Isolation and Identification of Halophiles from Sundarbans Estuary and Study of their Molecular Osmoadaptation Strategy

M. SC SUMMER TRAINING PROJECT WORK,

UNDER THE GUIDANCE OF

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INTRODUCTION

The Sundarbans, formerly Sunderbunds, are a vast tract of forest and salt water swamp forming the lower part of the Padma (Ganga)- Brahmaputra river delta in south-eastern West Bengal and southern Bangladesh. The tract extends approximately 160 miles (260 km) west-east along the Bay of Bengal from the Hooghly estuary in India to the western segment of the Meghna river estuary in Bangladesh, and reaches inland for about 50 miles (80 km) at its broadest point. A network of estuaries, tidal rivers, and creeks, intersected by numerous channels, it encloses flat, densely forested, marshy islands. The total area of Sundarbans, including both land and water, is roughly 3,860 sq. miles (10,000 sq. km), about three-fifths of which is in Bangladesh.[1] Indian Sundarbans is known as Hoogly-Matla estuary (Hooghly is the Lower part of River Ganges), where apart from Hoogly and Matla, there are innumerable big & small rivers criss-crossing the Sundarbans namely Bidya, Saptamukhani, Raimangal, Muriganga, Thakuran, Gomor etc. Many rivers have become almost completely cut off from the main freshwater sources (Sanyal & Bal, 1986) as for example Bidya, Matla are devoid of freshwater connection due to siltation in the upstream region and are converted into tidal creeks.[2]

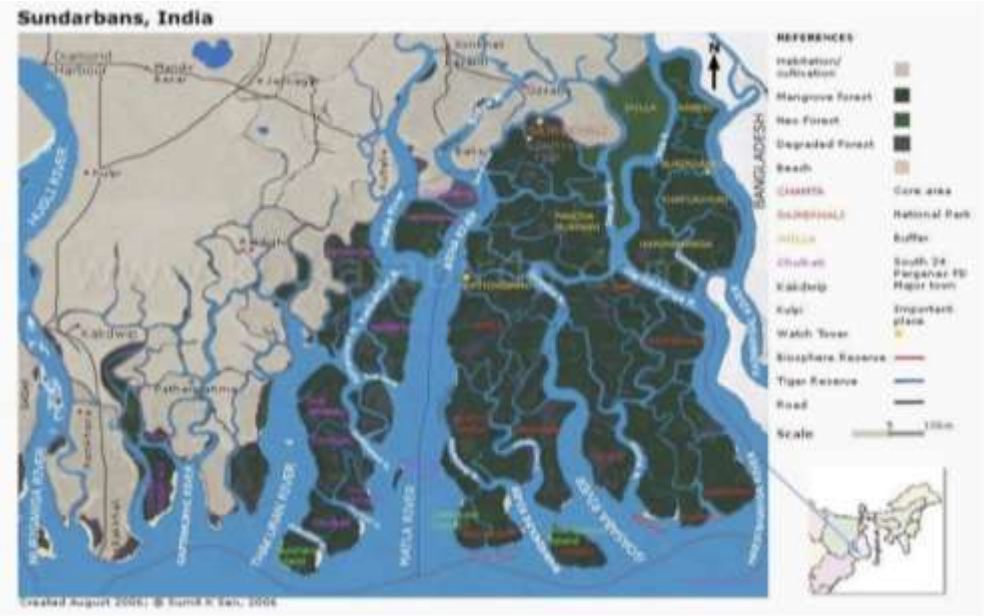


Figure 1: Geographical location of Sundarbans estuary, India

Sundarbans is the world's largest tidal halophytic mangrove ecosystem situated in the delta of Ganges, Meghna, and Brahmaputra rivers on the Bay of Bengal,

and has been recognized as a UNESCO World Heritage site [3]. It plays critical roles on the sea-continent interface providing environmental conditions for the development of important marine and estuarine species.

