Isolation and Identification of Halotolerant Bacteria from Patenga Area, Beach Soil and Land Soil

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

Submitted by,
Nafisa Tabassum
Student ID: 12136011
17th August2016
Department of Mathematics and Natural Sciences
BRAC University
66, Mohakhali, Dhaka-1212
Bangladesh

Declaration

I hereby declare that the thesis work titled "Isolation and Identification of Halotolerant Bacteria" has been written and submitted by me, Nafisa Tabassum (ID-12136011) Department of Mathematics and Natural Sciences under the supervision of Ms. Romana Siddique, Senior Lecturer Department of Mathematics and Natural Sciences without the use of other sources than those mentioned.

It is further asserted that this Bachelor's Thesis has never been submitted in the same or substantially similar version to any other examinations office. All explanations that have been adopted literally or analogously are marked as such.

(Nafisa Tabassum)
Candidate

CERTIFIED BY

Ms. Romana Siddique
Supervisor
Senior Lecturer
Department of Mathematics and Natural Sciences,
BRAC University
Dhaka, Bangladesh

Acknowledgement

I might want to thank the greater part of the general population who contributed somehow to the work depicted in this study. Above all else, I would like to show endless gratitude towards the Almighty for His endowments that gave me the quality to persevere against all chances and perform well and complete this postulation.

I might want to extend my gratefulness to the numerous individuals including my family members, companions, employees and lab associates, who so liberally added to the work, exhibited in this thesis. Special notice goes to my motivation, Professor Dr. A. A. Ziauddin Ahmad and Professor Naiyyum Choudhury. Without their bolster it would have been difficult to complete my paper and graduate on time. I am everlastingly appreciative to them for his backing and direction.

I am likewise appreciative to my Supervisor, Ms. Romana Siddique, Senior Lecturer, Department of Mathematics and Natural sciences, BRAC University, particularly to share her significant time and information about this thesis. I am enchanted to have her as my aide and I can't express gratitude toward her enough for the challenges she confronted for me. So also, significant appreciation goes to Associate Professor Dr. Mahboob Hossain and Assistant Professor Jebunnesa Chowdhury, who helped by drawing in me with new thoughts, and requesting a high caliber of work from every one of my attempts.

I might likewise want to take this chance to express my sincerest appreciation to the Laboratory Officer Asma Binte Afzal, Teaching Assistants Nahreen Mirza, Salman Khan Promon for their unremitting help; determined backing and interminable consolation that has made this study a lovely adventure.

Table of Contents

]

Abstract

Salinity provides an unhealthy environment that restricts normal crop production in Bangladesh. The saline soil lack Nitrogen and Phosphorous and become nutrient deficient and the organic matter content of the soils is also reduced. Micronutrients, such as Cu and Zn are widespread. Therefore the possibilities of increasing potential of these saline lands for increased production of crops is by the isolation of halotolerant bacteria that contains the salt tolerant gene which when transferred into the plants uptake salt as nutrient and store in them for higher yield. The study focused on the isolation and identification of the halotolerant bacteria. Three samples were taken from the Patenga area, beach soil and land soil near the coastal area of Chittagong. The samples were inoculated in nutrient media containing wide range of salt concentrations. All the samples showed that they are 2% (w/v), 4% (w/v) and 6 %(w/v) salt tolerant. Total 18 isolates were subcultured and 9 of them were further tested. The soil sample collected from Patenga with 4%(w/v), 6%(w/v) salt tolerance and the isolate from beach soil with 2% (w/v)salt tolerance showed catalase activity and all the isolates showed negative result for oxidase activity, indole production, phenol red lactose and motility. In addition to these, all the samples provided positive result for phenol red dextrose. Other biochemical test provided mixed result for the samples. Based on the morphological characteristics, biochemical test and ABIS software analysis the isolates fall within the *Enterobacteriaceae, Clostridium* and *Corynebacterium,* with a predominance of *Pastuerellaceae* and *Vibrios*. Overall the isolates were widely halotolerant, with best growth observed at lower salinities and no halophilism. The bacterial strains *Volucribacterpsittacicida*, Pantoea*stewartii subsp.stewartii*, *Clostridiuminnocuum*, *Brevibacillusagri*, *Aggregatibacter (Haemophilus) segnis*, *Corynebacteriumxerosis, Vibrio metschnikovii* were predicted to be present in the sample. The gene responsible for the salt tolerant trait in these bacteria can be identified, extracted and finally inserted into the crop plants to form a transgenic plant. These transgenic plants will now be enriched with this new trait the ability to resist wide range of salt concentrations. The plants will uptake the salt as nutrient and will store them to grow and enrich for high crop production for the rest of the year.

List of Figures:

List of abbreviations

Abbreviations	Descriptions
MIU	Motility Indole Urease Test
et al	And others
NA	Nutrient agar
Mg	Milligram
sp.	Species
Ml	Milliliter
TSI	Triple Sugar Iodine
MR	Methyl red
VP	Vogues proskauer
ppm	Parts per million
w/v	Weight by volume
g/l	Gram per liter
Nm	Nanometer
TNTC	Too numerous to count

List of tables:

CHAPTER ONE: Introduction

1.1 Introduction

Salinization is one of the root reasons for harming the crop production in Bangladesh. 20% of Bangladesh is covered as coastal area from which about 53% is affected by salinity (Haque, 2006). Salinity results in undesirable environment that harm the normal crop production throughout the year. During the rainy season (June-October) there is flooding in Bangladesh, the upward movement of the saline ground water in the dry season (November-May) are the factors that initiate the development of saline soil. The crops are being affected depending on the extend of salinity which decreases the yield and in worst cases yield is lost and results in negative nutrient balance. This problem was not given much attention previously. The population growth has increased the pressure for the demand of food. Thus food security problem has reached an alarming rate in the country. The hindrance for crop production in the coastal areas is high levels of salts in the root zone of the soil. The salts penetrate inland through rivers and channels in the dry (winter) season, when fresh water flow downwards becomes very low. That is when the salinity of the river water increases. Firstly the salts penetrate the soil by flooding with saline river water or by seeping from the rivers, and then the concentration of salt intensify in the surface layers through evaporation. The ground water also gain salinity and make it difficult for irrigation. Therefore it has become important to discover the possible ways of increasing the potential of these lands for high production of crops. Nevertheless, the intrusion of the seawater also increases the degree of salinity of the coastal drinking water. These cause severe health problems of the Bangladeshi people. The consumption of too much salt result in hypertension or high blood pressure, stroke, heart failure, other heart diseases, Pre-eclampsia a multi organ disorder causes swelling and convulsions in the body. It also poses danger for the expecting mothers and their children. Skin diseases, common cold and diarrheal dysfunction occurs due to salinity exposure.

Fig: Saline water affected areas hampers rice production (Daily star.net)

1.2 Extent of salinity

The coastal saline soils form in the river deltas of the sea coast up to 180 kilo meters. The findings showed that about 1.02 million hectares of the cultivated lands are affected by salinity. About 0.282, 0.297, 0.191, 0.450 and 0.087million hectares of lands are affected by very slight, moderate strong and very strong salinity (Haque, 2006).However the crop production can be increased by proper soil and water management

practices and by implanting salt tolerant varieties of different crops in slightly alkaline areas.

Table 1. Salinity affected areas in the coastal and offshore regions of Bangladesh

Description	Total cultivated area	Saline area	Area of each salinity class (ha) S1 4.0)	S2 (4.1-8.0)	S3 (8.1-12.0)	S4 (12.0-16.0)	S5 (2.0- (>16.0)
Non-saline with very slightly saline	4,25,490	1,15,370 (27%)	82,260 (72%)	31,590 (27%)	1,520 (1%)	0	0
Non-saline with very slightly saline	4,20,420	3,09,190 (73%)	1,70,380 (55%)	1,10,390 (35%)	29,420 (10%)	0	0
Slightly saline with moderately saline	2,57,270	2,40,220 (93%)	35,490 (15%)	1,13,890 (47%)	61,240 (26%)	25,870 (11%)	2650 (1%)
Moderately saline with strongly saline	1,98,890	1,98,890 (100%)	1,630 (1%)	36,060 (18%)	73,400 (37%)	55,130 (28%)	32,750 (16%)

Source: Soil salinity in Bangladesh (SRDI) 2000

1.3 Fertility status of the saline coastal soil

The soil pH value in Chittagong and Potuakhali is 6-7.8 and it is moderately alkaline to strongly alkaline. There are micronutrients' deficiencies in the places with higher pH values. The organic matter content is low in the soils with the exception of Paikgachha upazila of Khulna district, where the topsoil contain high organic matter (7%). The organic matter content of the top soils ranges from less than 1% to 1.5% (Haque, 2006).The poor physical condition of the coastal soils is characterized by low organic content. The CEC of the soil range from 9.4-40.6 m.e. %. In Khulna and Bagerhat soils the CEC values are higher because of finer texture and higher organic matter contents. There are different levels of exchangeable bases in the soil but in most of the soils generally the higher Ca and K saturation of the exchange complex is compared to Na and Mg. The property of the soil and the plant nutrition is destroyed by Na and Mg saturation of the exchange complex. Magnesium is essential for both the plant uptake of Na, Ca and K .The soils are usually consist of very low total nitrogen content resulting to low organic matter contents of most of the soils. The phosphorous status of the soils should normally range from 15-25 ppm. It was researched that Chittagong, Barguna, Satkhira and Patuakhali districts have phosphorous deficient soils. The coastal regions were observed to have Zn and Cu deficiencies.

1.4 Current agricultural land use:

In different regions soils, rice, jute, sugarcane, pulses, oilseeds, spices, vegetables and fruits are cultivated. In Barisal, Khulna and Patuakhali regions Aman rice and HYV Aman rice in the Chittagong region are grown as the major crop. In Barisal, Khulna, Noakhali, Patuakhali and Chittagong regions the dominant crop is Aman rice in the medium highlands. In Chittagong region aus rice is the major crop where as in the Khulna, Barisal and Patuakhali regions aman fallow is produced mainly. Aus-local transplanted aman covers 25-28% area in the Noakhali and Chittagong area. 18-20% area is covered by the transplanted aman fallow pattern. In the Noakhali district aman, wheat, potato and vegetables crops are cultivated it covers 11.5% area (Haque, 2006). During both aus and aman seasons the HYV rice are grown in high and medium lands in the Noakhali and Chittagong regions. HYV rice during aman season is also found in the highlands of Khulna, Barisal and Patuakhali regions. HYV aus rice is not cultivated in Khulna, Barisal and Patuakhali

regions . Nevertheless in the highlands there is possibility for HYV aman rice cultivation. The coastal areas are usually situated on the medium highlands and it is perfect for minimum two crops and sometimes three crops with winter wheat or other winter crops because the flooding depth ranges from 0.3-0.9 meter.

1.5 Halotolerant Bacteria

Bacteria that grow in the absence of salt and also in the presence of high salt concentrations are known as halotolerant. There are different types of halotolerant bacteria .Non halotolerant which can grow in low salt concentration about 1% w/v .Slightly tolerant they are able to survive in up to 2-8%, moderately tolerant 18-20% and extremely tolerant the microbes grow over the whole range of salt concentrations from zero to saturation. Mostly the spore formers are found to be halotolerant they can grow up to 15% concentration and the yeast fungi and algae are also quite halotolerant. The spoilage bacteria usually represents either the non tolerant or the slightly tolerant bacteria. This group also consists of known types of *psuedomonads, enterobacteria,* and *vibrios.* Halotolerant bacteria consist of three domains, Archaea, Bacteria and Eucarya; they represent many different types and can survive at different salt concentrations as well as outside this environment. They are found in environments such as salt lakes, saline soils, and salted food products. The halotolerant organisms maintain low level of ionic concentrations to synthesize compatible solutes to balance the osmotic level inside the cytoplasm with the outer medium. The adjustment of the concentration of solutes and fluid to maintain the osmotic pressure to keep the fluids from becoming too dilute or concentrated is osmoregulation. These mechanisms of maintenance of the internal environment and the properties of the cytoplasmic membrane help them to adapt to changes in the salt concentration of the environment. The salt tolerant microbes have unique adaptations, a salty cytoplasm, unique salt-requiring proteins, and light-driven proton and chloride pumps bacteriorhodopsin and halorhodopsin. Adaptation is the modification and adjustment of the structure or habit in the bacteria which make them better suited to thrive and reproduce in a particular environment. The levels of the tolerance and salt concentrations vary depending on the species, growth conditions like temperature and medium composition. The organisms that grow in low salt concentration require a specific temperature for growth. For example *Marinococcus halophilus* grows at 0.01 M salt concentration at 20°C but minimum 0.5 M is required at 25°C. Likewise *S. costicola* grow between 0.5 and 4 M NaCl at 30°C and can also grow to 0.2 M at 20°C.

1.6 List of Halotolerant Bacteria

Organism Name: ***Halomonas elongata CHR63***

Habitat: Soil
The taxonomy of *Halomonas elongate CHR63* is that they belong to the

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Oceanospirillales
Family: Halomonadaceae
Genus: Halomonas

Organism Name: ***Thioalkalivibrio versutus***

Habitat: Aquatic and terrestrial

The taxonomy of ***Thioalkalivibrio versutus*** is that they belong to the

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Chromatiales
Family: Ectothiorhodospiraceae
Genus: Thioalkalivibrio

Organism Name: ***Sporosarcina pasteurii***

Habitat: Soil
The taxonomy of ***Sporosarcina pasteurii*** is that they belong to the

Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Planococcaceae
Genus: Sporosarcina

Organism Name: ***Methanosarcina mazei Go1***

Habitat: Marine
The taxonomy of ***Methanosarcina mazei Go1*** is that they belong to the
Kingdom: Bacteria
Phylum: Euryarchaeota
Class: Methahomicrobia
Order: Methanosarcinales
Family: Methanosarcinaceae
Genus: Methanosarcina

Organism Name: ***Sulfolobus solfataricus***

Habitat: Geothermal habitat
The taxonomy of ***Sulfolobus solfataricus*** is that they belong to the

Kingdom: Bacteria
Phylum: Crenarchaeota
Class: Thermoprotei
Order: Sulfolobales
Family: Sulfolobaceae
Genus: Sulfolobus

Organism Name: ***Pseudomonas aeruginosa PA01***

Habitat: Soil
The taxonomy of ***Pseudomonas aeruginosa PA01*** is that they belong to the

Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Pseudomonadaceae
Genus: Pseudomonas

Organism Name: ***Thermoproteus tenax***

The taxonomy of ***Thermoproteus tenax*** is that they belong to the
Habitat: Acidic hot springs and water holes
Kingdom: Bacteria
Phylum: Crenarchaeota
Class: Thermoprotei
Order: Thermoproteales
Family: Thermoproteaceae
Genus: Thermoproteus

Organism Name: ***Methanocaldococcus jannaschii***
Habitat: Soil, Water
The taxonomy of ***Methanocaldococcus jannaschii*** is that they belong to the
Kingdom: Bacteria
Phylum: Euryarchaeota
Class: Methanococci
Order: Methanococcales
Family: Methanocaldococcaceae
Genus: Methanocaldococcus

Organism Name: ***Methanopyrus kandleri***
Habitat: Variety of habitats

The taxonomy of ***Methanopyrus kandleri*** is that they belong to the

Kingdom: Bacteria
Phylum: Euryarchaeota
Class: Methanopyri
Order: Methanopyrales
Family: Methanopyraceae
Genus: Methanopyrus

1.7 Strategy of osmoregulation in Halotolerant Bacteria

These bacteria have a unique property the ability to adapt themselves according to the outer environment. The water activity of cytoplasm of the bacterial cells decreases due to the exposure to salt stress which disrupts and changes the functions of their proteins and other macromolecules. Whereas the gradual plasmolysis restrict the physiological processes for example nutrient uptake inhibition of DNA replication and macromolecule biosynthesis. These bacteria have adopted two ways of osmoadaptation.Firstly they balance the osmotic level by maintaining the cytoplasmic KCl concentration equal to the outer environment and some of the modifications to protect the metabolic functions They generally do not synthesize organic solutes to maintain the osmotic equilibrium. Another way is to gather the compatible solutes in the cytoplasm to maintain high osmotic potential in the environment. The cell retains low salt concentration of salt in their cytoplasm by maintaining the osmotic potential by the uptake and synthesis of compatible solutes. Thus they become capable to adapt in high salt concentrations. The compatible solutes are the solutes which help and promote the enzymes to work properly in high concentrations.

