Biological Treatment of Saline Wastewater using Salt Tolerant Microorganisms

A thesis submitted for the partial fulfilment of
BACHELOR OF SCIENCE (BIOTECHNOLOGY)

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APRIL 2020

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CERTIFICATE

This is to certify that the thesis entitled **"Biological Treatment of Saline wastewater using salt tolerant microorganisms"** submitted by **Dhriti Seth (17-BT-024), Mishika Shah (17-BT-026), Drashti Mehta (17-BT-055)** to St. Xavier's College Ahmedabad (Autonomous) for partial fulfillment of the Degree of **Bachelor of Science (Biotechnology)** is a bonafide record of work carried out by the student under my supervision. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for award of any degree, diploma or titles.

Signature of the mentor

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DECLARATION

We hereby declare that the thesis **"Biological Treatment of Saline Wastewater"** submitted by **Dhriti Seth (17-BT-024), Mishika Shah (17-BT-026)** and **Drashti Mehta (17-BT-055)** to St. Xavier's College Ahmedabad (Autonomous) in partial fulfillment for the Degree of **Bachelor of Science in Biotechnology** is the record work carried out by us during the period from November 2019 to March 2020 under the guidance of **Ms Srishti Chabaria** . We also declare that this work has not formed the basis for the award of any degree, diploma or titles by any other Institute or University and all support received from various sources have been duly acknowledged. **All the pictures displayed are self-made.**

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ACKNOWLEDGEMENTS

As said, every achiever is inspired by a great mentor. With that, first and foremost we would like to express our sincere gratitude towards **Mrs. Shrishti Chabaria** and **Mr Dhavalkumar Patel** for their endless support and constant motivation throughout the project.

We are grateful to **Dr Avni Divatia** and **Dr Kinjal Bhatt** for guiding us during our ups and downs throughout our dissertation work.

We are glad to convey cordial thanks **to Dr Dweipayan Goswami, assistant professor Gujarat University** for guiding us throughout our project and showing us the right path.

We additionally appreciate **Mr Akshat Shah, head engineer of Shubham INC**. for helping us design our reactor.

Our heart-warming thanks to **Ms Nilam Vaghasiya** and **Mrs Naznin** for their help and support during experiments whenever needed.

We are grateful to **Dr. Sudeshna Menon**, Head of the department for providing us the facilities to carry-out this work. And most importantly we are thankful to God Almighty for bringing us to the platform where we are today and our family members for their patience, love and care without which we could not have completed our thesis work.

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ABBREVIATIONS

BOD: - Biological oxygen demand

COD: - Chemical oxygen demand

DO: - dissolved oxygen

d/w: - Distilled water

H_2SO_4: - Sulfuric acid

$K_2Cr_2O_7$: - Potassium dichromate

mg/L: - milligram per Litre

mL: - millilitre

mS/cm: - milli siemens per centimetre

NA: - Nutrient Agar

$Na_2S_2O_3$: - Sodium thiosulphate

NaCl:- Sodium chloride

ppm: - parts per million

ppt: - parts per thousand

Spp: - Species

TDS: - total dissolved solid

RBA: - Rose Bengal Agar

w/w: -Weight by weight

ABSTRACT

Biological aerobic treatment of saline wastewater provides the material of this study. Thirty-eight salt-tolerant microorganisms were isolated on various salt concentrations from sea water of Bedi port, Jamnagar. Selection, identification and characterization of the microorganisms were carried out. The isolate obtained on 20% NaCl was used as inoculum for biodegradation of wastewater. An activated sludge reactor operated in a batch reactor was used for the treatment of effluent from the textile industry using various concentrations of the isolated microbe. The results obtained showed that the Chemical Oxygen Demand of the black effluent was reduced by 98%. Also, the Biological Oxygen Demand of the pink waster was reduced by 33.33% and of black water was reduced by 50%. Since the impurities (organic matter) present in the effluent are consumed by the halophile hence the TDS is also reduced remarkably, by 21.05% and 40.91% for pink and black effluent respectively

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1. INTRODUCTION

1.1. OVERVIEW

Halophiles, salt-loving organisms that flourish in saline environments, are classified as slight, moderate or extreme, depending on their requirement for sodium chloride (Kanekar et al. 2012). While most marine organisms are slight halophiles, moderate and extreme halophiles are generally more specialised microbes inhabiting hypersaline environments found all over the world in arid, coastal and deep-sea locations, underground salt mines and artificial salterns. Halophilic microorganisms include heterotrophic, phototrophic and methanogenic archaea, photosynthetic, lithotrophic and heterotrophic bacteria and photosynthetic and heterotrophic eukaryotes (DasSharma et al. 2001). These microbes can be used in the treatment of wastewater coming from the textile industry which is mainly saline. Because of its remarkable ability to survive in saline water it is a better alternative than any other microorganism for the biological treatment of saline wastewater.

1.2. SOURCES OF SALINE WATER

Saline impaired water originates from many sources such as sea water, ground water, concentrate from decantation plants, effluent from sewerage treatment plants, brine from natural Salt Lake or saline effluent storage basins, brine from salt harvesting activities, effluent from farming and irrigation schemes, effluent from pulp and paper and food industry. (Butler et al. 2016). It also originates from fertilizers, chemicals, paint, ink, pharmaceuticals, textile industries which produces water from oil and gas production, effluent from mining and mineral processing industries, pickling processes, meat packing and dyestuff, pesticides, herbicides, polyhydric compounds, organic peroxides, and pharmaceuticals.

1.3. PROPERTIES OF SALINE WATER

Saline water (more commonly known as salt water) is water that contains a high concentration of dissolved salts (mainly sodium chloride). The salt concentration is usually expressed in parts per thousand (per milli or parts per million ,ppm) (Chen et al 2017). The United States Geological Survey classifies saline water in three salinity categories. Salt concentration in slightly saline water is around 1,000 to 3,000 ppm (0.1–0.3%), in moderately saline water 3,000 to 10,000 ppm (0.3–1%) and in highly saline water 10,000 to 35,000 ppm (1–3.5%). Seawater has a salinity of roughly 35,000 ppm, equivalent to 35 grams of salt per one litre (or kilogram) of water. The saturation level is dependent on the temperature of the water. At 20 °C one litre of water can dissolve about 357 grams of salt, a concentration of 26.3% w/w. At boiling (100 °C) the amount that can be dissolved in one litre of water increases to about 391 grams, a concentration of 28.1% w/w.

1.4. WHY IS TREATMENT OF SALINE WATER NECESSARY?

Proper disposal of industrial wastewater is crucial for the protection of the natural habitats of fish, bugs, bird, and plant communities. Chloride is categorized as a pollutant for many reasons. Chloride is necessary for water habitats to thrive, yet high levels of chloride can have negative effects on an ecosystem. Chloride may impact freshwater organisms and plants by altering

reproduction rates, increasing species mortality, and changing the characteristics of the entire local ecosystem (Protopopova et al. 2020). In addition, as chloride filters down to the water table, it can stress plant respiration and change the quality of our drinking water.

1.5. TREATMENT OF SALINE WASTEWATER

1.5.1. PHYSICAL TREATMENT

- **Sedimentation**

Sedimentation, which is a process of suspending the insoluble/heavy particles from the wastewater. Once the insoluble material settles down at the bottom, pure water can be separated. (Schleiss et al. 2016)

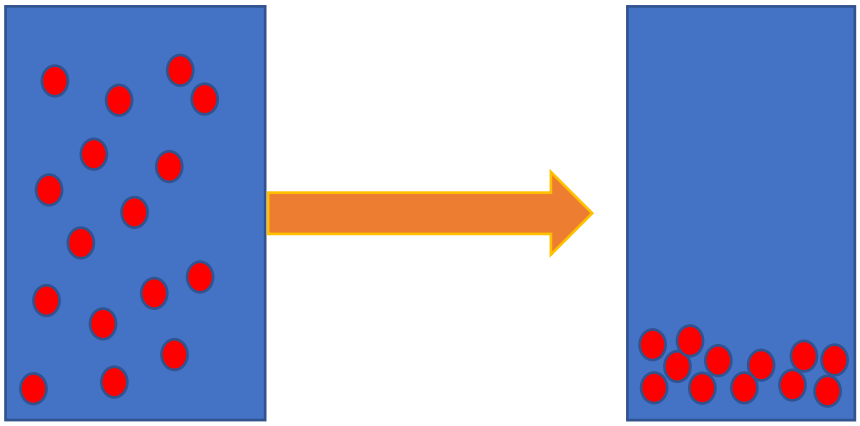

Figure 1 represents Sedimentation of the system

- **Grit chamber**

Grit chamber is a long narrow or circular tank in the primary sewage treatment plant that is designed to reduce the velocity of the flow of sewage to eliminate the girt materials such as sand, ash and clinkers, eggshells, bone chips and many inert materials inorganic in nature (Plana et al. 2020).

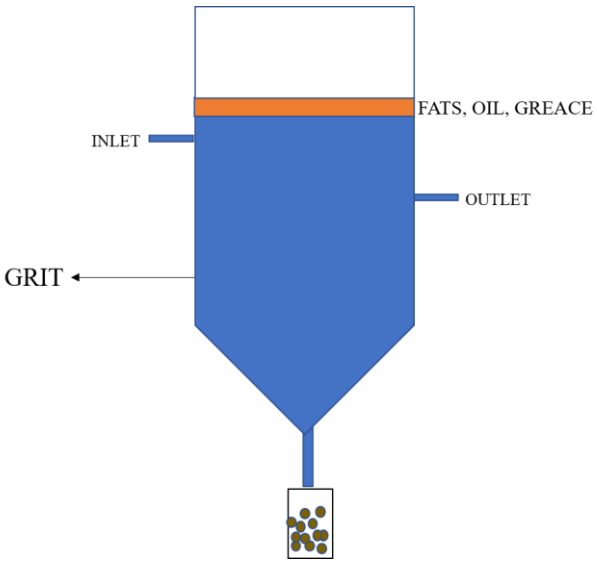

Figure 2 represents Grit chamber

- **Dissolved air flotation**

It clarifies wastewater by the removal of suspended matter such as oil or solids. The removal is achieved by dissolving air in the water or wastewater under pressure and then releasing the air at atmospheric pressure in a flotation tank basin. The released air forms tiny bubbles which adhere to the suspended matter causing the suspended matter to float to the surface of the water where it may then be removed by a skimming device (Zhang et al. 2016).

- **Reverse Osmosis**

Reverse osmosis is one of the most common physical water treatment methods employed in industrial water treatment. Reverse osmosis, also known as RO, filters contaminants out of water using applied pressure to force water through a semipermeable membrane. RO can remove impurities such as dissolved ions (e.g., sodium), bacteria, viruses, and other contaminants ranging from 0.005 to 0.0001 micron in size (Jiang et al 2017).

- **Multimedia Filtration (MMF)**

Multimedia filtration is a modern physical water treatment technique that uses at least three different layers of filtration media, typically anthracite, sand and garnet, to filter water. This filter arrangement allows for larger particulates to be trapped at the top of the filter while smaller particles are trapped deeper in the media (Maifadi et al. 2020). Suspended solids, including clay, algae, silt, rust, and other organic matter are removed as the water passes through each layer of media. This filtration method can remove particles from 10 to 25 microns in size. Multimedia filtration does not remove viruses, bacteria or smaller protozoans.

- **Microfiltration**

Microfiltration uses a barrier membrane to filter very small suspended solids from water. Microfiltration membranes are typically capable of removing contaminants ranging from 0.1 to 10 microns in size (Zeman 2017). This form of physical water treatment is ideal for

removing suspended solids, algae and protozoans from water but does not generally remove bacteria and viruses. Microfiltration does not remove dissolved contaminants from water

1.5.2. CHEMICAL TREATMENT

- **Chemical Precipitation**

Chemical precipitation is the most common method for removing dissolved metals from wastewater solutions containing toxic metals. To convert the dissolved metals into solid particle form, a precipitation reagent is added to the mixture. A chemical reaction, triggered by the reagent, causes the dissolved metals to form solid particles (Huang et al 2017). Filtration can then be used to remove the particles from the mixture. How well the process works is dependent upon the kind of metal present, the concentration of the metal, and the kind of reagent used. In hydroxide precipitation, a commonly used chemical precipitation process, calcium or sodium hydroxide is used as the reagent to create solid metal hydroxides. However, it can be difficult to create hydroxides from dissolved metal particles in wastewater because many wastewater solutions contain mixed metals (Lassoued et al. 2017).

