

ISOLATION AND PRODUCTION OF SINGLE CELL OIL PRODUCING BACTERIA FROM SOIL SAMPLE

**Submitted in partial fulfilment of the requirements for the award of Bachelor of
Science degree in Microbiology**

by

ROSHINI M (40300052)



DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY(DEEMED TO BE
UNIVERSITY)**

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI- 600 119

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This is to certify that this Project Report is the bonafide work of
ROSHINI M(Reg No. 40300052) who carried out the project entitled
ISOLATION AND PRODUCTION OF SINGLE CELL OIL
PRODUCING BACTERIA FROM SOIL SAMPLE under our
supervision from DECEMBER 2022 to MARCH 2023


INTERNAL GUIDE


EXTERNAL GUIDE


HEAD OF THE DEPARTMENT

Submitted for viva voce examination held on 09-05-2023


Internal examiner


External examiner
Dr. R. R. R.

DECLARATION

I, ROSHINI M hereby declare that the Project Report entitled ISOLATION AND PRODUCTION OF SINGLE CELL OIL PRODUCING BACTERIA FROM SOIL SAMPLE done by me under the guidance of Dr. JAYSHREE NELLORE (Internal) at DEPARTMENT OF BIOTECHNOLOGY and Dr. D SARAVANAN (External) at CENTRE FOR ANIMAL LABORATORY AND RESEARCH, SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY is submitted in partial fulfilment of the requirements for the award of Bachelor of Science degree in B.Sc.MICROBIOLOGY.

DATE: 3-05-2023

PLACE: CHENNAI



SIGNATURE OF THE CANDIDATE

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ABSTRACT

A brief overview is provided of the microbial oils (single cell oils - SCOs) that have been, or are still being, produced commercially. SCO was originally intended to denote the edible oils of microorganisms, that is the triacylglycerol fraction of the total cell lipid, and would thus be equivalent commercial plant and animal oils. Microbial lipids or single cell oils, produced by oleaginous microorganisms such as algae, fungi, and bacteria, are a promising source as well. These single cell oils can be used for many valuable chemicals with applications not only for nutrition but also for fuels and are therefore an ideal basis for a bio-based economy. Natural sources of oils and fatty acids are derived from plants, animals, and microorganism. The oils or lipids, thus produced from microorganisms are known as —Single Cell Oil. This present study aimed to isolate lipid accumulating bacteria for the production and optimization of single cell oil. In this preliminary investigation three soil samples were collected in around Chennai and Vellore Districts; 40 different bacterial strains were isolated and screened by Sudan black stain of that 8 strains showed maximum lipid accumulation. They were used for lipid production of by shake flask method. The lipid was extracted by Folch method using cell dry matters. Among 8 strains (B5) was identified as the efficient producers which produce about 23% of lipid content. It was confirmed as lipid by solubility, saponification test. Further the lipid was subjected to antimicrobial activity against human pathogens by well diffusion method. The strain B5 showed antibacterial activity against Lacto *Bacillus sp.*, *Staphylococcus sp.*, *Proteus sp.*, vibrio sp., E coli sp., Salmonella ., Candida sp., Pseudomonas sp., In optimization, the potent strains showed maximum lipid accumulation at 15.5 g/L of Dextrose, 0.4 g/L of ammonium nitrate, pH 8 and 72hours incubation time. The potential strain was identified as *Cellulomonas sp.*

Keywords: Single cell oil, Dry cell matter, lipid production, potential strain, optimization, Soil samples.

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LIST OF ABBREVIATIONS AND SYMBOLS:

gms	-	Grams
ml	-	Millilitre
mg	-	Milligrams
mg/ml	-	Milligram per millilitre
mM	-	Millimolar
%	-	Percentage
g/l	-	Gram per Litre
hrs	-	Hours
μL	-	Microlitre
°C	-	Degree Celsius
MSM	-	Mineral salt medium
pH	-	Power of hydrogen
CaSO ₄	-	Calcium sulphate
NaCl	-	Sodium Chloride

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CHAPTER 1

INTRODUCTION:

Single cell oil:

Single cell oil (SCO) is a valuable source of lipids that can be used for various applications such as biodiesel production, food and feed additives, and nutraceuticals. Oleaginous bacteria are known for their ability to accumulate large amounts of lipids in their cells, making them an attractive candidate for SCO production. The optimization of SCO production by oleaginous bacteria isolated from oil-contaminated environments is an important area of research (Murugan T et, al, 2012).

The production of SCO by oleaginous bacteria can be optimized by controlling various parameters such as carbon source, nitrogen source, temperature, pH, and dissolved oxygen concentration (Arshad M.U. et, al, 2007). The carbon source is the most important factor that affects the growth and lipid accumulation of oleaginous bacteria. Different carbon sources such as glucose, sucrose, fructose, and glycerol can be used for the cultivation of oleaginous bacteria. The nitrogen source is also an important factor that affects the growth and lipid accumulation of oleaginous bacteria. Organic nitrogen sources such as peptone, yeast extract, and tryptone are preferred over inorganic nitrogen sources (Abghari, A and Chen, S.,2014).

The temperature, pH, and dissolved oxygen concentration are other important parameters that affect the growth and lipid accumulation of oleaginous bacteria. The optimum temperature for most oleaginous bacteria is between 25-30°C, and the optimum pH is between 6-8. The dissolved oxygen concentration is also an important factor that affects the growth and lipid accumulation of oleaginous bacteria. The low dissolved oxygen concentration is preferred for the cultivation of oleaginous bacteria to avoid the formation of reactive oxygen species that can damage the cells. (Manabendra Mandal and Rupam Katakai 2023).

The isolation of oleaginous bacteria from oil-contaminated environments can be done by enrichment culture techniques. The samples can be collected from oil-contaminated soil, water, or sludge, and then the bacteria can be enriched by using specific carbon sources (Pritam Bardhan et, al, 2023).

In conclusion, the production and optimization of SCO by oleaginous bacteria isolated from oil-contaminated environments is a promising area of research. The optimization of various parameters such as carbon source, nitrogen source, temperature, pH, and dissolved oxygen concentration can lead to the efficient production of SCO (Bligh and Dyer 1959).

The isolated bacteria were found to belong to the genera *Acinetobacter*, *Pseudomonas*, and *Rhodococcus*(Ratledge 1982). The study shows that oleaginous bacteria isolated from oil-contaminated environments can be used for the efficient production of SCO.

Microbial oils, also known as single-cell oils (SCO), are a type of oil that is produced by microorganisms such as bacteria, fungi, and microalgae. These oils are composed of various fatty acids, like the oils found in plants and animals, and can be used for a wide range of industrial applications (Woodbine 1959).

Microbial oils have gained significant interest in recent years due to their potential as a sustainable and renewable source of energy. They can be produced using a variety of waste products, such as agricultural waste or wastewater, and do not require large areas of land or resources like traditional crop-based biofuels. Study of Oil Degrading Bacteria Isolated From (Arpita, et, al, 2015).

Furthermore, microbial oils can be tailored to meet specific industrial needs by adjusting the growth conditions of the microorganisms, such as temperature, pH, and nutrient availability. This makes microbial oils a highly versatile and customizable source of oil. Overall, microbial oils have the potential to play an important role in meeting the world's growing demand for sustainable energy and reducing our reliance on fossil fuels (Arjuna 2014).

Oleagenicity could be defined as the capacity of an organism to synthesize and accumulate significant amounts of lipids. The main trigger for TAG accumulation by *Rhodococcus* is the limitation of the nitrogen source and an excess of the available carbon source, or a high C/N ratio in the culture media. The imbalance between C and N in cells promotes profound changes in the metabolism with a redirection of the carbon flux to lipogenesis (Héctor and Alvarez 2012).

Oleaginous Microorganisms:

Oleaginous microorganisms are microorganisms that can accumulate large amounts of lipids or fats within their cells. These microorganisms can be bacteria, fungi, or microalgae. Biodiesel production from oleaginous microorganisms (Meng, et, al, 2009)

Microbial oils might become one of the potential feedstocks for biodiesel production in the future. To reduce the cost of microbial oils, exploring other carbon sources instead of glucose is very important especially for such oils applied to biodiesel production. It was reported that xylose, glycerol, corn straw, and other agricultural and industrial wastes could be used as the carbon sources for microbial oils accumulation (Liu and Sun 2005).

Oleaginous microorganisms have gained significant attention in recent years due to their potential to produce biofuels, as well as other high value products such as pharmaceuticals and nutraceuticals. The lipids accumulated by these microorganisms can be converted into biodiesel, which is a renewable and environmentally friendly alternative to traditional fossil fuels (C Ratledge and JP Wynn 2002).

Some examples of oleaginous microorganisms include the yeast species *Rhodospiridium torulosus*'s and *Yarrowia lipolytica*, as well as the microalgae species *Chlorella*, *Nannochloropsis*, and *Dunaliella*. These microorganisms can be grown in a variety of environments, including wastewater, agricultural runoff, and industrial waste streams, making them a promising source of biofuels and other valuable products (HU Cho and JM Park 2018)

Oleaginous microorganisms can convert numerous agro-industrial and municipal wastes into storage lipids (single cell oil (SCO) and are therefore considered as potential biofuel producers. While from an environmental and technological point of view the idea to convert waste materials into fuels is very attractive, the production cost of SCO is not currently competitive to that of conventional oils due to the low productivity of oleaginous microorganisms in combination with the high fermentation cost. Current strategies used to optimize the lipid-accumulating capacity of oleaginous microorganisms include the overexpression of genes encoding for key enzymes implicated in fatty acid (Dourou, et, al, 2018).

Oleaginous Bacteria:

Oleaginous bacteria are microorganisms that can accumulate large amounts of lipids, or oils, within their cells. These bacteria are of interest in various biotechnological applications, including the production of biofuels and other value-added products (Qadeer, et, al, 2017).

Some examples of oleaginous bacteria include species of the genera *Rhodococcus*, *Nocardia*, and *Acinetobacter*. These bacteria have been studied extensively for their ability to produce lipids, which can be converted into biodiesel or other renewable fuel (Shruthi, et, al, 2014).

Oleaginous bacteria can grow on a variety of substrates, including sugars, oils, and other organic compounds. They are able to accumulate lipids within specialized structures known as lipid droplets, which can account for up to 90% of the cell's dry weight (Kumar and Thakur 2018)

Research in this area is ongoing, as scientists continue to explore the potential of oleaginous bacteria for sustainable production of biofuels and other valuable products (Qadeer, et, al, 2017).

oleaginous bacteria also have an advantage over vegetable oil in fatty acid composition and fatty acids composition can modify to the desired level by changing the source of nutrients or substrates and metabolic engineering strategy. It also reported that oleaginous bacteria efficiently use high-carbon waste for lipid production (Koreti, et, al, 2022).

Oleaginous rhodococci are promising microbial cell factories to produce lipids to be used as fuels and chemicals. Cells could be engineered to create strains capable of producing high quantities of oils from industrial wastes and a variety of high-value lipids. The comprehensive understanding of carbon metabolism and its regulation will contribute to the design of a reliable process for bacterial oil production. Bacterial Oleagenicity requires an integral configuration of metabolism and regulatory processes rather than the sole existence of an efficient lipid biosynthesis pathway (Héctor, et, al, 2009)

Oleaginous Yeast:

Oleaginous yeast refers to a type of yeast that can accumulate large amounts of lipids or oils within its cells. These yeasts are of interest in industrial applications, particularly in the production of biofuels, since they can be used to produce high yields of lipid-based feedstocks (Spagnuolo, et, al, 2019).

Oleaginous yeasts are often classified as non-conventional yeasts, meaning they are not commonly used in traditional food and beverage fermentation processes. They have been studied extensively in recent years due to their potential use in biodiesel production, and as a source of high-value fatty acids and other lipids (S Papanikolaou and G Aggelis, 2011) Some examples of oleaginous yeasts include species of the genera *Rhodospiridium*, *Lipomyces*, and *Cryptococcus*. These yeasts have been shown to accumulate lipids up to 70% of their dry weight, making them an attractive option for industrial lipid production (Sitepu, et, al, 2014).

