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Bacterial diversity in south coast of the Caspian Sea: Culture-dependent and culture-independent survey

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ABSTRACT

Bacterial diversity in the south coast of the Caspian Sea was studied by analyzing 16S rDNA clone library and cultivation technique. Analysis of inserts of 30 clones revealed a total of 13 OTUs. All of these sequences were related to *Proteobacteria* including *Betaproteobacteria* (60%), *Gammaproteobacteria* (22%) and *Alphaproteobacteria* (18%). The majority of these sequences (40%) branched with member of *Limnobacter*. Within the cultivation effort, phylotypes related to *Gammaproteobacteria* (60%), *Firmicutes* (27%), *Actinobacteria* (9%) and *Bacteroidetes* (4%) were retrieved. Members of the *Bacillus* (14%) and *Rheinheimera* (18%) were the most common isolates. The secretions of eight hydrolytic enzymes and antibiotic compounds, as well as resistance to heavy metals were studied in these marine strains. Among them 46, 45, 38, 27, 19, 19, 7 and 4% of bacterial isolates were able to produce protease, pectinase, xylanase, amylase, cellulase, lipase, urease and DNase, respectively. Two strains which are phylogenetically related to *Streptomyces* and *Stenotrophomonas* produced antimicrobial compounds and could inhibit the growth of *Candida albicans* and *Bacillus subtilis*, respectively. A total of 2, 1, 2, 3 and 2 strains could survive in the presence of lead (1500 ppm), cadmium (1000 ppm), zinc (2000 ppm), copper (2000 ppm), and Chromium (2000 ppm), respectively. The investigation showed that this marine environment harbors a high bacterial diversity which is a potential source of hydrolytic enzymes and other valuable biotechnological activities.

Key words: Antimicrobial compounds, Bacterial diversity, Caspian Sea, Heavy metal resistance, Hydrolytic enzymes.

INTRODUCTION

Marine environments, the largest ecosystem on earth, harbor high microbial species (high evenness). This is because of the conditions in marine systems, which are neither selective nor inhibitory to specific groups of microorganisms (in contrast to the environments like hypersaline lakes harbor high species richness). The majority of metabolites isolated from marine microorganisms, as well as decreasing rate of discovery of novel compounds from soil led to interest in studying the marine microbial population (Lane & Moore 2011). There are various published reports about the biotechnological applications of marine microorganisms for novel bioactive compounds (Bhatnagar & Kim 2010; Imhoff *et al*. 2011) or biocatalysts (Zhang & Kim 2010, Trincone 2011). Ecological studies also emphasized on the potential applications of marine microorganisms in metal detoxification, nutrient cycling and greenhouse gas reduction (Zaky *et al*. 2014; Salazar & Sunagawa 2017). Hence, awareness of microbial variation in aqueous environments will provide great source for biotechnological application.

The Caspian Sea is the largest inland body of water in the planet with a surface area of about 373,000 km^2 (Dumont 1998). It is bordered by five countries including Azerbaijan, Iran, Kazakhstan, Russia and Turkmenistan. The water surface lies 28 m below sea level. It reaches its maximum depth of 1025 m in the south while the northern half averages only about 5 m depth. About 130 rivers of varying size drain freshwater into the sea thereby

decreasing its salinity to the maximum amount of 1.3 (% w/v) (one-third of the salinity of seawater) (Leroy *et al*. 2007). This landlocked system suffers from anthropogenic pollutants discharged from municipals and industrial wastes (Zonn 2005) which can influence the microbial population in coastal regions. Recently-published reports considered its sediments and brackish water microbial diversity by next generation sequencing (Mahmoudi *et al*. 2015, Mehrshad *et al*. 2016). However, there is no report about bacterial diversity in the coastal region of the sea. The following research focuses on bacterial diversity in the south coast of the Caspian Sea (Iranian part) and aims to a) describe its bacterial community using both cultivation and culture-independent approaches, b) survey on some biotechnological activities including: hydrolytic enzymes production, antimicrobial activity and resistance to heavy metals in the indigenous bacterial strains.

