EXPLORING THE POTENTIAL OF FISH GUT BACTERIAL FLORA IN DEGRADATION OF OIL

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "***EXPLORING THE POTENTIAL OF FISH GUT BACTERIAL FLORA IN DEGRADATION OF OIL***" is a bonafide work carried out by **Ms. Sherley Pereira** under my supervision in partial fulfilment of the requirements for the award of the degree of Masters in Microbiology at the School of Biological Sciences and Biotechnology, Goa University.

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DECLARATION

I hereby declare that the data presented in this Dissertation report entitled, *"**EXPLORING THE POTENTIAL OF FISH GUT BATERIAL FLORA IN DEGRADATION OF OIL**"* is based on the results of investigations carried out by me in Microbiology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Trupti Asolkar and the same has not been submitted elsewhere for the award of a degree by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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List of Abbreviations

Abbreviation	Full form
PAH	Polyaromatic Hydrocarbon
ZMA / ZMB	Zobell Marine Agar / Broth
NA / NB	Nutrient Agar / Broth
SS agar	Salmonella- Shigella agar
TCBS agar	Thiosulphate Citrate Bile salt Sucrose agar
MPN	Most Probable Number
TVC	Total Viable Count
Cfu/ml	Colony forming unit/ millilitre
rpm	Revolutions per minute
2,6- DCPIP	2,6- Dichlorophenolindophenol
SDS	Sodium Dodecyl Sulphate
E24	Emulsification Index
CTAB	Cetyltrimethylammonium bromide
BATH	Bacterial Adhesion to Hydrocarbon
IMViC	Indole, Methyl red, Voges Prausker, Citrate
g	gram
mm	millimetre

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INTRODUCTION

Countries of the third world continue to evolve in the major demographic transition from a rural to an urban and industrial society. Urbanisation and environment are related in a complex manner, as urbanisation surely creates jobs, ups the life style basic amenities and more are fulfilled but it does come at a cost, which creates the biggest challenge to the environment. Just to list a few we have, discharge of pollutants and generation of wastes (solid, liquid, gas), depletion or over exploitation of natural resources, it's relation to costs of social explosion, pollution, poverty and sustainable development (Kaur, S.,2017). Environmental pollution is a threatening reality. The air, land and water are all under a greater negative impact of human progress in industrial sectors, urbanisation and agriculture.

Marine Pollution as described by a group of experts is, " the introduction by man of substances into the marine environment resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of seawater and reduction of amenities" (Ricardo Beiras, 2018).

There are three major kinds of sources of pollution that make it to the ocean. One, is direct release of waste water or any such waste, second being land run-off due to rains, third is release of contaminants via atmosphere. 1)Run-off from agricultural lands, urban dwellings, constructing sites and more which introduce nutrients and minerals like phosphorus, Potassium, carbon, nitrogen etc. making the water excessively rich in nutrients giving way for algal blooms and consequently depletion of oxygen thus killing the aqua lifeforms. 2)Debris, plastics and dust is carried by air or human activity towards the sea. Inland mining leads to land wash of minerals like copper. 3)Deep sea mining is one such activity that is carried out on the ocean floor in order to extract minerals like manganese, gold, copper, silver, cobalt, even zinc via drilling of the seabed

about 1400 to 3700 m below the ocean's surface. Such activities cause perturbations to the habitats of the benthic organisms and also results in increased toxicity of the water column. Moreover these toxic metals get into the marine food webs causing changes in behaviour, reproduction, biochemistry and therefore suppresses growth in marine life.(Hulsey & Ludivina, 2012). 4) To push a little further, quite a significant increase in ocean temperature and the level of carbon dioxide in the atmosphere is a result of Climate Change. Ocean bodies being a large natural carbon sink absorbs carbon dioxide from the atmosphere and thus raises the level of carbon dioxide in the water which acidifies the ocean water. All of this contributes to changing aquatic ecosystems and directly impacting the sustainability of fisheries and thus the livelihood of the communities dependent on them. Alleviation of climate change can be also taken care of by maintaining a healthy ocean ecosystem (Ricardo Beiras, 2018).

Ships have long been a cause for marine pollution in various manners like production of garbage, sewage release, illegal discharge of many more toxic wastes, oil spills all that have a direct negative impact on the marine ecosystem. Introduction of such waste generates and spreads algal blooms, and other invasive species which will take over the natural ecosystem and boost the spread of new diseases and continues to jeopardise the native species by competing for food. Oils seeping into the waters via oil spills, leaks deteriorate the life as the crude oil components are difficult to degrade and clear up the vast area affected which also depends on the geographical conditions, lasting for long years and thus settling into sediments and marine environment.

Oil spill simply refers to release of oil into sea or ocean or coastal areas as a result of accidents such as collision of two oil carriers or tankers, explosions, hull failures, running on shore, and this

accounts for only a small percentage of global oil spill, other ways could be leaks from the oil extraction platforms. But the most common cause is land run-off. Engines used to run vehicles such as cars operate using petroleum fuels and other petroleum based lubricants. These begin to accumulate on land and in the ground thus slowly but progressively making its way to the ocean waters. Another being, Natural seepage - when tectonic plates shift and this gives way to oil from its natural reserves that is buried beneath the ocean bed , this is also accelerated by human activities by the action of drilling. It is the discharge of petroleum hydrocarbon, which again is a complex mixture.

Oil comprises of a concomitant of hydrocarbons. Oil Spills float as oil films over seawater. Such hydrocarbons happen to accumulate in the food webs, some deplete by vaporization and some enter sediments. These cause irreversible damage to the marine ecosystem. Corals for example of largely affected and the mangroves are highly susceptible to oil spills as oil deposits creating lethal impacts. Poly aromatic hydrocarbons are highly toxic which is found to be 12% of the spilled oil. Their accumulation in sediments and as suspended matter directly or indirectly affect the benthic organisms. On entering the food webs its affect magnifies posing a hazard to marine life and humans. In humans it raises the risk of cancer and cause other health issues. (Suneel, V., et al., 2019).

An immense rise in the amount if oil spill into sea due to operational discharges, collision and grounding of tankers, well blow-out and pipeline breaks. The Woods Hole Oceanographic Institute states " as much as one half of the oil that enters the coastal environment comes from natural seeps"(WHOI,2014).It is estimated that about 48% of marine pollution by oil is by fuels and around 29% by crude oil (Vethamony, P., et al., 2007). Accidents of tanks carrying oil and

transporting contribute only 5 % to marine pollution. Globally there is seen rise in economic growth and hence rise in the demand for petroleum products thus greater chances of future oils pills stand at hand especially along tanker routes and areas near the production platforms. It is clear that natural and anthropogenic activities serve as sources of oil spills in seas and oceans. (Suneel, V., et al., 2019)

Major oil spills in marine environment worldwide

As per statistics it is estimated that about 3.2 million tons of oil is released into the marina annually from all sources. Majorly due to shipping and other industrial activities. In the year 1978, the Amoco Cadiz oil tanker failed its steering in a severe storm leading it to run off the coast of Brittany, France, this got 1.8 million barrels of oil to spill on the shore. In the consecutive year the Atlantic Empress and the Aegean Captain, both loaded with approximately 2.1 million barrels of oil came head-on for 10 miles off coast of Tobago as a result of a tropical rainstorm, into the Caribbean. Around 2 million barrels of oil was released into the Arabian Gulf Sea during the Iran-Iraq war (1980-1988). The Cape Town coast faced an explosion of the Castillo de Bellver, which sank and introduced 250,000 tonnes of crude oil to the waters in the year 1983. A fully loaded Odyssey tanker sank on its explosion in the North Atlantic thus spilling 1 million barrels of oil for 700 miles off coast of Nova Scotia, Canada (1988). In the following year the Exxon Mobil Valdez oil tanker hit Prince William Sound in Alaska, pouring over 250,000 barrels of crude oil in the waters. Jump to the 90's, the ABT Summer oil tanker explosion, 900 miles off coast of Angola spilled 1.9 million barrels at sea. In 1996, the Sea Empress hit head-on with the rocks off Milford Haven at Britain's west coast that leaked more than 70,000 tonnes of oil spill. France faced a storm

in the year 1999 that took down the tanker Erika causing spill of thousands of tonnes of oil into the Bay of Biscay.

In the recent years, that is 2011/2012, New Zealand coastal waters and the beaches of Tauranga were struck by 47,230 ton container ship Rena. In 2018, the Sanchi tanker loaded with 136,000 tonnes of Iranian condensate hit the cargo ship at the Shanghai offshore that caught fire and continued to burn for a week.A super tanker the New Diamond (2020) holding 2 million barrels of oil caught fire offshore of Sri Lanka. The same year the Indian Ocean was contaminated by thousands of tonnes of oil spilled by the Wakashio a Japanese container off the Mauritius coast. (*Natural oil seeps,* 2019)

The MV Khalijia 3 and the MV MSC Chitra both oil containers collided off the coast of India in Mumbai in 2010 which gave way to 200 of 400 tonnes oil containers into the Arabian Sea(Mid-Day). Another collision in 2017 occurred a few miles away off the Kamarajar Port in Ennore, a natural Harbour in Chennai on the Coromandel coast, an area of 34000 Square meters was affected and the shoreline of the suburb Tiruvallur accumulated oil at a 3 kilometer stretch. The volume of the spill estimated to be 9.9 million gallons(*1978-2020: List of major oil spills,* 2020). An inland oil field gas and oil leak referred to as Baghjan gas leak at Tinsuki district, Assam, India. The blow-out took place in 2020 are reports of dead fish in local water bodies surfaced as a result (Assam Govt Sends Notice to OIL After Dead Fishes Found in Lake Near Blowout Site.,2020). India's giant oil field (Bombay High, BH) is located in the north-west Arabian Sea and it shares the long Indian coastline with several major and minor ports- Kandla Port, Bombay Port, New Mangalore port and Kochi Port, that handle crude oil among others. Here, therefore occurrence of oil spills in the offshore region and its effect across the coastline cannot be left unattended to. Goa,

a state along the east coast of India, is characterized as a major tourist destination fir its beauty of the shores and other recreational activities.

Tar ball deposition at the coast bring to attention how seriously the coastal health, marine life and as well as the tourism industry is affected (Suneel, V., et al., 2019). Tar balls are a result of oil spill due to its subsequent weathering. Just before the onset of monsoon season as a result of change in water currents the tarballs are transported to the shore (Fernandes, C.,et al., 2020).

Many methods have been tried, tested and used to avoid and / or cleanse petroleum pollution in the environment. In the course of extraction, refining, transport and storage of petroleum and petroleum products, accident spills into the sea water is prevalent. Crude oil being toxic, mutagenic and carcinogenic stands heavily as a serious threat to marine life (Bayat, Z.,et al., 2015).

Petroleum hydrocarbons adversely affect both terrestrial and aquatic ecosystems. While the aquatic habitat has drawn most attention in recent years as the impact of oil spills on marine life has been heavily threatened thereby affecting even the people that rely on its resources to make a livelihood. Oil is considered to have higher frequencies of being an organic pollutants of aquatic ecosystem among the rest.

Crude oil a complex of organic compounds- majorly, the hydrocarbons in addition to heterocyclic compounds (alkanes, napthenes, aromatics) whose content ranges from 30-100%. One of the important non-hydrocarbon components of oil are resins and asphaltenes. Heavy metals such as metal-porphyrin complexes and other trace elements are present in very minute quantities. Oil cannot be defined as a particular component with a specific physical property , behaviour but rather a concoction of chemicals components, each component thus eliciting its property in the oil. This

crude oil is thus processed as diesel fuel, lubricating oil or bitumen (diluted) which are components of the oil separated and modified for a desired produce.

One conventional method used to clearly illustrate the chemical components of crude oil is Gas Chromatography (GC) or GC coupled with Mass Spectrometry (GC-MS). Separation of the components is based on the London dispersive interactions seen as their differences in boiling points ,while in cases of two components sharing boiling points, a two-dimensional gas chromatography (GC x GC) is carried out where, in the first stationary phase it is separated by London interactions and then in the secondary stationary phase by polar-non polar interactions (Prendergast, D. P., & Gschwend, P. M., 2014)

It's toxicity hinges on varying factors like oil composition and characteristics (physical or chemical) and bioavailability. However one critical effect of it is narcosis, an anaesthetic effect due to cell membranes and nervous tissue partitioning leading to a dysfunctional central nervous system. In real time studies show when such hydrocarbons are ingested by marine animals they move to the liver and here enzymes activate Polycyclic Aromatic Hydrocarbons (PAHs) making them more toxic and reactive in nature. Metabolic by products of PAHs are huge contributors of toxicity and these have oxidative and carcinogenic properties stemming from the ability to attack and bind to DNA and proteins. Its volatile nature causes respiratory tract irritation and narcosis of mammals and birds on inhalation and absorption into the bloodstream via the gastrointestinal tract. Other adverse physical aspects include direct contact of fur and feathers, the layers on the body of mammals and birds that give them warmth and buoyancy. Consequently, falling prey to hypothermia and drowning when oil flattens and adheres to the outer layer (Saadoun, I. M. K., 2015).

