"Biosurfactant of Dairy and Fermented Products: Extraction and Characterization"

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By:

LALIT KUMAR CHAURASIA

Roll No. PDMB02

Reg. No.12SU6396

Under the Supervision of:

Dr. BUDDHIMAN TAMANG
(Assistant Professor)

DEPARTMENT OF MICROBIOLOGY

SIKKIM UNIVERSITY

GANGTOK, SIKKIM-737102

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DECLARATION

I declare that the thesis, entitled **"Biosurfactant of Dairy and Fermented Products: Extraction and Characterization"** submitted by me for the award of **Master of Philosophy** Degree in **Microbiology** of **Sikkim University,** is my original work. The content of this thesis is based on the experiments, which I have performed myself. This thesis has not been submitted for any other degree to any other University.

Date: 4/8/2014

Lalit Kumar Chaurasia

Roll No: 12PDMB02

Regn.No: 12SU6396

We recommend that the thesis be placed before the Examiners for evaluation.

HK

(Dr. H.K. Tiwari)

Head, Department of Microbiology

4/8/2014

(Dr. Buddhiman Tamang)

Supervisor

सिक्किम विश्वविद्यालय
(भारतीय संसद के अधिनियमद्वारा स्थापित केन्द्रीय विश्वविद्यालय)
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Certificate

This is to certify that the thesis entitled **"Biosurfactant of Dairy and Fermented Products: Extraction and Characterization"** submitted to the **Sikkim University** for the award of **Master of Philosophy Degree in Microbiology,** embodies the results of *bona fide* research work carried out by **Mr. Lalit Kumar Chaurasia** under my guidance and supervision. No part of the thesis has been submitted for any other degree, diploma, associate-ship and fellowship.

All the assistance and help received during the course of the investigation have been acknowledged by him.

Place: Gangtok, Sikkim
Date: 4/8/2014

4/8/2014
Dr. Buddhiman Tamang
(Supervisor)

6 माईल, सामदुर, तादोंग, गगंटोक - 737102 सिक्किम, भारत
दूराभाष : 00-91-3592 - 251067, 251403, फैक्स :- 251067/251757
6th Mile, Samdur, PO Tadong 737102, Gangtok, Sikkim, India
Phones : 00-91-3592-251067, 251403, Fax - 251067/251757
website : www.sikkimuniversity.in/www.sikkimuniversity.ac.in
Email : sikkimuniversity@gmail.com

Dedicated

To

My family

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Lalit Kumar Chaurasia

Contents

Chapter 1

Introduction

Chapter 1

Introduction

Biosurfactants are amphiphilic biochemical compounds produced on microbial cell surfaces, or are extracellularly secreted and contain hydrophobic and hydrophilic groups that confer the ability to accumulate between fluid phases, thereby reducing surface and interfacial tension at the surface and interface regions, respectively (Karanth *et al.*, 1999).

These compounds attribute to reduce the interfacial and surface tension in the same way as other chemical surfactants does. Biosurfactants are usually low molecular weight organic compounds, which mainly posses glycolipids or lipopeptide chains (Desai *et al.*, 1997). But certain high molecular weight lipopeptides and polymeric compounds have been isolated from microorganisms having surface active properties. Such compounds are described as bioemulsifiers (Fracchia *et al.*, 2012). The features that make them commercially promising alternatives to chemically synthesized surfactants are their lower toxicity, higher biodegradability and, hence, greater environmental compatibility, better foaming properties (useful in mineral processing), and stable activity at extremes of pH, salinity and temperature (Desai *et al.*, 1997). Primarily, biosurfactants attracted attention as hydrocarbon dissolution agents in the 1960s, and their applications have been greatly extended in the past five decades as an improved alternative to chemical surfactants (carboxylates, sulphonates and sulphate acid esters), especially in food, pharmaceutical and oil industry (Banat *et al*; 2000).

Biosurfactants have also been found to possess several properties of therapeutic and biomedical importance: they have antibacterial, antifungal and antiviral properties; they inhibit fibrin clot formation; and they have anti-adhesive action against several pathogenic microorganisms etc (Cameotra *et al.*, 2004, Singh *et al.*, 2004, Rodrigues *et al.*, 2006).

For the last thirty years, a lot of work has been done for the discovery of various microorganisms producing biosurfactants and extensive studies revels their various properties and applications in different commercial fields. As a result now we have a vast list of biosurfactants producing microorganisms and their applications. Though at present,

it is well established that biosurfactants possess promising commercially attractive properties, but its production at industrial level is still a great challenge. The two major challenges that prohibits it to be a successful industrial product are its low productivity and high production cost (Mukherjee, S. *et al.*, 2006).

Although a lot of work has been done regarding biosurfactants therapeutic properties and biomedical characteristics (Cameotra *et al.*, 2004, Singh. *et al.*, 2004, Rodrigues *et al.*, 2006), natural roles (Ron *et al.*, 2001), their production on cheap alternative substrates (Makkar *et al.*, 2002, Maneerat, 2005, Gautam *et al.*, 2005) and their commercial potential (Desai *et al.*, 1997, Banat *et al.*, 2000), still it strongly demands for a serious attempt for the strategies development in the direction of R&D to make it commercially attractive and cheaper product.

There is one more area related to biosurfactant production which is still in its infancy. Most of the biosurfactants have been isolated from petroleum or hydrocarbon contaminated soil, and successfully used for bioremediation. These bacteria include most of the species of *Bacillus* and *Pseudomaonas*. But there is a great scope in development of food grade biosurfactants which are non-toxic and hence can be applied in food and pharmaceutical products. Since Sikkim is rich in ethnic fermented food products and microbial diversity of these fermented foods are also very high. Considering this present study aims to isolate and identifty biosurfactants from the bacterial isolates of some of the fermented foods of Sikkim. From our literature survey, no such work has been carried out from the fermented foods of this region.

Aims and Objectives:

1. Isolation of *Bacillus sp.* and lactic acid producing bacteria from ethnic fermented food samples of Sikkim.
2. Qualitative screening of *Bacillus sp.* and Lactic acid bacteria (previously isolated from different sources) isolates for biosurfactant production.
3. Tentative identification of biosurfactant producers by biochemical methods.
4. Extraction and purification of biosurfactant.
5. Tentative characterization of biosurfactant with the help of Thin Layer Chromatography.

Chapter 2

Review of Literature

Chapter 2

Review Literature

Most of the surfactants are organic compounds which are amphiphilic in nature (i.e. it contains hydrophobic as well as hydrophilic groups both). The term *surfactant/surfactants* are a blend of *surface active agents* (Rosen *et al*, 2012). These compounds are called surface active compounds as they have the ability of lowering the surface tension and interfacial tension (Rosen *et al*, 2012). They also are characterized with the properties of making micro-emulsions and micelles between the two discrete phases of liquids (Fracchia *et al.*, 2012). In any surfactant the hydrophilic moiety is represented as head, on the other hand the tail designates its hydrophobic hydrocarbon chain of different length (Christofi N., 2002). Generally the surfactants are classified on the bases of the charges that they carry. So Surfactants are classified as anionic, cationic, non-ionic and zwitterionic, according to the ionic charge of the hydrophilic head of the molecule (Christofi N., 2002). The most common hydrophobic parts of chemically synthesized surfactants are paraffins, olefins, alkylbenzenes, alkylphenols and alcohols; the hydrophilic part is usually a sulphate, sulphonate or carboxylate group in anionic surfactants, quaternary ammonium group in cationic surfactants and polyoxyethylene, sucrose or a polypeptide in nonionic surfactants (Volkering F. *et al.*, 1998). An important factor that determines the characteristic of surfactant is the balance between their hydrophobic and hydrophilic moieties. Therefore surfactants are also be classified on the bases of their Hydrophile-Lipophile Balance (HLB) (Tiehm A., 1994). The HLB value indicates whether a surfactant will produce a water-in-oil or oil-in-water emulsion: emulsifiers with a lower HLB value of 3-6 are lipophilic and promote water-in-oil emulsification, while emulsifiers with higher HLB values between 10 and 18 are more hydrophilic and promote oil-in-water emulsions (Desai *et al.*, 1997). Classification of surfactants on the bases of their HLB values is useful for evaluating its application for various purposes. For example, it has been reported that the most successful surfactants in washing oil contaminated soils are those with a HLB value above 10 (Volkering F. *et al.*, 1998).

As the name suggests and due to their chemico-physical structure, "surfactants" partition preferentially at the interface between phases with different degrees of polarity and hydrogen bonding such as oil/water and air/liquid interfaces (Franzetti A. *et al.*, 2010). As the interfacial tension of the solution reduces, the molecules of the surfactants deposit at the interface of the two immiscible solvents. A number of different surfactants, both synthetic and of biological origin, are able to reduce the surface tension of water from 72 mN m-1 to 27-30 mN m-1 (Christofi N. *et al.*, 2002; Desai *et al.*, 1997). In the presence of a non-aqueous phase liquid (NAPL), the surfactant molecules also aggregate at the liquid-liquid interface, thus reducing the interfacial tension (Volkering F. *et al.*, 1998).

Forming micelles is another important property of a surfactant. It is the most important providing it dispersing properties and detergency characteristic to these compounds. When dissolved in water in very low concentrations, surfactants are present as monomers (Franzetti A. *et al.*, 2010). In such conditions, the hydrophobic tail, unable to form hydrogen bonding, disrupt the water structure in its vicinity, thus causing an increase in the free energy of the system (Franzetti A. *et al.*, 2010). At higher concentrations, when this effect is more pronounced, the free energy can be reduced by the aggregation of the surfactant molecules into micelles, where the hydrophobic tails are located in the inner part of the cluster and the hydrophilic heads are exposed to the bulk water phase (Franzetti A. *et al.*, 2010). The concentration above which the formation of micelles is thermodynamically favoured is called Critical Micelle Concentration (CMC) (Haigh, S.D., 1996). The number of molecules necessary to form a micelle generally varies between 50 and 100; this is defined as the aggregation number. As a general rule, the greater the hydrophobicity of the molecules in the aqueous solution, the greater is the aggregation number (Rosen M.J., 1989).

CMC is commonly used to measure the efficiency of a surface active agent ((Desai *et al.*, 1997). The value of CMC depends on various factors in water solution. At soil temperatures, the CMC typically varies between 0.1 and 1 mM (Volkering F. *et al.*, 1998). The size of the hydrophobic region of the surfactant is particularly important for the determination of the CMC: in fact the CMC decreases with increasing hydrocarbon chain length, i.e. increasing hydrophobicity (Franzetti A. *et al.*, 2010). The addition of a

CH2- group to the chain has been shown to decrease the CMC by a factor of 3, according to the Traube's rule (Fan A. *et al.*, 1997).

However, anionic surfactants have higher CMCs than nonionic surfactants even when they share the same hydrophobic group (Franzetti A. *et al.*, 2010). Electrolytes can reduce the CMC in solution by shielding the electrical repulsion among the hydrophilic heads of the molecules; such effect is more pronounced with anionic and cationic surfactants than with nonionic compounds (Haigh, S.D., 1996). If the concentration of the surfactant in water increased above the CMC, it enhances the more micelles formation. The formation of micelles leads to a significant increase in the apparent solubility of hydrophobic organic compounds, even above their water solubility limit, as these compounds can partition into the central core of a micelle (Franzetti A. *et al.*, 2010). The effect of such a process is the enhancement of mobilization of organic compounds and of their dispersion in solution (Perfumo *et al.*, 2010). This effect is also achieved by the lowering of the interfacial tension between immiscible phases. In fact, this contributes to the creation of additional surfaces, thus improving the contact between different phases (Christofi N. *et al.*, 2002).

The reduction effect of interfacial tension is particularly relevant when the pollutant is present in soil as a non-aqueous phase liquid (Franzetti A. *et al.*, 2010). In summary, the main surfactant mediated mechanisms which may potentially enhance hydrophobic organic compound remediation include the reduction of interfacial tension, micellar solubilization and phase transfer between soil particles and the pseudo-aqueous phase (Franzetti A. *et al.*, 2010).

2.1 Biosurfactants or bio-surface active compounds

Many microorganisms are able to produce a wide range of amphipathic compounds, with both hydrophilic and hydrophobic moieties present within the same molecule which allow them to exhibit surface activities at interfaces and are generally called biosurfactants or bioemulsifiers (Fracchia L. *et al.*, 2012). Biosurfactants or bio-surfactant active compounds are classified on the bases of their molecular weight, mode of action or general physio-chemical properties.

In literatures, the terms 'biosurfactants' and 'bioemulsifiers' are often used interchangeably, however in general those that reduce surface and interfacial tension at gas-liquid-solid interfaces are called biosurfactants and those that mainly reduce the interfacial tension between immiscible liquids or at the solid-liquid interfaces leading to the formation of more stable emulsions are called bioemulsifiers or bioemulsans (Fracchia L. *et al.*, 2012). The former group includes low molecular-weight compounds, such as lipopeptides, glycolipids, proteins, while the latter includes high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins (Smyth *et al.*, 2010a, 2010b).

In heterogeneous systems, biosurfactants tend to aggregate at the phase boundaries or interfaces. They form a molecular interfacial film that affects the properties (surface energy and wettability) of the original surface (Fracchia L. *et al.*, 2012). This molecular layer, in addition to lowering the surface tension in liquids, also lowers the interfacial tension between different liquid phases on the interfacial boundary existing between immiscible phases and therefore can have an impact on the interfacial rheological behavior and mass transfer (Fracchia, L. *et al.*, 2012).

When the biosurfactant compounds are introduces to a solid-liquid or liquid-vapor mixture, its hydrophobic tail faces to the hydrophobic phase (i.e. Hydrophobic solvent as oil), and hydrophilic head faces to the hydrophilic solvent (as water or polar solvent). Due to that it appears at the interphase of the two solvent systems. Their diverse functional properties namely, emulsification, wetting, foaming, cleansing, phase separation, surface activity and reduction in viscosity of heavy liquids such as crude oil, make them suitable for utilization for many industrial and domestic application purposes (Gautam 2005; Franzetti *et al.*, 2010a; Perfumo *et al.*, 2010a; Satpute *et al.*, 2010).

During the past three decades biosurfactants have been under continuous investigation as a potential replacement for synthetic surfactants and are expected to have several industrial and environmental applications mainly related to detergency, emulsification, dispersion and solubilization of hydrophobic compounds (Banat *et al.*, 2000). In addition, biosurfactants present several advantages over surfactants of a chemical origin, particularly in relation to their biodegradability, environmental compatibility, low toxicity, high selectivity and specific activity at extreme temperatures, pH and salinity

(Banat 1995a, 1995b). Due to all these properties, they have steadily gained increased significance in industrial and environmental applications such as bioremediation, soil washing, enhanced oil recovery and other general oil processing and related industries (Perfumo *et al.*, 2010b). Furthermore, potential commercial applications in several other industries including paint, cosmetics, textile, detergent, agrochemical, food and pharmaceutical industries begin to emerge (Banat *et al.*, 2000).

A number of research groups have explore the divers properties of biosurfactants or bioemulsifiers which reveals various interesting biological and chemical properties that express its great potential in prophylactic, therapeutic and biomedical fields.

2.2 Classification of biosurfactants

Large variety of microorganisms produces vast variety of structurally diverse group of surface active compounds. For the convenience of studies the biosurfactants are classified on the bases of its molecular weight and structural diversity. They are generally composed of a hydrophilic part, which may be amino acid or peptide anions or cations, mono- or polysaccharides, and a hydrophobic part consisting of saturated, unsaturated or fatty acids (Desai *et al.*, 1997). According to a classification proposed by Neu (Neu, 1996), the term "biosurfactants" should be correctly used to identify low-molecular-weight microbial surfactants. In contrast, high-molecular-weight polymers can be collectively defined as bioemulsifiers (Rosenberg,

et al., 1997) also otherwise known as bioemulsifiers (Smyth *et al.*, 2010a; Smyth *et al.*, 2010b). In fact, the former group includes molecules which efficiently lower surface and interfacial tension, while the latter is composed of amphiphilic and polyphilic polymers which are more effective in stabilizing oil-in-water emulsions but do not lower the surface tension as much (Franzetti A. *et al.*, 2010). The low-molecular-weight biosurfactants are generally glycolipids, such as rhamnolipids, trehalose lipids, sophorolipids and fructose lipids, or lipopeptides, such as surfactin, gramicidin S and polymixin (Smyth *et al.*, 2010a). The high molecular-weight bioemulsifiers are amphiphilic or polyphilic polysaccharides, proteins, lipopolysaccharides and lipoproteins (Smyth *et al.*, 2010a).

2.2.1 Low molecular weight surface active compounds

As it is mantioned previously that microbial surfactants are roughly classified as low molecular weight surface active compounds that have high efficiency of reducing the surface and interfacial tension (known as biosurfactants) and high molecular weight surface active compounds that have low capacity of lowering the surface tension but high efficiency of stabilizing the emulsions (known as bioemulsifiers) (Neu, 1996; Rosenberg, 2006; Rosenberg *et al.*, 1997, 2010).

Most of the low molecular weight surface active compounds studied are either lipopeptides or glycolipids (Fracchia *et al.*, 2012). The major source of lipopeptides is the members of *Bacillus* family; but with respect to various species and strains these lipopeptides varies in their peptide's amino acid composition and fatty acid chains (Dastgheib *et al.*, 2008; Thavasi *et al.*, 2008, 2011). Some most studied glycolipids surface active agents are well known as Emulsan produced by *Acinetobacter calcoaceticus*, Sophorolipids which is produced by several species of yeasts, *Candida* and *Starmerella clade* (Kurtzman *et al.*, 2010, Parekh *et al.*, 2011) and *Pseudomaonas aeruginosa* is the producer of Rhamnolipid (Ito *et al.*, 1971).

2.2.1.1 Glycolipids surface active compounds:

Glycolipids are monosaccharides or disaccharides which are acylated with hydroxyl fatty acids or fatty acids long chain (Fracchia *et al.*, 2012). But most studied glycolipid biosurfactants are rhamnolipids, sophorolipids mannosylerythritol lipids (or MELs) and trehalolipids (Fracchia *et al.*, 2012; Ito. *et al.*, 1971; Kurtzman *et al.*, 2010; Parekh *et al.*, 2011).

2.2.1.1.1 Rhamnolipids:

Rhamnolipids are the glycosides which are mainly produced by *Pseudomaonas aeruginosa* and *Burkholderia species*. Rhamnolipids are mainly composed of one or two rhamnose sugars (knows as momo-rhamnolipids or dirhamnolipids respectively) which are linked to β-hydroxyfatty acids chains (Perfumo *et al.*, 2006; Raza, 2009). These biosurfactants show very high surface activity and a number of potentials in the biomedical fields as they possess antifungal, antibacterial, antiviral and antiadhesive

properties (Abalos *et al.*, 2001; Cosson *et al.*, 2002; Kim *et al.*, 2000; Sotirova *et al.*, 2008; Yoo *et al.*, 2005). Some application of rhamnolipids has been noticed in nano-partical preparation (Palanisamy *et al.*, 2009; Xie *et al.*, 2006) and microemulsions (Nguyen & Sabatini, 2009; Xie *et al.*, 2007).

2.2.1.1.2 Mannosylerythritol (MELs):

The major producers of MELs glycolipids are *Pseudozyma sp., Ustilago sp.* and some other species of certain yeasts and fungi from soybean oil or n-alkane (Arutchelvi *et al.*, 2010). MELs are a mixture of partially acylated derivative of 4-*O*-*β*-D-mannopyranosyl-D-erythritol, containing C2:0, C12:0, C14:0, C14:1, C16:0, C16:1, C18:0 and C18:1 fatty acids as the hydrophobic groups (Bhattacharjee *et al.*, 1970, as cited in Arutchelvi *et al.*, 2010). On the bases of degree of acetylation at C4□ and C6□ position MELs are classified as MEL-A, MEL-B, MEL-C and MEL-D (Arutchelvi *et al.*, 2010). The structures which are completely de-acetylated are categorized as MEL-D (Rau *et al.*, 2005, as cited in Arutchelvi *et al.*, 2010).

2.2.1.1.3 Sophorolipids:

Sophorolipids are also a type of extracellular glycolipid surface active compound which is mostly synthesized by *Candida bombicola, C. batistae, C. apicola, Rhodotorula boqoriensis, and Wickerhaminella domercqiae* (Van Bogaert *et al.*, 2010). Their composition includes two glucose molecules linked with β-1,2 glycosidic bonds. It has acetylated 6- and 6□ hydroxyl groups (Van Bogaert *et al.*, 2010). The lipid moiety is linked to the reducing end of glucose with glycosidic bond (Rosenberg, 1999). Major application of this surface active compound in biomedical field is because of its antifungal activity, antibacterial property, antiviral property and anticancer property, beside that it is also useful for the synthesis of metal-bound nanopaticales in the pharmacodermatological and cosmetic products (Van Bogaert *et al.*, 2010).

2.2.1.1.4 Trehalose lipids:

Trehalose lipids are also a type of glycolipids that contains threhalose sugar as hydrophilic moiety. Threhalose are non-reducing disaccharide sugar which is composed

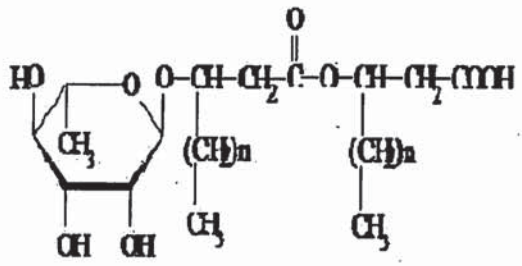
of two molecules of glucose linked with α, α-1,1-glycosidic bond (Franzetti *et al.*, 2010b). According to the most of the reports lrehalose 6,6-dimycolate, which represents the α branch chain of mycolic acid esterifies to C6□ position of the glucose. According to Lang and Philp (1998) there are various types of trehalose containing glycolipids which are produced by different mycolates group of microorganisms for example: *Nocardia, Arthrobacter, Gordonia* and *Rhodococcus* along with *Mycobacteria* and *Cornybacteria* (Franzetti *et al.*, 2010b). Their esterified fatty acids chain also varies in their number of carbon (C20-C90) (Lang *et al.,* 1998). Trehalose, not only have various industrial applications, but it is also well known for its cell membrane interaction potential and use in antitumor therapy (Harland *et al.*, 2009, Imasato *et al.*, 1990, Isoda *et al.*, 1995, as cited in Shao, 2010; Ortiz *et al.*, 2008, 2009; Zaragoza *et al.*, 2009, 2010).

2.2.1.2 Lipoprotein surface active agents:

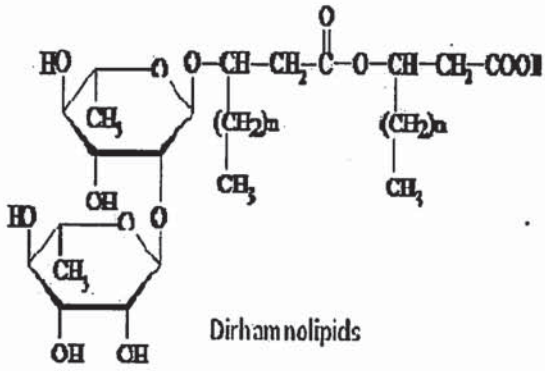
A number of cyclic lipopeptided, for example decapeptide antibiotics (gramicidins) and lipopeptides antibiotics (polymyxins), have been reported from many species of *Bacillus* and some other genera of microorganisms (Rosenberg *et al.*, 1999).

2.2.1.2.1 Surfactin:

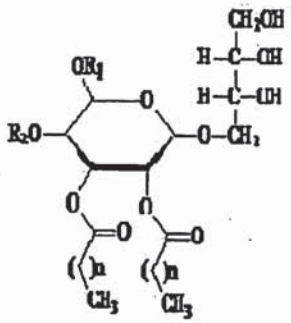
Surfactins have been reported as the most surface-active cyclic peptides, produced by *Bacillus subtilis* (Ron *et al.*, 2001). It was Arima *et al.*, (1968), who reported surfactin from the broth culture of *Bacillus subtilis* and because of its exceptional surface activity, it was named as surfactin (Peypoux *et al.*, 1999). Natural surfactins are the composition of various isoforms, as A, B, C, and D which are differs in their composition and sequence of amino acids (Shaligram *et al.*, 2010). Molecular structure and chemical composition of surfactin represents seven amino-acid ring structures, which is coupled to a fatty acid chain by lactonee linkage (Fracchia *et al.*, 2012). Surfactin-A has L-leucine, surfactin-B has L-valine and surfactin-C has L-isoleucine at the amino acid position involved in the lactonee ring formation with the C14–C15 β-hydroxy fatty acid (Fracchia *et al.*, 2012). However, the composition of amino acid residue may vary with respect to the culture conditions and carbon sources available in the media (Jacques, 2010).



