



Isolation and Characterization of Halophilic Bacteria from Salinity Soil of Shatkhira, Bangladesh

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MKM and SN designed the study, performed the statistical analysis and wrote the protocol and the first draft of the manuscript. Authors ASH and AKS managed analysis and the literature searches. Author MFH edited the manuscript and finalized it. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was undertaken for the isolation and molecular characterization of the halophilic bacteria from salt affected soils.

Place and Duration of Study: This study was conducted on the salinity affected area in Ramzan Nagor under Shamnagor Upazilla of Shatkhira District, Bangladesh. The collection of soil sample, isolation of halophilic bacteria and subsequent experiments were done from May to December 2018.

Methodology: Salt affected soil could be good source of halophilic bacteria as it contain high amount of salt. Hence, one gram of salt affected soil sample which was containing high amount of salt was suspended to 100 ml sterile distilled water and one ml of sample from the top of the suspension were taken in to 250 ml Erlenmeyer flaks containing 100 ml of mineral salts (MS) medium. The primary enrichment was incubated for several days at 37°C with shaking at 120 rpm (revolution per minute) on an orbital shaker. Cultures which were found to be turbid after a period of up to 4 days were used as inocula in subsequent experiments.

Results: Four halophilic bacterial strains viz. *Bacillus sp.* strain 8-15, *Enterobacter sp.* strain LCR75, *Acinetobacter sp.* strain 407 and *Acinetobacter junii* strain F27 were isolated from the saline

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soil and screened for their growth at a range of salt (NaCl) concentrations viz., 0%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5%. Out of four isolates, *Bacillus sp.* showed the highest capacity to tolerate 5% salt concentration whereas *Enterobacter sp.* and other two isolates (*Acinetobacter sp.* and *Acinetobacter junii*) showed the capacity to tolerate 4% and 2.5% salt concentration respectively. Based on their morphological and biochemical characteristics the isolates were found to be Gram-positive and Gram-negative, rod shaped, motile bacteria which were later identified on the basis of 16S rRNA gene sequencing. The optimum culture conditions of the isolates were pH 7.0 and temperature 37°C. Results on the antibiogram profiles of the isolates revealed that the strains were sensitive to most of the antibiotics used in this study except Kenamycin, Ceftazidime and Ampicillin.

Conclusion: The isolated halophilic bacteria may play an important role to optimize the salt affected soil; hence they could be important candidates for further research on salt bioremediation.

Keywords: Bioremediation; salinity; *Acinetobacter spp.*; *Bacillus sp.*; *Enterobacter sp.*

1. INTRODUCTION

Global warming is one of the leading threats to the human being in the near future because of its high impact on the rise of sea-level [1]. It is not a theoretical threat but a practical problem which have to be faced by mankind unless preventive actions are taken instantly. A significant part of Bangladesh is sea-side low land which is bordered with the Bay of Bengal. There are 19 districts in coastal areas of Bangladesh covering 32% of the total areas of country and accommodating around 35 million people [2]. Hence, these coastal areas of Bangladesh are significantly affected by the adverse impact of saltwater intrusion. Because of climate change, salt water contamination progressively extends to the inland soil. In 1973, the total amount of salt contaminated land was 83.3 million hectares, which had been enhanced to 102 million hectares in 2000 and 105.6 million hectares in 2009 in Bangladesh [3]. In the last 35 years, the salt contaminated area has been enlarged about 26 percent in Bangladesh [4]. Thus, growing salinity becomes a vital problem to the people who are living in the coastal region of Bangladesh. Because of growing soil salinity, the people of the coastal areas are suffering from shortage of land for cultivation of crops. Moreover, the soil salinity limits the development of cultivable crops and disturbs overall crop production [5,6]. In this circumstance, development of novel approach to manage the soil salinity for cultivation of crops in coastal areas is the crucial issue in Bangladesh.

