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Characterization of Halophilic Bacteria Isolated from Khewra Salt Mines

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ABSTRACT: *Halophilic bacteria can populate every niche of earth. These halophiles have a great potential of exopolysaccharides production that is of considerable importance in various industries. To screen EPS producing halophiles, saline soil samples were collected from Khewra salt mines, Jehlum, Pakistan. Twenty-two morphologically different bacterial strains were isolated by serial dilution method. All strains were considered as moderate halophilic bacteria as they could grow at 3-15% of NaCl concentration whereas only three strains could grow at 15% of NaCl, which belonged to the genus Bacillus and Pseudomonas. For screening of EPS production, P-medium was used. While for the estimation of slime production, congo-red agar was used that exhibited positive results by appearance of black colored colonies by many strains. Moreover, EPS production was analyzed quantitatively and qualitatively. Isolated Staphylococcus and Bacillus species produced high amount of EPS (20g/L). "Moderate halophiles" play an important role in therapeutics, bioremediation, food and medicine, petroleum and tanning industries by producing EPS. Recently, growth of many agriculture crops has been improved by using beneficial halophiles in saline soils. Consequently, with the help of these beneficial halophiles we can give benefit to mankind.*

Keyword: Halophilic bacteria, Exopolysaccharides, *Staphylococcus*, *Bacillus,* Bioremediation

INTRODUCTION

Khewra salt mines situated at the foothills of the salt range, nearby the Pind Dadan Khan, a city in Jehlum district, Punjab, Pakistan. This area is considered as salt-affected due to salts and its derivatives that accumulate in soils, which are harmful to crops. More than six million hectares of soil is affected by salinity and this emerging problem can be commonly seen in Sindh and southern Punjab. Most widespread soil degradation process on the Earth is "Salinity" (Dodd and Pérez-Alfocea, 2012). Saline soils have high salt concentrations of chloride ions, potassium, magnesium and sulfate ions and have specific water distribution. Increase in salinity directly link to increase in concentrations of these mentioned ions. Saline soils categorize into three different groups, i.e saline, sodic, and alkaline sodic soil (Doula and Sarris, 2016). Kushner and Kamekura in 1988 proposed the most acceptable classification of halophiles based on NaCl concentrations (Śliżewska et al., 2022). Slightly Halophiles include marine microbes, have optimum growth 1-3 % (0.2-0.5M) NaCl. Moderate Halophiles grow best in 3-15% (0.5-2.5M) NaCl concentration and extreme halophiles show maximum growth in 15-30% (2.5-5.2 M) NaCl containing media. Non-Halophiles are able to grow in the media having NaCl concentration less than 1% (<0.2 M) however, Halotolerant is group of those microbes which require or not high amount of NaCl for their growth (Roohi et al., 2012).

In study of soil, many cases have been reported that saline soils especially influence agriculture by affecting the production of crops and plants at high salinity (Allam et al., 2018). The microorganisms can populate every niche on earth. The microbes that live at high salt concentrations are widely distributed in three domains of life: *Archaea, Bacteria* and *Eukarya* (Ventosa et al., 2012).

In hypersaline environment, aerobic halophilic Archaea named as Haloarchaea (grow at 20-25% NaCl) and moderately halophilic bacteria (3-15% NaCl) are present in large numbers (Ventosa et al., 2012). Enzymatic activities of moderate halophiles change its importance in the commercial and agriculture areas. Halophiles have their enzymatic activities as: (i) Intracellular enzymes; that impart their role in intracellular environment of cell and maintain the osmotic solutes level. (ii)

Extracellular enzymes; truly work for external hyper saline environment. (iii) Membrane- bound enzymes; help in transport by using both external and inner medium and maintain overall cell metabolic processes. But extracellular enzymes as amylases, proteases and nucleases release by Halophilic bacteria, gain interest of study (Sahay et al., 2012). Many cases have been reported recently on the utilization of these extracellular enzymes in different fields i.e. biotechnology, agriculture, researches and commercial level.

