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ISOLATION AND CHARACTERIZATION OF BIOTECHNOLOGY RELEVANT BACTERIA FROM MARINE ENVIRONMENT

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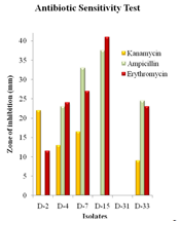
Graphical abstract



Abstract

Marine environment remained as largely unexplored source for researchers to discover marine microorganisms with novel properties. This study aims to isolate marine bacteria from the seashore of Desaru, Malaysia. Totally, six bacterial strains were successfully obtained and were identified by complete 16S rRNA sequencing. The characterizations of bacterial strains were performed based on morphological tests, Gram-staining, biochemical tests, and antibiotic sensitivity. 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 taxa belong to genera of *Pseudomonas, Marinomonas, Exiquobacterium, Micrococcus, Pseudoalteromonas,* and *Shewanella* respectively. Strain D-31 exhibited higher tolerance towards antibiotics kanamycin, ampicillin, and erythromycin while the growth of other strains were retarded by at least two of these antibiotics. We further characterized strain D-4 and D-31 that belonged to *Marinomonas* sp. and *Pseudoalteromonas* sp. Both genera are interesting as earlier researchers have discovered new antibacterial substances, industrial enzymes and unique secondary metabolites.

Keywords: Marine microorganisms, Antibiotic resistance, *Marinomonas*, *Pseudoalteromonas*, Antibacterial activity

Abstrak

Persekitaran marin kekal sebagai sumber yang belum diterokai oleh para penyelidik untuk mencari organisma marin novel. Kajian ini bertujuan untuk memencilkan bakteria marin dari pantai Desaru, Malaysia. Enam bakteria telah dikenal pasti melalui penjujukan 16S rRNA. Pencirian bakteria dilakukan berdasarkan ujian morfologi, pewarnaan Gram, ujian biokimia dan kepekaan bakteria terhadap beberapa antibiotik. Berdasarkan penjujukan 16S rRNA, bakteria D-2, D-4, D-7, D-15, D-31, dan D-33 masing-masing dikelaskan sebagai *Pseudomonas*, *Marinomonas*, *Exiquobacterium*, *Micrococcus*, *Pseudoalteromonas,* and *Shewanella*. Bakteria D-31 menunjukkan toleransi yang tinggi terhadap antibiotik dengan rintangan terhadap kanamycin, ampicillin, dan erythromycin. Pertumbuhan bakteria lain didapati terbantut terhadap sekurang-kurangnya dua jenis antibiotik yang diuji. Bakteria D-4 dan D-31 dari genus *Marinomonas* sp. dan *Pseudoalteromonas* sp. dipilih bagi pencirian lanjutan. Kedua-dua genera telah terbukti dengan penemuan antibakteria baru, enzim untuk aplikasi industri dan metabolit sekunder yang unik.

Kata kunci: Mikroorganisma marin, Toleransi antibiotik, *Marinomonas*, *Pseudoalteromonas*, Aktiviti antibakteria

1.0 INTRODUCTION

The ocean is the largest marine ecosystem, covering approximately 70% of the earth's biosphere [1]. Over the years, diversity of marine microorganisms has become an attractive research subject. Marine microorganisms have unique properties because they are able to adapt to extreme abiotic conditions such as salinity, temperature, saturated oxygen, limited nutrients, surface texture, UV, and higher pressure towards the bed of the sea [2, 3]. Besides, due to the wide range of ecological niches, for instance shallow ocean, deep ocean, open ocean, hydrothermal vent, mangroves, polar regions, and coral reefs provide, the microorganisms in ocean are more diverse and require more ecological adaptations than normal soil biome [4].

Marine microorganisms exhibit these unique abilities: antibacterial activity, biosynthesis of pigments and vitamins, antifungal activity, various industrial enzyme and antibiotics production [5]. These various bioactive metabolites are highly required in pharmaceutical, medical, food, cosmetic, therapeutic, and agricultural industries. This study aimed to isolate and characterize Malaysian halophiles from Desaru seashore. We hope that the isolates could be used as a source to discover novel enzymes and secondary metabolites.

2.0 EXPERIMENTAL

2.1 Sample Collection

The marine samples include seawater; sediments, sea grass and mud were collected from Desaru seashore, Kota Tinggi, Malaysia. The samples were collected in sterile falcon tubes and brought to the laboratory within 24 hours and kept at 4°C prior to bacteria isolation.

