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Optimization and characterization of antibacterial agents from marine bacteria

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Abstract

Marine bacteria were isolated from Red Sea in Egypt. Antimicrobial activities of the isolated bacteria were investigated. The agar diffusion method was used to screen ethyl acetate extracts from isolated marine bacterial fermentation to verify antibacterial activity. The study results revealed that 20 strains of isolates have antibacterial activity. The isolated bacteria that showed wide antibacterial spectrum were belonged to *Pseudomonas*, *Actinomycetes*, *Bacillus* and *Brevibacterium* genera. TLC Bioautography agar overlay assay underlying that antibacterial metabolites created from the active strains have broad-spectrum antimicrobial activity had been different. The isolated bacteria were suggested to be potential sources of natural antibiotics. It is concluded that marine bacteria can introduce some bacterial strains to be sources of new biomolecules.

Key words: Antibacterial agents, Marine bacteria.

1. Introduction

Currently, marine organisms are of great interest to scientists and researchers as they are a promising new source of bioactive compounds [1]. They produce a wide range of metabolites, some of which can be used to develop drugs. These microorganisms are an unlimited source of new compounds that have many therapeutic applications [2].

Antimicrobial resistance is an important global threat that reduces the chances for the infectious diseases treatment caused by microorganisms [3]. WHO global surveillance report showed increased morbidity and mortality of infectious diseases resulted from Antimicrobial resistance that may result in estimated economic losses up to 100 trillion U.S Dollars by 2050 because of 2–3% decrease in domestic product worldwide [4]. Thus, discovery of novel types of clinically important antimicrobial substances is necessary and critical [5]. Earth resources have been intensively examined for bioactive compounds detection. However, novel sources of antimicrobials and the other biologically active substances are increasingly isolated from marine environments [6].

Marine environment covers about 70% of the Earth's surface and has a high biodiversity; it has 36 phyla of known living organisms, thirty-four of which have been found in the marine environments with more than three hundred thousand known species of plants and animals [7].

Mayer et al. (2013) reported that from 2009 to 2011 about 230 natural marine biotic products were isolated, most of which (102 compounds) showed antimicrobial activities [8].

The Red Sea is characterized by physical and geochemical characteristics that make it unique environment compared to other marine ecosystems [9]. Red Sea has many characteristics such as high temperature that ranges from 24- 35 °C according to the season (at the surface), while it is about 22 °C (at 200 m depth); has high salinity [10], as a result of the high evaporation rate, low precipitation level and lack of inflows [9] and seasonal fluctuation of air and unique coral reef systems [11].

Due to these factors, it has a unique microbial variety that possess a unique metabolites either primary or secondary products [12].

2. Materials and Methods

2.1. Samples Collection

Twenty samples were collected from three locations at the red sea. Coastal water was collected for studying the antimicrobial properties and morphological characteristics of the marine bacterial species. Water samples were collected in clean and sterilized glass bottles.

2.2. Marine bacteria isolation

The 0.5 g samples were crush, suspended in sterile seawater and then spread on 1/10 Marine Agar surface which is consist of the followings:

- Agar 15 g
- Yeast extract 0.1 g
- $FePO_4$ 0.1 g
- Peptone 0.5 g

Dissolved in one liter of seawater (with pH= 7.2-7.6)

Incubation was done at 25°C for 20 days then the colonies (have different morphology) were used for bacteria isolation.

2.3. Crude extracts and Bacterial cultures Preparation

Marine bacteria were cultured in Marine Broth (300 ml) which consisted of the followings:

- yeast extract 1 g
- peptone 5 g
- $FePO_4$ 0.1 g

Dissolved in one liter of seawater (with pH= 7.2-7.6)

Using 500 ml flasks for secondary metabolites production. Erlenmeyer flasks were incubated at 25 °C for 7 days using shaker with 220 rpm. Centrifugation of broth was done at 5000 x g for 30 min for removal of cells then broth was extracted three times using ethyl acetate (100 ml). The solvent was removed at 37 °C under reduced pressure then extracts were used as crude samples to be used in the bioactivity assay.

2.4. Antimicrobial activity Bioassay for marine isolated bacteria

Screening of antimicrobial activity of marine bacteria, which were isolated from 5 coastal sites of Abo Ramad, Red Sea, were done using earthen microbe including *Aeromonas hydrophila*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Aspergillus niger*, which are obtained from bacteriology department, Animal Health Research Institute, as a test microorganisms. Assaying of antimicrobial activity was done using standard paper disc assay (in duplicate) [13]. EtOAc was used for dissolving of dried crude extracts in 100 mg ml^{-1} concentration. Twenty µl of samples were used for saturation of 6 mm antimicrobial assay paper disks and drying between applications. The paper disks were put in agar surface contain the test bacteria then diffusion was done for 10 hours at 8°C then they were incubated at 37 °C for 24 hours. The inhibition zones diameters were measured.

