Isolation And Characterization Of pH And Salinity-Responsive Microbes From Coastal Rhizosphere Soil of Patenga, Chittagong

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A thesis submitted to Department of Mathematics and Natural Science in partial fulfillment of the requirements for the Degree of Bachelor of Science in Microbiology

Department of Mathematics and Natural Science
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Declaration

It is hereby declared that:

1. The thesis submitted is my own original work while completing my degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The thesis titled, "Characterization of PH and Salinity-Dependent Growth response of Coastal microbe isolated from Patenga, Chittagong"

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Ethics Statement

This research is done under proper supervision and is the author's original work. The article is written in a manner that the write-up does not contain material previously published or written by a third party, except where this is appropriately cited through complete and accurate referencing. The study was conducted in BCSIR Chittagong laboratories on their R&D project for financial year 2024-2025.

Abstract

Coastal ecosystems have diverse microbial communities that play a crucial role in plant growth and environmental stress tolerance. An investigation carried out functional characterize indigenous bacteria from the random rhizosphere soil of Patenga region of Chittagong. A total of 19 bacterial isolates were obtained and screened for salinity tolerance at different NaCl concentrations (0%, 5%, 7.5%, 10%, and 15%), pH-dependent growth response in five pH conditions (4.5, 6, 8, 9, 10.5, and 12). Out of these isolates 10 isolated were Selected based on their growth response in high salt concentration and extreme pH condition and sodium uptake pattern. Among the 10 selected bacteria 4 show starch hydrolysis and 6 show ammonium production activity. Based on the pH and salinity tolerant and biochemical response of bacteria 3 bacteria selected observed molecular characterization using 16s sequencing the isolates identified as *Bacillus marisflavi, Exiguobacterium indicum, Aeromonas caviae.* Therefore, the research contributes to identifying bacteria that can adapt to, extreme salinity, or pH level

Keywords

Rhizosphere bacteria, salinity tolerance, sodium uptake, pH response, *Aeromonas caviae, Exiguobacterium indicum, Bacillus marisflavi*, Optical density, Concentration, stress tolerant.

Dedication

To My Parents

Acknowledgement

It gives me great pleasure in expressing a deep sense of gratitude and sincere appreciation to my thesis supervisor Associate Professor Dr. Fahim Kabir Monjurul Hoque for his enthusiastic guidance, constant encouragement and support. I would like to thank the leader of R&D project of BCSIR the scientific officer Anika Tasnim. Her valuable guidance throughout the study and giving me the opportunity to work in the project. Cordial thanks to Farhana Yeasmin, MS thesis students of BCSIR for her generous help and cooperation. I would like to extend my gratitude to the department of Microbiology of BCSIR for providing thesis opportunities to work on their project. Finally, I express my deepest sense of regards to my parents and husband for their blessings and for being the chief source of inspiration, encouragement, sacrifice and immense support to carry out this thesis work to completion. Also, I appreciate all the good wishes from my close friends and relatives.

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List of Acronyms

NA	Nutrient Agar
NaCl	Sodium Chloride
nm	Nanometer
PCR	Polymerase Chain Reaction
KB	Kilobasepair
BP	Base Pair
NCBI	National Centre of Biotechnology Information

Chapter 1 - Introduction

1.1 Exploration of Potential Microbes in Adverse Environment

A rhizosphere area within soil where plant roots grow contains numerous bacteria that develop important relationships with plants to improve both nutrients and plant wellness (Berg et al., 2014). Several rhizospheric bacterial strains show stress-tolerant abilities since these microorganisms survive harsh environmental conditions while. The salt-resistant plant species adapt well to wet coastal conditions thus their growing soil contains the best conditions for activating stress-tolerant microorganisms. A vast variety of microorganisms from various bacterial, fungal, and archaeal groups can be found in soil. Some microorganisms are now well known for their innate ability to support plant growth and withstand varying salt concentrations. Agriculture greatly benefits from these salt-tolerant

plant-beneficial microorganisms. They have demonstrated the ability to increase crop yields in dry and semiarid areas. (Cho & Azam,1988). Most of the microorganism's life is isolated from sunlight, our planet's primary energy source. Besides living in permanent darkness, organisms have to cope and adapt to the high pressure and low temperature that characterize this adverse ecosystem. This fascinating habitat represents one of the largest biomes on Earth, mostly occupied by bacteria and archaea that play a fundamental yet often overlooked role in shaping ecosystems and influencing agricultural productivity (Cho & Azam,1988). The exploration of microbial communities thriving in adverse environments, such as extreme temperatures, pressures, salinity, and pH levels, offers a window into their remarkable adaptability and resilience (Munns,2002)

Climate change is a significant concern impacting life on Earth, with effects on photosynthesis, root activity, overall morphology, and functionality of plant specimens, as well as their interactions (kirschbaum,2004). Concurrently, the characterization of microorganisms in ecologically dominant coastal areas unveils the intricate web of interactions that sustain biodiversity and ecosystem health.

1.2 Habitat of Microbes in Rhizospheric Soil

Mangrove forests are unique ecosystems in tropical and subtropical coasts, crucial for dike fixation, carbon storage, wind protection, biodiversity preservation, and saltwater purification. Due to intensive salinization and recurring tidal seawater immersion, mangrove soil has high salinity. Avicennia marina, the forerunner of mangroves, has exceptional salt tolerance and salt glands.(2) (Yang et al., 2023c). Most rhizospheric bacteria serve as plant-assisted microorganisms which enhance plant salt tolerance through improved growth and better nutrient acquisition functions (Ruppel et al., 2013).

1.3 Salinity and Plant Growth

Rapid climate change brought on by rising temperatures causes water to evaporate from the soil, increasing the concentration of salt in the soil and water resources, converting productive fields into arid areas across the globe (FAO 2015; Shrivastava and Kumar, 2015). According to Amin et al. (2016), salinity is one of the most detrimental environmental factors that reduces agricultural crop output, and it appears to be much more detrimental for agro-based nations like Bangladesh. About 20% of the world's irrigated areas and 2% of its dry lands are severely impacted by salinity, which affects about 6% of the land (FAO 2015; Ilangumaran and Smith, 2017). According to Amin et al. (2016), the effects of high salinity have been found for about 45 million hectares of all cultivable land worldwide. The soil salinity in Bangladesh's interior coastal regions has increased during the past forty years, rising from 2.96 mha in 2000 to 3.76 mha in 2007. Sea Level Rise (SLR), which impacts plant growth. In Bangladesh, almost 30% of arable land is located along the coast, with 53% of the land being impacted by varying salinities (Haque, 2006). A major threat to agriculture and food security, the salinity of soils has risen by an additional 20% (SRDI, 2010). More crop losses and significant stress on crop yields are caused by soil salinity than by any other limiting factor (Vaishnav et al., 2018). It has a number of effects on both direct and indirect disruptions to the majority of crops' growth and output. As soon as it is exposed, it induces osmotic stress, which alters water balance, cell turgor pressure, cell elongation, and cell division rates. After a few days, it alters ion homeostasis within the cell, which alters hormone levels, transpiration, photosynthesis, nutrient translocation, and other metabolic processes (Munns, 2002). According to Hasegawa et al. (2000), adaptations to restore ionic homeostasis are therefore necessary for plant life and growth. Another consequence of excessive salinity is hyperosmotic shock, which reduces cell turgor and chloroplast stromal volume while producing reactive oxygen. This is a major factor in the reduction of plant

growth and photosynthetic ability. Conversely, the three halophytes are randomly selected which can endure and thrive in environments with high salt. It is simple to see that a plant's adaptation to salt is a culmination of multiple metabolic changes, novel pathways, and altered expression levels given the variety of detrimental consequences of salinity on plants. Furthermore, plant-associated microorganisms are essential to a plant's ability to survive under stressful circumstances (Choudhary, 2012). Investigating halophytic plants' tolerance to stress like salt and pH variation.

