Isolation of Halotolerant and Halophilic Bacteria from Goan Saltpans and Exploring its Potential for Biotechnological Applications

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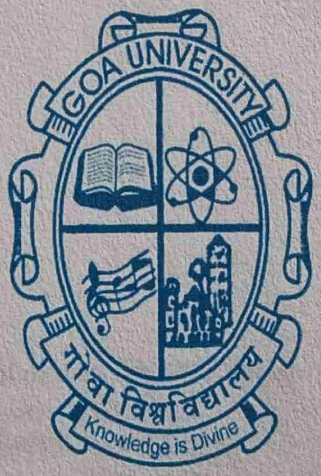
MISHA DENICA CARDOZO

Roll number: 21P042002
Under the supervision of

DR. MILIND M. NAIK

School of Biological Sciences and Biotechnology
Microbiology Programme



Goa University
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Microbiology Programme
School of Biological Sciences & Biotechnology
Goa University, Science Block E,
Taleigao Plateau, Goa - 403206

Examined by:

Seal of the school

CERTIFICATE

This is to certify that the dissertation report entitled "**Isolation of Halotolerant and Halophilic bacteria from Goan Saltpans and Exploring its Potential for Biotechnological Applications**" is a bonafide work carried out by **Ms. Misha Denica Cardozo** under my supervision in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology, in the Microbiology programme at the School of Biological Sciences and Biotechnology, Goa University in the academic year 2022-23.

17/4/2023

Dr. Milind M. Naik

Assistant professor

Microbiology program

School of Biological Sciences and Biotechnology

Date

Microbiology Programme
School of Biological Sciences & Biotechnology
Goa University, Science Block E,
Taleigao Plateau, Goa - 403206

School stamp

Sanitaharkar

Dr. Savita S. Kerkar

Dean of School of Biological Sciences & Biotechnology
Goa University 403206
Office Ph. 8669609246

School of Biological Sciences and Biotechnology

Biotechnology

Date: 16/5/23

Place:

STUDENT DECLARATION

I hereby declare that the data presented in this dissertation entitled "**Isolation of Halotolerant and Halophilic bacteria from Goan Saltpans and Exploring its Potential for Biotechnological Applications**" is based on the results of investigations carried out by me in the Microbiology programme at the School of Biological Science and Biotechnology, Goa University under the supervision of Dr. Milind M. Naik, Assistant Professor in Microbiology, Goa University 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 not be responsible for the correctness of observations/experimental or other findings given in the dissertation.

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Misha Denica Cardozo
21P042002
Microbiology programme
School of Biological Sciences and Biotechnology

Place: Goa University
Date: 1/05/2023

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CONTENTS

ABBREVIATIONS

NTYE	Nutrient yeast extract tryptone
CMC	Carboxy methyl cellulose
NH	Norberg and Hofstein
MSM	Mineral salt medium
OD	Optical density
MIC	Minimum inhibitory concentration
MTC	Maximum tolerance concentration
TBE	Tris borate EDTA
EDTA	Ethylenediaminetetraacetic acid
mM	Millimolar

1. Introduction

Microorganisms are known to inhabit different types of extremities like high temperature, pH, pressure, salt concentration, low water activity, and so on. The survival of these organisms at such extreme environmental niches has piqued the interest of the scientific community for the usage of these wonderous organisms and their bioactive molecules for potential use in agriculture, medicine, biofuel, bioenergy, bioremediation, and other industries. Halophilic microorganisms are potential candidates for such applications. Halophiles and halotolerant microorganisms survive, grow and proliferate at extreme concentrations of salt (>1M), hence these are dominantly found in saline and hypersaline econiches like the ocean, salterns, estuaries, mangroves, and others. Isolation from such habitats implies that halophiles, other than being salt tolerant are also capable of withstanding low and high temperatures, low water activity, fluctuating pH, and other parameters, hence they can be utilized for a wide range of applications.

One of the major applications of halophiles could be its potential role in the bioremediation of saline environments. Saline ecosystems have been under constant abuse due to the discharge of various effluents from industries. Similarly, saline wastewater is produced as a waste product in various industries like tanneries, dye, petroleum, polyurethane production etc (Mainka et al. 2021), and it has been determined that 5% of industrial by-products conation huge concentration of salt, along with several other xenobiotic substances like heavy metal, aromatic hydrocarbons, dyes, etc. the release of such effluents without proper pre-treatment into aquatic bodies can cause a serious environmental threat, as well have adverse effects on the aquatic flora and fauna. Wastewater from the textile industries has been a major environmental threat and has upgraded to become one the most hazardous pollutant of its sheer quantity of contaminants and toxicity level (Mishra & Maiti, 2018). The pollutants comprise dyes, heavy metals, organic compounds, toxic chemicals, etc. Azo dyes account for 70% of the dye load, and the release of azo dyes into water bodies causes aesthetic problems, deteriorates the quality of water as well as being carcinogenic, and mutagenic (Tian et al. 2021). Halophilic microorganisms with their ability to grow at such high salt concentrations could provide an eco-friendly, cost-effective way to deal with the bioremediation of saline wastewater. Enzymes obtained from halophiles such as proteases, lipases, amylases, and xylanases are beneficial in the bioremediation of contaminants in saline environments as they can be stable and function at a wide range of pH, temperature, and salt concentrations (Ibrahim et al. 2019). These enzymes, also called haloenzymes have commercial value and are highly sought after in industries like food, detergent, pharmaceutical,s, etc. Halophiles also

exhibit resistance to heavy metals and aromatic hydrocarbons, which otherwise prove to be fatal to other microorganisms even in trace quantities. This resistance ensures that halophiles and their bioactive molecules can function in wastewater containing such recalcitrant compounds. Due to survival under such harsh conditions which are common in dye-polluted wastewaters, halophiles have been known as the best candidates for the bio-decolorisation of azo dyes (Amoozegar et al. 2010).

In this study, I aimed to isolate halotolerant or halophilic bacteria from a saltpan sediment sample and check if it exhibits characteristics that could possibly be used as a tool for the bioremediation of such saline wastewater. The isolated organisms were checked for their salt tolerance, enzyme activity (amylase, lipase, cellulase, xylanase, protease, and agarase), azo dye decolorization activity, heavy metal tolerance (Cd and Hg), and aromatic hydrocarbon degradation (sodium benzoate).

2. LITERATURE REVIEW

2.1. Saline environments

Saline niches are widely distributed across the globe. These include marine environments, solar salterns, hypersaline lakes, soda lakes, salt marshes, acidic hypersaline lakes, deep ocean brine pools, etc. Seawater salinity is around 33-37‰. The Lake Van of Turkey has the highest recorded salinity of 330‰, followed by the Dead Sea, Israel at 240‰, and the Great salt lake, Utah, USA at 220‰. Other saline environments originated mostly from seawater's evaporation referred to as thalassohaline environments. The composition of such habitats is mainly sodium chloride and other dissolved salts of K, Mg, Ca, S, and Br. The pH of such environments is usually neutral to slightly alkaline (A. Oren, 2002).

Saline habitats that do not stem from seawater are referred to as athalassohaline environments and hence have a different ionic composition than seawater-based environments. The composition of these environments depends on the surrounding topology, geology, and climatic conditions (Rodriguez-Valera, 1988).

Salt pans are areas where water bodies have evaporated, leaving behind salt and other minerals. Salt pans occur naturally and are usually replenished by estuarine water or seawater. Salt pans in Goa are called mithache agor, and are usually located around waterlogged areas (khazan lands) surrounded by estuaries. These areas are used for the production of salt as a source of income. The process of salt production includes the preparatory phase where the embankments which hold the water in are built and the salterns are ploughed. 3 different kinds of pans are prepared; reservoir pans, evaporator pans and crystallizer pans. Reservoir pan receives water from rain or through tidal efflux of water. This pan is connected to several evaporator pans which are then connected to crystallizer pans (Mani et al. 2012). Sodium chloride precipitates out only once the salinity is high enough. Therefore, in the evaporator pans, calcium carbonate is precipitated out, followed by the precipitation of calcium sulphate as gypsum. these happen in different evaporator pans and the water is transferred from one pan to the other. Sodium chloride crystallizes at around 27°Bé and this occurs in the crystallizer pans (Mani et al. 2012).

2.2. Diversity of living organisms in saline environments

Hypersaline environments prove to be challenging to most organisms due to high salt concentration as well as due to high/low-temperature exposures, high osmolarity, extremely high/low pH values, and low oxygen concentrations (Rodriguez-Valera, 1988). Environments with high salinity are

inhabited by halophiles which are a group of extremophilic organisms that thrive and survive in such extreme environments. These organisms can be found across all three domains of life, namely- Bacteria, Archaea, and Eukarya. On the basis of their optical NaCl concentration for growth, different categories are established by Kushner and Kamekura (1988): i) non-halophilic requiring <1% NaCl ii) slight halophiles requiring 1%-3% NaCl iii) moderate halophiles requiring 3%-15% NaCl iv) extreme halophiles growing best in media containing 15%-30% NaCl (Moreno, et al., 2013). V) halotolerant grows optimally at <1% salt but is able to tolerate a wide range of salt concentration.

Halophilic archaea, under the order *Halobacteriales,* are quintessential examples of halophily. Examples: *Halobacterium salinarium, Haloferax volcanii,* and others belonging to genera *Halorubrum, Haloarcula,* etc. Halophilic organisms are scant under the eukaryotic domain. These include the marine shrimp- *Artemia salina*, photosynthetic flagellate *Dunaliella*, yeasts (e.g., *Hortaea werneckii*), etc. Under domain bacteria, we have a wide variety of halophilic (moderate) and halotolerant organisms like *Halorhodospora, Bacillus, Candidatus salinibacter* and other species belonging to Proteobacteria, Firmicutes, and Actinobacteria are some examples (A. Oren, 2002 and Ruginescu, et al., 2020).

2.3. Adaptation mechanisms of halophilic and halotolerant microorganisms

The adaptation of halophiles in these extreme environments depends on the organism's capability to cope under the osmotic pressure instigated by the high NaCl concentration of the niche they inhabit. Halophiles have 2 strategies for this purpose.

i. Salt-in strategy: in this strategy, they sequester inorganic ions (K^+, Cl^-,) intracellularly to equipoise the ionic concentration of the external environment and to maintain a higher osmotic potential intracellularly (to maintain positive turgor pressure. The transport of ions across membranes is through ion channels and pumps like Cl^- pumps in synergy with arginine and lysine residues for uptake of Cl^- ions or k^+ channel or ATP-dependent transport systems (Gunde-Cimerman et al., 2018). These ion exchange channels are energy-driven mechanisms. The energy required could be provided by light-driven processes using membrane-bound pigments like bacteriorhodopsin and halorhodopsin (e.g., found in *Halobacterium salinarium)*, etc.

ii. Compatible solute strategy: these are small, low molecular weight organic molecules called osmolytes. The compatible solutes can be taken up from the immediate environment or

synthesized *de novo* by the organism. Accumulation of compatible solutes and simultaneous extruding of salt by the cells help reduce osmotic potential and maintain turgor pressure (Iqbal, M.J 2018; Oren, 2002) . apart from maintaining osmotic potential, compatible solutes are also involved in DNA, enzyme stabilization, and protectors from stresses like freezing, desiccation, and heating. Osmolytes are at times referred to as chemical chaperones as they assist in the proper folding of polypeptide chains (Shivanand & Mugeraya, 2011). Include highly water-soluble sugars, amino acids, alcohols, betaine, ectoins, or their derivatives (Shivanand & Mugeraya, 2011).

Halophiles that use salt-in strategy for haloadaptation have enzymes that are dependent on salt for functioning, unlike normal enzymes (from non-halophilic organisms) which are rendered inactive through protein aggregation and collapse. Haloenzymes bind to salt due to their acidic amino acid makeup on the protein surface. These electrostatic interactions between proteins are detrimental to their stability and proper folding (Siglioccolo et al., 2011).

2.4. Applications of halophilic and halotolerant microorganisms

2.4.1. Production of Haloenzymes

In 2021, the global industrial enzyme market was valued at 6.6 billion USD and is predicted to reach a mark of 9.1 billion by 2026 (Markets and Markets, 2022). Industries such as biofuel production, detergents, textiles, and food are instigating demand for industrial enzymes (Markets and Markets, 2020). The role of enzymes in industries is affected by various factors like thermal agitation, temperature drops, pH, etc. Therefore, the market needs enzymes that can work over a dynamic range of external characteristics, without considerable loss of enzyme activity (Markets and Markets, 2022). Enzymes obtained from halophilic/halotolerant microorganisms-haloenzymes, possess optimum activity and stability at high salt and other stresses and therefore make these enzymes potential candidates for operational use, especially in harsh industrial conditions. Most of these enzymes' captivating aspects are not just high salt tolerance but also exhibit tolerance to high temperatures and stability in organic solvents (Oren, 2010) along with resistance towards denaturation, activity at low water activity, and the role of salt in maintaining structure (Karan et al, 2012). Lipases, amylases, cellulases, pectinases, proteases, and others are the most frequently sought-after industrial enzymes (Rathakrishnan & Gopalan, 2022).

2.4.1.1. Amylases

Belong to class of hydrolase enzymes and are involved in the hydrolysis of starch. Starch is a polymer of amylose (linear polymer of glucose) and amylopectin (branched polymer of glucose).

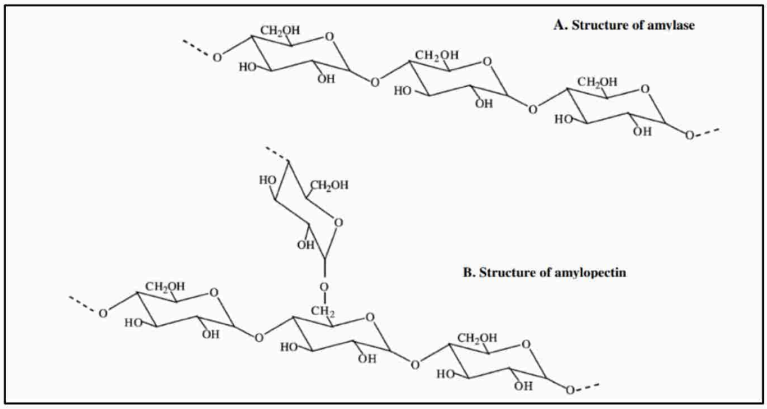

Figure 2.4.1.1. Two types of glucose polymers present in starch

(Souza & Magalhaes, 2010)

Amylases act by hydrolyzing glycosidic bonds (α-1,4 and α-1,6) between adjacent glucose units, with short dextrins as yield. Sources of amylase include plants, fungi, bacteria, archaea as well as animals (humans- secreted in the salivary gland). Amylases can be divided into two classes: endoamylases and exoamylases. Endoamylases hydrolyze the starch molecule in a random manner, yielding different lengths of linear as well as branched short chains(Gupta, et al,2003). Example: α amylase whose action on starch yields maltose, glucose, or limit dextran by hydrolyzing α 1,4 glycosidic bonds. Exoamylases hydrolyze from the non-reducing ends of the starch molecule yielding short-end products. Example: β amylase which yields glucose units or maltose and limit dextran, by cleaving either α-1,4 or both α-1,4 and α-1,6 glycosidic bonds (Tiwari, et al,2015). Applications of amylases starch saccharification, and in industries affiliated with textile, food brewing, and distillation. One example of an extracellular halophilic amylase isolated was from *Thalassobacillus* sp. LY18. This enzyme was stable over a dynamic range of temperatures (around 30 -90C) NaCl concentrations (0%-20%) and pH conditions (6-9) , demonstrating excellent halotolerant, thermotolerant and alkali tolerant properties (Li & Yu, 2012). These factors justify the demand for halophilic amylases in industries. *Haloarcula sp.* Produce an amylase active at 3M NaCl and temperature of 45°C, and could be used in food, textile and brewing industry (Siroosi et al. 2021).

2.4.1.2. Proteases

These are enzymes that catalyze the hydrolysis of peptide bonds inherent in proteins to yield smaller polypeptide chains and amino acids. Proteases are ubiquitous in nature and are produced by animals, plants, archaea, bacteria, and fungi. Proteases can be classified into two categories: endo proteases which cleave the internal peptide bonds of the protein, and exo proteases which act only at the ends of the polypeptide chain. Endo proteases are further classified as serine, cysteine, aspartic, and metalloproteases. The majority of the halophilic proteases belong to serine endo-proteases and are active at salt concentrations of around 4-5 M NaCl, pH of 5-10, and tolerate temperatures from 40-75'C. These salt-stable groups of serine protease are called halolysins, detected in *Halobacterium* sp (Mokshe, et al, 2018). Proteases from halotolerant species belong majorly to metalloproteases and serine proteases (Mokshe, et al., 2018). Bacillus luteus is an alkalohalophilic bacterium, which produces an extracellular serine endoprotease and is active at a broad range of temperatures and pH and is stable at around 5M NaCl (Kalwasinska et al. 2018). Proteases are key industrial enzymes and makeup about 20% of commercially useful enzymes and the global market for these enzymes is increasing rapidly (Mokshe, et al, 2018). These enzymes have applications in the food industry (cheese production, meat tenderization, dough activation, etc.), leather industry, detergent industry, and textile industry (Okpara., 2022). Commercially available proteases include Alcalase, Esperase (Novozymes, Denmark) FoodPro subtilisin (DuPont, USA) (Kalwasinska et al. 2018).

2.4.1.3. Lipases

These are enzymes that catalyze the hydrolysis of tri-, di-, monoglycerides, and glycerol by acting on its ester bond (Casas-Godoy, et al.,2012). These hydrolytic reactions occur at the lipid-water interface, as the substrates are immiscible in water. Apart from hydrolysis, lipases also catalyze esterification and transesterification reactions (Casas-Godoy, et al.,2012). Halophilic lipases, due to their adaptation to high salinity, elevated temperatures, and organic solvents, and considered excellent candidates for several industrial processes with harsh conditions (Que, et al, 2021). lipases are used for biodiesel production, for example, organic-solvent tolerant, alkali-stable, extracellular lipase isolated from *Idiomarina sp.* W33 using jatropha oil (Li, et al., 2014) and another lipase isolated from *Haloarcula sp*. G41 (Li & Yu., 2014). Apart from this lipases also have applications in food, detergents, cosmetics, dairy, production of biopolymers, and textile industries (Casas-Godoy, et al., 2018).

Cellulases

These are enzymes that catalyze the hydrolysis of cellulose polysaccharides by acting on β 1,4-glycosidic bonds. Cellulose is a linear polymer of D glucose, attached together by β 1,4-glycosidic bonds. Cellulase doesn't act as a single enzyme, but as a synergy of enzymes. These include-endoglucanase, exoglucanase and β glucosidase. Endoglucanase and exoglucanases yield glucose units and cellobiose units and products. β-glucobiose converts cellobiose to glucose, thus contributing to the complete breakdown of cellulose to its monomer units (Jayasekara & Ratnayake., 2019). Applications of cellulases in industries are extensive. These enzymes are used for biostoning and biopolishing in textile industries; pulping and deinking in paper and pulp industries; stain removal in detergent and laundry industries; food processing industries; and agriculture along with others (Jayasekara & Ratnayake., 2019). Example: alkali-tolerant, solvent-tolerant cellulase isolated from halotolerant Bacillus subtilis SU40 (Asha & Sakthivel., 2014) and CelR5 cellulase isolated from soil metagenome which is determined to be high salt-tolerant (Garg, et al., 2016).

2.4.1.5. Agarases

Agarases are enzymes that catalyse the hydrolysis of agar. Agar is a polymer made up of β-D galactose and anhydro α L galactose. Agar is extracted from Rhodophyceaea which is group of red algae, specifically from *Gracilaria* and *Gelidium.* α agarases cleave the α1-3 linkages to yield product agar oligosaccharides, which are related to agarose and β agarases cleave β 1-4 linkages to produce neoagarobiose (Fu & Kim, 2010). Agarases are used widely in the food industry, medical fields and cosmetics. Agarase activity has been identified in various halophilic organisms like *Thalassomonas sp.; Vibrio sp.; Agarivorans sp.;* and others.

2.4.2. Bioremediation of azo dyes from wastewater.

Dyes are substances that impart colour to materials and are widely used in the food, plastic, leather, fibre, cosmetics, and printing industries. Dyes are composed of two components: chromophore-which is composed of different functional groups and is responsible for the dyeing of the fibres and auxophore- functional groups that increase the affinity of the fibre to make up the dye. Dyes are classified into two categories on the basis of water solubility. Water soluble dyes include acid(cationic), basic(anionic), reactive and direct dyes. Insoluble dyes include sulphur, disperse, vat, azo dyes, and pigments.