Bacteria play an important role for enhancement of soil fertility [7,8], production of antimicrobial products [9,10,11] and bioremediation of toxic substances in soil and water [12,13,14,15]. Moreover, soil bacteria are playing very vital role

in biogeochemical cycles resulting in better crop production. Hence, demand of eco-friendly and sustainable agriculture with emphasis on the application of beneficial microorganisms is increasing day by day [16]. Moreover, native adaptation of floras to their environment is regulated by genetic variation of microorganisms which are in closely association with plants [16]. Similarly, microorganisms available in Rhizosphere of a plant play a vital role to improve the growth of various crops which are cultivated in wide-ranging root-zone salinities. Thus, there is high potential for bioremediation of salt by using halophilic microbes in rhizosphere. Hence this study was undertaken for the isolation and molecular characterization of the halophilic bacteria from salt affected soils.

2. MATERIALS AND METHODS

2.1 Study Area and Duration

This study was conducted on the salinity affected area in Ramzan Nagor under Shamnagor Upazilla of Shatkhira District, Bangladesh. The study area is located at 22.3306°N 89.1028°E. The collection of soil sample, isolation of halophilic bacteria and subsequent experiments were done from May to December 2018.

2.2 Collection of Samples

Soil samples were collected from soil of salinity affected study area. The collected soil samples were transferred to the Genetics and Molecular Biology Laboratory, Department of Zoology, University of Rajshahi, Bangladesh for conducting required experiments to isolate and characterize bacteria which were able to tolerate salinity. The soil samples used for physicochemical analysis were kept at room

temperature while the soil samples used for isolation of bacteria were kept in refrigerator at 4°C until used.

2.3 Analysis of Soil pH, Salinity and Conductivity of the Saturation Extract

For analysis of soil samples, digestion was done according to the method of McGrath and Cunliffe [17]. Briefly, the collected soil samples were dried at room temperature (30±3°C) and finely crushed into minute size (<0.1 mm). Then, 1 gram of each soil sample was burnt into ashes in a container. These ashes of soil sample which were kept in a new clean beaker were moistened with a small amount of double distilled water. Then concentrated HNO₃ and HCl (at 3:1 ratio) were added consecutively into soil sample. Soil sample were then heated mildly with a heating plate until the soil samples were digested, which was showed by the development of a clear solution above the soil residue.

2.4 Enrichment Culture of Organisms

1 gram of collected soil sample kept in a sterile 250ml Erlenmeyer flasks was suspended to 100 ml sterile distilled water and then vortexed well for 30 minutes. From the top of the suspension, one ml of samples were taken in to 250ml Erlenmeyer flasks containing 100 ml of mineral salts (MS) medium. Control flasks without an inoculum were also prepared. The primary enrichment was incubated at 37°C with shaking at 120 rpm for several days on an orbital shaker. The incubated liquid culture was observed for turbidity and it was used as an inocula in subsequent experiments when it was turned into turbid form.

2.5 Isolation and Screening of Bacteria

For the isolation of bacteria Nutrient agar was only used in this study because of its universal application as an all-purpose medium for the culture of wide range of bacteria. Loop full of primary enrichment culture was streaked on Nutrient agar plates (Hi-media) and incubated for 24h at 37°C temperatures. The single colonies were found to grow on the medium. Four isolates were found from primary screening and further grown on nutrient agar plate containing 0%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5%, and 5% sodium chloride concentrations. The plates were incubated for 48 h at 37°C. After incubation the

single colonies were selected for streaking on nutrient agar plates in a biosafety cabinet. Pure cultures were developed and stored on slants by stab and streak method which were then used subsequently for their identification and biochemical characterization.

2.6 Microscopic Examination and Identification of Bacterial Cells

For the identification of the halophilic bacteria, morphological characterization, microscopic observation, gram staining, growth characteristics, bio-chemical tests and antibiotic sensitivity tests were performed. Microscopic observation was done with a binocular light microscope (Labomed, USA). The microorganisms were identified according to Bergey's Manual of Systematic Bacteriology [18].