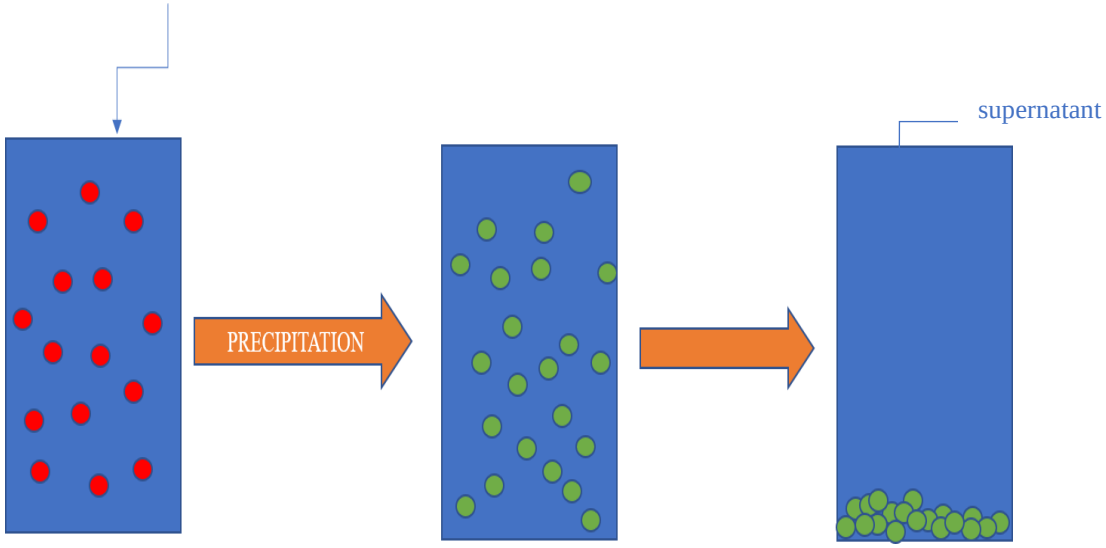

Figure 3 Process of chemical precipitation

- **Chemical Coagulation**

This chemical process involves destabilizing wastewater particles so that they aggregate during chemical flocculation. Fine solid particles dispersed in wastewater carry negative electric surface charges (in their normal stable state), which prevent them from forming larger groups and settling (Tang et al. 2016). Chemical coagulation destabilizes these particles by introducing positively charged coagulants that then reduce the negative particles' charge. Once the charge is reduced, the particles freely form larger groups. Next, an anionic flocculant is introduced to the mixture. Because the flocculant reacts against the positively charged mixture, it either neutralizes the particle groups or creates bridges between them to bind the particles into larger groups. After larger particle groups are formed, sedimentation can be used to remove the particles from the mixture (Shamaei et al. 2018).

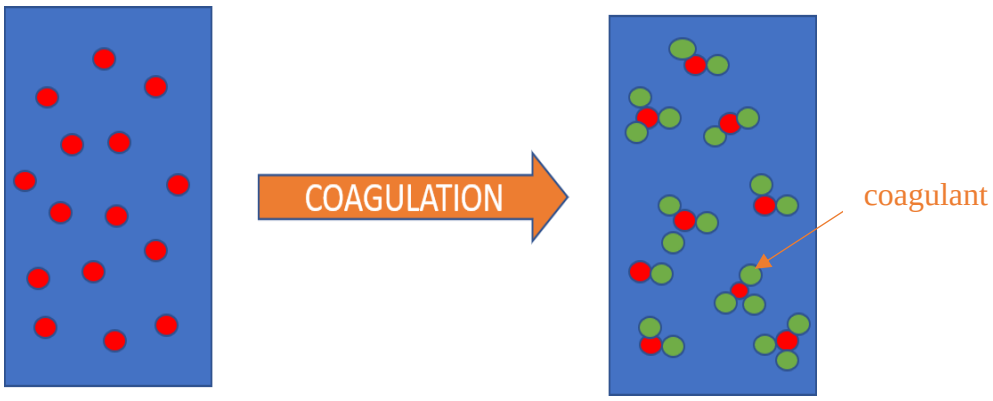

Figure 4 represents Chemical coagulation

- **Chemical Oxidation and Advanced Oxidation**

With the introduction of an oxidizing agent during chemical oxidation, electrons move from the oxidant to the pollutants in wastewater. The pollutants then undergo structural modification, becoming less destructive compounds. Alkaline chlorination uses chlorine as an oxidant against cyanide (Achour et al. 2017). However, alkaline chlorination as a chemical oxidation process can lead to the creation of toxic chlorinated compounds, and additional steps may be required. Advanced oxidation can help remove any organic compounds that are produced as a by-product of chemical oxidation, through processes such as steam stripping, air stripping, or activated carbon adsorption (Hamlet et al. 2017).

- **Ion Exchange**

When water is too hard, it is difficult to use to clean and often leaves a grey residue. (This is why clothing washed in hard water often retains a dingy tint.) An ion exchange process, like the reverse osmosis process, can be used to soften the water. Calcium and magnesium are common ions that lead to water hardness (Ran et al. 2017). To soften the water, positively charged sodium ions are introduced in the form of dissolved sodium chloride salt or brine. Hard calcium and magnesium ions exchange places with sodium ions, and free sodium ions are simply released in the water. However, after softening a large amount of water, the softening solution may fill with excess calcium and magnesium ions, requiring the solution to be recharged with sodium ions (Luo et al. 2018).

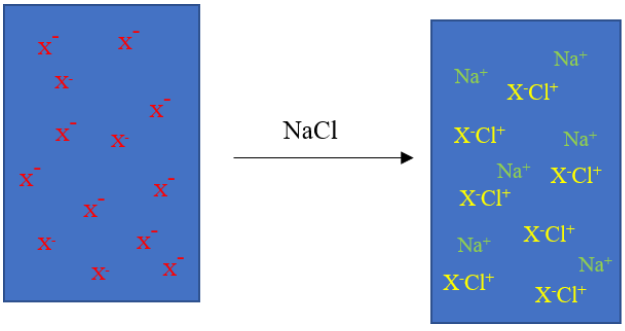

Figure 5 represents Ion exchange

- **Chemical Stabilization**

  This chemical wastewater treatment process works in a similar fashion as chemical oxidation. Sludge is treated with a large amount of a given oxidant, such as chlorine (Conner et al. 2017). The introduction of the oxidant slows down the rate of biological growth within the sludge and helps deodorize the mixture. The water is then removed from the sludge. Hydrogen peroxide can also be used as an oxidant and may be a more cost-effective choice.

1.5.3. BIOLOGICAL TREATMENT

Biological treatments rely on bacteria, nematodes, or other small organisms to break down organic wastes using normal cellular processes. Wastewater typically contains a buffet of organic matter, such as garbage, wastes, and partially digested foods. It also may contain pathogenic organisms, heavy metals, and toxins (Paz et al. 2017). Biological treatment usually is divided into aerobic and anaerobic processes. "Aerobic" refers to a process in which oxygen is present, while "anaerobic" describes a biological process in which oxygen is absent. Scientists have been able to control and refine both aerobic and anaerobic biological processes to achieve the optimal removal of organic substances from wastewater (Haydar et al. 2016).

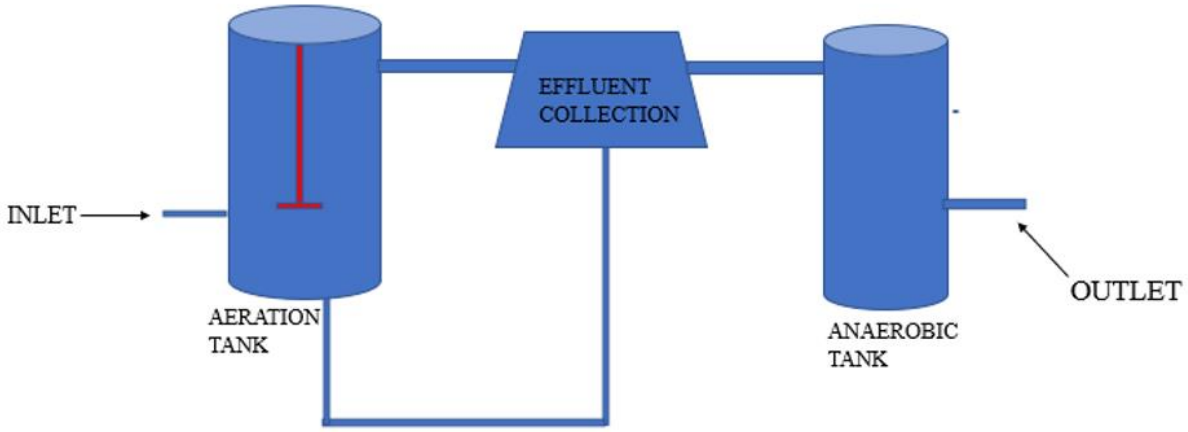

Figure 6 represents Biological oxygen demand of wastewater

1.6. PROBLEMS FACED

Biological treatment of saline wastewater has not been easy and by far the most popular treatment method. Salt removal operations by physicochemical processes such as reverse osmosis, ion exchange or electro dialysis before biological treatment are rather expensive. The performance of the biological treatment process for saline wastewater usually has low chemical oxygen demand removal due to adverse effects of salt on microbial flora. High salt concentrations (> 1%) cause disintegration of cells because of the loss of cellular water (plasmolysis) or recession of the cytoplasm which is induced by an osmotic difference across the cell wall and cause of outward flow of intracellular water resulting in the loss of microbial activity and cell dehydration. As a result, low removal performance of chemical and biological oxygen demands and increases of the effluent suspended solids especially at high salt concentrations (> 2%) occur.

1.7. MICROBIAL TREATMENT OF SALINE WASTEWATER

The use of a salt-tolerant organism in biological treatment units seems to be a more reasonable approach for saline wastewater treatment. Salt-tolerant halophilic organisms may be used singly or in activated sludge culture for effective biological treatment of the saline wastewater (Gulshan et al. 2019). Biological treatment of hyper-saline wastewater by pure halophilic bacteria has been studied in biofilms and in sequencing batch reactors. Inclusion of halophilic bacteria in an activated sludge culture was shown to improve COD removal efficiency at high salt content in a rotating biological contactor. High salt contents also adversely affect nitrification and denitrification of saline wastewater (Wang et al. 2017).

- **Reactors**

Wastewater treatment reaction vessel is operated with repeated sequences of aeration and non-aeration, using a single vessel or multiple vessels alternately, activated sludge which is acclimated for BOD reduction, nitrification or biological denitrification and phosphorus removal is absorptivity reacted with influent wastewater, and the combined flow is passed into subsequent absorptive reactor volumes, reducing the BOD such that the effluent BOD is less than twenty percent that of the influent.

- **Sequencing batch reactor**

Sequencing batch reactors (SBR) or sequential batch reactors are industrial processing tanks for the treatment of wastewater. SBR reactors treat waste water such as sewage or output from anaerobic digesters or mechanical biological treatment facilities in batches. Oxygen is bubbled through the waste water to reduce biochemical oxygen demand (BOD) and chemical oxygen demand (COD) to make it suitable for discharge into sewers or for use on land. While there are several configurations of SBRs the basic process is similar. The installation consists of at least two identically equipped tanks with a common inlet, which can be switched between them. The tanks have a “flow through” system, with raw wastewater (influent) coming in at one end and treated water (effluent) flowing out the other. While one tank is in settle/decant mode the other is aerating and filling. At the inlet is a section of the tank known as the bio-selector. This consists of a series of walls or baffles which direct the flow either from side to side of the tank or under and over consecutive baffles. This helps to mix the incoming Influent and the returned activated sludge, beginning the biological digestion process before the liquor enters the main part of the tank (U. satyanarayana 2008).

