Study on bacterial biosurfactants and characterization

A Dissertation Report

Submitted in partial fulfilment of Master's Degree in Biotechnology

By

RIYA MORESHWAR POTFODE

21P047015

Under the Supervision of

MS. SNESHA BHOMKAR

School of Biological Sciences and Biotechnology

GOA UNIVERSITY

Date: April 2023

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11/5/2023

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CERTIFICATE

This is to certify that the dissertation entitled "**Study on bacterial Biosurfactants and Characterization**" submitted by Ms. Riya Potfode is an authentic piece of work carried out in partial fulfilment of the requirement for the degree of Master of Science in Biotechnology, Goa University in the year 2022-2023, is according to the results obtained by her under my guidance and supervision.

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DECLARATION

I hereby declare that the work incorporated in her dissertation entitled "**Study on Bacterial Biosurfactants and their Characterization".** Submitted in partial fulfilment of the degree of Master of science in Biotechnology in original and carried out by me at the School of Biological Sciences and Biotechnology, Goa University.

It has not been submitted in part or full for any degree in this or any other university. 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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INDEX

LIST OF TABLES

LIST OF FIGURES

ABBREVIATION

Abbreviation	Full forms
CMC	Critical Micelle Concentration
BTEX	Benzene Toluene Ethylbenzene Xylene
MEOR	Microbial enhanced oil recovery
SDS	Sodium dodecyl sulphate
ONGC	Oil and Natural Gas Corporation
MSM	Mineral Salt Medium
BHB	Bushnell Haas Broth
NA	Nutrient Agar
ZMA	Zobell Marine Agar
E_{24}	Emulsification Index
OD	Optical Density
HCL	Hydrochloric Acid
H_2O_2	Hydrogen Peroxide
(v/v)	Volume by Volume
Rpm	Revolutions per minute
BATH	Bacterial Adhesion to Hydrocarbons
nm	Nanometer
µl	Micro liter
mg	Milligram
µg	Micro gram
g	Gram

INTRODUCTION

1.1 BIOSURFACTANT: NATURE AND CHARACTERISTICS

Biosurfactants are chemical substances made up of amphipathic molecules with hydrophilic and hydrophobic moieties that separate at physical interaction. They are typically non-toxic and biodegradable and have the properties of lowering surface tension, stabilizing emulsions, and inducing foaming (Nasr&Soudi,2009). The diversity, adaptability, and environmental friendliness of biosurfactants have recently increased interest in them over artificial surfactants. Microbes frequently adhere to surfaces and gather at interfaces. It is therefore not surprising that bacteria with a high surface-to-volume ratio produce a wide range of surface-active substances (Font & Gea, 2022).

The polar moieties can be cationic, anionic, non-ionic or amphoteric molecules, while the non-polar moieties are generally chains of hydrocarbons. They can produce microemulsions in which hydrocarbons can be dissipated by water or vice versa depending on the mixture of hydrophobic and hydrophilic moieties. The best approach to describe a surfactant is to measure its ability to alter surface and interfacial tensions by measuring the force of attraction between the molecules of liquids (Sarubbo & Silva, 2022).

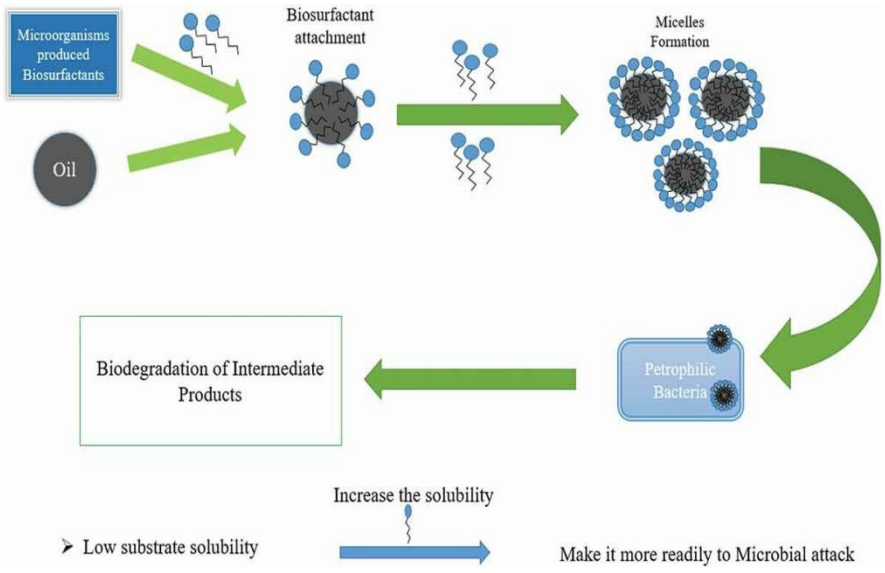


Fig. 1: Mechanisms of microbial degradation of the hydrophobic compound with the aid of biosurfactants (Bami & Estabragh, 2022)

The Critical micelle concentration (CMC) is the concentration of biosurfactants above which the micelles will form. When the biosurfactant molecules are at the surface at a low concentration they arrange themselves, as the concentration increases the surface tension decreases. At a point is the saturation, where the addition of more molecules leads to micelle formation. The critical micelle concentration can be found by using a tensiometer (Sobrinho & Luna, 2014).

Many environmental uses for biosurfactants exist, including the bioremediation and dispersion of oil spills, improved oil recovery, and the transfer of crude oil. The food, cosmetic, pharmaceutical, and agricultural industries are just a few other potential uses for biosurfactants. Some substances tend to be multifunctional agents as well: emulsifiers, wetting agents, antibacterial agents, and anti-adhesive agents (Banat & Makkar, 2000).

1.2 BIOSURFACTANTS IN BIOREMEDIATION

The existence of biosurfactants may lead to a possible improvement in the efficiency of biodegradation. According to this theory, biosurfactant molecules function as mediators, enhancing the mass transfer rate by increasing the bioavailability of hydrophobic contaminants to microorganisms (Kosaric, 2001).

In contrast, biosurfactants may also cause alterations in the characteristics of cellular membranes, increasing microbial adherence. When there are two immiscible phases (oil and water), and direct substrate uptake is possible, this method is significant (Ławniczak, 2013).

Because of their solubilization of hydrophobic compounds as well as environmental friendliness, biosurfactants are now acknowledged as effective agents for enhancing the bioremediation of contaminated environments (Kosaric, 2001).

Based on their capacity to complex heavy metal ions, biosurfactants have another important environmental application that may enhance the removal or extraction of these metals by biological treatment (Mishra & Lin, 2021).

However, factors like oxygen, pH, the presence of macro- and micronutrients, the physicochemical properties of the contaminant's history of pollution, and

particles to which the organisms and toxins may be adsorbed all affect how quickly these contaminants degrade. (R & Schinner F, 2001)

Through a variety of methods, biosurfactants can improve hydrocarbon bioremediation. By emulsifying molecules, the solubility is increased, making it susceptible to microbial attack (Bami & Estabragh, 2022). They can either interact with the cell surface to raise the cell surface hydrophobicity, allowing hydrophobic substrates to associate more readily with bacterial cells, or they can increase the substrate bioavailability for microorganisms. In the hydrophilic environment, biosurfactants with an amphiphilic structure build up in bulk quantities above the CMC and form micelles (Bami & Estabragh, 2022). Micelle creation is an equilibrium process, and micelles are thermodynamically stable structures. Hydrophobic contamination becomes diffused and soluble in the aqueous solvent as a result of surfactant hydrophobic groups contacting the aqueous phase in micelle formations. However, a micelle can speed up the pace at which a compound is absorbed by microbial cells. So, this allows for the creation of micelles to make it easier to transfer hydrophobic contaminants while giving bacterial cells more access (Jahan, Bodratti, Tsianou, & Alexandridis, 2020).

1.3 TYPES OF BIOSURFACTANT

1.3.1 Glycolipid

The most popular and accessible class of biosurfactants is glycolipids. Glycolipids, which are composed of monosaccharides, disaccharides, and polysaccharides, are lipids that are covalently bound to a carbohydrate. Rhamnolipids, Sophorolipids, and Trehalolipids are three of the most well-known glycolipids among them (Drakontis & Amin, 2020).

1. Rhamnolipid

Carbohydrate moiety in rhamnolipid is made of rhamnose bonded to β-hydroxy fatty acids. Rhamnolipids, a subclass of glycolipids, have been recognized as being among the greatest bacterial surfactants because of their superior physicochemical characteristics. (Drakontis & Amin, 2020) They are useful for direct application to many different industries due to their outstanding surface

and biological activity. These are mostly made by *Pseudomona*s among other microbes (Busi & Rajkumari, 2017).

2. Trehalolipid

Trehalose bonded to long chain α-branched-β-hydroxy fatty acid has high structural diversity, and is mainly produced by *Rhodococcus, Mycobacterium* and *Corynebacterium*. In culture, it has been observed that *Arthrobacter sp.* and *Rhodococcus* elaborate with reduced surface and interfacial tension (Asselineau & Asselineau, 1978).

3. Sophorolipid

Sophorose bonded to a long-chain hydroxy fatty acid. Numerous non-pathogenic yeast species can synthesize sophorolipids. They most frequently come from the genus *Candida*, and one of the most notable yeasts utilized to make sophorolipids is *Candida bombicola* (Shah, Nikam, & Gaikwad, 2016).

1.3.2 Lipopeptides and Lipoproteins

In these biosurfactants, lipids are attached to a polypeptide chain.

1. Surfactin

Seven amino acid ring structures coupled to the fatty acid chain via lactone linkage. Surfactin is known to be one of the most potent biosurfactants reported. Surfactin can function as an effective emulsifier, stabilizer, and surface moderator in the food sector and has anti-bacterial, anti-viral, anti-fungal, and actions ideal for health-related applications (Sarubbo, Silva, & Durva, 2022).

2. Lichenysin

Bacillus licheniformis produces this biosurfactant that exhibit excellent stability under extreme temperature, pH, and salt conditions and its structure is similar to surfactin (Vijayakumar & Saravanan, 2015).

1.3.3 Fatty acids and Phospholipids

Many bacteria and yeast produce large quantities of fatty acids and phospholipid surfactants during their growth on n-alkanes substrate. Microorganisms produce complex fatty acids containing OH groups and alkyl branches. These biosurfactants are of major importance in medical applications (Busi & Rajkumari, 2017)

1.3.4 Polymeric Surfactants

These polymeric biosurfactants, which also include various polysaccharide-protein complexes and emulsan, and liposan, have attracted the most attention. Even at a concentration, emulsan is a powerful emulsifying agent for hydrocarbons in water. *Candida* lipolytica produces the extracellular water-soluble emulsifier identified as liposan, which is mostly made up of 83% carbohydrates and 17% proteins. Liposan, a polymeric biosurfactant, is used in the food and cosmetics sectors as an emulsifier (Gakpe & Hatha, 2007).

