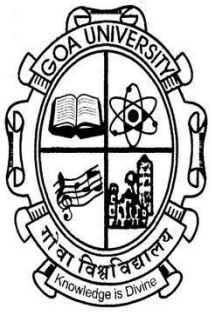
Bacteria from Goan salterns as producers of a biosurfactant

A Thesis submitted to Goa University



for the Award of the Degree of

DOCTOR OF PHILOSOPHY

In

BIOTECHNOLOGY

By

Ruchira Malik

Research Guide

Prof. Savita Kerkar

Goa University

Taleigao, Goa

February, 2021

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DECLARATION

I hereby declare that the thesis entitled **— Bacteria from Goan salterns as producers of a biosurfactant"**, submitted for the degree of **Doctor of Philosophy (Ph.D.)** in **Biotechnology** to Goa University, is based on studies carried out by me at the Department of Biotechnology, Goa University, under the supervision of **Prof. Savita Kerkar** (Research Guide).

The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University / Institute. Materials obtained from other sources have been duly acknowledged in the thesis.

Place:
Date:

Ruchira Malik

(Research Scholar)

Dedicated to

My Family

ACKNOWLEDGEMENT

At the outset I thank God for bestowing me with the physical and mental capacity to carry out my research work. It is because of His grace that I've been able to accomplish everything that I have today.

I thank and appreciate the valuable time set aside for me by my guide **Prof. Savita Kerkar**, Professor, Department of Biotechnology. Her constant support, encouragement and trust helped shape me into a better person professionally and personally. No words can describe how grateful I am having gained immensely from her profound knowledge in the subject; her positive attitude, commitment, generosity, patience, enthusiasm, constant encouragement and guidance at every stage during the course of my research.

I would also like to thank **Prof. Varun Sahni,** the Vice Chancellor of Goa University and **Dr. Satish Shetye** (former Vice Chancellor) of Goa University for the access to the University Laboratory and all the required apparatus, facilities and infrastructure that allowed me to effectively and efficiently conduct my research.

I express my gratitude to the present and former Deans of Faculty of Life Sciences, **Prof. P. K. Sharma**, **Prof. M.K. Janarthanam**, and **Prof. Saroj Bhosle** for their valuable advice and helpful encouragement.

I am extremely thankful to **Dr. Maria Judith Gonsalves and Prof. Usha D. Muraleedharan** (Vice Chancellor's Nominee) for their constant guidance and critical evaluation of my research.

My profound gratitude to the, **Head**, **Department of Biotechnology**, for providing me with laboratories well equipped with the necessary staff, instruments and chemicals needed for my research.

I take this opportunity to thank **Prof. Urmila Barros**, **Prof. Sanjeev Ghadi, Dr. Dharmendra Tiwari**, **Dr. Sanika Samant**, **Dr. Trupti Asolkar Dr. Abhishek Mishra, Dr. Meghnath Prabhu Ms. Diviti Mapari** and **Dr. Manisha Tiwari** for their valuable advice and feedback all throughout my tenure at the university.

A special thanks to **Prof. Santosh Tilve** and **Dr. Mahesh Majik** for their invaluable guidance through the compound purification and interpretation of data.

I appreciate the ever willing and cheerful assistance of the non-teaching staff of our Department. I express my heartfelt gratitude to **Mr. Serrao, Mrs. Sanjana, Dr. Sandhya, Mr. Parijat, Mr. Tulsidas, Mr. Ulhas, Mrs. Ruby, Mr. Sameer, Mr. Rahul, Mr. Aashish and Ms. Jaya.** I also fondly remember the very helpful **Late. Mr. Martin** who would go out of his way to help us out with anything, he will always be missed. I would also like to thank **Mrs. Kunda** and **Mrs. Manda** for ensuring a very clean atmosphere for researchers to work.

In the world of selfishness and contentment, I was blessed to have a laboratory with helpful, and dedicated lab mates and friends with whom I have had the pleasure of spending all these years with – **Dr. Kirti, Dr. Shuvankar, Dr. Priyanka, Dr. Amruta, Dr. Michelle, Dr. Srijay, Dr. Preethi, Dr. Imran, Dr. Surya, Judith, Delicia, Sreekala, Nuha, Deepti, Rakshita, Veda, Varsha, Anchit, Kunal** and **Suman** thank you so much for all the help, support and for creating a cheerful work environment. You all have left me with so many wonderful memories that I will cherish for life. A special thank you to my seniors **Dr. Tonima, Dr. Kuldeep Dr. Flory Dr. Krupali** and **Dr. Rupesh Kumar Sinha** for their excellent research work served as a benchmark and a constant reference throughout my research.

My deepest thank to my friends and fellow researchers, **Dr. Samantha, Alisha, Perantho, Pingal, Nicola, Manasi and Priti.** This would not have been possible without their insights, expertise, and constant support at every stage during the course of my research. Words can’t be enough to express my gratitude towards their love, care, and encouragement, in times much needed.

I am extremely thankful to research scholars from other department **Mr. Ketan, Ms. Lima,** and **Mr. Shashank** for their constant help and support.

I am extremely thankful to the **Indian Institute of Technology, Mumbai** for providing the ICP-AES facilities for the metal analysis and **CSIR-Central Leather Research Institute, Chennai** for providing the LC-MS facilities for the identification of the compound that was a part of my research.

I am greatly thankful to **Ms. Karishma Cotta** for her help at IIT, Mumbai in times much needed.

To my parents **Mrs. Jayanti** and **Mr. Gokuldas Malik**, my uncles **Mr. Rajaram, Mr. Shivanand,** and **Mr. Santosh,** my aunts **Mrs. Anuradha, Mrs. Shamal and Mrs. Suchan,** my sister **Jayashri**, my brothers in law **Suraj**, my brothers **Anant, Hemnandan, Charudatta, Smital and Naveen,** my sister in laws **Diksha** and **Sneha,** my darling nieces **Avani, Veera and Adhira**; words cannot express how grateful I am to all of you. Your love, support and sacrifice all throughout my Ph. D. journey has been the greatest blessing of all and I am forever indebted to all of you. I would also like to mention, **Sachin** whose support, belief in me, constant motivation and help in every possible way in completion of my work.

Lastly, I place on record, my gratitude to all those who have directly or indirectly lent a helping hand in my research and in the completion of my thesis in some way or the other, whether directly or indirectly and my sincere apologies to all those whom I could not mention individually.

---- ***Ruchira Malik***

CONTENTS

ABBREVIATIONS

%	Percentage
˚C	Degree Celsius
µL	Micro litre
µm	Micrometer
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
CaCl	Calcium chloride
CCD	Central composite design
CFS	Cell-free supernatant
Da	Dalton
DNA	Deoxyribonucleic acid
ESI	Electrospray ionization
g	Gram(s)
g/L	Gram per litre
g/mol	Gram per mole
GA	Genetic algorithm
h	Hour(s)
H^+ ions	Hydrogen ions
H_2O	Water
HCl	Hydrochloric acid
IBM	International Business Machines
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
IIT	Indian Institute of Technology
K_2HPO_4	Dipotassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
KX	1000 times
M	Massachusetts
m/z	Mass by charge
MEGA	Molecular evolutionary genetics analysis
mg/L	Milligram per litre

mg/ml	Milligram per millilitre
$MgCl_2$	Magnesium chloride
min	Minute(s)
mL	Millilitre
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometer
OD	Optical density
OFAT	One factor at a time
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
psu	Percentile salinity unit
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Rotations per minute
RSM	Response surface methodology
RT	Room temperature
s	Second(s)
SD	Standard deviation
SEM	Scanning electron microscopy
sp.	Species
spp.	Species (plural)
SPSS	Statistical Package for the Social Sciences
USA	United States of America
w/v	Weight per volume

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Surfactants are the most versatile products of the chemical industry. The term surfactant comes from the words surface active agent. Surfactants are utilized in every industrial area ranging from household detergents, food items to pharmaceuticals (Fait et al., 2019). They are a primary component of cleaning detergents. Without surfactants, soaps do not coalesce with water, but would instead just repel the water, making the cleaning process much more difficult. Therefore surfactants in combination with soaps are more effective in the removal of dirt from skin, clothes and household articles.

Surfactants are amphiphilic molecules consisting of a hydrophilic and a hydrophobic moiety that interacts with the phase boundary in heterogeneous systems. They are surface active compound having wide variety of applications in household products like soaps and detergents with the ability to concentrate at the air – water interface (Desai and Banat, 1997). The foremost application of surfactant is to take apart oily substances owing to the ability to reduce the surface or interfacial tension at the interfaces. They are broadly utilized as a formulation aid to promote emulsification dispersion, and solubilisation in product range from cosmetics, textiles, chemicals, foods and pharmaceuticals (Fenibo et al., 2019). TSurfactants have wide applications however when synthesized chemically are very toxic to the environment due to their recalcitrant and persistent nature (Varjani and Upasani 2017). The synthetic surfactants are routinely deposited in numerous ways on land and into water systems, whether as part of an intended process or as industrial and household waste. Some of them are known to be toxic to animals, ecosystems, and humans, and can increase the diffusion of other environmental contaminants. To meet this increasing demand there is a need to replace a surfactant with a natural product which is environmentally friendly.

Biosurfactants are produced by various microorganisms that comprise both hydrophilic and hydrophobic moieties. They are produced on the cell surface or excreted extracellularly. They consists of a hydrophilic moiety, consist of a peptide or polysaccharide and a hydrophobic moiety comprising of hydrocarbon chains or fatty acids. Biosurfactants have an ability to accumulate between liquid phases, capable of reducing surface and interfacial tension and to form micelles and micro-emulsions between two different phases (Shekhar et al., 2015). They are classified according to

their chemical structures and microbial origin. The wide range of structural diversity results in a broad spectrum of potential industrial applications including production of food, cosmetics, and pharmaceuticals, agriculture, mining, enhanced oil recovery, transportation of crude oil, cleaning oil storage tanks and pipelines and soil remediation (Pastewski et al., 2006).

Microorganisms produce these surface active compounds to enhance both the bioavailability of hydrophobic immiscible and mostly inaccessible substrates allowing better survival under low moisture conditions. They are being various preferred over chemically synthesized surfactants being less toxic, biodegradable, environmentally friendly, with lower critical micelle concentration, and highly selective in addition to have enhanced activity at extreme pH, temperatures, and salinity (Mukherjee et al., 2006). They are expected to become "multifunctional materials" of the 21st century as they have applications in different industrial processes as well as potential novel future uses (Marchant and Banat, 2012). Also the increasing environmental concerns and emergence of more stringent laws have led to biosurfactants being a potential alternative to the chemical surfactants available in the market.

Marine microorganisms possess an inexhaustible source of useful chemical substances for the development of new drugs and are being exploited for centuries for newer compounds. They are often under extreme conditions of pressure, temperature, salinity, and micronutrients, with survival often depending on the ability to produce biologically active secondary metabolites. In recent years, microorganisms from extreme environments have attracted considerable attention as source of novel bioactive compounds due to constant need for new and improved drugs (Neifar et al., 2015). These secondary metabolites have been recognized as a major source of compounds endowed with ingenious structures and potential biological activities (Maurya et al., 2020). One of the extreme environments is a hypersaline ecosystem, with high salinity, high temperature, and low oxygen concentrations, which limits the species diversity.

Marine salterns are one of the extreme coastal ecosystems which generally originate as a result of evaporation of seawater which harbors halotolerant and halophilic bacteria. The salinity decreases as low as 5-10 psu during the monsoon seasons and reaches high values up to 350 psu during the non-monsoon or salt manufacturing season. In the salt

crystallizer ponds, sea water gains entry during the high tide and is concentrated up to saturation levels by evaporation. These crystallizer ponds represent a unique marine hypersaline environment, with salinity levels from 10 to 350 psu; pH 6 to 9 and temperature 10 °C to 42 °C (Ballav et al., 2015). Halotolerant bacteria can grow in varying concentration of salt and can also grow in its absence and halophilic bacteria have an obligate requirement of salt for their growth. These diverse microorganisms are repositories of stable compounds that function at a wide range of salinity, temperature, pH and extreme conditions (Kerkar 2004). These bacteria are known to produce several important biomolecules such as enzymes, antibiotics, compatible solutes, etc. in order to survive in this environment. The search for biosurfactants from these microbes is very promising as they have a unique lipid composition which may have an important role to play as surface-active agents (Sarafin et al., 2014).

Goa is a state in western India with coastlines stretching along the Arabian Sea. Most of the Goa's rivers form estuaries and have saltpans surrounding the estuaries. These salt pans are interconnected multi-pond systems with a constant influx of sea water that is evaporated for the manufacture of natural salt (Mani et al., 2012). Saltpans are disturbed in both the districts of Goa, the majority being in the North district. Saltpans of Goa harbor diverse organisms from bacteria (aerobic and as well anaerobic), fungi, algae, etc.

Over the years, our research group has explored various aspects of the marine salterns of Goa and the bacteria in these salterns. These halotolerant and halophilic bacteria are known to produce pharmaceutically important compounds (Kamat and Kerkar, 2011). The interaction of the heterotrophic bacteria to the varying metal concentrations in the surface sediments of Ribandar saltern has also been studied (Pereira et al., 2013). Biodiversity of halotolerant and halophilic actinobacteria has been studied profoundly along with their anti-bacterial property (Ballav et al., 2015). Some halotolerant bacteria have been used as biofertilizers in agriculture, probiotics in shrimp aquaculture and also as a source of antifungal agents against mushroom pathogens (Bartakke 2018; Fernandes et al., 2019; Fernandes and Kerkar, 2019). The biodiversity of anaerobic sulfate-reducing bacteria (SRB) has been studied in depth along with its biotechnological applications (Kerkar and Bharathi, 2011; Das et al., 2018). These hypersaline bacteria have many interactions with trace metals and SRB play an

important role in detoxifying these soluble metal concentrations by precipitating them as their metal sulphides in the sediment. In the present study we were inquisitive to explore the potential of unexplored hypersaline bacteria as producers of biosurfactants.

Thus, the aim of our research work was to probe the potential of halotolerant bacteria isolated from the salt pans of Goa for the production of biosurfactants.

Our work was carried out with the following objectives:

1. Screening saltpan bacteria for production of biosurfactants.
2. Optimization of the biosurfactant production.
3. Application of the bacterial biosurfactants in heavy metal bioremediation
4. Characterization of the biosurfactant

SIGNIFICANCE OF THE THESIS

Over the years the surfactant industry has been a vast and dynamic business with markets everywhere from household detergents to pharmaceuticals. Surfactants are used as foaming agents, dispersants, emulsifiers, solubilizing agents, cleansers, and conditioners. The surfactants market size is projected to reach USD 52.4 billion by 2025 from USD 42.1 billion in 2020, at an annual growth rate of 4.5%. Asia-Pacific is the largest consumer as well as producer of surfactants. The growth of surfactant market is determined by the growing population and increasing urbanization. Due to current pandemic, the growing awareness regarding cleaning and hygiene are other factors driving its demand. However, these surfactants are of many different chemical types which are toxic to the environment and are hardly degraded by microorganisms. Nowadays, due to global environmental awareness and also to meet this increasing demand, the utilization of biological surface-active agents produced from microorganisms has attracted scholarly attention.

Biosurfactants are referred as green chemicals, microbial – derived surface active molecules which are equally diverse in terms of structure and function. Biosurfactants display a number of advantages over the synthetic counterparts with regards to biodegradability, specificity and low toxicity. They are biocompatible which make them excellent candidates for use in varied fields including usage in detergent and cleaning solutions. The microorganisms in extreme environments such as marine salterns have unique adaptation strategy making them useful candidates for biotechnological applications. These salterns are coastal ecosystems which harbour halotolerant and halophilic microbial communities. The bacteria from these environments have been reported to produce a wide range of biomolecules with potential applications and their ability to function in wide and extreme conditions. The potential of these halotolerant bacteria for the production of biosurfactants so far remained unexplored. The present study focused on exploring the potential of bacteria isolated from salterns of Goa for the production of biosurfactant and its application in heavy metal bioremediation. The data procured would probably provide biosurfactants with enhanced properties and enhanced application in remediating heavy metals and would also serve as a baseline study for other possible applications in Biotechnology.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

In recent years increasing global environmental awareness has led to interest in microbial surfactants compared to their chemical counter parts. Microorganisms produce a wide range of extracellular products includes surfactant which has gained attention in these recent years due to their diversity and environmentally friendly nature. Biosurfactants are structurally diverse group of surface active agents produced by diverse microorganisms such as bacteria, fungi and yeasts on the cell surface or excreted extracellulary. They are amphiphilic in nature i.e. they have both hydrophilic (water-loving) and hydrophobic (water hating) moieties. They have an ability to accumulate between liquid phases, capable of reducing surface and interfacial tension. Biosurfactants can be produced by cheap raw material which increases its utility with the increasing demand. In recent years they are widely used for numerous applications right from household detergents to pharmaceuticals. In addition, biosurfactants can be customized based on the applications by modifying the genes of the organism concerned or by optimizing the production conditions (Thenmozhi et al., 2011).

2.1. Classification of biosurfactats:

Biosurfactants are classified according to their molecular structure and microbial origin (Table 1). Generally, they are composed of hydrophilic moiety (peptides or amino acids or polysaccharides) and hydrophobic moiety (unsaturated or saturated fatty acids) (Desai and Banat, 1997). There are five major categories of biosurfactants viz. glycolipids, phospholipids and fatty acids, lipopeptides and lipoproteins, polymeric biosurfactants and particulate biosurfactants. They can also be classified as based on the molecular mass. Glycolipids, lipopeptides and phospholipids are low molecular surfactants whereas high molecular mass surfactants consist of polymeric and particulate surfactants (Ron and Rosenberg, 2001, Nitschke and Pastore, 2006).

2.1.1. Glycolipids:

Among biosurfactant classes, the most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids.

Among the glycolipids, the best known are rhamnolipids sophorolipids and mannosylerythritol lipids.

2.1.1.1. Rhamnolipids:

Rhamnolipids are the most characterized biosurfactant among biosurfactant classes. They are said to be the successive generation of biosurfactants to achieve the market. Rhamnolipids are predominantly produced by *Pseudomonas aeruginosa* and are classified as mono and di-rhamnolipids (Lang et al., 1987; Parra et al., 1989; Rashedi et al., 2005; Robert et al., 1989 and Siegmund and Wagner, 1991). They are composed of β-hydroxy fatty acid connected by carboxyl end to a rhamnose sugar molecule. Other *Pseudomonas* species that produce rhamnolipids are *Pseudomonas chlororaphis, Pseudomonas plantarii, Pseudomonas putida, and Pseudomonas fluorescens* (Randhawa and Rahman, 2014). They have broad range of applications in various industries.

2.1.1.2. Sophorolipids:

Sophorolipids, consist of a disaccharide sophoroses linked to a long-chain hydroxy fatty acid. They are produced mainly by yeasts such as *Candida bombicola*. They are combination of six to nine different hydrophobic sophorosides (Desai and Banat 1997). They can be classified as anionic (acidic) or non-ionic (lactonic). Among all the biosurfactants, the yield of sophorolipids is reported to be highest. They are considered among promising biosurfactants as they have been used for commercial production and applications.

2.1.1.3. Mannosylerythritol lipids:

Biosurfactant containing 4-O-β-D-mannopyranosylmeso-erythritol as the hydrophilic group and a fatty acid and/or an acetyl group as the hydrophobic moiety is known as Mannosylerythritol lipid (MEL). MEL is reported to be secreted by Ustilago sp. (as a minor component along with cellobiose lipid (CL) and Pseudozyma sp. (as a major component). Since the synthesis of MEL is not growth associated, it can also be produced by using resting (stationary phase) cells of yeast. MEL acts as an energy

storage material in the yeast cells similar to triacylglycerols. MELs are shown to reduce the surface tension of water to less than 30 mN m^{-1} (Arutchelvi et al., 2008)

2.1.2. Lipopeptides

Lipopepties are the most popular biosurfactants. They consist of a fatty acid in combination with a peptide moiety. The isoform differs by the peptide moiety, the length of the fatty acid chain, and with the linknge between the two groups (Mnif and Ghribi 2015). Among the lipopeptides, surfactin is the most powerful lipopeptide type biosurfactant produced by *Bacillus subtilis* (Wei et al., 2004; Wei et al., 2003; Yeh et al., 2005).

2.1.2.1. Surfactin:

Surfactin is a cyclic lipoheptapeptide, containing seven residues of D- and L-amino acids and one residue of a β-hydroxy fatty acid with an amino acid sequence completely different from the iturin group (Shaligram et al., 2010). It consists of four isomers, Surfactin A–D and exhibits various physiological activities. Owing to the exceptional surfactant activity, it is named as surfactin (Arima, 1968).

2.1.2.2. Iturin:

Iturin is a group of cyclic lipopeptides with a peptide moiety and a b-amino fatty acid linked by amide bonds to the constituent amino acid residues. They share a common sequence (bhydroxy fatty acid-Asx-Tyr-Asx) and show variation at the other four positions. Iturin A, C, D, and E, bacillomycin D, F, and L, bacillopeptin, and mycosubtilin belong to the iturin group (Jacques, 2011).

2.1.2.3. Fengycin:

Fengycin are lipopeptides with 10 amino acids and a lipid attached to the N-terminal end of the molecule. They differ from iturin and surfactin by the presence of unusual amino acids such as ornithine and allo-threonine. The diversity of the peptide moiety (variants which have a characteristic Ala-Val dimorphy at position 6 in the peptide ring) permits to classify the fengycin family into Fengycin and B and Plipastatin A and B (Moyne et al., 2001).

Table 1: List of Biosurfactants produced by different microorganisms

Biosurfactant class	Microorganism	References
Glycolipids		
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas sp.*	Guerra-sanstos et al., 1986, Hiratsuka et al., 1971, Koch et al., 1988, Rashedi et al., 2005, Robert et al., 1989, Suzuki et al., 1965
Trehalose lipids	*Rhodococcus erythropolis, Nocardia erythropolis, Arthobacter sp., Mycobacterium sp.*	Abu-Ruwaida et al., 1991, Bryant 1990, Cooper et al., 1981, MacDonald et al., 1981, Rosenberg et al., 1979
Sophorolipids	*Candida bombicola, Candida apicola, Rhodotorula muciliginosa and Candida rugosa*	Daverey and Pakshirajan, 2009, De Oliveira et al., 2014, Desai et al., 1997, Deshpande and Daniels, 1995
Mannosylerythritol lipids	*Pseudozyma (Candida) sp, Ustilago sp*	Arutchelvi et al., 2008, Morita et al., 2015, Yu et al., 2015
Lipopeptides		
Surfactin/iturin/fengycin	*Bacillus subtilis*	Arima, 1968, Bernheimer and Avigad, 1970, Cooper et al., 1981, Rosenberg & Ron 1999, Wei et al., 2004
Viscosin	*Pseudomonas fluorescens*	Neu et al., 1990
Lichenysin	*Bacillus licheniformis*	Grangemard et al., 2001, Madslien et al., 2013, Nerurkar 2010,
Serrawettin	*Serratia marcescens*	Matsuyama et al., 1987, Thies et al., 2014
Subtilisin	*Bacillus subtilis*	Bernheimer and Avigad, 1970
Gramicidins	*Bacillus brevis*	Marahiel et al., 1977

Biosurfactant class	Microorganism	References
Polymyxins	*Bacillus polymyxa*	Suzuki et al., 1965
Arthrofactin	*Arthrobacter* sp.	Morikawa et al., 1993
Bamylocin A	*Bacillus amyloliquefaciens*	Lee et al., 2007
Fenzycin S	*Bacillus amyloliquefaciens*	Lee et al., 2010
Fatty acids/neutral lipids/ Phospholipids		
Phospholipids	*Acinetobacter sp., Corynebacterium lepus, Thiobacillus thiooxidans*	Beebe and Umbreit, 1971, Dehghan-Noudeh et al., 2009, and Finnerty, 1979, Knoche and Shively, 1972, Rosenberg et al., 1999, Rosenberg et al., 1988, Zosim et al., 1982
Fatty acids	*Corynebacterium lepus*	Chandran and Das, 2011, MacDonald et al., 1981
Neutral lipids	*Nocardia erythropolis*	MacDonald et al., 1981
Corynomicolic acid	*Corynebacterium insidibasseosum*	Nitschke and Costa, 2007
Polymeric surfactants		
Emulsan	*Acinetobacter calcoaceticus*	Cirigliano and Carman, 1984, Cirigliano and Carman, 1985
Biodispersan	*Acinetobacter calcoaceticus*	Nitschke and Costa, 2007
Liposan	*Candida lipolytica*	Bernheimer and Avigad, 1970, Bryant 1990
Particulate biosurfactants		
Vesicles	*Acinetobacter calcoaceticus*	Gutnick & Shabtai 2017 Kappeli and Finnerty, 1979
Whole microbial cells	*Cyanobacteria, variety of bacteria*	Fattom & Shilo, 1985, Nitschke & Costa, 2007, Rosenberg et al., 1986

2.2 Applications of biosurfactants:

Over the years biosurfactants are becoming broadly applicable in various industries and are posing a serious threat to the synthetic surfactants. Biosurfactants are currently venturing into production economics because of their major applications that make them noticeable. They have a wide range of applications from pharmaceuticals to agriculture industries.

2.2.1. Cosmetics:

Many researchers have highlighted the huge potential of biosurfactants for cosmetic applications. Among all biosurfactants, glycolipids are the most studied in cosmetic and personal care formulations (Lourith & Kanlayavattanakul, 2009). Glycolipid was used in combination with a synthetic anionic surfactant at least 50% (w/w) of the total surfactant combination in products for skin washing such as shampoo formulations (Parry and Stevenson, 2014). For instance, rhamnolipids are used in` anti-wrinkle and anti-ageing products cosmetics. Piljac and Piljac (1999) patented a cosmetic product containing one or more rhamnolipid biosurfactants (from 0.001% up to 5%) to treat signs of aging. Rhamnolipid are also used to formulate a shampoo comprising 2% of rhamnolipid dissolved in water. This formulation showed antimicrobial effect left the scalp free from odor for three days maintaining a luster (Desanto 2008). Trevor et al., (2013) sophorolipid biosurfactant in combination with an anionic surfactant was used to prepare a mild formulation suitable for personal wash, shower gel and shampoo. This patented formulation was composed of 1–20% (w/w) sophorolipid. Rhamnolipids and sophorolipids were used in combination with 10% of oleic oil, in different cosmetic formulations like conditioning anti-dandruff shampoo, moisturizing skin cleanser, body cleanser, shower gel (Allef et al., 2014)

It has been also reported that Mannosylerythritol lipids (MEL) have potential as anti-aging skin care ingredients. Takahashi et al., (2012) evaluated the antioxidant capacity of MEL derivatives (A, B and C) by using a 1, 1-diphenyl-2-picryl hydrazine (DPPH) free-radical method and superoxide anion scavenging assay with fibroblasts NB1RGB cells. MEL-C showed the highest antioxidant activity (50.3% at 10g/L) and also presented good protective effects in cells against oxidative stress (30.3% at 10lg/mL of MEL-C). Kitagawa et al., (2015) patented a makeup product containing cosmetic

pigments, consisting of particles of pigment coated with MEL. Das et al., (2013) reported a biosurfactant obtained from Nocardiopsis VITSISB as alternative to SDS in cosmetic toothpaste formulation. Based on the results, the authors suggested that biosurfactants could replace synthetic surfactants, SDS because they are more effective and less toxic. Also lipopeptides have been used in the cosmetics industry due to their exceptional surface properties, having anti-wrinkle and moisturizing activities on human skin. They are also well utilized in dermatological products and in cleansing cosmetics for their highly washable capability (Montanari and Guglielmo 2008, Kanlayavattanakul Lourith , 2010)

2.2.2. Pharmaceuticals and therapeutics:

The demand for new antimicrobial agents has increased as a result of resistance shown by pathogenic microorganisms against existing antimicrobial drugs has drawn attention to biosurfactants as antibacterial agents. The most widely reported class of biosurfactants with antimicrobial activity are lipopeptides produced by *Bacillus* sp. They have been found to be active against a range of multidrug-resistant pathogenic strains. Surfactin, produced by *Bacillus subtilis*, is the best-known antimicrobial lipopeptide (Arima, 1968). Other antimicrobial lipopeptides includes fengycin, iturin, and mycosubtilins produced by *Bacillus subtilis*; lichenysin, pumilacidin and polymyxin B are produced by *Bacillus licheniformis, Bacillus pumilus* and *Bacillus polymyxa*, respectively (Naruse et al., 1990; Yakimov et al., 1995; Grangemard et al., 2001; Vater et al., 2002 and Landman et al., 2008). A cyclic lipopeptide biosurfactant daptomycin produced by *Streptomyces roseosporus* has been also reported having antimicrobial activity. This *lipopeptide antibiotic* has been approved in the USA in 2003 for the treatment of skin and skin structure infections caused by Gram-positive pathogens (Baltz et al., 2005). There are lipopeptides biosurfactants such as viscosin, a cyclic lipopeptide produced *Pseudomonas* sp and rhamnolipids produced by *Pseudomonas aeruginosa* (Neu & Poralla, 1990; Abalos et al., 2001; Benincasa et al., 2004 and Saini et al., 2008). Recently, a lipopeptide biosurfactant produced by a marine organism, *Bacillus circulans*, was found to be active against *Proteus vulgaris, Alcaligens faecalis*, methicillin resistant *Staphylococcus aureus* (MRSA) and other multidrug-resistant pathogenic strains (Das et al., 2008) while not having any haemolytic activity. Mannosylerythritol lipids (MEL-A and MEL-B) produced by *Candida antarctica*

strains have also been reported to exhibit antimicrobial action against Gram-positive bacteria (Kitamoto et al., 1993). Sophorolipids produced by *Candida bombicola* have also been reported to exhibit antimicrobial activity (Kim et al., 2002; Van Bogaert et al., 2007). They also have antitumor, antiviral and anti adhesive activities thereby showing promising applications in pharmaceuticals and therapeutics (Cao et al., 2009; Lee et al., 2010; Donio et al., 2013).

2.2.3. Enhanced oil recovery (EOR):

Oil recovery from oil wells is facing a huge problem either due to low permeability of the rocks forming the reservoir or due to the high viscosity of the crude oil. To overcome this problem use of biosurfactants can improve the process efficiency as they have an ability of to reduce the oil/water interfacial tension and form stable emulsions (Rahman et al., 2003 and Costa et al., 2010). The use of biosurfactants in microbial enhanced oil recovery (MEOR) has been extensively reviewed (Banat et al., 2000 and Singh et al., 2007). Rhamnolipids have been most frequently used, lipopeptides, such as surfactin, lichenysin and emulsan have also proved very effective in enhancing oil recovery (Alvarez et al., 2015). Pornsunthorntawee et al., (2008) demonstrated that both *Bacillus subtilis* PT2 and *Pseudomonas aeruginosa* SP4 biosurfactants were more effective than three synthetic surfactants in oil recovery from a sand-packed column, the *Bacillus subtilis* product being the most effective with an oil removal of 61% against 57% for the *Pseudomonas aeruginosa* surfactants and about 4% when distilled water was used. Lipopeptides derived from *Bacillus subtilis, Bacillus siamensis and Fusarium* sp. BS-8 enhanced oil recovery in sand pack column by 43, 60, and 46%, respectively (Pathak and Keharia, 2013; Varadavenkatesan and Murty, 2013 and Qazi et al., 2013). A mixture of lipopeptides produced by *Bacillus subtilis* B30 improved the recovery by 17–26% of light oil and by 31% of heavy oil in core-flood signifying it's prospective in the development of ex situ MEOR processes (Al-Wahaibi et al., 2014). Biosurfactants produced by *Rhodococcus erythropolis* and *Rhodococcus ruber* were used to extract hydrocarbons from oil shale in flask experiments; the maximum recovery was 25% and 26% for the two strains, respectively, with even lower recovery when a high percentage of asphaltenes and resin compounds were present in the oil (Haddadin et al., 2009). Biosurfactant produced by *Bacillus subtilis* isolated from Brazilian crude oils at a concentration of 1 g l^{-1} recovered between 19% and 22% of oil, whereas the recoveries

obtained with the chemical surfactants at the same concentration were between 9% and 12%. From the results obtained it can be concluded that these biosurfactants are more effective in oil recovery when compared with the chemical surfactants Enordet and Petrostep (Pereira et al., 2013). Biosurfactant-producing *Bacillus subtilis* strains was used to enhance oil recovery in laboratory sand-packed columns Injection of the CFS with 600.0 mgl^{-1} of the biosurfactant resulted in approximately 69% recovery of residual oil (Gudina et al., 2013).

2.2.4. Bioremediation of heavy metals:

Among biosurfactant classes, Rhamnolipids are the most characterized biosurfactant for its potential to remove heavy metals. They are anionic in nature so they are applied to remove cationic metal ions, such as Zn, Cu, Pb, Cd, Ni, Fe (Dahrazma and Mulligan 2007; Khan et al., 2015). Though so far, bioremediation potential of rhamnolipids has been extensively studied and is the major topic for publication. In 2007, Juwarkar et al reported removal of heavy metals (Cd and Pb) using rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* strain BS2 from artificially contaminated soil. The study showed that washing the soil with rhamnolipid removed 92% of Cd and 88% of Pb after 36 hr. Also the treatment with rhamnolipid solution enabled the soil to regain its fertility and soil microflora which were lost due to the inhibitory and toxic effect of heavy metals. Thus the study showed that biosurfactant technology is efficient method for bioremediation. The feasibility of rhamnolipid foam for the removal of cadmium (Cd) and Nickel (Ni) was evaluated from sandy soil by Wang and Mulligan (2004). The foam generated by a 0.5% rhamnolipid solution removed 73.2% of Cd and 68.1% of Ni with an initial pH value of 10. Rhamnolipid-mediated desorption of heavy metals from representative soil components were also examined. In 2007, Asci et al., evaluated the Cd(II) removal potential of rhamnolipid from kaolin (soil component). In this study the effects of pH and rhamnolipid concentration on desorption efficiency were also evaluated. The maximum removal of Cd(II) was observed to be 71.9% with optimum pH 6.8 and 80 mM concentration of the rhamnolipid. Another study by Asci et al., (2008a) reported the removal of zinc from Na-feldspar (a soil component). It was found that the best recovery efficiency from Na-feldspar using 25 mM rhamnolipid concentration was 98.83% of the sorbed Zn (II) (2.19 mmol kg^{-1}) at optimal pH 6.8. The recovery of Cd(II) from sepiolite and K-feldspar (soil components) using rhamnolipid biosurfactant was also reported by Asci et al (2008b). The desorption

efficiency from K-feldspar was approximately 96% whereas only 10.1% from sepiolite. In 2012, Venkatesh & Vedaraman, used synthesized rhamnolipids at different concentrations from 0.5 to 2% with and without 1% sodium hydroxide at a fixed ratio of 4:1. It was found that 2% rhamnolipids removed 71% and 74% of copper from soil with initial concentrations 474 and 4,484 ppm, respectively. Akintunde et al., (2015) reported remediation of Fe using rhamnolipid from aqueous solution. In this study potential of the biosurfactant to remove iron was studied and the result revealed that rhamnolipid was able to remove 60.34% of iron indicating it as an effective iron remediating agent. Also among the glycolipids, sophorolipids are also utilised in bioremediation of heavy metals. Sophorolipids produced by *Torulopsis bombicola* were utilized in the removal of metal ions from metal contaminated sediment. The study showed that a single washing with 4% sophorolipids removed 60% of Zinc and 25% of copper (Mulligan et al., 2001). The removal of cadmium and lead from artificially contaminated soil was also studied by using sophorolipids produced by *Starmerella bombicola* CGMCC 1576. Crude acidic sophorolipid was able remove 83.6% of Cd and 44.8% of Pb at concentration of 8%. The removal efficiency of sophorolipids was better than the synthetic surfactants. Moreover, the study also showed that the acidic sophorolipid were more effective than lactonic sophorolipid in remediation of heavy metal from the soils (Qi et al., 2018). *Candida guilliermondii* UCP 0992 was able to produce a biosurfactant with low-cost production medium. The extracted biosurfactant (0.42 %) was able to remove 99.9 % Zn, 98.6 % Fe and 93.8 % Pb from the soil comprising of heavy metal concentrations of 3038, 1877 and 1470 mg/l of Pb, Fe and Zn respectively. Further the toxicity of this biosurfactant was also studied on the germination of seeds of cabbage (*Brassica oleracea*). The study showed that the biosurfactant had no toxic effect on the seed germination (Sarubbo et al., 2018).

Recently lipopeptides are also becoming popular due to its anionic character they can form complex with the positively charged metal which makes them a metal sequestering candidate for remediating heavy metals. Das et al., (2009) reported the efficiency of a biosurfactant produced from a marine bacterium in removing heavy metals from solutions. Biosurfactant with a concentration of 0.5X critical micelle concentration (CMC) was able remove 76.6% and 42.74% of 100 ppm of lead and cadmium. As the concentration of biosurfactant was increased to 5X CMC, there was almost complete removal of 100 ppm of lead and cadmium. The efficiency of metal removal depended

on the concentration of a metal and the biosurfactant. In 2013, Singh and Cameotra studied the efficiency of surfactin and fengycin obtained from *Bacillus subtilis* A21 in remediating cadmium, cobalt, copper, iron, lead, zinc and nickel from the soil collected from industrial dumping site. Soil washing was carried out with mixture of surfactin and fengycin at a concentration of 50 CMC which were able remove metals namely cadmium (44.2 %), cobalt (35.4 %), lead (40.3 %), nickel (32.2 %), copper (26.2 %) and zinc (32.07 %) in a period of 24 h. Further biosurfactant washed soil was for mustard seed germination to check its ability for plant growth. The biosurfactant washed soil showed 100 % mustard seed germination compared to water washed soil where no germination was observed. Bioreduction of Cr (VI) using surfactin was also reported by Swapna et al., (2016). Chromium solution (100ppm) was treated with surfactin (10mg/ml) over the period of 72 hr. There was 38% removal of Cr (VI) at 12 h which was increased to 74% on incubation upto 72 h. The advantage of biosurfactant is that they can be produced by using cheap raw materials which can be cost effective. Hisham et al (2019) reported biosurfactant production by *Bacillus* sp. HIP3 by means of used cooking oil. The produced lipopeptide biosurfactant was able to remove copper (13.57 %), chromium (1.68 %), lead (12.71 %), zinc (2.91%), and cadmium (0.7%) respectively, stressing on its prospective for bioremediation.

2.2.5. Agriculture:

Agriculture has faced up drastic decrease in yields due to the outbreak of fungal diseases from ancient times. Use of agrochemicals has certainly decreased the fungal diseases, but at the same time has contributed to the development of resistant pathogens. Moreover, such chemicals can be lethal to beneficial microorganisms in the rhizosphere and useful soil insects, and they may also enter the food chain and accumulate in the human body (Godfray et al., 2016). To overcome the above problems, a non-hazardous alternative such as biological control has been extensively studied. The use of biosurfactants to combat plant disease has become of great interest because of their low toxicity, biodegradability and environmentally friendly nature. Biosurfactants have been studied for its antifungal activity towards phytopathogenic fungi and stimulation of plant defense.

Lipopeptide biosurfactants produce by *Bacillus subtilis* strain SPB1 was able to inhibit phytopathogenic fungi *Rhizoctonia bataticola and Rhizoctonia solani* (Mnif et al.,

2016). The co-production of surfactin, iturin, and fengycin isoforms involved in the biocontrol of *Plasmodiophora brassicae* and *Fusarium solani* and *Penicilium digitatum*, respectively (Li et al., 2014). Waewthongrak et al., (2014) suggested that fengycin and surfactin act as elicitors of defense-related gene expression in "Valencia" fruit following infection by *Penicilium digitatum*. The involvement of three isoforms, surfactin, iturin, and fengycin, affected spore germination and membrane permeability of spores from four fungal plant pathogens *Alternaria solani, Fusarium sambucinum, Rhizopus stolonifer, and Verticillium dahlia* (Liu et al., 2014). *Bacillus subtilis* 916 was able to produce a new family of lipopeptide called locillomycin which was active against *Fusarium oxysporum* (Luo et al., 2015). Cao et al., (2012) reported that fengycin and bacillomycin produced by *Bacillus subtilis* SQR 9 was involved in the inhibition of mycelial growth and spore germination of *Fusarium oxysporum*.

According to studies sophorolipid biosurfactants were active against numerous fungi, including *Saccharomyces, Cladosporium, Aspergillus, Fusarium, Penicillium, Gloeophyllum and Schizophyllum* as well as *Botrytis cineria* (Yanagisawa et al., 2014, Kim et al., 2002). Sophorolipid biosurfactant produced by *Rhodotorula babjevae* YS3 displayed potential antifungal activity against broad group of pathogenic fungi viz. *Corynespora cassiicola*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, *Trichophyton rubrum* and *Fusarium verticilliodes* (Sen et al., 2017). Another study reported that sophorolipid derivatives and combinations of sophorolipids derivatives exhibited significant antifungal activity against 18 plant fungal pathogens (Schofield et al., 2013)

Rhamnolipids produced from *Pseudomonas aeruginosa* have protective effects on plants against phtopathogenic fungi and bacteria infestation via stimulation of the plant immune system in tobacco, wheat and Arabidopsis thaliana (Vatsa et al., 2010). Moreover, rhamnolipids trigger strong defense responses in grapevine, including early events of cell signaling, such as Ca^{2+} influx, reactive oxygen species production and mitogen-activated protein kinase activation, and they also induce a large battery of defense genes, including some pathogenesis-related protein genes and genes involved in oxylipin and phytoalexin biosynthesis pathways (Varnier et al., 2009). Rhamnolipids also induced the biosynthesis of the plant hormones salicylic acid, jasmonic acid and ethylene, which are key players in the signaling networks involved in plant immunity.

Moreover, they elicited plant defense responses and the enhancement of resistance against the necrotrophic fungi *B. cinerea*, conferring the protection of grapevine leaves and berries (Sanchez et al., 2012). Also, rhamnolipds are able to stimulate defence genes in tobacco, wheat and *A. thaliana* and are also potent protectors in monocotyledonous plants against biotrophic fungi (Mnif et al., 2016).

Owing to this wide array of functional properties and diverse potential applications there is enormous demand for biosurfactants in the recent years, which has lead to the exploration of unexplored environments which have been found to be potential sources of bioactive compounds. A huge and extensive source of natural compounds can be retrieved from the marine environment (Bhatnagar and Kim, 2010). Microbial communities in the marine environments are often under extreme conditions such as pressure, temperature, salinity, and depletion of micronutrients and have an immense potential as a source of novel microbes of taxonomic significance. These microbes exhibit unique metabolic and physiological capabilities conferring them the ability to survive in this extreme conditions and consequently produce novel metabolites that cannot be found elsewhere (De Carvalho & Fernandes, 2010; Satpute et al., 2010). Hence, the marine environment holds a great promise towards the discovery of novel bioactive compounds including antibiotics, enzymes, vitamins and drugs among others (Kubicki et al., 2019). Due to their unique environmental conditions and properties, the marine environments are a good source for the isolation of new biosurfactant-producing microorganisms.

Marine salterns are coastal ecosystems which generally originate as a result of evaporation of seawater which harbors halotolerant and halophilic bacteria. The evaporation of water in different ponds leads to the progressive development of diverse microbial species that get adapted to varying concentrations of salinity (Das Sarma and Das Sarma, 2017). Hypersaline microorganisms produce several important biomolecules such as enzymes, antibiotics, compatible solutes, etc. in order to survive in this environment. The search for biosurfactants from these microorganisms is very promising as they have adapted to be stable in adverse environments and their products are highly stable and important in biotechnology (Sarafin et al., 2014).

According to Post and Collins (1982) Halophiles have a unique lipid composition which may have an important role to play as surface-active agents. The Archae bacterial ether-

linked phytanyl membrane lipid of the extreme halophilic bacteria has been shown to have surfactant properties. Yakimov et al., (1995) reported the production of biosurfactant by the halotolerant *Bacillus* sp. and it's prospective in enhanced oil recovery. Studies have reported that *Streptomyces* sp. isolated from the soil samples of marine saltpans showed production of biosurfactant (Lakshmipathy et al., 2010; Khopade et al., 2012). *Streptomyces sp.* isolated from from the salt pan showed the production of biosurfactant and showed promising activity in oil recovery. The biosurfactant was identified as "Decanal" by comparison of mass spectral data and retention times (Shubhrasekhar et al., 2013). Donio et al., (2013) reported biosurfactant production by halophilic *Bacillus sp.* BS3 isolated from solar salt works from southern India. The biosurfactant was characterized as lipopeptide. The biosurfactant showed antiviral activity against white spot syndrome virus by suppressing the viral replication in the shrimp and significantly raised shrimp survival. It also showed anticancer in mammary epithelial carcinoma cell at different concentrations. In another study, Donio et al. (2013) revealed that *Halomonas sp* BS4 isolated from solar salt works from southern India also produced a biosurfactant which also showed antibacterial, antifungal, antiviral and anticancer. Further the partially purified biosurfactant was characterized by TLC, GC-MS and FTIR analysis. GC-MS analysis revealed that, the biosurfactants contains polymers, fatty acids and other compounds including 1, 2-Ethanediamine N, N, N, N'-tetra, 8-Methyl-6-nonenamide, (Z)-9-octadecenamide etc. 1,2-Ethanediamine, N, N, N, N'-tetramethyl- is a polymeric biosurfactant. The study concluded that *Halomonas* sp BS4 produces a mixture of glycolipids, polymeric substances and lipopeptides. In 2014, Sarafin et al. reported production of biosurfactant which belonged to a lipopeptide group from halophilic *Kocuria marina* BS-15. The GC–MS analysis confirmed that the biosurfactant contains compounds such as Nonanoic acid and cyclopropane.

