IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL USEFUL BACTERIA FROM MARINE ENVIRONMENT

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ABSTRACT

Marine environment remained as largely unexplored source for researchers to discover novel properties from marine organisms which can benefits human kind. The study aims to isolate marine bacterium from various source of marine environment. Six bacterial strains were successfully isolated from marine samples from seashore of the Desaru, Malaysia and identified by 16S rRNA sequencing. The characterizations of bacterial strains were also performed based morphological tests, Gram staining, biochemical tests and antibiotic sensitivity against several antibiotics by disc diffusion method. The 16S rRNA sequence of D-2, D-4, D-7, D-15, D-31 and D-33 revealed a high identity of 97 to 99% with 16S rRNA sequence belong to genera *Pseudomonas, Marinomonas, Exiquobacterium,Micrococcus, Pseudoalteromonas*and *Shewanella* respectively. Strain D-31 exhibited higher tolerance towards antibiotic with resistance to Kanamycin, Ampicillin and Erythromycin. However, the growth of other strains was retarded by at least two of the antibiotics on their normal growth. The isolation of marine bacterial strain belongs to *Marinomonas* sp. and *Pseudoalteromonas*sp. create of interest for further biological characterization as the strains from these two genera have been proven for the discovery of new antimicrobial substances, enzymes for industry application and unique secondary metabolites.

Keywords: -Marine microorganisms, Antibiotic resistance, *Marinomonas*, *Pseudoalteromonas*

INTRODUCTION

The blue ocean is the largest marine ecosystem, covering 70% of the earth's biosphere and frames up >90% of the volume of its crust (Satpute*et al*., 2010). As investigation into marine ecosystem still in early stages, many unaccountable flora and fauna present themselves as an unexplored source that brings new insights to explore function of marine organisms. Marine microbes can be the valuable and perfect source to discover novel function and metabolisms which can benefits human kinds and environments. This is because, naturally marine microbes habit in a biologically competitive environment in the ocean with different water temperature, pH, salinity, saturated oxygen, amount of nutrients, surface, gradient change of lights and pressure (Wang *et al*., 2013, Zhang *et al*., 2005). The wide range of ecological niches, starting from shallow ocean, deep ocean, open ocean, hydrothermal vent, mangroves, polar regions, coral reefs and so many. This extent living conditions prove endurance, special biological activities and vary ecological adaptation of marine microorganisms.

Thus, these organisms are expected to possess the unusual metabolism, novel capability for various enzyme productions which can be industrial interest (Bornscheuer, 2005). To the date, marine bacteria provides magnificent opportunity to treasure unique bioactive compounds and proven to be a rich source of biologically active substances such as vitamins, drugs, antibiotics, enzymes, biosurfactant, which directed towards possible applications in pharmaceutical, medical, food, cosmetic, therapeutic, and agricultural industry.

AIM: To isolate and characterize marine bacteria from marine samples that can be explored in the future for potential benefits.

MATERIAL AND METHODS

1. Sample collection

The marine samples include sea water, sediments, sea grass and mud was collected from Desaru sea shore, Kota Tinggi, Malaysia. The samples were collected in the sterile falcon tubes and brought to the laboratory within 24 hours for isolation.

2. Isolation of bacteria

Isolation of bacteria were carried out by subjected the marine samples for simple serial dilution and followed by spread plate method. A 0.1 ml from each dilution factor was transferred on Marine Agar (MA) and other selective plates by spread plate method. The culture plates were incubated for 24-48 h at 37°C. After incubation, bacterial colonies were selected based on colony morphology including appearance, margin and elevation. Isolated strains were further purified and stored at -80°C with 20% (v/v) glycerol.

3. Biochemical characterization of bacteria

The isolates were characterized by several biochemical tests. Detection of various enzymes includes catalase, oxidase, urease, amylase, gelatinase,indole and utilization of different carbon source such as glucose, sucrose, lactose, H_2S production and oxidation-fermentation were tested as mentioned by Lemos (1985). Gliding motility also was tested based on standard method.

4. Morphology characterization

The isolated bacterial were subjected to Gram staining based on standard method to differentiate the Gram reaction based on their cell wall composition (Claus, 1992).

5. Identification of isolates

The bacterial strain was identified using 16S ribosomal RNA (16S rRNA) analysis. The genomic DNA of the isolates was isolated using genomic DNA purification kit (Promega). Approximately 1.5 kb long fragment of 16S rRNA gene was amplified from the extracted DNA template using universal primers [Forward primers: 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and Reverse primers: 1525 R (5'-AAGGAGGTGWTCCARCC -3')]. The amplified PCR product was purified using PCR purification kit (Promega) according to the manufacturer's instructions. Determined sequence of approximately 1500 bp long was obtained and sequence homologies were analysed by comparative studies using "The National Centre for Biotechnology Information (NCBI) database and Basic Alignment Search Tool (BLAST). The Clustal W Multiple Sequences Alignment with other closest sequences and phylogenetic tree was constructed using the neighbour-joining method with a bootstrap value of 1000 replicates using software package Mega 5.2.2.

