation refers to the productive use of degradative processes to remove or detoxify pollutants that have found their way into the environment and threaten public health [19]. Thus, there is a need to identify the types of microbes which can specifically degrade oil in order to minimize oil-pollution.

Many microorganisms present in the natural surroundings are beneficial to environment. According to Sanchez and co-workers (2006) [12], characterization of oil-utilizing microorganisms has become crucial to our understanding of the biological processes responsible for oil biodegradation in natural environment. Prior to the screening step, the isolation of colonies should be done.

Microbial identification is done to identify species of the microbes. Accurate identification of bacterial isolates poses a greater importance in environmental microbiology laboratories. Identification of bacteria is based on their phenotypic and/or genotypic features. Phenotypic feature involved conventional methods such as morphological characterization under microscope and gram staining. Different types of microbes possess different types of morphology and physiology.

The 16S rRNA gene is used for phylogenetic studies as it is highly conserved between bacteria and archaea species. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxanomy has been by far the most common housekeeping genetic marker used for numerous reasons. Universal PCR primers are used to amplify the 16s rRNA gene providing the phylogenetic information. The development of the techniques for analysis of 16S rRNA gene sequence in natural sample has gently enhanced our ability to detect and identify bacteria in nature [6].

The recent application of molecular phylogeny to environmental sample has resulted in the discovery of abundance unique and previously unrecognized microorganisms. However, there are many differences in number between isolated and naturally occurring microorganisms present in various habitat [11]. The vast majority of this microbial diversity has proven obstinate to cultivation [20].

In biology, phylogenetic is the study of evolutionary relatedness among various groups of organisms (for example, species or populations), which is discovered

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through molecular sequencing data and morphological data matrices. The term phylogenetics is of Greek origin from the terms phyle/phylon, means "tribe, race" and genetikos means "relative to birth" from genesis [21]. Taxanomy, the classification, identification and naming of organisms, has been richly informed by phylogenetic but remains methodologically and logically distinct. Phylogenetic analyses have become essential in researching the evolutionary tree of life. Evolution is regarded as a branching process, whereby populations are altered over time and may speciate into separate branches, hybridize together, to terminate by extinction. This may be visualized in a phylogenetic tree.

A phylogenetic tree of evolutionary relationships among various biological species or other entities is based upon similarities and differences in their physical and/or genetic characteristics. The taxa joined together in the tree are implied to have descended from a common ancestor. In a rooted phylogenetic tree, each node with descendants represents the inferred most recent common ancestor of the descendants and the edge lengths in some trees may be interpreted as tie estimates. Each node is called a taxonomic unit. Internal nodes are generally called hypothetical taxanomic units (HTUs) as they cannot be directly observed. Trees are useful in fields of biology such as systematic and comparative phylogenetics [15]. Cladistics is the current method of choice to infer phylogenetic trees. The most commonly used methods to infer phylogenies that include parsimony, maximum likelihood.

Hence, this study was conducted to identify the evolutionary relationships among indigenous microorganisms, specifically at Tanah Rata that are presumably capable of degrading oil. The microbial community at Tanah Rata is expected to be changed from time to time due to substantial human activities. Hence, this study might be useful to further examine the degradation ability of existing indigenous isolates which of moderate temperature environment (20-24 ^{o}C).

2. Methodology

2.1 Sampling

Samples were collected on September 2010 at construction site, Tanah Rata, Camreron Highland, Pahang. The sampling details are as follows:

- Location : Tanah Rata Cameron Highland
- Temperature : 24.4 ℃
- Types of samples: -Soil sediment

The samples were collected using scoop and were kept in ice. The samples were stored at 4 ^{o}C prior processing.

2.2 Isolation of Microorganism

Sediment sample

The bacteria strains were isolated by using enrichment culture technique from soil sediment collected from Tanah Rata, Cameron Highland.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 for four days at five different temperatures14℃, 18℃, 22 ℃, 24 ℃ and 37 ℃. After 5 cycles (4 days per cycle) of enrichment, a serial dilution was carried out and plated on nutrient agar. After an overnight of incubation at respective temperatures, bacteria colonies formed on the agar surface were selected and subsequently subcultured onto MSM agar with 1% (v/v) crude oil. Growth observed on the MSM plate were then morphologically examined.

2.3 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.

2.4 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).

2.5 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. Primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (3'-TACGGYTACCTTGTTACGACTT-5') specifically amplifying 16s rRNA sequences were used. DNA amplification was performed using aMastercycler (Eppendorf). The machine was set up according to the standard PCR profile.

