REVIEW OF LITERATURE

2.1. Biosurfactant

Biosurfactants are amphiphilic molecules mainly produced by microorganisms as a secondary metabolite. They possess both hydrophilic and hydrophobic moieties and are able to display a variety of surface activities and helps to solubilize hydrophobic substrates (Desai and Banat, 1997; Kokare et al., 2007, 2009; Mnif et al., 2013; Sarafin et al., 2014).

Microbial biosurfactants are heterogeneous in nature, complex and have structurally different group of surface active agents. These are produced by a wide variety of microbes inhabiting different environmental niches which either adhere to cell surface or excreted extracellular (Rosenberg and Ron, 1999; Mishra and Jha, 2009; Kavitha et al., 2011; Singh et al., 2011; Jain et al., 2012, 2013).

A characteristic feature of biosurfactants is hydrophilic-lipophilic balance (HLB) which makes surfactants acquiring excellent detergency, emulsifying, foaming and dispersion (Desai and Banat, 1997; Lang, 2002; Karanth et al., 2005; Sarafin et al., 2014).

Nowadays, biosurfactants are used in cosmetic, pharmaceutical, chemical, food, agriculture, cleansers, enhanced oil recovery industries, and in bioremediation of oil contaminated sites, considering the advantages and characteristics as thermostability, tolerance to ionic strength, biodegradability and low toxicity (Cameotra and Makkar, 1998; Bognolo, 1999; Makkar and Cameotra, 2002; Gautam and Tyagi, 2006; Singh et al., 2007; Sekhon et al., 2011; Chen et al., 2012; Jain et al., 2013; Jara et al., 2013; Rufino et al., 2014).

Although more attractive than their synthetic counterparts, biosurfactants are not yet competitive in the market due to functional reasons and high production costs. Thus, the success of biosurfactant production depends on the development of less costly
processes, especially with regard to substrates, which account for 10–30 per cent of the
total production cost. Low-cost or under-utilized substrates, such as industrial waste,
can be used to address this problem (Sobrinho et al., 2008; Gusmao et al., 2010; Luna
et al., 2013; Maria et al., 2014).

The biosurfactant production can be achieved by novel production strategies
such as the formulation of novel production media, production on low-cost substrates
and development of novel production approaches (Muthusamy et al., 2008; Ghribi et
al., 2011). Several studies reported biosurfactant production on various agro-industrial
solid byproducts under solid state fermentation (Das and Mukherjee, 2007; Kiran et al.,
2009; Neto et al., 2009; Ghribi et al., 2012; Mnif et al., 2013).

2.2. Classification of biosurfactants

Unlike chemically synthesized surfactants, which are usually classified
according to the nature of their polar grouping, biosurfactants are generally categorized
mainly by their chemical composition and microbial origin. Table 1 depicts the
classification of biosurfactant produced by various microorganisms and its application.
The microbial surfactants are complex molecules covering a wide range of chemical
types including peptides, fatty acids, phospholipids, glycolipids, antibiotics,
lipopeptides, etc. Microorganisms also produce surfactants that are in some cases
combination of many chemical types: referred to as the Polymeric Microbial
Surfactants (PMS) (Banat et al., 2000; Karanth et al., 2005; Muthusamy et al., 2008;
Sarafin et al., 2014).

Rosenberg and Ron (1999) suggested that biosurfactants can be divided into
low-molecular-mass molecules, which efficiently lower surface and interfacial tension
and high-molecular-mass polymers, which are more effective as emulsion-stabilizing
agents. The major classes of low-mass surfactants include glycolipids, lipopeptides and
phospholipids, whereas high-mass surfactants include polymeric and particulate surfactants.

Karanth et al. (2005) reported that many microbial surfactants have been purified and their structures were elucidated by many researchers. While the high molecular weight microbial surfactants are generally polyanionic heteropolysaccharides containing polysaccharides and proteins, the low molecular weight microbial surfactants are often glycolipids.

Nitschke and Coast (2007) observed that most biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long-chain fatty acids or fatty acid derivatives, whereas the hydrophilic portion can be a carbohydrate, amino acid, phosphate or cyclic peptide.

2.2.1. Low molecular-weight biosurfactants

The low molecular-weight biosurfactants are generally glycolipids or lipopeptides. They are disaccharides acylated with long-chain fatty acids or hydroxyl fatty acids. These type biosurfactants are efficient in lowering surface and interfacial tension at the air/water interface (Rosenberg and Ron, 1999; Calvo et al., 2008; Salihu et al., 2009).

2.2.1.1. Glycolipids

Glycolipids are commonly mono or disaccharides compounds acylated with long chain fatty acids or hydroxyl fatty acids. This linkage is by means of either ether or an ester group. Among them, the subclasses are rhamnolipids, mannosylerythritol lipids (MELs), sophorolipids and trehalolipids are the best-studied (Desai and Banat, 1997; Muthusamy et al., 2008).
Table - 1 Classification of biosurfactants produced by various Microorganisms

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Biosurfactant</th>
<th>Microorganism</th>
<th>Applications in environmental biotechnology</th>
<th>References</th>
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<tr>
<td></td>
<td>Group</td>
<td>Class</td>
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<tr>
<td>1</td>
<td>Glycolipids</td>
<td>Rhamnolipid</td>
<td><em>Pseudomonas aeruginosa,</em> <em>Pseudomonas</em> sp.</td>
<td>Enhancement of the degradation and dispersion of different classes of hydrocarbons and vegetable oils; Removal of metals from soil.</td>
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<td></td>
<td>Trehalolipds</td>
<td><em>Mycobacterium tuberculosis,</em> <em>Rhodococcus erythropolis,</em> <em>Arthrobacter</em> sp., <em>Nocardia</em> sp., <em>Corynebacterium</em> sp.,</td>
<td>Enhancement of the bioavailability of hydrocarbons.</td>
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<td></td>
<td>Sophorolipids</td>
<td><em>Torulopsis bombicola,</em> <em>Torulopsis petrophilum,</em> <em>Torulopsis apicola</em></td>
<td>Recovery of hydrocarbons from dregs and muds; Removal of heavy metals from sediments; Enhancement of oil recovery.</td>
</tr>
<tr>
<td></td>
<td>Fatty acids, phospholipids and neutral lipids</td>
<td>Corynomycolic acids</td>
<td><em>Corynebacterium lepus</em></td>
<td>Enhancement of bitumen recovery</td>
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<tr>
<td></td>
<td></td>
<td>Spiculissporic acid</td>
<td><em>Penicillium spiculisporum</em></td>
<td>Removal of metal ions from aqueous solution; dispersion action for hydrophilic pigments; Preparation of new emulsion-type organo gels, superfine microcapsules (vesicles or liposomes), heavy metal sequestrants.</td>
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<td></td>
<td>Phosphate-dylethanolamine</td>
<td><em>Acietobacter</em> sp., <em>Rhodococcus erythropolis</em></td>
<td>Increasing the tolerance of bacteria to heavy metals</td>
</tr>
<tr>
<td>3</td>
<td>Lipopeptides</td>
<td>Surfactin</td>
<td>Bacillus subtilis</td>
<td>Enhancement of the biodegradation of hydrocarbons and chlorinated pesticides. Removal of heavy metals from a contaminated soil, sediment and water. Increasing the effectiveness of phytoextraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lichenysin</td>
<td>Bacillus licheniformis</td>
<td>Enhancement of oil recovery.</td>
</tr>
<tr>
<td>4</td>
<td>Polymeric biosurfactants</td>
<td>Emulsan</td>
<td>Acinetobacter Calcoaceticus RAG-1</td>
<td>Stabilization of the hydrocarbon-in-water emulsions.</td>
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<tr>
<td></td>
<td></td>
<td>Alasan</td>
<td>Acinetobacter radioresistens KA-53</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Biodispersan</td>
<td>Acinetobacter calcoaceticus A2</td>
<td>Dispersion of limestone in water.</td>
</tr>
<tr>
<td></td>
<td>Liposan</td>
<td>Candida lipolytica</td>
<td>Stabilization of hydrocarbon-in-water Emulsions.</td>
<td>Cirigliano and Carman, 1984</td>
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<td></td>
<td>Mannoprotein</td>
<td>Saccharomyces Cerevisiae</td>
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<td>Cameron et al., 1988</td>
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</table>
Glycolipids were found in a variety of microorganisms such as the yeast *Torulopsis* (sophorolipids, which consist of two glucose units linked β-1,2 and a lipid portion connected to the reducing end through a glycosidic linkage) (Inoue and Itoh, 1982) and *Rhodococcus erythropolis* (trehalose dimycolates) (Kim et al., 1990). One of the best studied glycolipids is rhamnolipid, produced by several species of Pseudomonads (Perfumo et al., 2006; Costa et al., 2010; Pacheco et al., 2012).

### 2.2.1.1.1. Rhamnolipids

Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (1949).

Rhamnolipid consists of 2 mol of rhamnose and 2 mol of β-hydroxy-deconoic acid (Lang and Wullbrandt, 1999), while the OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the OH group of the second acids is involved in ester formation (Karanth et al., 1999).

Rhamnolipids are glycosides, produced mainly by *Pseudomonas aeruginosa* and *Burkholderia* genus. Those are composed of one (mono-rhamnolipids) or two (di-rhamnolipids) rhamnose sugar moieties linked to one or two β-hydroxyfatty acid chains (Perfumo et al., 2006; Raza et al., 2009).

These molecules display high surface activities and many potential applications in the biomedical field due to their antibacterial, antifungal, antiviral, anti-adhesive properties (Kim et al., 2000; Abalos et al., 2001; Cosson et al., 2002; Yoo et al., 2005; Remichkova et al., 2008; Sotirova et al., 2008).

They have also been used in the preparation of nanoparticles (Xie et al., 2006; Palanisamy and Raichur, 2009) and microemulsions (Xie et al., 2007; Nguyen and Sabatini, 2009).
Pacheco et al. (2012) reported the production of rhamnolipid under oxidative stress condition by *Pseudomonas aeruginosa* PA1 and identified the proteins which are potentially involved in the rhamnolipid production and responsible for the oxidative stress.

Sodagari et al. (2013) studied the possible effects of rhamnolipids on the attachment of bacteria and investigated in flow chambers with either hydrophilic glass walls or hydrophobic walls of octadecyl-trichlorosilane (OTS) modified glass. The antimicrobial activity of rhamnolipids, cell hydrophobicity, and wet ability of substratum surface were helpful in understanding their mechanism of action.

Long et al. (2013) studied that the rhamnolipid treatment could obtain over 90 per cent of dewatering efficiency on refractory waste crude oil. It seems that rhamnolipids as bio-demulsifiers were of great prospects in the industrial demulsification of waste crude oil.

Saikia et al. (2013) proposed a method to synthesize colloidal silver nanoparticles in presence of rhamnolipid and further investigated their stability with respect to time and salt concentration.
Patil et al. (2014) optimized the rhamnolipid production by *Pseudomonas aeruginosa* F23 isolated from oil contaminated soil sample and checked the antimicrobial activity of rhamnolipid against *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, *Escherichia coli*, *Shigella schmitzii*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Corynebacterium diphtheriae* and *Candida albicans*.

### 2.2.1.1.2. Mannosylerythritol

The mannosylerythritol (MELs) glycolipids are produced by yeasts strains of the genus *Pseudozyma* sp. and *Ustilago* sp. from soybean oil or n-alkane. 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. Based on the degree of acetylation at C4 and C6 position and their order of appearance on the thin layer chromatography, the MELs are classified into MEL-A, -B, -C and -D. MEL-A representing the diacetylated compound while MEL-B and MEL-C are monoacetylated at C6 and C4, respectively. The completely de-acetylated structure is known as MEL-D. Rau et al. (2005) studied the downstream processing of mannosylerythritol lipids produced by *Pseudozyma aphidis* (Arutchelvi and Doble, 2010).

MELs have recently gained attention due to their environmental compatibility, structural diversity, self-assembling properties and versatile biochemical functions. In particular, interesting applications have been described in the biomedical field as antimicrobial, antitumor and immunomodulating molecules, in the biotechnological field for gene and drug delivery and in cosmetic applications as skin moisturizers (Arutchelvi and Doble, 2010).
Morita et al. (2011a) studied the production and characterization of a glycolipid biosurfactant, mannosylerythritol lipid B, from Sugarcane Juice by *Ustilago scitaminea* NBRC 32730.

Recke et al. (2013) produced mannosylerythritol lipids MEL-A and MEL-B by culturing *Pseudozyma aphidis* on media with glucose as main carbon source and soybean oil as co-substrate.

![Figure 2: Structure of Mannosylerythritol lipid](image)

2.2.1.1.3. Sophorolipids

Sophorolipids consist of two glucose units linked β-1, 2. The 6- and 6’-hydroxyl groups are generally acetylated. The lipid portion is connected to the reducing end through a glycosidic linkage. The terminal carboxyl group of the fatty acid can be in the lactonic form or hydrolyzed to generate an anionic surfactant (Rosenberg and Ron, 1999).

Schippers et al. (2000) reported that the Critical Micelle Concentration (CMC) and the solubilization ratio of the sophorolipids biosurfactant were found to be in a good range compared with synthetic surfactants.
Hirata et al. (2009) observed that sophorolipids are lowfoaming surfactants with high detergency, which also exhibit both low cytotoxicity and readily biodegradable properties.

