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Biochemical and Molecular Profiling of Novel Halophiles Isolated from Coastal Region of South Gujarat

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ABSTRACT

Halophiles are excellent sources of enzymes that are not only salt stable but also can survive and carry out reactions efficiently under extreme circumstances. The aim of the study was to isolate and study the diversity among halophilic producing enzymes of industrial value. Screening of halophiles from various saline habitats of South Gujarat region, India led to isolation of Five potential halophilic producing industrially important enzymes. Characterization of two potential isolates as bacteria by morphological, biochemical and 16S rRNA gene analysis further nomenclature as *Brachybacterium paraconglomeratum* strain CHPBMCST 1 and *Kocuria polaris* strain CHPBMCST 2. Both were extreme halophiles, that showed growth at 35 % NaCl w/v concentration. It was concluded that these halophilic isolates are not only diversed in phylogeny but also in their enzyme characteristics. CHD B isolate showed positive for amylase & cellulase, while CHD A only showed positive for amylase and organic acid activity. Both CHD A and CHD B may able to utilize the complex molecules efficient, compare to simple carbon molecules as an energy source. These enzymes may be potentially valuable for catalysis under harsh operational circumstances encountered in industrial processes. Such microbes can also be used as a source of gene(s) that can increase salt tolerance in different crop species through genetic transformation.

Key words *Kocuria Polaris*, *Brachybecterium peraconglomeratum*, Halophiles, rRNA, NaCl

Screening new source of novel and industrially helpful enzymes is a key research pursuit in enzyme biotechnology. For applications in industrial processes, the enzymes should be steady at high temperature, pH, presence of salts, solvents, toxicants *etc*. In this context, the halophiles have emerged as a vast repository of novel enzymes in recent years (Oren 2002(a); Surve *et al*. 2012; Kumar *et al*. 2012). Bacteria survive in diverse ecological circumstances from extreme cold (Antarctica) to hot conditions (hot springs), mesophilic soil to extreme saline conditions of different pH (Gibbons, N. E. 1969; Vreeland, R. H. 1993; Ventosa et. al. 1988). Adaptability of the bacteria to such various circumstances has massive primary and applied significance. Besides naturally occurring saline environment, in excess of utilization of ground water raising the percentage of saline area. On an average about 25% of global cultivated area shows severe salinity (Chowdhury *et al*. 1993).

Enzymes derived from halophiles are endowed with unique structural features and catalytic power to maintain the metabolic and physiological processes beneath high salt conditions. Some of these enzymes have been reported to be active and stable under more than one atmospheric pressure (Gomes and Steiner,2004; Karan and Khare 2010; Sellek and Chaudhuri 1999; Li and Yu, 2012)

Therefore, there was need for the study of saline environment and its ecology. The halotolerant and moderately halophilic eubacteria are more analogous to non halophilic bacteria and these organism have contributed considerably to our knowledge of different stage behavior of physiological changes during adaptation to different NaCl concentrations and function of compatible solutes in defensive cells from salts (Spring *et al* 1996; Roessler & Müller 1998; Ara *et al*. 2013; Vreeland RH *et al*. 1993). Although halophilic bacteria have been studied for basic scientific interests, their biotechnological prospective has been largely unobserved.

Halophiles are extremophile organisms that flourish in environments with very high concentrations of salt. Halophiles are categorized as slight, moderate or extreme, based on the extent of their halotolerance. Slight halophiles prefer 0.3 to 0.8 M (1.8 to 4.7% - seawater is 0.6 M or 3.5%), moderate halophiles 0.8 to 3.4 M (4.7 to 20%), and extreme halophiles 3.4 to 5.1 M (20 to 30%) NaCl. Halophiles can be found in areas where the concentration of salt is five times greater than salt concentration of the ocean, such as the Great Salt Lake in Utah, Owens Lake in California, the Dead Sea, and in evaporation ponds. (Oren, 2002b; Roohi *et al*.,2012). They comprise mainly prokaryotic and eukaryotic microorganisms with the capacity to equilibrium the osmotic pressure of the environment and oppose the denaturing effects of salts. Halophilic microorganisms are variety of heterotrophic and methanogenicarchaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes. Halophilic microorganisms have several biotechnological applications like â carotene production of fermented foods. In recent years, uses of halophilic microorganisms have significantly increased. Many enzymes, stabilizers and valuable compounds from halophiles may present advantages for the progress of biotechnological product (Azar, M.*et al*. 2014).

In India, halophilic micro flora have been reported from natural hyper saline habitats from the coastal regions of Maharashtra, Gujarat, Tamil Nadu, Goa and desert state of Rajasthan (Kokare *et al*. 2004, Dodia *et al*. 2006, Vijayanand *et al*. 2012, Surve *et al*. 2012, and Nigam *et al*. 2013).

The aim of this research was to aimed to assess the

Table 1. Categorized of Potential halophilic sample on basis of pH, Temperature and Source

Name of sample	Dandi Soil (DS)	Dansi water (DW)	Salt Making Company (SMC)	Ubharat soil (US)	Ubharat water (SW)
pH of sample	8	8	7	8	8
Temp. of sample	35	35	35	35	35
Source of sample	Soil	Water	Water	Soil	Water

bacterial biodiversity of Halophilic bacteria isolated from different coastal regions of south Gujarat. It was also aim to explored novel Halophilic, extreme or moderate bacteria, and to examine their morphology, cultural characteristics as well as their biochemical characters.

MATERIALS AND METHODS

Sample Collection

The survey was carried out major chances for obtain halophilic bacteria at Surat, South Gujarat, Dandi beach (20.8866° N, 72.7971° E), Salt Making Company Olpad (21° 19' 43.8816'' N, 72° 45' 16.398'' E) and Ubharat beach Navsari (21° 0' 35.9388'' N,72° 44' 36.8448'' E). The survey was conducted to know the high salt concentration. Collected samples were immediately transferred to autoclaved plastic bottle to avoid cross contamination and transported to the laboratory for further analysis.

Enrichment and isolation of bacteria

Enrichment cultures and techniques to isolate moderately to extremely halophilic microorganism are performed in Halophilic Agar medium and Halobacterium medium. pH was adjusted to 7.2±0.1 before autoclaving. Enrichment cultures were subcultured several times under the same conditions with different NaCl concentration (0%, 5%, 10%, 20%). Aliquots (100 il) of 10^{-3} to 10^{-6} dilutions were plated on to agar medium. After two weeks of incubation at 37ºC, there were red, orange red, pale pink, yellowish, cream, transparent colonies. Different colonies were picked and streaked several times to obtain pure cultures. The isolates were stored at 4 ºC and sub-cultured at 15 days intervals (Sambrook *et al*. 2009).

