2. REVIEW OF LITERATURE

Extremophiles are microbes adapted to grow in conditions such as extreme pH, temperature, salinity, pressure, UV and ionizing radiations etc. In general, it has been believed that they survive in extreme environments in which they had adapted to grow. However, many extremophiles have been isolated from other extreme conditions. For example, alkaliphiles have been isolated from acidic and neutral environments places in which they would not be expected to grow. Alkalophilic bacteria are widely distributed in naturally occurring alkaline or non-alkaline environments (Desai et al., 2004). Stable alkaline conditions are primarily due to the unusual combination of climatic, geological and topological conditions. Soda lakes represent the most stable high pH environments on Earth and commonly have pH values above 11.5 (Pedersen et al., 2004). These environments are characteristically associated with low Mg and Ca concentration together with rates of evaporation that exceed any inflow. Such environments are found in arid and semi-arid areas of tropical or subtropical rain-shadow deserts such as in North America or in the continental interiors of Asia (Grant et al., 1990). Alkaliphilic cyanobacteria drive these systems, providing fixed carbon that is utilized by a vast range of alkalophilic aerobic and anaerobic chemoheterotrophs, notably Halomonads, Bacilli, Clostridia and methanogens (Grant et al., 1990). The Kenyan-Tanzanian Rift Valley contains a number of hyper saline lakes, with salt concentrations ranging from 5 to 35% w/v (saturation) and pH values of 8.5 to >11.5. The microbial community of Soda Lake contains alkaliphilic representatives of all the major trophic groups of bacteria and archaea. Between these groups, there is constant recycling of carbon, sulfur and nitrogen under aerobic and anaerobic conditions (Rees et al., 2004). Cyanobacteria, notably Arthrospira platensis and...
Cyanospira rippkae are responsible for photosynthetic primary production in dilute lakes (Rees et al., 2004) and similarly also an unquantifiable contribution to primary productivity is made by anoxygenic phototrophic bacteria of the genus Ectothiorhodospira (Jones et al., 1998). In hyper saline lakes, cyanobacteria and anoxygenic phototrophs from the genus Halorhodospira and Rhodobaca were reported to be may be responsible for primary effectiveness (Milford et al., 2000).

Commercial processes such as chlor-alkali, soda ash, textile, beverages, food, cement manufacturing and paper and hide processes generate alkaline conditions because of the chemistry of the components used. However, such environments have a relatively restricted range of alkaliophilic inhabitants, usually Bacillus or related species. A far more diverse population of alkaliophiles can be found where stable, naturally occurring alkaline conditions are maintained. There are two such kinds of environments caused by a combination of geological, geographical and climatic conditions. Naturally occurring stable alkaline environments such as eutrophic soda (Na₂CO₃) lakes harbour a much wider range of alkaliophilic microbes (Grant et al., 1990; Joshi et al., 2008). A similarly wide range of alkaliophiles may exist in oligotrophic [Ca(OH)₂] dominated ground water in certain parts of the world. From culture-dependent studies, the soda lakes of the East African Rift Valley have been shown to support a dense and diverse population of aerobic, organotrophic, halophilic, alkaliophilic and alkali tolerant representatives of major bacterial and archaeal phyla. Phylogenetic diversity of alkaliophiles from East African Soda Lake has revealed the presence of aerobic chemoorganotrophic bacteria and archaea (Duckworth et al., 1996; Grant et al., 1999; Humayoun et al., 2003; Joshi et al., 2008). The study of microbial diversity with respect to the depth of Mono lake in California has revealed the sequences related to five major lineages—α- and β- proteobacteria, CFB, high G+C,
low G+C, chloroplasts and candidate divisions (Humayoun et al., 2003; Joshi et al., 2008). The diversity and activity of microorganisms were studied in hyper alkaline spring waters in Maquqrin, Jordan where the diversity of aerobic heterotrophic populations from nonsaline alkaline environment was assessed (Tiago et al., 2004). Also, diversity of the Inner Mongolian Baer Soda Lake and Kenyan Soda Lake were studied by molecular methods (Ma et al., 2004; Jones et al., 1998).

The microbial diversity of saline lakes has been studied primarily by focusing on the isolation and characterization of individual organisms with potential industrial applications. Lipase producing microorganisms from Lake Bogoria were isolated and characterized, while starch-hydrolyzing microorganisms were isolated from a Kenyan alkaline soda lake as well as from Ethiopian soda lakes (Vargas et al., 2004; Martins et al., 2001). As far as Indian soda lakes are concerned, a culture-dependent approach has been applied to analyze the bacterial diversity of Lonar Lake (Joshi et al., 2008). The alkaline Lonar Lake is a unique basaltic rock meteorite impact crater, ranking third in the world. The Lonar crater is filled with saline water. The distinctiveness of the lake water is its salinity and high alkalinity. Extensive literature review revealed that its salinity was 40.78, 31.52 and 30.87% in 1910, 1958 and 1960 respectively. The salinity of the lake has now decreased to 7.9%. The observed alkalinity is ascribed to an interaction between sodium chloride, calcium carbonate and water over a long period. Some geological and chemical reports are available on Lonar Lake. However, there is meager data available on its bacterial diversity. Eutrophication and presence of blue green algae in Lonar Lake have also been described (Wani et al., 2006; Joshi et al., 2008). Some researchers have studied the alkaline metalloprotease from alkaline Streptomyces and Indibacter alkaliphilus gen. nov., sp. nov., an alkaliphilic bacterium, isolated from Lonar lake silt sample (Anilkumar et al., 2010). Bioremediation using
phenol utilizing alkaliphilic bacteria, isolated from its sediments was another interesting finding (Kanekar et al., 1995). A preliminary account of bacterial diversity of the Lonar Lake ecosystem has been reported, which includes some of the biochemically identified isolates. Culture dependent phenotypic characterization and 16S rRNA based phylogenetic analyses were applied to study aerobic, cultivable bacterial populations present in the alkaline Lonar Lake (Wani et al., 2006). The isolates were further studied for their biotechnological potential (Joshi et al., 2008).

