Isolation, Characterization of Halotolerant bacteria and its biotechnological potentials

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Abstract— Marine microbes represent a potential source for commercially important bioactive compounds and their bioremediation capabilities are also remarkable. Hence for the present study the bacterial strains were isolated from salt enriched soils which were collected from the natural saline habitats from Bhitarkanika coastal region of Orissa, India. The phenotypic characters of the isolates conclusively proved that isolates S1-4 belongs to *Bacillus spp.* and S5 belongs to *Micrococcus luteus* and S6 could not be identified, it would be a new isolate, which changed their shape to spherical forms when grown in NaCl which is a halotolerant character. The bacteria isolates (S 1-4) produced gummy (except S1) colonies of different shape, size margin and elevation. Bacterias were motile except S5, aerobic, gram +ve except S6, spherical and elliptical spores forming rods and cocci of (2.26 - 4.25) x (0.70-0.85) µm size range and catalase (+)ve. However, the organisms differed in some physiological and biochemical characters among themselves but the less distinguishing characters like oxidase, anaerobic (microaerobic) growth, acid and no gas formation from different sugars, protease, amylase, lipase, chitanse, NO_3 reductase production, fermentation of organic carbon sources, antibiotic sensitivity etc. Growth kinetics of the organisms (S1-3) in NB and (S4-6) in HPM showed wide variation having lag phase for 4-12 hours and exponential phases up to about 5-30 hours based on turbidity measurements. Stationary phases of the organisms were variable and results did not show typical declining trend, rather became unstable and the organisms attained the turbidity of about 0.38-0.45. The results obtained from the studies revealed that, the isolates are moderately halotolerant organisms. These organism could be exploited directly or transgenic microbes for environment safety for industrial waste treatment for degradation of toxic compound under saline conditions, leather, food, enzyme and polymer industries for the production of different stress compatible solutes, Oil fields, saline agricultural fields and transgenic plants for stress tolerance and also several other potential applications.

Index Terms— Halotolerant. Phenotypic. Physiological. Biochemical. Biotechnology. Industrial waste.

1 INTRODUCTION

Bacteria live in diverse ecological conditions from extreme cold (Antarctica) to hot conditions (hot springs), mesophilic soil to extreme saline conditions of different pH [1, 2, 3]. Adaptability of the bacteria to such diverse conditions has immense fundamental and applied importance. Besides naturally occurring saline environment, over utilization of ground water is increasing the saline area and about 25% global cultivated area shows excessive salinity [4]. Therefore, investigation on bacteria of saline environment has significant roles. The halotolerant and moderately halophilic eubacteria are more similar to non-halophilic bacteria and these organism have contributed significantly to our knowledge of the phase behaviour of lipids, physiological changes during adaptation to different NaCl concentrations and function of compatible solutes in protecting cells from salts [5]. Although halphilic bacteria have been studied for basic scientific interests, their biotechnological potential has been largely ignored. Halophilic bacteria are important for numerous industrial processes.

Moderate halophiles and halotolerant bacteria are needed for production of wide range of salty foods such as Thi fish sauce, pickling brines, salt-cured bacon and oil field production brines [2]. The halotolerant and halophilic organisms are important for maintenance of soil health and nutrition recycling in saline environment too [2].

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Mangroves are unique inter-tidal ecosystems of the tropics, which support genetically diverse groups of aquatic and terrestrials organisms. This ecosystem is ideally situated at the inter-phase between the terrestrial and marine environment and supports a rich and diverse group of microorganisms. They have a great role in current science and technology by using them can breed salt tolerant crops, many industrial important enzymes, proteins and antibiotics. Marine microbes represent a potential source for commercially important bioactive compounds and their bioremediation capabilities are also remarkable. They also play a crucial role in decomposition of organic matter and cycling of nutrients. Due to their large requirement and uses in vast area of biotechnology it is too important now a day.