Exopolysaccharides (EPS) are the glycopolymers that microbes release in the environment. EPS is of two types i.e. capsular polysaccharides (CPSs) and medium released exopolysaccharides (MRPs). Diversity in structure and composition, they may be homo and heteropolysaccharides and few may have organic and inorganic additional components. Many EPSs are showing emulsifying property having deoxyhexoses, uranic acids and fatty acids in their composition. A number of bioemulsifiers contain polysaccharides moiety attached to lipids or proteins. To improve the emulsifying properties, we use microbial EPS because it is a cheap substrate (Sałek and Euston, 2019). Microbes from marine environment have a great potential for secondary metabolites production especially bioactive metabolites against antimicrobial activity. They have also potential in biofuel production (Amoozegar et al., 2019). These bioactive metabolites may be used as drug directly or used as lead source for drug because of its richness. Microbes from saltpans are potential producers of antimicrobial, antifungal and antibacterial agents (Donio et al., 2013). Due to high salinity, growth of crops and plant is affected. For this, halotolerant bacteria are mostly used in recent time. Inoculations with plant growth promoting bacteria (PGPB), a technique used to reduce the harmful effects of salinity on plant growth. The study reported that at high salinity or salt stress only these bacteria (PGPB) affect the growth of plant very efficiently (Ansari, 2019).

The main objective of the study was to determine the diversity of halophillic bacteria from Khewra salt mines and to characterize them based on their ability to tolerate different salt concentration. Screening of the halophilic bacteria for EPS production might have potential applications in industrial and agriculture sector was another aim of the study.

MATERIALS AND METHODS

Sampling

Two different types of soil samples i.e. wet and dry soil were collected aseptically from Khewra mine, Jehlum, Pakistan in sterilized glass jars and were safely carried to the laboratory for further experiments. Physiochemical properties i.e. temperature, pH, color and texture of both soil samples were recorded.

Isolation and Purification

Soil samples were serially diluted in autoclaved distilled water up to the dilution of 10^{-5}. From each dilution, 50µl was spread on solidified LB-agar supplemented with 1%NaCl and without stress plates. After incubation of 2 days at 37°C, 22 morphologically different strains were appeared. For further purification, quadrant streak method was done on LB-agar plates to obtain single isolated colony of these 22 strains.

Morphological and Biochemical characterization

These strains were morphologically characterized by Gram's staining and spore staining while biochemical characterization was performed by following Bergey's identification scheme. For this purpose, catalase test, oxidase test, mannitol salt agar test, nitrate reduction test, Voges Proskaeur test, oxidative fermentative test and pigment production tests were performed (Gerhardt, 1994; Vos et al., 2011; Cappuccino and Sherman, 2014).

Minimum Inhibitory Concentration of NaCl

To screen halophiles, N-agar medium was supplemented with NaCl in variable concentrations i.e. 1%, 2%, 5%, 7%, 10%, 12%, and 15%. On agar plates, 24 hours old culture of bacterial strains was streaked and incubated at 37°C for 24-48 hours. The concentration of NaCl at which bacterial strains could not grow was considered as its minimum inhibitory concentration (Canfora et al., 2014).

Screening for EPS and Slime Production

P-medium is specifically used to determine the EPS production (Batool et al., 2017). P agar medium plates were prepared with and without NaCl stress and isolated bacterial strains were streaked on these plates. Bacterial strains which showed growth over these plates were considered as EPS producers. While, determination of slime production was done at Congo red agar medium. On these plates bacterial

colonies were streaked and observed for blackening of the medium. Bacteria that are capable of producing slime, change the color of agar to black (Fabres-Klein et al., 2015).