MATERIALS AND METHODS

Site description, samples collection and analysis

The Caspian Sea water was sampled from 0.5 and 30 m depth, and about 200 m away from the sea shore of four cities including Behshahr (36°41′40″ N, 53°32′33″ E), Babolsar (36°42′43″ N, 52°38′44″ E), Nowshahr (36°66′04″ N, 51°48′12″ E) and Tonekabon (36°48′31″ N, 51°52′55″ E) in Mazandaran Province, north of Iran in October 2013. The salinity and pH of the samples was determined *in situ* with SevenMulti dual meter pH/conductivity (Mettler). The anions and cations present in the samples were analyzed using titration and atomic absorption methods, respectively (Saad *et al*. 1998). Samples were pooled for culture dependent and culture-independent analyzes.

Culture media and growth conditions

Marine bacteria were isolated under aerobic conditions on different growth media: The marine agar (Merck); SW medium contained (g L^{-1}): NaCl 9.1, $MgSO_4.7H_2O$ 0.75, $MgCl_2.6H_2O$ 0.17, KCl 0.13, $CaCl_2.2H_2O$ 0.22, $(NH_4)_2SO_4$ 0.86, $FeSO_4.7H_2O$ 0.009, peptone 10.0, yeast extract 2.0, and agar 15.0; pH 8.2; sea water salt medium consisted of filtered Caspian Sea water supplemented by (g L^{-1}): peptone 1.0, yeast extract 0.2 and agar 15.0; pH 8.2. All samples were spread plated to culture media. The plates were incubated aerobically at 20 °C for 8 weeks. After successive cultivation, pure isolates were obtained.

DNA extraction, amplification of 16S rRNA genes and gene library construction

Genomic DNA was extracted using a Genomic-DNA extraction kit (Thermo Scientific, Lithuania), according to the manufacturer's instructions.

A modified method of Benlloch *et al*. (1996) including freezing and thawing step was applied for environmental DNA extraction. The 16S rRNA genes were amplified using Bacteria-specific froeward, 5′-AGAGTTTGATCATGGCTCAG-3′, and reverse, 5′- GGTTACCTTGTTACGACTT-3′, primers (Lane et al. 1985). Following PCR conditions were employed: 94 °C for 2 min, followed by 30 cycles of 94 °C for 60 s, 55 °C for 60 s and 72 °C for 60 s, with final 7 min extension at 72 °C. In amplifications involving environmental DNA, mentioned PCR condition varied with different annealing temperatures (48-60 °C). The PCR products of expected size (1500 bp) were gel purified (DNA extraction kit, Roche, Germany), pooled and ligated into pGEM-T cloning vector (Promega, USA) and employed to transform *E.coli* DH5α cells according to the manufacturer's instructions.

Sequencing and sequence analysis

The sequencing was conducted on ABI 3730XL DNA sequencer at Macrogen (Seoul, South Korea). The 16S rRNA genes of isolates were sequenced directly. The nucleotide sequences of the cloned products were determined from plasmid preparations (Miniprep plasmid extraction kit, Bioneer, South Korea). Putative chimeric sequences were recognized using Bellerophon server [8]. Relevant sequences were extracted from GenBank (www.ncbi.nlm.nih.org) using BLASTN and

through EzTaxon server (Chun *et al*. 2007). The sequences were considered to belong to an operational taxonomic unit (OTU) if they shared ≥97 % sequence identity. The alignments were generated using MUSCLE web server (http://www.ebi.ac.uk/Tools/msa/muscle/). Phylogenetic analysis was performed using the software package MEGA version 6 (Tamura *et al*. 2013).

Screening for extracellular hydrolytic activities

Qualitatively detection of extracellular amylase, cellulase, DNase, lipase, pectinase, protease, urease and xylanase production was performed in solid suitable SW medium. After 5 days incubation at 20 °C standard methods were applied for screening approach as described by Rohban *et al*. (2009) and Ramesh *et al*. (2014)

Screening for heavy metals (Cd, Cr, Cu, Pb and Zn) resistance

MIC (Minimum inhibitory concentration) determination was conducted in SW medium following the method described by Khammar *et al*. (2015).