A considerable number of marine microorganisms able to produce biosurfactants with different structures have been reported. However, it has to be taken into account that the great majority of the marine microbial diversity remains unexplored, mainly due to the difficulty of growing marine microorganisms under laboratory conditions (Gudina et al., 2016). There are very few reports on biosurfactant producers in hypersaline environments and in the recent years, there has been a great increase in interest and importance in halophilic and halotolerant bacteria for biomolecules.

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Growth and maintenance of salt pan bacteria

The bacterial isolates used in the present study were isolated from water, sediment and biofilms and selected Actinomycetes from the marine salterns of Goa situated in Ribandar, Nerul, Batim, Agarwaddo, and Curca and designated with codes SK, BGUMS, SBSK, TSK, FSK, MFSK, ABSK and RMSK followed by a number (Kamat, 2012; Pereira 2013; Ballav, 2016; Bartakke, 2018; Fernandes, 2019a). The bacteria were maintained on their respective media of isolation viz. Zobell marine agar, Nutrient agar, Media D agar and Actinomycetes isolation agar (Appendix A) at 4ºC in Prof. Savita Kerkar's Departmental collection). All the media components and chemicals used were of Analytical Grade.

3.2. Screening for biosurfactant production:

3.2.1. Primary screening*:*

The halotolerant bacteria (300 nos.) were individually inoculated in 50 mL Nutrient broth (Appendix A) in 150 ml Erlenmeyer flasks and were incubated on an orbital shaker for 24 h at 120 rpm and 28 ± 2 °C. The culture supernatant of each isolated was collected after centrifugation at 6000 rpm at 4°C for 15 minutes (Remi cooling centrifuge, India) and was filtered through 0.22 µm pore size filter paper (Millipore). This cell free culture supernatant was further used to check for the production of biosurfactant using drop collapse method and parafilm M test (Thavasi et al., 2011).

3.2.1.1 Emulsification assay

Emulsification assay was carried out following the procedure by Cooper and Goldenberg (1987). One millilitre of hydrocarbon i.e., kerosene was taken in a test tube (10 mL) to which 0.5 ml of cell free culture supernatant was added and was vortexed for 2 minutes to ensure homogenous mixing of both the liquids. The emulsification activity (E24) was observed after 24 h and it was calculated by using the formula:

$$E24 = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100$$

3.2.1.2 Drop collapse method

The drop collapse test was carried out with a modified procedure described by Jain et al (1991). Crude oil (20 µL) was applied to the each wells of 96-well micro-titre plate and allowed to equilibrate for 24 h. The cell free culture supernatant (5 µL) was added to the centre of oil coated regions and then the drop size was measured after 1 min using a ruler. Equal volumes of distilled water and Triton X-100 (1 mg/ml) were used as a negative and positive control respectively. The results were considered positive for biosurfactant production when the drop diameter was at least 1 mm larger than that produced by distilled water (negative control).

3.2.1.3 Parafilm M test

Cell-free culture supernatant (20 µL) was added to the hydrophobic surface of the parafilm M. The diameter of the drop was measured after 1 min with the help of a ruler. Distilled water and Triton X-100 (1mg/ml) were used as a negative and positive control respectively (Yalçın et al., 2018).

3.2.2 Secondary Screening

The results obtained from the primary screening were tabulated, and the isolates showing maximum activity (based on the drop size) were selected for secondary screening.

A total 44 microbial cultures showing promising activity in the primary screening were selected and further screened using the oil spreading method.

3.2.2.1 Oil spreading method

Oil spreading experiment was carried out as described by Morikawa et al. (1993). Here, 20 mL of distilled water was added to a plastic petri plate followed by addition of 20 µL of crude oil which formed a layer on the water surface. To this layer, 10 µL of cell free culture supernatant was then added to the oil surface. The Petri plate was left undisturbed for 2-3 seconds at RT ±2 and observed for oil displacement (oil free clearing zone). The diameter of the clearing zone is directly proportional to the biosurfactant concentration (Yousef et al., 2004). The size of this clearing zone indicates

the surfactant activity, also denoted as oil displacement activity. Distilled water was used as negative control (without surfactant) and Triton X-100 (1 mg/mL) was maintained as the positive control.

3.3. Optimization of biosurfactant production

3.3.1. One factor at a time method (OFAT):

OFAT method involves changing of factors one at a time while holding all other factors constant in order to determine the optimum conditions. To determine the optimum conditions required for biosurfactant production, glucose fermentation medium was used as a basal medium (Appendix A). Various cultural and nutritional parameters where changed one at a time, keeping other factor constant. The factor checked for the optimal production of biosurfactant were incubation temperature, pH, carbon and nitrogen source and their concentration, concentration of NaCl. Each factor was assessed based on the concentration of the biosurfactant produced in the supernatant by oil spreading method as described above (section 3.2.2.1).

3.3.1.1. Determination of optimum incubation temperature

The optimal incubation temperature required for biosurfactant production was determined by varying temperatures (10, 15, 20, 25, 30, 35, 40, 45 and 50°C). Cultures were inoculated in basal medium (50 ml) and incubated in orbital shaker at respective temperatures at 120 rpm for 24 h. The culture supernatant was collected after centrifugation at 6,000 rpm, 15 min at 4°C. The supernatant was further used to check for the presence of biosurfactant using the oil spreading method as described above (section 3.2.2.1).

3.3.1.2. Determination of optimum pH

The optimum pH required for biosurfactant production was studied by varying the initial pH of the basal medium. The initial pH of the medium was adjusted from 4.0 to 9.0 (with increments of 0.5 unit), respectively. Cultures were inoculated in basal medium (50 ml) and incubated at the optimal temperatures at 120 rpm for 24 h. The culture supernatant was collected by centrifugation at 6,000 rpm for 15 min at 4°C. The supernatant was used to check for the presence of biosurfactant by oil spreading method as described above (section 3.2.2.1).

3.3.1.3. Effect of carbon sources on the production of biosurfactant

To evaluate the effect of different carbon sources on the production of biosurfactant by SK27 and RMSK 10, the basal media was altered separately with 10 different carbon sources (glucose, glycerol, fructose, sodium citrate, mannitol, starch, mannose, lactose, paraffin and sucrose) at 2 % (w/v) concentration, respectively. The carbon sources were selected based on literature and availability (Pereira et al., 2013; Joice et al., 2014). Each carbon source (2 %) was added to the basal media containing fixed nitrogen source, with optimum pH. Flasks were inoculated by adding 100µL of each bacterial cell suspension and incubated at optimum temperatures and 120 rpm for 24 h. The culture supernatant was collected by centrifugation at 6,000 rpm for 15 min at 4°C and checked for the presence of biosurfactant.

3.3.1.4. Effect of nitrogen sources on the production of biosurfactant

To evaluate the effect of different carbon sources on the production of biosurfactant by SK27 and RMSK 10, the basal media was altered separately with 10 different nitrogen sources (yeast extract, meat extract, peptone, urea, beef extract, tryptone, sodium nitrate, glycine, soya peptone and casein) at 0.4 % (w/v) concentration, respectively. The nitrogen sources were selected based on literature and studies (Pereira et al., 2013, Joice & Parthasarathi, 2014). Each nitrogen source (0.4%) was added to the basal media containing selected carbon source, with optimum pH. Flasks were inoculated by adding 100 µL of each bacterial cell suspension and incubated at optimum temperatures and 120 rpm for 24 h. The culture supernatant was collected by centrifugation at 6,000 rpm for 15 min at 4°C. The supernatant was used to check for the presence of biosurfactant using the oil spreading method as described above (section 3.2.2.1).

3.3.1.5. Effect of concentration of carbon source on the production of biosurfactant

To study the effect of concentration of selected carbon source on the biosurfactant production by SK27 and RMSK10, was assessed using basal media with the optimized factors as the base. Carbon source concentration in the medium was adjusted in the range 0 to 2% (with an increment of 0.25 units) respectively. Flasks were inoculated by adding 100µL of each bacterial cell suspension and incubated at optimum temperatures

and 120 rpm for 24 h. The culture supernatant was collected to confirm the presence of biosurfactant

3.3.1.6. Effect of concentration of nitrogen source on the production of biosurfactant

To study the effect of concentration of selected nitrogen sources on the biosurfactant production by SK27 and RMSK10, the basal media with the optimized factors was used as the base. The nitrogen source concentration of the medium was adjusted in the range 0 to 1.8 % (with an increment of 0.2 units) respectively. Flasks were inoculated by adding 100 µL of each bacterial cell suspension and incubated at optimum temperatures and 120 rpm for 24 h. The supernatant was further used to check for the presence of biosurfactant.

3.3.1.7. Effect of sodium chloride (NaCl) concentration production of biosurfactant

The effect of the sodium chloride on the production the biosurfactant by SK27 and RMSK10 was assessed by varying the concentration of NaCl in the basal media ranging from 0 to 4% respectively. The basal media with the selected carbon source, nitrogen source and optimum pH was used. Flasks were inoculated by adding 100µL of each bacterial cell suspension and incubated at optimum temperatures and 120 rpm for 24 h. The culture supernatant was collected by centrifugation at 6,000 rpm for 15 min at 4°C, to confirm the presence of biosurfactant.

3.3.1.8. Growth profile and production of biosurfactant

The bacteria (SK27 and RMSK 10) were inoculated in the basal media with the optimized factors and incubated at 28 °C for 24 h. When the absorbance of the starter culture was 0.9 ± 0.02 at 630 nm, the culture was inoculated in basal media in triplicates and placed on an orbital shaker at 120 rpm. The initial absorbance was noted at 630 nm. The medium (2 mL) was withdrawn every 4 h for a period of 48 h and the absorbance was measured. The culture supernatant was collected by centrifugation at 6,000 rpm for 15 min at 4°C. The supernatant was further checked for the presence of biosurfactant every 4 h using oil spreading method described above (section 3.2.2.1).

3.3.2. Statistical approach

3.3.2.1. Optimization of biosurfactant production using Response surface methodology (RSM)

Once the critical factors were identified using OFAT method, optimization was carried out by a statistical approach, using the central composite design (CCD) and analyzed by response surface methodology (RSM). RSM was used to study the interaction between the three effective parameters selected from OFAT on the production of biosurfactant by SK27 and RMSK10. The three effective parameters for SK27 were, Sucrose (A), Yeast extract (B), Sodium chloride (C) and for RMSK 10 were, Starch (A), peptone (B), Sodium chloride (C). Each factor in the design was studied at three different levels (−1, 0, +1) as shown in Table 1 and Table 3. The other medium components of the OFAT optimized medium were kept constant varying only these three influential factors. The experimental design was analyzed by a statistical software package Design Expert 11.0 (Minneapolis, USA). The design was used to identify the effect of these variables on each other for determining the optimum fermentation condition, thereby maximizing the biosurfactant production.

According to this design, 20 experimental setups with various combinations of factors A, B, and C were generated containing six replicates at the centre point as controls to maximize the production of biosurfactant (Table 2 and Table 4). A set of 20 experiments were carried out in triplicates using basal media. Flasks were inoculated by adding 100 µL of each bacterial cell suspension and incubated at optimum temperatures at 120 rpm for 24 h. The culture supernatant was collected by centrifugation at 6,000 rpm for 15 min at 4 °C. The supernatant was further used to check for the production of biosurfactant by oil spreading method. The response value in each trial was calculated as the average of triplicates measured in cm.

Table 1: Coded and real values of variables for SK27 selected for CCD

Variables	Code	Units	Coded values		
			-1	0	+1
Sucrose	A	%	0.5	1	1.5
Yeast extract	B	%	0.6	0.9	1.2
NaCl	C	%	0.2	0.6	1

Table 2: Central composite design of experimental design for SK27

Run	A	B	C
1	0	0	0
2	-1	0	0
3	+1	+1	0
4	0	+1	+1
5	0	0	0
6	0	0	+1
7	0	0	0
8	-1	0	0
9	0	0	+1
10	0	0	-1
11	0	0	0
12	+1	+1	0
13	0	0	-1
14	-1	+1	0
15	-1	+1	-1
16	+1	0	+1
17	0	0	0
18	+1	-1	0
19	-1	-1	+1
20	0	0	0

Table 3: Coded and real values of variables for RMSK10 selected for CCD

Variables	Code	Units	Coded values		
			-1	0	+1
Starch	A	%	0.5	1	1.5
Peptone	B	%	0.4	0.9	1.4
NaCl	C	%	0.2	0.6	1

Table 4: Central composite design of experimental design for RMSK10

Run	A	B	C
1	-1	-1	+1
2	0	0	0
3	-1	+1	-1
4	0	0	0
5	0	+1	0
6	0	+1	+1
7	+1	+1	+1
8	+1	0	0
9	-1	-1	-1
10	0	0	-1
11	0	0	0
12	+1	-1	0
13	0	0	0
14	+1	-1	-1
15	0	0	+1
16	0	-1	0
17	-1	0	0
18	0	0	0
19	0	0	0
20	+1	+1	-1

The statistical significance of the model was verified by applying the analysis of variance (ANOVA). The second order model equation was determined by Fischer's test and the quality of the fit of the polynomial model equation was determined by the coefficient of determination, R^2. The polynomial equation was then expressed in the form of 3D and contour graphs to illustrate the relationship between the responses and the experimental levels of each independent variable.

The model was further validated experimentally by shake flask method. The cultures SK27 and RMSK10 were inoculated in media prepared using optimum values of the factors generated by the software and incubated at 30°C at 120 rpm for 24 h. The culture supernatant was collected by centrifugation at 6,000 rpm for 15 min at 4°C. The supernatant was further used to check for the production of biosurfactant by oil displacement method.

3.3.2.2. Optimization using Genetic algorithm

The Genetic algorithm (GA) is a technique for solving both constrained and unconstrained optimization problems that is based on natural selection, the process that drives biological evolution. It consists of creation of a population of experiments, evaluation of those experiments, selection of best experiments and breeding from there, a parent population and genetic manipulation to create the new population of experiments. The whole process continues until a suitable result was achieved[7]. GA was used to evaluate the fitness of the polynomial equation for maximizing the production of biosurfactant by SK27 and RMSK10 generated by RSM, using the GA toolbox of MATLAB 9.4 (The MathWorks, Inc., Natick, MA, USA).

3.4. Identification of the bacterial isolates

The halotolerant bacteria SK27 and RMSK10 were characterized and identified based on biochemical, morphological and molecular characteristics as illustrated in Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986).

3.4.1. Cell morphology and Gram characteristic

The cultures were Gram stained according to Hans Christian Gram (1884). The bacteria were also characterized as cocci (spherical cells) or rods (cylindrical cells) after observing their morphology.

3.4.2. Spore staining

The Gram positive cultures were stained according to Schaeffer-Fulton‘s method (1933). The position of the spore was observed and recorded as central, sub-terminal or terminal.

3.4.3. Cell size

The size of the bacterial cell was measured by Scanning Electron Microscopy (Bratbak, 1993). To prepare the samples, a clean coverslip (1 cm^2) was fixed with cellotape on a glass slide. Freshly grown bacterial culture (10 µL) was spread onto the coverslip, air dried and fixed with 2.5 % glutaraldehyde overnight. The next day, the coverslip was washed with sterile phosphate buffered saline (pH 7.4) followed by successive dehydration with 30 %, 50 %, 70 %, 80 % and 90 % acetone for 10 min each and 100 % acetone for 30 min. The slides were subsequently air dried. The coverslips were cut into small pieces and attached to a stub with cellotape, and sputter coated with gold (SC7620 Mini sputter coater, Quorum technologies, UK). The cells were viewed under a Scanning Electron Microscope (Evo 18, Carl Ziess, Germany) with magnification from 2 KX to 40 KX. The size of the cells was measured and the detailed morphology was recorded.

3.4.5. Motility test

The motility of the cultures was determined by hanging drop method (Collins et al, 1984).

3.4.6. Catalase test

The bacterial culture was grown on Nutrient agar plates for 24 h. A loopful of the culture was taken on a glass slide and 3 % hydrogen peroxide (2 drops) was added. The bacterial culture was observed for production of effervescence (Vasanthakumari, 2009).

3.4.7. Oxidase test

Oxidase test was carried out by oxidase discs (HiMedia DD018). The bacterial culture (10 µL) was inoculated on a disc and the colour change was observed. Microorganisms were documented as oxidase positive when the disc colour changed from white to

purple within 5 to 10 s. Change beyond 10 seconds or no colour change was considered oxidase negative.

3.4.8. Biochemical tests

The biochemical tests were carried out using Hi-media kits as per manufacturer‘s instructions. KB013 HiBacillusTM identification kit was used for Bacillus spp.,

3.4.9. Carbohydrate utilization test

Studies on carbohydrate utilization of the bacteria were carried out using KB009 HiCarboTM kits (HiMedia KB009A/ KB009B1 / KB009C). The kit contains a total of 35 tests for utilization of carbohydrates. The utilization profile was determined as per the manufacturer‘s instructions (HiMedia Laboratories Pvt. Ltd, India). A pure colony of the bacteria under study was inoculated in 50 mL Nutrient broth and incubated on an orbital shaker for 18 h at room temperature. Each well of the strip was inoculated with 50 µL of bacterial suspension aseptically. The strips were incubated for 24 - 48 h at room temperature. The colour change was noted and results were interpreted according to the chart provided with the strips.

3.4.10. Metabolic fingerprinting

Metabolic fingerprinting was carried out using the BIOLOG assay kit according to the manufacturer‘s instructions (Hayward, CA94545, USA). Biolog GN2 MicroPlateTM and Biolog GP2 MicroPlateTM comprising of 95 biochemical tests were used for Gram negative and Gram positive cultures respectively. The metabolic activity was also studied using Biolog GEN III MicroPlateTM containing 71 carbon source utilization tests and 23 chemical sensitivity assays. Freshly grown bacterial culture (150 µL) was added to each well and incubated for 48 h at room temperature. The colourless wells which resembled the blank were recorded as negative, whereas the wells that turned purple were recorded as positive.

3.4.11. Molecular identification

The genomic DNA of the four bacteria was isolated using HiPurATM Bacterial Genomic DNA purification kit (HiMedia, India) according to manufacturer‘s

instructions. The isolated genomic DNA was used as template to amplify the 16S rRNA gene by Polymerase chain reaction. The universal bacterial primers for 16S rRNA gene: forward primer 27F: AGAGTTTGATCCTGGCTCCAG and reverse primer 1492R: TACGGTTACCTTGTTACGACTT were utilized for PCR reaction. The PCR reaction mixture contained 20 µL MilliQ water, 3 µL template, 1 µL forward primer, 1 µL reverse primer and 25 µL 2X Taq polymerase mix. PCR was performed with an initial denaturation at 95 °C of 5 min followed by 35 cycles of 95 °C for 1 min; 54 °C for 1 min and 72 °C for 1 min. A final extension step was carried out at 72 °C for 10 min. The PCR amplified products were run on 0.8 % low melting agarose gel along with StepUp™ 500 bp DNA ladder (Genei, India). The expected 1.5 kb length band was excised using a sterile scalpel and eluted by AxyPrepTM DNA Gel Extraction Kit following the manufacturer‘s instructions (Axygen Biosciences, USA). The DNA sequence of the amplified excised gene segment was obtained from AgriGenome Labs, Pvt. Ltd, India. The obtained raw sequences were used to assemble the contigs using BioEdit software, version 7.2.5 (Hall, 1999) and aligned. The contigs (approximately 1.5 kb) were matched with GenBank database using NCBI-BLAST (Altschul et al., 1990). MEGA X software was used to construct the 16S rRNA gene sequence based phylogenetic tree (Kumar et al., 2018) by Neighbour-joining method. The evolutionary distances were computed by Tamura-Nei method with 1000 bootstrap replicates. The analysis included 21 nucleotide sequences. The positions consisting of less than 95 % site coverage were eliminated. Therefore, fewer than 5 % alignment gaps, ambiguous bases and missing data, were allowed at any position. The sequence of the bacterial isolate has been deposited in GenBank.

3.5. **Extraction and Purification of biosurfactant**

For a seed culture, the cultures were inoculated in 20 mL of respective RSM-GA optimized media and incubated in an orbital shaker (120 rpm) at 30˚C for 24 h. The seed culture was then transferred into each 1000 mL RSM-GA optimized production media. Fermentation was carried out under shaking conditions for 24 h (SK27) and 28 h (RMSK10). The culture supernatant was obtained by centrifugation at 10,000 rpm for 15 min, 4 °C, and was filtered through 0.22 µm filter paper (Millipore) to obtain cell-free supernatant (CFS). The supernatant was collected and acidified to pH 2.0 with conc. HCl, and kept overnight (4°C). The precipitate was centrifugated (10,000 rpm,

4°C, 15 min) and washed three times with acidic water (pH 2.0 with 12 N HCl), and dried by vacuum. The dried surfactant was extracted three times with methanol (100%) for 3 h. The methanol was removed with the aid of a rotary evaporator (Equitron® Rotaeva) under reduced pressure. The dried surfactant was extracted three times with butanol (100%) for 3 h. The crude biosurfactant was obtained as a brown-colour material using a rotary evaporator.

3.5.1. Gel filtration chromatography

The Butanol extracts of SK27 and RMSK10 were dried under vacuum (Equitron® Rotaeva) and were re-dissolved in methanol. The extracts were then loaded over Sephadex LH20 (Sigma) in 3x90 cm column and eluted with methanol. The fractions (1 ml) were collected at a flow rate of 0.7 ml/min were monitored for biosurfactant activity. The fractions were further analyzed by thin layer chromatography (TLC) in the solvent system Butanol: Acetic acid: water (5:1:4) and developed using ninhydrin reagent (Appendix B). The active fractions were pooled and were further purified using Preparative thin layer chromatography (PTLC) (UNIPLATE, ANALTECH).

3.6. Bioremediation of heavy metal using biosurfactants

To study the heavy metal bioremediation efficiency of biosurfactant, five different metals (Pb, Fe, Cr, Cd, and Zn) at 3 different concentrations (100 ppm, 200 ppm and 300 ppm) were used (Appendix B). Biosurfactant solutions at different concentrations i.e. 40, 80, 120, 160 and 200 mg/L were inoculated in the metal solutions and incubated in an orbital shaker at 120 rpm at 30 °C for 24 h (Das et al. 2009). EDTA (Himedia) a commercial surfactant was used with same concentrations. The resulting solution was centrifuged (10,000 rpm, 10 min) to separate metal-biosurfactant precipitate. The supernatant was analysed using ICP-AES analysis (IIT Mumbai, Powai) to quantify the unbound metals. The percentage of heavy metal removal was calculated by the following equations:

$$\% \text{ remediation} = \frac{\text{Initial conc. of heavy metal} - \text{Final conc. of heavy metal}}{\text{Initial conc. of heavy metal}} \text{ X } 100$$

3.6.1. Effect of temperature on bioremediation efficiency:

To evaluate the effect of temperature on the bioremediation efficiency of the biosurfactants, studies were carried out at different temperatures (4, 10, 20, 30, 40, 50 60 and 70 °C). The concentration of heavy metals (Fe and Pb) used was 300 ppm and the biosurfactant concentration was 100 mg/L (CB1) and 120 mg/L (CR1).

3.7. Determination of critical micelle concentration (CMC) of biosurfactant

To evaluate the CMC values of the biosurfactant, concentrated solutions of purified biosurfactant (5 g/L) were prepared in distilled water. These biosurfactants solutions were then progressively added to Milli-Q water (Millipore) so that the final concentration of biosurfactant increases by 5 mg/L with each addition (Mukherjee et al. 2009). The change in surface tension of the water was noted with a Stalagmometer (Borosil). The surface tension was calculated using the following formula:

$$\sigma_L = \frac{\sigma_w . N_w . \rho_L}{N_L . \rho_W}$$

Where: σ_L is the surface tension of the liquid under investigation, σ_W is the surface tension of water, N_L is the number of drops of the liquid, N_W *is* the number of drops of water, ρ_L is the density of the liquid and ρ_W is the density of water.

The concentration at which the surface tension is lowered abruptly and reaches its minimum value was considered as the CMC for the biosurfactant respectively.

3.8. Stability studies of biosurfactant

Stability studies were done using purified compound (CB1 and CR1). The compounds were incubated at different temperatures (4, 10, 20, 30, 40, 50, 60, 70, 80 and 90 °C) for 1 h and were also subjected to autoclaving at 121 °C, 15 lbs for 20 min. The pH stability was performed by adjusting the solution to different pH values (4 – 14). For studying the stability towi salt concentration, different concentrations of NaCl (0 – 20 % w/v) were added to biosurfactant solution and mixed until complete dissolution (Nitschke

and Pastore 2006). The biosurfactant activity was checked by oil spreading method described above (Section 3.2.2.1).

3.9. Mass spectrometric analysis

ESI–MS analysis was carried out at Centre for Analysis, Testing, Evaluation and Reporting Services, CSIR-Central Leather Research Institute, Chennai. Characterization of the biosurfactant (CB1) was carried out through direct injection into an ESI–MS in positive ionization modes (Waters Acquity UPLC, XevoTQD2000).

3.10. Statistical analysis of data

Statistical analysis was performed by using IBM© SPSS version 23.0 statistical software (IBM Corporation, USA, 2015). The results were expressed as mean with their corresponding standard deviation (S.D.). The results were subjected to one-way analysis of variance (ANOVA). Levene‘s test was performed to check homogeneity of variance. Subsequently, *post-hoc* multiple comparisons with Tukey HSD test was used to assess the significant difference between the treatment groups. The data were considered to be statistically significant at $p < 0.05$.

CHAPTER 4

RESULTS

4. RESULTS

4.1 Saltpan bacteria

The halotolerant bacteria and actinomycetes (300 nos.) from the marine salterns of Goa were selected based on their consistency in growth and morphological differences. It was noticed that the morphological diversity was highest in the overlying saltpan water and least in the sediments (Table 5).

Table 5: List of bacterial isolates used in the present study

Source	Culture code	Total cultures
Saltpan water	BGUMS 5, BGUMS 6, BGUMS 7, BGUMS 9, BGUMS 14, BGUMS 14A, BGUMS 14B, BGUMS 16, BGUMS 38, BGUMS 46, BGUMS 59, BGUMS 62, BGUMS 93, BGUMS 94, BGUMS 100, BGUMS 101, BGUMS 103, BGUMS 107, BGUMS 109, BGUMS 113, BGUMS 136, BGUMS 158, BGUMS 165, BGUMS 186, BGUMS 254, BGUMS 256, BGUMS 257, BGUMS262, BGUMS 264, BGUMS 265, BGUMS 284, BGUMS 296, BGUMS 299, BGUMS 304, BGUMS 305, BGUMS 312, BGUMS 313, BGUMS 315, BGUMS 317, BGUMS 346, BGUMS 348, BGUMS 359, BGUMS 360, BGUMS 370, BGUMS 373, BGUMS 376, BGUMS 384, BGUMS 386, BGUMS 388, BGUMS 396, BGUMS 397, BGUMS 400, BGUMS 434, BGUMS 435, BGUMS 436, BGUMS 440, BGUMS 451, BGUMS 457, BGUMS 459, BGUMS 471, BGUMS 472, BGUMS 473 A, BGUMS 473 B, BGUMS 473 C, BGUMS 474, BGUMS 475, BGUMS 527, BGUMS 740, BGUMS 744, BGUMS 799, BGUMS 837, BGUMS 873, BGUMS 465, BGUMS 806, BGUMS 1091, SK 07, SK 27, MFSK 01, MFSK 03, MFSK 04, MFSK 05, MFSK 08, MFSK 13, MFSK 23, MFSK 26, MFSK34, MFSK 35, MFSK 36, MFSK 38, MFSK 39, MFSK 41, MFSK 43, MFSK 46, MFSK 50, MFSK 52, MFSK 53, MFSK 55, MFSK60, MFSK 62, MFSK 77, TSK 01, TSK 03, TSK 06, TSK7, TSK 09, TSK10, TSK 11, TSK 12, TSK 13, TSK 15, TSK16, TSK 17, TSK 18, TSK19, TSK 20, TSK 21, TSK 22, TSK 32, TSK 71, RMSK 1, RMSK 2, RMSK 3	141

Source	Culture code	Total cultures
Saltpan water	RMSK 4, RMSK 5, RMSK 6, RMSK 7, RMSK 8, RMSK 9, RMSK 10, FSK 2.42, FSK 3.35, FSK 4.6, FSK 1.4, FSK 1.3, FSK 4.11, FSK 2.2, FSK 5.2, FSK 3.38, FSK 2.41, FSK 3.5, FSK 3.27, FSK 5.13, FSK 3.6, FSK 4.44, FSK 0.3	
Saltpan sediment	BGUMS 59, BGUMS 46, BGUMS 107, MFSK 09, MFSK15, MFSK 16, MFSK 62, MFSK 84, MFSK 81, RMSK 11, RMSK 12, RMSK 13, RMSK 14, RMSK 15, RMSK 16, RMSK 17, RMSK 18, RMSK 19, RMSK 20	19
Biofilm associated	ABSK 1, ABSK 2, ABSK 4, ABSK 5, ABSK 6, ABSK 8, ABSK 9, ABSK 11, ABSK 12, ABSK 13, ABSK 14, ABSK 15, ABSK 16, ABSK 17, ABSK 23, ABSK 31, ABSK 32, ABSK 33, ABSK 34, ABSK 35, ABSK 36, ABSK 37, ABSK 38, ABSK 39, ABSK 40, ABSK 41, ABSK 42, ABSK 43, ABSK 46, ABSK 48, ABSK 49, ABSK 50, ABSK 51, ABSK 52, ABSK 53, ABSK 54, ABSK 55, ABSK 56, ABSK 57, ABSK 58, ABSK 59 , ABSK 60, ABSK 61, ABSK 62, ABSK 63, ABSK 64, ABSK 66, ABSK 67, ABSK 71, ABSK 72, ABSK 75, ABSK 76, ABSK 78, ABSK 109, ABSK 186, ABSK 29, ABSK 9, ABSK 88, ABSK 89, ABSK 90, ABSK 91, ABSK 95, ABSK 97, ABSK 98, ABSK 99, ABSK 103, ABSK 106, ABSK 107, ABSK 108, ABSK 110, ABSK 112, ABSK 113, ABSK 114, ABSK 115, ABSK 116, ABSK 117, ABSK 118, ABSK 119, ABSK 120, ABSK 123,ABSK 124, ABSK 125, ABSK 127, ABSK 128, ABSK 129B, ABSK 129 C, ABSK 130, ABSK 131, ABSK 132, ABSK 133, ABSK 137, ABSK 138, ABSK 139, ABSK 140, ABSK 141, ABSK 142, ABSK 143, ABSK 144, ABSK 145, ABSK 146, ABSK 147, ABSK 152, ABSK 156, ABSK 159, ABSK161, ABSK 162, ABSK 164, ABSK 171, ABSK 172, ABSK 174, ABSK 182, ABSK 183, ABSK 184, ABSK 185, ABSK 186, ABSK 188, ABSK 192, ABSK 193, ABSK 194, ABSK 196	120
Actinomycetes	SBSK 4, SBSK 8, SBSK 83, SBSK 114, SBSK 115, SBSK 116, SBSK 201, SBSK 201, SBSK 203, SBSK 205, SBSK 285, SBSK 335, SBSK 351, SBSK 356, SBSK 359, SBSK 364, SBSK 392, SBSK 404, SBSK 431, SBSK 502, SBSK 569, SBSK 572, SBSK 576, SBSK 594, SBSK 600, SBSK 701, SBSK 702, SBSK 703, SBSK 704, SBSK 708	30

4.2 Primary Screening

4.2.1 Emulsification assay (*E24*)

Emulsification assay depends on the formation of a stable emulsion or 24 hr with the hydrocarbon. Out of 300 saltpan bacteria and actinomycetes screened for biosurfactant production, as seen in Fig. 1, 54 showed positive results for emulsification test out of which 42 were from saltpan water, 7 from sediment and 5 from biofilm associated bacteria. Plate 1 show a positive emulsification test by SK 27 which is representative bacteria showing a positive test. A positive emulsification assay was noted based on a white emulsified layer formed on the top of the transparent layer. The emulsification activity which was calculated by the E24 index was extrapolated based on the height of the emulsion formed with reference to the total height of the solution, where the height of the solution was measured from the top of the emulsified layer to the bottom centre of the test tube (since the test tube had a spherical bottom). The negative control exhibited a clear solution and the positive control with triton X-100 revealed an emulsification index which was lower than SK27.

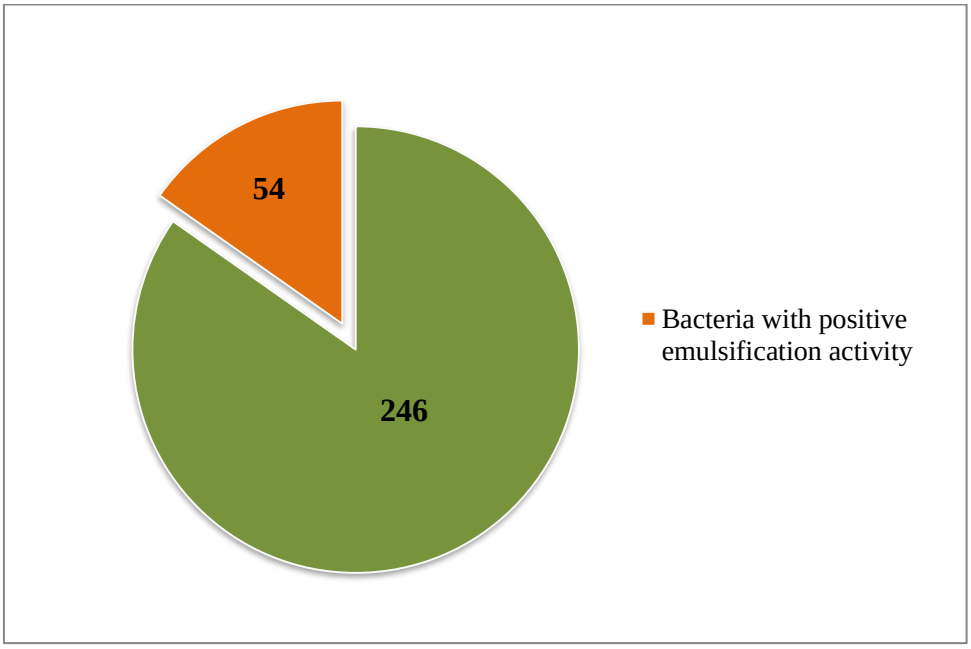

Fig. 1: Halotolerant isolates showing production biosurfactant by emulsification test

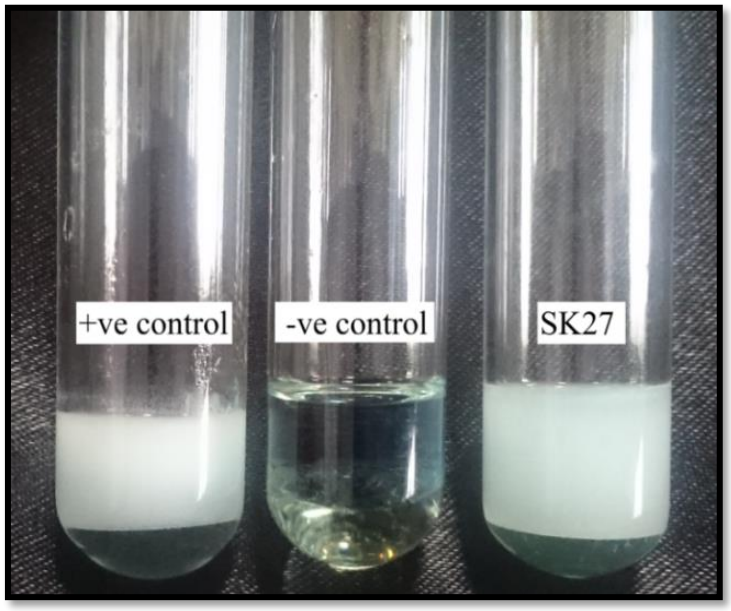

Plate 1: Production of biosurfactant by emulsification test with positive control and negative control

4.2.2 Drop collapse method

Drop collapse method relies on the destabilization of liquid droplets by surfactants (Walter et al., 2010). Since, this method consists of a hydrophobic surface, i.e. oil coated into the well. If the liquid contains surfactant, the drop spreads or collapses as the interfacial tension or force between the hydrophobic surface and the liquid drop is reduced (Plate 2). Out of 300 saltpan bacteria and actinomycetes screened for biosurfactant production, as seen in Fig. 2, 52 isolates showed positive results for drop collapse method out of which 38 were from saltpan water, 8 from sediment and 6 from biofilm associated bacteria. Plate 2 represents RMSK10 showing the production of biosurfactant indicated by increase in the diameter of the drop collapsed with positive and negative control. The RMSK10 revealed a higher drop diameter than that in the positive control.

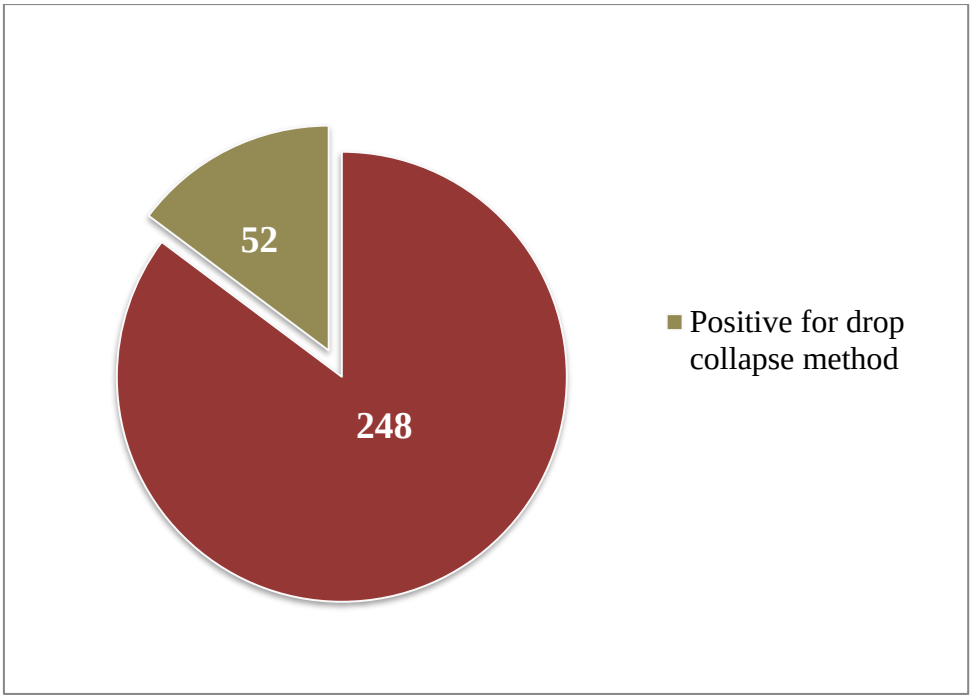

Fig. 2: Halotolerant isolates showing production of biosurfactant by Drop collapse method

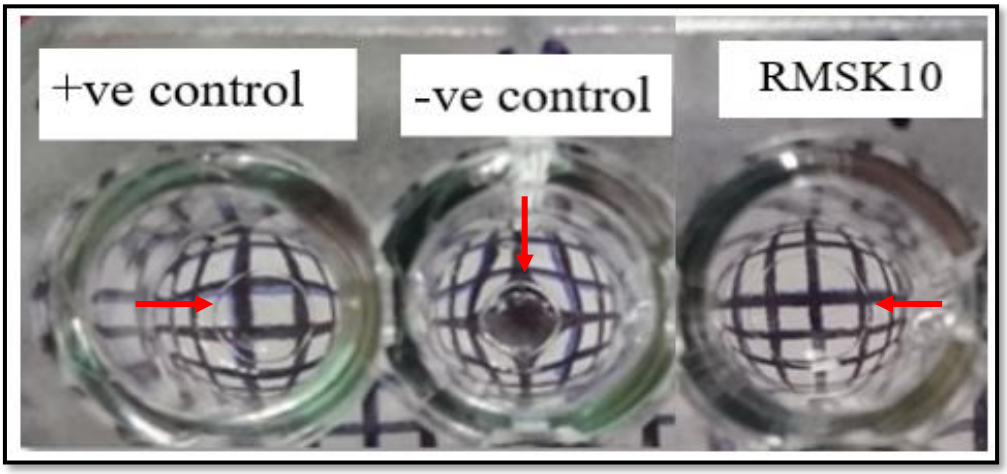

Plate 2: Production of biosurfactant by drop collapse method of RMSK 10 with positive control and negative control

4.2.3 Parafilm M test

Parafilm M test also depends on destabilization of liquid droplets by surfactants (Walter et al., 2010). This method consists of a hydrophobic surface similar to that in case of drop collapse method. The hydrophobic surface in this test is Parafilm M. Out of 300

saltpan bacteria and actinomycetes screened for biosurfactant production, as seen in Fig. 3, 48 cultures showed positive results for Parafilm M test out of which 36 were from saltpan water, 7 from sediment and 5 from biofilm associated bacteria. Plate 3 shows the parafilm M test carried out with ABSK 109 and the two controls. The plate shows that the cell free supernatant of ABSK 109 isolate revealed the presence of a biosurfactant due to the consistency in the triplicate tests and increase in the diameter of the drop when compared to the negative control.

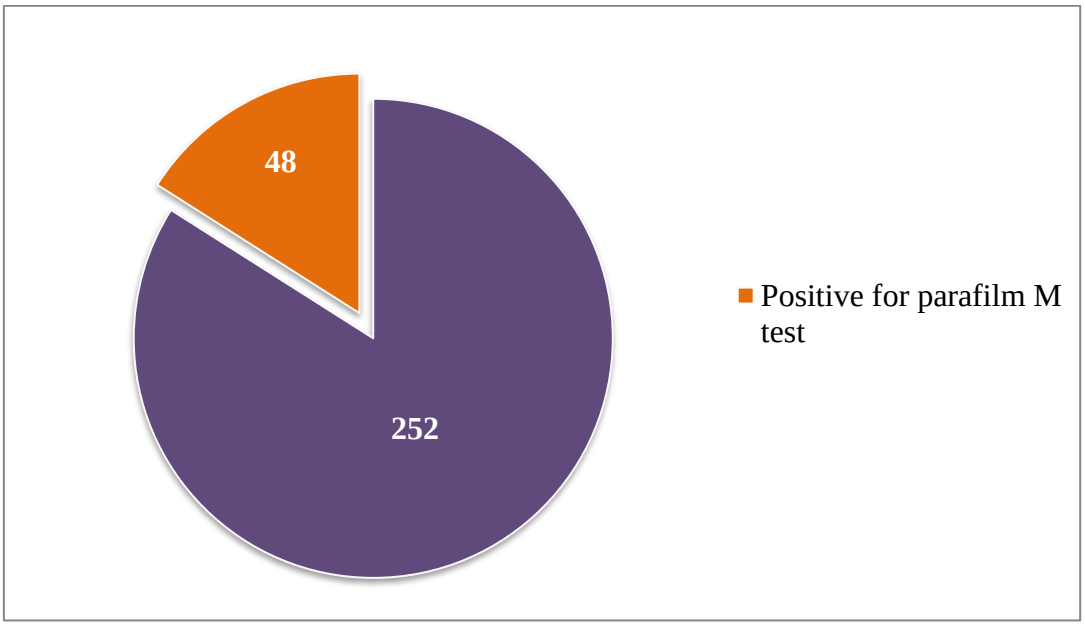

Fig. 3: Halotolerant isolates showing production biosurfactant by Parafilm M test

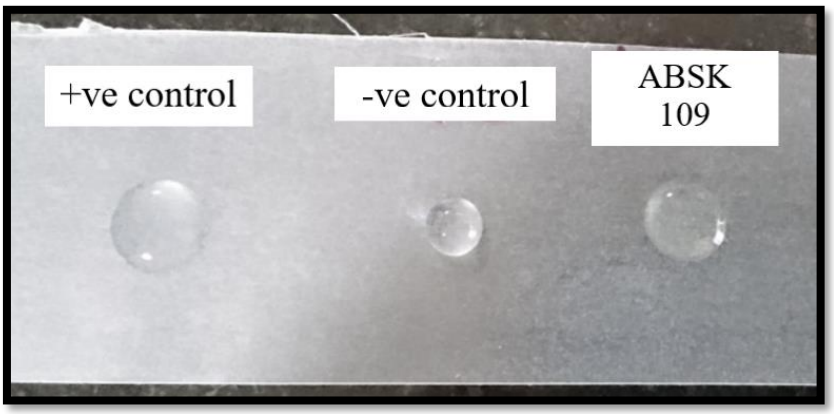

Plate 3: Production of biosurfactant by Parafilm M test of ABSK 109 with positive control and negative control

Amongst the 3 niches selected, bacterial isolates from the overlying saltpan water showed the highest biosurfactant production followed by sediment and biofilm associated bacteria. The actinomycetes isolates did not show biosurfactant production. Out of 300 cultures screened, 44 cultures showed positive results in all the three tests (Fig. 4). These cultures (44) were further subjected to secondary screening. Table 6 represents the halotolerant cultures showing activity in the all the three test.

