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Screening of Actinomycetes From Lipar Area of Oman Sea to Investigate the Antibacterial Compounds

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Background: Actinomycetes are one of the most important sources for the production of antibacterial compounds. Marine environments, due to their unique characteristics, are considered a good option to search for bacteria with the capability of producing antimicrobial compounds.
Objectives: The purpose of this study was to isolate the actinomycetes producing antibacterial compounds.
Materials and Methods: A total of 35 actinomycetes were isolated from Oman Sea (Lipar Area). To investigate antibacterial activity, the isolated actinomycetes were assessed against reference and pathogenic bacteria, including *Staphylococcus epidermidis*, *Staphylococcu intermedius*, *Staphylococcu chromogenes*, *Staphylococcu saprophyticus*, *Bacillus cereus* and methicillin-resistance *Staphylococcu aureus*, *Pseudomonas*, *Listeria*, *Klebsiella*, *Salmonella*, *Acinetobacter*, and *Escherichia coli* O157:H7, using the cross streak method.
Results: Based on the morphological characterization, 35 isolated cases belonged to actinomycetes and %94 of them had the ability to produce antibacterial compounds. In the cross streak method, most of the isolated bacteria have antibacterial activity against reference *S. aureus* among Gram-positive bacteria and *Acinetobacter* among Gram-negative bacteria. Inhibition zone diameters were measured between 2-25 and 1-20 mm for Gram-positive and -negative bacteria, receptivity.
Conclusions: Preliminary results indicate that the native Iranian *Actinobacteria* could be considered a suitable option for screening of the new antibacterial compounds. Molecular research and antibacterial compound extraction against the aforementioned pathogenic strains are also being conducted.

Keywords:Actinobacteria; Anti-Bacterial Agents; Oman Sea

1. Background

More than 70% of the surface of the planet is covered by sea water. These waters are home to an exceptionally diverse biological world which include 95% of biodiversity of the whole biosphere (1). Experts estimate that the biological diversity in some marine ecosystems, such as the deep sea floor and coral reefs, is higher than in the tropical rainforests (2). The ability to recover actinomycetes from sea water and deep ocean trenches is nothing new. What is more ambiguous is the performance nature of these marine-based strains and whether they are metabolically active or just adapted to life in the sea (3). Actinomycetes are the most economically and biotechnologically valuable prokaryotic microorganisms. They were found to produce nearly half of the discovered bioactive secondary metabolites. These secondary metabolites include antibiotics, antitumor agents, immunosuppressive agents and enzymes (4). Among 22500 biologically active substances acquired from microbes, actinomycetes account for 45%, fungi account for 38% and the rest are from other forms of bacteria. The culture of Gram-positive and Gram-negative bacteria and filamentous fungi has resulted in identifying north of 5000 antibiotics. More than 70% of the total antibiotic production is directly related to different species of *Streptomyces*. The measured amount of *Micromonospora*, compared to *Streptomyces*, was less than one-tenth (5). Nowadays, the number of discovery of new metabolites from terrestrial actinomycetes has been decreased. On the other hand, there is an increase in the rate of reisolation of known compounds (6). Therefore, thorough the examination of new groups of actinomycetes based on unexplored habitations as sources of fresh bioactive secondary metabolites proves crucial.

2. Objectives

The aim of the present study was to investigate the antibacterial compound from marine actinomycetes isolated from Oman Sea. This subject has been so little studied.

3. Materials and Methods

3.1. Sample Collection

The water samples were collected from the Oman Sea and particularly from Lipar Area, Iran. Field collection of samples carried out in September 2013 from the depth of 5-10 m.