Sophorolipids are synthesized by some yeast species including *Candida bombicola, Candida apicola, Rhodotorula bogoriensis, Wickerhaminella domercqiae* and *Candida batistae*. Sophorolipids have been reported suitable for a number of applications in the biomedical field including use as antimicrobial, antiviral and anticancer. They also have been used in the synthesis of metal-bound nanoparticles in cosmetic and pharmacodermatological products (Van Bogaert and Soetaert, 2010).

Van Bogaert et al. (2011) reported that several non-pathogenic yeast species are able to synthesize sophorolipids. It is a glycolipid molecule with surface tension lowering capacities and they offer environmental friendly, an alternative for the petrochemical derived surfactants used in various sectors and gained industrial attention due to the high yield (400 g/l by *Candida bombicola*).

![Structure of Sophorolipid](image)

**Figure 3: Structure of Sophorolipid**

Van Bogaert et al. (2013) studied the biosynthetic gene cluster for sophorolipids produced by *Starmerella bombicola* and hampering sophorolipid production does not
affect cell growth or cell viability in laboratory conditions similar to the secondary metabolites.

2.2.1.1.4. Trehalose

Trehalose lipids are also a glycolipids containing trehalose as the sugar moiety which is a non-reducing disaccharide in which the two glucose units are linked in an α,α-1,1-glycosidic linkage. The most reported trehalose lipid is trehalose 6,6'-dimycolate, which is a α-branched chain mycolic acid esterified to the C6 position of each glucose. These glycolipids vary in the number and overall chain length (C20–C90) of the esterified fatty acids. Different trehalose containing glycolipids are known to be produced by several other microorganisms belonging to mycolates group, such as *Arthrobacter*, *Nocardia*, *Rhodococcus* and *Gordonia*. *Rhodococcus* genus in particular produced several types of trehalose lipids as reported by Lang and Philp (1998). Trehalolipd is the basic component of the cell wall glycolipids in *Mycobacteria* and *Corynebacteria* (Franzetti et al., 2010b).

Zaragoza et al. (2009, 2010) reported that apart from their usual industrial applications, trehalose lipids recently attracted attention to their functions in cell membrane interaction and their potential as antitumor therapeutic agents. The amphiphilic nature of trehalose lipids points to the membrane as their hypothetical site of action and the details regarding the interaction between these biosurfactants and biological membranes are less. The trehalose lipids permeabilize phospholipid membranes and induce hemolysis of human red blood cell.

Ortiz et al. (2011) studied the effects of a bacterial trehalose lipid from *Rhodococcus* sp., on phosphatidylglycerol membranes of microorganisms because it has been suggested that the lipid composition of bacterial membranes plays an important role in the interaction with antimicrobial compounds.
White et al. (2013) studied the biosurfactant production by a novel marine *Rhodococcus* sp., strain PML026 and characterized the chemical nature and properties of the biosurfactant. The biosurfactant exhibited high surfactant activity under a wide range of conditions.

![Figure 4: Structure of Trehalose](image)

2.2.1.2. Lipopeptides

Neilsen et al. (1999) reported that the lipopeptides are composed of a peptide moiety that can be cyclized to form a lactone ring between two amino acids in the peptide chain and a fatty acid chain at the N-terminal amino acid, both varying in length, which could account for different properties of the lipopeptides.

Lipopeptides are mainly produced by members of the *Bacillus* species; they are composed of different families and each family is constituted of several variants, which can differ in their fatty acid chain and their peptide moiety (Dastgheib et al., 2008; Jaques, 2010; Thavasi et al., 2008, 2011a).

2.2.1.2.1. Surfactin

Surfactin was discovered by Arima et al. (1968) from the culture broth of *Bacillus subtilis* and it was named thus due to its exceptional surfactant activity (Peypoux et al., 1999).
A cyclic lipopeptide produced by *Bacillus subtilis* is called surfactin considered the most active biosurfactant discovered so far (Ron and Rosenberg, 2001). Natural surfactins are a mixture of isoforms A, B, C and D with various physiological properties obtained from *B. subtilis* BC 1212 which are classified according to the differences in their amino acid sequences and possess various physiological properties (Shaligram and Singhal, 2010).

![Surfactin](image)

**Figure 5: Structure of Surfactin**

Surfactin is composed of a seven amino-acid ring structure coupled to a fatty-acid chain via a lactone linkage. Surfactin-A has L-leucine, surfactin-B has L-valine and surfactin-C has L-isoleucine at the amino acid position involved in the lactone ring formation with the C14-C15 β-hydroxy fatty acid. The amino-acid residues may vary and the presence of these variants can be related to alterations in the culture conditions such as providing substrate containing some specific amino-acid residues in the culture media (Jaques, 2010).

Zeriouh *et al.* (2014) reported that the surfactin biosurfactant can triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity.
2.2.1.2.2. Lichenysin

Surfactin related compound is lichenysin, a lipopeptide discovered in the supernatant of *Bacillus licheniformis* culture (Horowitz *et al.*, 1990). Its chemical structure and physio-chemical properties are similar to surfactin (McInerney *et al.*, 1990). In particular, lichenysin has Glutamine amino-acid in position 1 while surfactin has Glutamic acid.

Nerurkar (2010) reported that the lichenysins are most potent anionic cyclic lipoheptapeptide biosurfactants produced by *Bacillus licheniformis* on hydrocarbonless medium with mainly glucose as carbon source. They have the capacity to lower the surface tension of water from 72 to 27 mN/m. Based on species specific variations they are named lichenysin A, B, C, D, G and surfactant BL86.

Madslien *et al*. (2013) elucidated that the prevalence of lichenysin production in *Bacillus licheniformis* and identified whether the feature was restricted to certain genotypes and studied the interaction between the concentration and cytotoxicity level.

2.2.1.2.3. Pumilacidin

These are compounds which resembles surfactin called pumilacidin A, B, C, D, E, F and G, a complex of acylpeptide antibiotics isolated from *Bacillus pumilus* culture supernatants with interesting antiviral properties (Naruse *et al.*, 1990; Morikawa *et al.*, 1992).

2.2.1.2.4. Iturin

Iturin belongs to the family of lipopeptide and classified as iturin family, among them iturin A is the most studied compound. It is a heptapeptide interlinked with a β-amino-acid fatty acid with carbon chain length from C14 to C17 produced by *Bacillus subtilis* strain (Peypoux, 1978).
These molecules have antifungal activities. Other members of the iturin family are iturin C, bacillomycin D, F and Lc and mycosubtilin (Besson, 1976; Bonmatin et al., 2003; Jaques, 2010).

2.2.1.2.5. Fengycins

Fengycin is a lipodecapeptide containing β-hydroxyl fatty acid in its side chain and comprises of C\textsubscript{15} to C\textsubscript{17} variants which have a characteristic Alanine-Valine dimorphy at position 6 in the peptide ring (Schneider et al., 1999).

![Figure 6: Structure of Iturin A](image)

Wang et al. (2004) demonstrated that, the identification of fengycin homologues produced by *Bacillus subtilis* by using Electrospray Ionization Spectrometry (ESI-MS) techniques.

Jaques (2010) reported that the family of fengycins includes fengycins A and B, lipodecapeptides which differ by their amino-acid residue in position 6 that can be Alanine or Valine and are known for their interesting fungitoxic and immunomodulating activities.
2.2.1.2.6. Serrawettins

Other interesting lipopeptides are Serrawettins, nonionic cyclodepsipeptide biosurfactants produced by *Serratia marcescens* (Matsuyama *et al*., 2010) and implicated with antitumor and antinematode activities.

2.2.2. High molecular weight biosurfactants

These are generally grouped together as polymeric biosurfactants. They are produced by a number of different bacteria and are composed of lipoproteins, proteins, polysaccharides, lipopolysaccharides or complexes containing several of these structural types (Rosenberg and Ron, 1997, 1999; Ron and Rosenberg, 2001).

The most commonly studied biopolymer is emulsan, a lipopolysaccharide isolated from *Acinetobacter calcoacetius* RAG-1 ATCC 31012 with a molecular weight of around 1,000 kDa (Rosenberg *et al*., 1979).

A property of biosurfactant RAG-1 emulsan is a complex of an anionic heteropolysaccharide and protein. Its surface activity is due to the presence of fatty acids, comprising 15 per cent of the emulsan dry weight, which are attached to the polysaccharide backbone via O-ester and N-acyl linkages (Rosenberg and Ron, 1999).

They are highly efficient emulsifiers that work at low concentrations and exhibit considerable substrate specificity and it was referred as bioemulsans (Dastgheib *et al*., 2008; Salihu *et al*., 2009).

2.2.2.1. Alasan

Another high molecular weight biosurfactant is alasan, a complex of an anionic polysaccharide and a protein with a molecular weight of around 1,000 kDa isolated from *Acinetobacter radioresistens* (Smyth *et al*., 2010). These high molecular weight biosurfactants generally possess effective emulsifying activity and are called bioemulsifiers.
2.2.3. Polymeric microbial surfactants

Cirigliano and Carman, (1984) reported that the yeasts produce a number of emulsifiers, which are particularly interesting because of the food-grade status of several yeasts which allows use in food related industries. Liposan is an extracellular emulsifier produced by *Candida lipolytica*. It is composed of 83 per cent carbohydrate and 17 per cent protein.

Cameron *et al.* (1988) reported that the mannanprotein emulsifiers are produced by *Saccharomyces cerevisiae*, this protein showed excellent emulsifying activity towards several oils, alkanes and organic solvents.

*Pseudomonas tralucida* produced an extracellular acetylated polysaccharide that was effective in emulsifying several insecticides. Several bioemulsifiers are effective at high temperature, including the protein complex from *Methanobacterium thermoautotrophium* and the protein-polysaccharide-lipid complex of *Bacillus stearothermophilus* ATCC 12980 (Rosenberg and Ron, 1999).

Little is known in general about these bioemulsifiers other than the producing organism and the overall chemical composition of the crude mixture.*Halomonas eurihalina* produces an extracellular sulfated heteropolysaccharide (Rosenberg and Ron, 1999).

A large number of other polymeric compounds have been discovered but remain partially or totally uncharacterized (Smyth *et al.*, 2010).

2.2.4. Particulate biosurfactant

This type of biosurfactant produced by *Acinetobacter* sp. when grown on hexadecane it is accumulated in the extracellular vesicles of 20-50mm diameter with a buoyant density of 1.158 g/cm$^3$. They are composed of protein, phospholipid and lipopolysaccharide (Kappeli and Finnerty, 1979). Extracellular membrane vesicles partition hydrocarbons forming micro emulsion which plays an essential role in alkane
uptake by microbial cells. The purified vesicles are composed of proteins, phospholipids and lipopolysaccharide (Kappeli and Finnerty, 1979; Neu, 1996; Desai and Banat, 1997; Perfumo et al., 2010a).

2.3. Properties of biosurfactant

Biosurfactants are of increasing interest for commercial use because of the continually growing spectrum of available substances. There are many advantages of biosurfactants compared to their chemically synthesized counterpart. The main distinctive features of biosurfactants and a brief description of each property are given below.

2.3.1. Surface and interface activity

Rhamnolipids from *P. aeruginosa* decrease the surface tension of water to 26 mN/m and the interfacial tension of water/hexadecane to <1 mN/m (Hisatsuka et al., 1971). Surfactin from *B. subtilis* can reduce the surface tension of water to 25 mN/m and interfacial tension of water/hexadecane to <1 mN/m (Cooper et al., 1981).

In general, biosurfactants are more effective and efficient and their Critical Michelle Concentration (CMC) is about 10–40 times lower than that of chemical surfactants, i.e. less surfactant is necessary to get a maximum decrease in surface tension (Desai and Banat, 1997).

The sophorolipids from *T. bombicola* have been reported to reduce the surface tension to 33 mN/m and the interfacial tension to 5 mN/m (Cooper and Cavalero, 2003). A good surfactant can lower surface tension of water from 72 to 35 mN/m and the interfacial tension of water/hexadecane from 40 to 1 mN/m (Mulligan, 2005).

2.3.2. Temperature, pH and ionic strength tolerance

Many biosurfactants and their surface activities are not affected by environmental conditions such as temperature and pH. McInerney et al. (1990) reported
that lichenysin from \textit{B. licheniformis} JF-2 was not affected by temperature (up to 50°C), pH (4.5–9.0) and by NaCl and Ca concentrations up to 50 and 25 g/l respectively. A lipopeptide from \textit{B. subtilis} LB5a was stable after autoclaving (121°C/20 min) and after 6 months at –18°C; the surface activity did not change from pH 5 to 11 and NaCl concentrations up to 20 per cent (Nitschke and Pastore, 1990).

2.3.3. Biodegradability

Unlike synthetic surfactants, microbial-produced compounds are easily degraded (Mohan \textit{et al.}, 2006) and particularly suited for environmental applications such as bioremediation (Mulligan, 2005) and dispersion of oil spills (Mohan \textit{et al.}, 2006).