2.7 Antibiotic Sensitivity Test

Antibiotic sensitivity test of the isolates was accomplished as stated by Saha and his colleagues [19]. Briefly, 1ml of fresh broth culture of the bacteria isolated from soil sample was spread homogeneously with a germ-free glass spreader on a nutrient agar plate. Then, the inoculated plates were air-dried for few minutes in a biosafety cabinet. The antibiotic discs *viz.* Doxycycline, Ceftazidime, Cephadrine, Neomycin, Amoxycillin, Erythromycin, Tetracycline, Kenamycin, Cefixime, Ampicillin were placed on air dried plates which were incubated at 37°C for 24 hours. After incubation, clear zone around the disc which was indicating the inhibition of growth of the isolate was measured and recorded.

2.8 Effects of pH and Temperature of the Isolates

Optimum growth of bacterial isolates were evaluated at various pH 5.0, 6.0, 7.0 and 8.0 and temperature (28°C, 37°C, 42°C) in a medium supplemented with 2% sodium chloride. The pH was adjusted by using concentrated NaOH or HCl. The optical density of bacterial culture was measured by photoelectric colorimeter (AE-11M, Erma Inc., Tokyo) at 660 nm wavelength at regular interval for evaluating optimal bacterial growth [20].

2.9 Identification of the Isolates by 16S rRNA Gene Sequence

Identification of the isolated strain was performed by 16S rDNA sequence analysis. Genomic DNA

was extracted from the bacterial cells using Maxwell® RSC Cultured Cells DNA Kit (Model: AS1620, Origin: Promega, USA). The 16S rDNA gene was amplified by PCR using the specific primers, 27F and 1492R which are capable of amplifying 16S from a wide variety of bacterial taxa [21]. The sequence of the forward primer was 16SF 5'- AGA GTT TGA TCM TGG CTC AG-3' and the sequence of the reverse primer was 16SR 5'- CGG TTA CCT TGT TAC GAC TT-3'. The PCR amplicons are separated electrophoretically in a 1% agarose gel and visualized after Diamond™ Nucleic Acid Dye (Cat: H1181, Origin: Promega, USA) staining. The PCR products were purified using SV gel and PCR Clean Up System (Cat: A9281, Origin: Promega, USA) according to the manufacturer's protocol. The total DNA yield and quality were determined spectrophotometrically by Nano Drop 2000 (Thermo Scientific, USA). PCR amplified 16S rDNA of the screened isolates was sent for automated sequencing (Applied Biosystem 3130) to the Centre for Advanced Research in Science (CARS) under Dhaka University, Bangladesh. The sequence generated from automated sequencing of PCR amplified DNA was analyzed through NCBI BLAST (<http://www.ncbi.nlm.nih.gov>) program to find out possible similar organism through alignment of homologous sequences. Finally, the isolates were identified based on alignment of partial sequence of 16S rDNA with the existing sequences available in the database.

3. RESULTS

3.1 Soil pH, Salinity and Conductivity of the Saturation Extract

The results of the analysis of soil pH and salinity revealed that pH of soil was 7.5 and soil salinity was found to be moderate. Due to moderate salinity yields of many crops are restricted in the study area (Table 1).

3.2 Isolation and Characterizations of Halophilic Bacterial Strains

In this study, four salt tolerant bacterial strains viz., *Bacillus sp.*, *Enterobacter sp.*, *Acinetobacter sp.* and *Acinetobacter junii* were isolated and screened for growth at different salt concentrations ranging 0% -5% of NaCl. Out of four isolates, *Bacillus sp.* showed tolerance to 5% salt concentration whereas *Enterobacter sp.* showed tolerance to 4% salt concentration. The

other two isolates (*Acinetobacter sp.* and *Acinetobacter junii*) were found to tolerate 2.5% salt concentrations (Table 2). Results of morphological characteristics and microscopic analysis of bacterial strains are presented in Table 3 (a) and 3(b) while the biochemical and antibiotic sensitivity tests of the isolates are presented in Table 4 (a) and 4(b), respectively. This was accomplished simultaneously by gross colony morphology and a number of biochemical tests on the basis of presence (+) or absence (-) criteria.

