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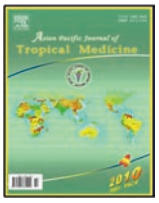

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Isolation of an antibiotic producer *Pseudomonas* sp. from the Persian Gulf

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ABSTRACT

Objective: To investigate the isolation of marine antibiotic-producing bacteria from the Persian Gulf, as an untapped source for searching new natural antibiotics. **Methods:** Initially water and sediment samples were collected from 18 study sites in the some northern areas of Persian Gulf. All of the bacterial isolates using Marine Agar 2216 were inoculated into Marine broth and incubated on a rotary shaker at 28 ℃ for 2-7 days. Bioactivity of their ethyl acetate extract was assessed at 100 mg/mL concentration in disc diffusion method against 6 gram-positive and 5 gram-negative bacteria. Synthetic antibiotics were used as control. **Results:** Altogether, 46 bacterial colonies were isolated. Only one isolate from a marine sediment sample collected at a depth of 10 m, identified as *Pseudomonas aeruginosa* PG-01, was exhibited the capability of antibiotic production. The obtained raw extract from intended bacterium was effective against all tested gram positive bacteria while gram negative bacteria were resistance. Methicillin resisitant *Stapuylococcus aureus* (MRSA), *Streptococcus pyogenes (S. pyogenes)*, *Staphylococcus epidermidis (S. epidermidis)* and *Bacillus cereus (B. cereus)* were the most sensitive strains. All of tested pathogens were multidrug resistant. The antibacterial compound from this bacterium was active even at 120 ℃. The optimized temperature and time for antibacterial metabolite production were 37 ℃ and 72 hrs, respectively. **Conclusions:** Considering the antibacterial effect of *Pseudomonas aeruginosa* PG-01 especially against MRSA, it can be regarded the intended bacterium as a valuable strain and can give hope for treatment of diseases caused by multidrug resistant bacteria.

1. Introduction

Today, increase in the number of drug-resistant pathogens particularly the acquired multi-drug resistant (MDR) strains such as methicillin resistant *Staphylococcus aureus* (MRSA) cause serious public health problem throughout the world. On the other hand, the limited success of strategies such as combinatorial chemistry in providing new agents indicates an uncertain forecast for further antimicrobial therapy[1]. Although the majority of natural products has been isolated from terrestrial sources, but in the last two decade several bioactive substances have been isolated from marine bacteria and are new resources for the development of medically useful compounds. For the first time, in 1947, Rosenfeld and Zobell demonstrated that marine bacteria produce antimicrobial substances[2]. Nowadays, several antibiotic compounds from marine microorganisms have been reported, including Pyron from *Pseudomonas*[3], Loloatin from *Bacillus*[4], Tiomarinol from *Altromonas*[5], Marinopyrroles from *Streptomyces*[6], Agrochelin from *Agrobacterium*[7], Koromicin from *Pseudoalteromonas*[8] and *Pelagiomicin* from *Pelagiobacter variabilis*[9] and so on. Because of the enormous biodiversity in the marine environment, in the case of microorganisms, sea water is composed of 78 million microscopic cell per ounce or the bottom, which mimic the soil, contain more than 1 billion cells in the volume of an ordinary cube of sugar[10]; this environment as an amuzing unique community provides the most prolific sources for new natural drugs. The present investigation was focused on the isolation of potent antibiotic-producing bacteria from several water and sediment samples collected form the Persian Gulf.

2. Materials and methods

2.1. Sampling procedure

From March to June 2009, samples were collected from surface seawater, deep seawater, coastal seawater, marine bed sediment and mangrove forest sediment at 18 study

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sites in the some northern areas of Persian Gulf, including Mahshahr port, Bahrakan port and Qeshm Island. Water samples were collected by niskin bottles (water sampler) which were sterilized by 75% ethanol prior to water collection and washed by seawater. Samples were collected by water sampler in sterilized glass bottles. Sediments were collected by sterilized van veen grab in sterilized plastic bags. All of the samples were kept at 4 ℃ up to reach to the laboratory.

2.2. Isolation of bacterial strains from marine samples

The water samples (8 μL) were inoculated into Marine Agar 2216 (High Media). Sediment samples after making serial dilution using sterilized sea water were spread on the entire surface of Marine Agar 2216. After incubation at 28 ℃ for 3 days, bacterial colonies with different morphology and pigmentation were chosen for further process.