Chapter 2 - Materials and Methods

The present investigation on characterization of salinity and different pH tolerant bacteria associated with rhizosphere soil from the coastal area Patenga, Chittagong.

2.1 General Procedures and Equipment

2.1.1 Sterilization

All equipment and glassware which had to be sterilized were autoclaved (CLG-322, GOV) at 121°C under 15 psi for 20 minutes. All the growth media and solutions were sterilized by the same conditions of autoclaving for 15 minutes. Fresh agar plates were prepared, dried at room temperature, and all inoculations were performed under aseptic conditions in a biosafety cabinet.

2.1.2 Preparation of Solutions

All the chemicals used for preparation of solutions were of analytical grade or guaranteed reagents (Merk, Roth, HiMedia, and others). Accurate weights of the components of various solutions were measured using a balance (Shimadzu ELB200, Japan) and for approximate weight a standard top pan laboratory balance was used. A pH meter (Hanna Instruments Edge, China) was used for determining pH values of different solutions and media. Media for cultivation of bacteria Various selective, non-selective and differential culture media were obtained commercially (Oxoid, HiMedia, Roth), and used for the growth and characterization of the isolates of rhizospheric soil.

2.1.3 Growth of Bacteria

For growth of bacteria in liquid media in shake flasks 37°C, a lateral shaking incubator (ShelLab, USA) was used. The inoculated solid and liquid culture media for static growth conditions were incubated at 37°C under aseptic conditions in an incubator (Memmert, Germany).

2.1.4 Maintenance of Cultures, Reagents and Solutions

Maintenance and preservation of different materials including microbial strains, heat labile chemicals, reagents, etc. were carried out in a refrigerator (Kelvinator, Sweden) at 4°C. The pure bacterial cultures were also kept in glycerol containing ampoule stocks at -80°C in the ultra-low maintaining freezer refrigerator (Telstar, Sapin) for long term preservation

2.2 Sampling and Isolation of Rhizosphere Bacteria

2.2.1 Collection of Samples

The sample was collected from random location of the rhizosphere soil of Chittagong local coastal sediments at Patenga. Soil collected from three spots on 10th October, 2024.



2.2.2 Isolation of Bacteria from Rhizosphere Soil

For isolating bacteria, a protocol as described by Anjum and Chandra (2015) was followed. Soil from three spots mixed (50 gm from each sample total 150 gm).

From that 150 gm soil, 1 gram separated and mixed with 9 ml saline to make 0.9% saline solution. Vortex for 1 minute, Diluted in two, three and four fold.

From three dilution factors, 10^-2,10^-3, 10^-4, 1 ml solution of each solution spread into three types of media, PCA with distilled water, PCA with Marine water, ZMA with distilled water. After incubation, bacterial colonies were differentiated on the basis of colonial characteristics. For isolation as pure culture, the bacterial isolates from the distinctly differentiated colonies were picked up from the culture plates and sub-cultured on fresh ZMA plates by streak-plate technique.

2.3 Morphological Characterization

2.3.1. Cultural Characteristics

The morphological study was performed on the basis of shape, elevation, texture, margin, color, size and pigmentation, etc. of colonies of endophytic bacteria grown on PCA medium and incubated at 37°C for 2-3 days.

2.3.2 Gram Staining

Gram staining was performed to differentiate bacterial isolates into Gram- positive and Gram-negative on the basis of growth in Selective McConkey agar medium.

2.3.3 Determination of the Salinity Dependent Growth Response of the Isolated Bacteria

Salt tolerance test was conducted by determining the growth of the isolated bacteria in different concentrations of sodium chloride in LB broth to observe the salt tolerance of those bacteria Sodium Chloride was added with LB broth in four different concentrations, they are 5%,7.5%,10%,15%. Inoculation of single colony from fresh culture in nutrient agar medium according to Macfarland standard in spectrophotometer. Then inoculate 100 microlitre in each broth and keep this at rotary shaker at 28°C in 150 rpm for 24 hours.

After incubation, 3 ml of uninoculated LB broth used to make zero the spectrophotometer to measure the optical density at wavelength 500nm.On the observation of bacterial growth under saline condition, the isolates were selected for further experiments.

2.3.4 Determination of Sodium Uptake Pattern of Isolated Bacteria

LB broth (10 mL) was supplemented with 5.0% (0.5 g), 7.5% (0.75 g) and 10.0% (1.0 g) NaCl. All the isolates were cultured in tubes in an incubator at 37°C. After 24 h of incubation the bacterial cells were harvested by centrifugation and the bacterial pellet was washed with sterile distilled water to remove the traces of medium. Washed pellets were digested overnight with 0.1 N HCl at room temperature. Samples were centrifuged and supernatant was taken for the estimation of sodium uptake by bacterial cells using flame photometer.

2.3.5 Determination of the pH Dependent Growth Response of the Isolated Bacteria

The pH of soil affects physical, chemical and biological characteristics of soil. pH under 7 considered as an acidic environment and pH above 7 considered as alkaline environment. The LB broth has pH range 8. To make the pH condition 4.5 and 6 as they are acidic conditions HCL added and measured with a pH pen tester. And to make pH condition 9, 10.5 and 12, NaOH has been added Bacteria isolated from pure culture and one loopful bacteria inoculated into LB broth then vortex the Test tube for mixing. Then measure the optical density of LB broth with inoculum with un-inoculated LB in 500 nm. If the OD value of inoculated LB broth is 0.1-0.28 nm then it would equal to mcfarland standard. Then 100 microlitre inoculate in all test tubes with different pH range and keep them in a rotary shaker at 28°C at 150 rpm for 24 hours to enrich the bacteria. After incubation the optical density is measured of the inoculums and observe the pH dependent growth response of bacterial isolates.

2.3.6 Physiological and Biochemical Characterization

Several physiological and biochemical tests were conducted to characterize and differentiate the isolated bacteria. All the tests were performed according to the procedures as described by Cappuccino and Welsh, 2017. Fresh cultured bacterial samples (18-hour culture) were used for the biochemical tests.

2.3.7 Starch Hydrolysis Test

This test was used to determine the ability of the isolated bacteria to utilize starch as carbon and energy source (Collins and Lyne, 1984). The medium was prepared with 1% starch and the agar plate was inoculated with the test bacteria using an inoculating loop by streaking in the plate. After incubation at 37°C for 24 hours, the plates were flooded with Gram's iodine solution and were left untouched for a few minutes. A clear zone observed around the inoculated area indicated starch hydrolysis, implying a positive reaction.

2.3.8 Citrate Utilization Test

The test was conducted to determine the ability of the isolated bacterial isolates utilization citrate as sole carbon source for metabolism with resulting alkalinity. Simmon's citrate agar (Appendix A.4) tube was inoculated with the test organisms by stabbing the butt and streaking the slant. The inoculated tube was then incubated at 37°C for up to 24 hours. A color change of the medium from forest-green to deep blue was observed, denoting alkalization.

2.3.9 Ammonium Production Test

The test was conducted to determine the ability of the isolated bacterial isolates to produce ammonium. Freshly cultured single loopfull bacterial colonies inoculate in alkaline peptone water and keep in rotary shaker for 48 hours at 30°C.After incubation 500µl nessler's reagent added color change observed.

2.4 Molecular Identification of Bacteria

2.4.1 Genomic DNA Extraction

A modified alkaline lysis method was applied for isolation of bacterial genomic DNA. The bacteria for study were cultured in NA plates in an incubator 37°C for 24 hours. A sterile inoculating-loop was used to pick a small colony of the bacterium from each plate and transferred to labelled Eppendorf tubes containing 400µl of TE buffer vortex for 15 seconds. The tube centrifuge at 13000 rpm for 10 minutes at 25°C.The supernatant discard and again 400µl TE buffer added to the pellet in eppendorf tubes and vortex for 10 minutes and put them in heat block for 10 minutes at 100°C.After the time it kept in room temperature for 5-10 minutes. After that centrifuge it at 13000 rpm for 10 minutes at 25°C.Then collect 200µl supernatant into another sterilized eppendorf where DNA present which use as a template of PCR

2.4.2 PCR Amplification of 16S rRNA Gene

A pair of universal primers was used to amplify the extracted 16S rDNA through Polymerase chain reaction.

