Bioprocessing of Biosurfactants from Microbial Sources and their Applications

by

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Islamabad

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A thesis submitted in partial fulfillment of the requirements for the Degree of

Doctor of Philosophy

In

Microbiology

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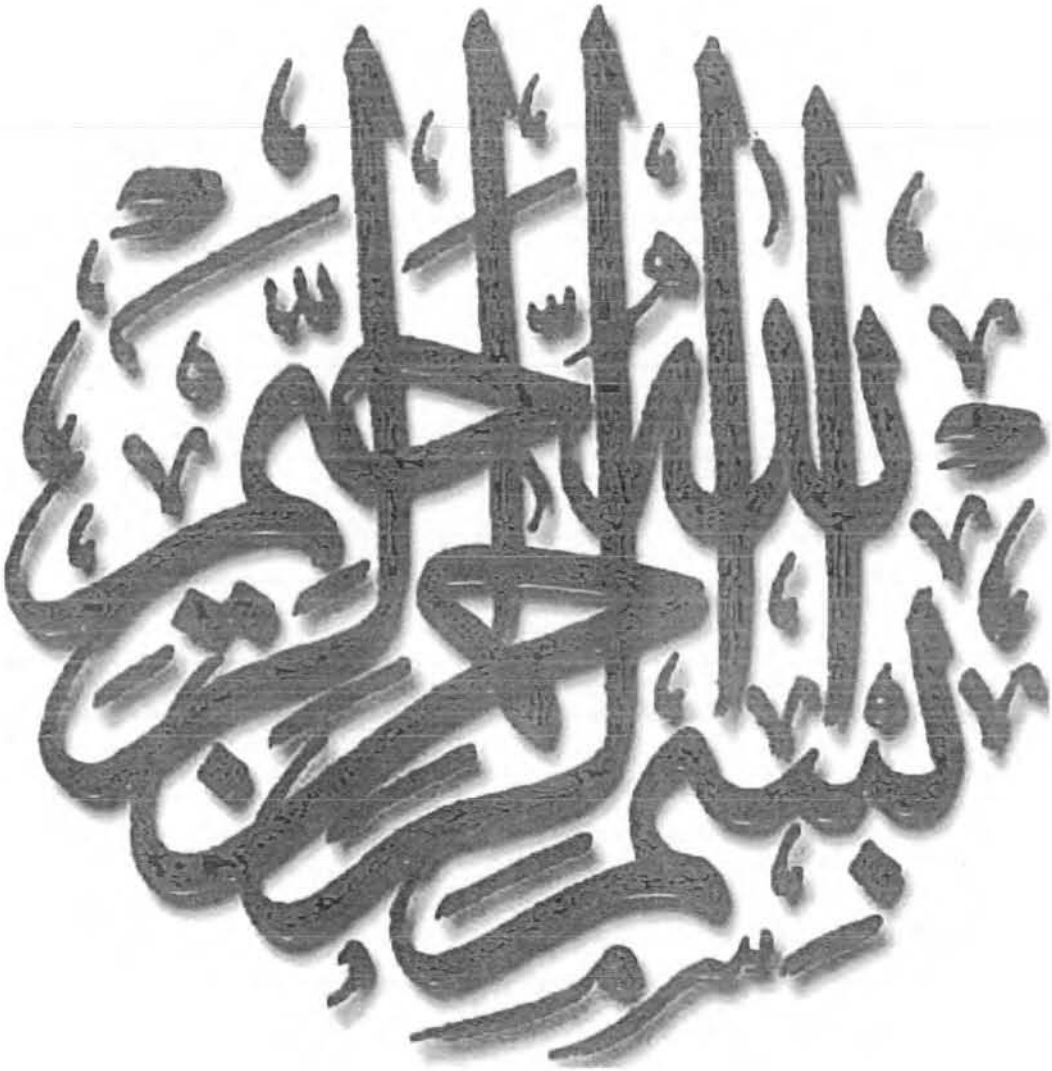

"In the name of Allah, the most Gracious and most Merciful"

DEDICATED

TO

MY BELOVED PARENTS

SYED UBAID-UR-REHMAN & AISHA AZAD

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LIST OF ACRONYMS/ABBREVIATIONS

AAS	Atomic Absorption Spectrophotometer
ANOVA	Analysis of Variance
B.E	Biodegradation Efficiency
cmc	Critical Micelle Concentration
CSH	Cell Surface Hydrophobicity
CTAB	Cetyl Trimethyl Ammonium Bromide
DF	Degree of Freedom
DLS	Dynamic Light Scattering
$E.I_{24}$	Emulsification Index
EC	Electrical Conductivity
Fig.	Figure
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
h	hours
K_2HPO_4	Di-potassium Hydrogen Phosphate
KNO_3	Potassium Nitrate
LC-ESIMS	Liquid Chromatography-Electrospray Ionisation Mass Spectrometry
M	Molar
m/z	Mass to charge ratio
MATH	Microbial Adhesion to Hydrocarbons Assay
MHA	Mueller Hinton Agar
MIC	Minimal Inhibitory Concentration
mM	milli molar
NA	Nutrient Agar
NaCl	Sodium Chloride
NaH_2PO_4	Sodium Di-hydrogen Phosphate
NCBI	National Center for Biotechnology Information

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Ramla Rehman

Abstract

Biosurfactants are extracellular microbial products that show a high degree of structural, functional and chemical heterogeneity. In comparison with synthetic surfactants, biosurfactants exhibit environmental compatibility, biodegradability, high reaction rate, astonishing chemical diversity, and better physiochemical characters. In the first phase of study, physicochemical characterization of crude oil affected and unaffected soil of Chak Naurang, Punjab, Pakistan was done. Results demonstrated that the contaminated soil was slightly alkaline clay-loam with TPH of 22.2 g/kg and TOC of 23.11 g/kg. The concentration of nitrogen, phosphorus and potassium was found to be quite low i.e. 0.644 g/kg, 12.46 mg/kg and, 20 mg/kg, respectively. Enrichment and isolation experiments were typically designed to obtain indigenous biosurfactants producing microorganisms of crude oil contaminated soil. Amongst forty isolates, qualitative and quantitative screening for biosurfactants production confirmed fourteen potential microorganisms, which were identified through morphological, microscopic, biochemical and molecular studies. The two most efficient biosurfactants producing microorganisms i.e. *Pseudomonas aeruginosa* MF069166 with 84 % of $E.I_{24}$, 26.6 mN/m of S.T, 8 cm of oil displacement zone and, *Meyerozyma* spp. MF138126 with 82 % of $E.I_{24}$, 26 mN/m of S.T and 7.4 cm of oil displacement zone were selected for further studies of bioprocess optimization, characterization and environmental applications of biosurfactants.

In the second phase of study out of five different fermentation media, M 2 was found to be the most suitable for *P. aeruginosa* MF069166 whereas, M 5 for *Meyerozyma* spp. MF138126. Plackett-Burman optimization showed that glycerol, K_2HPO_4, peptone, KNO_3, NaCl, yeast extract and $MgSO_4$ were significant components of M 2 for maximum rhamnolipids production (3.46 g/L) from *P. aeruginosa* MF069166 whereas, peptone, glycerol, NaH_2PO_4, yeast extract and $MgSO_4$ were significant components of M 5 for maximum sophorolipids production (4.02 g/L) from *Meyerozyma* spp. MF138126. Optimization of culture conditions using Response Surface Methodology revealed that the predicted values of pH; 6.5, T; 35 °C, SOI; 3.4 % and agitation speed of 120 RPM resulted in maximum growth of 3.06 and rhamnolipids production of 4.31 g/L from *P.*

aeruginosa MF069166. In case of *Meyerozyma* spp. MF138126, the predicted values of pH; 5.5, T; 33.2 °C, SOI; 3.3 % and agitation speed of 161 RPM resulted in maximum growth of 3.17 and sophorolipids production of 6.9 g/L.

Chemical characterization techniques of TLC, FTIR, RP-HPLC, ^{1}H and $^{13}CNMR$ and LC-ESI-MS confirmed that biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were rhamnolipids and sophorolipids, respectively. The stability of rhamnolipids was witnessed from 15-121 °C T, 4-11 pH and 2-10 % NaCl whereas, the stability of sophorolipids was observed from 5-115 °C T, 3-10 pH and 2-10 % NaCl. Cell surface hydrophobicity (CSH) studies showed more than 50 % affinity of the two isolates for crude oil, hexadecane and dodecane whereas, more than 70 % emulsification activity was observed by their respective rhamnolipids and sophorolipids in the presence of different hydrocarbons. *cmc* of rhamnolipids was found to be 40 mg/L whereas, the *cmc* of sophorolipids was 50 mg/L. z-average diameter of purified rhamnolipids and sophorolipids was noted under the effect of varying *cmc*, pH and electrolytes through Dynamic Light Scattering technique.

In the final phase of study, role of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and, their respective rhamnolipids and sophorolipids was evaluated in biodegradation of crude oil and bioremediation of heavy metals. In crude oil degradation experiments, gravimetric and GC-MS analysis demonstrated that the bacterial strain degraded 90 % of the petroleum hydrocarbons while the yeast species showed 85 % biodegradation efficiency after 14 days of incubation period. Results of heavy metals bioremediation studies indicated more than 75 % removal of zinc from aqueous phase and contaminated soil through metal chelating activity of rhamnolipids. Similarly, sophorolipids were able to precipitate out more than 80 % of lead from the two phases. Kinetic study of biosurfactants mediated soil washing demonstrated a gradual increase in percentage removal of lead and zinc with the passage of time. These findings suggested that biosurfactants producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 have high potential to be used in different environmental applications and can be considered as suitable candidates for field scale bioremediation studies.

1- INTRODUCTION

include amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers (Bezza et al., 2015).

1.2. Glycolipid biosurfactants

Glycolipids are the most important class of biosurfactants due to their diverse physio-chemical properties and biological activities (Reynolds and McIver, 2018). Structurally, they comprise of sugars like mono-, di-, tri- and tetra-saccharides representatives of which include glucose, mannose, galactose, glucuronic acid, rhamnose and galactose sulphate (Haloi and Medhi, 2019). Fatty acid portion of glycolipids normally depends on the specific lipid moiety secreted by particular microbial specie. Moreover, long chain aliphatic or hydroxyaliphatic acids have also been reported to form ester linkages with carbohydrates to maintain the structural integrity of glycolipids (Usman et al., 2016a). Some of the low molecular weight glycolipids i.e. sophorolipids, rhamnolipids and trehaloselipids effectively fulfil the eco-friendly criteria of sustainability (Sharma et al., 2014, Pocock et al., 2016). In comparison with the synthetic surfactants, BS offer better chemical properties, environmental compatibility, high reaction rate at low *cmc*, higher surface activity, and multiple functional groups with varying chiral centres, and ease of production using low cost raw materials (Pinheiro and Faustino, 2017). Taking all together, microbial surfactants are considered as the potential future replacement of the synthetic surfactants.

1.2.1. Properties of glycolipid biosurfactants

Surface active glycolipids are among the most comprehensively studied microbial products. These molecules display a wide spectrum of unique chemical, physical and biological properties (Safdel et al., 2017). When these biosurfactants are introduced into the aqueous system, surfactants monomers start self-aggregation and produce various potent microstructures such as micelles, lamellar sheaths, twisted and helical ribbons (Rodrigues, 2015). Above *cmc* value, surfactant shows a drastic change in surface properties, adsorption rate, detergency, electric conductivity, density and solubility of immiscible substrates in the system (Myers, 2017). It has been reported that *cmc* of the microbial glycolipids is far less (20-500 mg/L) which gives them

functional advantage over synthetic surfactants (Paulino et al., 2016). Hydrophobic-lipophilic balance (HLB) is another important feature of the biosurfactants that help in emulsion formation (Haq et al., 2018). The HLB of rhamnolipid, is 10.07 which is fairly higher than the chemical surfactants making glycolipids highly suitable for environmental applications (Long et al., 2016).

Glycolipid biosurfactants have excellent anti-bacterial, antifungal, anti-viral, anti-adhesive and anti-proliferative properties. In addition, the synthesis of biosurfactants also supports microbial survival in a particular environment (Vecino et al., 2017). It also helps the producer microorganism to organize various sub-cellular systems such as biofilm formation, cell to cell communication, pathogenesis, substrate accession and cellular defence (Ndlovu et al., 2016). In summary, considering the unique properties and molecular profile, the number of the applications of glycolipids is continued to grow. In spite of various process specific limitations, about 110 patents have been granted on glycolipids biosurfactants depicting their huge future potential (Kumar and Das, 2018b).

1.2.1.1. Rhamnolipids (RLs)

Rhamnolipid is a class of glycolipid biosurfactants principally produce by different strains of *Pseudomonas aeruginosa* (Mondal et al., 2017). Some other bacterial strains such *P. putida*, *B mallei*, *P. chlororaphis*, *B. thailandensis* and *A. calcoaceticus* have also been known as RL producers (Kumar and Das, 2018c). Rhamnolipid was first reported by Jarvis and Johnson in 1949 and its chemical structure was revealed by Edwards in 1965. RL is a combination of rhamnose sugar (head) attached with 3-hydroxydecanoic acid lipid moiety (tail) (Irorere et al., 2017). However, RLs show excellent chemical diversity due to the variation in chain length of the hydrophobic lipid moiety. For example, rhamnolipid (RL-1) is composed of a rhamnose molecule attached with one fatty acid. Whereas Rhamnolipid RL-2 contains two rhamnose molecule bonded with either one or two fatty acids (Kiran et al., 2016). Till recent, more than sixty RLs with different molecular weight and properties have been reported from different strains of *P. aeruginosa*. However, RL-1 and RL-2 remained the two most prevalent RL species produced during the fermentation process (Elshikh

et al., 2017). Rhamnolipid is anionic surfactant with a pKa of 4.1 to 5.6, due to carboxylic acid in their structure. The molecular mass of different RL congeners ranges between 467 to 776 g/mol (Kumar and Das, 2018a). In aqueous solution it undergoes self-assembly to form various kinds of nanostructures such as micelles, helixes, monolayers, lamella and sheaths which give unique properties and new outlook to the RL molecules (Chen et al., 2010). Rhamnolipids are capable to reduce the surface tension of the aqueous solution to 25 mN/m and interfacial tension of oil water system to less than 1 mN/m. In addition to these, *cmc* of RL molecules is in the range between 20-500 mg/L which is quite low than the commercial surfactants (Mendes et al., 2015).

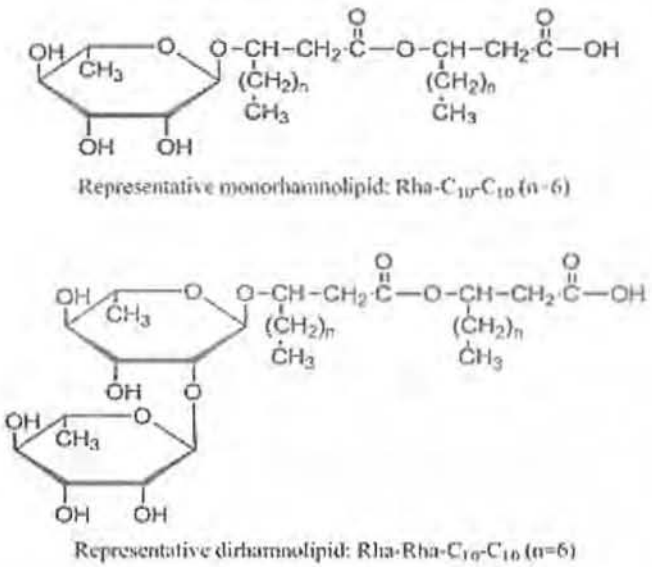
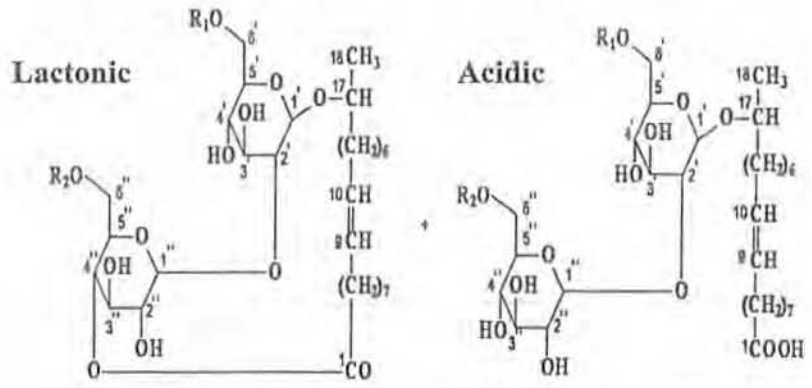


Figure 1.1: Biochemical structures of Rhamnolipids (Abdel-Mawgoud et al., 2010)

1.2.1.2. Sophorolipids (SLs)

Sophorolipids are one of the most promising type of biosurfactants due to higher cellular productivity and ease of product recovery. Yeast species in general and *Candida bombicola* in particular is well known for extracellular production of sophorolipids (De Graeve et al., 2018). Chemically, SLs molecules are comprised of hydrophilic disaccharide sophorose sugar forming β-glycosidic linkages with long

chain hydrophobic hydroxyl fatty acids. Two conformational isomers of sophorolipids have been reported and are named as open and cyclic SLs (Konishi et al., 2018). Acidic, non-lactonic or open SLs has a free carboxylic acid functional group attached to hydroxyl group of fatty acid while lactonic or closed SLs forms intra-molecular ester linkages with 4''-hydroxyl group of sophorose sugar resulting in the formation of a macrocyclic lactone ring. This structural diversity attributes a number of unique properties to SLs as they exhibit tolerance for a wide range of pH, temperature and varying salt concentrations (Kurtzman et al., 2016). They possess the ability of lowering surface tension to a value of 33 mN/m. Moreover, synergism between lactonic and non-lactonic forms further improves the surface activities of theses glycolipids. Currently, 30 different isoforms of sophorolipids are known (de Oliveira et al., 2015). Sophorolipids were first reported in 1961 by Gorin and co-workers as an extracellular nonionic product of *Torulopsis magnolia.* However, some other fungal species like *Candida apicola, Rhodotorula bogoriensis and Starmerella bombicola* also produce SLs (De et al., 2015). SL molecules offer several advantages over the petrochemical based surfactants such as biodegradability, high performance at low *cmc* value, low toxicity and antimicrobial activity against various pathogenic bacteria and fungi. Like other glycolipid surfactants, SL molecules lower the interfacial tension of oil water system to 5 mN/m (Ahn et al., 2016).



i. $R_1 = R_2 = COCH_3$ (Main component)
ii. $R_1 = COCH_3, R_2 = H$
iii. $R_1 = H, R_2 = COCH_3$
vi. $R_1 = R_2 = H$

v. $R_1 = R_2 = COCH_3$
vi. $R_1 = COCH_3, R_2 = H$
vii. $R_1 = H, R_2 = COCH_3$
viii. $R_1 = R_2 = H$

Figure 1.2: Biochemical structures of Sophorolipids (Claus and Van Bogaert, 2017)

1.3. Production of biosurfactants

The overall production of chemical surfactant has reached up to 12 million tons with an expected annual increase of 3 % (Pal and Pal, 2018). In comparison with synthetic surfactants, production cost of biosurfactants is 20 to 30 times higher and poses a significant challenge for their commercial viability (Luna et al., 2015). Number of cultivation strategies has been reported to achieve optimum yield of biosurfactants such as strain improvement, medium optimization, altering the bioreactor design and utilization of cheap carbon substrates (Singh et al., 2019). Since carbon substrate accounts for 50 % of total production cost, therefore, the main factor governing high volumetric production of biosurfactants is effective bioprocess design and use of low cost substrate. In some previous attempts various carbon substrates have been used for biosurfactants from different microorganisms (Makkar et al., 2011). The use of oily waste such as waste frying oil, oil refinery waste and other complex substrates like biodiesel waste glycerol, molasses, cassava waste, and distillery waste have been used for the production of biosurfactants (Kaur et al., 2015). When compared with the bacteria, application of mixture of hydrophilic and hydrophobic carbon substrate in case of yeast remained more effective for biosurfactants production. In a previous study, production of SLs having excellent detergent properties was achieved by feeding the yeast with a combination of non-traditional oils such as jatropha oil and neem oil along with glycerol (Bhangale et al., 2014). Apart from these, various simple hydrocarbons like glucose, sucrose and glycerol have also been reported for biosurfactants production. The carbon source not only affects the process efficiency but also has profound effect of the chemical composition of the biosurfactants (Nicolò et al., 2017). Apart from carbon substrate, other nutrients such as N^{+1}, K^{+1}, P^{+2}, Mg^{+2}, Fe^{+2}, and Ca^{+2} and their ratio affects overall efficiency of the bioprocess for the production of biosurfactants. Other bioprocess variable such as pH, temperature, shaking speed, aeration and multivalent ions also effect the production of biosurfactants. It is widely suggested that combination of relatively cheap fermentation substrates could be very promising for the production of cost effective biosurfactants (Sharma and Oberoi, 2017). In the light of the above facts, optimization of bioprocess variables is a critical aspect for achieving higher yield of biosurfactants.

The conventional method of bioprocess optimization, which implicates changing one variable at a time while keeping others constant, is time consuming and lacks the essentials for optimal metabolite production (Bertrand et al., 2018). These problems can be addressed by the application of statistical optimization strategy known as Response Surface Methodology (RSM) in which a mathematical relationship is being developed between several explanatory and response variables (Said and Amin, 2016). The implementation of these approaches would enable the industry to establish the best combinatorial proportion of media substrates and environmental conditions for improved biosurfactants production (Latha et al., 2017). It has been suggested that the production of RLs from *P. aeruginosa* was increased many folds by the application of response surface methodology (El-Housseiny et al., 2016). In case of sophorolipids, the RSM application improved the yield of the product up to 18.2 g/L by *Candida* species (Almeida et al., 2017). In another study, the production of SLs by *Starmerella bombicola* was enhanced up to 54 g/L that is quite higher than the un-optimized conditions (Sonawane et al., 2015). Besides various claims, high volumetric production and application of the biosurfactants still remain challenging because of; a) less production microbial strains, b) cost of fermentation media c) expensive downstream processing d) unavailability of information regarding the bioprocess conditions and e) patent and copy rights issues (Rebello et al., 2018). Therefore, there is a dire need to improve the productivity of the biosurfactants from the native strains to make them commercially attractive.

1.4. Environmental pollution

Environmental pollution is one of the most important challenges of the world specifically for the developing economies (Giles-Corti et al., 2016). During last three decades, extensive increase in global population has resulted in urbanization and industrialization which caused high demand of various trade commodities such as fossil fuel (Drakakis-Smith, 2017). Crude oil serves as predominant energy resource and to date the largest trade commodity (Sharmina et al., 2017). However, on the other hand, its exploration, transport, storage and utilization have been associated with generation of huge quantities of oily waste (Bayat et al., 2015). Crude oil is a highly complex and heterogenic mixture of many compounds ranging from simple

hydrocarbons to highly complex aromatics (Bagby et al., 2017). Therefore, contamination of soil and water with crude oil hydrocarbons can be very devastating and causes severe ecological toxicity. Furthermore, its constituents are reported to cause substantial damages to the life because of their long term persistence in the environment (Yavari et al., 2015). Heavy metals are among the most threatening environmental pollutants. They are naturally present in the ecosystem; however, their concentration is continuously increasing both in soil and water due to anthropogenic activities (Fashola et al., 2016). Heavy metals enter in the ecosystem by variety of sources such as from mining operations, industrial wastes, vehicle emissions, batteries and from microplastics (Clemens and Ma, 2016). Due to the simple structure, heavy metals are more toxic than other pollutants and can cause cancer, and other deformities like proteinuria and osteomalacia (Mahurpawar, 2015). Taking together, addition of hydrocarbons and heavy metals in the environment is considered as serious risk for all life forms. Conventional remediation methods are usually based on the application of chemical surfactants which are highly toxic in nature. According to various studies, chemical surfactants pose severe environmental toxicity and health risks (Akcil et al., 2015). Therefore, sustainable methods should be devised in order mitigate environmental contamination and potential risk of these contaminants. Considering aforementioned limitations, use of biosurfactants and biosurfactants producing microorganisms for the reclamation of the environment has been steadily growing all over the world.

1.4.1. Environmental applications of biosurfactants and BS producing microorganisms

Biosurfactants are mostly produced by hydrocarbons degrading microorganisms thus plays an important role in their bioremediation (Shekhar et al., 2015). Hydrocarbons contaminated sites are the prime locations for the isolation of potent biosurfactants producing microorganisms. In these contaminated environments surfactant production give competitive advantage to producers and help in substrate accessibility, colonisation, mobility and cell defence (De et al., 2015). The biosynthesis of biosurfactants is strongly associated with solubilisation of the complex hydrocarbons thereby, making them more accessible to the microbial cell by improving cell surface

hydrophobicity (Floris et al., 2018). Collectively, these processes facilitate inter-membrane transport of the hydrophobic pollutants and cause their degradation in the cell. In agriculture soils, release of biosurfactants has been associated with improved nutrient uptake for plants and enhances quality of soil by degrading organic pollutants (Singh et al., 2018). It has been reported that rhamnolipids produced by *P. aeruginosa* and sophorolipids by *Candida* species significantly increase degradation rates of organic pollutants such as crude oil, hexadecane, cholorophenol, phenanthrene and polyaromatic hydrocarbons (Karlapudi et al., 2018). Ma et al. (2016) have reported that rhamnolipid producing *P. aeruginosa* caused 90.52 % degradation of the crude oil hydrocarbons. In another study He et al., (2017) have suggested that *P. aeruginosa* supplemented with optimization of cultivation conditions degraded most of the crude oil components after 14 days of incubation period. Similarly, sophorolipids from *Candida* removed 80 % crude oil after 10 days under optimized conditions (Kang et al., 2010). In addition to their role in biodegradation of hydrocarbons, glycolipids have been extensively used in the remediation of metals contaminated sites. The heavy metals contaminants are removed from soil through ion-exchange and surfactants associated complexation (Santos et al., 2016). Moreover, the biodegradability, ionic nature and excellent surface active properties of glycolipids facilitate the dispersion and desorption of metal ions from soil. The potential of glycolipids biosurfactants particularly rhamnolipids and sophorolipids has been reported for the bioremediation of heavy metals (Usman et al., 2016b). It has been observed that increase in concentrations of sophorolipids above the *cmc* improve the metals removal percentage from soil. Wen et al. (2016) reported the enhanced phytoextraction of cadmium and zinc in the presence of rhamnolipids. Therefore, the significance of these molecules in research field has been acknowledged throughout the world.

Pakistan is an agricultural country and major part of its economy depends upon the principal natural resources of arable land and water (Azam and Shafique, 2017). However due to rapid industrialization, various post-industrial sites are unproductive for decades because of their incompatibility for agriculture or commercial use. In Punjab, province of Pakistan, these sites are mostly located in close proximity of the suburban or rural agriculture settings posing a serious threat to the public health due

to high concentration of pollutants (Zia et al., 2017). The restorations of these sites face serious challenges due to unavailability of indigenous technical resources. In addition, the available site remediation technologies are either very expensive or not compatible with the local climate conditions. These issues can be addressed by the exploitation of indigenous microflora having the ability to degrade the crude oil hydrocarbons. In spite of lab scale success in biosurfactants production and their applications, the large scale production of these molecules still remains a challenge because of the unavailability of productive strains and suitable bioprocess design (Singh et al., 2019). Keeping in view these limitations, the current research work was focused to isolate crude oil degrading and biosurfactants microorganisms from the contaminated site. The bioprocess was designed in order to increase the production of biosurfactants from the selected isolates and their role in biodegradation and bioremediation of the pollutants was also evaluated.

1.5. Aim and Objectives

Aim

To design process for optimal production of biosurfactants from microbial sources and their environmental applications.

Objectives

To achieve the aim of proposed study, followings are the major objectives

- Isolation and screening of biosurfactants producing microorganisms from crude oil contaminated soil.
- Optimization of bioprocess using design of experimental approach for biosurfactants production from selected microbial isolates.
- Downstream processing and characterization of biosurfactants.
- Investigation of environmental applications of biosurfactants.

2- REVIEW OF LITERATURE

Since the beginning of the millennium, environmental legislation has been changing rapidly for the development of sustainable products. This has motivated the modern day researchers to explore natural resources for industrial and domestic applications. Amongst various synthetic products, surface active compounds are being used extensively throughout the globe with an increasing trend of their production and consequent utilization (Akcil et al., 2015). Chemical surfactants are synthetic molecules which are mostly used in different industrial products such as washing powders, detergents, toothpastes, soaps and shampoos. However, their implication is associated with various environmental concerns due to their source of origin. Most of the commercially available surfactants are the derivatives of petroleum based organics therefore, scientists are now looking forward for their possible replacement (Bezza et al., 2015). Some recent scientific evidences have suggested the use of bio-based surface active compounds as an innovative and green replacement of their chemical counterparts. Biosurfactants are the amphiphilic secondary metabolites that possess significant surface and interfacial activity due to the presence of both hydrophobic and hydrophilic moieties in a single molecule (Banat and Rengathavasi, 2018). Due to these properties, biosurfactants tend to solubilize complex hydrophobic compounds and other contaminants in aqueous system. Considering the potential of biosurfactants, their applications are growing rapidly specifically in the field of environmental remediation (Abdel-Megeed et al., 2014). It has been reported that biosurfactants have generated about 30 million US $ revenues in 2015, which is likely to increase in upcoming years by 2.8 billion US $.

2.1. Microbial sources of biosurfactants

Biosurfactants are produced by both prokaryotic and eukaryotic species which make them ubiquitous in nature (Soberón-Chávez, 2010). These surface active molecules are either produced on the surfaces of living cells or secreted extracellularly. A number of plant, animal and microbial species have been reported as potential producers of biosurfactants. Microorganisms utilize biosurfactants to facilitate the diffusion of insoluble substrates like hydrocarbons (CxHy) into the cell (De et al., 2015). Scientists have declared the production of microbial derived biosurfactants as one of the key factors contributing towards the

development of modern era. Although, quite a few plant-based biosurfactants for example saponins, lecithins, and soy proteins have excellent emulsification properties however, their production process is not quite economical to be upgraded at industrial scale and have other debatable issues like hydrophobicity and solubility (Ławniczak et al., 2013). It has been observed that microbial biosurfactants are advantageous over other biological surfactants because of their multi-functional properties, rapid production, ease of availability and scale-up capacity. The studies involving microbial biosurfactants started in 1940s, however, the industrial use of these molecules has expanded since last few decades (Soberón-Chávez and Maier, 2011). So far, more than 250 patents have been obtained on these wonder biodegradable molecules. Some of these molecules have been formerly exploited in different fields while others are expecting to be discovered and developed (Kaur et al., 2015). Due to variety of experimental procedures being used, it is not easy to estimate the prevalance of biosurfactants producers within a microbial community. Reports are available that differentiated the percentage abundance of biosurfactants producing microorganisms in the contaminated and un-contaminated soils. Previously, 2-3 % of biosurfactants producing microorganisms were screened out from the non-polluted soil whereas, the number increased up to 25 % in polluted soil (Olivera et al., 2009). The bacterial genera that have been reported as eminent producers of biosurfactants include; *Pseudomonas*, *Bacillus*, *Actinobacteria*, *Sphingomonas*, *Halomonas*, *Pseudoalteromonas*, *Acinetobacter* and *Alcanivorax*, *Rhodococcus*, *Mycobacterium* and *Arthrobacter* (Shekhar et al., 2015, Biniarz et al., 2017, Perfumo et al., 2018a) Although the bacterial species have been extensively studied for biosurfactants production, however very little research work has been conducted to exploit the biosurfactants producing potentials of fungi. Amongst various fungal species, some eminent producers include *Candida bombicola*, *Candida lipolytica*, *Candida batistae*, *Candida ishiwadae*, *Trichosporon ashii* and *Aspergillus ustus* (Bhardwaj et al., 2013, Vijayakumar and Saravanan, 2015). It is to be noted that the nature of produced surfactants depends upon the composition of growth substrate provided. Some of the major biosurfactants and their microbial sources have been enlisted in Table 2.1.

Table 2.1: List of biosurfactants producing microorganisms

Biosurfactants	Microbial Sources	Reference
Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas chlororaphis*	(Rikalovic et al., 2017, Silva et al., 2017, Araújo et al., 2018)
Sophorolipids	*Candida bombicola, Torulopsis petrophilum, Starmerella bombicola, C. antartica,*	(Shah et al., 2017, Claus and Van Bogaert, 2017)
Trehaloselipids	*Arthrobacter* sp., *Corynebacterium* sp., *Nocardia erythropolis,*	(Roy, 2017, Wagner and Lang, 2017)
Surfactin	*B. subtilis, B. amyloliquefaciens*	(Liu et al., 2015, Montagnolli et al., 2015)
Emulsan	*A. calcoaceticus*	(Yi et al., 2019)
Monnosylerythritol lipids	*Pseudozyma siamensis, C.antartica*	(Arutchelvi et al., 2008, Bezerra et al., 2018)
Phospholipids	*Acinetobacter sp.*	(Banat and Rengathavasi, 2018)
Peptide lipids	*B. licheniformis*	(Bento et al., 2005)

2.2. Properties of biosurfactants

Biosurfactants exhibit diversity in their molecular structures and functions. The nonpolar hydrophobic tail is made up of hydrocarbon chains of varying length and complexity, whereas the polar hydrophilic head comprises of either peptide, carbohydrate, amino acid, alcohol or phosphate carboxyl acid (De Almeida et al., 2016). These molecules are known to create preferential partition between liquid interfaces of different polarities such as oil/water/air and improvises the bioavailability of substrates by reducing the surface and interfacial tension (Floris et al., 2018). Their various physiological attributes include low toxicity, greater biodegradability, environmental compatibility, structural diversity, functional stability at extreme pH, temperature and salinity, low *cmc* and broader substrate specificity. Wetting, phase separation, detergency, foaming, micro-emulsification and selective tension-active properties are also comparatively better than many synthetic surfactants (Santos et al., 2016). The production of biosurfactants from microbial sources can be either inducible or constitutive which makes them one of the most versatile

bioproducts of the modern day biotechnology (Fechtner et al., 2017). Moreover, the process economics of biosurfactants for bulk production can be optimized through the utilization of cheap and renewable raw materials, industrial wastes or other by-products (Nurfarahin et al., 2018). Following are some of the key features of biosurfactants that make them unique and adds to their functional diversity.

2.2.1. Critical micelle concentration (*cmc*) and self-assembly

Critical micelle concentration (*cmc*) is defined as the minimum concentration of a surfactant required for maximum reduction in the surface and interfacial tension. *cmc* has direct implications on performance and surface properties of surfactants (Perfumo et al., 2018b). Viscosity, osmotic pressure, density, turbidity, conductivity and chemical shifts are known to abruptly change at concentrations above and below *cmc*. Despite of their aqueous solubility, *cmc* of natural surfactants is comparatively low than many chemical surfactants (Chaprão et al., 2015). At concentrations above *cmc*, biosurfactants monomers organized into unique molecular aggregates known as micelles. A typical micelle is defined as a supramolecular assemble that disperse in a colloidal solution with the hydrophilic head orienting towards the solvent whereas the hydrophobic tail being sequestered inside the core (Soberón-Chávez and Maier, 2011). Micelles are usually spherical in shape with size several nanometers in diameters however, their intermolecular aggregation results in a number of unique structural patterns such as vesicles (P_β), lamella (L_α), sheath (L_β), cubic (V_2), sponge (L_3), hexagonal (H_2) and crystalline phases (Fig. 2.1). The properties of these self-assembled structures are governed by the hydrophilic-lipophilic balance (HLB) of biosurfactants (Corti et al., 2007). The efficiency of a good biosurfactant is determined by evaluation of *cmc* whereas its effectiveness is a measure of ability to reduce surface and interfacial tensions. The *cmc* of biosurfactants normally ranges between 1 to 2000 mg/L with low molecular weight glycolipids depicting significantly low *cmc*. Lin et al., (2017) has reported the *cmc* of rhamnolipids to be 56 mg/L whereas, in another study Elshikh et (al., 2017) has evaluated the *cmc* of lactonic sophorolipids as 48 mg/L. Similarly, in some other investigations the *cmc* of rhamnolipids, sophorolipids, surfactin and trehaloselipids

was found between 40 to 170 mg/L (Andersen et al., 2016, Jin et al., 2016, Narimannejad et al., 2019).

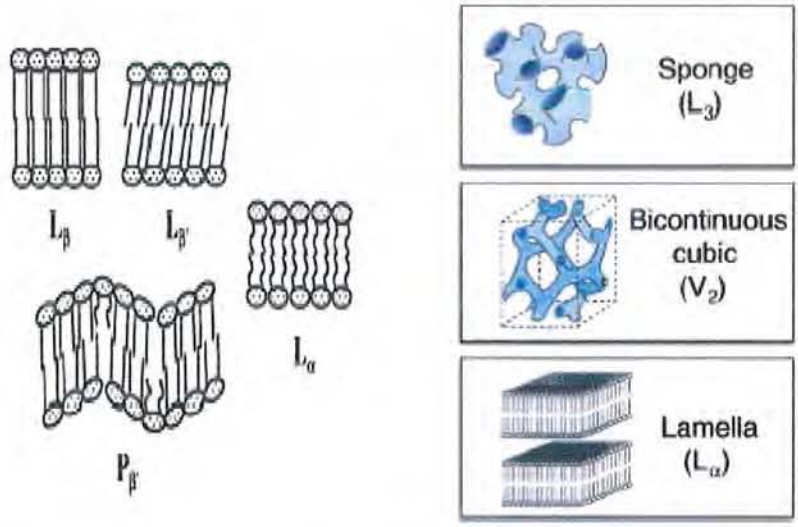


Figure 2.1: Pictorial sketch of self-assembled aggregates of biosurfactants (Corti et al., 2007)

2.2.2. Emulsification and de-emulsification

Emulsification is the dispersion of one immiscible liquid into another through the formation of micro-droplets. This process generates micelles solubilized particles which are more than 0.1 mm in size (Mnif and Ghribi, 2016). Whereas, de-emulsification process destabilizes the emulsion by disrupting the stable surface between the bulk phase and internal phase. The surface active properties of biosurfactants have been studied in detail through the understanding of their emulsification and de-emulsification behaviors. The heterogeneous system of emulsion is basically of two types; oil in water emulsion (o/w) and water in oil emulsion (w/o). The stability in emulsion system can be enhanced from a few days to few weeks by the addition of biosurfactants (De et al., 2015). Many glycolipids have been known to possess significant emulsification activity and can easily solubilize hydrocarbons in water. Some examples include sophorolipids and rhamnolipids for which highest emulsification indices and lowest surface tensions are reported. In a study, mono-

rhamnolipids congeners of *P. aeruginosa* showed different emulsification activities for hydrocarbons of varying complexities (Perfumo et al., 2006). High molecular weight biosurfactants are well acclaimed for emulsification activity however, are not very effective in reducing surface tension (Smyth et al., 2010). Liposan, a surface active compound secreted by *C. lipolytica* is known to stabilize oil in water emulsions and has been used in food and cosmetic industries (Wagner and Syldatk, 2017). The use of de-emulsifiers for disturbing the stable emulsions has also been reported. Mohebali et al., (2012) has suggested the use of de-emulsifiers for the treatment of emulsions containing waste generated from the crude oil industry. Similarly, the use of a novel rhamnolipids molecule as a potential destabilizer of crude oil waste has been reported (Aparna et al., 2012).

2.2.3. Specificity and structural diversity

One of the unique features of biosurfactants which makes them more effective than conventional surfactants is their substrate specificity. In case of microbial surfactants, excellent substrate specificity has been observed for a broad range of compounds (Khan and Butt, 2016). For instance, emulsans display specificity for aliphatic and aromatic hydrocarbons whereas rhamnolipids from *Pseudomonas* PG- 1 showed high degree of solubilization of pristine (Nwaguma et al., 2016). In addition to high substrate specificity, biosurfactants also show specificity in their mode of action. It has been reported that the specificity and selectivity of a biosurfactant molecule is due to their molecular structures and associated functional groups present (Saha and Rao, 2017). This property governs the broad spectrum use of surface active compounds in the process of de-emulsification of industrial emulsions, detoxification of hazardous chemicals and bioprocessing of cosmetics and drugs. To overcome, bioprocess associated limitations, scientists are searching for microbial surfactants with exceptional diversity. The presence of variety of functional groups in a single class of biosurfactants endow diversity to the surface active compounds (Mukherjee and Das, 2010). This is the reason why lab scale optimization experiments are necessary to ensure the maximal production of biosurfactants under different conditions.

The ability of these molecules to tolerate pH, temperature and high amount of NaCl can be attributed to the structural and chemical diversity.

2.2.4. Biodegradability

The chemical surfactants being xenobiotic and recalcitrant in nature are very resistant to the natural process of degradation. This results in accumulation of these chemicals in environment and cause eco-toxicity. Microbial surfactants, due to their natural origin are prone to biodegradation and does not mount up in soil and water (Rahman et al., 2017). These surface active molecules are degraded by the enzymatic actions of different microorganisms which first cleave and subsequently inactivate the surfactant monomers. Different enzymes have been reported for the degradation of surfactant monomers *e.g.* emulsan polymerase which breaks the polysaccharide backbone of emulsans, rendering the molecule inactive (Santos et al., 2016). Moreover, these surfactant degrading enzymes could be easily isolated and purified for further studies. Biodegradability of sophorolipids produced by a non-pathogenic strain of *C. bombicola* was checked and results revealed the immediate biodegradation of biosurfactants in comparison to synthetic surfactants, as the latter remained active even after 8 days (Ahn et al., 2016). It was observed that rhamnolipids biosurfactants were fully degraded under aerobic as well as anaerobic conditions whereas, the chemical surfactant Triton X-100 were partially degraded under aerobic conditions and did not degrade anaerobically (Liu et al., 2018).

2.2.5. Dispersion

Biosurfactants act as natural dispersants and find applications in petroleum and petro-chemical industry. A dispersant is a chemical agent that reduces the cohesive forces between particles of similar kind. This prevents the interaction of insoluble particles and does not allow the formation of aggregates (Luna et al., 2015). The property of dispersion allows hydrophobic organic compounds like crude oil to easily desorb from the rock surfaces and results in enhanced recovery. The surface area of oil could be increased after being dispersed into smaller droplets (Silva et al., 2018). Many previous reports have

shown that glycolipids particularly rhamnolipids biosurfactants exhibit excellent dispersion properties and can easily disrupt the biofilms of other bacterial species (Santos et al., 2017, Ma et al., 2018). Zhang et al., (1997) observed an increase in the dispersion of octadecane after the addition of rhamnolipids in the solution. According to Andrade et al., (2018) sophorolipids biosurfactants were able to disperse crude oil and was selected for field scale bioremediation studies.

2.3. Types of biosurfactants

The criteria for classification of microbial surface active compounds include diversity in their chemical and biological diversity (Banat and Rengathavasi, 2018). Primarily, these compounds are being characterized as low molecular weight biosurfactants which include glycolipids, lipoproteins and phospholipids and, high molecular weight bioemulsifiers such as lipopolysaccharides proteins, polymeric and particulate surfactants (De Almeida et al., 2016).

2.3.1. Glycolipids

Glycolipids are some of the most frequently reported microbial surfactants. These include rhamnolipids, sophorolipids, trehalolipids and mannosylerythritol lipids. In these molecules, the hydrophobic portion is either long chain aliphatic or hydroxyaliphatic acids connected to the mono, di, tri and tetra-saccharide hydrophilic sugar through an ester or ether linkage (Mnif and Ghribi, 2016, Paulino et al., 2016). The role of glycolipids in producer microorganisms has not been fully understood, however, some of the key cellular functions have been associated with these compounds. Physiologically, glycolipids are known to facilitate microbial mobility in complex environment. They reduce interfacial tension between cell and external environment thereby, help in promoting the growth, reproduction and colonization of microbial communities. Some of the microbial glycolipids are known to be used as energy sources and store extracellular carbon (Inès and Dhouha, 2015). Moreover, the complex carbohydrate-carbohydrate interactions within the sugar head-groups endow peculiar characteristics to these glycol-conjugates. This

maintains the hydrophilic-hydrophobic balance and helps these molecules to achieve different self-assembly patterns (Corti et al., 2007).

2.3.1.1. Rhamnolipids

Rhamnolipids contain a hydrophilic head composed of one or two rhamnose molecules, respectively known as mono-rhamnolipids (monoRL) and di-rhamnolipids (diRL) whereas, the hydrophobic tail can be either one or two fatty acid chains of varying length and complexity (Varjani and Upasani, 2017). Through different analytical techniques, approximately 60 homologues and congeners have been detected for rhamnolipids at varying concentrations in fermentation media. The different types and proportions of congeners usually depends upon the substrate composition, producer microorganisms, culture age, and media specific conditions (Kiran et al., 2016). *P. aeruginosa* is known as the chief producer of rhamnolipids and can produce up to 100 g/L of compound, making the production process quite cost-effective in comparison to many synthetic surfactants. Other *Pseudomonas* species that produce rhamnolipids are *P. fluorescens, P. plantarii, P. chlororaphis* and *P. putida* (dos Santos et al., 2016). In recent years, many other bacterial genera like *Acinetobacter*, *Bulkhorderia*, *Pseudoxanthomonas*, *Myxococcus*, *Enterobacter* etc. have been reported as producers of rhamnolipids (Sekhon Randhawa and Rahman, 2014). RL molecules were first discovered by Johnson and Jarvis in 1949 who reported the production of rhamnose containing glycolipids by *P. aeruginosa* whereas Kaeppeli, and Guerra-Santos, obtained their first patent (US 4628030) in 1986 for their work on *Pseudomonas aeruginosa* DSM 2659. To date, the highest number of patents and research publications have been reported for rhamnolipids (Kaskatepe and Yildiz, 2016). This is because of the tremendous research that has been conducted on rhamnolipids due to their eco-friendly properties and diverse biotechnological applications.

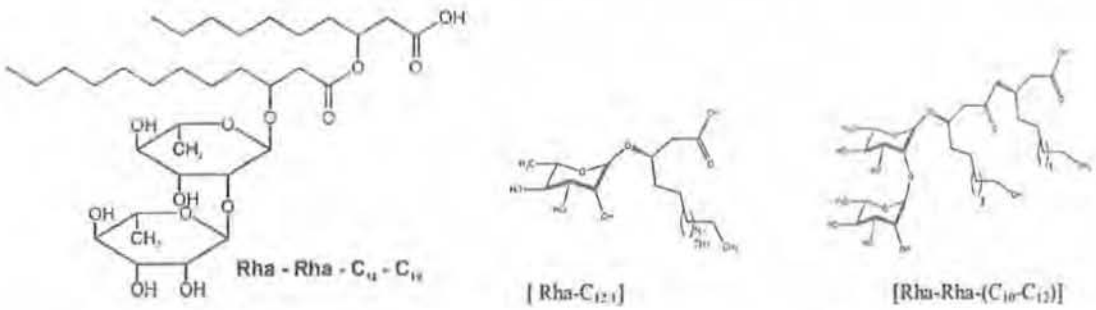


Figure 2.2: Biochemical structures of RL congeners (Abdel-Mawgoud et al., 2010)

2.3.1.1.1. Biosynthesis of rhamnolipids

The RL molecules are reported as anionic in nature and the biosynthesis of both mono and di RL moieties involves the catalytic role of rhamnosyl transferase I. The sugar component (L-rhamnose) is derived from 6-C glucose scaffold that yields deoxythymidine di-phospo (dTDP) L-rhamnose. Genes involved in the synthesis of these molecules are localized on *rmlBDA* operon. In the first step of the L-rhamnose synthesis, *rmlA* transfers the thymidyl monophosphate nucleotide to glucose-1-phosphate. Then, *rmlB* catalyzes both oxidation and dehydration of the OH group of C_4 of D-glucose simultaneously which results in the production of dTDP-4-keto-6-deoxy-D-glucose. After that *rmlC* causes a dual epimerization process at C_3 and C_5 of the glucose. Ultimately *rmlD* carries out reduction of C_4-keto-6-deoxy L-rhamnose resulting in the final product. Biosynthesis of rhamnolipids cannot be accomplished without *rhlA*, *rhlB* and *rhlC* genes. The enzyme produced by *rhlA* gene carries out synthesis of the fatty acid portion of rhamnolipids and a free 3-(3-hydroxyalkanoyaloxy) alkanoic acid (HAA). Rhamnosyl transferases encoded by *rhlB* and *rhlC* are responsible for the transfer of dTDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkanoic acid (Dobler et al., 2016, Chong and Li, 2017).

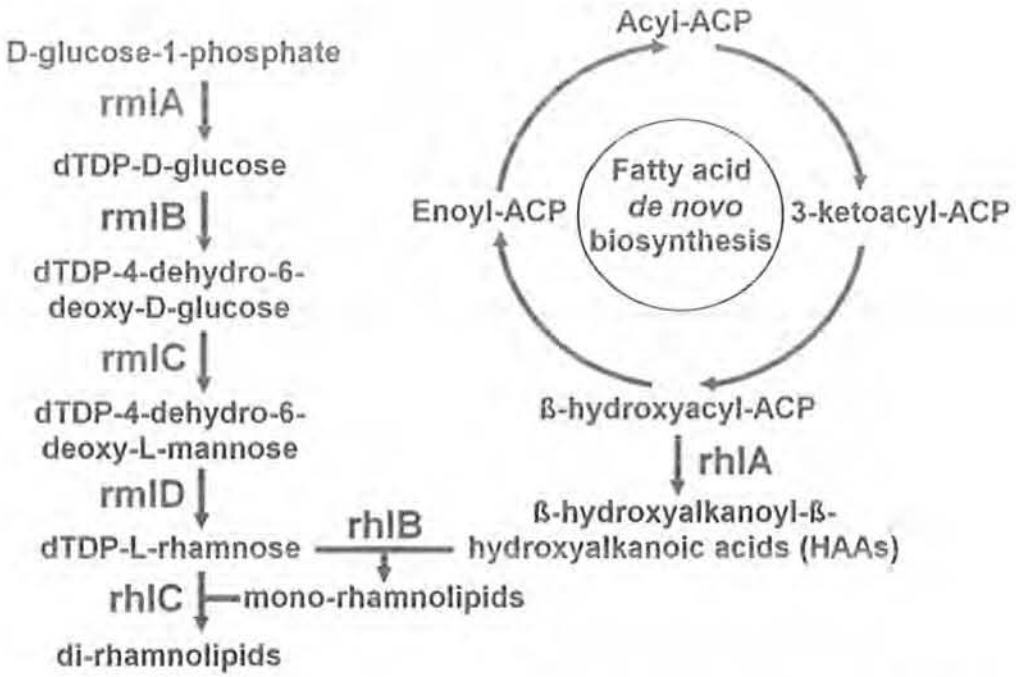


Figure 2.3: Biosynthesis pathway of rhamnolipids (Bahia et al., 2018)

2.3.1.1.2. Properties of rhamnolipids

The physico-chemical properties of rhamnolipids differ even with small changes in the molecular composition of congeners. In terms of surface active properties, rhamnolipids are considered as one of the most promising types of biosurfactants. They can effectively reduce the surface tension of water from 72 mN/m to 27 mN/m with a varying critical micelle concentration of 5-200 mg/L (Mendes et al., 2015). The self-assembly patterns of rhamnolipids are significantly affected by slight change in pH of solutions. They form vesicles of 50-100 nm in diameter at pH 4.3-5.8, lamellar structures at pH 6.0-6.5, lipid particles at pH 6.2-6.6 and micelles at pH more than 6.8 (Penfold et al., 2012). Rhamnolipids producing hydrocarbon degrading bacteria normally grow at neutral pH and even a slightest change in media pH disturb their growth patterns. Under acidic conditions the extracellularly released rhamnolipids aggregates protect the bacterial cell membrane by adapting vesicle or lamellar assembly whereas, in neutral or alkaline environments, the regular cell associated physiological roles are being performed by these surface active compounds (Nitschke et al., 2005). These unique molecular attributes of rhamnolipids are

due to the strong cohesive forces between the hydroxyl groups of the polar head groups, linked through H-bonds. This complexity in interactions is responsible for diverse and dynamic structures of these glycolipids at different temperatures, pH and hydration conditions (Shekhar et al., 2015).

2.3.1.1.3. Physiological roles of rhamnolipids

The physiological roles of rhamnolipids are best executed at their critical micelle concentration. The extracellular release of RL molecules in the medium is responsible for the emulsification of complex hydrophobic substrates by increasing their bioavailability for cellular metabolism. This process of substrate solubilization can be quite effective in the removal of contaminants and revitalization of age old brownfields (Rahman and Randhawa, 2015). However, an inhibitory effect was observed on biodegradation and microbial cellular metabolism for the exogenous supply of rhamnolipids above than *cmc*. This might be due to the mass transfer of emulsified droplets into the microbial cell that proves to be toxic and disrupts growth. In addition, distinguishing biological roles as in anti-microbial activity, anti-proliferative activity against human breast cancer cell lines, zoosporicidal activity, anti-phytoviral activity and wound healing activities have been associated with mono and di RL molecules (Chen et al., 2017).

2.3.1.1.4. Production of rhamnolipids

Media formulation plays a pivotal role in rhamnolipids production both at lab and industrial scale. Various studies were performed in order to optimize different macro and micronutrients such as carbon, nitrogen, and phosphate and some multivalent ions and, their concentrations for enhanced production of rhamnolipids. It has been reported that *P. aeruginosa* produces a mixture of RL molecules in the culture media with different molar ratio (Chong and Li, 2017). The nature of fermentation substrate is considered as one of the most important variables that affects the production yield. In some previous attempts carbon substrates like waste frying oil, oil refinery waste, molasses and distillery waste were used in batch scale experiments (Gudiña et al., 2016). Moreover, simple organic

compounds like glucose, sucrose, alkanes, and glycerol have also been reported for enhanced production of rhamnolipids (Bahia et al., 2018). In some studies, concentrations of nutrients in the given fermentation process significantly affected rhamnolipids production (dos Santos et al., 2016, Nickzad et al., 2018). For example, low C: N ratio was suggested to stimulate biosynthesis of rhamnolipids by *P. aeruginosa*. Moreover, K^{+1}, P^{+2}, Mg^{+2}, Fe^{+2}, and Ca^{+2} ratios also influence cellular productivity of RLs (Moussa et al., 2014). However, in order to meet the increasing commercial demands volumetric production of rhamnolipids is still a challenge and requires more dedicated attempts.