2.3.4. Low toxicity

Very little data are available in the literature regarding the toxicity of microbial surfactants. They are generally considered as low or non-toxic products and therefore, appropriate for pharmaceutical, cosmetic and food uses. When comparing the toxicity of six biosurfactants, four synthetic surfactants and two commercial dispersants, it was found that most biosurfactants degraded faster, except for a synthetic sucrose-stearate that showed structure homology to glycolipids and was degraded more rapidly than the bio-genic glycolipids (Poremba \textit{et al.}, 1991).

A biosurfactant from \textit{P. aeruginosa} was compared with a synthetic surfactant (Marlon A-350) widely used in the industry, in terms of toxicity and mutagenic properties. Both assays indicated higher toxicity and mutagenic effect of the chemical-derived surfactant, whereas the biosurfactant was considered slightly non-toxic and non-mutagenic (Flasz \textit{et al.}, 1998).

2.3.5. Emulsion forming and Emulsion breaking

Liposan does not reduce surface tension, but has been used successfully to emulsify edible oils (Cirigliano and Carman, 1985).
Stable emulsions can be produced with a lifespan of months and years (Velikonja and Kosaric, 1993). Biosurfactants may stabilize (emulsifiers) or destabilize (de-emulsifiers) the emulsion. High molecular mass biosurfactants are in general better emulsifiers than low-molecular-mass biosurfactants. Sophorolipids from *T. bombicola* have been shown to reduce surface and interfacial tension, but are not good emulsifiers (Cooper and Cavalero, 2003). Polymeric surfactants offer additional advantages because they coat droplets of oil, thereby forming stable emulsions. This property is especially useful for making oil/water emulsions for cosmetics and food.

2.3.6. Chemical diversity

The chemical diversity of naturally produced biosurfactants offers a wide selection of surface-active agents with properties closely related to specific applications.

2.4. Isolation and screening of biosurfactant producing organism

Hydrocarbon-degrading bacterial populations are generally dominated by a few main bacterial genera such as *Pseudomonas*, *Bacillus*, *Sphingomonas* and *Actinobacteria* in soils and sediments and *Pseudoalteromonas*, *Halomonas*, *Alcanivorax* and *Acinetobacter* from marine ecosystems. It is not surprising that a lot of biosurfactant or bioemulsifier producers belong to these same genera (Bodour et al., 2003).

In natural environments, microbes occur almost always in a mixed population composed of a multitude of different strains and species. For analyzing the properties of a defined organism out of such a mixed population, a pure culture is required. Apart from direct isolation of strains by diluting and plating, enrichment cultures with hydrophobic substrates are very promising for the isolation of biosurfactant producing microbes.

For the screening of biosurfactant producing microbes, enrichment cultures utilizing hydrophobic compounds as the sole carbon sources were tested (Schulz et al.,
1991; Mercade et al., 1996; Willumsen and Karlson, 1997; Giani et al., 1997; Huy et al., 1999; Rahman et al., 2002; Bento et al., 2005).

This is an indirect screening method as the growth on hydrophobic compounds indicates the production of biosurfactants, but not always correlates with this trait (Mercade et al., 1996; Willumsen and Karlson, 1997). Moreover, the applied screening medium and conditions will influence whether or not surfactants are produced (Bodour et al., 2003).

Schulz et al. (1991) isolated three bacterial strains of marine origin during a screening for biosurfactants among \textit{n}-alkane degrading microorganisms. As enrichment medium, they used mineral media with C$_{14}$ and C$_{15}$\textit{n}-alkanes and also agar plates with an alkane-soaked filter in the lid.

Mercade et al. (1996) isolated biosurfactant producing strains from petroleum-contaminated soil samples by using waste lubricating oil as the sole carbon source. They isolated 44 strains which were able to grow on hydrocarbons. Out of 44 strains, five of them produced biosurfactant.

Willumsen and Karlson (1997) isolated biosurfactant producing bacteria from soil which was contaminated with Poly Aromatic Hydrocarbons (PAHs). They used PAH-amended liquid minimal medium for enrichment culture. Furthermore, they used agar-plates coated with different PAHs and agar-plates with a PAH-soaked filter in the lid of the Petri dish for the selection. The degradation of PAHs by the microorganisms then leads to a clearing zone agar around the colonies in the PAH coated agar. As result, they isolated 57 strains of which only 4 strains showed surface activity.

Rahman et al. (2002) isolated 130 oil-degrading isolates from hydrocarbon-polluted environments by enrichment techniques. A mineral salts medium containing
crude oil as the sole carbon source was applied. Two of these strains were found to produce biosurfactants.

Pornsunthorntawee et al. (2008) isolated two types of biosurfactant producing bacteria, *Bacillus subtilis* PT2 and *Pseudomonas aeruginosa* SP4 from sludge oil and petroleum-contaminated soil. They found that both organisms producing biosurfactants could recover oil more effectively than three synthetic surfactants like polyoxyethylene sorbitan monooleate (Tween 80), Sodium Dodecyl Sulfonate (SDS) and sodium alkyl polypropylene oxide sulfate.

Saimmai et al. (2012b) suggested that, the isolation and functional characterization of a biosurfactant produced by a new and promising strain of *Oleomonas sagaranensis* AT18 from mangrove sediments and evaluated their antimicrobial activity and Microbially Enhanced Oil Recovery (MEOR) potentials.

Hoskova et al. (2013) studied the characterization of rhamnolipids produced by two non-pathogenic bacterial strains *Acinetobacter calcoaceticus* and *Enterobacter asburiae*.

Sarafin et al. (2014) isolated the *Kocuria marina* BS-15 a biosurfactant producing halophilic bacteria from solar salt works in India. The novel biosurfactants in extremophiles seems to be particularly promising since they have particular adaptations like increased stability in adverse environments and their microbial products are highly stable and important in various fields.

Bao et al. (2014) isolated a lipopeptide biosurfactant producing bacteria *Acinetobacter* sp. D3-2 and studied the biodegradation of crude oil. *Acinetobacter* sp. D3-2 could grow at 30°C in 3 per cent NaCl solution with a preferable ability to degrade 82 per cent hydrocarbons, showing that bioremediation does occur and plays a profound role during the oil separation process.
2.5. Mangroves Ecosystem for the isolation of biosurfactant producers

Mangrove ecosystem is a bridge between terrestrial and marine ecosystem. It harbours unique microbial diversity. Mangroves are the coastal wetland forests generally found near the intertidal regions of estuaries between creeks, lagoons, marshes etc. Mangroves provide a unique ecological site to different microbes. Because of richness in carbon and other nutrients mangrove ecosystem harbours diverse microbial communities which can adapt themselves in the extreme conditions there. Microorganisms forms integral part of the mangrove ecosystem. They help in recycling and transformation of various nutrients and thus make the mangrove ecosystem more productive (Bhat and Leena, 2013).

There are reports on the biosurfactants produced by microorganisms from mangrove sediments (Maneerat et al., 2006; Rodrigues et al., 2006; Maneerat and Phetrong, 2007; Kebbouche et al., 2009; Anandaraj and Thivakaran, 2010; Gudina et al., 2010; Burgos et al., 2011; Darvishi et al., 2011).

Saimmai et al. (2012c) reported that the 89 sediment samples screened for the biosurfactant producers collected from the east and west coasts of southern Thailand by an enrichment culture technique. Ninety five isolates tested positive for biosurfactant production according to the qualitative drop-collapsing test. Those 95 isolates also showed promising biosurfactant activity by exhibiting a surface tension reduction of more than 10 mN/m.

Govindammal and Parthasarathi (2013) isolated five strains and selected the best biosurfactant producing Pseudomonas fluorescens MFS03 for further studies.

Parthasarathi et al. (2014) reported that 63 isolates were isolated and screened for the biosurfactant production from mangrove and adjacent ecosystem. Among the 63 isolates two strains were showed greater biosurfactant activity.
2.6. Screening methods for the production of biosurfactant

Due to their unique properties and vast array of application, identification of new biosurfactant producing microbes is in great demand. There are several screening methods known for detection of biosurfactant producers. These methods includes Cell surface hydrophobicity (Rosenberg et al., 1980), Tilted glass slide method (Peerson and Molin, 1987), Direct colony chromatographic (TLC) technique (Matsuyama et al., 1987), Blue agar plate method (Siegmund and Wagner, 1991), Agar plate method (Morikawa et al., 1992), Haemolytic activity (Banat, 1993; Carrillo et al., 1996), Drop collapse method (Bodour and Miller-Maier, 1998), Oil spreading technique (Morikawa et al., 2000) and Emulsification activity (Ellaiah et al., 2002).

Satpute et al. (2010) studied all the above said methods to screen, detect and evaluated potential of biosurfactant producing microorganisms and studied both their advantages and disadvantages.

2.6.1. Haemolytic activity

Mulligan et al. (1984) recommended that, the blood agar method as a preliminary screening method for identifying biosurfactant producers. It is a qualitative screening test for detection of biosurfactant producers. Solid media such as Luria agar (LA), nutrient agar (NA), supplemented with 5 per cent fresh whole blood are used (Banat, 1993; Carrillo et al., 1996). Isolates are streaked and incubated at required temperature for 48 h. Visual inspection for haemolysis may be an indication of red blood cell lysis due to cell membrane rupture caused by the presence of surface active molecules. Blood agar is a complex medium hence; it is very difficult to test the biosurfactant productivity of a culture at different culture conditions directly on the agar (Youssef et al., 2004).
Haemolytic activity however has been considered an unreliable criterion for the detection of biosurfactant activity (Satpute et al., 2008). Thavasi et al. (2011c) also reported that the hemolytic assays are not reliable and sensitive, because this method will categorize microbes in two groups as hemolytic and non-hemolytic. Strains that are hemolytic are believed to be biosurfactant producers, but there are other products such as virulence factors that can lyse the blood cells and also biosurfactants with poor diffusion in agar may not be able to lyse the blood cells.

2.6.2. Drop collapse method

Drop collapse method is one of the qualitative methods used to determine the presence of biosurfactant. The drop collapse method is developed by Jain et al. (1991). This assay relies on the destabilization of liquid droplets by biosurfactants. Therefore, drops of a cell suspension or culture supernatant are placed on an oil coated solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. The stability of drops is dependent on surfactant concentration and correlates with surface and interfacial tension (Jain et al., 1991).

Bodour and Miller-Maier (1998) suggested that this assay can even be quantitative by measuring the drop size of the pure surfactant using a micrometer. An important distinction of this assay is that it can be transferred to an automated screening in microplates. This assay has been applied for the screening of biosurfactant producing microorganisms by several researchers (Bodour et al., 2003; Youssef et al., 2004; Batista et al., 2006; Plaza et al., 2006; Maczek et al., 2007).
2.6.3. Oil spread method

Biosurfactant producers are found by observation of droplet collapsing down (Peerson and Molin, 1987). The oil spreading is a quantitative method for the screening of biosurfactant producers and it was developed Morikawa et al. (2000).

Morikawa et al. (2000) reported that the area of oil displacement in oil spreading assay is directly proportional to the concentration of the biosurfactant in the solution. Oil spreading assay was also reported by Youssef et al. (2004) while screening bacteria for biosurfactant production and they recommended that both drop collapse and oil spreading assay methods as reliable techniques for testing biosurfactant production. Colony surrounded by an emulsified halo is considered positive for biosurfactant production (Morikawa et al., 2000).

It can also be applied when the activity and quantity of biosurfactant is low (Plaza et al., 2006). This technique is effectively a modification of the drop collapse method.

2.6.4. Cetyl Trimethyl Ammonium Bromide (CTAB) assay (or) Blue agar plate method

This technique was specially developed for detection of glycolipids such as rhamnolipids by Pseudomonas sp. It can be applied for detection of similar type of biosurfactant from other Gram negative isolates. The CTAB agar plate method is a semi-quantitative assay for the detection of extracellular glycolipids or other anionic surfactants and developed by Siegmund and Wagner, (1991).

Anionic biosurfactant forms insoluble ion pair with the cationic Cetyl Trimethyl Ammonium Bromide and methylene blue and formation of dark blue halo around the culture is considered as positive for biosurfactant production. It is an excellent technique that has been used generally for detection of glycolipids biosurfactants. This
method is a reliable screening method, but it is specific for anionic biosurfactants. This method was used as a screening method by many authors (Tahzibi et al., 2004; Gunther et al., 2005; Tuleva et al., 2005; Satpute et al., 2010).

2.6.5. BATH assay/Cell surface hydrophobicity technique

Cell hydrophobicity was measured by bacterial adherence to hydrocarbons according to a method described by Rosenberg et al. (1980).

Bouchez-Naïtali et al. (1999) demonstrated that microbes show low surface hydrophobicity when biosurfactant are released extracellular, where hydrocarbon uptake is mediated through the biosurfactant.

There is a direct correlation between cell surface hydrophobicity and biosurfactant production. This method is applied as one of the screening method to select an efficient biosurfactant producer (Pan et al., 2006; Maneerat and Dikit, 2007). Depending upon the hydrocarbon uptake behaviour, microorganisms may have high and/or low surface hydrophobicity. Generally, those microbes which can take hydrocarbon by direct uptake mode, shows high surface hydrophobicity. Cell bound biosurfactant production is also associated with hydrocarbon uptake. This phenomenon is discussed in detail by Franzetti et al. (2008) who had worked on Gordonia. The cell hydrophobicity index was used as a screening method by Parthasarathi (2010).