This mangrove ecosystem of Indian subcontinent is well known not only for the aerial extent, but also for the species diversity. The biodiversity of Sundarbans includes numerous species of phytoplankton, zooplankton, micro-organisms, benthic invertebrates, mollusks, amphibians and mammals. It is the only mangrove tiger land on the earth. It has been declared as a world heritage site by International Union for Conservation of Nature (1987).[2]

Sundari (*Heritiera fomes*), gewa or gengwa (*Excoecaria agallocha*), nipa palms (*Nypa fruticans*) and other halophytic (salt-tolerant) species are the dominant flora in the mangrove swamps.

The Sundarbans region is renowned as a refuge for a variety of animal species, many of them rare and endangered. Notably, it is one of the last preserves of Bengal tigers (*Panthera tigris tigris*), which are found in relative abundance there. Other animals include spotted deer, wild boars, otters, wild cats and Ganges river dolphins (*Platanista gangetica*). Several dozen reptiles and amphibian species are found in the Sundarbans, notably crocodiles, Indian pythons, cobras, and marine turtles. The region is home to more than 250 bird species- both seasonal migrants and permanent residents- including hornbills, storks and other waders, kingfishers, white ibis, and raptors such as sea eagles.[1]

The microbial population is mainly dominated by Archaea and after the analysis of metagenome sequences, a total of 44 phyla were found to be present in two seasons (July 13 and Dec 13) at Sundarbans. Proteobacteria is the most dominant phyla. The abundance of Proteobacteria was found to be 53% at 2 cm_Dec 2013 and 31% at 32 cm_Dec 2013 (postmonsoon) and 44% at 2 cm_ July 13 and 38% at 32 cm_July 13 (monsoon) respectively. Besides Proteobacteria, Bacteroidetes formed 15% and 5% bacterial population in post-monsoon (Dec 13) and 19% and 2% in monsoon (July 13) respectively. The third abundant phyla were Firmicutes in bothseasons. Insignificant abundance was detected in both sampling seasons for the phyla Nitrospirae. Most interesting
phyla, Actinobacteria accounted 7% for the post-monsoon at two different depth samples (2 cm_Dec 13 and 32 cm_Dec 13) and only 5% for monsoon sample only in surface sample (2 cm_July 13). Planctomycetes were found to be the less abundant phyla for both seasons (Dec: 6% and July: 5%).[4]

