Isolation of a potent antibiotic producer bacterium, especially against MRSA, from northern region of the Persian Gulf

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Abstract

Nowadays, emergence and prevalence of MRSA (Methicillin Resistant *Staphylococcus aureus*) strain have become a great global concern in 21st century, so, it is necessary to discover new antibiotics against this pathogen. The aim of this study was isolation and evaluation marine bacteria from the Persian Gulf in order to finding antibiotic compounds against some pathogenic bacteria. For this purpose, water and sediment samples were collected from the Persian Gulf during March to October 2009. The antibacterial activity of the isolated bacteria was assessed using disc diffusion method. The Growth Curve Interference (GCI) parameter against MRSA was determined for the high potential antibiotic producing strain. The most important factors affecting fermentation conditions in antibiotic production were also optimized. Definite identification of intended isolate was confirmed by 16S rRNA sequencing. Altogether, 51 bacterial colony was isolated and among them only 3 bacterium showed antibacterial activity. *Pseudoalteromonas piscicida* PG-01 isolated from a sediment sample was chosen as the best antibiotic producing strain. This strain was effective against all tested Gram-positive bacteria, had good anti-MRSA activity and also GCI value against MRSA was two times lower than MIC value. Among the optimized fermentation parameters, carbon and nitrogen sources play major role in efficacy of optimized antibiotic production. Ultrastructural study on the effect of intended antibiotic compounds on MRSA using TEM revealed that the target site for this compound is cell wall. Considering the antibacterial effect of PG-01 strain especially against MRSA, intended antibiotic compounds can gives hope for treatment of diseases caused by multi-drug resistant bacteria.

KEY WORDS: MRSA, natural antimicrobial, *Pseudoalteromonas piscicida* PG-01, marine environment

INTRODUCTION

Despite the use of antibiotics for nearly eight decades, infectious diseases continue to have an impact on human health and cause morbidity and mortality worldwide. In recent decades, the growing emergence of multi-drug resistant bacteria (MDRB) has caused one of the major challenges for infectious disease treatment. Methicillin Resistant *Staphylococcus aureus* (MRSA) causes a wide range of diseases from skin infections to invasive diseases such as necrotizing pneumonia, has become one of the greatest challenges for modern antimicrobial therapy in both industries and developing countries, particularly because due to multi-drug resistant strains. Presently, three different types of MRSA have been described: hospital-acquired MRSA (HA-MRSA), community-acquired MRSA (CA-MRSA) and lifestock-associated MRSA (LA-MRSA). These strains are world-widely distributed and cause medication failure in clinical cases. MRSA strains have acquired a mobile genetic element called staphylococcal cassette chromosome (SCC*mec*), carrying *mec*A encoding a penicillin-binding protein (PBP2a) with low affinity to beta-lactam antibiotics [1, 2]. The most frequent SCC*mec* types found in hospital isolates are I, II and III; also type IV is associated with community-acquired strains. Now, however, nosocomial infections are major problem around the world due to increasing bacterial resistance to classical antimicrobials and searching for new antibacterials with new antibacterial mechanisms is of great urgent. From the early 1960s, research groups began to concentrate on the oceans for novel bioactive compounds [3]. Marine microorganisms can be considered as an untapped source of new bioactive molecules because 1) certain classes of marine bacteria have developed unique adaptation mechanisms [4] to the physiological, physical, chemical and biological conditions e.g., salinity, pressure, temperature, and depletion of micronutrients found in the oceans, which is reflected in their physiology and biochemical properties [2, 5]; 2) competition among microbes for space and food [6] in some marine niches especially about of the particle associ-

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ated bacteria and sediment inhabiting bacteria has exerted a driving force on bacterial selection leading to new adaptive strategies and the synthesis of new metabolites, and 3) some bacteria have developed certain adaptation mechanisms resulting in production of bioactive metabolites which may be useful for their defense against predators such as protozoans. Furthermore, more than 95% of earth's biosphere is oceans and during the past five decades more than 10,000 marine metabolites have been isolated and characterized, which 18% of these bioactive compounds were obtained from bacterial sources [3]. Today, one of the main goals of the marine biotechnology is finding natural substances originated from marine microorganisms with pharmaceutical applications such as anti-cancer, anti-infection and anti-inflammation activities. Till now, several marine-derived antibiotics with anti-MRSA activity has been reported. These novel antibiotics have been isolated from five genera of marine bacteria including *Pseudoalteromonas, Pesudomonas, Bacillus, Marinospora*, and *Streptomyces* [7]. Persian Gulf, in southwest Asia, is a relatively shallow and extension of Indian Ocean located between Iran and the Arabian Peninsula; it is bordered by Iran on the entire north-eastern coastline. This study mainly focused on 1) isolation and identification of potent antibiotic producer bacteria, especially against MRSA, from the northern regions of Persian Gulf, 2), optimization of antibiotic production and preliminary purification and characterization of intended antibiotic compound, and 3) determination the mechanism of action for intended antibiotic compound against MRSA using Transmission Electron Microscopy (TEM).

MATERIALS AND METHODS

Sample collection

During March to October 2009, 3 sampling regions in Persian Gulf were selected (Figure 1) and samples of coastal water; surface water, deep water, coastal sediment, bed sediment and mangrove forest sediment were collected from 17 study sites in some northern area of Persian Gulf. Water samples were collected using sterilized-niskin bottle (using 70% ethanol prior to sampling); subsequently, samples were collected in sterilized glass bottles. Sediment samples were collected by sterilized van veen grab in to sterilized plastic bags. These samples were kept at 4˚C (placed on ice) until delivery to laboratory.

Isolation procedure

In order to isolation of marine bacteria from water samples, 8 µl of samples were spread on agar plates with marine agar 2216 (Himedia, India). Sediment samples (1 g) were transferred to test tube containing 1ml of sterilized sea water, vigorously mixed, and finally ten fold serial dilutions were prepared and aliquots (20 µl) of each dilution were spread

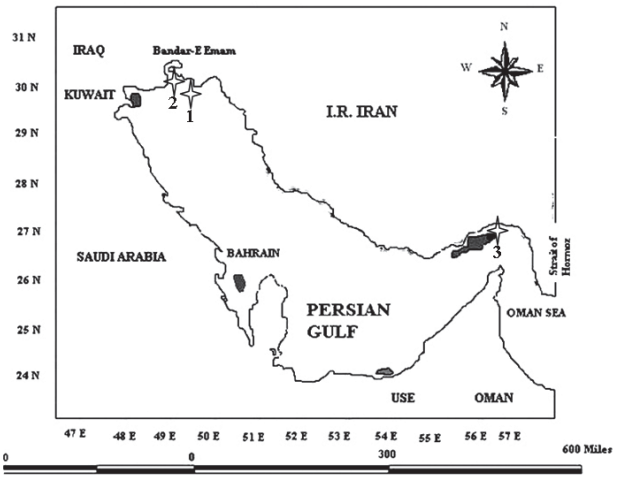


FIGURE 1. Map of sampling sites in the Persian Gulf. 1: Bahrakan port, 2: Mahshahr port, 3: Qeshm Island

on the entire surface of marine agar 2216 [8]. Isolation of marine bacteria from both water and sediment samples was performed using pour plate technique, too. The inoculated plates were incubated at 24, 30 and 37˚C for 3-5 days, developed colonies were purified by repeatedly subculturing on marine agar and finally, those colonies with distinct characteristics such as pigmentation, size, opacity, elevation, margin and surface were chosen for further processing [9].

Screening of isolates for antibacterial activity

All of the bacterial isolates from different samples were screened for the production of antimicrobial substances by primary screening method. Pure colonies were grown in maine broth medium (MB) (Laboratorios CONDA, Spain) at 30˚C for 5-7 days. The broth cultures were centrifuged at 10000 rpm for 15 min at 4˚C, and the cell free supernatants were filtered through 0.22 µm pore size filter (Millipore, Italy) and it was surveyed for antibacterial activity. Antibacterial activity was assayed by standard agar disk diffusion test against standard test organisms including *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus subtilis* (ATCC 12711), *Listeria monocytogenes* (ATCC 19112), *Escherichia coli* (ATCC 11303), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (ATCC 19430), and with Methicillin Resistant *Staphylococcus aureus* (MRSA) as clinical strain. Standard bacterial strains were obtained from Iranian Research Organization for Science and Technology (IROST) and MRSA that was also MDR (multi-drug resistant) was collected from the Golestan Hospital, Ahavz, Iran. Tested bacteria were cultured in Muller Hinton Broth (MHB, Merck, Germany) medium at 37˚C for 5-8 h until adjusted to the 0.5 Mc Farland turbidity (10^8 cfu/ml) [10]. 100 µl of these suspensions was inoculated on Muller Hinton Agar (MHA, Merck, Germany) plates as lawn culture by sterile cotton swab and remained for 15 min for absorption of ex-

cess moisture. The sterile filter paper discs (6 mm diameter) [11] were saturated by 50 µl of cell free supernatants of the marine isolates and then were placed on lawn cultures. After a diffusion period of 1h, the plates were incubated at 37˚C for 24h and the inhibition zone around each disc was measured in mm. This experiment was carried out in triplicate.