2.6.6. Emulsification assay

Cooper and Goldenberg (1987) developed the emulsification assay. Emulsification assay is an indirect method used to screen biosurfactant production.

Patil and Chopade (2001a) introduced emulsification assay based on emulsification units of the tested oils. Ellaiah et al. (2002) observed that the potent biosurfactant producing cultures can be detected through the emulsification assay. The cultures showing greater than 30 per cent emulsification activity were also positive for
biosurfactant production in two or three other methods. It is also possible to detect biosurfactant production and hydrocarbon degrading activity simultaneously on agar plate by overlaying with hydrocarbon (Kokare et al., 2007).

Emulsification activity is one of the criteria to support the screening and selection of potential biosurfactant producers (Bonilla et al., 2005). The surface activity and emulsification capacity do not always correlate (Plaza et al., 2006). Maximum number of isolates positive for kerosene, hexadecane, benzene, toluene and crude oil degradation were also positive for diesel utilization. Measurement of emulsification units help to choose the carbon and energy source for biosurfactant production. It was assumed that if the cell free culture broth used in this assay contains biosurfactant then it will emulsify the hydrocarbons present in the test solution. Over the long time it had been utilized in many screening methods (Bento et al., 2005; Plaza et al., 2006; Chen et al., 2007; Thavasi et al., 2011c).

2.6.7. Surface/Interfacial Activity

The majority of screening methods for biosurfactant producing microbes are based on the interfacial or surface activity. Various methods have been developed for measuring this property.

2.6.7.1. Direct Surface/Interfacial Tension Measurements

The direct measurement of the interfacial or surface activity of the culture supernatant is the most straightforward screening method and very appropriate for a preliminary screening of biosurfactant producing microbes (Lin, 1996). This gives a strong indication on biosurfactant production. The interfacial or surface tension of a liquid can be measured by a variety of methods. However, there is a restriction in the range of measurement. The surface tension decreases with increasing surfactant concentration until the CMC is reached. If the concentration of biosurfactant is above
the CMC, an increase in the concentration cannot be detected. Consequently, two cultures with very different concentrations of biosurfactant may display the same surface tension. This problem can be solved by serial diluting until a sharp increase in surface tension is observed (Duvnjak et al., 1982; Peerson and Molin, 1987; Bosch et al., 1988; Jain et al., 1991; Makkar and Cameotra, 1997a; Morikawa et al., 2000; Batista et al., 2006).

The corresponding dilution of the supernatant is called Critical Micelle Dilution (CMD) and correlates to the concentration of biosurfactant. Furthermore, the measurements are strongly affected by factors such as pH and ionic strength. For screening purposes, the following methods are established. They can all be used for measuring the surface and interfacial tension of a liquid. Especially the Du-Nouy-Ring method is quite easy and most frequently applied.

2.6.7.2. Du-Nouy-Ring Method

Cooper (1986) considered a culture as promising if it reduces the surface tension of a liquid medium to 40 mN/m or less. Willumsen and Karlson (1997) give a similar definition: a good biosurfactant producer is defined as one being able to reduce the surface tension of the growth medium by ≥20 mN/m compared with distilled water.

The advantage of this method is the accuracy and the ease of use. However, it requires specialized equipment. A disadvantage is that measurements of different samples cannot be performed simultaneously. Other limitations of this assay include the volume of sample required for analysis, usually some millilitres and the restricted range of concentrations that can be analyzed without dilution (Bodour and Miller-Maier, 1998).

The Du-Nouy-Ring method is based on measuring the force required to detach a ring or loop of wire from an interface or surface (Tadros, 2005). The detachment force
is proportional to the interfacial tension. It can be measured with an automated tensiometer which is available from many manufacturers. The ring must be free from contaminant, which is usually achieved by using a platinum ring that is flamed before use. Instead of a ring, a platinum plate, a so called Wilhelmy plate, can be applied in the same manner (Tuleva et al., 2005; Wei et al., 2005; Chen et al., 2007).

2.7. Other screening methods for the selection of biosurfactant producers

2.7.1. Agar plate overlaid with hydrocarbons

Pure isolates are streaked on oil coated agar plates and incubated for one week at desired temperature. Colonies surrounded by an emulsified halo are detected as biosurfactant producers (Morikawa et al., 1992). This is the efficient method where observation of emulsified halo around the culture is the direct indication of biosurfactant producer.

2.7.2. Direct colony-thin layer chromatographic (TLC) technique

This method characterizes biosurfactant producers. In this technique, a bacterial mass is directly placed on pre developed (chloroform; methanol; 2:1) TLC plate. After drying the bacterial mass, the plate is run in chloroform; methanol; 5 M ammonia (85:25:4 v/v) and developed with developers. Resulting chromatograph indicates the characteristic lipid compositions of organism (Matsuyama et al., 1987). The method is fast and easy to perform without any special requirement.

2.8. Production of biosurfactant

According to Desai and Banat (1997), the biosurfactant has a amphiphilic structure and in that the hydrophobic moiety is either a long-chain fatty acid, a hydroxy fatty acid, or a-alkyl-b-hydroxy fatty acid and the hydrophilic moiety may be a carbohydrate, carboxylic acid, phosphate, amino acid, cyclic peptide, or alcohol. Two primary metabolic pathways, namely, hydrocarbon and carbohydrate, are involved in
the synthesis of their hydrophobic and hydrophilic moieties, respectively. The pathways for the synthesis of these two groups of precursors are diverse and utilize specific sets of enzymes.

In many cases, the first enzymes for the synthesis of these precursors are regulatory enzymes; therefore, in spite of the diversity, there are some common features of their synthesis and regulation. The detailed biosynthetic pathways for the major hydrophobic and hydrophilic moieties have been extensively investigated and are well documented by many scientists and Hommel and Ratledge (1993) gave a brief description about the biosynthesis.

Syldatk and Wagner (1987) described that there are some possibilities exist for the synthesis of different moieties of biosurfactants and their linkage.

1. The hydrophilic and hydrophobic moieties are synthesized de novo by two independent pathways.
2. The hydrophilic moiety is synthesized de novo while the synthesis of the hydrophobic moiety is induced by substrate.
3. The hydrophobic moiety is synthesized de novo, while the synthesis of the hydrophilic moiety is substrate dependent.
4. The synthesis of both the hydrophobic and hydrophilic moieties is substrate dependent.

2.9. Regulation of biosurfactant production

In general three mechanisms, namely induction, repression and effect of nitrogen and multilevel ions, operate in the regulation of biosurfactant production. Induction is the mechanism which creates stress to the microorganism to synthesize the biosurfactant. It is the general regulation mechanism used to control the onset of synthesis of most lipopeptide biosurfactants (Besson and Michel, 1992).
2.9.1. Induction

The induction of sophorolipid synthesis by the addition of long chain fatty acids, hydrocarbons or glycerides to the growth medium of Torulopsis magnoliae (Tulloch et al., 1962), Trehalolipid synthesis in Rhodococcus erythropolis by the addition of hydrocarbons (Rapp et al., 1979) and glycolipid in Bacillus subtilis by addition of alkanes (Chakrabarty, 1985) has been reported. The inducible nature of biosurfactant in Endomycopsis lipolytica has also been confirmed (Roy et al., 1979).

2.9.2. Repression

Repression of biosurfactant production may happen with the presence of specific chemicals. Drastic reduction in synthesis of rhamnolipids by Bacillus subtilis (Hauser and Karnovsky, 1958) and liposan by Candida lipolytica (Cirigliano and Carman, 1985) upon the addition of D-glucose, acetate and tricarboxylic acids has been reported. Repression of biosurfactant production by Arthrobacter parafineus (Dujnjak et al., 1982) and Acinetobacter calcoaceticus (Gobbert et al., 1984) on hydrocarbon substrates has been observed with organic acids and D-glucose, respectively.

2.9.3. Nitrogen or metal ion-dependent regulation

Limiting the concentrations of salts of magnesium, calcium, potassium, sodium and trace elements, a higher yield of rhamnolipids can be achieved in Bacillus subtilis DSM 2659 (Guerra-Santos et al., 1984).

Nitrogen or metal ion-dependent regulation also played a prominent role in the synthesis of biosurfactants. Addition of nitrogen source caused an inhibition of rhamnolipid synthesis in resting cells of Pseudomonas sp. strain DSM-2874 (Syldatk et al., 1985).
The synthesis of rhamnolipids using *Bacillus subtilis* upon exhaustion of nitrogen and commencement of the stationary phase of growth has been observed by several investigators (Guerra-Santos *et al*., 1984, Ramana and Karanth, 1989).

The limitation of multivalent cations also causes overproduction of biosurfactants. Iron limitation can be achieved in *Pseudomonas fluorescens* (Persson *et al*., 1988) whereas addition of iron and manganese salts stimulates biosurfactant production in both *Bacillus subtilis* (Cooper *et al*., 1981) and *Rhodococcus* sp. (Abu-Ruwaida *et al*., 1991a).

Ochsner *et al*. (1994) observed the expression of genes from *Bacillus subtilis* for rhamnolipid synthesis in *Pseudomonas fluorescens* and *Pseudomonas putida* only under nitrogen-limiting conditions.

### 2.10. Use of alternative resources for the production of biosurfactant

Improvement in production and technologies has helped to some extent and can lead to further improvements. Researchers have emphasized the key parameters affecting the efficiency of biosurfactant production in terms of higher yields and lower production cost (Bognolo, 1999; Kosaric, 1992; Mukherjee *et al*., 2006). Table 2 depicts the different types of agroindustrial wastes used by various authors.

The main strategy to achieve the high yielding with low cost is as follows

1. Assessment of the substrate and product output with focus on appropriate organism, nutritional balance and the use of cheap or waste substrates to lower the initial raw material costs involved in the process.

2. Development of efficient bioprocesses, including optimization of the culture conditions and cost effective separation processes to maximize recovery.

3. Development and use of overproducing mutant or recombinant strains for enhanced yields. (Bognolo, 1999; Kosaric, 1992; Mukherjee *et al*., 2006).
### Table - 2 Potential substrates for the biosurfactant production

<table>
<thead>
<tr>
<th>S. No</th>
<th>Agro Industrial wastes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oil Seed cake</td>
<td>Ramakrishnan and Banerjee, 1952; Benjamin and Pandey, 1996; Ramachandran</td>
</tr>
<tr>
<td></td>
<td></td>
<td>\textit{et al.}, 2007; Kamini \textit{et al.}, 1998; Kiran \textit{et al.}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2010a; Thavasi \textit{et al.}, 2011b; Nadhem and Gumaa, 2012</td>
</tr>
<tr>
<td>2</td>
<td>Potato processing waste water</td>
<td>Natu \textit{et al.} 1991; Fox and Bala, 2000; Noah \textit{et al.}, 2002;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whang \textit{et al.}, 2008</td>
</tr>
<tr>
<td>3</td>
<td>Pineapple juice</td>
<td>Rangan, 1984; Kroyer, 1991; Lee and Kim, 2001; Correia \textit{et al.}, 2004;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Abdullah, 2007; Govindammal, 2013</td>
</tr>
<tr>
<td>4</td>
<td>Vegetable oils and used vegetable oils</td>
<td>Haba \textit{et al.}, 2000; Thaniyavarn \textit{et al.}, 2006; Dumont and</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Narine, 2007; Pornsunthornfootawee \textit{et al.}, 2008; Oliveira \textit{et al.}, 2009; Pantazaki \textit{et al.}, 2010</td>
</tr>
<tr>
<td>5</td>
<td>Dairy and sugar industry wastes</td>
<td>Daniel \textit{et al.}, 1998; Maneerat, 2005; Abdel-Mawgoud \textit{et al.}, 2010; Daverey and Pakshirajan, 2010</td>
</tr>
<tr>
<td>6</td>
<td>Ligoncellulosic waste</td>
<td>Taherzadeh and K K, 2007; Portilla-Rivera \textit{et al.}, 2008</td>
</tr>
<tr>
<td>7</td>
<td>Starch richy substrates</td>
<td>Nitschke and Pastore, 2003; Nitschke and Pastore, 2006</td>
</tr>
<tr>
<td>8</td>
<td>Cashew Apple Juice</td>
<td>Oliveira \textit{et al.}, 2013; Parthasarathy, 2010</td>
</tr>
<tr>
<td>9</td>
<td>Clarified Molasses</td>
<td>Makkar and Cameotra, 1997; Raza \textit{et al.}, 2007; Parthasarathy, 2010;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Raza \textit{et al.}, 2009</td>
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</table>

The use of alternative substrates such as agro industrial wastes is one of the attractive strategies for economical biosurfactants production. A wide variety of alternative raw materials are currently available as nutrients for industrial fermentations, namely various agricultural and industrial byproducts and waste materials (Deleu and Paquot, 2004; Ferreira, 2008; Makkar and Cameotra, 2002; Savarino \textit{et al.}, 2007; Da Silva \textit{et al.} 2009; Montoneri \textit{et al.}, 2009a,b; Parthasarathy, 2010; Oliveira \textit{et al.}, 2013; Amodu \textit{et al.}, 2014).

### 2.11. Process optimization using Response Surface Methodology (RSM)

Still expensive raw material, low production output and high purification cost in surfactin fermentation process have limited its commercialization in the manufacture.
To overcome these obstacles, it is necessary to select cheap substrates and to optimize the nutrient levels and fermentation conditions during production (Mukherjee et al., 2006).