6. Antibiotic sensitivity test

Antibiotic susceptibility of the isolates was determined on Marine Agar by disc diffusion method. All the six strains were tested against 3 antibiotics includes Kanamycin, Ampicillin and Erythromycin. All antibiotics were tested at concentration of 1mg ml^{-1}. The bacterial suspension containing 10^8 CFU/ml of bacteria was spread on Marine Agar and sterile disc impregnated with individual antibiotic were placed on the inoculated agar. Negative control was prepared using sterile distilled water. The inoculated plates were incubated at 37℃ for 24 hours. The zone of inhibition formed by each antibiotic was measured using a millimetre scale.

RESULTS

Six bacterial strains were successfully isolated based on distinguishable characters as shape, size and pigment productions. Each of the strains was tested for morphological, biochemical and antibiotic sensitivity. The strains grew well on BD Difco Marine Agar (MA) at room temperature and 37℃ after 24 hours of incubations. The results of morphological and biochemical tests were presented in Table 1 and 2 respectively. The cell shape and Gram staining of the six bacteria were recorded. *Pseudomonas* sp., *Marinomonas* sp., *Pseudoalteromona*s sp., and *Shewenella* sp., were found to be rod shaped Gram negative bacteria whereas *Exiguobacterium* sp., and *Micrococcus* sp., were Gram positive bacteria. The results demonstrated various distributions of bacterial genera in marine environment and four isolates were Gram negative.

Based on biochemical analysis all the isolated strains showed positive for catalase and only D-33 found to reduce sulphur to hydrogen sulphide. Interestingly, three marine isolates includes D-7, D-15 and D-33 showed positive result for hydrolysis of gelatin which indicates the presence of gelatinase (proteolytic enzyme).

Table 1: Morphological characterization of marine isolates

Strains	Colony morphology	Gram staining	16S rRNA identification
D-2	Creamy, irregular, flat, undulate	-ve rod cells	*Pseudomonas* sp.
D-4	Creamy, punciform, flat, shinny	-ve rod cells	*Marinomonas* sp.
D-7	Orange, circular, entire	+ve rod cells	*Exiguobacterium* sp.
D-15	Yellow, large, grow with entire margin	+ve, spherical cells	*Micrococcus* sp.
D-31	Creamy with purple pigment, flat, entire	-ve rod cells	*Pseudoalteromonas*sp.
D-33	Brownish, small circular, flat, entire	-ve rod cells	*Shewenella* sp.

Table 2: The summary of biochemical test results for all isolates.

strain	Oxidase	Catalase	Urease	Lactose utilization	Triple sugar iron test	Indole test	Motility test	Starch hydrolysis	Glucose fermentation test	Gelatine hydrolysis
D-2	+	+	-	+	K	-	-	-	+	-
D-4	-	+	-	-	K	-	-	-	+	-
D-7	+	+	-	-	A	-	-	+	+	+
D-15	-	+	-	-	A	-	-	-	-	+
D-31	+	+	-	-	K	-	-	+	-	-
D-33	+	+	-	+	H_2S	-	-	-	-	+

'**+**' = positive; '**-**'= negative ; '**A**' = Acid production; '**K**' = Alkaline reaction; '**H_2S**'= Sulfur reduction

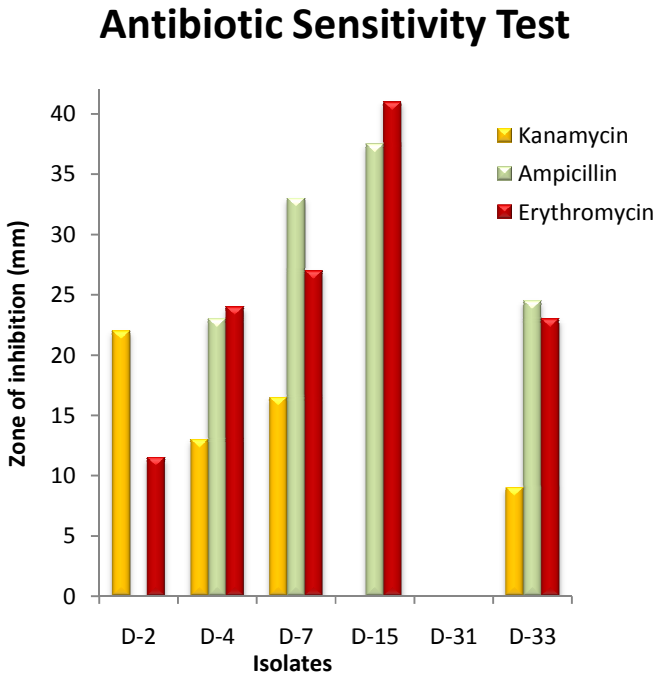

Figure 1: Antibiotic sensitivity test on Marine agar for six isolates against three antibiotics