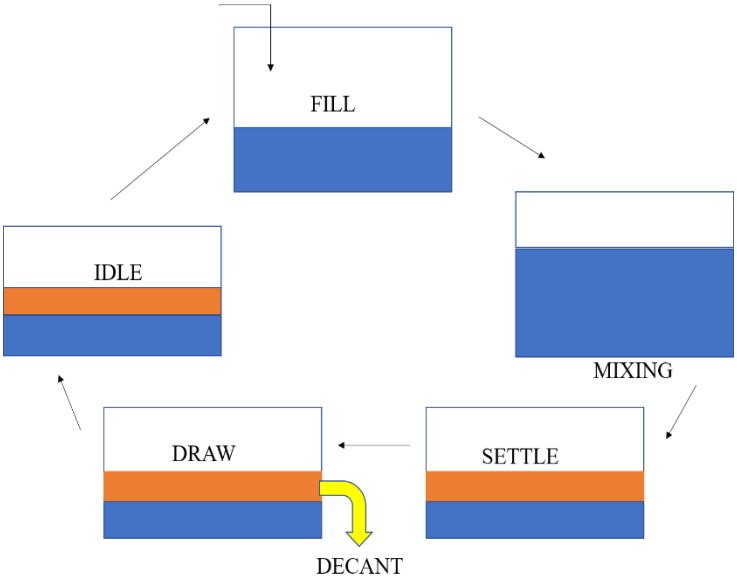

Figure 7 represents Sequential Batch Reactor

- **Rotating batch bioreactor**

Rotating biological contactor works on the principle of aerobic attached growth system operated on the moving media. It is composed of closely spaced and lightweight circular discs. They are made up of inert materials such as polystyrene or polyvinyl chloride or polyethylene. These discs are mounted on a horizontal shaft in a tank through which waste water flows. The shaft is rotated slowly (less than 10 revolutions per minute) by a low speed motor. The disc of the shaft, referred to as biodiscs, are partially (40%-60%) submerged in sewage. As the biodiscs are rotated, the biomass attached to them is alternately submerged in sewage. This enables the discs to pick up a thin layer of wastewater, and then to oxidize the absorbed substrates. The unoxidized substrates fall back (U. satyanarayana 2008).

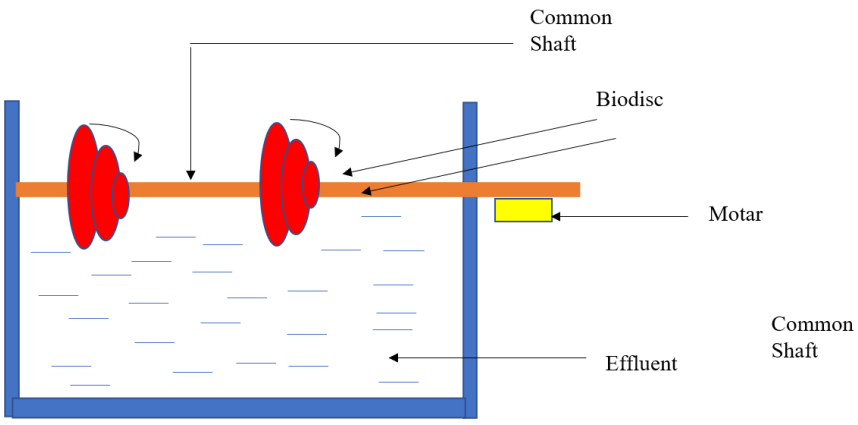

Figure 8 represents Rotating batch reactor

- **Fed batch bioreactor**

  Fed-batch culture is, in the broadest sense, defined as an operational technique in biotechnological processes where one or more nutrients (substrates) are fed (supplied) to the bioreactor during cultivation and in which the product(s) remain in the bioreactor until the end of the run (Patel et al. 2016)

2. LITERATURE REVIEW

2.1. ISOLATION OF HALOPHILIC MICROBES

Rodriguez et al. (1979) first isolated extreme halophilic bacteria from the ocean of Spain at the department of microbiology, University of Granada, Spain. All the isolates were found to be gram-negative *Cocci* and one isolate was compared to *Halococcus* sp.

Amoozegar et al. (2008) carried out isolation and initial characterization of the tellurite reducing moderately halophilic bacterium, *Salinicoccus* sp. strain QW6. Among the 49 strains of moderately halophilic bacteria isolated from the salty environments of Iran, a Gram-positive *Coccus* designated as strain *QW6* showed high capacity in the removal of toxic oxyanions of tellurium in a wide range of culture medium factors including pH (5.5–10.5), temperature (25–45 °C), various salts including NaCl, KCl, and Na2SO4 (0.5–4 M), seleno oxyanions (2–10 mM), and at different concentrations of potassium tellurite (0.5–1mM) under aerobic condition.

Fawzy et al. (2008) isolated salt-tolerant microorganism (*Staphylococcus xylosus*) from a vegetable pickled plant containing about 7.2% salt. They used these strains in activated sludge reactors and treated synthetic saline water using the isolated bacterial culture.

Hollis et al. (2010) isolated halophilic vibrio species from the blood cultures. They found 38 cultures of a halophilic bacterium which mainly belonged to *V. parahaemolyticus* and *V. alginolyticus.*

Delgado-García et al. (2010) isolated halophiles from the alkaline-sodic soils or saline soils of Cuatro Cienegas, Sayula and San Marcos lakes. They identified 23 cultivable halophilic bacteria, being *Halobacillus* sp., *Marinococcus* sp., and *Alkali Bacillus* sp. as the predominant genus.

Ghasemi et al. (2011) isolated and characterized some moderately halophilic bacteria with lipase activity. The bacterium *Bacillus vallismortis* BCCS 007 with 3.41 ± 0.14 U/mL lipase activity was selected as the highest lipase producing isolate. This is the first report of isolation and molecular identification of lipase producing bacteria from the Maharla lake.

Hamid et al. (2013) isolation and identification of moderately halophilic bacteria producing hydrolytic enzymes from the largest hypersaline playa in Iran. The moderately halophilic strains belonged to the genera *Halobacillus, Thalassobacillus, Bacillus, Salinicoccus, Idiomarina, Salicola*, and *Halomonas*.

Azhar et al. (2014) at Department of Microbiology, Baba Farid Institute of Technology, Suddhowala, Dehradun, India isolated halophiles and classified them into the Archaea Domain and they also isolated certain bacterial halophiles and some eukaryotes, such as the alga *Dunaliella Salina.*

Satbhail et al. (2015) isolated halophilic archaea and bacteria from the Dead lake. Majority of the genera found was *Haloferax, Haloarcula, Halobaculum, Halorubrum, Halomonas, Chromohalobacter* and *Salibacillus* at Department of Microbiology, Modern College of Arts, Science and Commerce, Ganeshkhind, Pune, India.

Javad et al. (2017) isolated and identified Halophilic and Halotolerant Bacteria from Badab-e Surt Travertine Spring, Kiasar, Iran. The isolates they found mainly belonged to *Roseovarius, Labrenzia, Erythrobacter, Erythromicrobium, Massilia, Marinobacter, Halomonas, Shewanella, Pseudomonas, Flavobacterium, Bacillus, Brevibacterium, Staphylococcus, Microbacterium, Kocuria,* and *Streptomyces* genera.

Delgado-García et al. (2018) studied isolation of halophilic bacteria associated with saline and alkaline-sodic soils by culture dependent approach of Cuatro Cienegas, Sayula and San Marcos lakes. *Alkalibacillus* sp. was predominant in Sayula and San Marcos lakes and was related to the high Na^+ content; while *Bacillus* sp. and *Halobacillus* sp. were predominant in Cuatro Cienegas, their occurrence was related to a high content of Ca^{2+}, Mg^{2+}, and SO_4^{2-}

2.2. GROWTH AND CHARACTERISATION OF HALOPHILIC MICROBES

Muńoz et al. (2001) studied growth of moderately halophilic bacteria isolated from sea water using phenol as sole carbon source. The isolate (Gram-negative motile rods) was identified as *Deleya venusta*. It grew well in the presence of up to 1600 mg/L of phenol and 8% NaCl under aerobic conditions.

Ventosa et al. (2003) studied diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. These strains were identified as members of the genera: *Salinivibrio (55 strains), Halomonas (25 strains), Chromohalobacter* (two strains), *Bacillus-Solibacillus* (29 strains), *Salinicoccus* (two strains) and *Marinococcus* (one strain), as well as eight non-identified isolates.

Birbir et al. (2004) isolated halophilic microbes from large and unpolluted salt mines in Central Anatolia, Turkey. DNA sequences and phylogenetic analyses revealed that the isolated strains were mainly species of the genera *Halobacterium, Haloarcula, Natrinema* and *Halorubrum.* In addition, enzymatic activity tests were also conducted to evaluate the salt quality for industrial applications. Results of gelatinase, caseinase, amylase, cellulase and lipase activity tests revealed that the isolated strains produced hydrolytic enzymes, which could cause deterioration in salt-treated food and hide. Moreover, β-galactosidase activity has been reported in some *Haloferax* and *Halorubrum* species but not in the genus *Halobacterium.*

Kamekura (2008) studied the history of the classification of the family *Halobacteriaceae*, the extremely halophilic aerobic Archaea with some emphasis on the recently described new genera *Halobaculum, Halorubrum, Natrialba, Natronomonas* and *Haloterrigena.* Speculation is made about the evolutionary relationship between members of the *Halobacteriaceae* and the extremely halophilic, anaerobic methanogens of the genera *Methanohalobium* and *Methanohalophilus.*

2.3. APPLICATIONS OF HALOPHILIC MICROBES

Bertrand et al. (1990) used an archaebacterium (strain *EH4*) which was able to biodegrade saturated and aromatic hydrocarbons has been isolated from a sail-marsh.

Thongthai et al. (1991) isolated *Halobacterium spp*. and *Halococcus spp.* and reported them for the first time in fermenting Thai fish sauce, fish residue and salt grain specimens. Other genera of bacteria including the moderate halophiles and halotolerant were also isolated and characterized. The salt responses and hydrolytic enzyme activities including gelatinolytic, caseinolytic, amylolytic and lipolytic activities of the halophilic bacteria were discussed in relation to their possible roles in fish sauce fermentation.

Cameotra et al. (1998) studied about biosurfactants and the microbes that produce them which have numerous industrial, medical and environmental applications, which frequently involve exposure to extremes of temperatures, pressure, ionic strength, pH and organic solvents.

Amoozegar et al. (2010) studied the Azo dye decolorization by halophilic and halotolerant microorganisms. *Shewanella putrefaciens* was determined to be capable of the complete removal of Reactive Black-5, Direct Red-81, Acid Red-88 and Disperse Orange-3 (all 100 mg l^{-1}) within 8 h in the presence of 40 g l^{-1} NaCl. Another halophilic example is *Halomonas spp.* GTW which has shown a remarkable performance in the removal of different azo dyes within 24 h in the presence of 150 g l^{-1} NaCl.

Tiquia-Arashiro et al. (2016) studied about certain halophiles being explored as potential sources of metal tolerant microorganisms with the ability to synthesize metallic nanoparticles. The various halophilic organisms and their by-products that have been exploited for nanomaterial synthesis, the mechanisms that may be involved in the nanomaterial fabrication and the possible applications of the fabricated nanoparticles were studied.

2.4. BIOLOGICAL TREATMENT OF WASTEWATER USING HALOPHILIC BACTERIA

Woolard (1994) did the biological treatment of hypersaline water using the halophilic bacteria. The results of his studies illustrate that biomass accumulation increases oxygen demand but does not significantly improve overall reactor performance.

Kargi (2002) enhanced biological treatment of saline wastewater by using halophilic bacteria. *Halobacter halobium* was used in activated sludge culture for COD removal from saline wastewater (1–5% salt) by fed-batch operation of an aeration tank

Lefebvre et al. (2005) used Halophilic biological treatment of tannery soak liquor in a sequencing batch reactor.

Olivier et al. (2006) performed treatment of organic pollution in industrial saline wastewater. They performed the combination of physico-chemical/biological treatment of saline industrial effluents.

Sohair et al. (2010) performed the biological treatment of saline wastewater using a salt-tolerant microorganism. They performed a fed batch method for the biological treatment using the activated sludge which had a mixture of *Staphylococcus* and pure *S. xylosus* at different salt concentrations ranging from 0.5 to 3% NaCl. The use of *S. xylosus* alone proved to be capable of biological treatment of vegetable pickled wastewater containing 7.2% salinity. The removal efficiency of COD was seen to be 88%.