RSM is not only used for optimization of culture parameters in the fermentation process, but also for studying the combined effects of media components (Dutta et al., 2004). This method was successfully used to determine the optimum media, inoculums and environmental factors for the enhanced production of surfactin by *Bacillus subtilis* (Sen and Swaminathan, 2004).

In the course of the whole fermentation, process optimization is a topic of central importance. Response surface methodology (RSM) is an efficient statistical experimental strategy by which the optimal conditions of a multivariable system may be determined (Oskouie et al., 2008).

RSM helps to optimize physical and chemical parameters, such as initial water content, incubation temperature, fermentation period, substrates and additional nutrients required in the fermentation process, thereby saving time and labour by minimizing the number of required experiments (Li et al., 2007; Sharma and Arora, 2010).

Response surface methodology (RSM) is an empirical technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivalent equations simultaneously. The graphical representations of these equations are called response surfaces. A $2^3$ full-factorial central composite design for two test variables with corresponding star points and centre points was employed to fit the procedure and analyzed using the Design Expert Software (version7.0). Based on the ANOVA of the quadratic regression model demonstrates that the model is highly significant, as is evident from the Fischer F test (Kiran et al., 2009).
RSM makes it possible to represent independent process parameters in quantitative form as:

\[ Y = f(X_1, X_2, X_3, \ldots, X_n) \pm \varepsilon \] \hspace{1cm} (1)

Where, \( Y \) is the response (yield), \( f \) is the response function, \( \varepsilon \) is the experimental error and \( X_1, X_2, X_3, \ldots, X_n \) are independent parameters.

By plotting the expected response of \( Y \), a surface known as the response surface is obtained. The form of \( f \) is unknown and may be very complicated. Thus, RSM aims at approximating “f” by suitably lowering-ordered polynomial in some region of the independent process variables.

If the response can be well modeled by a linear function of the independent variables, the function (Eq. 1) can be written as:

\[ Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \ldots + \beta_n x_n \pm \varepsilon \] \hspace{1cm} (2)

However, if a curvature appears in the system, then a higher order polynomial such as the quadratic model (Eq. 3) may be used.

\[ Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n} \beta_{ij} x_i^2 \pm \varepsilon \] \hspace{1cm} (3)

The objective of using RSM is also to investigate the response over the entire factor space, but also to locate the region of interest where the response reaches its optimum or near optimal value. By carefully studying the response surface model, the combination of factors, which gives the best response, can then be established.

The success of the RSM depends on the approximation by a low order polynomial in some region of the independent variables. In Eq. (2), \( \eta \) is the response, \( x_i \) and \( x_j \) are variables, \( k \) is the number of independent variables (factors), \( \beta_0 \) is the constant coefficient, \( \beta_i \)'s, \( \beta_{ij} \)'s and \( \beta_{ij} \)'s are interaction coefficients of linear, quadratic and the second-order terms, respectively and \( \varepsilon \) is the error. The quality of the fit of polynomial model was expressed by the coefficient of determination \( R^2 \) and \( R^2_{adj} \) in Eq.
(3) and (4), respectively. The statistical significance was checked with adequate precision ratio in Eq. (5) and (6) and by $F$ values and $P$ values:

$$x_i = \frac{X_i - X_0}{\Delta x}$$

(1)

$$\eta = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} x_i x_j + e_i$$

(2)

$$R^2 = 1 - \frac{SS_{\text{residual}}}{SS_{\text{model}} + SS_{\text{residual}}}$$

(3)

$$R^2_{\text{adj}} = 1 - \frac{SS_{\text{residual}}/DF_{\text{residual}}}{(SS_{\text{model}} + SS_{\text{residual}})/(DF_{\text{model}} + DF_{\text{residual}})}$$

(4)

$$\text{adequate precision} = \max (\bar{Y}) - \min (\bar{Y})$$

$$\sqrt{\bar{V}(\bar{Y})}$$

(5)

$$\bar{V}(\bar{Y}) = \frac{1}{n} \sum_{i=1}^{n} \bar{V}(\bar{Y}) = \frac{P \sigma^2}{n}$$

(6)

In Eq. (3) - (6), SS is the sum of squares, DF; the degrees of freedom, $P$ the number of model parameters, $\sigma^2$ the residual mean square from ANOVA table and “n” is the number of experiments.

Ablos et al. (2001) applied the RSM to enhance the biosurfactant production by *Pseudomonas aeruginosa* AT 10.

This CCD is an effective design ideal for sequential experimentation and allows a reasonable amount of information for testing lack of fit, not involving large number of design points (Montgomery, 1996; Myers and Montgomery, 2001).

The optimization and analysis of the age and size of the two-stage inocula for the production of surfactin in batch reactor using *Bacillus subtilis* DSM 3256 and found the interaction between primary inoculum size and secondary inoculum age (Sen and Swaminathan, 2004).
Joshi et al. (2008) proposed that the RSM’s greatest applications particularly have been in situations where a large number of variables influencing the Central Composite Design system (CCD) were used for the RSM in the experimental design, which is well suited for fitting a quadratic surface and usually works well for the process optimization. Parthasarathi (2010) studied the optimization of biosurfactant production using cashew apple juice media by *P. fluorescens* MFS-1.

Najafi et al. (2011) described the combination of Central Composite Rotatable Design (CCRD) and RSM to optimize the biosurfactant production by *Paenibacillus alvei* ARN63 isolated from an Iranian oil well.

Chen et al. (2012) used Response Surface Methodology (RSM) for medium optimization to enhance biosurfactant production by *Acinetobacter* genus and the first report that biosurfactant may be more sensitive to Ca (2+) than Na (+). A quadratic response model was constructed through RSM designs, leading to a 57.5 per cent increase of the growth associated biosurfactant production by *Acinetobacter* sp. YC-X 2 with an optimized medium.

Ghribi et al. (2012) examined the influence of interaction of various physicochemical parameters on biosurfactant production by *B. subtilis* was investigated by plotting the response surface curves. *B. subtilis* SPB1 biosurfactant production yield was increased significantly through application of response surface methodology. The results revealed that the optimising the medium composition and the culture conditions, the production of SPB1 biosurfactant were enhanced from 12.5 mg/g to 20.8 mg/g.

Abbasi et al. (2013) used the response surface optimization of biosurfactant produced by *Pseudomonas aeruginosa* MA01 isolated from spoiled apples. The medium components soybean oil was the best carbon source and sodium nitrate being the most effective nitrogen source on biosurfactant production. Biosurfactant
production at the optimum value of fermentation processing factor (15.68 g/l) was 29.5 per cent higher than the biosurfactant concentration obtained before the RSM optimization (12.1 g/l). The two stages of optimization using RSM could increase biosurfactant production by 1.46 times, as compared to the values obtained before optimisation.

Chandankere et al. (2014) observed that the validation of the model proposed revealed that after optimization process, strain Bacillus amyloliquefaciens USTBb increased the biosurfactant production more than 3-folds (2.2 to 6.85 g/l) when compared with un-optimized conditions.

Praveen Kumar et al. (2014) studied the optimization and production of biosurfactant by Pseudomonas aeruginosa 2297. The cultural condition and medium optimization was found that the maximum predicted biosurfactant production using pH: 7.37, sawdust: 7.656 g/l and glycerol: 1.5 ml /l was obtained using RSM.

Kiran et al. (2014) studied the biosurfactant production by marine Nocardiopsis sp. MSA13A. The optimization results revealed that the $R^2$ value of 0.9406 which was closer to 1 showed the model to be stronger and it can better predict the response. The model was found to be significant with $p < 0.0001$ and insignificant lack of fit. The glucose and yeast extract interactively reached a central value to influence the production maxima over a stable area.

**2.12. Extraction of biosurfactant**

Even if optimum production is obtained using optimal media and culture conditions, the production process is still incomplete without an efficient and economical means for recovery of the products. Recovery and/or purification of biotechnological products in downstream processing costs usually account for approximately 60 per cent of the total production costs which make commercial
production of biosurfactant quite expensive. Methods to reduce costs through the use of inexpensive and renewable substrates are therefore necessary (Desai and Banat, 1997; Makkar and Cameotra, 1997: Banat et al., 2000).

The most common biosurfactant recovery methods are either extracted with solvents (chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether) or acid precipitation or ammonium sulphate precipitation at low temperature (Boothroyd et al., 1956; Spencer et al., 1979). Crystallization and centrifugation, has been widely reported in the literature (Kosaric, 1992).

There is few unconventional but interesting recovery methods have also been reported like, the ultrafiltration (Reiling et al., 1986), adsorption–desorption on polystyrene resins and ion exchange chromatography (Reiling et al., 1986), foam fractionation (Davis et al., 2001; Noah et al., 2002) and adsorption–desorption on wood-based activated carbon (Dubey et al., 2005) are those unconventional recovery methods (Table 3).

The main advantages of these methods are their ability to operate in a continuous mode for recovering biosurfactants with high level of purity. However, the solvents that are generally used for biosurfactant recovery, for example, acetone, methanol and chloroform, are toxic in nature and harmful to the environment. Cheap and less toxic solvents such as methyl tertiary-butyl ether have been successfully used in recent years to recover biosurfactants produced by *Rhodococcus* (Kuyukina et al., 2001). However, a great deal of monetary input is required in the purification processes (Rodrigues et al., 2006).
Table - 3 Extraction methods for the recovery of biosurfactant from culture filtrate

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Methods</th>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adsorption on wood</td>
<td>Adsorption</td>
<td>Heyd <em>et al</em>., 2008, Dubey, 2005</td>
</tr>
<tr>
<td>2</td>
<td>Adsorption on polystyrene</td>
<td>Adsorption</td>
<td>Reiling, 1986</td>
</tr>
<tr>
<td>3</td>
<td>Ion exchange chromatography</td>
<td>Charge separation</td>
<td>Reiling, 1986</td>
</tr>
<tr>
<td>4</td>
<td>Solvent extraction</td>
<td>Dissolves in organic solvents</td>
<td>Kuyukina, 2001</td>
</tr>
<tr>
<td>5</td>
<td>Centrifugation</td>
<td>Due to centrifugal force</td>
<td>Nitschke and Pastore, 2003</td>
</tr>
<tr>
<td>6</td>
<td>Acid precipitation</td>
<td>Insoluble at low pH</td>
<td>Sen and Swaminathan, 2004</td>
</tr>
<tr>
<td>7</td>
<td>Membrane ultrafiltration</td>
<td>Micelles formation</td>
<td>Sen and Swaminathan, 2005</td>
</tr>
<tr>
<td>8</td>
<td>Selective crystallization</td>
<td>Re-dissolution in organic solvents</td>
<td>Satpute <em>et al</em>., 2010</td>
</tr>
<tr>
<td>9</td>
<td>Ammonium sulphate precipitation</td>
<td>Salting out of protein</td>
<td>Satpute <em>et al</em>., 2010</td>
</tr>
<tr>
<td>10</td>
<td>Organic solvent extraction</td>
<td>Solubility in organic solvents</td>
<td>Dubey, 2005</td>
</tr>
<tr>
<td>11</td>
<td>Foam fractionation</td>
<td>Surface activity</td>
<td>Sarachat <em>et al</em>., 2010</td>
</tr>
<tr>
<td>12</td>
<td>Thin layer chromatography</td>
<td>Difference in relative flow against solvent</td>
<td>Ismail <em>et al</em>., 2013</td>
</tr>
<tr>
<td>13</td>
<td>Dialysis</td>
<td>Difference in solute concentration</td>
<td>Satpute <em>et al</em>., 2010</td>
</tr>
<tr>
<td>14</td>
<td>Lyophilization</td>
<td>Cryodesiccation</td>
<td>Satpute <em>et al</em>., 2010</td>
</tr>
<tr>
<td>15</td>
<td>Isoelectric focusing</td>
<td>Electric charge difference</td>
<td>Satpute <em>et al</em>., 2010</td>
</tr>
</tbody>
</table>

2.12.1. Ammonium precipitation

Generally, purification and precipitation of high molecular weight biosurfactant is carried out using ammonium sulphate, followed by dialysis to remove any small molecules. In ammonium sulphate precipitation high molecular weight bioemulsifier such as emulsan, biodispersion (protein rich compounds) are precipitated using \((\text{NH}_4)_2\text{SO}_4\). This method was basically introduced by Rosenberg *et al*. (1979) for precipitation of biosurfactant from *Arthrobacter* RAG-1.
2.12.2. Acetone precipitation

Acetone precipitation is one of the method for the extraction and has been used by several workers, to purify biosurfactant (Rosenberg et al., 1979; Patil and Chopade, 2001a,b, 2003; Anna Joice and Parthasarathi, 2014).

2.12.3. Ethanol precipitation

Ethanol precipitation also involved in the extraction of biosurfactant from cell free culture filtrate. The acetone, ethanol is a popular solvent for obtaining crude extract of biosurfactant from the culture supernatant of microbes such as *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Cyanobacterium* and yeast, species. Phetrong et al. (2008) found that precipitation of emulsifier from *A. calcoaceticus* subsp. *Anitratus* SM7 with ethanol was the most efficient method when compared with other precipitation methods.