These primers are:

27F 5'- AGAGTTTGATCCTGGCTCAG- 3'

1492R 5'- GGTTACCTTGTTACGACTT- 3’

The PCR was conducted using a thermo-cycler machine.

Table 1: PCR Reaction Mix

Components	**Volume**
Master mix (Thermo Scientific DreamTaq Green PCR Master Mix 2x**)**	12µl
Nuclease free water	5µl
DNA Template	4µl
Forward primer (10mM) 1µl FP	2µl
Reverse primer (10mM) 1µl RP	2µl
Total	25µl

Table 2: PCR Cycle Time frame

Primer	**Initial Denaturation (1x)**	**Denaturation (30x)**	**Annealing (30x)**	**Extension (30X)**	**Final Extension (1x)**	**Hold (∞)**
27F **1492R**	96°C 4min	94°C 30 sec	57°C 30 sec	72°C 1min	72°C 10min	4°C

2.4.3 Agarose Gel Electrophoresis

The quality and quantity of the genomic DNA was checked on agarose gel electrophoresis. The 50X TAE buffer was prepared by adding 2 ml 50X TAE buffer with 98 ml distilled water. 1% agarose added and melting in a microwave oven. After cooling 4µL ethidium bromide was added in the solution and poured into a casting tray containing a sample comb and allowed to solidify at room temperature. Quality of DNA obtained was checked by loading 5 µl DNA on 1% agarose gel at the well and 1KB DNA ladder used to observe the band. The gel on the tray was placed horizontally into the electrophoresis chamber and flooded the top of the gel with a fresh running buffer (TAE) to cover the gel to a depth of about 1 mm. The solution (DNA sample with dye) was gently expelled into the well using a micropipette and 100 V current was applied for 45 minutes. After that the gel was carried and viewed in the UV trans illuminator.

2.4.4 DNA sequencing for identification of bacterial isolates

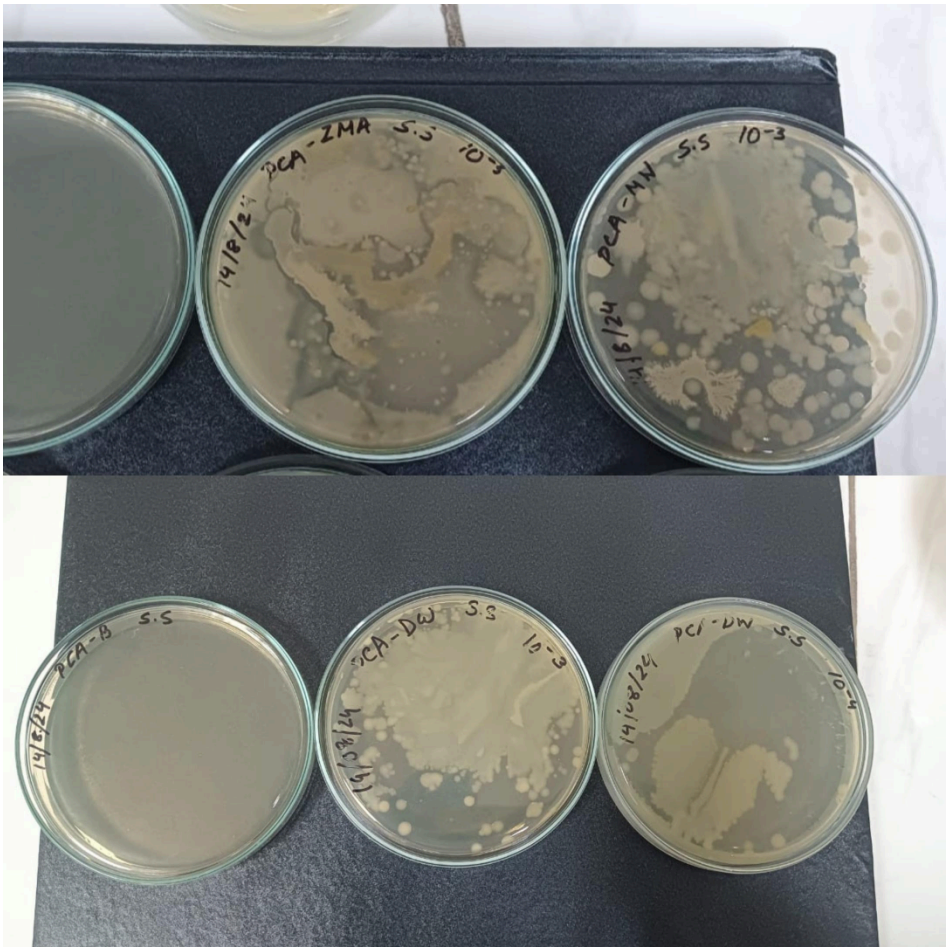
The amplified PCR products were sent to Child Health Research Foundation (CHRF), Dhaka for sequencing. Nucleotide sequencing was done by Sanger dideoxy sequencing method. After receiving the sequences in FASTA format, the online NCBI nucleotide BLAST tool (blast n) was used to scan the NCBI database for possible matches of the 16S rDNA partial query sequence. The BLAST-entries with the maximum blast scores (highly similar sequences) for both primers (F27 and R1492) were selected for identification of the target microorganism. Only entries with an E value of 0.0 and query coverage above 90% were considered for this purpose.

Chapter 3 - Results

The study was conducted to determine the stress tolerance of diverse bacterial isolates associated with rhizosphere soil in coastal areas.

3.1 Isolation of Bacteria from Rhizosphere soil

A total of 19 bacterial isolates were obtained from the soil collected from Patenga,



Chittagong.

Table 3: Isolated Different Bacteria from Soil Sample

Location	IDs of Isolated Bacteria
Rhizosphere Soil at Patenga, Chittagong	A1, A2, A3, A4, A5, A6 B1, B2, B3 C1 D1, D2, D3, D4, D5, D7, D8 E1

Table 4: Morphological Characteristics and Gram Staining of the Bacterial Isolates

ID of Isolates	Gram Staining	Colony Morphology		
		Size	Shape	Color
A1	Positive (+)	Medium	Circular Flat Round Ring round	White
A2	Negative (-)	Large	Rhizoid Irregular Flat	White
A3	Negative (-)	Large	Rhizoid Irregular Flat	White
A4	Negative (-)	Large	Irregular moist flat	Orange
A5	Negative (-)	Large	Round	Transparent
A6	Positive (+)	Large	Flat	White
B1	Negative (-)	Mucoid Large	Round	Milky white
B2	Negative (-)	Medium	Round Flat	White
B3	Negative (-)	Long	Flat	White
C1	Negative (-)	Small	Long Rhizoid Flat	White
D1	Positive (+)	Small	Round Flat	Orange
D2	Positive (+)	Medium	Round	Pink
D3	Negative (-)	Small	Round	Transparent

D4	Positive (+)	Large	Round Flat	Yellow
D5	Positive (+)	Small	Irregular	Yellow
D6	Positive (+)	Medium	Round	Transparent orange dot in middle
D7	Positive (+)	Small	Round Flat	Yellow
D8	Positive (+)	Small		Off-white
E1	Negative (-)	Large	Irregular	White

3.2 Screening the salinity dependent growth response of bacterial Isolates

All the bacterial isolates were screened to detect high salinity tolerant bacteria by inoculate the bacteria in different concentrations of NaCl with LB broth.