Kai-Shi et al.(2012) studied the performance of halophilic marine bacteria inoculated on nutrient removal from hypersaline wastewater in an intermittently aerated biological filter. The results of dehydrogenase activity assays demonstrated that inoculation with marine bacteria improved the activity of biofilm in hypersaline environments. Inoculation with marine bacteria improved the degradation of nitrogenous organics and denitrification in nitrogen transformation. In hypersaline environments biofilter recovery after backwashing was significantly prolonged whereas the time required in the bioaugmented IABF was comparatively short.

Laura et al. (2014) used halophilic organisms for biodegradation of organic pollutants in saline wastewater. They used halotolerant and halophilic organisms for the removal of organic pollutants such as organic matter in terms of chemical oxygen demand (COD), dyes, hydrocarbons, N-aliphatic and N-aromatic compounds, and phenols, in conditions of high salinity.

Sharghi et al. (2014) studied the organic pollutant removal performance and the mixed liquor characteristics of a membrane bioreactor (MBR), employing a moderately halophilic bacterial consortium, for the treatment of hypersaline synthetic produced water containing 100–250 g L^{-1} NaCl. The COD and oil and grease (O&G) removal efficiencies in the range 81.6–94.6% and 84.8–94.0% respectively.

Zhao et al. (2020) performed biotreatment of high-salinity wastewater. They used biological techniques as they are inexpensive and environment friendly. They treated saline wastewater of various industries, including the chemical, pharmaceutical, agricultural, and aqua-culture industries.

OBJECTIVES

- Isolation, Identification and purification of halophilic microbes.
- Characterisation of the isolated halophile
- Biochemical test for metabolic analysis of bacterial isolate isolated at 20% NaCl
- Physicochemical analysis of effluent and sea water:
    - BOD
    - COD
    - TDS
    - pH
    - Salinity
- Treatment of wastewater by batch reactor
- Physicochemical analysis of effluent after treatment:
    - BOD.
    - COD
    - TDS
- 16S rRNA sequencing of potent halophilic bacteria

3. METHODS AND MATERIAL

3.1. MICROORGANISMS AND GROWTH CONDITIONS

Salt tolerant microorganisms were isolated from the marine water (used as a sample) which was collected from the Bedi Port situated at Jamnagar, Gujarat, India (22°30120 N 70°02256 E). The pH of the sample water was found to be 8. The microbes were isolated on the Nutrient Agar (NA) and Rose Bengal Agar (RBA). 5%, 7%, 9%, 12%, 15% and 20% of salt concentration were taken into consideration and agar-agar was added along with the NA for bacteria and RBA for fungi. These media were autoclaved and poured into petri-plates.

Sample was serially diluted; a drop of this diluted sample was put on the plates and were spread uniformly with a spreader. The RBA plates also contained streptomycin to prevent growth of bacteria. The plates with NA media were kept at 37℃ and RBA plates were kept at room temperature (25°C). Different colonies were picked, and their five-sector streaking was done for sub-culturing of the bacteria. The slants were also prepared to preserve culture for a longer period in the same manner and then stored at 4℃.

3.2. STUDY OF MORPHOLOGICAL CHARACTERS OF BACTERIAL STRAINS

3.2.1. MORPHOLOGICAL CHARACTERISTICS

The morphology of the isolates was studied. Colonies were studied based on shape, size, elevation, margin and pigment (Cappuccino and Sherman 2008).

3.2.2. NEGATIVE STAINING

Negative stain is particularly useful for determining cell size and arrangement of bacterial cells. It can also be used to stain cells that are sensitive to heat. Nigrosin is an acidic stain. It means that the stain readily gives up a hydrogen ion and becomes negatively charged. Since the surface of most bacterial cells is negatively charged, the cell surface repels the stain. The background will stain but the bacterial cells will not. The bacteria will be seen clear against a dark background (Norris and Swain 1971).

PREPARATION OF STAIN

1g of nigrosin was taken with 100ml of sterile distilled water. It was kept boiling for 30 minutes so that nigrosin dissolved in water. 0.5ml of 40% formaldehyde was added as preservative. It was filtered twice and then stored.

PROCEDURE

A single drop of nigrosin was added at one end of a test slide. An isolated colony was picked with a wire loop and was dispersed in the stain. Use another clean slide to spread the drop of stain containing the organism. Allow the smear to air dry. Add a drop of oil and observe under oil immersion lens (McManus et al. 1960).

3.2.3. GRAM STAINING:

It is useful to determine whether the colonies are of gram-negative or gram-positive bacteria. The preparation of stains is as follows:

1. The primary stain (crystal violet reagents for staining)

- *Solution A*

2 g of crystal violet
20ml of ethanol (95percent vol/vol)

- *Solution B*

0.8 g of ammonium oxalate
80ml of distilled water
Mix A and B so as to obtain crystal violet staining reagent and store for 24 hours.

2. Mordant (gram's iodine)

1g of Iodine
2g of Potassium iodide
100 µL of sterile distilled water
Mix and store

3. Decolorizing agent

Ethanol, 95 percent (vol/vol)

4. Counterstain (safranin)

1g of Safranin O
100ml sterile distilled water

PROCEDURE

A bacterial smear was prepared, and heat fixed on a clean slide. A drop of Crystal Violet was added and left for one min. The stain was washed with tap water and Gram iodine was added. After 1 min it was washed with water and decolorized with alcohol. It was then washed with tap water and stained with Safranin which was washed after 45 seconds. The slide was dried and a drop of oil was added and observed under oil immersion lens (McManus et al. 1960).

When the bacteria are decolourised with alcohol, some of the bacteria might retain the primary stain whereas others get decolourised. The cells that retain the stain are Gram Positive since they have a thick peptidoglycan layer. Decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores of the cell wall and prevents the stain from exiting the cell. So, the alcohol cannot remove the Crystal Violet-Iodine (CVI) complex that is bound to the thick layer of peptidoglycan of gram-positive bacteria and appears blue or purple in colour.

In case of gram-negative bacteria, the cell wall also takes up the CVI complex but due to the thin layer of peptidoglycan and thick outer layer of lipids, CVI complex gets washed off. When they are exposed to alcohol, decolourizer dissolves the lipids in the cell walls, which

allows the crystal violet-iodine complex to leach out of the cells. Then when again stained with the counter stain Safranin, they take the stain and appear reddish in colour (Scherrer 1984).

3.2.4. STAINING WITH METHYLENE BLUE

Methylene Blue is an alkaline stain. The stain readily takes up hydrogen ions and becomes positively charged. Since the surface of most bacterial cells is negatively charged, the cell surface attracts the stain. Thus, the fungal cells will stain. For this, 1g of methylene blue was weighed. 100ml of sterile distilled water was added. It was mixed and then filtered, and the stain was obtained.

PROCEDURE

On a clean slide, a drop of methylene blue stain is put. The fungal isolates are picked up by a piece of cello-tape and are placed very gently on the slide over the drop of the stain. This is then observed under the Bright field compound microscope.

3.2.5. BIOCHEMICAL TESTS FOR METABOLIC ANALYSIS

3.2.5.1 *Carbohydrate Fermentation Test*

Media Preparation:

Nutrient broth

20% salt that is 20 gm in 100 ml

Different sugars in the amount of 1g containing glucose, fructose, xylose, mannitol and sucrose in different test tubes.

Procedure

Durham's tube was put inside a test tube in such a way that no bubbles are formed. The autoclaved media containing different sugars were added with a loopful of bacterial culture (colony obtained at 20%) in each tube. It was performed under sterile conditions. It was then kept at 37°C for 24 hours and observations were made.

3.2.5.2 *Oxidation-Fermentation Test*

Media Preparation

-Hugh and Leifson's OF basal medium; the constituents are as follows:

- Sodium chloride: 5 g
- Di-potassium phosphate: 0.3 g
- Peptone: 2 g
- Bromothymol blue: 0.03 g
- 20% salt
- Sterile paraffin oil (after autoclaving)
- Agar: 3 g
- Glucose: 10 g
- Water: 1000 ml

The pH should be adjusted to 7.1 prior to autoclaving. After the medium is autoclaved, a filter sterilized solution of 10% solution of carbohydrate is aseptically added to the medium to a final concentration of 1%. Dipotassium phosphate promotes fermentation and acts as a pH control buffer.

Procedure

a. Inoculate two tubes of OF test medium with the test organism using a straight wire by stabbing "halfway to the bottom" of the tube.
b. Cover one tube of each pair with 1 cm layer of sterile mineral oil or liquid paraffin (it creates anaerobic condition), leaving the other tube open to the air.
c. Incubate both tubes at 37°C for 24 hours.

3.2.5.3 *Citrate Utilization Test*

Media Preparation
Simmon's citrate agar
20% salt
Bromoethanol blue

Procedure: The Citrate media was autoclaved and slant is prepared. A loopful of bacteria is taken and streaked on the surface of the slant. Later, it is kept at 37°C for 48 hours

3.2.5.4 *Haemolysin Production Test*

Media Preparation
Blood agar
20% salt

Procedure
Autoclaved blood agar media was poured in a petri-plate. A loopful of culture of bacteria was taken and streaked on the plates. It was kept for incubation at 37°C for 24-48 hours and observations were made.

3.2.5.5 *Triple Sugar Iron Agar Test*

Media Preparation: TSI media

- Beef extract: 3 gm
- Yeast extract: 3 gm
- Peptone: 15 gm
- Proteose peptone: 5 gm
- Lactose 10 gm
- Glucose: 1 gm
- Ferrous sulphate: 0.2 gm
- Sodium chloride: 5 gm
- Sodium thiosulfate: 0.3 gm

- Agar: 12 gm
- Phenol red: 0.024 gm
- Distilled water to equal 1 L
- Final pH: 7.4
- 20% NaCl

Procedure: The TSI media was autoclaved and poured in sugar tubes. Once slants were prepared, a loopful of bacteria was streaked on the surface of slants and also the same colony was stabbed at the butt of the slant. It was kept at 37°C for 24 hours.

3.2.5.6 *Catalase Test*

Media Preparation
Nutrient agar
20% salt
Hydrogen peroxide

Procedure: The autoclaved media was poured in petri-plates. A loopful of inoculum was streaked on the plate and kept at 37°C for 1 week. we Add 2 drops of hydrogen peroxide onto it and observations were made.

3.3. ANALYTICAL METHODS

Physiological analysis of water before and after the treatment

3.3.1. BIOLOGICAL OXYGEN DEMAND (BOD)

Preparation of reagents:

- $FeCl_3$ solution: 0.25 g of $FeCl_3\,6H_2O$ in 1L distilled water (DW)
- $CaCl_2$ solution: 27.5 g anhydrous $CaCl_2$ in 1L DW
- Phosphate buffer: 8.5g KH_2PO_4 + 21.75 g K_2HPO_4 + 33.4 g Na_2HPO_4 + 1.7 g NH_4Cl + 500ml DW
- pH 7.2
- $MgSO_4$ solution: 22.5 g $MgSO_4 \cdot 7H2O$ in 1L DW
- Alkaline-Azide solution
- Conc. H_2SO_4
- Starch indicator: 1g soluble starch in 100ml DW
- $Na_2S_2O_3$ solution: 0.025N $Na_2S_2O_3$ i.e. 6.205g $Na_2S_2O_35H2O$ 1L DW

DILUTION WATER:

- 1ml $FeCl_3$
- 1 ml $CaCl_2$
- 1 ml Phosphate Buffer
- 1ml $MgSO_4$
- Make volume to 1L

Procedure: -

Dilution water was filled in a BOD bottle (300ml) and the stopper was closed such that there is no air bubble. 2ml of $MgSO_4$ and 2ml of alkaline-azide solution was added in the BOD bottle and the bottle was sealed. Due to the alkaline condition, manganese sulphate produces a white precipitate of manganese hydroxide. After the white flocs settle 2ml of sulphuric acid is added to the bottle and resealed again. Under acidic condition and dissolved oxygen, the white flocs turn brown and manganese diverts to its divalent state and releases iodine. This released iodine is titrated against sodium thiosulphate using starch as an indicator (Ponomareva et al. 2011).

