STUDIES ON THE PRODUCTION OF BIOSURFACTANT

Thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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DECLARATION

I, **Aparna A.**, hereby declare that the Research Thesis entitled **"Studies on the Production of Biosurfactant"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in **Chemical Engineering** is a bonafide report of the research work carried out by me. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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Chairman - DRPC

DEDICATED TO
MY PARENTS

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ABSTRACT

Petroleum hydrocarbons are an integral part of modern developed society as various petroleum fractions provide essential resources for energy, transportation, synthesis of plastics and chemicals, etc. They constitute a large and diverse class of around 250 hydrocarbon compounds consisting of varying constituents and molecular complexity ranging from complex mixture of saturates, aromatics, resins and asphaltenes. The extensive production and use of these hydrocarbons has resulted in widespread environmental contamination. These hydrocarbons reach the environment from leaking underground storage tanks, petroleum refineries and bulk storage facilities, harbour operations, broken oil pipelines, effluent discharges from petroleum industries, spills of petroleum products in chemical plants and transportation processes. Moreover, these hydrocarbons fall into the category of persistent pollutants. When these pollutants are released into the environment; they cause air, water as well as soil pollution.

Contamination by petroleum hydrocarbons is a major environmental concern since many of its constituents are highly toxic, carcinogenic and are poorly biodegradable in nature. The contamination of environment by these hydrocarbons can also result in uptake and accumulation of these contaminants in food chains, thereby causing harm to the flora and fauna. Many of these contaminated sites threaten to become sources of contamination to drinking water supplies and thereby, constitute substantial health hazards. Due to the serious and long-term damage caused to the ecosystems, terrestrial life, human health and natural resources; there is a need to remediate the sites which are by petroleum hydrocarbons.

The processes leading to the eventual removal of hydrocarbon pollutants from the environment involves various physical, chemical and biological methods or a combination of them. Physical and chemical remedial methods include adsorption, incineration, thermal desorption, solvent extraction, evaporation, etc. These methods are expensive, requires high energy input and also results in significant greenhouse gas

emissions. Moreover, they involve the transfer of the contaminant to another medium rather than eliminating the contaminant. Bioremediation has proven to be an efficient, ecofriendly and cost-effective approach to alleviate petroleum hydrocarbon contamination from the environment. The driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons for their growth and energy needs. One of the widely accepted bioremediation methods of petroleum hydrocarbons is biodegradation. The biodegradation of petroleum hydrocarbons is affected by many factors such as water, oxygen and nutrients. In addition, the lack or reduced bioavailability of petroleum hydrocarbons to the microorganisms affects the biodegradation of these hydrocarbons.

One of the options to increase bioavailability of the petroleum hydrocarbons to the microorganism is the use of surfactants. Surfactants emulsify the petroleum hydrocarbons, increase the surface area and thereby, increase the rate of biodegradation of these hydrocarbons. Surfactants used in the remediation of petroleum hydrocarbons earlier were synthetic surfactants which are synthesized from petroleum based products. Since synthetic surfactants are derived from petroleum based products, they are commonly toxic to ecosystems and resistant to complete degradation. Moreover, they act like secondary pollutants in the environment.

An increase in the concern about environmental protection has recently caused the consideration of alternatives to synthetic surfactants. Surfactants produced by microorganisms, called biosurfactants, are gaining importance as they exhibit lower toxicity, higher biodegradability, better environmental compatibility and selectivity. They are versatile process chemicals used in various industries such as cosmetic, petroleum, pharmaceutical, etc.

While reviewing the literature, it has been observed that there is less data with respect to isolation and identification of potential surfactant producing microorganisms,

studies on conditions required for the maximum production of surfactant and utilization of the surfactants in the removal of petroleum hydrocarbons from the affected medium. In this context, the objectives of the present research were formulated. Studies were initiated for the isolation and screening of surfactant potential producing microorganisms, identification of a potential surfactant producing bacteria, studies on the effect of various process parameters on surfactant production by the potential surfactant producer and the usage of surfactant in the biodegradation of crude oil.

Reports in the literature suggest that the prior exposure of microbial community in the soil as well as water environments to the petroleum hydrocarbon contaminant increases the incidences of the isolation of surfactant producing microorganisms due to the acclimatization of microorganisms to the contaminated environment. It has been postulated by various researchers that the function of biosurfactant is related to hydrocarbon uptake and therefore, a spontaneous release of biosurfactant occurs in the presence of the hydrocarbon substrate. Hence, in the present study, soil and water samples were collected from various petroleum hydrocarbon contaminated localities in and around Mangalore, Karnataka.

The soil and water samples were subjected to enrichment with crude oil in order to increase the chances of isolating microorganisms possessing the ability to produce surfactant. The study resulted in isolation of several microorganisms which were further screened for their ability to produce surfactant. Among several isolates, a bacterial isolate, designated as potential extracellular surfactant producer based on its ability to produce halos on selective Cetyl Trimethyl Ammonium Bromide (CTAB)-methylene blue agar medium, rapid drop collapse reaction and reduction in surface tension from 71.39 mN/m to 29.33 mN/m.

Based on microscopic studies, biochemical tests and 16S ribosomal DNA sequencing, the candidate bacterial strain 2B, was identified as a novel *Pseudomonas sp.*

Hence, the 16S ribosomal DNA sequence of the novel isolated bacterium was submitted in the GenBank database with an accession number JF683582. In the present research, we report surfactant production by the novel *Pseudomonas sp.* 2B.

In the present research work, we also have compared the data of *Pseudomonas sp.* 2B. with that of already reported surfactant producer, *Pseudomonas aeruginosa* (ATCC 10145), that was procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, Maharashtra, India. The bacterial strain, *Pseudomonas aeruginosa* (ATCC 10145), was selected based on references cited in the literature.

The effect of various process parameters influencing surfactant production by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was studied. The process parameters assessed for their ability to produce maximum surfactant by the bacterial strains included inoculum size, initial medium pH, incubation temperature, agitation speed, type and concentration of carbon source, type of nitrogen source, inducer, buffer and salinity. *Pseudomonas sp.* 2B produced maximum surfactant at 2% (v/v) inoculum size, initial production medium pH 7, incubation temperature of 37^{o}C, agitation speed of 150 rpm, 30 g/L (w/v) glucose as carbon source, using a combination of peptone and potassium nitrate, olive oil as inducer, Tris HCl buffer and 1% (w/v) NaCl concentration. Maximum surfactant was produced by *Pseudomonas aeruginosa* at 3% (v/v) inoculum size, initial production medium pH 7, incubation temperature of 37^{o}C, agitation speed of 150 rpm, 30 g/L (w/v) glucose as carbon source, using a combination of yeast extract and ammonium chloride, n-hexadecane as inducer, Tris HCl buffer and 0.5% (w/v) NaCl concentration.

Plackett-Burman method was used to screen process variables affecting surfactant production by the bacterial strains. Glucose as carbon source, potassium nitrate as nitrogen source and olive oil as inducer had significant effect on surfactant production by *Pseudomonas sp.* 2B whereas glucose as carbon source, ammonium chloride as nitrogen

source and n-hexadecane as inducer had significant effect on surfactant production by *Pseudomonas aeruginosa.* To obtain the optimal concentrations of these process variables leading to maximum surfactant production by the bacterial strains, Response Surface Methodology (RSM) was used. The optimum concentration of factors leading to maximum surfactant production by *Pseudomonas sp.* 2B was found to be 35.7645 g/L of glucose, 3.5% of olive oil and 5.5425 g/L of potassium nitrate. A maximum of 14.63 g/L of surfactant was produced by *Pseudomonas sp.* 2B using the RSM studies, the corresponding surface tension of the cell-free broth showed lowest value, *i.e.* 21.98 mN/m. The optimum concentration of factors leading to maximum surfactant production by *Pseudomonas aeruginosa* was found to be 35.7645 g/L of glucose, 3.5% of n-hexadecane and 5.6274 g/L of ammonium chloride. Using the RSM studies, a maximum of 10.69 g/L of surfactant was produced by *Pseudomonas aeruginosa,* the corresponding surface tension of the cell-free broth was found to be 25.31 mN/m.

During the production of surfactant by the bacterial strains, the quantity of cellular biomass, specific growth rate (μ), maximum growth rate (μ_{max}) and substrate utilization constant (Ks) were determined. In addition, kinetic parameters were evaluated in terms of yield factors-surfactant production to substrate utilization ($Y_{P/S}$), dry cell biomass to substrate utilization ($Y_{X/S}$) and surfactant production to dry cell biomass ($Y_{P/X}$). The study further revealed that the surfactant produced by both the bacterial strains were "primary metabolites" since the production of surfactant coincided with exponential growth phase of the bacterial strains.

The surfactant produced by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, were subjected to extraction as well as partial purification. Acidification followed by chloroform: methanol mixture (2:1) extraction was effective in the extraction of the extracellular surfactant from the cell-free broth of 2B and *Pseudomonas aeruginosa,* respectively, as both polar and non-polar components present in the surfactant could be extracted as compared to other extraction methods. The

results of the column chromatography experiments indicated that the surfactants produced by the bacterial strains were made up of different moieties as suggested by the surface tension values of the different fractions eluted during the experiments.

The surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, were characterized using thin layer chromatography, biochemical analysis, fourier transform infrared spectroscopy and liquid-chromatography-mass spectrometric techniques. The results revealed that the surfactant produced by both the bacterial strains were rhamnolipoproteins.

The cell-free broth containing the surfactant as well as partially purified surfactant produced by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, were found to be stable over a wide range of temperature, pH and salinity. The study also revealed that the cell-free broth could be directly applied without any purification step since the surface tension of the cell-free broth did not vary significantly from that of the partially purified surfactant in varied environmental conditions.

The efficiency of surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was tested in the biodegradation of crude oil by *Nocardia hydrocarboxydans* NCIM 2386. 95.5% and 93.5% of crude oil degradation was achieved over a span of 42 days in the presence of surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. In the control flask, 65.25% of crude oil biodegradation was observed. This suggests that the surfactant produced by the bacterial strains can be used for the remediation of petroleum hydrocarbon contaminated sites.

Keywords: Biosurfactant; *Pseudomonas sp.* 2B; surface tension; statistical optimization; partial purification; characterization; crude oil biodegradation

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NOMENCLATURE

Description	Symbol
Atmosphere	atm
Base pairs	bp
Biomass yield coefficient	$Y_{(X/S)}$
Centimeter	cm
Colony Forming Units	CFU
Daltons	Da
Degree Centigrade	^{o}C
Emulsification Index (%)	EI
Gram per litre	g/L
Grams	g
Gravitational force	g
Greater than	<
Hour	h
Initial biomass concentration	X_0
Lesser than	>
Litre	L
Logarithm	Log
Maximum specific growth rate	μ_{max}
Meter	m
Micro litre	µl

Micro grams	μg
Milligrams	mg
Milli Newton per meter	mN/m
Millimeter	mm
Millimolar	mM
Minute	min
Moles	M
Nanometer	nm
Optical density	OD
Parts per billion	ppb
Parts per million	ppm
Percentage	%
Revolutions per minute	rpm
Specific growth rate	μ
Substrate concentration	S
Substrate utilization constant	Ks
Surface tension	γ
Time	t

ABBREVIATIONS

Abbreviation	Description
ABS	Alkyl Benzene Sulphonate
ANOVA	Analysis of Variance
APE	Alkyl Phenol Ethoxylate
BDL	Below Detectable Level
CMC	Critical Micelle Concentration
CTAB	Cetyl Trimethyl Ammonium Bromide
FTIR	Fourier Transform Infra Red spectroscopy
MS	Mass Spectroscopy
NP	Nonyl Phenol
NPEO	Nonyl Phenol Ethoxylate
OSAT	Open Science Advisory Team
OPE	Octyl Phenol Ethoxylate
PPGAS	Proteose Peptone Glucose Ammonium Salt medium
PBD	Plackett-Burman Design
RSM	Response Surface Methodology
TLC	Thin Layer Chromatography
TPAS	Tetra Propylene Aryl Sulphonate
UST	Underground Storage Tank

CHAPTER 1

INTRODUCTION

Contamination of soil and water by different contaminants is a worldwide problem which may lead to the uptake and accumulation of toxic chemicals in food chains. This inturn can harm the flora as well as fauna of affected habitats. Although substantial progress has been made in reducing release of contaminants over recent years, major releases and accidents still occur. In addition, a considerable number of contaminated sites have been identified and new ones are continually being discovered. Many of these sites threaten to become sources of contamination to drinking water supplies and thereby, constitute substantial health hazards for current and future generations.

A class of pollutants facing particular attention today is the petroleum hydrocarbons. The development of petroleum industry into new frontiers, the apparent inevitable spillages that occur during routine operations and records of accidents during transportation has called for more studies into oil pollution problems (Timmis et al. 1998), which has been recognized as the most significant contamination problem on the continent (Snape et al. 2001). Also, the extensive use of petroleum products leads to the contamination of almost all compartments of the environment. During the previous years, the frequency and risk of oil pollution has led to extensive research. The quantity of natural crude oil spillage was estimated to be around 600,000 metric tons per year with a range of uncertainty of around 200,000 metric tons per year (Kvenvolden and Cooper 2003). This fact increased the interest of scientists and researchers to investigate the oil distribution, its fate in the environment and possible cleanup mechanisms for already existing contaminated sites.

1.1 PETROLEUM HYDROCARBONS

Petroleum hydrocarbons constitute a large and diverse class of around 250 hydrocarbon compounds consisting of varying constituents and molecular complexity.

They represent: saturates, aromatics, resins and asphaltenes. Saturates are defined as hydrocarbons containing no double bonds. They are categorized according to their chemical structures into alkanes (paraffins) and cycloalkanes. Saturates represents the highest percentage of crude oil constituents. Aromatic hydrocarbons with one or several aromatic rings are usually substituted with different alkyl groups. In comparison to the saturated and aromatic fractions, the resin and asphaltenes contain non-hydrocarbon polar compounds. Resins and asphaltenes have very complex and mostly unknown carbon structure with addition of many nitrogen, sulphur and oxygen atoms (Harayama 2004). Petroleum hydrocarbons recovered from different reservoirs varies widely in compositional and physical properties. The composition of particular petroleum product ranges from the low to the high molecular weight hydrocarbons. They are widespread in the environment as fuels, solvents and chemical compounds. Due to their complex nature, they are difficult to degrade in the environment.

1.2 PETROLEUM HYDROCARBONS AS POLLUTANT

A pollutant is classified as persistent and recalcitrant pollutant based on its ability to undergo degradation. Persistent pollutants are those which can undergo degradation, but they persist in the environment for a prolonged time due to non-biodegradation. Recalcitrant pollutants are those which may undergo a few steps of degradation, but they never undergo mineralization to carbon dioxide and water. Petroleum hydrocarbons fall into the category of persistent pollutants when they are released into the environment; they cause air, water as well as soil pollution.

Uncontrolled and catastrophic releases of the petroleum hydrocarbons into the environment whether accidentally or due to human activities are the main cause of water, air and soil pollution (Holliger et al. 1997). The extensive production and use of these hydrocarbons has resulted in widespread environmental contamination. The contamination can result from leaking Underground Storage Tanks (UST), petroleum refineries, bulk storage facilities, harbour operations, broken oil pipelines, effluent discharges from petroleum industries, spills of petroleum products in chemical plants

and transportation processes (Sherman and Stroo 1989). Many manufacturing processes necessarily produce water and sludge that are contaminated with hydrocarbons. Extensive changes in marine as well as terrestrial ecosystems resulting from the grounding of the Exxon Valdez (1989), the Nahodka oil spill (1997), the Erica spill (1999), the Prestige spill (2002) and the Gulf of Mexico oil spill (2010) have been reported due to the release of petroleum hydrocarbons. According the report of Open Science Advisory Team (OSAT) in 2010, oil remains have been observed in the sandy soil of the contaminated shoreline of Gulf of Mexico, due to the persistent nature of petroleum hydrocarbons. Physical properties of petroleum hydrocarbons such as density and viscosity of the hydrocarbons, formation of water in oil emulsions, etc, affect its persistence. Light petroleum fractions such as kerosene tend to evaporate and dissipate naturally and rarely need cleaning up. These fractions are termed as non-persistent hydrocarbons.

Petroleum hydrocarbon pollution problems often result in huge disturbances of both the biotic and abiotic components of the ecosystem (Mueller et al. 1992), more so that some hydrocarbon components have been known to belong to a family of carcinogenic and neurotoxic organopollutants (Hallier-Soulier et al. 1999). The compounds in different petroleum hydrocarbon fractions affect the living organisms in different ways. Important sources of exposure of petroleum hydrocarbons include inhalation, ingestion and dermal exposure through the use of petroleum hydrocarbon contaminated surface water or groundwater in domestic potable water applications. Some of the compounds, particularly the smaller compounds such as benzene, toluene and xylene, can affect the human central nervous system. Breathing toluene at concentrations greater than 100 parts per million (100 ppm) for several hours can cause fatigue, headache, nausea and drowsiness. One of petroleum hydrocarbons, n-hexane (concentration of 500-2000 ppm) affects the central nervous system in a different way, causing a nerve disorder called "peripheral neuropathy", characterized by numbness in the feet and legs and in severe cases, leads to paralysis. Some petroleum products such as gasoline and kerosene cause irritation of the throat and stomach, central nervous

system depression, difficulty in breathing and pneumonia. The compounds in some petroleum hydrocarbon fractions also affect the immune system, blood, liver, spleen, kidneys, developing foetus and lungs (Agency for Toxic Substances and Disease Registry 1999). Due to the serious and long-term damage caused to the ecosystem, terrestrial life, human health and natural resources; there is a need to remediate the petroleum hydrocarbons.

1.3 REMEDIATION OF SITES CONTAMINATED WITH PETROLEUM HYDROCARBONS

The process leading to the eventual removal of petroleum hydrocarbon pollutants from the environment has been extensively documented and involves the usage of physical, chemical and biological alternatives, or a combination of them (Okoh and Trejo-Hernandez 2006). The physical and chemical methods generally employed in the treatment of petroleum waste generally include incineration, thermal desorption, refinery coke use, burning in cement kilns, evaporation and the usage of solvents. Incineration and thermal desorption are regarded to be among the most expensive treatment methods; the high temperatures involved in these processes require high energy input and results in significant greenhouse gas emissions (Rasmussen 1994). Moreover, these technologies are expensive and can lead to incomplete decomposition of contaminants. Primarily due to cost and time considerations, landfilling is currently the most widely used remediation method. The conventional remedial processes also involve the transfer of the contaminant to another medium rather than eliminating the pollutant. Nevertheless, biological remediation in combination with other methods, has gained an established place in the restoration of ecosystem.

The process of bioremediation is defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry (Medina-Bellver et al. 2005). This has been claimed to be an inexpensive, natural method of cleanup of petroleum contaminated soil or water.

Moreover, the microorganisms involved in biodegradation process often do not generate secondary pollutants. The interest to use these microorganisms as tools for petroleum hydrocarbons remediation is increasing and the exploitation of these microbes has opened up a new avenue for completely eliminating the hydrocarbons from the environment. Bioremediation can completely destroy contaminants, converting them to carbon dioxide, water and new cell mass, or convert them to non-toxic products some of which may even be useful to the ecosystem. The biological decontamination of petroleum polluted soil has been assessed to be an efficient, economic, eco-friendly and versatile alternative to physicochemical treatment methods (Bartha 1986).

1.4 BIODEGRADATION OF PETROLEUM HYDROCARBONS

One of the widely accepted bioremediation methods of petroleum hydrocarbons is biodegradation. The process of conversion of an organic pollutant into environmentally acceptable form by microorganisms is termed as biodegradation. The microorganisms transform the substance through metabolic or enzymatic processes. The driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons for their cell growth and energy needs.

Microorganisms, owing to their biodiversity and vast catabolic potential, have been enormously harnessed for the biodegradation of toxic pollutants since long time (Dua et al. 2002). These diverse catabolic activities are due to the presence of catabolic genes and enzymes (van der Geize and Dijkhuizen 2004; de Carvalho and da Fonseca 2005; Khomenkov et al. 2008). Furthermore, microorganisms possess other adaptation strategies such as the ability to modify the cellular membrane to maintain the necessary biological functions (Isken and de Bont 1998; de Carvalho et al. 2009), the production of surface active compounds as biosurfactants (Ron and Rosenberg 2002) and of the use of efflux pumps to decrease the concentration of toxic compounds inside the cells (Isken and de Bont 1998; Van Hamme et al. 2003). All these mechanisms and metabolic abilities make microorganisms an interesting tool for the bioremediation of contaminated sites.

Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6% (Jones et al. 1970) to 82% (Pinholt et al. 1979) for soil fungi, 0.13% (Jones et al. 1970) to 50% (Pinholt et al. 1979) for soil bacteria and 0.003% (Hollaway et al. 1980) to 100% (Mulkins and Stewart 1974) for marine bacteria. Many scientists reported that mixed populations with overall broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil (Bartha and Bossert 1984), fresh water (Cooney 1984) and marine environments (Floodgate 1984; Atlas 1985). Among the different microorganisms, bacteria play an important role as they get acclimatized easily to the varying environmental conditions and grow rapidly (Riser-Roberts 1992; Bundy et al. 2004).

The petroleum hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Perry 1984; Ulrici 2000). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all (Atlas and Bragg 2009). Molecular stability and hydrophobicity are two primary factors that contribute to the persistence of high molecular weight petroleum pollutants in the environment, as shown by multiple studies correlating biodegradation rates and molecular size of the hydrocarbons (Heitkamp and Cerniglia 1987; Kanaly and Harayama 2000). Because of their lipophilic nature, high molecular weight petroleum hydrocarbons have a high potential for biomagnifications through trophic transfers (Clements et al. 1994; Twiss et al. 1999).

To improve the bioremediation process, besides a competent microbe able to degrade the contaminant carbon source, other parameters must be taken into account. These parameters include water, oxygen and utilizable nitrogen as well as phosphorous sources (Rosenberg et al. 1992). Lack of any of the mentioned parameters makes the remediation process under natural conditions inefficient. Strategies involving the

addition of seeded cultures, bioaugmentation, or the addition of nutrients, biostimulation, hold the promise of fostering degradation rates (Atlas 1995; Jimenez et al. 2006).