2.4.2.1. Azo dyes

Azo dyes are water-soluble, aromatic compounds containing one or more functional azo (-N=N-) groups bonded to benzene rings, aromatic heterocycles, naphthalene or phenyl linkages. In general, azo dyes include an azo functional group, an auxochrome group, and a chromophore group, attached to a backbone. The azo group, auxochrome and chromophore determine the colour of the azo dye (Benkhaya, et al, 2020). Azo dyes constitute the largest class of artificial dyes utilized for commercial applications and 70% of all dyes used in industry are azo dyes. Azo dyes are widely used in a variety of industries like paper, textile, fabric dyeing, pharmaceuticals and others. Synthesis of azo dyes occurs through diazotisation of aromatic primary amines, reduction of nitroaromatic derivatives or nitroso compounds, condensation of primary amines with nitroso compounds, etc. depending on the number of azo groups: -N=N- present azo dyes can be classified as monoazo, disazo, trisazo, polyazo, and azoic dyes (Benkhaya, et al, 2020).

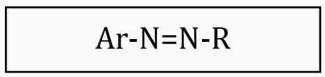

Figure2.4.2.1.1. General structure of azo dyes, where, R can be an aryl, hetero-aryl or alkyl derivative(Shah, 2019).

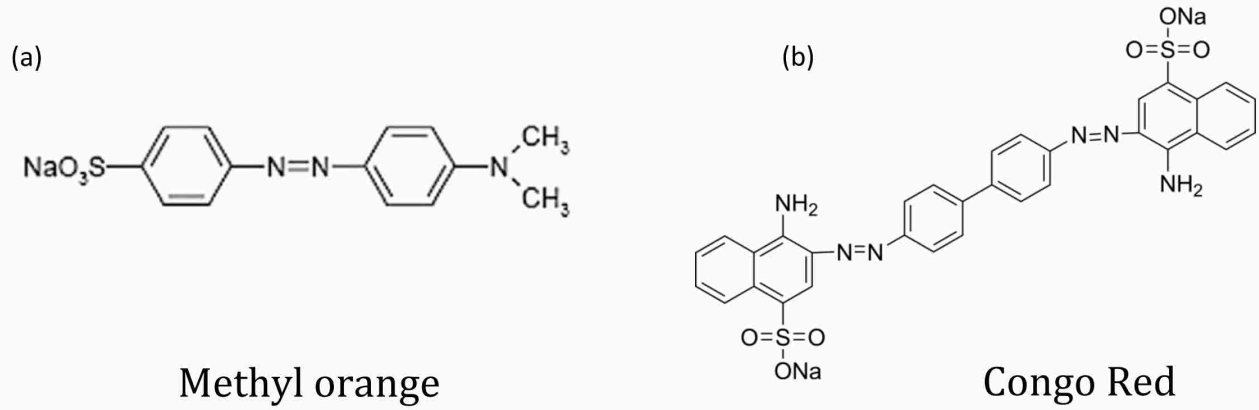

Figure2.4.2.1.2. structures of azo dye (a) Methyl Orange (Shah, 2019) *(b) Congo Red (https://file.medchemexpress.com/product_pic/hy-d0236.gif)*

2.4.2.2. Ecotoxicity of azo dyes.

Due to their chemical structure, most azo dyes (60-70%) are toxic, carcinogenic, and recalcitrant. Direct Black 38, an azo dye has been known to be carcinogenic to humans as it can be converted to benzidine through biological reactions. The textile industry has been extensively involved in the generation of effluent contaminants, surfacing from dyeing and related processes. For instance, the dyeing of 1 kg of cotton fibre with dye requires 0.6-0.8 kg of NaCl, 70-140 L of water and 70-80g of dyestuff, the end products of such processes usually consist of a highly concentrated dye solution which has a high concentration of organic molecules and salt((Babu et al., 2007). This has a major impact on water pollution, due to the release of these contaminants into open waters without prior treatment (Solis et al., 2012). Azo dyes have been studied to be extremely toxic towards aquatic life including fishes, algae, and crustaceans (Bafana et al., 2011). Azo dyes have been shown to have an impact on the feeding efficiency, procreation, and survivability of cladoceran *Moina macrocopa* (Wong et al., 2006). Apart from these, azo dyes can inhibit light penetration, hindering algal photosynthesis and deeply impacting the respiratory processes of the microbiota of the aquatic system like disrupting biogeochemical cycling. (Bafana et al., 2011). Water pollution manifests itself through its impact on total organic content, chemical oxygen demand, increasing levels of salinity, negative impacts on the aesthetic appearance, biological oxygen demand, drastic pH changes, etc. The effect of these dyes poses a serious threat to aquatic niches. It interferes with the equilibrium of the aquatic ecosystem and triggers serious risky outcomes like eutrophication of lakes, increase in turbidity, foul odours and long-term effects like mutagenicity and bioaccumulation (Berradi et al., 2019).

2.4.2.3. Removal of azo dyes

A wide variety of techniques are being utilized for the elimination of azo dye contaminants from wastewater. These could include physical processes like membrane filtration, flocculation-coagulation using inorganic coagulants like lime, aluminium, and magnesium salts, and ion exchange(Shah, 2019). Chemical methods for azo dye removal include photochemical treatment using UV/H_2O_2, and UV/O_3 where the method depends on the formation of free radicles and UV radiation which causes the decomposition of dye into CO_2 and H_2O (Shah, 2019). Other chemical methods include electrolysis, oxidation processes, etc (Shah, 2019). The problem with using such methods is the generation of waste sludge which usually requires further treatment before disposal, besides the generation of toxic sludge the processes themselves are high cost, dye specific, energy

requirement, low pH requirements and sensitivity towards molecules present in the dye wastewater (Shah, 2019).

Biological treatment of azo dyes is preferred as its environmentally friendly, cost-effective, and sludge generation is minimum (Singh et al., 2015). Biological methods could include using bio-adsorbents like corn, wheat, rice, and bagasse which are applied In a flocculation and coagulation process (Shah, 2019), or using whole microbial cells as biomass or yeast biomass and fungal biomass as adsorbents of the dye from wastewater (Shah, 2019). Microbial decolourisation of azo dyes can occur through either bio-sorption or enzymatic decolourisation and degradation of azo dyes.

2.4.2.4. Enzymatic decolourisation and degradation of azo dye

The initial step in azo dye bioremediation by enzymatic action is the cleavage of the azo bond by an azoreductase enzyme which leads to the formation of aromatic amines, these products are then further degraded by enzymes like monooxygenases, dioxygenases and hydrolases under aerobic conditions (Misal & Gawai, 2018). Azoreductases are enzymes expressed by dye-degrading microbes and can decolourise azo dyes into aromatic amino acids by the cleavage of -N=N- (azo bond) by the reduction process. Their activity depends on the presence of reducing agents like NADH, $FADH_2$, and NADPH, which act as electron donors for the breakdown process (Singh et al., 2015). Lacasses belong to oxidoreductases and are also called multicopper oxidases. Laccases are also involved in the decolourisation of azo dyes and are of great value in biotechnological processes due to their co-factor non-dependence and non-specific oxidation capacity (Singh et al., 2015). Due to their non-specificity, they have wide potential in the degradation of xenobiotic compounds including industrial dye effluents (Singh et al., 2015). Peroxidases and phenol oxidases are other enzymes involved in azo dye degradation mostly produced by fungal species and require H2O2 (Singh et al., 2015).

The effectiveness of biological treatments depends on many factors like temperature, oxygen levels, pH, the complexity of the dye, and others(Ajaz et al., 2020) Apart from these another important limiting factor for the activity or dye degrading bacteria and enzyme is the salinity of the dye effluent. Salt concentrations of around 150-200g/l have been detected in dyestuff industry wastewaters (U.S environmental protection agency) (Amoozegar et al., 2011). These salt concentrations limit the type of microorganisms that can degrade textile effluents, however halotolerant and halophilic microorganisms prove to be ideal for dye bioremediation in such highly saline environments (Qiu et al., 2022). *Halomonas elongata*, a halophilic bacterium was found to

have the ability to decolourise mono and di- azo dyes under anoxic conditions by the production of an azoreductase (AzoH) enzyme which requires NADH as an electron donor for function(Eslami et al., 2016). AzoH showed azo dye decolourisation activity up to 15% of NaCl concentrations(Eslami et al., 2016).

2.4.3. Bioremediation of sites with heavy metal contamination

Heavy metals comprise of naturally occurring substances with rather high densities, >50 atomic weight, and high atomic numbers (Sayqal and Ahmed 2021). Copper, cobalt, cadmium, nickel, zinc, mercry, selenium, etc are some metals that comprise the heavy metal clade. At micro concentration levels, heavy metals serve as micronutrients for the running of certain biological processes, metabolic reactions, and others(Bruins Kapil and Oehme 2000). Some heavy metals like iron , manganese- are used as electron acceptors for aerobic respiration, in their oxidised forms (Voica et al. 2016). However, when the concentration of heavy metals in the environment is high (>1mM), they prove to become toxic to the components of the ecosystem, including humans, microorganisms, and plants (Tchounwou et al. 2012; Chaudhary et al.2014) . Being recalcitrant in nature, heavy metals tend to accumulate in the organisms through bioconcentration or accumulate within the food chain, called as biomagnification (Tchounwou et al. 2012). Heavy metals like Ag, Pb, Al, Hg, Cd, Au, and As have no biological roles and are regarded as poisonous for all microbes, substituting themselves for the essential nutrients by competing for binding sites or through ligand interactions (Voica et al. 2016).

Nowadays, the concentration level of heavy metals in the environment has increased aggressively due to anthropogenetic activities like mining, smelting, metal processing, agricultural practices, and ceaseless fossil fuel combustion, in addition to the natural weathering of surfaces and volcanic eruptions, which has substantially contributed to heavy metal pollution of marine ecosystems (Tchounwou et al.2012). Most heavy metal polluted sites include closed drainage systems like saltpans, solar salterns and effluent discharges from industries (Pereira etal, 2012; Uratani et al. 2014). The Minamata disease caused due to methyl mercury poisoning in Japan in the 1950s is a notable example of the horrors of heavy metal contamination and the urgency to find ways to deal with heavy metal contamination (Lars Järup, 2003).

2.4.3.1. Methods for heavy metal extraction from contaminated sites

Varied processes can be used in heavy metal removal from contaminated sites, which include physiochemical methods and biological methods. Physiochemical incorporate methods like precipitation, reverse osmosis, leaching, electrochemical methods, landfilling, etc but these are mostly associated with incomplete removal of the heavy metal and sometimes add on to the problem through the production of toxic waste by-products. Apart from that these methods tend to be high cost, high energy consumption and unpredictability. Biological methods seem like a more reliable and efficient way for decontamination of heavy metal-compromised environments. Biological method application is divided into two main categories: in situ bioremediation and ex-situ bioremediation. In-situ bioremediation is dealing with the contaminant at the contamination site itself and involves methods like bioaugmentation, bespangling, biostimulation, and bioventing which are cost-effective and have fewer requirements. Ex-situ bioremediation involves the extraction of the contaminated resource from the original site and treating it at a facility, before placing it back in the extraction site. It includes processes like composting, land farming, and bioreactors (Sayqal and Ahmed 2021).

Microorganisms are the key players of heavy metal bioremediation. Many of these bacteria carry genes and plasmids coding for heavy metal resistance and comprise of several metabolic pathway modifications and mechanisms for the uptake of heavy metal ions to use it as sources of electron acceptors, and as raw materials for biological processes by chemical transformation of the metals inside cells (Sayqal and Ahmed 2021). Notably, halophiles and halotolerant microorganisms showed such patterns of metal tolerance, mostly due to the fact that some hypersaline environments naturally contain heavy metals (e.g. Searles and Mono lakes, western USA)and therefore these organisms have mechanisms to tolerate such conditions for basic survival (Kulp et al. 2006; Voica et al.2016). Halophiles are also being used as models for the molecular basis of heavy metal resistance.

2.4.3.2. Strategies for heavy metal tolerance used by halophiles.

Halophiles adopt various strategies for heavy metal resistance. Synthesis of biopolymers or exopolysaccharides (EPS) produced by halophilic bacteria and archaea are known to secrete negative residue-rich EPS which enhances binding to metal cations (Poli et al. 2011). In gram-positive bacteria, heavy metals are captured with cell wall trapping (Massadeh, Al-Momani, and Haddad 2005). Whereas in gram-negative bacteria heavy metals were concluded to be sequestered by periplasmic proteins e.g., *Halomonas sp.* (Osman et al. 2009). Metal transport across cell

membranes occurs via transport systems of the cell (ion transport e.g., phosphate, organic molecules transport e.g., glycerol) followed by the intracellular efflux or sequestration of the metal (Voica et al. 2016). Metallochaperones sense and modulate the expression of and transport of sequestered metal ions to set internal locations and the cell's inherent chelatants (Voica et al. 2016). Chelatants produced by haloarchaea include cysteine rich-peptides (like glutathione analogues) or polyphosphate (Voica et al. 2016; Orell et al. 2012). 3. Reduction of metal ions by enzyme-mediated reactions to convert them to fewer toxic forms is another strategy employed by these microorganisms (Voica et al. 2016). Various halophiles also carry plasmids that have metal resistance genes on operons. For instance, ars- arsenic resistance, cad- cadmium resistance, or mer-mercury resistance operons that contain genes for metallochaperones, transporters, proteins, enzymes, and sensors employed in the heavy metal tolerance, resistance, and detoxification (Voica et al. 2016).

Strains of Halobacillus and Halococcus are known to assist in the phytoremediation of saline soils polluted by heavy metals (Desale et al. 2014). Moreover, halophiles have been used to make whole-cell biosensors for the monitoring of heavy metal contamination (Nakayama et al. 2010). During detoxification, the sequestered heavy metal ions can be converted to nanoparticles. Strains of *Haloferax* and *Halogemetricum* are known to produce silver and selenium nanoparticles (Abdollahnia et al. 2020). Biosynthesis of nanoparticles by heavy metal-resistant halophiles in collaboration with detoxification and bioremediation of toxic heavy metals can be an economical and productive use of resources (Voica et al. 2016).

2.4.4. Aromatic hydrocarbon bioremediation by halophilic microorganisms

Aromatic compounds are organic molecules that contain one or more aromatic rings. Many aromatic hydrocarbons are ubiquitous and considered as a persistent recalcitrant, widespread in the environment. Several aromatic hydrocarbons like polycyclic aromatic hydrocarbons, toluene, phenol, and others have been known to be toxic, carcinogenic, and mutagenic due to their frequent occurrence in natural ecosystems. Aromatic hydrocarbons present themselves in aquatic systems through natural sources which include biodegradation of vegetative matter, crude oil maturation, and others. But the abundance of the hydrocarbons in these ecosystems is majorly due to anthropogenic activities like oil spills, and incomplete combustion of fossil fuels, sewage sl,udge and so on, which arise from sources like oil refineries, tanneries, food processing industries, etc. Hydrocarbons have been known to be a significant pollutant in saline water systems. The presence

of these xenobiotic compounds in the aquatic ecosystem affects the metabolism and photo-oxidation processes of aquatic life (Abdel-Shafy & Mansour, 2015). Some of these effects include tumors, compromised immunity, impairment of reproductive development, and others (Abdel-Shafy & Mansour, 2015). Polycyclic aromatic hydrocarbons have a moderate to high range of toxicity to aquatic life and birds (Abdel-Shafy & Mansour, 2015). Therefore, an efficient method of clearance of organic substances from industrial and saline wastewater is extremely beneficial from a conservation perspective (Mainka et al. 2021).

Removal of aromatic hydrocarbons from the natural system comprises several methods including chemical oxidation, leaching, photooxidation, bioaccumulation, adsorption to soil particles, etc. These methods for removal are usually expensive, inefficient, and slow (Mandri and Lin, 2007). Bioremediation is a desirable option, mainly with regard to its low cost and higher efficiency (Stallwood et al., 2005). Using microorganisms for the bioremediation of aromatic hydrocarbons focuses on the conversion of the toxic contaminant into a harmless product rendering it environmentally safe (Kumar et al., 2011). Microorganisms degrade aromatic hydrocarbons by breaking the energy-stable aromatic rings. This is done through metabolic pathways. The most known one includes the β-ketoadipate pathway when hydrocarbons like benzene, benzoate or phenol is converted to catechol, whichise then acted on and cleaved by oxygenases. The end products are further processed by the TCA cycle and transformed into pyruvate and acetaldehyde (Fuchs et al., 2011). The treatment of wastewater with high salinity requires the action of organisms and enzymes that are adapted to such challenging parameters. Moreover, the degradation of aromatic hydrocarbons has been frequently identified for the halophilic microbial community. These oorganismsare capable of using tromatic hydrocarbon as their sole source of carbon and in this process of utiliutilizationhis pollutant as an energy source, it removes it from the contaminated site. The pathways reported for aromatic compound degradation have been described in case of *Halomonas* and *Marinobacter* species, which are halophilic, for compounds like phenol, benzene and aniline. Toluene degradation was shown by halophilic bacteria like *Planococcus, Marinobacter*, and *Arhodomonas* (Mainka et al., 2021).

3. Materials and methods

3.1. Sampling

The sample was collected from the salt pans at Curca-Batim, Goa Velha, Goa India. The sampling site receives heavy annual rainfall during the monsoon season, with the average climate being warm and humid. The sample was collected during the month of May 2022. The coordinates of the location are Lat 15.458193° and Long 73.881239°. The sediment sample was collected in a sterile centrifuge tube and the salt pan water sample was collected in a clean plastic bottle.

Figure 3.1. (a) and (b) Map of the area from where the salt pan sediment was collected, Curca, Batim Goa Velha Lat 15.458193° and Long 73.881239°

3.2. Isolation, purification, and maintenance of purified isolates

*3.2.*1. Preparation of media:

The media used for the isolation of halophilic/halotolerant organisms was NTYE- NaCl tryptone yeast extract media. The pH of the media was adjusted to 7±0.2.

NTYE plates containing 5%, 10%, 15%, 20% and 25% NaCl concentrations were prepared(Salgaonkar & Bragança, 2017).

3.2.2. Isolation of potential halophilic/halotolerant cultures

1g of the sediment sample was added to 10 ml sterile saline and tenfold serial dilutions were carried out (up to 10^{-4}). 0.1 ml of the 10^{-4} dilution was spread plated onto the prepared NTYE plates. The plates were then incubated at room temperature (28±2°C) for around 20 days until diverse colonies appeared on the plates.

3.2.3. Purification of isolates

Colonies of interest from the 5%, 10%, 15%, 20%, and 25% plates were chosen and carefully picked up from the master plates and streaked onto fresh NTYE plates of the corresponding NaCl concentration. Repeated streaking was carried out multiple times until pure isolates were obtained. The colony characteristics of each isolate were noted down.

3.2.4. Maintenance of isolates

Isolates were maintained by periodic transfer onto fresh NTYE agar plates.

Glycerol stocks of the isolates were also prepared.

400µl of 60% glycerol solution with 600µl of NTYE broth were added to Eppendorf tubes. A loopful of culture was inoculated into the respective tube, marked appropriately and stored at freezing temperature (-20°C).

3.3. Determination of salt tolerance

The isolates obtained were streaked onto NTYE plates with different NaCl concentrations (5%, 10%, 15%, 20%, 25%) i.e., pure isolates obtained on 5% were streaked onto NTYE plates containing 10%, 15%, 20% and 25% NaCl, to check the salt tolerance of the selected isolates and to determine the best salt concentration for optimum growth. The plates were incubated at 28°C for approximately 15 days. Similarly, the pure cultures obtained on 10% NaCl concentration were streaked onto 5%, 15%, 20%, and 25% NaCl-containing NTYE

plates to determine the best salt concentration for optimum growth. The same procedure was repeated for pure cultures obtained from 15%, 20%, and 25% NaCl-containing NTYE plates.

3.4. Screening for enzyme activity

3.4.1. Amylase activity

Plate assay was carried out using a slightly modified NTYE medium supplemented with 1% soluble starch. NTYE medium was prepared based on the optimum concentration of NaCl required by the isolate known by the determination of salt tolerance. The cultures were spot inoculated onto these plates. The plates were then incubated at 28 ±2°C for 3-15 days. After the incubation period, the plates were flooded with 1% Lugol's iodine solution. Amylase production is indicated by the presence of clear zones at the periphery of the spot-inoculated culture in contrast to a blue-black background(Das et al., 2019).

*3.4.*2. Protease activity

10ml of skimmed, fat-free milk was sterilized separately and used as substrate stock solution. 1 ml of the stock was added to sterilized 99 ml modified NTYE media (under sterile conditions) as a substrate to check for protease activity. NTYE medium was prepared based on the optimum concentration of NaCl required by the isolate known by the determination of salt tolerance. The cultures were spot inoculated onto the media plates and incubated at 28 ±2°C for 3-15 days. The formation of clear zones/halo around the colonies indicates positive protease production (Das et al., 2019).

