

Evaluation of Bacterial resources for biosurfactant production

Submitted in partial fulfillment of the requirements for the
award of Bachelor of Technology degree in Biotechnology

by

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**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 | 12B Status by UGC| Approved by AICTE
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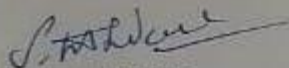
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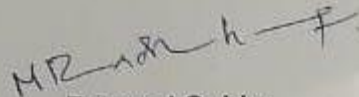
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This is to certify that this Project Report is the bonafide work of **ROJA G (41770026)**, who carried out the project entitled "**EVALUATION OF BACTERIAL RESOURCES FOR BIOSURFACTANT PRODUCTION**" under my supervision from **January 2024 to April 2024.**



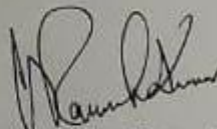
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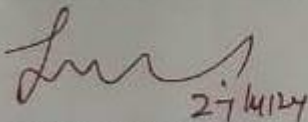
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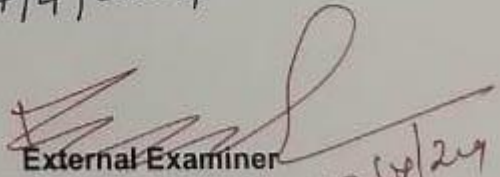
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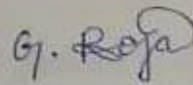
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DECLARATION

I Roja G(41770026) hereby declare that the Project Report entitled "**Evaluation of bacterial resources for biosurfactant production**" done by me under the guidance of **Dr. S Usha Nandhini** (Internal) and **Dr. M Radhakrishnan** (External) at Sathyabama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Bachelor of Science Degree in Biotechnology.

DATE: 24/04/2024

PLACE: CHENNAI



SIGNATURE OF THE CANDIDATE

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ABSTRACT

This study seeks to assess various bacterial resources for their capacity to produce biosurfactants, which are surface-active compounds generated by microorganisms. The focus is on exploring the surfactant properties and potential industrial uses of these bacterial resources. Specifically, the investigation targets microbial sources isolated from Kitchen Chimney in Kancheepuram district. A total of 31 distinct bacterial cultures were isolated using Actinomycetes Isolation Agar (AIA) and Nutrient Agar (NA), followed by screening for biosurfactant production. Among these cultures, 15 yielded positive results in both primary and secondary screenings. KC-15 and KC-23 gives positive results in screening methods. The biosurfactant-producing bacterial strains KC15 and KC23 were identified using various methods including oil displacement assay, drop collapse test, phenol red method, phenol sulfuric acid test, emulsification assay, and the parafilm method. Further tests were conducted on biosurfactant-positive cultures to evaluate their efficacy against various bacterial and fungal pathogens for potential non medical applications. The dye degradation method was employed with methylene blue and malachite green in strains KC-15 and KC-23. Optimization procedures were performed using methylene blue in strains KC-15 and KC-23 under various sources. Separation and extraction of potential bacterial cultures producing biosurfactants were carried out. Additionally, 16sRNA sequencing was employed to analyze potential biosurfactant-producing cultures, and LC-MS analysis provided insights into the presence of different bioactive cultures.

Keywords: Biosurfactant, Kitchen Chimney, LC-MS Bioprospecting, Surface tension.

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LIST OF ABBREVIATIONS

AIA Actinomycetes Isolation Agar

NA Nutrient agar

MB Methylene Blue

MG Malachite Green

AM Aerial Mycelium

SM Substrate Mycelium

RSP Reverse Side Pigment

KC Kitchen Chimney

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CHAPTER-1

INTRODUCTION

1.1. BIOSURFACTANT PRODUCTION

The extensive exploitation of natural resources and the expansion of industrial activities have led to significant environmental challenges. Among the most prevalent energy sources is petroleum and its derivatives, whose transportation and utilization frequently result in various forms of pollution. This includes heightened biological and chemical oxygen demands in soil and groundwater environments. The term "biosurfactant" denotes surface-active substances that bolster surface interactions by forming micelles, sourced from natural origins such as plants, microorganisms, and animals.

Some microorganisms utilize the metabolic pathways of petroleum-derived compounds. However, these compounds are not always fully degradable by microorganisms, leading to their persistence in soil and seawater for extended periods. Biosurfactants are categorized based on mass into two groups: low-mass biosurfactants, comprising glycolipids, phospholipids, lipopeptides, lipoproteins, and fatty acids, and high-mass biosurfactants, which encompass polymeric and particulate surfactants.

The term "surfactants" was coined by Antara Products in 1950, encompassing all substances with surface activity, including wetting agents, detergents, foaming agents, emulsifiers, and dispersants. Surfactants exhibit both polar and nonpolar characteristics, allowing them to decrease surface tension, which is the cohesive force between molecules in liquids, particularly in the interaction of oil and water. Biosurfactants can be categorized based on their microbial origin and chemical structure, with bacteria being the primary biosurfactant producers among various microorganisms. The production of biosurfactants by microbes or bacteria is influenced by fermentation conditions, environmental factors, and nutrient availability such as carbon and nitrogen sources.

Biosurfactants hold significant potential due to their advantages over synthetic surfactants across various fields such as environmental, food, agriculture, biomedical, and beyond. These compounds possess amphiphilic properties, featuring both hydrophilic and hydrophobic components, allowing them to exhibit tensioactive properties. This includes molecules like long-chain fatty acids, hydroxy fatty acids, and alkyl hydroxy fatty acids, which orient themselves at diverse interfaces among air, water, oil, and solid phases, influencing the properties of these phases. Surfactants constitute a versatile group of chemicals with widespread applications, ranging from household detergents, personal care products, pharmaceuticals, and agricultural chemicals to oilfield and food processing agents, industrial additives, and environmental remediation agents. The effectiveness of a surfactant in reducing surface tension and interfacial tension between two immiscible phases determines its efficacy. With over 2000 different biosurfactant structures identified, their utilization as carbon sources by soil-dwelling bacteria presents an opportunity to replace harsh surfactants currently employed in the pesticide industry.

1.1.1. Economic factor of biosurfactant production

To address the cost constraints associated with biosurfactant production, two main strategies are commonly employed worldwide to enhance cost-effectiveness: Utilizing inexpensive and waste substrates for formulating fermentation media, thereby reducing the initial raw material cost, Developing efficient bioprocesses through optimizing culture conditions and implementing cost-effective recovery processes to maximize biosurfactant production and recovery.

Given the substantial annual generation of millions of tons of hazardous and non-hazardous waste worldwide, there is a pressing need for proper management and utilization of these resources. Various residues from tropical agronomic crops such as cassava peels, soybean hulls, sugar beet, sweet potato peels and stalks, sweet sorghum, rice and wheat bran, rice straw, corn hulls, and rice husks, along with by-products from the coffee processing industry like coffee pulp, coffee husks, and spent coffee grounds, as well as residues from fruit processing such as pomace,

grape waste, pineapple waste, and carrot waste, have been identified as potential substrates for biosurfactant production. Additionally, waste from oil processing mills including coconut cake, soybean cake, peanut cake, canola meal, and palm oil mill waste, as well as sawdust, corn cobs, carob pods, tea waste, chicory roots, and other substrates, have been reported for biosurfactant production. Furthermore, water miscible wastes, molasses, whey milk, and distillery wastes are among the additional substrates utilized for biosurfactant production.

1.1.2. Microorganisms producing biosurfactant

Biosurfactants, synthesized by a variety of microorganisms including bacteria, fungi, and yeasts, exhibit diverse chemical compositions influenced by the producing microorganism's type. These microorganisms thrive in industrial effluents and wastewater sources, demonstrating an ability to grow on substrates typically considered noxious for non-producing microorganisms.

Microorganisms utilize a broad array of organic compounds as carbon and energy sources for growth. When the carbon source is insoluble, such as hydrocarbons, microorganisms facilitate their uptake by producing biosurfactants. Some bacteria and yeasts excrete ionic surfactants, like rhamnolipids produced by various *Pseudomonas* species, which emulsify hydrocarbon substances in the growth medium. Others modify their cell wall structure by producing nonionic or lipopolysaccharide surfactants, such as nonionic trehalose corynomycolates produced by *Rhodococcus erythropolis* and various *Mycobacterium* and *Arthrobacter* species, or lipopolysaccharides like emulsan from *Acinetobacter* species, and lipoproteins like surfactin and subtilisin from *Bacillus subtilis*.

While the production of biosurfactants by bacterial species is well-researched, fewer fungi are known to produce them. *Candida bombicola*, *Candida lipolytica*, *Candida ishiwadae*, *Candida batistae*, *Aspergillus ustus*, and *Trichosporon ashii* are among the explored fungal species, many of which can produce biosurfactants from low-cost raw materials, with sophorolipids being the major type produced by these strains.

Candida lipolytica, for instance, produces cell wall-bound lipopolysaccharides when growing on n-alkanes.

Many biosurfactant-producing microorganisms are also hydrocarbon degraders. Over the past decades, numerous studies have highlighted the effects of microbially produced surfactants not only on bioremediation but also on enhanced oil recovery.

Table.1.1. Microorganisms producing biosurfactants

SI. NO	BIOSURFACTANT	MICROORGANISMS	CURRENT ECONOMIC IMPORTANCE
1.	Cellobiose lipids	<i>Ustilago maydis</i>	Antifungal compounds
2.	Serrawettin	<i>Serratia marcescens</i>	Emulsification of hydrocarbons
3.	Polyol lipids	<i>Rhodotorula glutinis</i> , <i>R. graminis</i>	Anti-proliferative activity
4.	Trehalose lipids	<i>Rhodococcus erythropolis</i> , <i>Arthrobacter</i> sp., <i>Nocardia erythropolis</i> , <i>Cornibacterium</i> sp., <i>Mycobacterium</i>	Dissolution of hydrocarbons
5.	Ornithine lipids	<i>Pseudomonas</i> sp. <i>Thiobacillus thiooxidans</i> <i>Agrobacterium</i> sp.	Bioemulsifiers
6.	Viscosin	<i>Pseudomonas fluorescens</i> <i>Leuconostoc mesenteroides</i>	Surface active lipopeptides
7.	Rhamnolipids	<i>Pseudomonas aeruginosa</i> , <i>Pseudomonas chlororaphis</i> , <i>Serratia rubidea</i>	Bioremediation, Antimicrobial and biocontrol properties
8.	Carbohydrate lipid	<i>P. fluorescens</i>	Bioemulsifiers

9.	Protein PA	P.aeruginosa	Bioemulsifiers
10.	Diglycosyl diglycerides	Lactobacillus fermentum	Bioremediation
11.	Whole cell	Cyanobacteria	Bioflocculent
12.	Fatty acids/ neutral lipids	Clavibacter michiganensis subsp, insidious	Bioemulsifier
13.	Sophorolipids	C.antartica,C.apicola	Antimicrobial, Antiviral,spermicidal
14.	Liposan	C.tropicalis	Bioemulsan
15.	Monosylerythritol lipids	C.antartica,P.siamensis	Antifungal compounds
16.	Surfactin	B.subtilis	Antimicrobial properties
17.	Subtilisin	B.subtilis	Antimicrobial properties
18.	Peptide lipids	B.licheniformis	Antimicrobial properties
19.	Phospholipids	Acinetobacter sp.	Bioremediation
20.	Emulsan	A.calcaoacetious	Microbially enhanced oil recovery(MEOR)

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a prevalent encapsulated, gram-negative bacterium, exhibiting aerobic-facultatively anaerobic traits, and rod-shaped morphology. It is known to cause diseases in both plants and animals, including humans. Recognized for its medical significance, *P. aeruginosa* is a multidrug-resistant pathogen with well-established antibiotic resistance mechanisms, making it associated with severe illnesses, particularly in hospital settings such as ventilator-associated pneumonia and various sepsis syndromes. The bacterium selectively inhibits the penetration of antibiotics through its outer membrane and displays high resistance to numerous antibiotics, as acknowledged by the World Health Organization.

P. aeruginosa presents one of the most significant challenges to human health in terms of antibiotic resistance. Moreover, *P. aeruginosa* is highly prolific in producing exoproducts and secondary metabolites, including antimicrobials/toxins such as pyocyanin, proteases, exotoxin A, and hydrogen cyanide. Many of these are regulated by quorum sensing mechanisms. Additionally, it synthesizes two siderophores, pyoverdine and pyochelin, essential for scavenging iron in low-iron environments. These secondary metabolites play crucial roles in *P. aeruginosa*'s fitness across various environments and are implicated in social behaviors like biofilm formation.

