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Antimicrobial Activity of Marine Microorganisms Isolated from the Coast of the Arabian Gulf

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Forty-seven isolates of marine microorganisms were isolated from water, sediment and leaf litter, analyzed using the halo zones on cross streak plate assay, agar-overlay plate assay, agar diffusion method and Liquid (turbidity) assay in terms of their general inhibition effects to test bacteria (*Campylobacter jejuni*, *Bacillus cereus*, *Pseudomonas. aeroginosa* ATCC 15442, *Salmonella enteric* ATCC 13311 and *Haemophilus infuenzae*). The results demonstrated that most isolates are effective against pathogens bacteria. Based on biochemical and physiological properties, they were classified as *Bacillus licheniformis* KSAWD3. This isolate exerted board spectrum antibiotic activities against pathogenic bacteria when compared with the commercial antibiotics used in this study.

Key words: Antimicrobial, Marine microorganism, Pathogenic bacteria.

The appearance of drugs resistance between bacterial pathogens from the hospital, home and industrial environments and in biofilms is reported worldwide. Therefore, effective treatment systems with conservative antibiotics are failed. Since, detection rate of new pharmaceutical discovery is slower than the rate of appearance of resistance, consequently once easily treated infections are now becoming untreatable and fatal[1-3]. Therefore, screening of effective antibiotic producing microorganisms from the natural environment and improvement of novel, broad-spectrum antibiotics particularly targeting the individual pathogen virulence factors is needed as for alternative strategies of antimicrobial treatment[3]. In spite of the fact that the ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity, with estimates ranging between 3 and 5×10^8 different species, till date, the biodiversity of marine microbes and versatility of the their bioactive metabolites have not been fully explored. Recent studies around the world have revealed that the marine organisms syntheses structurally unique bioactive secondary metabolites and small molecules of therapeutic application. Several of these compounds show pharmacological activities and are helpful for the invention and discovery of bioactive compounds, primarily for deadly diseases like cancer, acquired immunodeficiency syndrome (AIDS), arthritis, etc., while other compounds have been developed as analgesics or to treat inflammation, etc[4]. Around 2500 new metabolites were reported from marine organisms ranging from

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microbes to fish, during the period 1977 to 1987, which accounts from less than 1% of the total marine organisms[5]. Nevertheless, the search of new metabolites from marine organisms has resulted in the isolation of more or less 10.000 metabolites many of which are endowed with pharmacodynamic properties[6]. To date approximately 16.000 marine natural products have been isolated from marine organisms and several bioactive compounds had antiviral, antibacterial, antimalarial, anti-inflammatory, antioxidant and cancer potentials. The marine biodiversity of Saudi Arabia have not yet been explored for biomedical molecules. In this context, the present study was initiated towards screening of microorganisms associated with marine environments, including mangrove, around the Saudi Arabia for bioactive molecules active against well known human pathogens.

MATERIALS AND METHODS

Isolation and identification

5 samples each of water, sediment and leaf litter (leaves decayed and remain as a suspension in the water) at intervals of 200 meter at each site were collected from the mangrove area of Tarot Island, Qatif and Dammam on the Arabian Gulf coast. A total of 45 samples were transported to laboratory on ice condition and placed on ZoBells marine agar medium (peptic digest of animal tissue 5g, yeast extract 1g, $Fe(C_6H5O_7).xH_2O$ 0.1g, NaCl 19.45g, $MgCl_2$ 8.8g, Na_2SO_4 3.24g, $CaCl_2$ 1.8g, KCl 0.55g, $NaHCO_3$ 0.16g, KBr 0.08g, $SrCl_2$ 0.034g, H_3BO_3 0.022g, Na_2SiO_3 0.004g, NaF 0.0024g, NH_4NO_3 0.0016g, Na_2HPO_4 0.008g, agar 15g, final pH 7.6±0.2 at 25 °C), Actinomyctes agar medium (Sodium caseinate 2g, L-Asparagine 0.1g, $Na(C_3H_5COO)$ 4g, K_2HPO_4 0.5g, $MgSO_4$ 0.1g, $FeSO_4$ 1mg, agar 15 g, Final pH 8.1±0.2 at 25°C), Brain heart infusion (BHI) (Calf Brains, Infusion from 7.7 g, Beef Heart, Infusion from 9.8 g, Proteose Peptone 10 g, Dextrose 2 g, NaCl 5 g, Na_2HPO_4 2.5g final pH 7.4±0.2 at 25 °C) and Mycological agar medium (enzymatic digest of soybean meal 10g, dextrose 10g, agar 16g final pH 7.4±0.2 at 25 °C) for screening heterotrophic bacteria, actinomycetes and fungi, respectively employing pour plate techniques. Inoculated plates were incubated at 28 °C for 5-10 days. All the single cell colonies were purified and stocked as 40% glycerol mixed cell culture and preserved at -80 °C. one set of culture was stored at 4 °C until use. Marine bacteria were identified based on its morphological, biochemical and physiological properties.