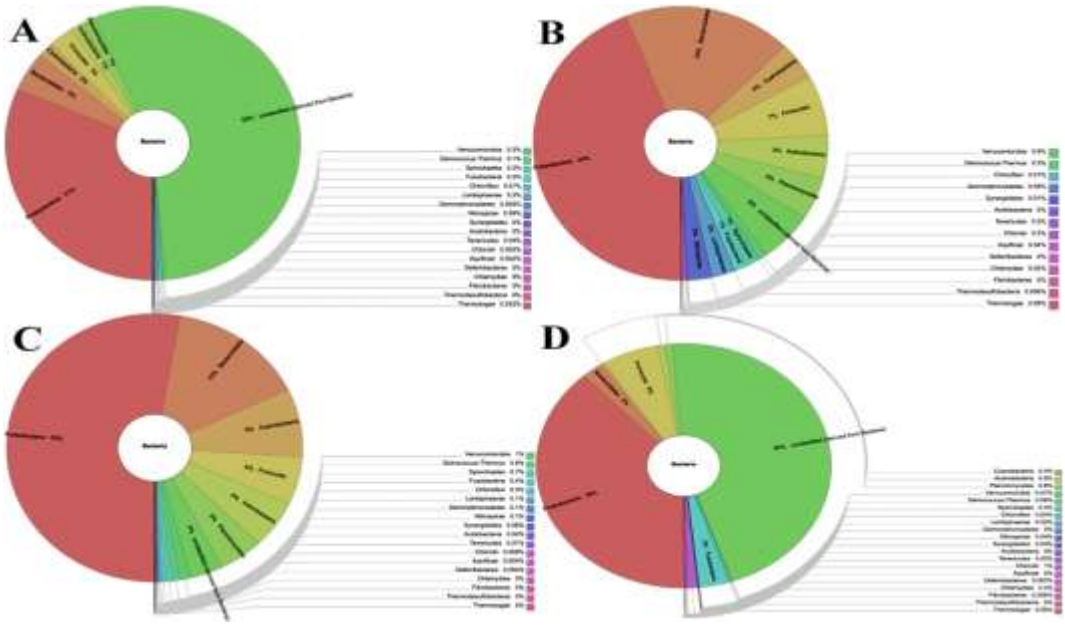


Figure 2: Taxonomic distribution of the metagenome sequences of the bacterial communities dominant at Sundarbans. The figure was prepared by the Krona interactive visualization program built within the MG-RAST portal. Shown are A (32 cm_Dec 12), B (2 cm_July 13), C (2 cm_Dec 12) and D (32 cm_July 13), respectively.[4]

Of the domain Bacteria, the species of interest for our project purpose is *Halobacillus* sp.[1]

AIMS AND OBJECTIVES

1. Isolation and identification of Halobacteria from Sundarbans, India
2. Effect of Sodium Chloride concentration on growth of bacteria
3. To know about the protein expression profile of bacterial cell on NaCl stress condition
4. To explore any structural alteration in bacterial cell due to salt stress.

MATERIALS REQUIRED

a) Chemicals

1. Zobelle Marine Broth 2216 (Himedia)

2. Sodium Chloride

3. Phosphate buffer

4. SDS-PAGE chemicals

b) Bacterial Strain

Halobacillus sp. GSS1 (NCBI Accession No. MH298009)

c) Instrument

1. Sartorius BSA 2245-CW weighing machine/ common balance

2. Thermo Scientific Sorvall Legend X1R ultracentrifuge

3. Hielscher Ultrasound Technology UP200S ultrasonic processor (sonicator)

4. Tarsons Rockymax dancing shaker

5. Varian Cary 50 Bio UV-Visible spectrophotometer

6. Eppendorf Centrifuge 5415R microcentrifuge

METHODS

1. Sample collection Site and Isolation of Halobacteria

Soil samples were collected from 'Bonnie Camp', Sundarbans, India, and stored in refrigerator until their further use. Soil sample was serially diluted up to 10^{-6} dilution and spread on Zobelle Marine broth (ZMB) plate to isolate pure colony of bacteria. Four different colonies were isolated according to their colour and morphology. The colonies were kept in refrigerator as pure culture stocks for further use. Each culture was spread on ZMB agar plate at 5 different concentrations of NaCl (e.g.-0M, 1M, 2M, 3M & 4M). Of these, only one culture showed growth on the 4M NaCl plate. This strain was isolated and the organism was primarily characterized as a halobacterium, *Halobacillus* sp. GSS1, based on its morphological characteristics and microscopic observation. [5]

2. Molecular Identification

Isolated bacterial strain was grown on ZMB 2216 at 37^0C with shaking (120 rpm) for 5-7days. The biomass was separated from the culture medium by centrifugation at 10,000g for 10 min and washed thoroughly with deionized water and used for DNA extraction. Chromosomal DNA was extracted using MasterPureTM Bacterial DNA Purification Kit (EPICENTRER Biotechnologies, Madison, WI, USA) according to the instructed protocol.

The 16S rRNA gene was amplified using universal forward primer 27F and reverse primer 1492R. The PCR amplification was carried out for 35 cycles in a thermocycler (Gene AmpR PCR System 9700, Applied Biosystems, USA) using a thermal cyclic condition. After PCR was completed the product was purified using purification kit (High Pure PCR Product Purification Kit, Roche, Germany). 500 mL of binding buffer was added to each 100 mL PCR tube and mixed well. The sample was transferred to the upper reservoir of the filter tube and centrifuged at 13,000g. The filter tube was disconnected and the flowthrough solution was discarded. New filter tube was reconnected and same procedure was repeated twice with 500 mL and 200 mL wash buffer respectively. The flowthrough solution was discarded and reconnected the filter tube to a clean 1.5 ml microcentrifuge tube. 50 mL elution buffer was added to upper reservoir of filter tube and centrifuged for at 13,000g, to get the purified DNA. The purified product was subjected to electrophoresis in 1% (w/v) agarose gels. The purified amplicons were sequenced in both forward and reverse direction by using an automated DNA sequencer, Genetic Analyzer 3130 (Applied Biosystems, USA) using the Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, USA). The recovered 16S rRNA gene sequences were compared with known sequences from NCBI database by using BLAST algorithm to identify the most similar sequence for the identification of the isolate.[5]