Systematic and phylogenetics studies have defined a large number of species to be included within the moderately halophilic bacteria, distributed over at least half of the major phylogenic branches of the bacteria. Molecular ecology techniques available nowadays should be used to determine in more detail the ecological distribution of these halophilic microorganisms and the role they play in hypersaline environments, as well as their contribution to microbial transformation processed. The use of such techniques would enable the elucidation of the biodiversity of moderately halophilic bacteria and the identification of species that constitute the predominant populations in these extreme habitats. Hence, it was decided to investigate the systematic morphophysiological, biochemical characters studies on natural saline habitats from Bhitarkanika coastal region of Orissa, India.

2. Materials and Methods

Site description and Sample collection

A mangrove of Bhitarkanikais situated in Kendrapara district of Orrisa. It extends about 432 Sq.Km. the Brahmani and Baitarani deltaic region of Orissa. The core of the Sanctuary was declared as a National Park for the better protection of flora and fauna. For present study, the samples were collected from different places of National park**.** Top layer of soil sample (about 1 cm) was removed. In total five soil samples were collected from five different spot of each location [Sample-A (Rangani), Sample-B (Mainsamunda), Sample-C (Dangmal), Sample-D (Habalganda), Sample-E (Kalibhanjadia)]. Sample were mixed thoroughly and put in sterile polythene packets with proper levels.

Population Dynamics of Different types of Microorganism in Soil

Population dynamic of different types of micro organisms in soil were studied from soil suspensions. Soil suspensions were diluted to 10^{-5} level, 100 µl suspension was added to 100 ml of the desired medium separately and bulk pour plated in each five Petri plates. The plates were incubated at 30± +0.1ºC generally for 3 to 7 days in a BOD incubator and the numbers of the colonies were counted [6,7, 8].

Determination of Heterophilic Bacterial population on Nutrient Agar (NA) medium

In 100 ml nutrient agar (NA) medium (±45ºC), 1.0 µl of soil suspension was added separately, mixed thoroughly and plated. The plates were incubated at 30± 0.1ºC for 72 hours in a BOD incubator and the bacterial colonies i.e. the colony formation units (CFU) were counted. Some colonies of different bacteria were picked up and were preserved on NA slants to check their sodium chloride and common salt tolerance. Resistant colonies were preserved.

Gram-Negative Bacterial Population Determination

To assess the gram–negative bacterial population, soil suspension were plated on NA medium containing crystal violet (added during plating), incubated in BOD incubator at 30± +0.1ºC for 72 hours. The bluish violet colonies were counted.

Denitrifying (NO_3Reducing I.E. NO_3 to NO_2 Producing) Bacteria population determination

The denitrifying (NO3 reducing) bacterial population was determined on Winogradsky's medium replacing ammonium sulphate with potassium nitrate or sodium nitrate. The plates were inoculated with soil suspension, incubated 72 hours at 30± 0.1ºC, flooded with sulfanillic acid reagent and the pink colonies were counted.

Phosphate (Inorganic) solubilizing bacterial population determination

The phosphate solubilizing bacteria were determined on phosphate (PBS) agar medium. Plating was done after sterilization without allowing the medium to solidify. The soil suspension were plated and the plates were incubated for 72 hours or more at 30± 0.1ºC. The colonies having a clear zone around them were counted.

Asymbiotic (free living) nitrogen fixing bacterial population determination

The symbiotic nitrogen fixing bacteria were determined on nitrogen free medium. The plates inoculated with soil suspensions were incubated for 72 hours at 30± 0.1ºC and the developed colonies were counted.

Sulphur oxidizing (Thaobqaacillus Group) Bacterial population determination

To determine the population of sulphur oxidizing bacteria, soil suspension were plated in Thaobqaacillus medium, incubated in BOD at 30± 0.1ºC for 7 days or more. The organism produce black (or brownish black) colonies due to sulphur deposition were counted.

Spore forming Bacterial population determination

Spore forming bacterial population was determined from pasteurized soil suspensions. Soil suspension were plated on NA medium and incubated in BOD at 30±0.1ºC for 72 hours. The colonies were counted.