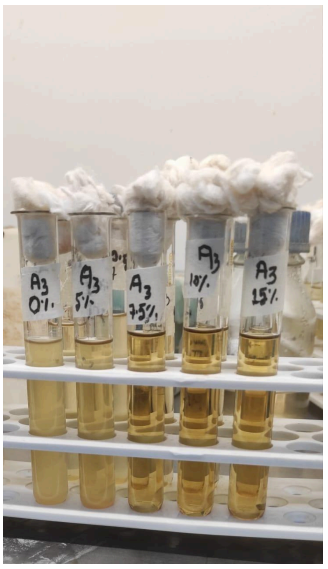


Figure 4: Salinity Test

Table 5: OD Value of Inoculum Under Different Salt Concentration

ID of Isolates	OD value of the following Concentration in 500nm wavelength				
	0%	5%	7.50%	10%	15%
A1	0.7848	0.2346	0.1784	0.2083	0.0196
A2	0.9728	0.1911	0.1038	0.1925	-0.0012
A3	1.0978	1.0978	1.987	-0.7458	0.0139
A4	0.6761	0.4237	0.0544	0.089	0.0195
A5	0.5779	0.2518	0.079	0.0144	-0.007
A6	1.3481	0.2585	0.1152	0.0308	-0.0012
B1	0.9751	0.0168	0.0169	0.0065	0.0115
B2	1.4173	0.1282	0.002	0.0145	0.0002
B3	0.247	0.1156	0.0225	0.0181	0.0063
C1	1.0803	0.529	0.1128	0.409	0.0014

D1	1.4442	0.0229	0.0323	0.0164	0.0073
D2	1.1279	0.0394	0.0102	0.0076	-0.0036
D3	0.8958	0.0595	0.1056	0.0256	-0.0003
D4	0.0082	0.4922	0.4794	0.3241	-0.0092
D5	0.7892	0.0394	0.0102	0.0076	-0.0036
D6	0.9313	0.4167	0.435	0.3036	0.2674
D7	0.2938	0.3406	0.3226	0.5127	-0.0139
D8	0.343	0.2192	0.0234	0.0245	-0.0158
E1	1.6304	0.0646	0.0188	0.0212	0.0357

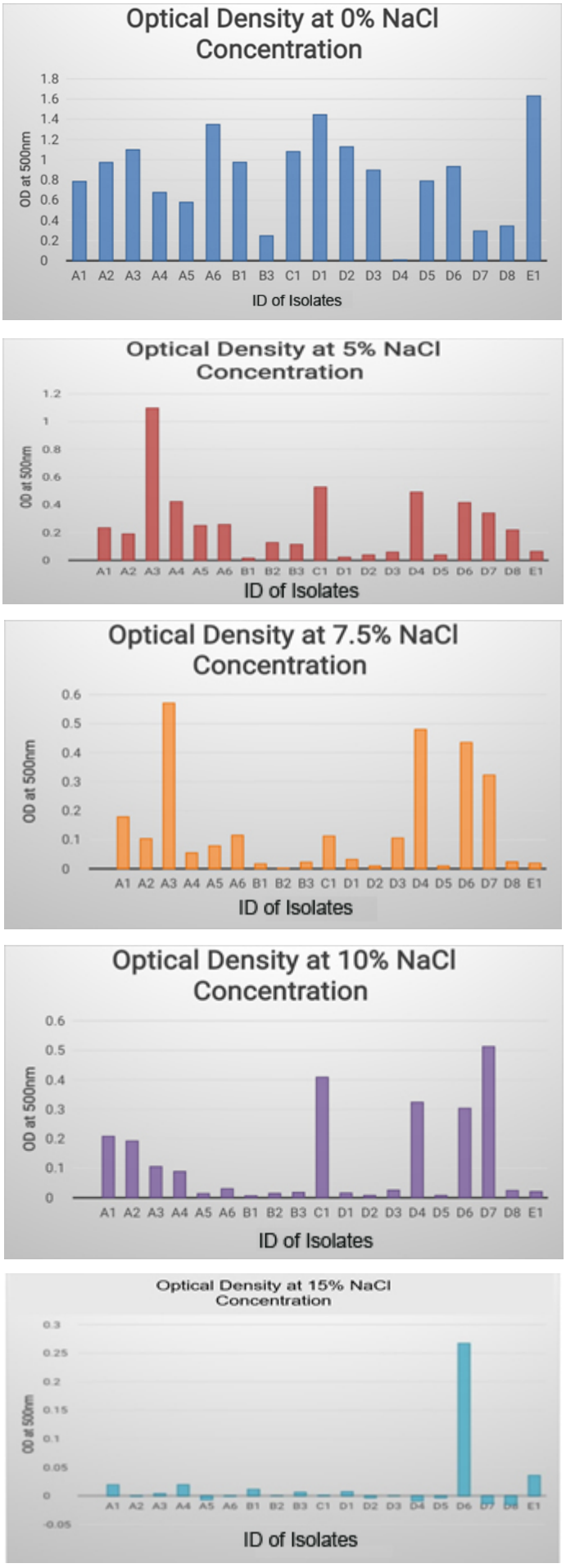


Figure 5: Analysis of Bacterial Growth Response under Different Salt Stress

3.3 Screening the Sodium uptake pattern of isolated bacteria

All the bacterial isolates were screened to absorbance rate of sodium according to its concentration. Bacteria inoculate in different concentrations of NaCl with LB broth

Table 6: Na Uptake Pattern of Bacterial Isolates

Name of Bacteria	Na Concentration	Abs	Concentration mg/L
B1	Na 5% B1	0.227	3.405
	Na 7.5% B1	0.516	5.16
	Na 10%B1	0.57	14.25
	Na 15%B1	0.17	17
D6	Na5% D6	0.305	10.56
	Na 7.5%D6	0.349	16.9695
	Na 10% D6	0.236	7.08
	Na 15%D6	0.782	27.37
A5	Na 5%A5	0.172	41.373
	Na 7.5%A5	0.299	26.9865
	Na 10% A5	0.855	41.1444
	Na 15%A5	0.455	32.8617
A1	Na 5%A1	0.322	15.471
	Na 7.5%A1	0.566	40.8348
	Na 10%A1	0.409	29.4975
	Na 15%A1	0.69	49.7718
D8	Na 5%D8	0.258	18.62271
	Na 7.5%D8	0.357	25.7445
	Na 10%D8	0.515	37.2006
	Na 15%D8	0.968	69.9111
D7	Na 5%D7	0.168	14.0019
	Na 10%D7	0.686	57.5652
	Na 15%D7	0.676	56.7732

D1	Na 5%D1	0.384	49.6551
	Na 7.5%D1	0.052	55
	Na 10%D1	0.04	67.749
	Na 15% D1	0.082	73.15
A6	Na 5%A6	0.532	6.1889 CU
	Na 7.5%A6	0.523	66.9922
	Na 10%A6	0.495	