3. Effect of Sodium Chloride concentration on growth of bacteria

The effect of NaCl concentration on growth of Halobacillus was studied by monitoring its growth in 50 ml ZMB medium containing 0, 1M, 2M, 3M, and 4M NaCl at 37^0C for 5-7 days. The growth was measured in terms of absorbance at 600 nm using UV-visible spectrophotometer. A growth curve was plotted with the data.[6]

4. Preparation of cell lysate for SDS-PAGE

The cell pellet obtained after centrifugation of the ZMB media for the previous experiment was washed with 10 ml distilled water and vortex. Then centrifugation was carried out as before. The washing process was repeated thrice. The centrifuged pellet was dissolved in 2 ml phosphate buffer, and then transferred to 1.5 ml centrifuge tubes. The samples were then sonicated till frothing (70 amplitude = 15 cycles approximately, 1 minute/cycle). The sonicated samples were centrifuged at 10,000 rpm for 10 minutes at 4^0C. The pellet was kept, supernatant was discarded. Then the tubes were kept in the refrigerator.

6. Protein Expression Profile by SDS-PAGE

10% polyacrylamide gel was prepared according to protocol. 40µl of refrigerated samples and 15µl of Bromophenol Blue dye were added, and boiled for 7-8 mins. Then they were centrifuged at 4,000 rpm for 1 min (approximately). Then sample were loaded in the gel wells. After gel run was complete, the gel was kept in a staining solution containing Coomassie Blue dye for overnight, then the destaining was done until the band was observed. Image was captured using Gel-Doc (Biorad) instruments.[7]

7. Bio-SEM

Bacterial cells were fixed with glutaraldehyde (1% solution) and paraformaldehyde (2%) buffered in phosphate buffer saline (0.1 M, pH 6.8) for 12-18 hrs at 4^0 C; washed and fixed for 2 hrs in osmium tetroxide (1%) in PBS at 4^0C. The fixed cells were smeared with poly-L-lysin for 30 mins under wet condition. The specimen was washed with phosphate buffer ; dehydrated in a series of ethanol-water solutions (30%, 50%, 70% and 90% ethanol, 5 mins each) and dried under a liquid CO_2 atmosphere for 20 mins. Mounting was performed on aluminium stubs, and the cells were coated with 90 A^0 thick gold-palladium coating in polar on Sc 7620 sputter coater (VG Microtech, UK) for 10 mins. Coated cells were viewed at 15kV with scanning electron microscope equipped with EDAX (FEI Quanta 200).[5, 8]