Growth morphological and staining characters of the organisms

Morphological characters of the colonies and the bacteria were studied following the standard microbiological methods from the bacteria grown on NA [6, 7, 8].

Colony characters of the isolates

The isolates were diluted streak on NA plates, incubated 72 hours at 30± +0.1ºC. The shape, size, colour, margin and opacity were recorded from isolated colonies.

Growth characters on NA slant, stab and NB

The isolates were streaked on NA slant and stab cultured with a straight needle pierced through the centre of the NA stab tubes, incubated at 30± 0.1ºC for 72 hours and growth of the organism were recorded.

Morphology of cells and spores and motility

Morphology of the vegetative cells and spores were observed under a Phase contrast microscope under a 100X objective from <18 hours (for vegetative cells and motility) and ≥5days old (spores) cultures grown on NA plates at 30± 0.1ºC in a BOD incubator.

Staining characters of vegetative cells and spores

Gram's stain.

To study the Gram's stain (crystal violet) i.e. Gram (+ve) or Gram (-) characters of the isolates, diluted suspensions of the bacteria (8-12h old) were smeared on clean slides, air dried, heat fixed by passing over a flame for 2-3 times. The slides were flooded with crystal violet solution for 1 minute, washed

with water and flooded with Gram's iodine for 1 minute. The slides were washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet colour was visible from drain off alcohol. The slides were washed with water and counter stained with safranin stain for about 30 seconds and washed with water. The slides were air dried an examined under a microscope using 100X objective using a daylight filter.

Spores stain.

The spores were stained with malachite green stain. The smear was flooded with the stain, steamed (avoid boiling) over a flame for 10 minutes, washed under tap water, counter stained with safranin and observed under 100X objective. The sores take green stain.

Physiological and Biochemical characters

The activities of various physiological and biochemical characters by the isolates were studied by the following tests viz., i) Na-acetate tolerance test; ii) Oxidase test; iii) Catalase test; iv) Urease test; v) Indole production test; vi) Methyl red test; vii) Voges-Proskauer (Acetoin production) test; viii) Nitrate Reduction test; ix) Citrate utilization test; x) Hydrogen sulphide (H_2S) production test.

Assays of Extracellular enzymatic activities

The activities of various extracellular enzymes produced by the isolates were studied by the following tests viz., i) Starch hydrolysis test; ii) Lipase test; iii) Tributyrin test (or vegetative oil) hydrolysis test; iv) Tween-80 hydrolysis test; v) Cholesterol hydrolysis test; vi) Protein hydrolysis test; vii) Gelatin hydrolysis test; viii) Casein hydrolysis test; ix) Pectin hydrolysis test; x) Chitin hydrolysis test; xi) Lecithin hydrolysis test; xii) DNase test.

Antibiotic sensitivity test for the organism

Response of the organism to different antibiotics was tested on NA medium. NA plates were surface seeded with concentrated bacterial suspension. Different antibiotic discs with effective concentrations were placed over the plates. Inhibition of growth depicted by a clear zone formation around the discs indicated sensitive reaction otherwise the organism was resistant to the antibiotic.

Growth curve and measurement of generation Time

The organism were grown in 250 ml nephalflasks containing 50 ml NB medium. Over night grown bacteria at 100 rpm and 30± 0.1ºC temperature on a rotary shaker was used for growth kinetics and generation time. In duplicates of nephaloflasks, 100µl inoculum was added and both turbidity and cell numbers of the flasks were recorded. One flask was immediately put on the shaker to grow at 30± 0.1ºC temperature at 100 rpm speed. Another flask was placed after 12 hours growth of the first flask. Both turbidity and cell numbers were recorded at 1 hour interval. Turbidity of growth was measured through a colorimeter at 540 nm (for colourless medium) or 660 nm (for yellowish medium) and cell number was counted through a haemocytometer under a phase contrast microscope. The turbidity and cell numbers (semilogarithmic plot) were plotted against time function to determine the growth kinetics. From the cell count of exponential growth phase and generation time were calculated.