2.12.4. Acid precipitation

Acid precipitation is easy inexpensive and readily available to recover crude biosurfactant such as surfactin, lipopeptides, glycolipids etc., *Bacillus* sp. produces different types of surface active peptides (Arima et al., 1968) which are purified from cell free supernatant. Acid hydrolysis is carried out by using concentrated HCl to bring down the pH 2.0. Biosurfactant becomes insoluble at lower pH and precipitates proteins and lipid containing biosurfactant at 4°C overnight (Cooper et al., 1981).

It is followed by centrifugation and pellet is further extracted by using various solvents (Thaniyavarn et al., 2003; Nitschke and Pastore, 2006; Mukherjee et al., 2006). Acidification, centrifugation and extraction procedure are similar for other glycolipids. However, different solvents *Viz.*, chloroform, methanol, ethyl acetate are used generally to purify rhamnolipids. Organic phase is removed separately and mixed
with Na$_2$SO$_4$ to remove water and can be concentrated in a rotary evaporator at 40°C to obtain crude product. Residue is further dissolved in NaHCO$_3$ to purify biosurfactant. There are a number of publications reporting on rhamnolipid purification by acid precipitation (Haba et al., 2000).

Sophorolipids (Nunez et al., 2001), trehalose lipids, mannosyl erythritol lipids (MELs) (Rapp et al., 1979) are extracted similarly to that of rhamnolipids.

Noah et al. (2002) conducted a combined experiment of surfactin production from potato process effluents with direct foam fractionation techniques to enhance the yield of biosurfactant.

Depending upon the type of biosurfactant, (NH$_4$)$_2$SO$_4$ with different concentrations were used for the extraction of biosurfactants. In A. ventianus RAG, 60 per cent (NH$_4$)$_2$SO$_4$ was added to the cell free supernatant for the extraction of biosurfactant whereas 65 per cent of (NH$_4$)$_2$SO$_4$ was added to precipitate Alasan (Toren et al., 2001, 2002; Bach et al., 2003).

Extracted material is filtered for removal of residues and evaporated to dryness using rotary evaporator. Lipopeptide biosurfactant from microbes grown under simple or complex growth conditions are also purified by this method (Jennings and Tanner, 2004).

Youseef et al. (2005) reported that lipopeptide has been partially purified from Bacillus sp. by precipitating with 40 per cent (NH$_4$)$_2$SO$_4$. Acid hydrolysis is carried out by using concentrated HCl to bring down the pH 2.0, biosurfactant becomes insoluble at lower pH (Mukherjee, et al., 2006).

Phetrong et al. (2008) found that precipitation of emulsifier from A. calcoaceticus sub sp. Anitratus SM7 with ethanol was the most efficient method when compared with other precipitation methods.
Hydrophobic moieties of biosurfactant are soluble in some solvents which help in extraction and separation of crude product. Several types of biosurfactant such as rhamnolipid, trehalose lipids, sophorolipids, cellobiolipids, liposan produced by different microbial population have been purified by solvent extraction (Desai and Banat, 1997; Smyth et al., 2010a, b).

2.13. Characterization of biosurfactant

2.13.1. Thin layer chromatography

Thin layer chromatography is one of the most commonly used techniques to detect biosurfactant. It is based on the principle that the solutes compete with the solvent for the surface sites of the adsorbent. Depending upon the distribution coefficient, compounds are distributed on surface of the adsorbents. To detect biosurfactant, solvent system depends upon the type of compound of interest. Organic and inorganic solvents which can be dissolved and are non-volatile are preferred. Solvents which cannot be used with HPLC, UVdetection due to interference can be used in TLC. Sometimes acetic acid, diethyl ether, ethyl acetate, n-hexane, pyridine are needed for the mobility of functional groups of biosurfactant. Spot detection of samples from the TLC plates is done by destructive and non-destructive techniques. Non-destructive techniques involve iodine, water methods and UV radiation. Destructive method involves use of H$_2$SO$_4$. Various developers such as orcinol, resorcinol, iodine, sulphuric acid, ninhydrin are used for detection of carbohydrate, lipid and protein. Occasionally single solvent systems are enough for mobilization of different functional groups which can be sequentially identified with different developing reagents as reported of biosurfactant obtained from *Bacillus* sp. (Makkar and Cameotra, 1997; Haba et al., 2000).
The biosurfactant extracted from the cell-free broth was developed by thin-layer chromatography and visualised with specific reagents, producing spots with different retention factors (Rf). The results demonstrated positive reactions to sugars using Molish reagents (Rf = 0.7) and to lipids using iodine vapours (Rf = 0.8), but negative reactions to amino groups using ninhydrin. The presence of both glycosyl units and lipids indicates that the sample is a glycolipid (Juliana et al., 2013).

A sample of the crude biosurfactant recovered from the I-15glucose (Bacillus sp.) culture revealed yellow spots with iodine vapour when the mobile phase chloroform:methanol:water (65:35:4) was used. This suggests the presence of polar lipids. Treatment with ninhydrin did not reveal any pink or red spots. Moreover, no pink spots were detected when the TLC plate was treated with a-naphthol, indicating lack of carbohydrates (Ismail et al., 2013).

2.13.2. Infra Red (IR) spectroscopy

Absorption of different IR frequencies of a sample positioned in the path of an IR beam is measured. It is a form of radiation that can travel through a vacuum while heat is associated with the motion and kinetic energy of molecules. The spectrometer consists of a radiation source, monochromator and detector. The term “infrared” covers the range of the electromagnetic spectrum between 0.78 and 1000 mm. Absorption of IR is restricted to compounds with small energy differences in the possible vibrational and rotational states. This technique determines the functional groups of gases, liquids and solids samples and gives a structural elucidation of compound. Surfactin, lichenysin and rhamnolipids have been characterized by the IR technique (Das et al., 2008).
The IR Spectrophotometer used is in the range of 4000–400 cm\(^{-1}\). For each spectrum 100 scans are used in 0.23 mm KBr liquid cell. Alkyl, carbonyl, ester compounds of BS are detected clearly (Tuleva et al., 2002).

Thavasi et al. (2007) reported that the IR spectrum for Freeze-dried crude biosurfactant (10 mg) which was added to potassium bromide (100 mg) and pressed in a mechanical press (7500 kg for 30 sec) to obtain translucent pellets. The IR absorption spectrum was recorded on a Thermo Niocolet, AVATAR 330 FTIR systems.

Thavasi et al. (2010) reported that the biosurfactant produced by \textit{B. megaterium} was classified as a glycolipid with carbohydrate and lipid combination of 28:70 percent. The FTIR analysis of the biosurfactant revealed that, the most important bands were located at 2929 cm\(^{-1}\) (for the CH aliphatic stretching), 1700 cm\(^{-1}\) (for the C=O ester bond), 1066 cm\(^{-1}\) (PII band: polysaccharides) and 764, 699 cm\(^{-1}\) (for the CH2 group) and 3342 cm\(^{-1}\) (for O–H bonds) confirming the presence of glycolipid moieties. In addition, the mass spectrometric analysis of the biosurfactant also confirmed the above results with peaks observed at m/z = 326.5, 413.3, 429.3 for lipids and at 663.4 cm\(^{-1}\) for carbohydrate moieties.

Rahman et al. (2010) studied the molecular structure of the rhamnolipids with the help of FTIR spectroscopy. Strong and broad bands of the hydroxyl group free (-OH) stretch due to hydrogen bonding were observed in the region (3368 cm\(^{-1}\)). The presence of carboxylic acid functional group in the molecule was confirmed by the bending of the hydroxyl (O-H) of medium intensity bands in the region of 1455-1380 cm\(^{-1}\). The aliphatic bonds CH3, CH2 and C-H stretching with strong bands are shown in region of 2925 - 2856 and 1455 - 1380 cm\(^{-1}\). The carbonyl (C=O) stretching was found in the region of 1737 cm\(^{-1}\) with strong intensity bands. Two other strong peaks between 1300 and 1033 in the region due to C-O stretch are characteristic
of an ester functional group in the molecule. The peak in the range of 1121 - 1033 cm$^{-1}$ was also reported as C–O–C stretching in the rhamnose. Moreover, we noticed stronger bands of pyranyl I sorption band in region at 918 - 940 cm$^{-1}$ and α- pyranyl II sorption band in region of 838 - 844 cm$^{-1}$ that showed the presence of di-rhamnolipid in the mixture.

Sriram et al. (2011) reported that the FTIR revealed the presence of carboxyl group and peptide component in the biosurfactant. The compound showed the C-H stretching vibrations in the transmittance range 2930 cm$^{-1}$ indicating the aliphatic chain. The distinct peak values observed at 1540 cm$^{-1}$ and 3420 cm$^{-1}$ corresponded to the deformed and strong N-H bond respectively. The transmittance at 1400 cm$^{-1}$ referred to the aliphatic chain of C-H group and he confirmed that the biosurfactant was lipopeptide in nature.

Rikalovic et al. (2012) studied the IR spectrum of rhamnolipid from P. aeruginosa san-ai organism. The study revealed that the fingerprint areas between 400 - 1500 cm$^{-1}$ showed the deformation C–OH band at 1384 cm$^{-1}$, the O–H in plane deformation at 1315 cm$^{-1}$, the O–C–O symmetric band at 1047 cm$^{-1}$, the C–O stretching at 1168, 1127 and 1047 cm$^{-1}$, C–H deformations at 1451, 1238 and 808 cm$^{-1}$ and CH$_3$ rocking at 983 cm$^{-1}$ for rhamnolipid. There are also the typical stretching vibrations of the COO– group. The strong symmetric stretching C=O band of the carboxylate group of RL was at 1739 cm$^{-1}$. The IR spectra of rhamnolipid gave absorption bands at 3360 cm$^{-1}$ for symmetric O–H stretching. The spectrum also showed vibrations at 2928 cm$^{-1}$ and 2856 cm$^{-1}$ typical for the C–H stretching vibrations of CH$_2$ and CH$_3$ groups. The results are in a good agreement with a typical IR spectrum of rhamnolipids.
Ismail et al. (2013) observed the peaks are those commonly found in the IR spectra of lipopeptide biosurfactants produced by several Bacillus species. The broad strong band in the range of 3000 to 3700 cm\(^{-1}\) with a maximum at 3417 cm\(^{-1}\) represents –OH, –CH and –NH stretching vibrations. This is characteristic of carbon-containing compounds with amino groups. Another strong sharp band was observed at 1659 cm\(^{-1}\), which signifies CO–N stretching vibration. Moreover, absorption in the region 1600 - 1700 cm\(^{-1}\) is characteristic for amide I vibrations in proteins, thus indicating the presence of peptide groups in the biosurfactant.

### 2.13.3. Nuclear Magnetic Resonance spectroscopic studies

Nuclear magnetic resonance (NMR) is based on transitions in atoms with a magnetic moment when an external magnetic field is applied. It is the absorbance of radio frequency radiation by a nucleus in a strong magnetic field. Radiation absorption causes the nuclear spin to realign or flip in the higher-energy direction. Once the energy is absorbed, the nuclei will re-emit radiation and return to the lower-energy state. NMR transition energy totally depends on the magnetic-field strength and a proportionality factor for each nucleus called, magnetogyric ratio. NMR provides information regarding the functional groups as well as the position of linkages within the carbohydrate and lipid molecules. Exact location of each functional group can be obtained and information about the structural isomers is also possible with the help of series of NMR experiments. A detailed analysis of glycolipid biosurfactant was carried out with the help of NMR methodology and has recently been reported in the literature (Smyth et al., 2009a).

\(^1\)H-NMR for detection the hydrocarbon chains and rhamnose rings is indicated by the appearance of the characteristic chemical shifts in the region of 0.8 - 1.4 and 3.3 - 5.5 ppm, respectively (Wei, 2005).
Complete assignments of the glycolipid signals were carried out using 1D 1H and $^{13}$C NMR (coupled and decoupled) by 1H, 1H correlation spectroscopy (COSY), 1H, 1H total correlation spectroscopy (TOCSY) and 1H, 13C heteronuclear multiple quantum coherence (HMQC) programs (Monteiro et al., 2007).

The characterization of biosurfactants produced by *B. subtilis* strains using NMR spectroscopy has been described in the literature (Kowall et al., 1998; Tang et al., 2007; Liu et al., 2009).

The structural identification of lipopeptide by NMR produced by *Selenomonas ruminantium* CT2 isolated from mangrove sediment. The result obtained from $^1$H-NMR clearly indicated that the molecule being studied is a lipopeptide. The spectrum confirms the presence of a long aliphatic chain ($\text{CH}_2$ at 1.55 - 1.25 ppm) and a peptide backbone ($\text{NH}$ at 8.00 - 7.24 ppm and $\text{CH}$ at 4.8 - 4.2 ppm) (Saimmai et al., 2012a).

The differences observed in the spectra are in chemical shifts from the region correspondent to the amide protons (6.5–10 ppm), in which the NMR spectrum is less defined. All spectra showed some similarity with the standard surfactin (Pereira et al., 2013).