Alkaliphiles isolated from such environments show considerable phylogenetic diversity. Many physiologically active bacterial and archaeal species have also been isolated and characterized. Cultured microorganisms represent only a minor but crucial component in the existing diversity of soda lakes. In alkaline environments such as soils, increase in pH is due to microbial ammonification and sulfate reduction and by water derived from leached silicate minerals. The pH of these environments fluctuates due to their limited buffering capacity and therefore, alkalitolerant microbes are more abundant in these habitats than alkaliphiles. The best studied alkaline environments are soda lakes and soda deserts (e.g. East African Rift valley, Indian Sambhar Lake). The nutrient levels are very low in certain freshwater lakes known as oligotrophic lakes. Desert soils in particular, represent very low-nutrient environments, which have negligible and unpredictable rainfall resulting in poorly developed soils. Such soils are generally low in organic matter and water and range from acidic to strongly alkaline on the surface. From neutral soils, alkaliphilic Gram-positive and endospore forming Bacillus spp., and non-sporing species of Pseudomonas, Paracoccus, Micrococcus, Aeromonas, Corynebacterium and Actinopolyspora, and alkalitolerant fungi have been isolated. Alkaliphilic Exiguobacterium auranticum and Exiguobacterium sp. are described from man-made alkaline environment such as potato processing waste and
textile industry effluent (Kumar et al., 2005a, b; Kumar and Kumar, 2008; Dafale et al., 2010; Kulshreshtha et al., 2010; Yang et al., 2011). Calcium springs in Oman support the growth of aerobic species of *Bacillus*, *Vibrio*, *Flavobacterium*, *Pseudomonas* and members of enterobacteria (Tiago et al., 2004; Satyanarayana et al., 2005). Red colored water in the lakes is due to large numbers of haloalkalophilic archaea such as *Natronobacterium pharaonis*, *N. gregoryi* and *Natronococcus occultus*. Haloalkalophilic *methanotrophs* such as *Methylobacter alcalophilus* and *Methylomicrobium alcaliphilum* were reported from Lake Khadyn and Kenyan soda lake sediment, respectively. Anaerobes, *Alkalithermophiles*, *Clostridium*, *Thermoanerobacter* sp. and *Thermopallium natronophilum* were isolated from lake sediments (Satyanarayana et al., 2005). *Bacillus alcalophilus*, the first aerobic alkaliphilic bacterium isolated during 1934 by Vedder, was exploited by Horikoshi (1999) for its potential in bioremediation applications for the industrial effluents. The use of alkaliphilic microorganisms has a long history in Japan. From ancient times, indigo leaves have been naturally reduced under alkaline conditions in the presence of sodium carbonate as part of a traditional process known as indigo fermentation. Alkaliphiles were isolated as early as 1968 by Horikoshi et al. The first paper concerning an alkaline protease was published in 1971 by the same group (Horikoshi, 1999). Another alkaliphilic bacterium, *Halanaerobium hydrogeniformans*, was isolated from Soap Lake, WA, USA for production of biohydrogen (Begemann et al., 2012). *Bacillus marmarensis* sp. nov., an alkaliphilic bacterium and protease producer was isolated from mushroom compost from Marmara region, Turkey. (Denizci et al., 2010). The cyclodextrin glycosyltransferase production was carried out using *Bacillus agaradhaerens* KSU-A11 isolated from Egyptian soda lakes, Egypt (Ibrahim et al., 2010). Purple sulfur bacteria of the family *Ectothiorhodospiraceae*
(Ect.haloalkaliphila, Ect.magna and Ect.variabilis) were isolated from brackish, moderately saline steppe and hypersaline lakes (Gorlenko et al., 2009). Haloalkaliphilic Bacilli consists of different genera such as Alkalibacillus, Gracilibacillus and Halobacillus were isolated from various different saline environments (Jeon et al., 2005; Romano et al., 2005; Echigo et al., 2010) Some haloalkaliphilic strains such as Alkalibacillus silvisoli (Usami et al., 2007), Bacillus oshimensis (Yumoto et al., 2005), Halalkalibacillus halophilus (Echigo et al., 2007) and Geomicrobium halophilum gen. nov., sp. nov. (Echigo et al., 2010) were isolated from forest and garden (non-saline) soils of Japan.

Alkaliphilic diversity studies have also been reported from other sources include spring waters (Pedersen et al., 2004), washwaters of edible olive oil and olive mill wastes (Ntougias and Russell, 2000; Ntougias et al., 2006), carbonate leachate from uranium heap (Ghauri et al., 2006), tanneries and salt mine (Lefebvre et al., 2006), ground water (Roadcap et al., 2006), oil contaminated coasts (Al-Awadhi et al., 2007) and soda ponds (Rusznyák et al., 2008).

**Alkaliphiles for biotechnological applications**

Alkaliphilic bacteria are promising producers of organic acids (Paavilainen et al., 1995). Halolactibacillus halophilus, an alkaliphilic microorganism isolated from a marine environment, produced L-lactic acid at pH 9, with low yield and optical purity. Instead, alkaliphilic Bacillus sp. WL-S20 was the most potent strain for the production of L-lactic acid with high optical purity (Calabia et al., 2011; Meng et al., 2012).

A boron-tolerant alkaliphilic bacteria, Chimaereicella boritolerans sp. nov., was isolated from Kutahya Province, Turkey which had a naturally high concentration
of boron minerals (Ahmed et al., 2007). As and Se reducing haloalkaliphilic bacteria were isolated from Mongolian soda lakes (Hamamura et al., 2012); Amphibacillus sp. KSUCr3, isolated from hypersaline soda lakes, and Bacillus sp. isolated from salt flat, Soap Lake, exhibited high Cr(VI) and Fe(III) reducing capacity, respectively, under alkaline conditions (Pollock et al., 2007; Ibrahim et al., 2011). Anaerobic utilization of pectinous substrates at extremely haloalkaline conditions was reported by Natranaerovirga pectinivora gen. nov., sp. nov., and Natranaerovirga hydrolytica sp. nov., isolated from alkaline black liquor of pulp mill wastewater and hypersaline soda lakes (Sorokin et al., 2012).

