Chapter 1

Introduction and Literature survey

1.1. Introduction

Surfactants are the wetting agents that lower the surface tension of water and also the interfacial tension between oil and water by absorbing at liquid-liquid interface. They are amphiphilic in structure consisting of both hydrophobic (tail) and hydrophilic (head) groups that have the property of being soluble in both organic solvents and water respectively.

1.1.1. Surface tension

It is the property of the liquid caused by cohesive attraction i.e. intermolecular attraction between the molecules within the substances. It has the dimensional unit of force per unit length and it is represented in SI units as Newton per meter and in CGS unit as dynes per centimetre (cm). Surface energy quantifies the disruption of intermolecular bonds that occurs when a surface is created. It is the interactions between the forces of cohesion and the adhesion which determines the wetting property, the spreading of a liquid over a surface, occurs. The surface tension (force per unit length) and the surface energy density are identical for the liquid samples.

1.1.2. Interfacial tension

It is the property of the interface between liquid and some other substance caused by adhesive force. It can be measured by the contact angle measurement. Contact angle (θ) is the angle at which the two phases meet on a solid phase indicated by θ.

1.2. Properties of surfactants

Surfactants are capable of reducing the surface tension of water by partitioning at the interface between air and water. In water, they tend to form molecular aggregates called vesicles and micelles. The concentration at which the surfactant molecules start forming micelles is called the critical micelle concentration (CMC). Once the surfactants are added in to the system they will partition in to the interface of the water molecules which reduce the surface tension. Vesicle, a supramolecular structure made up of many aggregates of the surfactant molecules. They formed naturally due to the properties of lipid molecules. The CMC of the solution containing surfactants are determined by

measuring the surface tension of the solution. The surface tension of the liquids is routinely measured by du-Nouy ring method and wihelmy plate method.

1.2.1. Du-Nouy ring method

In this method, a platinum ring is dipped in to the liquid phase and then slowly lifting the ring from the surface of the liquid. The force required to raise the ring from the liquid surface is measured as surface tension and expressed in dynes per centimeter or milli newton per meter.

1.2.2. Wilhelmy plate method

In this method, the thin plate is used to measure the surface or interfacial tension. The plate is fixed perpendicular to the liquid interface and moved towards the surface of the water until the meniscus connects it. The force exerted on it measured and expressed as same.

1.3. Types of surfactants

Surfactants are categorized in to two type's namely synthetic surfactants and biosurfactants.

1.3.1. Synthetic surfactants

Chemically derived surfactants are called as synthetic surfactants and they are classified in to four groups such as cationic, anionic and non-ionic. Cationic surfactants are dissociated into water according to the positive charge neutralization. This class of surfactants mainly has nitrogen compounds such as fatty amine salts and quaternary ammoniums, with long alkyl chains. The hydrophilic parts of the non-ionic surfactants are composed of polyethylene glycol chain and lipophilic portions composed of alkyl or alkylbenzene type chains. These non-ionic surfactants do not have any electric charge, which makes them resistant to water hardness deactivation. Anionic surfactants are composed of alkaline metals like Na^+, K^+. The head portion of the anionic surfactants is negatively charged. Most commonly used anionic surfactants are alkly sulphates, alkyl ethoxylates sulphates and soap. Amphoteric/zwitterionic surfactants can anionic, cationic or non-ionic in solution, mainly depends on the acidity or pH of the solution.

1.3.1.1. Uses of synthetic surfactants

Surfactants have wide range of industrial applications like petroleum refinery, cosmetics and detergent. Surfactants may be applied to advantage in many parts of the petroleum production process: in reservoirs, in oil wells, in surface processing operations, and in environmental, health, and safety applications (Makkar and Rockne 2003). Though there are broad spectrum applications of synthetic surfactants in various process industries, several drawbacks like toxicity, environmental in-compatibility, and stability problems are causes of major concern.

As alternative to synthetic surfactants, in recent times, considerable attention has been paid to the production of microbially derived biosurfactants. Microbial surfactants are non-toxic and easily biodegradable as opposed to synthetic surfactants and require a green process for their fermentative production.

1.3.2. Microbial surfactants

Microbial surfactants are mostly anionic amphipathic surface active molecules produced by various microorganisms. The structure of these molecules is basically composed of hydrophobic and hydrophilic moieties that help these molecules to partition preferentially at the interface between two liquid phases (Das *et al*., 2008c). These properties cause reduction in the surface tension of water approximately 45 units from the original surface tension values. Microbial surfactant molecules possess several advantages over the chemically derived surfactants i.e. lower toxicity, higher biodegradability, environmental compatibility, potential stability and activity at high temperature, extremes of pH values and high salinity conditions (Desai and Banat 1997; Makkar and Cameotra 1998; Rahman *et al*., 200; Joshi *et al*., 2008; Feng *et al*., 2010). It has the ability to emulsify aqueous, non-aqueous phase, to biodegrade the hydrocarbons and also used for bioremediation purpose.

1.3.2.1. Classification of microbial surfactant

Depending on the chemical nature of the two distinct moieties, microbial surfactants are broadly classified into different diverse groups such as glycolipids, lipopeptides, lipoproteins, fatty acids, neutral lipids, phospholipids, particulate biosurfactants and polymeric biosurfactants (Ron and Rosenberg 1999; Mukherjee *et al*., 2006). The

structural classifications of these molecules include hydrophobic tail portion (consisting of saturated, unsaturated or fatty acids) and hydrophilic head moieties (comprised of amino acids or peptides or polysaccharides (Ron and Rosenberg 2002; Vater *et al*., 2002; Das *et al*., 2008a; 2008b).