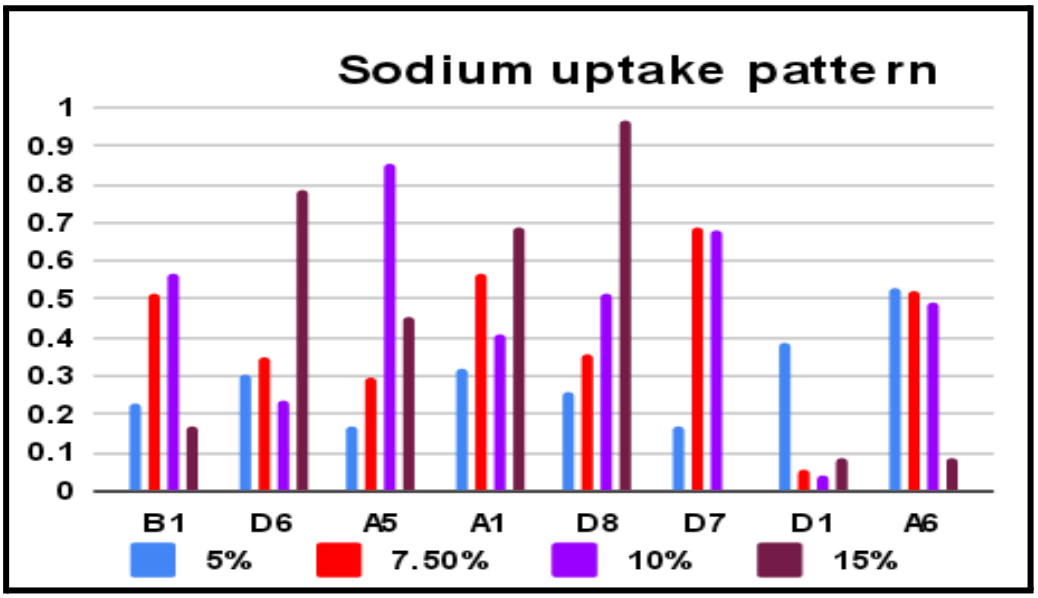
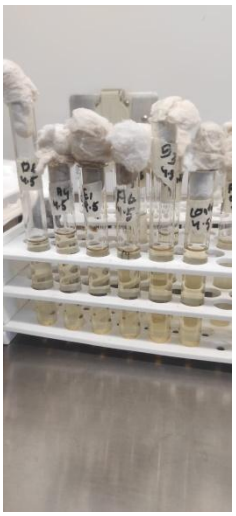
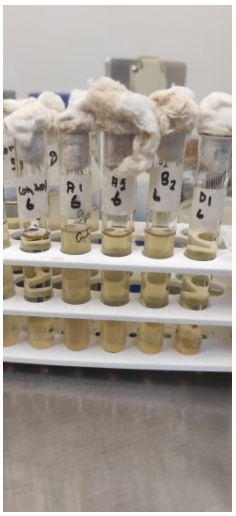


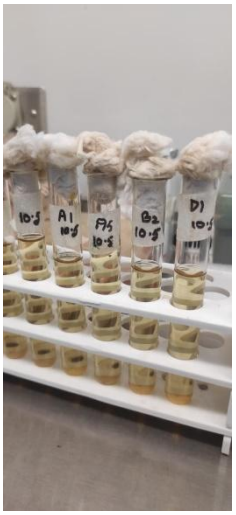
Figure SEQ Figure * ARABIC 6: Analysis of Sodium Uptake Pattern

3.4 Screening the pH-Dependent Growth Response of Bacterial Isolates

All the bacterial isolates were screened to detect pH dependent growth response by adding Sodium hydroxide (NaOH) and Hydrochloric acid HCl to change the pH of LB broth as pH range of LB broth is 8.







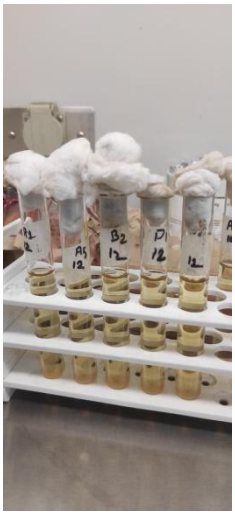


Table 7: OD Value of Inoculum Under pH Variation

Name of Bacteria	OD value of the growth response of following pH range in 500nm					
	pH- 4.5	pH-6	pH-8	pH-9	pH-10.5	PH-12
A1	0.0003	0.7569	0.7848	1.014	-0.0035	0.0001
A3	0.0033	0.761	0.735	0.5326	0.8448	0.065
A4	0.0032	0.2326	0.86	0.7134	-0.0122	-0.139
A5	0.0018	0.197	0.5779	0.9142	0.0326	0.0024
A6	0.0022	0.8665	0.5597	0.5257	0.0929	-0.1197
B1	0.0007	1.19	0.7897	1.1755	0.0687	0.0228
B2	-0.0014	0.3227	0.426	0.3342	-0.0013	-0.0024
B3	0.0034	0.2733	0.8257	0.6855	0.0111	-0.1276
C1	0	1.2094	1.0921	1.1672	0.0273	-0.0226
D1	0.0118	-0.0029	0.2579	0.1254	-0.002	0.0012
D2	0.006	1.0126	0.9583	1.0929	0.0331	0.0126
D3	0.0007	1.25	0.8958	1.1644	0.1929	0.026
D4	-0.003	0.3755	0.0082	0.3872	0.1791	0.0865
D5	-0.009	0.137	0.0363	0.182	0.0918	0.1777
D6	0.0003	-0.0034	0.01	0.0823	0.0146	-0.128
D7	0.0006	0.5263	0.2938	0.2512	0.5064	0.0056
D8	0.0028	0.1174	0.343	0.6143	0.2886	0.0152
E1	0.0032	0.2326	0.86	0.7134	-0.0122	-0.139

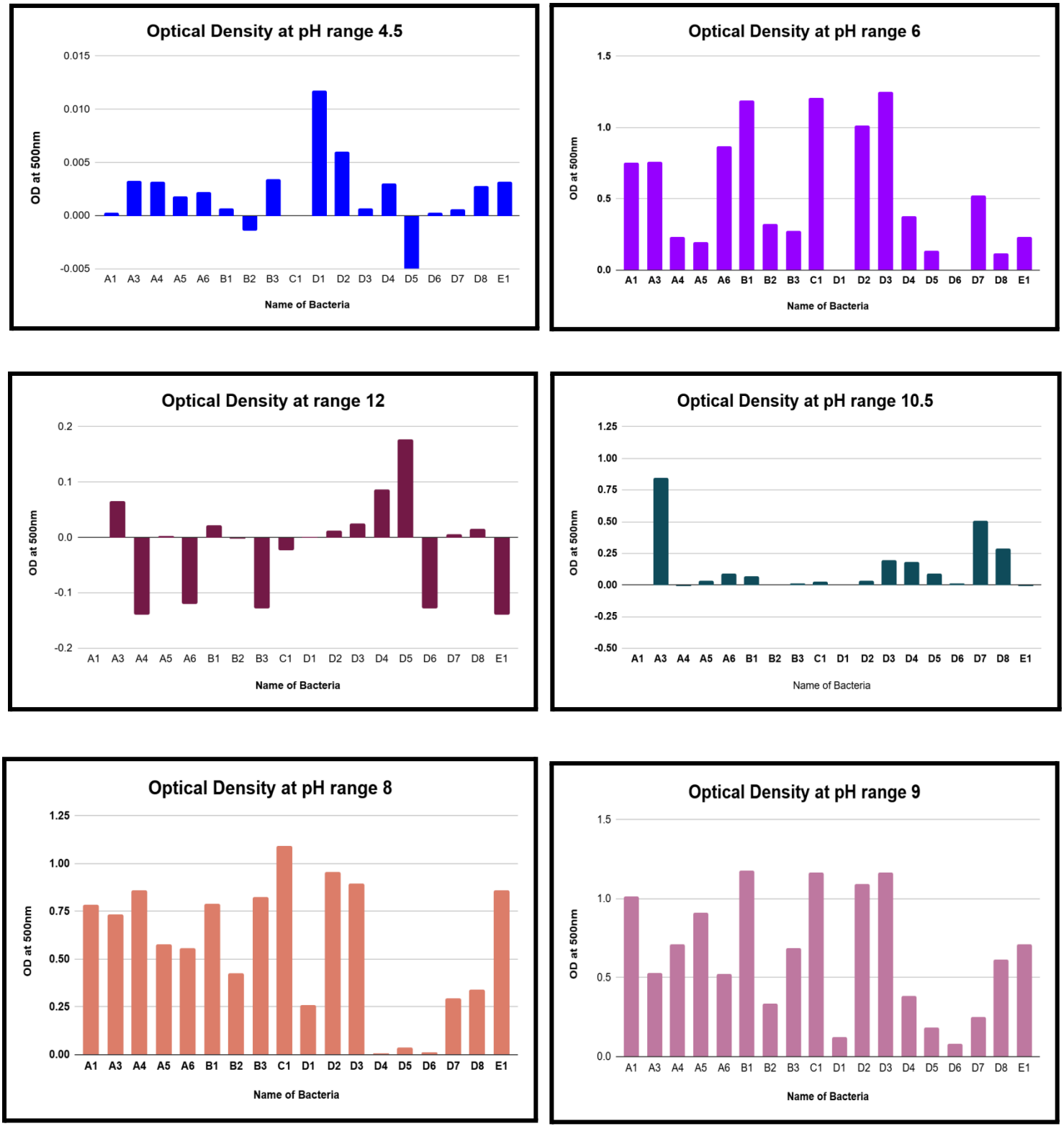
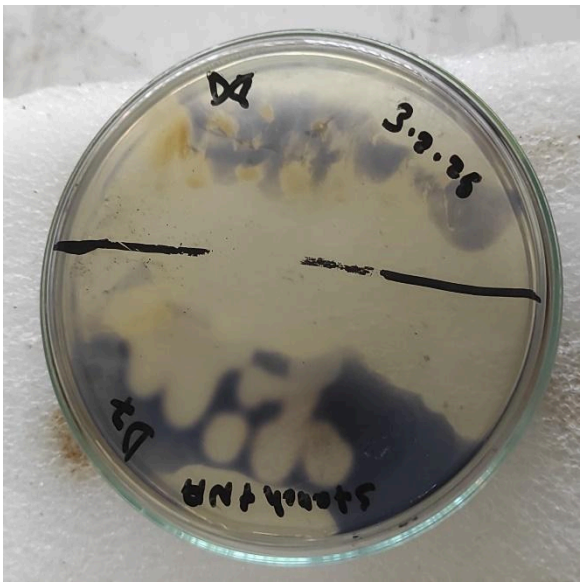
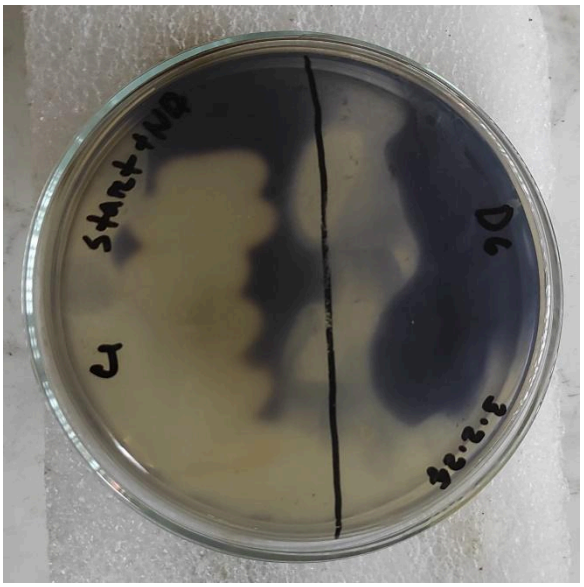