Antibacterial activity of the highest potential producer

After primary screening, the isolate responsible for the greatest zone of inhibition was chosen for further study. In order to test the antibacterial activity, after 3 days of incubation, the broth culture was centrifuged at 8000 rpm for 15 min at 4˚C, and then supernatant was extracted using equal volume of ethyl acetate. Solvent was removed at 37˚C. The dried crude extract was dissolved in ethyl acetate at concentration of 50 mg/ml. The antibacterial activity of the obtained raw extract was evaluated at this concentration using disc diffusion method against Gram-positive microorganisms including *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228), *B. subtilis* (ATCC 12711), *L. monocytogenes* (ATCC 19112), MRSA, and *L. monocytogenes* (clinical isolate) and Gram-negative bacteria including *E. coli* (ATCC 11303), *P. aeruginosa* (ATCC 27853), *S. typhi* (ATCC 19430), *Bordetella bronchiseptica* (clinical isolate) and *Brucella melitensis* (clinical isolate). The clinical isolates were prepared from the Golestan Hospital, Ahvaz, Iran. A disc soaked in ethyl acetate was kept as negative control. The following antibiotics were used as control (µg/disc): vancomycin (VA) 30 µg, methicillin (MT) 5 µg, nitrofurantoin (FM) 300 µg, penicillin (P) 10 µg, and colistin (CL) 10 µg. All of these synthetic antibiotic discs were produced by Difco, USA. The MIC (Minimal Inhibitory Concentration) of the antimicrobial agent from the best producer strain was determined against two somewhat important and more sensitive bacteria by macro broth dilution assay method [12,13]. MBC (Minimal Inhibitory Concentration) was also determined.

Determination of GCI (Growth Curve Interference) parameter

GCI is a parameter which defined as the lowest concentration of an antibiotic compound that modifies the growth curve of a tested pathogen comparing to a control without antibiotic [14]. Tested pathogen in this study was MRSA. For determination of GCI, MIC was determined by the macro broth dilution assay method. At first, 15 ml of extract at four concentrations including equal to MIC and sub-MICs was prepared. In the next step, four flasks containing 15 ml Muller Hinton Broth medium of 0.5 Mc Farland turbidity of MRSA culture was prepared. Finally, each of the prepared different extracts were added to above flasks and then incubated at 37˚C on a rotatory shaker; biomass accumulation was measured from 0 till 10 h (every 1 h) by spectrophotometry reading of optical density (OD) at 600 nm until tested bacterium was entered in stationary and death phase of growth. A flask containing 30 ml MHB with the 0.5 Mc Farland turbidity of MRSA but without extract was used as control. Related curves were drawn and GCI was determined.

Optimization of the culture conditions and processing parameters for antibiotic production

The preliminary fermentation conditions for production of antibiotic by PG-01 isolate was prepared in Marine Broth (as control medium) by incubating at 30˚C for 3 days. Also, a synthetic medium containing of glucose (primary carbon source) 1 g/l and peptone (primary nitrogen source) 5 g/l of sterilized-sea water with initial pH 7.5 was prepared in order to achievement a medium as an alternative for Marine Broth (as un-optimized medium). The required sea water for the optimization process was collected from a site with 2 km distance from seashore and then sterilized by 0.22 µm Millipore filter. Furthermore, in order to access to the optimum NaCl concentration, both seawater and distilled water were separately used for preparation of synthetic medium. Optimization process for antibacterial compound production was studied based on carbon and nitrogen sources, optimum temperature, optimum initial pH, optimum NaCl concentration and optimum incubation time. In these experiments one variable was changed at a time but in order to achieve a suitable medium for antibiotic production by this marine isolate, once a factor will be optimized it was used for further optimization process; the carbon source and NaCl concentration were the first and the last optimized factors, respectively. The bacterium was grown at different conditions such as different temperatures (25, 28, 31, 34, 37, 40 and 43˚C), pH values (6, 6.5, 7, 7.5, 8, 8.5 and 9), NaCl concentration (0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8 and 10% (w/v)), various carbon sources (sorbitol, dextrose, galactose, fructose, glycerol, xylose, glucose and starch), various nitrogen sources (peptone, tryptone, yeast extract, meat extract, KNO_3, casein, L-arginine and tyrosine) and different incubation times (12, 24, 36, 48, 60, 72, 84, 96 and 120 h), and then the efficiency of the optimized parameters was discovered using disc diffusion method against MRSA [15]. For anti-MRSA assay, 35 µl of the filter-sterilized (0.45 µm) supernatant prepared from each of the harvested samples from production media under optimized conditions was tested. Also, the best solvent for the antibiotic extraction was optimized.

Kinetics of growth and antibiotic production

Co-variation between bacterial growth and antibiotic production was investigated in a batch system. The culture was undertaken in marine broth medium. A 10 ml inoculum prepared from a fresh overnight culture (was grown in a 250 Erlenmeyer containing 100 ml medium on a rotary

shaker at 30˚C, 140 rpm) was inoculated in this medium and incubated at 37˚C on a rotatory shaker (140 rpm). Cell growth was measured at OD_{600}. Samples were harvested from production media at 2-h intervals from 0 till 6h and 4h intervals from 6h till stationary and death phase of growth. Supernatants were harvested from these samples by centrifugation at 8000 rpm at 4˚C for 10 min and subsequently were sterilized by filtration 0.45 µm and then used for antibacterial assay. Anti-MRSA activity of the harvested samples was assessed using disc diffusion method [16].

Purification of antibacterial compound

To reveal the antimicrobial constituents clearly by bioautography, the supernatant and the raw ethyl acetate extract (prepared from broth culture) possessing antibacterial activity were partially purified by TLC plates (Merck, Germany) using n-butanol: acetic acid: distilled water (v/v 4:4:1) as mobile phases. The separated components were visualized under visible and ultraviolet light (254 and 366 nm). The retardation factor (R_f) values of the spots were also determined. For bioautographic analysis, developed TLC plate were dried overnight and the respective bands were scraped out separately along with silica and dissolved in ethyl acetate, and then centrifuged at 5000 rpm for 4 min and supernatants were passed from filter (0.45 µm) [17] . All of the bands were subjected to antibacterial activity assay against MRSA using disc diffusion method and also their activity was compared with the whole extract. Oxytetracycline was used as control.

Thermal and enzymatic stability of the intended antibacterial compound

For determining the effect of temperature on stability of the antibiotic, 1 ml of supernatant was harvested from broth culture and was added to sterile screw capped ampoules and treated at 4˚C in refrigerator for 24h, at 25, 37, 56, 70 and 90˚C in a water bath for 30 min and at 120˚C in autoclave for 15 min [18, 19]. Enzymatic stability was also examined by treatment of 200 µl of supernatant by 20 µl from each of pepsin (Merck, Germany), Proteinase K (Fermentas, Canada) and Pancreatin enzymes (Philip Harris, UK) at 100 mg/ml final concentration [20]. Finally, the residual antibacterial activity of heat- and enzymatic-treated samples was determined by measurement of the inhibition zone against MRSA. Untreated preparations were used as controls. For all enzymes a positive enzymatic effect was demonstrated by the lack of an inhibition halo around the enzyme-impregnated paper [21].

Determination the site of antibacterial compound accumulation

In order to finding the possible intra-cellular location of the intended active compounds with anti-MRSA activity, 5 ml of broth culture (containing whole cell) was harvested form 3 day's age culture and then sonicated (Bandelin Sonoplus, Germany) at 120 MHz for 40 s at 4˚C [22]. 1 ml of this sonicated sample was filter-sterilized using 0.45 µm filter. An un-sonicated sample was prepared, too. Finally, for antibacterial activity assay, 35 µl of both samples was tested against MRSA using disc diffusion method [21].

Ultrastructural study on the effect of intended antibacterial compound against MRSA

Determination the mechanism of action of intended antibacterial compound on MRSA was done by Transmission Electron Microscope (TEM) (Philips, Netherlands). After determination of MIC value of the ethyl acetate extract of PG-01 isolate, MRSA cells were grown in MHB medium to 0.5 Mc Farland turbidity, then immediately these cells were treated with MIC concentration (40 mg/ml) and sub-MIC concentrations (37, 34 and 30 mg/ml) for 7 h. Samples were harvested at 2, 4 and 7 h. Finally, all of the samples were mixed together on a vortex mixer and centrifuged at 4500 rpm, and then the cells were fixed in 4% glutaraldehyde [23]. MRSA cells without the extract were considered as control [24] . Then, treated and control cells were post-fixed in 1% osmium tetroxide. Washing was done by sodium cacodylate buffer and then these samples were dehydrated in gradualed gold ethanol series (30-100%) [25]. Dried cell blocks were infiltrated by epoxy resin and Ultrathin sections were prepared. Subsequently, the sections were stained by Uranyl acetate and lead citrate. Ultimately, the ultrathin sections were analyzed using TEM.