3.4.3. Lipase activity

Tween 80 was supplemented to a modified NTYE medium as the substrate for screening of lipase production. NTYE medium was prepared based on the optimum concentration of NaCl required by the isolate known by the determination of salt tolerance The cultures were spot inoculated onto the media plates and incubated at 28°C for 3-15 days. The presence of white precipitate around the vicinity of the culture colony indicates lipase production (Das et al., 2019).

3.4.4. Cellulase activity

1% carboxymethylcellulose (CMC) was added to modified NTYE media as the substrate for screening cellulase production. NTYE medium was prepared based on the optimum

concentration of NaCl required by the isolate known by the determination of salt tolerance. The cultures were spot inoculated onto the media plates and incubated at 28±2°C for 3-15 days. Following incubation, the plates were flooded with 0.1% Congo red solution. The presence of clear zones around the spotted culture indicates cellulase production (Das et al., 2019) .

3.4.5. Agarase activity

NTYE media devoid of any carbon source was prepared, having 2% agar as the only source of carbon and poured into sterile plates. NTYE medium was prepared based on the optimum concentration of NaCl required by the isolate known by the determination of salt tolerance. The cultures were spot inoculated onto the media plates and incubated at 28±2°C for 3-15 days. Following incubation, the plates were flooded with 1% Lugol's iodine solution. Clear zones around the colony indicate agarose activity.

3.4.6. Xylanase activity

1% xylan was supplemented to modified NTYE media as a substrate to check for xylanase production. NTYE medium was prepared based on the optimum concentration of NaCl required by the isolate known by the determination of salt tolerance. The cultures were spot inoculated onto the media plates and incubated at 28°C for 3-15 days Following incubation, the plates were flooded with 0.1% Congo red solution. The presence of clear zones around the spotted culture indicates xylanase activity.

3.5. Screening for potential azo dye decolorizing halophiles

3.5.1. Qualitative screening for azo dye decolourisation

NTYE agar medium supplemented with 0.1% dye- methyl orange and Congo red- was prepared and poured into sterile Petri plates. The cultures to be screened for dye decolourisation activity were then spot-inoculated on these plates separately. The plates were incubated at 28±2°C, isolates with 5% NaCl concentration for growth were incubated for 4 days, 10% were incubated for 7 days and 15% were incubated for 10days. The presence of zones of clearance indicates a positive result.

3.5.2. Quantitative screening of azo dye decolourisation activity

The cultures that showed zones of clearance around colonies on qualitative screening plates were selected. NTYE broth with 0.1% dye was prepared and inoculated with the screened isolates and

incubated at 28±2°C, isolates with 5% NaCl concentration for growth were incubated for 4 days, 10% incubated for 7 days and 15% were incubated for 10 days, on a shaker incubator. The maximum absorbance of the dyes; Congo red and methyl orange was determined by a pigment scan using a spectrophotometer. The wavelength at which the peak was obtained was used to determine the residual dye concentration.

Following incubation the broths were centrifuged at 8000 rpm for 5 mins. The supernatant was used to check the residual dye concentration in the broths, spectrophotometrically at the wavelength showing peak absorbance. The optical density of the broths was checked using uninoculated broth containing dye as a control. The percentage dye decolourisation of each isolate was determined using the formula:

$$\% \text{ decolourisation} = \text{Initial OD- final OD/ initial OD} \times 100$$

Initial OD= control OD

Final OD= OD of culture supernatant

3.5.3. Cross-inhibition test for consortium formulation

10 best, potential dye degrading isolates were checked for cross-inhibition. The test isolate was streaked in one vertical line on an NTYE media plate and the other isolates were streaked perpendicular to the test isolate (as shown in Figure 3.5.3.1). The plates were incubated at 28°C for 2 days. The presence of zones of inhibition at the junction of the vertical and perpendicular isolates was indicated as a positively cross-inhibiting culture. The isolates that inhabit each other cannot be used for consortia formulations. Isolates that don't show cross-inhibition were further selected to formulate a consortium.

3.5.4. Azo dye decolourisation using microbial consortia

4 different combinations of the selected isolates were put together to form 4 different microbial consortia depending on the cross-inhibition test. NTYE broth was prepared with the optimum NaCl concentration required for growth and the isolate amalgam was inoculated into the broth and incubated at 28±2°C for 2 days on a shaker incubator. After the incubation period, 200µl of the

consortia was added to tubes containing 0.1% dye-supplemented NTYE broth and incubated at 28°C for 4 days on a shaker incubator, along with an uninoculated dye broth as control.

The broths were then centrifuged at 8000 rpm for 5 mins. The supernatant was used to check the residual dye concentration in the broths by spectrophotometric analysis. The optical density of the broth supernatant was determined. The uninoculated broth has used a control (blank/initial OD). % decolourisation was determined.

3.6. Screening for heavy metal tolerance

3.6.1. Preparation of metal stock solution

The two metals chosen to check for resistance were mercury and cadmium which were used in their salt form - $HgSO_4$ and $CdSO_4$.

An initial concentration of 50mM stock solution was prepared of the metal salts using deionised water.

The stock was autoclaved and used for further tests.

3.6.2. Determination of metal tolerance

NTYE medium was prepared by weighing the individual components, with NaCl concentration corresponding to optimum growth conditions, pH was adjusted to 7±0.2 and sterilised by autoclaving. The autoclaved medium was then supplemented with 0.1 mM metal salt solution from the previously autoclaved stock solution. The medium was then poured into sterile Petri plates and the cultures were streaked onto the media plates and incubated at room temperature for 7 days. Growth on the media plates indicates tolerance of culture towards the metal salt.

3.6.3. Determination of minimum inhibitory concentration (MIC)

The cultures that initially grew on 0.1mM metal salt concentration were selected for this test. NTYE medium containing different concentrations of metal salt was prepared. The concentrations ranged from 0.15mM to 0.6mM, with 0.05mM intervals between the concentrations. The cultures were streaked onto the media plates and incubated at room temperature for 7 days. The concentration at which the growth of the organism was halted was noted down.

3.7. Plasmid isolation

Culture isolates showing considerable resistance to heavy metals were screened for the detection of plasmids. Plasmids could be the characteristic responsible for the resistance of the organism to metal toxicity. Mostly metal resistance and dye degradation genes are expected to be present on plasmids.

The plasmid isolation was done using **Thermo Scientific GeneJET Plasmid Miniprep Kit #k0502**. The components of the kit included:

Resuspension solution
Lysis solution
Neutralisation solution
Concentrated wash solution
(10mg/ml) RNase A
Elution buffer- Tris-HCl, pH 8.5
GeneJET Spin columns
Collection tubes

Initially, RNase A was added to the resuspension solution and mixed to prepare the working resuspension solution. Also 96% ethanol solution was added to the wash solution, prior to use.

3.7.1. Growth of culture isolates and preparation for plasmid isolation.

The selected culture isolates were grown in 10ml NTYE broth at room temperature for 48 hours under shaker incubator conditions. The culture was then harvested by centrifuging the incubated broth in microfuge tubes at 8000rpm for 5 mins at room temperature. The supernatant was discarded and the cell pellet was used for the further process.

3.7.2. Purification of plasmid DNA

The cell pellet obtained after centrifugation of the broth was resuspended in 250µl of **Resuspension solution** and vortexed to dissolve the cell pellet completely. No residual lumps were left behind.

To this 250µl of **lysis solution** was added and mixed thoroughly. Mixing down by gently inverting the tube 4-6 times, until the solution becomes clear and viscous. This solution wasn't allowed to incubate for more than 5 mins, proceeded to the next step quickly.

350µl of the **Neutralisation solution** was added to the tube and mixed immediately by inverting the tubes for a few times. Mixing should be through and gentle.

The tube was then centrifuged at 12,000 rpm for 5mins at 20°C, to pellet out the cell debris and the chromosomal DNA. Leaving behind the plasmid DNA in the supernatant.

The supernatant was then transferred to the provided GeneJET spin column by pipetting. Care was taken so as to not disturb the pellet. The spin column consists of the column portion and a collection tube. It was then centrifuged at 12,000 rpm for 1 minute at 20°C. The flow through was discarded and the column was placed back in the collection tube.

500µl of **wash solution** was added to the GeneJET Spin column, followed by centrifugation for 30-60 seconds. The flow through was discarded. This step was repeated using 500µl of wash solution. The spin column was centrifuged for another extra minute to remove any residual wash solution.

The GeneJET Spin column was then transferred to a microfuge tube (1.5ml). the elution buffer was prewarmed to 70°C before usage. Addition of 50µl of the **Elution buffer**, directly at the centre of the column avoiding contact with the column membrane. This was incubated for 2mins at room temperature. After incubation time the tube with the column was centrifuged for 2 minutes. The column was discarded and the purified plasmid was stored at -20°C.

3.7.3. Agarose gel electrophoresis

0.8g of agarose was added to 100 ml of 1X TBE buffer, to prepare 0.8% agarose molten gel after microwaving the solution. To this 2µl of ethidium bromide was added from an ethidium bromide stock of 10µl/ml and swirled swiftly. The gel casting tray and comb were properly arranged and the molten agar was poured into the gel casting tray. Once the gel was set, the comb was removed and the gel was placed in an electrophoretic chamber. The chamber was then filled with 1X TBE solution, upto a level where the gel is submerged.

The plasmid samples were mixed with a loading dye containing bromophenol blue and 40% sucrose and loaded into the wells made on the gel. A 100bp marker lane was loaded alongside the samples. The gel was run at 50V until the tracking dye reached $3/4^{th}$ of the length of the gel. After the electrophoretic run, the gel was viewed under UV transillumination.

3.8. Screening for biodegradation of hydrocarbon.

3.8.1. Initial screening for medium optimization.

The hydrocarbon selected for the test was sodium benzoate. Two different mediums were used for the experiment, minimal salt medium(MSM) and diluted NTYE (devoid of tryptone and a trace amount of yeast extract). The Individual components of both the media were weighed with 5% NaCl and were supplemented with 1% sodium benzoate for initial screening of growth on the hydrocarbon. 10 isolates with optimum NaCl concentration of 5% (SpWI-SpWX), were streaked onto both the medium plates i.e. NTYE and MSM and incubated at room temperature (28±2°C) for 4 days. The appearance of colonies on the media plates indicates that the culture isolate is not inhibited by the hydrocarbon and could potentially use the hydrocarbon as a carbon source. The cultures were also initially screened for agarase activity for making sure that the isolate is utilizing the hydrocarbon as the sole source of carbon and not the agar present in the medium.

3.8.2. Determination of minimum inhibitory concentration (MIC) of sodium benzoate.

After observing the growth onto MSM and NTYE plates, the isolates were selected for further screening, to check the maximum tolerance concentration (MTC) and the minimum inhibitory concentration (MIC) of sodium benzoate, which is the concentration at which sodium benzoate is toxic, and inhibits the growth of the culture isolate.

NTYE medium was prepared with varying concentrations of Sodium Benzoate. MSM medium was not utilized because no growth of the isolates was seen on MSM during the preliminary test. The concentrations utilized were; 1 mM, 5 mM, 10 mM, 20 mM, and 40 mM of Sodium Benzoate. The plates were prepared and the culture isolates were streaked onto the plates, followed by incubation at room temperature (28±2°C) for 4 days. The results were tabulated as displayed in table

3.9. Morphological characterisation

The isolates having 5% NaCl concentration as the optimum salt requirement for growth were selected for morphological characterisation.

3.9.1. Colony characteristics

The selected isolates were streaked onto sterile NTYE plates and incubated at room temperature for 48 hours. The colony characteristics of the grown isolates were then recorded in terms of size, shape, colour, margin, opacity, consistency, and elevation

3.9.2. Light microscopy

The 48 hour old culture of the isolates was smeared onto a clean, grease-free slide and heat fixed. The slide was then flooded with crystal violet and held for 1 min. the stain was discarded and followed by flooding with Gram's iodine for about 30 seconds. The slide was then rinsed with water. The slide was then decolourised with ethanol for about 30 seconds. The slide was then counterstained with saffranine for 1 min, the slide was washed, air-dried and observed under an oil immersion lens (100X).

3.9.3. Biochemical tests

Carbohydrate utilisation

Norberg-Hofsten (NH) medium (Das et al. 2019) Click or tap here to enter text.was used as the basal medium, and this 0.01% phenol red was added as an indicator. The pH of the medium was adjusted to neutral using NaOH solution. Stock solutions (10%) of different sugars were made and sterilised separately. 9 ml of the basal media was added in tubes and Durhams tubes were inserted into the tubes for the detection of gas formation. The tubes were autoclaved and 1 ml of the stock solution was added to each tube, to make a 1% concentration of sugar in the basal medium. The cultures were inoculated into the tubes and incubated at room temperature for 48 hours. The colour change of the medium from red to yellow and/or gas bubble formation in Durham's tube indicates a positive test.

3.9.4. Citrate test

Simmons citrate agar was used to detect the utilisation of citrate as the carbon source.

The required quantity of medium was weighed and the medium was prepared by autoclaving. The colour of the medium is bright green. The agar was then poured into test tubes and kept in a tilted formation so they solidify to form slants. The cultures were then

streaked onto the slants and incubated at room temperature for 2 days. The colour change of the medium from green to blue indicated a positive test.

3.9.5. Methyl red (MR) and Vogues Proskauer (VP) test

The cultures were inoculated into a 10ml glucose phosphate broth medium and incubated at room temperature for 48 hours. After incubation, the medium is separated into two parts. To one part, 3 drops of methyl red solution were added. The colour change to red indicates a positive MR test. To the other part, 3 drops of O'Meara's reagent were added and mixed well. The tubes were then incubated for 1 hour. A positive test was indicated by the pink colouration of the broth.

3.9.6. Indole test

10 ml of tryptone water broth medium was inoculated with the culture isolates. The tubes were then incubated at room temperature for 48 hours. Followed by incubation 5 drops of Kovac's reagent were added to the broth tubes. The red or pink colouration of the broth in the reagent layer at the top of the medium broth indicates a positive test.

3.9.7. Urease test

10ml of urea broth was inoculated with the culture isolates and incubated at room temperature for 48 hours. The pink colouration of the broth indicates a positive test.

3.9.8. Catalase test

A suspension of the culture isolates was prepared using 2% saline. A drop of the suspension was placed on a clean grease-free slide and a drop of 3% hydrogen peroxide was added to the suspension on the slide. Emission of effervescence indicates a positive a catalase test.

The test results were tabulated and tentative conclusions were drawn using Bergy's Manual of determinative bacteriology as reference.

4. RESULTS AND DISCUSSION

4.1. Sampling

The salt pan sediment sample was collected from the salt pans at Curca-Batim, Goa Velha, Goa India during the month of May 2022. The coordinates of the location are Lat 15.458193° and Long 73.881239°. The water from river Mandovi enters saltern creek during high tide and is drained out during low tide. The water is drained from this creek and is allowed to flow from one saltpan bed to the next, finally leading to the crystallizer pond. Here evaporation occurs and crude salt is produced (Kerkar & Loka Bharathi, 2007). The sediment sample was collected in a sterile centrifuge tube. The collected sample was stored under refrigeration conditions (around 4°C).

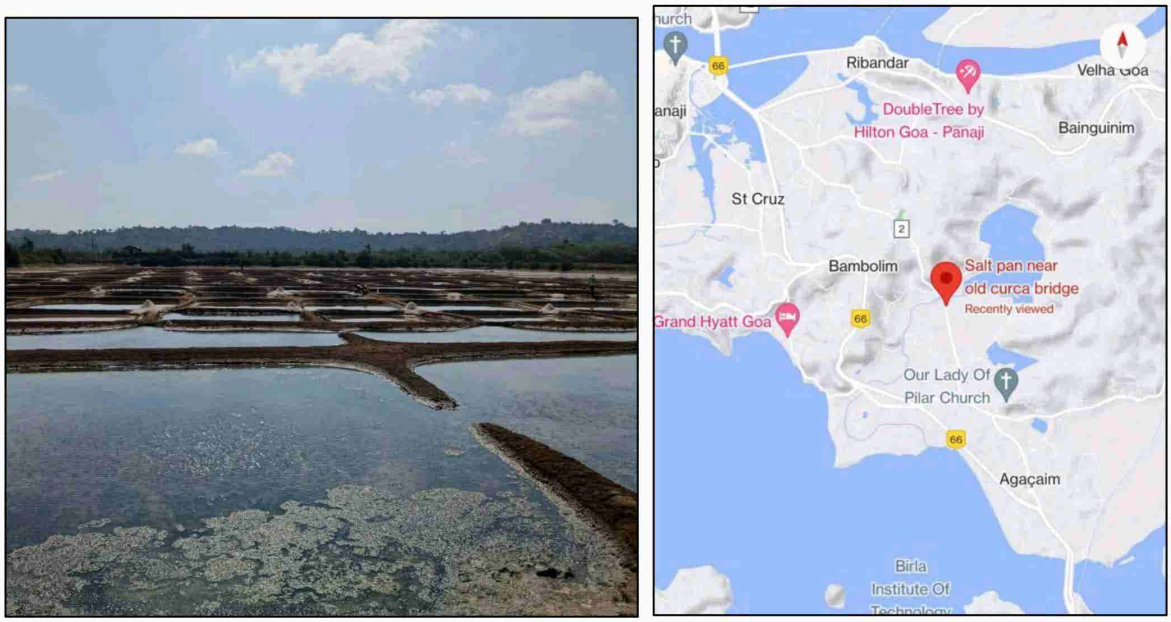

Figure 4.2.1.1 (a) Curca, Batim salt plan, Goa Velha (b) Map of Goa indicating the salt pan location

4.2.1. Preparation of media

NTYE medium was used for the isolation of potential halophilic or halotolerant bacterial species. Different concentrations of crude salt stock solution were used to make NTYE agar medium plates. The concentrations prepared were 5%, 10%, 15%, 20,%, and 25%.

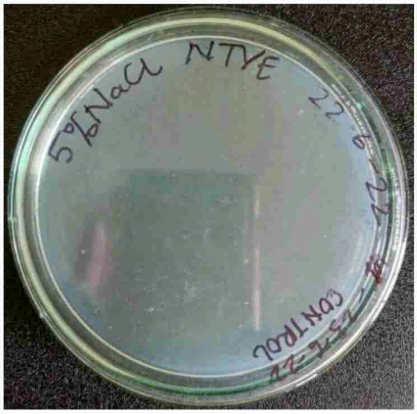

Figure 4.2. NTYE media plate

4.2.2. Isolation of potential halophilic/halotolerant cultures

The saltpan sediment sample was serially diluted and spread plated and streaked onto sterile NTYE plates. The 1st of the colonies started appearing after 4 days of incubation on the medium supplemented with 5% crude salt concentration, with subsequent growth on the higher salt concentration NTYE plates. The higher the concentration of NaCl, more was the more time required forcolonieslnies to surface on the NTYE medium plates.

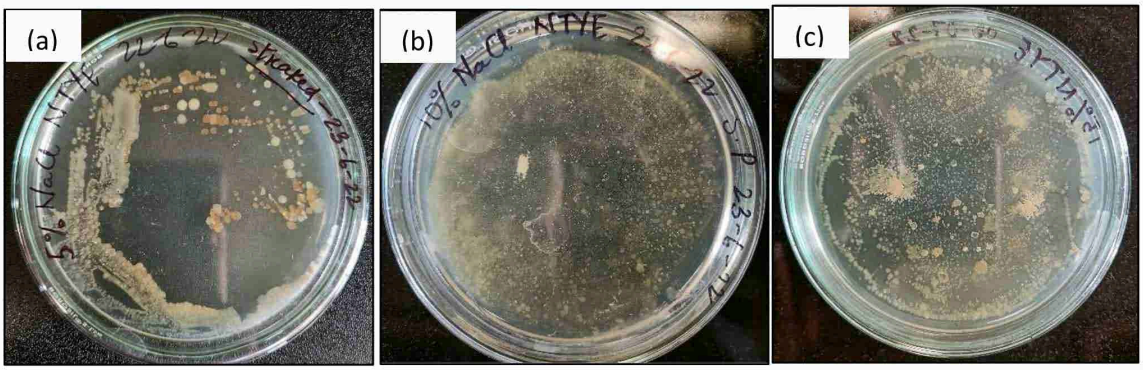

Figure 4.3 (a) 5% NTYE master plate after 4 days of incubation ; (b) 10% NTYE master plate after 7 days of incubation; (c) 15% NTYE master plate after 10 days of incubation.