### 2.14. Silver nanoparticles

The use of nanoparticles is gaining impetus in the present century as they possess’ defined chemical, optical and mechanical properties. The metallic nanoparticles are most promising as they show good antibacterial properties due to their large surface area to volume ratio, which is coming up as the current interest in the researchers due to the growing microbial resistance against metals, antibiotics and the development of resistant strains (Gong et al., 2007; Rai et al., 2009; Lima et al., 2012).
The novel properties of nanoparticles have been exploited in a wide range of potential applications in medicine, cosmetics, renewable energies, environmental remediation and biomedical devices (Lu et al., 2008; De et al., 2010; Ghosh et al., 2011; Tran et al., 2013).

There are various types of nanoparticles and specifically metal nanoparticles play an important role. Among them, silver nanoparticles (Ag-NPs or nanosilver) are attracted increasing interest due to their unique physical, chemical and biological properties compared to their macro-scaled counterparts (Sharma et al., 2009; Prabhu and Poulose, 2012; Kulkarni and Muddapur, 2014).

Ag-NPs have distinctive physico-chemical properties, including a high electrical and thermal conductivity, surface-enhanced Raman scattering, chemical stability, catalytic activity and nonlinear optical behaviour (Bloemer et al., 1990; Chang and Yen, 1995; Sun and Seff, 1994; Shiraishi and Toshima, 2000; Krutyakov et al., 2008; Tran et al., 2013).

Feng et al. (2008) reported mechanistic study of inhibition of silver ions against two strains of bacteria, S. aureus and E. coli. The silver ions enter into the bacterial cells by penetrating through the cell wall and consequently turn the DNA into condensed form which reacts with the thiol group proteins and result in cell death. The silver ions also interfere with the replication process.

Kazachenko et al. (2000) investigated the synthesis and antimicrobial activity of silver complexes with histidine and tryptophan. The histidine complex with silver compound showed good antimicrobial activity against gram-negative bacteria while, the tryptophan complex with silver compound showed higher antimicrobial activity and broad spectrum of action.
Sondi and Salopek-Sondi (2004) reported antimicrobial activity of silver nanoparticles against *E. coli* as a model for gram-negative bacteria. It was also observed that the silver nanoparticles interact with the building elements of the bacterial membrane and cause damage to the cell.

Butkus *et al.* (2004) studied the synergistic effect of silver ions and UV radiation on a RNA virus, which can efficiently enhance the effectiveness of UV radiation.

Baker *et al.* (2005) reported the synthesis of nanoparticles by inert gas condensation and co-condensation techniques. The antibacterial efficiency of nanoparticles was tested against *E. coli*. The nanoparticles were observed to exhibit antibacterial activity at low concentrations. The mechanism behind the antibacterial activity of silver nanoparticles was assumed to be related to the surface area to volume ratio of nanoparticles. The smaller sized particles possessed larger surface area to volume ratio and hence efficient antibacterial activity.

Morones *et al.* (2005) studied the effect of silver nanoparticles in the size range of 1 - 100 nm on Gram-negative bacteria using high angled annular dark field microscopy (HAADF) and TEM. High angled annular dark field (HAADF) images show that the smaller sized nanoparticles (~ 5 nm) depicted efficient antibacterial activity thus concluding that the activity of silver nanoparticles is size-dependent.

Yamanaka *et al.* (2005) investigated the antibacterial efficacy of silver ions using *E.coli* with the help of energy-filtering TEM (EFTEM), two dimensional electrophoresis (2-DE) and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). It was concluded that bactericidal action of silver ions is basically caused due to the interaction of silver ions with ribosome and the suppression and expression of enzymes and proteins necessary for ATP production.
Panacek et al. (2006) reported a one step protocol for synthesis of silver colloid nanoparticles. They found high antimicrobial and bactericidal activity of silver nanoparticles on Gram-positive and Gram negative bacteria including multi resistant strain such as methicillin resistant *S. aureus*.

Leaper (2006) studied the use of silver dressings and their role in wound healing, the role of nanocrystalline silver dressings in wound management. The topical delivery of silver nanoparticles promotes healing of burn wounds with better cosmetic appearance and provides an effective therapeutic direction for scarless healing of wounds (Tian et al., 2006).

Shahverdi et al. (2007) investigated the combination effects of silver nanoparticles with antibiotics. The silver nanoparticles were synthesized using *Klebsiella pneumoniae* and evaluated its antimicrobial activity against *S. aureus* and *E. coli*.

Shrivastava et al. (2007) reported synthesis of silver nanoparticles in the size range of 10 - 15 nm and its dose dependent effect on the Gram-negative and Gram-positive microorganisms. From the results it was found that the dose dependent silver nanoparticles have marked activity against Gram-negative organisms than the Gram-positive organisms.

Pal et al. (2007) investigated the antibacterial properties of silver nanoparticles of different shapes and found that the antibacterial efficacy of silver nanoparticles was shape dependent.

Gong et al. (2007) synthesized bifunctional Fe$_3$O$_4$@Ag nanoparticles possessing super paramagnetic and antibacterial properties and showed excellent activity against *E.coli*, *S. epidermis* and *Bacillus subtilis*. The Fe$_3$O$_4$@Ag nanoparticles possessed broad antibacterial activity and could be recycled due to their super paramagnetism.
Maneerung et al. (2008) suggested a novel technique for preparation of wound dressings using bacterial cellulose and the antibacterial effect of silver nanoparticles impregnated on the wound dressing. The silver nanoparticles impregnated with bacterial cellulose demonstrated efficient antimicrobial activity against *E. coli* and *S. aureus*.

Gade et al. (2008) studied the *Aspergillus niger* mediated synthesis of silver nanoparticles. The electron microscope study demonstrated the presence of silver nanoparticles in the cell membranes of the test bacteria.

Kumar et al. (2008) investigated an environmental friendly method for the synthesis vegetable oil based metal nanoparticles embedded paints.

Jha et al. (2010) reported that green, low-cost and reproducible *Lactobacillus*-mediated biosynthesis of silver nanoparticles. Individual nanoparticles having the dimensions of 10 - 25 nm (n-Ag) was obtained.

Kaviya et al. (2011) studied the synthesis of silver nanoparticles (AgNPs) using *Polyalthia longifolia* leaf extract as reducing and capping agent along with D-sorbitol used to increase the stability of the nanoparticles. The synthesized silver nanoparticles are found to be highly toxic against Gram-positive bacteria than Gram-negative bacteria.

Nagajyothi and Lee (2011) studied that the synthesis of plant-mediated silver nanoparticles using *Dioscorea batatas* rhizome extract and evaluation of their antimicrobial activities.

Saklani et al. (2012) reviewed that interesting facts about silver nanoparticles synthesis and the mechanism involved in the antimicrobial activity of silver ions. Dhoondia and Chakraborty (2012) reported the synthesis of silver oxide nanoparticles using *Lactobacillus mindensis*, isolated using fixer solution from an X-ray photographic
laboratory. *Lactobacillus mindensis* served as a promising candidate in the quest to synthesize silver oxide nanoparticles through green chemistry.

Korbekandi *et al.* (2013) studied the optimization of production of silver nanoparticles using biotransformations by *Fusarium oxysporum* and a further study on the location of nanoparticles synthesis in this microorganism. The results revealed that silver nanoparticles are synthesized intracellular and not extracellular.

Das *et al.* (2014) reported the extracellular synthesis of silver nanoparticles by the *Bacillus* strain CS 11. The size of silver nanoparticles obtained was in 42 - 92 nm range.

Pettegrew *et al.* (2014) Silver nanoparticle synthesis using monosaccharides and their growth inhibitory activity against Gram-Negative and Positive Bacteria. The observations suggested that the growth inhibition of Ag NPs is mediated by interfering with the bacterial cell wall peptidoglycan.

### 2.15. Preparation of silver nanoparticles

Currently, many methods have been reported for the synthesis of silver nanoparticles by using chemical, physical, photochemical and biological routes. Each method has advantages and disadvantages with common problems being costs, scalability, particle sizes and size distribution.


2.15.1. Microemulsion techniques

Krutyakov et al. (2008) described that the uniform and size controllable silver nanoparticles can be synthesized using microemulsion techniques. The nanoparticles preparation in two-phase aqueous organic systems is based on the initial spatial separation of reactants (metal precursor and reducing agent) in two immiscible phases. The interface between the two liquids and the intensity of inter-phase transport between two phases, which is mediated by a quaternary alkyl-ammonium salt, affect the rate of interactions between metal precursors and reducing agents. Metal clusters formed at the interface are stabilized, due to their surface being coated with stabilizer molecules occurring in the non-polar aqueous medium and transferred to the organic medium by the inter-phase transporter. One of the major disadvantages is the use of highly deleterious organic solvents.

For instance, Zhang (2007) used dodecane as oily phase (a low deleterious and even nontoxic solvent), but there was no need to separate the prepared silver solution from the reaction mixture. On the other hand, colloidal nanoparticles prepared in non-aqueous media for conductive inks are well-dispersed in a low vapour pressure organic solvent, to readily wet the surface of polymeric substrate without any aggregation. The advantages can also be found in the applications of metal nanoparticles as catalysts to catalyze most organic reactions, which have been conducted in non-polar solvents. It is very important to transfer metal nanoparticles to different physicochemical environments in practical applications (Cozzoli et al., 2004).

2.16. Mechanism of action of silver nanoparticles

The silver nanoparticles show efficient antimicrobial property compared to other salts due to their extremely large surface area, which provides better contact with microorganisms. The nanoparticles get attached to the cell membrane and also penetrate
inside the bacteria. The Figure 7 depicts the mode of action of silver nanoparticles against the bacteria.

The bacterial membrane contains sulphur containing proteins and the silver nanoparticles interact with these proteins in the cell as well as with the phosphorus containing compounds like DNA. When silver nanoparticles enter the bacterial cell it forms a low molecular weight region in the centre of the bacteria to which the bacteria conglomerates thus, protecting the DNA from the silver ions. The nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death. The nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity (Feng et al. 2000; Sondi and Salopek-Sondi, 2004; Morones et al., 2005; Song et al., 2006; Rai et al., 2009; Prabhu and Poulose, 2012; Tran et al., 2013).

Figure 7: Various modes of action of silver nanoparticles on bacteria

(Courtesy: Prabhu and Poulose, 2012)
2.17. Nanotechnological applications of surface-active compounds

Besides the application in environment the biosurfactant play a vital role in the field of nanotechnology as a stabilizing agent in the preparation of various nanoparticles (Kitamoto et al., 2005).

Xie et al. (2005) reported that the rhamnolipid biosurfactants can be used to form microemulsions at room temperature and obtained the phase diagram of rhamnolipid/n-butanol/n-heptane/water pseudoternary system and found that reverse micelles can be formed within larger region.

Xie et al. (2006) studied the synthesis of silver nanoparticles in water-in-oil microemulsion stabilized by rhamnolipid. Silver nanoparticles obtained in this system are of spheric and uniform and rhamnolipid was identified as a good stabilizer for the silver nanoparticles in heptane.

Xu et al. (2006) reported that the silver nanoparticles were prepared by the reduction of silver nitrate using NaBH₄ as reducing agent in water-in-oil reverse micelles system, in which gemini surfactant 2-hydroxy-1, 3 bis (octadecyldimethylammonium) propane dibromide (18-3(OH)-18) was used as stabilizer.

Biswas and Raichur (2008) studied the effect on the synthesis and stabilization nanozirconia particles by rhamnolipid biosurfactant.

Kasture et al. (2008) discussed the synthesis of silver nanoparticles by sophorolipids and effect of temperature and sophorolipid structure on the size of particles. Sophorolipids were used as capping and reducing agent in nanoparticle synthesis.

Reddy et al. (2009) reported that the surfactant mediated synthesis of nanoparticles is emerging as a potential method for the stabilization of nanoparticles.
Although the chemical and physical processes are potentially useful in the synthesis of nanoparticles, the size-controlled synthesis is still remaining as a challenge in material science. The size and shape are critical factors that decide the biological activity and specificity. In this context, the surfactants are emerging as potential stabilizing agents, however, the synthetic surfactants are are not environmentally friendly. Therefore, the biosurfactants are emerging as a “green” alternate for the synthesis and stabilization of nanoparticles.

Lee et al. (2009) reported the synthesis of silver nanoparticles using non-ionic surfactants such as polysorbate 20, polysorbate40, polysorbate 60, polysorbate 80 and Brij 97, under microwave irradiation and fullerene [C60]-linked silver nanoparticles.

Kiran et al. (2010) reported the synthesis of silver nanoparticles by glycolipid biosurfactant produced from marine Brevibacterium casei MSA19. It was found that the nano-scalesilver can be synthesized in reverse micelles using the glycolipid as stabilizer. The silver nanoparticles synthesized were uniform and stable for two months.

Liao et al. (2010) described the use of the natural surfactin to stabilize super paramagnetic iron oxide nanoparticles (SPION) which can serve as a sensitive contrast agent for Magnetic Resonance Imaging (MRI). It showed that the organic magnetic nanoparticles were transferred into water by the surfactin, without particles aggregation and size change.

Lah et al. (2011) studied the anisotropic mono-dispersed silver nanoparticles synthesis via a simple chemical reduction method and assisted by synthetic commercial Daxad 19 surfactant.

Singh et al. (2011) reported the synthesis of stable cadmium sulfide nanoparticles using surfactin produced by Bacillus amyloliquifaciens strain KSU-109.
Kiran et al. (2014) studied the effect of Fe nanoparticle on growth and glycolipid biosurfactant production under solid state culture by marine *Nocardiopsis* sp. MSA13A.