Screening for antimicrobial activity

For preliminarily antimicrobial activity study, marine strains were streaked onto TSA (Merck) and incubated at 20 °C for 5 days. Test strains including *Staphylococcus aureus* (PTCC1431), *Bacillus subtilis* (PTCC1023), *Pseudomonas aeruginosa* (PTCC1074), *Escherichia coli* (PTCC 1330) and *Candida albicans* (PTCC 5027) were streaked perpendicular to the marine strains and incubated overnight. Inhibitory activity was indicated by inhibited growth of pathogenic strains as compared to the negative control. Selected marine strains (based on the zone of inhibition) were inoculated into 100 mL of SW broth medium and incubated at 20 °C in a rotary shaker at 150 rpm for 5 days. The broth culture was extracted using equal volume of ethyl acetate and then solvent was removed by evaporation.

The antibacterial effects of crude extract were compared with tetracycline (30 µg $disc^{-1}$) and cycloheximide (100 µg $disc^{-1}$) as positive control against bacterial and fungal test strains, respectively.

RESULTS

Diversity of bacterial isolates and sequences of environmental 16S rRNA genes

The physicochemical properties of water samples collected are presented in Table 1. We identified Na^+ and Cl^- as major ions in the samples followed in abundance by SO_4^{2-} and Mg^{2+}. The temperature of samples was about 19 °C.

Table 1. Chemical characterization of the Caspian Sea water.

Ion	Concentration (g L^{-1})
Na^+	3.41
Mg^{2+}	0.83
Ca^{2+}	0.34
K^+	0.079
Mn^{2+}	>0.001
Fe^{2+}	>0.001
Cl^-	5.33
SO_4^{2-}	3.88
HCO_3^-	0.26

After eight weeks of incubation, viable counts obtained on different media were comparable and ranged from 2.5 - 4 × 10^6 CFU mL^{-1}. A total of 124 isolates were obtained and subsets of 30 different strains were analyzed. Bacterial isolates clustered into 17 OTUs (Table 2) and belonged to *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Gammaproteobacteria* phyla (Fig. 1). They were phylogenetically related to the following genera: *Aeromonas* (3.5 % of bacterial isolates obtained), *Bacillus* (14%), *Cyclobacterium* (3.5%), *Halomonas* (3.5%), *Jeotgalicoccus* (3.5%), *Kocuria* (3.5%), *Marinobacter* (7%), *Micrococcus* (3.5%),

Paenibacillus (7%), *Pseudoalteromonas* (7%), *Pseudomonas* (7%), *Psychrobacter* (3.5%), *Rheinheimera* (18%), *Sporosarcina* (3.5%), *Staphylococcus* (3.5%), *Stenotrophomonas* (3.5%) and *Streptomyces* (3.5%). A total of 30 bacterial clones were selected randomly and sequenced. Chimeric sequences (3 sequences) were removed, while others were assigned to OTUs sequences and used for phylogenetic analysis (Table 2). Environmental sequences of bacteria, which formed 13 OTUs, belonged to *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* (Fig. 1). None of the detected groups by cultivation were identified in culture-independent approach. The majority of sequences (40%) branched with member of *Limnobacter*. Other phylotypes were related to *Citreimonas* (4.5% of sequences analyzed), *Acinetobacter* (4.5%), *Acidovorax* (4.5%), *Spongiibacter* (4.5%), *Perlucidibaca* (4.5%), *Seohaeicola* (4.5%), *Youngimonas* (4.5%) and *Sulfitobacter* (4.5%). A remarkable part of recovered sequences (20%) were unrelated to any previously reported sequences.

Table 2. Comparison of isolates and clones *16S rRNA* sequences obtained from the Caspian Sea with those available in EzTaxon (Chun *et al*. 2007).