The drug resistance of the isolated strains were screened by disc diffusion method against three commonly used antibiotics. The results of antibiotic susceptibility on MA showed that strain D-4, D-7 and D-33 were sensitive to ampicillin, kanamycin and erythromycin. However, strain D-2 and D-15 were resistant to kanamycin but sensitive to ampicillin and erythromycin respectively. Among the six strains tested in this study, only strain D-31 showed multiple resistances against the three antibiotics used (Figure 1).

Approximately 1500 bp of the 16S rRNA gene fragment of six isolates was sequenced (Figure 2). Analysis of 16S rRNA showed that all strains shares 97-99% of similarities to closet homology strains which confirmed their identity in the particular genera. Phylogenetic tree for the isolates was constructed using Neighbour-Joining method (Saitou &Nei, 1987).The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site (Nei& Kumar 2000). The analysis involved 26 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1557 positions in the final dataset. Evolutionary analyses were conducted in MEGA 5.2.2 software (Tamura *et al*., 2011).Strain D-2, D-4, D-7, D-15, D-31 and D-33 were found to be closely related to *Pseudomonas borbori*, *Marinomonas*sp., *Exiguobacteriumindicum*, *Micrococcus* sp., *Pseudoalteromonasnigrifaciens* and *Shewenellachilikensis* respectively with 99% of similarity (Figure 3).

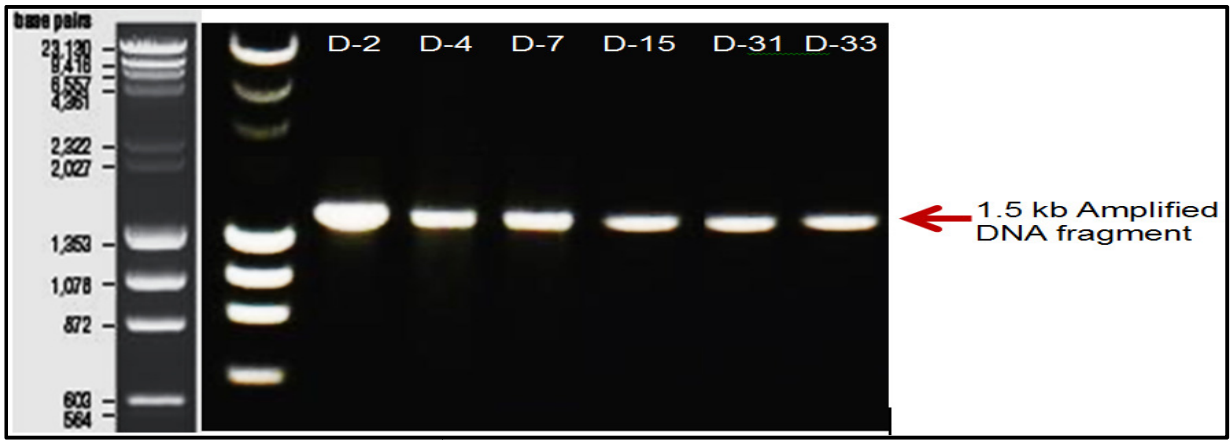

Figure 2: PCR product of 16S rRNA gene on 1% agarose gel. PCR was done as described in materials and