India being a subtropical region provides a natural habitat for wide diversity of alkaliphiles. Sporadic attempts have been made till now to isolate alkaliphiles from extreme Indian environments. Twenty-eight obligate alkaliphiles from various estuarine mangrove regions of Goa were reported based on morphological, biochemical and physiological characteristics (Desai et al., 2004). Extracellular enzymes like amylase, lipase, protease, xylanase (Menon et al., 2010) and cellulase producing Bacillus cereus, Bacillus firmus, Enterococcus caseliflavus, Bacillus fusiformis, Bacillus cohnii, Bacillus horikoshii and Bacillus odysseyi and methanol degrading microorganisms Methanotrophs were isolated from water and sediment of alkaline Lonar Lake (Tambekar and Tambekar, 2012). Studies related to extracellular alkaline enzymes, pigments and antibiotics have also been reported from different parts of India. Red pigment producing, extremely haloalkaliphilic bacteria were isolated from Sambhar lake (salt lake and solar pans) in Rajasthan (Upasani and Desai, 1990). An alkaliphilic, thermophilic Bacillus sp. isolated from a hot-water spring (Vajreshwari, Bombay) was found capable of producing D-xylose isomerase (Chauthaiwale and Rao, 1994) and xylanase II with a distinctly different structure.
from other xylanases (Kulkarni et al., 1999). Extracellular protease and carboxymethylcellulase producing *Alcaligenes faecalis* and an alkalothermophilic actinomycete, respectively, were reported from the Indian peninsula (Thangam and Rajkumar 2000; George et al., 2001). The production, optimization and characterization of extracellular protease obtained from an alkaliphilic strain, *Arthrobacter ramosus*, MCM B-351, isolated from the alkaline lake of Lonar, Maharashtra, was studied and reported by Nilegaonkar et al., and Kanekar et al., (2002). Das et al., (2004) and Singh et al., (2004), purified and characterized a thermostable, alkaliphilic, extracellular α-amylase from *Bacillus subtilis* DM-03 and an alkaline cellulase from, a novel isolate, *Bacillus sphaericus* JS1 respectively. An alkaline α-amylase from *Bacillus* sp. AB 04 was characterized by Behal et al., (2006). Purification and analysis of pigment (melanin) produced by *Streptomyces* was reported by Dastager et al., (2006). A *Bacillus licheniformis* N-2 strain isolated from decaying organic soil from Punjab was found to produce alkaline protease (Nadeem et al., 2007). The study of the antimicrobial potential of *Bacillus clausii* MB9 from the southeastern coastal regions of India was done by Devi et al., (2008). The production of extracellular protease was reported from *Streptomyces albidoslavus* isolated from Guntur, Andhra Pradesh. (Narayan and Vijayalakshmi, 2008). Many novel alkaliphiles with novel properties like radiation and arsenic resistance have also been found in India. *Deinococcus mumbaiensis* sp. nov., a radiation-resistant pleomorphic bacterium was isolated from Mumbai by Shashidhar and Bandekar, (2006). *Streptomyces deccanensis* sp. nov., a novel alkaliphilic actinomycete, was isolated from Gulbarga, Karnataka (Dastager et al., 2008).

Gujarat is one of the most industrialized states of India, having several soda ash factories that provide good habitat for the alkaliphiles. Patel et al., (2005) have
reported an extracellular alkaline protease from a newly isolated haloalkaliphilic Bacillus sp. An alkaliphilic actinomycetes, isolated from soil samples of the arid region of Saurashtra, capable of producing alkaline protease, was reported by Mehta et al., (2006). A potent antibiotic producing salt-tolerant and alkaliphilic actinomycete, Streptomyces sananensis strain RJT-1, was isolated by Vasavada et al., (2006) from alkaline soil of Saurashtra University Campus, Rajkot. The bacterial strain was found to grow optimally at 5% (w/v) NaCl and pH 9 and produced antibiotics against Gram-positive organisms i.e. S. aureus, B. cereus, B. megaterium and B. subtilis. Production of extracellular alkaline proteases from halophilic and alkaliphilic bacteria, isolated from saline habitat of coastal Gujarat, was reported by Dodia et al., (2006). An alkaliphilic, salt- tolerant actinomycetes, Streptomyces clavuligerus strain Mit-1 from Mithapur, was exploited for its potential for the production of alkaline protease having organic solvent tolerance (Thumar et al., 2009) From the same soil, a bacterium, identified as Bacillus agaradhaerens Mi-10-62 strain; producing an organic solvent tolerant α-Amylase was reported by Pandey and Singh, (2012). Halotolerant alkaliphilic actinomycete, Streptomyces aburaviensis Kut-8, was isolated from Kutch, Western India and were found to produce an antimicrobial agent (Thumar et al., 2010). Halobacterium sp. including, Halobacterium halobium (Akolkar et al., 2008), Halobacterium sp. Js1 (Vijayanand et al., 2010), and many strains of Halobacterium salinarum and Bacillus halodurans WN-SK5 were isolated from habitats like soda lakes, salt pan of coastal Tamil Nadu, India, Thai fish sauce, salted fish and hides, solar salterns and estuaries polluted with crude oil etc. (Thongthai and Suntinanalert, 1991).
Bioremediation

Bioremediation is the use of living organisms, to degrade the environmental contaminants into less-toxic or non-toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. In order to boost the bioremediation process, biostimulation (addition of specific nutrients) or the bioaugmentation strategy (addition of microorganisms exogenous and/or endogenous with effective degradation capacity of the contaminated sites) has been proposed and evaluated (Vidali, 2001; Mukred et al., 2008; Janbandhu and Fulekar, 2011). The problems associated with contaminated sites are increasing in many countries. Contaminated lands generally result from the past industrial activities when awareness of the health and environmental effects connected with the production, use and disposal of hazardous substances were less recognized than today. The problem is worldwide, and the estimated number of contaminated sites is significant. It is now widely recognized that, contaminated land is a potential threat to human health and its continual discovery over recent years has led to international efforts to remediate many of these sites, either as a response to the risk of adverse health or environmental effects caused by contamination or to enable the site to be redeveloped for use (Vidali, 2001). The soil washing method is cost-effective and relatively fast, thereby having potential to be applied in treating and removing a large amount of pollutant (Lai et al., 2009). The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminants are transformed by living organisms through reactions that take place as a part of their metabolic processes. For bioremediation to be effective, microorganisms must attack the pollutants enzymatically and convert them to harmless products. As bioremediation can be effective only where
environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to make the atmosphere conducive for microbial growth and degradation to proceed at a faster rate. Like other technologies, bioremediation has its limitations. Some contaminants, such as chlorinated organic compounds or aromatic hydrocarbons, are resistant to microbial attack. On the other hand, bioremediation techniques are more economical as compared to traditional methods as some pollutants can be treated on site, thus reducing the exposure risks. Since bioremediation is based on natural attenuation, the public considers it more acceptable than other technologies. Most bioremediation systems run under aerobic conditions, but running a system under anaerobic conditions may permit organisms to degrade otherwise recalcitrant molecules.

