ASSESSING THE POTENTIAL OF SALT PAN BACTERIA ISOLATED FROM SALT PANS OF GOA AS A PROBIONT

A Dissertation

Course Code and Course Title: MBO381 Dissertation

Credits: 8

Submitted in partial fulfilment of Master's Degree of

Master of sciences in Marine Biotechnology

By

LOVELY ANAND

21P050012

Under Supervision of

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DATE: APRIL 2023

Examined by

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DECLARATION

I hereby declare that the data presented in this Dissertation / Internship report entitled, **"Assessing the Potential of Salt Pan Bacteria Isolated from Salt pans of Goa as a Probiont"** is based on the results of investigations carried out by me in the Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision/Mentorship of **Dr. Savita S. Kerkar** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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CERTIFICATE

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LIST OF ABBREVIATION

Abbreviation	Definition
mm	Millimetre
S. No	Serial Numbers
cm	Centimetre
l	Litres
kg	Kilograms
g	Grams
cfu	Colony forming unit
MD	Media D
GIT	Gastrointestinal tract
CNS	Central nervous system
M	Molarity
µg	microgram
FAO/WHO	Food and Agricultural organisation / World health organisation

Introduction

Introduction

Salt pans are shallow, artificial ponds designed to commercial production of salt from sea water. They are inhabited by a rich variety of microorganism, from halotolerant to moderately. Different saltpan bacteria offer an array of natural products with diverse application.

Halophilic and halotolerant bacteria secrete a wide range of hydrolytic enzymes e.g., amylases, proteases, xylanases and cellulases, into their surrounding environment useful for many industrial processes (e.g., agriculture, food, feed and drinks, detergents, textile, leather, pulp and paper. Halophiles also produces bioactive compounds which are of with medicinal importance.

Several studies have demonstrated the importance of secondary metabolites produced by marine bacteria in medicinal applications such as antidepressants, anxiety medications, dementia treatments, and cancer treatments. Secondary metabolites' effects on gut and brain health have also been explored. Gut microbiota can be controlled through the administration of probiotics and prebiotics, which can also affect brain health and be employed as a possible therapeutic agent by activating brain health (Parker et al., 2019).

Manipulation of human microbiota has already been shown to have a favourable influence on human health. Probiotics are one potential positive approach to enhancing the human microbiome.

Probiotics can be defined as living microorganisms that have a variety of health benefits when administered in sufficient quantities (Bajagai et al., 2016). Probiotics' in treating conditions such as inflammatory disease, constipation, antibiotic-associated diarrhoea, allergy-related conditions, hypertension, and diabetes have been well studied and documented (Hill et al., 2014).

Recent studies have demonstrated the potential of salt pan bacteria as probiotics in aquaculture, medicine, agriculture, and other fields (Pawaskar et al., 2022; Fernandes et al., 2019). The selection of the probiotic strain is crucial since it must be safe and beneficial to the host.

The bacterial extract with antidepressant activity pointing out the importance of bioactive compound secreted by bacteria. Taking live bacteria as probiotic can be another approach to resist from the disease.

For the assessment of the probiotic activity of bacterial strains, particular requirements have been established. It must be capable of withstanding conditions in the digestive tract such as an acidic pH and high concentrations of bile salts and digestive enzymes. The study of probiotics is mainly based on the culture-dependent method, which is mostly associated with the identification, isolation, and growth parameters in labs using conventional morphological and biochemical assays (AlKalbani et al., 2019; Mirbakhsh et al., 2022).

11 (MD100, MD107, MD256, MD264, MD265, MD327, MD461, MD471, MD473, MD837, and MD1091) different strains of halotolerant bacteria isolated from different salt pans of Goa [Ribandar and Agarwada (Pernem's salt pan)] were chosen for screening and assessment of probiotic activity potential.

The aim of this study was to explore and identify the probiotic potential of the salt pan bacteria. The work was carried out with the following objectives

1. Revival of 11 strains of salt pan bacteria from the culture collection.
2. Partial characterisation of the 11 isolates.
3. Checking the compatibility of isolated cultures to grow synergistically.
4. In vitro screening of probiotic attribute of selected bacterial isolates.
5. In vivo screening of probiotic attribute of selected bacterial isolates with Zebra fish as a host.
6. Identification of isolates by 16sRNA Sequencing.

Literature Review

Literature Review

Goa's marine salt pans are interconnected thalassohaline multi-pond systems that allow for a discontinuous salinity gradient. These ponds receive a continual influx of sea water, which is evaporated for commercial manufacture of sodium chloride, also known as natural salt. The oceans are the largest bodies of saline water, with typical salinities ranging from 32 to 35 psu (3.2% - 3.5%). Hypersaline habitats, with salinities significantly higher than normal seawater salinities, are typically formed as a result of seawater evaporation. Halophiles, or salt-loving creatures, inhabit these kinds of environments. They can endure high saline conditions and manage osmotic pressure, allowing them to resist the denaturing effects of salt in their environment. *Archeal halobacterium* sp., Cyanobacteria such as *Aphanothece halophytica*, and the green algae *Dunaliella salina* are examples of the most widely dispersed halophilic microorganisms. (Kerkar & Ranjan Das, 2016) (Kerkar Savita, 2004). Halophiles are classified based on their salt requirement for growth in hypersaline circumstances. Halotolerant bacteria, on the other hand, do not require NaCl to grow and can grow in high salinity and low salt concentration conditions.

Halophiles are categorised into three classes based on their reaction to NaCl.

Category of bacteria	NaCl% required for the growth
Slight halophiles	2-5% NaCl (0.2-0.85 M).
The moderate halophiles	5- 20% NaCl (0.85-3.4 M).
The extreme halophiles	20-30% NaCl (3.4-5.1 M).

Halotolerant organisms maintain low ionic concentrations in order to synthesise compatible solutes to maintain the osmotic level within the cytoplasm with the surrounding medium. These internal environment maintenance mechanisms and cytoplasmic membrane features assist them in adapting to changes in the saline environment like as salt lakes, salty soils, and salted food sources.

Halotolerant organisms maintain low ionic concentrations in order to synthesise compatible solutes to maintain the osmotic level within the cytoplasm with the surrounding medium. These internal environment maintenance mechanisms and cytoplasmic membrane features assist them in adapting to changes in the saline environment like as salt lakes, salty soils, and salted food sources (Rahman et al., 2017).

Characterisation of the isolates is very crucial and gram staining is a method for identifying bacteria based on their capacity to maintain the primary stain and classifying them as gram-positive or gram-negative. Gram-positive bacteria have a higher peptidoglycan content in their cell walls, which allows them to maintain a crystal violet stain during solvent treatment, whereas gram-negative bacteria have a higher lipid content.

Gram Staining Method: Gram-positive bacteria have a thicker peptidoglycan cell wall than gram-negative bacteria. It is a polymer that is 20 to 80 nm thick, whereas the peptidoglycan layer of the gram-negative cell wall is 2 to 3 nm thick and protected by an outside lipid bilayer membrane. We discovered that the majority of the supplied rods are gram positive using gram staining and KOH techniques. Gram-positive bacilli (rods) are classified based on their ability to generate spores. *Listeria* and *Corynebacterium* are spore-forming rods, whereas *Bacillus* and *Clostridia* are not. Spore-forming rods can persist in harsh settings for many years. *Nocardia* and *actinomyces* are also included in the branching filament rods. (CG; *Gram positive bacteria*)

Gram negative rod: gram negative bacteria are those which do not retain the primary stain and studies have found out that they the group of bacteria which causes major health problems examples- *E. coli*, *Psedomonas aeruginosa*

Studies have found that bacterial compatibility in combined inoculation is critical for improving growth of the host due to the synergistic effects of compatible bacteria, which may be attributed to increased production of host growth-promoting substances as well as the co-existence of bacteria in the host system (Santiago, et al., 2017).

"Probiotics are live microbial feed additives that improve microbial balance in the host animal." (FAO/WHO ,2002)

Lactobacillus, Bifidobacterium, Escherichia, Enterococcus, Bacillus, and Streptococcus are the most common bacterial genera utilised in probiotic formulations. Some *Saccharomyces* fungal strains have also been employed. Probiotics have been shown to be effective in a variety of clinical conditions, including infantile diarrhoea, antibiotic-associated diarrhoea, necrotizing enterocolitis, relapsing Clostridium difficult colitis, inflammatory bowel disease, and *Helicobacter pylori* infections as well as cancer, female uro-genital infection, and surgical infections. Lactobacillus rhamnoses strain GG has been shown to improve gut immunity (Gupta & Garg, 2009).

Probiotic properties

A dose of ($5x10^9$ CFU/day) has been recommended for at least five days to provide adequate health benefits. The microorganisms used in probiotic preparations should be generally recognised as safe, resistant to bile, hydrochloric acid, and pancreatic juice, have anti-carcinogenic activity and stimulate the immune system, have reduced intestinal permeability, produce lactic acid, and be able to survive both acidic stomach conditions and alkaline duodenum conditions.

Fermented milks, cheeses, fruit juices, wine, and sausages are examples of human foods that include mostly lactic acid bacteria. Probiotic medicines contain single and mixed cultures of living microorganisms (Gupta & Garg, 2009)

Mechanism of probiotic activity

Studies have shown that Lactic acid bacteria create a variety of metabolites such as fatty free acids, hydrogen peroxide, bacteriocins, and others that inhibit the growth of food-borne pathogens in dairy products. Enzymatic pathways can also be used by probiotics to change toxin receptors and inhibit toxin-mediated disease. Probiotic treatments also limit pathogen colonisation by competitive inhibition (Gupta & Garg, 2009).

Additional mechanisms proposed for the influence on intestinal microbiota include lower intestinal pH, release of gut protective metabolites, modulation of intestinal motility, and mucus production.

For probiotic potential the culture must withstand the different environmental factors of the gut system and must have the good enzymatic potential. Various enzymes that are necessary for the metabolic process are Amylase, cellulase, protease and lipase.

Amylase is a digestive enzyme that is mostly released by the pancreas and salivary glands but is also found in other tissues at extremely low levels. Amylases' primary job is to hydrolyse glycosidic linkages in starch molecules, reducing carbohydrates that are complex in nature to simple sugars. There are three types of amylase enzymes: alpha-, beta-, and gamma-amylase, which each work on a distinct portion of the carbohydrate molecule. Alpha-amylase is present in people, animals, plants, and microorganisms. Microbes and plants contain beta-amylase. Gamma-amylase is found in both mammals and plants (Zakowski & Bruns, 1985). Cellulase is a type of enzyme that the human body does not

produce. Its capacity to degrade cellulose aids in the digestion of cellulose, which, while not immediately assimilable, is an important plant fibre for the healthy functioning of the flora of the intestines (Ejaz et al., 2021). Proteolytic enzymes (also known as peptidases, proteases, and proteinases) are enzymes that can hydrolyse peptide bonds in proteins. They are found in all living creatures, including viruses, animals, and humans. Because of their critical function in biological processes and the life-cycle of many pathogens, proteolytic enzymes are extremely important in medicine and pharmaceuticals (Mótyán et al., 2013). Lipase is an enzyme that catalyses the hydrolysis of triglyceride ester linkages, converting them to free fatty acids and glycerol.[5] Lipases are enzymes found in pancreatic secretions that aid in fat breakdown and metabolism. They are involved in lipid transport and have specific activities in different tissues, such as hepatic lipase in the liver, hormone-sensitive lipases in adipocytes, lipoprotein lipase in endothelial cells, and lipase from the pancreas in the small intestinal tract (Salah et al., 2006).

Microbes are generally found in complex ecosystems with numerous different species. These microbial communities are critical for humans because they reside in and on our bodies and have the ability to influence our health and well-being.

On the other hand, because many metabolic events involve the turnover of protons, microorganisms affect the pH around them. pH plays a vital role for all microorganisms, this regulates their growth behaviour and the interactions between different bacterial species based on how each species reacts to pH variations in the environment (Ratzke & Gore, 2018). Additionally, ingestion of microbes as probiotic need to pass through different extreme pH variation of gut. pH tolerance test of microbes is thus an essential test to understand its efficacy as probiont.

The gastrointestinal mucosa serves as the major interface between the outside world and the immune system. Antigen transit increases when intestinal microbiota is reduced, demonstrating that normal gut microflora maintains gut defences. To initiate immunological signals, non-pathogenic probiotic bacteria interact with gut epithelial cells and immune cells. These bacteria must interact with M cells in Peyer's patches, gut epithelial cells, and immune cells linked with them. It has been demonstrated that probiotic bacteria can influence immunoglobulin synthesis (Madsen et al., 2001).

Secretory IgA is vital in mucosal immunity, helping to form a barrier against harmful bacteria and viruses. The most notable attribute caused by probiotic organisms and fermented milk yoghurt was an increase in the number of IgA generating cells (Szajewska et al., 2001).

Antibiotic susceptibility test is very important it can help us to identify which antibiotic is most effective for treating specific infection caused by bacteria. Antibiotic susceptibility testing is an in vitro test that uses the diffusion technique on agar media to determine a bacteria's sensitivity to one or more antibiotics. Its goal is to select an antibiotic to treat a bacterial infection and to use the data to track bacterial resistance to antibiotics.

Bacteria exhibit two types of antibiotic resistance: intrinsic resistance and acquired resistance. Intrinsic resistance occurs when a species was resistant to an antibiotic prior to its introduction. The term "acquired resistance" refers to a species that was once susceptible to an antibiotic but later became resistant. This is because due to the mutations and genetic variation between the closely related species which makes it difficult for the treatment.

Antimicrobial susceptibility test determines if bacteria are susceptible (can be treated with the treatment), intermediate (may be curable with the drug, but

dosage may need to be altered), or resistant (cannot be treated with the drug). (Delgado, 2020).

To establish safety guidelines for probiotic organisms, FAO and WHO recommend that probiotic strains be characterised at a minimum with a series of tests such as antibiotic resistance patterns, metabolic activities, toxin production, hemolytic activities, infectivity in immunocompromised animal models, side effects in humans, and adverse consumer outcomes.

16sRNA sequencing was done to identify bacterial diversity of the given sample up to genus level of the selected culture. The historical technique relies on a comparison of an exact morphologic and phenotypic description of type strains or typical strains with an exact morphologic and phenotypic description of the isolate to be identified. These results then compare with the standard references given in Bergey's Manual of Systematic Bacteriology or the Manual of Clinical Microbiology. (Clarridge, 2004)

The sequence of the 16S rRNA gene has been established for a vast number of bacteria. GenBank, the world's biggest database of nucleotide sequences, contains over 20 million sequences, 90,000 of which are of the 16S rRNA gene. This means that there are numerous previously recorded sequences against which one can evaluate an unknown strain's sequence. (Clarridge, 2004)

In general, 16S rRNA gene sequence comparison allows differentiation between organisms at the genus level across all major phyla of bacteria, as well as identifying strains at numerous levels, including what we now term the species and subspecies level. (Clarridge, 2004).