Bioaugmentation or seeding is the addition of highly concentrated and specialized populations (single strains or consortia) to the site contaminated with recalcitrant toxic compounds (Leahy and Colwell 1990; Gentry et al. 2004). This technique is best suited for sites that (i) do not have sufficient microbial cells or (ii) the native population does not possess the metabolic routes necessary to metabolize the compounds under concern. On the other hand, biostimulation involves the identification and adjustment of factors such as nutrients that may be looming the biodegradation rate of the contaminants by the indigenous microorganism at the affected site (Swannell et al. 1996). Besides the type and concentration of nutrients, physical and environmental parameters also influence the mineralization rate of hydrocarbons by the degrading microorganism. These factors include the chemical composition, physical state and concentration of the crude oil or hydrocarbons; along with the temperature, oxygen availability, salinity, pressure, water activity and pH on the contaminated site (Leahy and Colwell 1990).

Although biodegradation of petroleum hydrocarbon contaminated environments is a promising alternative remedial strategy (Cerniglia 1992), the biodegradation rate of these hydrocarbons in the environment is limited by various factors (Providenti et al. 1993). One of the major factors is limited availability of the hydrocarbons to microbes because of their low solubility and strong and/or irreversible sorption to soil (Rockne et al. 2002). Petroleum hydrocarbon compounds bind to soil components and they are difficult to be removed or degraded (Barathi and Vasudevan 2001). One of the options to increase the bioavailability of these pollutants is the use of surfactants.

Several reports have shown enhanced biodegradation of petroleum contaminants in lab and field studies by adding synthetic surfactants (Aronstein et al. 1991; Laha and Luthy 1991). Surfactants have been suggested as possible enhancers of desorption, biodegradation of petroleum hydrocarbons and other organics in the soil environments

(Sanseverino et al. 1994). Nakahara et al. (1981) suggested that surfactants may stimulate degradation of hydrocarbons by making more of the surface of the chemical available. In another report, the process of bioremediation was enhanced by using surfactants to increase the availability of the polyaromatic hydrocarbon compounds to the microorganisms (Carriere and Mesania 1995).

1.5 SURFACTANTS

Surfactants are surface active amphiphilic molecules consisting of a hydrophilic and a hydrophobic part (Banat et al. 2000). The hydrophobic moiety of the surfactant is usually the equivalent of an 8 to 18 carbon atoms. It can be aliphatic, aromatic, or a mixture of both. The hydrophilic end of the surfactant is strongly attracted to the water molecules and the force of attraction between the hydrophobic groups and water is only slight. As a result, the surfactant molecules align themselves at the surface and internally so that the hydrophilic end is towards water and the hydrophobic moieties are squeezed away from water. Hence, they are known to reduce the surface tension of liquids and emulsify the hydrocarbon contaminant. Therefore, these compounds improve the microbial utilization rates of the hydrocarbons.

The surfactants used in different applications are derived from petrochemical raw materials. One of the predominant reasons for the ubiquitous deployment of surfactants is their remarkable ability to influence the properties of surfaces and interfaces and to thereby, have an impact on industrial and environmental processes. The applications of surfactants in industry area are quite diverse and have a great practical importance. Surfactants are applied in the production and processing of foods, agrochemicals, pharmaceuticals, personal care and laundry products, petroleum mineral ores, fuel additives and lubricants, paints, coatings and adhesives as well as in photographic films. They are also found throughout a wide spectrum of biological systems as well as medical applications, soil remediation techniques and other environmental, health as well as safety applications (Schramm et al. 2003).

1.6 ENVIRONMENTAL POLLUTION BY SYNTHETIC SURFACTANTS

Earlier, the surfactants used in the remediation of petroleum hydrocarbons were synthetic surfactants synthesized from petroleum based products. A standard synthetic surfactant type used in many cleansers and detergents is alkylphenol ethoxylates (APEs), with the most common members of the family being octylphenol ethoxylate (OPE) and nonyl phenolethoxylate (NPEO). APEs are versatile, non-ionic surfactants used for more than half a century in a variety of applications. They have excellent detergency, wetting, solubilization and emulsification properties. They are one of the lowest priced bulk surfactants in the market. A drawback of APEs, though, is that they biodegrade slowly and persist in the environment. They are made from alkyl phenols and ethylene oxide polymer chains, both derived from petrochemicals. The use of these chemicals further adds pollutant load to the environment since they are synthesized from petroleum based compounds. During the biological degradation, alkyl phenol ethoxylates are transformed to alkyl phenols, e.g. nonyl phenol ethoxylate (NPEO) degrades to nonyl phenol (NP), which is known to be toxic and have hormone like effects. Even small amounts of the surfactant are known to affect the endocrine systems of animals.

Another example of a highly effective surfactant that persists in the environment is branched tetrapropylene aryl sulfonates (TPAS). Over time, foaming was discovered in sewage treatment plants. TPAS was identified as the culprit and the branched nature of the molecule was found to be the cause of the persistence. Most surfactants are more or less toxic to aquatic organisms due to their surface activity which reacts with the biological membranes of the organisms.

As a result, a linear version of the surfactant, linear alkylbenzenesulfonate (ABS), was developed. ABS is biodegradable, although slowly. ABS has been under some debate over the recent years due to the fact that it does not biodegrade under anaerobic conditions. In the early 1960's many rivers and lakes receiving the waste waters from

large cities started to be covered by persistent foams, which resulted in ecological damage because the thick layer curtailed photosynthesis and oxygen dissolution. The culprit was found to be the branching of the alkylate group of the ABS made from propylene, whose polymerization follows Markovnikoff's rule. It was found that branching confers to the alkylate group a resistance to biodegradation. As a consequence, environmental protection laws were passed around 1965 to restrict and forbid the use propylene-based alkylate in USA and Europe.

Being an important group of products of the chemical industry, synthetic surfactants play a significant role in economic and socio-economic terms. Their production and use implies environmental and health impacts. Moreover, synthetic surfactants exhibit a low rate of biodegradation and a high potential to aquatic toxicity (Magali and Michel 2004). Surfactant pollution of rivers and oceans is attributable to the high demand of surfactant use and the improper control or wastewater management. They act like secondary pollutants in the environment. Since surfactants are derived from petroleum based products, they are commonly toxic to ecosystems and resistant to complete degradation.

Consequently, industry and research organizations are currently active in finding out new ways of producing surfactants which are environmentally friendly and safer, which entail a minimum health risk and can be produced from domestic renewable resources (Karsa et al. 1999; Ehrenberg 2002). An increase in the concern about environmental protection has recently caused the consideration of alternative to synthetic surfactants. Since these chemicals must be both effective and compatible with the environment, it is natural to turn to the microbial world, which is the only source to fulfill this demand (Makkar and Cameotra 2002). However, it was only in past few decades that surface active molecules of biological origin, referred to as biosurfactants, have gained considerable interest (Desai and Banat 1997).

Microbes produce largely unexplored variety of chemicals, such as biosurfactants, that have exciting potential for application in biotechnology and industry. Although

biosurfactants are just one class of natural products, they are a representative example of the challenges that exist in discovery, analysis, production and use of natural products in various industries. In this era of green technology, biosurfactants are highly sought biomolecules for present and future applications as fine specialty chemicals, biological control agents and new generation molecules for pharmaceutical, cosmetic and health care industries. From an environmental standpoint, biosurfactants are more acceptable for the remediation process both at sea and land (Cameotra and Bollang 2003; Cameotra and Makkar 2010).

1.7 BIOSURFACTANTS

Biosurfactants are an important class of environment friendly surface active products which are produced on microbial cell surfaces or secreted extracellularly. Some structural types of surfactant are produced using biological systems and cannot easily be synthesized by chemical processes (Gerson and Zajic 1979). These molecules can be tailor-made to suit different applications by changing the growth substrate or growth conditions (Fiechter 1992). Interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications.

In terms of structure, biosurfactants are polymers, totally or partially extra-cellular, with an amphipathic configuration, containing distinct polar and non-polar moieties which allow them to form micelles that accumulate at interface between liquids of different polarities such as water and oil. This process is based upon the ability of biosurfactants to reduce surface tension, blocking the formation of hydrogen bridges and certain hydrophilic and hydrophobic interactions. They form microemulsion where hydrocarbons can solubilize in water or where water can solubilize in hydrocarbons. Such characteristics confer excellent detergency, emulsifying, foaming and dispersing traits, which makes biosurfactants some of the most versatile process chemicals.

Biosurfactants have several advantages over their synthetic counterparts, such as lower toxicity, higher biodegradability, better environmental compatibility, higher

foaming, higher selectivity and specific activity at extreme temperature, pH and salinity as well as the ability to be synthesized from renewable feedstock and industrial waste. They are required in small quantities for bringing about reduction in surface tension. These unique properties allow the use of biosurfactants and possible replacement of chemically synthesized surfactants in a great number of industrial applications. This makes microbial surfactants even more promising (Turkovskaya et al. 2001). The main differences between the chemical and biosurfactants are presented in Table 1.1.

Table 1.1: Differences between synthetic surfactant and biosurfactant

Synthetic surfactant	Biosurfactant	References
Categorized according to the nature of their polar group	Categorized according to their chemical composition and microbial origin	Desai and Banat (1997)
Specific for certain temperature, pH and salinity	High selectivity and specificity at extreme temperature, pH and salinity	Banat et al. (2000)
High toxicity	Low toxicity	Edwards et al. (2003)
Low biodegradability	High biodegradability	Banat (1995)
Chemically synthesized mostly from petroleum resources	Mostly synthesized from renewable feed-stock	Fox and Bala (2000)
Specific for particular industrial application	Broad spectrum of industrial application	Cameotra and Makkar (1998)
Low production cost	High production cost	Banat (1995)

In the recent years, biosurfactants are required in large number of diverse applications due to their broad range of functional properties (Cooper 1986; Desai and Desai 1993). They are potentially useful in every industry, due to their basic structure that contains both hydrophilic and lipophilic portions (Desai and Banat 1997). Biosurfactants are also used in food and cosmetic industries, industrial cleaning of products and in agricultural chemicals to dilute and disperse fertilizers and pesticides (Kosaric et al. 1987). Ishigami (1997) has speculated various potential applications of biosurfactants in bioengineering including their use as cryopreservatives, protein solubilizers, enzyme stabilizers, DNA isolating agents, preservatives for cut flowers, growth enhancers for plants, recovery enhancers for wounds and swelling.

The largest possible market for biosurfactant is the oil industry, both for petroleum production and for incorporation into oil formulations (Van Dyke et al. 1991). Other applications related to the oil industries include oil spill bioremediation/dispersion, both in land and at sea, removal/mobilization of oil sludge from storage tanks and enhanced oil recovery (Georgiou et al. 1992; Khire and Khan 1994). The second largest market for biosurfactants is emulsion polymerization for paints, paper coatings and industrial coatings. Layman (1985) described other uses of surfactants including asphalt, cement, textile and rubber manufacturing, in addition to metal treatment, mining, water treatment, coal slurry defoamers and as wood preservatives.

In addition, biosurfactants are used as enhancers of biodegradation of petroleum hydrocarbons instead of synthetic surfactants. One of the limiting factors in petroleum biodegradation by microorganisms is the bioavailability of the many fractions of the oil to microorganisms. There must be a form of emulsification to make various components of petroleum hydrocarbons available to microorganisms for effective degradation. In order to cope with this challenge, some hydrocarbon degrading microorganisms produces biosurfactants of diverse chemical nature and molecular size. These surface

active compounds increase the surface area of hydrophobic insoluble petroleum hydrocarbons and their bioavailability, thereby, enhance the growth of microorganisms and rate of biodegradation (Rosenberg et al. 1988; Ron and Rosenberg 2002).

Biosurfactants enhance petroleum hydrocarbon bioremediation by two mechanisms. The first includes the increase of substrate bioavailability for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with microbial cells (Mulligan and Gibbs 2004). By reducing surface and interfacial tensions, biosurfactants increase the surface areas of insoluble compounds leading to increased mobility and bioavailability of hydrocarbons. In consequence, biosurfactants enhance biodegradation and removal of hydrocarbons. Addition of biosurfactants can be expected to enhance hydrocarbon biodegradation by mobilization, solubilization or emulsification (Deziel et al. 1996; Bai et al. 1997; Urum and Pekdemir 2004; Nievas et al. 2008).

Many scientists have employed the use of biosurfactants to facilitate the process of bioremediation (Banat 1995; Cameotra and Bollang 2003). Biosurfactant enhanced bioremediation approach has been frequently used to treat hydrocarbon pollution in some sensitive environments like the mangrove swamp where accessibility and human intrusion is difficult (Teas et al. 1993). Bayoumi and Nagar (2009), while monitoring a bioremediation program on the Egyptian red sea mangrove discovered that surface mangrove sediments harboured diverse petroleum hydrocarbon degrading bacteria that produced biosurfactant. They further observed that the biosurfactant accelerated the rate of degradation of petroleum hydrocarbons. Banat (1995) demonstrated the use of biosurfactants in pollution control, oil tank clean up and enhanced oil recovery. Enhanced bioremediation of n-alkane in a sludge using bacterial consortium amended with biosurfactant and macronutrients has been reported by Rahman et al. (2003). Millioli et al. (2009) have also observed that addition of biosurfactant to hydrocarbon

contaminated soil significantly increased the biodegradation of the hydrocarbon by the indigenous hydrocarbon degrading microbial community.

The research pertaining to bioremediation of petroleum hydrocarbons in the present day is focusing on the identification of new surfactant producing microorganisms as well as the utilization of the surfactants in the bioremediation of hydrocarbons since there is frequent accidental oil spills and petroleum hydrocarbon pollution around the world. The cost involved in the production of synthetic surfactants is high. In this regard, there is a need to initiate studies on the isolation of novel microorganisms with the ability to produce surfactant, study the effect of various factors on surfactant production so as to increase the concentration of surfactant. The surfactant synthesized could be utilized to remediate sites contaminated with petroleum hydrocarbons.

The underlying hypothesis of the present study was to isolate and screen an efficient surfactant producer that produces acellular biodegradable surfactant which could be used in environmental applications.

1.8 OBJECTIVES OF THE STUDY

The objectives of the present research are

1. Isolation of microorganisms from the soil and water samples which were exposed to petroleum hydrocarbons for prolonged lengths of time.
2. Screening surfactant producing microorganisms from petroleum hydrocarbon contaminated soil and water samples.
3. Identification of the potential surfactant producer.
4. Studies on the effect of various process parameters on surfactant production.
5. Statistical optimization of process variables for the enhanced production of surfactant.
6. Characterize the kinetics of surfactant production.
7. Recovery/partial purification of surfactant.

8. Characterization of surfactant.
9. Studies on the effect of various environmental factors on the stability of surfactant.
10. Studies on the efficiency of the surfactant in the biodegradation of crude oil.

1.9 ORGANIZATION OF THE THESIS

Chapter 1 presents the introduction as well as application of biosurfactants in petroleum hydrocarbon remediation and the objectives of the present investigation. Chapter 2 deals with extensive literature review on classes and advantages of biosurfactants, isolation and screening of surfactant producers, effect of various process parameters on the production of biosurfactants, recovery/ partial purification of the surfactants, characterization of surfactants and application of biosurfactants in various fields. Chapter 3 presents the various materials and methods employed in the experimental work to achieve the stated objectives. Chapter 4 deals with the results and discussion of the experimental work. Chapter 5 presents the summary of the work along with conclusions.

CHAPTER 2

REVIEW OF LITERATURE

The present chapter presents the relevant literature information with respect to studies carried out on the production, characterization and applications of biosurfactant. Recently, there has been a growing interest in studying biosurfactant production by a wide array of microorganisms not only from the stand point of comparative biology but also with the expectation of finding candidate biosurfactant producers as well as the usage of different biosurfactants in various biotechnological applications. The review of literature on biosurfactant, different classes of biosurfactant and their applications is presented. The identification of the sampling sites for the isolation of biosurfactant producing microorganisms, isolation techniques, screening procedures and identification of the biosurfactant producers has been discussed. Reports on studies on various factors affecting the production of biosurfactant, optimization of process variables using statistical method and kinetics of biosurfactant production is presented. The partial purification steps, various biosurfactant characterization techniques and effect of various environmental factors affecting biosurfactant stability are discussed. The advantages and numerous applications offered by biosurfactants in various fields are also presented.

In the recent years, contamination of land and water by the petroleum hydrocarbons has been identified as one of the most significant contamination problems on the continent. The extensive usage of petroleum hydrocarbons as fuels, process chemicals, solvents, etc, and also occurrence of frequent oil spills and accidents has led to increase in the number of contaminated sites. Various physicochemical and biological methods have been used for the restoration of these contaminated sites. Due to increased environmental awareness, biological remediation methods have gained more importance in the recent past. In order to increase the availability of the petroleum hydrocarbons by the microorganisms, synthetic surfactants have been traditionally used. Synthetic surfactants are surface active agents which are derivatives of petroleum hydrocarbons.

Since the synthetic surfactants are derived from petroleum based compounds, they add more pollutant load to the environment and lead to the production of secondary pollutants. Moreover, they are toxic and exhibit low-biodegradability. In an effort to restore the petroleum hydrocarbon polluted environments, an effort has been made for the production of surfactants using biological routes due the numerous advantages they offer. Surfactants produced by the biological sources exhibit low toxicity, high specificity and biodegradability. Moreover, they exhibit significant reduction in surface tension when applied in low concentration.

2.1 BIOSURFACTANTS

Increasing environmental awareness and emphasis on sustainable society in harmony with the global environment, during the recent years, has led to serious consideration of biosurfactants as possible alternative to synthetic surfactants as they cause environmental problems due to their resistance to biodegradability as well as toxicity to ecosystems (George and Jayachandran 2009). In the recent years due to their diversity, environmentally friendly nature, the possibility of their production through fermentation and their potential applications in the environmental protection, crude oil recovery, health care and food-processing industries, the demand for biosurfactants is steadily increasing.

Biosurfactants are surface active molecules of biological origin. They are synthesized by wide variety of bacteria, yeast, fungi which either adhere to cell surfaces or are excreted in growth medium and confer the ability of reducing the surface and interfacial tension (Muthusamy et al. 2008). They have recently received much more attention in concern with the protection of environment, making them “green” chemicals, primarily because of their inherent good biodegradability, low toxicity, higher foaming, high selectivity and effectiveness at extreme pH, temperature and salinity. Also, they have unique structures which provide new properties that classical surfactants may lack (Abouseoud et al. 2008; Abdel-Mawgoud et al. 2010).

2.2 CLASSES OF BIOSURFACTANTS

Biosurfactants are categorized mainly by their chemical composition and microbial origin. They 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 molecular mass surfactants include glycolipids, lipopeptides and phospholipids, whereas high molecular mass surfactants include polymeric and particulate surfactants. 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 (Rosenberg and Ron 1999). A brief discussion about each class of biosurfactant is given below:

2.2.1 GLYCOLIPIDS

Most known biosurfactants are glycolipids. They are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. The linkage is by means of either ether or an ester group. Among the glycolipids, the best known are rhamnolipids, trehalolipids and sophorolipids.

2.2.1.1 RHAMNOLIPIDS

Rhamnolipids are well known glycolipids. They contain one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid (Fig. 2.1). They are the most common glycolipids produced by the genus *Pseudomonas* (Syldatk and Wagner 1987; Maier and Soberón-Chávez 2000; Rahman and Gakpe 2008). Production of rhamnose-containing glycolipids was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (1949). Rhamnolipids produced by *Pseudomonas sp*. have been

demonstrated to lower the interfacial tension against n-hexadecane to 1 mN/m and the surface tension to 25-30 mN/m (Guerra-Santos et al. 1986; Parra et al. 1989). L-Rhamnosyl-L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate are the principal rhamnolipid congeners produced by *Pseudomonas aeruginosa*. Up to 28 analogues of rhamnolipids have been detected by various characterization techniques. The congeners of rhamnolipids differ in the amount of rhamnose sugar and fatty acid chain present, with one or two rhamnose and fatty acid chains (C_8-C_{12}) being predominant (Benincasa et al. 2004). Rhamnolipids are also used as a source of L-rhamnose. L-rhamnose is a sugar which is commonly extracted from plant sources; it is used as a starting material in the synthesis of organic compounds. Rhamnolipids from *Pseudomonas aeruginosa* are currently commercialized by Jeneil Biosurfactant, USA, mainly as a fungicide for agricultural purposes or an additive to enhance bioremediation activities.

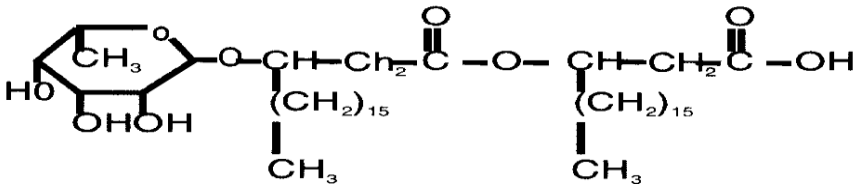


Fig. 2.1 Structure of rhamnolipid

2.2.1.2 TREHALOLIPIDS

Several structural types of microbial trehalolipid biosurfactants have been reported which consists of trehalose (a disaccharide of two glucose molecules) connected to long chain fatty acids (Fig. 2.2). Disaccharide trehalose linked to mycolic acids is associated with most of the species. Mycolic acids are long chain, branched-β-hydroxy fatty acids. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms and the degree of unsaturation (Asselineau and Asselineau 1978). Trehalolipids are produced by a number of different microorganisms, such as *Mycobacterium sp.* (Karanth et al. 1999), *Nocardia sp.* and *Corynebacterium sp.*

(Banat et al. 2010). However, the most extensively studied compounds in this class are trehalose dimycolates produced by *Rhodococcus erythropolis* (Rapp et al. 1979). Trehalolipids from *Rhodococcus erythropolis* and *Arthrobacter sp.* were found to lower the surface and interfacial tension of culture broth ranging from 25-40 mN/m and 1-5 mN/m, respectively (Li et al. 1984).