The organic osmolytes are of three categories:
(i) zwitterionic solutes, (ii) noncharged solutes, and (iii) anionic solutes

Zwitterionic solutes:	**Occurrence:**
Betaine	Halotolerant: *Thioalkalivibrio versutus;*
Ectoine	Halotolerant: *Sporosarcina pasteurii.*
Ng-acetyldiaminobutyrate	Halotolerant: *Halomonas elongata CHR63*
Ne-acetyl-b-lysine	Halotolerant:*Methanosarcina mazei Gö1*
Uncharged solutes:	**Occurrence:**
a-glucosylglycerol	Halotolerant: *Pseudomonas mendocina*
a-mannosylglyceramide	Halotolerant: *Rhodothermus marinus*
Trehalose	Halotolerant: *Sulfolobus solfataricus*
Sucrose	Haloterant: *proteobacteria*
N-acetylglutaminylglutamine amide	Halotolerant*Pseudomonas aeruginosa PAO1*
Anionic solutes (carboxylates):	**Ocurrence:**
L-a-glutamate	Many halotolerant bacteria and methanogens
b-glutamate	Halotolerant; *Methanothermococcus*
Hydroxybutyrate	Halotolerant: *Photobacterium profundum*

poly-b-hydroxybutyrate	Halotolerant: *Photobacterium profundum*
a-glucosylglycerate	Halotolerant: *Agmenellum quadruplicatum;*
a-mannosylglycerate	Halotolerant: *Methanothermus fervidus;*
Anionic solutes (phosphate, sulfate):	**Occurrence:**
a-diglycerol phosphate	Halotolerant: *Archaeoglobus fulgidus*
di-myo-inositol-1, 1'-phosphate	Halotolerant: *Archaeoglobus fulgidus;*
mannosyl-DIP	Halotolerant: *Thermotoga maritima*
cyclic-2, 3-diphosphoglycerate	Halotolerant: *Methanopyrus kandleri*

Noncharged solutes

Carbohydrate

Uncharged amino acids and peptides

Organic anions

β-Glutamate

β-Hydroxybutyrate and derivatives

Anionic polyols and carbohydrates

K+ and other inorganic ions

Halotolerant archae gather organic anions and the negative charge is supplied by the phosphate moiety and also by the sulphate in addition to the noncharged solute. This class of compounds consists of the glycerol derivative α-diglycerol phosphate and a series of myoinositol phosphodiesters based on di-myo-inositol 1, 1'phosphate (DIP).Phosphomonoesters have strong interactions with the cat ions compared to the phosphodiesters.The concentration of the solutes inside the cell increase with the outside NaCl. The increase is significant with the growth temperatures.

1.8 Role of the Halotolerant bacteria

1.8.1 Food biotechnology

Halotolerant bacteria are essential for production salty foods such as Thi fish sauce, pickling brines, salt-cured bacon and oil field production brines. In the product Thi fish sauce they are mixed with concentrated brine (25–30% NaCl) and allowed to ferment for around a year (Margesin, 2001).

Halotolerant fermentative bacteria are used to produce food products fermented fish, shrimp, meat, fruits, and vegetables (pickles), Asian fish and meat sauces, rice noodles and flours, and Indonesian soy sauce. The bacteria involved are non-obligate halophiles, including species of the genera *Lactobacillus, Halobacterium, Halococcus, Bacillus, Pediococcus,* and *Tetragenococcus*

1.8.2 Decolorization of Textile Azo Dyes

There were 27 strains of halophilic and halotolerant bacteria isolated from effluents of textile industries, three of them showed that they are capable of decolorizing the utilized azo dyes. (Asad, 2006).They are able to decolorize azo dyes in different NaCl concentrations (up to 20% w/v), temperature (25–40ºC), and pH (5–11) after 4 days of incubation in culture; they can even decolorize a mixture of dyes. The decolorization occurs for biodegradation by a reduction of the azo bond, followed by cleavage.

1.8.3 Composting process

Salt-tolerant bacteria were screened in the Organic Composting Production Unit (OCPU) of São Paulo Zoological Park Foundation (Lilian C.G. Oliveira et.al 2015). The results of the study show these halotolerant bacteria are able to produce some classes of hydrolases, lipases, proteases, amylases and cellulases, and biopolymers. The results depict the biotechnological potential of certain microorganisms recovered from the composting process, including halotolerant species, are capable of producing enzymes and biopolymers.

1.8.4 Removal of Hydrocarbon contamination

Four of the halotolerant bacteria *Bacillus atrophaeus, Halomonas shengliensis, Halomonas koreensis,* and *Virgibacillus* salaries (Kothari ,2014) have the ability to metabolize hydrocarbons. The results showed that as the *V. salarius* was able to grow at high salt concentration, alkaline pH, hydrocarbon degradation, in presence of various metal ions, it can be used for bioremediation of marine oil spills.

1.8.5 Production of enzymes

Halophilic and halotolerant bacteria were found to have enzymatic characteristics. They can be used for production of enzymes with different immunological properties. (Shirazian, 2016)The halotolerant is also essential for nutrient recycling and for maintaining the soil health in salty environment.

1.9 Literature Review

History Background

It was reported that the amelioration of salt stress inhibitory effect on the canola seed germination was attributed to the inoculation of ACC deaminase-producing halotolerant bacteria modulating ethylene emission and inducing hydrolytic enzymes (Siddikee,2015) .It was done by using 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing plant growth promoting halotolerant bacteria

A research was executed on plant growth-promoting rhizobacteria (PGPR) containing aminocyclopropane-1-carboxylate (ACC) deaminase, (Habib, 2016) examined their effect on salinity stress tolerance in okra through the induction of ROS-scavenging enzyme activity. PGPR inoculated okra plants exhibited higher germination percentage, growth parameters, and chlorophyll content than control plants. Increased antioxidant enzyme activities (SOD, APX, and CAT) and up regulation of ROS pathway genes (CAT, APX, GR, and DHAR) were observed in PGPR inoculated okra plants under salinity stress.

Simultaneously in a research the mechanism of salt stress ameriolation in red pepper plants by 1-aminocyclopropane-1-carboxylicacid (ACC) deaminase producing halotolerant bacteria was studied(Siddikee,2011).The result showed salt stress ethylene production by increasing enzyme activities of biosynthetic pathway. Inoculation with ACC delaminate producing halotolerant bacteria reduces the ACC concentration although a direct effect on reducing ACO activity was also observed. It was also reported that the growth promotion in inoculated red pepper plants under inhibitory levels of salt stress is due to ACC deaminase activity present in the halotolerant bacteria.

1.10 Objectives of the study:

The goal of the research was to isolate and identify halotolerant bacteria from natural sources like soil and water. The soil samples were studied in this paper. The aim also focuses on the possibilities to reduce the effects of salinity in crop production. This can be done by transferring the salt tolerant gene into the plants so that these plants uptake salt as nutrient and store in them thus resulting in good yield throughout the year.

CHAPTER TWO: MATERIALS AND METHODS

2. Materials and Method

2.1 Sources

Three different soil samples were taken. The soil was taken from the coastal area Chittagong Patenga Beach soil, Land soil and Patenga area soil.

2.2 Methods

2.2.1Collection of the soil sample

The soil sample was collected from 10-12inch depth in a sterile polythene packet and then kept at the room temperature until it was further used.

2.2.2 Isolation and screening of bacteria

5g of each of the soil samples were taken in a conical flask and mixed with 20ml of distilled water to prepare a soil suspension. Then serial dilution was performed three dilution factors was taken 10^{-3},10^{-5} and 10^{-7}.100µl of the each of the diluted samples was spread on the nutrient agar plates containing 2%(w/v),4%(w/v) , 6%(w/v),8%(w/v) and 10%(w/v) NaCl for 24hr at 37˚C.There were no bacterial growth observed for 8%(w/v) and 10%(w/v) NaCl plates so further tests were conducted with 2%,4% and 6%Two isolates was taken from each of the plates from primary screening. Total 18 organisms isolated and streaked in nutrient agar plates for performing further biochemical tests. The spread plate method was used for all the three dilution factors and for the raw sample as well. The screening procedure was carried out determining the size and morphological characteristics of the colonies. Each of the colony was marked as per the concentration from which they were obtained.

2.2.3 Confirmation of bacteria

After 24hrs of incubation all the nutrient agar plates containing different concentration was observed and also realized that the number of colonies decreased per dilution. Secondly the isolation of the two colonies was streaked into the plain nutrient agar media without any salt concentrations. Streak plate method was used to observe single isolated colonies. After 24hrs of incubation at 37˚C the biochemical tests were done.

2.2.4 Biochemical tests

Biochemical tests were done to detect and confirm the presence of microorganisms after the observation of the single isolated colonies. Several biochemical tests were done among them were Gram staining, Starch hydrolysis, TSI test, Simmon's citrate test, Oxidase test, Catalase test, Nitrate reduction, Nitrate Broth media , MRVP, Indole test, Urease, Acid production from carbohydrate and growth at 6.5% NaCl solution.

2.2.4.1 Catalase test

The hydrogen peroxide used here is broken down to water and oxygen. The test is done in a slide one drop of the 3% hydrogen peroxide was given over the surface of a glass slide. Test organism was taken from the nutrient agar plates and places it on the reagent drop. The presence or the absence of the bubbles or foam was observed. This determines whether the organism was capable of catalase activity.

2.2.4.2 Oxidase test

This test is done for the morphological identification of *Pseudomonas aeruginosa* or *Neisseria species*. It is a filter paper spot test. Two drops of oxidase reagent p-aminodimethylaniline oxalate were added to the surface of growth of test organisms onto the filter paper. The test organisms were picked from nutrient agar plates and smeared in the filter paper. Two drops of oxidase reagent p-aminodimethylaniline oxalate were added to the surface of growth of test organisms onto the filter paper. The colour changes from pink to maroon and to purple were observed. Positive test colour change will take place in 10-30second, negative test no colour change or light pink colour.

2.2.4.3 Nitrate reduction test

Nitrate broth consists of beef extract (3g/l), peptone (5g/l), potassium nitrate (5g/l). Nitrate broth was prepared in which the inoculums from the culture was transferred and incubation done for 24hrs at 37℃ .Secondly five drops of nitrate reagent A was added followed by five drops of nitrate reagent B after the incubation period. Observation of red colour indicates a positive result. The cultures in which red colour did not appear minute amount of zinc were added. Then the conversion of the red colour is observed. Based on this the organisms capable of nitrate reduction is determined.

2.2.4.4 TSI test

The triple sugar iodine agar is prepared and the filled in the test tubes 7ml per test tube. Test organisms was picked up from the nutrient agar plates by needle and stabbed into the TSI consisting of dextrose, lactose and sucrose butt. The tubes are kept for incubation for about 24hrs at 35˚C. If the organism is capable of fermenting all three sugars then it will produce yellow (acidic) colour in the butt, whereas if the slant and the butt appears red (alkaline) colour then the organism is a non fermentor.A Black precipitation in the butt indicates the formation of hydrogen sulphide.If CO_2 is produced there would be crack and bubbles in the medium. The absence of yellow colour in the butt and slant indicates negative result.

2.2.4.5 Gram Staining

A smear is prepared by mixing loopful of culture with a drop of saline. It was then left to air dry for some time with some heat fixation. Then the crystal violet was added on the smear and washed with tap water after 60 seconds. Gram's Iodine was also added on the fixed culture and after 60 seconds the solution was poured off and the slide washed with tap water. After that few drops of ethanol was added for decolourization. After 5 seconds it was rinsed off. Safranin was used as a counter stain for 40 to 60 seconds and washed off. The whole slide is air dried and was observed under microscope.

2.2.4.6 MRVP Test

The test organism from fresh culture was inoculated into the test tube containing 10ml MR-VP broth. The broth consists of peptone, dextrose and potassium phosphate. It was kept for inoculation at 37˚C for 24hrs.5ml of the inoculum was transferred into another test tube for VP test. In 5ml of MR-VP broth containing tube 5 drops of Methyl Red was added. Positive result shows a red ring in the test tube. Negative result gives a yellow colour. The other test tube containing 5ml of MR-VP broth was used here. The test tube contains 5ml of the desired bacterial culture. Thirdly 10 drops of Baritt's reagent A was added and the culture

shaken. Next the Baritt's reagent B was added and the culture shaken again. They were kept for 15minutes to allow them to react with each other. The red colour shows a positive result and yellow or no colour changed is indicated as negative result.

2.2.4.7 Indole production test

The indole is produced when the organism can hydrolyse the tryptophan. The test organism are inoculated into SIM agar deep tube by stab inoculation and kept for incubation for 24hrs at 37˚C. Kovac's reagent is used to detect the indole production.

2.2.4.8 Urease test

MIU test was done for urease test, indole and motility. Organisms that utilize urea produce ammonia which makes the medium alkaline, showing pink-red colour by change in the phenol red indicator.

2.2.4.9 Simmon's Citrate test

This test indicates the organism capacity to utilize citrate as a carbon source. Simmon's citrate agar consists of sodium citrate as the sole source of carbon, ammonium dihydrogen phosphate as the sole source of nitrogen, other nutrients, and the pH indicator bromthymol blue. Firstly the test organism is inoculated into the Simmon's agar slants by the means of streak inoculation. The cultures are incubated for 24hrs at 37℃.The citrate positive cultures show blue coloration and the citrate negative cultures will show no growth and medium remain green. The organism that show positive results use the enzyme citrase or citrate-permease to transport the citrate into the cell. They transform the ammonium dihydrogen phosphate to ammonia and ammonium hydroxide, this produce an alkaline environment in the medium. At pH 7.5 or above, bromthymol blue turns blue and at neutral pH, bromthymol blue is green.

2.2.4.10 Carbohydrate fermentation

The ingredients are trypticase- 10g/l, NaCl-5g/l, Phenol red 0.018/l, sugar (Glucose, Lactose and Sucrose) 5g/l .The experimental organism is inoculated into the phenol red lactose, dextrose and sucrose broth by loop inoculation. In this step shaking of the fermentation tube may force a bubble of air into the inverted gas vial displacing the medium. They are incubated at 37˚C for 24hrs.Based on the colour change of the carbohydrate broth cultures and the presence or absence of gas bubbles the organism capable of fermenting carbohydrate substrate with the production of acid or acid and gas is determined.