Morphological and Biochemical identification different isolates:

These isolates were grown on selective media and were chosen for further characterization (Domsch *et al* 1980; von Arx, 1981; Kiffer and Morelet, 1997). Isolates were examined for colony and cell morphology. Colony morphology was described with special emphasis on pigmentation, colony elevation and opacity. These characteristics were described from cultures growth at optimum temperature, pH, salt concentration.

Biochemical test were performed for the identification of bacterial isolates. Acid/gas production, casein hydrolysis, catalase test, gelatin hydrolysis, H_2S production test, indole production, motility test, nitrate reduction test, oxidase test, starch hydrolysis and urease test were carried out for biochemical characterization. Different parameters like pH tolerance, salt tolerance, temperature tolerance were also analyzed (Smibert and Krieg ,1994).

Metabolic profiling of Novel Halophiles:

Advance Biochemical analysis

Advance biochemical analysis was done in Advanced Diagnostic Laboratory, Param doctor house, Lal Darvaja, Near Surat Railway Station, Surat, Gujarat. All the different isolates were purified on LB Agar plates. For identification purpose the purified isolates were streaked on LB agar plate by four flame method to obtain isolated colony (Fader *et al* 2013).

Specific Enzymatic Activity

For determination of the various enzyme production, enzyme activity was checked for amylase, protease, lipase, cellulose & organic acid (Atef Jaouani , *et al*., 2014; Gaur, *et al*., 2015). For that plates of starch, skimmed milk, tri butyric acid, carboxy methyl cellulose & calcium carbonate agar plate were prepared respectively (Rakesh & Kiran Patel, 2004; Fader *et al* 2013).

Antibiotic resistant activity

All the bacterial isolates were tested for their resistance to 19 different antibiotics on N-agar plate using the discs of antibiotics (Himedia manufactured) with standard concentration (ig/disc) were used. The plates were incubated at 37°C for 24-48 h. (Fader *et al* 2013;Patel H & Mahatma L, 2015).

Optimization of growth conditions

Bacterial isolates were evaluated at various pH (4-11), sodium chloride concentrations (1-40%) and temperature (4, 10, 20, 28, 35, 37, 40, 45 and 50ºC) to find out the optimum growth conditions. The optical density at 600 nm wavelength was measured for evaluating bacterial growth in broth culture.

Identification and characterization of potential halophilic isolates:

Genomic DNA extraction for amplification of 16S rRNA gene was performed as described previously (Ahmed *et al*., 2007) by suspending few well isolated colonies in TE buffer in a micro-centrifuge tube. These cells were heated for 10 minutes at 95ºC and were centrifuged at 6,000 rpm for 5 minutes. The supernatant was used as template DNA for the amplification of 16S rRNA gene (Sambrook *et al*. ,2009).

The 16S rRNA gene of all the isolated strains were amplified by polymerase chain reaction using primers 9F (5'- GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') using PreMix ExTaq (Takara, Japan) as described previously (Ahmed *et al*., 2007). The polymerase chain reaction was carried out in ABI Veriti PCR Machine (Applied Biosystems, USA) using optimized PCR

Program: initial denaturation at 94ºC for 2 min; 30 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1:30 min. The final extension was performed at 72ºC for 5 min. The amplified PCR products of 16S rRNA gene of bacterial strains were purified and sequenced using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT-3') using commercial service of Macrogen Inc. Korea (www.dna.macrogen.com) (Lane, 1991).

Sequence comparison and Phylogenetic analysis of bacterial isolates:

The nucleotide sequence thus obtained were manually analysed, overlapping sequences were removed and the complete 16S rDNA sequence for each bacterial strain was generated. Nucleotide sequences were analysed by BLAST (blastn) search and compared against bacterial 16S rDNA sequences available in the Gene bank data base (Altschul *et al*. 1990). The sequences were aligned by using Clustal W 1.74 (Thompson *et al*. 1994), followed by construction of neighbour joining phylogenetic tree using MEGA4 (http://www.megasoftware.net) (Tamura *et al*. 2007). The nucleotide sequences were submitted in the GenBank data base (https://www.ncbi.nlm.nih.gov/genbank/).

Phylogenetic trees were constructed using full optimal alignment in the Clustal_X version 1.83 Software (Thompson *et al*., 1997) and neighbor-joining method with 1000 bootstrap replications available in the MEGA version 4.0 (Tamura *et al*., 2007). All the sequences presented in Table 1 and Table 2 were used for the analysis.

Table 2. Characterization of halophilic from different location sample

Location	Sample	Fungi	Actinomyces	Bacteria
Dandi, olpad	DS	0	0	5
	DW	0	0	3
Ubharat, Navsari	US	0	0	7
	UW	0	0	5
Olpad	SMC	0	1	7

RESULTS AND DISCUSSION

Recent decades have seen a rise in studies on microorganisms isolated from extreme environments, including hyper saline ecosystems. Both molecular and microbiological studies have revealed the existence of moderate to extremely halophilic micro and some macro-organisms in a wide range of these saline environments (Ventosa *et al*. 1998; Roohi *et al*. 2012; Hedi *et al*. 2014). In the present study, five bacterial strains have been isolated from different coastal region of south Gujarat region.

Sample collection and isolation

Five potential samples were collected in sterilized plastic bottle from the different site of the sea area and Salt Making Company (Table 1) from the South Gujarat region near to Surat. Collected sample were immediately transferred to the laboratory for the avoiding contamination of non-halophilic bacteria. (Grant, 1993; Madern *et al.,* 2004). On the basis of halophiles habitat characteristics, sample collection site were selected, which show high salinity or marine area or salt industrial area. Table 2 indicated that among all the microbes, bacteria were dominated. Ubharat and Olpad sample showed highest seven number of bacteria when cultivated on L.B agar.

Enrichment of sample

One ml from the all sample collected from the south Gujarat region were inoculated in to different nutrient broth (NB) medium supplemented with 5% w/v NaCl for enrichment of halophiles. Again 5% NaCl containing culture was transfer till 35% NaCl containing LB media. 35% NaCl containing culture may takes up to 7-8 days for the growth (Abdul karim *et al*, 2005). Further screening indicated that DS shows growth at highest concentration of NaCl and it was 35% w/v. At 25% NaCl concentration US and UW was stopped showing growth of bacteria or it was not detectable (Table 3). Further, at 30% NaCl concentration and 35% NaCl concentration SMC sample and DW sample was stopped showing growth respectively. Then culture were streak on LB agar media at 37°C incubation. After 7 days, only two different colonial bacteria may able to grow on 35% NaCl concentration. For further characterization both different colonial bacteria named as CHD A and CHD B.

