CHARACTERIZATION AND EMULSIFYING ACTIVITIES OF A QUORUM SENSING BIOSURFACTANT PRODUCED BY A MARINE BACTERIUM

A THESIS

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ABSTRACT

CHARACTERIZATION AND EMULSIFYING ACTIVITIES OF A QUORUM SENSING BIOSURFACTANT PRODUCED BY A MARINE BACTERIUM

Our understanding about the ecology of hydrocarbon (oil) degrading microorganisms which are mostly bacteria, fungi have greatly enhanced in recent periods. Biosurfactants and bioemulsifers are the amphiphilic compounds that are produced extracellular or as a part of the cell membrane by bacteria. Hydrosphere consists of marine ecological environment which is more prone to oil pollution by contamination. Understanding how microorganisms degrade hydrocarbons, and thereby mitigate ecosystem damage is important to target the problem and to plan the strategy. Biosurfactants produced by biofilm-producing bacteria has a profound impact in medical biotechnology, food production, pharmaceutical, bioremediation and hydrometallurgy. Biofilms are the bacterial communities that are regulated by many signaling processes. Biofilms have the exopolysaccharide (EPS) sheath which protects the cells of the bacteria from various adverse conditions. The typical ExoPolysaccharide formed from the biofilm bacteria has the maximum carbohydrate portion and considered to be the main ingredient in the biofilm. Research suggests that amount of EPS produced by different bacteria possess different concentration and composition. Generally, the kind of bacteria which produces Exopolymeric materials such as biosurfactant, has the role in antibiotic resistance, as it offers many regulatory pathways to act against antibiotics. Natural plant extracts which contains several phenolic compounds have great role in pharmaceutical field. Due to the safety issues concerned with the natural extracts which could be a characteristic feature to become a drug, our present work deals with the screening antibiofilm agents using natural extracts and disturbing the quorum sensing signal which is responsible for the formation of the biofilm. Garlic offered a promising approach in the field of quorum sensing signal blocking mechanism. Antimicrobial compounds from the marine aquatic ecosystems delivering the promising results, bacteria live in the marine environment are deeply subjected to stress that leads to the release of various compounds in order to sustain under adverse conditions. Current work focuses on the extraction of the antimicrobial compounds from the biosurfactant producing bacteria and finding the potential bacteria as a source of novel Exopolysaccharide producing

biofilm bacteria, in the role of oil biodegradation. Screening procedures were done to detect EPS producing biofilm bacteria. The isolate sample-M, identified as *Acinetobacter* species by 16S rDNA analysis, submitted to NCBI as *Acinetobacter M6* strain (Accession no: KR559749). The polymer produced by *Acinetobacter M6* has significant emulsification activity similar to some of the commercial emulsifiers, and also have the good surfactant activity, which appears to be a glycolipoprotein as shown by FTIR analysis. This is one of the few reports of a *Acinetobacter* species producing EPS with surfactant properties. The biosurfactant has shown promising results in terms of antimicrobial activity against *Pseudomonas aeruginosa*, therefore we can develop the drug by using pure form of biosurfactant. Bacteria produces many kinds of molecules that allow bacteria to communicate about population size, metabolic states or producing end products that initiate some activities such as bioluminescence. These molecules are generally regarded as auto inducer peptides that serves as a signal carriers. Quorum sensing reveals the fact that bacteria have the capacity to assess the number of other components they can activate, once the threshold number is reached. Acyl homoserine lactones are present mainly in gram negative bacteria and they control their own synthesis. In AHLs, the head group consists of homoserine lactones and the tail region determines the specificity of the receptor. Oligopeptide molecules are present mainly in gram positive bacteria, their synthesis is dependent on ribosomes. In the present study it is hypothesized that the production of autoinducing peptides and biofilm formation are interlinked. The small peptides which may have antimicrobial activity will also act as signals for the bacteria to form biofilm when they reach considerable cell density.

Keywords: Biosurfactant, Biodegradation, Bioremediation, Hydrocarbon, Marine microbiology, Auto inducers, AHLs, Gram specific strains, oligopeptides, signaling molecules, stationary phase, quorum sensing, Exopolysaccharide **(**EPS)

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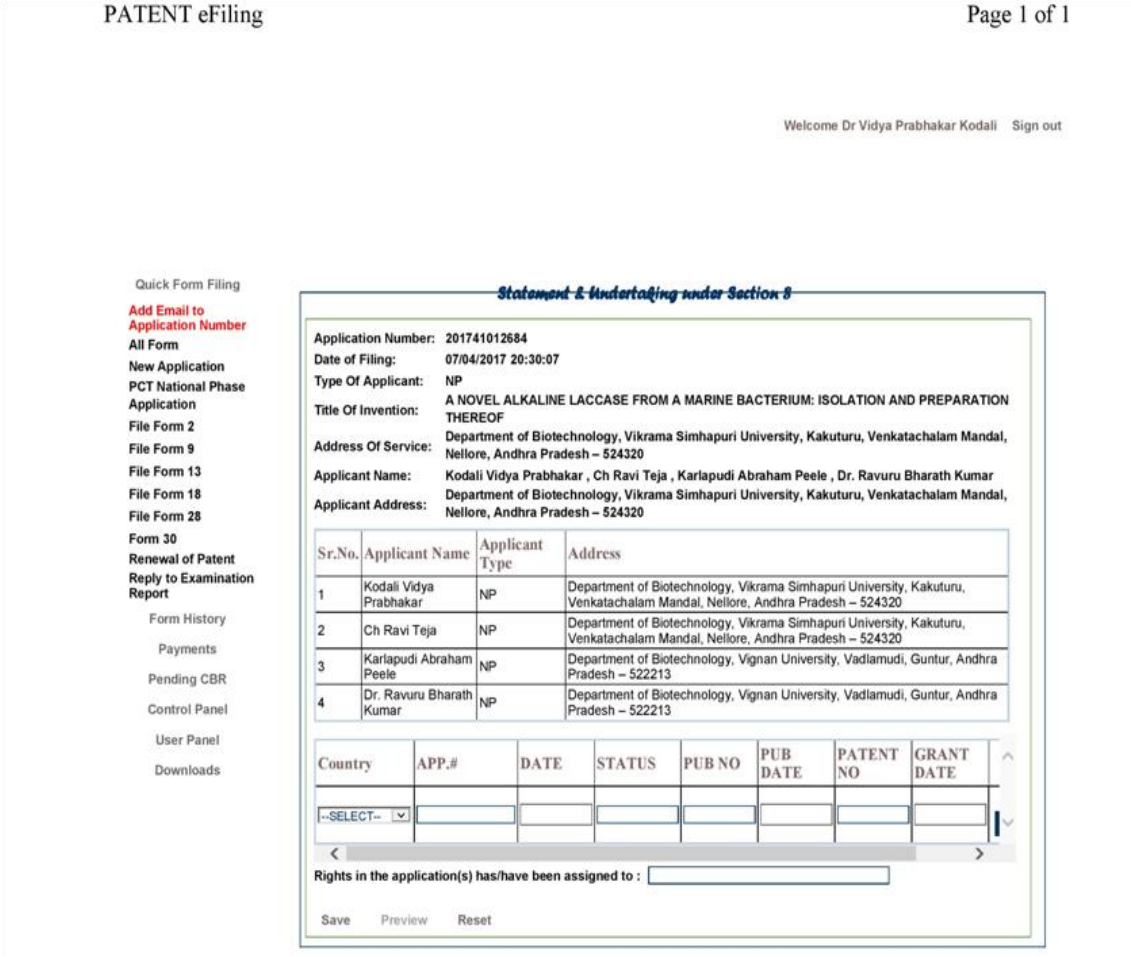
LIST OF SYMBOLS AND ABBREVIATIONS

°C – degree Celsius
ANOVA – Analysis Of Variance
CMC – Critical Micelle Concentration
CMD - Critical Micelle Dilution
kDa – Kilo Dalton
FTIR – Fourier Transform Infrared spectroscopy
g/l – gram per liter
h - hour
LB – Luria Bertani
BH- Bushnell Haas
mg/ml – milligram per milliliter
min - minute
ml – milliliter
mN/m – milli Newton per meter
nm – nanometer
OD600 – Optical Density at 600 nm
PCR – Polymerase Chain Reaction
rpm – revolutions per minute
sp. – species
ST - Surface Tension
TLC – Thin Layer Chromatography
v/v – volume by volume
vvm – volume per volume per minute
w/v – weight by volume
w/w – weight by weight
µg/ml – microgram per liter
CBB - Coomassie Brilliant Blue
CEOR - Chemically enhanced oil recovery
EOR - Enhanced Oil Recovery
EPS - Exopolysaccharide
IPG - Immobilized pH gradient
IR - Infrared spectrum
MEOR - Microbial Enhanced Oil Recovery
MALDI - Matrix-Assisted Laser Desorption Ionization
PCR - Polymerase chain reaction
PAS - Periodic Acid Schiff's
TLC - Thin layer chromatography



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CHAPTER-I

INTRODUCTION

1.1 HYDROCARBON CONTAMINATION SITES

Soil and groundwater contamination by organic hydrocarbons, which are the reasons for majority of environmental problems worldwide, affects the health of living organisms and the quality of the environment they are surviving in (Yadav, et al., 2015). The main and constant sources are organic hydrocarbons, such as the hydrocarbons of petroleum products and solvents, and poly aromatic hydrocarbons; these sources are usually persistent in the list of soil contaminants (Wu & Coulon, 2015). Industrial activities are also under the category of contamination sources as the emissions of various levels of hydrocarbons are observed. Transportation and refining of petroleum are considered major contributors to environmental contamination. However,organic hydrocarbons could be released accidentally or deliberately (Tornero & Hanke, 2016). The physical nature of the contaminant classification is determined based on whether it is in a solid or a liquid state. Organic contaminants of liquid nature have low solubility and remainin a different phase and are called non-aqueous phase liquids (NAPLs). Differences exist between liquids that are lighter than water and those that are heavier than water.This condition implies that liquids are heavier than water.Lighter liquids will float in water and spread on water bodies. One of the examples of light non-aqueous phase liquid (LNAPL) is diesel that contains a homogenous mixture of complex compounds that are aromatic in nature. Normally branched cyclic alkenes are extracted from distillation by the fraction of the gasoline during the petroleum separation process (Matara, 2016). A frequently reported hydrocarbon pollutant is diesel oil, which when leaked from pipelines or storage tanks causes accidental spills. Diesel oil is the common pollutant of groundwater,which is a result of underground storage tank and pipeline leakage (Gadhamshetty, et al., 2015). The negative influence on water and soil properties caused due to the contamination of diesel oil, resistance to various types of degradation, toxicity to the living biota, and intrinsic chemical stability (Shah, et al., 2016). Different technologies such as flushing, bioremediation, chemical treatment as well as incineration are used for the site remediation that contains diesel oil contaminated soil. One of the best approaches is bioremediation among different technologies for cleanup of soil and ground water

which is contaminated (Koshlaf, et al., 2016). Franzetti et al. (2010) has reported the most economical tool is bioremediation, that could be used for contamination management of the polluted sites. Chen et al. (2011) has categorized bioremediation as eco friendly and effective technology for the sites which are contaminated mainly with hydrocarbons. It involves in increasing the pace of the process which is naturally occurring biodegradation (Usman, et al., 2016). Bioremediation majorly consists of phosphorus and nitrogen fertilizer applications, pH management of the effluent and addition of the bacteria, but the availability of hydrocarbons to microorganisms is the most important limiting factor (Vijayakumar & Saravanan, 2015). Diesel is hydrophobic and has less water solubility, hence less available to microorganism. The availability of diesel is limited due to the adsorption quality of the soil (Duan, et al., 2015). The subjects with high hydrophobicity as well as low solubility such as hydrocarbons have the ability to adhere strongly to particles of soil. The compounds slowly released into the water phase that could cause the time to be the factor for the proceed of bioremediation (Trellu, et al., 2016).

1.2 SURFACTANTS

Surfactants are used for bioremediation of the hydrocarbons and made the hydrocarbons available for the microorganisms to degrade. Hence the transfer of the hydrocarbons to the aqueous phase in bulk is the important process for its bioavailabity (Adrion, et al., 2016). Among various methods, surfactants can be seen as the promising method for bioavailability related problems. There are many uses of surfactants for the increase in the hydrocarbons solubility, the use of surfactants could increase the hydrocarbons mobility as well as the bioavailability which promotes the rate of biodegradation (Haftka, et al.,2015). Mulligan (2001) stated that industry of petroleum has been using surfactants majorly as they can increase the solubility of petrol and its byproducts. The diverse group of surfactants are divided on structural basis depend on the type of microorganisms that produced them (Cheng, et al., 2016).

1.3 BIOSURFACTANTS AND ITS CLASSIFICATION

Biosurfactants produced mostly by microorganisms, are the biological active surface-molecules with vast applications in the field of industries, as they possess many of the versatile properties of specificity, minute toxicity and biological acceptability (Shivlata, et al., 2015). They are used as an additives for the production of organic chemicals, petro-derivatives, petrochemicals. They possess several advantages over chemical surfactants. Bioremediation of waste water effluents can be done effectively

by using biosurfactant producing microorganisms due to their specificity of utilizing the organic waste and hydrocarbon waste as raw materials. Biosurfactants bear surface activity, high tolerance to various environmental factors, withstand from mean to extreme conditions, such as acidity or basicity of an aqueous solution, temperature, salt concentration, ionic strength, biodegradable nature, demulsifying-emulsifying ability, anti-inflammatory potential and anti-microbial activity. Microorganisms living in extreme environments such as extremophiles have gained much attention for the last few decades as they possess different properties by producing certain useful compounds. Surfactants derived from chemicals expose severe environmental problems, hence there is a need to screen the biodegradable surfactants from the extreme marine environments for the biosorption of hydrocarbons of polyaromatic cyclic compounds (Tiquia-Arashir & Rodrigues, 2016). Biosurfactants consists of two different parts as they are amphiphilic compounds which possess hydrophilic polar moiety as well as a non polar group which is hydrophobic. The hydrophilic group has oligo or monosaccharide and proteins as well as polysaccharides or peptides and the hydrophobic moiety has unsaturated, saturated fatty alcohols or hydroxylated fatty acids (Rodrigues, 2015). One of the key features of biosurfactant is the hydrophilic-lipophilic balance which causes the hydrophobic as well as hydrophilic portions to be determined in substances that are surface active. Because of the amphiphilic structure, biosurfactants not only have the ability to increase the hydrophobic substance surface area but also have the ability to change the property of cell surface of the microorganisms along with the ability in increasing the bioavailability of substances. Because of the surface activity, surfactants behave as excellent foaming agents, emulsifiers and dispersing agents. Naturally occurring surfactants are better and have many advantages when compared to their chemical versions. The naturally derived surfactants are eco-friendly, low toxicity and biodegradable (De, et al., 2015). They show good foaming capacity and selectivity of the substrate to degrade and are functionally active at extreme conditions of high temperatures, high salt concentrations, as well as pH which is caused by the by products and generated waste from industries. Due to the long lasting characteristics, the biosurfactants are cheap and reduces the cost and time of effect of biodegradation of the polluted soils and water bodies (Nercessian, et al., 2015). Due to their enormous advantages, they are widely used in many of the food production, pharmaceutical, agricultural and cosmetic industries. Different properties of surfactants are dispersion, emulsification

or de-emulsification, wetting, foaming as well as coating due to which they are useful in bioremediation and physiochemical technologies of metal and organic contaminants (Wu & Lu, 2015). Biosurfactants show increase in the bioavailability of hydrocarbons which results in degradation of contaminants by the hydrocarbon degrading bacteria and enhanced growth of the bacteria in the polluted soil (fig.1.1). In the soils with heavy-metal pollution, the biosurfactants form different complexes with metals and perform surface removal of heavy metals which causes in the increase of ion concentration of metals and the bioavailability (Sarma, et al., 2015). The pollutants which are hydrophobic that are present inside hydrocarbons, water and soil have the necessity to be solubilize before they were degraded by the microorganisms. Surfactants have the property of increasing hydrophobic particle surface area like pesticides applied in the soil and water, which in turn increases the solubility (Neitsch, et al., 2016). Increase in the microbial production of surfactants and the wide use of biosurfactants for the degradation of harmful compounds like chemicals that kill pest and insecticides in different kind of environment like soil, water has gained attention in the past few years (Shah, et al., 2016). The biosurfactants which are produced by various microorganisms are identified and characterized by Lin, (1996), Desai, (1987) and Parkinson, (1985). Hence there are various types of biosurfactants based on the properties such as characterization, antimicrobial activity, production, efficiency of hydrocarbon removal from environment and its ability of reducing the surface tension (Tabatabaei, et al., 2015).

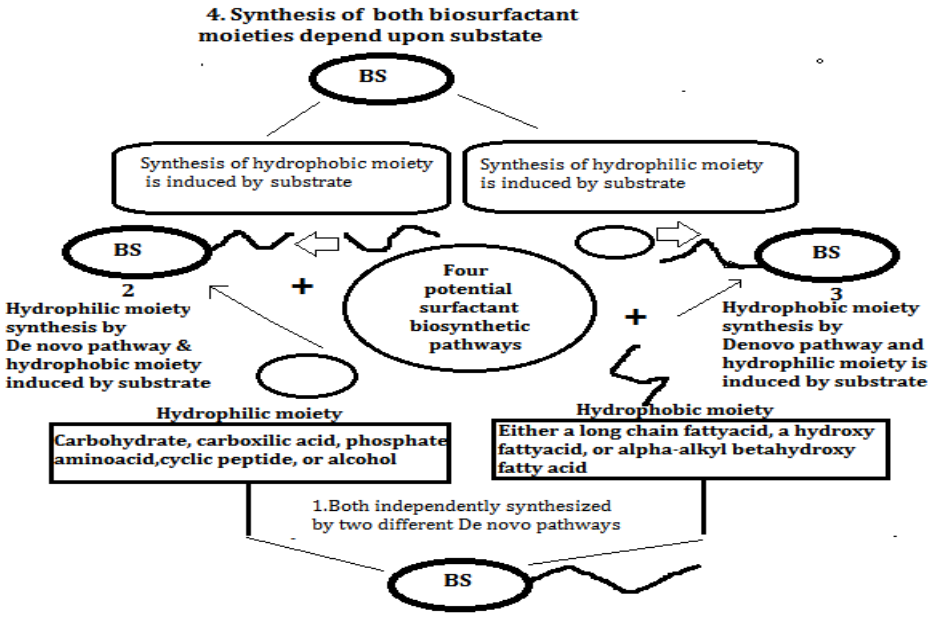


Fig.1.1 Biosynthetic pathway of biosurfactant in bacteria

A wide range of compounds that are organic were used by microorganisms as the energy rich source and as the carbon source for their growth. but if carbon is insoluble hydrocarbon the microorganisms diffuse various substances that are called as biosurfactants where as some of the yeast and bacteria diffuse biosurfactants that can emulsify the hydrocarbons available in the medium (Leuchtle, et al., 2015). Some examples for this type are different species of *Pseudomonas* producing rhamnolipids and sophorolipids which is produced by different species of *Torulopsis*. Most of the microorganisms could change the cell wall structure which was caused by the production of lipopolysaccharides in the cell wall (Saenz-Marta, et al., 2015). *Candida lipolytica* produce lipopolysaccharide which are cell wall-bound when the medium contains n-alkanes. *Rhodococcus erythropolis* along with different *Mycobacterium species* and *Arthrobacter* species produce non-ionic trehalose corynomycolates. *Acinetobacter* species Produce emulsan as well as lipoproteins like Subtilisin, are produced by *Bacillus subtilis*. *Rhodococcus sp.* synthesises Mycolates, Corynomycolates synthesized by *Pseudomonas rubescens, Thiobacillus ferroxidans* and *Gluconobacter cerinus* synthesizes ornithinlipids. Classification of the biosurfactants are mainly based on the origin of the microbes and their chemical composition. Biosurfactants are classified not like the artificial chemical surfactants which are categorized based on the polarity of the functional group (Sharma, et al., 2016). Biosurfactants are divided into two types based on the molecular weight, low molecular weight compounds which lower the interfacial surface tension, polymers of high molecular weight that are most of the efficient stabilizing agents. Glycolipids, lipopeptides and phospholipids constitute the majority of low mass biosurfactants, while particulate and polymeric surfactants come under the large mass biosurfactants (Saenz-Marta, et al., 2015). Mostly are anionic biosurfactants and some are neutral, while hydrophobic moiety is based on the derivatives of fatty acid long chains and have the hydrophilic moiety that could be an amino acid, phosphate group, carbohydrate part and a cyclic peptide (Harvey, et al., 2015).

1.3.1 Glycolipids

Glycolipids are a group of carbohydrates which has a long-chain of aliphatic acids. They form a connection of either ester group or ether group. Some of the glycolipids are sophrolipds, rhamnolipids and trehalolipids **(**Rikalovic, et al., 2015).

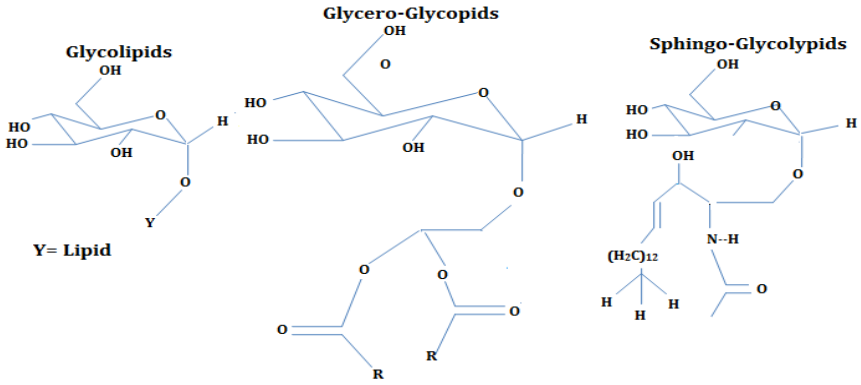


Fig.1.2 Structure of glycolipids

1.3.2 Rhamnolipids

These are the glycolipids in which any of the rhamnose sugar moieties linked to the myrmicacin, which is a derivative of β-hydroxycarboxylic acid hydroxyl group at the reducing end of rhamnose disaccharide, or present as one of the hydroxyl group is occupied by ester formation (Nickzad, et al., 2016).

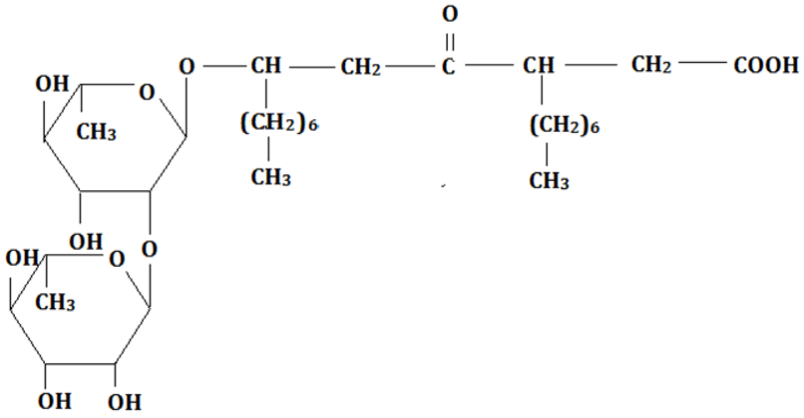


Fig.1.3 Structure of rhamnolipids

1.3.3 Trehalolipids

Trehalolipids are present in most of the species such as *Corynebacterium sp.*, *Mycobacterium sp.*, and *Nocardia sp.* Trehalose is a disaccharide sugar which is linked at 6th position of the carbon backbone to long chain fattyacids of mycolic acid. The structure and size of the mycolic acid vary from organism to organism by the different number in the presence of atoms of carbons and its unsaturation rate. Trehalose lipids obtained from *Arthrobacter sp.* and *Rhodococcus erythropolis* decreased the interfacial as well as surface tension in the growth medium (Sharma, et al., 2016).

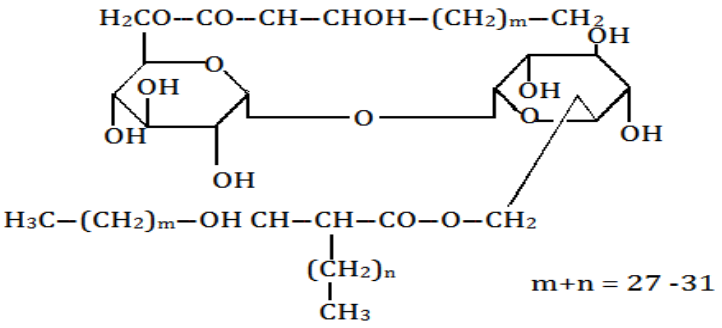


Fig.1.4 Structure of trehalolipids

1.3.4 Sophorolipids

Torulopsis bombicola synthesizes three types of glycolipids. *T. Petrophilum* as well as *T. apicola* contains a carbohydrate sophorose that is dimeric through the glycosidic linkage attached to the hydroxyl fatty acid. Generally sophorolipids are heterogenous mixture of macrolactones and a free acidic group. Lactones, ester groups of hydroxycarboxylic acids extracted from sophorolipid molecules are required for various biomedical applications as polymers (Jimenez-Penalver, et al., 2016).

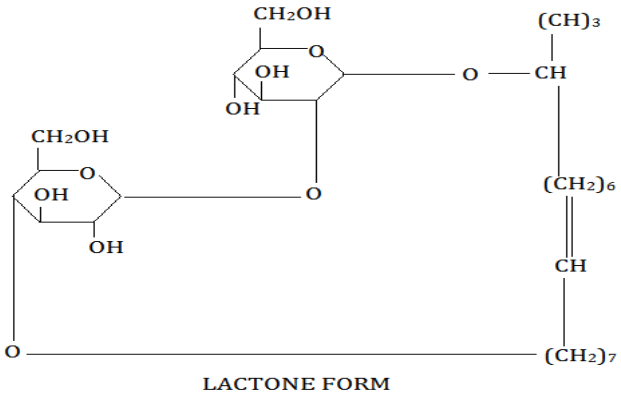


Fig.1.5 Structure of sophorolipids

1.3.5 Lipoproteins and Lipopeptides

Cell walls of wide range of microorganisms have cyclic lipopeptides which triggers the responses of immune system, they include decapeptide-lipopeptide antibiotics. Lipopeptides and lipoproteins contain lipid as the functional group linked to the polypeptide chain. *Bacillus subtilis* synthesizes the cyclic lipopeptide surfactin which is the most effective biosurfactant. Surfactin is made of seven ring structure of amino-acid which is joined to fatty acid chain with the help of a lactone linkage. Surfactin was reported that it has reduced the surface tension below 28 mN/m (Nguyen, et al., 2016).

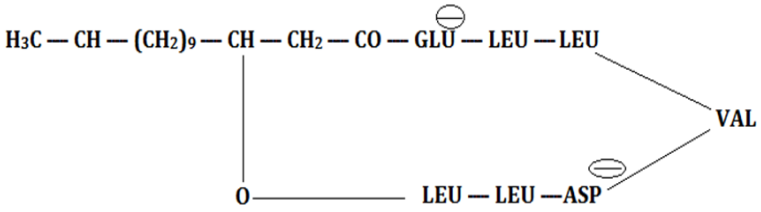


Fig.1.6 Structure of surfactin

1.3.6 Lichenysin

Several of the biosurfactants synthesized by *Bacillus licheniformis* have exhibited great stability towards salt, temperature as well as pH and have same structural as well as physio-chemical properties that of surfactin. Surfactant of *Bacillus licheniformis* is capable of lowering the surface tension of various liquids (Ronning et al., 2015).

1.3.7 Phospholipids and Fatty acids

Yeast and bacteria when grown on n-alkane medium synthesize a large number of phospholipid and fatty acid molecules. *Acinetobacter* sp. produces rich vesicles of phosphatidylethanolamine which form microemulsions that are clear in water. *Rhodococcus erythropolis* produce phosphatidylethanolamine when grown on n-alkane decreases the surface tension of water and hexadecane (Helfrich, et al., 2015).

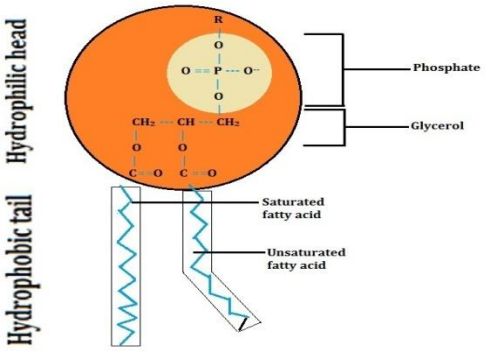


Fig.1.7 Structure of phospholipids

1.3.8 Polymeric biosurfactants

Liposan and Alasan are some of the most popular polysaccharide–protein complexes. Heteropolysaccharide biosurfactants show extracellular polyanionic activities that are synthesized by most of the *Acinetobacter species.* Emulsan is used to emulsify hydrocarbons present in water which is considered to be one of the effective emulsifying agents even if the concentration is lesser than 0.01%. Extracellular polymeric emulsifier, liposan is a water soluble emulsifier synthesized by *C. lipolytica*

which consists more than 80% of carbohydrate and less than 20% of protein part (Wilton, et al., 2016).

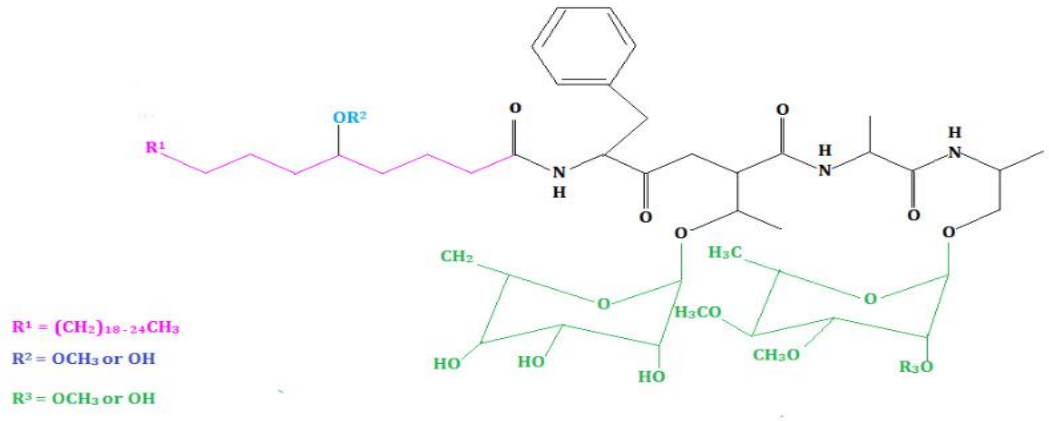


Fig.1.8 Structure of Glycolipopeptide

1.4 PROPERTIES OF BIOSURFACTANTS

1.4.1 Surface and interface activity

An effective surfactant or a biosurfactant is the one that lowers the surface tension of water. *Bacillus Subtilis* produces surfactin that lowers surface tension of liquids most effectively even at adverse extreme conditions. *Pseudomonas aeruginosa* produces biosurfactant of rhamnolipid nature that decreases the water surface tension effective than many other surfactants (Kim, et al., 2015). Sophorolipids produced by *T. bombicola* reduces the surface tension. Biosurfactants are effective as well as efficient, their CMC is from 10 to 40 times lower than chemical surfactants, because of the reason very less amount of biosurfactant is required to reduce the surface tension (Anjum, et al., 2016).

1.4.2 Temperature, pH and ionic strength tolerance

Functions and parameters such as temperature and pH of most of the biosurfactants are not altered by the environmental conditions. Research studies suggesting that lichenysin which is produced by *B. licheniformis* was less affected by pH (4.5–9.0), temperature (up to 50°C) and by NaCl as well as Ca concentrations. At high temperatures beyond autoclavable temperature (121°C) and at low temperatures below minus 15°C, lipopeptides produced by *Bacillus subtilis* found to be stable when stored for 180 days. At NaCl concentrations, greater than 15% and pH range between 4 and 12 , the activity was found to be stable (Mujumdar, et al., 2016).

1.4.3 Biodegradability

Biosurfactants are regarded as non-toxic agents, they are one of the best options to use in cosmetic, food and pharmaceutical fields. One of the recent studies suggest that the polyanionic surfactant named emulsan has shown LC_{50} against *Photobacterium phosphoreum* which is much lesser than *Pseudomonas* rhamnolipids. Commercially, if we compare ten of the biosurfactants based on the toxicity, seven of them were synthetic surfactants while others are dispersants, most of the biosurfactants are easily degradable in nature (Gregorich, et al., 2015). Biosurfactants produced by *Pseudomonas* species are widely in use in industries because of its wide applications and environmental toxic friendly nature compared with artificial surfactants. Many of the laboratory tests were available to assess the toxicity levels of biosurfactant and chemical surfactant. Studies indicated the range of mutagenic and toxicity effects of biosurfactant when compared to that of chemical surfactant was less (Shah, et al., 2016). Formation and breaking of emulsion could be produced within a month, emulsion may be stabilized or destabilized by the biosurfactants. Emulsifiers are generally a class of biosurfactants with high molecular weight compared with low mass biosurfactants. *T. bombicola* produces sophorolipid surfactant was able to lower the surface tension and surface area. Stable emulsions were formed by the use of polymeric biosurfactants and have the additional advantage that they consists of oil coat droplets to form oil/water emulsions for cosmetics and food that are stable. Liposan produced by *C. lipolytica* can emulsify edible oils but does not reduce surface tension effectively.

1.5 BIOFILM

Biofilm formation is a complex process of surface attached community transition from numerous free floating cells. Based on the planktonic cells engaged, the biofilms can be of different types. Biofilms formed by single species are highly regulated by signal circuits relating the same species of organisms. Multiple species that generate specific signals are responsible for the formation of surface attached community of various bacterial planktonic cell species. The Biofilm development factors such as surface area, smoothness, flow velocity, nutrients influence the biofilm by providing suitable environment for bacterial growth and attachment (Donlan & Costerton, 2002). Biofilms are varied by the mechanisms that different bacteria should employ and depend on environmental conditions in regard to the specific strain features. The formation of biofilm is a complicated process involves the following crucial steps:

The organic molecules adhere to the material submerged in water and neutralize the surface charge repelling the bacteria. Planktonic bacteria temporarily attach by electrostatic and physical forces. The permanent attachment can be created by producing extracellular polymeric substances (EPS). The EPS cements the cell to the substratum material forming an ion exchange system entrapping nutrients. The adequate nutrients can lead to doubling of the organisms by reproduction. The slimy nature of the biofilm was due to the presence of maximum percentage of EPS and water. The metabolites produced by the primary colonizers were utilized by the secondary colonizers and grow on them to settle, thereby forming a biofilm. Bacteria secreting extra cellular polysaccharides (EPS) by the regulation of respective genes via Quorum Sensing (QS) systems in forming biofilms has been a fabulous asset to microbes (Fletcher, et al., 1991). QS systems assist the microrganisms in the quorum to survive against antimicrobial compounds, and also to avail nutrients in a nutrient limited condition. The well studied multiple species type of biofilms are the dental plaques/ biofilms. The oral bacteria interact competitively and cooperatively to exhibit the most sophisticated communication of metabolites released by them.

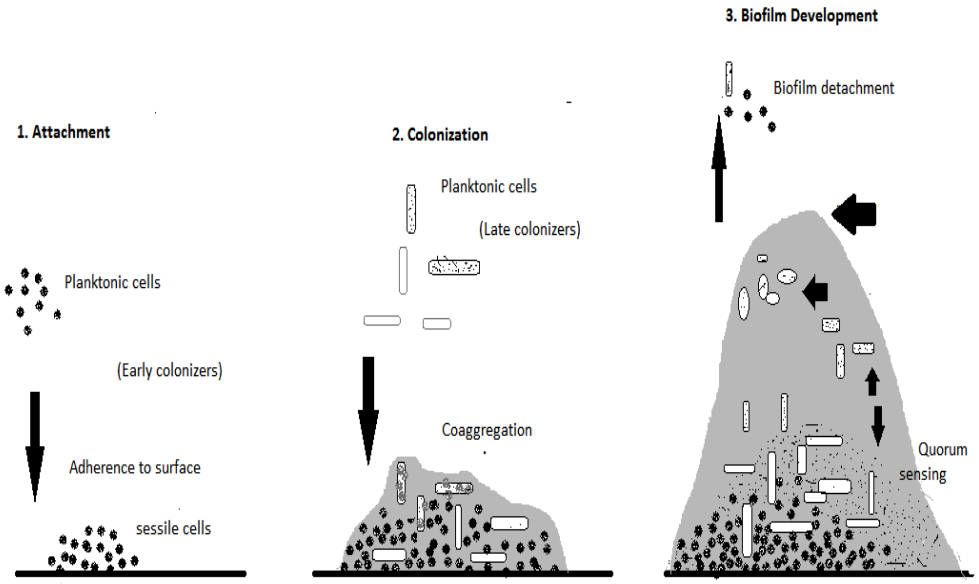


Fig.1.8 Biofilm formation (from adhesion to dispersal)

As a consequence, biofilms that contaminate medical devices, manufacturing surfaces and fluid systems were extremely difficult to eliminate. Several mechanisms of biofilm resistance have been described and those are believed to work in synergy to bring about reduced susceptibility in biofilms (Kumar & Anand, 1998). The existence of cells in a biofilm allows a community response which would be greater when

compared to that from a single cell (planktonic). In addition, the three dimensional structures provide protection of the persisted cells, which, when these are disrupted, might result in the cells becoming susceptible to the antimicrobial agents. More recently high magnetic field and ultrasound have been reported to be useful in eradicating biofilms (Jeng, et al.,1990). The use of chemical biocides (disinfectants, sanitizers and detergents) was also common in the control of biofilms (Kumar & Anand,1998). These are divided into two main groups; oxidizing and non-oxidising agents. The commonly used oxidizing agents include chlorine, ozone, iodine and hydrogen peroxide. These agents can act by depolymerising the EPS matrix, thereby disrupting the biofilm integrity. Non-oxidising agents include quaternary ammonium compounds (QACs), formaldehyde, anionic and non-ionic surface active agents were widely used (Dreeszen, 2003).