Recent years have seen an increase in investigation of basidiomycetous oleaginous yeasts. For example, the genome of basidiomycetous yeast *Rhodospiridium torulosus*'s was sequenced, and genetic tools are being developed .The metabolic and genetic factors that are responsible for increased fatty acid synthesis and lipid accumulation in oleaginous species have been identified in some yeast species (Luis A Garay, et, al, 2007).

The phylogenetic diversity of oleaginous species, beyond the few species commonly studied; and physiological characteristics that should be considered when choosing yeast species and strains to be utilized for conversion of a given type of plant biomass to oleochemicals. Standardized terms are proposed for units that describe yeast cell mass and lipid production. the phylogenetic diversity of oleaginous species, beyond the few species commonly studied; and physiological characteristics that should be considered when choosing yeast species and strains to be utilized for conversion of a given type of plant biomass to oleochemicals. Standardized terms are proposed for units that describe yeast cell mass and lipid production (Garay, et, al, 2014).

Oleaginous algae :

Microalgae can use carbon dioxide as the carbon sources and sunlight as the energy for photoautotrophic culture, or use organic carbon as the carbon sources instead of sunlight for heterotrophic culture. And they can also use light with organic carbon as supplementary carbon sources for mixotrophic culture. Scaling up for autotrophic microalgae is more complicated, since light is needed during the cultivation process. To minimize the cost, oil production from microalgae must rely on available free sunlight, despite daily and seasonal variations in light levels (Thevenieau and Jean-Marc, 2022).

Heterotrophic microalgae are easily cultivated and controlled in normal fermenters. But they require organic carbon sources for oil accumulation, which might limit the application of such microalgae used for high value products. The average lipid contents of algal cells vary between 1% and 70% but can reach 90% of dry weight under certain conditions. Oil levels of 20–50% are quite common (Peng X-W and Chen H-Z. 2007).

Microalgae, such as *Chlorophyta*, *Bacillariophyceae*, have higher oil contents, and they are easier to cultivate, especially chlorella, which could be applied to industrial production, so they are the ideal energy microalgae resources. acquired high lipid content of heterotrophic chlorella by heterotrophic transformation cell engineering technology, containing the lipid compounds as high as 55% of cell dry weight, which was 4 times of the autotrophic one (14%) (Miao XL and Wu QY 2004).

The growth of cells and lipid accumulation by algae under phototrophic conditions is influenced by the intensity of light, pH, dissolved oxygen concentration, fraction of carbon dioxide in sparing gas, concentration of nutrients such as nitrogen, phosphorous, silicon, and iron, and presence of organic carbon sources (Liang M-H and Jiang J-G, 2013).

To enhance the economic feasibility of algal oil production, biomass productivity (production per unit volume per unit time), cellular lipid content, and overall lipid productivity are the three key parameters that need to be improved. These requirements are not always compatible, and in general, conditions favouring a high growth rate of cells result in a low lipid fraction in the cells and vice versa (O'Brien RD.2008).

SOIL BACTERIA :

Bacteria are some of the smallest and most abundant microbes in the soil. In a single gram of soil, there can be billions of bacteria. There are an estimated 60,000 different bacteria species, most which have yet to be even named, and each has its own roles and capabilities. Most live in the top 10cm of soil where organic matter is present. Furthermore, the diversity of the microbial community in soil is closely related to the function and structure of its surrounding ecosystem, and is one of the components to maintain soil productivity (Mu Peng, et, al, 2022).

Some bacteria species are very fragile and can be killed by slight changes in the soil environment. Other species are extremely tough, able to withstand severe heat, cold or drying. Some can lie dormant for decades waiting for favourable conditions. Others can extract nitrogen directly from the air or break down some toxic substances. Populations of microbes can boom or bust in the space of a few days in response to changes in soil moisture, soil temperature or carbon substrate. To gain advantage in this process, many microbes release antibiotic substances to suppress competitors. In this way some species can suppress other disease-causing microorganisms. (Hoorman, et, al, 2011).

Microbes in the soil are directly tied to nutrient recycling especially carbon, nitrogen, phosphorus, and sulphur. Bacteria are a major class of microorganisms that keep soils healthy and productive. Most soils are simply a graveyard for dead bacteria cells. Bacteria are so simple in structure that they have often been called a bag of enzymes and/or soluble bags of fertilizer. Bacteria population may easily double in 15-30 minutes. Flourishing microbial populations increase soil productivity and crop yields over time. (Dick, R. 2009).

Bacteria are the smallest and most hardy microbe in the soil and can survive under harsh or changing soil conditions. Bacteria are only 20–30% efficient at recycling carbon, have a high N content (10 to 30% N, 3–10 C:N ratio), a lower C content, and a short life span. There are basically, four functional soil bacteria groups including decomposers, mutualists, pathogens, and lithotrophs. Decomposer bacteria consume simple sugars and simple carbon compounds, while mutualistic bacteria form partnerships with plants including the nitrogen-fixing bacteria (*Rhizobia*) (Reeder, R.C., 2012).

Bacteria can also become pathogens to plants and lithotrophic bacteria convert nitrogen, sulphur, or other nutrients for energy and are important in nitrogen cycling and pollution degradation. Actinomycetes are classified as bacteria but are very similar to fungus and decompose recalcitrant (hard to decompose) organic compounds. Bacteria can adapt to many different soil microenvironments (wet vs. dry, well oxygenated vs. low oxygen). They also can alter the soil environment to benefit certain plant communities as soil conditions change. (Sylvia, et al, 2005)

The most effective oil producing bacterial species present in soil bacteria are species of Cellulomonas, Arthrobacter, Acromicrobium, Pseudomonas.

Description of Cellulomonas:

Cellulomonas persica (per«si.ca. L. fem. adj. *Persica* Persian, from Persia, classical name of Iran, where the organism was isolated). Irregular, straight or slightly curved rods, which occasionally exhibit typical coryneform branching. Cells are motile, with one to four peritrichous flagella. Gram-positive, but readily decolourized. Colonies are light yellow, ca. 1 mm in diameter, circular, convex, and glossy. Optimal growth temperature is 30 °C. Optimal pH for growth is 7. Additional characteristics that differentiate this species include the ability to produce acid from dextrin and the inability to utilize d-ribose or raffinose. Starch and gelatin are hydrolysed, the latter weakly. Nitrate is reduced to nitrite; DNase produced. Urea hydrolysed as source in cellulose fermentation. Cell wall is of the L-Orn-d-Asp type and contains the sugars rhamnose, glucosamine and minor amounts of mannose (Stackebrandt, et al, 1986).

Description of Arthrobacter:

Gram-stain-negative, rod-shaped (about 0.5–0.6 mm wide and 1.1–1.8 mm long). Colonies on LB medium appeared to be light yellow, round, and convex with regular edges. The genus *Arthrobacter* is a member of the family Micrococcaceae and compared to other genera of the family, it contains the highest number of species. The genus cannot be considered monophyletic because within the clade embracing all *Arthrobacter* species also members of other genera of the family are present. Based on quinone system and peptidoglycan structure, the genus *Arthrobacter* can be subdivided into two major groups. One group is characterized by a quinone system with monosaturated menaquinone [MK-8(H₂) and/or MK-9(H₂)] and peptidoglycan type A3α. The second group contains completely unsaturated menaquinones (MK-8, MK-

9, and/or MK-10) and peptidoglycan type A4 α . Combining chemotaxonomic and 16S rRNA based data, the genus can be subdivided at least into 11 subgroups. Most established species have been isolated from soil and sediments, but some were recovered from clinical specimens, as well. *Arthrobacter* are heterotrophic bacteria that do not require fastidious growth conditions. Most of them are mesophilic with growth optima below 30 °C, but some strains isolated from cold environments (Arctica, Antarctica, glaciers) are psychrotolerant or even psychrophilic. Numerous *Arthrobacter* have been studied that are able to degrade harmful compounds such as 4-chlorophenol, 4-fluorophenol, 4-nitrophenol, or phenanthrene. Some strains have been identified as a source of enzymes including cold-adapted β -galactosidases. Since the genus *Sinomonas* harbors three former *Arthrobacter* species, its species will be dealt with here without separating from *Arthrobacter* species (Zhenshan Deng†, et al, 2012)

Description of *Acromicrobium* :

Small irregular rods (mostly 0.3–0.6 μ m in diameter) to coccoid forms. Elongated rods, branched filamentous elements, and V-forms rarely occur. A marked rod-to-coccoid growth cycle can be observed. Endospores are not formed. Nonmotile or motile. Extracellular diffuse haloes or capsules can be produced. Gram-positive cell-wall chemotype, non-acid-fast. Colonies are non-pigmented, yellow, or beige to amber-beige in colour. Chemo-organotrophs, metabolism primarily respiratory with oxygen as the terminal electron acceptor. Mostly catalase-positive; negative catalase reaction may occasionally occur. Nitrate can be reduced by some species. Acids are produced oxidatively from some carbohydrates. Generally, nutritionally exacting; grows aerobically on complex media based on peptone, yeast extract, and similar sources of nutrients. Some species can grow in chemically defined media. Mesophilic, optimal growth at 25–37°C; growth range is ~ 4–42°C. Mostly non-halophilic or slightly halophilic; some species are salt-requiring. Prefer a neutral to mildly alkaline pH. The cell-wall peptidoglycan contains L-diaminopimelic acid, along with alanine, glutamic acid, and glycine (Ratkowsky, et al, 1982).

CHAPTER 2

Review of Literature

1. This study suggests that the addition of *Teucrium polium* L. aqueous extract to the growth medium can increase the production of single cell oil by *Yarrowia lipolytica*. Single cell oil, which is produced by certain microorganisms, has potential as a renewable and sustainable source of lipid-based products, including biofuels and food additives. This study highlights the potential of using plant extracts to enhance the production of single cell oil and promote sustainable production practices. Lipid accumulation of *Candida* 107, grown at dilution rates from 0.03 to the maximum of 0.21/h, with carbon, nitrogen, phosphate, and magnesium limitations in a chemostat, was maximal at about 40% (wt/wt) with nitrogen-limited medium at a dilution rate of 0.06/h, giving an efficiency of substrate conversion of 22 g of lipid per g of glucose consumed. At higher dilution rates the lipid content decreased. With carbon-limited growth, the highest lipid content (14%, wt/wt) was at the maximum dilution rate. High lipid contents also occurred with phosphate + nitrogen as double limitations of growth, with the lipid content of the yeast (about 35%, wt/wt) continuing to be near maximum at dilution rates also near maximum (0.17/h), thus giving the highest specific rate of lipid formation of any growth conditions (0.59 g of lipid/g of yeast per h). However, the efficiency of substrate utilization was only 5.2 g of lipid formed per 100 g of glucose consumed (Aggelis and Komaitis 1999).
2. The study you are referring to focuses on the production of microbial oil by newly isolated oleaginous yeasts using various carbon sources. The authors of the study are Sofia Maina, Chrysanthi Pateraki, Nikolaos Kopsahelis, Spiros Paramithiotis, Eleftherios H. Drosinos, Seraphim Papanikolaou, and Apostolis Koutinas. Microbial oil, also known as single-cell oil, is a type of oil that is produced by microorganisms such as yeasts, fungi, and bacteria. It can be used as a sustainable alternative to traditional vegetable oils in various industries, including food, cosmetics, and biofuel production. In this study, the researchers isolated six oleaginous yeasts from various sources, including soil, sewage sludge, and plant material. They then evaluated the ability of these yeasts to produce microbial oil using different carbon sources, including glucose, xylose, sucrose, and glycerol. The results showed that all the isolated yeasts were able to produce microbial oil,

with some yeasts producing higher yields than others. The researchers also found that the carbon source used had a significant impact on the amount and composition of the microbial oil produced. Overall, the study suggests that newly isolated oleaginous yeasts have the potential to be used for the sustainable production of microbial oil, and that the choice of carbon source can play an important role in optimizing the production process (Sofia Maina, et, al, 2000).