Purification and DNA Sequencing

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

2.6 Computational Analysis of DNA Sequences

The sequences similarity was done 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. The 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 achieved by using Clustal X version 1.81 software. The evolutionary relationship was determined by comparing the DNA sequences obtained from the sequencing step with the BLAST results obtained from NCBI. These relationships were constructed using two approaches: (Neighbor-Joining and Maximum Parsimony (MP) and Maximum Parsimony applied in Molecular Evolutionary Genetics Analysis (MEGA5) software. Bootstrap replicates were performed to estimate the reliabilities of the nodes in the phylogenetic trees. Trees were drawn with TREEVIEW 3.2 software.

3. Results and Discussion

Media used in this experiment was MSM which contained minimal nutrients needed by the microbes. Besides, sterile engine oil was mixed in the broth as the sole C source. This will also lead to the preselection process since only microbes that tolerate to engine oil can survive in the selective culture condition. An enrichment culture technique was applied in order to maximize the amount of dominant microbes that can survive in the culture.

Seven genera were identified. They were *Pseudomonas, Bacillus, Exiguobacterium, Stenotrophomonas, Acinetobacter, Serratia* and *Gamma Proteobacterium*. Based on the finding, the microbial population in that area was more diverse than expected.

Most of the 16S rRNAgene clones differed from the nearest phylogenetic neighbor by 2 to 16 % at the rRNA gene level. A few but a significant number were very closely related (99 %) to the reported valid species (Shivaji, et. Al., 2004).

FASTA sequences for the 18 samples obtained from DNA sequencing were used to identify the species through BLAST. All sequences retrieved were having Expected (E) value 0.0, which means the matches were significant. The samples were then identified up to their species as per summarized in Table 1.

The choice of method for tree reconstruction depending on the size and the quality of the dataset. In this project, three methods of constructing phylogenetic trees were applied which were-Neighbour-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML). The three phylogenetic trees were constructed to examine the consistency of the species obtained at the sampling sites. Although the data analyzed here gave rather similar phylogenetic trees, it can be concluded that there was a constant relationships pattern among isolates.

Table 1: Possible species of the samples obtained from NCBI database.

Samples	Possible species	Accession No.	Max Identity (%)
A1	Pseudomonas sp. S27	EU747694.1	99
A2	Bacillus cereus strain Aj0803191A	HQ727973.1	98
A3I	Stenotrophomonas sp. 412(2010)	GU814023.1	98
A3II	Stenotrophomonasmaltophilia	EU244771.1	96
A4	Psedomonas putida strain BJ10	HQ848377.1	97
B1	Pseudomonas sp. G60	FN557187.1	90
B2	Exiguobacterium sp. D25(2010)	GU566358.1	98
B4	Acinetobacter baumannii strain Ab8	AY847284.1	100
C1	Pseudomonas sp. CMR12a	FJ652622.1	96
C2	Serratia sp. endosymbiont of Nilaparvatalugens clone M149	GU124496.1	99
C3	Stenotrophomonas sp. Pm3	GU391493.1	98
D1	Pseudomonas aeruginosa clone AZ130	GU414568.1	99
D2	Pseudomonas aeruginosa starin E70	HQ407247.1	97
D3	Acinetobacter sp. WJ07	HM045831.1	99
E1	Pseudomonas taiwanensis strain CAIM 837	HM584012.1	96
E2	Gamma proteobacteriumectosymbiont of Symmetromphalusaff. hageni clone C5	GU253374.1	73
E3	Acinetobacter johnsonii strain GRA732	HM209770.1	84
E4	Serratiamarcescens strain JNB5-1	HQ260324.1	99

Referring to Figure 1., neighbor-joining result shows that isolates A3i, A3ii and C3 were rooted similarly with 100% relationship between samples. In addition, neighbor-joining test showed that isolates B4 and E3 were 99 % similar but not for D3 even though from similar genus (*Acinetobacter*). *Pseudomonas* genus is highly related to each other since they are clustered under same root except for sample D2. D2 sample was far away from other *pseudomonas* genus. For *Serratia*genus, relationship between E4 and C2 can be clearly seen, but C2 sample is more likely to be related towards D3 samples, which belongs to *Acinetobacter* genus. The samples A2 and B2 are highly related to each other where the percentage of relatedness was 99 %.

Comparing all trees, isolates A1, A4, C1 and E1 (Pseudomonas sp.) were consistently grouped together in the bootstrap test. However, *Pseudomonas aeruginosa* strain E70 (D2) was continuously clustered with *Bacillus cereus* strain Aj0803191A (A2), *Acinetobacter sp.* WJ07 (D3) and *Serratiasp*, endosymbiont of Nilaparvatalugens clone M149 (C2) for all tests. On the other hand, C2 was observed to be grouped with (D3) for Neighbour-joining, Maximum parsimony and Maximum likelihood trees.