3.3 Effects of pH and Temperature of the Isolates

To verify the effects of pH and temperature of medium on the growth rate of the isolates, a number of experiments were carried out which are presented in Fig. 1 respectively. The optimal pH for the growth of the isolates was 7.0 while extreme pH was 5.0 and 8.0 which restricted the bacterial growth. The optimal temperature for the growth of the isolates was found to be 37°C while the extreme temperatures were between 28° and 42°C which restricted the bacterial growth. At 37°C the rate of the best growth was found (OD 0.97) after 24hr of incubation.

3.4 Identification of the Bacteria

The 4 isolated bacterial strains identified by morphological and biochemical tests were subjected to 16S rRNA gene sequence analysis. Analysis of 16S rRNA gene sequence revealed that maximum sequence similarity of the 4 isolates were found to that of *Bacillus sp.* strain 8-15, *Enterobacter sp.* strain LCR75, *Acinetobacter sp.* strain 407 and *Acinetobacter junii* strain F27 respectively. The homologous identities of the four bacterial isolates are presented in Table 5.

4. DISCUSSION

Salt contamination in soil and water is a major abiotic stress that decreases productivity of crop both in semiarid and arid soils. In the world 33% of irrigated land and 20% of the total cultivated agricultural land are suffering from high salinity [22]. Recently, it was reported that that typical crop production becomes more limited because of increasing salinity of some areas as well as extension of salt affected area resulted from further invasion of saline water [23]. But, more crop production is required to meet the demand of growing population which in turn is urging for

more research on crop production in adverse environmental condition. In this situation, appropriate biotechnology can help to increase crop productivity by improvement of soil health through interactions of plant roots and microbes in rhizosphere [24].

Table 1. Analysis of soil sample

pH	Conductivity of the saturation extract (ds/ml)	Soil salinity class	Effects of crop plants
7.5	4.7	Moderate saline	Yields of many crops are restricted

(0-2) = Non saline, (2-4) = Slight saline, (4-8) = Moderate saline, (8-16) = Strongly saline, (>16) = Very strongly saline

Table 2. Growth pattern of isolated bacterial strains on nutrient agar medium containing different concentrations of sodium chloride

Strain No.	0%	2%	2.5%	3%	3.5%	4%	4.5%	5%
<i>Bacillus sp.</i>	+++	+++	+++	+++	+++	++	++	++
<i>Enterobacter sp.</i>	+++	+++	+++	++	+	+	-	-
<i>Acinetobacter sp.</i>	+++	++	+	-	-	-	-	-
<i>Acinetobacter junii</i>	+++	++	+	-	-	-	-	-

("+++” = High growth, “++” = Moderate growth, “+” = Low growth, “-” = No growth)

Table 3(a). Colony morphology of the selected isolates

Morphological Tests	Colony morphology			
	Isolated microorganism			
	<i>Bacillus sp.</i>	<i>Enterobacter sp.</i>	<i>Acinetobacter sp.</i>	<i>Acinetobacter junii</i>
Colony shape	Round	Round	Round	Round
Color	Pale Yellow	Creamy	White	White
Surface	Smooth	Smooth	Smooth	Smooth
Opacity	Opaque	Opaque	Opaque	Opaque
Consistency	Sticky	Sticky	Sticky	Sticky

Table 3(b). Microscopic observations of the isolated bacterial strains

Characteristics	Bacterial strains			
	<i>Bacillus sp.</i>	<i>Enterobacter sp.</i>	<i>Acinetobacter sp.</i>	<i>Acinetobacter junii</i>
Gram staining	+ve	-ve	-ve	-ve
Shape	Rod	Rod	Rod	Rod
Motility	Motile	Motile	Non motile	Non motile