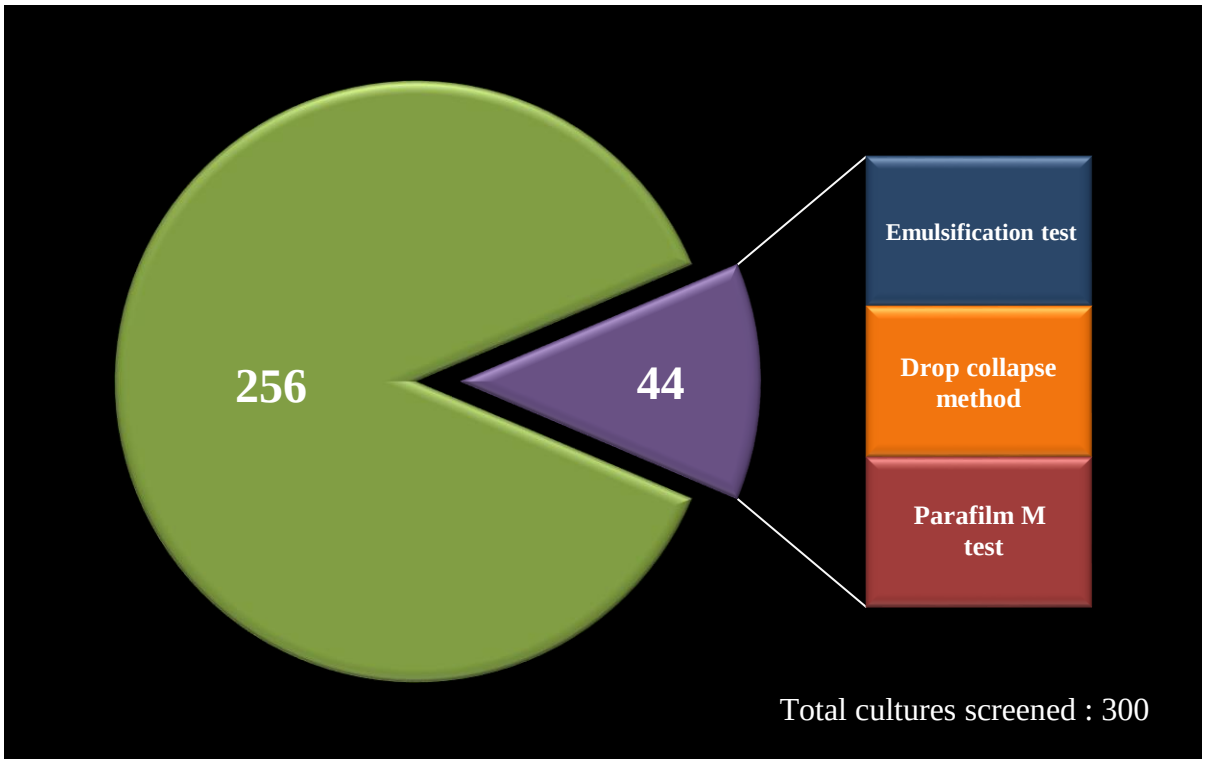

Fig. 4: Halotolerant isolates showing production of biosurfactant by Emulsification test, Drop collapse method and Parafilm M test

Table 6: Production of biosurfactant by Drop collapse method, Parafilm M test, Emulsification index

Sr. No.	Cultures	Drop collapse method	Parafilm M test	Emulsification index (%)
1.	SK 27	+++	+++	73.33
2.	BGUMS 93	++	++	20.00
3.	BGUMS 14 A	+++	+++	60.00
4.	BGUMS 14 B	+++	+++	73.33
5.	BGUMS 1091	+++	+++	60.00
6.	BGUMS 473 A	+	+	20.00
7.	BGUMS 473 C	+++	+++	20.00
8.	SK 07	+	+	13.00
9.	BGUMS 46	+	+	26.00
10.	BGUMS262	+	+	10.40
11.	BGUMS 256	+	+	13.00
12.	BGUMS 472	+++	+++	13.00
13.	BGUMS 457	+	+	13.44
14.	BGUMS 107	+	+	13.36
15.	BGUMS 370	+++	+++	16.00
16.	BGUMS 359	++	++	20.00
17.	BGUMS 315	++	++	33.33
18.	BGUMS 527	++	++	26.69
19.	BGUMS 265	+	+	33.33
20.	BGUMS 740	++	++	33.33
21.	BGUMS 109	+++	++	13.33
22.	RMSK 3	+	+	13.47
23.	RMSK 5	+	+	16.80
24.	RMSK 7	++	++	13.00
25.	RMSK 8	+	+	16.00
26.	RMSK 10	+++	+++	73.33
27.	RMSK 14	++	++	20.67
28. `	RMSK 19	++	++	13.33
29.	MFSK 09	+	+	10.66
30.	MFSK 16	+	+	10.66
31.	MFSK 53	+	+	14.00
32.	MFSK 46	+++	+++	40.00
33.	MFSK 50	+	+	13.33
34.	MFSK 34	+	+	15.77
35.	MFSK 55	+	+	13.00
36.	MFSK 26	+++	+++	26.66
37.	MFSK 62	+++	+++	60.00
38.	TSK 21	++	+++	26.66
39.	TSK 32	+	+	6.00
40.	TSK 11	+	+	13.33
41.	TSK 09	+	+	13.33
42.	TSK 06	+	+	6.00
43.	TSK 17	+	+	6.00
44.	ABSK 109	+++	+++	13.34

*Drop collapse assay: '+++'- drop size > 0.6 cm, '++'- drop size 0.6 cm and '+' - drop size > 0.4 cm of CFS addition.
*Parafilm M test: '+++'- drop size > 0.8 cm, '++'- drop size 0.8 cm and '+' - drop size 0.6 cm

4.3 Secondary screening

4.3.1. Oil spreading method

The 44 isolates were further tested by oil spreading method to ascertain their biosurfactant producing activity which was dependent on the oil clearing zone. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. Plate 4A shows the intact oil layer on a water surface and 4B shows a clearance zone after addition of cell free supernatant of SK27. This zone is proportional to the biosurfactant concentration. Based on the oil spreading method, cultures showed a zone diameter ranging from 2 cm to 4.2 cm (Table 7). Out of 44 cultures, the biosurfactant from SK27 and RMSK10 demonstrated the primary ability of oil displacement at 4.2 cm respectively. These isolates showed consistent activity in all the 4 screening methods i.e. maximum drop size, maximum oil clearing zone and highest emulsification index. SK27 and RMSK10 were therefore selected for further studies. The 2 isolates produced maximum yield of the biosurfactant as compared to the other isolates.

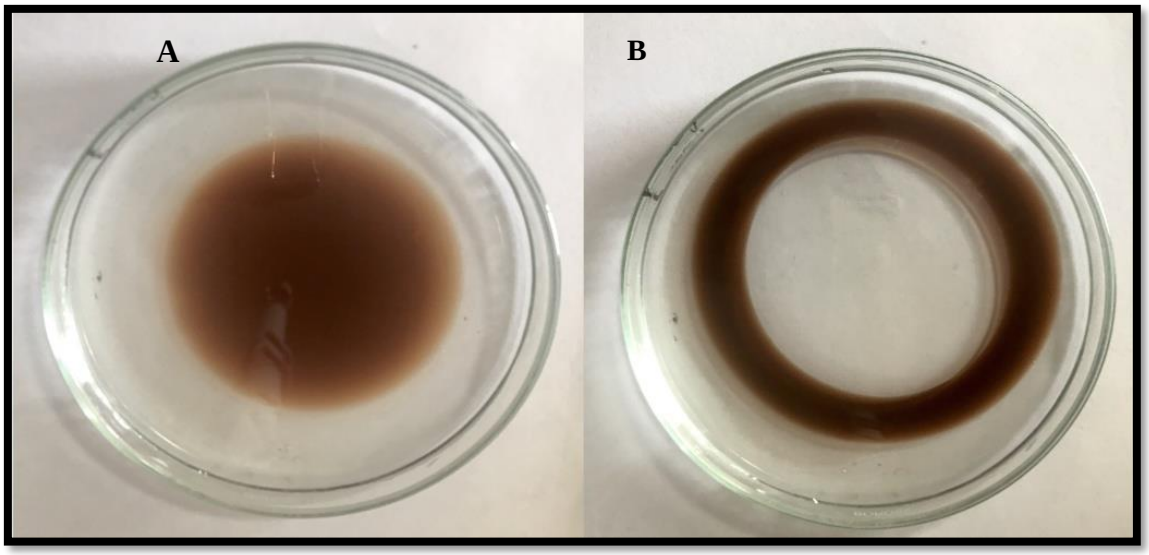

Plate 4: Production of biosurfactant by Oil spreading method **A.** Oil layer on the surface of the water **B.** Zone of clearance after addition of CFS

Table 7: Production of biosurfactant by oil spreading method

Sr. No.	Cultures	Oil clearing zone (cm)
1.	**SK 27**	**4.2 ± 0.1**
2.	BGUMS 93	2± 0.1
3.	BGUMS 14 A	3.2± 0.3
4.	BGUMS 14 B	2± 0.2
5.	BGUMS 799	2± 0.1
6.	BGUMS 473 A	2.1± 0.2
7.	BGUMS 473 C	3.3± 0.2
8.	BGUMS 1091	3.4± 0.2
9.	BGUMS 07	2± 0.3
10.	BGUMS 46	2± 0.1
11.	BGUMS262	2± 0.1
12.	BGUMS 256	2.4± 0.3
13.	BGUMS 472	2.3± 0.2
14.	BGUMS 457	2± 0.1
15.	BGUMS 107	2.4± 0.2
16.	BGUMS 370	2± 0.2
17.	BGUMS 359	3± 0.3
18.	BGUMS 315	2.5± 0.2
19.	BGUMS 527	3.2± 0.1
20.	BGUMS 265	2± 0.1
21.	BGUMS 740	2± 0.1
22.	BGUMS 109	2± 0.2
23.	RMSK 3	2± 0.2
24.	RMSK 7	2± 0.3
25.	RMSK 8	2.1± 0.1
26.	**RMSK 10**	**4.2± 0.1**
27.	RMSK 14	2± 0.2
28.	RMSK 19	2.2± 0.3
29.	MFSK 09	2± 0.1
30.	MFSK 16	2± 0.1
31.	MFSK 53	2± 0.1
32.	MFSK 46	3.4± 0.2
33.	MFSK 50	2.3± 0.1
34.	MFSK 34	2± 0.2
35.	MFSK 55	2.4± 0.2
36.	MFSK 26	3.3± 0.3
37.	MFSK 62	3.2± 0.2
38.	TSK 21	3.1± 0.2
39.	TSK 32	2.5± 0.2
40.	TSK 11	3.3± 0.2
41.	TSK 09	3.1± 0.2
42.	TSK 06	3.0± 0.2
43.	TSK 17	2± 0.2
44.	ABSK 109	2.4± 0.2

4.4 Optimization of Media for production of biosurfactant

4.4.1 One factor at a time method (OFAT)

4.4.1.1 pH optimization for maximum biosurfactant production

Fig 5 shows the effect of pH on the biosurfactant production from SK27 and RMSK10. The results revealed that biosurfactant production in SK27 gradually increased from pH 5.5 to 6.5 and maximal production occurred at pH 6.5. Increase in pH beyond pH 6.5 to 7.5 decreased the concentration of the biosurfactant produced. The biosurfactant production in RMSK10 gradually increased from pH 6 to 7 and maximum production observed at pH 7. A further increase from pH 7 to 7.5 decreased the concentration of the biosurfactant produced. Thus pH 6.5 and pH 7 was selected as the optimum pH for fermentation of SK27 and RMSK10 respectively.

4.4.1.2 Temperature optimization for maximum biosurfactant production

Results showed that the biosurfactant production from SK27 and RMSK10 gradually increased from 20°C to 30°C and maximal production occurred at 30°C. Increase in temperature beyond 30°C to 50°C decreased the concentration of the biosurfactant produced. Fig 6 shows the effect of temperature on the biosurfactant produced by SK27 and RMSK10 which exhibited a uniform trend though SK27 was from Ribandar saltern and RMSK10 was from Agarwado saltern. Thus 30 °C was selected as the optimum temperature for fermentation of these bacteria.

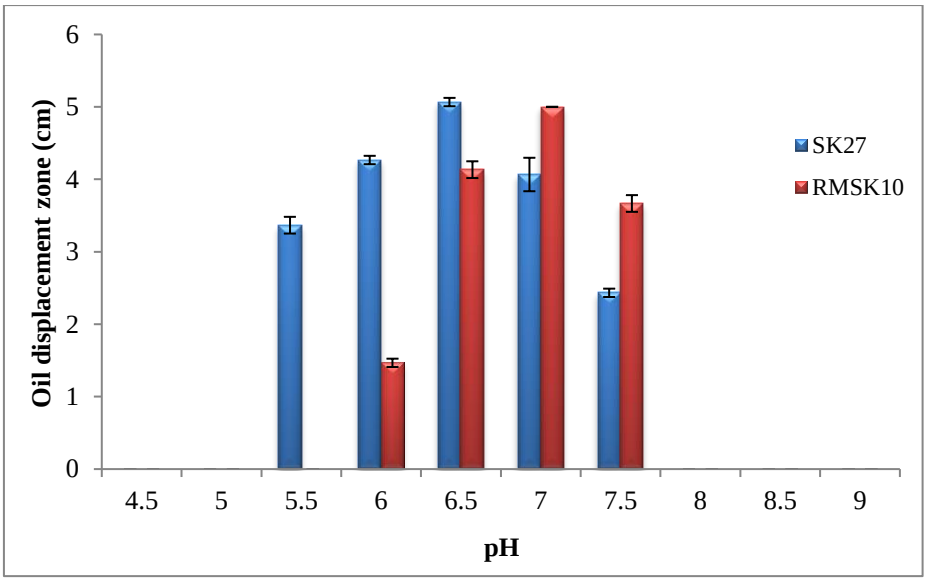

Fig.5: Effect of pH on production of biosurfactant by SK27 and RMSK10

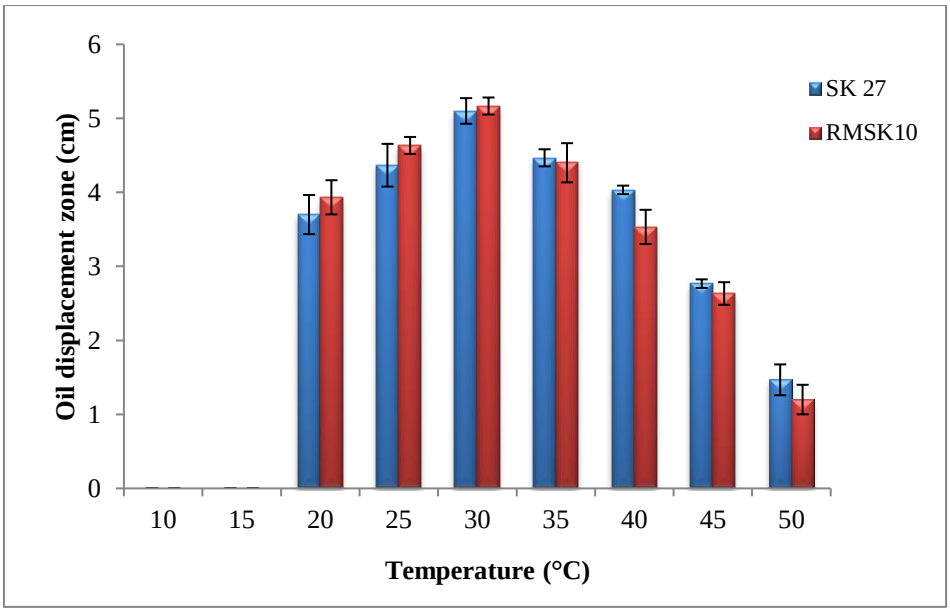

Fig.6: Effect of Temperature on production of biosurfactant by SK27 and RMSK10

4.4.1.3 Carbon source optimization for biosurfactant production

Various carbon sources such as glucose, glycerol, fructose, sodium citrate, mannitol, starch, mannose, lactose, paraffin and sucrose were used to evaluate the biosurfactant production by SK27 and RMSK10 in shaker condition. Results (Fig. 7) indicate that for SK27 the maximum oil displacement activity was seen when media was supplemented with sucrose as a carbon source followed by mannitol, mannose and glucose. On the other hand, for RMSK10 the maximum oil displacement activity was seen when media was supplemented with soluble starch as a carbon source followed by mannose, glucose and sucrose (Fig.8).

4.4.1.4 Nitrogen source optimization for biosurfactant production

The strains SK27 and RMSK10 were able to grow and produce biosurfactant with all the nitrogen sources with sucrose and starch as a carbon source respectively (Fig 9). However, maximum oil displacement activity for SK27 was observed when the media was supplemented with yeast extract as a nitrogen source followed by tryptone, beef extract and peptone. While for RMSK10 maximum oil displacement activity was with peptone as a nitrogen source followed by yeast extract and meat extract (Fig. 10). Thus, yeast extract and peptone was the preferred nitrogen source for SK27 and RMSK10 respectively.

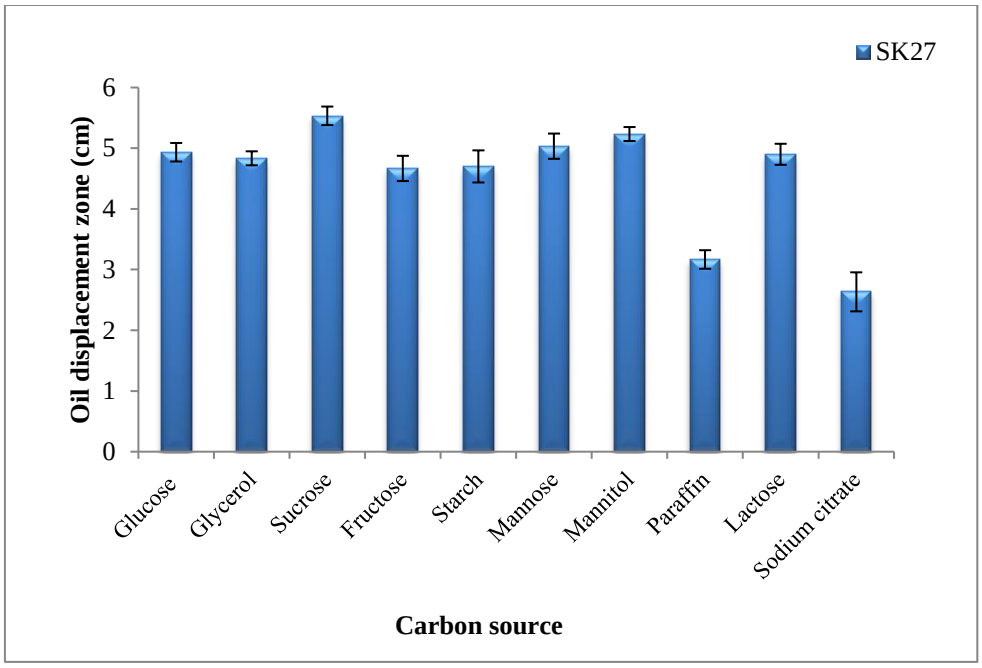

Fig.7: Production of biosurfactant by SK27 in different carbon sources

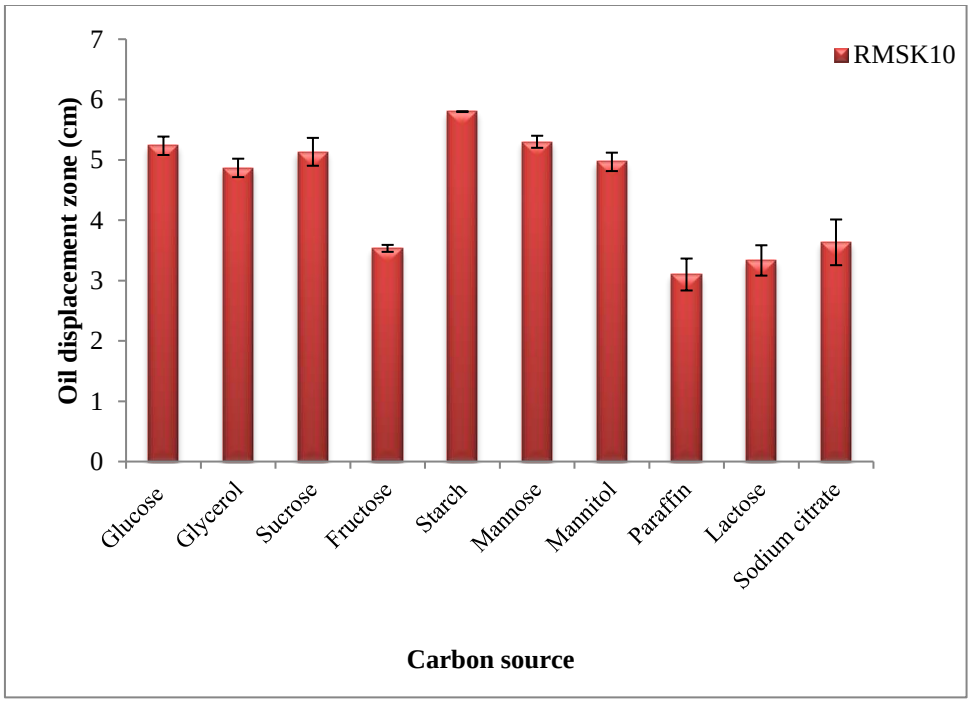

Fig.8: Production of biosurfactant by RMSK10 in different carbon sources

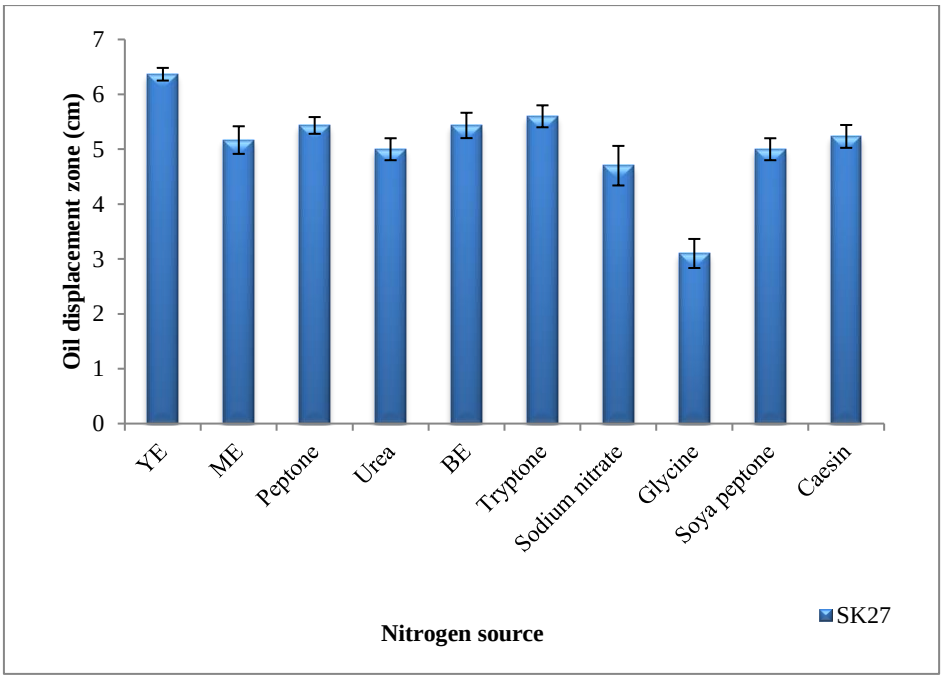

Fig.9: Production of biosurfactant by SK27 in different nitrogen sources

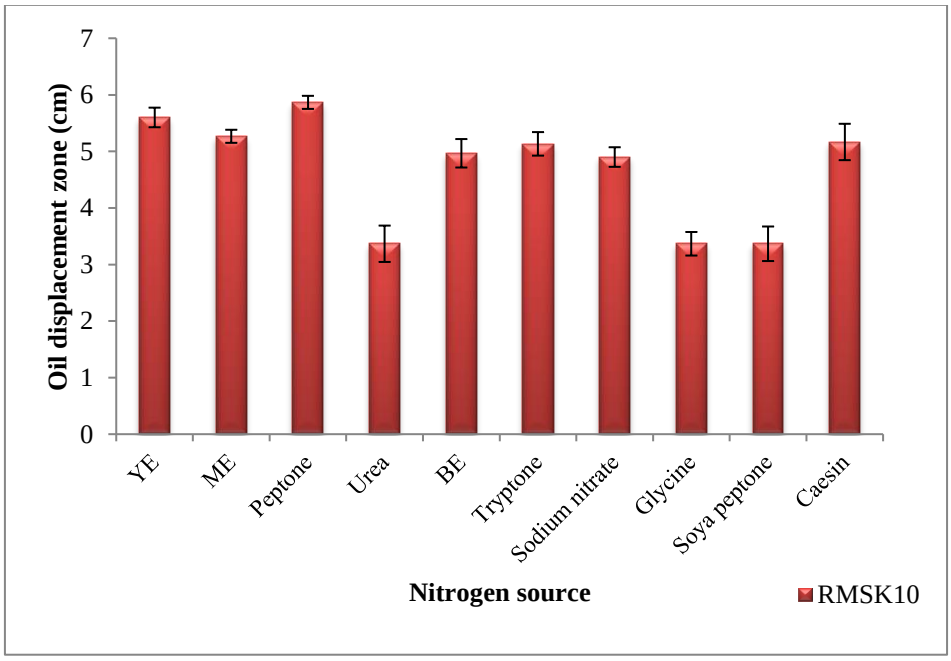

Fig.10: Production of biosurfactant by RMSK10 in different nitrogen sources

4.4.1.5 Carbon source concentration

As indicated in Fig. 11, the biosurfactant production (SK27) varied with the sucrose concentration. Of all the tested concentrations, 1% of sucrose showed maximum oil displacement activity which was also directly proportional to the biosurfactant production. As the concentration of sucrose was increased above 1%, a gradual decrease in the biosurfactant production was observed. Biosurfactant production by RMSK10 was also studied by changing the concentration of the respective carbon source (starch) in the production medium which showed a variation with change in the starch concentration. Maximum oil displacement activity was observed with 1% starch in the medium. However, the activity did not increase with further increase in starch concentration (Fig. 12).

4.4.1.6. Nitrogen source concentration

Further the optimum concentration of the nitrogen source, i.e. yeast extract for SK27 and peptone for RMSK10 was also checked by changing the concentration of yeast extract and peptone in the production medium. It varied with the respective concentration of the nitrogen source concentration. The optimum concentration of yeast extract for SK27 was found to be 0.8% and the optimum concentration of peptone for RMSK10 was found to be 1%. The activity gradually decreased as the concentration of yeast extract and peptone was increased above this concentration (Fig.13 and 14).

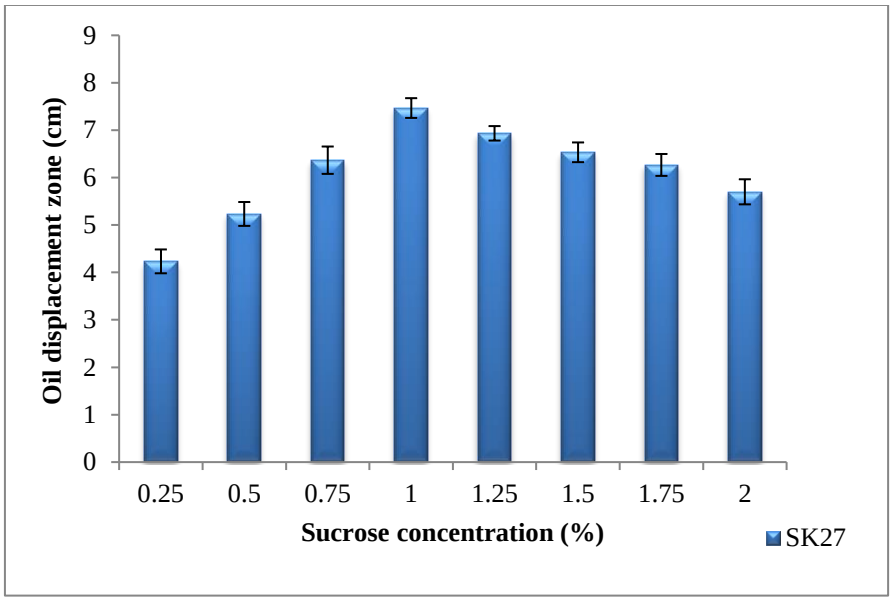

Fig.11: Production of biosurfactant at different concentration of sucrose

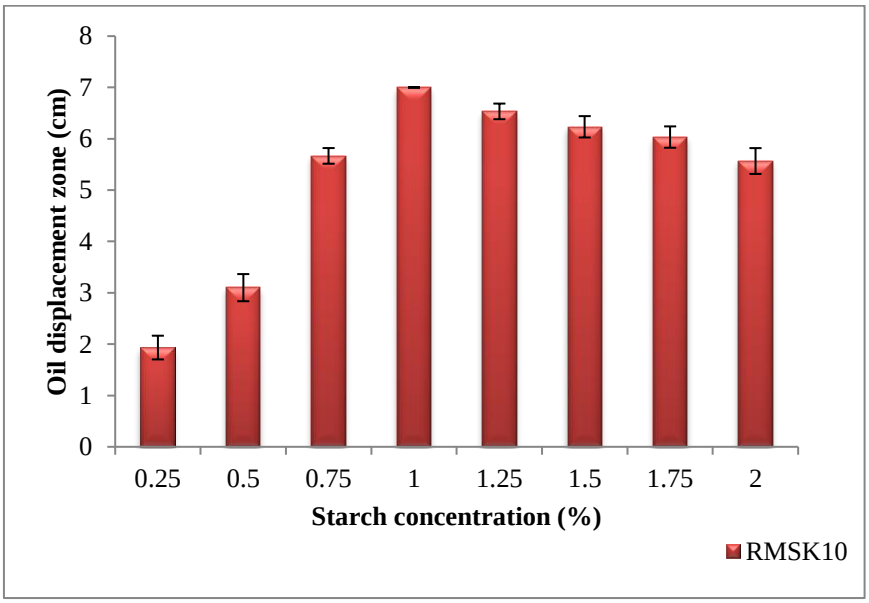

Fig.12: Production of biosurfactant at different concentration of starch

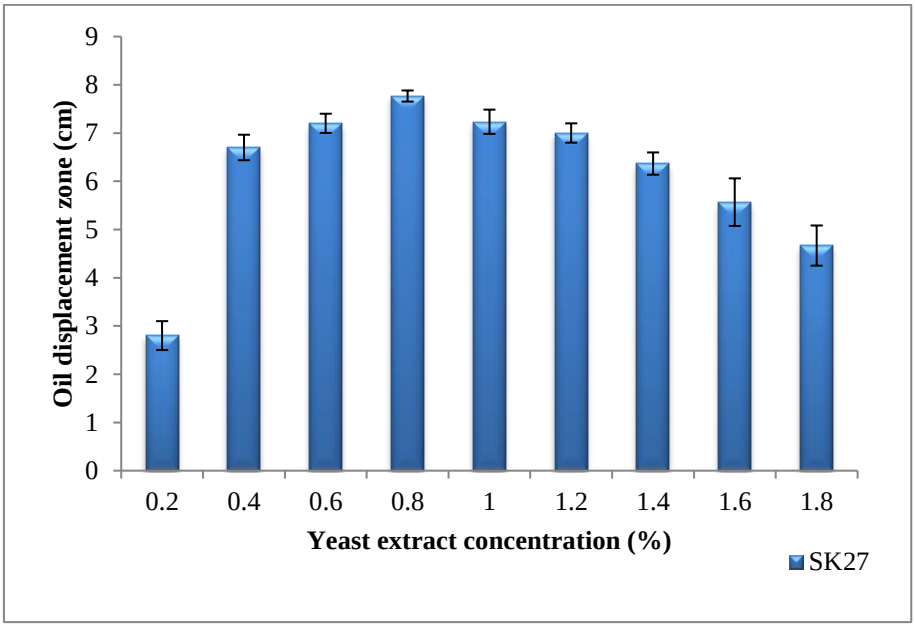

Fig.13: Production of biosurfactant at different concentration of yeast extract

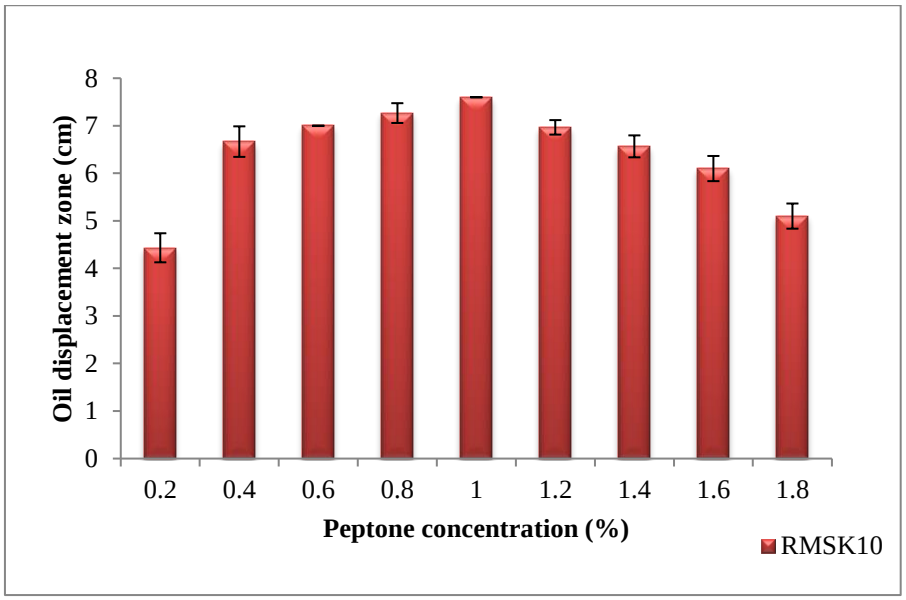

Fig.14: Production of biosurfactant at different concentration of peptone

4.4.1.7 NaCl optimization for biosurfactant production

The production of the biosurfactant was examined by supplementing varied concentrations of NaCl in the production medium. The optimum NaCl concentration for biosurfactant production was found to be 0.6% for both SK27 and RMSK10. As the concentration of NaCl was increased above this concentration, there was a decrease in the biosurfactant production (Fig 15). When the concentration of NaCl was decreased to 0.4 %, there was a slight decrease in the biosurfactant produced however at 0.2% NaCl, the biosurfactant production was reduced to the half the volume which was deduced by the oil displacement zone as seen in Fig 15. When the medium was devoid of NaCl there was no biosurfactant production.

4.4.1.8 Growth profile and production of the biosurfactant

SK27 and RMSK10 exhibited a sigmoidal curve of growth, with each culture having a distinct lag and log phase. Culture SK27, showed biosurfactant production within 20 – 48 h. The highest biosurfactant production was observed at 24 h during early stationary phase (Fig.16a) whereas RMSK 10, exhibited biosurfactant production from 24 – 48 h, with the highest biosurfactant production at 28 h during stationary phase (Fig.16b).

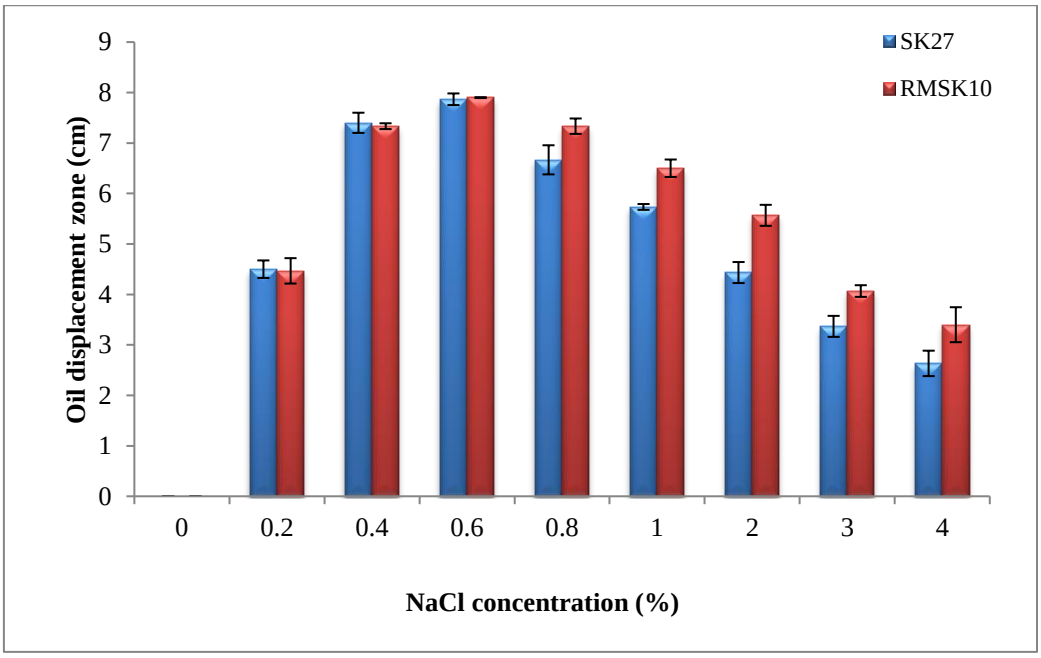

Fig.15: Production of biosurfactant at different NaCl gradients

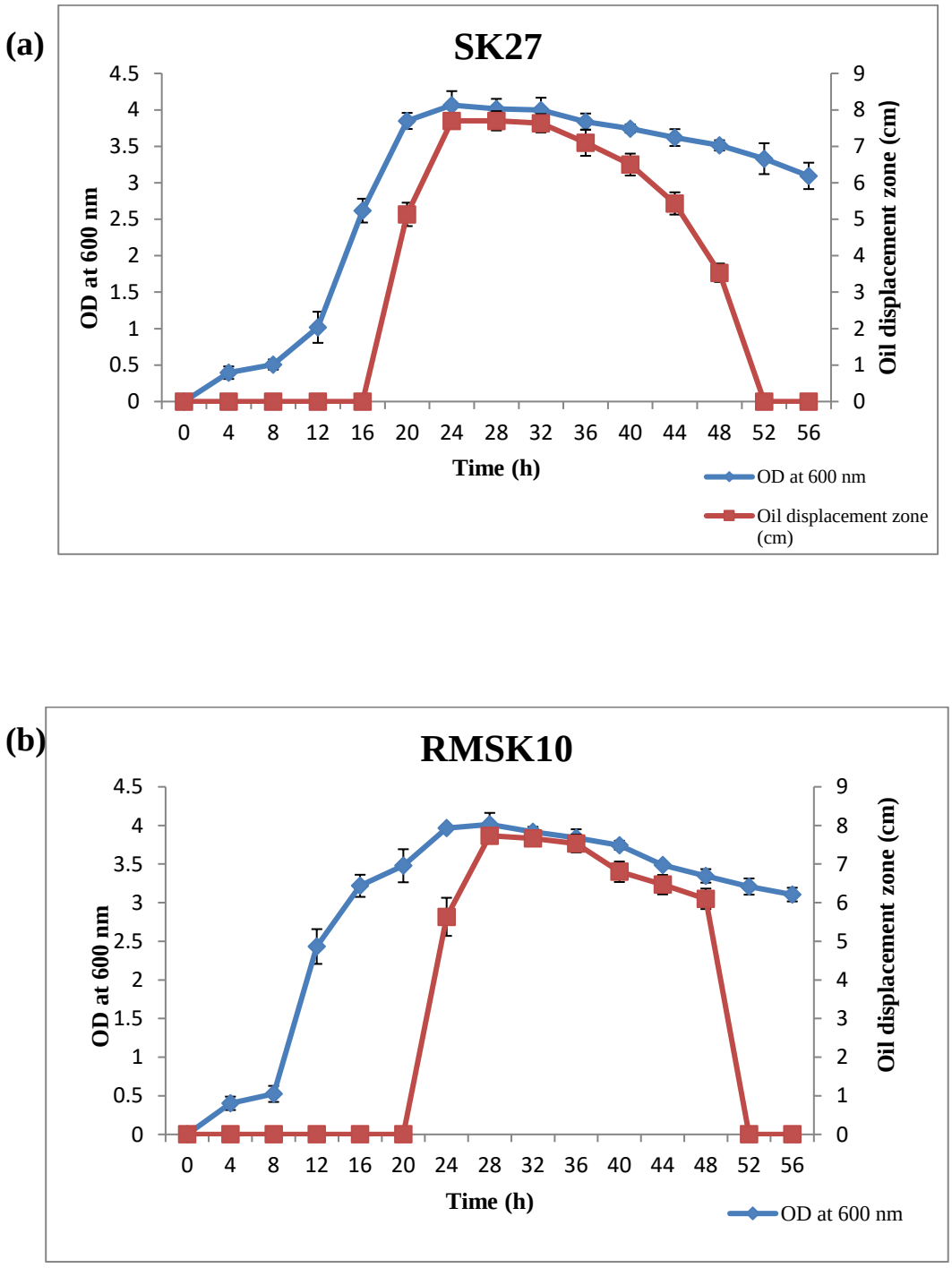

Fig. 16: Growth profile and biosurfactant production of (a) SK27 (b) RMSK10

4.4.2 Response surface methodology and Genetic algorithm (RSM-GA)

4.4.2.1 SK27

The OFAT approach of enhancing production media and physical conditions was effectively applied for the production of biosurfactant by SK27 by which there was 1.85-fold increase in biosurfactant production expressed as oil displacement activity. The oil displacement activity increased from 4.2 to 7.8 cm.

The central composite design (CCD) was applied to the most influencing media components selected from the OFAT experimental observation. The composition of the variables used for optimization of the medium for SK27 were: sucrose (1%), Yeast extract (0.8%), sodium chloride (0.6%), pH 6.5; temperature 30°C. Based on the identification of the effective variables by one variable approach, a central composite design was developed for the variables affecting the maximum production of the biosurfactant by SK27 the most. The experimental results of the biosurfactant production by three factor-two level factorial experiment design with six replications of central point and fourteen axial points are shown in Table 8.

In order to determine the maximum oil displacement activity corresponding to the optimum levels of sucrose, yeast extract and sodium chloride in the culture media, a second order polynomial model was proposed to calculate the optimum levels of these variables. The production of biosurfactant was best predicted by the model generated by applying multiple regression analysis which explains the role of each variable and their interactions:

$$Y = +8.79 - 0.0540*A - 0.016*B - 0.014*C - 0.00225*A*B - 0.0025*A*C - 0.0525*B*C -0.6064*A^2 - 0.5564* B^2 - 0.4064*C^2 \quad (1)$$

where Y, biosurfactant activity measured by oil clearing zone (cm); A: Sucrose concentration (%); B: Yeast extract concentration (%); C: NaCl concentration (%). The quadratic model in equation (1) has 9 terms contains three linear terms, three quadratic terms and three two factorial interactions.

ANOVA results revealed that the model is highly significant with an F – value of 661.63 and probability value of <0.0001 (Table 9). The Fisher's F-test with a very low probability value (<0.0001) and Model F-value of 661.63 implies that the model is

significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of P > F less than 0.05 indicates model terms are also significant (Table 10). The "Lack of Fit F-value" of 0.0153 implies the Lack of Fit is insignificant relative to the pure error. Non-significant lack of fit is good, as we want the model to fit. This implies that the model is adequate for prediction within the range of variables employed.

The coefficient of determination (R^2) was calculated to be 0.9983, to examine the fitness of the model for maximum biosurfactant production.The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1.00, the stronger the model is and the better it predicts the response . When expressed as a percentage, R^2 is interpreted as the percent variability in the response explained by the statistical model. This model implies that the sample variation of 99% for biosurfcatnt production was attributed to the independent variables and only 1% of the total variation was not explained by the model. This ensured a satisfactory adjustment of the quadratic model to the experimental data. The adjusted determination coefficient (Adj R^2= 0.9968) and predicted determination coefficient (R^2=0.9680) was also satisfactory to confirm the significance of the model. Adjusted Precision, measures the signal to noise ratio, ratio >4 being desirable.The ratio of 62.501 indicates an adequate signal and therefore the model is significant for the process. A low value of the coefficient of variation (CV= 0.4889%) demonstrated that the experiment conducted was precise and reliable.

By substituting the equation representing the model, values are predicted for each run. A linear graph of predicted and actual values thus proves that the model can correctly represent the experiment. The normal plot of residuals is important diagnostic for the model. A linear pattern demonstrated that there are no signs of any problems in the data (Fig. 17).