LITERATURE REVIEW

Although there has been a significant amount of research over the past 20 years on the economics of producing biosurfactants, their commercial success in comparison to their synthetic equivalents still poses a financial hurdle (Mariano & Kataoka, 2007).

The goal of the current study is to investigate the many microorganisms that produce biosurfactants. Several bacterial strains were identified and tested in the current investigation for their potential as biosurfactant agents. The current experiment focuses on comparing how different bacteria produce biosurfactants. The advancement of this field of study is crucial, especially regarding the current environmental safety issues (Sivapathasekaran, 2010).

Furthermore, the possibility of their production on a large scale, selectivity, performance under intense conditions and their future applications in environmental fortification also has been increasingly attracting the attention of the scientific and industrial community. These molecules have the potential to be used in a variety of industries like cosmetics, pharmaceuticals, food preservatives and detergents (Saharan & RK Sahu, 2011).

Ghayyomi Jazeh, M., *et al*. (2012) extracted biosurfactant-producing bacteria from petroleum-contaminated soil, and they reported that 160 strains were able to produce biosurfactant, of which 59 strains positively demonstrated blood hemolysis and 45 strains showed oil-spreading in a positive manner. They discovered that the maximal emulsion and foaming activity occurred at pH 7 and 37 °C. The isolation culture media was created in a lab using the Banat method (Oh & Jazeh, 2012).

According to Kaustuvmani Patowary et.al.(2017), *P. aeruginosa* was found to be an efficient crude oil degrader and could produce rhamnolipid biosurfactant using crude oil as the sole carbon and energy source (Patowary R. , 2017).

Although the use of biosurfactants in bioremediation has been thought to be hugely valuable, a variety of shortcomings and limitations have quickly been identified when putting the theory into reality. While there was some potential improvement during the initial short-term trials, little beneficial or even retardation was frequently seen, particularly with in situ treatment. The emerging gap may be caused by a considerable lack of consistency between

laboratory experiments and actual environmental clean-up efforts (Ławniczak, 2013).

Another study shows that *Bacillus subtilis* and *Pseudomonas aeruginosa* use diesel as the best carbon source for biosurfactant production (Usharani, 2009).

The production of biosurfactants by *B. sphaericus* and *B. azotoformans* was demonstrated in a particular study. The best substrate for huge amounts of surfactants turned out to be glucose, followed by diesel and crude oil. Moreover, the biosurfactants were primarily composed of phospholipids and were stable at a variety of pH, temperature, and salinity levels, while they could still be used efficiently under some circumstances. The two *Bacillus* species produced a significant amount of biosurfactants; therefore, it is crucial to use their products in bioremediation and MEOR (Adamu & ijah, 2015).

The compounds from the BTEX group, which are present in petrol and diesel, have a higher solubility in water than the other elements of these fuels, which explains why they are the contaminants found in greater amounts on the water table when underground tank leaks, being led by groundwater. These hydrocarbons are harmful to human health in their current forms; hence it is important to research ways to get rid of or reduce their presence in the environment (Souza, 2014).

Sohnger and Kserer started researching how microorganisms consume hydrocarbons in about 1906. (Souza & Vessoni-Penna, 2014) After some time, Sohnger demonstrated in 1913 that some bacteria, mostly belonging to the genera *Mycobacterium* and *Pseudomonas*, were capable of oxidizing petrol, kerosene, paraffin and paraffin oil, CO2, water, and minute amounts of organic acids (Zobell, 2023).

Following that, several experiments were conducted to identify the species. Grey and Thornton (1928) isolated bacteria from the genera *Micrococcus*, *Mycobacterium*, *Bacterium*, *Bacillus*, and *Spirillum* that could break down naphthalene, toluene, cresol, and phenol (H.G. & Gray , 1928).

Kevin B. Cheng and colleagues researched the emulsion properties of bacterial biosurfactants in 2008. They isolated three unidentified biosurfactant-producing

bacteria and compared their emulsification activity to that of the two synthetic surfactants SDS and Triton X-100.In hexadecane, two biosurfactants had better emulsion activity than synthetic surfactants, however, in diesel, synthetic surfactants had superior emulsion activity than all of the extracted biosurfactants (Kevin B. Cheng, Jian , & Wang, 2008).

FACTORS AFFECTING BIOREMEDIATION OF HYDROCARBONS

Several factors influence the hydrocarbon degradation rate and the hydrocarbon availability to the microorganism. According to Varjani (2017), microorganism are highly responsive to changes in environmental conditions that affect the rate of degradation of hydrocarbons. to create a sustainable environment, microorganisms are crucial for preserving the ecosystem and biosphere (Varjani, 2017).

Mittal & Singh (2009), have discovered nine isolates from oil-contaminated soil in the nearby Haridwar area and eleven isolates from the oil production site of the Lingala oil field, ONGC. (Mittal & Singh, 2009) Bayoumi and Abul-Hamd (2010) , have documented the isolation of 109 pure bacterial cultures in mineral salt medium (MSM), with toluene and phenol serving as the only sources of carbon and energy (Bayoumi & Hamd, 2010).

A contaminant is biodegraded across many phases across using several enzyme. Individual microorganisms or groups of microorganisms from the same or disti nct genera can preferentially metabolize hydrocarbons(Abbasian, Lockington, 2015).However, it has been demonstrated that consortiums have greater capaci ty than individual cultures for metabolizing/degrading broad ranges of hydroca rbons (Boopathy, 2000). This may be caused by the existence of petroleum hydrocarbon molecules (the only source of carbon and energy) that are subject to bacterial decomposition, the adaptation of hydrocarbon pollutant degraders to polluted environments, and the presence of enzymes that contribute to several pathways for biodegradation (Atlas, 1991).

A certain type of bacterial species uses a few hydrocarbons as its preferred food source, and when they are combined, they have a synergistic impact (Sugiura &

Ishihara, 1997). Petroleum hydrocarbon breakdown may be mediated by a particular enzyme system (Widdel & Rabus, 2001). The generation of biosurfactants/bioemulsifiers, biopolymers, solvents, gases, and acids, as well as the attachment of microbial cells to the substrates, are two common processes used to launch an initial attack (Banat I. , 1995). Crude oil is a mixture of simple and complex hydrocarbons that are broken down by a number of native microorganisms, each of which is capable of destroying a particular class of molecules (Varjani, 2017).

Numerous factors affect the rate of biodegradation, including (a) pollutant characteristics such as availability, type, and length of hydrocarbons, dispersion into the aqueous phase, and volatilization (b) microorganisms, cell metabolic pathways, and several structural changes from inclusions to the complex extracellular polymeric texture (Leahy and Colwell, 1990) (c) environmental conditions, such as pH, temperature, water content, salinity; oxygen availability and nutritional factors viz. carbon and nitrogen source and other nutrients (Aislabie ,2006) (d) physio-chemical properties of soil such as density water holding capacity, pH moisture and texture etc (Leahy & Colwell, 1990).

According to Okoh (2006), have noted that higher temperatures make hydrocarbon pollutants more soluble, reduce viscosity, and transfer long-chain n-alkanes from the solid phase to the liquid phase. According to Thamer *et al*. (2013), salt and high temperatures inhibit the growth of microorganisms and the production of their by-products (Okoh, 2006).

For successful biodegradation of hydrocarbons all the essential nutrients like nitrogen and phosphorus is important. Some of the nutrients are limiting factors that affect the process and rate of biodegradation (Varjani, 2017). The occurrence of oil spills increases the supply of carbon but the availability of nitrogen and phosphorus becomes the limiting factor for degradation. Therefore, nitrogen and phosphorus should be supplied based on the number of carbons that are to be metabolized (Atlas, 1991).

AIMS

AND

OBJECTIVES

1. Enrichment and Isolation of potential biosurfactant producing isolates.

2. Screening for biosurfactant production.

3. Extraction and Purification of biosurfactant.

4. Chemical characterization of purified biosurfactants.

5.Biochemical characterization and tentative Identification of potential biosurfactant producing isolates.

MATERIALS

AND

METHODS

4.1 COLLECTION OF SAMPLES

In the present study, samples were collected from the following different sites of Goa :

Sample 1 : Water sample from Dona Paula Jetty

Sample 2 : Soil sample from Dona Paula Jetty

Sample 3 : Soil sample from a Petrol pump

Sample 4 : Swab from a Petrol tank

Sample 5: Soil sample from Oil Mill

The Samples were collected aseptically using a sterile spatula for soil in zip lock bags and water samples in sterile screw cap bottles. The pH of water sample was determined using a pH strip at the time of collection. The samples were then stored at 4˚C for further use.

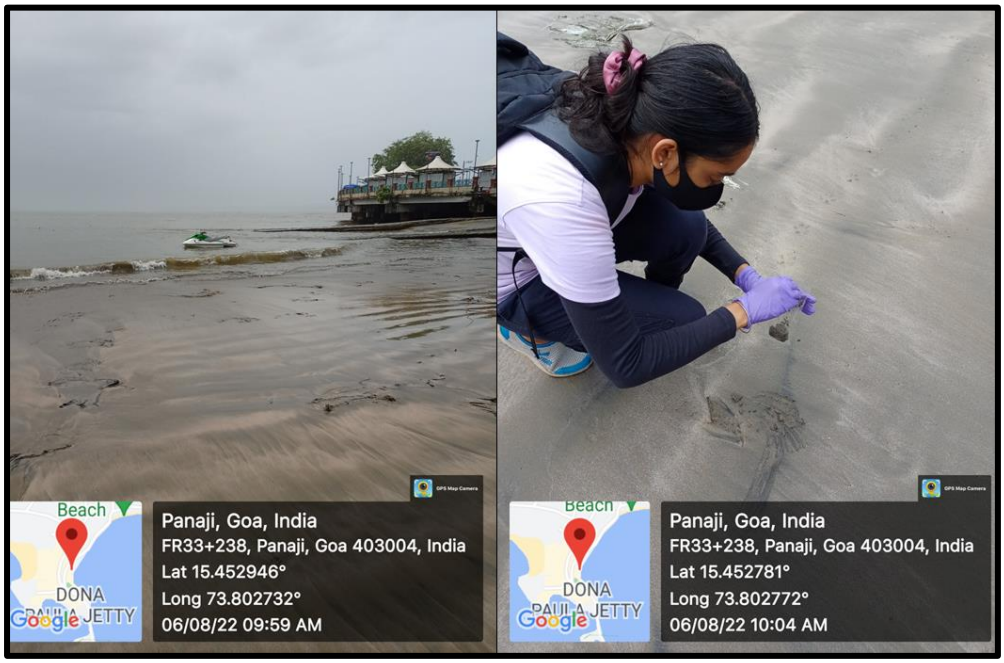


Fig 2 : Collection of samples

4.2 ENRICHMENT OF POTENTIAL BIOSURFACTANT PRODUCING ISOLATES

Bushnell Haas broth (BHB) was prepared and was enrichment with 1% mineral oil as the sole carbon source. Each flask of 100 ml BHB liquid media was prepared (pH 7) and sterilized by autoclaving at 121 psi for 15 mins and was inoculated with the 1ml of the water sample and 1 g of soil sample separately. The flasks were incubated in shaking conditions at room temperature for a period of 7 days.