Quantitative Analysis of EPS

For the extraction of EPS, 100ml of LB-broth without NaCl and with 1%NaCl stress was autoclaved at 121°C and inoculated with 24 hours old bacterial culture. Cultures were incubated at 37°C for 5-7 days at shaking incubator for proper oxygenation and uniform growth. After incubation, inoculated cultures were centrifuged at 10,000 rpm for 15 minutes. Following centrifugation, supernatant was separated and mixed with chilled ethanol in ratio 1:2 (supernatant: ethanol). For EPS precipitation, these tubes were then placed at 4°C for 24 hours. Precipitated EPS was separated by centrifugation at 15000 rpm for 20 minutes. The pellet was allowed to dry properly and the tubes were weighed afterwards. EPS produced by strains was determined by subtracting the weight of dried EPS containing tubes from the weight of empty tubes (Banerjee, 2019).

Qualitative Estimation of EPS

For the qualitative estimation of EPS, protein and carbohydrate content of EPS was estimated. Carbohydrate content was analyzed by phenol sulfuric acid precipitation method while for protein content Lowry's method was used (Lowry, 1951; Gerhardt, 1994).

RESULTS

Sampling Site

For the isolation of halophilic bacteria, soil samples were collected from two different sites of Khewra salt (mayo salt) mine, Jehlum, Pakistan. Physiochemical properties of soil sample were recorded as given in Table 1.

Table 1: Physiochemical characteristics of soil samples

Sample	Temperature	pH	Color	Appearance
Soil (k)	18.5°C	8.0	Light Brown	Dry
Soil (s)	18.0°C	7.6	Dark Brown	Wet

Isolation and Purification of Halophilic Bacteria from Khewra Salt Mine

By serial dilution method, 22 morphologically different strains were isolated. These strains were designated as KA, KB, KC, KD, KE, SF, SG, SH, SI, SJ, SK, SL, SM, SN, SP, SQ, SQ1,

SQ2, SR, ST, SU, SV and purified by the quadrant streak method. These purified strains were used for further studies.

Morphological and Biochemical Characterization of Halophilic bacteria

Most of the isolated colonies showed off-white color and entire margin. Based on gram staining, there were 54.54% gram-positive rods, 31.8% gram- negative rods, 9% gram positive cocci and 4.5% gram negative cocci. All gram-positive rods/ bacilli were spore formers. Biochemical characterization revealed that 86% of bacterial strains were catalase positive while, only 31% were oxidase positive. In 66% of bacterial strains mannitol fermentation potential was observed. Out of total 22 strains, 30% of bacterial strains were aerobes while, 70% were facultative anaerobes or anaerobes. All of the isolates showed positive results (100%) for nitrate reduction. In MRVP test, all of the strains were positive for MR while, only 67% showed positive VP test. Most of the identified strains belonged to *Bacillus, Pseudomonas* and *Staphylococcus* species based on Bergey's manual identification scheme.

Minimum Inhibitory Concentration of NaCl

Isolated 22 different strains were grown on LB-agar plates supplemented with 1%, 2%, 5%, 10%, 12%, and 15% NaCl, respectively. All the strains gave maximum growth in 1% and 2% of NaCl stress. At stress of 5% NaCl, SL gave no growth. There was no growth of bacterial strains KC, KE, SI, SQ at 7% NaCl and by KA, KD, SF, SK, and SM at 10% NaCl. Bacterial strains SG, SH, SJ, SP, SU, SV showed no growth while, strains ST and SQ2 showed slight growth on 12% of NaCl stress. At 15% NaCl, three bacterial strains KB, SN and SQ1 survived.