In 2002, FAO/WHO developed Operating Standards, which provided rules for all enterprises producing probiotic products.

These guidelines include the following:

1) Implementation of probiotics use guidelines

2) studies to identify mechanism of action in-vivo;

3) Informative/precise labelling;

4) Good manufacturing practise and production of high-quality products;

5) Phase I, II, and III clinical trials to demonstrate health benefits that are as good as or better than standard preventive or treatment for a specific ailment or disease;

6) Expansion of proven strains to benefit the oral cavity, nasopharynx, respiratory tract, stomach, vagina, bladder and skin as well as for cancer, allergies and recovery from surgery/injury.

7) development of probiotic organism that can carry vaccines to hosts and /or antiviral probiotics.

Relation between probiotic and gut- brain health.

The gut microbiota is a diverse community of bacteria, viruses, protozoa, archaea, and fungus that live in the human gastrointestinal tract (GIT).

The GIT, the microorganisms that inhabit it, and the peripheral and central nervous systems (CNS) all communicate via a complicated communication system. The microbiota-gut-brain axis (MGBA) is continually transmitting and interpreting signals from the periphery to the brain and back.

Studies have also shown that the gut health can be modified by inducing the manipulation in the gut microbiota with the help of probiotic bacteria. It can also lead to the epigenetic modification which could be used to treat psychiatric disorders (KUWAHARA et al., 2020).

The use of probiotics to treat depression and anxiety was initially proposed in 1910 and then reviewed in 2005. Probiotics are dosed in 'colony forming units' (CFU), which are living bacteria with health-improving characteristics.

The gut-brain axis (GBA) is a bidirectional communication system that connects the central and enteric nerve systems, connecting emotional and cognitive centres of the brain with peripheral digestive processes. Recent scientific discoveries have shown the relevance of gut bacteria in modulating these interactions. This relationship between microbiota and GBA appears to be bidirectional, with signalling from gut microbiota to brain and from brain to gut microbiota via neurological, endocrine, immunological, and humoral linkages (Quigley, 2011).

Halotolerant bacteria as a potential probiotic

Various studies have shown the potential probiotic effect of halotolerant one of them is three halotolerant lactobacilli (Lactobacillus plantarum, Lactobacillus pentosus, and Lactobacillus acidipiscis) isolated from ripened Mexican tropical cheese (double cream Chiapas cheese) were assessed as potential probiotics and compared to two commercial probiotic strains (Lactobacillus casei Shirota and Lactobacillus plantarum 299v) of human origin. (Melgar-Lalanne et al., 2013).

It has been already proven that salt pan bacteria have positive role in increasing the feed utilisation capacity of Litopenaeus vannamei. (Fernandes et al., 2019). Which clearly shows that salt pan bacteria can be used as a potential probiotic bacterium.

Materials and Methods

3.1. Cultures and growth conditions under different salt concentration

3.1.1 Culture streaking

Cryopreserved bacterial culture were revived in media D broth (composition:). 50ul of stock culture was added in freshly prepared sterile broth and kept for overnight incubation at 37ºC in incubator shaker. The recovered bacteria (MD100, MD107, MD256, MD264, MD265, MD327, MD461, MD464, MD471, MD837, and MD1091) were streaked on media D plates containing [composition: Trypton 15 g/L, Soyaton 5 g/L, Nacl (3.5%) 5 g/L, and Agar 15 g/L] and incubated at 37°C for 24 hours.

The growth of the cultures was examined after 24 hours. Plates stored in 4ºC for future use.

3.1.2: Identifying halotolerant or halophilic bacterial isolates.

To assess whether the isolates are halophilic or halotolerant, they were streaked on medium D plates with varying NaCl concentrations (0.5%, 3.5%, 5% and 10 %) and incubated at 37°C for 24 hours. After 24 hours the growth of the cultures was analysed.

3.2: Partial characterisation of the 11 isolates.

3.2.1 Gram staining

A thin smear of fresh culture was applied to the slide and heat fixed with a burner or spirit lamp.

Gram staining protocol kit (Himedia) was utilised to evaluate the bacteria's gram nature. The slide was flooded with Crystal violet (the primary solvent) and kept for 1 minute. It was then drained off and an iodine solution (mordant) was applied to the smear to "fix the dye.". After 1 minute, decolorizer was applied to the slide dropwise until the stain was totally removed. The slide was rinsed with water and the counterstain safranin was applied; after 1 minute, the slide was washed with water and air dried and examined under a microscope (100X oil emission).

3.2.2 Confirmatory test using KOH method

A loopful of culture was placed on a slide with a drop of 3% KOH and thoroughly mixed. The slide was then examined for sting formation.

3.3: Antibiogram for eleven bacterial isolates.

The antibiogram study was carried out according to Ribbon method to check the Compatibility of the cultures to grow together. A thick line of one bacteria culture (primary culture) was streaked over a media D agar plate and incubated at 37°C for 48 hours. After 48 hours, other test cultures were streaked perpendicular to the primary culture on the same medium D agar plate and incubated for more 24 hours at 37°C. After 24 hours, the zone of inhibition was measured, and culture showing compatibility with other culture were selected for further study.

Control plate was streaked with 11 culture and used to determine which culture showed the zone of inhibition in the presence of other culture.

3.4: Evaluation of Enzymatic Activity of Selected Strains.

Different enzyme activities (viz. Amylase, cellulase, protease and lipase) produced by bacteria were measured. Cultures were grown on Media D agar with 2 different salts concentrations, 3.5% and 0.5% representing sea water salinity and body salinity respectively.

3.4.1: Amylase activity Assay:

Cultures were spotted on medium D agar plates containing 1% starch and incubated cultured for 24 hours at 37°C. After 24 hours of incubation, plates were flooded with grams iodine, and the zone of inhibition was measured by ruler (Chamekh et al., 2019; Elyasi Far et al., 2020; Oliviera et al., 2019)

3.4.2: Cellulase activity assay:

The cultures were spotted on a 25% nutrient agar plate (composition: beef extract 10 g/L, peptone 10 g/L, sodium chloride 8 g/L, agar 15 g/L) containing 1% carboxy-methyl cellulose (pH 7–7.5) was incubated for 48 hours at 37°C and flooded with grams iodine solution and the zone of inhibition was measured by ruler (Lokapirnasari et al., 2015).

3.4.3: Lipase activity Assay:

Cultures were spotted on 1% tributylin agar plate and incubated for after 48–72 hours of incubation at 37°C (Zheng, 2018; Patel et al., 2016). Zone of clearance was measured using ruler.

3.4.4: Protease activity assay:

Skimmed milk agar plate was made by autoclaving media separately (121°C, 15 psi) for 20 minutes and autoclaving 1% skimmed milk for 10-15 minutes, allowing the media to cool to 50°C before adding skimmed milk. Cultures were spotted on 1% skimmed milk agar plate and incubated for 24 hours at 37°C. Protease activity was measured by measuring the zone of clearance created by the respective culture on plate. (Abdelmoteleb et al., 2017)

3.4.5: Hemolytic Activity

Hemolytic activity determines the hemolytic ability of the bacterial culture. To test hemolytic activity, blood agar was prepared by using 5% human sterile blood (sheep blood agar base). Cultures were spotted on blood agar plates and incubated for 24 hours at 37°C. After 24 h plates were observed for zone produced by bacteria.

3.5: Bacterial growth assessment under In Vitro conditions

3.5.1 pH tolerance:

Media D agar plates were prepared with different pH ranges (3, 7, and 9). pH was adjusted using 1N NaOH and 1N HCl. Cultures were streaked on plate and incubate overnight. The growth of the cultures was observed after 24 hours of incubation at 37°C.

3.5.2. Bile salt tolerance:

Media D agar plates were prepared with different concentrations of bile salt (0.5%, 3%, and 9%). Cultures were streaked on plate and incubate overnight. The growth of the cultures was observed after 24 hours of incubation at 37°C.

3.5.3. Antibiotic susceptibility test

The Kirby-Bauer disc diffusion method was used to test an organism's antibiotic resistance. Selected cultures were disseminated on medium D agar plates with sterile swabs, and different antibiotic discs (Himedia) were placed on the plate after 24 hours of incubation at 37°C. Ampicillin (10g), chloramphenicol (30g), erythromycin (15g), streptomycin (10g), penicillin (10g), tetracycline (30g), and kanamycin (30g) were employed in the antibiotic susceptibility test. After appropriate incubation, plates were observed and the diameter of the zone of inhibition were measured. The bacterial strains were classified as susceptible, intermediate, or resistant according to the chart given by clinical and laboratory standard institute.

3.6: Assessment of bacterial activity on Zebra Fish (*Danio rerio*).

3.6.1. Preparation of consortium.

Selected strains were inoculated in 100 ml of medium D broth and incubated for 24 hours at 37°C in a shaker incubator. After 24 hours, media was centrifuged at 10000 rpm for 20 minutes. after 24 hours; The supernatant was discarded, and a pellet of mixed bacterial strains were obtained. The pellet was resuspended in sterile 0.5% NaCl and aliquoted in microfuge tubes. These tubes were again centrifuges at 10000 for pellet extraction. The supernatant was discarded, and the pellet can be kept in 4^{o}C for future use.

3.6.2. Maintenance of fish and treatment (Experimental setup preparation).

Healthy wild type zebra fish (*Danio rerio*) were used for study. Fishes were purchased from commercial fish breeder and kept in tank for 15 days for acclimatization. Fish were maintained at 25-27^{o}C temperature and 14:10-h light-dark cycle. Fish were fed commercial food twice daily.

Two experimental tanks were set to investigate the impact of a prepared consortium. Set 1 is for control, and Set 2 is used for treatment. 5 fish were used for each set. Weight and length of fish were measured before placing the fish in respective tank. The control tank fish receive only commercial feed while treatment tank fish receive bacterial consortia and commercial feed once daily. Bacterial pellet was resuspended in 0.5 % NaCl solution, cfu/ml were counted using hemocytometer and 109 cfu were added to tank water daily for 7 days.

cfu/ml was calculated by using the following formula.

Cfu/ml = average no. of cells in 4 sets of 16 squares *10^4* dilution factor.

3.6.3. Calculating the growth indices.

At the end of the experiment tenure (7 days). Based on the observations specific growth rate (SGR), relative growth rate (RGR), survival Rate (SR) were calculated. Following parameters were calculated (final and initial), food conversion rate (FCR), relative growth rate (RGR), survival Rate (SR): (Venkat et al., 2004) (Mirbakhsh et al., 2022).

1. Specific Growth rate = $\frac{\ln [\text{final weight(g)} - \ln [\text{initial weight(g)}]}{7 \text{ days}} * 100$

2. Relative Growth Rate = $\frac{\text{Final Weight (g) – initial weight(g)}}{\text{Initial weight (g)}} * 100$

3. Food conversion rate = $\frac{\text{Food consumed (ml)}}{\text{Final weight – initial weight}}$

4. Survival Rate = $\frac{\text{No. of fish at the end of experiment (pieces)}}{\text{No. of fish at the beginning of experiment (pieces)}}$

Objective 3.7: Identification of bacterial culture.

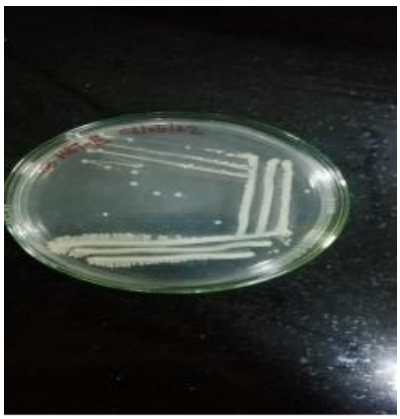
For the identification of the selected strains, 16sRNA Sequencing was done by HiMedia Sequencing kit.

Results and Discussion

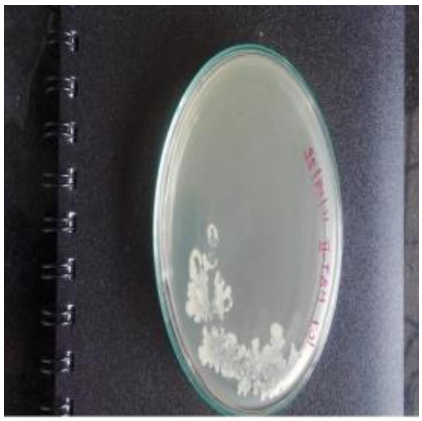
4.1: Growth of the cultures

4.1.1 Isolate recovery by using streaking method:

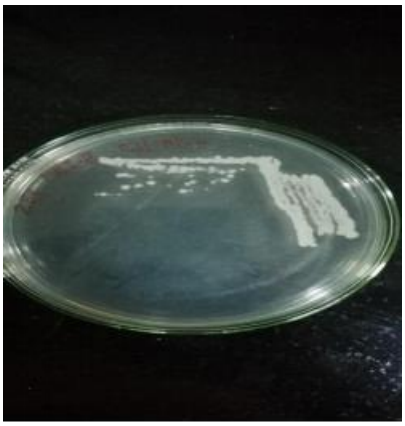
All eleven cultures were recovered on Media D agar plates containing 3.5% NaCl incubated at 37°C for 24 hr. 3.5% NaCl was the standard salt concentration of sea. All 11 cultures showed growth on media D agar plates containing 3.5% NaCl.