4.3 ISOLATION AND PURIFICATION OF POTENTIAL BIOSURFACTANT PRODCUING ISOLATES

4.3.1 Serial dilution and isolation of colonies

Each of the enriched broths was serially diluted aseptically by transferring 1 ml from each sample to 9 ml of 0.8% and 3% saline. The Soil and Marine Water sample were serially diluted up to 10^{-6} dilutions.100 µl of the dilution 10^{-4},10^{-5} and 10^{-6} were aseptically transferred to prepared NA and ZMA plates respectively and spread plated using a sterile glass spreader. The spread-plated plates were incubated at the room temperature for 24 hours.

4.3.2 Purification of the cultures

The isolated colonies from the spread plates were picked aseptically using sterile loop and were quadrant streaked on freshly prepared Nutrient Agar and Zobell Marine Agar plates. Morphologically distinct colonies were selected. The selected isolates were named as DPW1,DPW2,DPS1,DPS2,PS1,PS2,PS3,PT1,OM1,OM2,OM3,OM4.

Where,

DPW - Dona Paula water sample

DPS - Dona Paula soil sample

PS - Petrol Pump soil sample

OM – Oil Mill soil sample

The cultures were later maintained on agar slants for further use.

4.4 SCREENING OF BIOSURFACTANT PRODUCING BACTERIA

4.4.1 Drop Collapse Assay

Drop collapse assay was performed by using the protocol described by Jain *et al* (1991). All the isolates were grown in BHB with 1% oil as a carbon source for 7 days in shaking conditions. The microbial cells were separated by centrifugation at 10000 rpm for 15 mins to obtain cell-free supernatant. 2 µl of oil was loaded on a glass slide and then to the oil surface 5 µl of the cell free supernatant of the isolates was added. The drop's shape on the surface was inspected for 3 minutes. Biosurfactant producing cultures gave flat drops, with the scoring system ranging from "+" to "+++". Cultures that gave rounded drops were scores "-". Distilled water and Triton x-100 served as a negative and positive control respectively.

4.4.2 Oil Displacement Assay

The oil displacement method, developed by Morikawa *et al* (2000), was used to determine the efficiency of the biosurfactant by evaluating the diameter of the clear zone, that occurs after the addition of a surfactant-containing solution on oil-water interphase. To a clean Petri plate, 25 ml of distilled water was added which was then surface loaded with 20 µl of oil. Then 10 µl of cell-free supernatant was placed at the centre of the oil surface. The oil displacement zone was noted. Biosurfactant producing cultures showed oil displacement, with the scoring system ranging from "+" to "+++". Cultures with no oil displacement were scores "-". Distilled water and Triton x-100 served as a negative and positive control respectively.

4.4.3 Emulsification Assay

The assay developed by Cooper and Goldenberg (Cooper DG ,1987) was conducted to measure the emulsification capacity of the biosurfactant produced by the bacterial isolates. The cell-free supernatant was used as the biosurfactant source to check the emulsification of crude oil (Engine oil). To 2 ml of cell-free

supernatant of each culture in a test tube,2 ml of crude oil was added. The tubes were then vortexed for 1-2 minutes, at maximum speed, and were then allowed to remain in a stationary position for 24 hours. After 24 hours the total height of the solution and the height of emulsified layer were measured. The emulsification activity E_{24} (%) was determined using the following equation:

Emulsification index (E24) = (Height of emulsified layer/Total height) ×100

Distilled water and Triton x-100 served as a negative and positive control respectively.

4.4.4 BATH Assay

Bacterial Adhesion to Hydrocarbons uses to calculate the cell hydrophobicity of the bacterial isolates as explained by Rosenberg *et al*, (Rosenberg M,1980). Bacterial isolates were inoculated in BHB media and were incubated for two days at room temperature in shaking conditions. After incubation, the cells were harvested by centrifugation at 10000 rpm for 15 minutes. The cells were suspended in 0.8% saline to obtain cell suspension. To 2 ml of cell suspension in a test tube, 200 µl of oil was added and vortex shock for 3-5 minutes. After shaking the oil was allowed to separate from the aqueous phase, by placing it in a stationary position for 1 hour. The absorbance of the aqueous phase was then measured using a spectrophotometer at 600 nm. The bacterial cell adherence to hydrocarbon was determined by calculating the cell hydrophobicity.

Hydrophobicity % =1- (Optical density of aqueous phase/Optical density of initial phase) ×100

Distilled water and Triton x-100 served as a negative and positive control respectively.

4.5 EXTRACTION OF BIOSURFACTANT

Flasks containing 100 ml BHB media with 1% oil as a carbon source were prepared and autoclaved at 121˚C for 15 minutes. After autoclaving each flask was inoculated with a 24-hour fresh culture of the isolates. The flasks were then incubated for 7 days in shaking conditions. After 7 days, bacterial cells were separated to form the pellet by centrifugation at 10000 rpm at 4˚C for 20 minutes. After centrifugation 6 N HCL was added to the recovered cell-free supernatant till the pH was adjusted to pH 2 using a pH meter. It was then kept

overnight at 4˚C. To the supernatant, an equal volume of chloroform: acetone (2:1) was added. Using a rotary evaporator at 40˚C the solvent was evaporated to obtain the crude biosurfactant. The dry weight of the extracted biosurfactant produced by the bacterial isolates was then determined.

Dry Weight = (Weight of Eppendorf tube with dry biosurfactant – Weight of empty Eppendorf tube) (El-Sheshtawy, Mahdy, Sofy, & Sofy, 2022).

4.6 PURIFICATION OF BIOSURFACTANT

The Purification of the biosurfactant was done by column chromatography. The column was prepared by mixing 20 g of silica gel (Merck, Germany) in 100 mL of chloroform and then poured into the glass column. The column was left overnight to ensure good packing of the silica gel as the stationary phase. The column was washed with chloroform three times. The solvent was allowed to flow down to beaker. One gram of extracted biosurfactant was taken as a sample.

Initially, the sample was mixed with 10 mL of chloroform before passing it through the silica gel column. Multiple fractions of the eluate were collected in aliquots of 1 ml each . Each fraction was tested for the presence of biosurfactant using E_{24} Assay. Fractions that showed positive result for E_{24} were then dried in a hot air oven to obtain purified biosurfactant which was further characterized (Rusly, 2019).

4.7 CHARACTERISATION OF BIOSURFACTANT

4.7.1 Protein Estimation

The test solution containing biosurfactant made to a final volume of 1 ml. 1% Ninhydrin was prepared of which 1 ml was mixed into the sample by vortexing. The tubes were kept for 10 minutes in a boiling water bath and were cooled. The tubes were observed for colour change from colourless to blue. The standard was prepared using Bovine Serum Albumin. The absorbance was taken at 570 nm (Sun, Lin, Weng, & Chen, 2006).

4.7.2 Carbohydrate test

The test solution containing biosurfactant was made to 1 ml with distilled water and to this 2 ml of Benedict’s solution was added. The tubes were then kept for

10 minutes in a boiling water bath. The samples were for colour change from colourless to red.. The absorbance was taken at 750 nm (Katoch, 2011).

4.7.3 Lipid test

The test solution was made to 1 ml with distilled water and a few drops of 5% Potassium dichromate solution was added. To that 5 ml of concentrated Nitric Acid was added. Mineral oil was used as standard. It was then observed for colour change from colourless to blue (Rathore, *et al*., 2021).

4.8 TENTATIVE IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF THE BACTERIAL ISOLATES

4.8.1 Morphological identification potential biosurfactant producing isolates

The morphological different isolates were observed on the plate and identified.

A. Gram staining

To the centre of a clean dry slide, a small amount of bacterial suspension of the selected isolates was transferred aseptically. A thin smear of the bacterial suspension was made by spreading the drop with a sterile loop. The slide was allowed to air dry and was fixed by passing the slide rapidly over the flame. The slide was then flooded with crystal violet stain for 1 minute and washed gently with distilled water to remove excess stain. The slide was then flooded with grams iodine for 1 minute. Later the decolourizer was added drop by drop until no more colour flows from the smear. The decolourizer was washed off. Then the slide was flooded with the counterstain safranin for 30 seconds and later washed with distilled water. After air drying the slide, it was observed under the microscope with an oil immersion objective at 100 X (Smith & Hussey, 2005).

B. Motility

With the help of a sterile toothpick spread petroleum jelly on the four corners of the clean coverslip. Using an inoculating loop, aseptically place a drop of bacterial suspension in the centre of a coverslip. The cavity slide was placed facing down onto the coverslip so that the drop protrudes into the centre of the cavity slide. Press gently to form a seal. Turning of hanging drop slide over and

placing it on the stage of the microscope for observation (Shields & Cathcart, 2011).

4.8.2 Biochemical analysis

Four isolates were selected OM1, OM2, PS1 and PT1 based on the amount of biosurfactant they produced and were subjected to biochemical analysis for further identification. Selected isolates were inoculated in NB media. After 24 hours OD was taken at 600 nm, and when the OD was ≥ 0.5 then, 50 µl of the culture was inoculated in HICarbohydrate KB009 kit and incubated for 24-48 h at 37˚C. The results were compared with the interpretation chart.

4.8.3 Enzyme assay

A. Lipase Assay

The isolates were spot inoculated with 24 hr fresh culture onto the tributyrin agar plates and were incubated at 37˚C for 24-48 hours. The defined zone of lysis was later observed around the colonies after the incubation time. The zone of clearance around the colonies represents the production of Biosurfactant. The diameter of the zones depending on concentration was represented as '++' large and complete clearance, '+' small and incomplete clearance, '-' no clearance around the colonies. (Bharathi, Rajalakshmi, & Komathi, 2019)

B. Protease Assay

The isolates were spot inoculated with 24 hr fresh culture onto NA plates containing 1% Casein and were incubated at 37˚C for 24-48 hours. The zone of clearance observed around the colonies represented the production of Biosurfactant. The diameter was measured and represented as '++' large and complete clearance '+' small and incomplete clearance, '-' no clearance around the colonies (Zhang, Shuai, Yao, Li, & He, 2021).