Lipopolysaccharide (LPS) is a major component found in *P. aeruginosa*'s outer membrane, comprising lipid A and a polysaccharide composed of O-antigen, outer core, and inner core linked by covalent bonds. LPS contributes to the negative charge of *P. aeruginosa* cells, provides structural integrity, and shields the membrane from various chemicals. Notably, *P. aeruginosa* strains can simultaneously produce two different types of O-antigen (A and B bands) within the same cell. The A band, also known as the common antigen, is produced by many *P. aeruginosa* strains, eliciting a relatively weak antibody response

Stenotrophomonas maltophilia

Stenotrophomonas, a group of Gram-negative bacteria, includes at least ten species and is primarily found in soil and plants. These species vary from typical soil organisms like *S. nitritireducens* to potential human pathogens like *S. maltophilia*. Though the molecular taxonomy of the genus remains somewhat uncertain, its presence and diversity in various environments are notable.

Stenotrophomonas spp. demonstrate versatile colonization abilities across plants, humans, and marine environments. In plant environments, they efficiently metabolize various organic compounds, including phenolic compounds from root exudates. Notably, *S. maltophilia* can degrade several pollutants and produce indole-3-acetic acid (IAA), promoting plant growth through nitrogen fixation and sulfur oxidation.

Additionally, many *S. maltophilia* strains exhibit resistance to heavy metals and produce antifungal compounds and enzymes such as proteases and lipases.

1.1.3. Surface and interface activity

Surfactants play a crucial role in reducing surface tension and interfacial tension, such as between water and hexadecane, to less than 1 millinewton (Cooper et al., 1981). Rhamnolipids produced by *P. aeruginosa* have been observed to achieve a similar reduction in surface tension of water/hexadane to less than 1 millinewton (Syldatk et al., 1985). Generally, biosurfactants exhibit greater effectiveness and efficiency compared to chemical surfactants, with their critical Micelle Concentration (CMC) being several times lower. This means that for achieving maximum reduction in surface tension, a lesser amount of biosurfactant is required (Desai and Banat, 1997).

1.1.4. Biodegradability

Microbially derived compounds exhibit easier degradation compared to synthetic surfactants (Monan et al., 2006), making them well-suited for environmental applications such as bioremediation and biosorption (Mulligan et al., 2010). With growing environmental concerns, there is a heightened focus on seeking alternative products like biosurfactants (Cameotra and Makkar, 2004). Synthetic chemical surfactants pose environmental challenges, prompting the consideration of biodegradable biosurfactants from marine microorganisms for biosorption of poorly soluble polycyclic aromatic hydrocarbons, such as phenanthrene, found in aquatic surfaces (Olivera et al., 2003). Lee et al. (2008) demonstrated the control of marine algae blooms, specifically *Cochlodinium*, using the biodegradable biosurfactant sophorolipid, achieving a removal efficiency of 90%.

1.1.5. Low toxicity

Although there is limited literature available on the toxicity of biosurfactants, they are generally regarded as low or non-toxic substances and are suitable for use in pharmaceuticals, cosmetics, and food products. Poremba et al. (1991) demonstrated the higher toxicity of the chemical-derived surfactant Corexit, showing a LC50 against *Photobacterium phosphoreum* that was 10 times lower than that of rhamnolipids.

Flasz et al. (1998) conducted a comparison of the toxicity and mutagenicity profiles of biosurfactants from *Pseudomonas aeruginosa* and chemically derived surfactants, indicating the biosurfactant as non-toxic and non-mutagenic.

The low toxicity profile of sophorolipids from *Candida bombicola* has made them valuable in food industries (Cavalero and Cooper, 2003).

1.1.6. *Temperature and PH tolerance*

In recent decades, there has been growing interest in the commercial potential of biosurfactant production from extremophiles. Many biosurfactants and their surface activities exhibit resilience to environmental factors such as temperature and pH. McInerney et al. (1990) noted that lichenysin from *Bacillus licheniformis* remained stable at temperatures up to 50°C, pH levels ranging from 4.5 to 9.0, and NaCl and Ca concentrations of up to 50 and 25 g/L, respectively. Another biosurfactant produced by *Arthrobacter protophormiae* was found to be both thermostable (30- 100°C) and pH stable (pH 2 to 12) (Singh and Cameotra, 2004). Given that industrial processes often involve exposure to extreme conditions of temperature, pH, and pressure, there is a necessity to isolate novel microbial products capable of functioning under these challenging conditions (Cameotra and Makkar, 2004).

CHAPTER-2

REVIEW OF LITERATURE

2.1. BIOSURFACTANT PRODUCTION

Banat et al – 2010, the production, applications, and future potential of microbial biosurfactants were explored in a report detailing the current understanding of biosurfactants, their production methods, recent advancements in applications, and biotechnological strategies aimed at enhancing production efficiency and unlocking future potential.

Priyam Vandana and Dinesh singh et al- 2018, the review delved into biosurfactant production and its diverse applications, focusing on extraction methods for various microbial biosurfactants from cell-free supernatants. It covered the solvent separation process and detailed qualitative as well as quantitative analysis techniques.

BS Saharan, RK Sahu, and D Sharma's -2011, the review explores multiple facets of biosurfactant production, encompassing fermentation methodologies, recent advancements, production techniques, and genetic engineering strategies. It is anticipated that this review offers a thorough examination of the cutting-edge techniques and obstacles in biosurfactant research and manufacturing.

Katz and Demain -1977, offering insights into the chemistry, biogenesis, and potential functionalities of peptide antibiotics synthesized by *Bacillus* species, this exploration could bear significance for biosurfactant manufacturing and microbial interrelations.

Delorme et al.- 2011, this investigation is expected to delve into the impacts of surfactants on lipase structure, activity, and inhibition. Although lipases do not serve as biosurfactants, comprehending their interplay with surfactants holds relevance for diverse industrial applications.

Desai and Banat -1997, the review likely delves into the microbial production of surfactants and their commercial viability.

It is expected to offer a thorough examination of the process involved in producing microbial biosurfactants and their various industrial applications, including those directly related to biosurfactants.

Kalyani and Sireesha -2014, They might have identified actinomycetes capable of producing biosurfactants from both terrestrial and marine soils, thereby aiding in the discovery of new and diverse reservoirs of biosurfactants.

Noel and George et al – 2005, a strain demonstrating rapid growth and enhanced biosurfactant production was identified and designated as 3B-2. Cultures of strain 3B-2 were purified and stored in cryovials with 15% glycerol, then promptly preserved at -20°C. Morphological characteristics of strain 3B-2 were examined through gram staining and photomicroscopy, while its morphological traits were scrutinized using a scanning electron microscope. Phenotypic analysis and various biochemical tests were conducted according to the protocols outlined in Bergey's Manual of Systematic Bacteriology.

Rau et al. -2005, discuss the downstream processing of mannosylerythritol lipids produced by *Pseudozyma aphidis*. This research likely delves into the purification and isolation of these biosurfactants for industrial applications.

Johnson et al. -1992, investigate the production of bioemulsifiers by the yeast *Rhodotorula glutinis* IIP-3. Bioemulsifiers, a subtype of biosurfactants, play a role in stabilizing emulsions. This study likely delves into the process of bioemulsifier production and examines their properties.

Arguelles-Arias et al. -2009, this study is expected to offer valuable insights into biosurfactant synthesis, examining the molecular mechanisms and genetic regulation underlying biosurfactant production in microorganisms.

Roy et al - 2018, this review likely presents a thorough examination of biosurfactants, covering their characteristics, varieties, and utilization across various sectors.

It is expected to offer valuable insights into the extensive applications of biosurfactants and potential advancements in future research.

Campos et al. -2014, the study likely emphasizes the development of a bioemulsifier geared towards the food industry. It is expected to delve into the isolation and characterization of a bioemulsifier sourced from microorganisms, evaluating its emulsification capabilities and its compatibility for various food-related uses, including emulsions and formulations.

Andrade et al. -2018, The research likely introduces a promising biosurfactant sourced from *Cunninghamella echinulata* UCP 1299, utilizing renewable resources. It likely delves into the production procedure, the physicochemical characteristics of the biosurfactant, and its efficacy in cleansing cotton fabric. This study underscores the potential of microbial-derived biosurfactants for environmentally friendly cleaning tasks, emphasizing their sustainability and biodegradability advantages over conventional chemical surfactants.

2.2. BIOSURFACTANT PRODUCTION BY PSEUDOMONAS SPECIES

R.M.Patel,A.J.Desai et al-2003, The discussion focused on biosurfactant production by *Pseudomonas aeruginosa* GS3 using molasses as a substrate. It detailed the properties of *Pseudomonas aeruginosa* GS3, highlighting its ability to produce rhamnolipids. These rhamnolipids demonstrated the capability to create stable emulsions with both crude oil and olive oil. These findings suggest that utilizing renewable and relatively inexpensive substrates such as molasses holds promise for significant advancements in biotechnology processes.

Wen -jiexia et al-2011, Biosurfactant-producing bacteria were isolated and characterized from oil-contaminated soil, yielding three strains: *B. subtilis*, *P. aeruginosa*, and *R. erythropolis*. These bacteria were obtained from reservoir formation water and were cultivated using crude oil as a carbon source. Notably, *P. aeruginosa* exhibited exceptional biosurfactant production rates, as well as high levels of resistance and stability.

Eman El-Gebaly -2020, The screening of biosurfactant production was conducted using bacterial strains isolated from oil-contaminated sites near gas stations in Egypt. The study identified 30 biosurfactant-producing bacteria from oil-contaminated soils, with varying percentages of positive results in different screening assays. These bacteria were selected for further characterization and extraction procedures. Among them, *Stenotrophomonas maltophilia*, two *Bacillus* spp., two *Achromobacter* strains, *Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa* exhibited activity. The biosurfactants produced by these organisms hold potential for use in combination with antibiotics to treat bacterial infections.

Hisatsuka et al. -1971, The study explores the synthesis of rhamnolipids by *Pseudomonas aeruginosa* and their role in hydrocarbon fermentation. Rhamnolipids are robust biosurfactants with diverse industrial uses, such as improving oil recovery and facilitating bioremediation processes.

2.3. SCREENING METHODS FOR BIOSURFACTANT PRODUCTION

Bodour, A. A., & Miller-Maier, R. M et al 1998- The study discusses the utilization of a modified drop-collapse technique for quantifying surfactants and screening microorganisms capable of biosurfactant production. The procedure involved applying 2 microliters of oil to 96-well plates, allowing it to settle for 24 hours, and then adding 5 microliters of culture supernatant to the oil-coated areas. Results were observed after 1 hour, with deionized water serving as the negative control.

Ranjini dhiman, Khen raj meena, Abhishek sharma, S.kanwar-2016, Biosurfactants and their screening methods were discussed about classification of biosurfactant and explained about screening methods for biosurfactant production.

2.4. OPTIMIZATION FOR BIOSURFACTANT PRODUCTION

Foukia E. Mouafi et al 2016 The investigation delves into the optimization of biosurfactant production by *Bacillus brevis* employing response surface methodology. Twenty bacterial isolates underwent screening for biosurfactant production, with BS identified as exhibiting promising potential as an emulsifier.

Wuyang sun et al 2021 The study focused on optimizing biosurfactant production from *Pseudomonas* sp. using carbon dioxide and its application for remediating heavy metal-contaminated soil. Biosurfactant production was tested, and ultimately, it was found that CQ2 was effective in removing Cd, Cu, and Pb from the soil.

Khuri and Cornell- 1996, Response surface design, a statistical approach frequently employed in optimization studies concerning biosurfactant production, was utilized.

Onwosi and Odibo -2013, Employing response surface design, the optimization of starter cultures was conducted to enhance rhamnolipid production by *Pseudomonas nitroreducens*.

Choi et al. -1996, The investigation focuses on exploring the impact of ethanol and phosphate on the production of emulsan by *Acinetobacter calcoaceticus* RAG-1. Emulsan, a biosurfactant synthesized by specific bacteria, is the subject of this research, which aims to understand the factors that affect its production and optimize the process.

2.5. APPLICATIONS OF BIOSURFACTANT

Andrea F, Isabila G, Giuseppina B, and Lbrahim Met al – 2010, In their Environmental Science paper, the authors examine both the accomplishments and setbacks of biosurfactant-based bioremediation. This research likely offers valuable insights drawn from case studies and experimental results concerning the effectiveness of biosurfactants in remedying environmental pollutants, including oil spills.

Amalesh et al. -2012, It's possible that the researchers explored the biosurfactant capabilities of bacteria that produce alkaline protease, which were isolated from municipal solid waste.

This investigation might provide insights into the origins of microorganisms capable of producing biosurfactants and how they can be utilized in waste management practices.

Christofi and Ivshina -2002, The research probably offers valuable insights into the application of microbial surfactants, encompassing biosurfactants, in studies focused on soil remediation. This investigation holds significance in comprehending the potential uses of biosurfactants in endeavors aimed at environmental restoration.

Maneera and Dikit -2007, The aim of the study is to characterize cell-associated bioemulsifiers derived from *Myroides* sp. SMI, a marine bacterium. Gaining insights into the properties of bioemulsifiers sourced from marine bacteria is crucial for their potential utilization across diverse industries, such as bioremediation and pharmaceuticals.