Table 8: Central composite design of experimental design for SK27

Run	Sucrose (%)	Yeast extract (%)	NaCl	Oil displacement zone (cm)
1	1	0.9	0.6	8.8
2	0.5	0.9	0.6	8.8
3	1.5	1.2	0.6	8.2
4	1	1.2	1	7.1
5	1	0.9	0.6	8.2
6	1	0.6	1	8.36
7	1	0.9	0.6	8.26
8	0.5	0.6	0.6	8.8
9	1	0.9	1	7.36
10	1	0.9	0.2	8.4
11	1.5	0.9	0.6	8.8
12	1.5	1.2	0.6	8.16
13	1	0.9	0.2	7.16
14	0.5	1.2	0.6	8.8
15	0.5	1.2	0.2	7.4
16	1.5	0.6	1	7.2
17	1	0.9	0.2	7.2
18	1.5	0.6	0.6	8.8
19	0.5	0.6	1	7.2
20	1	0.9	0.2	7.2

Table 9: ANOVA for full quadratic model

Source	Sum of squares	Mean square	DF	F-value	P>F
Model	9.13	1.01	9	661.63	< 0.0001
Residual	0.0153	0.0015	10		
Lack of Fit	0.0153	0.0031	5		
Pure error	0.0000	0.0000	5		
Total	9.15		19		

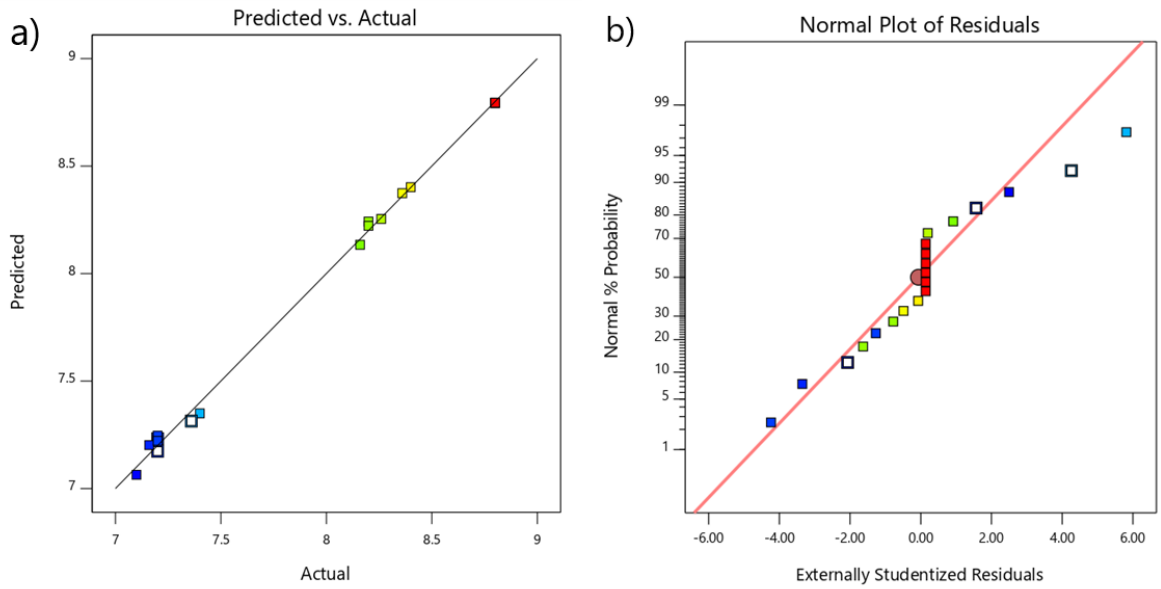

Fig.17: a) Plot of Predicted values verses actual values **b)** Normal plot of residuals

Another method in RSM which gives a potential relationship between the variables is the three-dimensional (3D) surface plots and two dimensional (2D) contour plots. The 3D response surface curve plot are a graphical representation of the regression equation (Fig.18-20). Each figure represents the effect of two variables, while the third being at zero level for maximum biosurfactant production and each contour curve in the graph represents a number of combinations of two influencing variables with the other being constant; with maximum predicted value confined in the smallest ellipse. Fig 18a. shows the effect of sucrose and yeast extract on the production of biosurfactant keeping NaCl concentration constant at the middle level. When yeast extract concentration was kept at low concentration and the sucrose concentration was increased, the activity increased upto a point and a further increase in the sucrose concentration, the activity decreased. A similar trend was observed vice versa and also in Fig. 19 and Fig. 20.

Perturbation graph compares the effect of every factor on a particular point using space design, suggesting that all three variables show significant mutual interaction with each other (Fig. 21).

4.4.2.1.1 Validation of the model

The optimal combination of the major media constituents for biosurfactant production as evaluated from the response surface and contour plots was Sucrose 0.97 %, yeast extract 0.89 % and NaCl 0.59 %. The model predicted optimum activity to be 8.79 cm using the above mentioned optimum concentrations of the variables. The verification of the results was carried out by shake flask method using optimum concentrations of each parameter predicted by the model, and the maximum zone was found to be 8.8 cm, close to the predicted value. A 2.1-fold increase in yield was observed after optimization with RSM as compared to the un-optimized medium, thereby proving the effectiveness of the model in media optimization.

4.4.2.1.2 Genetic algorithm

The non-linear equation (1) of oil displacement activity was used as fitness function for optimization using GA tools. The fitness of individual population is represented in Fig. 22. The oil displacement zone was found to be 8.76 cm under optimal process conditions, close to the predicted RSM activity.

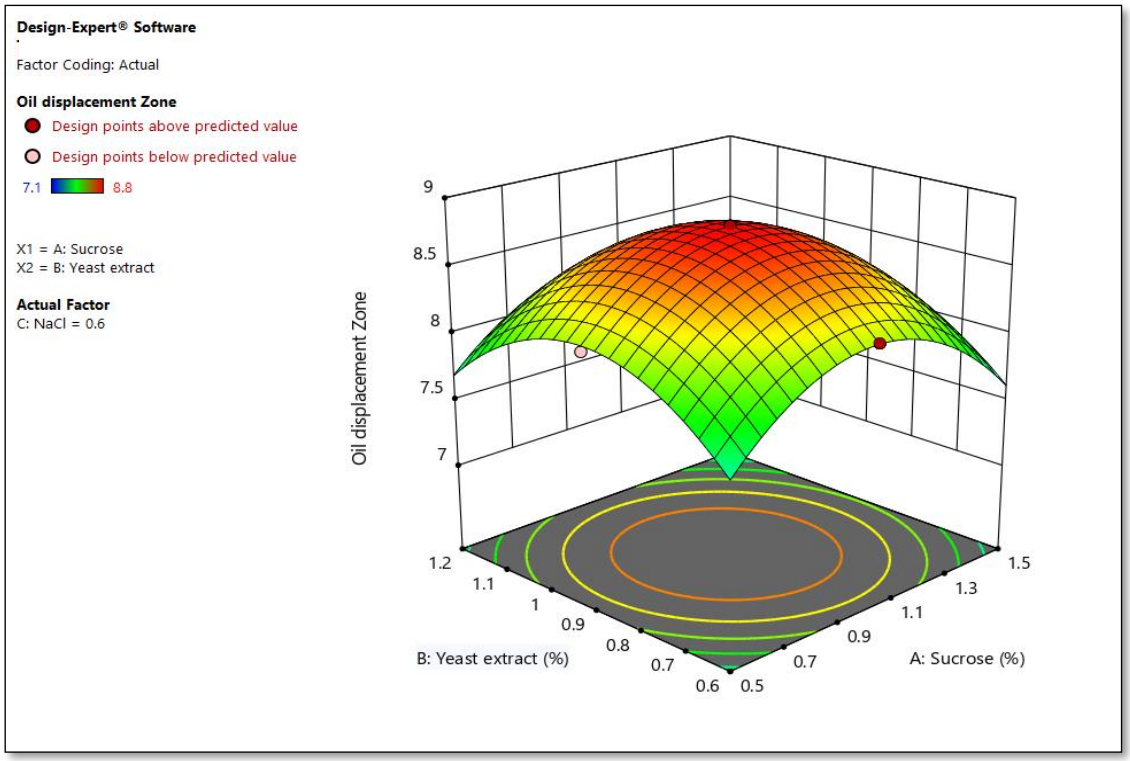

Fig. 18a: Surface plot showing effect of sucrose and yeast extract on biosurfactant production; keeping NaCl constant.

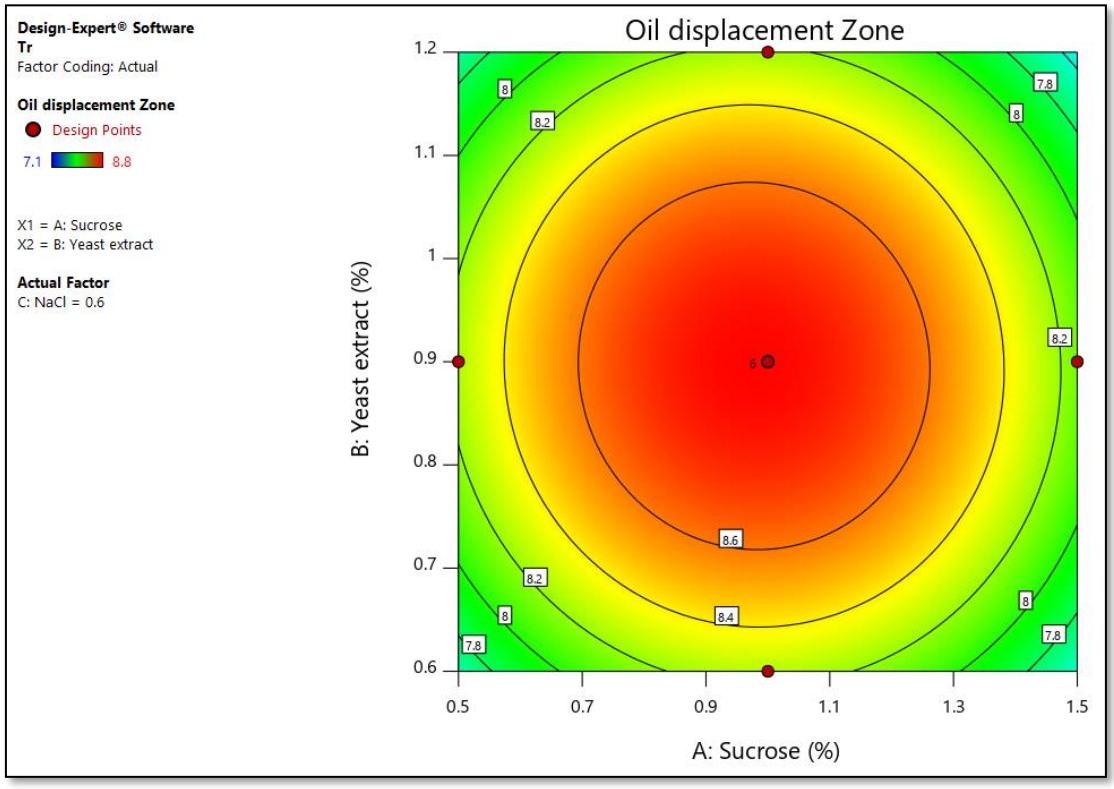

Fig. 18b: Contour plot showing effect of sucrose and yeast extract on biosurfactant production; keeping NaCl constant.

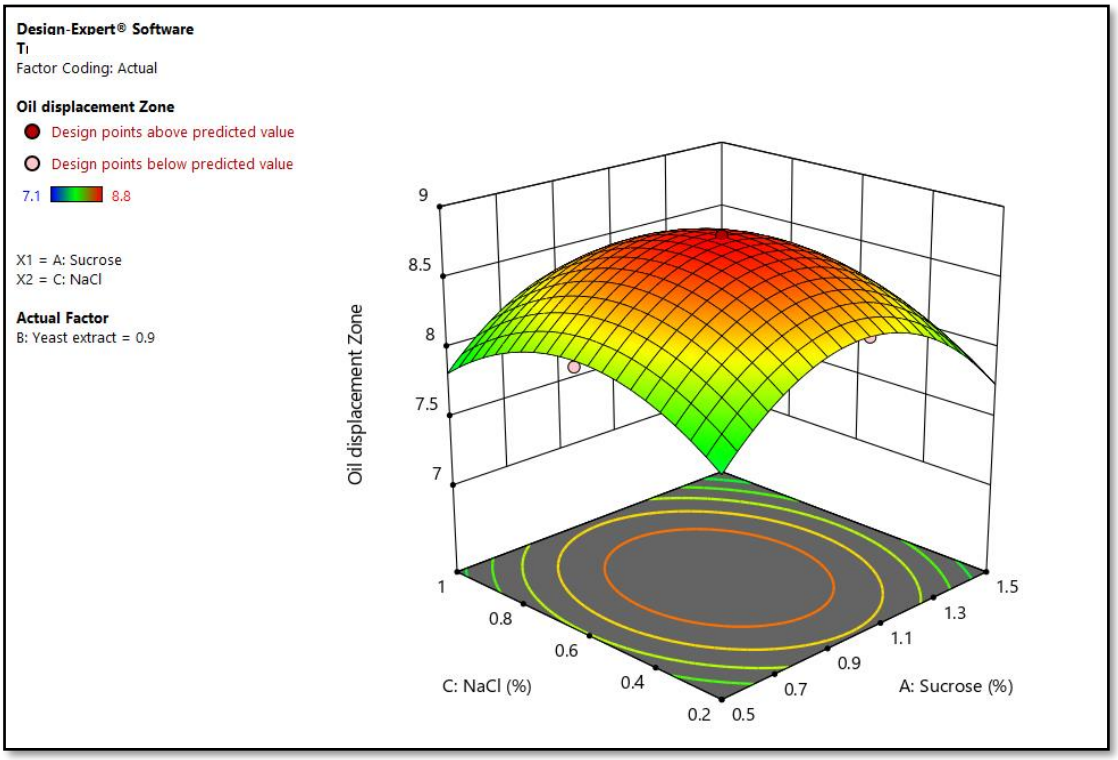

Fig. 19a: Surface plot showing effect of sucrose and NaCl on biosurfactant production; keeping yeast extract constant.

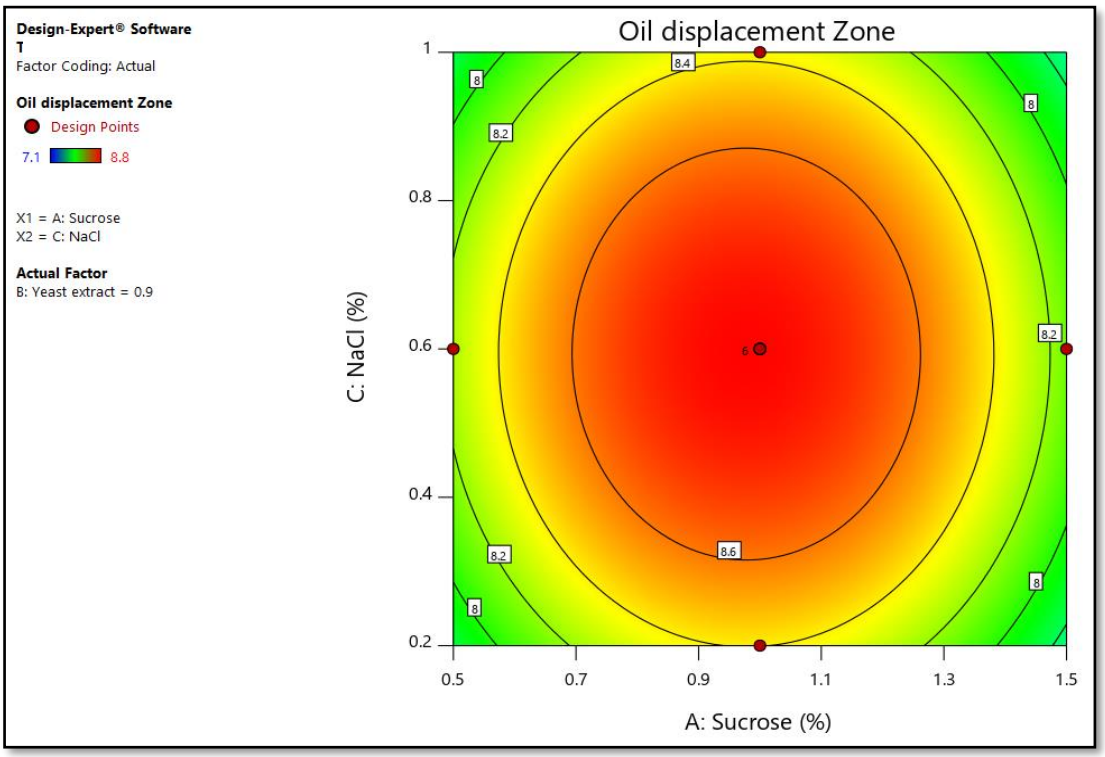

Fig. 19b: Contour plot showing effect of sucrose and NaCl on biosurfactant production; keeping yeast extract constant.

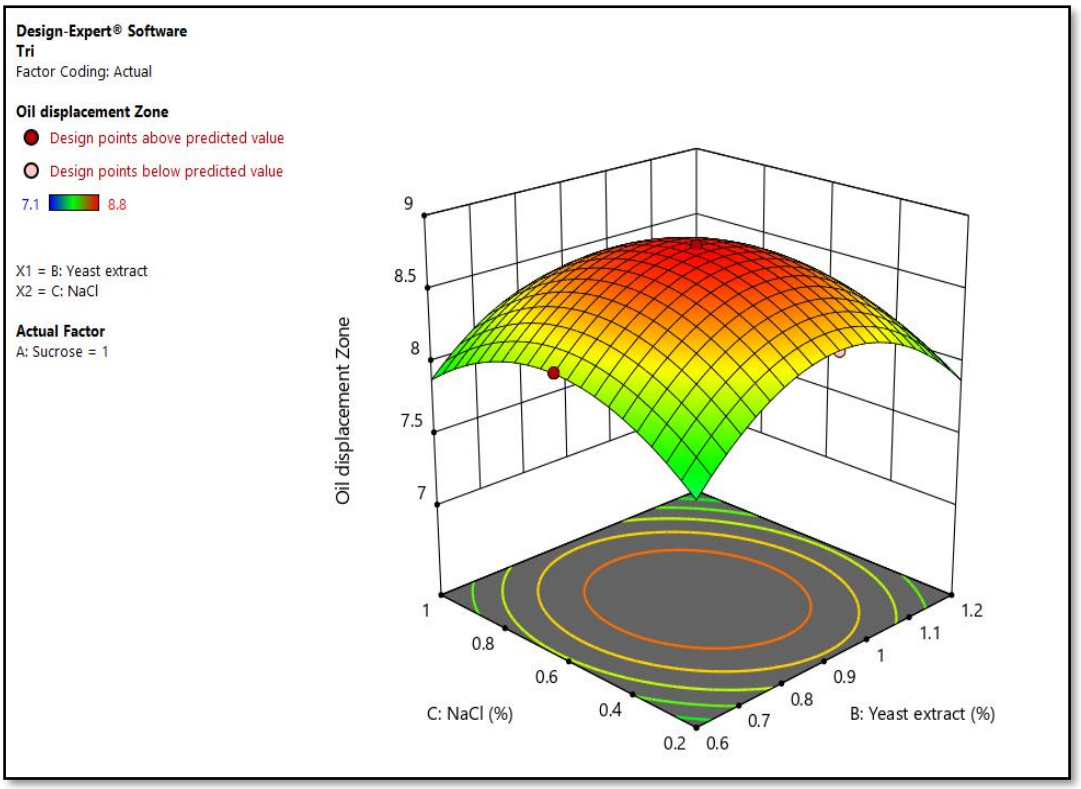

Fig. 20a: Surface plot showing effect of yeast extract and NaCl on biosurfactant production; keeping sucrose constant.

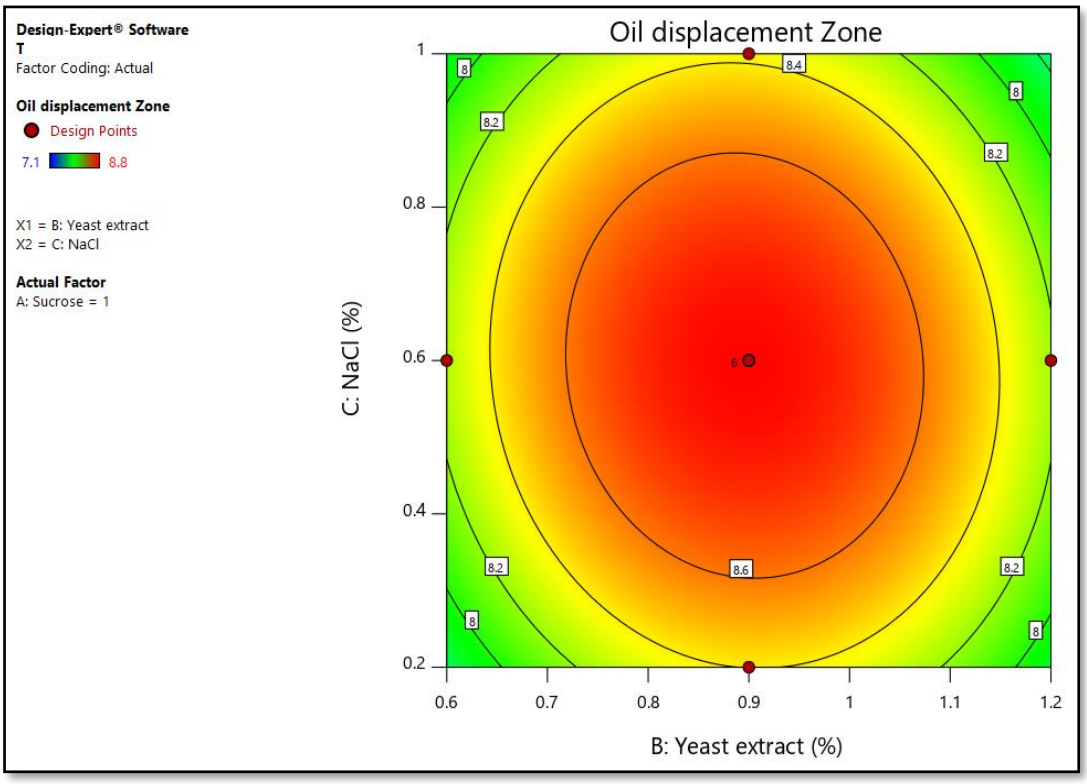

Fig. 20b: Contour plot showing effect of yeast extract and NaCl on biosurfactant production; keeping sucrose constant.

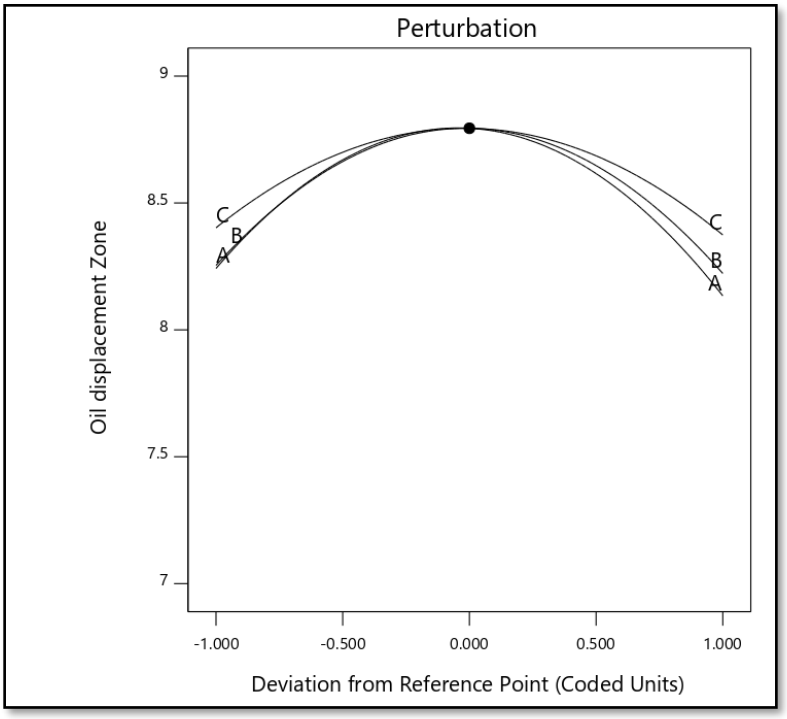

Fig.21: Pertubation graph showing effect of each of the independent variables on biosurfactant production

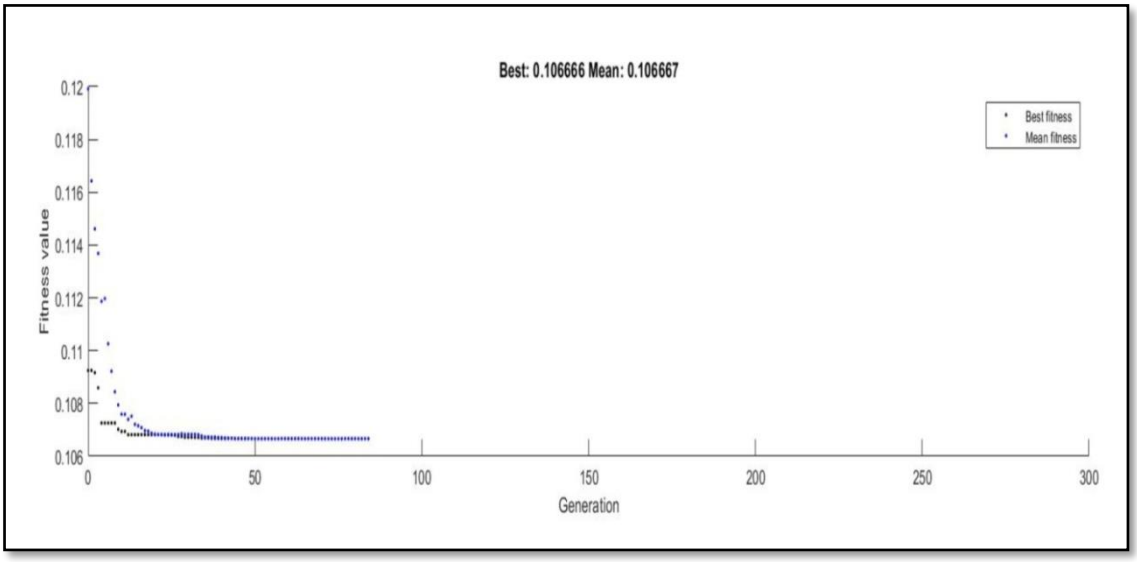

Fig. 22: Fitness of the individual population

4.4.2.2 RMSK10

Based on the identification of the effective variables, a central composite design was developed for the components affecting the production of the biosurfactant i.e. carbon source: Starch (A), nitrogen source: Peptone (B) and sodium chloride (C).

The experimental results of the biosurfactant production by three factor-two level factorial experiment design with six replications of central point and fourteen axial points are shown in Table 10.

Response surface methodology (RSM) suggested a quadratic model to predict the responses by applying multiple regression analysis.

$$Y = +8.69 +0.1260*B + 0.0760*C + 0.0075*AB - 0.1675*AC + 0.1575*BC - 0.5273*A^2 -0.6773*B^2 - 0.5273*C^2 \quad (1)$$

where Y, biosurfactant activity measured by oil clearing zone (cm); A: Starch concentration (%); B: Peptone concentration (%); C: NaCl concentration (%).

The ANOVA results revealed that the model is highly significant with an F value of 76.34 and probability value of <0.0001(Table 11). Further, the coefficient of determination (R^2) was calculated to be 0.9857, to examine the fitness of the model. The R^2 value was closer to 1.00 which suggest that the model is stronger and the better to predict the response.The adjusted determination coefficient (Adj R^2= 0.9727) and predicted determination coefficient (R^2=0.9183) was also satisfactory to confirm the significance of the model. Adjusted precision assess the signal to a noise ratio, a ratio of 23.154 shows an satisfactory signal and therefore the model is significant for the process. A low value of coefficient of variation (CV= 1.68%) demonstrated that the experiment conducted was precise and reliable.

Table 10: Central composite design of experimental design for RMSK10

Run	Starch (%)	Peptone (%)	NaCl (%)	Oil displacement zone (cm)
1	0.5	0.4	1	6.8
2	1	0.9	0.6	8.76
3	0.5	1.4	0.2	6.5
4	1	0.9	0.6	8.76
5	1	1.4	0.6	8
6	0.5	1.4	1	7.4
7	1.5	1.4	1	7.36
8	1.5	0.9	0.6	8.1
9	0.5	0.4	0.2	6.6
10	1	0.9	0.2	8.1
11	1	0.9	0.6	8.76
12	1.5	0.4	1	6.8
13	1	0.9	0.6	8.76
14	1.5	0.4	0.2	7.2
15	1	0.9	1	8
16	1	0.4	0.6	7.8
17	0.5	0.9	0.6	8
18	1	0.9	0.6	8.76
19	1	0.9	0.6	8.76
20	1.5	1.4	0.2	7.2

Table 11: ANOVA for full quadratic model

Source	Sum of squares	Mean square	DF	F-value	P>F
Model	11.85	1.32	9	76.34	< 0.0001
Residual	0.1725	0.1725	10		
Lack of Fit	0.1725	0.0345	5		
Pure error	0.0000	0.0000	5		
Total	12.02		19		

From substituting the equation representing the model, values are predicted for each run. A linear graph of predicted and actual values thus proves that the model can correctly represent the experiment. The normal plot of residuals is an important diagnostic for the model. A linear pattern demonstrated that there are no signs of any problems in the data (Fig. 23).

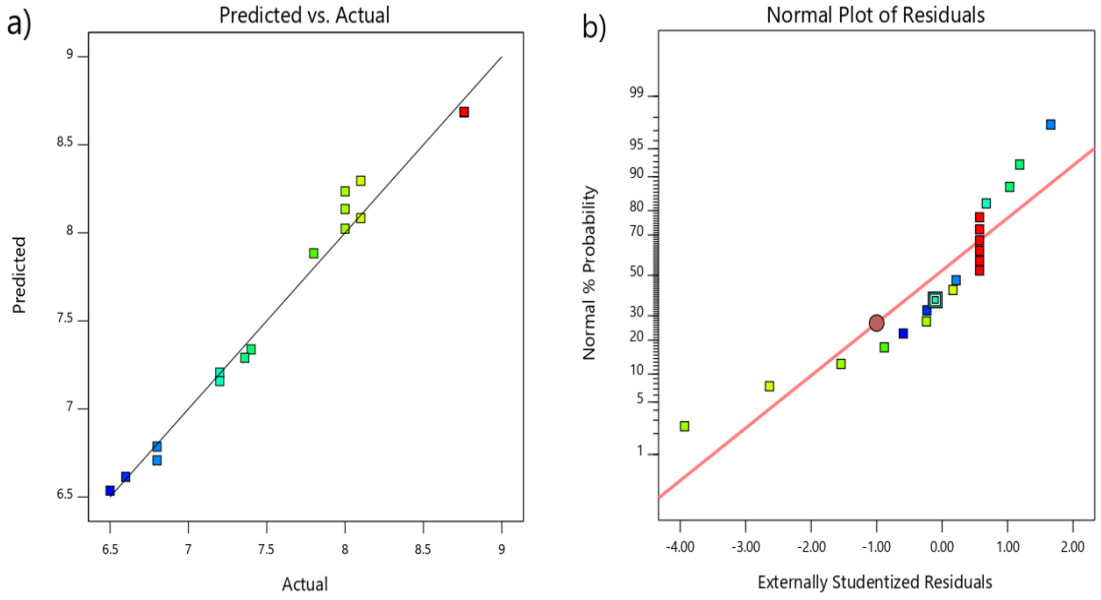

Fig.23: a) Plot of Predicted values verses actual values **b)** Normal plot of residuals

Another method in RSM which gives a potential relationship between the variables is the three-dimensional (3D) surface plots and two dimensional (2D) contour plots. The 3D response surface curve plot are a graphical representation of the regression equation (Fig 24 – 26). It displays the three dimensional relationship with predictor variables on the *x* and *y* scales, and the response (*z*) variable.

Each figure represents the effect of two variables, while the third being at zero level for maximum biosurfactant production. Fig 24a. shows the effect of starch and peptone on

the production of biosurfactant keeping NaCl concentration constant at the middle level. A similar trend was observed in Fig. 25a and Fig. 26a.

Pertubation graph shows the effect of each of the independent variables on biosurfactant production while keeping other variables at their respective middle point levels.The response surface plots and pertubation graph shows that all the three variables (Starch, Peptone and NaCl) have significant role in the response (Fig.27).

Also the pertubation graph showed the effect of each of the independent variables on the production of the biosurfactant. Based on the response values, 3D plots and pertubation graph, it can be concluded that all the factors investigated in the present study had a positive effect on the biosurfactant production.

4.4.2.2.1 Validation of the model

The optimal combination of the major media constituents for biosurfactant production as evaluated from the response surface and contour plots was Starch 1.05 %, Peptone 0.95 % and NaCl 0.63 %. The model predicted optimum activity to be 8.7 cm using the above mentioned optimum concentrations of the variables. The verification of the results was carried out by shake flask method using optimum concentrations of each parameter predicted by the model, and the maximum zone was found to be 8.7 cm, same as the predicted value. A two - fold increase in yield was observed after optimization with RSM as compared to the un-optimized medium, thereby proving the effectiveness of the model in media optimization.

4.4.2.2.2 Genetic algorithm

The non-linear equation (1) of oil displacement activity was used as fitness function for optimization using GA tools. The fitness of individual population is represented in Fig. 28. The oil displacement zone was found to be 8.7 cm under optimal process conditions, close to the predicted RSM activity.

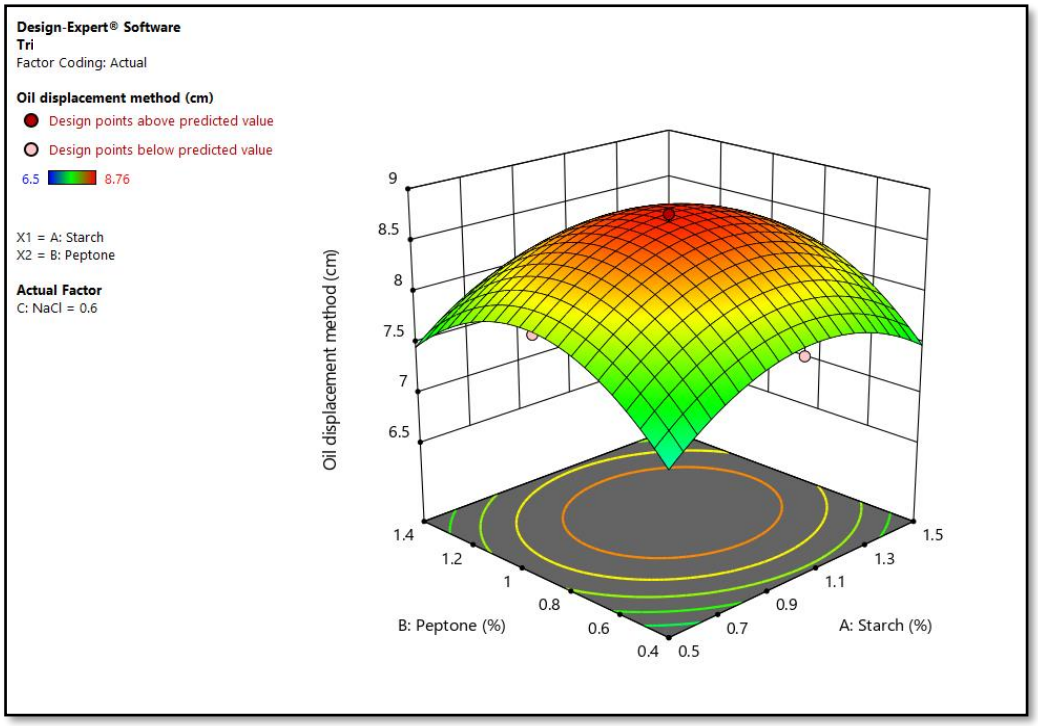

Fig. 24a: Surface plot showing effect of starch and peptone on biosurfactant production; keeping NaCl constant.

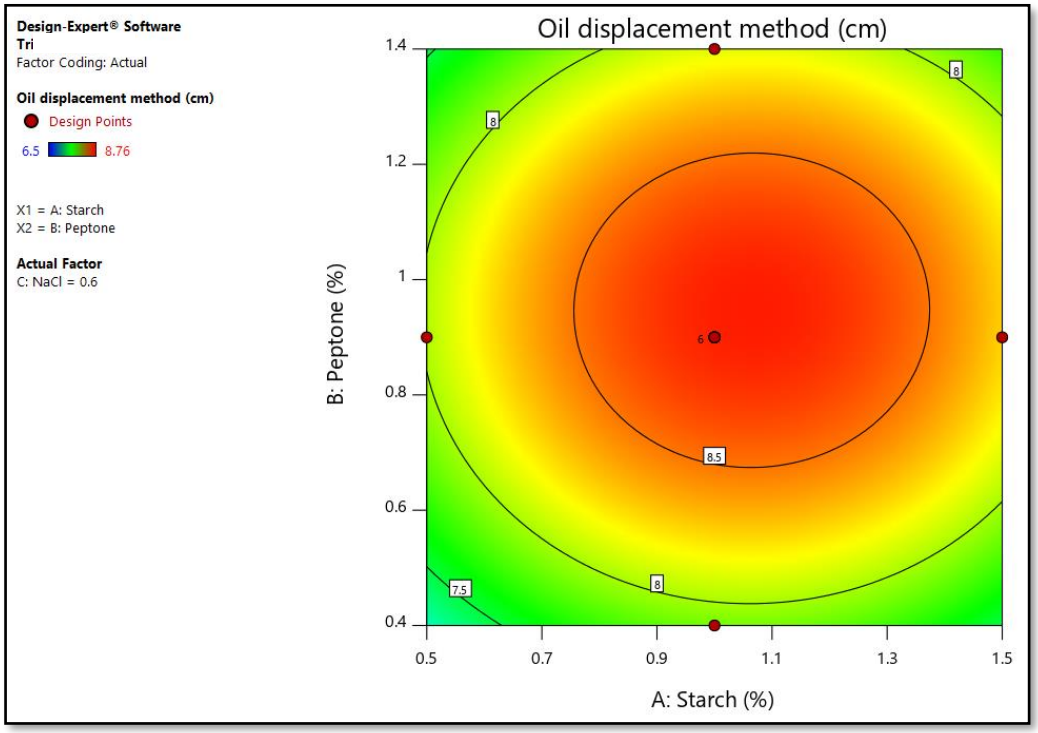

Fig. 24b: Contour plot showing effect of starch and peptone on biosurfactant production; keeping NaCl constant.

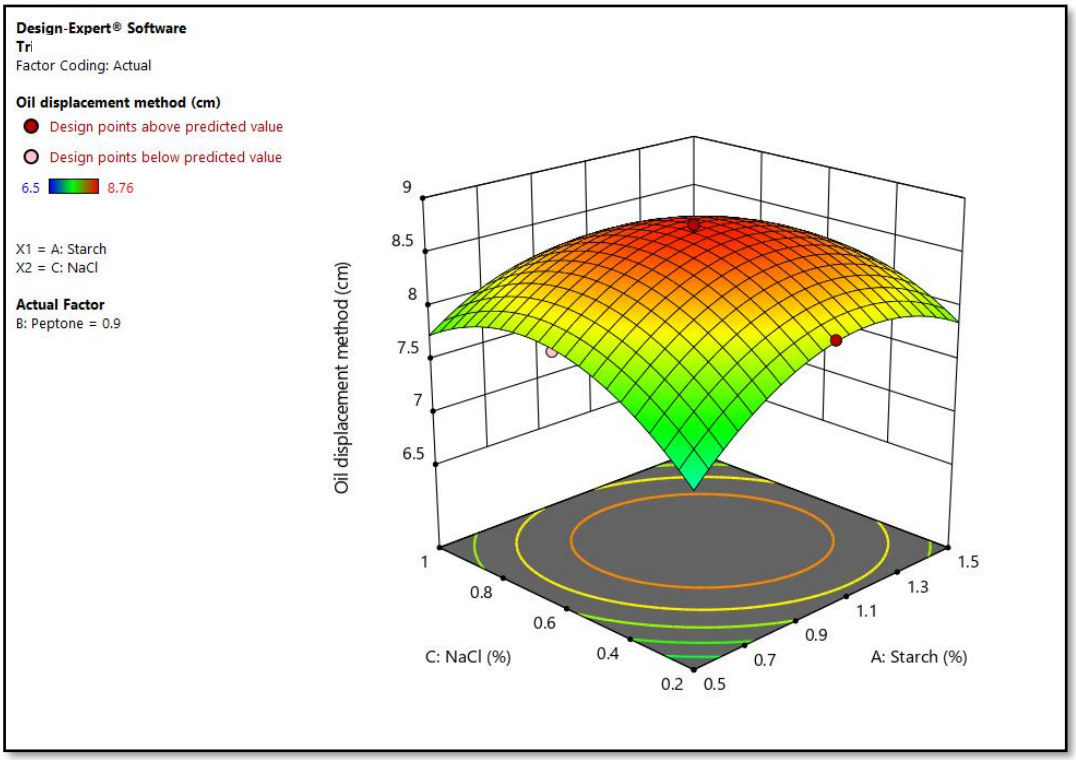

Fig. 25a: Surface plot showing effect of starch and NaCl on biosurfactant production; keeping peptone constant.

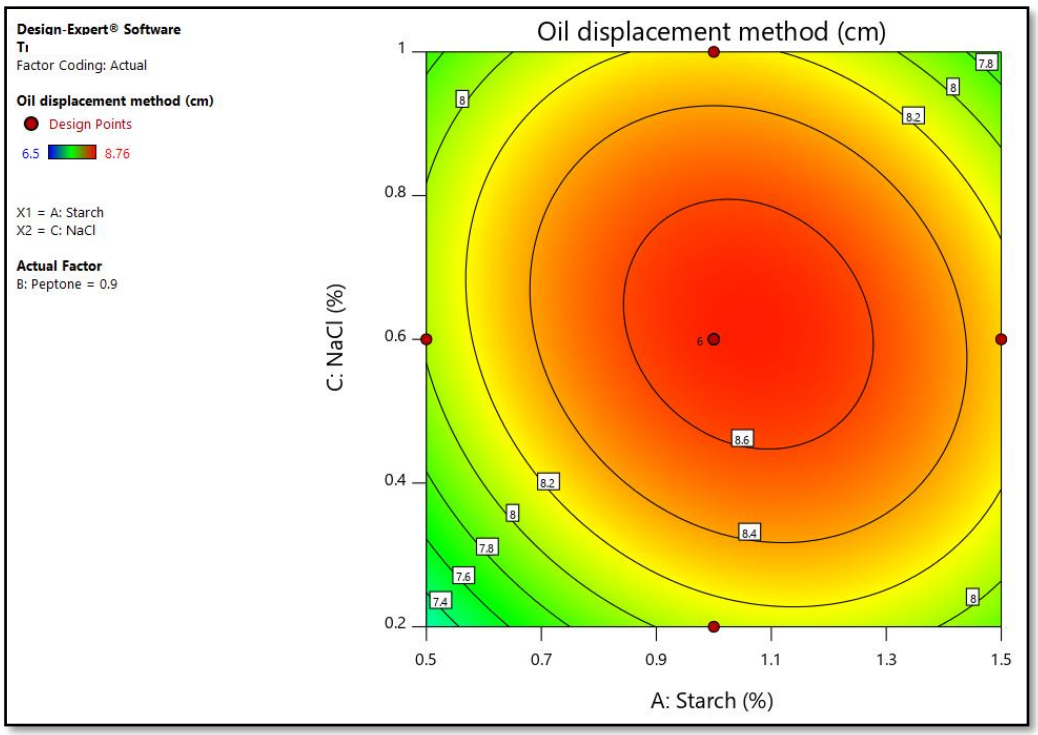

Fig. 25b: Contour plot showing effect of starch and NaCl on biosurfactant production; keeping peptone constant.

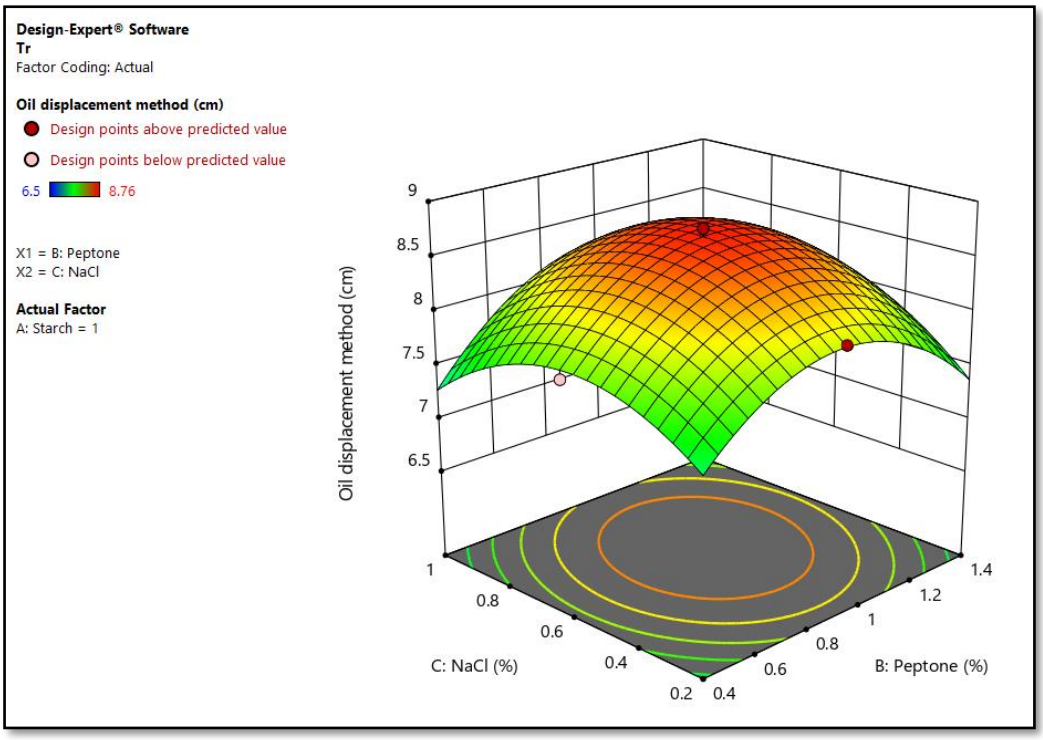

Fig. 26a: Surface plot showing effect of peptone and NaCl on biosurfactant production; keeping starch constant.