2.3.1.2. Sophorolipids

Sophorolipids, the second most studied class of glycolipids in terms of properties and applications, contain the disaccharide sophorose linked through β-glycosidic linkage to a long hydroxy fatty acid chain (Van Bogaert et al., 2011). These molecules either exist in acidic form; where the fatty acid tail is free or lactonic form; in which the carboxyl group of fatty acid chain is linked through an intra-molecular ester bond with the hydroxyl group of sophorose sugar. In different studies, up to 40 different types of structurally identical isomers have been identified for sophorolipids in the fermentation medium (Jonas et al., 2018). These differences in the structure arise due to the different acetaylation patterns of the hydroxyl group of sophorose moiety, presence of either acidic or lactonic form or both in different proportions, length of the fatty acid chain usually ranging between C_6 to C_{18} and the β-glycosidic linkage with terminal or sub-terminal C-atom of fatty acid chain (Claus and Van Bogaert, 2017).

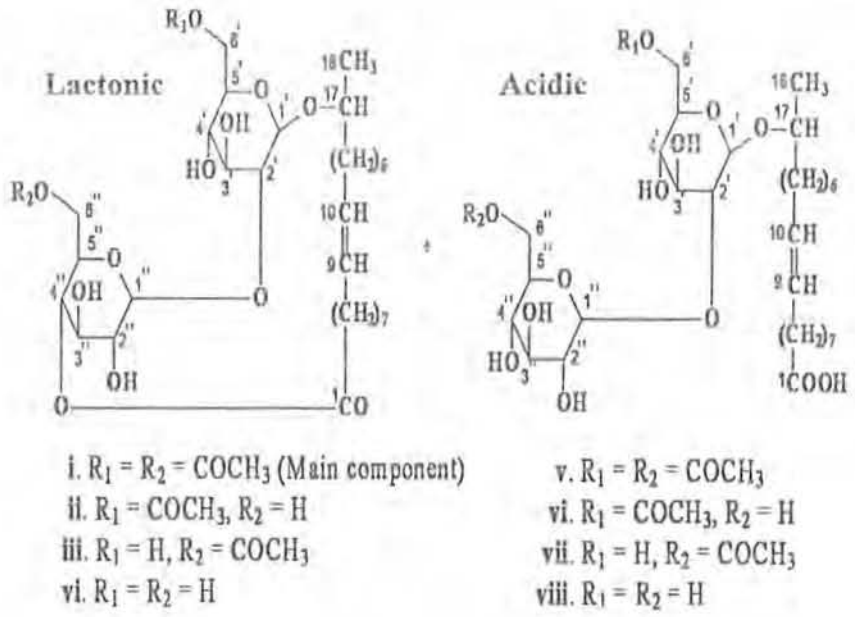


Figure 2.4: Biochemical structures of SL congeners (Claus and Van Bogaert, 2017)

2.3.1.2.1. Biosynthesis of sophorolipids

Sophorolipids were first reported to be produced by two yeast species; *Candida apicola* and *Candida bombicola* in 1961 and 1970, respectively. Within the last few years, biotechnological interest in these surface active molecules has been increased exponentially, with more than 200 citations being indexed in the year 2016 and 2017 (Rebello et al., 2018). Up to now, some new microbial species like *Rhodotorula bogoriensis*, *Wickerhamiella domericqiae* and *Wickerhamomyces anomalus* have been reported as prominent producers of SLs. The optimal yield of SL molecules has been reported up to 400 g/L (Van Bogaert et al., 2011). The biosynthesis of sophorolipids initiates with glucose as precursor molecules for hydrophilic portion of SL whereas, alkanes or triglycerides or fatty acid methyl esters as precursors for hydrophobic moiety. The hydrophobic carbon sources yield fatty acid chain with 16 or 18 C-atoms. The fatty acids are subsequently oxidized into hydroxylated fatty acids through cytochrome P450 monooxygenase enzyme. The fatty acid chains are linked to the first glucose molecule through glycosyltransferase I, whereas the second glucose molecule is attached through glycosyltransferase II. Afterwards, the acetylation of the sophorose head group is

accomplished by acetyl-CoA dependent acetyl transferase. This acidic sophorolipids, when released into the fermentation medium, is then esterified through extracellular esterase to the lactonic form of SL (Delbeke et al., 2018).

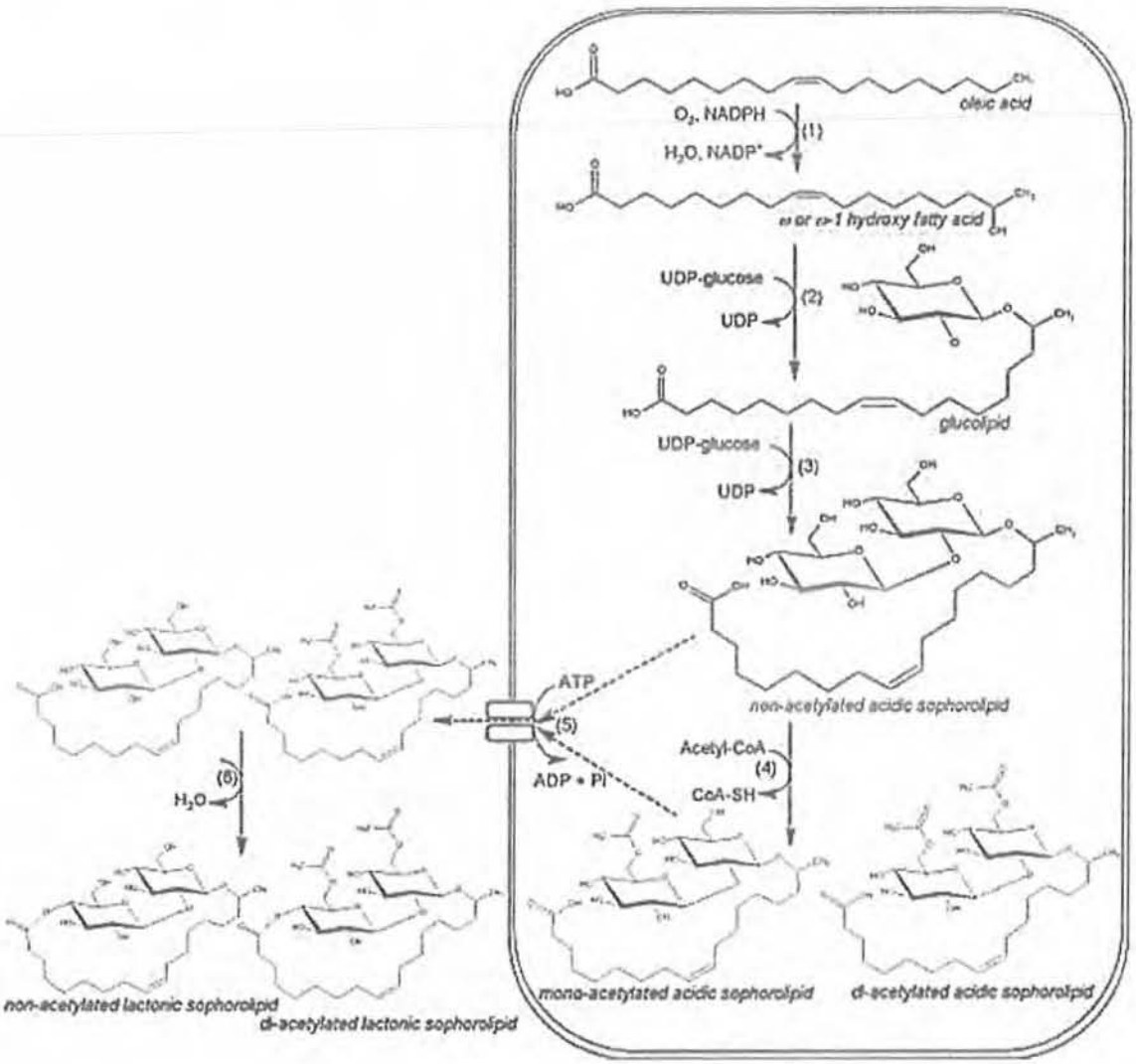


Figure 2.5: Biosynthesis pathway of sophorolipids (Saerens et al., 2015)

2.3.1.2.2. Properties of sophorolipids

The physico-chemical properties of sophorolipids depend upon the mixture composition and the relative proportion of acidic and lactonic forms (Valotteau et al., 2017). Lactonic forms, due to structural modifications, are more hydrophobic and exhibit pro-

inflammatory, cytotoxic, spermicide antimicrobial activities and lowers the surface and interfacial tension. On the other hand, acidic form of SLS, have high water solubility and the resulting foaming activity is responsible for efficient biodegradation of hydrophobic contaminants and other industrial applications (Develter and Lauryssen, 2010). The presence of acetyl groups significantly effects the properties of SL molecules by lowering the hydrophilicity and stimulating cell proliferating activities. Sophorolipids when added in water, reduce the surface tension from 72 mN/m to 30-40 mN/m and possess a critical micelle concentration of 40-100 mg/L (Hirata et al., 2009). Pure sophorolipids form a white colorless powder in their dry form, whereas the partially purified brown yellowish honey-like viscous product might be due to the high content of water and other impurities. However, to study the dynamic properties and advance scale applications, high level of purity is required which could be attained either through lyophilization, flash chromatography or ice-water crystallization (K Morya et al., 2013).

2.3.1.2.3. Physiological roles of sophorolipids

The lactonic sophorolipids possess tissue repairing and restructuring properties as they stimulate the metabolism of dermis fibroblasts cells and help in synthesis of collagen (de Oliveira et al., 2015). These molecules also exhibit depigmenting, melanogenesis inhibiting and desquamating activities by detaching the corneocytes. Moreover, the use of SLs as anti-wrinkle, anti-dandruff and for the lipolysis of adipocytes highlights the dermatological activity of sophorolipids (K Morya et al., 2013). In one of the studies, spermicidal and virucidal potential of SLs was reported. The ethyl ester SLs derivative showed significant virucidal activity against HIV. Moreover, the sperm-immobilizing activity against human semen was comparable with non-oxynol 9 (Shah et al., 2005).

2.3.1.3. Trehalolipids

Trehalolipids also represent a major portion of glycolipids and is composed of disaccharide trehalose attached to mycoilic acid (α-branched β-hydroxy fatty acid chains). These molecules were first noticed by Anderson and Newman in 1933 (Franzetti et al., 2010).

Mostly Gram positive bacteria with high GC content such as *Mycobacteria*, *Nocardia* and species of *Corynebacteria* are considered as major producers of trehalose biosurfactants. These molecules exhibit varying degree of saturation along with various shapes and sizes and reveal promising physiological activities under extreme conditions. The trehalolipids produced by *Rhodococcus* form emulsions and are known to be stable at extreme conditions (pH; 2-10), temperatures from 20 °C-100 °C and salt concentration of 5-25 % w/v (Marqués et al., 2009). The two molecular forms of TLs known as Trehalose mono-corynomycolate (TL-1) and di -corynomycolate (TL-2) reduce the surface tension of water from 72 mN/m to 32 and 36 mN/m and interfacial tension of water/n-hexadecane system from 43 to 14 and 17 mN/m, respectively. The critical micelle concentration of these molecules has been detected in the range of 4-200 mg/L (Jana et al., 2017). The physiological role of trehaliolipids is elucidated in terms of their bioremediation potentials as these molecules increase the bioavailability of contaminants. Moreover, the significant antimicrobial activity against Gram positive bacteria and some pathogenic fungal species are also some of the key attributes of TLs (Shekhar et al., 2015).

2.3.2. Lipopeptides

Lipopeptides are produced by fungus, bacteria and yeast. Surfactin, inturin, and fengycin are known to be most studied lipopeptides. They tend to reduce viscosity, enhance mobility, solubilisation and good metal sequestering agents. Their capability to form pore and disrupt biological membrane make them potential candidate to be used as haemolytic, anti-viral, antibacterial and anti- carcinogenic agents (Hamley, 2015). Surfactin, a lipopeptide was discovered from the culture broth of *Bacillus subtilis*. Surfactins are observed to be a blend of various isoforms A, B, C and D. Surfactin structure possess seven amino-acid ring structure linked to a fatty-acid chain by a lactone linkage. Surfactin-A contains L-leucine, surfactin-B has L-valine and surfactin-C has L-isoleucine in ring structure .The diversity in structure is because of alteration in culture conditions such as substrate i.e. amino acid. They have at least eight dipeptides with almost 1316 carbon atoms arranged in a ring system (Mnif and Ghribi, 2015). Surfactin possess ability to inhibit fibrin clot formation and effectively lyse Red blood cells, protoplasts and spheroplasts. The

literature suggests that surfactin can lower the surface tension of media up to 27 Nm/m therefore, proves to be an effective biosurfactant (Meena and Kanwar, 2015). Surfactins are produced by non-ribosomal peptide system where multi-enzymatic thio templates synthesizes surfactin synthetase enzyme for surfactin formation. The surfactin synthetase complex has four enzymatic subunits. SrfA, SrfB and SrfC and SrfD are enzymatic subunits for surfactin production, srf D (40 kDa) plays major role in initiation cycle of surfactin production. The modules of the peptide synthetase has various domains which incorporates and modifies specific amino acid into the growing peptide chain and module's sequence is compatible with the sequence of the peptide product. Srf A is an inducible operon for surfactin synthetase, which also activates sporulation and competence development (Płaza et al., 2015).

Lichenysin, another surfactin lipopeptide was firstly found in the supernatant of *Bacillus licheniformis* culture and possess huge similarity to surfactin. There is minor difference between two as lichenysin has Glutamine while surfactin has Glutamic acid at amino acid position 1. The production of lichenysin is catalyzed by lichenysin synthetase complex (LchA/ Lic), encoded by lichienysin operon (32.2kb) (Grangemard et al., 2001). Cytotoxicity of lichenysin molecules is observed at a concentration more than 10 µg/mL. This is the reason why lichenysin and surfactin are used in pharmaceutical industry as hemolytic, antimicrobial and chelating agents (Coronel-León et al., 2016). Pumilacidin, another lipopeptide surfactant exist in various forms i.e. A, B, C, D, E, F and G acyl peptide, and show resemblance with surfactin (Banat et al., 2010). The optimization of media conditions for maximal production of lipopeptides yielded 860 mg/L of surfactants from *Bacillus licheniformis*. Iturin A, a lipopeptide synthesized by *Bacillus subtilis* and possess antiviral activity. Iturin is composed of heptapeptide attached with L-amino-acid fatty acid with carbon chain length from C_{14} to C_{17}. Other inturin compounds are iturin C, bacillomycin D, F, L and mycosubtilin (Aranda et al., 2005). Fengycin family of lipopeptides includes fengycins A and B, lipodecapeptides. They have different amino acid at position 6 *i.e.* Alanine and Valine. These molecules show remarkable fungitoxic and immuno-modulating activities (Płaza et al., 2015).

2.3.3. Polymeric microbial surfactants

Polymeric bio-surfactants are likewise important high molecular weight BS. Various genera of microorganisms are known for maximal production of these types of surface active molecules which include; *Arthrobacter*, *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Halomonas* and *Candida*. Amongst different types, the most studied polymeric biosurfactants are emulsans, liposans, mannoprotein and polysaccharide protein complexes (Rodrigues, 2015). The surfactant properties of particulate biosurfactants are responsible for reducing the IFT between two immiscible liquids that results in the formation of stable emulsions. However, comparatively less reports are available on these molecules for the significant reduction in ST. Emulsans, the hetero-lipopolysaccahrides microbial surfactants and contains a fatty acid linked through ester and amide bonds, are majorly produced by *Acinetobacter calcoaceticus* with a molecular weight of 1000 kDa (De et al., 2015). In a study conducted by Mercaldi, (2009) it was observed that emulsans are comprised of 80 % w/w lipopolysaccahrides and 20 % w/w exopolysaccahrides. Moreover, due to unique properties of substrate specificity and surfactant activity even in dilute concentrations, emulsans are usually considered for advance biotechnological applications. Liposan, normally produced by *Candida lipolytica* is another effective emulsifier and belongs to the category of polymeric microbial surfactants. It contains 83 % carbohydrates and 17 % proteins, with the carbohydrate portion quite similar to that of emulsans. Other physiological functions associated with these molecules are their roles as bio-flocculants and growth stimulators (Amaral et al., 2010).

2.3.4. Particulate biosurfactants

Particulate biosurfactants exist either as extracellular membrane vesicles like those produced by *Acinetobacter* or sometimes, the whole microbial cell acts as surfactants e.g. different species of *Cyanobacteria* and *Aeromonas*. There are several bacteria that has the ability to form extracellular membrane vesicles with 20 to 50 nm diameter and form micro emulsions (Gharaei-Fathabad, 2011). These micro-emulsions play a key role in uptake of alkanes by bacteria by fusing with cell envelope and transferring the hydrocarbons to the

inclusion body. These extracellular vesicles when purified are composed of phospholipids, polysaccahrides and proteins. The major phospholipids were phosphatidylethanolamine and phosphatidylglycerol. The protein components of these vesicles were quite similar to those present in outer membrane. The lipopolysaccharide content was determined through the quantification of 2-keto-3-deoxyoctulosonic acid, and turned out to be 360-times enriched than the outer membranes (Bhardwaj et al., 2013). In order to categorize a specific microorganism as surfactant, various techniques have been used to determine the cell surface hydrophobicities of the whole cell. These include microbial adhesion to hydrocarbons (MATH), adherence to solid surfaces, salting-out aggregation, hydrophobic interaction chromatography, binding of radiolabeled dodecanoic acid, two-phase partitioning and contact angle measurements (Hatha et al., 2007). In different microbial species, various cellular components are known to contribute towards the surface active properties and ensure high cell surface hydrophobicity. Some of the consolidated reports are available on the surface active roles of 1) "A protein" of *Staphylococcus aureus* 2) "A layer" present in *Aeromonas salmonicida* 3) gramicidin S in the spores of *B. brevis* 4) lipoteichoic acid and M protein of group A streptococci and 5) thin fimbriae in *A. calcoaceticus* (Roy, 2017).

2.3.5. Fatty acids, phospholipids and neutral lipids

Few bacteria and some species of yeasts have been reported to produce huge quantities of fatty acids and phospholipids surfactants, when grown on n-alkanes substrates of varying complexity and nature. The synthesis of fatty acids surface active agents takes place through the terminal or sub-terminal oxidation of alkanes (Shekhar et al., 2015). Phospholipids are one of the vital structural components of microbial membranes. When alkanes are utilized by certain hydrocarbon dependent bacteria or yeast, the yield of the phospholipids increases rapidly (Singh et al., 2018). When hexadecane was used as a substrate by *Acinetobacter* sp HOI-N it produces phosphatidyl ethanolamine type of phospholipids. Apart from these aliphatic acids, microbes also produce complex fatty acids comprising of OH groups and alkyl branches, example of such compounds are corynomuolic acids (Shah et al., 2016).

2.4. Metabolic regulation of biosurfactants production

Diverse metabolic pathways are involved in the biosynthesis of precursor metabolites for biosurfactants production. These pathways are solely dependent upon the nature of carbon sources provided in the culture medium. Amongst the variety of growth substrates available, hydrophilic compounds are specifically consumed by microorganisms for the synthesis of polar components of biosurfactants whereas, the hydrophobic substrates are utilized to generate the fatty acid moiety of biosurfactants (Reis et al., 2013). Sydatk and Wagner suggested that the biosynthesis of a biosurfactant molecule takes place through one of the four different approaches: (a) carbohydrate and lipid synthesis; (b) synthesis of the carbohydrate portion while the synthesis of the lipid portion depends upon the length of the chain of the carbon substrate present in the medium; (c) synthesis of the lipid part while the synthesis of the carbon half depends on the substrate consumed; and (d) synthesis of carbon and lipid components, which are both dependent on the nature of growth substrate provided (Santos et al., 2016). The detailed mechanism of cellular metabolism with respect to biosurfactants production has already been reported. It has been observed that when carbohydrates are provided as the sole carbon source for glycolipids production, the carbon flow is maintained in such a way that both the lipogenic pathways (for the generation of lipophilic moiety) as well as the glycolytic pathways (for the generation of hydrophilic moiety) are restrained by microbial metabolism. In the glycolytic pathway, the hydrophilic substrate, particularly sugars are degraded till the formation of intermediates, such as glucose 6-phosphate. The presence of glucose-6-phosphate triggers the formation of carbohydrates found in the hydrophilic portion of biosurfactants. The complete oxidation of glucose results in the formation of pyruvate. Pyruvate is then converted into acetyl-CoA, which after reacting with oxaloacetate produce malonyl-CoA. Malonyl-CoA through TCA cycle is converted into fatty acid molecule, the precursor of lipid moiety of biosurfactants. Besides, when hydrocarbon molecules are provided as the only carbon source, the microbial metabolism is primarily directed to gluconeogenesis (formation of glucose through different hexose precursors) and lipolytic pathways, which subsequently allows the synthesis of fatty acids and sugars. The gluconeogenesis pathway is functionalized to produce sugars through the β-oxidation of fatty acids to acetyl-CoA (or

propionyl-CoA if fatty acids are present in odd number). The pathway of gluconeogenesis is generally considered as the reverse of glycolysis however, some of its enzyme catalyzed steps are irreversible (Kosaric and Sukan, 2010). It has been suggested that the chain length of n-alkanes not only induce alterations in cell surface hydrophobicity but also triggers different metabolic pathways for biosurfactants production. One of the *Dietzia* sp. has been reported to produce three different types of biosurfactants when grown on three hydrophobic substrates of varying chain length (Wang et al., 2014). In another study, Kitamoto et al., (2001) has examined the production of manosilerythritol lipids (MELs) from the yeast *C. antarctica* in the presence of different n-alkanes and concluded that the strain could not grow and yield biosurfactants when fermentation medium was supplemented with 10 to 18 carbon growth substrates. However, quite high yield of biosurfactants was obtained when the microbial species were grown in the presence of C_{12} to C_{18} carbon substrates. The susceptibility of hydrophobic organic compounds to microorganisms decrease with increasing chain length and complexity. The general order has been categorized as linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Ye et al., 2017). Once biosurfactants monomers are synthesized, the microbial multi-enzyme complexes are required for structural modifications of these bioproducts. The production of rhamnolipids was observed through the sequential catalysis of two different rhamnosyl transferase whereas, in another study the production of surfactins was catalyzed through non-ribosomal peptide synthesis with the aid of enzyme peptide synthetase complex called as surfactin synthetase (Soberón-Chávez et al., 2005).

2.5. Bioprocess optimization for biosurfactants production

Biosurfactants are generally secreted by aerobic microorganisms in submerge fermentation system. The downstream processing of biosurfactants costs up to 60 % of the total production cost. Moreover, the usage of expensive substrates, formation of variety of side products and low quantity of purified end products are some of the constraints associated with bioprocessing of biosurfactants (Sharma et al., 2016). To overcome these limitations, researchers have proposed different alternates in terms of utility of cheap substrates, optimization of the cultivation conditions and improvement of the downstream recovery

processes. Amongst the variety of options, formulation of an optimized medium containing adequate nutrients in appropriate quantity provides an ideal micro-environment for the microbial specie to grow and continue biosurfactants production (Singh et al., 2019).

2.5.1. Media optimization

Optimization of the cultivation conditions using different statistical tools as in central composite design, Plackett-Burman design, factorial design and response surface methodology not only ensures the better productivity through higher cellular growth but also helps the biotechnological industries to overcome certain economic constraints (Singh et al., 2017). For biosurfactants production various media components play an essential role. The varying concentration of macro and micro nutrients in the medium directly or indirectly affect the bacterial growth and metabolism (Rahman et al., 2017). The reason to use Plackett Burman design is to find out best media composition for maximum yield of biosurfactants. Basic factors which are involved for initial screening were identified by method of Burman (Ekpenyong et al., 2017). Large scale production of glycolipids particularly rhamnolipids and sophorolipids is achieved using different fermentation strategies. The factors that affect the production have previously been optimized using conventional one dimensional approach. But drawback of these methods includes prolong time periods and inability to explain the interactive effect of process variables (Banat et al., 2010). On the other hand, computer assisted optimization methods particularly Response Surface Methodology (RSM) is responsible for improving the competence of biological system. This is an outstanding statistical tool that offers better understanding of interaction between different parameters with lowest time requirement. Another advantage of RSM is the provision of optimum area where maximal results can be predicted (Saharan et al., 2011).

In a study conducted by El-Sersy, (2012) the *Bacillus subtilis* N10 strain was studied for production of biosurfactants. The production media used for growth was LB and for increasing the productivity of biosurfactants Plackett-Burman design was utilized. Results of post optimization analysis revealed that the emulsification index increased up to 1.14

fold in contrast to basal media conditions. In another research, optimization of fermentation conditions was done to increase the concentration of biosurfactants in production media. The statistical software of Plackett-Burman design integrated in Minitab 17 trial version was used in order to screen twelve trace nutrients in 20 experimental sets. The concentration of biosurfactants was studied as the only available response. In comparison to un-optimized media, the yield of biosurfactants increased significantly. The studies showed that highest concentration of biosurfactants of 36.02g/L was obtained at 10^{th} run. Moreover, the results indicated that amongst 12 nutrients, only 5 significantly affected the glycolipids production from the selected microorganism. Statistical analysis of the remaining trace elements suggested that their contribution in biosurfactants production was non-significant as *p*-value was more than 0.05. The optimal production of biosurfactants for their successful applications in pharmaceutical industries have been reported in many studies. Scientists have encountered the process associated constraints in terms of expensive substrates through the optimization of cost effective and cheap raw materials using statistical tools (Kalyani et al., 2014). In a study conducted by Mabrouk et al., (2014) the maximum production of biosurfactants was obtained from *B. subtilis* through the optimization of 12 different substrates. In another experiment, the effect of five selected variables including temperature, pH, inoculum size, moisture and age of inoculum was studied using central composite design and response surface methodology. The statistical analysis of data depicted that inoculum size, temperature and incubation period had beneficiary effect on biosurfactants production. Results showed that the optimal conditions for maximum production of biosurfactants were 37 °C, inoculum age of 14 h and 88 % moisture content (IA Haddad et al., 2014). In another study conducted by Hassan et al., (2016), the experimental design developed through Plackett Burman revealed that glycerol, size of inoculum, water content and temperature play efficient role in biosurfactants production. Fontes et al., (2010) selected ten different variables for production of biosurfactants by using central composite design. The *p*-value for every component was being analyzed. The significant factors showed *p*-value of less than 0.05, whereas the results were further analyzed by using response surface methodology. The analysis of calcium, magnesium, arginine, and maltose showed their significant effect on

biosurfactants production however, ferrous sulphate had an insignificant effect. The production yield reached to a maximum level post media optimization however, the un-optimized media depicted less yield.

2.5.1.1. Effect of carbon source on biosurfactants production

The metabolic nature of biosurfactants producing microorganisms favors the heterotrophic mode of nutrition. Since they specifically consume organic carbon sources for growth and secondary metabolism therefore, in production economics 30-40 % of cost is consumed in the preparation of fermentation media. However, the production cost can be reduced significantly by using different kind of raw and cheap feedstocks. The rate of microbial growth and type of biosurfactants produced directly depends upon the carbon source provided in media (Md, 2012). Generally, three types of carbon sources has been reported for the production of biosurfactants. These include carbohydrates, fats and oils and different complex hydrocarbon compounds. Amongst the different carbohydrates, glucose is one of the most common sugar that is readily available and easily utilized by microorganisms for biosurfactants production through energy yielding glycolytic pathway (Onwosi and Odibo, 2012). Santa Anna et al., (2002) reported that a strain of *P. aeruginosa* utilized glucose and showed high production of biosurfactants, growth, EI_{24} and minimum surface tension in comparison to other carbon sources. Glycerol is another substrate that enhances the production of biosurfactants and enters into central carbon metabolism in the form of glyceraldehyde-3-phosphate. Studies report the improved production of biosurfactants by treated *Y. lipolytica* NCIM 3589 with glucose, glycerol, and sodium acetate which were present in media as soluble carbon source (Zinjarde and Pant, 2002). Conversely, *Y. lipolytica* 1055 has been reported for production of biosurfactants independent of hydrocarbon requirements (Saharan et al., 2011). The role of fats and oil for the production of biosurfactants has been reported, as well. It was observed that in the presence of 2 % waste frying coconut surface tension of the fermentation broth decreased considerably for *P. aeruginosa* (George and Jayachandran, 2013). In case of *K. marxianus* increased production of mannan proteins was recorded when media was supplemented with fresh coconut oil (Lukondeh et al., 2003). It was also observed that the addition of 7.5 %

cotton seed oil and 5.0 % glucose in the fermentation media produced 10 g/L of bioemulsifiers in 5 days of incubation for one of the yeast specie. The strain of *C. glabrata* yielded 7 g/L biosurfactants on 5th day of incubation after the supplementation of 5 % vegetable fat waste in the culture broth (Asfora Sarubbo et al., 2006). Similarly, there are reports available that state the production of biosurfactants from the diverse microbial species in the presence of hydrocarbons. These complex hydrocarbons of varying chain lengths can be easily degraded through emulsification and are incorporated into the different metabolic pathways (Santos et al., 2016). Glycolipid biosurfactants like mannosylerthritol lipids (MEL) was produced only when n-alkanes were present as carbon source in the fermentation broth. The length chain of n-alkanes also affected MEL production and n-octadecane was the hydrocarbon responsible for highest yield (Rau et al., 2005). In another study, increase in the production of sophorolipids from *C. bombicola* 22214 was observed with the increasing complexity of n-alkanes. The yield of sophorolipids recorded up to 21 g/L, with the surface reduction value up to 37 Nm/m at pH 6 and 38 Nm/m at pH 9 by adding soy molasses supplemented with oleic acid. The critical micelle concentration values were 6 mg/L at pH 6 and 13 mg/L at pH 9 were also recorded (Cavalero and Cooper, 2003). The exploitation of cheap carbon sources and waste materials like rice bran, corn steep liquor, rice husk etc. also increase the production of biosurfactants. These studies suggested the significant effect of carbon sources on different microbial species and the importance of carbon source in biosurfactants production cannot be overlooked.

2.5.1.2. Effect of nitrogen source on biosurfactants production

Nitrogen plays an important role in microbial growth and synthesis of primary and secondary metabolites. The supplementation of appropriate nitrogen source in the fermentation medium ensures maximum microbial growth and efficient production of surface active compounds (Gautam and Tyagi, 2006). In the medium, nitrogen helps in assimilation of essential protein components which are important for the structural and functional physiology of microorganisms. Nitrogen sources are categorized as organic and inorganic on the basis of unit structure present in them. Meat extract, peptone, yeast extract,

tryptone, beef extract are the substrates having organic unit structures whereas, the inorganic salts like ammonium nitrate, potassium nitrate, ammonium sulphate etc. contain the inorganic unit structure of nitrogen (Bustamante et al., 2012). The most common nitrogen source involved in the production of biosurfactants is yeast extract however, it has varying effects on the metabolism of different microbial species. Studies have reported that the emulsification properties of biosurfactants usually increase in the presence of complex nitrogen sources however, it decreases the economic efficiency of process in case of MEOR applications (Amaral et al., 2010). Since the production of biosurfactants comes under the category of secondary metabolism therefore, maximum yield is attained during stationary phase of microbial growth when the nitrogen sources are depleted in the fermentation media. The microorganisms also show high affinity for nitrate based nitrogen sources. The nitrate would be first reduced to nitrite and then converted into ammonium ion which is then assimilated to form glutamate or glutamine (Gautam and Tyagi, 2006). In a study, the sub specie of *L. paracasei* showed maximum production of biosurfactants in the presence of yeast extract, followed by meat extract whereas, peptone turned out to be least significant (Gudiña et al., 2011). In another study, better production of biosurfactants was observed by the yeast *R. glutinis* IIP30 when potassium nitrate was used as nitrogen source however, the supplementation of urea and ammonium sulphate did not increase the yield (Vigneshwaran et al.). It was reported in some studies that *K. marxianus* FII 510700 produced 2 g/L of biosurfactants while assimilating yeast extract whereas, the addition of ammonium sulphate enhanced the yield up to 5g/L (Lukondeh et al., 2003). The production of biosurfactants has also been reported from the waste nitrogenous materials e.g. *P. aeruginosa* OG1 yielded 7 g/L of biosurfactants in the presence of chicken feather peptone (Ozdal et al., 2017). Similar reports are available that show the production of biosurfactants through the utilization of corn steep liquor along with $NaNO_3$ (Novik et al., 2018, López-Prieto et al., 2019).

2.5.1.3. Effect of minerals on biosurfactants production

It has been observed that both macro and micronutrients minerals affect the production of biosurfactants. Calcium (Ca^{2+}), Potassium (K^{2+}), Magnesium (Mg^{2+}) and Iron (Fe^{2+}) are

some of the macronutrients that play pivotal role in cell wall communication and protein synthesis (De et al., 2015). FengXia and XiaoMei, (2012) reported that Mn^{2+}, Fe^{2+} and Mg^{2+} act as cofactors of enzymes involved in the production of surfactin from *B. subtilis*. It has also been observed that the production of biosurfactants was improved when iron containing salt was supplemented in the MSM. Moreover in another study, the presence of Ca^{2+} and Mg^{2+} ions significantly enhanced the production of biosurfactants (Hogan, 2016). Calcium ions normally act as mediator and facilitates the signal delivery processes from the extracellular environment to intracellular microbial cell (Dominguez, 2004). The role of potassium and calcium ions have been described to balance the osmotic pressure within the cell and monitoring the membrane potential so that cell lysis could be avoided (Meena et al., 2016). Magnesium ions specifically bind with ATP and form Mg-ATP complex. This complex acts as an indicator to measure the cellular metabolic activities. As high metabolism requires high ATP therefore, the amount of ADP and free Mg^{2+} ions would increase representing the microbial log phase (Swaminathan, 2003). Iron acts as a co-factor in microbial metabolism and is utilized in the form of Fe^{2+} or Fe^{3+}. However, most of the researchers have reported the utilization of iron as Fe^{2+} for the enhanced production of biosurfactants (Muthusamy et al., 2008). Micro-nutrients also affect the production of biosurfactants and their unavailability in the medium might reduce the yield. Some of the micro-nutrients or trace elements are zinc (Zn^{2+}), cobalt (Co^{2+}), manganese (Mn^{2+}), copper (Cu^{2+}) and molybdenum (Mo^{2+}). Different reports are available which show increase in biosurfactants production after the addition of trace elements in the fermentation medium (Makkar and Cameotra, 2002, Wei et al., 2007).

2.5.1.4. Effect of salts on biosurfactants production

Salinity of the production medium is very important in order to optimize the microbial growth as well as to ensure the biosurfactants stability. NaCl is mostly used to adjust the salinity of the fermentation medium as it regulates the osmolarity for microbial growth. Most of the bacteria have been reported to produce maximum amount of biosurfactants in the presence of 2-5 % NaCl however, halo-tolerant bacteria have also been studied as eminent biosurfactant producers. In a study conducted by Ilori et al., (2005) *Aeromonas*

spp. produced maximum amount of biosurfactants and with highest $E.I_{24}$ and least S.T value in the presence of 5 % NaCl. Similarly, *Bacillus* spp. yielded maximum amount of biosurfactants in the presence of 4 % NaCl (Rivardo et al., 2009) whereas, the *Pseudomonas* spp. produced biosurfactants when MSM was supplemented with 0.4 % of NaCl (Pornsunthorntawee et al., 2009). In another study, it was observed that the production of biosurfactants from a strain of yeast was increased with the increasing salt concentration up to 5 % however, further increase in salinity reduced the emulsification and surface active properties of the tested microorganism (Rufino et al., 2007). Besides, a strain of *B. licheniformis* was able to tolerate 35 % NaCl and reduced the surface tension of water up to 35 mN/m (Gudiña et al., 2012). This broad range of halo-tolerance exhibited by most of the biosurfactants producing microorganism make them promising candidates for certain field scale applications like MEOR.

2.5.1.5. Effect of pH on biosurfactants production

pH is one of the most important physio-chemical factor that affects the metabolic potential of microorganisms. This is the reason why its effect on biosurfactants production has been studied in detail. It has been observed that pH 6-6.5 is the most effective range for maximum production of biosurfactants, however depending upon the phylogenetic origin of microorganisms, the tolerance range varies from 4-8 (Gautam and Tyagi, 2006). Further increase in pH usually causes a significant decline in the yield of biosurfactants. Similarly, acidic pH can be quite detrimental for microbial growth and very little or no biosurfactants production is mostly recorded (Shekhar et al., 2015). In a study conducted by Amaral et al., (2010), pH range from 4 to 8 was investigated for sophorolipids production from *C. antractica* and a significant decrease in the yield was observed at basic pH. Similarly in another study the impact of pH was observed on biosurfactants production from *Y. lipolytica* and the yield of biosurfactants was recorded till pH 8.0 (Fontes et al., 2012). Muthusamy et al., (2008) reported that changes in pH of production media resulted in acidification of broth when checked for the quantification of glycolipids from *C. antractica* and *C. apicola*. The maximum yield of glycolipids was observed at pH ranging from 5-

5.5. Conclusively, significant reduction in the yield of biosurfactants due to lack of pH control system in the production medium states its importance in fermentation kinetics.

2.5.1.6. Effect of temperature on biosurfactants production

An optimum temperature is required for maximum microbial growth and stability of surface active compounds in the fermentation broth. In many studies, maximum production of biosurfactants was achieved within the temperature range of 25 to 30 °C. An increase in the production of rhamnolipids was observed between the temperatures of 25 to 30 °C, followed by a significant decline at 42 °C (Rahman and Gakpe, 2008). The quantity of biosurfactants produced from *C. bombicola* was maximum around the temperature range of 25 to 30 °C however, a difference was observed in the two fermentation processes as high utilization of glucose and low quantity of biomass was detected at 25 °C rather than 30 °C. In another study, *C. bombicola* shows maximum growth and efficient yield of biosurfactants at 30 °C whereas, in case of *C. antarctica* variation in biosurfactants production was recorded at different ranges of temperature (Amaral et al., 2010). In another study, bioprocess optimization of MELs showed that maximum yield of biosurfactants was observed at 25 °C with those cells that were both in growing and resting phase (Kim et al., 2002).

2.5.1.7. Effect of aeration and agitation on biosurfactants production

Aeration is associated with foam accumulation in the fermentation broth whereas, the process of agitation is responsible for mass transfer of media components and oxygen to and from the cell. Therefore, both these factors holds great importance in microbial growth and biosurfactants production particularly in case of aerobic microorganisms. It has been reported that the rate of biosurfactants production decreases with the increase in shear stress however, the yield of biosurfactants from one of the yeast specie increases as aeration and stirring increases (Md, 2012). For *C. antarctica* the aeration directly affected the production yield which reached to a maximum value of 45.5 g/L when the flow rate of air was 1vvm and the dissolved oxygen concentration was reserved at saturation level of 50

%. However, when the flow rate varied up to 2 %, intense foam was formed and 84 % reduction in biosurfactants yield was noted (Saharan et al., 2011). In another study the effect of agitation speed was studied on growth of biosurfactants producing microorganisms. It was observed that increase in agitation rate from 50 to 200 rpm resulted in enhanced growth rate to 0.72/hour. Moreover, the yield of biosurfactants was increased up to 80 % under the aforementioned conditions (De et al., 2015). So far, various attempts have been made to reduce the product inhibition during biosurfactants production by separating the foam from aqueous phase through two phase cultivation technique.

2.6. Downstream processing of biosurfactants

Efficient and economic means of downstream processing are very important for the successful accomplishment of a bioprocess. Once the maximum yield is obtained through the optimization of cultivation conditions, an efficient bio-recovery process ensures the collection of biological product from the fermentation broth without compromising its quality and quantity. In case of many microbiological products, 60 % of the total production cost is liable for the downstream processing (Webera et al., 2012). Biosurfactants being amphiphilic in nature, shows affinity for a number of polar and non-polar solvents therefore, different downstream processing techniques have been reported for these molecules (Franzetti et al., 2010). Some of the conventional approaches that have been in use for recovery of biosurfactants from the production medium include acid precipitation, salting out of biosurfactants and organic solvent extraction. These procedures are feasible for the recovery because of the ability of biosurfactants to form micelles or vesicles when present in the supernatant. This also ensures the applicability of these conventional approaches at industrial scale for continuous recovery processes (Makkar et al., 2011). Some other latest techniques have also been reported for the biorecovery of biosurfactants. These include ultrafiltration, adsorption, fractionation, desorption on polystyrene resins, ion exchange chromatography and High pressure Liquid Chromatography (HPLC). However, these techniques require a great amount of monetary input which reduces the cost efficiency of the production process (Behrens et al., 2016). Amongst the many aforestated methods, the most commonly used technique for the

recovery of biosurfactants from batch mode process is the solvent extraction. For this particular purpose a number of cheap, inexpensive and less toxic organic solvents are generally used. Moreover, the compatibility of a particular type of biosurfactants with a particular solvent is also very important. Some commonly used organic solvents involved in the extraction of biosurfactants are chloroform-methanol, ethyl acetate, hexane, pentane, diethyl ether etc (Varjani and Upasani, 2017). Fernandes et al., (2016) separated the crude biosurfactants from the fermentation broth by using a mixture of hexane and ethyl acetate. Centrifugation is also one of the common and practical approach to separate biosurfactants from the fermentation broth, particularly in continuous mode operations. In a study conducted by Satpute et al., (2016) precipitated biosurfactants molecules were collected through the process of centrifugation at 10,000 rpm. High molecular weight compounds particularly those of protein in nature are generally separated through the salting out process. In this process different salts like ammonium sulphate is directly added into the fermentation broth without removing the microbial cell. The mixture is then incubated overnight followed by the addition of more salt and then the resultant solution is centrifuged and supernatant is clarified. More ammonium sulphate is then added into the culture supernatant from which the biosurfactants-salt complex is removed through centrifugation and solvent extraction (Chen et al., 2015). The concentration of ammonium sulphate depends upon the nature of surface active compounds present. In the technique of adsorption-desorption, some biosurfactants molecules exhibit the potential to adsorb and desorb on certain polystyrene resins which subsequently helps in the purification of the molecules. Different organic solvents can be used for desorption of biosurfactants from the polymer resin. This is one of the most efficient technique for the high quality recovery of biosurfactants in a single step (Mnif and Ghribi, 2016).

2.7. Applications of biosurfactants

Over the years, biosurfactants particularly glycolipids have become widely applicable in numerous industries, out-competing synthetic surfactants in terms of advantages and safety. Before venturing into production economics, it is imperative to evaluate their major

applications which distinguish these surface active molecules from other biosurfactants. A list of major applications that caters to the wide range of industrial demands is as follows;

2.7.1. Biodegradation of crude oil

Biosurfactants are suitable for both in-situ and ex-situ bioremediation studies. Due to ecofriendly nature, these molecules exhibit a unique ability to emulsify organic hydrocarbons and enhance their degradation rate in environment. When used in low concentration with other surfactants for target remediation, biosurfactants improve the performance of other surface active agents as well as economize their usage, saving costs without compromising their performance (Das and Chandran, 2011). In the recent era, exploration of natural resources has been increased to fulfill the increasing demand of energy and fuel. The preexisting oil wells produces huge amount of fossil water mixed with crude oil. The release of petroleum hydrocarbons along with produced water causes severe health related issues for the local populations, destroys the agriculture land and ground water reservoirs. The recalcitrant hydrophobic organic compounds (HOCs) when release into the environment, are first adsorbed on soil particles through capillary forces induced kinetics and are finally entrapped in a water-hydrocarbon immiscible phase. This process of molecular entrapment reduces the water solubility and hinders the natural process of contaminant degradation (Santos et al., 2016). To overcome these limitations, the indigenous microbial flora of contaminated sites have adapted different physiological strategies that improvise the cell-surface contact with hydrophobic substrates (Ahmed et al., 2014). The two commonly observed phenomena for enhanced microbial-hydrocarbon interaction and ultimate substrate assimilation include 1) Biosurfactants/bioemulsifiers facilitated pseudo solubilization that employs the use of surface active compounds (SACs) for the emulsification of complex chemicals into simple monomers for cellular absorption; and 2) Biosurfactants mediated enhanced Cell Surface Hydrophobicity (CSH) of the outer membrane that allows the adherence of whole cell to the composite substrate followed by the terminal/sub-terminal oxidation through membrane-bound oxygenases (Karlapudi et al., 2018).

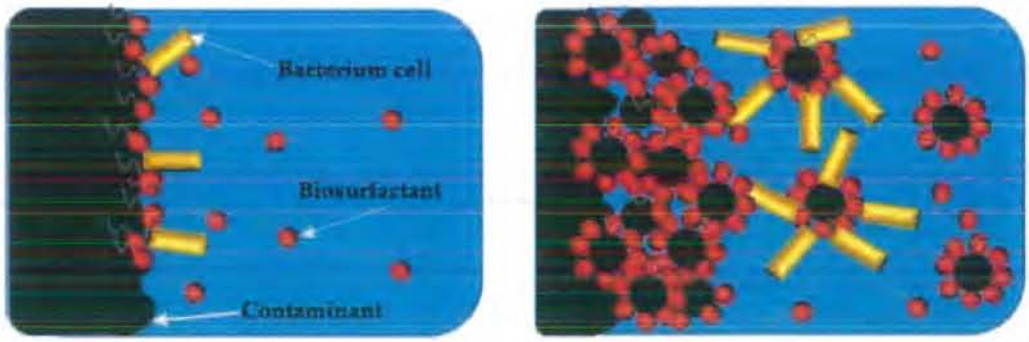


Figure 2.6: Diagrammatic representation of biosurfactants mediated biodegradation of crude oil (Santos et al., 2016)

Amongst bacteria, one of the best hydrocarbon degrader so far recognized is the *Pseudomonas* specie, which produces biosurfactants such as rhamnolipids. Some reports are also available on the yeast species that has the ability to produce biosurfactants. These molecules has the ability to decrease the surface tension, which depends upon the different types of interphases involved i.e. water/oil or water/air. In the interface of water/air, molecules of biosurfactants increases the surface area through formation of surfactants oriented monolayer across the particle of hydrocarbons with hydrophobic tail of surfactants pointing out to the liquid phase. The primary interaction between microbes and oil droplets is achieved through direct contact (Lin et al., 2017). This type of direct interaction depends upon the cell surface hydrophobicity. During direct contact, hydrocarbons penetrate into the cell as submicroscopic drops. Surfactants activity and hydrophobicity favor interaction between microbes and insoluble substrate, overwhelming diffusion limitation during substrate transport to the cell. Microbes that degrade crude oil and produce biosurfactants in oxygen depleted environments are considered effective for the formation of methane in oil fields. The efficiency of biosurfactants to remove different hydrophobic organics like crude oil also depends upon the pH as well as the ionic content of solution. These factors significantly alter the aggregation patterns of micelles which further affects the rate of absorption of surface active molecules on the soil particles (Mnif et al., 2011). This also limitize the biosurfactants induced mobility of hydrophobic organics. Many previous studies have shown the enhancement in biodegradation rate after the addition of glycolipids

biosurfactants. Rhamnolipids, sophorolipids and trehaloselipids have been reported to significantly enhance the hydrocarbons emulsification through their micelles induced entrapment of the hydrophobic organics (Tahseen et al., 2016, Karlapudi et al., 2018). In a study conducted by Kang et al., (2010), beneficial effects of sophorolipids were evaluated on biodegradation of hexadecane and pristane. Similarly, an increase in biodegradation of hydrocarbons was observed when rhamnolipids were introduced in the crude oil containing fermentation medium (Ma et al., 2016). Andrade Silva et al., (2014) suggested the use of glycolipid biosurfactants produced by a yeast strain in future bioremediation studies. The bioemulsions BS29 produced from a strain of *Gordonia* was found to be a promising washing agents for the bioremediation of hydrocarbon-contaminated soils (Franzetti et al., 2009).

2.7.2. Bioremediation of heavy metals

Unlike organic pollutants, the non-biodegradable nature and extremely toxic properties of heavy metals even at trace concentrations make them a constant threat to the public health and surrounding environment. These noxious heavy metals are mostly released into the environment through different sources which include mines, tanneries, electroplating facilities and by manufacture of paints, metal pipes, batteries, and ammunition. The higher concentration of these inorganic pollutants is very lethal as they cause oxidative stress through the formation of free radicals (Mahurpawar, 2015). Biosurfactants when present in solution facilitate desorption, solubilization and dispersion of heavy metal ions and metalloids therefore, allow the reuse of soil and water. The anionic nature, low toxicity, excellent surface active properties and biodegradability of glycolipids make them efficient bioremediation agents. The negatively charged biosurfactants form neutral complexes after binding to the positively charged metal ions (Santos et al., 2016). This property allows the use of biosurfactants as metal sequestering agents therefore aiding in desorption of metals from the contaminated soil and water.

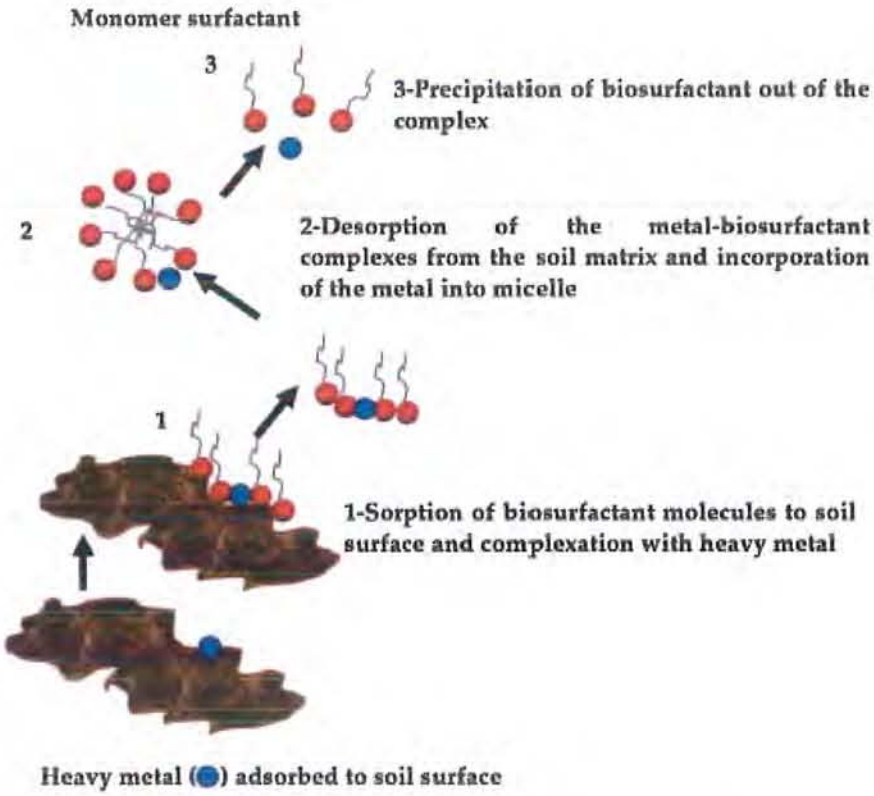


Figure 2.7: Mechanism of heavy metals removal by biosurfactants (Santos et al., 2016)

It has been observed that glycolipids i.e. rhamnolipids and sophorolipids considerably enhance the heavy metals solubility at high concentrations and particular *cmc* (Hogan, 2016). The solubilization of heavy metals is accomplished through the process of bioleaching which is defined as the dissolution of the metals either by the action of microbial species or their biological products. In all these processes biosurfactants-associated complexation is required for the effective removal of heavy metals from the contaminated soil (Mulligan et al., 2001). Wang and Mulligan, (2009) reported a number of rhamnolipids associated processes that might be involved in the removal of cationic heavy metals i.e. chromium and arsenic from the soil. In another study, Mulligan, (2009) studied the effect of different concentrations of biosurfactants on the removal rate of heavy metals. Das et al., (2009) stated the feasibility of using biosurfactants produced by marine bacterium *B. circulans* for removal of heavy metals (lead and cadmium) from solutions. In

another study, surfactin and lichenysin were employed by Zouboulis et al., (2003) for the removal of zinc and chromium from the metal contaminated wastewater. In another study, acidic sophorolipids effectively removed 100 % zinc from the contaminated soil after washing treatments (Mulligan et al., 2001).

2.7.3. Biosurfactants in pharmaceuticals and therapeutics

Glycolipids exhibit low toxicity, enhance surface active properties and act as potent antimicrobial agents against several pathogens depicting diverse applications in pharmaceuticals and therapeutics. Many studies have been conducted on anti-bacterial, anti-viral, fungicidal and insecticidal applications of biosurfactants. In past few years, use of biosurfactants as the possible alternatives to control biofilms has been scrutinized extensively (de Jesus Cortes-Sanchez et al., 2013). Biosurfactants alter the membrane associated surface properties of bacterial cells and inhibit their adhesion to other substrates. Moreover, biosurfactants produced by Gram's negative bacteria have found to obstruct the development of biofilms and cell to cell communication in microorganisms (Muthusamy et al., 2008). An ointment containing rhamnolipids has been reported in treating a chronic decubitus ulcer as these molecules significantly increased the amount of neutrophils and monocytes in bone marrow, stimulated the proliferation of keratinocytes, facilitated wound re-epithelialization and diminished fibrosis (Randhawa and Rahman, 2014). In another experiment, sophorolipids derived from a strain of *C. bombicola* exhibited MIC of 1 mg/L and inhibited the growth of many Gram negative and Gram positive bacteria (K Morya et al., 2013). Similarly, the anti-adherent activity of biosurfactants has been reported which can significantly reduce several nosocomial infections without the usage of any chemical disinfectants. Meylheuc et al., (2006) reported the anti-adherent property of glycolipids produced by *P. fluorescens* whereas, Pirog et al., (2016) stated the role of yeast derived lunasan in inhibiting the attachment of *P. aeruginosa*, and *S. sanguis*.