1.6 THESIS OBJECTIVES

The objectives of the present work are

1. Screening and characterization of biosurfactant producing marine bacterium
2. Study of oil degradation ability of biosurfactant
3. Biological characterization of the biosurfactant
    a. Anti-Inflammatory activity of the biosurfactant
    b. Antimicrobial activity of the biosurfactant
4. Quorum sensing studies of biosurfactant production

1.7 THESIS ORGANIZATIONS

The thesis has five main chapters, Chapter 1 dealt with introduction of Biosurfactants and majorly focused on the oil spills that can influence the pollution of the ground and water resources. The review of literature discussed in Chapter 2 has been related to biosurfactant classification, economic importance of biosurfactant, chemical nature of biosurfactant, recovery and applications of biosurfactants. Methodology, reagents for experimental work were discussed in Chapter 3, Chapter 4 and Chapter 5. The conclusion and scope of work were summarized in chapter 6, and the thesis would be completed with references and appendices.

CHAPTER- II

REVIEW OF LITERATURE

2.1 INTRODUCTION

Rapid growth of industries lead to the environmental pollution and other environmental hazards. One of the prevalent ecological hazards is petroleum pollution which show harmful effects on all aquatic living organisms particularly microbial population (Shan, et al., 2013). The first step in this effect is hydrocarbon transportation to the surface of the microbial cell from oil phase to cell surface through the contact and then transportation across the cell membrane. Even though a great amount of work was done in this area, n-alkane transportation into the bacterial cell and assimilation mechanism of the hydrocarbons in the microbial cells were poorly understood (Ampelli, et al., 2016). It has already been reported that some bacterial populations exhibited resistance to oil transportation and also few bacterial population efficiently degrade oils/hydrocarbons. Two different types of interactions normally observed in the processes of oils/hydrocarbon biodegradation. Oil adhesion, pseudo solubilization and degradation of hydrocarbons to form small droplets of oils are the sequential steps involved in one of the mechanisms. There are several reports which support this theory. Kirschner et al. (1980) proposed that microbial cells adhere to the drops of hydrocarbons whose size was less than the cells and the substrate uptake has taken place by active transport or by diffusion at the point of interference between cells and hydrocarbons.

There are two models of the hydrocarbon interactions with the cells of the microbes

- Unmediated interaction directly between microorganisms and the substrate which is insoluble
- Interaction between the microorganism and the substrate (Palecek, et al., 2015)

It has been shown that the microorganisms solubilize the hydrocarbons/oils by a group of molecules produced by bacteria, called as bioemulsifiers and the mechanism is called as emulsification. Bioemulsifiers that reduce the surface tension are termed as biosurfactants. Biosurfactants may be located inside the cells (intracellular) or secreted outside the cells (extracellular) (Antoniou, et al., 2015 & Sharma, et al., 2016). There are many reports available on bacterial biosurfactants, but the spectrum of activity depends on their chemical composition. A strain of *Pseudomonas aeruginosa* was reported to produce the rhamnolipid type biosurfactant which was

mono as well as di-rhamnolipid (Patel, et al., 2015). It has been proved that the rhamnolipids and its producing microorganisms specifically degraded hexadecane, hence there is a clean correlation exists between the type of surfactant and the type of hydrocarbon/oil that gets degraded (Salek & Gutierrez, 2016). It has been noted that several studies were done on phenanthrene degradation by various chemical surfactants. It was also indicated that the increased phenanthrene degradation when it was associated with bacterial isolate that produced a non-ionic surfactant (Itrich, et al., 2015). In another instance, oil degradation capacity of a chemical surfactant 'Finasol OSR-5' was multiplied when supplemented with a biosurfactant trehalose-5,5'-dicorynomycolates and reported to be the complete removal of aromatic hydrocarbons from the contaminated soil within a given period (Itrich, et al., 2015). In another study, polycyclic aromatic hydrocarbons (PAHs) were significantly degraded by a group of bacteria that produced glycolipids and sophorose lipids (Chakrabarti, et al., 2012). Surface active glycolipids when added to the hydrocarbon sites have increased the biodegradation of 2,4-DCPIP. In the presence of glycolipids, most of the PAH's are almost removed completely in less than a month in soil contaminated sites. Bacteria produce biosurfactants in the form of biofilm which interacts with an interface and alters the surface properties such as wettability and other properties. A marine bacterium isolated from sea water polluted with oil, *Pseudomonas aeruginosa*, has shown the ability to break hexadecane, octadecane, heptadecane as well as nonadecane after 28 days of incubation. The degradation ability of this bacterium has been proved due to the production of a biosurfactant. It was also proved that *Pseudomonas aeruginosa* has effectively degraded a range of hydrocarbons like 2-methylnaphthalene, tetradecane and pristine (Zhuang, et al., 2002). In another experiment, the hydrocarbon contaminated soil was inoculated with *Acinetobacter haemolyticus* and *Pseudomonas* ML2 (biosurfactant producing strains) and the degradation of hydrocarbons were studied, after the completion of the 2 months period of incubation, a tremendous reduction of hydrocarbons (39-71%) and (11-71%), was achieved by *Acinetobacter haemolyticus* and *Pseudomonas* ML2 respectively. These results suggested that cell free biosurfactant produced by bacteria had the remarkable hydrocarbon degradation ability. The rhamnolipid content of *Pseudomonas aeruginosa* was extensively characterized for its hydrocarbon degradation ability. The growth of economy of any country increases along with the demand for oil which should be met by all the new discoveries and technologies. The major pollutants from

the oil production companies lead to the deposition of oil sludge which gets strongly bound to the effluents during conditioning and treatment by the treatment process. As the sludge deposition increases, the hydrocarbons penetrate through the top layer of soil and then slowly diffuses into subsoil which causes high risk of contamination to the ground water. Hence, the oil sludge needs to be treated to prevent the environmental toxicity. Even if the sludge is burned, it would cause undesirable air pollution (Tian & Yun, 2016). There are two major factors in the formation of the oil sludge. The first factor is residual inorganic substances which has scales, sand and dust while the second major factor is the precipitation of paraffin wax, as the paraffin wax was in less soluble form. Oxidation of organic heavy material present in the crude oil leads to various climatic changes. These changes cause changes in material balance of various components resin, polymeric compounds and asphaltenes of the oil sludge. There are many technologies being used for the cleaning up of the contaminated sites include thermal evaporation, excavation and soil vapour extraction. Bioremediation is the most important method which has been accepted treatment by using indigenous microbial flora. Certain biosurfactant producing bacteria can metabolize several classes of hydrocarbons. Technologies have already been developed and used in middle east and Canada for bioremediation of hydrocarbon contaminated soil by using biosurfactant producing bacteria Most of the hydrocarbon contaminated soil sites in middle east and Canada were added with biosurfactant producing microorganisms for the bioremediation since glycolipid rich biosurfactants act as the nutrients to the soil. Microorganisms oxidize the organic hydrocarbon compounds by dissolving or emulsifying them while the major limiting factor of the biodegradation of the oil is its solubility rate, biosurfactants increase the rate of biodegradation of the organic compounds by increasing their solubility by emulsification. Most of the crude oil-degrading bacteria release extracellular biosurfactants to facilitate microbial oil uptake and facilitate degradation by emulsifying the hydrocarbon (Morikawa, et al., 2000). Biosurfactants can increase the pseudo-solubility due to their specificity and degradability. Biosurfactants were in different complex nature namely rhamnolipids, trehalolipids, sophorolipids, peptide-lipid complexes and carbohydrate-peptide-lipid complexes. They were produced from different sources that differ in their physicochemical properties include the following properties.

- Modification of the reservoirs wettability
- Reduction of the viscosity of oil

- Drilling of mud
- Control on the deposition of Paraffin or asphalt
- Increase of oil displacement
- Stabilization of the oil emulsion
- Emulsification of the Oily sludge
- Extraction and Transportation of Oil
- Cleaning of the container or oil tanker

Biosurfactants contain hydrophilic group which may be a sugar, or a protein, where as hydrophobic group usually contains fatty acids or fatty alcohols. Biosurfactants perform several functions as they increase the surface area, thus increase the bioavailability of water-insoluble complexes and finally bound to heavy metals for removal (Rodrigues, et al., 2006). Biosurfactants have been shown to possess antioxidant, antimicrobial and anti-inflammatory activities (Williams, 2009). Different complexes involve in versatile biological functions and the common characteristic was to reduce the surface tension of liquids (Table 2.1). Bioactive surfactant molecules were potent to perform several functions include inhibition, fibrin clot formation, antimycoplasmic, antitumorigenic and insecticidal activities. Microorganisms that produce surfactants were used for nanoparticle synthesis, tend to give different applications in the field of biology.

Table 2.1 Surface tension values from the selected biosurfactant producing strains

Biosurfactant	**Organism**	**Surface Tension ($mN\ m^{-1}$)**	**Reference**
Rhamnolipids	*P. aeruginosa*	29	Amani, et al., 2013
Trehalolipids	*Rhodococcus sp.*	36	Kuyukina, et al., 2015
Sophorolipids	*T. bombicola*	33	Imura, et al., 2014
Peptide-lipid	*B. licheniformis*	27	Yakimov, et al., 1995
Serrawettin	*S. marcescens*	33	Thies, et al., 2014
Viscosin	*P. fluorescens*	26.5	Bak, et al., 2015
Surfactin	*B. subtilis*	27-32	Liu, et al. , 2015
Emulsan	*A. calcoaceticus*	32	Goldman, et al., 1982
Mannan-lipid-protein	*C. tropicalis*	30	Chen, et al., 2011
Liposan	*C. lipolytica*	29	Anaukwu, et al., 2015
Carbohydrate-protein-lipid (GLP)	*Microbacterium* sp.	27	Camacho, et al., 2014
Carbohydrate-protein-lipid (Current work)	*Acinetobacter* M6	30	Abraham, et al., 2016

Polyphilic polymers contain deoxy sugars and hydrophobic constituents. Bioemulsan is the best ever studied polymer produced by *Acinetobacter*. Microbes use many of the pathways including de-novo pathway. Most of the amphipathic polysaccharides were produced by *Acinetobacter* species. Rhamnolipids which are carbohydrate-lipid derivatives has been produced by *Pseudomonas* sp., and showed good emulsification ability, peptide linked bioemulsifiers produced by *Methyl bacterium* sp., and *Methanobacterium sp*., *A.calcoaceticus* has carbohydrate-protein derivative. Lipid-protein derivatives produced by *Bacillus velezensis* and *Streptococcus gordonii*. Lipid-fatty acid derivatives produced by *Myroides* species. Surface active agents show the surface property are made up of biological molecules such as carbohydrates, lipids and proteins in various combinations and compositions (Table 2.2). Microorganisms that produce bioemulsifiers have typical physiological behavior which was poorly understood by researchers as they perform definite functional roles in the microbes.

Table 2.2 Economic importance of the selected biosurfactant producing strains

Biosurfactant	**Microorganisms**	**Economic importance**	**References**
Cellobiose lipids	*Ustilago maydis*	Antifungal compounds	Morita, et al., 2013
Rhamno lipids	*Pseudomonas aeruginosa*	Bioremediation	Amani, et al., 2013
Trehalose lipids	*Rhodococcus erythropolis*	Dissolution of hydrocarbons	Urum, et al., 2004
Sophoro lipids	*Candida bombicola*	Antimicrobial activity	Solaiman, et al., 2007
Surafactin	*Bacillus subtilis*	Antimicrobial property	Lee, et al., 2004
Lichenysin	*Bacillus licheniformis*	Microbially enhanced oil recovery	Qiu, et al., 2014
Emulsan Glycolipopeptide	*Acinetobacter calcoaceticus*	Microbially enhanced oil recovery	Goldman, et al., 1982
Microbactan Glycolipopeptide	*Microbacterium*	Emulsifier	Camacho, et al., 2014

Biosurfactants play a role in bioremediation by increasing the surface area of substrates. Biosurfactant producing microorganisms create their own micro-environment and promotes emulsification by the release of certain compounds through various mechanisms such as quorum sensing. Compounds exhibit hydrophobicity show poor water solubility and prolonged environmental persistence. Alasan, a known bioemulsifier has increased the solubility of polyaromatic compounds (PAHs) by

many folds. *Alcanivorax borkumensis* and *A. calcoaceticus* RAG-1 were well known standard bioemulsifiers. Surface active biomolecules could replace chemical analogues offer various advantages in various ecological aspects. The activity and application attributed to the use of biosurfactant in oil industry has been presented by many researchers (Pereira, et al., 2004). Biological processing was being considered as a suitable constituent due to its less severity and more selectivity to specific reactions (Prince, 1993). The low water solubility nature of hydrocarbon compounds limited the capability of microorganisms to emulsify. The microorganisms that degrade the hydrocarbons normally produce a variety of extracellular biosurfactants and were observed when mixed with chemical surfactants, increased the efficiency of the hydrocarbon removal from solid or soil surfaces, but the inhibition and enhancement of the hydrocarbon degradation was observed (Sarafzadeh, et al., 2013). Many biosurfactants with low molecular weight such as lipopeptides and glycolipids are lot effective in decreasing the surface tension. Biosurfactants emulsify the compounds, increase the water solubility and make the compounds more accessible for the microorganisms. In the past few years, a lot of research being focused on the study of biosurfactants for their spreading, emulsifying, wetting and foaming properties, but recently biosurfactants have been extensively studied for their applications in oil and food industries.

2.2 MICROBIAL ENHANCED OIL RECOVERY (MEOR)

Microbial enhanced oil recovery has been extensively used for the recovery of oil. Some bacteria mobilize the sediments of oil trapped in the reservoirs and rocks for their metabolism to produce various metabolites (Kirschner, et al., 1980). Recently, it had been shown that the interfacial tension reduction and alteration in wettability were two important mechanisms of microbial enhanced oil recovery. Sarafzadeh et al, (2013) reported that biosurfactants played an important role on adsorption of oil from the rocks. It had been shown the effect of biosurfactant producing bacteria on laboratory sand packed columns to demonstrate the effectiveness of microbially enhanced oil recovery and been reported that surfactin from *Bacillus coagulans* 30 could form emulsions with crude oil, which in turn increased the recovery of oil from 17 to 31% (Chaprao, et al., 2015), meanwhile, surfactin formed an emulsion which was stable at different pH, temperature and salinity ranges. Dinger et al. (2002) justified that the surfactin produced by *B. subtilis* was active even at high pH, temperature and salt concentration ranges (pH 3–10, temperature 21–70°C and NaCl

0%–10%). Many studies had revealed that surfactants showed potential uses in microbially enhanced oil recovery. *Bacillus* species produced lipopeptides at a range between 85 and 95 mg/l in the reservoirs of oil. During the last decade, around ten of the microbially enhanced oil recovery methods had been implemented in USA, Malaysia, China and Argentina. Maudgalya reviewed about 26 different types of biosurfactants in field trialed of microbially enhanced oil recovery and found out 20 of the biosurfactants were capable of oil recovery (Chaprao, et al., 2015) and most promising results of the microbially enhanced oil recovery were seen in Shengli oil field of China till the date. Microbially enhanced oil research is the promising field of research and was known to show the high potential in increasing the oil production and extending the life of the oil field economically (Chaprao, et al., 2015).

2.3 OIL CONTAMINATION AND OIL REMEDIATION

Oil spills cause devastating effect on aquatic life on marine environment. Chemically synthesized surfactants had been reported for their toxicity on aquatic organisms, so were, treated them unsuitable for remediation. One of the inherent alternatives for this purpose was to find the biomolecules which had surface activity as well as the emulsifying activity along with the low Critical Micelle Concentration (CMC) characteristics. The biosurfactants emulsify the hydrocarbons in water to form various mixtures and make them water soluble. Lichenysins, rhamnolipids and surfactin are the few surfactants which are found to be successful in the remediation of the oil contamination. Kim et al, (1997) isolated a bacterium from a crude oil sample which produced a biosurfactant that had good emulsifying properties on crude oil and paraffin. Literature suggested that biosurfactants produced from marine bacterium were capable enough to destroy the oil slicks which float on the surface of water inorder to promote the dispersion of oil in water by forming a stable emulsion thereby enhancing the rate of biodegradation. Due to these factors, biosurfactants had shown potential in its applications of cleaning up the oil spills on shorelines and in the sea. The ubiquitous presence of the marine bacteria which degrade hydrocarbons have been recognized as hydrocarbonoclastic bacteria. These bacteria degrade the hydrocarbons present in the polluted sites of marine environments. Different studies revealed that the mixture of the biosurfactants stimulated the degradation of hydrocarbons in the marine environment. Hydrocarbonoclasticity bacterial consortium has a wide range of degradation capabilities on both aliphatic as well as aromatic fractions of crude oil. In general, biosurfactants produced by oil degrading bacteria

can enhance the assimilation of the hydrocarbons as well as the nutrients available in the environment. Some groups of microorganisms synthesize emulsifying agents that could help in hydrocarbon degradation, hence emulsifiers have been used for cleaning up the oil (Kim, et al., 1997). Biosurfactants can be largely produced in the industrial scale by fermentation process; Lichenysins were produced from *B.licheniformis* JF-2 which was isolated from the well water, Lichenysin even at lower concentrations (10-60 mg/l) was able to reduce the surface tension between the interfacial surfaces into ultra lesser values (10^{-2} mN/m). The range of temperature (≤ 140°C), pH (6 -10), and salinity (up to 10% w/v NaCl) variation had no effect on its activity. Biosurfactant adsorbs the oil by altering the wettability capacity of the porous media. The emulsion produced by *Acinetobacter venetianus* ATCC 31012 at 0.1 mg/ml removes 89% of crude oil which had been reabsorbed to the samples of limestone and 98% of removal was achieved are used at 0.5 mg/ml concentration (Bach, et al., 2003). Majority of the studies had focused on the possibility of introducing the bacteria which produce biosurfactants in to the infeced sites, so that they can utilize the nutrients present in the oil well for their growth, but it was more suitable for the strategy of microbially enhanced oil recovery where the bacteria would metabolically active even at extreme conditions in the petroleum reservoirs. Many bacterial species that produce biosurfactants had been described for the microbially enhanced oil recovery in-situ applications that belong to *Bacillus* sps. because of their thermal and halotolerance abiity. A typical *Bacillus* strain was grown and produced lichenysin by both anaerobic and aerobic processes at relatively high temperatures ranging from 40-60°C (Yakimov, et al., 1995). Different processes can be approached to exploit the biosurfactant producing strains in oil recovery applications. A biosurfactant composed of rhamnolipid had the CMC of 70 mg/l, was stable even at 90°C and had shown good emulsifying activity at the low pH of 2.0, but it was slightly affected by the calcium ions and salinity. In another study, it had been demonstrated that the use of *Pseudomonas aeruginosa* for microbially enhanced oil recovery had limitations due to several reasons as mentioned below:

1. *P.aeruginosa* has been classified as a Group-2 risk organism that has regulation and restriction in handling along with the dispersion of the biosurfactants in the environment.
2. The synthesis of rhamnolipid had been controlled by a several series of systems of quorum sensing which are related to the stimuli of environment

3. *P. aeruginosa* was not able to grow actively under the conditions of the reservoir.

Cloning of the biosynthetic genes had been attempted to overcome the limitation possibilities of the microorganism. Biosurfactant synthesis under strict anaerobic conditions was desirable and essential characteristic for aerobic microbes in a microbially enhanced oil recovery procedure. *Anaerophaga thermohalophila* (DSM 12881T), a well known anaerobic bacterium which was able to grow at high temperatures like 50°C and high salinity such as 7.5%, produced the low molecular weight peptide which was a surface active compound. There were several factors that affect microbial degradation of crude oil such as nature, ratio of the structural classes of the hydrocarbons and bioavailability of the substrate. One of the vital features of microbial genetic engineering in oil industry was to increase the biosurfactant secretion and to provide the bioavailability of hydrocarbons, specially, the heavy fractions to be converted, or for use in bioremediation of hydrocarbon infected soils. Poly Aromatic Hydrocarbons (PAHs) always impose harm to aquatic creatures and human fitness, in addition, their removal capacity might have constrained with the aid of using low mass transfer phases at Poly Aromatic Hydrocarbons-contaminated soils. A lot of research was being focused to investigate novel molecules that improve the bioavailability on increasing solubility of hydrocarbon contaminating compounds. Bioremediation of PHAs was considered to be the most promising and environmentally useful cleanup approach as it involves the microbial transformation of pollutants to useful metabolites. In 2002, Zhuang et al, isolated and characterized a bacterium which degrades naphthalene contaminated site present in marine sediments.

2.4 PETROLEUM HYDROCARBON DEGRADATION

The word "oil" outlined a vast form of hydrocarbon based materials. Hydrocarbons constitute chemical substances comprise the elements of hydrogen and carbon. Simple unit of fat as well as oils consisted of one glycerol molecule blended with three fatty acid molecules, called a triglyceride. At ambient temperature, if any of the material is in liquid form it would be traditionally called oil; if it would be in the form of a solid, considered as fats. Hydrocarbon biodegradation had immense ecological importance, because it incorporates the fundamental process for remediation of infected areas. Microorganisms having capability to degrade the number of hydrocarbon chains were described, and also their mode of action had been studied. The soil infected with hydrophobic pollutants was restrained via negative availability of such contaminants

to the micro-organisms. Surfactants ease the process of solubilization, stabilization emulsification and deliver the processed hydrocarbons occluded to the soil's natural rely. Microorganisms such as yeast, bacteria and filamentous fungi have been studied as remodeling representative way towards their capability to degrade a huge variety of pollutants. For this reason micro-organisms were considered to be the most effective option for traditional techniques in solving environmental troubles. Oil contaminated sites that are infected with hydrocarbons could serve as the enrichment environments to the hydrocarbon degrading biosurfactant delivering microbial strains. Production and manufacturing of the biosurfactants with the aid of soil borne microorganisms isolated from the infected sites was based on the fact that they can utilize hydrocarbons as a carbon source which were water insoluble. In fact, very little research has been carried out on marine bacteria that degrade hydrocarbons and PAHs, hence suggested that bacteria belonged to genera *Cycloclasticus*, *Vibrio* and *Pseudalteromonas* had the ability of degrading hydrocarbons through biosurfactant production. Pereira and Mudge (2004) carried out experiments on microbial degradation of biodiesel and observed that biodiesel was completely degraded by a group of microorganisms.

2.5 ANTIBIOTIC DEGRADATION

There was a huge concern on the usage of antibiotics to treat various human ailments, because antibiotics may cause various adverse effects on human health. Recently, it had been indicated that antibiotics used to treat fish and shrimp might deposit in the bottom of the pond and damaged the herbal habitat found for shrimp, fish, hen and human race. Ponds that were used to culture shrimps could contaminate the water as it consists of number of materials which include nutrients like Phosphorous, Potassium, metabolic wastes, antibiotics, different drugs defending shrimp and suspended soil debris due to erosion. Focus on the fish farms had proven that most of the antibiotics were delivered into the feed were not passed by the fish but they entered into the environment causing damage to the ecosystem in the tropical mangroves. The only safest way for the elimination of these antibiotics was by the natural biodegradation, many of the antibiotics were absorbed in the nature so as many of the microorganisms that are found in nature make food out of these antibiotics and they can have a number of antibiotic resistance genes in common. In addition to this, a few soil bacteria can also live by the use of antibiotics serving as energy source of carbon. In order to remove the pollutants involved in chemical pollution and toxicity in the environment,

biotransformation approach was always a great kind of ecofriendly process (Johnsen, et al., 2005).

2.6 CASE RESEARCH

Biodegradation of hydrocarbons can be achieved by many microorganisms as they have regulatory mechanism of systems that produce enzymes which are useful in biodegradation, these enzymes attack the hydrocarbons and process them, many of the indigenous microorganisms found in soil and water had the ability to degrade hydrocarbons as they feed on these things**.** Proper documentation should be done to know the bacterial diversity in hydrocarbon contaminated soils and it was important to know the diverse group of microorganisms to isolate and identify novel bacterial strains that can potentially degrade hydrocarbons like petroleum and their derivatives. Bioremediation offer remedy performance and economical ease of biodegradation both in-situ and ex-situ relevant, then again, introducing bacteria that degrades hydrocarbons into the infected sites which are contaminated with oil should not guarantee the complete degradation, as few components found to be difficult to degrade. By the use of bacteria, fungi and yeast, hydrocarbons in the polluted environment can be degraded, but, there was difference in the abilities of the biodegradation rate. Incase of marine bacteria it was found to be 0.003% - 100%, for soil fungi it was 6% - 82% and for soil bacteria, 0.13% to 50%. For the degradation of complicated cluster of hydrocarbons like heavy metals and crude oil present in soil, marine and fresh water, some blended populations of microbes along with overall extensive enzymatic capacities were needed. Numerous bacteria and fungi fed exclusively on hydrocarbons, but the bacteria was considered as the maximum primary hydrocarbon degrader in the areas of oil spills because of its capability to enhance the degradation rate. As there was an uptake of hydrocarbons by the microbes, similar group of microbial clusters would perform complete degradation of hydrocarbons. Activation of these microbes happened by the insertion of oxygen atoms doanted by bacteria and algae to give it to phenol cis-dihydrodiols, for rapid biodegradation in the aquatic environment dispersion and emulsification were needed. There was always an excessive concentration of tar and other crude oils, mousse, tar balls, on the surface of the water which aid the harboring of microbes. Microbial degradation has been considered to be the only natural mechanism that easily removes the hydrocarbon pollution from the environment. In order to achieve a successful biodegradation process there are some crucial requirements like optimum nutrient

concentration, oxygen, pH, etc. Optimum pH conditions was mentioned as between 6 to 9. If the microbes are given with these optimum conditions then there will be a successful degradation process with reduced costs, which was an another important determinant for the biodegradation. Experiments conducted at Baffin island to assess the effect of mineral fertilizers on biodegradation of Lago Medio crude oil buried under gravel and sand were discussed by Prince et al. in 1993. A fivefold increase in the marine oil degradation rate was inferred as compared to untreated area. All the above field trials, on a small or pilot scale, indicated the need for a liquid fertilizer that allows slow release of nitrogen and phosphorus, and as such, these formulations remain attached to the oil to be degraded. Inipol EAP22 was one such fertilizer developed by Elf Aquitaine 7, which forms an oil external micro-emulsion that contains nitrogen and phosphorous nutrients, its outer oil surface was known to stick to pollutant oil residues, and therefore, it was described as an oleophillic fertilizer. In an experimental oil spill at Norway, inipol-EAP22 was first compared with water-soluble agricultural fertilizer, a three-fold increase in alkane biodegradation of Stafford oil was observed in both Inipol-EAP22 and agricultural fertilizers-containing sites as compared to the untreated areas. Although inorganic fertilizers were much cheaper, because of their high solubility, their concentration in the water column decreased very fast, limiting their use in water bodies and in marine environment. Further, excessive use of any kind of fertilizers was always a cause of concern for environmentalists. On the other hand, slow release formulations such as inipol was used in naval material research. Bioremediation on actual oil spill was first used in November 1985 of Ny Alesund, Spitsbergen, where above 88,000 liters of marine gas oil was spilled parallel to the shoreline. Inipol was applied 4-5 months after the oil spill had occured and a 6 to 9 fold higher rate of biodegradation was observed. The largest and the most extensively studied incident was of Exxon Valdez, Alaskan oil spill, in which a tanker ran around Bligh Reef on 24 March 1989, spilling 1.8 million tons of north slope crude oil, on this Knight island, bioremediation was approved for large-scale application on 1 August 1989 and by the summer, 118 km of shoreline had been treated. The cleanup workforce employed over 11,000 workers, 1,400 boats, and 84 aircraft. By 1990, shoreline oil decreased substantially as a result of the cleanup exercise and natural cleansing. In 1990, stand-alone bioremediation in combination with mechanical cleanup techniques were used. By 1991, oil was further reduced significantly and by May-June 1992, as per Joint Federal report, it was confirmed that

all the oil had been removed from the shoreline. Experiments on oil-contaminated coastline, after an oil spill occurrence was less difficult, however, once more, scientists had little desire inside the form of seaside, awareness, form of oil, degree of weathering, and emulsification. Consequently, the mission had to address oil spills under the existing environmental conditions and tried to layout cautiously-controlled experiments. The bioremediation research below field situations can be considered as shoreline studies and open-water studies. In shoreline studies, both the inorganic and the natural fertilizers were used for bioremediation of oil slick along side or without an oil-degrading bacterium. Literature suggested that much research has been directed toward preparing formulations of biosurfactant for toxic heavy metal removal from soil and water. In that scenario, mixture of chemicals added to the biosurfactant formulations and their physical form of the formulations have been considered. Biosurfactants production was studied in terms of the microbial cellular growth and activity when we supply different carbon and hydrogen sources in the media (Pavitran, et al., 2006). Process optimization was based on the key parameters like oxygen transfer ratio to scale-up the production of biosurfactant. The concentration of nitrogen limited the biosurfactant production and sometimes overproduction by micro-organisms (Suzuki, et al., 1974). Critical micelle concentration (CMC) of a surfactant is the concentration at which micelle starts formation. The CMC of biosurfactants is many times lower than that of chemical surfactants (Desai & Banat 1997), beyond the CMC value the molecules tend to form different large molecular structures. Micelles generally form when lipid moiety of the surfactant unable to form hydrogen bonds. Biosurfactants that have low CMC increase the solubility of hydrocarbon substrates by mixing with hydrophobic moieties (Zhang & Miller, 1994). Stable emulsions were not a usual trait of these surfactants. Bioemulsifiers were high molecular weight compounds produce stable emulsions than lowering surface tension and made bacteria to bound the hydrophobic surfaces (Rosenberg, 1981 & Neu, 1996).

2.7 BIOFILM FORMATION

Biosurfactants like bioemulsifiers made use of wettability property by creating a suitable environment for the attachment of bacterial adhesion. Biofilms were the microbial communities that produces extracellular matrix (Ortega-Morales, et al., 2010). Bioemulsifiers were exopolymeric substances that help the bacteria in the biofilm formation, these substances help the cells in survival and protect themselves

from adverse extreme conditions, predators and especially from the loss of water from the cell (Das, et al., 2009). Bacterial adhesion occurs in mobile and stagnant phases.

2.8 PURIFICATION PROCEDURES

Production and purification of biosurfactants were based mainly on their charge, solubility and selection of solvents. Biosurfactants that were secreted into the supernatant are extracted from centrifugation of the culture. Purification of individual components include acid hydrolysis, solvent extraction, filtration, chromatography and lyophilization methods (Desai and Banat, 1997). Based on the species that produce biosurfactants of glyco and lipo conjugates, the selection of the solvent should be used for the precipitation of active fraction. Solvent mixtures like chloroform/methanol (2/1), acetone and ethyl acetate are used (Mata-Sandoval, et al., 1999). Generally most of the biosurfactants were less soluble in water due to their complex structure. Culture supernatant was applied to the column and different fractions were eluted with suitable solvents based on adsorption was one of the advanced separation techniques. Purification of the biosurfactants involved chromatography (Reiling, et al., 1986) separated by anion exchangers, preparative TLC using silica gel column, membrane filtration with a cut-off range of 10 kDa, foam fractionation through a column in a bioreactor, TLC offered simple and economic feasibility for the purification of biosurfactants.

2.9 IDENTIFICATION AND STRUCTURAL ANALYSIS

Mass spectrometry was one of the best proteomic analytical techniques that ionizes chemical groups based on their mass to charge ratio. Biosurfactant structural analysis can be done by tandem quadrupole mass spectrometry (TQMS), electro spray ionization (ESI). Identification of the target ions can be expertised by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS)/MS analysis using MASCOT search, the database that quantifies proteins using peptide mass spectrometry data. FTIR spectroscopy use the Infra red light for the irradiation of molecules that gives the characteristic frequencies of every molecule for the identification of chemical compound. Infra red spectra gives information about functional groups in given molecules (Heyd, et al., 2008). Structural confirmation should be done using Nuclear Magnetic Resonance (NMR) spectroscopic analysis, as it was based on transitions in atoms and chemical shifts in their frequency of absorption. It allows more accurate structure and purity analysis than IR spectroscopy. Bacteria produce a number of biological active compounds that are aggregates of

different molecules with different properties. Lipopeptides showed antibiotic property and were resistant to peptidases and proteases (Nagorska, et al., 2007). Biosurfactants exhibited many pharmacological activities: antibacterial (Stein, 2005), antifungal (Thimon, et al., 1992), antiviral (Kracht, et al., 1999), anti-mycoplasma properties (Vollenbroich, et al., 1997), and biocontrol of plant pathogens.

2.10 BIOLOGICAL ACTIVITY OF SURFACTANTS

2.10.1 Inflammation

Inflammation is triggered by the production of numerous inducers of inflammatory mediators derived from cell plasma proteins. Based on the biochemical characteristic features, inflammatory mediators can be classified into seven groups and they show effects on the vasodilation, vasoconstriction (Strassheim, et al., 2002) in sensitized organisms, resulting in respiratory failure during anaphylatic shock. Vasoactive peptides are generated through proteolysis by thrombin and plasmin in the secretory vesicles. These factors activate a series of cascades, that affects the vasculature (Chow, et al., 2007).

2.10.2 Cyclooxygenases and Lipoxygenases

Cyclooxygenases (COX) are glycoproteins exists in two isoforms COX-1 and COX-2 which are expressed in tissues and cells (Kujubu, et al., 1991). Almost in all mammalian tissues COX-1 is expressed and is the source of prostaglandins to perform the metabolic functions, such as reabsorption of renal water, homeostasis of vascular tissue and protection of gastrointestinal tract (Bubenik, et al., 1996). COX-2 can be induced in many cell types when treated with cytokines, growth factors, promoters and inducers of tumour (Coussens & Werb, 2002). COX isozymes show significant differences between their pharmacological profiles and in physiology of cell. They share amino acids required for prostaglandin H_2 synthesis (Simmons, et al., 2004). Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin possess many side affects related to arthritis, gastric ulceration. COX-2 inhibitors have benefits of NSAIDs with minimized gastric side effects. Lipoxygenase play a key role in the disorders of asthma, hypersensitivity, psoriasis and tumour induction. Leukotrienes (LTs) synthesized via the 5-LOX pathway have a great role in the inflammatory pathway (Morham, et al., 1995). Regulation of oxygen free radicals play an important role in inflammation as the nitric oxide O_2 induces the fibroblast proliferation and H_2O_2 in the activation of transcription factors like NFkB via tumour necrosis factor and IL-1 which cause inflammation by triggering several pathways. The free radicals

generated by leukocytes in the process of phagocytosis is one of the hallmarks in inflammatory process. Reactive oxygen species production by macrophages cause damage to lipid membranes, lipoproteins of the cells. Targeting the cause for the generation of oxygen free radicals is the way to treat many of the inflammatory diseases (Shen, et al., 2002).

2.11 MOTIVATION

There is always a maximum probability of certain research gaps when we encounter a global problem, current research poses in a way to bridge a gap between the review of literature and our objective of study. The objectives for the current study were specified to fill the content as one of the minor constituents in the biosurfactant research. Researchers continuously accepting the challenges on isolating a potent novel biosurfactant-producing bacteria that is capable of degrading hydrocarbons in any harsh kind of environment. The information regarding biosurfactant-producing and hydrocarbon-degrading bacteria with anti-inflammatory potential is limited in literature. In addition, work related to how quorum sensing mechanism effects the biosurfactant production which was also limited. Hence, the current work was designed to probe the role of biosurfactant production and to emulsify different hydrocarbon substrates. Hydrocarbons throw a challenge to the researchers due to their resistance against degradability. There are a few reports available on the production of novel biosurfactants from marine bacteria. The present study was aimed to isolate a new strain from marine source which was capable of emulsifying hydrocarbons. Biosurfactants were effective emulsifiers and can reduce the surface tension. Remarkable crude oil recovery emulsifying activities were shown by the glycolipopeptides belonging to the class of biosurfactants. Hence, the study was focused on identification of a novel emulsifier from a marine bacterium.