Factors affecting bioremediation

The control and optimization of bioremediation processes is a complex system and depend on many factors. These factors include: the existence of desired microbial population; the availability of contaminants to the microbial population and conducive environmental conditions (temperature, pH, the presence of oxygen or other electron acceptors and nutrients). Environmental biotechnology is not a new field; composting and wastewater treatments are known examples of environmental biotechnological applications. However, recent studies in molecular biology and ecology offer opportunities for more efficient biological processes. Notable accomplishments of these studies include the clean-up of polluted water and land areas. Although the microorganisms are present in contaminated soil and wastewater, they may not necessarily be there in the numbers required for bioremediation of the site. Their growth and activity must be roused. Biostimulation usually involves the addition of nutrients and oxygen to help indigenous microorganisms for growth. Microbial growth
and activity are readily affected by pH, temperature, and moisture. The amount of available oxygen will determine whether the system is aerobic or anaerobic. Hydrocarbons are readily degraded under aerobic conditions whereas chlorinated compounds are degraded only in anaerobic ones.

Generally, the neutralization of alkaline wastewaters is being done purely by chemical means where a huge amount of acid is used which is neither economically feasible nor safe as it poses serious health hazards to the workers. Conversely, neutralization of alkaline wastewater by biological processes is quite safe. Common bioremediation processes with terrestrial microbes are unsuitable for the effluents having high salinity and alkalinity, as they cannot survive under such extreme conditions, hence, prior neutralization is essential. Alternatively, isolation and utilization of microbes, which are best, adapted to the extreme conditions of salinity and pH would aid in providing an economic and safe process for neutralization of alkaline waste-waters. Though Gujarat has several chlor-alkali industries that provide a good habitat for the alkaliphiles, only a few alkaliphiles were studied and characterized (Upasani and Desai, 1990). Moreover, a very few biological processes have been developed for neutralizing highly alkaline, beverage, textile and other alkaline industrial waste waters using different bacterial strains isolated in India (Kumar et al., 2005a; 2005b; 2008; Kulshreshtha et al., 2010; Yang et al., 2011). According to these studies, alkaliphilic bacteria, could reduce the pH of alkaline wastewaters of textile and beverage industry from 12.0 to 7.0 within two to three hours using Exiguobacterium sp. and Kurthia sp. (Kumar et al., 2005a; 2005b; 2008; Kulshreshtha et al., 2010).

Textile industrial effluent can pollute underground water or water bodies once discharged without proper treatment, leading to adverse effect on aquatic eco-systems.
2. Review of literature

(Brik et al., 2006; Tufekci et al., 2007). The textile industry is a tremendous water user and polluter (Robinson et al., 2001; Babu et al., 2007). The major issues with this effluent are color, high pH, TDS, organic contents and toxic wastes. Traditional methods for the cleaning up of pollutants usually involve removal of unwanted materials through sedimentation, filtration and subsequent chemical treatments such as flocculation, neutralization and electrodialysis before disposal. These processes are laborious, expensive and hazardous which can further pollute the environment (Babu et al., 2007). The biological treatment can be used as an alternative to these processes as this can be the basis for a cost effective, environment friendly and publicly accepted technology (Dafale et al., 2010). There are many reports on bioremediation of textile industrial effluent using microorganisms, specifically fungi where focus was on decolorization and degradation (Asamudo et al., 2005; Verma et al., 2010) of dyes however; reports on biological neutralization of these effluents using bacteria are relatively rare. Several reports are available on the isolation of haloalkaliphiles which can grow up to pH 8-11 and 3.5-10% salt concentration.

Bioremediation studies regarding methyl violet removal of dye-industry effluent by Lonar lake alkaliphilic bacteria is reported by Sarnaik and Kanekar (1995). Under extreme alkaline conditions, microbial utilization of benzoate, m-hydroxybenzoate and thiocyanate was reported, delineating the probable use of alkaliophiles in effluent treatment rich in such compounds (Sorokin et al., 2001; Yumoto et al., 2003; Celso et al., 2007). Degradation of aromatic compounds and acetonitrile at either high salinity or alkalinity was reviewed by Peyton and Alva, (2003) and Dimitry et al., (2007). Degradation of a four ring compound, pyrene, under alkaline condition, was reported by Habe et al. (2004). Biodegradation of aniline in an
alkaline environment by a novel strain of the halophilic bacteriaum, *Dietzia natronolimnaea* JQ-AN reported by Jin *et al.*, (2012).

The major difficulty in bioremediation of oil-contaminated soil is the bioavailability or mass transfer limitation of the oil pollutants in the soil, causing poor food-microorganism contact and thus poor biodegradation efficiency. Oil penetration through soil is an extremely complex process related to physical, chemical and biological factors. Petroleum hydrocarbons are highly hydrophobic material with low water solubility and those components attach to soil particles, reducing the bioavailability of oil to microorganisms and thus limiting the rate of biodegradation. One of the effective ways to increase the bioavailability (or solubility) of petroleum hydrocarbon in soil is to use surfactants to enhance desorption and solubilization of petroleum hydrocarbons, thereby facilitating their assimilation by microorganisms. In particular, recent studies showed that biosurfactant (a more environmental friendly type of surfactant) has the ability to effectively solubilize and mobilize organic compounds adsorbed on soil constituents. On the other hand, some synthetic surfactants, such as Triton X-100, Tween 80 and SDS are also shown to enhance the concentration of non polar compounds in the aqueous phase.