3.2. Isolation of Actinomycetes

To prevent other bacterial flora, the heat treatment was performed by holding the water samples in a water bath at 60° C° for 10 minutes. All samples were diluted with sterile 0.5% saline prior to inoculation into the isolation. To maximize the isolation of actinomycetes, the process was done with different media, such as starch casein agar, starch nitrate agar and glycerol glycine agar with two salt concentrations (20 and 26 g/L). The diluted samples were placed on the starch-casein agar medium, starch-nitrate agar medium and glycerol-glycine agar. The media were supplemented with 100 μg/mL of cycloheximide to eliminate fungal contamination. All experiments were carried out in triplicates. All culture media and their supplements were commercially purchased from Merck (Merck, Germany) and Qulab (Quelab, Canada).

3.3. Screening for Antibacterial Activity

The antibacterial activity was studied preliminarily using the cross streak method (Figure 1) against bacteria. Different test organisms were used, including *Staphylococcus aureus* (PTCC1431), *Staphylococcus epidermidis* (PTCC1435), *Staphylococcus saprophyticus* (PTCC1440), *Staphylococcus intermedius* (from patient), *Staphylococcus chromogenes* (from patient), methicillin-resistance *Staphylococcus aureus* (MRSA), *Bacillus cereus* (PTCC1274), *Pseudomonas aeruginosa* (PTCC1074), *Kelebsiella* (from patient), *Salmonella typhimurium* (ATCC14028), *Listeria monocytogenes* (PTCC1298), *Escherichia coli* O157 (NCTC12900), *E. coli* (resistance), *E. coli* (resistance), *Acintobacter* (resistance), *Acintobacter* (resistance). After preliminary testing of the isolates for their antibacterial activities, the most active isolates were selected for further examination. The antibiotic resistance strains were collected from Ghaem Hospital of Mashhad.

3.4. Morphological and Biochemical Characterization

Morphological characterizations of selected actinomycetes strains including colony characteristics, type of aerial hyphae, spore formation, gram staining, catalase and hemolysis were examined.

3.5. Exo-enzymatic Assay

The selected actinomycetes were screened on the media starch, skim milk, gelatin, urease and Simmons' citrate supplemented by agar for hydrolytic enzymes including amylase, protease, geletinase, urease and citratase, respectively.

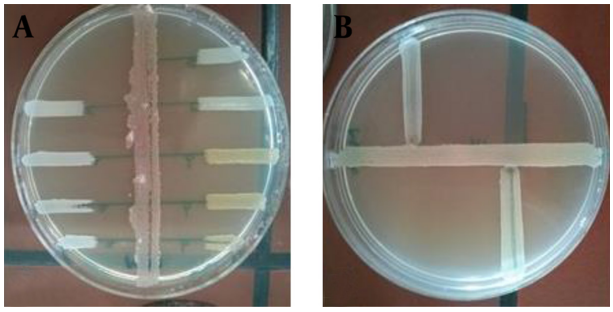

Figure 1. (A) vertical line is related to Actinomycete which produces antibacterial compound. horizental lines are related to pathogenic strains which are affected by Actinomycete. (B) vertical lines are related to pathogenic strains. horizental line is related to Actinomycete without any antibacterial compound production.

4. Results

Among the 177 isolated bacteria, 35 isolates belonged to the actinomycetes group. Among these isolates 94% showed a significant antibacterial activity. The results are summarized in Table 1. In the cross streak method examination, most of the isolated bacteria have antibacterial compounds against reference *S. aureus* among Gram-positive bacteria and *Acinetobacter* among Gram-negative bacteria (Table 1). Inhibition zone diameters were measured between 2 - 25 and 1 - 20 mm for Gram-positive and -negative bacteria, respectively. The best antibacterial compound producers (14 isolates) were selected for further thorough analysis. Biochemical tests were conducted for casein, starch, gelatin, urea and citrate hydrolysis, catalase and hemolysis. The biochemical characterization and the production of different enzymes are shown in Table 2.

Enzymatic activities of 14 selected actinomycetal isolates were performed and results are presented in Table 2. It was found that 10 (71.4 %), 3 (21.4%), 5 (35.7%), 1 (7%) and 9 (64.3%) number of actinomycetes were possessing protease, amylase, gelatinase, urease and citratase activity, respectively.