Screening bioactive strains from all marine isolates

Pathogen used for the screening in this study

Screening of bioactive microbial cultures was performed in three phases against standard reference strains obtained from ATCC, USA following thee procedures suggested by Att-ur-Rahman et al.[7], clinical pathogens which were obtained from the Military Hospital in Riyadh, which included, *Salmonella enteric* subsp enteric serovar *Typhimurium* (ATCC 13311), *Shigella somei* (11060), *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC 33591-MRSA), *Campylobacter jejuni*, *Streptococcus pyogens*, *Pseudomonas aeruginosa* (ATCC 15442), *Streptococcus pneumonia*, *Haemophilus infuenzae* and *Salmonella* sp.

Cross streak plate assay

During the first, phase all those presumptive cultures, which showed halo zones around them on Zobell's agar and actinomycetes agar were tested for bioactivity against selected pathogens mentioned in the previous sections cross streaking. Science there was no fungus that showed bioactivity only marine bacterial isolates, obtained were streaked on the plates and test pathogens were streaked diametrically across the streak of the marine isolates as single streak on BHI as well as in Zobell's medium at 37 °C for 3 days and were observed for presence of any inhibition zone at the junction of the cross streak.

Agar-overlay plate assay

Agar-overlay method was also tried in order to ascertain the antibacterial activity of the marine isolates. The marine bacterial isolates were spot inoculated on Zobell's and incubated at 37 °C for 12 hours. While the pathogenic bacteria were grown overnight in BHI broth and cell suspension was prepared in physiological saline after centrifugation at 10000, rpm at 4 °C for 15 min. under aseptic conditions. The prepared cell suspension was mixed with freshly prepared soft BHI (1.2% agar). The prepared soft agar-cell suspension was overlaid on Zobell's in which the marine isolates were already spot inoculated and

colony growth was noted. The agar-overlaid plates were inoculated at 37 °C for 48 hours and growth of pathogens on the pates and probable zone of inhibition around the spot of colony was observed.

Agar well diffusion assay

In the second phase, standard agar well diffusion assay was performed against the pathogens. This test was done on BHI agar plates. A well with 1 cm. diameter was made in agar plate, after inoculating the pathogen on the plate by speared plate, and 100 µl of the cell extract (centrifuged and filtered) obtained from the test isolates grown in the Zobell's broth for 48 hours was added to the well. The plates were incubated at 37 °C for 48 hours, the halo zones formed around the agar well were measured, and the positive cultures were selected.

Liquid (turbidity) assay

During the third phase, inhibition of pathogens growth in BHI broth added with cell free extract of the test cultures at 37 °C for 48 hours was determined by turbidity assy. Growth was measured in terms of turbidity in the flask in UV-Visible spectrophotometer at 600 nm. The strategy behind this experiment was to assess the growth of bacterial pathogens in the presence of bioactive molecules produced by marine bacteria and estimating the resultant turbidity as absorbance at 600 nm.

Protein precipitation for the bioactive antibacterial compound

Two different protocols were followed for precipitation the bioactive supernatant:- 1) four volume of ice cold acetone was added to one volume of supernatant and incubated at -20 °C in a deep freezer for one hour. After incubation, the contents were harvested by centrifugation at 10.000 rpm for 15 min., decant the supernatant, and the above said step was repeated with the pellet. Later the pellets in two tubes were air-dried. The pellet in one tube was dissolved with 15% NaCl solution and with sterile distilled water in the second tube. 2) One volume of supernatant was mixed with 9 volume of ice-cold 100% ethanol and kept in -20 °C in a deep freezer overnight. The tubes were centrifuged at 10.000-rpm for15 min., supernatant decanted, and pellet washed with 90% ice-cold ethanol. The pellet was air-dried and the pellet in one tube was dissolved in15 percentage NaCl solution and other sterile distilled water.