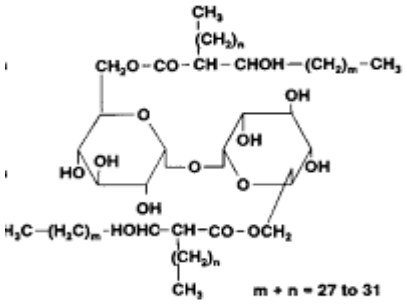


Fig. 2.2 Structure of trehalolipid

2.2.1.3 SOPHOROLIPIDS

Sophorolipids consists of a disaccharide sophorose (consists of two β-1, 2- linked glucose units) by a glycosidic bond through a hydroxyl group located at the penultimate position of an 18-carbon hydroxyl fatty acid (Fig. 2.3). This type of biosurfactant occurs as a mixture of macrolactone and open-chain (free acid) forms and may be acetylated at the primary hydroxyl positions of the sophorose (Daverey and Pakshirajan 2009). These are produced mainly by yeasts, such as *Candida bombicola* (also known as *Torulopsis bombicola*), *Centrolene petrophilum, Candida apicola* and *Rhodotorula bogoriensis* (Banat et al. 2010). In addition to reducing surface tension of the broth, they are also effective emulsifying agents (Cooper and Paddock 1984; Karanth et al. 1999). They are known to possess biocidal activity (Lang et al. 1989) and are used in cosmetic industries as antidandruff formulations as well as in deodorants (Mager et al. 1987).

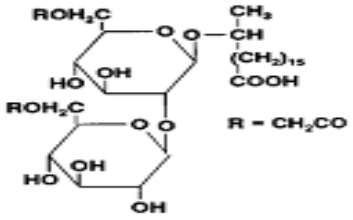


Fig. 2.3 Structure of sophorolipid

2.2.1.4 MANNOSYLERYTHRITOL-LIPIDS

The other high yielding glycolipids from yeasts are mannosylerythritol-lipids (MEL) which are receiving much attention owing to their biomedical applications (Kitamoto et. al. 1993; Kitomato et al. 2002). Mannosylerythritol lipid (MEL), 2,3-di-O-alka(e)noyl-β-D-mannopyranosyl-(1→4)-O-meso-erythritol partially acetylated at C_4 and/or C_6, is a glycolipid that contains mannose, the sugar alcohol erythritol as hydrophilic moiety, acetyl groups as well as fatty acids as the hydrophobic moiety (Fig. 2.4). MELs are produced by *Pseudozyma* yeasts, *Pseudozyma aphidis*, *Pseudozyma antarctica* and *Pseudozyma rugulosa* (Konishi et al. 2007).

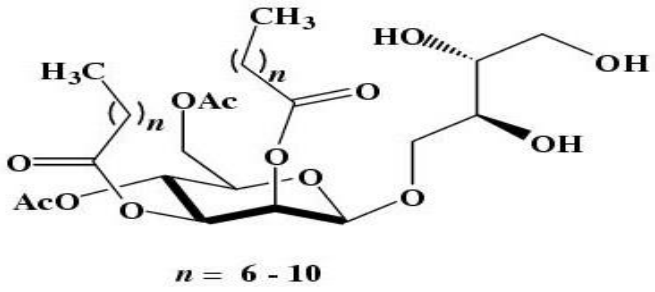


Fig. 2.4 Structure of mannosylerythritol-lipid

2.2.2 LIPOPEPTIDES AND LIPOPROTEINS

Lipopeptides consists of fatty acids attached to a chain of amino acids. The cyclic lipopeptide surfactin, produced by *Bacillus subtilis* ATCC 21332, is one of the well known biosurfactants (Fig. 2.5). This anionic cyclic lipopeptide is constituted by a heptapeptide interlinked with a β-hydroxy fatty acid. Surfactin reduces the surface tension of the broth from 72 mN/m to 27 mN/m, making it one of the most powerful biosurfactants (Rahman and Gakpe 2008). A large number of cyclic lipopeptides, including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) belong to this category of biosurfactants. Surfactins are also used for the synthesis of spheroplasts as they can lyse the erythrocytes (Rahman and Gakpe 2008).

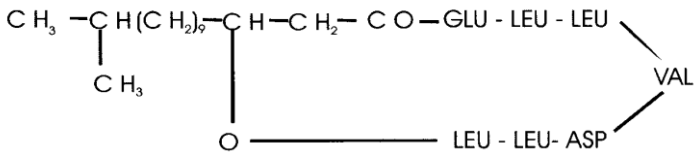


Fig. 2.5 Structure of cyclic lipopeptide

2.2.3 FATTY ACIDS, PHOSPHOLIPIDS AND NEUTRAL LIPIDS

Several bacteria and yeast produce large quantities of fatty acids and phospholipid surfactants during growth on n-alkanes (Cooper and Paddock 1984). Fatty acids are produced from alkanes as a result of microbial oxidations has been considered as surfactants (Rehn and Reiff 1981). In *Acinetobacter sp.* strain HO1-N, phosphotidylamine rich vesicles are produced (Kappeli and Finerty 1979), which form optically clear microemulsions in water (Fig. 2.6). Phospholipids are known to form major components of microbial membranes. The quantitative production of phospholipids has also been detected in *Thiobacillus thiooxidans* (Beeba and Umbriet 1971) and *Aspergillus sp.* (Kappeli and Finerty 1979). Phospholipids produced by *Thiobacillus thiooxidans* have been reported to be responsible for wetting elemental sulphur, which is required for growth (Beeba and Umbriet 1971). *Arthrobacter* strain AK-19 (Wayman et.

al., 1984) and *Pseudomonas aeruginosa* 44T1 (Robert et al. 1989) accumulate up to 40 to 80% (w/w) of such lipids when cultivated on hexadecane and olive oil, respectively.

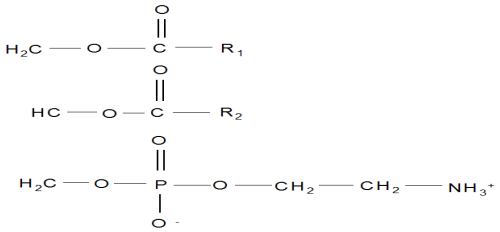


Fig. 2.6 Structure of phosphatidylethanolamine

2.2.4 POLYMERIC BIOSURFACTANTS

These are polymeric heterosaccharide containing proteins which are efficient in solubilizing the hydrocarbons (Karanth et al. 1999). The best studied polymeric biosurfactants are emulsan, liposan, alasan, mannoproteins and other polysaccharide-protein complexes (Desai and Banat 1997). *Acinetobacter calcoaceticus* RAG-1 produces an extracellular potent polyanionic amphipathic heteropolysaccharide bioemulsifier called 'emulsan' (Rosenberg et al. 1979). It contains heteropolysaccharide backbone having a repeating trisaccharide of N-acetyl-D-galactosamine, N-acetylgalactosamine uronic acid and an unidentified N-acetyl amino sugar (Fig. 2.7). Emulsan is an effective emulsifying agent for hydrocarbons in water (Zosim et al. 1982) even at a concentration as low as 0.001 to 0.01%. Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and is composed of carbohydrates and proteins (Muthusamy et al. 2008). Biodispersan is an extracellular, nondialyzable dispersing agent produced by *Acinetobacter calcoaceticus* A2. It is an anionic heteropolysaccharide that contains four reducing sugars, namely, glucosamine, 6-methylaminohexose, galactosamine uronic acid, and an unidentified amino sugar (Rosenberg et al. 1988). Mannoprotein produced by *Saccharomyces cerevisiae* is a polysaccharide-protein complex, the polysaccharide is made up of mannose, accounting upto 44% of the complex and protein, accounting upto 17% of the complex (Karanth et al. 1999; Rahman and Gakpe 2008).

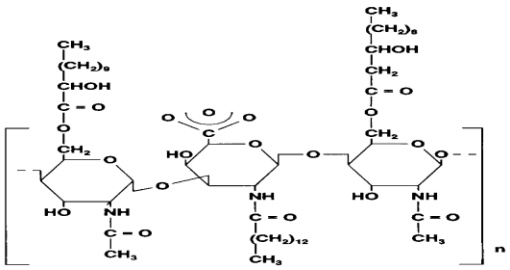


Fig. 2.7 Structure of emulsan

2.2.5 PARTICULATE BIOSURFACTANTS

Particulate biosurfactants include extracellular membrane vesicles which partition hydrocarbons to form a microemulsion that plays an important role in alkane uptake by microbial cells. Vesicles of *Acinetobacter sp.* strain HO1-N with a diameter of 20-50 nm are composed of protein, phospholipids and lipopolysaccharide (Kappeli and Finnerty 1979).

2.3 ADVANTAGES OF BIOSURFACTANTS

There are various advantages of biosurfactants compared to their chemically produced counterpart. The major distinctive features of biosurfactants and a brief description of each property are given below:

2.3.1 SURFACE AND INTERFACE ACTIVITY

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). Surfactin produced by *Bacillus subtilis* is able to reduce the surface tension of water to 25 mN/m and interfacial tension of water/hexadecane to <1 mN/m (Cooper et al. 1981). Rhamnolipids produced by *Pseudomonas 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). The sophorolipids produced by *Torulopsis bombicola* reduce the surface tension to 33 mN/m and the interfacial tension to 5 mN/m (Cooper and Cavalero

2003). Benincasa et al. (2004) reported that *Pseudomonas aeruginosa* LB1 strain produced a rhamnolipid surfactant which reduced the surface tension of the broth from 70 mN/m to 24 mN/m. In general, biosurfactants are more effective and efficient and their Critical Micelle Concentration (CMC) is about 10-40 times lower than that of chemical surfactants, *i.e*. less amount surfactant is required to get a maximum decrease in surface tension (Desai and Banat 1997).

2.3.2 TEMPERATURE, pH AND IONIC STRENGTH TOLERANCE

In the literature, different trends have been observed by various authors with respect to effect of environmental factors on the stability of biosurfactant. Many biosurfactants and their surface activities are not affected by environmental conditions such as temperature, pH and NaCl concentrations. McInerney et al. (1990) suggested that lichenysin produced by *Bacillus licheniformis* was not affected by temperature (up to 50°C), pH (4.5-9.0) and by NaCl up to 50 g/L, respectively. A lipopeptide produced by *Bacillus subtilis* 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% (Nitschke and Pastore 1990).

2.3.3 BIODEGRADABILITY

Unlike synthetic surfactants, compounds produced by microbial route are easily degraded (Mohan et al. 2006) since they are synthesized from easily degradable substrates unlike synthetic surfactants which are produced from petroleum derivatives that are persistent in environment. Hence, biosurfactants are chiefly suited for environmental applications such as bioremediation (Mulligan 2005) and dispersion of oil spills.

2.3.4 LOW TOXICITY

Biosurfactants are in general considered as low or non-toxic products and therefore, are appropriate for pharmaceutical, food and cosmetic uses. The surfactant produced by *Pseudomonas aeruginosa* was compared to a synthetic surfactant that is widely used in the industry, regarding toxicity and mutagenic properties. Both assays indicated a higher level of toxicity and mutagenic effect of the chemically derived surfactant, whereas the biosurfactant exhibited low toxicity and was found to be non-mutagenic (Flasz et al. 1998).

2.3.5 CHEMICAL DIVERSITY

The chemical diversity of naturally produced biosurfactants offers a wide selection of surface-active agents with properties closely related to specific applications. Microbial biosurfactants include a wide variety of chemical structures, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids and fatty acids (Georgiou et al. 1992). It is, therefore, reasonable to expect diverse properties and physiological functions for different families of biosurfactants.

2.4 PHYSIOLOGICAL FUNCTION OF BIOSURFACTANTS

The physiological function of biosurfactants is not clear. Although most biosurfactants are considered as primary/secondary metabolites, some may play essential roles for the survival of the biosurfactant producing microorganisms either through facilitating nutrient transport or microbe-host interactions, or as biocides. There has been speculation about their involvement in emulsification of water-insoluble substrates (Harvey et al. 1990; Francy et al. 1991; Zhang and Miller 1992). Direct contact of cells with hydrocarbon droplets and their interaction with emulsified droplets have been described (Rosenberg 1986; Francy et al. 1991). In addition, biosurfactants have been shown to be involved in cell adherence which imparts greater stability under hostile environmental conditions and virulence (Rosenberg and Rosenberg 1981; Rosenberg 1986), in cell desorption to find new habitats for survival (Rosenberg and Rosenberg

1981), in antagonistic effects toward other microbes in the environment (Lang et al. 1989; Kitamoto et al. 1993), etc.

Some cell-bound biosurfactants may be responsible for hydrocarbon transport and the attachment of the cells to interface (Kappeli and Fiechter 1977; Bar-Ness et al. 1988). This mechanism is supported by the observation that 2.5% fatty acid was isolated in the polysaccharide moiety from the cell surface of *Candida tropicola* grown on alkanes, while only a trace amount of fatty acid was detected in the corresponding polysaccharide fraction from the cells grown on glucose. This indicated that the cell-bound polysaccharide-fatty acid complex might be involved in the direct transportation of hydrocarbon substrates into the cells. Surfactant produced by *Serratia marcescens* presumably modulates the hydrophobicity of the cell surface, which appears to be an important factor for cell adhesion and colonization of various interfaces (Bar-Ness et al. 1988). Anionic phospholipids are believed to play a critical role in the membrane insertion of proteins. It has been recently demonstrated that anionic phospholipids might be responsible for mediating the membrane insertion of protein toxin (Leenhouts et al. 1995).

2.5 ROLE OF BIOSURFACTANTS IN BIOREMEDIATION

The extensive production and use of petroleum hydrocarbons has resulted in widespread environmental contamination by these chemicals. Due to their toxicity, persistent and negative influence on living organisms, it is important to clean-up the polluted sites. Microbial remediation of hydrocarbons and crude oil-contaminated soils is an emerging technology involving the application of biosurfactants. The interactions between bacteria, contaminants and biosurfactant can be interpreted from a functional perspective, considering that the main natural role attributed to biosurfactants is their involvement in hydrocarbon uptake (Perfumo et al. 2010). Removal and biodegradation of hydrocarbon from soil is enhanced by the ability of biosurfactant to stabilize oil/water emulsions and increase hydrocarbon solubility (Franzetti et al. 2010).

The spilling of oil into the sea is a major problem that can destroy coastlines. The Amoco Cadiz spill at the Brittany coast in 1978; the Exxon Valdez spill near Prince William Sound in 1989 and Gulf of Mexico oil spill in 2010, are examples of significant coastline contamination. Biosurfactants can be useful for oil spills since they could be less toxic and persistent than synthetic surfactants. Chakrabarty (1985) showed that surfactant produced by *Pseudomonas aeruginosa* SB30 could disperse oil into fine droplets which could enhance biodegradation. Shafeeq et al. (1989) showed that biosurfactants were produced during biodegradation of a hydrocarbon mixture by *Pseudomonas aeruginosa* S8. Chhatre et al. (1996) showed that four bacterial isolates from crude oil were able to degrade 70% of the Gulf and Bombay High Crude Oil. One of the isolates produced a rhamnolipid biosurfactant that enhanced biodegradation by the emulsification of the crude oil.

Biodegradation of hydrocarbons by native microbial populations is the primary mechanism by which hydrocarbon contaminants are removed from the environment (Atlas and Bartha 1992). The effectiveness of enhancing hydrocarbon degradation through addition of microbial inoculum prepared from non-indigenous populations (bioaugmentation) has been ambiguous (Atlas 1991). However, the addition of biosurfactant stimulated the indigenous bacterial population to degrade hydrocarbons at rates higher than those which could be achieved through addition of nutrients alone (Desai and Banat 1997). Thus, a promising method that can improve bioremediation effectiveness of hydrocarbon contaminated environments is the use of biosurfactants. They can enhance hydrocarbon biodegradation by reducing the surface tension and interfacial tension, thereby, increasing the bioavailability, solubility and mobility of the hydrocarbon contaminants (Urum and Pekdemir 2004; Nievas et al. 2008).

The capability of biosurfactants and biosurfactant-producing bacterial strains to enhance organic contaminants availability and biodegradation rates was reported by many authors (Déziel et al. 1996; Rahman et al. 2004; Inakollu et al. 2004). Obayori et al.

(2009) investigated the biodegradative properties of biosurfactant produced by *Pseudomonas* sp. LP1 strain on crude oil and diesel. The results obtained confirmed the ability of strain LP1 to metabolize the hydrocarbon components of crude and diesel oil. They reported 92.34% degradation of crude oil and 95.29% removal of diesel oil. Biodegradative properties of biosurfactant producing *Brevibacterium* sp. PDM-3 strain were tested by Reddy et al. (2010). They reported that this strain could degrade 93.92% of phenanthrene and also had ability to degrade other polyaromatic hydrocarbons such as anthracene and fluorene.

The usefulness of biosurfactant producing strains in bioremediation of sites highly contaminated with crude petroleum-oil hydrocarbons was confirmed by Das and Mukherjee (2007). The ability of three biosurfactant producing strains: *Bacillus subtilis* DM-04, *Pseudomonas aeruginosa* M and *Pseudomonas aeruginosa* NM, respectively, to remediate petroleum crude-oil contaminated soil samples was investigated by treating the soil samples with aqueous solutions of biosurfactants obtained from the respective bacterial strains. Additionally, the tested soil was inoculated with mineral-salts media containing a specified amount of *Bacillus subtilis* DM-04 or *Pseudomonas aeruginosa* M and NM strains. In the presence of surfactant produced by *Pseudomonas aeruginosa* M and NM consortium and *Bacillus subtilis* strains, TPH levels were reduced from 84 to 21 and 39 g/kg of soil, respectively. In contrast, the TPH level was decreased to 83 g/kg in control soil.

Joseph and Joseph (2009) separated the oil from the petroleum sludge by induced biosurfactant production by bacteria. Petroleum sludge is generated in significant amount in the refineries during crude oil processing. Crude oil is usually stored in storage tanks. Pollutants present in the oil are deposited at bottom of the tank. During cleaning of the tank, the sludge is recovered and is treated as a waste. The sludge used for the investigation contained TPH in the concentration range of 850 ± 150 g/kg. In this study, the sludge was inoculated directly with *Bacillus* sp. strains and by addition of the cell-

free supernatant. Un-inoculated sludge was also taken as a control. Upon inoculation of the supernatant to the sludge slurry, oil separation and reduction of TPH was observed. The oil separation process was slow initially in the test supplied with the fresh inoculation of the bacterium compared to the samples inoculated with the supernatant, but the residual TPH of both became equal within 48 hours. The efficiency of removal of the various isolates ranged from 91.67% to 97.46%. Therefore, it has been observed that the surfactant produced by the primary inoculums remained in the supernatant and it was enough to continue the reaction. The biosurfactant displayed the property to reduce surface and interfacial tensions in both aqueous and hydrocarbon mixtures and hence, had potential for oil recovery.

Kang et al. (2010) used sophorolipid in studies on biodegradation of aliphatic and aromatic hydrocarbons and Iranian light crude oil under laboratory conditions. Addition of this biosurfactant to soil increased also biodegradation of tested hydrocarbons with the rate of degradation ranging from 85% to 97% of the total amount of hydrocarbons. Their results indicated that sophorolipid may have potential for facilitating the bioremediation of sites contaminated with hydrocarbons having limited water solubility and increasing the bioavailability of microbial consortia for biodegradation.

2.6 IDENTIFICATION OF DIFFERENT ECOLOGICAL SOURCES FOR THE ISOLATION OF POTENTIAL SURFACTANT PRODUCING MICROORGANISMS

Surfactant producing microorganisms are ubiquitous in nature and they have been isolated from different ecological sources. Literature has supported several instances where samples exposed to hydrocarbons resulted in higher number of surfactant producing microorganisms. Prior exposure of a microbial community to hydrocarbons results in production of the surfactants during the utilization of the hydrocarbons for the growth of the microbes. The environmental samples usually considered for the isolation

of potential biosurfactant producers include petroleum hydrocarbon contaminated soil and water samples.

Soil is known to contain different microorganisms capable of synthesizing surfactant. The localities of soil contaminated with petroleum hydrocarbons are known to contain higher number of surfactant producing microorganisms than the soil which is not exposed to petroleum hydrocarbons (Balogun and Fagade (2008). Prior exposure of the microorganisms (either from petroleum exploration, transportation activities, waste oil disposal and accidental oil spills) to hydrocarbons in the soil has profound influence on the production of surfactants by microbes. The microorganisms undergo necessary changes in the metabolic activities in order to survive in the presence of the contaminant by using the contaminant as carbon and energy source. Apart from soil, water is also a rich source of potential biosurfactant producers. With the increasing incidence of oil spillage in the environment, novel microorganisms are being isolated especially from the marine water resources.

Various researchers have isolated potential surfactant producers from various hydrocarbon contaminated regions such as oil soaked soil (Samadi et al. 2007), soil around oil well (Elouzi1 et al. 2009), marine water sources (Bertrand et al. 1993; Rahman et al. 2002), soil samples of petrol pumps (Ray 2012), soil and water samples around petroleum refining industries as well as garage (Batista et al. 2006, Balasubramaniam et al. 2011), effluents from food and oil industries (Mercade et al. 1993; Yalcin and Ergene 2009), oil spilled sea water (Dhail and Jasuja 2012), oil sewage and sludge dumps (Jazeh et al. 2012), etc. Hydrocarbon contaminated sites can be considered as enrichment environment for selection of hydrocarbon degrading and/or surfactant producing microbial strains (Viramontes-Ramos et al. 2010).

In a study by Turkovskaya et al. (2001), cultures producing surfactants were isolated from petroleum-polluted soils and microbial associations formed in pilot plant

devices. Among eighteen strains isolated, *Pseudomonas aeruginosa* 50.3 was identified as surfactant producer based on the reduction of surface tension to 25 mN/m.

Batista et al. (2006) collected terrestrial (soil and water) and marine (sediment and water) samples at three Brazilian sites historically contaminated by crude oil or its byproducts : (1) REGAP-Gabriel Passos Refinery, owned by Petroleo Brasileiro S/A (2) REDUC-Duque de Caxias Refinery owned by Petroleo Brasileiro S/A and (3) UFV-the motor vehicle maintenance garage. 192 morphologically distinct microbial colonies were isolated, including 185 bacteria, 3 filamentous fungi and 4 yeast isolates. Of these, 103 microorganisms were isolated from the REGAP samples, 75 from REDUC samples and 14 from the UFV sample. Eighty-four percent of the bacterial isolates (155 of 184) were Gram-negative. It has previously been reported that most bacteria isolated from sites with a history of contamination by oil or its byproducts are Gram-negative, and this may be a characteristic that contributes to survival of these populations in such harsh environments (Bicca et al. 1999).