Oil recovery is one of peak concerns for oil companies. An increase in demand for energy in the coming decades is growing and hence to satisfy these needs an increasing number of recovery factors from mature fields under primary and secondary production is critical. Improved oil recovery (IOR) methods includes drilling and well technologies, reservoir management and control, reservoir monitoring techniques and Enhanced Oil Recovery (EOR) methods (Alvarado, V., et al. 2010). Oil clean-up has advanced along the years to being more and more effective with the inclusion of a variety of approaches. Techniques are classified as mechanical/ physical, chemical, and biological. Recovery capacity of oil spill remediation techniques, mostly fail to be effective as they do not take into account variable oil viscosity and thickness, emulsified oil, dispersant addition or some other complicating factors. It is seen that recovery of oil by a device is lower than the amount of oil it first encounters, as it depends on the distribution of the oil spill and the devices capacity to carry out the remediation (Prendergast, D. P., & Gschwend, P. M., 2014).

Some of the various oil spill recovery methods involve mechanical means via the use of skimmers and booms, on-site burning of oil, and oil dispersion by dispersant. Each of the methods have its own boons and bane's, mechanical means of recovery for example is quite efficient in skimming off the oil from water surface but deemed to be a very slow process further limitation posed by weather. On site burning again has a good potential to remove oil but the oil needs to be fresh and enough thick. Chemical dispersant too treat large areas and volumes of oil, and for this to be a complete removal it relies on ,microbial degradation. Chemical dispersant also can add to the environmental pollution if it does not pass certain guidelines laid by the experts.

New age formulations comprise of more than one surfactants which align at the interface (oil-water) which on the physical action of the water such as the wave and other turbulence creates droplets of the size 70 microns or less. Thus the dispersed droplets either sink in down the water column as they become less dense than the water around them but eventually move upwards through the water column as a result of the physical aspects attributed to its size. A significant negative impact was recorded on fish, corals and even birds. It is noted that such chemicals put out even toxic break down products which by itself or in combination with oil droplets and chemical dispersant, hence making dispersed oil more toxic than untreated oil. If the oil is left untreated , it remains on the surface and eventually washes off, as a result of ocean currents, on shore. The coastal area is thus polluted putting at danger coastal organisms like invertebrates, mammals and birds. On the other hand if the oil spill is treated with chemical dispersants, the marine life including fish, planktons and larvae are faced with the toxic oil droplets. The key behind such concept is that an increased surface-to-area ratio with respect to the dispersed oil droplets thus makes it a perfect substrate for the microorganisms to interact and utilise the oil for its metabolism in-turn acting as a mechanism of bioremediation (Alvarado, V., et al., 2010).

Importance of Marine Organisms

Plants, algae, invertebrates, vertebrates, microorganisms and more wider range of diverse forms of life house the marine ecosystem. Among these plants, algae and some plankton serve as primary producers in major food webs, even in the case of higher trophic level organisms. The marina is a major commercial source of fisheries as for human consumption as well as for eco-tourism purposes. Zooplanktons and the microorganisms aids in biogeochemical cycling. Marine invertebrates like crustaceans play a crucial role in pelagic food web, by providing for the large vertebrates and other invertebrate predators. Crabs on the other hand escalate free oxygen

availability by causing sediment turnover. Amphipods act as herbivores and are food to higher vertebrates and invertebrates and also help in degradation of plant and animal matter making nutrients accessible to other members of the ecosystem. Molluscs function by enhancing quality of the water thus giving fish hunting places and a commercial source of fishery (Snyder, S. M.et al., 2015)

Oil spills invade fishes variably. The major route of oil uptake depends on the behaviour of the species of fish and the physical and chemical properties of the Poly Aromatic Hydrocarbons (PAH). Exposure can be via ingestion, ventilation of the gills and dermal uptake. One factor is the habitat of the fish where in cases of a fish that swims at the marine surface there's higher risk of dermal exposure while in cases of a benthic fish or benthic consuming fish, they are likely to ingest the PAHs depending on its exposure to PAH-contaminated sediments and the diet of the benthic fish(Snyder, S. M.et al., 2015)

Fish waste now a valuable resource : In the name of sustainability

The United Nations (UN) in the year 2016 set up goals aiming for environmental, social and economic growth by taking on green methods and cleaner production technologies. These target the fulfilment of basic human, and needs more for the planets health. The foundation of the Sustainable Development Goals (SDG) lies in protecting and fulfilling the earth and its beings by making mindful use of sustainable science.

In the current scenario, the endangerment of the environment is visible and the affects are felt to great extents, as a result of human activities including the indiscriminate use of chemicals, choice of non- renewable sources of energy over sustainability, huge yields of waste products and mismanagement locally, industrially and globally. Thus the need for the world to shift the gear and adopt more sustainable measures, cleaner production and green technologies has never been more

lucid. 193 countries have ergo agreed onto collaboratively working on the 17 Sustainable Development Goals. These can be categorised as economic, social and environmental dimensions, which remain and will continue to be the integrated and the indivisible balance of the 17 SDGs. It aims at generating mechanisms and solutions so as to enable economic as well as social development without having to trade the environmental well being for it. The emphasis is clearly on making greater efforts to protect the environment by preventing and controlling illegal activities like exploitation of natural resources (Akinsemolu, A. 2018).

How can micro-organisms play their role in aiding us to accomplish certain goals towards creating a sustainable present and future? Microbes have long known to be survivors of extreme and constantly changing environmental conditions. They adapt and thrive knowing no boundaries or limitations. From the perspective of the layman, microbes only property is to cause disease and end human life, but in actuality just a fraction of them is pathogenic and a great majority of them being beneficial or neutral but having a definite and essential role to play for life (American society of microbiology). They are known to be a very crucial part of the series of green processes and cleaner technologies which ranges from bio-geochemical cycles to varying industrial processes.

Microbial diversity is imperceptibly humongous with approximately 10^{12} microbial species on earth, the bacterial and archeal micro-organisms making up about 10^{30} cells of it. The ecological and environmental processes drive biodiversity and essentially the global threat over it deteriorates the vital ecosystem on which all life relies upon. Developments in microbial ecology is promising and will provide innovative and alternate methods (Vuong, P., et al., 2000). Bioprospecting, a process involving in-depth search of microbes that are potential holders of gene that code for key enzymes or molecules for bioprocesses or bioproducts that may be of commercial or industrial interest, can give way and contribute crucially to understand and create nature-based solutions by

continually searching for metabolic potential which can be utilised to boost present microbial course to build a sustainable industry. At this point arises one question and that is, “Where do we begin to search?” or “ which could be the best spot to search?” Experts suggest that the best place to bioprospect is the system “where the need for it is”. Thus any area rather a system that is continually under ecological transition like in the case of marine ecosystem, agricultural and urban expansion, come directly under the radar of bioprospecting (Vuong,P.,et al.,2000). Marine ecosystem is vast and holds a very significant value to humankind. One of the biggest resource yield is fishery which makes up for the largest sector of our economy. At the same time it should be noted that a huge quantity of waste in the form of by-products, which takes into account gut, head, scales, bones etc. These have not yet been identified for any real time application as yet. The heaps of collection of such waste is now a matter of concern and giving rise to more and more space for suitable solutions. Some suggestive solutions such as use of these discards as secondary raw material which would both, manage waste collection and its adverse environmental impact. The other solution of interest is the that the micro-biota that are housed by such tissues or organs. Such marine microorganisms along with their by-products have been identified as a unique source of novel bioactive molecules.(Floris, R., et al.,2013). The fish gastrointestinal tract (GIT) is capturing the interests of researchers due the variability and the complexity of the bacterial biome that it inhabits, it is a hotspot for diverse metabolic processes and synthesis of bioactive molecules . Some of the many bioactive metabolites include digestive enzymes, polysaccharides, short fatty acid chains (SCFAs)- antibacterial and antitumoral activities(Chen, J. et al., 2019). Biosurfactants have a range of properties attributed to them like anti-fouling, anti-bacterial, anti-thrombotic, anti-adhesive, anti-cancer, etc. Biosurfactants has drawn new interest due to its multi functionality and a commercial product for pharmaceutical companies, industrial, cosmeceutical

and environmental. To accelerate the expression and improve the yield by manipulating the genetic code via genetic engineering and recombinant DNA technology.

In a study conducted by Floris, R., et al (2021), concerning bacterial isolates picked from different species of wild mullets sampled in different seasons, the results of cell-free supernatant analyses in autumn samples indicated good emulsifying properties and noteworthy reduction in surface tension, while those that were sampled in winter showed erratic regularity in the surface tension activity in the supernatants. All through the study it was noted that the interfacial activity and the emulsification capacity were not always coherent. On a whole, the best producers of biosurfactants were marked as belonging to *Pseudomanas* spp. Further the preliminary analysis of the chemical structures of biosurfactants from a group of representative strains were carried out. The data thus attested that the bioactive compounds belong to two classes of glycolipids : rhamnolipids and less polar compounds.

In a previous study by Floris, R., et al (2017), on fish gut bacterial flora of Mediterranean gilthead sea bream (*Sparus aurata* Linnaeus), different screening tests proved 17 out of 100 bacterial isolates to be biosurfactant producers in three and more independent experiments. Emulsification index evaluation being the first screening method to identify the surfactant producers. The values of emulsification index (E-24), ranged from 0% to 44% on 48 or 72 hr of incubation. It was also observed that the emulsions remained stable for about 1-2 months. The next text was drop -collapse method where surface activity of varying intensity was observed in 15 isolates. Two isolates identified as belonging to *Pseudomonas* spp. (strains MA67 and MA94) were scored as strong positive (+++), the next six again found to be belonging to *Pseudomonas* spp. (strains MA68, MA88, 69PP, 53T, 56T,and 75T) were marked as good surface active (++), another four of the 17 isolates, also *Pseudomonas* spp. (strains 38T, 43T, 47T, AND 122T) showed weak activity (+).

Strains identified as, *Acinetobacter* sp. MA72, Pseudomonas spp. (64PP) and *Sphingomonas* sp. 46T too showed very weak activity (w), while isolates identified as *Aeromonas* sp. 1T and *Sphingomonas* sp. 40T did not show any activity (-). Looking across the tests it was pointed that Pseudomonas strain 53T showed to be a good surfactant producer in most tests performed except the emulsification index (E24) that was seen to be 0%. Thus indicating that the surfactant from such strains are not as good of an emulsifying agent. As for oil spread method the cell free supernatant of each of the 17 bacterial 'active' isolates created a clear zone of varying diameters implying surface tension activity. Blue halos were produced by most of isolates on CTAB agar proving the anionic nature of the surfactants produced.

Both of the above papers aimed at understanding biosurfactant producing bacteria from a novel source such as fish intestine. The relevance is in understanding the interaction of the bacterial flora of the gut and its ecological role as with the host and among themselves. Such analysis of different fish species from different ecological niches thus can lay down answers to certain questions and bring to light as to whether or not such bioactive compounds may be a biological mechanism for survival.

This particular study deals with the isolation and characterisation of marine microorganisms in particular, fish gut micro-flora as a novel source for biosurfactant production. Biosurfactants are known to have potential anti-microbial, anti-tumor properties and more. The complexity of the gut micro-biome due to the diverse microorganisms and their interactions among themselves as well as with the environment thus makes it an interesting area for research. The marine waters are more often than ever polluted with oil in forms of household dumping, car leaks from land to water, industries, factories, ship leaks, ship wrecks and during oil extractions . It may not be visible as a real-time issue but the fish especially the ones that feed on remnants suspended in water face

grievous consequences. These then have very adverse effects over time and needs attention. The objectives of this study include 1) Isolation and partial characterisation of the diversity of the bacteria present in fish gut , 2) Screening the bacterial isolates for commercially important enzymes, 3) Screening and characterisation of bacteria for degradation of oil.

Chapter 02.

Isolation and partial characterisation of the bacterial diversity from fish gut

2.1 Methods and Materials

2.1.1 Sample collection:

Two types of fresh fish was collected from the fish market, Margao, Salcette-Goa. The fish was shipped in from Karwar beach.

Sample 1 : Indian Mackerel fish (*Rastrelliger kanagurta*)

Sample 2: Grey Mullet (*Mugil cephalus*)

Samples were collected in the months of July and August respectively i.e. during the monsoon season. The samples were collected in autoclaved bags with enough ice for transport. The samples were brought in the morning and immediately sampled for the gut tissue in sterile conditions.

Under sterile conditions, the surface of the fish was swabbed with ethanol to surface sterilise it. With the help of sterilised scissors the fish was cut open on the ventral side, starting from the upside then through the length making sure not to tear off the inner tissue. Then the whole gut was carefully removed and placed on a sterile plate. Only the intestine was kept of it and the rest discarded safely.

2.1.2 Total Viable Count :

The gut tissue was placed in a sterilised mortar and ground well with a pestle to get a uniform tissue sample. One gram of the tissue was then weighed in a sterile, pre-weighed 2ml eppendorf tube. This 1 gm was added to sterile 9ml of 0.1% Saline Peptone water. Serial dilutions were made by transferring one aliquot from previous

tube to the next 9 ml 0f saline peptone water, up to 10^{-5} dilutions. 0.1ml of each of the dilutions 10^3, 10^4, 10^5 were then spread plated on Zobell Marine Agar and R2A plates, in triplicates and incubated at room temperature for 24 hours.