CHAPTER -III

EMULSIFICATION POTENTIAL OF BIOSURFACTANT PRODUCED FROM ACINETOBACTER M6

3.1 INTRODUCTION

Biosurfactants are surface-active, structurally diverse group of molecules that are synthesised by the microbial cells. Surfactants being used are mostly chemically synthesized. Observable increase in the interest among the scientists on microbial emulsifiers is seen due to their potential applications in environmental protection, low toxicity, high biodegradability and high foaming capacity. Biosurfactants get accumulated at the interphase between the two immiscible phases by which they can reduce the surface tension, thereby resulting in the increased solubility and emulsification of the immiscible phases. Biosurfactants have a property of converting the insoluble substrate into soluble ones, which can be utilised by the microorganisms for their metabolism (Cheng, et al., 2016). Biosurfactants with the above surface properties stand as a good example for Enhanced Oil Recovery (EOR). Some of the biosurfactants are very effective as they have an ability to reduce the surface tension of water from 72 dynes/cm to value range of 25-30 dynes/cm (Satpute, et al., 2010). Biosurfactants and bioemulsifiers differ in their molecular weights as they are composed of lipopolysaccharide, lipoprotein, polysaccharides or complex mixtures of polymers. Lipopeptides belonging to the class of biosurfactants show remarkable surface active properties viz., surplus crude oil recovery, food processing, de-emulsification, antimicrobial, antitumor, antiviral and antiadhesive activities (Bodour, et al., 2004). Dispersant activity is shown by biosurfactants as they reduce the surface tension of oil-water interface effectively. Many bacteria like *Acinetobacter*, *Rhodococcus*, *Actinomycetes* and other biofilm forming bacteria have been reported to produce biosurfactants, but, only less abundance of biosurfactant-producing microorganisms are grown in natural environments compared with contaminated environments. Biofilm formation and growth pattern may depend on various development factors such as surface area, smoothness, flow velocity and nutrients. Typical exopolysaccharides (EPS) formed from the biofilm producing bacteria show maximum carbohydrate portion and can be considered as the main ingredient in the biofilm. Resistance is offered by several bacteria that show response to different types of antibiotics by exhibiting different mechanisms such as quorum sensing, regulation

of signal peptide molecules, efflux pumps and release of antagonistic compounds. The strategies that are being followed by the researchers to inhibit the microorganisms have led to gain the resistance among them, hence there is a need to develop novel mechanisms which could regulate the genetic and molecular levels of the bacteria. Surfactants produced from the bacterial and fungal origin have been isolated and tested for their antimicrobial, anti-inflammatory and anti-tumorigenic activities. These kind of emulsifiers have a great market value as they show promising results in many of the medical applications. Marine and aquatic ecosystems offer rich biodiversity of species like pigment containing coral reef fishes. Researchers suggest that the amount of EPS production and its composition produced by marine bacteria is highly strain specific. EPS produced by diverse microorganisms play an important role in antibiotic resistance (Lee, et al., 2011).

3.2 METHODOLOGY AND RESEARCH DESIGN

3.2.1 Isolation and screening of biosurfactant producing bacteria and Biofilm Assay

Marine water samples were collected from the Bay of Bengal at Bapatla, Guntur Dist., Andhra Pradesh. Serial dilutions were performed for the screening of bacteria with highest biosurfactant producing ability. The marine isolates were grown using nutrient broth (HiMedia, Mumbai). Crystal violet based tube staining method was used to test the biofilm producing ability of the microbes (Abraham, et al., 2012). After the screening procedure, six strains were retained for EPS production on the basis of the highest amount of exopolysaccharide produced. EPS production was carried out at 37°C in 500 ml Erlenmeyer flasks containing 200 ml of Luria Bertani (LB) broth. Batch fermentation was initiated by inoculating 5 ml of a suspension of cells grown overnight in the same culture medium at 180 rpm for 168 h. The growth of the bacteria was determined spectrophotometrically at 600 nm at regular intervals. Biosurfactant production was measured at 490 nm.

3.2.2 Estimation of crude oil degradation

Bacterial isolates were inoculated with 50 ml Bushnell Haas (BH) medium and 1% crude oil as the sole source of carbon and were incubated on rotary shaker at a stirring rate of 180 rpm for 168 h. The degradation of the crude oil was assessed in terms of its cell concentration by taking absorbance at 600 nm (A_{600}) as the cell mass directly indicates the consumption of crude oil by the bacteria. The estimation of growth of screened bacteria in terms of whole cell protein was done by hydrolyzing 1.0 ml of

cell suspension with 1N NaOH at 100°C for 10 min followed by quantitative estimation of protein by Folin-Lowry's method (Hanson, 1993).

3.2.3 Screening of hydrocarbon degraders by 2,6-DCPIP oxidation test

This method consisted of 2,6-dichlorophenolindophenol (DCPIP), an electron acceptor dye to test the ability of the microorganism to utilize the hydrocarbon. The color change of DCPIP from blue (oxidized) to colorless (reduced) indicates the dye degrading capability of the bacteria. Bacterial isolates were inoculated in a 250 ml Erlenmeyer flasks containing 50 ml of BH medium, 1% of crude oil and 0.2% DCPIP, incubated at 30°C with a stirring rate of 180 rpm and observed for the de-colorization of dye (Hanson, 1993).

3.2.4 Biochemical Characterization and Hemolysis test

The biochemical characters of marine bacterium were determined using different biochemical tests. The tests done were catalase, citrate utilization, H_2S production, starch hydrolysis, indole production, MR-VP, urease production, laccase and lactase. Hemolysis test was performed on nutrient agar containing 5% blood, as a primary screening method for biosurfactant producing bacteria (Carrillo, et al., 1996). Isolates were streaked onto the agar, observed for 48 h to determine the hemolytic potential.

3.2.5 16S rDNA sequence analysis

The 16S rDNA analysis of the marine bacterium (Sample-M) was analyzed. DNA purification kit (PureFast® Bacterial Genomic DNA kit), 16S Bac specific Primer - forward (10 picomoles/µl), 16S Bac specific Primer- reverse (10 picomoles/µl) were used to amplify the 16S rDNA gene by PCR. The PCR mixture contained 50 µl final volume; 25 µl of Master Mix contains (10X *Taq* buffer, 2 mM $MgCl_2$, 0.4 mM dNTPs mix, 2U Proofreading *Taq* DNA polymerase, 1 µl Genomic DNA). The DNA amplification was performed using Eppendorf thermal cycler. The PCR program was as follows: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, 30 cycles each. The PCR products were visualised under UV light after electrophoresis on a 2% (w/v) agarose gel containing ethidium bromide. The PCR product was sent to Helini Biomolecules for Sequence analysis. The nucleotide sequence of the 16S rDNA genes were aligned using BLAST-N program (Lee, et al., 1993).

3.2.6 Biofilm assay and EPS estimation

Luria Bertani medium was inoculated with marine bacterium, incubated for 168 h at a stirring rate of 180 rpm at room temperature. The tubes were decanted and washed with phosphate saline buffer to remove the planktonic bacteria. The dried tubes were

stained with 0.1% crystal violet solution and excess stain was removed using distilled water. The tubes were dried in inverted position and observed for biofilm production. After the cultures reached the exponential phase of growth (24–36 h), the culture broths were heated at 100°C for 15 min to inactivate the enzymes capable of degrading the polymer (Cerning, et al., 1995). The cells were then removed by centrifugation at 6000 rpm for 20 min at 4°C, then the EPS was precipitated by adding two volumes of cold absolute ethanol. The EPS was collected by centrifugation at 5000 rpm for 30 min at 4°C and dissolved in small volume of distilled water to repeat the precipitation procedure as described previously. It was subsequently dialysed (molecular weight cut-off 6000–8000 Da) against deionized water for 48 h, precipitated and dried at 40°C. Cell bound EPS was studied by centrifuging cells at 13,000 rpm for 10 min, and the pellet was treated with a mixture of 0.5 M NaCl and 0.05 M of EDTA for 2 h at 50°C. The mixture was centrifuged, dialyzing (membrane with 3,500 Da cutoff) against distilled water for 72 h. The amount of EPS was quantified using phenol-sulphuric acid method. The protein content was determined by following the method proposed by Lowry et al. (1951) using albumin as the standard protein.

3.2.7 Estimation of Biomass

Culture flasks were inoculated with marine bacterium, incubated for 168 h on shaker at 37°C. Biomass was extracted by mixing equals volumes of acetone to the cell free supernatant, followed by centrifugation at 10000 rpm for 20 min. Dry biomass can be obtained by drying at 80°C overnight.

3.2.8 Effect of Media, pH, temperature, carbon, nitrogen and sodium chloride on biosurfactant Production

To observe the effect of media, different media were tested and LB medium was selected. To study the effect of different temperatures on biosurfactant production, LB broth was inoculated with marine bacterium, incubated at different temperature ranges between 15 and 50°C. The effect of pH on biosurfactant production was studied using LB broth, adjusted to different pH ranges from 4 to 12. Similarly the effect of salt concentration was determined by screening varied concentrations of NaCl (w/v). Effect of carbon source was studied using glucose, lactose, maltose, sucrose, fructose, galactose, ribose and mannose at 1% (w/v) concentration. Effect of nitrogen source was studied using yeast extract, beef extract, peptone, alanine, casein and glycine at

1% (w/v) concentration. Biosurfactant production was measured after 168 h of incubation at 490 nm spectrophotometrically.

3.2.9 Surface tension and the Critical Micelle Concentration (CMC)

The concentration at which micelles began to form was constituted as the CMC. Biosurfactant was dissolved in double distilled water (pH 7.0) at concentrations of 0, 50, 100, 150, 200, 250, 300, 500, 750 and 1000 mg/l (Gadelha, et al., 2014)**.** Surface tension measurements were carried out with a K_6 tensiometer (Kruss, Germany) using measured by using Ring method at room temperature. The CMC was determined by plotting the surface tension as a function of the biosurfactant concentration, and surface tension.

3.2.10 Emulsifying potential of the biosurfactant

The emulsifying potential of the crude and purified biosurfactants were found against hydrocarbons (benzene and xylene), vegetable oils (olive and sunflower) and crude oils (kerosene, diesel and petrol). All the hydrocarbons were of analytical grade (Fischer Scientific, USA) and rest of the oils were bought from local suppliers. The emulsifying activity was measured by combining equal volumes of biosurfactant solution (1% w/v) and the hydrophobic substrates, mixed by using a vortex for 2 min and left to stand for 168 h at 30°C. Emulsifying activity was determined by calculating the percentage of the total height occupied by the emulsion (Table-1).

3.2.11 Staining and Visualization of EPS with fluorescently labeled lectins

For the visualization of EPS of sample-M, glass slides were immersed in bacterial suspension and kept in Petri dishes for 7 days at 37°C for the biofilm formation, then the glass slide surface is covered with 50 µl of 300 nM DAPI solution dissolved in PBS for 15 min and the slide was rinsed several times with PBS. Drain excess buffer from the cover slip and the slide was observed under fluorescence microscope using blue/cyan filters (excitation at 360 nm; emission at 470 nm), Olympus at 100X after drying (Pal, et al., 2013).

3.2.12 Gel Filtration Chromatography, TLC and FTIR analysis

Biosurfactant (10 mg/ml) dissolved in de-ionized water was filtered through 0.22 µm membrane filter, loaded onto a Sephadex G-100 column. The column was equilibrated using de-ionized water and the sample was eluted using de-ionized water. Fractions of 1 ml were collected and pooled. Total carbohydrate content of the fractions was determined by phenol-sulphuric acid method. The purified biosurfactant was dissolved in methanol and spotted on a 10 x 10 cm precoated silicagel GF 254. Test samples

were prepared by the homogeneous dispersal of 1 mg of the biosurfactants in pellets of potassium bromide. IR Spectra were recorded in the range of 400-4000 cm^{-1} using OPUS v5.0 software averaging 64 scans at a resolution of 4 cm^{-1} (Talari, et al., 2016).

3.3 RESULTS AND DISCUSSION

3.3.1 Isolation and screening of Biosurfactant producer

After screening, out of 37 isolates, six were found positive for test of crude oil degradation through whole cell protein estimation, one isolate was selected based on its highest exopolysaccharide producing ability, hemolysis capacity, and dye degrading capability, which are indicators of the biosurfactant production of *Acinetobacter* M6 after characterization using biochemical methods and 16s rDNA sequencing (Carrillo, et al., 1996). The preliminary identification was done based on the morphological and biochemical characteristics of the bacteria. The ability of the isolate to use crude oil as the sole source of carbon is estimated in terms of its increase in turbidity and indicated a variety of results, ranging from luxuriant to moderate growth (Hanson, 1996). The other method of screening for hydrocarbon degrading efficiency was the estimation of whole cell protein. This method directly indicates the increase in whole cell protein implies the ability of bacteria to grow on crude oil as the sole carbon and energy source (fig.3.1), (Table.3.1). A redox indicator dye dichlorophenol indophenol (2,6, DCPIP) was used to assess the potential of isolates to degrade oil. The isolate discolored the dye after a period of 72 h at 37°C, indicating a slow response to the biological oxidation. In a drop-collapsing test, a flat drop was seen; and in the oil displacement method, a clear zone was observed. The assessment of whole cell protein concentration based on bacterial growth indicates the potential for crude oil degradation.

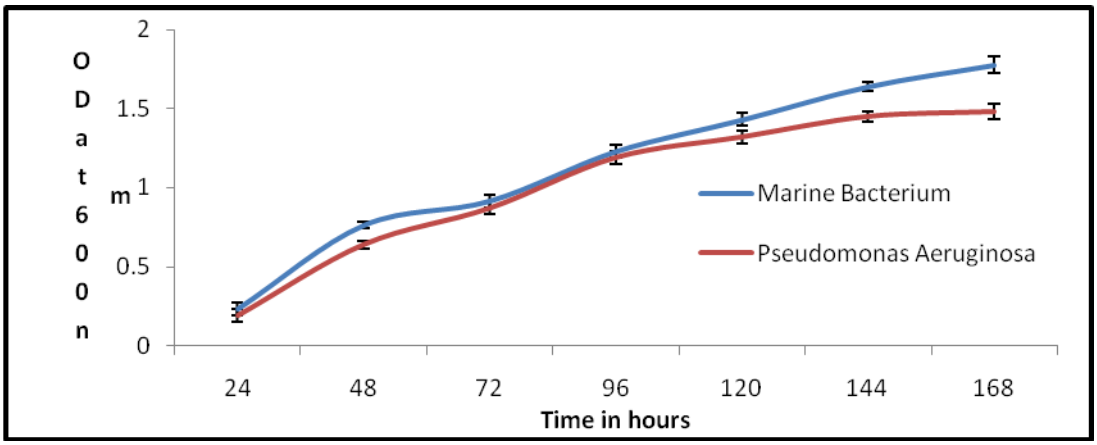


Fig.3.1 Growth profiles of *Pseudomonas* and marine bacterium in the BH medium with 2% kerosene as a sole carbon source at 37°C and 200 rpm .

Table.3.1 represents the whole cell protein concentration of the marine bacterium

Organism	Turbidity due to utilization of Kerosene (2%)	Whole cell Protein (µg/ml)
Marine bacterium	++++	356 ± 4.0
Pseudomonas aeruginosa	++++	347 ± 3.1

The biochemical test results for the marine isolate are given in Table 2. The strain showed positive to biofilm formation Catalase, Laccase and negative for Hydrolysis of starch, Lactase, Methyl Red, Voges-Proskauer, Indole production, Utilization of citrate, Urease production and HydrogenbSulphide production tests (fig.3.2), (Table.3.2). Fermentation test is always an important step to differentiate between the closely related strains. The isolate has the capacity to produce acid from glucose, xylose, galactose, mannose and negative reaction observed in sucrose, maltose, lactose and mannitol tests. *Acinetobacter* group was often difficult to identify because of the additional tests involved. At the same time, nucleic acid hybridization and 16s rDNA sequencing studies would provide the best available methods for determining the species relationships between microorganisms. The isolates were screened for their ability to carry out lysis of RBCs, which might be related to lowering the surface tension due to growth of biosurfactant-producing isolates. It was observed that 40% of the isolates grown on blood agar plates were positive for hemolysis i.e. lysis of RBCs. There are reports in literature regarding the use of blood agar lysis method to screen microorganisms for biosurfactant production (Joshi, et al., 2008; Plaza, et al., 2006)

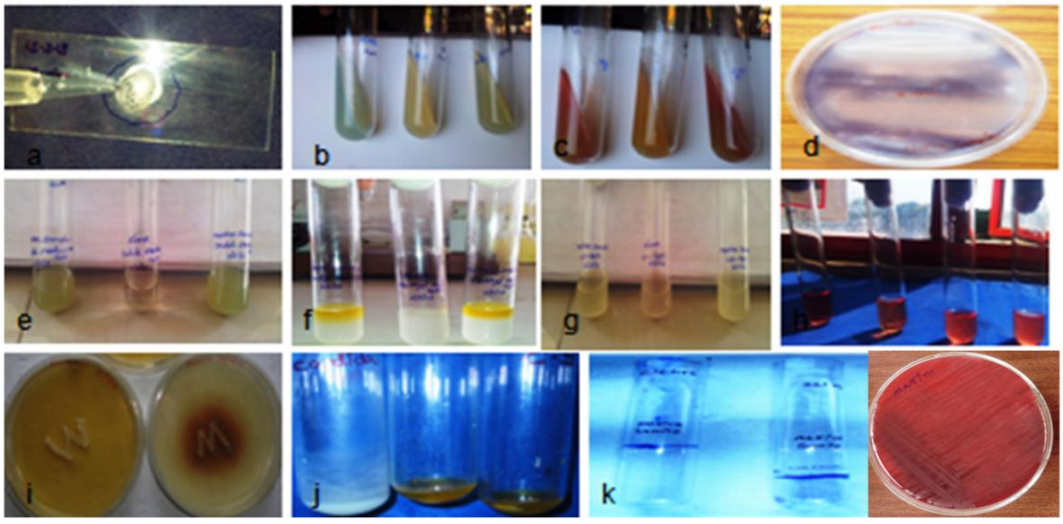


Figure 2: Images of results of various biochemical tests- (a) Catalase (b) Citrate Utilization (c) H_2S production (d) Starch Hydrolysis (e) Indole production (f) MR test (g) VP test (h) Urease production (i) Laccase (j) Lactase (k) Biofilm formation (L) Hemolysis

Fig.3.2. Biochemical characteristics of Marine bacterium

Table.3.2 Biochemical characteristics of Marine bacterium

S.no	Biochemical tests	Sample-M
1.	Catalase	+
2.	Citrate utilization	-
4.	H_2S production	-
5.	Starch hydrolysis	-
6.	Indole production	-
7.	MR	-
8.	VP	-
9.	Urease	-
9.	Laccase	+
10.	Lactase	-
11.	Glucose oxidation	+
12.	Xylose oxidation	+
13.	Mannitol oxidation	-
14.	Sucrose oxidation	-
15.	Galactose oxidation	+
16.	Mannose oxidation	+
17.	Lactose oxidation	-
18.	Maltose oxidation	-

3.3.2 16S rDNA sequence analysis

The Phylogenetic position of isolated sample-M based on its 16S rDNA gene sequence confirmed that its closest relative was genus *Acinetobacter* (fig.3.3). However, definitive species identification of this bacterial isolate requires an approach including biochemical, physiological and nucleic acid-based methods. Several genotypic based methods developed for the identification of *Acinetobacter* species. Recent taxonomic studies suggested that genus *Acinetobacter* belonged to subclass Gammaproteobacteria, family Moraxellaceae. Recent molecular studies have shown 31 distinct *Acinetobacter* species. Only a few phenotypic techniques have been validated to identify important *Acinetobacter* species. In our study, genus was identified as *Acinetobacter* by conventional method and was further confirmed by molecular method (16s RNA sequencing). Genotypic identification gives faster turnaround time and high sensitivity and plays an important role in the identification of isolates with distinct biochemical profiles. Genera *Acinetobacter* has been reported with many species and 16S rDNA sequences of it are available in GenBank. After alignment with Clustal W, using neighbor-joining method uncorrected distances and pair wise deletion options were selected to generate a conservative estimate of

divergence among the selected sequences. The evolutionary distances were computed using the p-distance method. The analysis involved 5 nucleotide sequences. When analyzed for evolutionary relationships, all the four strains of *Acinetobacter* sp. namely *indicus, radioresistens, baumanii and junii* clustered in a single group with respect to our marine isolate, sample-M (Chang, et al., 2005). The sequence of Acinetobacter M6 was submitted to NCBI and alloted with Accession no: KR559749 (fig.3.4)

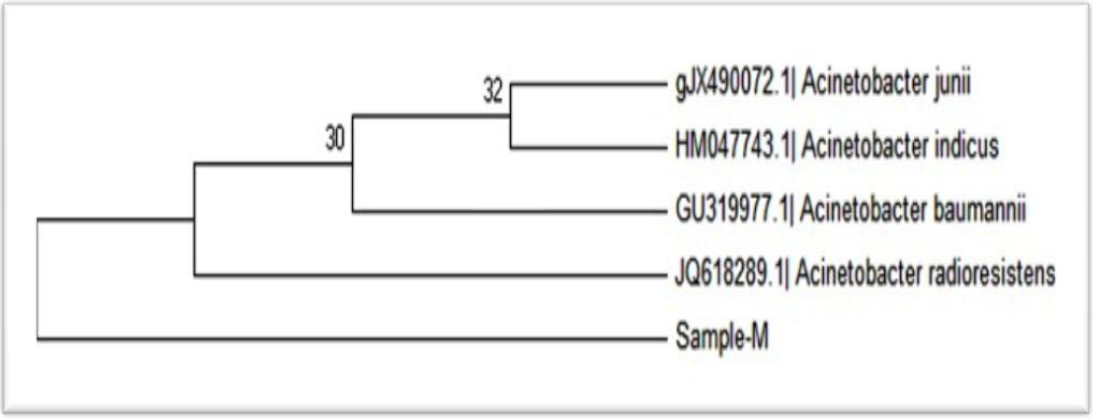
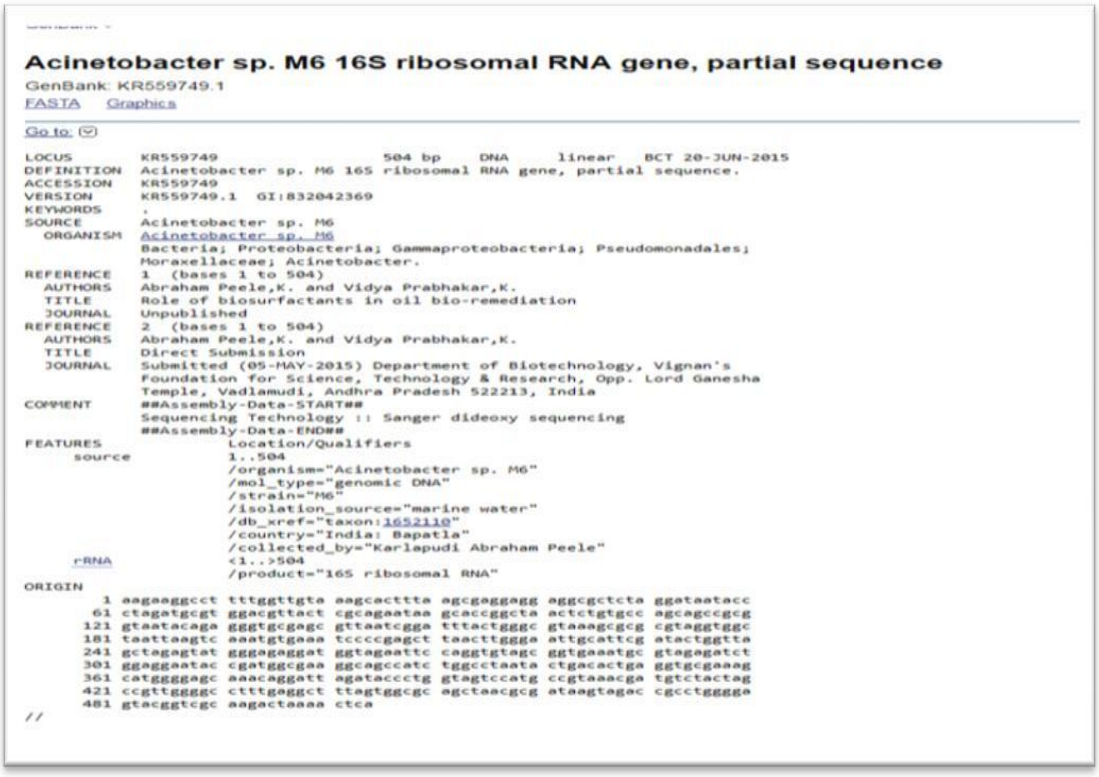


Fig.3.3 Phylogenetic tree based on 16S rDNA gene sequences, showing the positions of strain sample-M (A*cinetobacter M6*, Accession no: KR559749) relative to all known *Acinetobacter* species. Numbers above the branches denote posterior probabilities to percentage converted.



Acinetobacter sp. M6 16S ribosomal RNA gene, partial sequence

GenBank: KR559749.1

FASTA Graphics

Go to:

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LOCUS       KR559749                 504 bp    DNA     linear   BCT 20-JUN-2015
DEFINITION  Acinetobacter sp. M6 16S ribosomal RNA gene, partial sequence.
ACCESSION   KR559749
VERSION     KR559749.1  GI:832042369
KEYWORDS    .
SOURCE      Acinetobacter sp. M6
  ORGANISM  Acinetobacter sp. M6
            Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
            Moraxellaceae; Acinetobacter.
REFERENCE   1  (bases 1 to 504)
  AUTHORS   Abraham Peele,K. and Vidya Prabhakar,K.
  TITLE     Role of biosurfactants in oil bio-remediation
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 504)
  AUTHORS   Abraham Peele,K. and Vidya Prabhakar,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAY-2015) Department of Biotechnology, Vignan's
            Foundation for Science, Technology & Research, Opp. Lord Ganesha
            Temple, Vadlamudi, Andhra Pradesh 522213, India
COMMENT     ##Assembly-Data-START##
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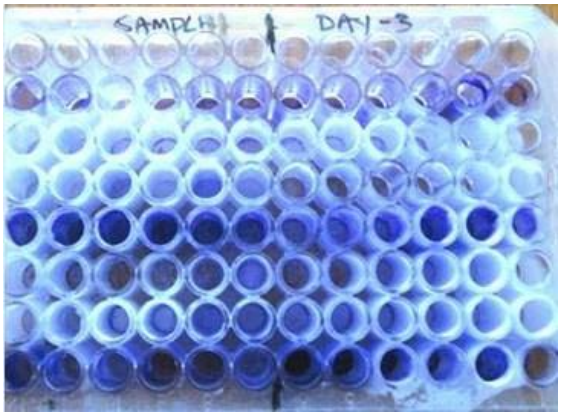
      181 taattaagtc aaatgtgaaa tccccgagct taacttggga attgcattcg atactggtta
      241 gctagagtat gggagaggat ggtagaattc caggtgtagc ggtgaaatgc gtagagatct
      301 ggaggaatac cgatggcgaa ggcagccatc tggcctaata ctgacactga ggtgcgaaag
      361 catggggagc aaacaggatt agataccctg gtagtccatg ccgtaaacga tgtctactag
      421 ccgttggggc ctttgaggct ttagtggcgc agctaacgcg ataagtagac cgcctggggа
      481 gtacggtcgc aagactaaaa ctca
//
```

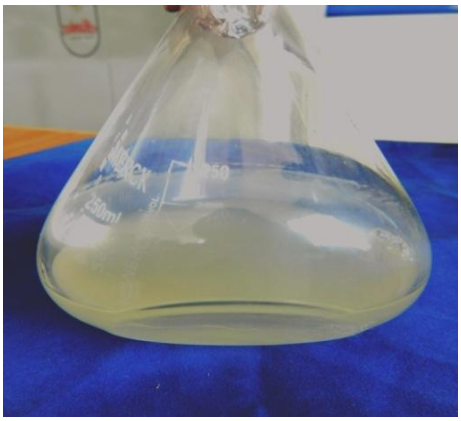
Fig.3.4 A*cinetobacter M6*, KR559749 gene sequence submitted to NCBI

3.3.3 Staining and visualization of biofilm EPS with fluorescent stain

One of the most important tools for the detection of components of the extracellular polymeric substances (EPS) in biofilms is staining through fluorescence microscopy in combination with DAPI. Exopolysaccharides are visualized according to their interaction with specific target sugars that explores complex arrangement of EPS in biofilms. Crystal Violet staining is the preliminary method of visualization (fig.3.5), later the biofilm was stained with DAPI, visualized under fluorescence microscope (fig.3.6), DAPI stains the cells and extracellular matrix by passing through the cell membrane and allows the microscopic detection of the EPS in the biofilm.



(a)



(b)

Fig.3.5 (a) Biofilm assay by crystal violet staining (b) EPS layer formation after addition of Acetone

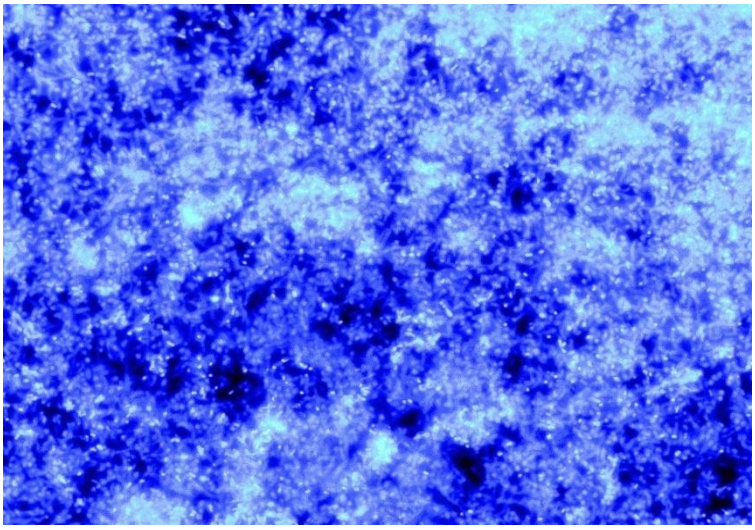


Fig.3.6 Visualization of bacterial cells and EPS staining (excitation at 360 nm; emission at 470 nm) with DAPI by fluorescence microscopy at 100x, Olympus.

3.3.4 Biosurfactant production and quantification

Biosurfactant production was started after 12 h of incubation at which 0.85 g/l dry weight were produced during 1st day of incubation, these values progressively

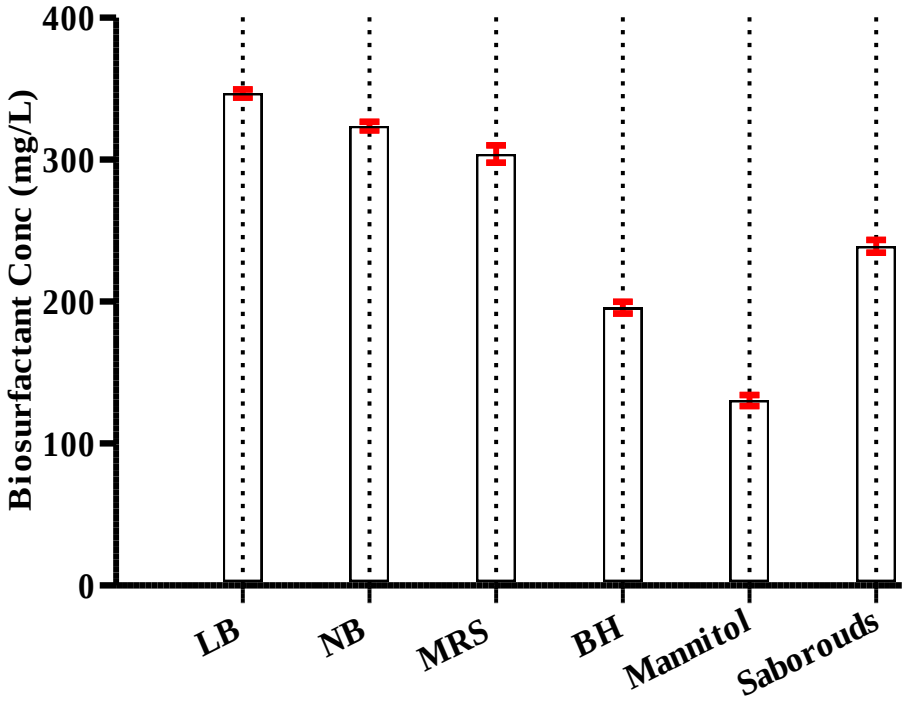
increased with a maximum biomass yield of 8.8 g/l at 7 days (168 h) of culture and a maximum biosurfactant concentration of 370 mg/l during early stationary phase and it is evident that concentration is directly dependent on the yield of the culture (fig.3.7). The concentration of biosurfactant varied with time. The EPS content which was expressed as the biosurfactant concentration reported in the present study was substantially higher than previously reported values for other *Acinetobacter sp.* For biosurfactant production, *Bacillus* species reported from hydrocarbon (Klawech, et al., 2013) utilized soybean oil and waste lubricating oil respectively as the carbon sources for biosurfactant production. *Bacillus* strains reported for bioemulsifier production (Patel & Gopinathan, 1986) were grown in the presence of fenthion as the sole carbon source while *B. stearothermophilus* VR-8, (Gurjar, et al., 1995) produced bioemulsifier in presence of 4% crude oil as a carbon source. Optimizing the production of metabolites was done by modeling tools available with respect to the typical physical and nutritional factors. Designing and formulating the basic and needed components of the media is the challenging task to accomplish and developing a database of the microbial response growth production mediated studies with respect to the number of factors tested is the main criteria.

(a) (b) (c)

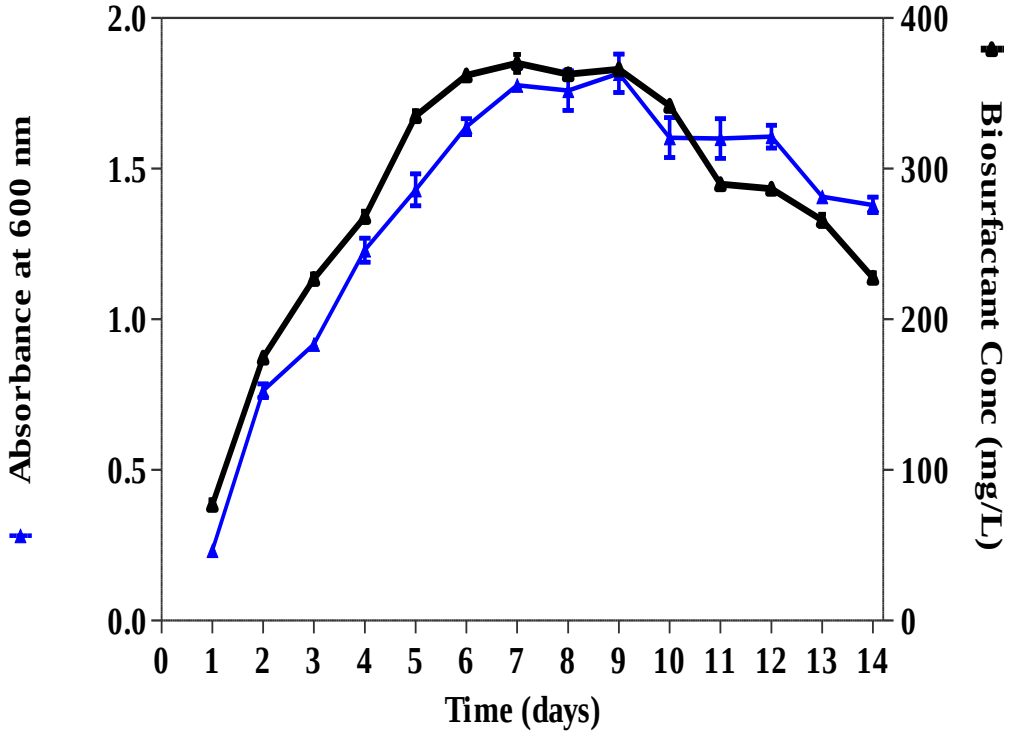
Fig.3.7. (a) crude biosurfactant (b) drop-collapse method (c) Purified Biosurfactant

3.3.5 Effect of Media, pH, temperature, carbon, nitrogen and sodium chloride on biosurfactant Production

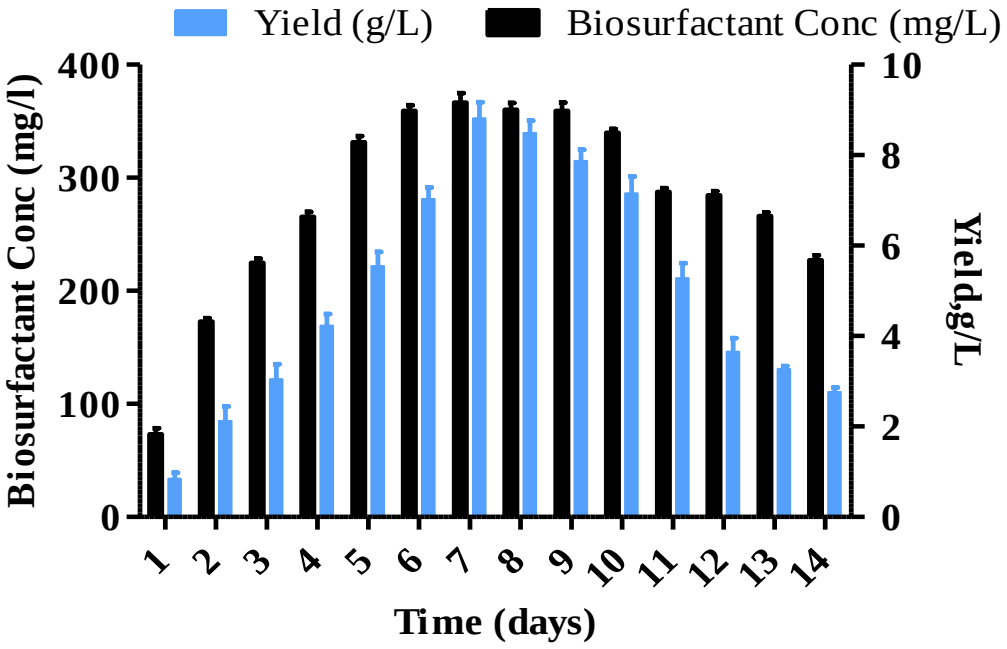
The marine isolate selected for the study was grown on LB media and found to be more selective for the production of biosurfactant. Biosurfactant production was not drastically affected data temperature range between 30 and 40°C, but its concentration was reduced at above 40°C. Optimum growth was at pH 7.0 and 35°C. NaCl concentrations at 1% were observed as the highest for growth (fig.3.8, 3.9).


Biosurfactant Conc (mg/L)
400
300
200
100
0
LB
NB
MRS
BH
Mannitol
Saborouds

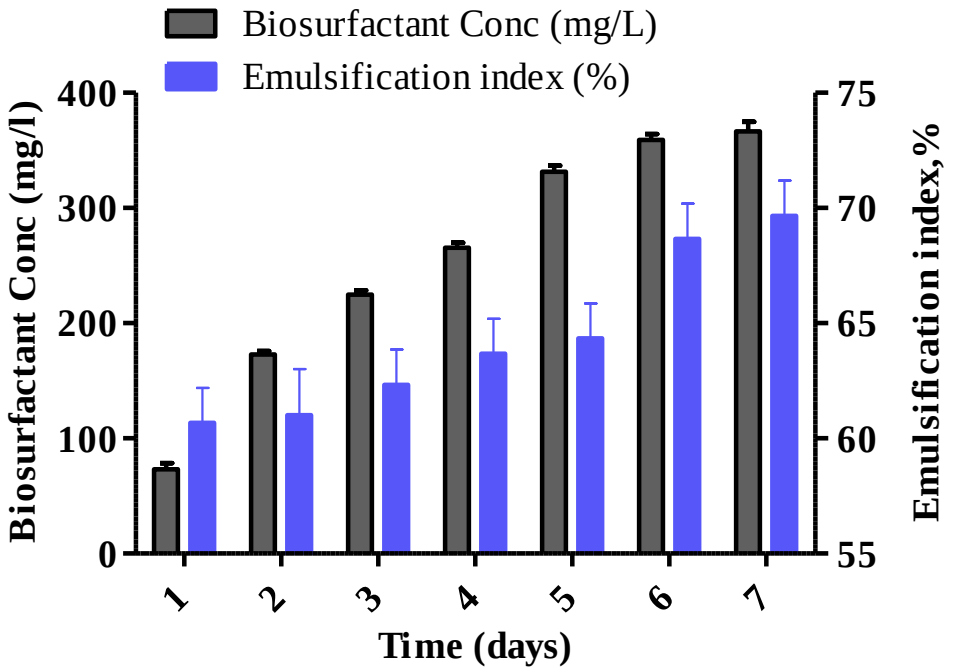
(a)


Absorbance at 600 nm
Biosurfactant Conc (mg/L)
2.0
1.5
1.0
0.5
0.0
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (days)

(b)

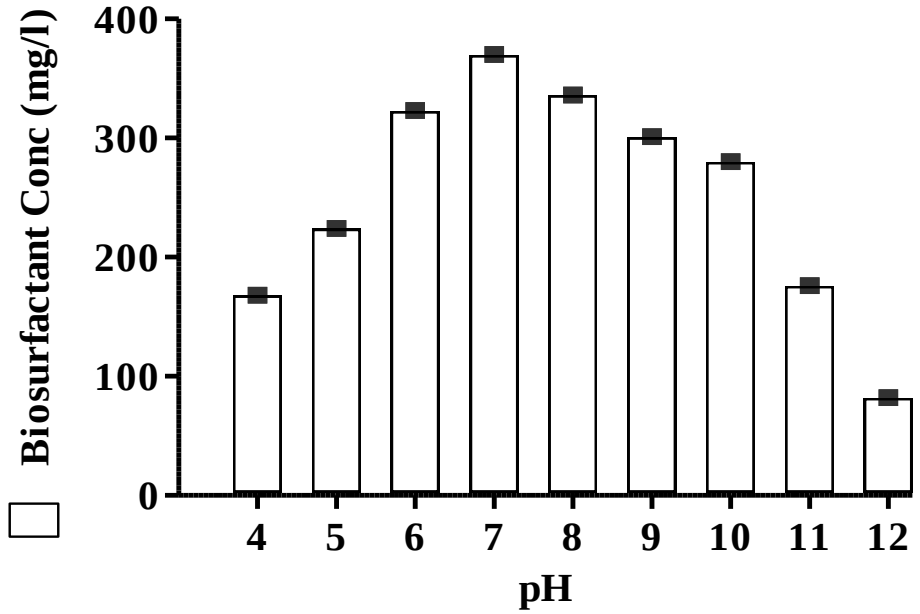


(c)

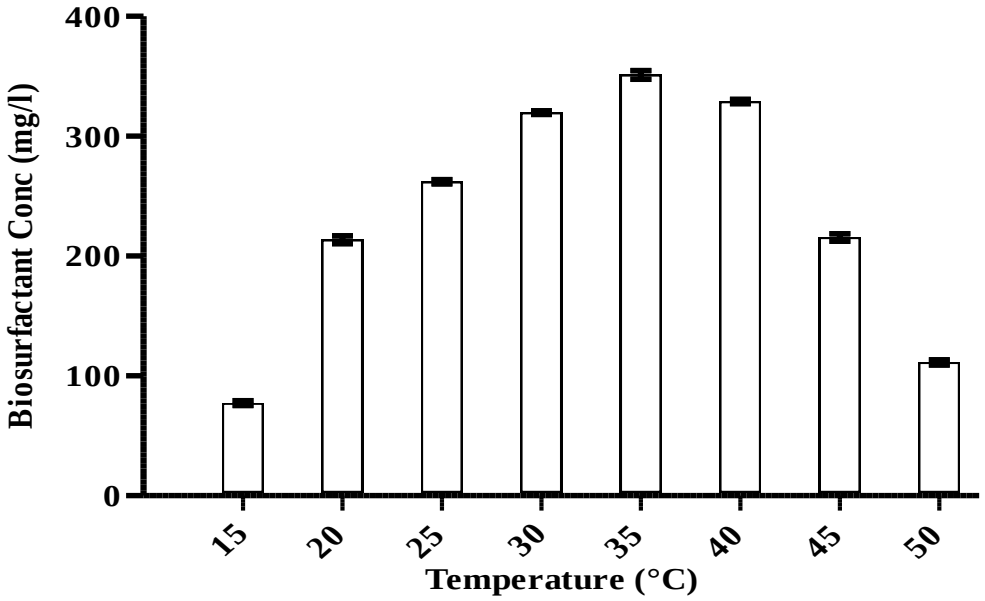


(d)

Fig.3.8 (a) Effect of Media on biosurfactant production, (b) Time course (growth kinetics) of biosurfactant production, (c) Effect of biomass yield on biosurfactant production, (d) Emulsification index of *Acinetobacter M6* produced biosurfactant



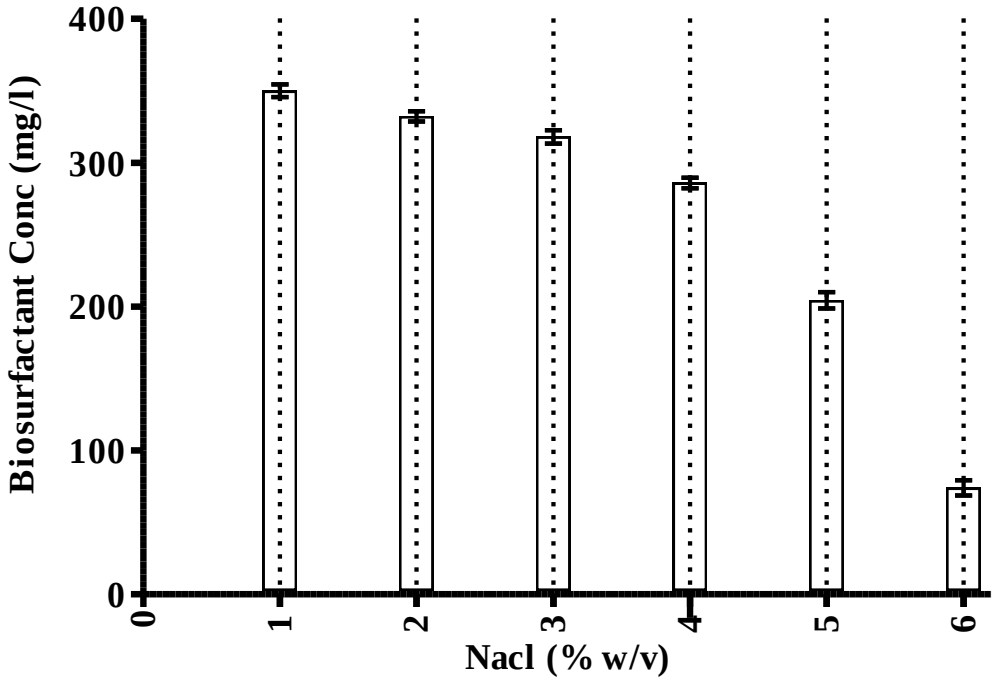
(a)



(b)

Mozzi et al. (1996) measured the maximum polysaccharide synthesis at 488 mg/l at a constant pH of 6.0 for *L. casei* CRL 87. In their research, they observed that the amount of biosurfactant was 3.6 times as high in pH control as in those without pH control. In the case of *L. sakei*, the optimum pH for EPS production was 5.8, but higher cell numbers were achieved at pH 6.2. The maximum production of

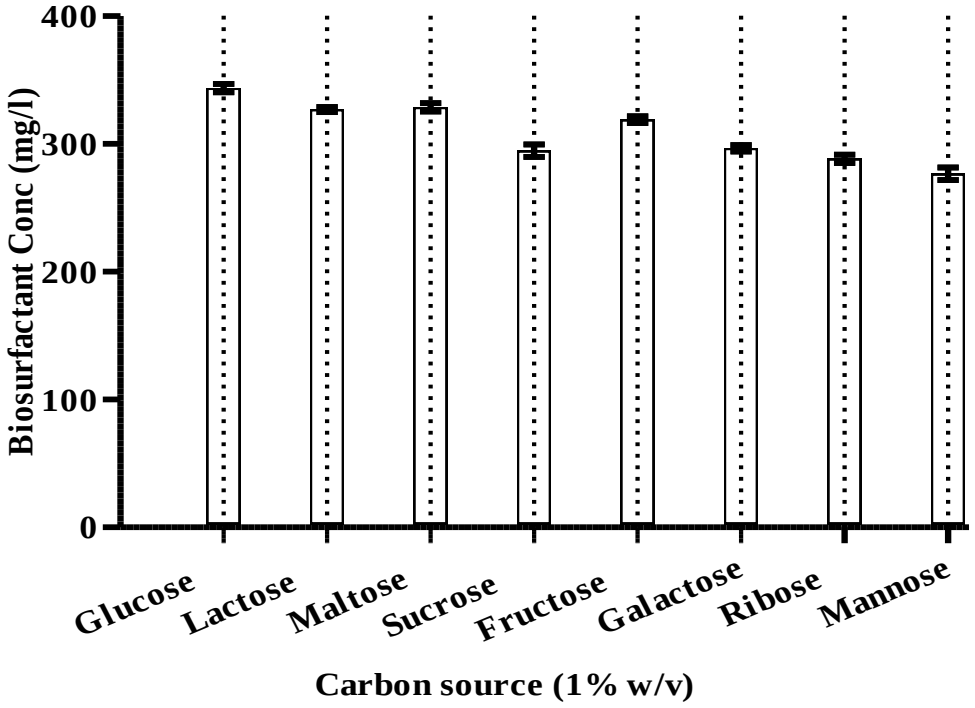
biosurfactant was observed at 7 day time interval when Luria Bertani medium was used compared to the other media (Nutrient Broth medium, MRS, BH, Mannitol and saborouds). It should be evident from the obtained data that a high amount of biosurfactant production results in high emulsification indices (fig.3.8).



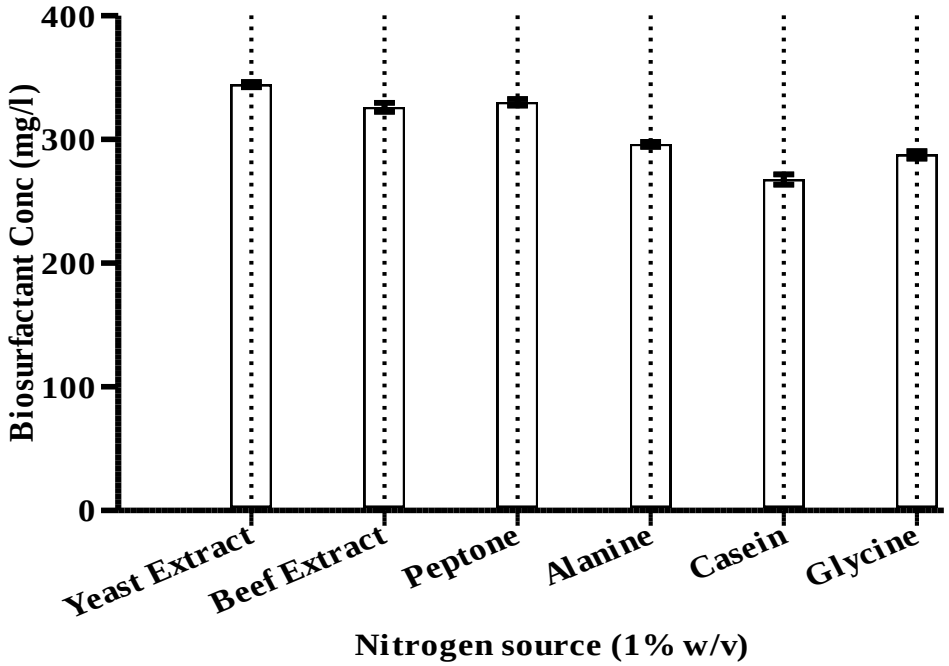
(c)

Fig.3.9 (a) Effect of pH, (b) Temperature, (c) salt concentration on biosurfactant production by *Acinetobacter M6* in LB medium

Addition of carbon and nitrogen sources (C/N) to the LB medium did not induce biosurfactant production as we compared the control with that of without addition of the C/N source. Here, the inability of sugars and nitrogen sources to enhance biosurfactant production was observed (fig.3.10). Glucose was one of the few sugars that caused a slight increase in the production of biosurfactant, but this resulted in a small increase of 0.1%–0.3% only. In our present study, among the carbon and nitrogen sources tested, glucose showed the highest biosurfactant concentration of 361 mg/l at 0.2% (w/v) concentration, whereas yeast extract at 0.4% (w/v) showed a maximum concentration of 365 mg/l (fig.3.11). Always, the amount of biosurfactant produced depends not only on the bacterial strain, the energy source used and the culture conditions maintained, but also by the genetic regulation of the signal molecules involved in synthesis of biosurfactant genes.

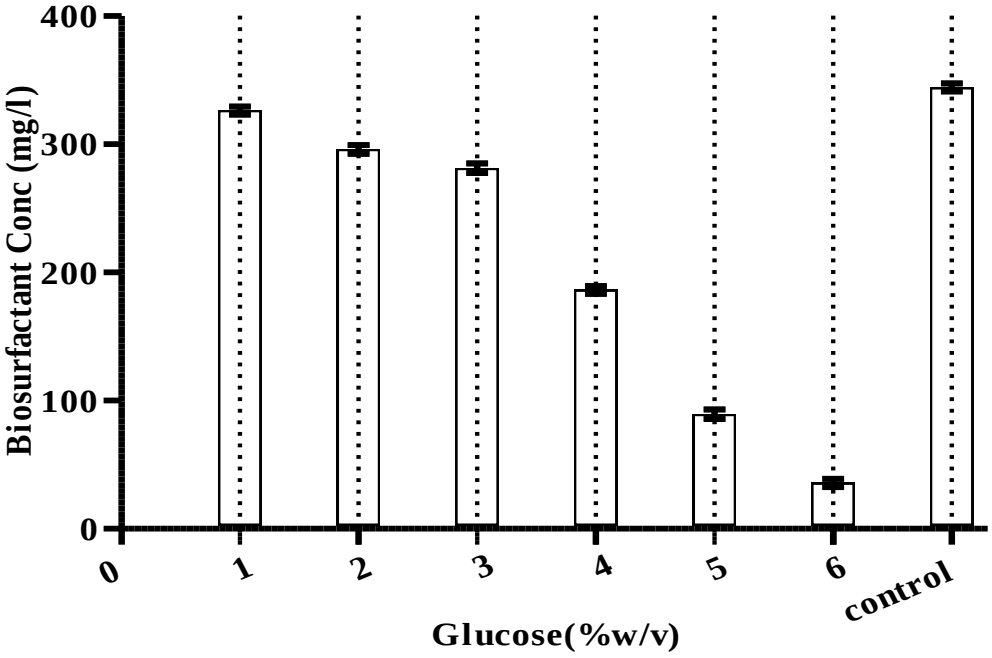


(a)

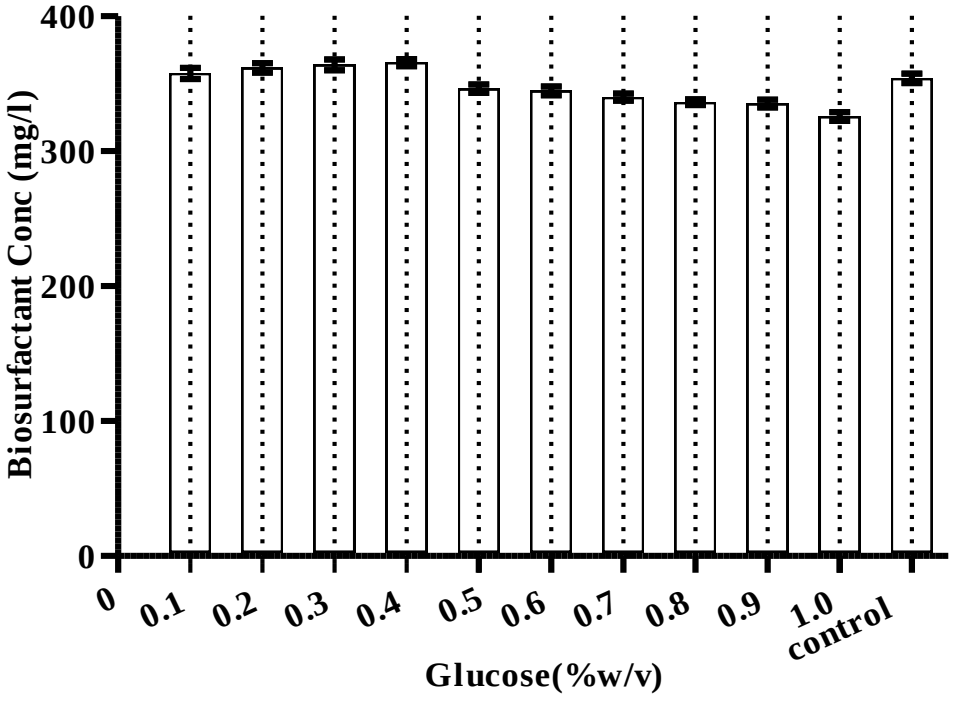


(b)

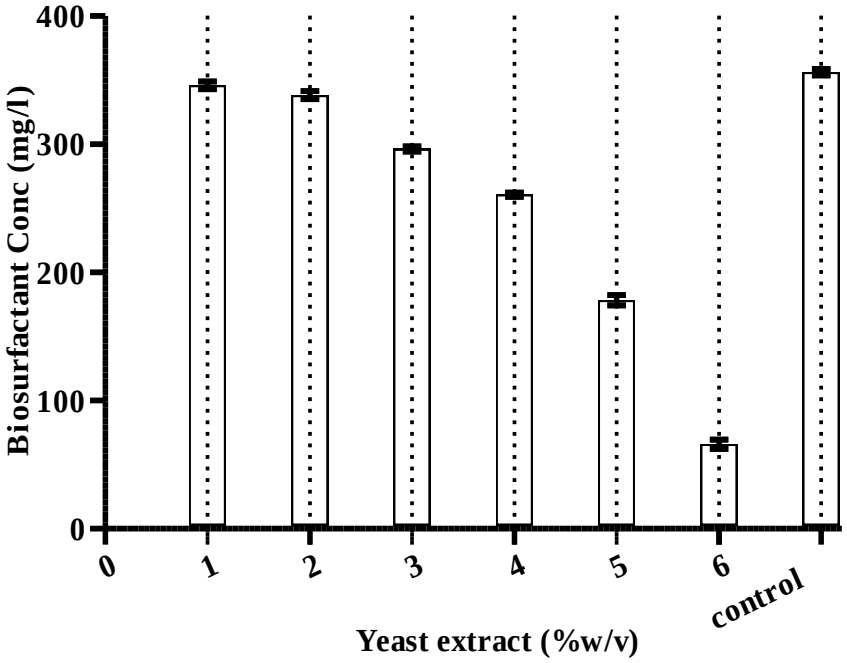
Fig.3.10 Effect of different (a) carbon and (b) nitrogen source on biosurfactant production by *Acinetobacter M6* in LB medium


Biosurfactant Conc (mg/l)
400
300
200
100
0
0
1
2
3
4
5
6
control
Glucose(%w/v)

(a)


Biosurfactant Conc (mg/l)
400
300
200
100
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
control
Glucose(%w/v)

(b)



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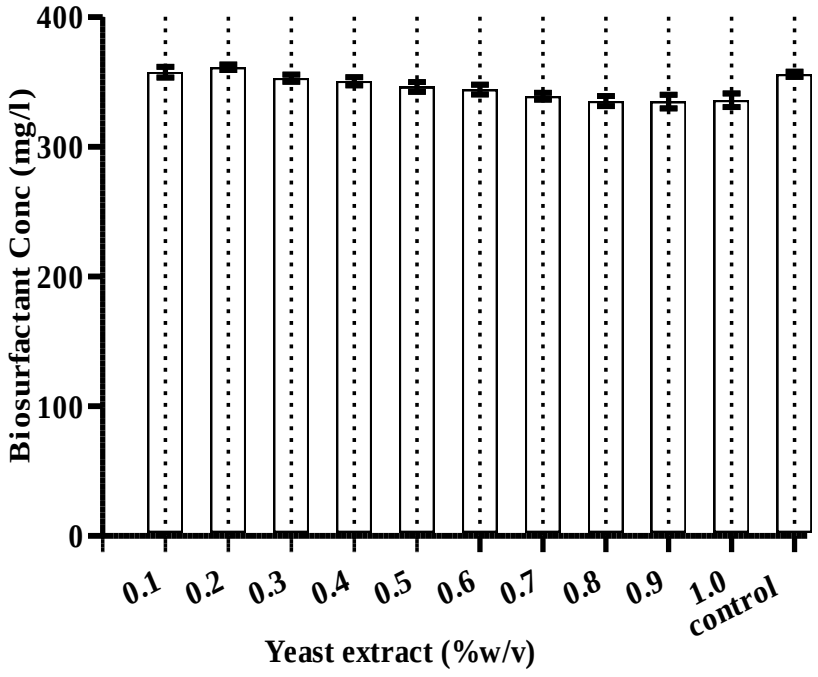


(d)

Fig. 3.11 Effect of different concentrations of carbon and nitrogen sources (a, b, c, d) on biosurfactant production

The present study showed that glucose and yeast extract were considered as the preferred carbon and nitrogen sources, but, interestingly, at high concentrations the production rate decreased and the reason might be the exopolysaccharide produced

between early logarithmic and late stationary phase was a typical glycoprotein. The addition of carbon and nitrogen sources even at lower concentrations may act as limiting substrates due to the production of glycoprotein. In bacteria, glucose and yeast extracts were excellent sources for growth and even regulate product formation. Regulation at the level of gene may depend on the use of preferred source. Different mechanisms in bacteria have been described to explain the negative effects of carbon and nitrogen sources in metabolite production. Biosurfactant production involves a quorum-sensing mode of regulation mediated by induction peptides. When the peptide reaches a critical threshold concentration, it triggers a series of reactions that could regulate promoters to activate all set of genes involved in biosurfactant production. In many cases bacteria grown with various carbon and nitrogen sources were observed with greater cell mass than those grown without them. However, even at a high cell density, the culture yielded lower surfactant production and vice versa (Sanchez et al., 2010). The biosurfactant concentration by *Acinetobacter* M6 from the carbon sources, galactose, ribose and mannose were lower than that from lactose, maltose, and fructose. The product concentration from yeast extract, peptone and beef extract was higher than that of alanine, casein and glycine.

3.3.6 Surface tension and the Critical Micelle Concentration (CMC)

One of the key characteristic features of biosurfactant is to lower the surface tension. As surfactant concentration increases, the surface tension of the solution initially decreases and then become almost constant due to the interface saturation with surfactant. The concentration at which this phenomenon occurs is known as the critical micelle concentration (CMC). Efficiency is measured by the surfactant concentration required to produce a significant reduction in the surface tension of water, whereas effectiveness is measured by the minimum value to which the surface tension can be reduced (Parkinson, 1985). Therefore, the important characteristic properties of potent surface-active agents are their abilities to lower the surface tension in aqueous solutions, and to possess a low CMC (Sheppard and Mulligan, 1987). Biosurfactants are quite effective even at low concentrations than chemically made surfactants, so small amounts of biosurfactants are needed to reduce surface tension. The CMC of the biosurfactant preparation was 300 mg l^{-1} (fig.3.12) and the corresponding γCMC was 39 mNm^{-1} (Khopade, et al., 2012). Thus, the biosurfactant produced by Acinetobacter M6 is effective biosurfactant as compared to a range of CMC between 200-300 mg/l reported in literature (Pornsunthorntawee, et al., 2008;

Wei, et al., 2005). According to Haba et al. (2003), the differences between the CMC values reported in the literature are generally related to the ratio and composition of homologues, the presence of unsaturated bonds, the branching and length of the aliphatic chain of the biosurfactant. Thus, there is a need to further purify the components of the surface-active preparation of in order to understand the composition of each component.

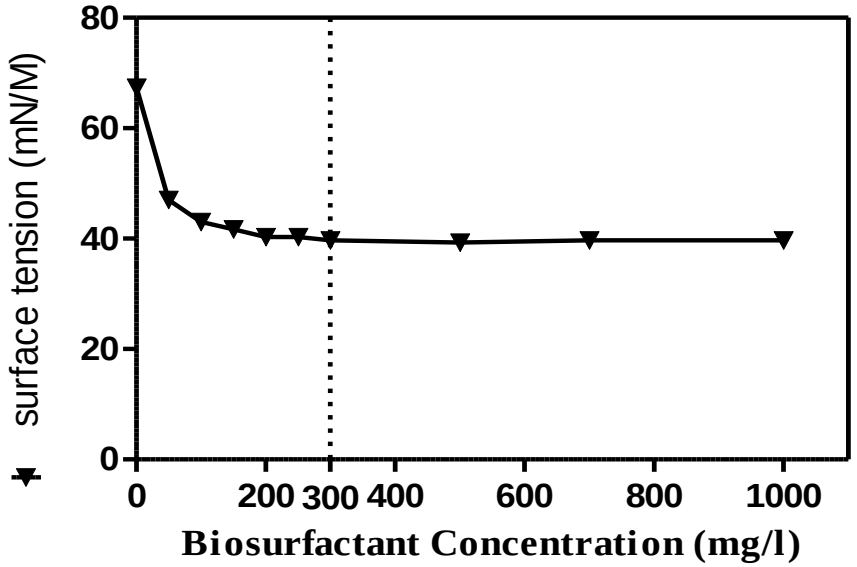


Fig.3.12 Critical micelle concentration of biosurfactant produced by *Sample-M*

3.3.7 Emulsifying potential of biosurfactant

The emulsifying activity of crude biosurfactant and purified biosurfactant was tested against different hydrocarbon substrates after 24, 96, and 168 h of incubation at 37°C. These emulsions remained stable even after 7 days of evaluation. The synthetic surfactants Triton X-100 and Tween 20 were more efficient than the biosurfactant, and the two natural biopolymers showed emulsification activities at 100% against oils after 168 h (Table 3.3). The biosurfactant showed stronger emulsifying activities than gum arabic, and the emulsifying activity of the biosurfactant was dependent on the type of substrate. These results indicate that the biosurfactant of sample-M was a good emulsifier. In the present study, the strong co-relation between the biosurfactant production and surface tension reduction was determined. All experiments were conducted in triplicate and analyzed with ANOVA, a t-test, using the Graph Pad Prism 5 software. The results represented a standard error mean.

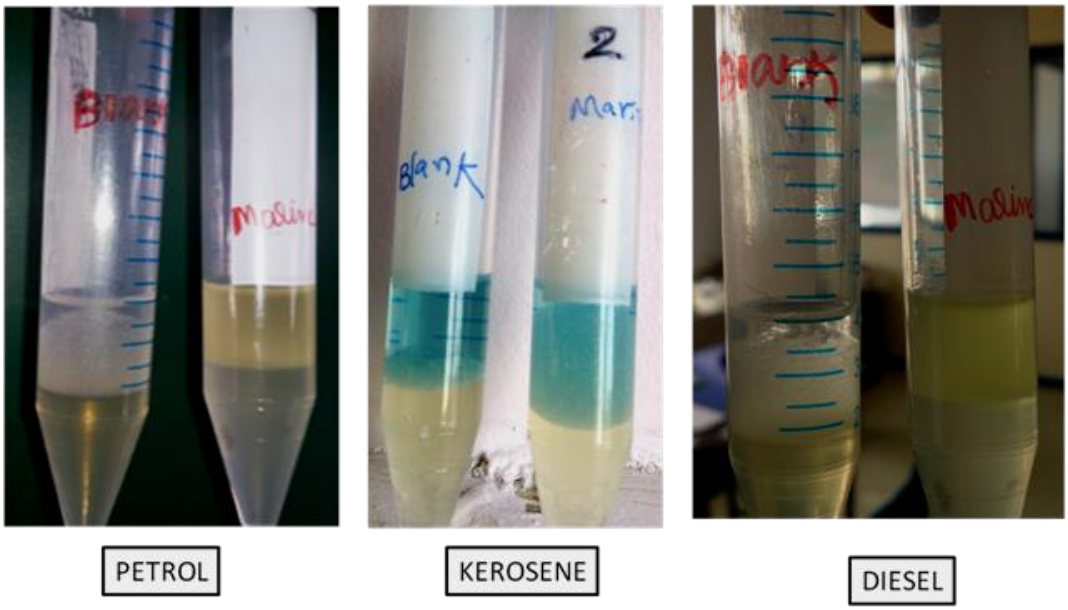


Fig.3.13 Emulsification activity of crude biosurfactant using different substrates (1% w/v) after 24 hrs of incubation at 37°C.

(a) (b) (c)

Fig.3.14 Emulsification potential of biosurfactant in kerosene (1% *w/v*) at 0 hrs (a), 24 hrs (b) 168 hrs (c)

The activity of the surface-active compounds produced by microbes can be determined by evaluating their ability to emulsify different water-insoluble substrates (Makkar & Cameotra, 1998; Van Dyk et al., 1993). In the present work, the produced biosurfactant was evaluated for emulsification of hydrocarbon substrates such as xylene, benzene, toluene, petrol, diesel, kerosene, motor oil, olive oil, and sunflower oil (fig.3.13, 3.14). The stability of emulsion varied from 63 to 95% (Table 3.4). The biosurfactant of *Acinetobacter* M6 was used to prepare the emulsions of different

hydrocarbons, which were found to remain stable for up to 7 days, while the emulsion formed with motor oil remained stable for more than a week (Table 3.5). The emulsification index was found to be least with kerosene (67%) and maximum with motor oil (81%). The other emulsification index values were found to be higher than those reported for *B. subtilis* LB5a (Nitschke & Pastore, 2006).

Table.3.3 Emulsifying activity of Biosurfactant, synthetic surfactants and biosurfactants on various hydrophobic substrates after 24 h of evaluation

Hydrophobic substrate	Biosurfactant	Synthetic surfactants		Biopolymers	
		Tween 20	Triton-X-100	Gum arabic	Xanthan gum
Xylene	76 ± 0.91	93 ± 0.60	94 ± 0.89	78 ± 2.25	78 ± 1.10
Benzene	69 ± 1.23	93 ± 0.10	67 ± 0.70	82 ± 2.13	72 ± 0.66
Toluene	73 ± 0.97	81 ± 0.20	81 ± 1.89	72 ± 1.05	71 ± 0.45
Diesel	78 ± 0.45	72 ± 0.34	69 ± 1.33	89 ± 0.40	89 ± 1.19
Petrol	76 ± 0.31	69 ± 0.65	71 ± 1.45	88 ± 1.94	89 ± 1.10
Kerosene	69 ± 0.53	67 ± 1.38	69 ± 0.25	77 ± 0.75	75 ± 0.89
Motor oil	79 ± 0.64	69 ± 1.31	67 ± 0.46	94 ± 0.49	91 ± 0.75
Olive oil	71 ± 1.40	98 ± 0.26	99 ± 0.20	98 ± 0.41	82 ± 0.74
Sunflower oil	75 ± 0.80	98 ± 0.37	97 ± 0.52	98 ± 0.2	86 ± 0.40

Table.3.4 Emulsifying activity of Biosurfactant, synthetic surfactants and biosurfactants on various hydrophobic substrates after 96 h of evaluation

Hydrophobic substrate	Biosurfactant	Synthetic surfactants		Biopolymers	
		Tween 20	Triton-X-100	Gum arabic	Xanthan gum
Xylene	76 ± 0.9	92 ± 0.28	94 ± 1.21	77 ± 2.21	78 ± 1.1
Benzene	67 ± 1.2	92 ± 0.7	67 ± 0.52	81 ± 2.08	71 ± 0.4
Toluene	74 ± 0.97	81 ± 0.35	81 ± 1.93	71 ± 1.0	71 ± 0.2
Diesel	79 ± 0.45	72 ± 0.57	68 ± 2	90 ± 0.69	89 ± 1.33
Petrol	77 ± 0.30	69 ± 1.1	72 ± 0.67	88 ± 2.13	87 ± 0.75
Kerosene	69 ± 0.52	68 ± 1.53	69 ± 0.25	77 ± 0.40	74 ± 0.60
Motor oil	79 ± 0.64	70 ± 1.13	67 ± 0.49	94 ± 0.89	92 ± 1.1
Olive oil	71 ± 1.4	98 ± 0.5	100	99 ± 0.462	81 ± 0.56
Sunflower oil	76 ± 0.83	99 ± 0.43	98 ± 0.87	98 ± 0.4	86 ± 0.85

Table.3.5 Emulsifying activity of Biosurfactant, synthetic surfactants and biosurfactants on various hydrophobic substrates after 168 h of evaluation

Hydrophobic substrate	Biosurfactant	Synthetic surfactants		Biopolymers	
		Tween 20	Triton-X-100	Gum arabic	Xanthan gum
Xylene	74 ± 0.91	94 ± 0.72	95 ± 0.96	78 ± 1.16	78 ± 1.19
Benzene	67 ± 1.42	94 ± 0.97	68 ± 0.70	82 ± 2.13	72 ± 1.35
Toluene	71 ± 0.97	82 ± 0.24	81 ± 1.93	70 ± 1.85	70 ± 3
Diesel	76 ± 1.23	73 ± 0.37	68 ± 2	89 ± 0.48	89 ± 1.2
Petrol	78 ± 0.78	68 ± 2.2	72 ± 0.67	88 ± 0.84	87 ± 3
Kerosene	67 ± 0.82	67 ± 1.46	69 ± 0.25	78 ± 0.87	75 ± 0.89
Motor oil	81 ± 0.94	69 ± 1.32	67 ± 0.49	93 ± 0.71	91 ± 0.75
Olive oil	73 ± 2.05	100	100	99 ± 0.49	83 ± 0.84
Sunflower oil	76 ± 0.89	100	98 ± 0.82	98 ± 0.2	86 ± 0.41

A higher emulsification index with motor oil suggests its applications in oil recovery. A significantly higher emulsification activity was observed with petrol, diesel, olive oil, and sunflower oil, indicating their application in the preparation of olive oil–based cosmetics. Furthermore, the emulsification activity of the biosurfactant from *Acinetobacter* M6 was significantly better than that of Tween 20 and Triton X-100 in the case of diesel, petrol, kerosene, and motor oil emulsions, and gum arabic and xanthan gum in the case of toluene; and the remaining were found to be the same or poorer in comparison with the stability of the emulsion formed by the known surfactants used. The stability of the emulsion of hexadecane formed by the biosurfactant of *B. subtilis* K1 was poorer than that reported for the emulsion stability of the surfactant produced by *B. subtilis* A8-8. However, since the substrates used for emulsion in both cases are different, such comparison does not explain the superiority of the biosurfactant. Prieto et al. (2008) reported the ability of the biosurfactant produced by *P. aeruginosa* to emulsify a variety of hydrophobic compounds, such as n-hexane, soybean oil, fish oil, diesel oil, crude oil, gasoline, BTX, cyclohexane, and chlorobenzene with E_{24}, ranging from 39% to 59%.

3.3.8 Gel filtration chromatography, TLC and FTIR

The biosurfactant concentration in the crude sample was found to be 357 mg/l, the sample was further dialyzed against distilled water for the removal of the medium salts and residual sugars was loaded onto a Sephadex G-100 column. The

biosurfactant started eluting from fraction no. 5 and the maximum elution was observed in the fraction no.9. Table 3.6 shows the purification profile. The fractions containing high total carbohydrate content were pooled.

Table.3.6 Purification profile of biosurfactant from *Acinetobacter M6*

Volume (ml)	Sample	Total Carbohydrate (mg/l)	Total Protein (mg/l)	Emulsification index (in kerosene)
25	Crude Biosurfactant (Dialyzed)	357 ± 3.1	156 ± 2.3	67 ± 0.82
25	Sephadex G-100	87 ± 2.9	47.98 ± 1.4	73 ± 1.33

The purified solution again precipitated, dried and stored at -20°C which was used for further characterization. Purified fraction of biosurfactant was shown maximum emulsion formation in kerosene as 72% when compared with crude biosurfactant 67% in 168 hours. The ninhydrin and anthrone reagent developed plates showed the confirmation of peptides and lipids as red and yellow spots providing the preliminary result analysis of the biosurfactant were lipopeptide in nature (fig.3.16).

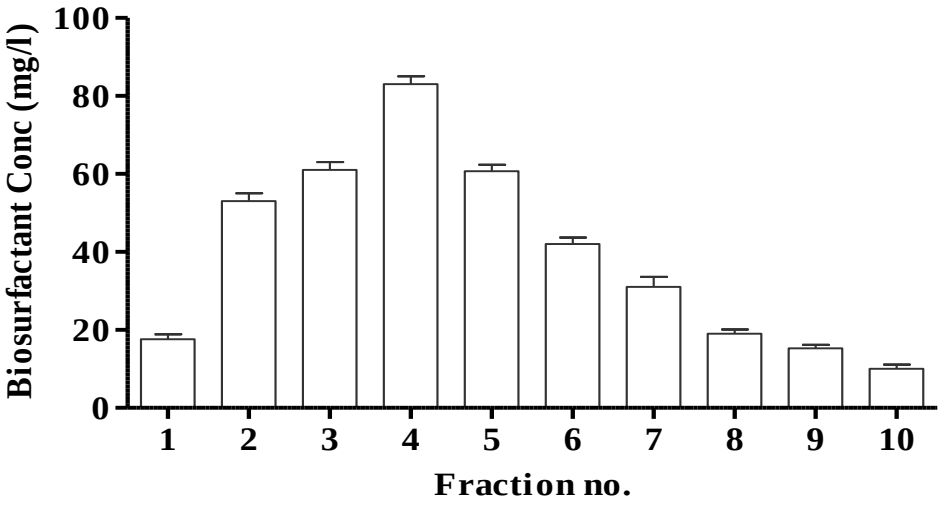


Fig.3.15 Elution profile of biosurfactant on Sephadex G-100 column chromatography

The FTIR spectrum of the biosurfactant showed a broad O-H stretching frequency at 3346 cm^{-1} and an intense frequency at 1070 cm^{-1} which is an indicative for typical carbohydrates. In addition, frequencies at 1637 and 1530 cm^{-1} indicating the presence of pepides, the peak at 2941 cm^{-1} in the FTIR analysis (Table.3.7) represent the asymmetric stretch of $–CH_2$ groups combined with that of $–CH_3$ groups in lipids

(Fig.3.17). The presence of lipid, carbohydrate and protein functional groups suggesting that biosurfactant was a class of glycolipoprotein.

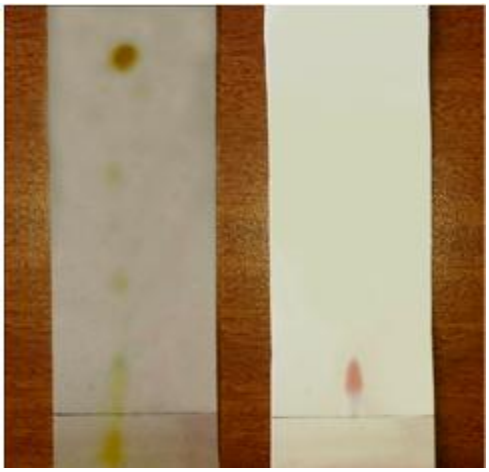


Fig.3.16 TLC analysis of biosurfactant produced by *Acinetobacter M6*