C. Amylase Assay

Starch agar was prepared and poured in plates and later spot inoculated with 24 hr fresh culture. Incubated at 37˚C for 24-48 hours. After incubation, the surface of the plates was flooded with iodine solution using a dropper for 30 seconds. After 30 seconds the excess iodine was poured off. A clear zone around the colonies was observed. (Lal & Cheetham, 2012)

D. Catalase Test

To a clean slide, a drop of H_2O_2 was added. A loop full of 24-hour fresh culture was then mixed with H_2O_2 using a sterile loop. A positive catalase test was indicated by the production of effervescence (Khatoon, Anokhe, & Kalia, 2022).

E. Oxidase Test

Whatman's filter paper was moistened with drops of freshly prepared reagent 1% solution of NNN'N'- tetra-methyl-p-phenylene-diamine dichloride. It is then air dried, after which a loopful of 24 hours old culture of the bacterial isolate grown on nutrient agar slant was rubbed against this moistened filter paper. If the test is positive then it is indicated by an intense deep-purple color, appearing within 5-10 seconds, a "delayed positive" reaction by coloration in 10-60 seconds, and a negative reaction by the absence of coloration or by coloration later than 60 seconds (Shields & Cathcart, 2010).

RESULTS

5.1 ISOLATION AND PURIFICATION OF THE POTENTIAL ISOLATES

After enrichment incubation 11 morphologically different colonies were observed. They were purified on NA and ZMA plates using quadrant streaking shown in figure 5. They were designated as DPW1 ,DPW2, DPS1, DPS3, PS1, PS2, PS3, PT1, OM1, OM2, OM3. These isolates were then preserved on slants at 4˚C for further studies as in figure 6.

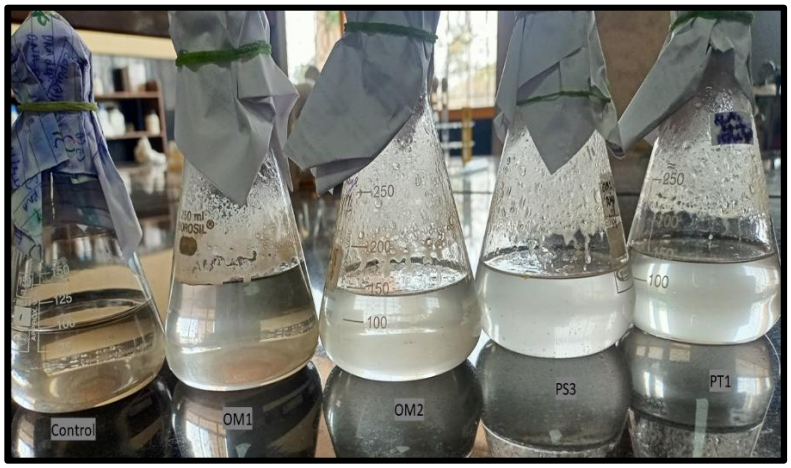


Fig 3: Growth of individual isolates in BHB with 1% Oil as carbon source after 7 days of enrichment

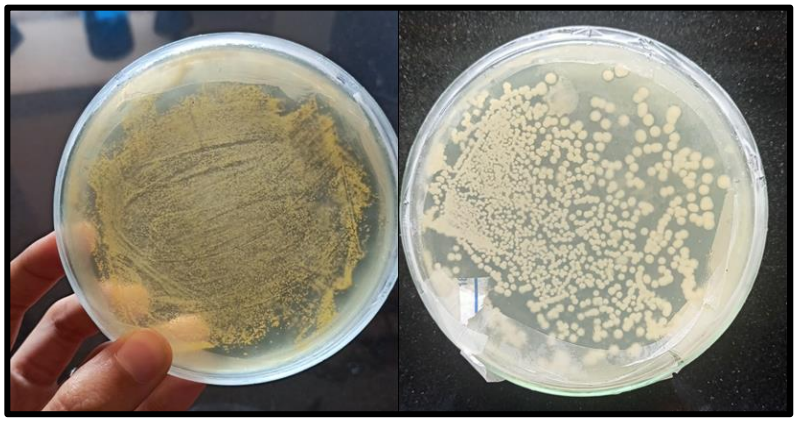


Fig 4 : Isolation of bacterial isolates

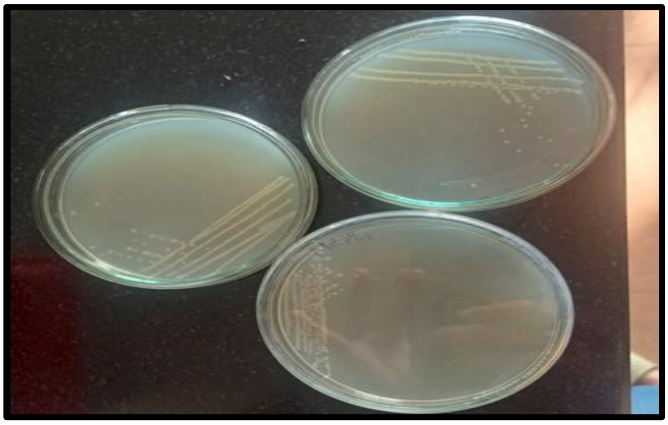


Fig 5: Purification of selected bacterial isolates

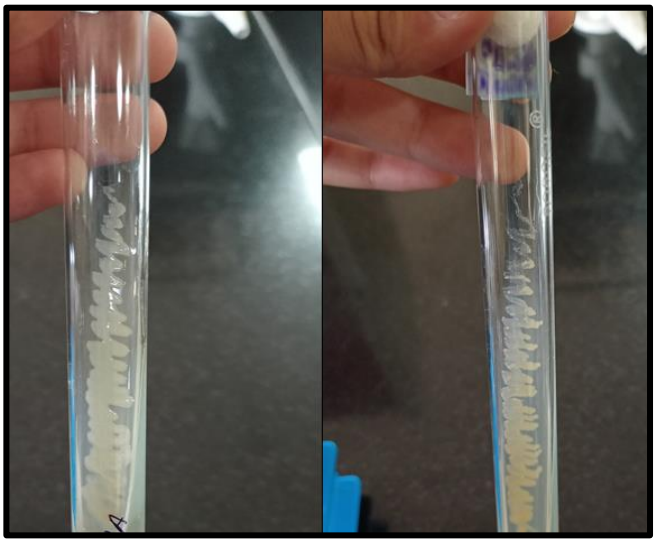


Fig 6: Preservation of bacterial isolates on slants

5.2 SCREENING FOR BIOSURFACTANT PRODUCING BACTERIAL ISOLATES

5.2.1 Drop Collapse Assay

Table 1 : Drop Collapse Assay of the isolates

Isolate	Result
DPS1	-
DPS2	-
DPW1	-
DPW2	-
OM1	-
OM2	-
OM3	-
PT1	++
PS1	-
PS2	-
PS3	++

The eleven selected isolates were subjected to drop collapse assay, by placing a drop of cell-free supernatant of each isolate on crude oil. It is a qualitative test where the collapse of the drop detects the production of biosurfactants. Out of all the isolates tested 2 isolates namely PT1 and PS3, showed the positive result as in figure 5 showing the drop collapse rapidly when compared to positive control i.e. Triton x-100 and negative control i.e. Distilled water.

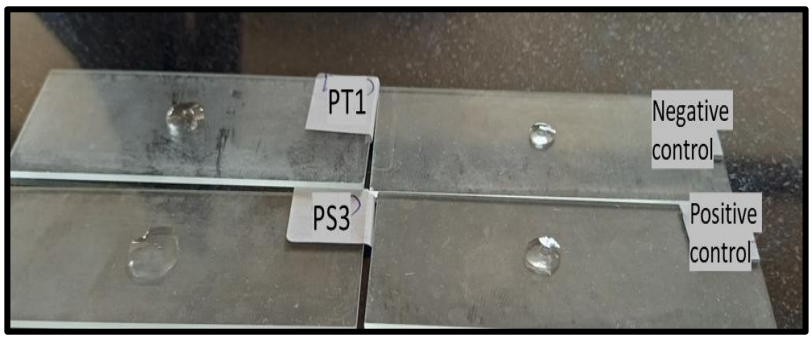


Fig 7 :Drop Collapse:The collapse of the drop indicates a reduction of interfacial tension by the produced biosurfactant

5.2.2 Oil Displacement Assay

Table 2: Oil Displacement Assay of the isolates

Isolate	Result
DPS1	+
DPS2	-
DPW1	-
DPW2	-
OM1	++
OM2	+
OM3	-
PT1	+
PS1	++
PS2	+
PS3	++

The oil displacement assay was applied to all 11 isolates to check for the production of biosurfactant production. After adding the cell-free supernatant to the oil layer, it was observed that OM1, OM2, PT1, PS1, PS2, PS3 could displace the oil layer and produce clear zones shown in figure 8 indicating surface activity when compared to positive control i.e. Triton x-100 and negative control i.e. Distilled water respectively .

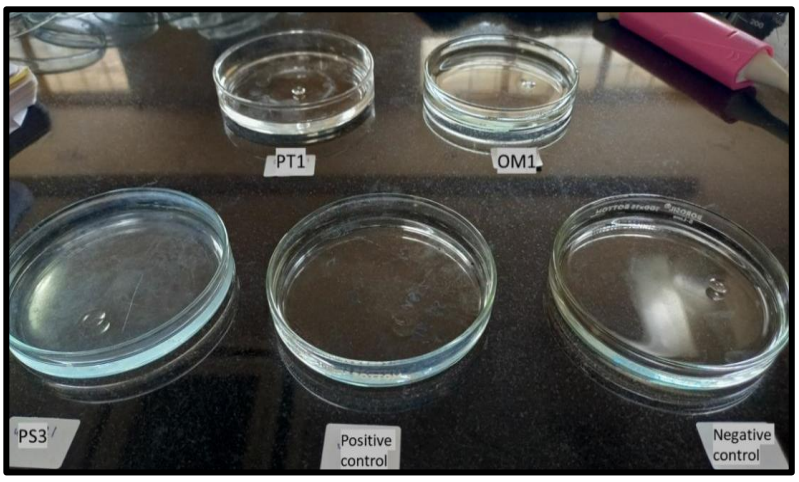


Fig 8 : Oil Displacement Assay: A clear zone observed due to biosurfactant activity displayed by the bacterial isolate

5.2.3 Emulsification assay

Table 3: Emulsification index of the isolates

Isolates	Total Height	Height of emulsified layer	E_{24} Index(%)
DPS1	2.5	0	0
DPS2	2.5	0	0
DPW1	2.5	0	0
DPW2	2.5	0	0
OM1	2.5	0.4	16
OM2	2.5	0.5	20
OM3	2.5	0	0
PT1	2.5	0.2	8
PS1	2.5	0.3	12
PS2	2.5	0.1	4
PS3	2.5	0.3	12

The emulsification index of each selected isolate was measured using the emulsification assay. Out of 11 isolates, 6 showed emulsification after vortexing and letting the emulsified layer settle for 24 hours as shown in figure 9. The formation of the emulsified layer between the biosurfactant containing cell supernatant and the engine oil, confirms the ability of the isolates to emulsify hydrocarbons. Triton X-100 was used as a positive control whereas Distilled water was used as a negative control respectively.