OTU-97 %	No. of isolates	Closest identified species	Similarity (%)
Isolates			
1	1	*Cyclobacterium caenipelagi*	98.8
2	2	*Paenibacillus barcinonensis*	99.7
3	1	*Streptomyces hydrogenans*	99.7
4	2	*Pseudoalteromonas lipolytica*	99.1
5	5	*Rheinheimera aquimaris*	98.5
6	1	*Halomonas aquamarina*	98.7
7	4	*Bacillus aryabhattai*	99.8
8	1	*Staphylococcus equorum*	99.9
9	1	*Aeromonas caviae*	99.1
10	1	*Kocuria rosea*	99.6
11	1	*Stenotrophomonas rhizophila*	99.7
12	1	*Pseudomonas hibiscicola*	98.6
13	2	*Marinobacter lipolyticus*	98.6
14	1	*Jeotgalicoccus coquina*	99.7
15	1	*Micrococcus terreus*	99.8
16	1	*Psychrobacter faecalis*	99.7
17	1	*Sporosarcina psychrophila*	99.9
clones			
1	9	*Limnobacter thiooxidans*	99.1
2	1	*Citreimonas salinaria*	97.2
3	2	*Limnobacter thiooxidans*	92.5
4	1	*Acinetobacter johnsonii*	98.2
5	1	*Acidovorax temperans*	99.7
6	1	*Spongiibacter tropicus*	95.2
7	1	*Perlucidibaca piscinae*	95.3
8	1	*Limnobacter thiooxidans*	87.5
9	1	*Porticoccus litoralis*	92.2
10	1	*Oleiphilus messinensis*	98.8
11	1	*Seohaeicola saemankumensis*	99.7
12	1	*Youngimonas vesicularis*	97.1
13	1	*Sulfitobacter marinus*	99.2

Enzymes production by marine isolates

Survey on enzymes production was tested qualitatively among strains isolated from the Caspian Sea (Fig. 2). A total of 12, 12, 10, 7, 5, 5, 2 and 1 bacterial isolates were able to produce protease, pectinase, xylanase, amylase, cellulose, lipase, urease and DNase, respectively.