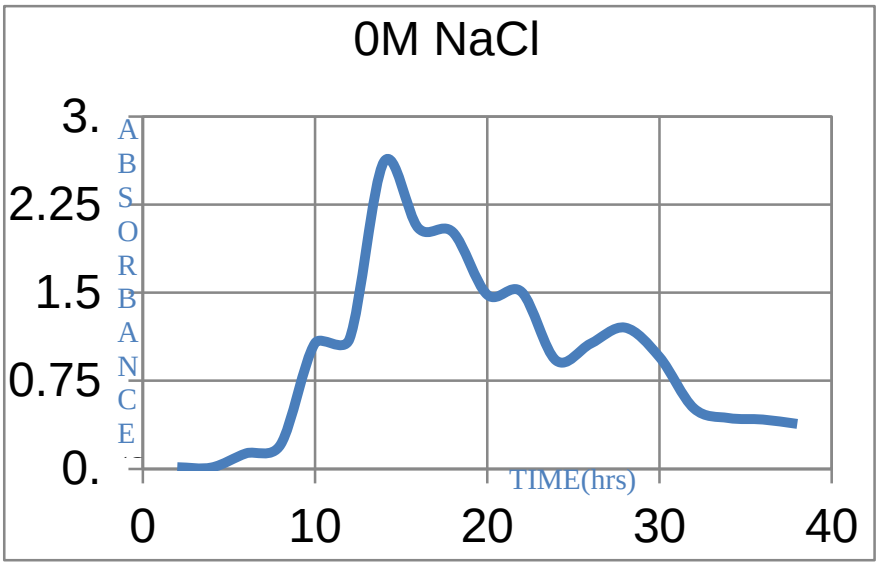
RESULTS & DISCUSSIONS:

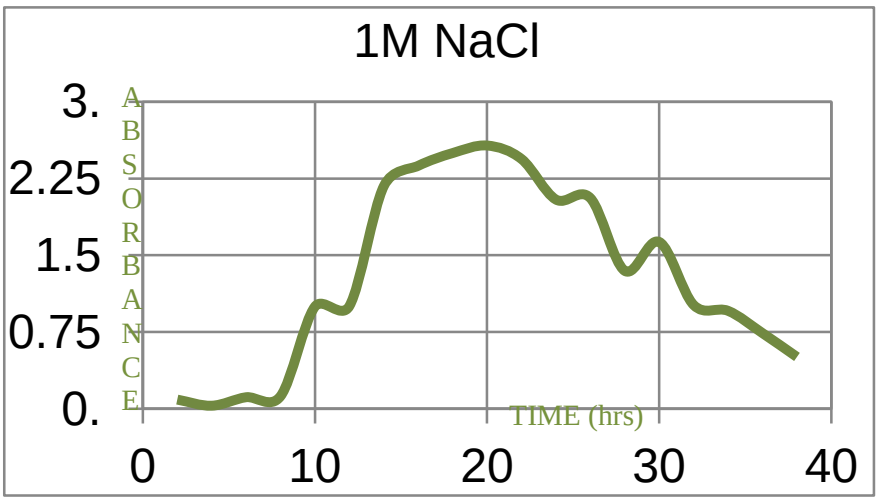
Identification of potential bacterial isolate

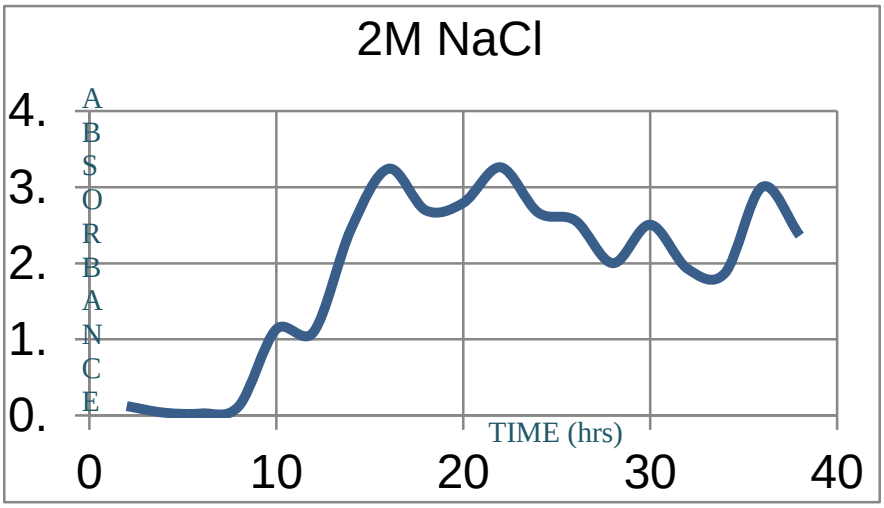
The bacterial cultures were isolated and purified from soil samples by single colony techniques using ZMB 2216. After getting the 16S rRNA gene sequence, the sequence was subjected to BLAST in NCBI database to identify the most similar sequence for the isolate, when 99% similarity was observed with the Halobacillus genus. Thus we inferred the inference the isolated strain GSS1 is belongs to the Halobacillus genus. Analysis of the 16S rRNA gene sequence was recognized by NCBI Sequence Viewer, accession number MH298009.