2.7.4. Biosurfactants in agriculture and food Industry

Due to high emulsification potentials, glycolipids biosurfactants are used in food industry for processing of different raw materials. Consistency in fat based products, stability in aerated systems and molecular agglomeration can be easily controlled by the anionic biosurfactants (Mujumdar, 2015). In some studies, rhamnolipids were reported to improve the emulsifying properties of butter, desserts etc. In another example, sophorolipids from *Candida utilis* were used in the salad dressings (Campos et al., 2015). In the field of agriculture, use of biosurfactants has been mentioned for remediation purposes in improving soil quality, plant pathogen elimination, aiding the absorption of fertilizers and nutrients through roots and, as bio pesticides. They are environmentally safe and act as effective alternative in agricultural pest and disease control (Md, 2012). They can be used for the protection of many fruits and vegetables crops as well as ornamental plants like grapevine and in the composting of green waste for fertilizer. In case of biological control, these surface active molecules are reported to control damping-off disease as an anti-fungal agent (Saharan et al., 2011). In horticulture, biosurfactants are used to increase the wettability and hydrophilicity of deteriorated soil. It has been observed that their presence in pesticides ease out the movement of toxicants to their respective target. In addition, rhamnolipids and sophorolipids produced by different microbial species have also been used to stimulate immunity in plants and animals and for washing the ultrafiltration membranes in dairy industry (Borsanyiova et al., 2016, Chen et al., 2017).

2.7.5. Biosurfactants in Enhanced Oil Recovery (EOR)

Enhanced oil recovery (EOR) employs the use of different physical, chemical and microbial processes to recover 50-65 % oil that has been trapped in microporous rocks due to high interfacial tension between hydrocarbons and aqueous phase. Recently, the high use of biosurfactants to reduce the interfacial tension between different phases has been reported. Amongst the various types of biosurfactants, glycolipids are preferentially applied in EOR processes due to their broad thermal stability and peculiar surface active properties (Gao and Zekri, 2011). Rhamnolipids are used in different formulations along

with chemical surfactants for decreasing the interfacial tension (IFT) between water and crude oil as well as altering the wettability of rocks, sand and brine (Patel et al., 2015). Sophorolipids have been reported to enhance the solubility, permeability and fluidity of crude oil in water; decreases the saline effect and emulsifies immiscible oil in water (Rashedi et al., 2012). Different reports are available that show the successful implementation of biosurfactants in EOR field trials (Safdel et al., 2017).

2.7.6. Biosurfactants as detergents and cleaners

Biosurfactants are natural emulsifiers and their surface active properties are responsible for their wide applications in detergents, laundry products, shampoos and soaps. Cleanup based on biosurfactants successfully removes oily sludge from tank bottoms and recovers more than 90 % of the hydrocarbon trapped in the sludge (Santos et al., 2016). At the same time these biologically synthesized molecules also help in avoiding the harmful environmental impact of toxic wastes as they significantly reduce its volume at the end of the cleaning process (Marchant and Banat, 2012). Besides, broad thermal and pH stability ranges allows biosurfactants to be used instead of chemical surfactants at industrial level without losing surface dynamic properties. In one of the study strength of these molecules as clothing cleaners has also been reported (Sajna et al., 2013).

2.7.7. Applications in cosmetics industry

Comprehensive studies of glycolipids demonstrated their excellent surface active properties for applications in various cosmetics formulations. The role of sophorolipids and rhamnolipids as active ingredients for several skin treatments like wound healing with reduced fibrosis and cure of burn shock has been reported time and again (Lourith and Kanlayavattanakul, 2009). Moreover, these molecules have also been used for the treatment of wrinkles. Their proliferative and hygroscopic properties help them to interrupt the dermal fibroblast metabolism and prevent the formation of free radicals that can damage skin (Piljac and Piljac, 2007).

2.7.8. Applications in nanotechnology

Recent studies have enlightened the role of biosurfactants in the field of nanoscience. The synthesis of nanoparticles has been facilitated through the diverse micellization patterns of different types of surface active compounds especially glycolipids (Rangarajan et al., 2014). In one experiment, rhamnolipids mixture was employed for the synthesis of nickel oxide (NiO) showing excellent antimicrobial activities against some human, plants and animals pathogens (Motahari et al., 2014). Moreover, the discrete polymeric microstructures of sophorolipids have been successfully used as DNA delivery agents into host cell (de Oliveira et al., 2015).

3- MATERIALS AND METHODS

3.1. Crude oil and soil sampling

The crude oil and soil samples were collected from the oil field of Chak Naurang, District Chakwal, Punjab, Pakistan with the co-ordinates 32°59'30"N and 72°55'41"E. This field was discovered in 1986 and started regular production in July, 1987. According to the data, the field produces heavy crude oil also known as black crude oil. The crude oil was collected from well no. 1-A and stored in sterile Duran bottles. The crude oil contaminated soil samples were collected from the waste pit of the same field at the depth of about 10-30 cm. The control/uncontaminated soil sample was obtained from the agricultural land adjacent to the oil field. All soil samples were placed in sterile zipper plastic bags, labeled and kept in the ice box and brought into the lab where they were stored at 4 °C till further processing.

3.2. Soil analysis

The contaminated and uncontaminated soil samples were analyzed for their physicochemical properties. Pre-analysis, the soil samples were air dried and filtered through mesh sieve to remove any kind of stones and debris. Soil texture was determined using hydrometer method proposed by Kettler et al., (2001), pH and Electrical Conductivity-E.C (dS/m) through a digital pH and conductivity meter, water content (%) by the oven drying method, Total Petroleum Hydrocarbons-TPH (g/kg) with the help of gravimetric method, Total Organic Carbon-TOC (g/kg) through the heat colorimetric method of potassium dichromate dilution used by Wang et al., (2013), total Nitrogen-N (mg/kg) by applying Kjeldahl method (Sainju, 2017), available Phosphorous-P (mg/kg) as extractable phosphorus using sodium bicarbonate ($NaHCO_3$) (Uddin et al., 2012) and available Potassium-K (mg/kg) using suitable extractants and flame photometer (Behera et al., 2011). All readings were taken in triplicates.

3.3. Soil enrichment

Soil enrichment technique was used for the isolation of microorganisms from the samples. For this purpose, 1 g of contaminated soil sample was inoculated in the sterilized 100 ml

MSM (minimal salt medium) with the following composition; K_2HPO_4 (10 g/L), NaH_2PO_4 (5 g/L), $NaNO_3$ (2 g/L), $MgSO_4.7H_2O$ (0.2 g/L), $CaCl_2.2H_2O$ (0.01 g/L), $FeSO_4.7H_2O$ (0.08 g/L) and 1 % (v/v) crude oil (carbon source) (Song et al., 2006). The flask containing culture medium was incubated at 37 °C and 150 RPM for a time period of 30 days. After every 5 days, 1 ml of inoculum was transferred into the freshly prepared minimal salt medium containing 1 % (v/v) crude oil as sole carbon source and then re-incubated at aforementioned conditions. During the process of soil enrichment, pH of the medium was kept neutral.

3.4. Isolation of crude oil degrading and biosurfactants producing microorganisms

After enrichment, the microorganisms were isolated by the application of serial dilution method also used by Maddela et al., (2016). The inoculum was spread on already prepared nutrient agar (NA) and sabouraud dextrose agar (SDA) plates followed by incubation at 37 °C and 25 °C, respectively. The bacterial colonies appeared on NA plates after almost 48 h of the incubation period. The bacteria with different colony morphology and pigmentation were considered as a distinct bacterial type and were further purified using sub-culturing technique. Same was the case for fungi as discrete colonies of distinct color and different morphologies started appearing on SDA plates after 96 h. Replica Plate Method was used for further purification of fungal colonies (Walter et al., 2010).

3.5. Screening of biosurfactants producing microorganisms

The isolated bacterial and fungal strains were initially screened qualitatively for the production of biosurfactants on the basis of different tests.

3.5.1. Qualitative screening of biosurfactants producing microorganisms

For qualitative screening of biosurfactants producing microorganisms, following plate assays were performed.

3.5.1.1. Blood Agar Hemolysis Assay

In blood agar hemolysis assay, blood agar plates were prepared that contained 5 % fresh sheep blood. Afterwards, individual bacterial and fungal colonies were inoculated on these plates using spot inoculation method. After incubation of 24-96 h, visual inspection of plates was done and the observed hemolytic activity was designated as α (alpha), β (beta) or γ (gamma) depending upon the diameter of zones of hemolysis (Varjani et al., 2014).

3.5.1.2. CTAB Methylene Blue Agar Assay

This assay is a priority screening method to detect the production of anionic surfactants specifically glycolipids produced by different microbial species (Roy, 2017). CTAB Methylene Blue agar plate contains the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue mixed with 1 % nutrient agar. After inoculation and incubation, if the strain produces anionic surfactants, an insoluble complex is formed around the microbial colony due to bonding between anionic and cationic moieties and, is visible in the form of a dark blue halo. Post incubation of the plates for approximately 72-96 h, zones of clearance were observed.

3.5.1.3. Crude Oil Overlay Agar Assay

Mueller Hinton Agar plates were prepared and coated with 1 % crude oil. Individual bacterial and fungal strains were streaked over the plates followed by an incubation period of 72-96 h. An emulsified zone of clearance was observed around the colony of biosurfactants producing microorganisms and results were recorded (Shoeb et al., 2015).

3.6. Identification of potential biosurfactants producing microorganisms

The initial screening assays resulted in the selection of potential biosurfactants producing bacteria, yeast and fungi. These selected microorganisms were then subjected to morphological, biochemical and molecular identification studies.

3.6.1. Morphological identification

For morphological identification, the bacterial and fungal strains were sub-cultured on nutrient agar and sabouraud dextrose agar plates, respectively. The bacterial isolates were observed for their morphological characteristics mentioned in Table 3.1. Similarly, for fungal species, the color, margins and texture was noted after the colonies attained mature growth on sabouraud dextrose agar plates.

Table 3.1: Morphological characteristics of bacterial strains

Size	Pinpoint, small, medium, large
Pigmentation/ Color	White, Creamish, other
Shape	Circular, Irregular
Opacity	Opaque, Translucent, Transparent
Margin	Regular, Irregular (Lobate, Undulate, Filamentous)
Textures	Smooth, Mucoid
Elevation	Flat, Slightly Raised, Raised

3.6.2. Microscopy

The 24 h old cultures of bacterial strains and yeast species was subjected to Gram's staining technique in order to examine the microbial cells microscopically. For fungal isolates, Lactophenol Blue staining was done and the characteristics patterns of fungal hyphae were observed.

3.6.3. Biochemical identification

The potential biosurfactants producing bacterial isolates were further checked for oxidase, catalase, urease, H_2S and indole production, motility, sugars fermentation and citrate utilization. Methyl Red and Voges Proskauer tests were also performed.

3.6.4. Molecular identification

The selected bacterial and fungal isolates were identified through sequencing of the conserved 16S and 18S rRNA sequences by following the protocol of Wilson (2001).

3.6.4.1. Gel Electrophoresis

Isolated genome was stored at -4 °C for 24 hours and then gel electrophoresis was performed. Gel was made by dissolving 1% agarose in TBE buffer and stained by ethidium bromide (1µg ml^{-1} in TBE buffer) (Wilson, 2001).

3.6.4.2. Sequencing and phylogenetic analysis

Extracted DNA samples were sent to Macrogen Sequencing Service, Korea for sequencing purposes. After sequencing, similarity of the sequences was determined through BLAST search tool. Phylogenetic tree was made using MEGA 7 software. Afterwards, the sequences were submitted in NCBI database and accession number for each sequence was obtained.

3.7. Quantitative screening of potential biosurfactants producing microorganisms

Quantitative estimation of biosurfactants was carried out through various assays frequently reported in the literature. For this purpose, the identified strains were inoculated separately in 100 ml of MSM medium (section 3.3) containing 1 % (v/v) Glycerol as sole carbon source. For this purpose, flasks containing the media was inoculated with isolated microorganisms and incubated in orbital shaker at 150 RPM and 37 °C for 72-96 h. After incubation, culture medium from each flask was centrifuged at 10,000 RPM for 20 min. Pellet was discarded and supernatant was collected and further tested through following assays to enumerate production capability of biosurfactants from the tested strains.

3.7.1. Emulsification Index ($E.I_{24}$) Analysis

The culture supernatant of every strain was studied for emulsification properties using kerosene oil as standard (Walter et al. 2010). For this purpose equal volume of supernatant and kerosene oil was added in the test tube, vortexed for 1 minute and placed at room temperature for 24 h. After 24 h, the emulsification index was calculated by the following equation:

$$E.I_{24} = \frac{Height\ of\ emulsion\ layer\ (cm)}{Total\ Height\ of\ the\ liquid\ column\ (cm)} \times 100 \ldots\ldots Equation\ 3.1$$

3.7.2. Surface Tension Measurement

Surface tension of the culture supernatant was measured using plate method as reported by Marajan et al., (2015). In plate method, Easy Dyne K20, KRÜSS GmbH Tensiometer (Germany) was set on surface tension mode, the platinum plate was carefully cleaned and attached to the hook. The plate was then inserted into the liquid and surface tension was automatically calculated. All readings were taken in triplicates.

3.7.3. Oil Displacement Assay (ODA)

The oil displacement activity of biosurfactants present in culture supernatant was detected through oil displacement assay reported by Youssef et al., (2004). 20 ml of distilled water was taken in a petri plate and 20 µl of crude oil was gently added on the surface of water. After that, 20 µl of supernatant was dispensed onto the oil film. After 30 seconds, zone of displacement was observed and measured accordingly.

3.8. Selection of the most efficient biosurfactants producing microorganisms

On the basis of results obtained from various assays employed for the qualitative and qualitative determination of the biosurfactants, two strains i.e. RB 27 and RF 38 were considered as the most productive biosurfactants producers and selected for further study. The identification confirmed RB 27 as *Pseudomonas aeruginosa* MF069166 whereas, the yeast species was named as *Meyerozyma* spp. MF138126.

3.9. Effect of fermentation media on growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

In order to evaluate the role of media composition on growth and biosurfactants production, five different types of fermentation media were used (Table 3.2). For each formulation, ingredients were mixed and homogenized using stir plate. After mixing, each medium was

supplemented with 0.1 ml of the micronutrients solution. The solution of micronutrients was prepared separately and then filter sterilized. After autoclaving the medium, 1% inoculum was added and flasks were incubated for 96 h at 37 °C and 150 RPM. The experiment was run in triplicates and, microbial growth and biosurfactants production was calculated after every 24 hours.

Table 3.2: Formulations of different fermentation media

Media Components	Medium 1 (g/L)	Medium 2 (g/L)	Medium 3 (g/L)	Medium 4 (g/L)	Medium 5 (g/L)
$CaCl_2.7H_2O$	0.01	0.01	0.01	0.01	-
$FeSOS_4.7H_2O$	0.04	0.08	0.04	0.08	-
Glucose	20	10	20	20	2.5
Glycerol	10	20	10	10	5
K_2HPO_4	10	5	5	10	0.4
$MgSO_4.7H_2O$	0.1	0.2	0.1	-	0.2
NaCl	1	1	1	-	0.5
NaH_2PO_4	5	5	2.5	2.5	1.4
$NaNO_3$	-	2	-	2	-
$(NH_4)_2SO_4$	-	-	2	2	-
Peptone	20	10	20	-	20
Yeast Extract	-	1	-	1	1
Distilled H_2O	1000	1000	1000	1000	1000
pH	7	7	7	7	7

The composition of micronutrients solution is as follows;

$CoCl_2.6H_2O$	1.2 mg/L
$CuSO_4.5H_2O$	1.2 mg/L
$MnSO_4.2H_2O$	0.8 mg/L
$ZnSO_4.7H_2O$	1.4 mg/L

3.9.1. Measurement of growth

Growth of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was determined by measuring the absorbance of culture medium at 600 nm using UV-visible spectrophotometer. Briefly, 150 ml of production media was poured in 250 ml of

Erlenmeyer flasks and autoclaved at 121 °C for 15 minutes at 15 lbs pressure. After cooling, the flasks were inoculated with 1 % of inoculum and the microbial cultures were placed in shaking incubator at 37 °C. Samples were collected after every 24 h and readings were taken in triplicates.

3.9.2. Quantification of glycolipids biosurfactants

Since, bacteria and yeast produce chemically different types of glycolipid biosurfactants, therefore, two previously reported methods were employed for their quantitative determination. For biosurfactants from *Pseudomonas aeruginosa* MF069166 orcinol assay was used. In case of, *Meyerozyma* spp. MF138126, anthrone assay was applied for the estimation of extracellular biosurfactants.

3.9.2.1. Orcinol Assay

Method of Smyth et al., (2010) was used to quantify the rhamnolipids produced by bacterial isolate. Briefly, 100 µl of the culture supernatant taken after every 24 h was mixed with 900 µl of orcinol reagent in a 2 ml eppendorf tube. The tube was then incubated at 80 °C for 30 min. The mixture was then allowed to cool at ambient temperature and absorbance was detected at 421 nm using UV-visible spectrophotometer. Readings were taken in triplicates.

3.9.2.2. Preparation of orcinol reagent

0.19 g of orcinol (5-methyl resorcinol), purchased from Sigma Aldrich, was dissolved in 47 ml of distilled autoclaved water. To prepare the orcinol reagent, 0.19 % orcinol solution was added carefully in 53 % H_2SO_4 within an ice box and few ml at a time.

3.9.2.3. Preparation of standard rhamnolipids solution

The standard "Rhamnolipids" was purchased from Sigma Aldrich. To prepare the stock solution, 0.5 mg of the standard was dissolved in 100 ml of distilled autoclaved water and standard curve was prepared by making different dilutions of the stock solution.

3.9.2.4. Preparation of standard curve

The standard curve of rhamnolipids was made using orcinol reagent. For this purpose, the stock solution of rhamnolipids was serially diluted to attain 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µl/ml concentrations. 100 µl of each dilution was treated with 900 µl of orcinol reagent and incubated at 80 °C for 30 min. The mixture was then allowed to cool at room temperature and the absorbance was detected at 421 nm. Readings were taken in triplicates. The concentrations of rhamnolipids in the bacterial cultures were determined by the calibrated standard curve.

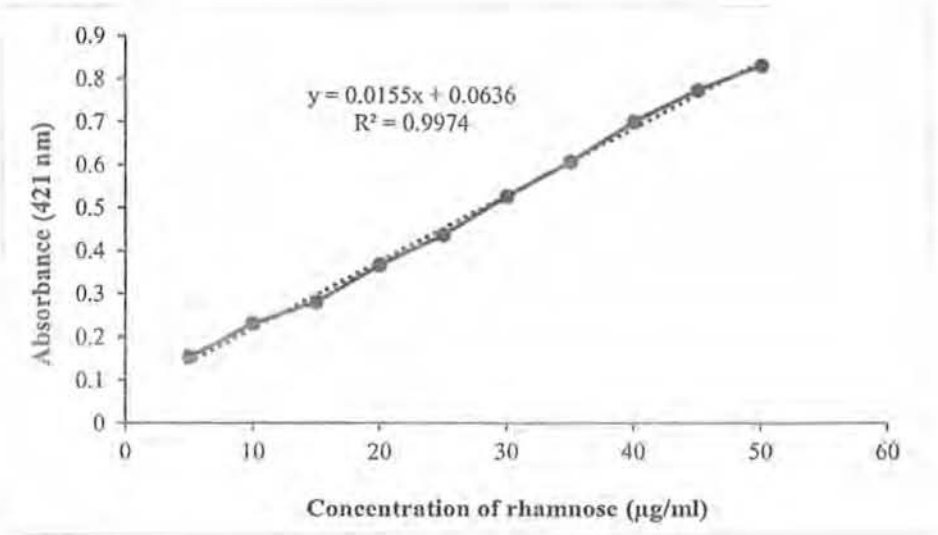


Figure 3.1: Standard curve of rhamnose

3.9.2.5. Anthrone Assay

The sophorolipids produced by the yeast species were quantitatively estimated using Anthrone assay reported by Smyth et al., (2010). 200 µl of cell free supernatant taken after every 24 h was mixed with 1000 µl of anthrone reagent in 2 ml eppendorf tube. After mixing, the tube was heated in the boiling water for 9 min. After cooling the reaction mixture, absorbance was measured at 625 nm through UV-visible spectrophotometer. All readings were taken in triplicates.

3.9.2.6. Preparation of anthrone reagent

The anthrone reagent was prepared using 200 mg of anthrone (9, 10-dihydro-9-oxoanthracene) dissolved in 5 ml of absolute ethanol in a 100 ml flask. The volume was raised up to 100 ml through the addition of 75 % H_2SO_4 in an ice box and few ml at a time.

3.9.2.7. Preparation of standard sophorolipids solution

The standard "Sophorolipids" was purchased from Sigma-Aldrich. To prepare the stock solution, 0.5 mg of the standard was dissolved in 100 ml of distilled autoclaved water and standard curve was prepared by making different dilutions of the stock solution.

3.9.2.8. Preparation of standard curve

The standard curve of sophorolipids was made using anthrone reagent. For this purpose, the stock solution of sophorolipids was serially diluted to attain 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µl/ml concentrations. 200 µl of each dilution was treated with 1000 µl of anthrone reagent and incubated at 100 °C for 10 min. The mixture was then allowed to cool at room temperature and the absorbance was detected at 625 nm. Readings were taken in triplicates. The concentrations of sophorolipids in the yeast cultures were determined by the calibrated standard curve.

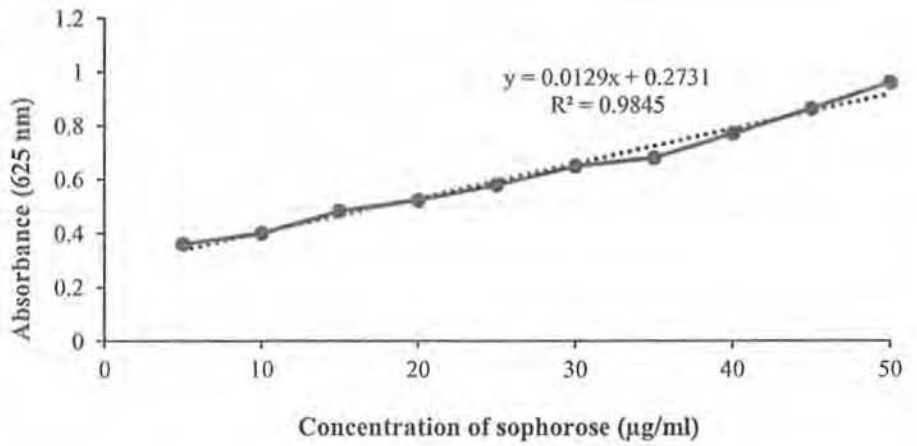


Figure 3.2: Standard curve of sophorose

3.10. Optimization of media components through Plackett-Burman design for growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

Results suggested that medium no. 2 showed maximum growth and biosurfactants production for *P. aeruginosa* MF069166 whereas medium no. 5 was found to be the most suitable for *Meyerozyma* spp. MF138126. In order to further consolidate these findings, Plackett-Burman design was used to select the appropriate media components and their concentration. The non-coded and coded values of media components are presented in Table no. 3.3 and 3.5. Factorial design was made by selecting the option of Plackett-Burman design using Minitab software (version 16.0). In the software, names of the media elements were written and in the adjacent columns, their maximum and minimum ranges were added. After data input, the software generated a design with 16 experimental runs having different combinations of the factors (Table no. 3.4 and 3.6). Similar design was made for both microbial candidates however, the reaction conditions/variables were different with different levels. The experiments were performed according to the prescribed design and, microbial growth and concentration of biosurfactants (g/L) were selected as responses. Each experiment was performed for 96 h and the values of both responses were recorded at the end of experiment. The experiment was performed in triplicates and results represented the mean reading. After conducting the experiments, the data was statistically analyzed using the same software. Plackett-Burman's technique is based on the principle of Hadamard metrics in which N variables are reflected at two levels (+1 and -1). These variables are presented in orthogonal arrangement in such a way that the entries of +1 in the column are equal to those of -1. For screening of N number of variables, this design strategy is very effective and comprehensive to calculate main effects. The design based on the fact that it undermines the interactive effect between the variables while considering only linear trends.

$$Y = \beta_o + \sum \beta_i X_i \ (i = 1, \ldots\ldots, K) \quad \ldots\ldots\ldots\ldots \textit{Equation 3.2}$$

Where Y is the response and β_i represents the regression coefficients. In order to screen N variables, N+ 1 experiments are required enabling screening up to 100 factors at a time with high level of accuracy using this factorial design. The effect of individual parameters on growth and concentration of biosurfactants was estimated by equation 3.3.

$$Effect = 2\,[\sum R\ (H) - \sum R\ (L)]\ /N \ldots\ldots\ldots\ldots\ldots\ldots \text{ Equation 3.3}$$

In the above equation, R (H) represents responses when variables were at +1 and R (L) denotes variables at -1 level, Where N is the total experimental runs. The standard error and significance level of each variable (*p*-value) was determined by *t*-test (Equation 3.4).

$$t(X_i) = \frac{E(X_1)}{S.E} \ldots\ldots\ldots\ldots\ldots\ldots \text{Equation 3.4}$$

In the above equation, E (X_i) represents effect of variables X_i on the factors with their levels (high or low) have been presented.

Table 3.3: Input variables with their coded and non-coded values along with their levels for *P. aeruginosa* MF069166

Variables	Variable code	Low level (-1) (g/L)	High level (+1) (g/L)
Glycerol	X1	10	20
Glucose	X2	10	20
K_2HPO_4	X3	5	10
NaH_2PO_4	X4	2.5	5
Peptone	X5	1	2
KNO_3	X6	1	2
NaCl	X7	0.5	1
Yeast Extract	X8	0.5	1
$MgSO_4$	X9	0.1	0.2
$FeSO_4$	X10	0.004	0.008
$CaCl_2$	X11	0.001	0.005

Table 3.4: Plackett-Burman design for media formulation for *P. aeruginosa* MF069166

Run Order	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11
1	1	1	1	-1	1	-1	-1	-1	-1	1	1
2	-1	1	-1	-1	-1	-1	1	1	1	-1	-1
3	1	1	-1	-1	-1	1	1	-1	1	1	-1
4	1	-1	1	1	1	1	-1	-1	1	-1	-1
5	-1	1	1	1	-1	1	-1	-1	-1	-1	-1
6	-1	-1	-1	1	-1	1	1	1	-1	1	-1
7	-1	1	-1	1	1	-1	1	-1	1	-1	1
8	1	-1	1	-1	-1	1	-1	1	1	-1	1
9	1	1	-1	1	1	1	1	1	1	1	1
10	1	-1	1	1	-1	-1	1	-1	-1	-1	1
11	-1	1	-1	-1	1	1	-1	1	-1	-1	1
12	-1	-1	-1	-1	-1	-1	-1	-1	1	1	-1
13	-1	-1	1	1	1	-1	-1	1	1	1	-1
14	1	1	-1	1	-1	-1	-1	1	-1	1	-1
15	-1	-1	-1	-1	1	1	1	-1	-1	1	-1
16	1	-1	-1	-1	1	-1	1	1	-1	-1	-1

Table 3.5: Input variables with their coded and non-coded values along with their levels for *Meyerozyma* spp. MF138126

Variables	Variable code	Low Level (-1) (g/L)	High Level (1) (g/L)
Peptone	X_1	10	20
Glycerol	X_2	2.5	5
Glucose	X_3	1.5	3
NaH_2PO_4	X_4	0.7	1.4
Yeast Extract	X_5	0.25	0.5
K_2HPO_4	X_6	0.2	0.4
$MgSO_4$	X_7	0.1	0.2
NaCl	X_8	0.1	0.5

Table 3.6: Plackett-Burman design for media formulation for *Meyerozyma* spp. MF138126

Run Order	X1	X2	X3	X4	X5	X6	X7	X8
1	1	1	-1	1	1	-1	-1	1
2	-1	1	-1	-1	-1	1	-1	1
3	-1	1	1	1	-1	1	-1	-1
4	1	1	-1	-1	1	1	1	-1
5	1	-1	-1	1	1	1	-1	-1
6	-1	-1	1	1	1	-1	-1	1
7	1	-1	1	-1	-1	1	-1	1
8	-1	1	-1	1	1	-1	1	-1
9	-1	-1	1	-1	1	1	1	-1
10	1	1	1	-1	1	-1	-1	-1
11	-1	1	1	-1	-1	-1	1	1
12	1	-1	-1	-1	1	-1	1	1
13	-1	-1	-1	-1	-1	-1	-1	-1
14	-1	-1	-1	1	-1	1	1	1
15	1	-1	1	1	-1	-1	1	-1
16	1	1	1	1	1	1	1	1

3.11. Optimization of cultivation conditions through Response Surface Methodology (RSM) for growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

After selecting the appropriate media components through Plackett-Burman design, cultivation conditions were optimized using central composite design (CCD) and response surface methodology (RSM). For this purpose four bioprocess variables *vis* pH, temperature (T), size of inoculum (SOI) and agitation speed (RPM) were selected for both microorganisms. However, the ranges selected for each variable was different for bacterial and fungal strains (Table 3.7 and 3.8). For this purpose, the data was input in the Minitab software (version 16). The response surface with four factors was selected as an option. The minimum and maximum ranges of each parameter were adjusted and software generated a full factorial design with 30 experimental runs for each microbial strain. Growth and concentration of biosurfactants were selected as responses. As there was no increase in biosurfactants production from both *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 after 96 h therefore, all results were recorded for 96 hours of the

fermentation period. After performing experiments, results were obtained and analyzed statistically using the same software. Equation 3.5 was used for estimation of results.

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_{i^2} + \sum \beta_{ij} X_i X_j \ldots\ldots\ldots \textit{Equation 3.5}$$

Table 3.7: Layout of central composite design for optimization of cultivation conditions for *P. aeruginosa* MF069166

No. of Runs	pH	T	SOI	RPM
1	6	20	5	120
2	7.5	30	3	210
3	7.5	30	3	150
4	7.5	30	3	150
5	6	20	5	120
6	9	40	1	120
7	10.5	30	5	150
8	9	40	1	180
9	7.5	30	3	150
10	9	40	1	180
11	7.5	30	3	150
12	9	40	1	180
13	6	20	5	180
14	4.5	30	3	150
15	7.5	30	3	150
16	9	40	1	120
17	9	40	1	180
18	7.5	10	3	100
19	7.5	30	7	150
20	7.5	30	2	150
21	7.5	50	3	150
22	6	20	1	180
23	6	20	1	180
24	6	20	5	180
25	9	40	1	120
26	7.5	30	3	150
27	6	20	1	150
28	7.5	30	3	150
29	9	40	5	100
30	6	20	1	120

Table 3.8: Layout of central composite design for optimization of cultivation conditions for *Meyerozyma* spp. MF138126

Run	A:pH	B:T	C:SOI	D:RPM
1	8	45	1	180
2	5.5	32.5	3	150
3	3.5	20	1	180
4	5.5	32	3	150
5	3.5	57.5	1	120
6	5.5	7.5	3	120
7	5.5	32.5	7	150
8	8	45	2	120
9	5.5	32.5	3	150
10	8	45	1	120
11	8	45	5	180
12	5.5	32.5	3	150
13	8	45	5	210
14	5.5	32.5	3	150
15	5.5	32.5	3	150
16	3.5	20	5	180
17	8	45	1	180
18	3.5	20	5	120
19	5.5	32.5	3	150
20	3.5	20	5	120
21	8	45	5	120
22	3.5	20	5	180
23	10.5	32.5	1	180
24	0.5	32.5	1	150
25	8	45	1	180
26	8	45	1	120
27	5.5	32.5	3	150
28	5.5	32.5	3	150
29	3.5	20	1	100
30	5.5	32.5	3	150

3.12. Extraction and recovery of biosurfactants

The extraction of biosurfactants was carried out through solvent extraction method proposed by De Rienzo et al., (2016). Briefly, after inoculating and incubating the two strains in previously optimized fermentation medium under optimized conditions, culture medium was centrifuged at 10,000 RPM for 20 min. Pellet was discarded and pH of the

supernatant was adjusted at 2 by the addition of 6 N HCl. This suspension was then incubated at 4 °C for 24 h to precipitate out the surface active compounds. Initially, the precipitates were separated through centrifugation at 10,000 RPM for 20 minutes followed by the treatment of acidified supernatant with ethyl acetate to ensure maximum recovery. From the surfactants-ethyl acetate mixture, organic phase was collected and air dried to remove any traces of solvent. The partially purified biosurfactants were used for chemical characterization studies.

3.12. Characterization of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

3.12.1. Thin Layer chromatography (TLC)

TLC analyses of biosurfactants was performed on silica gel 60 TLC plates through the method reported by Kumar and Das, (2018). For rhamnolipids, the solvent system of chloroform:methanol:acetic acid in the ratio of 6.5:1.5:0.2 (v/v/v) was used whereas, for sophorolipids, chloroform:methanol:water 6.5:1.5:0.2 (v/v/v) was applied. The extracted rhamnolipids and sophorolipids were spotted near the bottom of plates at the point of origin and, then dipped in the solvent systems for 15 minutes. Once the plates were developed and air dried, anthrone reagent was sprayed on the plates and bands were visualized under UV light. For every sample Rf values were recorded.

3.12.2. FTIR Spectroscopy

FTIR spectroscopy was used to elucidate the variety of functional groups present in the partially purified biosurfactants extracted from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126. The compounds were analysed in a Thermoscientific 4700 FTIR spectrometer. The samples were analyzed whereas, results were shown in the form of spectra with peaks ranging between 400-4000 cm^{-1} at a resolution of 4 cm^{-1} (Talukdar et al., 2017). The resultant peaks of microbial biosurfactants were compared with the standard rhamnolipids and sophorolipids, respectively.

3.12.3. Reverse Phase HPLC

The partially purified aliquots of biosurfactants were further analyzed through reverse phase HPLC by the method proposed by Gupta and Prabhune, (2012). For this purpose samples were run on an Agilent series HPLC instrument with Eclipse Plus C18 column (3.5 µm solid support size and dimensions of 4.6 mm width X 150 mm length) equipped with a UV detector system. The peaks were detected at 220 nm wavelength. The gradient elution profile for the molecules and their respective standards was as follows: acetonitrile (solution A) and 0.05 % Formic acid (solution B) in the ratio of 30:70 v/v holding for 10 min to a final composition of 70:30 v/v with a linear gradient for 30 min at a flow rate of 0.8 ml/min.

3.12.4. Nuclear Magnetic Resonance (NMR)

The NMR spectra of samples were acquired on a Bruker Advance III HD spectrometer equipped with an inverse detected TCI probe with cryogenic enhancement on ^{1}H and ^{13}C, operating at 599.90 MHz and 150.86 MHz for ^{1}H and ^{13}C, respectively. Samples were prepared in chloroform-*d* and methanol-d_4, and recorded at 298 K (Perinelli et al., 2017, Konishi et al., 2016).

3.12.5. Liquid Chromatography Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

Biosurfactants from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and, their respective standards were analyzed through LC-MS using a Dionex Ultimate 3000 UHPLC coupled to a Bruker Maxis Impact QTOF in negative ESI mode. 1 µl of each sample was separated on a Phenomenex Luna C18 (2) column (5 µM 100A 2.0 X 50 mm) using a gradient of 98 % mobile phase A (0.1 % formic acid in water) and 2 % mobile phase B (0.1 % formic acid in acetonitrile) to 20 % mobile phase A and 80 % mobile phase B in 9 minutes, then kept at 100 % mobile phase B for 2 minutes at a flow rate of 0.3 ml/min (Mainez et al., 2017). The data was processed using the Bruker Data Analysis software version 4.2.

3.13. Stability of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

The stability of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was checked under varying effects of temperature, pH and salinity. After treatment, Emulsification Index ($E.I_{24}$) and Surface Tension (mN/m) was detected for every treatment through the methods described in section 3.7. All readings were taken in triplicates.

3.13.1. Preparation of biosurfactants solution

A loop full of bacterial and yeast culture was inoculated in 250 ml of their aforementioned optimized media, respectively. The flasks were then kept under optimized conditions to ensure optimal production of compounds. Post incubation, the culture media was centrifuged at 10,000 RPM for 20 min at 4 °C to obtain the cell free supernatants.

3.13.2. Thermal stability

The thermal stability of rhamnolipids and sophorolipids was evaluated by incubating the culture supernatants from both the isolates at varying temperatures between 5 °C to 115 °C for one hour. In addition, both the supernatants were tested after autoclaving conditions (121 °C, 15 psi for 20 min). The samples were then allowed to cool at room temperature and then tested for their emulsification and surface active properties (Almeida et al., 2017). The experiment was run in triplicates.

3.13.3. pH stability

The effect of both acidic and basic conditions was checked on the stability of rhamnolipids and sophorolipids. For this purpose, the pH of cell free supernatants was adjusted from 1-14 using 1N HCl and 1N NaOH and then incubated at 37 °C for one hour. $E.I_{24}$ and S.T was recorded for every sample (Almeida et al., 2017).

3.13.4. Stability under varying NaCl concentrations

To evaluate the effect of salts on stability of rhamnolipids and sophorolipids, the supernatants from both strains were treated with 2-14 % w/v concentrations of NaCl. After treatment, the triplicate runs of every sample was checked for emulsification indices and surface tension measurements (Almeida et al., 2017).

3.14. Properties of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

3.14.1. Determination of critical micelle concentration (*cmc*)

cmc is defined as the particular concentration of surfactants at which micelles are formed and is one of the most important property of surfactants. The *cmc* of rhamnolipids and sophorolipids was determined by making different dilutions (in autoclaved distilled water) from 250 mg/L stock solutions of the purified biosurfactants. Surface tension of each dilution was recorded through Whimley Plate Method using Easy Dyne K20, KRÜSS GmbH Tensiometer (Germany) according to the protocol of García-Reyes et al., (2017). After obtaining the results, a graph was plotted for ST values against different surfactant concentrations and *cmc* was noted.

3.14.2. Size distribution through Dynamic Light Scattering (DLS)

The size of particles and polydispersity index was measured using a Nano S90 (model ZEN 1690) from Malvern Instruments (U.K.). The solution of rhamnolipids and sophorolipids were prepared in ultrapure water and the variability in size of micellar aggregates was observed at ½ *cmc*, *cmc* and 2 *cmc*; different pH and, 250 mM, 500 mM and 1000 mM concentrations of NaCl. Post treatment, 1 mL of sample was loaded to the cell and analyzed through DLS at 632.8 nm with Red laser at 25 °C. The scattered light was collected by receptor at angle of 90° from light path. Every sample was analyzed 10 times and readings were recorded. The size of the aggregates was expressed in terms of hydrodynamic

diameter calculated by using the Zetasizer software associated with the instrument (Zhong et al., 2016).

3.15. Environmental applications of biosurfactants and BS producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

The potential role of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and their biosurfactants i.e. rhamnolipids and sophorolipids was monitored in the biodegradation of crude oil and bioremediation of heavy metals.

3.15.1. Role of biosurfactants and BS producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 in biodegradation of crude oil

In order to check the efficacy of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and their biosurfactants for biodegradation of crude oil, cell surface hydrophobicity and emulsification activity of biosurfactants was determined. This was followed by the crude oil degradation experiment of three weeks during which microbial growth, biosurfactants production and crude oil removal percentage was noted.

3.15.1.1. Determination of Cell Surface Hydrophobicity (CSH)

Biosurfactants production and degradation of hydrocarbons is strongly associated with the cell surface hydrophobicity of microorganisms. Therefore, the cell surface hydrophobicity of both the isolates were assessed against crude oil, n-Hexadecane, Dodecane and Toluene using MATH (Microbial Adhesion to Hydrocarbons) assay reported by Obuekwe et al., (2009) with slight modifications. Briefly, microbial cells were separated from the culture medium through centrifugation at 5000 RPM for 15 min. The collected pellet was re-suspended into 10 mm diameter test tubes containing 5 ml sterile MSM. Absorbance of cell suspension was recorded at 600 nm using UV-visible spectrophotometer. Afterwards, 0.5 ml aliquot of hydrocarbons was added to this suspension and vortexed for 2 min. The mixture was allowed to settle at room temperature for hydrocarbon separation. Turbidity of aqueous phase was recorded by measuring the absorbance at 600 nm. MATH is

expressed as the percent loss in the absorbance of aqueous phase in comparison to the absorbance of initial cell suspension (Equation 3.6).

$$MATH = \{1 - (A_{600}\ of\ the\ aqueous\ phase / A_{600}\ of\ the\ initial\ cell\ suspension)\} \ldots Equation\ 3.6$$

3.15.1.2. Effect of hydrocarbons on emulsification activity of biosurfactants

The emulsification properties of rhamnolipids and sophorolipids obtained from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were evaluated under the effect of different hydrocarbons. For this purpose, 2 ml of different oils including crude oil, kerosene oil, diesel, petrol, mustard oil, canola oil and olive oil were added into equal volume of cell free supernatants obtained from both microbial isolates, separately. Experiment was performed in triplicates and emulsification index values were recorded after 24 h (Mendes et al., 2015).

3.15.1.3. Biodegradation studies

In order to evaluate the potential role of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 in biodegradation of crude oil, shake flask experiments were conducted. Seed cultures of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were inoculated in 1 % v/v concentration in 250 ml Erlenmeyer flasks containing 100 ml of sterilized minimal salt medium (MSM) with 1 % crude oil as sole carbon source. Uninoculated flasks were kept as control. All the flasks were incubated at 37 °C on 150 RPM for 21 days. Samples were withdrawn on 7^{th}, 14^{th} and 21^{st} day of incubation for the estimation of biodegradation rate through the assays reported by Patowary et al., (2017).

3.15.1.3.1. Biomass estimation

The effect of crude oil on microbial growth was estimated through calculation of the dry biomass in g/L. Samples were collected at regular intervals and centrifuged at 10,000 RPM for 20 min at 4 °C. Post centrifugation, supernatant was discarded and pellet was washed first with distilled water and then with chloroform to remove any traces of hydrocarbons.

Pellet was then dried at 100 °C till constant weight was achieved. Readings were taken in triplicates.

3.15.1.3.2. Surface tension measurement

To examine the production of biosurfactants in fermentation broth, samples were withdrawn after every 7, 14 and 21 days. Surface Tension of the cell free supernatants was measured by the method already described in section 3.7.2.

3.15.1.3.3. Gravimetric analysis of residual crude oil

Percentage concentrations of residual crude oil in the test flasks were determined after 7, 14 and 21 days through gravimetric analysis. The crude oil from culture broth of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was extracted in ratio of 3 sample: 1 n-hexane. Whole content of the flask was placed in a separating funnel along-with a known concentration of solvent. After continuous shaking for 5-10 min, the mixture was allowed to settle and the layer of solvent containing residual crude oil was collected in a pre-weighed beaker. Extraction was repeated twice to ensure complete recovery of crude oil. After extraction, n-hexane was evaporated in an oven at 70–75 °C and, the beaker was cooled down and re-weighed. The sterile control was processed under similar conditions. Percentage degradation was calculated by using the following formula.

(Weight of Crude oil degraded/Weight of Crude Oil added in the medium) X100 ..*Equation 3.7*

3.15.1.3.4. GC-MS analysis of residual crude oil

After an incubation period of 21 days, crude oil extracted from the control and test flasks were subjected to Gas Chromatography-Mass Spectrometric analysis using SCHIMADZU instrument model QP5050 having an Elite-5MS with specifications 30 m X 0.25 mm I.D. fused silica, 0.25 µm diameter and DB-5. Helium was used as the carrier gas with a flow rate of 1.0 mL/min. 1 µl of sample was injected via split less injection. The injection temperature was maintained at 250 °C whereas, the oven temperature was set at 60 °C with

an initial hold time of 5 min. The temperature was further increased upto 300 °C with the final hold of 30 min. The electron ionization mode was used to obtain the mass spectrometric data with the mass range (m/z) of 50-650. GC-MS solution software was used for the analyses of chromatograms whereas, the crude oil components were identified using the NIST 11 library database. The percentage Biodegradation Efficiency was calculated by the method described by Michaud et al., (2004)

3.15.2. Role of biosurfactants and BS producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 in bioremediation of heavy metals

The potential of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and their biosurfactants i.e. rhamnolipids and sophorolipids was determined in the bioremediation of heavy metals through following assays.

3.15.2.1. Heavy metals tolerance through Plate Diffusion Method

The preliminary tests for heavy metals tolerance of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was carried out through plate diffusion method proposed by Neethu et al., (2015). For this purpose, 100-1000 ppm dilutions (in sterile deionized water) of five heavy metal salts i.e. cadmium chloride, chromium sulphate, copper sulphate, lead nitrate and zinc chloride were tested against the two strains. 500 µl aliquot of each concentration of metals was dispensed into central wells of 1 cm in diameter and 4 mm in depth. The wells were sealed at the bottom with soft agar on Mueller Hinton Agar (MHA) plates. The two strains were streaked onto the plates followed by incubation at 37 °C for 96 h. The metal resistance activity was measured in terms of Minimal Inhibitory Concentration (MIC). MIC was estimated through measuring the diameter of zones appeared on MHA plates after incubation. Sterile deionized water was used as control.

3.15.2.2. Biosurfactants activity in the presence of heavy metals

To evaluate the surface active potential of biosurfactants in the presence of heavy metals, 100, 500 and 1000 ppm concentrations of the aforementioned heavy metals salts were

added in MSM containing 1 % glycerol as sole carbon source. The two sets of flasks were inoculated with the two strains separately and, incubated at 37 °C at 150 RPM for 72 h. Post incubation, samples were withdrawn and $E.I_{24}$ of culture supernatant was measured using kerosene oil as standard. Flasks without heavy metals were used as negative control. All readings were taken in triplicates.

Results of above mentioned assay suggested that rhamnolipids from *P. aeruginosa* MF069166 showed maximum resistance against zinc chloride followed by lead nitrate whereas, sophorolipids from *Meyerozyma* spp. MF138126 exhibited maximum activity against lead nitrate followed by zinc chloride. Therefore, the following experiments were conducted with respect to lead nitrate and zinc chloride to evaluate their maximum percentage removal through biosurfactants.

3.15.2.3. Metal chelating activity of biosurfactants

The metal chelating activity of biosurfactants was checked through overnight incubation of ½ *cmc*, *cmc* and 2 *cmc* concentrations of biosurfactants with 100, 500 and 1000 ppm solutions of lead nitrate and zinc chloride. After incubation, the solution was centrifuged at 10,000 RPM for 10 min to separate the metal-biosurfactant precipitates and the resulting supernatant was checked for the presence of unbound metals through atomic absorption spectrophotometer. The percentage removal of heavy metals in the biosurfactants treated solutions was determined through running the appropriate blanks and standards on atomic absorption spectrophotometer. All readings were taken in triplicates (Luna et al., 2016).

3.15.2.4. Conductivity measurements

To evaluate and compare the metals removal efficiency and ionic content of biosurfactants and chemical surfactants treated samples, electrical conductivity was measured. The solutions of biosurfactants and chemical surfactants i.e. SDS, Tween 20 and Tween 80 were prepared in ½ *cmc*, *cmc* and 2 *cmc* concentrations. The *cmc* of SDS, Tween 20 and Tween 80 is 0.234 %, 0.007 % and 0.012 %, respectively. The prepared concentrations of surface active compounds were mixed with 500 ppm solutions of lead nitrate and zinc

chloride followed by 24 h incubation at room temperature. The metal-biosurfactants/surfactants complex was then removed from the samples through centrifugation. The conductivity meter was first calibrated with deionized water and then used to measure the conductivity of the resulting supernatants. All readings were taken in triplicates (Luna et al., 2016).

3.15.2.5. Washing of heavy metals contaminated soil using biosurfactants

3.15.2.5.1. Soil spiking

The unaffected and pre-characterized soil obtained from the agricultural field of Chak Naurang, Punjab, Pakistan was artificially spiked with 1000 ppm solutions of lead nitrate and zinc chloride. 1 kg of soil was filtered through mesh sieve to remove any stones and debris, autoclaved and contaminated with metals solutions and, finally placed in a shaker incubator at 150 RPM for 96 h to allow the homogenous mixing of metals in soil. Soil samples were then air dried to remove any traces of solvent (water) so that predetermined concentration of metals could be achieved.

3.15.2.5.2. Soil washing

A series of soil washing experiments were conducted using the ½ *cmc*, *cmc* and 2 *cmc* concentrations of biosurfactants extracted from the culture supernatant of two strains through the method reported by Luna et al., (2016). Deionized water was used as control. 5 g of the spiked soil was transferred into 250 ml Erlenmeyer flasks followed by the addition of 50 ml biosurfactants solutions of aforestated concentrations. The flasks were then kept under shaking conditions at 150 RPM for 24 h at 30 °C. After 24 h, the whole content of each flask was subjected to centrifugation at 10,000 RPM for 10 min. This procedure was repeated thrice to ensure the complete removal of soil particles from supernatant. The resulting supernatant was then analyzed through Atomic Absorption Spectrophotometer to determine the concentration of heavy metals in each run.

In another set of experiment, the kinetics of heavy metals removal was studied using the cell free broth (crude biosurfactants) of the two isolates. 50 ml of culture supernatant was added in 250 ml Erlenmeyer flasks containing 5 g of artificially spiked soil followed by incubation of flasks under shaking conditions for 15 days. Flask without the culture supernatant was run as control. Samples were withdrawn every second day and the percentage removal of metals was calculated through atomic absorption spectrometry. All readings were taken thrice.

4- RESULTS

A- Isolation and screening of biosurfactants producing microorganisms from crude oil contaminated soil

In the first phase of study, isolation and screening of biosurfactants producing microorganisms was done from the crude oil contaminated soil. For this purpose soil samples were collected from a crude oil contaminated site located near the Chak Naurang oil field, District Chakwal, Punjab. The isolation of biosurfactants producing microorganisms was done through a number of qualitative and quantitative screening assays. The efficient biosurfactants producing isolates were selected and subsequently identified using morphological, biochemical and molecular identification tools.

4.1. Soil analysis

The crude oil contaminated soil samples taken from Chak Naurang oil field were analyzed in order to estimate the effect of petroleum hydrocarbons on soil properties in comparison with the control (Fig. 4.1). The results of soil analysis have been summarized in Fig. 4.2. It was observed that the crude oil contaminated soil was clay-loam in texture with slightly alkaline pH. The moisture content was found to be 46 % in the control (unaffected) soil which significantly decreased up to 18 % in the crude oil contaminated soil. Crude oil contaminated soil showed less electrical conductivity (4.3 dS/m) as compared to the agricultural soil (24.81 dS/m). However, considerably high quantity of Total Petroleum Hydrocarbons (up to 22.56 g/kg) and Total Organic Content (23.11 g/kg) was detected in the contaminated soil as the recorded value of TOC for agricultural soil was 4.03 g/kg. Contrary to aforestated parameters, reduced concentration of nitrogen (0.644 g/kg), phosphorous (12.46 mg/kg) and potassium (20 mg/kg) was detected in the soil samples collected from crude oil affected brownfield.

Figure 4.1: Different regions of crude oil affected and unaffected agricultural land of Chak Naurang

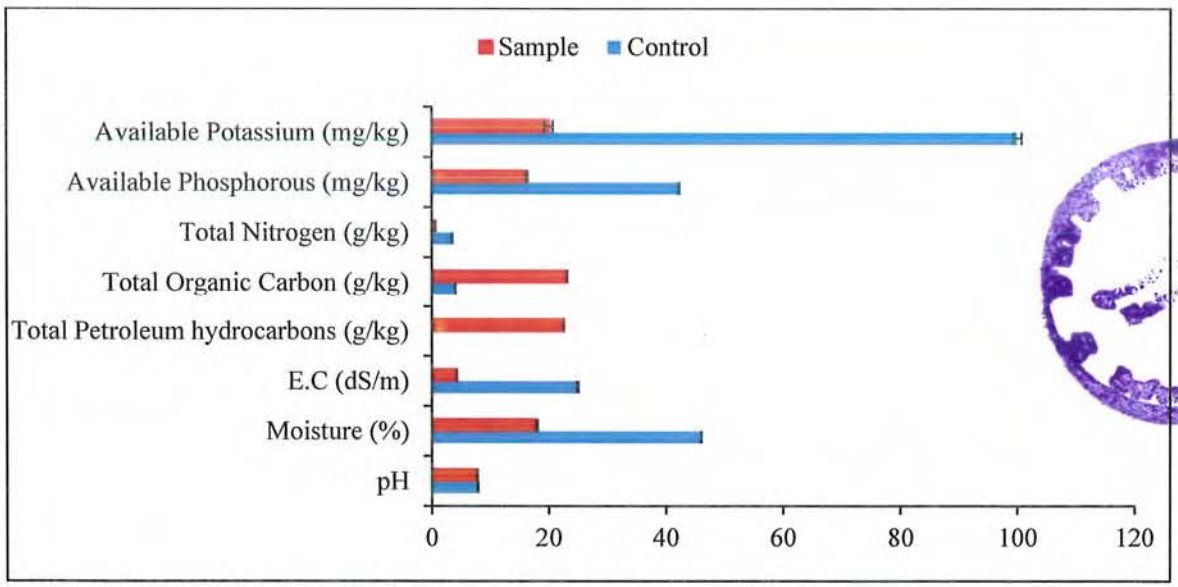


Figure 4.2: Comparative analysis of physicochemical properties of crude oil affected and unaffected soil

4.2. Isolation of crude oil degrading and biosurfactants producing microorganisms

After soil analysis, the crude oil contaminated soil was used for the isolation of crude oil degrading and biosurfactants producing microorganisms. After enrichment of soil for 30

days, thirty-four bacterial and six fungal strains including one yeast species was obtained that were able to use crude oil as sole source of carbon and energy. Isolated microbial strains were further purified on nutrient agar and sabouraud dextrose agar plates and, codded as RB and RF for representation of bacterial and fungal species, respectively. In the next step, all purified isolates were tested for their metabolic capability to produce biosurfactants using following assays.