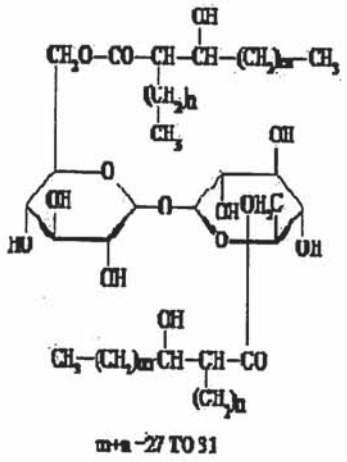
Monorhamnolipid



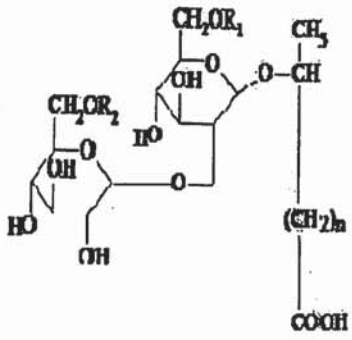
Dirhamnolipids



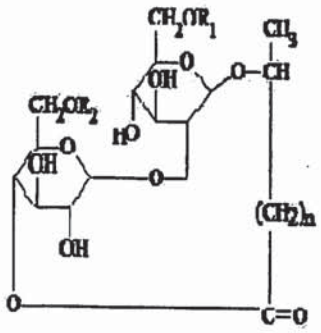
Mannosylerythritollipid



Trehalose dimycolate



Acidic Sophorolipid



Lactonic Sophorolipid

Fig: 1- Chemical structure of low molecular weight glycolipid microbial biosurfactants: Mono and di-rhamnolipids, Mannosylerythritollipid, Trehalose dimycolate, Acidic Sophorolipid, Lactoneic Sophorolipid. (Fracchia *et al.*, 2012)

2.2.1.2.2 Lichenysin:

Lichenysin is another surfactin related biosurfactant which was discovered in supernatant of *Bacillus licheniformis* broth culture (Horowitz *et al.*, 1990). Regarding to the chemical structure and physio chemical properties it resembles to surfactins (Mc Inerney *et al.*, 1990). Lichenysin posses Glutamine at position 1 while in case of surfactin Glutamic acid is found at the same position (Morikawa *et al.*, 1992; Naruse *et al.*, 1990).

2.2.1.2.3 Pumilacidin:

Pumilacidin is another surfactin like compound. Pumilacidin is also the complex of isoforms A, B, C, D, E, F and G. It is the complex of acylpeptide antibiotics, which are isolated form *Bacillus pumilus* culture and showing increasing antiviral activities (Morikawa *et al.*, 1992; Narse *et al.*, 1990).

2.2.1.2.4 Iturin:

Iturin A is the most studied compound in iturin family (Fracchia *et al.*, 2012). Iturin is a heptapeptide which is interliked with β-amino acid and carry fatty acid with C14 to C17 carbon chain (Peypoux, 1999). It is frequently isolated from *Bacillus subtilis* and a strong antifungal activity has been reported in it (Fracchia *et al.*, 2012).

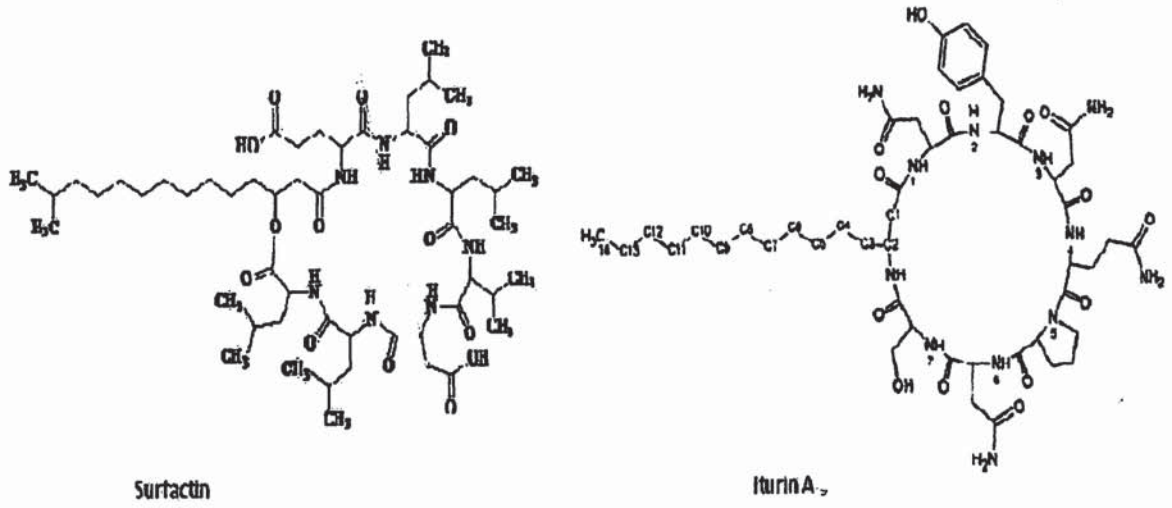


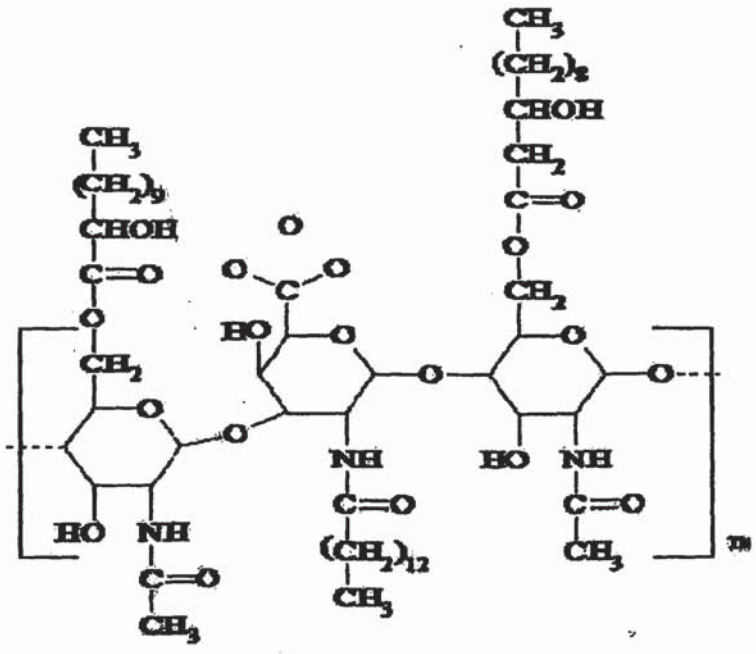
Fig: 2- chemical structure of low molecular weight lipopeptide/lipoproteins surface active compounds: Surfactin and Lturin A (Fracchia, L. *et al.*, 2012).

2.2.2 High molecular weight surface active compounds:

High molecular weight biosurfactants are generally designated as polymeric biosurfactants (Fracchia *et al.*, 2012). There are a number of bacteria which are observed to produce this category of compounds and their composition includes lipoproteins, polysaccharides, proteins, lipopolysaccharides and many other complex structures (Ron *et al.*, 2001; Rosenberg, 1997, 1999). Most of the biopolymers are reported as bio-emulsan (Rosenberg *et al.*, 1979). One of such lipopolysaccharide, which is isolated from *Acinetobacter calcoacetius* RAG-1 ATCC 31012, has molecular weight of around 1000 kDa (Rosenberg *et al.*, 1979).

2.2.2.1 RAG-1 Emulsan:

Major components of RAG-1 emulsan are protein and anionic heteropolysaccharide (Rosenberg, 1987, as cited in Rosenberg, 1999). 15% of the emulsan dry weight is its fatty acids which provide it high surface active property. This fatty acid chain is attached to polysaccharide backbone with the help of O-ester and N-acyl linkages (Rosenberg, 1987, as cited in Rosenberg, 1999).



Emulsan

Fig: 3- Chemical structure of high molecular weight biosurfactant: Emulsan (Fracchia *et al.*, 2012).

2.2.2.2 Alasan:

Alasan is another example of high molecular weight biosurfactant, which is the complex of proteins and anionic polysaccharides with molecular weight of 1000kDa (Smyth *et al.*, 2010b). It is isolated from *Acinetobacter rodioresistens* (Smyth *et al.*, 2010b). Alasan is also a good bioemulsifier like other high molecular weight surface active compounds (Smyth *et al.*, 2010b). Very little information is available about the high molecular weight biosurfactants; most of the papers report only the chemical composition of the crude and producing microorganism (Rosenberg *et al.*, 1999).

There are many high molecular biosurfactant which are effective at even high temperature though they have complex protein structures, for example biosurfactant isolated from *Methanobacterium thermoautotrophium* (Rosenberg *et al.*, 1999) and protein polysaccharide lipid complex isolated from *Bacillus stearothermophilus* ATCC1280 (Gunjar *et al.*, 1995, Rosenberg *et al.*, 1999). *Candida lipopytica* produces Liposan, which is an extracellular emulsifier (Rosenberg, 1999). Liposan is composed of carbohydrate 83% and protein 17% (Rosenberg *et al.*, 1999). Many of these biosurfactants represents high commercial potential in cosmatics, food and petroleum industries (Rosenberg, 1999).

2.3 Important terms related to biosurfactant:

Major terms associated to biosurfactants have been derived from the chemical detergents and soaps industries. Some of the important terms are given as followed-

2.3.1 Surfactant:

Surfactants are the compounds, which have ability to modify its chemical interaction with polar and non-polar liquids at the interphase of two immiscible liquid boundaries (Satpute *et al.*, 2010).

2.3.2 Emulsifier:

Those surface-active compounds that have ability to make emulsion between two immiscible liquids are called emulsifiers. It is not necessary that an emulsifier have efficiency to reduce the surface tension. Emulsifier can just bind to polar and non-polar

solvent with their respective polar and non-polar moieties in its molecule (Satpute *et al.*, 2010).

2.3.3 Surface tension:

Surface tension is the force per unit area exerted by a liquid in contact with a solid or other liquid. It can also be defined as the measurement of free energy per unit area associated with the surface or interface of the liquid (Satpute *et al.*, 2010).

2.3.4 Interfacial tension:

It is the intermolecular force of attraction within a liquid. Higher is the interfacial force of a liquid, lower the ability of the liquid to be emulsified (Satpute *et al.*, 2010).

2.3.5 Critical micelle concentration:

It represents the initial value of minimum surface tension while the micelle term is given by Mc Brian in 2013 (Mc Brian, 2013). Blow the critical micelle concentration surfactant molecules remain as monomer because the molecules remain loosely integrated in the water structure (Margaritis *et al.*, 1979). But when the concentration of the surfactant increase to the critical micelle concentration the surfactant and water integrate to form micelles, this also lead to the change in certain physical properties of the solution like conductivity, density, viscosity, turbidity, osmotic pressure and chemical shifts (Margaritis *et al.*, 1979).

2.3.6 Hydrophilic and lipophilic balance:

This term is denoted by Griffin (1946, 1954) for nonionic surfactants. It represents the relative simultaneous attraction of any emulsifier for the two different phases of oil and water system (Attwood *et al.*, 1987). The value of hydrophilic and lipophilic balance is expressed by arbitrary scale with value from 0-20, with respect to the most hydrophilic materials having highest value (Attwood *et al.*, 1987). Hydrophilic and lipophilic balance us useful to represent the relative ability of a surfactant to form emulsion of water in non-polar solvent (oil) or oil in water by comparing with the surfactants of known hydrophilic and lipophilic balance value and relative properties (Attwood *et al.*, 1987).

2.3.7 Emulsion:

Emulsion can be defined as the colloidal suspension of a liquid with another immiscible liquid (for example oil and water) (Satpute *et al.*, 2010).

2.3.8 Microemulsion:

When two immiscible liquids (polar and non polar solvents) are dissolved vigorously one of the solvent forms microscopic droplets dispersed in another liquid, this type of colloidal dispersion is called as microemulsion (Satpute *et al.*, 2010). Such microemulsion stabilized in the system due to its thermodynamic energy (Goma *et al.*, 1973). Here the size of the droplet is about 10nm to 100nm. This property of forming microemulsion reduces the interfacial tension of the liquid (Goma *et al.*, 1973).

2.4 General properties of Bio-surface active agents:

The properties of varies surfactants differs with respect their size of hydrophilic and hydrophilic functional groups. This is because various surfactants are explored to different applications in different industries as detergents, cosmetics, agriculture, oil recovery, mining and pharmaceutics (Desai, 1997; Jagtap *et al.*, 2009).

2.4.1 Surface and interfacial tension:

Surface tension and interfacial tension are the primary properties of any surfactant. As it is known to all that water molecules are hold together by intermolecular cohesive force. This cohesive force of attraction on the surface of the liquid creates a tension that binds the water molecules together. This tension on the surface of liquid is called surface tension of the liquid (Satpute *et al.*, 2010). Distilled water has the highest surface tension with value of 72mN/m (Thaniyavarn *et al.*, 2003). When surfactant is added to the water or any other liquid its surface tension and interfacial tension reduces (Thaniyavarn *et al.*, 2003). One biosurfactant unit can be defined as the amount of biosurfactant needed for forming 1 cm^3 of oil displaced area (Thaniyavarn *et al.*, 2003). Surfactin is a type of biosurfactant, which can reduce the surface tension of water most efficiently from 72 dynes/cm to 27 dynes/cm (Cooper *et al.*, 1987; Banat, 1993).

2.4.2 Emulsification:

When two immiscible liquids are mixed together with vigorous shaking, the drops of one liquid are dispersed in another liquid. It shows the microcellular solubilization while the resultant solubilized particles are bigger in size (Satpute *et al.*, 2010).

2.4.3 Wetting:

It is the penetrating power and spreading power of a substance of the biosurfactant due to which surface tension of the liquid reduces (Satpute *et al.*, 2010). It reduces the cohesive forces between the similar molecules of the liquid and increases the attraction force between the unlike surface of the liquid and solid (Pastewski *et al.*, 2006).

2.4.4 Foaming property:

It is already mentioned that surfactants have property to concentrate at the interface of polar and non-polar liquids or liquid and gas surface. When the surfactant concentrates at the interface surface of liquid and gas it forms bubbles through the liquid this lead to the foam formation (Dubey *et al.*, 2005).

2.4.5 Adsorption:

Because of this property, surfactants are adsorbed on the hydrophobic surface (Satpute *et al.*, 2010). Mei, Mather, and Fotheringham in 2005 first of all recovered Biosurfactant JBR215 rhamnolipid using this technique for the Jeneil BS Company, USA (Wei *et al.*, 2005). Till that time about 95% of the biosurfactants have been isolated through this technique. Adsorption property of the biosurfactants provides the biosurfactant the potential for oil recovery (Curbelo *et al.*, 2007). Due to the adsorption property the surfactant interacts with rock so firmly, which enhances the oil recovery from the rock (Curbelo *et al.*, 2007).

2.4.6 Dispersion:

Dispersion is the property due to which the attraction force of cohesion reduces between the similar molecules of the solvent (Satpute *et al.*, 2010). This property of the surfactant allows the insoluble particles remain in suspension in any solvent (Satpute *et al.*, 2010).

This property of the biosurfactant also allows desorption of hydrophobic substance from the rock by enhancing the mobility, so it shows high application in oilfield chemistry (Satpute *et al.*, 2010).

2.4.7 Detergency:

Detergency represents the cleansing property of the surfactant. Biosurfactants shows the cleansing property like the chemical surfactants (Satpute *et al.*, 2010).

2.4.8 Flocculation:

Flocculation is the process in which the emulsion droplets stick together to form a cluster. This of emulsion can be broken by the mechanical forces to restoring the emulsion again (Satpute *et al.*, 2010). Optical microscopy and micro-electrophoresis are the major techniques to study the flocculation (Satpute *et al.*, 2010).

2.4.9 Phase separation:

When two different immiscible liquids are mixed together, it forms emulsion (Satpute *et al.*, 2010). But when it is kept undisturbed for some time, after a short period, the droplets assembles together again and forms two different phases of different liquids (Satpute *et al.*, 2010). This process of the liquid is called phase separation (Satpute *et al.*, 2010).

2.4.10 Solubilization:

Surfactant has also efficiency to solubilize the insoluble substance in a solvent. At high concentration of the surfactant it forms micelles in the solvent, which encapsulates the insoluble particles of the solute and increase the solubility of the solvent for the respective solute. Biosurfactants and bioemulsifiers are the more successful solubilizing agent than any synthetic surfactants (Perfumo *et al.*, 2009, Wong *et al.*, 2004).

2.5 Advantages of biosurfactants

Biosurfactants represents many advantages over synthetic surfactants or detergents.

2.5.1 Biodegradability:

As compare to the synthetic surfactants biologically originated surfactants are easily degraded (Mohan *et al.*, 2006). That is why it is suitable for the application of bioremediation (Mulligan, 2005). In a comparative study of six biosurfactant and synthetic surfactants regarding to its degrading property, dispersion property and toxicity, it was found that most of the biosurfactant have faster biodegradability and stronger dispersion property than commercial dispersants and synthetic surfactants (Kapadia *et al.*, 2013).

2.5.2 Low Toxicity:

There is less data reported that shows its toxicity of the microbial biosurfactants therefore it is consider as nontoxic or very low toxic compound, hens it is useful in food industry and pharmaceuticals (Kapadia *et al.*, 2013). Corexit, which is a synthetic anionic surfactant displays 50% lethal dose against *Ptotobacterium phosporium* (Kapadia *et al.*, 2013). This is about 10 times lower than rhamnolipids. It is also a batter dispersant as it shows to decrease effective concentration up to 50% of the test sample (i.e. EC50) against synthetic dispersants (Flasz *et al.*, 1998, Poremba *et al.*, 1991). Morlon A-350 (a synthetic surfactant) is used with biosurfactant isolated from *Pseudomaonas aeruginosa* in many industries. Many research shows that Marlon A-350 is highly toxic and mutagenic than biosurfactant (Flasz *et al.*, 1998).

2.5.3 Availability of raw material:

A lot of work has been done to produce high quantity of biosurfactant with low cost easily available raw material. There are a number of vegetable oil and other plant derived oils, like rapeseed oil (Kosaric, 2001), Babassu oil (Trummler *et al.* 2003, Vance-Harrop *et al.* 2003) and corn oil, are very useful as raw material for the production of biosurfactant at very low cost (Pekin *et al.*, 2005, Rahman *et al.*, 2002, Ferraz *et al.*, 2002). We can get high production of rhamnolipids, mannosylerythritol lipid and sophorolipid with soy bean oil and sunflower oil as raw material (Pekin *et al.*, 2005, Rahman *et al.*, 2002, Ferraz *et al.*, 2002). The waste product of potato industry is the

good source of starch so it is also used as the cheap raw material for biosurfactant production (Thompson *et al.*, 2000; Thompson *et al.*, 2001; Noah, 2005; Noah, 2002).

2.5.4 Physical factors:

There are a number of biosurfactants have been reported that are highly stable at high temperature, low and high pH and ionic strength. Biosurfactant produced by *Bacillus licheniformis* (known as Lichenysin) can sustain at 50°C, pH upto 2-9 and NaCl concentration about 50g/l (Noah, 2005).

2.6 Biosynthesis and molecular regulation of biosurfactants

Biosurfactants represents large variety of chemical differences in its structure, for example lipopeptides (Arima *et al.*, 1968; Gurjar *et al.*, 1995; Hasumi *et al.*, 1995; He *et al.*, 2001; Lee *et al.*, 2007; Morikawa *et al.*, 1993; Rahman *et al.*, 2006; Thaniyavarn *et al.*, 2003; Tran *et al.*, 2007; Trischmann *et al.*, 1994; Yakimov *et al.*, 1995), glycolipids (Benincasa *et al.*, 2002; Guerra-Santos *et al.*, 1984; 1986; Hisatsuka *et al.*, 1971; Kitamoto *et al.*, 1990a; Kobayashi *et al.*, 1987; Mercade *et al.*, 1993; Morita *et al.*, 2007; Patel *et al.*, 1997; Raza *et al.*, 2007; Robert *et al.*, 1989; Wu *et al.*, 2008;), flavolipids (Bodour *et al.*, 2004), and polymeric biosurfactants (Deshpande *et al.*, 1995; Franzetti *et al.*, 2008; Panilaitis *et al.*, 2006; Shabtai, 1990).

Though there are a number of biosurfactants has been reported, but most intensive studies has been conducted on rhamnolipids and surfactin, as well these are the two biosurfactants, which molecular aspect of biosynthesis has been studied (Das *et al.*, 2008). But up to this time there are a number of other biosurfactants which biosynthesis and molecular control mechanisms has been studied, for example arthrofactin (produced *by Pseudomaonas sp*), lichenysin and iturin has been isolated form *Bacillus sp.*, mannosylerythritol lipids (from *Candida*) and emulsan (from *Acinetobacter sp.*) (Das *et al.*, 2008). There is a list of some other biosurfactants like alasan, amphisin, hydrophobin, lokisin, putisolvin, serrawettin, tensin and viscosin etc which molecular mechanism of biosynthesis is still unknown (Das *et al.*, 2008). It also has been reported that in case of some bacteria, quorum sensing play an important role to express the gene for the synthesis of certain biosurfactants (Daniels *et al.*, 2004). These bacteria actually produce

certain low molecular weight signaling molecules that regulates the synthesis of the biosurfactant (Daniels *et al.*, 2004).

2.6.1 Molecular mechanism of biosynthesis of glycolipids biosurfactant:

The credit of glycolipid biosurfactant discovery was marked to Jarvis and Johnson in 1949, when they have isolated rhamnolipids and reported its properties similar to chemical surfactants and detergents (Jarvis *et al.*, 1949). They have observed the similarities between his compounds and polymer and higher rhamnose hydroxyacid ratio which were previously isolated by Bergstrom *et al* in 1947 (Bergstrom *et al.*, 1947). Finally Hauser and Karnovsky discovered the biosynthetic pathway of mono as well as di-rhamnolipids from *Pseudomaonas aeruginosa* (Hauser *et al.*, 1957; Burger *et al.*, 1957).

2.6.1.1 Rhamnolipid biosynthesis by *Pseudomaonas spp.*

However, the rhamnolipid has been discovered in 1949 but its process of biosynthesis has been discovered in the end of 20th century by Ochnser and his co-workers (Ochsner *et al.*, 1994a; Ochsner *et al.*, 1994b) and Latifi with his team independently (Latifi *et al.*, 1995). They have explained the role of quorum sensing system in biosynthesis of rhamnolipid from *Pseudomaonas sp* (Satoyte *et al.*, 2010). There are two quorum sensing system regulates the biosynthesis of rhamnolipid and their genes are located at the two different sites of the chromosomes (Stover *et al.*, 2000). Mono-rhamnolipids formation is regulated by rhamnosyltranferase I while the formation of di-rhamnolipid synthesis is mediated by rhamnosyltransferase II (Stover *et al.*, 2000). Regulation of rhamnolipid synthesis is associated with nitrogen (Mulligan *et al.*, 1989) and phosphate (Bazire *et al.*, 2005) limitation in the cell.

Deep studies shows that rhamnosyltransferases I protein is coded by four genes, which are designated as *rhlA, rhlB, rhlR, rhlI* (Ochsner *et al.*, 1995a). *rhlA* and *rhlB* genes are located at the upstream of the structural genes while the *rhlR* and *rhlI* are positioned on the downstream to the structural genes (Ochsner *et al.*, 1995a). Here *rhlA* and *rhlB* codes for rhamnosyltransferase I, so these two genes transcribe bi-cistronic RNA (Ochsner *et al.*, 1994a; 1994b; Ochsner *et al.*, 1995a). *rhlA* codes for structural protein which is

present in periplasm and *rhlA* code for the inner membrane proteins which is essential for the synthesis of rhamnolipids transport and solubilization of the rhamnosyltransferases (Ochsner *et al.*, 1995b).