Strain identification with 16S rRNA gene sequencing

The highest potential antibiotic producer strain (PG-01 isolate) was selected for molecular identification by 16S rRNA sequencing. In order to genomic DNA extraction, a single colony of intended bacterium was subcultured in MB at 30°C for 48 h and DNA was extracted by a commercial kit (Gen Fanavaran, Iran), according to the manufacture instructions. Full length sequence of 16S rRNA gene was amplified from the isolated genomic DNA using the following universal bacterial 16S rRNA primers: Forward strand: 5′-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG and Reverse strand: CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT-5′ [26]. Polymerase chain reaction (PCR) was performed in a 50 µl mix reaction containing 50 mM MgCl2, 10 mM of each deoxynucleoside triphosphates (dATP, dTTP, dGTP and dCTP), 10×PCR buffer, 10 ρmol/µl of forward and reverse primers and 5 U of Taq polymerase. The PCR thermal profile was consisted of denaturation at 94˚C for 5 min followed by 30 cycles of amplification, each consisting of denaturation at 94˚C for 1 min, annealing at 62˚C for 40 sec, and elongation at 72˚C for 2.5 min. A fi-

nal elongation step at 72˚C for 20 min was also included. The Electrophoresis of PCR product was done on 1% agarose gel for 55 min at 95 v. PCR product was sequenced by Gen Fanavaran biotech corp. The obtained sequences were compared in BLAST analysis with available data of Genbank, NCBI. Finally, phylogenetic tree was constructed by CLC Main Workbench 6.0 software program using the neighbour-joining algorithm [27]. Also, the obtained sequence from PG-01 isolate was compared to the sequence of its closest phylogenetic neighbour strain by chimera check/pintail program (http://www.bioinformatics-toolkit.org/Web-Pintail/).

Phenotypic characterization of PG-01 isolate

Different characteristics of the PG-01 isolate including Gram stain, pigmentation, motility and utilization of different carbon sources were determined. Morphological study of this isolate was done using scanning electron microscopy (SEM). A single colony of antibiotic producer bacterium from 24h culture on marine agar was dissolved in 5 ml sterile distilled-water and subsequently 2 µl from this suspension was harvested and fixed in 1% glutaraldehyde and then, cells were dehydrated using a graded acetone series. Finally the air-dried prepared sample was coated by silver for 7 min and surface morphology was studied by a Leo1455 Vp scanning electron microscope.

RESULTS

Fifty one bacterial isolates were isolated from different seawater and sediment samples collected from Persian Gulf. The 2, 9, 13, 3, 18 and 6 isolates were obtained from coastal water, surface water, deep water, coastal sediment, bed sediment and mangrove forest sediment, respectively (Table 1). Of these, three stains have the capability of antibiotic production (Table 1) which were named as PG-01, PG-02 and PG-03 isolates. The PG-01 and PG-02 isolates were from bed sediment (at a depth of 10 m) and surface water, respectively and both samples were collected from Mahshahr port, north of Persian Gulf. But PG-03 was isolated from a mangrove forest sediment sample collected from Qeshm Island, south of Persian Gulf. These three bacteria were maintained at 15˚C on marine agar slants and frozen in marine broth with 20% glycerol. Primary assays showed that PG-01 and PG-02 were effective only against tested Gram-positive bacteria while PG-03 isolate presented a low level of antibacterial activity against some Gram-positive and Gram-negative bacteria. However, PG-01 strain considered as the most potent antibiotic producer isolate; it was effective on all of the tested Gram-positive bacteria and the obtained DIZ (Diameter of inhibition zone) of this strain comparing to the PG-02 and PG-03 isolates was remarkable (Table 2). The obtained raw extract from the PG-01 isolate was effective against all tested Gram-positive bacteria while Gram-negative bacteria showed resistance to it (Table 3). Among tested pathogens, *S. aureus* ATCC and MRSA were the most sensitive strains to this extract; however, its antibacterial activity against other Gram-positive bacteria was remarkable, too. Furthermore, *L. monocytogenes* was the most resistant Gram-positive bacterium to the PG-01 extract. All tested clinical

TABLE 1. Number of bacterial strains isolated from different samples collected from the northern regions of Persian Gulf

Sampling site	Number of bacterial strains isolated from different marine samples						Isolated strain (No.)	Strain with antibacterial activity (No.)
	coastal water	surface water	deep water	coastal sediment	bed sediment	mangrove forest sediment		
Bahrakan port	-	2	4	1	4	-	11	0
Mahshahr port	-	4	5	-	7	-	16	2
Qeshm island	2	3	4	2	7	6	24	1

TABLE 2. Results of the determination of spectrum and potential activity of three antibacterial compound producer bacteria isolated from Persian Gulf.

Marine strain	Sample (origin)	Spectrum activity	Antibacterial activity
PG-01	Bed sediment	Gram-positive	+++
PG-02	Surface water	Gram-positive	++
PG-03	mangrove forest sediment	Gram-positive and negative	++

(+): DIZ > 9 mm; (++): Against Gram-positive bacteria: DIZ:9-14 mm; (++): Against Gram-negative bacteria: DIZ:9-12 mm; (+++): Against Gram-positive bacteria: DIZ>14 mm; (+++): Against Gram-negative bacteria: DIZ> 12 mm

FIGURE 2. Anti-MRSA activity of PG-01 strain extract compared with vancomycin. (The diameter of disc is 6 mm)

TABLE 3. Antibacterial activity of ethyl acetate extract of *Pseudoalteromonas piscicida* PG-01 against some clinical and standard pathogens compared with commercial antibiotics.

	Concentration of extract (mg/ml) PG-01			Antibiotic disc		
Bacterial species	50	MT	VA	P	FM	CL
Gram-positive bacteria						
MRSA	32	R	20	R	23	R
L. monocytogenes	14	R	17	R	16	R
S. aureus ATCC	34	13	18	12	23	R
S. epidermidis ATCC	25	15	15	R	28	R
B. subtilis ATCC	23	18	24	13	23	14
L. monocytogenes ATCC	15	11	R	R	14	R
Gram-negative bacteria						
B. bronchiseptica	R	30	R	R	17	R
Br. melitensis	R	R	R	R	28	R
E. coli ATCC	R	R	18	R	R	R
P. aeruginosa ATCC	R	R	R	R	R	R
S. typhi ATCC	R	R	10	R	20	R

*(6mm) diameter disc
R: Resistant

pathogenic bacteria were MDR strains; also among standard strains, except *S. aureus* ATCC and *B. subtilis* ATCC, other bacteria were MDR strains. All the tested pathogenic microorganisms, except *B. subtilis* ATCC, were resistant to colistin; meanwhile most of the tested clinical pathogenic bacteria revealed resistance to penicillin and methicillin. *P. aeruginosa* ATCC was the most resistant bacterium. The diameter of the inhibition zones for vancomycin and PG-01 against MRSA were 20 and 32 mm, respectively (Figure 2 and Table 3). MIC and MBC values for ethyl acetate extract of PG-01 broth culture against MRSA were 30 and 60 mg/ml, respectively. Meanwhile MIC and MBC values against *B. subtilis* ATCC were the same (20 mg/ml). Antibacterial activity of indented antibacterial compound against MRSA was maintained with sub-MIC concentrations even with two times below the MIC value (GCI: 20 mg/ml) (Figure 3). In the presence of the PG-01 extract at 40, 50 and 60 mg/ml concentrations, the growth curve of MRSA was not reach to stationary

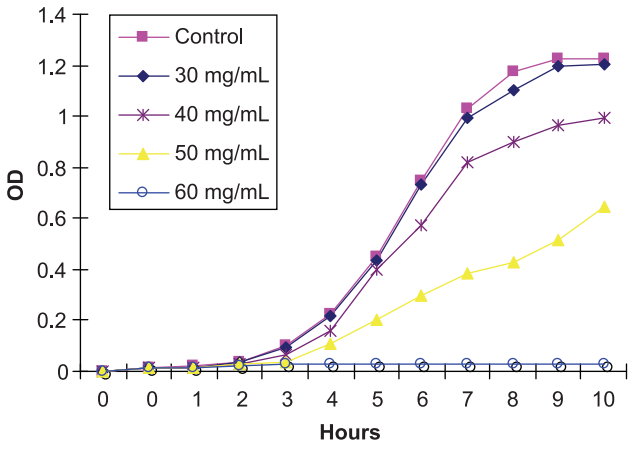


FIGURE 3. Determination of GCI parameter for P*seudoalteromonas piscicida* PG-01 broth culture extract against MRSA.