4.3. Qualitative screening of biosurfactants producing microorganisms

Initially, the isolated microorganisms were qualitatively screened for biosurfactants production. For this purpose, three plate assays were used which include blood agar hemolysis assay, CTAB methylene blue agar assay and crude oil overlay agar assay.

4.3.1. Blood Agar Hemolysis Assay

The inoculation of isolated microorganisms on blood agar plates resulted in the lysis of erythrocytes present in the medium. After incubation of 72-96 h, different zones of lysis were recorded in order to differentiate biosurfactants producing and non-producing microorganisms. The results are presented in Fig. 4.3. It was noted that amongst forty isolates, 45 % of strains showed α-hemolysis, 37 % of strains showed β- hemolysis and 18 % of strains showed no hemolytic activity on blood agar plates.

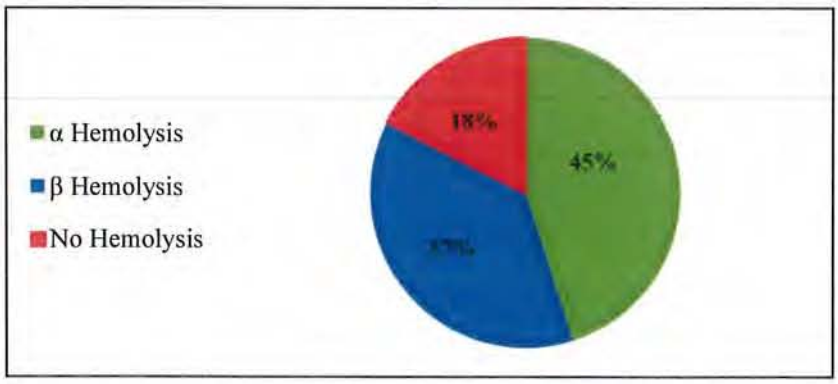


Figure 4.3: Blood agar hemolysis assay of biosurfactants producing microorganisms

4.3.2. CTAB Methylene Blue Agar Assay

CTAB Methylene blue agar assay displayed varying percentages of biosurfactants producing and non-producing microorganisms with respect to their ability to release charged biosurfactants molecules in the medium. Results showed that 32 % of strains produced a dark blue halo around the point of inoculation, 38 % of strains produced a comparatively lighter zone whereas the remaining 30 % of strains displayed no zone after 72-96 h of incubation (Fig. 4.4).

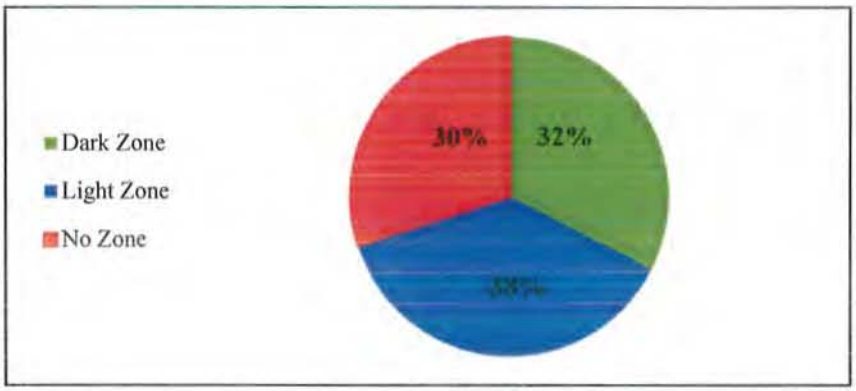


Figure 4.4: CTAB methylene blue agar assay of biosurfactants producing microorganisms

4.3.3. Crude Oil Overlay Agar Assay

The ability of isolated microorganisms to degrade crude oil through the production of biosurfactants was checked in crude oil overlay agar assay. Results of the assay revealed that 65 % of strains formed emulsified halos around their colonies whereas, no emulsification activity was observed on MHA plates for the remaining 35 % of isolates (Fig. 4.5).

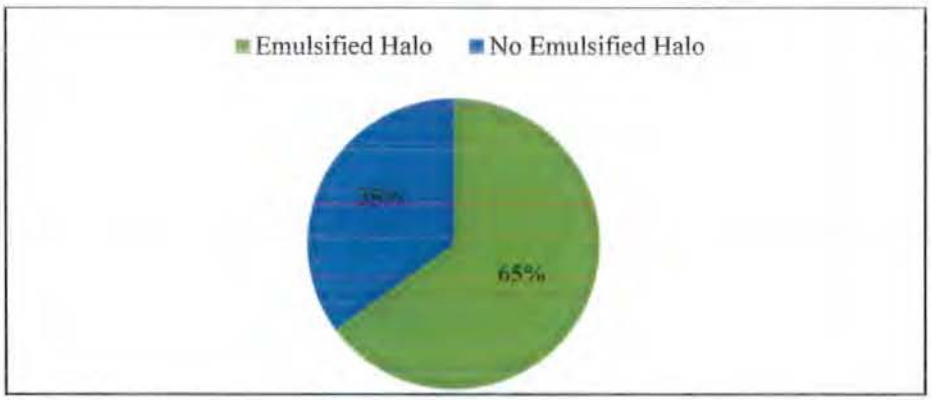


Figure 4.5: Crude oil overlay agar assay of biosurfactants producing microorganisms

4.4. Identification of potential biosurfactants producing microorganisms

On the basis of qualitative plate assays, potential biosurfactants producing microorganisms were enlisted and subsequently subjected to identification studies. The selected fourteen microorganisms were initially identified through morphological, biochemical and microscopic tests and later on, their percent homology was confirmed through molecular tools. Initial studies revealed diverse phenotypic properties of the microorganisms. Most of the bacterial isolates formed off white to cream colored colonies on nutrient agar plates and were positive for Gram staining, catalase, oxidase and motility tests. However, two of the isolates showed characteristics of a typical Gram negative bacteria. In case of the two fungal isolates, the colony morphology and cellular staining exhibited characteristic eukaryotic properties (Table 4.1).

Table 4.1: Morphological identification of potential biosurfactants producing microorganisms

BACTERIAL ISOLATES										
Isolates	Colony Morphology	Gram Staining	Biochemical Tests							
			CAT	OX	CIT	URE	MR	VP	IND	Motility
RB3	White color colony	+ive	+ive	-ive	+ive	-ive	-ive	+ive	-ive	+ive
RB5	Off white color rough	+ive	+ive	+ive	-ive	+ive	-ive	-ive	-ive	+ive
RB6	Greenish color rough	-ive	+ive	+ive	+ive	-ive	-ive	-ive	-ive	+ive
RB7	Off white color smooth	+ive	+ive	-ive	+ive	-ive	-ive	+ive	-ive	+ive
RB9	White color rough threadlike	+ive	+ive	-ive	+ive	-ive	-ive	+ive	-ive	+ive
RB11	Cream color irregular	-ive	+ive	+ive	+ive	+ive	-ive	-ive	-ive	+ive
RB12	Light yellow color smooth	+ive	+ive	-ive	+ive	-ive	-ive	+ive	-ive	+ive
RB27	Olive green color smooth	-ive	+ive	+ive	+ive	-ive	-ive	-ive	-ive	+ive
RB29	Off white color filamentous	+ive	+ive	variable	+ive	-ive	-ive	+ive	-ive	+ive
RB31	White color rough	+ive	+ive	-ive	+ive	-ive	-ive	+ive	-ive	+ive
RB32	Light yellow color rough	+ive	+ive	-ive	+ive	-ive	-ive	+ive	-ive	+ive
RB33	Light green color smooth	-ive	+ive	+ive	+ive	-ive	-ive	-ive	-ive	+ive

FUNGAL ISOLATES		
	Morphology	**Staining**
RF36	Yellow color colony	Lactophenol Blue staining showed the characteristic fungal hyphae
RF38	Off white sticky, creamish and slimy	Grams's staining showed round Gram positive cells

Molecular identification of the selected microorganisms was carried out through the isolation and characterization of conserved 16S and 18S rRNA sequences. Results revealed diversity in their phylogenetic origin. Amongst bacterial isolates, six strains belonged to the genus *Bacillus*, two to *Pseudomonas*, one to *Paenibacillus* and one to *Achromobacter*. The yeast species showed 99 % homology with the members of genus *Meyerozyma* whereas, the other fungal isolate was a member of genus *Aspergillus*. Accession number of every strain was obtained after submitting the characterized sequence in NCBI. The

scientific names and respective accession numbers of microorganisms have been enlisted in Table 4.2. The phylogenetic tree was constructed using MEGA 7 software (Fig. 4.6 a and b).

Table 4.2: Molecular identification of potential biosurfactants producing microorganisms

Strain Name	Scientific Name	Accession #
RB 3	*Bacillus pumilus*	**MF138116**
RB 5	*Paenibacillus azoreducens*	**MF138117**
RB 6	*Pseudomonas stutzeri*	**MF138118**
RB 7	*Bacillus licheniformis*	**MF138121**
RB 9	*Bacillus thuringiensis*	**MF138122**
RB 11	*Achromobacter xylosoxidans*	**MF138123**
RB 12	*Bacillus cereus*	**MF138124**
RB 27	*Pseudomonas aeruginosa*	**MF069166**
RB 29	*Bacillus subtilis*	**MF138125**
RB 31	*Bacillus* spp.	**MF138130**
RB 32	*Bacillus amyloliquefaciens*	**MF138127**
RB 33	*Pseudomonas* spp.	**MF099829**
RF 36	*Meyerozyma* spp.	**MF138126**
RF 38	*Aspergillus terreus*	**MF138128**

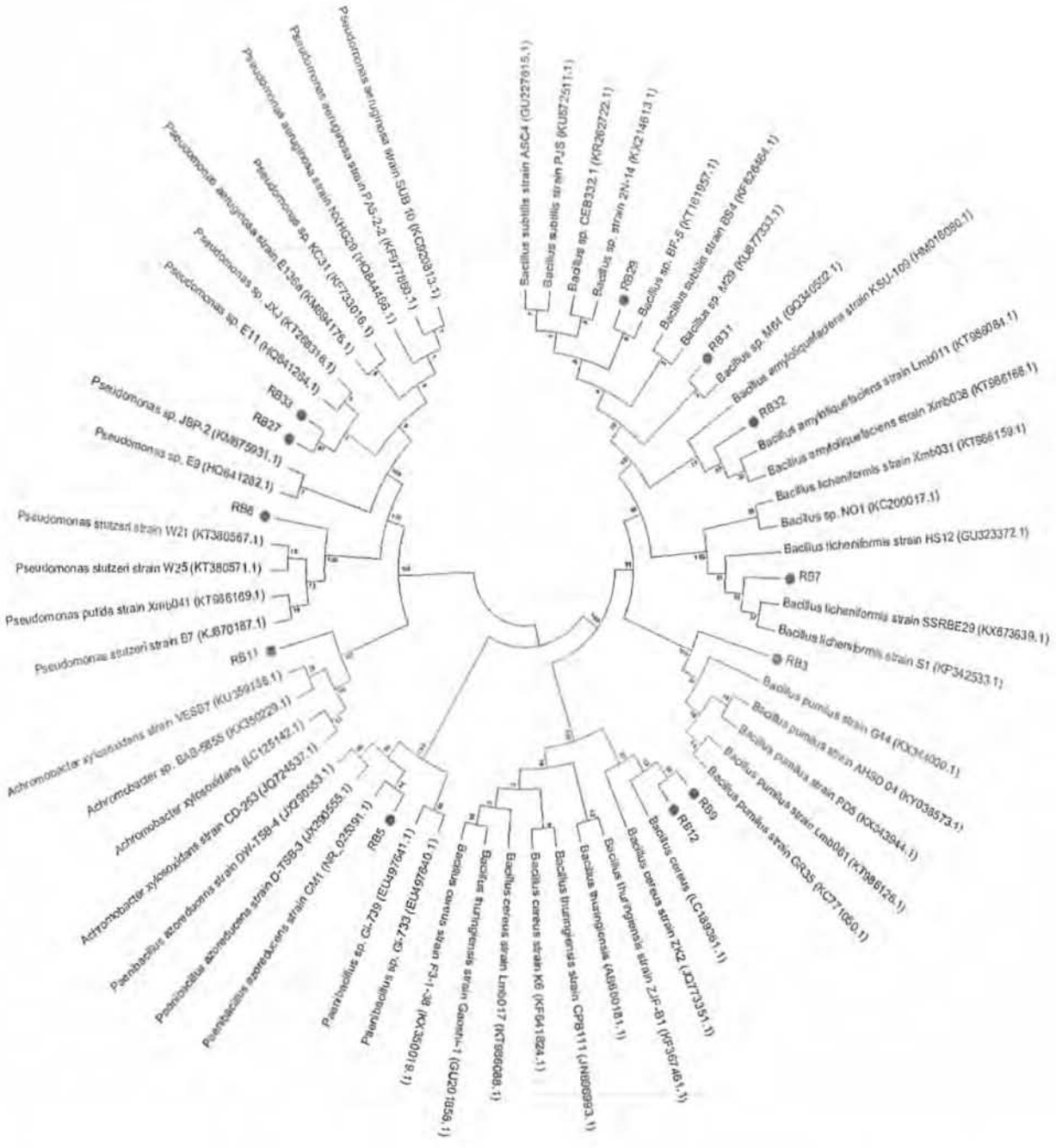


Figure 4.6 a: Phylogenetic characterization of potential bacterial biosurfactants producing microorganisms

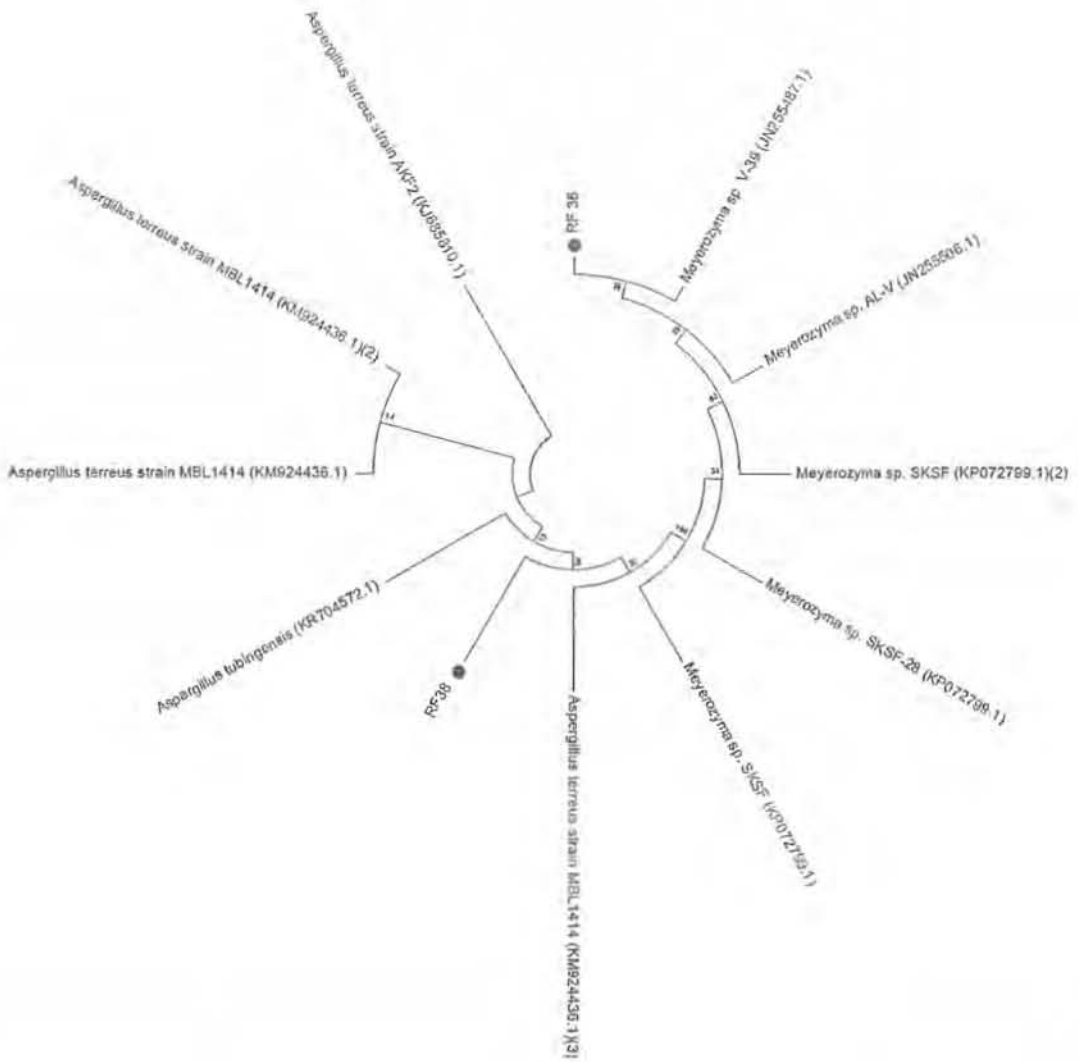


Figure 4.6 b: Phylogenetic characterization of potential fungal biosurfactants producing microorganisms

4.5. Quantitative screening of potential biosurfactants producing microorganisms

Followed by identification studies, the quantitative analyses of biosurfactants production was performed for fourteen isolates. The culture supernatant from each strain was tested for the presence of surface active compounds through determination of emulsification index, surface tension measurement and oil displacement assay.

4.5.1. Emulsification Index (E.I24) analysis

Emulsification index of the selected microorganisms was determined using kerosene oil as standard. All the characterized strains displayed more than 70 % emulsification of kerosene oil. *P. aeruginosa* MF069166 turned out to be the best emulsifier with 84 % E.I24 followed by *Meyerozyma* spp. MF138126 which showed 82 % E.I24. Besides these strains, *Pseudomonas* spp. MF099829, *B. amyloliquefaciens* MF138127, *B. thuringensis* MF138122, *Bacillus* spp. MF138130, *P. stutzeri* MF138118, *A. tereus* MF138128 and *B. licheniformis* MF138121 also stabilized oil in water emulsion and showed promising emulsification activities of 79 %, 77 %, 75 %, 72 %, 70 % and 69 %, respectively (Fig. 4.7).

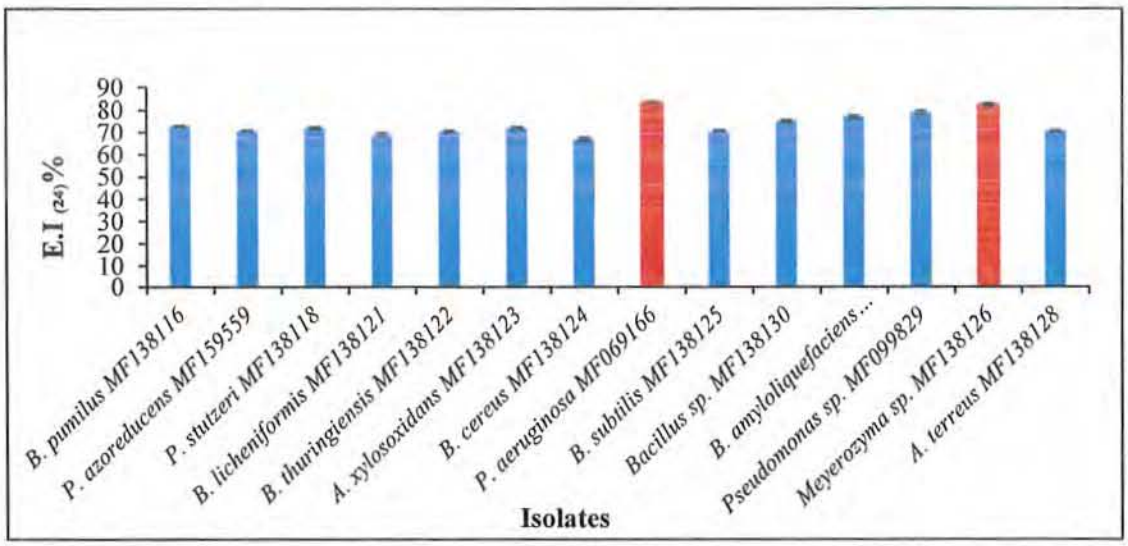


Figure 4.7: Emulsification Index (E.I24) of potential biosurfactants producing microorganisms

4.5.2. Surface Tension Measurement

The surface tension (ST) of culture supernatant is also considered as one of the best confirmatory assay for the identification of biosurfactants producing microorganisms.

Results showed a substantial decrease in the surface tension which confirmed the presence of surface active compounds in the culture supernatant. The least ST value of 25 mN/m was recorded for *Meyerozyma* spp. MF138126 whereas, *B. amyloliquefaciens* MF138127 reduced the ST of culture media from 72 mN/m to 39.8 mN/m. Other strains like *P. aeruginosa* MF069166, *P. azoreducens* MF159559 and *P. stutzeri* MF138118 significantly reduced the surface tension of fermentation broth and the recorded ST values were 26.6, 33.7 and 34.3 mN/m, respectively (Fig.4.8). These results suggested that amongst 14 strains, *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were comparatively more effective in reducing the surface tension of culture medium.

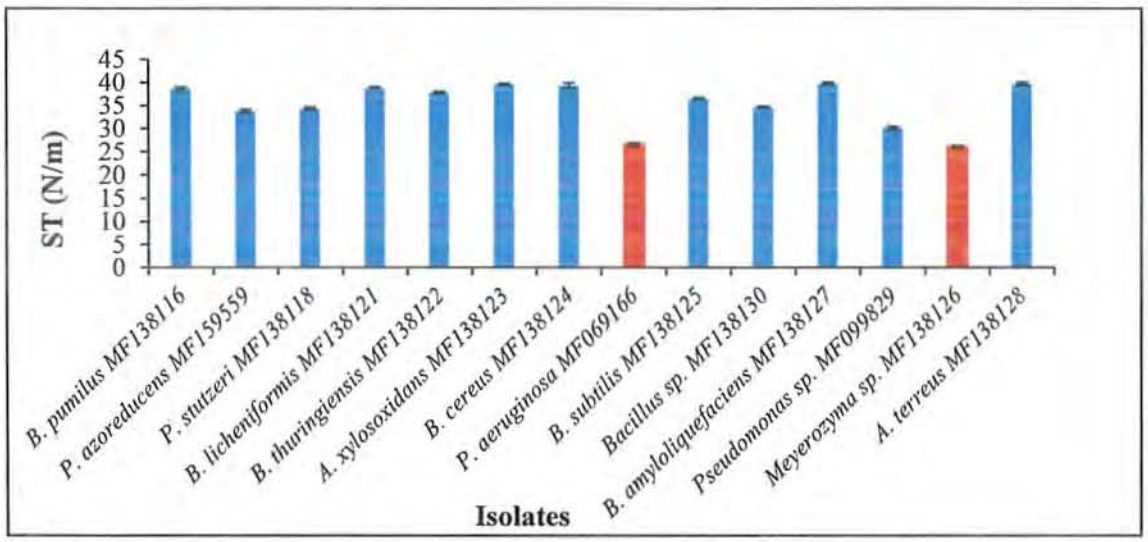


Figure 4.8: Surface Tension (ST) of potential biosurfactants producing microorganisms

4.5.3. Oil Displacement Assay (ODA)

In oil displacement assay, biosurfactants from the isolated strains exhibited varying degrees of surface active properties and displaced crude oil layer from the surface of water. *P. aeruginosa* MF069166 displaced the thin film of crude oil up to 8 cm whereas *B. pumilus* MF138116 produced a zone of 3.5 cm. Other strains displayed remarkable surface active

properties by forming displacement zones ranging between 3.9 cm to 7.4 cm. However, *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were able to displace oil film much better than remaining isolates (Fig. 4.9).

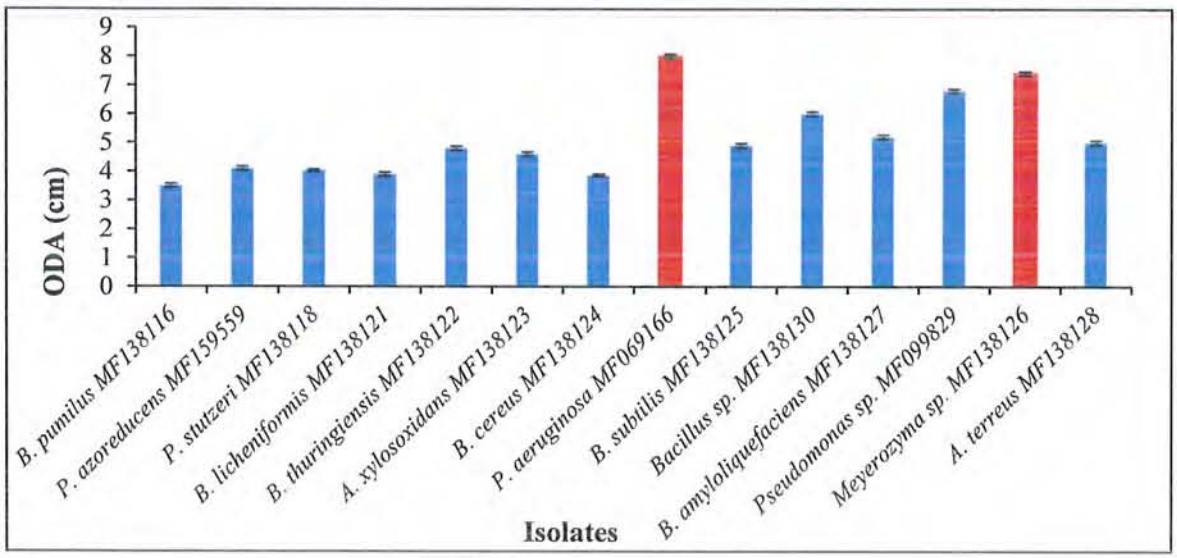


Figure 4.9: Oil Displacement Assay (ODA) of biosurfactants producing microorganisms

4B

B- Optimization of bioprocess using design of experimental approach for biosurfactants production from selected microbial isolates

In the second phase of study, one bacterial strain i.e. *P. aeruginosa* MF069166 and one fungal strain i.e. *Meyerozyma* spp. MF138126 was selected amongst the fourteen potential biosurfactants producing microorganisms. For the two isolates, optimization of bioprocess was done through design of experimental approach. Plackett-Burman design was used to optimize the media components whereas, the physico-chemical conditions were optimized using Response Surface Methodology (RSM). Post optimization studies, high microbial growth and improved yield of biosurfactants was obtained.

4.6. Selection of the most efficient biosurfactants producing microorganisms

On the basis of various testes applied for qualitative and qualitative determination of the biosurfactant production, the bacterial strain *P. aeruginosa* MF069166 and the fungal strain *Meyerozyma* spp. MF138126 were found to be the most productive and selected for further studies.

4.7. Effect of fermentation media on growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

After selection of the most productive strains, the appropriate media formulation for growth and biosurfactants production was screened. For this purpose, five different previously reported fermentation media with varying combination of nutrients were tested for *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 (Table 3.2). The experiments were conducted in triplicates and microbial growth and biosurfactants production was monitored for 96 h.

4.7.1. Effect of fermentation media on growth and rhamnolipids production from *P. aeruginosa* MF069166

In case of *P. aeruginosa* MF069166, a gradual increase in growth and rhamnolipids concentration was observed after every 24 h which reached up to its maximum value after 96 h of incubation period. Highest bacterial growth (2.53) and rhamnolipids production (2.31 g/L) was obtained in M 2 followed by M 4 (2.01, 1.86 g/L), M 3 (1.54, 1.66 g/L), M 1 (1.16, 0.72 g/L) and M 5 (1.03, 0.64 g/L). These results suggested that M 2 was the most effective medium for growth and biosurfactants production from *P. aeruginosa* MF069166 (Fig. 4.10).

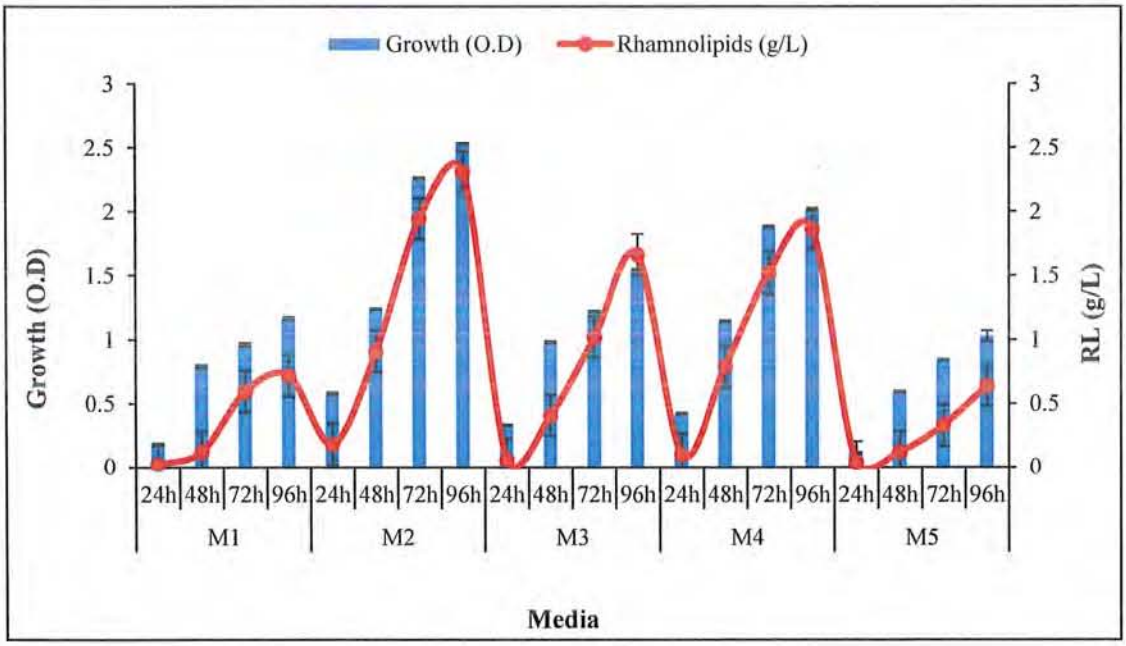


Figure 4.10: Effect of different fermentation media on growth and rhamnolipids production from *P. aeruginosa* MF069166

4.7.2. Effect of fermentation media on growth and sophorolipids production from *Meyerozyma* spp. MF138126

In another set of experiment, the effect of five different media formulations was checked on growth and sophorolipids production from *Meyerozyma* spp. MF138126. Highest growth and yield of sophorolipids was obtained in M 5. After 96 h of incubation, the recorded values for growth and SL concentration were 2.94 and 2.88 g/L, respectively.

Almost similar type of growth was observed in M 1 and M 2 however, better yield of SLs was obtained in M 2. M 3 was also quite effective in increasing the yield of sophorolipids up to 2.01 g/L whereas 1.98 g/L of SLs were produced in M 4 (Fig. 4.11). These observations suggested that M 5 was the most productive fermentation medium for *Meyerozyma* spp. MF138126.

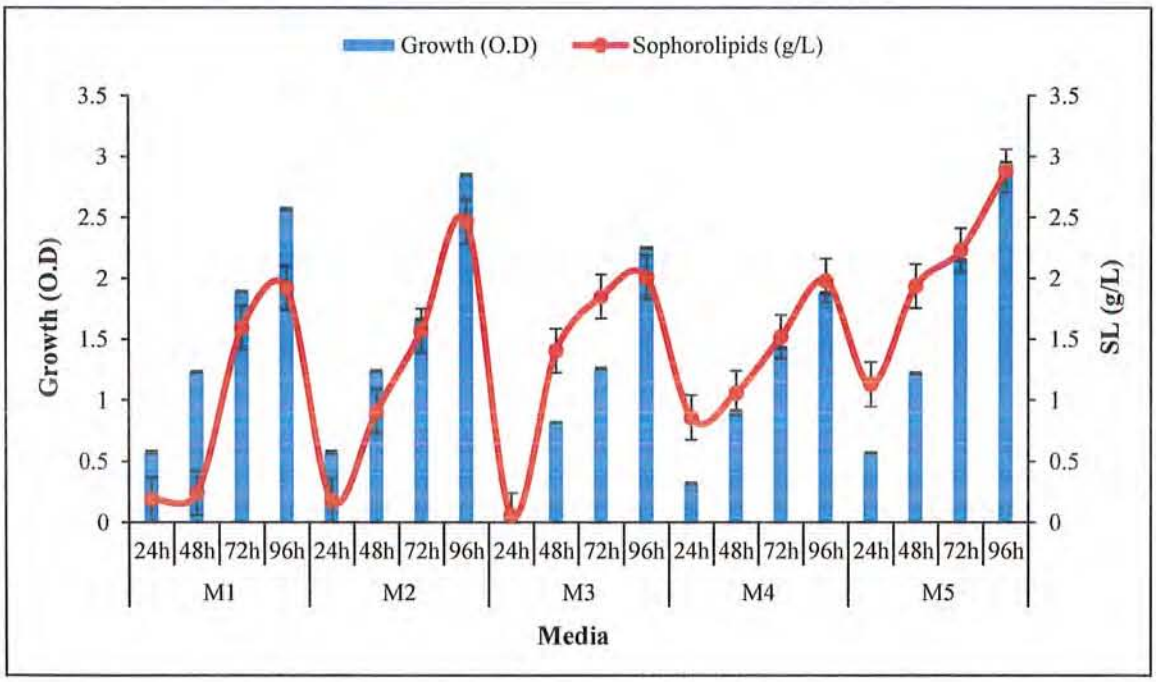


Figure 4.11: Effect of different fermentation media on growth and sophorolipids production from *Meyerozyma* spp. MF138126

4.8. Optimization of media components through Plackett-Burman design for growth and rhamnolipids production from *P. aeruginosa* MF069166

After selection of the appropriate media (M 2) for rhamnolipids production from *P. aeruginosa* MF069166, the optimum concentrations of the nutrients was determined using Plackett-Burman design. For this purpose, randomized design was generated with different concentrations of nutrients. After performing experiments for 96 h the effect of different media components on growth and rhamnolipids production from the bacterial strain was

determined (Table 4.3). The statistical analysis of results indicated that media composition and concentrations of the variables had significant effect on bacterial growth and rhamnolipids production.

In case of growth, the experimental design was statistically significant with a *p*-value of 0.001 indicating that the design explains significant variability in the factors. Glycerol, NaH_2PO_4, peptone, KNO_3, NaCl, yeast extract, $MgSO_4$ and $FeSO_4$ were found to be significant model terms with the *p*-values of 0.001, 0.027, 0.003, 0.003, 0.003, 0.001, 0.009 and 0.014, respectively. Whereas, glucose, K_2HPO_4 and $CaCl_2$ were statistically non-significant model terms as their obtained *p*-values were greater than 0.05. Regression analysis indicated that glycerol, NaCl, yeast extract and $FeSO_4$ had positive effect on bacterial growth at high concentrations whereas, NaH_2PO_4, peptone, KNO_3 and $MgSO_4$ were effective at low concentrations (Equation 4.1). The R^2 value obtained for growth of *P. aeruginosa* MF069166 was 0.99 which depicts that the experiments were performed with reasonable accuracy and lies in close conformity with the predicted values (Table 4.4). This confirms that 99 % variability in bacterial growth can be explained by the model. On the basis of *p*-values and regression analysis, the optimum medium composition for maximum growth of *P. aeruginosa* MF069166 was found to be; glycerol (2 %), NaH_2PO_4 (2.5 g/L), peptone (1 g/L), KNO_3 (1 g/L), NaCl (1 g/L), yeast extract (1 g/L), $MgSO_4$ (0.2 g/L) and $FeSO_4$ (0.008 g/L) (Fig. 4.12).

In case of rhamnolipids production; glycerol, K_2HPO_4, peptone, KNO_3, NaCl, yeast extract and $MgSO_4$ were significant model terms with the *p*-values of 0.001, 0.032, 0.008, 0.022, 0.032, 0.001 and 0.005. The remaining nutritional parameters i.e. glucose, NaH_2PO_4, $FeSO_4$ and $CaCl_2$ were statistically non-significant with the *p*-values of 0.290, 0.069, 0.149 and 0.193, respectively. Furthermore, 0.99 R^2 depicted reliability and adequate precision of the experimental setup and showed that 99 % variability in production of rhamnolipids from *P. aeruginosa* MF069166 can be precisely elucidated by the model (Table 4.5). These observations also suggested that the predicted and experimental values were closely related. *p*-values and regression analysis showed that glycerol, K_2HPO_4, NaCl and yeast

extract enhanced rhamnolipids production from *P. aeruginosa* MF069166 when added in high concentrations whereas, peptone, KNO_3 and $MgSO_4$ had a positive effect on RLs yield when added in low concentrations (Equation 4.2). The maximum yield of 3.51 g/L of rhamnolipids was obtained in the presence of 2 % glycerol, 10 g/L of K_2HPO_4, 1 g/L of peptone, 1 g/L of NaCl, 1 g/L of yeast extract, 1 g/L of KNO_3 and 0.2 g/L of $MgSO_4$ (Fig. 4.13).

Taking together, application of the Plackett–Burman design provided significant media components along with their relevant concentrations where maximum growth and rhamnolipids production was achieved. Furthermore, Plackett–Burman design also pointed out the path of steepest ascent and center point for maximum growth and improved yield of rhamnolipids from *P. aeruginosa* MF069166.

Table 4.3: Experimental design and results of Plackett-Burman design in terms of responses (growth and rhamnolipids production) for *P. aeruginosa* MF069166

Run Order	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Growth	RL (g/L)
1	1	1	1	-1	1	-1	-1	-1	-1	1	1	3.05	3.45
2	-1	1	-1	-1	-1	-1	1	1	1	-1	-1	2.32	2.84
3	1	1	-1	-1	-1	1	1	-1	1	1	-1	3.01	3.46
4	1	-1	1	1	1	1	-1	-1	1	-1	-1	1.98	2.42
5	-1	1	1	1	-1	1	-1	-1	-1	-1	-1	1.03	1.52
6	-1	-1	-1	1	-1	1	1	1	-1	1	-1	1.89	2.59
7	-1	1	-1	1	1	-1	1	-1	1	-1	1	1.06	1.53
8	1	-1	1	-1	-1	1	-1	1	1	-1	1	2.77	3.13
9	1	1	-1	1	1	1	1	1	1	1	1	3.02	3.46
10	1	-1	1	1	-1	-1	1	-1	-1	-1	1	2.81	3.16
11	-1	1	-1	-1	1	1	-1	1	-1	-1	1	1.19	1.42
12	-1	-1	-1	-1	-1	-1	-1	-1	1	1	-1	0.91	0.51
13	-1	-1	1	1	1	-1	-1	1	1	1	-1	1.02	0.48
14	1	1	-1	1	-1	-1	-1	1	-1	1	-1	3.02	3.51
15	-1	-1	-1	-1	1	1	1	-1	-1	1	-1	0.72	0.23
16	1	-1	-1	-1	1	-1	1	1	-1	-1	-1	2.85	3.12

Table 4.4: ANOVA of selected model terms for growth of *P. aeruginosa* MF069166

Source	DF	Seq SS	Adj SS	Adj MS	F	*p*
Main Effects	11	11.8068	11.8068	1.07334	330.50	0.001
A:Glycerol	1	9.2039	7.2510	7.25095	2232.69	0.001
B:Glucose	1	0.2262	0.0295	0.02952	9.09	0.057
C:K_2HPO_4	1	0.4225	0.0006	0.00057	0.17	0.704
D:NaH_2PO_4	1	0.0024	0.0535	0.05350	16.47	0.027
E:Peptone	1	0.3411	0.2427	0.24266	74.72	0.003
F:KNO_3	1	0.2198	0.2612	0.26125	80.44	0.003
G:NaCl	1	0.1570	0.2414	0.24138	74.32	0.003
H:Yeast Extract	1	1.0305	1.1218	1.12181	345.42	0.001
J:$MgSO_4$	1	0.1167	0.1153	0.11526	35.49	0.009
K:$FeSO_4$	1	0.0800	0.0859	0.08586	26.44	0.014
L:$CaCl_2$	1	0.0067	0.0067	0.00668	2.06	0.247
Residual Error	3	0.0097	0.0097	0.00325		
Total	15	12.5309				

R-Sq = 99.92 %	R-Sq (adj) = 99.61 %

Table 4.5: ANOVA of selected model terms for rhamnolipids production from *P. aeruginosa* MF069166

Source	DF	Seq SS	Adj SS	Adj MS	F	*p*
Main Effects	11	17.7477	17.7477	1.6134	141.70	0.001
A:Glycerol	1	12.3841	10.7462	10.7462	943.77	0.001
B:Glucose	1	0.7224	0.0187	0.0187	1.64	0.290
C:K_2HPO_4	1	0.8806	0.1627	0.1627	14.29	0.032
D:NaH_2PO_4	1	0.1933	0.0881	0.0881	7.74	0.069
E:Peptone	1	0.6934	0.4709	0.4709	41.36	0.008
F:KNO_3	1	0.1258	0.2206	0.2206	19.37	0.022
G:NaCl	1	0.2311	0.1627	0.1627	14.29	0.032
H:Yeast Extract	1	1.8234	1.8207	1.8207	159.90	0.001
J:$MgSO_4$	1	0.6025	0.6214	0.6214	54.58	0.005
K:$FeSO_4$	1	0.0592	0.0425	0.0425	3.73	0.149
L:$CaCl_2$	1	0.0319	0.0319	0.0319	2.80	0.193
Residual Error	3	0.0342	0.0342	0.0114		
Total	15	20.7458				

R-Sq = 99.84 %	R-Sq (adj) = 99.18 %

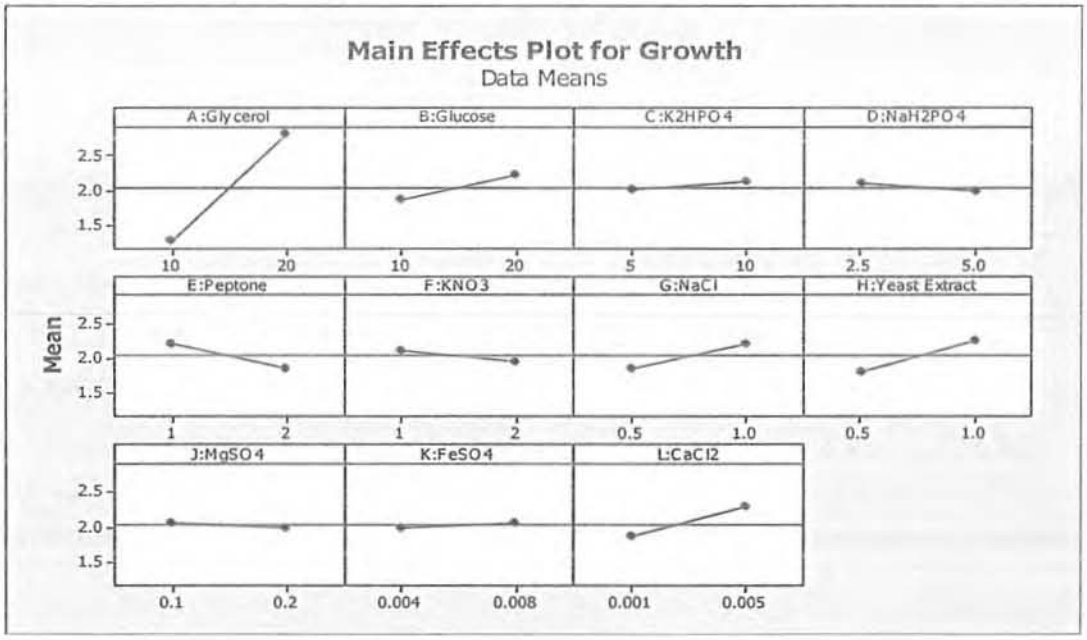


Figure 4.12: Main effect plots for growth of *P. aeruginosa* MF069166

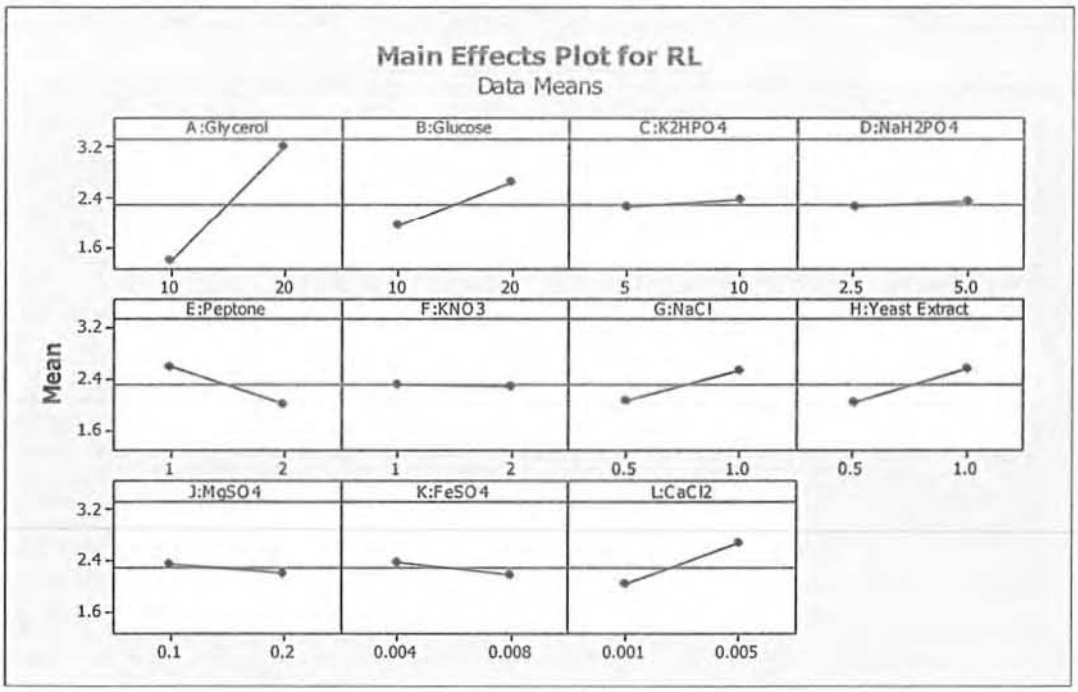


Figure 4.13: Main effect plots for rhamnolipids production from *P. aeruginosa* MF069166

4.8.1.1 Regression equation for growth of *P. aeruginosa* MF069166

Growth = - 1.88 + 0.146 A: Glycerol + 0.0429 B: Glucose + 0.0703 C: K_2HPO_4 - 0.0847 D: NaH_2PO_4 - 0.361 E: Peptone - 0.179 F: KNO_3 + 1.03 G: NaCl + 1.05 H: Yeast Extract - 0.59 J: $MgSO_4$ + 42.3 K: $FeSO_4$ + 2.5 L: $CaCl_2$....*Equation 4.1.*

4.8.1.2. Regression equation for rhamnolipids production from *P. aeruginosa* MF069166

RL = - 2.49 + 0.174 A: Glycerol + 0.0770 B: Glucose + 0.065 C: K_2HPO_4 - 0.007 D: NaH_2PO_4 - 0.581 E: Peptone - 0.046 F: KNO_3 + 1.31 G: NaCl + 1.23 H: Yeast Extract - 1.46 J: $MgSO_4$ - 23.9 K: $FeSO_4$ + 5 L: $CaCl_2$....*Equation 4.2.*

4.9. Optimization of cultivation conditions through Response Surface Methodology (RSM) for growth and rhamnolipids production from *P. aeruginosa* MF069166

After selecting optimum media composition, growth and production of rhamnolipids from *P. aeruginosa* MF069166 was further improved by using Response Surface Methodology (RSM) and Central Composite Design (CCD) with four bioprocess variables i.e. pH, temperature (T), size of inoculum (SOI) and agitation speed (RPM). The experiments were performed in line with the prescribed design and results/responses were evaluated after 96 h of incubation period (Table 4.6). Results indicated that the model for cellular growth and production of rhamnolipids was fitted reasonably with a confidence level of 99.9 %. Significance of the process variables was determined after statistical analyses with reference to obtained *p*-values. The results revealed that for growth and rhamnolipids production from *P. aeruginosa* MF069166; pH, T, SOI and RPM were significant model terms with the *p*-values less than 0.05 (Table 4.7 and 4.8).

Analysis of variance test (ANOVA) was performed in order to estimate the role of each selected model term. Our results indicated that overall the model was statistically significant with a *p*-value of 0.001 (Table 4.7 and 4.8). The adequacy of the bioprocess was

estimated with reference to the coefficient of regression R^2. The R^2 value for both cellular growth and rhamnolipids production was found to be 0.99, which suggested that the predicted and experimental responses were in close conformity with each other. Similarly, it shows that 99 % of the variability in the selected responses can be explained by the given model. Response surface graphs demonstrated the co-relation between the actual and predicted values for growth and sophorolipids production. Besides individual model terms, the interactive effect of bioprocess variables was also significant. For growth of *P. aeruginosa* MF069166, AD, BD, CD, A^2, B^2, C^2 and D^2 were significant model terms with *p*-value less than 0.05 whereas, AB was non-significant model term with *p*-value of 0.16 (Table 4.7). Similarly, for RL production, the interactive and quadratic effect i.e. AD, BD, A^2, B^2, C^2 and D^2 were found to be significant (Table 4.8).

Table 4.6: Experimental design and results of central composite design in terms of responses (growth and rhamnolipids production) for *P. aeruginosa* MF069166

No. of Runs	pH	T	SOI	RPM	Growth	RL (g/L)
1	6	20	5	120	2.17	2.82
2	7.5	30	3	210	1.84	1.58
3	7.5	30	3	150	2.65	3.13
4	7.5	30	3	150	2.66	3.14
5	6	20	5	120	2.16	2.85
6	9	40	1	120	0.59	0.12
7	10.5	30	5	150	0.09	0
8	9	40	1	180	0.55	0.15
9	7.5	30	3	150	2.65	3.13
10	9	40	1	180	0.61	0.21
11	7.5	30	3	150	2.65	3.12
12	9	40	1	180	0.56	0.16
13	6	20	5	180	2.15	2.85
14	4.5	30	3	150	1.52	1.01
15	7.5	30	3	150	2.64	3.13
16	9	40	1	120	0.48	0.19
17	9	40	1	180	0.44	0.18
18	7.5	10	3	100	0.86	0
19	7.5	30	7	150	1.32	1.43
20	7.5	30	2	150	2.62	3.15
21	7.5	50	3	150	0.23	0.1
22	6	20	1	180	2.76	2.12
23	6	20	1	180	2.75	2.85
24	6	20	5	180	2.24	2.87
25	9	40	1	120	0.54	0.22
26	7.5	30	3	150	2.65	3.15
27	6	20	1	150	2.16	2.85
28	7.5	30	3	150	2.66	3.15
29	9	40	5	100	0.52	0.23
30	6	20	1	120	2.15	2.85

Table 4.7: ANOVA of selected model terms for growth of *P. aeruginosa* MF069166

Source	DF	Sum of Squares	Mean Square	F	*p*
Model	12	27.02	2.25	189.88	< 0.0001
A-pH	1	1.44	1.44	121.15	< 0.0001
B-T	1	0.45	0.45	37.99	< 0.0001
C-SOI	1	0.064	0.064	5.38	0.0330
D-RPM	1	0.088	0.088	7.38	0.0146
AB	1	0.026	0.026	2.15	0.1606
AD	1	0.085	0.085	7.17	0.0159
BD	1	0.26	0.26	21.59	0.0002
CD	1	0.31	0.31	25.74	< 0.0001
A^2	1	4.36	4.36	367.71	< 0.0001
B^2	1	0.62	0.62	52.57	< 0.0001
C^2	1	0.82	0.82	69.28	< 0.0001
D^2	1	0.47	0.47	39.23	< 0.0001
Residual	17	0.20	0.012		
Pure Error	14	0.026	1.850E-003		
Total	29	27.22			

R-Sq = 99.26 %	R-Sq (adj) = 98.74 %

Table 4.8: ANOVA of selected model terms for rhamnolipids production from *P. aeruginosa* MF069166

Source	DF	Sum of Squares	Mean Square	F	*p*
Model	12	52.70	4.39	157.00	< 0.0001
A-pH	1	5.02	5.02	179.46	< 0.0001
B-T	1	1.92	1.92	68.51	< 0.0001
C-SOI	1	0.21	0.21	7.51	0.0139
D-RPM	1	1.22	1.22	43.45	< 0.0001
AB	1	0.018	0.018	0.65	0.4327
AD	1	1.27	1.27	45.48	< 0.0001
BD	1	1.47	1.47	52.45	< 0.0001
CD	1	2.153E-003	2.153E-003	0.077	0.7848
A^2	1	9.76	9.76	348.80	< 0.0001
B^2	1	0.50	0.50	17.87	0.0006
C^2	1	2.50	2.50	89.22	< 0.0001
D^2	1	1.53	1.53	54.84	< 0.0001
Residual	17	0.48	0.028		
Pure Error	14	0.28	0.020		
Total	29	53.18			

R-Sq = 99.11 %	R-Sq (adj) = 98.47 %

4.9.1. Effect of bioprocess variables on growth of *P. aeruginosa* MF069166

The effect of bioprocess variables on growth of *P. aeruginosa* MF069166 was estimated with the help of regression equation. A second order polynomial equation was generated by the software (Equation 4.3). It was observed that temperature had positive effect on growth of the bacterial strain whereas, negative effect of pH, size of inoculum and RPM was noted when their value was fluctuated from an optimum central point. To further consolidate these, results 3D response surface and contour plots were made in order to demonstrate the role of significant parameters on bacterial growth.

4.9.1.1. Regression equation for growth of *P. aeruginosa* MF069166

Growth = + 2.12 + 1.34 A - 0.77 B - 0.084 C - 0.45 D - 0.25 AB + 0.43 AD - 0.52 BD - 0.16 CD - 1.15 A^2 - 0.44 B^2 - 0.29 C^2 - 0.18 D^2..... *Equation 4.3.*

4.9.1.2. Effect of pH

The effect of pH on growth of *P. aeruginosa* MF069166 was noted. Our results indicated that at pH 4.5, the optical density of the culture medium was 1.01 which significantly increased up to a maximum value of 2.65 at pH 7.5. This increase in culture turbidity was due to high growth of bacterial strain at neutral pH. Afterwards, a significant decrease in culture turbidity was witnessed at pH 9 whereas, at pH 10.5, no growth was observed (Table 4.6 and 4.7). These results showed that *P. aeruginosa* MF069166 can show optimum growth at a pH range of 4.5-7.5 and slight fluctuation in values negatively affected the bacterial growth (Fig. 4.14).