3. The authors collected soil samples from an oil-contaminated area and isolated 32 hydrocarbon-degrading bacteria strains. They then screened these strains for their ability to degrade crude oil by measuring their growth and hydrocarbon degradation rates. The authors found that four strains of bacteria, identified as *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Bacillus cereus*, and *Staphylococcus saprophyticus*, showed the highest rates of crude oil degradation. The study also investigated the effects of different environmental factors, such as pH, temperature, and salinity, on the growth and hydrocarbon degradation of the selected bacterial strains. The results showed that the optimum conditions for the growth and crude oil degradation of the strains were pH 7.0, temperature 30°C, and salinity 2%. Overall, the study provides valuable information on the isolation, screening, and crude oil degradation characteristics of hydrocarbon-degrading bacteria for the treatment of oily wastewater, which could be useful in the development of bioremediation technologies for oil-contaminated environments (Xiumei Tian, et, al, 2015).
4. The article discusses various aspects of microbial production of different compounds, including flavors and fragrances, fats and oils, dyes, bioplastics (PHAs), polysaccharides, pharmacologically active substances from marine microbes, anti-cancer agents, and microbial biotransformation. The authors describe the importance of these compounds in various industrial and medical applications and discuss the potential of microbial production for sustainable and efficient manufacturing. Overall, the article provides a comprehensive overview of microbial production of various compounds and highlights the potential of this approach to produce important compounds with industrial and medical applications (Bhalla, et , al, 2007).
5. The study aimed to develop a simple and efficient method for the purification of arachidonic acid (AA) from fungal single cell oil (SCOs) obtained from the fungus *Mortierella alpina*. AA is a polyunsaturated fatty acid (PUFA) that has several

important physiological functions, including its role as a precursor for the synthesis of eicosanoids, which are potent signaling molecules in the body. The authors used a combination of chromatographic techniques, including silica gel column chromatography and preparative high-performance liquid chromatography (HPLC), to purify AA from the SCO extract. They optimized the purification conditions, such as the solvent system, column size, and flow rate, to achieve a high yield of AA with a purity of over 99%. The authors also characterized the purified AA using various analytical techniques, including gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), and Fourier transform infrared (FT-IR) spectroscopy. They confirmed the identity of AA and showed that the purified AA had a high degree of unsaturation and a low level of impurities. Overall, the study provides a reliable and efficient method for the purification of AA from fungal SCOs, which could have important applications in the production of AA for nutritional and pharmaceutical purposes (Vali, et al, 2003).

6. The study aimed to isolate and identify bacterial strains capable of producing biosurfactants from oil reservoirs. Biosurfactants are natural surfactants produced by microorganisms that have applications in various industrial processes, including oil recovery, bioremediation, and food processing. The authors collected soil and water samples from several oil reservoirs in Iran and screened them for biosurfactant-producing bacteria using a drop-collapse test. They identified several bacterial strains that showed strong biosurfactant production activity and further characterized them using various biochemical and molecular methods. The authors showed that the isolated bacterial strains belonged to various genera, including *Pseudomonas*, *Bacillus*, and *Staphylococcus*. They also demonstrated that the biosurfactants produced by these strains had a low critical micelle concentration (CMC) and high emulsification activity, indicating their potential for use in oil recovery and other industrial applications. Overall, the study provides evidence for the presence of biosurfactant-producing bacteria in oil reservoirs and highlights their potential to produce biosurfactants with industrial applications. The isolation and characterization of these bacterial strains could lead to the development of new and more efficient biosurfactants for various industrial processes (Tabatabaee, et al 2005).

7. The paper highlights the potential of spore-forming bacteria as a promising tool in heavy oil recovery, particularly in harsh environments where other methods are not feasible. The paper begins by providing an overview of heavy oil reservoirs and the challenges associated with their exploitation. It then introduces the concept of MEOR, which involves the use of microorganisms to improve oil recovery. The authors explain how spore-forming bacteria can be effective in MEOR due to their ability to withstand harsh environmental conditions and remain dormant until suitable conditions for growth are available. The authors then discuss the different mechanisms by which spore-forming bacteria can enhance oil recovery, including the production of biosurfactants, biopolymers, and gases, as well as the reduction of interfacial tension between oil and water. The paper also highlights the importance of selecting the appropriate bacterial strain for MEOR, based on factors such as the reservoir conditions, the type of oil, and the properties of the bacteria. The review further discusses the various laboratory and field studies that have been conducted to evaluate the effectiveness of spore-forming bacteria in MEOR. The authors summarize the findings of these studies, which indicate that spore-forming bacteria can significantly improve oil recovery in heavy oil reservoirs. Finally, the paper discusses the challenges associated with the commercialization of MEOR using spore-forming bacteria, including the need for further research and development, regulatory issues, and economic viability. Overall, the review provides a valuable insight into the potential of spore-forming bacteria in MEOR, highlighting the need for further research and development to fully exploit their potential in heavy oil recovery (Biji Shibulal, et al, 2008).
8. Oleaginous *Rhodococcus* spp. are bacteria that can accumulate large amounts of intracellular lipids, which make them promising candidates to produce biofuels and other high-value products. The metabolism of these bacteria is complex and involves multiple pathways for the synthesis and degradation of fatty acids, as well as the mobilization of energy from various carbon sources. One of the key features of the metabolism of oleaginous *Rhodococcus* spp. is their ability to utilize a wide range of carbon sources, including both simple and complex compounds such as glucose, glycerol, fatty acids, and even aromatic compounds. This versatility is due to the presence of multiple transporters and enzymes that allow the bacteria to convert these substrates into the precursors needed for lipid biosynthesis. The biosynthesis of lipids in oleaginous *Rhodococcus* spp. is a complex process that

involves the conversion of acetyl-CoA into long-chain fatty acids through a series of enzymatic reactions. The fatty acids are then incorporated into triacylglycerols (TAGs) and stored in lipid droplets within the cytoplasm. The TAGs can be later mobilized and utilized as an energy source under nutrient-limiting conditions. In addition to lipid biosynthesis, oleaginous *Rhodococcus* spp. also possess multiple pathways for the degradation of fatty acids, which allow them to obtain energy from these compounds. These pathways involve the beta-oxidation of fatty acids, which leads to the production of acetyl-CoA and other intermediates that can be further oxidized in the citric acid cycle. Overall, the metabolism of oleaginous *Rhodococcus* spp. is highly adaptable and allows these bacteria to thrive in a wide range of environments. Understanding the metabolic pathways involved in lipid biosynthesis and degradation in these bacteria is crucial for the development of efficient biotechnological processes to produce biofuels and other high-value products (Hector, et al, 2008).

9. The article "An Efficient Method for the Purification of Arachidonic Acid from Fungal Single-Cell Oil (ARASCO)" describes a new method for isolating and purifying arachidonic acid (ARA) from a fungal single-cell oil (SC-Oil) source. ARA is an important polyunsaturated fatty acid that has a variety of physiological functions and is used in the production of pharmaceuticals, dietary supplements, and infant formulas. The authors first extracted the SC-Oil from the fungal biomass and then subjected it to a series of purification steps, including saponification, acidification, and chromatography. They found that using a combination of silver nitrate-impregnated silica gel column chromatography and preparative high-performance liquid chromatography (HPLC) was an effective way to isolate and purify ARA from the SC-Oil. The purity of the final ARA product was confirmed using gas chromatography-mass spectrometry (GC-MS) analysis. The authors also evaluated the yield of the purification process and found it to be higher than previous methods reported in the literature. Overall, this study provides a new, efficient method for isolating and purifying ARA from fungal SC-Oil that could be useful in the production of ARA-based products (Shaik Ramjan Vali, et al, 2016).
10. The article "FTIR spectroscopy for evaluation and monitoring of lipid extraction efficiency for oleaginous fungi" by Forfang et al. (2017) describes the use of Fourier Transform Infrared (FTIR) spectroscopy as a tool for evaluating and monitoring the efficiency of lipid extraction from oleaginous fungi. The authors used three different

solvents (chloroform, methanol, and ethanol) for lipid extraction and analysed the extracted lipids using FTIR spectroscopy. They found that the peak intensity of the ester carbonyl stretching vibration (1745 cm^{-1}) was directly proportional to the amount of extracted lipids. The study also demonstrated that FTIR spectroscopy could be used to monitor the lipid extraction process in real-time. Overall, this study suggests that FTIR spectroscopy can be a valuable tool for monitoring and optimizing lipid extraction processes, which is important to produce biofuels and other value-added products from oleaginous fungi (Forfang, et al, 2017).

11. The article focuses on the production of microbial lipids from beet molasses, which is a by-product of sugar beet processing. The authors conducted experiments to evaluate the potential of using beet molasses as a substrate for lipid production by microorganisms. The authors found that beet molasses could be used as a source of carbon and energy to produce microbial lipids. They identified several microorganisms that could produce lipids from beet molasses, including *Candida lipolytica*, *Candida tropicalis*, and *Rhodotorula glutinis*. The article provides important insights into the use of by products from the sugar industry as potential sources of microbial lipids. The production of microbial lipids has gained attention in recent years as a potential source of biofuels and other valuable products. The findings of this study could have implications for the development of sustainable and eco-friendly industrial processes (Sabry, et al, 1990).
12. The study aimed to screen bacterial isolates from marine sponges for their potential to produce single cell oil (SCO) and polyunsaturated fatty acids (PUFA). SCO and PUFA have several industrial applications, including in the production of biodiesel and nutritional supplements. The researchers collected samples from six different marine sponges and isolated 64 bacterial strains. They then screened these strains for their ability to produce SCO and PUFA by growing them in nutrient-rich media and analyzing the fatty acid profiles of the cells. The results showed that 33 of the 64 bacterial strains could produce SCO and/or PUFA. The most promising strain was identified as *Pseudomonas aeruginosa*, which produced high levels of both SCO and PUFA. Overall, the study demonstrated that bacterial isolates from marine sponges have the potential to be used as a source of SCO and PUFA, which could have important industrial applications (Patnayak and Sree, 2004).
13. The article discusses the process of fatty acid biosynthesis in microorganisms that are being used to produce single cell oil. Single cell oil refers to the oil that is

produced by certain microorganisms, such as yeasts, fungi, and algae, which can be used as a source of biofuel and other industrial applications. The article provides an overview of the different pathways involved in fatty acid biosynthesis in microorganisms, including the type II fatty acid synthesis pathway and the polyketide synthase pathway. The article also discusses the regulation of fatty acid biosynthesis and the factors that influence the accumulation of oil in microorganisms. Overall, the article provides valuable insights into the process of fatty acid biosynthesis in microorganisms that are being used to produce single cell oil, and highlights the potential of these microorganisms as a source of renewable energy and other industrial applications (Ratlidge, 2004).