3. Statistical Analysis

All the Statistical analysis was done by Student's test. Growth rate was calculated using the following equation, $k = log_{10}N_t - log_{10}N_0 / 0.301t$, where, k=exponential growth rate constant i.e., number of generations per hour; N_0 = population size at a certain time of exponential phase; N_t = population time at a subsequent time; t = time lapse between N_t and N_0; 0.301 = constant for $log_{10}2$; generation time = l/k .

4. Results

Colony characters of the isolates viz.S1-S6 were either irregular, filamentous, punctiform or circular form; yellowish brown, brown, creamy_white or pale_brown colour; convex, flat or raised elevation; entire, undulate or filamentous margin; range of colony size was 1.0 – 4.0 mm on NA and HPM after 2 days having gummy and sticky consistency (Table 1 & 2).

Table 1 : Population dynamics

Population = Colonies x 10^{-5} g^{-1} soil

Soil	Type	Heterophilic Bacteria	Dentrifying bacteria	Gram Negative bacteria	Phosphate solubilizing bacteria	Nitrogen fixation bacteria	Sulfur oxidizing bacteria	Spore forming bacteria
A	Moist	402	9	63.66	29	6.66	13	11.33
B	Moist	407	14.66	175	40.66	17.66	41.33	11.66
C	Moist	282	23	44	91.66	31.33	24.33	11
D	Moist	–	139	129	70.33	24	27.33	18.33
E	Moist	317	0	55.66	63.33	35.65	32	21.66

*Average of three replicates.

Sample-A (Rangani), Sample-B (Mainsamunda), Sample-C (Dangmal), Sample-D (Habalganda), Sample-E (Kalibhanjadia)

Table 2 : Colony characters of the bacteria on Nutrient Agar & Halophilic Media Plates

Bacteria	Form	Colour	Elevation	Margin	Size (mm)	Consistency
S1	Irregular	Yellowish brown	Raised	Undulate	1.4 -2.3	Sticky
S2	Filamentous	Creamy white	Flat	Filamentous	1.5 – 2.5	Gummy
S3	Irregular	Creamy white	Flat	Undulate	2.5 – 4.0	Gummy
S4	Circular	Yellowish brown	Convex	Entire	2.5 – 4.5	Gummy
S5	Punctiform	Brown	Convex	Entire	1.0 – 1.5	Gummy
S6	Punctiform	Light brown	Convex	Entire	1.0 – 2.0	Gummy

*Results are from 5 colonies.

The characteristics of vegetative cells and spores of the isolates are given in Tables 3 and 4. Vegetative cells of the isolates (rod

forms) were 0.51 x 2- 4µm. Diameter of the spherical cell was 1-4 µm. The isolates S1-4 formed elliptical and S5-6 produced round spores. Dimensions of the spores ranged from 1.8-3.0 x 0.8-5.0 µm for the elliptical spores and diameter of the spherical spores were 0.8 µm. The bacteria were positive Gram stain, except for S6 and motile except for S5 which was nonmotile.

Table 3: Characters of vegetative cells of Bacterial isolates

Bacteria no.	Shape	Length (µm)		Breadth (µm)		Motility	Gram Stain
		Range	Mean	Range	Mean		
S1	Rod	2 -3	2.5	0.5 - 1.0	0.75	Motile	+ (w)
S2	Rods in chain	2- 3	2.5	1.0 – 1.50	1.25	Motile	+
S3	Rod	3.5 – 4	3.75	0.75 – 1.0	0.87	Motile	+
S4	Rod	2.5 – 3	2.75	0.45 – 0.75	0.60	Motile	+
S5	Cocci	3.5 – 4	3.75	0.75 – 1.0	0.87	Non Motile	+
S6	Rod	2.5 – 3.5	3.0	0.45 – 1.5	0.97	Motile	-

+ = Positive result; - = Negative result. Results are means of 5 observations.