2.2 Isolation of Bacteria

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

2.3 Biochemical Characterization of Bacteria

The isolates were examined using several biochemical analyses which included of tests for catalase, oxidase, urease, amylase, gelatinase, indole and utilization of different carbon source such as glucose, sucrose, lactose, H_2S production, and oxidation-fermentation [6]. Gliding motility also was tested.

2.4 Morphology Characterization

The isolated bacterial were subjected to Gram staining based on standard method [7].

2.5 Identification of Bacteria

The bacterial strains were identified using 16S rRNA analysis. The genomic DNA of the isolates were 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 PCR product was purified using PCR purification kit (Promega) and sent for sequencing. The results from sequencing were analyzed by comparative studies using "The National Centre for Biotechnology Information (NCBI) database and Basic Alignment Search Tool (BLAST). The ClustalW Multiple Sequences Alignment with other closest sequences and phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value of 1000 replicates using software package Mega 5.2.2.

2.6 Antibiotic Sensitivity Test

Antibiotic susceptibility of the isolates were determined on Marine Agar by disc diffusion method. All the six strains were tested against 3 antibiotics includes kanamycin, ampicillin and erythromycin 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°C for 24 hours. The zone of inhibition formed by each antibiotic was measured using a millimeter scale.

3.0 RESULTS AND DISCUSSION

Six bacterial strains were successfully isolated based on distinguishable characteristics such as shape, size and pigment productions. These bacteria were designated as strain D-2, D-4, D-7, D-15, D-31, and D-33. Each of the strains was tested for morphological, biochemical and antibiotic sensitivity. The strains grew well on BD Difco Marine Agar at room temperature and 37°C 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. Strain D-2, D-4, D-31, and D-33 were found

to be rod shaped Gram negative bacteria whereas strain D-7 and D-15 were Gram positive bacteria.

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	*Exiguobacteriu m* sp.
D-15	Yellow, large, entire margin	+ve, spherical cells	*Micrococcus* sp.
D-31	Creamy, purple pigment, flat, entire	-ve rod cells	*Pseudoalteromo nas*sp.
D-33	Brownish, small circular, flat, entire	-ve rod cells	*Shewanella* sp.

Table 2 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$_2$S	-	-	-	-	+

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

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

The antibiotics resistance of the isolated strains was screened by disc diffusion method. 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).

The phylogenetic tree for the isolates was constructed using Neighbor-Joining method [8]. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site [9]. The analysis involved 26 nucleotide sequences. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA 5.2.2 software [10]. 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., *Exiguobacterium indicum*, Micrococcus sp.*Pseudoalteromonas nigrifaciens*, and *Shewanella chilikensis* respectively with 99% of similarity (Figure 2).

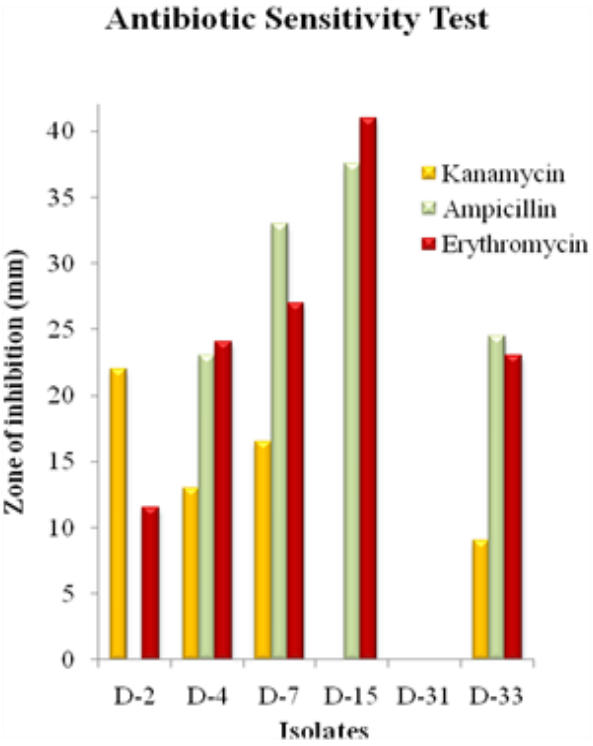


Figure 1 Antibiotic sensitivity test on Marine agar for six isolates against three antibiotics

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

Shewanella sp., belong to a single family, the Gamma Proteobacteria, often found in marine environments. All the three isolates were Gram negative bacteria with different morphological and biochemical characteristics. According to Das [11], 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. D-4 and *Pseudoalteromonas* sp. D-31, are expected to be potential source for further applications. In other study, *Marinomonas primoryensis* was reported as the producer of anti-freeze protein, which the protein could be used in preservation of tissue, organs and in food industry [12]. In addition, *Pseudoalteromonas flavipulchra* and *Marinomonas mediterranea* were reported with ability to produce protein with antibacterial activity. Proteins from both strains were successfully purified and characterized [13]. Moreover, Dong [15] stated that *Marinimonas* sp. could degrade a wide variety of PAHs (polycyclic aromatic hydrocarbon) such as naphthalene, fluorene, phenanthrene, anthracene, and pyrene at 25°C.