2.5 TLC Bioautography agar overlay assay

Four of the wide antibacterial spectrum marine bacteria (Isolate 15, Isolate 17, Isolate 18 and Isolate 20) were used as crude extracts to be assayed in TLC Bioautography overlay assay [14]. 100 mg ml^{-1} concentration was made from each crude extract after its dissolving in EtOAc. 2 µl solution was subjected to analysis of TLC on 3.5 x 5 cm silicagel plate (Merck Co, USA) using to some chemicals such as dichloromethane: EtOAc: Methanol; 5:5:1, v/v) as a mobile phase. UV/V was used at 254 nm and 365 nm wavelengths. Developed TLC plates were enchased in 9 mm Petri-dish with base nutrient agar then Sterilized using UV lamp for thirty minutes. The plates were covered with melting nutrient agar (at 46 °C) that contain test bacteria.

The plates left for diffusion process at 8 °C for 10 h then plate was incubated for 24 h at 37°C, the upper agar was soaked with methylthiazoletetrazolium (5 mg ml^{-1}) (MTT, Sigma, USA) to be converted to formazan dye by test bacteria. Clear spots against purple background were observed as recorded as inhibition zones. Rf values of inhibition zones were calculated.

2.6. Bacterial strains identification

According to the schemes of Mearns-Spragg et al. (1998) [15] and Schwartsmann et al. (2001) [16], isolated bacteria with antibacterial activity were identified to genus level through observation of the morphological and biochemical characteristics of each isolate. The isolated marine bacteria with broad antibacterial spectrum were identified to species level by BLAST analysis, 16S rRNA gene amplification using PCR and sequences comparison with GenBank nucleotide database. The PCR was done according to Acinas et al. (1999) [17]. Purification and sequencing of PCR products were done in RLQCP.

3. Results

3.1. Characterization and Antibacterial activity

Twenty marine bacteria were isolated from red seawater samples. The antibacterial assay revealed that twenty strains inhibited at least one-tested bacteria (Table 1). Taxonomic study showed that the marine bacteria with antibacterial activity belong to *Pseudomonas* (eight strains), *Actinomycetes* (six strains), *Bacillus* (two strains) and *Brevibacterium* (four strains). Four strains inhibited all test bacteria. Five marine bacteria were preserved in slant and broth cultures to be used for studying the morphology, colony, microscopical characteristics, motility, gram staining and other biochemical characteristics.

Table 2 shows the marine bacteria colony shape and motility in the medium. While Table 3 shows oxidase, catalase and IMVIC reactions in isolate 15 and isolate 17.

According to sequence analysis of 16S rRNA, the isolate 15 and isolate 17 strains were identified as *Pseudomonas putida* and *Brevibacterium frigoritolerans*, respectively. The identification and separation of the bioactive compounds with broad antibacterial spectrum from the isolated marine bacteria were afforded.

Table (1): Results of antibacterial activity of isolated marine bacteria using agar diffusion and taxonomic characterization of active bacteria

Where (−) means no inhibition, (+) means inhibition zone of (0-3 mm), (++) means inhibition zone of (3-5 mm), (+++) means inhibition zone more than five mm.

3.2 Antibacterial metabolites of isolated strains

Crude extracts of isolate 15 and isolate 17 that showed wide antibacterial spectrum were assayed by TLC Bioautography agar overlay assay; the study results, done at Nawah Scientific Lab, were illustrated in figure (1). Each crude extract of isolated strains showed at least 1-inhibition spots in TLC development system and Rf values were different in each strain. In case of isolate 15 and 17, the inhibition spots Rf values were 0.69 and 0.61 respectively.

Table (2): Isolated marine bacteria according to colony Morphology and other characters

No.	Bacterial strain	Colonial morphology	Bacteria cell shape	Motility	Gram staining
1	**Isolate 15**	smooth, shiny, cream color, circular, convex, entire with green soluble Pigments	Rod	+	-

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Isolate No.	**Antimicrobial activity**					
	Genus	***Aeromonas hydrophila***	***E.coli***	***Klebsiella pneumoniae***	***Staph. aureus***	***Aspergillus niger***
Isolate 1	*Actinomycetes sp.*	+	++	–	++	+
Isolate 2	*Pseudomonas sp.*	–	–	–	+	–
Isolate 3	*Actinomycetes sp.*	+	–	+	–	–
Isolate 4	*Pseudomonas sp.*	–	+	–	+	–
Isolate 5	*Pseudomonas sp.*	+	+	–	-	–
Isolate 6	*Actinomycetes sp.*	–	+	–	+	–
Isolate 7	*Actinomycetes sp.*	+	–	+	-	–
Isolate 8	*Actinomycetes sp.*	+	+	–	-	–
Isolate 9	*Pseudomonas sp.*	+	–	–	+	–
Isolate 10	*Bacillus sp.*	+	–	+	–	–
Isolate 11	*Pseudomonas sp.*	+	–	–	+	–
Isolate 12	*Brevibacterium sp.*	–	–	–	+	–
Isolate 13	*Pseudomonas sp.*	+	++	–	–	–
Isolate 14	*Brevibacterium sp.*	+	–	–	+	–
Isolate 15	*Pseudomonas sp.*	+++	++	+++	+++	+++
Isolate 16	*Brevibacterium sp.*	+	-	+	-	-
Isolate 17	*Brevibacterium sp.*	+++	++	+++	+++	+++
Isolate 18	*Pseudomonas sp.*	+	+	++	-	+
Isolate 19	*Actinomycetes sp.*	-	-	+	-	-
Isolate 20	*Bacillus sp.*	++	++	+	++	+

