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Isolation of Halophilic Bacteria from Maharlu salt Lake - Iran and their evaluation for the production of bioactive compounds

Toktam Hashemi[1] (M.Sc), Majid BaseriSalehi[1] (Ph.D), Nima Bahador[2] (Ph.D)

1.Department of Microbiology, Kazeroon Branch, Islamic Azad University of Kazeroon, Kazeroon, Iran
2.Department of Microbiology, Shiraz Branch, Islamic Azad University of Shiraz, Shiraz, Iran

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ABSTRACT

Halophilic bacteria grow over a wide range of salt concentrations. In this study we aimed to isolate and screen out the halophilic bacteria and to determine their activity for production of the bioactive compounds. A total of 50 water, sediments and soil samples were collected from Maharlu salt lake in southern region of Fars-Iran and subjected for isolation of the bioactive compound producing Halophilic bacteria. The results obtained indicated that out of all isolates, three strains could produce the bioactive compounds. The isolates were molecular (16SrRNA) identified as *Bacillus licheniformis*, *Bacillus subtilis* and *Bacterium Culaeen*. Furthermore, structural analysis of the bioactive compounds was carried out in order to achieve maximum information concerning to them. The results obtained illustrated the existence of glycoprotein in all the bioactive compounds. Although, *Staphylococcus aureus*, *Aspergillusniger* and *Mucor*…. were sensitive to all the bioactive compounds, *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus cereus* were resistant to them. In addition, the bioactive compound producing isolated strains by *Bacillus licheniformis* and *Bacillus subtilis* showed antifungal activity against *Aspergillusniger* and *mucor* sp. In total, our finding illustrated that the maharlu salt lake might be considered a source of halophilic bacteria with potent activity for production of the bioactive compounds. In addition, isolation and characterization of these compounds culminate in the achievement of the new drugs.

1. Introduction

As crystalline salt (NaCl) is normally considered to be hostile to most forms of life, it has been used for centuries as a foodstuff preservative. However, Halophilic bacteria can contaminate food them (Gunde-Cimerman *et al*., 2011). These bacteria are living in the hypersaline environments around the world. They could grow even in the extreme situations such as saturtated salt concentration. In fact, cell structures, proteins and enzymes of Halophilic bacteria organized basaed on their ability to tolerance to high salt concentrations (Ventosa, 2006).

Halophilic bacteria classified based on their optimal salt concentration, and divide into four categories: slight halophiles, which grow best in media with 1% to 3% NaCl, moderate halophiles, growing best in media with 3% to 15% NaCl, and extreme halophiles, which show most favorable growth in media containing 15% to 30% NaCl. non-halophilic organisms are defined as those requiring less than 1% NaCl,

*Corresponding author: Dr. Majid Baserisalehi
Tel: 09173043055
E-mail address: majidbaserisalehi@hotmail.com

whereas if they can endure high salt concentrations are considered as halotolerant microorganisms (Kushner and Kamekura., 1988; Mellado et al., 2013).

Although, it was proved that Halophilic bacteria have potent activity for production of antimicrobial compounds, their antimicrobial spectrum against pathogenic microorganisms are differ. Therefore, these bioactive compounds might consider as therapeutics directly or used as lead structures for drug innovation (Proksch et al., 2002; Kamat and Kerkar., 2004).

Based on foregoing evidence the present study conducted to isolate and identified Halophilic bacteria from Maharlu Lake in the south of Shiraz-Iran and evaluate their potential for production of the bioactive compounds. Furthermore, the bioactive compounds were characterised based on their physical and chemical properties.

2. Materials and Methods

2.1. Sample collection

In total 50 water, soil and sediment samples were collected from the Maharlu salt lake in the southern region of Fars-Iran. All collected samples were subjected to isolation of the bioactive compound producing halophilic bacteria. To perform the experiment, 10 ml of each sample was added into six tubes containing nutrient broth with various concentrations (0.5, 1, 1.5, 2, 3, 4 M) of salt. The suspensions were kept in a shaker incubator at 35°C, 130 rpm for 24 hours.