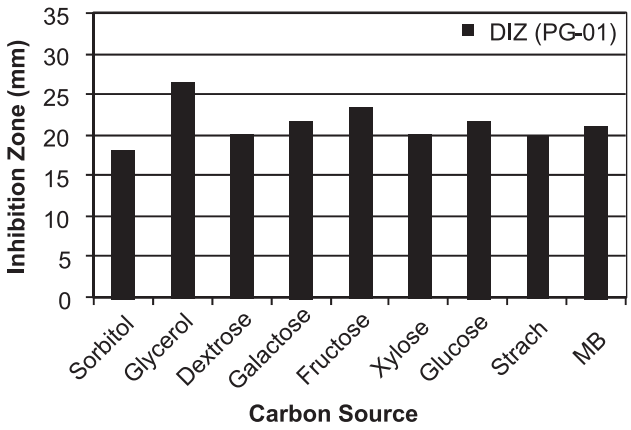


FIGURE 4. Effect of various carbon sources on antibiotic production. (The diameter of disc is 6 mm), DIZ: Diameter of Inhibition Zone

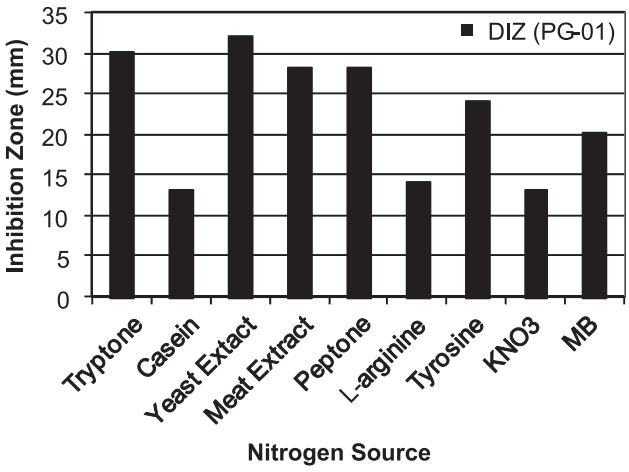


FIGURE 5. Effect of various nitrogen sources on antibiotic production; (The diameter of disc is 6 mm), DIZ: Diameter of Inhibition Zone

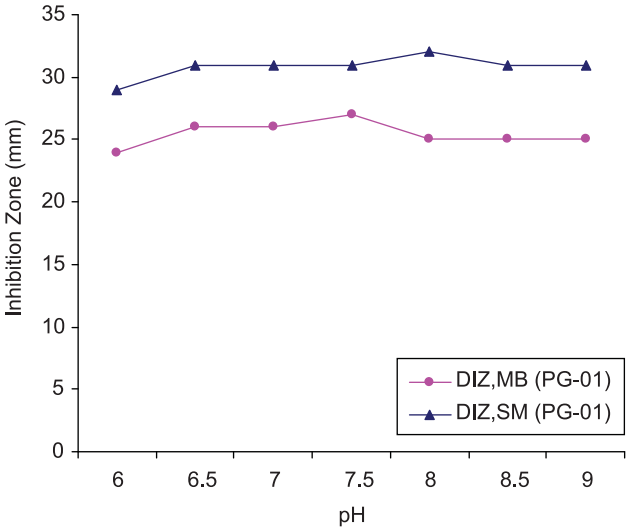


FIGURE 6. Effect of pH on antibiotic production. (The diameter of disc is 6 mm), MB: Marine Broth, SM: Synthetic Medium, DIZ: Diameter of Inhibition Zone

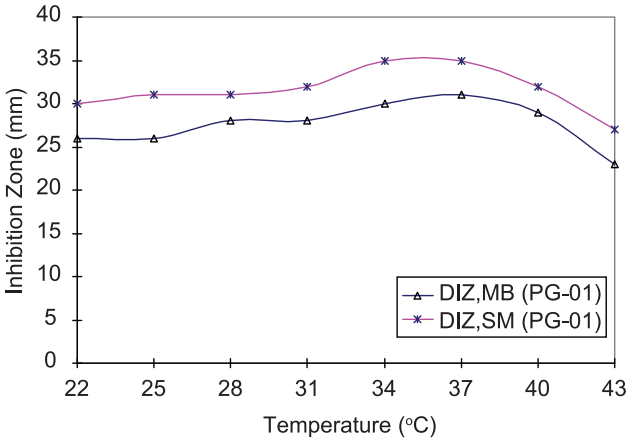


FIGURE 7. Effect of temperature on antibiotic production. (The diameter of disc is 6 mm), MB: Marine Broth, SM: Synthetic Medium, DIZ: Diameter of Inhibition Zone

phase. Also, partially interference in growth curve was observed at 30 mg/ml concentration compared to control. The optimization results revealed that glycerol and yeast extract are the best carbon and nitrogen sources for production of antibiotic by PG-01 isolate, respectively, (Figures 4 and 5). In the case of synthetic medium the maximum antibiotic activity was achieved at initial pH 8 (Figure 6), at temperature 34-37˚C (Figure 7), 0% NaCl (w/v) (Figure 8), and 48 h incubation time (Figure 9). For marine broth medium, the optimum conditions for the antibiotic production were initial pH 7.5, temperature 37˚C (Figure 7), 2% NaCl (w/v) (Figure 8), and 60 h incubation time (Figure 9). Antibacterial activity of harvested samples was observed after 12h for both production medium, but at this time DIZ for the harvested sample from marine broth and synthetic medium were 14 and 25 mm, respectively (Figure 9). The best NaCl concentration for antibiotic production by PG-01 isolate in synthetic medium prepared by distilled water was 2% (w/v) while about of synthetic medium prepared with natural seawater adding of NaCl even at the lowest concentration cause to blocking of the antibiotic production by PG-01 isolate (Figure 8). Also, ethyl acetate was chosen as the best solvent for antibiotic extraction (Figure 10). Sorbitol as carbon source presented the lowest efficiency for antibiotic production by this isolate; furthermore, casein and KNO_3 were the least effective nitrogen sources for production by PG-01 isolate. About of the temperature optimization, the lowest antibiotic production was observed at 43°C. Among the optimized fermentation parameters, carbon and nitrogen sources have a significant effect on antibiotic production while pH has only a slight favorable effect on production. On the other hand, NaCl concentration was found as a

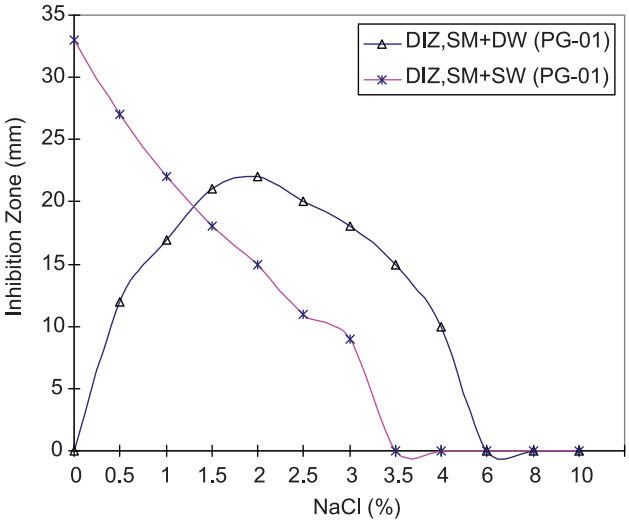


FIGURE 8. Effect of salinity on antibiotic production. (The diameter of disc is 6 mm), DW: Distilled Water / SW: Sea Water, DIZ: Diameter of Inhibition Zone

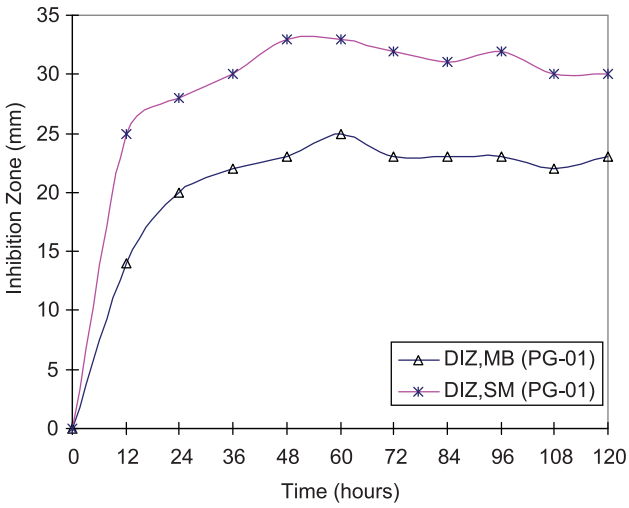


FIGURE 9. Effect of incubation time on antibiotic production. (The diameter of disc is 6 mm), MB: Marine Broth. SM: Synthetic Medium, DIZ: Diameter of Inhibition Zone

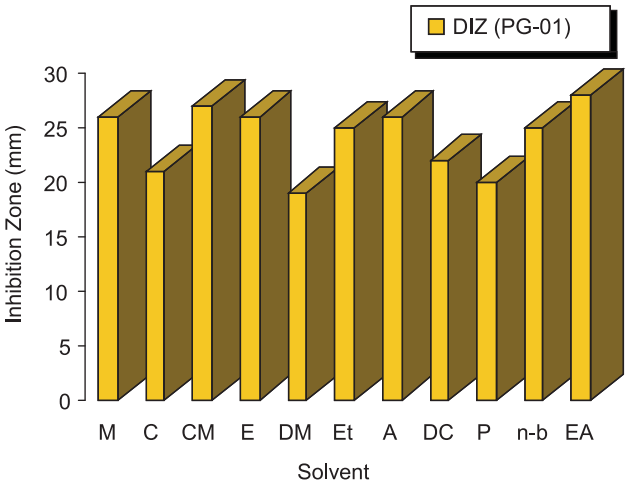


FIGURE 10. Optimization of solvent for antibiotic extraction
M: Methanol, **C**: Chloroform, **CM**: Chloroform-Methanol (1:2 (v/v)), **E**: Ethanol, **DM**: DMSO, **Et**: Ether, **A**: Aceton, **DC**: Dichloromethane, **P**: Propanol, **n-b**: n-butanol, **EA**: Ethyl Acetate. (The diameter of disc is 6 mm), DIZ: Diameter of Inhibition Zone