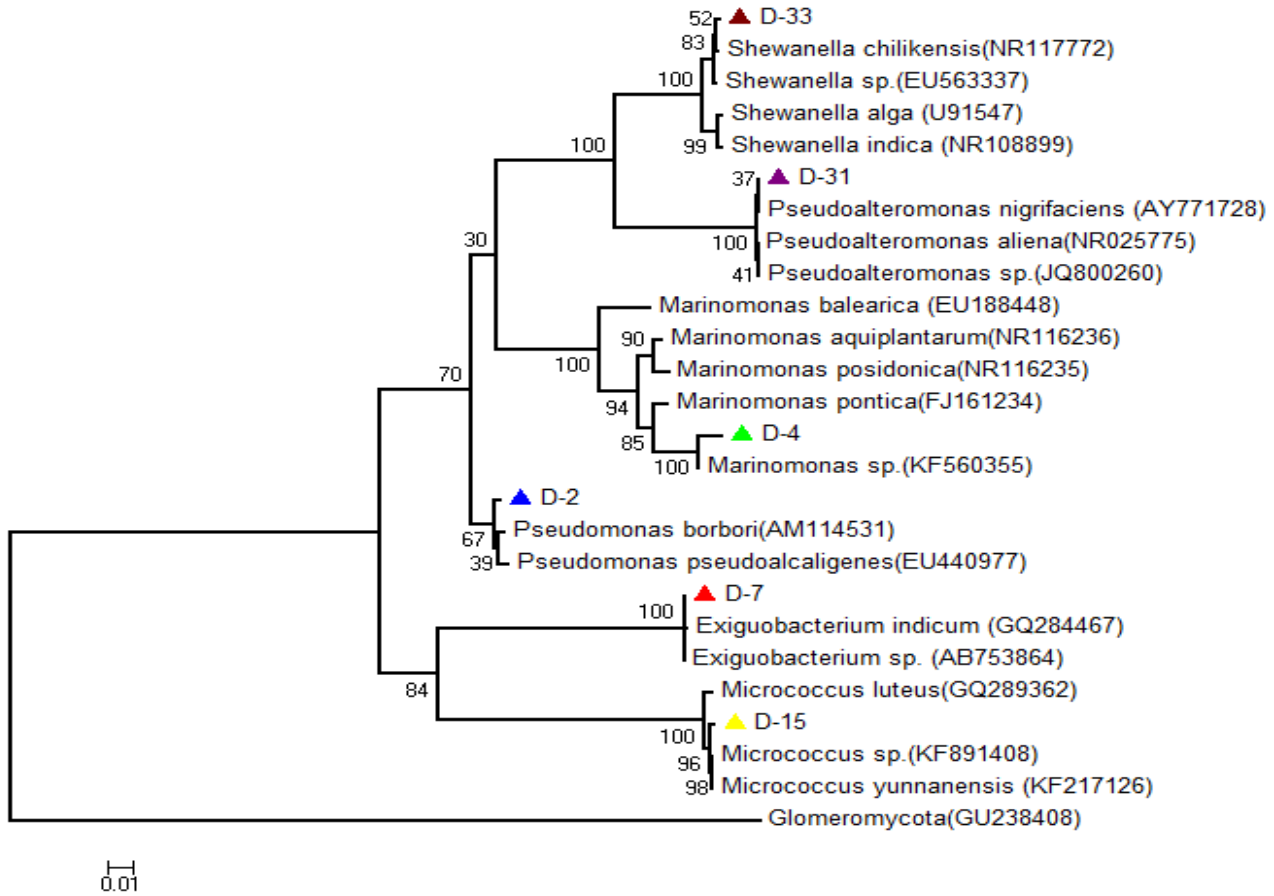

Figure 3: Phylogenetic tree showing the position of strain D-2, D-4, D-7, D-15, D-31 and D-33 and representative members of from the genus of each strain. The sequences were taken from GenBank. The tree was constructed by the neighbour-joining method with a bootstrap value (%) of 1000 replicates. The scale bar represents 0.01 nucleotide substitutions per position.

DISCUSSION

In this study, six bacterial isolates were successfully isolated, identified and characterized from marine samples. Three of the isolates which are *Pseudoalteromonas* sp., *Marinomonas* sp., and *Shewenella* sp., belong to a single family, the Gamma proteobacteria, which was most common type that can be found in marine environments. All the three isolates were Gram negative bacteria with different morphological and biochemical characteristics. According to Das (2006), Gram negative bacteria are dominant microflora in marine environment, since the cell wall composition is better adapted for extreme survival in marine ecosystem.

Isolation of *Marinomonas* sp. and *Pseudoalteromonas* sp., are expected to be potential source for further applications. Strains *Marinomonasprimoryensis* framed up with valuable application include production of AFP (anti-freeze protein) which has applications in preservations tissue, organs and in food industry (Sharp 2011). Besides that, both strains proved with highest antimicrobial activity, where PfaP and MMB-1(antimicrobial proteins) successfully purified from *Pseudoalteromonasflavipulchra* and *Marinomonas mediterranea* (Yu *et al*., 2012). More interestingly, Dong (2014) stated that *Marinimonas*sp.can degrade a wide variety of PAHs (polycyclic aromatic hydrocarbon) such as naphthalene, fluorene, phenanthrene, anthracene, and pyrene at 25℃.

In conclusions, *Marinomonas* sp. and *Pseudoalteromonas* sp. have created interest for further biological characterization as the strains are potential source for discovery of new antimicrobial substances, enzyme for industrial application.

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