2.1.3 Total Pathogen count:

2.1.3.1 Most Probable Number:

Preliminary test was carried out. For this sterilised, 5 tubes of, 10 ml each, double strength MacConkeys broth and 10 tubes of, 10 ml each, single strength MacConkeys broth was prepared and inverted Durham's tube was added to the tubes. Three sets were thus made, wherein first 5 tubes with double strength medium 10ml of 10^{-1} dilution of the sample was added. Next, to 5 tubes of single strength broth 1ml of 10^{-2} dilution of the sample was added and in the last set of 5 tubes of single strength broth, 0.1 ml of 10^{-3} dilution of the sample was added. Incubated at 37℃ for 24 hours. Observation based on change in colour, from purple to yellow, were noted. The Durham's tubes were checked for gas bubble. For the Completed test, O.1ml aliquot from each of the sets was then spread plated on EMB agar plates. Colonies showing green metallic sheen on the plates confirm the test for *E.coli.*

2.1.3.2 TVC on TCBS and SS agar:

0.1ml of 10^{-1} dilution was spread plated on pathogen specific media I.e. Thiosulphate Citrate Bile salt Sucrose agar and *Salmonella Shigella* agar, duplicates were maintained and incubated at room temperature. On TCBS agar, yellow and green colonies and on SS agar colourless and black centered colonies, with respect to the pathogens were checked for. Colony characteristics and total viable count as CFU/ml were the noted.

2.1.4 Glycerol Stock Preparation:

Each of the unique isolates were carefully picked up and purified on ZMA plates. To purify, the culture was streaked and subcultured 4 times to ensure the presence of the single desired isolate. The isolates were labelled as, Mac (1 - 19) representing isolates from Mackerel fish gut sample and GM (1-49) representing isolates from Grey Mullet fish gut sample .

These were then used to prepare glycerol stocks. To prepare the stock, 60% glycerol was made by adding 60 ml of glycerol to 40 ml of distilled water. This was the sterilised. In 2ml eppendorf tubes 400µL of Zobell Marine Broth of put and to this 600µL of 60% glycerol was added. Purified cultures from the agar were inoculated scrapped and inoculated in the 30% glycerol stock which was then mixed well. The eppendorfs tubes were labelled as per the given isolate name and carefully taped to avoid smudging. Each isolate were maintained in duplicates. These were then stored in the freezer at -3℃ and -20℃ for future use.

2.2 Results and discussion:

2.2.1 Isolation of Fish Gut Microorganisms

Total viable count (TVC) of bacterial colonies was averaged and recorded as colony forming units per ml. A total of 19 different bacterial isolates from Mackerel sample and 46 different bacterial isolates from Grey Mullet, were isolated, purified and glycerol stocks were prepared. The isolates were labelled as, for Mackerel sample- **Mac** (**1-19**) and for Grey Mullet- (**1-46**) **GM.**

Table 1 :

Sample	Total Viable Count (CFU/ml)	
	ZMA	R2A
Mackerel	1243×10^4	1533×10^4
Grey Mullet	2000×10^3	636×10^3

2.2.2. Isolation on Pathogenic Specific Media:

A total viable count was recorded as colony forming units per ml. Each of the different colonies were then recognised and the table following records the colony characteristics of the colonies. On purifying the isolates, it remained unculturable and hence further tests couldn't be carried out.

Table 2: Mackerel Sample

Colony character	**Salmonella- Shigella agar**		
Shape	circular	circular	circular
Size	7mm	8mm	5mm
Elevation	flat	flat	convex
Consistency	butyrous	butyrous	mucoid
Colour	Yellow (ringed)	Yellow with black centre	Pale yellow

Table 3: Mackerel Sample

Colony character	**Thiosulphate Citrate Bile salt Sucrose agar**	
Shape	circular	circular
Size	4mm	3mm
Elevation	flat	flat
Consistency	butyrous	butyrous
Colour	yellow	green

Table 4: Grey Mullet

Colony character	**Salmonella Shigella agar**		
Shape	circular	circular	circular
Size	3mm	5mm	6mm
Elevation	flat	Raised	flat
Consistency	butyrous	butyrous	butyrous
Colour	Black centred	yellow	peach

Table 5 : Grey Mullet

Colony character	**Thiosulphate Citrate Bile salt Sucrose agar**	
Shape	circular	circular
Size	2mm	2mm
Elevation	Raised	raised
Consistency	butyrous	butyrous
Colour	yellow	green

2.2.3. Most Probable Number:

Presumptive and Completed test was carried out to confirm the number of coliforms and its presence. For the presumptive test the results confirmed with McCrady's table for MPN are as follows, the most probable number of organisms per 100ml of Mackerel sample was 49 cells and of Grey Mullet sample was more than 1800 cells. As for the completed test, on plating each of the different dilutions on Eosin Methylene Blue agar, matt growth of green metallic sheen colonies interspersed with pinpoint purple colonies indicated high presence of *E.coli* hence the completed test was found to be positive.

Table 6:

Media Volume	10ml (2X strength)	10 ml (1X strength)	10ml (1X strength)	**MPN index/100ml (95% confidence**
Sample Volume	10 ml	1 ml	0.1 ml	
Dilution factor	10^0	10^1	10^2	

Sample **Number of tubes** **positive**				**interval)**
Mackerel sample	5	2	-	49
Grey Mullet Sample	5	5	5	>1800

Figure 1: Diversity on Zobell Marine Agar (dilutions- 10^3, 10^4 , 10^5)

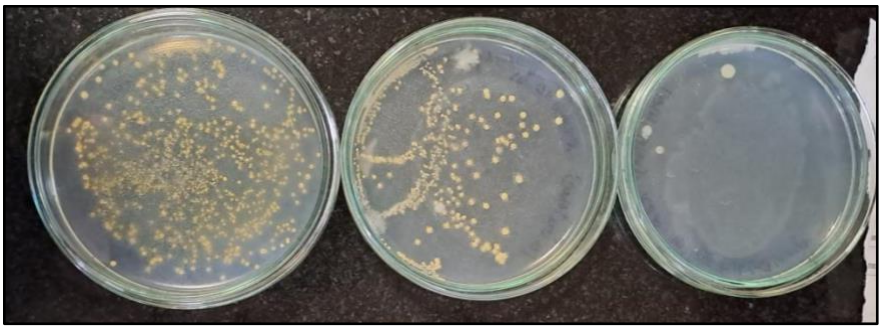


Figure 2: Diversity on R2A Agar (dilutions - 10^3, 10^4, 10^5)

Figure 3 : Purification of bacterial isolates on Zobell Marine Agar media

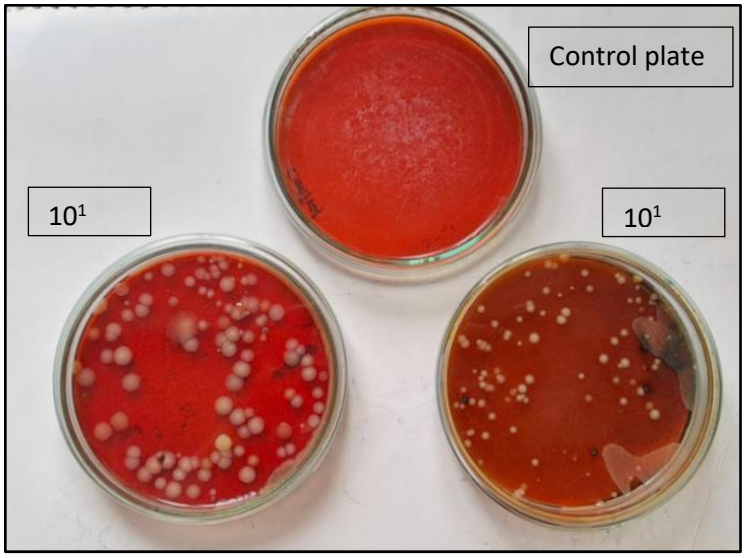


Figure 4 : Diversity on Salmonella Shigella agar

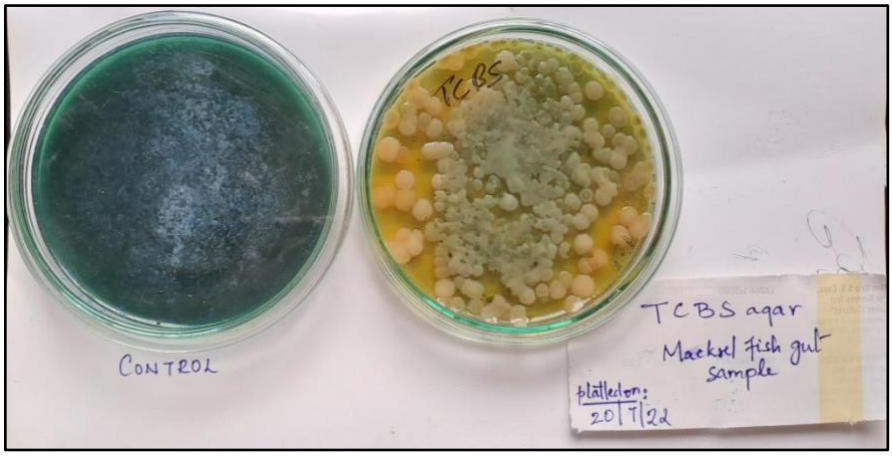


Figure 5: Diversity on Thiosulphate Citrate Bile Sucrose Agar

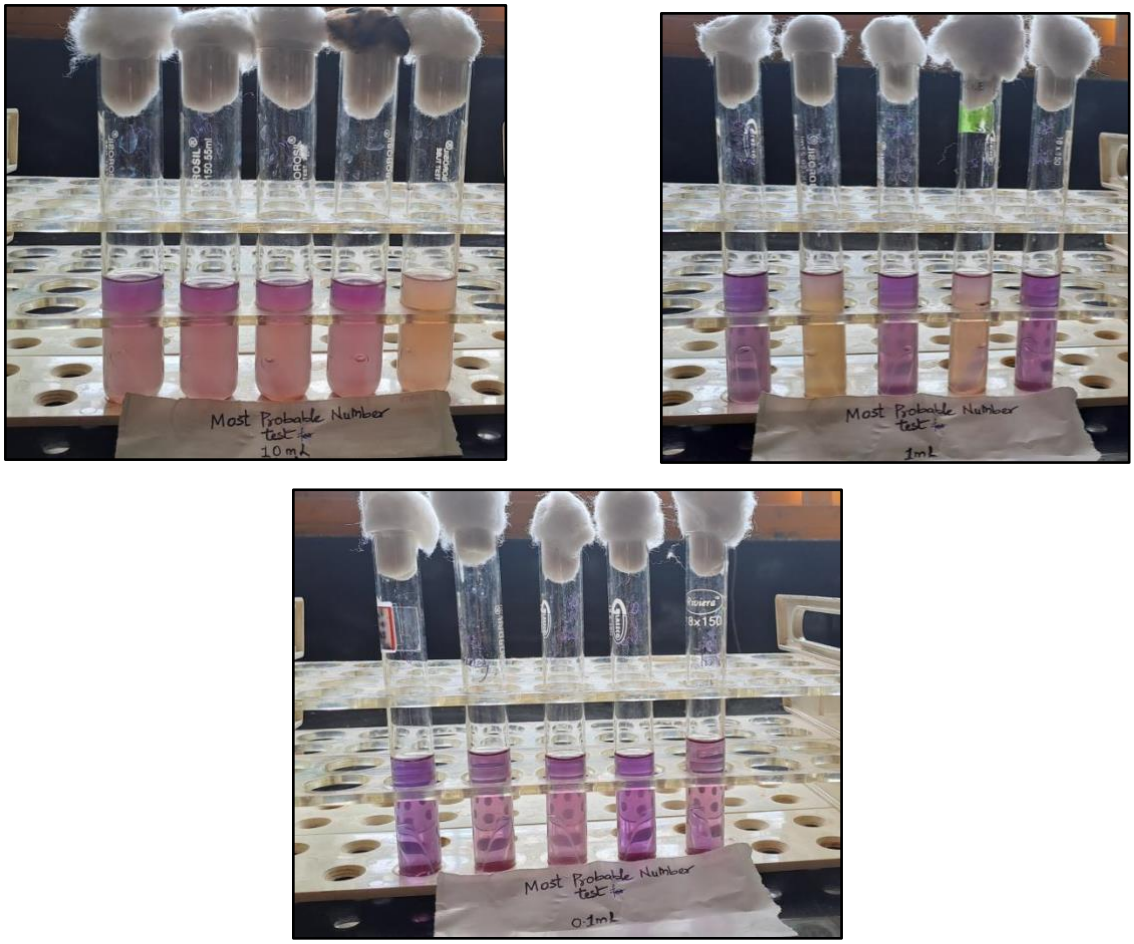


Figure 6: Most Probable Number - Presumptive test

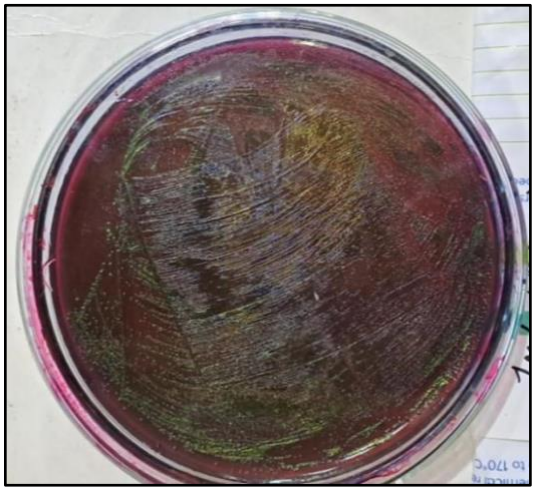


Figure 7 : Most Probable Number - Completed test

Chapter 03

Screening the bacterial isolates for commercially important enzymes

3.1 Methods and Materials:

3.1.1 Protease activity

24 hour old cultures were spot inoculated on saturated skim milk agar with ZMA being the base medium. Incubation of plates were done at 37℃ for 24-48 hours. Colonies with a halo of clearance were checked for to confirm protease activity.