1.3.2.2. Commercial and health care applications of biosurfactants

Microbial surfactants are potentially more applicable in industrial processes because of their desirable properties as mentioned earlier (Bongolo 1999; Banat *et al*., 2000; Nitschke and Costa 2007; Singh *et al*., 2007; Rahman *et al*., 2008). Some of the potential applications are microbial enhanced oil recovery and use as emulsification agents in pharmaceutical, food, dye, cosmetic, and agrochemical industries (Singh *et al*., 2007). These molecules also exhibit the properties of improved degradation of hydrocarbon contaminants and heavy metal removal (Das *et al*., 2008a; Das *et al*., 2009a). The potential usage of biosurfactants in the medical field has increased rapidly in recent years. Biosurfactants of terrestrial origin have been reported to show environmental bioremediation properties (Sen 2008; Ron and Rosenberg 2010) and potential therapeutic applications as antiviral, antitumor and antimicrobial agents (Banat *et al*., 2000; Cameotra and Makkar 2004; Rodrigues *et al*., 2006a). Considering increasing menace of drug resistance against pathogenic microorganisms, these microbial surface active molecules may find potential applications as drug candidates for new age chemotherapy and may occupy an important place in biopharmaceutical drug discovery and development program.

1.4. Marine biosurfactants

The marine environment comprises of 70% of the earth surface and is a bounty of natural products. There still remains a largely unexplored reservoir of the diverse microorganisms and their products. The current menace of multidrug resistance among pathogenic organisms has instigated the scientific community to focus on the marine habitat in search of novel bioactive compounds like lipopeptides (Jensen and Fenical, 1996; Das *et al*., 2008b).

1.5. Literature survey

Microbial surfactants are produced by a variety of microorganisms derived from terrestrial contaminated environments (Table 1.1) (Desai and Banat, 1997; Mukherjee *et al*., 2006) and are amphiphilic molecules with hydrophilic and hydrophobic domains, thereby facilitating uptake of hydrocarbons into living cells. Microbial surfactants have the ability to reduce the surface tension of water from 72 mNm^{-1} to 27 mNm^{-1} almost 45 units, with critical micelle concentration (CMC) of lower to higher ranges (Sen and Swaminathan 2005; Das and Mukherjee 2005; Ron and Rosenberg 2002). At the CMC, the surfactant molecule aggregates and associates to form a macromolecular structure, with molecular weight generally ranging from 500 Da – 1500 Da. Microbial surfactants habitually possess the property of surface tension reduction and emulsification of hydrocarbon substrates due to their property of forming micelles at the critical micelle concentration (CMC) (Mulligan 2005; Sen 2008).

Table 1.1: Production of biosurfactants by *Bacillus* sp. isolated from different terrestrial habitats

Microorganism	Origin	Reference
Bacillus sp.	Hydrocarbon contaminated sites	Banat 1993
Bacillus subtilis DSM 3256	Terrestrial	Sen 1997
Bacillus subtilis C-1	Petroleum sludge	Vater *et al*., 2002
Bacillus licheniformis JF-2	Fermented food	Lin *et al*., 1993; Thaniyavarn *et al*., 2002
Bacillus amyloliquefaciens FZB42; *Bacillus subtilis* MZ-7	Environmental isolate	Koumoutsi *et al*., 2004; Ajlani *et al*., 2007
Bacillus coagulans	Soil	Huszcza *et al*., 2003
Bacillus thuringiensis CMB26		Kim *et al*., 2004
Bacillus amyloliquefaciens ES-2	Scutellaria baicalensis (Chinese medicinal plant)	Sun *et al*., 2006
Bacillus licheniformis HSN221	Oil field	Li *et al*., 2008
Bacillus megaterium	Soil	Pueyo *et al*., 2009

In general biosurfactants have lower CMC values compared to their chemical counterparts, which make them potential agents in emulsification, degradation of hydrocarbons, environmental remediation, enhanced oil recovery and health care applications (Makkar and Cameotra 1997; Rahman *et al*., 2002; Nitschke and Pastore 2006; Joshi *et al*., 2008). These molecules are structurally diverse and each one has its own unique properties and applications. In search for novel lipopeptide biosurfactants with better properties and greater application potentials, so far unexplored marine niche has recently been targeted.

The largely unexplored marine niche may serve as a rich source and repertoire of microbial lipopeptide biosurfactants with diverse structure and novel properties. In the recent years, several biosurfactants such as glycolipids, glycolipopeptides, carbohydrates-lipid-protein complexes, exopolysaccharides and lipopeptides have been isolated from the various genera of microorganisms of marine origin (Table 1.2). Surface-active agents, glycolipids and lipopeptides, from the marine microorganisms thus have tremendous potential to be used in industrial processes, environmental remediation and as drugs (Table 1.3) (Das *et al*., 2008a & 2008b; Das *et al*.; 2009a & 2009b; Tonkawa *et al*., 2005). This information coupled with our urge to discover some new bioactive lipoeptides justify our drive to embark on a journey to the Andaman Islands for collecting samples for subsequent isolation of biosurfactant-producing marine bacteria.

Table 1.2: Different types of biosurfactants derived from marine isolates

Type of biosurfactant	Producer organism	References
Glycolipid	Bacterial strain MM1	Passeri *et al*., 1992
	Nocardiodes sp.	Tonkawa *et al*., 2005
	Pantoea sp.	Tonkawa *et al*., 2007
	Rhodococcus erthrophili	Peng *et al*., 2007
Polysaccharides	*Alcaligenes sp.PHY 9L-86*	Goutx *et al*., 1987
	Antarctobacter sp.	Gutierrez *et al*., 2007
Lipopeptides	*Pseudomanas* sp.	Gerard *et al*., 1997
	Bacillus pumilus KMM 150	Kalinovskaya *et al*., 1995
	Brevibacillus laterosporus	Desjardine *et al*., 2007

	Bacillus circulans	Das *et al*., 2008a
	Bacillus sp.	Mukherjee *et al*., 2008
	Nocardiodes alba MSA10	Gandhimathi *et al*., 2009
	Bravibacterium aureum MSA 13	Kiran *et al*., 2010