14. The article "Microbial lipid production from potato processing wastewater using oleaginous filamentous fungi *Aspergillus oryzae*" published in Water Research in 2013 investigated the potential of using *Aspergillus oryzae*, an oleaginous filamentous fungi, to produce microbial lipids from potato processing wastewater. The study found that *Aspergillus oryzae* was able to grow and accumulate lipid content in potato processing wastewater under optimized conditions. The optimal conditions for lipid production were found to be pH 6.0 and a temperature of 30°C. The study also found that the lipid content of *Aspergillus oryzae* increased with increasing concentrations of potato processing wastewater. The lipid content of *Aspergillus oryzae* was found to be as high as 31.1% of its dry weight, which is comparable to the lipid content of other oleaginous microorganisms such as algae. The fatty acid profile of the microbial lipids produced by *Aspergillus oryzae* was also analysed and found to be suitable for biodiesel production. Overall, the study suggests that *Aspergillus oryzae* has the potential to be used as a cost-effective and sustainable source of microbial lipids for biodiesel production, particularly in the context of potato processing wastewater treatment. The study highlights the importance of exploring the use of unconventional feedstocks for microbial lipid production in order to improve the sustainability of the biofuels industry (oryzae Warer, 2013).
15. The authors highlight the importance of lipids in human nutrition and the growing demand for functional foods and nutraceuticals. They discuss how microbial lipids, which are lipids produced by microorganisms such as bacteria, yeast, and microalgae, have gained attention as a potential source of food supplements due to their unique characteristics. The article provides an overview of various microbial

lipids, including triglycerides, phospholipids, sphingolipids, and sterols, and their potential applications in food supplements. The authors discuss how microbial lipids can be modified to enhance their nutritional value and functionality, such as improving their fatty acid profile or increasing their bioavailability. The article also discusses the different methods for producing microbial lipids, including fermentation and bioconversion processes, and their advantages and limitations. The authors highlight the potential of using various substrates, including agro-industrial wastes and by-products, as feedstocks for microbial lipid production, which could contribute to the sustainability of the process. Furthermore, the article discusses the potential health benefits of microbial lipids, including their role in promoting cardiovascular health, brain function, and immune system function. The authors also highlight the potential of microbial lipids in weight management and as a source of omega-3 fatty acids, which are essential for human health. In conclusion, the article "Microbial lipids as potential source to food supplements" discusses the potential of microbial lipids as a source of food supplements, highlighting their unique characteristics, production methods, and potential health benefits. The article provides valuable insights into the potential applications of microbial lipids in the development of functional foods and nutraceuticals, and their role in promoting human health (Bélignon, 2016).

16. The article you are referring to is a study conducted by M.J. Hall and C. Ratledge and published in the journal *Applied and Environmental Microbiology* in 1977. The study investigated the lipid accumulation in an oleaginous yeast, *Candida* 107, grown on glucose under various conditions in a one- and two-stage continuous culture. The researchers found that the lipid accumulation in *Candida* 107 was affected by various factors such as the glucose concentration, the dilution rate, the aeration rate, and the nitrogen source. They also found that a two-stage continuous culture system was more effective in producing lipid than a one-stage system. Overall, the study provided insights into the conditions that can optimize lipid production in *Candida* 107, which has potential applications in the production of biodiesel and other biofuels (Hall and Ratledge, 1977).
17. The article *Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production* focuses on the biochemistry of the production of single cell oil (SCO) by oleaginous yeasts. SCO refers to the oil that is accumulated within the cytoplasm of certain microorganisms, which can be used as a source of renewable energy

and valuable products. The article provides an overview of the biochemical pathways involved in the synthesis and accumulation of lipids in oleaginous yeasts. It describes how the metabolism of these yeasts is optimized for the accumulation of lipids, which are stored as triacylglycerols (TAGs) in lipid droplets within the cells. The article also discusses the factors that affect SCO production, including the composition of the growth medium, the culture conditions, and the genetic background of the yeasts. It highlights the potential of genetic engineering and metabolic engineering to increase the yield and quality of SCO. Overall, the article provides important insights into the biochemistry of SCO production by oleaginous yeasts, which has significant implications for the development of sustainable and renewable sources of energy and products (Seraphim Papanikolaou and George Aggelis, 2011).

18. The article focuses on the production of surfactants by microorganisms, which are compounds that reduce the surface tension of liquids and can be used in a wide range of applications, including cleaning products, personal care products, and agricultural formulations. The authors discuss the various types of microbial surfactants, including glycolipids, lipopeptides, and polymeric surfactants, and describe their properties and potential uses. The article also provides an overview of the commercial potential of microbial surfactants, highlighting their advantages over chemically synthesized surfactants, such as their biodegradability, low toxicity, and high specificity. The authors discuss the challenges associated with scaling up microbial surfactant production and suggest strategies for overcoming these challenges. Overall, the article provides a comprehensive review of the microbial production of surfactants and their potential applications, and is a valuable resource for researchers and industry professionals working in this field (Desai and. Banat, 1997).
19. The article discusses the concept of peak oil, which refers to the point at which global oil production reaches its maximum level and begins to decline. The authors argue that the IEA's reference scenario does not adequately account for the decline in oil production that is likely to occur after peak oil is reached. They also criticize the IEA for relying on optimistic assumptions about the availability and accessibility of oil reserves, as well as the rate at which new oil fields will be discovered and developed. The authors present an alternative scenario based on a more conservative estimate of global oil reserves and a slower rate of production growth.

They argue that this scenario is more realistic and provides a better basis for energy policy planning. Overall, the article provides a critical analysis of the IEA's projections for future oil production and highlights the importance of considering the finite nature of oil reserves in energy policy planning (Aleklett, 2008).

20. The researchers investigated the growth and lipid accumulation of *C. podzolicus* DSM 27192 under different conditions and found that the optimal temperature for growth was 28°C, while the optimal temperature for lipid accumulation was 22°C. They also found that the highest lipid content (57.5%) was obtained when the yeast was grown in a medium containing 70 g/L glucose and 5 g/L yeast extract. In addition, the researchers explored the production of GA by *C. podzolicus* DSM 27192 during growth on glucose. They found that the highest GA concentration (118.2 g/L) was obtained when the yeast was grown in a medium containing 150 g/L glucose and 10 g/L yeast extract. The study demonstrated that *C. podzolicus* DSM 27192 can efficiently co-produce SCO and GA, with lipid accumulation and GA production occurring simultaneously during growth on glucose. The results suggest that this yeast has potential for use in the production of both SCO and GA, which have a variety of industrial applications (Xiujuan Qian, et, al, 2019).

CHAPTER 3

AIM AND OBJECTIVE :

3.1 AIM :

To Isolate And optimize single cell oil producing bacteria from soil samples.

3.2 OBJECTIVE:

1. Collection of samples from oil contaminated sites and areas.
2. Isolation of morphologically different bacteria by spread plate method.
3. Screening of single cell oil producing bacteria.
4. Purification of Single cell oil producing bacteria.
5. Selection of the potent isolate of single cell oil producing bacteria.
6. Production of the potent single cell oil producing isolate.
7. Optimization of Single cell oil bacteria
8. Characterization of potential isolate.

CHAPTER 4

MATERIALS AND METHODOLOGY

MATERIALS:

1. Soil from different oil contaminated areas.
2. Nutrient agar and differential agar.
3. Mineral salt medium Broth.
4. Staining : Sudan B Black stain , Gram's stain , Capsule stain , spore stain, Acid fast stain and other required stains.
5. Glassware's and other essentials laboratory products.



Fig 1 Material Required

METHODOLOGY:

4.1 Collection of sample:

Soil is generally sampled by using a soil auger into the soil, usually to 10cm depth and extracting a soil core. A soil auger was used in collection sample for analysis. The samples were collected from oil contaminated soils such as oil grinding machine oil refineries oil and gas refineries a crude oil extraction plants and automobiles mechanic shop. 3 soils were isolated from three different places. The first soil was isolated from the ground soil of CPCL Chennai and the second soil was isolated from an oil refinery, Vellore and the third sample was isolated from an automobile mechanic shop near Manali, Chennai. These samples were collected aseptically in zip lock bags and brought to the lab for isolation of single cell oils.

4.2 Preparation of Nutrient Agar :

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth.

Procedure:

1. Using a weighing dish and balance mass 23 g of nutrient agar.
2. Transfer the nutrient agar solid to a 2-L beaker
3. Using a 1000-mL graduated cylinder, measure 1000 mL of distilled water. Transfer the water to the beaker of nutrient agar.
4. Stir the solution until the solid is evenly distributed.
5. Place the solution in an autoclave for 15 minutes at 121 °C (15 lbs of pressure).
Note: Nutrient agar must be sterilized if it will be used as culture media.
6. Allow the solution to cool to 50–55 °C and pour into sterilized culture dishes.

4.3 Isolation of Bacterial Strain:

4.3.1 Serial dilution method :

For isolation of the bacteria from soil sample, the basic method that is the serial dilution Method was used. Then these serially diluted water blanks were streak on the nutrient agar plates for the growth and isolation of the bacteria. Serial dilution was done by taking 10 test tube along with one test tube having 1grams soil with 9ml of water. Then serially the concentration was raised to the power of tens. Then using micro pipette, 0.1ml of the serially diluted water blanks with soil were spreaded on nutrient agar plates. Which were prepared before the serial dilution. Spread plates was made on nutrient agar medium and incubated for 24 ~ 48 hours at room temperature.

4.3.2 Spread plate method

100 µl of the individual sample was spread into the solidified agar medium using L-rod. The plates were then incubated at 37°C for 24 hours. The colonies were studied for their characteristics.

4.4 Screening for potential bacteria:

4.4.1 Sudan BLACK B staining:

Sudan black is slightly basic dye and will combine with acidic in compound lipids, thus staining phospholipid also. An attentive stain to the B stain. Black Sudan B is used for the staining of a wide variety of lipids such as phospholipid, sterols and neutral triglycerides. Black Sudan B is not lipid specific like Sudan dye and can also be used for chromosome lipids are present in azurophilic and secondary granules of myelocytic cells. Hence these are Sudan B Black positive Sudan Black B dye is fat soluble, then it stains fat particles which present in the primary and secondary of myelocytic cells. It stains intracellular lipids and phospholipids. Positive staining of granulocytic cell and eosinophils is seen as the granules are membrane bound / contain phospholipids. Weak monocytic staining. Sudan B Black has an advantage over myeloperoxidase.

Procedure:

1. The isolated stains were stained with Sudan B Black staining.
2. The smear was fixed in the slide. Then the slide was flooded with Sudan B stain for 15min and rinsed twice in xylene.
3. Then it was followed by counter stain with diluted safranin for 15 second.
4. Then it was washed with distilled water. the air-dried slide was observed under compound microscope. On oil immersion for presence of blue or greyish coloured fat globules within the cell.

4.5 Purification for the potent isolate :

MSM: Mineral Salts Medium

This is a carbon-free mineral salts medium for enrichment / isolation / growth of specific nutritional types of microbes. A carbon source (e.g., glucose) must be added to MSM before use. Usually best to add carbon sources from filter-sterile stock solutions after autoclaving to avoid possible problems. The ratio of phosphate salts has been chosen so that the medium should be already close to pH 7 without need to adjust. MSM without nitrogen can be prepared (e.g., for isolation of N₂-fixers) simply by omitting ammonium sulphate. MSM recipe is from (Coleman et al 2002) (AEM, JS666), originally adapted from Hartman's et al (Appl.Microbiotech.1992). Many bacteria will grow fine in plain MSM, but some may also require the vitamin solution.

MSM bulk medium

Deionized water:	1 litre
Dipotassium hydrogen phosphate:	2.27 g
Potassium dihydrogen phosphate:	0.95 g
Ammonium sulphate:	0.67 g
Metals solution (Trace elements solution):	2 ml
Agar	17.0 g
pH	7.0 ± 0.2

Procedure:

1. The Positive 8 bacterial stains were further processed in lab under mineral salt medium.
2. The mineral salt medium was prepared using sterile conditions
3. The positive isolated bacterial strains were inoculated using the inoculum loop
4. Then the medium along with the bacteria were incubated around 37 Degree Celsius
5. After 24- 48 hrs the growth in the medium was absorbed
6. The growth of the potent isolate was seen more.
7. This strain was used for production and further related studies.