2.2.4.11 MIU Test

This test indicates the motility, urease and indole production in one go. In this method the test organism is taken from the fresh subculture by a needle. The needle is stabbed into the MIU media. This test is done for motility, indole and urease test. After incubation of 24hr if the stab line of the media turn hazy then it is motility positive, if the media turns pink it is urease positive and after adding 10 drops of Kovacs reagent a red ring appears in the media then it is considered as indole positive.

2.2.4.12 Starch hydrolysis test

Starch agar was prepared by adding 3 g/l of beef extract, 10g/l of soluble starch and 15g/l of agar then it was autoclaved at 37˚C for 24 hours. An inoculum from a pure culture is streaked on sterile plates of starch agar. Then the plates are incubated for 24hr.Finally Iodine reagent is added to flood the growth. The presence of clear zone is positive that means they can digest starch thus indicates presence of alpha amylase.

2.2.4.13 Growth at 6.5% NaCl solution

Firstly a pure inoculum is transferred aseptically to a sterile tube of 6.5% NaCl solution. Then the tubes are kept for incubation for 24hr.A positive result is indicated by the presence of turbidity. And ultimately the turbidity measurements are taken by measuring their OD.

Table 2.2.4.13 OD Measurement

Sample	Concentration	OD(nm)
Control	nil	0.131
Patenga area	2%	0.148
Patenga area	4%	0.384
Patenga area	6%	0.012
Land soil	2%	0.157
Land soil	4%	0.136
Land soil	6%	0.188
Beach soil	2%	0.042
Beach soil	4%	0.015
Beach soil	6%	0.129

CHAPTER THREE: RESULTS AND OBSERVATIONS

3.1- Isolation of Bacteria

Table: 3.1.1 Isolation of bacteria (Land Soil)

Morphology	2% NaCl			4% NaCl			6 % NaCl		
Concentration	10-3	10-5	10-7	10-3	10-5	10-7	10-3	10-5	10-7
Size									
Large	X	✓	✓	X	✓	✓	✓	✓	x
Small	✓	✓	✓	✓	✓	✓	✓	✓	✓
Surface									
Smooth	✓	✓	✓	✓	✓	✓	✓	✓	✓
Dull/rough	X	X	X	X	x	x	x	x	x
form									
Circular	✓	✓	✓	✓	✓	✓	✓	✓	✓
Irregular	X	X	X	X	x	x	x	✓	x
Clustered	✓	✓	✓	✓	✓	✓	x	x	x
Colour									
Buttery	✓	✓	✓	✓	✓	✓	✓	✓	✓
Opaque	X	X	X	X	x	x	x	x	x
Translucent	X	X	X	X	x	x	x	x	x
Elevation									
Flat	✓	✓	✓	✓	✓	✓	✓	✓	✓
Raised	X	X	X	X	x	x	x	x	x
Margin									
Entire	✓	✓	✓	✓	✓	✓	✓	✓	✓
Undulate	X	X	X	X	x	x	x	x	x
Lobate	X	X	X	X	x	x	x	x	x
Total Colony No	TNTC	TNTC	TNTC	TNTC	TNTC	137	214	80	110

Table: 3.1.2 Isolation of bacteria (Patenga Area)

Morphology	2% NaCl			4% NaCl			6 % NaCl		
Concentration	10-3	10-5	10-7	10-3	10-5	10-7	10-3	10-5	10-7
Size									
Large	X	✓	✓	X	✓	x	x	✓	x
Small	✓	✓	✓	✓	X	✓	✓	✓	✓
Surface									
Smooth	✓	✓	✓	✓	✓	✓	✓	✓	✓
Dull/rough	X	X	X	X	x	x	x	X	x
form									
Circular	✓	✓	✓	✓	✓	✓	✓	✓	✓
Irregular	✓	✓	✓	✓	x	x	x	✓	✓
Clustered	✓	X	✓	X	x	x	x	X	x
Colour									
Buttery	✓	✓	✓	✓	✓	✓	✓	✓	✓
Opaque	X	X	X	X	x	x	x	X	x
Translucent	X	X	X	X	x	x	x	X	x
Elevation									
Flat	✓	✓	✓	✓	✓	✓	✓	✓	✓
Raised	X	X	X	X	x	x	x	x	x
Margin									
Entire	✓	✓	✓	✓	✓	✓	✓	✓	✓
Undulate	X	X	X	X	x	x	x	x	x
Lobate	X	X	X	X	x	x	x	x	x
Total Colony No	182	68	246	TNTC	1	1	1	TNTC	TNTC

Table: 3.1.3 Isolation of bacteria (Beach Soil)

Morphology	2% NaCl			4% NaCl			6 % NaCl		
Concentration	10-3	10-5	10-7	10-3	10-5	10-7	10-3	10-5	10-7
Size									
Large	✓	✓	✓	✓	✓	✓	✓	✓	✓
Small	✓	✓	✓	✓	✓	✓	✓	✓	✓
Surface									
Smooth	✓	✓	✓	✓	✓	✓	✓	✓	✓
Dull/rough	X	X	X	X	x	x	X	X	x
form									
Circular	✓	✓	✓	✓	✓	✓	✓	✓	✓
Irregular	X	X	X	X	x	x	X	X	x
Clustered	X	X	X	X	x	x	X	X	x
Colour									
Buttery	✓	✓	✓	✓	✓	✓	✓	✓	✓
Opaque	X	X	X	X	x	x	X	X	x
Translucent	X	X	X	X	x	x	X	X	x
Elevation									
Flat	✓	✓	✓	✓	✓	✓	✓	✓	✓
Raised	X	X	X	X	x	x	X	x	x
Margin									
Entire	✓	✓	✓	✓	✓	✓	✓	✓	✓
Undulate	X	X	X	X	x	x	X	x	x
Lobate	X	X	X	X	x	x	X	x	x
Total Colony No	13	19	23	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

Table: 3.1.4 Morphology of all the three samples before dilution

Morphology	Beach Soil								Land Soil								Patenga area							
Concentration	2%	4%	6%	8%	10%	12%	14%	16%	2%	4%	6%	8%	10%	12%	14%	16%	2%	4%	6%	8%	10%	12%	14%	16%
Size																								
Large	✓	✓	✓	X	x	x	X	X	X	✓	✓	x	x	X	x	✓	X	✓	✓	✓	x	x	x	x
Small	✓	✓	✓	✓	✓	✓	X	X	X	✓	✓	✓	✓	✓	x	✓	X	✓	✓	✓	✓	✓	x	✓
Surface																								
Smooth	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Dull/rough	x	X	x	x	x	x	X	X	X	X	X	x	x	X	x	x	X	x	X	x	x	x	x	x
form																								
Circular	✓	✓	✓	✓	✓	x	✓	X	X	✓	✓	✓	✓	✓	x	✓	X	✓	✓	✓	✓	✓	x	✓
Irregular	✓	✓	✓	x	x	x	X	X	X	✓	✓	x	x	X	x	✓	X	✓	✓	x	x	x	x	
Colour																								
Cloudy	x	X	x	x	x	x	X	X	X	X	X	x	x	X	x	x	X	x	X	x	x	x	x	x
Opaque	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Translucent	x	X	x	x	x	x	X	X	X	X	X	x	x	X	x	x	X	x	X	x	x	x	x	x
Elevation																								
Flat	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Raised	x	X	x	x	x	x	X	X	X	X	X	x	x	X	x	x	X	x	X	x	x	x	x	x
Margin																								
Entire	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Undulate	x	X	x	x	x	x	X	X	X	X	X	x	x	X	x	x	X	x	X	x	x	x	x	x
Lobate	x	X	x	x	x	x	X	X	X	X	X	x	x	X	x	x	X	x	X	x	x	x	x	x

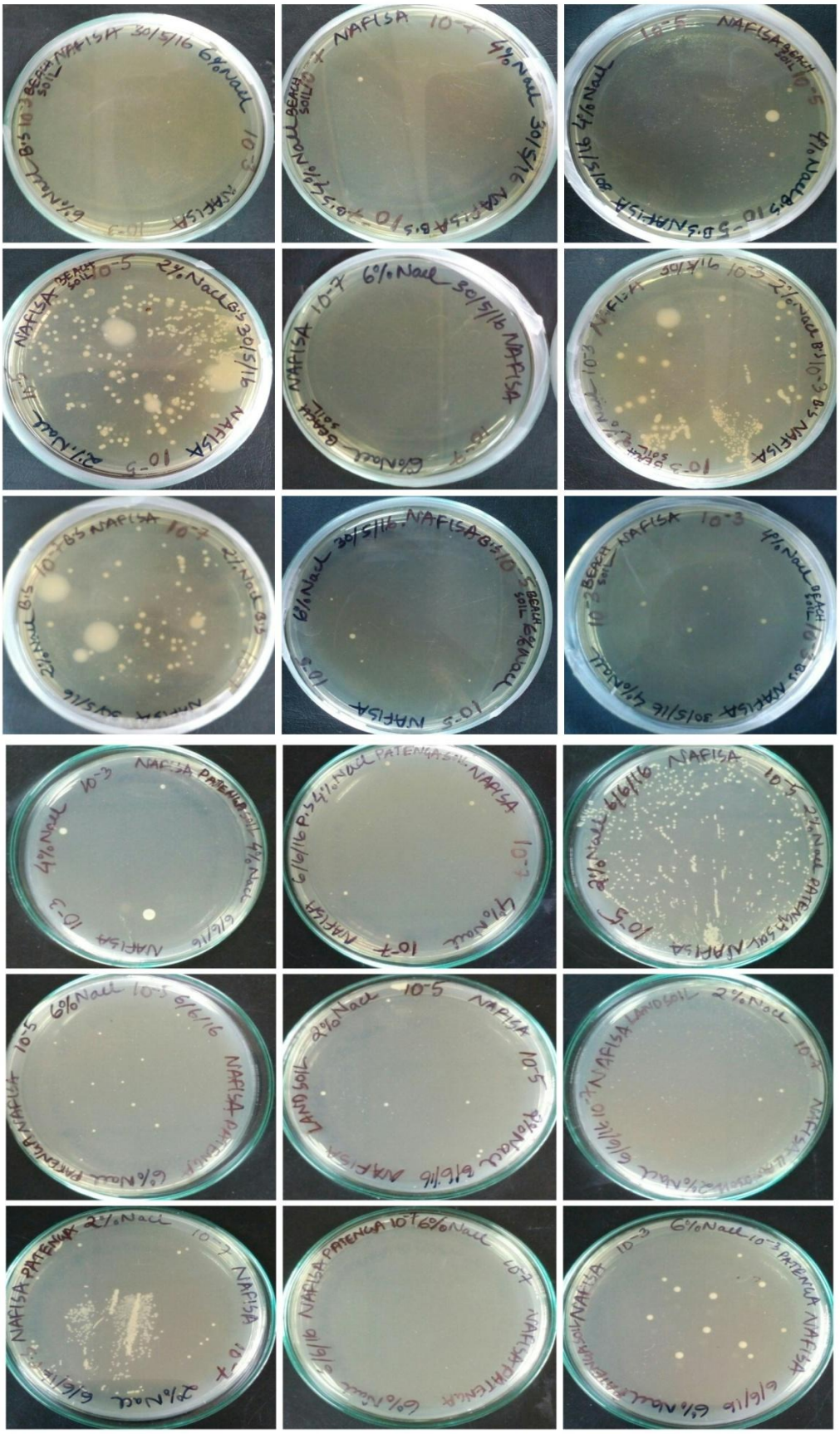

Fig: 3.1a Colony Morphology of isolation of bacteria from i) Beach soil ii) Patenga area iii) Land soil

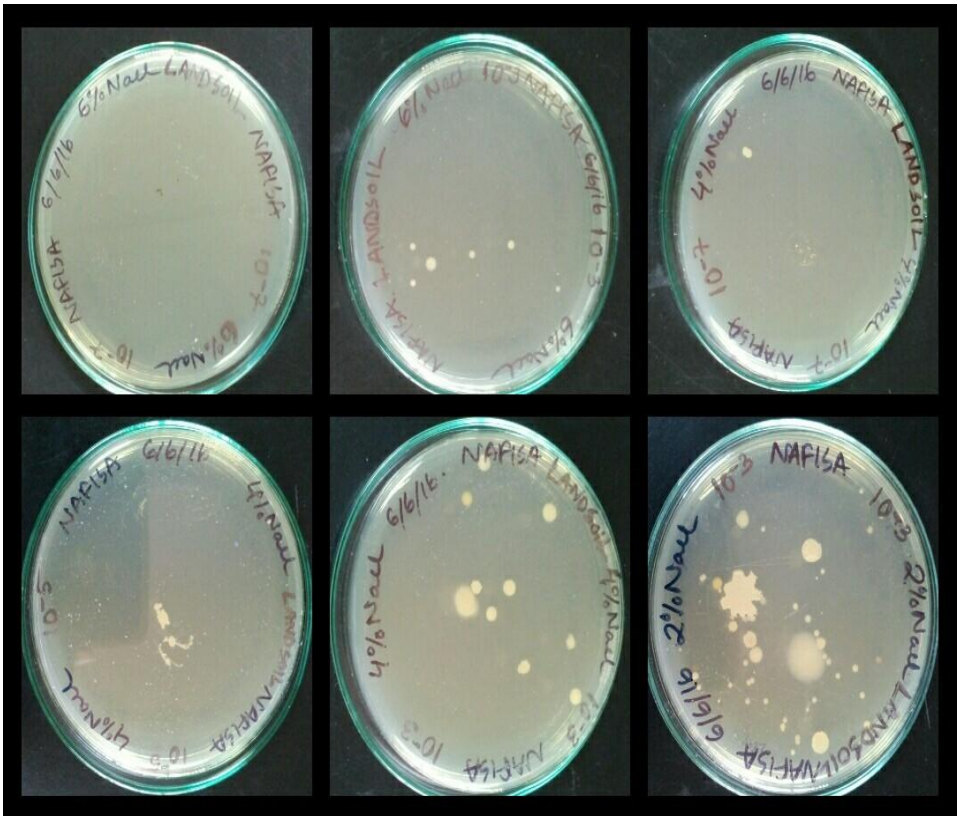

Fig: 3.1b Colony Morphology of isolation of bacteria from i) Beach soil ii) Patenga area iii)Land soil