3.1.2 Lipase activity

Tween 80 as a source of lipid with ZMA being the basal medium was prepared. 24 hour old cultures were spot inoculated. The plates were incubated at 37℃ for 24-48 hours. White precipitate around the colony were looked for to confirm lipase activity.

3.1.3 Esterase Activity

Tween 20 as a source of lipid with ZMA being the basal medium was prepared. 24 hour old cultures were spot inoculated. The plates were incubated at 37℃ for 24-48 hours. White precipitate around the colony were looked for to confirm esterase activity.

3.2 Results and Discussion :

On screening all the isolates from both the samples, Mac 2, Mac 16, 10GM and 22GM, out of which 10GM and 22GM portrayed the best protease, lipase and esterase activity making them potential producers of lipo-protease enzymes can be thus used in detergent, leather, baking and pharmaceutical industries. While among Mac isolates, 4 of the isolates showed , exclusively, protease activity and 3 isolates were lipase and esterase positive. In GM isolates, 4 of the isolates were positive for protease activity, 3

isolates were exclusively esterase producers and 6 of them being both lipase and esterase activity. Overall, Mac 11, Mac 12 and 42GM isolates had the best lipase-esterase activity, these isolates can be good candidates with reference to food, cosmetic and pharmaceutical industries.

Table 7: Mackerel Sample

Culture Media	Skimmed milk agar	Tween 20	Tween 80
Mac 2	+++	++	++
Mac 7	-	++	++
Mac 11	-	++++	++++
Mac 12	-	++++	++++
Mac 13	++	-	-
Mac 14	++	-	-
Mac 16	++	+++	+++
Mac 17	+++	-	-
Mac 18	++	-	-

Key: Weakly positive (++)

Moderately positive (+++)

Strongly positive (++++)

Negative (-)

Table 8: Grey Mullet Sample

Culture Media	Skimmed milk agar	Tween 20	Tween 80
1 GM	-	++	-
3 GM	-	+++	-
7 GM	-	+++	-
10 GM	++++	++++	++++
17 GM	++	-	-
18 GM	-	+	+
19 GM	-	+	+
22 GM	++++	+++	+++
24 GM	++	-	-
26 GM	-	++	+
27 GM	-	++	+
34 GM	+++	-	-

39 GM	++	-	-
42 GM	-	+++	+++
44 GM	-	++	++

Key: Weakly positive (++)

Moderately positive (+++)

Strongly positive (++++)

Negative (-)

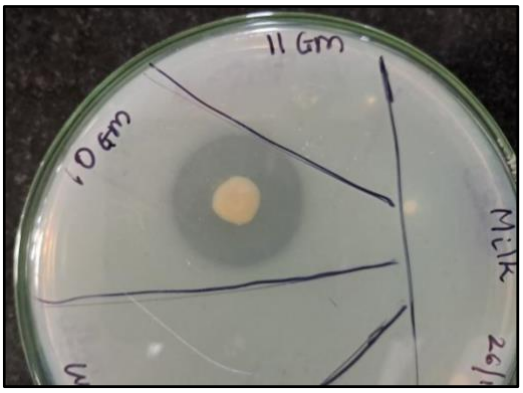


Figure 8: Protease activity on Skimmed milk Agar

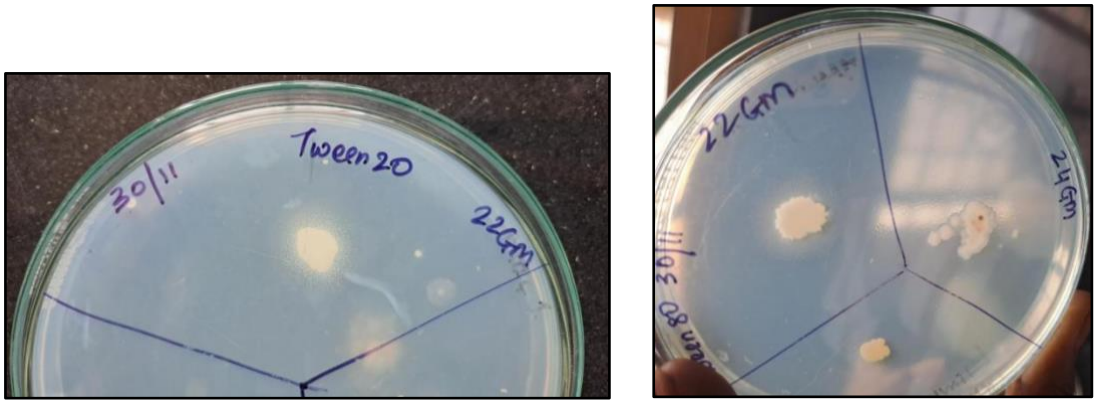


Figure 9 : Lipase and Esterase activity on Tween 80 and Tween20 Agar media respectively

Chapter 04

To evaluate the ability of fish gut based bacteria in degradation of oil

4.1 Methods and Materials

4.1.1 Hydrocarbon Overlay method

Nutrient agar plates were coated with 100 µL of burnt engine oil and the plates was spot inoculated with overnight grown cultures and the plates were then incubated at 37℃ for 24 hours. Emulsified halo around the colony were considered positive for biosurfactant activity. The positive cultures were then used for further screening.

4.1.2 Emulsification index

The emulsification index (E24) of the biosurfactant was determined by adding to a test tube 2ml of burnt engine oil and 2ml of cell free supernatant. The tube was then vortexed for 2 minutes and allowed to stand for 24 hours at room temperature. Following is the formula applied to determine the E24 index :

E24 index(%) = height of emulsified layer(mm) / total height of the liquid column (mm) x 100

4.1.3 Drop Collapse method:

To clean grease free slide 20 µL of burnt engine oil was placed and this was kept in the incubator at 37℃ for 1 hour to equilibrate the oil. To this 25 µL of cell free supernatant was added . SDS was used as a positive control and sterile nutrient broth used as negative control. A collapse in the drop, placed on the oil, was observed for 1 minute to confirm biosurfactant production.

4.1.4 Oil spread method

50ml of distilled water was added to the bottom of a clean petriplate and 180µL of burnt engine oil was placed such that it makes a thin layer over the water. Over the oil, 160µL of cell free supernatant was added. Occurrence of clear zone indicated positive results. SDS was used as a positive control and distilled water as a negative control.

4.1.5 2,6-DICHLOROPHENOLINDOPHENOL (DCPIP) ASSAY

Cultures inoculated in nutrient broth and incubated on shaker at 100rpm for 24 hours at room temperature. Of this broth 2 ml was taken in eppendorf tube and centrifuged at 1000 rpm for 10 minutes at 4℃. The supernatant was carefully discarded and pellet washed with 0.9% saline and then suspended in 0.9% saline and vortexed. To another 2ml eppendorf tube 750 µL of half strength nutrient broth was added followed by the addition of 100 µL of 2,6-DCPIP solution. Next, 200 µL cell suspension followed by 10 µL burnt engine oil was pipetted. These tubes were then placed horizontal for ease of mixing on a shaker at 100 rpm at room temperature for 24 hours. The tubes were checked for de-colourisation.

4.1.6 SIGMUND WAGNER TEST

Cetyl Trimethyl Ammonium Bromide (CTAB) Agar method

Cultures were spot inoculated in CTAB agar media, containing half strength Nutrient agar as basal medium, CTAB as the cationic surfactant, burnt engine oil acting as a carbon source and methylene blue as an indicator. This was then incubated at 37℃ for 24-48 hours. Occurrence of blue coloured colonies and blue halo are considered positive for the test.

4.1.7 GRAVIMETRIC ANALYSIS - QUANTITATIVE ANALYSIS OF OIL DEGRADATION:

Cultures were grown in 50ml half strength Nutrient broth, supplemented with 5 grams of burnt engine oil and incubated on shaker at 100 rpm at 37℃ for 48 hours. Bacterial activity was then stopped on addition of 1% 1N HCl. 50ml of the culture was mixed with 20ml of petroleum ether:acetone in 1:1 ratio, as a non-polar solvent, in a separating funnel. The contents of it were mixed well in order to get an emulsified layer. In a clean beaker(pre-weighed), the topmost layer I.e. oil extract was collected. For the non-polar solvent to evaporate leaving behind just the residual oil, the beaker was placed in the oven at 60℃. With this the gravimetric value was calculated using the following formula. Same procedure was followed for the consortium study.

Percentage of oil degraded = weight of oil degraded/ original weight of oil x 100

Note : Weight of oil degraded= original weight of oil - weight of the residual oil obtained after evaporating the solvent

4.1.8 COMPATIBILITY TEST:

To carry out the gravimetric analysis of oil degradation using a consortium of the best isolates, the isolates were tested for their compatibility with each other. This was done by streaking the test isolate as a vertical line in the centre of the nutrient agar plate and the other isolates were then streaked perpendicular to the test isolate. To maintain duplicates, the isolates were streaked across on either side of the test isolate. The plates were incubated at 37℃ for 24 hours. In case of antagonism, a zone of inhibition around the test culture will be visible such cultures are not taken into consideration. Isolates that grew well with other isolates were chosen for the consortium study.

4.1.9 GRAVIMETRIC ANALYSIS - QUANTITATIVE ANALYSIS OF OIL DEGRADATION: (Consortium)

Consortium of the best oil degrading isolates were grown in 50ml half strength Nutrient broth, supplemented with 5 grams of burnt engine oil and incubated on shaker at 100 rpm at 37℃ for 48 hours. Bacterial activity was then stopped on addition of 1% 1N HCl. 50ml of the culture was mixed with 20ml of petroleum ether:acetone in 1:1 ratio, as a non-polar solvent, in a separating funnel. This was then mixed vigorously to get a single emulsified layer. The contents of it were mixed well in order to get an emulsified layer. In a clean beaker(pre-weighed), the topmost layer I.e. oil extact was collected. For the non-polar solvent to evaporate leaving behind just the residual oil, the beaker was placed in the oven at 60℃. With this the gravimetric value was calculated using the following formula.

Percentage of oil degraded = weight of oil degraded/ original weight of oil x 100

Note : Weight of oil degraded= original weight of oil - weight of the residual oil obtained after evaporating the solvent

4.1.10 Bacterial adhesion to hydrocarbon (BATH) assay

2ml of the cell suspension was taken in a test tube previously grown in half strength nutrient broth. The initial optical density at 600nm was noted. To the suspension 100µL of burnt engine oil was added and vortexed for 3 minutes. The emulsion was then allowed to stand and separate out for an hour. Optical density of the lower aqueous layer at 600nm after 1 hour of separation was then noted. Following formula was applied to calculate the hydrophobicity percentage of adherence to the oil.

Hydrophobicity percentage of adherence to the oil = {1 - O.D. (aqueous)/O.D.(initial)} x 100

Note : O.D. (aqueous)= optical density of the aqueous phase

O. D. (initial)= optical density of the initial cell suspension

4.2 Results and Discussion

4.2.1 Hydrocarbon Overlay Method:

All the purified cultures from both the samples were subjected to hydrocarbon overlay method, as a qualitative test. Occurrence of emulsified halos around the colony indicated positive for biosurfactant production. Thus 14 isolates that were positive as seen in table 15, from both the samples were then subjected for further biosurfactant screening.

Table 9:

Culture	Result
Mac 7	+++
Mac 9	+++
Mac 10	++
Mac 12	++
Mac 15	++
Mac 17	++++
Mac 18	++
24 GM	++++

27 GM	+++
32 GM	++
33 GM	++++
38 GM	++++
43 GM	++++
44 GM	++++

Figure 10: Hydrocarbon Overlay Method

4.2.2 Emulsification Index:

14 best isolates were tested for its potential emulsification index. A clear emulsified layer at the interface was visible. The index was calculated using a formula wherein,

the E24 is related to the surfactant concentration, thus confirming it ability to emulsify oil. The resulting indices ranged from 0% to 56% with Mac 9 showing the highest emulsification index of 56% among other isolates.