TABLE 4. Results of determination of R_f values and anti-MRSA activity for each of the separated bands using TLC

Spot	R_f	DIZ (mm)
6A	0/77	10
6B	0/91	R
7A	0/95	24
8A	0/44	17

TABLE 5. Antibacterial activity of anti-MRSA compound produced by *Pseudoalteromonas piscicida* PG-01 after enzymatic treatment

Strain	Control	Pancreatin	Pepsin	Proteinase K
PG-01	22	11	22	22

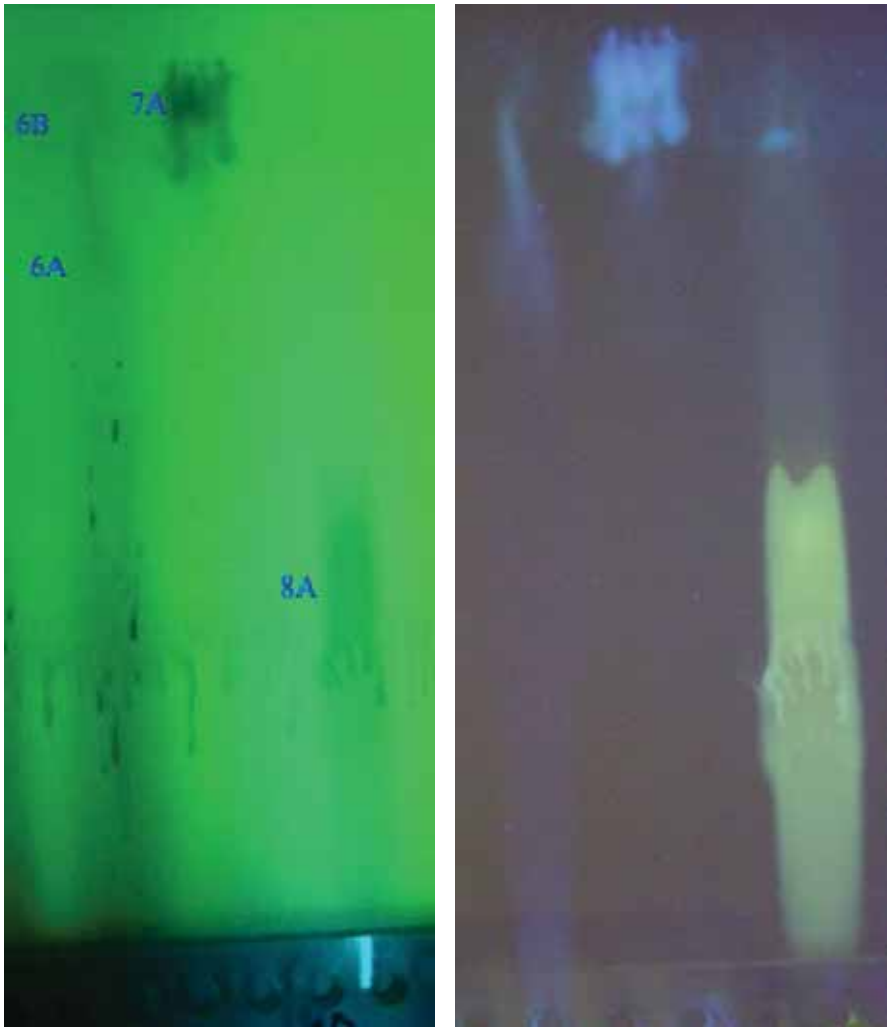
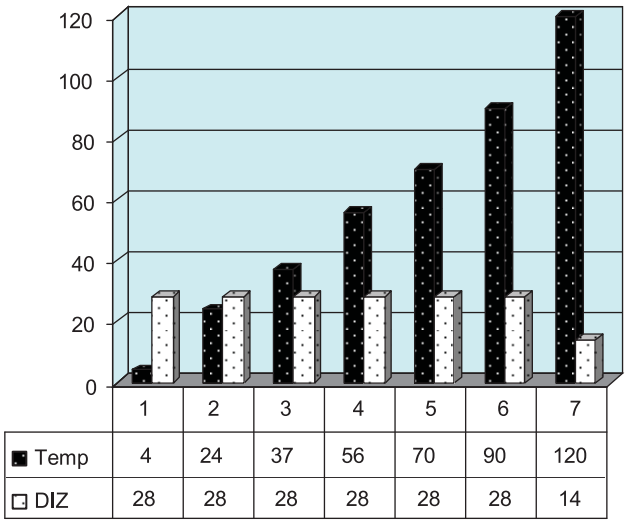


FIGURE 11. TLC analysis for the supernatant and ethyl acetate extract samples of *Pseudoalteromonas piscicida* PG-01
a: Tlc (visualized at 254 nm) , **b:** Tlc (visualized at 366 nm), 6: Sample 6 (the supernatant sample prepared from broth culture), 7: Sample 7 (extract sample prepared from broth culture), 8: Sample 8 (Oxytetracycline as control), A and B: Separated bands

critical factor on the antibiotic production. In general, antibacterial activity (inhibition zone diameter) of PG-01 against MRSA at un-optimized (marine broth) and optimized (synthetic medium) conditions was 16 and 31 mm, respectively. PG-01 isolate was found to produce only one antimicrobial component by bioautography. Results from bioautographic analysis revealed that the two spots 6A and 7A which were obtained from supernatant and extract, respectively were presented anti-MRSA activity (Table 4, Figure 11); however, the spot 7A with a R_f value of 0.95 exhibited the strongest antibacterial activity against MRSA so that when activity of this component was assessed against MRSA compared to the whole extract, the obtained DIZ values were relatively equal. Likewise, the intended two active components and oxytetracycline have remarkably different polarities (Figure 11 a, b). Thermostability studies exhibited that this antibacterial substance was a thermostable compound. However, the antibacterial activity remained unaltered after treatment at 4-90˚C, it started to decrease after being autoclaved (121˚C) but even at this temperature it didn't completely loose its activity too (Figure 12). The anti-MRSA activity of this antibacterial compound was decreased only



	1	2	3	4	5	6	7
Temp	4	24	37	56	70	90	120
DIZ	28	28	28	28	28	28	14

FIGURE 12. Results of heat treatment of an anti-MRSA compound produced by *Pseudoalteromonas piscicida* PG-01, (The diameter of disc is 6 mm), Temp: Temperature (°C)

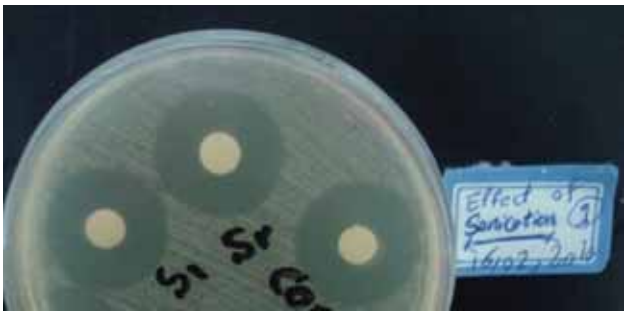
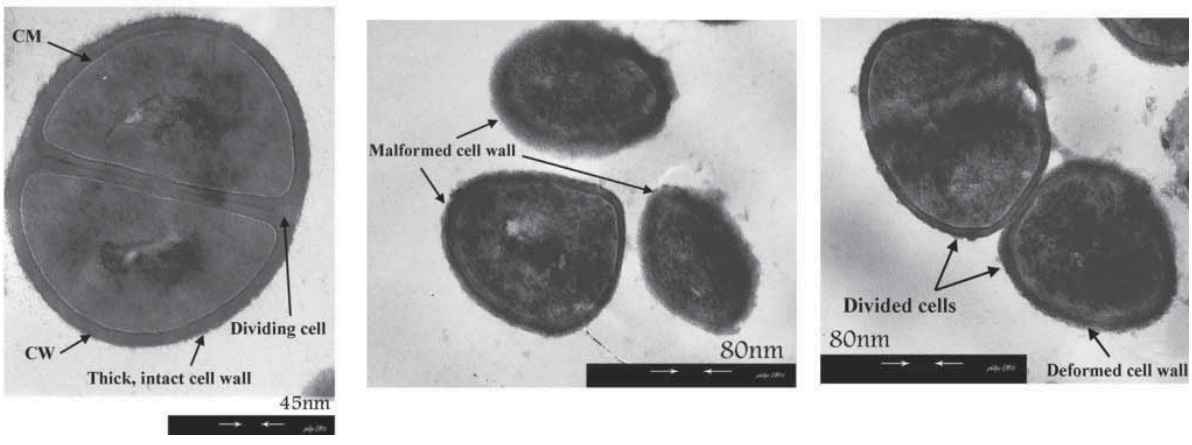