Mary Swarnalatha S and Jaqueline Chinna Rani I -2019, The Pharma Innovation Journal may feature a publication discussing innovative discoveries or applications concerning biosurfactants. Evaluating the study's specific emphasis is necessary to determine its significance within the field.

Mulligan et al -2005, A review likely examines the environmental uses of biosurfactants, emphasizing their potential roles in bioremediation, soil reinforcement, and treating wastewater. This thorough overview probably discusses different biosurfactant varieties and their efficacy in environmental restoration endeavors.

Satpute et al. -2010, Offering techniques for exploring biosurfactants and bioemulsifiers, this resource is beneficial for researchers keen on analyzing and assessing the attributes of these compounds.

CHAPTER-3

AIM AND OBJECTIVES

3.1. AIM

To investigate the assessment of bacterial reservoirs for biosurfactant synthesis.

3.2. OBJECTIVES

- To collect the sample in different places of kitchen chimney from kanchipuram
- To isolate the bacterial culture
- To the screening for potential culture
- To the production of biosurfactant
- To the characterization of Biosurfactant
- To the molecular Identification of bacterial culture

CHAPTER-4

MATERIALS AND METHODOLOGY

4.1. ISOLATION OF BACTERIAL CULTURE FROM KITCHEN CHIMNEY OIL

4.1.1. Collection of sample

Oil samples were taken from the kitchen chimney tray after settling for three months. These samples were then serially diluted, and *Pseudomonas* and *Stenotrophomonas* were identified using 16srna sequencing. Subsequently, dilutions of 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were employed for further analysis.

4.1.2. Isolation of Bacteria from oil sample

Bacterial strain were isolated by spread plate method using Actinomycetes Isolation Agar (AIA), Nutrient agar (NA). About 1 gram of kitchen chimney oil was heat treated for moisture removal and was added in 10 ml distilled water. The suspension was serially diluted up to 10^{-5} . 100 μ l of solution from 10^{-3} , 10^{-4} and 10^{-5} dilutions was taken and spread over agar plates using sterile L-rod. The plates were incubated at 28°C for 7-14 days. After incubation, the bacterial colonies were identified and then sub cultured in AIA and NA plates and stored for further use.

4.2. INVITRO SCREEING OF BIOSURFACTANT PRODUCING CULTURES

4.2.1. Oil Displacement Assay

A standard assay for biosurfactant producers involved adding 20ml of distilled water to a petri plate. Then, 1ml of used engine oil was spread over the surface of the water. Subsequently, 100 μ l of cell-free culture broth was carefully dropped onto the surface of the crude oil. The diameter of the clear zone formed on the oil surface was measured. This measurement was compared to the clear zone formed by adding 10 μ l of distilled water as a negative control.

4.2.2. Drop Collapse Test

In a 96-well microtiter plate, 2µl of mineral oil was dispensed into each well. Subsequently, 5µl of culture supernatant was added onto the oil in each well. The plate was then incubated at 37°C for 1 hour. The shape of the oil drop on the surface was examined to determine the result. Biosurfactants alter the shape of the oil drop and reduce the interfacial tension between the drop and the hydrophobic surface, causing the drop to collapse. While this method is dependable for detecting large unknown consortia, it may lack sensitivity in detecting low levels of surfactant.

4.2.3. Emulsification Activity

This method relies on the capacity of biosurfactants to emulsify hydrocarbons, enhancing their accessibility for cellular uptake and forming emulsions. In each of four sterile glass test tubes, 2ml of petrol was added, followed by 2ml of culture supernatant (KC-15, KC-23). The contents were then vigorously vortexed for 2 minutes at high speed. Afterward, the tubes were left to stand for 24 hours. The emulsification index percentage was calculated using the formula:

Emulsification index = (Height of emulsion layer/Total height) × 100.

4.2.4. Phenol Sulfuric Acid Method

In each of 10 glass test tubes, 1ml of 5% phenol and 1ml of culture supernatant were added. Sulfuric acid was then cautiously added drop by drop, up to 5ml, until a color change occurred. The presence of glycolipid-type biosurfactants was confirmed by the formation of an orange color in the reaction mixture. This test relies on sulfuric acid's ability to interact with carbohydrates, resulting in the formation of furfural compounds through the removal of water molecules. These compounds subsequently react with phenol, producing a dark yellow color indicative of the presence of glycolipid-type biosurfactants.

4.2.5. Phenol Red Method

Phenol red media was prepared to observe the cultures. Using an inoculation loop, the cultures were spotted onto the phenol red media. A change in color from red to yellow indicates positive results. Phenol red serves as a pH indicator dye, enabling the detection of pH changes resulting from acidity. When extracellular lipases act on triglycerides, fatty acids are released, leading to a change in the medium's acidity.

This method is employed to detect lipolytic activity, which signifies the presence of biosurfactants.

4.2.6. Parafilm Method

Culture supernatant (25µl) was dispensed onto a parafilm sheet for each culture. Similarly, 25µl of distilled water and glycerol were separately dispensed onto the parafilm sheet. Following the droplet placement, the shape of each drop was observed. A concave shape indicated a negative result, while a flat shape indicated a positive result. Biosurfactants have the ability to destabilize drops of polar liquid by reducing the surface tension and interfacial tension between the drop and the hydrophobic surface of the parafilm strip.

4.3. DEGRADATION OF THE DYE USING THE STRAIN KC-15, KC-23 WITH METHYLENE BLUE AND MALACHITE GREEN

A total of 240ml of AIA Broth was prepared and divided into 12 Falcon tubes, with each tube containing up to 20ml of AIA broth. Methylene Blue and Malachite Green were selected for dye degradation, with concentrations set at 100ppm (0.002g), 250ppm (0.005g), and 500ppm (0.01g) for each dye. The culture was then inoculated using an inoculating loop, and readings were taken once inoculation occurred, using 1ml of Eppendorf tube to collect the sample. After inoculation, the broth was centrifuged at 8500 rpm for 15 minutes. Subsequently, 100µl of supernatant was added to a 96-well microtiter plate for UV-Visible Spectroscopy analysis. The degradation of dyes at different concentrations over a period of 4 days was measured using UV-Visible Spectroscopy, with methylene blue at 660nm and malachite green at 620nm. The percentage of dye degradation was calculated using the formula:

$$\% \text{ of degradation} = (\text{Initial absorption} - \text{Final absorption}) / \text{Initial absorption} * 100$$

4.4. EFFECT OF DIFFERENT SOURCES ON DECOLORIZATION OF METHYLENE BLUE

4.4.1. Effect of Carbon Sources on degradation of Methylene Blue by strain KC15, KC23 Samples.

To optimize carbon sources, AIA broth was prepared with various sugars including glucose, sucrose, xylose, inositol, mannitol, fructose, lactose, starch, cellulose, and a control with no sugar. Methylene blue dye was added to each broth at a concentration of 100mg/l and autoclaved. Initial readings were taken using UV-Visible Spectroscopy at their respective wavelengths (MB-660nm, MG-620nm). The broths were then inoculated with bacterial strains (KC-15, KC-23) and incubated in a shaker at 120 rpm. After 7 days of incubation, samples were drawn aseptically and centrifuged at 8500 rpm for 10 minutes. The estimation of dye decolorization was observed using UV-Visible Spectroscopy by adding 100µl of sample supernatant to a 96-well microtiter plate. The carbon source that exhibited the highest percentage of dye degradation was considered the most effective for degrading methylene blue dye.

4.4.2. Effect of Nitrogen Sources on degradation of Methylene Blue by strain KC-15, KC23 Samples.

To optimize nitrogen sources, AIA broth was prepared using various nitrogen sources including malt extract, yeast extract, peptone, soybean meal, KNO₃, and a control with no nitrogen source. Methylene blue dye was added to each broth at a concentration of 100mg/l and autoclaved. Initial readings were taken using UV-Visible Spectroscopy at their respective wavelengths. The broths were then inoculated with bacterial strains (KC-15, KC-23) and incubated in a shaker at 120 rpm. After 7 days of incubation, samples were drawn aseptically and centrifuged at 8500 rpm for 10 minutes. The estimation of dye decolorization was observed using UV-Visible Spectroscopy by adding 100µl of sample supernatant to a 96-well microtiter plate. The nitrogen source that exhibited the highest percentage of dye degradation was considered the most effective for degrading methylene blue dye.

4.4.3. Effect of Mineral Sources on degradation of Methylene Blue by strain KC-15, KC-23 Samples.

To optimize mineral sources, AIA broth was prepared with various mineral sources including NaCl, CaCl₂, MnCl₂, FeSO₄, MgSO₄, and a control with no mineral source. Methylene blue dye was added to each broth at a concentration of 100mg/l and autoclaved. Initial readings were taken using UV-Visible Spectroscopy at their respective wavelengths. The broths were then incubated in a shaker at 120 rpm after inoculation with bacterial strains (KC-15, KC-23). After 7 days of incubation, samples were aseptically drawn and centrifuged at 8500 rpm for 15 minutes. The estimation of dye decolorization was observed using UV-Visible Spectroscopy by adding 100µl of sample supernatant to a 96-well microtiter plate. The mineral source that exhibited the highest percentage of dye degradation was considered the most effective for degrading methylene blue dye.

4.4.4. Effect of pH on degradation of Methylene Blue and Malachite Green by strain KC15, KC23 Samples.

To optimize pH conditions, AIA broth was prepared with different pH levels including pH 4, pH 5, pH 6, pH 7, pH 8, and pH 9. Methylene blue dye was added to each broth at a concentration of 100mg/l and autoclaved. Initial readings were taken using UV-Vis Spectroscopy at their respective wavelengths. The broths were then incubated in a shaker at 120 rpm after inoculation with bacterial strains (KC-15, KC- 23). After 7 days of incubation, samples were aseptically drawn and centrifuged at 8500 rpm for 15 minutes. The estimation of dye decolorization was observed using UV-Visible Spectroscopy by adding 100µl of sample supernatant to a 96-well microtiter plate. The pH range that exhibited the highest percentage of dye degradation was considered the optimal pH for the degradation of methylene blue dye.

4.5. SOLVENT SEPARATION AND EXTRACTION OF KC15, KC23 SAMPLES.

This procedure utilized the cell-free supernatant technique. Initially, the pH of the supernatant was adjusted to 2.0 using concentrated HCl. Subsequently, 5ml of ethyl acetate was added to the supernatant in a 2:1 ratio. The mixture was thoroughly shaken for proper mixing and left overnight to allow color sediments, indicative of biosurfactants, to form. These sediments were then dried and mixed with 1ml of DMSO. The oil displacement activity was assessed using dried crude extracts with DMSO in three plates, employing KC15 and KC23.

4.6 CHARACTERIZATION OF BIOSURFACTANT BACTERIAL STRAINS

The microscopic, physiological, and cultural characteristics of the potential actinobacterial strain HRB1 were examined following the methods outlined by Shirling and Gottlieb (1966) and Radhakrishnan et al. (2013). Molecular characterization was performed through 16S rRNA gene sequence analysis. Genomic DNA was extracted from the HRB1 strain using the Solute Ready Genomic DNA Kit (GenElute™). PCR amplification of the 16S rRNA gene was conducted using the Eppendorf Master Cycler Gradient with primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (Lane, 1991).

The PCR reaction mixture (50 µl) contained 100 ng of genomic DNA, 10x buffer, 1 mg/ml of BSA, 2.5 mM of dNTP mixture, 3.5 U of Taq DNA polymerase, and 10 µM of each primer. The PCR conditions were as follows: initial denaturation at 94°C for 6 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 1.5 min, and final extension at 72°C for 8 min. Purified PCR products were subjected to 16S rRNA gene sequencing using the Sanger sequencing method at Eurofin Genomics, Bangalore. The sequences were edited and contigs assembled using DNA Baser software version 3. Edited sequences were compared with sequences deposited in the GenBank database using BLAST analysis. Alignment of 16S rRNA gene sequences with selected sequences from GenBank was performed using CLC Sequence Viewer 6.0 software.

A phylogenetic tree was constructed using the neighbor-joining algorithm with bootstrap analyses of 1000 replications to determine confidence values for the tree branches using MEGA software version 7.0. The partial 16S rRNA nucleotide sequence of strain HRB1 was deposited in the GenBank database.

CHAPTER – 5

RESULTS AND DISCUSSIONS

5.1. COLLECTION OF SAMPLE

The oil extracted from the kitchen chimney underwent heat treatment and was subsequently isolated on various media plates. A total of 28 morphologically distinct strains were obtained from the kitchen chimney oil and subcultured. All the strains exhibited robust growth. The morphological characteristics of these 31 strains are detailed in Table 5.1. Römling et al. isolated 41 bacterial and fungal strains for biosurfactant production in 1998.