1.6. Marine biosurfactants

1.6.1. Glycolipids

Glycolipid is the most known biosurfactant composed of carbohydrates and long chain aliphatic acids. These molecules are low-molecular weight and the best known in these groups are rhamnolipid, sophorolipids, trehalolipids. The *Halomonas*, *Pantoea*, *Nocardioides*, *Rhodococcus* are the few genera that produce glycolipid type of biosurfactants. *Halomonas* sp. ANT-3b isolated from Ross Sea, Antarctica, was able to degrade the *n*-hexadeccane for biosurfactant production (Pepi *et al*., 2005). Similarly, *Pseudomonas aeruginosa* A41 isolated from gulf of Thailand (Thaniyavarn *et al*., 2006), *Nocardioides* sp. isolated from Antarctic soil (Tonkawa *et al*., 2005), *Pantoea* sp. isolated Frazier Islands (Tonkova *et al*., 2007) and *Rhodococcus erythropolis* 3C-9 (Peng *et al*., 2007) isolated from the soil sample obtained from Islands of Xiamen, Taiwan, produced glycolipid type of biosurfactant. These microorganisms degrade and utilize the hydrocarbons as carbon source during the fermentation and produced biosurfactant which can be used for environmental applications.

1.6.2. Exopolysaccharides

Marine microbes such as *Alcaligenes* sp. PHY 9L.86 (Goutx *et al*., 1987), *Planococcus maitriensis* Anita I from sea water collected from costal area of Gujarat, India (Kumar *et al*., 2007) and marine strain *Antarctobacter* (Gutierrez *et al*., 2007a) are the main producers of this type of biosurfactants. The biosurfactant produced by these microorganisms have been used for the degradation of tetradeccan, aliphatic and aromatic hydrocarbons (Goutx *et al*., 1987; Bonilla *et al*., 2005). Owing to the good emulsification property and oil dispersion potential of this type of biosurfactants, they have been employed as an emulsifier in food and metal industries (Gutierrez *et al*., 2007a; Gutierrez *et al*., 2007b).

1.6.3. Glycolipopeptides

Another class of biosurfactants and emulsifiers from marine bacteria are the glycolipopeptides being produced from the marine bacterium such as *Corynebacterium kutscheri* isolated from Tuticorin harbor, India (Thavasi *et al*., 2007) and marine *Halomonas* sp (Pepi *et al*., 2005) as well as yeasts like *Yarrowia lipolytica* NCIM 3589 (Zinjarde *et al*., 1997). Emulsifier consists of water loving hydrophilic part and an oil-loving hydrophobic part which helps to emulsify the two immiscible liquid phase.

1.6.4. Lipopeptides

Lipopeptides are the most effective biosurfactants which are having potential environmental and health care applications. These molecules have a hydrophilic peptide head group and a hydrophobic lipid tail. Several strains of *Bacillus* sp have been reported to be major producers of lipopeptides such as surfactin, lichenysin, fengycin, bacillomycin and iturin (Arima *et al.,* 1968; Sen 1997; Peypoux *et al*. 1999; Vater *et al*., 2002). These molecules are comprised of liphophlic (hydrocarbon fatty acid chain) and hydrophilic moieties (carboxyl fatty acid chain), which are synthesized by the multimodular enzyme complexes known as non-ribosomal peptide synthetases (NRPs), conserved naturally in various *Bacillus* sp. (Kim *et al*. 2002; Das *et al*. 2008c; Sen, 2010). Arima *et al*. (1968) reported the first biologically active lipopeptide from *Bacillus subtilis*, named as surfactin. Surfactin, a cyclic lipopeptide, consists of a heptapeptide and *β*-hydroxy fatty acid with acyl chain length ranging from 12–18 carbons (Vater *et al*., 2002; Sen 2010). The various isoforms of surfactin thus produced have molecular mass in the range from (*m/z*) 900–1095 Da (Kowall *et al*., 1998). This surfactin product (*m/z* 1034 Da) is commercially available with Sigma, USA. Fengycin is also another biologically active lipopeptide having antifungal property, is composed of *β*-hydroxy fatty acid chain attached to a peptide part comprising of 10 amino acids (Sun *et al*., 2006; Romera *et al*., 2007; Deleu *et al*., 2008). Two variants of fengycin, fengycin A and fengycin B have been reported in literature (Vater *et al*., 2002; Romera *et al*., 2007). The basic difference among these isoforms is the presence of either valine or alanine at the position six of the lactone ring (Sun *et al*., 2006). The lipopeptide molecules are detected, in their protonated form or as Na^+ or K^+ adducts, by MALDI-ToF mass spectrometry in

the m/z range of 1400–1550 Da (Vater *et al*., 2002; Sun *et al*., 2006 Romera *et al*., 2007; Deleu *et al*., 2008).

Some of the marine strains also have been reported to produce surface active lipopeptides such as *Pseudomonas* sp. MK90e85 and MK91CC8, *Myroides* and *Bacillus.* A mixture of cyclic depsipeptides originally named as bacircines was produced by *Bacillus pumilus* KMM 150, isolated from an Australian marine sponge *Ircinia* sp (Kalinovskaya *et al*., 1995). Another *Bacillus pumilus* KMM 1364 isolated from the surface of the ascidian *Halocynthia aurantium* also produced lipopeptide type of biosurfactant (Kalinovskaya *et al*., 2002).

1.7. Physiochemical properties

Biosurfactants have lower critical micelle concentration (CMC) values; surfactin (Sigma, USA) CMC is 13 mgL^{-1}(Sen and Swaminathan 2005), and hence it has great commercial potential. As these molecules possess several advantages over the chemically derived surfactants For example, biosurfactant from *Bacillus subtilis* exhibits excellent stability at 100ºC and wide ranges of pH (3.0–11.0) (Makkar and Cameotra 1998). Lipopeptide obtained from *Bacillus subtilis* C9 displayed the stability from pH 5.0–9.0, in incubation at 100ºC and stability was also observed up to 1000 mM for NaCl and 10 mM for $CaCl_2$ (Kim *et al*., 1997; Nitschke and Pastore 2006). Lipopeptide produced by the marine isolate *Azetobacter chroococum* also displayed similar kinds of properties (Thavasi *et al*., 2009) as shown by the biosurfactant from the terrestrial origin.