3.2 Subculture

Fig: 3.2 Subculture of a) Beach soil 4% 10^{-5} b) Beach soil 2% 10^{-5}

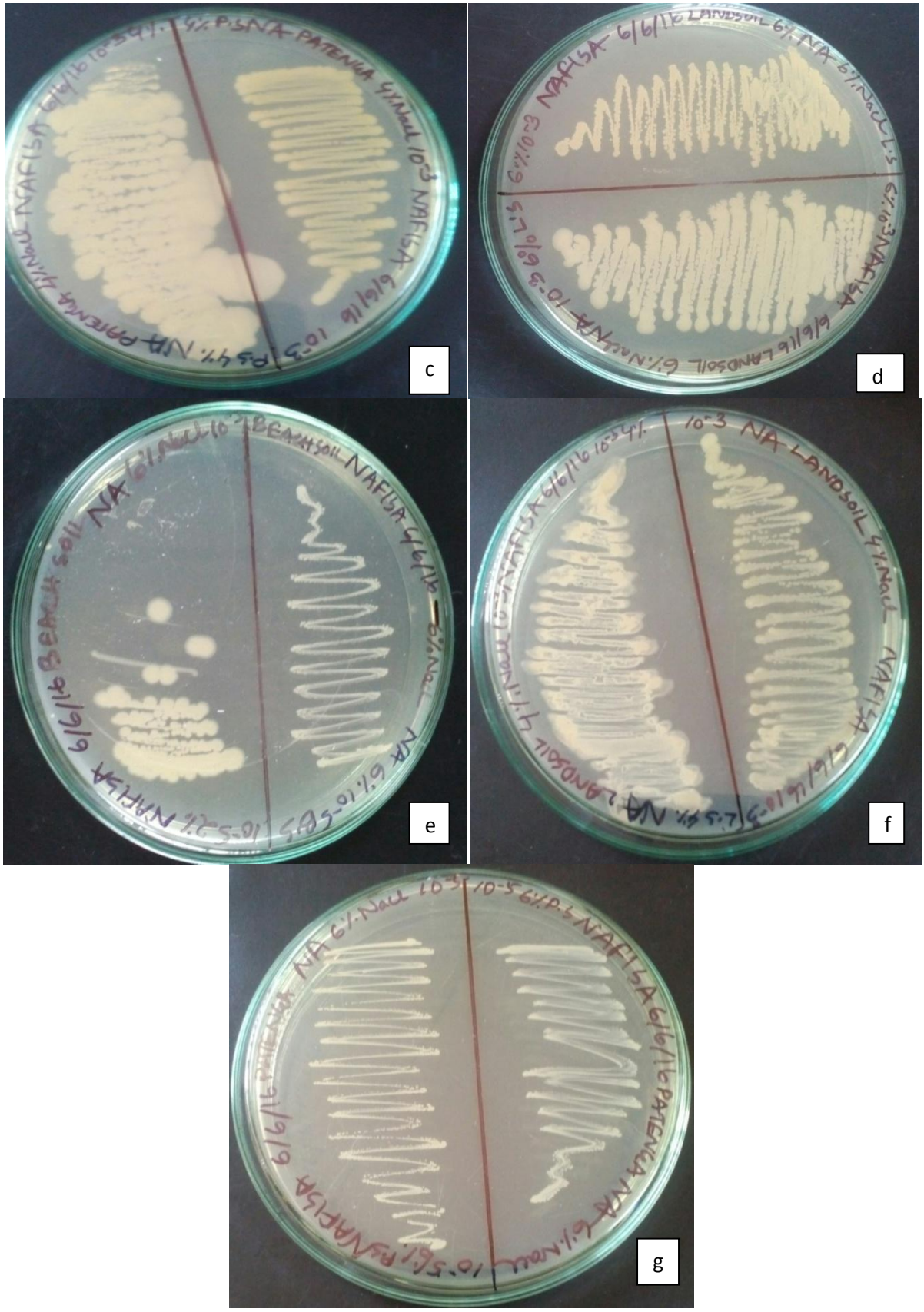

Fig: 3.2 Subculture of c) Patenga 4% 10^{-3} d) Land soil 6%10^{-3} e) Beach soil 6%10^{-7} f) Land soil 4%10^{-3} g) Patenga6%10^{-5}

3.3Biochemical Test

3.3.1Gram Staining

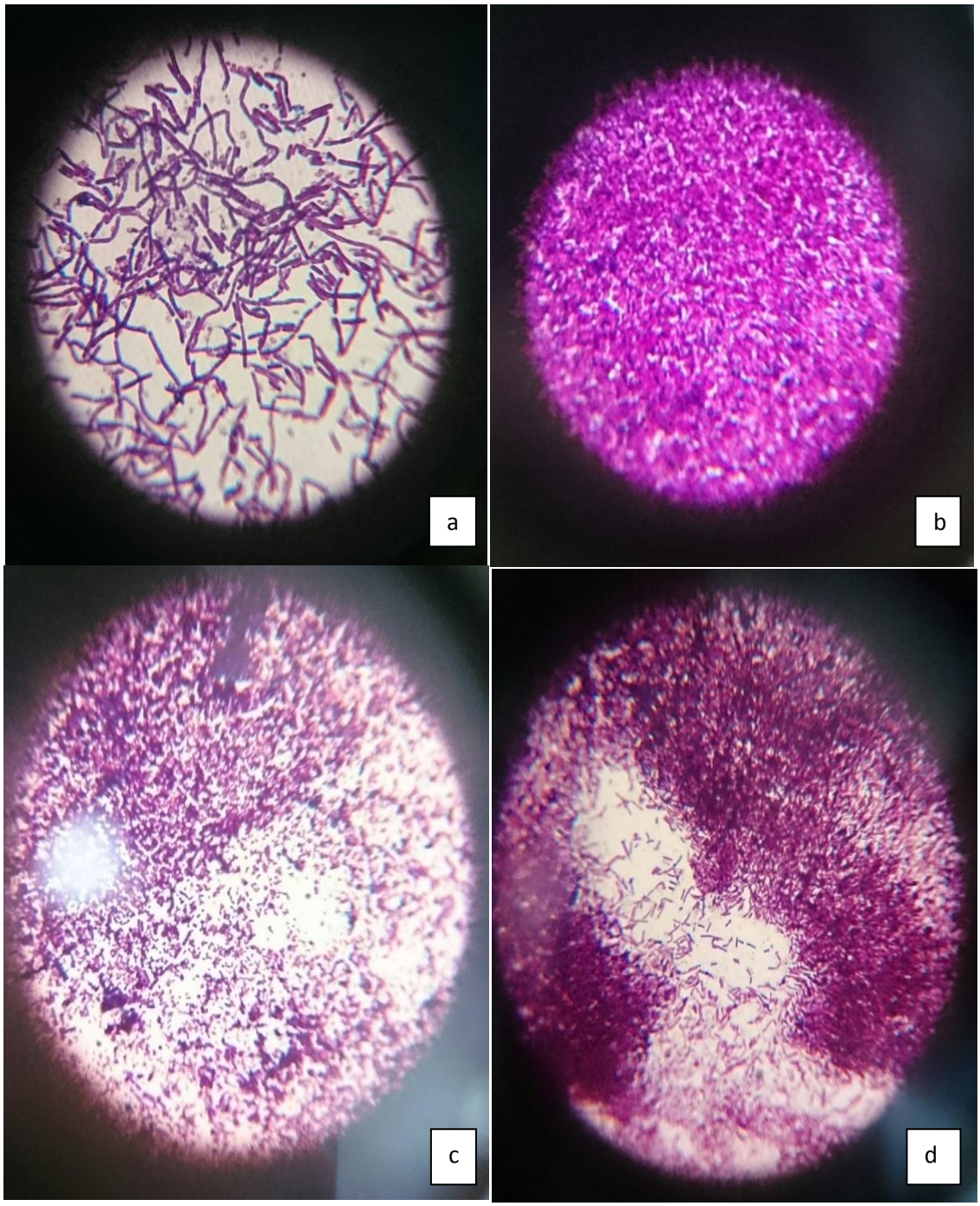

Fig: 3.3.1a) Beach soil 4% b)Land soil2% c)Beach soil2% d)Land soil6%

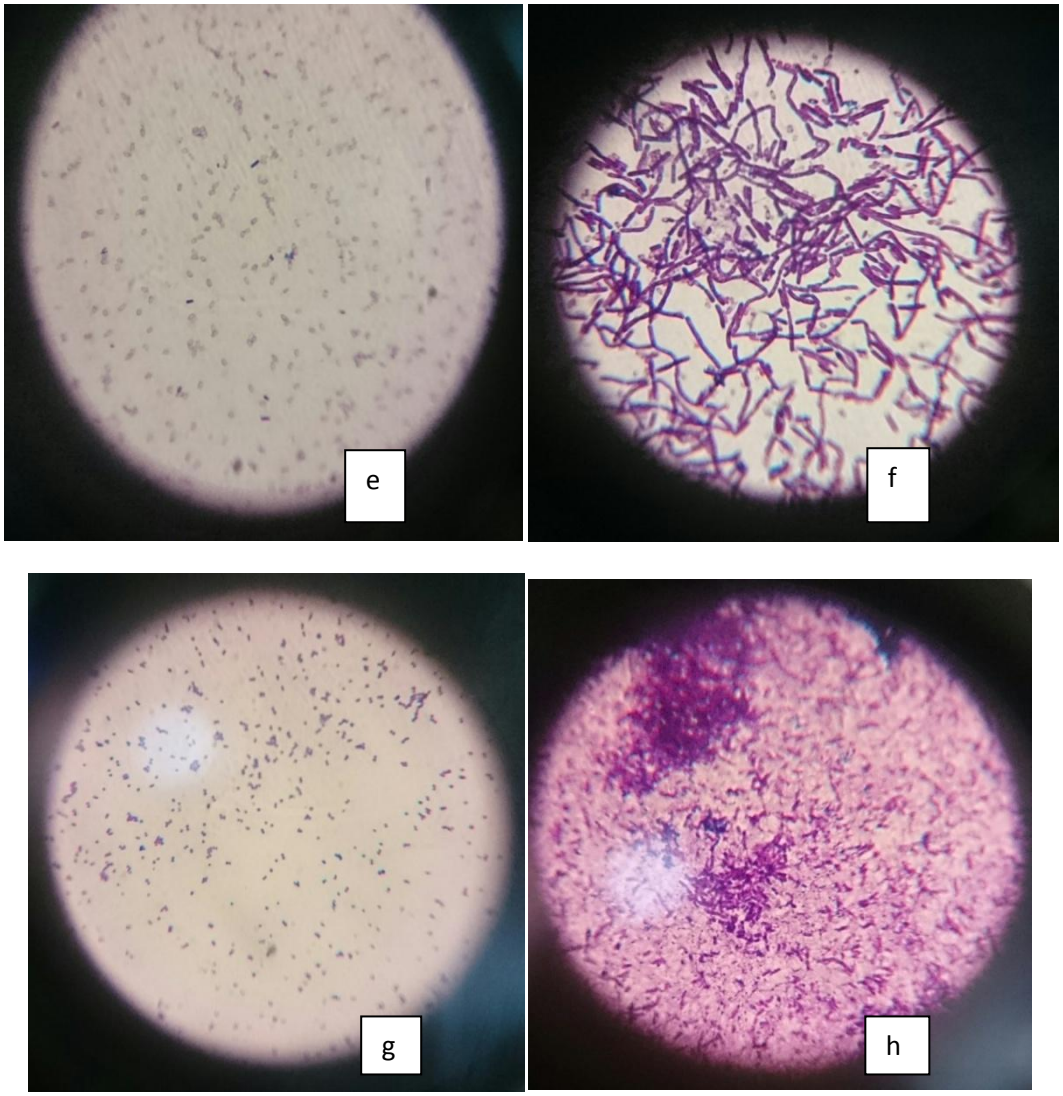

Fig: 3.3.1e) Patenga 4% f) Land soil4% g) Patenga6% h) Beach soil6%

The samples were all Gram Positive. Patenga 4%, 6% are cocci shaped others are all rod shaped

3.3.2 Triple sugar iodine Test

Table: 3.3.2 Triple sugar iodine Test

Sample	Concentration	Slant colour	Butt colour
Patenga area	2%	Yellow	Yellow
Patenga area	4%	Red/Orange	Yellow
Patenga area	6%	Yellow	Red/Orange
Beach soil	2%	Yellow	Yellow
Beach soil	4%	Yellow	Yellow
Beach soil	6%	Yellow	Yellow
Land soil	2%	Yellow	Red/Orange
Land soil	4%	Yellow	Red/Orange
Land soil	6%	Red/Orange	Yellow

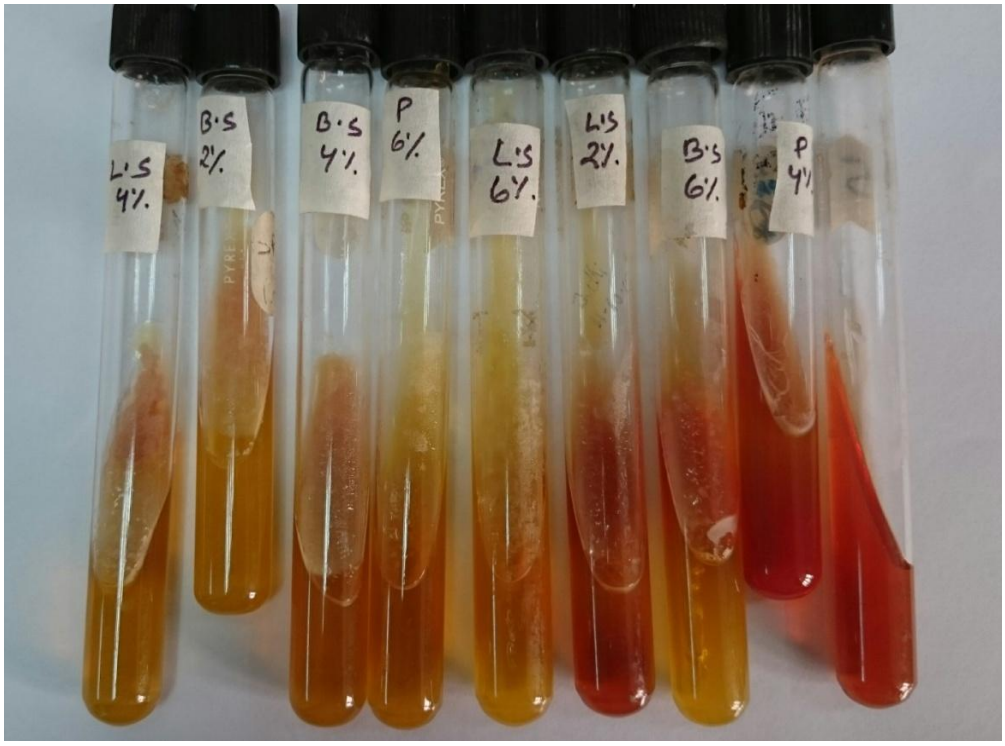

Fig: 3.3.2 Triple sugar iodine (TSI) Test

3.3.3 Simmon's Citrate Test

Table: 3.3.3 Simmon's Citrate test

Sample	Concentration	Colour
Patenga area	2%	No change
Patenga area	4%	No change
Patenga area	6%	No change
Beach soil	2%	Blue
Beach soil	4%	Blue
Beach soil	6%	No change
Land soil	2%	No change
Land soil	4%	Blue
Land soil	6%	Blue