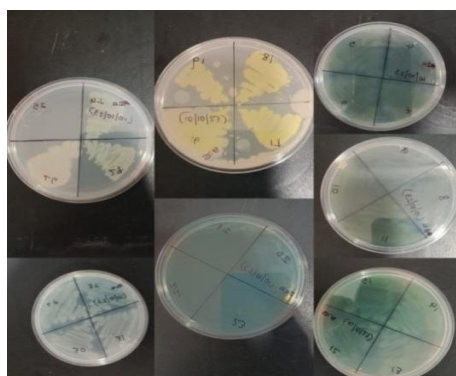


Fig:5.1 Morphologically different strains sub cultured in AIA and NA me

Table:5.1 Morphological Characteristics of isolated strain from KC

STRAIN NO	GROWTH	AERIAL MYCELIUM (AM)	SUBSTRATE MYCELIUM (SM)	REVERSE SIDE PIGMENT (RSP)	COLONY CONSISTA-NCY	COLONY COLOR
KC4	GOOD	-	+	GREYISH GREEN	CREAMY	PALE WHITE
KC5	GOOD	-	+	GREYISH GREEN	CREAMY	PALE WHITE

KC6	GOOD	-	+	GREYISH GREEN	CREAMY	PALE WHITE
KC7	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC8	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC9	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC10	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC11	GOOD	-	-	GREYISH GREEN	CREAMY	PALE WHITE
KC12	GOOD	+++	+	GREYISH GREEN	CREAMY	PALE WHITE
KC13	GOOD	++	+	GREYISH GREEN	CREAMY	PALE WHITE
KC14	GOOD	++	+	GREYISH GREEN	CREAMY	PALE WHITE
KC15	GOOD	++	+	GREYISH GREEN	CREAMY	PALE WHITE
KC16	GOOD	-	-	GREYISH WHITE	CREAMY	PALE YELLOW
KC17	GOOD	-	-	GREYISH WHITE	CREAMY	PALE YELLOW
KC18	GOOD	-	-	GREYISH WHITE	CREAMY	PALE YELLOW
KC19	GOOD	-	-	GREYISH WHITE	CREAMY	PALE YELLOW

KC20	GOOD	-	-	GREYISH GREEN	CREAMY	PALE WHITE
KC21	GOOD	-	+	PALE GREEN	CREAMY	PALE WHITE
KC22	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC23	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC24	GOOD	++	-	GREYISH GREEN	CREAMY	PALE YELLOW
KC25	GOOD	-	-	PALE GREEN	CREAMY	PALE WHITE
KC26	GOOD	-	-	GREYISH GREEN	CREAMY	PALE YELLOW
KC27	GOOD	-	-	GREYISH GREEN	CREAMY	PALE YELLOW
KC28	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC29	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC30	GOOD	++	-	GREYISH GREEN	CREAMY	PALE WHITE
KC31	GOOD	-	+	GREYISH GREEN	CREAMY	PALE WHITE

Out of the total 28 strains, 21 exhibit pale white colony morphology, while the remaining 7 display pale yellow colonies. M. Cristina Diez et al. have characterized their isolated bacterial strains, identifying 1 with a red colony, 1 with a yellow colony, 1 with a dark pink colony, and 1 with a light pink colony.

5.2. INVITRO SCREEING OF BIOSURFACTANT PRODUCING CULTURES

5.2.1. *Oil Displacement Assay*

The assessment and observation of the clear zone on the oil surface were carried out for KC-15 and KC-23, as shown in Figure 5.2.



Fig: 5.2. oil displacement assay for KC-15 and KC-23 samples

Among the 28 bacterial strains tested, 2 exhibited the most favorable results in the oil displacement assay. Moussa et al provided a detailed explanation of the oil displacement assay in the year 2017, The favorable outcomes for them involve bacillus strains MG 13 and MG 20.

5.2.2. *Drop Collapse Test*

The shape of the oil drop on the surface was evaluated to determine the results. KC-15 and KC-23 yielded positive results, as illustrated in Fig 5.3.

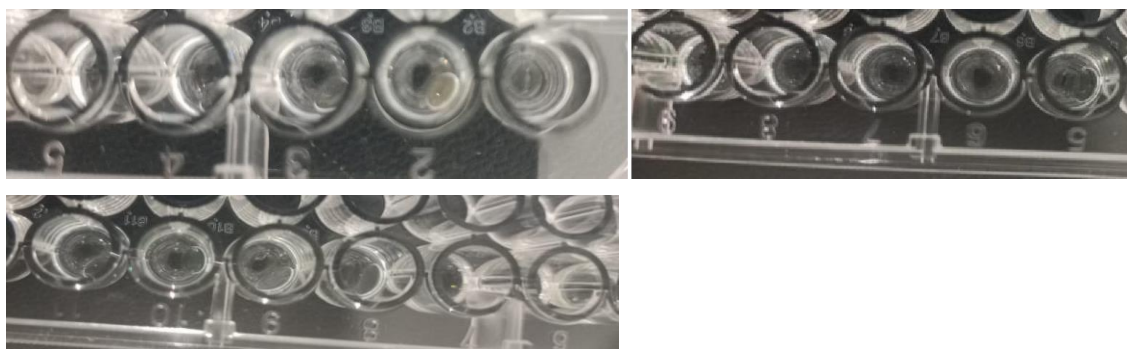


Fig:5.3 Bacterial strain showing drop collapse test

The strains CH 2 and CH 5 show promise as potential candidates for biosurfactant production in the study by Mounira Arieche et al.

5.2.3. Emulsification Activity

The calculation of the emulsification index percentage is determined using the formula. Positive results for KC-15 and KC-23 are depicted in Fig 5.4.

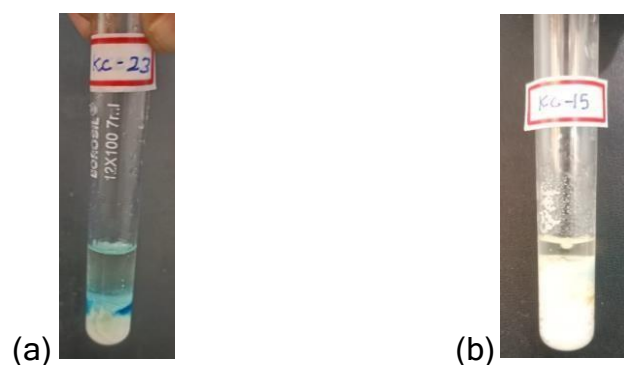


Fig:5.4. KC-15, KC-23 showing emulsification activity

Among all the strains examined, KC-15 and KC-23 exhibited positive results in emulsification activity. Camacho Cab et al. found that Triton X-100 and Tween 20 were more effective in this assay.

5.2.4. Phenol Sulfuric Acid Method

The presence of glycolipid type was confirmed by the formation of an orange color in the reaction mixture. Positive results for KC-15 and KC-23 are illustrated in Fig 5.5.

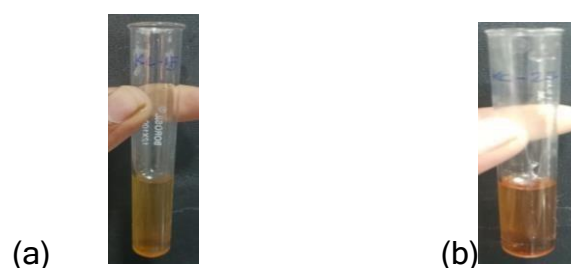


Fig:5.5 KC15, KC23 reacts to phenol sulfuric acid test

5.2.5. Phenol Red Method

A change from red to yellow color indicates positive results for the bacterial strain producing biosurfactant. The results are depicted in Fig 5.6.

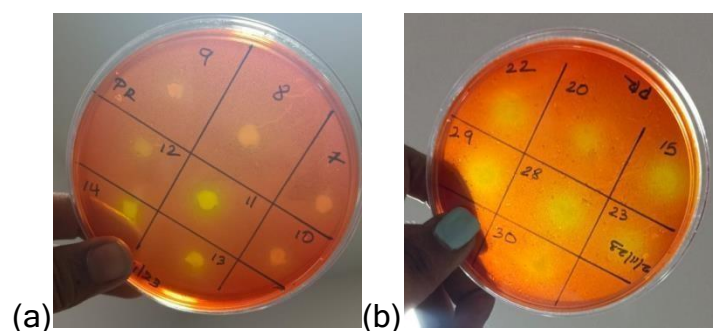


Fig:5.6 Bacterial strains showing positive results in phenol red method

Out of 28 bacterial strains, positive results were obtained from 2 strains (KC15, KC23).

5.2.6. Parafilm Method

After dropping, the shape of the drop is observed, with a concave shape indicating a negative result, while a flat shape indicates a positive result. KC15 and KC23 exhibited positive results, indicating biosurfactant production, as depicted in Fig 5.7.

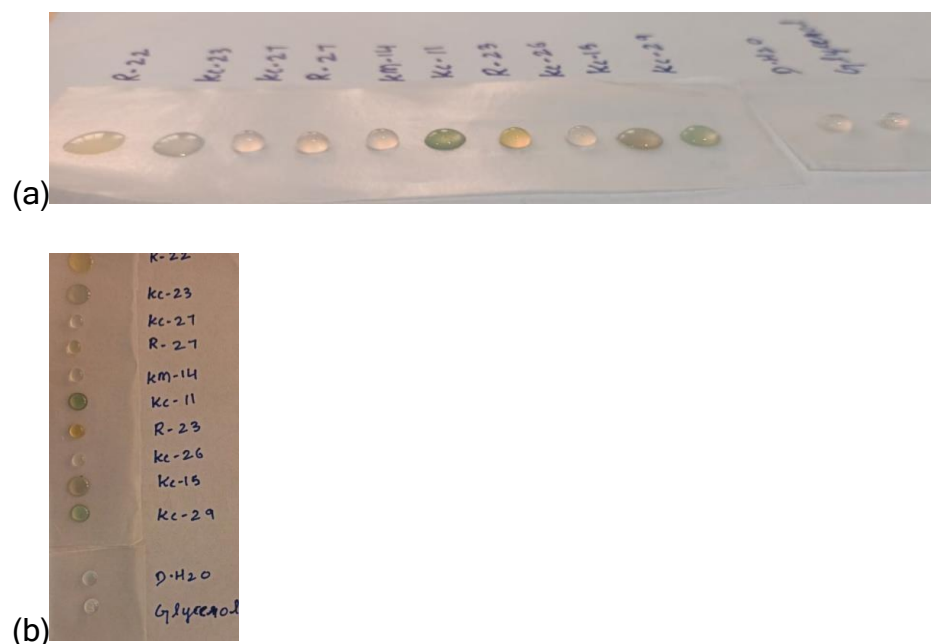


Fig:5.7. KC15, KC23 samples showing biosurfactant production

Khyati Patel has discovered six isolates—Ky-48, Ky-53, Ky-54, Ky-84, Ky-86, and Ky-87—that exhibit encouraging biosurfactant activity.

5.3. DYE DEGRADATION TEST

The bacterial strain was subjected to a dye degradation test using methylene blue and malachite green at varying concentrations (100ppm, 250ppm, 500ppm).

5.3.1. Degradation of different concentrations of methylene blue using the strain KC15, KC23 Samples

The degradation of methylene blue was assessed at various concentrations (100ppm, 200ppm, and 500ppm), with 0.002mg, 0.005mg, and 0.01mg added to each 20 ml of AIA broth by the KC15 strain, as illustrated in Fig 5.8. The percentage of degradation of methylene blue increased from the 2nd day to the 3rd day and from the 3rd day to the 4th day. The degradation percentage of methylene blue was determined using a degradation percentage formula.

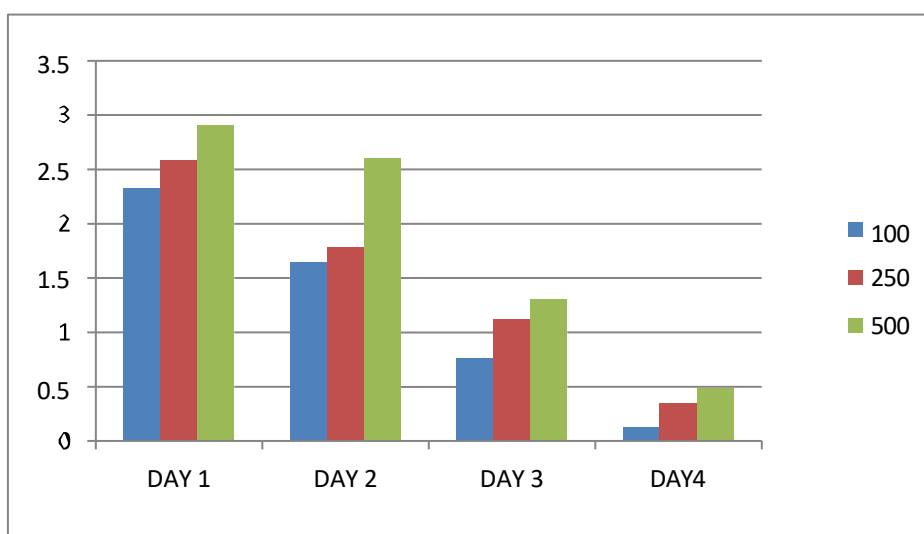


Fig:5.8 Degradation of different concentration of methylene blue using the strain KC15

The degradation of methylene blue was conducted at varying concentrations (100ppm, 200ppm, and 500ppm), with 0.002mg, 0.005mg, and 0.01mg added to each 20 ml of AIA broth by the KC23 strain, as depicted in Fig 5.9.