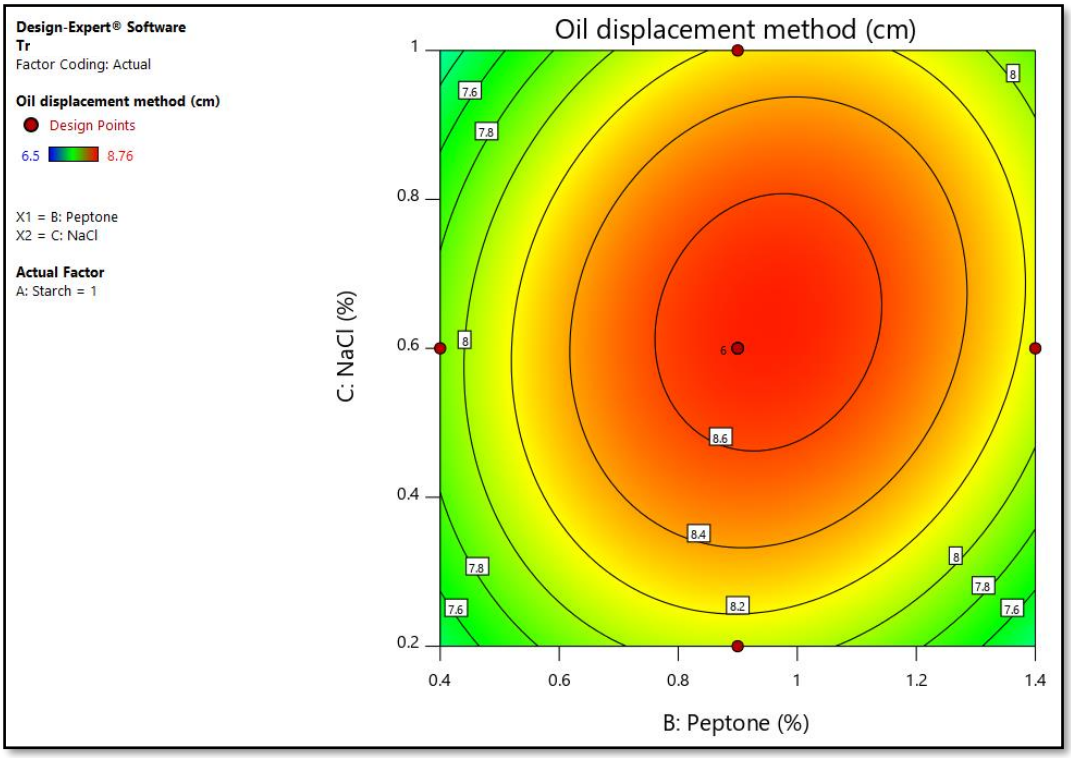

Fig. 26b: Contour plot showing effect of peptone and NaCl on biosurfactant production; keeping starch constant.

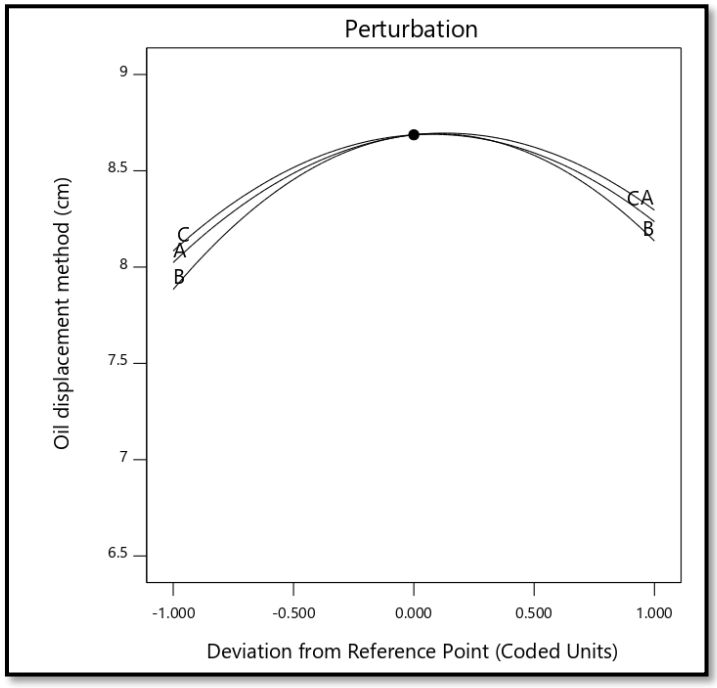

Fig.27: Pertubation graph showing effect of each of the independent variables on biosurfactant production

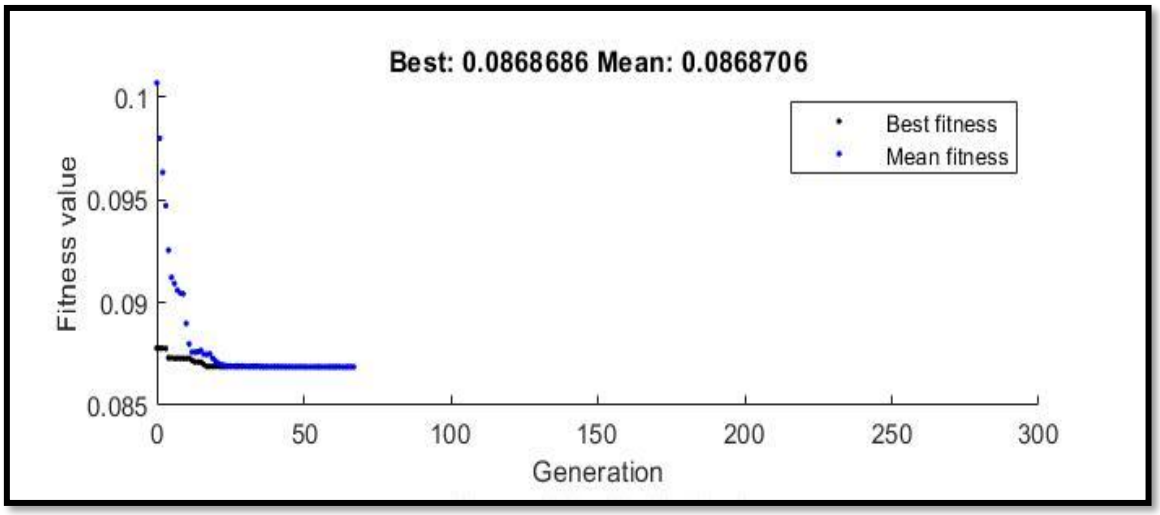

Fig. 28: Fitness of the individual population

Table 12: Composition of fermentation media for SK27 optimized using OFAT and RSM-GA methods

Media composition	Optimization methods	
	OFAT (g/L)	RSM-GA (g/L)
Sucrose	10	9.7
Yeast extract	8	8.9
KH_2PO_4	5	5
K_2HPO_4	5	5
$MgSO_4.7H_2O$	0.25	0.25
NaCl	2	5.9
$CaCl_2$	0.08	0.08

Table 13: Composition of fermentation media for RMSK10 optimized using OFAT and RSM-GA methods

Media composition	Optimization methods	
	OFAT (g/L)	RSM-GA (g/L)
Starch	10	10
Peptone	10	9.5
KH_2PO_4	5	5
K_2HPO_4	5	5
$MgSO_4.7H_2O$	0.25	0.25
NaCl	6	6.3
$CaCl_2$	0.08	0.08

Table 14: Comparative assessment of the optimized media for biosurfactant production by SK27 and RMSK10

Sr. No.	Fermentation media	SK27		RMSK10	
		Activity (cm)	Fold Increase	Activity (cm)	Fold Increase
1	Un-optimized media	4.2	-	4.2	-
2	OFAT	7.8	1.85	7.9	1.88
3	RSM-GA	8.8	2.1	8.7	2.0

4.5. Identification of potential isolates

SK27 was identified as *Bacillus amyloliquefaciens* subsp. plantarum strain FZB42, GenBank accession numbers MF599413 (Fernandes, 2019c)

The potential halotolerant bacterium RMSK10 was identified using classical and molecular methods. Plate 5 illustrates the morphology of the respective bacterial colony on agar plate along with the bacterial morphology by SEM analysis. Table 15 further describes the morphological and biochemical characteristics of each individual culture. Subsequently the biochemical characterisation was confirmed by BIOLOG plates which briefly outlined the substrates utilized, bacterial tolerance to NaCl and pH concentrations and the reaction to antibiotics (Table 16 to 17).

Table 18 describes the molecular identification details of the respective bacteria. Salt pan bacteria RMSK10 (1417 bp) showed 99 % similarity with *Bacillus subtilis* strain SBTS18. The sequence has been submitted to GenBank database under accession numbers MN901611 (RMSK10). The taxonomic position of the salt pan bacteria in the phylogenetic tree along with their closest relatives is illustrated in Fig. 29. The figure validates the results of identification as the bootstrap values were found to be close to 100. Neighbor-Joining method was used to infer the evolutionary history of the respective cultures. The percentage of replicates in which the related taxa clustered together in the bootstrap test (1000 replicates) is depicted next to the nodes. The tree has been drawn to scale, using the same units of branch lengths as that of the evolutionary distances used to deduce the phylogenetic tree.

Plate 5: Colony morphology and scanning electron micrograph of RMSK10

Table 15: Morphological and biochemical characteristics of the selected isolates

RMSK 10	
Gram character	Gram positive
Shape	Rods
Size	3.776 µm x 0.797 µm
Catalase	+
Oxidase	+
Endospore	Sub-terminal
Motility	Motile
Carbohydrate utilizated	Cellobiose, Esculin, Galactose, Fructose, Glucose, Glycerol, L-Arabinose, Maltose, Mannitol, Mannose, Rhamnose, ONPG, Salicin, Sucrose, Trehalose
H2S production	+
Nitrate reduction	+
Voges proskauer	-
Arginine utilization	-
Ornithine utilization	-
Lysine utilization	-
Urease	+
Phenylalanine deaminase	-

Table 16: Metabolic profile of RMSK10 on BIOLOG GEN III MicroPlateTM.

1 Negative Control	**A2 Dextrin**	**A3 D-Maltose**	**A4 D-Trehalose**	**A5 D-Cellobiose**	**A6 Gentiobiose**	**A7 Sucrose**	**A8 D-Turanose**	**A9 Stachyose**	**A10 Positive Control**	**A11 pH 6**	**A12 pH 5**
B1 D-Raffinose	**B2 D-Lactose**	**B3 D-Melibiose**	**B4 Methyl-D Glucoside**	**B5 D-Salicin**	**B6 N-Acetyl-D-Glucosamine**	**B7 N-Acetyl- D-Mannosamine**	**B8 N-Acetyl-D-Galactosamine**	**B9 N-Acetyl-Neuraminic Acid**	**B10 1% NaCl**	**B11 4% NaCl**	**B12 8% NaCl**
C1 D-Glucose	**C2 D-Mannose**	**C3 D-Fructose**	**C4 D-Galactose**	**C53 Methyl Glucose**	**C6 D-Fucose**	**C7 L-Fucose**	**C8 L-Rhamnose**	**C9 Inosine**	**C1 01% Sodium Lactate**	**C11 Fusidic Acid**	**C12 D-Serine**
D1 D-Sorbitol	**D2 D-Mannitol**	**D3 D-Arabitol**	**D4 myo-Inositol**	**D5 Glycerol**	**D6 D-Glucose- 6-PO4**	**D7 D-Fructose- 6-PO4**	**D8 D-Aspartic Acid**	**D9 D-Serine**	**D10 Troleandomycin**	**D11 Rifamycin SV**	**D12 Minocycline**
E1 Gelatin	**E2 Glycyl-L-Proline**	**E3 L-Alanine**	**E4 L-Arginine**	**E5 L-Aspartic Acid**	**E6 L-Glutamic Acid**	**E7 L-Histidine**	**E8 L-Pyroglutamic**	**E9 L-Serine**	**E10 Lincomycin**	**E11 Guanidine HCl**	**E12 Niaproof 4**
F1 Pectin	**F2 D-Galacturonic Acid**	**F3 L-Galactonic Acid Lactone**	**F4 D-Gluconic Acid**	**F5 D-Glucuronic Acid**	**F6 Glucuronamide**	**F7 Mucic Acid**	**F8 Quinic Acid**	**F9 D-Saccharic Acid**	**F10 Vancomycin**	**F11 Tetrazolium**	**F12 Tetrazolium**
G1 p-Hydroxy-Phenylacetic Acid	**G2 Methyl Pyruvate**	**G3 D-Lactic Acid Methyl Ester**	**G4 L-Lactic Acid**	**G5 Citric Acid**	**G6 Keto-Glutaric Acid**	**G7 D-Malic Acid**	**G8 L-Malic Acid**	**G9 Bromo-Succinic Acid**	**G10 Nalidixic Acid**	**G11 Lithium Chloride**	**G12 Potassium Tellurite**
H1 Tween 40	**H2 Amino-Butryric - Acid**	**H3 Hydroxy-Butyric Acid**	**H4 β-Hydroxy-D,L-Butyric Acid**	**H5 Keto-Butyric Acid**	**H6 Acetoacetic Acid**	**H7 Propionic Acid**	**H8 Acetic Acid**	**H9 Formic Acid**	**H10 Aztreonam**	**H11 Sodium Butyrate**	**H12 Sodium Bromate**

Table 17: Metabolic profile of RMSK10 on BIOLOG GP II MicroPlateTM.

A1 Water	**A2 α-cyclodextrin**	**A3 B-cyclodextrin**	**A4 dextrin**	**A5 glycogen**	**A6 Inulin**	**A7 Mannan**	**A8 Tween 40**	**A9 Tween 80**	**A10 N- Acetyl-D-Glucosamine**	**A11 N-Acetyl-β-Mannosamine**	**A12 Amygdalin**
B1 L-Arabinose	**B2 D-Arabitol**	**B3 Arbutin**	**B4 D-Cellobiose**	**B5 D-Fructose**	**B6 L-Fucose**	**B7 D-Galactose**	**B8 D-Galacturonic Acid**	**B9 Gentiobiose**	**B10 D-Gluconic Acid**	**B11 α-D-Glucose**	**B12 m-Inositol**
C1 α-D-Lactose	**C2 Lactulose**	**C3 Maltose**	**C4 Maltotriose**	**C5 D-Mannitol**	**C6 D-Mannose**	**C7 D-Melezitose**	**C8 D-Melibiose**	**C9 α-Methyl-D-Galactoside**	**C10 β-Methyl-D-Galactoside**	**C11 3-Methyl Glucose**	**C12 α-Methyl-D-Glucoside**
D1 β-Methyl-D-Glucoside	**D2 α-Methyl-D -Mannoside**	**D3 Palatinose**	**D4 D-Psicose**	**D5 D-Raffinose**	**D6 L-Rhamnose**	**D7 D-Ribose**	**D8 Salicin**	**D9 Sedoheptulosan**	**D10 D-Sorbitol**	**D11 Stachyose**	**D12 Sucrose**
E1 D-Tagatose	**E2 D-Trehalose**	**E3 Turanose**	**E4 Xylitol**	**E5 D-Xylose**	**E6 Acetic Acid**	**E7 α-Hydroxybutyric**	**E8 β-Hydroxybutyric**	**E9 γ-Hydroxybutyric**	**E10 p-Hydroxy-Phenylacetic**	**E11 α- Ketoglutaric**	**E12 α-Ketovaleric**
F1 Lactamide	**F2 D-Lactic Acid**	**F3 L-Lactic**	**F4 D-Malic**	**F5 L-Malic**	**F6 Pyruvatic Acid**	**F7 Succinic Acid**	**F8 Propionic**	**F9 Pyruvic**	**F10 Succinamic**	**F11 Succinic**	**F12 N-Acetyl-L-**
G1 L-Alaninamide	**G2 D-Alanine**	**G3 L-Alanine**	**G4 L-Alanyl-**	**G5 L-Asparagine**	**G6 L-Glutamic**	**G7 Glycyl - L-**	**G8 L-Pyroglutamic**	**G9 L-Serine**	**G10 Putrescine**	**G11 2,3-Butanediol**	**G12 Glycerol**
H1 Adenosine	**H2 2'-Deoxy Adenosine**	**H3 Inosine**	**H4 Thymidine**	**H5 Uridine**	**H6 Adenosine-5'-Monophosphate**	**H7 Thymidine-5'-Monophosphate**	**H8 Uridine-5'-Monophosphate**	**H9 D-Fructose-6-Phosphate**	**H10 α-D-Glucose- 1-Phosphate**	**H11 D-Glucose- 1-6-Phosphate**	**H12 D-L-α-Glycerol Phosphate**

Table 18: Molecular identification of the selected bacterial isolate

Culture code	GenBank accession number	Length of the sequence	Closest match Percent similarity	Percentage similarity	Culture identification
RMSK10	MN901611	1418	*Bacillus subtilis* strain SBTS18	99 %	*Bacillus subtilis* strain RMSK10

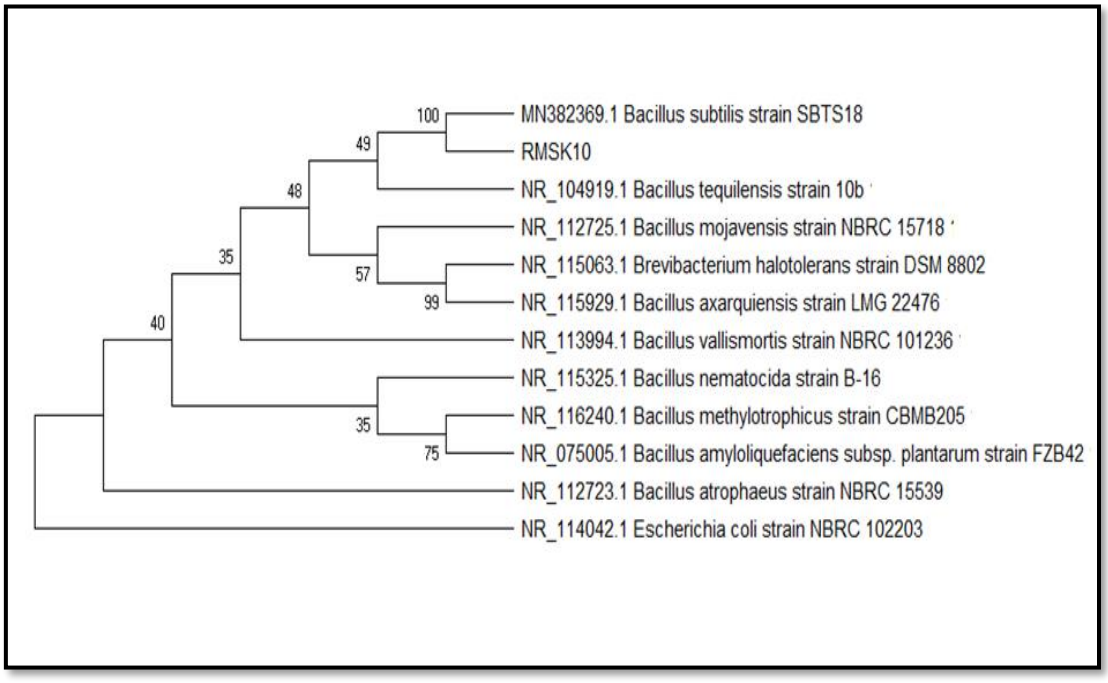

Fig. 29: Phylogenetic tree depicting the relationship of the halotolerant bacteria RMSK10 with other isolates based on 16S rRNA sequence similarity.

4.6 Extraction and Purification of biosurfactant

Biosurfactant extraction was carried out using HCl fractionation for both the cultures SK27 and RMSK10. Further, TLC of the extracts was carried out which showed 6 pinkish purple spots in SK27 and 4 in RMSK10 suggesting the presence of amino acids and could probably be components of a peptide or lipopeptide (Plate 6). Further these compounds were purified using Gel filtration chromatography by using Sephadex LH20 column. The column chromatography was repeated to ensure proper purification of the compound. The elution profile revealed that fractions from 15 – 41 were active fractions in SK27 and fractions from 43 – 67 were active in RMSK10. These fractions collected from the column having biosurfactant activity were pooled and concentrated as mentioned in Table 19.

Further these fractions were purified using PTLC (Plate 7). Maximum activity was found in pooled fractions CB1 and CR1 suggesting the presence of the active compound.

Table 19: Fractions and biosurfactant activity profile from Sephadex LH20

Pooled Fractions	Cumulative eluted volume (mL)	Oil displacement zone (cm)
CB1 (elute tube 15-41)	13	8.9
CR1 (elute tube 43-55)	12	8.8

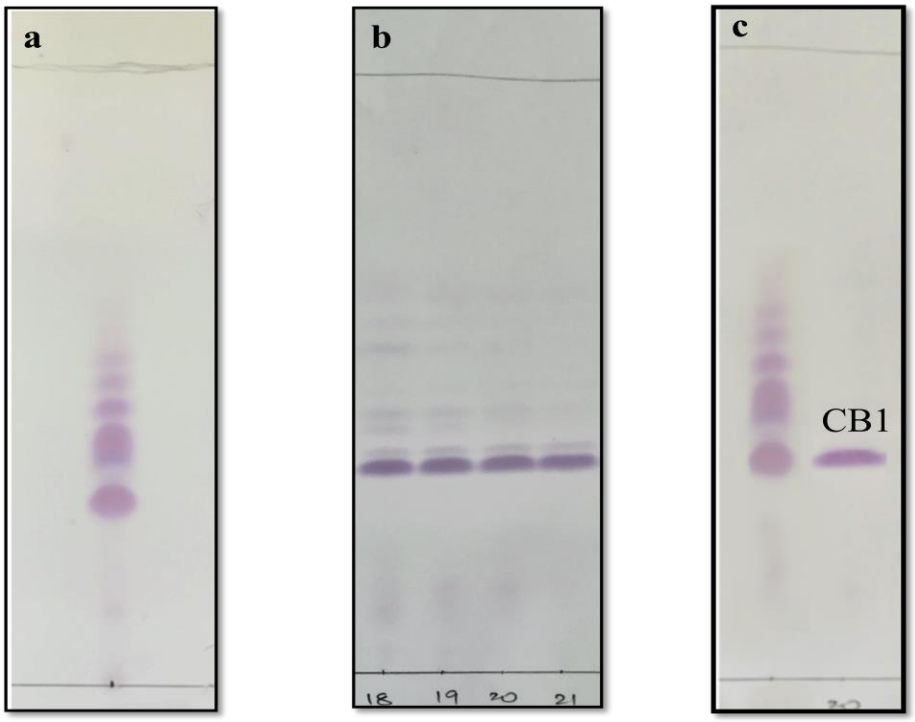

Plate 7: TLC profile of fractions of SK27 (a) after extraction (b) fraction after column (C) after PTLC with crude

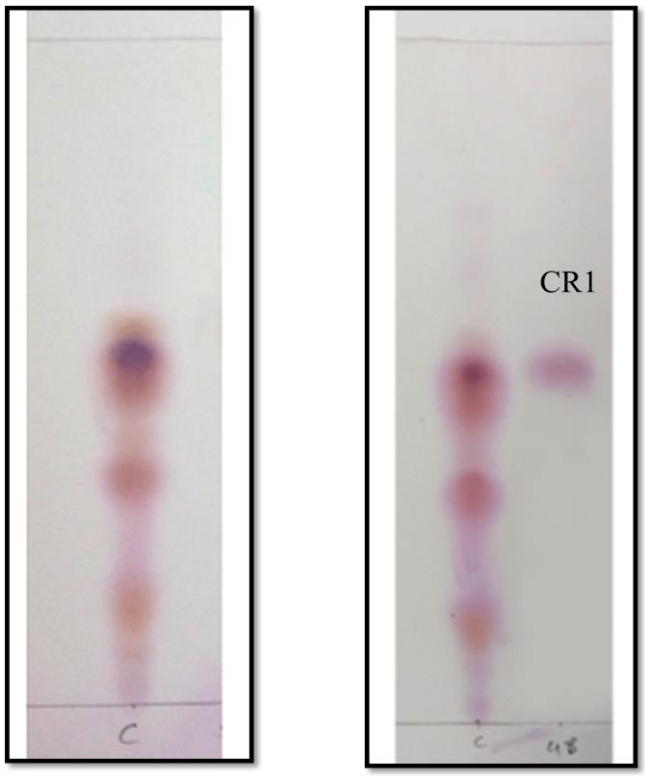

Plate 8: TLC profile of fractions of SK27 (a) after extraction (b) after column

4.7 Bioremediation of heavy metal using biosurfactant

The ICP-AES results showed that the Fe and Pb concentration in the solution obtained after biosurfactant treatment (CB1 and CR1) were significantly lower as compared to the initial concentration of the metal, suggesting the metal co-precipitated with the biosurfactant. The metal-biosurfactant precipitate was observed after the addition of the biosurfactant to metal solutions followed by incubation (24 h). Whereas the concentration of the other 3 metals (Cr, Cd, and Zn) in the solution was similar to the starting concentration of the metals which indicated that the biosurfactant (CB1 and CR1) could be specific in its binding to Fe and Pb. In case of EDTA, it did not show remediation at 200 mg/L however when used at a concentration of 2000 mg/L the remediation efficiency increased to 88.5% for Fe and 29% for Pb.

Fig. 30 represents the effect of the biosurfactant concentration (CB1) on the bioremediation efficiency of the Fe. As seen in the graph, an increasing trend in the bioremediation efficiency was observed across all the treatment groups as the biosurfactant concentration increased. CB1 at 40 mg/L was able to remediate 97.5% of 100 ppm, 72 % of 200 ppm and 45 % of 300 ppm of Fe. The bioremediation efficiency increased to 98.4 % for 200 ppm of Fe at 80 mg/L which was significantly higher ($p<0.05$) as compared to 40 mg/L. At 100 mg/L, there was almost a complete removal of 300 ppm of Fe and Pb which was significantly higher ($p<0.05$) as compared to 40 mg/L and 80 mg/L. The results revealed that when the concentration of the metals is high, there is a need to increase the concentration of the biosurfactant proportionally.

Fig. 31 represents the effect of the biosurfactant concentration on the bioremediation efficiency of the Pb. As seen in the graph, CB1 at 40 mg/L was able to remediate 91 % of 100 ppm, 67.6 % 200 ppm, and 41 % of 300 ppm of Pb respectively. The bioremediation efficiency increased to 100 % for 100 ppm, 88.2 % for 200 ppm at 80 mg/L which was significantly higher ($p<0.05$) as compared to 40 mg/L. At 100 mg/L, there was almost complete removal of 200, 300 ppm of Fe and Pb which was significantly higher ($p<0.05$) as compared to 40 mg/L and 80 mg/L.

Similarly, the effect of the biosurfactant concentration (CR1) was also checked on the bioremediation efficiency. Fig. 32 and 33 represents the effect of the CR1 on the bioremediation efficiency of the Fe and Pb. It was seen from the graph that, bioremediation efficiency of CR1 at 40 mg/L was lower as compared to CB1 for both

the metals. When the concentration of CR1 was increased to 80 mg/L, the bioremediation efficiency increased to 99.2 %, 67.5 %, 41.4 % for 100, 200, 300 ppm of Fe. There was complete removal of 100 ppm and 200 ppm of Fe at 100 mg/L which was significantly higher ($p<0.05$) as compared to 40 mg/L. Further when concentration of CR1 was increased to 120 mg/L, there was almost complete removal of 300 ppm of Fe which was significantly higher ($p<0.05$) as compared to 100 mg/L.

Fig. 33 represents the effect of the CR1 on the bioremediation efficiency of the Pb. the bioremediation efficiency of CR1 at 80 mg/L was 92.5%, 50.7% 29.7 % for 100, 200, 300 ppm Pb respectively, which was significantly higher ($p<0.05$) as compared to 40 mg/L. Further when concentration of CR1 was increased to 100 mg/L, the bioremediation efficiency increased to 100% for 100 ppm and 96% for 200 ppm which was significantly higher ($p<0.05$) as compared to 80 mg/L. At 120 mg/L, there was almost 96 % removal of 300 ppm Pb which was significantly higher ($p<0.05$) as compared to 100 mg/L.

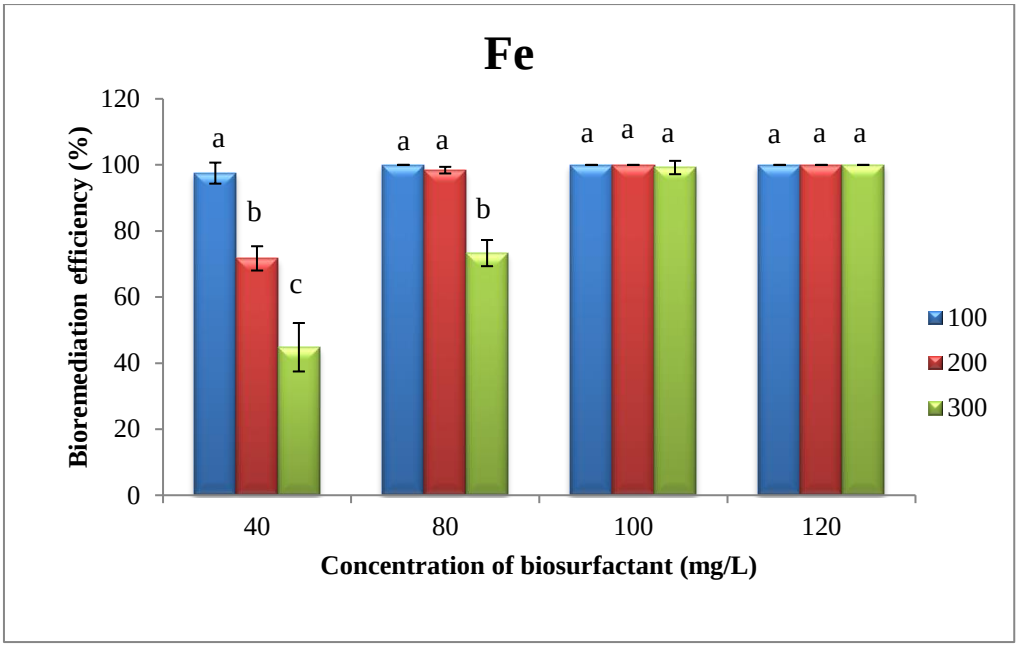

Fig. 30: Effect of the biosurfactant concentration on the bioremediation efficiency of Fe

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

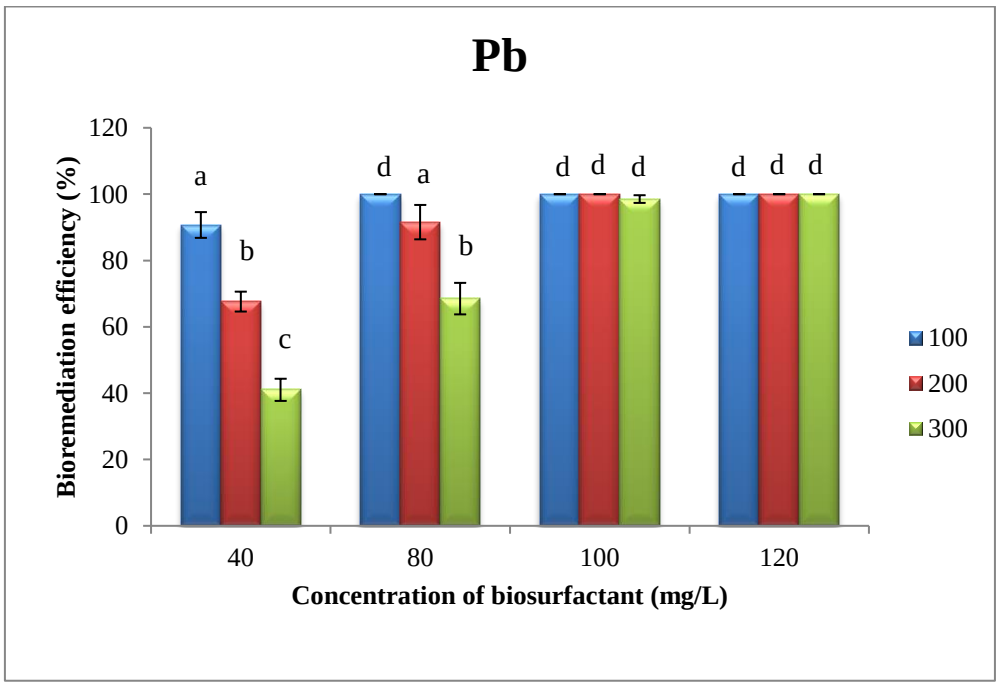

Fig 31: Effect of the biosurfactant concentration on the bioremediation efficiency of Pb

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

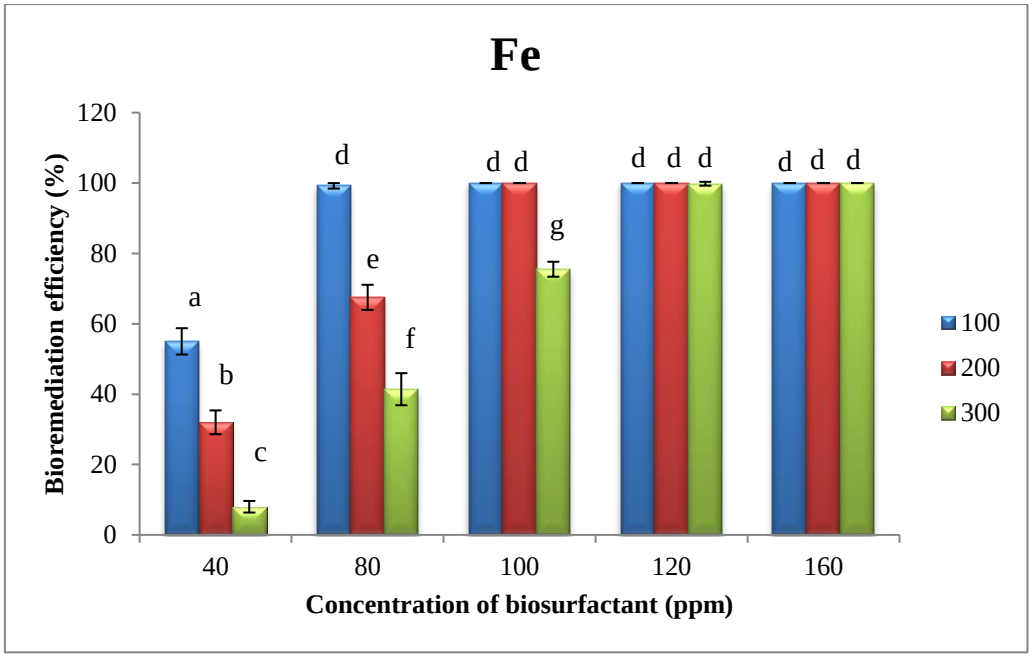

Fig 32: Effect of the biosurfactant concentration on the bioremediation efficiency of Fe

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

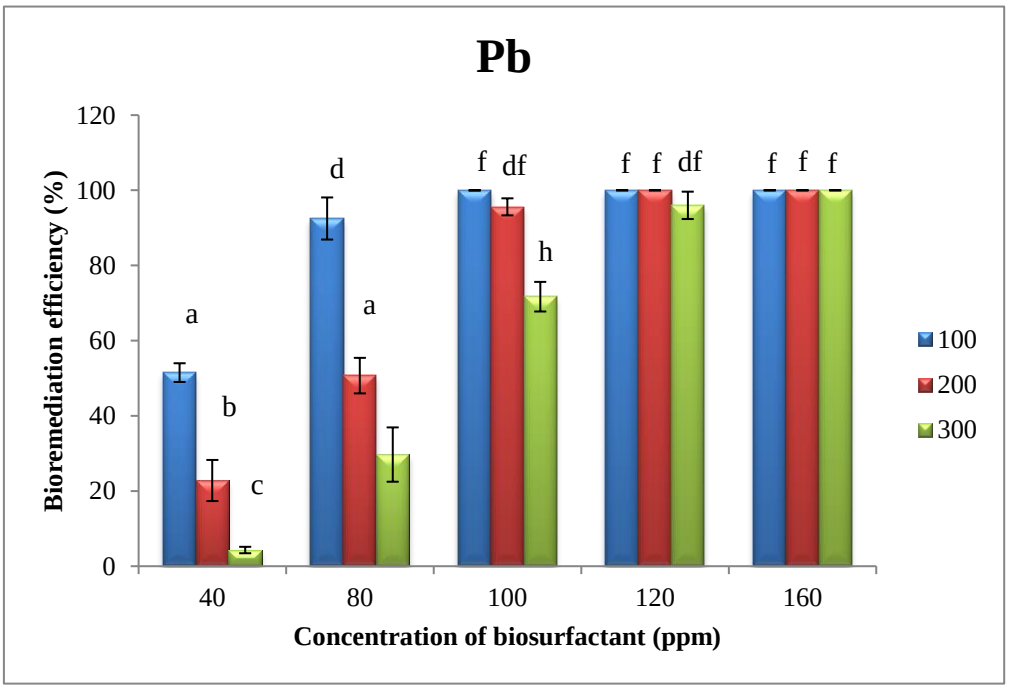

Fig 33: Effect of the biosurfactant concentration on the bioremediation efficiency of Pb

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

4.7.1 Effect of temperature on bioremediation efficiency of the biosurfactant

Fig. 34 represents the effect of temperature on bioremediation efficiency of CB1. As seen in the graph, the bioremediation efficiency of CB1 on Fe varied with the varying temperature. It was seen that as the temperature was lowered to 4°C, 10°C, the bioremediation efficiency was observed to be 80.5% and 81.6% which was significantly lower ($p<0.05$) as compared to 30°C. As the temperature was increased to 60°C and 70°C, the bioremediation efficiency of CB1 was 92.5% and 89.2% respectively which was lower as compared to 30°C but was significantly higher ($p<0.05$) as compared to 4 and 10°C. Similar results were obtained for Pb (Fig. 35). The bioremediation efficiency decreased to 75.4% at 4°C and 76% 60°C which was significantly lower ($p<0.05$) as compared to 30°C. As the temperature was increased to 50°C, The bioremediation efficiency decreased 91.6% which was lower as compared to 30°C but was significantly higher ($p<0.05$) as compared to 4 and 10°C.

In case of CR1, the bioremediation efficiency declined with the varying temperature. The bioremediation efficiency was higher at 30°C followed by 20°C. When the temperature was decreased to 4 and 10°C, the bioremediation of Fe decreased to 14% and 28.4% respectively which was significantly low ($p<0.05$) compared to 30°C. At 40°C, the efficiency was observed to be 19.2% and with further increase temperature there was almost a complete decrease in the bioremediation efficiency was seen (Fig. 36). Similar results were observed for Pb. The bioremediation efficiency was higher at 30°C followed by 20°C. When the temperature was decreased to 4 and 10°C, the bioremediation of Pb decreased to 4.4% and 5% respectively which was significantly low ($p<0.05$) compared to 30°C. At 40°C the efficiency was observed to be 13.6 % and it was seen that with further increase in temperature there was almost a complete decrease in the bioremediation efficiency (Fig. 37).

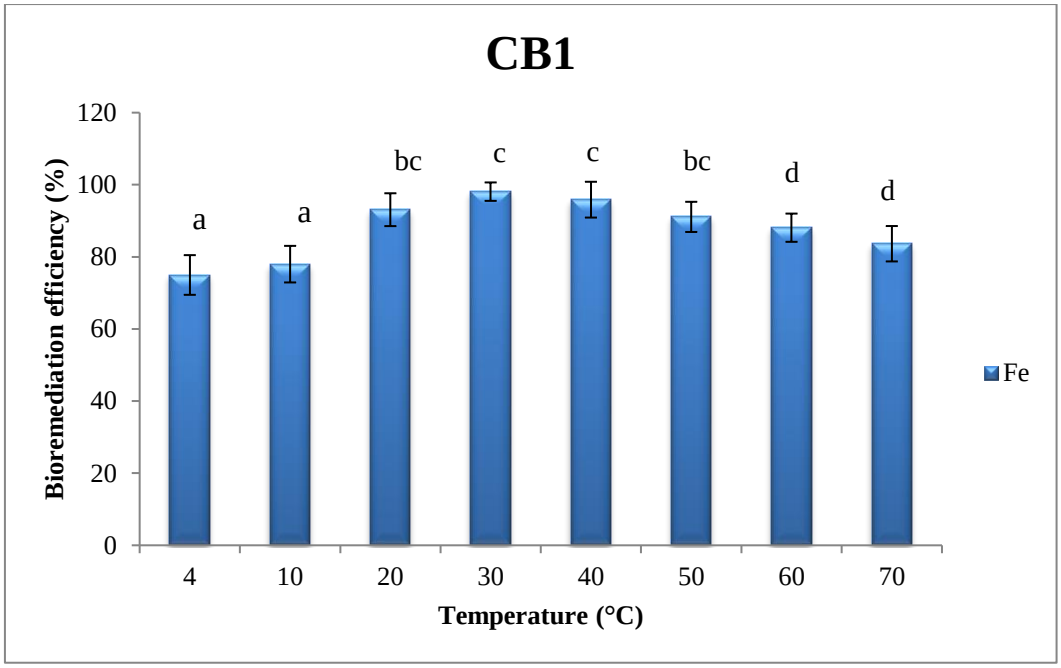

Fig 34: Effect of temperature on bioremediation efficiency of CB1 on Fe

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

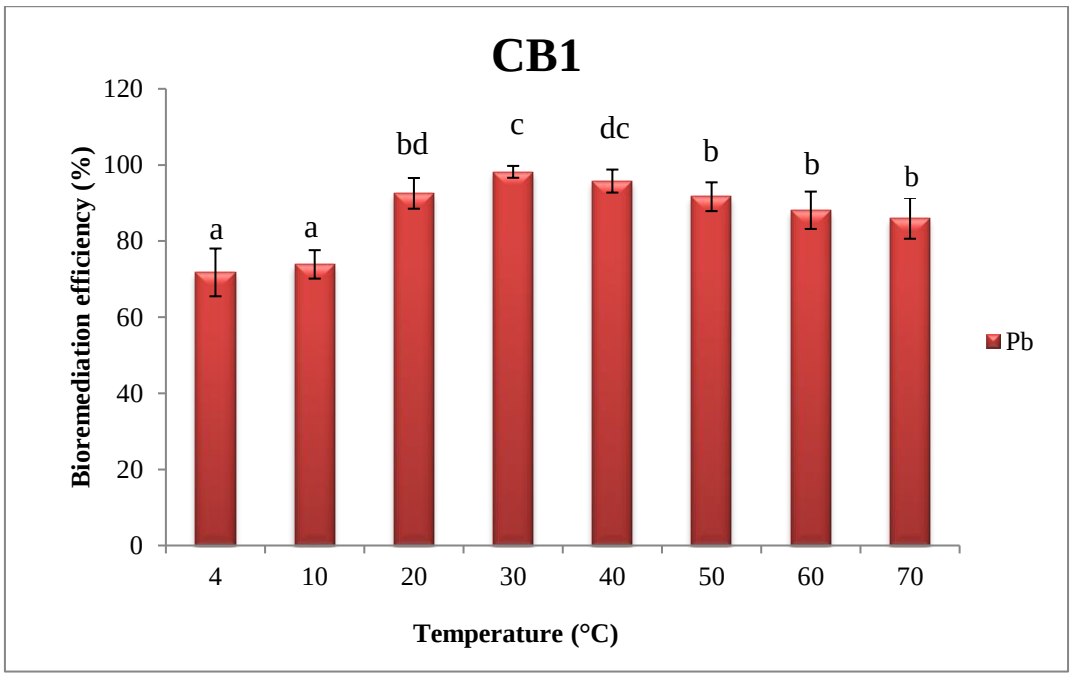

Fig 35: Effect of temperature on bioremediation efficiency of CB1 on Pb

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

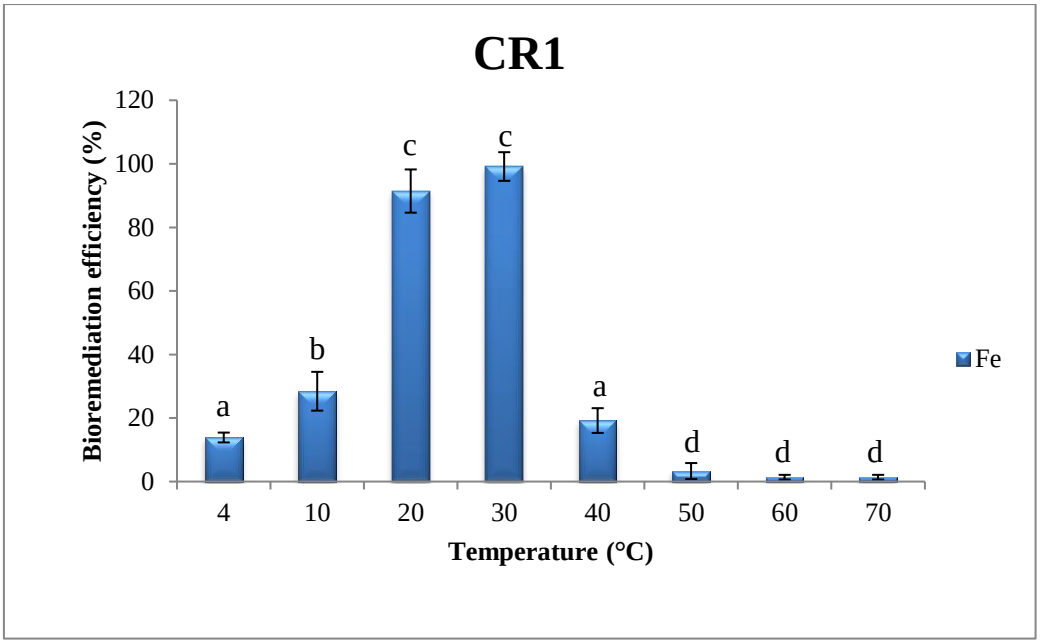

Fig 36: Effect of temperature on bioremediation efficiency of CR1 on Fe

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

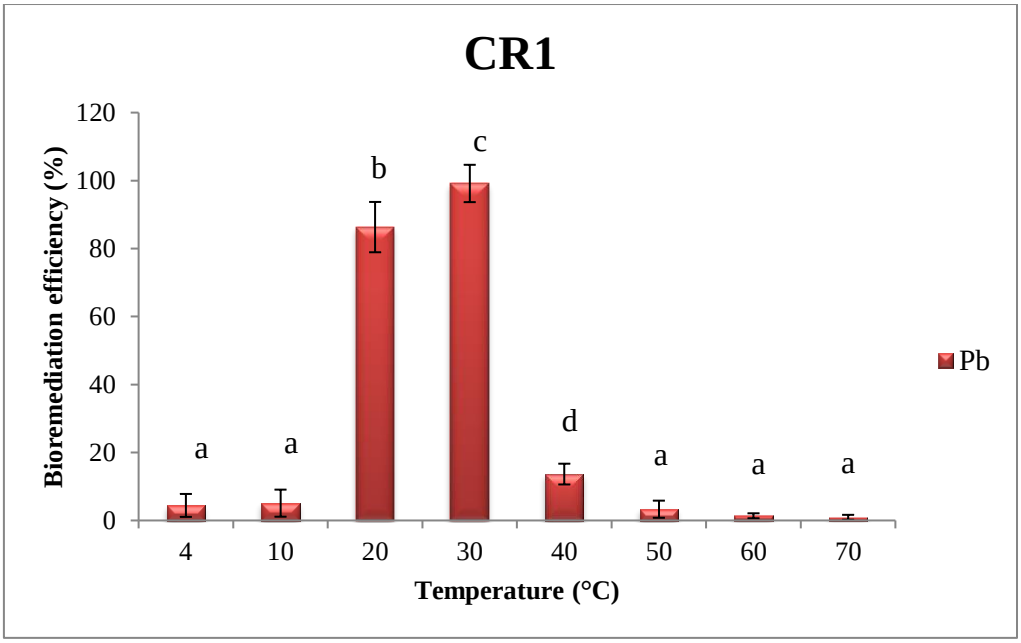

Fig 37: Effect of temperature on bioremediation efficiency of CR1 on Pb

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

4.8 Determination of critical micelle concentration (CMC) of biosurfactant

The effect of biosurfactant concentration (CB1) on surface tension of the water is represented in Fig. 38. As seen from the graph, the surface tension of water decreased on addition of the biosurfactant. The biosurfactant CB1 at concentration of 20 mg/l was able to reduce the surface tension of water from 72 mN/m to 25.56 mN/m which was significantly higher as compared to other concentrations ($p<0.05$). Once the concentration reached 20 mg/L, there was no further significant decrease in the surface tension of water. This concentration was considered as the critical micelle concentration (CMC) of CB1. Similarly Fig. 39 represents the effect of CR1 concentration on surface tension of the water. The surface tension of water reduced to maximum i.e. 28 mN/m at 60 mg/l concentration of CR1 which was significantly higher as compared to other concentrations ($p<0.05$). Once the concentration reached to 60 mg/l, there was no further significant decrease in the surface tension of water. In case of CR1 the critical micelle concentration (CMC) was 60 mg/l.