1.8. Biological activities

Among several types of biosurfactants, lipopeptides are particularly interesting because of their high surface activities and antibiotic potential (Table 1.3). So, these molecules have gained importance in therapeutics and biomedical applications. The cyclic lipopeptide (CLPs) produced from *Pseudomonas* spp. (Nielsen *et al*., 2002) and iturin from *Bacillus subitlis* (Besson *et al*., 1976) showed potent antifungal activities. Lipopeptide from the marine strain *Bacillus circulans* also exhibits potent antimicrobial, antiadhesive and antifungal activities (Das *et al*., 2008b; Das *et al*., 2009b).

Table 1.3: Biological activities of the lipopeptide biosurfactants derived from terrestrial and marine habitats

Biosurfactants	Biological activities	References
Terrestrial lipopeptides	Antibiotic; antiadhesive; antitumor; antifungal	Nielsen *et al*., 2002; Kim *et al*., 2004; Koumoutsi *et al*., 2004; Fernandes *et al*., 2007; Liu *et a*l., 2009; Cao *et al*., 2009; Kim *et al*., 2009
	Caused hemolysis	Moran *et al*., 2002; Noudeh *et al*., 2005; Dufour *et al*., 2005; Liu *et al*., 2008; Huang *et al*., 2009
Marine lipopeptides	Antimicrobial, Antiadhesive	Das *et al*., 2008b; Das *et al*., 2009b
	Non-hemolytic property	

Lipopeptides have been reported to possess haemolytic activities, for example lipopeptide produced from *Bacillus subtilis* ATCC 6633 (Noudeh *et al*., 2005), *Bacillus subtilis* S499 (Jacques *et al*., 1999) and lipopeptide by a marine sponge-associated *actinomycetes Nocardiopsis alba* MSA10 (Ghandhimathi *et al*., 2009) having the hemolytic properties. However, the non-hemolytic property of the lipopeptide was first described by Das *et al*. (2008b). On the other hand, antimicrobial activities of the lipopeptides have also been studied extensively (Fernandes *et al*., 2007; Das *et al*., 2008; Cao *et al*., 2009). In addition to this, lipopeptides have also been reported for cytotoxic activities against different cancer cell lines (Kim *et al*., 2007; Thanomsub *et al*., 2007). In a previous study, surfactin, a cyclic lipopeptide (CLP) from *Bacillus subtilis* and lipopeptide produced from *Bacillus natto* KMD 1126 showed a potential antitumor activity (Kameda *et al*., 1974; Kim *et al*., 2007; Wang *et al*., 2007).

1.9. Production of biosurfactants

Economical production of biosurfactants is the major bottleneck as in any biotechnological field. Biosurfactant production can be characterized as growth-associated, pseudo growth associated, non-growth associated, growth limiting conditions

and precursor supplementation (Desai and Banat 1997; Kranth *et al*., 1999; Banat *et al*., 2010). The microbial products are classified in to three categories, namely growth associated, non-growth associated and mixed growth associated production. The characteristic nature of the microbial production can be represented by Luedeking-Piret (L-P) model given in the Equation 1.1,

$$q_p = \alpha\mu + \beta \quad (1.1)$$

q_p, specific product rate formation; μ- specific growth rate and α, β- empherical constants.

1.9.1. Growth associated production

The products are produced simultaneously with the microbial growth and the specific product formation (q_p) is rate is proportional to the specific growth rate (μ) and L-P model for this type of production is given below, (Equation 1.2)

$$q_p = \alpha\mu \quad (1.2)$$

Most of the biosurfactant production is reported to be growth associated which showed the relationship between the growth, substrate utilization and biosurfactant production. Production of lipopeptides form *Bacillus subitlis* LB5a (Nitschke and Pastore 2004) and *B. licheniformis* JF-2 (Lin and Sharma 1993) are examples of growth associated biosurfactant production.

1.9.2. Mixed growth associated production

The product formation takes places during the growth phase and exponential phas. In this case the L-P model having the both empherical constant values and the Equation (1.3) is given below,

$$q_p = \alpha\mu + \beta \quad (1.3)$$

Biosurfactant production can also be found to be pseudo metabolite during the growth of the microorganisms. Surfactin from *Bacillus subtilis* showed pseudo metabolic production (Sen 1997).

1.9.3. Non-growth associated production

Non-growth associated product formation occurs at the stationary phase of the microbial when the growth rate is zero. The L-P model for this type of production is given in the Equation (1.4)

$$q_p = \beta \qquad (1.4)$$

The biosurfactant production occurs in the stationary phase considered to be non-growth associated production. Biosurfactant from *Pseudomonas* sp. displayed the non-growth associated production (Babu *et al*., 1996). Mostly the glycolipid production from the various microorganism and yeasts are found to be non-growth associated (Persson *et al*., 1988; Davila *et al*., 1992).

1.9.4. Production under growth-limiting conditions

In some cases, biosurfactant production mainly depends on the growth limiting substrates; carbon is the main source for biosurfactant production.

1.9.4.1. Carbon source

Carbon source helps to attain higher cell concentrations and biosurfactant yield. Rodrigues *et al*. (2006b & 2006c) described the importance of the carbon source in biosurfactant production from the probiotic bacteria, *Lactobacillus lactis* 53 and *Streptococcus thermophilis A*. The carbon source present in the medium was optimized for improved biosurfactant production by 23 % (Sen 1997). Most of the microorganisms produce biosurfactant by utilizing the hydrocarbon substrates as carbon sources. For example, polyaromatic hydrocarbon anthracene was utilized as carbon source by the marine strain of *Bacillus circulans* (Das *et al*., 2008a). Effect of carbon sources such as n-hexadecane, olive oil and glucose on biosurfactant production from *Pseudomonas fluorescens* has been studied (Abouseoud *et al*., 2008). In this study, better production was observed in n-hexadecane and olive oil as compared to glucose. For *Bacillus subtilis* S499, the best carbon source was found to be glucose, fructose and sucrose (Sandrin *et al*., 1990). Agro-industrial product/by-product, starch was used in the biosurfactant production from *Bacillus coagulans* (Huszcza and Burczyk 2003). *Candida antarctica* KCTC 7804, yielded maximum biosurfactant concentration of 41 g L^{-1} when glycerol and

olive oil were used as carbon source in initial and a feeding carbon source (Kim *et al*., 2002).

