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Isolation and characterization of bacterial strains from shoreline waters of Caspian Sea with novel antibacterial activity

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ABSTRACT

In this study, a total 108 bacterial strains with potential of antibacterial activity were isolated from 145 shoreline water samples of Caspian Sea. The isolated bacteria were: Gram negative and positive bacilli, Gram negative and positive cocci and Gram negative coccoid. Amongst 108 isolated bacterial strains, 12 strains (11%) showed significant antibacterial activity against 10 reference strains. Morphological, physiological, biochemical features and 16S rRNA analysis were used to identify the isolated strains with antibacterial activity. Amongst the isolated strains, six isolates possessed typical cellular, colonial morphologies, physiological, biochemical and nutritional features that resembled *Bacillus* spp. Four strains were identified as *Bacillus cereus*. The two other isolates belonged to *Bacillus subtilis* group. Two gram positive rod shape isolates were identified as *Exiguobacterium acetylicum*. The rest of isolates with antibacterial potency were gram negative bacteria. They were identified as *Pseudomonas aeruginosa* (3isolate) and *Alcaligenes faecalis* (one isolate). Crude extracts of *Pseudomonas aeruginosa* strains showed significant antimicrobial activity against all reference strains. Also, *E.acetylicum* and *A. faecalis* showed antibacterial activity against some reference strains such as *Escherichia coli* PTCC 1533, *Shigella flexneri* PTCC 1234 and *Salmonella enterica subsp.enterica Paratyphi* B PTCC 1231. The crude extracts of four isolates with wide antibacterial activity were used in TLC autobiography overlay assay. The TLC autobiographic overlay assay implied that the antimicrobial metabolites produced by the strains with wide antimicrobial spectrum were different.

1. Introduction

Over the past decades, we have seen the rapid emergence of antibiotic-resistant bacteria and there is an urgent need for discovering novel antimicrobial compounds (Vicente et al., 2006; Wagner-Döbler and Biebl, 2006). Marine ecosystems represent 95% of the biosphere and coastal regions are very important due to different adapted species in these environments. In the last few years, marine microorganisms have emerged as new source for discovery of novel biologically active compounds (Fenical, 1993, Fenical, 1997). Some marine bacteria are inhibitory to other bacteria (Barja et al., 1989; Burkhold et al., 1966; Gauthier and Flatau, 1976), suggesting that bacterial interactions

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could play an important role in marine ecology (Long and Azam, 2001; Strom, 2008).

Marine microorganisms are also the focus of attention due to their production of metabolites that may have a range of pharmaceutical and biotechnological applications (Blunt et al., 2008; Bull and Stach, 2007; Burgess et al., 1999; Debnath et al., 2007; Egan et al., 2008; Fenical and Jensen, 2006; Jensen and Fenical, 1996). Marine bacteria are mainly isolated from sediments, but can be obtained from open oceans or marine surfaces including marine live organisms (Jensen and Fenical, 1994). They are either planktonic, associated with inert or biotic surfaces, or they inhabit the sediments. Due to ability to occupy in different niches, there is possible to isolate bacterial strains from shorelines of the seas. These bacteria may not be typically originated from marine environments but they can share some properties of marine forms. Nowadays, several antibiotic compounds from marine bacteria have been reported, including Pyron from *Pseudomonas*, Loloatin from *Bacillus*, Tiomarinol from *Altromonas*, Marinopyrroles from *Streptomyces*, Agrochelin from *Agrobacterium*, Komaricin from *Pseudoalteromonas* and Pelagiomicin from *Pelagiobacter variabilis*.

The Caspian Sea is the largest inland body of water in the world and accounts for 40 to 44 percent of the total lacustrine waters of the world. It is bigger than the Great American lakes and Lake Victoria in Africa by surface area. As distinct from other lakes, the water of the Caspian is not fresh, but brackish. Each liter of Caspian water contains 10-13 grams of salt making this water unsuitable for drinking or irrigation. An important feature of the Caspian Sea is the extreme diversity of biotopes, biotic and abiotic conditions (Zenkevich, 1963). First of all, water salinity in different parts of this lake is quite different (Kosarev and Tuzhilkin, 1995). However, earlier, when the level of the Caspian was much higher than now, and there was strong vertical salinity stratification, the oxygen was practically absent at the bottom (Kosarev and Tuzhilkin, 1995; Dumont, 1998). That is why, presently, there is poor life at the depths of more than 100m. There is no abyssal fauna and flora in this lake. It is possible to assume that this ancient natural ecological catastrophe heavily reduced modern diversity of the Caspian fauna and flora. We assume that bacterial strains with high capability adaptations to hostile environments are more populated in Caspian Sea. To the best of our knowledge, there is little information concerning isolation and characterization of bacterial strains with antibacterial activity from this lake. In the present study we focused on isolation of cultivable bacteria from shorelines of Caspian Sea to identify organisms which could be further explored from a biotechnological perspective with antibacterial activity.