Table 4(a). Biochemical test results for the isolated bacterial strains

Bacterial strain	Biochemical tests																
	TSI test	H ₂ S Production	Methyl Red	Nitrate reduction	Urease test	Citrate	SIM	Oxidase test	KOH	Catalase Test	Mac Conkey Agar	Fructose	Maltose	Cellulose	Galactose	Glucose	Sucrose
<i>Bacillus sp.</i>	-	+	+	+	+	+	-	+	-	+	-	+	+	+	+	+	+
<i>Enterobacter sp.</i>	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
<i>Acinetobacter sp.</i>	-	+	+	+	+	+	-	-	+	+	+	-	-	+	-	+	+
<i>Acinetobacter junii</i>	-	+	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+

("+" symbol used for the positive reaction of biochemical tests and/or bacterial growth, and "-" symbol used for negative reaction and/or growth)

Table 4(b). Antibiotic sensitivity tests

Antibiotics	Isolates with antibiotics range(mm) and the level of sensitivity			
	<i>Bacillus sp.</i>	<i>Enterobacter sp.</i>	<i>Acinetobacter sp.</i>	<i>Acinetobacter junii</i>
Doxycycline	30(H)	14(I)	30(H)	19(S)
Ceftazidime	1 (R)	2(R)	1(R)	1(R)
Cephadrine	47(H)	1(R)	27(H)	16(S)
Neomycin	20(S)	19(S)	15(S)	16(S)
Amoxycillin	39(H)	1(R)	18(S)	4(R)
Erythromycin	37(H)	7(R)	28(H)	21(S)
Tetracycline	32(H)	17(S)	28(H)	20(S)
Kenamycin	10(R)	8(R)	5(R)	6(R)
Cefixime	35(H)	25(H)	23(H)	1(R)
Ampicillin	21(S)	6(R)	17(S)	2(R)

** (5-10mm) = Resistance to antibiotic(R); (10-15mm) = intermediate resistance (I); (15-20mm) = Sensitive to antibiotic(S), (>20mm) = Hyper sensitive (H)