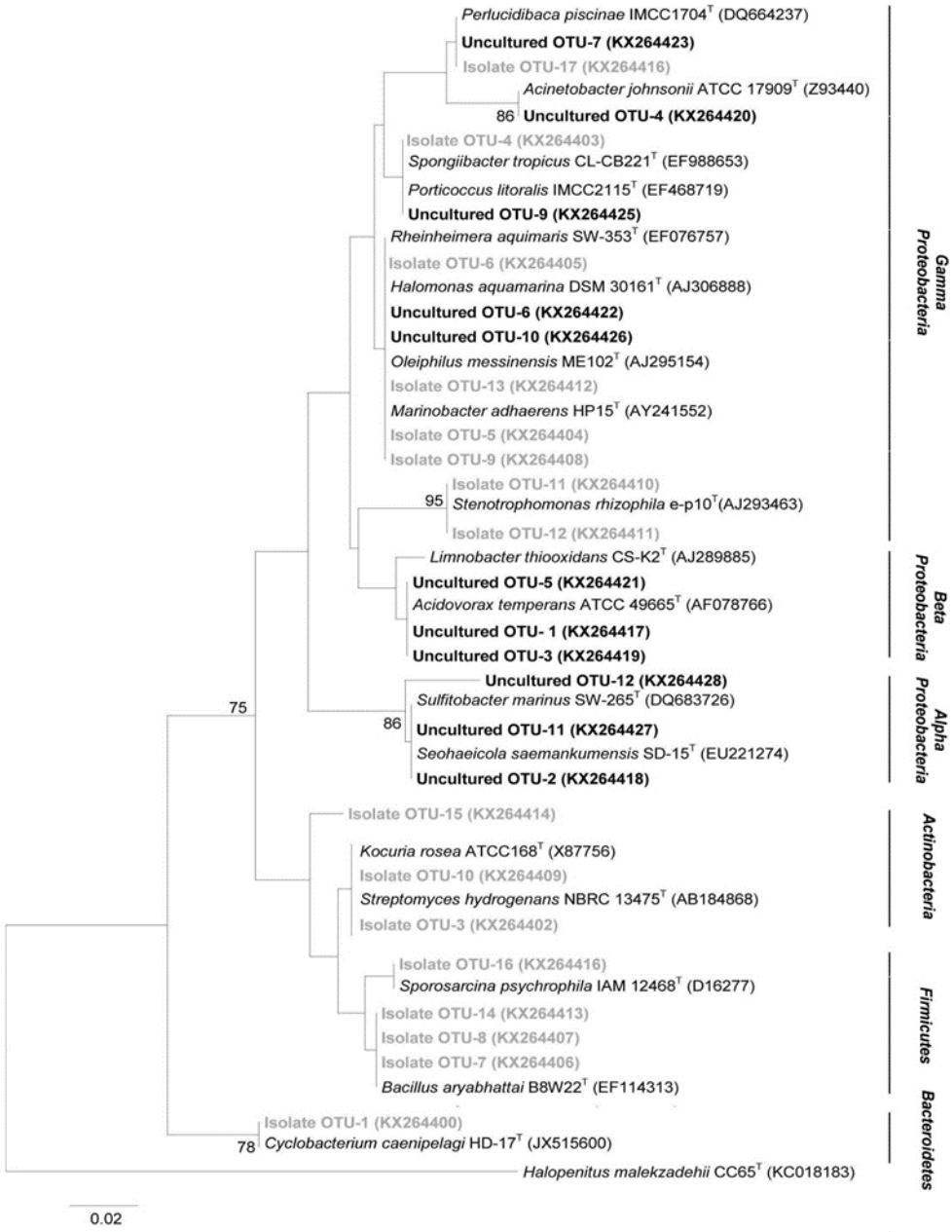

Fig. 1. Neighbor-joining phylogenetic reconstruction of 16S rRNA of bacterial sequences recovered from the Caspian Sea. Gray: isolate sequences; bold black: environmental sequences. The sequence of the halophilic archaeon *Halopenitus malekzadehii* CC65T (KC0180183) was used as an outgroup. Bootstrap values (%) are based on 1000 replicates. Only values higher than 70% are shown. Bar, equals 0.02 substitutions per nucleotide position.

Combined hydrolytic activity was also detected in many marine strains. Two strains (belonged to *Pseudomonas* and *Streptomyces* genera) presented 6 hydrolytic activities. Protease, pectinase and xylanase were the most common hydrolytic enzymes among strains by 46, 38 and 33% abundance, while amylase (25%), cellulase (20%) and lipase (20%), had an intermediate diversity. The rare rate of urease (6%) and DNase (3%) were observed.

Screening of heavy metal tolerant in marine bacteria

Marine isolates were screened to identify most potential metal resistant bacteria. All of the bacterial strains could survive at: cadmium (Cd) (100 ppm), chromium (Cr) (100 ppm), copper (Cu) (100 ppm), lead (Pb) (100 ppm) and zinc (Zn) (250 ppm). Strain ZP37 (*Pseudomonas*) could survive in 1000 ppm cadmium. Strains ZC15 and KP10 which is phylogenetically related to *Jeotgalicoccus* and *Psychrobacter* could tolerate up to 2000 ppm chromium. High level of copper resistance (2000 ppm) were observed in strains MN24 (*Micrococcus*), KP10 (*Psychrobacter*) and FZC6 (*Bacillus*). Strains Mn23 (*Kocuria*) and KC19 (*Sporosarcina*) could tolerate 2000 ppm zinc. Finally two strains including KP10 (*Psychrobacter*) and ZP35 (*Stenotrophomonas*) could grow in the presence of 1500 ppm of lead (Fig. 3).