FIGURE 13. The obtained results from sonication treatment of broth culture (containing whole cell) of PG-01 isolate; as we can see, inhibitory activity of the treated sample against MRSA remained unaltered, that is to say, there is no intracellular anti-MRSA compound in the cells of PG-01 isolate. S1: Sample (un-filtered), S2: Sample (filtered), Con: Control (un-sonicated)

after treatment with Pancreatin by 50% compared with control (22 mm) (Table 5). Based on these findings, the active molecule may not be proteinaceous in nature, but a glycolipid nature for this antibiotic can be suggested. Sonication treatment indicated no intracellular active compound, because inhibitory activity of the treated sample against MRSA remained unaltered (Figure 13). So, it can be concluded that the antibacterial compound produced by PG-01 is completely secretory. TEM results of ultrathin section of MRSA treated with the antibacterial compound produced by PG-01 isolate were shown in Figure 14. A comparison of transmission electron micrograph results of the treated cells with untreated cells, revealed deformities caused in the cell wall structure (weakened and deformed cell wall) by this marine-derived antibacterial compound while un-treated cells have a thick and intact cell wall (Figure 14). Lysis of cell with residual membranes and empty protoplasmic space and numerous membrane blebs are shown in Figure 15, too. So, the target site for antibacterial compound produced by PG-01 isolate is cell wall and this antibacterial compound can be considered as a bactericidal agent against MRSA. In the case of treated cells, also, because of decrease in the thickness of the cell wall we can see that the electron density of this part is decreased. Considering growth of PG-01 and time course of its antibacterial compound production revealed that the growth of intended marine isolate increased exponentially at 4 to 28h of incubation (Figure 16). The anti-MRSA activity of the culture supernatant was detectable as early as at 12 h of incubation and reached the highest activity at 100h of incubation (Figure 16). So, in marine broth culture, production of antibacterial compound with PG-01 isolate was started in the early stage of logarithmic phase and increased to a maximal production level until the stationary phase; that it can be said that antibacterial production occurs during the whole growth phase.



(a) **(b)** **(c)**

FIGURE 14. Transmision electron micrographs of MRSA. **a** Cell grown in the absence of antibacterial compound produced by *Pseudoalteromonas piscicida* PG-01; **b, c** Cells grown in the presence of antibacterial compound produced by *Pseudoalteromonas piscicida* PG-01

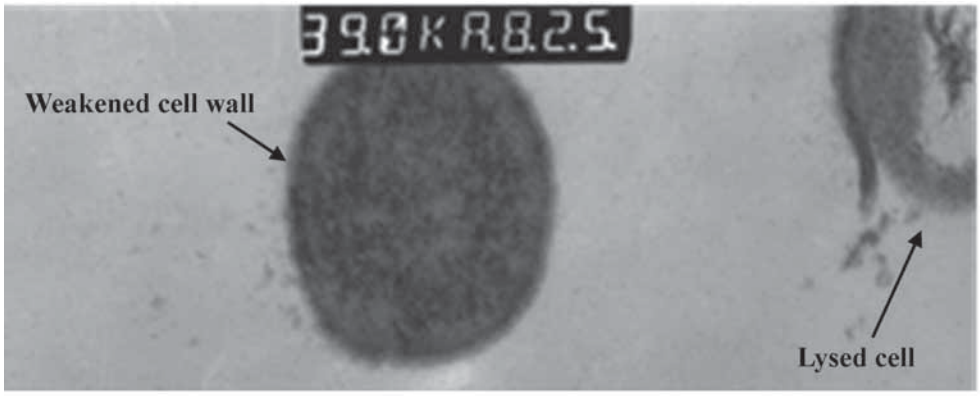


FIGURE 15. Transmision electron micrographs of MRSA; **a** cell with weakened cell wall; **b** lysis of cell due to bactericidal effect of antibiotic produced by PG-01 Strain.

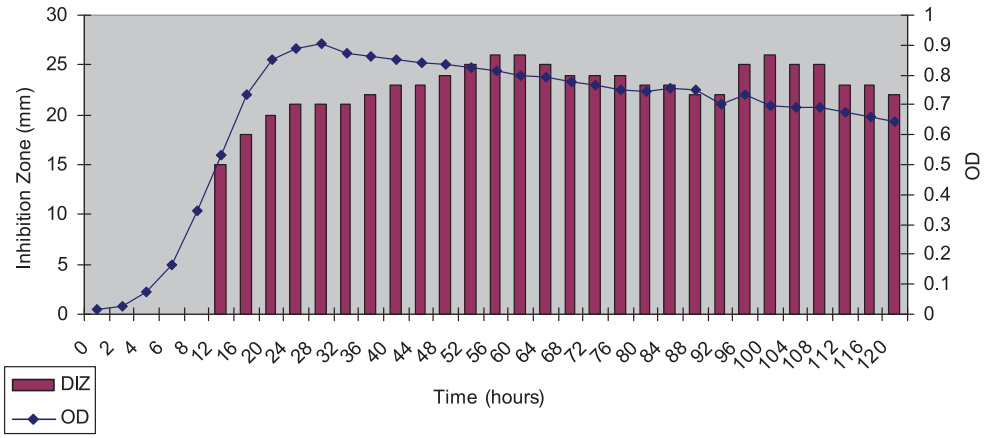


FIGURE 16. Results of Co-variation between cell growth of *Pseudoalteromonas piscicida* PG-01 and its antibiotic production

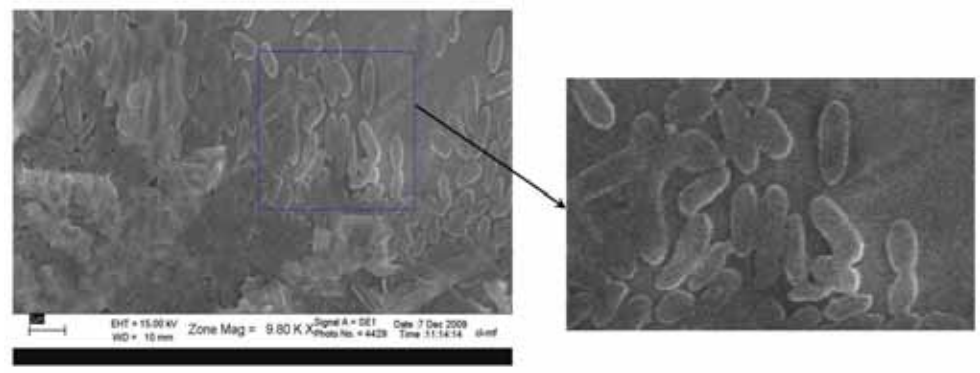


FIGURE 17. Scanning electron micrographs of PG-01 isolate in order to study of its morphology. (**a, b**)

The marine PG-01 isolate was Gram-negative, motile, catalase positive and strictly aerobe. The colonies of this isolate on marine agar were smooth, convex, and brownish with entire edges. The SEM micrograph results presented that this marine isolate is typically a rod-shape bacterium and its surface is smooth (Figure 17 a, b). This isolate didn't growth on more than 5% (w/v) NaCl concentration and also, was able to utilize a range of carbohydrates such as D-glucose, xylose, and maltose and glycerol as carbon substrates. Furthermore, this strain was resistant to the commercial antibiotics including polymixin B, tetracycline, and penicillin. In molecular diagnosis, a 1500 bp PCR product was amplified successfully (Figure 18) and on the basis of the BLAST search, the PG-01 isolate showed the highest 16S rRNA gene sequence similarity (97%) to *Pseudoalteromonas piscicida* 1314. The phylogenetic tree analysis showed also that this isolate had the most phylogenetic homogeneity with *P. piscicida*; the other two *Pseudoalteromonas* species including *P. viridis* and *P. rubra* were placed within the intra-cluster branches related to the location of PG-01 isolate (Figure 19). Also, the mean of the observed nucleotide differences between *Pseudoalteromonas piscicida* PG-01 and 1314 (Figure 20) was 1.24%, and is roughly equivalent to evolutionary distance. The 16S rRNA gene sequence of PG-01 strain was deposited in GenBank with accession number JF737768.

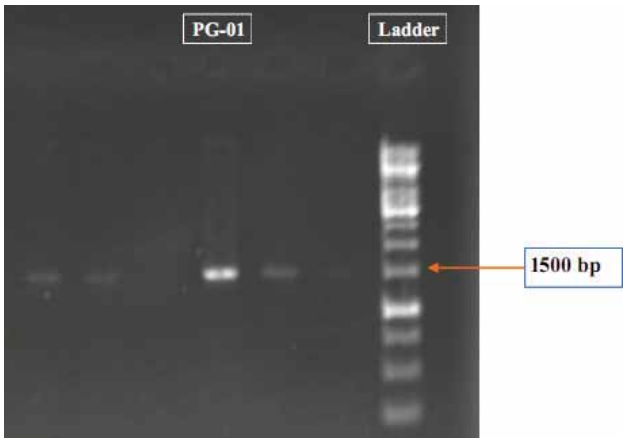


FIGURE 18. Agarose gel electrophoresis of amplified 16S rRNA fragment (1500 bp) of PG-01 isolate.