Isolates, namely AC 112, 113, 114, 117, 122, 127, 130, 131, 135 and 275 showed positive results in casein hydrolysis. The isolates namely AC111, AC117 and AC275 exhibited positive results in starch hydrolysis. As an insoluble polymer of glucose, starch provides microorganisms with a source of carbon. The following five isolates showed positive results in gelatin hydrolysis: AC111, AC112, AC113, AC135 and AC275. Many microorganisms, including actinomycetes produce exo-enzymes that are capable of hydrolyzing gelatin and liquefying the nutrient gelatin medium. All fourteen isolates had catalase activity and none of them had urease activity except for AC275. Isolates AC117, 122, 141 and 275 showed beta hemolysis and others were gamma hemolysis.

Table 1. Inhibition Zone of Different Bacteria Against Selected Actinomycetes Isolates by Cross Streak Method (cm) [a]

	Sa	Se	Ss	Si	Sc	MRSA	Bc	Pa	K	St	Lm	Ec 157	EcR	EcR2	AR	AR1
111	2.1					2	0.6	0.4	0.4		1.2	0.4				
112	1.1	0.3		0.5	1.1	1.7					0.6					
113		1	1	1.1	1.5	1.7										
114	1.5	1.5	1.7	0.5	1.5	1.4										
115	1.3	1.5	1.7	1.5	1.5	1.4	0.4				1.2	0.3				
116	1.7	1.5	1.6	1.5	1.5	1.1									0.5	
117	2.1	0.8	2	0.5	0.9	1.8	0.4				1					0.5
122			0.9			0.9									2	
127		1.5	1.5	1.5	1.5	1.7	0.3	0.1	0.4	0.1	1.2	0.5			0.5	
130	1.6	1.8	2	1.8	1.8	1.5								0.5	0.5	0.5
131	1.3	1.8	2	1.5	1.7	1.2					0.3			0.5	0.5	0.5
135	2	2	1.3	1.2	2	1.1	0.2	0.1	0.2		0.6	0.1	0.1			
141	1.2					1.3	0.9		0.9	0.9	1.1		0.9			
275	1.2	1.4	1.7	1.5	1.5	1.1	1.1				1.3					

[a] Abbreviations: Sa, *Staphylococcus aureus*; Se, *Staphylococcus epidermidis*; Ss: *Staphylococcus saprophyticus*; Si, *Staphylococcus intermedius*; Sc, *Staphylococcus chromogenes*; MRSA, Methicillin resistance *Staphylococcus aureus*; Bc, *Bacillus cereus*; Pa, *Pseudomonas aeruginosa*; K, *Kelebsiella*; St, *Salmonella typhimurium*; Lm, *Listeria monocytogenes*; Ec157, *E. coli* O157; EcR, *E. coli* (resistance); EcR2, *E. coli* (resistance); AR, *Acintobacter* (resistance); AR1, *Acintobacter* (resistance).

Table 2. The Biochemical Characterization and Production of Different Enzymes by Selected Isolates

Isolates	Protease	Amylase	Gelatinase	Urease	Citratase	Catalase	Hemolysis	Reverse color	Aerial mycelia
AC 111	-	+	++	-	-	+	Gamma	Grayish-white	Grayish-white
AC112	+	-	+	-	+	+	Gamma	White	Grayish-white
AC113	+	-	++	-	+	+	Gamma	White	Grayish-white
AC114	+	-	-	-	+	+	Gamma	Cream	Light gray
AC115	-	-	-	-	+	+	Gamma	Brown	Dark gray
AC116	-	-	-	-	+	+	Gamma	Brown	Dark gray
AC 117	+	+	-	-	-	+	Beta	Dark gray	Dark gray
AC 122	+	-	-	-	+	+	Beta	White	White
AC127	+	-	-	-	-	+	Gamma	Yellow	Dark gray
AC130	+	-	-	-	-	+	Gamma	Light gray	Dark gray
AC 131	+	-	-	-	+	+	Gamma	Light gray	Dark gray
AC135	+	-	+	-	+	+	Gamma	Brown	Dark gray
AC141	-	-		-	+	+	Beta	Yellow	Yellowish-white
AC275	+	++	++	+	-	+	Beta	Pink	Pink