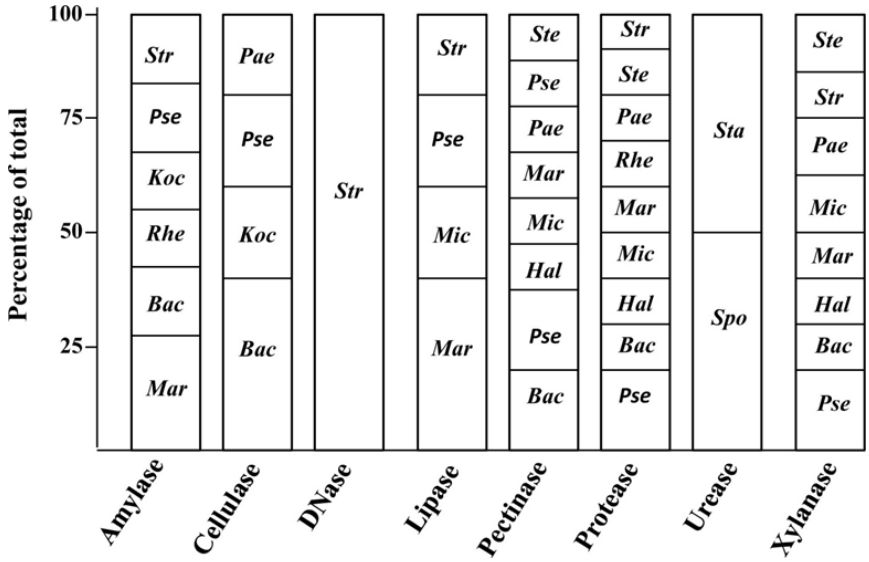

Fig. 2. Phylotype diversity for enzyme-producing bacteria in the Caspian Sea. The bar chart compares 16S rRNA sequence diversities of strains produced various hydrolytic enzymes. Abbreviations; *Bac*: *Bacillus*; *Hal*: *Halomonas*; *Koc*: *Kocuria*; *Mar*: *Marinobacter*; *Mic*: *Micrococcus*; *Pae*: *Paenibacillus*; *Pse*: *Pseudomonas*; *Rhe*: *Rheinheimera*; *Ste*: *Stenotrophomonas*; *Str*: *Streptomyces*.

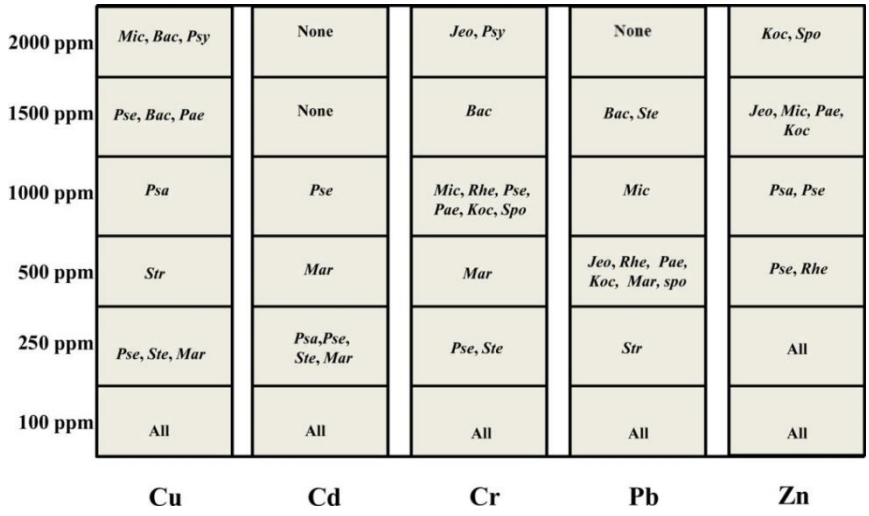

Fig. 3. Phylotype diversity for heavy metal resistance bacteria in the Caspian Sea. The bar chart compares 16S rRNA sequence diversities of strains resistant to various amount of metals. Abbreviations; *Bac*: *Bacillus*; *Hal*: *Halomonas*; *Koc*: *Kocuria*; *Mar*: *Marinobacter*; *Mic*: *Micrococcus*; *Pae*: *Paenibacillus*; *Pse*: *Pseudomonas*; *Rhe*: *Rheinheimera*; *Ste*: *Stenotrophomonas*; *Str*: *Streptomyces*.

Antimicrobial activity of marine isolates

Only two strains, ADR10 and ZP35 which are phylogenetically related to *Streptomyces* and *Stenotrophomonas,* represented antimicrobial activity against test strains according to the cross-streak method. The growth inhibition was also observed by well diffusion assay in the cultures of *C. albicans* and *B. subtilis* by strains ADR10 and ZP3, respectively. The high antifungal activity was observed by a crude extract of strain ADR10 culture (22 mm inhibition zone compared to 11 mm zone of cycloheximide [100 µg mL^{-1}]) (Fig. 4). However ZP35 crude extract represented low antibacterial activity (8mm inhibition zone compared to 20 mm zone by tetracycline [30 µg mL^{-1}]).