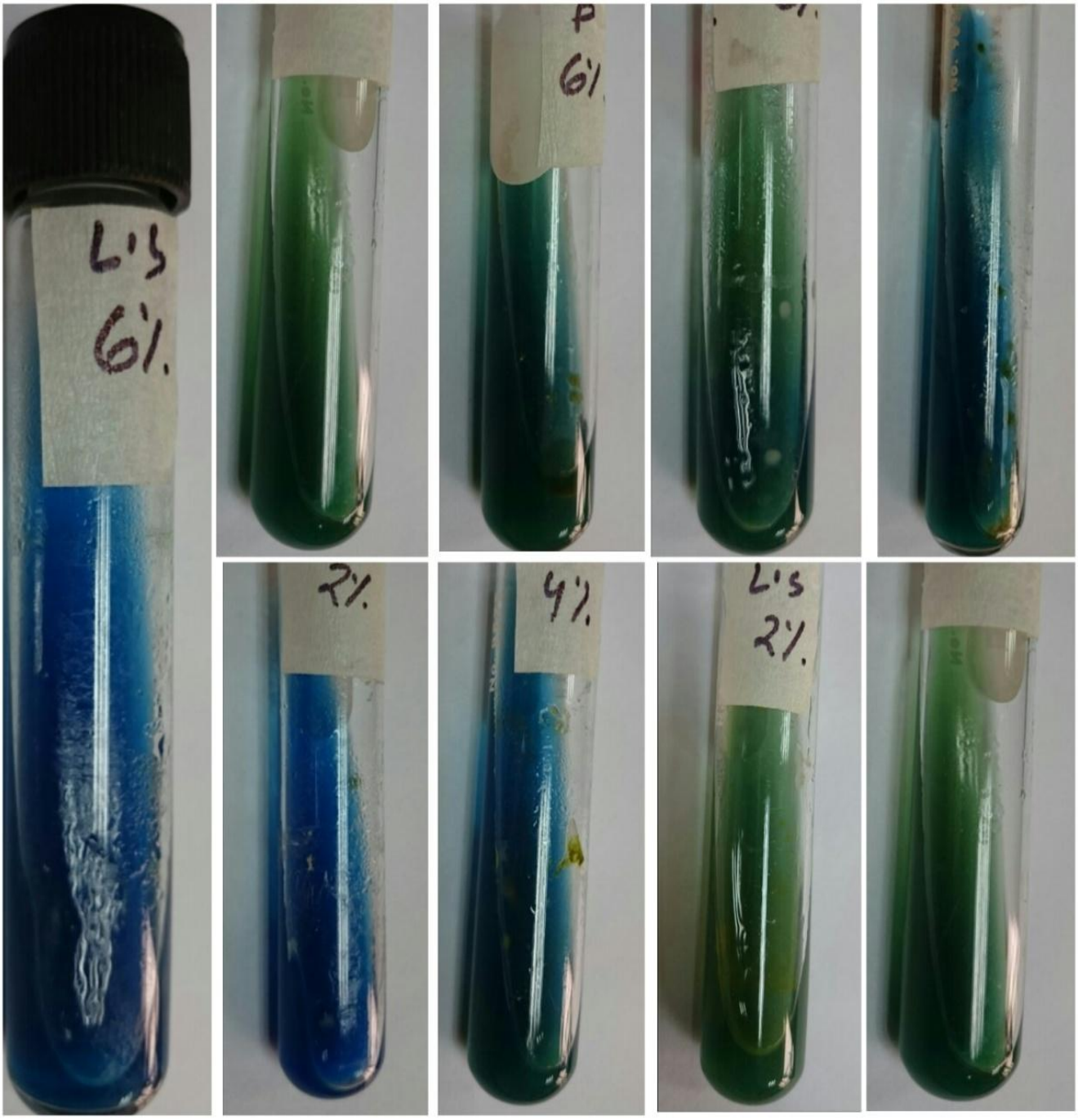

Fig: 3.3.3 Simmon's Citrate Test

Positive result

Beach soil 2%, 4% and Land soil 4%, 6%

3.3.4 MR Test

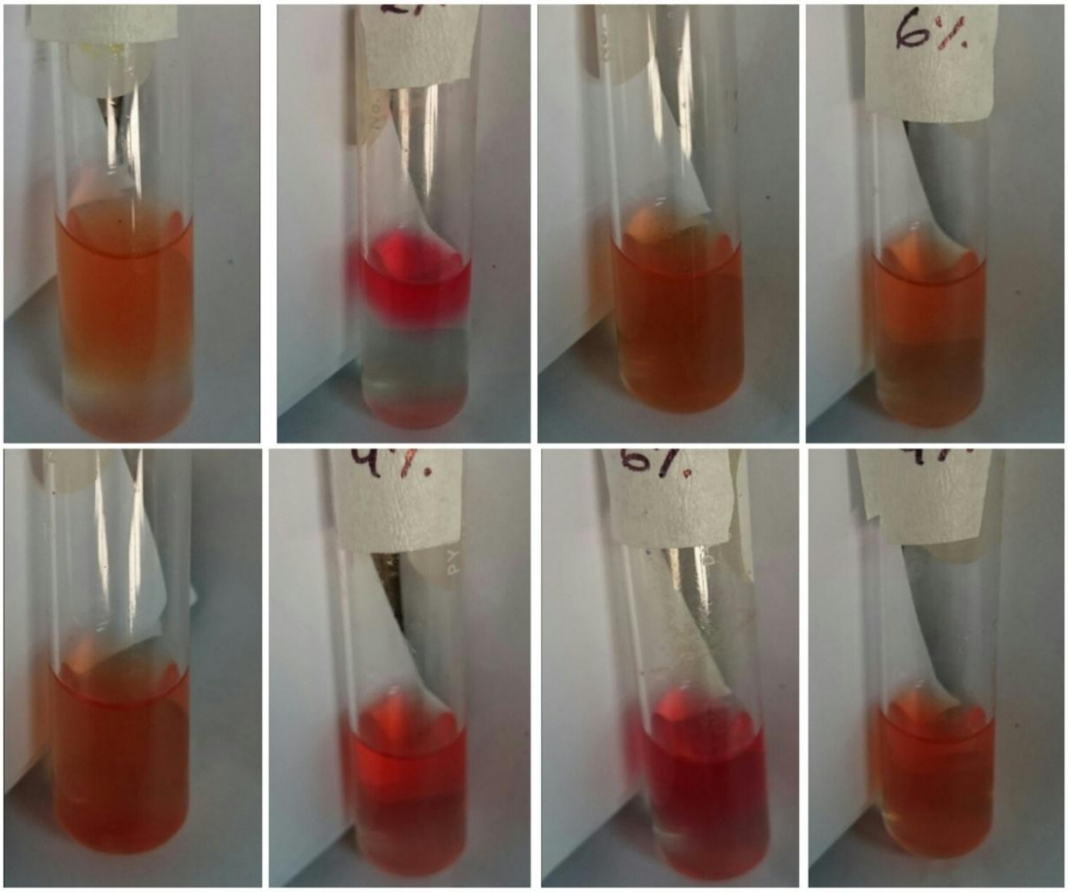

Fig: 3.3.4 Methyl Red Test

Table: 3.3.4 Methyl Red Test

Sample	Concentration	Colour	Result
Patenga area	2%	Red	Positive
Patenga area	4%	Orange	Negative
Patenga area	6%	Orange	Negative
Beach soil	2%	Red	Positive
Beach soil	4%	Red	Positive
Beach soil	6%	Red	Positive
Land soil	2%	Orange	Negative
Land soil	4%	Orange	Negative
Land soil	6%	Orange	Negative

Positive result

Beach soil 2%, 4% and 6%

3.3.5 VP Test

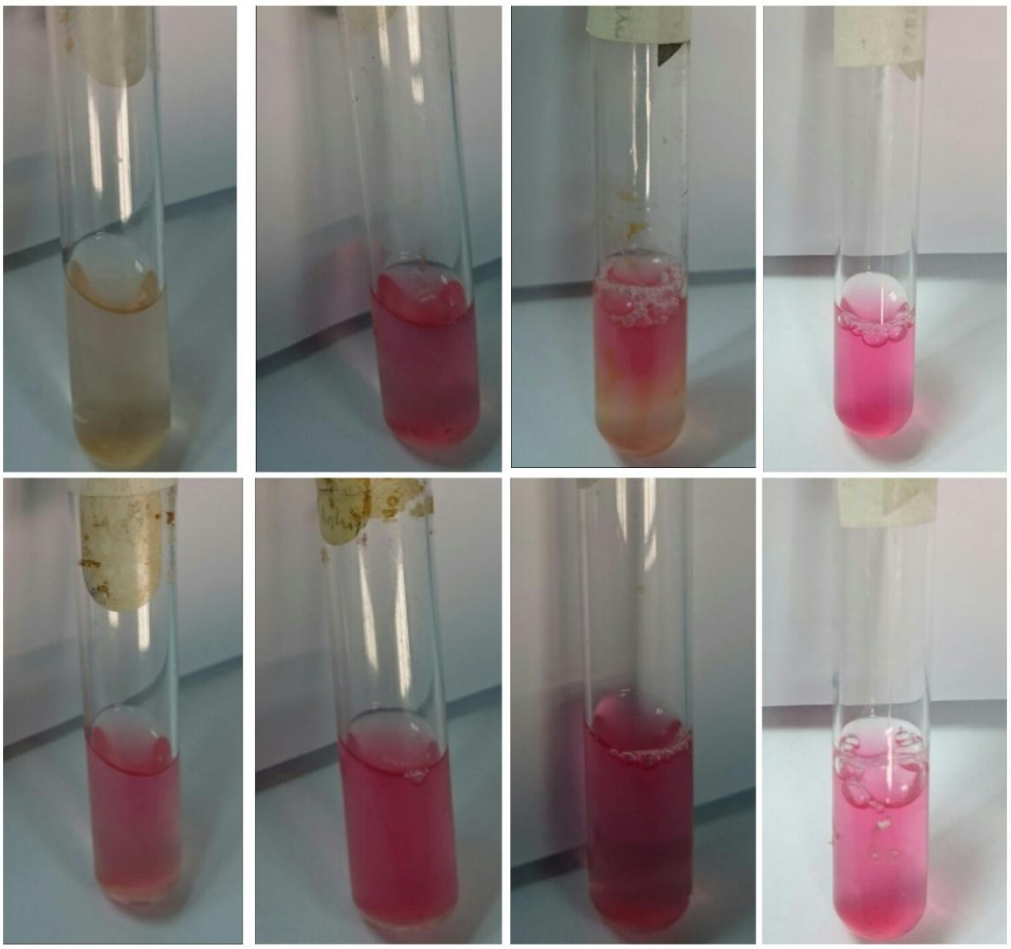

Fig: 3.3.5 Voges Proskauer Test

Table: 3.3.5 Voges Proskauer Test

Sample	Concentration	Colour	Result
Patenga area	2%	No change	Negative
Patenga area	4%	Pink	Positive
Patenga area	6%	Pink	Positive
Beach soil	2%	Pink	Positive
Beach soil	4%	Pink	Positive
Beach soil	6%	No change	Negative
Land soil	2%	Pink	Positive
Land soil	4%	Pink	Positive
Land soil	6%	Pink	Positive

Positive result

Patenga 4%, 6%, Beach soil 2%, 4% ,Land soil 2%, 4%, and 6% because the colour did not change to pink.

3.3.6 Indole Production Test

All sample result is negative. No pink ring was visible

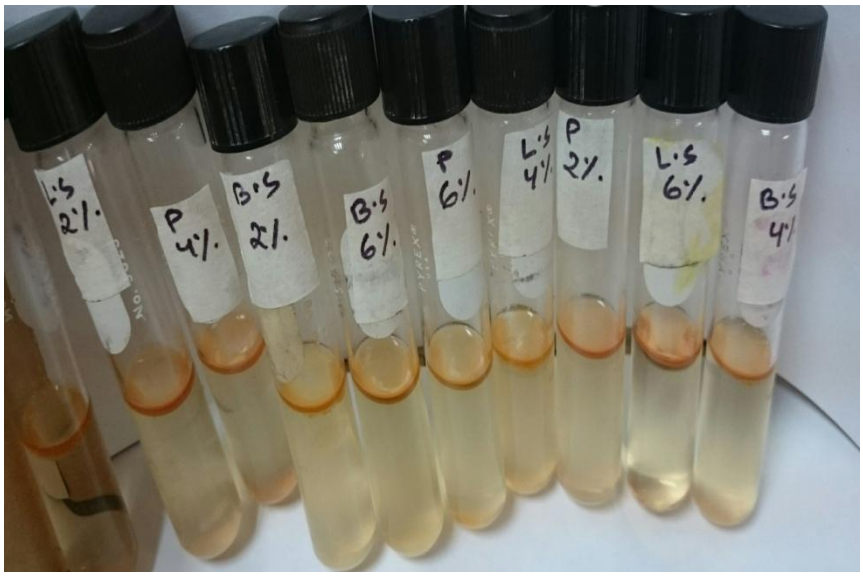

Fig: 3.3.6 Indole Production Test

3.3.7 Carbohydrate Fermentation

3.3.7.1 Phenol Red Sucrose

Table: 3.3.7.1 Phenol Red Sucrose

Sample	Concentration	Result
Patenga area	2%	Yellow
Patenga area	4%	Yellow
Patenga area	6%	Yellow
Beach soil	2%	Yellow
Beach soil	4%	Yellow
Beach soil	6%	Yellow
Land soil	2%	Red
Land soil	4%	Yellow
Land soil	6%	Yellow