The percentage of degradation of methylene blue exhibited an increase from the 2nd day to the 3rd day and from the 3rd day to the 4th day. Calculation of the degradation percentage of methylene blue was performed using a degradation percentage formula.

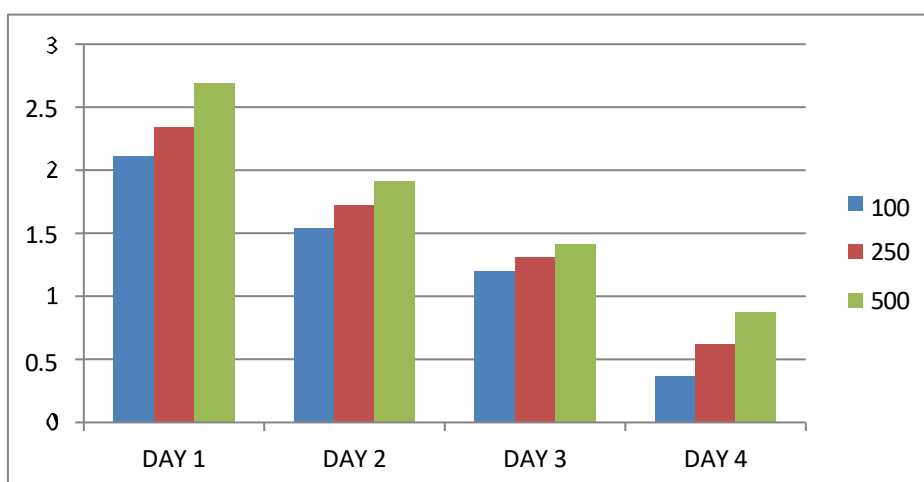


Fig:5.9 Degradation of different concentration of methylene blue using the strain KC23

5.3.1. Degradation of different concentrations of malachite green using the strain KC15, KC23 samples

The degradation of malachite green was conducted at various concentrations (100ppm, 200ppm, and 500ppm), with 0.002mg, 0.005mg, and 0.01mg added to each 20 ml of AIA broth by the KC15 strain, as illustrated in Fig 5.10. The percentage of degradation of malachite green exhibited an increase from the 2nd day to the 3rd day and from the 3rd day to the 4th day. Calculation of the degradation percentage of malachite green was performed using a degradation percentage formula.

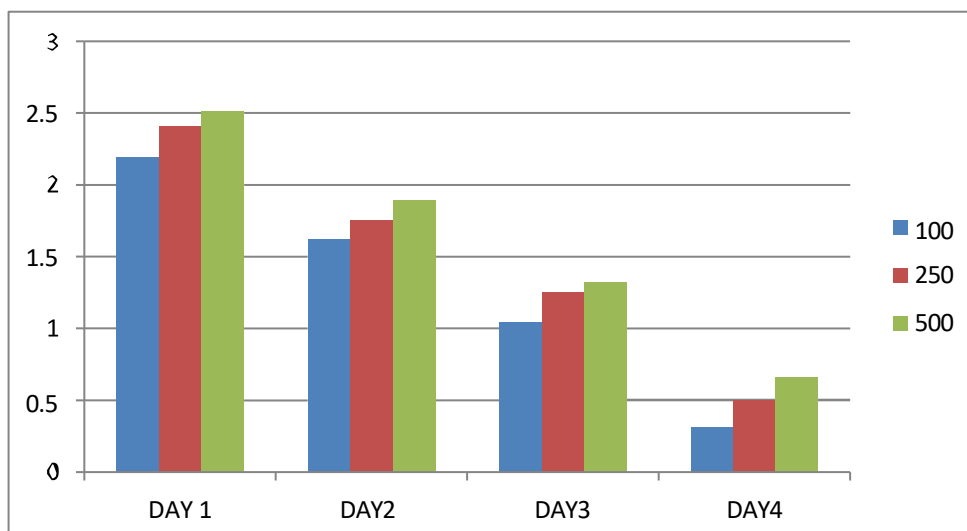


Fig:5.10 Degradation of different concentration of malachite green using the strain KC15

The degradation of malachite green was performed at varying concentrations (100ppm, 200ppm, and 500ppm), with 0.002mg, 0.005mg, and 0.01mg added to each 20 ml of AIA broth by the KC23 strain, as depicted in Fig 5.11. The percentage of degradation of malachite green exhibited an increase from the 2nd day to the 3rd day and from the 3rd day to the 4th day. Calculation of the degradation percentage of malachite green was carried out using a degradation percentage formula.

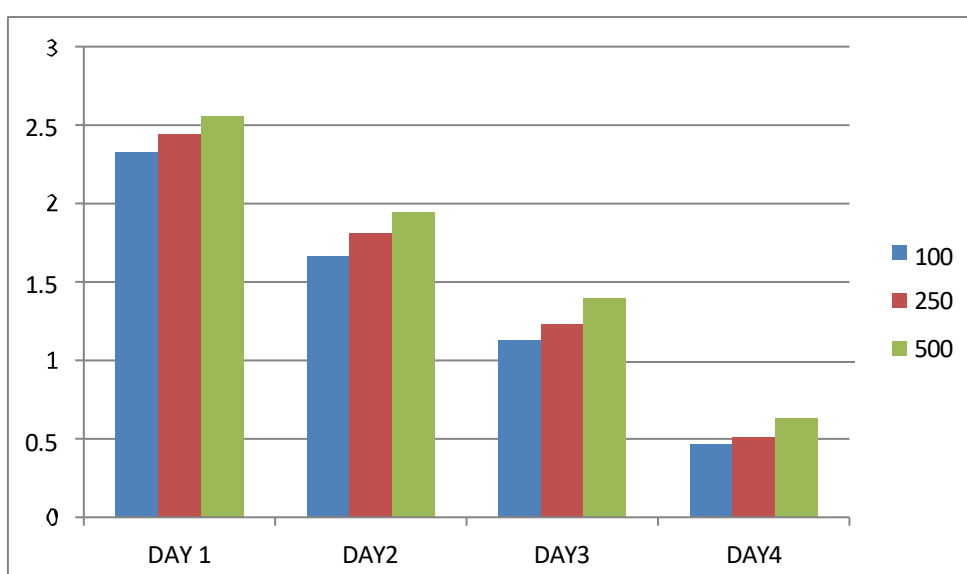


Fig5.11 Degradation of different concentration of malachite green using the strain KC23 sample

5.4. EFFECT OF DIFFERENT SOURCES ON DECOLORIZATION OF METHYLENE BLUE.

5.4.1. Effect of Carbon Sources on degradation of Methylene Blue by strain KC15, KC23 samples

5.4.1.1. Degradation of methylene blue by strain KC15 and KC23 under different carbon Sources

The degradation percentage of methylene blue in various carbon sources is illustrated in Fig 5.12. According to this investigation, cellulose exhibited the highest percentage of degradation of methylene blue compared to other carbon sources.

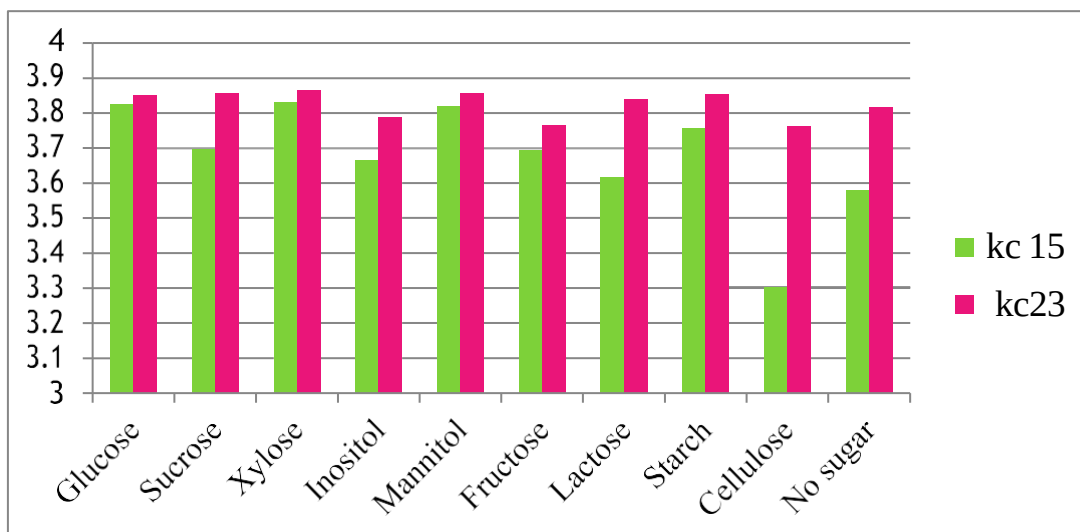


Fig:5.12 Degradation of methylene blue by strain KC15 and KC23 under different carbon sources

5.4.2. Effect of nitrogen source in methylene blue degradation by strain KC15, KC23 samples

5.4.2.1. Degradation of methylene blue by strain KC15 and KC23 under different nitrogen sources:

The degradation percentage of methylene blue in different nitrogen sources is presented in Fig 5.13. According to this analysis, yeast extract showed the highest percentage of degradation of methylene blue compared to other nitrogen sources.

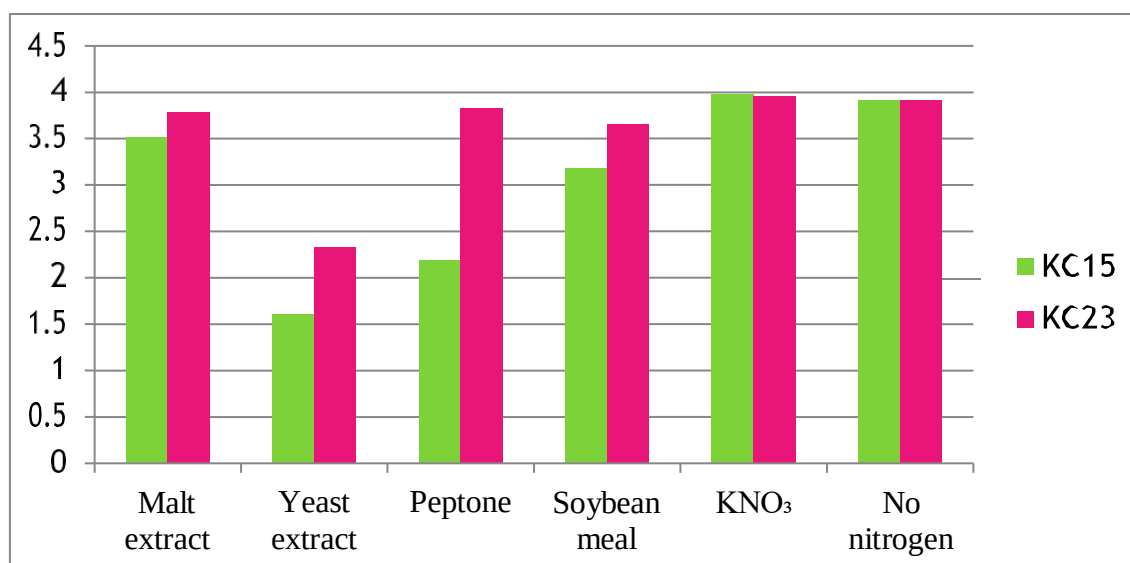


Fig:5.13 Degradation of methylene blue by strain KC15 and KC23 under different nitrogen sources

5.4.3. Effect of mineral source in methylene blue degradation by strain KC15, KC23 samples

5.4.3.1. Degradation of methylene blue by strain KC15 and KC23 under different mineral sources

The degradation percentage of methylene blue in various mineral sources is illustrated in Fig 5.14. According to the findings, the degradation of methylene blue in MnCl_2 exhibited the highest percentage compared to other mineral sources.