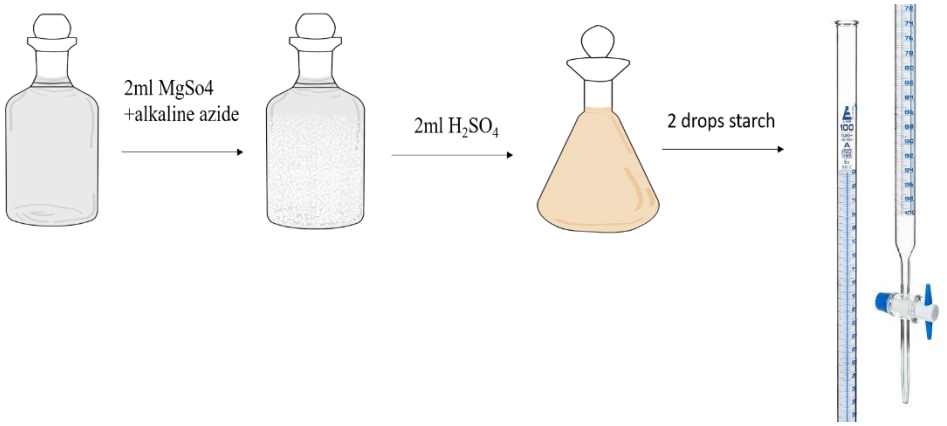

Figure 9 Biological oxygen demand (BOD) analysis

3.3.2. CHEMICAL OXYGEN DEMAND (COD)

Cod refers to the oxygen equivalent of organic matter that can be oxidised by using strong chemicals.

In this method organic matter is first oxidized with known volume of $K_2Cr_2O_7$ and excess of O_2 is allowed to react with KI to liberate iodine in an amount equal to the excess O_2 and is estimated titrimetric ally with sodium thiosulphate using starch as an indicator.

Reagents used

KI: - 135.5 gm of KI in 500ml of d/w

$HgSO_4$: - 10gm $HgSO_4$ in 90ml d/w

1% starch: - 1 gm starch in 100ml d/w

0.1N $Na_2S_2O_3$: - 24.72 gm sodium thiosulphate in 1000ml d/w

Procedure: -

One ml of $HgSO_4$ solution is added to 5ml of sample water and d/w respectively. In each 20ml of potassium dichromate solution is added and mixed thoroughly. They are then heated for 10 min at 92°C. The flasks are then cooled by adding 150ml d/w and then we add 10ml KI. The sample is titrated against sodium thiosulphate using starch as an indicator. End point is marked by pale green colour (Boyles et al. 1997)

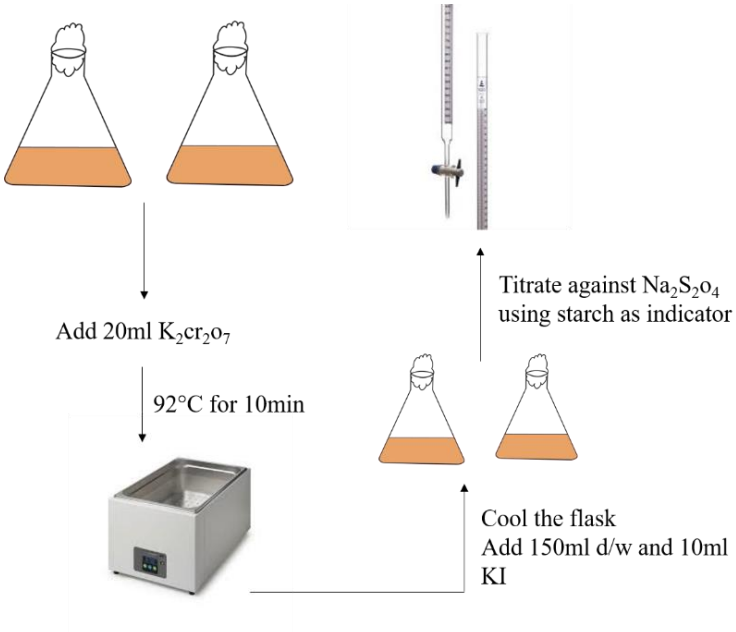

Figure 10 represent Schematic diagram of Chemical oxygen demand analysis

3.3.3. TOTAL DISSOLVED SOLIDS

Effluents can be treated by biological means to achieve carbon, nitrogen and phosphorous removal thus leading to lowering of TDS from the effluents.
The TDS was measured using LAQUA 100 series Instrument

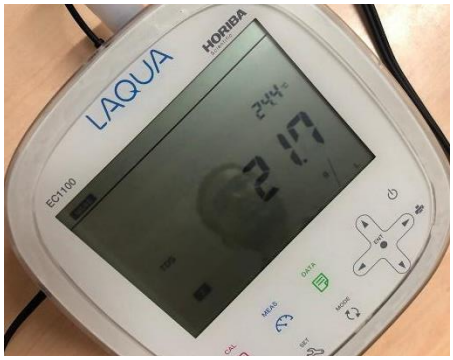

Figure 11 LAQUA 100 series instrument used to measure TDS

3.3.4. SALINITY

Salinity is the saltiness or dissolved inorganic salt content of a body of water. Substances that are dissolved in water are usually called solutes. The typical seawater has a salinity of 35 ppt or 35%.

Salinity was measured using LAQUA 100 series Instrument

Figure 12 LAQUA 100 series instrument used to measure salinity in parts per trillion(ppt)

3.3.5. CONDUCTIVITY

Conductivity is a measure of water's capability to pass electrical flow. This ability is directly related to the concentration of ions in the water. These conductive ions come from dissolved salts and inorganic materials such as alkalis, chlorides, sulphides and carbonate compounds
Conductivity was measured using LAQUA 100 series Instrument

3.4. 16S rRNA SEQUENCING OF POTENT HALOPHILIC BACTERIA

16S rRNA gene sequencing will be used for identification, classification and quantitation of isolate found in 20% NaCl. The 16S rRNA gene is a highly conserved component of the transcriptional machinery of all DNA-based life forms and thus is highly suited as a target gene for sequencing DNA in samples containing up to thousands of different species (Emery et al 2017). Universal PCR primers can be designed to target the conserved regions of 16S making it possible to amplify the gene in a wide range of different microorganisms from a single sample. Conveniently, the 16S rRNA gene consists of both conserved and variable regions the conserved region makes universal amplification possible. Sequencing the variable regions allows discrimination between specific different microorganisms such as bacteria, Archaea and microbial Eukarya (Schloss et al. 2016).

4. RESULTS AND DISCUSSION

4.1. ISOLATION AND SUB CULTURING OF FUNGAL STRAINS ISOLATED FROM SALINE WATER

The fungal strains were isolated from the saline water of Bedi port, Jamnagar at different salt concentrations such as 5% NaCl, 7% NaCl, 12% NaCl. 18 fungal isolates were found to be present in these water samples at different salt concentrations. It was sub cultured using the streaking method technique. Morphological and Microscopic (Methylene Blue staining) analysis were conducted

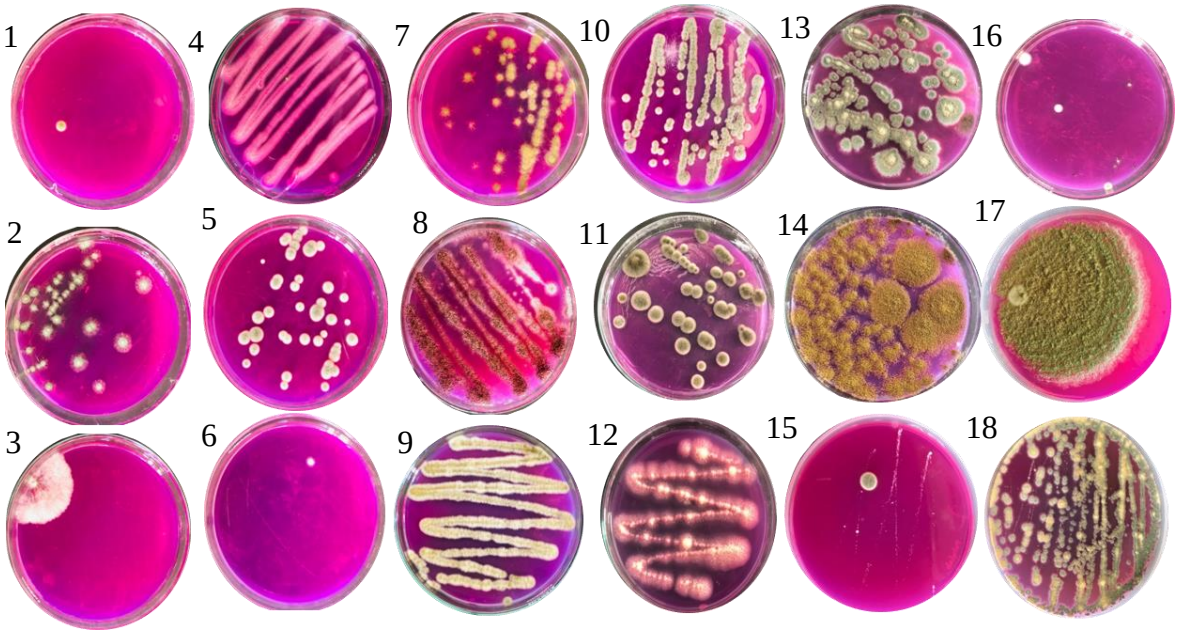

Figure 13 represents Fungal isolates, 1-7 fungi are isolated obtained on 5%NaCl, 8-17 are isolated obtained on7% NaCl and 18 is the fungal isolate obtained on 12% NaCl

4.1.1. MORPHOLOGICAL ANALYSIS

The nineteen colonies of the fungi obtained after streaking on Rose Bengal Chloramphenicol Agar (RBCA) media were having the following characteristics.

Table 1 Characterisation of fungi isolated at 5% and 7% NaCl

Colony	Form	Elevation	Margin	Colour	Outline
1	Circular	Raised	Entire	Green spores	Not present
2	Circular	Flat	Filiform	Black spores	Not present
3	Circular	Raised	Lobate	Green spores	Not present
4	Circular	Flat	Entire	White spores	Not present
5	Circular	Umbonate	Undulate	Green	Not present
6	Circular	Flat	Entire	Light green	Not present
7	Circular	Flat	Filiform	Green	White
8	Irregular	Raised	Entire	Brown	Not present
9	Irregular	Raised	Lobate	Green	White
10	Circular	Convex	Entire	Dark green	Not present
11	Circular	Convex	Entire	Light green	Not present
12	Filamentous	Raised	Filiform	White	Not present
13	Circular	Umbonate	Filiform	Green	White
14	Filamentous	Raised	Filiform	Light brown	White
15	Irregular	Flat	Undulate	Lightgrey	Dark grey
16	Irregular	Convex	Filamentous	Olive green	White
17	Filamentous	Umbonate	Flat form	Grey	Not present
18	Irregular	Flat	Undulate	Green	Not present

4.1.2. METHYLENE BLUE STAINING

This technique is used to differentiate between the bacterial, viral and fungal strains. It is a cationic dye that stains cells blue because the positively charged dye is attracted to negatively charged particles such as polyphosphates, DNAs, and RNAs. The cells of the fungi are stained blue while the spores are stained deep blue.