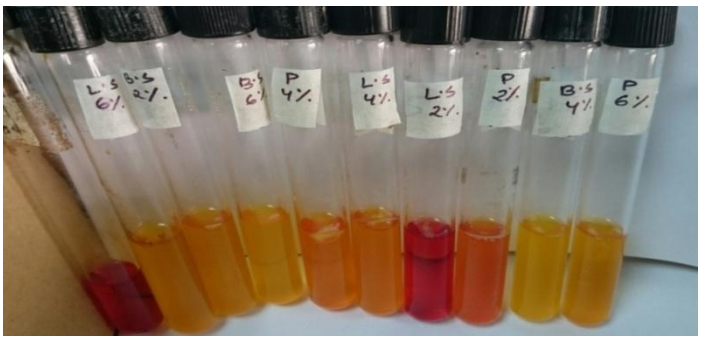

Fig: 3.3.7.1 Phenol Red Sucrose

Positive result

Land soil 4%, 6%, Patenga area 2%, 4%, 6%, Beach soil 2%, 4%, and 6%

3.3.7.2 Phenol Red Lactose

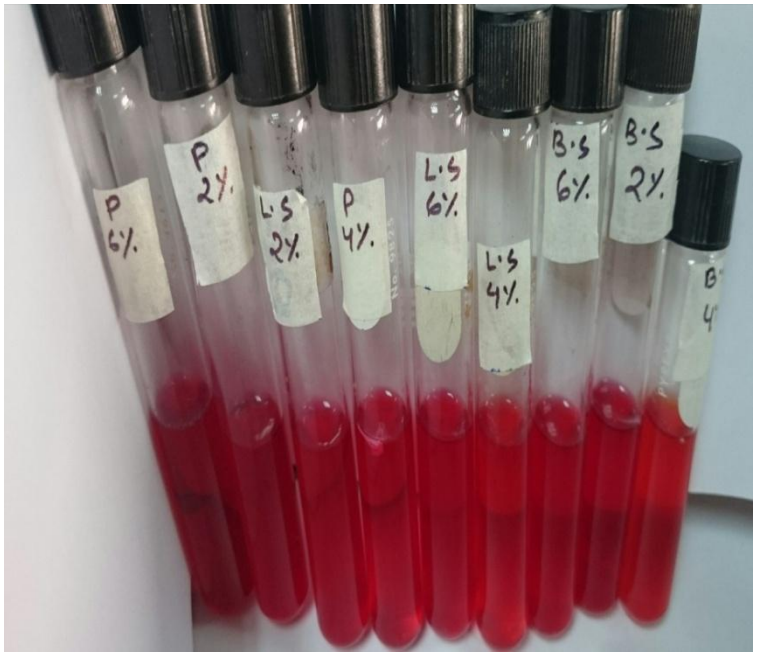

Fig: 3.3.7.2 Phenol Red Lactose

No positive result. The colour remains red

3.3.7.3 Phenol Red Dextrose

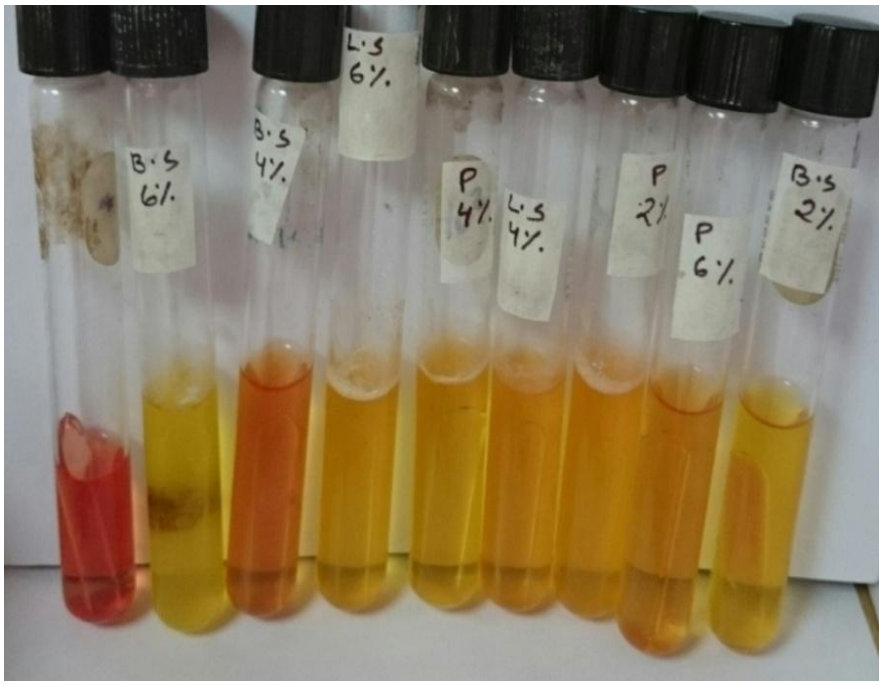

Fig: 3.3.7.3 Phenol Red Dextrose

Table: 3.3.7.3 Phenol Red Dextrose

Sample	Concentration	Colour	Gas Formation
Patenga area	2%	Yellow	Positive
Patenga area	4%	Yellow	Negative
Patenga area	6%	Yellow	Negative
Beach soil	2%	Yellow	Negative
Beach soil	4%	Yellow	Positive
Beach soil	6%	Yellow	Negative
Land soil	2%	Yellow	Negative
Land soil	4%	Yellow	Negative
Land soil	6%	Yellow	Negative

All the sample result is positive. For gas formation positive is Patenga area 2% and Beach soil 4%.

3.3.8 Nitrate Reduction

Fig: 3.3.8 Nitrate Reduction Test

Table: 3.3.8 Nitrate Reduction

Sample	Concentration	Colour	Addition of zinc powder
Patenga area	2%	Pink	No change
Patenga area	4%	Pink	Red
Patenga area	6%	Yellow	No change
Beach soil	2%	Yellow	No change
Beach soil	4%	Yellow	No change
Beach soil	6%	Yellow	No change
Land soil	2%	Red	No change
Land soil	4%	Red	No change
Land soil	6%	Pink	Red

Only the sample Land soil 2% and 4% is positive because the colour changed to red.

3.3.9 MIU Test

All negative result that is they are nonmotile

Patenga 6% is urease positive

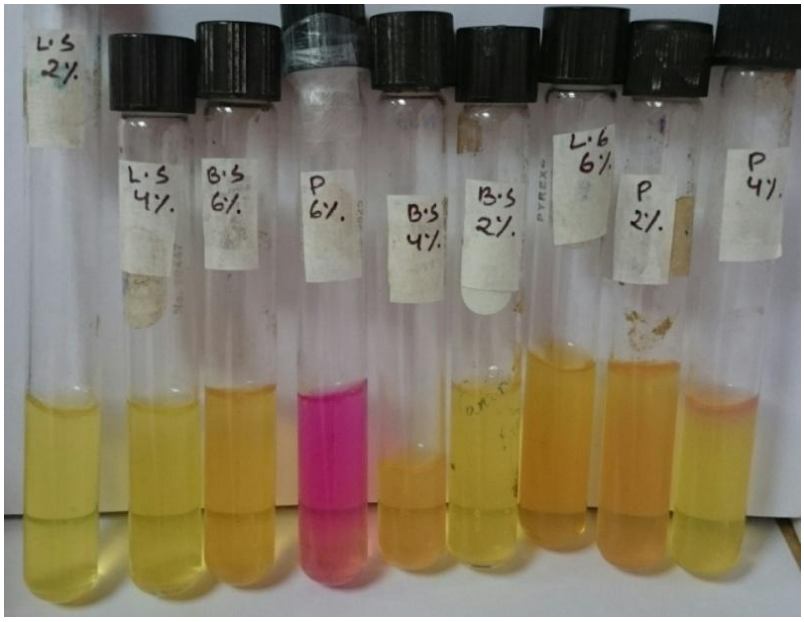

Fig: 3.3.9 MIU Test

3.3.10 Oxidase Test

All negative result.

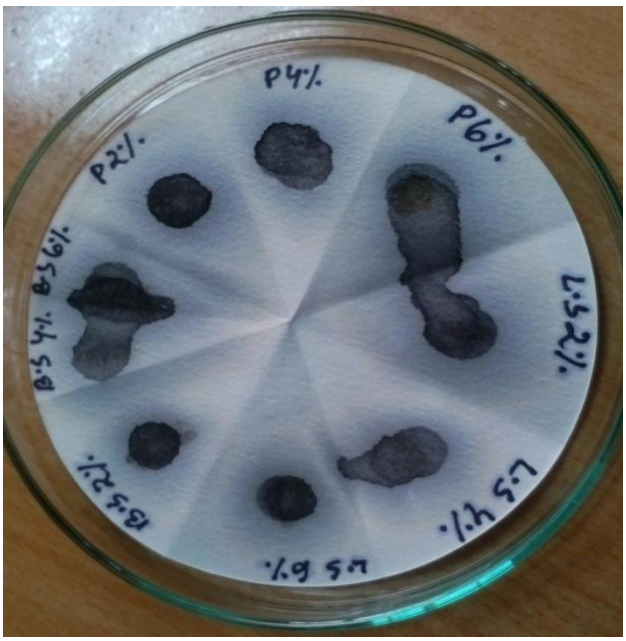

Fig: 3.3.10 Oxidase Test

3.3.11 Starch Hydrolysis

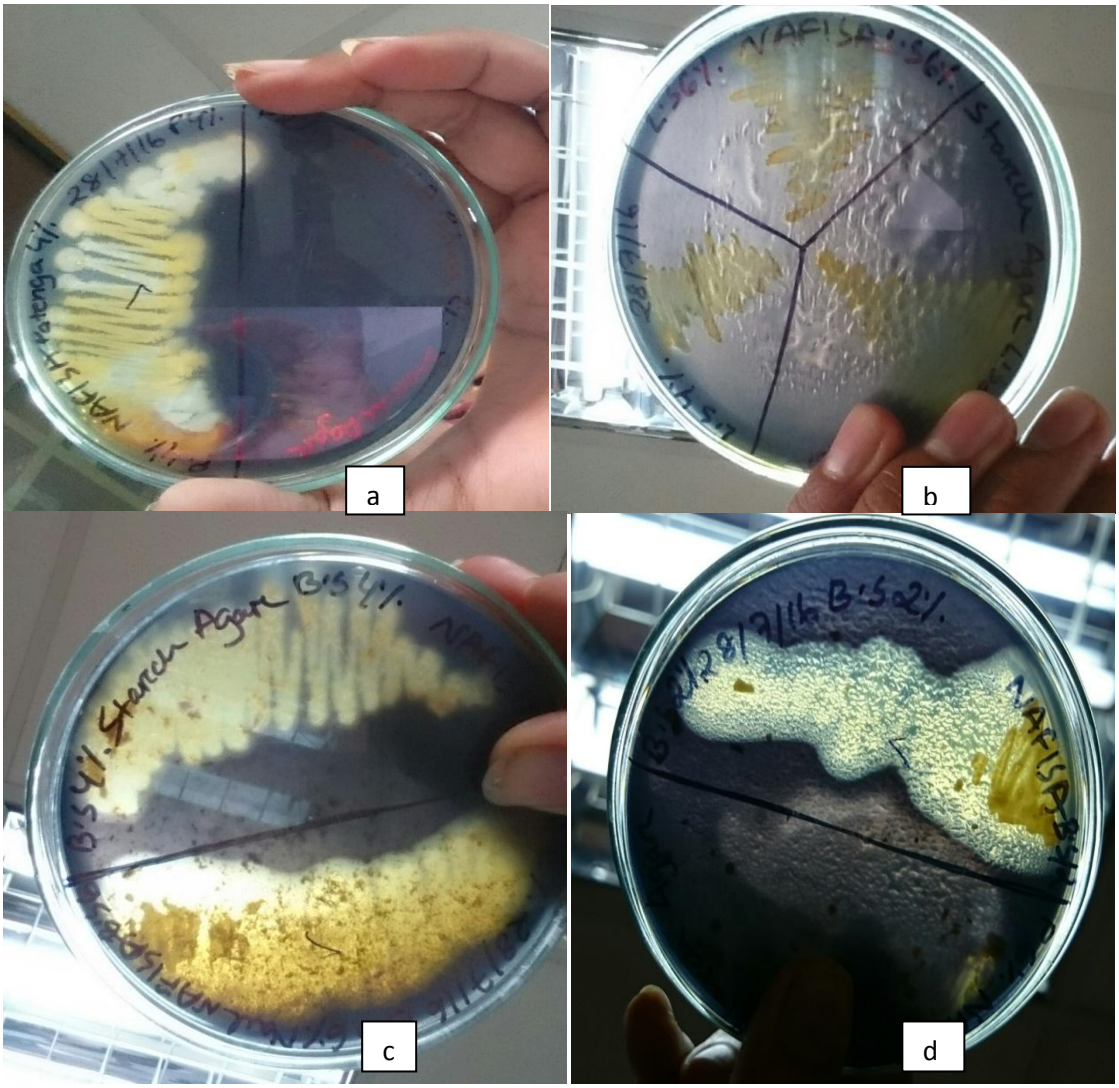

Fig:3.3.11a) Patenga 4%&2% b) Land soil2%,4%&6% c) Beach soil 6%&4%d)Beachsoil2%& Patenga6%

Table: 3.3.11 Starch Hydrolysis

Sample	Concentration	Observation	Result
Patenga area	2%	No clear zone	Negative
Patenga area	4%	Clear zone	Positive
Patenga area	6%	No clear zone	Negative
Beach soil	2%	Clear zone	Positive
Beach soil	4%	Clear zone	Positive
Beach soil	6%	Clear zone	Positive
Land soil	2%	No clear zone	Negative
Land soil	4%	No clear zone	Negative
Land soil	6%	No clear zone	Negative

Positive result

Patenga area 4% and Beach soil 2%, 4%, 6%.

3.3.12 Catalase Test

Table: 3.3.12 Catalase Test

Sample	Concentration	Observation	Result
Patenga area	2%	Bubble	Positive
Patenga area	4%	No bubble	Negative
Patenga area	6%	Bubble	Positive
Beach soil	2%	Bubble	Positive
Beach soil	4%	No bubble	Negative
Beach soil	6%	No bubble	Negative
Land soil	2%	No bubble	Negative
Land soil	4%	No bubble	Negative
Land soil	6%	No bubble	Negative

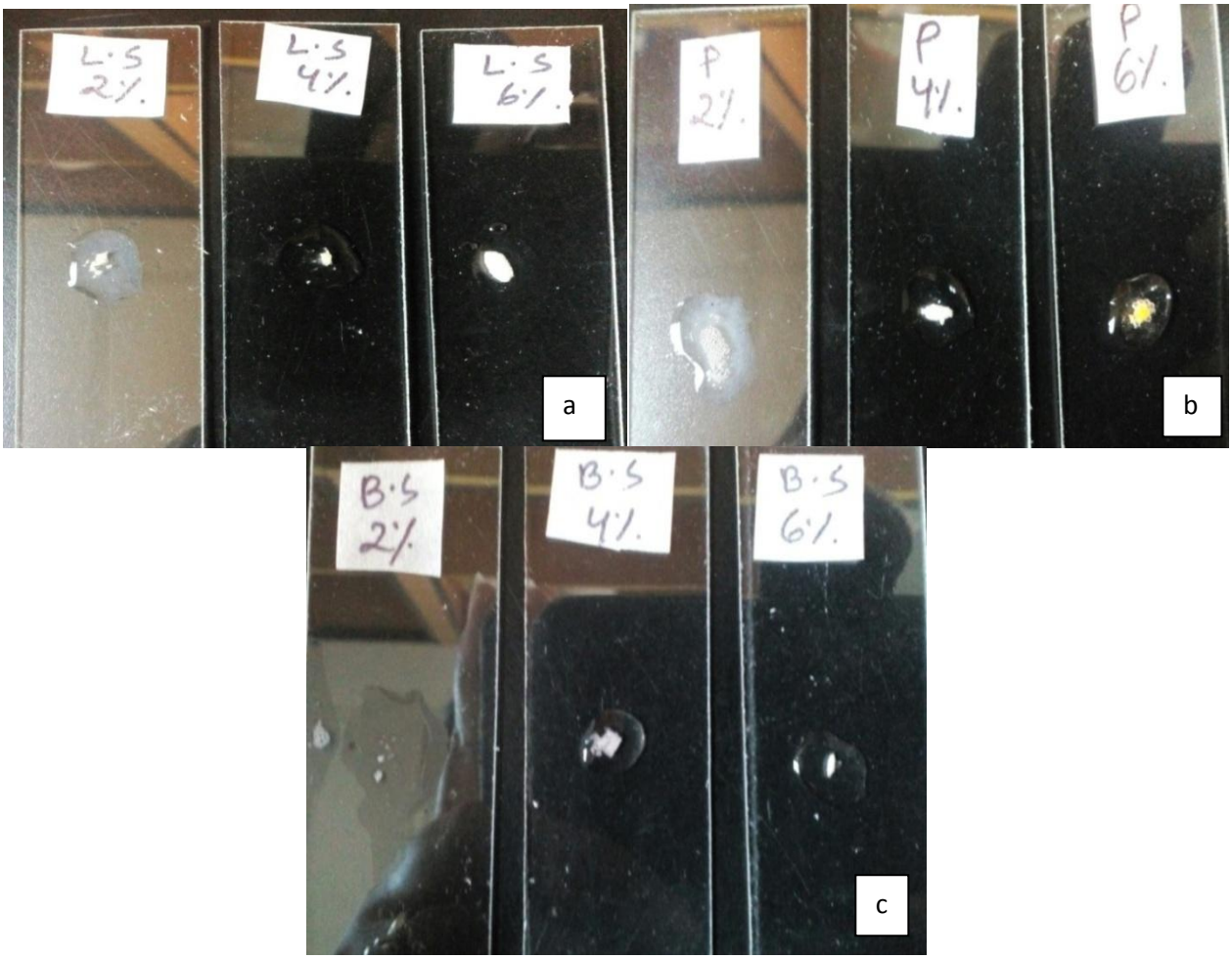

Fig:3.3.12a)Land soil 4%,2%&6% b)Patenga 2%,4%&6% c)Beach soil 2%,4%&6%

Positive result-Patenga 4%, 6% and Beach soil 2%.