4.6 Selection of the potential bacterial strain:

The cell density can be quantified in two basic ways: as grams of dry or wet weight per litre of sample, or as number of viable/dead cells per ml. The cells in a sample can be separated from the broth and weighed while they are wet, or the cells may be thoroughly dried before weighing. The dry weight measurement usually gives a much more consistent result than the wet weight. Biomass concentration is one of the most critically needed measurements in fermentation studies. Reliable estimates of bacterial biomass (as carbon content) are essential to determine the quantitative importance of bacteria in many ecosystems. The dry weight/wet weight ratio, and the carbon weight/dry weight ratio of bacterial cells. It states that about 20% is a reasonable average estimate of bacterial dry weight. Percent dry matter of bacterial cells is usually determined by weighing a bacterial pellet before and after drying to constant weight. Available information on the water content in bacterial pellets, however, suggests that >60% of the total water in the pellets may be intercellular

water. Thus, proper corrections for intercellular water in the pellets seem to be crucial when determining the dry weight/wet weight ratio to the bacterial cells. If a bacterial pellet contains 20% dry matter, and if 60% of the water in this pellet is assumed to be intercellular water, then the bacterial cells must have a dry-matter content of about 38%. This is nearly double the commonly assumed value of 20% dry matter for bacterial cells. Based on the dry weight percentage of total lipid extracted from the cell dry matter of isolates, the potential isolates were selected. The isolated strain was further used for maximum production of single cell oil and optimization studies.

PROCEDURE :

1. Dry in an air with an empty Eppendorf. Weigh them and store them in a desiccator lined with Drierite (anhydrous CaSO_4).
2. Stir the Eppendorf to suspend the culture evenly.
3. Separate the cells from the Eppendorf by centrifugation at 10,000 g for 5 minutes. Then carefully discard the clear broth and scrape the cell paste from the centrifuge tube. Rinse the centrifuge tube with a few ml of water.
4. Rinse the reservoir with a few ml of water and scrape any paste adhering to the glassware. The cell weight of the culture is measured immediately after all the water has been pulled through.
5. Dry the cell paste in an oven set at 100°C. The cells will be charred and the filter membrane will be burned if the temperature of the oven is set too high. Measure the weight of Eppendorf plus the cell paste periodically until there is no further decrease in the dry weight. It will take 6-24 hours to dry the sample completely, depending on the oven temperature and the thickness of the paste. Calculate the difference in the weight, and express the dry weight in g/l.

4.7 Confirmation analysis of lipids:

Solubility test: A small amount of extracted lipid from all bacterial isolated were taken in three test tubes and each of three tubes was added with water, alcohol, chloroform and vortexed well to detect the solubility nature.

Saponification test: about 2 ml of 2% NaOH solution was added to all the small amount of lipid extracted from potential isolated and mixed well to emulsify the lipid. Positive result observed by the formation of soapy solution.

Antibacterial activity of lipids:

The single cell oil (lipid) extracted from the potential isolates were tested for antimicrobial activity against human bacterial pathogens by well diffusion method. The 18 hours culture of *Bacillus sp.*, *Staphylococcus sp.*, *Escherichia coli* and *Proteus sp.*, were swabbed on MHA plates. In each well, the crude lipid (50µl) was added and incubated at 30° C for 24 hours.

4.8 Production methods:

4.8.1 Inoculum Preparation:

All the Sudan black positive isolates were studied to produce lipids. About 2ml of 24 hours bacterial culture were inoculated in 100 ml of sterilized Supplemented Nutrient Broth (SNB) medium and placed in a shaker at 200 rpm for 3 to 5 days at 30° C.

4.8.2 Production of the potent isolate in the fermentation medium:

For production of the bacteria, the recommended methodology was followed. Each 100 ml of MBM (Mineral Broth Medium) medium at pH 7 was prepared in 500 ml Erlen Meyer flask and used to produce single cell oils. After sterilization, 5% of potent bacterial inoculum was transferred in to MBM production medium and incubated at 28°C in rotary shaker for 120 hours. After incubation, fermentation medium was removed from shaker and centrifuged by using cooling centrifuge at 10,000 rpm for 30 minutes at 4°C. The clear debris was collected in screw cap tube and stored at 4°C.

4.8.3 Obtaining dry cell matter:

The cell density can be quantified in two basic ways: as grams of dry or wet weight per litre of sample, or as number of viable/dead cells per ml. The cells in a sample can be separated from the broth and weighed while they are wet, or the cells may be thoroughly dried before weighing. The dry weight measurement usually gives a much more consistent result than the wet weight.

Procedure :

1. After three days of incubation, the broth was centrifuged at 5000 rpm for 15 minutes for separation of pellet.
2. The pellet was washed three times with sterile distilled water and dried overnight at 80°C .
3. Then the biomass was weighed for the determination of cell dry matter

4.8.4 Total lipid extraction:

Folch method:

A mixture of chloroform and methanol in the ratio of 2:1 (v/v) extract lipid more exhaustively from animal cells than most other simple solvents systems. With most cells, the lipids are removed almost completely after two or three treatments with the mixture. Most of the contaminating compounds in the extract can be removed from the chloroform-methanol (2:1 v/v) mixtures simply by shaking the combined solvents with a quarter of the total volume of water. The lower phase which comprises 60% of the total volume contains the purified lipid. This extraction yields approximately a 95-99% recovery of lipids.

Procedure:

1. Weigh the chopped sample into the homogenizer cup.
2. Add C-M mixture volume of about 3.5 times the weight of sample, and 2-3 drops of antioxidant solution.
3. Homogenize for 1 min and filter with Whatman No. 1 filter paper using a Buchner funnel and vacuum pump.
4. Transfer the residue into the cup and repeat homogenization twice.
5. Transfer the combined filtrate into a separating flask.
6. Pour distilled water, volume approximately a quarter of that of the extract, into the separating flask.
7. Shake very gently 2-3 times, and stand overnight.
8. Drain off the chloroform phase through a Whatman No. 1 filter paper into an Erlenmeyer flask containing about 2-5 g anhydrous sodium sulphate. Shake well and leave for about 5 min. Decant into an evaporating flask.
9. Wash the filter paper 2-3 times with C-M Mixture.

10. Concentrate the extract with a rotary evaporator under reduced pressure at 40°C (water-bath temperature).
11. Dissolve the concentrated extract with C-M Mixture and transfer to 50 ml volumetric flask using a pipette.
12. Make up to the mark with C-M Mixture.
13. Then remove the supernatant and use the debris or pellets for lipids extraction.

4.9 Optimization of the potent isolate:

To enhance production of single cell oil, the potential strains TH2 were subjected to optimization studies. Production of SCO was optimized by studying various nutritional factors (such as carbon and nitrogen) and environmental (such as pH, incubation time, temperature, inoculum size) was incorporated in Minimal unbalanced agar media. To identify the maximum lipid accumulation in potential strains, the incubated plates were flooded with Sudan black staining solution (0.02 % of Sudan black + 96 % Ethanol) for 30 – 60 minutes and washed with 96 % ethanol and observed for the maximum stain absorption.

Bacterial inoculum was prepared by inoculating potential bacteria culture on nutrient broth and kept in rotary shaker for 18 hours at 28°C. Each 5 ml of nutrient broth culture was used as inoculum.

Effect of carbon source: To study the effect of carbon source about 5 ml of 18hours potential bacterial culture inoculum was inoculated into series of flasks containing 100 ml of minimal broth medium (replacing with carbon source) with 1% different carbon sources (dextrose, glucose, fructose, sucrose, and maltose). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

Effect of nitrogen source: To study the effect of nitrogen source about 5 ml of 18hours potential bacterial strain inoculum was inoculated into series of flasks containing 100 ml of Minimal broth medium (replacing with nitrogen source) with 1% different nitrogen sources (Ammonium nitrate, yeast extract, beef extract, peptone, Urea). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

Effect of pH: To study the effect of pH about 5 ml of 18hours inoculum was inoculated into series of flasks containing 100 ml of minimal broth medium with different pH (5, 6, 7, 8 and 9). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

Effect of temperature: To study the effect of temperature about 5 ml 18hours potential bacterial strain inoculum was inoculated into series of flasks containing 100 ml of minimal broth medium with different temperature (25°C, 30, 35, 40 and 45°C). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

Effect of incubation time: To study the effect of incubation period, about 5 ml 18hours potential bacterial strain inoculum was inoculated into series of flasks containing 100 ml of minimal broth medium with incubation duration such as 24, 48, 72, 96 and 120 hours. All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

Effect of inoculum size: To study the effect of inoculum size, about 5 ml 18hours potential bacterial strain inoculum was inoculated into series of flasks containing 100 ml of minimal broth medium with inoculum size such as (75 µL, 150,275, 300 375 µL). All the flasks were incubated at 28°C in rotary shaker with 120 rpm for 120 hours.

4.10 Characterization of the potent isolate:

Microscopic, cultural, and biochemical characteristics of potential strain were studied by adopting standard procedure and the potential strain was identified with the help of Bergey's manual of Systematic Bacteriology.

4.10.1 Microscopic identification:

4.10.1.1 Gram Staining:

Bacterial smears of isolated old cultures were made on clean grease-free slides, heat-fixed, and stained as follows. Add about 5 drops of crystal violet solution to the slide, drain, and rinse with water .crystal violet dye work as an intercalating agent means adheres to the cell membrane. then add a drop of Grams iodine solution for one minute, drain, and rinse with water. It acts as a mordant which was fix the crystal violet in the cell wall of bacteria. Decolourized with ethyl alcohol for 30 Sec which dehydrates the layer of peptidoglycan and shrinks it and later counterstained with safranin for one minute colouring the cell and to detection of mast cell also used to distinguish gram-negative bacteria from gram-positive bacteria. and observed under an oil immersion microscope. The study revealed gram-negative bacteria.

Crystal violet : 2.0 g in 20 ml 95% ethanol

Ammonium Oxalate : 8.0 g in 80ml distilled water.

Gram's Iodine

Iodine : 1.0 g

Potassium Iodine:	2.0 g
Distilled Water:	300 ml
Acetone Alcohol	
95% ethanol :	70 ml
Distilled water:	30 ml
Gram's Safranin	
Safranin:	0.25 g
95% ethanol:	10 ml
Distilled Water:	100 ml

4.10.1.2 Acid Fast Stain :

Some bacteria produce the waxy substance mycolic acid when they construct their cell walls. Mycolic acid acts as a barrier, protecting the cells from dehydrating, as well as from phagocytosis by immune system cells in a host. This waxy barrier also prevents stains from penetrating the cell, which is why the Gram stain does not work with mycobacteria such as *Mycobacterium*, which are pathogens of humans and animals. For these bacteria, the acid-fast staining technique is used.

To perform the acid-fast stain, a heat-fixed smear is flooded with the primary stain carbol fuchsin, while the slide is heated over a steaming water bath. The heat “melts” the waxy cell wall and permits the absorption of the dye by the cells. Then the slide is allowed to cool and a solution of acid and alcohol is added as a decolourizer. Cells that are “acid-fast” because of the mycolic acid in their cell wall resist decolourization and retain the primary stain. All other cell types will be decolourized. Methylene blue is then used as a counterstain. In the end, acid-fast bacteria (AFB) will be stained a bright pink colour, and all other cell types will appear blue.

4.10.1.3 Capsule staining:

The polysaccharide goo that surrounds some species of bacteria and a few types of eukaryotic microbes are best visualized when the cells are negative stained. In this method, the bacteria are first mixed with the stain, and then a drop of the mixture is spread across the surface of a slide in the thin film. With this method, capsules appear as a clear layer around the bacterial cells, with the background stained dark.

4.10.1.4 Endospores staining :

Endospores are dormant forms of living bacteria and should not be confused with reproductive spores produced by fungi. These structures are produced by a few genera of Gram-positive bacteria, almost all bacilli, in response to adverse

environmental conditions. Two common bacteria that produce endospores are *Bacillus* or *Clostridium*. Both live primarily in soil and as symbionts of plants and animals, and produce endospores to survive in an environment that change rapidly and often. The process of endospore formation (the formation of endospores) involves several stages. After the bacterial cell replicates its DNA, layers of peptidoglycan and protein are produced to surround the genetic material. Once fully formed, the endospore is released from the cell and may sit dormant for days, weeks, or years. When more favourable environmental conditions prevail, endospores germinate and return to active duty as vegetative cells.