Figure 8: Analysis of Bacterial Growth Response under different pH Range

3.5 Biochemical Characteristics of the Bacterial Isolates

3.5.1 Starch Hydrolysis





A1, C1, D7, D8 these four bacteria can hydrolyze starch. Here the clear zone around the bacterial colony shows positive results and no zone shows negative results.

3.5.2 Ammonium Production

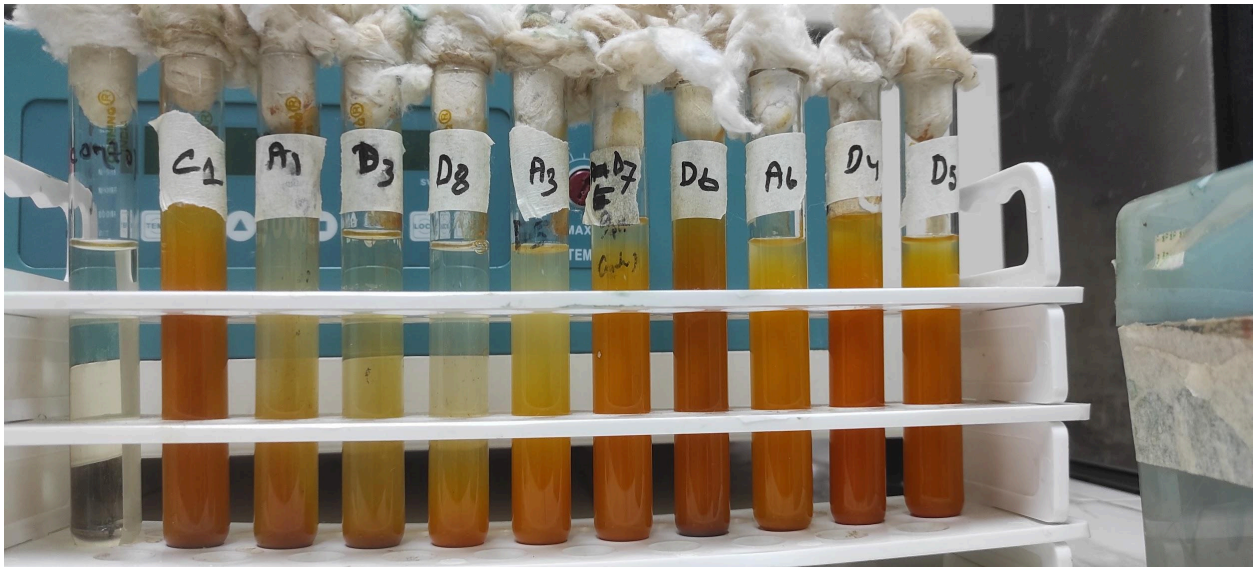


Figure 10: Ammonium Production

C1, D7, D6, A6, D4, D5 these six bacteria can produce ammonium. The color change from yellow to red indicates ammonium production

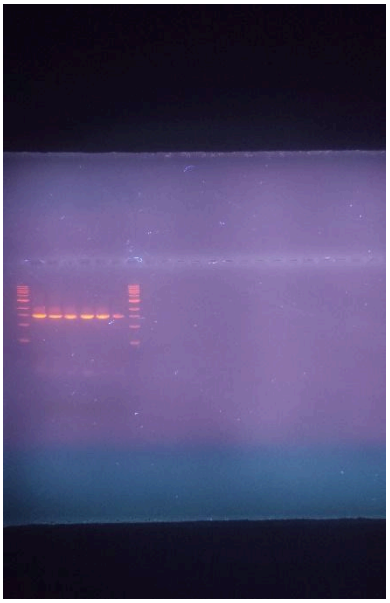
3.6 Molecular Identification of the Bacterial isolates

Based on 16S rRNA Sequencing among the three bacterial isolates, it was possible to identify the genera and species, through sequencing of their 16S rRNA genes and through BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/) using NCBI database. The isolated bacterial colonies were amplified by universal 16S rRNA specific primers referred to in Chapter-2 Sladder.

The amplified 16S rRNA genes were sequenced to identify the bacterial colonies. All the sequencing results were analyzed with the database of NCBI using the NCBI BLAST TOOL (blastn).

3.6.1 PCR Amplification of 16S rRNA Gene

Genomic DNA of all three bacterial isolates were extracted using a modified alkaline lysis method. For molecular identification of bacterial isolates, PCR reaction was performed and the product was evaluated in 1% agarose gel to confirm the size of the PCR products. Gel electrophoresis analysis revealed that the size of the PCR product was approximately 1500 when compared with the 1kb DNA ladder



3.6.2 Analysis of sequencing results through BLAST

The BLAST algorithm was used to search publicly accessible nucleotide databases for similar sequences. The sequencing results of 16S rRNA gene of unknown bacteria were analysed using the online Nucleotide BLAST tool (blastn) of National Center for Biotechnology Information (NCBI). All the identified strains had a sequence query coverage of 98% and above, while having an E value of 0.0. The sequencing results for molecular identification of the 3 bacterial isolates up to species levels are presented in Table -.