2. Materials and Methods

2.1. Materials

All media and materials used in this study were obtained from (Merck Co. Darmstadt, Germany). DNA purification kit was provided from QIAGEN. GmbH. All reference strains used for antibacterial activity and phenotypic characterization studies were obtained from Persian Type Culture Collection (PTCC, Tehran, Iran).

2.2. Sample Collection

One hundred and forty five water samples were collected from 29 regions (five sample for each) across the shorelines of Caspian Sea. The sampling regions were, Chaboksar, Kelachai, Gaskar, Hasanak Sara, Rodsar, Chamkaleh, Chaf, Amirabad, Anbarsar, Vajehsar, Kiashar, Zibakenar, Haj bekandeh, Jafrod, Hassanrod, Talebabad, Bandaranzali, Boshman, Kaporchal, Paresar, Gisom, Asalem, Talesh, Jokandan, Mahmodabad, Nargesabad, Havig, Lavanvil and Astara in Gilan province/Iran, respectively. Surface water samples were taken at 50 cm depth. Samples were collected in 50 ml clean, sterile polypropylene round-bottom tubes in kept in ice box and transported to the laboratory.

2.3. Isolation of bacterial strains from marine samples

Two methods were used for isolations of bacterial strains from marine samples. In the first method, five milliliter of each water samples was inoculated into 250ml-Erlynmeyer flasks containing 50 ml of Marine broth. In the second method, 10 ml of water samples were centrifuged at 15000 rpm for 20 minutes. The supernatants were discarded and the plletes were

resuspended in 5ml of PBS, and inoculated into 250ml-Erlenmeyer flasks containing 50 ml of Marine broth. Incubation was carried out at 30 ° C for 48 h. 100µl of each grown cultures was spread on Marine Agar plates and incubated at 30°C until visible colonies growth forms. Single colonies were purified by restreaking on marine agar plates. The pure culture was preserved by freezing in 20% glycerol (v/v) at -70°C for further studies.

2.4. Phenotypic and biochemical characterization of isolated strains

The following reference strains were used for the phenotypic characterization studies: *Bacillus subtilis* subsp. subtilis PTCC 1720^{T}, *Bacillus cereus* PTCC 1247^{T}, *Pseudomonas aeruginosa* PTCC 1599, *Staphylococcus aureus* PTCC 1112^{T}. The isolates strains were characterized using the procedures in Bergey's Manual of Systematic Bacteriology (Sneath, 1986). The following characteristics were studied for the isolated gram positive bacillus strains: Morphology of vegetative cells and sporangia, shape and position of spores, catalase production, motility, anaerobic growth , Voges-Proskauer test, pH in Voges-Proskauer test, acid production from D-glucose, L-arabinose , D-xylose, and D-manitol, degradation of casein, gelatin, starch and, utilization of citrate, deamination of phenylalanine, formation of indole, formation of dihydroxyacetone, nitrate reduction test, production of lecithinase, growth at 5°, 10°, 40°, 50°, 60°C and growth in sodium chloride (0, 2, 5, 7, 10%). Following characteristics were studied for the isolated gram negative bacillus :production of catalase and oxidase enzymes, growth on MackConkey agar, growth on KIA, oxidation-fermentation tests, H_2S production, formation of indole, motility, argenine dihydrolase production, production of pyocyanin and pyoverdin, degradation of gelatin, starch, growth at 4°, 41°C, nitrate denitrification.