Table 10:

Culture	Height of emulsified layer (mm)	Total height (mm)	E24 (%)
Mac 7	-	25	0%
Mac 9	14	25	56%
Mac 10	-	22	0%
Mac 12	-	20	0%
Mac 15	5	20	25%
Mac 17	5	22	22.7%
Mac 18	5	20	25%
24 GM	10	22	45.45%
27 GM	6	30	24 %
32 GM	10	23	43.4%
33 GM	5	22	22.7%
38 GM	2	22	9%
43 GM	-	20	0%

44 GM	6	20	30 %

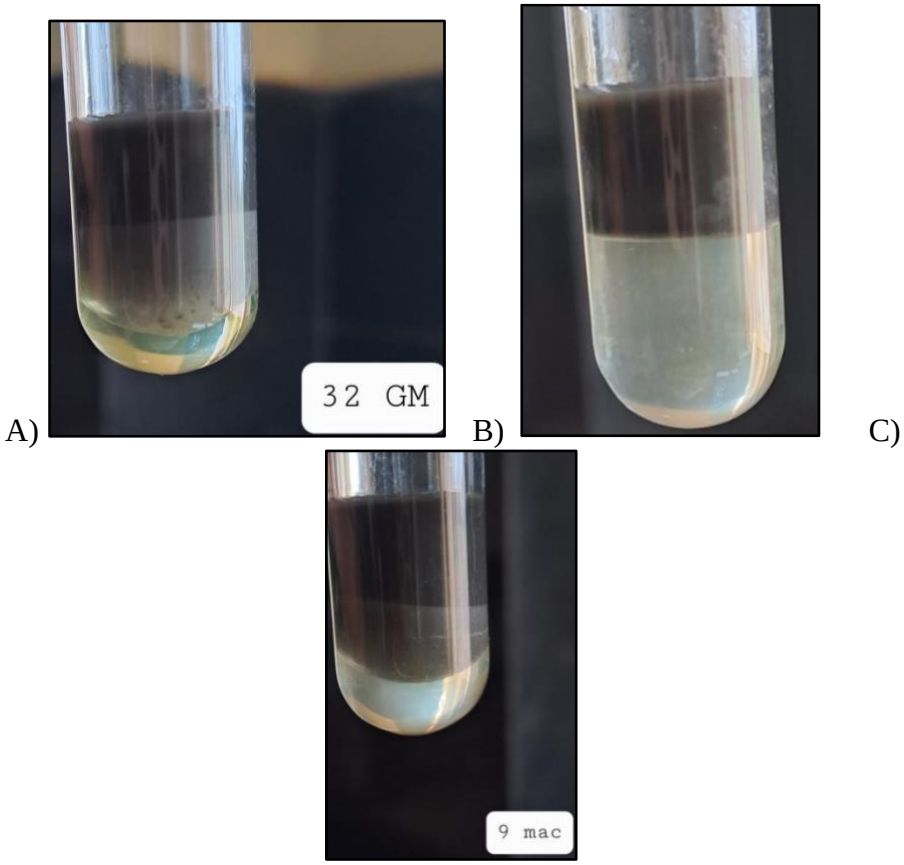


Figure 11: Emulsification Index- A) Isolate 32GM, B) Negative Control, C) Isolate Mac 9

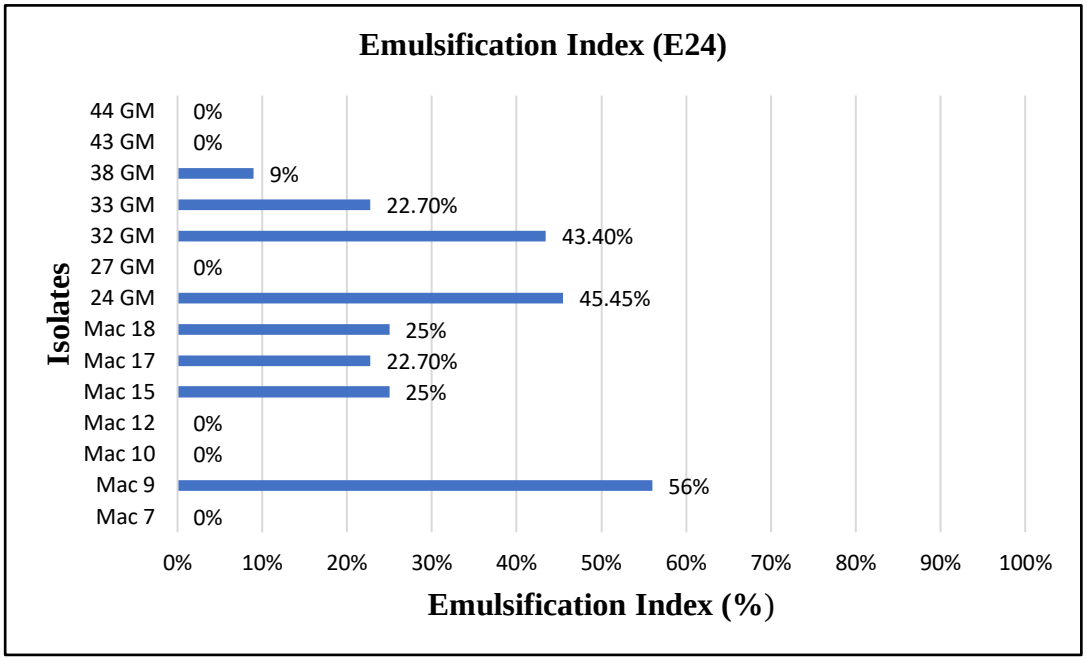


Figure 12 : Emulsification Index (%)

4.2.3 Drop Collapse Method:

This test is based on the property of destabilisation of the oil-water interface by the biosurfactants produced. All the cultures were found to be positive under 2 minutes of application. except mac7 & mac 12.

Table 11:

Culture	Result
Mac 7	-
Mac 9	+
Mac 10	+
Mac 12	-
Mac 15	+

Mac 17	+
Mac 18	+
24 GM	+
27 GM	+
32 GM	+
33 GM	+
38 GM	+
43 GM	+
44 GM	+

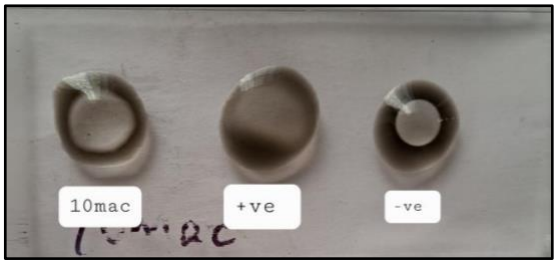


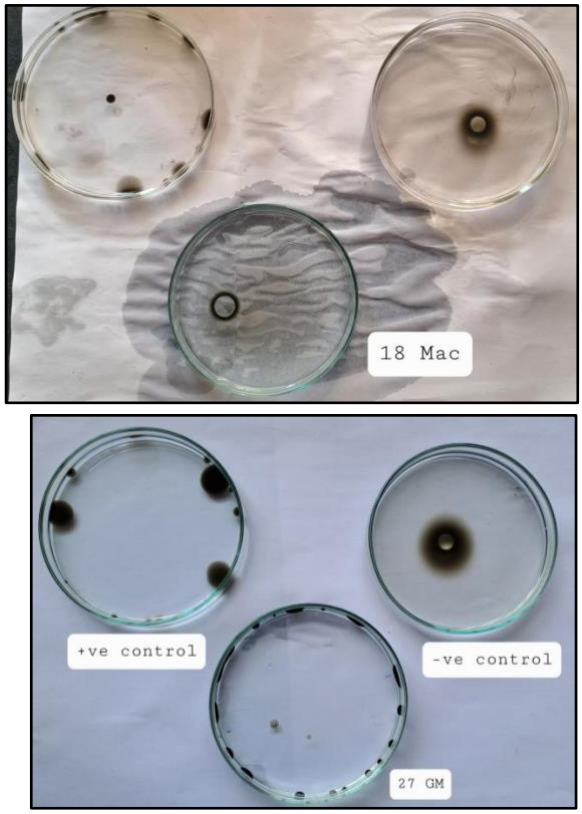
Figure 13: Drop Collapse Method

4.2.4 <u>Oil Spread Method:</u>

This qualitative test checks for the oil displacement that is directly related to the surface active compound in the supernatant. 12 isolates out of 14 were seen to have a clear zone when the cell free supernatant was place over the thin oil film over the water, as compared to the control. Isolates 24 GM,27 GM, 44 GM proved to show the best results on excess addition of the cell free supernatant, just like the positive control I.e. SDS.

Table 12:

Culture	Result
Mac 7	-
Mac 9	+
Mac 10	+
Mac 12	-
Mac 15	+
Mac 17	+
Mac 18	+
24 GM	+
27 GM	+
32 GM	+
33 GM	+
38 GM	+
43 GM	+
44 GM	+

A) 

B)

Figure 14: Oil Spread Method- A) Isolate Mac 18, B) Isolate 27 GM

4.2.5 2,6- DICHLOROPHENOLINDOPHENOL (DCPIP) ASSAY:

DCPIP plays the role of an electron acceptor where initially in its oxidised form it is blue in colour at pH 7.0 but when reduced completely it de-colourises. Thus in this test the ability of the isolate to degrade the burnt engine oil via the mechanism of biodegradation and consequent de-colourisation of the indicator I.e. DCPIP is observed.

All 14 cultures have shown varying degree of de-colourisation with Mac 10 completely reducing the indicator and 38 GM showing the next best de-colourisation of the reagent.

Table 13:

Culture	Result
Mac 7	++
Mac 9	++
Mac 10	++++
Mac 12	++
Mac 15	++
Mac 17	++
Mac 18	++
24 GM	++
27 GM	++
32 GM	++
33 GM	+++
38 GM	+++
43 GM	+++
44 GM	++

Key: Weakly de-colourised (++)

Moderately de-colourised (+++)

Completely de-colourised (++++)

Negative (-)

A) 

B) 

Figure 15: DCPIP assay- A) Isolate 38 GM and Negative control, B) Isolate Mac 10

4.2.6 CETYL TRIMETHYL AMMONIUM BROMIDE (CTAB) AGAR ASSAY

This is a semi-quantitative assay done to check the production of specific anionic biosurfactants by bacterial isolates. CTAB behaves as a cationic surfactant and the blue colour to the agar is imparted on the addition of methylene blue. The isolates were found to be fastidious due to their poor growth even after 48 - 72 hours of incubation at 37°C. Despite poor growth 9 out of 14 isolates showed positive results on the appearance of blue halos.

Table 14:

Culture	Result
Mac 7	+
Mac 9	-
Mac 10	+

Mac 12	+
Mac 15	+
Mac 17	-
Mac 18	+
24 GM	-
27 GM	+
32 GM	+
33 GM	-
38 GM	+
43 GM	-
44 GM	-

Key: Blue Colonies (+)

No growth (-)

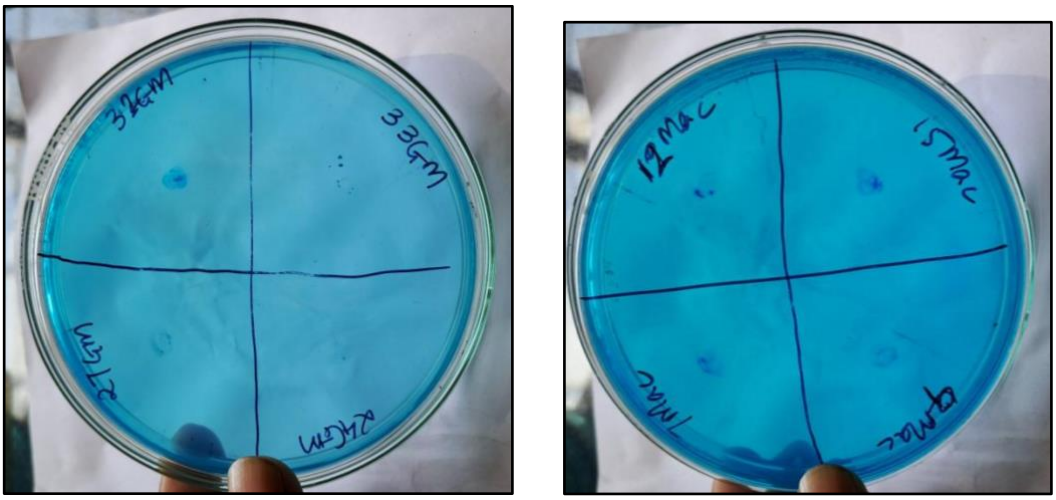


Figure 16: Cetyltrimethylammoniumbromide (CTAB) Agar

4.2.7 GRAVIMETRIC ANALYSIS - QUANTITATIVE ANALYSIS OF OIL DEGRADATION:

The isolates demonstrated the ability to degrade oil at different rates From the 14 isolates, four isolates (Mac 15, Mac 18, 32GM and 38GM) were found to have degraded higher percentage of oil than the rest.

Table 15:

Culture	Weight of beaker containing the extract (g)	Weight of empty beaker (g)	Weight of oil added (g)	Weight of residual oil (g)	Amount of oil degraded (g)	Percentage of oil degradation (%)
Mac 7	22.6	17.68	5	4.92	0.08	1.6%
Mac 9	22.41	17.68		4.73	0.27	5.4%
Mac 10	37.17	32.4		4.77	0.23	4.6%
Mac 12	21.2	16.5		4.62	0.38	7.6%
Mac 15	37.4	32.4		4.2	0.8	16%
Mac 17	22.6	17.62		4.98	0.02	0.4%

Mac 18	36.43	33.01		3.42	1.58	31.6%
24 GM	21.16	16.58		4.58	0.42	8.4%
27 GM	22.7	17.68		4.58	0.42	8.4%
32 GM	34.8	30.51		4.29	0.71	14.2%
33 GM	21.69	17.04		4.65	0.35	7%
38 GM	21.9	17.62		4.28	0.72	14.4%
43 GM	35.1	30.15		4.95	0.05	1%
44 GM	22.1	17.16		4.94	0.06	1.2%

The four best isolate I.e. Mac 15, Mac 18 , 32 GM and 38 GM showed 16%, 31.6%, 14.2% and 14.4% of oil degradation. These four isolates were then subjected to compatibility test for consortium, BATH assay and gravimetric analysis of degraded oil by consortium. Further biochemical tests, colony characteristics and microscopy of each of the four isolates were carried out for identification.