**Biosurfactants**

Biosurfactants, heterogenous and structurally diverse group of surface active molecules, synthesized by variety of microorganisms are amphilic compounds that partition at the interface between fluid phases with different degrees of polarity and hydrogen bonding in oil/water or water/oil interfaces. With an increased environmental awareness among the users, emphasis on a sustainable harmony with global environment and stringent new legislations aiming at ecosystem protection, have
prompted consideration of natural biosurfactants as an alternative to the existing petroleum based synthetic chemical surfactants. Recently, biosurfactants have increased the attraction of pharmaceutical, food processing and cosmetic industries for their applications as multifunctional materials due to unique features such as:

1. possibility of using cheap renewable resources
2. stability under extreme environmental conditions
3. functionality in lower quantity
4. potential for tailor made applications
5. lower toxicity and higher biodegradability
6. eco-friendly nature

Microbial biosurfactants are produced by a wide variety of bacteria, yeast, phytoplankton, algae, cyanobacteria and filamentous fungi from different environmental habitats which either adhere to cell surface or are excreted extracellularly. All biosurfactants are amphiphiles, they consist of two parts: a polar (hydrophilic) moiety and a non polar (hydrophobic) group. A hydrophilic group consists of mono, oligo or polysaccharides, peptides or proteins, various functional groups including carboxyl, amino, phosphate and sulphur and a hydrophobic moiety usually containing aliphatic chains, saturated, unsaturated and hydroxylated fatty acids or fatty alcohols (Desai and Banat, 1997; Brahmachari et al., 2007) and hence can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility and mobility of hydrophobic or insoluble organic compounds and toxic metals (Mulligan, 2005). Generally, bioavailability and biodegradation kinetics of the hydrophobic pollutants are affected variably by the
surfactants. Surface activity makes surfactants excellent emulsifiers, foaming and dispersing agents (Desai and Banat, 1997). Both stimulating and inhibiting effects of surfactants on bioremediation of pollutants are known depending on the chemical characteristics of the surfactant, pollutant and physiology of the microorganism (Banat et al., 2000). In nature, biosurfactants play a physiological role in increasing bioavailability of hydrophobic molecules, promoting the swarming motility of microorganisms and participating in cellular physiological processes of signaling and differentiation (Kearns and Losick, 2003). Surfactants can interact with microbial proteins and can be manipulated to modify enzyme conformation in a manner that alter enzyme activity, stability and specificity (Kamiya et al., 2000). Chemical surfactants can imitate the latter effects of biosurfactants and have been exploited, for example, as antimicrobial agents in disease control and to improve degradation of chemical contaminants. Both chemical surfactants and biosurfactants are potentially toxic to specific microbes and may be exploited as antimicrobial agents against plant, animal and human microbial pathogens (Cameotra and Makkar, 2004).

**Classification of biosurfactants**

Compared to chemical surfactants, which are classified according to their dissociation pattern in water, biosurfactants are categorized by their microbial origin, chemical composition, molecular weight, physico-chemical properties and mode of action. According to molecular weight they are divided into low-molecular-mass biosurfactants like glycolipids, phospholipids and lipopeptides and high-molecular-mass biosurfactants/bioemulsifiers containing amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers (Table 2.1).
## Table 2.1: Microbial biosurfactants and its applications

<table>
<thead>
<tr>
<th>Type of biosurfactants</th>
<th>Microorganism</th>
<th>Applications</th>
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2. Review of literature

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<td>Celllobiose lipids</td>
<td><em>Trichosporon porosum</em>, <em>Cryptococcus humicola</em> JCM 1461 and <em>Ustilago maydis.</em></td>
<td>Bioemulsifier. Stabilization of the hydrocarbon in water. Dispersion of limestone in water.</td>
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<tr>
<td>Lipo polysaccharides (Emulsan, Alasan, Biodispersan and Liposan)</td>
<td><em>Acinetobacter calcoaceticus</em> RAG-1 ATCC 31012, <em>A. radioresistens</em>, <em>A. calcoaceticus A2</em> and <em>Candida lipolytica.</em></td>
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<tr>
<td>Phospholipids</td>
<td><em>T. thiooxidans</em>, <em>Corynebacterium alkanolyticum</em>, <em>Rhodococcus erythropolis</em></td>
<td>Enhancement of metal tolerance for bacteria,</td>
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| Cameotra and Makkar, 2004; Singh and Cameotra, 2004; Thavasi et al., 2008, Whang et al., 2008; Calvo et al., 2009; Mulligan, 2009; Banat et al., 2010; Pacwa-Płociniczak et al., 2011; Thavasi et al., 2011). |

Low-molecular-mass biosurfactants efficiently decrease the surface and interfacial tensions, whereas high-molecular-mass biosurfactants are more effective at stabilizing oil-in-water emulsions (Rosenberg and Ron., 1999; Banat et al., 2000; Calvo et al., 2009). The biosurfactants accumulate at the interface between two immiscible fluids or between a fluid and repulsive forces between two dissimilar phases and allow these
two phases to mix and interact more easily. Biosurfactant activities depend on the concentration of the surface-active compounds until the critical micelle concentration (CMC) is obtained. At concentrations above the CMC, biosurfactant molecules associate to form micelles, bilayers and vesicles. Micelle formation enables biosurfactants to reduce the surface and interfacial tension and increase the solubility and bioavailability of hydrophobic organic compounds (Whang et al., 2008). The CMC is commonly used to measure the efficiency of surfactant. Efficient biosurfactants have a low CMC, which means that less biosurfactant is required to decrease the surface tension (Desai et al., 1997). Micelle formation has a significant role in microemulsion formation. Microemulsions are clear and stable liquid mixtures of water and oil domains separated by monolayer or aggregates of biosurfactants. Micro emulsions are formed when one liquid phase is dispersed as droplets in another liquid phase, for example oil dispersed in water (direct micro emulsion) or water dispersed in oil (reversed microemulsion) (Desai et al., 1997) (Figure 2.1).