Table 4 : Characters of spores of Bacterial isolates

Bacteria no.	Shape	Length (µm)		Breadth (µm)		Spore Stain
		Range	Mean	Range	Mean	
S1	E	1.25 - 2	1.62	0.25 - 0.75	0.5	+
S2	E	1 – 1.5	1.25	0.25- 1	0.67	+
S3	E	1.5 – 2	1.75	0.5 – 1.25	0.87	+
S4	E	1 –2	1.5	0.5 – 1	0.75	+
S5	S	1.5	0.25			+
S6	S	1.25	0.45			+

+ = Positive result. Results are means of 5 observations. E = Elliptical ; S = Spherical

The organism were isolated from NA and HPM containing 12% NaCl and could tolerate atleast up to 10% NaCl except S4 and S5 which tolerated up to 25% NaCl (Table 4) which not the normal character of none of the identified species. Probably, as the organism were continuously growing in saline or coastal soil (Table 5) they might have acquired the salt tolerant character in course of time. As the organisms S4 – 5 (except 1,2,3 and 6) could grow in the presence of more than 10–20% NaCl, so the isolates could be grouped as moderately halotolerant but not halophilic. Change of shape into spherical form is well known to be the stress tolerant character of bacteria, thus the observation proved conclusively that they are moderately osmotolerant non-halophilic microbes.

Table – 5 : Growth characteristics of Bacterial isolates :

Growth Medium	Bacterial Number					
	S1	S2	S3	S4	S5	S6
NA	+	+	+	+	+	+
NA+NaCl (%)						
5	-	-	-	+	+	-
7	+	+	+	+	+	-
10	+	+	+	+	+	+W
12	-	-	-	+	+	+
13	-	-	-	+	+	+
15	-	-	-	+	+	+
20	-	-	-	+	+	+
25	-	-	-	+	+	-
NA+Sea salt (%)						
10	+	+	+	+	+	+
15	-	-	-	+	+	+
NB+Sea salt (%)						
10	+	+	+	+	+	+
15	-			+	+	+
Anaerobic growth in NB	-	-	-	+	+	+
Growth at Temperature						
30C	+	+	+	+	+	+
35C	+	+	+	+	+	+

NA – Nutrient Agar; NB = Nutrient broth ; + = Positive result ; - = Negative result.

Enzymatic activities of different organisms are given in Table 6 (few representative results are shown) None of the organisms were positive for Cholesterol (except S2-3), Chitin, Pectin, Lecithin, Gelatin (except S1), Casein, Tween 80, Tributyrin (except S1,4) hydrolysis, DNase activity (except 3,5).

Table 6 : Extracellular enzymatic activities of Bacterial isolates

Test	Bacteria Number					
	S1	S2	S3	S4	S5	S6
Protease :						
Gelatinase	+	-	-	-	-	-
Casein hydrolysis	-	-	-	-	-	-
Lecithinase	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-

+ = Positive result ; - = Negative result

Fermentation (aerobic) reactions of different organisms were not identified (Table 7). Most of the organisms were negative for the test, except for S5 (Melobiose, Lactose, Dextrose), S6 (Lactose, Dextrose, Fructose, Arabinose), S4 (Lactose, Dextrose, Fructose), S1 (Mannose).

Table 7: Fermentation / utilization of organic carbon sources.

Carbon source (1%)	Bacteria Number					
	S1	S2	S3	S4	S5	S6
Melibiose	-	-	-	-	+	-
Fructose	-	-	-	+	-	+
Mannose	+	-	–	-	+	-
Lactose	-	-	-	+	-	+
Arabinose	-	-	-	-	-	+
Dextrose	-	-	-	+	+	+

+ = Positive result ; - = Negative result

Some of the physiological and biochemical properties of the cultures are presented in Table 8. All of the organisms were Catalase positive, nitrate reductase negative and urease (except S3) positive, oxidase (except S5-6) positive, indole negative, VP positive (except S3-4), citrate negative (except S2-3), H_2S positive (except 5,6), ADH positive.