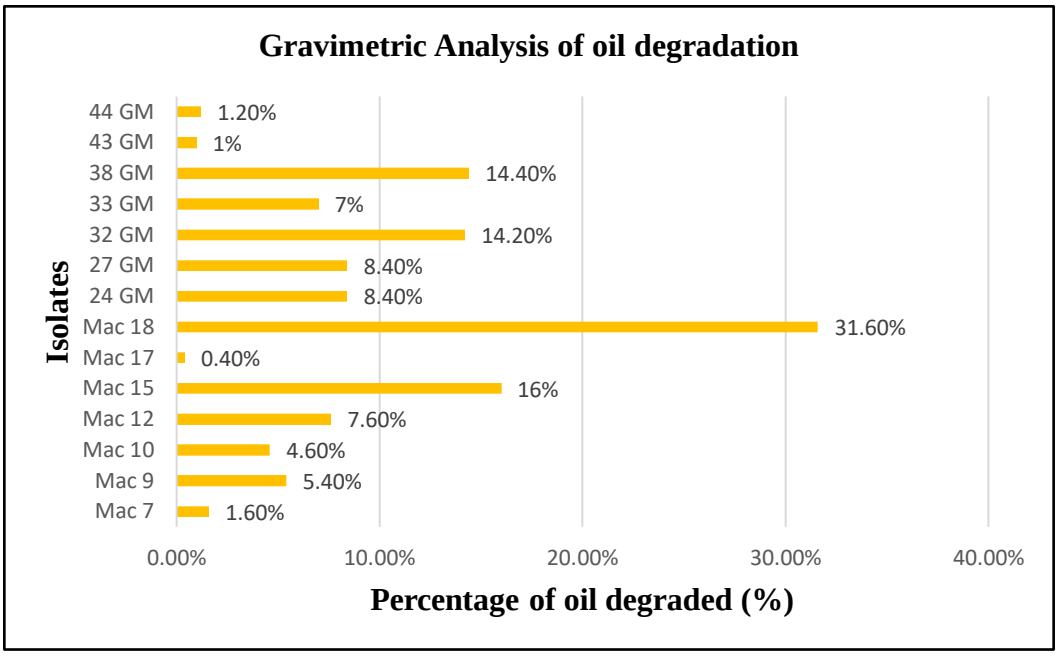


Figure 17: Percentage of oil degraded(%) by selected bacterial isolates

4.2.8 COMPATIBILITY TEST:

The four isolates were thus found to be very compatible with each other and hence gravimerty analysis of oil degradation with the consortium was carried out.

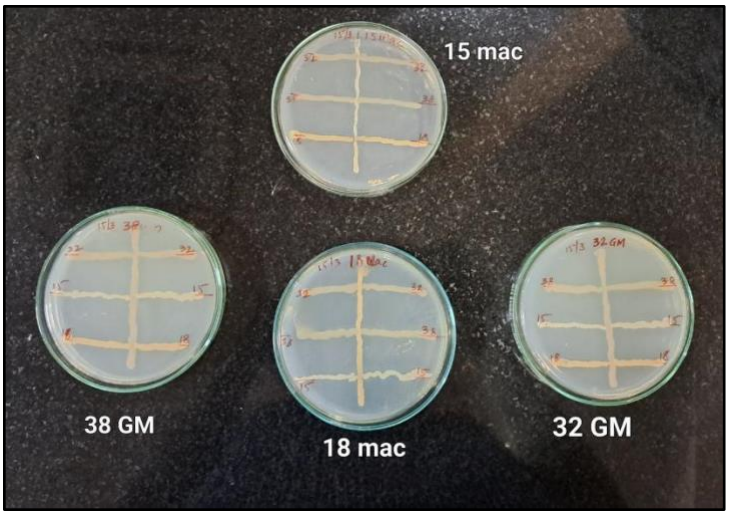


Figure 18: Compatibility test

4.2.9 GRAVIMETRIC ANALYSIS - QUANTITATIVE ANALYSIS OF OIL DEGRADATION: (Consortium)

Oil degradation by the consortium was not found to be very significant as compared to the individual isolates. This may be due to the increase in competition for oil despite being compatible and therefore cancelling out the higher potential efficiency of the consortia.

Table 16:

Culture	Weight of beaker containing the extract (g)	Weight of empty beaker (g)	Weight of oil added (g)	Weight of residual oil (g)	Amount of oil degraded (g)	Percentage of oil degradation (%)
Consortium	21.31	17.62	5	3.7	1.3	26%

Figure 19: Estimation of quantity of oil by gravimetric method

4.2.10 ANALYSIS OF CELL SURFACE HYDROPHOBICITY:

Bacterial adhesion to hydrocarbon assay (BATH)

The BATH method is a photometric analysis used for measuring the hydrophobicity of the bacterial cell. A decrease in the optical density of the aqueous phase is seen, on separation of the phases as the cells adhere to the burnt engine oil droplets and remain in the non-polar phase thus indicating the hydrophobicity of the cells. Among the four best isolates, maximum hydrophobicity was seen in 38 GM with a hydrophobicity percentage of 12%.

Table 17:

Culture	O.D. of the initial cell suspension	O.D. of the aqueous phase	Hydrophobicity percentage (%)
Mac 15	1.64	1.53	6.8%
Mac 18	1.82	1.71	6.1%
32 GM	1.47	1.3	11.6%
38 GM	1.26	1.11	12%

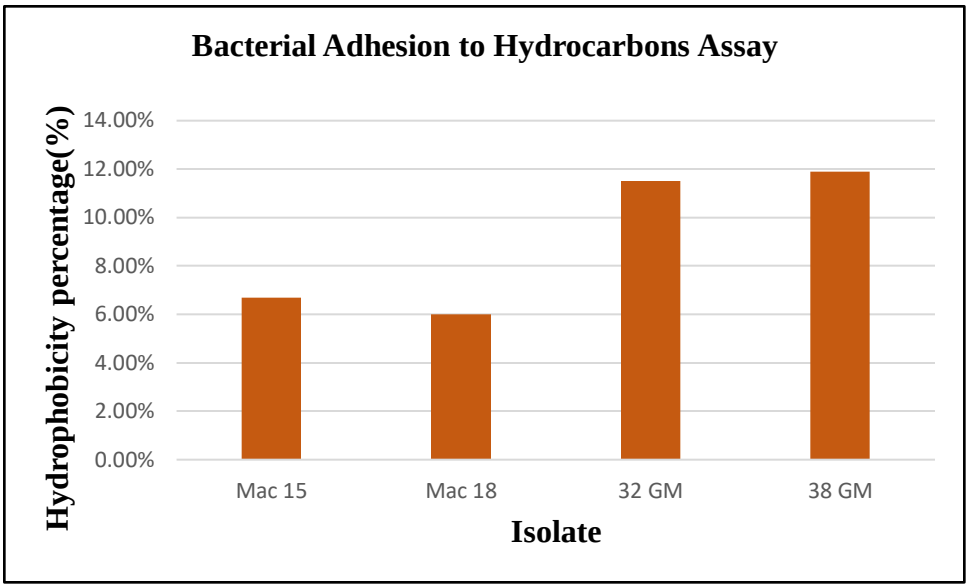


Figure 20: Hydrophobicity Percentage (%) of the four best isolates

Chapter 05

IDENTIFICATION OF THE OIL DEGRADING ISOLATES :

5.1 Methods and Materials

5.1.1 BIOCHEMICAL TESTS:

Identification of pure cultures was done by performing tests like IMViC, catalase test, nitrate reduction test, Hugh Leifson's test for aerobic and anaerobic test, motility test to check if organisms are motile.

A) Indole test:

Test tubes with 5ml aliquot of indole broth were prepared. Autoclaved and allowed to cool. The tubes were lightly inoculated with a 24 hour old pure culture. The tubes were then incubated at 37℃ for 24 hours. To this then, 10-12 drops of Kovacs reagent was added to the broth and stirred. Results were noted within 3-5 minutes. Appearance of a red ring at the top of the broth indicates positive result, if not it's a negative result.

B) Methyl red and Voges Proskauer

Glucose phosphate water was prepared and five ml was dispensed in the tubes. The tubes were then incubated at 37℃ for 24 hours. After incubation the broth was divided in two parts. In first part 1-2 drops of methyl red was added and colour change was observed and noted. In second part, O'Meara's reagent was added and tubes incubated for 2 hours at 37℃ and colour change was observed.

C) Citrate utilisation test:

Simmons Citrate agar was prepared and poured in tubes to make slants. The tubes were autoclaved and inoculated with overnight grown culture. Incubation was carried out at 37℃ for 24 hours. Blue colouration was observed for a positive test.

D) Hugh Leifson's test (aerobic and anaerobic growth conditions) :

Tubes of the medium were inoculated by stabbing the agar. Hugh Leifson's media of one tube was layered with sterile paraffin oil as deep as 5mm to 10mm. The tubes were incubated at 37℃ for 24 hours.

E) Nitrate reduction test:

A loopful of 24 hour old culture was inoculated in Nitrate Peptone water and incubated 37℃ for 24 hours. After incubation few drops of Sulfanilic acid and alpha-Napthylamine was added. Tubes were checked for red colouration. In the absence of red colouration, zinc dust was added, to confirm the presence of unreduced nitrate, on appearance of red colour.

F) Catalase test:

A drop of H2O2 was placed on a clean slide, with the help of a sterile nichrome loop the culture was placed in the drop. Bubbling or effervescence of the drop confirmed a positive test.

G) Motility test:

Five ml of soft nutrient agar was prepared in test tubes, autoclaved and cooled. The media was then stabbed to inoculate with a 24 hour old culture. The tubes were then incubated at 37℃ for 24 hours. Results were then noted.

H) Fermentation of sugars:

Loopful of 24 hour old culture inoculated into the fermentation base with the respective sugar. The tubes were then incubated at 37℃ for 24 hours. Acid production indicated by yellow colouration of the broth and gas production indicated by gas bubble in Durham's tube.

Sugar analysed: Glucose, Sucrose, Lactose and Maltose

5.1.2 Microscopy: Gram staining

The bacterial isolates giving the best results for oil degradation were subjected to Gram staining to determine their Gram character.

On a clean grease free slide a drop of normal saline was placed. With a sterile loop, the surface of the colony of the isolate was lightly touched to obtain few cells. This was than thoroughly mixed with the saline on the slide in order to prepare a thin smear. The slides were then allowed to air dry and then were heat fixed. This smear was then stained, first by flooding with crystal violet letting it sit for 1 minute. The slides were then washed under a gentle stream of water followed by addition of Grams iodine and keeping it for 1 minute and then draining off the iodine. The slides were then subjected to de-staining solution I.e. 95% ethanol for 30 seconds. Lastly, a counter stain, safranine was added to the slides and left for 30 seconds. This was then washed off under a gentle stream of water. The slides were then allowed to air dry. A drop of immersion oil was placed on the slides and were then observed under 100x.

5.2 Results and Discussion

5.2.1 BIOCHEMICAL CHARACTERISTICS

Table 18:

Test **Culture**	**Mac 15**	**Mac 18**	**32 GM**	**38 GM**
Indole	-	-	-	-
Methyl Red	++++	++	-	-
Voges Proskauer	-	-	-	-
Citrate	-	-	+	+
Catalase	+	+	+	+
Urease	+	+	-	-
Hugh-Leifson's aerobic	oxidative	oxidative	oxidative	oxidative
Hugh-Leifson's anaerobic	fermentative	fermentative	inert	fermentative
Nitrate reduction	++ No gas	++++ No gas	+++ Gas	- (Red on adding

				Zn)
Motility	Non-motile	Non-motile	Non-motile	Non-motile
Gram character	Gram positive cocci	Gram positive cocci	Gram positive rods	Gram positive rods

5.2.2 Fermentation of sugars:

Table 19:

Sugar Culture	Mac 15	Mac 18	32 GM	38 GM
Glucose	++++	++++	-	+++
Sucrose	++	++++	-	-
Lactose	+	+++	-	-
Maltose	++++	++++	+	+

The identification of the four best oil degrading isolates were carried by performing various biochemical tests and microscopy. This was then compared with the Bergery's Manual of Determinative Bacteriology for determination up to genus level, tentatively. Mac 15 and Mac 18 were Gram positive cocci and thus belong to the group 17 on Bergey's Manual. On testing for catalase activity it was noted to be positive and hence following the Gram Positive cocci flowchart, it indicated to be belonging either to *Staphylococcus* spp. Or *Micrococcus* spp. Since the Mac 15 colony is non-pigmented it can be said it belongs to *Staphylococcus* spp. And Mac 18 being lightly pigmented as creamy yellow and since it tested positive for glucose fermentation it could be *Micrococcus varians*.

Isolates 32 GM and 38 GM were found to be Gram positive rods, regular and non-sporulating and thus belong to Group 19 on Bergey's Manual. The isolates were catalase positive and thus could belong to *Corynebacterium* spp. as per Gram positive rods flowchart.