Multiple colonies were obtained on all 5 concentrations of NaCl (5%, 10%, 15%, 20%, and 25%) by the 40th day of incubation.

4.2.3. Purification of isolates

Colonies showcasing distinct colony characteristics and pigments were selected from each master plate and purification was done by repeated streaking onto NTYE plates. 10 colonies were isolated from each salt concentration (5%,10%, 15%, 20%, 25%) NTYE master plates. Isolates obtained from 5%, 10%, and 10% NTYE plates were selected for further studies. Isolates obtained from 5% were named with the acronym SpWI, SpWII, SpWIII, up to SpWX. Isolates from 10% were named with the acronym SppW1 up to SppW10. Isolates from 15% were denoted acronym SpW1 upto SpW10.

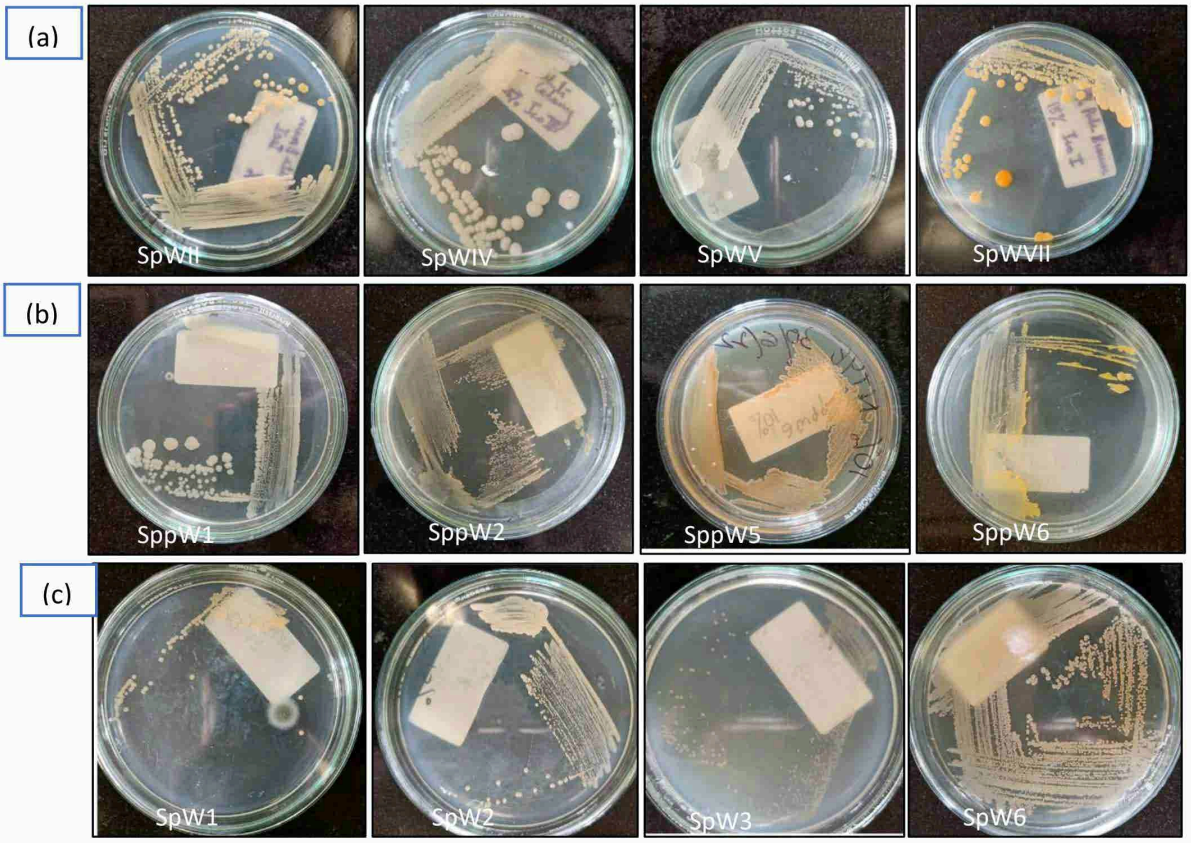

Figure 4.3 Few of the purified isolates (a) isolated from 5% NTYE (b) isolated from 10% NTYE (c) isolated from 15% NTYE

4.2.4. Maintenance of isolates

The culture isolates were maintained on NTYE slants and preserved as glycerol stocks.

4.3. Determination of salt tolerance

The culture isolates were grown on NTYE medium containing different concentrations of salt and grown in NTYE medium without salt to check if the organism necessarily required salt for growth. The time taken for the cultures to grow, the size of the colonies, the intensity of pigment formation was all recorded. These observations were used to determine the optimum salt concentration of the isolates for growth.

Table 4.3.1. Salt tolerance of 10 isolates obtained from NTYE medium containing 5% NaCl concentration.

Salt concentration	SpWI	SpWII	SpWIII	SpWIV	SpWV	SpWVI	SpWVII	SpWVIII	SpWIX	SpWX
No salt	No growth	++++	++++	No growth	++++	++++	++++	++++	No growth	No growth
5%	+++	+++	+++	+++	++++	+++	+++	+++	+++	+++
10%	++	++	++	++	+++	++	++	++	++	++
15%	+	+	+	+	++	+	+	+	+	+
20%	no growth	no growth	no growth	no growth	+	no growth	no growth	no growth	no growth	no growth
25%	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth

Key : (+) ok growth; (++)good growth; (+++) excellent growth; (++++)best growth.

Isolates SpWI, SpWIV, SpWIX, and SpWX showed no growth in the absence of salt, hence this indicates that these isolates are halophilic and strictly require salt for growth and survival. Isolates SpWII, SpWIII, SpWV, SpWVI, SpWVII, and SpWVIII showed the best growth at 0% NaCl concentration as well as considerable growth on increasing salt concentration in the NTYE medium. This is indicative, that these cultures are halotolerant in nature. The survivability of cultures was inversely proportional to the salt concentration. The growth of the isolates decreased with an increase in salt (NaCl) concentration, and was inhibitory at concentrations above 15%. The isolates SpWI, SpWIV, SpWIX, and SpWX obtained and isolated at 5% NaCl concentration on salt are moderately halophilic in nature.

Table 4.3.2. Salt tolerance of 10 isolates obtained from NTYE medium containing 10% NaCl concentration

Salt concentration	SppW1	SppW2	SppW3	SppW4	SppW5	SppW6	SppW7	SppW8	SppW9	SppW10
5%	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
10%	++	++	++	++	++	++	++	++	++	++
15%	+	+	+	no growth	+	+	+	+	+	+
20%	no growth	no growth	no growth	no growth	+	no growth	no growth	no growth	no growth	no growth
25%	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth

Key : (+) ok growth; (++)good growth; (+++) excellent growth; (++++)best growth.

All 10 isolates obtained from the NTYE medium with 10% NaCl concentration showed the best growth at 5% NaCl concentration, indicating that they optimally grow at 5% NaCl concentration. These isolates showed a gradual decrease in growth as the NaCl concentration in the NTYE medium increased. None of the isolates grew at NTYE medium with NaCl concentration above 15%.

Table 4.3.3. Salt tolerance of 10 isolates obtained from NTYE medium containing 10% NaCl concentration

Salt concentration	SpW1	SpW2	SpW3	SpW4	SpW5	SpW6	SpW7	SpW8	SpW9	SpW10
5%	+++	+++	+++	+++	+++	+++	+++	+++	no growth	+++
10%	++	++	++	++	++	++	++	++	no growth	++
15%	++	++	++	++	++	++	++	++	++	+
20%	no growth	no growth	no growth	no growth	+	no growth	no growth	no growth	no growth	no growth
25%	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth	no growth

Key : (+) ok growth; (++)good growth; (+++) excellent growth; (++++)best growth

All 10 isolates except SpW9, obtained from the NTYE medium with 15% NaCl concentration showed excellent growth at 5% NaCl concentration, with a gradual decrease in growth as the NaCl concentration in the NTYE medium increased. Isolate SpW9 showed growth on NTYE containing 15% NaCl concentration only, this indicates that it strictly requires 15% NaCl concentration for growth, and hence 15% is its optimum growth condition and is strictly halophilic.

Most of the 30 isolates screened for salt tolerance exhibited 5% NaCl concentration as the optimal concentration of NaCl for growth.

Figure 4.3.1. The growth difference among isolates obtained from NTYE medium containing 5% NaCl concentration, on NTYE medium having varying concentrations of salt.

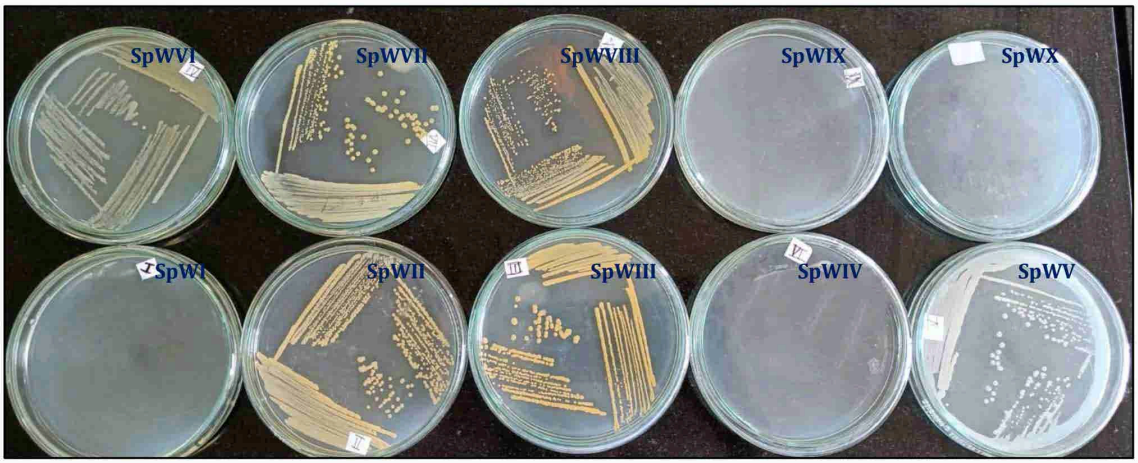

Figure 4.3.2. Culture isolates obtained from NTYE medium containing 5% NaCl concentration, grown on NTYE medium with no crude salt added (0% NaCl concentration)

4.4. Screening for enzymes

30 isolates obtained from the saltpan sediment sample were checked for the production of hydrolytic enzymes.

4.4.1. Amylase activity:

30 isolates were screened for amylase activity, using an NTYE medium supplemented with 1% starch. Out of the 30 isolates screened, 3 isolates- SpWIX, SpW9 and SpW10 showed moderate amylase activity. Among these 3 isolates, SpWIX showed the widest zone of clearance of .

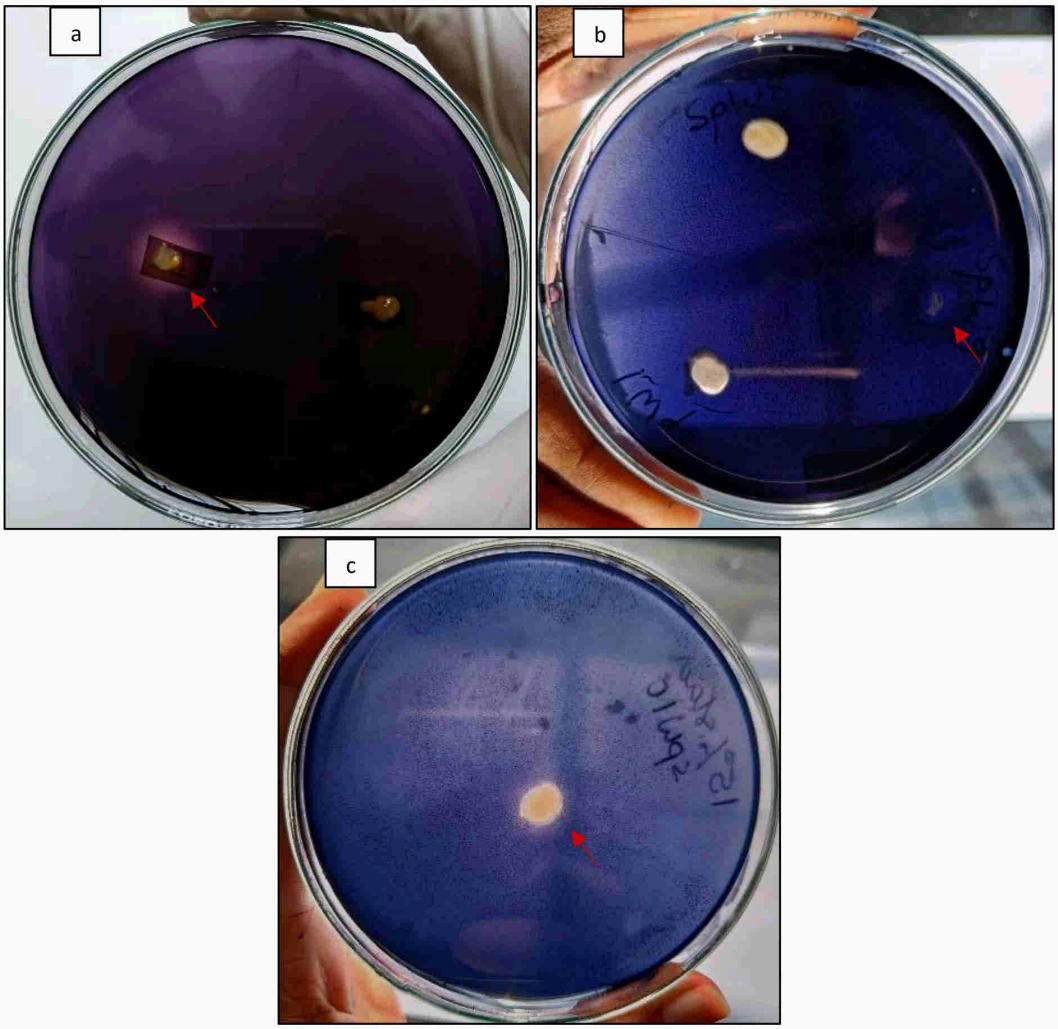

Figure 4.4.1.1. Zone of clearance detected after flooding inoculated NTYE medium plate with 1% Lugol's iodine (a) SpWIX on NTYE with 5% NaCl ; (b) SpW9 on NTYE medium with 15% NaCl; (c) SpW10 on NTYE medium conatining 15% NaCl

The extent of amylase activity of isolate SpWIX was checked by checking the amylase activity by growing the isolates on different concentrations of salt- 5%, 10% and 15%. The enzyme activity at 5% salt concentration was the highest by comparison.

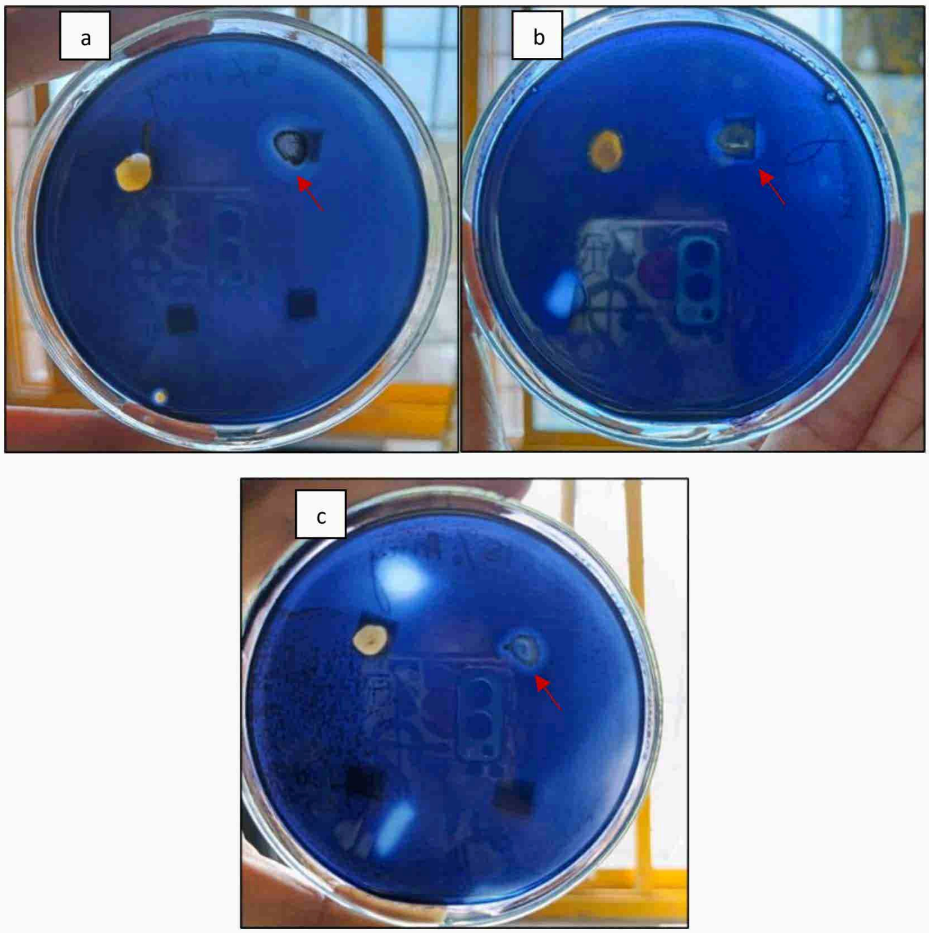

Figure 4.4.1.2. Zones of clearance of amylase activity were observed after inoculation of SpWIX at different salt concentrations. (a) 5%; (b) 10%; (c) 15%

4.4.2. Protease activity

30 isolates were screened for protease activity using an NTYE medium supplemented with skim milk. Out of the 30 isolates, 5 isolates- SpWII, SpWIII, SpWV, SpWVII, and SppW4, showed zones of clearance around the vicinity of the colonies after the incubation period, indicating protease production. SpWV showed the highest protease activity among the isolates. Protease activity, by comparison, was highest at 5% salt concentration.

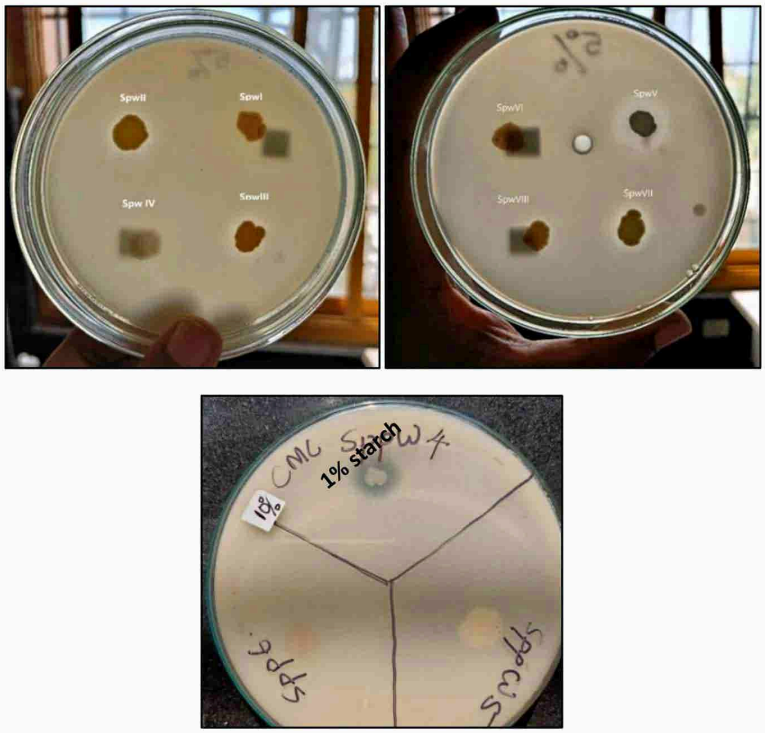

Figure 4.4.2.1. Zones of clearance observed on NTYE media supplemented with 1% skim milk

4.4.3. Lipase activity

30 isolates were screened for lipase activity, using NTYE medium supplemented by tween80, to check for lipase activity. Out of the 30 isolates, 3 isolates- SppW7, SppW9, and SppW10, showed precipitate formation around the vicinity of the colony, indicating lipase activity. Lipase activity was observed only from isolates grown on an NTYE medium containing 10% NaCl concentration.