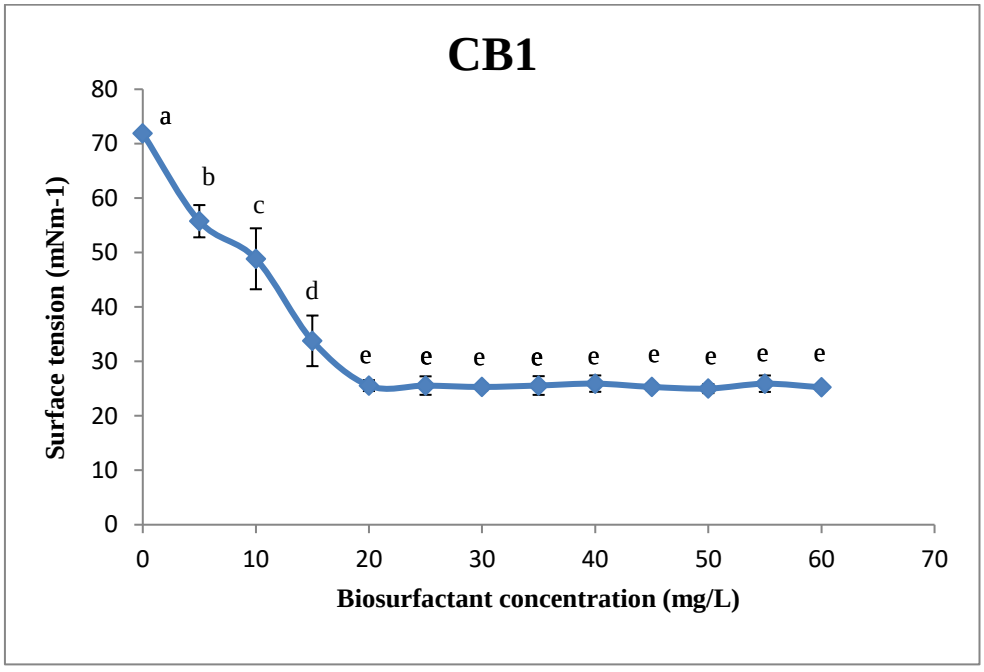

Fig 38: Determination of the critical micelle concentration (CMC) of CB1

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; p < 0.05 and subsequent post hoc multiple comparison with Tukey HSD test).

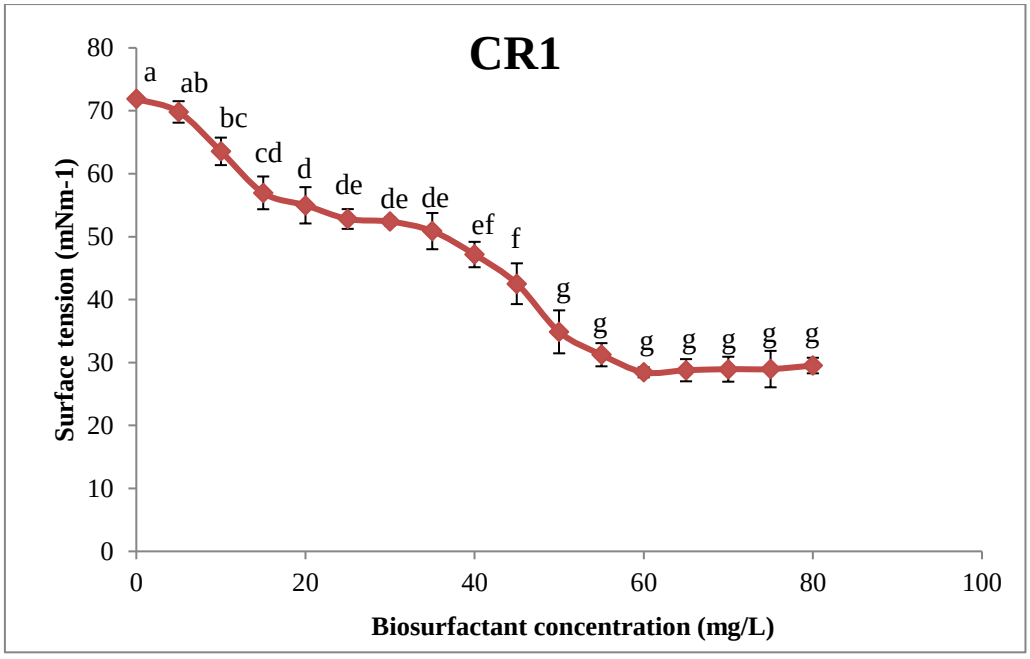

Fig 39: Determination of the critical micelle concentration (CMC) of CR1

Each value is the mean ± SD of three replicates (n = 3).Within each column, means with different superscript letters are statistically significant (ANOVA; $p < 0.05$ and subsequent post hoc multiple comparison with Tukey HSD test).

4.9 Stability studies of biosurfactants

The effect of temperature showed that there was no change in biosurfactant activity of compound CB1 with a change in temperature. The activity remained stable over a range of temperature i.e. 4 to 90°C. It was also stable on autoclaving at 121°C, 15 lbs pressure for 20 min. On other hand, the activity of CR1 remained stable at 4 – 70 °C but lost its activity when exposed to high temperature i.e. 90°C. The activity of CR1 decreased completely when exposed to pH 10 whereas CB1 retained its activity over a pH range of 4 to 14. At the lower end of pH scale (<4) the activity was decreased due to precipitation of surfactant. The effect of NaCl showed that the activity of CB1 remained stable even after an exposure to high NaCl range i.e. up to 20 % (w/v) whereas CR1 shown gradual decrease in its activity with increasing NaCl concentration and lost its activity when exposed to 10 % (w/v) sodium chloride.

4.10 Mass spectrometric analysis

Intense signals in the m/z ranges 1,000–1,100 were obtained in the MS spectra of the lipopeptide mixture. As seen in Fig. 40, peaks 1008.67, 1022.65, corresponds to $[M+H]^+$ of C_{13} and C_{14} surfactin, Peaks 1030.65, 1044.7, 1058.69, 1072.6, corresponds to $[M+Na]^+$ C_{13}, C_{14}, C_{15} and C_{16} surfactin and peaks 1046.59, 1074.63 corresponds to $[M+K]^+$ of C_{13} and C_{15} surfactin. Out of all the peaks 1030.65, 1044.7, 1058.69 and 1072.68 were prominent peaks of which 1044.7, 1058.69 were most intense peaks. When the monoisotopic mass was calculated of the obtained peaks, they were similar to the mass of Surfactin isomers A-D (Table 20 – 22). The difference between the precusor ions $[M + Na]^+$ at m/z 1030.6, 1044.7, 1058.7 and 1072.7 is 14 Da correspond to Surfactin isoforms differing by an acid chain length (m/z of CH_2 = 14 Da). Thus, biosurfactant (CB1) produced by halotolerant *Bacillus amyloliquefaciens* SK27 was characterized as Surfactin homologue.

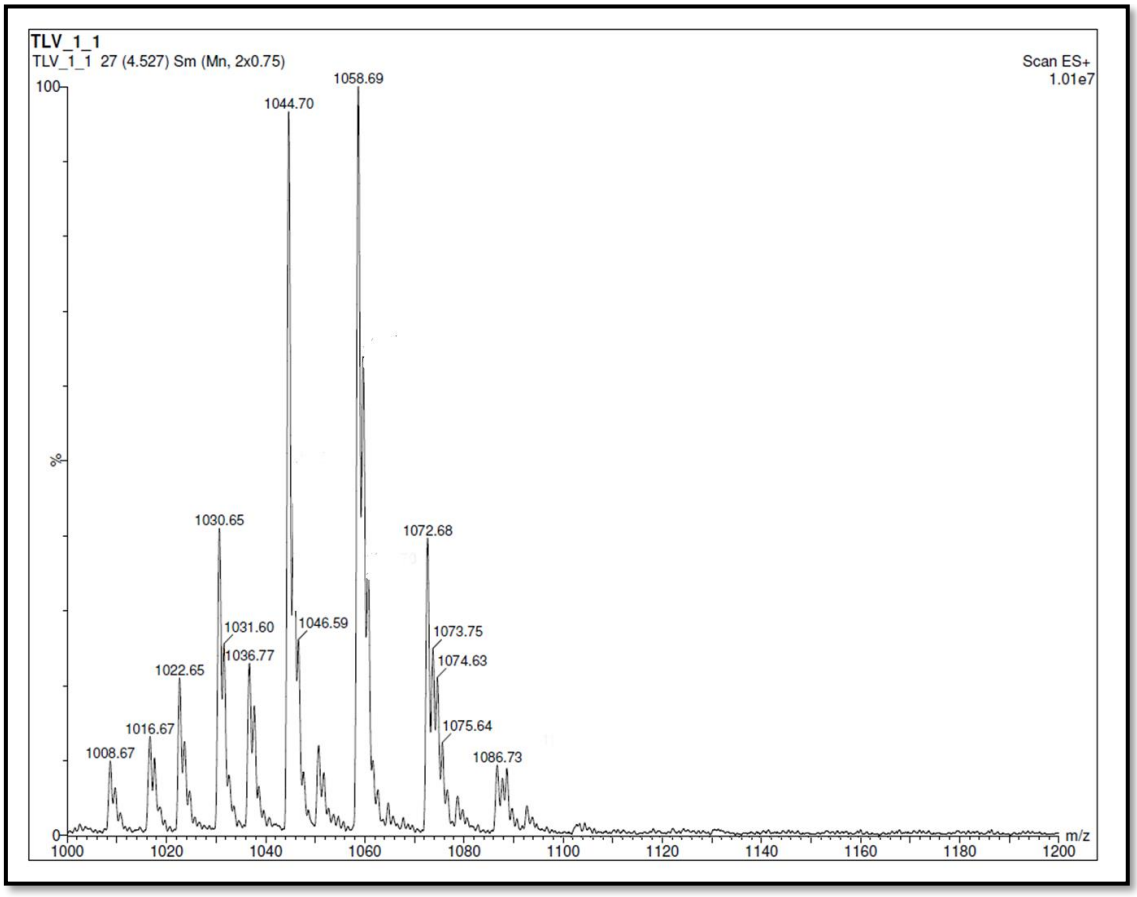

Fig 40: ESI-Mass spectra of fraction CB1 from SK27

Table 20: Comparison of obtained peaks $[M+H]^+$ with the database

Sr. No	Obtained $[M+H]^+$	Compound	PubChem CID	Exact Mass (g/mol)	Monoisotopic mass (g/mol)
1	1008.65	Surfactin A (C_{13})	70789014	1007.65	1007.64
2	1022.65	Surfactin B (C_{14})	46226665	1021.67	1021.64

Mass of H^+ – 1.00784

Table 21: Comparison of obtained peaks $[M+Na]^+$ with the database

Sr. No	Obtained $[M+Na]^+$	Compound	PubChem CID	Exact Mass (g/mol)	Monoisotopic mass (g/mol)
1	1030.65	Surfactin A (C_{13})	70789014	1007.65	1007.65
2	1044.7	Surfactin B (C_{14})	46226665	1021.67	1021.7
3	1058.69	Surfactin C (C_{15})	443592	1035.68	1035.69
4	1072.68	Surfactin D (C_{16})	70789015	1049.69	1049.68

Mass of Na^+ – 22.989769

Table 22: Comparison of obtained peaks $[M+K]^+$ with the database

Sr. No	Obtained $[M+K]^+$	Compound	PubChem CID	Exact Mass (g/mol)	Monoisotopic mass (g/mol)
1	1046.59	Surfactin A (C_{13})	70789014	1007.65	1007.63
2	1074.63	Surfactin C (C_{15})	443592	1035.68	1035.67

Mass of K^+ – 38.963706

CHAPTER 5

DISCUSSION

5. DISCUSSION

Surfactants are one of the most versatile groups of chemicals used in various industrial processes. The market is competitive, and there is a need to expand eco-friendly and cost effective for the production of surfactants. The focus presently is to increase the production of biosurfactants. The structural diversity and functional properties of various reported biosurfactants make them an attractive set of compounds used for a broad range of industrial, biotechnological and environmental applications.

Screening methods make the task easier for the isolation of the biosurfactant producing microorganisms. However due to the diversity of biosurfactants, the screening methods differ. There are several methods (qualitative and quantitative) available with variable precisions and aim for screening of potential biosurfactant producers. The most common methods for screening the producing strains are based on the physical effects of biosurfactants. Therefore, choosing a right set of screening methods is crucial.

In the present study, 300 nos. halotolerant bacteria were screened using 3 methods i.e. emulsification index, drop collapse method and parafilm M test. One of the functions of biosurfactants is to enhance the contact between two immiscible liquids which is measured by an emulsification index. The use of one single method that measures properties other than the surface activity can be challenging. The ability of a molecule to form a stable emulsion is not always associated with surface activity. Studies have suggested that the biosurfactant producing isolates can also produce bioemulsifiers but not vice versa. Bioemulsifiers emulsify two immiscible liquids such as hydrocarbons or other hydrophobic substrates even at low concentrations but in contrast are less effective in reducing the surface tension. Therefore, they possess emulsifying activity and no surface activity (Uzoigwe et al., 2015). Thus emulsification activity gives a preliminary indication for biosurfactant production by the isolates and can be used only for primary screening of biosurfactant.

Bacteria (300 nos.) were further screened for production of biosurfactants using drop collapse method and parafilm M test which gave us an indication of surface and interfacial activity. Some of the cultures with high emulsification exhibited a decreased activity in drop collapse method and parafilm M test. Previous reports by Satpute et al., (2008) and Thavasi et al., (2011) have suggested that drop collapse method and parafilm

M test are reliable methods for screening biosurfactant production from diverse microorganisms. However, these methods signify the presence of a biosurfactant in the culture supernatant and thus can be used as a qualitative method.

The oil spreading method relies on oil clearing zone. Formation of a clear zone on the oil zone is a unique feature of surfactant which provides an indicator to evaluate the performance of biosurfactant producing bacteria. Studies have shown a linear correlation between quantity of surfactant and clearing zone diameter (Walter et al., 2010). Youssef et al., (2004) reported increase in the diameter of the clear zone with the increasing concentration of surfactin over a wide range. Also it demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms (Plaza et al., 2006). In our study, 44 cultures were found positive for primary screening and were also positive for oil spreading assay. Further, some of the bacterial isolates which showed good activity in primary screening showed a low oil clearing zone which indicates low concentrations of biosurfactant. Hence, using this method for secondary screening would help in eliminating any false positives with low concentrations of biosurfactant.

Thus we deduced that, combining methods was a better strategy for a successful screening of biosurfactants. Our study suggests that all the 4 methods are easy and reliable for screening halotolerant bacteria for biosurfactant production.

5.1. FACTORS AFFECTING BIOSURFACTANT PRODUCTION

Despite the numerous advantages and industrial applications, the use of biosurfactants has been limited because of their low yields and high production cost (Mukherjee et al., 2006). One of the methods to overcome these limitations is the selection of appropriate nutrients at their right concentration and ideal culture conditions for growth and biosurfactant production (Kim et al., 2013). Media composition not only influences the growth and metabolism of the culture, but also increases the product yield. For designing a production medium, the most suitable fermentation conditions (e.g., pH, temperature, agitation speed, etc.) and the appropriate medium components (e.g., carbon, nitrogen, etc.) must be identified and optimized accordingly (Singh et al., 2019).

Thus in the present study, we studied the biosynthesis of the biosurfactant produced by SK27 and RMSK10 identified as *Bacillus amyloliquefaciens* and *Bacillus subtilis* respectively and the physiological parameters and nutrients affecting its production with a view to maximise the production of the biosurfactant, thereby maximizing its surface activity and determination of optimum fermentation conditions.

It was observed that both SK27 and RMSK10 showed maximum biosurfactant production at 30±2°C. Previous reports suggest that the temperature for maximum biosurfactant productivity by *Bacillus* sp was found to be between 25 °C – 37 °C (Lee et al., 2007, Mouafi et al., 2016, Barakat et al., 2017). Previous reports suggest that highest yield of biosurfactant by *Bacillus amyloliquefaciens, Bacillus subtilis, Pseudomonas aeruginosa* was observed at the range of pH 5–7. Any change to either lower or higher pH values caused an substantial drop in biosurfactant production (Daverey et al., 2009, Sahoo et al., 2011, Sharma et al., 2020) Although SK27 and RMSK10 could grow in a medium of a broad range of pH (5 to 9), biosurfactant production varied from pH 5.5 to 7.5 i.e. mildly acidic to mild alkaline medium. The maximum biosurfactant production for SK27 was observed at pH 6.5 and for RMSK10 pH 7.

Out of the ten different carbon sources used, the maximum biosurfactant production by SK27 was observed in sucrose followed by mannitol and glucose. Liu et al., (2012) too reported that sucrose was the best carbon source for *Bacillus amyloliquefaciens* among the water soluble carbon sources like glucose, galactose, maltose, glycerol, mannitol, soluble starch and dextrin for biosurfactant production. In addition, Singh et al., (2014) reported that Sucrose is the most suitable carbon source for production of a lipopeptide biosurfactant. Presence of an array of glycoside hydrolases in *Bacillus amyloliquefaciens* species bestows these microorganisms to utilize diverse range of carbohydrates. In RMSK10, the maximum biosurfactant production was observed when starch was used as a carbon source followed by glycerol and glucose. This observation is contrary to the various reports where glycerol and glucose was highlighted as the most efficient carbon source for biosurfactant production by *Bacillus subtilis* (Das et al., 2009, Al-Wahaibi et al., 2014, Zhu et al., 2016). Strain SK27 formed minimum clear zone when grown on paraffin and sodium citrate whereas for RMSK10 a minimum clear zone was observed in paraffin, lactose, sodium citrate and fructose. This indicates that these bacteria exhibited a preference to selective carbon sources for an optimal

biosurfactant production showing that sucrose and starch were the most preferred carbon sources for the production of biosurfactant. The majority of known biosurfactants are synthesized from water-immiscible hydrocarbons; however, *Bacillus subtilis* strains are able to produce surfactants from water-soluble substrates. Moreover, it was previously reported that the addition of a hydrocarbon to culture medium completely inhibited surfactin production (Cooper et al., 1981). Water-soluble substrates are cheaper than hydrocarbons and are preferred because single-phase fermentation is simpler than biphasic fermentation (Makkar and Cameotra, 1997). Different water soluble carbon source not only influenced the biosurfactant yield but also exerted note worthy effect on lipopeptides composition, surface activity, emulsification behaviour (Singh et al., 2014). Moreover, hydrocarbon substrates are unacceptable for many applications, such as in food, cosmetics, and pharmaceutical products. (Nitschke and Pastore, 2006).

The synthesis of biosurfactant is closely related to the metabolism of nitrogen resources provides raw materials to produce the protoplasm and other cellular structures. The nitrogen resources have a critical role in microbial growth and biosurfactant production. Ten different nitrogen sources, both organic and inorganic, were also evaluated individually for biosurfactant production by SK27 and RMSK10. SK27 showed maximum biosurfactant production in yeast extract, as also observed by Sharma et al., 2020, Sharma and Pandey, 2020 in *Bacillus* sp whereas other researchers presented sodium nitrate as the optimal nitrogen source for biosurfactant production (Venkatesh and Vedaraman, 2012; Pereira et al., 2013). On the contrary, the preferred nitrogen source for RMSK10 was peptone followed by yeast extract. Fig 9 and 10 thus shows that organic nitrogen sources were more conducive to bacterial growth than inorganic nitrogen sources tested. Higher oil displacement activity was obtained with organic nitrogen sources consistently. These results showed that strain SK27 and RMSK10 preferentially utilized organic nitrogens viz. yeast extract and peptone respectively to produce biosurfactant and showed a lower production with inorganic nitrogen sources.

Thus our study revealed that carbon and nitrogen concentrations influence the biosurfactant production. The ratio of carbon source to nitrogen source (C/N ratios) is a fundamental factor in improving the yield of biosurfactants (Khopade et al., 2012, Liu et al., 2017). Maximum activity was found at 0.8% yeast extract with 1% sucrose in the medium for SK27 and 1% peptone with 1% starch for RMSK10. The growth of bacteria is influenced by salt concentration which also affects biosurfactant production. Since the

cultures SK27 and RMSK10 were halotolerant, the influence of salinity on the production of biosurfactant was evaluated. The osmotic pressure of culture solution increases greatly with increase in NaCl concentration (Greenway and Osmond, 1972). Microorganisms generally grow well in isotonic solution. Under hypotonic conditions a large number of water molecules penetrate in microbial cell, leading to swelling and rupture, under hypertonic conditions, cells will undergo plasmolysis. Fig 15 shows that in the absence of NaCl strain SK27 and RMSK10 almost had minimal growth and its biomass was substantially lower than that of other NaCl concentrations. The poor growth leads to less production of biosurfactant from bacterium. When 2 g/L was supplied, the diameter of clear zone was only 4.5 cm and the highest salinity of 40 g/L might have negatively affected the surface activity of biosurfactant produced exhibiting a zone of 2.6 cm and 3.4 cm for SK27 and RMSK10 respectively. In this experiment the goal was the optimal NaCl concentration. According to the results 6 g/L was selected as optimum NaCl concentration for biosurfactant production from SK27 and RMSK10 respectively. It was observed that when the growth medium was devoid of NaCl there was an absence in biosurfactant production which implies the need of NaCl for the biosynthesis for the biosurfactant. Though, both the isolates SK27 and RMSK 10 were halotolerant. This complements the study by Ballav (2016) and Fernandes (2019), where they suggested that the production of bioactive compounds of marine microorganisms is too affected by the sodium chloride concentration.

It is intriguing finding that the production of the biosurfactant by SK27 was initiated within 24 h and was constantly produced during the stationary phase with an optimum production in the stationary phase and remained constant from 24 h to 40 h which is a quite early production as compared to other reports. Similarly, the biosurfactant produced by RMSK10 initiated within 28 h and the production was stable up to 48 h. Lee et al. (2007) reported the production of biosurfactant by *Bacillus amyloliquefaciens* strain LP03 after 5 days. Sharma et al. (2020) reported that good lipopeptide production by *Bacillus amyloliquefaciens* SAS-1 and *Bacillus subtilis* BR-15 was observed from 48–96 h. In 2015, Zhang et al. reported that the maximum production of biosurfactant from *Bacillus amyloliquefaciens* was observed at 48 h. This property gives the biosurfactant produced by SK27 and RMSK10 an added value in accelerating the production of the biosurfactant and thus in-turn reducing the economics of the fermentation process.

One-factor-at-a-time method (OFAT) was used to choose the most influential parameters which affect the biosurfactant production. The OFAT analysis concluded that carbon and nitrogen source concentrations as well as sodium chloride concentrations plays a significant role in the production of biosurfactant. According to the results of OFAT, 1% sucrose, 0.8 % yeast extract and 0.6 % NaCl were selected to improve the biosurfactant production of SK27 however 1 % starch, 1 % peptone and 0.6 % NaCl were selected for RMSK10. This method successfully led to about 1.8 fold increase in the yield by SK27 and RMSK10 as compared to the basal medium. A numbers of studies (Jamal et al., 2012, Irfan et al., 2014) have shown that OFAT is very useful for preliminary screening of appropriate nutritional and cultural conditions for maximizing the production. However, OFAT alone failed to extrapolate the combination of all the factors together in the media. Thus, a combination of Response Surface Methodology (RSM) and Genetic Algorithm (GA) tools together made it easier to evaluate the interaction of all the variables on the biosurfactant production by SK27 and RMSK10. We proposed a significant and consistent quadratic model for the production of the biosurfactant with 0.97 % sucrose, 0.89 % yeast extract and 0. 56% sodium chloride for SK27 and 1.05 % starch, 0.95 % peptone and 0.62 % sodium chloride for RMSK10 with keeping pH (6.5 for SK27 and 7 for RMSK10) and temperature (30±2 °C) constant. A high R^2 value of 0.9983 and 0.9857 also supported the significance of the model and substantiated a good correlation between the individual factors. The interactive effects of all the three variables were found to be significant in case of SK27 and RMSK10 and thus can be used as limiting nutrients for altering the biosurfactant yield. The concentration of each variable above the optimized concentration showed a decline in activity.

The RSM model was also validated using Genetic algorithm to obtain the best fermentation parameters which provides a fitness function to determine the optimum concentration of the media components. The nonlinear regression equation of oil displacement activity was optimized using GA optimization tools. The activity was found to be 8.76 for SK27 and 8.7 for RMSK10 which was close to that predicted by RSM. The application of RSM–GA hybrid statistical approaches thus resulted in a significant enhancement in biosurfactant production, which gave a 2 – fold increase in the yield of biosurfactants. Statistical approaches for optimization bioprocess for antibiotic or other metabolites and growth yield have been performed frequently

(Sathiyanarayanan et al., 2014, Manivasagan et al., 2014). However, there are no reports available on media optimization for maximizing the yield of biosurfactant from *Bacillus* spp. using RSM–GA hybrid method, which we found as a valuable tool for enhancing production of biosurfactant.

5.2. BIOSURFACTANT FROM *BACILLUS spp.*

Over the years *Bacillus* is known to produce several important biomolecules with applications in various fields of biotechnology. Many strains within *Bacillus* genus are able to produce various bioactive compounds (e.g. proteins, peptides, and lipopeptides) of commercial importance (Jacques 2011). They are considered to be prodigious producers of industrial important enzymes such as proteases, α-amylases, and other macromolecular hydrolases. Some of these compounds are of ribosomal origin like subtisilin, subtilosin A, TasA, and sublancin and other compounds such as bacilysin, chlorotetain, mycobacillin, rhizocticins, bacillaene, difficidin, and lipopeptides are formed by non-ribosomal peptide synthetases and/or polyketide synthases (Urdaci and Pinchuk, 2004, Leclere et al., 2005) A large proportion of the secondary metabolites produced by the *Bacillus* isolates are cyclic lipopeptides termed as biosurfactants as they exhibit excellent surface and emulsification activity.

Lipopeptide biosurfactants from *Bacillus* species are known as one of the most efficient microbial surfactants. They are classified into three families i.e. Surfactin, Iturin and Fengycin. There structure includes a fatty acid in combination with a peptide moiety. The length of the fatty acid chain varies from C_{13} to C_{16} for surfactins, from C_{14} to C_{17} for iturins, and from C_{14} to C_{18} in the case of fengycins (De Souza et al., 2018, Bartal et al., 2018). The surfactin family encompasses the heptapeptide variants of the lichenysin, pumilacidin and surfactin groups (Raaijmakers et al., 2010, Miljakovic et al., 2020). Within the iturin family, iturin A and C, bacillomycin D, F and L, and mycosubtilin are the six main variants. The third family comprises fengycins A and B, also referred to as plipastatins if Tyr9 is D-configured (Olishevska et al., 2019). Due to their natural diversity lipopeptides may be synthesized as a mixture of homologues and isoforms differing in the length of the fatty acid chain and in the amino acid composition of the peptide sequence (Pecci et al., 2010).

The *Bacillus amyloliquefaciens* SK27 isolate produced 4 isomers which were synergistically active as a biosurfactant. The ESI-MS of the most active fraction (CB1) gave a mixture of compounds, and the analysis suggested metabolites ranged with the m/z ratio between 1008 to 1074 Da. The m/z ratios of the metabolites were also compared to other studies which suggested that the obtained peaks correspond to protonated, sodiated and potassium adducts of C_{13}, C_{14}, C_{15} and C_{16} Surfactin, where they also compared the peaks with the Surfactin standard (Sigma) (Vater et al., 2002, Jemil et al., 2018, Sarwar et al., 2018, Yuan et al., 2018). Further, the exact mass of the each compound was calculated and compared to the database (PubChem) which showed that the compounds obtained corresponds to the Surfactin A-D with fatty chain C_{13} - C_{16}. Additionally, among the four isomers, Surfactin B and Surfactin C were present in higher quantities as compared to Surfactin A and D.

Among the lipopeptides, Surfactin is known as the most powerful biosurfactants. Surfactin as a secondary metabolite was first reported from a cultivation broth of *Bacillus subtilis* in 1968 and consists of four isomers (A-D) and exhibits various physiological activities, including as an inhibitor of fibrin clotting and as a cell lysate agent (Arima et al., 1968). It is produced as a result of non ribosomal biosynthesis catalysed by a large multienzyme complex consisting of four modular building blocks, called Surfactin synthetase (Płaza et al., 2015). The chemical structure of surfactin has a common peptide loop of seven amino acids (l-asparagine, l-leucine, glutamic acid, l-leucine, l-valine and two d-leucines) with a long hydrophobic fatty acid chain. Also according to the literature data, Surfactin is synthesized as a mixture of the three or four homologues which are synergistically active as a biosurfactant (Liu et al., 2015, Wu et al., 2017, Yan et al., 2020).

Li et al., (2012) reported the production of four surfactin homologues (from C_{13} to C_{16}) by *Bacillus licheniformis* cultivated in medium with glucose, yeast extract and ammonium chloride. Bacon et al., (2012) showed that some of 29 studied *Bacillus mojavensis* strains synthesized even seven surfactin homologues (with acyl chain lengths ranging from C_{11} to C_{17}). In 2018, Sarwar et al. also reported that *Bacillus* subtilis produces isomers of Surfactin with C_{12} to C_{16} acyl chains. Additionally, Laird et al., (2019) also revealed the presence of three surfactin homologues (C_{13} to C_{15}) from *Bacillus* velezensis wherein surfactin B and surfactin C were present in greater quantities than surfactin A. Biosurfactants produced by Marine *Bacillus* sp. CS30

isolated from cold seep in deep sea belonged to the category of surfactin with presence of four isoforms of Surfactin (C_{13} to C_{16}) (Wu et al., 2019).

5.3. SURFACE PROPERTIES OF BIOSURFACTANTS

Because of their amphiphilic nature, biosurfactants accumulate at the interface between two immiscible liquids or between a liquid and air. At the interface, surfactants align themselves so that the hydrophobic part is in air (or oil) and hydrophilic part in water. By reducing surface tension (liquid-air) they reduce the repulsive forces between two dissimilar phases and allow these two phases to mix and interact more easily (Whang et al., 2008; Varjani et al., 2014). The most efficient biosurfactant decreases the surface tension of water to a value less than or equal to 30 mN/m from 72 mN/m. In the present study, the Surfactin produced by halotolerant *Bacillus amyloliquefaciens* (SK27) was able to reduce the surface tension of water from 72 mN/m to 25.56 mN/m at a concentration of 20 mg/L. This concentration of Surfactin was denoted as the critical micelle concentration (CMC) which is the minimum concentration required reducing maximum surface tension of water to 25.56 mN/m.

Surface active agents at low concentrations are present as individual molecules and bring about changes in surface tension (Batista et al., 2006; Rabiei et al., 2013). However, as the surfactant concentration is increased, a critical concentration is reached beyond which surfactant molecules aggregate to form micelles and further no change in surface properties is noticed. This concentration of the surfactant is known as critical micelle concentration (CMC). The CMC of surfactant is an indicator of its surfactant capacity (Desai and Banat, 1997; Satpute et al., 2010; Souza et al., 2017).

Cooper et al., (1981) reported a surfactin from *Bacillus subtilis* (ATCC 21332) with a CMC of 25 mg/l and minimal surface tension of 27.9 mN/m. Nitschke and Pastore, (2006) reported that surfactin from *Bacillus subtilis* strain decreased the surface tension of water from 72 mN/m to 26.6 mN/m at a CMC of 33 mg/L. Also the surfactin obtained from *Bacillus subtilis* was able reduce the surface tension of water to 28.69 mN/m from 70.54 at a CMC of 20.73 mg/L (Rocha et al., 2020). Jakinala, et al., (2019) has reported a Surfactin obtained from *Bacillus velezenis* reduced the surface tension of water to 33.2 mN/m from 72.12 with CMC of 40 mg/L. Hentati et al., (2019) have

reported surface tension reduction value of 34 mN/m and a CMC of 50 mg/L for surfactin produced by *Bacillus stratosphericus*.

Thus we conclude that the Surfactin produced by SK27 comparatively has an much enhanced surface activity than the reported values; suggesting it to be a potential surface-active agent.

5.4. BIOSURFACTANT MEDIATED BIOREMEDIATION OF HEAVY METALS

Several studies have been executed to evaluate the possibility of metal removal by biosurfactants from the soil as well as from aqueous solutions due to their anionic characteristics and properties like emulsification, solubilization, and complex formation with metal ions (Sarubbo et al., 2015).

Preliminary bioremediation studies were carried out using five different heavy metals viz. cadmium, chromium, iron, lead and zinc at a concentration of 100 ppm. The metal-biosurfactant precipitate was observed after addition of biosurfactants (CB1 and CR1) to Fe^{+3} and Pb^{+2} metal solutions followed by proper incubation (24 h). The bioremediation efficiency of the biosurfactants CB1 and CR1 was validated by using ICP-AES. The ICP-AES results showed that the Fe^{+3} and Pb^{+2} concentrations in the solution obtained after biosurfactant treatment (CB1 and CR1) was significantly lower compared to initial concentration of the metal while the concentration of other 3 metals (Cr, Cd, and Zn) in the solution was similar to that of the starting concentration of metals. Thus, biosurfactants (CB1 and CR1) were able to selectively remediate Fe^{+3} and Pb^{+2}. Further, studies were carried out by varying the concentration of Fe^{+3} and Pb^{+2} (100, 200 and 300 ppm) and also the concentration of the biosurfactants.

The ICP-AES analysis showed that the bioremediation efficiency of biosurfactant CB1 was better as compared to that of CR1. CB1 and was able to remediate 97.5% of Fe^{+3} and 91% of Pb^{+2} at concentration of 40 mg/L whereas the bioremediation efficiency of CR1 was lower at 40 mg/L. The bioremediation of CR1 increased as its concentration was increased to 80 mg/L. CB1 at 100 mg/L and CR1 at 160 mg/L were able to remediate completely 300 ppm of Fe^{+3} and Pb^{+2}. When a chemical surfactant i.e. EDTA was used for bioremediation of Fe^{+3} and Pb^{+2}, it didn't not show any remediation till 200

mg/L concentration however when the concentration increased to 2000 mg/L, it was able to remove 88.5% of Fe and 29% of Pb^{+2}. Studies showed that a lipopeptide biosurfactant produced by marine *Bacillus circulans* at 200 mg/L was able to remediate 100 ppm of lead (Das et al., 2009). Another study showed that when 1,000 mg/L of the lipopeptide was used to treat 100 mg/L of Pb^{2+}, Fe^{3+}, Fe^{2+}, the highest removal rate was found for Pb^{2+} (80%) and Fe^{3+}, Fe^{2+} was around 40% (Zhao et al., 2020). The result shows that the biosurfactant obtained from our *Bacillus* spp. from marine salterns has much enhanced bioremediation efficiency for Fe^{+3} and Pb^{2+} as compared to the previously reported ones, showing its selectivity to only remediate certain metals from aqueous solutions. It is imperative to decrease the cost of the bioremediation process, one of which is very effective is using microorganism for the production of biosurfactant. Biosurfactant which generally are lipopeptide have characteristics of low cost, are environmentally friendly and are produced in abundant. The fermentation of *Bacillus amyloliquefaciens* and *Bacillus subtilis* can be carried out with cheap substrates like agricultural and industrial by products.

In general, temperature affects the removal efficiency of heavy metal ions in water (Li et al., 2017). Thus in the present study, we considered studying the effect of temperature on bioremediation efficiency of biosurfactant essential. The remediation of Fe^{3+} and Pb^{2+} with the biosurfactant CB1 to remained stable from temperature 4°C to 70°C with a slight decrease in the 25% of efficiency at low temperature (4°C) and 12 % at high temp of 70°C. The decrease in the bioremediation efficiency at low temperature may be due the decrease in diffusion coefficient of metal ion, which also affects the ability of lipopeptides to remove metal ions. Previous studies have shown that at 4°C, the ability of lipopeptides to remove Pb^{2+} reduced to 35% and was minimal as the temperature was increased (60°C), which could be due to the denaturation of lipopeptides thus reducing their ability to remove metal ions (Zhao et al., 2020, Yuan et al., 2019). In our study, the biosurfactant CB1 was able to remediate heavy metals at a range of 4°C to 60 °C and even at high temperatures (70 °C) which suggest that the biosurfactant is stable and also retains its activity at high temperature. This bioremediation study provides an economically viable method in the removal of Pb^{2+} and Fe^{3+} from water bodies, aquifers and impoundments.

As biosurfactants are often involved in natural and industrial processes under extreme conditions of pressure, ionic strength, pH, salinity, and organic solvents, it is necessary to evaluate the stability of the biosurfactant under such conditions (Makkar and Cameotra, 1998). When the stability of the biosurfactant at different temperatures, pH and NaCl concentration was carried out the biosurfactant CB1 was stable even after autoclaving at 121°C, 15 lbs pressure for 20 min. Additionally, it was also stable from pH 4-14 and 0-20% NaCl concentration. It has been reported by Sarwar et al., (2018) that the activity of surfactin purified from *Bacillus subtilis* reduced by 17% upon autoclaving. The surfactin produced by *Bacillus velezensis* was stable till 6% of NaCl and the surfactin activity decreased with an increase in NaCl concentrations with an 80 % loss in the activity at 20% NaCl. Similarly with a variation in pH, the surface activity of surfactin remained stable from pH 6 to 12. However, at pH 4 the activity decreased completely (Guimaraes et al., 2019).

These findings revealed that the biosurfactant obtained from our *Bacillus* spp. could be very useful in extreme conditions of temperature, salinity and alkaline pH and could be explored on operations for enhancing oil recovery and bioremediation of marine environments containing heavy metals.

SUMMARY

SUMMARY

The thesis on **—** "**Bacteria from Goan salterns as producers of a biosurfactant"** explores the potential of bacteria from marine salterns producing biosurfactants. The salient features of our research are presented below:

1. Bacteria (300) from marine salterns were screened for the production of biosurfactants.
2. Three different methods viz. drop collapse method, parafilm M test and emulsification assay were used to detect the presence of biosurfactants.
3. Secondary screening was carried out using oil spreading method where the diameter of the clearing zone was proportional to the biosurfactant concentration where isolates SK27 and RMSK10 were found to be the potential isolates producing maximum amounts of the biosurfactants.
4. Media optimization for biosurfactant production of SK27 and RMSK10 was carried out using Classical approach, Statistical approach and Genetic algorithm where the bacteria was able to produce the biosurfactant at 24 hr and 28 hr incubation time respectively.
5. Bacterial isolates SK27 and RMSK10 were both spore forming bacteria and were characterized as *Bacillus amyloliquefaciens* and *Bacillus subtilis* by classical and molecular methods.
6. This is the first report which uses a Statistical approach along with genetic algorithm (RSM-GA) for enhancing the production of the biosurfactant by *Bacillus* sp.
7. The Biosurfactants (CB1 and CR1) were able to remediate Fe and Pb upto 300 ppm from aqueous solutions at concentrations of 100 mg/L and 120 mg/L respectively.
8. ESI-MS analysis demonstrate that halotolerant *Bacillus amyloliquefaciens* SK27 produced isomers of surfactin with C_{13}, C_{14}, C_{15} and C_{16} acyl chains with m/z 1030.6, 1044.7, 1058.6, 1072.6 which was synergistic in its action as a biosurfactant.

9. On comparison with other biosurfactants from literature, the biosurfactant produced by *Bacillus amyloliquefaciens* SK27 was characterized as a Surfactin homologue with C_{14}-surfactin, C_{15}-surfactin as the most abundant components.
10. The biosurfactant CB1 from *Bacillus amyloliquefaciens* SK27 was novel in being active even after autoclaving (121°C, 15 lbs for 20 min), and was active at a broad pH range of 4 to 14, salinity (0 – 200 psu) and bioremediation efficiency up to 70 °C.
11. The activity of the biosurfactant was assessed using a stalagmometer and was found to decrease the surface tension of water only with 20 mg/L which is a very low concentration and is less than the values so far reported of biosurfactants produced by *Bacillus subtilis.*

CONCLUSION

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1. The halotolerant bacterial isolates viz. *Bacillus amyloliquefaciens* and *Bacillus subtilis*, isolated from Ribandar and Agarwado salterns respectively produced biosurfactants with enhanced activity.
2. The result of this study indicates that the biosurfactant from SK27 can efficiently reduce the surface tension and also has high oil displacement efficiency. This finding has a practical advantage as this kind of biosurfactant promotes the degradation of oil which may result in a wider application of the biosurfactant for bioremediation .
3. The biosurfactant from *Bacillus amyloliquefaciens* strain SK27 was identified as "Surfactin" comprising of 4 isomers with synergistic activity.
4. "Surfactin" as a biosurfactant exhibited unique properties in having a CMC of 20 mg/L, temperature stability at 121°C at 15 lbs, pH stability from 4 to14 and tolerance to NaCl from 0 to 200 psu.
5. The bioremediation potential of CB1 & CR1 biosurfactants to 300 ppm of dissolved Pb^{2+} and Fe^{3+} was almost 100%, confirming its function as a potential metal bioremediator.

BIBLIOGRAPHY

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- Abalos, A., Pinazo, A., Infante, M. R., Casals, M., Garcia, F., & Manresa, A. (2001). Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soybean oil refinery wastes. *Langmuir*, *17*(5), 1367-1371.
- Abu-Ruwaida, A. S., Banat, I. M., Haditirto, S., & Khamis, A. (1991). Nutritional requirements and growth characteristics of a biosurfactant-producing *Rhodococcus* bacterium. *World Journal of Microbiology and Biotechnology*, *7*(1), 53-60.
- Acmite Mark (2016). Global surfactant market. Market report. In: Acmite Mark. Intell. http://www.acmite.com/brochure/Brochure-GlobalSurfactant-Market-Report.pdf. Accessed 6 Sept 2018
- Akintunde, T. A., Abioye, O. P., Oyeleke, S. B., Boboye, B. E., & Ijah, U. J. J. (2015). Remediation of iron using rhamnolipid-surfactant produced by *Pseudomonas aeruginosa*. *Research Journal of Environmental Sciences*, *9*(4), 169.
- Allef, P., Hartung, C., Schilling, M. (2014.) Aqueous hair and skin cleaning compositions comprising biosurfactants. *Patent US 20140349902 A1*.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, *215*(3), 403-410.
- Alvarez, V. M., Jurelevicius, D., Marques, J. M., de Souza, P. M., de Araújo, L. V., Barros, T. G. & Seldin, L. (2015). *Bacillus amyloliquefaciens* TSBSO 3.8, a biosurfactant-producing strain with biotechnological potential for microbial enhanced oil recovery. *Colloids and Surfaces B: Biointerfaces*, *136*, 14-21.
- Al-Wahaibi, Y., Joshi, S., Al-Bahry, S., Elshafie, A., Al-Bemani, A., & Shibulal, B. (2014). Biosurfactant production by *Bacillus subtilis* B30 and its application in enhancing oil recovery. *Colloids and Surfaces B: Biointerfaces*, *114*, 324-333.
- Arima, K. (1968). Surfactin, acrystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun.*, *31*, 488-494.