Illori et al. (2008) collected water samples containing waste oils discharged from ships and ferries into the lagoon from Lagos lagoon, South Africa. They isolated 2 yeast strains, characterized as *Saccharomyces cerevisiae* LW1 and *Candida albicans* LW2. They further reported the production of surfactants in diesel and crude oil containing media. The organisms showed emulsification activity (E24) of 61.4 and 64.2%, respectively. In addition, the organisms exhibited antimicrobial activity on *Escherichia coli* and *Staphylococcus aureus*.

Pornsunthorntawee et al. (2008) isolated 120 microorganisms from oil sludge, petroleum contaminated soil and sea water. 6 organisms isolated from oil sludge, 16 strains isolated from petroleum-contaminated soil and 4 strains isolated from sea water showed surfactant production ability. Among these strains, *Bacillus subtilis* isolated from oil sludge was identified as potential surfactant producer based on reduction of surface tension to 30 mN/m.

Satpute et al. (2008) isolated *Acinetobacter sp.* and other marine bacterial strains from sea water, sediment and shell samples from West Bengal, Mumbai, Chennai and Calicut, India. They identified a Gram negative bacterium MM73 isolated from Mumbai mussel as surfactant producer based on positive drop collapse test and maximum emulsification activity (68%).

The production of biosurfactant by a flowering plant that parasitizes members of Pinaceae and Cupressaceae, Dwarf mistletoes (*Arceuthobium americanum*), as reported by Vaudry et al. (2010) is an exception. The plant depends on the host for water, mineral nutrients and carbon. The authors suspected that Dwarf mistletoes necessarily affect the water relation of its host by producing surfactants. The surfactant was extracted from the stem tissue as indicated by the reduction of surface tension from 72 mN/m to 30 mN/m.

Tambekar and Gadakh (2012) collected 15 oil contaminated soil samples from various automobile workshops and petrol pumps of Amravati city, India. Totally 9 isolates were identified as potential surfactant producers based on screening tests. Out of the 9 surfactant producers, 3 strains were isolated from automobile workshops and 6 strains were isolated from petrol pump. Out of all the isolates, the strain *Pseudomonas aeruginosa* G2 isolated from automobile workshop was identified as potential surfactant producer based on reduction in surface tension (47.12 mN/m).

Jazeh et al. (2012) collected samples from 8 different areas of oil contaminated soils, oil pipeline leakages, oil sewage and sludge dumps in Sirri Island, Persian Gulf, Iran. A total number of 160 strains were isolated in the study, out of which 18 strains showed surfactant production ability based on surface tension reduction (29-56 mN/m) and emulsification activity (E24) (51.54-80.36%). Based on the screening tests, S7, No. 7 and S5, No. 6 were identified as potential surfactant producers. They further compared the total surfactant producers in the control soil sample (uncontaminated soil sample) with that of oil contaminated samples. Oil contaminated soils contained doubled number of surfactant producing bacteria than the control sample.

In a report by Khopade et al. (2012), they described the production of surfactant by marine actinomycetes, *Nocardiopsis sp.* B4, isolated from marine sediment sample of Mumbai coastal region of India. The organism lowered the surface tension of the cell-free broth from 68 mN/m to 29 mN/m and 80% emulsification activity (E24) during its growth on olive oil containing medium.

From the reports in the literature, it is evident that microorganisms producing surfactants were isolated mainly from petroleum hydrocarbon contaminated regions. This usually occurs since, in nature, surfactants play a physiologic role in increasing bioavailability of hydrophobic molecules which are involved in cellular signaling and differentiation processes, which facilitate the consumption of carbon sources (Singh et al. 2007). This permits the microorganisms to grow on such carbon substrates by reducing the surface tension, thereby making the hydrophobic substrate more readily available for uptake and metabolism (Calvo et al. 2004).

2.7 ISOLATION OF SURFACTANT PRODUCING MICROORGANISMS

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 a mixed population, a pure culture is required. The samples collected can be subjected to random isolation or isolation using enrichment technique. Whether random or enrichment technique, the basic procedures involved are serial dilution, spread plate, streak plate and pour plate technique (Cappuccino and Sherman 1986; Aneja 2003). Serial dilution of any sample collected reduces the load of the organisms and enables the isolation of pure cultures.

Random isolation involves the isolation of the microorganisms first and in the second step, the microorganisms are screened for biosurfactant producers. Initial isolation is not carried out in any enriched medium and the organisms are not acclimatized with the hydrocarbon of interest. After isolation of the microorganisms by serial dilution from

soil or water samples, the pure cultures are isolated by spread plating on a nutrient medium. Some colonies are randomly picked based on the colony characteristics and ability of the organisms to produce surfactant. Further, shake flask experiments are conducted for the screening of the surfactant producers (Stanbury et al. 2005). The major problem faced by researchers in this situation is the re-isolation of the microorganisms which have been already screened before. This results in the waste of labour, time and chemicals. Hence, most of the researchers look for rapid, accurate and reliable isolation techniques which would result in the isolation of novel microorganisms with desired properties (Stanbury et al. 2005).

Apart from random isolation of strains by diluting and plating, enrichment technique is promising for the isolation of surfactant producing microbes as suggested by various reports in the literature. The principle of enrichment culture is to provide growth conditions that are favourable for the organisms of interest possessing industrially important properties. Hence, the microbes of interest are selected and enriched (Walter et al. 2010). It has been observed in various reports that prior exposure of the organisms to hydrophobic compounds increases the number of potential surfactant producers. Most of the work in the literature has been confined to isolation of surfactant producing microorganisms by enrichment technique.

For the isolation of surfactant producing microbes, enrichment cultures utilizing hydrophobic compounds as the sole carbon source were used (Schulz et al. 1991; Mercade et al. 1996; Willumsen and Karlson 1997; Giani et al.1997; Rahman et al. 2002; Bento et al. 2005). It is known that most of organisms take up insoluble hydrocarbons by producing surfactants that promote substrate emulsification and/or solubilization and/or enhance cell hydrophobicity thus allowing the cells to get into direct contact with the oil phase (Rosenberg and Ron 1999). This is an indirect screening method as the growth of microorganisms on hydrophobic compounds indicates the production of surfactants.

Kumar et al. (2007) collected soil samples from hydrocarbon and asphalt contaminated regions of Guanoco Lake, Sucre State, Venezuela. 5g of the soil sample was suspended into 100 ml of basal salt medium containing 2% (v/v) crude oil. The samples were subjected to enrichment five times so as to select strains that could produce surfactant. A candidate surfactant producer, identified as *Bacillus sp.* strain DHT, could degrade crude oil and produce surface active agent as indicated by high emulsification activity (71 ± 4 %).

Deka and Das (2009) collected 4 oil contaminated soil samples from Gauhati Refinery, Assam, India, each sample having a varied level of oil contamination. 1g of the soil sample was added to 100 ml of defined medium with 2% (v/v) liquid light paraffin. By enrichment culture, altogether 17 isolates were isolated. Among the 17 isolates, 2 isolates could reduce the surface tension of culture medium from 69 mN/m to around 32 mN/m. The enrichment culture particularly makes the selection of hydrocarbon (liquid light paraffin) degrading bacteria which was focused here for the production of surfactant.

Dubey et al. (2012) isolated 23 different isolates from soil samples contaminated with lube oil and distillery spent wash by using selective enrichment procedure. Soil used for isolation purpose was collected from an area just below the spent wash pumping device of a distillery unit. Among the 23 isolates, 2 bacterial cultures, *Kocuria turfanesis* and *Pseudomonas aeruginosa,* showed decrease in surface tension value from 58 mN/m to 27 mN/m indicating production of the surfactants.

As a conclusion, sampling of hydrocarbon contaminated sites combined with direct isolation or enrichment culture is an approved strategy for discovering new surfactant producing strains. However, as the proportion of positives is only in the range of a few percent, several dozen of isolates have to be tested for every hit.

2.8 SCREENING POTENTIAL SURFACTANT PRODUCING MICROORGANISMS

A variety of new surfactant producing strains is the key issue in overcoming the economic obstacles of the production of surfactants. Considering the demand of surfactant producing microorganisms in various sectors, it is mandatory to screen potential surfactant producing strains from the non-producers. The diverse applications of surfactant necessitate easy, rapid and reliable methods to screen the surfactant-producing microorganisms. Therefore, increased efforts in the discovery of new surfactant producing microbes must be made by applying a broad range of different screening methods. The cultures following enrichment are subjected to the screening tests. The principle aim in screening is the discovery of potential surfactant producing strains. The second aim in screening is to find new surfactant structures with strong surface activity, high emulsion capacity, etc., which are of interest for further investigation.

There are a number of screening approaches that measure directly the surface / interfacial activity of the culture supernatant, which indicate the production of surfactant. The screening tests reported in the literature include screening on selective Cetyl Trimethyl Ammonium Bromide (CTAB)-methylene blue agar medium (Lin et al. 1998), surface and/or interfacial tension measurement (McInerney et al. 1990; Mercade et al. 1996; Haba et al. 2000), drop collapse method (Jain et al. 1991; Bodour and Maier 1998), measuring emulsification index (Van Dyke et al. 1993; Makkar and Cameotra 1998), or measurement of cell surface hydrophobicity (vander Mei et al. 1987; Mozes and Rouxhet 1987; Neu and Poralla 1990; Pruthi and Cameotra 1997). The following methods of screening candidate surfactant producing strains have been explained in the literature:

2.8.1 SCREENING POTENTIAL SURFACTANT PRODUCERS USING THE SELECTIVE CETYL TRIMETHYL AMMONIUM BROMIDE (CTAB)-METHYLENE BLUE AGAR PLATE METHOD

The increased interest in surfactant production and applications warrant the development of fast and effective strain-screening methods for surfactant productivity. For this purpose, Siegmund and Wagner (1991) proposed the use of agar plates containing methylene blue and cetyl trimethyl ammonium bromide (CTAB). It is an excellent technique that has been used generally for detection of glycolipid (anionic) surfactant (Satpute et al. 2010). The microorganisms of interest are grown on the selective CTAB-methylene blue medium containing plates. The plates are incubated for 48-72 hours. If surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cationic surfactant, CTAB and methylene blue. The biosurfactant interacts with CTAB of the CTAB-methylene blue complex, there by releasing methylene blue which is visualized as dark blue halo produced in this medium.

The CTAB-methylene blue agar medium is the preliminary screening method for surfactant production. It has been applied in several screenings (Christova et al. 2004; Tahzibi et al. 2004; Gunther et al. 2005). In a study by Tuleva et al. (2002), among the 14 strains isolated from industrial waste water samples, 5 strains demonstrated the ability to produce surfactant as indicated by halo formation on CTAB-methylene blue agar medium. Plaza et al. (2006) reported that among several isolates screened using CTAB-methylene blue agar plates; *Alcaligenes piechaudii* and *Ralstonia picketti* colonies produced halos, indicating the synthesis of surfactant. Leonardo et al. (2010) screened 31 strains for surfactant production using the selective CTAB-methylene blue agar plates, 16 strains showed halo formation on the medium indicating the production of surfactants. The surfactant production ability of *Pseudoxanthomonas sp.* PNK-04 and *Pseudomonas desmolyticum* NCIM 2112 (Pd 2112) was identified based on the formation of dark blue halos around the colonies in CTAB-methylene blue agar plates (Nayak et al. 2009; Jadhav et al. 2011).

2.8.2 DROP COLLAPSE METHOD

Drop collapse technique has been defined as a qualitative assay to screen surfactant-producing bacteria. This method is simple, sensitive, easy to perform, reproducible, requires little specialized equipment and can be used to screen large number of samples (Bodour and Miller 1998). Moreover, the test volume required in this technique is much smaller (5µl). This assay consists of applying a drop of a culture supernatant to be tested (for surfactant production) over a polystyrene plate containing shallow wells covered with oil. The drop collapse method is based on the ability of surfactants to destabilize liquid droplets on an oily surface and surface tension reduction. In the presence of surfactant, the liquid droplet spreads over the hydrophobic surface due to the reduction of interfacial tension between the liquid droplet and the hydrophobic surface. Otherwise, the droplet remains beaded in the absence of surfactant due to the repellence of water molecules from the hydrophobic surface (Bodour and Maier 1998). The correlation between the drop collapsing with the spreading tension between aqueous and hydrocarbon phases also has been discussed previously by Jain et al. (1991). Drops with higher spreading tension and lower surface tension will collapse on oily surfaces. In contrast, drops with lower spreading tension or higher surface tension do not have the ability to spread on an oily surface.

Various reports on drop collapse method suggest that it is a widely accepted method. Bodour et al. (2003) screened a total of 1,305 isolates by drop collapse method on 10W-40 motor oil (Pennzoil) coated polystyrene plates and of these, 45 isolates demonstrated the ability to produce surfactant. Nasr et al. (2009) isolated 102 culture isolates from oil contaminated soil samples, among which 16 isolates showed positive result for drop collapse method on mineral oil coated plate indicating these strains possessed the ability to produce extracelluar surfactant. Viramontes-Ramos (2010) screened 324 bacterial strains isolated from motor oil and fuel oil contaminated soil samples for surfactant production using drop collapse method. They reported that 17 strains showed positive result for drop collapse method on 10W-40 motor oil (Pennzoil) coated plate. In a study by Thavasi et al. (2011), among 105 strains screened for

surfactant production, 82 strains were positive for drop collapse activity on crude oil coated micro-well plates. Patil et al. (2012) screened a total of 129 bacterial strains for surfactant production, 3 bacterial cultures identified as *Stenotrophomonas koreensis*, *Pseudomonas aeruginosa* and *Rhodococcus sp.* showed positive result for drop collapse method on 10W-40 motor oil coated plates indicating the extracellular surfactant production.

2.8.3 SURFACE TENSION MEASUREMENT

Biosurfactant is defined as the one that can reduce the surface and interfacial tension of aqueous medium. The direct measurement of the surface tension of the culture supernatant is the most straightforward screening method and appropriate for a screening of surfactant producing microbes (Lin 1996). The measurement of surface tension has traditionally been used to detect surfactant production and most of the other methods that measure the surface properties of surfactant use surface tension reduction as the standard (Persson and Molin 1987; Willumsen and Karlson 1997; Makkar and Cameotra 1998). A good surfactant producer is defined as one being able to reduce the surface tension of the growth medium to 40 mN/m compared with distilled water (Willumsen and Karlson 1997).

Efficiency of biosurfactant is measured by the surfactant concentration required to produce a significant reduction in the surface tension of water, whereas effectiveness is measured by the minimum value, to which the surface tension can be reduced (Kim et al. 2000). Surface-tension of the culture-free broth was measured by methods like symmetric drop shape analysis (Rotenberg 1983), Du Nouy ring method (Schulz et al. 1991; Mercade et al. 1996; Rahman et al. 2002), stalagmometric method (Plaza et al. 2006), etc. The widely used method for measuring the surface tension of the cell-free supernatant is

the Du Nouy Ring method (Willumsen and Karlson 1997; Bodour and Miller-Maier 1998; Samadi et al. 2007) which is based on the measurement of the force required to detach a loop or ring of wire from the surface or interface (Tadros 2005). This method of surface tension measurement has proved to be accurate and easier to use.

Nasr et al. (2009) isolated 102 culture isolates from oil contaminated soil samples, among which 13 isolates showed reduction in surface tension values of the cell-free broth indicating that these strains possessed the ability to produce extracelluar surfactant. The surface tension values of the cell-free broth varied from surface tension varied from 23.3-57.6 mN/m. The organism that showed surface tension value of 23.3 mN/m was identified as a potential surfactant producer and it was characterized as *Bacillus subtilis*. Viramontes-Ramos (2010) screened 324 bacterial strains isolated from motor oil and fuel oil contaminated soil samples for surfactant production. They screened the bacterial strains by measuring the surface tension and reported that 17 strains decreased surface tension of the cell-free broth from 56.6 mN/m to 26.7 mN/m, which further confirmed the results of the drop-collapse technique.

Surface tension measurement of cell-free culture broth revealed that out of 105 strains screened for surfactant production by Thavasi et al. (2011), 79 strains showed reduction in surface tension. Highest surface tension reduction was observed with 5 strains, namely, *Bacillus megaterium* (30.8±1.13 mN/m), *Bacillus subtilis* (38.75±0.3 mN/m), *Corynebacterium kutscheri* (36.9±0.77 mN/m), *Corynebacterium* xerosis (37.8±0.42 mN/m), *Lactobacillus delbrueckii* (32.5±0.70 mN/m), *Pseudomonas aeruginosa* (28.7±0.98 mN/m) and *Pseudomonas fluorescens* (34.7±0.35 mN/m). They further reported that there was a direct correlation between drop collapse method and surface tension measurement. In a study by Jazeh et al. (2012), a total of 160 strains were isolated from the oil contaminated soil samples and they were further screened for their potential to produce surfactant. Among these, 18 strains showed decrease in surface tension in the range of 29-56 mN/m.

2.8.4 OIL SPREADING ASSAY

The oil spreading assay was developed by Morikawa et al. (2000). For this assay, 10 µl of crude oil is added to the surface of 40 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant is gently placed on the centre of the oil layer. If surfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity. For pure biosurfactant, a linear correlation between quantity of surfactant and clearing zone diameter can be given. The oil spreading method is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample (Plaza et al. 2006). It can be applied when the activity and quantity of biosurfactant is low. Youssef et al. (2004) and Plaza et al. (2006) demonstrated that the oil spreading technique was a reliable method to detect surfactant production by diverse microorganisms.

Among 102 strains screened for surfactant production using oil displacement test by Nasr et al. (2009), 32 strains tested positive for oil displacement test. In a similar study conducted by Thavasi et al. (2011), among the 105 strains screened for surfactant production by using oil displacement method, 82 strains showed positive result for oil displacement method. Techaoei et al. (2011) reported that among 197 strains screened by oil displacement method for surfactant production, 25 strains demonstrated the ability to displace oil suggesting the production of biosurfactant. Further, it was observed that there was corroboration of oil displacement results with drop collapse method results.

2.8.5 HEMOLYSIS TEST

Biosurfactants can cause lysis of erythrocytes. This technique was first discovered by Bernheimer and Avigad (1970) who reported that the production of surfactant (surfactin) by *Bacillus subtilis* caused the lysis of red blood cells on the blood agar medium. Positive strains would cause lysis of the blood cells and exhibit a colourless,

transparent ring around the colonies. Hemolysis can also be shown with purified biosurfactant. Many researchers have used this technique to screen for surfactant production by new isolates (Carrillo et al. 1996; Yonebayashi et al. 2000; Patil et al. 2012).

Blood agar is a rich growth medium for many microorganisms. But the method has some limitations (Schulz et al. 1991; Plaza et al. 2006). First, the method is not specific, as lytic enzymes can also lead to clearing zones. Second, hydrophobic substrates cannot be included as sole carbon source in this assay. Third, diffusion restriction of the surfactant can inhibit the formation of clearing zones. Mulligan et al. (1984) recommended the blood agar method as a preliminary screening method which should be supported by other techniques based on surface activity measurements. In addition, Schulz et al. (1991) showed that some biosurfactants do not show any hemolytic activity. Youssef et al. (2004) also confirmed the poor specificity of this method. They reported that this test gave a lot of false negative and false positive results.

Takeyama et al. (2002) reported that among the strains screened for surfactant production by hemolysis test, 58 strains formed clear zones around the colony on blood agar plates, indicating the production of surfactant. In a study by Nasr et al. (2009), among the 102 pure isolates screened for surfactant production by hemolysis test, 16 isolates were positive for hemolysis. However, other screening tests such as drop collapse assay, oil displacement test and surface tension measurement were performed to confirm the results obtained after hemolysis test. They observed that there was no correlation between the hemolysis test and the other complimentary tests in some of the cases. Thavasi et al. (2011) screened 105 bacterial strains for surfactant production by hemolysis test, 101 strains were positive for hemolysis as indicated by clear zone around the colony. However, they used other screening tests such as drop collapse assay, surface tension measurement and oil spreading assay to confirm the production of surfactant by the cultures.

2.8.6 EMULSIFICATION INDEX (EI) OR EMULSIFICATION CAPACITY ASSAY

This technique was developed by Cooper and Goldenberg (1987). The emulsification index is measured by mixing supernatant containing biosurfactant or purified biosurfactant with equal volume of hydrocarbon. The mixture is vortexed for 2 minutes at high speed. After 24 hours, the height of the stable emulsion is measured. It is measured in terms of the percentage of emulsification index. EI is calculated as the ratio of height of the emulsion layer to the total height of the liquid (Cooper and Goldenberg 1987).

$$\text{Emulsification Index (EI)} = \frac{\text{Height of emulsion layer}}{\text{Total height of the liquid}} \times 100$$

The EI can be also calculated at regular time intervals in order to test the stability of the emulsion formed. The emulsion formed by the surfactant produced by *Acinetobacter venetianus* RAG-1 was stable for several months whereas that formed by *Rhodococcus erythropolis* 20S was less stable as indicated by decrease by 30% from initial volume (Dorobantu et al. 2004). The more stable the emulsion formed, more efficiency is shown by the biosurfactant. This screening technique has been used by many researchers in the literature (Willumsen and Karlson 1997; Bento et al. 2005; Chen et al. 2007). Different hydrocarbons have been tested for the emulsion formation by various researchers. In the literature, benzene, xylene, n-pentane, n-nonane, gasoline, diesel oil, kerosene, hexane, octane, heptadecane, dodecane, paraffin, olive oil, sunflower oil, corn oil, crude oil were used for emulsion forming capacity by various researchers (Sifour et al. 2007; Liu et al. 2010; Li et al. 2011). The emulsification activity of the surfactant produced by *Pseudomonas aeruginosa* RB 28 was studied by Sifour et al. (2007). The various hydrocarbons tested were kerosene, hexane, octane, heptadecane, dodecane, paraffin, olive oil, sunflower oil, corn oil, gasoline and crude oil. Among the various hydrocarbons tested, sunflower oil, heptadecane and paraffin were efficiently emulsified

as indicated by EI values which ranged from 70-90%. Least EI was observed with hexane (13%).