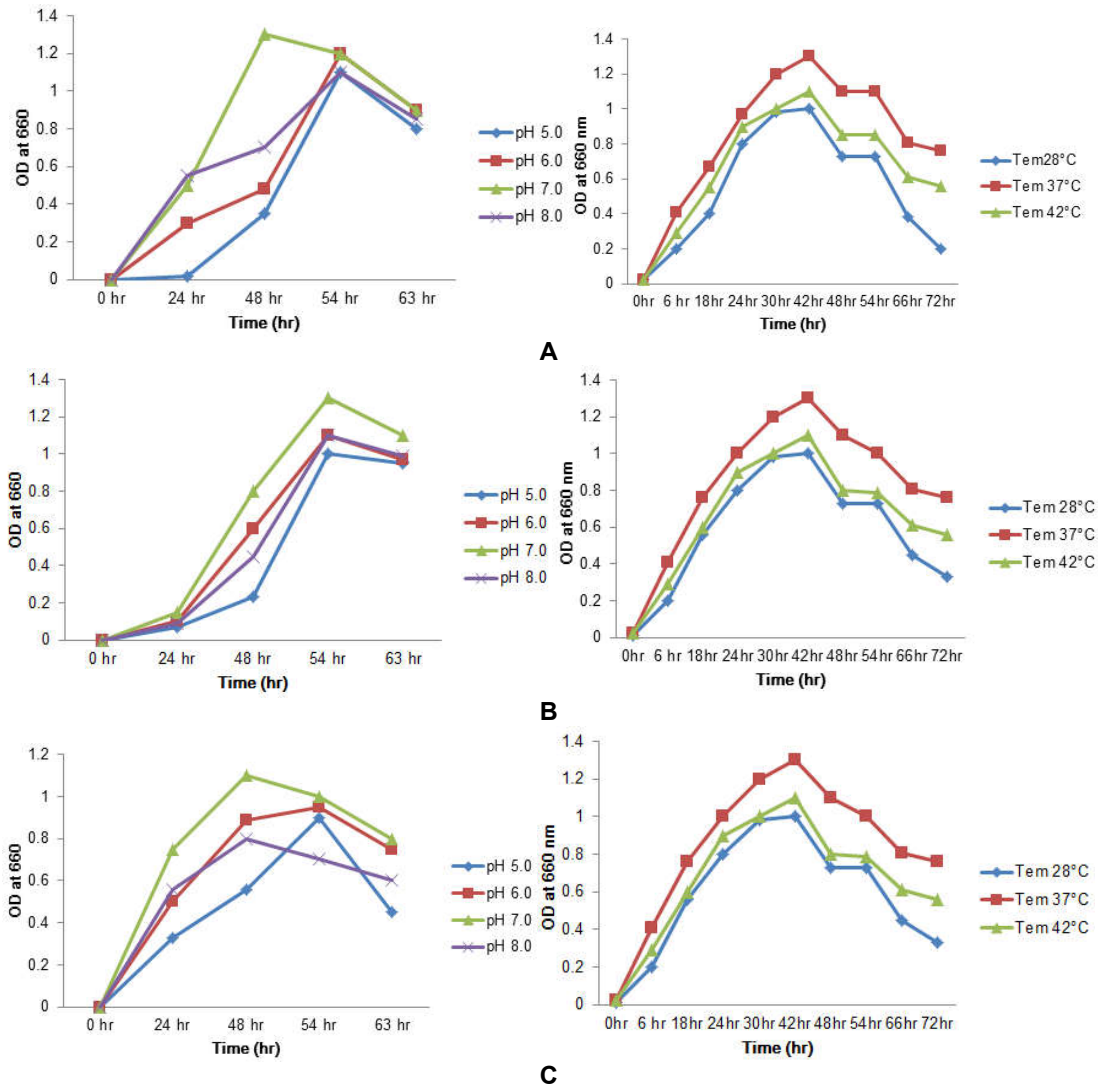


Fig. 1. Optimum pH and temperatures for growth of bacterial isolates A (*Bacillus sp.*), B (*Enterobacter sp.*) and C (*Acinetobacter sp.*)

Table 5. Homologous identity of the bacterial isolates

Tested strain and accession number	Related species	bacterial	Accession number	Max. score	Total score	Query cover	Identity
P-228883	<i>Bacillus</i> sp. strain 8-15		KX806623.1	1575	1575	71%	93%
	<i>Bacillus persicus</i> strain W13118		KJ676970.1	1573	1573	71%	93%
L-70225	<i>Enterobacter</i> sp. LCR75		FJ976584.1	2026	2026	94%	98%
	<i>Enterobacter cloacae</i> complex sp. 35734		CP012162.1	2022	16042	94%	98%
O-127043	<i>Acinetobacter</i> sp. 407		EU841481.1	2022	2022	97%	98%
	<i>Acinetobacter baumannii</i> strain S-X9A		KJ806391.1	2010	2010	96%	98%
S-171313	<i>Acinetobacter junii</i> strain F27		MF681999.1	2012	2012	96%	98%
	<i>Acinetobacter</i> sp. strain W1		KX622562.1	2012	2012	96%	98%

In this study, the halophilic bacteria were isolated soil samples were collected from different salinity affected areas. Based on 16S rRNA gene sequence analysis the isolates were identified as *Bacillus*, *Enterobacter*, *Acinetobacter* spp. and *Acinetobacter junii* respectively. In the present study, *Bacillus* sp. showed 5% salt tolerance activity whereas *Enterobacter* sp. showed 4% and other two isolates (*Acinetobacter* sp. and *Acinetobacter junii*) were found which could stand for at 2.5% NaCl concentrations. Similarly, five salt tolerant bacteria were isolated from salinity affected areas viz. Khulna and Satkhira, Bangladesh by other researchers [25]. They found that the isolate was *Azotobacter* species and showed growth in medium supplemented up to 10% salt.