Table 8 : Some physiological and biochemical properties of Bacterial isolates.

Test	Bacteria Number					
	S1	S2	S3	S4	S5	S6
Catalase	+	+	+	+	+	+
Indole production	-	-	-	-	-	-
Methyl red test	-	-	-	-	-	-
Vogus Proskauer (AMC) test	+	+	-	-	-	+
Nitrate Reduction	-	-	-	-	-	-
Urease Production	+	+	-	+	+	+
Citrate Utilization	-	+w	+	-	-	-
Oxidase	+	+	+	+	-	-
Arginine dihydrolase	+	+	+	+	+	+
Deoxyribose nuclease	-	-	+	-	+	-

+ = Positive result ; - = Negative result . AMC = Acetyl Methyl Carbinol.

Acid and gas production by the isolates mostly negative (Table 9). The organism did not produce gas from any one of the carbon sources but S2 produced acid from Sucrose, Glucose, Galactose, Mannose, Maltose and Mannitol, but S1 and S5

produced acid from Maltose.

Table 9 : Acid and gas production by the isolates from different carbon sources.

Bacteria number	Glucose		Sucrose		Arabinose		Arginine		Mannose		Mannitol		Galactose		Maltose	
	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
S1	+	-	-	-	-	-	-	-	-	-	-	-	-	+w	-	
S2	+	-	+	-	-	-	-	-	+	-	+	-	+w	-	-	-
S3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S5	-	-	-	-	-	-	-	-	-	-	-	-	-	+w	-	
S6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Positive result ; - = Negative result . +w = weakly positive

The response of the organisms to the recommended doses of different antibiotics (Table 10) shows that all of them were sensitive to all of the antibiotics except the antifungal antibiotic Nystatin.

Table 10 : Antibiotic assay of Bacterial isolates.

Antibiotic	S1		S2		S3		S4		S5		S6	
	S/R	C	S/R	C	S/R	C	S/R	C	S/R	C	S/R	C
Nystatin (10µg)	R	20	R	16	R	21	S	18	S	16	R	16
Penicillin G (10U)	S	36	S	16	S	21	S	40	S	32	S	31
Polymyxin B (300U)	S	15	S	13	S	16	R	19	S	15	S	17
Norfloxacin (10µg)	S	18	S	25	S	18	S	20	S	21	S	24
Bacitracin (10U)	S	32	S	11	S	10	S	39	S	15	S	25
Chloropheni col (30µg)	S	21	S	35	S	24	S	18	S	22	S	16
Erythromycin (15µg)	S	14	S	6	S	14	S	39	S	25	S	12
Gentamycin (10µg)	S	39	S	39	S	29	S	30	S	25	S	20
Tetracycline (30µg)	S	24	S	30	S	27	S	18	S	22	S	22
Methicycline (30µg)	S	21	S	24	S	27	S	18	S	22	S	16
Ciprofloxacin (30µg)	S	32	S	40	S	36	S	23	S	20	S	24
Chlorotetracycline (30µg)	S	34	S	37	S	38	S	20	S	16	S	22
Kanamycin (30µg)	S	31	S	32	S	21	S	18	S	16	S	14
Vancomycin (30µg)	S	20	S	19	S	7	S	20	S	24	S	16

Growth kinetics of the organisms (S1-3) in NA and (S4-6) in HPM showed wide variation having lag phase for 4-12 hours and exponential phases up to about 5-30 hours based on turbidity measurements. Stationary phases of the organisms were variable and results did not show typical declining trend, rather became unstable and the organisms attained the turbidity of about 0.38-0.45.

Table 11 : Identification scheme of the isolates up to Genus Level based on Morphological and Physiological characters.