In a study by Li et al. (2011), the different oils evaluated for the determination of EI were palm oil, soybean oil, peanut oil, diesel, gasoline, toluene and liquid paraffin. The surfactant produced by *Pseudomonas aeruginosa* GIM 32 showed highest EI of 71% and 73% with peanut oil and palm oil, respectively. They suggested that it could be having a potential application as a cleaning and emulsifying agent in the food industry. Moreover, the emulsification indexes for diesel (68%) and gasoline (64%) suggested that this surfactant had the potential to be applied in bioremediation and oil recovery operations.

Many researchers have found that surface activity and emulsification activity do not always correlate (Van Dyke et al. 1993; Willumsen and Karlson 1997; Plaza et al. 2006). Hence, this method just gives an indication on the presence of biosurfactant produced by a microorganism.

The development of rapid and reliable methods for screening and selection of microbes from thousands of potentially active organisms and the subsequent evaluation of surface activity holds the key to the discovery of new surfactant producing strains. According to Chen et al. (2007), a screening method for the selection of surfactant producing microbes must fulfill three requirements:

• The ability to identify potential organisms

• The ability to assess quantitatively the effectiveness of the surfactant

• The ability to screen many candidates quickly.

2.9 SURFACTANT PRODUCING MICROORGANISMS

The surfactant-producing microbes are distributed among a wide variety of genera. They are produced by a wide variety of microorganisms, which include bacteria, yeasts and filamentous fungi (Mulligan 2005; Chen et al. 2007; Abouseoud et al. 2008). A variety of microbial species producing microorganisms has been reported in the literature which is presented in section 2.9.1 and 2.9.2, respectively.

2.9.1 SURFACTANT PRODUCING BACTERIA

Increasing environmental awareness has led to the discovery of candidate bacterial strains with pronounced surfactant production capacity. By evolution, bacteria have adapted themselves to feeding on water-immiscible materials by synthesizing and producing surfactant that helps the bacteria, which are in the aqueous phase to adsorb, emulsify, wet, and disperse or solubilize the water-immiscible material (Healy et al. 1996).

Bushnell and Hass (1941) were among the first to demonstrate bacterial production of surfactant by *Corynebacterium simplex* and *Pseudomonas sp.* in a mineral media containing either kerosene, mineral oil or paraffin. Since then, a large number of surfactant-producing microorganisms have been isolated and studied in greater detail, culminating in elucidation of the structure and mechanisms involved in their production and action (Banat 1995; Lin 1996; Richter et al. 1998; Tuleva et al. 2002).

Tabatabaee et al. (2005) isolated 45 Gram positive and Gram negative bacterial isolates from crude oil samples collected from oil reservoirs in Iran. Among them, 8 isolates showed the ability to lower surface tension to values below 40 mN/m. Among isolated strains, one of the strains, identified as *Bacillus sp.*, showed high salt tolerance and production of surfactant in a wide range pH and temperature. They concluded that the strain could be a potential candidate for microbial enhanced oil recovery operations.

Pseudomonas fluorescens HW-6 was isolated from lubricating oil polluted water by Vasileva-Tonkova et al. (2006). The strain produced surfactant during growth on hexadecane as sole source of carbon and energy. It decreased the surface tension of water from 72 mN/m to 32 mN/m. The organism efficiently emulsified aromatic hydrocarbons, kerosene, n-paraffin and mineral oil. They reported that *Pseudomonas fluorescens* HW-6 produced glycolipid with effective surface and emulsifying properties which represented a promising potential for application in bioremediation of polluted wastewaters containing hydrocarbons.

Haddad et al. (2008) isolated biosurfactant-producing bacteria from the water of an oil field. Isolates were screened for biosurfactant production based on reduction of surface tension. The highest reduction of surface tension was achieved with a bacterial strain *Brevibacilis brevis* HOB1. They observed that the strain was able to grow and reduce the surface tension of the broth to 29 mN/m in commercial sugar and maltose containing media.

Kebbouche-Gana et al. (2009) isolated 5 halobacterial isolates from hypersaline ponds in Ain Salah, Algeria. The strains reduced surface tension below 40 mN/m, and two of them exhibited high emulsion stabilizing capacity. The two halobacterial strains with enhanced surfactant producing capacity were identified as *Halovivax sp*. and *Haloarcula sp.* These strains also produced surfactant above 15% (w/v) salt concentration.

In a study by Anyanwu and Chukwudi (2010), *Pseudomonas aeruginosa* LS-1 was isolated from petroleum contaminated soil of Nsukka, South East Nigeria. The organism was able to produce extracellular surfactant in different carbon substrates such as glucose, fructose, glycerol, n-hexadecane and paraffin oil, containing medium. During the growth of the organism on different substrates, it reduced the surface tension of the cell free broth in the range 34.5-56.4 dynes/cm. The surfactant exhibited a high level of thermal , pH stability and salinity tolerance. These

observations show perspectives for the use of the products in extreme environmental conditions in bioremediation, pharmaceutical formulations.

Patil et al. (2012) collected soil samples contaminated with petroleum and its products from eastern Maharashtra region at Jawaharlal Nehru Port Trust, Navi Mumbai and oil contaminated sites in north Maharashtra region. For enrichment of surfactant producers, 5% (v/v) of diesel, petrol and dodecane were added in mineral salts medium. *Stenotrophomonas koreensis, Pseudomonas aeruginosa* and *Rhodococcus sp.* were identified as surfactant producers based on reduction in surface tension. Among the 3 isolates, *Stenotrophomonas koreensis* reduced the surface tension of the broth from 62.4 mN/m to 27.8 mN/m.

2.9.2. SURFACTANT PRODUCING YEASTS AND FUNGI

The study of surfactant production by yeasts and fungi has been growing in importance, with production being reported mainly by the genus *Candida sp*., *Pseudozyma sp., Yarrowia sp., Aspergilus sp., Ustilago sp.,* etc. Though there are many reports on surfactant production by yeasts, reports on surfactant production by fungi is meager.

Zinjarde and Pant (2002) isolated a tropical marine strain of *Yarrowia lipolytica,* NCIM 3589 that produced an emulsifier in the presence of alkanes or crude oil. In the stationary phase, the organism produced the emulsifier extracellularly under carbon excess and nitrogen limitation conditions. Other requirements for extracellular emulsifier production included an initial pH of 8.0 and the presence of sodium chloride at a concentration of 3% (w/v).

Ilori et al. (2008) isolated yeast strains, *Saccharomyces cerevisiae* and *Candida albicans,* respectively, from polluted lagoon water. Both strains were able to grow effectively on crude oil and diesel as sole sources of carbon and energy. Although *Candida albicans* appeared to be a better diesel-utilizer and surfactant-producer, the

potency of its surfactant was smaller than that of *Saccharomyces cerevisiae*. The partially-purified surfactant exhibited antimicrobial activities by completely inhibiting the growth of clinical strains of *Escherichia coli* and *Staphylococcus aureus*, respectively.

Morita et al. (2008) isolated basidiomycetous yeast, *Pseudozyma graminicola* CBS 10092 which was found to accumulate a large amount of glycolipids in the culture medium when grown on soybean oil as sole source of carbon. The glycolipids produced by this strain were identified as mannosylerythritol lipids which showed antitumour and cell differentiation activities with respect to human leukemia.

A smut fungus *Ustilago scitaminea* NBRC 32730 was isolated by Morita et al. (2009) and they found that the fungus accumulated a large amount of glycolipid biosurfactant in the culture medium. The glycolipids were sufficiently produced in the presence of variety of sugars such as sucrose, glucose, fructose, mannose, methyl oleate and olive oil. However, maximum surfactant (12.8 g/L) was produced in sucrose containing medium.

Kiran et al. (2009) isolated a surfactant producing fungal strain, *Aspergillus ustus* from the marine sponge *Fasciospongia cavernosa* collected from the Bay of Bengal region of the Indian peninsular coast. The organism produced maximum surfactant in glucose containing medium as indicated by emulsification activity (E24) of 35%. The surfactant produced by the strain showed the ability to recover hydrocarbons from contaminated sand.

The yeast species, *Rhodotorula muciliginosa and Candida rugosa,* respectively, isolated from hydrocarbon contaminated sites were capable of producing surfactants in the presence of 2% (v/v) diesel as sole source of carbon and energy (Chandran and Das 2011). The surfactant produced by *Rhodotorula muciliginosa and Candida rugosa,* respectively, reduced the surface tension of the broth to 33 mN/m and 34 mN/m, respectively. In addition, the surfactants produced by these strains could effectively

emulsify and stabilize emulsions with diesel (86±0.7 and 78±0.7%) as well as various types of hydrocarbon substrates.

Due to the increasing demand of microbial surfactants, there is a need to isolate efficient surfactant producers. It can be concluded that the search for potential surfactant producing microorganisms still continues though there are numerous studies reported by various authors.

2.10 FERMENTATIVE PRODUCTION OF BIOSURFACTANTS

There are basically two methods of surfactant production. Most of the reports in the literature report the production of surfactant production by submerged fermentation processes (Mulligan and Gibbs 1993). The other method rarely used is solid state fermentation; this method is not widely used because of problems during downstream processing.

Various reports on biosurfactant production suggest that both submerged fermentation (Duvnjak et. al. 1982; Yakimov et. al. 1995; Dubey and Juwarkar 2004; Hewald et. al. 2005; Kumar et al. 2008; Obayori et al. 2009; Alcantara et al. 2012) and solid state fermentation (Neto et al. 2008; Neto et al. 2009; Kiran et al. 2010; Colla et al. 2010) methods have been used to produce surfactant by various microorganisms. However, reports on surfactant production by solid substrate fermentation are meager because of the following disadvantages (Mienda et al. 2011):

• Ease of recovery of biosurfactant

• Microorganisms like bacteria which may require high moisture levels can perform poorly in solid state fermentation.

• Difficulties are usually encountered in biomass determination.

• Monitoring of process parameter such as pH, moisture content, substrate, oxygen and biomass concentration becomes a problem because of solid nature of the substrate.

• Aeration may be difficult sometimes due to high solid concentrations.

Hence, most of the reports in the literature are focused on surfactant production by submerged fermentation since they offer the following advantages:

• Ease of recovery of biosurfactant

• Harvesting of the cells from the culture broth is easier; hence biomass determination is also easier.

• Various parameters such as oxygen, pH, moisture content, substrate, etc can be monitored and controlled easily.

2.10.1 PRODUCTION OF SURFACTANT BY SUBMERGED FERMENTATION

Most of the researchers have reported surfactant production by submerged fermentation using various microorganisms.

In a study by Cooper et al. (1981), surfactant production by *Bacillus subtilis* was carried out under submerged condition. They reported that the bacterial strain produced a maximum of 0.l g/L of surfactant in nutrient broth whereas in the minimal medium containing glucose, the surfactant quantity obtained was 0.8 g/L. They further found that when hexadecane was substituted in the medium, the surfactant production was inhibited though there was biomass synthesis.

Patel and Desai (1997) reported the production of surfactant in molasses containing medium using submerged fermentation by *Pseudomonas aeruginosa* GS3. They reported a maximum of 0.24 g/L of surfactant quantity was produced by the

bacterial strain. The surfactant produced by the strain reduced the interfacial tension against crude oil from 21 mN/m to 0.47 mN/m.

During surfactant production by *Pseudomonas aeruginosa* strain BS2 isolated from oily sludge, Dubey and Juwarkar (2001) reported that the isolate utilized distillery and whey waste for its growth and surfactant production. The surfactant yield obtained in distillery and whey waste containing medium were 0.91 and 0.92 g/L, respectively. The surfactant possessed potent surface active property, as it effectively reduced the surface tension of water from 72 mN/m to 27 mN/m.

In a study by Vasileva-Tonkova (2006), surfactant production was carried using submerged fermentation by *Pseudomonas fluorescens* strain HW-6 that was isolated from industrial wastewater. The strain produced glycolipid biosurfactant at high concentrations (1.4-2.0 g/L) when grown on hexadecane as a sole carbon source. The surfactant decreased the surface tension of water to 35 mN/m.

George and Jayachandran (2009) carried out production of surfactant by *Pseudomonas aeruginosa* MTCC 2297 by submerged fermentation using various cost-effective waste materials such as orange peelings, carrot peel waste, lime peelings, coconut oil cake and banana waste. The addition of orange peel to the medium resulted in maximum amount of surfactant production (9.18 g/L) which reduced the surface tension of water to 31.3 mN/m.

2.10.2 PRODUCTION OF SURFACTANT BY SOLID STATE FERMENTATION

Most current research into the production of surfactants involves submerged fermentation conditions. However, this method for surfactant production creates serious problems with foam formation, especially when aerated bioreactors are used (Veenadig et al. 2000; Lee and Kim 2004; Yeh et al. 2006). The use of solid-state fermentation, in which the microorganism is cultivated on moist particles of solid substrate, can avoid

these problems with foaming (Neto et al. 2008). Foaming reduces the productivity of the process and increases the risks of contamination. Although the foam can be combated by the addition of anti-foaming agents or by mechanical means, these strategies are not highly effective and they increase the costs of bioreactor operation or downstream processing. Hence, few researchers have used solid state fermentation for the production of surfactants.

Neto et al. (2008) reported surfactant production by *Pseudomonas aeruginosa* UFPEDA 614. The organism was grown on a solid medium impregnated with a solution containing glycerol. On the basis of the volume of impregnating solution added to the solid support, the surfactant quantity obtained was of the order of 46 g/L. They demonstrated that this method has good potential for the production of surfactant on the basis of the volume of liquid added to the system; surfactant production was as high as that obtained during submerged fermentation. They obtained surfactant with excellent surfactant properties as indicated by emulsification indices (E24) of over 90% with kerosene, gasoline and diesel after 24 hours.

Neto et al. (2009) studied surfactant production by *Pseudomonas aeruginosa* UFPEDA 614 by solid state fermentation using sugarcane bagasse impregnated with a solution containing glycerol. Surfactant levels reached 40 g of surfactant/ kg of dry initial substrate after 12 days. On the basis of the volume of liquid used, the surfactant level was similar to that obtained during submerged fermentation. The properties of the surfactant were similar to that obtained in submerged condition with an emulsification index of over 90% against gasoline at 24 hours.

Kiran et al. (2010) reported surfactant production by marine *Brevibacterium aureum* MSF13 by solid state fermentation using industrial and agro-industrial solid waste residues as substrates. To develop the solid state culture, 5 g of the dried substrate was transferred to 250 ml Erlenmeyer flasks and mixed with 5 ml of moistening media

(salt solution) consisting of NH_4NO_3 0.5%, NaCl 0.9%, $MgSO_4.7H_2O$ 0.1% (pH 7.0). The production of surfactant under solid state cultivation by the culture isolate was maximum as indicated by emulsification index (E24) value of 86% with pre-treated molasses as substrate and glucose, acrylamide, $FeCl_3$ and inoculums size as critical control factors. The surfactant produced by the culture showed a broad spectrum of activity against the pathogenic microbes such as *Escherichia coli, Klebsiella pneumonia, Micrococcus luteus, Staphylococcus epidermidis, Enteribacter faecalis, Proteus mirabilis*, etc.

Surfactant was produced by *Bacillus subtilis* SPB1 using solid state fermentation (Ghribi et al. 2012). Solid-state fermentation was conducted using 15 g of the substrate, 75 µL of KH_2PO_4 (1 M), 225 µL of $MgSO_4$ (1 M) and 367 µL of deionised distilled water were aseptically added for the fortification of nutrients. Among the various substrates screened, maximum surfactant (0.012 g/g of the substrate) was produced when millet was used as the substrate. Further, it was reported that the surfactant exhibited antimicrobial activities against *Staphylococcus aureus*, *Staphylococcus xylosus*, *Enterococcus faecalis*, *Klebsiella pneumonia*, *Enterobacter cloacae, Penicillium notatum, Penicillium italicum*, etc.

Based on the various reports in the literature, it can be concluded that both submerged as well as solid state fermentation conditions have been used for the production of surfactants by different microorganisms. Production of surfactant is the most preferred over solid state fermentation condition for the surfactant production due to the numerous advantages over the latter.

2.11 PRODUCTION MEDIA FOR SURFACTANT PRODUCTION

The surfactant media components are reported to affect the process of surfactant production and the final quantity as well as the quality of surfactant produced by the microorganisms (Mukherjee et al. 2006). Biosurfactant production media consisting of

varied components have been reported in the literature in order to obtain maximum quantity.

Arima et al. (1968) reported the production of surfactant by *Bacillus subtilis* IAM 1213 in nutrient broth. A maximum of 0.1 g/L of surfactant was produced by the bacterial strain during its growth in the nutrient broth. Robert et al. (1989) reported surfactant production by *Pseudomonas aeruginosa* 44T1 in a medium consisting of the following medium components (g/L): 4g $NaNO_3$, 0.1 g KCl, 0.1 g KH_2PO_4, 1.0 g K_2HPO_4, 0.01 g $CaCl_2$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.1 g yeast extract, 1 ml of trace element solution and 2% carbon source. They screened various carbon sources for surfactant production that included glucose, fructose, sodium acetate, sodium succinate, sodium pyruvate, sodium citrate, mannitol, glycerol and olive oil. Maximum surfactant quantity (7.65 g/L) was obtained when olive oil as carbon source.

Peptone Glucose Ammonium Salt (PPGAS) medium consisting of 0.02 M NH_4Cl, 0.02 M KCl, 0.12 M Tris-HCl, 0.0016 M $MgSO_4$, 1% Proteose Peptone, 0.5% glucose was used was used as production medium for *Pseudomonas aeruginosa* ATCC 9027 (Zhang and Miller 1992). They observed that the surfactant produced by the bacterium reduced the surface tension from 72 mN/m to 29 mN/m.

Both Kay's minimal medium consisting of 0.3% $NH_4H_2PO_4$, 0.2% K_2HPO_4, 0.2% glucose, 0.005 g $FeSO_4$, 0.1% $MgSO_4$ in g/L and a mineral salts medium consisting of 0.7 g KH_2PO_4, 0.9 g Na_2HPO_4, 2 g $NaNO_3$, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 2 ml of trace element solution/L, 2 g $FeSO_4 \cdot 7H_2O$, 1.5 g $MnSO_4 \cdot H_2O$, 0.6 g $[(NH_4)_6Mo_7O_{24} \cdot 4H_2O]$ in g/L were used for surfactant production by *Pseudomonas chlororaphis* strain NRRL B-30761 by Gunther et al. (2005). They reported that maximum surfactant production was observed in mineral salts medium as indicated by reduction in surface tension (from 65 mN/m to 25 mN/m).

Three different surfactant production media were screened by Oliveira et al. (2009). They were coded as MM#1, MM#2 and MM#3, presenting the following compositions in g/L of distilled water: (a) MM#1: 0.5 Na_2HPO_4, 4.5 KH_2PO_4, 2.0 NH_4Cl, 0.1 $MgSO_47H_2O$; (b) MM#2: 1.0 $(NH_4)_3PO_4$; (c) MM#3: 3.0 inorganic commercial fertilizer N:P:K (10% N, 10% P_2O_5, 10% K_2O) and 0.5% (v/v) palm oil. Among these production media, *Pseudomonas alkaligenes* showed maximum growth and surfactant production in MM#3; the surface tension of the cell-free broth was reduced to 38 mN/m. In the MM#1 and MM#2, the surface tension of the cell-free broth was 50 mN/m.

Kiran et al. (2009) screened four different media compositions for surfactant production by *Aspergillus ustus* MSF3. The four different production medium screened were Sabouraud dextrose broth (peptone-10.0 g, dextrose-40.0 g, agar-15.0 g, distilled water-1000 ml), potato dextrose broth (PDB) (potato peeled-200.0 g, dextrose-20.0 g, distilled water-1000 ml), Zobell Marine broth 2216 (ZMB 2216) (glucose-10.0 g, peptone-5.0 g, yeast extract-1.0 g, ferric citrate-0.1 g, sodium sulphate-3.24 g, calcium chloride-1.50 g, Na_2HCO_3-0.16 g, H_3BO3-0.22 g, $SnCl_2$-0.034 g, sodium silicate-0.004 g, sodium fluorate-0.0024 g, $NaNO_3$-0.0016 g, Na_2PO_4-0.008 g, NaCl-19.45 g, $MgCl_2$-8.8 g, KCl-0.55 g, KBr-0.08 g, pH 7.6 ±0.2, distilled water-1000 ml) and MSM ($NaNO_3$-15 g, KCl-1.1 g, $FeSO_4 \cdot 7H_2O$-0.00028 g, KH_2PO_4-3.4 g, K_2HPO_4-4.4 g, $MgSO_4 \cdot 7H_2O$-0.5 g, yeast extract-0.5 g). Trace elements-$ZnSO_4 \cdot 7H_2O$-0.29 g, $CaCl_2 \cdot 4H_2O$-0.24 g, $CuSO_4 \cdot 5H_2O$-0.25 g, $MnSO_4 \cdot H_2O$-0.17 g/100 ml) medium in order to obtain maximum surfactant production. Among the different production media evaluated, maximum surfactant was observed in Sabouraud dextrose broth as indicated by emulsification index (E24) value (45%).

Surfactant production by the yeast, *Trichosporon asahii*, was carried out in Bushnell Hass medium containing 50 ml mineral salt medium (Bushnell-Haas medium containing g/L of KH_2PO_4- 1.0 g, K_2HPO_4 – 1.0 g, NH_4NO_3–0.05g, $MgSO_4.7H_2O$–0.2 g

and 0.5% (v/v) diesel oil (Chandran and Das 2010). A maximum of 1.2 g/L of surfactant quantity was produced by the yeast.