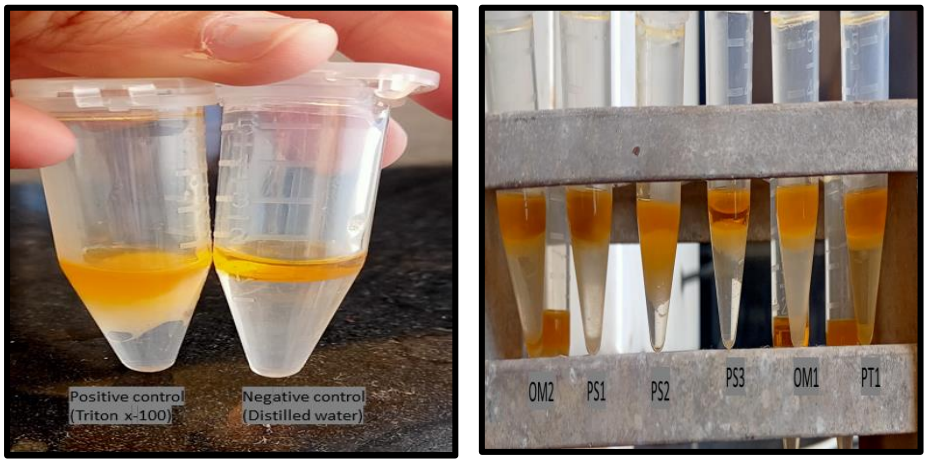


Fig 9 : Emulsification assay: formation of a stable emulsified layer of oil by the bacterial isolates

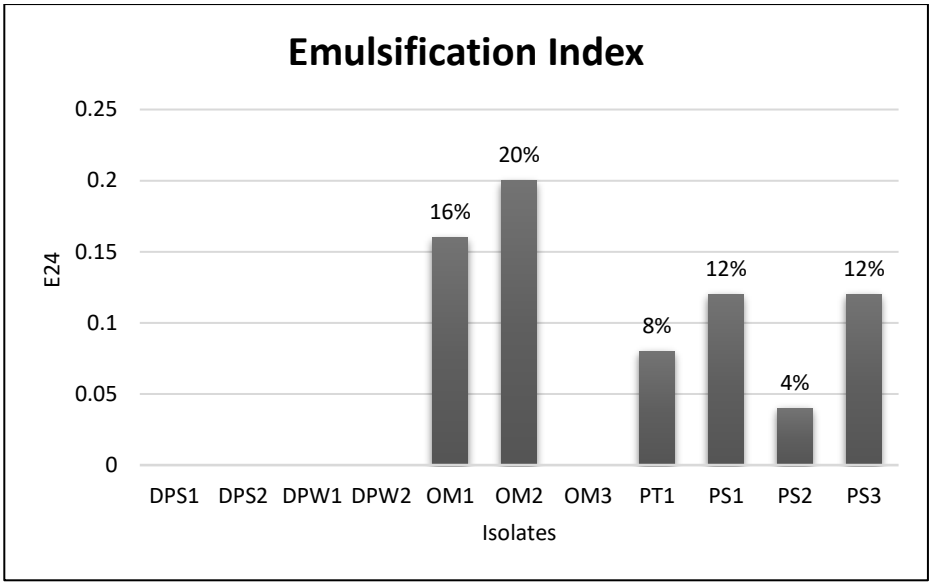


Fig 10 : Emulsification index of the isolates

5.2.4 BATH Assay

Table 4: BATH Assay of the isolates

Isolates	OD of the cell suspension(600 nm)		OD of the aqueous phase(600 nm)	Hydrophobicity (%)
DPS1	0.023	200 µl Oil and Vortex	0.022	5%
DPS2	0.054		0.053	2%
DPW1	0.101		0.102	0
DPW2	0. 199		0.197	2%
OM1	0.234		0.123	48%
OM2	0.174		0.079	55%
OM3	0.200		0.172	14%
PT1	0.307		0.117	62%
PS1	0.200		0.140	30%
PS2	0.112		0.104	8%
PS3	0. 326		0.175	47%

The Bacterial Adhesion to hydrocarbons assay was used to determine the cell surface hydrophobicity of the bacterial isolates. The reduction in the optical density confirmed the bacterial cell adherence to the hydrocarbon. The degree

of hydrophobicity of each sample was calculated. The results of the BATH Assay are represented in Table 4.

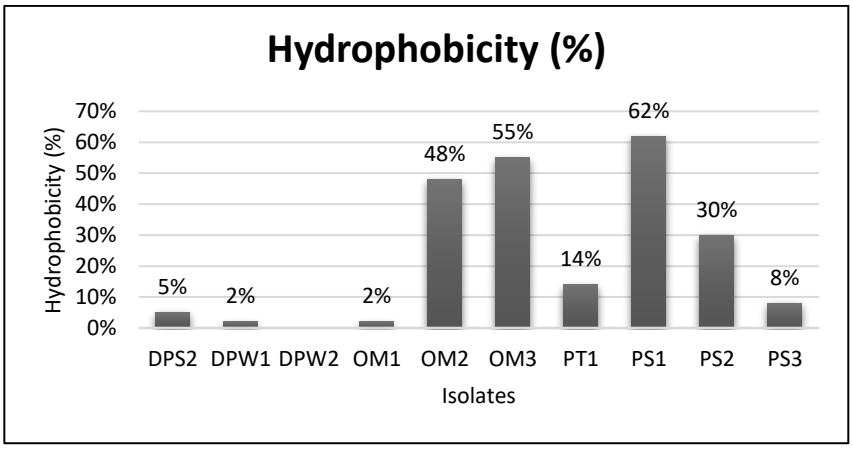


Fig 11: Hydrophobicity of the isolates determined by BATH assay

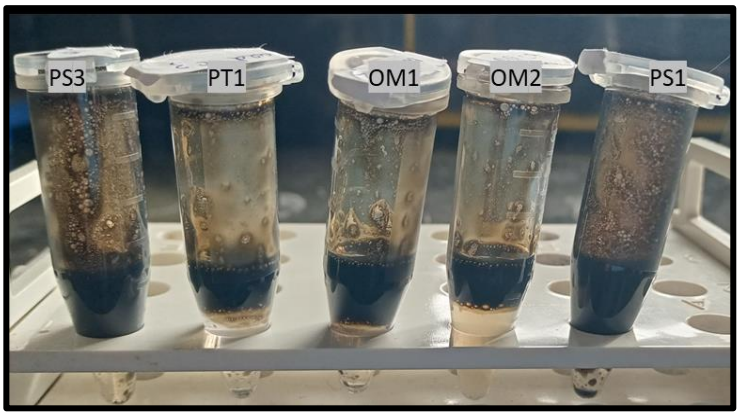


Fig 12: Bath assay

5.3 EXTRACTION AND PURIFICATION OF BIOSURFACTANT

Fig 13: Crude biosurfactant obtained in 10 ml and 150 ml supernatant

Table 5 : Dry weight of the biosurfactant production

Isolates	Weight of empty Eppendorf tube	Weight of empty Eppendorf tube with biosurfactant	Dry weight of biosurfactant for 20 ml of supernatant	Estimated Dry weight of biosurfactant for 150 ml of supernatant	Amount of biosurfactant after purification
OM1	1.12	1.37	0.25	0.63 g	0.38 g
OM2	1.12	1.34	0.22	0.61 g	0.25 g
PS3	1.12	1.31	0.19	0.53 g	0.33 g
PT1	1.12	1.26	0.14	0.53 g	0.14 g

Fig 14: Purified biosurfactant using column chromatography

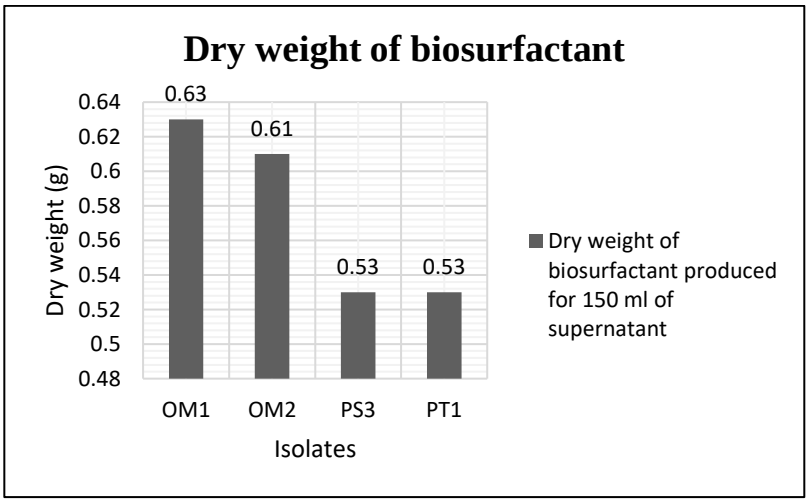


Fig 15 : Dry weight of biosurfactant produced by isolates

The best 4 isolates OM1, OM2, PS3 and PT1 were selected based on the screening method and the amount of biosurfactant in 20 ml of supernatant. The biosurfactant was extracted from the cell-free supernatant using a mixture of chloroform: methanol (2:1,v/v). The overnight evaporation resulted in the formation of white-coloured sediments which is the biosurfactant. On determining the dry weight, it was seen that isolates OM1 and OM2 produced the highest amount of biosurfactant i.e., 0.63 g and 0.61 g respectively for 150 ml of cell-free supernatant.

The crude biosurfactant obtained was then subjected to column chromatography for purification of obtained biosurfactant. After purification, OM1 and PS3 showed the highest production i.e., 0.38g and 0.33g.