5.2.3 Colony Characteristics:

Following is a table of colony characteristics of the isolates as observed on Zobell Marine Agar

Table 20:

	Mac 15	Mac 18	32 GM	38 GM
Shape	circular	circular	circular	irregular
Size	1mm	1mm	1mm	1mm
Colour	white	Off-white	Pale brown	brown

Margin	entire	entire	entire	undulated
Elevation	convex	mbonate	flat	flat
Opacity	opaque	opaque	translucent	translucent
Consistency	butyrous	butyrous	butyrous	butyrous
Texture	dry	dry	dry	dry
Gram Character	Gram positive cocci	Gram positive cocci	Gram positive Short rods	Gram positive Short rods
Motility	Non-motile	Non-motile	Non-motile	Non-motile

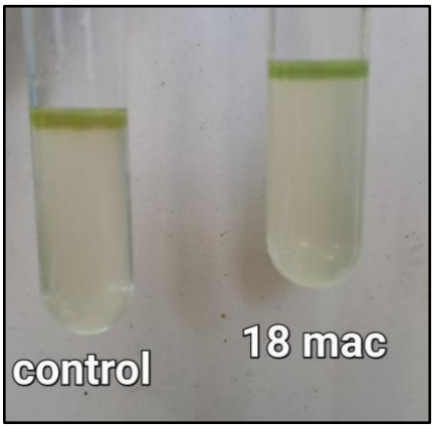


Figure 21: Indole test

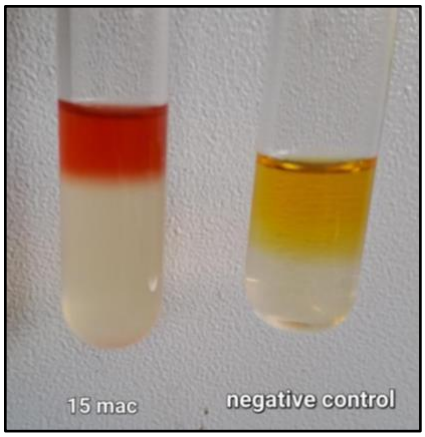


Figure 22: Methyl Red test

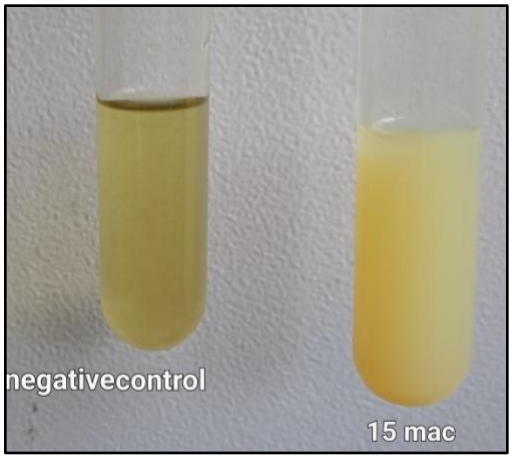


Figure 23: Voges Prausker test

Figure 24: Citrate test

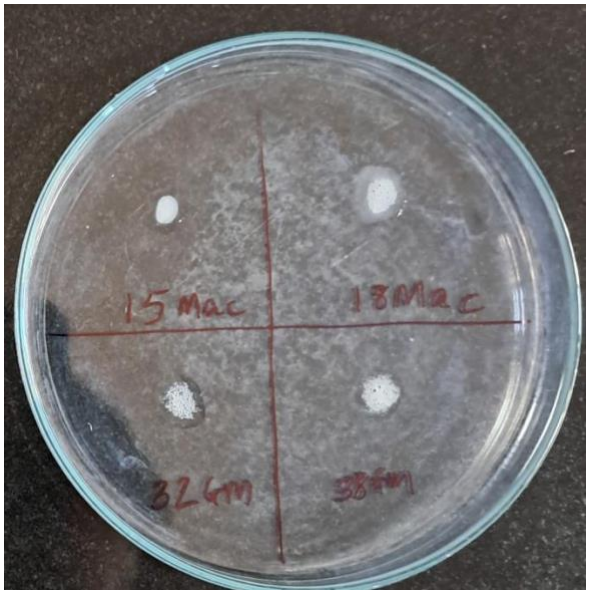


Figure 25: Catalase test

Figure 26: Urease test

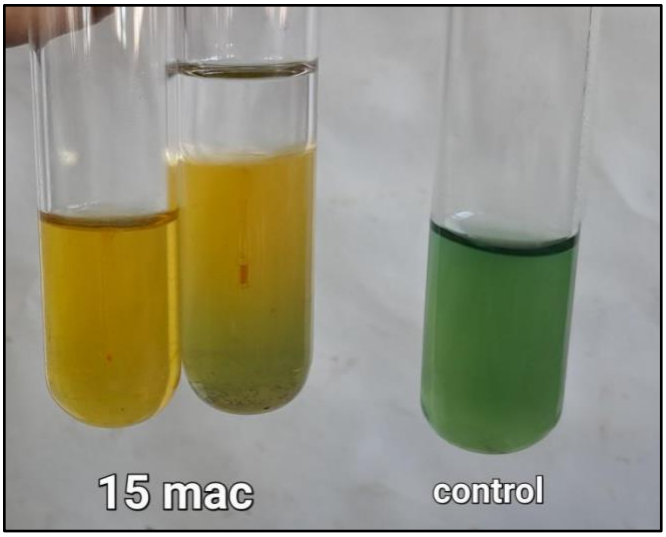


Figure 27: Hugh Leifsons (aerobic and anaerobic)

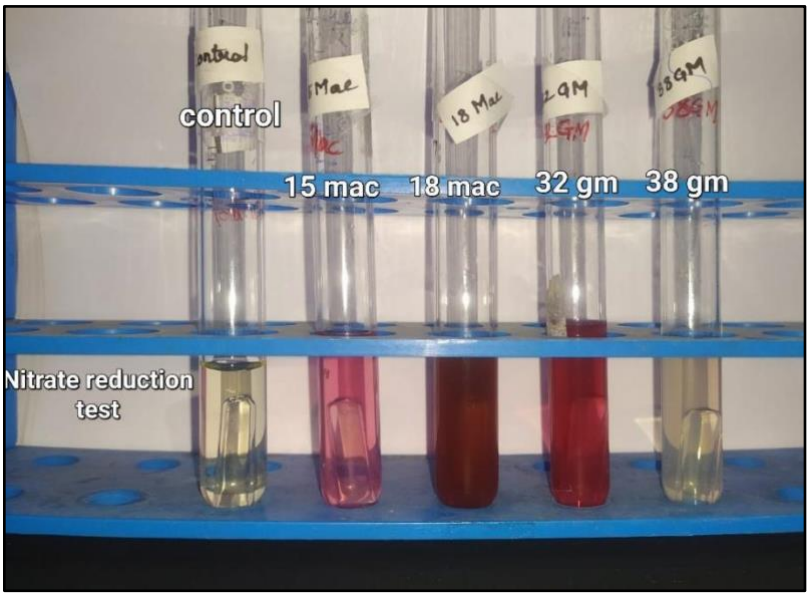


Figure 28: Nitrate reduction

Figure 29: Motility test

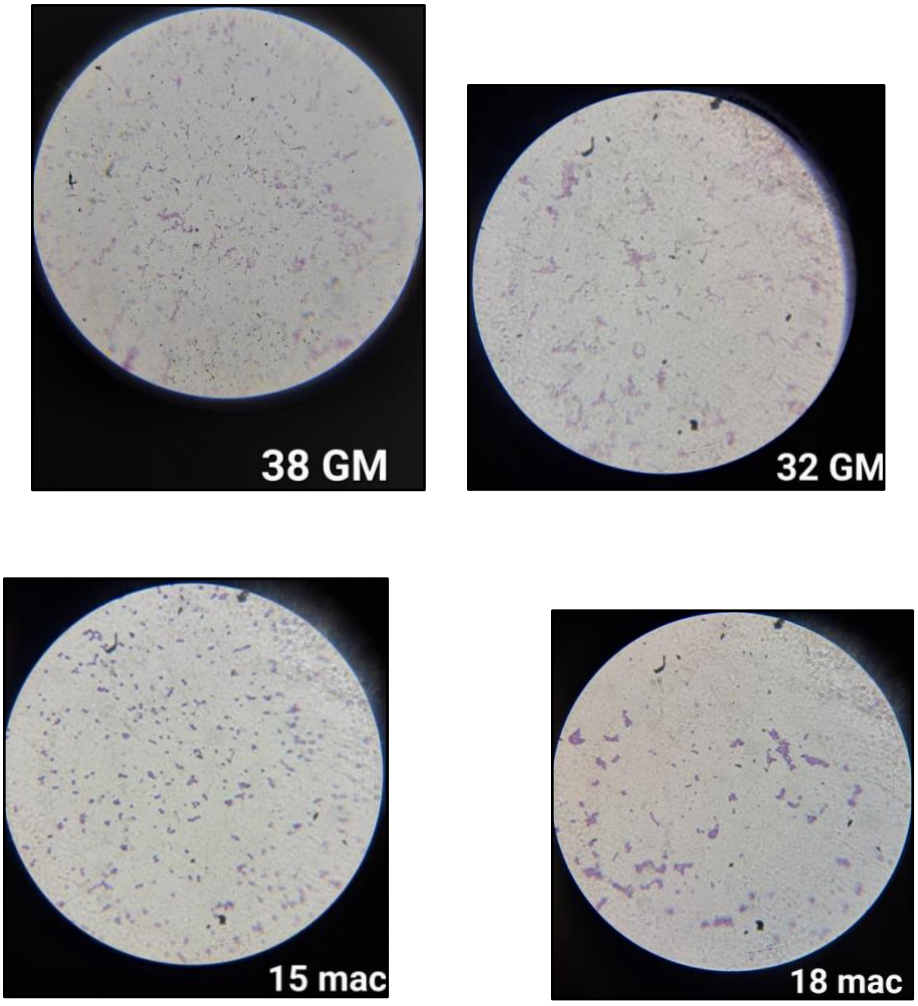


Figure 30: Microscopy

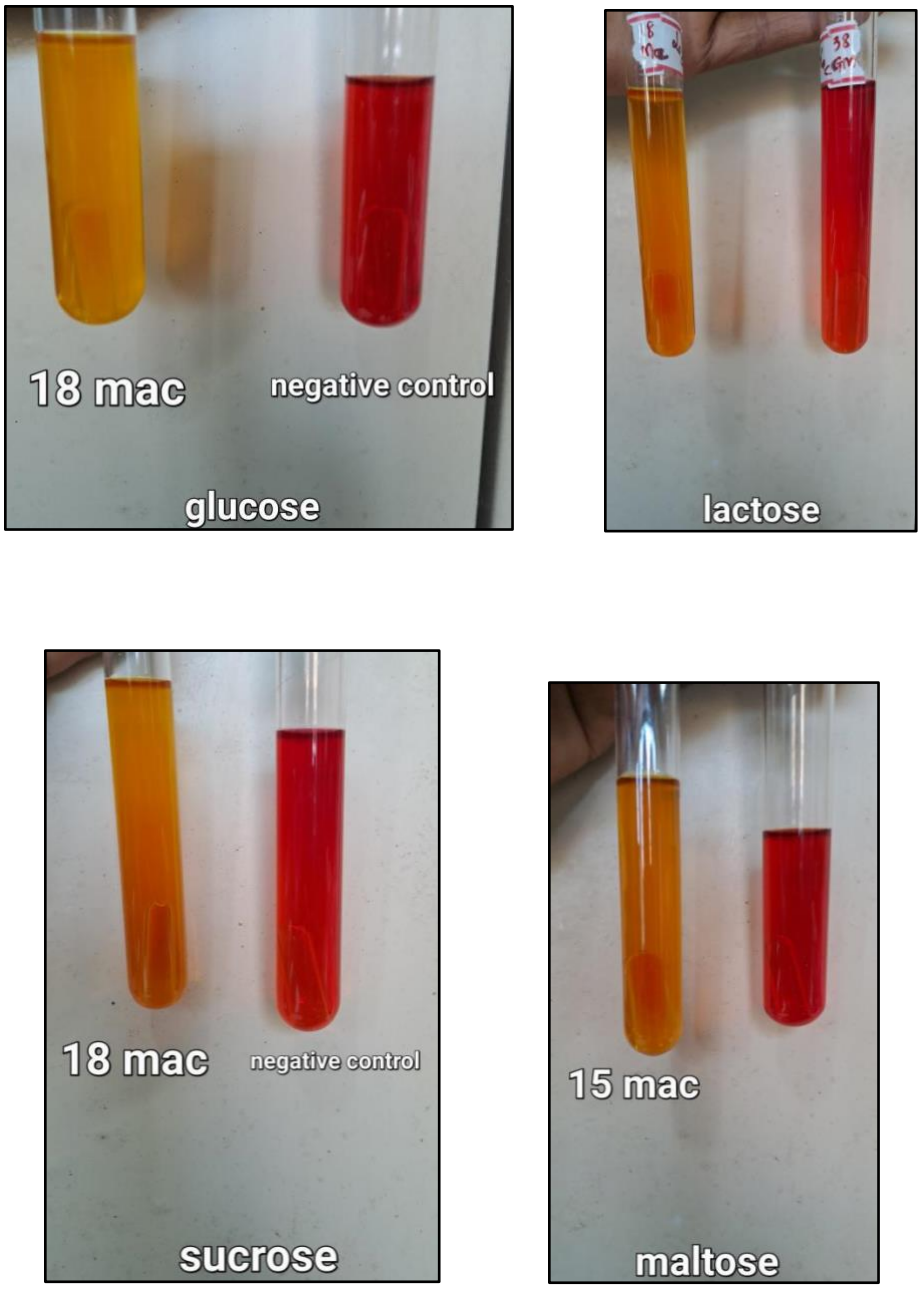


Figure 31: Sugar Fermentation

SUMMARY

In this study, bacteria from fish gut was isolated and screened for biosurfactant producers. It was also screened for three commercially important enzymes namely protease, lipase and esterase. Tentative identification of the best oil degrading bacteria was carried out by performing biochemical tests and microscopy.

Indian Mackerel (*Rastrelliger kanagurta*) and Grey Mullet (*Mugil cephalus*) were the two kinds of fish selected as samples for the study. Both were chosen for there ,contrasting yet relevant to the matter of interest , habits in the water column. Mackerel is found in shallow coastal waters and feed on planktons while, Grey Mullet is found at the shore as well as in the benthic zone of the water column where it avails and feeds on benthic detritus and sediment. Thus covering a broader range of water column and the feeding habits of the fish.