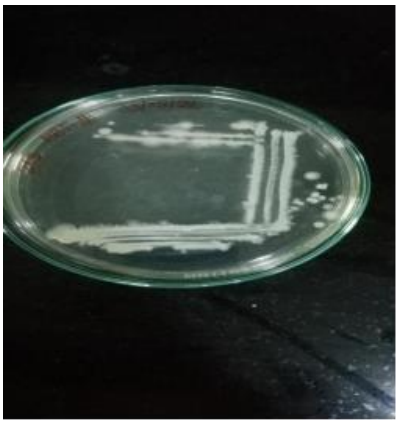
(a) MD100



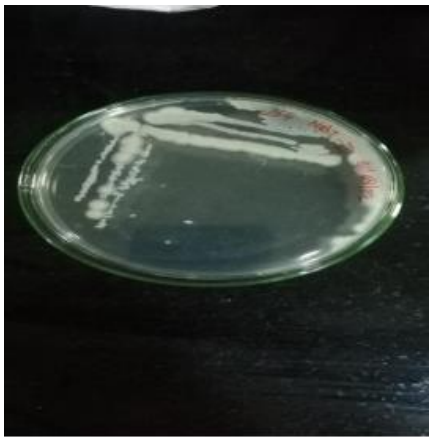
(b) MD107



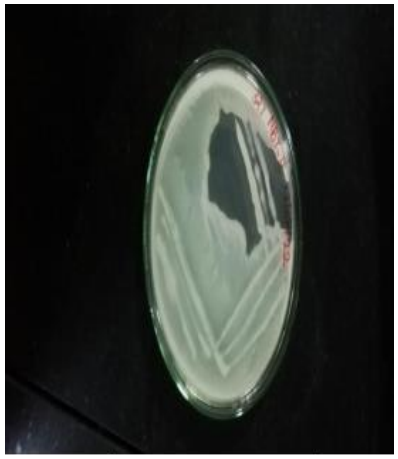
(c) MD256



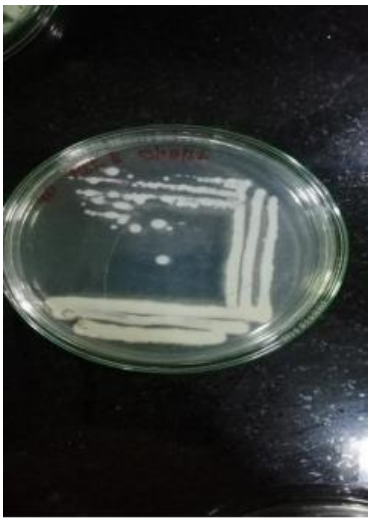
(d) MD264



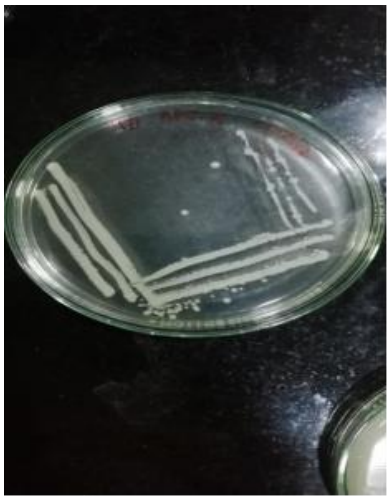
(e) MD265



(f) MD327

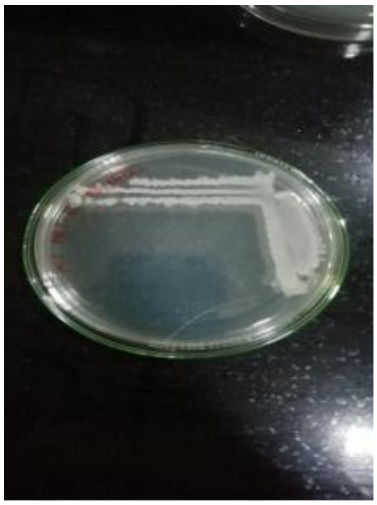


(g) MD461

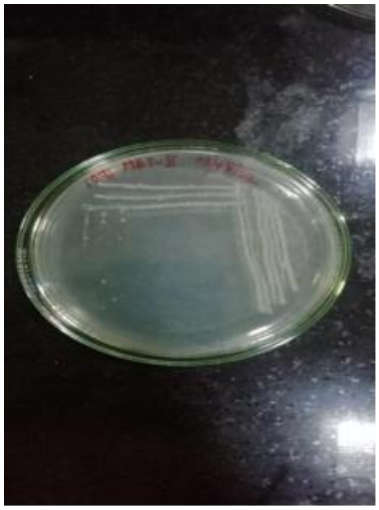


(h) MD471

(i) MD473



(j) MD837

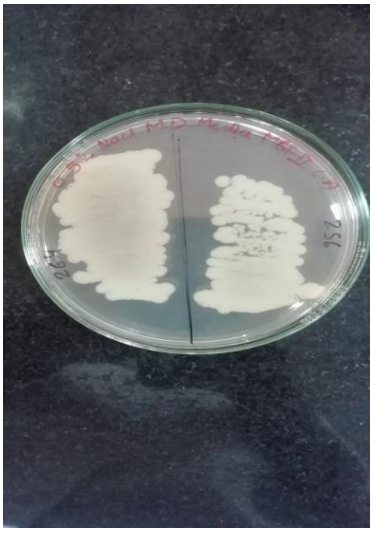


(k) MD1091

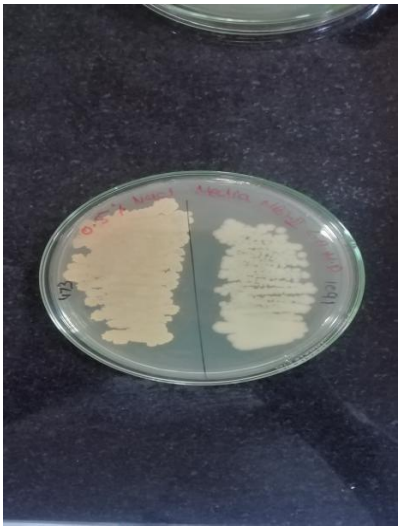
Figure 4.1.1: Plates of eleven cultures MD100, MD107, MD 256, MD264, MD265, MD327, MD461, MD471, MD473, MD837, MD1091) after 24 h incubation at 37°C .

4.1.2 Halotolerant and halophilic bacterial culture

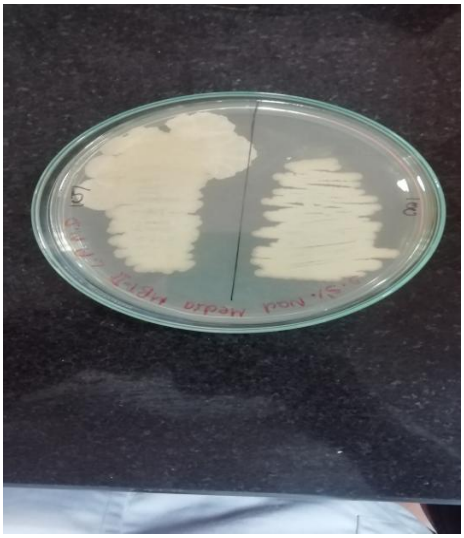
4.1.2 (a): Growth of isolates on Media D agar plate containing 0.5% Nacl.



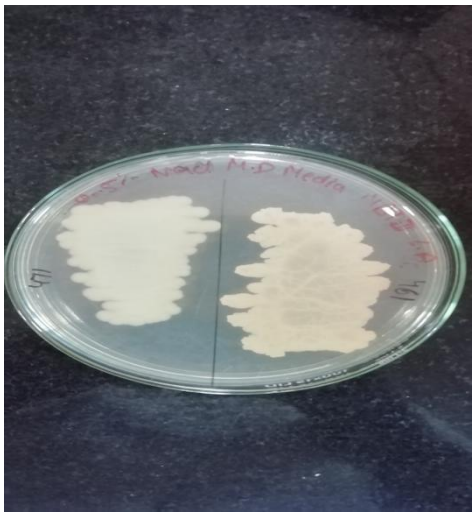
(a) (MD 264, MD256)



(b)(MD473, MD1091)

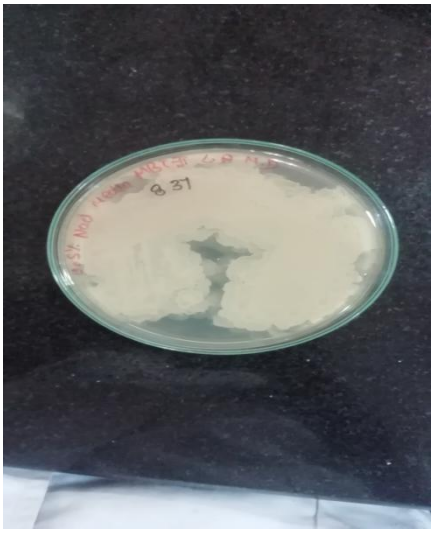


(c)(MD107, MD100).



(d) (MD471, MD461)

(e)(MD265, MD327)



(f)(MD837)

Figure 4.1.2.(a): Growth of cultures on media D agar plates containing 0.5% NaCl after 24 hours of incubation at 37°C.

All 11 cultures showed growth on agar plates containing 0.5% NaCl. Suggested that all bacterial cultures are halotolerant bacteria.

4.1.2.(b): Assessing the growth of isolates on Media D agar plate containing 10% of NaCl

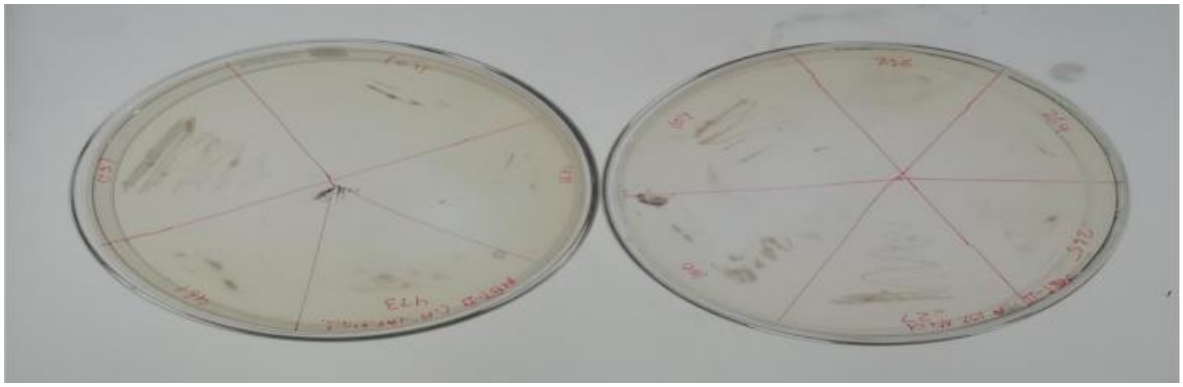


Figure 4.1.2.(b): Growth of isolated cultures on Media D agar plates containing 10% NaCl After 24 hours of incubation at 37°C.

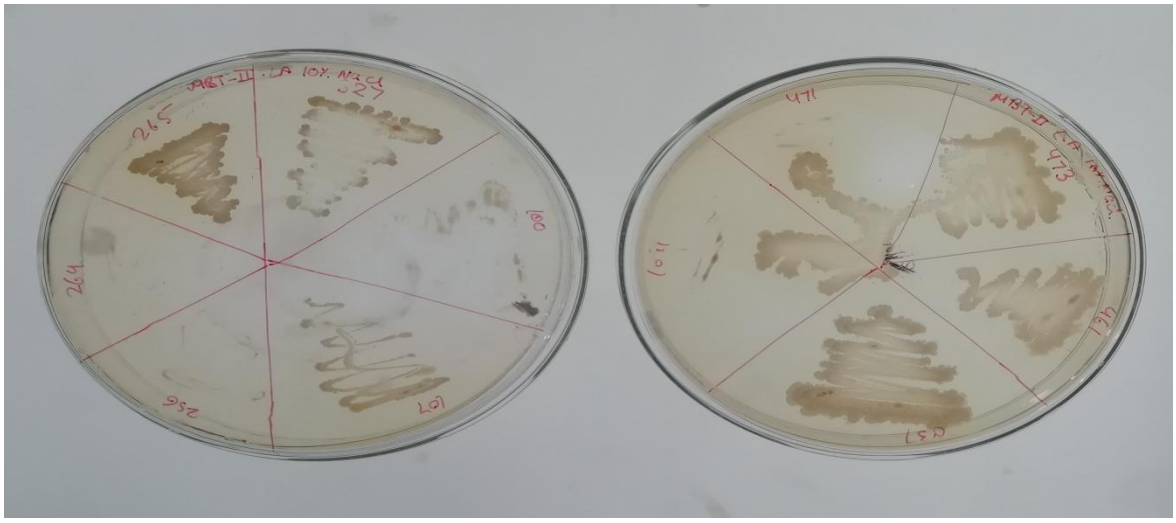


Figure 4.1.2.(b): Determination of growth of isolated cultures on Media D agar plates containing 10% NaCl after 7 Days of incubation at 37°C.

Media D plates containing 10% NaCl, after 24 hr of incubation growth was not observed. However, after 7 days of incubation growth was showed by (MD265, MD327, MD100, MD837, MD461, and MD473).

These results suggest that as all cultures are potent enough to grow within 24h under wide range of salinity (0.5 – 3.5), these cultures are halotolerant cultures. Growth of some cultures in 10% salt concentration in 7 days indicate they can withstand that much salinity and manage to grow.

4.2 Partial characterisation of isolates.

4.2.1 Gram's Staining

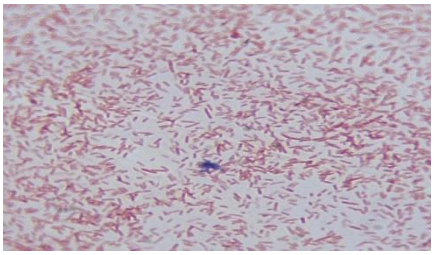
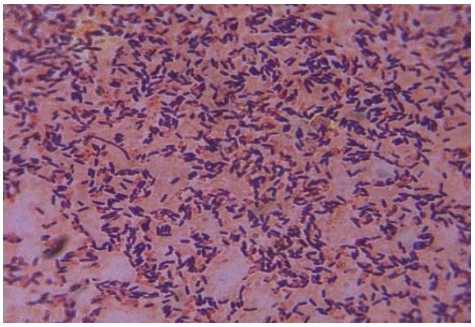
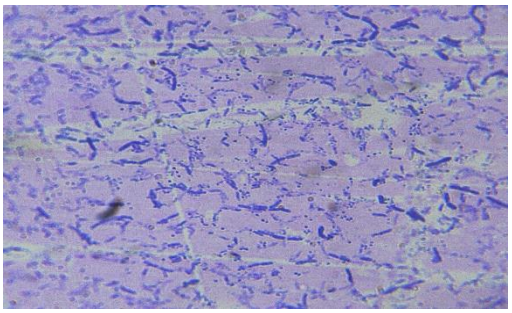


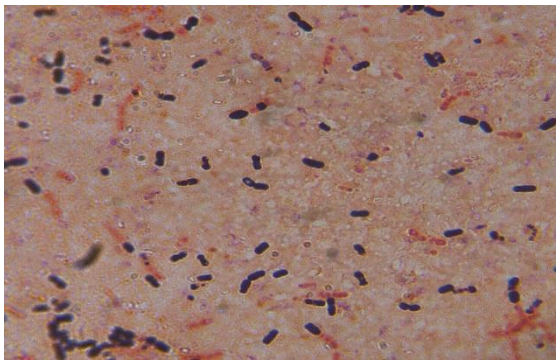
Figure 4.2.1 (a) Control E. coli (gram negative)



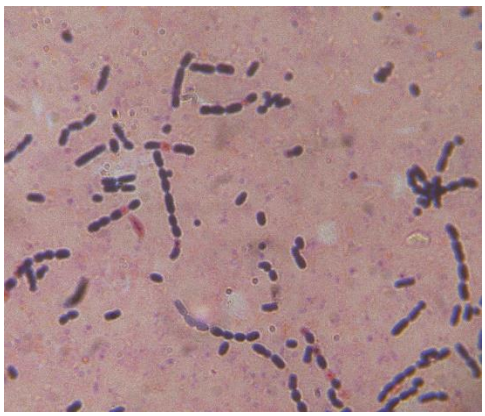
MD100(Gram positive rod)