2.3. Preparation of extract

Pure colonies of bacterial isolates were transferred to Erlenmeyer flasks containing Marine Broth (Laboratorios CONDA) medium and were incubated on a rotatory shaker (150 rpm) at 28 ℃ to produce secondary metabolites. After 2-7 days, the broth culture first was centrifuged at 1 300 rpm (×g) for 20 mins at 4 ℃, and then supernatant was extracted using same volume of ethyl acetate (EtoAc). Solvent was removed at 37 ℃.

2.4. Antibacterial activity assay

All of the test strains were grown in Muller Hinton Broth (MHB, Merck) medium at 37 ℃ for 24 hrs. Final inoculums sizes were adjusted to 10^8 cfu/mL with reference to the McFarland turbidometery[11,12]. Antibacterial activity was assessed two times by disc diffusion method on Mulller Hinton Agar (MHA, Merck) using gram positive bacteria including MRSA, *Staphylococcus epidermidis (S. epidermidis)*, *Bacillus anthracis (B. anthracis)*, *Bacillus cereus (B. cereu)*, *Bacillus pumilus (B. pumilus)*, *Listeria monocytogenes (L. monocytogenes)*, *Streptococcus pyogenes (S. pyogenes)* and gram negative bacteria including *Pseudomonas aeruginosa (P. aeruginosa)*, *Escherichia coli (E. coli)*, *Proteus mirabilis (P. mirabilis)*, *Salmonella typhi (S. typhi)* and *Klebsiella pneumoniae (K. pneumoniae)* as test microorganisms. The dried crude extract was dissolved in methanol to a concentration of 100 mg/mL. 35 μL volume samples were added to the sterile filter paper disc (6 mm in diameter) to assay the antibacterial activity[13], and the disc was dried for 30 mins. Then, discs were placed on lawn cultures. Zone of inhibition was measured in mm after incubation for 24 hrs at 37 ℃. Eight different antibiotics including Vancomycin (VA) 30 mcg, Erythromycin (E) 15 mcg, Methicillin (MT) 5 mcg, Carbenicillin (CB) 100 mcg, Nitrofurantion (NF) 300 mcg, Penicillin (P) 10 mcg, Oxacillin (OX) 1 mcg and Streptomycin (s) 10 mcg were used as control. All these synthetic antibiotic discs were produced by Difco.

2.5. Effect of the time and temperature on the production of antibacterial metabolite

To find out the optimum temperature for production of antibacterial metabolite by isolated marine bacterium, this bacterium was grown in Erlenmeyer flasks containing Marine Broth at 25 ℃, 28 ℃, 31 ℃, 34 ℃, 37 ℃, 40 ℃ and 43 ℃; after 3 days, ethyl acetate extract prepared from each flask was tested at 100 mg/mL concentration against MRSA. Also, for determination of optimum intubation time, this isolate was grown in Marine Broth at optimum temperature and samples were harvested after every 12 hrs from 0 till 120 hrs, and then ethyl acetate extract prepared from each sample was tested at 100 mg/mL against MRSA.

2.6. Heat and protease treatment on antibacterial metabolite

To find out thermostability of this antibacterial metabolite, 1 mL of supernatant was harvested from broth culture and treated by temperature at 25 ℃, 37 ℃, 56 ℃, 70 ℃ and 90 ℃for 10 mins and 120 ℃ for 15 mins; also 200 μL of supernatant was treated by 20 μL of pepsin (Merck) at final concentration 100 mg/mL[14]. Finally, the result of these treatments was found by measurement of the inhibition zone against MRSA.

2.7. Identification of antibiotic-producing strain

The isolated marine bacterial strain with antibacterial activity was identified to the species level by observing its morphology and biochemical reactions according to the methods described by Lyudmila *et al* and Bergey´s manual for systematic of bacteriology[15,16].

3. Results

3.1. Isolation and identification of antibiotic-producing strain

Overall, 46 strains of marine bacteria were isolated from water and sediment samples of the Persian Gulf, but only one brown-pigmented bacterium isolated from a marine sediment sample collected at a depth of 10 m was exhibited the capability of producing antibiotic compounds (Table 1). On the basis of biochemical diagnostic tests (Table 2), this bacterium was identified as *Pseudomonas aeruginosa* PG-01.

3.2. Antibacterial activity test

The antibacterial compound produced by intended bacterium was effective against all of the tested gram positive bacteria while gram negative bacteria showed resistance to it (Table 1). MRSA, *S. pyogenes*, *S. epidermidis* and *B. cereus* exhibited the most sensitivity to *Pseudomonas aeruginosa* PG-01. All tested bacteria were resistant to Oxacillin and Penicillin and most of the bacterial species were resistance to Methicillin. The inhibition zone diameter for Vancomycin and *Pseudomonas aeruginosa* PG-01 against MRSA were 22 and 30 mm (Figure1), respectively.