Then, the suspensions were cultivated on the nutrient agar with various concentrations (0.5, 1, 1.5, 2, 3, 4 M) of salt and incubated at 35°C for 48 hours under aerobic conditions.

2.2. Screening of bioactive compound producing strains

The bacterial colonies obtained from the samples separately were inoculated into hundred milliliters of nutrient broth with various concentrations of salt (0.5, 1, 1.5, 2, 3, 4 M) and incubated at 35°C, 200 rpm for 24h. Then, 5ml of each culture broth was centrifuged at 12000 rpm for 30 min. Supernatant of each suspension was assessed for antimicrobial property against bioassay strains of bacteria viz., *E.coli* PTCC 1330, *Staphylococcus aureus*, PTCC 1337 *Bacillus cereus* PTCC 1137, *Pseudomonas aeruginosa* PTCC1556, *Aspergillus niger* and *Mucor*……. To perform the test, bioassay strain was cultivated on Mueller Hinton agar and wells (5 mm in diameter) were made in plate agar using sterile sharp borer. Then, 100 µl of each supernatant was added into the each well and the plates were incubated at 35°C for 24h. Afterward, exhibition of a clear zone of growth inhibition considered bioactive compound activity.

*2.3. Phenotypic identification of the bioactive producing strain*s

Bioactive producer strains of halophilic bacteria were identified by catalase, oxidase, nitrate, indole, urease, starch and gelatin hydrolysis tests and fermentation of glucose, sucrose, lactose, mannitol and xylose.

2.4. Authentication of the bioactive producing strains)

Identification of antimicrobial producing strains was verified by Gene sequencing of 16SrRNA. This method was carried out as follows: DNA was extracted from the halophilic bacteria isolates by standard kit (Roche-Germany). Amplification of 16SrRNA was performed using universal perimers with Forward and Reverse of 5'-TTG GAG AGT TTG ATC CTG GCT C-3' and 5'-AGG AGG TGA TCC AAC CGC A-3', respectively.

Each reaction was performed in a total volume of 25.5 µl contained 14.5 µl of molecular biology-grade water (Sigma Aldrich Company Ltd.), 2.5 µl of 10×PCR buffer (Cinagen-Iran), 1 µl of each forward and reverse PCR primers, 1 µl of a 10 mM dNTPs (Cinagen-Iran), 0.5 µl of Smar taq polymerase (cinagen-Iran), 1 µl of 50mM $MgCl_2$ (cinagen-Iran) and 5 µl of DNA template. PCR amplification conditions on a thermocycler were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 40 s, 60°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 10 min and stored at

4°C. All PCR products obtained were run on a 1% (w/v) agarose gel with a 1000 bp DNA ladder. PCR products were visualized follow by electrophoresis and staining with ethidium bromide. The purified PCR products have been sent to the National center of the Iranian Genetic Bank for DNA Gene sequencing.

2.5. Determination of Growth phase for production of the bioactive compound

Production of the bioactive compound during growth of the halophilic strains estimated using the optical density. To perform the test, 5 ml of the culture at time intervals of 12, 24, 36, 48, 60 and 72 hrs was withdrawn, and the cells measured by the optical density at 620nm. The suspension was centrifuged at 12000 rpm for 10 min, then; their supernatant was assessed against *Staphylococcus aureus* PTCC 1337as mentioned above.

2.6. Extraction of the Bioactive compounds by different solvents

The extraction was performed using different solvents viz., xylene, ethanol, methanol, ethyl acetate and chloroform. The solvents separately were added to the filtered supernatant in 1:1 proportion, and mixed by homogenizer for 45 min. Then, the mixtures were centrifuged at 5000 rpm for 15 min and the solvent parts were separated and evaporated at 70 and 80^{o}C. The dark, brown and gummy compounds from each solvent was separated and subjected to further study (Dehnad et al., 2010).