Total viable count (TVC) on ZMA (nutrient rich) and R2A (nutrient deficient media) and pathogen count was carried out for each. The TVC in Mackerel sample for ZMA was found to be 1243 x 10^4 CFU/ml and for R2A was found to be 1533 x 10^4 CFU/ml. TVC in Grey Mullet sample for ZMA was found to be 2000 x 10^3 CFU/ml and for R2A was found to be 636 x 10^3 CFU/ml. Followed by enumeration of coliforms by carrying out MPN- presumptive and completed test. In the presumptive test most probable number of organisms per 100ml of Mackerel sample was 49 cells and of Grey Mullet sample was more than 1800 cells and for the completed test on plating 0.1 ml aliquot from the MPN tubes on EMB agar gave a matt of green metallic sheen colonies and purple colonies thus confirming the presence of E.coli. Two other pathogen specific media namely TCBS and SS agar were utilised for partial identification of pathogens like *Vibrio* spp. on TCBS agar and *Salmonella* sp., *Shigella* sp. on SS agar.

Colony characteristics of typical *Vibrio* spp. colonies and *Salmonella* sp. and *Shigella* sp. were noted down.

Screening of commercially important enzymes namely, protease, lipase and esterase on Skimmed milk agar, Tween 80 agar and Tween 20 agar respectively was done. Among Mac isolates, 4 of the isolates showed , exclusively, protease activity and 3 isolates were lipase and esterase positive. Among GM isolates, 4 of the isolates were positive for protease activity, 3 isolates were exclusively esterase producers and 6 of them being both lipase and esterase activity. Overall, Mac 11,

Mac 12 and 42GM isolates had the best lipse-esterase activity, these isolates can be good candidates with reference to food, cosmetic and pharmaceutical industries. 10GM and 22GM portrayed the best protease, lipase and esterase activity making them potential producers of lipo-protease enzymes.

Further Mac isolates and GM isolates were screened and characterised for oil degradation. Hydrocarbon overlay method was used to select the best of the isolates. Thus 14 isolates that tested positive for the test were then subjected to other tests that checked for biosurfactant production. Emulsification capacity, where the biomolecules produced can efficiently emulsify two immiscible liquids but are known to have lesser surface tension reduction capacity (Uzoigwe et al, 2015), is tested by calculating its Emulsification Index (E24). The resulting indices ranged from 0% to 56% with Mac 9 showing the highest emulsification index of 56%, among other isolates. Drop collapse assay, a sensitive test where with a little amount of cell free supernatant the destabilisation of the oil-water interface was observed for about a minute. All the cultures were found to be positive under 2 minutes of application except Mac 7 and Mac 12. The next qualitative test that held evidence for biosurfactant production by the

isolates was oil spreading assay, this is done to check the displacement of the oil layer by the CFS which is directly related to the surface-acitve compound in the supernatant.12 isolates out of 14 were seen to have a clear zone when the cell free supernatant was place over the thin oil film over the water, as compared to the control. Isolates 24 GM,27 GM, 44 GM proved to show the best results on excess addition of the cell free supernatant .DCPIP plays the role of an electron acceptor where initially in its oxidised form it is blue in colour at pH 7.0 but when reduced completely it decolourises. Thus in 2,6- dichlorophenolindophenol (dcpip) assay, the ability of the isolate to degrade the burnt engine oil via the mehanism of biodegradation and consequent decolourisation of the indicator I.e. DCPIP, is observed. All 14 cultures have shown varying degree of decolourisation with Mac 10 completely reducing the indicator and 38 GM showing the next best decolourisation of the reagent.

Cetyltrimethylammonium bromide (CTAB) agar assay is a semi-quantitative assay done to check the production of specific anionic biosurfactants by bacterial isolates. CTAB behaves as a cationic surfactant and the blue colour to the agar is imparted on the addition of methylene blue. 9 out of 14 isolates showed positive results on the appearance of blue halos. The isolates displayed poor growth in such minimal growth medium, the same was observed when they were inoculated for quantitative analysis of oil degradation where oil is the only carbon source. The minimal medias tried out were Mineral Salt Medium 9 and Bushnell Hass agar/broth, which had poor growth and O.D. at 600nm was noted as 0.1 even on four days of incubation, thus the following gravimetric analysis for oil degradation was done by inoculating in half strength nutrient broth medium supplemented with burnt engine oil.

The isolates demonstrated the ability to degrade oil at different rates. From the 14 isolates, four isolates (Mac 15, Mac 18, 32GM and 38GM) were found to have

degraded higher percentage of oil than the rest, on subjecting them to gravimetric analysis of oil degradation.The four best isolate I.e. Mac 15, Mac 18 , 32 GM and 38 GM showed 16%, 31.6%, 14.2% and 14.4% of oil degradation. These four isolates were then subjected to compatibility test for consortium. Percentage of oil degradation was calculated as 26% for the consortium. Oil degradation by the consortium was not found to be very significant as compared to the individual isolates. This may be due to the increase in competition for oil despite being compatible and therefore cancelling out the higher potential efficiency of the consortia.

BATH method is a photometric analysis used for measuring the hydrophobicity of the bacterial cell. Separation of the two phases, the hydrophobic cells were seen to become bound to burnt engine oil droplets and rise with it. The turbidity of the aqueous phase was measured. The decrease in turbidity of the aqueous phase indicated the hydrophobicity of the cells to the burnt engine oil. The hydrophobicity percentage (%) was calculated. The maximum hydrophobicity was seen in 38 GM with a hydrophobicity percentage of 12%.

Biochemical characterisation and microscopy of these four isolates was carried out for tentative identification of the bacterial isolates. Mac 15 colony is non-pigmented it can be said it belongs to *Staphylococcus* spp. And Mac 18 being lightly pigmented as creamy yellow and since it tested positive for glucose fermentation it could be *Micrococcus varians*. 32 GM and 38 GM isolates were catalase positive and thus could belong to *Corynebacterium* spp. as per Gram positive rods flowchart.

CONCLUSION

This study in its minimal capacity for analysis preliminary tests, screening tests were carried out, wherein the fish gut was checked for, as a potential source of biosurfactant producing bacteria. The tests were seen to have been backing up the fact that the selected isolates did indeed naturally produce surface active compounds as biosurfactants or bioemulsifiers.

Among the four best isolates based on gravimetric analysis, Mac 18 tentatively identified as belonging to *Micrococcus* spp. proved to have degraded the highest percentage of burnt engine oil compared to the rest. A consortium of the four didn't show significant increase in oil degradation even though they were tested positive for compatibility. The nutrient optimisation of the media needs more study and further tests to enhance its potential as a consortium. Another alternate could be testing different combinations of the four isolates to see the best results. Other factors such as pH, temperature also need to be optimised.

Isolates producing biosurfactants and other enzymes like protease, lipase and esterase can be great candidates as probiotics in aquaculture or other such ecosystems. Biosurfactant producers would immensely boost fish health as it confers properties such as anti-microbial, anti-inflammatory among others. These can also attract as an MEOR applicant, with in-depth studies carried out (Giri, S., et al. 2020)

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APPENDEX

Appendix 1 (Media)

1.1) Zobell Marine agar 2216 (HiMedia):

Ingredients	Gms / Litre
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004

Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.000
Final pH	7.6±0.

1.2) R-2A agar (HiMedia)

Ingredients	**gm / L**
Casein hydrolysate	0.500
Yeast extract	0.500
Proteose peptone	0.500
Glucose	0.500
Starch	0.500
Dipotassium hydrogen phosphate	0.300
Magnesium sulphate anhydrous	0.024
Sodium pyruvate	0.300
Agar	15.000

Final pH (at 25°C)	7.2±0.2

1.3) EMB (HiMedia)

Ingredients	**Gms / Litre**
Peptone	10.000
Dipotassium hydrogen phosphate	2.000
Lactose	5.000
Saccharose (Sucrose)	5.000
Eosin - Y	0.400
Methylene blue	0.065
Agar	13.500
Final pH (at 25°C)	7.2±0.2

1.4) MacConkey broth(HiMedia)

Ingredients	Gms / Litre
Gelatin peptone	20.000
Lactose monohydrate	10.000
Dehydrated bile	5.000
Bromo cresol purple	0.010
pH after sterilization (at 25°C)	7.3±0.2

1.5) SS agar (HiMedia)

Ingredients	Gms / Litre
Peptone	5.000
HM peptone B #	5.000
Lactose	10.000
Bile salts mixture	8.500
Sodium citrate	10.000

Sodium thiosulphate	8.500
Ferric citrate	1.000
Brilliant green	0.00033
Neutral red	0.025
Agar	15.000
Final pH (at 25°C)	7.0±0.2

1.6) TCBS (HiMedia)

Ingredients	**gm / L**
Proteose peptone	10.000
Yeast extract	5.000
Sodium thiosulphate	10.000
Sodium citrate	10.000
Bile	8.000
Sucrose	20.000
Sodium chloride	10.000

Ferric citrate	1.000
Bromo thymol blue	0.040
Thymol blue	0.040
Agar	15.000
Final pH (at 25°C)	8.6±0.2

1.7) Nutrient broth

Ingredients	g/L
Peptone	5
NaCl	5
Meat Extract	3
Distilled water	1000
pH	7.2

1.8) Simmons Citrate agar

Ingredients	g/L
Sodium Chloride (NaCl)	5.0
Sodium Citrate (dehydrate)	2.0
Ammonium Dihydrogen Phosphate	1.0
Dipotassium Phosphate	1.0
Magnesium Sulfate (heptahydrate)	0.2
Bromothymol Blue	0.08
Agar	15.0

1.9) Glucose peptone broth

Ingredients	g/L
Peptone	10
Glucose	10
Sodium Chloride	8.5
Final pH (at 25℃)	7±0.2

1.10) Hugh-Leifsons's Medium (HiMedia)

Ingredients	Gms / Litre
Peptone	2.000
Sodium chloride	5.000
Dipotassium hydrogen phosphate	0.300
Dextrose (Glucose)	10.000
Bromothymol blue	0.050

Agar	2.000
Final pH (at 25°C)	6.8±0.2

1.11) Nitrate Peptone water

Ingredients	**g/L**
Peptone	5
KNO_3	0.2
Distilled water	1000ml
pH	7.4

1.12) Glucose Phosphate Peptone water

Ingredients	**g/L**
(A)Peptone	2.5
K2HPO4	2.5
(B) Glucose	5

Distilled water	1000
pH	7.6

Dissolve the components of solution (A) in distilled water adjust the pH to 7.6. Sterilize this for 20 minutes at 121˚ C and pressure of 15 pounds per square inch by autoclaving. Dissolve the glucose in distilled water and autoclave separately. Mix the two solutions after sterilization.

1.13) Tryptone water

Ingredients	**g/L**
Tryptone	100
NaCl	5
Distilled water	1000
pH	7.2

1.14) 1%Skimmed milk agar

Ingredients	g/100ml
Whole skim milk	1ml
Yeast extract	0.25
Dextrose	0.1
Agar	2

1.15) Tween 20 Agar

Ingredients	g/L
Peptone	10
NaCl	5
$CaCl_2$ H_20	0.1
Tween 20	10ml
Agar	20
Distilled water	1000ml

1.16) Tween 80 Agar

Ingredients	**g/L**
Peptone	10
NaCl	5
$CaCl_2$ H_20	0.1
Tween 80	10ml
Agar	20
Distilled water	1000ml

1.17) Cetyl Trimethyl Ammonium Bromide (CTAB) agar

Ingredients	**g/250ml**
Peptone	1.25
Meat Extract	0.75

NaCl	1.25
Cetyltrimethylammonium Bromide	0.05
Burnt engine oil	0.5
Methylene blue	0.00125
Distilled water	250
pH	7.2

APPENDIX 2 (REAGENTS)

2.1) 0.5% Iodine solution

Igredients	g/100ml
KI	0.5
I2	0.05
Distilled water	100 mL

2.2) O'Meara's Reagent

Ingredients	g/100ml
KOH	4
Creatine	0.3
Distilled water	100

2.3) Kovac's Reagent

Ingredients	g/200ml

Amyl alcohol	150
P-dimethyl-amino benzaldehyde	10
Concentrated HCL	50ml

Dissolve aldehyde in alcohol and slowly add acid. Shake gently before use.

2.4) Methyl Red Solution

Ingredients	**g/500ml**
Methyl Red	0.1
95% ethanol	300
Distilled water	200

2.5) 2, 6- Dichlorophenolindophenol (DCPIP) solution

Ingredients	**µg/ml**
2, 6- DCPIP	37.5

Distilled water	1

2.6) 20 mM Sodium dodecyl sulfate (SDS) solution

Igredients	**mg/ml**
Sodium Dodecyl Sulphate	5.67
Distilled water	50

APPENDIX 3 (STAINS)

3.1) GRAM'S STAIN

(A) Crystal Violet stain

Ingredients	g/100 mL
(A)Crystal violet	2
Absolute alcohol	20
(B) (NH4)2C2O4	0.8
Distilled water	80

Solution (A) and (B) to be mixed and stored for 24 hours and then used.

(B) Grams Iodine

Ingredients	g/100ml
Iodine	0.3

KI	1
Distilled water	100

C) Safranine stain

Ingredients	**g/100ml**
Safranine	0.25
Ethanol	10
Distilled water	100

(D) Destaining solution

95% ethanol

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