3.3.13 Growth at 6.5% NaCl solution

Table: 3.3.13a Growth at 6.5% NaCl solution

Sample	Concentration	Observation	Result
Patenga area	2%	Turbid	Positive
Patenga area	4%	Turbid	Positive
Patenga area	6%	Clear	Negative
Beach soil	2%	Turbid	Positive
Beach soil	4%	Clear	Negative
Beach soil	6%	Turbid	Positive
Land soil	2%	Turbid	Positive
Land soil	4%	Turbid	Positive
Land soil	6%	Turbid	Positive

Positive result

Patenga 2%, 4%, Beach soil 2%, 6%, Land soil 2%, 4% and 6%.

Table: 3.3.13b OD Measurement

Sample	Concentration	OD (nm)
Control	Nil	0.131
Patenga area	2%	0.148
Patenga area	4%	0.384
Patenga area	6%	0.012
Beach soil	2%	0.042
Beach soil	4%	0.015
Beach soil	6%	0.129
Land soil	2%	0.157
Land soil	4%	0.136
Land soil	6%	0.188

3.4 Identification Results

Finally the test results were analyzed in ABIS software. This software is used to analyze the genus of the organism. The following are the results:

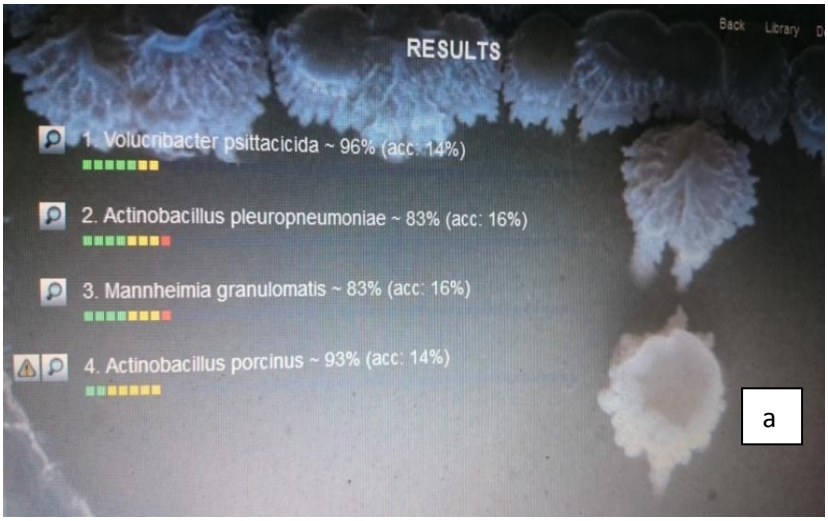

Fig: 3.4a Beach soil 6% -*Volucribacter psittacicida*

Fig3.4b Beach soil 2%- *Vibrio metschnikovii*

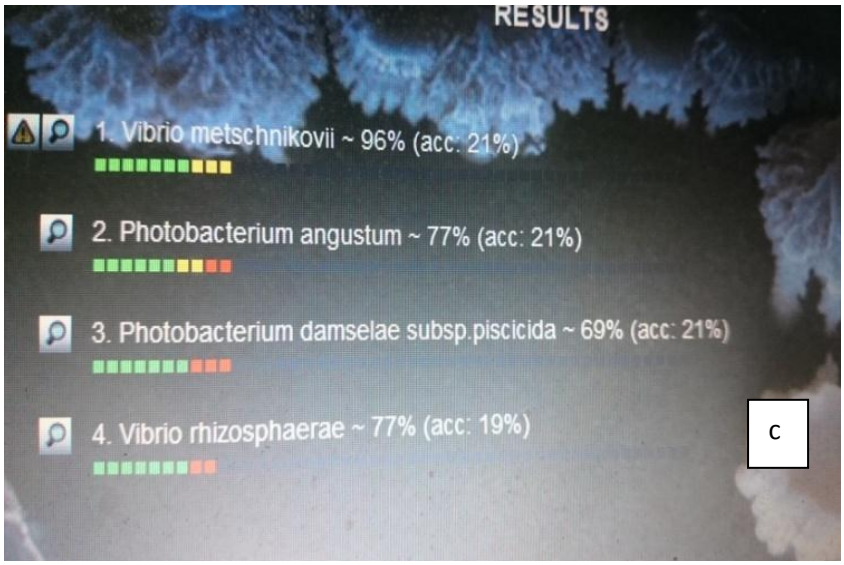

Fig: 3.4c Beach soil 4%- *Vibrio metschnikovii*

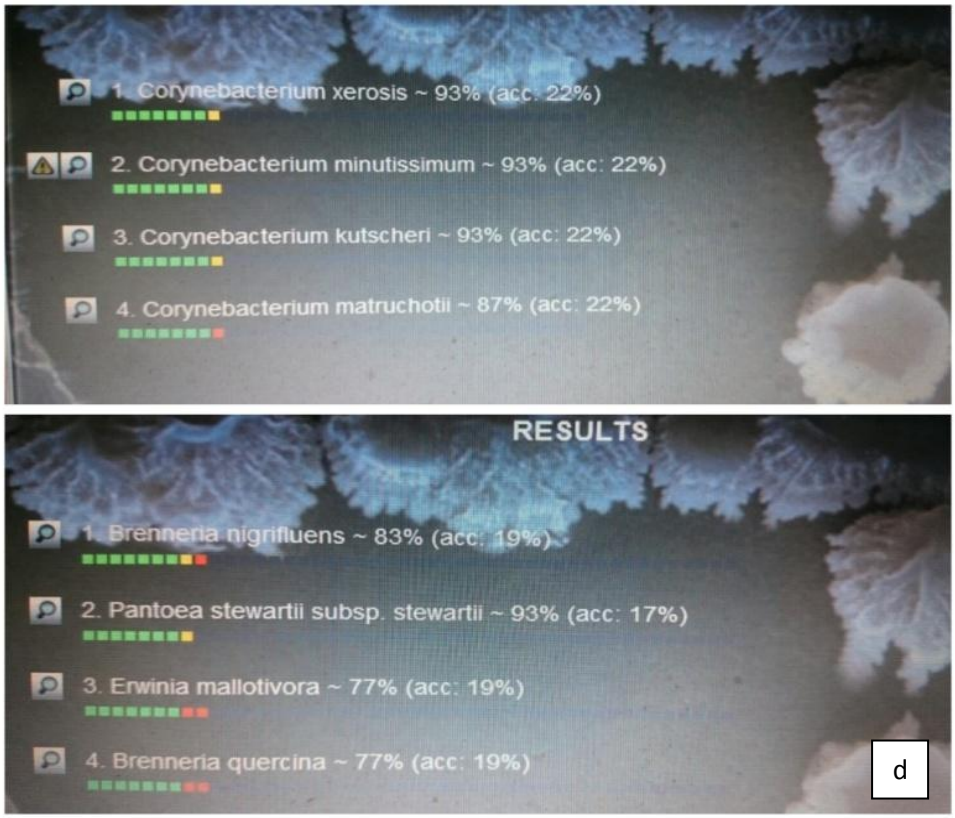

Fig: 3.4d Patenga 6%- *Corynebacterium xerosis*

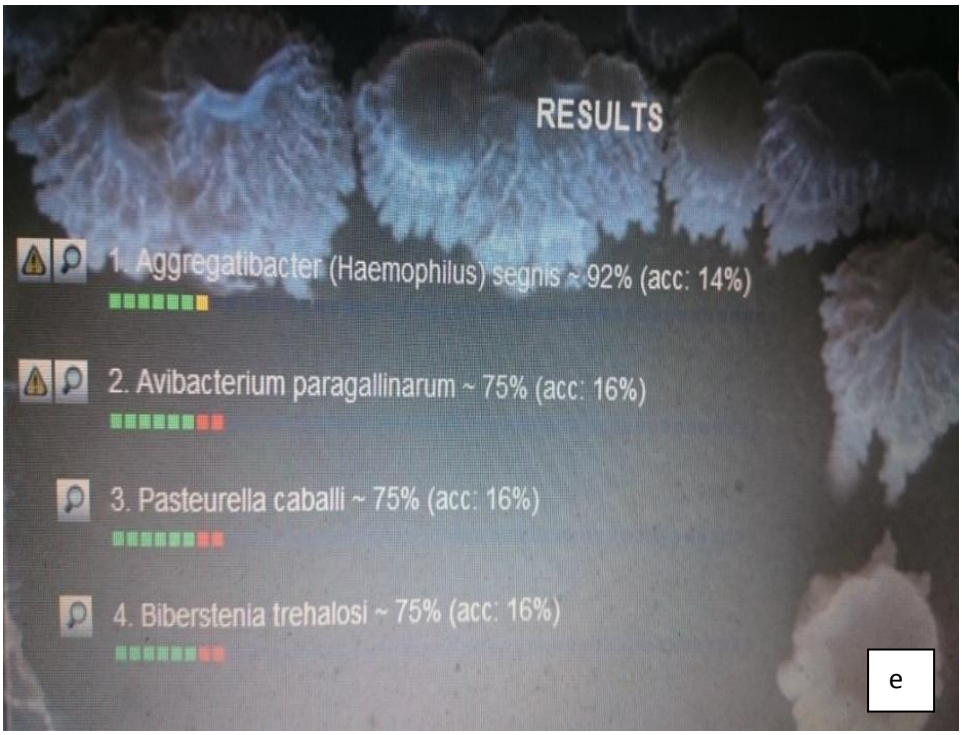

Fig: 3.4e Land soil 2%- *Aggregatibacter (Haemophilus) segnis*

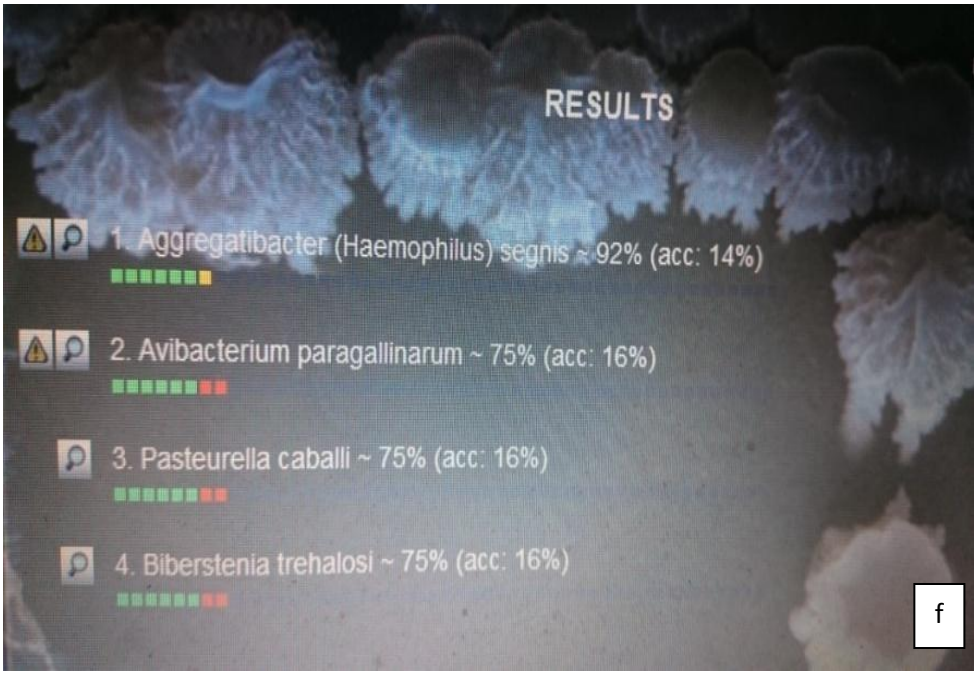

Fig: 3.4f Land soil 4%- *Aggregatibacter (Haemophilus) segnis*

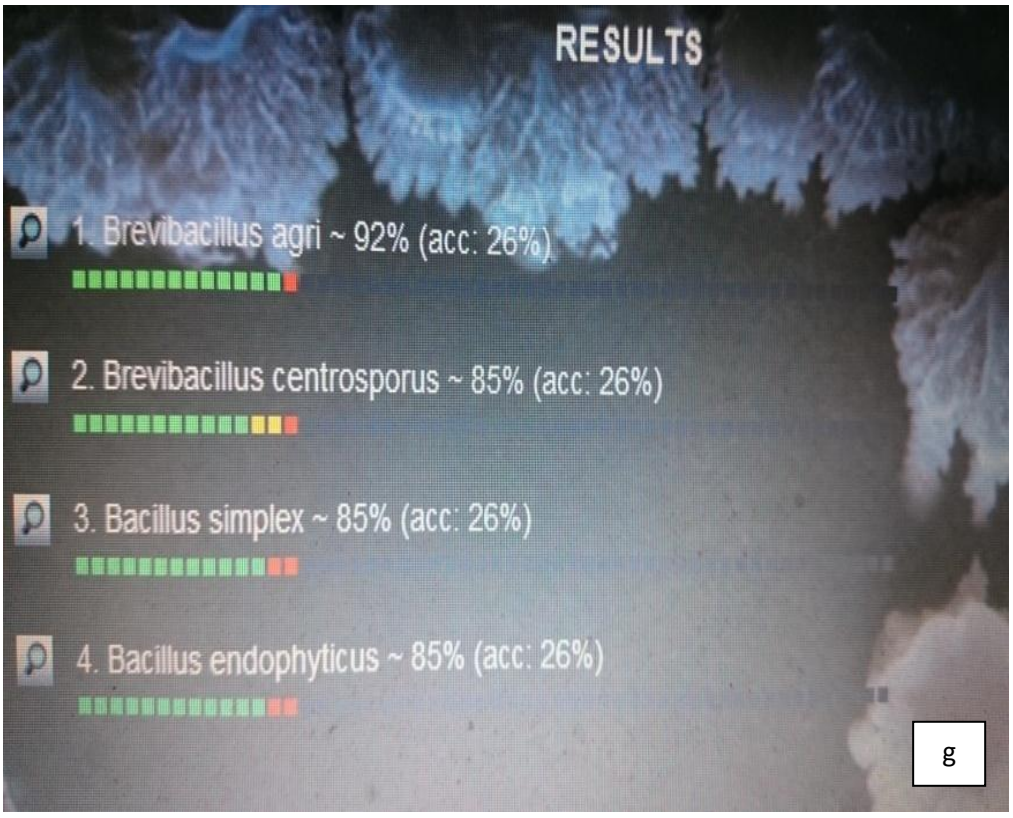

Fig: 3.4gPatenga 2%- *Brevibacillus agri*

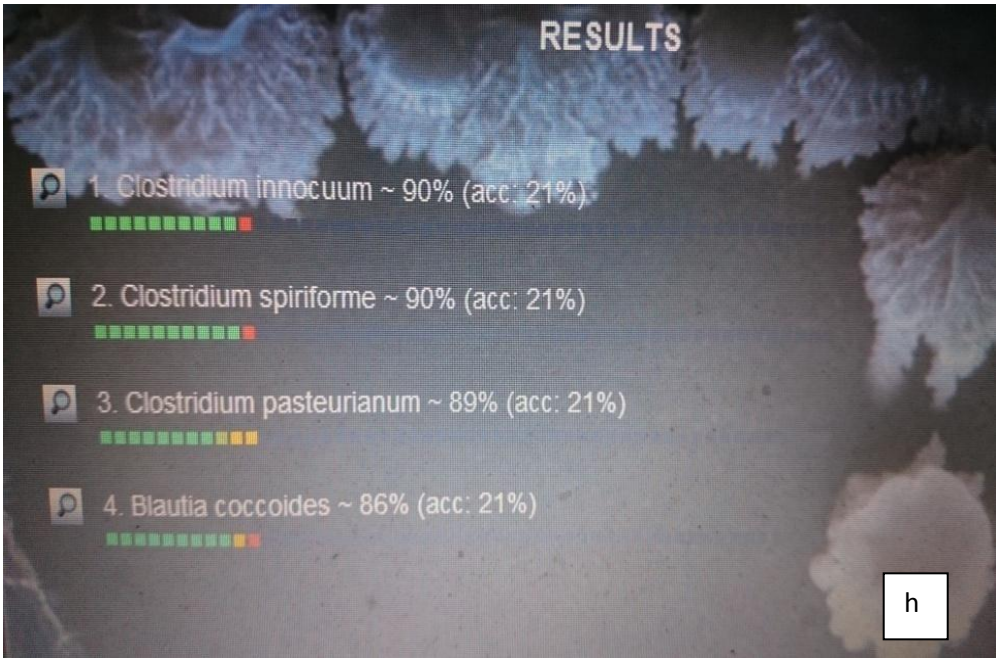

Fig: 3.4hLand soil 6%- *Clostridium innocuum*

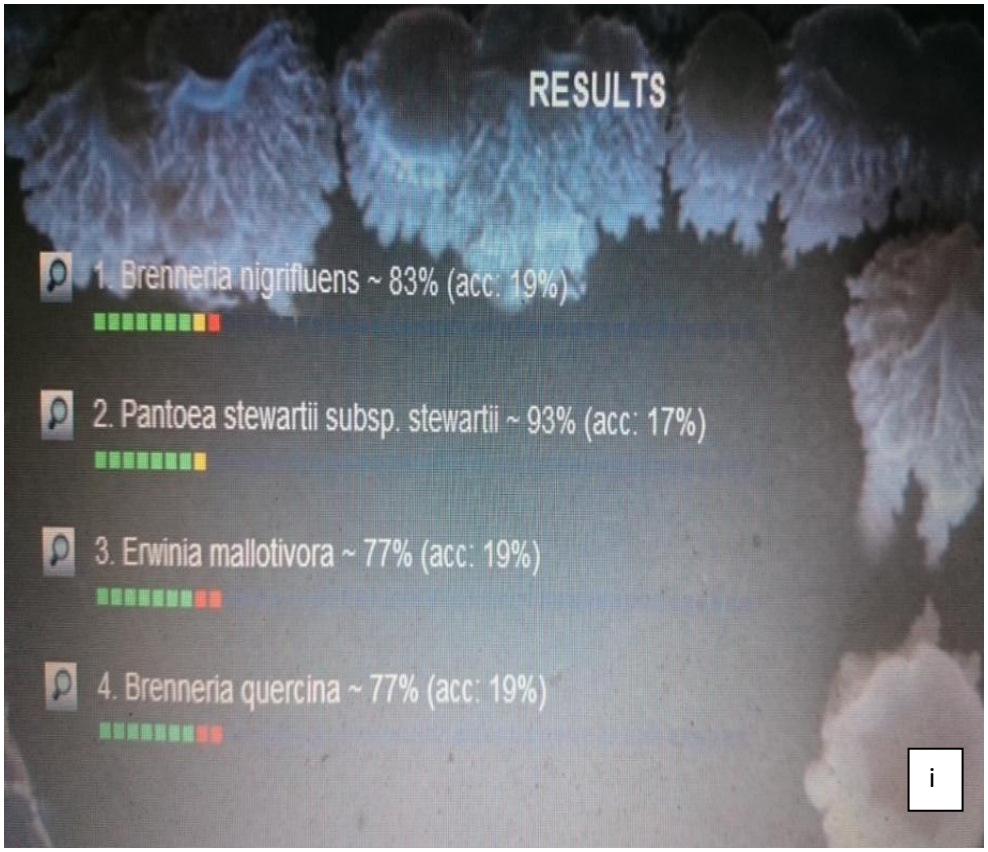

Fig: 3.4i Patenga 4%-*Pantoea stewartii subsp.stewartii*

Based on the morphology characteristics, Biochemical test and ABIS software the following results were predicted:

Beach soil 6% -*Volucribacter psittacicida*

Patenga 4%-*Pantoea stewartii subsp.stewartii*

Land soil 6%- *Clostridium innocuum*

Patenga 2%-*Brevibacillus agri*

Land soil 4%-*Aggregatibacter (Haemophilus) segnis*

Land soil 2%-*Aggregatibacter (Haemophilus) segnis*

Patenga 6%-*Corynebacterium xerosis*

Beach soil 4%-*Vibrio metschnikovii*

Beach soil 2%-*Vibrio metschnikovii*

CHAPTER FOUR: DISCUSSION

4. Discussion

Three samples were collected from the coastal area of Chittagong. The bacteria have successfully survived in wide range of salinities. The dilution factor is inversely proportional to the no of colonies. No. of colonies decreases as the dilution factor increases. The study revealed the abundance of gram positive bacteria. All the isolates showed salt tolerance to 2% (w/v), 4% (w/v) and 6% (w/v) of NaCl. The isolates that were extracted from Patenga area that are 4%, 6% salt tolerant and the sample from Beach that are 2% tolerant showed catalase activity and all the isolates showed negative result for oxidase activity, indole production, phenol red lactose and motility. Aerobic and facultative aerobes exhibit oxidase activity whereas *Enterobacteriaceae* are oxidase negative. This proves *Enterobacteriaceae* was present in the samples. In the MIU test only the isolates extracted from Patenga area that are 4% (w/v) NaCl tolerant showed urease positive result. In addition to this, all the isolates provided positive result for phenol red dextrose that is carbohydrates have been fermented. *Pastuerellaceae* and *Vibrios* were predominating in the investigated soil along with *Corynebacterium, Clostridium* and *Enterobacteriaceae* were also detected.

In a study *Brevibacillus* sp. KUMAs1 in the rhizosphere of chilli plant (Mallick, 2015) showed the possibility of using this isolate for successful bioremediation of arsenic-contaminated crop fields. Nevertheless, *Corynebacterium xerosis* was the potent degraders of hydrocarbons (petrol and diesel) (Jyothi, 2012). The isolates were capable of degrading hydrocarbons. Halophilic and halotolerant bacteria were found to have enzymatic characteristics. They can be used for production of enzymes with different immunological properties. (Shirazian, 2016).The halotolerant bacteria produce enzymes so they can use in food, pharmaceutical industry and bioenergy industries. They can be used in the food biotechnology. In the product Thi fish sauce they are mixed with concentrated brine (25–30% NaCl) and allowed to ferment for around a year (Margesin, 2001). Halotolerant bacteria are able to produce some classes of hydrolases; lipases, proteases, amylases and cellulases, and biopolymers (Lilian C.G. Oliveira et.al 2015).There are scope of application in the composting process.

CHAPTER FIVE: CONCLUSION

5. Conclusion

The isolates extracted in this study were widely halotolerant, with best growth observed at lower salinities and no halophilism. All the isolates showed that they are 2% (w/v), 4% (w/v) and 6 %(w/v) salt tolerant. The bacterial strains *Volucribacter psittacicida*, Pantoea *stewartii subsp.stewartii*, *Clostridium innocuum*, *Brevibacillus agri, Aggregatibacter(Haemophilus)segnis ,Corynebacterium xerosis, Vibrio metschnikovii* were predicted to be present in the soil sample collected from Patenga area, beach soil and land soil. The future prospective of the research is to build up awareness. The salinity problems in Bangladesh can be reduced. Salinity hampers the crop production. In Bangladesh nearly 53% of the coastal area is affected by salinity (Haque, 2006).The agricultural land use is very poor in the coastal area. The possible remedy can be the plantation of the transgenic plants in the coastal area for better agricultural use. The salt tolerant gene can be added into the crop plants by modification. Through this study the specific gene for the halotolerant properties can be isolated and later transplanted to produce the transgenic plants. These plants can be further planted to increase the crop production rate in the coastal area. The plants give higher yield by two mechanism they uptake the salts as nutrients and store them and they can also accumulate the salts to reduce the level of salt for better growth. The procedure is cost efficient and also less time consuming but it is environmentally stable and economically efficient. The research provides future aspects of the soil microorganisms that they can adapt and can be used as a natural fertilizer when natural calamity hit the coastal region. These microbes have the capability to survive in high salt condition and this result is quite significant.