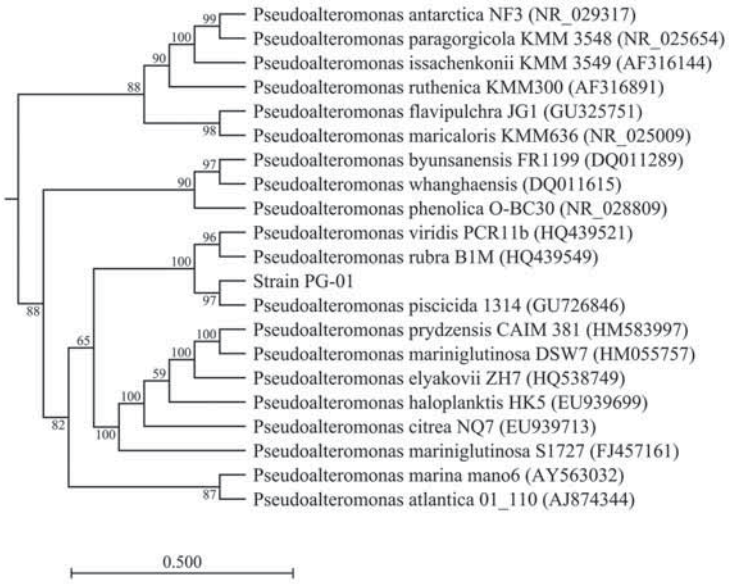


FIGURE 19. Phylogenetic relationships of Strain PG-01 and some related *Pseudoalteromonas* species on the basis of 16S rRNA gene sequence analysis. Numbers at branch nodes are bootstrap values (expressed as a percentage of 1000 replicates) based on the neighbour-joining algorithm. Bar represents approximately 0.5 % nucleotide sequence difference.

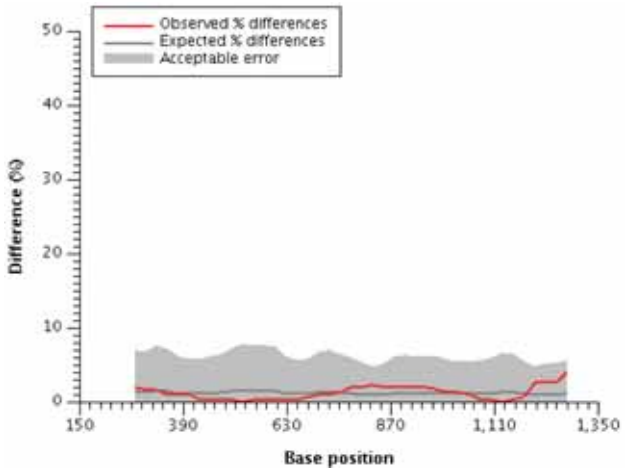


FIGURE 20. Variation in % difference between *P. piscicida* 1314 (1372 nt) and *P. piscicida* PG-01 (1248 nt) determined with a 300 base sliding window, moving 25 bases at a time along the sequences.

DISCUSSION

During two previous decades, studying about marine microorganisms has been raised significantly due to their very high capability of producing secondary metabolites. In fact, the strong selective pressure resulted from competition between bacteria in the marine environment for space and nutrition and also metabolic and physiological differences between marine and terrestrial microorganisms arising from different conditions in marine would result in that these microorganisms produce a great amount of natural products applicable in medicine and industry [6]. The emergence and dissemination of resistant pathogenic bacteria to current antibiotics particularly MRSA (Methicillin Resistant *Staphylococcus aureus*) has become a great global concern in 21st century, especially for the hospitalized patients. Today, vancomycin has become the drug of choice for treating MRSA infections; however, treatment failures, adverse side effects and the emergence of vancomycin resistant bacteria are leading to urgent needs for alternative anti-MRSA therapies [28]. So, it seems that finding new antibiotic against this rebel pathogen named superbug, especially from natural resources is vital. Marine ecosystem as an untapped resource can be surveyed for natural antimicrobials. For the first time, antibiotic production by marine bacteria has been documented by Rosenfeld and Zobell, 1946 [29]. It is noticeable that a sea with somewhat special climate such as Persian Gulf with relatively high temperature (average 35˚C) and salinity (~4%) must be more focused to finding new and special natural metabolites. Meanwhile, Persian Gulf is a rich source of marine micro- and macro-organisms with high biodiversity. In our research, two of the three obtained marine antibiotic producer bacteria were isolated from sediment samples; this result may be due to existence of competition on space and nutrients at this habitant. However, there are many reports that marine colonized bacteria consist a percentage of antibiotic producing bacteria higher than observed in free-living state isolated from marine environment [30]. The activity of the crude extract of PG-01 strain was restricted to Gram-positive bacteria; however, it was highly active against human pathogenic strains, including clinical and standard strains. 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 pres-

ence of enzymes in periplasmic space which are able to break down foreign molecules introduced from outside [31]. All of the studied pathogenic clinical strains were MDR bacteria even against the well-known synthetic antibiotics; furthermore, nearly 55% of all isolates studied were resistant to methicillin. Also, it seams that penicillin and colistin must be gradually go away form the effective antibiotics list. Based on the obtained results from GCI parameter determination, antibacterial activity of intended antibacterial compound against MRSA was maintained with sub-MIC concentrations, this outcome has a pharmaceutical importance about using antibacterial compound, especially for treatment of high-risk patients and also preventing of emergence of new antibiotic resistances. Results from optimization process revealed that among the optimized fermentation parameters, carbon and nitrogen source or C/N ratio plays the most important role in efficacy of optimization for antibiotic production by intended marine strain. Glycerol and yeast extract were chosen as optimum carbon and nitrogen sources for antibiotic production by *Pseudoalteromonas piscicida* PG-01; there are several reports about selection of these substrates as the best sources in order to antibiotic production by different bacteria [32, 33]. Considering of these results, the culture of this strain in complex medium revealed more than 100% higher antibiotic production compared to marine broth. Commonly, chemically defined media as production media aren't used in industrial fermentation process to obtain high yields and hence this improvement of cultivation parameters is one of the main objectives in industrial research [34]; So, the obtained result from optimization is very usable for improving supply of the intended marine antibiotic for its industrial production in future. The results of heat and enzymatic treatment show that the active constituent of the antibacterial compound produced by *Pseudoalteromonas piscicida* PG-01 is thermostable substance and also is not sensitive to tested proteolytic enzymes; so, this antibacterial compound maybe not peptide in nature. However, this marine antibiotic compound loose its activity by 50 % when treated by pancreatin; since this enzyme has amylase, lipase and proteinase activity, a glycolipid nature of active molecule can be suggested. On the other hand, the findings obtained from the solvent optimization revealed that some solvents specific for lipid extraction such as Chloroform-Methanol (1:2 [vol/vol] had a excellent efficiency for extraction of antibiotic produced by PG-01, suggests that there was substantial lipid fraction in this inhibitory substance. TLC and bioautography results revealed that the intended antimicrobial compound and oxytetracycline are with remarkably different polarities; consequently they have different antimicrobial profiles. In study the co-variation between cell growth and antimicrobial compound production test, we found that production of antibiotic with PG-01 strain was started at the mid-logarithmic phase while the most of the antimicrobials as secondary metabolites usually will be produced at the late exponential or stationary phase; however there are several reports that indicates antibiotic production can be triggered at the early stages of cell growth [8, 35]. Moreover, with alteration in culture condition such as carbon source and so on, production of secondary metabolites may be started at the logarithmic growth phase; that is to say, under this condition in logarithmic phase, cell division will be stopped and subsequently antibiotic production will be occurred [35, 36]. TEM results showed that the target site for antibacterial compound produced by *Pseudoalteromonas Piscicida* PG-01 is cell wall; so, this antibiotic compound can be considered as a bactericidal agent against MRSA and can give hope for treatment of diseases caused by this rebel pathogen. It seems likely this antibacterial substance deforms the cell wall of target cells, influnce proton motive force and subsquently increase osmotic pressure due to influx of water and finally cell lysis occurs [25]. This strain was characterized by both biochemical and as well as molecular methods. The PG-01 isolate exhibited some phenotypic differences from its closest phylogenetic neighbor *P. piscicida* strain that noted in Bergey's manual for systematic of bacteriology [37] including ability to grow at 40°C, produces a brownish diffusible pigment, inability to use of ammonia, and ability to nitrate reduction. However, based on Phylogenetic analysis of the 16S rRNA gene sequencing and phenotypic characteristics, this marine isolate maybe proposed to represent a novel species or sub-species of the *Pseudoalteromonas* genus, we named as *P. piscicida* PG-01. It has been reported that different strains belonging to *Pseudoalteromonas sp.* have antibacterial activity, antiviral activity and agarolytic activities [38]. Finally, determination of the structures and pharmacological study of the active compound produced by intended marine bacterium is necessary that will be reported in future.