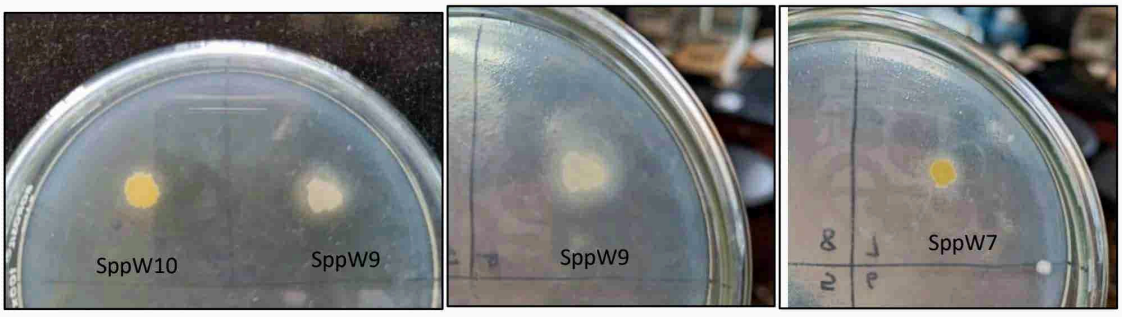

Figure 4.4.3.1. Precipitate formation around the vicinity of colonies, indicating lipase activity on NTYE media with Tween80 as substrate

4.4.4. Cellulase activity

30 isolates were screened for cellulase activity, using NTYE medium supplemented with carboxymethyl cellulose (CMC) as substrate. No zones of clearance were observed on the plates after flooding with 0.1% Congo red solution post-incubation, for any of the 30 isolates. Therefore, none of the 30 isolates showed cellulase activity.

4.4.5. Agarase activity

30 isolates were screened for agarose activity, using NTYE medium devoid of any carbon source other than agar. Isolate- SpWIX , out of 30 isolates was the only culture that showed agarase activity, observed as a zone of clearance around the vicinity of the colony on flooding with 1% Lugol's iodine solution.

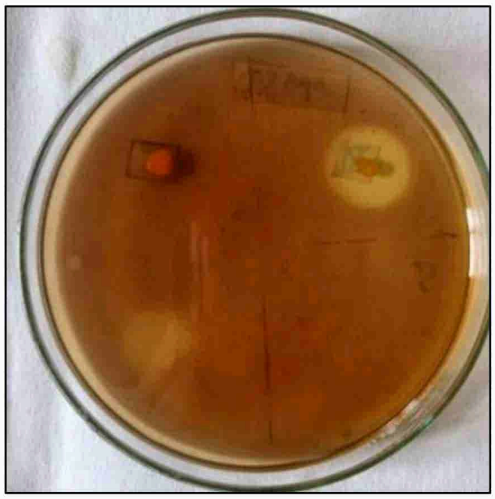

Figure 4.4.5.1. Zone of clearance around SpWIX isolate on the addition of iodine, indicating agarase activity.

The extent of agarase activity of isolate SpWIX was checked on different concentrations of salt- 5%, 10% and 15%. The optimum agarase activity was at 5% salt concentration. The agarase activity showed a gradual decline as the salt concentration increased.

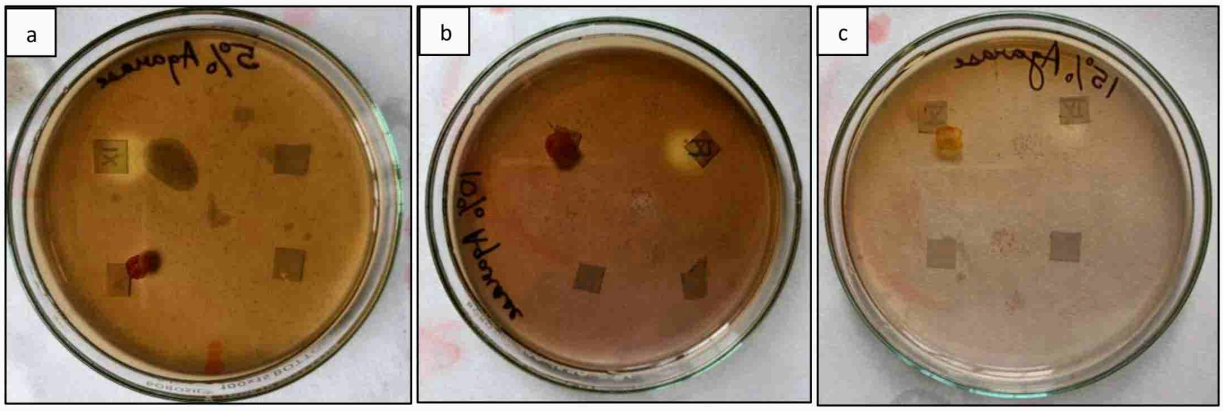

Figure 4.4.5.2. Zones of clearance of agarase activity observed after inoculation of SpWIX at different salt concentrations. (a) 5%; (b) 10%; (c) 15%

4.4.6. Xylanase activity

30 isolates were screened for xylanase activity, using an NTYE medium supplemented with 1% xylan. No zones of clearance were observed on the flooding of the post-incubation plates by 0.1% Congo red solution, for any of the 30 isolates. This indicates no xylanase activity.

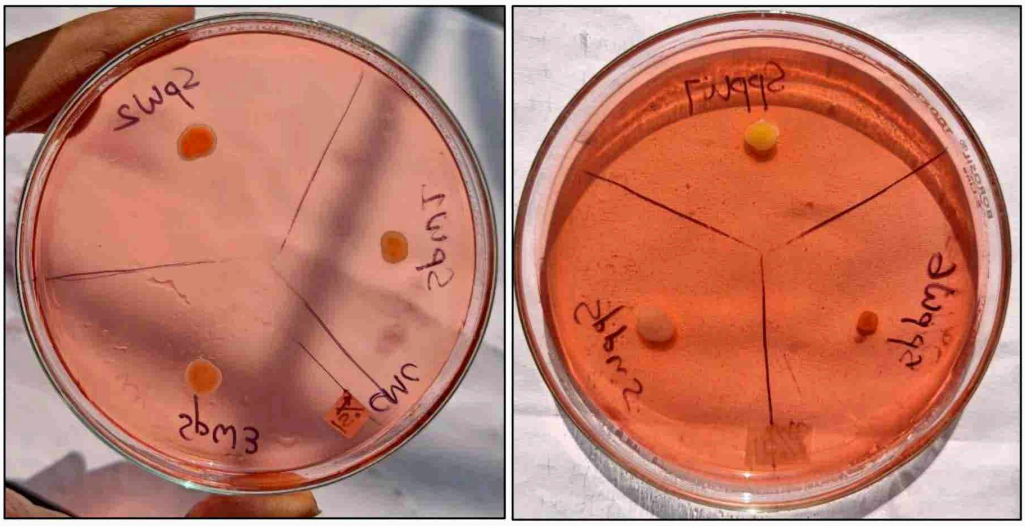

Figure 4.4.6.1 No zones of clearance were seen after flooding with Congo red on NTYE medium supplemented with 1% xylan, indicating a negative result for xylanase activity.

4.5. Azo dye decolorization

4.5.1. Qualitative screening for azo dye decolorization

30 culture isolates were screened for azo dye decolorization. Two azo dyes were used for the experiment- Congo red and Methyl orange.

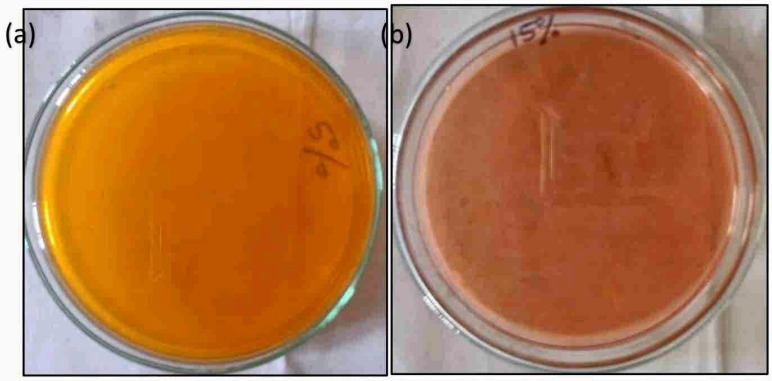

Figure 4.5.1.1 Control plates (a) Methyl orange and (b) Congo red

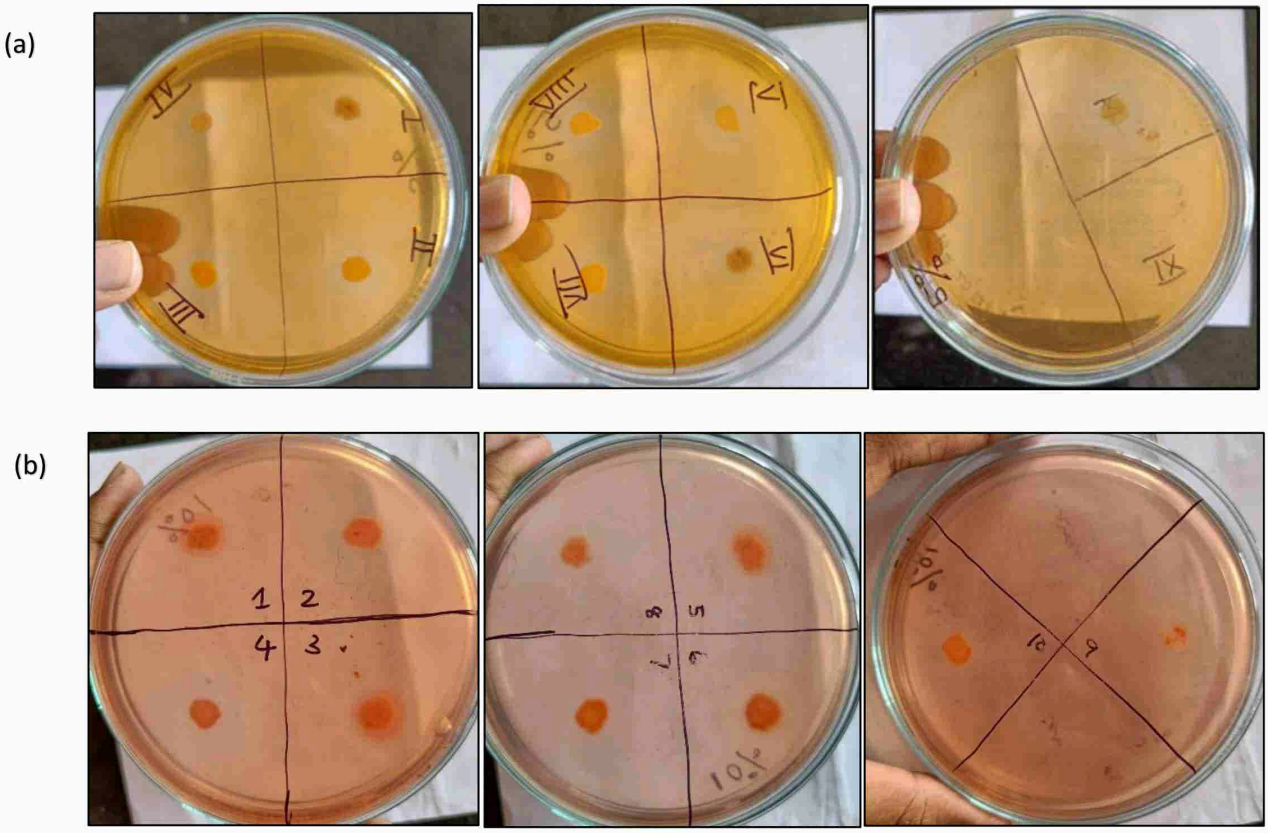

Figure 4.5.1.2 Decolourisation of azo dye in the vicinity of the colonies. (a) isolates obtained from 5% NaCl concentration grown on NTYE medium supplemented with 0.1% Methyl orange and (b)) isolates obtained from 10% NaCl concentration grown on NTYE medium supplemented with 0.1% Congo red

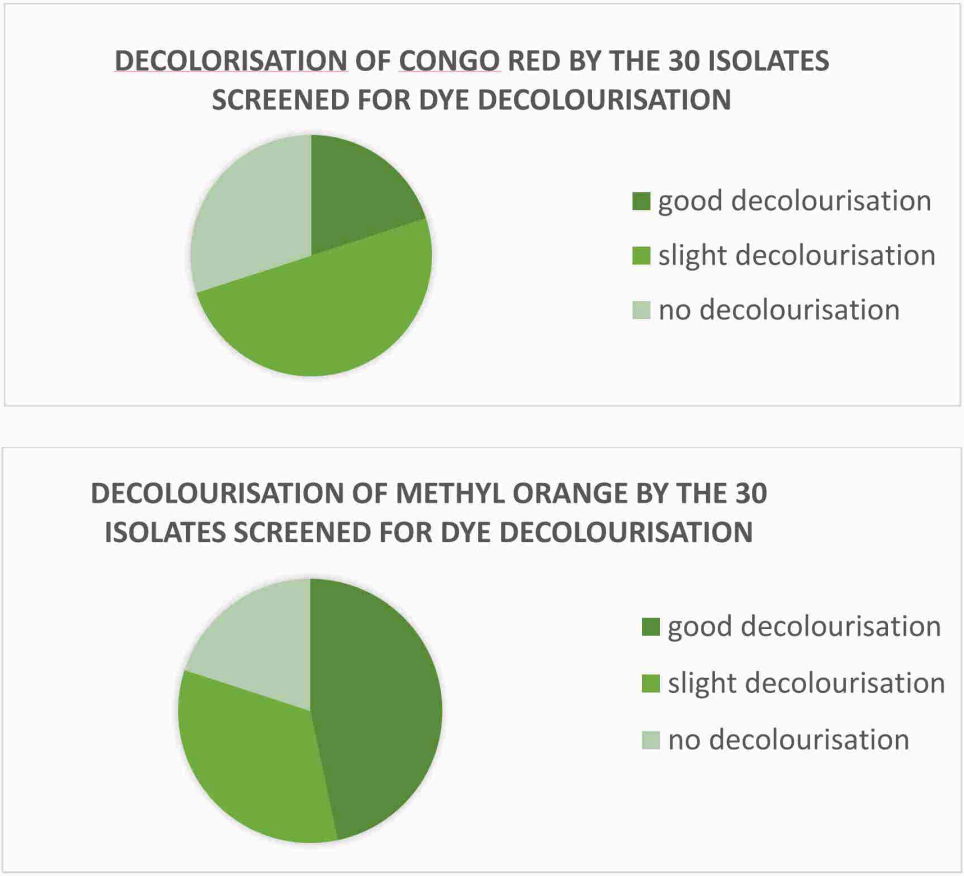

Figure 4.5.1.3 Total consensus of decolorization of methyl orange and Congo red, by the selected 30 isolates.

Out of the 30 isolates screened for methyl orange and congo red dye decolourisation, most of the isolates showed a greater efficacy of decolourising Methyl orange dye compared to congo red dye.

4.5.2. Quantitative screening of azo dye decolorization activity

The cultures that showed zones of clearance around colonies on qualitative screening plates were selected for quantitative analysis. The isolates were grown on dye-supplemented (methyl orange and congo red) NTYE media broth. The maximum absorbance peaks of the dyes were determined before the checking optical density of the culture broths to determine the peak absorbance wavelength. The peak absorbance for methyl orange was at 483nm and for congo red was at 471nm.

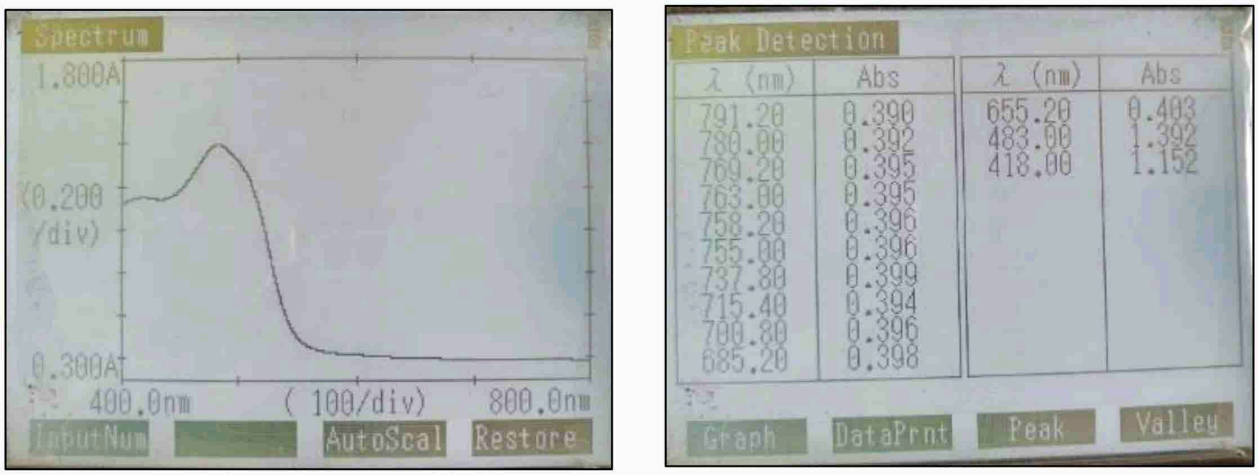

Figure 4.5.2.1 Peak detection of methyl orange in NTYE medium

Peak absorbance observed at 483nm

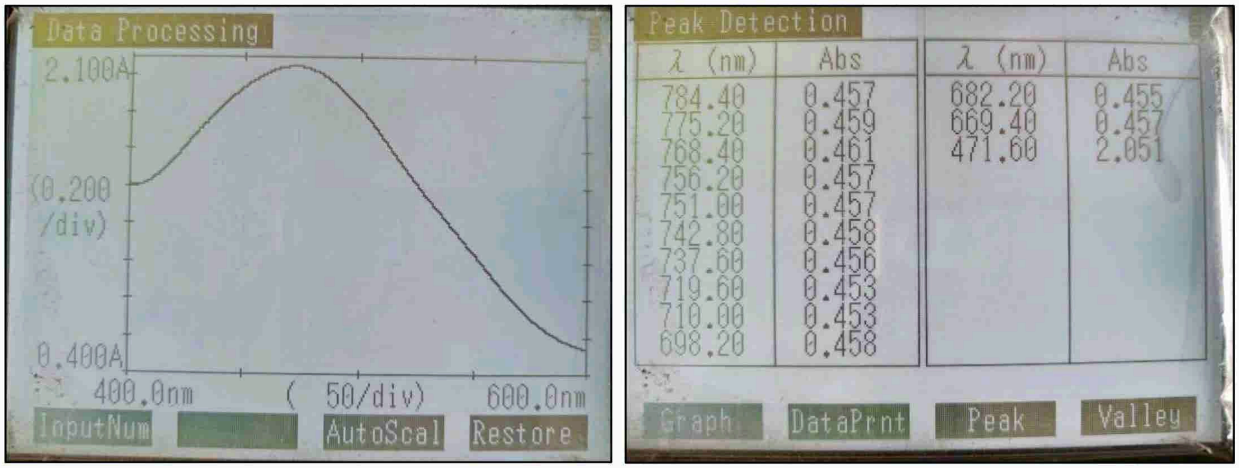

Figure 4.5.2.2 Peak detection of Congo red in NTYE medium

Peak absorbance observed at 471 nm.