1.9.4.2. Nitrogen source

Surfactin produced by *Bacillus subtilis* ATCC 21332 in a batch culture was strongly influenced by nitrogen metabolisms (Davis *et al*., 1999). The nitrogen and C/N ratio was used as trhe nutritional source for the production of rhamnose from *Pseudomonas aeruginosa* (Lang 2002). NH_4HCO_3 when used as nitrogen source for the growth of *Bacillus subtilis* C9 yielded 13.5 gL^{-1} of lipopeptide biosurfactant (Kim *et al*., 1999). The best nitrogen source for *Bacillus subtilis* S499 for the production of biosurfactant were found to be L-amino acids especially L-glutamic acid (Sandrin *et al*., 1990) L-glutamic acid, L- valine, L-lysine and β-alanine were reported to be good nitrogen substrates for biosurfactant production from *Bacillus subtilis* (Sandrin *et al*., 1990).

1.9.4.3. Role of precursor in biosurfactant production

Many authors reported that the addition of the precursor in the medium during the fermentation process will induce the biosurfactant production. The addition of the precursor plays a main role in the biosynthesis pathway of the microorganisms which improved the production of biosurfactant (Cameotra and Makkar 1998). When vegetable oil was used as precursor during the fermentation of *Candida antarctica* KCTC 7804, maximum biosurfactant concentration of 31 g L^{-1} was obtained (Kim *et al*., 2002). The addition of lipophilic compounds to the culture medium of *Torulopsis* sp. resulted in increased biosurfactant yield of about 120–150 gL^{-1} (Tulloch *et al*., 1962; Stuwer *et al*., 1989; Cooper and Paddock 1984). Marine bacterium are known to have the potential to degrade the lipophilic compounds, they enhance their bioavailability (Harayama *et al*., 2004). *Alcanivorax borkumensis* uses aliphatic hydrocarbons as its main carbon source for growth and produces an anionic glucose lipid biosurfactant (Abraham et al., 1998). The growth of the microorganism on liphophlic compounds are escorted by the metabolic path way and structural alteration of the cell which help to improve the biosurfactant production (Hommel 1990).

1.10. Fermentation processes

Normally, biosurfactant production has been carried out in two ways (1) submerged fermentation and (2) solid state fermentation. Most of the authors describe biosurfactant production in submerged fermentation process while the solid state process has been described in few reports (Ohno *et al*., 1995; Nitschke and Pastore 2004; Mizumoto ans Shoda 2007; Das and Mukherjee 2007).

Table 1.4: Biosurfactants yield obtained from terrestrial and marine microorganisms

Microorganisms	Origin	BS Yield	References
Bacillus licheniformis 86	Terrestrial	0.6–1.2 mg of surfactant	Horowitz *et al*., 1989
Bacillus licheniformis JF-2		13 μg ml^{-1}	McInerney *et al*., 1990
Bacillus subtilis MTCC 2423, thermophilic strain		0.74 gL^{-1}	Makkar and Cameotra 1997
Bacillus sp. LB5a		2.0 gL^{-1}	Nitschke *et al*., 2004
Bacillus subtilis ATCCC 21332		2.2 gL^{-1}	Nitschke and Pastore 2004
Bacillus sp. LB5a		3.0 gL^{-1}	Nitschke and Pastore 2006
Pseudomonas 300 B-mutant		4.1 g/L	Raza *et al*., 2006
Bacterial strain MM1	Marine	1.7 g L^{-1}	Passari *et al*., 1992
Pseudomonas aeruginosa A41		2.91 gL^{-1}, 2.93 gL^{-1} and 6.58 gL^{-1}	Thaniyavarn *et al*., 2006
Bacillus sp.		2.42 gL^{-1}	Mukherjee *et al*., 2008

In the submerged fermentation the biosurfactant production has been carried out using defined medium whereas in case of solid state the production is carried out using cheap substrates and raw materials. Biosurfactant production in submerged fermentation using glucose mineral salt medium has been studied extensively. Copper *et al*. (1981) introduced the glucose mineral salt medium for the biosurfactant production from

Bacillus subtilis, which yielded 0.8 gL^{-1} of surfactin. *Bacillus subtilis* prototroph strain ATCC21332 produced 328 mg of surfactant using the same mineral salt medium (Mulligan *et al*., 1989). *Pseudomonas aeruginosa* UW-1 produced the maximum rhamnolipid concentration of 24.3 gL^{-1} by using the salt medium containing 6% canola oil (Sim *et al*., 1997). Similarly, there are several microorganisms have been found to produce biosurfactants using different medium compositions (Table 1.4). Table 1.4 shows that good amount of biosurfactant production was observed in the marine microorganisms as compared to the terrestrial origin.

However the large scale production of these molecules has not been realized because of low yields in production process and high recovery and purification cost. Mukherjee *et al*. (2006) described the practical approaches that have been adopted to make the biosurfactant process economically such as use of cheap resource in the media, optimized and efficient bioprocesses and overproducing mutant and recombinant strain for obtaining maximum biosurfactant production.

1.11. Bioprocess optimization

Unlike their chemical counter parts these molecules can be produced under mild conditions by microbial fermentation using cheap agro-based media formulations (Mukherjee *et al*., 2006). Despite their multifarious advantages and diverse potential applications, their production on a commercial level could not be achieved due to the low yields in production process (Mukherjee *et al*., 2006). One of the primary approaches applied for obtaining increased yields in fermentative production is the medium optimization. The most effective method used for the optimization of factors is the statistical approach (Table 1.5).