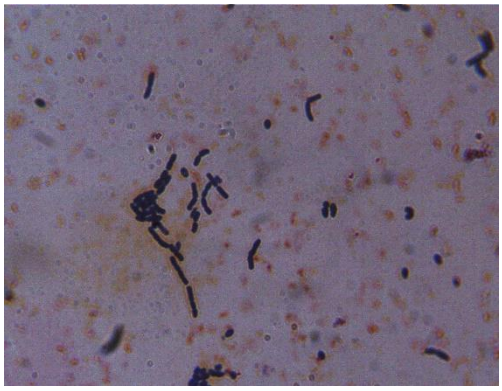
MD107(Gram positive rod)



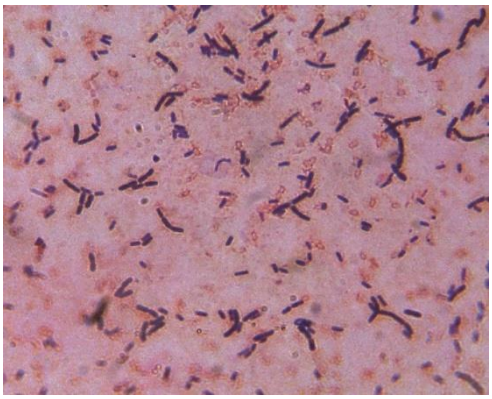
MD256(Gram positive rod)



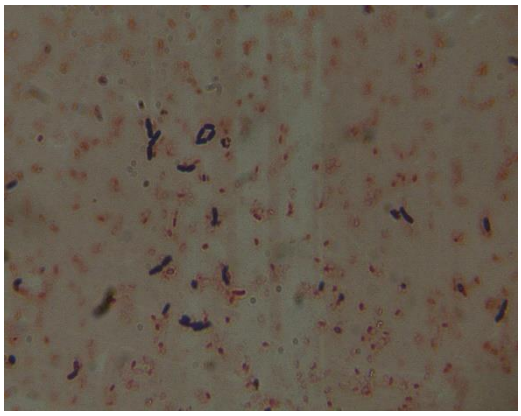
MD265 (Gram positive rod)



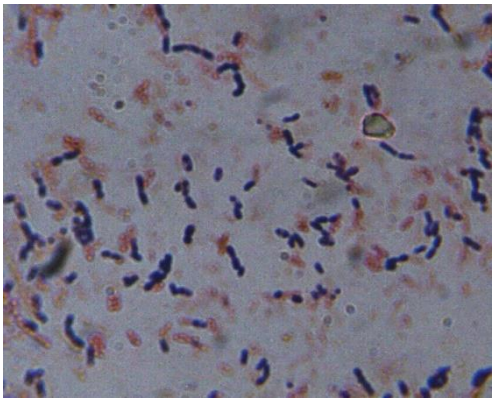
MD264 (Gram positive rod)



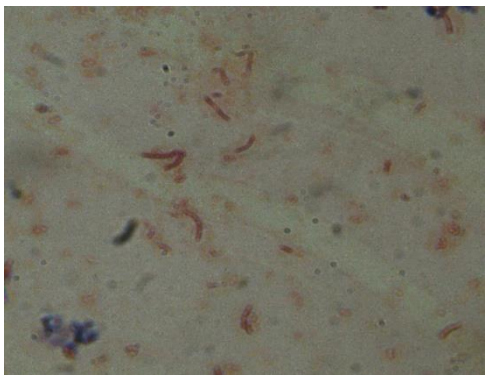
MD461 (Gram Positive rods)



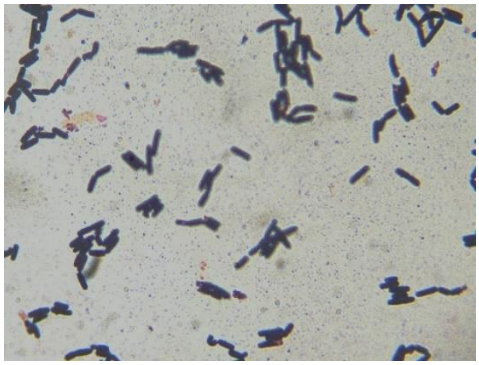
MD327 (Gram-positive rod)



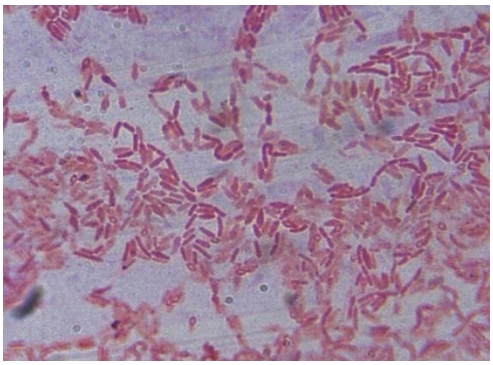
MD471 (Gram positive rod)



MD473 (Gram negative rod)



MD837(Gram Positive rod)



MD1091(Gram negative rod)

Figure 4.2.1 (b) Gram staining of isolated strains

4.2.2: 3% KOH Method

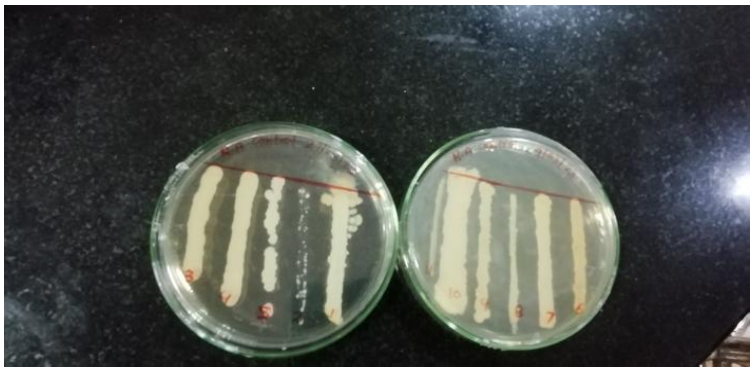
Table 4.2.2: Gram character of the given cultures by using 3% KOH

Cultures	string formation/ no string formation	Interpretation
MD100	No String formation	Gram positive
MD107	No String Formation	Gram positive
MD256	No String Formation	Gram positive
MD264	No String Formation	Gram positive
MD265	No String Formation	Gram positive
MD327	No String Formation	Gram positive
MD461	No String Formation	Gram positive
MD471	No String Formation	Gram positive
MD473	String Formation	Gram Negative
MD837	No String Formation	Gram positive
MD1091	String Formation	Gram Negative

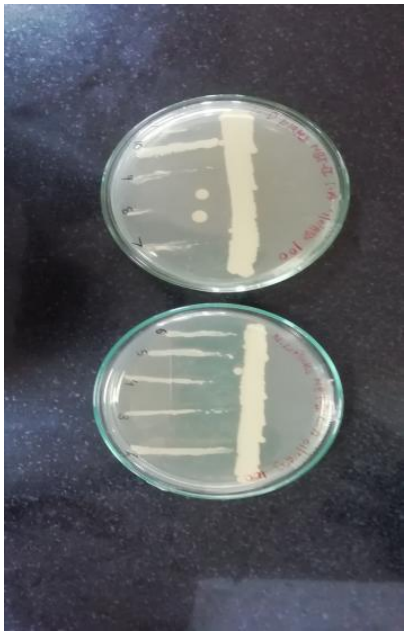
With the help of Gram's staining method and 3% KOH method we observed that most of the isolates were Gram positive (MD100, MD107, MD256, MD264, MD327, MD461, MD471, MD837). And few of them were Gram Negative (MD473, MD1091).

4.3: Compatibility between isolated cultures

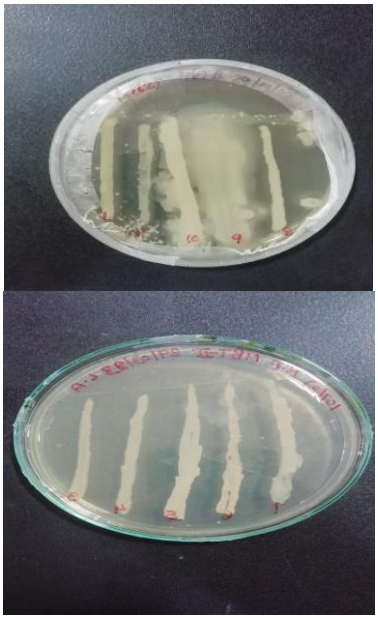
4.3.1. Antibiogram for eleven bacterial isolates.



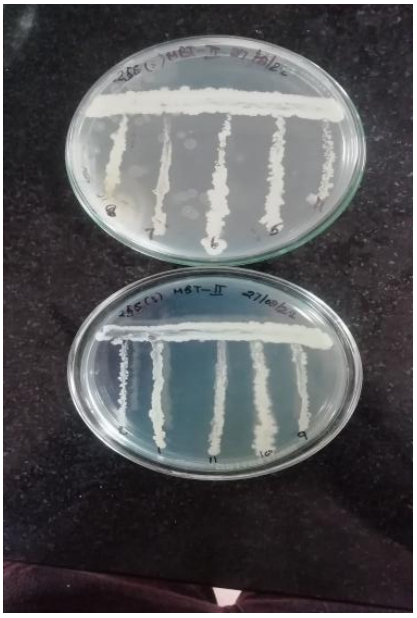
Control



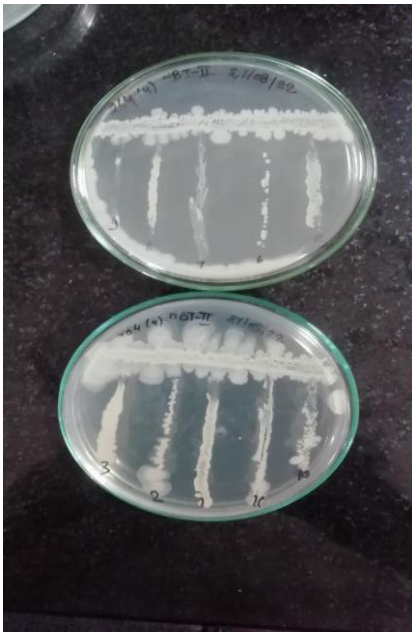
MD 100



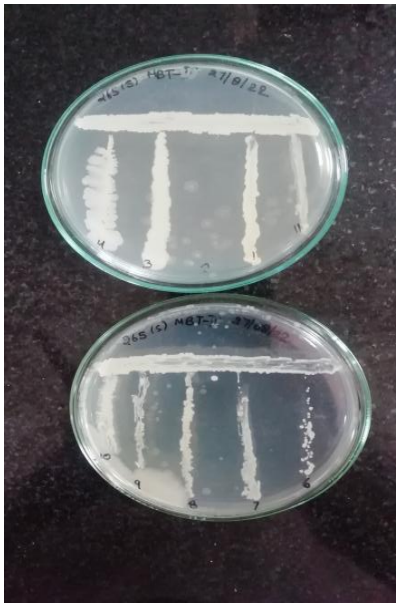
MD 107



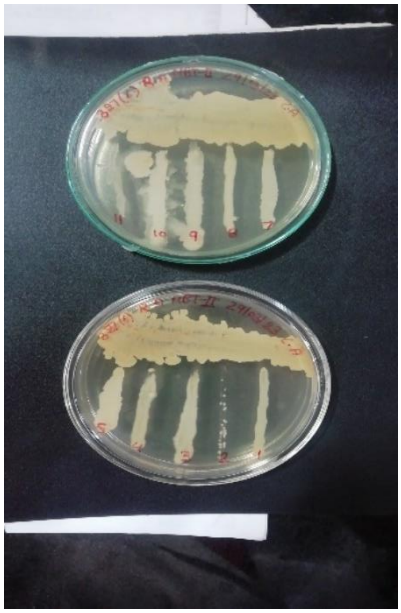
MD 256



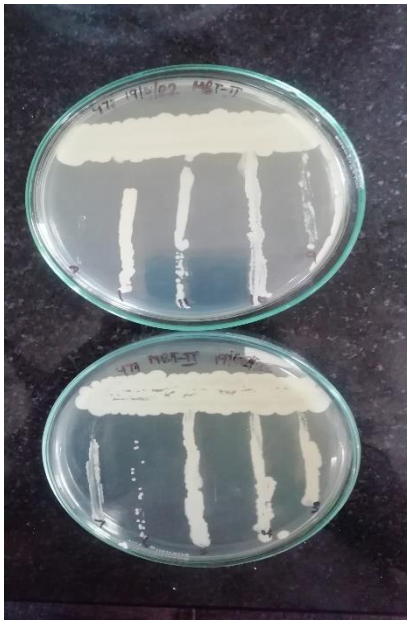
MD 264



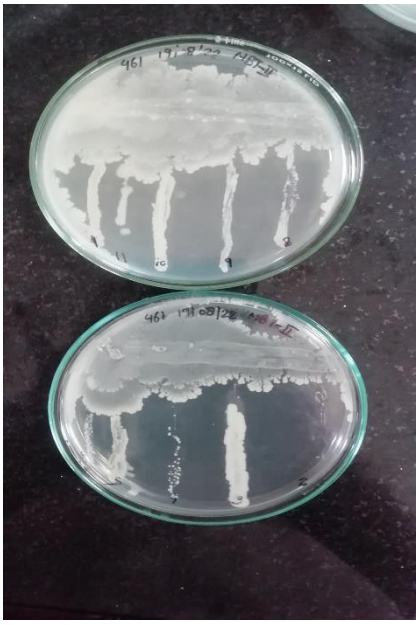
MD 265



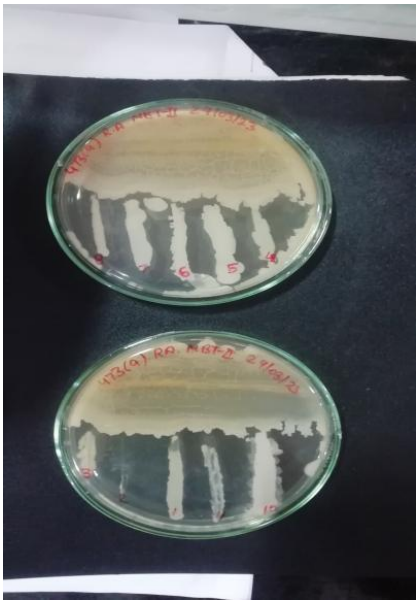
MD 327



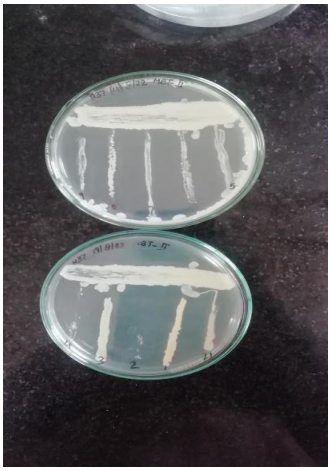
MD 471

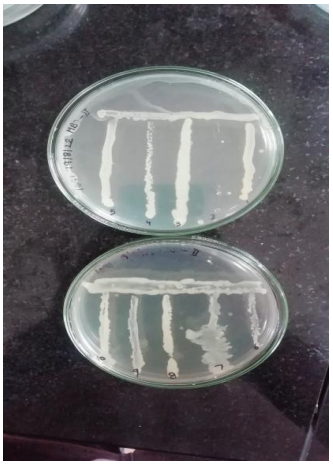


MD 461



MD 473





MD 837 MD 1091

Figure 4 3.1: Ribbon assay of 11 isolated cultures.