Table 2: Minimum inhibitory concentration of NaCl for selected bacterial strains

Strains	NaCl Concentrations						
	1%	2%	5%	7%	10%	12%	15%
KA	+	+	+	+	-	-	-
KB	+	+	+	+	+	+	+
KC	+	+	+	-	-	-	-
KD	+	+	+	+	-	-	-
KE	+	+	+	-	-	-	-
SF	+	+	+	+	-	-	-
SG	+	+	+	+	+	-	-
SH	+	+	+	+	+	-	-
SI	+	+	+	-	-	-	-
SJ	+	+	+	+	+	-	-
SK	+	+	+	+	-	-	-
SL	+	+	-	-	-	-	-
SM	+	+	+	+	-	-	-
SN	+	+	+	+	+	+	+
SP	+	+	+	+	+	-	-
SQ	+	+	+	-	-	-	-
SQ1	+	+	+	+	+	+	+
SQ2	+	+	+	+	+	+	-
SR	+	+	+	+	+	-	-
ST	+	+	+	+	+	+	-
SU	+	+	+	+	+	-	-
SV	+	+	+	+	+	-	-

Screening for Exopolysaccharide (EPS) and Slime Production

In this study, 77% bacterial strains gave positive results while only 23% were negative for slime production as shown in Fig. 1. All strains were streaked on solidified P- medium for the qualitative estimation of EPS production. All strains showed slight to moderate growth on medium that was considered as a positive result given in Fig. 2.

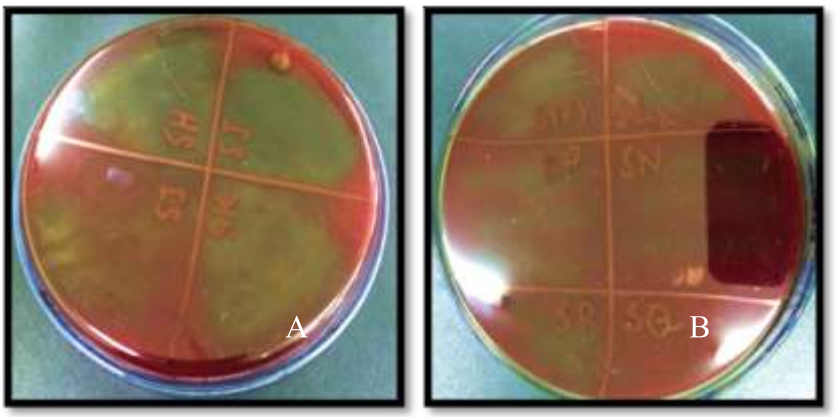

Fig. 1. (A) All strains showed black coloration (positive result) In (B) SQ strain showed negative result

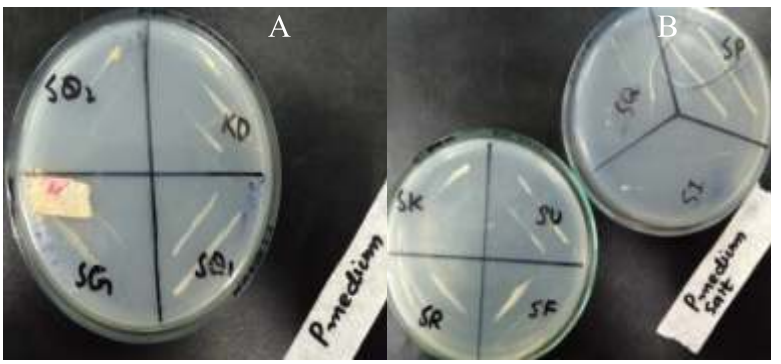

Fig. 2. Growth on P-media (a) without stress (b) with stress

Quantitative Analysis of EPS production

Mostly halophilic bacteria (KB, KE, SF, SQ1, SQ2, SR, SU, SV) produced EPS more than 20g/L in stress while these strains gave EPS below 20g/L in without stress condition. Bacterial strains (SH, SI, SL) produced more than 5g/L of EPS within stress and without stress. SQ was only halophilic bacterial strain, which produced 20g/L EPS in without stress condition and 10g/L within stress. SN only gave 5g/L

of EPS within stress and 7g/L without stress. While KA, SM, and SP gave EPS extraction more than 10g/L in without stress condition and more than 5g/L within stress.

Qualitative Estimation of EPS

Protein content of EPS ranged from 709-1129µg/ml without stress while with stress conditions it had a range of 840-1170µg/ml. Moreover, carbohydrate content of the EPS was also higher in stress conditions i.e. 283-610mg/ml as compared to without stress condition i.e. 181-539 mg/ml (Table 3).