![Figure 2.1: Effect of different concentration of biosurfactant on the solubility, surface tension and critical micelle concentration.](image)

**Figure 2.1:** Effect of different concentration of biosurfactant on the solubility, surface tension and critical micelle concentration.
Economics of biosurfactant production

In spite of numerous advantages of biosurfactants over the synthetic chemical surfactants the problems related with the large scale and cheap production still exists and is a major hurdle in economic competitiveness. This has led to concentrated efforts on minimizing production cost using the cheaper carbon sources in order to facilitate wider commercial use.

Limited success of large scale production has been realized for most of the biosurfactants due to expensive raw material, low production yield and high purification cost. The production medium, carbon source, and the growth conditions (pH, temperature, limiting nutrients, and trace elements) can influence the types and yields of biosurfactants. Carbon is an important substrate for production medium of microbial surfactants to influence both the quality and quantity of biosurfactants. To address this problem, evaluation of carbon sources was conducted to obtain high yield of biosurfactant (Raza et al., 2005). Several studies have been carried out to define the best ratio between carbon, phosphorous, nitrogen and metal ions as well as the conditions such as pH and temperature needed to obtain high production yields (Das et al., 2009; Freitas et al., 2009). Thus, temperature, pH of the medium, medium composition and salinity were proved to be of prime importance for control and optimization of biosurfactant production. At present, biosurfactants are unable to compete with the chemical surfactants due to their high production costs. As biosurfactants are readily biodegradable and can be produced from renewable and cheaper substrates, they might be able to replace their chemically synthesized counterparts (Patel and Desai, 1997). The rhamnolipids produced by Pseudomonas species from different carbon sources have been extensively studied. The genus Pseudomonas is capable of using different substrates, such as glycerol, mannitol,
fructose, glucose, n-paraffins and vegetable oils, thereby producing rhamnolipid biosurfactants. Several types of substrates used for production of biosurfactant were water miscible/soluble (ethanol, glucose, glycerol, starch, fructose, maltose etc) and immiscible (lubricants, olive and vegetable oils and hydrocarbons) carbon and nitrogen sources (peptone, yeast extract, ammonium salts, urea and nitrate salts) (Desai and Banat, 1997; Abouseoud et al., 2008; Das et al., 2009). Some recent studies on biosurfactant production found that biosurfactants produced from hydrophobic carbon sources were superior to those produced from hydrophilic carbon sources in terms of surface activity and productivity (Das et al., 2009). On the other hand, when hydrophilic carbon sources are used, the extraction of biosurfactants can be simplified resulting in the substantial reduction in production cost (Liu et al., 2009). The molecular weight of the alginate from *Pseudomonas fluorescens* produced on fructose was much higher than that produced on glucose indicating influence of different carbon sources on physico-chemical properties (Staudt et al., 2012).

Production economy is the major bottleneck in biosurfactant production as is the case with most biotechnological processes. More often, the amount and type of raw materials contribute significantly to the production cost. It is estimated that raw material accounts for 10–30% of the total production costs in most biotechnological processes. Hence, in order to reduce this cost, it is desirable to use low cost raw materials (Kosaric 1992; Makkar and Cameotra, 2002; Mukherjee et al., 2006; Makkar and Cameotra, 2011). Thus, one of the possibilities explored extensively is the use of cheap, agro-based raw materials or industrial wastes, rich in organic matter, as substrates for biosurfactant production. This approach will help to achieve double benefits of reducing the pollutants while producing useful products. A variety of these cheap raw materials supporting biosurfactant production includes plant derived oils
like sunflower oil, soybean oil, oil wastes like soapstock and oil refinery wastes (Makkar and Cameotra, 2002; Bednarski et al., 2004; Nitschke et al., 2005; Makkar and Cameotra, 2011). They can potentially induce microbial growth and metabolite production due to their typical fatty acid composition. The effluent from dairy industry known as dairy wastewater supported good microbial growth and has been used as a cheap raw material for biosurfactant production (Dubey and Juwarkar, 2001). Potential use of dairy wastewaters in biosurfactant production provided a strategy for economical production of biosurfactants along with efficient dairy wastewater management. Starchy substrates like potato process effluents (Thompson et al., 2000; 2001) and cassava wastewater have acted as attractive substrates for biosurfactant production (Nitschke and Pastore, 2006). As millions of tons of hazardous and non-hazardous wastes are generated each year throughout the world, a great need exists for their proper management and utilization (Kosaric, 1992; Mukherjee et al., 2006).

Plant biomass is a valuable and sustainable resource of organic fuels, chemicals and bio-materials. In addition, bioconversion of waste materials into value added products is considered to be of prime importance for the near future because of its favorable economics, low capital and energy cost, reduction in environmental pollution and relative ease of operation. Producing usable products from agro-industrial waste is therefore a feasible and favorable option (Makkar and Cameotra, 2002, 2011). The low-cost agro-industrial waste substrates include lignocellulosic residues (barley bran husks, trimming vine shoots, corn cobs and Eucalyptus globules chips), jute (Chowdhury et al., 2011), plant polymer and oil extracts and waste, distillery and whey wastes, potato process effluent and pea nut cake (Vedyashkina et al., 2005; Thavasi et al., 2011). These waste materials are some examples of food industry wastes that can be used as feedstock for biosurfactant production (Nitschke
and Pastore, 2006). Sugars, sarch and cellulose are the major components of agricultural crops like *Madhuca indica*, corn, tapioca, wheat, soyabean and potatoes. Other sources include sugar plants such as dates syrup, sugar beet, sugar cane or sugar sorghum (Moosavi-Nasab *et al*., 2008). Sugar and starch processing industries also produce large amounts of solid residues and wastewater rich in starch (Onbasli and Aslim, 2009). The carbohydrates rich solid wastes or wastewater are suitable substrates for the production of microbial products, like biosurfactant (Maneerat, 2005). Vegetable oils are a lipidic carbon sources, generally comprised of saturated or unsaturated fatty acids with 16-18 carbon atoms chain. For biomaterial production, variety of oils were used from canola, corn, sunflower, olive, rape seed, grape seed, palm, coconut and fish (Makkar and Cameotra, 2002, 2011; Thavasi *et al*., 2011). The dairy industry has a considerable amount of byproducts; whey and wastewater. Whey is a liquid by product of cheese production, rich in lactose (75% of dry matter) and containing other organic water-soluble components (12-14% protein). The high yield of sophorolipids was achieved with whey concentrate and rape seed oil as substrate (Daniel *et al*., 1998). Molasses was used as carbon source since it is economical (Raza *et al*., 2007). Molasses, a sweet, dark brown thick liquid that is produced in the process of beet contains high sucrose concentration, another important substance for the fermentation process, is a low cost option. The main problem related to the use of alternative substrates as culture medium is to obtain a waste with the right balance of nutrients that permits cell growth and product accumulation (Makkar and Cameotra, 1999).