Table 3. Identified Potential halophilic bacteria at highest 35 % NaCl concentration

NaCl Conc. (%)	DS	DW	SMC	US	UW
25	+ 2	+ 1	+ 2	-	-
30	+ 2	+ 1	-	-	-
35	+ 2	-	-	-	-

+ =indicate No. of potential bacterial growth

Several hyper saline environments have been studied worldwide, of which Dead Sea has the maximum salt content (DasSarma and Arora 2001; Grant 2004). It has been reported that the concentration of NaCl and KCl in Dead Sea ranges from 7.1 to 9.1% and 1.5 to 1.7%, respectively (Wisniak 2002; Gupta et. al, 2015).

Morphological and biochemical characterization of isolated halophiles

Colony characters of the isolates shape, slight viscous, smooth, flat and small in size. After streaking on agar plate both shows different pigmentation like CHD A shows golden yellow coloured colonies and CHD B was shows pale brown coloured colonies.; range of colony size was 1.0 – 4.0 mm on LB. Both the isolates showed different morphologies when viewed under the microscope. CHD A showed gram's negative, rod shape and non motile, while CHD B showed gram's positive, cocci shape and motile. The Growth conditions of both the strains were optimized for pH, NaCl tolerance and temperature. The purpose of

optimization of the strains was to find their optimum growth on different pH. From the results it was concluded that the halophilic bacterial strains grow best on 6-9 pH. Similarly, NaCl tolerance was checked for these strains, both strains grow best in the range of 5-35% at the temperature range of 25-45°C (Table 4).

Amongst the hypersaline habitats in India, the Lonar Lake in Maharashtra has been reported to contain 0.35% Na+ and 0.0017% K+ ions (Pedge and Ahirrao 2013).similar result also reported by Vidyasagar *et al*. (2007) isolated gram negative, rod shaped halophilic strains from solar evaporation pond with optimum growth at 23% (w/v) NaCl. Spring *et al*. (1996) described the isolation of orange pigmented Halobacillus trueperi from Great Salt Lake in Utah, which exhibited growth at temperature range of 10–44°C and pH 6.0–9.5 in the presence of 0.5–30% (w/v) NaCl. Recently, rod shaped, creamish pigmented Halobacillus sp. Has been reported from Sehline Sebkha salt lake, Tunisia, which showed growth in presence of 5–25% (w/v) NaCl at 37°C (Hedi *et al*. 2014,Gupta et. al, 2015).

Table 4. Morphological characterization of two potential halophilic bacteria CHD A and CHD B

Characteristic	CHD A	CHD B
Growth rate	Slow	Slow
Grams staining	Gram negative	Gram positive
Shape	Rod	Cocci
Motility	Negative	Negative
Temp	30-45^{0}C	22-45^{0}C
pH range	6-8	6-9
NaCl (%)	5-35%	5-35%
Colonies size	Small	Small
Colonies shape	Round	Round
Colonies edge	Entire	Entire
Colonies elevation	Flat	Flat
Colonies opacity	Opaque	Opaque
Colonies pigmentation	Golden yellow	Pale brown
Colonies consistency	Slight viscous	Slight viscous
Colonies surface	Smooth	Smooth

Biochemical characterization

Biochemical test was performing for identification of the genus of isolated halophiles from secondary screening. Strong acidification of glucose, maltose, manitol and lactose was done by CHD A, while weak acid production was noticed on fermenting sucrose. On the contrary, negative results were found on fermenting xylose. Acid produce in case of lactose and sucrose. CHD B fermented glucose, xylose and starch, but did not shows fermentation from lactose, maltose and sucrose (table 5). Hedi *et al*. 2009 also used API 20E® strips to characterize halophilic bacteria from a salt lake in Tunisianand observed similar results.

Enzymatic and Organic activity

Enzymatic activities of both isolates are given in table 6. None of the isolate was positive for lipase and protease activity. CHD B isolate showed positive for amylase & cellulose, while CHD A only showed positive for amylase and organic acid activity. Similar work also done by Gaur *et al* 2015. The isolate Salinicoccus roseus showed highest extracellular enzymes activity as Amylase, Protease and Gelatinase.

Table 5. Biochemical characterization of two potential halophilic bacteria CHD A and CHD B

Substrate	CHD A	CHD B	Substrate	CHD A	CH D B
Gelatine	-	-	Sucrose	+	-
Urea production	+	+	Xylose	-	+
H2S production	-	-	Fructose	+	+
VP test	-	+	Citrate	-	-
Indole test	-	-	MR test	-	-
Glucose	+	+	Starch	+	+
Lactose	+	-	Catalase	+	+
Maltose	+	-	Nitrate reduction	+	-
Manitol	+	-			

Table 6. Enzymatic profiling of two potential halophilic bacteria CHD A and CHD B

Name of activity	CHD A	CHD B
Lipase activity	Negative	Negative
Amylase activity	Positive	Positive
Cellulase activity	Negative	Positive
Protease activity	Negative	Negative
Organic acid activity	positive	Negative

Metabolic Profiling

By performing the advance biochemical test it was found both CHD A and CHD B bacteria may not able to utilize normal source of carbon like maltose Trehalose, sucrose and dextrose with is a major characteristic of halophiles (table 7). Both CHD A and CHD B may able to utilize the complex molecules efficient, compare to simple carbon molecules. They may get their energy from the utilization of complex organic molecules.