4.9.1.3. Effect of temperature

The effect of temperature on bacterial growth was demonstrated through statistical analysis. Results indicated that significant growth of the bacterial strain was observed in the temperature range of 20-40 °C. Maximum optical density of 2.65 was recorded when culture medium was incubated at 30 °C for 96 h (Table 4.6 and 4.7). However, comparatively less bacterial growth was observed at the two extremes of 10 and 50 °C. These findings confirmed that *P. aeruginosa* MF069166 was mesophilic and can grow best at moderate temperatures (Fig. 4.14).

4.9.1.4. Effect of inoculum size

It was observed that an increase in inoculum size significantly affected the bacterial growth. At 1 % concentration of inoculum, average optical density of 0.5 was recorded which reached up to a maximum value of 2.65 at 3 % concentration of inoculum (Table 4.6 and 4.7). Further increase in inoculum size resulted in comparatively less bacterial

growth. 3-D response surface and contour plots further confirmed that the optimal value of SOI was around 2-3 % (Fig. 4.14).

4.9.1.5. Effect of agitation speed

The effect of agitation speed on growth of *P. aeruginosa* MF069166 was monitored at different RPM provided to the experimental runs. A linear increase in bacterial growth was observed with an increase in agitation speed upto 180 RPM whereas, maximum bacterial growth was recorded at 150 RPM (Table 4.6 and 4.7). It was further noted that very low and high RPM negatively affected the bacterial growth (Fig. 4.14).

4.9.1.6. Interactive or quadratic effects

The quadratic and interactive effect of bioprocess variables was evaluated through regression equation (Equation 4.3). It was observed that the interactive effect of pH and RPM and, temperature and RPM was significant with p-value less than 0.05 (Table 4.6 and 4.7). Therefore these factors must be controlled efficiently in the bioprocess for optimal growth of *P. aeruginosa* MF069166 (Fig. 4.14).

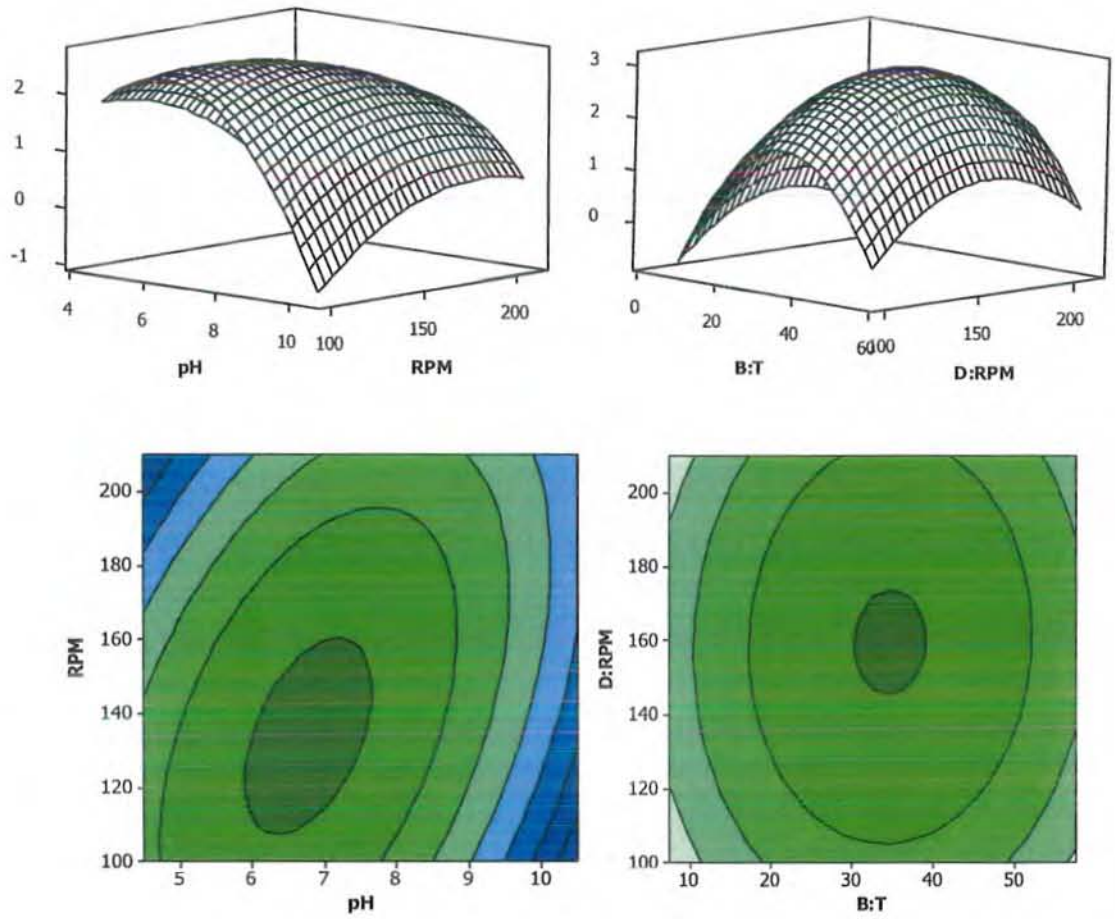


Figure 4.14: 3-D response surface and contour plots for the effect of different bioprocess variables on growth of *P. aeruginosa* MF069166

4.9.2. Effect of bioprocess variables on rhamnolipids production from *P. aeruginosa* MF069166

The effect of bioprocess variables on rhamnolipids production from *P. aeruginosa* MF069166 was estimated with the help of regression equation. A second order polynomial equation was generated by the software (Equation 4.4). It was observed that pH and size of inoculum positively affected rhamnolipids production from *P. aeruginosa* MF069166 however, negative effect of temperature and RPM was noted when their values were fluctuated from an optimum central point. To further consolidate these results 3-D response

surface and contour plots were made that depicted the role of selected parameters on rhamnolipids production from the bacterial strain (Fig. 4.15).

4.9.2.1. Regression equation for rhamnolipids production from *P. aeruginosa* MF069166

RL (g/L) = + 1.97 + 2.51 A - 1.60 B + 0.15 C - 1.66 D + 0.21 AB + 1.67AD - 1.26 BD - 0.013 CD - 1.71 A^2 - 0.40 B^2 - 0.50 C^2 - 0.33 D^2....*Equation 4.4.*

4.9.2.2. Effect of pH

The effect of pH on rhamnolipids production was studied trough RSM. After 96 h, more than 3 g/L yield of rhamnolipids was detected at pH 7.5 whereas, no RL production was witnessed at pH 9 and 10.5. Significant production of rhamnolipids was also observed at pH 6 (Table 4.6 and 4.8). These results showed that the optimum pH range for maximum rhamnolipids production was neutral (Fig. 4.15).

4.9.2.3. Effect of temperature

The effect of temperature on rhamnolipids production from *P. aeruginosa* MF069166 was demonstrated by constructing response surface and contour plots (Fig. 4.15). A significant increase in rhamnolipids production was observed at temperature from 20-40 °C. However, no RL production was witnessed at 10 and 50°C which shows the negative effect of low and high temperatures on biosynthesis of RL molecules. Maximum RL production of 3.15 g/L was recorded when culture medium was incubated at 30 °C for 96 h (Table 4.6 and 4.8).

4.9.2.4. Effect of inoculum size

Increase in inoculum size positively affected rhamnolipids production from *P. aeruginosa* MF069166. Minimum rhamnolipids production was detected in the culture medium administered with 1 % concentration of inoculum (Table 4.6 and 4.8). The experimental

runs that were provided with 3 % SOI showed more than 3 g/L yield of rhamnolipids whereas, further increase of 5 and 7 % SOI negatively affected the bioprocess (Fig. 4.15).

4.9.2.5. Effect of agitation speed

The effect of agitation speed on rhamnolipids production was monitored through RSM. Maximum yield of 3.15 g/L of rhamnolipids was detected at 150 RPM whereas, comparatively less RL production was observed at low and high RPMs (Table 4.6 and 4.8). Response surface and contour plots showed that the optimum agitation speed for maximum production of rhamnolipids was around 150 RPM (Fig. 4.15).

4.9.2.6. Interactive or quadratic effects

The interactive and quadratic effect of bioprocess variables was determined by regression equation (Equation 4.4). The interactive effect of pH and RPM and, temperature and RPM was found to be significant with *p*-values less than 0.05 (Table 4.6 and 4.8). Therefore, these parameters must be monitored efficiently in order to obtain maximum yield of rhamnolipids from *P. aeruginosa* MF069166. Moreover, the quadratic effect of bioprocess variables was also found to be significant and affected rhamnolipids production in fermentation medium (Fig. 4.15).

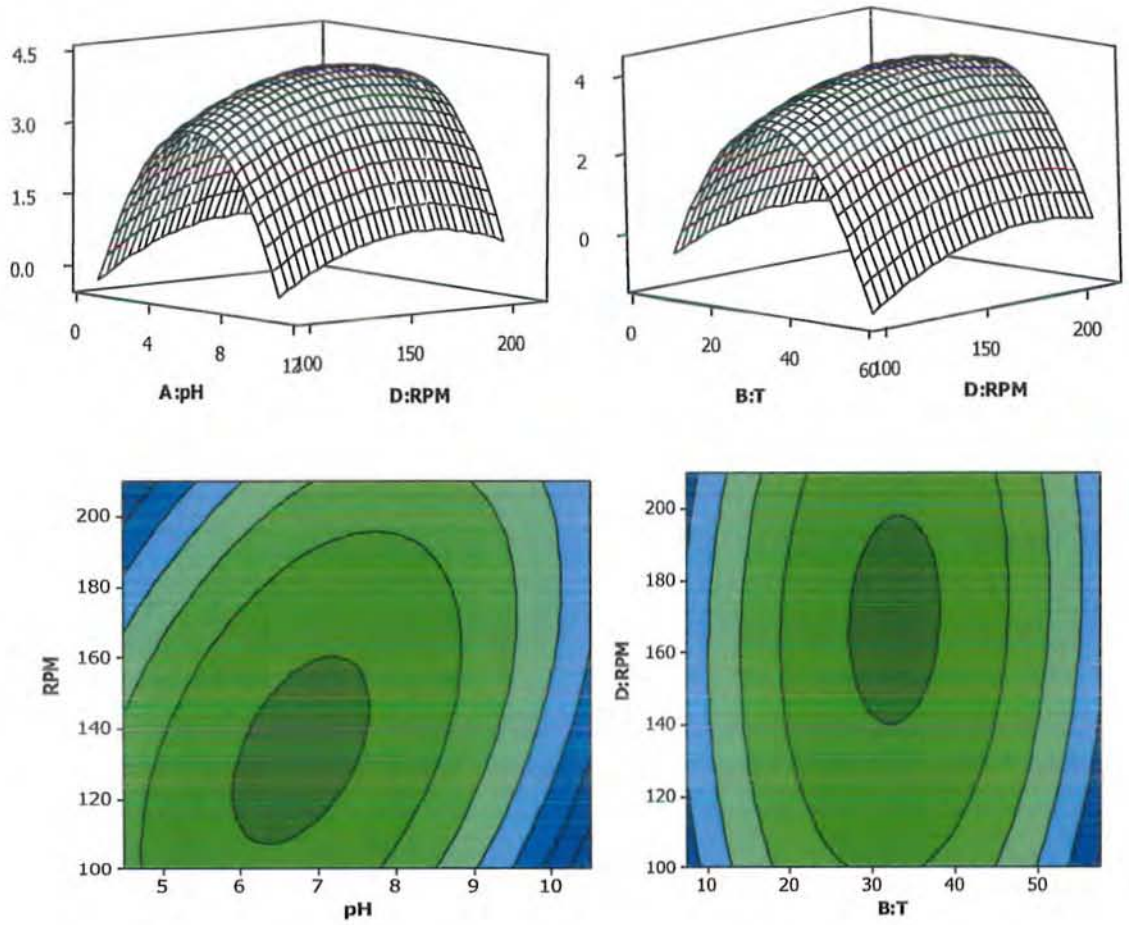


Figure 4.15: 3-D response surface and contour plots for the effect of different bioprocess variables on rhamnolipids production from *P. aeruginosa* MF069166

4.9.2.7. Numerical optimization of the bioprocess

After conducting the experiments according to Response Surface Methodology, the optimum values of each factor was also determined through numerical optimization. The criteria for finding the optimal conditions was set for microbial growth and rhamnolipids production. The software predicted a pH value of 6.5, T of 35 °C, SOI of 3.4, and RPM of 120 as the most suitable conditions for growth and rhamnolipids production from *P. aeruginosa* MF069166. In order to consolidate the aforesaid predicted conditions, a confirmatory experiment was run in triplicates where a yield of 3.97 g/L of rhamnolipids

should be produced. It was noted that under these conditions, 4.31 g/L of rhamnolipids was produced that was fairly higher than the prediction of software. This suggested that the values of the parameters were reasonably selected and had a significant effect on growth and rhamnolipids production from *P. aeruginosa* MF069166. Moreover, our results suggested that the application of mathematical modelling techniques were proved to be very efficient to enhance the productivity of a particular microbial metabolite in the fermentation process.

4.10. Optimization of media components through Plackett-Burman design for growth and sophorolipids production from *Meyerozyma* spp. MF138126

The medium composition for growth and sophorolipids production from *Meyerozyma* spp. MF138126 was evaluated by the application of Plackett-Burman design with 8 variables (Table 4.9). After performing experiments in triplicates, results were analyzed using Minitab 16.0 software. Statistical analysis of results indicated that media composition and concentrations of the variables had significant effect on growth and sophorolipids production.

In case of growth, the experimental design was statistically significant with a *p*-value of 0.001 indicating that the design explains significant variability in the factors. Peptone, glycerol, NaH_2PO_4, NaCl, and $MgSO_4$ were significant model terms with *p*-values of 0.001, 0.001, 0.040, 0.035 and 0.045, respectively. Whereas, glucose, K_2HPO_4 and yeast extract were statistically non-significant model terms as their obtained *p*-values were greater than 0.05 (Table 4.10). The analysis indicated that all the significant factors had positive effect on growth at high concentrations (Fig. 4.16). The R^2 value obtained for growth was 0.96 which depicts that the experiments were performed with reasonable accuracy and lies in close conformity with the predicted values. This confirms that 96 % variability in the growth of *Meyerozyma* spp. MF138126 can be explained by the model (Table 4.10). On the basis of *p*-values and regression analysis, the optimum medium composition for maximum growth of *Meyerozyma* spp. MF138126 was found to be;

peptone (20 g/L), glycerol (5 %), NaH_2PO_4 (1.4 g/L), NaCl (0.5 g/L) and $MgSO_4$ (0.2 g/L) (Equation 4.5).

In case of sophorolipids production, peptone, glycerol, NaH_2PO_4, yeast extract and $MgSO_4$ were significant model terms with the *p*-values of 0.001, 0.003, 0.049, 0.011 and 0.036, respectively. The remaining nutritional parameters i.e. glucose, K_2HPO_4 and NaCl were statistically non-significant as their *p*-values were greater than 0.05. Furthermore, 0.94 R^2 depicted reliability and adequate precision of the experimental setup and showed that 94 % variability in the production of sophorolipids from *Meyerozyma* spp. MF138126 can be precisely elucidated by the model (Table 4.11). These observations also suggested that the predicted and experimental values were closely related. *p*-values and regression analysis showed that peptone, glycerol, and yeast extract enhanced sophorolipids production from *Meyerozyma* spp. MF138126 when added in high concentrations whereas, NaH_2PO_4 and $MgSO_4$ had a positive effect on SLs yield when added in low concentrations (Fig. 4.17). The maximum yield of 4.01 g/L of sophorolipids was obtained in the presence of 5 % glycerol, 20 g/L of peptone, 0.5 g/L of yeast extract, 0.7 g/L of NaH_2PO_4 and 0.1 g/L of $MgSO_4$ (Equation 4.6).

Taking together, application of the Plackett–Burman design provided significant media components along with their relevant concentrations where maximum growth and sophorolipids production was achieved. Furthermore, Plackett–Burman design also pointed out the path of steepest ascent and center point for maximum growth and improved yield of sophorolipids from *Meyerozyma* spp. MF138126.

Table 4.9: Experimental design and results of Plackett-Burman design in terms of responses (growth and sophorolipids production) for *Meyerozyma* spp. MF138126

Run Order	X1	X2	X3	X4	X5	X6	X7	X8	Growth	SL (g/L)
1	1	1	-1	1	1	-1	-1	1	2.48	3.95
2	-1	1	-1	-1	-1	1	-1	1	1.96	2.88
3	-1	1	1	1	-1	1	-1	-1	1.92	2.96
4	1	1	-1	-1	1	1	1	-1	2.47	3.95
5	1	-1	-1	1	1	1	-1	-1	1.88	3.58
6	-1	-1	1	1	1	-1	-1	1	1.35	2.65
7	1	-1	1	-1	-1	1	-1	1	1.88	3.55
8	-1	1	-1	1	1	-1	1	-1	1.97	2.87
9	-1	-1	1	-1	1	1	1	-1	1.27	2.65
10	1	1	1	-1	1	-1	-1	-1	1.90	4.01
11	-1	1	1	-1	-1	-1	1	1	1.85	2.87
12	1	-1	-1	-1	1	-1	1	1	2.02	3.57
13	-1	-1	-1	-1	-1	-1	-1	-1	1.23	2.55
14	-1	-1	-1	1	-1	1	1	1	1.53	1.55
15	1	-1	1	1	-1	-1	1	-1	1.89	2.55
16	1	1	1	1	1	1	1	1	2.76	4.00

Table 4.10: ANOVA of selected model terms for growth of *Meyerozyma* spp. MF138126

Source	DF	Seq SS	Adj SS	Adj MS	F	*p*
Main Effects	8	2.61108	2.61108	0.32638	22.48	0.001
A:Peptone	1	1.10250	0.96469	0.96469	66.45	0.001
B:Glycerol	1	1.13422	1.12525	1.12525	77.51	0.001
C:Glucose	1	0.03240	0.03417	0.03417	2.35	0.169
D:NaH_2PO_4	1	0.09000	0.09219	0.09219	6.35	0.040
E:Yeast Extract	1	0.00718	0.00220	0.00220	0.15	0.709
F:K_2HPO_4	1	0.05544	0.05559	0.05559	3.83	0.091
G:$MgSO_4$	1	0.09013	0.08628	0.08628	5.94	0.045
H:NaCl	1	0.09920	0.09920	0.09920	6.83	0.035
Residual Error	7	0.10162	0.10162	0.01452		
Total	15	2.71270				

R-Sq = 96.25%	R-Sq (adj) = 91.97%

Table 4.11: ANOVA of selected model terms for sophorolipids production from *Meyerozyma* spp. MF138126

Source	DF	Seq SS	Adj SS	Adj MS	F	*p*
Main Effects	8	6.90845	6.90845	0.86356	14.01	0.001
A:Peptone	1	4.20250	2.38472	2.38472	38.68	0.001
B:Glycerol	1	1.47623	1.16942	1.16942	18.97	0.003
C:Glucose	1	0.00810	0.04419	0.04419	0.72	0.425
D:NaH_2PO_4	1	0.22563	0.34960	0.34960	5.67	0.049
E:Yeast Extract	1	0.55913	0.71908	0.71908	11.66	0.011
F:K_2HPO_4	1	0.01851	0.02275	0.02275	0.37	0.563
G:$MgSO_4$	1	0.40809	0.41057	0.41057	6.66	0.036
H:NaCl	1	0.01027	0.01027	0.01027	0.17	0.695
Residual Error	7	0.43155	0.43155	0.06165		
Total	15	7.34000				

R-Sq = 94.12%	R-Sq (adj) = 87.40%

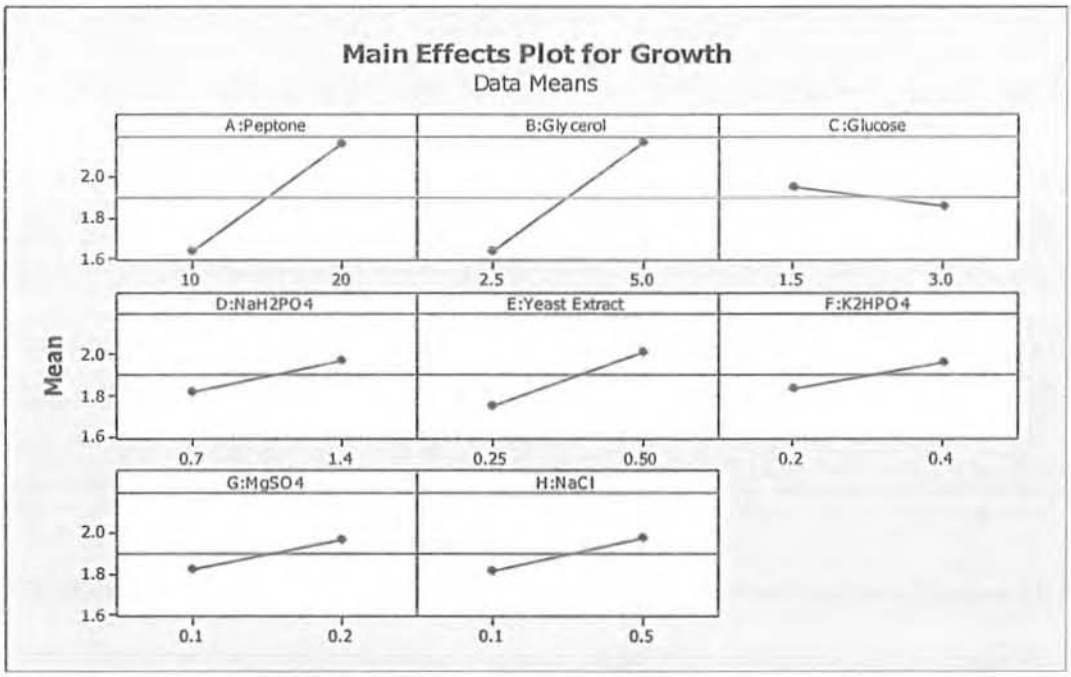


Figure 4.16: Main effect plots for growth of *Meyerozyma* spp. MF138126

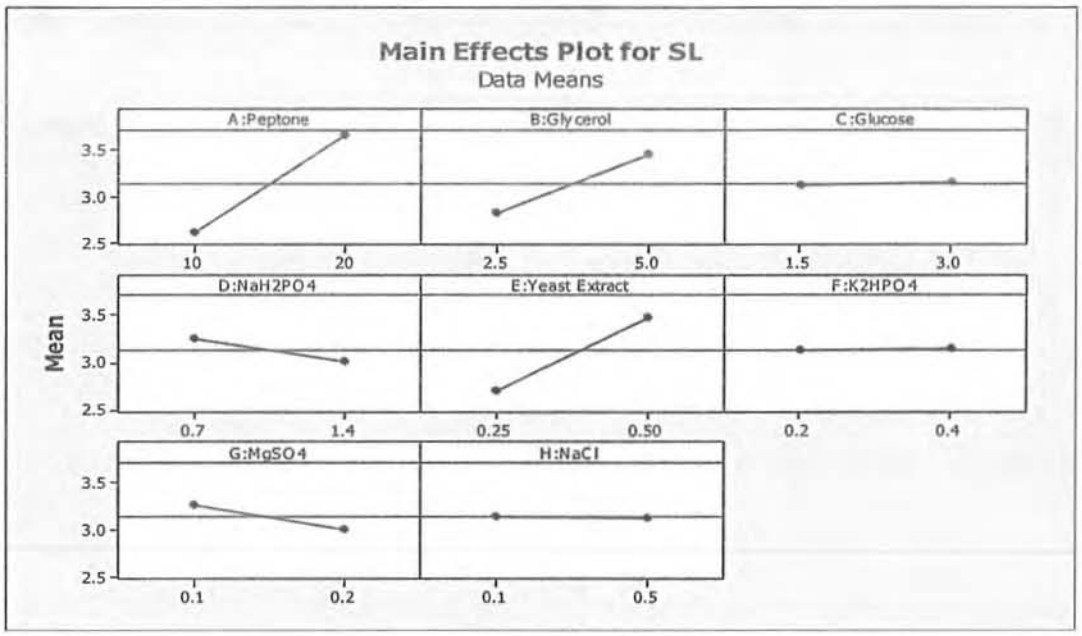


Figure 4.17: Main effect plots for sophorolipids production from *Meyerozyma* spp. MF138126

4.10.1.1. Regression equation for growth of *Meyerozyma* spp. MF138126

Growth = - 0.277 + 0.0535 A: Peptone + 0.214 B: Glycerol - 0.0623 C: Glucose + 0.219 D: NaH_2PO_4 - 0.108 E: Yeast Extract + 0.596 F: K_2HPO_4 + 1.48 G: $MgSO_4$ + 0.398 H: NaCl...*Equation 4.5.*

4.10.1.2. Regression equation for sophorolipids production from *Meyerozyma* spp. MF138126

SL = 0.910 + 0.0841 A: Peptone + 0.219 B: Glycerol + 0.0708 C: Glucose - 0.427 D: NaH_2PO_4 + 1.96 E: Yeast Extract + 0.381 F: K_2HPO_4 - 3.24 G: $MgSO_4$ + 0.128 H: NaCl... *Equation 4.6.*

4.11. Optimization of cultivation conditions through Response Surface Methodology (RSM) for growth and sophorolipids production from *Meyerozyma* spp. MF138126

The effect of different reaction conditions for growth and sophorolipids production from *Meyerozyma* spp. MF138126 was evaluated by using Response Surface Methodology (RSM) and Central Composite Design (CCD) with four bioprocess variables i.e. pH, Temperature (T), size of inoculum (SOI) and agitation speed (RPM). The experiments were performed in line with the prescribed design and results/responses were evaluated after 96 h of incubation period (Table 4.12). Results indicated that the model for cellular growth and production of sophorolipids was fitted reasonably with a confidence level of 99.9 %. The significance of the bioprocess variables was determined after statistical analyses with reference to obtained *p*-values. The results revealed that for growth and sophorolipids production from *Meyerozyma* spp. MF138126; pH, T, SOI and RPM were significant model terms with the *p*-values less than 0.05 (Table 4.13 and 4.14).

Analysis of variance test (ANOVA) was performed in order to estimate the role of each selected model term. Our results indicated that overall the model was statistically significant with a *p*-value of 0.001. The adequacy of the bioprocess was estimated with

reference to the coefficient of regression R^2. The R^2 values for cellular growth and sophorolipids production were found to be 0.992 and 0.994, respectively, which suggested that the predicted and experimental responses were in close conformity with each other. Similarly, it further shows that 99.2 % and 99.4 % variability in the selected responses can be explained by the given model (Table 4.13 and 4.14). Response surface graphs demonstrated the co-relation between the actual and predicted values for growth and sophorolipids production (Fig. 4.18). Besides individual model terms, interactive effect of the bioprocess variables was also significant. For growth of *Meyerozyma* spp. MF138126 AB, A^2, B^2, C^2 and D^2 were the significant model terms with *p*-values less than 0.0001. However, the factors BD and CD were non-significant with *p*-values of 0.325 and 0.101, respectively (Table 4.13). Similarly, for SL production, the interactive and quadratic effect i.e. AB, CD, A^2, B^2, C^2 and D^2 was found to be significant whereas, the interaction of BD was found to be non-significant (Table 4.14).

Table 4.12: Experimental design and results of central composite design in terms of responses (growth and sophorolipids production) for *Meyerozyma* spp. MF138126

Run	A:pH	B:T	C:SOI	D:RPM	Growth	SL (g/L)
1	8	45	1	180	1.23	0.51
2	5.5	32.5	3	150	3.05	4.15
3	3.5	20	1	180	1.26	1.01
4	5.5	32	3	150	3.02	4.17
5	3.5	57.5	1	120	0.25	0.09
6	5.5	7.5	3	120	0.12	0.15
7	5.5	32.5	7	150	2.02	2.28
8	8	45	2	120	1.58	0.51
9	5.5	32.5	3	150	3.06	4.14
10	8	45	1	120	0.98	0.25
11	8	45	5	180	1.63	1.12
12	5.5	32.5	3	150	3.04	4.16
13	8	45	5	210	0.96	1.02
14	5.5	32.5	3	150	3.06	4.15
15	5.5	32.5	3	150	3.07	4.17
16	3.5	20	5	180	1.22	1.52
17	8	45	1	180	1.21	0.27
18	3.5	20	5	120	0.95	0.96
19	5.5	32.5	3	150	3.05	4.15
20	3.5	20	5	120	0.95	0.97
21	8	45	5	120	0.92	0.2
22	3.5	20	5	180	1.22	0.91
23	10.5	32.5	1	180	0.05	0.02
24	0.5	32.5	1	150	0.17	0.05
25	8	45	1	180	1.21	0.21
26	8	45	1	120	0.95	0.22
27	5.5	32.5	3	150	3.01	4.15
28	5.5	32.5	3	150	3.05	4.17
29	3.5	20	1	100	0.16	0.23
30	5.5	32.5	3	150	3.16	4.18

Table 4.13: ANOVA of selected model terms for growth of *Meyerozyma* spp. MF138126

Source	DF	Sum of Squares	Mean Square	F	*p*
Model	11	34.97	3.18	216.12	< 0.0001
A:pH	1	0.079	0.079	5.37	0.0325
B:T	1	1.63	1.63	110.54	< 0.0001
C:SOI	1	0.087	0.087	5.93	0.0255
D:RPM	1	0.45	0.45	30.61	< 0.0001
AB	1	0.33	0.33	22.61	0.0002
BD	1	0.015	0.015	1.02	0.3251
CD	1	0.044	0.044	2.99	0.1010
A^2	1	6.72	6.72	456.80	< 0.0001
B^2	1	5.13	5.13	348.96	< 0.0001
C^2	1	1.24	1.24	84.40	< 0.0001
D^2	1	0.64	0.64	43.74	< 0.0001
Residual Error	18	0.26	0.015		
Pure Error	14	0.016	1.109E-003		
Total	29	35.24			

R-Sq = 99.25 %	R-Sq (adj) = 98.79 %

Table 4.14: ANOVA of selected model terms for sophorolipids production from *Meyerozyma* spp. MF138126

Source	DF	Sum of Squares	Mean Square	F	*p*
Model	11	89.32	8.12	279.91	< 0.0001
A:pH	1	0.69	0.69	23.65	0.0001
B:T	1	1.29	1.29	44.47	< 0.0001
C:SOI	1	0.46	0.46	16.01	0.0008
D:RPM	1	0.53	0.53	18.41	0.0004
AB	1	6.92	6.92	238.41	< 0.0001
BD	1	0.13	0.13	4.59	0.0562
CD	1	0.16	0.16	5.61	0.0292
A^2	1	12.27	12.27	423.13	< 0.0001
B^2	1	16.44	16.44	566.71	< 0.0001
C^2	1	3.89	3.89	134.03	< 0.0001
D^2	1	0.25	0.25	8.68	0.0086
Residual Error	18	0.52	0.029		
Pure Error	14	0.24	0.017		
Total	29	89.84			

R-Sq = 99.42 %	R-Sq (adj) = 99.06 %

4.11.1. Effect of bioprocess variables on growth of *Meyerozyma* spp. MF138126

The effect of bioprocess variables on growth of *Meyerozyma* spp. MF138126 was estimated with the help of regression equation. A second order polynomial equation was generated (Equation no 4.7). It was observed that pH, temperature, size of inoculum and RPM had positive effect on growth however, their negative effect was noted when their concentration fluctuated from an optimum central value. To further consolidate these results 3-D response surface and contour plots were constructed that showed the role of selected parameters on growth of *Meyerozyma* spp. MF138126.

4.11.1.1. Regression equation for the growth of *Meyerozyma* spp. MF138126

Growth = + 3.00 + 0.096 A+ 0.48 B + 0.084 C+ 0.16 D - 0.30 AB + 0.036 BD + 0.062 CD - 0.61 A^2 - 0.79 B^2 - 0.32 C^2 - 0.32 D^2....*Equation 4.7.*

4.11.1.2. Effect of pH

The effect of pH on growth of *Meyerozyma* spp. MF138126 was monitored through RSM. Our results showed that there was no microbial growth at the two pH extremes i.e. 0.5 and 10.5. However, when the pH was increased up to 3.5, a slight increase in medium turbidity was witnessed and culture density was recorded up to 1.26. Maximum growth was detected at pH 5.5 and optical density of the fermentation medium was more than 3.0. This was followed by a gradual decline in the O.D of growth medium at pH 8.0 (Table 4.12 and 4.13). Therefore, it can be concluded that the optimum pH range for maximum growth of *Meyerozyma* spp. MF138126 was from 4.5-5.5 (Fig. 4.18).

4.11.1.3. Effect of temperature

The effect of temperature on growth of *Meyerozyma* spp. MF138126 was demonstrated through statistical analysis. Results indicated that significant growth of the yeast species was observed in the temperature range of 20-45 °C. Maximum density of 3.16 was recorded when culture medium was incubated at 32.5 °C for 96 h. However, very low turbidity in culture medium was witnessed at 57.5 °C (Table 4.12 and 4.13). Similarly, the temperature 7.5 °C did not support the growth of yeast hence, very low culture density was recorded (Fig. 4.18).

4.11.1.4. Effect of inoculum size

Increase in inoculum size affected the microbial growth and an increase in density of culture medium was observed. 1 % concentration of inoculum resulted in the average 1.22 O.D, which attained a maximum value of 3.16 in the presence of 3 % SOI. However, further increase in inoculum size resulted in a gradual decrease in turbidity of culture medium and 5 % SOI resulted in comparatively less microbial growth (Table 4.12 and 4.13). These findings indicated that an adequate inoculum size is necessary for efficient growth of *Meyerozyma* spp. MF138126 (Fig. 4.18).

4.11.1.5. Effect of agitation speed

The effect of agitation speed on growth of *Meyerozyma* spp. MF138126 was monitored through RSM. Results suggested significant increase in the growth of yeast species with an increase in RPM. Maximum optical density of 3.16 was recorded at 150 RPM whereas minimum growth was witnessed at the two extremes of 100 and 210 RPM (Table 4.12 and 4.13). Significant microbial growth was also observed at 180 RPM (Fig. 4.18).

4.11.1.6. Interactive or quadratic effects

The quadratic and interactive effect of the bioprocess variables was evaluated through regression equation (Equation 4.7). It was observed that the combinatorial effect of pH and temperature was significant with the *p*-value less than 0.05 (Table 4.12 and 4.13). These findings suggested that these factors must be controlled efficiently in the bioprocess for maximum growth of *Meyerozyma* spp. MF138126. Moreover, the quadratic effects were also found to be significant and affected the microbial growth in fermentation medium (Fig. 4.18).

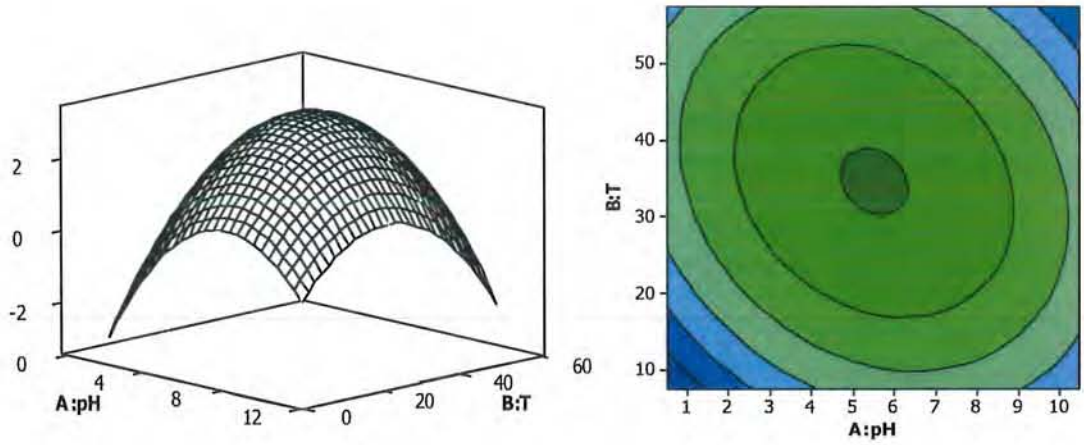


Figure 4.18: 3-D response surface and contour plots for the effect of different bioprocess variables on growth of *Meyerozyma* spp. MF138126

4.11.2. Effect of bioprocess variables on sophorolipids production from *Meyerozyma* spp. MF138126

The effect of bioprocess variables on sophorolipids production from *Meyerozyma* spp. MF138126 were estimated with the help of regression equation. A second order polynomial equation was generated by the software for the description of bioprocess variables (Equation no. 4.8). It was observed that pH, temperature, size of inoculum and RPM had positive effect on sophorolipids production from *Meyerozyma* spp. MF138126 however, negative effects were observed when parameters were fluctuated from an optimum central value. To further consolidate these results 3-D response surface and contour plots were made in order to demonstrate the role of selected parameters on sophorolipids production from the yeast species (Figure 4.19).

4.11.2.1. Regression equation for sophorolipids production from strain MF138126

$SL\ (g/L) = +4.12 + 0.28\ A + 0.43\ B + 0.19\ C + 0.18\ D - 1.35\ AB + 0.11\ BD + 0.12\ CD - 0.82\ A^2 - 1.42\ B^2 - 0.57\ C^2 - 0.20\ D^2$.... *Equation 4.8.*

4.11.2.2. Effect of pH

The effect of varying pH on sophorolipids production was studied trough RSM. Minimum SL production was witnessed at the two pH extremes (0.5 and 10.5) however, significant increase in the yield was observed at pH 3.5. Maximum yield of 4.28 g/L of sophorolipids was obtained at pH 5.5 and an increase in the concentration of SL molecules was detected with an increase in pH up to a certain point (Table 4.12 and 4.14). These results showed that the optimum pH for maximal yield of sophorolipids from the yeast species was around 5.5 (Fig. 4.19).

4.11.2.3. Effect of temperature

The effect of temperature on sophorolipids production from *Meyerozyma* spp. MF138126 was demonstrated through statistical tools. Results showed that significant production of

SLs was obtained at temperatures ranging between 20-32.5 °C. However, no SL production was witnessed at 7.5 °C and 57.5 °C that reflected the negative effect of extremely low and high temperatures on the sophorolipids biosynthesis pathway (Table 4.12 and 4.14). Maximum SL production of 4.28 g/L was recorded when culture medium was incubated at 32.5 °C for 96 h (Fig. 4.19).

4.11.2.4. Effect of inoculum size

Increase in inoculum size positively affected sophorolipids production from *Meyerozyma* spp. MF138126. Minimum production was detected in the culture flasks that were administered with 1 % concentration of inoculum whereas, an increase in inoculum size significantly enhanced SLs production in fermentation medium. For the experimental runs provided with 3 % SOI, more than 4 g/L production of sophorolipids was detected (Table 4.12 and 4.14). These results suggested that the optimum percentage of inoculum for maximum SLs production was around 3 % (Fig. 4.19).

4.11.2.5. Effect of agitation speed

The effect of agitation speed on sophorolipids production was monitored in the current study. Very low yield of sophorolipids was obtained at 100 RPM whereas, maximum production of 4.28 g/L was witnessed at 150 RPM. Significant amount of SLs was also detected in the experimental runs that were provided with an agitation speed of 180 RPM for 96 h. However, further increase in RPM caused reduction in the production of sophorolipids (Table 4.12 and 4.14). The optimum level of shaking speed for maximum production of sophorolipids could be observed at the central point of response surface and contour plots near 150 RPM (Fig. 4.19).

4.11.2.6. Interactive or quadratic effects

The interactive and quadratic effect of bioprocess variables was determined by regression equation (Equation 4.8). It was observed that the interactive effect of pH and temperature

and, size of inoculum and RPM was significant. Therefore, these parameters must be monitored efficiently in order to obtain maximum yield of sophorolipids from *Meyerozyma* spp. MF138126 (Table 4.12 and 4.14). Moreover, the quadratic effect was also found to be significant and affected the production of sophorolipids in fermentation medium (Fig. 4.19).

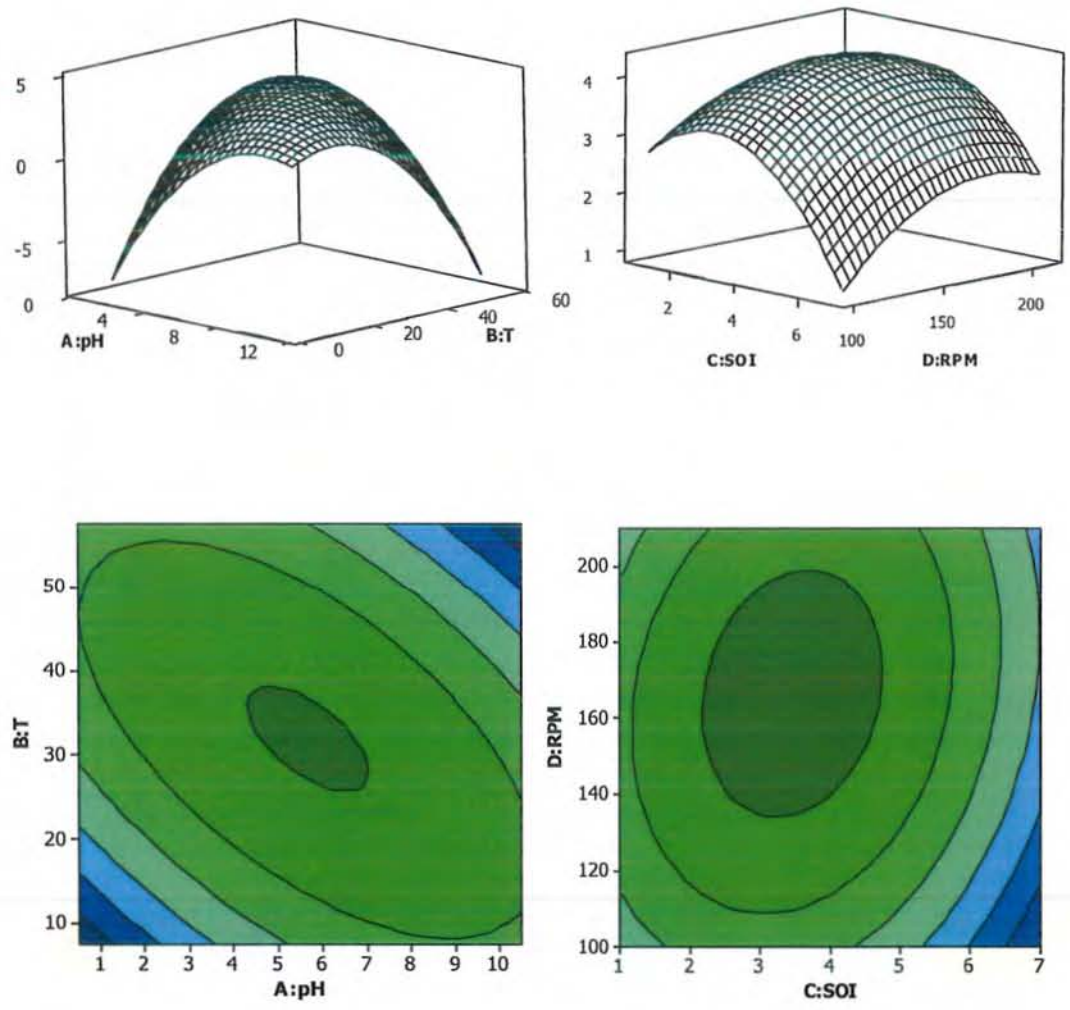


Figure 4.19: 3-D response surface plots for the effect of different bioprocess variables on sophorolipids production from *Meyerozyma* spp. MF138126

4.11.2.7. Numerical optimization of the bioprocess

After conducting the experiments according to Response Surface Methodology, the optimum values of each factor was also determined through numerical optimization. The criteria for finding the optimal conditions was set for microbial growth and sophorolipids production. The software predicted a pH value of 5.5, T of 33.2 °C, SOI of 3.3, and RPM of 161 as the most suitable conditions for growth and sophorolipids production from *Meyerozyma* spp. MF138126. In order to consolidate the aforesaid predicted conditions, a confirmatory experiment was run in triplicates where a yield of 5.5 g/L of sophorolipids should be produced. It was noted that under these conditions, 6.9 g/L of sophorolipids was produced that was fairly higher than the prediction of software. This suggested that the values of the parameters were reasonably selected and had a significant effect on growth and sophorolipids production by *Meyerozyma* spp. MF138126. Moreover, our results suggested that the application of mathematical modelling techniques were proved to be very efficient to enhance the productivity of a particular microbial metabolite in the fermentation process.

C- Downstream processing and characterization of biosurfactants

In the third phase of study, the biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were extracted and purified through solvent extraction method. The purified biosurfactants were chemically characterized through Thin Layer Chromatography (TLC), Fourier Transform Infrared Spectroscopy (FTIR Spectroscopy), Reverse Phase- High performance Liquid Chromatography (RP-HPLC), Nuclear Magnetic Resonance (NMR) and Liquid chromatography Electrospray Ionization-Mass Spectrometry (LC-ESI-MS). Stability of the biosurfactants was checked at varying pH, temperature and NaCl concentrations. Furthermore, the properties of biosurfactants were studied through *cmc* (critical micelle concentration) determination and DLS (Dynamic Light Scattering) analysis.

4.12. Characterization of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

4.12.1. Thin Layer chromatography (TLC)

The chemical characterization of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was done through TLC. Results showed that the partially purified rhamnolipids produced by the bacterial strain contained two major congeners as two bright spots were detected on TLC plate. The Rf values of the two spots were found to be 0.36 and 0.84. Similarly for sophorolipids produced by the yeast, five spots were detected on TLC plate that showed the presence of various structural isomers of SLs in the extract. The Rf values of the bioproduct ranged between 0.17 to 0.65 (Fig. 4.20 a and b).

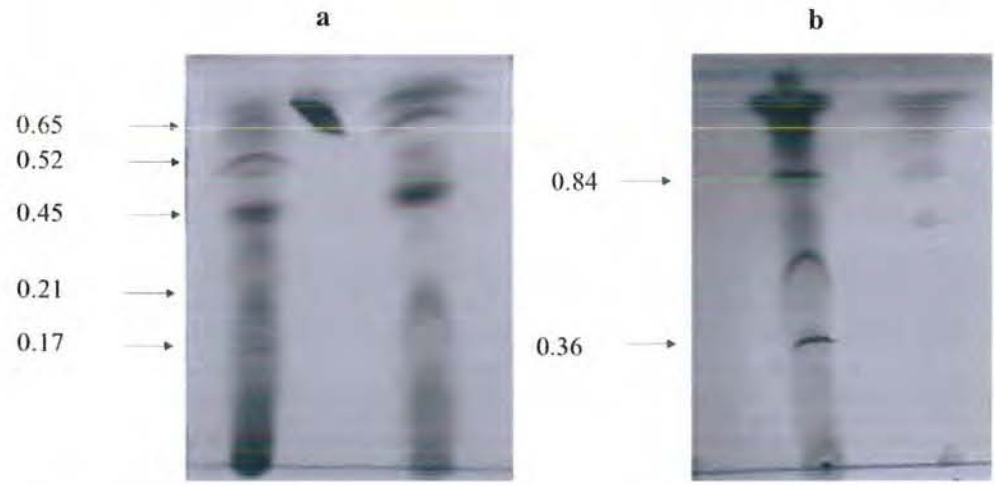


Figure 4.20: Thin Layer Chromatography of a) rhamnolipids from *P. aeruginosa* MF069166 b) sophorolipids from *Meyerozyma* spp. MF138126

4.12.2. FTIR Spectroscopy

FTIR analysis was done in order to confirm the chemical nature of biosurfactants. FTIR spectroscopy of rhamnolipids from *P. aeruginosa* MF069166 revealed correlation between absorbance bands and absorbance frequencies of the functional groups (Fig. 4.21 a and b). Both the test and standard RL molecules showed similar bands at 3200-3300, 2927 and 2853 cm^{-1} due to symmetric C-H stretching vibrations of aliphatic groups, particularly those present in the hydroxydecanoic acid chain tails of rhamnolipids. A carbonyl (C=O) stretching band at 1728 cm^{-1} is a characteristic of carboxylic acid groups and ester bonds. In the fingerprint region of the spectra, the area between 1239–1457 cm^{-1} represents C-H and O-H deformation vibrations, typical for carbohydrates present in the rhamnose moieties of the molecules. The lower range of the fingerprint region i.e. below 1200 cm^{-1} represents different kinds of C-H, C-O and CH_3 vibrations.

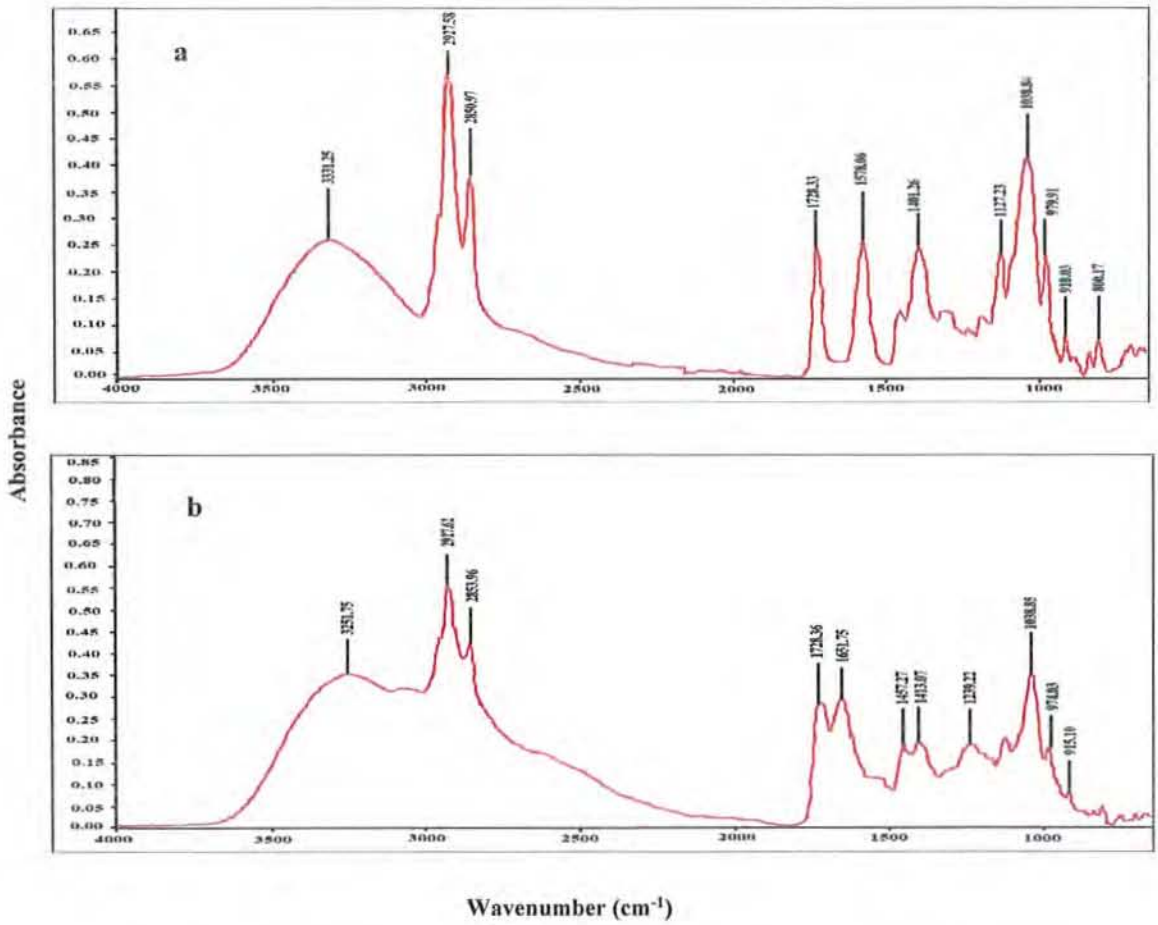


Figure 4.21: FTIR spectrum of a) standard rhamnolipids; b) rhamnolipids extracted from *P. aeruginosa* MF069166

Sophorolipids from *Meyerozyma* spp. MF138126 displayed the absorption bands quite similar to the standard SLs (Fig. 4.21 c and d). The broad peak observed at 3250-3450 cm^{-1} relates to the characteristic O-H stretch of SLs. The peaks in the range of 2800 cm^{-1} and 2900 cm^{-1} represents the symmetrical and asymmetrical stretching of methylene groups, respectively. The strong absorption bands from 1720-1740 cm^{-1} arise due to C=O stretching in the molecules and might contain contributions from lactones, acids or esters. The stretch at 1410 cm^{-1} is due to the characteristic C–O–H group of carboxylic acid (COOH) and indicates that a portion of extracted compound from the yeast strain is

comprised of acidic S.Ls. The absorption band at about 1200 cm^{-1} for both test and standard molecules corresponds to the stretch of C–O group from C (=O)–O–C present in lactones.