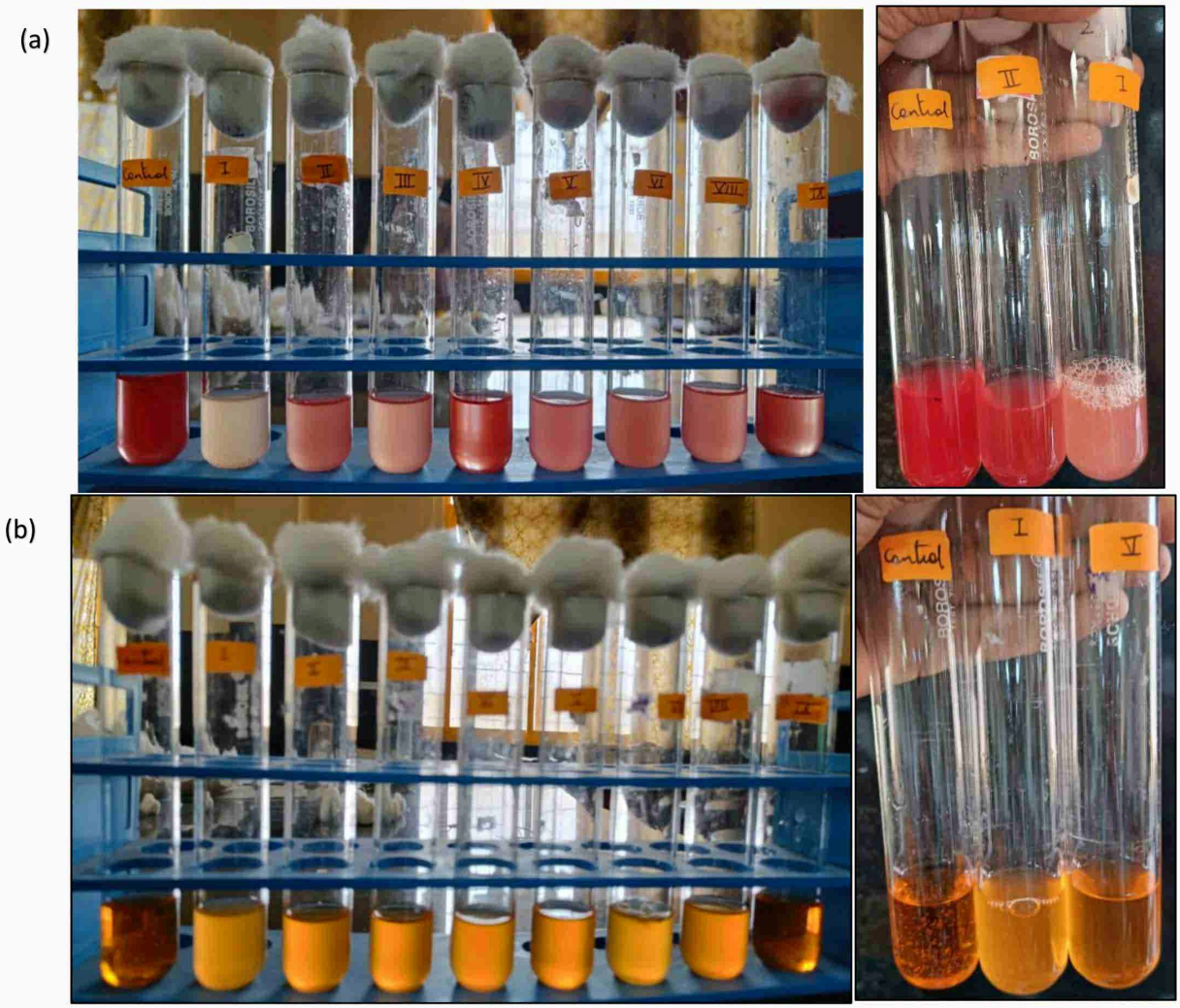

Figure 4.5.2.3 Decolorization of dyes by the isolates obtained from 5% NaCl concentration grown in NTYE medium broth supplemented with (a) 0.1% Congo red (b) 0.1% Methyl orange after 72 hours of incubation

The decolourisation of the dye supplemented in the NTYE broth by isolates (with 5% NaCl concentration as an optimal requirement for growth), was clearly visible after 72 hours of incubation when compared with the control tube. Following incubation, the broths were centrifuged and the absorbance of the supernatant was determined spectrophotometrically. Uninoculated NTYE broth supplemented with 0.1% dye was used as the initial absorbance reading. The residual dye concentration was determined using absorbance values, which were used to find % decolourisation.

The % decolorization results were noted down graphically.

From the ten isolates obtained from NTYE medium with 5% NaCl concentration, isolate SpWX showed the highest decolourisation activity of methyl orange, of about 49.86% while simultaneously, showing the least % decolourisation for Congo red. Isolate SpWVIII showed the best overall decolourisation of both the dyes, methyl orange and Congo red. Overall, isolates with 5% as the optimum concentration of salt for growth showed the best decolourisation activity towards methyl orange dye, ranging from 27.03% - 78%, while showing poor decolourisation potential for Congo red.

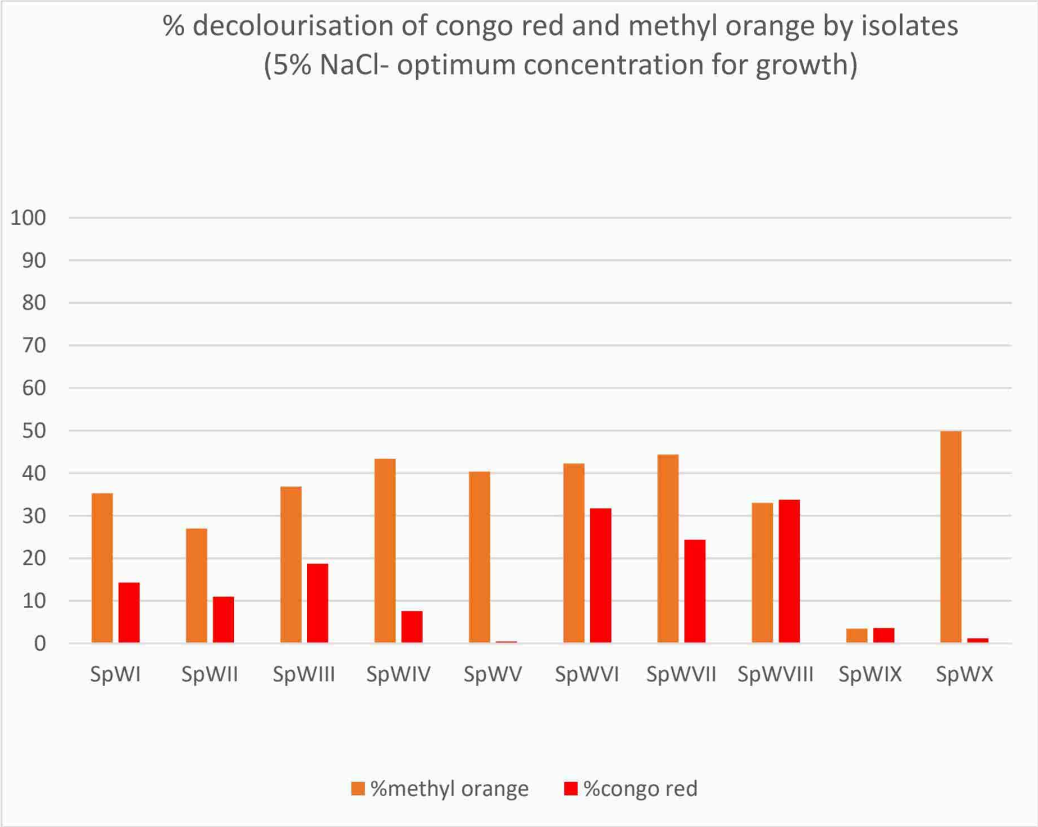

Figure 4.5.2.5 Graphical representation of percentage decolorization of the dyes based on absorbance values for isolates obtained from 5% NaCl concentration NTYE medium.

% decolorization of Congo red and methyl orange by isolates obtained from 10% NaCl concentration medium, showed good decolorization of methyl orange, but Congo red was sparingly decolorised. Methyl orange decolourisation ranged from 3.8% - 46%. Congo red decolourisation was observed only for 7 out of 10 isolates.

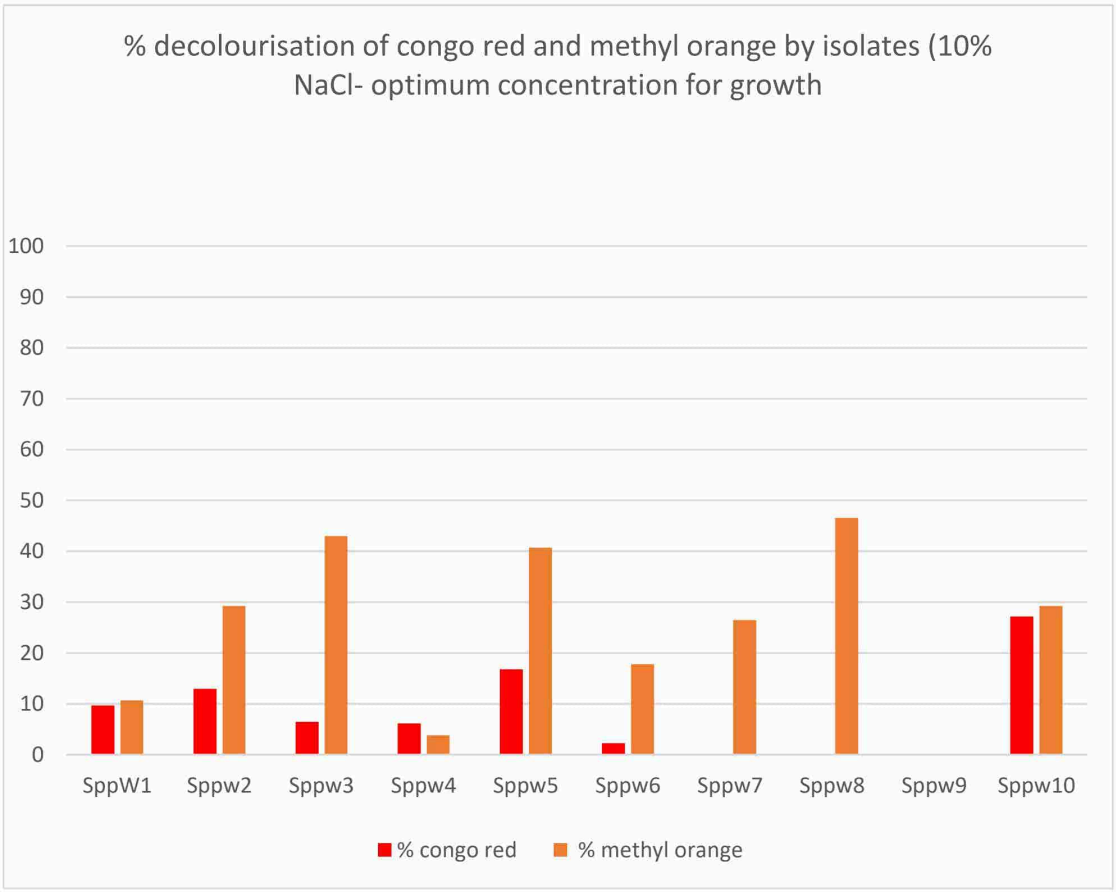

Figure 4.5.2.6 Graphical representation of percentage decolorization of the dyes based on absorbance values for isolates obtained from 10% NaCl concentration NTYE medium

Isolates obtained from a 15% NaCl concentration medium showed better overall decolourisation of Congo red than methyl orange. With Congo red decolourisation ranging from 34.4%-78%. Isolate SpW9 showed the highest Congo red decolourisation activity of about 78%.

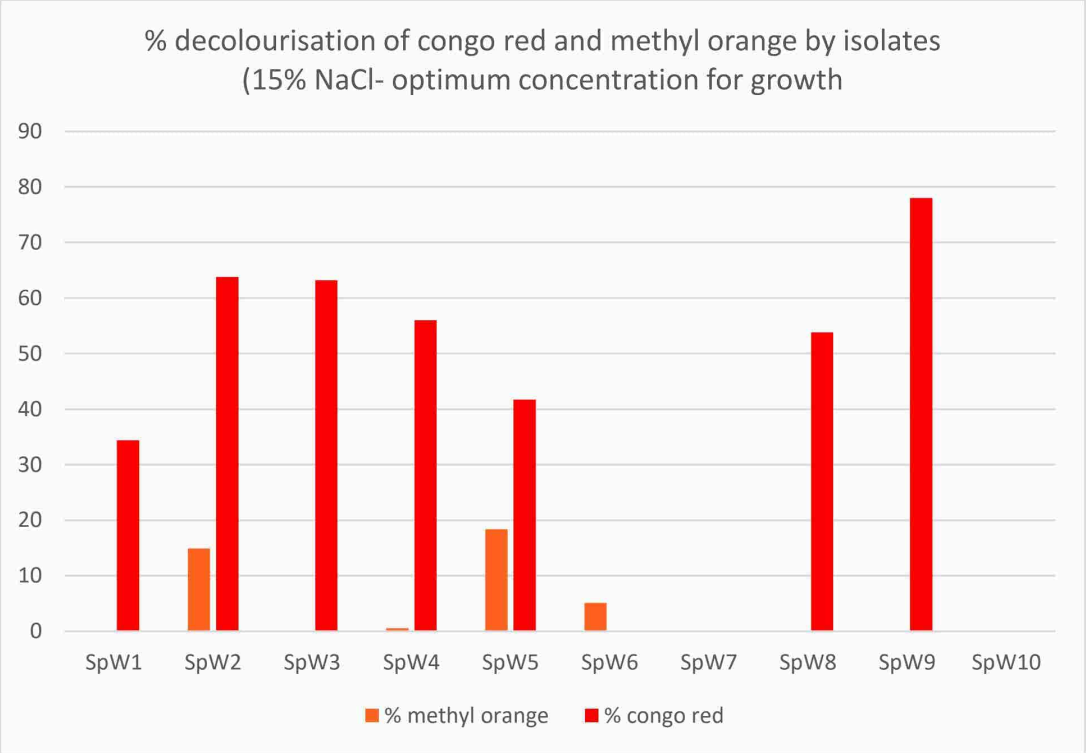

Figure 4.5.2.6 Graphical representation of percentage decolorization of the dyes based on absorbance values isolates obtained from 15% NaCl concentration NTYE medium.

4.5.3. Cross-inhibition test for consortium formulation

Out of 30, 10 isolates were checked for cross-inhibition. The test isolate was streaked in one vertical line on an NTYE media plate and the other isolates were streaked perpendicular to the test isolate.

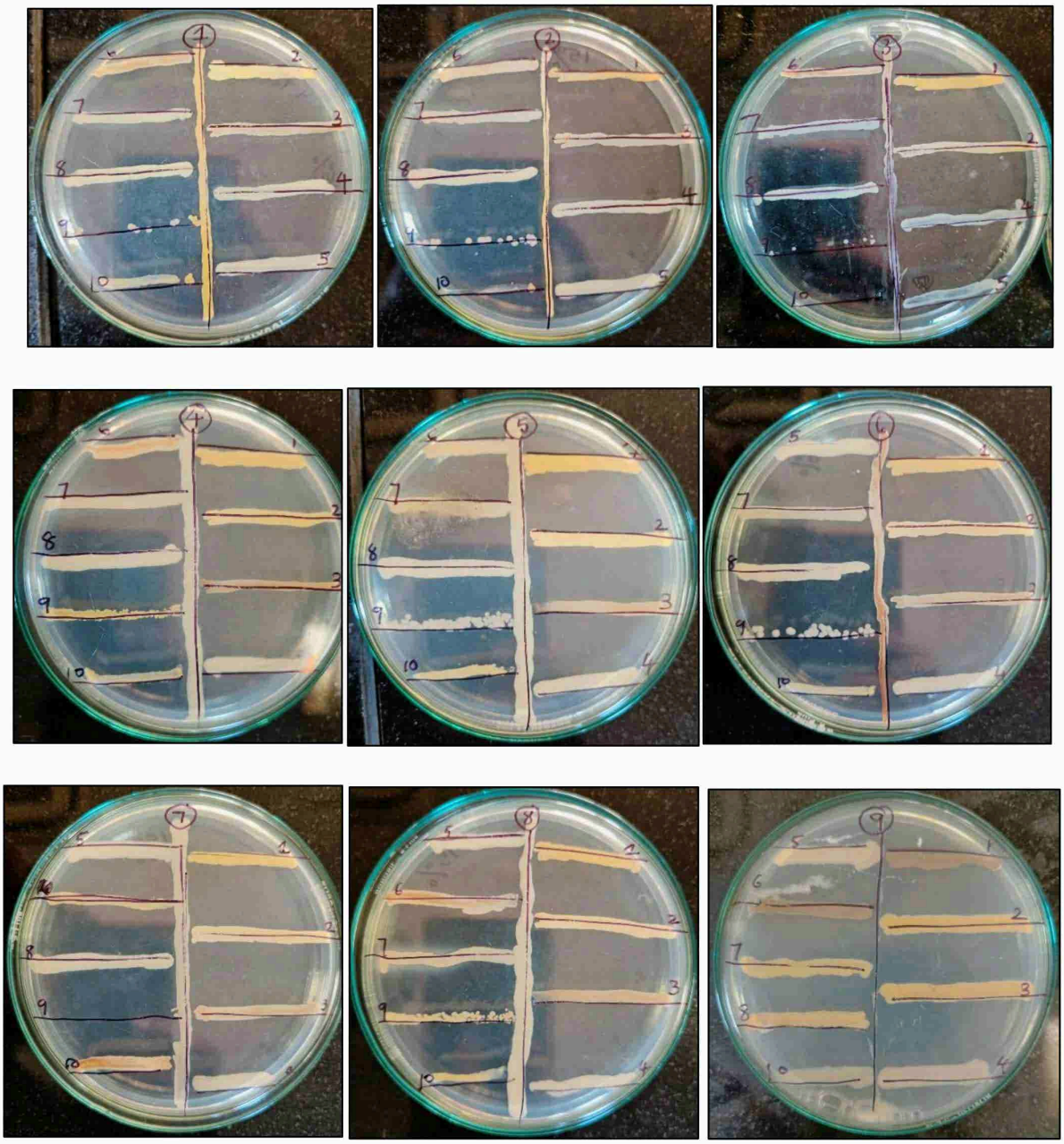

Figure 4.5.3.1 Microbial cross-inhibition test.

The presence of zones of inhibition at the interface of the cultures streaked in vertical and perpendicular line indicates inhibition. But the ten isolates, examined showed no zones of clearances, this indicates that the cultures do not inhibit each other's growth and can be used for formulate microbial consortia.

4.5.4. Azo dye decolorization using microbial consortia

4 different combinations of the selected isolates were put together to form 4 different microbial consortia. The consortiums were formulated based on their qualitative and quantitative decolourisation analysis of methyl orange and congo red. From the results obtained from the previous experiment, the isolates obtained from NTYE medium with 5% NaCl concentration showed the best overall decolourisation activity for both the dyes. Hence, these isolates were used to formulate the consortia.

Table 4.5.4. Consortia formulations assessed for dye decolourisation

	Consortia members
Consortium I	SpWI, SpWII, SpWIII, SpWIV and SpWV
Consortium II	SpWVI, SpWVII, SpWVIII, SpWIX, and SpWX
Consortium III	SpWIV, SpWVII, and SpWX
Consortium IV	SpWV, SpWIV, and SpWVII

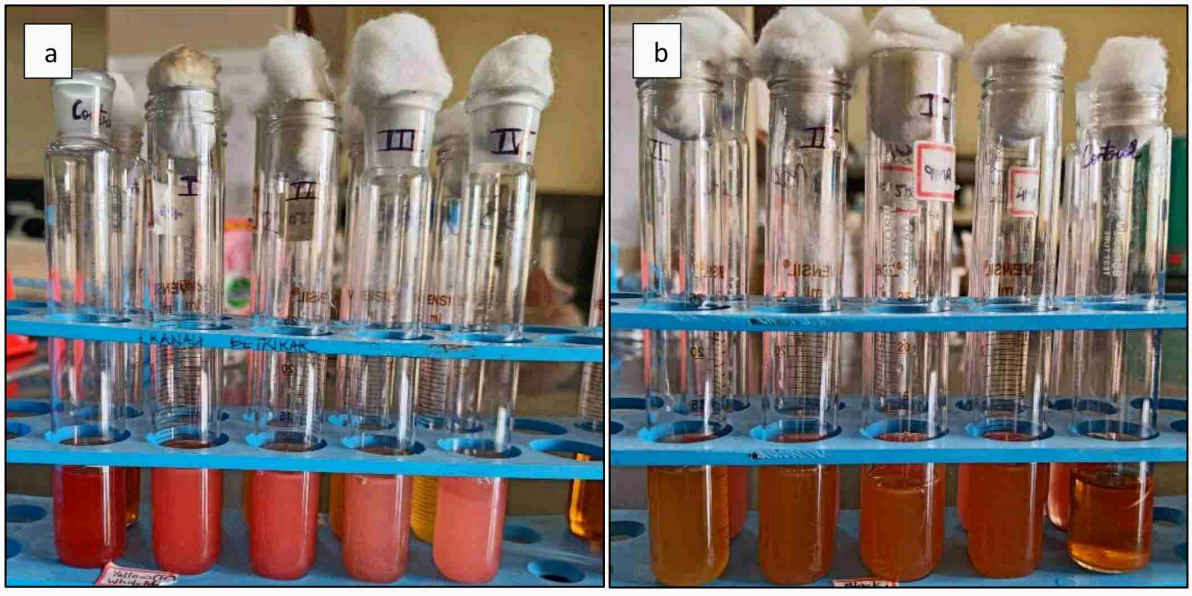

Figure 4.5.4.1 Decolorization of dye by various consortia formulations. (a)Congo red and (b)Methyl orange

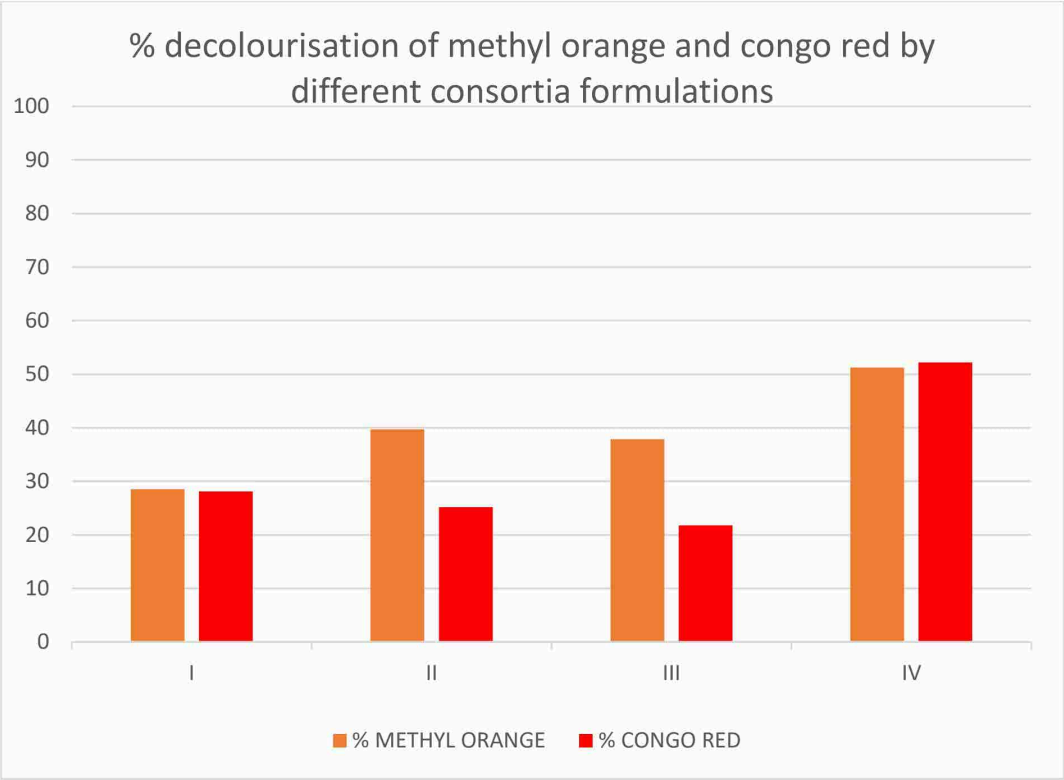

Figure 4.5.4.2. % decolorization of dye by various consortia formulations.