These results indicate that the identified bacteria belong to different bacterial species.

The identified diverse bacterial endophytes were:

C1-*Aeromonas caviae*

D4- *Bacillus marisflavi*

D7- Exiguobacterium indicum

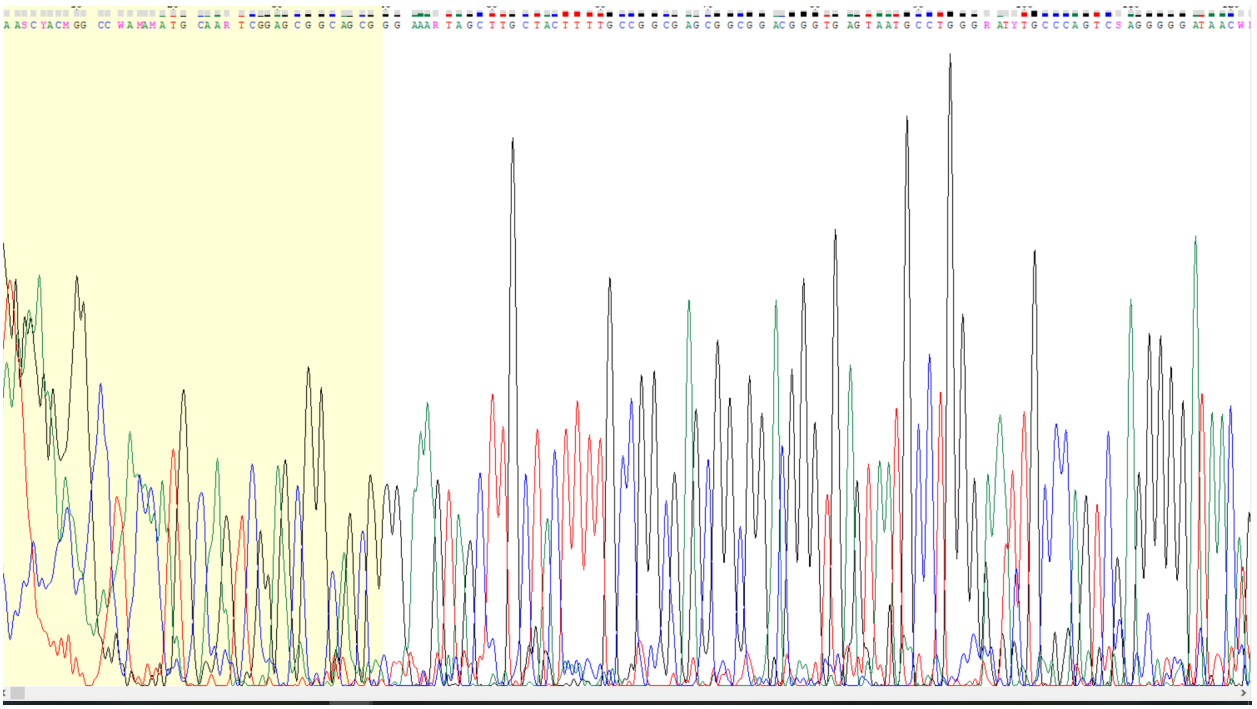


Figure 12: Alignment in Chromas of Bacteria C1

Figure 13: Alignment in Chromas of Bacteria D4

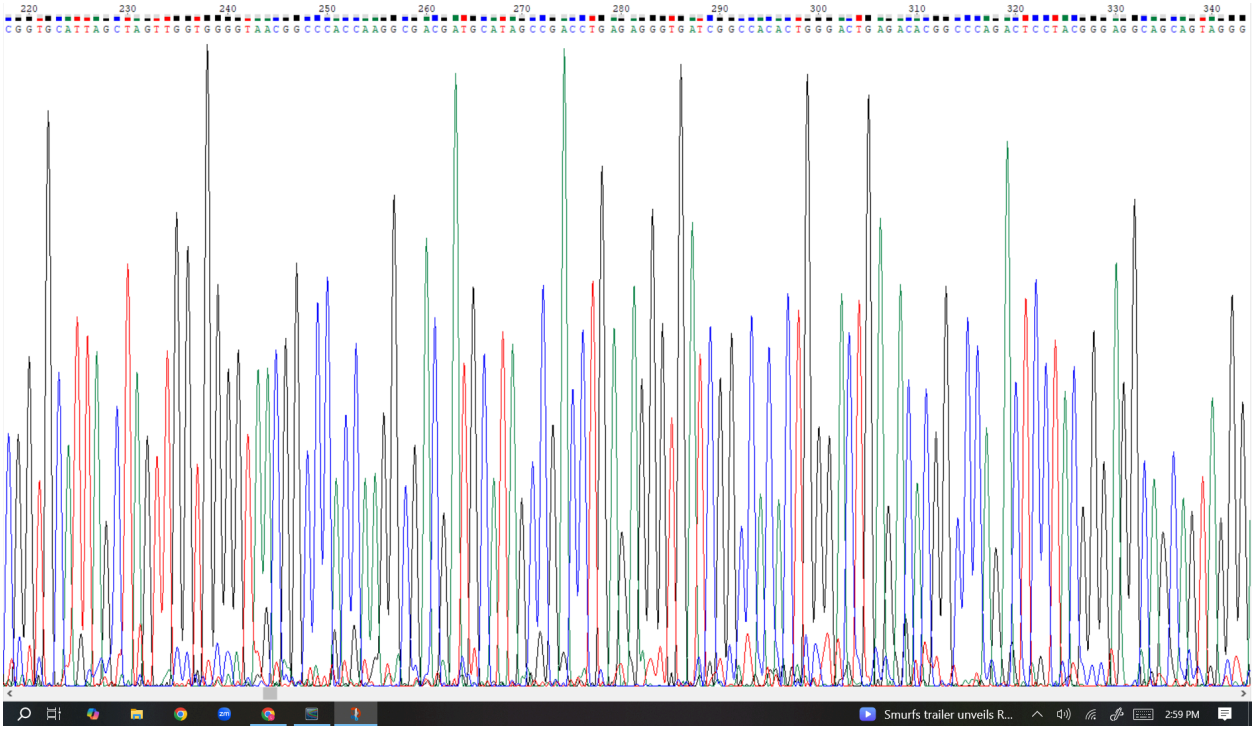


Figure 14: Alignment in Chromas of Bacteria D7

```
>L1
GGAAARTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGRATY
TGCCCAGTCSAGGGGGATAACWRYTGGAAACGRCTGCTAATACCGCATACGCCCTACGGG
GGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATRTGCCCAGGTGGGATTAGCTAGT
TGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAA
GCACTTTCAGCGAGGAGGAAAGGTCRGTAGCTAATATCTGCYGGCTGTGACGTTACTCGC
AGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTT
AATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCC
CCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGG
TAGAATTCCAGGTGTAGCGGTGAAATGCGTAG
```

Figure 15: Sequence of C1 in FASTA format

>L2
GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGGATAACACCTACCCCCGCATGGGGGAAGGTTGAAAGGYGGCTTCGGCTATCACTTA
CAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGSGACGATG
CGTASCCRACCTGARAGGGTGATCGGCCACACTGGGACTGARACACGGCCCARACTCCTA
CGGGAGGCAGCAKTAGGGAATCTTCCSCAATGGACGAAAGTCTGACGGARCAACGCCGCG
TGAGTGAARAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCCGTTC
KRATAGGGCGGCGCCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACRTGCCAGCA
GCCGCGGTAATACKTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCA
GGTGGTTTCTTAAGTYTGATGTGAAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAA
CTGGGGAACTTGAGTGCAGAARAGGAAAGTGGAWTTCCMAGTGTAGCGGTGAAATGCGTA
GATATTTTGGAGGAAACACCAGTGGCGAAGGCGACTTTTCTGG