Table 4.3.1 (a): Ribbon assay and measurement of zone of inhibition.

Culture	Spotted against	Length of inhibition (mm)
MD100(1)	MD107(2)	0
	MD256(3)	15
	MD264 (4)	15
	MD265(5)	5
	MD327(6)	3
	MD461(7)	20
	MD471(8)	25
	MD473(9)	20
	MD837(10)	0
	MD1091(11)	15

Culture	Spotted against	Length of inhibition (mm)
MD107(2)	MD256(3)	0
	MD264 (4)	2
	MD265(5)	0
	MD327(6)	0
	MD461(7)	0
	MD471(8)	0
	MD473(9)	0
	MD837(10)	0
	MD1091(11)	0
	MD100(1)	0

MD100 showed resistance toward MD107and MD837 and slight resistance showed toward MD265 and MD327 whereas, MD107 showed resistance toward MD256, MD265, MD327, MD461, MD471, MD473, MD837, MD1091, MD100

Culture	Spotted against	Length of inhibition (mm)
MD256(3)	MD264 (4)	0
	MD265(5)	0
	MD327(6)	0
	MD461(7)	10
	MD471(8)	0
	MD473(9)	0
	MD837(10)	0
	MD1091(11)	0
	MD100(1)	0
	MD107(2)	50

Culture	Spotted against	Length of inhibition (mm)
MD264(4)	MD265(5)	10
	MD327(6)	15
	MD461(7)	0
	MD471(8)	7
	MD473(9)	30
	MD837(10)	10
	MD1091(11)	0
	MD100(1)	10
	MD107(2)	2
	MD256(3)	5

MD256 showed resistance toward MD264, MD265, MD327, MD471, MD473, MD837, MD1091, MD100, whereas MD264 showed resistance toward MD461, MD1091 and slight resistance showed by MD107, MD256 and MD471.

Culture	Spotted against	Length of inhibition (mm)
MD265(5)	MD327(6)	20
	MD461(7)	10
	MD471(8)	0
	MD473(9)	0
	MD837(10)	0
	MD1091(11)	0
	MD100(1)	0
	MD107(2)	50
	MD256(3)	0
	MD264(4)	2

Culture	Spotted against	Length of inhibition (mm)
MD327(6)	MD461(7)	0
	MD471(8)	0
	MD473(9)	2
	MD837(10)	0
	MD1091(11)	2
	MD100(1)	0
	MD107(2)	15
	MD256(3)	2
	MD264(4)	2
	MD265(5)	5

MD265 showed resistance toward MD471, MD473, MD837, MD1091, MD100, MD256 slight resistance toward MD264. Whereas MD327 showed resistance toward MD461, MD471, MD837, MD100 and slight resistance shown toward MD473, MD1091, MD256, MD264, MD265.

Culture	Spotted against	Length of inhibition (mm)
MD461(7)	MD471(8)	10
	MD473(9)	5
	MD837(10)	0
	MD1091(11)	7
	MD100(1)	0
	MD107(2)	50
	MD256(3)	15
	MD264(4)	40
	MD265(5)	0
	MD327(6)	20

Culture	Spotted against	Length of inhibition (mm)
MD471(8)	MD473(9)	0
	MD837(10)	0
	MD1091(11)	7
	MD100(1)	10
	MD107(2)	50
	MD256(3)	14
	MD264(4)	0
	MD265(5)	0
	MD327(6)	50
	MD461(7)	13

MD461 showed resistance toward MD837, MD100, and MD265 and slight resistance showed toward MD473. Whereas MD471 showed resistance toward MD473, MD837, MD264, MD265.

Culture	Spotted against	Length of inhibition (mm)
MD473(9)	MD837(10)	7
	MD1091(11)	0
	MD100(1)	0
	MD107(2)	0
	MD256(3)	0
	MD264(4)	30
	MD265(5)	20
	MD327(6)	5
	MD461(7)	0
	MD471(8)	10

Culture	Spotted against	Length of inhibition (mm)
MD837(10)	MD1091(11)	11
	MD100(1)	2
	MD107(2)	18
	MD256(3)	2
	MD264(4)	15
	MD265(5)	0
	MD327(6)	0
	MD461(7)	0
	MD471(8)	0
	MD473(9)	2

MD473 Showed resistance toward MD1091, MD100, MD107, MD256, MD461. Whereas, MD837 showed resistance toward MD265, MD327, MD461, MD471 and slight resistance toward MD100, MD256, MD473.

Culture	Spotted against	Length of inhibition (mm)
MD1091(11)	MD100(1)	0
	MD107(2)	50
	MD256(3)	0
	MD264(4)	15
	MD265(5)	0
	MD327(6)	0
	MD461(7)	0
	MD471(8)	0
	MD473(9)	2
	MD837(10)	0

MD 1091 showed resistance toward MD100, MD256, MD265, MD327, MD461, MD471, MD837 and slight resistance showed by MD473.

Table 4.3.1 (b): Chart showing compatibility of the given culture.

culture	compatibility tested against the cultures										
	MD 100	MD 107	MD 256	MD 264	MD 265	MD 327	MD 461	MD 471	MD 473	MD 837	MD 1091
100	-	+	-	-	-	-	-	-	-	+	-
107	+	+	+	-	+	+	+	+	+	+	+
256	+	-	+	+	+	+	-	-	+	+	+
265	-	+	+	-	-	+	+	+	-	+	+
264	+	-	+	-	+	+	-	+	-	-	-
327	+	+	-	-	-	-	-	-	+	+	+
461	+	-	-	-	-	-	-	-	-	+	-
471	-	-	+	+	+	+	-	-	-	+	+
473	+	+	+	-	+	-	+	+	-	-	+
837	+	+	+	-	-	+	+	+	+	+	+
1091	+	-	+	+	+	-	+	+	+	-	-

(+) sign indicates the compatibility which means that it is resistant to the antibiotic produced by bacterial culture; (-) sign indicates the susceptibility of the bacterial culture against the culture, which means they are not compatible to grow as their growth was inhibited by other culture. According to the ribbon assay 7 cultures (MD100, MD107, MD256, MD265, MD471, MD837 and MD1091) were compatible and can be grown together.

4.4 : Enzymatic activity of the selected strains.:

All bacterial strains used in this study are halotolerant and they produce bioactive compound with antidepressant activity. All the previous studies performed with these cultures to check the efficacy to produce antidepressant metabolite was done with 3.5% salt concentration. However, nature of secondary metabolites in 0.5% salt concentration is not known. As salt concentration is one of the major regulators of enzyme activity, we compared all the enzyme activity in both salt concentration (3.5% as well as 0.5%).

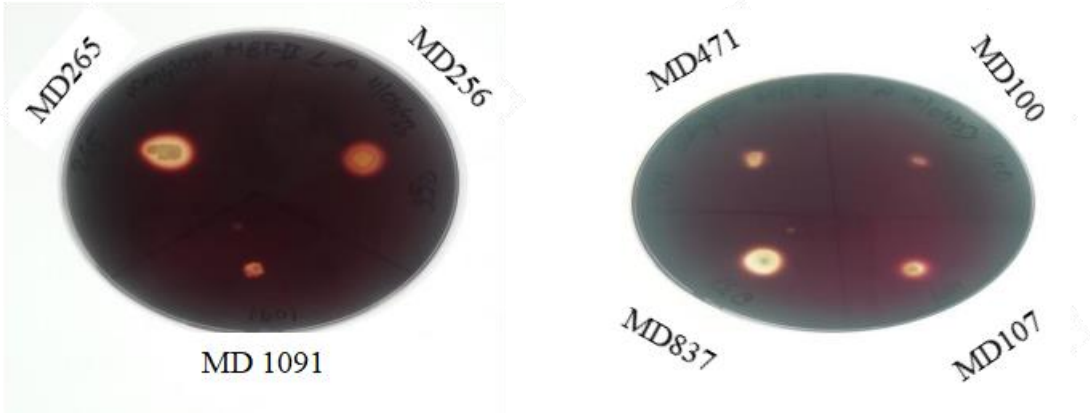
4.4.1: Amylase activity

4.4.1: (a) Amylase activity was observed on Medium D agar plates containing 3.5% NaCl and 1% starch, incubated at 24 hours at 37°C for selected isolates.

(a) (b) (c)

Figure 4.4.1: (a): Amylase activity test 3.5% NaCl [(a) MD 265, MD 100, MD 256 (b) control and (c) MD471, MD837, MD1091]

4.4.1.1 (b): Amylase activity was observed on Medium D agar plates containing 0.5% NaCl and 1% starch incubated at 24 hours at 37℃ for selected isolates.



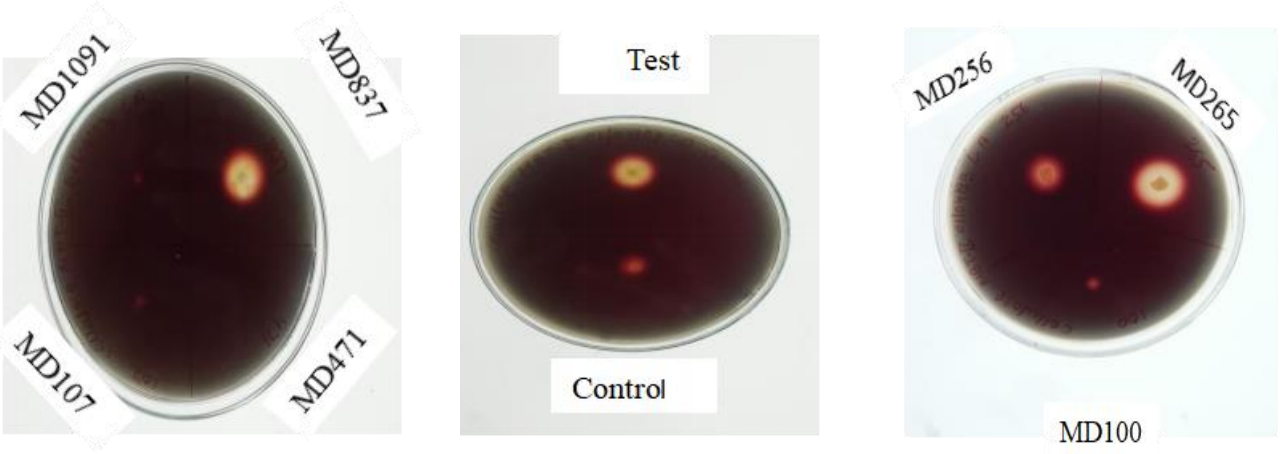
(a) (b)

Figure 4.4.1.1.(b): Amylase activity test 0.5% NaCl [(a) MD265, MD256, MD1091 (b) MD837, MD471, MD100, MD107)]

On the basis of amylase activity maximum zone of clearance was shown by MD837 and MD265 when flooded with gram iodine. While slight zone of clearance was shown by MD1091 and MD256 and no zone of clearance was shown by MD100, MD471 and MD107 on plate containing 3.5% NaCl. On 0.5% NaCl concentration maximum zone od clearance was shown by MD837, MD256, MD265 and slight zone of clearance was shown by remaining cultures MD100, MD107, MD471, MD1091 which conformed that on 3.5% NaCl concentration the amylase activity shown by the culture was limited and they were not able to hydrolyse starch into its monomeric compounds. While on agar plate containing 0.5% NaCl most of the culture were found be effective in hydrolysis of starch efficiently and presence of amylase activity was confirmed.

4.4.2: Cellulase Activity

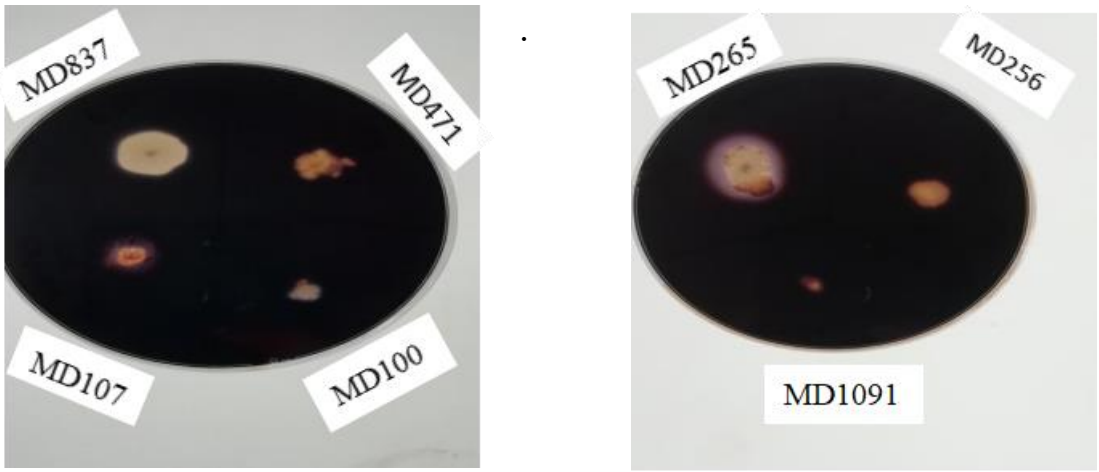
4.4.1.2 (a): Cellulase activity was shown by selected cultures on 25% Nutrient Agar plate containing 1% Carboxy-methyl cellulose and 3.5% NaCl, incubated for 48 hours at 37°C.



(a) (b) (c)

Figure 4.4.2 (a): Cellulase activity test [(a) MD107, MD1091, MD837, MD471 (b) control plate (c) MD100, MD256, MD256].

4.4.2: (b): Cellulase activity was shown by selected cultures on 25% Nutrient Agar plate containing 1% Carboxy-methyl cellulose and 0.5% NaCl, incubated for 48 hours at 37°C.



(a) (b)

Figure 4.4.2: (b): Cellulase activity test [(a) MD 837, MD471, MD107, MD100 (b) MD1091, MD265, MD256]

For cellulase enzyme maximum zone of inhibition was shown by MD837, MD256, and MD265 and slight zone of inhibition was shown by remaining other culture MD100 and no zone of inhibition was shown by MD471, MD1091 and MD107 on agar plate containing 3.5% NaCl while on agar plate containing 0.5% NaCl, maximum zone of inhibition was shown by MD265 and MD837 while slight zone of inhibition was shown by MD256, MD471 and no inhibition was shown by MD100 and MD1091.