Consortium IV was determined to be the best formulation among the 4 formulated consortia. Showing almost 50% methyl orange and Congo red, dye decolourisation. Consortium IV comprising of SpWV, SpWIV, and SpWVII showed a higher decolourisation activity for both methyl orange and Congo red, compared to the individual decolourisation activity of the isolates.

4.6. Screening for heavy metal tolerance

4.6.1. Determination of metal tolerance

The screening for heavy metal tolerance was done for 10 isolates with 5% NaCl concentration as optimum requirement for growth , SpWI- SpWX. A concentration of 0.05mM of $HgCl_2$ and $CdSO_4$ were added respectively to the NTYE medium. The 10 cultures were streaked onto the media plates and incubated at 28°C for 7 days. After 7 days, the results were noted down as depicted in table 3.5.1.

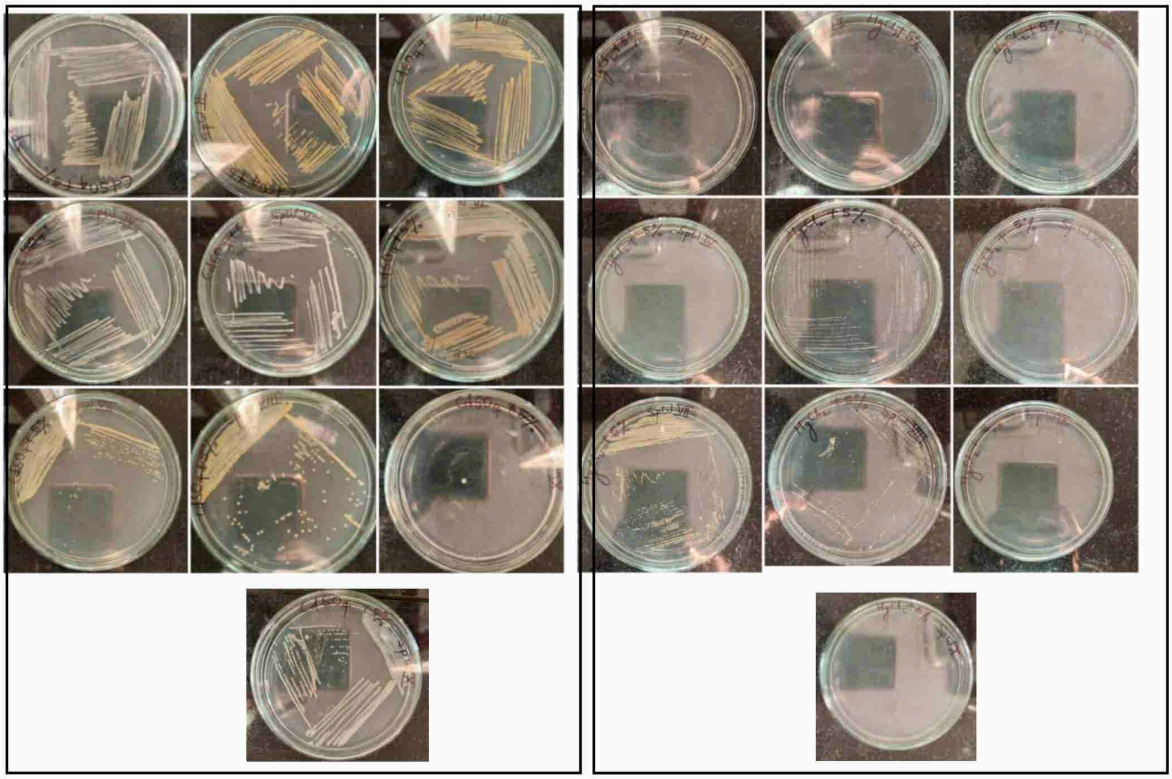

Figure 4.6.1. Growth of cultures on 0.05 mM concentration of heavy metals Hg and Cd

Nine out of ten isolates showed tolerance towards a minimum 0.05mM concentration of $CdSO_4$. Isolate SpWIX showed susceptibility towards 0.05mM of$CdSO_4$. However, isolate SpWV, SpWVI, and SpWVII grew on 0.05mM $HgCl_2$, while the remaining 7 isolates were susceptible at 0.05mM $HgCl_2$ in the NTYE medium, indicative of low tolerance $HgCl_2$.

Table no. 4.6.1. Growth of cultures on 0.05 mM concentration of heavy metal salt $HgCl_2$ and $CdSO_4$

5% NaCl NTYE	0.05 Mm $CdSO_4$	0.05mM $HgCl_2$
SpWI	+	-
SpWII	+	-
SpWIII	+	-
SpWIV	+	-
SpWV	+	+
SpWVI	+	-
SpWVII	+	+
SpWVIII	+	+
SpWIX	-	-
SpWX	+	-

KEY: (+) growth; (-) no growth

4.6.2. Determination of minimum inhibitory concentration (MIC)

The cultures that initially grew on 0.05mM metal salt concentration were selected for this test. NTYE medium containing different concentrations of metal salts ranging from 0.15mM to 0.6mM.

Table 4.6.2.1. Minimum inhibitory concentration table of isolates obtained from NTYE medium with 5% NaCl concentration for cadmium sulfate tolerance

Metal salt	Conc (mm)	Isolate								
		SpWI	SpWII	SpWIII	SpWIV	SpWV	SpWVI	SpWVII	SpWVIII	SpWX
$CdSO_4$	0.1	-	-	-	-	+	-	-	+	-
	0.15	-	-	-	-	-	-	-	+	
	0.2	-	-	-	-	-	-	-	-	-
	0.25	-	-	-	-	-	-	-	-	-
	0.3	-	-	-	-	-	-	-	-	-
	0.35	-	-	-	-	-	-	-	-	-
	0.4	-	-	-	-	-	-	-	-	-

Table 4.6.2.2. Minimum inhibitory concentration table of isolates obtained from NTYE medium with 5% NaCl concentration for mercuric chloride tolerance

Metal salt	Conc (mM)	Isolates		
		SpWV	SpWVII	SpWVIII
$HgCl_2$	0.1	+	+	+
	0.15	+	+	+
	0.2	+	+	+
	0.25	+	+	+
	0.3	+	+	+
	0.35	+	+	+
	0.4	+	+	+
	0.45	-	-	-
	0.5	-	-	-

KEY: (+) growth(-) no growth

The isolates which showed tolerance at 0.05mM of $HgCl_2$ and $CdSO_4$ were selected and checked for the maximum tolerance concentration (MTC) and minimum inhibitory concentration (MIC).

The MTC for $CdSO_4$ was found out to be 0.05mM for 9 isolates out of ten. Isolate SpWIX showed zero tolerance to $CdSO_4$. The minimum inhibitory concentration of $CdSO_4$ for isolates SpWI, SpWII, SpWIII, SpWIV SpWVI, SpWVII, and SpWX was determined to be 0.05mM. For isolate SpWV , the MIC was determined to be 0.15mM and for SpWVIII, MIC was determined to be 0.2mM.

Out of ten isolates, only three isolates- SpWV, SpWVII, and SpWIX showed tolerance towards $HgCl_2$. The minimum inihibitory concentration of $HgCl_2$ for these isolates was determined to be 0.45mM and the maximum tolerance concentration was determined to be 0.4mM. thus, these 3 isolates show a considerably high degree of resistance to $HgCl_2$.

4.7. Plasmid isolation

The plasmid isolation was carried out using Thermo Scientific GeneJET Plasmid Miniprep Kit #k0502**.** Three isolates showing good dye decolourisation activity as well as heavy metal tolerance were selected for plasmid detection. The isolates chosen were SpWI, SpWV, and SpWVIII.

The cells were pelleted out after optimal growth was achieved and were resuspended in solution, followed by subjecting the cells to alkaline lysis using SDS, which liberates the plasmid DNA from the cell. The lysate obtained was neutralized using the neutralisation solution provided in the kit, this was used to make sure that the conditions are appropriate for the plasmid to bind to the silica membrane in the spin column. Through centrifugation, cell debris and SDS is pelleted out and the supernatant which contains the plasmid DNA is loaded on the GeneJET Spin Column. The colum is washed to remove contaminants and the plasmid DNA was eluted out using elution buffer (10mM Tris-HCL). This extracted plasmid DNA was then stored at -20°C until further analysis.

The sample obtained after running plasmid isolation procedure was verified for presence or absence of plasmid using agarose gel electrophoresis followed by visualization through UV transillumination. After visualisation a two-band pattern was observed for isolate SpWV. Uncut plasmid DNA usually produces two bands on a gel, representing the open-circular, and supercoiled plasmid DNA also referred to as covalently closed circular DNA. This indicates that the isolate may conatin a plasmid and the plasmid could be the reason it shows $HgCl_2$ tolerance of upto 0.4mM or responsible for 40% methyl orange dye degradation.

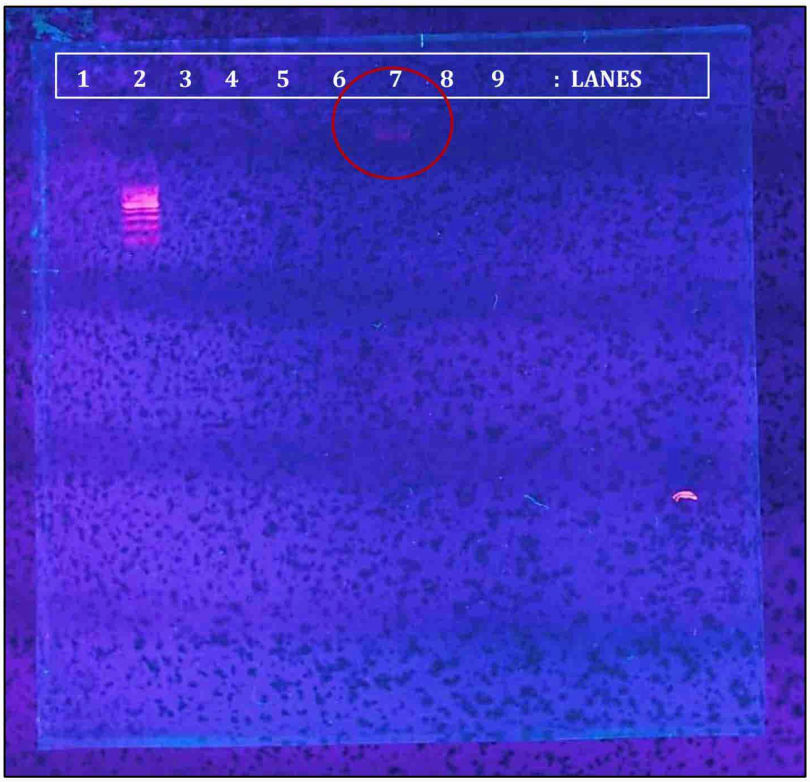

Figure 4.7.1. Agarose gel after UV transillumination, post electrophoresis. Lane 2 – DNA marker lane; lane 6- plasmid isolation sample from isolate SpWI; lane 7-- plasmid isolation sample from isolate SpWV; lane 8- - plasmid isolation sample from isolate SpWVIII; The red circle indicates the bands obtained after running the plasmid isolation sample from isolate SpWV

4.8. Screening for biodegradation of aromatic hydrocarbon- Sodium benzoate

4.8.1. Initial screening for medium optimization.

Ten isolates with 5% as the optimum NaCl concentration for growth were selected for screening for sodium benzoate tolerance and biodegradation. Two media: Minimal salt medium with 0.05mM sodium benzoate and diluted NTYE media with 0.05mM sodium benzoate were utilized. Both media plates were streaked with the isolates and incubated for the same time period. No growth was observed on MSM plates with 0.5mM sodium benzoate. Whereas growth was observed on diluted NTYE plates with 0.5mM sodium benzoate. Hence, NTYE media was selected as the default medium for the determination of minimum inhibitory concentration and maximum tolerance concentration of the isolates towards sodium benzoate.

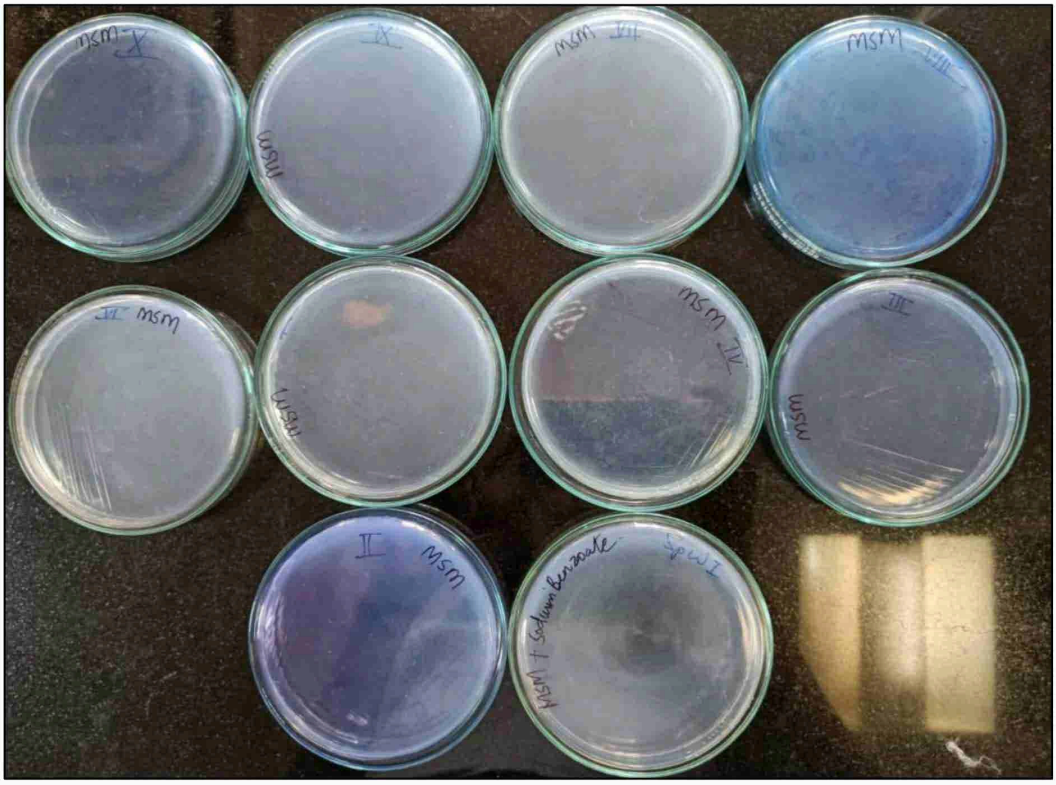

Figure 4.8.1.1. Minimal salt medium with 0.05mM sodium benzoate streaked with the test isolates(after 4 days of incubation)

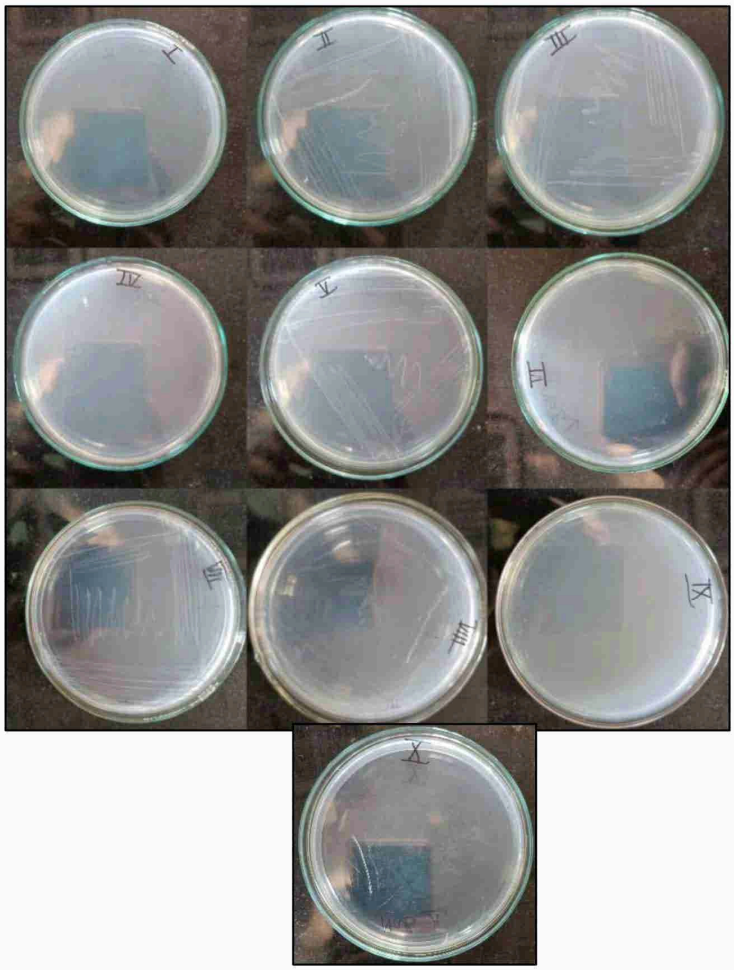

Figure 4.8.1.2. diluted NTYE medium with 0.05mM sodium benzoate streaked with the test isolates (after 4 days of incubation)

Table 4.8.1. summary of the growth observed on diluted NTYE and MSM medium with 0.05mM sodium benzoate in bot

Isolate	SpWI	SpWII	SpWIII	SpWIV	SpWV	SpWVI	SpWVII	SpWVIII	SpWIX	SpWX
Diluted NTYE medium with 0.05mM sodium benzoate	-	+	+	-	+	-	+	+	-	-
Minimal salt medium with 0.05mM sodium benzoate	-	-	-	-	-	-	-	-	-	-

4.8.2. Determination of minimum inhibitory concentration and maximum tolerance concentration of the isolates towards sodium benzoate.

NTYE medium was prepared with varying concentrations of Sodium Benzoate, the concentrations utilized was; 1 mM, 5 mM, 10 mM, 20 mM, and 40 mM of Sodium Benzoate. MSM medium was not utilized because no growth of the isolates was seen on MSM during the preliminary test. The plates were streaked with the isolates and incubated at 28°C±2 for 4 days. The growth pattern of the isolates is as tabulated in Table4.8.2.