RESULTS

Water, sediment and leaf litter samples from mangrove environments and seawater from Tarot Island, Al-Qatif and Dammam were screened for microorganisms. Unfortunately, no fungi were detected among the cultures that developed on agar medium. Only bacteria and actinomycetes cultures appeared on the plates and focus was given only to bacteria and showed visible halo zones around the colonies developed on the agar medium. Among the 320 marine bacterial isolates that showed halo zones around their colonies. When subjected for further screening process by testing against well known human pathogens, only 47 strains showed activity against the test organisms, *C. jejuni*, *Bacillus cereus*, *P. aeroginosa*, *S. enteric* and *H. infuenzae* (Table 1), subsequent screening through bioassays was performed to select a potential strain that showed relatively maximal bioactivity against the human pathogens. Out of the 47 stains, which showed activity, isolate *B. licheniformis* KSAWD3 was selected as potential strain for further screening and identification.

Identification of the strain

The results obtain for the various characteristics are presented in Table (2). Based on the morphological and biochemical characteristics recorded the marine bacteria KSAWD3 was tentatively paced under the genera *Bcillus* sp.

Re- confirmation of anti bacterial activity by *B. licheniformis*

B. licheniformis KSAWD3 that was selected as potential strain with bioactivity was reconfirmed for its potential for production of bioactive substance as intracellular fraction. After ultra-sonication, the cell soup was centrifuged and filtered and using the filtrate bioassay was performed to reconfirm bioactivity following the same methods mentioned above.

From the results obtained it was observed that the supernatant could demonstrate inhibitory activity against *C. jejuni*, *Bacillus cereus*, *P. aeroginosa*, *S. enteric* and *H. infuenzae,* when tested by agar well diffusion assay. Further, the pathogenic cultures were also tested for growth inhibition by addition *B. licheniformis* culture extract containing prospective bioactive

Table 1. The 47 isolates which showed bioactivity against selected pathogens

Test culture	No. of sensitive pathogens	Bioactivity	Test culture	No. of sensitive pathogens	Bioactivity
KSAWD1	1	++	KSAST7	1	++
KSAWD2	1	+	KSAST8	1	++
KSAWD3	4	++++++	KSAST9	1	+
KSAWD4	2	+	KSAST10	2	+
KSAWD5	1	+	KSAST11	1	+
KSASD1	2	+	KSAST12	2	+
KSASD2	1	++++	KSAST13	1	+++
KSASD3	2	+	KSAST14	2	++
KSAWT1	2	+	KSAST15	2	+
KSAWT2	1	+	KSALT1	1	+
KSAWT3	1	+	KSALT2	1	+
KSAWT4	1	++	KSALT3	1	+
KSAWT5	2	+	KSALT4	1	+
KSAST1	1	+	KSALT5	1	+
KSAST2	1	+	KSALT6	1	+
KSAST3	1	+	KSASQ1	1	+
KSAST4	2	+	KSASQ2	2	+
KSAST5	1	+++	KSASQ3	1	++
KSAST6	1	++	KSASQ4	2	+
KSASQ1	1	++	KSASQ10	1	+
KSASQ2	2	+	KSASQ11	1	++
KSASQ3	1	+	KSASQ12	2	+
KSASQ4	1	++	KSALQ1	1	+
KSASQ5	2	+			

KSU= Kingdom of Saudi Arabia
W=Water S=Sediment L=Leaf litter D=Dammam Q=Al-Qatif T=Tarot Island

Table 2. Characteristics of *B. licheniformis* KSADW3 isolated from water sea of Arabian Gulf, Tarot Island, Saudi Arabia

Variable	Characteristics
Colony	White cream colored, Round, moist
Cell morphology	Small rod
Gram reaction	Positive
Oxidation/Fermentation	Fermentative
Endospora formation	Positive
Relation of free oxgen	Aerobic
Pigment production	Negative
Nitrate reduction	Positive
Indole test	Positive
Citrate utilization test	Positive
Acid production from oxygen	Positive
Gas production from glucose	Positive
Voges prosjaur test	Positive
Methyl red test	Positive

substances in BHI broth and measuring growth in terms of absorbance using a UV spectrophotometer at 600 nm. Results obtained for growth are presented in Table (3) and Fig. 21. From the results, it was inferred that there was significant growth inhibition by *B. licheniformis* culture extract indicating potential bioactivity against human pathogens. However, other methods of bioassay cross streaking against the pathogens as well as the agar-overlaid method did not show any satisfactory results for bioactivity against the tested pathogens.