Because the endospore coat is highly resistant to staining, a special method was developed to make them easier to see with a brightfield microscope. This method, called the endospore stain, uses either heat or long exposure time to entice the endospores to take up the primary stain, usually a water-soluble dye such as malachite green since endospores are permeable to water. Following a decolourization step which removes the dye from the vegetative cells in the smear, the counterstain safranin is applied to provide colour and contrast. When stained by this method, the endospores are green, and the vegetative cells stain pink.

4.10.2 Cultural characteristics identification:

When grown on a variety of media, microorganisms will exhibit the difference in the microscopic appearance of their broth. These differences are called cultural characteristics and are used as a basis for separating microorganism into taxonomic groups. The cultural characteristics for all taxon microorganisms are contained in Bergey's Manual of systematic bacteriology.

The following characters of colony are noted:

1. Size: in millimetre
2. Shape: circular / irregular
3. Surface : smooth, rough, granular
4. Elevation : flat, low convex, high convex, raised, umbonate, umbulate
5. Edge: entire, undulate, lobate, crenated, fimbriate, ciliate
6. Opacity : opaque, translucent, transparent
7. Colour of colony
8. Consistency : mucoid, friable
9. Other properties : haemolysis, pigmentation, swarming.

4.10.3 Biochemical test:

4.10.3.1 INDOLE TEST

Tryptone broth was inoculated with the test isolates and incubated at 37°C for 24-48 hours. About 0.2-0.3 ml of Kovac's reagent was then added to the test tube, shaken, and allowed to stand. The formation of the brown ring on the surface of the broth confirmed the production of indole.

Tryptone broth

Tryptone	10.0gms
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Distilled water	1000ml
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Kovac' Reagent

Benzaldehyde

Butyl alcohol

Cone of hydrochloric acid

4.10.3.2 METHYL RED TEST

Methyl red – Voges Proskauer (MR-VP) broth inoculated with the culture was incubated for 24-48 hrs at 37°C. The appearance of red colour on the addition of methyl red solution was considered as positive.

Glucose – Phosphate broth (MR – VP)

Buffered Peptone	7.0gms
------------------	--------

Dextrose	5.0gms
----------	--------

Dipotassium	5.0gms
-------------	--------

Sodium chloride	5.0gms
-----------------	--------

Distilled water	1000ml
-----------------	--------

Ph	7.2
----	-----

MR reagent

Methyl red	0,1 g
------------	-------

95 % ethanol	300ml
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Distilled water	200ml
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4.10.3.3 VOGES - PROSKAUER TEST

MR- VP medium inoculated with culture was incubated at 37°C for 24-48 hrs. After incubation, 3 ml of Barrit's reagent A and one ml of Barrit's reagent B were added. The tubes were shaken and allowed to stand for 15 minutes and observed for colour change. The development of red colour was considered as positive.

Barrit's Reagent

5% alpha naphthol	50gms
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Absolute ethanol	95m
------------------	-----

Reagent B

Potassium hydroxide	40gms
---------------------	-------

Creatine	3gms
----------	------

Distilled water	1000ml
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4.10.3.4 CITRATE UTILIZATION TEST :

Citrate utilization test was used to detect the ability of an organism to utilize citrate as the sole carbon source for its growth, lightly inoculate Simmon Citrate Agar (Hi media, Mumbai) with a loop (pure culture) of culture. Record the visible growth as positive and no growth as negative. Incubated at 37°C for 24 hrs. Record a blue colour growth on a slant as positive and the absence of growth as negative.

Sodium Chloride	5gms
-----------------	------

Magnesium Sulphate	0.2gms
--------------------	--------

Ammonium dihydrogen phosphate	1gm
-------------------------------	-----

Dipotassium phosphate	1gm
-----------------------	-----

Sodium citrate	2gms
----------------	------

Bromothymol blue	0.08gms
------------------	---------

Agar	15gms
------	-------

Distilled water	1000ml
-----------------	--------

pH	6.8
----	-----

4.10.3.5 TRIPLE SUGAR IRON TEST

The Triple Sugar Iron (TSI) test is to find the ability to test a microorganism ferment sugars and to produce hydrogen sulphide. Acidic by-product gives yellow colour whereas alkaline by-products give red colour. The black colour indicated hydrogen sulphide formation. Gas production is indicated by the lifting of the butt from the surface of the test tube. TSI medium inoculated with the culture was incubated for 24 hrs at 37°C.

Beef extract	3gms
Peptone	20gms
Yeast extract	3gms
Lactose	10gms
Sucrose	10gms
Dextrose monohydrate	1gms
Ferrous sulphate	0.2gms
Sodium chloride	5gms
Sodium thiosulphate	0.3gms
Phenol red	0.024gms
Agar	12gms
Distilled water	1000ml
pH	6.8

4.10.3.6 UREASE TEST

When urea is utilized, ammonia is formed during incubation which makes the medium alkaline, showing a pink-red colour. Urease medium inoculated with the culture was incubated for 24 hrs at 37°C.

UREASE AGAR MEDIUM :

Dextrose	1gms
Peptic digest of animal tissue	1.5 gms
Sodium chloride	5 gms
Monopotassium phosphate	2gms
Phenol red	0.012 gms
Agar	15 gms
pH	6.8

4.10.3.7 CATALASE TEST

A small amount of culture was placed over a clean slide. A drop of 3% hydrogen peroxide was placed over the culture and observed for effervescence. The production of effervescence showed the ability to produce the enzyme catalase.

4.10.3.8 OXIDASE TEST

The organism was spotted on an oxidase disc, blue or purple colour change was observed within 10 seconds.

CHAPTER 5

RESULT AND DISCUSSION :

1. **Collection of soil samples :** Soils were collected from 3 different places like the oil refinery companies , oil mills and mechanical workshop.



Fig 1.1 soils collected

2. **Preparation of the nutrient agar:** The nutrient agar was prepared using sterile conditions:



Fig 2.1 nutrient agar

3. **Isolation of the bacterial strains :** For isolation of the bacteria from soil sample, the basic method that is Serial dilution method, along with the Spread plate method was used. Morphologically 40 different bacterial isolates were selected.

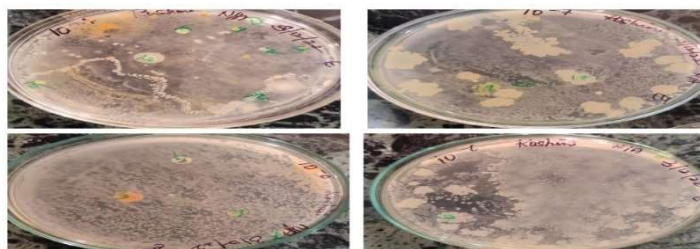


Fig 3.1 Bacterial colonies

4. Screening for the potent isolate Bacteria:

Among 40 different isolates, primary screening was done using Sudan B Black staining. This stain helps in identification of single cell oil bacteria, by staining the microbial oil and lipid content within the bacteria. These 40 different morphologically bacteria isolates were stained and seen under microscope, where 8 isolates were postically stained, showing presence of blue and greyish globules. These 8 isolates were used for further studies.

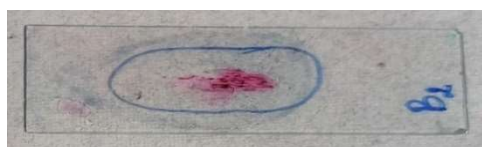


Fig 4.1 Sudan B black staining slide

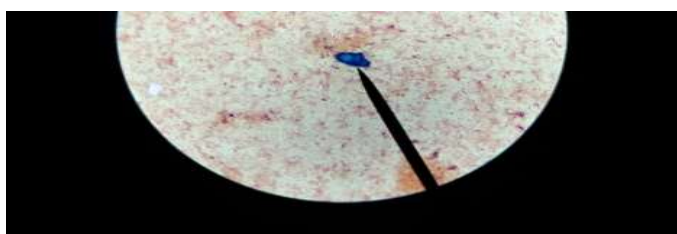


Fig 4.2 Microscopic visualization of the stain

Table 4.1 Sudan B Black staining results

B1 = - ve	V1= - ve	TH1= - ve
B2 = +ve	V2= - ve	TH2= +ve
B3= +ve	V3= - ve	TH3= - ve
B4= - ve	V4= - ve	TH4= - ve
TH2= - ve	V5= +ve	TH5= - ve
B6= - ve	V6= +ve	TH6= +ve
B7= - ve	V7= - ve	TH7= - ve
B8= - ve	V8= - ve	TH8= +ve
B9= +ve	V9= - ve	TH9= - ve
B10= - ve	V10= - ve	TH10= - ve

5. Purification of the potent isolate :

The positive 8 bacterial strains were purified using the mineral salt medium. These bacteria strains were grown in flask along with the mineral salt broth. The strain TH2 showed maximum growth under this condition. This strain was used for further studies.

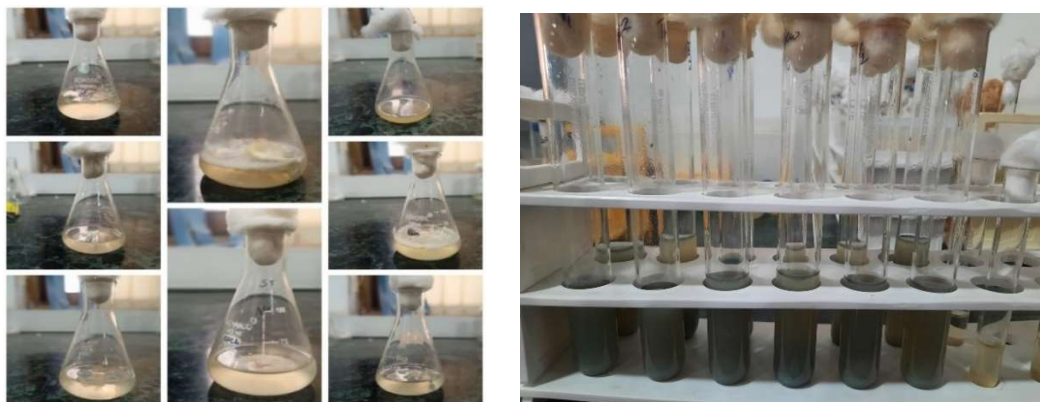


Fig 5.1 Mineral salt medium in flask **Fig 5.2 Mineral salt medium in test tube**

6. Selection of the potential isolate :

Based on the percentage of dry lipid, the positive isolate named TH2 was selected as potential strain.



Fig 6.1 Separated pellet from the medium



Fig 6.2 Dry cell matter



Fig 6.3 Pellets collected in tube

Table 6.1 Cell dry matter of potential strain

S. No.	Strain no.	Total cell dry matter (mg/100ml)	Total lipid dry weight	Total lipid (% of w/w of CDM)
1.	TH2	325	75	23%

7. Confirmation for lipids analysis:

7.1 Solubility test : The extracted lipids were insoluble in water but soluble in alcohol and chloroform.



Fig 7.1.1 solubility test

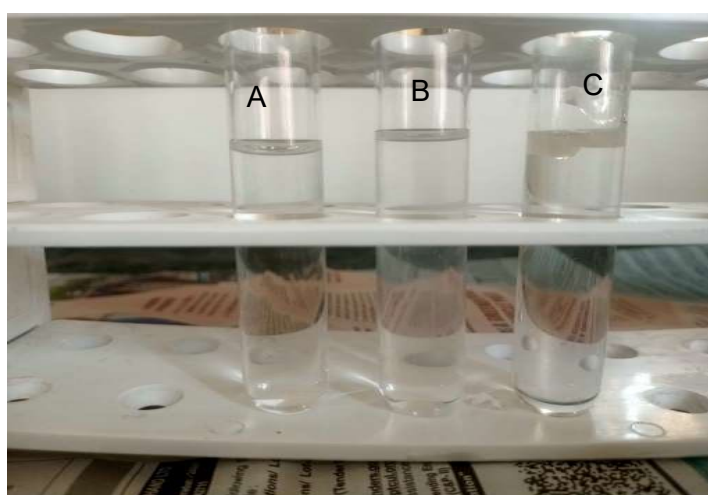


Fig 7.1.2 Lipid insoluble in water (c)

7.2 Saponification Test : The NaOH solution saponifies the lipids presented in the extracted compound by the formation of soapy solution indicating the presence of lipids.