- Arutchelvi, J. I., Bhaduri, S., Uppara, P. V., & Doble, M. (2008). Mannosylerythritol lipids: a review. *Journal of industrial microbiology & biotechnology*, *35*(12), 1559-1570.
- Aşçı, Y., Nurbaş, M. A. C. İ. D., & Açıkel, Y. S. (2007). Sorption of Cd (II) onto kaolin as a soil component and desorption of Cd (II) from kaolin using rhamnolipid biosurfactant. *Journal of Hazardous Materials*, *139*(1), 50-56.
- Aşçı, Y., Nurbaş, M. A. C. İ. D., & Açıkel, Y. S. (2008b). A comparative study for the sorption of Cd (II) by K-feldspar and sepiolite as soil components, and the recovery of Cd (II) using rhamnolipid biosurfactant. *Journal of Environmental Management*, *88*(3), 383-392.
- Aşçı, Y., Nurbaş, M., & Açıkel, Y. S. (2008a). Removal of zinc ions from a soil component Na-feldspar by a rhamnolipid biosurfactant. *Desalination*, *223*(1-3), 361-365.
- Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E. M., Linder, T., Wawrosch, C., Uhrin, P. & Stuppner, H. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology advances*, *33*(8), 1582-1614.
- Ayangbenro, A. S., & Babalola, O. O. (2018). Metal (loid) bioremediation: strategies employed by microbial polymers. *Sustainability*, *10*(9), 3028.
- Bacon, C. W., Hinton, D. M., Mitchell, T. R., Snook, M. E., & Olubajo, B. (2012). Characterization of endophytic strains of *Bacillus mojavensis* and their production of surfactin isomers. *Biological Control*, *62*(1), 1-9.
- Ballav, S. (2016). Exploring the biodiversity of culturable actinomycetes in the saltpans of Goa and their antimicrobial potential. Goa University, Doctoral Thesis, Goa India.
- Ballav, S., Kerkar, S., Thomas, S., & Augustine, N. (2015). Halophilic and halotolerant actinomycetes from a marine saltern of Goa, India producing anti-bacterial metabolites. *Journal of bioscience and bioengineering*, *119*(3), 323-330.
- Baltz, R. H., Miao, V., & Wrigley, S. K. (2005). Natural products to drugs: daptomycin and related lipopeptide antibiotics. *Natural product reports*, *22*(6), 717-741.

- Banat, I. M., Makkar, R. S., & Cameotra, S. S. (2000). Potential commercial applications of microbial surfactants. *Applied microbiology and biotechnology*, *53*(5), 495-508.
- Barakat, K. M., Hassan, S. W., & Darwesh, O. M. (2017). Biosurfactant production by haloalkaliphilic *Bacillus* strains isolated from Red Sea, Egypt. *The Egyptian Journal of Aquatic Research*, *43*(3), 205-211.
- Bartakke, A. (2018) Formulation of a biofertilizer for salt tolerant rice grown in khazan soils, using salt pan bacteria. Thesis, Goa University.
- Bartakke, A. (2018). Formulation of a biofertilizer for salt tolerant rice grown in khazan soils using salt pan bacteria. Goa University, Doctoral Thesis, Goa India.
- Bartal, A., Vigneshwari, A., Bóka, B., Vörös, M., Takács, I., Kredics, L., Manczinger, L., Varga, M., Vágvölgyi, C. & Szekeres, A. (2018). Effects of different cultivation parameters on the production of surfactin variants by a *Bacillus subtilis* strain. *Molecules*, *23*(10), 2675.
- Batista, S. B., Mounteer, A. H., Amorim, F. R., & Totola, M. R. (2006). Isolation and characterization of biosurfactant/bioemulsifier-producing bacteria from petroleum contaminated sites. *Bioresource technology*, *97*(6), 868-875.
- Beebe, J. L., & Umbreit, W. W. (1971). Extracellular lipid of *Thiobacillus thiooxidans*. *Journal of bacteriology*, *108*(1), 612.
- Benincasa, M., Abalos, A., Oliveira, I., & Manresa, A. (2004). Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. *Antonie Van Leeuwenhoek*, *85*(1), 1-8.
- Bernheimer, A. W., Avigad, L. S. (1970). Nature and properties of a cytological agent produced by *Bacillus subtilis*. *Journal of General Microbiology*, 61, 361–369.
- Bhambure, A. B., Mahajan, G. R., & Kerkar, S. (2018). Salt tolerant bacterial inoculants as promoters of Rice growth and microbial activity in coastal saline soil. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *88*(4), 1531-1538.
- Bhatnagar, I., Kim, S. K. (2010). Immense essence of excellence: marine microbial bioactive compounds. *Marine drugs*, 8(10), 2673-2701.

- Bratbak, G. (1993). Microscope methods for measuring bacterial biovolume: epifluorescence microscopy, scanning electron microscopy, and transmission electron microscopy. *Handbook of methods in aquatic microbial ecology*, 309-317.
- Bryant, F. O. (1990). Improved method for the isolation of biosurfactant glycolipids from *Rhodococcus* sp. strain H13A. *Applied and environmental microbiology*, *56*(5), 1494-1496.
- Cao, X. H., Liao, Z. Y., Wang, C. L., Yang, W. Y., & Lu, M. F. (2009). Evaluation of a lipopeptide biosurfactant from *Bacillus natto* TK-1 as a potential source of anti-adhesive, antimicrobial and antitumor activities. *Brazilian Journal of Microbiology*, *40*(2), 373-379.
- Cao, Y., Xu, Z., Ling, N., Yuan, Y., Yang, X., Chen, L. & Shen, Q. (2012). Isolation and identification of lipopeptides produced by *B. subtilis* SQR 9 for suppressing Fusarium wilt of cucumber. *Scientia horticulturae*, *135*, 32-39.
- Chandran, P., & Das, N. (2011). Characterization of sophorolipid biosurfactant produced by yeast species grown on diesel oil. *International journal of science and nature*, *2*(1), 63-71.
- Chang, C. L., Lin, Y., Bartolome, A. P., Chen, Y. C., Chiu, S. C., & Yang, W. C. (2013). Herbal therapies for type 2 diabetes mellitus: chemistry, biology, and potential application of selected plants and compounds. *Evidence-Based Complementary and Alternative Medicine*, *2013*.
- Cirigliano, M. C., & Carman, G. M. (1984). Isolation of a bioemulsifier from *Candida lipolytica*. *Applied and Environmental Microbiology*, *48*(4), 747-750.
- Cirigliano, M. C., & Carman, G. M. (1985). Purification and characterization of liposan, a bioemulsifier from Candida lipolytica. *Applied and environmental microbiology*, *50*(4), 846-850.
- Claus D, Berkeley RCW (1986) Genus Bacillus In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey‘s Manual of Systematic Bacteriology vol 2. The Williams & Wilkins Co, Baltimore, pp 1105-1139.
- Collins, C. H., Lyne, P. M., & Grange, J. M. (1984). Microbiological methods 5th Ed. *Butter worth and co-publisher Ltd. Ibadan*, 300-341.
- Cooper, D. G., & Goldenberg, B. G. (1987). Surface-active agents from two *Bacillus* species. *Applied and environmental microbiology*, *53*(2), 224-229.

- Cooper, D. G., Macdonald, C. R., Duff, S. J. B., & Kosaric, N. (1981). Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. *Applied and Environmental microbiology*, *42*(3), 408-412.
- Costa, S. G., Nitschke, M., Lépine, F., Déziel, E., & Contiero, J. (2010). Structure, properties and applications of rhamnolipids produced by *Pseudomonas aeruginosa* L2-1 from cassava wastewater. *Process Biochemistry*, *45*(9), 1511-1516.
- Dahrazma, B., & Mulligan, C. N. (2007). Investigation of the removal of heavy metals from sediments using rhamnolipid in a continuous flow configuration. *Chemosphere*, *69*(5), 705-711.
- Das, I., Roy, S., Chandni, S., Karthik, L., Kumar, G., & Rao, K. V. B. (2013). Biosurfactant from marine actinobacteria and its application in cosmetic formulation of toothpaste. *Pharm Lett*, *5*(5), 1-6.
- Das, K. R., Kowshik, M., Kumar, M. P., Kerkar, S., Shyama, S. K., & Mishra, S. (2018). Native hypersaline sulphate reducing bacteria contributes to iron nanoparticle formation in saltpan sediment: A concern for aquaculture. *Journal of environmental management*, *206*, 556-564.
- Das, P., Mukherjee, S., & Sen, R. (2008). Antimicrobial potential of a lipopeptide biosurfactant derived from a marine *Bacillus circulans*. *Journal of applied microbiology*, *104*(6), 1675-1684.
- Das, P., Mukherjee, S., & Sen, R. (2009). Biosurfactant of marine origin exhibiting heavy metal remediation properties. *Bioresource technology*, *100*(20), 4887-4890.
- DasSarma, S., & Arora, P. (2001). Halophiles. *e LS*. Wiley, UK, pp 1–13.
- DasSarma S, DasSarma P. 2017. Halophiles, encyclopedia of life science. In eLS. John Wiley & Sons Ltd., Chichester, United Kingdom
- Daverey, A., & Pakshirajan, K. (2009). Production of sophorolipids by the yeast *Candida bombicola* using simple and low cost fermentative media. *Food Research International*, *42*(4), 499-504.

- De Carvalho, C. C., & Fernandes, P. (2010). Production of metabolites as bacterial responses to the marine environment. *Marine Drugs*, *8*(3), 705-727.
- De Oliveira, M. R., Camilios-Neto, D., Baldo, C., Magri, A., & Celligoi, M. A. P. C. (2014). Biosynthesis and production of sophorolipids. *Int J Sci Technol Res*, *3*(11), 133-146.
- de Souza, C. G., Martins, F. I. C. C., Zocolo, G. J., Figueiredo, J. E. F., Canuto, K. M., & de Brito, E. S. (2018). Simultaneous quantification of lipopeptide isoforms by UPLC-MS in the fermentation broth from *Bacillus subtilis* CNPMS22. *Analytical and bioanalytical chemistry*, *410*(26), 6827-6836.
- Dehghan-Noudeh, G., Moshafi, M. H., & Masoumi, M. A. (2009, July). Studies on biosurfactant production by *Acinetobacter calcoaceticus* (ptcc 1318). In *The Annual Meeting and Exhibition 2009*.
- Desai, J. D., & Banat, I. M. (1997). Microbial production of surfactants and their commercial potential. *Microbiology and Molecular biology reviews*, *61*(1), 47-64.
- Desanto, K. (2008). Rhamnolipid-based formulations. *Patent WO 2008013899 A2*.
- Deshpande, M., & Daniels, L. (1995). Evaluation of sophorolipid biosurfactant production by *Candida bombicola* using animal fat. *Bioresource technology*, *54*(2), 143-150.
- Donio, M. B. S., Ronica, F. A., Viji, V. T., Velmurugan, S., Jenifer, J. S. C. A., Michaelbabu, M., Dhar, P. & Citarasu, T. (2013). *Halomonas* sp. BS4, A biosurfactant producing halophilic bacterium isolated from solar salt works in India and their biomedical importance. *SpringerPlus*, *2*(1), 149.
- Donio, M. B. S., Ronica, S. F. A., Viji, V. T., Velmurugan, S., Jenifer, J. A., Michaelbabu, M., & Citarasu, T. (2013). Isolation and characterization of halophilic *Bacillus* sp. BS3 able to produce pharmacologically important biosurfactants. *Asian Pacific journal of tropical medicine*, *6*(11), 876-883.
- Fait, M. E., Bakas, L., Garrote, G. L., Morcelle, S. R., & Saparrat, M. C. (2019). Cationic surfactants as antifungal agents. *Applied microbiology and biotechnology*, *103*(1), 97-112.
- Fattom, A., & Shilo, M. (1985). Production of emulcyan by *Phormidium* J-1: its activity and function. *FEMS Microbiology Letters*, *31*(1), 3-9.

- Fenibo, E. O., Douglas, S. I., & Stanley, H. O. (2019). A review on microbial surfactants: production, classifications, properties and characterization. *Journal of Advances in Microbiology*, 1-22.
- Fernandes, M. (2019a). Bioprospecting halotolerant bacteria for useful and promising antimicrobial compounds and enzyme inhibitors to improve quality and shelf life of Agaricus bisporus. Goa University, Doctoral Thesis, Goa India.
- Fernandes, M. S., & Kerkar, S. (2019b). Enhancing the anti-tyrosinase activity of a hypersaline *Kitasatospora* sp. SBSK430 by optimizing the medium components. *Current Science 116*(4): 649-653
- Fernandes, S. (2019c). Halotolerant bacteria as probiotics in shrimp aquaculture. Goa University, Doctoral Thesis, Goa India.
- Fernandes, S., Kerkar, S., Leitao, J., & Mishra, A. (2019d). Probiotic role of salt pan bacteria in enhancing the growth of whiteleg shrimp, *Litopenaeus vannamei*. *Probiotics and antimicrobial proteins*, *11*(4), 1309-1323.
- Godfray, H. C., Mason-D'Croz, D., & Robinson, S. (2016). Food system consequences of a fungal disease epidemic in a major crop. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *371*(1709), 20150467.
- Gomaa, E. Z., & El-Meihy, R. M. (2019). Bacterial biosurfactant from *Citrobacter freundii* MG812314. 1 as a bioremoval tool of heavy metals from wastewater. *Bulletin of the National Research Centre*, *43*(1), 69.
- Grangemard, I., Wallach, J., Maget-Dana, R., & Peypoux, F. (2001). Lichenysin. *Applied biochemistry and biotechnology*, *90*(3), 199-210.
- Greenway, H., & Osmond, C. B. (1972). Salt responses of enzymes from species differing in salt tolerance. *Plant Physiology*, *49*, 256–259.
- Gudina, E. J., Pereira, J. F., Costa, R., Coutinho, J. A., Teixeira, J. A., & Rodrigues, L. R. (2013). Biosurfactant-producing and oil-degrading *Bacillus subtilis* strains enhance oil recovery in laboratory sand-pack columns. *Journal of hazardous materials*, *261*, 106-113.
- Gudina, E., Teixeira, J., & Rodrigues, L. (2016). Biosurfactants Produced by Marine Microorganisms with Therapeutic Applications. *Marine Drugs, 14(2), 38.* doi:10.3390/md14020038

- Guerra-Santos, L. H., Käppeli, O., & Fiechter, A. (1986). Dependence of *Pseudomonas aeruginosa* continous culture biosurfactant production on nutritional and environmental factors. *Applied Microbiology and Biotechnology*, *24*(6), 443-448.
- Guimarães, C. R., Pasqualino, I. P., da Mota, F. F., de Godoy, M. G., Seldin, L., de Castilho, L. V. A., & Freire, D. M. G. (2019). Surfactin from *Bacillus velezensis* H2O-1: Production and Physicochemical Characterization for Postsalt Applications. *Journal of Surfactants and Detergents*, *22*(3), 451-462.
- Gutnick, D. L., & Shabtai, Y. (2017). Exopolysaccharide bioemulsifiers. In *Biosurfactants and biotechnology*. Routledge, 211–246
- Haddadin, M. S., Abou Arqoub, A. A., Reesh, I. A., & Haddadin, J. (2009). Kinetics of hydrocarbon extraction from oil shale using biosurfactant producing bacteria. *Energy conversion and management*, *50*(4), 983-990.
- Hall, T.A. (1999) BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95-98.
- Hentati, D., Chebbi, A., Hadrich, F., Frikha, I., Rabanal, F., Sayadi, S., Manresa, A. & Chamkha, M. (2019). Production, characterization and biotechnological potential of lipopeptide biosurfactants from a novel marine *Bacillus stratosphericus* strain FLU5. *Ecotoxicology and environmental safety*, *167*, 441-449.
- Hiratsuka, K., Nakahara, T., Sano, N., & Yamada, K. (1971). Formation of rhamnolipid by *Pseudomonas aeruginosa*: its function in hydrocarbon fermentations. *Agricultural* and *Biological Chemistry*, *35*, 686-692.
- Hisham, M. B., Hanisah, N., Ibrahim, M. F., Ramli, N., Abd-Aziz, S. (2019). Production of Biosurfactant Produced from Used Cooking Oil by *Bacillus* sp. HIP3 for Heavy Metals Removal. *Molecules 24*(14), 2617.
- Irfan, M., Nadeem, M., & Syed, Q. (2014). One-factor-at-a-time (OFAT) optimization of xylanase production from *Trichoderma viride*-IR05 in solid-state fermentation. *Journal of radiation research and applied sciences*, *7*(3), 317-326.

- Jacques, P. (2011). Surfactin and other lipopeptides from *Bacillus* spp. In *Biosurfactants* (pp. 57-91). Springer, Berlin, Heidelberg.
- Jain, D. K., Collins-Thompson, D. L., Lee, H., & Trevors, J. T. (1991). A drop-collapsing test for screening surfactant-producing microorganisms. *Journal of Microbiological Methods*, *13*(4), 271-279.
- Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary toxicology*, *7*(2), 60-72.
- Jakinala, P., Lingampally, N., Kyama, A., & Hameeda, B. (2019). Enhancement of atrazine biodegradation by marine isolate *Bacillus velezensis* MHNK1 in presence of surfactin lipopeptide. *Ecotoxicology and Environmental Safety*, *182*, 109372.
- Jamal, P., Nawawi, W. M. F. W., & Alam, M. Z. (2012). Optimum medium components for biosurfactant production by *Klebsiella pneumoniae* WMF02 utilizing sludge palm oil as a substrate. *Australian Journal of Basic and Applied Sciences*, *6*(1), 100-108.
- Jemil, N., Hmidet, N., Ayed, H. B., & Nasri, M. (2018). Physicochemical characterization of *Enterobacter cloacae* C3 lipopeptides and their applications in enhancing diesel oil biodegradation. *Process Safety and Environmental Protection*, *117*, 399-407.
- Joice, P. A., & Parthasarathi, R. (2014). Optimization of biosurfactant production from *Pseudomonas aeruginosa* PBSC1. *Int J Curr Microbiol Appl Sci*, *3*, 140-151.
- Juwarkar, A. A., Nair, A., Dubey, K. V., Singh, S. K., & Devotta, S. (2007). Biosurfactant technology for remediation of cadmium and lead contaminated soils. *Chemosphere*, *68*(10), 1996-2002.
- Kamat, T., & Kerkar, S. (2011). Bacteria from Salt Pans: a potential resource of antibacterial metabolites. *Recent Research in Science and Technology* 3(9): 46-52.
- Kamat T (2012) A study of an antibacterial compound from salt pans of Goa. Thesis, Goa University.
- Kanlayavattanakul, M., & Lourith, N. (2010). Lipopeptides in cosmetics. *International journal of cosmetic science*, *32*(1), 1-8.

- Käppeli, O., & Finnerty, W. R. (1979). Partition of alkane by an extracellular vesicle derived from hexadecane-grown *Acinetobacter*. *Journal of bacteriology*, *140*(2), 707-712.
- Kerkar, S. (2004). Ecology of hypersaline microorganisms. National Institute of Oceanography, Goa.
- Kerkar, S., & Bharathi, P. L. (2011). G Model Revisited: Seasonal Changes in the Kinetics of Sulphate-reducing Activity in the Salterns of Ribander, Goa, India. *Geomicrobiology journal*, *28*(3), 187-197.
- Khan, M. Y., Swapna, T. H., Hameeda, B., & Reddy, G. (2015). Bioremediation of heavy metals using biosurfactants. *Adv Biodegrad Bioremediat Ind Waste*.
- Khopade, A., Biao, R., Liu, X., Mahadik, K., Zhang, L., & Kokare, C. (2012). Production and stability studies of the biosurfactant isolated from marine *Nocardiopsis* sp. B4. *Desalination*, *285*, 198-204.
- Kim, H. S., Jeon, J. W., Kim, S. B., Oh, H. M., Kwon, T. J., & Yoon, B. D. (2002). Surface and physico-chemical properties of a glycolipid biosurfactant, mannosylerythritol lipid, from *Candida antarctica*. *Biotechnology letters*, *24*(19), 1637-1641.
- Kim, K., Dalsoo, Y., Youngbum, K., Baekseok, L., Doonhoon, S., & Eun-Ki, K. I. M. (2002). Characteristics of sophorolipid as an antimicrobial agent. *Journal of microbiology and biotechnology*, *12*(2), 235-241.
- Kim, B. & Kim, J. (2013) Optimization of culture conditions for the production of biosurfactant by *Bacillus subtilis* JK-1 using response surface methodology. *J. Kor. Soc. Appl. Biol. Chem.*, **56**, 279−287
- Kitagawa, M., Nishimoto, K., Tanaka, T. (2015). Cosmetic pigments, their production method, and cosmetics containing the cosmetic pigments. Patent US 9181436 B2.
- Kitamoto, D., Yanagishita, H., Shinbo, T., Nakane, T., Kamisawa, C., & Nakahara, T. (1993). Surface active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by *Candida antarctica*. *Journal of Biotechnology*, *29*(1-2), 91-96.
- Knoche, H. W., & Shively, J. M. (1972). The structure of an ornithine-containing lipid from *Thiobacillus thiooxidans*. *Journal of Biological Chemistry*, *247*(1), 170-178.

- Koch, A. K., Reiser, J., Käppeli, O., & Fiechter, A. (1988). Genetic construction of lactose-utilizing strains of *Pseudomonas aeruginosa* and their application in biosurfactant production. *Bio/Technology*, *6*(11), 1335-1339.
- Kubicki, S., Bollinger, A., Katzke, N., Jaeger, K.-E., Loeschcke, A., & Thies, S. (2019). Marine Biosurfactants: Biosynthesis, Structural Diversity and Biotechnological Applications. *Marine Drugs, 17(7), 408*
- Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, *35*(6), 1547-1549.
- Laird, M., Piccoli, D., Weselowski, B., McDowell, T., Renaud, J., MacDonald, J., & Yuan, Z. C. (2019). Surfactin-producing *Bacillus velezensis* 1B-23 and *Bacillus* sp. 1D-12 protect tomato against bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis*. *Journal of Plant Pathology*, 1-8.
- Lakshmipathy, T. D., Prasad, A. A., & Kannabiran, K. (2010). Production of biosurfactant and heavy metal resistance activity of *Streptomyces* sp. VITDDK3-a novel halo tolerant actinomycetes isolated from saltpan soil. *Biological Research*, *4*(2), 108-115.
- Landman, D., Georgescu, C., Martin, D. A., & Quale, J. (2008). Polymyxins revisited. *Clinical microbiology reviews*, *21*(3), 449-465.
- Lang, S., & Wullbrandt, D. (1999). Rhamnose lipids–biosynthesis, microbial production and application potential. *Applied microbiology and biotechnology*, *51*(1), 22-32.
- Leclere, V., Béchet, M., Adam, A., Guez, J. S., Wathelet, B., Ongena, M., Thonart, P., Gancel, F., Chollet-Imbert, M. & Jacques, P. (2005). Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. *Applied and environmental microbiology*, *71*(8), 4577-4584.
- Leclere, V., Béchet, M., Adam, A., Guez, J. S., Wathelet, B., Ongena, M., Thonart, P., Gancel, F., Chollet-Imbert, M. & Jacques, P. (2005). Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. *Applied and environmental microbiology*, *71*(8), 4577-4584.

- Lee, D. W., Lee, H., Kwon, B. O., Khim, J. S., Yim, U. H., Kim, B. S., & Kim, J. J. (2018). Biosurfactant-assisted bioremediation of crude oil by indigenous bacteria isolated from Taean beach sediment. *Environmental pollution*, *241*, 254-264.
- Lee, S. C., Kim, S. H., Park, I. H., Chung, S. Y., & Choi, Y. L. (2007). Isolation and structural analysis of bamylocin A, novel lipopeptide from *Bacillus amyloliquefaciens* LP03 having antagonistic and crude oil-emulsifying activity. *Archives of microbiology*, *188*(4), 307-312.
- Lee, S. C., Kim, S. H., Park, I. H., Chung, S. Y., Chandra, M. S., Choi, Y. L. (2010) Isolation, purification and characterization of Novel Fenzycin S from *Bacillus amyloliquefaciens*LSC04 Degrading-Crude Oil. *Biotechnology and Bioengineering 15*: 246 – 253
- Lee, S.-C., Kim, S.-H., Park, I.-H., Chung, S.-Y., & Choi, Y.-L. (2007). Isolation and structural analysis of bamylocin A, novel lipopeptide from *Bacillus amyloliquefaciens* LP03 having antagonistic and crude oil-emulsifying activity. *Archives of Microbiology, 188(4), 307–312.*
- Li, L. L., Feng, X. Q., Han, R. P., Zang, S. Q., & Yang, G. (2017). Cr (VI) removal via anion exchange on a silver-triazolate MOF. *Journal of hazardous materials*, *321*, 622-628.
- Li, X. Y., Yang, J. J., Mao, Z. C., Ho, H. H., Wu, Y. X., & He, Y. Q. (2014). Enhancement of biocontrol activities and cyclic lipopeptides production by chemical mutagenesis of *Bacillus subtilis* XF-1, a biocontrol agent of *Plasmodiophora brassicae* and *Fusarium solani*. *Indian journal of microbiology*, *54*(4), 476-479.
- Li, X.Y., Mao, Z.C., Wang, Y.H., Wu, Y.X., He, Y.Q. and Long, C.L. (2012) ESI LC-MS and MS/MS characterization of antifungal cyclic lipopeptides produced by *Bacillus subtilis* XF-1. J Mol Microbiol Biotechnol 22, 83–93.
- Liu, C., You, Y., Zhao, R., Sun, D., Zhang, P., Jiang, J., Zhu, A. and Liu, W., (2017). Biosurfactant production from *Pseudomonas taiwanensis* L1011 and its application in accelerating the chemical and biological decolorization of azo dyes. *Ecotoxicology and Environmental safety*, *145*, 8-15.
- Liu, J., Hagberg, I., Novitsky, L., Hadj-Moussa, H., & Avis, T. J. (2014). Interaction of antimicrobial cyclic lipopeptides from *Bacillus subtilis* influences

their effect on spore germination and membrane permeability in fungal plant pathogens. *Fungal biology*, *118*(11), 855-861.

- Liu, W., Wang, X., Wu, L., Chen, M., Tu, C., Luo, Y., & Christie, P. (2012). Isolation, identification and characterization of *Bacillus amyloliquefaciens* BZ-6, a bacterial isolate for enhancing oil recovery from oily sludge. *Chemosphere*, *87*(10), 1105-1110.
- Liu, Q., Lin, J., Wang, W., Huang, H., & Li, S. (2015). Production of surfactin isoforms by Bacillus subtilis BS-37 and its applicability to enhanced oil recovery under laboratory conditions. *Biochemical engineering journal*, *93*, 31-37.
- Lourith, N., & Kanlayavattanakul, M. (2009). Natural surfactants used in cosmetics: glycolipids. *International journal of cosmetic science*, *31*(4), 255-261.
- Luo, C., Zhou, H., Zou, J., Wang, X., Zhang, R., Xiang, Y., & Chen, Z. (2015). Bacillomycin L and surfactin contribute synergistically to the phenotypic features of *Bacillus subtilis* 916 and the biocontrol of rice sheath blight induced by *Rhizoctonia solani*. *Applied microbiology and biotechnology*, *99*(4), 1897-1910.
- Macdonald, C. R., Cooper, D. G., & Zajic, J. E. (1981). Surface-active lipids from *Nocardia erythropolis* grown on hydrocarbons. *Applied and Environmental Microbiology*, *41*(1), 117-123.
- Madslien, E. H., Rønning, H. T., Lindbäck, T., Hassel, B., Andersson, M. A., & Granum, P. E. (2013). Lichenysin is produced by most *Bacillus licheniformis* strains. *Journal of Applied Microbiology*, *115*(4), 1068-1080.
- Makkar, R. S., & Cameotra, S. S. (1997). Biosurfactant production by a thermophilic Bacillus subtilis strain. *Journal of Industrial Microbiology and Biotechnology*, *18*(1), 37-42.
- Makkar, R. S., & Cameotra, S. S. (1998). Production of biosurfactant at mesophilic and thermophilic conditions by a strain of Bacillus subtilis. *Journal of Industrial Microbiology and Biotechnology*, *20*(1), 48-52.
- Mani, K., Salgaonkar, B. B., Das, D., & Bragança, J. M. (2012). Community solar salt production in Goa, India. *Aquatic biosystems*, *8*(1), 30.

- Manivasagan, P., Sivasankar, P., Venkatesan, J., Sivakumar, K., & Kim, S. K. (2014). Optimization, production and characterization of glycolipid biosurfactant from the marine actinobacterium, *Streptomyces sp*. MAB36. *Bioprocess and biosystems engineering*, *37*(5), 783-797.
- Marahiel, M. A., Danders, W., Krause, M., & Kleinkauf, H. (1979). Biological Role of Gramicidin S in Spore Functions: Studies on Gramicidin-S-Negative Mutants of *Bacillus brevis* ATCC9999. *European Journal of Biochemistry*, *99*(1), 49-55.
- Marchant, R., & Banat, I. M. (2012). Biosurfactants: a sustainable replacement for chemical surfactants? *Biotechnology letters*, *34*(9), 1597-1605.
- Matsuyama, T., Sogawa, M., & Yano, I. (1987). Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents. *Applied and environmental microbiology*, *53*(5), 1186-1188.
- Maurya, I. K., Dilawari, R., Singh, D., & Singh, R. P. (2020). Bioactive Compounds from Extremophiles. In *Microbial Versatility in Varied Environments* (pp. 117-134). Springer, Singapore.
- Miljaković, D., Marinković, J., & Balešević-Tubić, S. (2020). The significance of *Bacillus* spp. in disease suppression and growth promotion of field and vegetable crops. *Microorganisms*, *8*(7), 1037.
- Mnif, I., & Ghribi, D. (2015). Review lipopeptides biosurfactants: mean classes and new insights for industrial, biomedical, and environmental applications. *Peptide Science*, *104*(3), 129-147.
- Mnif, I., Grau-Campistany, A., Coronel-León, J., Hammami, I., Triki, M. A., Manresa, A., & Ghribi, D. (2016). Purification and identification of Bacillus subtilis SPB1 lipopeptide biosurfactant exhibiting antifungal activity against Rhizoctonia bataticola and Rhizoctonia solani. *Environmental Science and Pollution Research*, *23*(7), 6690-6699.
- Montanari, D.; Guglielmo, M. World patent 2008/080443 2008; Labo Cosprophar Ag, Basel. 126.
- Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y., & Imanaka, T. (1993). A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. *Journal of bacteriology*, *175*(20), 6459-6466.

- Morita, T., Fukuoka, T., Imura, T., & Kitamoto, D. (2015). Mannosylerythritol lipids: production and applications. *Journal of Oleo Science*, *64*(2), 133-141.
- Mouafi, F. E., Elsoud, M. M. A., & Moharam, M. E. (2016). Optimization of biosurfactant production by *Bacillus brevis* using response surface methodology. *Biotechnology Reports*, *9*, 31-37.
- Moyne, A. L., Shelby, R., Cleveland, T. E., & Tuzun, S. (2001). Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *Journal of applied microbiology*, *90*(4), 622-629.
- Mukherjee, S., Das, P., & Sen, R. (2006). Towards commercial production of microbial surfactants. *Trends in Biotechnology*, *24*(11), 509-515.
- Mukherjee S, Das P, Sivapathasekaran C. and Sen R. (2009) Antimicrobial biosurfactants from marine Bacillus circulans: extracellular synthesis and purification, Letters in Applied Microbiology 48 (2009) 281–288.
- Mulligan, C. N., Yong, R. N., & Gibbs, B. F. (2001). Heavy metal removal from sediments by biosurfactants. *Journal of hazardous materials*, *85*(1-2), 111-125.
- Naruse, N., Tenmyo, O., Kobaru, S., Kamei, H., Miyaki, T., Konishi, M., & Oki, T. (1990). Pumilacidin, a complex of new antiviral antibiotics. *The Journal of antibiotics*, *43*(3), 267-280.
- Neifar, M., Maktouf, S., Ghorbel, R. E., Jaouani, A., & Cherif, A. (2015). Extremophiles as source of novel bioactive compounds with industrial potential. *Biotechnology of bioactive compounds: sources and applications. Wiley, Hoboken*, 245-268.
- Nerurkar, A. S. (2010) Structural and molecular characteristics of lichenysin and its relationship with surface activity. *Biosurfactants, Advances in Experimental Medicine and Biology*, 672:304-15.
- Neu, T. R., & Poralla, K. (1990). Emulsifying agents from bacteria isolated during screening for cells with hydrophobic surfaces. *Applied microbiology and biotechnology*, *32*(5), 521-525.
- Neu, T. R., Härtner, T., & Poralla, K. (1990). Surface active properties of viscosin: a peptidolipid antibiotic. *Applied microbiology and biotechnology*, *32*(5), 518-520.
- Nitschke, M., & Costa, S. G. V. A. O. (2007). Biosurfactants in food industry. *Trends in Food Science & Technology*, *18*(5), 252-259.

- Nitschke, M., & Pastore, G. M. (2006). Production and properties of a surfactant obtained from *Bacillus subtilis* grown on cassava wastewater. *Bioresource technology*, *97*(2), 336-341.
- Olishevska, S., Nickzad, A., & Déziel, E. (2019). *Bacillus* and *Paenibacillus* secreted polyketides and peptides involved in controlling human and plant pathogens. *Applied microbiology and biotechnology*, *103*(3), 1189-1215.
- Pagare, S., Bhatia, M., Tripathi, N., Pagare, S., & Bansal, Y. K. (2015). Secondary metabolites of plants and their role: Overview. *Current Trends in Biotechnology and Pharmacy*, *9*(3), 293-304.
- Parra, J. L., Guinea, J., Manresa, M. A., Robert, M., Mercade, M. E., Comelles, F., & Bosch, M. P. (1989). Chemical characterization and physicochemical behavior of biosurfactants. *Journal of the American Oil Chemists Society*, *66*(1), 141-145.
- Parry, N. J., Stevenson, P. S. (2014). Personal care compositions. Patent WO 2014095367 A1.
- Pastewski, S., Hallmann, E., & Medrzycka, K. (2006). Physicochemical aspects of the application of surfactants and biosurfactants in soil remediation. *Environmental engineering science*, *23*(4), 579-588.
- Pathak, K. V., & Keharia, H. (2013). Identification of surfactins and iturins produced by potent fungal antagonist, *Bacillus subtilis* K1 isolated from aerial roots of banyan (*Ficus benghalensis*) tree using mass spectrometry. *3 Biotech*, *4*(3), 283–295.
- Pathak, K. V., & Keharia, H. (2014). Application of extracellular lipopeptide biosurfactant produced by endophytic *Bacillus subtilis* K1 isolated from aerial roots of banyan (*Ficus benghalensis*) in microbially enhanced oil recovery (MEOR). *3 Biotech*, *4*(1), 41-48.
- Pecci, Y., Rivardo, F., Martinotti, M. G., & Allegrone, G. (2010). LC/ESI-MS/MS characterisation of lipopeptide biosurfactants produced by the *Bacillus licheniformis* V9T14 strain. *Journal of mass spectrometry*, *45*(7), 772-778.
- Pereira, F., Kerkar, S., & Krishnan, K. P. (2013). Bacterial response to dynamic metal concentrations in the surface sediments of a solar saltern (Goa, India). *Environmental monitoring and assessment*, *185*(5), 3625-3636.

- Pereira, F.S.S. (2013). Metal tolerance in heterotrophic bacteria from marine salterns of Ribandar Goa. Doctoral thesis, Goa University, Goa.
- Pereira, J. F., Gudiña, E. J., Costa, R., Vitorino, R., Teixeira, J. A., Coutinho, J. A., & Rodrigues, L. R. (2013). Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. *Fuel*, *111*, 259-268.
- Piljac, T., Piljac, G. (1999). Use of rhamnolipids in wound healing, treating burn shock, atherosclerosis, organ transplants, depression, schizophrenia and cosmetics. *Patent WO1999043334 A1*.
- Płaza, G. A., Zjawiony, I., & Banat, I. M. (2006). Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated and bioremediated soils. *Journal of Petroleum Science and Engineering*, *50*(1), 71-77.
- Płaza, G., Chojniak, J., Rudnicka, K., Paraszkiewicz, K., & Bernat, P. (2015). Detection of biosurfactants in *Bacillus* species: genes and products identification. *Journal of applied microbiology*, *119*(4), 1023-1034.
- Pornsunthorntawee, O., Arttaweeporn, N., Paisanjit, S., Somboonthanate, P., Abe, M., Rujiravanit, R., & Chavadej, S. (2008). Isolation and comparison of biosurfactants produced by *Bacillus subtilis* PT2 and *Pseudomonas aeruginosa* SP4 for microbial surfactant-enhanced oil recovery. *Biochemical Engineering Journal*, *42*(2), 172-179.
- Post, F. J., & Al-Harjan, F. A. (1988). Surface activity of halobacteria and potential use in microbially enhanced oil recovery. *Systematic and Applied Microbiology*, *11*(1), 97-101.
- Post, F. J., & Collins, N. F. (1982). A preliminary investigation of the membrane lipid of *Halobacterium halobium* as a food additive. *Journal of Food Biochemistry, 6* (1), 25–38
- Qazi, M. A., Subhan, M., Fatima, N., Ali, M. I., & Ahmed, S. (2013). Role of biosurfactant produced by *Fusarium* sp. BS-8 in enhanced oil recovery (EOR) through sand pack column. *International Journal of Bioscience, Biochemistry and Bioinformatics*, *3*(6), 598.
- Qi X, Xu X, Zhong C, Jiang T, Wei W, Song X (2018) Removal of Cadmium and Lead from Contaminated Soils Using Sophorolipids from Fermentation

Culture of *Starmerella bombicola* CGMCC 1576 Fermentation. *International journal of environmental research and public health*, *15*(11): 2334.

- Raaijmakers, J. M., De Bruijn, I., Nybroe, O., & Ongena, M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS microbiology reviews*, *34*(6), 1037-1062.
- Rabiei, A., Sharifinik, M., Niazi, A., Hashemi, A., & Ayatollahi, S. (2013). Core flooding tests to investigate the effects of IFT reduction and wettability alteration on oil recovery during MEOR process in an Iranian oil reservoir. *Applied microbiology and biotechnology*, *97*(13), 5979-5991.
- Rahman, K. S., Rahman, T. J., Kourkoutas, Y., Petsas, I., Marchant, R., & Banat, I. M. (2003). Enhanced bioremediation of n-alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. *Bioresource technology*, *90*(2), 159-168.
- Rashedi, H., Jamshidi, E., Bonakdarpour, B., & Mazaheri, A. M. (2005) Isolation and production of biosurfactant from *Pseudomonas aeruginosa* isolated from Iranian southern wells oil. *International Journal of Environmental Science and Technology.* 2(2): 121-127
- Robert, M., Mercade, M. E., Bosch, M. P., Parra, J. L., Espuny, M. J., Manresa, M. A., & Guinea, J. (1989). Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. *Biotechnology Letters*, *11*(12), 871-874.
- Rocha, P. M., dos Santos Mendes, A. C., de Oliveira Júnior, S. D., de Araújo Padilha, C. E., de Sá Leitão, A. L. O., da Costa Nogueira, C., de Macedo, G.R. & Dos Santos, E. S. (2020). Kinetic study and characterization of surfactin production by *Bacillus subtilis* UFPEDA 438 using sugarcane molasses as carbon source. *Preparative Biochemistry & Biotechnology*, 1-9.
- Ron, E. Z., & Rosenberg, E. (2001). Natural roles of biosurfactants: Minireview. *Environmental microbiology*, *3*(4), 229-236.
- Rosenberg, E., & Ron, E. Z. (1999). High-and low-molecular-mass microbial surfactants. *Applied microbiology and biotechnology*, *52*(2), 154-162.
- Rosenberg, E., Rubinovitz, C., Legmann, R., & Ron, E. Z. (1988). Purification and chemical properties of *Acinetobacter calcoaceticus* A2 biodispersan. *Applied and environmental microbiology*, *54*(2), 323-326.

- Rosenberg, E., Zuckerberg, A., Rubinovitz, C., & Gutnick, D. (1979). Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. *Applied and Environmental Microbiology*, *37*(3), 402-408.
- Sahoo, S., Datta, S., & Biswas, D. (2011). Optimization of culture conditions for biosurfactant production from *Pseudomonas aeruginosa* OCD1. *Journal* of *Advanced Scientific Research*, *2*(3), 32-36.
- Saini, H. S., Barragán-Huerta, B. E., Lebrón-Paler, A., Pemberton, J. E., Vázquez, R. R., Burns, A. M., & Maier, R. M. (2008). Efficient purification of the biosurfactant viscosin from *Pseudomonas libanensis* strain M9-3 and its physicochemical and biological properties. *Journal of natural products*, *71*(6), 1011-1015.
- Sanchez, L., Courteaux, B., Hubert, J., Kauffmann, S., Renault, J. H., Clément, C., Baillieul, F. & Dorey, S. (2012). Rhamnolipids elicit defense responses and induce disease resistance against biotrophic, hemibiotrophic, and necrotrophic pathogens that require different signaling pathways in *Arabidopsis* and highlight a central role for salicylic acid. *Plant physiology*, *160*(3), 1630-1641.
- Sarafin, Y., Donio, M. B. S., Velmurugan, S., Michaelbabu, M., & Citarasu, T. (2014). *Kocuria marina* BS-15 a biosurfactant producing halophilic bacteria isolated from solar salt works in India. *Saudi journal of biological sciences*, *21*(6), 511-519.
- Sarubbo, L. A., Rocha Jr, R. B., Luna, J. M., Rufino, R. D., Santos, V. A., & Banat, I. M. (2015). Some aspects of heavy metals contamination remediation and role of biosurfactants. *Chemistry and Ecology*, *31*(8), 707-723.
- Sarubbo, L., Brasileiro, P., Silveira, G., Luna, J., & Rufino, R. (2018). Application of a low cost biosurfactant in the removal of heavy metals in soil. *Chemical Engineering Transactions*, *64*, 433-438.
- Sarwar, A., Hassan, M. N., Imran, M., Iqbal, M., Majeed, S., Brader, G., Sessitsch, A. & Hafeez, F. Y. (2018). Biocontrol activity of surfactin A purified from *Bacillus* NH-100 and NH-217 against rice bakanae disease. *Microbiological research*, *209*, 1-13.
- Sathiyanarayanan, G., Gandhimathi, R., Sabarathnam, B., Kiran, G. S., & Selvin, J. (2014). Optimization and production of pyrrolidone antimicrobial

agent from marine sponge-associated *Streptomyces* sp. MAPS15. *Bioprocess and biosystems engineering*, *37*(3), 561-573.

- Satpute, S. K., Banat, I. M., Dhakephalkar, P. K., Banpurkar, A. G., & Chopade, B. A. (2010). Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. *Biotechnology advances*, *28*(4), 436-450.
- Satpute, S. K., Bhawsar, B. D., Dhakephalkar, P. K., & Chopade, B. A. (2008). Assessment of different screening methods for selecting biosurfactant producing marine bacteria. *Indian Journal of Marine Sciences*, *37*(3), 243-250.
- Schofield, M. H., Thavasi, R. & Gross, R. A. (2013). Modfied sophorolipids for the inhibition of plant pathogens. *US Patent: 20130085067 A1*.
- Sekhon Randhawa, K. K., & Rahman, P. K. (2014). Rhamnolipid biosurfactants—past, present, and future scenario of global market. *Frontiers in microbiology*, *5*, 454.
- Sen, S., Borah, S. N., Bora, A., & Deka, S. (2017). Production, characterization, and antifungal activity of a biosurfactant produced by *Rhodotorula babjevae* YS3. *Microbial cell factories*, *16*(1), 1-14.
- Shaligram, N. S., & Singhal, R. S. (2010). Surfactin–a review on biosynthesis, fermentation, purification and applications. *Food technology and biotechnology*, *48*(2), 119-134.
- Sharma, R., Singh, J., & Verma, N. (2020). Statistical optimization and comparative study of lipopeptides produced by *Bacillus amyloliquefaciens* SAS-1 and *Bacillus subtilis* BR-15. *Biocatalysis and Agricultural Biotechnology*, 101575.
- Sharma, S., & Pandey, L. M. (2020). Production of biosurfactant by *Bacillus subtilis* RSL-2 isolated from sludge and biosurfactant mediated degradation of oil. *Bioresource Technology*, 123261.
- Shekhar, S., Sundaramanickam, A., & Balasubramanian, T. (2015). Biosurfactant producing microbes and their potential applications: a review. *Critical Reviews in Environmental Science and Technology*, *45*(14), 1522-1554.
- Shubhrasekhar, C., Supriya, M., Karthik, L., Gaurav, K., & Bhaskara Rao, K. V. (2013). Isolation, characterization and application of biosurfactant produced by

marine Actinobacteria isolated from saltpan soil from costal area of Andhra Pradesh, India. *Research Journal of Biotechnology Vol*, *8*, 1.