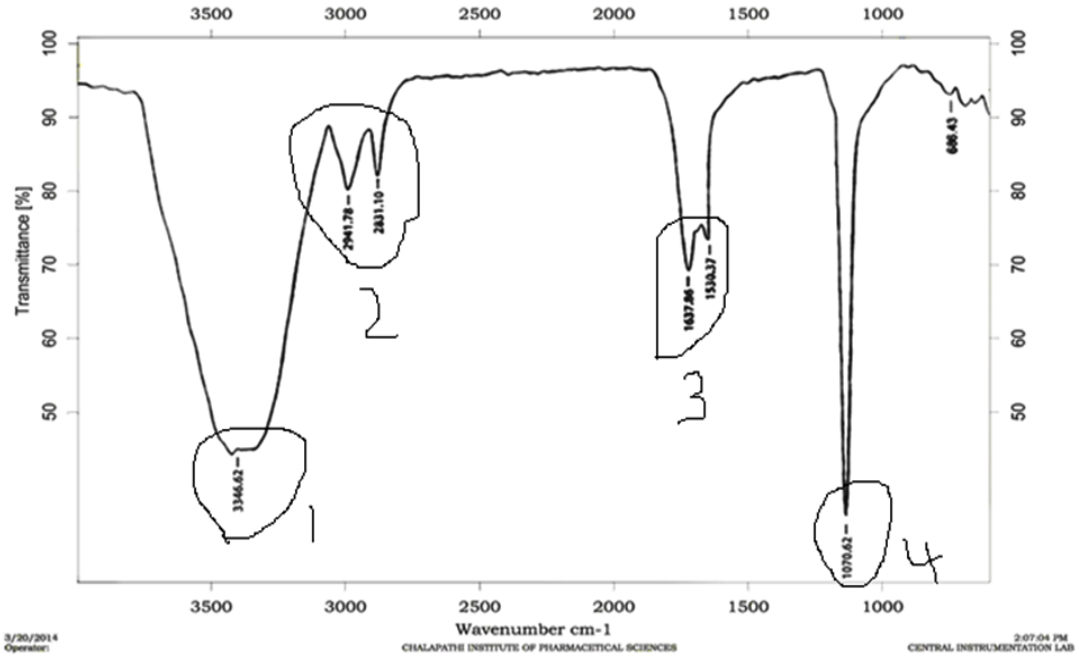


Fig.3.17 FTIR analysis of biosurfactant mixed with KBR pellet at 4cm^{-1} resolution; Instrument model: Bruker ATR spectrophotometer system.

Table.3.7 Identification of the compounds using FTIR analysis

S.no	Stretching frequency	Compounds
1	O-H stretch at 3346 cm^{-1}	Carbohydrates
2	CH_2 stretch at 2941 cm^{-1}	Lipids
3	N-H stretch at 1637 and 1530 cm^{-1}	Peptides
4	C-O stretch at 1070 cm-1	Carbohydrates

3.4 SUMMARY

Developing methods to assess the crude oil degradation and to evaluate the efficacy of microbes in terms of hydrocarbon degradation led to adopt new bioremediation technologies. Major studies on biosurfactants has always been with respect to their wide range of industrial and environmental applications so far. Genus *Acinetobacter* had been studied for their functions in detail of its ability to produce high molecular weight biosurfactants known as bioemulsifiers. Different microorganisms were studied for how the quorum sensing mechanism influence biofilm formation. There was quite insufficiency in studies on various roles or functions of biosurfactant among different species, as only 1% of the total marine bacteria have been studied. Present study focused on ecological potential of biosurfactant from Marine isolate, sample-M which was identified as genus *Acinetobacter*, submitted to Genbank of NCBI as *Acinetobacter* M6. Strain *Acinetobacter* M6 that produce biosurfactant which was a surface active agent considered to be an eco-friendly, exopolymeric glycolipoprotein and had a good role in future for industrial and environmental applications. Maximum of biosurfactant production was observed after 7 days of incubation. The total carbohydrate and protein concentrations were observed to be 310 and 150 µg/ml respectively. *Acinetobacter* M6 showed maximum biosurfactant production at pH 7.0 and 37°C. The addition of biosurfactant at the concentration of 300 mg/l to PBS buffer reduced the surface tension value to 39 mN/m as CMC value and the functional stability of this biosurfactant was retained for long periods of time. This study extends a great knowledge of marine bacteria that produce biopolymers. *Acinetobacter* M6 that was considered to be a new species, producing a novel biosurfactant specially selected for its high emulsification activity, has been Isolated from Bapatla, India. *Acinetobacter* M6 produced biosurfactant was found to be a glycolipoprotein of more than 10 kDa in size. The biosurfactant showed some interesting results when checked for FTIR. Biosurfactant showed a broad range of emulsification activity with key aromatic hydrocarbons like benzene, toluene as well as in aliphatic hydrocarbons like oils. Highest stability of the emulsions was observed in xylene stabilizing the emulsion and was also found to be function as a solubilizer of hydrocarbons.

CHAPTER-IV

ANTI- INFLAMMATORY STUDIES OF A BIOSURFACTANT

4.1 INTRODUCTION

Due to the potential advantages of biosurfactants, they are more widely used in several industries such as food production, pharmaceutics, agricultural as well as cosmetic industries (Nitschke, et al., 2007). Various properties of these surfactants which are dispersion, emulsification, de-emulsification, wetting, foaming and coating were widely useful in bioremediation and physiochemical technologies of metal and organic contaminants. In the soils contaminated with heavy-metal pollution, biosurfactants form different complexes with metals, followed by removal of metal from the surface which cause increase in ion concentration of metals and the bioavailability (Pacwa-Płociniczak, et al., 2011). Most of the bacteria have the property of producing biosurfactants (EPSs) with physiological and therapeutic activities, this make researchers to pay much attention in their production. Especially, EPS from lactic acid bacteria possess to have antitumor effects, capable of lowering blood cholesterol (Patel, et al., 2010). Bacteria utilize simple to complex substrates and produce diverse biopolymers with varied chemical properties. Out of all, some have same function while others have specificity in their function (Vu, et al., 2009). They have been categorized into two types, intracellular and extracellular on the basis of their cellular location. Intracellular biopolymers are few and have only limited use, whereas extracellular biopolymers are numerous and vastly used (Dashtban, et al., 2009). Again extracellular biopolymers are divided into four classes; polysaccharides, inorganic polyanhydrides (such as polyphosphates), polyesters, and polyamides. All these are collectively called as extracellular polymeric substances. Some of their functions include adherence of cells to surfaces, protection from engulfment. Extracellular biopolymers have abundant polysaccharide components and their location relative to the cell forms the basis of their classification. Their cell wall constitutes teichoic acids and serve as structural and protective purpose (Nwodo, et al., 2012). They use to form a covalently bound cohesive layer, a capsule that was excreted as slime into the environment, these capsules serve as adherents and are overproduced in the abundance of sugars (Wingender, et al., 1999). Advancement in the technology led to the understanding of biopolymers usage for the mankind on providing various industrial and medical applications. Some of the inherent properties

like biocompatibility and non-toxic nature makes polysaccharides dealing with numerous applications as scaffolds or matrices in tissue engineering, drug delivery and wound dressing etc. (Goeln, et al., 2003). These advantages make bacterial polymers more attractive than polysaccharides from plants and algae. The biological activities of microbial extracellular glycolipids, mannosylerythritol lipids-A, mannosylerythritol lipids-B, rhamnolipids, polyol lipids, sophorose lipids have been well studied (Chakrabarti, et al., 2012). Some of the Inflammatory diseases, such as chronic asthma, rheumatoid arthritis (RA), multiple sclerosis, psoriasis and inflammatory bowel disease are widely spread in the world on large scale (Lees, et al., 2011). Drugs like cyclooxygenase inhibitors are often used to alleviate pain and stiffness in patients, but most of such drugs are expensive and show side effects, emerging an alternate drugs having minimal side effects and cost reduction (Russell, et al., 2004). EPS is produced by a diverse microorganisms, most of the biofilm producing bacteria secrete EPS into the growth media with novel functions and structures. This biofilm development process may differ from one bacterium to other.

4.2 METHODOLOGY AND RESEARCH DESIGN

4.2.1 Extraction and Quantification of Biosurfactant from *Acinetobacter M6*

The *Acinetobacter* M6 strain was isolated from marine source. Biofilm formation was tested by crystal violet assay. When the culture attained the stationary phase, centrifuged at 6000 rpm for 10 min to pellet down the cells (Anderl, et al., 2003). Three volumes of chilled acetone was added to the supernatant and left overnight at 4°C. Once emulsifier got precipitated, washed and dialysed at 4°C against chilled deionised water for 24 h (Gregor, et al., 1986). The dialyzed sample was washed with distilled water, lyophilized and dissolved in sterile distilled water (Hi Media). The Lyophilized sample was analyzed for its carbohydrate (Dubois, et al., 1951) and protein contents (Bradford method).

4.2.2 Protein Purification using 2D clean up

Biosurfactant was subjected to 2D clean up by using a commercial kit obtained from GE Healthcare, USA. Briefly, 1 mg of biosurfactant crude sample was added to 0.5 ml of acetone and three volumes of precipitant, vortexed and incubated on ice for 15 min, followed by the addition of co-precipitant. The tubes were centrifuged at 14,100 rpm for 10 min. One milliliter of pre-chilled wash buffer, 5 µl of wash additive was added and vortexed. The tubes were incubated at -20°C for 30 min and vortexed for 20-30 sec for every 10 min interval. Centrifugation was done at 14,100 rpm for 10 min. A

white pellet was observed at the bottom of the tube, subjected to 2D clean up which was stored at -20°C for further use.

4.2.3 Iso-electrofocusing (IEF) and Two Dimensional (2-D) SDS-PAGE

The IPG strip was rehydrated with the protein sample in order to re-swell the gel side of the strip as well as to imbibe the proteins in the mixture on to the strip. It was done with gel side down in the appropriate volume of rehydration solution using immobiline drystrip reswelling tray, strip was overlaid with cover fluid for rehydration. The pellets obtained after 2-D clean up were suspended in 125 µl 2-D buffer. A 7 cm 2-D strip was subjected to overnight passive rehydration by adding the sample mixed in 2-D buffer of bromophenol blue. The strip was transferred to manifold containing mineral oil and allowed to Iso-electrofocusing, according to the program set previously. Iso-electrofocusing was performed in Ettan™ IPGphor™ 3, (Serial Number: 1285200 Firmware version: A13 02/13/06) with a 7 cm linear IPG strip of pH range 3-10 in a programmed manner. The gels were allowed to run at 35 V on the 7 cm gel till the dye reaches the bottom of the plates (Lafitte, et al., 2013).

4.2.4 Coomassie Brilliant Blue (CBB) and Periodic Acid Schiff's (PAS) staining

SDS-PAGE gels were carefully taken out after running. Proteins on the gel were fixed by adding a fixative, then CBB was applied to the gel, incubated overnight at room temperature. The presence of glycoproteins was detected by running another SDS-PAGE gel which was incubated with 1% periodic acid in 3% acetic acid for an hour at room temperature. The periodic acid solution was removed, washed for one hour with double-distilled water. Gel was stained with Schiff's reagent for 30 min by wrapping the box with aluminum foil and kept in a dark place. After 30 min, Schiff's reagent was removed, and the gel was destained with 10% acetic acid until clear spots appeared on the gel, then it was scanned at a visible range and stored in 3% acetic acid (Yagi, et al., 2003).

4.2.5 Protein in gel-digestion and identification

The obtained spots on the 2-D gels were excised. The excised gel spots were destained using 1.5 ml of 50 mM ammonium bicarbonate and 50% acetonitrile solution, vortexed at 450–500 rpm at room temperature for overnight (Zhang et al, 2005). After destaining, the gel spots were washed with 100% acetonitrile for 3–5min. An amount of 100 µl DTT was added to the tubes, incubated for 1 h at 56°C and 950 rpm. The spots containing gel was made completely dry before trypsin treatment. The digestion step was performed by adding a small volume of trypsin to cover the gel

pieces, followed by incubation at 37°C on ice. The tubes were subjected to alternate vortexing for 30 to 60 min. The extraction buffer was added and subjected to SpeedVac to extract the peptides. Peptides obtained from in-gel digestion were suspended in 5 µl of extraction buffer and sonicated for 15 min. The peptides were vortexed for 5 min prior to mixing with the matrix α-Cyano-4-hydroxycinnamic acid (α-CHCA). Matrix-assisted laser desorption ionization (MALDI) mass spectrometric analysis was performed on an Ultraflex TOF-TOF instrument. Spotting was done by mixing 1.5 µl of sample and 1.5 µl of the α-CHCA matrix on the MALDI target plate, the spot position was noted. The plate was allowed to dry and fix in the slot of the mass spectrometer. The selected spot was subjected to a high-intensity laser beam to get the spectrum. The obtained mother peak was picked for MS/MS analysis to obtain the amino acid sequence of the peptide, which helped to identify a particular protein. Identification was performed using peptide mass fingerprint data (Solazzo et al., 2014). Database search was performed using the Mascot algorithm (Matrix Science).

4.3 ANTI-INFLAMMATION STUDIES

4.3.1 Solvent extractions of biosurfactant fractions

Two mg of biosurfactant was weighed and dissolved in 100 µl of 1x PBS (Samal, et al., 2012). Anti-inflammatory activity of purified biosurfactant was checked for each individual fraction. Carbohydrate was extracted using ethanol, protein using ammonium sulphate precipitation and acetone method. Lipid extraction was carried out using Bligh Dyer method (Parsons et al. 1984). The carbohydrate, protein pellets were dissolved in 100 µl of 1x PBS (pH 7.4), lipid pellet was suspended in solution of 100 µl chloroform: methanol in 2:1 ratio (Keller, et al., 2013).

4.3.2 Inhibition of 5-Lipoxygenase (5-LOX) activity of biosurfactant

Enzyme activity was measured using Polarigraphic method with a clark's oxygen electrode (Strath kelvin Instruments Limited, model: 782). The substrate solution contained 133 µM (final concentration) of arachidonic acid in the reaction mixture. Reactions were carried out without inhibitor which contain 2 ml of 100 mM PBS buffer (pH-6.3), Milli-Q water: 970 µl, 5-LOX enzyme: 20 µl, arachidonic acid (substrate): 10µl, with the presence of standard inhibitor (NDGA; 30 µl from 1 mg/ml working stock), and test inhibitor (biosurfactant; 30 µl from 10 mg/100µl working stock). All the reactions were allowed to proceed for 1 min. The initial (at 0^{th} second) and final (after 1 min) O_2 concentrations were noted (Sanchez, et al., 2011) as the

difference of the two would give the amount of O_2 that the enzyme has incorporated into the substrate in 1 min time interval. The obtained difference value is compared with 100% activity value (Zhang, et al., 2015).

4.3.3 Inhibition of Cyclooxygenase (COX-1 & COX-2) activity

The enzymatic activity of COX-1 and COX-2 were measured using a chromogenic assay based on the oxidation of N, N, N′, N′-tetramethyl-p-phenylenediamine (TMPD) during the reduction of Prostaglandin G_2 (PGG_2) to Prostaglandin H_2 (PGH_2). The chromogenic assay buffer consisited of 100 mM tris, pH 8.0, 3 µM EDTA and 16 µM hematin. Cyclooxygenase metabolized the arachidonic acid and formed PGG_2, which was finally reduced to PGH_2 with the release of one oxygen molecule. The oxygen molecule oxidized TMPD, resulting in the formation of a colored product, which showed absorbance at 610 nm. Auto zero was done with the assay buffer, and absorbance remained at 610 nm. The reaction time was 1 min, followed by absorbance at zero and 60 sec. The difference between the two values was considered to calculate the enzyme activity (Table-3). As the reaction proceeded, a hyperbolic curve was obtained. An inhibitory activity on COX indicated the inhibition of the enzyme to synthesize PGG_2, followed by the synthesis of PGH_2 (Rouzer et al, 2003).

$$\textbf{Enzyme activity} = \frac{(\text{Volume of the reaction mixture}) \times (\text{Absorbance diff./min})}{(\varepsilon) \times \text{volume of the enzyme in ml}}$$

Where, ε = Molar extinction coefficient of the TMPD=0.073/µM

Reaction mixture for enzyme activity: (941 µl, 100 mM Assay buffer, pH 8 + 50 µl enzyme + 5 µl, 20 mM AA + 4 µl TMPD).
Reaction mixture with Indomethacin: (931 µl, 100 mM Assay buffer, pH 8 + 50 µl enzyme + 10 µl inhibitor + 5 µl, 20 mM AA + 4 µl TMPD).
Reaction mixture with Crude BIOSURFACTANT (1mg/ml): (841 µl, 100 mM Assay buffer, pH 8 + 50 µl enzyme + 100 µl inhibitor + 5 µl, 20 mM AA + 4 µl TMPD).
Reaction mixture for Carbohydrate analysis (1 µl /ml): (841 µl, 100 mM Assay buffer, pH 8 + 50 µl enzyme + 100 µl PBS suspended carbohydrate (inhibitor) + 5 µl, 20 mM AA + 4 µl TMPD).
Reaction mixture for Protein analysis (1 µl /ml): (841 µl, 100 mM Assay buffer, pH 8 + 50 µl enzyme + 100 µl PBS suspended proteins (inhibitor) + 5 µl, 20 mM AA + 4 µl TMPD).
Reaction mixture for lipid analysis (1 µl /ml): (841 µl, 100 mM Assay buffer, pH 8 + 50 µl enzyme + 100 µl (Chloroform:Methanol 2:1) mixture + 5 µl, 20 mM AA + 4 µl TMPD).

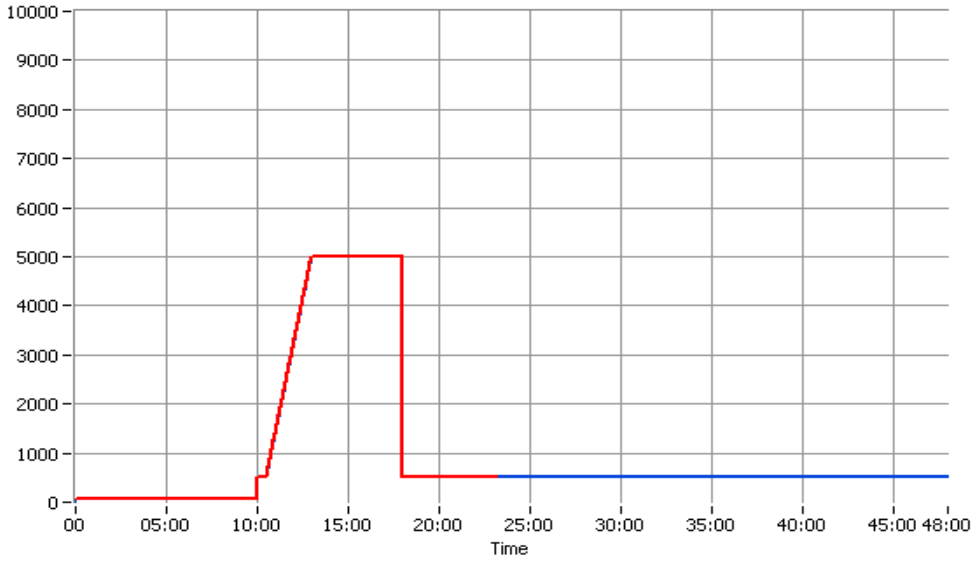
4.4 RESULTS AND DISCUSSION

4.4.1 Biofilm formation and quantification of Biosurfactant

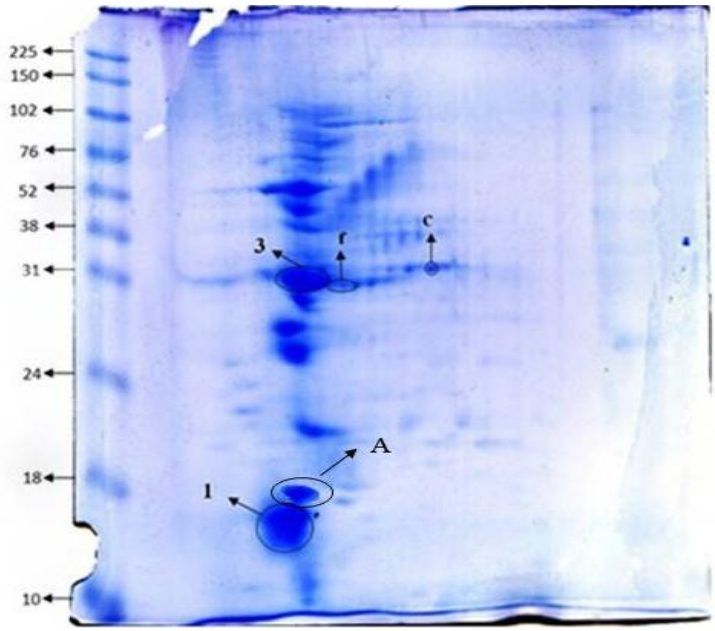
The biofilm thickness was observed to be greater during 48 h period, indicating the biosurfactant concentration was more in the medium when the bacterial culture attained stationary phase. Here, based on the results, the biosurfactant concentration was taken as the measure of biofilm thickness. The total carbohydrate concentration of the *Acinetobacter* M6 produced biosurfactant was found to be 340 µg/ml, caluculated using glucose as a standard (Dubois, et al., 1951). Total protein concentration was found to be 160 µg/ml using BSA as a standard by Bradford method.

4.4.2 SDS-PAGE

Glycoproteins that bear different carbohydrate groups have diverse functions. Carbohydrates influence protein folding and conformation. PAS stain was mainly used to detect the high amount of carbohydrate containing molecules such as glycoproteins. Periodic acid act as oxidizing agent which oxidize hydroxyl group of carbohydrates resulting in reduced aldehydes. When Schiff's reagent was added, an insoluble colored complex formed and indicating the presence of glycoprotein conjugate. The gel was scanned and the considerable number of spots were observed on the 2-D gel at the region between pH 4 and 5, the molecular weights obtained were 16 KDa and 30 KDa approximately (fig.4.4.1). The spots were excised and processed for in-gel trypsin digestion to identify the proteins on MALDI. After destaining faint spots were observed at the region in between pH 4 and 5 on the gel. It may be due to the low excess of glycosylation of proteins in biosurfactant.

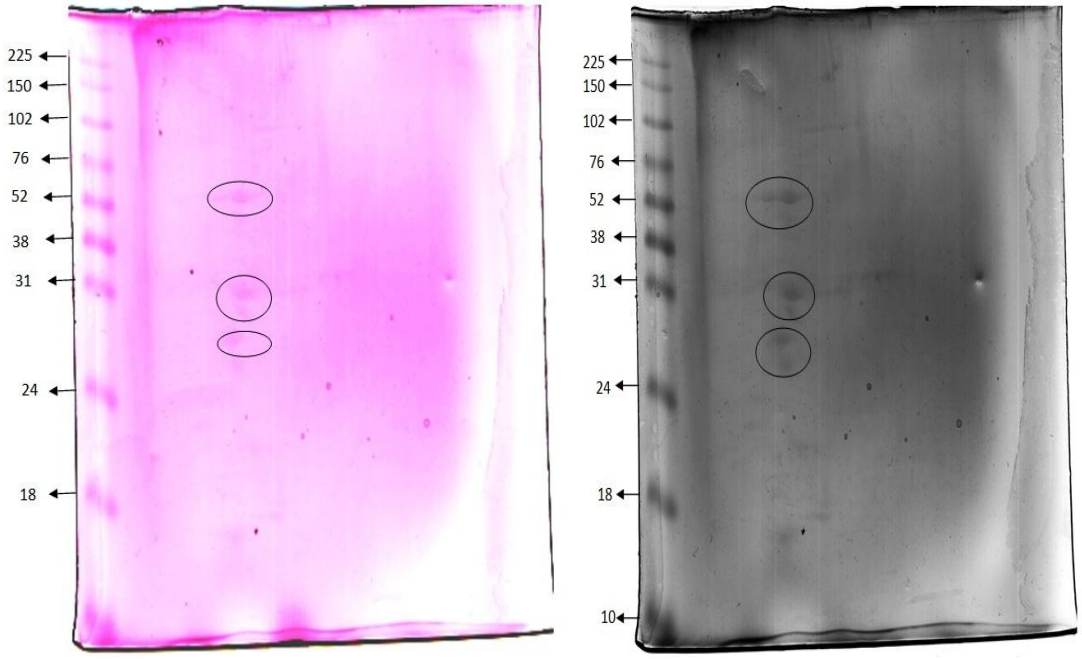


(a) IEF graph



(b) (CBB stained gel)

Fig.4.1 Spots were observed on the CBB stained gel (b) having molecular weights between 16 KDa and 30 KDa approximately.



(a) PAS stained gel **(b) grey scale**

Fig.4.2 Glycoproteins were identified as spots on the PAS stained gel (a) grey scale (b)

4.4.3 MALDI-TOF analysis of peptides

In this particular context , five proteins were identified by MS/MS. **Tetraacyl disaccharide 4ˈ-kinase** plays a key role in the formation of outer membranes of gram negative bacteria by phosphorylating the 4-position of tetraacyl disaccharide lipid A, thereby maintaining the surface integrity of cell membrane. **Serine hydroxymethyl**

transferase is involved in the biosynthesis of purines by SOG pathway, which will be helpful in the survival of bacteria. **Homoserine-o-acetyl transferase** is involved in the methionine biosynthesis. **Acyl-CoA dehydrogenase**, makes the cell capable of surviving under long-term starvation by fatty acid β-oxidation in the mitochondria of cell, implicating its role in lipid metabolism. This makes the cell to remain in the stationary phase for a long time when the biofilm formation is observed to be high. **Fimbriae Z protein** is the protein present in the fimbriae of aerobic bacteria, which carry adhesins that attach them to the substratum during biofilm formation so that the bacteria can withstand shear forces and obtain nutrients. Thus, fimbriae allow the aerobic bacteria to remain on the broth, from which they take nutrients, while they congregate near the air.

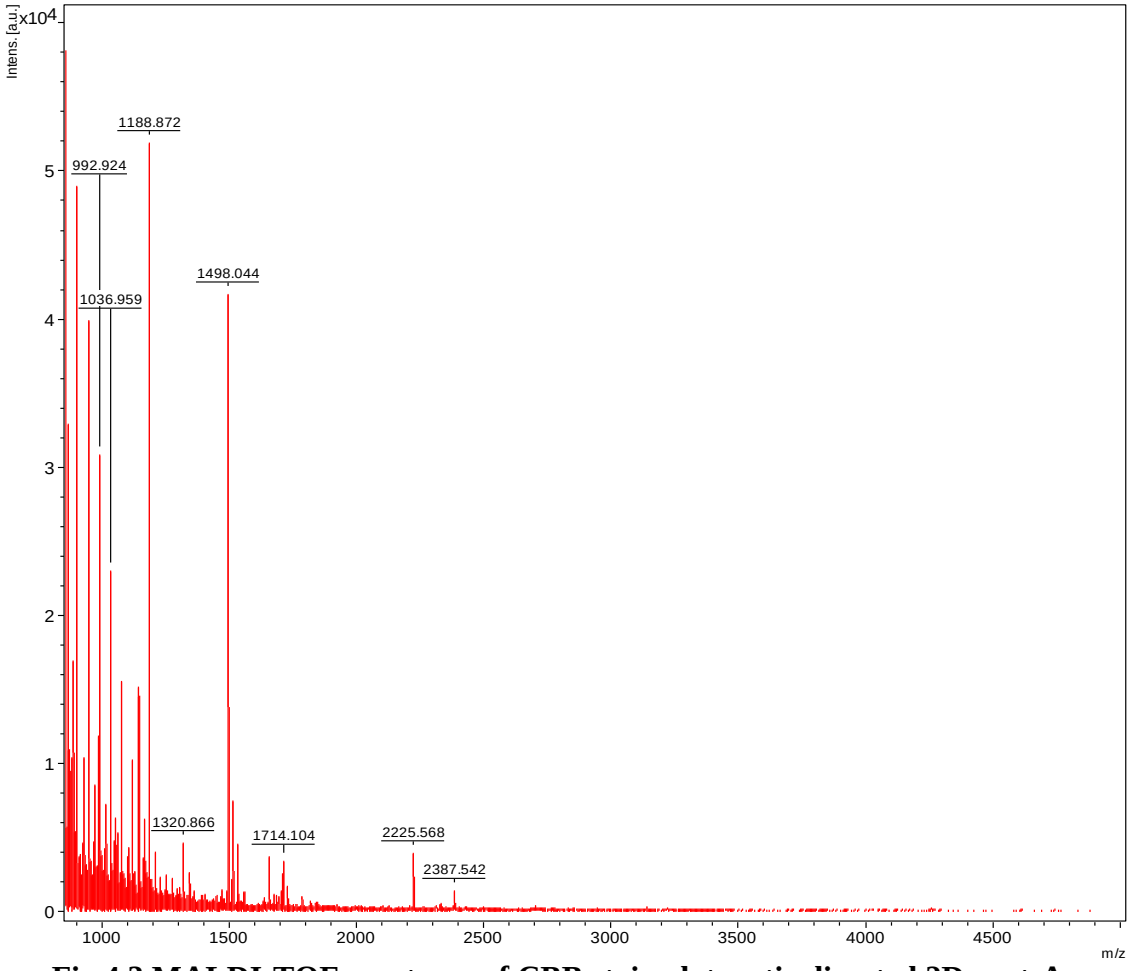
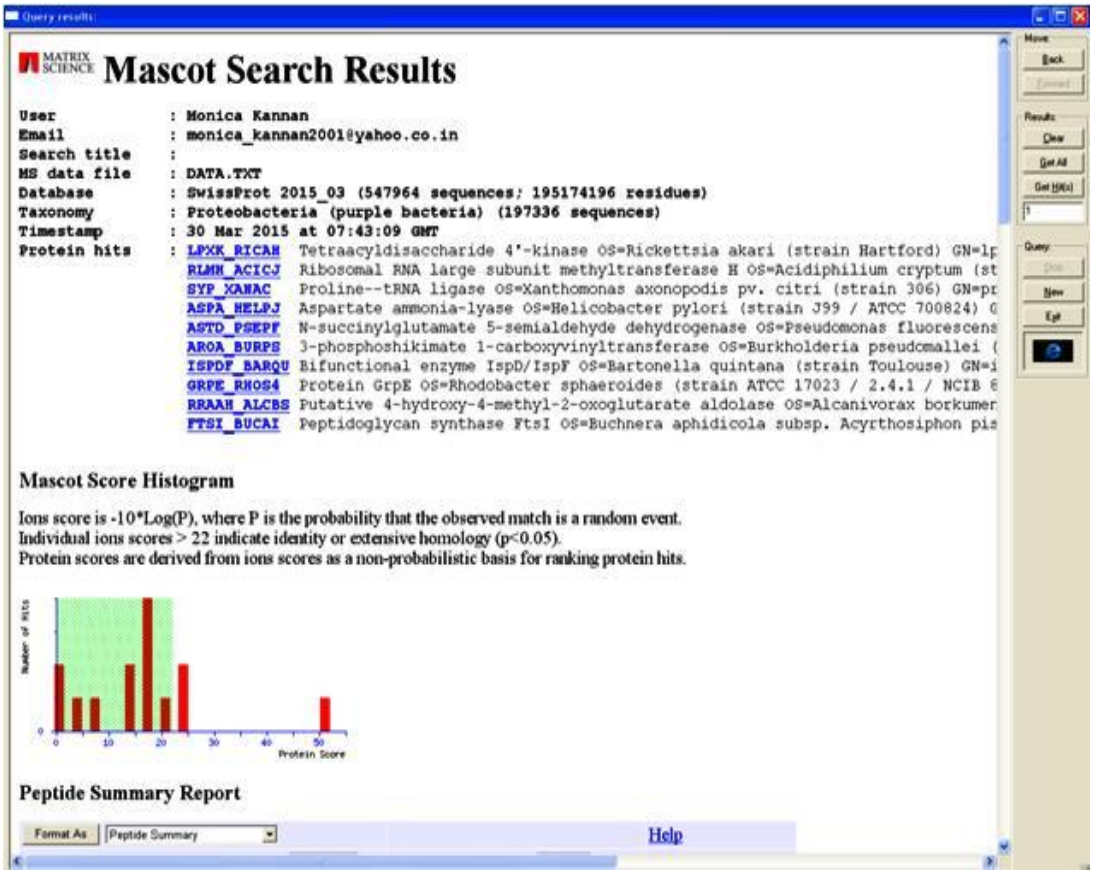


Fig.4.3 MALDI-TOF spectrum of CBB stained, tryptic digested 2D spot-A

MALDI-TOF analysis of proteins is one of the accurate methods so far to know the sequence information and structure. Many copies of the protein are fragmented at the peptide back bones to generate peptides, the TOF (time of flight) mass analyzer generates the spectrum when the ions reached the detector. In fig 4.3 the spectrum

shows the peaks from 2500 da to 1000 da. The prominent peak at 1498.044 was selected for measuring MS/MS spectrum. The main idea behind producing MS/MS spectrum is to know the mass difference between two fragment ions to calculate the mass of the aminoacids in the selected peptide. Mascot algorithm gives the Protein sequence coverage information from the Mascot database (fig.4.4 - 4.7).