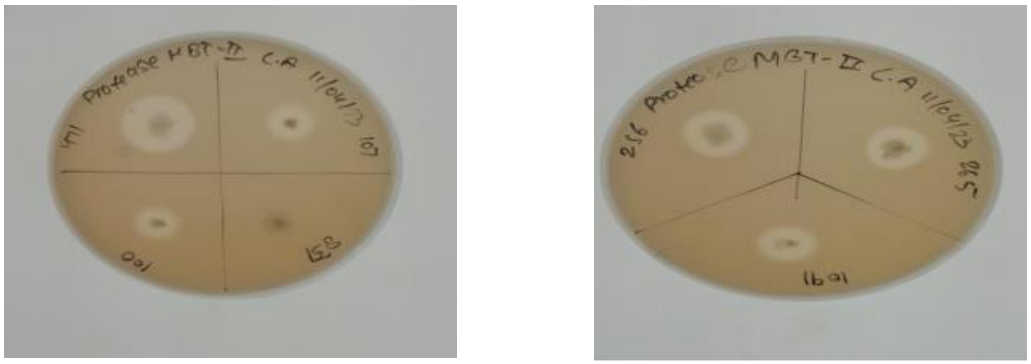
4.4.3: Protease Activity

4.4.3: (a): Protease activity was shown by selected cultures on Media D agar plate containing 1% Skimmed milk and 3.5% NaCl, incubated for 24 hours for 37°C

a) b) c)

Figure 4.4.1.3.(a): protease activity test [(a) MD256, MD100, MD1091, MD471 (b) control plate (c) MD837, MD107, MD].

4.4.3: (b): Protease activity was shown by selected cultures on Media D agar plate containing 1% skimmed milk and 0.5% NaCl, incubated for 24 hours at 37°C.



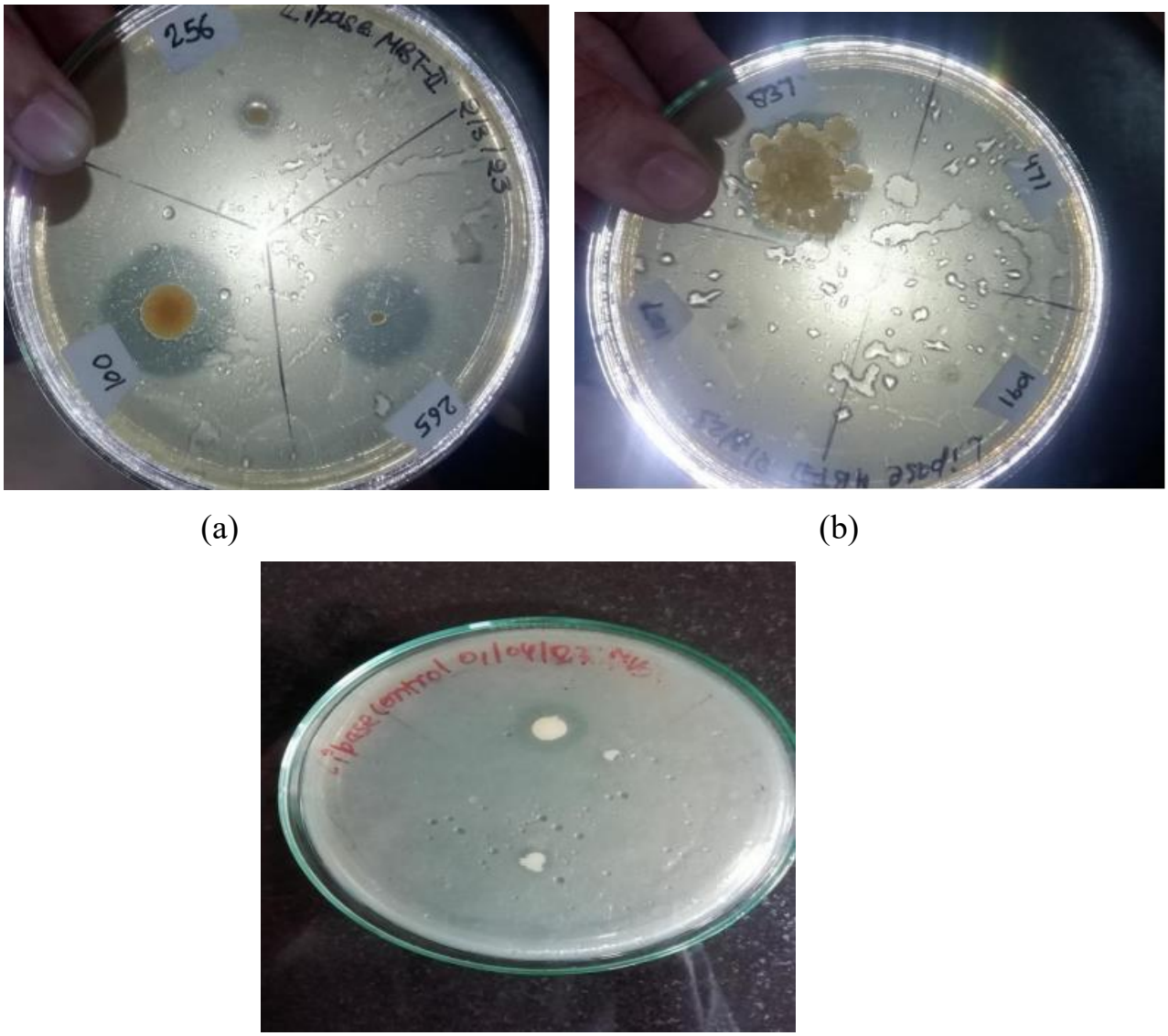
(a) (b)

Figure 4.4.3: (b): protease activity test [(a) MD837, MD471, MD107, MD100 (b) MD265, MD256, MD1091].

By protease activity analysis plate containing 3.5% NaCl, zone of clearance was measured on the Skimmed milk agar plate. Maximum zone of clearance was observed for cultures MD256, MD100 and MD471 while no zone of clearance was observed for MD837, MD107, MD265 and MD1091. On plates containing 0.5% NaCl maximum protease activity was shown by cultures (MD256, MD265, MD1091 and MD471) and slightly less zone of clearance was shown by MD100 and MD107 and no zone of clearance was shown by MD837 which depicts that cultures which showed maximum zone of clearance has the maximum proteolytic activity and cultures which showed no zone of clearance have no proteolytic activity.

4.4.4: Lipase Activity

4.4.1.4 (a) Lipase activity was shown by selected cultures on Media D agar plate containing 1% tributylin agar plate and 3.5% NaCl, incubated for 48 hours for 37°C.

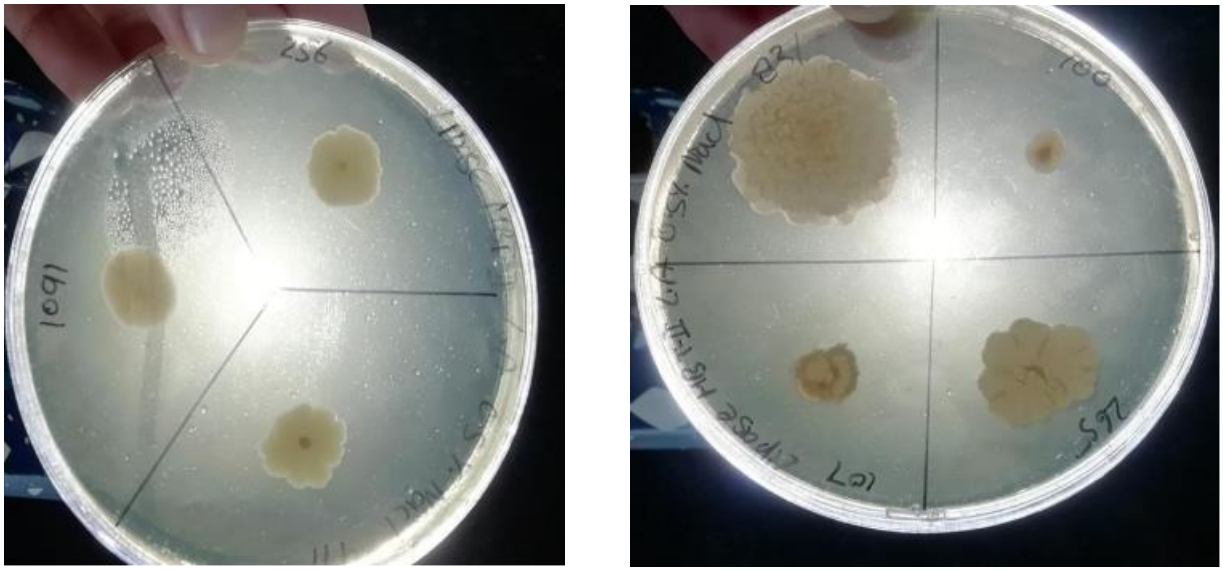


(a) (b)

(c)

Figure 4.4.1.4 (a): Lipase activity test [(a) MD256, MD265, MD100, (b) MD837, MD471, MD1091, MD107]

4.4.4: (b): Lipase activity shown by selected cultures on Media D agar plate containing 1% tributylin agar plate and 0.5% NaCl and incubated for 48 hours for 37℃.



(a) (b)

Figure4.4.1.4 (b): Lipase activity test [(a) MD1091, MD471, MD265 (b) MD265, MD100, MD107, MD837]

Maximum lipase activity was shown by MD265, MD100 and MD837 after 48 hours of incubation and slight lipase activity was shown by MD256 whereas other cultures MD107, MD471, MD1091 no lipase activity was observed on agar plate containing 3.5% NaCl and no specific lipase activity was shown by any cultures expect MD265, slight lipase activity was observed on plate containing 0.5% NaCl. Which depicts that cultures showing maximum zone of inhibition have potential to hydrolysis triglyceride ester linkages into free fatty acids and glycerol's. It was observed that selected cultures showed maximum lipase activity on agar plate containing 3.5% NaCl.

Some cultures showed good enzymatic activity on agar plate containing 0.5% NaCl while some cultures showed good enzymatic activity on agar plate containing 3.5% NaCl.

Table 4.4.1 Enzymatic activity of selected cultures on different concentration of NaCl

Enzymatic activity	Zone of inhibition (mm) for 3.5% NaCl (Cultures)						
	100	107	256	265	471	837	1091
Amylase	2 mm	0 mm	2 mm	7 mm	0 mm	5 mm	0 mm
cellulase	2 mm	0 mm	3 mm	10 mm	0 mm	7 mm	0 mm
protease	3mm	0 mm	2 mm	0 mm	2mm	0 mm	0 mm
Lipase	7 mm	0 mm	3 mm	10 mm	0 mm	2 mm	0 mm
Zone of inhibition (mm) for 0.5% NaCl							
Amylase	1 mm	2 mm	3 mm	3 mm	1 mm	4 mm	0 mm
Cellulase	0 mm	1 mm	0 mm	3 mm	0 mm	0 mm	0 mm
Protease	5 mm	7 mm	5 mm	5 mm	15 mm	0 mm	5 mm
Lipase	0 mm	0 mm	0 mm	2 mm	0 mm	0 mm	0 mm

4.4.5: Hemolytic activity of the selected strains.

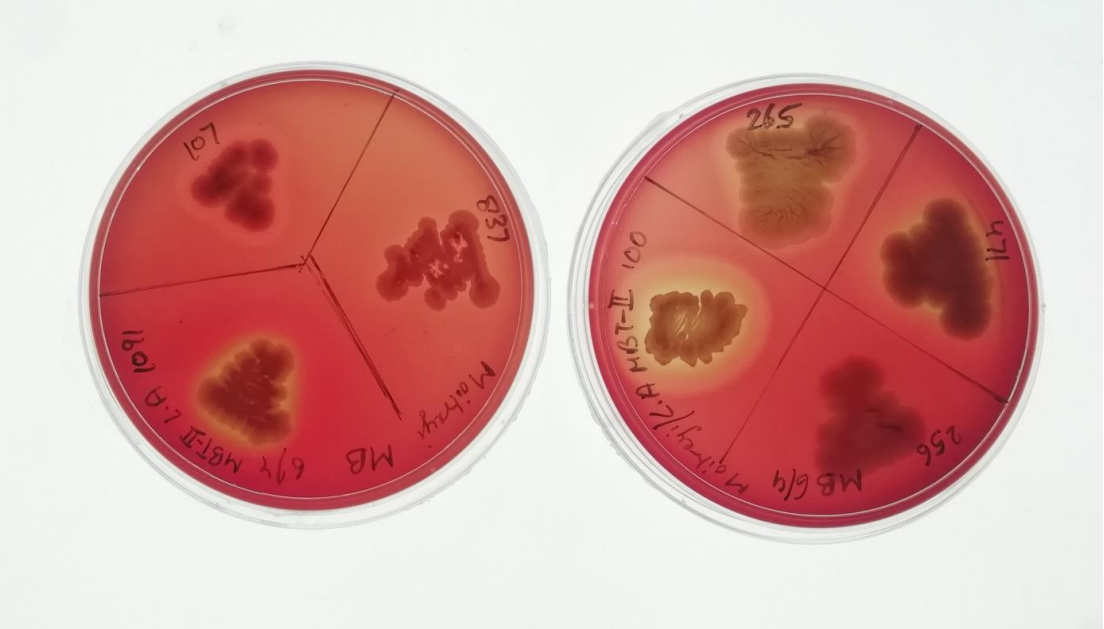


Figure 4.4.2: Hemolysis activity shown by selected culture

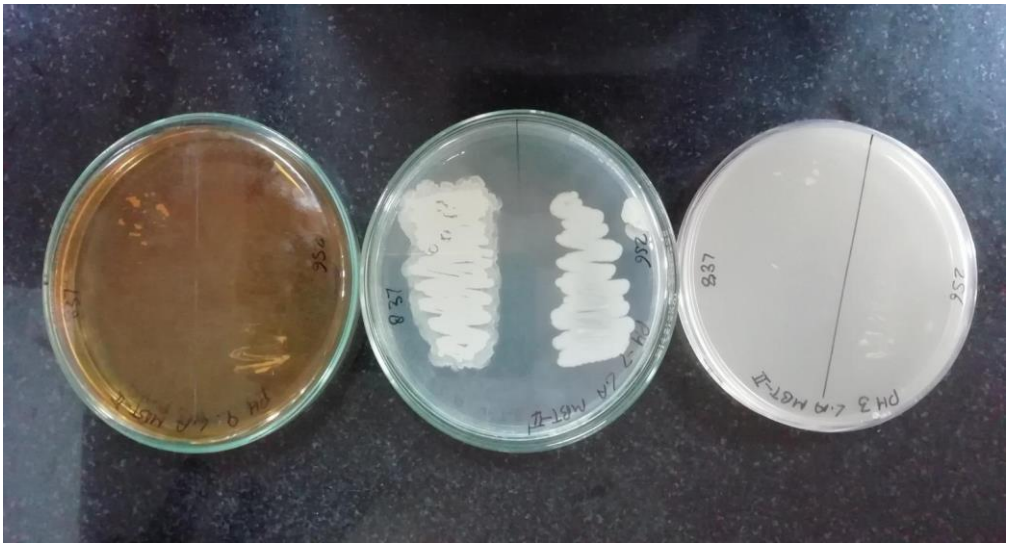
Alpha hemolysis (α) is the reduction of the red blood cell haemoglobin to methaemoglobin in the medium surrounding the colony. This causes a green or brown discoloration in the media hemolysis showed by MD471.