2.7. Arbitrary Unit (AU) of bioactive compound

To determine an Arbitrary Unit of the bioactive compound produced by halophilic bacteria isolates, the bacterial culturewas serially diluted (10^{-2}, 10^{-4}, 10^{-8}, 10^{-16}, 10^{-32}, 10^{-64}, 10^{-128} and 10^{-256}) then 100 µl of each dilution was added into the wells of seeded Muller Hinton agar by *Staphylococcus aureus* PTCC 1337. The plates incubated at 35°C for 24hrs and the Arbitrary Unit of each bioactive compound was determined by the reciprocal of the highest dilution exhibiting the antimicrobial effect.

2.8. Optimization of bioactive compound production

Production of the bioactive compounds was optimized by different temperatures, pHs, carbon and nitrogensources.

To determine the best temperature, 10ml of each bacterial culture containing 1M NaCl was kept at 40, 50, 60 and 70 for 24 h. To achieve the optimum pH for production of the bioactive compound, pH of each bacterial culture containing 1M NaCl (10 ml) was adjusted to 5, 6, 7, 8 and 9 using KOH and HCl. Furthermore, the best carbon and nitrogen sources for production of the bioactive compound was determined after cultivation of the isolates in present of glucose, lactose and glycerol as carbon sources and urea and peptone as nitrogen sources. In all cases, the bioactive compound production was evaluated by well agar diffusion against *Staphylococcus aureus* PTCC 1337.

2.9. Chemical analysis of the bioactive compound prodced by the Halophilic bacteria isolates

Chemical analysis of the bioactive compound was determined using the protocol recommended by Fiedler (Fiedler, 1993). Commercially available readymade TLC sheets (Silica gel 60-F254 nm) spotted with the bioactive compounds obtained after extraction by the solvents. The sheets were dried and the following reagents were spray on them and kept in hot air oven at 120°C to observe the colour changing of the spots.

Spraying reagents

One gram of dimethyl amino benzaladehyde was mixed with 25 ml of 36% HCl and 75 ml of methanol. The stained sheets were heated to about 120° for a few minutes till maximal colouration. This reagent was specific for primary amines. Reagent A consisted of 0.5 g of blue tetrazolium mixed with 100 ml of methanol. Reagent B was prepared by mixing 24 g of sodium hydroxide in 50 ml water with 50 ml methanol. Reagent A and B were mixed 1:1 before use. The stained sheets were heated to

about 120°C for few minutes till maximal colouration. Blue or violet colour zone formation or a light background will indicates the positive result. This reagent was specific for steroids and reducing compounds.

One gram vanillin was mixed with 100 ml concentrated sulphuric acid. The stained sheets were heated to about 120°C for few minutes till maximal colouration. Coloured zones produced on a pale background were the indication of positive result. This reagent was specific for higher alcohols, phenols, steroids.

Reagent 1 consisting of 0.2 g naphthalene-1,3-diol in 100 ml ethanol was mixed 1:1 with reagent 2, which is 20% Sulphuric acid, just before use. The stained sheets were heated to about 120°C for few minutes till maximal colouration. Red, blue, green, violet, brown, orange or yellow coloured zone formation were the indication of positive results. This reagent was relatively specific for sugars.

3. Results

3.1. Isolation and screening of the bioactive producing Halophilic bacteria

In Total fifteen strains of Halophilic bacteria were isolated and assessed for the production of bioactive compounds. Out of all, three isolated strains could produce the bioactive compounds (*a1*, *f3* and *m4*). The results obtained from antimicrobial activity of the bioactive compounds indicated that *Staphylococcus aureus*, *Mucor* and *Aspergillus niger* were sensitive and *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus cereus* were resistant to all the bioactive compounds. In addition, the bioactive compound of *a1*and *m4* showed high and less antimicrobial activity respectively (table 1).

3.2. Identification of bioactive producing Halophilic bacteria

The results obtained from identification of Halophilic bacteria isolates illustrated that antimicrobial producing Halophilic bacteria were *Bacillus licheniformis* strain KIBGE, *Bacillus subtilis* subsp. natto BEST195 and *Bacterium Culaeen* E9M. They produced the bioactive compounds of a1, f3 and m4 respectively.