5.4 CHEMICAL CHARACTERIZATION OF PURIFIED BIOSURFACTANTS

1. Protein estimation

Table 6 : Protein estimation

Concentration (mg/ml)	Volume of stock(ml)	Volume of diluent (ml)	Absorbance (570 nm)
0.1	0.1	0.9	0.071
0.2	0.2	0.8	0.114
0.3	0.3	0.7	0.142
0.4	0.4	0.6	0.222
0.5	0.5	0.5	0.265
0.6	0.6	0.4	0.468
0.7	0.7	0.3	0.324
0.8	0.8	0.2	0.412
0.9	0.9	0.1	0.456
Blank	1	0	0
0.42040272	-	-	0.228
0.4645396	-	-	0.254

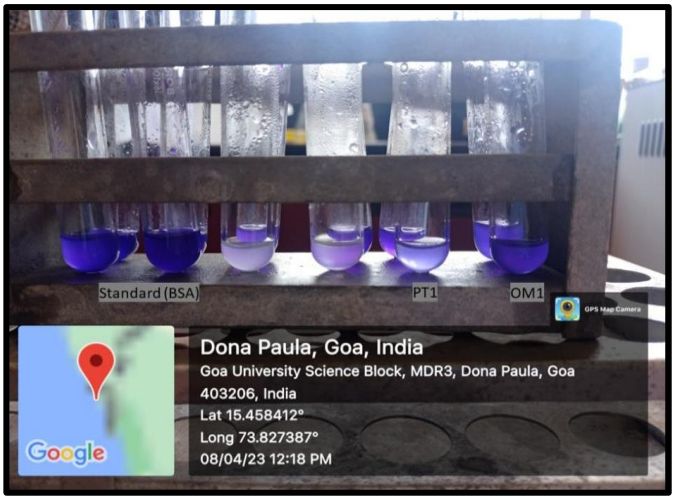


Fig 16 : Protein estimation test (Ninhydrin test)

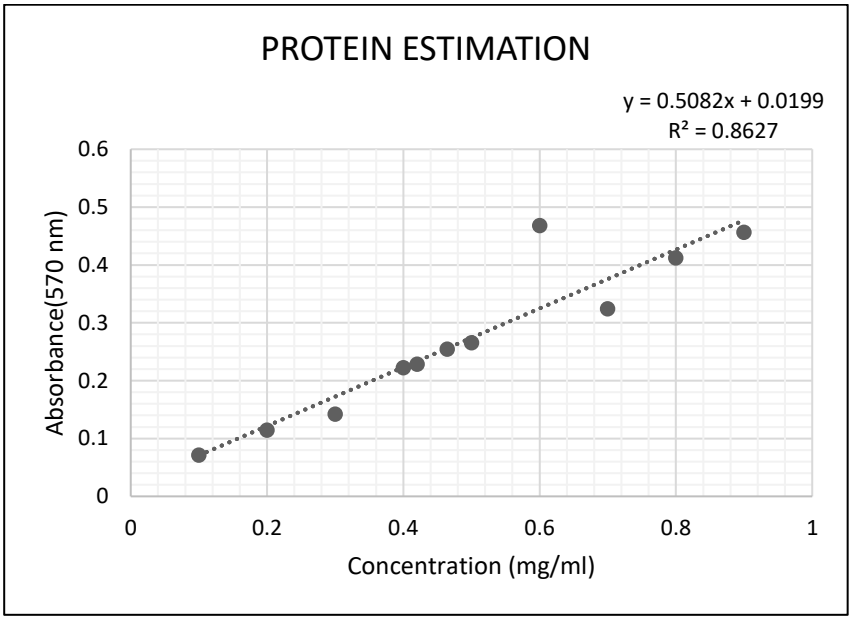


Fig 17: Estimation of protein concentration

The unknown concentration of the isolate OM1 and PT1 were found to be 0.4204 mg/ml and 0.4645 mg/ml respectively.

2 . Carbohydrate test

Table 7 : Carbohydrate estimation

Concentration (mg/ml)	Volume of stock(ml)	Volume of diluent (ml)	Absorbance (750 nm)
0.1	0.1	0.9	0.015
0.2	0.2	0.8	0.032
0.3	0.3	0.7	0.061
0.4	0.4	0.6	0.067
0.5	0.5	0.5	0.092
0.6	0.6	0.4	0.091
0.7	0.7	0.3	0.109
0.8	0.8	0.2	0.176
0.9	0.9	0.1	0.201
Blank	1	0	0
0.813048	-	-	0.167

Fig 18 : Carbohydrate estimation (Benedict's test)

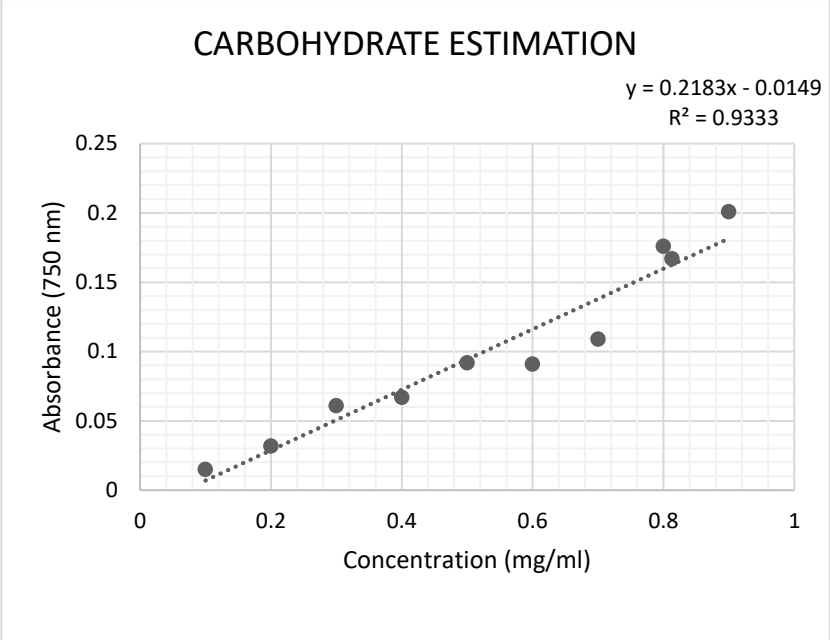


Fig 19 : Estimation of Carbohydrate Concentration

The unknown concentration of PT1 for is found to be 0.8130 mg/ml.

3 . Lipid test

Fig 20: Lipid test

5.5 TENTATIVE IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION OF THE BACTERIAL ISOLATES

5.5.1 Morphological identification potential biosurfactant producing isolates

A. Gram nature

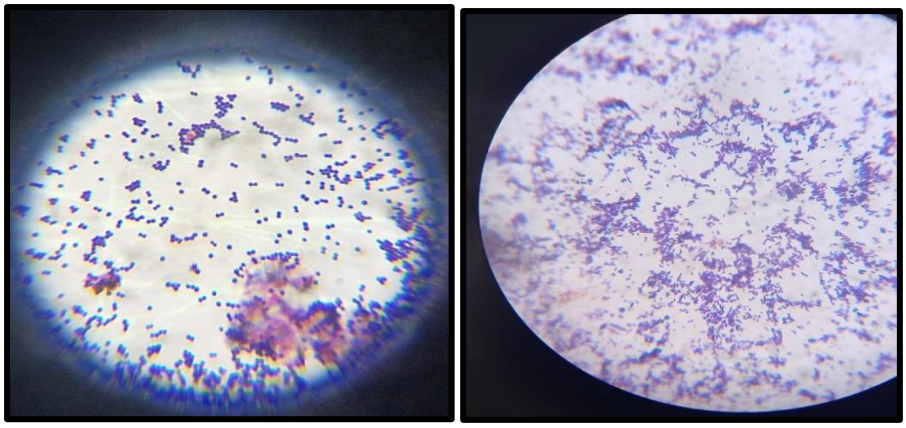
All four isolate were gram stained and was microscopically observed PT1, OM1 and OM2 were found to be Gram-positive rods while PS3 was found to be Gram-positive cocci.

Table 8: Gram nature of the isolates

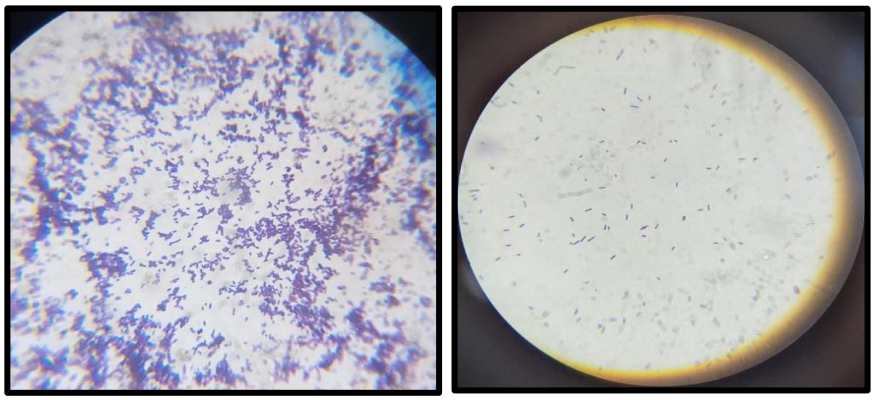
Isolates	Gram's Nature
OM1	Gram Positive rods
OM2	Gram Positive rods
PT1	Gram Positive rods
PS3	Gram Positive cocci

Table 9: Colony Characteristics of the isolates

Isolates	Color	Shape	Size	Elevation	Margin	Opacity	Texture
OM1	White	Round	Large	Flat	Curled	Opaque	Rough
OM2	White	Round	Small	Flat	Entire	Translucent	Smooth
PT1	Yellow	Round	Pinpoint	Raised	Entire	Opaque	Smooth
PS3	White	Round	Medium	Flat	Entire	Opaque	Smooth



PS3 **PT1**



OM1 **OM2**

Fig 21 : Gram staining of the isolates

B. Motility

Out of 4 isolates, PS3 was motile .