Table 3: Qualitative analysis of EPS produced by selected bacterial strains

Strains	Carbohydrate content (mg/ml)		Protein content (µg/ml)	
	Without stress	With stress	Without stress	With stress
KB	450±0.32	565±0.97	1051.34±1.02	1022.67±1.59
KC	500±0.65	465±0.24	1051.34±1.64	1022.67±1.52
KD	489±0.84	459±0.61	1051.34±1.27	1170±1.68
KE	300±0.24	376±0.51	985.87±0.94	1070.92±0.82
SF	438±0.56	577±0.28	985.87±1.92	1119.59±0.94
SG	181±0.51	450±0.94	1051.34±0.84	1070.92±1.03
SH	489±0.54	398±0.65	1051.34±0.93	1127.59±1.49
SI	355±0.8	438±0.35	1022.67±0.76	1123.59±1.61
SJ	398±1.5	610±0.41	985.87±1.24	985.87±1.72
SK	467±0.8	355±0.61	1051.34±1.49	1064.34±1.46
SL	258±0.84	367±0.54	1051.34±0.92	1051.34±1.69
SM	398±0.46	355±0.32	1129.59±0.81	1070.92±1.71
SN	300±0.54	500±0.94	1051.34±1.03	1070.92±0.73
SP	438±0.32	300±0.67	1022.67±1.07	1022.67±0.84
SQ	529±0.72	300±0.83	1022.67±1.37	1022.67±1.67
SQ1	300±0.61	283±0.46	1119.59±1.61	944.28±0.80
SQ2	467±0.49	471±0.57	985.87±1.27	985.87±1.54
SR	300±0.91	355±0.92	1022.67±1.68	944.28±0.65
ST	438±0.82	300±0.34	1022.67±1.91	1022.67±1.24
SU	258±0.68	287±0.16	709.56±1.08	852.65±0.84
SV	283±0.52	300±0.51	852.65±1.64	840.15±1.65
KA	539±0.59	519±0.29	1022.67±1.43	1070.92±1.32

Mean of replicates ± standard error

DISCUSSION

Saline soils have been widely studied with reference to diverse functions of indigenous microbes in various fields as microbiology, agriculture, biotechnology and

medicine, etc. There is a large number of microbiota including bacteria, archea, fungi, protists and viruses present in saline soils. So, microbiologists have been putting their effort to find out various helpful microbes, among them bacteria and actinomycetes have attained greater heed in this regards. In the present case, the Khewra salt mine was selected which is the second largest mine of the world, located in the province Punjab, Pakistan (Aftab et al., 2015) Recently, a few studies reported the isolation of halophilic bacterial strains from the salt rich environment that are helpful in plant growth promotion (Bahar et al., 2015; Mukhtar et al., 2018).

In the present study, isolated bacteria were mostly gram positive. There were a high number of gram-positive bacteria because they have high resistance towards physical disruption and drying of cell. The cell wall of gram-positive bacteria is composed of multiple layers of peptidoglycan, which protect the cell interior from hazards and maintain the cell integrity (Xia et al., 2019).

Halophiles are differently categorized based on different salt concentrations to determine their stress tolerance potential, which is helpful in their mechanism and in biochemical reactions against host cells or harsh environments (Pérez-Llano et al., 2020). Few of isolated strains grew well on 15% NaCl stress i.e. moderate halophiles. These halophiles belong to *Bacillus* group. This finding has been reported by Javid et al. that *Bacillus species* are known to tolerate saline stressed environment because of their genomic and metabolic background as well as their spore formation potential (Javid et al., 2020).