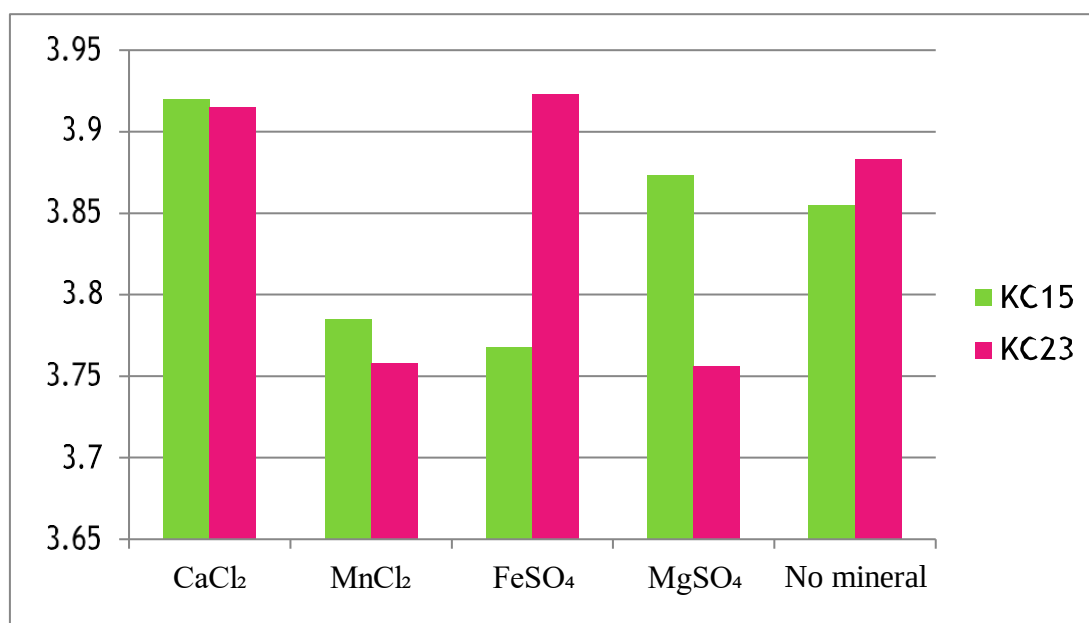


Fig:5.14. Degradation of methylene blue by strain KC15 and KC23 under different mineral sources

5.4.4. Effect of pH in methylene blue degradation by strain KC15, KC23 samples

5.4.4.1 Degradation of methylene blue by strain KC15 and KC23 under different PH Sources

The degradation percentage of methylene blue across different pH levels is depicted in Fig 5.15. The results indicate that methylene blue degradation is most pronounced at pH 6 compared to other pH values.

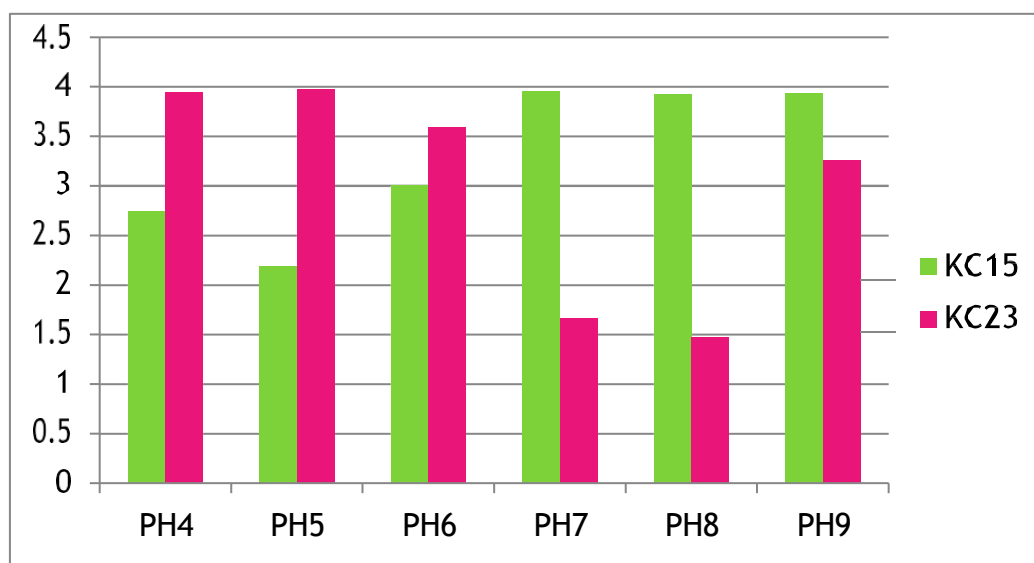


Fig: 5.15 Degradation of methylene blue by strain KC15 and KC23 under different pH sources

5.5. Solvent separation and extraction of KC15, KC23 samples

The DMSO negative control shows no reaction, while KC15 and KC23 exhibit a positive result, as depicted in Fig 5.16, indicating biosurfactant production.



Fig:5.16 Bacterial strain KC15, KC23 shows biosurfactant production

5.6 Characterization of KC-15 and KC-23 samples

The physicochemical properties of the oil samples were analyzed, leading to the isolation of two distinct actinobacterial strains: KC 15 and KC 23.

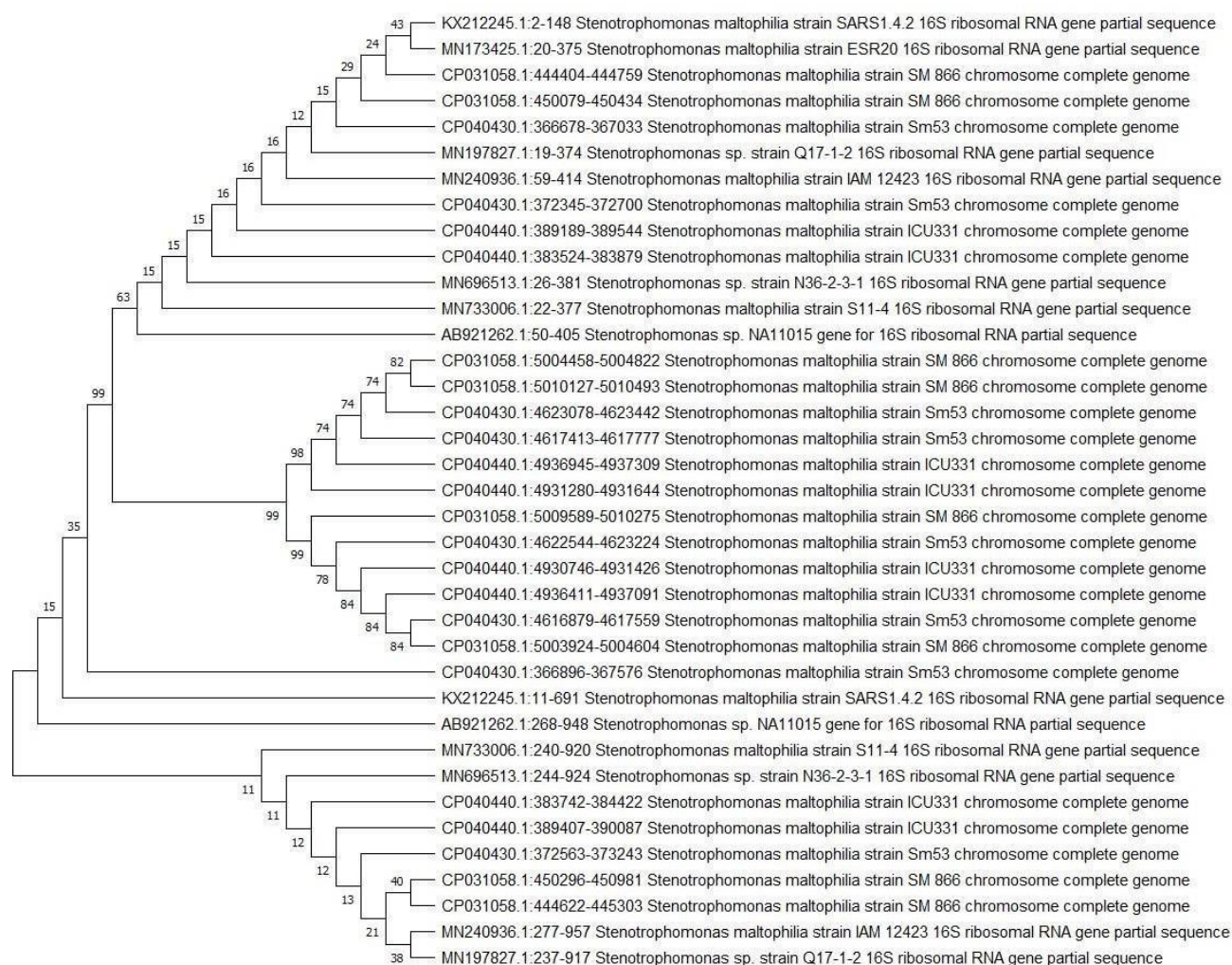


Fig:5.17 Characterization of Bacterial strain KC-15 sample

Subsequent characterization revealed that KC 15 belonged to the species *Stenotrophomonas maltophilia*.

gctcctggattcagcggcggacgggtgagtaatgcctaggaatctgcctggtagtggggg
ataacgtccggaaacgggcgctaataccgcatacgtcctgagggagaaagtgggggatct
tcggacctcacgctatcagatgagcctaggtcggattagctagttggtggggtaaaggcc
taccaaggcgacgatccgtaactggtctgagaggatgatcagtcacactggaactgagac
acggtccagactcctacgggaggcagcagtggggaatattggacaatgggcgaaagcctg
atccagccatgccgcgtgtgtgaagaaggctctcggattgtaaagcactttaagttggga
ggaagggcagtaagttaataccttgctgtttgacgttaccaacagaataagcaccggct
aacttcgtgccagcagccgcggttaatacgaagggtgcaagcgtaatcggaattactggg
cgtaaagcgcgctaggtggttcagcaagttggatgtgaaatccccgggctcaacctggg
aactgcatccaaaactactgagctagaGtaGtacttctggagcaaccactcccatggt
gtgacgggcggtgtgtacaaggcccggaacgtattcaccgtgacattctgattcacgat
tactagcgattccgacttcacgcagtcgagttgcagactgcgatccggactacgatcgg
tttatgggattagctccacctcgcggttggaaccccttgtagccaccattgtagcacg
tgtgtagccctggccgtaagggccatgatgacttgacgtcatccccaccttctccggtt
tgtcaccggcagtccttagagtgtccacccgagggtgctggtaactaaggacaaggggt
gcgctcgttacgggacttaaccaacatctcacgacacgagctgacgacagccatgcagc
acctgtgtctgagttcccgaaggcaccaatccatctctggaagttctcagcatgtcaag
gccaggttaaggttctcggtgtctcgaattaaaccacatgtccaccgcttggtcggg
ccccgtaattcatttgagtttaaccttgcgccgtactcccaggcggtcgacttat
cgcgtagctgcgccactaagatctcaaggatcccaacggctagtcgacatcgtttacgg
cgtggactaccagggtatctaactctgtttgctccccacgcttcgcacctcagtgtag
tatcagtcagggtggtgccttcgccactggtgttccttctatatctacgcatttcacc
gctacacaggagattccaccacctctaccgtactctagctcagta

Fig:5.18 16s RNA sequencing of Bacterial strain KC-15 sample

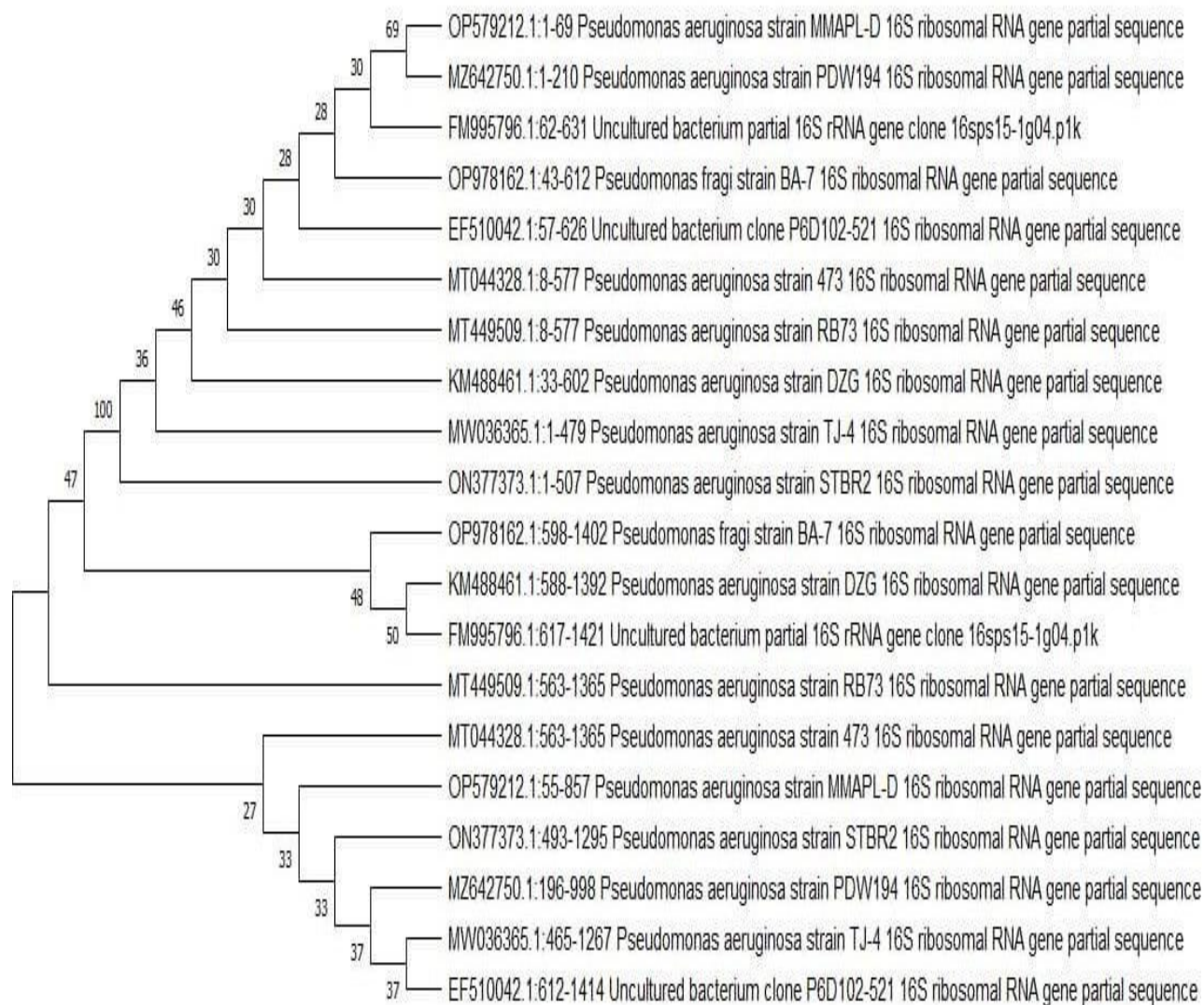


Fig:5.19 Characterization of Bacterial strain KC-23 sample

KC 23 was identified as belonging to the species *Pseudomonas aeruginosa*.