CHAPTER SIX: REFERENCES

6. References from Journals

S. A. Haque , 2006, Salinity Problems and Crop Production in Coastal Regions of Bangladesh. Review Article. Department of Soil Science, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Md. Lokman Hossain,2010, Effects of salinity Institute of Forestry and Environmental Science.

Elaine Colligan , 2011,Georgetown Journal of International Affairs , Salinity Issues in Bangladesh.

H. M. Rasel, M. R. Hasan, B. Ahmed1and M. S. U, 2013, Miah Investigation of soil and water salinity, its effect on crop production and adaptation strategy Vol.5 (8), pp.475-481,

M. Sarmast, M. H. Farpoor, M. Sarchesh mehpoor, and M. K. Eghbal,2014 , Micromorphological and Biocalcification Effects of Sporosarcina pasteurii and Sporosarcina urea in Sandy Soil Columns. J. Agr. Sci. Tech. Vol. 16: 681-693 681

Bassam Oudh Aljohny,2015, Halophilic Bacterium - A Review of New Studies Biological Science Department, Faculty of Science, King Abdulaziz University, P, O, Box 80203, Jeddah 21589, Kingdom of Saudi Arabia.DOI : http://dx.doi.org/10.13005/bbra/1874

Lilian C.G. Oliveira , Patricia Locosque Ramos , Alyne Marem , Marcia Y. Kondo , Rafael C.S. Rocha , Thiago Bertolini, Marghuel A.V. Silveira , João Batista dam Cruz , Suzan Pantaroto de Vasconcellos , Luiz Juliano, Debora N Okamoto, 2015, Halotolerant bacteria in the São Paulo Zoo composting process and their hydrolases and bioproducts.

Vijay Kothari, Meera Panchal, Namrata Srivastava, 2014, Hydrocarbon Degradation Potential of Halotolerant Bacteria Paperback.

Pejman Shirazian, Sedigheh Asad, Mohammad Ali Amoozegar, 2016, The Potential of Halophilic and Halotolerant Bacteria for the Production of AntineoplasticEnzyme: L-Asparginase and L-Glutaminase-

Huber, R. Huber, H. Stetter, K.O., 2000, Toward the ecology of hyperthermophiles: biotypes, new isolation strategies and novel metabolic properties. FEMS Microbiology Reviews. Volume 24. p. 615-623

Lavik, G. Stuermann, T. Bruechert, V. Van der Plas, 2009, A. Detoxification of sulphidic African shelf waters by blooming chemolithotrophs. Nature. Volume 457. p. 581-586

Messner, P. Pum, D. Margit, S. Stetter, K. Sleytr, U. 1986, Ultra structure of the Cell Envelope of the Archaebacteria Thermoproteus tenax and Thermoproteus neutrophilus. Journal of Bacteriology. Volume 166 Number 3 p. 1046-1054

Stetter, K.O. Fiala, G. Huber, G. Huber, R. Segerer, 1990, A. Hyperthermophilic Microorganisms. FEMS Microbiology Reviews. Volume 75. p. 117-124.

Strauss, G. Eisenreich, W. Bacher, A. Fuchs, 1992, G. 13C-NMR Study of autotrophic CO_2 fixation pathways in the sulfur-reducing Archaebacterium Thermoproteus neutrophilus and the in the phototrophic Eubacterium Chloroflexus aurantiacus. European Journal of Biochemistry. Volume 205. p. 853-866

W. Hugo Ramos-Vera, Valérie Labonté, Michael Weiss, Julia Pauly, and Georg Fuchs, 2010, Regulation of Autotrophic CO_2 Fixation in the Archaeon Thermoproteus neutrophilus Journal of Bacteriology, p. 5329-5340, Vol. 192, No. 20

Shiladitya Das Sarma, 2012, Halophiles, University of Maryland,Baltimore,Maryland,USA Priya Das Sarma, University ofMaryland,Baltimore,Maryland,USA.

Elhafid Nabti, Michael Schmid and Anton Hartmann,2015, Application of Halotolerant Bacteria to Restore Plant Growth under Salt Stress.

Mary F Roberts, 2015, Organic compatible solutes of halotolerant and halophilic microorganisms, Review.

Jyothi K, K Surendra Babu, Nancy Clara. K, Amit Kumar, 2012, Identification and Isolation of Hydrocarbon Degrading Bacteria by Molecular Characterization.

References from Websites

http://www.imb.nrc.ca/imb/sulfolob/sulinf_e.html

http://phylogeny.arizona.edu/tree/archaea/archaea.html#TOC3

http://www.sanger.ac.uk/Users/pmr/hot/organisms.html

http://megasun.bch.umontreal.ca/People/ragan/research1.html

http://members.tripod.com/~Cooldude5000/extremo.html

http://www.sciam.com/0497issue/0497marrs.html

Appendices:

Apparatus:

1. Aluminum foil papers
2. Beaker
3. Bunsen burner
4. Conical flask
5. Durham tubes
6. Filter paper
7. Falcon tubes
8. Filter papers
9. Glass pipette
10. Glass slides
11. Masking tape
12. Measuring cylinder
13. Micropipette
14. Para film
15. Petri dish
16. Spatula
17. Spirit lamp
18. Test tubes
19. Vials

Chemicals:

1. Agar powder
2. Beef extract
3. Simmon's citrate
4. Distilled water
5. Di potassium hydrogen phosphate
6. Ethanol
7. Fructose
8. Glucose
9. Lactose
10. Maltose

11 MIU agar

12. Nutrient agar
13. Peptone
14. Phenol

18. Potassium di hydrogen phosphate
19. Potassium nitrate
20. Savlon
21. Sodium chloride
22. Starch agar
23. Trypticase
24. Urea
25. Zinc powder

Machineries:
1. Autoclave machine
2. Centrifuge
3. Incubator
4. Laminar air flow
5. Microscope
6. pH meter
7. Refrigerator
8. Shaker
9. Spectrophotometer
10. Vortex machine

Soil sample is the source of the bacterial cultures which were isolated and identified with the aid of biochemical tests.

Various types of reagents were used while carrying out the biochemical tests to identify the bacterial cultures.
For catalase test:
1. Hydrogen peroxide (H_2O_2)

For Gram staining
1. Crystal violet
2. Grams iodine
3. 100% ethanol
4. Safranin

For starch test:
1. Indole

For vogues proskauer:
1. Baritt's reagent A= 5% napthol in absolute ethanol
2. Baritt's reagent B= 40% KOH in deionized water.

For oxidase test:
1. 1% kovacs oxidase reagent.