Antibiotic profiling

41 different antibiotics having specific concentration

Table 7. Different metabolic profiling of two potential halophilic bacteria CHD A and CHD B

Advance Biochemical Test	CHD A	CHD B
A_Arginine-Arginine 7-Amido-4-Methylcoumarin Trihydrochloride	+	-
A_Glycine-Proline 7-Amido-4-Methylcoumarin Hydrobromide	+	-
A_L-Arginine 7-Amido-4-Methylcoumarin Trifluoroacetate Salt	+	-
A_L-Arginine 7-Amido-4-Methylcoumarin Hydrochloride	+	-
A_L-Histidine 7-Amido-4-Methylcoumarin Trifluoroacetate Salt	-	-
A_L-Isoleucine 7-Amido-4-Methylcoumarin	+	-
A_L-Leucine 7-Amido-4-Methylcoumarin Hydrochloride	+	-
A_L-Phenylalanine 7-Amido-4-Methylcoumarin Trifluoroacetate Salt	+	+
A_L-Proline 7-Amido-4-Methylcoumarin Hydrobromide	+	-
A_L-Pyroglutamic Acid 7-Amido-4-Methylcoumarin	-	-
A_L-Tryptophane 7-Amino-4-methylcoumarin	+	-
A_Methionine 7-Amino-4-methylcoumarin Acetate	+	-
C_3-Methyl Glutaric Acid	+	+
C_Colistin	+	+
C_D-Fructose	+	+
C_D-Gluconic Acid	+	+
C_D-Mannitol	+	+
C_D-Iminodiacetic Acid	+	+
C_D-Ketoglutaric Acid [A]	+	+
C_3-Methyl Adipic Acid	+	+
C_Polymyxin B	-	-
C_Thymidine	+	+
M_4-Methylumbelliferyl a-D-Glucoside	+	-
M_4-Methylumbelliferyl B-D-Cellobioside	+	-
M_4-Methylumbelliferyl B-D-Galactoside	+	+
M_4-Methylumbelliferyl B-D-Glucuronide	+	-
M_4-Methylumbelliferyl N-Acetyl-B-D-Glucosaminide	+	+
M_4-Methylumbelliferyl Phosphate at pH 7.2	+	-
M_4-Methylumbelliferyl Phosphate at pH 7.2 w/ 3% Trehalose	-	+
N_L-Proline-p-Nitroanilide Trifluroacetic acid salt	-	+
N_Alanine-Alanine p-Nitroanilide Hydrochloride	-	-
N_p-nitrophenyl-p-nitroanilide Trifluro	+	-
N_Valine-Alanine p-Nitroanilide Acetate salt	-	-
P_p-Nitrophenyl Phosphate at pH 6.5 w/ 3% Trehalose	+	-
R_B-Gentiobiose	-	-
R_Dextrose	-	-
R_D-Sucrose	-	-
R_D-T agatose	-	+
R_D-T rehalose	-	-
R_Maltose	-	-
R_Maltotriose	-	-
R_N-Acetyl-Glucosamine	+	-
S_Urea	-	-
T_Esculin	-	-

Table 8. Antibiotic profiling of two potential halophilic bacteria CHD A and CHD B

Sr. No	antibiotic	Code	Conc (mg)	zone of diameter(mm)			Antibiotic sensitivity (mm)	
				I	R	S	CHD A	CHD B
1	Cefotaxime	CTX	30	=14	15-22	=23	20	21
2	Cefadroxil	CFR	30	=14	15-22	=18	12	10
3	Ampicillin	AX	10	=13	14-16	=17	23	31
4	Penicillin/Cloxacillin	P	10	=14	14-15	=15	29	12
5	Ampicillin/sulbactam	A/S	10/10	=11	12-14	=15	32	20
6	Cephalothin	CEP	30	=14	15-17	=18	32	12
7	Cefazolin	CZ	30	=14	15-17	=18	30	23
8	Oxacillin	OX	1	=10	11-12	=13	26	30
9	Teicoplanin	TEI	30	=10	11-13	=14	13	18
10	Methicillin	MET	5	=9	10-13	=14	25	11
11	Amoxyclav	AMC	30	=13	14-17	=18	30	25
12	Imipenem	APM	10	=10	14-15	=16	34	25
13	Aztreonam	AT	30	=15	16-21	=22	10	15
14	Ceftriaxone	CTR	30	=13	14-20	=21	24	25
15	Ceftriaxone	CPD	10	=17	18-20	=21	19	11
16	Gentamicin	GEN	10	=12	13-14	=15	26	24
17	Netilmycin	NET	30	=12	13-14	=15	36	16
18	Azithromycin	AZM	15	=13	14-17	=18	13	20
19	Amikacin	AK	30	=14	15-16	=17	22	19
20	Streptomycin	S	10	=11	12-14	=15	13	28
21	Doxycycline HCl	DO	30	=10	11-13	=14	24	32
22	Tetracycline	TE	30	=11	12-14	=15	25	35
23	Chloramphenicol	C	30	=12	13-17	=18	11	25
24	Clarithromycin	CLR	15	=10	11-15	=16	27	21
25	Clindamycin	CD	2	=14	15-20	=21	21	22
26	Cefoperazone	CPZ	75	=15	16-20	=21	28	27
27	Ceftazidime	CAZ	30	=14	15-17	=18	14	19
28	Cefaclor	CF	30	=14	15-17	=18	20	25
29	Cefepime	CPM	30	=14	15-17	=18	27	28
30	Cefoxitin	CX	30	=14	15-17	=18	11	13
31	Norfloxacin	NX	10	=12	13-16	=17	12	8
32	Ciprofloxacin	CIP	5	=15	16-20	=21	23	15
33	Sparfloxacin	SPX	5	=15	16-18	=19	27	17
34	Nalidixic acid	NA	30	=13	14-18	=19	13	8
35	Gatifloxacin	GAT	5	=14	15-17	=18	24	16
36	Moxifloxacin	MO	5	=20	21-23	=24	27	21
37	Co-trimoxazole	COT	25	=13	14-17	=18	19	18
38	Vancomycin	VA	30	=14	15-16	=17	22	14
39	Linezolid	LZ	30	=20	21-22	=23	20	13
40	Tobramycin	TOB	10	=10	13-14	=15	33	12
41	Nitrofurantoin	NIT	300	=14	15-16	=17	10	24