Farias et al. (2014) reported the simpler route for nanoparticle synthesis compared to existing systems using whole organisms or partially purified biological extracts, showing that the low-cost biosurfactant can be used for nanoparticle synthesis as a non-toxic and biodegradable stabilizing agent.

### 2.18. Textile Industry

The term 'Textile' is a Latin word originating from the word 'texere' which means 'to weave'. Textile refers to a flexible material comprising of a network of natural or artificial fibres, known as yarn. The textile and clothing industry normally seen as “traditional industry” is an important part of the European and Asian manufacturing industry. Because of the increased competition, the industries have to move towards more innovative, high quality products in order to differentiate themselves and compete with other competitors. In the development of fabrics, functional aspects such as antibacterial and UV protection are playing an increased important role (Ramachandran et al., 2004; Kwong, 2006; Rajendran et al., 2012).

Textile consumers are now becoming much more aware of the deleterious effect that microorganisms may have upon textiles and human hygiene. In particular, the medical textile sector has welcomed the greater applicability of antimicrobial finishes to stem the possibility of infections arising from the presence of microorganisms. Several researchers have used antimicrobial finishes with barriers against microorganisms (Holme, 2008).

Natural cellulosic fibers due to their hydrophilic nature support the growth of microorganisms such as bacteria and fungi. In order to inhibit the growth of
microbes on cellulosic fabrics, several eco-friendly bioactive agents are effectively used for antimicrobial finish (Joshi et al., 2009; Nithya et al., 2012). Combining the nanotechnology and textile industry to provide the antimicrobial textiles is the main scenario at present.

2.19. Medical textiles

Combination of textile technology and medical sciences has resulted into a new field called Medical textiles. New areas of application for medical textiles have been identified with the development of new fibers and manufacturing technologies for yarns and fabrics. Development of medical textiles can be considered as one such development, which is really meant for converting the painful days of patients into the comfortable days (Ram Meena, 2011).

2.20. Application of antimicrobial textiles

The number of applications of textiles with antimicrobial activity has increased dramatically. A brief summary of the various fields of applications and associated products is shown Table 4.

<table>
<thead>
<tr>
<th>Medicine</th>
<th>Sport and leisure</th>
<th>Outdoor</th>
<th>Technology</th>
<th>Domestic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Support stocking</td>
<td>Shoes</td>
<td>Jackets</td>
<td>Wall hangings</td>
<td>curtains</td>
</tr>
<tr>
<td>Antidecubitus mattress</td>
<td>Shocks</td>
<td>Tents</td>
<td>Roof coverings</td>
<td>coverings</td>
</tr>
<tr>
<td>Incontinence liners</td>
<td>T-shirts</td>
<td>Uniforms</td>
<td>Facade linings</td>
<td>cloths</td>
</tr>
<tr>
<td>Encasings</td>
<td>Cycle wear</td>
<td>Personal protective</td>
<td>Air filters</td>
<td>Bath mats</td>
</tr>
<tr>
<td>Bedding filling</td>
<td>Team kit</td>
<td>Astro turf</td>
<td>Automotive</td>
<td>Sanitizers</td>
</tr>
<tr>
<td>Pillows</td>
<td>Jogging suits</td>
<td>Sunshades</td>
<td>Geotextiles</td>
<td>Underwear</td>
</tr>
<tr>
<td>Implants</td>
<td>Awnings</td>
<td></td>
<td></td>
<td>Carpets</td>
</tr>
</tbody>
</table>
2.21. Silver nanoparticles coated textile fabrics

In the past few decades, researchers are taking interest in the development of textile fabrics containing antibacterial agents. As, silver is non-toxic and possesses antimicrobial properties it has encouraged workers to use silver nanoparticles in different textile fabrics. In this direction, silver nanocomposite fibres were prepared containing silver nanoparticles incorporated inside the fabric. The scanning electron microscopic study revealed that the silver nanoparticles incorporated in the sheath part of fabrics possessed significant antibacterial property compared to the fabrics incorporated with silver nanoparticles in the core part (Yeo and Jeong, 2003).

Therefore the development of AgNPs based on environmental benign natural polymers is considered as most appropriate method for environmental reasons (Raveendran et al., 2003).

Duran et al. (2007) studied the usage of silver nanoparticles on polyester nonwovens. It was reported that silver nanoparticles coated textile fabrics possess antibacterial activity against *S. aureus*.

Ghosh et al. (2010) also studied that the silver nanoparticles synthesized and were found to have stronger antibacterial activity against the Gram negative bacteria than the Gram positive ones and biological synthesized silver nanoparticles and their incorporation in fabrics cotton, providing them sterile properties. The cotton fabrics incorporated with these silver nanoparticles exhibited antibacterial activity against *S. aureus* and *E. coli*.

Mahltiga et al. (2011) prepared the silver nanoparticles suitable for textile finishing processes to produce textiles with strong antibacterial properties against different bacterial types.
Hebeish et al. (2011) prepared highly effective antibacterial textiles containing green synthesized silver nanoparticles. Results explored that, regardless of the concentration of AgNPs used, the bacterial reduction, in presence/absence of binder was always higher than 95 per cent without washing. However, binder retains excellent antibacterial properties even after 20 washing cycles reflecting the significance of binder in fixation of AgNPs deposits on the surface of the fabrics.

Youbo et al. (2012) studied the silver nanoparticles-chitosan composite using microcrystalline chitosan gelatinous water dispersion at ambient temperature and its aqueous solution was applied to the antibacterial finishing of Tencel/cotton nonwoven fabric. The finished nonwoven fabric showed excellent water absorption ability, air permeability and antibacterial activity against *E. coli*.

Budama et al. (2013) reported that the silver nanoparticles have been fabricated within reverse micelle cores of Polystyrene-block-Poly Acrylic Acid (PS-b-PAA) copolymer synthesized by the Atom Transfer free Radical Polymerization (ATRP) method. Significant antibacterial activity against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* has been determined. The antibacterial activity is permanent up to five washings against *E. coli* and up to twenty washings against *S. aureus*.

Nateghi et al. (2014) studied the effect of different morphologies of silver nanoparticles such as spherical, polygonal, disk, prism and hierarchical on antimicrobial characteristics of cotton fabrics. Nonspherical morphologies such as polygonal, prism and hierarchical like shapes in comparison with spherical and disc morphologies exhibited stronger growth inhibitory effect against Gram-negative bacterium *Escherichia coli* and Gram-positive *Staphylococcus aureus* after five replicate standard
washing processes. In addition, among various tested morphologies, the hierarchical like morphology showed a very well antimicrobial activity over 91 per cent after five washing cycle.

Li et al. (2014) reported a new approach for fabrication of a long-term and recoverable antimicrobial nanostructure/textile hybrid without increasing the antimicrobial resistance. New Ag/ZnO/textile antimicrobial composites can be used for wound dressings and medical textiles for topical and prophylactic antibacterial treatments.

2.2.2. Tests used to check the antimicrobial activity

Several test methods have been developed to determine the efficacy of antimicrobial textiles (Gao and Cranston, 2008). The tests to evaluate the antibacterial properties generally fall into two categories: Agar diffusion test (qualitative method) and Dynamic shake test (quantitative method). The bacterial species Escherichia coli (Gram negative) and Staphylococcus aureus (Gram positive) are used in most test methods (Table 5).

2.2.2.1. Qualitative methods

2.2.2.1.1. Agar diffusion test

The agar diffusion tests include AATCC 147-2004 (American Association of Textile Chemists and Colourists), JIS L 1902-2002 (Japanese Industrial Standards), SN 195920-1992 (Swiss Norm) and ISO20645:2004 (International Organization for Standardization). They are only qualitative, but are simple to perform and are most suitable when a large number of samples have to be screened for the presence of antimicrobial activity (Mucha et al., 2002; Hasabo et al., 2012).
2.22.1.2. Parallel Streak Method

“Parallel Streak Method” AATCC Test Method 147-2004 has the aim to determine the antibacterial activity of diffusible antimicrobial agents on treated textile fabric. The method is used for obtaining an estimate of activity, in that the growth of the inoculum organism decreases from one end of each streak to the other and from one streak to the next resulting in increasing degrees of sensitivity. The size of the zone of inhibition and the narrowing of the streaks caused by the presence of the antibacterial agent allow an estimate of the residual antibacterial activity after multiple washings.

The average width (W) of an inhibition zone, along a streak, on either side of the test specimen is calculated by the following Eq. (1):

\[ W = \frac{T - D}{2} \]

Where, T is total diameter of test specimen and clear zone (in mm) and D is diameter of the test specimen (in mm) (Rajendran et al., 2010; Rajendran et al., 2012)

2.22.2. Dynamic shake tests (Quantitative method)

The Dynamic shake tests include ASTM E 2149-01 (American Society for Testing and Materials) and AATCC Test Method 100-1999 (American Association of Textile Chemists and Colourists). They provide quantitative values on the antimicrobial finishing, but are more time-consuming than agar diffusion tests.

“Standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions” ASTM E 2149-01 is designed to evaluate the resistance of non-leaching antimicrobial treated specimens to the growth of bacteria under dynamic contact conditions. This dynamic shake flask test was developed for routine quality control and screening tests in order to overcome difficulties in using classical antimicrobial test methods to evaluate substrate-bound antimicrobials.
### Table 5 Various tests used to check the antimicrobial property of the fabrics

<table>
<thead>
<tr>
<th>Test</th>
<th>Description</th>
<th>Examples of textile tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>AATCC-147-1998 (USA)</td>
<td>Qualitative antibacterial activity assessment of diffusible antibacterial agents (quick method)</td>
<td>Clothing: socks, tee shirts, training shoe, interlinings Other: dish cloths, foot coverings, bedding and towels, wall papers, upholstery, leather, plastics and rubber materia</td>
</tr>
<tr>
<td>SNV-195 920, 1994 (Swiss)</td>
<td>Qualitative - agar diffusion test: assessment of antibacterial effect of agents and impregnated textiles</td>
<td>Clothing: socks, tee shirts, training shoe, interlinings Other: dish cloths, foot coverings, bedding and towels, wall papers, upholstery, leather, plastics and rubber materia</td>
</tr>
<tr>
<td>SNV-195 921, 1994 (Swiss)</td>
<td>Qualitative - agar diffusion test: assessment of antifungal agents and fabrics impregnated by them - antimycolic effect</td>
<td>Clothing: swimwear, clothing liable to wet Other: textiles, upholstery, leather, plastics and rubber materials</td>
</tr>
<tr>
<td>AATCC-100-1998 (USA)</td>
<td>Quantitative assessment of antibacterial finishes on textiles - measures the degree of antibacterial activity</td>
<td>Clothing: socks, tee shirts, training shoe interlinings Other: Dish cloths, floor coverings</td>
</tr>
<tr>
<td>JIS L 1902-1998 (Japan)</td>
<td>Quantitative assessment of fibres and fabrics with inherent antibacterial properties (static and cidal) (e.q. zeolites)</td>
<td>Clothing: socks, tee shirts, training shoe interlinings Others: furnishings, bedding</td>
</tr>
<tr>
<td>BS 6085 part 5, 1992</td>
<td>Mildew and fungal growth analysis</td>
<td></td>
</tr>
<tr>
<td>BS 6085 part 4, 1992</td>
<td>Resistance of textiles to bacterial degradation</td>
<td>Clothing: woollen articles</td>
</tr>
<tr>
<td>BS EN ISO 11721, 2001</td>
<td>Soil Burial test Severe test conditions</td>
<td>Cellulose containing products in contacts with soil-sandbags, shoe liners, tarpaulins, textile based sports equipment</td>
</tr>
</tbody>
</table>
These difficulties include ensuring contact of inoculum to treated surface (AATCC 100), flexibility of retrieval at different contact times, use of inappropriately applied static conditions (AATCC 147), sensibility and reproducibility. The antimicrobial activity is expressed in per cent reduction of the organisms after contact with the test specimen compared to the number of bacterial cells surviving after contact with the control. The percentage reduction is calculated using the following Eq. (2):

$$100 \left( \frac{CFU}{ml} \right) \% \ Reduction = \frac{B - A}{B} \times 100$$

where A are the surviving cells (CFU/ml) for the flasks containing the treated substrate after the specified contact time and B are “0” contact time CFU/ml for the flasks used to determine A before the addition of the treated substrate (Su et al., 2011; Hasabo et al., 2012; Xue et al., 2012; Nithya et al., 2012).

2.22.3. Antifungal tests

There are various tests for the determination of antifungal characteristics of treated fabrics. BS EN ISO-14119:2003, BS 6085 part 5:1992, BS EN ISO 11721: 2001 are the basic protocol for the assessment of antifungal activity of fabrics (Saponjic et al., 2008; Vankar and Shukla, 2012).

Silver nanoparticles synthesized using biosurfactant are coated to cotton textiles and checked for the antibacterial property. The compact coating of silver nanoparticles imparts not only the metallic feature to the fibers rendering the textiles conductive, but also the antibacterial property to the textiles. This method to multifunctionalizing conventional textiles with one material is useful in the textile industry and this strategy is expected to become a powerful platform for the fabrication of multifunctional materials.