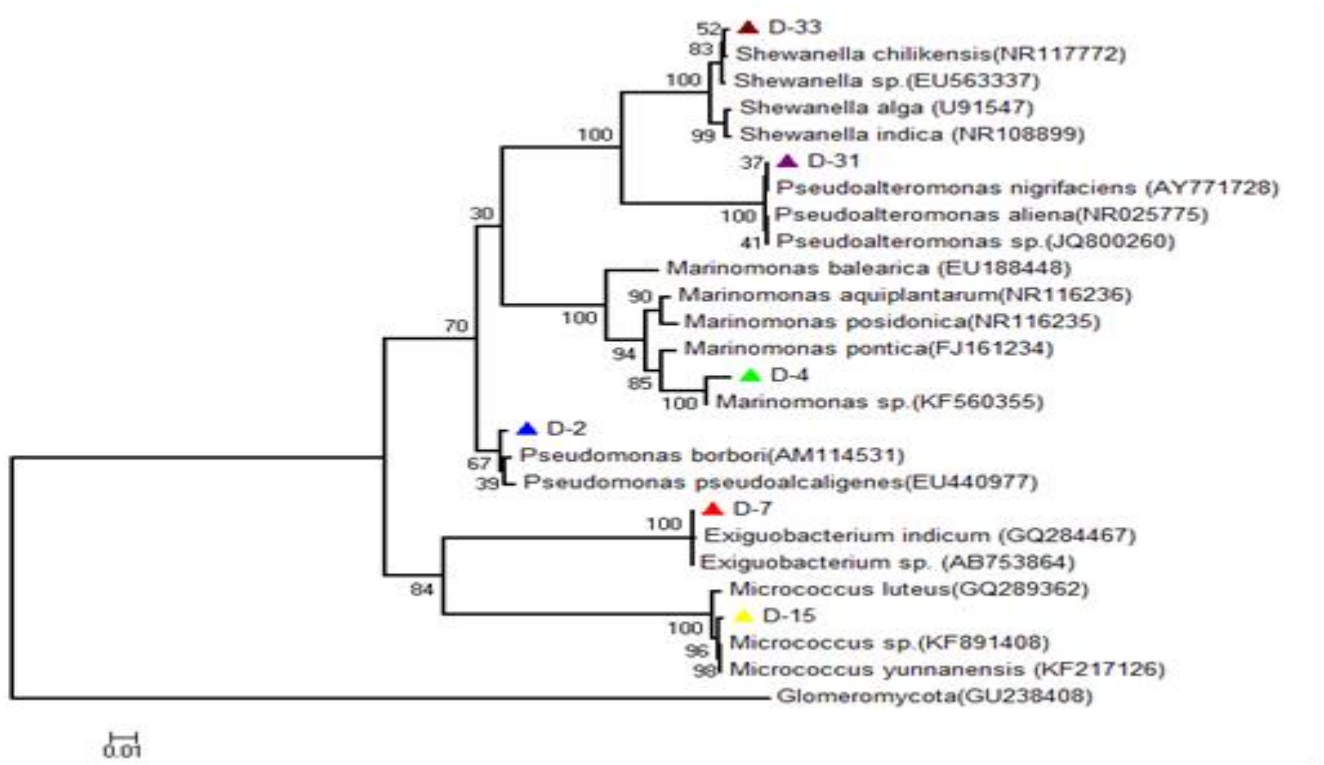


Figure 2 Phylogenetic tree showing the position of strain D-2, D-4, D-7, D-15, D-31 and D-33 and representative members of from the genus of each strain. The sequences were taken from GenBank. The tree was constructed by the neighbor-joining method with a bootstrap value (%) of 1000 replicates. The scale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

4.0 CONCLUSION

Marinomonas sp. D-4 and *Pseudoalteromonas* sp. D-31 have created interest for further biological characterization as these bacteria are potential source for discovery of new antibacterial substances and enzymes for industrial applications. Future work of this research can be focused on isolation and purification of potential enzymes with commercial importance.

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