3.3. Optimization of temperature and time for production of antagonistic action in Pseudomonas aeruginosa PG-01

Results of the effect of time and temperature on the production of antibacterial metabolite based on disc diffusion showed that although *Pseudomonas aeruginosa* PG-01 was able to grow from 25 ℃ to 43 ℃, the optimum temperature for production of the antagonistic component was 37 ℃ (Figure 2), with a maximum inhibitory zone of 33

Table 1

Inhibition zones of ethyl acetate extract of *Pseudomonas aeruginosa* PG-01 against some clinical pathogens compared with commercial antibiotics (mm)*.

Bacterial species(100 mg/mL)	Antibiotics Disc (mm)								
	NF	VA	CB	P	FM	MT	OX	S	E
B. pumilus	16	15	13	R	17	23	R	16	15
B. anthracis	18	20	16	R	21	23	R	14	21
B. cereus	20	15	12	R	18	R	R	15	15
S. aureus	30	22	31	R	R	R	R	12	16
S. epidermidis	27	14	25	R	23	R	R	13	14
Str. Pyogenes	32	18	26	R	23	R	R	18	25
E. coli	R	R	22	R	20	R	R	R	R
S. typhi	R	10	28	R	21	R	R	R	R
P. mirabilis	R	24	28	R	10	R	R	R	R
K. pneumoiae	R	13	26	R	18	R	R	R	R
P. aeruginosa	R	R	25	R	R	R	R	R	R

*: (6mm) diameter disc;
R: Resistant; –: Not used.

mm on the 5th days; also, maximum antibacterial compound production at 37 ℃ temperature was achierved in 72 hrs culture-old broth (Figure 3).

Table 2

Biochemical tests of antibiotic-producing bacterium isolated from sediment of Persian Gulf, Iran.

Test	Result	Test	Result
Gram´s reaction	–	Acid from	
Cell shape	rod	D-glucose	+
Motility	+	L-arabinose	–
Indol production	–	D-mannitol	–
O-F test	+	Maltose	–
Catalase	+	Xylose	+
Oxidase	+	Growth at 4 ℃	–
Citrate Utilization	+	Growth at 42 ℃	+
Urease	+	Growth at 8% Nacl	–
Nitrate reduction	+	Sensitivity to antibiotics	
Gelatin hydrolysis	+	Penicillin	–
TSI	+	Tetracylnine	–

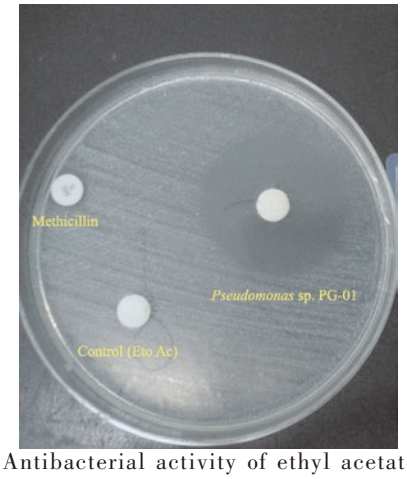

Figure 1. Antibacterial activity of ethyl acetate extract of *Pseudomonas aeruginosa* PG-01 against MRSA.

3.4. Preliminary characterization of antibacterial metabolite

The antibacterial component didn´t completely loose its activity even after the cell free supernatant was autoclaved for 15 mins at 121 ℃. The zone of inhibition produced by heat-treated culture supernatant decreased to 14 mm, compared to the control (28 mm at 24 ℃) (Figure 4). The antibacterial activity of the cell free supernatant was not affected after enzymatic treatment. The zone of inhibition (28 mm) observed after treatment with the Pepsin was equal to the untreated cell free supernatant that served as a control.

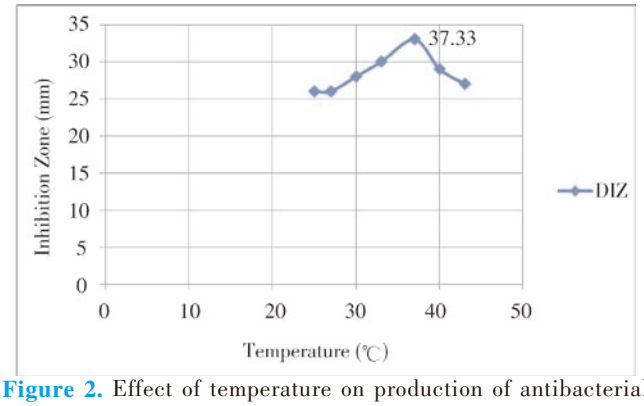

Figure 2. Effect of temperature on production of antibacterial metabolite by *Pseudomonas aeruginosa* PG-01.
DIZ: Diameter of Inhibition Zone.