Figure 16: Sequence of D4 in FASTA format

>L3
CTCTTCGGAGGGAAGGCAGCGGAATGAGCGGCGGACGGGTGAGTAACACGTAAGGAACCT
GCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGATAGTTCATCGGACC
GCATGGTCCGYTGATGAAAGGCGCTYCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTA
GCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGA
TCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATC
TTCCACAATGGACGAAAGTCTGATGGAGCAACGCCMCGTGAGTGATGAAGGTTTTCGGAT
CGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGGGAATGCTCGTACCTTGACGGT
ACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTRAGTCTGATGTG
AAAGCCCCCGGCTCAACCGGGGAGGGSCATTGGAAACTGGAAGGCTTGAGTACAGAAGAG
AAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGC
GAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAA

Figure 17: Sequence of D7 in FASTA format

Table 8: Result of NCBI Blastn

Name of Bacteria	Present Identification	E value
Aeromonas caviae(C1)	98.10%	0.0
Bacillus marisflavi(D4)	96.58%	0.0
Exigobacterium indicum(D7)	99.29%	0.0

Chapter 4 – Discussion

The study obtained three bacterial strains from coastal rhizosphere soil at Patenga in Chittagong named Aeromonas caviae (C1), Exiguobacterium indicum (D7) and Bacillus marisflavi (D4). The bacteria maintained resistance in environments containing different combinations of salt and acidity levels while showing biochemical properties that supported plant development. Research proves that these resilient bacterial strains demonstrate strong agricultural potential because they can survive in salinity-affected coastal regions. The research of microbial communities in such soils provides potential sustainable methods to improve both plant growth and restore soil fertility in degraded lands.

Plant growth continues to be restricted by salinity stress which becomes particularly prominent in coastal environments because soil sodium accumulation causes water use and nutrient intake difficulties (Shrivastava & Kumar, 2015). The salt-tolerant characteristics demonstrated by Exiguobacterium indicum (D7) and Bacillus manisflavi (D4) during NaCl tests up to 15% indicate their capacity to enhance soil productivity in hale environments. Experimentations have shown that salt-tolerant bacteria improve plant growth efficiency through exopolysaccharides that manage soil aggregation and trap sodium ions thus decreasing salinity toxicity (Mapelli et al., 2013). Extreme environmental conditions pose no challenge to Exiguobacterium indicum which retains its capacity to contribute to bioremediation while thriving in harsh environments (Vishnivetskaya et al., 2009).

Soil pH serves as a vital element that controls how microbes survive together with their functional capabilities in the environment. At this wide pH scale from 4.5 to 12 the isolates exhibited noticeable growth responses where Bacillus manisflavi (D4) and Exiguobacterium indicum (D7) showcased outstanding resilience. These microorganisms can operate at

different pH levels which makes them potential candidates as biofertilizers for using in environments with acidic and alkaline soils according to Rousk et al. (2010). Soil nutrient availability suffers from extreme pH but bacteria that thrive in various pH levels substantially improve both the soil environment-stability and nutrient cycling systems (Tripathi et al., 2007). These strains show strong potential for sustainable agricultural use because they have proven resistance against environmental pH changes.

The biochemical tests demonstrated that the chosen bacterial strains could bring potential agricultural advantages. Aeromonas caviae (C1) together with Exiguobacterium indicum (D7) and Bacillus manisflavi (D4) generate ammonium thus contributing to soil nitrogen enrichment that supports plant nutrition requirements (Dobbelaere et al., 2003). The important function of ammonium-producing bacteria in nitrogen cycling involves their transformation of organic matter into usable bioavailable nitrogen which supports plants growing in nutrient-poor soils (Hayat et al., 2010). Aeromonas caviae (C1) together with Exiguobacterium indicum (D7) displays starch hydrolysis ability that supports their participation in organic matter decomposition leading to better soil fertility (Behera & Ray, 2021). Soil microbial ecology benefits from the starch hydrolysis ability because this capability helps microbes convert hard-to-digest carbohydrates into simpler compounds that plants together with other microbes can better use.

The research shows that these bacterial isolates play a vital role in increasing plant tolerance against stress situations. The bacteria demonstrate excellent survival in harsh soil conditions of extreme salinity and pH which establishes their potential to serve as bio fertilizer and bioremediation agents. Further research should conduct in-field experiments to prove their capability in enhancing plant growth within changing soil salinity conditions and inconstant pH levels of agricultural fields. The research can advance with additional molecular and

metabolomics studies to reveal how genetics and metabolism contributes to their stressful environment adaptations as well as their plant helpful properties. Thorough comprehension of these mechanisms stands essential to reach the best possible outcomes when deploying them for sustainable agricultural purposes.

Chapter 5 – Limitations, Prospects and Conclusion

Limitations and Prospects

A fundamental limitation of this study analyzed microbial diversity from a single location which may not fully represent the broader microbial diversity in coastal soil. A larger sampling area could yield more diverse and functionally distinct bacterial strains (He et al.,2022)

While the study identified salinity and pH tolerant bacteria it did not explore their mechanism of stress tolerance such as specific gene expression metabolic Pathways or enzyme activity in a stress adaptation (Munns,2022).

The study focused on starch hydrolysis and ammonium production but other important plans growth promoting trade suggest phosphate solubilization siderophore production were not evaluated

Conclusion

The study successfully isolated and characterized PH and salinity responsive bacteria from the coastal rhizosphere soil Patenga, Chittagong. Three bacterial strains where molecularly identified as Aeromonas Ceviae,Bacillus marisflavi and Exiguobacterium Indicum. All of which exhibited high salinity and pH tolerance along which beneficial by a chemical trait like ammonium production and starts hydrolysis. The groundwork of using halotolerant and pH adaptive microbes to improve soil health crop yield and environmental sustainability but the research necessary to optimize their application in agriculture and bioremediation

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Appendix - A

Zobell Marine Agar (ZMA) Media

Ingredients	**Amount (Per 1000mL)**
Peptone	5.0 g
Yeast extract	1.0 g
Ferric Citrate	0.1 g
Sodium Chloride (NaCl)	19.45 g
Magnesium Sulphate ($MgCl_2$)	8.8 g
Sodium Sulfate(Na_2SO_4)	3.24 g
Calcium Chloride ($CaCl_2$)	1.8 g
Potassium Chloride(KCl)	0.55 g
Sodium bicarbonate ($NaCO_3$)	0.16 g
Agar	15.0 g
Final pH	7.0 g

Luria-Bertani (LB) Broth

Ingredients	**Amount (Per 1000 mL)**
Tryptone	10.0 g
Yeast extract	5.0 g
Sodium Chloride (Nacl)	10.0 g
pH	7.0

Nutrient Agar (NA media)

Ingredients	**Amount (per 1000 mL)**
Peptone	5.0 g
Beef extract	3.0 g
Sodium Chloride (NaCl)	5.0 g
Agar	15.0 g
Final pH	7.0

Alkaline Peptone Water (APW)

Ingredients	**Amount (Per 1000 mL)**
Peptone	10.0 g
Sodium Chloride (Nacl)	10.0 g
Adjusted final pH	8.6

Appendix - B

Chemicals and Reagents

B-1 Gram's iodine

Iodine 1.0g

Potassium Iodide 2.0g

Sodium bicarbonate 3.0g

All the components were dissolved in 300ml distilled water.

B-2 NaOH solution (25mM)

Preparation: 1g of NaOH (in pellet form) was dissolved in 1000 ml distilled water.

B-3 TAE buffer (1x solution)

50x TAE stock 20ml

Distilled water 980ml

B-4 Nessler reagent

Mercuric iodide (HgI_2): 10 g

Potassium iodide (KI): 7 g

Sodium hydroxide (NaOH): 10 g

Distilled water: 100 mL