5. Discussion

Actinomycetes account for 10% of the total bacteria in marine habitats. There is no doubt that the environment exhibits excellent characteristics for the development of fresh and strong bioactive producing microorganisms. The diluting effect of sea water, which provides the high potency required for the effectiveness of bioactive substances in the marine environment, has made marine microbes specifically interesting to observe. Few reports are available regarding the observation of the actinomycetes whose habitats are aquatic environments and thus, their characteristics are poorly understood. Actinomycetes accounts for 70% of the surface of the earth and provides a marvelous source for the isolation and thorough examination of fresh microorganisms. The environment is also excellent for the production of potent bioactive secondary metabolites (7, 8). The present study aimed to isolate actinomycetes from marine environments and thoroughly observe them in regards to the production

of antibacterial substances. Meena et al. observed that *Streptomyces* species show efficient antagonistic activity compared to other actinomycetes (7). This was similar to the present investigation which also showed efficient antagonistic activity of the isolates which may belong to *Streptomyces* species upon preliminary results. The identification of the extracted actinomycetes was carried out using the colony morphology and gram staining. In the present study, the actinomycetes were identified by observing whether or not powdered colonies are present on the surface of agar plate. The filamentous nature of Gram-positive actinomycetes is also stated by Meena et al. (7). While screening the novel secondary metabolites, Meena et al. observed that actinomycetes extracts are often present. This shows there is a greater active antimicrobial activity against Gram-positive bacteria, compared to the Gram-negative ones (7). In the current study, the isolates showed a good antibacterial activity against Staphylococcus species than Gram-negative bacteria including *E. coli*, *Listeria*, etc.

The results showed that high numbers of actinomycetes were biochemically active in marine water. These actinomycetes play a significant role in decomposition of complex organic matter. A number of research workers in earlier investigations have also reported that actinomycete from soil and water bodies possess high number of enzymatically active actinomycetes. Protease, amylase, gelatinase, urease and citratase activity from marin actinomycetes were also reported by numerous studies (9, 10).

There has generally been little attention towards marine actinomycetes. Recent examinations show an excellent potential for marine actinomycetes, and *Streptomyces* species in particular, to be used as a maintainable source of novel bioactive natural products. The results of the present study clearly demonstrate marine actinomycetes characteristics as a powerful novel source of antibiotics. Since actinomycetes are the most important resources of the secondary metabolites, the isolation, characterization and study of actinomycetes is expected to be considerably influential in the discovery of new species.

The recent study has found the starch nitrate agar suitable for the isolation of actinomycetes isolated from Lipar area of The Oman Sea in Iran. Out of 35 isolated actinomycetes, 94% exhibited significant antibacterial activity. The utilization of marine actinomycetes as a production source for novel secondary metabolites is a relatively new concept. However, it is worth mentioning that the recent discovery rate of novel secondary metabolites from marine-based actinomycetes has exceeded the rate of similar discoveries in terrestrial habitat (6). The present study aimed to isolate antibiotic producing actinomycetes from marine environments. Molecular research and antimicrobial compounds extraction against the aforementioned pathogenic strains are also being conducted.

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Authors' Contributions

Mahsa Shams performed the screening of actinomycetes, culture experiments and analysis, and wrote the paper with Bahar Shahnavaz; Bahar Shahnavaz coordinated the project, developed the original idea and wrote the paper with M.S.; Kiarash Ghazvini was responsible for preparation of pathogenic bacteria from hospital; Toraj Valinasab was responsible for water sampling and site characterization.

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