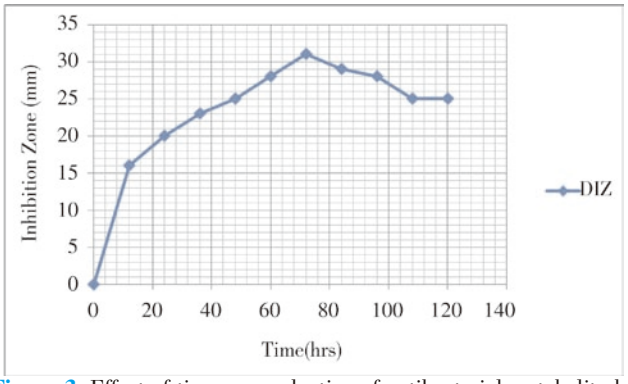

Figure 3. Effect of time on production of antibacterial metabolite by *Pseudomonas aeruginosa* PG-01.
DIZ: Diameter of Inhibition Zone.

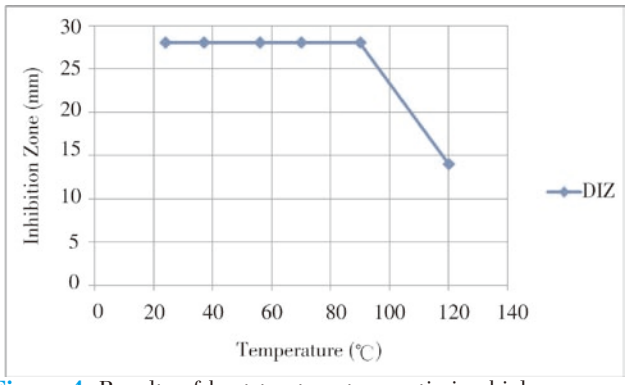

Figure 4. Results of heat treatment on antimicrobial compound produced by marine *Pseudomonas aeruginosa* PG-01.
DIZ: Diameter of Inhibition Zone.

4. Discussion

The emergence of resistant strains to commonly used antibiotics among human microbial pathogens has necessitated the researches to discovery the new antimicrobial agents that are produced in natural way. The oceans, which cover almost 70% of the earth's surface[17], contain a variety species, many of which have no terrestrial counterparts. So, marine bacteria as a largely untapped source are a good choice for searching novel biologically active secondary metabolites. In the present study, we have attempts to isolate marine antibiotic-producing bacteria from the Persian Gulf, as an untapped source for searching new natural drugs. Among the isolates, only one bacterium identified as *Pseudomonas aeruginosa* PG-01 was found to be effective against clinical pathogens. Pseudomonas are well-known widespread microorganism, which have been isolated from variety of natural sources, soil, plants, mineral waters and clinical specimen and they are characterized by a high level of metabolic diversity[15]. Based on these results, the antibacterial compound produced by *Pseudomonas aeruginosa* PG-01 had antibacterial effect on all of the tested gram-positive bacteria while all of the gram-negative bacteria were resistant to it. This difference may be due to several possible reasons such as permeability barrier provided by the presence of cell wall with multilayer structure in gram-negative bacteria or the membrane accumulation mechanisms or presence of enzymes in periplasmic space which are able to break down foreign molecules introduced from outside[18]. Antibacterial activity of intended marine isolate against MRSA was remarkable, while this strain was resistant to broad spectrum antibiotics, so, this suggests new hopes for treatment of the infections caused by MRSA. Furthermore, all of the tested bacillus strains as resistant spore forming bacteria showed a high degree of sensitivity to the raw extract of *Pseudomonas aeruginosa* PG-01; so, the active compound from this extract can be used as antiseptic compound. Treatment of the cell free supernatant with Pepsin or heating even up to 121 ℃ didn't cause to active supernatant completely loose its activity. These results show that the active constituent of the antibiotic produced by *Pseudomonas aeruginosa* PG-01 maybe not proteinaceous in nature, like these results about of antibacterial compounds produced by *Pseudomonas* sp. has been reported previously[19,20]. Finally, Comparing the antibacterial effect of the obtained extract from *Pseudomonas* sp. PG-01 especially against MRSA, it can regard the intended bacterium as a valuable strain and further studies should be performed in order to purify and identify the chemical structure of its antimicrobial compound.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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