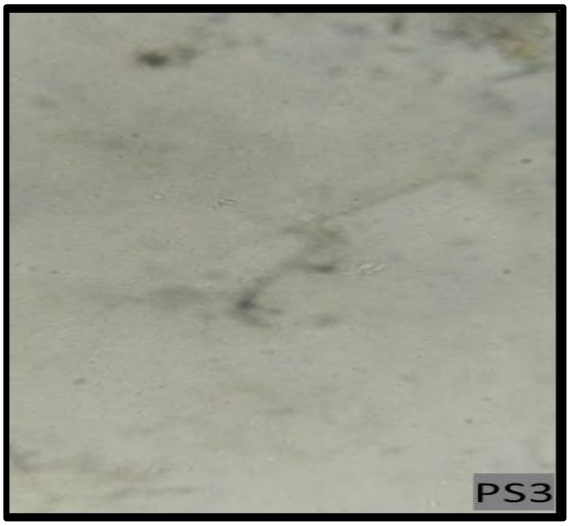


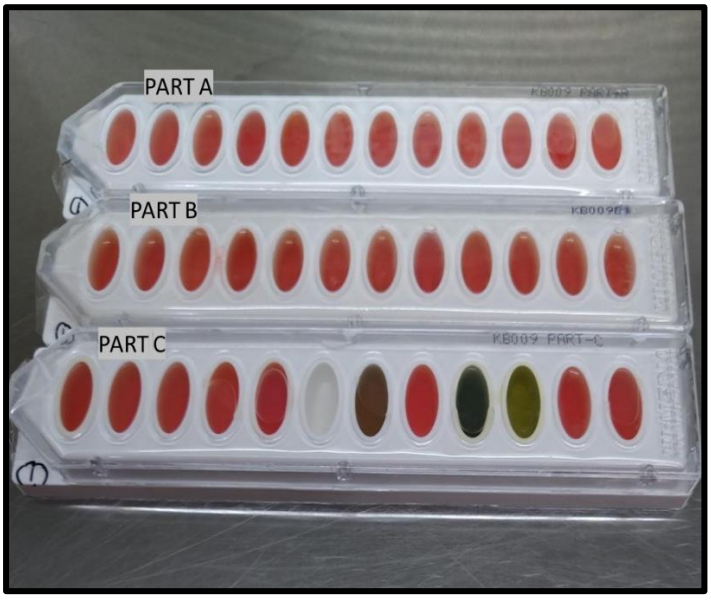
Fig 22: Motility test by hanging drop method

5.5.2 Biochemical assay

Table 10 : Biochemical analysis

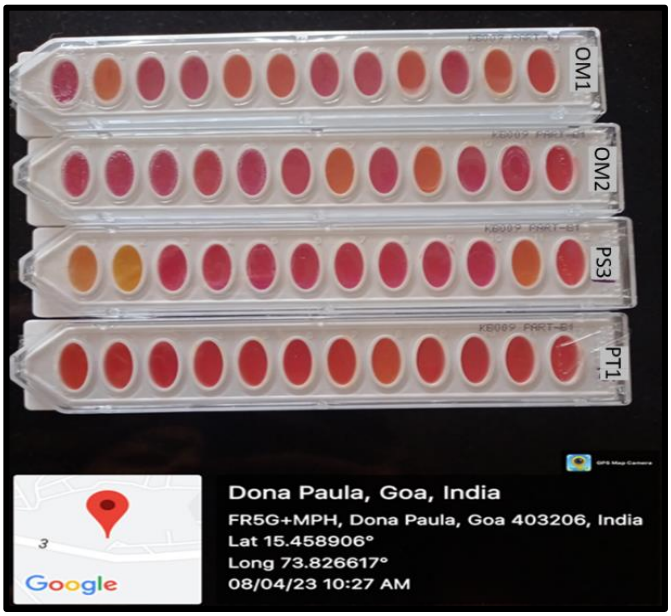
Test	OM1	OM2	PT1	PS3
Lactose	-	-	+	-
Xylose	+	+	+	+
Maltose	-	-	-	-
Fructose	-	-	-	-
Dextrose	+	+	+	+
Galactose	+	+	+	-
Raffinose	-	-	-	-
Trehalose	-	+	-	-
Melibiose	-	-	+	-
Sucrose	-	-	+	-
l-arabinose	+	-	+	+
Mannose	+	-	-	-
Inulin	-	-	+	-
Sodium gluconate	-	-	+	-
Glycerol	-	-	+	-
Salicin	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Sorbitol	-	-	-	-
Mannitol	-	-	-	-
Adonitol	-	-	-	-
Arabitol	-	-	-	-
Erthritol	-	-	-	-
α -methyl-D-glucoside	-	-	-	-
Rhamnose	-	-	-	+
Cellobiose	-	-	+	-
Melezitose	-	-	-	-
α -methyl-D-mannoside	-	-	-	-

Xylitol	-	-	-	-
ONPG	-	-	+	-
Esculin Hydrolysis	-	+	+	+
D-arabinose	-	-	-	-
Citrate utilization	+	+	-	+
Malonate utilization	-	-	-	-
Sorbose	-	-	-	-

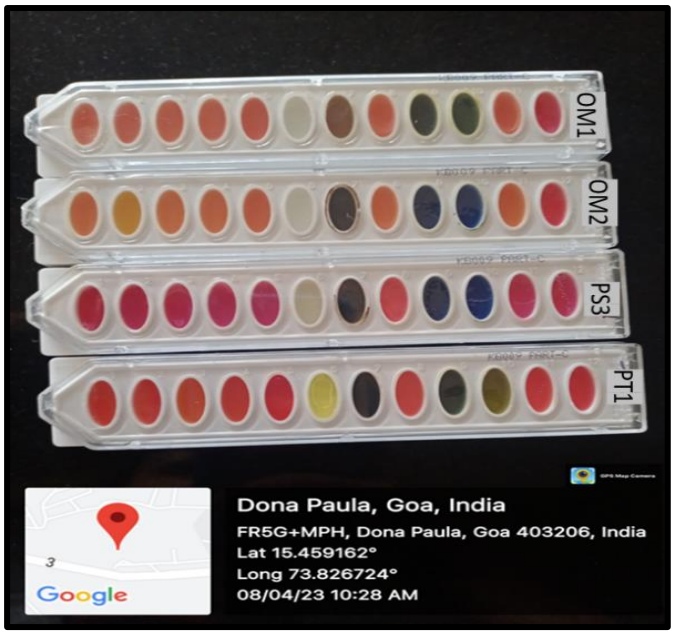


Before inoculation

Part A (After 48 hrs)



Part B (After 48 hrs)



Part C (After 48 hours)

Fig 23: Carbohydrate Utilization Kit

5.5.3 Enzyme Assay

A. Oxidase test

For oxidase test all four isolates were negative.

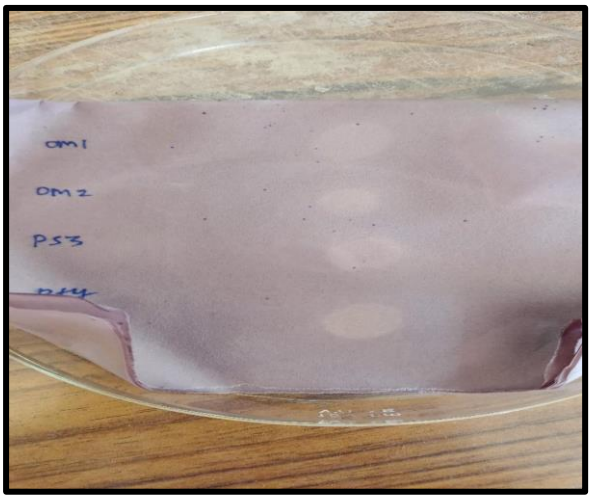


Fig 24: Oxidase test

B. Catalase

All four isolates were positive. The effervescence was seen when tested with H_2O_2.

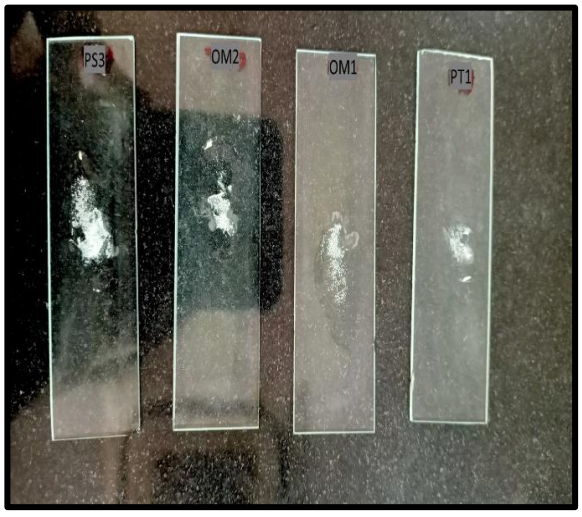


Fig 25: Catalase test

C. Lipase Assay

Table 11: Lipase Assay of the isolates

Isolate	Zone of Clearance
OM1	30 mm
OM2	15 mm
PT1	20 mm
PS3	32 mm

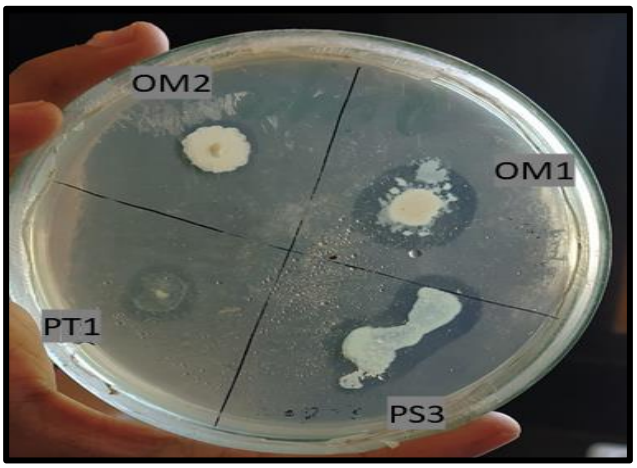


Fig 26 : Lipase assay : zone of clearance observed on the tributyrin agar plate confirming lipase activity

The selected isolates were subjected to tributyrin agar to determine their ability to produce lipase. The isolates OM1, OM2, PS3, PT1 showed a zone of clearance as in figure 26 confirming the production of lipase.

D. Protease Assay

Table 12:Protease Assay of the isolates

Isolate	Zone of Clearance
OM1	-
OM2	-
PT1	-
PS3	-

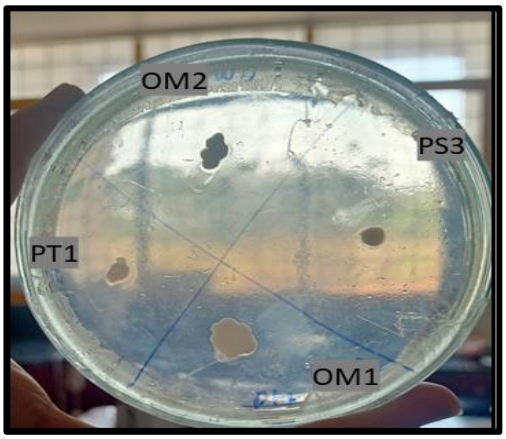


Fig 27: Protease assay: No zone of clearance indicates bacterial isolated cannot degrade casein

The selected isolates were subjected to determine protease assay. The protease producing will degrade casein and will show a zone of clearance as in figure 27. No isolates showed zone of clearance.