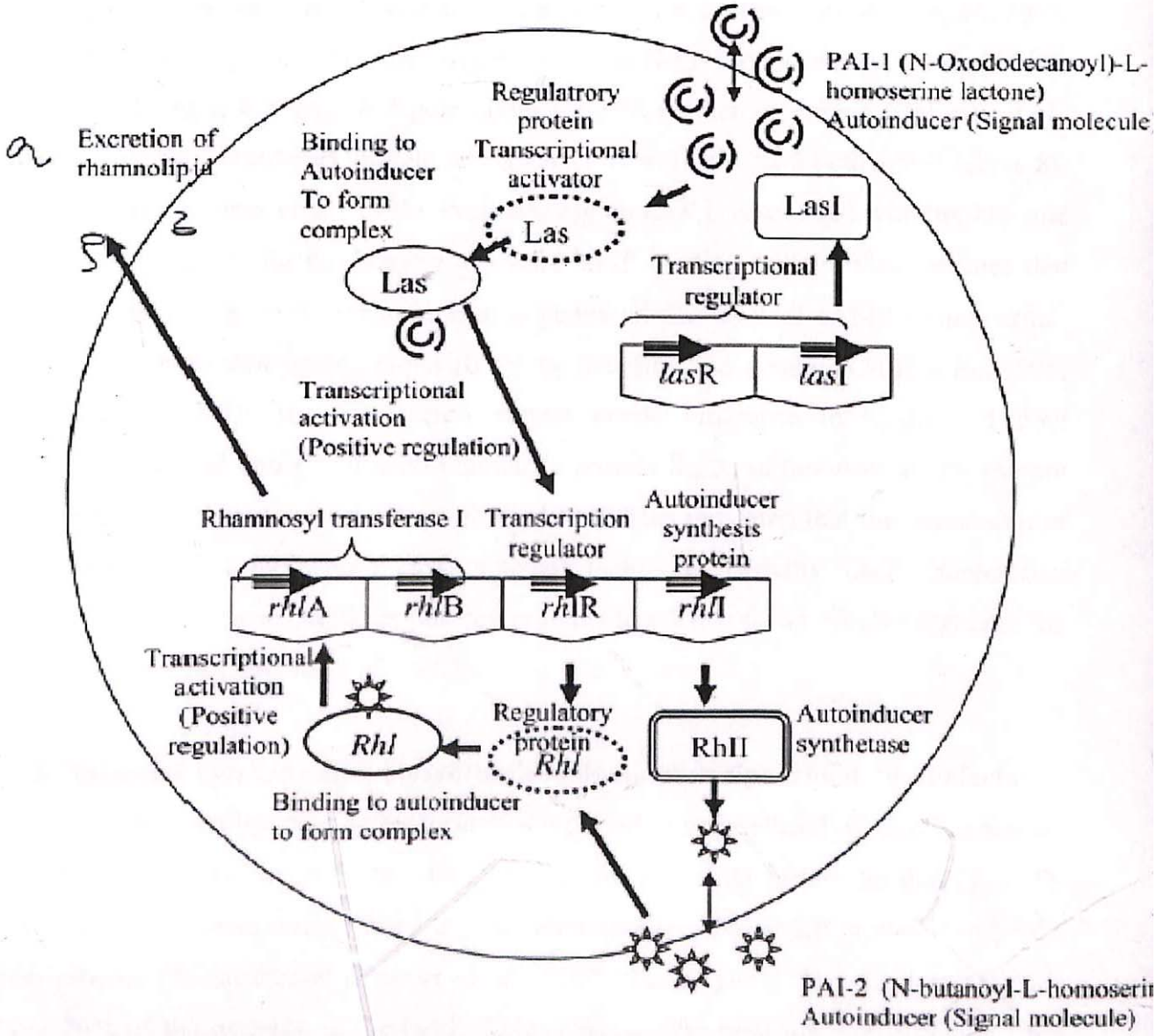


Figure:4- Digramatic presentation of two quorum sensing system for the synthesis of Rhamnolipid: representing signal transduction to activate the genes at two different sites of the chromosomes. Here thick black bold arrows show genes on chromosome of *Pseudomaonas*; Black arrows: protein synthesis for gene; dotted oval indicates regulatory protein; continuous oval: Active complex of regulatory protein and auto-inducer (Lang *et al.*, 1999; Bodour *et al.*, 2002; Sullivan, 1998) Fig is adapted from (Satput *et al.*, 2010).

In first quorum sensing system, rhlR protein works as positive regulator for *rhlA* and *rhlB* genes (Satoyte *et al.*, 2010). Transcriptional activator is encoded by *rhlR* gene and *rhlI* encodes autoinducer (Satoyte *et al.*, 2010). *rhlI* codes for the signaling molecules i.e. N-butanoyl-L-homoserine (PAI-2) and hexanoyl-1-homoserine lactonee (Satoyte *et al.*, 2010).

Transcriptional activator (produced by rhlR) binds to N-butanoyl-L-homoserine (i.e. PAI-2), and activation of rhlR (transcriptional activator) lead to the activation of structural genes rhlA and rhlB for the transcription of rhamnosyltransferase I (Satoyte *et al.*, 2010). Second quorum sensing system deals with two genes known as *lasR* and *lasI* (Pesci *et al.*, 2007; Lazdunski *et al.*, 2004). Here autoinducer named as N-(3-oxododecanoyl)-HSL (PAI-1) is encoded by gene *lasI* and *rhlR* gene codes for rhlR regulatory protein (Ochsner *et al.*, 1995b). This rhlR regulatory protein needs PAI-1 as well as PAI-2 both autoinducers for its activation (Ochsner *et al.*, 1995). Promoter region *lasR* is associated with lux-box and consensus sequences for the binding of cyclic AMP binding protein; this indicates that quorum sensing system 2 activation also depends on the level of cAMP (Albus *et al.*, 1997). The whole *rhlR* operon is positively regulated by *Las* system (Latifi *et al.*, 1996; Pesci *et al.*, 1997). The *las* operon system create hindrance in binding of both autoinducers PAI-2 and PAI-1 with regulatory protein RhlR, in this way the *las* system controls the *rhl* operon system (Pesci *et al.*, 1997). This indicated that the expression of *RhlR* is influenced by certain environmental factors in partially LasR independent conditions, beside those certain regulatory proteins like Vfr σ factor 54 also regulates the expression of *rhlR* (Medina *et al.*, 2003).

2.6.2 Molecular mechanism of biosynthesis of lipopeptide/lipoprotein biosurfactant:

Surfactin is the first lipopeptide biosurfactant reported to be produced by *Bacillus* species (Arima *et al.*, 1968). Surfactin has the efficiency to reduce the surface tension up to 72-27dyns/cm and shows critical micelle concentration very low which proves it to be the most effective biosurfactant (Cooper *et al.*, 1987; Banat, 1993). For the last 45 years about 70% of the research in the field of biosurfactant has been done on surfactin from *Bacillus sp.* for exploring the detail information about surfactin production, structural details, enzymes responsible for biosynthesis and genetic regulation (Peypoux *et al.*, 1999). Research on molecular and genetic regulation of surfactin production remain halted till 1988 but with the foundation of the molecular studies proposing non-ribosomal protein synthesis for surfactin biosynthesis provide the great success in understanding the whole mechanism (Kluge *et al.*, 1988).

2.6.2.1 Surfactin biosynthesis:

Surfactin is biosurfactant produced by *Bacillus sp.*, contains β-hydroxyl fatty acid, mostly β-hydroxytetradecanoic acid, which is synthesized by *srfA* operon of 27kb (Satput *et al.*, 2010). Like glycolipid biosurfactants biosynthesis, surfactin synthesis is also regulated by quorum sensing system (Satput *et al.*, 2010). This quorum sensing system regulates unique non-ribosomal protein synthesis mechanism, which is operated by four open reading frames of *srfA* operon system (Fabret *et al.*, 1995; Cosmina *et al.*, 1993).

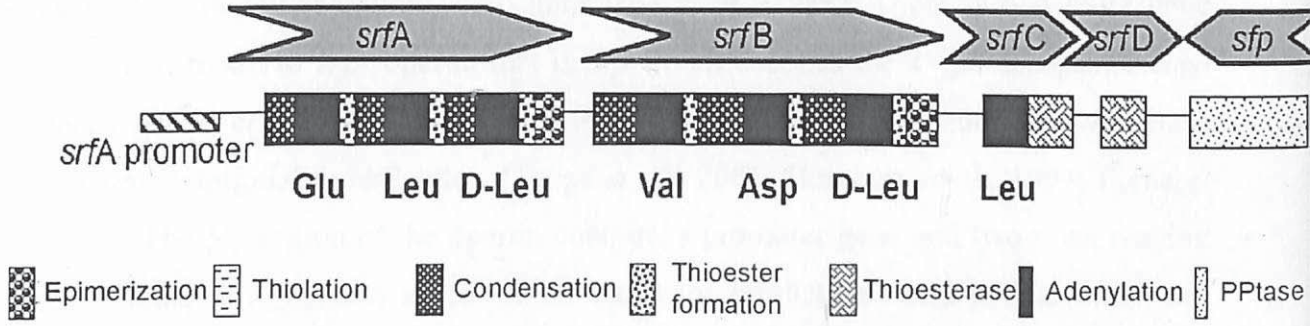


Figure: 5-Structural organization of genes which encods various modular domains of surfactin synthetase. (Das *et al.*, 2008).

The *srfA* operon regulates the transcription of RNA for three important multifunctional enzymes essential for surfactin synthesis (Cosmina *et al.*, 1993). These three genes on *srfA* operon are represented as *srfA, srfB* (now known as *ComA*) and *srfC* which synthesize the three different modular proteins and all the three these modular proteins together called as surfactin synthetase (Nakano *et al.*, 1994).

Each module of surfactin peptide synthetase is constructed with different domains and each domain incorporates and modifies one of the specific amino acid to the peptidyl moiety in surfactin (Shaligram *et al.*, 2010). The module consists of the co-linear sequences with respect to the amino acid sequence of the peptidyl product (Shaligram *et al.*, 2010). The module is made of three domains, where first one is adenylation domain which adenylates the cognate amino acid from adenosine tri-phosphate (ATP) and forms aminoacyl adenosine and pyrophosphate is released (Reuter *et al.*, 1999). Second domain is responsible for providing covalent linkage between activated amino acid and 4-phosphopantatheinyl prosthetic group of the peptidyl carrier protein with the help of thioester linkage (Reuter *et al.*, 1999). Third domain is conceder as the condensation

domain, which catalyzes the condensation of thioesterified intermediates of the modified amino acid to the peptidyl chain of the surfactin (Reuter *et al.*, 1999).

Here *srfA* gene codes for the template enzyme for surfactin synthesis (Nakano *et al.*, 1994). There are four open reading frames in *srfA* region named as *SrfAA, SrfAB, SrfAC* and *SrfAC* (also known as *SrfA-TE*) (Das *et al.*, 2008). Either in or out of the frame of *srfB* there is one more gene, which is designated as *ComS* (Das *et al.*, 2008). Till this date the exact function of *srfAD* is not well understood but it is though that this gene takes part in the process of loctonization (Venkataramana *et al.*, 1989). There is one more gene located downstream to *srfA* operon that is *Sfp* which encodes the 4′- phosphopantotheinyl transferase (Hsieh *et al.*, 2004). *Sfp* gene essential for the activation of surfactin synthetase by post transcriptional modification (Tsuge *et al.*, 2001; Borchert *et al.*, 1994; Fuma *et al.*, 1993). The 5□ region of the operon contains a promoter gene and two open reading frames marked as *srfAA* and *srfAB* which codes for synthetase I and II (Nakano *et al.*, 1988). The region *srfAA* carries genes that codes for the domains which need for activating Glu, Leu and Val, on the other hand *srfAB* synthesize the peptide domain that activates Val, d-Leu and Asp (D'Souza *et al.*, 1993). The *srfC* gene codes for the activation domain for Leu (Galli *et al.*, 1994). This region also codes for the thioesterase type I enzyme that needs for the termination of the peptide (De Ferra *et al.*, 1997).

The *srfB* gene in third locus of *srfA* operon is also essential for the synthesis of surfactin (Satput *et al.*, 2010; Das *et al.*, 2008). *ComA* gene regulates the *srfA* expression at transcriptional level (Das *et al.*, 2008). The product of *comA* functions as the positive regulator for *srfA* (Nakano *et al.*, 1991; 1989). Whereas the *srfD* is responsible in stimulating the initiation process (Steller *et al.*, 2004). It is still a mystery "how the surfactin release from the cell?" (Satput *et al.*, 2010). But it is assumed that the surfactin is released from the cell membrane by passive diffusion (Stein, 2005). As the bacterial cells in the medium reaches to the maximum density, the level of ComX in the medium becomes so high that it interacts with histidine kinase ComP (a membrane bound protein) and ComA (a response regulator) (Hamoen *et al.*, 2003). ComP activates the ComA by phosphorylating it then active ComA binds to promoter srfA to start the transcription (D'Souza *et al.*, 1994). Along with this system there are certain other proteins like ComR and SinR which influence the expression of *srfA* (Liu *et al.*, 1996).

ComP functions as positive regulator as well as negative regulator for ComA, while ComP itself is regulated by ComX (Cosby *et al.*, 1998).

2.6.2.2 Lichenysin biosynthesis

Lichenysin is a lipopeptide biosurfactant which is synthesized by *Bacillus licheniformis* JF2 strain (Yakimov *et al.*, 1995). Genetic composition of the lichenysin has been studied intensively and it is found that it shows the great homology with surfactin (Das *et al.*, 2008). So it is assumed that biosynthesis of surfactin and lichenysin follows almost similar metabolic pathway (Das *et al.*, 2008). Like surfactin lichenysin is also synthesized by non-ribosomal multienzyme peptide synthetase complex (Das *et al.*, 2008). The most studied lichenysin A synthetase is also synthesized by operon system which possess seven amino acid activation thiolation domains, two epimerization domains and one thioesterase domain same as surfactin (Yakimov *et al.*, 1998). The lichenysin synthesis operon carries three genes for peptide synthesis which are designated as *lic*A, *lic*B and *lic*C which are transcribed in same direction (Marahiel *et al.*, 1999). According the sequencing studies of *lic* operon in *B. licheniformis* it is 26kb long which is divided into three major genes *lic*A with three modules, *lic*B with three modules and *lic*C represents of one module (Yakimov *et al.*, 1998). These all genes and module shows high similarities with the genes and modular structures of the surfactin operon system (Yakimov *et al.*, 1998).

2.7 Natural role of biosurfactants

As it is already considered that there are a number of microorganisms which produces large variety of biosurfactants. These biosurfactants supports various growth and life processes of microorganisms (Fracchia *et al.*, 2012). As there is a large verity of biosurfactants produced by such a diverse range of bacteria so it is quiet difficult to generalize the role of biosurfactant in microbial physiology of bacterial cells (Fracchia *et al.*, 2012). Because of their large diversity in their chemical and physical properties different biosurfactant plays different role to supports the growth of the respective producer microorganism (Fracchia *et al.*, 2012). In case of certain bacteria biosurfactants helps in motility of bacterial cells by

gliding or swarming (Ron *et al.*, 2001; Van Hamme *et al.*, 2006). One of the well studied examples is *Serratia marcescens* which produces serrawettin (biosurfactant produced by *S. marcescens*), and serrawettin is important for accessing to water repelling surface and help in surface locomotion (Arutchelvi *et al.*, 2008). In the same the *Bacillus subtilis* produces surfactin which is important for the flagellar biosynthesis (Van Hamme *et al.*, 2006). In many cases biosurfactants regulates the attachment and detachment of microorganism on the surface of host or habitat (Van Hamme *et al.*, 2006).

There are strong evidences of biosurfactant role in cell to cell interaction for example microbial pathogenesis, biofilm formation and quorum sensing, cell maintenance and maturation (Fracchia *et al.*, 2012). For instance, *Pseudomaonas* produce rhamnolipids which is necessary to uphold the architecture of bacterial biofilm and provide it virulence factor too (Arutchelve *et al.*, 2008; Ron *et al.*, 2001; Van Hamme *et al.*, 2006). Surfactin, mannosylerythritol lipid and rhamnolipids represents antimicrobial properties so it confers an intra-species competitive property of the organism at the time of colonization (Arutchelve *et al.*, 2008). Beside that biosurfactant leads to cellular differentiation, increase the bioavailability of the substrate and attribute to provide the toxin resistance for the producer microorganism (Neu, 1996). The most remarkable role of biosurfactant is to help in interaction of microbial cells and insoluble hydrocarbon and other substrates (Neu, 1996). Biosurfactants are also very important as it increase the bioavailability by increasing the surface area and allow the growth of bacteria even on hydrophobic and water insoluble substrates (Neu, 1996 and Ron *et al.*, 2001; Van Hamme *et al.*, 2006).

2.2.8 Commercial potentials of biosurfactants:

According to the various reports of 2000, the amount of surfactant produced worldwide is about seventeen million metric tons and in future it is expected to be three to four percent per year globally (Rahman, 2008). As most of the chemical surfactants are petroleum products, they are usually non-biodegradable therefore they are toxic to the environment where they are deposited (Fakruddin *et al.*, 2012). Many of these compounds are bio-accumulate, not only that its production process also produces many environmentally hazardous by-products (Benincasa, 2007). These all facts made the environmental

scientists to produce non-toxic environmental friendly surfactants and detergents to save our environment. There are a number of industrial potential in biosurfactants to take the place of chemically derived surfactants.

2.8.1 Biosurfactant in agriculture

Being green surfactants against the chemical surfactants they have greater advantages in the agriculture field (Singh *et al.* 2007). As biosurfactants has potential of enhancing the fertility of soil by remediation and biodegradation of pollutants (Dhara *et al.*, 2013). Biosurfactants also have antimicrobial activity so it is also protects crops from pathogens and it also increases the interaction of plants and friendly microorganisms (Lima *et al.*, 2011). Chemical surfactants used in pesticides can be changed with biosurfactants as these biosurfactants can also be used as carbon sources by the microorganisms present in the soil (Takenaka *et al.*, 2007; Lima *et al.*, 2011).

2.8.1.1 Bioremediation of the soil

Bioremediation is the process of removal of pollutants like hydrocarbon and heavy metals form the contaminated soil. Biosurfactant producing microorganisms are quiet efficient in removing the heavy metals and hydrocarbons from the soil (Sun *et al.*, 2006). As it is well known that biosurfactant have tremendous efficiency to enhance the bioavailability of the microorganism it is quiet good for biodegradation of various complex hydrocarbons with the help of washing technology or clean up combined technology etc (Pacwa-Plocineczak *et al.* 2011; Liu *et al*, 2010; Partovinia *et al.* 2010). There are a number of insecticides which are decomposed by biosurfactants from the agriculture soil (Zhang *et al.*, 2011; Singh *et al.*, 2009; Wattanaphon *et al.*, 2008; White *et al.*, 2006; Neilson *et al.*, 2003). Many reports shows that surfactin are good in biodegradation of the pesticides (Moldes *et al.*, 2011) and glycolipids promotes the biodegradation of chlorinated hydrocarbons (Awashti *et al.*, 1999). Lacto*Bacillus* pentosus produces biosurfactant which degrades the 58.6% to 62.8% octane hydrocarbon from the contaminated soil (Moldes *et al.*, 2011).

2.8.1.2 Elimination of plant pathogens

As it is well established, that many of the biosurfactants have strong antimicrobial activity, so they have a great potential to be used as biocontrol molecules to obtain sustainable crop on the agriculture field (Dhara *et al.*, 2013). Biosurfactant isolated from rhizobacteria shows strongest antagonistic property (Nihorimber *et al.*, 2011). According to Jazzar and Hammad (2003) and Kim *et al* (2003) studies many surfactants have great insecticidal activity (Jazzar *et al.*, 2003; Gronwald *et al.*, 2002; Krishanayya and Grewal 2002). Not only that, certain surfactants in combination with some fungi (for example Myrothecium verrucaria) work as an excellent weedycide (Boyette *et al.*, 2002).

2.8.1.3 Beneficial in microbial-plant interaction

It is essential for the plant to interact with the rhizobacteria to get its benefits (Nihorimbere *et al.*, 2011). Mobility of bacteria, biofilm formation and quorum sensing all these factors are essential for the interaction of microbes with plants (Nihorimbere *et al.*, 2011).

According to certain studies of Dusane *et al.*, (2010), biosurfactants produced by *Pseudomaonas spp.* controls the process of quorum sensing in bacteria. Many reports also reveals that many microbial activities like motility, biofilm formation, signal transduction, bacterial differentiation and formation of biofilm is also affected by biosurfactant secretion by bacteria (Berti *et al.* 2007; Kearns *et al.*, 2003; Ron *et al.*, 2011; Van Hamme *et al.* 2006). So we can conclude that biosurfactant play a crucial role in many factors that are responsible for the plant microbial interaction to provide the suitable benefits to the plant growth. As biosurfactant increases the bioavailability it helps in uptakes of hydrophobic molecules to the plant which is necessary for plants nutrition (Van Hamme *et al.* 2006). Biosurfactants also increase the wettability of the soil which favors in proper distribution of fertilizers (Kearns *et al.*, 2003).

2.8.2 Biosurfactant in pharmaceutical

Maximum work on biosurfactants has been concentrated to the bioremediation only but it has a great potential to be used as therapeutic agent also (Cameotra *et al.*, 2010).

Biosurfactants like rhamnolipids (from *Pseudomaonas*), many lipopeptides (from *Bacillus*) and glycolipids like mannosylerythritol lipid (from yeast) shows great antimicrobial and other medicinal properties (Cameotra *et al.*, 2004; Kitamoto *et al.*, 2002; Maier *et al.*, 2000; Singh, 2004).

2.8.2.1 Antibacterial activity of biosurfactants:

Lipopeptide biosurfactants represents the most potent antimicrobial property and therefore is been the topic of various studies of new antibiotics discovery (Fracchia *et al.*, 2012).

The basic mechanism behind its antibacterial activity is that the biosurfactants associates to the cell membrane and form pores in the sensitive bacterial cell (Carrillo *et al.*, 2003). Biosurfactant sometimes accumulates in the form of micelles into the bacterial cells (Deleu *et al.*, 2008). It makes hydrophobic interaction with the phospholipids and penetrates the bacterial cell up to the various thicknesses (Bonmatin *et al.*, 2003). This mode of action is nonspecific and useful for gram positive as well as gram negative bacterial cells (Lu. *et al.*, 2007).

2.8.2.2 Antiviral activity of biosurfactant:

Surfactin and some other analogues biosurfactant also show certain antiviral properties too (Naruse *et al.*, 1990). Enveloped viruses are more effective as compare to the non enveloped viruses; it may be because of the physico-chemical interaction of lipopeptides with envelop of viruses (Vollenbroic *et al.*, 1997). Lipopeptides biosurfactant disintegrates the viral envelops and sometimes capsids also by making ion channels (Jung *et al.*, 2000; Seydlová & Svobodová, 2008).

2.8.2.3Antiadhesion activity of biosurfactants:

Formation of microorganism's biofilms on technical and medical equipments is a critical and hazardous problem (Fracchia *et al.*, 2012). Most of these biofilms includes the consortium of highly drug resistant bacteria (Falagas *et al*, 2006a). Biosurfactants has the great potential to inhibit the adhesion of microorganisms from the solid surface or

infection site (Rodrigues *et al.*, 2006a). Rodrigues demonstrated that if the pre coated vinyl urethral catheter is run with surfactin solution before inoculation with media, the rate of *Salmonella typhimurium, E coli, Salmonella enteric* and *Proteus mirabilis* biofilm formation is remarkably reduced (Rodrigues *et al.*, 2006a). It has also been reported that if the silicone runner is treated with *S. thermophilus* biosurfactant, it also reduces the adhesion of *C. albicans* upto 85% (Krishnaswamy *et al.*, 2008).