CONCLUSION

In conclusion, The PG-01 strain exhibited antibacterial activity against all tested Gram-positive bacteria. PG-01 isolate had good anti-MRSA activity and also GCI value against MRSA was two times lower than MIC value. Among the optimized fermentation parameters, carbon and nitrogen sources found to have a significant effect on antibacterial compound production by intended marine bacterium. TLC results for ethyl acetate extract of PG-01 showed that there is only a single fraction with antibacterial activity. We found that intended marine antibacterial compound is no proteinaceus in nature; also, it is completely secretory and has extra-cellular location.

Ultrastructral study on the effect of intended antibacterial compound on MRSA using TEM revealed that the target site for this is cell wall. Finally, *P. piscicida* PG-01 can be regarded as a valuable strain for discovery of new weapon in fighting against multi-drug resistant bacteria, especially MRSA.

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DECLARATION OF INTEREST

The authors declare no conflict of interest for this study.

REFERENCES

[1] Katayama Y, Ito T, Hiramatsu K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother. 2000;44(6):1549-55
[2] Utsui Y, Yokota T. Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcusaureus*. Antimicrob Agents Chemother 1985; 28:397-403.
[3] Bhatnagar I, Kim SK. Immense essence of excellence: marine microbial bioactive compounds. Mar Drugs 2010; 8: 2673-2701.
[4] Schäberle FT, Goralski E, Neu E, Erol O, Hölzl G, Dörmann P, et al. Marine Myxobacteria as a source of antibiotics -- comparison of physiology, polyketide-type genes and antibiotic production of three new isolates of *Enhygromyxa salina*. Mar Drugs 2010; 8: 2466-2479.
[5] Carvalho CCCR, Fernandes P. Production of metabolites as bacterial responses to the marine environment. Mar Drugs 2010; 8: 705-727.
[6] Zheng L, Han X, Chen H, Lin W, Yan X. Marine bacteria associated with marine microorganisms: the potential antimicrobial resources. Ann Microbiol 2005; 55:119-124.
[7] Rahman H, Austin B, Mitchell WJ, Morris PC, Jamieson DJ, Adams DR et al. Novel anti-infective compounds from marine bacteria. Mar Drugs 2010; 8:498-518.
[8] Brinkhoff T, Bach G, Heidorn T, Liang L, Schlingloff A, Simon M. Antibiotic production by a Roseobacter clade-affiliated species from the German Wadden Sea and its antagonistic effects on indigenous isolates. Appl Environ Microbiol 2004;70: 2560-2565.
[9] Yeon SH, Jeong WJ, Park JS. The diversity of culturable organotrophic bacteria from local solar salterns. J Microbiol 2005; 43:1-10.
[10] Asthana RK, Deepali, Tripathi MK, Srivastava A, Singh AP, Singh SP. Isolation and identification of a new antibacterial entity from the Antarctic *Cyanobacterium Nostoc* CCC 537. J Appl Phycol 2009; 21:81-88.
[11] Xiong H, Qi S, Ying XuY, Miao L, Qian P. Antibiotic and antifouling compound production by the marine-derived fungus Cladosporium sp. F14. J Hydro-environ Res 2008;2: 264-270.
[12] NCCLS. Performance Standards for Antimicrobial Susceptibility Testing; Ninth Informational Supplement. NCCLS document M100-S9. National Committee for Clinical Laboratory Standards, Wayne, PA, 2008; pp. 120-126.
[13] Motamedi H, Darabpour E, Gholipour M, Seyyednejad SM. In vitro assay for the anti-brucella activity of medicinal plants against tetracycline-resistant *Br. melitensis*. J Zhejiang Univ-SCIENCE B 2010;11: 506-511.
[14] Castillo A, Liebana J, Lopez E, Baca P, Liebana JM, Liebana MJ et al. Interference of antibiotics in the growth curves of oral streptococci. Int J Antimicrob Agent 2006; 27:263-266.
[15] Vijayan KK, Bright SIS, Jayaprakash NS, Alavandi S, Somnath PS, Preetha R et al. A brackishwater isolate of Pseudomonas PS-102, a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. Aquaculture 2006; 251:192-200.
[16] Shibl AM, Al-Sowaygh IA. Antibiotic inhibition of protease production by Pseudomonas aeruginosa. J Med Microbiol 1980;13: 345-349.
[17] El-Naggar MY, El-Assar SA, Abdul-Gawad SM. Meroparamycin production by newly isolated *Streptomyces* sp. strain MAR01: taxonomy, fermentation, purification and structural elucidation. J Microbiol 2006; 44: 432-438.
[18] Manilal A, Sujith S, Selvin J, Shakir C, Kiran GS. Antibacterial activity of Falkenbergia hillebrandii (Born) from the Indian coast against human pathogens. Int J Exp Bot 2009; 78:161-166.
[19] Amin M, Kalantar E, Mohammad-Saeid N, Ahsan B. Antibacterial effect and physicochemical properties of essential oil of *Zataria multiflora* Boiss. Asian Pac J Trop Med 2010; 3:439-442.
[20] Mirhossini M, Nahvi I, Emtiazi G, Tavasoli M. Incidence and antibiotic susceptibility of bacteriocin-producing lactic acid bacteria from dairy products. Int J Dairy Technol 2008; 61: 391-396.
[21] Braja JL, Lemos ML, Toranzo AE. Purification and characterization of an antibacterial substance produced by a marine *Alteromonas* species. Antimicrob Agent Chemoter 1989;33:1674-1679.
[22] McArthur M, Bibb MJ. Manipulating and understanding antibiotic production in *Streptomyces coelicolor* A3 (2) with decoy oligonucleotides. Proc Natl Acas Sci 2008;105:1020-1025.
[23] Guignard B, Entenza JM, Moreillon P. β lactams against methcillin resistant *Staphylococcus aureus*. Curr Opin Pharmacol 2005;5: 479-489.
[24] Kersten A, Poitschek C, Rauch S, Aberer E. Effects of penicillin, ceftriaxone, and doxycycline on morphology of *Borrelia burgdorferi*. Antimicrob Agent Chemoter 1995; 39:1127-1133.
[25] Chen H, Chan SC, Lee JC, Chang CC, Murugan M, Jack RW. Transmission Electron *Microscopic* observations of membrane effects of antibiotic Cecropin B on *Escherichia coli*. Microsc Res Tech 2003; 62:423-430.
[26] Papizadeh M, Roayaei Ardakani M, Ebrahimipour G, Motamedi H. Utilization of dibenzothiophene as sulfur source by Microbacterium sp. NISOC-06. World J Microbiol Biotechnol 2010; 26:1195-1200.
[27] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-425.
[28] Chopra I. Antibiotic resistance in *Staphylococcus aureus*: concerns, causes and cures. Expert Rev Anti Infect Ther 2003;1: 45-55.
[29] Asha Devi NK, Balakrishnan K, Gopal R, Padmavathy S. *Bacillus clausii* MB9 from the east coast regions of India: Isolation, biochemical characterization and antimicrobial potentials. Curr Sci 2008;95:5-10.
[30] Armstrong E, Yan L, Boyd KG, Wright PC, Burgess JG. The symbiotic role of marine microbes on living surfaces. Hydrobiol 2001;461: 37-40.
[31] Motamedi H, Darabpour E, Gholipour M, Seyyednejad SM. Antibacterial effect of ethanolic and meyhanolic extract of *Plantago ovata* and *Oliveria decumbens* endemic in Iran against some pathogenic bacteria. Int J Pharmacol 2010;6: 117-122.
[32] Preetha R, Jayaprakash NS, Philip R, Singh ISB. Optimization of carbon and nitrogen sources and growth factors for the production of an aquaculture probiotic (Pseudomonas MCCB 103) using response surface methodology. J Appl Microbiol 2007; 102:1043-1051.
[33] El-Banna NM. Effect of carbon source on the antimicrobial activity of *Corynebacterium kutscheri* and *Corynebacterium xerosis*. Afric J Biotechnol 2006; 5:833-835.
[34] Nair R, Murphy T, Roy I, Keshavarz T. Optimization studies on multiple elicitor addition in microbial systems: *P. chrysogenum* and *B. Licheniformis*. Chem Eng Trans 2008; 14:373–380.

[35] Oyama M, Kubota K. Induction of antibiotic production by protease in Bacillus brevis (ATCC 8185). J Biochem 1993; 3: 637-641.
[36] Dewi AS. Biologically active secondary metabolites from tropical marine invertebrates. MSc Thesis. University of British Columbia. Canada. 2009; pp. 43-72.
[37] Garrity GM, Bell JA, Lilburn TI. Pseudoalteromonas. In:, Bergey's manual of systematic bacteriology. Bownan JP and McMeekin TA, editors. 2nd Edition, New Yourk: Springer, 2006; pp. 467-477.
[38] Castillo CSD, Wahid MI, Takeshi Y, Taizo S. Isolation and inhibitory effect of anti-Vibrio substances from Pseudoalteromonas sp. A1-J11 isolated from the coastal sea water of Kagoshima Bay. Fish Sci 2008;74: 174-179.