A similar study achieved by Roohi [26] showed that twenty one halotolerant and halophilic bacterial strains viz., *Bacillus*, *Enterobacter*, *Virgibacillus*, *Staphylococcus*, *Pseudomonas*, *Halobacillus*, *Terribacillus* etc. were isolated from salt mines of Karak region, Pakistan. These isolates showed grow in media with 5-40% NaCl concentrations. Islam [27] also noticed that *A. chroococuum*, *A. vinelandii* and *A. armeniacus* can tolerate more than 1% salt concentration. Arora [28] reported that a salt tolerant *Bacillus* sp. isolated from coastal region of Gujrat which can grow in media 10% NaCl concentration.

Mendpara [29] studied that two salt tolerant bacterial strains viz. GSD1 (*Exiguobacterium* sp.) and GSD2 (*Serratia* sp.) were isolated from agricultural soil and the species were able to adapt up to 10% salt concentration. A number of salt tolerant strains of *Rhizobium trifolii* have

been reported by Mandal [30]. It was also found that the strains of *Rhizobium trifolii* showed 3% salt stress tolerance.

Pourbabae [31] reported 167 halotolerant bacterial strains were isolated from different saline habitats and screened for growth at different salt concentrations. The strains belonged to species *Bacillus mojavensis* and the strains showed grow well at 20% salt concentration. Ramadoss [32] found that only 25% (21 out of 84) isolates showed growth at 20% salt concentration.

Rodriguez-Valera [33] noticed that genera of *Bacillus*, *Micrococcus*, *Alcaligenes* and *Pseudomonas* were dominant types halophilic bacteria available in saline soil. Likewise, 71 halotolerant endospore forming gram-positive rods were isolated from saline soils which were tentatively assigned to the genus *Bacillus* and the majority were able to grow in soil with 25% salts [34]. Similarly, *Marinococcus halotolerans* which was motile cocci that grow over a wide range of salt concentrations and up to 20% NaCl was one of the extremely halophilic bacteria isolated from soil samples [35]. Recently, bacteria of different genera including *Rhizobium*, *Pseudomonas*, *Bacillus*, *Pantoea*, *Burkholderia*, *Paenibacillus*, *Azospirillum*, *Achromobacter*, *Azotobacter*, *Methylobacterium*, *Microbacterium*, *Enterobacter*, *Variovorax* etc. have been stated to help in developing capacity tolerance of host plant under different abiotic stress environments [36].

The bacterial isolates could be characterized depending on their capacity to utilize different

carbon sources [37,38]. In the present study sucrose, maltose, fructose, glucose and cellulose (25 mg Hi-media, India) were utilized for experiments. It was found that the bacterial isolates could utilize all the five sugar which was comparable to the results reported by Akhter [25].

In this study, the highest growth was observed at pH 7.0 and temperature 37°C. This result is supported by the findings of Hongyu [39] who isolated the halophilic bacteria from some salt ponds in China and revealed that the growth of these bacteria was optimal at the temperature of 35-40°C and pH 7.0-8.0 with 20% NaCl. Rohban [40] also reported that the halophilic bacterial strains showed the best growth on pH 7-9 and 28°-37°C temperature.

5. CONCLUSION

Plant-microbs interaction is beneficial association which could be used as a more efficient method for the reclamation of salt affected soils. Hence, four halophilic bacteria viz. *Bacillus sp.* strain 8-15, *Enterobacter sp.* strain LCR75, *Acinetobacter sp.* strain 407 and *Acinetobacter junii* strain F27 were isolated from the saline soil of Shatkhira District, Bangladesh. It was found that these halophilic isolates can tolerate up to 5% salt concentration. The optimum culture conditions of the isolates were pH 7.0 and temperature 37°C. They were sensitive to the most of the studied antibiotics except Kenamycin, Cefazidime and Ampicillin. Altogether, it can be concluded that the isolated bacteria could survive in highly salt affected soil indicating their importance as candidates for further research on their role on improvement of soil quality by bioremediation of salt which in turn may contribute to increase crop production in coastal areas in Bangladesh.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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