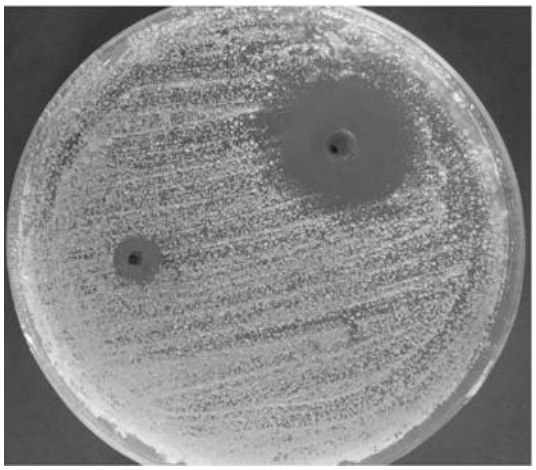

Fig. 4. Antifungal effect of strain ADR10. Culture extract (right), cycloheximide (left).

DISCUSSION

Oligotrophy (limited amounts of nutrients) is a common condition in aquatic environments (Bristow *et al.* 2017). However, meso- to eutrophic are usual in coastal waters due to incoming nutrients from anthropogenic origins (Smith 2003). Therefore, microbial populations near the shore may be different from offshore regions with respect to different amounts of nutrients. Over half of the sequences retrieved from meso- to eutrophic water in the present study belonged to *Betaproteobacteria* (60 %), followed by clones related to *Gammaproteobacteria* (22 %) and *Alphaproteobacteria* (18 %). The recently- reported ratio of these classes of *Proteobacteria* in the offshore water of the Caspian Sea (15 - 150 m in depth) was different, while the *Alphaproteobacteria* (60%) and *Gammaproteo- bacteria* (30%) were dominant; but only 10% of retrieved sequences belonged to *Betaproteobacteria* (Mehrshad *et al.* 2016). The proportion of this class of *Proteobacteria* was also different in the other report of bacterial diversity in sediments of the Caspian Sea (Mahmoudi *et al.* 2015). In the latter report, most retrieved sequences in this groups belonged to the *Gammaproteobacteria* (over 75% in depth of 140 m), whereas the two other classes were equal (about 12.5% each). Abundance of *Betaproteobacteria* in the present study may refer to the chemiolithotrophic condition in this coastal water. Most *Betaproteobacteria*-related sequences obtained were related to *Limnobacter thiooxidans,* a thiosulfate-oxidizing bacterium isolated from freshwater lake. However the limited number of sequences in the present study in comparison with afore-mentioned reports may also bias the results.

With respect to the culture-dependent approach, retrieved *Proteobacteria*-related sequences had no overlaps with clone sequences at the sub phylum levels (Table 2). Furthermore, sequences related to *Firmicutes*, *Actinobacteria* and *Bacteroidetes* could only be obtained in the culture base approach. Interestingly, *Firmicutes* were not reported in