Table 1.5: Statistical methods used for the bioprocess modeling and optimization for enhancing the production of biosurfactants

Statistical methods	References
Plackett-Burman design	Mukherjee *et al*., 2008
Taguchi experimental design	Wei *et al*., 2006
Fractional Factorial design	Rodrigues *et al*., 2006
Single-variable at a time experiments	Sen 1997

Response surface methodology	Sen 1997; Sen and Swaminathan 1997; Joshi *et al*., 2008; Mutalik *et al*., 2008
Artificial neural network modelling and genetic algorithm	Pal *et al*., 2009

1.11.1 Plackett-Burman design

Several nutrients, media components and trace elements are reported to affect the biosurfactant production process. The Plackett-Burman is a well established and widely used technique for the screening of medium components (Jacques *et al*., 1999; Mukherjee *et al*., 2008). Two-level fractional factorial design and Plackett-Burman, can screen up to *n*-variables with *n*+ 1 experiment while the multifactor design will be difficult because of more of experiments. The experimental designs help to screen the critically influencing variables on biosurfactant production with less number of experiments. Joshi *et al*. (2007) screened nearly fourteen nutritional components and studied their effects on the biosurfactant production from *Bacillus licheniformis*. This study showed that nearly thirteen nutritional parameters were highly influencing the biosurfactant production. Mukherjee *et al*. (2008) used this statistical technique to identify the nutritional parameters which critically influence the biosurfactant production from marine *Bacillus* sp. In that study the authors screened nearly eleven factors and studied their effects on biosurfactant production.

1.11.2. Taguchi experimental design

Taguchi experimental design, a standard orthogonal array of L_9, L_{18}, L_{27} and L_{36} ($N^{(n+1)}$) is used to examine the experimental design. Here the L and the subscript represent the Latin square and the number of experimental runs respectively. The analysis of the design is performed using the analysis of variance (ANOVA). Taguchi experimental design is a good positive option for the optimization of biotechnological processes, however the model is complex. There is only one report available in biosurfactant production using this design (Wei *et al*., 2007). By using this design, Wei *et al*. (2007), optimized the trace elements such as Mg^{2+}, K^{+}, Mn^{2+} and Fe^{2+} for improved production of surfactin from *Bacillus subtilis* ATCC 21332.

1.11.3. Fractional factorial design

This design can be used for screening many factors to find the significant factors. The interactions between the variables will be determined by the *R*-n factors. Fractional designs are expressed using the notation l^{k-p}, where l is the number of levels of each factor investigated, k is the number of factors investigated, and p describes the size of the fraction of the full factorial used. The use of this model in biosurfactant research is scarce; optimization of biosurfactant production from probiotic bacteria (Rodrigues *et al*., 2006b) and biosurfactant production from *Candida bombicola* (Casas *et al*., 1997) using the fractional factorial experimental design approach have been reported. Fontes *et al*. (2010) used this design to optimize the biosurfactant production from *Yarrowia lipolytica*. The effects of aeration and agitation, carbon and nitrogen sources were studied for the optimization of biosurfactant production using this method (Fontes *et al*., 2010).

1.11.4. Single variable at a time experiments

The critical media components that influence the production processes are identified by single-factor-at-a-time optimization strategy. The experiments (n-1), n-single variable, will be designed by leaving out one of the components present in the medium, keeping all the other components are present. The results obtained from these experiments helped find out the effect of the different nutrients on biosurfactant production. Based on the single variable experiments, critical experimental variables are identified and their range and levels are also determined to design the optimization experiments. This experimental strategy was first adopted for the optimization of surfactin production from *Bacillus subtilis* (Sen 1997). Sen (1997) identified that four critical media components influenced the surfactin production. Similar kind of study was reported by several authors for biosurfactant production (Sen and Swaminathan 1997; Sen and Babu 2005; Rodrigues *et al*., 2006; Mutalik *et al*., 2008; Joshi *et al*., 2008; Sivapathasekaran *et al*., 2010b & 2010d).

1.11.5. Response surface methodology

Response surface methodology (RSM) consists of a group of empirical techniques that explores the relationship between the independent variables and output values. This method is employed with multiple regression analysis by using quantitative data obtained from the properly designed experiments to solve multivariate equations (Carley *et al*., 2004). Thus performance measure is called the response and input variables are sometimes called independent variables**.** In terms of coded variables the response function is given in equation 1.5

$$Y = f(X_1, X2, \ldots\ldots, X_k) \quad (1.5)$$

f, is the true response which will be function of first or second polynomial quadratic model. Second order polynomial function is widely used in the response surface methodology for several reasons like very flexible, method of least square can be used for this purpose and it works well in solving response surface problems. The empirical equation of the second order polynomial function is given in equation 16.

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j \chi_j + \sum_{j=1}^{k} \beta_{jj} \chi_j^2 + \sum_{i<} \sum_{j=2}^{k} \beta_{ij} \chi_i \chi_j \quad (1.6)$$

RSM has been used to optimize the critical process variables for enhancing biosurfactant production. Sen (1997) used this optimization technique for the optimization of the critical medium components such as glucose ($C_6H_{12}O_6$), ammonium nitrate (NH_4NO_3), manganese sulphate ($MnSO_4$) and iron sulphate ($FeSO_4$) for the biosurfactant production from *Bacillus subtilis*. Medium components such as carbon source, sodium nitrate ($NaNO_3$), phosphate and $FeSO_4$ were optimized for the enhanced rhamnolipids production from *Pseudomonas aeruginosa* AT10 (Abalos *et al*., 2002). Similarly, medium components were optimized for the rhamnolipid production from *Pseudomonas aeruginosa* S2 (Chen *et al*., 2007) and *Pseudomonas aeruginosa* J16 (Wei *et al*., 2008). Critical medium components which highly influenced the lipopeptide biosurfactants from *Bacillus licheniformis* RG1 (Ramnani *et al*., 2005), *Bacillus subtilis* MO-01 (Gu *et al*., 2008) and *Bacillus licheniformis* R2 (Joshi *et al*., 2008) were optimized using this method for enhanced production. In our study also the lipopeptide

biosurfactants from *Bacillus circulans* MTCC 8281 was optimized using RSM (Sivapathasekaran *et al*., 2010). The effect of inoculum age and size were optimized for the enhanced surfactin production from *Bacillus subtilis* DSM 3256 using this statistical technique (Sen and Swaminathan 2004).