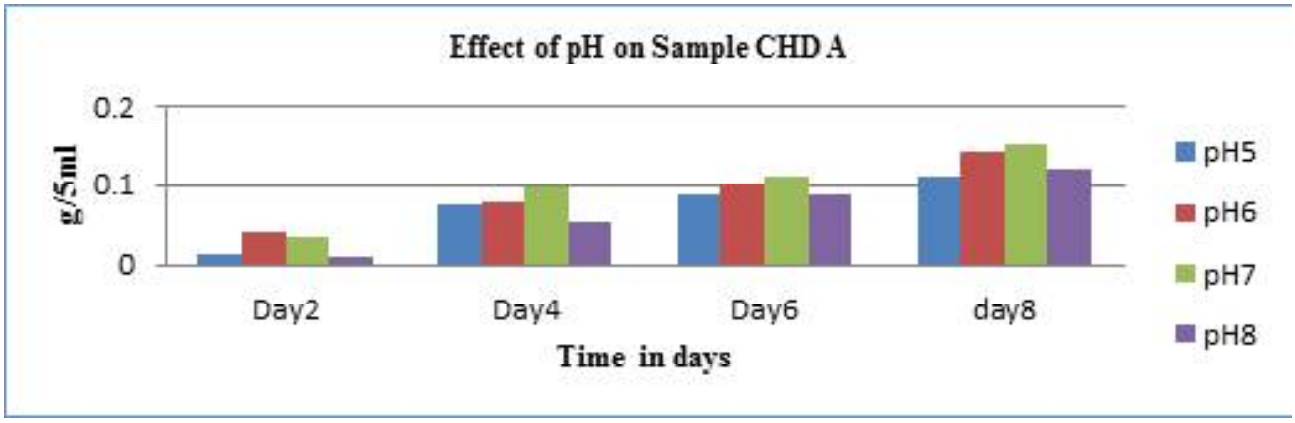


Fig 1. Biomass characterization (in g/5ml) of different pH for CHD A

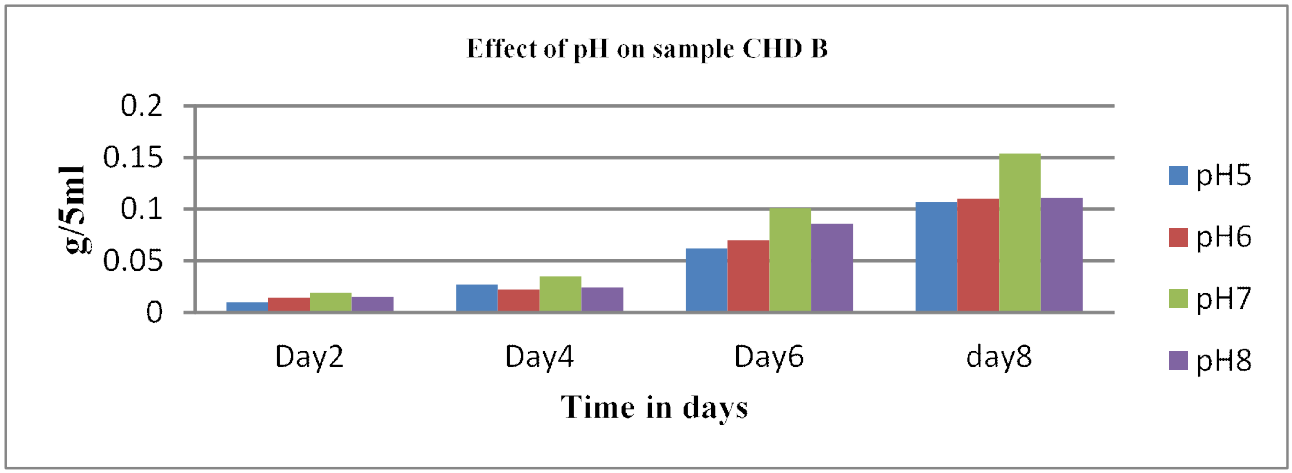


Fig 2. Biomass characterization (g/5ml) of different pH for CHD B

were placed in the three different categories viz., resistant (R), intermediate resistance (I) and susceptible (S) as per the standard method and the criteria given for the each antibiotic specifically.

Data in table 8 indicated that. Isolates CHD A and CHD B showed susceptible against three antibiotics (Ampicillin, penicillin/cloxacillin, and Teicoplanin) and intermediate resistant against two antibiotics (cefotaxime and aztreonam, while it showed resistant to others antibiotics. CHD A and CHD B showed resistant against only one azithromycin antibiotics, while showed susceptible against three antibiotics (netilmycin, amikacin and doxycycline HCl).

MIC values are presented in ìg /mL

Growth kinetics

Optimization of the physicochemical parameters is considered as key factors to maximize the growth and activity. Optimization by one parameter at a time is still considered as good method for optimization.

Results of pH optimization studies indicated that pH 8 was the optimum for growth of CHD A and CHD B (Fig 1 and Fig 2).

The bacterial sources show drop in production media pH during the course of the experiment. The ability of the CHD A and CHD B culture to grow and produce enzyme in media of high salt concentration that makes value of this enzyme more suitable for industrial applications.

The growth pattern of isolates CHD A and CHD B determined over a range of temperature (20-40^{0}C). Data in Fig.3 & 4 show that isolate CHD A and CHD B grew best at 25^{0}C followed by 30^{0}C and 20^{0}C. The lowest growth was observed at 40^{0}C. It is worth mentioning that maximum growth was recorded after 8 days, these results were in close agreement with those reported by Nedwell & Rutter (1994) and Ruger *et al* (2000).

16 s r-RNA sequencing

Further sequencing of CHD A and CHD B isolates identified as Brachybacterium spp. and Kocuria spp. respectively(Table 9 and Table 10). Original sequence are submitted to NCBI with accession no KU878971.1 and KU878972.1 (Appendix 1 and appendix 2). KU878971.1 showed 95 % similarity with KU894797.1 (Brachybacterium paraconglomeratum strain SL-88 16S ribosomal RNA gene, partial sequence) while KU878972.1 showed 95 % similarity with KM186611.1(Kocuria polaris strain XJB-YJ7-2 16S ribosomal RNA gene, partial sequence).

Both sequence were further carried forwarded for multiple sequence alignments taking all sequence showing similarity with KU878971.1 and KU878972.1. The evolutionary distance was inferred using Neighbor-Joining