Characters	Bacteria number (TB)					
	S1	S2	S3	S4	S5	S6
Rod shaped	+	+	+	+	-	+
Cell length (µm)	1.5	1.5	2	3	3	4
Diameter >2.5µm	+	+	+	+	+	+
Filaments	-	-	-	-	-	-
Rods or filaments curved	+	+	+	+	-	+
Cocci in tetrads or packets	-	-	-	-	Packets	-
Endospore produced	+	+	+	+	+	+
Motile	+	+	+	+	+	+
Strict aerobes	-	-	-	-	-	-
Facultative anaerobe or Microaerophilic	-	-	-	+	+	+
Strict anaerobes	+	+	+	-	-	-
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	-	-
Marked acidity from glucose	-	-	+	-	+	-
Nitrate reduced to nitrite	-	-	-	-	-	-
Genus	*Bacillus*	*Bacillus*	*Bacillus*	*Bacillus*	*Micrococcus*	unidentified

+ = Positive ; - = negative.

Comprehensive results of biochemical characters of the results of the investigation on phenotypic and molecular characters of the isolates for identification up to genus level are presented in Table 11. All of the isolates were Gram positive (except S6), facultative anaerobic, spore forming motile (except S5), rods (except S5), width of rods was <2.5 µm, catalase positive, nitrate reductase and no gas production (Table 8 & 9). The organisms showed variable results for the other tests.

Results on species level identification of the isolates are presented in Table 12. The organism S1-4 produced elliptical spores of <1 µm diameter and S5-6 produced spherical spores. Sporangia were not swollen. All the organisms were catalase, citrate utilization (except S2 and S3), lecithinase, indole production, chitin hydrolysis, pectin hydrolysis negative; tributyrin hydrolysis (lipase) positive for S1 and S4 but negative for S2, S3, S5,S6 and could grow in presence of 25% (S4and S5), 20% (S6) and 10% (S1-3) NaCl. The organisms did not produce gas and the S2 only was highly acidic in presence of glucose, sucrose, galactose,mannose, maltose and mannitol and did not hydrolyze casein and Tween 80. However, other tests were variable.

Table 12 : Identification scheme of the isolates up to Species level based on Morphological and Physiological characters.

Character	Bacteria Number					
	S1	S2	S3	S4	S5	S6
Cell diameter >1µm	+	+	+	+	+	+
Spores round	-	-	-	-	+	+
Sporangium swollen	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Anaerobic growth	-	-	-	+	+	+
VP test	+	+	-	-	-	+
Acid Production :						
Melibiose	-	-	-	-	+	-
L-Arabinose	-	-	-	-	-	+
Fructose	-	-	-	+	-	+
Dextrose	-	-	-	+	+	+
Lactose	-	-	-	+	-	+
Gas from glucose	-	-	-	-	-	-
Hydrolysis of :						
Casein	-	-	-	-	-	-
Gelatin	+w	-	-	-	-	-
Starch	+	-	-	+	-	+
Citrate utilization	-	+w	+	-	-	-
Egg yolk lecithinase	-	-	-	-	-	-
Indole production	-	-	-	-	-	-
Growth in NaCl (20%)	-	-	-	+	+	+
Growth at :						
35°C	+	+	+	+	+	+
40 °C	+	+	+	+	+	+
Lipase Test :						
Tributyrin test	+w	-	-	+w	-	-
Tween 80	-	-	-	-	-	-
Hydrolysis of :						
Chitin	-	-	-	-	-	-
Pectin	-	-	-	-	-	-
Urease test	+	+	-	+	+	+
Genus	*Bacillus*	*Bacillus*	*Bacillus*	*Bacillus*	*Micrococcus*	unidentified
Species	*Sp.*	*Sp.*	*Sp.*	*Sp.*	*luteus*	