- Shukla, K. P., Sharma, S., Singh, N. K., Singh, V., Bisht, S., & Kumar, V. (2013). Rhizoremediation: a promising rhizosphere technology. *Applied Bioremediation: Active and Passive Approaches*, 331.
- Siegmund, I., & Wagner, F. (1991). New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. *Biotechnology Techniques*, *5*(4), 265-268.
- Singh, A. K., & Cameotra, S. S. (2013). Efficiency of lipopeptide biosurfactants in removal of petroleum hydrocarbons and heavy metals from contaminated soil. *Environmental Science and Pollution Research*, *20*(10), 7367-7376.
- Singh, A. K., Rautela, R., & Cameotra, S. S. (2014). Substrate dependent in vitro antifungal activity of *Bacillus* sp strain AR2. *Microbial cell factories*, *13*(1), 67.
- Singh, A., Van Hamme, J. D., & Ward, O. P. (2007). Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnology advances*, *25*(1), 99-121.
- Singh, P., Patil, Y., & Rale, V. (2019). Biosurfactant production: emerging trends and promising strategies. *Journal of applied microbiology*, *126*(1), 2-13.
- Souza, K. S. T., Gudiña, E. J., Azevedo, Z., de Freitas, V., Schwan, R. F., Rodrigues, L. R., ... & Teixeira, J. A. (2017). New glycolipid biosurfactants produced by the yeast strain *Wickerhamomyces anomalus* CCMA 0358. *Colloids and Surfaces B: Biointerfaces*, *154*, 373-382.
- Suzuki, T., Hayashi, K., Fujikawa, K., & Tsukamoto, K. (1965). The chemical structure of polymyxin E: the identities of polymyxin E1 with colistin A and of polymyxin E2 with colistin B. *The Journal of Biochemistry*, *57*(2), 226-227.
- Swapna, T. H., Papathoti, N. K., Khan, M. Y., Reddy, G., & Hameeda, B. (2016). Bioreduction of Cr (VI) by biosurfactant producing marine bacterium *Bacillus subtilis* SHB 13. *Journal of Scientific & Industrial Research. 75*, 432-438.
- Takahashi, M., Morita, T., Fukuoka, T., Imura, T., & Kitamoto, D. (2012). Glycolipid biosurfactants, mannosylerythritol lipids, show antioxidant and

protective effects against H2O2-induced oxidative stress in cultured human skin fibroblasts. *Journal of Oleo Science*, *61*(8), 457-464.

- Thavasi, R., Sharma, S., & Jayalakshmi, S. (2011). Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria. *J Pet Environ Biotechnol S*, *1*(2).
- Thenmozhi, R., Sornalaksmi, A., Praveenkumar, D., & Nagasathya, A. (2011). Characterization of biosurfactant produced by bacterial isolates from engine oil contaminated soil. *Advances in Environmental Biology*, 2402-2409.
- Thies, S., Santiago-Schübel, B., Kovačić, F., Rosenau, F., Hausmann, R., & Jaeger, K. E. (2014). Heterologous production of the lipopeptide biosurfactant serrawettin W1 in *Escherichia coli*. *Journal of Biotechnology*, *181*, 27-30.
- Trevor, F., Crawford, R., Garry, L. (2013). Mild to the skin, foaming detergent composition. *Patent US 8563490 B2*.
- Urdaci, M. C., & Pinchuk, I. (2004). Antimicrobial activity of Bacillus probiotics. Bacterial spore formers–Probiotics and emerging applications. *Norfolk, UK: Horizon Bioscience*, 171-82.
- Uzoigwe, C., Burgess, J. G., Ennis, C. J., & Rahman, P. K. (2015). Bioemulsifiers are not biosurfactants and require different screening approaches. *Frontiers in microbiology*, *6*, 245.
- Van Bogaert, I. N., Saerens, K., De Muynck, C., Develter, D., Soetaert, W., & Vandamme, E. J. (2007). Microbial production and application of sophorolipids. *Applied microbiology and biotechnology*, *76*(1), 23-34.
- Varadavenkatesan, T., & Murty, V. R. (2013). Production of a lipopeptide biosurfactant by a novel *Bacillus* sp. and its applicability to enhanced oil recovery. *International Scholarly Research Notices*, *2013*.
- Varjani, S. J., & Upasani, V. N. (2017). Critical review on biosurfactant analysis, purification and characterization using rhamnolipid as a model biosurfactant. *Bioresource technology*, *232*, 389-397.
- Varjani, S. J., Rana, D. P., Bateja, S., Sharma, M. C., & Upasani, V. N. (2014). Screening and identification of biosurfactant (bioemulsifier) producing bacteria from crude oil contaminated sites of Gujarat, India. *Int J Inno Res Sci Eng Technol*, *3*(2).

- Varnier, A. L., Sanchez, L., Vatsa, P., Boudesocque, L., Garcia-Brugger, A. N. G. E. L. A., Rabenoelina, F., Sorokin, A., RENAULT, J.H., Kauffmann, S., Pugin, A. and Clément, C & Dorey, S. (2009). Bacterial rhamnolipids are novel MAMPs conferring resistance to *Botrytis cinerea* in grapevine. *Plant, cell & environment*, *32*(2), 178-193.
- Vasanthakumari R (2009) Practical microbiology. BI Publications, New Delhi
- Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N., & Cameotra, S. S. (2002). Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. *Applied and Environmental Microbiology*, *68*(12), 6210-6219.
- Vatsa, P., Sanchez, L., Clement, C., Baillieul, F., & Dorey, S. (2010). Rhamnolipid biosurfactants as new players in animal and plant defense against microbes. *International journal of molecular sciences*, *11*(12), 5095-5108.
- Venkatesh, N. M., & Vedaraman, N. (2012). Remediation of soil contaminated with copper using rhamnolipids produced from *Pseudomonas aeruginosa* MTCC 2297 using waste frying rice bran oil. *Annals of microbiology*, *62*(1), 85-91.
- Villaescusa, B. P., Rangel-Huerta, O. D., Aguilera, C. M., Gil, A. (2015). A systematic review of the efficacy of bioactive compounds in cardiovascular disease: carbohydrates, active lipids and nitrogen compounds. *Annals of Nutrition and Metabolism*, *66*(2-3), 168-181.
- Waewthongrak, W., Leelasuphakul, W., & McCollum, G. (2014). Cyclic lipopeptides from *Bacillus subtilis* ABS–S14 elicit defense-related gene expression in citrus fruit. *PloS one*, *9*(10), e109386.
- Walter, V., Syldatk, C., & Hausmann, R. (2010). Screening concepts for the isolation of biosurfactant producing microorganisms. In *Biosurfactants* (pp. 1-13). Springer, New York, NY.
- Wang, S., & Mulligan, C. N. (2004). Rhamnolipid foam enhanced remediation of cadmium and nickel contaminated soil. *Water, Air, and Soil Pollution*, *157*(1-4), 315-330.

- Wei, Y. H., Wang, L. F., & Chang, J. S. (2004). Optimizing iron supplement strategies for enhanced surfactin production with *Bacillus subtilis*. *Biotechnology Progress*, *20*(3), 979-983.
- Wei, Y. H., Wang, L. F., Changy, J. S., & Kung, S. S. (2003). Identification of induced acidification in iron-enriched cultures of *Bacillus subtilis* during biosurfactant fermentation. *Journal of bioscience and bioengineering*, *96*(2), 174-178.
- Whang, L. M., Liu, P. W. G., Ma, C. C., & Cheng, S. S. (2008). Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. *Journal of hazardous materials*, *151*(1), 155-163.
- Wu, Q., Zhi, Y., & Xu, Y. (2019). Systematically engineering the biosynthesis of a green biosurfactant surfactin by *Bacillus subtilis* 168. *Metabolic engineering*, *52*, 87-97.
- Wu, Y. S., Ngai, S. C., Goh, B. H., Chan, K. G., Lee, L. H., & Chuah, L. H. (2017). Anticancer activities of surfactin and potential application of nanotechnology assisted surfactin delivery. *Frontiers in pharmacology*, *8*, 761.
- Yakimov, M. M., Timmis, K. N., Wray, V., & Fredrickson, H. L. (1995). Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Applied and environmental microbiology*, *61*(5), 1706-1713.
- Yalçın, H. T., Ergin-Tepebaşı, G., & Uyar, E. (2018). Isolation and molecular characterization of biosurfactant producing yeasts from the soil samples contaminated with petroleum derivatives. *Journal of basic microbiology*, *58*(9), 782-792.
- Yan, L., Liu, G., Zhao, B., Pang, B., Wu, W., Ai, C., Zhao, X., Wang, X., Jiang, C., Shao, D. & Shi, J. (2020). Novel biomedical functions of surfactin A from *Bacillus subtilis* in wound healing promotion and scar inhibition. *Journal of agricultural and food chemistry*, *68*(26), 6987-6997.
- Yanagisawa, S., Kawano, S. & Yasohara, Y. (2014). Method for producing sophoroselipid. *US Patent: 8664373B2*.

- Yeh, M. S., Wei, Y. H., & Chang, J. S. (2005). Enhanced Production of Surfactin from *Bacillus subtilis* by addition of solid carriers. *Biotechnology progress*, *21*(4), 1329-1334.
- Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R. M., & McInerney, M. J. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal of microbiological methods*, *56*(3), 339-347.
- Yu, M., Liu, Z., Zeng, G., Zhong, H., Liu, Y., Jiang, Y., Li, M., He, X. & He, Y. (2015). Characteristics of mannosylerythritol lipids and their environmental potential. *Carbohydrate research*, *407*, 63-72.
- Yu, Z., Lang, G., Kajahn, I., Schmaljohann, R., & Imhoff, J. F. (2008). Scopularides A and B, cyclodepsipeptides from a marine sponge-derived fungus, *Scopulariopsis brevicaulis*. *Journal of Natural Products*, *71*(6), 1052-1054.
- Yuan, L., Zhang, S., Wang, Y., Li, Y., Wang, X., & Yang, Q. (2018). Surfactin inhibits membrane fusion during invasion of epithelial cells by enveloped viruses. *Journal of virology*, *92*(21).
- Yuan, J., Yang, Y., Zhou, X.H., Ge, Y.C., Zeng, Q.R. (2019). A new method for simultaneous removal of heavy metals and harmful organics from rape seed meal from metal contaminated farmland. *Sep. Purif. Technol*. 210, 1001–1007.
- Zhang, W., Zhang, X., & Cui, H. (2015). Isolation, fermentation optimization and performance studies of a novel biosurfactant producing strain Bacillus amyloliquefaciens. *Chemical and Biochemical Engineering Quarterly*, *29*(3), 447-456.
- Zhao, P., Xu, X., Zhao, X., Ai, C., Xu, K., Li, M., Jiang, C. & Shi, J. (2020). Capability of *Bacillus Subtilis* to remove Pb2+ via producing lipopeptides. *Science of the Total Environment*, 138941.
- Zhu, Z., Zhang, B., Chen, B., Cai, Q., & Lin, W. (2016). Biosurfactant production by marine-originated bacteria *Bacillus Subtilis* and its application for crude oil removal. *Water, Air, & Soil Pollution*, *227*(9), 328.
- Zosim, Z., Gutnick, D., & Rosenberg, E. (1982). Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. *Biotechnology and bioengineering*, *24*(2), 281-292.

APPENDIX

APPENDIX A: MEDIA COMPOSITION

Sr. No	Name of media	Composition	
		Ingredients	**g/L**
1	**Nutrient Agar**	Peptone Sodium chloride Beef extract Yeast extract Agar Seawater Final pH (at 25°C)	5.000 5.000 1.500 1.500 15.000 1000 mL 7.4 ± 0.2
2	**Media D Agar**	Soy Peptone Sodium chloride Tryptone Distilled water Final pH (at 25°C)	0.500 0.500 1.500 1000 mL 7.4 ± 0.2
3	**Actinomycetes isolation agar**	Sodium caseinate L-Asparagine Sodium propionate Dipotassium phosphate Magnesium sulphate Ferrous sulphate Agar Distilled water Final pH (at 25°C)	2.000 0.100 4.000 0.500 0.100 0.001 15.00 1000 mL 8.1 ± 0.2

Media	Name of media	Composition	
		Ingredients	**g/L**
4	**Nutrient Broth**	Peptone Beef extract Sodium chloride Distilled water Final pH (at 25°C)	10.00 10.00 5.000 1000 mL 7.2 ± 0.2
5	**BASAL MEDIA**	Glucose Peptone Potassium Dihydrogen Phosphate Dipotassium Hydrogen Phosphate Magnesium Sulphate heptahydrate Sodium Chloride Calcium Chloride Final pH (at 25°C)	20.00 4.00 5.00 5.00 0.25 2.00 0.08 6.7 ± 0.2

APPENDIX B: REAGENTS, STAINS

Sr. No	Name of Reagent	Composition	
		Ingredients	**g/L**
1	**Ninhydrin**	Ninhydrin 90% (v/v) ethanol Glacial acetic acid	0.2 g 95 mL 5 mL
2	**0.4% Safranin stain**	Safranin 95% (v/v) ethanol	0.4 g 100 mL
3	**2.5% Safranin stain**	Stock solution Distilled water (*Stock solution - 2.5% (w/v) safranin in 95% (v/v) ethanol)	10 mL 90 mL
4	**Crystal violet stain**	Crystal violet 95% (v/v) ethanol Ammonium oxalate Distilled water	2 g 20 mL 0.8 g 80 mL
5	**Gram's Iodine**	Iodine Potassium iodide Distilled water	1 g 2 g 300 mL
6	**Decolorizer for Gram's staining**	Acetone 95% (v/v) ethanol	50 mL 50 mL
7	**0.5 % Malachite green stain**	Malachite green Distilled water	0.5 g 100 mL

Sr. No	Name of Reagent	Composition	
		Ingredients	**g/L**
8	**Lead acetate** **(Stock solution – 1000 ppm)**	Lead acetate Distilled water	1.83 1000 mL
9	**Ferric chloride** **(Stock solution – 1000 ppm)**	Ferric chloride Distilled water	4.84 1000 mL
10	**Cadmium sulphate** **(Stock solution – 1000 ppm)**	Cadmium sulphate Distilled water	3.42 1000 mL
11	**Zinc sulphate** **(Stock solution – 1000 ppm)**	Zinc sulphate Distilled water	2.19 1000 mL
12	**Potassium dichromate** **(Stock solution – 1000 ppm)**	Potassium dichromate Distilled water	5.65 1000 mL

PUBLICATIONS

MANUSCRIPT PUBLISHED

- Malik, R., & Kerkar, S. (2019). Enhancing biosurfactant production by hypersaline *Bacillus amyloliquefaciens* SK27 using response surface methodology and genetic algorithm. Current science. 117(5); 847-852. doi: 10.18520/cs/v117/i5/847-852

BOOK CHAPTER IN PRESS

Title: Biosurfactant- mediated remediation of heavy metals - a review
Authors: Ruchira Malik and Savita Kerkar*
Book: Rhizobiont and Attenuation of Precarious Waste, Springer NATURE

MANUSCRIPT PREPARATION

- Response surface methodology and genetic algorithm for enhancing the biosurfactant production by saltpan bacteria. Ruchira Malik and Savita Kerkar*

- Biosurfactant production from hyersaline *Bacillus amyloliquefaciens* SK27 and its potential applications. Ruchira Malik, Manasi Pawaskar, Dikshita Temkar, Ajeet Mohanty, Santosh Tilve and Savita Kerkar*

ABSTRACTS PUBLISHED IN INTERNATIONAL AND NATIONAL CONFERENCES

- Presented a poster entitled " **Saltpan bacteria as producers of Biosurfactants**" in the National conference on Recent Trends in Biotechnology (BioTrends-2016) organized by National Institute of Ocean Technology and the Society for Biotechnology held on 19th to 21st October 2016 at NIOT Chennai.

- Presented a poster entitled **"Biosurfactants From Hypersaline Bacteria and Process Optimization"** in the 3rd International Conference on Innovative Research in Science and Technology, ICIRST2017, Kolhapur from 7-8 November, 2017

Enhancing biosurfactant production by hypersaline *Bacillus amyloliquefaciens* SK27 using response surface methodology and genetic algorithm

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The use of biosurfactants has been limited because of their low yield and high production cost. A central composite design was used to study the interactive effect of sucrose, yeast extract and sodium chloride which were the most influencing variables. Response surface analysis showed that the quadratic model with R^2 value of 0.9983 was fit for biosurfactant production. When genetic algorithm was used for maximization, the optimal activity (oil displacement zone) was found close to that obtained by response surface methodology, both of which were close to the predicted value. Biosurfactant production was enhanced by 1.2-fold using these approaches.

Keywords: *Bacillus amyloliquefaciens*, biosurfactants, central composite design, genetic algorithm, response surface methodology.

BIOSURFACTANTS are surface-active amphiphilic molecules produced by various microorganisms. They are produced on the cell surface of the organism or excreted extracellularly, and have hydrophilic and hydrophobic moieties. Biosurfactants have the ability to accumulate between liquid phases and are capable of reducing surface and interfacial tension[1]. They are classified according to their chemical structure and microbial origin. In recent years, work is focused on their applications in various areas, such as oil recovery, food, cosmetics, agriculture, pharmaceutical industry, petro-chemistry and so on[2,3]. Biosurfactants have numerous advantages compared to chemically synthesized surfactants, which include lower toxicity, biodegradability, being environment-friendly, having better stability and tolerance to a wide range of pH, temperature and salinity values[4].

Despite numerous advantages and industrial applications, the utilization of biosurfactants has been restricted because of their low yield and high production cost[5]. One of the methods to overcome these limitations is to determine the appropriate nutrients at their optimal levels and ideal culture conditions for growth and production of biosurfactants[6]. Medium composition not only influences the growth and metabolism of the culture, but also increases the product yield. Thus, medium optimization is a standout amongst the most crucially explored phenomena, that is completed before any large-scale metabolite production. For structuring a production medium, the most suitable fermentation conditions (e.g. pH, temperature, agitation speed, etc.) and medium components (e.g. carbon, nitrogen, etc.) must be recognized and optimized accordingly[7]. This can be accomplished utilizing a wide range of strategies, from a classical 'one-factor-at-a-time' to present-day statistical and mathematical techniques, viz. genetic algorithm (GA), etc.

Optimization of culture conditions by the classical 'one-factor-at-a-time' (OFAT) method involves changing one factor while keeping all other factors constant. The advantage of this method is its simplicity. The outcomes can be analysed by means of simple graphs without using any statistical analysis. This method primarily helps in the selection of the most influential parameters affecting production significantly; however, it is incapable for studying the interactive effects of several nutritional parameters[8]. This limitation can be overcome by the use of a statistical approach like response surface methodology (RSM).

RSM is an extensively applied statistical approach for designing experiments, assessing interactive effects of variables, and foreseeing the ideal conditions through least number of experiments[9]. It helps develop a numerical model portraying the compound or biochemical system considering the variables and their interactive influence simultaneously and in a few experiments[10]. It is also easy and efficient compared to methodologies because of decrease in the quantity of experimental runs, making it an excellent statistical tool[11,12].

GA is a metaheuristic method inspired from Charles Darwin's theory of natural evolution to solve both constrained and unconstrained optimization problems. It develops solutions to given problems by mimicking the metaphor of the natural biological evolutionary process. Each GA works on an underlying population of potential solution to evaluate fitness and enhance a new population through the use of genetic operators. This method has a fitness value, a real number which will evaluate how good a solution is to the given problem[13]. Currently, utilization of GA to the quadratic equation of RSM is well-known for the optimization of process parameters[14–16]. Thus this study is aimed at optimizing the fermentation medium for enhancing biosurfactant production by hypersaline *Bacillus amyloliquefaciens* SK 27 using statistical approaches.

The bacterial isolate, *B. amyloliquefaciens* SK27 (MF599413) used in the study was previously isolated from water sample of Ribandar saltpans of Goa, India (15°30.166N and 73°51.245E). The isolate was maintained on nutrient agar (HiMedia M001) prepared with sea water[17].

The basic medium used for optimization of the biosurfactant was prepared according to Zhang *et al.*[18]. A

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loopful of the bacterial culture was inoculated in 50 ml basic medium (glucose 20 g/l, peptone 4 g/l, potassium dihydrogen phosphate 5 g/l, potassium phosphate dibasic 5 g/l, calcium chloride 0.08 g/l, magnesium sulphate heptahydrate 0.25 g/l and sodium chloride 4 g/l) and incubated on an orbital shaker (30°C, 120 rpm). After 24 h, the culture supernatant was collected by centrifugation (6000 rpm, 15 min and 4°C), and used further to check the presence of the biosurfactant[19]. All the chemicals used were of analytical grade (Hi-Media, India).

The production of biosurfactant was determined by oil spreading method, as explained by Morikawa *et al.*[20]. In a petri plate containing 20 ml of distilled water, 20 µl of crude oil was added, which formed a layer on the water surface. To this, 10 µl of culture supernatant was added. The petri plate was left undisturbed for 2–3 sec at room temperature and observed for oil displacement (oil-free clearing zone). The diameter of this clearing zone is directly proportional to the biosurfactant concentration[21]. The size of this clearing zone indicates the surfactant activity, also denoted as oil displacement activity. Distilled water was used as negative control (without surfactant) and Triton X-100 (1 mg/ml) was maintained as the positive control.

In order to screen the most influential variables for the production of biosurfactant, one-factor-at-a-time method (OFAT) was carried out[22]. Various process parameters such as pH of the medium (5–9), temperature (10–50°C), carbon source (glucose/glycerol/fructose/sodium citrate/mannitol/starch/mannose/lactose/paraffin/sucrose), nitrogen source (yeast extract/meat extract/peptone/urea/beef extract/tryptone/sodium nitrate/glycine/soya peptone/casein), concentration of carbon (0–2%), concentration of nitrogen (0–1.8%) and NaCl concentration (0–4%) were analysed one at a time, keeping other factors constant.

Once the critical factors were identified, RSM approach was used to study the interaction among three influencing variables, i.e. carbon source: sucrose (*A*), nitrogen source: yeast extract (*B*) and sodium chloride (*C*) selected from OFAT for biosurfactant production using *B. amyloliquefaciens* SK27. The other media components were kept constant, with a variation in the three influential components[23]. Central composite design was utilized to get a quadratic model, comprising tryouts in addition to a star arrangement to gauge quadratic effects and central points to appraise the pure process variability, with biosurfactant production as response. The software package 'Design Expert 11.0' (Minneapolis, USA) was used to analyse the experimental design by central composite design[24]. As indicated by this design, 20 experiments were carried out in triplicate for estimating the experimental uncertainty variance. The response value in each trial was the average of triplicates measured in centimetres. Each factor in the design was considered at three different levels (–1, 0, +1). The levels of three independent variables used were: sucrose (*A*), yeast extract (*B*) and sodium chloride (*C*), each at three coded levels (Table 1). The relationships and interrelationships of these variables were determined by fitting the second-degree polynomial equation to data obtained from 20 experiments using mean values of triplicate of each experiment conducted thrice at different occasions. Response surface plots were generated using the Design-Expert software. The second-order model equation was determined by Fischer's test and the coefficient of determination, R^2 determined the fitness of the polynomial model equation.

GA is a strategy for solving both constrained and unconstrained optimization problems which is based on natural selection, the process that drives biological evolution. It is a simple cycle of four stages consisting of creation, selection, crossover, mutation. The process begins with the creation operator generating an initial population, once the initial population is generated; the selection operator selects the fittest candidates to breed and further alter the population by applying crossover and mutation operator to develop the new population. The entire procedure proceeds until an appropriate outcome is achieved[7]. Optimum conditions were selected after evaluation of GA to attain adjusted fermentation conditions in the specified range of input parameters. The GA toolbox of MATLAB 9.4 (MathWorks, Inc., Natick, MA, USA) was applied in the modelling studies[25].

RSM consists of a pragmatic method for developing, advancing and optimizing processes influenced by variable responses. It defines the effect of independent variables, alone or in combination, on the process and generates a mathematical model[26,27]. Prior knowledge of the process and process variables is important for achieving a realistic

Table 1. Central composite design of the experimental design

Run	*A*	*B*	*C*	Oil displacement zone (cm)
1	0	0	0	8.8
2	–1	0	0	8.2
3	+1	+1	0	8.2
4	0	+1	+1	7.1
5	0	0	0	8.8
6	0	0	+1	8.36
7	0	0	0	8.8
8	–1	0	0	8.26
9	0	0	+1	7.36
10	0	0	–1	8.4
11	0	0	0	8.8
12	+1	+1	0	8.16
13	0	0	–1	7.16
14	–1	+1	0	8.26
15	–1	+1	–1	7.4
16	+1	0	+1	7.2
17	0	0	0	8.8
18	+1	–1	0	7.2
19	–1	–1	+1	7.2
20	0	0	0	8.8

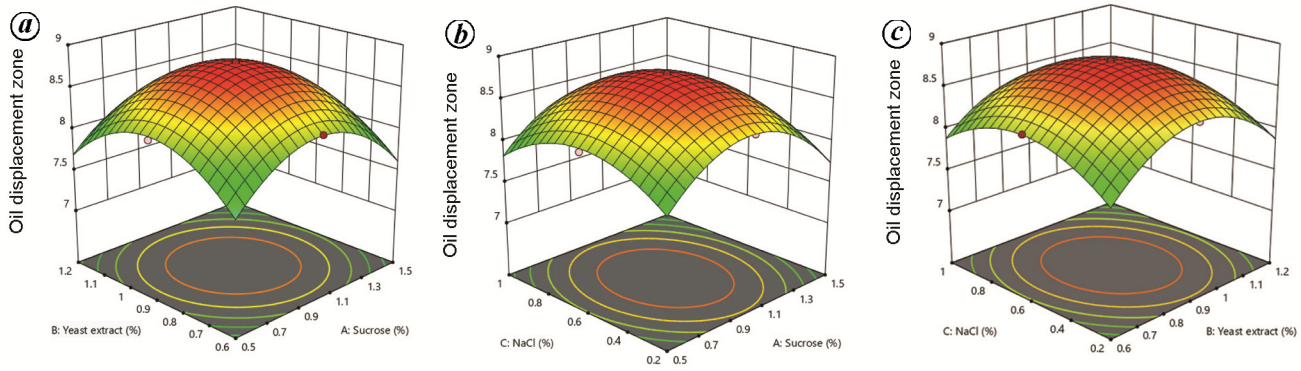

Figure 1. Surface plots of biosurfactant production (oil displacement zone). ***a–c***, 3D response surface curve plot of (***a***) sucrose and yeast extract; (***b***) sucrose and NaCl; (***c***) yeast extract and NaCl.

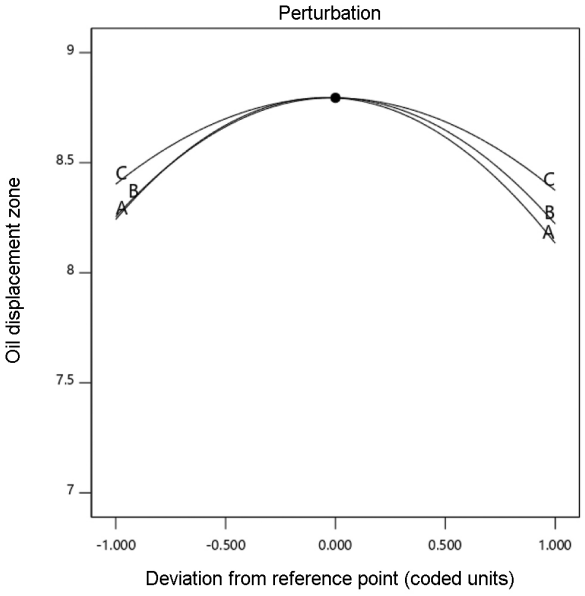

Figure 2. Pertubation graph showing the effect of each of the independent variables on biosurfactant production.

Table 2. Results of regression anaysis of experimental design

Source	Mean square	*F*-value	Prob > *F*
Model	1.01	661.63	<0.0001
A – Sucrose	0.0292	19.02	0.0014
B – Yeast extract	0.0026	1.67	0.2254
C – NaCl	0.0020	1.28	0.2846
AB	0.0040	2.64	0.1352
AC	0.0000	0.0326	0.8603
BC	0.0221	14.38	0.0035
A^2	1.01	659.41	<0.0001
B^2	0.8512	555.14	<0.0001
C^2	0.4541	296.15	<0.0001

model[28]. For this, the effect of different factors such as pH, temperature, carbon and nitrogen sources, and concentration of carbon and nitrogen sources and sodium chloride was evaluated by OFAT. Sucrose and yeast extract had a significant effect on biosurfactant production compared to the other nine carbon and nitrogen sources. Factors such as pH and temperature did not have a considerable effect on biosurfactant production. Based on the identification of the effective variables, a central composite design was developed for the components affecting biosurfactant production, i.e. carbon source: sucrose (*A*), nitrogen source: yeast extract (*B*) and sodium chloride (*C*).

Table 1 shows the experimental results of biosurfactant production by three-factor-two-level factorial experiment design with 6 replications of central point and 14 axial points.

RSM suggested a quadratic model to predict the responses by applying multiple regression analysis (Table 2).

$$Y = +8.79 - 0.0540*A - 0.016*B - 0.014*C - 0.00225*A*B - 0.0025*A*C - 0.0525*B*C - 0.6064*A^2 - 0.5564*B^2 - 0.4064*C^2, \quad (1)$$

where Y is the biosurfactant activity measured by oil clearing zone (cm); A the sucrose concentration (%), B the yeast extract concentration (%) and C is the NaCl concentration (%).

The ANOVA results revealed that the model is highly significant with an *F*-value of 661.63 and probability value of <0.0001 (Table 3). Further, the coefficient of determination (R^2) was calculated to be 0.9983, to examine the fitness of the model. The R^2 value was closer to 1.00, which suggests that the model is stronger and better to predict the response[29]. The adjusted determination coefficient (adj $R^2 = 0.9968$) and predicted determination coefficient ($R^2 = 0.9680$) were also satisfactory to confirm the significance of the model. Adjusted precision assesses the signal-to-noise ratio; a ratio of 62.501 shows satisfactory signal and therefore the model is significant for the process. A low value of

Table 3. ANOVA for full quadratic model

Source	Sum of squares	Mean square	DF	*F*-value	*P* > *F*
Model	9.13	1.01	9	661.63	<0.0001
Residual	0.0153	0.0015	10		
Lack of fit	0.0153	0.0031	5		
Pure error	0.0000	0.0000	5		
Total	9.15		19		

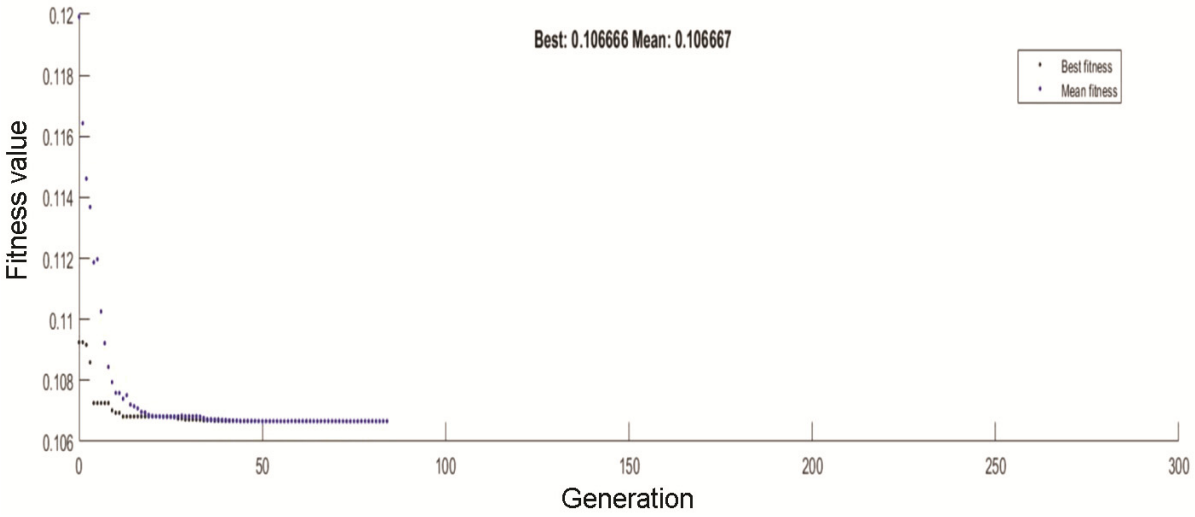

Figure 3. Fitness of the individual population.

coefficient of variation (CV = 0.4889%) demonstrated that the experiment conducted was precise and reliable[30].

Another method in RSM which gives a potential relationship between the variables is the three-dimensional surface plots. It displays the 3D relationship with predictor variables on the *x* and *y* scales, and the response (*z*) variable.

The 3D response surface curve plots in Figure 1 *a–c* are a graphical representation of the regression equation. Each figure represents the effect of two variables, while the third is at zero level for maximum biosurfactant production. Figure 1 *a* shows the effect of sucrose and yeast extract on the biosurfactant production, keeping NaCl concentration constant at the middle level. Figure 1 *b* and *c* also shows a similar trend. Perturbation graph shows the effect of each of the independent variables on biosurfactant production, while keeping the other variables at their respective middle point levels. The response surface plots and perturbation graph show that all the three variables (sucrose, yeast extract and NaCl) play a significant role in the response (Figure 2).

Also, the perturbation graph shows the effect of each of the independent variables on biosurfactant production. Based on the response values, 3D plots and perturbation graph, it can be concluded that all the factors studied have a positive effect on biosurfactant production.

The optimal combination of the major media constituents for biosurfactant production as evaluated from the response surface and contour plots was sucrose 0.97%, yeast extract 0.89% and NaCl 0.59%. The model-predicted optimum activity was found to be 8.79 cm using the above-mentioned optimum concentration of the variables. Verification experiment was carried out using optimized conditions, which gave an activity of 8.8 cm, close to the predicted value. A 1.2-fold increase in yield was observed, i.e. production was increased for 6.9 to 8.7 mg/ml after optimization with RSM, thereby proving the effectiveness of the model in media optimization.

GA is an optimization method which provides a fitness function to determine the optimum concentration of the media components. It is based upon the principle of 'survival of the fittest'. The RSM output data were further optimized using GA to obtain the best fermentation parameters. The nonlinear regression equation of oil displacement activity was optimized using GA optimization tool. Figure 3 represents fitness of the individual population. The oil displacement activity was found to be 8.76 at optimum process conditions which was close to that predicted by RSM. The application of RSM–GA hybrid statistical approaches thus resulted in a significant enhancement in biosurfactant production by *B. amyloliquefaciens* SK27.

Saibaba *et al.*[31] applied hybrid RSM–GA-based technique for modelling and optimization of methylene blue dye removal. This method was found to be efficient and resulted in maximum dye removal of 96.9% (ref. 31). Shirodkar *et al.*[32] used RSM–GA hybrid method for enhancing the α-amylase production by *Thraustochytrium* sp. AH-2. The study showed that there was 1.2-fold increase in α-amylase production, which proved that these statistical experimental designs are efficient and valuable tools in optimizing medium composition[32].

Several studies have been carried out on production of biosurfactants as they are environment-friendly compared to their synthetic counterparts. However on-field application is limited because of their low yield and high production cost. Thus, optimizing biosurfactant production is crucial for up-scaling the process. Optimization of the media components was carried out in the present study using RSM–GA amalgamated approach, which gave a 1.2-fold increase in the yield of biosurfactants. Thus, the statistical approach proved to be efficient, simple as well as time-saving for enhancing biosurfactant production from *B. amyloliquefaciens* SK27. To the best of our knowledge, there are no reports available on media optimization for maximizing the yield of biosurfactant from *B. amyloliquefaciens* using RSM–GA hybrid method, which is as a valuable tool for enhancing biosurfactant production.

1. Karnath, N. G. K., Deo, P. G. and Veenanadig, N. K., Microbial production of biosurfactant and their importance. *Curr. Sci.*, 1999, **77**, 116–123.
2. Khopade, A., Ren, B., Liu, X. Y., Mahadik, K., Zhang, L. and Kokare, C., Production and characterization of biosurfactant from marine *Streptomyces* sp. B3. *J. Colloid Interface Sci.*, 2012, **367**, 311–318.
3. Satpute, S. K., Banpurkar, A. G., Dhakephalkar, P. K., Banat, I. M. and Chopade, B. A., Methods of investigating biosurfactants and bioemulsifiers: a review. *Crit. Rev. Biotechnol*., 2010, **30**, 127–144; doi:10.3109/07388550903427280.
4. Mukherjee, S., Das, P. and Sen, R., Towards commercial production of microbial surfactants. *Trends Biotechnol.*, 2006, **24**(11), 509–515; doi:10.1016/j.tibtech.2006.09.005.
5. Mukherjee, S., Das, P., Sivapathasekaran, C. and. Sen, R., Enhanced production of biosurfactant by a marine bacterium on statistical screening of nutritional parameters. *Biochem. Eng. J*., 2008, **42**, 254–260.
6. Kim, B. and Kim, J., Optimization of culture conditions for the production of biosurfactant by *Bacillus subtilis* JK-1 using response surface methodology. *J. Kor. Soc. Appl. Biol. Chem.*, 2013, **56**, 279−287; doi:10.1007/s13765-013-3044-6.
7. Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M. and Tripathi, C. K. M., Strategies for fermentation medium optimization: an in depth review. *Front. Microbiol.*, 2017, **7**, 2087; doi:10.3389/fmicb.2016.02087.
8. Alikunju, A. P. *et al.*, A statistical approach to optimize cold active β-Galactosidase production by an arctic sediment pscychrotrophic bacteria. *Enterobacter ludwigii* (MCC 3423) in cheese whey. *Catal. Lett.*, 2018, **148**(2), 712–724; doi:org/10.1007/s10562-017-2257-4.
9. Myers, R. H. and Montgomery, D. C., *Response Surface Methodology: Product and Process Optimization Using Designed Experiments*, New York, John Wiley, USA, 2002, 2nd edn.
10. Vohra, A. and Satyanarayana, T., Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. *Process Biochem.*, 2002, **37**, 999–1004.
11. Liyana-Pathirana, C. and Shahidi, F., Optimization of extraction of phenolic compounds from wheat using response surface methodology. *Food Chem*., 2005, **93**, 47–56.
12. Montgomery, D., *Design and Analysis of Experiments*, John Wiley, New York, USA, 2005, 6th edn.
13. McCall, J., Genetic algorithms for modelling and optimisation. *J. Comput. Appl. Math.*, 2005, **184**(1), 205–222; doi:10.1016/j.cam.2004.07.034.
14. Gaitonde, V. N., Karnik, S. R., Achyutha, B. T. and Siddeswarappa, B., GA application to RSM based models for burr size reduction in drilling. *J. Sci. Indust. Res.*, 2005, **64**, 347.
15. Franco-Lara, E., Link, H. and Weuster-Botz, D., Evaluation of artificial neural network for modelling and optimization of medium composition with a genetic algorithm. *Proc. Biochem.*, 2006, **41**, 2200–2206.
16. Moorthy, I. M. G. and Baskar, R., Statistical modeling and optimization of alkaline protease production from a newly isolated alkalophilic *Bacillus* species BGS using response surface methodology and genetic algorithm. *Prep. Biochem. Biotechnol.*, 2013, **43**, 293–314.
17. Fernandes, S., Kerkar, S., Leitao, J. and Mishra, A., Probiotic role of salt pan bacteria in enhancing the growth of Whiteleg Shrimp, *Litopenaeus vannamei*. *Probiot. Antimicrob. Proteins*, 2019, 1–15; doi.org/10.1007/s12602-018-9503-y.
18. Zhang, W., Zhang, X. and Cui, H., Isolation, fermentation optimization and performance studies of a novel biosurfactant producing strain *Bacillus amyloliquefaciens*, *Chem. Biochem. Eng. Q.*, 2015, **29**(3), 447–456.
19. Thavasi, R., Sharma, S. and Jayalakshmi, S., Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria. *J. Pet. Environ. Biotechnol.*, 2011, **S1**(001), 1–6; doi:10.4172/2157-7463.S1-001.
20. Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y. and Imanaka, T., A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS 38. *J. Bacteriol*., 1993, **175**, 6459–6466.
21. Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R. M. and McInerney, M. J., Comparison of methods to detect biosurfactant production by diverse microorganisms. *J. Microbiol. Meth.*, 2004, **56**, 339–347; doi:10.1016/j.mimet.2003.11.001.
22. Irfan, M., Nadeem, M. and Syed, Q., One-factor-at-a-time (OFAT) optimization of xylanase production from *Trichoderma viride*-IR05 in solid-state fermentation. *J. Radiat. Res. Appl. Sci*., 2014, **7**, 317–326; doi:10.1016/j.jrras.2014.04.004.
23. Fernandes, M. S. and Kerkar, S., Enhancing the anti-tyrosinase activity of a hypersaline *Kitasatospora* sp. SBSK430 by optimizing the medium components. *Curr. Sci.*, 2019, **116**(4), 649–653; doi:10.18520/cs/v116/i4/649-653.
24. Lee, N. K., Statistical optimization of medium and fermentation conditions of recombinant *Pichia pastoris* for the production of xylanase. *Biotechnol. Bioprocess Eng.*, 2018, **23**(1), 55–63; doi:10.1007/s12257-017-0262-5.
25. Srivastava, A. *et al.*, Response surface methodology-genetic algorithm based medium optimization, purification, and characterization of cholesterol oxidase from *Streptomyces rimosus*. *Sci. Rep.*, 2018, **8**(1), 10913
26. Anjum, M. F., Tasadduq, I. and Al-Sultan, K., Response surface methodology: a neural network approach. *Eur. J. Oper. Res.*, 1997, **101**, 65–73.
27. Bas, D. and Boyac, I. S., Modeling and optimization I: usability of response surface methodology. *J. Food Eng.*, 2007, **78**, 836–845.

28. Akhnazarova, S. and Kefarov, V., *Experiment Optimization in Chemistry and Chemical Engineering*, Mir Publishers, Moscow, Russia, 1982.
29. Zhu, Z., Zhang, G., Luo, Y., Ran, W. and Shen, Q., Production of lipopeptides by *Bacillus amyloliquefaciens* XZ-173 in solid state fermentation using soybean flour and rice straw as the substrate. *Bioresour. Technol*., 2012, **112**, 254–260; doi:10.1016/j.biortech.2012.02.057.
30. Box, G. E. and Wilson, K., On the experimental attainment of optimum conditions. *J. R. Stat. Soc. Series. B*, 1951, **13**, 1–45.
31. Saibaba, K. V. N. and King, P., Modelling and optimization of dye removal process using hybrid response surface methodology and genetic algorithm approach. *J. Fundam. Renew. Energy Appl.*, 2014, **4**(01), doi:10.4172/2090-4541.1000126.
32. Shirodkar, P. V. and Muraleedharan, U. D., Enhanced α-amylase production by a marine protist, *Ulkenia* sp. using response surface methodology and genetic algorithm. *Prep. Biochem. Biotechnol.*, 2017, **47**(10), 1043–1049; doi:10.1080/10826068.

ACKNOWLEDGEMENT. We thank the Head, Department of Biotechnology, Goa University for providing funds and facilities.

Received 26 March 2019; revised accepted 20 June 2019

doi: 10.18520/cs/v117/i5/847-852

South Asian subtropical low-level jet: influence on regional hydrology and aerosol optical depth

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A 38-year high-resolution wind climatology shows a consistent occurrence of hitherto unreported South Asian subtropical low-level jets (SASLLJs) over the 31°–35°N and 60°–62°E latitude–longitude region, occurring throughout the year are the northerly LLJs (NLLJs) and southerly LLJs (SLLJs). The NLLJ is persistent mostly during the monsoon season. The NLLJ is frequent, unlike the SLLJ. The synoptic heat low in South Asia (62°–66°E and 27°–32°N) and the anticyclone over Turkmenistan (40°–50°N and 50°–60°E) are critical in inducing the NLLJ. Regional topography permits direct mid-latitude air intrusion into the subtropical region. Importantly, we find that the NLU results in enhancing the transportation of dust to the Indian subcontinent and the northern part of the Arabian Sea due to wind blows. Long-term NLLJ intensity displays waning trend because of the weakening of pressure gradients between the heat low in South Asia and over Turkmenistan.

Keywords: Aerosol optical depth, low-level jet, regional hydrology, rainfall.

REGIONAL wind systems play a critical role in the climate and hydrology of an area[1–3]. The importance of low-level jets (LLJs) in impacting regional climate has been well recognized in many areas around the world[4]. The occurrence of regional wind patterns in Asia has an enormous consequence on oil exploration, agriculture and the economy of a region[5–7]. The monsoon LLJ and Shamal wind over the Asian region have been well documented[3,5,8]. Previous studies suggest the existence of low-level northerly winds from June to September over Southwest Asia in the Iran–Afghanistan border area[9,10]. The northerly wind over Iran region is known as the Sistan wind or the 'wind of 120 days'[10]. A recent study has examined the influence of Asian winds on the ecosystem in South Asia[11]. It also discusses about a Levar wind near the Iran–Afghanistan region reaching up to the northern part of the Arabian Sea. However, these studies are based on a dataset for a shorter period during the monsoon season. The analyses of 38-year multiple atmospheric reanalysis data showed two LLJ spells in the region, and we report them here as South Asian Subtropical LLJ (SASLLJ). The SASLLJ spells can be categorized into two types based on the direction as northerly and southerly (NLLJ and SLLJ respectively).

South Asia has relatively high population density, where freshwater availability mainly depends on the precipitation over the region. It has been recognized that even a small rainfall anomaly can influence the economy of South Asia[12,13]. Many regional winds influence the precipitation pattern and hydrology of the region[1,14,15]. On a different note, a study showed that aerosols from the Sahel region influence the Indian summer monsoon climate[16]. In the present study, potential influence of the aforementioned wind patterns in West Asia, and its synoptic pattern on rainfall anomalies and aerosol transport into South Asia are examined. The study also analyses aerosol optical depth (AOD) as observed by the MODIS satellite[17], which realized in understanding composite anomalies over South Asia.

Here we analyse the long-period characteristics of SASLLJs near the Iran–Afghanistan region using multiple reanalysis data. The role of SASLLJ synoptic conditions on regional hydrology and AOD over Asia is also examined. The long-term trend of SASLLJ intensity and its causes are presented. The European Centre for Medium-Range Weather Forecasts (ECMWF), the ERA

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