2.5. Antibacterial activity assay for the isolated bacteria

Overnight culture of pure colonies of the isolated bacterial strains were transferred to 250ml-Erlenmeyer flasks containing 50 ml of Marine broth medium and incubated on a rotary shaker 150 rpm at 30 °C for 96 h to produce antibacterial metabolites. For antibacterial activity assay, the broth culture was centrifuged at 12000rpm for 25 min at 4°C, the supernatant used for possible antibacterial activity.Following reference strains were used for assessing the antibacterial activity: *Escherichia coli* PTCC 1533, *Salmonella enterica* subsp.enterica Paratyphi B PTCC 1231, *Shigella flexneri* PTCC1234, *Shigella dysenteriae* PTCC 1188, *Proteus vulgaris* PTCC1312, *Pseudomonas aeroginosa* PTCC1599, *Staphylococcus aureus* PTCC1112 ^{T}, *Entrococcus hirae* PTCC1239, *Streptococcus pyogenes* PTCC1447 and *Bacillus cereus* PTCC1247.

2.6. Antibiotic sensitivity

Sensitivity of reference strains to antibiotics was determined by using the routine agar diffusion plate technique. At first, standard strains were grown on NB medium, and were used to prepare suspensions with optical density of 0.5McFarland Turbidity Standard (1.5×10^{8}). A 0.1-ml portion of suspension was cultured on Muller-Hinton Agar (MHA) and disks containing antibiotic were placed onto the surface of the medium. After incubation, the zones of inhibition surrounding the disks were measured and compared with the standard for each antibiotic according to CLSI. All incubations were performed at 30°C for an overnight. The following antibiotics were used (µg/disk): Tetracycline (30 µg), Nitrofurantoin (300 µg), Rifampicin (30 µg), Nalidixic acid (30 µg), Erythromycin (15 µg), Gentamycin (10 µg), Cephalexin (30 µg), Vancomycin (10 µg), Trimethoprim sulfamethoxazol (1.25/23.75 µg).

2.7. Hemolytic activity

Hemolytic activity of the isolated strains was performed using agar gel diffusion method. Isolated bacterial strains were cultured in nutrient broth (NB) and incubated for 24 hours at 30°C. One milliliter of medium culture of each strain was transferred into sterile 1.5 ml microtubes and centrifuged at 14000g for 20 min. Two hundred microliter of supernatant of centrifuged tubes was transferred on blood agar medium and incubated at 37°C for 72 hours. The plates were examined for possible hemolytic activity.

2.8. Isolation of genomic DNA from bacterial isolates

For the isolation of genomic DNA from bacterial isolates, a single colony of each bacterial strain inoculated into 100 ml-Erlenmeyer flasks containing 10 ml of Luria broth medium and incubated on a rotary shaker 150 at rpm and 30°C for overnight. One ml of growth culture was centrifuged and the pellets were washed twice with 0.01 M phosphate buffer, pH 7.5. Genomic DNA was isolated using Qiagen DNeasy blood and tissue kit following the manufacturer's instructions. For 16S ribosomal RNA (rRNA) gene amplification, primers 27f (5'-GAG TTT GAT CCT GGC TCA G -3') and 1541r (5'- AAG GAG GTG ATC CAG CCG CA - 3') were used. PCR was carried out under the following conditions: initial denaturation at 92°C for 3 min, followed by 30 cycles of 92°C for 1 min, 50°C for 1 min, and 72°C for 2 min, with a final extension 72°C for 10 min.

2.9. Sequencing of 16S rRNA for some bacterial isolates

PCR products were purified using Qiaquick gel extraction kit (Qiagen) and was sequenced directly using chain- terminator sequencing (Sanger) method by (Genetic Analyzer 3130XL, ABI, America). The following primers were used 16R339: 5'- ACT GCT GCC TCC CGT AGG AG -3' 16F358: 5'- CTC CTA CGG GAG GCA GCA GT -3'704 F: 5GTA GCG GTG AAA TGC GTA GA- 3' for sequencing procedures. The 16S rRNA gene sequences were analyzed using BLAST (Altschul et al., 1997) to identify the closest bacterial neighbors.