Patil et al. (2012) used mineral salt medium for surfactant production by *Stenotrophomonas koreensis*. The medium consisted of the following components (g/L): glucose: 10.0 g $(NH_4)_2SO_4$, 1.0 g Na_2HPO_4, 4.0 g yeast extract, 5.0 g KH_2PO_4, 3.0 g NaCl, 2.7 g $MgSO_4$, 0.6 g and 5 ml/L of trace element solution containing 5.0 mg $FeSO_4.7H_2O$, 3.34 mg $ZnSO_4$, 1.56 mg $MnSO_4.7H_2O$ and 2.0 mg $CoCl_2.2H_2O$. During the growth of the bacterium, surfactant was produced extracellularly in the medium which reduced the surface tension of the broth from 62.4 mN/m to 27.8 mN/m.

These reports suggest that the selection of appropriate production medium composition is a necessity in order to obtain high concentrations of surfactant.

2.12 EFFECT OF PROCESS PARAMETERS ON SURFACTANT PRODUCTION

An efficient and economical bioprocess is the foundation for every profit-making biotechnology industry. Hence, bioprocess development is the primary step towards commercialization of all biotechnological products, including biosurfactants. Any attempt to increase the yield of a biosurfactant demands optimal addition of media components and selection of the optimal culture conditions that will induce the maximum or optimum productivity. The classical method of medium optimization involves changing one variable at a time, while keeping the others at fixed levels (Mukherjee et al. 2006). In general, it was necessary to optimize the conditions for surfactant production because the quality and the quantity of biosurfactants were strongly dependant on factors, such as fermenter design, pH, temperature, nutrient composition, production media and types of substrate used (Mulligan and Gibbs 1993).

Biosurfactant production like any other chemical reaction is effected by a number of factors that either increase its productivity or inhibit it. Environmental factors and growth conditions such as intial medium pH, incubation temperature, agitation and

oxygen availability affect biosurfactant production through their effects on cellular growth or activity (Mahdy et al. 2012). Cell growth and the accumulation of metabolic products were also strongly influenced by medium composition such as carbon sources, nitrogen sources, salinity and other factors, thus the optimization of organisms can give results in the high yield of biosurfactants (Sen et al. 1997). In this regard, various researchers have emphasized the need to study the effect of process parameters such as type of carbon and nitrogen sources, concentration of carbon and nitrogen sources, inducers, trace elements, buffer, etc on biosurfactant production (Cooper et al. 1981, Makkar and Cameotra 1997; Desai and Banat 1997; Khopade et al. 2012).

The following factors affecting biosurfactant production have been reported in the literature:

2.12.1 EFFECT OF INTIAL pH OF THE MEDIUM ON SURFACTANT PRODUCTION

The effect of pH can be apportioned to its effects on substrate as well as its direct influences on the growth of microorganism as well as surfactant production. On increasing pH, the whole cell protein was improved; making the substrate more available to be utilized (Cooper and Goldenberg 1987; Makkar and Cameotra 1997).

Control of initial pH of the medium was necessary in most of the reports in the literature for increased biosurfactant production. Makkar and Cameotra (1997) varied the initial pH of the medium from 4.5 to 10.5 in order to study the effect of pH on surfactant by *Bacillus subtilis* strain. They reported that maximum surfactant production occurred at pH 7, a maximum of 0.84 g/L of surfactant was produced. In the acidic pH range, *i.e.*, 4.5-6.5, the concentration of surfactant produced was in the range of 0.108-0.604 g/L.

The concentration of biosurfactant produced in the alkaline pH (8.5-10.5) ranged from 0.604-0.648 g/L.

The influence of medium pH on surfactant production by the marine bacterial strain, *Azotobacter chroococcum*, was studied by Thavasi et al. (2009). The pH was varied from 5-9. At pH 5, 6 and 7, the quantity of surfactant produced by the isolate was 0.7 g/L, 0.8 g/L and 0.9 g/L, respectively. However, maximum quantity of surfactant was synthesized at pH 8, a maximum of 1.2 g/L of biosurfactant concentration was recorded. There was decrease in surfactant quantity (1 g/L) at pH 9.

Kiran et al. (2009) studied the effect of medium pH on surfactant production by *Aspergillus ustus* MSF3. The pH range considered in the study was 4 to 9. The strain MSF3 produced the highest yield of surfactant as indicated by emulsification activity (E24=15%) at pH 7. The emulsification activity (E24) in the acidic pH range (4-6) ranged from 0-10% whereas in the alkaline pH range (8-9), the emulsification activity dropped to 0% suggesting that both acidic and alkaline pH affected the surfactant production.

The pH of the surfactant production medium of *Pseudomonas aeruginosa* OCD1 was varied from 4.5-8 by Sahoo et al. (2011). The surface tension of the cell-free broth varied from 34 mN/m to 47 mN/m in the pH range 4.5-5.5. Maximum surfactant production as indicated by surface tension reduction (33 mN/m) was reported at pH 6. Further increase in pH resulted in increase in surface tension values. The surface tension value varied from 34-37 mN/m in the pH range 6.5-8.

Praveesh et al. (2011) studied the influence of medium pH on surfactant synthesis by *Pseudomonas sp.*, the pH of the production medium was varied from 6 to 10. The organism synthesized 6.8 g/L of surfactant at pH 6. At pH 7, maximum surfactant quantity (8.6 g/L) was obtained. The surfactant concentration obtained in the alkaline pH range (8-10) varied from 2.3-6.6 g/L indicating that neutral pH supported surfactant production by the organism.

Khopade et al. (2012) studied the effect of intial medium pH on surfactant production by *Nocardia sp.* B4. The intial pH of medium was adjusted in the range of 4 and 12. The strain showed gradual increase in surfactant production with increase in pH till pH 7. Maximum surfactant was observed at pH 7 as indicated by decrease in surface tension (28 mN/m); with further increase in pH, there was increase in surface tension values. The surface tension value in the acidic pH range (4-6) was in the range 56-64 mN/m whereas in the alkaline pH range (8-12), the surface tension values were in the range of 42-50 mN/m.

2.12.2 EFFECT OF INCUBATION TEMPERATURE ON SURFACTANT PRODUCTION

Incubation temperature is an important parameter that affects the growth of the microorganism as well as production of surfactant (Sahoo et al. 2011). Temperature may cause alteration in the composition of the surfactant (Syldatk et al. 1985). Hence, it is important to control the incubation temperature in order to prevent decrease in the concentration of surfactant. Most of the reports in the literature suggest the maintenance of mesophilic temperature range (30-38ºC) for the production of surfactant by bacterial cultures whereas temperature ranging from 20-25ºC was favourable for surfactant synthesis by fungal and yeast cultures. However, there are few reports on surfactant production at thermophilic temperature.

Makkar and Cameotra (1997) reported surfactant production by thermophilic *Bacillus subtilis* strain. They observed that the strain was able to grow and produce surfactant on 2% (w/v) sucrose at 45ºC. As a result of surfactant synthesis, the surface tension of the medium was reduced from 68 mN/m to 28 mN/m.

The effect of incubation temperature on the cultivation as well as surfactant production by the marine bacterial strain, *Azotobacter chroococcum*, was studied by Thavasi et al. (2009). Temperatures considered in the study were 30ºC, 34ºC, 38ºC, 42ºC and 46ºC. The isolate showed maximum biomass and surfactant production at 38ºC, a maximum of 3.0 g/L of biomass and 1.2 g/L of biosurfactant was produced. At other incubation temperatures considered in the study, the quantity of biomass and surfactant varied from1.8-2.8 g/L and 0.7-0.8 g/L, respectively.

Kiran et al. (2009) emphasized the role of incubation temperature on surfactant production by *Aspergillus ustus* MSF3. The temperature at which the culture flasks were incubated was varied from 10-50ºC. The surfactant production was calculated in terms of the emulsification index. At 10ºC, the emulsification activity (E24) was found to be 0%. Emulsification activity reached the highest (20%) when the strain was grown at 20ºC. When the incubation temperature was increased from 30 to 50ºC, there was decrease in the emulsification activity, *i.e.*, the emulsification activity dropped from 15% to 0%.

The effect of incubation temperature on surfactant production by *Pseudomonas sp.* was studied by Praveesh et al. (2011). Incubation temperature was varied from 20-45°C. 4.8-6.7 g/L of surfactant quantity was obtained when the incubation temperature of the culture flasks ranged from 20-30ºC. However, the maximum production of surfactant occurred at 35ºC, a maximum of 9.2 g/L of surfactant was obtained. The surfactant quantity dropped to 5.8 g/L and 2.2 g/L at 40ºC and 45ºC, respectively.

Incubation temperature was varied from 25-40ºC by Sahoo et al. (2011) during surfactant production by *Pseudomonas aeruginosa* OCD1. At 25ºC, the growth of the organism as indicated by optical density reading at 600 nm was 0.7; the surface tension of the cell-free broth was 38 mN/m. The biomass and surfactant production was maximum at 30ºC, the optical density was 1.6 and surface tension value was 32 mN/m. At 35ºC, there was decrease in biomass (optical density 1.5) and increase in surface tension value

(33 mN/m), which suggested that maintenance of incubation temperature was important during surfactant production. At 45ºC, the biomass and surface tension value were found to be 0.5 and 42 mN/m, respectively.

In order to test the effect of incubation temperature on surfactant production by *Nocardia sp.* B4, the incubation temperature was set to 4, 15, 25, 30, 35 40, 45 and 60ºC (Khopade et al. 2012). Maximum reduction in surface tension (32 mN/m) was observed at 30ºC. The surface tension at 4 and 15ºC was found to be 72 and 70 mN/m. Further, it was observed that increase in temperature caused decrease in surface tension value of the cell-free broth. At 25ºC, the surface tension value was 34mN/m. With further increase in temperature, *i.e.*, from 35-60ºC, the surface tension values ranged from 40-50 mN/m.

2.12.3 EFFECT OF INOCULUM VOLUME/SIZE ON SURFACTANT PRODUCTION

An important characteristic of most microorganisms is their dependence on the inoculum volume for growth and production of surfactants (Sahoo et al. 2011). Adequate density of the inoculum or seed culture was determinant for high concentration of surfactant production (Sen and Swaminathan 2004).

The inoculum size was maintained as 0.0016 g/L, 0.004 g/L and 0.008 g/L by Anna et al. (2001) during the production of surfactant by *Pseudomonas aeruginosa* PA1. 0.004 g/L of inoculum size resulted in maximum production of surfactant. A maximum of 0.90 g/L of surfactant quantity was obtained. The surfactant quantity obtained for 0.0016 g/L and 0.008 g/L were 0.70 g/L and 0.48 g/L, respectively.

Abushady et al. (2005) considered different inoculum sizes from an overnight broth culture of 10^6 colony forming units/ml in volumes from 0.25-3.0 ml in order to study the effect of inoculum volume on surfactant production by *Bacillus subtilis* (BBk1) AB01335. With increase in inoculum size, there was increase in surfactant synthesis till 2

ml inoculum size. The surfactant quantity increased from 1.5 to 1.8 g/L with increase in inoculum size from 0.25-1.5 ml. Maximum concentration of surfactant (2.3 g/L) was obtained at 2 ml inoculum volume. Thereafter, the concentration of surfactant remained constant, *i.e.*, 1.6 g/L at 2.5 ml and 3.0 ml, respectively.

Sahoo et al. (2011) investigated the effect of inoculum volume on surfactant production by *Pseudomonas aeruginosa* OCD1. Inoculum volume ranging from 0.2-10% (v/v) was considered in the study. At 0.2% and 0.5% (v/v) inoculum volume, the surface tension values were 38 mN/m and 36 mN/m, respectively. Maximum reduction in surface tension (32 mN/m) was observed at 1% (v/v) inoculum volume. With increase in inoculum size from 3-10% (v/v), the surface tension varied from 35-39 mN/m.

To study the effect of inoculum volume on surfactant production by *Oleomonas sagaranensis* AT18, Saimmai et al. (2012) varied the inoculum volume from 2 to 12 % (v/v). With increase in inoculum size from 2 to 6% (v/v), growth and surfactant production by the bacterial strain was enhanced, a maximum of 6.0 g/L of biomass and 5.0 g/L of surfactant, respectively, was produced at 6% (v/v) inoculum volume. The biomass and surfactant quantity remained constant at higher inoculum size (8-12% v/v).

2.12.4 EFFECT OF AGITATION ON SURFACTANT PRODUCTION

Agitation rate influences the production of surfactants by facilitating the oxygen transfer from the gas phase to the aqueous phase and it may also be linked to the physiological function of surfactants. It has been suggested that the production of surfactants can enhance the solubilization of water‑insoluble substrates and consequently, facilitate nutrient transport to microorganisms (Amaral et al. 2008). Agitation rates have been shown to affect biosurfactant production in various microorganisms.

Abushady et al. (2005) varied the agitation speed of the culture flasks from 0 to 200 rpm in order to study its effect the surfactant synthesis by *Bacillus subtilis* (BBk1) AB01335. Without the agitation of the culture flask (static condition), the surfactant quantity synthesized by the culture isolate was 0.5 g/L. With increase in agitation speed, the surfactant quantity produced by the isolate also increased, the surfactant synthesized ranged from 0.52-1.4 g/L. Maximum surfactant production (1.42 g/L) occurred at 200 rpm.

The effect of agitation speed during the production of surfactant by *Nocardia amarae* was reported by Moussa et al. (2006). The agitation speed was varied from 0-250 rpm. 0.6 g/L of surfactant was synthesized in static condition whereas agitation of the culture flasks improved the concentration of surfactant. At 100 rpm, the concentration of surfactant obtained was 0.7 g/L. The surfactant quantity increased to as high as 4.3 g/L at 150 rpm. There was decrease in surfactant concentration at 200 rpm and 250 rpm, the corresponding amount of surfactant recorded were 2.5 g/L and 0.4 g/L, respectively.

Sahoo et al. (2011) emphasized the influence of agitation speed on surfactant production by *Pseudomonas aeruginosa* OCD1. In their study, the agitation speed was varied from 0-150 rpm. At 0 rpm, 50 rpm and 100 rpm, the surface tension values recorded were 40 mN/m, 37 mN/m and 35 mN/m, respectively. However, maximum reduction in surface tension (33 mN/m) was observed at 125 rpm. At 150 rpm, the surface tension of the broth increased to 35 mN/m.

2.12.5 EFFECT OF CARBON SOURCE ON SURFACTANT PRODUCTION

Surfactant production like any other chemical reaction is effected by a number of factors that either increase its productivity or inhibit it. Often the amount and type of a raw material can contribute considerably to the production cost; it is estimated that raw materials account for 10-30% of the total production cost in most biotechnological processes. Thus, to reduce this cost it is desirable to use low-cost raw materials for the

production of surfactants (Makkar and Cameotra 2002). One possibility explored extensively is the use of cheap and agro-based raw materials as substrates for surfactant production. A variety of cheap raw materials, including plant-derived oils, oil wastes, starchy substances, lactic whey and distillery wastes have been reported to support surfactant production.

Carbon source is one of the important chemical parameters in the production of surfactant. Several reports in the literature address the influence of the carbon source in surfactant production by different microorganisms showing the possibility to use a wide variety of substrates. It also has been observed that the presence of carbon source, particularly the carbohydrate used, has a great bearing on the type of biosurfactant produced (Amaral et al. 2008). These changes may be welcomed when some properties are sought for a particular application (Cooper 1986). Both water soluble substrates such as glucose (Duvnjak et al. 1982; Makkar and Cameotra 1997), ethanol (Mulligan and Gibbs 1993), molasses (Patel and Desai 1997), glycerol (Silva et al. 2010), whey (Praveesh et al. 2011), etc and water-immiscible carbon substrates such as n-alkanes (Duvnjak et al. 1982) and olive oil (Haba et al. 2000), etc, have been used by several researchers in order to maximize surfactant concentration by various microorganisms. Recently, renewable low cost substrates such as vegetable oils (olive oil, soybean oil, palm oil, coconut oil, fish oil, soybean oil, etc), frying oil wastes, dairy and sugar industry wastes (such as whey, molasses, etc), lignocellulosic waste (starch from corn, wheat, potatoes), cassava waste water, cashew apples, glycerol, etc have been utilized for surfactant production in a report by Makkar et al. (2011).

The earliest study on surfactant production by *Pseudomonas aeruginosa* was carried out by Jarvis and Johnson (1949) in 30 g/L of glycerol containing medium. They reported a maximum of 2.5 g/L of surfactant production by the bacterium. Similar study was reported by Hauser and Karnovsky (1954), they reported that *Pseudomonas*

aeruginosa produced a maximum of 2 g/L of surfactant quantity during its growth in 30 g/L of glycerol containing medium.

Cooper and Goldenberg (1987) reported production of 1.6 g/L of surfactant by *Bacillus cereus* using sucrose as a carbon source. In a study by de Roubin et al. (1989), a maximum of 1.1 g/L of surfactant was produced by *Bacillus subtilis* ATCC 51338 during the growth of the bacterium in glucose containing salts medium. In a similar study by Lindardt et al. (1989), surfactant production by *Pseudomonas aeruginosa* KY4025 was reported. They reported that the bacterial strain produced a maximum of 8.5 g/L of surfactant during its growth on n-paraffin (90 g/L) containing medium.

In a study by Ohno et al. (1992), along with surfactant, the strain *Bacillus subtilis* RB 14 also produced iturin during the growth of the bacterium in glucose containing medium. In a study by Makkar and Cameotra (1997), the different carbon sources used in the production of surfactant by *Bacillus subtilis* were sucrose, glucose, sodium, pyruvate, sodium acetate, tri-sodium citrate, hexadecane, dodecane, decane, kerosene and pristine. Maximum surfactant (0.744 g/L) was produced in sucrose containing medium whereas least surfactant (0.093 g/L) was produced in kerosene containing medium.

Haba et al. (2000) reported surfactant production by *Pseudomonas aeruginosa* 47T2 in medium containing 40 g/L of waste olive oil. They reported that a maximum of 2.7 g/L of surfactant was synthesized by the bacterium. In another study by Dubey and Juwarkar (2001), distillery wastes, whey wastes and glucose were used as carbon sources for surfactant production by *Pseudomonas aeruginosa* BS2. They observed that a maximum of 0.91 g/L, 0.92 g/L and 0.97 g/L of surfactant was produced by the bacterial strain during its growth in distillery wastes, whey wastes and glucose containing medium.

Benincasa et al. (2002) added sunflower oil, olive oil, soybean oil, olein and soapstock as carbon sources in mineral medium for the synthesis of surfactant by *Pseudomonas aeruginosa* LB1. Maximum surfactant was obtained in the soapstock

containing medium, a maximum of 12 g/L of surfactant was produced. The surfactant concentrations in sunflower oil, olive oil, soybean oil, olein and soapstock were 4.9 g/L, 5.4 g/L, 4.8 g/L and 4.5 g/L, respectively.

Corn oil was used as carbon source by Amezcua-Vega et al. (2007) to synthesize surfactant in the production medium of *Candida ingens* CB-216. A maximum of 5.6 g/L of surfactant was produced by the yeast strain. In a similar study, Youssef et al. (2007) reported maximum surfactant production of 0.09 g/L by *Bacillus subtilis* using glucose as carbon source. In a study by Thavasi et al. (2009), *Azotobacter chroococcum* isolated from the marine environment produced a maximum of 4.6 g/L of surfactant during the growth of the bacterium in peanut oil containing medium.

Glucose, starch, mannitol, maltose, sucrose, hexadecane and crude oil were tested as carbon sources by Haddad et al. (2009) during the synthesis of surfactant by *Bacillus subtilis* HOB2. It was observed that glucose as carbon source supported maximum surfactant production as indicated by reduction in surface tension value from 65 mN/m to 28 mN/m. Maltose supplemented medium showed surface tension value of 29 mN/m. The surface tension values for other carbon sources ranged from 32-54 mN/m.

Among the various carbon sources (galactose, olive oil, glucose, starch, mannitol, sucrose, trehalose, hexadecane, fructose and maltose) screened by Khopade et al. (2012) for surfactant synthesis by *Nocardia sp.* B4, maximum surfactant production was observed in olive oil containing medium. The surfactant produced decreased the surface tension to 30 mN/m and the emulsifying activity was 80%.

2.12.6 EFFECT OF NITROGEN SOURCE ON SURFACTANT PRODUCTION

Nitrogen is important in the surfactant production medium because it is essential for microbial growth as protein and enzyme synthesis that take part in the formation of different biosurfactant moieties (Amaral et al. 2008). Different nitrogen compounds have

been used for the production of biosurfactants, such as ammonium sulphate (Zinjarde et al. 1997), urea (Vance-Harrop et al. 2003) peptone (Kim et al. 2006) yeast extract (Casas and Ochoa 1999; Amezcua-Vega et al. 2007), meat extract and malt extract (Mata‑sandoval et al. 2001), ammonium nitrate (Thanomsub et al. 2004) sodium nitrate (Bednarski et al. 2004), etc.

Cooper and Paddok (1984) studied the effect of the nitrogen source on surfactant production by *Torulopsis bombicola.* They evaluated sodium nitrate, ammonium chloride, ammonium nitrate, urea or yeast extract as nitrogen source. They observed that nitrate was not a good nitrogen source since it affected the biomass growth while yeast extract promoted higher surfactant production (10 g/L). When yeast extract was substituted by peptone, the surfactant concentration obtained was reduced to half and a low concentration of surfactant was obtained when urea was used.

Makkar and Cameotra (1997) studied the effect of different organic as well as inorganic nitrogen sources on surfactant production by *Bacillus subtilis.* Urea, peptone, yeast extract, beef extract and tryptone were the organic nitrogen sources considered in the study. The inorganic nitrogen sources included in the study were potassium nitrate, sodium nitrate, ammonium nitrate and ammonium sulphate. Among the various nitrogen sources evaluated, maximum surfactant (1.012 g/L) was produced in urea containing medium whereas least surfactant (0.170 g/L) was produced in medium with ammonium sulphate.

Surfactant production by *Candida apicola* ATCC 20509 was studied by Bednarski et al. (2004). A combination of organic and inorganic nitrogen source was supplemented in the production medium to maximize the production of surfactant. Supplementation of yeast extract and sodium nitrate yielded a maximum of 10.3 g/L of surfactant during the growth of the yeast species. Corn steep liquor and urea were supplemented as nitrogen sources by Thanomsub et al. (2004) during surfactant

production by *Candida bombicola* ATCC 22214. A maximum of 120 g/L of surfactant production was recorded for the yeast species after 68 hours of incubation.