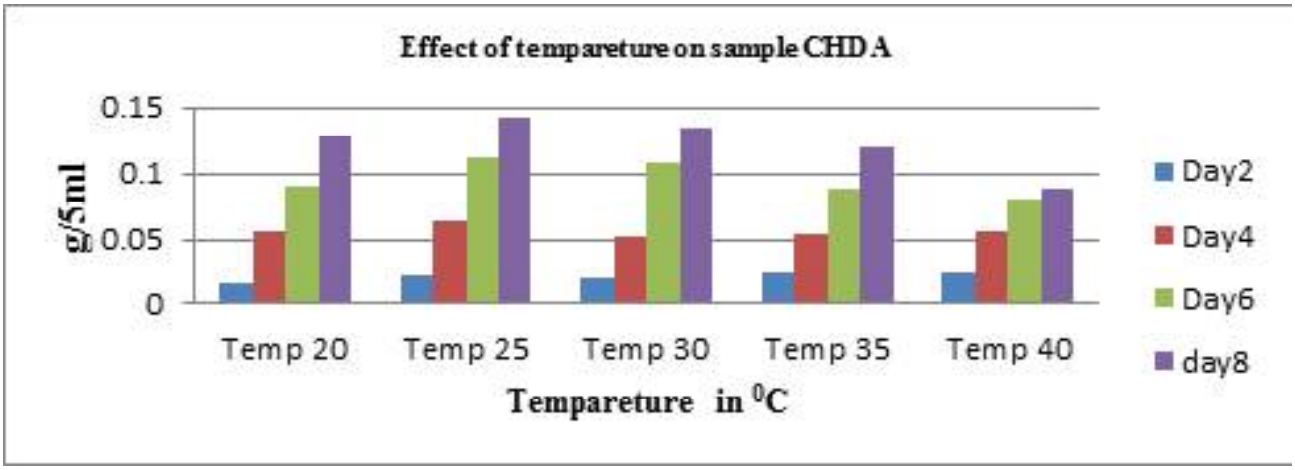


Fig 3. Biomass characterization at different Temperature for CHD A

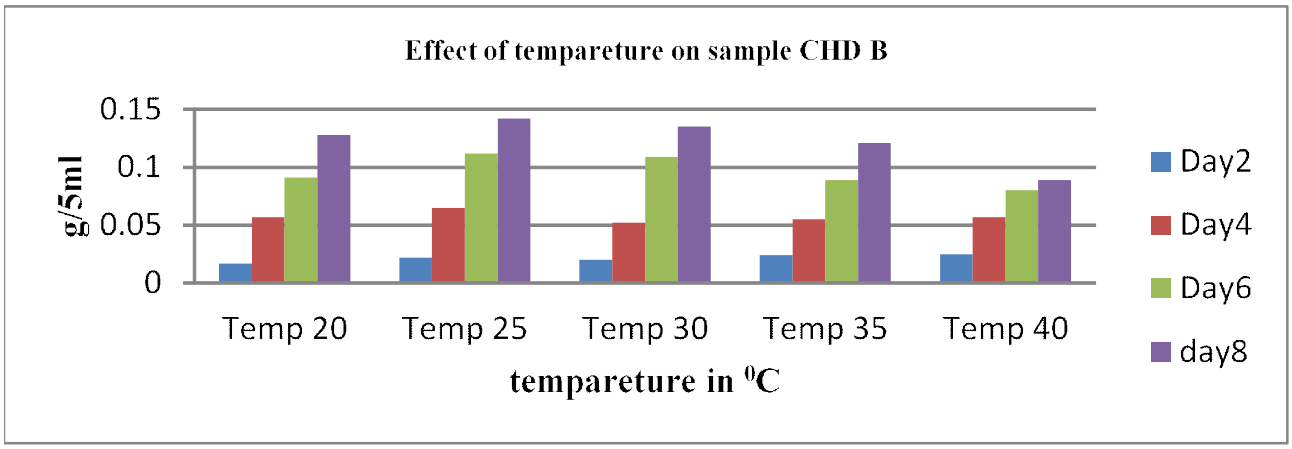


Fig 4. Biomass characterization at different Temperature for CHD B

method (Saitou and Nei, 1987). The evolutionary distances were computed using Maximum Composite Likelihood method (Tamura *et al*., 2004) and were in units of number of base substitutions per site. On comparative Phylogenic analysis result suggested Brachybacterium peraconglomeratum strain CHPBMCST 1 showed closest proximity with HQ455045.1 and Kocuria polaris strain CHPBMCST 2 showed closest proximity with KJ187440.1 (Fig 5). Similar work was carried out by Sarwar *et al* 2014.Two strains FA 2.2 and FA 3.3 were selected for 16s rRNA amplification. On phylogenetic analysis, named as Bacillus spp.FA2.2 and Staphylococcus spp.FA3.3.

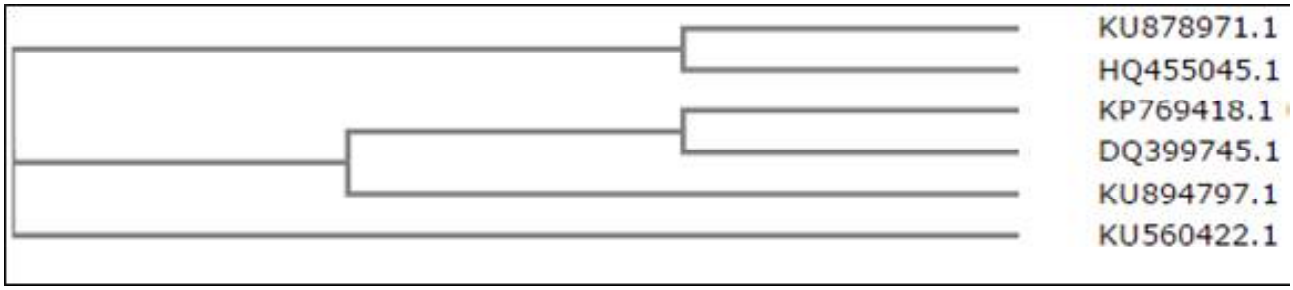
Culturing microbes and molecular analysis give us an edge to have more cultured microorganisms with their taxonomy from the extreme environments. The results from

Table 9. Results of similarity searches between *Brachybacterium paraconglomeratum* strain CHPBMCST 1 with other sequence of *Brachybacterium* spp. GenBank accessions using BLASTX algorithm.

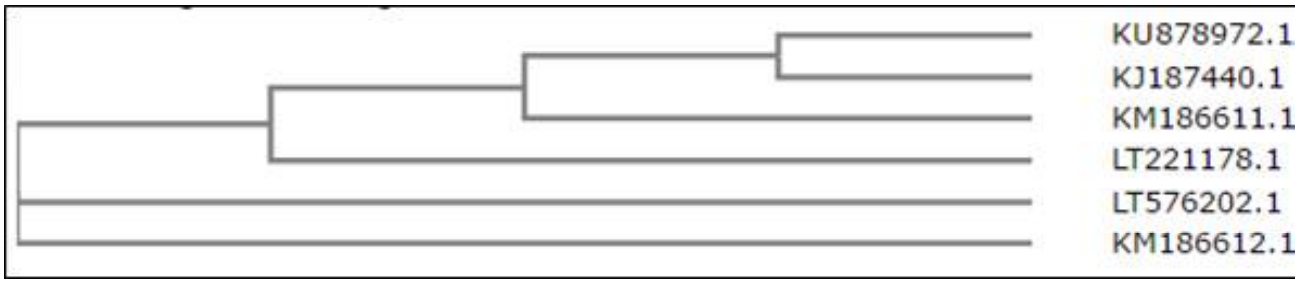
Sr. No.	accession no.	Sequence name	Percentage identity	Query coverage	E value
1	KU894797.1	KU894797.1 Brachybacterium paraconglomeratum strain SL-88 16S ribosomal RNA gene, partial sequence	95%	95%	0.0
2	HQ455045.1	Brachybacterium sp. CJ-6 16S ribosomal RNA gene, partial sequence	95%	95%	0.0
3	DQ399745.1	Brachybacterium sp. B-116 16S ribosomal RNA gene, partial sequence	95%	95%	0.0
4	KU560422.1	Brachybacterium sp. MCCC 1A11196 16S ribosomal RNA gene, partial sequence	95%	94%	0.0
5	KP769418.1	Brachybacterium paraconglomeratum strain DN73_5E10 16S ribosomal RNA gene, partial sequence	95%	94%	0.0