2.10. Preparation of crude extracts and TLC autobiography overlay assay

Four marine bacteria (CSS-3, CSS-6, CSS-8, and CSS-10) with wide antibacterial activity against reference strains were inoculated in 500ml Erlenmeyer-flasks containing 300 ml of marine broth. Flasks were incubated on a rotatory shaker 220 rpm for 2days at 30°C. Fermentation broth was first centrifuged at 5000×g for 30 min to remove the cells, then supernatant were extracted 3 times with 100ml ethylacetate (EtoAC). Solvent removal were done under reduced pressure at 37°C (until reach to a concentration about 5ml), these extracts were used as the crude sample for TLC autobiography overlay assay. In this regards, 5µl of each crude extracts were submitted to TLC analysis on two (8×9cm and 4×9cm) silica gel plates (TLC aluminium sheet, 20×20 cm, Silica Gel $60F_{254}$, Merk Co, USA) using dichloromethane (DCM): EtoAc: Methanol (MeOH) (5:5:1, v/v) as the mobile phase, UV/Vis absorption was used for detection at wave lengths of 254 nm. The developed TLC plates were sterilized by UV lamp for 30 min, then plates were transferred to a petri dish with the base of 5mm depth of nutrient agar, it was then covered by melting nutrient agar (40°C) containing test microorganism *Staphylococcus aureus* PTCC1112 ^{T}. After 24h incubation at 37°C, inhibition zones were observed as clear spot against background with grown test strain. Then their R_f values were calculated.

3. Results

Marine microorganisms have become an important point of study in searching for novel antibiotics. This is consequent to the decrease in discoveries from terrestrial sources, as well as, the emergence of antibiotic resistant clinical pathogens such as *Mycobacterium tuberculosis*, *Enterococcus*, *Pseudomonas* sp., *Streptococcus pneumoniae*, and *Staphylococcus aureus* led to constant need to find new sources of effective antibiotics.

In this study, a total 108 bacterial strains were isolated from 145 shoreline water samples of Caspian Sea. Figure 1 shows 29 regions for sample collection across the shorelines of Caspian Sea, the regions were shown with red circles. Most of the isolated bacteria were gram positive and negative bacilli. Amongst 108 isolated bacterial strains, 12 strains (11%) showed significant antibacterial activity against 10 reference strains. The strains were named CSS-1 to CSS-12 regarding to Caspian Sea abbreviation. Table 1 shows antibacterial activity of the isolated strains against the reference strain. As shown in table 1, CSS-3, CSS-11 and CSS-12 strains showed the best antibacterial activity against all reference strains. Table 2 shows antibacterial activity of some selected antibiotics against the same reference strains. As shown in table 2 Gentamycin,

Erythromycin and Nailidixic acid showed the highest antibacterial activity against most of the reference strains but not on all of them.

Amongst the isolated strains, six isolates (CSS- 1, CSS- 2, CSS- 4, CSS-5, CSS-7 and CSS-10) possessed typical cellular, colonial morphologies, physiological, biochemical and nutritional features that resembled *Bacillus* spp. These bacillus strains were motile and produced endospores located at sub terminal, terminal or central positions in the sporangia. As shown in Table 3, six strains have been identified as belonging to the species *B.cereus* and *B.subtilis*, respectively.

Fig 1. Sample sites across the shorelines of Caspian Sea. Twenty nine sampling regions are shown in red circles.