gctcctggattcagcggcggacgggtgagtaatgcctaggaatctgcctggtagtggggg
ataacgtccggaaacgggcgctaataccgcatacgtcctgagggagaaagtgggggatct
tcggacctcacgctatcagatgagcctaggtcggattagctagtgtgggtgggtaaaggcc
taccaaggcgacgatccgtaactggctgagaggatgatcagtcacactggaactgagac
acggtcagactcctacgggaggcagcagtggggaatattggacaatgggcgaaagcctg
atccagccatgccgcgtgtgtgaagaaggctctcggattgtaaagcactttaagttggga
ggaagggcagtaagttaataccttgctgtttgacgttaccaacagaataagcaccggct
aacttcgtgccagcagccgcgtaatacgaagggtgcaagcgtaatcggaattactggg
cgtaaagcgcgctaggtggttcagcaagttggatgtgaaatccccgggctcaacctggg
aactgcatccaaaactactgagctagaGtaGtacttctggagcaaccactcccatggt
gtgacgggcggtgtgtacaaggcccggaacgtattcaccgtgacattctgattcacgat
tactagcgattccgacttcacgcagtcgagttgcagactgcgatccggactacgatcgg
tttatgggattagctccacctcgcggttggaaccccttgtagccaccattgtagcacg
tgtgtagccctggccgtaaggccatgatgacttgacgtcatccccacctcctccggtt
tgtcaccggcagtccttagagtgtcccacccgagggtgctggtaactaaggacaaggggt
gcgctcgttacgggacttaaccaacatctcacgacacgagctgacgacagccatgcagc
acctgtgtctgagttcccgaaggcaccaatccatctctgaaagtctcagcatgtcaag
gccaggtgaaggttctcgctgtctcgaattaaaccacatgtccaccgcttgtagcggg
ccccgtaattcatttgagtttaaccttgccggccgtactccccaggcggtcgacttat
cgcgtagctgcgccactaagatctcaaggatcccaacggctagtcgacatcgtttacgg
cgtggactaccaggggtatctaactctgtttgctccccacgcttcgcacctcagtgtag
tatcagtcagggtggtgccttcgccactggtgttccttctatatctacgcatttcacc
gctacacaggagattccaccacctctaccgtactctagctcagta

Fig:5.18 16s RNA sequencing of Bacterial strain KC-15 sample

CHAPTER- 6

SUMMARY AND CONCLUSION

The rapid expansion of industries has exacerbated environmental pollution, with petroleum contamination being a particularly prevalent ecological hazard. This pollution poses significant threats to aquatic ecosystems, especially microbial populations. Biosurfactants, serving as alternatives to chemical surfactants, are employed in environmental applications, particularly in the remediation of oil pollution. These substances, possessing surface-active properties, play crucial roles in bioremediation processes by aiding in the emulsification, dispersion, and degradation of oil and hydrocarbon contaminants.

The use of biosurfactants in oil pollution remediation offers several advantages, including biodegradability and low toxicity. *Pseudomonas aeruginosa*, for instance, has been identified as a producer of rhamnolipid-type biosurfactants, comprising both mono and di-rhamnolipids. Studies have shown a direct correlation between the type of biosurfactant and the hydrocarbon or oil it effectively degrades, with rhamnolipids demonstrating efficacy in hexadecane degradation.

Biosurfactants encompass a wide range of products with surface-active properties, serving as wetting agents, detergents, foaming agents, emulsifiers, and dispersants. Their versatility extends to various applications, including household and personal care products, pharmaceuticals, agricultural chemicals, oilfield and food processing agents, industrial additives, and environmental remediation agents.

Oil samples were collected from kitchen chimneys in Kanchipuram, Tamil Nadu, leading to the isolation, morphological identification, and subculturing of 31 strains. In the primary screening, 15 strains exhibited positive results, with strains KC15 and KC23 identified as potential candidates. These strains underwent secondary screening using methods such as drop collapse, emulsification activity assessment, phenol sulfuric acid, and phenol red assays, confirming biosurfactant production.

Dye degradation tests were conducted using methylene blue and malachite green dyes at three concentrations for strains KC15 and KC23. Percentage decolorization increased with time but decreased with higher dye concentrations. Optimization studies using KC15 and KC23 with methylene blue at 100ppm concentration indicated that yeast extract, MnCl_2 , and pH 6 were effective parameters for dye degradation. The strain was taxonomically identified KC-15 as *Stenotrophomonas maltophilia* and KC-23 as *Pseudomonas aeruginosa* through the characterization of its 16S rRNA using sequencing methods.

REFERENCES

- Abdel-Mawgoud, A.M., Aboulwafa, M.M., Hassouna, N.. Characterization of surfactin produced by *Bacillus subtilis* isolate BS5. *Applied biochemistry and biotechnology*.2008.150,289-303.
- Andrea F, Isabila G, Giuseppina B, Lbrahim M. Biosurfactant and bioremediation, successes and failure. *Environ Sci*, 2010:145-156.
- Amalesh S,Pinski P, Anupam M.Chandrima S, Asif L, Das M. Estimation of biosurfactant activity of Alkaline protease producing bacteria isolated from municipal solid waste, *Cent Europ J of Exper Bio*, 2012;1:26-35.
- A.A.Jimof and J.Lin, Biosurfactant: A new frontier for greener technology and environmental sustainability,” *Ecotoxicol. Environ. Saf.*, vol. 184, no. June, p. 109607, 2019, DOI: 10.1016/j.ecoenv.2-19.109607.
- Andrade RF, Silva TA, Ribeaux DR, Rodriguez DM, Souza AF, et al. Promising biosurfactant produced by *Cunninghamella echinulata* UCP 1299 using renewable resources and its application in cotton fabric cleaning process. *Advances in Material Science and Engineering* 1-12.(2018)
- Amaral PFF, da Silva JM, Lehocky M, et.,. Production and characterization of a bioemulsifier from *Yarrowialippolytica*. *Process Biochemistry*, 2006, 41(8):1894-1898.
- Arguelles-Arias A, Ongena M, Halimi B, Lara Y, Brans A, Biosurfactant Synthesis, *Microb Cell Fact*,2009;(8);63.
- A. Roy, “A review on the biosurfactants: properties, types and its applications,” *Journal of Fundamentals of Renewable Energy and Applications*, vol.8,2018.

Ayed HB, Azabou MC, Hmidet N, Triki MA, Nasri M(2019) Economic production and biocontrol efficiency of lipopeptide biosurfactants from *Bacillus mojavensis*A21. Biodegradation 30(4): 273-286.

A.P.Karlapudi et al., "Role of biosurfactants in nioremediation of oil pollution-a review,"Petroleum,vol.4, no.3, pp. 241-249, 2018,doi: 10.1016/j.petlm.2018.03.007.

A.R. On et al., " A Review on Applications of Biosurfactants produced from unconventional Inexpensive Wastes in food and," vol. 4, no. 8, pp.827-842,2015.

Benincasa M., Rhamnolipid production by *P.aeruginosa* LB1 growing on soapstock as the sole carbon source. Journal of Food Engineering, 2002; 54:283-288.

Bodour, A. A., & Miller-Maier, R. M. (1998). Application of a modified drop-collapse technique for surfactant quantitation and screening of biosuractant producing microorganisms. Journal of Microbiological Methods,32(3), 273-280.

Biosurfactant producers from oil reservoirs in China (She et al., 2005 ; Wang et al., 2011 ; Xia et al., 2013), Brazil (Gudina et al., 2012), India (Pruthi and Cameotra, 2003), Iran (Lotfabad et al., 2009) and Germany (Yakimo et al.,1995), most of whichare from inland reservoirs.

BS saharan, RK Sahu, D Sharma,. A Review on Biosurfactants; Fermentation, Current Developments and production , Genetic Engineering and Biotechnolgy Journey.2011.

Choi WJ Choi HG, Lee WH. Effects of ethanol and phosphate on emulsan production by *Acinetbactercalcoaceticus*RAG-1.J Biotechnol,1996;93),217-225.

C.N. Mulligan, "Environmental applications for biosurfactants," Environmental pollution, vol.133, no. 2, pp.183-198, 2005.

Christofi, N., Ivshina .,. Microbial surfactants and their use in the field studies of soil remediation. Journey of Applied Microbiology 2002; 93,915-929.

Cooper, D, G., & Goldenberg, B. G. Surface- active agents from two *Bacillus* species . Applied and environmental microbiology,1987; 53(2), 224-229.

C. Liao et al., "Biosurfactant-enhanced phytoremediation of soils contaminated by crude oil using maize (*Zeamays.L*)," Ecol.Eng., vol. 92, pp. 10-17,2016,doi: 10.1016/j.ecoleng.2016.003.041.

C.E. Drakontis and S. Amin, "Biosurfactants: Formulations, properties, and applications," Curr. Opin. Colloid Interface Sci., vol. 48, pp.77-90,2020, doi: 10.1016/j.cocis.2020.03.013.

Campos JM, Stamford TL, Sarubbo LA Production of a bioemulsifier with potential application in the food industry. Appl BiochemBiotechnol(2014) 172(6): 3234-3252.

D.C. Panadare and V.K.Rathod, "Applications of Waste Oil other than biodiesel: A Review," vol.12, no.3, pp.55-76,2015.

Delorme V, Dhouib R, Canaan S, Fotiadu F, Carriere J, Effects of surfactants on lipase structure, activity, and inhibition. Pharm. Res. 2011;28(8):1831-1842.

Desai, J. D., & Banat, I.M. (1997). Microbial production of surfactants and their commercial potential. Microbiology and Molecular biology reviews,61(1), 47-64.
Kalyani, A. L. T., Sireesha, G. NIsolation of bio-surfactant producing actinomycetes from terrestrial and marine soils. .(2014).

^Diggle SP, Whiteley M (January 2020). "Microbe Profile: *Pseudomonasaeruginosa*: opportunistic pathogen and lab rat". Microbiology. 166 (1): 30-33.

Deak NA, Johnson LA, Functional properties of protein ingredients prepared from high source/low-starchose soybeans. *Journal of American Oil Chemistry Society*, 83: 811-818.2006.

D.G. Hayes, *Fatty Acids-Based Surfactnts and their uses*. Elseveir Inc., 2017.

D.Sharma et al., “Biosurfactant production by *Pseudomonas aeruginosa* DSVP20 isolated from petroleum hydrocarbon-contaminated soil and its physicochemical characterization,” *Enviro. Sci. Pollut. Res.*, vol.22, no.22, pp.17643,2015, doi: 10.1007/s11356-015-4937-1.

Diggle SP, Griffin AS, Campbell GS, West SA. Cooperation and conflict in quorum-sensing bacterial populations. *Nature*, 2007;450:411-414. Doi:10.1038/nature06279.

Edward B, Shinichiro H. A. Saccharogenic Method for Estimating Electrophoretic and Chromatographic Distribution of Human Serum Amylase *Clinical Chemistry*.1965;11:97-112.

Eliora ZR, Eugene R, Biosurfactant and bioremediation *Curr Opini in Biote*. 2002;13:249-252.

E.C. Souza, T.C. Vessoni- Penna, and R.P. De Souza Oliveira, “Biosurfactant-enhanced hydrocarbon bioremediation: An overview,” *Int.Biodeterior, Biodegrad.*, vol. 89, pp. 88-94, 2014, doi: 10.1016/j.ibiode.2014.01.007.