E. Amylase Assay

Table 13 :Amylase Assay of the isolates

Isolate	Zone of Clearance
OM1	-
OM2	10 mm
PT1	9 mm
PS3	-

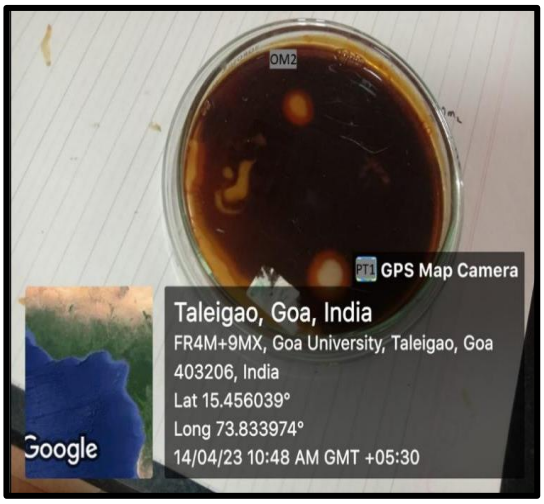


Fig 28: Amylase assay : Starch hydrolysis

The selected isolates were taken to determine their ability to degrade starch by producing amylase enzyme. Out of all the isolates , two isolates showed zone of clearance shown in figure 28.

DISCUSSION

A total of 11 isolates were obtained from different Soil and Water samples. On performing Drop Collapse, Oil displacement, E_{24} and BATH assay isolate PS3 and PT1 gave positive result for drop collapse assay, isolate OM1,OM2,PT1,PS1,PS2 and PS3 showed positive result for oil displacement assay, while for E_{24} assay isolate OM1,OM2,PT1,PS1,PS2 and PS3 gave positive result and for BATH assay except for DPW1 all the isolates were negative. Hence the extraction of Biosurfactant was proceeded using OM1, OM2,PS3 and PT1 isolate.

As reported by Jain *et al*.(1991), drop collapse assay and Oil displacement assay are sensitive to detect the production of biosurfactant. This was evident as the appearance of the flat drop as a result of collapsing, in drop collapse assay confirmed the biosurfactant production activity.

Rosenberg (1980) have reported that bacterial adhesion is crucial for growth on hydrocarbons when mixing is poor and the hydrophobic properties of the bacteria can enhance adhesion and promote growth on hydrocarbons. There is direct correlation between cell surface hydrophobicity and Biosurfactant production (Pendse, 2018).The bacterial isolates in the present study displayed a wide range of hydrophobicity, demonstrated using BATH assay, confirmed the ability of isolates to adhere to hydrocarbon.

Surfactants can display emulsifying activity thus enhancing the dispersion of hydrocarbons in water and further reduce the surface tension (Rahman & Banat, 2007).The rate and extent of hydrocarbon degradation can therefore be enhanced by biosurfactants (Oberbremer, 1990). In the present study, the isolated bacterial culture could emulsify oil and displayed results similar to the results reported by V Saravanam *et al*.(2012).However the emulsification index displayed was not as high as obtained in studies reported earlier (Aparna, 2012).

On extraction of crude biosurfactants from each of the isolate using chloroform: acetone solvent system, these biosurfactants were then purified using column chromatography. To produce biosurfactants with a high level of purification, column chromatography is used (Venkataraman, 2021).

On purification of the biosurfactant isolate OM1 showed 0.38g of biosurfactant production which was the highest followed by isolate PS3 ,OM2 AND PT1 that gave 0.33g,0.25g and 0.14 g of biosurfactant respectively.

Each of the purified biosurfactant was then analysed for their lipid ,protein and carbohydrate contents.All purified biosurfactant were negative for lipid . 0.42 mg/ml and 0.46mg/ml protein concentration was estimated in the biosurfactant produced by isolate OM1 and PT1 respectively.While the carbohydrate concentration was found to be 0.813mg/ml for isolate PT1.

The isolates that showed highest production of biosurfactant were then tentatively identified morphologically and biochemically . Isolate PS3 was found to be Gram positive cocci while other isolate OM1,OM2 and PT1 were Gram positive rods .

For biochemical test , it was performed with carbohydrate utilization kit. For Xylose and Dextrose all the isolates showed positive result ,while for lactose PT1 was positive and for galactose all isolates were positive except for PS3.

Finally enzyme assays were performed , wherein all isolates were negative for Oxidase and Protease assay as there was no zone of inhibition seen. For Catalase test all isolates were positive due to formation of effervescence , while for lipase assay OM1,OM2,PS3 and PT1 showed 30mm,15mm,32mm and 20 mm diameter for zone of clearance. Only two isolates OM2 and PT1 were positive for amylase assay with 10mm and 19mm diameter of zone of clearance for isolate OM2 and PT1.

SUMMARY

Different potential isolates were selected. Oil spreading technique, drop collapse assay, BATH assay, emulsification activity were performed for the screening of the biosurfactant producing bacteria.

Extraction, purification and characterization of the biosurfactant produced by the isolates explained its significance in enhancing the adherence of the bacteria to hydrocarbons and in emulsifying the crude oil.

The emulsification assay confirmed the ability of the isolates to emulsify the engine oil forming an emulsification layer. The maximum emulsification showed by the isolates was 20%.When subjected to BATH assay the ability of the isolates to adhere to the hydrocarbon was confirmed as optical density of the aqueous layer decreased. Maximum hydrophobicity of 62% was showed by the isolate PT1.

The dry weight of the biosurfactant produced by each isolate was determined and calculated after extracting the crude biosurfactant using chloroform: Acetone method.

Biochemical analysis and characterization were done of the selected isolates. Different tests like gram staining,motility were performed for their characterization along with the biochemical assays of the isolates using Carbohydrate utilization kit.

Enzyme assay was done wherein isolates were tested for lipse, amylase,protease,catalase and oxidase production by testing their zone of clearance.

FUTURE PROSPECTS

Because of its adaptability, biodegradability, ecological safety, and environmental acceptance, using biosurfactants is a desirable option. Biosurfactant use is limited because of its higher production costs, purifying requirements, and low yield. Renewable substrates, alternative purification processes, genetic and metabolic engineering tools, and statistical methodologies can all be used to manufacture biosurfactants with a high yield and cheaper cost. Evaluation of biosurfactants in situ and their impact on local microorganisms need to be done more thoroughly. (Bami M. S., 2022)

The use of biosurfactant-producing bacterial inoculants in the phytoremediation of hydrocarbon-polluted soil appears to be a potential strategy for increasing the effectiveness of this method. In order to use biosurfactants in phytoremediation on a broad scale, research is needed to determine whether or not they could be poisonous to plants. (Sivapathasekaran, 2010)

Although biosurfactants are supposed to be environmentally harmless, certain investigations showed that they can actually be detrimental to the environment in specific situations. However, cautious and controlled application of these intriguing surface active molecules would undoubtedly aid in the improved removal of harmful environmental contaminants and give us a clean atmosphere (Vijayakumar & Saravanan, 2015).

Biosurfactants should be scaled up and increased annually to reduce the price and allow entry into the mass market, and foam control should be improved to remove unwanted side products. Research should focus on exploring suitable microbes with high-level metabolic activities to make biosurfactants more economically viable for application in different sectors (S & Soudi M R, 2009).

Biosurfactants need to be studied to understand their biosurfactant pathway, safety, antimicrobial activities, medicinal and industrially important characteristics, and anti-cancer and anti-biofilm activities. (Kosaric, 2001)

Biosurfactants have a huge hidden potential not yet revealed, and future research will focus on increasing product efficiency, enhancing productivity, and reducing the high costs of fermentation and downstream processing. Governments should create incentives to promote economic production and market entry of biosurfactants (Sarubbo & Silva, 2022).

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APPENDIX

APPENDIX – I (MEDIA)

1) Nutrient Agar

Components	Concentration (Gram/litre)
Peptone	5
Meat extract	5
Sodium Chloride	3
Agar	15
Distilled water	1000 ml
pH	5 ± 0.2

2) Bushnell Haas Broth

Components	Concentration (Gram/litre)
Magnesium Sulphate	0.2
Calcium chloride	0.02
Monopotassium phosphate	1.0
Dipotassium phosphate	1.0
Ammonium nitrate	1.0
Ferric chloride	0.05
Distilled water	1000 ml
pH	0 ± 0.2

3) Tributyrin Agar

Components	Concentration (Gram/100 ml)
Yeast extract	0.5
Peptone	0.3
Tributyrin	1 ml
Distilled water	100
pH	7

4) Casein Agar

Components	Concentration (Grams/100 ml)
Nutrient Agar	100 ml
1 % Casein	1
Distilled water	100 ml
pH	7

5) Starch Agar

Components	Concentration (Grams/litre)
Meat Extract	3
Peptic digest of animal tissue	5
Starch	2

Agar	15
Distilled water	1000 ml
pH	7.2 ± 0.1

APPENDIX – II (BIOCHEMICAL REAGENTS)

1) Crystal Violet

Components	Concentration (Gram/100 ml)
Iodine	2
Absolute alcohol	20
Ammonium monohydrate	0.8
Distilled water	80

2) Destaining Solution

95% ethanol

3) Saffranine

Components	Concentration (Gram/100 ml)
Saffranine	0.25
Ethanol	10
Distilled water	90

4) Catalase reagent

3% Hydrogen Peroxide

5) 3% H_2O_2

Components	Concentration (ml)
Hydrogen Peroxide	3
Distilled Water	100

6) Oxidase reagent

Components	Concentration(Gram/litre)
Tetramethyl-p-phenylenediamine dihydrochloride	1
Distilled water	1000 ml

SOLUTIONS

1) 0.8% Saline

Components	Concentration (Grams/100 ml)
Sodium Chloride	0.8
Distilled water	100

2) 1.5% Saline

Components	Concentration (Grams/100 ml)
Sodium Chloride	1.5
Distilled water	100

3) Phosphate buffered saline

Components	Concentration (Grams/litre)
Sodium Chloride	8
Potasssium chloride	0.2
Disodium hydrogen phosphate	1.44
Potassium dihydrogen phosphate	0.22
Distilled water	1000 ml
pH	7.4

4) Ninhydrin solution

Components	Concentration (Grams/10 ml)
Ninhydrin	2
Absolute ethanol	10 l

5) Potassium dichromate (5%)

Components	Concentration (Grams/100 ml)
Potassium dichromate	5
Absolute ethanol	100 ml