The term beta hemolysis (β) refers to the complete or genuine breakdown of red blood cells. The colony is surrounded by a clear zone that is similar in colour and transparency to the underlying medium. Many bacteria species produce toxic byproducts capable of damaging red blood cells. β hemolysis showed by MD1091, MD100, MD265, slight β hemolysis showed by MD107.

Gamma hemolysis (γ) Gamma represents the absence of hemolysis. The surrounding medium should not react. γ hemolysis showed by MD256 and MD837 and these two cultures can be used for further studies.

4.4.6 Growth of selected strains on different pH concentrations

On the basis of hemolytic activity selected strains MD256 and MD837 were selected for further analysis.



(a) (b) (c)

Figure 4.4.6: (a): Growth Analysis of selected isolates (MD837, MD256 on Media B agar plate containing different pH [(a) pH 9, (b) pH 7.5 (c) pH 3]

Growth was observed for the Selected culture (MD256, MD837) on pH 7.5 after incubating for 24 hours at 37℃, while there was no growth observed on plates containing pH 9 and pH 3 after 24 hours of incubation.

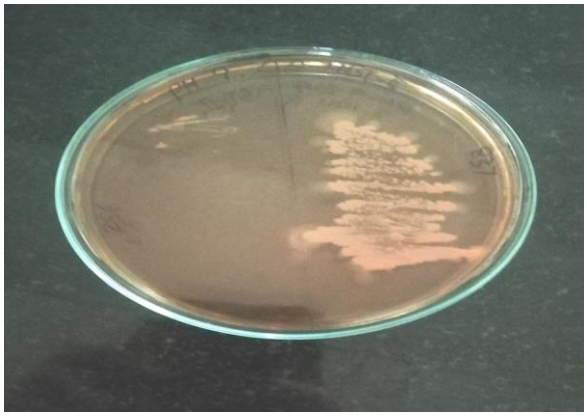
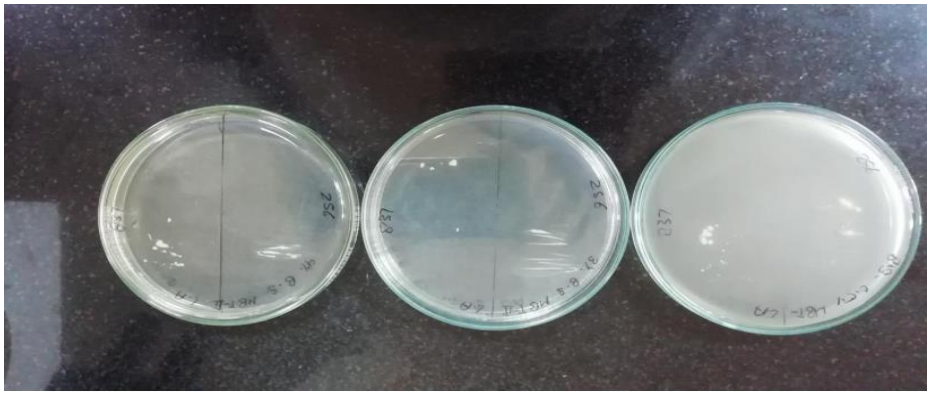


Figure 4.4.6: (b): Growth was shown by MD837 after 7 days of incubation.

The growth was shown by MD837 after incubating for 24 hours at 37°C. we can conclude that pH 7.5 is optimum for growth of isolates but growth was shown by MD837 at pH 9 so it can tolerate high pH level of gut system.

4.4.7: Growth of selected strains on different bile salt concentration

Bile acids may trigger cell death by damaging the bacterial cell membrane. As a result, bile acid tolerance of probiotic bacteria has been extensively studied. (Hagi et al., 2020).



(a) (b) (c)

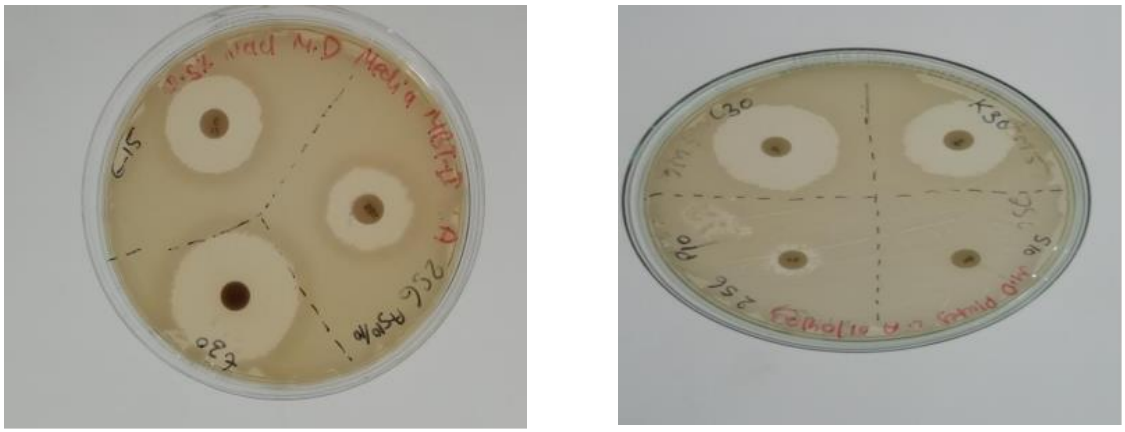
Figure 4.4.7: Growth analysis of selected bacteria on media D agar plates containing different bile salt concentration [(a) 0.5%, (b) 3%, (c) 9% .

There was no growth observed for selected culture (MD837, MD256) on different bile salt concentration which depicts that the selected culture was not tolerant to different bile salt concentration. Even After incubating for 7 days at 37℃ no significant growth was seen on media D plates having different concentration of bile salt.

4.4.8: Antibiotic susceptibility test

(a) (b)

Figure 4.4.8: (a): Antibiotic Susceptibility was shown by MD837 (a), (b) against Ampicillin ($As^{10/10}$) Penicillin (P10), Chloramphenicol (C30), Erythromycin (E15), Kanamycin (K30), Streptomycin (S10), tetracycline (T30)



(c) (d)

Figure 4.4.8: (b): Antibiotic Susceptibility was shown by MD256 (a), (b) against Ampicillin ($As^{10/10}$ Penicillin (P10), Chloramphenicol (C30), Erythromycin (E15), Kanamycin (K30), Streptomycin (S10), tetracycline (T30)

Table 4.4.9: Antibiotic susceptibility test

Antibiotics	Zone of inhibition (mm)		
	MD256	MD837	Interpretive criteria (mm)
Inhibition of cell wall			
Ampicillin (10µg)	14I	5R	S (≥ 17), R (≤13), I (14-17)
Penicillin (10 µg)	2R	3R	S (≥ 22), R (≤12), I (12-21)
Inhibition of protein synthesis			
Chloramphenicol (30µg)	18S	13I	S (≥ 18), R (≤13), I (13-17)
Erythromycin (15µg)	15I	0R	S (≥ 23), R (≤13), I (14-22)
Kanamycin (30 µg)	18S	14I	S (≥ 18), R (≤13), I (14-17)
Streptomycin (10µg)	0R	0R	S (≥ 15), R (≤11), I (12-14)
Tetracyclin (30µg)	18S	18S	S (≥ 15), R (≤11), I (12-14)

Susceptible (S), Resistant (R) and Intermediate (I) (Delgado, 2020)

According to the Antibiotic susceptibility test MD 256 Showed the resistance toward penicillin(cell wall inhibition), and streptomycin (protein synthesis), while it has shown susceptibility toward chloramphenicol, kanamycin, and tetracycline (all belongs to protein synthesis inhibitory family) and MD 837 Showed the resistance toward Ampicillin, Penicillin, Erythromycin and streptomycin while it has shown Intermediate susceptibility toward chloramphenicol and Kanamycin and susceptibility toward tetracycline.

According to the findings, the halotolerant bacteria were susceptible to various antibiotics from different groups. When exposed to certain antibiotics, certain bacteria were somewhat sensitive, with an intermediate breakpoint. Some bacteria are showed resistant to some of the antibiotics and according to research, certain ‘intrinsic type' resistance was found in some bacteria, due to membrane permeability or lack of affinity of the antibiotic to the target bacteria or creation of innate enzymes that inactivate the antibiotic. Bacteria with this sort of resistance pose no risk of horizontal multiplication and are thus safe to use (Fernandes et al., 2019).

4.5: 16sRNA sequencing of MD837

16sRNA sequencing was done by HiMedia 16sRNA sequencing to identify the genus of the culture. To identify the culture phylogeny tree was studied along with fasta sequencing.

Phylogeny tree of MD837

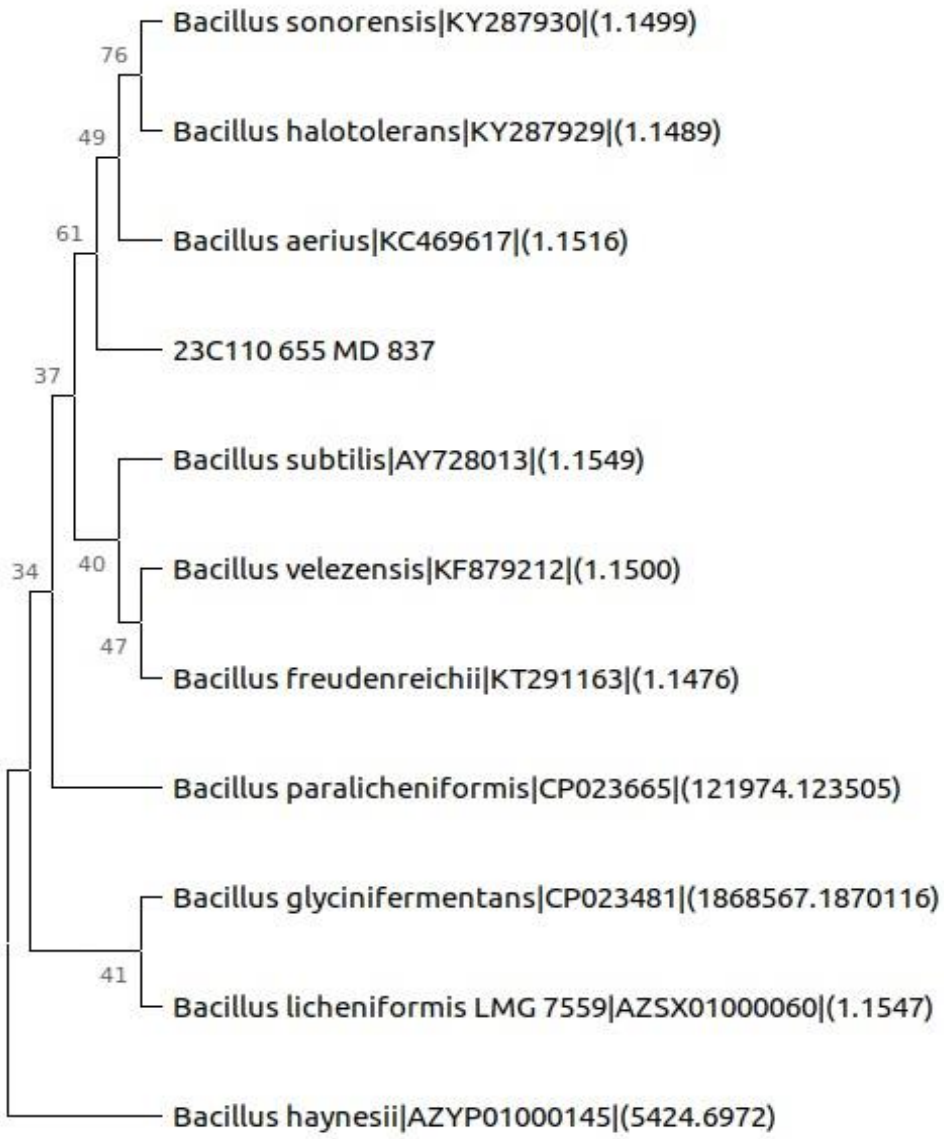


Figure 4.5(a): phylogeny tree showing the relationship between MD837 with other isolates based on 16sRNA sequencing.

Fasta Sequence of MD837

>23C110_655_MD_837_907R.ab1

GKCGTMTCCAGGCGGAGTGCTTAATGCGTTTGCTGCAGCACTAAAG

GGCGGAAACCCTCTAACACTTAGCACTCATCGTT

TACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCT

TTCGCGCCTCAGCGTCAGTTACAGACCAGAGAG

TCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCT

ACACGTGGAATTCCACTCTCCTCTTCTGCACT

CAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTT

TCACATCAGACTTAAGAAACCGCCTGCGCGCGC

TTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCG

CGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCT

GGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTGTTCT

TCCCTAACAACAGAGTTTTACGATCCGAAAACC

TTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGA

AGATTCCCTACTGCTGCCTCCCGTAGGAGTCT

GGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGC

TACGCATCGTTGCCTTGGTGAGCCGTTACCTCA

CCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCTAAAA

SCCACCTTTTATAATTGAACCATGCGGTTCAAT

CAAGCATCCGGWATWAGCCCCGGTTTCCCGGAGTTATCCCAGTCTT

ACAGGCAGGTTACCCACGTGTTACTCACCCGTCC

GCCGCTRACATCAGGGAGCAAGCTCCCATCTGTCCGCTCGACTTGCA

TGTATTAGGCACGCCGCCAGCGTCGTCTGAKAG

ARRAAAAAAAAWYMTATAA

Figure 4.5(b): Fasta sequence of MD837.

Salt pan bacteria MD837 (907 bp) showed 98% nBLAST similarity with *Bacillus paralicheniformis*. Bacillus is capable of spore formation, can resist hight temperature and pH which makes them suitable to be used as probiotics.

4.6: The effect of live bacteria on in-vivo system.

4.6.1: consortium of the selected strains as a feed for *Danio rerio* (zebra Fish).