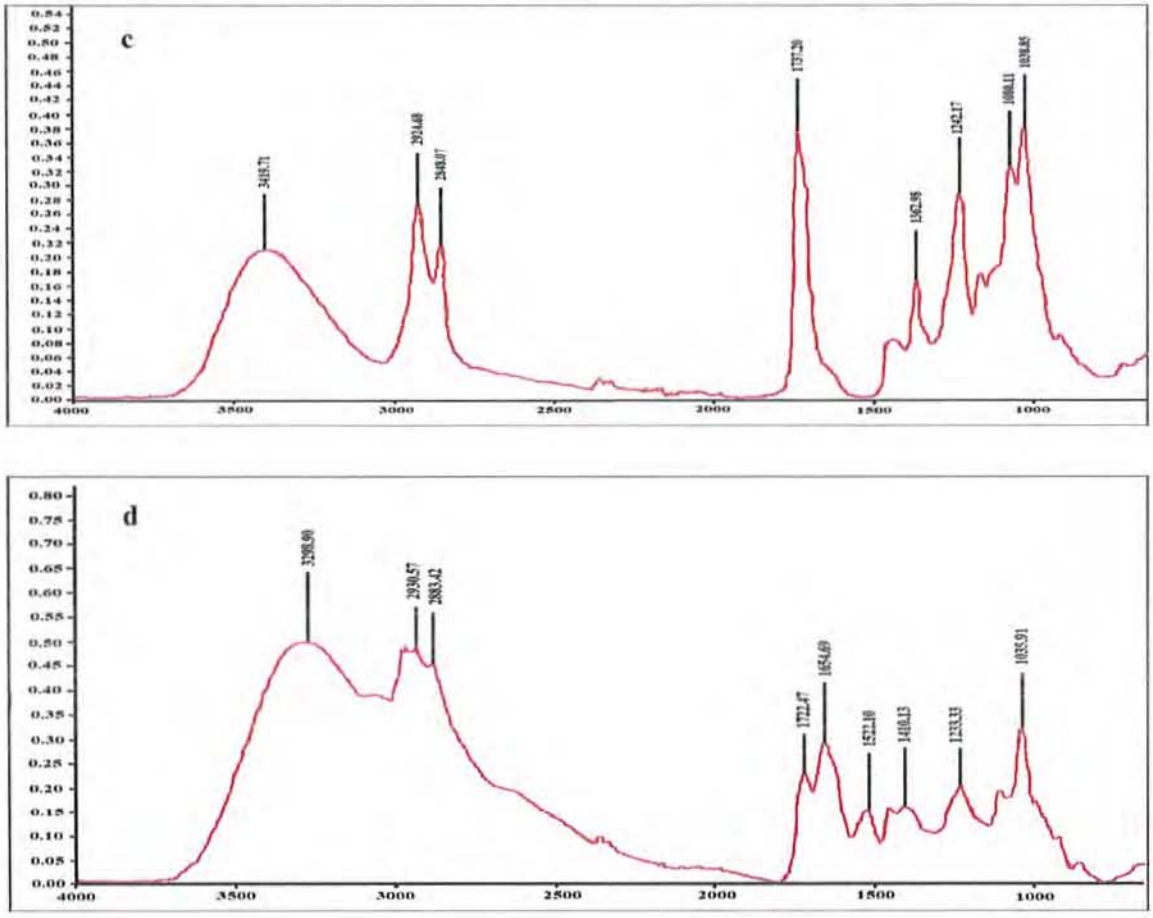


Figure 4.21: FTIR spectrum of c) standard sophorolipids; d) sophorolipids extracted from *Meyerozyma* spp. MF138126

4.12.3. Reverse Phase HPLC

The chemical structure of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were studied through reverse phase HPLC. Elution profiles of rhamnolipids and sophorolipids were compared with their respective standards (Fig. 4.22 a and b). HPLC chromatogram of rhamnolipids from bacterial strain displayed five

major fractions eluting at the retention times: 2.59, 2.85, 16.13, 18.45, 20.50, 22.59 and 24.30 min.

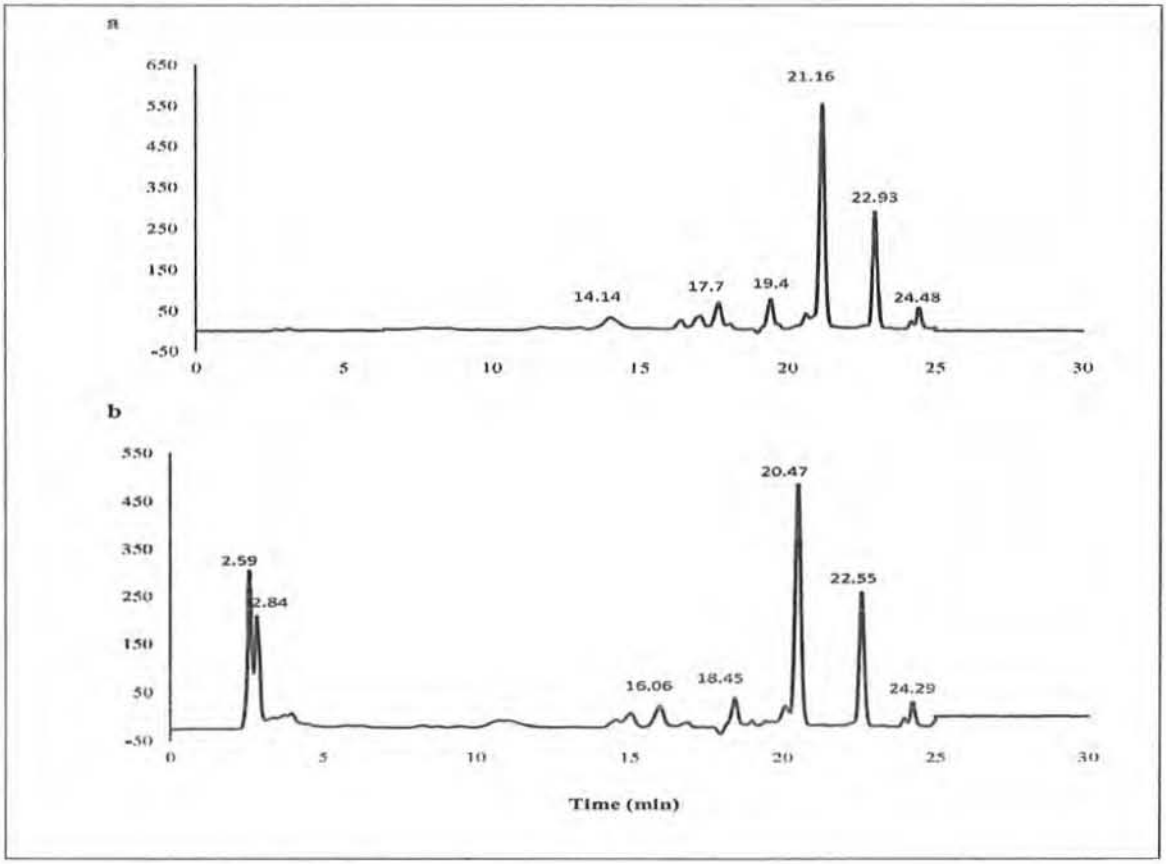


Fig. 4.22: Reverse Phase HPLC analysis of a) standard rhamnolipids b) rhamnolipids extracted from *P. aeruginosa* MF069166

For *Meyerozyma* spp. MF138126, chromatograms of standard and test SLs displayed comparable elution profile. The extracted sophorolipids of yeast species yielded seven fractions at 1.69, 1.98, 2.75, 6.18, 13.26, 15.48 and 19.16 min as illustrated in Figure Fig. 4.22 c and d. All the purified fractions were stored in separate vials and then used for detailed characterization of biosurfactants through further techniques.

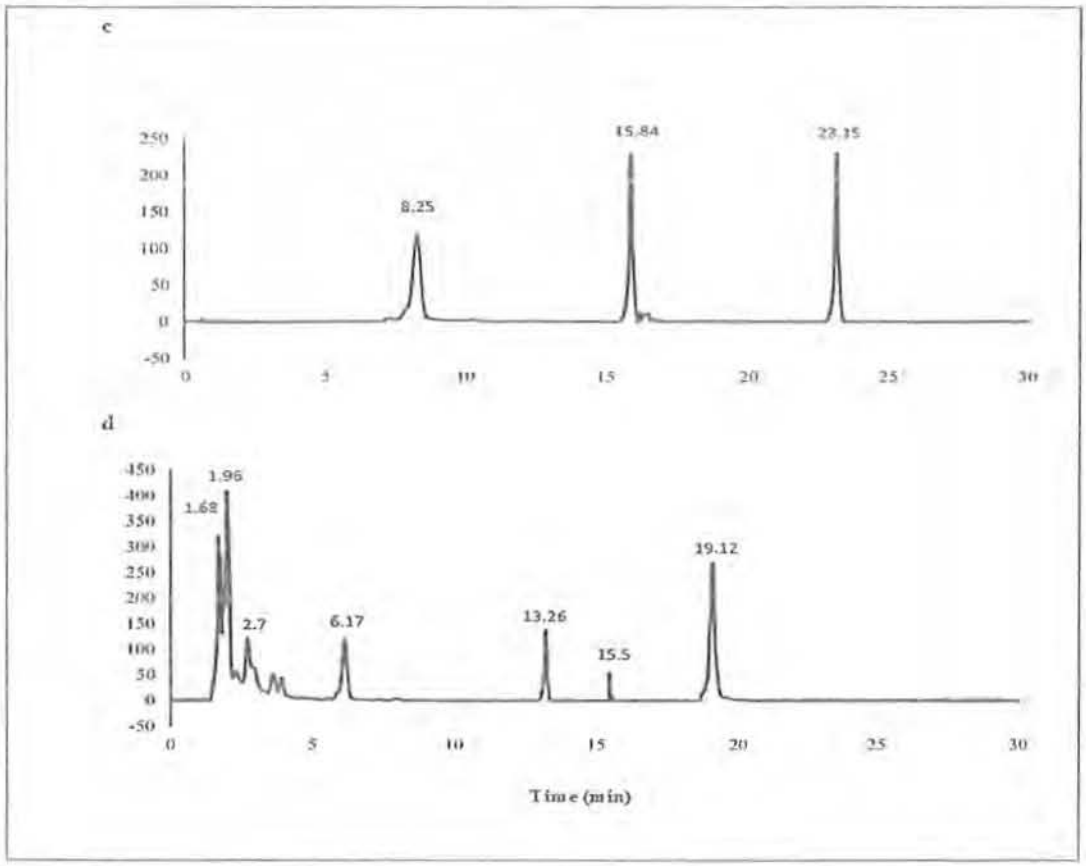


Fig. 4.22: Reverse Phase HPLC analysis of c) standard sophorolipids d) sophorolipids extracted from *Meyerozyma* spp. MF138126

4.12.4. Nuclear Magnetic Resonance (NMR)

4.12.4.1. Rhamnolipids

The chemical structure of rhamnolipids from *P. aeruginosa* MF069166 was further confirmed through ^{1}H and ^{13}CNMR. Peaks were identified through previous reports. The characteristics chemical shifts indicated the presence of both mono and di rhamnolipids (Table 4.15). In ^{1}HNMR spectrum, the chemical shifts detected at 0.88 ppm, 1.27 ppm, 2.55 ppm, 4.14 ppm, 4.90 ppm and 5.47 ppm displayed the resonated protons of rhamnose and fatty acid moieties (Figure 4.23 a). The ^{13}CNMR spectrum indicated that the chemical

shifts observed at 17.22, and 17.42 ppm were due to the methyl groups present on rhamnose moieties whereas, the methyl groups on fatty acid chains resonated at 13.57 ppm. Next to methyl groups, the methylene groups of fatty acid chains resonated within a range of 22.4-33.9 ppm. The signals from 68.84-71.01 ppm were due to the carbon atom attached to oxygen atom of rhamnose head group. Moreover, the peaks obtained at 95.30 ppm belonged to RL1 molecules whereas, those at 94.88 ppm and 102.72 ppm showed the presence of RL2 molecules (Figure 4.23 b).

Table 4.15: ^{1}H and ^{13}C NMR assignments for rhamnolipids extracted from *P. aeruginosa* MF069166

Assignment	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)
$-CH_3$ (on β-hydroxyfatty acids)	0.88	13.57
$-CH_3$ (on rhamnose moiety)	1.18, 1.19	17.22, 17.42
$-(CH_2)_5-$ (on β-hydroxyfatty acids)	1.27	22.4-33.9
$-(CH_2)-CH(O)-CH_2COO$ (on β-hydroxyfatty acids)	1.57	36.1-38.9
$-CH(O)-CH_2COO$ (on β-hydroxyfatty acids)	2.55	39.5
$-(CH_2)-CH(O\text{-}Rha)-CH_2COO$ (on β-hydroxyfatty acids)	3.4	68.84
$-(CH_2)-CH(-O-C=O)-CH_2COO$ (on β-hydroxyfatty acids)	4.14	71.15
–CH–OH (on rhamnose moiety)	4.9	94.88, 95.30, 102.72
–CH–O–C (on rhamnose moiety)	5.47	71.01

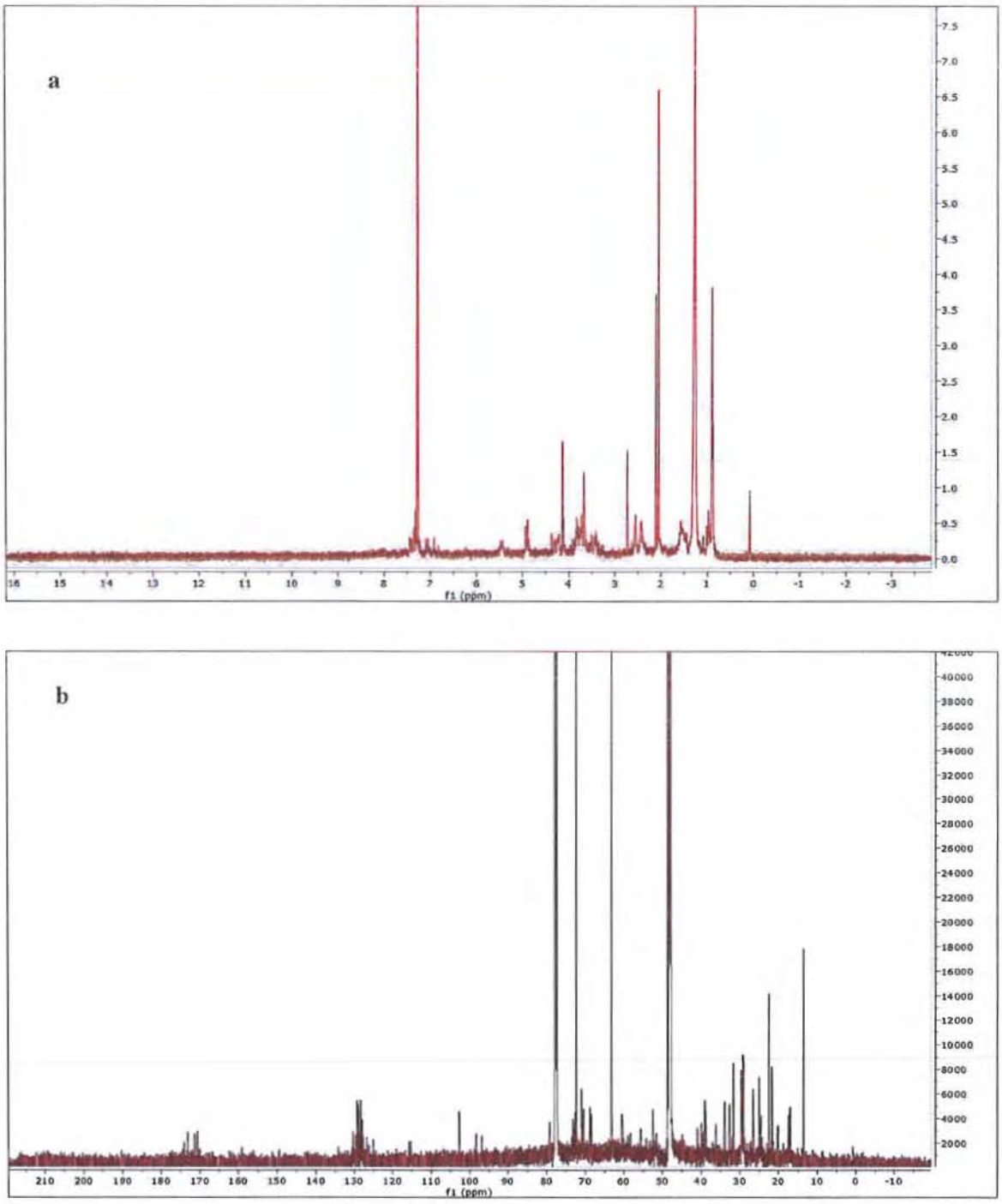


Figure 4.23: a) ^{1}HNMR spectrum and, b) ^{13}CNMR spectrum of rhamnolipids extracted from *P. aeruginosa* MF069166

4.12.4.2. Sophorolipids

The ^{1}H and ^{13}C NMR analysis of sophorolipids from *Meyerozyma* spp. MF138126 displayed characteristic chemical shifts of a typical glycolipid molecule (Table 4.16). Peaks were assigned on the basis of aforementioned data. In ^{1}HNMR spectrum, the protons H-1' and H-1" of sophorose moiety were resonated within the region of 4.14-4.29 ppm whereas, the other protons of sugar molecule were detected between 3.7-3.92 ppm. Protons of $-CH_3$ group of fatty acid moiety and the $-CH_2$ group attached to carboxylic group of fatty acid moiety resonated at 1.26 and 1.84 ppm, respectively. Moreover, multiple signals of protons were also obtained between 1.26 and 1.55 ppm (Fig. 4.23 c). The ^{13}CNMR spectrum revealed that the peaks at 21.73 ~ 38.81 ppm belonged to $-CH_2$ groups in fatty acid moiety whereas, the two =CH– groups in fatty acid chain were resonated at 129.18 and 130.31. The multiple peaks observed at 170.61 ~ 174.08 were the characteristics of carbonyl groups (–CO–) present. The –C–1' and –C–1" of sugar molecule were detected at 102.72 and 103.51 ppm whereas, the other carbon atoms of sugar resonated between 60.49-73.24 ppm (Fig. 4.23 d).

Table 4.16: ^{1}H and ^{13}C NMR assignments for sophorolipids extracted from *Meyerozyma* spp. MF138126

Assignment	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)
C_{18}-O-H of Hydroxyoleic acid	1.26-1.55	28.99-31.61
$-COCH_3$	2.063	26.52
D-Glu 1', 1"	4.25, 4.29	102.72, 104.36
D-Glu 6', 6"	4.39, 3.88	63.16, 60.49
–CO– of fatty acid	4.14	170.61-174.08

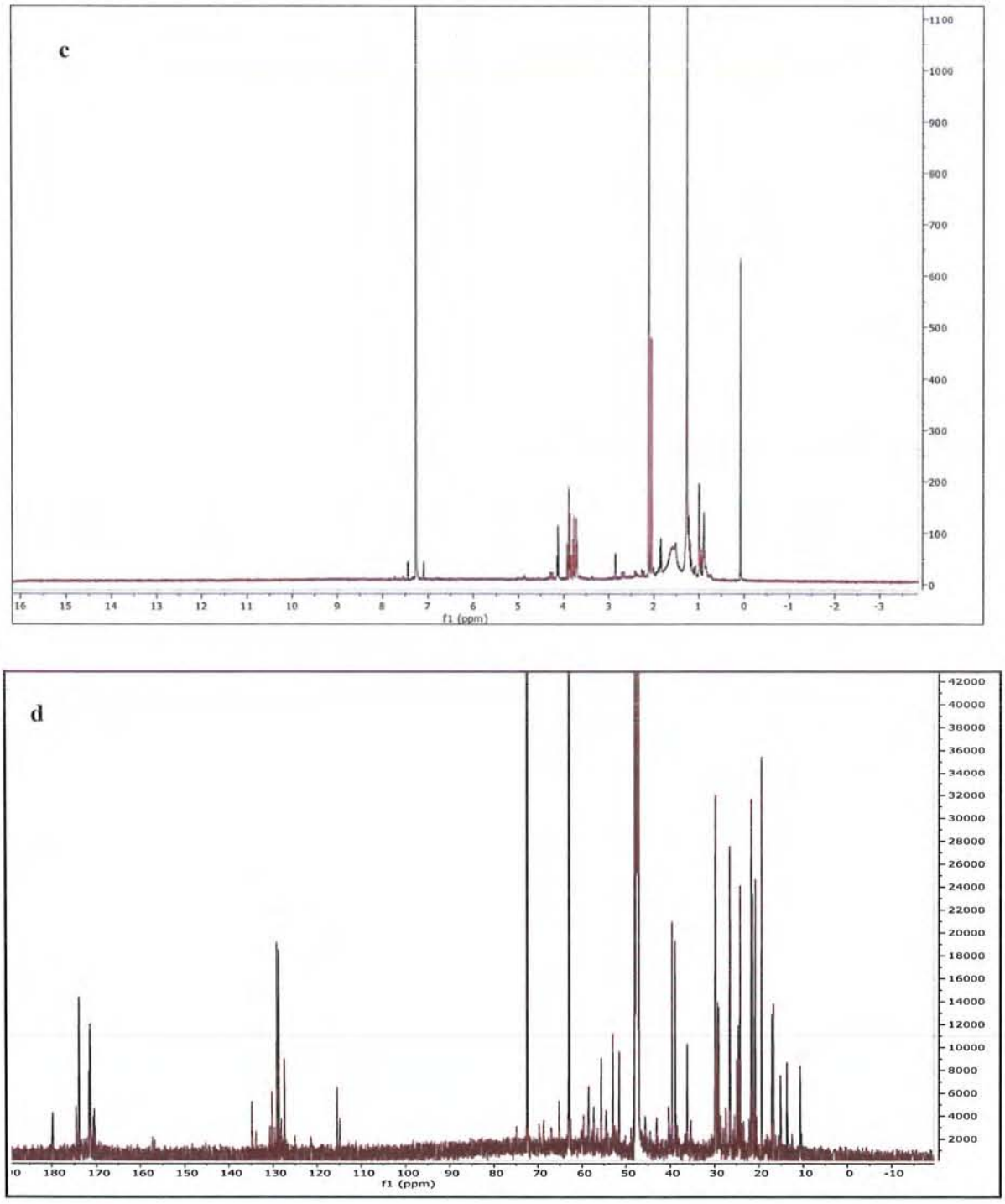


Figure 4.23: c) ^{1}HNMR spectrum and d) ^{13}CNMR spectrum of sophorolipids extracted from *Meyerozyma* spp. MF138126

4.12.5. Liquid chromatography Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

4.12.5.1. Rhamnolipids

The chemical structure of rhamnolipids from *P. aeruginosa* MF069166 was compared with the standard RL molecules through mass spectrometric analysis. Test and standard molecules were subjected to LC-ESI-MS technique that was run in positive mode. Standard RL molecules displayed major peaks at 679.42, 506.33 and 360.27 *m/z* that corresponds to the pseudomolecular ions of Rha-Rha-C_{10}-C_{12}, Rha-Rha-$C_{12:1}$ and Rha-$C_{12:2}$. In addition, minor quantities of other mono and dirhamnolipids congeners were also present (Fig. 4.24 a). In case of RL molecules extracted from *P. aeruginosa* MF069166, peaks representing different structural homologues of mono and dirhamnolipids were detected in the chromatogram. Amongst the identified congeners, the predominant protonated molecular ions belonged to Rha-Rha-C_{10}-C_{12} (679.42 *m/z*), Rha-Rha-$C_{12:1}$ (506.33 *m/z*), Rha-$C_{12:2}$ (360.27 *m/z*) and Rha-Rha-$C_{12:1}$-C_{10} (677.83 *m/z*) (Fig. 4.24 b). Moreover, low relative abundance of Rha-Rha-C_{10}-C_{10} was also detected at *m/z* of 651.79 (Table 4.17).

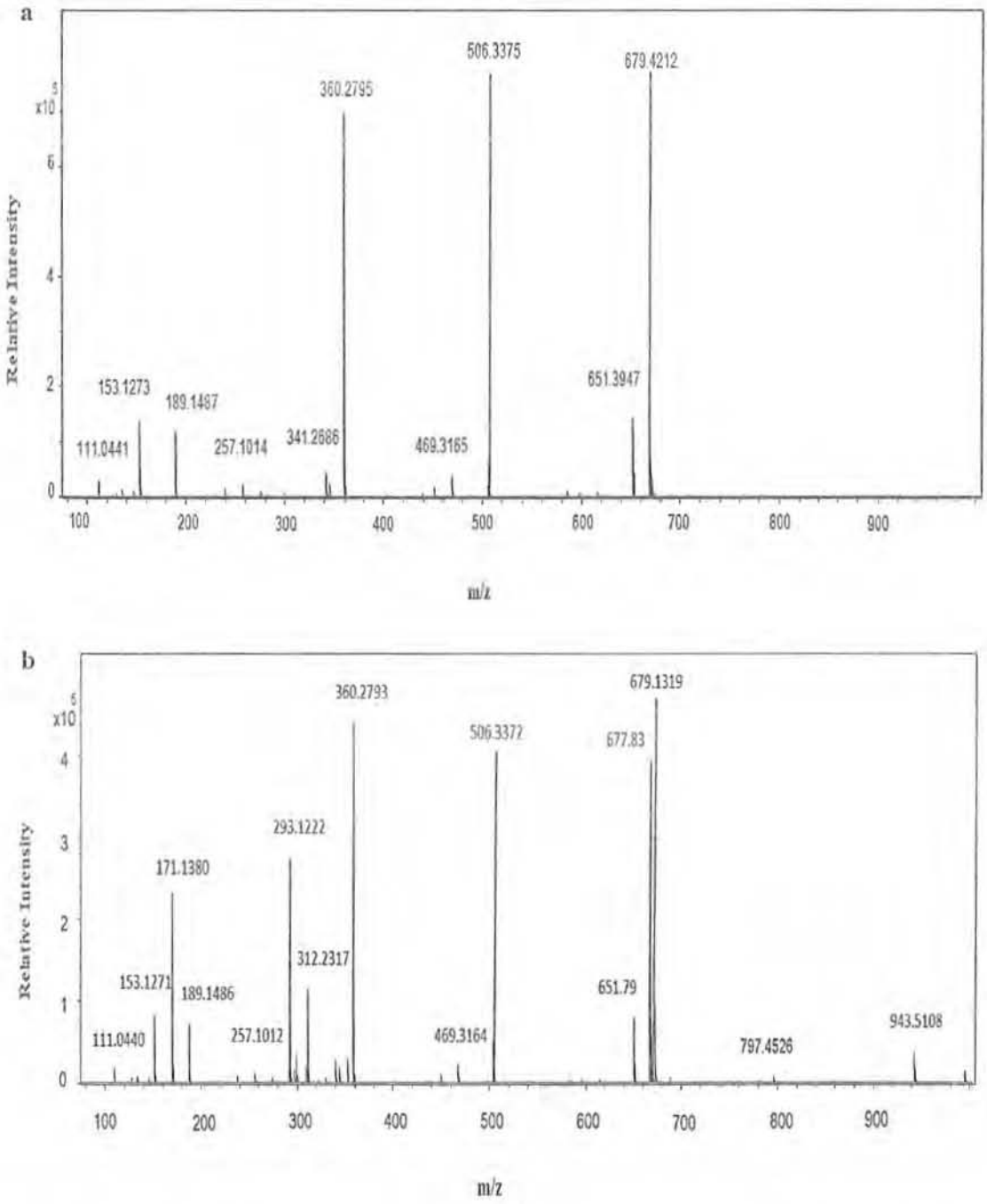


Figure 4.24: LC-ESI-MS profile of a) standard rhamnolipids b) rhamnolipids extracted from *P. aeruginosa* MF069166

Table 4.17: Identification and relative abundance (%) of major rhamnolipids congeners using LC-ESI-MS

Rhamnolipids congeners	Pseudomolecular ion (*m/z*)	Relative abundance (%)	Ionic Fragments
Rha-Rha-C_{10}-C_{12}	679	24.5	312, 293, 189, 171, 153
Rha-Rha-$C_{12:1}$-C_{10}	677	22.4	
Rha-Rha-C_{10}-C_{10}	651	5.61	
Rha-Rha-$C_{12:1}$	506	22.7	
Rha-$C_{12:2}$	360	24	

4.12.5.2. Sophorolipids

The mass spectrum of sophorolipids produced by *Meyerozyma* spp. MF138126 showed the corresponding ionic fragments of protonated and sodiated SL molecules. LC-ESI-MS was applied in the positive mode. The obtained pseudomolecular ions were compared with commercially available standard as well as previously reported molecules. The mass spectrum of standard SLs represented major fragments of diacetylated acidic ($C_{18:1}$), diacetylated lactonic ($C_{18:1}$) diacetylated lactonic ($C_{16:0}$) and monoacetylated lactonic ($C_{18:1}$) fragments at *m/z* values of 706, 711, 685 and 669 (Fig. 4.24 c). In case of sophorolipids extracted from *Meyerozyma* spp. MF138126, major fragments obtained at 711 and 689 *m/z* were identified as sodiated and protonated forms of diacetylated lactonic ($C_{18:1}$) SLs whereas, the peak at 515 *m/z* represented the protonated acidic form ($C_{11:0}$) of SLs. The minor fractions at 788, 623, 601 and 535 *m/z* correspond to $C_{22:0}$ diacetylated lactone, sodiated $C_{18:2}$ lactone, protonated $C_{18:2}$ lactone and $C_{13:1}$ lactone present in the fungal SLs. The signals at 432 and 409 *m/z* were the characteristic peaks of sodiated and protonated form of diacetylated disaccharide sophorose after losing hydroxylated fatty acid moiety from diacetylated lactone SLs of *m/z* 689 (Fig. 4.24 d). The fragment at 373 *m/z* was the protonated ion generated after the loss of terminal hexose from acidic SLs whereas, the peak at 325 *m/z* was obtained due to the presence of non-lactonized sophorose ions in the extract. The other prominent peaks at 227 and 188 *m/z* represented the hexose fragments created due to molecular ionization (Table 4.18).

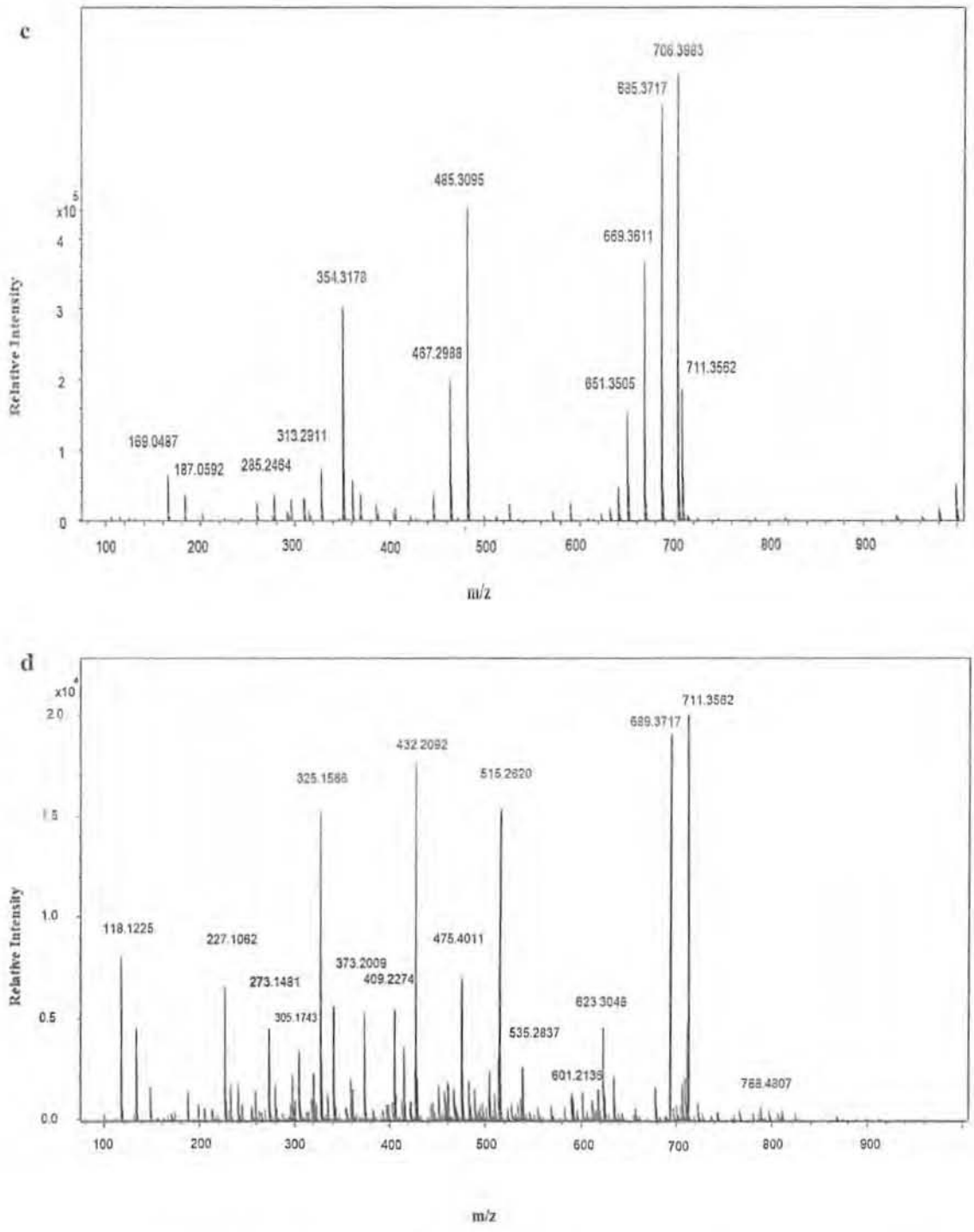


Figure 4.24: LC-ESI-MS profile of c) standard sophorolipids b) sophorolipids extracted from *Meyerozyma* spp. MF138126

Table 4.18: Identification and relative abundance (%) of major sophorolipids congeners using LC-ESI-MS

Sophorolipids congeners	Pseudomolecular ion (*m/z*)	Relative abundance (%)	Ionic Fragments
Diacetylated Lactone ($C_{22:0}$)	788	1.55	432, 409, 373, 325, 305, 273, 227, 118
Diacetylated Lactone ($C_{18:1}$)	711	31	
Diacetylated Lactone ($C_{18:1}$)	689	29.4	
Deacetylated Lactone ($C_{18:2}$)	623	7.7	
Deacetylated Lactone ($C_{18:2}$)	601	2.32	
Lactone ($C_{13:1}$)	535	4.65	
Acidic ($C_{11:0}$)	515	23.2	

4.13. Stability of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

In order to determine the industrial efficacy of rhamnolipids and sophorolipids obtained from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126, stability in their surface active properties was checked under varying conditions of pH, temperature and salinity. For every treatment, emulsification index ($E.I_{24}$) and surface tension was recorded thrice.

4.13.1. Thermal stability

The effect of temperature on crude biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was studied at a temperature range of 5 °C to 121 °C. Results of ST and $E.I_{24}$ assay demonstrated that rhamnolipids produced by bacterial strain were stable at a temperature range of 15 °C to 100 °C. Maximum $E.I_{24}$ of 82 % and minimum ST of 29.3 mN/m was recorded at 35 °C followed by a gradual decline in surface activity at high temperatures. Post autoclaving, very low emulsification activity was observed, however, no significant change in surface tension was detected (Figure 4.25 a).

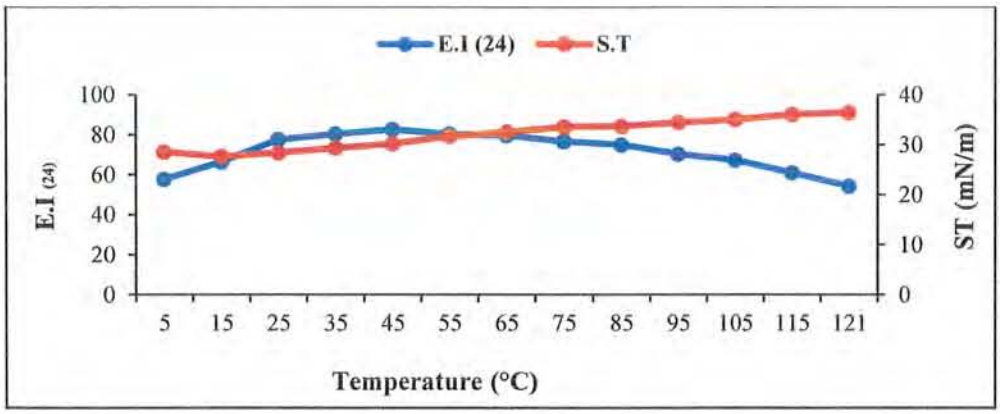


Figure 4.25 a: Thermal stability of rhamnolipids from *P. aeruginosa* MF069166

Sophorolipids produced by *Meyerozyma* spp. MF138126 showed significant thermal stability at a wide range of temperature. Highest $E.I_{24}$ of 86 % and lowest ST of 27 mN/m was recorded at 35 °C however, satisfactory surface active properties were also witnessed at 25 °C and 45 °C. The molecules were effective even at 115 °C with 61 % of $E.I_{24}$ and 35 mN/m of ST. Moreover after autoclaving the culture supernatant, no sudden decline in the surface active properties of SLs was recorded (Fig. 4.25 b).

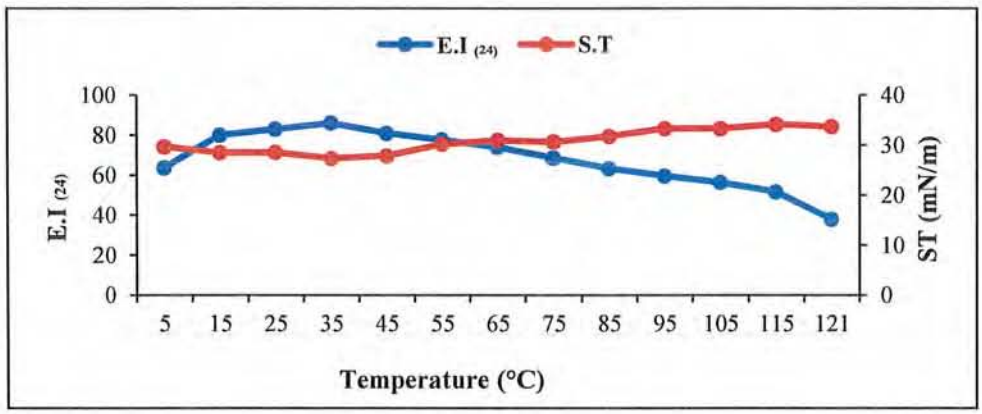


Figure 4.25 b: Thermal stability of sophorolipids from *Meyerozyma* spp. MF138126

4.13.2. pH stability

The stability of biosurfactants at was evaluated at acidic and basic conditions. For this purpose, a pH gradient of 1-14 was selected. Emulsification and surface active properties of biosurfactants was evaluated through the analysis of $E.I_{24}$ and surface tension measurement. It was observed that rhamnolipids exhibited more than 50 % of $E.I_{24}$ at pH 4-11, however, very low emulsification activity was noted at both acidic and basic extremes. Likewise, the molecules effectively reduced ST of water from 72 mN/m to less than 30 mN/m when incubated at pH 4-11 whereas, no significant reduction in surface tension was witnessed at extremely high and low pH (Fig. 4.25 c).

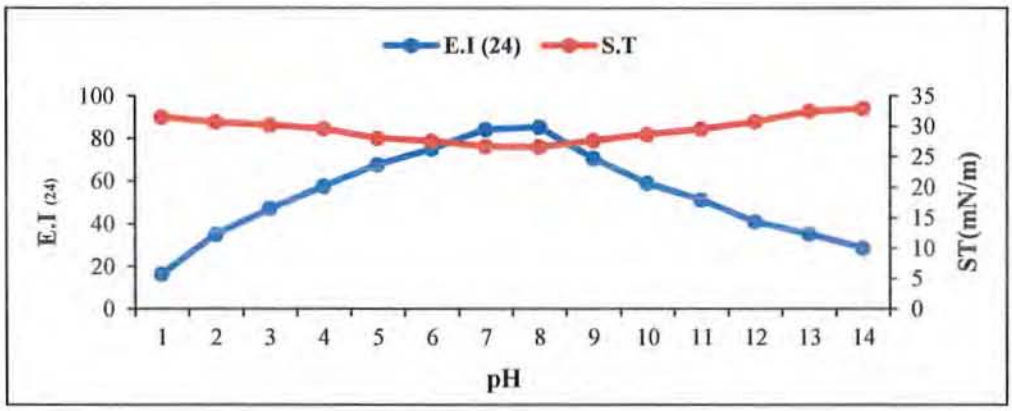


Figure 4.25 c: pH stability of rhamnolipids from *P. aeruginosa* MF069166

The stability of sophorolipids produced by *Meyerozyma* spp. MF138126 was checked at a pH range of 1-14. The molecules were able to effectively emulsify kerosene oil and reduce surface tension of water from pH 3-10. Highest emulsification activity was observed at pH 5 and 6 followed by a continuous decline in $E.I_{24}$ after pH 8. In case of surface activity, sophorolipid molecules significantly decreased the surface tension of water from 72 to 28 mN/m at pH 4, whereas comparatively higher ST of 34.5 and 35.5 mN/m were recorded at pH 1 and 14, respectively (Figure 4.25 d).

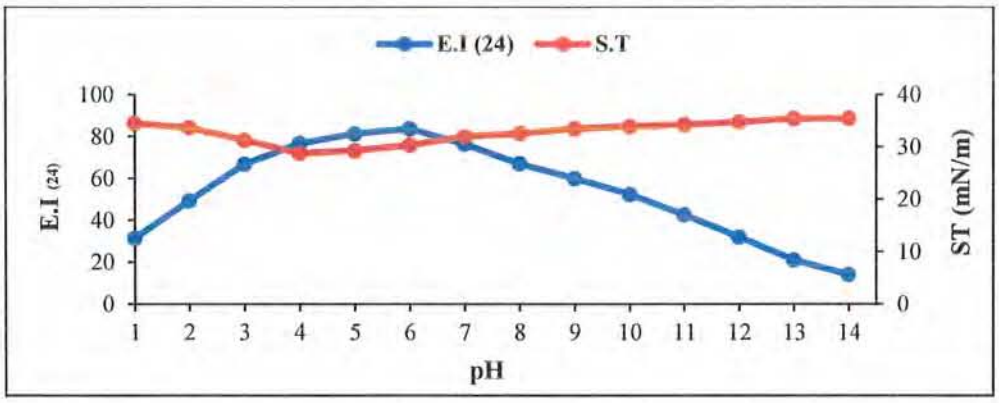


Figure 4.25 d: pH stability of sophorolipids from *Meyerozyma* spp. MF138126

4.13.3. Stability under varying NaCl concentrations

The stability of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was determined at different concentrations of NaCl. Results of $E.I_{24}$ and ST showed that rhamnolipids were able to withstand a broad range of salt concentrations, and retained their surface activity till 10 % w/v NaCl however, comparatively low salt concentration was effective in maintaining the stability of rhamnolipids (Fig. 4.25 e).

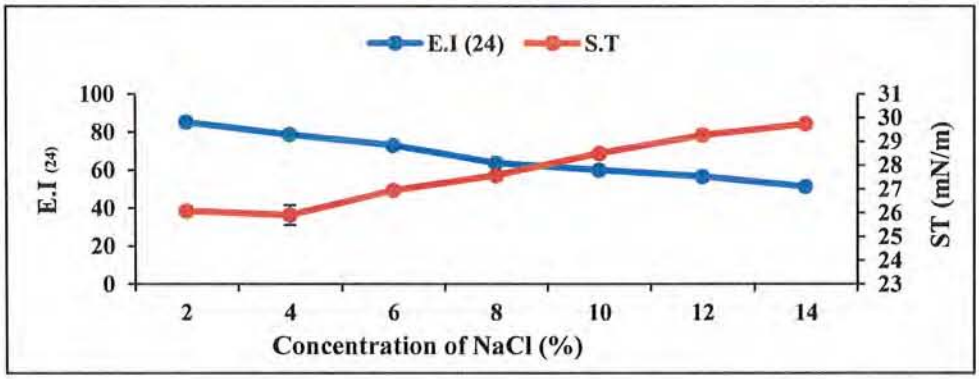


Figure 4.25 e: Stability of rhamnolipids from *P. aeruginosa* MF069166 under varying NaCl concentrations

Sophorolipid molecules produced by *Meyerozyma* spp. MF138126 effectively reduced the surface tension of water and displayed promising emulsification activity in the presence of

2-12 % NaCl. No significant decline in surface ativity of sophorolipids was witnessed upto 12 % increase in salt concentration however, minimum surface activity of SLs was witnessed at the highest concentration of 14 % (w/v) (Fig. 4.25 f).

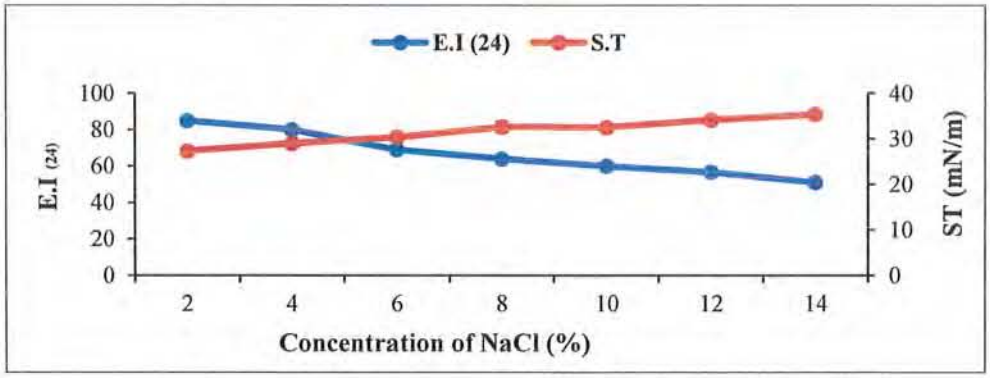


Figure 4.25 f: Stability of sophorolipids from *Meyerozyma* spp. MF138126 under varying NaCl concentrations

4.14. Properties of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

4.14.1. Determination of critical micelle concentration (*cmc*)

cmc of rhamnolipids and sophorolipids was determined by making different dilutions of purified biosurfactants in distilled water. The *cmc* of rhamnolipids from *P. aeruginosa* MF069166 was found to be 40 mg/L after which no significant decrease in surface tension was detected and the solution rettained a constant ST value of 29 mN/m (Fig. 4.26 a).

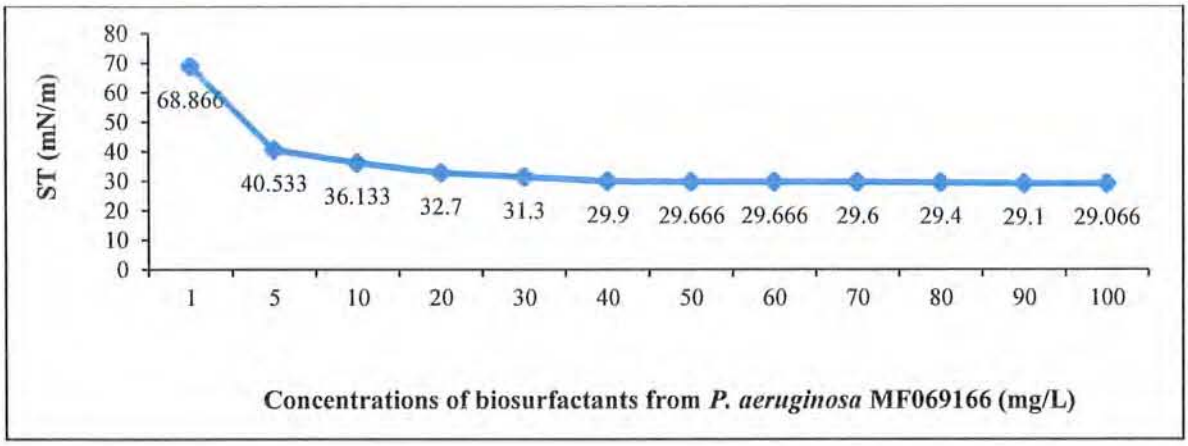


Figure 4.26 a: *cmc* of rhamnolipids extracted from *P. aeruginosa* MF069166 (mg/L)

The *cmc* of sophorolipids from *Meyerozyma* spp. MF138126 was calculated as 50 mg/L. The surface tension of solution reduced up to a constant value of 33 mN/m of ST from 65.76 mN/m after this particular concentration of sophorolipids was achieved. No significant decline in surface tension of the solution was witnessed afterwards (Fig. 4.26 b).

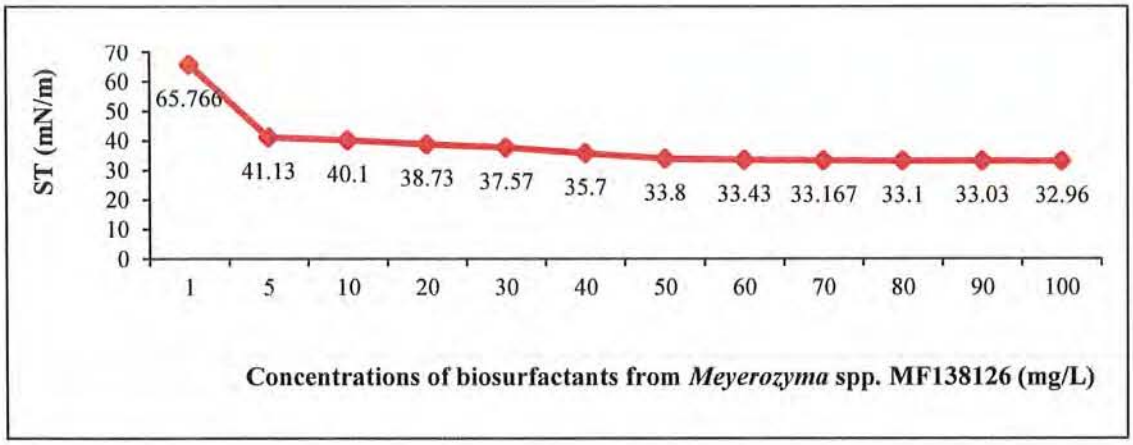


Figure 4.26 b: *cmc* of sophorolipids extracted from *Meyerozyma* spp. MF138126 (mg/L)

4.14.2. Size distribution through Dynamic Light Scattering (DLS)

The glycolipids self-assemble into variety of supra molecular forms having different shapes and sizes. These properties have direct connection with the application of biosurfactants in the field of biodegradation and bioremediation. Therefore, in present study the self-assembly and micellization behavior of the extracted biosurfactants was evaluated using Dynamic Light Scattering technique under the effect of concentrations, pH and electrolytes.

4.14.2.1. Effect of concentration on micellar size distribution

The effect of concentration on size of purified biosurfactants was monitored using dynamic light scattering. pH of rhamnolipids solution was maintained at 7. The diameter of average micellar aggregates of rhamnolipids ranged between 300-350 nm at ½ *cmc* and *cmc*. However, when the critical micelle concentration was doubled, an increase in micellar size was witnessed and 712 nm of z-average diameter was recorded. The heterogeneity in samples was determined through the evaluation of polydispersity index. The minimum PDI of 0.027 observed at 2 *cmc* of RLs whereas, maximum PDI of 0.192 was observed at *cmc* (Fig. 4.27 a).

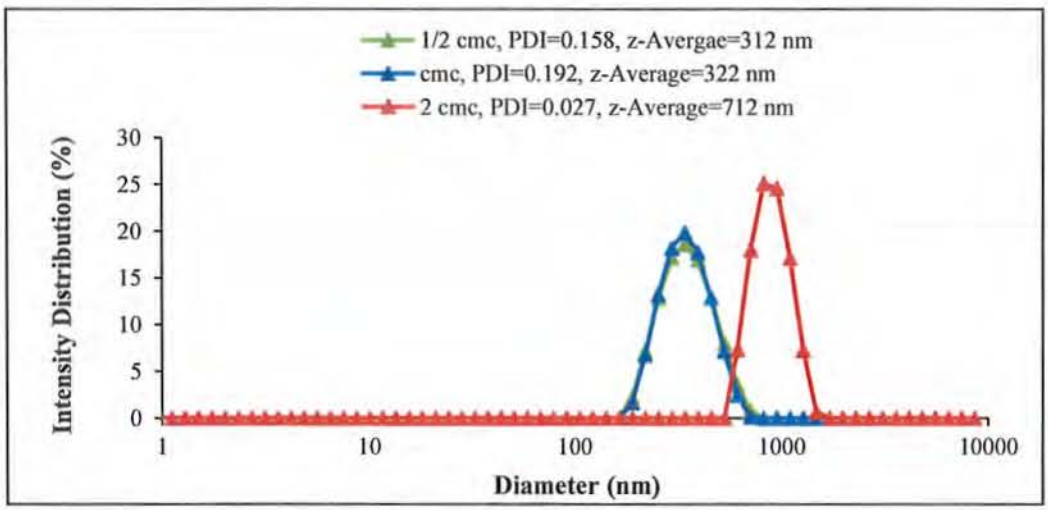


Figure 4.27 a: Size distribution of RLs aggregates at different concentrations

The size of purified sophorolipids was checked with respect to the varying concentration of molecules. pH of sophorolipids solution was maintained at 5. The z-average diameter of SLs micellar aggregates was increased with an increase in their critical micelle concentration. Average size of purified molecules was found to be 309 nm and 380 nm for ½ *cmc* and *cmc,* which drastically increased up to 1217 nm at 2 *cmc* of SLs. Maximum PDI of 0.296 was observed when the *cmc* was doubled whereas, minimum PDI of 0.109 was witnessed at ½ of *cmc* (Fig. 4.27 b).