Haddad et al. (2008) evaluated different nitrogen sources (ammonium nitrate, ammonium sulphate, sodium nitrate, potassium nitrate and soybean flour) during surfactant production by *Bacillus subtilis* HOB2. In the ammonium sulphate supplemented medium, maximum quantity of surfactant was produced as indicated by reduction in surface tension value (27.3 mN/m). The surface tension values obtained for other nitrogen sources ranged from 32.5-45.6 mN/m.

Peptone, yeast extract, beef extract, sodium nitrate and urea were used as nitrogen sources (1% v/v) during surfactant production by *Aspergillus ustus* MSF3 (Kiran et al. 2009). Among the nitrogen sources considered in the study, maximum surfactant production as indicated by maximum emulsification activity (E24=25%) was obtained when yeast extract was used. The emulsification activity recorded for peptone, beef extract and urea supplemented culture medium was 0% and sodium nitrate supplemented medium showed emulsification activity of 10%.

Various nitrogen sources such as ammonium sulphate, phenyl alanine, alanine, ammonium chloride, urea, asparagine and sodium nitrate were screened by Khopade et al. (2012) during surfactant production by *Nocardia sp.* B4. Among the various nitrogen sources screened, maximum surfactant was synthesized in alanine containing medium, as indicated by surface tension value (30 mN/m) whereas in phenyl alanine containing medium, least surfactant was produced (60 mN/m) by the bacterial strain.

2.12.7 EFFECT OF INDUCER ON SURFACTANT PRODUCTION

Hydrocarbons added to the fermentation medium are known to induce the production of biosurfactant (Bento and Gaylarde 1996). It was postulated by Mutalik et al. (2008) that an appropriate combination of a simple carbon source and an inducer

enhance the production of biosurfactant. In this regard, few researchers have studied the role of inducers on surfactant production by different microorganisms.

Mutalik et al. (2008) in their study used mannitol as carbon source and n-hexadecane as the inducer. In the absence of the inducer, the emulsification activity (E24) was 31.5%, whereas in the presence of the inducer, the emulsification activity increased to 78.9%. They also speculated that mannitol acted as a primary carbon source during the initial growth phase, while in the later stages of fermentation, *Rhodococcus* cells probably used n-hexadecane as a carbon source.

In a study by Pal et al. (2009), seven inducers (n-hexadecane, n-octadecane, dodecane, paraffin, toluene, olive oil and castor oil) were screened for their ability to induce surfactant production by *Rhodococcus erythropolis* MTCC 2794. Among the various inducers screened, toluene enhanced surfactant synthesis as suggested by high emulsification activity of 53.84%.

The inducers screened for surfactant production by *Acinetobacter lwoffii* TA 38 included almond oil, castor oil, coconut oil and soybean oil (Jagtap et al. 2010). Castor oil supplemented medium enhanced surfactant synthesis, showing emulsification activity of 110.3 EU/ml. The other inducers supplemented medium showed emulsification activity ranging from 45.1 to 108 EU/ml.

Among the various inducers (such as castor oil, olive oil, eucalyptus oil, clove oil, coconut oil, cod liver oil, salmon oil, toluene, n-hexane, xylene, kerosene, petrol and diesel) tested by Khopade et al. (2012) for surfactant synthesis by *Nocardia sp.* B4, n-hexane induced surfactant production as indicated by surface tension value (28 mN/m).

2.12.8 EFFECT OF TRACE ELEMENTS ON SURFACTANT PRODUCTION

The addition of various metal supplements greatly affects the growth and surfactant production by microorganisms (Makkar and Cameotra 1997). The type of trace element supplementation depends on the nutritional requirement of the surfactant producer.

Makkar and Cameotra (1997) studied the effect of supplementation of trace elements in surfactant production medium of *Bacillus subtilis*. The trace elements tested for increased production of surfactant by the isolate included magnesium sulphate, calcium chloride, ferrous sulphate and trace element solution consisting of 2.32 g $ZnSO_4 \cdot 7H_2O$, 1.78 g $MnSO_4 \cdot 4H_2O$, 0.56 g H_3BO_3, 1.0 g $CuSO_4 \cdot 5H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.42 g $CoCl_2 \cdot 6H_2O$, 1.0 g EDTA, 0.004 g $NiCl_2 \cdot 6H_2O$ and 0.66 g KI. In the absence of the trace element, the surfactant quantity was 0.342 g/L. However, maximum surfactant (1.23 g/L) was observed when the trace element solution (containing all the trace elements) was used. In the presence of individual trace element such as magnesium sulphate, calcium chloride and ferrous sulphate, the amount of surfactant produced were 0.792 g/L, 0.624 g/L and 0.814 g/L, respectively.

To examine the effect of trace elements on surfactant production, Kiran et al. (2009) added trace elements such as ferrous sulphate, calcium chloride, copper sulphate, magnesium chloride and manganous chloride in the production medium of *Aspergillus ustus* MSF3. Among the trace elements, ferrous sulphate showed maximum surfactant production as indicated by maximum emulsification activity (70%). The emulsification activity recorded for media with other trace elements ranged from 0-60%.

Trace elements such as ferrous sulphate, manganous sulphate, magnesium sulphate and potassium dihydrogen phosphate were added into surfactant production medium of *Bacillus subtilis* PTCC 1023 (Noudeh et al. 2010). The organism showed preference to ferrous sulphate as indicated by high emulsification activity (72%) compared to other trace elements. The emulsification activities shown in the presence of

other trace elements were 58% for manganous sulphate, 36% for magnesium sulphate and 28% for potassium dihydrogen phosphate containing medium, respectively.

The supplementation of trace elements such as aluminium sulphate, manganous sulphate, zinc sulphate and barium sulphate in the surfactant production medium of *Pseudomonas aeruginosa* OCD1 was studied by Sahoo et al. (2011). Among the trace elements considered, zinc sulphate resulted in maximum reduction in surface tension (31.6 mN/m) by the bacterial isolate. The surface tension values recorded for other trace elements were 31.9 mN/m for manganous sulphate, 32 mN/m for barium sulphate, 32.2 mN/m for aluminium sulphate supplemented media, respectively.

2.12.9 EFFECT OF SALINITY ON SURFACTANT PRODUCTION

Salt concentration also affects surfactant production depending on its effect on cellular activity of the microorganism (Tabatabaee et al. 2005). Various reports in the literature suggest that salinity plays an important role in surfactant production by microorganisms especially of marine origin.

Makkar and Cameotra (1997) studied the effect of salinity on surfactant production by *Bacillus subtilis* strain. The concentration of NaCl was varied from 0.01-4% (w/v). With increase in NaCl concentration, there was decrease in the concentration of surfactant produced by the bacterial strain. At 0.01% NaCl concentration, 0.936 g/L of surfactant was produced. 0.694 g/L of surfactant was produced at 1% NaCl concentration and at 4% NaCl concentration; 0.408 g/L of surfactant was produced.

The effect of salinity on surfactant production by the marine fungal isolate, *Aspergillus ustus* MSF3 was investigated by Kiran et al. (2009). The concentration of NaCl was varied from 1-3.5% (w/v). With increase in salt concentration, there was increase in emulsification activity (E24 ranged from 0-15%) till 3% salt concentration, thereafter, the emulsification activity dropped to 0%. Since the fungus was a marine

isolate, it showed maximum emulsification activity (E24=20%) when the production medium contained 3% NaCl.

The influence of salinity on surfactant production by the marine bacterial strain, *Azotobacter chroococcum*, was studied by Thavasi et al. (2009). The salt concentration was varied from 10-40% (w/v). At 10% and 20% NaCl concentrations, the surfactant yield produced by the marine isolate were 0.9 g/L and 1.1 g/L, respectively. Maximum surfactant production (1.2 g/L) was recorded at 20% NaCl concentration. However, there was a decrease in surfactant production (0.8 g/L) at 40% NaCl concentration. In the absence of the salt, 0.8 g/L of surfactant was synthesized by the isolate.

To examine the effect of sodium chloride (NaCl) on surfactant production by *Nocardia sp.* B4, sodium chloride was added in the production medium to achieve final concentrations of 1-10% (w/v) (Khopade et al. 2012). At 1% NaCl concentration, the surface tension of the cell-free broth was found to be 38 mN/m. Maximum decrease in surface tension (28 mN/m) was observed at 2% NaCl concentration. With further increase in NaCl concentration, there was increase in surface tension of cell-free broth. The surface tension values ranged from 36 to 58 mN/m when the salinity was increased from 3 to 10% NaCl concentration.

2.13 LARGE SCALE PRODUCTION OF BIOSURFACTANT

Production of biosurfactant is being mostly carried out by shake flask method. However, recently, higher volume bioreactors have been used in laboratory condition. The large scale production of biosurfactants is important in meeting the demand for biosurfactant in various sectors.

Banat et al. (1991) conducted laboratory surfactant production studies in a 7 L capacity fermenter operating at pH 6.8, temperature 34-37°C, oxygen saturation level 20% and working volume of 4 L. Bacterial strain Pet 1006 was employed for surfactant production studies using basal salt medium containing 2% glucose. 2% hydrocarbon was

added after glucose consumption. Complete glucose exhaustion was observed at 14 hours, the surface tension of the cell-free broth decreased from 76 mN/m to 37 mN/m. However, the organism utilized hydrocarbon after 14 hours, the maximum reduction in surface tension (33.2 mN/m) was observed at 30 hours. Using the same cultural and medium conditions, the biosurfactant production was scaled up in 1500 L fermenter containing 900 L of working volume media. In this study, glucose was completely utilized after 8 hours and maximum reduction in the surface tension (37.1 mN/m) was achieved after 19 hours of incubation.

In a study by de Lima et al. (2009), 6 L (3.3 L working volume) capacity fermenter was used for the synthesis of surfactant by *Pseudomonas aeruginosa*. Experiments were carried out in mineral medium containing residual brewery yeast and waste fried soybean oil at 30°C, pH 7 and by varying the agitation as well as aeration. They observed that maximum surfactant production was achieved using 0.5 vvm of aeration and agitation speed of 550 rpm, a maximum of 3.3 g/L of rhamnolipid biosurfactant was produced which reduced the surface tension to 26 mN/m.

Rahman et al. (2010) used bench scale bioreactor of 7.5 L containing 3 L of glycerol containing nutrient broth for surfactant production by *Pseudomonas aeruginosa* DS10-129. The operating conditions of the bioreactor, notably 30°C, pH 7 and agitation speed of 200 rpm, respectively. Maximum production of surfactant was achieved at 96 hours; a maximum of 0.04 g/L of surfactant concentration was produced by the bacterial culture. The surfactant produced reduced the surface tension of distilled water from 72 to 27.9 mN/m.

42 L capacity fermenter was used by Muller et al. (2010) for the production of surfactant by *Pseudomonas aeruginosa* PAO 1. A working volume of 30 L was maintained and medium containing sunflower oil was added as sole carbon source. Stirrer speed was set fix at 400 rpm, temperature at 37°C and dissolved oxygen was set at

minimum 5%. The bacterium produced a maximum of 39 g/L of surfactant after 90 hours of incubation.

2.14 OPTIMIZATION OF THE MEDIUM COMPONENTS USING STATISTICAL EXPERIMENTAL DESIGN METHODS

The classical method of medium optimization involves changing one variable at a time, while keeping the others at fixed levels; however, this method is laborious, time consuming and does not guarantee the determination of the optimal conditions for biosurfactant production. To tackle this problem and make the optimization process easier, a statistical optimization strategy based on response surface methodology (RSM) has been used by various researchers in the literature. The objective of RSM is to develop an empirical model of the process and to obtain a more precise estimate of the optimum media composition. This approach to media optimization is called response surface methodology and the design is a central composite design, one of the most important experimental designs used in optimization studies. The application of statistical design for screening and optimization of process parameters allows quick identification of important factors and interactions between them (Parekh and Pandit 2011). The combined effect of the variables can be predicted and optimization can be achieved with the help of experimental design tool, which is practically difficult in conventional experimentation.

RSM was successfully used to determine the optimum media, inoculum and environmental conditions for the enhanced production of surfactin by *Bacillus subtilis* (Sen 1997; Sen and Swaminathan 1997; Sen and Swaminathan 2004). RSM has also been applied to enhance surfactant production by *Pseudomonas aeruginosa* AT 10 (Abalos et al. 2002) by the probiotic bacterial strains, *Lactococcus lactis* and *Streptococcus thermophilus* (Rodrigues et al. 2006), respectively and by *Bacillus licheniformis* for the concomitant production of surfactants and protease RG1 using agro-products such as cornstarch and soy flour as carbon and nitrogen sources (Ramnani et al. 2005). An increase in productivity of biosurfactant was observed in all these cases. These

optimization methods would help the industry to design the best media containing cheaper substrates and to use the most favourable environmental conditions for improved biosurfactant production.

The use of RSM effectively enhanced the production of surfactant by *Rhodococcus spp*. MTCC 2574 growing on n-hexadecane with yield of surfactant increasing from 3.2 g/L to 10.9 g/L (Mutalik et al. 2008). The chemical variables used in the RSM study were mannitol (10-40 g/L), yeast extract (2-8 g/L), meat peptone (5-20 g/L) and n-hexadecane (20-80 g/L). 10.9 g/L of surfactant quantity was produced following optimization at 20 g/L of mannitol, 2 g/L of yeast extract, 5 g/L of meat peptone and 20 g/L of n-hexadecane.

Oliveira et al. (2009) chose RSM in order to increase surfactant productivity by *Pseudomonas alcaligenes*. In their study, palm oil concentration, initial medium pH and agitation speed were considered; the corresponding ranges were 0.5-5.5% (v/v), 6-8 and 50-250 rpm, respectively. The response was evaluated in terms of decrease in surface tension. The maximum decrease in surface tension (28 mN/m) and surfactant concentration (2.3 g/L) was obtained when the concentration of palm oil was 3%, pH was 7 and the agitation speed was 150 rpm. The surface tension value obtained prior to optimization study was 39 mN/m. This suggests that RSM is useful in increasing the biosurfactant productivity as indicated by surface activity.

Response surface methodology (RSM) was used for the surfactant production by *Aspergillus ustus* MSF3 (Kiran et al. 2009). The chemical variables considered in the study were glucose and yeast extract, the concentration of both the variables were varied from 0.5-1% (w/v). During their study, the emulsification activity was as high as 55% when the concentration of glucose and yeast extract was 0.5%.

2.15 KINETICS OF BIOSURFACTANT PRODUCTION

The kinetics of biosurfactant production exhibit many variations among various systems and only a few generalizations can be derived. However, for convenience, kinetic parameters can be grouped into the following types: (i) growth-associated production, (ii) production under growth-limiting conditions, (iii) production by resting or immobilized cells and (iv) production with precursor supplementation.

2.15.1 GROWTH-ASSOCIATED PRODUCTION

For growth-associated biosurfactant production, parallel relationships exist between growth, substrate utilization, and biosurfactant production (Fig 2.8 A). The production of rhamnolipid by *Pseudomonas sp.* (Yamaguchi et al. 1976; Syldatk et al. 1985; Robert et al. 1989), glycoprotein AP-6 by *Pseudomonas fluorescens* 378 (Persson et al. 1988), surfactant by *Bacillus cereus* IAF 346, biodispersan by *Bacillus sp.* strain IAF-343 (Cooper and Goldenberg 1987), surfactant by *Lactobacillus paracasei sp. paracasei* A20 (Gudina et al. 2011), are all examples of growth-associated surfactant production. The production of cell-free emulsan by *Acinetobacter calcoaceticus* RAG-1 has been reported to be a mixed growth-associated and non-growth-associated type (Gutnick et al. 1980; Goldman et al. 1982). Emulsan-like substance accumulates on the cell surfaces during the exponential phase of growth and is released into the medium when protein synthesis decreases (Goldman et al. 1982; Pines et al. 1983; Shabtai et al. 1985).

2.15.2 PRODUCTION UNDER GROWTH-LIMITING CONDITIONS

Production under growth-limiting conditions is characterized by a sharp increase in the biosurfactant level as a result of limitation of one or more medium components (Fig 2.8 B). A number of investigators have demonstrated an overproduction of surfactants by *Pseudomonas sp.* when the culture reaches the stationary phase of growth due to the limitation of nitrogen and iron (Suzuki et al. 1974; Guerra-Santos et al. 1986; Mulligan and Gibbs, 1989; Ramana and Karanth 1989). Production of surfactant by

Torulopsis apicola (Hommel et al. 1987), bioemulsifier by *Candida tropicalis* IIP-4 (Singh et al. 1990) and glycolipid by *Nocardia sp*. strain SFC-D (Kosaric et al. 1990) have also been reported to follow this pattern. In contrast to the observation in *Pseudomonas aeruginosa* (Mulligan et al. 1989), low phosphate concentration stimulated bioemulsifier production in a Gram-negative bacterium during cultivation on ethanol (Palejwala and Desai 1989). Phosphate, iron, magnesium, and sodium were all important elements for surfactant-producing *Rhodococcus sp*., much more than either potassium or calcium (Abu-Ruwaida et al. 1991).

2.15.3 PRODUCTION BY RESTING OR IMMOBILIZED CELLS

Production by resting or immobilized cells is a type of biosurfactant production in which there is no cell multiplication (Fig 2.8 C). The cells nevertheless continue to utilize the carbon source for the synthesis of biosurfactants. Several examples of biosurfactant production by resting cells are known. They include production of sophorolipid production by *Torulopsis bombicola* (Inoue and Itoh 1982), rhamnolipid production by *Pseudomonas sp.* (Syldatk et al. 1985; Reiling et al. 1986; Syldatk and Wagner 1987), trehalose tetraester production by *Rhodococcus erythropolis* (Margaritis et al. 1980; Syldatk et al. 1985; Syldatk and Wagner 1987), cellobiolipid production by *Ustilago maydis* (Fautz et al. 1986), surfactant by *Candida apicola* (Hommel and Huse 1993) and mannosylerythritol lipid production by *Candida antarctica* (Kitamoto et al. 1992; Kitamoto et al. 1993). Biosurfactant production by resting cells is important for the reduction of cost of product recovery, as the growth and the product formation phases can be separated.

2.15.4 PRODUCTION WITH PRECURSOR SUPPLEMENTATION

Many investigators have reported that the addition of biosurfactant precursors to the growth medium causes both qualitative and quantitative changes in the product. For example, the addition of lipophilic compounds to the culture medium of *Torulopsis*

magnoliae (Tulloch et al. 1962), *Torulopsis bombicola* (Margaritis et al. 1979; Cooper and Paddock 1984) and *Torulopsis apicola* IMET 43747 (Stuwer et al. 1987) resulted in increased surfactant production (Lee and Kim 1993). Similarly, increased production of surfactants containing different mono-, di-, or trisaccharides was reported to occur in *Corynebacterium sp., Nocardia sp.* and *Brevibacterium sp.* through supplementation with the corresponding sugar in the growth medium (Brennan et al. 1970; Itoh and Suzuki 1974; Suzuki et al. 1974).

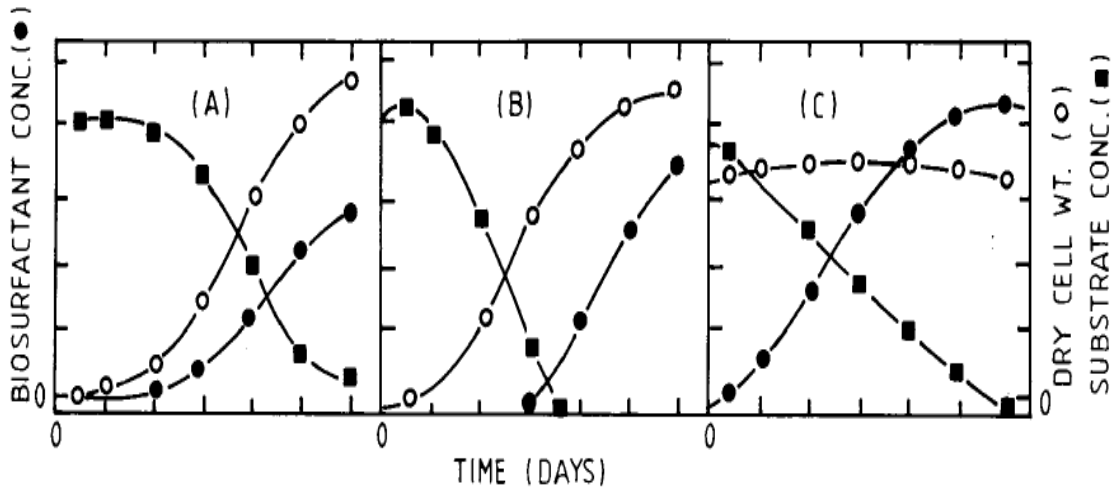


Fig 2.8 Schematic illustration showing different types of fermentation kinetics for biosurfactant production (A) Growth-associated production in which a parallel relationship is obtained between growth, substrate utilization, and biosurfactant production as observed in *Acinetobacter calcoaceticus*. **(B)** Production under growth-limiting condition in which biosurfactant production is observed only when growth limitation occurs by exhaustion of nutrients such as nitrogen as in the case of *Pseudomonas* spp. **(C)** Production of biosurfactant by resting or immobilized cells where cells continue to utilize substrate and produce biosurfactant without cell multiplication as observed for some *Torulopsis sp.*

2.16 BIOSYNTHETIC PATHWAY OF BIOSURFACTANT SYNTHESIS

Generally, biosurfactants are amphiphilic compounds with hydrophilic moiety consisting of carbohydrate, amino acid, cyclic peptide, alcohol or phosphate and hydrophobic moiety may be a long chain fatty acid, a hydroxy fatty acid or alkyl hydroxy fatty acid. They are synthesized by two primary metabolic pathways, namely hydrocarbon and carbohydrate pathways (Desai and Banat 1997). The metabolic pathways involved in the synthesis of these two groups of precursors are diverse and utilize a specific set of enzymes. Usually, regulatory enzymes are the first enzymes for the synthesis of these precursors in the biosynthetic pathways.