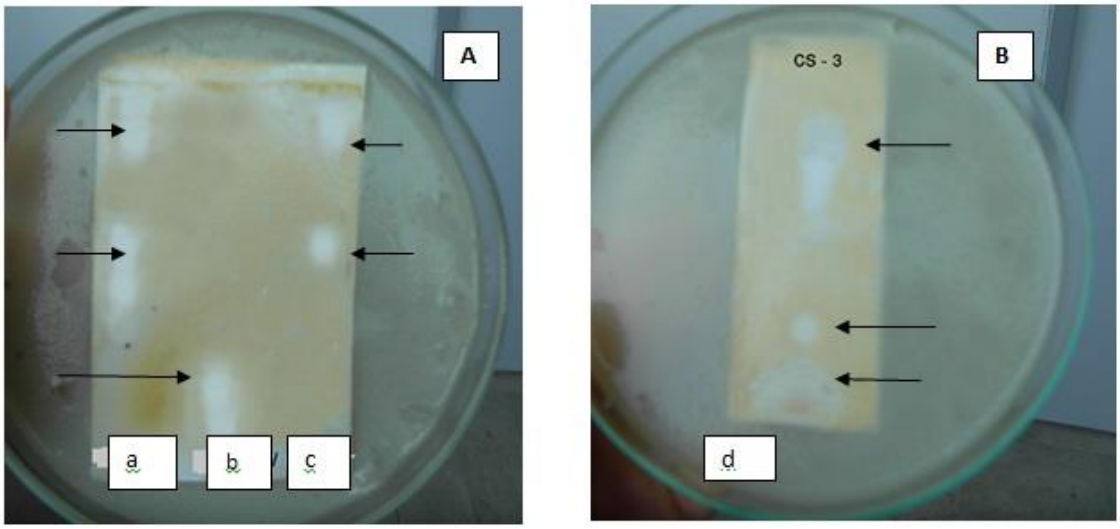

Fig 2. TLC autobiographic assay for broad antimicrobial spectrum of the isolated strains from shorelines of Caspian Sea against *Staphylococcus aureus* PTCC1112 ^{T} (A) and (B). The samples are extracted from the strains: a, CSS-6; b, CSS-8; c, CSS-10; d, CSS-3. The inhibition spots are marked with arrow-head.

Table 1. Antibacterial activities of 12 isolated strains from shorelines of Caspian Sea against some reference strains.

Isolated Strains	Reference strains									
	Salmonella enterica PTCC1231	*Shigella flexneri* PTCC1234	*Staphylococcus aureus* PTCC 1112^T	*Pseudomonas aeruginosa* PTCC 1566^T	*Escherichia coli* PTCC 1533	*Shigella dysenteriae* PTCC 1188^T	*Enterococc us hirae* PTCC 1239^T	*Proteus vulgaris* PTCC 1312	*Streptococcus pyogenes* PTCC 1447	*Bacillus cereus* PTCC 1565^T
CSS-1	10-15[a]	0	9-11	0	10-14	11-15	14-18	7-12	0	0
CSS-2	0	0	10-13	0	9-16	14-17	8-11	8-13	0	0
CSS-3	22-26	13-16	18-20	8	8-12	9-13	12-14	7-10	9-11	9-11
CSS-4	9-12	8-10	8-11	0	9-13	8-14	12-17	9-11	0	0
CSS-5	8-13	10-12	10-13	0	13-16	14-19	9-14	0	5-10	0
CSS-6	10-14	14-16	16-17	0	9-12	15-18	0	0	9-13	0
CSS-7	0	0	12-14	0	11-13	9-11	11-14	0	0	0
CSS-8	10-15	21-25	9-11	0	9-12	0	0	0	0	0
CSS-9	13-15	15-17	11-16	0	9-14	0	0	0	0	0
CSS-10	0	0	915	0	9-10	10-13	8-12	0	0	5-9
CSS-11	19-22	14-17	16-21	13-18	15-21	13-15	11-16	9-11	18-23	9-13
CSS-12	21-26	12-14	9-14	13-17	17-23	14-17	14-18	8-12	13-17	7-10

[a] renge of the mean diameter of inhibition zone, mm

Table 2. Antibacterial activity of some selected antibiotics against the reference strains.

Antibiotic	Reference strains									
	Salmonella enterica PTCC1231	*Shigella flexneri* PTCC1234	*Staphylococcus aureus* PTCC 1112^T	*Pseudomonas aeruginosa* PTCC 1566^T	*Escherichia coli* PTCC 1533	*Shigella dysenteriae* PTCC 1188^T	*Enterococcus hirae* PTCC 1239^T	*Proteus vulgaris* PTCC 1312	*Streptococcus pyogenes* PTCC 1447	*Bacillus cereus* PTCC 1565^T
Gentamycin GM10	10-25[a]	0	19-23	0	16-19	10-14	16-18	16-20	23-27	21-24
Nitrofurantoin FM300	15-18	13-15	0	0	16-22	13-18	18-24	0	21-26	18-25
Vancomycin V30	15-20	0	13-18	0	10-14	14-16	12-16	0	9-13	8-13
Cephalexin CN30	0	0	26-31	0	8-14	13-17	19-25	12-14	21-25	17-21
Rifampicin RA5	0	0	32-36	0	0	0	0	0	23-27	6-11
SXT	0	0	20-25	0	0	0	21-26	3-8	28-31	32-35
Tetracycline TE30	16-21	16-19	0	0	16-18	14-19	20-23	12-16	17-23	23-27
Erythromycin E15	23-28	13-16	0	0	23-28	18-24	20-24	0	13-16	18-22
Nailidixic acid NA30	18-25	17-21	0	0	18-22	18-22	20-26	24-26	12-16	19-25