Based on the phylogenetic tree constructed, it can be seen that the *Acinatobacter* genus is highly related to *Pseudomonas* because they were regularly clustered together under same root, while *Stenotrophomonas*genus is related to *Pseudomonas* and *Acinatobacter*. *Bacillus* (A2) and *Exiguobacterium*(B2) were repeatedly observed to be grouped together. This explains that they are highly related to each other due to their similarity in gram test; gram-positive group and were grouped away from other gram-negative bacteria.

It is predicted that microbial communities within contaminatedecosystems tend to be dominated by organisms capable of utilizing and/or surviving in toxic contamination. As the result, these communities were typically less diverse than those in nonstressed systems. According to the outcomes of this study, the isolates obtained were common to be associated with hydrocarbon utilization. This include *Acinatobacter, Bacillus and Pseudomonas* [7] and *Exiguobacterium* Chen et. Al., 2017, among others. In addition, *Bacillus sp.* has also known to capable of growing under a wide range of temperatures apart of possessing the capability in utilizing complex carbon sources [8].

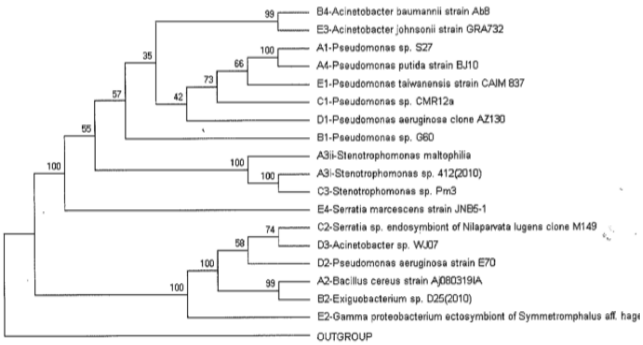

Fig. 1:Neighbour-joining

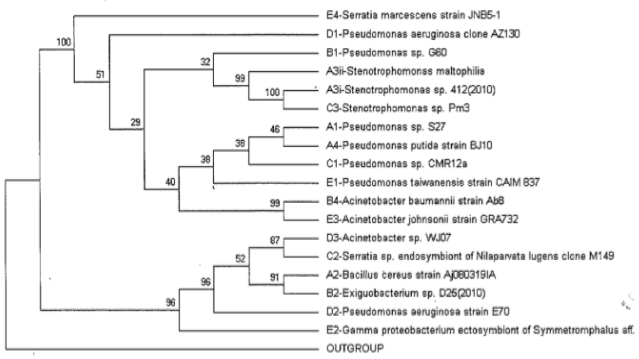

Fig. 2: Maximum Parsimony

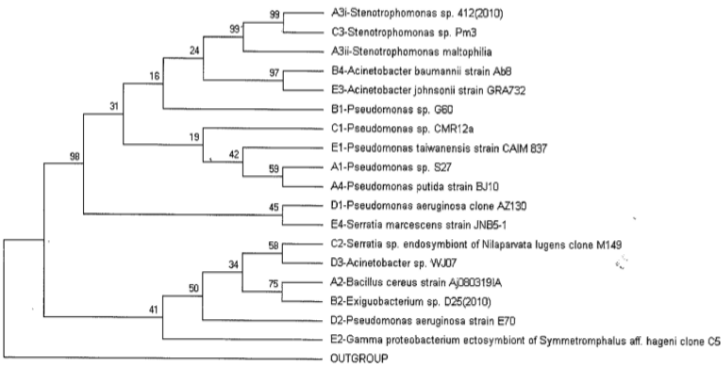

Fig. 3: Maximum Likelihood

4. Summary

The isolated bacteria in this study were considered presumptive hydrocarbon tolerant. They are capable to grow in the selective medium containing 1% engine oil. Seven genera were isolated and characterized as *Pseudomonas*, *Bacillus*, *Exiguobacterium*, *Stenotrophomonas*, *Acinetobacter*, *Serratia*and *Gamma Proteobacterium*.

The isolated and identified species were clustered in a tree. The relationship among them can be easily interpreted. Besides, the lineage of the species can be easily identified through the tree constructed.

All in all, it is expected for this study to contribute some insight in bioremediation technology specifically for a moderate weather of environment that perhaps may lead to a healthy environment with minimal cost using this 'green' technology.

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