The results obtained from the LC-MS/MS data search can be complex to interpret because the peptide sequence obtained did not provide the exact protein to which it belong to. The bold typefaces used in the results were intended to highlight the matched sequence of top scoring peptide match for a spectrum and are the most likely the best matches in the database. The score given was a function of the number of trials which was the number of times we tested for a match. If the false positive match of a peptide is 1 in a 20 chances in a MS/MS search of a database contains 5000 peptides that matches with our peptide, the probability was given by the formula P = 1/(20 x 5000) which was a Mascot score of S = -10LogP = 50. The expectation value (E) can be deduced directly from the score. It was the number of times you could expect to get this score. The better the match, the smaller the expectation value.

Protein View: LPXK_RICAH
Tetraacyldisaccharide 4'-kinase OS=*Rickettsia akari* (strain Hartford) GN=lpxK PE=3 SV=1

Query	Observed	Mr (expt)	Mr (calc)	ppm	Miss	Score	Expected (E)	Unique	Peptide
4	**1498.0444**	**1497.0371**	**1496.8198**	**145**	**0**	**51**	**0.00013**	**U**	**K.VISAQIVPSNNIDK.T**

Search parameters
Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 145.2 ppm
Fragment Mass Tolerance: ± 1.9 Da
Max Missed Cleavages : 0
Instrument type : MALDI-TOF-TOF
Number of queries : 4

Database:	SwissProt
Score:	51
Nominal mass (M_r):	36784
Calculated pI:	9.13
Taxonomy:	*Rickettsia akari* str. Hartford
MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)

Protein sequence coverage: 4% Matched peptides shown in **bold**.

MIKLLYPKFW QKRNIIAYLL LPIGLIYKFL GYLRDSLARP IMLPAKVICV
GNCSVGGTGK TQIVMYLAKL LRAKNVSFVI VTKAYGSNLK SATTIHPGHT
ALEVGDEGVI LAKYGTVIAT KNIKEILPLI NELKPDIIII DDFLQNPYFY
KDFTIVSVDS QRLFGNGFLI PAGPLRQDPN KALDAADLIF LVSSTNDKIP
NILTPYVNK**V** **ISAQIVPSNN** **IDK**TKNYFAF SGIGNPERFF STLKNYGLNI
TGYKIFPDHY NYLQEDLENL YSLAKEHNTT LITTRKDHIK FNDLNNNIVC
LDVELSINNH DLLNEKIFKK AQIFN

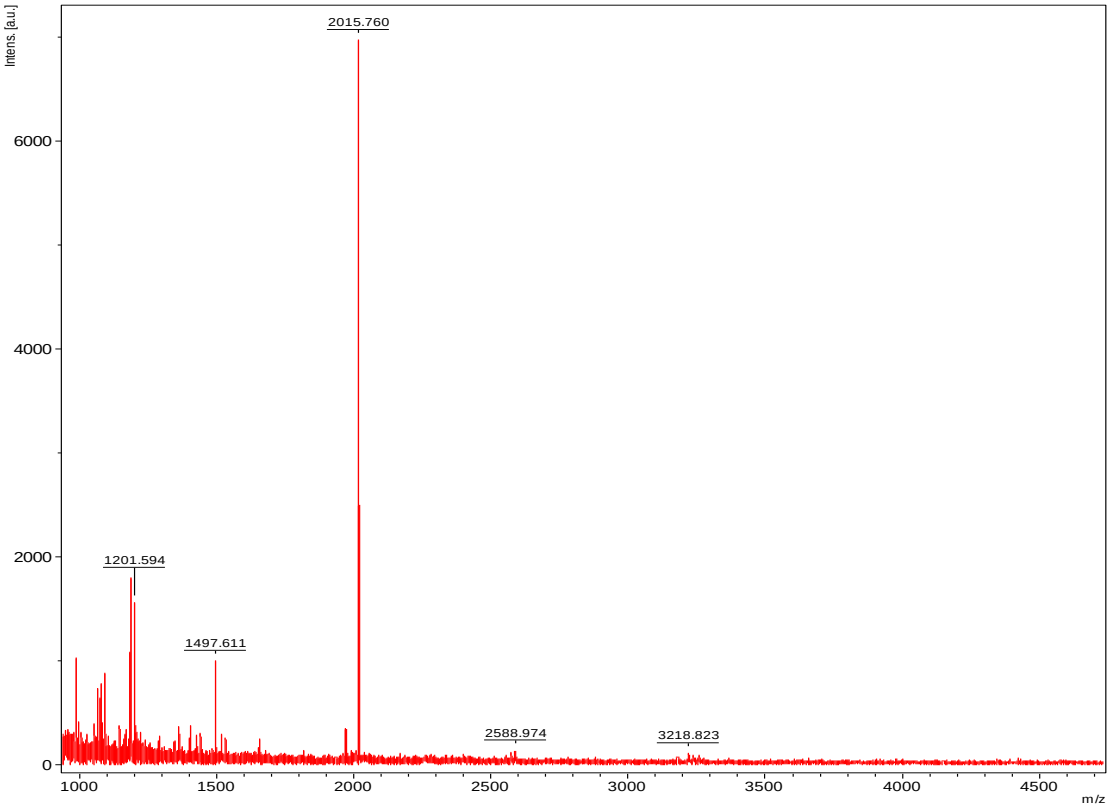
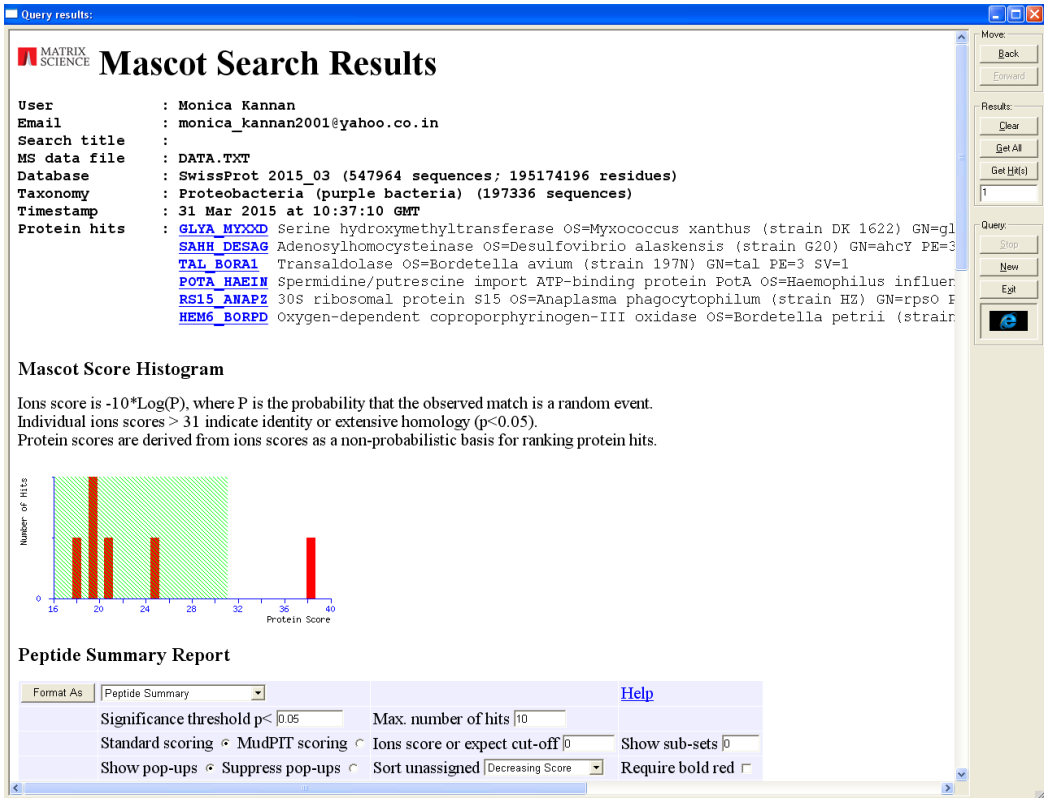


Fig.4.4 MALDI-TOF spectrum of CBB stained, tryptic digested 2D spot-1



Protein View: GLYA_MYXXD
Serine hydroxymethyltransferase OS=*Myxococcus xanthus* (strain DK 1622) GN=glyA PE=3 SV=1

Database:	SwissProt
Score:	37
Nominal mass (M_r):	44683
Calculated pI:	7.18
Taxonomy:	*Myxococcus xanthus* DK 1622
MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M), Phospho (ST), Phospho (Y)

Query	Start	–	End	Observed	Mr(expt)	Mr(calc)	M	Score	Expect	U	Peptide
1	344	–	360	2015.7600	2014.7527	2014.8893	0	37	0.056	U	K.NMIPFDPEKPMTTSGVR.V + Oxidation (M); Phospho (ST)

Search Parameters
Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M),Phospho (ST)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 67.82 ppm
Fragment Mass Tolerance: ± 1.55 Da
Max Missed Cleavages : 0
Instrument type : MALDI-TOF-TOF
Number of queries : 1

Protein sequence coverage: 4%

MENIRTLAEV DPEIARVLRE ETQRQEEGLE LIASENFVSP AVMEAVGS
TNKYAEGYPG KRYYGGCEV DVAENLAIAR AKDLFGADA NVQAHSGSQ
NMGAFMALM PGDTMLSLD NSGGHLTHGA TFNFSGKLYK VVHYGLTRD
ETIDFAQVES LAKEHKPKVI VVGASAYPRT LDFAKFREIA DAVGAAML
MAHIAGLVAA GVHPSPVPV DIVTSTTHKT LRGPRGGLVL SREPYAKAIN
SQIFPGIQGG PLMHVIAGK VAFKEALSPE FKAYQRQIVA NAKALAEAL
RAGLRLTSGG TDNHLMLVD RPKKLTGKVA EEVLDKAGIT VK**NMIPFDP**
EKPMTTSGVR VGTPAITTRG MREAEMAVV RLIGEALDAA QDAALARIK
GQVKELSQGF PLYASRLK

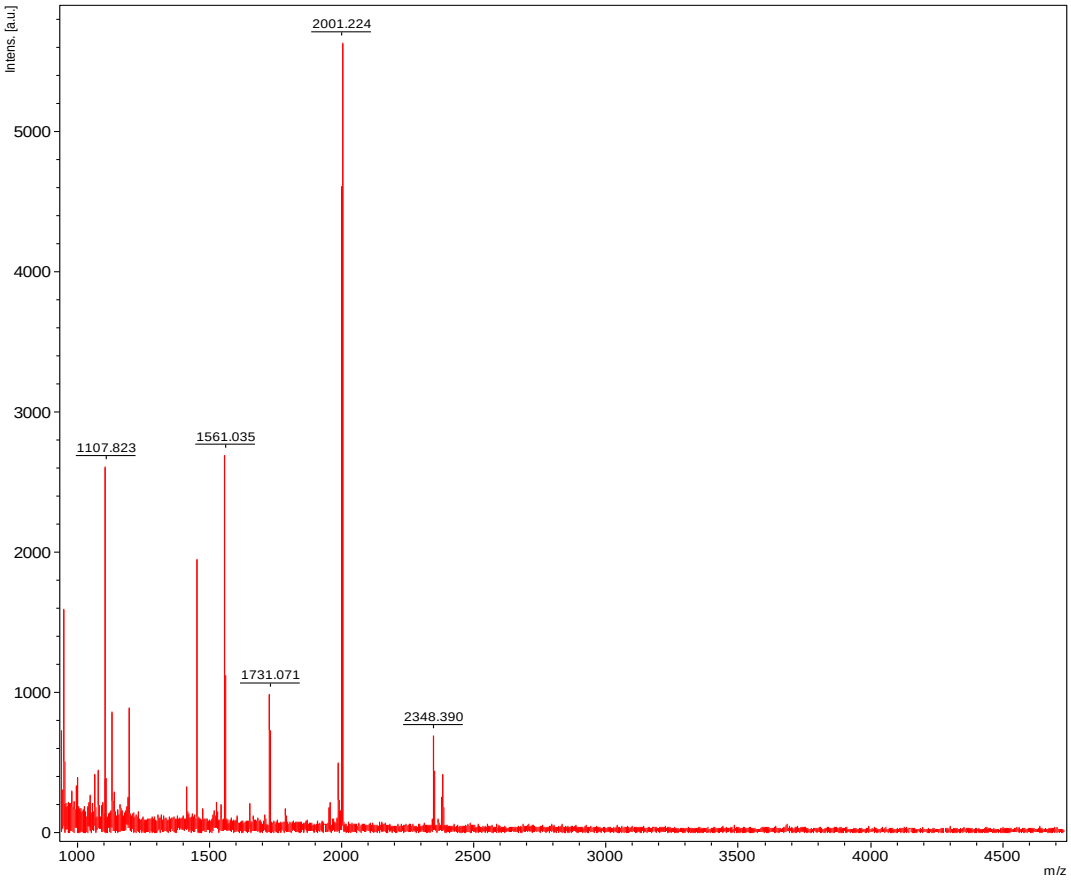
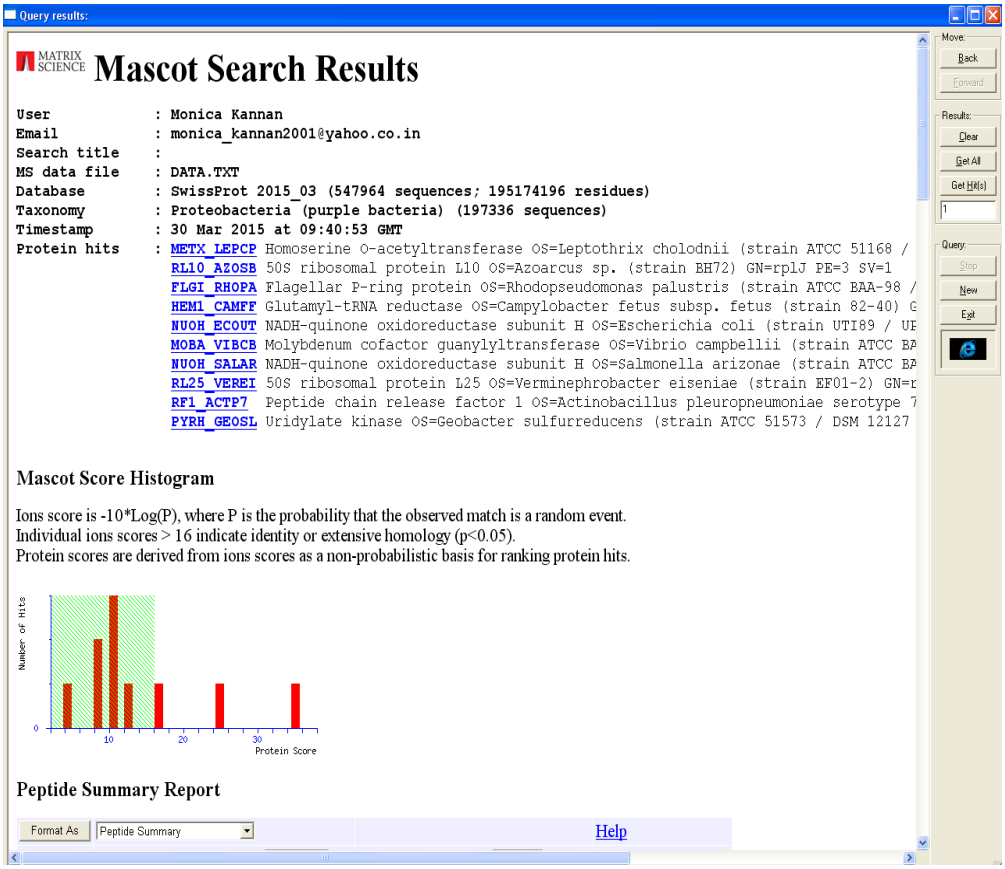


Fig.4.5 MALDI-TOF spectrum of CBB stained, tryptic digested 2D spot-C



Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
3	2001.2255	2000.2182	2000.0877	65.3	0	35	0.0021	1	U	R.LAAVIGGSLGGMQALSWTLR.H

Protein View: METX_LEPCP; Homoserine O-acetyltransferase OS=*Leptothrix cholodnii* (strain ATCC 51168 / LMG 8142 / SP-6) GN=metX PE=3 SV

Database:	SwissProt
Score:	35
Nominal mass (M_r):	41662
Calculated pI:	6.27
Taxonomy:	Leptothrix cholodnii SP-6
MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)

Search Parameters
Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 65.4 ppm
Fragment Mass Tolerance: ± 1.95 Da
Max Missed Cleavages : 0
Instrument type : MALDI-TOF-TOF
Number of queries : 3

Protein sequence coverage: 5%

MVTLGHVTAQ QMSFSDALPL RSGAALRDYT LVYETYGTLN ADRSNAVLVC
HALNASHHVA GTYADSDRSE GWWDNLIGPG KPLDTNRFFV IGVNNPGSCF
GSTGPTHPNP ATGRPYGADF PVVTVEDWVD AQARLLDGLG IER**LAAVIGG**
SLGGMQALSW **TLR**HPARVGH ALIIASAPNL SAQNIAFNEV ARRAIITDPD
FHAGHFYAHG VVPKRGLRVA RMIGHITYLS DDSMEAKFGR ALRSAELAYS
TQEIEFQIES YLRYQGDKFS EYFDANTYLL ITRALDYFDP AREFGGNLSA
ALAVARAKFL VVSFTTDWRF SPLRSREIVK ALLDNRRDVS YAEIAAPHGH
DAFLLDDPRY HGVLRAYFER VAQELPR

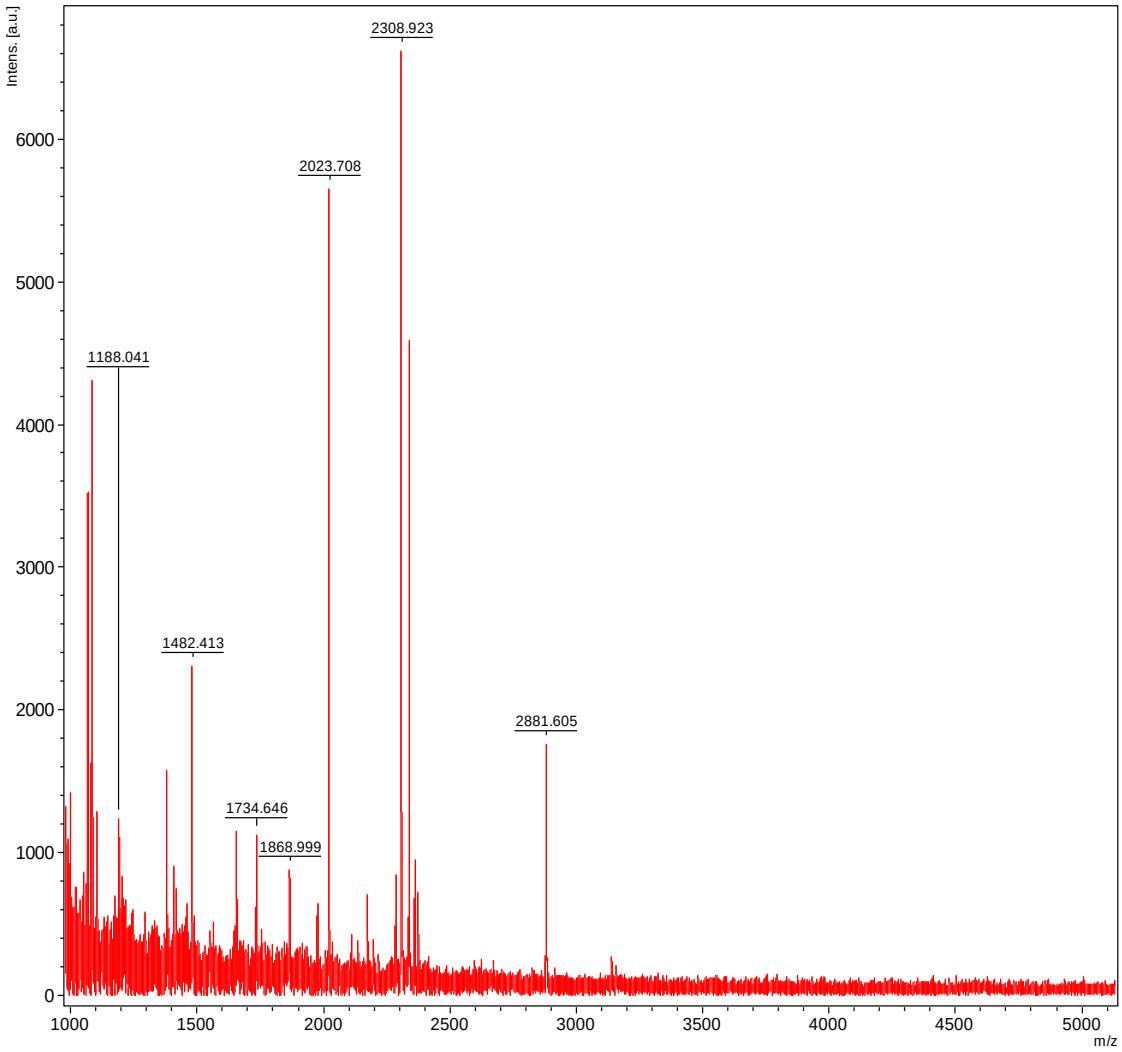
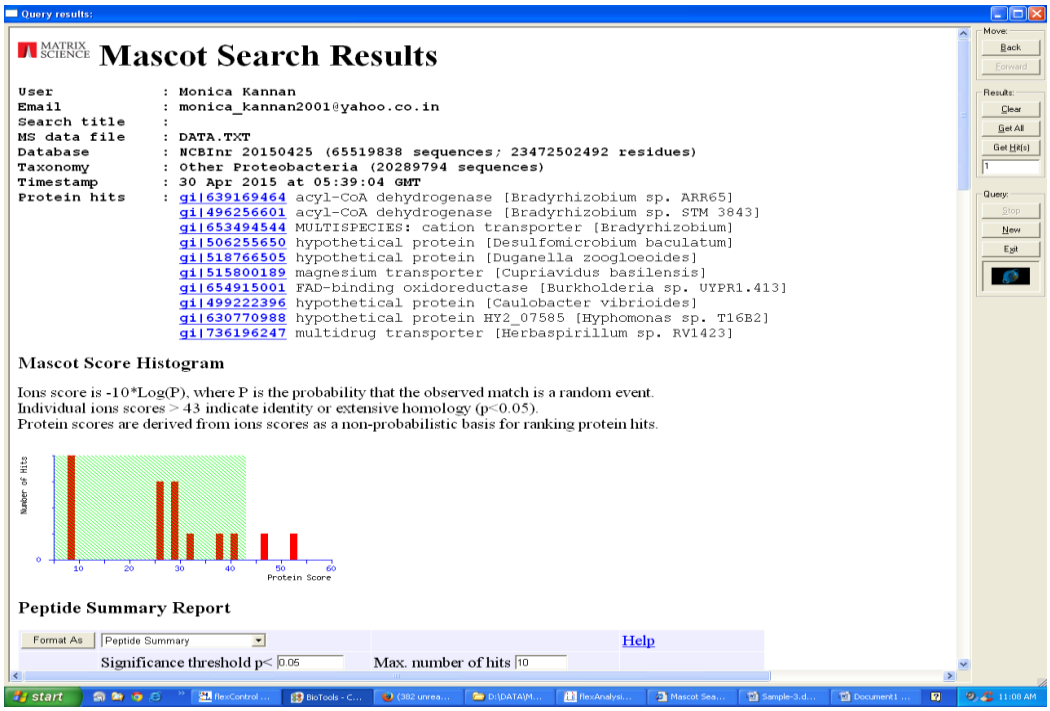


Fig.4.6 MALDI-TOF spectrum of CBB stained, tryptic digested 2D spot-3



Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
3	2339.1482	2338.1409	2338.1166	10.4	0	53	0.0097	1	U	R.VATEQLLLASSTTEGQNGGNIR.A

Protein View: gi|639169464; acyl-CoA dehydrogenase [*Bradyrhizobium* sp. ARR65]

Database:	NCBInr
Score:	53
Nominal mass (M_r):	43848
Calculated pI:	6.54
Taxonomy:	*Bradyrhizobium* sp. ARR65
MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Phospho (ST)

Protein sequence coverage: 5%. Matched peptides shown in **bold**.

MNVQHASFDK PAADDDHLLM DRGSLFLQRT AAVAVAAAAE AEEVDRDARF

PRAAIDAARE QKLLGMLIPV EFGGFGASIQ DVTEICYTLG RACASSAMIF

AMHQTKVACL VRHSVGSRYH QDLMRR**VATE** **QLLLASSTTE** **GQNGGNIR**AS

AAAVEHDANG ISLTRNATVI SYGAEADGIV TIARRASDAT ASDQVLLAVT

KDHYTLARGQ GWETLGMRGT CSAGFELKIR GPAEMIFPEA YERIHAQTMT

PVAHLTWSSV WAGIAAAAVE RAQRFIRKAA RGAGGQMPPG AAHYTAAKMS

LTKLRAMIAG HLDLYAVHEY DDRVLSSIDF QSAINLLKVQ ASELAVETAT

HAMRACGLAG YRNDGEFSIG RHLRDALSAP LMINNDRILA NIATASLMSA

VPTALRD

Search Parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Phospho (ST)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 10.5 ppm
Fragment Mass Tolerance: ± 1.75 Da
Max Missed Cleavages : 0
Instrument type : MALDI-TOF-TOF
Number of queries : 3

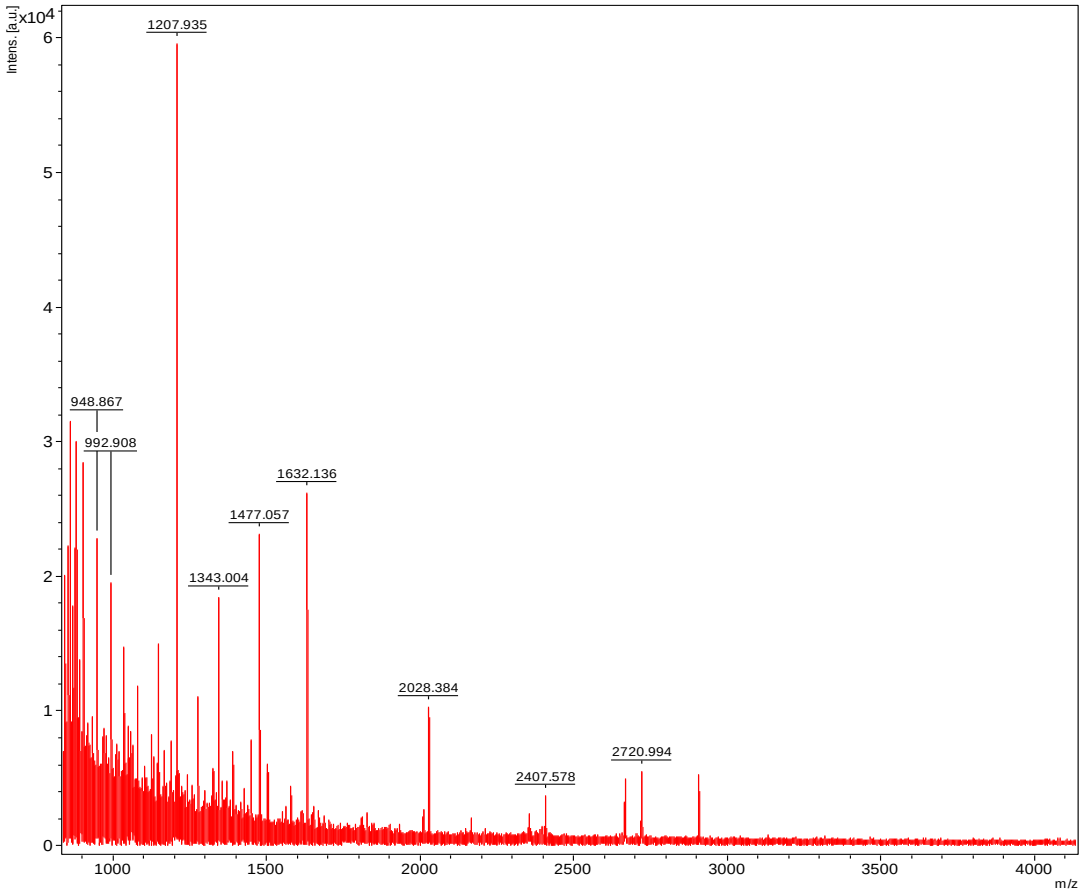
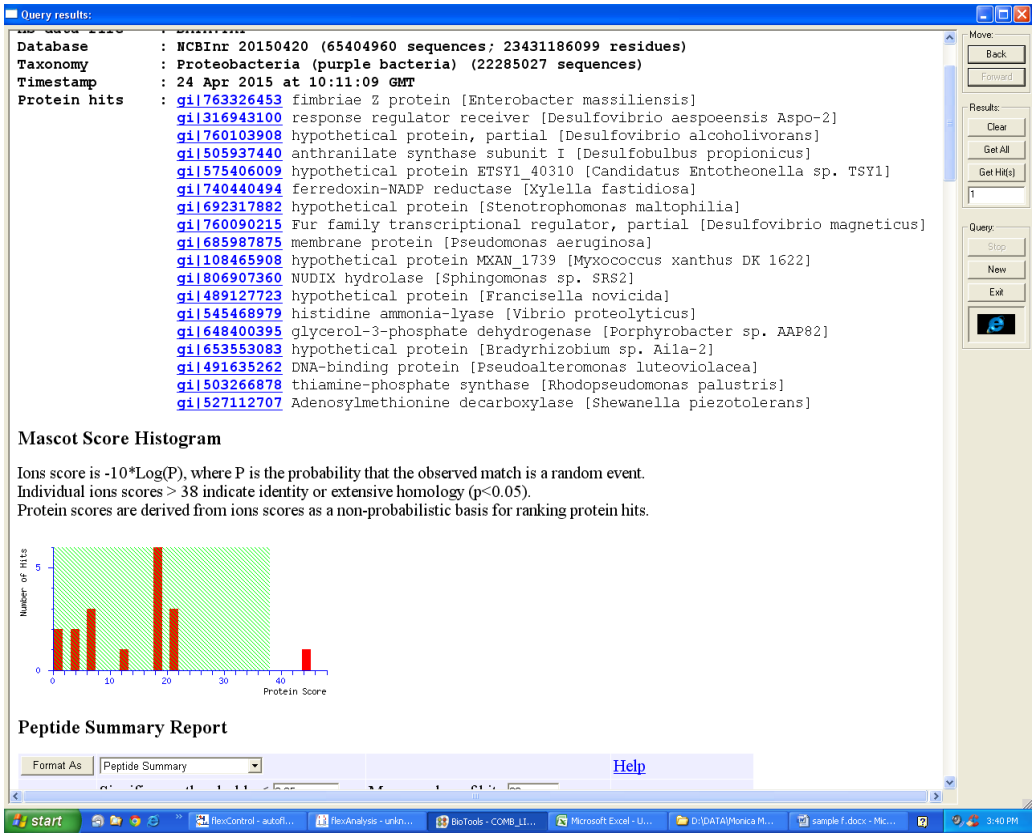


Fig.4.7 MALDI-TOF spectrum of CBB stained, tryptic digested 2D spot-f



Database:	NCBInr
Score:	44
Nominal mass (M_r):	23914
Calculated pI:	9.34
Taxonomy:	Enterobacter massiliensis
MS data file:	DATA.TXT
Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Oxidation (M)

Protein sequence coverage: 6%

1	MKTASVIIMD	EHPLIRMSIE	VLLKNNKNIN	VVLNTDDGHK	VLSYIRKHNV
51	ELVILDIELP	NSDGFTFLKR	IK**EIRPNTQV**	**LFLSSK**SESF	YAGRAIRAGA
101	NGFVSKKTDL	NEIYNAVEML	LAGYSFFPSE	ILIQFNQFPR	RLRERDDMPL
151	SNREVTVLRY	LANGLSNKEI	AKQLLLSNKT	ISAHKSNIFS	KLGVHSIVEL

Query	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expected	U	Peptide
3	1632.1361	1631.1288	1630.9042	138	0	44	0.027	U	K.EIRPNTQVLFLSSK.S

Search Parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 138.5 ppm
Fragment Mass Tolerance: ± 1.75 Da
Max Missed Cleavages : 0
Instrument type : MALDI-TOF-TOF
Number of queries : 3

In a MS/MS, many of the copies of the selected peptides were fragmented using short pulse of laser light at the peptide backbone to generate ions (b-ions and y-ions). The mass spectrum produced peaks at certain m/z (mass to charge) ratio values of the fragmented ions for the corresponding peptides. Sequence matched peptides and the protein identified using MASCOT search with their score were given in the table 4.1.

Table. 4.1 Proteins identified by MALDI-TOF based on peptide sequence match

S. no	Sample ID	Protein Identity from MASCOT search	Score	Mol wt in Da	pI	Sequence Coverage (%)	Sequence matched
1.	Spot-1	Serine hydroxymethyl transferase	37	44683	7.18	4	NMIPFDPEKP MTTSGVR
2.	Spot-A	Tetraacyl disaccharide 4-kinase	51	36784	9.13	4	VISAQIVPSN NIDK
3.	Spot-C	Homoserine O-acetyltransferase	35	41662	6.27	5	LAAVIGGSL GGMQALSW TLR
4.	Spot-3	acyl-CoA dehydrogenase	53	43848	6.54	5	VATEQLLLA SSTTEGQNG GNIR
5.	Spot-f	fimbriae Z protein	44	23914	9.34	6	EIRPNTQVLF LSSK

4.5 ANTI-INFLAMMATION STUDIES

4.5.1 Inhibition of 5-LOX activity by crude, partially purified pellets of biosurfactant

The aim of the objective was to evaluate the immunoregulatory potential of EPS derived from the high-exopolysaccharide producer, *AcinetobacterM6*. The immunoregulatory potential of EPS was compared with that of known inhibitors. All the values are the average of the three O.D difference values and the 100% activity of enzyme is calculated as 41.63 nmol/ml x min.

Table.4.2 5-LOX assay result for biosurfactant sample

Concentration (µg/ml)	Compound	Difference in Oxygen Conc. nmol/ml	%activity	%inhibition
5	Control	120.16 ± 6.1	100	0
100	Crude Biosurfactant	76.26 ± 3.2	61.05	38.95
10	NDGA	32.85 ± 2.9	20.98	79.1
1	Protein	101.17 ± 9.1	64.72	35.28
1	Lipid	110.1 ± 12.2	50.21	49.79
5	Carbohydrate	131.3 ± 4.6	83.88	16.12

Crude Biosurfactant showed 38.37% inhibition of 5-LOX enzyme activity. Protein part of the biosurfactant showed 35.28% inhibition at a concentration of 1 µg/ml and at the same concentration Lipid Portion of the biosurfactant showed 49.79% inhibition

of the enzyme and therefore the protein and lipid portions of the Biosurfactant contributing majorly in the inhibition of 5-LOX enzyme.

4.5.1 Inhibiton of Cyclooxygenase (COX-2) activity

The enzyme activity of COX-2 was observed to be 0.289 absorbance difference/min x ml and 100% activity of enzyme is deduced as 348 nmol/ml x min. Crude biosurfactant showed 43.37% inhibition of COX-2 enzyme activity which is a measure of decrease in oxygen transfer to the substrate arachidonic acid which further results in the synthesis of prostaglandins. Protein part of the biosurfactant showed 41.61% inhibition at a concentration of 1 µg/ml and at the same concentration lipid Portion of the biosurfactant showed 51.56% inhibition of the enzyme, therefore the protein and lipid portions of the biosurfactant contributing majorly in the inhibition of COX-2 enzyme.

Table. 4.3 COX-2 assay result for biosurfactant sample

S.no	Test (reaction mixture)	%activity	%inhibition
1.	TMPD (26.4 µg/ml) + AA (0.1 mM)+ Analysis buffer	Auto-Oxidation = 0.073	
2.	TMPD (26.4 µg/ml) + AA (0.1 mM) + COX-2 + Analysis buffer	100	0
3.	TMPD (26.4 µg/ml) + AA (0.1 mM) + COX-2 + Indomethacin (100 µg /ml) + Analysis buffer	1.35	98.65
4.	TMPD (26.4 µg/ml) + AA (0.1 mM) + COX-2 + Crude biosurfactant (100 µg /ml) + Analysis buffer	56.63	43.37
5.	TMPD (26.4 µg/ml) + AA (0.1mM) + COX-2 + Protein (1 µg/ml) + Analysis buffer	58.39	41.61
6.	TMPD (26.4 µg/ml) + AA (0.1mM) + COX-2 + Lipid (1 µg/ml) + Analysis buffer	48.44	51.56
7.	TMPD (26.4 µg/ml) + AA (0.1 mM) + COX-2 + Carbohydrate (5 µg/ml) + Analysis buffer	81.84	18.16

4.5.2 Inhibiton of Cyclooxygenase (COX-1) activity

The enzyme activity of COX-1 was observed to be 0.308 absorbance difference/min x ml and 100% activity of enzyme is deduced as 373 nmol/ml x min. Crude Biosurfactant showed 53.57% inhibition of COX-1 enzyme activity which is a measure of decrease in oxygen transfer to the substrate arachidonic acid which further results in the synthesis of prostaglandins. Protein part of the biosurfactant showed 48.61% inhibition at a concentration of 1 µg/ml and at the same concentration lipid Portion of the biosurfactant showed 61.63% inhibition of the enzyme, here also the protein and lipid the lipid portions of the biosurfactant showed good inhibition over COX-1 enzyme.

Table.4.4 COX-1 assay result for biosurfactant sample

S.no	Test (reaction mixture)	%activity	%inhibition
1.	TMPD (26.4 µg/ml) + AA (0.1 mM)+ Analysis buffer	Auto-Oxidation = 0.073	
2.	TMPD (26.4 µg/ml) + AA (0.1 mM) + COX-1 + Analysis buffer	100	0
3.	TMPD (26.4 µg/ml) + AA (0.1 mM) + COX-1 + Indomethacin (100 µg /ml) + Analysis buffer	0.649	99.35
4.	TMPD (26.4 µg/ml) + AA (0.1 mM) + COX-1 + Crude biosurfactant (100 µg /ml) + Analysis buffer	46.43	53.57
5.	TMPD (26.4 µg/ml) + AA (0.1mM) + COX-1 + Protein (1 µg/ml) + Analysis buffer	51.39	48.61
6.	TMPD (26.4 µg/ml) + AA (0.1mM) + COX-1 + Lipid (1 µg/ml) + Analysis buffer	38.37	61.63
7.	TMPD (26.4 µg/ml) + AA (0.1 mM) + COX-1 + Carbohydrate (5 µg/ml) + Analysis buffer	82.38	17.62

4.5 SUMMARY

Glycosylation that influences protein folding was the posttranslational modification event occurred in most of the eukaryotic cells, prokaryotic glycosylation has been somewhat understudied. The set of principles accepted for prokaryotes were not able to glycosylate proteins. Research focused on exploring the prokaryotic protein glycosylation and there has been a steady increase in the identification and the characterization of glycoproteins secreted from other bacteria (Messner, 2009). Important steps in pathogenesis have been linked to the glycan substituent of bacterial proteins, indicating that prokaryotic protein glycosylation has a key role to play in infection and pathogenesis, hence interference occured with host inflammatory immune responses. Prokaryotes produce a vast array of unusual monosaccharides and glycan structures that are often difficult to characterize using the analytical technologies developed for the more predictable eukaryotic glycans and glycoproteins. Mass spectrometry procedures were used to characterize prokaryotic glycoproteins and novel glycan moieties. Bacterial extracellular components (peptydoglycan, lipoteichoic acids and EPS) were (Zhang et al. (2005) reported that both live and heat killed bacteria may ameliorate inflammation by decreasing TNF-α-induced IL-8 production. Lipoteichoic acid (LTA) of lactobacilli activates macrophages and dendritic cells through TLR2, in a strain-specific manner (Matsuguchi, et al., 2003). Structural diversity derived from different bacteria give rise to immunoregulatory properties. Little is known about the immunoregulatory potential of exopolysaccharides (EPS) which is a key component of the biofilm matrix of bacteria.

Recently, it has been reported that EPS-producing probiotic bacteria significantly attenuate experimental colitis, in a dose-dependent manner (Kleniewska, et al., 2016). The proteins played a crucial role in the biochemical processes of any organism. Based on the results shown by MALDI-TOF analysis, the five identified proteins have a significant role in the growth and survival of *Acinetobacter* M6 strain and its biofilm formation. Eicosanoids formed via Lipoxygenase and Cyclooxygenase pathways have a key role in inflammation and cancer. NSAIDs are being used, but, had side effects, hence the development of natural drugs was essential. Cyclooxygenase (COX) has a physiological role, so there was a need to develop the selective COX inhibitors to minimize the side effects. Though there were selective inhibitors, COXIBs, but these also have fewer gastric side effects. Therefore there is a need for the development of natural compound which might be a selective COX inhibitor or a dual LOX and COX inhibitor. The assay results showed the crude biosurfactant was acting as dual inhibitor of both 5-LOX and COX, but its inhibition of COX was observed to be more when compared to 5-LOX quantitatively. Further the carbohydrate, protein and lipid extracts of biosurfactant had shown significant inhibition of 5-LOX. The above observations highlight the importance of biosurfactant to be considered as a potent dual inhibitor of COX and 5-LOX, as it showed anti-inflammatory and anti-oxidant properties. The above results clearly indicating that the purified fractions of protein and lipid components of biosurfactant were showing maximum inhibition when compared to carbohydrate portion and crude biosurfactant. This was observed to be the first report on 5-lipoxygenase and cyclooxygenases inhibitory activity of purified exopolysaccharide isolated from *Acinetobacter* M6**.**

CHAPTER-V

QUORUM SENSING STUDIES OF BIOSURFACTANT PRODUCTION

5.1 INTRODUCTION

Biosurfactants produced by different kinds of microorganisms show different surface active properties because of their amphipathic nature and unique structure. Biosurfactants and bioemulsifiers differ in their molecular weights only, and are composed of lipopolysaccharide, lipoprotein, polysaccharides or complex mixtures of polymers. Biosurfactants have huge pharmaceutical market due to low toxicity and biodegradable nature and can be used as most effective antimicrobial agents (Satpute, et al., 2010). The biosurfactant is an exopolymeric material that can tolerate extreme (pH and temperature) conditions and favors the bacteria to sustain under adverse conditions. Many of the exopolymeric substances producing bacteria lives in biofilm and the biofilm formation is typical feature that the bacteria resides inside the biofilm, escapes from the drugs as the exopolymeric substance acts as a protective sheath. Generally this kind of bacteria which produces exopolymeric materials such as biosurfactant has the role in antibiotic resistance, as this kind offers many regulatory pathways to act against antibiotics. Natural plant extracts which contains several phenolic compounds have great role in pharmaceutical field due to the safety issues concerned with the natural extracts and this could be the characteristic feature to become a drug. Researchers are aiming on the antibacterial, antibiofilm effects of the garlic as it contains allicin, which is an organosulphur compound that involves in various biochemical interactions. Garlic shows the mechanism of disrupting signal mechanism by inhibiting the Acyl Homoserine Lactone (AHL) peptide molecules as a part of quorum sensing regulatory mechanism. Quorum sensing is the regulation mechanism which is offered by the threshold concentration of the AHL peptides. Biofilm formation is typically regulated by the quorum sensing regulation mechanism; therefore researchers are targeting the AHLs, quorum sensing molecules, using various plant antagonistic compounds (Gonzalez, et al., 2006). In gram negative bacteria like Acinetobacter M6, the fatty acyl chain bound to homoserine lactone with an amide bond forms the basic structure of AHLs. Heteropolysaccharide type of emulsifiers derived from the bacterial origin have gained prior attention among researchers. Biosurfactant from the natural origin has less potential side effects as they

are least toxic by nature. There has always been few pages for the bioremediation research in the history of environmental toxicology and protection. The complex nature of the biosurfactant comes from the genetic diversity of the species that are adapted to the local conditions of the marine habitats and experience the stress type of conditions in order to survive under extreme situations by tolerating the pH and temperatures. Most of the biosurfactants are safe as they leave fewer side effects and are less toxic in nature. Several of the bacteria show resistance to different types of antibiotics by different mechanisms such as quorum sensing regulation of signal peptide molecules, efflux pumps and release of antagonistic compounds (Fajardo, et al., 2008). The strategies that are being followed by the researchers to inhibit the microorganisms has also led to the resistance of the microorganisms, there is a need to develop the novel mechanism which could regulate at the genetic level and molecular level. Emulsifiers from the natural sources are the potent tools for the degradation of oils by breaking down the chemical polymer chains of alkanes of hydrocarbons. The complexity of pollutants depends on the chemical nature of hydrocarbons and are digested by the typical polymers released by the bacteria which are heteropolysaccharide in nature, which have different properties of antimicrobial, anti-inflammatory and anti-tumorigenic activities, emulsifiers have great market value, as they show promising results in many of the medical applications. Several antimicrobial compounds have been synthesizing for years include chemical and natural molecules, researchers have been paying kind attention on isolating antimicrobial compounds from the extracellular polymers of the bacteria due to their diverse functional nature, although they do not know the correct mechanism of the molecules involved and their exact role in an activity, hence, the correct mechanisms should be elucidated with respect to their molecular level (Rietschel, et al., 1994). The structure that defines the function of the protein has the key role in inhibiting the microbes. Several crystallographic, diffractive, spectroscopic and chromatographic techniques are needed for the structural determination of the antimicrobial compound. Much focus has been thrown on the novel properties of bacteriocin molecules which show many of the antimicrobial properties; however the exact mechanisms of how these molecules interacting and blocking the molecules of targeted bacteria may be unknown. Emulsifiers from the bacterial and fungal origin have been isolated and tested for the antimicrobial, anti-inflammatory and anti tumorigenic activities; these kinds of emulsifiers have a great market value as they show promising results in many