All strains showed growth on P-media that would be considered as a positive result for EPS production. This finding is in accordance with another study which indicated that, many halophilic bacteria are capable of producing extra polymeric substances like exopolysaccharides (EPS). The bacterial EPS are complex polymers of carbohydrates, released from outside of their cell wells that are important in maintaining cellular integrity and provide successful survival from stress environment (Bahar et al., 2015). Biofilm formation leads to slime production of bacteria that is determined by Congo-red agar media. In this study 77% strains were positive for biofilm production. Slime- production aids bacterial cell for phagocytosis and better protection from opsonization (Rainard et al., 2021).

Quantitative analysis of EPS production was carried out by using cold ethanol precipitation method. EPS production depends on substrate and medium concentration. Some bacteria such as *H. aquamarina, H. meridiana* and *K. indalinina* give high production of EPS in stress environment from 0.5M to 1M NaCl (Qureshi and Sabri, 2011). Bacterial strains identified as *Pseudomonas* and *Bacillus* species (on the basis of Bergey's manual identification scheme) gave high EPS production (>20g/L) at high salt stress. The high value of EPS produced in stress gave idea that how would it be helpful in survival of strains under stress conditions by giving them protection (Malick and Khodaei, 2017).

In *Staphylococcus* species, EPS production shield them from antimicrobial agent and host defences and contributes to its virulence and give protection to them from abiotic stresses (Kaplan et al., 2018). Staphylococcal species *S. aureus* and *S. epidermidis* have been isolated from surgical devices being used for removal of infectious surgical wounds. The extracellular polymeric substances (EPS) released by these two species consist of poly-N-acetylglucosamine surface polysaccharide (PNAG) and extracellular DNA (eDNA), which mediate numerous virulence traits involving host colonization, adhesion to surfaces and antimicrobial resistance (Kaplan et al., 2018). Different strains of *Bacillus* species i.e. *B. amyloliquefaciens* and *B. licheniformis* yield the EPS value that is dependent on medium concentration and fermentation conditions (Malick and Khodaei, 2017).

Qualitative estimation of EPS depends upon its composition. As, we know that, extra polymeric substances are heterogeneous compounds released from bacteria that contain macromolecules like protein, lipids and other such molecules for extra cellular activities. For the carbohydrate content analysis of EPS, phenol-sulfuric acid method was done (Qureshi and Sabri, 2011). Whereas, Lowry's assay was used to estimate protein content. The proteins impart blue color to the mixture due to a specific reaction between Folin's reagent and amino acids. With the help of *Bergey's manual for systematic bacteriology* (Vos et al, 2011), isolated SJ and KA strains belong to *Staphylococcus* and *Bacillus* species which gave high carbohydrate content with stress and without stress respectively. *Bacillus* species impart their roles diversely in different industries by producing EPS. These proteins provide hydrophobicity to

bacterial cells in stress conditions to shield from desiccation. Moreover, halophiles have protein content slightly higher than non-halophiles for cellular protection.

In this study two bacterial genera i.e. *Pseudomonas* and *Bacillus* were found to tolerate maximum concentration of salinity. This finding is similar to other studies which reveal that these genera compensate the rhizosphere and saline soils by improving agriculture methods of crops (Liu, 2017). Halophilic bacteria are helpful in removal of hazardous compounds from the industrial wastes; therefore, they have huge applications in agro-food, textile, petroleum and tanning industries. They degrade the harmful organic pollutants such as aromatic hydrocarbons, nitro aromatics, phenols and azo dyes etc. due to their high efficiency. These halophiles are good candidates for the bioremediation of hypersaline environments and treatment of saline effluents. Non-halophilic organisms have lesser ability to degrade such harmful components at high salt concentrations (Bera et al., 2022).

CONCLUSION

Halophilic bacterial strains isolated from Khewra salt mines, Jehlum, Pakistan were found to possess exopolysaccharide production potential. Most of the strains producing EPS were identified as *Bacillus* and *Pseudomonas* species. Their potential to withstand high salt stress environment along with secondary metabolite production makes them suitable for a number of applications in agriculture, biotechnology, industries, food, medicine, and bioremediation fields.

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