5. DISCUSSION

The bacteria isolates (S1-4) produced gummy (except S1) colonies of different shape, size margine and elevation, bacteria were motile except S-5, aerobic, gram (+)ve except S6, spherical and elliptical spores forming rods and cocci of (2.26 - 4.25) x (0.70-0.85) µm size range and catalase (+)ve. However, the organisms differed in some physiological and biochemical characters among themselves but the less distinguishing characters like oxidase, anaerobic (microaerobic) growth, acid and no gas formation from different sugars, protease, aminlase,

lipase, chitanse, NO_3 reductase production, fermentation of organic carbon sources, antibiotic sensitivity also conform with characters of *Bacillus spp*. [8, 9]. Beside the characters of *Bacillus spp*. which is mentioned above, the organism produced gummy colonies, elliptical or spherical spores and non-swollen sporangia and positive for catalase test which confirmed that the organism belongs to either Group I of Bacillus spp. [9].

The morphophysiological, biochemical characters proved that the isolates S1, S2, S3 and S4 are *Bacillus spp*. The isolate S5 and S6 produced punctuate, gummy colonies; S5 was Gram (+)ve and S6 was Gram (-)ve ; Catalase, Urease, ADH positive; oxidase, methyl red, indole, nitrate reduction, citrate utilization, hydrogen sulfide production, lipase, protease lecithinase and pectin hydrolysis negative and other tests were variable. Based on the phenotypic characters, S5 was identified as *Micrococcu luteus* [10] and the isolate S6 remained unidentified. Bacteria are also identified on serological reactions, analysis of the component like aminoacid, lipids, phage typing etc. [9]. However, all these method have both inherent merits and demerits and some time one method may not suffice to classify all the new isolates. Therefore, biochemical grouping is widely followed for classification of bacteria and other methods are being used a supporting information.

The organism were isolated from NA and HPM containing 12% NaCl and could tolerate atleast up to 10% NaCl except S4 and S5 which tolerated up to 25% NaCl which not the normal character of none of the identified species [9, 10]. Probably, as the organisms were continuously growing in saline or coastal soil they might have acquired the salt tolerant character in course of time. Although the organism could grow in presence of 10% NaCl or common salt but NaCl or salt was not essential for the growth of organisms because organisms could grow both in HPM or NA although growth was better in the medium supplemented with NaCl or salt. As the organisms S4-5 (except 1,2,3 and 6) could grow in the presence of more than 10 – 20 % NaCl, so the isolates could be grouped as moderately halotolerant but not halophilic [11, 12]. Change of shape into spherical form is well known to be the stress tolerant character of bacteria [5, 12]. Thus the observation proved conclusively that they are moderately osmotolerant non-halophilic microbes. In Indian soil, so far no Bacillus has been isolated which can tolerate 10% salt or NaCl but for *B.coagulans* which tolerate up to 6% NaCl isolated from the desert soils of Rajasthan [13]. Thus the organisms isolated during the present study are unique organisms having moderate salt tolerance capacity. However, animal pathogens viz. *E.coli*, *Micrococcus*, *Staphylococcus*, *Vibrio* etc. were also known to be naturally halotolerant microbes (14, 15, 16]. Although several *Bacillus spp.* have salt tolerance character, none of these organisms were reported earlier to have salt stress tolerance capacity [3,12].

Growth kinetics of the organisms (S1-3) in NA and S4-6) in HPM showed wide variation having lag phase for 4-12 hours and exponential phases up to about 5-29 hours based on turbidity measurements. Stationary phases of the organisms were variable and results did not show typical declining trend, rather became unstable and the organisms attained the turbidity of about 0.38-0.45. The variations of growth kinetics of bacteria are usual because it is dependent on several complex factors and members of same group are unlikely to express identical growth. Instability of growth after stationary phases of the organisms could be explained from the fact that towards the end of the stationary phase all the organisms produced spores but turbidity became unstable because occasional germination of some of the spores altered could the turbidity.

The results from the studies revealed that, the *Bacillus* and *Micrococcus* spp. and the unidentified isolates are moderately halotolerant organisms. These organism could be exploited directly or transgenic microbes for environmental safety for industrial waste treatment (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.

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