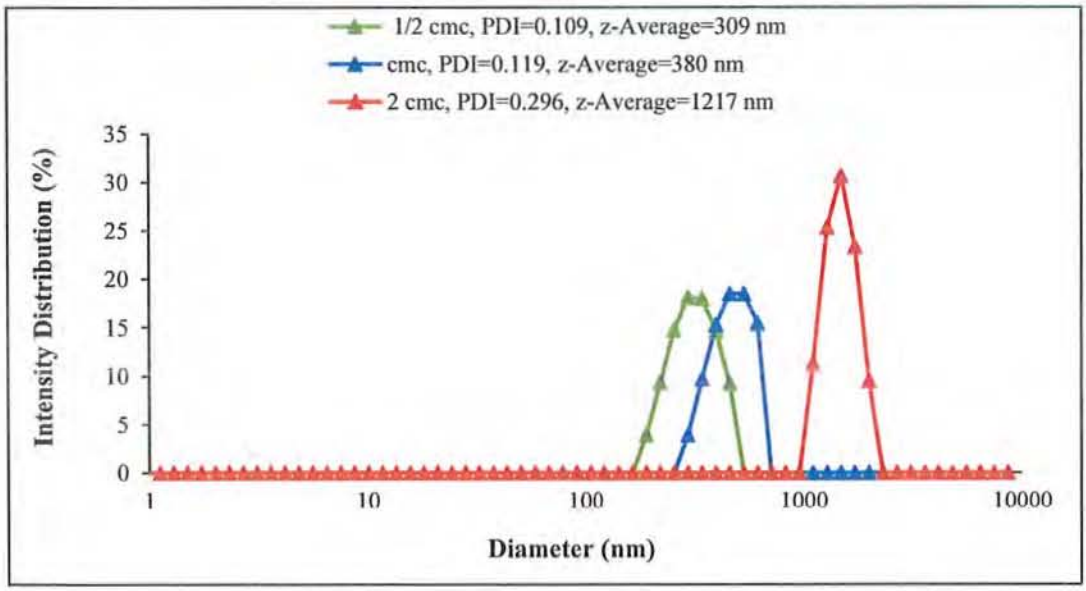


Figure 4.27 b: Size distribution of SLs aggregates at different concentrations

4.14.2.2. Effect of pH on micellar size distribution

It was observed that increase in pH affected the molecular assembly of purified RLs by causing a decrease in the size of structural aggregates. In solution form, rhamnolipids were present in a concentration above than *cmc*. For pH 5, z-average diameter of 388 nm was recorded which decreased up to 225 nm at pH 7 and 154.5 nm at pH 9. Varying PDI values of 0.174, 0.179 and 0.247 were recorded for pH 5, 7 and 9, respectively (Fig. 4.27 c).

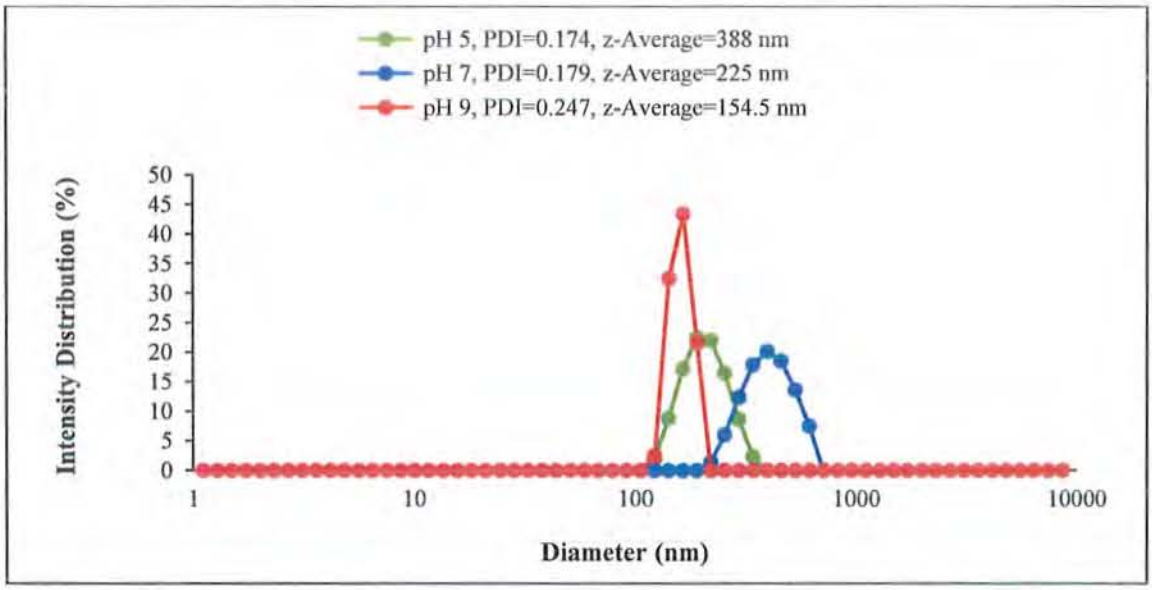


Figure 4.27 c: Size distribution of RLs aggregates at different pH

The self-assembly properties of sophorolipids extracted from *Meyerozyma* spp. MF138126 were checked at pH 3, 7 and 11. In solution, sophorolipids were present in a concentration above than *cmc*. At pH 3, the z-average diameter of molecules was 1154 nm suggesting the formation of large SL aggregates under acidic conditions. When the pH was raised to 7, a sudden decline in size was noted and maximum number of molecular aggregates exhibited the diameter of 727 nm. Further increase in pH was responsible for producing the micellar structures of average 240 nm diameter in solution (Fig. 4.27 d).

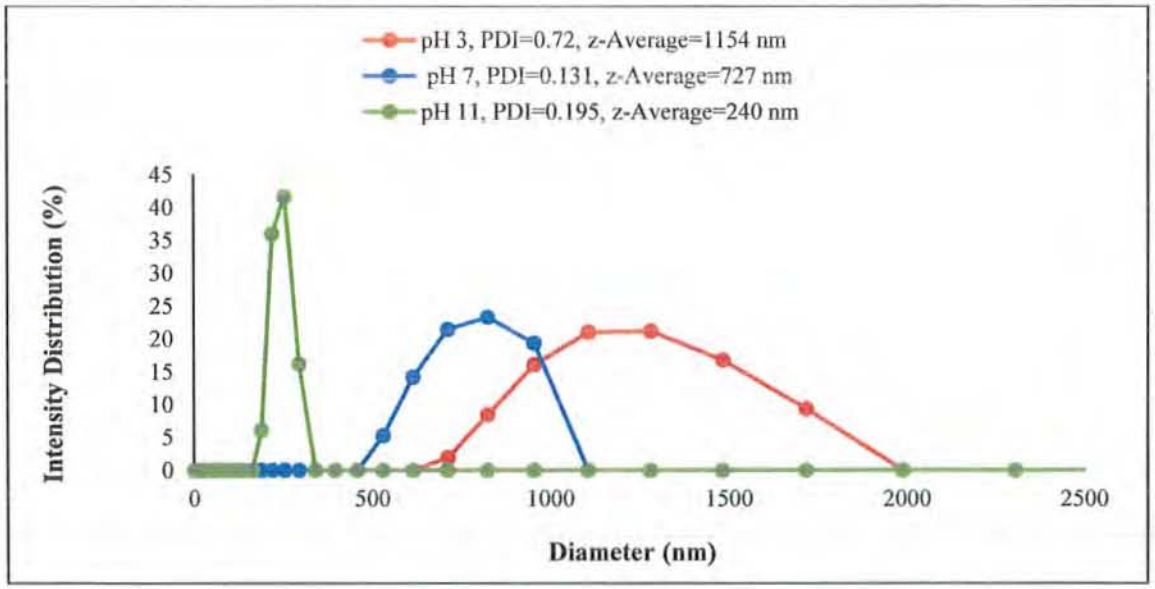


Figure 4.27 d: Size distribution of SLs aggregates at different pH

4.14.2.3. Effect of electrolytes on micellar size distribution

The increasing concentration of NaCl significantly affected the assembly of RL molecules. Low amount of NaCl favored the molecular assembles of smaller size. For 250 mM concentration of salt, the z-average diameter of RLs at their critical micelle concentration was 180 nm with a PDI value of 0.245 whereas, for 500 mM salt, the z-average diameter increased up to 465 nm. Finally for 1000 mM NaCl, very large aggregates of approximately 2000-2500 nm diameter was detected in the solution. PDI value of 1 confirmed the heterogeneity of sample (Fig. 4.27 e).

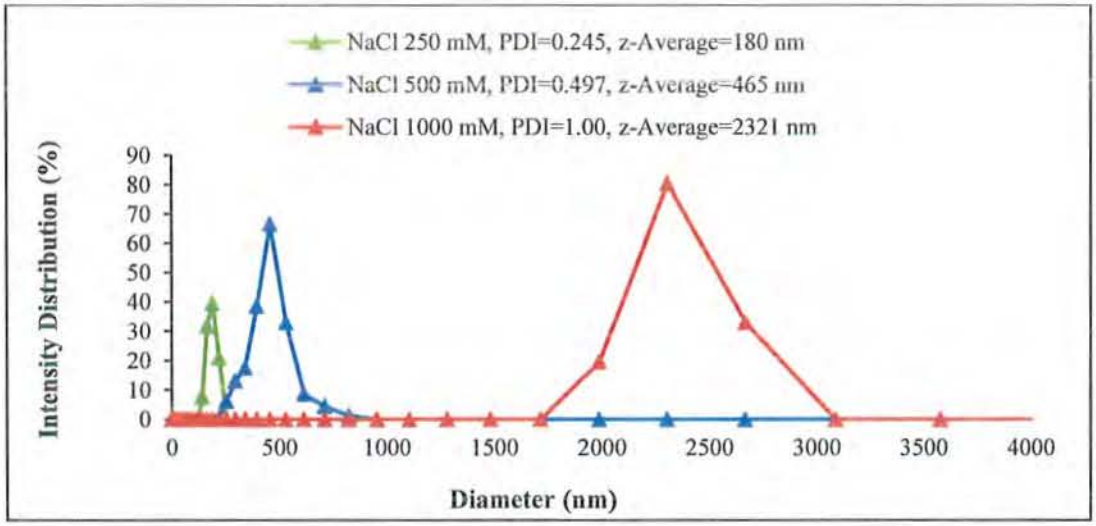


Figure 4.27 e: Size distribution of RLs aggregates at different NaCl concentrations

The effect of high concentrations of NaCl on self-assembly patterns of sophorolipids was monitored. At 250 mM NaCl, majority of molecular aggregates of SLs were 1175 nm in diameter. Likewise RLs, an increase in salt concentration was responsible for producing large molecular aggregate of diameter up to 4800 nm. Heterogeneity was detected in the samples as PDI value reached up to 1 (Fig. 4.27 f).

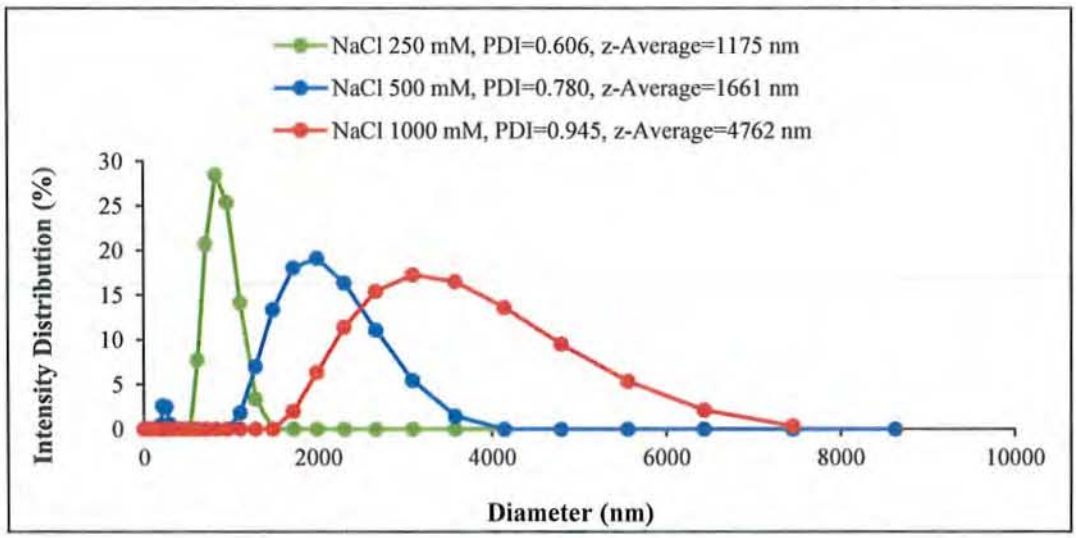


Figure 4.27 f: Size distribution of SLs aggregates at different NaCl concentrations

4D

D- Investigation of environmental applications of biosurfactants

In the fourth and final phase of study, the rhamnolipids produced by *P. aeruginosa* MF069166 and sophorolipids produced by *Meyerozyma* spp. MF138126 were investigated with respect to environmental applications. For this purpose, role of rhamnolipids and sophorolipids was studied in the biodegradation of crude oil and bioremediation of heavy metals.

4.15. Role of biosurfactants and BS producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 in biodegradation of crude oil

The potential role of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and their biosurfactants i.e. rhamnolipids and sophorolipids was studied in biodegradation of crude oil.

4.15.1. Determination of Cell Surface Hydrophobicity (CSH)

In order to check the efficacy of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 for biodegradation of crude oil, microbial cell surface hydrophobicity (CSH) was evaluated through MATH (Microbial Adhesion to Hydrocarbons) assay. Both isolates were individually treated with hydrocarbons of varying complexity and hydrophobicity. After treatment, percentage adherence for every hydrocarbon was recorded. The bacterial strain showed maximum adherence of 70 % to Toluene followed by 65 % affinity for hexadecane, 62 % for crude oil and 57 % for dodecane. Whereas, the yeast specied exhibited 64.66 % adherence to crude oil and 41 % to toluene. Besides, 63.66 and 52 % affinity was also noted for hexadecane and dodecane (Table 4.19).

Table 4.19: Adherence (%) of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 against different hydrocarbons estimated through MATH assay

Microorganisms	Hydrocarbons			
	Toluene	Dodecane	Hexadecane	Crude Oil
P. aeruginosa MF069166	70±0.694	57.33±1.44	65.33 ± 1.06	62.66±0.40
Meyerozyma spp. MF138126	41±0.694	52±0.694	63.66±1.44	64.66±0.40

4.15.2. Effect of hydrocarbons on emulsification activity of biosurfactants

Emulsification method was used to assess the activity of crude rhamnolipids and sophorolipids against complex hydrocarbons (Fig. 4.28). For rhamnolipids, highest $E.I_{24}$ of 87 % was recorded for crude oil followed by 86 %, 85 % and 81 % for diesel, mustard oil and kerosene oil, respectively. Sophorolipids also showed remarkable emulsification properties against the various hydrophobic organic compounds. The maximal $E.I_{24}$ of 90 % was recorded for crude oil whereas, diesel and petrol were emulsified up to 84 % and 81 %, respectively. More than 70 % of emulsification was observed in case of mustard oil, canola oil and olive oil.

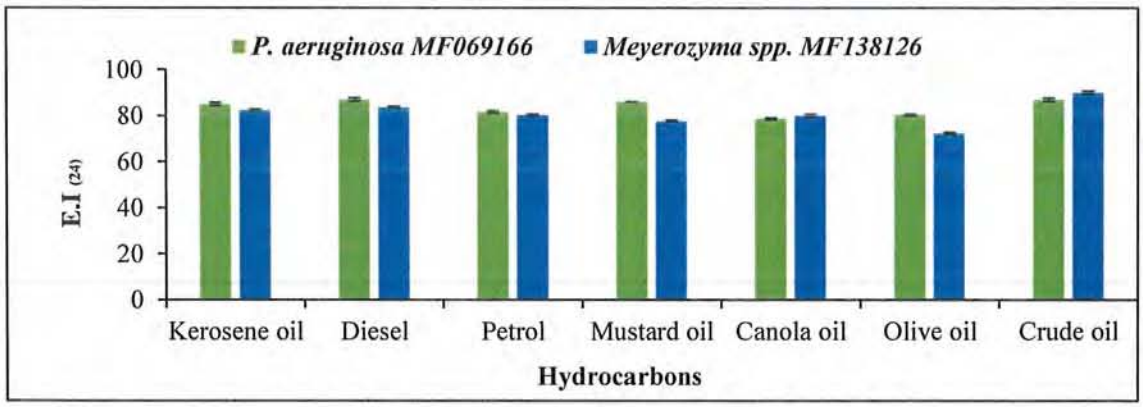


Figure 4.28: Emulsification activities of rhamnolipids containing supernatants of *P. aeruginosa* MF069166 and sophorolipids containing supernatants of *Meyerozyma* spp. MF138126 against hydrocarbons substrates after 24 hours

4.15.3. Biodegradation studies

After evaluating the role of biosurfactants in improving cell surface hydrophobicity of microbial strains and emulsification properties under the effect of various hydrocarbons, two sets of biodegradation experiments were conducted for three weeks. *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were inoculated separately in minimal salt medium containing crude oil as sole carbon source. During the entire biodegradation experiments, microbial growth and surface tension was monitored whereas, microbial degradation of crude oil in the biodegradation systems was determined through gravimetric and GCMS analysis.

4.15.3.1. Biomass estimation

During the course of biodegradation experiments, significant turbidity in culture medium was observed for *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 after 18 h and 24 h of inoculation, respectively. This was followed by an exponential increase in cellular biomass during initial fourteen days of incubation, however, no significant increase in growth was monitored after third week. Specifically, the bacterial strain yielded 5.38±0.043 g/L of biomass after two weeks which slightly increased up to 5.796±0.050 g/L after three weeks of incubation (Fig.4.29 a). Similar trend was observed for the yeast species which yielded higher biomass of 7.226±0.031 g/L after fourteen days, followed by a very insignificant increase of 0.514 g/L at the end of incubation period (Fig. 4.29 b).

4.15.3.2. Surface tension measurement

The production of biosurfactants from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was investigated through measuring the surface tension of crude oil containing culture supernatant after 7, 14 and 21 days of incubation. The recorded ST value for bacterial strain was 29.166±0.382 after 7 days which slightly increased up to 31.066±0.539 after 21 days (Fig. 4.29 a). In the other set of experiment, culture supernatant of yeast

species displayed minimum ST value of 28.6±0.138 mN/m after one week however, 35.33 mN/m of ST was witnessed after prolong incubation of three weeks (Fig. 4.29 b).

4.15.3.3. Analysis of residual crude oil

The degradation of crude oil was initially monitored gravimetrically and further confirmed through GC-MS analysis (Table 4.20) (Fig. 4.29 a and b). Gravimetric analysis revealed that *P. aeruginosa* MF069166 degraded 68 % of crude oil after first week, 85 % after two weeks and 91 % at the end of incubation period. In case of *Meyerozyma* spp. MF138126, varying degradation percentages of 64 %, 85 % and 87 % were recorded after 7, 14 and 21 days of incubation, respectively. After incubation, the residual crude oil extracted from biotic and abiotic samples was analyzed through GC-MS to understand the level of degradation and nature of different crude oil derivatives present in the culture medium (Fig. 4.34). The chromatogram of abiotic sample showed intense peaks of different crude oil constituents ranging between C_8-C_{44}. The chromatograms of both biotic samples were compared with that of abiotic control and Biodegradation Efficiency (B.E) of the two strains was determined. Results showed that the bacterial isolate completely utilized most of the low molecular weight compounds with 8 to 16 C-atoms whereas, the high molecular weight compounds (C_{17} to C_{44}) were degraded upto 60 %. The final Biodegradation Efficiency (B.E) of *P. aeruginosa* MF069166 was found to be 91 %. Chromatographic results showed that the Biodegradation Efficiency (B.E) of *Meyerozyma* spp. MF138126 was 100% for 15 C-alkanes whereas, most of the long chain alkanes were consumed up to 65 %. Besides, the composite hydrophobic organic compounds particularly some halogenated hydrocarbons like Bromo-dodecane, octadecane-1-chloro and tridecane, 1-iodo were utilized up to 75-85 % whereas, the degradation percentage of tetratetracontane was very low. The final Biodegradation Efficiency (B.E) of *Meyerozyma* spp. MF138126 was 85 %.

Figure 4.29 a: Biodegradation profile of *P. aeruginosa* MF069166

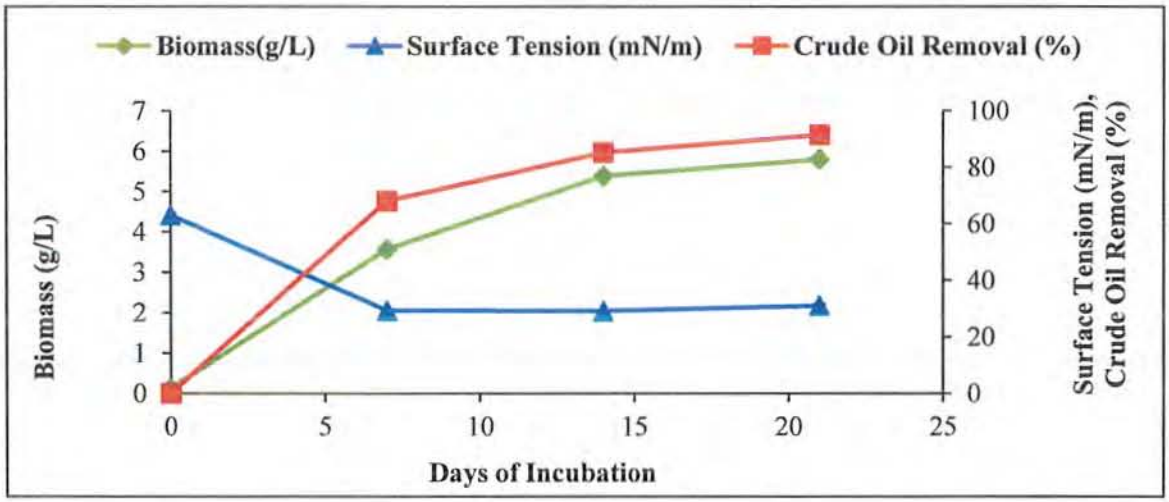


Figure 4.29 b: Biodegradation profile of *Meyerozyma* spp. MF138126

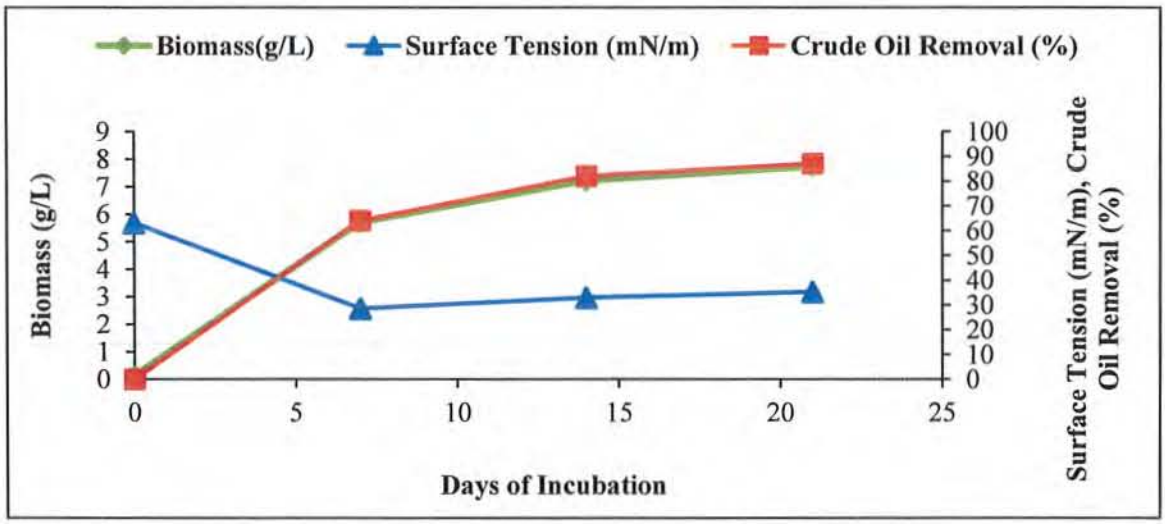


Table 4.20: Biodegradation Efficiency (B.E) of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 in the presence of crude oil

RT	Compounds	Molecular Weight	Chemical Formula	B.E (%) of *P. aeruginosa* MF069166	B.E (%) of *Meyerozyma* spp. MF138126
2.914	Octane	114	C_8H_{18}	100	100
3.68	Nonane	128	C_9H_{20}	100	100
4.386	Decane	142	$C_{10}H_{22}$	100	100
5.942	Undecane	156	$C_{11}H_{24}$	100	100
6.713	Tridecane	184	$C_{13}H_{28}$	100	100
7.242	2,3,5,8 tetramethyl decane	198	$C_{14}H_{30}$	100	100
8.025	3,5,-bis(1,1-dimethylethyl)	206	$C_{14}H_{22}O$	100	100
9.683	Pentadecane	212	$C_{15}H_{32}$	100	100
10.65	Dodecane 2,6,10 trimethyl	212	$C_{15}H_{32}$	100	61.07
11.858	Hexadecane	226	$C_{16}H_{34}$	100	100
12.233	2,4 ditertbutyl-7,7-dimethyl-1,3,5-cycloheptatriene	232	$C_{17}H_{28}$	79.88	74.22
13.275	2-Bromo dodecane	248	$C_{12}H_{25}Br$	100	87.06
13.767	Nonadecane	268	$C_{19}H_{40}$	63.1	70.86
14.242	Octadecane, 1-chloro-	288	$C_{18}H_{37}Cl$	81.37	75.66
15.275	Heptadecane	296	$C_{21}H_{44}$	69.29	90.95
16.242	Tridecane, 1-iodo	310	$C_{13}H_{27}I$	87.29	88.73
18.908	Tetratetracontane	618	$C_{44}H_{90}$	58.79	4.29
				Total B.E= (1539.72/1700)=90.57	(1452.84/1700)=85.46

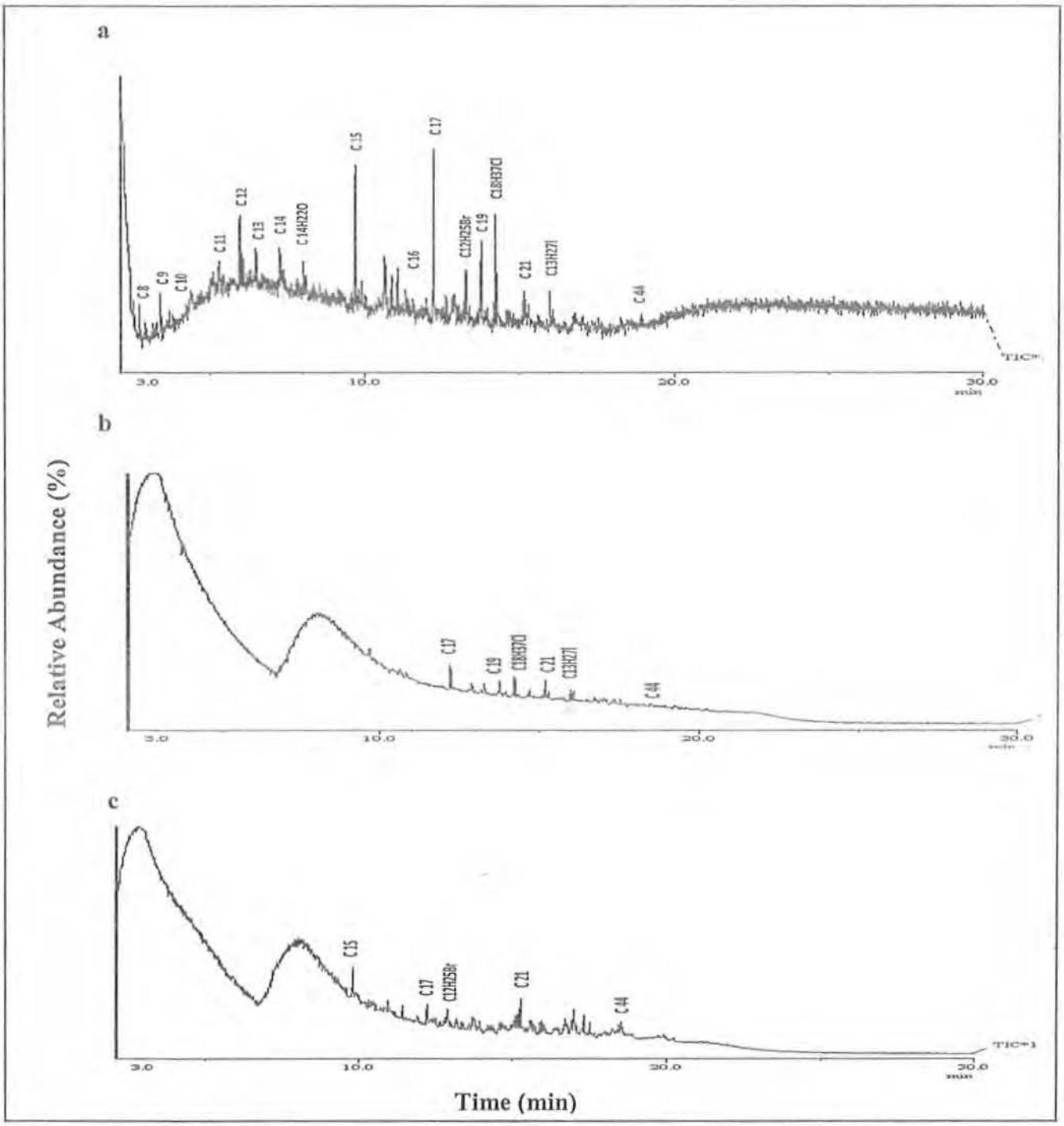


Figure 4.30: Gas chromatography-Mass Spectrometry (GC-MS) characterization of residual crude oil extracted from (a) Abiotic control (b) *P. aeruginosa* MF069166 (c) *Meyerozyma* spp. MF138126

4.16. Role of biosurfactants and BS producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 in bioremediation of heavy metals

The potential role of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and their biosurfactants i.e. rhamnolipids and sophorolipids was studied in biodegradation of crude oil.

4.16.1. Heavy metals tolerance through Plate Diffusion Method

The ability of two microorganisms to tolerate heavy metals was determined through plate diffusion method. Results showed that *P. aeruginosa* MF069166 displayed maximum resistance against zinc chloride followed by lead nitrate, cadmium chloride and copper sulphate whereas, no microbial growth was observed on the plates containing chromium sulphate. On the other hand, *Meyerozyma* spp. MF138126 displayed maximum growth in the presence of lead nitrate and was sensitive to a very minute concentration of cadmium chloride. Minimal Inhibitory Concentration (MIC) of the all the heavy metals tested against these two isolates have been summarized in Table 4.21.

Table 4.21: MIC of heavy metals for *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

Strains	Minimal Inhibitory Concentration (ppm)				
	Cd^{2+}	Cr^{3+}	Cu^{2+}	Pb^{2+}	Zn^{2+}
P. aeruginosa MF069166	600	100	400	700	800
Meyerozyma spp. MF138126	200	500	500	900	800

4.16.2. Biosurfactants activity in the presence of heavy metals

The surface active potential of crude biosurfactants was established in the presence of 100, 500 and 1000 ppm concentration of heavy metals through the determination of Emulsification Index ($E.I_{24}$). Results showed that zinc chloride had minimum effect on emulsification properties of crude rhamnolipids as highest $E.I_{24}$ of 83 % and 81 % was observed at 100 and 500 ppm concentrations after 72 h. However, significant reduction in

$E.I_{24}$ was observed when the fermentation broth was supplemented with different concentrations of chromium sulphate. In case of sophorolipids, lead nitrate showed least toxic effects as 81 % and 79 % of $E.I_{24}$ was observed in the presence of 100 and 500 ppm concentrations of lead nitrate. However, cadmium chloride had an inhibitory effect on the biosurfactants production capability as very low emulsification activities of 37 %, 17 % and 10 % were recorded (Fig. 4.31).

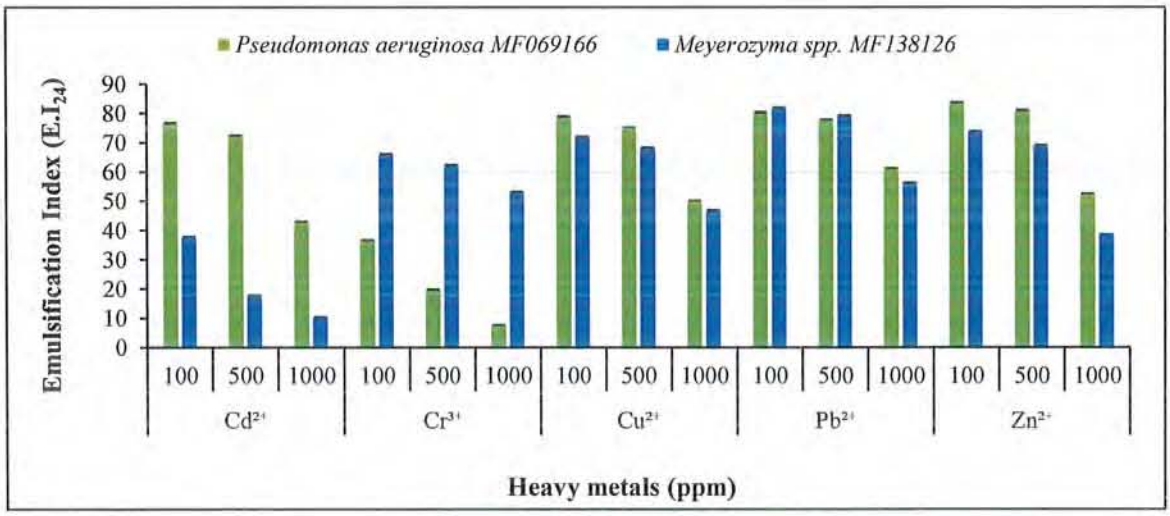


Figure 4.31: Emulsification Index ($E.I_{24}$) of rhamnolipids and sophorolipids in the presence of heavy metals

The initial assays showed that *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and their biosurfactants displayed maximum resistance against lead nitrate and zinc chloride therefore, the following experiments were conducted with respect to these two heavy metals.

4.16.3. Metal chelating activity of biosurfactants

The metal chelating activity of biosurfactants was determined through studies conducted using atomic absorption spectrophotometer (AAS). Results of AAS showed that biosurfactants produced by *P. aeruginosa* MF069166 efficiently chelated and consequently removed different concentrations of zinc chloride and lead nitrate. In case of zinc chloride,

very low amount of heavy metal was detected, post treatment, particularly in the solutions that were provided with *cmc* and 2 *cmc* of RLs. Maximum removal of 92 % was achieved when *cmc* of RLs was added in 100 ppm solution of zinc chloride. Moreover, 77 % removal of lead nitrate was also recorded when the *cmc* of RLs was doubled. These results also suggested that RLs even in their half of critical micelle concentration were able to successfully remove more than 60 % of both heavy metals from the treated solutions (Table 4.22).

Table 4.22: Percentage removal of zinc and lead by different concentrations of rhamnolipids produced by *P. aeruginosa* MF069166

Rhamnolipids solutions of *P. aeruginosa* MF069166	Concentrations of heavy metals (ppm)	Percentage Removal (%)	
		Zn	Pb
0.002 % (1/2 *cmc*)	100	81±2.1	72±1.6
	500	78.5±1.5	68±1.1
	1000	72±1.1	67±1.4
0.004 % (*cmc*)	100	92±1.3	76±1.8
	500	88±1.9	72±1.5
	1000	84±1.7	68±1.3
0.008 % (2 *cmc*)	100	91±2.1	77±1.5
	500	88±1.6	71±1.1
	1000	86±1.0	69±1.3

The percentage removal of heavy metals from solutions treated with sophorolipids produced by *Meyerozyma* spp. MF138126 was evaluated through atomic absorption spectroscopy. Results indicated that high concentrations of SLs were able to precipitate out both lead nitrate and zinc chloride from solution. The percentage removal of lead nitrate was comparatively higher than zinc chloride. Maximum removal of 95 % was detected in 100 ppm solution of lead nitrate when SLs were added in their *cmc*. Significant high removal of 73-80 % was achieved in the other solutions of lead nitrate that were provided with ½ *cmc* of the SLs. Moreover, different concentrations of SLs were also able to remove

more than 50 % of zinc chloride from the solutions. 81 % removal of zinc chloride was recorded using 2 *cmc* of SLs whereas, ½ *cmc* of SLs effectively chelated 52 % of the metal from 1000 ppm solution (Table 4.23).

Table 4.23: Percentage removal of lead and zinc by different concentrations of sophorolipids produced by *Meyerozyma* spp. MF138126

Sophorolipids solutions of *Meyerozyma* spp. MF138126	Concentrations of heavy metals (ppm)	Removal (%)	
		Pb	Zn
0.0025 % (1/2 *cmc*)	100	80±1.1	72±1.2
	500	79±1.7	66±1.8
	1000	73±1.8	52±1.1
0.005 % (*cmc*)	100	95±2.1	73±1.1
	500	92±1.9	71±1.5
	1000	84±1.5	67±2.2
0.01 % (2 *cmc*)	100	93±1.6	81±1.8
	500	87±1.3	77±1.5
	1000	85±1.2	75±1.4

4.16.4. Conductivity measurements

The conductivity of heavy metals solutions treated with both natural and chemical surfactants in their critical micelle concentration was recorded using a conductivity meter. After treatment, the resultant surfactant-metal complex was removed through centrifugation and the ionic content of supernatant was recorded. Results showed that increase in concentration of natural surfactants caused a decrease in the electrical conductivity of heavy metals solution. Rhamnolipids showed more affinity for zinc chloride as 2 *cmc* of RLs was able to reduce E.C from 582 µS to 5.4 µS. Likewise, significant reduction in E.C of heavy metals solution was observed when treated with sophorolipids. The conductivity of lead nitrate solution was decreased from 625 µS to 6.5 µS, when the *cmc* of SLs was doubled. SDS, the anionic chemical surfactant, was able to

reduce conductivity of both heavy metals solution, however, by increasing the surfactants concentration, an increase in conductivity was observed. No significant reduction in the conductivity measurements was witnessed for other two non-ionic surfactants therefore, E.C of the solution remained unchanged (Table 4.24).

Table 4.24: Electrical Conductivity (E.C) of lead and zinc solutions treated by different concentrations of natural and synthetic surfactants

Sample	Concentrations	Electrical Conductivity for 500 ppm Pb (µS)	Electrical Conductivity for 500 ppm Zn (µS)
Rhamnolipids	0.002 % (1/2 *cmc*)	13.6	12.5
	0.004 % (*cmc*)	10.9	6.7
	0.008 % (2 *cmc*)	8.5	5.4
Sophorolipids	0.0025 % (1/2 *cmc*)	11.1	15.6
	0.005 % (*cmc*)	9.3	14.8
	0.01 % (2 *cmc*)	6.5	11.2
SDS	0.117 % (1/2 *cmc*)	8.3	9.9
	0.234 % (*cmc*)	9.08	10.4
	0.468 % (2 *cmc*)	9.5	11.1
Tween 20	0.0035 % (1/2 *cmc*)	623	580
	0.007 % (*cmc*)	618	575
	0.014 % (2 *cmc*)	615	569
Tween 80	0.006 % (1/2 *cmc*)	619	573
	0.012 % (*cmc*)	609	562
	0.024 % (2 *cmc*)	608	559

4.16.5. Washing of heavy metals contaminated soil using biosurfactants:

Washing of artificially metals contaminated soil was done using crude biosurfactants and the half, full and double critical micelle concentrations of isolated biosurfactants. Deionized water was used as control. Results of soil washing showed that removal of heavy metals increased with an increase in concentration of isolated biosurfactants. In case of rhamnolipids produced by *P. aeruginosa* MF069166, 89 % removal efficiency was witnessed for zinc chloride when the *cmc* was doubled. The crude rhamnolipids were also

able to remove more than 80 % of heavy metals from the contaminated soil however, comparatively high removal rates were observed for zinc. Sophorolipids produced by *Meyerozyma* spp. MF138126 removed 89 % of lead nitrate in their 2 *cmc* concentration whereas 90 % metal removal was witnessed with their crude form. Moreover, 85 % removal of zinc chloride was observed with 2 *cmc* of SLs. However, physical treatment with deionized water could only remove 12 % and 13 % concentration of lead nitrate and zinc chloride, respectively (Table 4.25).

Table 4.25: Removal of heavy metals contained in soil by the washing solutions of rhamnolipids and sophorolipids

Sample	Treatments	Removal (%)	
		Pb	Zn
Deionized water	Control	12±1.5	13±2.2
P. aeruginosa MF069166	Crude rhamnolipids (cell free broth)	81±2.4	88±1.2
	0.002 % (1/2 *cmc*) rhamnolipids solution	75±1.3	74±2.1
	0.004 % (*cmc*) rhamnolipids solution	80±2.2	82±2.0
	0.008 % (2 *cmc*) rhamnolipids solution	82±1.5	89±1.7
Meyerozyma spp. MF138126	Crude sophorolipids (cell free broth)	90±1.6	88±2.1
	0.0025 % (1/2 *cmc*) sophorolipids solution	78±1.7	77±1.8
	0.005 % (*cmc*) sophorolipids solution	83±2.1	82±1.4
	0.01 % (2 *cmc*) sophorolipids solution	89±1.2	85±1.1

The kinetics of soil washing was studied by incubating the heavy metals contaminated soil with crude biosurfactants solutions of rhamnolipids and sophorolipids for 15 days. The rate of removal of lead nitrate and zinc chloride was measured at 1st, 3rd, 5th, 7th, 9th, 11th, 13th and 15th day of incubation. Results showed that an increase in removal kinetics of soil washing with an increase in incubation time. After 24 h of incubation, 10 % removal of zinc chloride was recorded for rhamnolipids produced by *P. aeruginosa* MF069166 which

increased up to 90 % at the end of incubation period. Likewise, a gradual increase in the percentage removal of lead nitrate was also witnessed after 15 days (Fig. 4.32 a).

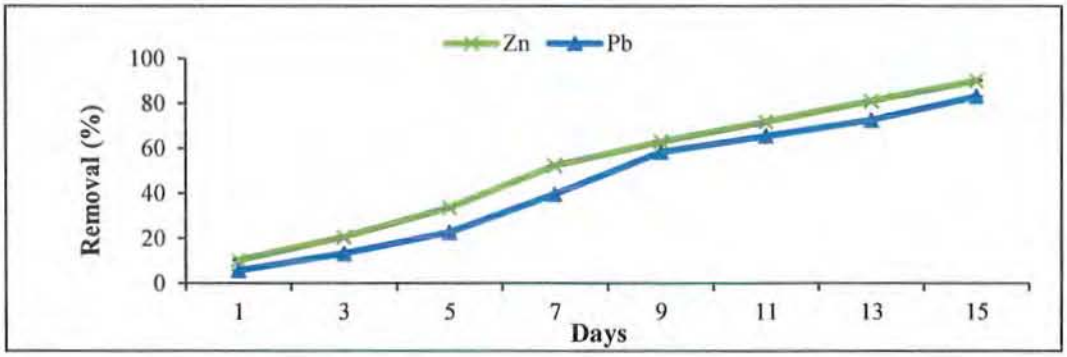


Figure 4.32 a: Removal of zinc and lead over time using the cell-free broth of *P. aeruginosa* MF069166

Crude sophorolipids produced by *Meyerozyma* spp. MF138126 also enhanced the removal of both heavy metals from contaminated soil with the passage of time. At the end of incubation period, lead nitrate was removed up to 92 % whereas, 80 % removal efficiency was noted for zinc chloride. It was further noted that after 24 h, removal percentages of lead nitrate and zinc chloride were 15 % and 6.3 %, respectively (Fig. 4.32 b).

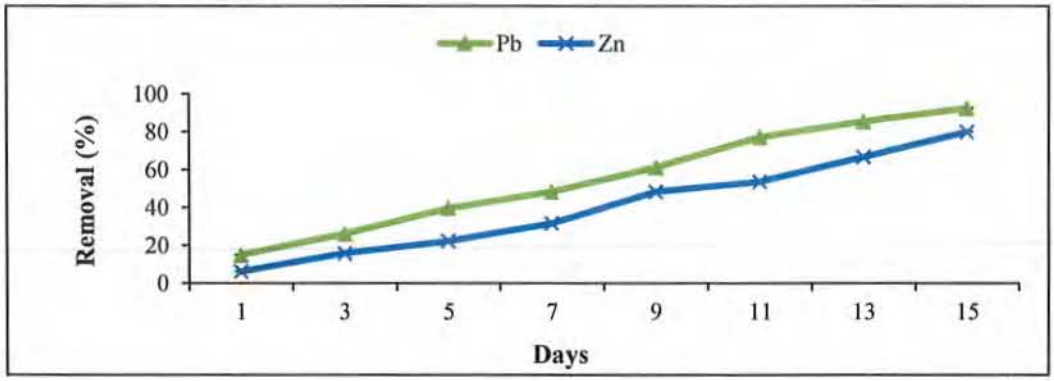


Figure 4.32 b: Removal of lead and zinc over time using the cell-free broth of *Meyerozyma* spp. MF138126

5- DISCUSSION

5A

A- Isolation and screening of biosurfactants producing microorganisms from crude oil contaminated soil

In recent years, biosurfactants characterization, production optimization and applications are growing in pursuing the objectives of sustainability. The glycolipid biosurfactants specifically produced by the bacteria and yeast provides excellent opportunity to replace petroleum based chemical surfactants because of their sustainability, better physiochemical and biological properties. Although, contemporary biotechnological methods enabled a significant improvement in the biosurfactants production up to 10-20 folds, however, still these molecules are not commercially viable (Barakat et al., 2017). Considering the limitations of bulk production, the future biosurfactants research will likely to focus on alternative low cost carbon substrates, search of efficient microorganisms, process design and optimization, product purification and biosurfactants with new structure and properties. Biosurfactants offer several advantages over the ordinary surfactants such as high emulsification, better surface and interfacial tension reduction, low toxicity, low *cmc*, optimum activity at extreme pH and temperatures. More importantly, these molecules are biodegradable and eco-friendly. Biosurfactants are versatile process chemicals having broad spectrum of applications, specifically for the mitigation of environmental pollutants through biodegradation and bioremediation (Karlapudi et al., 2018).

Besides, production and applications of the biosurfactants are facing various challenges, unique self-assembly and micellization behavior of glycolipids have changed traditional outlook of these molecules. Recently, various potent self-assembled lyotropic liquid crystalline phases including sponge, lamella, helices, nano-tubes and bicontinuous cubic structures have been reported for glycolipid biosurfactants (Corti et al., 2007). On the other side, metabolic diversity of biosurfactants microorganisms is very fascinating. It has been demonstrated that a single microbial strain could produce chemically diverse surfactants molecules under varying physiological conditions. These discoveries have made possible to use biosurfactants in induction of cellular differentiation against leukemia cells, as

immunity modulators and vector for gene delivery in mammalian cells. Due to these advance features, biosurfactants R&D is considered as one of the most exciting area of industrial biotechnology.

5.1. Soil analysis

The contamination of petroleum hydrocarbons negatively affects the physicochemical and biological properties of soil. The rehabilitation of these sites requires comprehensive information of the soil nutrient profile, contamination level and microbial flora (Wang et al., 2013). The analysis of contaminated soil from Chak Naurang oil fields, Punjab, Pakistan, was carried out and properties were compared with non-contaminated agricultural soil of the same region (Fig. 4.2). Our findings showed that the contaminated soil was saturated with petroleum hydrocarbons with a TPH value of 22.56 g/kg. The attachment of contaminants to clay particles created composite reservoirs of petroleum hydrocarbons in soil which could be the possible reason for high concentrations of TPH in affected soil (Gros et al., 2014, Lacatusu et al., 2017). Besides, the high quantity of Total Organic Carbon could be due to the elevated levels of petroleum hydrocarbons as the affected site was devoid of any vegetation (Masakorala et al., 2014). The soil was also found lacking essential nutrients for agriculture and biological activities. Active consumption of nitrogen, phosphorous and potassium in energy-yielding activities of indigenous micro-flora caused an imbalance of soil nutrients and C:N ratio (Gao et al., 2015). No major differences in the texture and pH of both affected and unaffected soil was noted which showed that petroleum contamination had partially affected the soil chemistry (Wang et al., 2010). Substantial decrease in E.C for contaminated soil samples could be associated with non-polar nature of crude oil that hindered the free movement of ions in soil and made it more hydrophobic (Vincent et al., 2011). Saturated hydraulic activity, clogged pore spaces, reduced water permeability or increased bulk density due to hydrophobic coating of oil on clay particles might be responsible for reduced water concentration in petroleum affected areas (Khamehchiyan et al., 2007). These findings

suggested that crude oil contaminated soil of Chak Naurang was deficient in nutrients, water content and salts and, was unfavorable for agriculture activities. Therefore, addition of nutrients and increasing the water content could be very useful strategy for improving the biodegradation rate of petroleum hydrocarbons. In some previous findings, success of bioremediation projects was based on the pre-site analysis (Liu et al., 2013, Sungur et al., 2015). In present finding, it was also suggested that the knowledge of site conditions is essential for field scale bioremediation.

5.2. Isolation of crude oil degrading and biosurfactants producing microorganisms

The principle aim of searching biosurfactants producing microorganisms from the environmental samples has been to find out more productive isolates and novel surfactants molecules with low *cmc*, strong surface activity, high solubility and emulsion capacity. In present study, crude oil contaminated soil of Chak Naurang, Punjab, Pakistan was selected for the isolation of crude oil degrading and biosurfactants producing microorganisms. Soil enrichment technique yielded thirty-four bacterial and six fungal strains. Previously, Vargas et al., (2017) reported twenty-four and Yadav et al., (2016) reported nineteen biosurfactants producing microorganisms from different oil spill impacted areas. In agricultural soils, biosurfactants production helps the microbial colonization in the rhizosphere and increases the bioavailability of nutrients for plants uptake. Due to their antimicrobial activity, biosurfactants protect the plant roots against the invading pathogens (Mnif and Ghribi, 2015). The hydrocarbons, specifically crude oil contaminated soils are important sources for the isolation of biosurfactants producing microorganisms and are frequently reported in the literature (Joy et al., 2017). In crude oil contaminated soil, production of biosurfactants by microorganisms decreases the surface and interfacial tension of petroleum compounds and make them soluble in the aqueous phase. Subsequently, these hydrophobic contaminants are solubilized and become biologically available to the microbial cells causing their mineralization/degradation. The recent reports of Zouari et al., (2018) and Ebadi et al., (2018) supported the findings of current study that

crude oil contaminated sites are prime sources for the isolation of efficient crude oil degrading and biosurfactants producing microorganisms.

5.3. Qualitative screening of biosurfactants producing microorganisms

The screening of biosurfactants producing microorganisms from growing cultures provide basic information regarding their metabolic abilities. Due to diverse chemical nature of biosurfactants, different assays have been used for screening purposes (Varjani et al., 2014). In present research, three qualitative plate assays were performed for initial screening of biosurfactants producing microorganisms. These include crude oil overlay agar assay, blood agar hemolysis assay and CTAB methylene blue assay. Results suggested that every isolate showed different patterns when subjected to screening. Blood agar hemolysis assay has been recommended as one of the primary screening method. It involves the lysis of erythrocytes present in the hydrophilic media which results in the formation of a transparent zone around the microbial colonies (Elazzazy et al., 2015, Sharma et al., 2015). In present study, 45 % of strains showed α-hemolysis whereas, 37 % of strains showed β- hemolysis (Fig. 4.3). Previously it has been reported that amongst 40 isolates, 10 strains formed hemolytic zones in blood agar assay (Walter et al., 2010). Similarly, in a study conducted by Hamed et al., (2012) 20 % isolates were found to be positive for this assay. CTAB methylene blue assay is a semi-qualitative method particularly used for the screening of anionic biosurfactants producing microorganisms. The negatively charged biosurfactants binds with the cationic CTAB present in the medium and the resultant insoluble complex is visible due to methylene blue (Pacwa-Płociniczak et al., 2016). It was noted that most of the isolates formed blue halos around their respective colonies that suggested the production of anionic biosurfactants (Fig. 4.4). Graziano et al., (2016) and Roy, (2017) suggested the use of CTAB assay for rapid identification of biosurfactants producing microorganisms. In case of crude oil overlay agar assay, distinct emulsified zones were observed around the colonies of 65 % isolates (Fig. 4.5). This assay stimulated the most positive response amongst the isolates which could be due to

biosurfactants mediated degradation of hydrocarbons. Kokare et al., (2007) has stated the efficacy of crude oil overlay agar assay for simultaneous detection of biosurfactants production and hydrocarbon degradation by the producer microorganisms. In a study conducted by Shoeb et al., (2015), 65.1 % isolates formed oil degradation zones around their respective colonies. In contrast to many previous findings of Ali et al., (2013), Santhini, (2014) and El-Gamal et al., (2015), the present research work showed that not even a single isolate was found to be negative for any qualitative plate assay. However, due to high possibility of giving both false negative and false positive results, these assays should be supported by quantitative confirmatory assays of biosurfactants production.

5.4. Identification of potential biosurfactants producing microorganisms

Fourteen potential biosurfactants producing microorganisms were selected on the basis of their ability to give positive results for qualitative screening assays. These microorganisms were then subjected to microscopic, biochemical and molecular identification studies. Morphological analysis revealed the diverse phenotypic properties of microorganisms (Table 4.1). Phylogenetic characterization revealed percentage homology at species level and the isolates were named as *Bacillus pumilus* MF138116, *Paenibacillus azoreducens* MF138117, *Pseudomonas stutzeri* MF138118, *Bacillus licheniformis* MF138121, *Bacillus thuringiensis* MF138122, *Achromobacter xylosoxidans* MF138123, *Bacillus cereus* MF138124, *Pseudomonas aeruginosa* MF069166, *Bacillus subtilis* MF138125, *Bacillus* spp. MF138130, *Bacillus amyloliquefaciens* MF138127, *Pseudomonas* spp. MF099829, *Meyerozyma* spp. MF138126 and *Aspergillus terreus* MF138128 (Table 4.2) (Fig. 4.6). It has been reported that *Pseudomonas* and *Bacillus* species are the most prevalent bacterial strains at crude oil contaminated sites. Besides, various biosurfactants producing fungi especially yeasts have also been screened from the hydrocarbons contaminated soil (Mnif et al., 2011, Bezza and Chirwa, 2015, Deng et al., 2016,). Present study also reports some recently discovered genera like *Meyerozyma* and *Paenibacillus* from the affected brownfield of Chak Naurang, Punjab which suggests that this site harbors diversified

biosurfactants producing microbial communities and can serve as an important source for the exploration of novel biosurfactants producing microorganisms.