The primary approaches applied for obtaining increased yields in fermentative production is the medium optimization. One of the most effective methods used for the optimization of bioprocess parameters is the statistical modeling and analysis based
2. Review of literature

RSM technique (Sen, 1997; Sen and Swaminathan, 1997, 2004). Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. By careful design of experiments, the objective is to optimize a response (output variable) which is influenced by several independent variables (input variables). An experiment is a series of tests, called runs, in which changes are made in the input variables in order to identify the reasons for changes in the output response. Originally, RSM was developed to model experimental responses (Box and Draper, 1987), and then migrated into the modelling of numerical experiments. The difference is in the type of error generated by the response. In physical experiments, inaccuracy can be due, for example, to measurement errors while, in computer experiments, numerical noise is a result of incomplete convergence of iterative processes, round-off errors or the discrete representation of continuous physical phenomena. The application of RSM to design optimization is aimed at reducing the cost of expensive analysis methods.

RSM has been extensively applied in many areas of biotechnology previously for the optimization of sorbitol, xylitol, lactic acid, protease and chitinase enzyme, chitosan, citric acid, biosurfactant, ethanol, bacetrocin and honey candy production (Sreekumar et al., 1999; Li et al., 2002; Gu et al., 2005; Cazetta et al., 2005; De Lima et al., 2010; Seth and Mishra, 2011, Dong et al., 2012; Selvaraj et al., 2012). RSM was used for gellan and pullulan production by Sphingomonas paucimobilis ATCC-31461, Aureobasidium pullulans and Aureobasidium pullulans P56 (Goksungur et al., 2005). Zunongwangia profunda SM-A87 isolated from deep-sea sediment was applied for EPS production by optimizing culture conditions using RSM. The conventional one-factor-at-a-time optimization method has been used to identify the critical medium components affecting biosurfactant production (Sen, 1997; Mutalik et al., 2008;
Sivapathasekaran et al., 2010). These critical nutritional parameters present in the medium need to be optimized in order to enhance the production level (Sen, 1997; Mutalik et al., 2008). Optimization of low-cost medium was done for biosurfactant production of *Bacillus subtilis* using response surface methodology (RSM) (Ghribi et al., 2011; Mnif et al., 2012). Biosurfactant from *Acinetobacter* sp. YC-X 2 was optimized successfully through RSM medium containing n-hexadecane as carbon source (Chen et al., 2011).

Some of the widespread downstream processing technologies used are solvent extraction (e.g. chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether, etc.) or acid precipitation, use of ammonium sulfate precipitation, crystallization, centrifugation, adsorption and foam fractionation (Mukherjee et al., 2006; Kumar et al., 2007). Recovery of extracellular microbial surfactants from culture broth is by procedures that involves: 1) cell removal, by centrifugation or filtration 2) biopolymer precipitation from the cell-free supernatant by the addition of a precipitating agent that consists of a water-miscible solvent in which the biopolymer is insoluble (e.g. methanol, ethanol, isopropanol or acetone) 3) freeze drying (laboratory scale) or drum drying (large scale) (Freitas et al., 2009; Kumar et al., 2007).

**Chemical characterization**

An overall characterization of biopolymers involves assessment of their chemical, physical and biological properties, which are the key factors to understand their behavior in different environments. Chemical characterization includes quantification of sugar contents, monomer composition, protein contents and presence of functional groups (acyl, carboxyl, phosphate and sulfur groups). Further characterization of
functional groups can be done using FT-IR and NMR. Monosaccharide composition and molecular mass can be monitored using GC-MS and MALDI-TOF, respectively. These techniques allow the evaluation of interactions of each carbon/hydrogen with adjacent atoms and chemical groups, and eventually determine their relative position in the structure. Various techniques have been used for the determination of average polymer molecular weight and polydispersity index. High-performance size-exclusion chromatography- GPC (Gel permeation chromatography) with multi-angle laser light scatter detection is a recent efficient method for the evaluation of absolute molecular weight of polysaccharide against standard polysaccharides, which provides greater resolution than traditional gel permeation chromatography. The mass spectrometric analysis is used to determine molecular mass of biosurfactant (Thavasi et al., 2008). A Differential Scanning Calorimetry (DSC), X-ray diffraction (XRD) and Thermal Gravimetric (TG) analysis were used to evaluate the thermal stability and crystalinity properties of biosurfactant (Mishra et al., 2011; Singh et al., 2011). Such properties are related to the molecular characteristics of biopolymers, such as intrinsic viscosity (hydrodynamic volume of a single molecule), surface tension, rheology, solubility, foaming, critical overlap concentration (concentration above which coil overlapping starts to occur), molecular conformation and flexibility, as well as the nature and number of intra/intermolecular interactions. In addition, TEM, AFM are rather versatile techniques which are able to assess the complex and intact 3D molecular structure of the biopolymer. It can identify the conformation of individual molecules and their assemblies (e.g. aggregation phenomena and gel systems) (Seviour et al., 2010; Mishra et al., 2011; Singh et al., 2011).
Applications of microbial biosurfactants in bioremediation