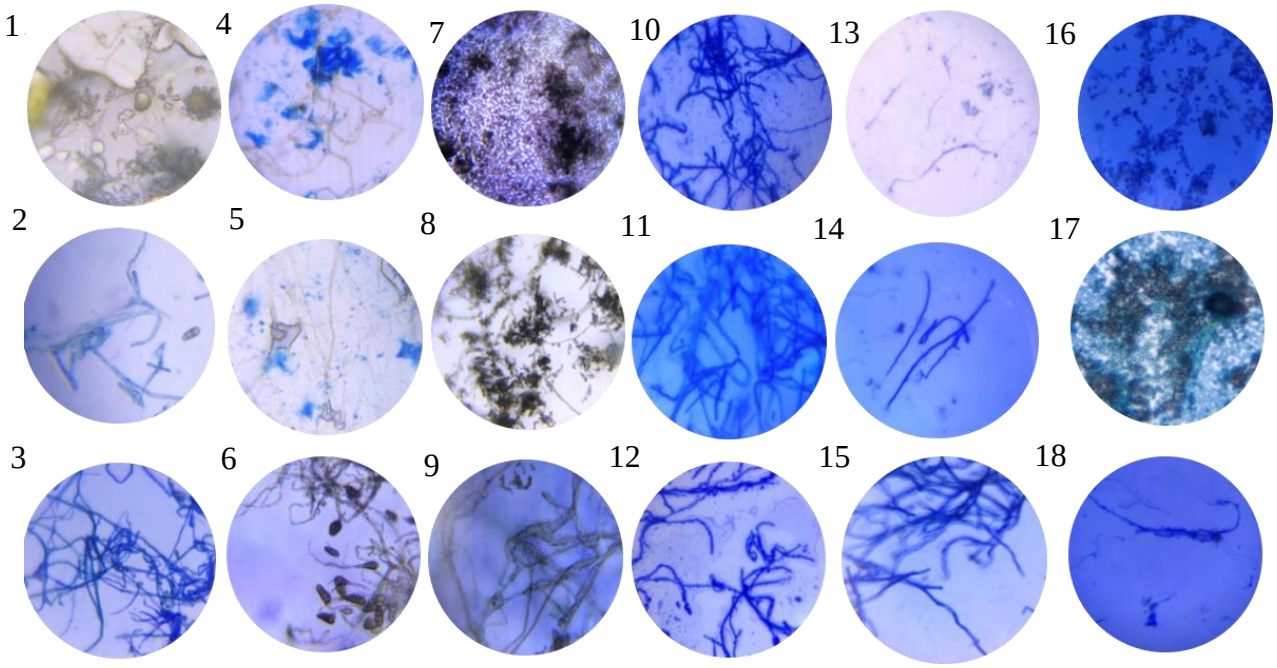

Figure 14 represents Methylene blue staining of the isolated fungi.

The figure shows the staining of fungi with the help of methylene blue dye. The thread-like structure seen in the figure are mycelia and the circular objects seen are the spores. The croissant-like structure in (6) is the head of the fungi and based on the position of the head and spore different types of fungi can be identified.

4.2. ISOLATION AND SUBCULTURING OF BACTERIAL STRAINS

4.2.1. ISOLATION OF BACTERIAL STRAINS AT 5% NaCl

Eight bacterial strains were isolated from the water sample collected from the Bedi port, Jamnagar at 5% NaCl. They were sub-cultured using 5 sector streaking techniques. Morphological and Microscopic analysis (Gram Staining and Negative staining) were studied

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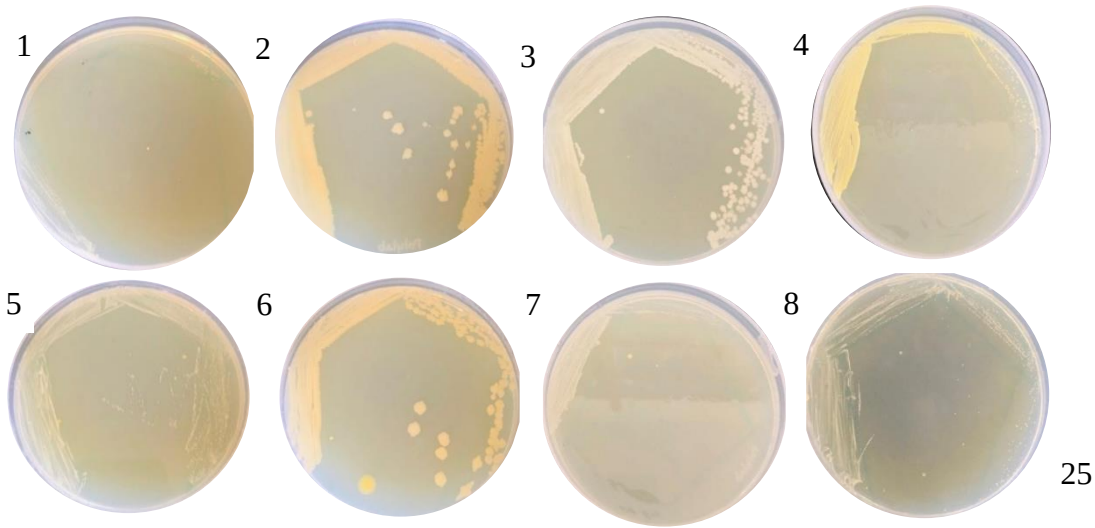

Figure 15 represents isolates obtained on 5% NaCl

4.2.1.1 *MORPHOLOGICAL ANALYSIS*

The eight colonies of the bacteria were obtained after 5 sectors streaking on nutrient media.

Table 2 Characterisation of bacterial strains isolated at 5% NaCl

Colony	Form	Elevation	Size	Margin	Colour
1.	Irregular	Flat	Small	Undulate	Yellow
2.	Circular	Convex	Small	Entire	White
3	Filamentous	Flat	Medium	Lobate	Yellow
4	Filamentous	Flat	Small	Lobate	Non pigmented
5	Circular	Flat	Medium	Entire	Non pigmented
6	Circular	Umbonate	Large	Undulate	Non pigmented
7	Circular	Umbonate	Small	Entire	Yellow
8	Circular	Umbonate	Small	Entire	Non pigmented

4 colonies were transparent with faint growth, one colony was white and opaque and the rest 3 were pigmented (yellow) out of which one was filamentous

4.2.1.2 *GRAM STAINING*

Out of the eight isolates, six of them were found to be gram positive as the bacterial strain appeared to be crystal violet in color while the rest two were gram negative as they gave pink stain. Due to the presence of smaller peptidoglycan layers in gram negative bacteria, they were able to wash out the crystal violet stain with ethanol and were stained pink with the help of safranin, a counterstain.

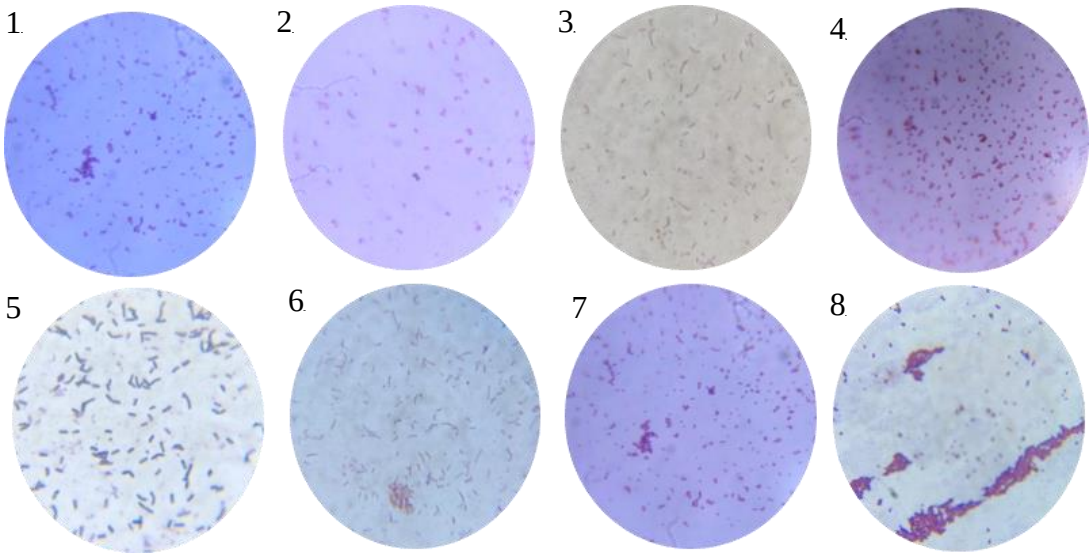

Figure 16 represents Gram staining of isolates grown at 5% NaCl

4.2.1.3 ***NEGATIVE STAINING***

The isolates appeared to have different shapes like *Cocci* and *Bacilli*. The background was stained purple and the bacterial species appeared colourless. Out of eight isolates, 6 isolates were gram positive, from which 3 were rod shaped i.e. *Bacilli*, and the rest 3 were round i.e. *Cocci*. 2 isolated were gram negative *Cocci.*

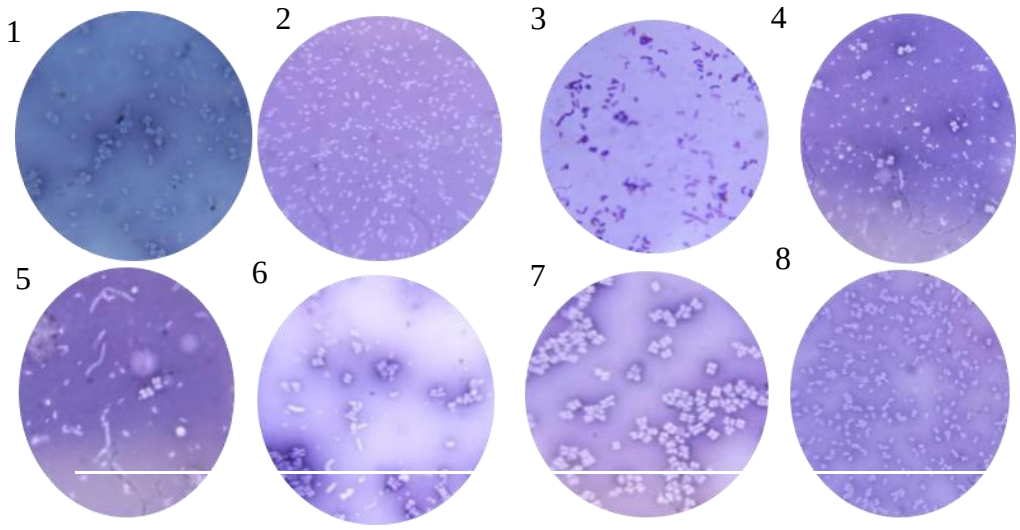

Figure 17 represents Negative staining of isolates grown at 5% NaCl

Fig 1: *Cocci* and gram positive
Fig 2: Cocci and gram negative
Fig 3: *Bacilli* and gram positive

Fig 4: *Cocci* and gram negative
Fig 5: *Bacilli* and gram positive
Fig 6: *Cocci* and gram positive
Fig 7: *Bacilli* and gram positive

4.2.2. ISOLATION AND SUBCULTURING OF BACTERIA ISOLATED AT 7%

Eight bacterial strains were isolated from the water sample collected from the Bedi port, Jamnagar at 7% NaCl. They were further sub-cultured using 5 sector streaking techniques. Morphological and Microscopic (Gram staining and Negative staining) studies were carried out.

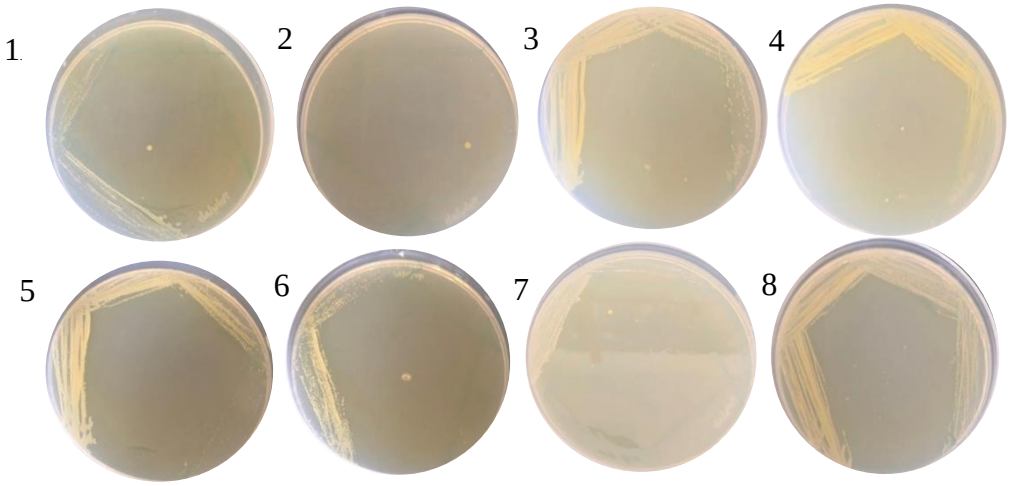

Figure 18 represents Five sector streaking of isolates grown at 7% NaCl

4.2.2.1 ***MORPHOLOGICAL ANALYSIS***

The eight colonies of the bacteria were obtained after 5 sector streaking on nutrient media with 7% NaCl were having following characteristics

Table 3 Characterisation of bacterial Isolates isolated at 7% NaCl concentration.