of the medical applications. Marine and Aquatic ecosystems have the rich biodiversity of species like coral reefs which contain pigments and blue blooded snails which have copper containing protein hemocyanin show antimicrobial effects. Besides that, there are wide ranges of microbial species which show a wide variety of mechanisms against the target organisms (Holmstrom, et al., 1999). Biosurfactants has been produced from many of the bacterial strains, majority of the *Acinetobacter* Species are good emulsifiers which can produce complex heteropolysaccharaides, glycoproteins, glycolipids, lipoproteins, polysaccharides (Rosenberg, et al., 1999).

5.1.1 Quorum sensing in bacteria

Bacteria have signalling mechanism to communicate among their cell communities. Gram-negative bacteria utilize acylated homoserine lactones (AHLs) as signalling molecules and gram-positive bacteria make use of small peptides (oligopeptides) as signalling molecules. These signal molecules enable specific intra species communication. Quorum sensing in gram positive bacteria involves signal oligopeptides. The biofilm producing organisms are highly resistant to antibiotics (Waters, et al., 2005). The use of drugs that destroy the biofilm producing pathogens is the major challenge in the current research scenario. The eradication of disease and infection causing pathogens is difficult, particularly pathogens that produce biofilm. Blocking quorum sensing (Quorum-quenching) signal mechanism can be one of the strategies to destroy the biofilm producing pathogens. Lot of research has been focused on finding natural quorum-quenching molecules that can be used along with new generation of antimicrobials. Quorum quenching compounds has to be designed from natural sources, various plant extracts have been reported to have antibiofilm activity against *Acinetobacter* species (Sujana, et al., 2013, Stanbury, et al., 2013). Theoretically, the microorganisms reach the stationary phase due to the depletion of nutrients or accumulation of nutrients. Most *Acinetobacter* sp. studies have focused on understanding the mechanism of antimicrobial resistance found within them. Biofilms are microbial colonies with their own network systems. Biosurfactant producing quorum sensing regulatory bacteria use the acyl carrier proteins and provide them to the homoserine moiety. Quorum-sensing signaling may be extracellular or intracellular and is often mediated by N-acyl-homoserine lactone (AHL) concentration In this case the microorganisms did not grow in the spent media, but a considerable microbial growth was observed in spent media indicating that the microorganisms released signalling molecules once they attain considerable cell density/number. The

idea of the proposal was originated by taking the culture from the growth of microorganisms in spent culture. Though there are many reports available on auto inducing peptides of gram positive bacteria (Waters, et al., 2006), very few reports were available on auto inducing peptides of gram negative bacteria. This may lead to identifying the anti-inducer molecules which can be targeted against virulence factors. Most biosurfactants are secondary metabolites, they play essential role through facilitating nutrient transport, also include bioavailability of hydrophobic water-insoluble substrates, quorum sensing and biofilm formation. Biofilm producing microorganisms are more resistant to antimicrobial substances (Nadell, et al., 2011). In the present study, it was hypothesized that the production of auto inducing peptides and biofilm formation were interlinked. The small peptides which may have antimicrobial activity would also act as signals for the bacteria to form biofilm when they reached considerable cell density.

5.2. MATERIALS AND METHODS

5.2.1 Biosurfactant extraction

The biosurfactant was extracted from the 24 h grown culture of *Acinetobacter* M6 strain, which was centrifuged at 9,200 rpm for 30 min. The centrifugation step left the supernatant, mixed with thrice the volume of acetone and kept the solvent/supernatant mixture at 4°C for 10 h. The solvent/supernatant mixture was subjected to centrifugation at 13,200 rpm for 5 min at 4°C. The precipitate was dissolved in 1 ml millipore water and the solution containing biosurfactant was dialyzed using 12-14 kDa cut-off membrane (Hi media). The biosurfactant concentration was expressed as the total carbohydrate content (Willumsen, et al., 1996).

5.2.2 Biofilm formation and Scanning Electron Microscopy (SEM)

Biofilm formation by *Acinetobacter* M6 isolate was observed for its adherence on glass surface by Scanning Electron Microscopy **(**SEM). The adherent cells form multilayer formation with in 168 h of deposition forming a mature biofilm surrounded by extracellular matrix (Kodali, et al., 2009). Biofilm developed for seven days, was fixed with 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1 h, washed gently for three times in phosphate buffer, and then post fixed in liquid propane/isopentane mixture (2:1) before freeze-drying under vacuum. Sample was coated with 10 nm gold/palladium, viewed under a Carl zeiss supra (Germany) scanning electron microscope at a low magnification scale of 4 μm.

5.2.3 Inhibition studies of the biofilm and biosurfactant production using plant extracts

Mentha pipertia, Pongamia pinnata, Azadiractha indica, Aloe vera, Camellia sinensis, Ocimum tenuiflorum, Cymbopogon citratus and Musa paradiciaca leaves were collected, air dried at 42^{o}C in a hot-air oven and grinded into fine powder. One gram of the powdered plant material was separately extracted by boiling with ethanol and water separately (10ml). Supernatants were filtered through a funnel with Whatman no.1 filter paper. All selected plant Extracts have been tested for the inhibition of biosurfactant (biofilm) production (Ainsworth, et al., 2007).

5.2.4 Study of the effect of plant extracts on biosurfactant production

The selected plant extracts for the inhibition of biosurfactant production were quantified. Formation of biofilm was tested by 96-microtitre well plate and tube methods. *Acinetobacter* M6 strain was inoculated into test tubes containing 50 ml of Luria Bertani broth and incubated for 18 h of biofilm development along with selected plant extracts. The selected plant extract concentrations ranged between 47 and 70 µg/ml were added to the tubes, incubated for 2-3 days.

5.2.5 Anti susceptibility test of the *Acinetobacter M6*

The Antimicrobial susceptibility test for the marine isolate *Acinetobacter* M6 was performed using 25 ml of Muller Hinton Agar per each test plate. The test Petriplates were spreaded with 100 µl overnight culture of *Acinetobacter* M6. The test strain was treated with the antibiotics Chloramphenicol, Kanamycin, Tetracycline and Ampicillin at different concentrations ranging from 1 to 500 µg. The plates were incubated at 37°C for one day and were checked for inhibition zones. Three values were recorded and the mean was calculated as the final measurement of the zone of inhibition (Cantisani, et al., 2014).

5.3 QUORUM SENSING STUDIES

5.3.1 Extraction of Ginger and Garlic Juice

One hundred grams each of ginger (*Zingiber officinale*) and garlic (*Allium sativum*) were taken and extracts were prepared separately. The extracts of garlic and ginger were centrifuged at 5000 rpm for 15 min to collect their juices. Concentrations of 0.1%, 0.5%, 1%, 1.5% and 2% of the garlic and ginger extracts were added to the culture supernatants that are inoculated with their respective strains. Growth was monitored and biofilm production was measured.

5.3.2 N-Acyl Homoserine Lactone peptide extraction from *Acinetobacter* M6

Supernatants were collected from the stationary phase grown cultures of *Acinetobacter* M6 after the addition of garlic and ginger extracts in different concentrations. Ethyl acetate was added to the supernatant and mixed for 15min in an orbital shaker. The mixture was taken in a separating funnel and observed till the mixture gets separated. The upper organic phase was collected and dried at 37^{o}C using rotary vacuum evaporator. The residues were dissolved in methanol and water, analysed using liquid chromatography mass spectroscopy (LC-MS) (Dong, et al., 2001).

5.3.3 The Antimicrobial Activity of biosurfactant

The antibacterial activity of biosurfactant was evaluated by disc diffusion method. The following bacteria (*P. aeruginosa* MTCC 6458, *P. Aeruginosa* 1688, *E. coli* MTCC 10312, *S. pneumoniae* MTCC 1936, *L. monocytogenes* MTCC 839, *E. coli* MTCC 9721, *K. pneumoniae* MTCC 9751, *B. cereus* MTCC 9490, *S. aureus* MTCC 3160) and fungal strains (*C. albicans* MTCC 7253, *C. albicans* MTCC 8332) were used for determination of antimicrobial activity of the biosurfactant. Bacteria and yeast were cultivated in Mueller Hinton (MH) broth and the inhibition zones were observed (Asimi, et al., 2013).

5.3.4 DPPH radical scavenging assay

The DPPH scavenging activity of biosurfactant extracted from *Acinetobacter* M6 was measured (Liu, et al., 2008). The reaction mixture contained 2 ml of 95% ethanol, 0.1 μM DPPH and 2 ml of the biosurfactant (50–400 mg/l). After 20 min of incubation at room temperature, the DPPH reduction was measured at 517 nm. Ascorbic acid at a concentration of 1 mM was used as a control.

5.4 RESULTS AND DISCUSSION

5.4.1 Effect of plant extracts on biofilm and biosurfactant Production

For biofilm growth, the eight plant extracts showed either little to no inhibition, or actually enhanced the formation of the biofilm mass when compared to the vehicle control. It was clearly shown and again proved the already proven concept that the concentration of the biosurfactant, which was an exopolymeric material produced by the bacteria depend upon its biofilm forming ability, which was mediated by the quorum sensing mechanism of the bacteria. The biofilm formation was the indication of the production of biosurfactant which was regulated by quorum sensing signaling molecules concentration during the stationary phase.

Table 5.1 The effect of plant extracts (Ethanolic) on biofilm (biosurfactant) formation

S. no	Plant source	Phenolic Concentrations used (µg/ml)	Biosurfactant concentrations (µg/ml)
1	*Ocimum tenuiflorum*	47.0	345 ± 2.6
2	*Mentha Pipertia*	70.0	310 ± 1.5
3	*Pongamia pinnata*	63.5	319 ± 1.0
4	*Azadiractha indica*	70.0	348 ± 6.0
5	*Aloe vera*	53.0	335 ± 7.0
6	*Camellia sinensis*	65.7	320 ± 2.5
7	*Musa Paradiciaca*	54.3	362 ± 0.5
8	*Cymbopogon citratus*	56.9	354 ± 1.5
9	Control(Without extracts)	NIL	357 ± 1.5

The leaves of *Mentha Pipertia, Pongamia pinnata* and *Camellia sinensis* showed little inhibition on the growth of biofilm, biosurfactant concentrations were measured as 311 ± 1.5, 319 ± 1.0 and 320 ± 2.5 µg/ml respectively. Remaining species showed insignificant inhibitory activities (Table. 5.1).

5.4.2 Anti susceptibility test of the *Acinetobacter* M6

Acinetobacter M6 strain was treated with different antibiotics such as Chloramphenicol, Kanamycin, Tetracycline and Ampicillin at different concentrations ranging from 1 to 500 µg (fig.5.1). The test was susceptible at higher concentrations and had larger zones of inhibition, the minimum inhibitory concentration (MIC) of these antibiotics fall under the range from 3 to 5 µg/ml. The MIC is the lowest concentration of the antimicrobial agent that results in inhibition of visible growth. Here, the MIC ranged from 3 to 5 µg/ml, the strain should be considered as semi-resistant organism. Hence there was a need to develop the antimicrobial drug from the natural approach.

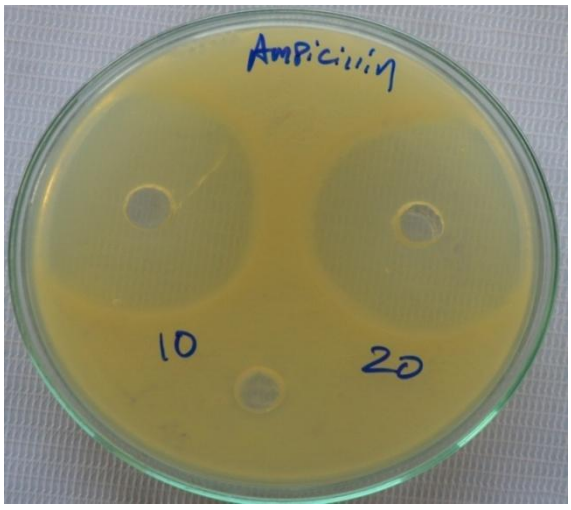


Fig.5.1 Anti-Susceptibility test against *Acinetobacter* M6 using Ampicillin (10 µg, 20 µg)

5.5 QUORUM SENSING STUDIES

5.5.1 Estimation of growth profiles in broth and supernatant inoculated cultures

In our experiments the obtained results showed that the gram negative *Acinetobacter* M6 strain when inoculated in their respective supernatants (spent media) showing less growth in autoclaved supernatant when compared to the without-autoclaved supernatants (Fig.5.2, 5.3), justifying the statement that the AHLs (peptides) responsible for growth regulation got denatured when supernatants were subjected to autoclave, there by provided a favourable environment for the bacteria to grow. In case of without-autoclaved supernatants, the AHLs restricted the growth of the bacteria to continue further even when there is nutrient availability, through the mechanism of quorum sensing. Normally the presence of AHLs, auto inducers of gram negative bacteria restricts the growth of the bacteria even there was nutrient availability. The initial concentrations of protein and carbohydrate in the supernatant broth before the inoculation were 540 µg/ml and 18.2 µg/ml respectively.

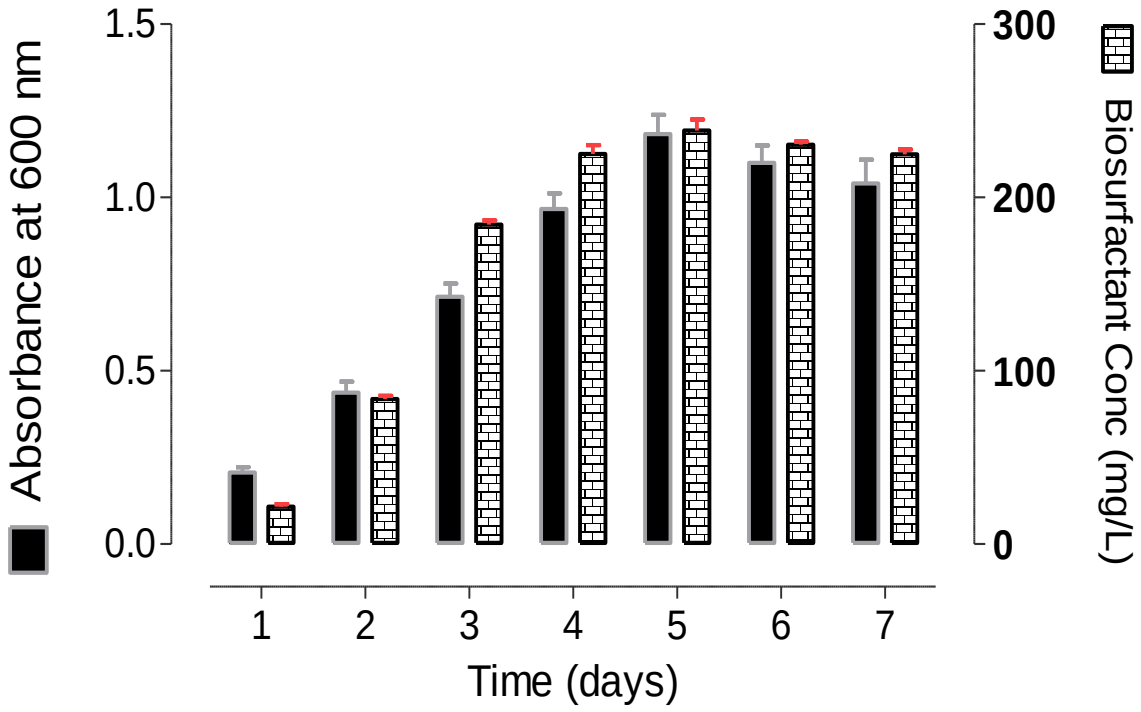


Fig.5.2 Growth profile of the gram negative *Acinetobacter* M6 (with autoclave) in the supernatant

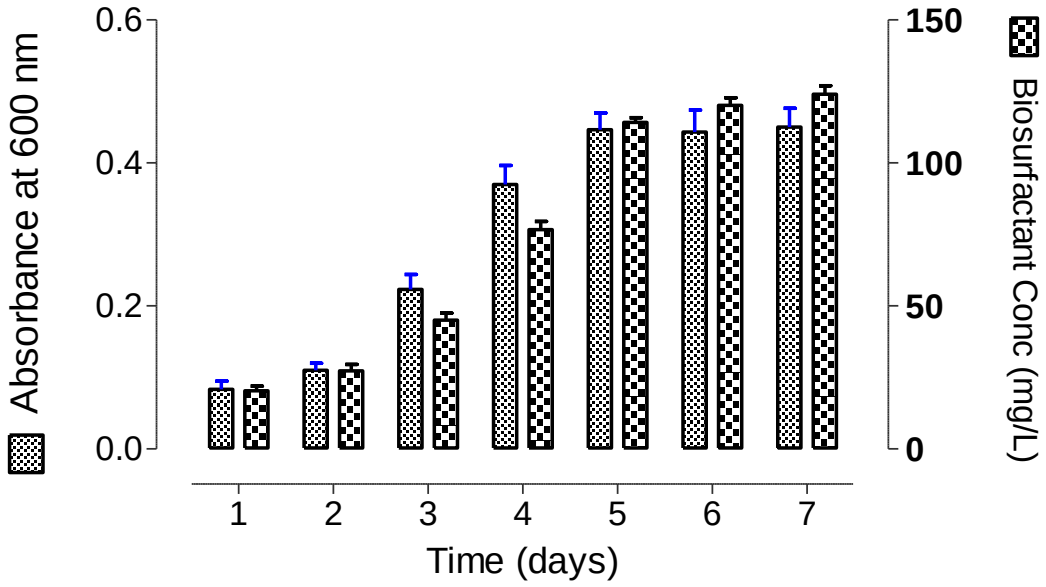
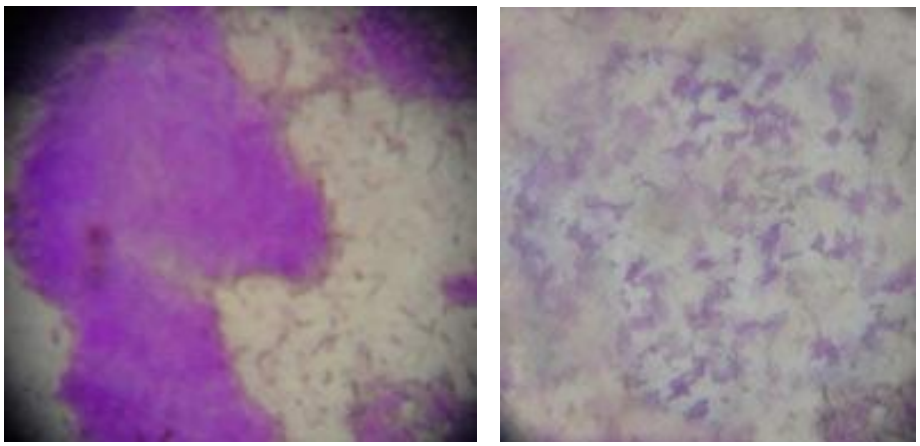


Fig.5.3 Growth profile of the *Acinetobacter* M6 (without autoclave) in the supernatant

5.5.2 Biofilm formation and Scanning Electron Microscopy (SEM)

The biofilm formation of *Acinetobacter* M6 was increased corresponding to the time of biosurfactant production. Due to increase in the hydrophobicity of the cell surface cell aggregation event occured, which is directly dependent on the production of cell bound EPS (fig.5.4). The results suggested the influence of exopolysaccharide biosurfactant on the surface of the bacteria and to determine the adhesion rate of the surface to which it formed the conditioning biofilm, it was clearly seen even under normal microscope and with better resolution at low magnification with scanning electron microscope (Fig.5.5).



7^{th} day (control) (a) **7^{th} day (1% garlic extract) (b)**

Fig.5.4 Biofilm formation during 7^{th} day was observed under microscope by crystal violet (0.1%) staining (a) and treatment with garlic extract (b)

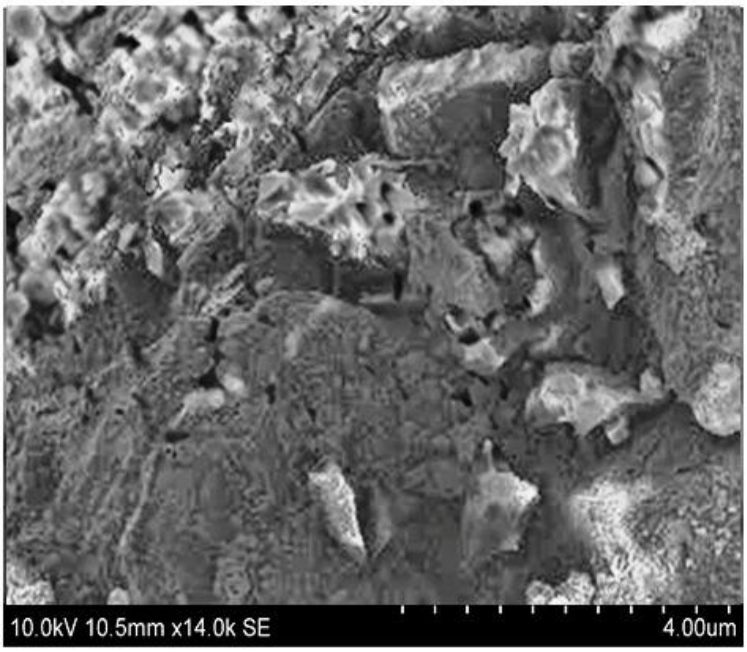


Fig.5.5 Scanning electron microscope image of *Acinetobacter* M6 biofilm at low magnification Scale bar: 4 µm.

5.5.3 Quorum sensing studies

LC-MS results suggested that, on the addition of garlic extract at 1% concentration targeted the quorum sensing molecules (AHL peptides) which are responsible for the biofilm formation in most of the gram negative bacteria. The peak at 279 remains same in before and after the treatment with 1% garlic extraction (Fig 1) and some of peaks were lost after the treatment indicating the loss of quorum sensing signal responsible for biofilm formation (fig.5.6).

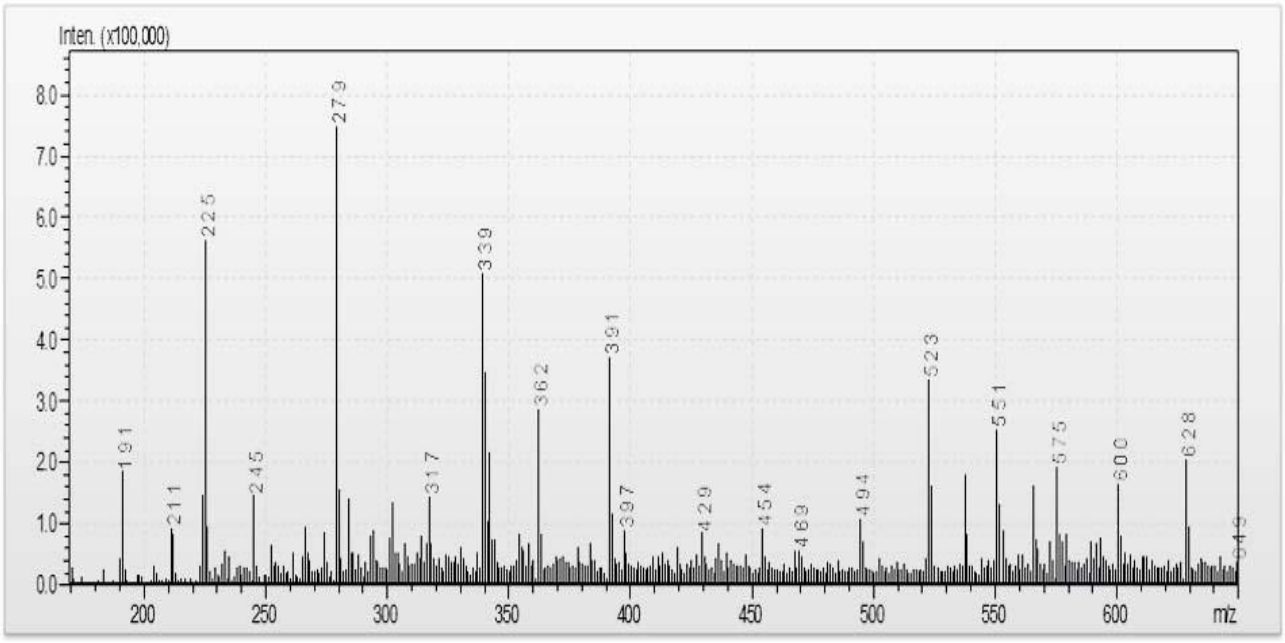


Fig (5.6a): LC-MS graph of AHL peptides before treatment with Garlic extract

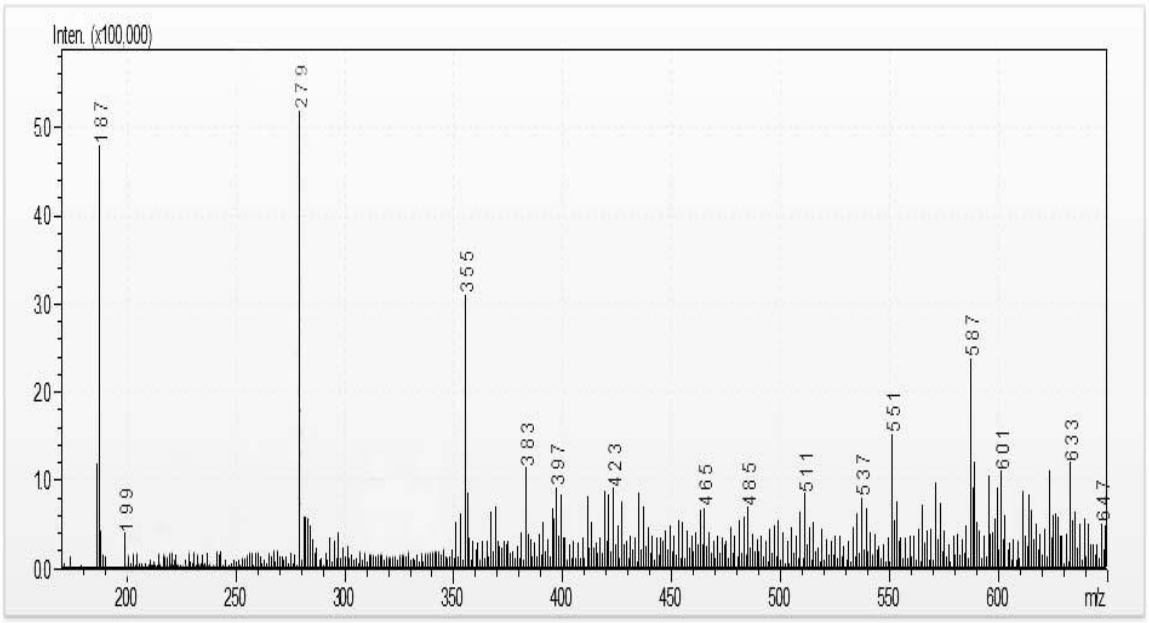


Fig (5.6 b): LC-MS graph of AHL peptides after treatment with garlic extract (1%)

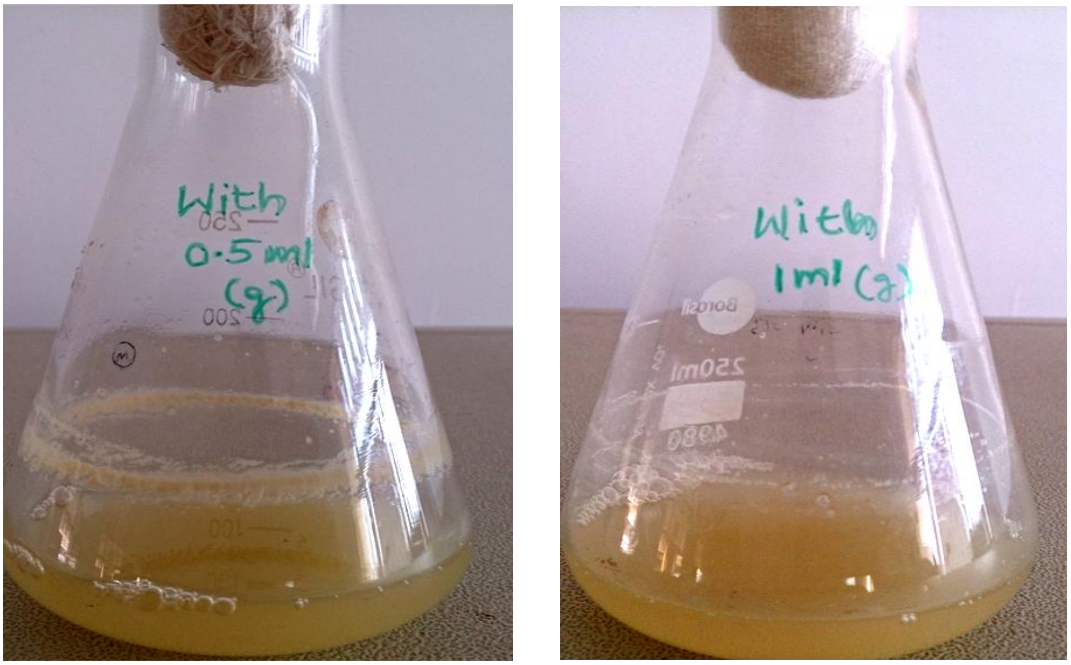


Fig 5.7 Biofilm inhibition at 1% garlic concentration after 7 days of incubation

Differenet concentrations (0.1%, 0.5%) of garlic extract has no effect and even high biofilm production was observed compared to control. It may be due to the stress conditions offered by garlic in the medium. Dispersed biofilm was observed when 1% garlic extract was used (fig.5.7). The biosurfactant was observed to be less (118 µg/ml), when compared with the control (362 µg/ml) after 7 days of incubation with 1% garlic extract. Ginger extract has no significant effect on biofilm production.

5.5.4 The Antimicrobial Activity of biosurfactant

The diameter of the Zones of inhibition produced by biosurfactant (25 to 100 µg/ml) were measured (fig.5.8). At higher concentrations (100 µg/ml) of biosurfactant, the antimicrobial activity was increased. Our biosurfactant showed higher activity against gram-positive *Pseudomonas aeruginosa* MTCC 6458 and *Pseudomonas aeruginosa* MTCC 1688 (Table 5.2). Biosurfactant offered one of the useful properties that has not been studied extensively by researchers was, its antimicrobial activity. In the present study, the glycolipopeptide biosurfactant produced by *Acinetobacter M6* exhibited antimicrobial activity against Gram-positive *Pseudomonas* species, remaining all tested microorganisms were resistant to the biosurfactant. Several lipopeptide biosurfactants produced by *B. licheniformis* have been shown to have antimicrobial activity (Jenny et al. 1991; Fiechter 1992; Yakimov et al. 1995). The ability to produce biofilm could be an important virulence factor by facilitating establishment of resistant infections. *Pseudomonas* has been reported to produce EPS wherever conditions are appropriate for bacterial colonization. Biofilm can physically protect *Pseudomonas* from antimicrobial exposure and it was the most sensitive strain to our *Acinetobacter* M6 extracted biosurfactant.

Fig 5.8 Anti microbial activity against *Pseudomonas Aeruginosa* MTCC 6458

Table 5.2 The zones of inhibition of selected microorganisms against biosurfactant

Microorganism MTCC	ANTIMICROBIAL ACTIVITY (ZONE OF INHIBITION mm)			
	Bio emulsifier Concentration in µg/ml			
	25	**50**	**75**	**100**
P. aeruginosa 6458	8 ± 1.1	12 ± 2.3	15 ± 0.9	17 ± 2.1
P. aeruginosa 1688	7 ± 0.6	10 ± 3.6	13 ± 3.2	15 ± 1.4
E. coli 10312	-	-	-	-
S. pneumoniae 1936	-	2 ± 0.5	-	4 ± 2.5
L. monocytogenes 839	-	-	-	-
E.coli 9721	-	-	-	4 ± 0.6
K. pneumoniae 9751	-	-	-	-
B. cereus 9490	-	5 ± 1.0	-	8 ± 1.8
S. aureus 3160	-	-	-	-
C. albicans 7253	-	-	-	-
C. albicans 8332	-	-	-	-

mm= millimeter, µg/ml = microgram per milliliter

5.5.5 DPPH radical scavenging assay

DPPH scavenging ability of biosurfactant was shown maximum at 500 mg/l with a scavenging rate of 68 ± 1.22%, which was much higher thaN our control ascorbic acid (53.5 ± 4.42 %). Biosurfactant extracted from *Acinetobacter* M6 has greater potential that can act as an antioxidant and an alternative to synthetic antioxidants (fig.5.9).

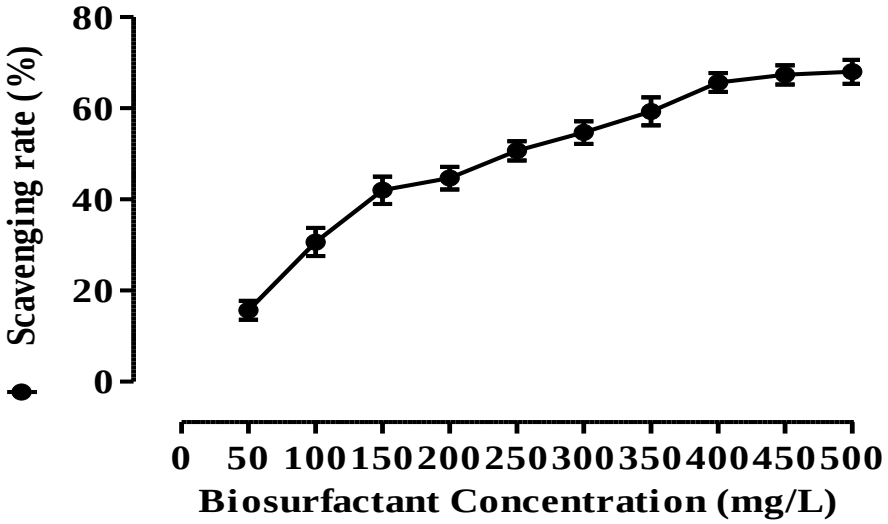


Fig.5.9 DPPH radical scavenging assay of biosurfactant from *Acinetobacter* M6

5.6 SUMMARY