Based on the safety evaluation (antibiotic susceptibility test and hemolystic test), two strains (MD256 and MD837) were chosen to prepare the consortium and employed as feed for Danio rerio to examine whether they can be used as a probiotic or not. The key effects of the bacterial consortia on fish were weight, size, and survival rate were assessed for 7 days. Studies have found out that the standard number of bacterial cells per ml should be given is between 10^7-10^9 cfu/gram (Valcarce et al., 2019). The average number of cells were calculated by using the hemocytometer.

Average Number of cells = 1097.5 cells per square of hemocytometer, Dilutions Factor= 25

Cfu/ml = average no. of cells in 4 sets of 16 squares * 10^4 * dilution factor.

$$= 4.39*10^9 \text{ cfu/ml.}$$

4.6.2: Experimental Setup

For control set up:

5 Fishes were taken, normal commercial feed was provided for test set up:

5 Fishes were taken, consortium of selected culture (MD256, MD837) along with commercial feed was given.

4.7.2 (a): Measuring the weight of the fish

Average of initial weight of fish

S. No	Weight of 50ml water and 100ml beaker(g)	Weight of fish+100ml beaker+50ml water(g)	Difference (g)
1.	99.2998	99.8566	0.5568
2.	100.4372	100.6321	0.1949
3.	99.8327	100.1231	0.2904
4.	99.6988	99.9530	0.2542
5.	99.5339	99.8535	0.3196
Average			0.3237

Average of final weight of fish after 7 days

S. No	Weight of 50ml water and 100ml beaker(g)	Weight of fish+100ml beaker+ 50ml water(g)	Difference (g)
1.	98.3226	99.7124	1.3898
2.	99.2672	99.8909	0.6237
3.	99.0902	99.3250	0.2348
4.	98.1140	98.3799	0.2659
5.	98.3475	98.5534	0.2059
Average			0.5440

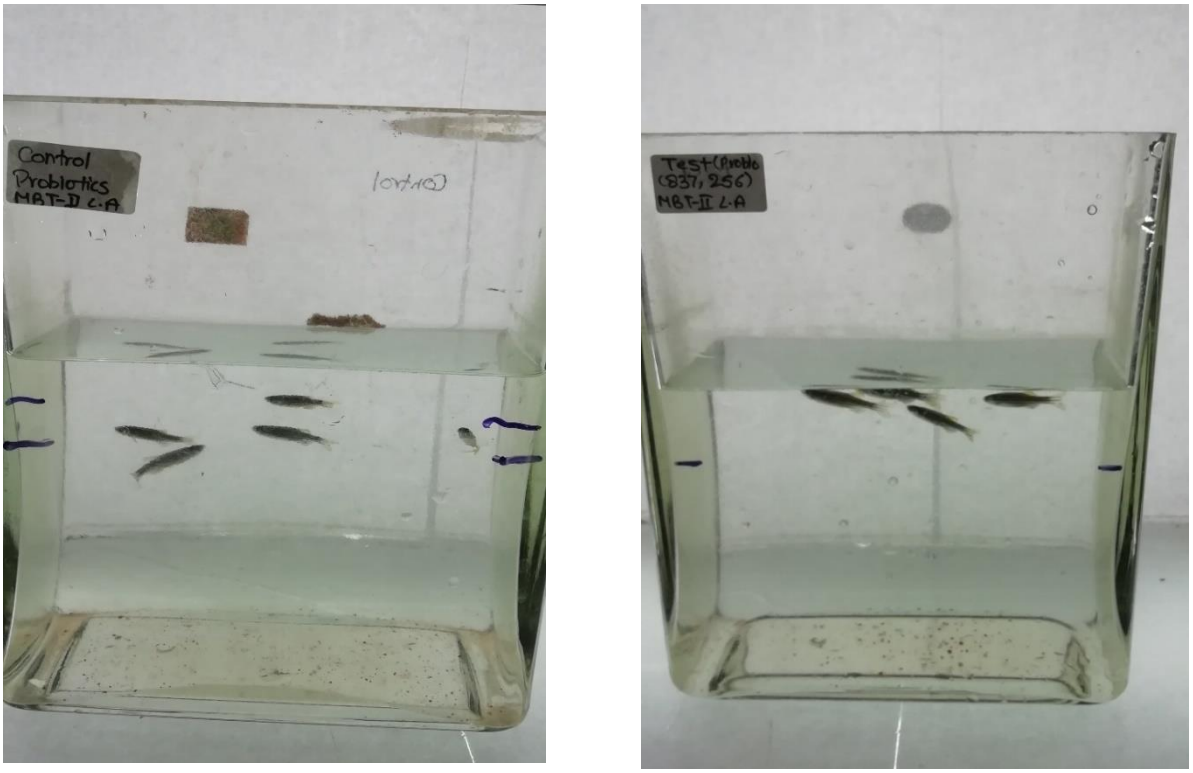
4.7.2 (b): Measuring of the size of fish

Initial size

S. No	Size of the fish (cm)
1.	2.5
2.	2.3
3.	2.0
4.	2.5
5.	1.7
Average	2.20

Final Size

S. No	Size of the fish (cm)
1.	2.0
2.	2.5
3.	2.8
4.	2.3
5.	2.5
Average	2.42



Control Test (MD837, MD256)

Figure 4.7.2: control and test set up to check effect of live bacteria on in-vivo system.

Probiotics are living bacteria that, when included in the diet, improve animal health. The gut microbiome is a complex ecosystem that serves several important roles for the host organism. These bacteria work by regulating intestinal transit, altering intestinal villi, and safeguarding nutrient digestion and absorption.

zebrafish (Danio rerio) used as a vertebrate model for the study of development, genetics, and illnesses, among other things. This teleost is also an excellent model for probiotic-related investigations since zebrafish microbiota and gut colonisation are comparable. the potential beneficial benefits of probiotics of halotolerant bacteria can be assessed through in vivo experiments. (Valcarce et al., 2019)

Average Initial weight of the fish = 0.3237g, Average final weight of the fish = 0.5440g

Size of the fish initially = 2.20 cm, size of the fish finally = 2.42 cm

$$1.\text{Specific Growth rate} = \frac{\ln[\text{final weight(g)}] - \ln[\text{initial weight(g)}]}{7 \text{ days}} * 100$$

$$= 7.3426\%$$

$$2.\ \text{Relative Gain Rate} = \frac{\text{Final Weight (g)} - \text{initial weight(g)}}{\text{Initial weight (g)}} \times 100$$

$$= 68.05\%$$

$$3.\text{Food conversion rate} = \frac{\text{Food consumed (ml)}}{\text{Final weight} - \text{initial weight}}$$

$$= 31.7748$$

$$4.\text{Survival Rate} = \frac{\text{No. of fish at the end of experiment (pieces)}}{\text{No. of fish at the beginning of experiment (pieces)}}$$

$$= 5/5$$

$$= 100\%$$

Despite short duration of our trial, our data revealed no statistical changes in size or weight between the experimental groups after one week. Control cultures are those that do not include probiotics, while test cultures contain probiotics. These findings support the use of halotolerant bacteria (MD254 and MD837) as carriers of probiotic potential.

Summary and Conclusion

- This study begins with revival of cultures and culturing with the respective media component. We used media D for culturing the cultures. The aim of this study was to explore the probiotic potential of the salt pan bacteria isolate from salt pans of Goa
- Growth of 11 isolates (MD100, MD107, MD256, MD264, MD265, MD327, MD461, MD471, MD473, MD837 and MD1091) was studied on different salt concentration (0.5%, 3.5% and 10%) and differentiated into halophilic and halotolerant bacteria.
- Partial characterisation was done to differentiate the isolate between Gram positive and Gram negative and confirmatory test was done by using 3% KOH method with this most the isolates were identified as Gram positive (MD100, MD107, MD256, MD264, MD265, MD327, MD461, MD471, and MD837) and Gram negative (MD473, and MD1091).
- To study their synergistical growth antibiogram test by ribbon method was done and 7 isolates (MD100, MD107, MD256, MD265, MD471 MD837 and MD1091) were selected for further assessment.
- The cultures were then studied to determine their enzymatic activity for Amylase, cellulase, protease and lipase at 0.5% and 3.5% salt concentration. some cultured showed better enzymatic activity at 0.5% NaCl and some showed better activity at 3.5% NaCl.
- Cellulase and lipase activity was observed better on plates containing 3.5% NaCl, while better protease and amylase activity was observed on plates containing 0.5% NaCl.

- Hemolysis activity test was done to identify which culture is safe for human utilisation. Alpha hemolysis was observed by MD471 and beta hemolysis was observed by MD1091, MD100, MD265, and MD107. Gamma hemolysis was observed by MD256 and MD837 and these two cultures were safe to use for utilisation.
- Growth of the selected isolates were observed under different pH and bile salt concentration. optimum pH for the growth of selected culture was 7.5 MD256 and MD837 while growth was observed for MD837 at pH 9 after 7 days of incubation which depicts that MD837 can tolerated high range of pH and can survive various pH level of gastrointestinal tract.
- While no growth was observed by any culture on plates containing different bile salt concentration.
- Antibiotic susceptibility test gives us insight that whether the given culture is susceptible or not. Cultures showing more susceptibility against specific antibiotic are better suited for consumption.
- Consortium of MD256 and MD837 was prepared, consortium was given as a feed to host and effect of bacteria on host model (Zebra fish) was observed for 7 days
- By 16SrRNA sequencing we found that MD837 belongs to *Bacillus paralicheniformis sp.* Bacillus species can withstand with physical and chemical stress and can be used as probiotics.
- Now a days probiotics are essential for the better gut health and studies have shown that salt pan bacteria have potential to use as a probiotic. Futher studies need to be done to ensure its ability as a probiotic.

Future Prospects

Various secondary metabolites are produced by halotolerant bacteria which can be used as anti-depressant and anti-anxiety have been reported in various studies. With this study we can conclude that halotolerant bacteria produced various enzymes and can with stand different stress environment of in vivo condition. On the basis of various preliminary safety examination, we can say that the cultures that were isolated from salt pan of Goa have potential probiotic activity and can be used as probiotics.

Appendix

7.1 Media Compositions

7.1.1 **Media D agar**: Media D was prepared and dissolved in 1000ml of distilled water. Sterilized by autoclaving at 15lbs pressure at 121°C for 20 min.

Constituent	Quantity and units
Tryptone	15g/l
Soyaton	5g/l
NaCl	5g/l
Agar	15g/l
Final pH	7.0- 7.5

7.1.2 Media D broth: Media D broth was prepared and dissolved in 1000ml of distilled water. Sterilized by autoclaving at 15lbs pressure at 121°C for 20 min.

Constituent	Quantity and units
Tryptone	15g/l
Soyaton	5g/l
NaCl	5g/l
Final pH	7.0- 7.5

7.1.3 Nutrient broth: 13g of nutrient broth powder was dissolved in 1000ml of distilled water. Sterilized by autoclaving at 15lbs pressure at 121°C for 20 min.

Constituent	Quantity and units
Peptone	5g/l
NaCl	5g/l
Yeast extract	1.5g/l
Beef extract	1.5g/l
Final pH	7.4

7.1.4 Nutrient agar: 28g of nutrient agar powder was dissolved in 1000ml of distilled water. Sterilized by autoclaving at 15lbs pressure at 121°C for 20 min.

Constituent	Quantity and units
Peptone	5g/l
NaCl	5g/l
Yeast extract	1.5g/l
Beef extract	1.5g/l
Agar	15g/l
Final pH	7.4

7.1.5 To study salt tolerance.

0.5% - 5g/l of NaCl was added.

3.5%- 35g/l of NaCl was added.

10% - 100g/l of NaCl was added (to the media constituents used)

7.1.6: To study bile salt tolerance.

0.5% - 5g/l of bile salt was added.

3.0% - 30g/l of bile salt was added.

9.0%- 90g/l of bile salt was added (to the media constituent).

7.1.7 Reagent used to adjust pH of the media

7.1.7.1 1M NaOH: 40g of sodium hydroxide pellets in 1000ml distilled water

7.1.7.2 1N HCl: 8.48 ml HCl dissolved in 1000ml of distilled water.

7.1.8: Gram Staining Reagents

7.1.8.1: Gram's crystal violet: Solution A: 2g of crystal violet was dissolved in 10ml of 95% ethanol and the total volume was brought up to 20ml with distilled water. Solution B: 0.8g of ammonium oxalate was dissolved in 10ml of distilled water and the total volume was made up to 80ml. Solution A and B were then mixed.

7.1.8.2: Gram's iodine: 1g of Iodine and 2g of KI was dissolved in distilled water and the volume was brought up to 300ml with distilled water.

7.1.8.3: Gram's Decolourizer: 50ml of acetone was mixed with 50ml absolute ethanol.

7.1.8.4: Gram's Saffronin: 0.25g of saffronin was dissolved in 10ml of 95% ethanol and the total volume was brought up to 100ml using distilled water.

7.1.9: Enzymatic test

Enzyme tested	Substrate used	Dye used
Amylase	Starch	Gram's Iodine
Cellulase	Carboxymethylcellulose	Gram's Iodine
Protease	Skimmed Milk	No dye
Lipase	Tributylin agar	No dye

7.1.10 Method for Consortium Preparation.

Step1: Inoculation of MD256 and MD837 and was kept for incubated for 24 hours at 37°C

Step 2: Aseptically media was transferred to the centrifuge tube and centrifuge at 10000rpm for 20 min.

Step 3: Supernatant was discarded and pellet was resuspended in 0.5% NaCl and aliquoted in eppendorf tubes.

Step 4: Eppendorf tube was again centrifuged at 10000rpm for 20 min for pellet extraction and supernatant was discarded and pellet was stored at 4°C for further use.

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Introduction Salt pans are shallow, artificial ponds designed to commercial production of salt from sea water. They are inhabited by a rich variety of microorganism, from halotolerant to moderately. Different saltpan bacteria offer an array of natural products with diverse application. Halophilic and halotolerant bacteria secrete a wide range of hydrolytic enzymes e.g., amylases, proteases, xylanases and cellulases, into their surrounding environment useful for many industrial processes (e.g., agriculture, food, feed and drinks, detergents, textile, leather, pulp and paper. Halophiles also produces bioactive compounds which are of with medicinal importance. Several studies have demonstrated the importance of secondary metabolites produced by marine bacteria in medicinal applications such as antidepressants, anxiety medications, dementia treatments, and cancer treatments. Secondary metabolites' effects on gut and brain health have also been explored. Gut microbiota can be controlled through the administration of probiotics and prebiotics, which can also affect brain health and be employed as a possible therapeutic agent by activating brain health (Parker et al., 2019). Manipulation of human microbiota has already been shown to have a favourable influence on human health. Probiotics are one potential positive approach to enhancing the human microbiome. Probiotics can be defined as living microorganisms that have a variety of health benefits when administered in sufficient quantities (Bajagai et al., 2016). Probiotics' in treating conditions such as inflammatory disease, constipation, antibiotic-associated diarrhoea, allergy-related conditions, hypertension, and diabetes have been well studied and documented (Hill et al., 2014).