In a fermenter, process variables play a critical role in the production as well as process economics and thus the importance to optimize the process conditions in order to maximize the product yield at very low cost (Panda *et al*., 2007). Likewise critical process variables in biosurfactant production in a fermenter were optimized by using this statistical technique. For example, the process variables such as temperature, pH, aeration and agitation were found to critically influence the biosurfactant production in the reactor (Sen and Swaminathan 1997). These process variables were optimized for the enhanced biosurfactant production from *Bacillus subtilis* DSM 3256 (Sen and Swaminathan 1997). Biosurfactant production from sponge associated marine fungus *Aspergillus ustus* MSF3 was also optimized using this method (Kiran *et al*., 2010). The main drawback of RSM is that the optimization is confined to the quadratic non-linear model whereas biological process consisting of many complex non-linear patterns. Other modern modelling and optimization tools such as artificial neural network coupled with genetic algorithm (ANN-GA) can also serve as powerful aids for bioprocess optimization to augment biosurfactant production (Pal *et al*., 2009).

1.11.6. Artificial Neural Network modeling (ANN) and Genetic algorithm (GA) optimization

ANN, a mathematical and computational tool, is a collection of interconnecting between the independent process variables (input) and dependent variables (output) without any prior knowledge of the relationship between them. GA is a globalized optimization technique which searches the global optima value of a complex objective function obtained from ANN by reproduction of the biological process such as genetics, crossover and mutation (Goldberg 1989). Imandi *et al*. (2008) first time described the optimization of critical medium components using ANN-GA for the enhanced production of lipopeptide from *Bacillus subtilis* MO-01. Later, same tool was adopted for the medium optimization for the biosurfactant production from *Rhodococcus erythropolis* MTCC

2794 (Pal *et al*., 2009). Sivapathasekaran *et al*., 2010b optimized the medium for the enhanced biosurfactant production from our marine isolate using this tool.

1.12. Batch and Fed batch/semi-continuous operations

Batch fermentation is a closed system where no nutrients are added during the time of fermentation. In case of fed batch operation, the growth limiting nutrients were added in to the medium by intermittent feeding during the fermentation to increase the biomass and the productivity. Initially the fed batch fermentation is operated at very low volume up to batch mode; once the feeding starts, the volume gets increased according to the feeding. Surfactin production from *Bacillus subtilis* ATCC 21332 was performed in a 2 L fermenter (MultiGen, New Brunswick Scientific, NJ, USA); biosurfactant production from *Bacillus subtilis* 21332 and *Bacillus licheniformis* 86 was carried out in two types of fermenters – 20 L and 16 L fermenter (L-H fermentation series 200) respectively in a batch mode (Horowitz *et al*., 1990) and surfactin from *Bacillus subtilis* DSM 2 and the production was done in 2 L Virtis Omni- culture fermenter (Sen and Swaminathan 1997). Small scale fermentation was carried in 3 L fermenter (MDL model, B.E. Marubishi, Japan) for the production of lipopeptides from *Bacillus subtilis* C9 (Kim *et al*., 1997). Similarly, the production was carried out in a 1 L bioreactor for the production of biosurfactant from *Lactobacillus lactis* 53 and *Streptocococcus thermophilis* (Rodrigues *et al*., 2006). The production of rhamnolipid from *Pseudomonas aeruginosa* (Das and Mukherjee 2005) and biosurfactant from food isolate *Bacillus subtilis* 20B (Joshi *et al*., 2008) were carried out in 5 L Bioflow 110 fermenter (New Brunchwich Scientific, NJ, USA). Few literatures are there in the fed batch production of biosurfactant from different microorganisms.

The pH fed batch mode was performed for the production of rhamnolipid from *Pseudomonas aeruginosa*, glucose was used as limiting substrate and the production was enhanced from 1.68 $gL^{-1}$to 4.4 g L^{-1} (Lee *et al*., 1999). Surfactin production from *Bacillus subtilis* ATCC 21332 was enhanced by feeding nitrogen source in the fed batch operation which yielded 439 mg L^{-1} surfactin (Davis *et al*., 1999). By feeding the nitrogen source for the glycolipid biosurfactant production from *Candida* sp. SY16 yielded 37 g L^{-1} whereas soybeans residual oil feeding as carbon source yielded 95 g L^{-1} surfactin (Kim *et*

al., 2004). The maximum biosurfactant concentration of 4.1 gL^{-1} was produced from gamma ray induced *Pseudomonas putida* mutant (Raza *et al*. 2007). These are the several approaches made by the experts working in this field to enhance the biosurfactant to commercial level.

1.13. Downstream processing and characterization

The production of biosurfactants has been enhanced by various optimization strategies but still the purification remains a major challenge due to the high cost downstream processing costs and purity requirements. The downstream processing cost of most biotechnological products is almost ~60% of the production cost (Mukherjee *et al*., 2006). Biosurfactants can be qualitatively and quantitatively analyzed by various analytical methods (Table 1.6).