2.8.2.4 Advantages of biosurfactants form probiotics:

Probiotics are the friendly microorganisms, which are directly consumed, in adequate amount to confer the health benefits to the host (Gupta, 2009). Many research papers shows that probiotics reduces the incidences and duration of antibiotic related diarrhea, and also helps in prevention of vaginal candidiasis, urinary tract infection and bacterial vaginosis (Falagas *et al.*, 2006a). It also improves our immunity and nuterilizes the antimatabolite toxins (Flavas *et al.*, 2006b, 2007).

There are a number of microorganisms which are used as probiotics and with respect to their divrsity the mode of action of probiotics varies, some are known for production of organic acids, while other are efficient in production of hydrogen peroxide or carbon peroxides, beside that some can produce bacteriocins and diacetyls (Merk *et al.*, 2005). It has also well established that probiotics also interferes in the biofilm formation with pathogens on epithelial cells of intestinal tracts and urogenital tracts (Reid *et al.*, 1998 and 2001). This is biosurfactant that probiotic bacteria produce to interfere in the biofilm formation (Gudina *et al.*, 2010; Rodrigues *et al.*, 2006b). *Bacillus* produces biosurfactants having antimicrobial properties which also lead to inhibition of biofilm formation of pathogens (Hong *et al.*, 2005). In the same way, Lacto*Bacillus* probiotics produces biosurfactants and crates the compitation for the pathogenic microorganism for making biofilms in vaginal tract (Barrons *et al.*, 2008; Falagas *et al.*, 2007).

2.8.2.5 Anti-tumor activity of biosurfactant:

Some of the current studies has reported that microbial biosurfactants has also the efficiency to control of mammalian cell division and other signaling functions (Fracchia *et al.*, 2012; Cameotra *et al.*, 2010). According to Osada, (1998) some biosurfactants

participates directly or indirectly in various signal transduction processes, immune responses and cell differentiations. Biosurfactant isolated from *Bacillus* (surfactin) induces apoptosis and cell death in human breast myeloma cells MCF-7 through ROS/JNK-mediated mitochondrial/caspase pathway (Cao *et al.*, 2010). According to the recent work of Cao *et al.*, (2011) surfactin induces production of reactive oxygen species (ROS) and Ca^{2+} to affect mitochondrial permeability transition pore (MPTP) which lead to the MCF-7 cells apoptosis. Cao *et al.*, (2011) demonstrated that surfactin induces the production of ROS, this results in the increase in the concentration of Ca^{2+}, in addition of that cytochrme c is realized from the mitochondria through MPTP and activates caspase-9, finally apoptosis takes places.

According to some studies biosurfactant, produced by *Pseudomaonas libanensis* M9-3, named as viscosin, inhibits the migration of metastatic PC-3M (prostate cancer cell line) without and toxic effect (Saini *et al.*, 2008). *Serratia marcescens* produces cyclic depsipeptide named as serratamolide AT514, which induces apoptosis in several cancer cell lines and B-chronic lymphocytic leukemia cells (Matsuyama *et al.*, 2010). It leads to mitochondrial mediated apoptotic pathway and interferes with Akt/NF-kB survival signals to induce apoptosis (Matsuyama *et al.*, 2010).

Among various glycolipids biosurfactants, Mannosylerythritol lipids (MEL), expresses great potential against anti-cancer properties (Kitamoto *et al.*, 2002). MEL-A and MEL-B both display strong inhibition of growth and differentiation inducing activities against myelogenous leukemia cells K562, human basophilic leukemia cells line KU812 and promyelocytic leukemia cell HL60 (Arutchelvi *et al.*, 2010; and Kitamoto *et al.* 2002).

2.8.2.6 Biosurfactant as anti-inflammatory agent:

Some recent reports show that some isomers of surfactin isolated from mangrove bacterium *Bacillus sp.* (No 061341) offers anti-inflammatory activities (Tang *et al.*, 2010). These lipopeptide biosurfactants inhibits the overproduction of nitric oxide and release of IL-6 in lipopolysaccharides induced murine macrophage cell RAW264.7 (Tang *et al.*, 2010). Park *et al.* (2010) has demonstrated that surfactin has anti-inflammatory activity a context of *Prophyromonas gingivalis* cause infection periodontitis. He has

observed that surfactant reduces the production of pro-inflammatory cytokines, which induces tumor narcosis factor (NF-α), interleukin (IL-1β), IL-6 and IL-12. Actually surfactin suppress the activity of nuclear factor-κB in *P. gingivalis* lipopolysaccharides stimulated THP-1 human macrophages cells (Park *et al.*, 2010).

2.8.3 Application of biosurfactants in food industry:

Biosurfactant shows great potential in food industries but its major applications are food formulation ingredients and anti adhesive agents (Faruddin, 2012). As biosurfactants have ability to reduce the surface tension or interfacial tension it can stabilize the emulsion of ingredient that lead to food formulation (Krishnaswamy *et al.*, 2008). Biosurfactants are useful for controlling the agglomeration of oil or fat globules, stabilizing the aerated system, improvement of texture and increasing the shelf life of starch products and perk up the consistency of fat based products (Kachholz *et al.*, 1987).

2.8.3.1 Application of biosurfactant in formulation of food:

Biosurfactants are used in ice cream and bakery industry for formulation of porducts, it controls the consistency, solubilization of flavor oils and retarding staling of the product; it also stabilizes and antispatters the fats at the time of cooking (Kosaric, 2001). To stabilize the texture stability, conservation and volume of dough rhamnolipid is added in the bakery industry to the product (Van Haesendonck *et al.*, 2004). Rhamnolipid also enhances the croissants and frozen confectionery of butter cream (Iyer *et al.*, 2006). According to the Iyer *et al.*, (2006) reports *Enterobacter cloacae* marine strains produces certain biosurfactants that has good properties to reduce surface tension at low pH so can be used in the production of ascorbic acid and citric acid based food products.

L-Rhamnose is already used as high quality flavoring agent in the food industry like furaneol (Linhardt *et al.*, 1989). Therefore use of rhamnolipids isolated from P. *aeruginosa* has great potential as a source of rhamnose in the food industry (Linhardt *et al.*, 1989).

2.8.3.2 Biosurfactant as anti-adhesive agent in food:

In food processing industry bacterial biofilm on the surface of fermenter, supply tubes and other equipments has been a great problem that leads to various food born disease transmission and food spoilage (Hood *et al.,* 1995). That is the reason food processing industries shows zero percent tolerance for the microorganisms like *Listeria monocytogenes* and *Salmonella* (Hood *et al.,* 1995). So it is essential to remove even single microbial cell from the system, which may lead to formation of whole biofilm, for the quality control and healthy food production (Hood *et al.,* 1995).

Therefore biosurfactant are also used for inhibition or control of biofilm formation on the various equipments in food industry. Biosurfactant isolated from *Streptociccus thermophilus* is very useful for control of fouling of heat exchanger plates in pasteurizers (Busscher *et al.*, 1996).

To reduce the adhesion of microorganisms and biofilm formation is control by the new biosurfactant as bioconditining agent (Nitschke *et al.*, 2007). If the silicone rubber is treated with biosurfactant isolated from *S. thermophilus* before use, it inhibits the biofilm of *Candida albicans* up to 85% (Busscher *et al.*, 1997). In the same way *Lactobacills fermentum and L acidophilus* produces biosurfactants that adsorbed on glass and reduce the adherence of uropathogenic bacterial cells of *Enterococcus faecalis* by 77% (Velraeds *et al.*, 1996).

The growth and adhesion *L. monocytogenes* L028 on stainless steel can be inhibited if the preconditioning of the steel surface is done with *Pseudomaonas fluorescens* biosurfactant (Nitschke, M. *et al.*, 2007). It reduces the adhesion more than 90% of pathogenic organisms (Meylheuc *et al.*, 2006). According to the recent investigation biosurfactants isolated from gram negative bacteria as *P. fluorescens* and gram positive bacteria as *LactoBacillus helveticus* have great potential to inhibit the adhesion of *L. monocytogenes* on stainless steel (Meylheuc *et al.*, 2006).

2.8.4 Application of biosurfactant in petroleum industry:

Biosurfactants are certain novel biomolecules which have most versatile and strong microbial biotechnology potentials and offers the diver's benefits in the fields of

befouling degradation of hydrocarbons oils recovery and bio corrosion beside that it also works as biocatalyst for petroleum up-gradation (Perfumo *et al.*, 2010). Biosurfactants are also very useful in petroleum extraction, upgradation, petrochemical manufacturing, refining and transportation (Fakruddin, 2012).

2.8.5 Application of biosurfactants in other commercial industries:

Most of the surfactants used in laundry are chemically originated. Though it offers quiet good detergence and cleansing properties but along with that it also brings its toxic effect and non biodegradability which is the matter of concern for the environmentalist. Now with the increasing problem of water and air pollution people are getting aware about the environmental hazards associated with non biochemical surfactants. This motivates the new researchers to find a new eco-friendly substitute of chemical surfactants, and the solution of this problem is biosurfactant (Fakruddin, 2012). Cyclic lipopeptides like biosurfactants offers a number of stable properties as wide range pH stability, heat stability and high surface activity (Mukherjee, 2007). They also express great emulsion formation efficienty with vegetable oils therefore it is considered as a compatible product for commercial laundry detergent industry (Das *et al.*, 2007).

There are a number of microorganisms and their metabolic pathways that are explored to the marginally producing reservoirs to increase the recovery of oil (Fakruddin, 2012). In present days a new strategy has been introduced in this direction which involves the use of biosurfactant to recover oil from the marginal sources (Fakruddin, 2012). This mechanism includes the acidification of the solid phase (Das *et al.*, 2007). *Bacillus subtilis*, Torulopsis bombicola and *Pseudomaonas aeruginosa* etc are some of the examples which can utilize crude oil and other hydrocarbons as their sole carbon source (Das *et al.*, 2007). So they are used to clean up of oil spills (Das *et al.*, 2007).

Chapter 3

Materials & Methods

Chapter 3

Material and methods

3.1 Material

3.1.1 Chemicals, Reagents, Plastic wares, Glassware and other instruments

Media and media components

S.No.	Product	Source
1	Nutrient agar media	Hi-Media
2	MRS media	Hi-Media
3	Anaerobic agar media	Hi-Media
4	Starch agar media	Hi-Media
5	Bacteriological Peptone	Hi-Media
6	Beef extract	Hi-Media
7	Sodium Chloride	Hi-Media
8	Agar-Agar	Hi-Media

Table: 3.1- List of used media and media components

Chemical reagents

S.No.	Product	Source
1	Ethanol	Merck
2	Glycerol	Merck
3	Liquid Paraffin	Merck
5	Mustard Oil	Dhara
6	Sunflower Oil	Fortune
7	Petrol	Bharat petroleum
8	Diesel	Bharat petroleum
9	Kerosene Oil	Unknown
10	Hydrochloric acid	Merck
11	Sodium-Hydroxide pallet	Merck
12	Ammonium sulfide dehydrated	Merck
13	Hydrogen Peroxide	Qualigens
14	Suffaranin	Hi-Media
15	Crystal Violet	Hi-Media
16	Grams Iodine	Hi-Media
17	Methanol	Merck
19	Chloroform	Merck
20	Ammonia water	Merck
21	Ninhydrin	Qualigens
22	Anthrone	Qualigens
23	Sulfuric acid	Merck
24	Acetone	Merck
25	Di-Chloromethane	Merck
26	Ethyl acetate	Merck

Table: 3.2- Showing the major chemicals and reagents used in the experiments

3.1.2 Biological Materials

S. No.	Microbial sample	Source of Sample	Number of isolates
1	Lactic acid bacteria	Churpi	8
2	Lactic acid bacteria	Yac Milk	5
3	Lactic acid bacteria	Meso (Bamboo shoot)	16
4	*Bacillus*	*Kinema* (Fermented soy-beans)	12
	Total sample		41

Table: 3.3- Representing the various types and number of microbial samples and their sources used in the experiments.

3.2 Methods

3.2.1 Collection of Samples and Bacterial Isolates

Total forty one (41) bacterial samples have been collected from various sources. 12 *Bacillus* isolates were isolated form *Kinema* samples obtained from Ranipool, Sikkim. 29 isolates of lactic acid bacteria have been collected from Department of Microbiology Laboratory, Sikkim University which were isolated from various ethnic fermented food of Sikkim as mentioned in table: 3.3.

3.3.2 Isolation and Revival of Bacterial Isolates

Ten gram of *Kinema* sample was suspended in ten ml of sterilized saline (0.85% NaCl) solution to make one percent sample solution. The serial dilution of the sample was made for the five times (as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) in normal saline. 100 µl of each diluted sample was inoculated on freshly prepared nutrient agar plates by spread plate method. Lactic acid bacteria were collected in the form of glycerol stock from microbiology laboratory of Sikkim University. A loop full glycerol stock culture was inoculated in the freshly prepaired MRS broth media under aseptic condition. Each culture was incubated for 24 hours at optimum temperature. Then from the MRS broth media the culture were streaked to the MRS agar plates to observe the purity of the culture. Purity of the culture was also analyzed under compound microscopy with the help of simple staining.

3.2.3 Primary Screening of the Biosurfactant

Primary screening for biosurfactant production was done by two methods using different types of oils. One technique was modified Drop Collapse Method and second technique was estimation of Emulsification Index (EI_{24}).

3.2.3.1 Drop Collapse Method

For the selection of biosurfactant producers, all the *kinema* isolates were transferred to Nutrient broth and isolates of lactic acid bacteria were transferred to the MRS broth. All the broth cultures were incubated for 72 hours at optimum temperature and pH conditions. All the cultured bacterial isolates were explore to the qualitative screening by drop collapse method (Youssef *et al.*, 2004). In this method a glass surface was coated with a layer of sunflower oil, kerosene oil, mustard oil, paraffin oil and diesel oil separately. Each bacterial cultures were centrifuged at 10,000 rpm for 15 m, at 4°C. A drop (about 8µl) of supernatant was placed on the oil coated surface. Broth containing surfactant interacts with the oily layer and spreads on the oily surface. While the supernatant without the biosurfactant will not spread and remain raised on the surface.

3.2.3.2 Estimation of emulsification efficiency of the biosurfactant

Emulsification of the biosurfactants can be determined in terms of 'Emulsification index' by method described by Pereira *et al.*, 2013. To estimate the emulsification efficiency, 5ml of oil was added to the same volume (5ml) of cell-free supernatant of broth culture in the test tube. Each test tube was vertex at high speed for two minutes. Each tube was then allowed to stand at room temperature under undisturbed condition for 24 hours. After 24 hours the stability of the emulsion formed by the emulsification of oil and the biosurfactant containing cell free supernatant can be determined (Pereira *et al.*, 2013). Emulsification index (EI_{24}) was calculated with the following formula-

$$EI_{24} = \frac{length\ of\ the\ emulsification\ layer}{total\ lengyh\ of\ the\ liquid\ in\ the\ tubes} \times 100$$

Length of the emulsification layer and liquids contents in the tubes were measured in mm and the whole experiment was conducted in triplicates. The emulsification index was

measured with four different types of oils (vegetable oils- mustard oil, sunflower oil and petroleum oil-kerosene oil and liquid paraffin).

3.2.4 Partial characterization of the bacterial isolates

Bacterial characterization of the biosurfactant producing isolates was done by Gram staining, endospore staining, catalase test, Voges-Proskauer test, growth in anaerobic conditions, growth at 65°C, growth of bacteria at 7% NaCl and starch hydrolysis following the key of Sarkar *et al*, (2007).

3.2.4.1 Microscopy

All the pure cultures of the biosurfactant producing bacteria were subjected to the Gram staining. Endospore staining was done to observe the position of endospore and the shape of sporangium of the bacteria. Motility of the bacteria was also observed under microscope.

3.2.4.2 Catalase test

A loop full of pure culture was spotted on a clean glass slide and a drop of 3% hydrogen peroxide was applied on the culture. Formation of bubbles indicated the catalase positive bacteria.

3.2.4.3 Voges-Proskauer test

Bacteria cultures were inoculated in freshly prepared sterilized triptic soy broth and were incubated at 36°C. After the incubation of 24 hours the 10 drops of 'Barritt reagent-A' (6g α-Naptholin in 100 ml 95% ethanol) and 'Barritt reagent-B' (16 g KOH in 100 ml H_2O) was added. Test tube was vertex second and kept for few minutes. Cultures positive to Voges-Proskauer test produce red color while negative cultures didn't produce any color.

3.2.4.4 Growth of bacteria in anaerobic condition, at 65°C and in 7% NaCl

Bacterial cultures were inoculated in anaerobic agar media and were incubated in anaerobic jar, at optimum temperature. One set of nutrient agar plates were prepared, inoculated with the bacterial culture and incubated at 65°C. To observe the growth of bacteria in high salt concentration, nutrient agar media was prepared with 7% NaCl concentration and bacteria was inoculated in it, it was allowed to incubate at optimum temperature.

3.2.4.5 Starch hydrolyses test

To observe the starch hydrolyzing property of bacteria, the culture was inoculated on starch agar media plates and incubated at optimum temperature for 24 hours. The plates were folded with iodine solution. Clear zone around the bacterial colonies indicated that the bacteria were utilizing the starch as carbon source

3.2.5 Production and Extraction of Biosurfactant

Pure colony of each culture was inoculated in 250 ml of sterilized nutrient broth, and incubated at optimum temperature for 48 hours. For the extraction of biosurfactant, first of all cell free supernatant is prepared, by centrifuging the broth culture at 10,000 rpm for 20 min, at 4°C. Then precipitation of biosurfactant was done by acidifying the supernatant with 4N HCl until the pH of the supernatant reached 2.0. The acidified suspension of the biosurfactant in culture supernatant was incubated for overnight at 4°C (Yin *et al.* 2008). Off white or gray color precipitate was collected at the bottom of the flask. The precipitate was collected by centrifugation at 10,000 rpm for 20 min. at 4°C. The extracted biosurfactant was further purified by suspending it in chloroform-methanol solution (2:1 v/v) and allow it to be incubated at 30°C on rotary shaker at the speed of 250 rpm. The mixture was further centrifuged at 10,000rpm for 20 min. at 4°C (Chander *et al.*, 2012). The biosurfactant was accumulated at the interphase of chloroform (lower layer) and methanol (upper layer). The chloroform was removed and biosurfactant in methanol was collected. Finally methanol was evopurated at room temperature.

3.2.6 Purification of Biosurfactant

Purification of biosurfactant was done by solvent extraction method described by Vanlerberghe *et al.*, (2009). The precipitated biosurfactant, obtained after overnight incubation at 4□C followed by acidification, was transferred to the separating funnel; equal amount of ethyl acetate was added to the separating funnel. Both organic solvent and aquas suspension of biosurfactant was shaken vigorously to mix the two solvent. The separating funnel was kept undisturbed for few minutes to allow separation of the liquid phases. This organic phase was eluted and this step was repeated two-three times to remove water as much as possible. Finally the organic phase was passed through sodium sulphate, to remove the residual water present in the solution. The ethyl acetate was evaporated with the help of rotary evaporator and biosurfactant was recovered.

3.2.7 Partial characterization of biosurfactant

Fundamental chemical nature of biosurfactant was analyzed by thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR).

3.2.7.1 Thin layer chromatography

The preliminary biosurfactant characterization was done by thin layer chromatography (Bayoumi *et al.*, 2010). About 10µl of biosurfactant solution in methanol was applied on silica gel TLC plate (Merck. TLC S Silica gel 60 F_{254}). The TLC tank was saturated with Chloroform: Methanol: 28%NH_4OH: : 65:35:5 vapors for 30 min. TLC plates for each biosurfactant sample was prepared in duplicates. The plates spotted with the biosurfactants were kept in the TLC tank in undisturbed condition. When the plates were developed one set of plate was treated with anthrone reagent (1g anthrone in 5ml H_2SO_4 and 95ml ethanol). Another set of the developed TLC plates were treated with ninhydrin solution (0.5g ninhydrin in 100ml of anhydrous acetone). After heating the plates at 110□C bands were observed. The R_f value was calculated with the following formula-

$$R_f value = \frac{Distance\ covered\ by\ the\ sample}{Total\ distance\ covered\ by\ the\ solvent}$$

3.2.7.2 Fourier Transform Infrared spectroscopy

Fourier transform infrared (FT-IR) spectroscopy was used to study the general chemical characteristic of the biosurfactant (Saravanand *et al.*, 2012). FT-IR analyses revels the details about chemical structures and atomic bonding in the test compound, therefore we can identified the various functional groups present in the compound. In this current study FT-IR spectrometer Model-Bruker, Germany/Alpha FTIR was used which was equipped with Opus graph plotter. The resolution of the instrument was $4cm^{-1}$ at 50 scans. The spectra were observed in the range of wavenumber 400 to $4000cm^{-1}$. Purified samples by acidification and solvent extraction methods were suspended in dichloromethane and spotted on the instrument. Dichloromethane was allowed to air dry so that a thin homogenous layer of the sample would be remained. The sample was scanned and the absorbance peaks were recorded.

Chapter 4

Results

Chapter: 4

Results

4.1 Collection of Samples and Bacterial Isolates

A total of 41 isolates of bacterial cultures were collected from various Sikkim fermented food for example *kinema*, milk, churpi and meso. All lactic acid bacteria isolated from milk, churpi and meso were cultured on MRS media, while twelve bacteria isolated from *kinema* were cultured on nutrient agar media as these bacteria belong to *Bacillus* species. Isolates were selected on the basis of morphological character and isolated colonies were maintained as pure culture on nutrient agar medium or MRS medium as per requirement in slants at 4°C. Pure cultures were maintained in 50% glycerol solution at -80°C also for long term use.

4.2 Primary screening for biosurfactant production:

Primary screening was done by modified drop collapse method on five different oils (Mustard oil, sunflower oil, diesel, petrol, and liquid paraffin oil). Out of 41 isolates only 12 isolates were found to be positive for biosurfactant production (Figure 4.2). All these bacterial isolates producing biosurfactant was tentatively identified as *Bacillus* from morphological and spore study.

4.3 Emulsification index estimation:

Emulsification index (EI_{24}) was observed from the cell free suparnatent of each *Bacillus* culture with sunflower oil, kerosene oil, liquid paraffin oil and mustard oil as per described in the methodology (Figure 4.3). Experiment was conducted in triplicate and emulsification index was calculated. Mean and standard deviation of EI_{24} for each culture with different types of oils is mentioned in the table 4.1. With sunflower oil maximum EI_{24} value was observed, while with kerosene oil the EI24 value was least (Figure 4.5). Isolate number LK.4.5 has given maximum EI_{24} value with most of all the oils (Table 4.1).

4.4 Morphology:

All good biosurfactant producers were subjected to the Gram staining. All bacteria were rod shaped and Gram positive (Figue 4.4). On endospore study endospores were also observed in 2-3 days old culture with bulging sporangium. Most of the cells had shown endospore in the middle of the cell but some cultures like LK.5.3.1 and LK.1 had shown endospore at the terminal position of the cell with bulging sporangium (Table 4.2). Lk.11 represented spherical cell, with Gram positive cell wall. It was non endospore forming bacteria. All isolates except LK.11 were motile bacteria.