Table 10. Results of similarity searches between *Kocuria polaris* strain CHPBMCST 2 with other sequence of *Kocuria* spp. GenBank accessions using BLASTX algorithm.

Sr. No.	accession no.	Sequence name	Percentage identity	Query coverage	E value
1	KM186611.1	Kocuria polaris strain XJB-YJ7-2 16S ribosomal RNA gene, partial sequence	88%	93%	0.0
2	LT576202.1	Uncultured Kocuria sp. partial 16S rRNA gene, clone W3R17	87%	93%	0.0
3	LT221178.1	Kocuria polaris partial 16S rRNA gene, isolate AT2NF2	87%	93%	0.0
4	KM186612.1	Kocuria polaris strain XJB-YJ7-3 16S ribosomal RNA gene, partial sequence	87%	93%	0.0
5	KJ187440.1	Kocuria sp. Act48 16S ribosomal RNA gene, partial sequence	87%	93%	0.0



a) KU878971.1(*Brachybacterium paraconglomeratum* strain CHPBMCST 1)



b) and KU878972.1 *(Kocuria polaris strain CHPBMCST 2)*

Fig 5. Phylogenetic tree of 16s r DNA sequences of *Brachybacterium paraconglomeratum* strain CHPBMCST 1 and *Kocuria polaris* strain CHPBMCST 2 with other similar sequence on basic of BLAST result

the studies revealed that, Two types of halophilic and halotolerant bacteria (CHD A and CHD B) have been isolated from soil which are phylogeneticaly different and names *Brachybacterium paraconglomeratum strain CHPBMCST 1* and *Kocuria polaris strain CHPBMCST 2* are moderately halotolerant organisms. The microbial diversity could prove to be a valuable future resource in various industrial and biotechnological processes. These organism could be exploited directly or transgenic microbes for environmental safety for industrial waste treatment as bioremediation (degradation of toxic compounds under saline conditions); leather, food, enzyme and polymer industries; production of different stress compatible solutes, oil fields, saline agricultural fields and transgenic plants for stress tolerance and had several other potential applications. Halophiles offer an added advantage to be a source of gene(s) that can increase salt tolerance in different crops through genetic engineering techniques. Thus these offer an important potential for application in microbial, enzyme and agricultural biotechnology.

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LITERATURE CITED

Abdulkarim S.M., Fatimah A.B. and Anderson J.G, 2005. Effect of salt concentrations on the growth of heat stressed and unstressed Escherichia coli. *J Food, Agric and Environ*, **7**(3): 51-54.

Ahmed, I., A. Yokota and T. Fujiwara 2007. A novel highly boron tolerant bacterium, Bacillus boroniphilus sp. nov., isolated from soil, that requires boron for its growth. *Extremophiles*, **11**(2): 217-224.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. 1990 Basic local alignment search tool. *J Mol Biol* **215**:403–410.

Ara, I., Daram, D., Baljinova, T., Yamamura, H., Bakir, M. A. and Suto, M. 2013 Isolation, classification, phylogenetic analysis

and scanning electron microscopy of halophilic, halotolerant and alkaliphilic actinomycetes isolated from hypersaline soil. *Afr J Microbiol Res* **7**:298–308.

Atef Jaouani, Mohamed Neifar, Valeria Prigione, Amani Ayari, Imed Sbissi, Sonia Ben Amor, Seifeddine Ben Tekaya, Giovanna Cristina Varese, Ameur Cherif, and Maher Gtari 2014. Diversity and Enzymatic Profiling of Halotolerant Micromycetes from Sebkha El Melah, a Saharan Salt Flat in Southern Tunisia BioMed Research International. 1-10.

Azhar, M., Uniyal, V., Chauhan, N. and Rawat, D. S. 2014 Isolation and biochemical characterization of Halophiles from Sahastradhara region, Dehradun, India *Int.J.Curr.Microbiol.App. Sci* **3**(12): 753-760.

Chowdhury J.B., Jain S., Jain R.K. 1993. Biotechnological approaches for developing salt tolerant field crops. *J.Plant Biochem., Biotechnol*. **2**: 1-7.

DasSarma, S., and Arora, P. 2001 Halophiles. In: Encyclopedia of Life Sciences. Nature Publishing Group, London.

Dodia, M.S., Joshi, H. R., Patel, K. R. and Singh, P.S. 2006 Characterization and stability of extracellular alkaline proteases from halophilic and alkaliphilic bacteria isolated from saline habitat of Coastal Gujarat, India. *Braz J Microbiol* 37:276–282

Domsch, K. H., Gams, W. and Anderson, T. H. 1980 Compendium of Soil Fungi, Academic Press, London, UK.

Fader R.C, Weaver E, Fossett R, Toyras M, Vanderlaan J. 2013. Multilaboratory study of the biomic automated well-reading instrument versus MicroScan WalkAway for reading MicroScan antimicrobial susceptibility and identification panels. *J Clin Microbiol* **51**: 1548-1554.

Gaur, A., Sharma, G. and Mohan, D. 2015 Isolation and characterization of moderate halophilic Salinicoccus sp. producing extracellular hydrolytic enzymes. *Journal of Global Biosciences*. **4**(9), 3379-3383.

Gibbons, N. E. 1969. Isolation, growth and requirements of halophilic bacteria . In : Methods in Microbiology, Vol.3 B. eds. Norris JR, Ribbons DW, Acad. Press, London, England.

Gomes, J. and Steiner, W. 2004. The biocatalytic potential of extremophiles and extremozymes. *Food Technol. Biotechnol*. **42**: 223-235.