Colony	Form	Elevation	Size	Margin	Colour
1.	Irregular	Flat	Small	Undulate	Yellow
2.	Circular	Convex	Small	Entire	White
3.	Filamentous	Flat	Medium	Lobate	Yellow
4.	Filamentous	Flat	Small	Lobate	Non pigmented
5.	Circular	Flat	Medium	Entire	Non pigmented
6.	Circular	Umbonate	Large	Undulate	Non pigmented
7.	Circular	Umbonate	Small	Entire	Yellow
8.	Circular	Umbonate	Small	Entire	Non pigmented

4.2.2.2 *GRAM STAINING*

Out of the eight isolates, six of them were found to be gram positive as the bacterial strain appeared to be crystal violet in color while the rest two were gram negative as they gave pink stain. Due to the presence of smaller peptidoglycan layers in gram negative bacteria, they were able to wash out the crystal violet stain with ethanol and were stained pink with the help of safranin a. counterstain.

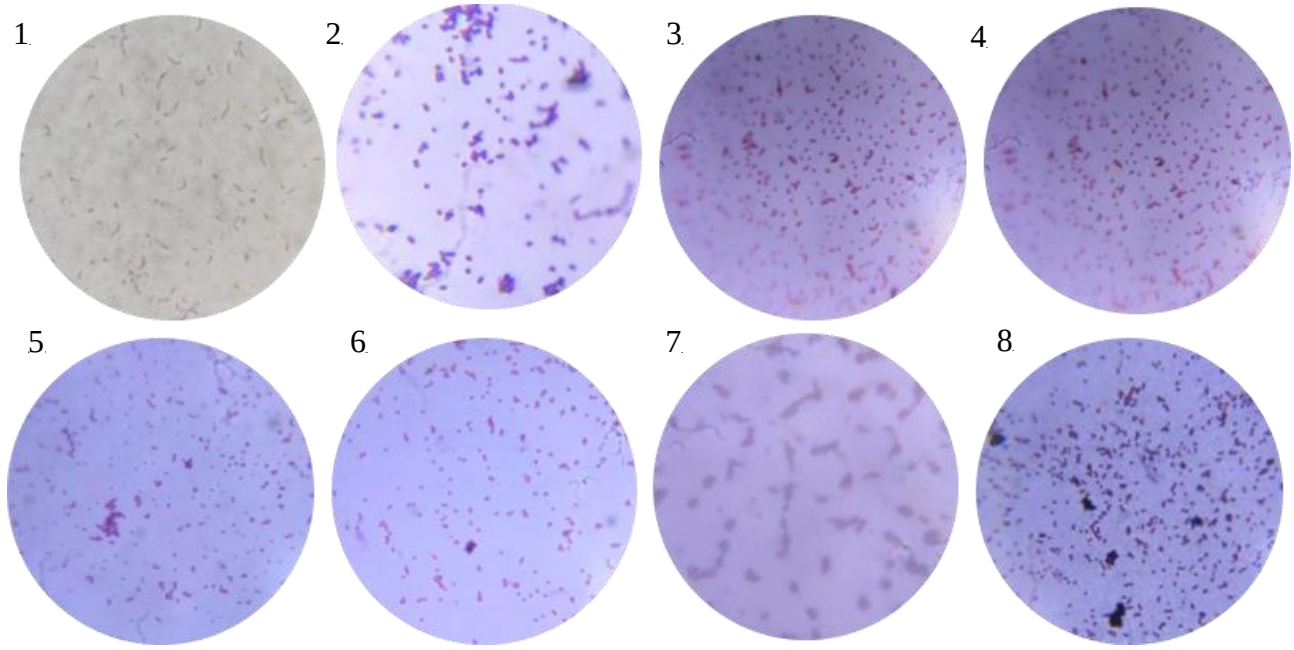

Figure 19 represents Gram staining of the isolates grown at 7% NaCl

4.2.2.3 ***NEGATIVE STAINING***

The isolates appeared to have different shapes like *Cocci* and *Bacilli*. The background was stained purple with the nigrosin stain, and the bacterial species were not stained which helped in identifying the shape of the isolates.
Out of eight isolates, 6 isolates were gram positive, from which 3 were rod shaped i.e. *Bacilli*, and the rest 3 were round i.e. *Cocci*. 2 isolated were gram negative *Cocci.*

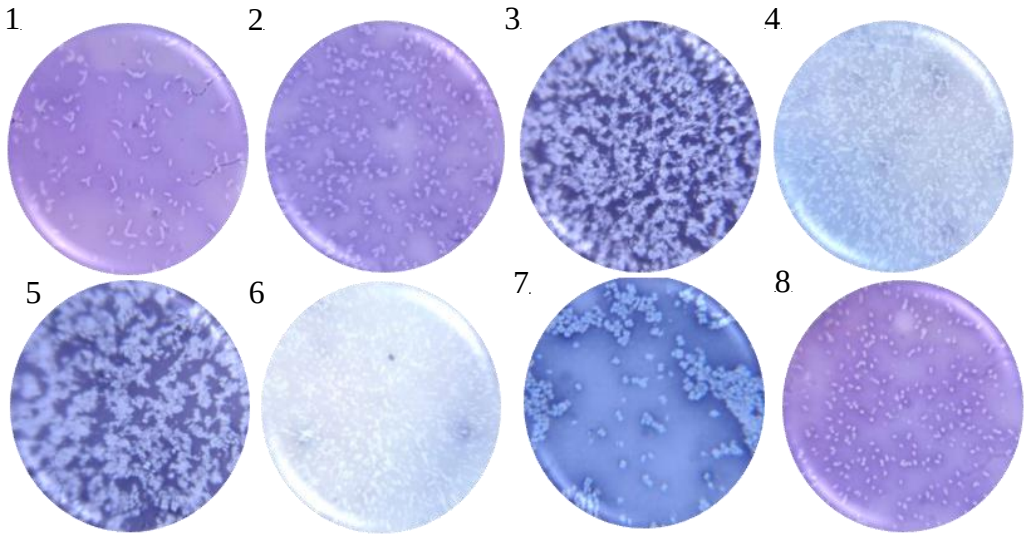

Figure 20represents Negative staining of the isolates grown at 7% NaCl

4.2.3. ISOLATION OF BACTERIAL STRAIN AT 12% NaCl

One bacterium strain was isolated from the water sample collected from the Bedi port, Jamnagar at 12% NaCl. They were further sub-cultured using 5 sector streaking techniques. Morphological and Microscopic analysis (Gram staining) were made.

Figure 21 represents Bacterial colonies seen at 12% NaCl concentration

4.2.3.1 *MORPHOLOGICAL ANALYSIS*

One colony of the bacterium obtained was subcultured by 5 sector streaking technique on nutrient media with 12% NaCl were having following characteristics

This bacterium appeared white opaque

Table 4 Characterisation of bacterial strains isolated at 12% NaCl concentration

colony	**Form**	**Elevation**	**Size**	**Margin**	**Colour**
1.	Circular	Umbonate	Small	Entire	White

4.2.3.2 *GRAM STAINING*

The isolate appeared to be gram positive *Bacilli* as short rods were visible. The bacteria were stained purple and the background was not stained .

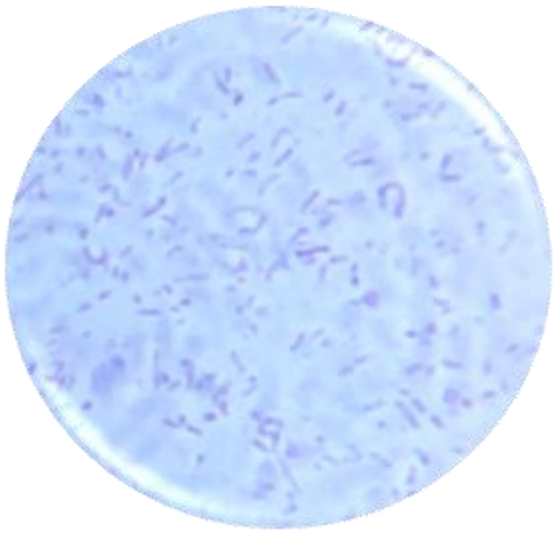

Figure 22 represents Gram staining of the isolates grown at 12% NaCl

4.2.4. ISOLATION OF BACTERIAL STRAIN AT 15% NaCl

One bacterium strain was isolated from the water sample collected from the Bedi port, Jamnagar at 15% NaCl. They were further sub-cultured using 5 sector streaking techniques. Morphological and Microscopic analysis (Gram staining) were made.

4.2.4.1 *MORPHOLOGICAL ANALYSIS*

One colony of the bacterium obtained was sub cultured by 5 sector streaking technique on nutrient media with 15% NaCl having following characteristics.
This bacterium appeared white.

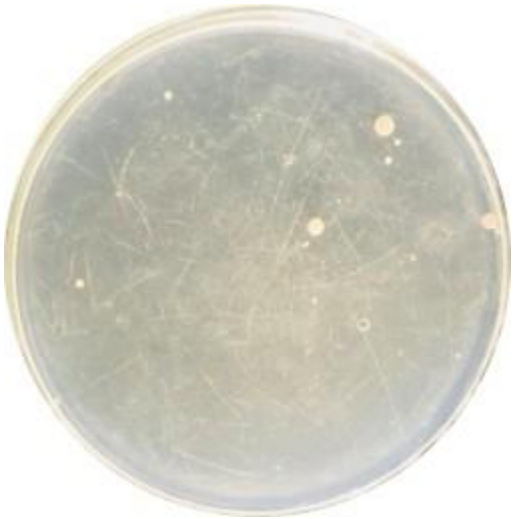

Figure 23 represents Bacterial isolates grown at 15% NaCl

Table 5 Characterisation of bacterial strains isolated at 15% NaCl

Colony	**Form**	**Elevation**	**Size**	**Margin**	**Color**
1.	Circular	Flat	Medium	Entire	White

4.2.4.2 ***GRAM STAINING***

The isolate appeared to be gram positive *Bacilli* as short rods were visible. The bacteria were stained purple and the background was not stained .

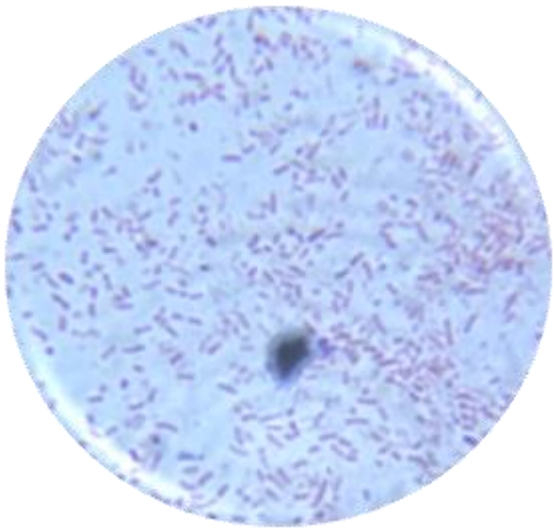

Figure 24 represents Gram staining of the isolate grown at 15% NaCl

4.2.5. ISOLATION OF BACTERIAL STRAIN AT 20% NaCl

One bacterium strain was isolated from the water sample collected from the Bedi port, Jamnagar at 20% NaCl. They were further sub-cultured using 5 sector streaking techniques. Further, the identification of the same was carried out by observing the morphological character, gram staining as well as negative staining.

4.2.5.1 ***MORPHOLOGICAL ANALYSIS***

One colony of the bacterium obtained was subculture by 5 sector streaking technique on nutrient media with 20% NaCl were having following characteristics.

This bacterium was transparent.