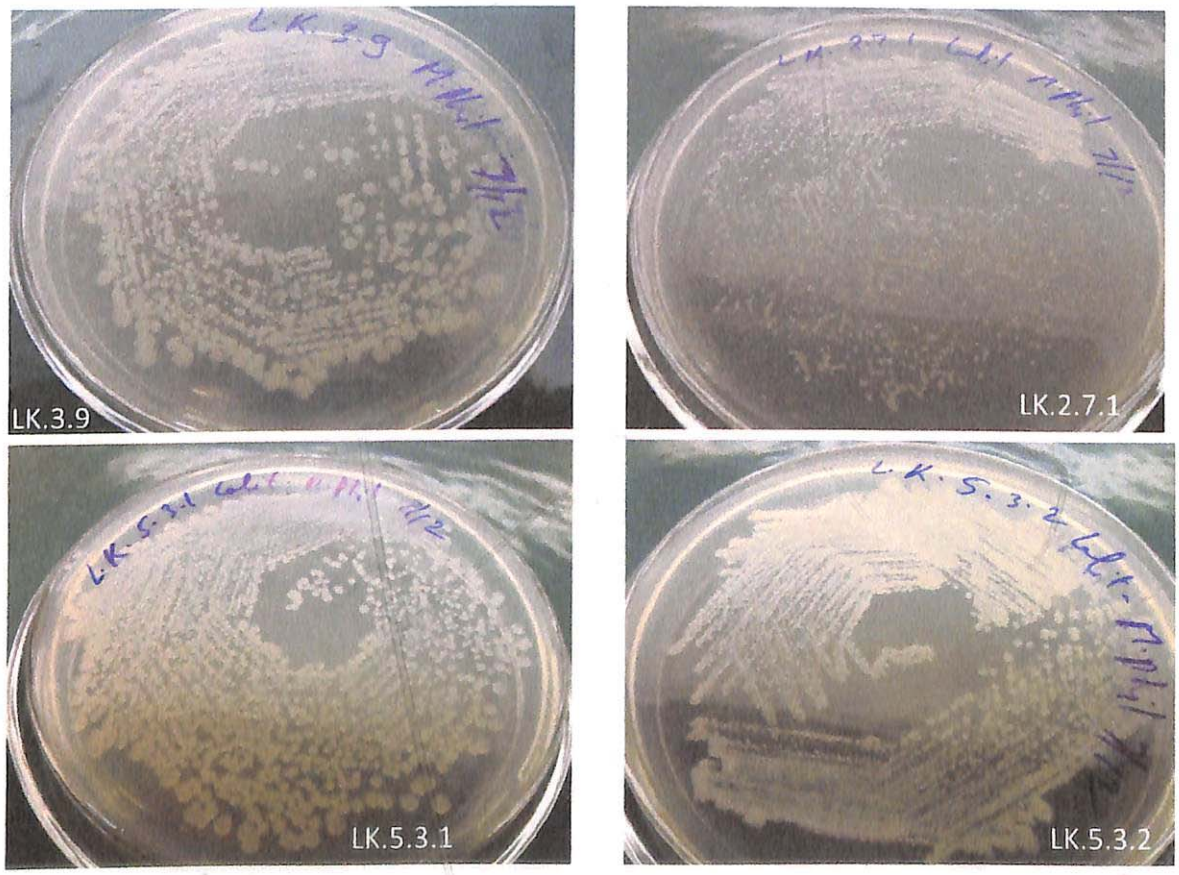


Figure: 4.1- Colony morphology of some *Bacillus* isolates on nutrient agar

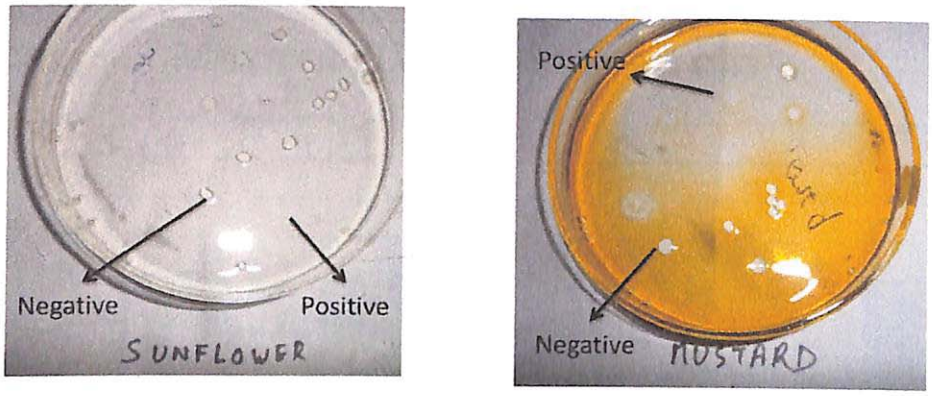


Figure: 4.2- Primary screening of biosurfactant production by drops collapse method on sunflower oil and mustard oil respectively.

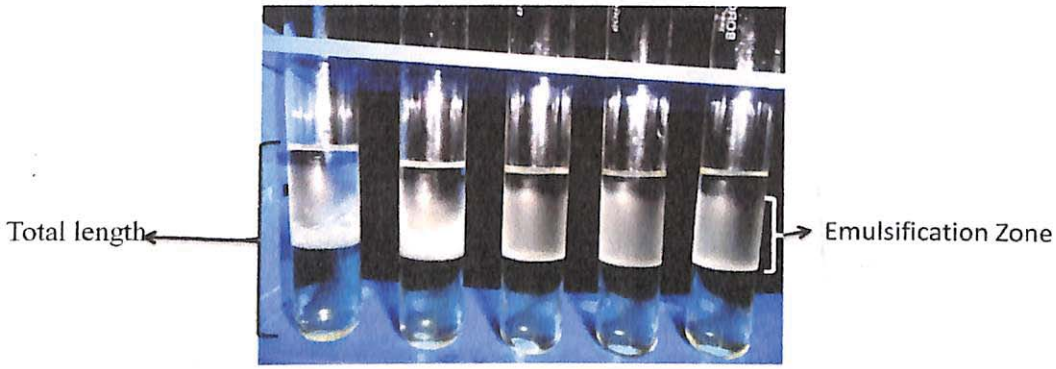


Figure: 4.3- Showing the lower layer of supernatant of culture, middle cloudy layer of emulsified oil and water and the upper layer of oil in the test of emulsification index estimation.

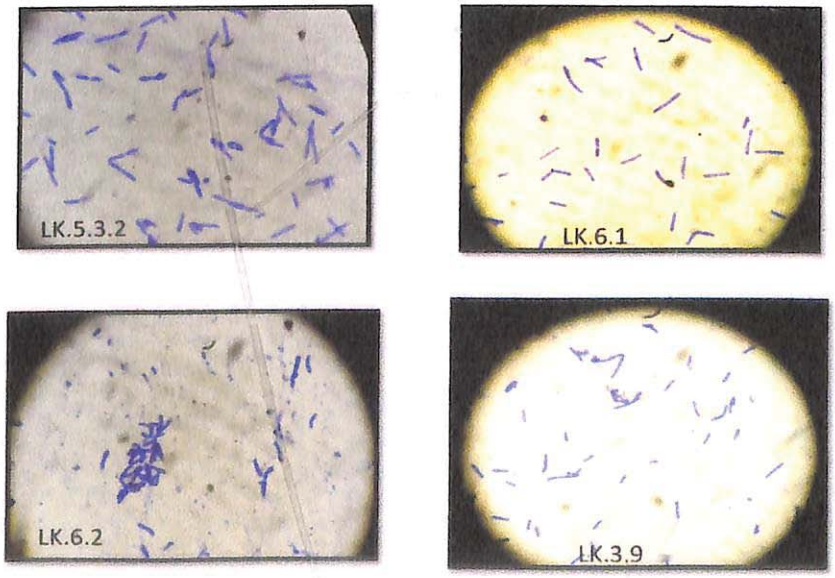


Figure: 4.4- Showing the Gram's staining of bacterial cells with compound microscope at 100x with oil immersion

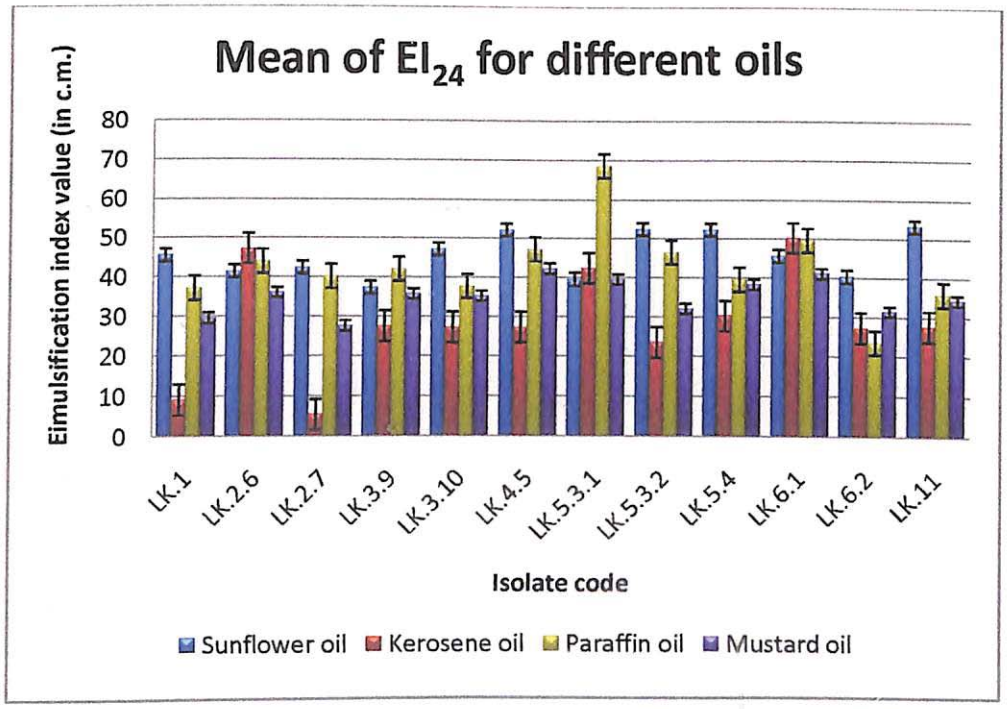


Figure: 4.5- Graph representing the comparative means of IE_{24} for different cultures.

Table: 4.1- Representing the mean and standard deviation of EI_{24} value for Sunflower oil, Kerosene oil, Paraffin oil and Mustard oil

Isolate code	Sunflower oil	Kerosene oil	Paraffin oil	Mustard oil
LK.1	45.51±1.11	9.09±0.00	37.11±1.09	29.54±1.19
LK.2.6	41.42±4.10	47.27±3.15	44.03±1.09	36.14±3.54
LK.2.7	42.46±4.89	5.45±0.00	40.25±3.93	27.67±1.09
LK.3.9	37.51±0.40	27.56±6.05	42.14±5.76	35.85±3.27
LK.3.10	47.27±0.00	27.27±0.00	37.74±0.00	35.32±8.26
LK.4.5	52.21±3.90	27.27±0.00	47.17±0.00	42.27±1.96
LK.5.3.1	39.53±0.81	42.42±1.05	68.55±2.18	39.53±0.81
LK.5.3.2	52.46±0.00	23.64±0.00	46.54±1.09	32.08±0.00
LK.5.4	52.31±6.32	30.30±1.05	39.62±0.00	38.37±4.36
LK.6.1	45.61±0.00	50.30±4.20	49.69±1.09	41.11±1.33
LK.6.2	40.39±0.00	27.27±0.00	23.64±0.00	31.45±5.45
LK.11	53.33±2.89	27.69±3.54	35.85±3.27	34.41±2.89

Data represents mean of three replicates

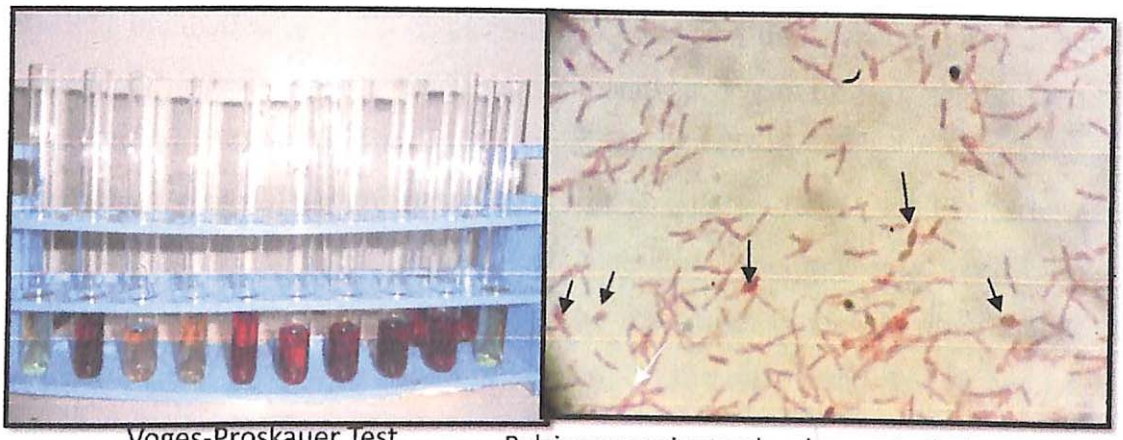
Table: 4.2- Representing the biochemical and physiological characteristics of the biosurfactant producing bacterial isolates (according to Appendix A).

Isolate Code	Gram Staining	Spor Formation	Spore Position	Swelling of Sporangium	Catalase test	Voges-Proskauer test	Growth in Anaerobic Condition	Growth at 65 °C	Growth in 7% NaCl	Starch Hydrolyses
LK.1	+	+	Central	-	+	-	+	-	+	-
LK.2.6	+	+	Central	-	+	+	+	+	+	-
LK.2.7	+	+	Central	-	+	+	+	+	+	-
LK.3.9	+	+	Central	-	+	+	+	+	+	+
LK.3.10	+	+	Central	-	+	+	+	+	+	+
LK.4.5	+	+	Central	-	+	+	+	+	+	+
LK.5.3.1	+	+	Subterminal	+	+	+	+	+	+	+
LK.5.3.2	+	+	Middle	+	+	+	+	+	+	+
LK.5.4	+	+	Central	-	+	+	+	+	+	+
LK.6.1	+	+	Subterminal	+	+	+	+	+	+	+
LK.6.2	+	+	Central	+	+	+	+	+	+	+

4.5 Bio-Chemical characterization:

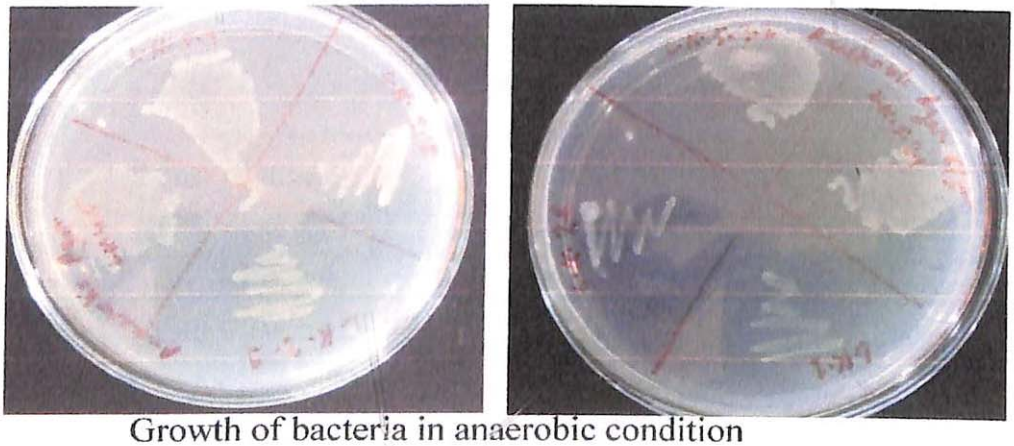
According to the bacterial identification key of *Bacillus* (Sharkar *et al.*, 2002) as per requirement Catalase test, Voges-proskauer test, growth at 65□C, growth in anaerobic condition, growth in 7% NaCl, starch hydrolyses tests were conducted. Its results were represented in the table 4.2. All the *Bacillus* isolates were found to be Voges-proskauer positive except isolate number LK.1. All the *Bacillus* isolates have shown prominent growth in the incubation of 24 hours at 65 □C, but *Bacillus* isolate LK.1 did not show any growth. All the *Bacillus* isolates were grown in anaerobic agar on agar plates as well as in stab covered with paraffin oil. Plates and stabs were incubated in anaerobic jar. All the *Bacillus* culture have shown prominent growth under anaerobic condition at 36 □C. In nutrient agar media with 7% NaCl concentration all the *Bacillus* culture have shown prominent growth within 24 hours. *Bacillus* isolates LK.1, LK.2.6 and LK.2.7 were

unable to hydrolyze starch, while other culture have give faint zone for the starch hydrolyses on starch agar media. According to *Bacillus* identification key (source: Sarkar *et al.*, 2002) most of the cultures were *B. licheniformis* (Appendix A).



Voges-Proskauer Test

Bulging sprongium and endospoer in the bacterial cell



Growth of bacteria in anaerobic condition

Starch Hydrolyses by Bacteria

Figure: 4.6- Results of biochemical tests

4.6 Extraction of biosurfactant:

Biosurfactant was isolated with acidification method. On reducing the pH of supernatant of 48 hours old culture, the supernatant converted to turbid and cloudy solution. On overnight incubation at 4 $^{\square}$C in undisturbed condition the suspended biosurfactant had precipitated. Which was purified in methanol and chloroform solution and white precipitate was extracted in the organic phase. On drying it, white-brown color biosurfactant was obtained (Figure 4.7). Isolate number LK.11 could not be extracted with acidification method, so solvent extraction method was used along with acidification. Isolate number LK.6.2 was the maximum producer, while isolate LK5.4, LK.4.5 and LK.2.6 were also good producers. On the other hand, LK.11 and LK.3.9 has produced very less amount (Table 4.3).

Table: 4.3- Quantity of biosurfactant isolated from different cultures

S.No.	Isolate	Vol. of Media	Dry weight of biosurfactant
1	LK.1	250 ml	26.8 µg
2	LK.2.6	250 ml	53.8 µg
3	LK.2.7	250 ml	34.4 µg
4	LK.3.9	250 ml	2.0 µg
5	LK.3.10	250 ml	22.9 µg
6	LK.4.5	250 ml	65.8 µg
7	LK.5.3.1	250 ml	12.5 µg
8	LK.5.3.2	250 ml	14.5 µg
9	LK. 5.4	250 ml	189.8 µg
10	LK.6.1	250 ml	20.9 µg
11	LK.6.2	250 ml	1633.0 µg
12	LK.11	250 ml	5.9 µg

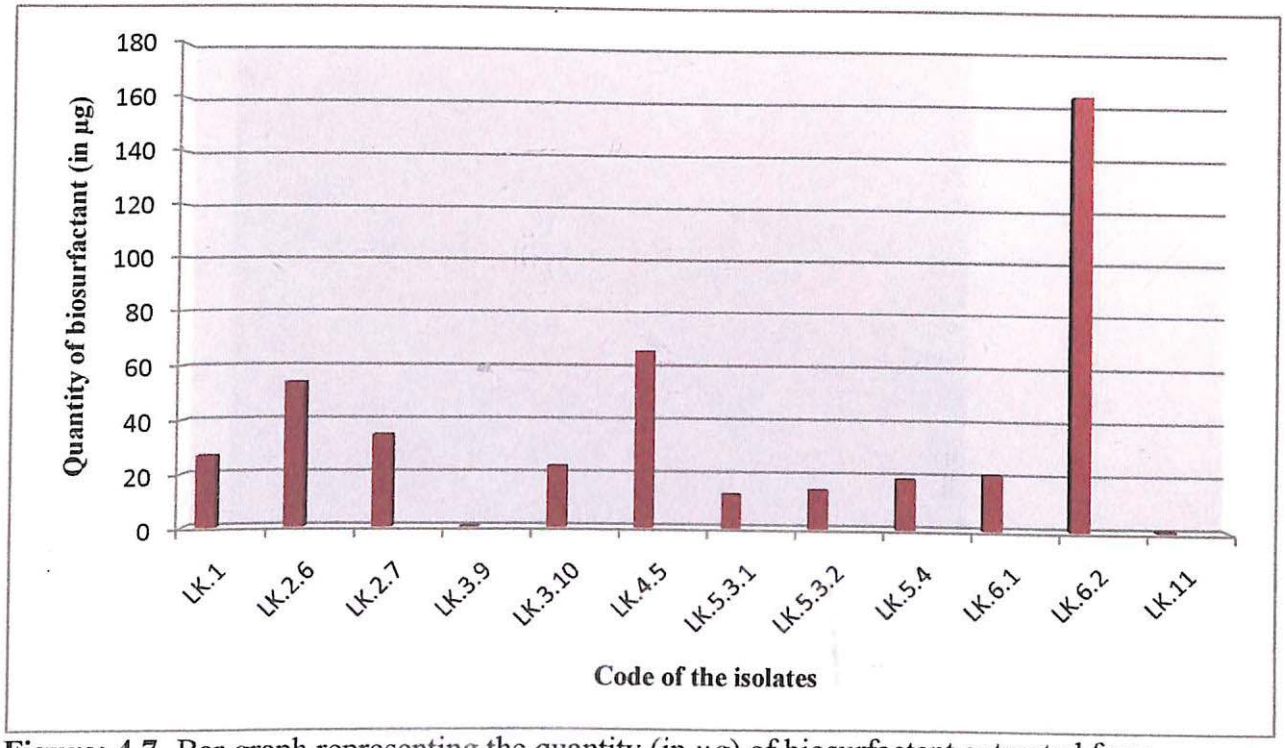


Figure: 4.7- Bar graph representing the quantity (in µg) of biosurfactant extracted from the different cultures in 250 ml Nutrient agar media after 48 hours of incubation.

4.7 Thin Layer Chromatography (TLC):

All the biosurfactant samples were subjected to TLC, in two sets. After running the sample with solvent having, composition of chloroform: methanol: 28% NH_4OH, one of the set of TLC plates were treated with anthrone reagent to study the presence of glycolipids and another set of samples were treated with ninhydrin solution to study the presence of lipoprotein. Plates treated with anthrone reagent did not show any band while all the plates treated with ninhydrin have given pink bands (Figure 4.8). This indicates that biosurfactants prodced by all the isolates were lipopeptide in nature.

Beside the spotted samples in TLC plate separated into two to four bands which shows each isolate produced more than one type of biosurfactants. Some similarity has also been observed among the TLC bands of biosurfactants isolated from different cultures. This indicates that biosurfactant produced from these *Bacillus* isolates have similar molecular mass, polarities and may have similar structural properties (Table 4.4).

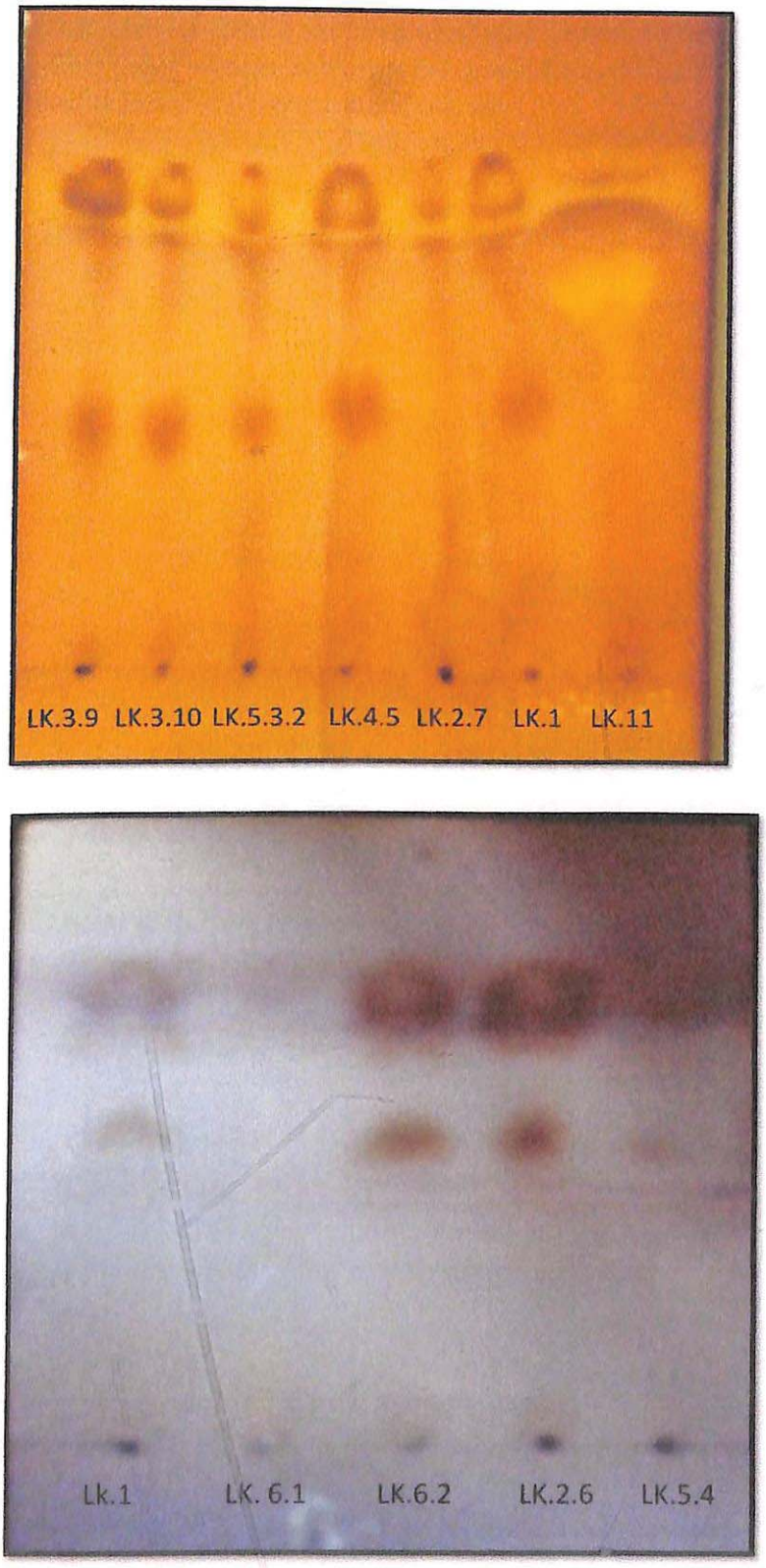


Figure: 4.8- TLC plate stained with Ninhydrin.