Our present work dealt with the screening of antibiofilm agents using natural extracts and disturbing the quorum sensing signal which was responsible for the formation of the biofilm and the extraction of the antimicrobial compounds from the biosurfactant producing bacteria. Garlic offered a promising approach in the field of quorum sensing signal blocking mechanism. Antimicrobial compounds from the marine aquatic ecosystems delivering the promising results, as the bacteria growing in the marine environments were deeply subjected to stress and led to the release of various compounds to sustain under adverse situations. The biosurfactant which was extracted from the bacteria had shown promising results, as it displayed antimicrobial activity against pseudomonas aeruginosa. In recent years researchers were much focusing on the antimicrobial compounds which originate from the natural plant sources. There was much information available on the antimicrobial properties of plant extracts. Here, the study aimed on the antibiofilm activities and the information is less available. Various biomolecular compounds such as saponins, tannins, phenols, alkaloids, flavonoids took part in antimicrobial mechanisms. It was always a great challenge to the researchers to develop the antibiofilm agents, as the biofilm matrix formed by the bacteria were complex in nature and adopt different types of protective mechanisms so that, the bacteria does not allow the drug and other foreign competent molecules to penetrate into their cells. Researchers found that there were some strong quorum sensing signaling pathways which were regulated by quorum sensing peptides. The target was now changed to the blocking of quorum sensing molecules at the level of gene or at the level of protein. Screening of anti-quorum sensing molecules gained much attention in the recent years. Garlic family takes the pride of blocking these quorum molecules, thereby regulating the pathways that led to the inhibition of biofilm formation. Generally most of the micro organisms grew comfortably in the nutrient broth for some period of time, till there is a rich nutrient availability. When there was depletion of nutrients in the medium, cells communicate among themselves by quorum sensing in which they would stop the growth to attained stationary phase. Assuming that there was a limited nutrient availability in the medium, we have tested the presence of carbohydrates and proteins in the spent media by phenol sulphuric acid and Folin-Lowry methods. After confirming the presence of carbohydrates and proteins in the medium, we had cultured respective organisms in their respective supernatants, which showed the results that there was not any

considerable growth in the without-autoclaved supernatant representing the auto inducers present in the supernatant were limiting the growth of bacteria, even in the presence of nutrients in the supernatant. On the other hand, the cultures which were inoculated in the autoclaved supernatants had shown a considerable growth in their respective supernatants representing that there are no auto inducers to limit their growth in the medium, so that they had grown by utilizing the nutrients in the supernatant. Further we had to focus on the concept that whether these AHLs or oligopeptides show any specificity or any other organism could grow in the supernatant of another organism. Much research is needed to understand the blocking mechanism and the molecule which is responsible for anti-quorum activity. The biosurfactant showed good antimicrobial activity against pseudomonas aeruginosa and we would develop the drug by using pure form of biosurfactants.

CHAPTER-6

CONCLUSIONS AND SCOPE FOR FUTURE WORK

6.1 CONCLUSIONS

- Marine watersamples were collected from 4 different sampling sites of India for the isolation of biosurfactant producing, biofilm forming and quorum sensing mediated bacteria.
- 37 isolates were screened to get 6 isolates giving positive test result for crude oil degradation by whole cell protein estimation and emulsification index (%EI) above 50%. One isolate was selected based on its highest exopolysaccharaide producing ability, hemolytic and dye degrading ability.
- Phenotypic characterization based on biochemical tests of isolate Marine bacterium (sample-M) supported 16S rDNA sequencing identification as *Acinetobacter* genus
- After clustering, the one isolate which was given the names as marine bacterium or sample-M was subjected to 16S rRNA gene sequencing, identified as *Acinetobacter* genus and its sequence was deposited in NCBI GenBank as *Acinetobacter species* M6 strain.
- This is the first time, biosurfactant production by *Acinetobacter* M6 strain with emulsification activity and Critical micelle concentration were reported.
- *Acinetobacter* M6 strain was checked with different hydrocarbons and the stability of emulsions vary from 63 to 95%.
- The biosurfactant production was optimum at 1% inoculum of 168 h old culture. Optimum conditions for biosurfactant production by *Acinetobacter* M6 was found to be pH 7 at a temperature of 35°C. The biosurfactant production was also not affected between pH range of 6 to 8 and temperature range of 30- 40°C.
- The biosurfactant was purified by gel filtration chromatography with Sephadex G-100 column after dialysis and microfiltration.
- The biosurfactant was found 500 μg of protein per mg of carbohydrate, lipid content was detected through TLC.
- The glycoprotein nature was indicated by glycoprotein staining using PAS.

- In FTIR, the biosurfactant showed a broad O-H stretching frequency at 3346 cm^{-1} and at an intense frequency at 1070 cm^{-1} which is an indicative for typical carbohydrates, 1637 and 1530 cm^{-1} indicating the presence of peptides and the lipids were indicated by the presence of peak at 2941 cm^{-1}. The presence of lipid, carbohydrates and protein functional groups suggesting that biosurfactant was a class of glycolipoprotein.
- The relative emulsion stability (% ES) of biosurfactant M6 was found maximum with motor oil as 81%.
- MALDI-TOF/MS-MS of the biosurfactant M6 was provided with five peptide sequences that have role in quorum sensing and biofilm formation.
- The isolate produced a surfactant which was composed of glycolipoprotein based on results shown by TLC, FTIR and electrophoretic techniques.
- The emulsification results showed that the biosurfactant had good surface active properties in industrial and environmental applications.
- Biofilm formation of *Acinetobacter* M6 was enhanced in presence of selected plant extracts and significantly reduced by garlic extract.
- Biosurfactant can be considered as a potent dual inhibitor of COX and 5-LOX, as it showed anti-inflammatory and anti-oxidant properties.
- The biosurfactant was shown good antimicrobial activity against *Pseudomonas aeruginosa* and could be developed as a drug.
- The diversity of biosurfactants produced by different kingdom of life showed the importance of biosurfactant molecules. In different microorganisms, biosurfactants have been studied for influencing adhesion and biofilm formation. Due to their direct interaction with the host and other microorganisms in the environment, biosurfactants were known to had ecophysiological potential to influence the microorganism in settling in a niche. Genus *Acinetobacter* will be studied in detail for their ability to produce biosurfactant and their functions.

6.2 SCOPE FOR FUTURE WORK

The microbial biosurfactant synthesis has important applications in bioremediation for a range of hydrocarbon pollutants and can be considered as a key strategy for bioremediation due to their biodegradability and low toxicity. If we take necessary steps for the large scale production of biosurfactants using cheap raw materials such as organic wastes that should be a cost effective over chemically derived surfactants. In the present study, biosurfactant from marine bacterium was isolated and explored

for various applications. Future research would be on complete structural characterization, genetic level studies and elucidation of the individual components of the biosurfactant extracted from *Acinetobacter* M6.

LIST OF PUBLICATIONS FROM THESIS

- **Peele, K. Abraham,** Ravi Teja Ch, and Vidya P. Kodali. "Emulsifying activity of a biosurfactant produced by a marine bacterium. 3 Biotech 6(2), 2016, 177.

- **Abraham Peele Karlapudi,** Vidya Prabhakar Kodali, Bharath Kumar Ravuru, Detection of Quorum Sensing in Bioemulsifier Producing Bacteria. International Journal of Pharmaceutical Sciences Review and Research, 2016, 38(1): 227-229.

- **Karlapudi Abraham Peele,** Indira Mikkili, T. C. Venkateswarulu, John Babu Dulla, and Ranganadha Reddy. Bioconcrete Build Buildings with Quorum Sensing Molecules of Biofilm Bacteria. Journal of Pharmaceutical Sciences and Research, 2015, 8, 10-12.

- Kota Krishna Priya, Rohini Krishna Kota, John Babu Dulla, and **Abraham Peele Karlapudi**. Bioconcrete Enhancement from Biofilm Producing Marine Bacterium. International Journal of Pharmaceutical Sciences Review and Research, 25(2), 2014, 276-279.

INTERNATIONAL CONFERENCE PRESENTATIONS

- Abraham Peele Karlapudi presented a poster on " Biosurfactant as an Emulsifier" in OMICS group International conference on Agriculture and Horticulture, 27-29 October 2014 in Hyderabad International Convention centre, India.

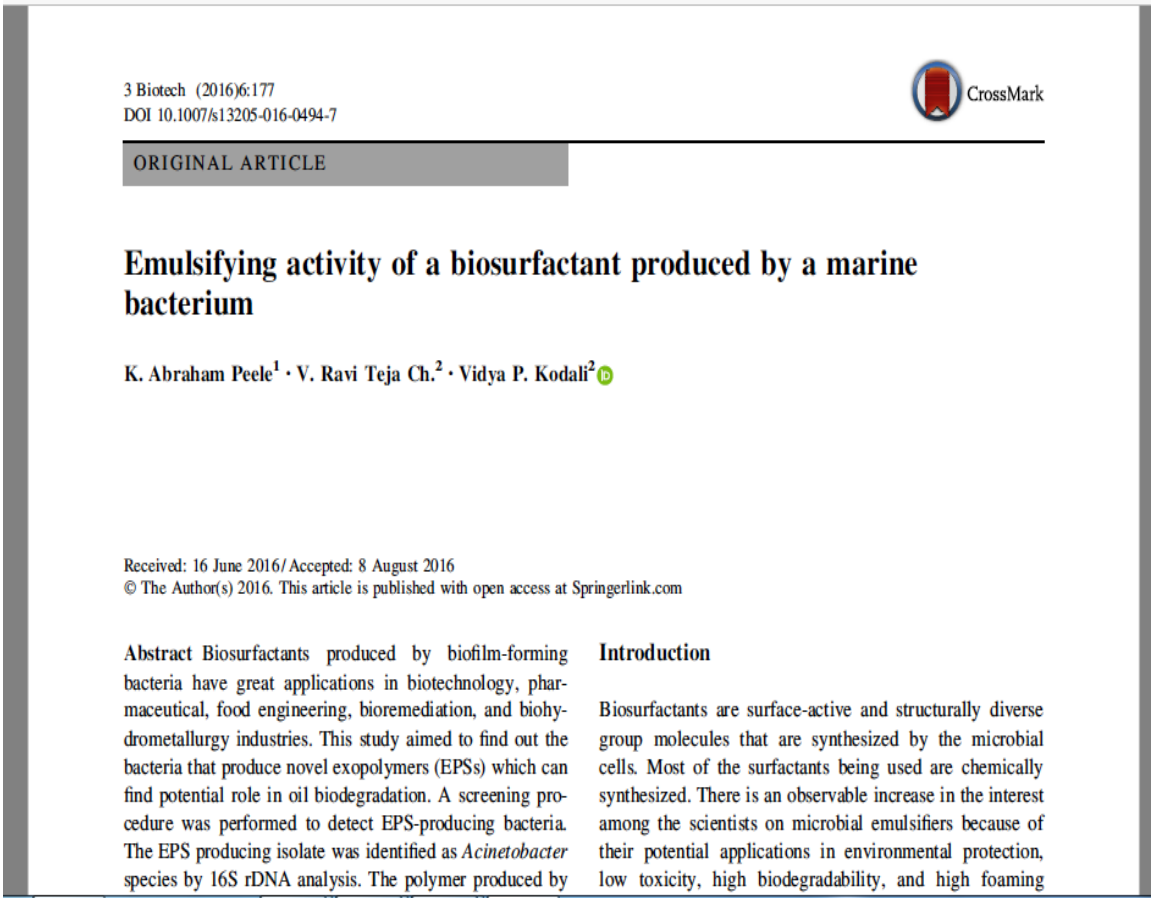
- Abraham Peele Karlapudi presented a paper on "Role of Biosurfactant in oil bioremediation" in an International conference (ESTIEH-2014), 1-3 August 2014, jointly Organized by Vikrama Simhapuri University, Nellore and University of Texas, USA.

- Abraham Peele Karlapudi and Vidya P Kodali presented Poster presentation in "International conference "Drugs for the Future: Infectious Diseases" at NIPER Hyderabad, during 27-28 March, 2014 titled "Antimicrobial activity of a bioemulsifier isolated from marine bacterium".

NATIONAL CONFERENCE PRESENTATIONS

- Abraham Peele Karlapudi and Vidya P Kodali presented a paper on "Bioemulsifier in oil bioremediation" in National conference "Biotrendz-2015" 27-Aug-2015 was organized by Department of Biotechnology, KL University.

- Abraham Peele Karlapudi presented a poster on "Bioconcrete build buildings using quorum sensing molecules of Biofilm producing bacteria" in SIPRA-2015 National conference held on July 2nd 2015 in SIPRA labs, Hyderabad.



3 Biotech (2016)6:177
DOI 10.1007/s13205-016-0494-7

ORIGINAL ARTICLE

Emulsifying activity of a biosurfactant produced by a marine bacterium

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Received: 16 June 2016 / Accepted: 8 August 2016

Abstract Biosurfactants produced by biofilm-forming bacteria have great applications in biotechnology, pharmaceutical, food engineering, bioremediation, and biohydrometallurgy industries. This study aimed to find out the bacteria that produce novel exopolymers (EPSs) which can find potential role in oil biodegradation. A screening procedure was performed to detect EPS-producing bacteria. The EPS producing isolate was identified as *Acinetobacter* species by 16S rDNA analysis. The polymer produced by

Introduction

Biosurfactants are surface-active and structurally diverse group molecules that are synthesized by the microbial cells. Most of the surfactants being used are chemically synthesized. There is an observable increase in the interest among the scientists on microbial emulsifiers because of their potential applications in environmental protection, low toxicity, high biodegradability, and high foaming

Cite reference: Peele, K. Abraham, Ravi Teja Ch, and Vidya P. Kodali. "Emulsifying activity of a biosurfactant produced by a marine bacterium, ***3 Biotech*** **,** 2016, **6(2) : 177. Springer.**

Int. J. Pharm. Sci. Rev. Res., 38(1), May – June 2016; Article No. 39, Pages: 227-229 ISSN 0976 – 044X

Research Article

Detection of Quorum Sensing in Bioemulsifier Producing Bacteria

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ABSTRACT

Bacteria produces many kinds of molecules that allow bacteria to communicate about population size, metabolic states or producing end products that initiate some activities such as bioluminescence. These signal molecules are generally called as autoinducers. Quorum sensing reveals the fact that bacteria have the capacity to assess the number of other components they can activate once the threshold number is reached. Acyl homoserine lactones are present mainly in gram negative bacteria and they control their own synthesis. In AHLs, the head group consists of homoserine lactones and the tail region determines the specificity of the receptor. Oligopeptide molecules are present mainly in gram positive bacteria. Their synthesis is dependent on ribosomes. In the present study it is hypothesized that the production of autoinducing peptides and biofilm formation are interlinked. The small peptides which may have antimicrobial activity will also act as signals for the bacteria to form biofilm when they reach considerable cell density.

Keywords: Auto inducers, AHLs, Gram specific strains, oligopeptides, signaling molecules, stationary phase, quorum sensing

INTRODUCTION

Bacteria have signalling mechanism to communicate

autoinducing peptides of Gram negative bacteria[9]. This may also lead to identifying the anti-inducer molecules which can be targeted against virulence factors. Biofilm

Cite reference: Abraham Peele Karlapudi, Vidya Prabhakar Kodali, Bharath Kumar Ravuru, Detection of Quorum Sensing in Bioemulsifier Producing Bacteria International Journal of Pharmaceutical Sciences Review Research, 38(1): 227-229 (Scopus cited)

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APPENDIX-A (Media)

Nutrient broth (in g%, wt/vol):
Beef extract, 0.3; Peptone, 0.5 and NaCl, 0.5

Luria Bertani (HiMedia, India) medium (in g%, wt/vol):
Casein enzymic hydrolysate, 1; Yeast extract, 0.5; NaCl, 0.5 and pH 7.0±0.2 in 100 ml Distilled water.

Phosphate Buffer Saline i.e.PBS
137 mM NaCl , 2.7 mM KCl ,10mM Na_2HPO_4 , 2 mM KH_2PO_4 at pH 7.3

Bushnell Haas Medium (BHM):
$MgCl_2$, 0.02g%; $CaCl_2$, 0.002g%; KH2PO4, 0.1g%; K_2HPO_4, 0.1g%; NH_4NO_2, 0.1g%; $FeCl_3$, 0.005g%; pH 7.0 (± 0.2).

Preparation of Bradford reagent: 10mg of CBB G-250 is weighed. 10ml of 85% phosphoric acid and 5ml of 95% ethanol is added to CBB and stirred to dissolve it. Volume is made up to 100 ml with Milli-Q water and stirred overnight to dissolve completely. It was filtered and stored in amber color bottle.

Preparation of BSA stock

Primary stock of BSA(5mg/ml) is prepared by dissolving 5mg BSA in 1ml Milli-Q water.

Secondary stock of BSA(1mg/ml) is prepared for 1ml by taking 200µl of primary stock of BSA and making up to 1ml with Milli-Q water. This will be the running concentration.

Reagents for 5-LOX assay

EPS stock (10mg/ml): EPS stock of concentration 10mg/ml is prepared and 300µl from the stock is added to the reaction mixtureof 3ml. Then the final concentration of EPS in the reaction mixture is 3mg/3ml i.e, 1mg/ml.

NDGA: NDGA stock of 1mg/ml is prepared and 30µl from the stock was added to the reaction mixture. Then the final concentration of NDGA in the reaction mixture is 30µg/3ml i.e, 10µg/ml. Phosphate Buffer (PB) primary stock of 100mM of pH 6.3. Working stock of PB buffer is 1mM of pH 6.3

Reagents for COX-1 assay

Enzyme (Cycloxygenase-1): Dilute the enzyme in solubilising buffer such that, its O.D difference to be 0.4 to 0.6.

COX activity = [((ΔA_{610}/ 2 min)/ 0.0826µM^{-1}) x (1ml/0.05ml)]/2

Substrate (Arachidonic acid): From 20 mM stock, 5µl is added to the reaction mixture of 1ml for COX assay. Its final concentration in reaction mixture is 0.1mM.

TMPD: 6.6 mg/ml in absolute alcohol (Stock). From this, 4µl is added to the reaction mixture. Its final concentration in reaction mixture is 26.4µg/ml.

Indomethacin: 6mg of indomethacin is dissolved in 600µl of DMSO to prepare stock. 10µl from the stock is added to the reaction mixture. Its final concentration in reaction mixture is, 0.1mg/ml.

APPENDIX-B (Compositions)

Colloidal Coomassie (G-250)

Chemicals	Final concentration	Amount for 1 litre	Amount for 500ml
Coomassie blue G-250	0.08%	800mg	400mg
Ethanol	20%	200ml	100ml
Orthophosphoric acid	0.35 M	23.2	11.6
Ammonium sulfate	8%	80gr	40gr
Double distilled water	-	Dissolve & make up to1000ml	Dissolve & make up to 500ml

10 X Laemmli SDS electrophoresis buffer or tank buffer

Chemical	Final Concentration	Amount
Tris base (FW 121.1)	250 mM	30.4 gr
Glycine	1.92 M	144.0 gr
SDS	1%(w/v)	10.0 gr
Milli - Q water	Dissolve & Make up To 1 lit	

12% resolving gel (10ml)

Chemical	Amount
Monomer	4 ml
1.5M tris pH 8.8	2.5 ml
10% SDS	0.1 ml
Milli Qwater	3.3 ml
10% APS	0.1 ml
TEMED	0.004 ml

5% stacking gel (5ml)

Chemical	Amount
Monomer	0.83 ml
1.5M tris pH 6.8	0.63 ml
10% SDS	0.05 ml
Milli Qwater	3.4 ml
10% APS	0.005 ml
TEMED	0.005 ml

6X SDS- Sample loading buffer (100ml)

Chemical	Amount
Tris HCl	5.91 g
SDS	6 g
100% glycerol	48 ml
1.47M 2- Mercaptoethanol	9 ml
Bromophenol blue	30 mg

Rehydration buffer

Chemical	Final Concentration	Amount (25ml)	Amount (10 ml)
Urea	7 M	10.5 g	4.2g
Thio urea	2 M	3.8 g	1.52g
Chaps	4%	1 g	0.4g
Pharmalyte or IPG buffer	0.5%	500 µl	250µl
1% bromophenol blue stock solution	0.002%	50 µl	25 µl

Equilibration buffer-1

Equilibration buffer	Dithiothreitol (DTT)	Amount
10ml	65mM	0.1 gr or 100mg
20 ml	65mM	0.2gr or 200 mg

Equilibration buffer-2

Equilibration buffer	Iodacetamide (IAA)	Amount
10 ml	135mM	0.25 gr or 250mg
20ml	135mM	0.5 gr or 500mg

0.5% Agarose sealing solution

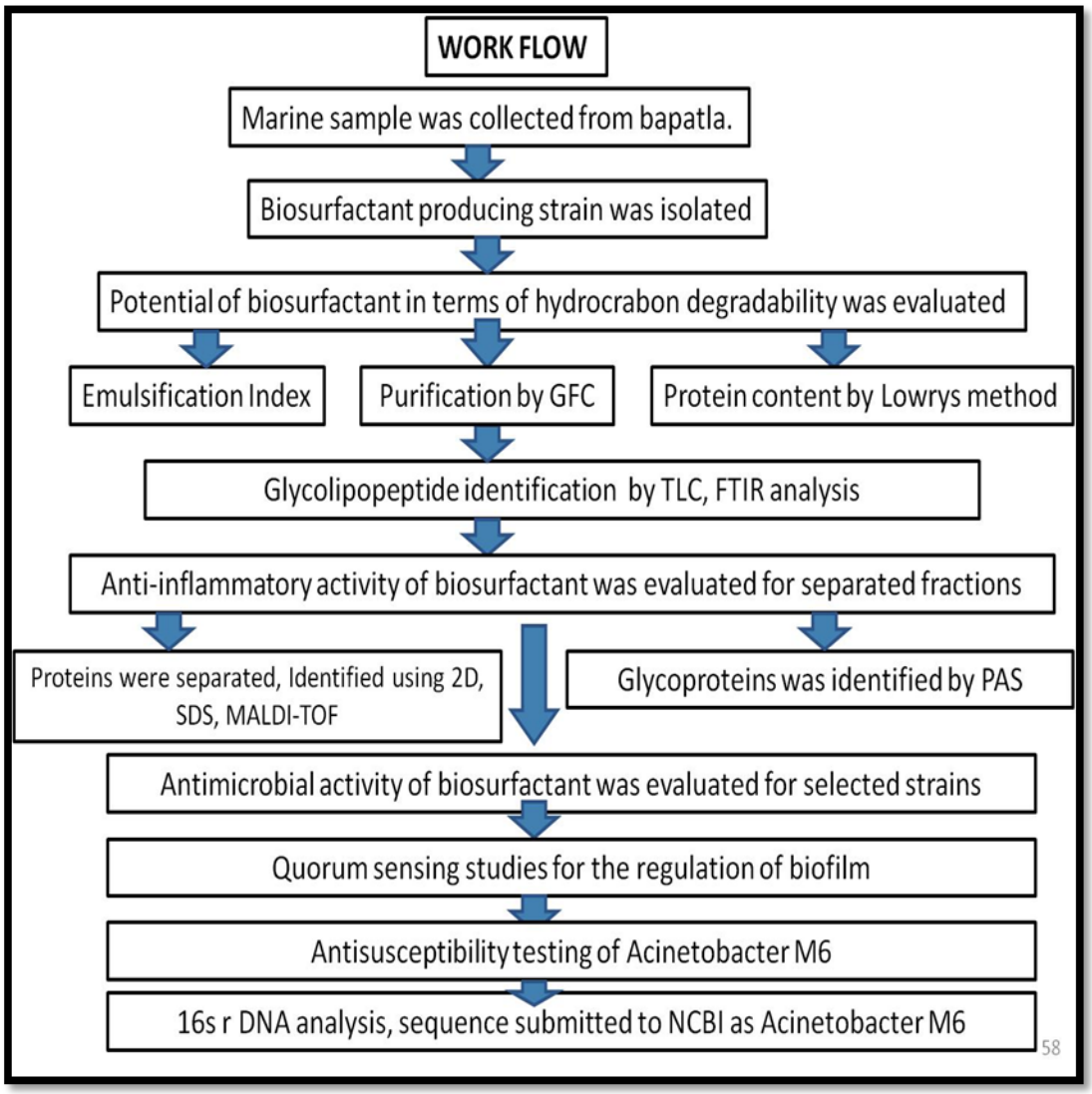
Chemical	Final Concentration	Amount
Laemmli SDS electrophoresis buffer	1X	100ml
Agarose	0.5%	0.5gr or 500mg
1% Bromophenol blue	0.002% (w/v)	200µl

Solubilizing buffer (pH-8.0)

Chemical	Final Concentration	Amount
Tris HCl	5mM	125µl
Glycerol	0.5%	25µl
Tween 20	0.8%	40µl
Phenol	1mM	50µl

Assay buffer

Chemical	Final Concentration	Amount
Tris HCl (pH-8.0)	100mM	2.5ml
EDTA	5mM	250µl
Hematin	5mM	50µl



Flow Chart of the Research work carried out

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CAREER OBJECTIVE:
To work in an environment that is innovative, challenging and build the career with a constant update of my knowledge and skills.

ACADEMIC PROFILE:

COURSE	YEAR	BOARD/ UNIVERSITY	STUDY OF INSTITUTION	% OF MARKS OBTAINED
Ph.D	(2012-TIL DATE)	Vignan's University	Vignan's University	-
M. Tech	2009-2012	**Vignan's University**	**Vignan's University**	85 %
B.Tech	2005-2009	Bharath University	Bharath University, Chennai	72 %
Intermediate	2003-2005	Board of intermediate education	Sri chaitanya junior college, Tenali.	79.4 %
S.S.C	2002-2003	Board of secondary education	Little citizens, Tenali.	69.4 %

ACADEMIC ACHIEVEMENTS:-

- Qualified in GATE 2012 and secured 2989 rank
- One patent applied
- No. of publications in International Journals: 13 (Eight are Scopus indexed, Two are SCIE indexed)
- JRF FELLOWSHIP from DST project (2013-2014)

- No of INTERNATIONAL CONFERENCE presentations: 07
- No. of National conference presentations: 04
- No of Workshops attended: 08

PUBLICATIONS

1. Abraham Peele Karlapudi, Sabiha Sultana Shaik , Vijaya Ramu, Kota, Krishna Priya, , Kodali vidya prabhakar and "Deciphering the nano particle property against acne causing bacteria propionibacterium." *3biotech* **2015. SPRINGER JOURNAL (SCIE Indexed) IMPACT FACTOR : 1 (ISI Thomson Reuters)**

2**. Abraham Peele Karlapudi**, Kodali vidya prabhakar Molecular Characterization of an Emulsifier Producing Marine Bacterium. *3biotech* **2016 (Accepted). SPRINGER JOURNAL (SCIE Indexed) IMPACT FACTOR : 1 (ISI Thomson Reuters)**

SCOPUS INDEXED

1. Kota, Krishna Priya, Rohini Krishna Kota, John Babu Dulla, and **Abraham Peele Karlapudi**. "Bioconcrete Enhancement from Biofilm Producing Marine Bacterium." *International Journal of Pharmaceutical Sciences Review & Research* 25, no. 2 (2014).

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3. Indira Mikkili, Abraham P karlapudi, venkateswarulu T.C, Babu, John, S. B. Nath, Vidya P. Kodali,"Isolation, Screening and Extraction of Polyhydroxybutyrate (PHB) producing bacteria from Sewage sample." International Journal of Pharm Tech Research, vol 6, No.2, 850-857 (2014)

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