E.J. Gudina, J.A. Teixeira, and L.R. Rodrigues, “Biosurfactant-Producing *Lactobacilli*: Screening, Production Profiles, and Effect of medium composition,” *Appl. Environ. Soil Sci.*, vol. 2011, pp. 1-9, 2011, doi: 10.1155/2011/201254.

Fortin J, Jury WA, Anderson MA. Enhanced removal of trapped nonaqueous phase liquids from saturated soil using surfactant solutions. *J. ContamHydrol*, 1997;24:247-267.

Franzetti A, Di Gennaro P, Bestetti G, Lasagni A, Pitea D, Collina E, Selection of surfactants for enhancing diesel hydrocarbons-contaminated media bioremediation. J. Hazard Mater. 2008;(152):1309-1316.

F. R. C. Damasceno, E. D. Calvalcanti-Oliveira, I. K. Kookos, A.A. Koutinas, M. C. Cammarota, and D.M.G Frieire, "Treatment of wastewater with high fat content employing an enzyme pool and biosurfactant ; Technical and economic feasibility," Brazillian J. Chem. Eng., vol. 35, no. 2, pp. 531-542, Apr. 2018, doi: 10.1590/0104-6632.20180352s20160711.

Ilori MO, Amund DI (2001) Production of a peptide glycolipid bioemulsifier by *Pseudomonas aeruginosa* grown on hydrocarbon. Z Naturforsch C J Biosci 56(97-8): 547-552.

I. Mnif , S. Ellouz-Chaabouni, and D.Ghribi, "Glycolipid Biosurfactants, Main Classes, Functional Properties and Related Potential Applications in Environmental Biotechnology," J. Polym. Environ., vol. 26, no. 5, pp. 2192-2206, 2018, doi: 10.1007/s10924-01-1076-4.

Ithoh, S. and T. Suzuki, 1972. Effect of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in n-paraffin-utilizing ability. Agri. Biol. Chem., 36: 2233-2235.

Gharaei-Fathabad E, Biosurfactants in pharmaceutical industry: A Mini - Review. Ame J Dr Discov and Dev. 2011;1(1):58-69.

Hisatsuka K, Nakahara T, Sano N, Yamada K, Formation of rhamnolipid by *pseudomonas aeruginosa* and its function in hydrocarbon fermentation, Agr Biol Chem, 1971;(35): 273-277.

Jarvis, F.G. and M.J. Johnson, A glycolipid produced by *pseudomonas aeruginosa*. J. A. Chem. Soc., 71:4124-4126.1949.

Jadhav, M., S. Kalme, D Tamboli and S. Govindwar,. Rhamnolipid from *Pseudomonasdesmolyticum* NCIM-2112 and its role in degradation of brown 3REL. J. Basic Microbiol., 51:385-396; 2011.

Joice, P.A. and Parthasarathi, R. Optimzation of Biosurfactant Production from *Pseudomonasaeruginosa* PBSC1, International Journal of Current Microbiology and Applied Sciences 3, 140-151.(2014)

Joshi P, Shekhawat D, Screening and isolation of biosurfactant producing bacteria from petroleum contaminated soil, Euro J Exp Bio. 2014;4(4): 164-169.

J.J. Sheng, "Status of surfactant EOR technology," petroleum, vol. 1,no. 2, pp. 97-105,2015.

Johnson V, Singh M, Saini VS. Bioemulsifier production by anpleaginous yeast *Rhodotorulaglutinis*IIIP-3. Biotechnol Lett, 1992; (14): 487-490.

J.D. Desai and I. M. Banat, "Microbiology and Molecular Biology Reviews: MMBR, vol.61, no. 1, pp.47-64,1997.

J. W. Liu et al., "Surfactant-enhanced remediation of oil-containted soil and groundwater: A review," Sci. Total Environ., vol. 756, p.144142, 2021, doi: 10.1016/j.scitotenv.2020.144142.

J.F.F. Secato, D.F. Coelho, N. G. J. Rosa, L. D. L. Costa, and E.B. Tambourgi, "Biosurfactant production by *Bacillus subtilis* using corn steep liquor as culture medium," Front. Microbiol., vol.6, no. FEB, pp.1-8, 2015, doi: 10.3389/fmicb.2015.00059.

Karanth, N.G.K., P.G. Deo and N.K. Veenanadig, Microbial production of biosurfactants and their importance. Curr. Sci., 77: 116-126.1999.

Katz E, Dermain AL. The peptide antibiotics of *Bacillus*; chemistry, biogenesis, and possible functions. *Bacteriol Rev.* 1977;449-474.

Kappeli O, Finnerty WR, Partition of alkane by an extracellular vesicle derived from hexadecane-grown *Acinetobacter*. *J of Bact.* 1997; (140):707-712.

Khuri, A.I. and Cornell, J.A. *Response Surfaces : Design and Analyse*. Marcel Dekker, New York.(1996)

Khanna S, Pattnaik P Production and functional characterization of food compatible biosurfactants. *Applied Food Sciences Journal* 3: 1-4.(2019)

King JD, Kocincova D, Westman EL, Lam JS. Review: Lipopolysaccharide biosynthesis in *Pseudomonasaeruginosa*. *Innate Immun.* 2009;15:261-312. Doi: 10.1177/1753425909106436.

K. Liu, Y. Sun, M.Cao, J. Wang, J.R. Lu, and H.Xu, “ Rational design, properties, and applications of biosurfactants: a short review of recent advances,” *Curr. Opin. Colloid Interface Sci.*, vol. 45, -pp.57-67, 2020, doi: 10.1016/j.cocis.2019.12.005.

Kumar, S., Stecher, G. and Tamura, K., MEGA7 Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33, 1870-1874.55.(2016)

L.W. Lake,“Fundamentals of enhanced oil recovery,” vol I, Society of Petroleum Engineers, Richardson, TX, USA, 2014.

Maneera S, Dikit P, Characterization of cell-associated bioemulsifer from *Myroides sp.* SMI, a marine bacterium Songklanakarin J Sci Technol, 2007;929): 7899-779.

Makkar R.S, Rockne KJ, Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. *Environ toxi and chem.* 2009;22(10):2280-92.

Maggot M, Washington DC, Ollivier B, Indigenous microbial communities in oil fields
In: Petroleum microbiology, ASM Press. 2005:21-33.

Mary Swarnalatha S and Jaqueline Chinna Ranil, The pharma Innovation Journal 2019;
8(2):684-687.

M. Maikudi Usman, A. Dadrasnia, K. Tzin Lim, A. Fahim Mahmud, and S. Ismail,
“Application of biosurfactants in environmental biotechnology; remediation of oil and
heavy metal,” AIMS Bioeng., vol. 3, no. 3, pp. 289-304, 2016, doi:
10.3934/bioeng.2016.3.289.

M. Henkeland R. Hausmann, Diversity and Classification of Microbial Surfactants,
Second Edi. Elsevier Inc., 2019.

Muthusamy K, Gopalakrishnan S, Ravi TK. Biosurfactants; properties, commercial
production and application. Cur Sci. 2008; 94:736-773.

M. S. Kuyukina. I.B. Ivshina, T.A. Baeva, O.A. Kochina, S. V. Gein and V.A.
Chereshnev, “Trehalolipid biosurfactants from nonpathogenic *Rhodococcus*
actinobacteria with diverse immunomodulatory activities,’ N. Biotechnol., vl.32, no.6,
pp. 559-568, 2015, doi: 10.1016/j.nbt.2015.03.006.

Neu TR, Significance of bacterial surface- active compounds in intraction of bacteria
with interfaces. Micro Rev. 1996;60:151-166.

Noah KS, Surfactin production from potato process effluent by *Bacillussubtilis* in
chemostat. Applied Biochemistry and Biotechnology, 122:465-474.2005.

Nurfaeahin, A.H., Mohamed, M.S. and Phang, L.Y. Culture Medium Developmet for
Microbial-Derived Surfactants Production- An Overview. Molecules (Base,
Switzerland), 23,1049.(2018)

Nitschke M, Silva SSE (2018) Recent food applications of microbial surfactants. Crit Rec Food Sci Nutr 58(4): 631-638.

Onwosi, C.O. and Odibo, F.J.C. Use of Response Surface Design in the Optimization of Starter Cultures for Enhanced Rhamnolipids Production by *Pseudomonas nitroreducens*. African Journal of Biotechnology 12,2611-2617.(2013)

Patel RM, Desai AJ, Biosurfactant production by *Pseudomonas aeruginosa* GS3 from molasses. Letters in Applied Microbiology, 25:91-94.1997..

Pekin G, Production of sophorolipids from *Candida bombicola* ATCC 22214 using Turkish corn oil and honey. English Life Science, 5:357-36.2005.

P. Jimenez-Penalver, A. Koh, R. Gross, T. Gea, and X. Font, "Biosurfactants from waste structures and Interfacial Properties of Sophorolipids Produced from a Residual Oil Cake," J. Surfactants Deterg., vol. 23, no. 2, pp. 481-486,2020, doi: 10.1002/jsde.12366.

Priyam vandhana^{1,2} and Dinesh Singh², International journal of current microbiology and Applied sciences.ISSN:2319-7706 Volume number 08(2018).

P.Singh, Y. Patil, and V. Rale, "Biosurfactant production: emerging trends and promising strategies," J.Appl. Microbiol., vol. 126, no. 1, pp. 2-13, 2019, doi: 10.1111/jam.14057.

Rau U, Nguyen AL, Roeper H, Koch H, Downstream processing ofmannosylerythritol lipids produced by *Pseudozyma aphidis*. Eur J Lip Sci Technolo, 2005;107(6):373-380.

R. Nelson, "Cosurfactant- enhanced alkaline flooding," in Proceedings of the SPE Enhanced Oil Recovery Symposium, OnePetro, Tulsa, OK,USA, April 1984.

R. Schmidt, "Thermal enhanced oil recovery current status and future needs," Chemical Engineering Progress; (USA), vol. 86,no. 1, 1990.

W.F. Ramirez, Application of Optimal Control Theory to enhanced oil recovery, Elsevier, Amsterdam, Netherlands, 1987.

Santos, D.K., Rufino, R.D., Luna, J.M., Santos, V.A. and Sarubbo, L.A. Biosurfactants : Multifunctional biomolecules of the 21st Century. International Journal of Molecular Sciences, 17,401.(2016)

Satpute, S.K., A.G. Banpurkar, P.K. Dhakephalkar, I.M. Banat and B.A. Chopade, Methods for investigating biosurfactants and bioemulsifiers: A review. Critt. Rev. Biotechnol., 30: 127-144.2010.

Sarubbo LA, Farias CBB, Campos-Takaki GM, Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*. CurrMicrobiol, 2007;(54): 68- 73.

S. De, S.Malik, A.Ghosh, R. Saha, and B.Saha, "A review on natural surfactants," RSC Adv., vol. 5, no.81, pp. 65757-65767, 2015, doi: 10.1039/c5ra11101c.

Shepherd R, Rockey J, Sutherland IW, Roller S. Novel bioemulsifiers from microorganisms for use in foods. J Biotechnol, 19;95;(40): 207-217.

S.K. Satpute, G. A. Plaza, and A.G. Banputkar, "Biosurfactants" Production from renewable natural resources: Exaple of innovative and Smart Technology in Circular Bioeconomy," Manag. Syst. Prod. Eng., vol. 25, no. 1, pp. 46-54, 2017, doi: 10.1515/mspe-2017-0007.

Smyth Tjp, Perfumo A, Mc Clean S, Marchant R, Banat IM, 2010b. Isolation and analysis of lipopeptides and high molecular weight biosurfactants, In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin.

Tamura, K., Dudley, J., Nei, M. and Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Molecular Biology and Evolution*, 24, 1596-1599.(2007)

Thompson DN, Biosurfactant from potato process effluent. *Applied biochemistry and biotechnology*, 84: 917-930.2000.

^"UK Standards for Microbiology Investigations: Identification of *Pseudomonas* species and other Non-Glucose Fermenters". Public Health England. 13 April 2015. From the original on 3 July 2022. Retrived 4 May 2022.

V. Bogaert, J. Zhang, and W. Soetaert, "Microbial synthesis of sophorolipids," *Process Biochemistry*, vol. 46, pp.821-833,2011.

Yadhav RP, Saxena RK, Gupta R, Davidson S, *Journal of Science and Industrial Research* 56: 479-248.1997.

Yaraguppi, D.A., Bagewadi, Z.K., Muddapur, U.M., and Mulla, S.I. Response Surface Methodology- Based Optimization of biosurfactant production from isolated *Bacillus aryabhattai* Strain ZDY2, *Journal of Petroleum Exploration Production Technology*, 10, 2483-2498.(2020)

Z. Liu et al., "Recent advances in the environmental applications of biosurfactant saponins: Areview," *J. Environ. Chem. Eng.*, vol.5, no. 6, pp. 6030, 2017, doi: 10.1016/j.jece.2017.11.021.