Antibiotic comparison with *B. licheniformis* KSAWD3 supernatant

The antibiotic activities of *B. licheniformis* KSAWD3 against *C. jejuni*, *P. aeroginosa*, *S. enteric* and *H. infuenzae* were compared with activity of well known antibiotics against the same strains, antibiotic disks were

tested for halo zones around them on the BHI agar medium plates cultured with the pathogens (Table 4).

Protein precipitation tests

The both ways used for protein precipitation did not give satisfactory results.

Table 3. Turbidity assay performed for evaluation growth of reference ATCC strains in the presence of cell free extracts of *B. licheniformis* (OD measured at 600 nm)

Pathogens	Control (OD)	100 µl (free extract)	Difference (OD)
C. jejuni	1.504	0.642	0.862
S. enteric	0.800	0.164	0.636
H. infuenzae	0.901	0.083	0.818
P. aeruginosa	1.658	1.130	0.528

Table 4. Comparative evaluation of bioactivity of substances isolated from *B. licheniformis* KSAWD3 against known pathogens with known antibiotics (mm.)

Treatments	*C. jejuni*	*S. enteric*	*H. infuenzae*	*P. aeruginosa*
B. licheniformis (100µl)	9	10	8	10
Ampcillin	0	14	6	8
pencillin	0	4	3	6
Tetracycline	5	13	7	6
Neomycin	0	4	10	0
Gentamycin	0	3	4	7
Chloroanphenicol	14	14	14	14

DISCUSSION

In the present study a large number of isolates with prospective bioactivity was isolated from marine sediment, sea water and leaf litter samples collected from mangrove areas of marine environments of Arabian Costal in Tarot Island, Al-Qatif and Dammam, Saudi Arabia. All of these isolates were subjected to cross streak assay against known pathogens obtained from ATCC and from Military Hospital in Riyadh. Cross streaking assay did not give satisfactory results since all the cultures recorded inhibitory zones in the range of 2-3 mm. All the isolates showed very small halo zones around the cross point and the results were not satisfactory. During the process of bioactivity evaluation of the extracts it was noted that cross streaking of standard strains against test isolates obtained from environment showed poor inhibition of growth by standard strains probably due to the inadequacy of the nutrients in the cultivation medium and the differential incubation temperature for the respective isolates of marine origin which grew well at 28 °C and the pathogens of human origin which required 37 °C. Likewise during the process of testing bioactivity of the culture extract by agar well diffusion technique there was very clear inhibition zone around the agar well. Hence, the agar well diffusion assay was performed to evaluate the potential of the cell free extract to inhibit the pathogens during growth. The agar well diffusion assay yielded satisfactory results and 47 isolates could show inhibition zones around the wells indicating strong bioactivity. The bioactivity test was repeated to confirm their activity. All of them showed inhibition activity (inhibition zones varying from 4 to 10 mm in diameter around the tested colonies) confirming their potential for production of bioactive molecules.

The current classification of species within the genus *Bacillus* and related genera is well established and is based on a combination of numerous experimental approaches[8]. Systemic studies of the *Bacillus* group have typically focused on terrestrial isolates, even though marine bacilli are noted for their ability to produce different

biologically active compounds [9]. Although the vast majority of bacterial diversity inhabiting marine sediments appears to be Gram-positive, there is evidence to suggest that Gram-positive bacteria comprise a relatively large proportion of these communities[10]. Most of the bacilli of marine origin belonged to the species *Bacillus subtilis*, according to their phenotypic characteristics, antibiotic susceptibility profile, and fatty acids patterns[9,11]. The strain KSADW3 selected as potential strain, and identified as *B. licheniformis* showed strong activity against common well known antibiotics as well as the pathogens *C. jejuni*, *S. enteric*, *H. influenza* and *P. aeruginosa*. The observations suggest that marine *B. licheniformis* KSAWD3 has potential for isolation and use of antibiotic molecule and subsequent use as drug. However further studies are required to characterize the molecule.

CONCLUSION

The results obtained during the course of the present study strongly indicated the potentials of marine bacteria for deriving potential antibiotic biomolecules active against clinical human pathogens at comparable and at enhanced levels when compared with commercially available antibiotics.

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