Grant, W. D. 2004 Life at low water activity. *Philos Trans R Soc Lond B Biol Sci* 359:1249–1267

Gupta, S., Sharma, P., Dev, K. Srivastava, M. and Sourirajan, A. 2015 A diverse group of halophilic bacteria exist in Lunsu, a natural salt water body of Himachal Pradesh, India *SpringerPlus* **4**:274-283.

Hedi, A., Sadfi, N., Fardeau, M.L., Rebib, H., Cayol, J.L., Ollivier, B. and Boudabous, A. 2009: Studies on the biodiversity of halophilic microorganisms isolated from El-Djerid Salt Lake (Tunisia) under aerobic conditions. *Int J Microbiol* ,**17**:256-272.

Hedi, A., Essghaier, B., Cayol, J., Fardeau, M. and Sadfi, N. 2014 Prokaryotic biodiversity of halophilic microorganisms isolated from Sehline Sebkha Salt Lake (Tunisia). *Afr J Microbiol Res* **8**:355–367

Karan, R. and Khare, S. K. 2010. Purification and characterization of a solvent stable protease from Geomicrobium sp. EMB2. *Environ. Technol*. **31**:1061-1072

Kiffer, E. and Morelet, M. 1997 Les deut´eromyc'etes: Classification et cl´es d'identification g'en´erique, INRA Editions, Paris, France.

Kokare, C. R., Mahadik, K. R., Kadam, S. S. and Chopade, B. A. 2004 Isolation, characterization and antimicrobial activity of marine halophilic Actinopolyspora species AH1 from the west coast of India. *Curr Sci* **86**:593–597.

Kumar, S.; Karan, R.; Kapoor, S.; Singh, S.P. and Khare, S. K. 2012 Screening And Isolation Of Halophilic Bacteria Producing Industrially Important Enzymes, *Brazilian Journal of Microbiology* **15**: 1595-1603.

Lane, D. J. 1991 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 115-175.

Li X and Yu HY 2012 Characterization of an organic solvent-tolerant á-amylase from a halophilic isolate, Thalassobacillus sp. LY18. *Folia Microbiol* **57**:447–453.

Madern D., Camacho M., Rodríguez-Arnedo A., Bonete M. J., Zaccai G. 2004. Salt-dependent studies of NADP-dependent isocitrate dehydrogenase from the halophilic archaeon Haloferax volcanii. *Extremophiles* **8**:377–384

Nigam, V. K., Singhal, P., Vidyarthi, A. S., Mohan, M. K. and Ghosh, P. 2013 Studies on keratinolytic activity of alkaline proteases from halophilic bacteria. *Int J Pharm Biol Sci* **4**:389–399

Oren, A. 2002(a) Molecular ecology of extremely halophilic archaea and bacteria. *FEMS Microbiol Ecol* **39**:1-7.

Oren, A. 2002(b). Diversity of halophilic microorganisms: Environments, phylogeny, physiology and applications. *Journal of Industrial Microbiology and Biotechnology*. **28**: 56-63.

Pedge, S.S. and Ahirrao, D.S. 2013 Assessment of environmental impact on Lonar Lake Water, (MS) India. *Middle East J Sci Res* **15**:1285–1289

Rakesh J. Patel, Kiran R. Patel, Experimental Microbiology Vol . I & II. Pub: Aditya, Ahmedabad

Roessler, M. and Müller, V. 1998 Quantitative and physiological analyses of chloride dependence of growth of Halobacillus halophilus. *Appl. Environ. Microbiol*. **64**: 3813-3817.

Roohi A, Ahmed, I., Iqbal, M. and Jamil, M. 2012 Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan. *Pak J Bot* **44**:365–370

Rutter M, Nedwell D.B. 1994. Influence of changing temperature on growth rate and competition between two psychrotolerant Antarctic bacteria: competition and survival in non-steady-state temperature environments. *Appl Environ Microbiol*. **60**(6):1993–2002.

Saitou N. and Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*: **4**: 406-425.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 2009 Molecular cloning: a laboratory manual by Joe Sambrook, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Sarwar, M. K., Anwer, F, Rashda, A., Azam, I, Iqbal, T., Nosheen, A., Iqbal, W, Azam, F., Zahra, A. and Atta, K. 2014 Isolation and characterization of halophilic bacterial strains from saline waters of khewra salt mines on the basis of 16s rRNA gene Sequence. *International Journal of Applied Biology and Pharmaceutical Technology*, **5**(1):142-150.

Sellek, G. A. and Chaudhuri, J. B. 1999. Biocatalysis in organic media using enzymes from extremophiles. *Enzym. Microb. Tech*. **25**:471-482.

Smibert R. M. and Krieg, N.R. 1994 phenotypic characterization. In: Gerhardt P, Murray RG, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology *ASM 5*, pp 611–654.

Spring, S.; Ludwig, W.; Marquez, M.C.; Ventosa, A. and Schleifer, K. H. 1996 Halobacillus gen. nov.,with descriptions of Halobacillus litoralis sp. nov. and Halobacilus trueperi sp. nov., and transfer

of Sporosarcina halophila to Halobacillus halophilus comb. nov. *Int. J. Syst. Bacteriol*., **46**:492-496.

Surve, V. V., Patil, M.U. and Dharmadekari, S.M. 2012. Moderately halophilic bacteria from solar salt pans of Ribander, Goa: a comparative study. *Intl J Adv Biotec Res* **3**:635–643

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007 MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24:**1596–1599

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**:4673–4680

Ventosa, A., Nieto, J. J. and Oren, A. 1988. Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.* **62**:504-544.

Vidyasagar, M., Prakash, S., Jayalakshmi, S.K. and Sreeramulu, K. 2007 Optimization of culture conditions for the production of halothermophilic protease from halophilic bacterium Chromohalobacter sp. TVSP101. *World J Microbiol Biotechnol* **23**:655–662

Vijayanand, S., Hemapriya, J., Selvin, J. and Kiran, S. 2012 Biodiversity of extremely halophilic bacterial strains isolated from Solar Salterns of Tuticorin, Tamilnadu, India. *Int J Water Res Environ* **2**:01–07

Von Arx, J. A. 1981. The Genera of Fungi Sporulating in Pure Culture, *J. Cramer, Vaduz*, Germany.

Vreeland, R. H. 1993 Biology of halophilic bacteria, Part I. Introduction, biology of halophilic bacteria : Research priorities and biotechnological potential for the 1990s. *Experientia **49***: 471-472.

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