Table: 4.4- Showing the R_f values of biosurfactants isolated from different bacterial cultures. This table is also showing the similarities in the R_f values among the biosurfactant isolated from different cultures.

Isolate No.	R_f Value				
	Band 1	*Band 2*	*Band 3*	*Band 4*	*Band 5*
LK.1	0.52	0.80	0.84	-	0.96
LK.2.6	0.62	0.80	0.86	0.90	0.96
LK.2.7	-	0.83	-	0.90	0.98
LK.3.9	0.48	0.83	0.88	-	0.96
LK.3.10	0.48	0.83	0.88	-	0.96
LK.4.5	0.50	0.83	0.88	-	0.96
LK.5.3.1	0.42	-	0.86	0.93	0.98
LK.5.3.2	0.48	0.75	0.86	-	0.96
LK.5.4	0.60	0.83	0.78	-	0.98
LK.6.1	-	0.84	-	0.91	0.95
LK.6.2	0.62	0.82	0.86	0.90	0.98
LK.11	-	-	0.87	0.93	-

-, Represent no TLC band at the respective position

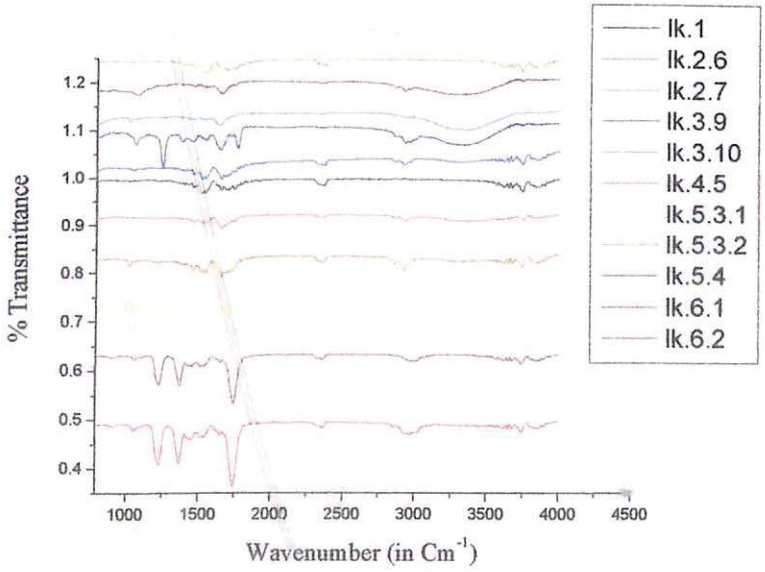
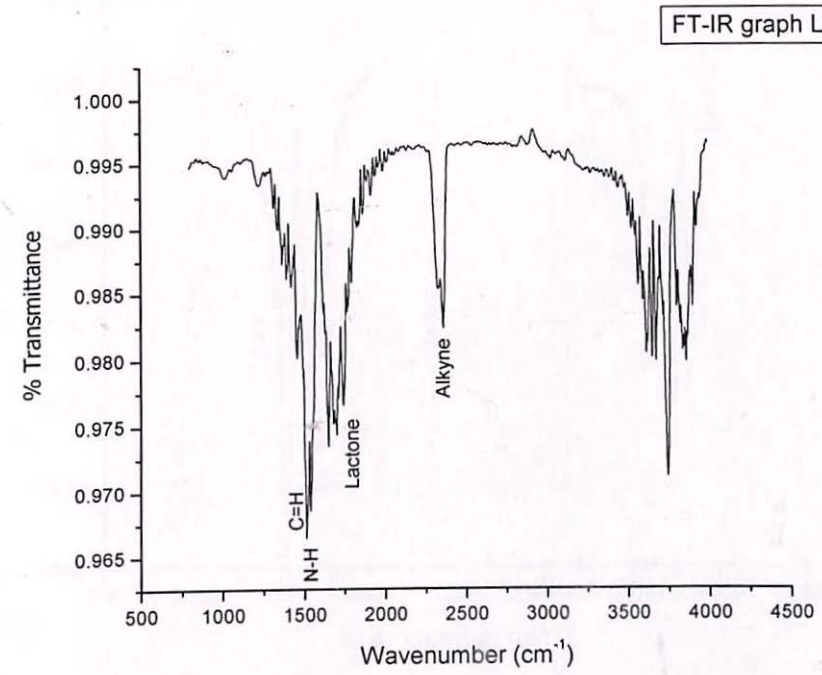


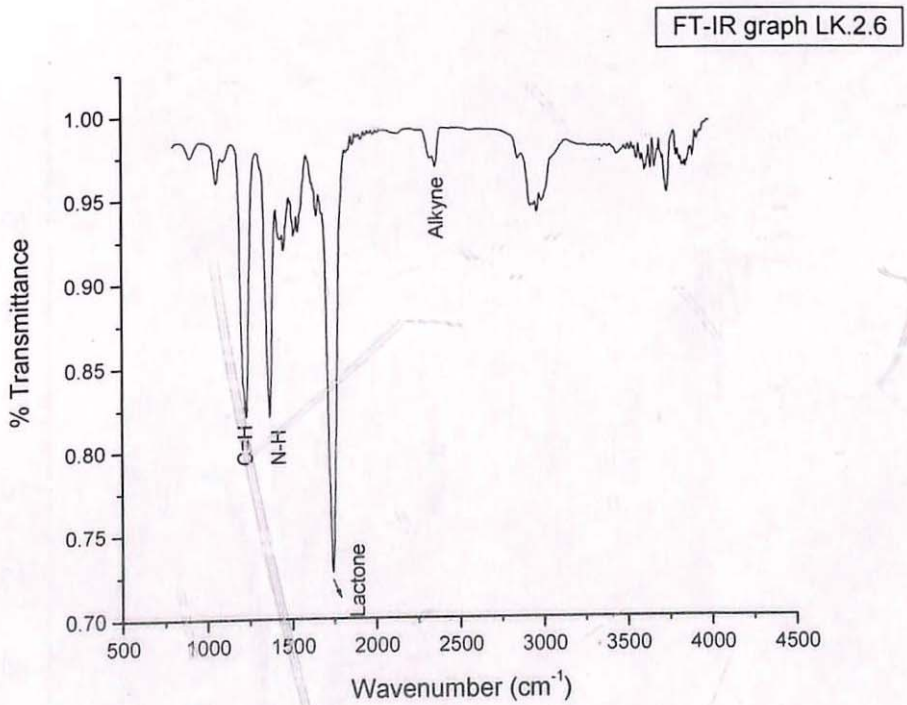
Figure: 4.9- FT-IR absorbance peaks of biosurfactants from *Bacillus* isolates

4.8 FT-IR analyses:

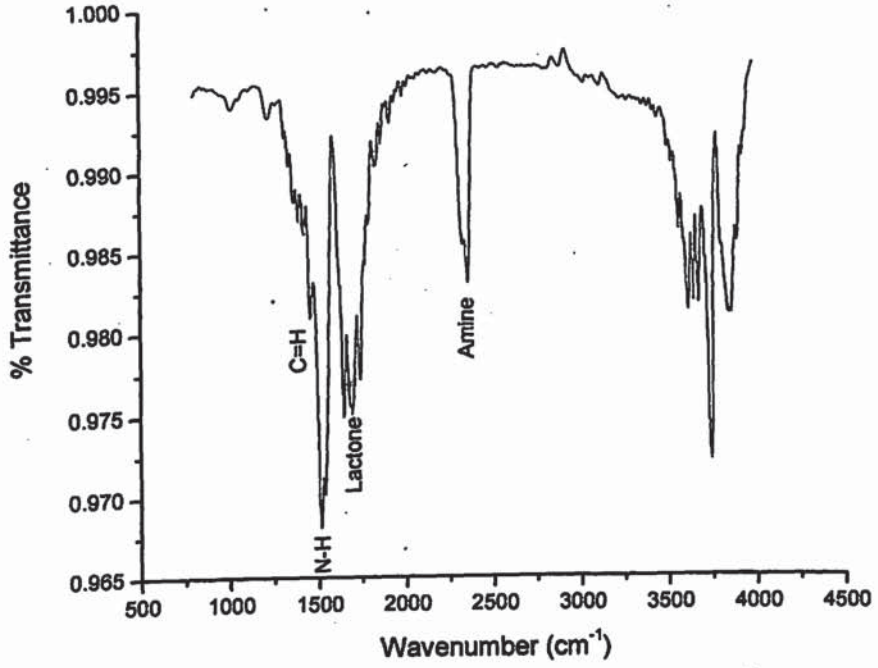
All the samples were subjected to FT-IR analyses and the charts showing respective cultures peaks pattern is given in the following figure 4.9 and table 4.5. Peaks has been observed in the range of 1365cm^{-1} to 1456 cm^{-1} wavenumber which indicated the presence of alkyn group deformation. Bands for C-H bond stretching have also been observed between the wavenumber of 2925 cm^{-1}-2965 cm^{-1}. Biosurfactant of isolate LK.4.5 have shown sharp peak at 3362 for N-H bond stretching. There are some absorption peaks been observed in the region of wavenumber 1508 cm^{-1} to 1561 cm^{-1}. Peaks for CO-N bonds has been noticed in the region of wavelength 1633 to 1718cm^{-1} for all the samples. A broad peak of –OH group has been observed for isolate LK.5.3.1 at 3345 cm^{-1}. Biosurfactant extracted from LK.1, LK.2.6, LK.2.7, LK.4.5, LK.5.3.1, LK.6.2 isolates have given the sharp peaks at 1701 cm^{-1} to 1740 cm^{-1} wavenumber for lactonee ring. Lactonee ring is the characteristic of surfactin. Peaks for the N-H bonds, CO-N bonds, NH bonds in combination of CN represents the presence of peptides or amino acids in the sample, on the other hand C-H bonds stretching shows the presence of fatty acids in the structure (Table 4.5). Some unidentified peaks have also been observed (Figure 4.10). Overall observation of the FT-IR analyses shows that the isolated biosurfactant was lipopeptides and many are closely related to surfactin (Figure 4.9).

Figure: 4.10- Representing the various FT-IR plots for different extracted biosurfactants