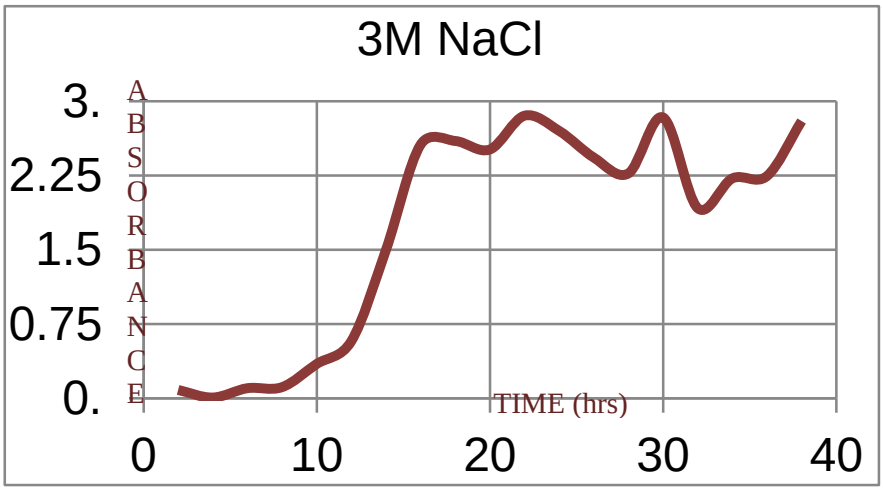
Absorbance and Growth Curve

The effect of different salinity concentrations in the range of 0M, 1M, 2M, 3M, and 4M NaCl on growth of *Halobacillus* sp. was studied and the results are depicted below. Fig 3. indicates the organism demonstrated extremely good growth in 1M and 2M NaCl within the first 24 hours, and in 3M and 4M, 48 hrs onwards. It is incapable of growth in medium without NaCl.









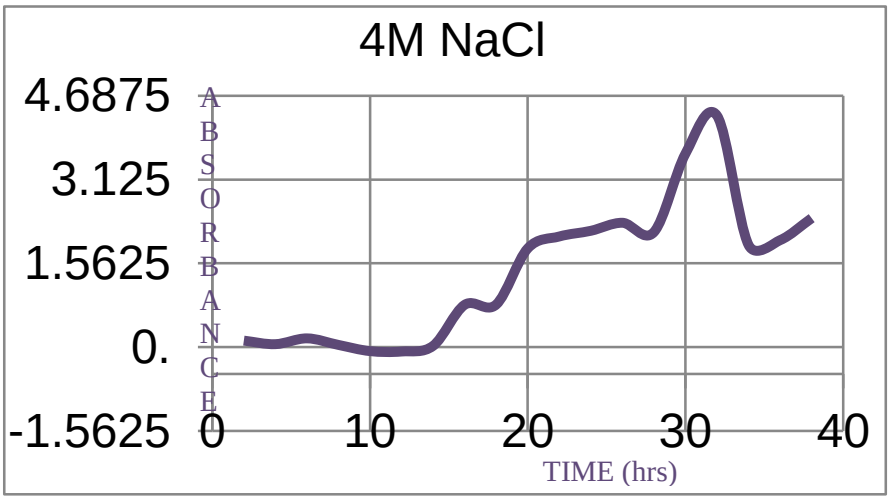


Figure 3: Growth curves of *Halobacillus* sp at 0, 1M, 2M, 3M, and 4M NaCl concentrations