Table 1.6: Analytical methods used for qualitative and quantitative analysis of biosurfactants

Analytical methods	References
Surface tension measurements , Critical micelle dilutions (CMD^{-1}), Critical micelle concentration (CMC^{-1})	Sen 1997, Makkar and Cameotra 1998; Sen and Swaminathan 2005; Pansiripat *et al*., 2009
Turbidometric method	Mukherjee *et al*., 2009a
Drop-collapse method	Bodour *et al*., 1998
Emulsification index	Deleu *et al*., 1999; Pal *et al*., 2009
High performance liquid chromatography (HPLC)	Lin *et al*., 1998; Vater *et al*., 2002; Das *et al*., 2008b; Sun *et al*., 2009
Thin layer chromatography (TLC)	Chrsitova *et al*., 2003; Das *et al*., 2008; Mawgoud *et al*., 2009
High performance thin layer chromatography (HPTLC)	
Mass spectral analysis, Mass assisted laser desorption ionization time of flight (MALDI-ToF)	Peypoux *et al*., 1999; Vater *et al*., 2002; Pueyo *et al*., 2009
Fourier transform infrared spectroscopy (FTIR)	

Surfactin concentration can be indirectly measured by measuring the surface tension of the serially diluted broth samples known as critical micelle concentration (CMC) (Sen 1997), critical micelle dilution (CMD^{-1} and CMC^{-2}) and emulsification index (Makkar and Cameotra 1997 & 1998; Nitschke and Pastore 2006; Ghojavand *et al*., 2008; Haddad *et al*., 2009). Mukherjee *et al*. (2009a) first time reported the quantification of biosurfactant from the *Bacillus megaterium* using simple turbidometric method. On other hand, High performance thin layer chromatography (HPTLC) method is used to detect the chemical nature of the biosurfactants and biosurfactant concentrations can be easily determined by this method with less amount of samples (Philip *et al*., 2002; Kartsova and Strel'nikova 2007; Mukherjee *et al*., 2008; Mawgoud *et al*., 2009). Similarly, high performance liquid chromatography (HPLC) is mainly used to analyze and separate the isoforms present in the crude mixture of the biosurfactants (Jenny *et al*., 1991; Lin et al., 1994 & 1998; Vater *et al*., 2002; Sivapathasekaran *et al*., 2009), which is not possible in any other analytical method. The functional groups present in these molecules are determined by FTIR analysis and the mass ranges are identified through MALDI-ToF mass spectral analysis (Peypoux *et al*., 1999; Vater *et al*., 2002; Thaniyavarn *et al*., 2003; Mukherjee and Das 2005; Pueyo *et al*., 2009; Sivapathasekaran *et al*., 2010a). A number of purification methods have been employed by many researchers in their purification studies (Table 1.7).

Table 1.7: Methods used for the purification of biosurfactants

Purification methods	Reference
Gel filtration chromatography (GFC)	Kim *et al*., 2000; Mukherjee *et al*., 2009b
High performance liquid chromatography (HPLC)	Lin *et al*., 1998; Das *et al*., 2008b; Sivapathasekaran *et al*., 2009
Thin layer chromatography (TLC)	Das et al., 2008; Heyd et al., 2008; Sivapathasekaran *et al*., 2010a
Ultrafiltration (UF)	Lin and Jiang 1997; Sen and Swaminathan 2005; Mulligan and Gibbs 2005; Isa *et al*., 2007

1.14. Purity determination

The percentage of the purity and effectiveness of the biosurfactant products were determined by critical micelle concentration (CMC) values. CMC of the biosurfactant is the minimum amount required for the onset of the process of micellization. At this and higher concentrations, the surface tension reaches a minimum value and biosurfactant molecules will form the macromolecular structure resulting in bigger micelles, vesicles and lamellae formation (Lin and Jiang 1997; Sen and Swaminathan 2005). The standard surfactin, 98% purity (Sigma, USA) showed the CMC value of 13 mg L^{-1} whereas CMC value of surfactin obtained from *Bacillus subtilis* showed 17 mg L^{-1}, 70% of the purity was achieved using ultrafiltration process (Sen and Swaminathan 2005). The CMC values indicate the efficiency of a surfactant molecule; lower the CMC, lower is the amount required to achieve the lowest surface-tension value and hence, higher is its efficacy. Similarly the purity achieved for the biosurfactant isoforms were determined using the CMC values which showed above 85% purity (Sivapathasekaran *et al*., 2009).

1.15. Lacuna and Challenges

Biosurfactants have been traditionally analyzed by surface tension measurement, CMC^{-1}, CMD^{-1} (Sen 1997; Nitschke *et al*., 2004; Pansiripat *et al*., 2009). There are some recent reports on the use of HPLC and HPTLC for small scale purification and characterizations of these molecules (Mawgoud *et al*., 2009).

- But complete analysis cum quantification using HPTLC for time course studies was not reported.

  Purification of biosurfactants by HPLC has been reported (Thaniyavarn *et al*., 2003; Smyth *et al*., 2010).
- But these methods were time consuming and not optimized, hence, developing an optimal purification strategy became a necessity.

  Process development and optimization in fermenter for the enhanced biosurfactants production by terrestrial strains was reported earlier (Sen and Swaminathan 1997; Rodrigues *et al*., 2006b; Joshi *et al*., 2008).
- However the systematic process development and optimization for the enhanced production of marine biosurfactants are very few.

Moreover, most of the optimization protocol reported so far has employed response surface methodology (RSM).

- But the application of other efficient methodologies for process optimization like Artificial Neural Network modelling and Genetic Algorithm optimization (ANN – GA) was not reported.

  There are reports on production of biosurfactants at lab fermenter scale (Sen & Swaminathan 1997, Chen *et al*., 2006).
- However, fermenter level production of marine biosurfactants in batch and fed batch mode/semi-continuous of operations have not been reported. There are reports on two stage ultrafiltration based recovery of biosurfactants with 70-90% purity (Sen and Swaminathan 2005; Isa *et al*., 2008).
- But the challenge stills remains to achieve same or higher degree of purity in a single stage ultrafiltration operation.

  Based on these lacuna and challenges, thesis objectives have been designed to solve these problems.

Objectives

1. Analytical method development for the assay of biosurfactants in high performance thin layer chromatography (HPTLC) and high performance liquid chromatography (HPLC)
2. Formulation of production medium and its optimization
3. Development of a bioprocess in bench top fermenter using batch and unsteady state fed batch operations
4. Process optimization in batch fermenter for the enhanced production of the biosurfactants
5. Purification of biosurfactant products by chromatographic and ultrafiltration methods and their characterization