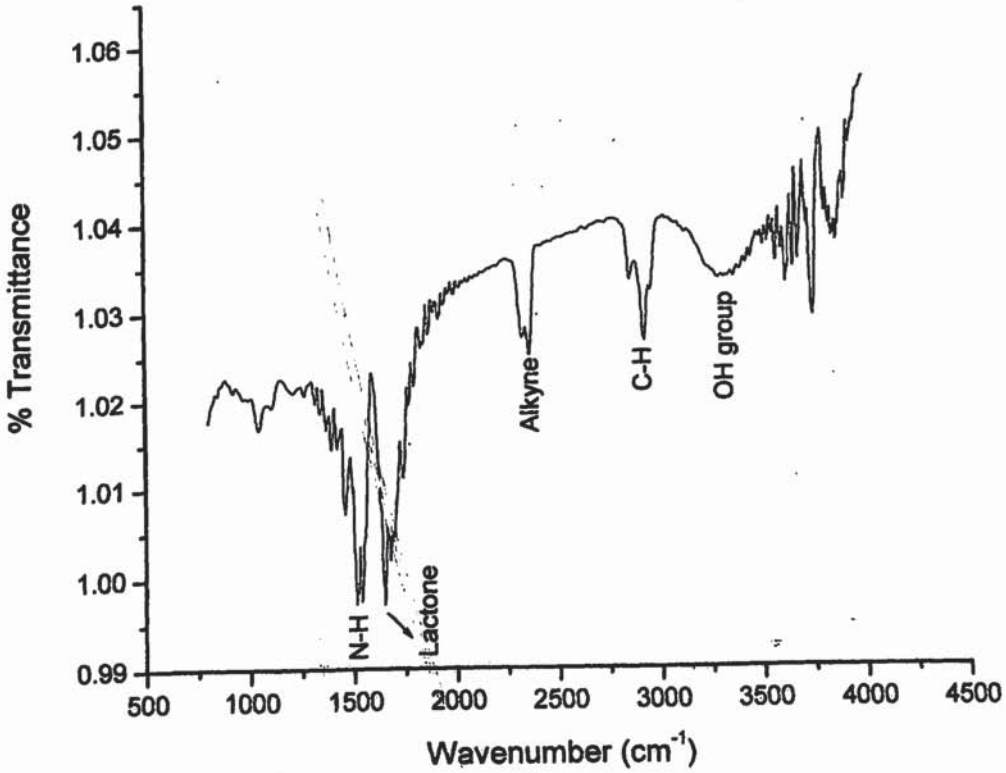




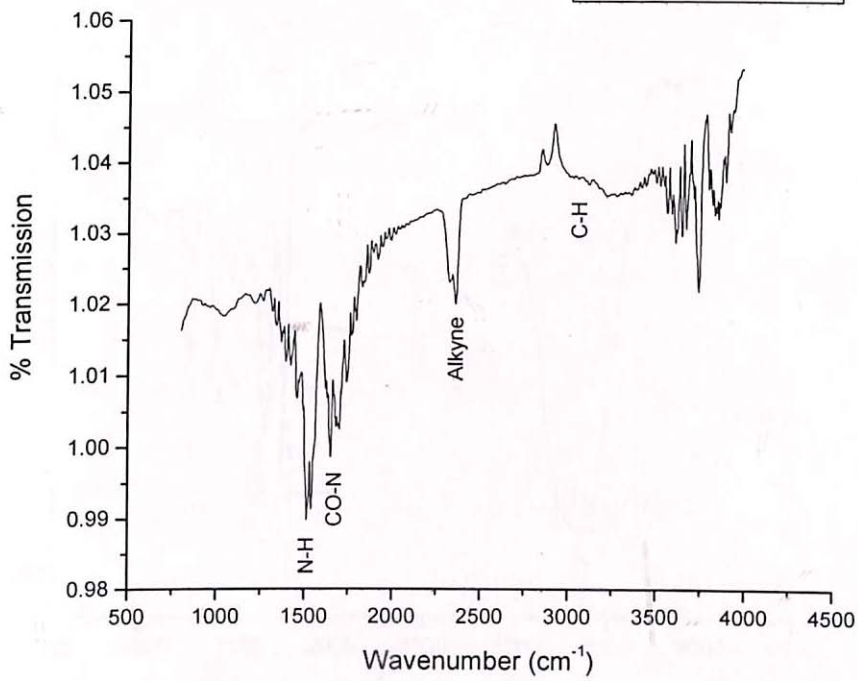
FT-IR graph LK.2.7



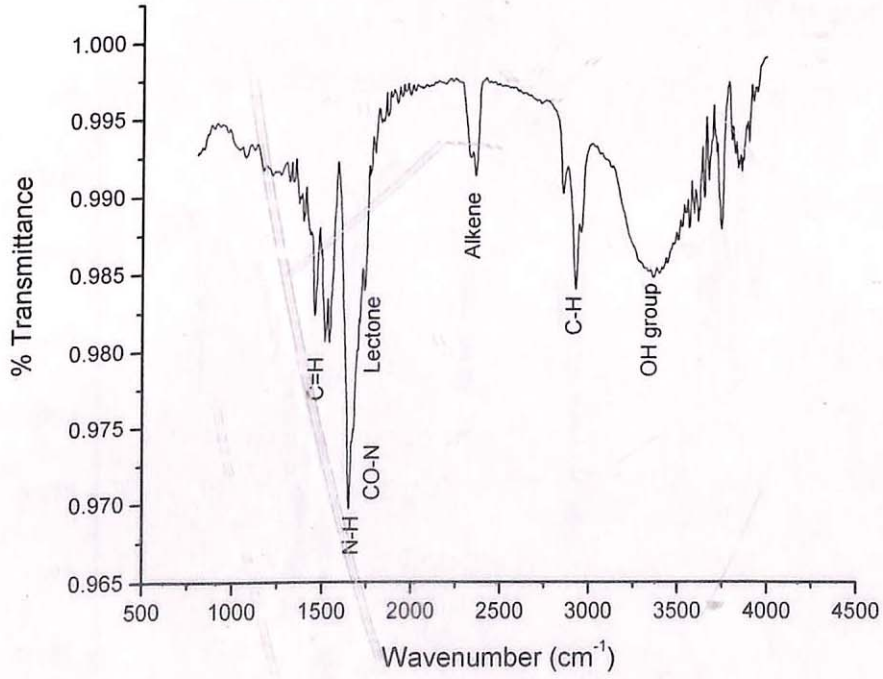
FT-IR graph LK.3.9



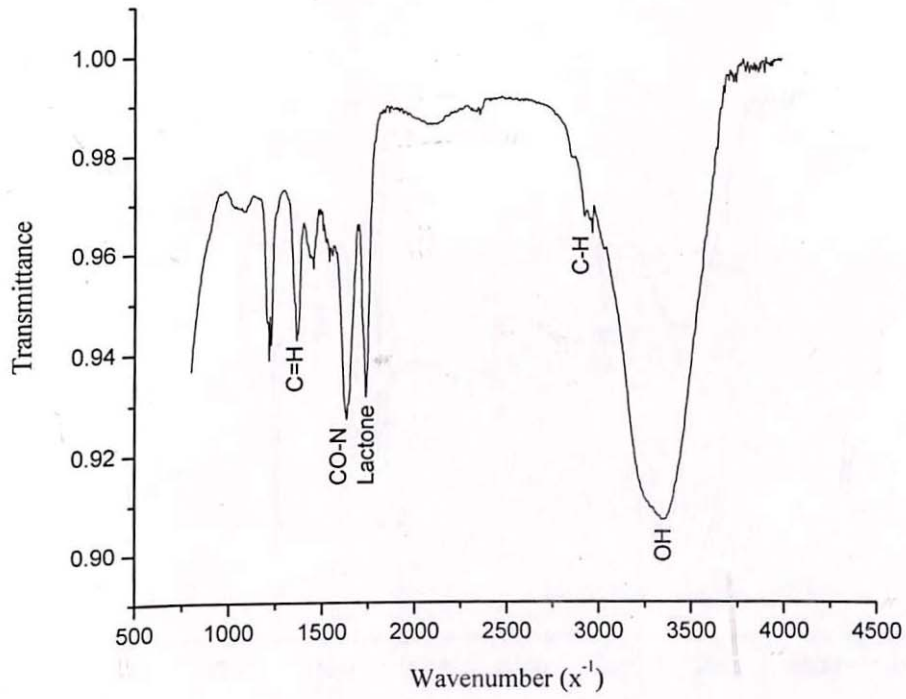
FT-IR graph LK.3.10



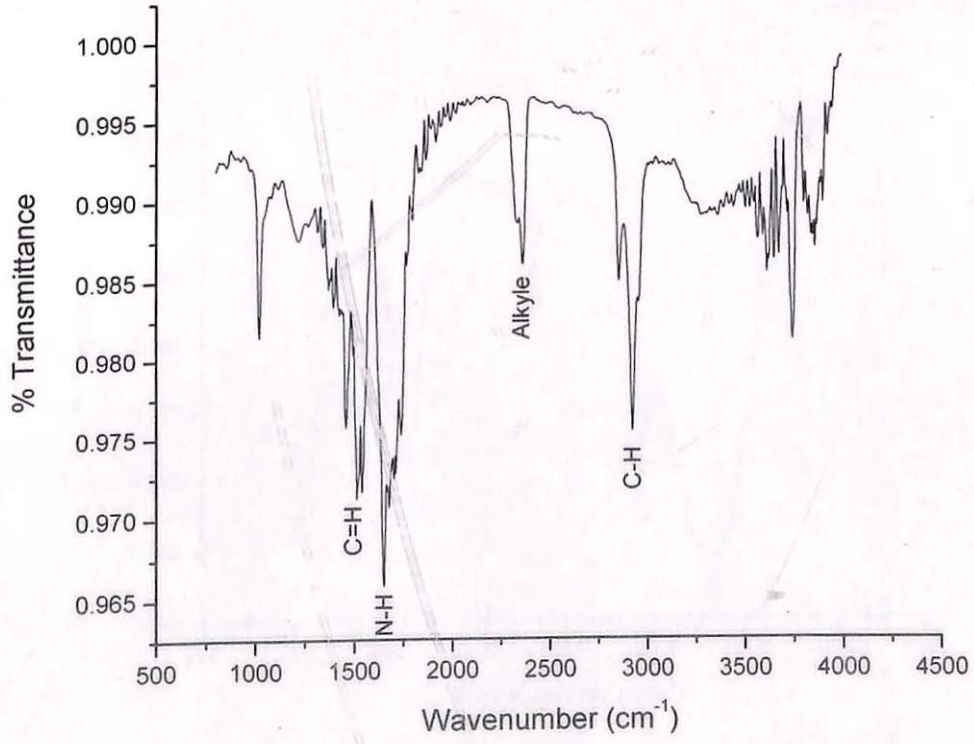
FT-IR graph LK.4.5



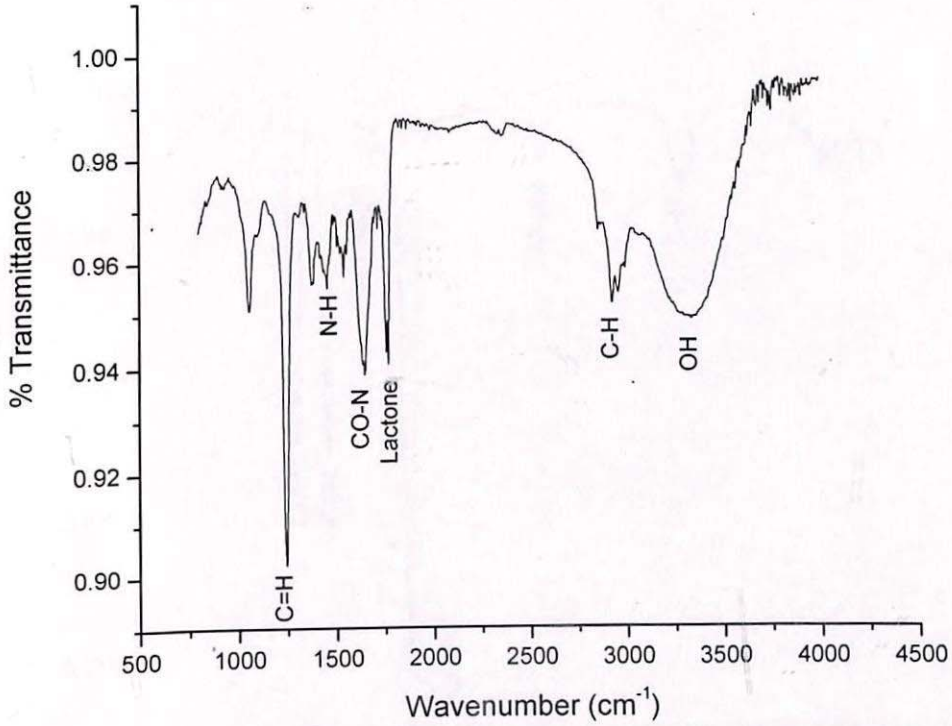
FT-IR graph LK.5.3.1



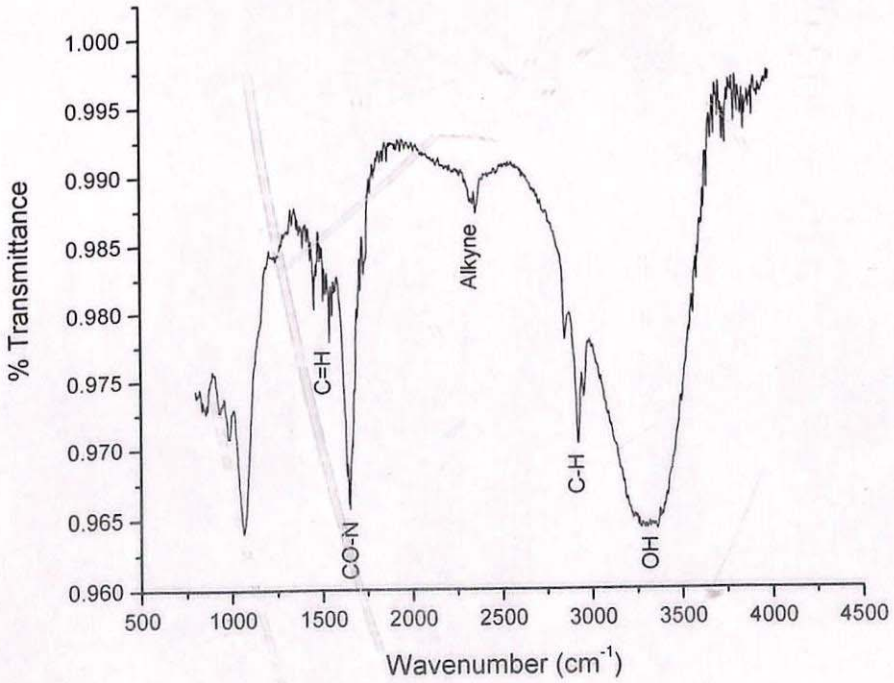
FT-IR graph LK.5.3.2



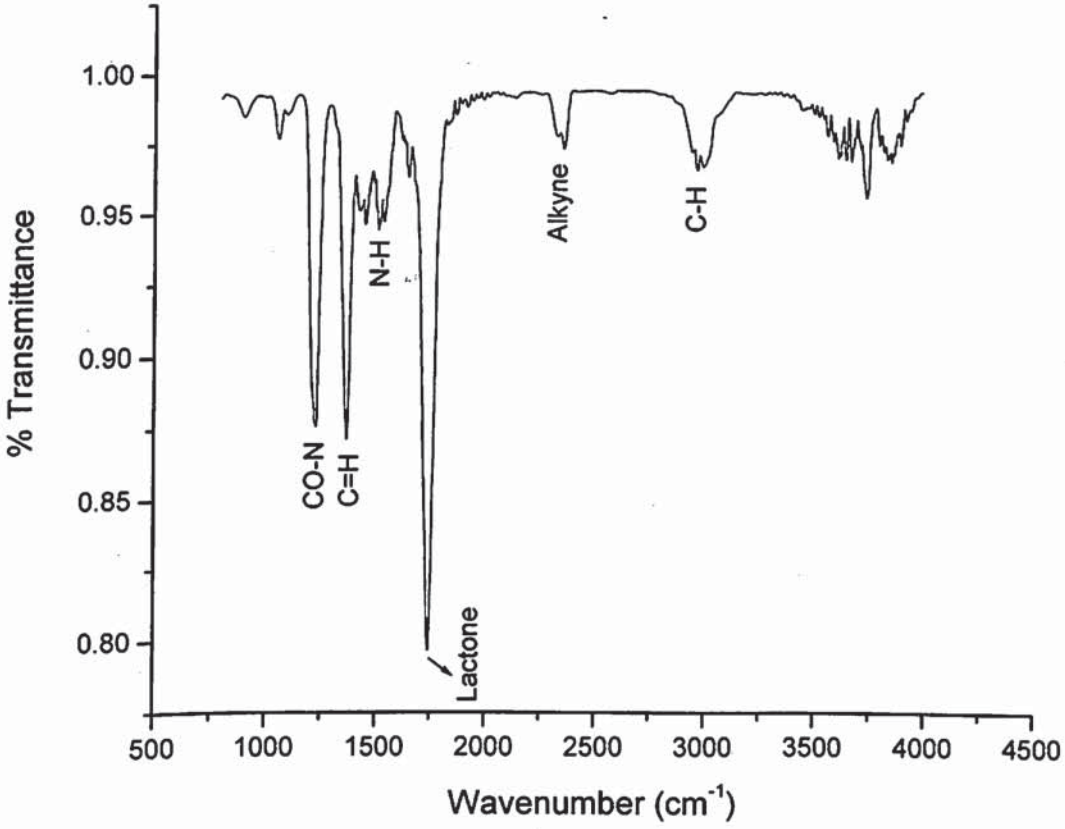
FT-IR graph LK.5.4



FT-IR graph LK.6.1



FT-IR graph LK.6.2



Chapter 5

Discussion

Chapter 5

Discussion

Sikkim lies in the hot spot zone of biodiversity. Sikkim is not only rich in flora and fauna but also known for its richness in the ethnic foods. Most of the people of Sikkim belonging to different ethnic groups such as Bhutia, Lepcha and Nepali has knowledge of indigenous ethnic food preparation and preservation. Rate of consumption of these ethnic foods are also significant in Sikkim. According to Tamang *et al* (2007) more than 90% of the people in Sikkim consume fermented food and beverages on regular basis. Even there are certain ethnic groups in Sikkim who economically depends on fermented food production for the sustenance of their livelihood. Some of the most familiar fermented foods of Sikkim are- *Kinema, Sinki, Chuurpi, Gundruk, Sel roti*, etc (Tamang *et al.*, 2012).

During the process of fermentation, microorganisms transform the substrate to increase its flavour, nutritional value and shelf-life of the food (Stiles *et al.*, 1997). Besides, fermented foods are good source of probiotic for the people in the rural area, which provide various health benefits to the consumers (Stiles *et al.*, 1997). This fermented food is a superior source of probiotics in Sikkim, for example *Chuurpi* contains lactic acid bacteria as the dominant microorganisms (Tamang *et al.*, 2012) and *Kinema* consist of various species of *Bacillus* (Tamang *et al.*, 2012). *Bacillus* species which are most commonly used as probiotics involves *Bacillus subtilis, B. coagulans, B. licheniformis, B. laterosporus* etc (Urdaci *et al.*, 2004).

Bacillus is also well known for the production of various types of biosurfactants. The first rod shaped endospore producing bacteria found to be synthesizing biosurfactant is *Bacillus subtilis*, and the biosurfactant it produces is a lipopeptide, named as 'surfactin' (Arima *et al.*, 1968). Later other biosurfactants like iturin, fengycin, and mycosubtilins were also discovered from *B. subtilis* (Vater *et al.*, 2002). In the same way *Bacillus licheniformis* produces lichenysin (a lipopeptide biosurfactant) (Yakimov *et al.*, 1995) and *Bacillus pumilus* produces pumilacidin (Naruse *et al.*1990). All these biosurfactants have antimicrobial properties (Das *et al.*, 2013). Probotic *Bacillus* produces these

antimicrobial biosurfactant lipopeptides to inhibit the growth of the other pathogenic microorganisms and inhibition of biofilm formation in the human gastrointestinal tract (Hong *et al.*, 2005).

Most of the microorganisms which are used as probiotics are species of *LactoBacillus, Bifidobacterium, Bacillus, Saccharomyces,* etc (Robert, 2006). As mentioned in the literature review section that *Bacillus* and *LactoBacillus* possess good probiotic properties and they are also the good sources of biosurfactants. Beside, *Bacillus* and *LactoBacillus* are the most common microorganisms which are used in the fermentation of food in Sikkim.

Biosurfactants are the biologically originated surface active agents, which are produced by number of microorganisms as secondary metabolites (Fiechter, 1992). These compounds possess many advanced properties like biodegradability, and less toxicity, which makes them a green detergent (Desai, 1997; Muller-Hurtig *et al.*, 1993). As biosurfactants have high efficiency to reduce the surface tension and interfacial tension, they can be used as the promising substitute of chemical surfactants (Fiedler, 1987; Kosaric, 1993).

Hence, it is possible to isolate certain good quality, non-toxic and food grade biosurfactants from the bacteria found in the unexplored regional fermented foods. As most of the Sikkim biodiversity is still unexplored for the research in the field of biotechnology and industrial microbiology, to select Sikkim's regional fermented food for isolation of biosurfactant, is a virgin area for research. These biosurfactant can be a noble product which can be used in food industry, therapeutic and pharmaceutical fields.

Saravanand *et al.* (2012) have demonstrated that most of the chemically derived surfactants used these days are derived from the petroleum resources. Because of their complex, long and cyclic structures these surfactants are non-biodegradable, therefore these polluting compounds are potential threat for the environment (Saravanand *et al.*, 2012). Therefore , there is a need to develop an eco-friendly surfactant or detergent that can be used in oil refineries, oil extraction industries, cleansing and laundry industries, pharmaceuticals, cosmetic industries, food industries and other chemical industries (Urum *et al.*, 2004).

Mbawala and Mouafo, (2012) has isolated 15 lactic acid bacteria species from fermented milk (*Penedidam*) of Cameroon, from which only 8 species were able to produce biosurfactant. In the same way Vanlerberghe *et al.*, (2009) has isolated 50 lactic acid bacterial strains and has reported only three biosurfactants producing isolates. Chauhan *et al.* (2010) has also isolated 52 different types of lactic acid producing bacteria, out of that only 23 were positive in the primary screening of biosurfactant. This shows that the number of biosurfactant producing Lacitc actid bacteria is relatively less than *Bacillus* and *Pseudomaonas*. During our work, 29 different lactic acid bacterial isolates were screened for biosurfactant following the method desccribed in the method section but none of them produced biosurfactant. On the other hand all 11 spore forming isolates and one non-spore forming isolate from *Kinema* produced biosurfactant (Table 4.2).

Bento and his co-workers (2005) have isolated 33 different bacterial cultures from the diesel contaminated soil, from which most of the isolates which showed best biosurfactant producing potential were *Bacillus* spp. In the same way, Nasr *et al.*, (2009) have found most of the isolates, from petroleum contaminated soil, having superior biosurfactant producing property are *Bacillus* spp. for example *Bacillus subtilis* (*B. subtilis*) and *B. cereus*. Bayoumi *et al.* (2010) during the structural characterization of biosurfactant, found 79% *Bacillus* spp. out of 27 different isolates of the bacterial cultures. These all research demonstrates that the most frequent species of bacteria that produces biosurfactant belong to the *Bacillus* species.

All the isolates showing positive results for the emulsification test, and all most all the isolates has given value of emulsification index. EI_{24} (Table 4.1) of more than 27 (except three isolates i.e. Lk.1, Lk.2.7 and Lk.5.3.2 with kerosene oil), which indicates that these biosurfactants are strong emulsifiers and possess food grade properties. Conducting more than one types of primary screening test is always advisable, because it make the selection of good biosurfactant producer easy. Utilization of different oils of different nature indicates that same biosurfactant shows the different emulsification efficiency with different oils. According to the experiment conducted, the maximum emulsification index has been obtained with sunflower oil. Out of 12 isolates 8 has given maximum emulsification value with sunflower oil, while isolate LK.2.6 and LK.6.1 has given maximum EI_{24} with kerosene oil, while for most of the isolates has shown minimum EI_{24}

with kerosene oil. Throughout this experiment LK.5.3.2 has shown maximum EI_{24} (EI_{24} = 68) with paraffin oil. This shows that sunflower oil and paraffin oil can be used in the media to promote the production of the biosurfactant with different *Bacillus* species.

All the cultured *Bacillus* isolates were subjected to identification according to *Bacillus* identification key (Sarkar *et al.*, 2002). However, *Bacillus subtilis, Bacillus cereus* and *Bacillus licheniformis* shows a lot of similarities in their physiological, biochemical and structural characteristics so it is quiet difficult to differentiate between closely related species like *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis*. According to the *Bacillus* identification key (Sarkar *et al.*, 2002) almost all the bacterial isolates were *Bacillus licheniformis*, but isolate number LK.3.10 and LK.6.1 could not be identified. However according to Sarkar and Tamang *et al.*, Bacillus subtilis is most dominant bacteria in *kinema*, but in this work *Bacillus licheniformis* was reported as most dominant bacteria, it may be because of limited sample collection and limited phylogenitic analyses. For more prominent bacterial identification, molecular analyses is strongly needed.

Biosurfactant was isolated from 12 different *Bacillus* isolates by acidification method. The mean dry weight of the biosurfactant from the different samples was 173.53 µg. The maximum production of biosurfactant was obtained with the isolate LK.6.2 which was 1.633 g (dry weight), while minimum production was obtained from LK.3.9, which gave only 2µg production of biosurfactant. Maximun production of biosurfactant can be obtained in stationary phase. But the production will not increase in death phase i.e. maximum production can be obtained in stationary phase and with the commencement of death phase the productivity will be reduced (Latifi *et al.*, 1995). Production of biosurfactant also depends on the other physical factors. During the process of biosurfactant extraction the pH of the media was also observed, and the pH was found alkaline. On alkaline pH the biosurfactant remain in soluble form in the supernatant of the media. When the pH of the supernatant is shifted to acidic pH the surfactant solubility reduces and the clear solution of the supernatant was converted to the suspension of biosurfactant particles. On keeping it at low temperature (at 4°C) the suspended particles of the biosurfactant settled down, which was collected by centrifugation. Remaining non-polar impurities can be removed by mixing it in the solution of methanol and chloroform

(1:2). All thee impurities dissolved in the chloroform layer and biosurfactant got accumulated at the interphase of chloroform and methanol. The biosurfactant was then extracted with methanol solution. Methanol was evaporated and biosurfactant was collected. On comparing with solvent extraction method, this is more cost effective and more sensitive technique.

Thaniyavarn and his co-workers (2003) have extracted biosurfactant with acidification method and solvent extraction method with hexane. They had further purified the biosurfactant with TLC with solvent mixture chloroform: methanol: water (65:25:4). They have found surface active compounds migrated to give spots at various points which were reflecting the R_f value from 0.56 to 0.94 (Thaniyavarn *et al.*, 2003). In the same way Priya *et al.* (2009) has done the preliminary characterization of the biosurfactant by TLC with the solvent system $CHCl_3$:CH_3OH:H_2O (70:10:0.5) (v/v/v). They have developed the spots with ninhydrin to detect the lipopeptide as red spots, which was showing the R_f values 0.48, 0.51, 0.52, 0.58 and concluded it to be the surfactin produced by *B. subtilis* (Priya *et al.*, 2009). Varadovenkatesan *et al.* (2013) have run TLC for their biosurfactant with solvent system of chloroform: methanol: water (64: 25:4) and observed the spots with short wavelength UV light and ninhydrin solution (Varadovenkatesan *et al.*, 2013). Lin *et al.* (1997) have purified surfactin up to 95% with TLC using mobile phase chloroform: methanol: water in the proportion of 65:25:4 (vol/vol/vol). Makkar *et al.* (1999) have uses TLC technique with chloroform, methanol and water in ratio of 65:15:1 respectively to identify the biosurfactant with its R_f value, and they have calculated the R_f value equal to 0.337 which was similar to surfactin (Makkar *et al.*, 1999).

But Hsuehy *et al.* (2007) has spotted the solution of biosurfactant and methanol (10µg/µl) on TLC plate and run the TLC with $CHCl_3$:CH_3OH:H_2O in 80:25:4 (v/v/v). With this mobile phase, on treatment with ninhydrin they have got the R_f value 0.69, which was identified as surfactin of *B. cereus* with the help of NMR analyses (Hsuehy *et al.* 2007). Matsuura *et al.* (2014) has develop TLC plate with mobile phase solution of chloroform: methanol: ammonium hydroxide (65:25:4) and observed the two distinct spots and labeled as type I and type II from the crude biosurfactant. The R_f value of type I spots was 0.44 and 0.62 and for type II spots R_f values were 0.52 and 0.62. After further

analyses with FT-IR and NMR they have concluded that R_f 0.62 corresponds to surfactin and R_f value 0.52 was for the biosurfactant which has closely similar structure to surfactin (Matsuura *et al.* 2014).

With our work no good results have been obtained on TLC plates using mobile phase solution of chloroform: methanol: water in various combinations. But with solvent system of chloroform: methanol: 20% NH_4OH we have got very clear and sharp separation of the various components of the crude biosurfactant. Two different types of spots have also been observed with 0.5g ninhydrin/100ml of acetone. For most of the biosurfactants R_f obtained were 0.42, 0.48, 0.52 which are suppose to be biosurfactant similar structure of surfactin (Matsuura *et al.* 2014). While the other groups of R_f value 0.91, 0.93, 0.96, 0.98 are suppose to be surfactin (Thaniyavarn *et al.* 2003; Hsuehy *et al.* 2007). Beside that the spots of R_f value 0.75, 0.86, 0.88 could not be assigned to any known biosurfactants from the literature.

Saravanan and Vijaykumar (2012) have explored their biosurfactant samples to FT-IR analyses under the spectral range of 400-4000 wavenumber (cm_{-1}). They have notice the bands for $\square CH_2$ and $-CH_3$ at 3000 to 2700 cm^{-1}. They have also observed the deformation variation in the region of 1467 and 1379 cm^{-1}, which indicates the presence of alkyl group. They have also observed the stretching bands at 1745 cm^{-1}, providing the hint for the presence of easter compound. They have concluded it as rhamnolipid (a glycolipid) (Saravanan *et al.,* 2012). On the other hand for lipopeptide biosurfactant Pereira *et al* (2013) have observed peak at 3305 cm^{-1} which is corresponding to NH-stretching mode, bands at 1643 cm^{-1} represented the stretch mode of CO-N bond; and deformation bands at 1543 cm^{-1} indicates N-H bond combined with C-N stretching mode. Beside that bands between 2957-2855 cm^{-1} showed the presence of –CH3; $-CH_2-$ aliphatic chain (Pereiya *et al.*, 2013). To comprehend the whole structure of biosurfactant Varadavenkatesan *et al* (2013) have studied the FT-IR peaks, and observed vibration at wavenumber 3433 cm^{-1} and 3600 cm^{-1} to 3100 cm^{-1} for the presence of C-H and N-H bonds. This is the characteristic of carbon containing compound with amino group. They have also received the sharp peaks at 1463 cm^{-1}, 1379 cm^{-1}, 2955 cm^{-1} and 2854 cm^{-1} which indicates the presence of aliphatic chains of $-CH_3$ and $-CH_2-$. These structures represent the presence of aliphatic chains of alkyl group. Strong peaks for carbonyl

groups have also been observed at 1741 cm^{-1}, 1726 cm^{-1} and 1713 cm^{-1} (Varadavenkatesan *et al.*, 2013). Like other workers Yalçın E. *et al.* (2010) have found peaks in the region of wavenumber 1450 cm^{-1}-1455 cm^{-1}, which is corresponding to CH_2 bending of lipids (Garip *et al.*, 2009). They have observed the bands for primary amide vibration at 1600 cm^{-1}-1700 cm^{-1} which is the characteristic of proteins. Along with that they have also got some characteristic peaks in the wavenumber region of 3300 cm^{-1} which indicates the stretching vibration of N-H and O-H in peptides (Kong *et al.*, 2007). They have observed some peaks at 1541 cm^{-1} indicating the presence of secondary amides and N-H bending and C-H stretching (Gomez *et al.*, 2003). Some research on *B. subtilis* MTCC2423 biosurfactant shows the FT-IR absorbance at 3310 cm^{-1}, which represents the presence of N-H stretching. While at 1650 cm^{-1}, CO-N stretching mode of NH bonds has been observed in combination of C-N stretching. Absorbance at wavenumber 2970 cm^{-1} to 2850 cm^{-1} 1450 to 1380 was observed, which indicate the presence of aliphatic chain of C-H (Emine *et al.*, 2010). They have also found the peak at 1730 cm^{-1} for lactone ring, which is the strong indicator of surfactin (Makkar *et al.*, 1999).

FT-IR analyses of our isolated biosurfactants have also been done to understand the basic biochemical nature and chemical composition of the compound. On comparing the analytical details of our biosurfactants with the data of other workers (as mentioned above), a lot of similarities have been observed. Infra-red absorption peaks obtained by FT-IR analyses were showing great similarities in the region of wavenumber at which the peaks have been obtained by other authors. Many reports have demonstrated the C-H stretching in the region of 2850 cm^{-1} to 3000 cm^{-1}, in the same way we also got absorption peaks in region of 2924 cm^{-1} to 2972 cm^{-1} for different biosurfactants isolated from different cultures. Here for most of the biosurfactants we have received the absorbance at 2925 cm^{-1} wavenumber, but *Bacillus* isolate LK.1 did not gave peak in this region but we have observed a characteristic deformation peak in the region of 1216 cm^{-1} to 1231 cm^{-1} which also corresponds to C-H bond stretching. This indicates the presence of lipid chain in the compound. On the bases of comparative analyses of FT-IR data alkyne group has also been detected, as absorption peaks have been obtained in the region of wavenumber of 1365 cm^{-1} to 1459 cm^{-1} in all the samples. Papers have

demonstrated the N-H stretching peaks in the region of 3300 cm^{-1} to 3600 cm^{-1} and we have recorded the sharp peak at 3362 cm^{-1} only for the biosurfactant of *Bacillus* isolate LK.4.5 only. Many workers have recorded the deformation region in 1600 cm^{-1} to 1741 cm^{-1} for CO-N bonds, we also have observed the peaks in the region of 1632 cm^{-1} to 1718 cm^{-1}, but for *Bacillus* isolate LK6.2 no peaks have been observed in this region but biosurfactant of this bacterial culture have given the peak at 1365 cm^{-1} which also correspond to the CO-N bond stretching. Absorbance peaks at 1508 cm^{-1} to 1542 cm^{-1} have also been recorded which corresponds to N-H bond deformation in combination of C-N bond (Saravanan *et al.*, 2012; Pereiya *et al.*, 2013, Varadavenkatesan *et al.*, 2013; Makkar *et al.*, 1999; Emine *et al.*, 2010). These details illustrate that the isolated compounds are lipopeptide. Peaks for lactone ring have been obtained in the region of 1701 cm^{-1} to 1740 cm^{-1} for *Bacillus* isolates LK.1, LK.2.6, LK.2.7, LK.4.5, LK.5.3.1 and LK.6.2. Lactone ring is the characteristic of surfactin. So it can be concluded that the biosurfactants isolated is either surfactin or have similar structure to that of surfactin.

The important fact that have been observed is that various biosurfactants isolated from different isolates of *Bacillus* show similarities in the bands of TLC and R_f value, and at the same time the biosurfactants are also showing the great similarities in the absorption peaks for FT-IR analyses. TLC analyses presented the bands close to the R_f value of surfactin and FT-IR peaks for lactone ring strength the hypothesis for the presence of surfactin in the sample. So all of the study represents that most the biosurfactant carry similar chemical, structural and physical properties to surfactin.

Chapter 6

Summary & Conclusion

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Probiotics are one of the most admirable subjects of research that attracts the academic and industrial scientists because of its promising health benefits. The 'probiotics' term was first of all coined by Lilly and Stillwell in 1965 in contrast to antibiotics, for the organisms that derives the factors for the growth of normal microflora of our gut (Lilly *et al.,* 1965). Probiotics can be defined as the microorganisms that can be excogitated in different types of food products, drugs or dietary supplements for human consumption in order to provide the health benefits. The most comman species of microorganisms that are used as probiotics are *LactoBacillus, Bifidobactrium, Saccharomyces cerevisiae* and some strains of *Bacillus* and *E. coli (*Urdaci *et al*., 2004*)*.