Table 3. phenotypic characteristics of gram positive rods isolated from shorelines of Caspian Sea

Characteristics	*Bacillus subtilis* PTCC 1720^T	*Bacillus cereus* PTCC 1247^T	CSS- 1	CSS- 2	CSS- 4	CSS-5	CSS-7	CSS-10
Cell								
diameter >1.0 μ	-[a]	+	+	+	-	-	-	+
Spore round	-	-	-	-	-	-	-	-
Sporangium swollen	-	-	-	-	-	-	-	-
Parasporal crystals	-	-	-	-	-	-	-	-
Catalase	+[b]	+	+	+	+	+	+	+
Anaerobic growth	-	+	+	+	-	-	+	+
VP	+	+	+	+	+	+	+	-
pH in V-P broth								
pH < 6	+	+	+	+	-	-	+	+
pH > 7	-	-	-	-	-	-	-	-
Acid from:								
D- Glucose	+	+	+	+	+	+	+	+
+L- Arabinose	+	-	-	-	+	+	-	-
D-Xylose	+	-	-	-	+	+	-	-
D-Mannitol	+	-	-	-	+	+	-	-
Hydrolysis of								
Casein	+	+	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+
Utilization of Citrate	+	+	+	+	+	+	+	-
Nitrate reduced to nitrite	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-
Hemolysis	+	-	+	+	-	-	+	+
Growth at pH 6.8	+	+	+	+	+	+	+	+
Growth at pH 5.7	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+
Egg-yolk Lecithinase	-	+	+	+	-	-	+	-
Deamination of								
Phenylalanine	-	-	-	-	-	-	-	+
Growth in NaCl								
2%	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+
7%	+	-	-	-	+	+	-	-
10%	+	-	-	-	+	+	-	-
Growth at								
5°C	-	-	-	-	-	-	-	-
10°C	-	-	-	-	-	-	-	-
30°C	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+
50°C	+	-	+	-	+	+	+	+

Table 4. phenotypic characteristics of isolated gram negative rods from shorelines of Caspian Sea

Characteristics	*Pseudomonas aeruginosa* PTCC 1466 ^{T}	CSS- 3	CSS- 11	CSS- 12
Catalase	+	+	+	+
Oxidase	+	+	+	+
Growth on MacConkey agar	+	+	+	-
KIA	Alk/Alk	Alk/Alk	Alk/Alk	Alk/Alk
OF	Oxidative	Oxidative	Oxidative	Oxidative
SH_2	-	-	-	-
Indol	+	+	+	+
Motility	+	+	+	+
Argenin dihydrolase	+	+	+	+
Pyocyanin Production	+	+	+	+
Gelatin hydrolase	+	+	+	+
Growth at				
4°C	-	-	-	-
41°C	+	+	+	+
Starch hydrolysis				
Denitrification	+	+	+	+
Hemolysis	+	+	+	+

4. Discussion

Bacillus species are well known to be the producers of metabolite with antimicrobial and antifungal activities (Zuber et al., 1993). Bioactive metabolites have also been reported in a variety of Bacillus sp. (Lebbadi et al., 1994; Hathout et al., 1999), rendering this genus one of the most active producer of bioactive metabolites. Antimicrobial metabolites such as macrolactin F, 7-O- succinylmacrolactin F and 7-O- succinylmacrolactin A, from *Bacillus* sp. (Sc 026) (Jaruchoktaweechai et al., 2000), new thiopepetide compounds from *Bacillus cereus* QN03323 (Nagai et al., 2003), and three bacteriocin-like peptides named Lichenin, Bacillocin 490 and P40 produced by *B.licheniformis* strain P40, (Cladera-Olivera et al., 2004) are unique in this genus.

Bacillus species have primarily been isolated from soil but have been frequently found also in several marine habitats (Trischman et al., 1994; Seifert et al., 2000) on multiple occasions. Also isolation of bacillus species such as *B. cereus*, *B.clausii*, *B.pumilus* and *B.subtilis* from marine macroalgae in Japan have been reported (Manmadhan et al., 2006).