5.5. Quantitative screening of biosurfactants producing microorganisms

The quantitative screening of biosurfactants producing microorganisms was ascertained by oil displacement assay, emulsification index analysis and surface tension measurement because of reliability, efficacy and sensitivity of these methods for detecting biosurfactants. In addition, the production of biosurfactants is directly related to the emulsification activity, reduction of surface tension and higher oil displacement (Al-Bahry et al., 2013, Dhail, 2013, Ahmad et al., 2016,). Results indicated that the biosurfactants producing microorganisms showed siginificant tensioactive potential and a positive correlation was observed between the emulsification behavior and surface active properties of biosurfactants. In contrast to previous reports, more than 65 % emulsification of kerosene oil was showed by biosurfactants producing microorganisms in present study (Fig. 4.7). Measurement of surface tension is one of the direct method to enumerate the surface activity of biosurfactants. Surface tension of a solution usually decreases with an increase in concentration of surfactants, therefore the microorganisms that can reduce the ST up to 25-40 mN/m are considered as eminent biosurfactants producers. Minimum surface tension of 25 mN/m was recorded for *Meyerozyma* spp. MF138126 (Fig. 4.8). Similarly, results of oil displacement assay exhibited potent surface active properties of strains and clearing zones of varying diameters were witnessed. The biggest zone of 8 cm was observed in case of *P. aeruginosa* MF069166 though, other isolates also produced significant zones ranging from 3.5-7 cm (Fig. 4.9). These findings showed that the biosurfactants producing microorganisms exhibited promising emulsification and surface active properties both qualitatively and quantitatively. Previously, Ahmed et al., (2014) reported 65 % $E.I_{24}$ for a bacterial strain whereas, Gautam et al., (2014) reported a clearing zone of 5 cm in ODA by the biosurfactants from fungi.

5B

B- Optimization of bioprocess using design of experimental approach for biosurfactants production from selected microbial isolates

5.6. Selection of *Pseudomonas aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

Results of qualitative and quantitative screening assays of biosurfactants showed that the two isolates i.e. *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 had comparatively better physiochemical properties and higher cellular productivity of biosurfactants. *P. aeruginosa* is known to produce rhamnolipid type of biosurfactants which have been used in wide range of the applications (Liu et al., 2018a). Due to ease of production, better physico-chemical properties and higher cellular yield as compared to the other rhamnolipid producers, *P. aeruginosa* is considered as a potential candidate for the high volumetric production of rhamnolipids (Araújo et al., 2018). As rhamnolipids show extraordinary stability under extreme temperature, pressure and other hostile conditions, most of the efforts are being made to make this biosurfactant commercially viable. Still, there are several impediments for the high volumetric production of rhamnolipids that invites future research. Regarding sophorolipids, it is one of the best studied fungal (eukaryotic) biosurfactants with similar properties to the commercial surfactants. Based on recent studies, sophorolipids have been found active against cancer in human cell lines and holds significant importance (de Oliveira et al., 2015). In addition, being an excellent emulsifier sophorolipids are amongst those biosurfactants which could possibly replace synthetic surfactants in future (Elshikh et al., 2017). Taking together, physico-chemical properties and high cellular yield, both rhamnolipids and sophorolipids show excellent potential in wide ranging commercial applications.

5.7. Effect of fermentation media on growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

Composition of fermentation media plays significant role in the production of various commercially important products including biosurfactants. Therefore, designing

phosphates in the medium enhances the yield of biosurfactants particularly in eukaryotic microorganisms, as majority of phosphate molecules are utilized in the assembly of cellular organelles. Previously, different media formulations have been reported for enhanced production of sophorolipids from the yeast cells (Rispoli et al., 2010, Parekh and Pandit, 2011). Current findings suggested that nutrients stressed conditions resulted in highest yield of biosurfactants and maximum microbial growth. Therefore, the use of media M 2 for *P. aeruginosa* MF069166 and M 5 for *Meyerozyma* spp. MF138126 was recommended and implemented in further studies. Biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126, was found to be associated with cellular growth and maximum yield was obtained when the microbial growth was increasing exponentially. The production of biosurfactants was found as a function of microbial growth in some previous reports and current research finds reasonable agreement with the findings of Wu et al., (2008) and Santos et al., (2016).

5.8. Optimization of media components through Plackett-Burman design for growth and rhamnolipids production from *P. aeruginosa* MF069166

There is an increasing demand of biosurfactants owing to their multifaceted properties and applications. As previously mentioned that commercial viability of biosurfactants mainly rely on the bioprocess cost therefore, optimization of the media is considered important for enhancing the product yield. Fermentation is the basic process pertaining to enhance the quality and quantity of biosurfactants. Generally, maximum yield is obtained when the cultivation conditions are most favorable. Previously classical optimization techniques were used to enhance the production of biosurfactants however more time consumption, variables associated expenses, poor interactive effect, and lack of prediction are some of the major drawbacks being associated with conventional optimization methods. These problems can be effectively addressed by the application of latest statistical optimization methods like Plackett-Burman and Box Bankham designs which allow the multi-factors variability at a time. These methods have several advantages such as accuracy of the

process information, optimum reaction conditions in less time, less number of experimental runs and reduction of the process cost (Bertrand et al., 2018).

In present studies, the two isolates i.e. *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were investigated under the effect of different concentrations of media nutrients already selected through aforementioned fermentation experiments. For this purpose, Plackett-Burman design was used to optimize and establish interactive effect amongst different media components. Optimization of media is a prerequisite that ensures the metabolic efficiency of microorganisms at lab scale and further aids in up gradation of bioprocess at industrial level. Besides the right selection of different media nutrients, it is necessary to determine their relative proportion in order to increase cellular growth and yield of bio-based products. Recent reports are available where interactive effects of different variables were studied through statistical optimization (Almeida et al., 2017, Bertrand et al., 2018). After experimental runs, the design and effect of each variable was evaluated through aforementioned statistical package and results were summarized in Table 4.3 and 4.9.

5.8.1. Significance of media components for growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

In order to estimate the effect of media composition on growth and biosurfactants production of both strains, Plackett-Burman design was used. For *P. aeruginosa* MF069166, glycerol, peptone, K_2HPO_4, KNO_3, NaCl, yeast extract and $MgSO_4$ were determined as significant factors for growth and rhamnolipids production whereas, glucose, NaH_2PO_4, $FeSO_4$ and $CaCl_2$ were non-significant terms (Table 4.4 and 4.5). Similarly, for *Meyerozyma* spp. MF138126, glycerol, peptone, NaH_2PO_4, yeast extract and $MgSO_4$ were significant media components whereas, the glucose and K_2HPO_4 were non-significant terms (Table 4.10 and 4.11). These findings also indicated the effect of essential macro and micronutrients on growth and biosurfactants production. Amongst the macro-nutrients, carbon is fundamental to all life forms and significantly affects growth and

metabolism of microorganism. It has been observed that carbon substrate affects the chemical composition of biosurfactants. Moreover, soluble and insoluble carbon substrates lead to different cellular productivity. In case of present research, glycerol was found to be a significant model term for microbial isolates both in terms of growth and biosurfactants production (Fig. 4.12, 4.13, 4.16 and 4.17). Putri and Hertadi, (2015) reported 2-6 % concentration of glucose for optimized production of biosurfactants from the strain BK-AB12 whereas, Eraqi et al., (2016) suggested 3 % glycerol for maximum yield of biosurfactants. In case of nitrogen, peptone, yeast extract and KNO_3 significantly affected the growth of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and, biosurfactants production (Fig. 4.12, 4.13, 4.16 and 4.17). However, the effect varied with respect to different concentrations of nitrogen sources provided in the fermentation broth. Nitrogen is the second most important macronutrient in maintaining growth and metabolism. As discussed previously, the production of both rhamnolipids and sophorolipids is associated to microbial growth therefore, the presence of both organic and inorganic nitrogen sources could be justified due to their fundamental role in synthesis of cellular materials and escalating the metabolic pathways. In some previous findings, high yield of rhamnolipids and sophorolipids has been reported in the presence of peptone, yeast extract and several inorganic nitrates (Jiménez-Peñalver et al., 2016, Ozdal et al., 2017). Phosphates are considered as essential components of cell membrane associated phospholipids, nucleic acids and ATP therefore, their role is considered vital in maintaining cell growth and enhancing biosurfactants production. Results of present study suggested the significant effect of phosphates containing salts on growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 (Fig. 4.12, 4.13, 4.16 and 4.17). These findings are in close conformity with previous reports where increase in biosurfactants concentration was witnessed when phosphates were supplemented in the production medium (Varjani and Upasani, 2017). Likewise, $MgSO_4$ was another significant factor and had a positive effect on growth and biosurfactants production from both isolates. Magnesium is one of the key micronutrient that plays a fundamental structural and functional role in different cellular systems. It is used in the

assembly of integral cellular structures like cell wall and ribosomes. Significance of magnesium for microbial growth and high yield of biosurfactants has also been reported by Wei et al., (2007), Rodrigues, (2015) and Gudiña et al., (2015). Collectively, the final medium formulation was selected on the basis of path of steepest ascent for both strains. After selection and initial optimization, for *P. aeruginosa* MF069166 aforementioned significant media components were added in the final media formulation and 3.6 g/L rhamnolipids was produced. Whereas, in case of *Meyerozyma* spp. MF138126 a yield of 4.9 g/L of sophorolipids was obtained. This indicates the significance of statistical optimization in enhancing the metabolite production as multiple fold increase in the quantity of biosurfactants was witnessed under optimized media conditions.

5.9. Optimization of cultivation conditions through Response Surface Methodology (RSM) for growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

Response surface methodology (RSM) is one of the most important statistical tool for improving the productivity of a given fermentation process. RSM is useful for predicting the response of interest and estimating the simultaneous effect of different parameters on product optimization. The application of RSM has been recommended in design and formulation of novel microbial metabolites and, to enhance the yield of existing products. This is the reason why bioprocess optimization through RSM has gained considerable appreciation.

In present study, the growth and biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was investigated under the effect of four process bioprocess variables i.e. pH (A), temperature (B), size of inoculum (C) and agitation speed (D). The significant effect of environmental conditions was determined through response surface methodology. 2^4 central composite design (CCD) was employed for the bioprocess optimization. This method ensures orthogonality, uniformity, rotatability and high quality

predictions for the bioprocess. After experimental runs, the effect of every parameter was statistically evaluated and results are presented in Table no. 4.7, 4.8, 4.13 and 4.14.

5.9.1. Effect of pH on growth and biosurfactants production

The effect of pH on microbial growth and biosurfactants production was investigated using RSM. After statistical analysis, results demonstrated a significant effect of pH on the metabolism of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126. The bacterial isolate showed maximum growth and rhamnolipids production (2.65, 3.16 g/L) within a pH range of 6-7.5 (Fig. 4.14 and 4.15) whereas, the pH range for optimum growth and sophorolipids production (3.16, 4.28 g/L) from *Meyerozyma* spp. MF138126 was found to be 4.5-6 (Fig. 4.18 and 4.19). It was further observed that acidic and basic extremes did not favor the microbial growth and biosurfactants production. This could be due to the denaturation of different enzymes necessary for microbial metabolism at high concentration of H^+ and OH^- ions in the fermentation media. Many previous findings has stated the role of pH to be the most significant for growth and biosurfactants production however, the exact role of pH on biosurfactants related metabolism has not been fully unnderstood. The present results are in close conformity with previous findings where neutral pH range was reported for growth and rhamnolipids production from *Pseudomonas* species whereas, different strains of yeast were known to grow under slightly acidic conditions. Saikia et al., (2012) reported an optimum pH of 7-8 for maximum rhamnolipids production from *P. aeruginosa* RS29. In another study, Parekh and Pandit, (2011) detected the maximum yield of sophorolipids from the strain of *C. apicola* at pH 5.5.

5.9.2. Effect of temperature on growth and biosurfactants production

The effect of temperature was studied on growth and biosurfactants production from the *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 through RSM. It was noted that different temperature treatments significantly affected the microbial responses however, optimum temperature range was found to be in mesophilic region for both strains. *P.*

aeruginosa MF069166 showed maximum growth O.D of 2.65 and 3.15 g/L of rhamnolipids production at a temperature range of 20-40 °C (Fig. 4.14 and 4.15) whereas for *Meyerozyma* spp. MF138126, growth O.D of 3.16 and sophorolipids production of 4.28 g/L was observed from 20-45 °C (Fig. 4.18 and 4.19). Low and high temperature extremes of 10 and 57 °C hampered the microbial metabolic processes therefore, very low turbidity of culture medium and almost no yield of biosurfactants was detected. In present study, production of biosurfactants was found to be growth associated, for that reason the physical conditions that restricted the growth of bacterial and yeast species negatively affected biosurfactants production. These findings are in close conformity with some previous reports where the direct effect of temperature on microbial growth and biosurfactants production was suggested by Souza et al., (2018) and Perfumo et al., (2018). Lan et al., (2015) reported a temperature range of 25-42 °C for maximum growth and rhamnolipids production from *Pseudomonas* SWP-4 whereas Zhou et al., (2019) reported an optimum temperature of 25 °C for highest yield of sophorolipids from *C. bombicola*.

5.9.3. Effect of inoculum size on growth and biosurfactants production

The significance of varying concentrations of inoculum (SOI) was studied by the application of Central Composite Design (CCD) strategy. Results of present research work suggested an average inoculum size of 2-3 % for maximum microbial growth and highest biosurfactants production from the two isolates. At 3 % inoculum size, 2.65 growth O.D and 3 g/L of RL was detected for the bacterial strain (Fig. 4.14 and 4.15) whereas, an O.D of 3.16 and 4 g/L of SL was estimated for the yeast species (Fig. 4.18 and 4.19). It was further noticed that low and high percentage of inoculum negatively affected the microbial metabolism. At 1 % SOI, very low culture density was observed which resulted in mild yield of rhamnolipids and sophorolipids in their respective fermentation media. High inoculum size of 5 % and more was not favored at all due to significant reduction in microbial food/mass ratio. This scarcity of nutrients in the fermentation broth could result in high competition amongst the microorganisms and switch off certain metabolic

pathways. Similar type of results were also reported previously where optimum microbial growth and biosurfactants production was detected at medium concentration of inoculum whereas, high and low SOI negatively affected the bioprocess (Brasileiro et al., 2015, Sharma et al., 2018).

5.9.4. Effect of agitation speed on growth and biosurfactants production

The effect of agitation speed on growth and biosurfactants production was monitored by providing different RPM to the experimental runs as prescribed by the statistical design. Results demonstrated a direct effect of RPM on microbial responses. It was noted that at low RPM, microbial growth and biosurfactants production was considerably less however, a significant increase was witnessed with an increase in shaking speed. 3.15 g/L RL (Fig. 4.14 and 4.15) and 4.28 g/L of SL was produced at an optimum agitation speed of 150 RPM (Fig. 4.18 and 4.19). Considerable yield of glycolipids biosurfactants was also obtained at 180 RPM however, further increase did not provide the desired results. The role of agitation in different fermentation systems has been well understood. Continuous agitation is necessary for efficient growth and metabolism of aerobic microorganisms. It facilitates the transfer of oxygen from gaseous phase to liquid phase in reaction mixture. Moreover, mass transfer rate of media components can be enhanced when good agitation speed is provided to fermentation broth. It has also been reported that microorganisms can alter certain metabolic pathways under the effect of static or shaking bioprocess conditions (De et al., 2015). Reports are available that show the importance of agitation speed on microbial growth and biosurfactants production. Generally, an average agitation speed of 140-170 RPM has been observed for maximum rhamnolipids production from *Pseudomonas* species (Kumar et al., 2012, Ji et al., 2016). Similarly, an increase in sophorolipids production from different strains of yeast was observed with an increase in shaking speed (Liu et al., 2018b, Souza et al., 2018).

The optimal conditions for growth and maximum biosurfactants production from *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were defined through *D*-

optimality method. It was observed that the software prescribed conditions resulted in a final yield of 4.31 g/L of rhamnolipids and 6.9 g/L of sophorolipids which was respectively 200 % and 400 % higher than the quantity of biosurfactants obtained after conventional optimization method. Therefore, it can be concluded that the application of mathematical modelling techniques were proved to be very efficient to enhance the productivity of rhamnolipids and sophorolipids in the fermentation process. These findings are in accordance with the previous reports of El-Housseiny et al., (2016) and Jiménez-Peñalver et al., (2016) who reported multifold increase in the production of rhamnolipids and sophorolipids respectively, after optimizing the cultivation conditions through RSM.

5C

C- **Downstream processing and characterization of biosurfactants**

5.10. Characterization of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

The chemical characterization of rhamnolipids and sophorolipids produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was done through five different analytical techniques.

5.10.1. Thin Layer chromatography (TLC)

Thin layer chromatography is one of the most simple analytical technique that results in the pure quality of biosurfactants. Silica-coated glass plates of variable thickness were applied with the crude extract of rhamnolipids and sophorolipids, and run in different solvent systems. Non-destructive technique of UV visualization was used to spot the bands (Fig. 4.20 a and b). In case of rhamnolipids, the obtained Rf values of 0.36 and 0.84 showed that crude biosurfactants extract from *P. aeruginosa* MF069166 was comprised of two RL species i.e. RL-1 and RL-2. These results are in close conformity with Das et al., (2014), and Noramiza Sabturani et al., (2016) who reported 0.9 and 0.83 Rf values for mono-rhamnolipids and 0.4 and 0.36 Rf values for di-rhamnolipids. Similarly, the biosurfactants extracted from *Meyerozyma* spp. MF138126 exhibited five bands of different Rf values ranging from 0.17 to 0.65. Under UV light three spots of lactonic sophorolipids (LS) were detected with Rf values of 0.45, 0.52 and 0.65 whereas, two bands of acidic sophorolipids (AS) were appeared with the Rf values of 0.17 and 0.21. These results are in accordance with previous findings of Konishi et al., (2016) and Sen et al., (2017) who reported Rf values for sophorolipids within a range of 0.20-0.75 and, 0.13-0.68, repectively.

5.10.2. FTIR Spectroscopy

FTIR spectroscopy is a very handy technique to detect the molecular structures that differ in their vibrational and rotational states of energy. Infra red is a type of radiation that can

pass through vaccuum and in turn alters the kinetic energy of molecules through the oscillation of chemical bonds at particular frequencies. IR region covers the electromagnetic spectrum ranging between 0.78-1000 mm therefore, functional groups associated with liquids, solids and gasses can be easily determined through IR spectroscopy. In present research, crude biosurfactants extracts of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were compared with their respective standards through FTIR spectroscopy. The standard molecules of rhamnolipids and sophorolipids were purchased from Sigma Aldrich. The resultant chromatograms revealed identical peaks for test and standard molecules. In case of rhamnolipids, the crude sample and purified standard gave bands within the region of 3200-2853 cm^{-1} that confirmed the presence of lipid moiety within the molecules. The carbonyl stretch, particular for carboxylic acids and esters, was present in both test and standard compounds. Similarly, the fingerprint region ranging from 1239–1457 cm^{-1} represented the carbohydrate portion of glycolipids (Fig. 4.21 a and b). This structural similarity of crude extract with the standard molecules and some of the previous reports showed that the biosurfactants produced by *P. aeruginosa* MF069166 were rhamnolipids in nature (Rahman et al., 2010, Rikalović et al., 2012, Bazsefidpar et al., 2019). Similarly, the crude biosurfactants extract of *Meyerozyma* spp. MF138126 gave peaks quite identical to the standard sophorolipids molecules. The broad band at 3200-3500 cm^{-1} showed the presence of OH groups whereas, the characteristic stretching of methylene groups was observed in the region of 2800-2900 cm^{-1}. Moreover, the structural similarities especially in the fingerprint region of lactones and acidic SLs confirmed that the biosuractants produced by yeast were sophorlipids in nature (Fig. 4.21 c and d). These results were further compared with the relavant literature and found in reasonable agreement with previous findings (Daverey and Pakshirajan, 2010, Chandran and Das, 2011, Shah et al., 2017).

5.10.3. Reverse Phase HPLC

Reverse phase HPLC was used for separation and identification of different structural congeners present in crude biosurfactants. This type of chroamtography involves a ploar mobile phase and a non-polar stationary phase. After injecting the sample, hydrophobic structural components binds with the hydrophobic stationary phase whereas, the hydrophilic molecules easily pass through the column and are normally eluted first. The fractions collected for individual peaks are used to analyze the structure of each moiety. In present research, standard rhamnolipids and sophorolipids were run alongwith the test samples. It was observed that the elution profile of crude rhamnolipids was comparable with the standard RL molecules (Fig. 4.22 a and b). The chromatogram of bacterial rhamnolipids displayed five major fractions eluting at retention time of 2.59, 2.85, 16.13, 18.45, 20.50, 22.59 and 24.30 min. It has been reported that because of low hydrophobicity molecular congeners with shorter fatty acid chains are eluted before the congeners with longer fatty acid chains (Twigg et al., 2018b). Besides, degree of saturation/unsaturation of rhamnose and HAA moieties also affect the rate of elution (Wei et al., 2008). Behrens et al., (2016) purified crude rhamnolipids produced by a strain of *P. putida* through Liquid Chromatography and characterized the resulting fractions through Mass Spectrometry.

For *Meyerozyma* spp. MF138126, elution profiles of standard and crude sophorolipids were also compared. Seven fractions were obtained for crude SLs at retention time of 1.69, 1.98, 2.75, 6.18, 13.26, 15.48 and 19.16 min (Fig. 4.22 c and d). According to literature, acidic forms of SLs are eluted before the lactonic SLs (Thaniyavarn et al., 2008). Structural differences in terms of saturation/unsaturation, position of hydroxylation, chain length of fatty acid moiety and, the lactonization and acetylation pattern of sophorose sugar determine the elution profile of sophorolipids (Ribeiro et al., 2012). 14 components of sophorolipids were separated on the basis of lactonization and acetylation pattern (Van Bogaert et al., 2007) whereas in another study, Davila et al., (1993) identified more than 20 isomers by analyzing the structural differences in fatty acid moiety through HPLC. Samtani et al., (2018) also purified and characterized the acidic and lactonic forms of sophorolipids through RP-HPLC.

5.10.4. Nuclear Magnetic Resonance (NMR)

To further consolidate the findings of LC-ESI-MS analysis, ^{1}H and $^{13}CNMR$ was done for rhamnolipids and sophorolipids. NMR technique is based on atomic transitions associated with magnetic moment when an external magnetic field is applied. Once the nucleus absorbs the radiation, it flips or spins in the direction of higher energy, re-emits the radiations and then return to the low-energy state. Magnetic field strength and magnetogyric ratio of every nucleus determine its NMR transition energy. For $^{1}HNMR$ spectroscopy of rhamnolipids produced by the *P. aeruginosa* MF069166, resonance obtained at 0.88 and 1.27 ppm indicated the adjacent presence of CH_3 and CH_2 groups in the fatty acid chain whereas, the multiple signals obtained at 2.55 ppm indicated that CH_2 groups were adjacent to carboxylic acids and esters, as well. The protons present on rhamnose moiety resonated within a range of 4.9-5.47. In $^{13}CNMR$ spectrum of rhamnolipids, the RL1 and RL2 molecules were detected at 95.30 ppm and, 94.48 and 102.72 ppm, respectively (Table 4.17) (Fig. 4.23 a and b). These results were in consistence with previous reports where mono and di-rhamnolipid molecules from different *Pseudomonas* species were detected at similar resonance frequencies through ^{1}H and $^{13}CNMR$ (Lan et al., 2015, Oluwaseun et al., 2017).

The presence of acidic and lactonic sophorolipid molecules in the SL extract of *Meyerozyma* spp. MF138126 was confirmed through ^{1}H and $^{13}CNMR$ spectroscopy. In $^{1}HNMR$ spectrum, multiple peaks were obtained for the protons attached to sugar and fatty acid moiety of SL molecules. Similarly, the presence of deacetylated and diacetylated lactonic and, acidic sophorolipids was confirmed through $^{13}CNMR$ spectroscopy. The spectrum revealed resonance at 102.72 and 103.51 ppm for sugar molecule whereas the presence of carbonyl group was detected at 170.61 ~ 174.08. Furthermore, the $-CH_2$ groups of hydroxyoleic acid resonated at 21.73 ~ 38.81 ppm (Table 4.18) (Fig. 4.23 c and d). These findings are in close conformity with the reports of Wadekar et al., (2012), Joshi-Navare and Prabhune, (2013) and Elshafie et al., (2015).

5.10.5. Liquid chromatography Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

LC-ESI-MS is a type of mass spectrometry where the sample is initially separated by liquid chromatography and then subjected to ionization in an electric field to produce a fine mist of charged droplets. This aerosol of multi-charged ions prevent the fragmentation of macromolecules and effectively extend the mass range of analyzer. This technique combines the resolving power of HPLC and high accuracy of a mass spectrometer. For this particular reason, the detailed characterization of test and standard rhamnolipids and sophorolipids was done using the positive mode of LC-ESI-MS. Results demonstrated that rhamnolipids produced by the *P. aeruginosa* MF069166 were structurally diverse containing a plethora of mono and di-RL congeners which further differed in the chain length and degree of saturation/unsaturation of lipid moiety (Fig. 4.24 a and b). The major congeners were di-rhamnolipidic in nature and identified as L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydodecanoate (Rha-Rha-C_{10}-C_{12}), L-rhamnosyl-L-rhamnosyl-β-hydroxydodecanoate mono-unsaturated (Rha-Rha-$C_{12:1}$), L-rhamnosyl-L-rhamnosyl-β-hydroxydodecanoyl-β-hydroxydecanoate (Rha-Rha-$C_{12:1}$-C_{10}) and L-rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (Rha-Rha-C_{10}-C_{10}) with relative abundance of 24.5, 22.7 22.4 and 5.61 %, respectively. However, 24 % of L-rhamnosyl-β-hydroxydodecanoate –di unsaturated (Rha-$C_{12:2}$) was also detected in the rhamnolipids mixture of the bacterial strain. Moreover, LC-ESI-MS analysis of standard RL molecules showed the predominance of aforementioned Rha-Rha-C_{10}-C_{12}, Rha-Rha-$C_{12:1}$ and Rha-$C_{12:2}$ congeners (Table 4.15). These results are also in line with Pantazaki et al., (2011), Ndlovu et al., (2017) and Twigg et al., (2018a) where they found similar RL congeners with identical structures and same range of molecular masses.

The positive mode of LC-ESI-MS aided in detailed structural elucidation of sophorolipids produced by *Meyerozyma* spp. MF138126 and standard molecules. A number of protonated and sodiated adducts of lactonic and acidic sophorolipids were detected in the

sophorolipids mixture of yeast (Fig. 4.24 c and d). Specifically, diacetylated lactone was present as major constituent in the form of $[M+H]^+$ and $[M+Na]^+$ with relative abundance of 29.4 and 31 %, respectively. Besides, acidic SL ($C_{11:0}$) was also present in a relative concentration of 23.2 % (Table 4.16). In addition to these pseudomolecular ions, various adducts of sophorose moiety were spotted in minor quantity. These results were in close conformity with the standard molecules in which varied fragments of diacetylated acidic ($C_{18:1}$), diacetylated lactonic ($C_{18:1}$) diacetylated lactone ($C_{16:0}$) and monoacetylated lactone ($C_{18:1}$) were present. Sen et al., (2017) reported different SL isomers of the acidic and lactonic sophorolipids with the same range of molecular masses from an YS3 strain of *Rhodotorula*. In another study, Konishi et al., (2016) reported the production of acidic and lactonic SLs from a yeast species with *m/z* values of 729 and 711, respectively.

5.11. Stability of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

5.11.1. Thermal stability

One of the important aspect of current investigation was to analyze thermal stability of biosurfactants for various bioprocess applications. Cell free broth containing crude biosurfactants was incubated at a temperature range of 5 °C to 121 °C for 1 hour. It was observed that rhamnolipids retained their surface activity at a temperature of 15 °C to 100 °C. Moreover, no significant loss in surface tension was observed after autoclaving the supernatant (Fig. 4.25 a). For sophorolipids, a similar stability range was recorded as rhamnolipids, however, SL molecules displayed considerable surface activity at slightly elevated temperatures. Autoclaving of culture supernatant didn't affect the surface and emulsification properties of sophorolipids, as well (Fig. 4.25 b). Previous studies showed that efficient biosurfactants retain their physico-chemical properties at a wide range of temperature and donot undergo considerable structural alterations (Kim et al., 2000, Abouseoud et al., 2008). This makes biological surfactants more advantageous than their chemical counterparts i.e. SDS and Tween 80, which have been reported to lose surface

activity at extreme temperatures due to hydrolysis of fatty acid chains. Recently Chen et al., (2018) reported excellent stability of rhamnolipids from 20-100 °C. In another study, Elazzazy et al., (2015) reported the production of thermally stable biosurfactants from the bacterial isolate of Jeddah region, Saudi Arabia. Thermal stability of sophorolipids from a strain of *C. bombicola* at the tested temperatures of 40-100 °C was observed which suggested the use of prticular SL molecules in MEOR applications (Daverey and Pakshirajan, 2010).

5.11.2. pH stability

pH stability of crude biosurfactants was checked at a gradient range of 1-14 to report industrial efficacy. Rhamnolipids showed significant emulsification and surface activity from pH 4 to 11 however, acidic and basic extremes affected their surfactant properties (Fig. 4.25 c). Likewise for sophorolipids, pH stability range was found to be 3 to 10 (Fig. 4.25 d). Instability in surface activities at extremely acidic or alkaline conditions could be due to protonation or alkylation of hydrophilic portion of biosurfactants that cause the molecular precipitation (Lovaglio et al., 2011). Results showed that rhamnolipid molecules displayed comparatively better surface active properties under basic conditions whereas, the surfactant potential of sophorolipid molecules were more inclined towards acidic conditions. This difference in affinity for acidic or alkaline environment could possibly be due to the different phylogenetic origin of biosurfactants producing microorganisms. Literature review showed that *Pseudomonas* species show better metabolic capabilities at neutral or slightly alkaline conditions whereas, the yeast strains being fungal in nature display better adaptability to acidic conditions (Chebbi et al., 2017). Elshafie et al., (2015) reported a pH stability range of 2-12 for sophorolipid molecules whereas, Lovaglio et al., (2011) reported the most stable emulsion of rhamnolipids-kerosene oil at pH 8 and 9. Likewise, in some previous findings stability of glycolipid molecules has been reported at high pH due to alkali-associated fatty acid stability (Mouafi et al., 2016, Barakat et al., 2017).

5.11.3. Stability under varying NaCl concentrations

In the concluding part of experiment, effect of different concentrations of NaCl was studied on stability of crude biosurfactants. Stability of biosurfactants at high salt concentrations is desirable for bioremediation of the hydrocarbons contaminated saline soil. Results revealed that different concentration of salt did not adversely affect the surface active properties of biosurfactants. Rhamnolipids were able to withstand 10 % of NaCl (Fig. 4.25 e) whereas, considerable activity of sophorolipids was witnessed up to 12 % NaCl (Fig. 4.25 f). According to (Helvacı et al., 2004), presence of an electrolyte shields the carboxylic groups of anionic surfactants and promotes micellar growth in the solution by reducing repulsive forces. This phenomenon stabilizes the surface tension of the solution and renders the surface activities unaltered. In another study, glycolipid biosurfactants showed stability till 15 % NaCl concentration. Similar results were also reported by Khopade et al., (2012) and Chen et al., (2018).

5.12. Properties of biosurfactants produced by *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

5.12.1. Determination of critical micelle concentration (*cmc*)

cmc is defined as the minimal amount of surface active compound required to reduce the surface tension of solution to its least value and initiate the formation of micelles (Mao et al., 2015). For various process applications, biosurfactants must usually be present in a concentration higher than *cmc* due to their unique molecular attributes which remarkably vary above and below this specific concentration (Mnif and Ghribi, 2015). At low concentrations, biosurfactants monomers are present in unassociated forms. However when their concentration is increased up to a particular level, the monomers due to certain attractive and repulsive forces self-assemble into different molecular aggregates known as micelles (De et al., 2015). In present study, *cmc* of rhamnolipids produced by *P. aeruginosa* MF069166 was found to be 40 mg/L at which surface tension of the solution was recorded

as 29 mN/m (Fig. 4.26 a). Similarly the sophorolipids produced by *Meyerozyma* spp. MF138126 showed *cmc* of 50 mg/L, after which the solvent system attained a constant ST value of 33 mN/m (Fig. 4.26 b). This data is in line with some previous findings which showed that low molecular weight biosurfactants particularly glycolipids usually display low *cmc* values (Bhardwaj et al., 2016, Silva et al., 2017, Ohadi et al., 2018).

5.12.2. Size distribution through Dynamic Light Scattering (DLS)

Dynamic light scattering is a technique that measures the rate of fluctuation in the intensity of scattered light due to rapid Brownian movements of particles. The hydrodynamic diameter of sub-micron range aggregates in the sample suspension can be easily detected. In current investigation, the particle sizing of ultra-purified samples of rhamnolipids and sophorolipids was done through conventional DLS. The experiment was conducted in order to check the effect of concentration, pH and electrolytes on size of micellar aggregates present in the solutions of rhamnolipids and sophorolipids. Zhong et al., (2015), Eismin et al., (2017) and Faas et al., (2017) used the technique of dynamic light scattering to explain the variability in size and shape of glycolipid biosurfactants.

5.12.2.1. Effect of concentration on micellar size distribution

To ascertain the effect of concentration, ½ *cmc*, *cmc* and 2 *cmc* solutions of rhamnolipids and sophorolipids were analyzed using DLS. For rhamnolipids, no effect of concentration was seen on the size of particles till *cmc* was achieved and an average z-diameter of 350 nm was recorded. However, when the *cmc* was doubled, majority of structural aggregates were in the range of 700 nm diameter (Fig. 4.27 a). In case of sophorolipids, an insignificant increase in size of micelles was witnessed when the concentration of SLs was increased from ½ *cmc* to *cmc* whereas at 2 *cmc*, the molecular aggregates of more than 1000 nm were detected (Fig. 4.27 b). High PDI values showed heterogeneity in particles size whereas low PDI values depicted that majority of aggregates in the sample were of uniform size. Reports are available that suggested the formation of small and thermodynamically stable micelles

when biosurfactants were added in a concentration below *cmc* (Kitamoto et al., 2009, Song et al., 2013). These micelles were then transformed into heterogeneous giant vesicles, spheroidal ribbons or microtubes when the concentration was increased above *cmc*. This increase in micelle growth and size could possibly be due to aggregate shape transition or intermolecular aggregation when a change in concentration of biosurfactants was detected in bulk. Sánchez et al., (2007) stated the micelle to vesicle transformation of di RL congener when the concentration of rhamnolipids was increased. Similarly, other investigations also confirmed increase in micellar size for different glycolipid molecules when the concentration was above *cmc* (Baccile et al., 2012, Zhong et al., 2015).

5.12.2.2. Effect of pH on micellar size distribution

DLS revealed the presence of diverse molecular aggregates in the purified solution of rhamnolipids and sophorolipids under the effect of varying pH. In light of existing literature, 5, 7 and 9 was the pH selected for rhamnolipids (Fig. 4.27 c) whereas, the values were slightly varied to 3, 7 and 11 for sophorolipids (Fig. 4.27 d). Generally for both glycolipids, increase in pH was responsible for reducing the size of micellar aggregates whereas, low PDI values showed homogeneity in sizes. The observed reduction in particle size could be due to the partial dissociation of carboxylic moiety of glycolipids at high pH. In a typical glycolipid molecule, the sugar head group endows hydrophilicity whereas, its amphipathic properties are functionally governed by the carboxylic moiety. Slight changes in pH affects the protonation state of carboxylic group which further induces certain alterations in hydrophilic head groups and affects its diameter (Sánchez et al., 2007). When the pH is high, the net negative charge on hydrophilic head group increases, which due to electrostatic repulsion between adjacent monomers, do not favor the formation of large molecular aggregates and arrange themselves into small micelles. The results herein obtained are in close conformity with Pornsunthorntawee et al., (2009), Mendes et al., (2015) and Peyre et al., (2017) who reported a decrease in micellar size with an increase in solution pH.

5.12.2.3. Effect of electrolytes on micellar size distribution

The effect of electrolytes on particle size distribution of rhamnolipids and sophorolipids was checked. The ultra-purified solution of both biosurfactants were separately treated with 250, 500 and 1000 mM concentration of NaCl (Fig. 4.27 e and f). Post DLS analysis, it was observed that increase in salt concentration favored the formation of vesicles with large hydrodynamic radius. High PDI values particularly at maximum concentration of NaCl showed the heterogeneous presence of small and large molecular aggregates of biosurfactants in the sample. When NaCl is added into solution, Na^{+1} ions acts as soft acids and easily bind to the negatively charged carboxylate ions of glycolipids. This linkage reduces electrostatic repulsion between the neighboring biosurfactants monomers and bridges the gap between their hydrophilic head groups (Helvacı et al., 2004). For that reason, with increase in concentration of salt more aggregation of anionic rhamnolipid and sophorolipid molecules was detected. Rodrigues et al., (2017) reported similar effect of NaCl on hydrodynamic radius of rhamnolipids and found out that the vesicles size of mono and di-RL congeners increased when the concentration of salt was high. However, in some other reports reduction in size of micellar aggregates was observed due to an increase in concentration of NaCl (Sánchez et al., 2007, Dahrazma et al., 2008).

5D

D- Investigation of environmental applications of biosurfactants

5.13. Environmental applications of biosurfactants and BS producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126

In order to determine the environmental efficacy, the potential of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 and their biosurfactants i.e. rhamnolipids and sophorolipids was checked in biodegradation of crude oil and bioremediation of heavy metals.

5.13.1. Role of biosurfactants and BS producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 in biodegradation of crude oil

5.13.1.1. Determination of Cell Surface Hydrophobicity (CSH)

Cell surface hydrophobicity (CSH) is a key factor that facilitates the adhesion of microorganisms to water-hydrocarbons interfaces. Microbial adhesion to hydrocarbons (MATH) assay is a simple photometrical assay that measures the degree of CSH through determining the percent adherence to hydrocarbons. It has been reported that biosurfactants producing microorganisms exhibit high affinity for hydrophobic organic compounds (HOCs) due to surfactants induced alterations in the microbial outer membrane (Krasowska and Sigler, 2014). The surfactant monomers when adsorb to cell surface, bind to the exposed hydrophobic groups through their lipophilic tails and subsequently mask the hydrophilic portions. This net increase in membrane hydrophobicity promotes cellular adhesion to hydrocarbons and stimulates substrate diffusion and utilization (Liu et al., 2014). Results demonstrated that *P. aeruginosa* MF069166 showed maximum adherence of 70 % to Toluene whereas, the *Meyerozyma* spp. MF138126 exhibited 64.66 % adherence to crude oil (Table 4.19). Obuekwe et al., (2009) stated a positive correlation between CSH and hydrocarbon degradation through MATH assay. Zhong et al., (2016) developed a correlation between the concentration of mono-rhamnolipids and reduction in cell surface

hydrophobicity. Similarly, Reddy et al., (2018) reported that a very low concentration of rhamnolipids was required to increase the microbial cell surface hydrophobicity and escalating the rate of PAHs degradation. These results suggested that high CSH can be used as an indirect measure to enumerate microbial capability for enhanced biosurfactants production and hydrocarbon degradation.

5.13.1.2. Effect of hydrocarbons on emulsification activity of biosurfactants

Emulsification assay was used to assess the surface active potentials of crude rhamnolipids and sophorolipids against complex hydrocarbons. Emulsions are the microscopic droplets formed due to homogenous dispersion of one liquid phase into another. They are thermodynamically unstable however, due to micelles induced kinetic restrictions, the stability of emulsions could be increased for a specific period of time (Bai and McClements, 2016). Salinity and pH of the aqueous and organic phases, nature of fine particulates, emulsion stabilizing nature of biosurfactants, threshold temperature and the chain length of hydrophobic substrates are some other factors that determine the kinetics of emulsion systems (de Sousa, 2017). In current study, it was observed that rhamnolipids produced by *P. aeruginosa* MF069166 emulsified 87 % of crude oil, 86 % of diesel and 85 % of mustard oil. Similarly, the sophorolipids produced by *Meyerozyma* spp. MF138126 showed maximal $E.I_{24}$ of 90 % for crude oil followed by 84 % and 81 % for diesel and petrol (Fig. 4.28). The results obtained were in accordance with the findings of Khopade et al., (2012) and Velmurugan et al., (2015) who reported significant emulsification of hydrocarbons through microbial surfactants. In another study, Pornsunthorntawee et al., (2009) found an increase in emulsification activity of glycolipid biosurfactants for complex hydrocarbons like vegetable and crude oil. The ability of biosurfactants to emulsify HOCs enhances the bioavailability of contaminants and improves their biodegradation rate. Therefore, it can be concluded that emulsification of hydrophobic compounds is a prerequisite that determines the efficacy of biosurfactants and their producer microorganisms in bioremediation of oil contaminated sites.

5.13.1.3. Biodegradation of crude oil

Crude oil is a mixture of aliphatic and aromatic hydrocarbons of varying complexity and hydrophobicity. Considering the chemical heterogeneity and associated toxicities, petroleum hydrocarbons are enlisted as priority pollutants by EPA (Rahman et al., 2017). Amongst the various crude oil remediation processes, biological practices are considered as most attractive because of their operational feasibility, cost effectiveness and capability to degrade pollutants with high efficiency. Bioremediation implies the application of indigenous microorganisms that can use hydrocarbons as sole source of carbon and energy and facilitate natural biodegradation process through the production of secondary metabolites like enzymes and biosurfactants (Santos et al., 2016). In present investigation, utilization of crude oil of Chak Naurang through biosurfactants producing microorganisms *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was monitored during the biodegradation process (Fig. 4.29 a and b).

Based on primary observation, obvious morphological changes were detected in crude oil during three weeks of incubation. First, it appeared as a thin film floating on the surface of aqueous medium. Later, this film was converted into small droplets that dispersed throughout the aqueous phase and lastly, these droplets were completely homogenized and eventually disappeared into the culture medium. A number of short and long chain alkanes were present in crude oil however, due to ease of availability, short chain alkanes were likely to be processed first. The degradation of low molecular weight components of crude oil lead to an increase in cellular biomass and emulsification droplets which resulted in a more turbid culture medium (Bagby et al., 2017). The growth pattern of the two isolates suggested that they harbored a very short lag phase followed by a longer log phase of almost two weeks. Subsequently, during third week of incubation microorganisms achieved a stable stationary phase and showed no considerable increase in cellular biomass. Moreover, it was noted that the yeast strain due to its eukaryotic origin yielded more biomass than the bacterial species. The display of conventional growth pattern showed that both isolates had not only effectively acclimatized themselves to the surrounding

hydrophobic organic compounds but also utilized these complex organics as primary growth substrates. Similar observations were made previously where simultaneous increase in microbial growth and crude oil degradation was detected (Guo-liang et al., 2005, Zavareh et al., 2016).

In biodegradation systems of the two isolates, significant increase in biosurfactants production was witnessed with the rapid transition of microbial growth from lag phase to log phase. At the end of log phase, bacterial strain reduced surface tension of culture medium to 29 mN/m (Fig. 4.29 a) whereas, in case of yeast species, minimum ST value of 28.6 mN/m was recorded (Fig. 4.29 b). However after three weeks, a slight reduction in concentration of biosurfactants was observed which could be due to degradation or utilization of surface active molecules in facilitated transport of complex organics. The current findings revealed a positive correlation between the consumption of crude oil, increase in cellular biomass and production of biosurfactants during the biodegradation process.

In the concluding part of experiment, crude oil degradation percentage of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 was determined (Table 4.20 and Fig. 4.30). Gravimetric analysis demonstrated a continuous decline in the quantity of crude oil in fermentation medium and the final degradation of 91 % and 87 % was recorded for *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126, respectively. Afterwards, residual crude oil extracted from biotic and abiotic samples was analyzed through mass spectroscopic studies. It was observed that the low molecular weight alkanes i.e. n-octane, nonane, decane, undecane tridecane, pentadecane and hexadecane, detected in the abiotic control sample, were completely absent from the two biotic samples. This showed that the Biodegradation Efficiency (B.E) of the two stains for 16-C-alkanes was 100 %. The remaining high molecular weight halogenated alkanes like Bromo-dodecane, octadecane-1-chloro and tridecane, 1-iodo were utilized up to 80 % by the two microorganisms. These chromatographic results confirmed the potential of biosurfactants producing microorganisms in facilitating the solubilization of hydrophobic organics and escalating

the biodegradation rate of petroleum hydrocarbons. These results were in alignment with Das and Mukherjee, (2007) who stated that production of biosurfactants boosts the overall degradation process of crude oil. In another study, Priya et al., (2016) related the production of biosurfactants with the increase in cellular biomass of *C. viswanathii*. Recently, (Patowary et al., 2017) reported 82 % B.E of a non-optimized biosurfactant producing isolate; *P. aeruginosa* PG1, after five weeks of incubation. Similarly, the B.E of a strain of *B. subtilis* was 82 % after a long incubation period of 18 days (Bezza and Chirwa, 2015). Parthipan et al., (2017a) reported 86 % B.E of a mixed microbial consortia containing two optimized biosurfactant producing microorganisms, whereas in a separate study, he stated 87 % B.E of an optimal biosurfactant producing strain *B. subtilis* A1 after seven days of incubation (Parthipan et al., 2017b).

5.13.2. Role of biosurfactants and BS producing *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 in bioremediation of heavy metals

Heavy metals are the second most persistent and recalcitrant pollutants to be found in soil and aquatic environment after petroleum hydrocarbons. In the last few decades, an alarming increase in heavy metals concentration has been reported in many industrialized cities (Liu et al., 2017). Different physical, chemical and biological approaches have been reported for the removal of heavy metals from the environment. Recently, the use of biosurfactants has been favored for removal of metals and metalloids through the phenomena of ion-exchange and surfactants associated complexation (Mondal et al., 2015). For this specific reason, the two biosurfactants producing microorganisms i.e. *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 were tested for their ability to decontaminate and detoxify heavy metals.

Preliminary assays were performed in order to screen out the varying toxic effects of heavy metals on microbial growth and biosurfactants production. Plate diffusion method was used to establish minimum inhibitory concentration (MIC) of cadmium, chromium, copper, lead and zinc for *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 (Table 4.21). High

incidence of heavy metals resistance in this assay suggested the potentials of two isolates in bioremediation studies. Moreover, high emulsification indices of rhamnolipids and sophorolipids in the presence of different concentrations of heavy metals exhibited their potential for decontamination purposes (Fig. 4.31). Results demonstrated that lead and zinc posed least toxic effect both on microbial growth and biosurfactants production therefore, further studies were performed in accordance with these two heavy metals. In the next experiment, biosurfactants mediated metal removal was evaluated through atomic absorption spectroscopy. After treatment, very low percentage of lead and zinc was detected in the solution which could be due to the co-precipitation of heavy metal ions with biosurfactant molecules (Table 4.22 and 4.23). It was noted that increase in concentration of biosurfactants resulted in high metals removal. Another noteworthy finding of present study is that significant removal percentage was detected by rhamnolipids and sophorolipids at concentrations below *cmc*. Literature survey suggested that anionic surfactants bind to cationic metal ions through strong ionic linkages and form non-ionic complexes which can be easily removed from the system through centrifugation. The metal ions are entrapped within the biosurfactants micelle and cannot be desorbed onto the soil. The resultant neutral complexes are then detached from soil or water surface due to reduction in interfacial tension (Sarubbo et al., 2015). This property of microbial surfactants to chelate heavy metal ions at different *cmcs* may find important applications in industrial wastewater treatment. The conductivity measurements were done in order to compare the surface active potentials of rhamnolipids and sophorolipids with chemical surfactants i.e. SDS, Tween 20 and Tween 80 at different *cmcs*. Electrical conductivity is defined as a measure of ionic content of the solution. Significant decrease was observed in the reduction of electrical conductivity with an increase in concentration of rhamnolipids and sophorolipids (Table 4.24). The electrical conductivity of zinc chloride solution decreased up to 5.4 µS at 2 *cmc* of rhamnolipids. Similarly, for lead nitrate, least value of 8.5 µS was recorded when *cmc* was doubled. This decline in electrical conductivity could be due to the encapsulation or chelation of free metal ions by biosurfactants which reduces their availability in the solution. Chemical surfactant i.e. SDS, reduced the E.C of solution

however, with an increase in their concentration high conductivities were detected. Identical charges and high charge to mass ratio could be the possible reasons behind this observation. In case of non-ionic surfactants, no significant reduction in E.C was observed due to their inability to bind with heavy metal ions. These findings are in close conformity with some previous reports. Mulligan, (2009) stated the positive role of different concentrations of biosurfactants in heavy metals removal. Similarly Das et al., (2009) reported insignificant changes in the solution E.C of heavy metals after the addition of Tween 20, Tween 60 and Tween 80. However, in a study conducted by Luna et al., (2016) increase in E.C of heavy metals solution was witnessed with an increase in sophorolipids concentration.

Latest biotechnological interventions have renewed the interest of modern day researchers in soil washing through various bio-based products. For this particular reason, the use of biosurfactants has been favored due to associated low environmental risks and high affinity for heavy metal ions. Therefore, in the concluding part of experiment, the potential of rhamnolipids and sophorolipids produced by the two microbial isolates was evaluated for soil washing. Results demonstrated high metal removal percentages for both compounds in their crude and partially purified forms (Table 4.25). For all the three concentration tested, more than 75 % removal of heavy metals was observed however, comparable percentages were also detected in case of cell free broths. These findings suggested the possible use of crude biosurfactants in heavy metals remediation studies which would decrease the production cost up to 60 %. The kinetics of soil washing studied with crude biosurfactants demonstrated significant increase in removal percentage with the passage of time (Fig. 4.32 a and b). Different mechanisms of precipitation-dissolution and counter ion-association have been reported for heavy metals removal from the soil surface through the action of biosurfactants (Mao et al., 2015). In case of anionic biosurfactants, the sequential extraction of metal ions occurs through the sorption of biosurfactants to the soil surface followed by the transfer of metals to the solution and their consequent binding to micelles through electrostatic interactions. Literature suggested minimal interaction of

biosurfactants with the ground for efficient remediation of heavy metals therefore, the biosurfactants should remain active in aqueous phase (Santos et al., 2016). Removal efficiency of biosurfactants depends upon their charge and hydrophobicity as well as structure, texture, clay content and ionic strength of the soil system. The present results are in accordance with the findings of Mulligan et al., (2001), Das et al., (2009), de França et al., (2015) and Sarubbo et al., (2018) where an increase in heavy metals removal was observed after treating the soil with microbial surfactants. However, the approach of process optimization for soil washing has also been suggested that could further enhance the heavy metals removal efficiency of biosurfactants and make the field trials cost effective.

6- CONCLUSIONS

On the basis of the results, following conclusions were made from the present work

- The soil of Chak Naurang, oil field was adversely affected by the petroleum hydrocarbons and lacked various nutrients as compared to the control soil sample.
- Crude oil contaminated soil harbored diverse microbial communities of hydrocarbons degrading and biosurfactants producing microorganisms.
- On the basis of various qualitative and quantitative tests, two efficient biosurfactants producing strains were screened and identified as *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126. The biosurfactants from *P. aeruginosa* MF069166 showed the emulsification activity of 84 % and reduced the surface tension of liquid media to 26.6 mN/m whereas, the biosurfactants from *Meyerozyma* spp. MF138126 exhibited an emulsification index of 82 % and reduced the S.T to 26 mN/m.
- The applications of statistical optimization tools were very effective to enhance the production of biosurfactants from *P. aeruginosa* MF069166 up to 4.31 g/L and 6.90 g/L from *Meyerozyma* spp. MF138126 which was 200 and 400 % higher than the yield obtained from conventional optimization methods.
- The chemical characterization of biosurfactants using a diverse array of analytical tools such as TLC, FTIR, RP-HPLC, ^{1}H and ^{13}C NMR and LC-ESI-MS showed that *P. aeruginosa* MF069166 produced rhamnolipids with higher molar ratio of dirhamnolipids (RL-2) congeners. In case of *Meyerozyma* spp. MF138126, both acidic and lactonic isomers were present however, higher molar ratio of acidic sophorolipids was detected.
- Rhamnolipids from *P. aeruginosa MF069166* and sophorolipids from Meyerozyma spp. MF138126 showed stability under broad ranges of pH, temperature and NaCl concentrations.
- Cell surface hydrophobicity (CSH) studies showed more than 50 % affinity of *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 for crude oil, hexadecane and

dodecane. Whereas, more than 70 % emulsification activity was observed by their respective rhamnolipids and sophorolipids in the presence of different hydrocarbons.

- The critical micelle concentration (*cmc*) of rhamnolipids from *P. aeruginosa* MF069166 was found to be 40 mg/L whereas, 50 mg/L for sophorolipids from *Meyerozyma* spp. MF138126.
- DLSD analysis demonstrated the effect of concentration, pH and electrolytes on micellar size distribution of purified rhamnolipids and sophorolipids.
- 91 % crude oil biodegradation efficiency (B.E) of *P. aeruginosa* MF069166 and 85 % crude oil biodegradation efficiency (B.E) of *Meyerozyma* spp. MF138126 demonstrated the potential of two strains and their respective rhamnolipids and sophorolipids for bioremediation of hydrophobic organic compounds.
- Heavy metals bioremediation studies showed the decontamination, detoxification and metal chelation activity of rhamnolipids and sophorolipids. Soil washing was done in order to study the kinetics associated with biosurfactants-metal complex removal with time.

7- FUTURE PROSPECTS

- Process scale up is required for commercial production of rhamnolipids from *P. aeruginosa* MF069166 and sophorolipids production from *Meyerozyma* spp. MF138126.
- The yield of rhamnolipids and sophorolipids could be enhanced by using molecular studies like recombinant DNA techniques, random and site directed mutagenesis, etc.
- Downstream processing and product recovery techniques should be improved to enhance the efficacy and reduce the cost of bioprocess for field scale applications.
- The role of different carbon substrates on chemical composition of biosurfactants is yet to be studied.
- The detailed chemical characterization of individual congeners of rhamnolipids and sophorolipids is yet to be done.
- Antimicrobial properties of rhamnolipids and sophorolipids should be studied.
- The potential of rhamnolipids and sophorolipids should be evaluated in other applications of nano-biotechnology and agriculture (biocontrol agents).
- The biosurfactants producing microorganisms i.e. *P. aeruginosa* MF069166 and *Meyerozyma* spp. MF138126 can be studied for the degradation of other pollutants.

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