Figure 25 represents Bacterial isolate grown at 20% NaCl

Table 6 Characterisation of bacterial strains isolated at 20% NaCl

Colony	**Form**	**Elevation**	**Size**	**Margin**	**Colour**
1.	Punctiform	Flat	Medium	Entire	Non pigmented

4.2.5.2 ***GRAM STAINING***

The isolate appeared to be gram positive *Bacilli*, long rods. The bacteria was stained purple and the background was not stained

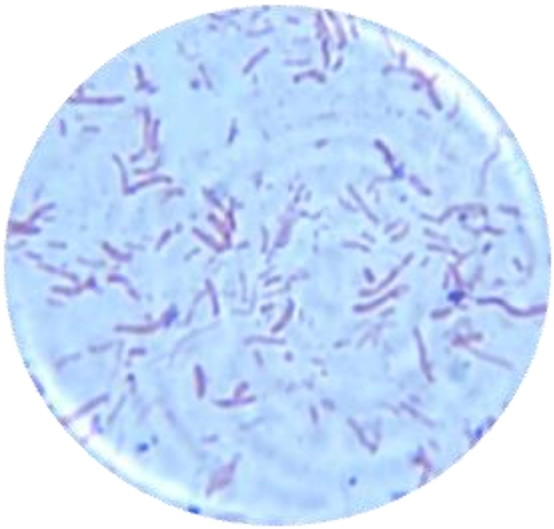

Figure 26 represents Gram staining of the bacterial isolate grown at 20% NaCl

4.3. BIOCHEMICAL TESTS FOR METABOLIC ANALYSIS

4.3.1. CARBOHYDRATE FERMENTATION TEST

The result of this test is observed by change in colour in the test tube. The colour is primarily red due to phenol red in an alkaline environment. As fermentation of sugar occurs, acid and/or gas is released which makes the environment acidic and turns the colour to yellowish. Thus, here results are observed as follows:

Table 7 Result of Carbohydrate fermentation test

Sugars	Result
Mannitol	-
Fructose	+
Glucose	++
Sucrose	++
Xylose	+++

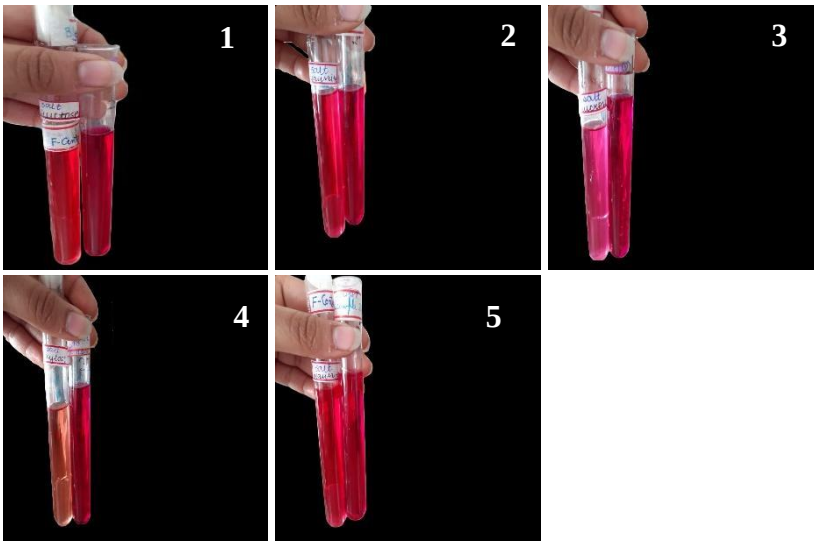

Figure 29 represents Carbohydrate fermentation of sugar.

Fig1. Fructose

Fig 2. Mannitol

Fig 3. Glucose

Fig4 Xylose

Fig 5. Sucrose

4.3.2. OXIDATION FERMENTATION TEST

The observation in this test is if the colour changes to yellow from green then the test is positive. Here in this case, there was no production of acid or gas. Hence, the result was negative.

4.3.3. TRIPLE SUGAR IRON TEST

The growth of colonies was found on the slant and no blackening was found at butt which shows absence of H_2S gas.

4.3.4. CATALASE TEST

In this test, effervescence was observed due to breaking down of H_2O_2 into H_2O and O_2. The effervescence was observed due to liberation of oxygen gas. The figure shown below is of the effervescence of O_2 on the petri-plate.

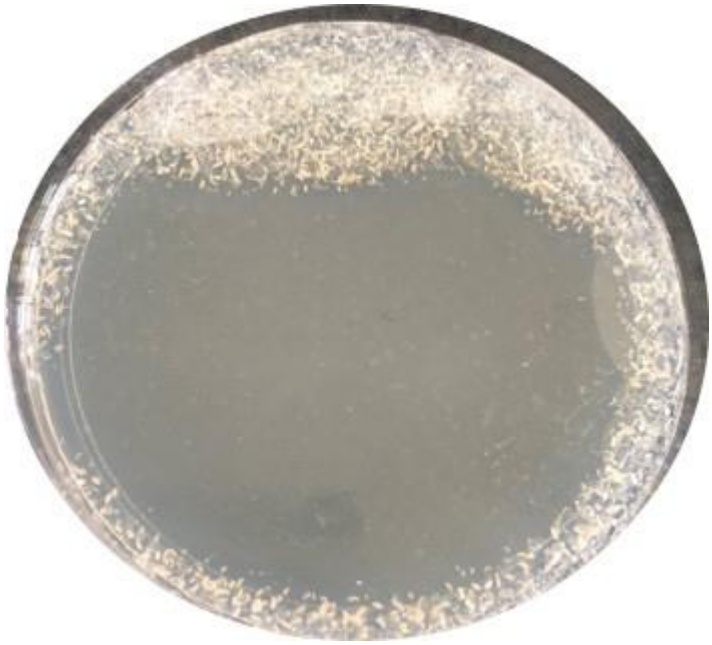

Figure 27 represents Effervescence seen in Catalase test

4.4. PHYSICOCHEMICAL ANALYSIS OF EFFLUENT WATER

The wastewater was taken from the textile industry for biological treatment. The physicochemical analysis of the same was performed before and then after the treatment for comparative studies. There were two types of wastewater: -

Pink water: - The wastewater obtained from textile industry appeared pink in colour hence it was named pink water
Black water: - the wastewater obtained from textiles industry appears black in colour hence it was named black water

Table 8 Physicochemical analysis of effluent water before biological treatment

NAME OF TESTS	PINK WATER	BLACK WATER	BEDIPORT WATER
BOD	60 ± 0.28 mg/L	88mg/L ± 0.41	-
COD	400 ± 0.21 ppm	1200 ± 1.60 ppm	-
SALINITY	2.12 ppt	3.803 ppt	22.989 ppt
TDS	2566.4 ± 2.4 ppm	4441.6 ± 4.31 ppm	23296 ppm
CONDUCTIVITY	4.01 ms/cm	6.34 ms/cm	36.4 ms/cm

4.5. BIOLOGICAL TREATMENT OF WASTEWATER

The treatment of the wastewater was performed at small scale i.e. 100ml.
The 20% NaCl bacterial isolate was used as an activated sludge for the biological treatment of the water. As this isolate was able to survive at such high salt concentration (extreme conditions) thus it was used to treat the industrial wastewater

4.5.1. BOD

Table 9 BOD analysis of the black water after the biological treatment

Dilution (DW)	Dissolved oxygen Day 1	Dissolved oxygen Day 5	Biological oxygen demand
Control	7.6 ± 0.28 mg/L	6 ± 0.28mg/L	80 ± 3.7 mg/L
1:9 dilution	6.8 ± 0.31 mg/L	1.2 ± 0.4 mg/L	280 ± 13.3 mg/L
1:19 dilution	7.6 ± 0.28 mg/L	3.2 ± 0.14mg/L	220 ± 9.3mg/L
1:34 dilution	8 ± 0.17 mg/L	4 ± 0.17 mg/L	50 ± 2.3 mg/L
1:49 dilution	7.2 ± 0.3.5 mg/L	6.4 ± 0.3mg/L	40 ± 1.8mg/L
1:99 dilution	7.6 ± 0.3.6mg/L	6 ± 0.27mg/L	80 ± 3.7 mg/L

Table 10 BOD analysis of the pink water after the biological treatment

Dilution (DW)	**Dissolved oxygen Day 1**	**Dissolved oxygen Day 5**	**Biological oxygen demand**
Control	7.6 ± 0.3.5 mg/L	6 ± 0.26 mg/L	80 6 mg/L
1:9 dilution	8 ± 0.37 mg/L	5.6 ± 0.25 mg/L	192 ± 9.5 mg/L
1:19 dilution	8.4 ± 0.4mg/L	5.2 ± 0.23 mg/L	40 ± 1.7 mg/L
1:49 dilution	8.4 ± 0.4 mg/L	6 ± 0.28 mg/L	120 ± 5.6 mg/L
1:99 dilution	8.8 ± 0.42 mg/L	6.4 ± 0.3 mg/L	120 ± 5.6 mg/L

Figure 28 represents (a) BOD bottle Day 1 (b) and (c) BOD bottle Day 5

The biological oxygen demand (BOD) is determined at different dilutions. Control is prepared to know the BOD of the distilled water which is used to dilute the sample for the BOD analysis of the sample and different dilutions.

As per the results, it is concluded that the biological treatment of wastewater is best at 1:49 dilution and least at 1:9 dilution of black water. The pink water showed the best treatment activity at 1:19 dilution while least at 1:9.

On treating the two effluent, the Biological Oxygen Demand of **pink wastewater** is reduced by **33.33%** and the **black water** is reduced by **50%.**

4.5.2. COD

The chemical oxygen demand (COD) of the treated water is found using the titrimetric method.

Figure 29 represents COD analysis of black water

Table 11 COD analysis of water before and after the treatment

Effluent	COD
Pink water (before treatment)	400 ± 0.21 ppm
Black water (before treatment)	1200 ± 1.60 ppm
Black water (after treatment)	16 ± 0.72 ppm

The COD of the black water decreased by **98%.**

4.5.3. TDS

The TDS was calculated by LAQUA 100 SERIES before and after the treatment of water. The TDS of the effluent water before treatment is 2566.4 ± 2.4 ppm (pink water) and 4441.6 ± 4.32 ppm (black water) and after treatment is 2026.3 ± 2.03 ppm (pink water) and 2624.8 ± 2.5 ppm (black water).

Hence, the TDS of pink water is reduced by 21.05% and black water by 40.91%.

4.5.4. Comparative studies

Pink water

Table 12 Comparative study of pink water before and after treatment

Name of the test	Untreated water	Treated water
BOD	60 ± 0.28 mg/L	40 ± 1.7 mg/L
COD	400 ± 0.21 ppm	-
TDS	2566.4 ± 2.4 ppm	2026.3 ± 2.03 ppm

Black water

Table 13 able 12 Comparative study of black water before and after treatment

Name of the test	Untreated water	Treated water
BOD	88 ± 0.41 mg/L	40 ± 1.7 mg/L
COD	1200 ± 1.60 ppm	16 ± 0.72 ppm
TDS	4441.6 ± 4.31 ppm	2624.8 ± 2.5 ppm

On treating the two effluent, the Biological Oxygen Demand of pink wastewater was reduced by 33.33% and the black water was reduced by 50%. Also, the TDS of pink water was reduced by 21.05% and black water by 40.91%. The Chemical Oxygen Demand was reduced by 98% efficiency of black water. Since the water got treated hence the organic waste reduced as a result Total Dissolved Solid also reduced.

The BOD of efficiently treated municipal sewage is 20 mg/L. we could reduce the BOD of our effluent till 40mg/L. So, by changing the dilution or increasing the incubation time or performing primary treatment before performing the biological treatment we might be able to lower down the BOD with greater efficiency and less cost.

5. CONCLUSION

Nineteen halophilic bacteria and 18 halophilic fungi were isolated from the water of Bedi port Jamnagar (22.5025137, 70.0268012). 8 isolates of bacteria were able to grow in media containing 5% NaCl (Figure 16), 8 bacteria were able to grow in media containing in 7% NaCl (Figure 19), 1 bacterium was able to grow in media containing in 12% NaCl (Figure 22), 15% NaCl (Figure 24) and 20% NaCl (Figure 26) respectively. 7 isolates of fungi were found in 5% (Figure 14), 10 isolates of fungi were able to grow in 7% NaCl (Figure 14) and 1 isolate of fungus could grow in 12% NaCl.

Out of 37 microbial isolates (bacteria and fungi) one bacterial isolate belonging to *Bacillus* sp. *which was* able to grow in 20% NaCl concentration was used in the treatment of wastewater. Two different effluents from the textile industry were treated. Viz, pink and black wastewater. The BOD of pink and black effluent reduced by 33.33% and 50% respectively. The TDS of pink water was reduced by 21.05% and black water by 40.91%. Since the water got treated hence the organic waste reduced as a result Total Dissolved Solid also reduced.

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