Three isolates (CSS- 3, CSS- 11 and CSS-12) were identified as *Pseudomonas aeruginosa* based on morphological and biochemical characteristics as shown in Table 4. These *Pseudomonas aeruginosa* strains showed highest antibacterial activity against reference strains. The antimicrobial activity by these strains was more than Gentamycin, Erythromycin and Nailidixic acid antibiotics. Marine *Pseudomonas* has been reported to produce an antibiotic metabolite which is inhibitory for both gram-negative and gram-positive bacteria (Nair and Simidu, 1987 Uzair et al., 2006; O'Grady et al., 1997). The production of inhibitory compound by fluorescent *Pseudomonas* is well documented (Leong, 1986; Isnansetyo et al., 2003; Sing et al., 2003). Also, fluorescent *Pseudomonads* have drawn attention worldwide because of the production of secondary metabolites such as siderophores, antibiotics, volatile compounds HCN, enzymes and phytohormones (Edward and Errington, 1997; Fenton et al., 1992). Antibacterial activity of *Pseudomonas* sp. isolated from spoiled iced fish and newly caught fish has also been demonstrated (Gram, 1993). One isolate (CSS-6) was identified as *Alcaligenes faecalis*. *Alcaligenes faecalis* is a gram negative rods or coccobacilli, motile with one to nine peritrichous flagella, obligately aerobic, possessing a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Our isolate was confirmed by Persian Type Culture Collection (PTCC,Tehran/Iran) as *Alcaligenes faecalis* PTCC 1757. Two isolates (CSS-8 and CSS-9) were identified as *Exiguobacterium acetylicum* based on biochemical and 16S rRNA analysis. This isolate deposited in Persian Type Culture Collection (PTCC) as *Exiguobacterium*

acetylicum PTCC 1756. The genus Exiguobacterium belongs to the expanding group of coryneform bacteria with thermophilic properties. The cells in genus *Exiguobacterium* are ovoid or short occurring singly, in pairs, or chains, nonspore forming, motile, facultatively anaerobic and catalase positive. The Centers for Disease Control has reported a number of *Exiguobacterium* strains from various clinical sources (e.g.skin, wounds, cerebrospinal fluid) (Funke et al.,1997). In one of the available studies, Barnet et al. (2006) have reported the suppression of *R.solani* AG-8 on wheat by an interaction between *Pantoea*, *Exiguobacterium* and *Microbacteria*, in disease suppressive soils, but they were able to prove the antagonistic effect of the *Exiguobacterium* strains isolated from disease suppressive soils, nor were they able to detect the reduction in pathogenic DNA levels as a result of interaction between the bacterial microflora. For the first time, Selvakumar et al., (2009) reported antagonistic effect of *Exiguobacterium acetylicum* strain IP (MTCC 8707) against fungal pathogens such as *Rhizoctonia solani*, *S. ralfisii*, *Phytium* and *F.oxyssporum*.

The crude extracts of four isolates (CSS-3, CSS-6, CSS-8 and CSS-10) with wide antibacterial activity were subjected to TLC autobiography overlay assay, and the results were presented in Fig 2. (A and B), respectively. Each extract of different strains showed one or two inhibition spots, and the R_f values of these spots were different from 0 to 0/93.

The results of TLC autobiographic overlay assay demonstrated that different species could produce different antimicrobial metabolites, and some had more than one antimicrobial substance. Antibacterial activity was detected in an ethyl acetate fraction of the crude extracts. This suggests that these antimicrobial compounds are not bound to cell surface and could be extracted from the fermentation broth by portioning organic solvents. In this study antibacterial effect of the isolated strains was evaluated after 96 hours, suggesting that these active compounds could be secondary metabolites.

Conclusion

From this study it could be concluded that a majority of bioactive compounds produced by the isolated bacteria from shoreline waters of Caspian Sea. Although there are lots of reports concerning bioactive compounds production by different microorganisms. The isolated *Pseudomonas aeruginosa* strains showed significant antimicrobial activity against all reference strains comparing to some potent antibiotics such as Gentamycin, Erythromycin and Nailidixic acid. To the best of our knowledge bioactive material production by *Alcaligenes* and *Exiguobacterium* genus is a new area for finding new antimicrobial compounds. In this study, we showed the first report on the antagonistic properties of *Exiguobacterium acetylicum* against different bacterial strains.

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