Up-regulation of proteomic profile under NaCl stress

An overview of the proteome response profile of Halobacillus sp. GSS1 was explored on NaCl exposure at different concentration by carrying out 10% SDS PAGE analysis. The cell samples were taken from the mid-log growth phase of the test and also for control (0M) experiment, to get maximum viability of the cell to adapt NaCl stress. There is no such variation of protein expression observed in comparison with 0, 1M, 2M, & 4M (Fig.4). Interestingly ~12kDa protein was found in 3M NaCl treatment cells, may be due to the up-regulation of protein in response to NaCl stress. As the 3M NaCl is the ideal concentration for the optimum growth of isolated Halobacillus sp. strain, the up-regulated ~12kDa protein was secreted by bacteria in this favourable environment to keep metabolically active. However, the role of proteins in bacterial adaptation into salt stress is yet to be unfolded.

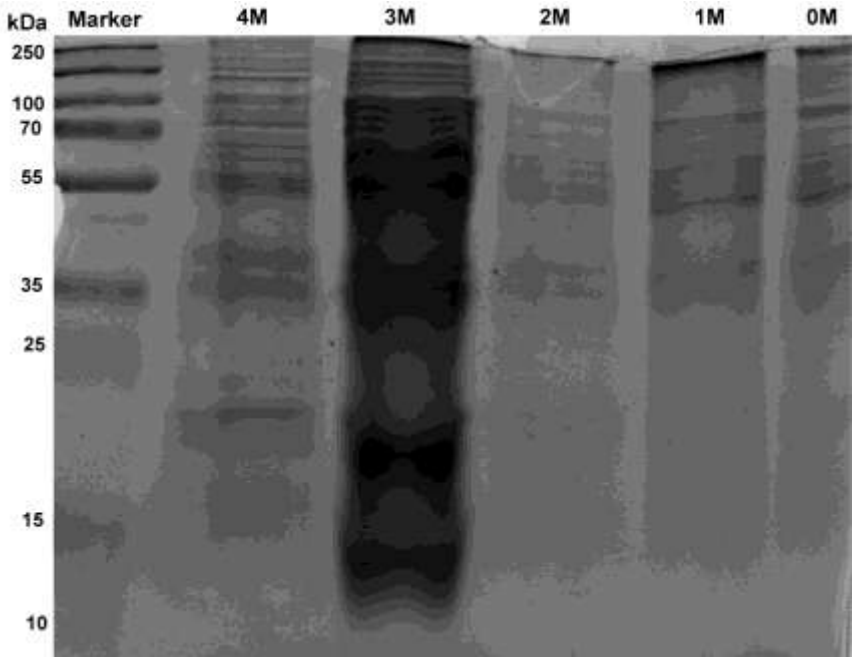
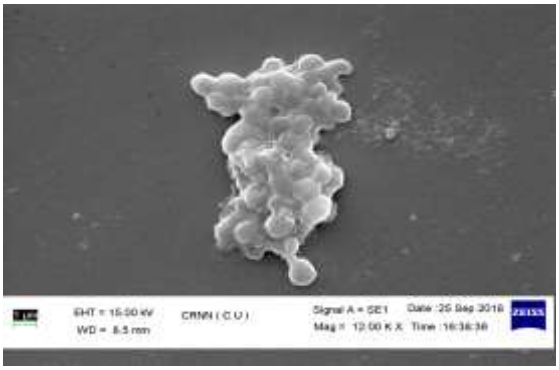


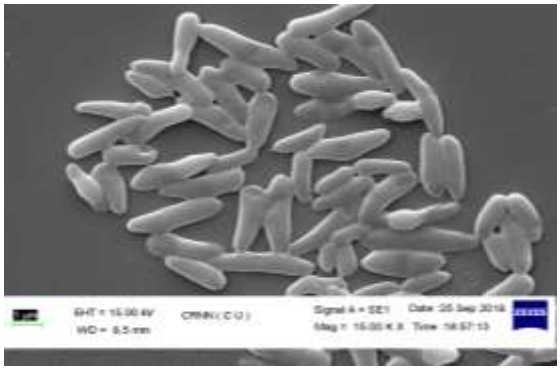
Figure 4: SDS-PAGE bands of protein expression profile from salt tolerant Bacterial isolate at the mid-log growth phase in different concentration of NaCl. Marker denotes protein maker.