3.3. Extraction of the bioactive compounds by different solvents

The result obtained from the extraction of the bioactive compounds of different solvents indicated that out of all solvents chloroform was the best solvents for extraction of all bioactive compounds.

3.4. Optimization of the bioactive compounds production

The results obtained from the optimization of bioactive compound production by the isolated strains showed that the best temperature and pH for the production was 35°C and 7 respectively. On the other hand glucose and peptone recognized as best carbon and nitrogen sources for production of the bioactive compounds. Arbitrary Units obtained for both bioactive compounds were 128 AU.

3.5. Bacterial Growth phase for production of the bioactive compounds

The results obtained from the production of bioactive compounds during growth of the isolates indicated that the phase production of the compounds was similar for all three strains and started on 36^{th} hours and reach to maximum level on 72^{th} of the bacterial growth. Hence, it can be interpreted that the stationary phase is a phase of the bioactive compound production for all isolates (Figure 1).

3.6. Chemical composition of the bioactive compounds

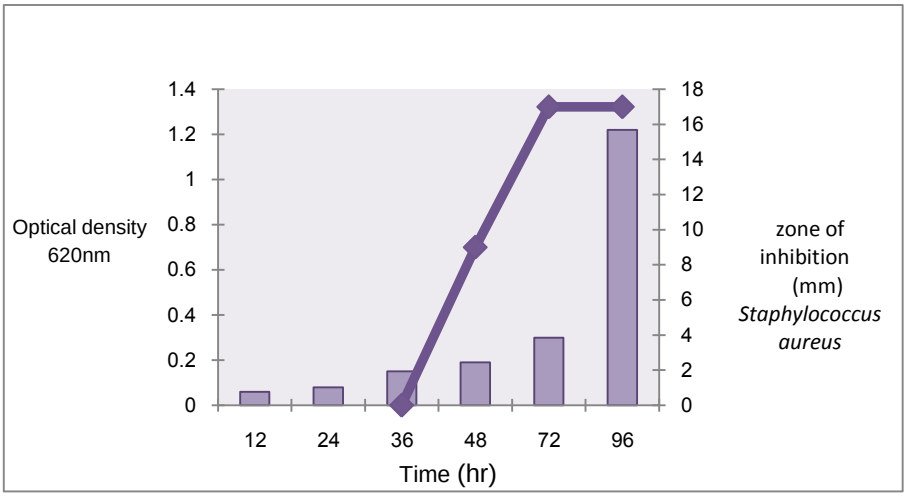
The structural analysis of the bioactive compounds by spraying reagents illustrated the existence of sugars and proteins in the structure of the compounds. In addition, among of sugar in the structure of the bioactive compound of *a1* was relatively more and in *m4* was less. Furthermore, alcohol, phenol and steroid groups existed in the bioactive compounds.

Table1. Antimicrobial activity of the compounds produced by Halophilic bacteria isolates

	Inhibition zone (mm) of the bioactive compounds of		
Microorganisms	*a1*	*f3*	*m4*
Escherichia coli	-	-	-*
Pseudomonas aeruginosa	-	-	-
Staphylococcus aureus	17	14	13
Bacillus cereus	-	-	-
Aspergillusniger	13	11	8
Mucor sp.	14	14	12

, no zone

Figure1. Growth curves of *Bacillus licheniformis* and its relations to bioactive compound production