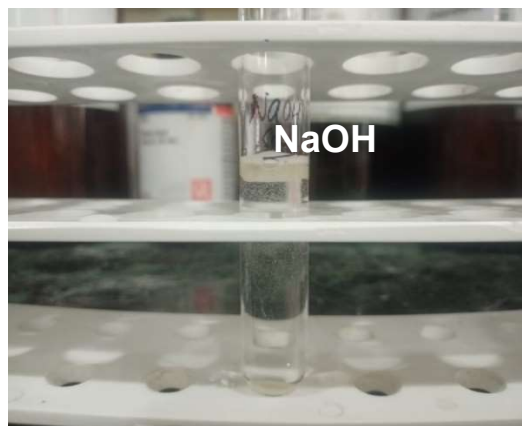
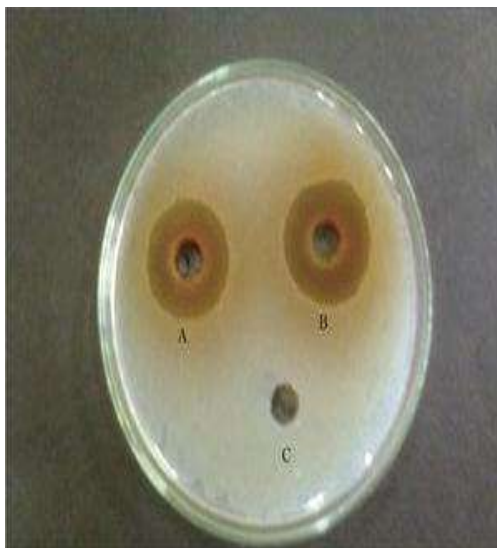


Fig 7.2 Saponification test (soapy formation)

7.3 Antibacterial activity of Lipids : The crude lipids extracted from potential strain TH2 showed antibacterial activity against Lacto Bacillus sp., Staphylococcus sp., Proteus sp., vibrio sp., E coli sp., Salmonella ., Candida sp., Pseudomonas sp.,



7.3.2 Antimicrobial activity

- A- Staphylococcus
- B- Proteus
- C- Lactobacillus



Fig 7.3.1 Antimicrobial activity

Fig

- 1- vibrio
- 2- E coli
- 3- salmonella
- 4- candida
- 5- pseudomonas

Table 7.3 Antimicrobial activity of lipids

Strain name	Test Organisms (zone of inhibition in mm)							
	Staphylococcus	Proteus	Lactobacillus	Vibrio	E coli	Salmonella	Candida	Pseudomonas
TH2	5	7	-	1	2	3	5	8

8. Production of single cell oil bacteria : The positive strain TH2 were used for the production single cell oil. The percentage of total lipid content was calculated from total cell dry matter and they showed 23% lipid content.



Fig 8.1 Production in 24 hrs

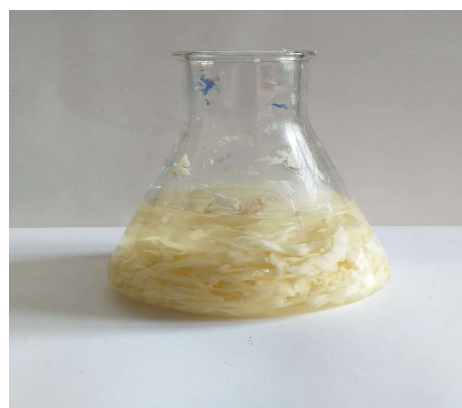


Fig 8.2 production in 96 hrs

Table 8.1 Production of single cell oil bacteria

S. no	Strain no	Total cell dry matter (mg/100ml)	Total lipid dry weight	Total lipid (% of W/w of CDM)
1.	TH2	325	75	23%

9. Optimization of single cell oil production:

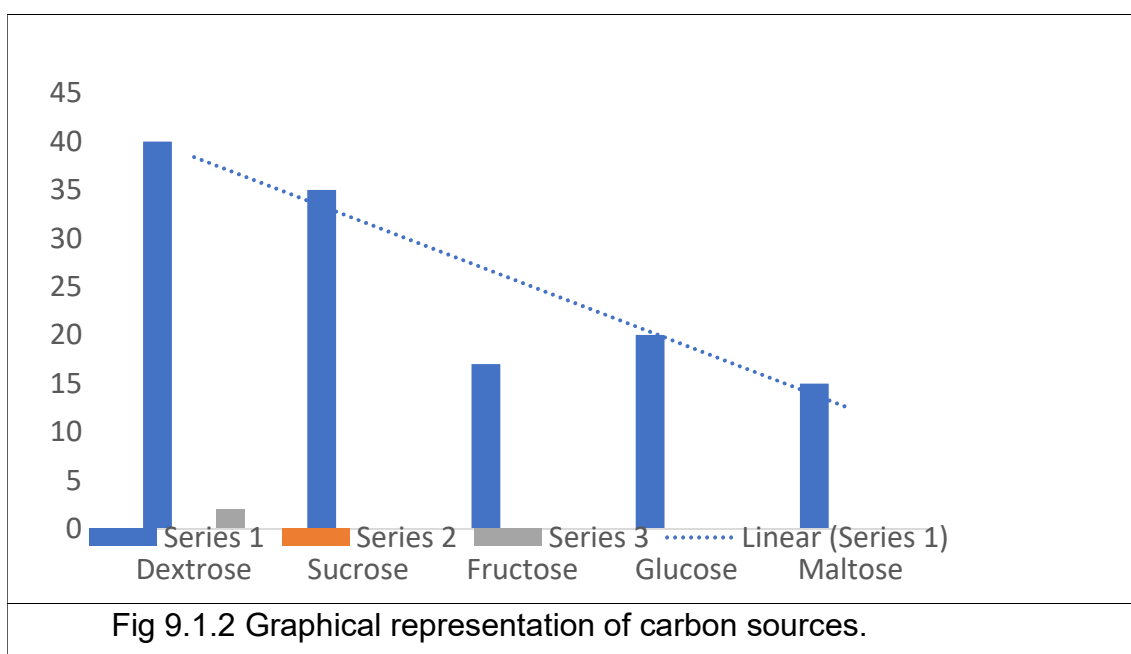
Effect of nutritional sources :

9.1 Effect of carbon sources:

Growth of all strains was observed in 5 different concentrations of. All strains showed maximum lipid accumulation at 1.55 g/L concentration. Strain TH2 showed maximum lipid accumulation at increased concentration of sucrose and dextrose. The maximum activity 40mm was obtained when dextrose (1%) was used as the carbon source and sucrose (1%) showed the second maxima enzyme production 35mm. The minimal production 17mm was obtained when fructose (1%) was used as carbon source and glucose (1%) showed the second minimal enzyme production 20mm and maltose showed 15mm zone of clearance.



Fig 9.1 Effect of carbon sources



9.2 Effect of Nitrogen sources:

In 5 different concentration of nitrogen source, maximum lipid accumulation was found at low concentration of nitrogen source beef extract and peptone. (0.4g/L). The maximum activity 30mm was obtained when ammonium nitrate (1%) was used as the nitrogen source and beef extract (1%) showed the second maximal enzyme production 23mm. The minimal production 12mm was obtained when peptone (1%) was used as nitrogen source and yeast extract showed the second minimal enzyme production 17mm.



Fig 9.2 Effect of nitrogen sources

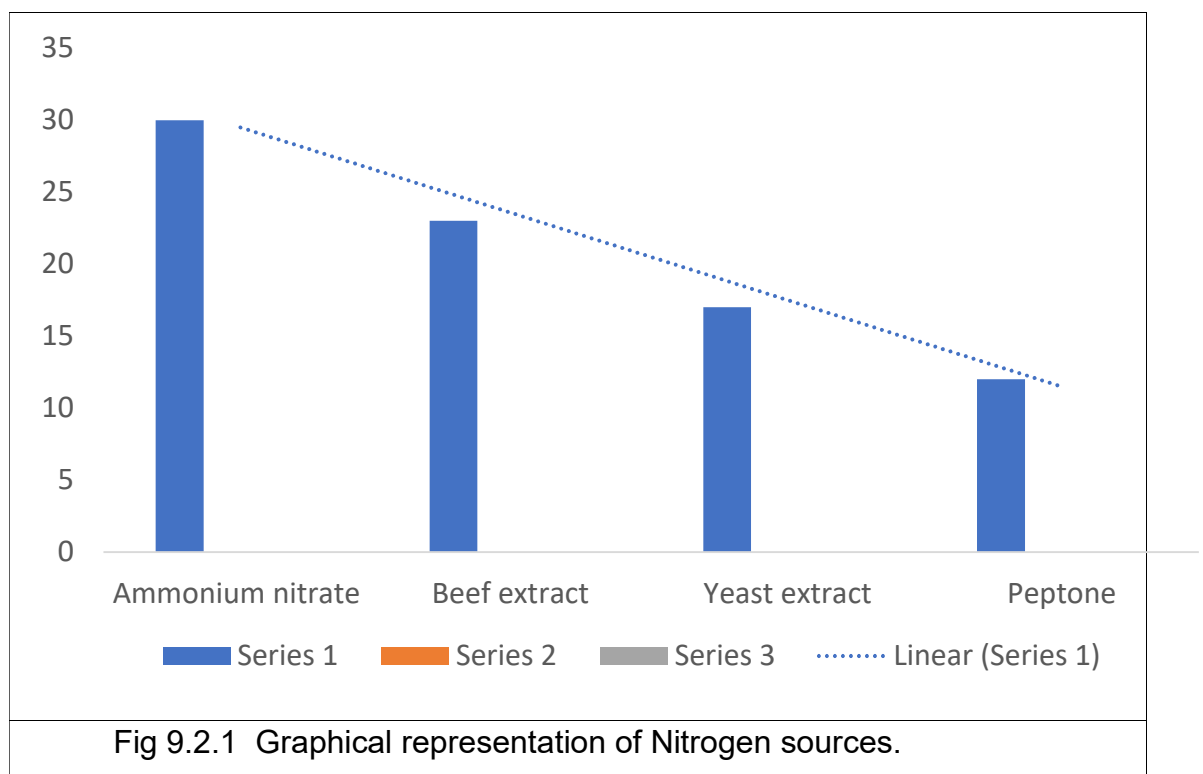


Table 9.1 Effect of Nutritional sources

Strain name		TH2	
Nutritional source (g/100ml)		Growth	Absorption of Strain
Carbon Sources	Maltose	Poor	+
	Fructose	Poor	++
	Glucose	Good	+++
	Sucrose	Good	++++
	Dextrose	Good	++++
Nitrogen Sources	Ammonium Nitrate	Good	++++
	Beef extract	Good	+++
	Yeast extract	Good	+++
	Peptone	Poor	+

Effect of environmental factors:

9.3 Effect of pH: All strains showed maximum growth at pH 8 and 7. Moderate growth was obtained at pH 6 and poor growth was obtained at pH 5.

Effect of incubation time: The growth was observed from 24 hours to 96 hours. The increasing hours of incubation showed strong staining ability which represents the maximum lipid accumulation. At 96 hours of time, it showed maximum accumulation.

9.4 Effect of Temperature: The growth was observed from 25 °C to 45 °C. The increasing temperature showed strong staining ability which represents the maximum lipid accumulation. At 35 °C it showed maximum accumulation.