2	**Isolate 17**	Smooth, white to gray-white. Pigment is light dependent. Have distinctive cheesy odor.	Irregular rods	-	+

Table (3): Biochemical Characteristics

No.	**Strain**	**Catalase**	**Oxidase**	**IMVC**			
				I	**M**	**V**	**C**
1	**Isolate 15**	+	+	-	-	+	+
2	**Isolate 17**	+	+	-	-	-	+

Where + means positive, - means negative

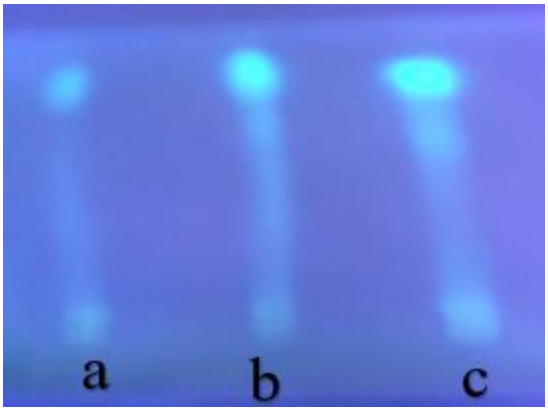

Figure (1): TLC Bioautography agar overlay assay

TLC Bioautography agar overlay assay for broad-spectrum antibacterial spectrum strains against *Staphy. aureus*. Samples were obtained from isolate 17 (a), 15 (b), 20 (c). Inhibition spot was detected.

4. Discussion

In this work, 20 strains were isolated from seawater where huge number of bacteria can live in it. These types of bacteria are generally not very symbiotic of the host but can be considered as bacteria associated with them [18] with relative relationship with the

hosts. Marine bacteria could get their necessary nutrition (vitamin, fatty acid and polysaccharide) from the plant or animal host however; marine bacteria can excrete some products (toxin propitious, amino acid and antibiotic) for the metabolism of their hosts or for improving of the capability of their chemical defense [19]. This study results come at line with that reported by previous investigations.

Köhler et al. (1999) investigated the antibacterial activity of the coastal seawater marine bacteria [20], the results of their study revealed that 17% of marine bacteria showed antimicrobial activity to *Staphy.aureus*. Tested bacteria showed pigmented colonies and the antibiotic producing marine bacteria belonged to *Pseudomonas putida.* In the present study,

The isolated bacteria from seawater showed antibacterial producing activity with 12% proportion in pigment producing colonies and no pigment producing. The isolated strains were identified as *Pseudomonas*, *Bacillus*, *Actinomycetes* and *Brevibacterium*. The results of molecular investigation of isolated strains revealed that the marine bacteria with broad spectrum antimicrobial activity were *Pseudomonas putida*, *Brevibacterium frigoritolerans* and *Bacillus licheniformis*. These results are come at line with the results of the several studies such as Hamid at al. (2013) [21], Ahmed et al. (2008) [22] and Mc Evoy (1993) [23]. This study results confirmed that the antibacterial spectra of active marine bacteria are varying from among species. In the twenty strains with antibacterial activity, 15 strains (75%) inhibited *Aeromonas hydrophila,* 10 strains (50%) inhibited *E.coli*, 8 strains (40%) inhibited *Klebsiella pneumoniae*, 11 strains (55%) inhibited *Staphylococcus aureus*, and 5 strains (25%) inhibited *Aspergillus niger*. the results showed that only four strains had inhibition activity for all tested bacteria. The intrinsic resistance of bacteria to the wide range of antibiotics may be due to the lower outer membrane permeability [24, 25].

However, there were several studies on the medical benefits of marine life and investigation of ecological system related to marine bacteria [26-34], the present study is one of the few studies reported marine bacteria with antibacterial activity in the red

sea. According to TLC Bioautography agar overlay assay it is concluded that different bacterial species produces different antibacterial metabolites and some species could produce more than one antibacterial substance. This study results revealed that some marine bacteria could produce different antibiotic substances for providing themselves the superiority of survival competition. Marine bacteria as model systems represents the potential for understanding and development of treatments for various disease depending on the physiological role of the secondary metabolites of these bacteria and they are intensively involved in developing new medications.

5. Conclusion

It is concluded that marine bacteria isolation can introduce different bacterial strains that are sources of biomolecules originated from marine system. It is observed that certain strains of marine bacteria could be induced for production of antibiotics.

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