4. Discussion

A great variety of microbial life could be observed in brine from marine salinity up to about 3–3.5 mol/l NaCl. Halophiles are saline loving organisms that living in hypersaline environments (Molinski, 1993; Salameh and Wiegel, 2007) .They include prokaryotic as well as eukaryotic microorganisms with the ability to balance internal osmotic pressure and resist to the denaturing effects of salts (Salameh and Wiegel, 2007). They belong to the genus; *Arhodomonas, Pseudomonas, Alcaligenes, Flavobacterium, Alteromonas, Acinetobacter, Halobacillus* and *Bacillus* (Mellado and Ventosa, 2003; Kamat and Kerkar, 2004). In the present study different genus of Halophilic bacteria were isolated and subjected for production of the bioactive compounds. Our finding illustrated the detection of three Halophilic strains of *Bacillus* viz., *Bacillus licheniformis*, *Bacillus subtilis* and *Bacterium Culaeen* with potent activity for production of the bioactive compounds. *Bacterium Culaeen* as intriguing bacterium, which first detected in India, but less information concerning to this bacterium is available. Only Based on its morphology, Gram stain and biochemical tests might be considered as the genus close to Halophilic *Bacillus*. Furthermore, *Bacillus licheniformis* (the bioactive compound producer of a1) showed relatively potent antimicrobial activity and *Bacterium Culaeen* (the bioactive compound producer of m4) exhibited less antimicrobial activity.

On the other hand, the results obtained from determination of growth phase of the bioactive compound production indicated that the production phase of the bioactive compounds for all the isolated strains was exponential phase. Therefore, it can be interpreted that the bioactive compounds are bacteriocin and probably they are Halocins. This claim could be verified by chemical analysis of the bioactive compounds and existence of proteins in the structure of the bioactive compounds.

Conclusion

These results show that the maharlu salt lake of fars-iranconstitutes an untapped source of bacterial diversity, and also that some halophilic bacteria isolates are capable of antibacterial and antifungal metabolite production. Overall, the bioactive compounds produced by Halophilic bacteria and might be used as remedy for treatment of the infections.

Refereces

Dehnad, A.R., Parsa, Y.R., Bakhshi, R., Mokhtarzadeh, A., Soofiani, S.A., Monadi, A.R., et al., 2010. Investigation antibacterial activity of Streptomycetes isolates from soil samples,West of Iran. African Journal of MicrobiologyResearch. 4: 1685-1693.

Fiedler, H.P., 1993. Biosynthetic capacities of actinomycetes.Screening for secondary metabolites by HPLC and UV-visible absorbance spectral libraries.J Natural Product Letters. 2(2):119-128.

Gunde-Cimerman, N., Sepsis, K., Zarar, P., 2011. Low water activity induces the production of bioactive metabolites in halophilic and halotolerant fungi. 9(1):59-70.

Kamat, T., Kerkar, S., 2004. Studies on a bioactive compound produced by a halotolerant salt pan isolate. In: Conference on Microbiology of the Tropical Seas (COMITS), National Institute of Oceanography, Goa. MB 10.

Kushner, D.J., Kamekura, M., 1988. Physiology of halophilic eubacteria. In Halophilic Bacteria; Rodríguez-Varela, F., Ed. CRC Press: Boca Raton, FL, USA. 1: 109-138.

Mellado, E., Garcia, M.T., Perez, D., Moreno, M.L., 2013. Halophilic Bacteria as a Source of Novel Hydrolytic Enzymes.journal life. 3: 38-51.

Mellado, E., Ventosa, A., 2003. Biotechnological potential of moderately and extremely halophilic microorganisms. In Microorganisms for Health Care, Food and Enzyme Production; BarredoJL(Ed). Research Signpost: Kerala, India. 233-256.

Molinski, T.F., 1993. Marine pyridoacridine alkaloids: structure, synthesis, and biological chemistry. J. Chem. Rev. 93:1825–1838.

Proksch, P., Edrada, R.A., Ebel, R., 2002. Drugs from the seas current status and microbiological implications.J Appl Microbial Biotechnol. 59: 125–134.

Salameh, M., Wiegel, J., 2007. Lipases from extremophiles and potential for industrial applications. Adv. Appl. Microbiol. 61:253-283.

Ventosa, A., 2006. Unusual microorganisms from unusual habitats: hypersaline environments. In Prokaryotic Diversity-Mechanism and Significance (Google eBook); Logan NA, Lppin-Scott HM, Oyston PCF(Eds). Cambridge University Press: Cambridge, UK.223-253.