Biosurfactants are widely used in different industries, such as cosmetics, special chemicals, food, pharmaceutics, agriculture, cleansers and microbial enhanced oil recovery (MEOR) (Cameotra and Makkar, 2004; Singh and Cameotra, 2004; Bhaskar and Bhosle, 2005; Mulligan, 2005; Rodrigues et al., 2006; Mulligan, 2009). Bioremediation processes provide cost effective, environmental friendly and contaminant-specific treatments to reduce the concentration of pollutants. Pollution of the sea by crude oil is one of the critical and serious environmental issues over the world. Ship operations also produce wastes that are collected in the lowest part of the hull, called the bilge area. This oil-containing bilge waste must be managed properly to avoid environmental pollution (Olivera et al., 2003; Maneerat and Phetrong, 2007). Several oil spill accidents in recent years have resulted in significant contamination of oceans and shoreline environments. Such incidents have intensified attempts to develop various chemicals, procedures and techniques for combating oil pollution both at sea and along the shoreline. Biosurfactants were applied to parts of the Exxon Valdez oil spill (Harvey et al., 1990). The ability of biosurfactants to emulsify hydrocarbon water mixtures enhances the degradation of hydrocarbons in the environment. The presence of hydrocarbon-degrading microorganisms in seawater renders biodegradation, one of the most efficient methods for removing pollutants (Kimura et al., 1989; Atlas, 1991; Saadoun and Al-Ghzawi, 2005). Most of the biosurfactants, as compared to chemical surfactants, have lower toxicity and shorter persistence in the environment. The ability of a surfactant to enhance the biodegradation of slightly soluble organic compounds depends on the extent to which it increases the bioavailability of the compound (Mulligan, 2005; 2009). Chemical surfactants and biosurfactants can increase the pseudo solubility of hydrocarbons in
water (Urum and Pekdemir, 2004). Surfactants are effective in reducing the interfacial tensions of oil and water in situ and they can also reduce the viscosity of the oil and remove water from the oil prior to processing (Al-sabagh, 2000; Liu et al., 2004). Biosurfactants can be as effective as the synthetic chemical surfactants and for certain applications; they have advantages such as high specificity.

Biosurfactants can also be used in microbial enhanced oil recovery (MEOR). MEOR methods are used for the recovery of oil left in reservoirs after primary (mechanical) and secondary (physical) treatments (Banat et al., 2000; Sen, 2008). It is an important tertiary process where microorganisms or their metabolites, including biosurfactants, biopolymers, biomass, acids, solvents, gases and also enzymes, are used to increase recovery of oil from depleted reservoirs. Application of biosurfactants in enhanced oil recovery is one of the most promising advanced methods to recover a significant proportion of the residual oil. The remaining oil is often located in regions of the reservoir that are difficult to access and the oil is trapped in the pores by capillary pressure (Sen, 2008). Biosurfactants reduce interfacial tension between oil-water and oil-rock. This reduces the capillary forces preventing oil from moving through sand pores. Biosurfactants can also bind tightly to the oil-water interface and form emulsions. This stabilizes the desorbed oil in water and allows removal of lubricant oil along with the injection water (Figure 2.2) (Mulligan, 2005; 2009).
Figure 2.2: Removal of lubricant oil from contaminated sand using biosurfactant solution.

Injection of biosurfactants and bacteria such as *Pseudomonas aeruginosa*, *Xanthomonas campestris*, *B. licheniformis* and *Desulfovibrio desulfuricans* along with nutrients showed increase in oil recovery. In some cases, analysis of the crude oil before and after microbial treatment revealed degradation of long chain alkanes and alkyl chains of aromatics, but no apparent degradation of aromatic ring structures.

The usefulness of biosurfactants for bioremediation of heavy metal contaminated soil is mainly based on their ability to form complexes with metals. The anionic biosurfactants create complexes with metals in a nonionic form by ionic bonds. These bonds are stronger than the metal bonds with the soil and metal-biosurfactant complexes are desorbed from the soil matrix to the soil solution due to the lowering of the interfacial tension. The cationic biosurfactants can replace the same charged metal ions by competition for some but not all, negatively charged surfaces (ion exchange). Metal ions can be removed from soil surfaces also by the biosurfactant micelles. The polar head groups of micelles can bind metals which mobilize the metals in water (Figure 2.3) (Mulligan, 2005; 2009).
Figure 2.3: Removal of metal from contaminated soil using biosurfactant solution.

Role of biosurfactants in mobilization and decontamination of heavy metal contaminated soil was confirmed by Juwarkar et al., (2008), using a biosurfactant, di-rhamnolipid, produced by *Pseudomonas aeruginosa* BS2. Similarly, use of marine microbes for remediation of polyaromatic hydrocarbons is reported by Das et al., (2008). Biosurfactants are effective in a wide range of extreme conditions including temperature, pH and salinity as compared to chemical (synthetic) surfactants (Banat et al., 2000; Mulligan, 2005; Bramhachari et al., 2007; Mukherjee, 2007 Banat et al., 2010). Environmental imbalance, created by crude oils, hydrocarbons and toxic metals can be remediated by biosurfactant effectively as it forms a stable emulsion, adsorbent or amalgam (Calvo et al., 2009).

In recent years, interest in the exploitation of valuable biosurfactants has been increasing for various industrial applications and the attention towards biosurfactant producing extremophilic bacteria has greatly enhanced. Extreme environments are bio-resources of potential microorganisms that secrete new compounds (Rodrigues et al., 2006). Biosurfactant production in extremophiles is reported by a few researchers. It
was observed that biosurfactants were able to remove significant amount of crude oil from the contaminated soil at different concentrations; rhamnolipid and SDS removed 80% oil while lecithin removed about 42% oil in a similar fashion whereas 84% oil removal from soil was observed by biosurfactant obtained from *Candida glabrata* UCP 1002 at 2.5% concentration (Luna *et al*., 2009). Previously, 57-82% oil removal was reported from contaminated soil using biosurfactant produced by *Pseudomonas aeruginosa* SP4, *Bacillus subtilis* PT2 and *Rhodococcus ruber* (Santa Anna *et al*., 2007; Whang *et al*., 2008; Lai *et al*., 2009). Chemically-synthesized surfactants have been used in the oil industry to aid in the cleanup of oil spills as well as to enhance oil recovery from reservoirs. These compounds are not biodegradable and can be toxic to environment (Wei *et al*., 2005). Biosurfactants can be used in many processes involving emulsification, foaming, detergent, wetting, dispersion and solubilization of hydrophobic compounds. These favorable features make biosurfactants potential alternatives which can replace chemically synthesized surfactants in a variety of applications.