Table 4.8.2. Minimum inhibitory concentration (MIC) of isolates with 5% NaCl as optimum salt concentration

	1mM	5mM	10mM	20mM	40mM
SpWI	-	-	-	-	-
SpWII	+	+	+	-	-
SpWIII	+	+	+	+	-
SpWIV	+	+	+	-	-
SpWV	+	+	+	+	-
SpWVI	+	+	+	-	-
SpWVII	+	+	+	+	-
SpWVIII	+	+	+	+	+
SpWIX	-	-	-	-	-
SpWX	+	-	-	-	-

c

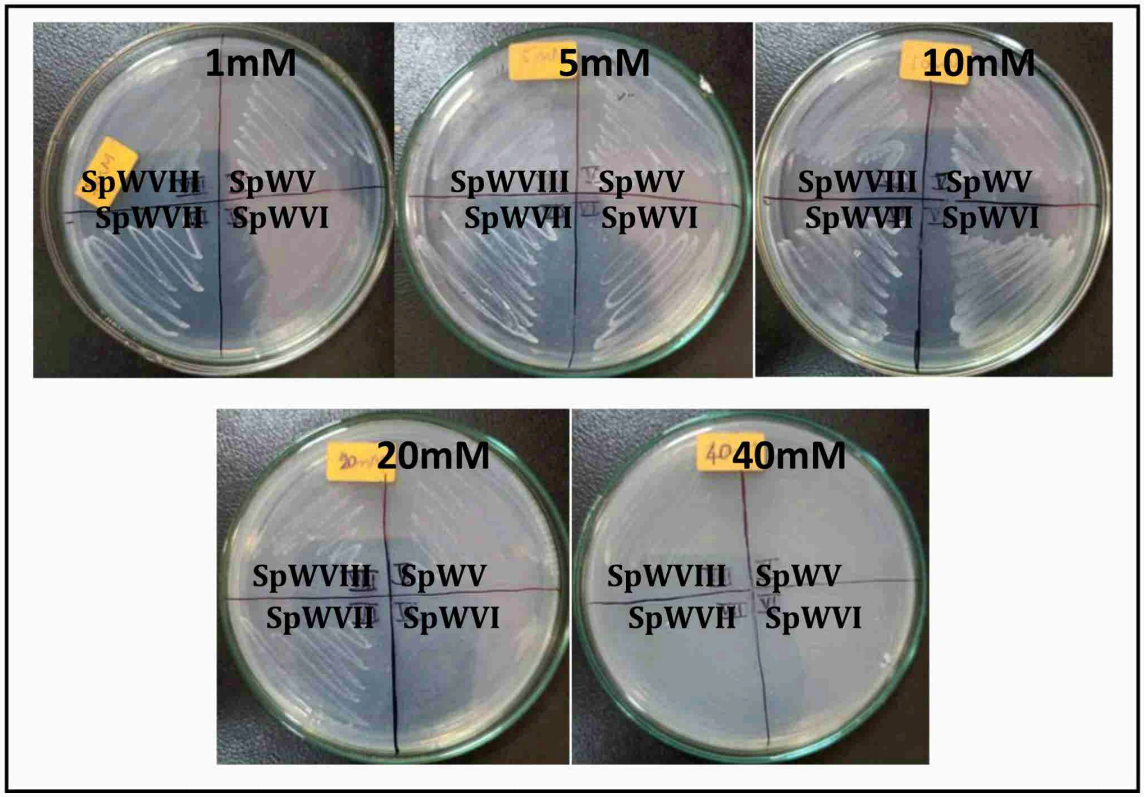

Figure 4.8.2. 5% NaCl containing NTYE medium plates prepared with varying concentrations of Sodium Benzoate- 1mM, 5mM, 10mM, 20mM,40mM., and streaked with isolates SpWV, SpWIV, SpWVII, and SpWVIII.

In the study, the bacteria isolated from the salt pan sediment sample growing optimally at 5% NaCl concentration were found to have a minimum inhibitory concentration of sodium benzoate to be 20mM. Out of the ten isolates screened for sodium benzoate tolerance and biodegradation, eight isolates were capable of growing onto the sodium benzoate containing NTYE medium. Isolate SpWVIII showed the highest tolerance at 40mM concentration. Isolates SpWIII, SpWV, and SpWVII, showed MTC at 20mM concentration. While isolates SpWII, SpWIV,SpWVI showed MTC at 10mM concentration of sodium benzoate. Isolate SpWX showed the least tolerance to sodium benzoate, and had an MTC of about 1mM. isolate SpWI and SpWIX didn't demonstrate any tolerance to sodium benzoate, hence no growth.

4.9. Partial identification of the isolates

The isolates having 5% NaCl concentration as the optimum salt requirement for growth were selected for morphological characterization.

4.9.1. Colony characteristics:

Table 4.9.1 a

NAME	SpWI	SpWII	SpWIII	SpWIV	SpWV
Media	NTYE	NTYE	NTYE	NTYE	NTYE
time	4 days	4 days	4 days	4 days	4 days
temperature	room temperature	room temperature	room temperature	room temperature	room temperature
size	1 mm	2mm	3mm	3mm	2mm
shape	circular	Circular	Circular	circular	Circular
color	dark brown	creamish	Off-white	palest brown	white
margin	entire	Entire	Entire	entire	Entire
elevation	convex	convex	raised	Flat	Flat
surface texture	smooth	matte	smooth	smooth	matte
opacity	opaque	opaque	opaque	opaque	opaque
consistency	butyrous	smooth	Butyrous	smooth	dry

Table 4.9.1 b

NAME	SpWVI	SpWVII	SpWVIII	SpWIX	SpWX
Media	NTYE	NTYE	NTYE	NTYE	NTYE
time	4 days	4 days	4 days	4 days	4 days
temperature	room temperature	room temperature	room temperature	room temperature	room temperature
size	2mm	2mm	3-4mm	1mm	2mm
shape	circular	circular	circular	circular	Circular
color	dark brown	yellow	Orange	pale beige	colourless
margin	entire	Entire	Entire	entire	entire
elevation	convex	Flat	Raised	raised	convex
surface texture	smooth	dry	smooth	smooth	smooth
opacity	opaque	opaque	opaque	opaque	translucent
consistency	smooth	dry	Mucoid	butyrous	smooth

Figure 4.9.2. (a) methyl red test (b) vogeus-proskauer test (c) urease test (d) catalase test (e) citrate test (f)citrate positive test tube (by isolate SpW5) against the control tube.

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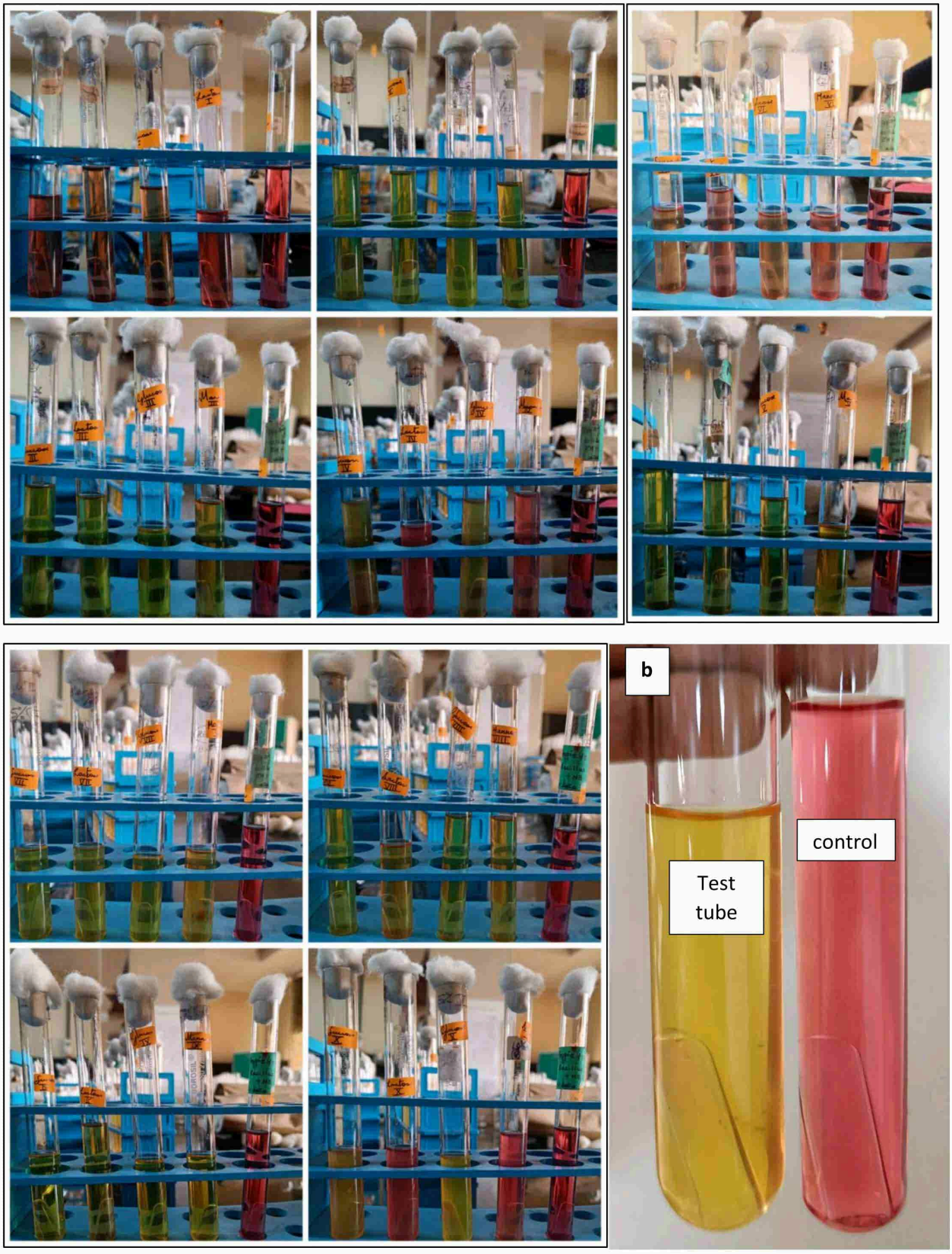

Figure 4.9.2. Sugar utilisation tests. (b) positive test for sugar utilization

4.9.2. Biochemical tests:

Biochemical tests were performed for the selected 10 isolates and the results were tabulated as shown in table 4.9.2a and 4.9.2b.

Table 4.9.2 a

Biochemical test	SpWI	SpWII	SpWIII	SpWIV	SpWV
Gram character	Gram positive cocci	Gram positive cocci	Gram positive cocci	Gram negative coccobacilli	Gram positive cocci
Methyl red	-	-	-	-	-
Voges-Proskauer	-	-	-	-	-
Indole	-	-	-	-	-
Catalase	-	+	+	+	+
citrate	-	-	-	+	-
Urease	-	-	-	-	+
Glucose	+	++	++	++	++
Sucrose	+	++	++	++	++
Lactose	-	++	++	-	++
Mannose	-	++	++	-	++
Maltose	-	++	++	++	++
Mannitol	-	++	++	+	++
Xylose	-	+	++	-	++
Galactose	-	+	++	+	-

Key: + weakly positive; ++ strongly positive; - negative test

Table 4.9.2 b

Biochemical test	SpWVI	SpWVII	SpWVIII	SpWIX	SpWX
Gram character	Gram negative coccobacilli	Gram positive cocci	Gram positive cocci	Gram positive rods	Gram positive rods
Methyl red	-	-	+	-	-
Voges-Proskauer	-	-	-	-	-
Indole	-	-	-	-	-
Catalase	-	+	+	+	-
citrate	-	-	-	+	-
Urease	-	+	+	-	+
Glucose	+	++	++	++	++
Sucrose	+	++	++	++	++
Lactose	-	++	++	++	-
Mannose	-	++	++	++	-
Maltose	-	++	++	+	++
Mannitol	+	++	++	+	+
Xylose	-	++	++	++	-
Galactose	-	-	+	+	++

Key: + weakly positive; ++ strongly positive; - negative test

Based on the results obtained after performing the biochemical tests, isolates SpWIX and SpWX were tentatively determined to belong to the genus *Bacillus.* Apart from them the results obtained for the other isolates, weren't conclusive enough to draw accurate results for tentative identification.

5. SUMMARY

In this study, the halophilic and halotolerant cultures obtained from Curca,Goa Velha saltpan were screened for its potential to be used as a tool for biotechnological purposes like bioremediation of saline and hypersaline wastewaters contaminated with azo dye, heavy metals, hydrocarbons as well as other organic pollutants. 30 isolates were obtained from the saltpan sample. The majority of these isolates showed optimum growth at 5% NaCl concentration but could survive up to 15% of NaCl concentration. Amylase, protease, agarase, and lipase activities were also detected among the purified isolates. Isolate SpWIX showed optimum amylase activity at 5% NaCl concentration. Lipase activity was observed at 10% NaCl concentration by isolates SppW7, SppW9, and SppW10. Further, the isolates were screened for azo dye decolorization capability. The azo dyes used in the study were Congo red and Methyl orange. The isolates on average showed a better decolorization potential towards methyl orange than Congo red, with the highest % decolorisation of methyl orange observed at around ~49% at 5% NaCl concentration by isolate SpWX. Moreover, isolates SpWV, SpWIV, and SpWVII were formulated into a consortium that showed ~50% decolorization of both Methyl orange and Congo red. Heavy metal tolerance and aromatic hydrocarbon tolerance and biodegradation activity were also checked for 10 isolates having 5% NaCl as optimum NaCl concentration for growth. Isolates SpWV, SpWVII, and SpWIX demonstrated tolerance to a concentration of 0.4 mM of $HgCl_2$. Isolate SpWVIII showed highest tolerance among the nine other isolates for $CdSO_4$, with an MTC of 0.2mM. tolerance towards sodium benzoate was also determined in the study. Nine out of ten isolates showed tolerance to a minimum of 0.05mM sodium benzoate. Out of which isolates SpWII, SpWIV, and SpWVI showed maximum tolerance concentration of sodium benzoate to be 20mM.

6. CONCLUSION

Halophilic and halotolerant microorganisms, capable of surviving and thriving in saline environments, extend abundant potential applications in biotechnological advancement (Margesin & Schinner.2001). From the production of bioactive molecules like hydrolytic enzymes which are active at high salt concentrations and other physically harsh conditions, to the bioremediation of saline wastewaters, containing a wide range of organic pollutants, heavy metals, dyes, and others. Halophilic microoragnisms are the best candidates to be used as solutions towards bioremediation of such contaminated sites due to their flexibility for survival at varied extreme conditions, and is not restricted only to high salt tolerance. The isolates obtained from saltplans in this study showed hydrolytic enzyme production. These enzymes are most sought after in industries due to their high-end stability and activity at extreme pH, elevated temperatures, etc. Furthermore the isolates also showed azo dye decolourisation of approximately ~50% as well as tolerance to heavy metals, $HgCl_2$ and $CdSO_4$ which could further aid in azo dye bioremediation in waters containing heavy metal contaminants. Tolerance to these indicates that dye decolourisation wouldn't be hindered by the presence of heavy metals in the effluent. The isolates obtained also showed considerable tolerance to sodium benzoate, which is a common component of most wastewaters.

7. FUTURE PROSPECTUS

- Standardisation, and optimisation of conditions for optimum enzyme activity of the isolates and extraction of the enzyme for potential commercial applications.
- Azo dye biodegradation screening.
- Textile effluent treatment design with intricately curated consortia of the isolates.

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9. APPENDIX

MEDIA COMPOSITION

1) Crude salt stock solution (25%)

Ingredients	**g/litre**
Crude salt	250
Distilled water	1L

Weigh 250g of crude salt and add to 1000ml of distil water. Filter the solution using a filter paper and funnel, after dissolving the crude salt completely. Use this solution as NaCl solution for preparation of NTYE medium.

2) Nutrient tryptone yeast extract medium (NTYE)

Ingredients	**g/litre**
NaCl	250.0
$MgSO_4.7H_2O$	20.0
KCl	5.0
$CaCl_2.2\ H_2O$	0.2
Yeast extract	3.0
Tryptone	5.0
Agar	20.0
pH	**6.8 ± 0.2**

Weigh all the components, and the required volume of NaCl as stock solution based on the concentration of NTYE medium required (volume determined using $C_1V_1=C_2V_2$). Adjust the pH of the medium to 6.8± 0.2 using 5M NaOH. Autoclave the medium at 121°C, 15lbs pressure for 20 minutes.

3) Mineral salt medium

Ingredients	g/litre
K_2HPO_4	1.5
KH_2PO_4	0.5
$MgSO_4.7H_20$	0.3
NaCl	100
NH_4Cl	3.0
$FeCl_3$	0.01
$CaCl_2.2H_2O$	0.01
Agar	20
D/W	1

Stock solutions of individual components were made and autoclaved separately (121°C for 15 mins). The appropriate volumes of stock solutions were combined to make the final medium composition.

BIOCHEMICAL MEDIUM COMPOSITION

1) Norberg Hofsten (NH) medium broth (for sugar fermentation test)

Ingredients	**g/litre**
NaCl	200.0
$MgSO_4.7H_2O$	10.0
KCl	5.0
Yeast extract	1.0
Phenol red	0.1

9ml of NH broth was added to a test tube with an inverted Durham's tube , plugged and autoclaved for 20 minutes at at 121°C. after autoclaving. 1ml of sugar stock solution was added to the broth.

2) Sugar stock solution (10%)

Ingredients	**g/l**
Sugar (glucose/galactose/xylose/mannose/maltose/lactose/sucrose)	10
Distilled water	1 L

The sugars to be used for the sugar fermentation test were prepared as 10% stocks and a\sterilized by autoclaving at 121°C for 10 mins.

3) Glucose phosphate broth (MR-VP medium)

Ingredients	**g/litre**
Peptone	5.0
Dipostassium phosphate	1.0
Dextrose	0.5
pH	**7.4 ±0.2**

4) Tryptone broth

Ingredients	g/litre
Tryptone	10
NaCl	5.0
Distilled water	1litre

5) Christensen's Urea medium

Ingredients	g/litre
peptone	1.0
KH_2PO_4	2.0
NaCl	5.0
Urea	20.0
Phenol red	0.1
Distilled water	1litre
pH	**6.8**

Urea was added separately to the medium, as it is prone to degradation during autoclaving. 20% urea stock solution was prepared and sterilized by filter sterilization method . The appropriate volume of urea stock was added to the above medium broth, after it has been sterilized by autoclaving at 121°C for 20 minutes.

1) Simmons citrate agar

Ingredients	g/100ml
NaCl	0.5
$MgS_4..7H_2O$	0.02
NaH_2PO_4	0.1
KH_2PO_4	0.1
Sodium citrate	0.5
Bromothymol blue (0.2%)	4.5
Agar	4
Distilled water	100
pH	**6.8**

REAGENTS AND STAINS

1) 1% Lugol's iodine solution

Ingredients	**g/litre**
Lugol's iodine	1ml
Distilled water	100ml

2) 0.1% congo red solution

Ingredients	**g/litre**
Congo red	0.1
Distilled water	100ml

3) 1M NaCl solution (for destaining)

Ingredients	**g/litre**
NaCl	24.72
Distilled water	100ml

4) 2% saline

Ingredients	**g/litre**
NaCl	2
Distilled water	100ml

5) Methyl red reagent (for MR test)

Ingredients	**g/litre**
Methyl red	6.2
Ethyl alcolol	600ml
Distilled water	400ml

6) Kovac's reagent (indole test)

Ingredients	g/litre
Isoamyl alcohol	150ml
p-dimethyl amino benzaldehyde	10.0
Concentrated HCl	50ml

7) Omeara's reagent (Vogues-Proskauer test)

Ingredients	g/litre
Creatine	0.15
KOH	20.0
Distilled water	40ml

8) Gram's Crystal violet

Ingredients	g/litre
Crystal violet	2
Ammonium oxalate	0.8
Ethyl alcohol	20ml
Distilled water	80ml

9) Safranine

Ingredients	g/litre
Safranine-O	0.5
Ethyl alcohol	50ml

10) Gram's iodine

Ingredients	g/litre
Iodine	1.0
Potassium iodide	2.0
Distilled water	300ml

Store in amber coloured bottle.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1) 5X TBE buffer (to prepare 500ml)

Ingredients	g/litre
Tris base	27g
Boric acid	13.75g
0.5M EDTA	10ml

Add 400 ml of distilled water to the flask and to it add tris base and EDTA. Keep on the magnetic stirrer to dissolve the powder completely. Adjust the pH to 8 using NaOH, followed by making up the volume to 500ml.

2) Agarose gel

Ingredients	g/litre
Agarose	0.8g
1X TBE buffer	100ml
Ethidium bromide	0.5 µl

Microwave the suspension until the agarose dissolves completely. On cooling a bit add ethidium bromide (0.5µl) from 10 µ/ml stock to the agarose suspension.

3) Running buffer (1X)

5X TBE buffer diluted to 1X using distilled water.

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