the two above metagenomics-based studies in the Caspian Sea. The findings supported the importance of cultivation approaches in high throughput sequencing ages (Prakash *et al.* 2013). Enzymes from marine microorganisms may differ from terrestrial homologous according to the special marine conditions (Zhang & Kim 2010). For all eight qualitatively-screened enzymes in the present study, the producing strains were detected. Protease was the most common enzyme and over half of analyzed taxa represented this activity. Xylanase had particular economic value but most of the reported marine xylanase belonged to fungi (Raghukumar *et al.* 2004) or *Bacillus* sources (Yin *et al.* 2010). However, eight strains from the present study related to different genera including *Bacillus*, *Halomonas*, *Marinobacter, Micrococcus*, *Paenibacillus, Pseudomonas*, *Stenotrophomonas* and *Streptomyces* could produce remarkable amount of xylanase. Interestingly two strains related to *Staphylococcus* and *Sporosarcina* showed urease activity at alkaline condition. These enzymes are very important for bioremediation and bio-consolidation (Anbu *et al.* 2016) in alkaline soils. As a land-locked system, discharged anthropogenically pollutants into the Caspian Sea are concentrated. All of the obtained strains in the present study could grow in remarkable concentrations (100 ppm) of heavy metals. The abundance of bacterial resistance against different heavy metals reveals the contaminated nature of the Caspian Sea. On the other hand, it may show the properties of aquatic systems, as a recombination soup that many traits could be distributed among their inhabitants. Emergence of antibiotic-resistant pathogens along with increasing the susceptibility in communities by extended life expectancy as well as prevalence of predisposing disease, such as cancer or AIDS make it necessary to develop new antibiotic classes. There are serious concerns about fungal infections and they are recognized as an important public health problem. In the present study, a novel *Streptomyces* sp. is introduced which its antifungal activity against *C. albicans* is twice in comparison with cycloheximide. As predicted recently, marine environments contain many unique forms of actinomycetes which are sources for very new antimicrobial compounds (Subramani & Aalbersberg 2012).

CONCLUSION

The sequences obtained in this study are not related to many groups previously obtained from metagenomics-based studies. Also, sequences from culture-dependent and molecular approach used in the present study are different. It could be concluded that the different methods applied to examine the microbial diversity of one ecosystem may result in various outcomes. Bacterial isolated from the Caspian Sea represent great potential for some biotechnological applications like enzyme production, antibiotic generation and bioremediation process.

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تنوع زیستی باکتریایی در ساحل جنوبی دریای خزر: ارزیابی وابسته به کشت و غیر وابسته به کشت

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چکیده

تنوع زیستی باکتری‌های ساحل جنوبی دریای خزر با دو روش کتابخانه ژنی *16S rRNA* و کشت ارزیابی شد. بررسی ۳۰ کلون از کتابخانه ژنی منجر به شناسایی ۱۳ گروه تاکسونومی متفاوت شد. تمامی این توالی‌ها متعلق به *Proteobacteria* و شامل *Betaproteobacteria* (۶۰٪)، *Gammaproteobacteria* (۲۲٪) و *Alphaproteobacteria* (۱۸٪) بودند. بخش اصلی این توالی‌ها مربوط به جنس *Limnobacter* بود. در روش وابسته به کشت، اعضای *Gammaproteobacteria* (۴۰٪)، *Firmicutes* (۲۷٪)، *Actinobacteria* (۹٪) و *Bacteroidetes* (۴٪) جداسازی شدند. بیشترین سویه‌ها متعلق به جنس های *Bacillus* (۱۴٪) و *Rheinheimera* (۱۸٪) بود. توانایی تولید هشت آنزیم هیدرولازی، تولید آنتی‌بیوتیک و مقاومت به فلزات سمی در این سویه‌های دریایی بررسی شد. به ترتیب ۴۶، ۴۵، ۳۸، ۲۷، ۱۹، ۱۹، ۷ و ۴ درصد از جدایه‌ها قادر به تولید پروتئاز، پکتیناز، زایلاناز، آمیلاز، سلولاز، لیپاز، اوره آز و دی ان آز بودند. دو سویه متعلق به جنس‌های *Streptomyces* و *Stenotrophomonas* با تولید ترکیبات ضد میکروبی قادر به مهار رشد *Bacillus subtilis* و *Candida albicans* بودند. در مجموع ۲، ۱، ۲، ۳ و ۲ سویه به ترتیب قادر به حفظ حیات در حضور سرب (۱۵۰۰ ppm)، کادمیوم (۱۰۰۰ ppm)، روی (۲۰۰۰ ppm)، مس (۲۰۰۰ ppm) و کروم (۲۰۰۰ ppm) بودند. پژوهش حاضر نشان داد که این محیط دریایی با تنوع باکتریایی بالا می‌تواند منبعی برای آنزیم‌های هیدرولازی مفید و همین طور دیگر توانمندی‌های مرتبط با زیست فناوری باشد.