SEM analysis

In 0M NaCl, the cells appear clumped due to their very short span of growth cycle. Within this growth phase, they have already entered into the death phase. In 1M and 2M NaCl, the cells appear healthy, indicating the salt concentration was favorable for bacteria for their growth. In 3M NaCl, some cells appear flattened and in 4M the cellular leakage occurred, due to high salt concentration.



(A)



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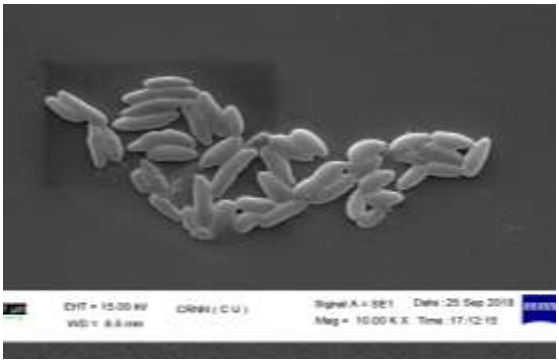

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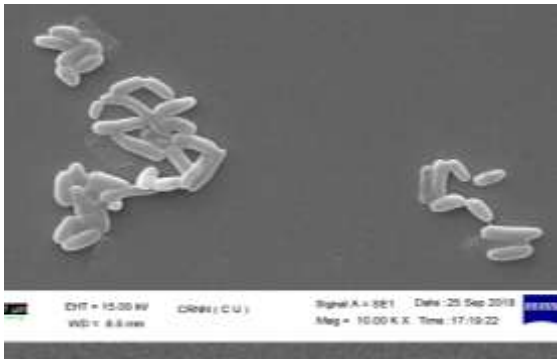

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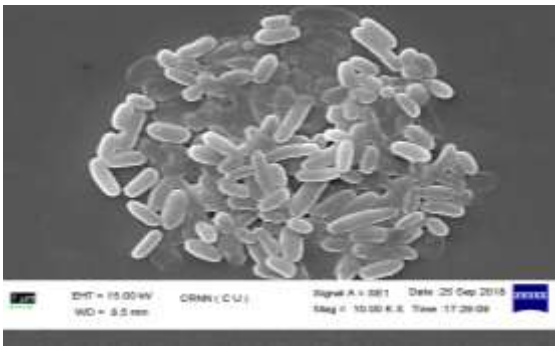

(E)

Figure 5: SEM pictures of bacterial cells in (A) 0M, (B) 1M, (C) 2M, (D) 3M, and (E) 4M NaCl concentrations

CONCLUSION

- The effect of different salinity concentrations in the range of 0, 1M, 2M, 3M, and 4M NaCl on growth of *Halobacillus* sp. was studied and it was observed that the organism demonstrated extremely good growth in 1M and 2M NaCl within the first 24 hours, and in 3M and 4M, 48 hrs onwards; it is incapable of growth in medium without NaCl
- In SDS-PAGE experiment, a ~12kDa protein was found in 3M NaCl treatment cells, which may be due to the up-regulation of protein in response to NaCl stress. As the 3M NaCl is the ideal concentration for the optimum growth of isolated Halobacillus sp. strain, the up-regulated ~12kDa protein was secreted by bacteria in this favourable environment to keep metabolically active
- From SEM analysis it was observed that inn 0M NaCl, the cells appear clumped due to their very short span of growth cycle. Within this growth phase, they have already entered into the death phase. In 1M and 2M NaCl, the cells appear healthy, indicating the salt concentration was favorable for bacteria for their growth. In 3M NaCl, some cells appear flattened and in 4M the cellular leakage occurred, due to high salt concentration.
- More experiments need to be performed to fully understand the osmoadaptive strategies of *Halobacillus* sp

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