9.5 Effect of Inoculum Size: The growth was observed from 75 μL to 375 μL . The increasing inoculum size showed strong staining ability which represents the maximum lipid accumulation. The maximum activity 45mm was obtained when 375 μL 10% was used as inoculum size.

Table 9.2 Effect of Environmental sources

Strain name		TH2	
Environmental source (g/100ml)		Growth	Absorption of Strain
pH	5	Poor	+
	6	Poor	++
	7	Good	+++
	8	Good	++++
Incubation Time	24	Good	++++
	48	Good	+++
	72	Good	+++
	96	Poor	+
Temperature	25°C	Poor	+
	30°C	Poor	++
	35°C	Good	+++
	40°C	Good	++++
	45°C	Good	++++
Inoculum Size	75 μL	Poor	+
	150 μL	Poor	++
	275 μL	Good	+++
	300 μL	Good	++++
	375 μL	Good	++++

10. Characterization of potential isolates:

The potent strain was done for cultural characteristics, microscopic, and biochemical test for identification:

10.1 MICROSCOPIC IDENTIFICATION :

Gram staining , cell arrangement , spore identification, capsule identification , flagella identification was done.

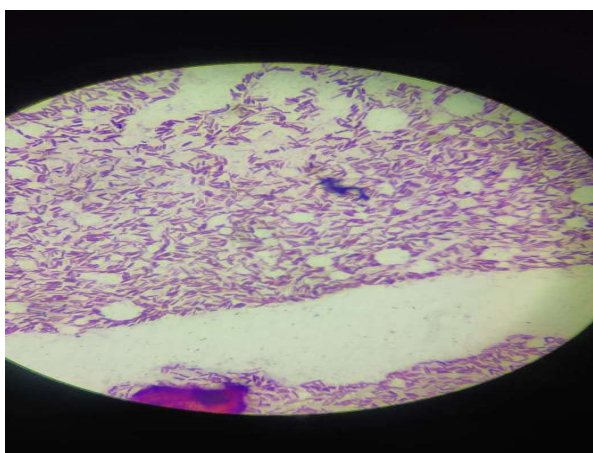


Fig 10.1 Gram Positive Rods

10.2 CULTURAL CHARACTERISTICS :

1. Size: 1.3 millimetre
2. Shape: Rod
3. Surface : smooth
4. Elevation : convex
5. Motility : Motile
6. Opacity : opaque, translucent, transparent
7. Colour of colony: yellow colour
8. Consistency : mucoid
9. Other properties : haemolysis

10.3 BIOCHEMICAL TESTS :

catalase, coagulase, oxidase, indole , methyl red, Voges Proskauer, citrate, urease , gelatin liquefaction , {Carbohydrate utilization test – glucose, maltose, lactose, fructose , sucrose} starch hydrolysis , nitrate reduction and mannitol test were done to the positive strain.

INDOLE TEST:



Fig 10.3.1 Indole Test

METHYL RED TEST:

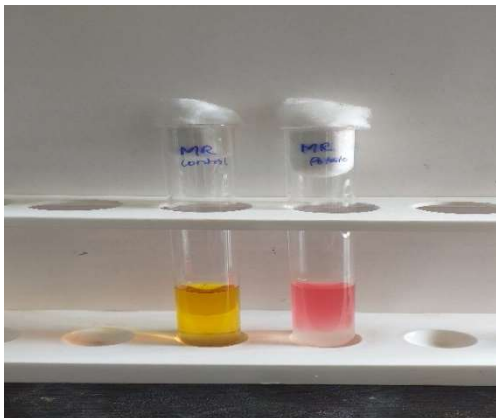


Fig 10.3.2 Methyl red test

VOGES - PROSKAUER TEST:

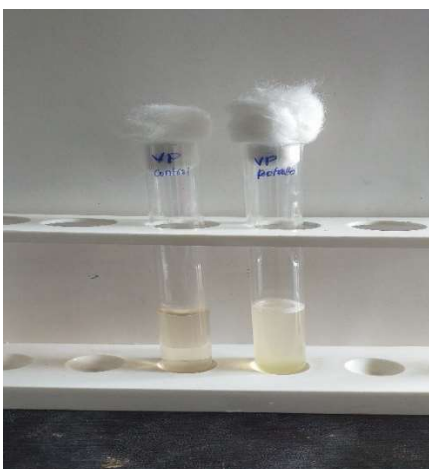


Fig 10.3.3 Voges Proskauer Test

CITRATE UTILIZATION TEST:



Fig 10.3.4 Citrate utilization Test

TRIPLE SUGAR IRON TEST :



Fig 10.3.5 Triple sugar test

UREASE TEST



Fig 10.3.6 Urease test

CATALASE TEST

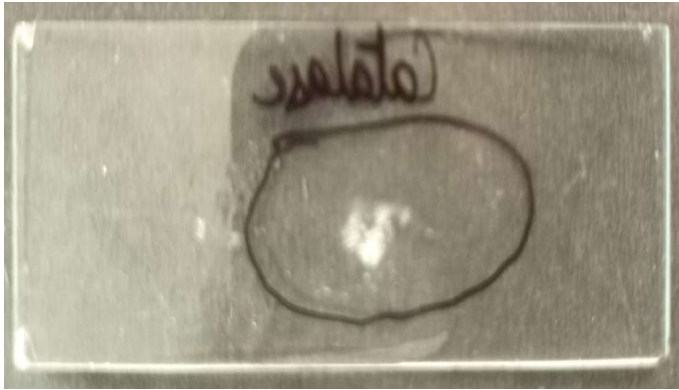


Fig 10.3.7 Catalase test

OXIDASE TEST



Fig 10.3.8 Oxidase test

GELATIN LIQUEFACTION:

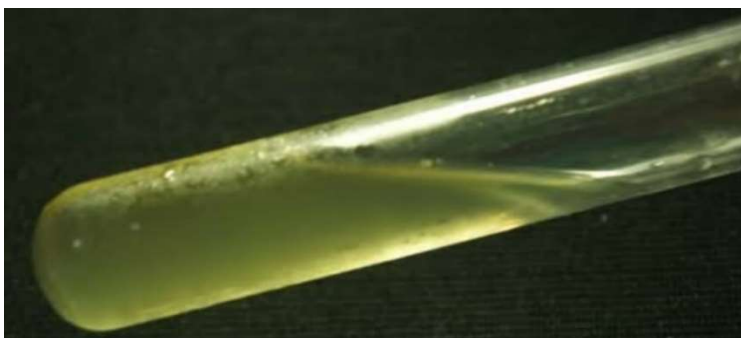


Fig 10.3.9 Gelatin Liquefaction

Table : 10.1 Characterization of the potent isolate

Characteristics	Strain-TH2
Colony morphology	Yellow colour, convex, smooth
Temperature	35° C
Gram staining	G+ ve, rods
Motility	Motile
Spore staining	Non spore bearing
Gelatin liquification	+
Starch hydrolysis	—
Nitrate reduction	—
Catalase	+
Oxidase	+
Glucose	+
Fructose	+
Maltose	+
Mannitol	—
Sucrose	+
Indole	+
MR	+
Citrate	+
Urease	—
VP	—

DISCUSSION

Microbial lipophilic compounds called single cell oils (SCO), has been the object of research and industrial interest for many years, due to their specific characteristics. Such SCO products are potential for using as alternative sources of animal or plant oils. According to (Gouda *et al.*, 2005) and (Patnayak and Sree 2005) single cell oil is mainly produced by Fungi and Yeasts, Bacteria for single cell oil production are not well explored. In this concern, the present study has aimed to isolate and identify single cell oil producing bacteria from different oil contaminated sites and to optimize for maximum production. For the collection of sample, three different oil contaminated sites were taken and analysed for isolation of bacteria from oil reservoirs (vali et, al, 2003). In their present study also totally 40 strains were isolated from three different oil contaminated sites. Of this sample-I contained large amount of microbial load when compared with sample-II and sample-III. The SCO producers were screened by Sudan black staining and by determination of total lipid content on cell dry matter (Patnayak S and Sree 2004) In their study also all the 37 strains were screened by Sudan black stain of that 6 isolates showed positive result for lipid accumulation. The production of bacterial SCO was done by shake flask method according to (Gouda *et al.*, 2002). The lipids were extracted by Folch method which is an efficient method to extract the total lipid content from cell dry matter (Aggelis and Komaitis 1999) (Ratledge 2004) After extraction, based on the percentage of total lipid content, 1 isolate were selected. Among 8 strains TH2 was identified as the efficient producers which produce about 23% of lipid content. For the confirmation of extracted compounds as lipids, lipid analyzing tests were done and confirmed the presence of lipids. The positive strain was taken up for further optimization studies and application studies. The lipids extracted from cell dry matter were analysed for their antimicrobial properties. In previous work, Gram positive and yeast only showed higher sensitivity to the lipid compounds (Kabara et, al, 1977) in the present study, the strain TH2 showed activity against Gram positive bacteria and Gram-negative . The potential strain was optimized for maximum lipid production and tested qualitatively by absorption ability of Sudan black stain. The nutritional factors such as carbon source and nitrogen source and environmental factors such as pH and incubation time were optimized for maximum production of lipid. This study showed the mild increased

concentration of glucose enhances the production of lipid. At 15.5 g/L concentration the strain TH2 showed maximum lipid accumulation when glucose (carbon source) used. (Papanikolaou *et al* 2002) reported 0.5g/L of ammonium sulphate had produced high lipid. In this study, 0.4 g/L had showed maximum lipid accumulation when ammonium sulphate (nitrogen source) used. (Hall and Ratledge) reported that pH had little influence on lipid accumulation. In this study pH had influence the accumulation of lipid. The strain showed maximum lipid accumulation at pH 8 . The effect of incubation time was previously reported by (Papanikolaou *et al* 2002). In this study, all the potential strains showed maximum lipid accumulation at an increased time of incubation. After 96 hours of incubation, the lipid accumulation remains constant. The potential Single cell oil producer was identified as *Cellulomonas sp.*

CHAPTER 6

SUMMARY AND CONCLUSION :

Summary :

The production and optimization of single-cell oil (SCO) by oleaginous bacteria isolated from oil-contaminated environments is a process of generating oil from microorganisms. This process is sustainable and can provide an alternative source of oil that can be used for various industrial applications.

Oleaginous bacteria can accumulate large amounts of lipids or oils within their cells, which can be extracted and used as a source of biofuel or other products. The isolation and selection of oleaginous bacteria from oil-contaminated environments is an important step in this process, as these bacteria are adapted to thrive in environments with high levels of hydrocarbons.

The production of SCO involves optimizing various parameters such as nutrient composition, temperature, pH, and aeration to maximize lipid accumulation in the cells. This optimization can be achieved through various methods, including statistical experimental design, response surface methodology, and mathematical modelling.

The resulting SCO can be used in a variety of industrial applications, including biodiesel production, animal feed, and food supplements. The use of SCO as a sustainable alternative to traditional oil sources is becoming increasingly important due to concerns over climate change and the environmental impact of fossil fuels.

Overall, the production and optimization of SCO by oleaginous bacteria isolated from oil-contaminated environments is a promising approach to generating sustainable oil sources for various industrial applications. In fact, Single Cell Oils represent intermediates for biodiesel production, polymers, biosurfactants. The exploitation of Single Cell Oils for other purposes was studied by (Simopoulos 2008) and (Bharathiraja et al. 2017) and related to the possibility of ensuring mono or polyunsaturation, known as EFAs, essential fatty acids.

Conclusion :

The present study can be further analysed by thin layer chromatography and Gas chromatography. In future, many unknown bacteria may result in the strains that are even better source of SCO producers than the ones we know. Efficient production techniques with detailed knowledge of lipid mechanism should be developed. This study offers an insight into exploring the possibility of producing such valuable added single cell oil at a high amount and to use it as a supplementary to other edible fats or to synthesis lipid-based products such as biosurfactant, bioplastics, etc.,

CHAPTER 7

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