Fermented food has been the primary and most popular source of probiotics. Even during the ancient time people have recognized the health benefits of the fermented food, and they were consuming the probiotics to take the advantages of microbial health enhancing properties. But about 40 years ago it was the first time when scientist archive success in the isolation of biosurfactant compounds from bacteria. Gradual study of biosurfactant properties and its applications reviled its great potential in the field of food industry and therapeutic fields, which promoted the scientist to isolate the food grade biosurfactant from probiotic microorganism. Some of the work has been done in the field of isolating biosurfactant from Lacto*Bacillus*. But till this date no great success has been achieved as the productivity is so low and production cost is so high.

Therefore there is great demand to isolate a cost effective food grade biosurfactant from new sources. This demand now motivates the scientist to isolate biosurfactant from various ethnic fermented food producing microorganisms. A lot of papers have reported the biosurfactant production from the bacteria found in milk fermented food like curd, yoghurt, cheese, butter etc.

In the same direction we have screen 29 lactic acid bacteria from *Chuurpi*, Yak milk and *Mesu* (Sikkim fermented food of Sikkim) and 12 isolates of *Bacillus* from *Kinema* (a soy-bean fermented food of Sikkim and Darjeeling hills). No biosurfactant producing

microorganisms have been isolated from the 29 lactic acid producing test microorganisms used in our work. While all 12 *Bacillus* isolates from *kinema* have shown positive results in primary screening of biosurfactant production. This clearly reviles that *Bacillus* spp. is more frequent source of biosurfactant in comparison to lactic acid bacteria. It has also been observed that product lose is minimum in the process of biosurfactant extraction by acidification method. Purification of biosurfactant is the most expensive part of downstream process in biosurfactant production. It have been found that, use of methanol and chloroform solution for biosurfactant purification is quiet cost effective, and give better results. *Bacillus licheniformis* isolate LK.6.2 was found to producer the highest amount of biosurfactant.

Differences in results were not observed when drop collapse method was used for screening of biosurfactants with five different thypes of oils. Howeveer, this method has proved to be fast, easy and most reliable for qualitative screening of bacteria for biosurfactant production. During the estimation of emulsification index, most of the biosurfactant have emulsify the sunflower oil most frequently and with maximum efficiency, while all the biosurfactant were able to emulsify the mustard oil and paraffin oil quiet efficiently but the emulsification index of biosurfactant with mustard oil and paraffin oil is relatively less than that of with sunflower oil.

Biosurfactant characterization by TLC have shown two major groups of biosurfactant on the bases of polarities, one group represented the biosurfactant which have low polarity and gave R_f value close to 50, while other group of biosurfactant represent the R_f value approximately 95. R_f value of biosurfactant has also proved that the biosurfactants are structurally similar to surfactin. FT-IR amylases have also proved that the biosurfactant is lipopeptide in nature and some of them contain lactone ring which is the characteristic of surfactin. So all results of TLC and FT-IR concluded that the biosurfactant isolated from *Bacillus* isolates are lipopeptides and structurally similar to surfactin.

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Appendix

Appendix A: *Bacillus* Identification Key

Table 1

Phenotypical key used for tentative identification of Gram-positive endospore forming rod-shaped bacteria[a]

(1) Allantoin or urate required	positive	*Bacillus fastidiosus*
	negative	2
(2) Catalase	positive	3
	negative	20
(3) Voges–Proskauer	positive	4
	negative	11
(4) Growth in anaerobic agar	positive	5
	negative	10
(5) Growth at 50 °C	positive	6
	negative	7
(6) Growth in 7% NaCl	positive	*Bacillus licheniformis*
	negative	*Bacillus coagulans*
(7) Acid and gas from glucose	positive	*Paenibacillus polymyxa*
	negative	8
(8) Reduction of NO_3^- to NO_2^-	positive	9
	negative	*Paenibacillus alvei*
(9) Parasporal body in sporangium	positive	*Bacillus thuringiensis*
	negative	37
(10) Hydrolysis of starch	positive	*Bacillus subtilis*
	negative	*Bacillus pumilus*
(11) Growth at 65 °C	positive	32
	negative	12
(12) Hydrolysis of starch	positive	13
	negative	17
(13) Acid and gas from glucose	positive	*Paenibacillus macerans*
	negative	14
(14) Width of rod ≥ 1.0 mm	positive	34
	negative	15
(15) Growth at pH 6.8	positive	16
	negative	*Bacillus alcalophilus*
(16) pH in VP broth < 6.0	positive	28
	negative	26
(17) Growth in 10% NaCl	positive	*Bacillus pasteurii*
	negative	18
(18) Growth in anaerobic agar	positive	*Brevibacillus laterosporus*
	negative	19
(19) Acid from glucose	positive	30
	negative	24
(20) Growth at 65 °C	positive	33
	negative	21
(21) Growth in anaerobic agar	positive	22
	negative	*Bacillus azotoformans*
(22) Decomposition of casein	positive	35
	negative	23
(23) Parasporal body in sporangium	positive	*Paenibacillus popilliae*
	negative	35
(24) Growth at 50 °C	positive	*Bacillus badius*
	negative	25
(25) Growth at 5 °C	positive	*Bacillus insolitus*
	negative	*Bacillus sphaericus*
(26) Acid from arabinose	positive	*Bacillus lentus*
	negative	27
(27) Growth at 5 °C	positive	30
	negative	31
(28) Growth at 5 °C	positive	*Paenibacillus macquariensis*
	negative	29
(29) Growth in 10% NaCl	positive	*Virgibacillus pantothenticus*
	negative	*Bacillus circulans*
(30) Hydrolysis of urea	positive	*Bacillus globisporus*
	negative	*Bacillus marinus*
(31) pH in VP broth > 7	positive	*Brevibacillus brevis*
	negative	*Bacillus firmus*
(32) Hydrolysis of starch	positive	33
	negative	*Bacillus schlegelii*
(33) Growth at pH 6.8	positive	*Bacillus stearothermophilus*
	negative	*Alicyclobacillus acidocaldarius*
(34) Growth in anaerobic agar	positive	*Bacillus thuringiensis*
	negative	*Bacillus megaterium*
(35) Growth in 10% NaCl	positive	*Bacillus pasteurii*
	negative	36
(36) Growth at 40 °C	positive	*Paenibacillus larvae*
	negative	*Paenibacillus lentimorbus*
(37) Colony rhizoidal	positive	*Bacillus mycoides*
	negative	38
(38) Cells motile	positive	Bacillus cereus
	negative	Bacillus anthracis

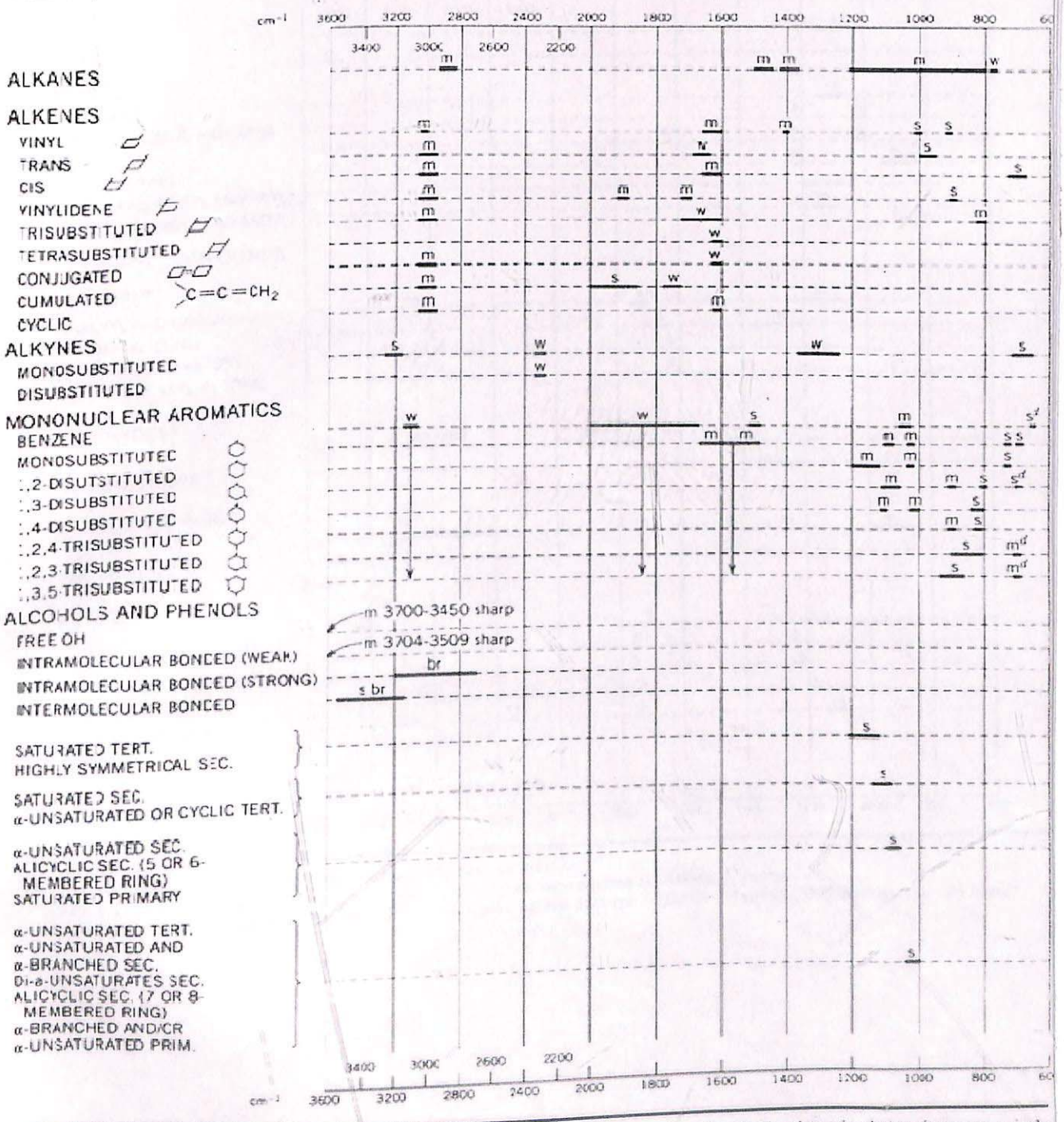
[a] Numbers on the right indicate the number (on the left) of the next test to be applied until the right-hand number is replaced by a species name (based on Claus and Berkeley, 1986; Seppeky and Hemphill, 1992; Euzéby, 1997)

and 25 pmol of each primer. The mixture was overlaid with a drop of sterile mineral oil (prod. no. M5904, Sigma, St. Louis, MO, USA) and capped. In each PCR assay, a negative control without any bacterial DNA was included. PCR with random primers was carried out in a DNA thermal cycler (Perkin-Elmer 480). The temperature profile started with initial denaturation for 5 min at 94 °C, followed by 40 amplification cycles each consisting of 1 min at 94 °C, annealing for 1 min at 35° (primer R1) or 30 °C (primer S1) and extension for 2 min at 72 °C. After completion, reaction mixtures were cooled to 4 °C.

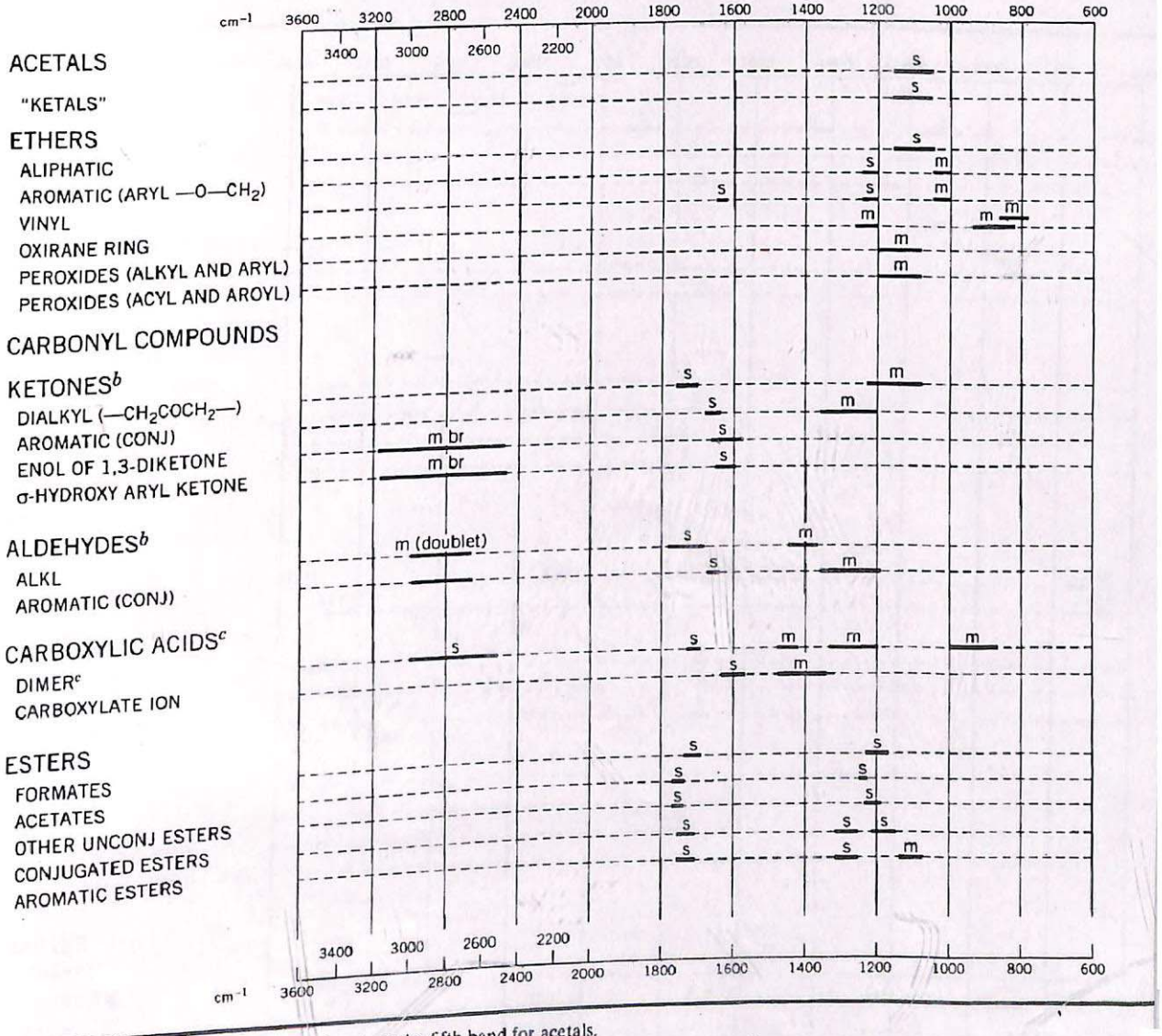
A 25-µl sample of the PCR product was mixed with 5 µl loading buffer consisting of 25% Ficoll-DL

Reference: Sarkar P.K. et al (2002)

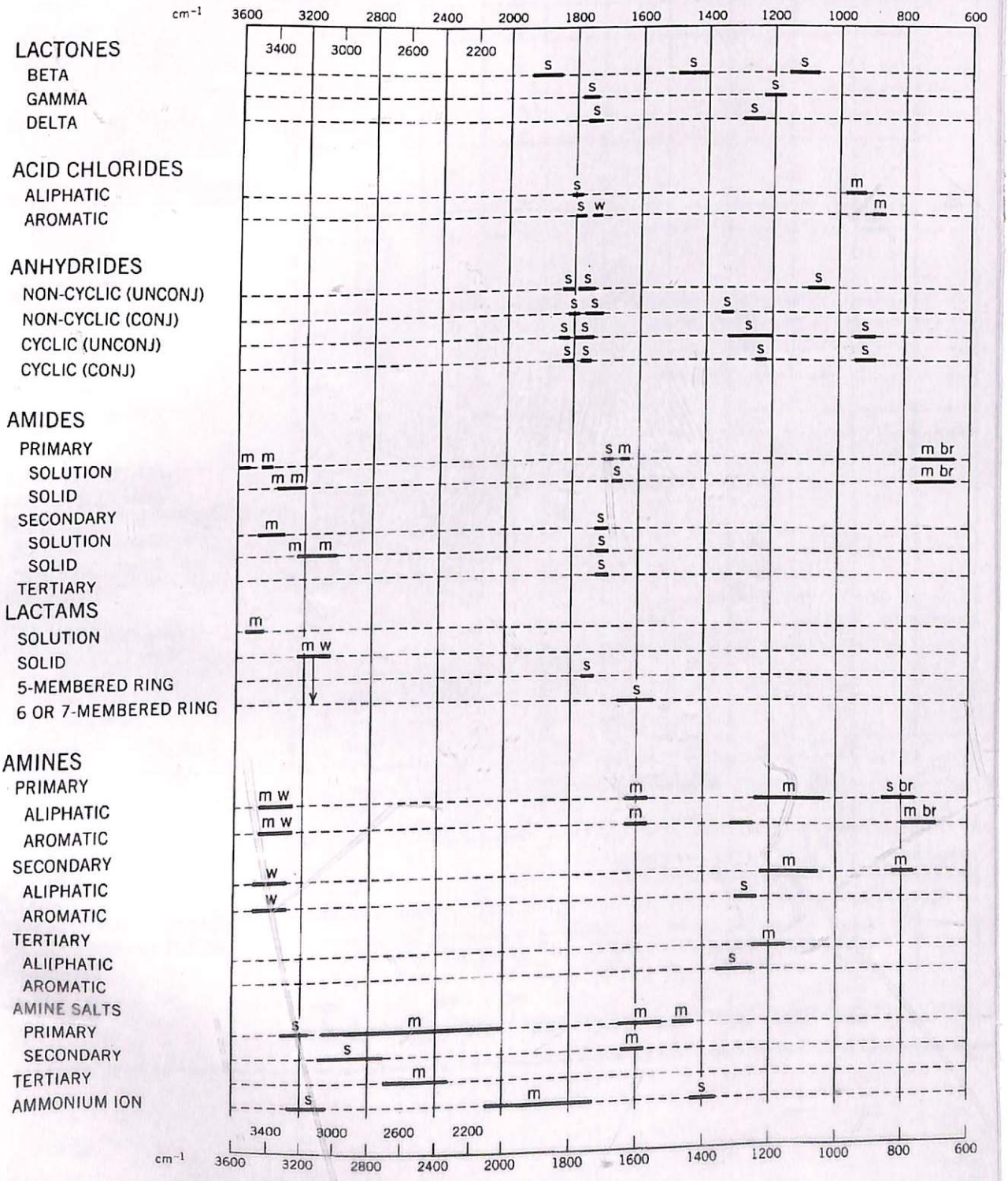
Appendix B: Characteristics Group Absorptions (FT-IR Spectra)

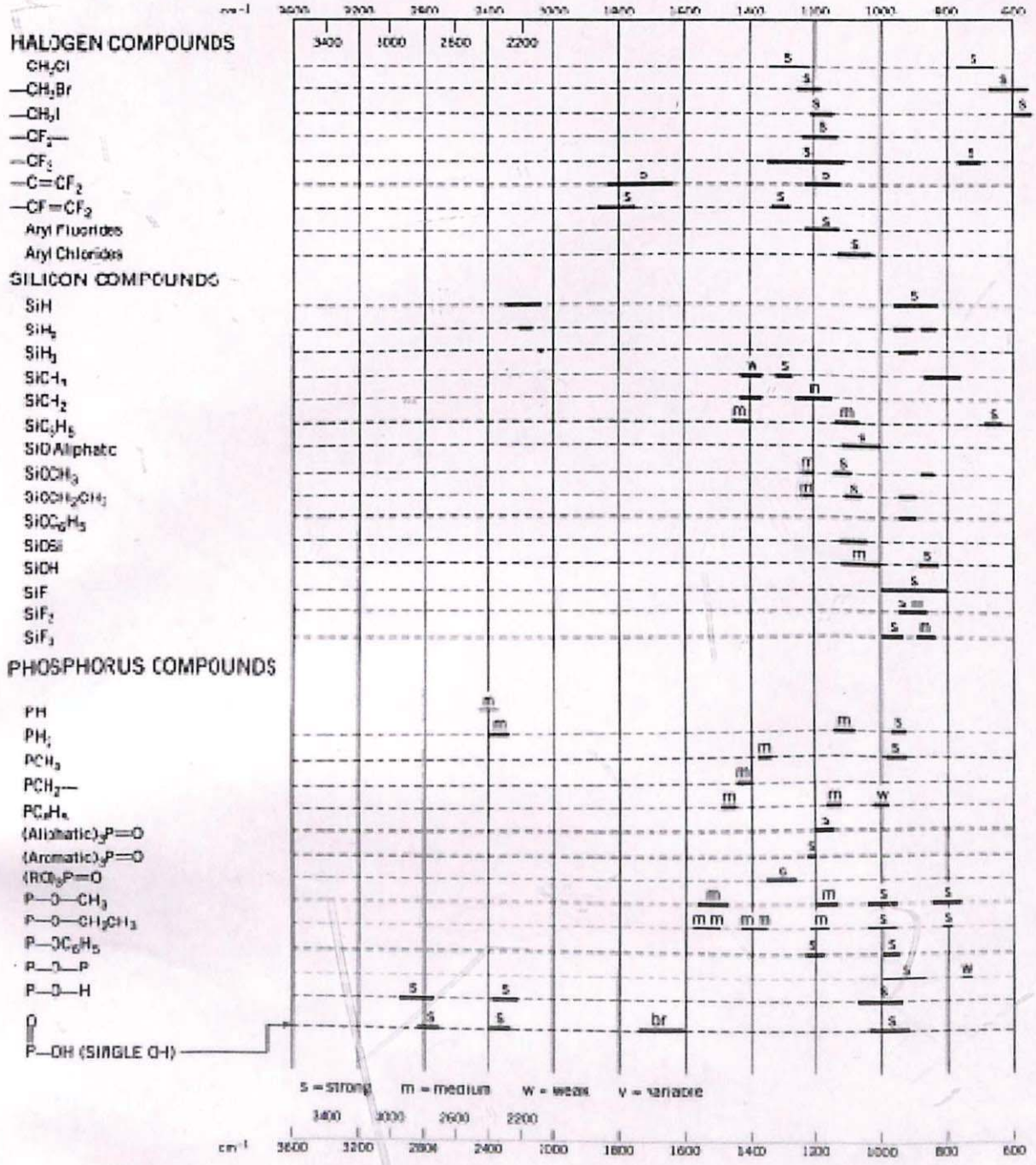


[a] Absorptions are shown by heavy bars. s = strong, m = medium, w = weak, sh = sharp, br = broad. Two intensity designations over a single bar indicate that two peaks may be present.
[b] May be absent.
[c] Frequently a doublet.
[d] Ring bending bands.



[a] Three bands, sometimes a fourth for ketals, and a fifth band for acetals.
[b] Conjugated aliphatic examples show C=O stretch at virtually the same position as aromatic structures.
[c] Conjugated examples show C=O stretch at lower wavenumbers (1710–1680 cm^{-1}). The O—H stretch (3300–2600 cm^{-1}) is very broad.


cm⁻¹
3600 3200 2800 2400 2000 1800 1600 1400 1200 1000 800 600
3400 3000 2600 2200
LACTONES
BETA
GAMMA
DELTA
ACID CHLORIDES
ALIPHATIC
AROMATIC
ANHYDRIDES
NON-CYCLIC (UNCONJ)
NON-CYCLIC (CONJ)
CYCLIC (UNCONJ)
CYCLIC (CONJ)
AMIDES
PRIMARY
SOLUTION
SOLID
SECONDARY
SOLUTION
SOLID
TERTIARY
LACTAMS
SOLUTION
SOLID
5-MEMBERED RING
6 OR 7-MEMBERED RING
AMINES
PRIMARY
ALIPHATIC
AROMATIC
SECONDARY
ALIPHATIC
AROMATIC
TERTIARY
ALIPHATIC
AROMATIC
AMINE SALTS
PRIMARY
SECONDARY
TERTIARY
AMMONIUM ION
3400 3000 2600 2200
cm⁻¹
3600 3200 2800 2400 2000 1800 1600 1400 1200 1000 800 600



Source: "Silverstein R.M. & Webster F. X. (2010).