Syldatk and Wagner (1987) documented the following possibilities could occur for the synthesis of biosurfactant and their linkage: (1) the hydrophilic and hydrophobic moieties are synthesized *de novo* by two independent pathways followed by their linkage to form a complete biosurfactant molecule, (2) the hydrophilic moiety is synthesized *de novo* while the hydrophobic moiety is substrate dependent synthesis followed by its linkage, (3) the hydrophobic portion is synthesized *de novo* while the synthesis of hydrophilic portion is induced by substrate, (4) both hydrophilic and hydrophobic moieties have substrate-dependent synthesis. The pathways involved in biosynthesis are dependent on the carbon source and the type of biosurfactant produced (Mulligan and Gibbs 1993). A glycolipid synthesized from a carbohydrate will be regulated by both the lipogenic pathway and glycolytic metabolism. By these mechanisms, the addition of lipophilic compounds to the carbohydrate will enhance the production of biosurfactant (Boulton and Ratledge 1987).

The metabolic pathways involved in the synthesis of the precursors of biosurfactants are diverse and to some extent, dependent on the nature of the principle carbon source. Sugars are all required either for the synthesis of structural entities of the cell or for the biosynthesis of amino acids, proteins and nucleic acids. Glucose may be regarded as the starting point for most microbial fermentations because it is the most universally used and cheapest carbons source available so far. Formation of disaccharides

and polysaccharides found in many biosurfactants follows the glycolytic pathway prior to sugar modification and transformation (Lang and Wagner 1993). A specific synthetase acts to ensure that the correct reactions occur. The phosphorylated disaccharide will then be used as the activated sugar for the formation of the glycolipid biosurfactant. Generally the metabolism of hydrocarbons such as alkanes to biosurfactant will consist of the alkane being oxidized to mono-alkanoic acids (fatty acids), which normally make up the hydrophobic moiety of the biosurfactant. The fatty acid can be further metabolized via β-oxidation to yield acetyl-CoA. The differences of the synthesized unsaturated fatty acids occur mainly because of the organization of the enzymes making up the individual fatty acid synthetase complexes. Acetyl CoA is converted further in the tricarboxylic acid cycle to sugars and amino acids, part of which forms the hydrophilic moiety of the biosurfactant (Hommel and Ratledge 1993).

As shown in Figure 2.9, pyruvate is the end product of glucose metabolism in glycolysis. If the energy level of the cell is high due to the glucose catabolism, the flow of carbon into the tricarboxylic acid cycle will be slowed and acetyl-CoA accumulated can be diverted into the biosynthesis of fatty acids (lipid). In this case, acetyl-CoA is formed directly in the cytoplasm from pyruvate by pyruvate dehydrogenase (Hommel and Ratledge 1993).

$$(\text{Glucose}) \longrightarrow \longrightarrow \text{pyruvate} + NAD^{+} + \text{CoA} \longrightarrow \text{Acetyl-CoA} + CO_2 + \text{NADH}$$

Acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACC) to produce malonyl-CoA, which then becomes the activated carbon donor for the biosynthesis of fatty acids.

$$\text{Acetyl-CoA} + HCO_3 + \text{ATP} \longrightarrow \text{Malonyl-CoA} + \text{ADP} + \text{Pi}$$

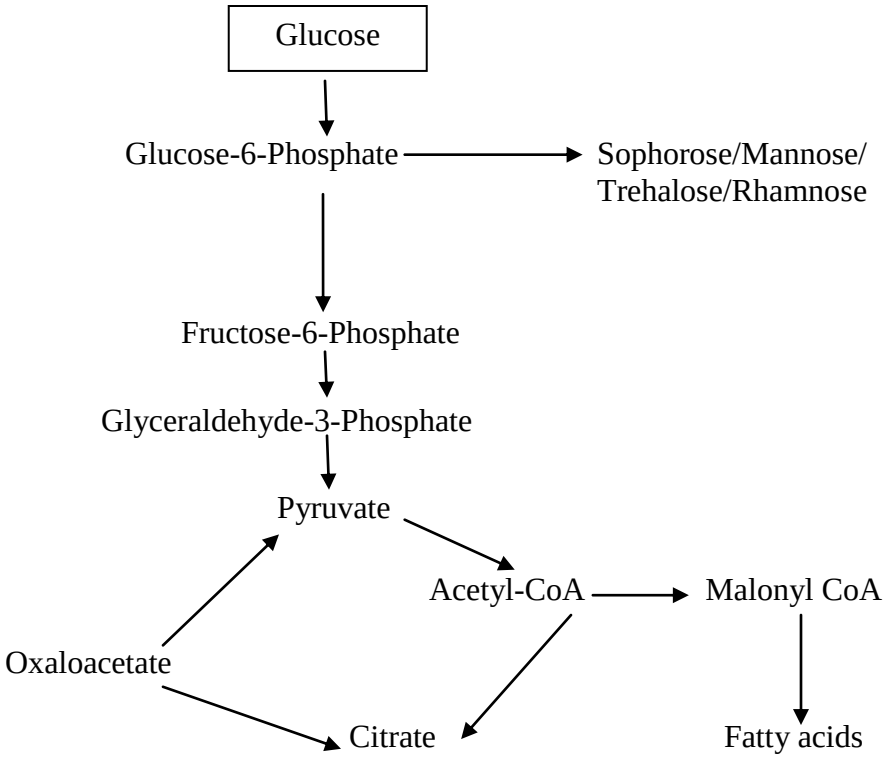
The elongation of acetyl-CoA to long chain fatty acids is a combination of various enzymatic reactions. The essential steps are the condensation of an acetyl group with a

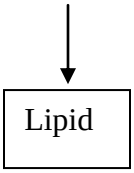
malonyl group to yield a C_4 unit by β-ketoacyl synthase; followed by reduction, dehydration and further reduction of the C_4 unit until a saturated C_4 (butyryl) group is formed. The cycle is then repeated by condensation of the butyryl group with a further malonyl group leading to a C_6 moiety. The reaction cycle continues until a long-chain fatty acyl group is formed (Hommel and Ratledge 1993).

The overall reaction may therefore be written as:

$$7\ \text{Malonyl-CoA} + \text{acetyl-CoA} + 14\ \text{NADPH} \xrightarrow{\text{Fatty acid synthetase}} \text{Palmitoyl-CoA} + 7\ CO_2 + 7\ \text{CoA} + 14\ NADP^+ + 7\ H_2O$$

Fig. 2.9: Metabolic pathway of biosurfactant synthesis (Mulligan and Gibbs 1993)





2.17 REGULATION OF BIOSURFACTANT PRODUCTION

A number of studies have indicated that the type of medium and growth conditions influence the type and yield of the biosurfactant. The carbon source influences the biosurfactant synthesis either by induction or repression. In some cases, addition of water-immiscible substrates results in induction of biosurfactant production. The induction of sophorolipid synthesis by addition of long-chain fatty acids, hydrocarbons, or glycerides to the growth medium of *Torulopsis magnolia* (Tulloch et al. 1962), trehalolipid synthesis in *Rhodococcus erythropolis* by addition of hydrocarbons (Rapp et al. 1979) and glycolipid in *Pseudomonas aeruginosa* SB-30 by addition of alkanes (Chakrabarty 1985) have been reported. Roy et al. (1979) confirmed the inducible nature of surfactants in *Endomycopsis lipolytica*. Synthesis of many lipopeptide biosurfactants is also regulated by induction (Kluge et al. 1989; Ullrich et al. 1991; Besson and Michel 1992).

Repression of surfactant production by *Arthrobacter paraffineus* (Duvnjak et al. 1982) and *Arthrobacter calcoaceticus* (Gobbert et al. 1984) on hydrocarbon substrates has been observed with D-glucose and organic acids, respectively. Similarly, a drastic reduction in the synthesis of rhamnolipids by *Pseudomonas aeruginosa* (Hauser and Karnovsky 1954, 1958) and liposan by *Candida lipolytica* (Cirigliano and Carman 1984) upon addition of D-glucose, acetate and tricarboxylic acids has been observed.

Nitrogen can be an important key to the regulation of biosurfactant synthesis and there is evidence that the nitrogen plays an important (definite) role in the production of surfactants by microorganisms. *Arthrobacter paraffineus* ATCC 19558 preferred ammonium to nitrate for surfactant production when inorganic salts were used as the nitrogen source. Urea also led to increased surfactant production (Duvnjak et al. 1983). Investigations on rhamnolipid production by *Pseudomonas sp.* 44T1 growing on olive oil showed that sodium nitrate was the best nitrogen source (Robert et al. 1989). Similar results were obtained using a *Pseudomonas aeruginosa* culture and a *Rhodococcus sp*., growing on olive oil and paraffin, respectively (Ramana and Karanth 1989; Abu-Ruwaida et al.1991). In *Corynebacterium lepus*, nitrate as a sole nitrogen source resulted in a fast increase of surfactant production during the mid-exponential growth phase, while ammonium plus nitrate resulted in a growth-associated surfactant production (Gerson and Zajic 1978). In *Pseudomonas aeruginosa*, a correlation between surfactant production and glutamine synthetase activity was observed in nitrogen-limited conditions (Mulligan and Gibbs 1989). Nitrogen limitation plays an important role in regulating surfactant synthesis in *Candida tropicalis* IIP-4 (Singh et al. 1990) and *Nocardia* strain SFC-D (Kosaric et al. 1990). Syldatk et al. (1985) showed that nitrogen limitation not only caused increased biosurfactant production but also changed the composition of the biosurfactant.

The limitation of multivalent cations also causes overproduction of biosurfactants (Itoh and Suzuki 1974; Guerra-Santos et al. 1984). Guerra Santos et al. (1986) demonstrated that by limiting the concentrations of magnesium, calcium, potassium, sodium and trace element salts, a better yield of rhamnolipid could be achieved in *Pseudomonas aeruginosa* DSM 2659. Surfactin production by *Bacillus subtilis* was stimulated by addition of either iron or manganese salts to the medium (Cooper et al. 1981). Similar results were obtained in the case of *Rhodococcus sp.* (Abu-Ruwaida et al. 1991).

2.18 RECOVERY/ PARTIAL PURIFICATION OF BIOSURFACTANTS

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 the recovery of the products. Thus, one important factor determining the feasibility of a production process on a commercial scale is the availability of suitable and economic recovery and downstream procedure (Mukherjee et al. 2006). Recovery and/or purification of biotechnological products in downstream processing costs usually account for approximately 60% of the total production costs which make commercial production of biosurfactants quite expensive (Satpute et al. 2010). Economically biosurfactant recovery processes are mainly dependent on its ionic charge, water solubility and its nature location (intracellular, extracellular or cell bound) (Desai et al. 1994). Most biosurfactants are secreted into the medium and they are isolated from the supernatant obtained after removal of cells. Several conventional methods for the recovery of biosurfactants, such as acid precipitation (Smyth et al. 2009; Mnif et al. 2012), solvent extraction, crystallization, ammonium sulphate precipitation, etc, have been widely reported in the literature (Desai and Banat 1997).

A few unconventional and interesting recovery methods have also been reported in recent years. These procedures take advantage of some of the other properties of biosurfactant, such as their surface activity or their ability to form micelles and/or vesicles and are particularly applicable for large-scale continuous recovery of extracellular biosurfactants from culture broth. A few examples of such biosurfactant recovery strategies include foam fractionation (Davis et al. 2001; Noah et al. 2002), ultrafiltration (Sen and Swaminathan 2005), adsorption-desorption on polystyrene resins and ion-exchange chromatography (Reiling et al. 1986) and adsorption-desorption on wood-based activated carbon (Dubey et al. 2005). One of the main advantages of these methods is their ability to operate in a continuous mode for recovering biosurfactants with high level of purity.

In the literature, various biosurfactant extraction methods have been employed. Surfactant produced by *Pseudomonas sp.* (Hisatsuka et al. 1971), *Arthrobacter paraffineus* (Itoh and Suzuki 1974), *Rhodococcus erythropolis* (Rapp et al. 1979), *Ustilago sp.* (Spencer et al. 1979), *Candida lipolytica* (Cirigliano and Carman 1985), *Mycobacterium sp.* (Lang and Wagner 1987), *Nocardia corynebacterioids* (Powalla et al. 1989), etc, are some of the well-known examples of surfactants recovered by solvent extraction. 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 (MTBE) have been successfully used in recent years to recover surfactants produced by *Rhodococcus sp.* (Kuyukina et al. 2001; Philp 2002). These types of low cost, less toxic and highly available solvents can be used to cut the recovery expenses substantially and minimize the environmental hazards.

Surfactant produced by *Myroides sp.* was extracted by acidification followed by ethyl acetate extraction (Maneerat and Phetrong 2007). Surfactant has been recovered from cell-free broth of *Lactobacillus paracasei* by acidification as reported by Gudina et al. (2010). For large scale downstream processing of biosurfactants, preparative column chromatography using silica gel has been used by researchers (Burger et al. 1966; Monteiro et al. 2007). Rodrigues et al. (2006) harvested *Lactobacillus sp.* cells by centrifugation (10,000×g, 5 minutes, 10°C), washed twice in demineralized water and resuspended in 20 ml of phosphate-buffered saline (PBS: 10mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) for the extraction of intracellular surfactant. The bacteria were left at room temperature up to 24 hours with gentle stirring for surfactant release.

Mukherjee et al. (2006) reported that crude or impure biosurfactants obtained at the initial stages of recovery process can be used for environmental applications, applications in oil recovery and the paint as well as textile industries; these will be

available at lower costs. Alternatively, the highly pure biosurfactants required for the pharmaceutical, food and cosmetic industries can be obtained by performing further purification steps. This type of multistep recovery technique would be useful in industries producing biosurfactants for wide range of applications.

2.19 CHARACTERIZATION OF BIOSURFACTANT

Various techniques have been employed for the characterization studies of surfactant synthesized by different microorganisms following the recovery/partial purification of the surfactant. The most commonly used methods in the literature by various investigators include fourier transform infrared spectroscopy (FTIR), mass spectroscopy, thin layer chromatography (TLC), nuclear magnetic resonance spectroscopy (NMR), etc.

2.19.1 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

A classic method for biosurfactant structure analysis is the FTIR. Irradiation of molecules with IR light induces an oscillation of chemical bonds at characteristic frequencies and, thus, energy is absorbed. The resulting transmission of radiation is measured over a frequency spectrum from about 400-4,000 cm^{-1}. The so-called fingerprint area between 400 and 1,500 cm^{-1} shows deformation bands which are characteristic of every molecule and allow for the chemical substances to be identified from spectrum files. Partial structures are analyzed by dilation oscillations in the area from 1,500 to 4,000 cm^{-1}, as chemical bonds generate distinct valency oscillation bands (Heyd et al. 2008). This technique has been frequently used by researchers as the spectrum gives information of the various functional groups present in the biosurfactant which helps in the identification of the biosurfactant.

Fourier transform infrared absorption spectroscopy was used by Oliveira et al. (2009) in order to characterize the surfactant produced by *Pseudomonas alcaligenes.* FTIR spectra were consistent with the rhamnolipid biosurfactant structures. In the 3000-2700 cm^{-1} region, several C-H stretching bands of methyl and methylene groups were observed. The deformation vibrations at 1465^{-1} and 1375 cm^{-1} also confirmed the presence of alkyl groups. The carbonyl stretching band was verified at 1740 cm^{-1} which is characteristic for ester compounds. The ester carbonyl group was identified from the band at 1250 cm^{-1} which corresponded to C-O deformation vibrations.

In a study by Sitohy et al. (2010), the characterization of surfactant produced by *Candida guilliermondii* and *Bacillus subtilis,* respectively was carried out. The spectral analysis results as indicated by distinct wave numbers revealed that the surfactant produced by the yeast and bacterial cultures were glycolipid and lipoprotein, respectively.

The molecular composition of the isolated surfactant of *Pseudomonas aeruginosa* strain OBP1 was evaluated by FTIR (Bharali and Konwar 2011). The important bands which were located at 2,927.03, 2,860.39, 1,725, and 1,300-1,100 cm^{-1} confirmed the presence of glycolipid-type surfactant. The bands appearing at 3,430.19 cm^{-1} denoted the presence of -OH stretching of the hydroxyl group. The strong adsorption peaks at 3,000-2,700 cm^{-1} showed the presence of -CH aliphatic stretching bands. The characteristic peaks for the ester compounds were observed at 1,725.32 cm^{-1}, conforming -C=O stretching vibrations of carbonyl groups. Another strong peak at 1,638.1 cm^{-1} showed the presence of -COO- stretching vibrations of carbonyl groups. Other absorption peaks between 1,300 and 1,000 cm^{-1} confirmed the presence of ester carbonyl groups which corresponded to the presence of -C-O- deformation vibrations

2.19.2 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) has been extensively used to characterize different moieties of the biosurfactant using different solvents and support matrix.

Samples are applied onto TLC plates after extraction from culture broth and concentration. The separated biosurfactant components are eluted on the solid matrix using suitable solvent mixtures. The advantage of this method being that large number of samples with small volumes can be analyzed by TLC.

Koch et al. (1991) eluted surfactant produced by *Pseudomonas aeruginosa* mutants on a silica gel plate using solvent mixture containing chloroform: methanol: 20% aqueous acetic acid (65:15:2). They characterized the surfactant as rhamnolipid after the separation of different moieties of surfactant.

The characterization of surfactant produced by *Pseudomonas aeruginosa* A41 was carried out by Thaniyavarn et al. (2006) using thin layer chromatography. Rf values of various biosurfactant components were 0.33, 0.61, 0.68, 0.75, 0.83 and 0.95. The results suggested that the surfactant was a glycolipid which is common with *Pseudomonas sp.* which usually produces rhamnolipid (Arino et al 1996; Lang and Wullbrandt 1999).

Surfactant produced by marine *Brevibacterium aureum* MSA13 was subjected to thin layer chromatography studies in order to characterize the surfactant (Kiran et al. 2010). Lipid and protein fractions were detected after the separation of the surfactant components indicating the lipopeptide nature of the surfactant.

2.19.3 LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROSCOPY

Direct coupling of reverse phase liquid chromatography to a mass spectrometer provides the advantages of characterizing a given biosurfactant by its retention time along with its mass spectral signature. This is normally done by splitting the flow coming from the high pressure liquid chromatography (HPLC) using a splitter that conveys only a fraction of the eluent into the mass spectrometer. Electrospray Ionization (ESI), and sometimes Atmospheric Pressure Chemical Ionization (APCI), has been mostly used to ionize biosurfactants prior to mass analysis (Deziel et al. 1999, 2000; Haba et al. 2003;

Benincasa et al. 2004; Monteiro et al. 2007). In negative ESI, the molecular weight of the pseudomolecular ion $[M-H]^-$ can be directly obtained. This provides some information on the nature of the biosurfactant eluting from the column at that retention time. In order to improve ionization, ammonium acetate is added to both solvents of the water/acetonitrile gradient (Deziel et al. 2000). Ionized molecules are selected by a mass analyzer according to their mass-to-charge ratio (m/z) and are subsequently detected.

In order to characterize the surfactant production by *Pseudomonas alcaligenes*, Oliveira et al. (2009) used mass spectroscopy. Based on the abundance of different fragments (pseudomolecular ions with m/z 503, 531, 621, 649 and 677), the surfactant was identified as a rhamnolipid. They further confirmed the presence of congeners of rhamnolipid (Rha-$C_{10}C_{10}$ and Rha-$C_{10}C_{12}$, Rha-Rha-C_8C_{10}, Rha-Rha-$C_{10}C_{10}$, Rha-Rha-$C_{10}C_{12}$ and Rha-Rha-$C_{12}C_{10}$). Similar results were reported in a study by Nie et al. (2010) during the characterization of surfactant produced by *Pseudomonas aeruginosa* strain NY3.

The surfactant produced by marine *Brevibacterium aureum* MSA13 was subjected to mass spectral analysis in order to characterize the surfactant (Kiran et al. 2010). Pseudomolecular ions of varied m/z values (% relative intensity), 300, 255, 200, 150, 85, 75, 55 and 40 were obtained. Based on the results obtained, the surfactant synthesized by the bacterium was characterized as a lipopeptide.

The surfactant secreted extracellularly into the culture medium was recovered by means of acid precipitation method directly from the culture supernatant by Bharali and Konwar (2011). The mass spectrum of the purified biosurfactant showed a mixture of rhamnolipid with a molecular weight between 333 and 649 with intense molecular ions at m/z 333.09, 503.18, 504.18, 531.20, and 649.20. The m/z values obtained were consistent with the molecular structure of Rha-C_{10}, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, and Rha-Rha-C_{10}-C_{10}, respectively.

2.19.4 NUCLEAR MAGNETIC RESONANCE (NMR)

NMR spectroscopy is based on transitions in atoms with a magnetic moment when an external magnetic field is applied. Structure information is obtained from three parameters: chemical shifts of the absorption frequency, coupling (mutual influence of adjacent nuclei) and integral height. One and two dimensional 1H and 13C NMR spectroscopy have already been performed for pure rhamnolipids (Choe et al. 1992; Monteiro et al. 2007). Measurements of deuterium-exchanged samples are generally carried out in chloroform-deuterated methanol (2:1 v/v) using tetramethylsilane as the internal standard. The chemical shifts and coupling constants obtained by the 1H NMR spectroscopy of biosurfactant was studied by Choe et al. (1992). In the last few years, NMR spectroscopic analysis (Tahzibi et al. 2004; Wei et al. 2005) was performed in principle to confirm the structure of rhamnolipids produced by isolated or mutant bacteria strains.

2.20 EFFECT OF VARIOUS ENVIRONMENTAL FACTORS ON THE STABILITY OF BIOSURFACTANT

The applicability of biosurfactants in several fields depends on their stability at different environmental conditions such as salinity, temperature and pH values. In this regard, the influence of environmental factors on the stability of biosurfactant has been studied by various researchers in the literature.

Makkar and Cameotra (1997) studied the effect of pH and temperature on the stability of the surfactant produced by the bacterial strain, *Bacillus subtilis*. They observed that the cell-free broth containing biosurfactant was pH stable as well as thermostable. The biosurfactant was stable over a pH range of 4.5 to 10 with minimal deviation in surface tension values (29-33 mN/m). When the cell-free broth was heated in a boiling water bath at 100°C, it retained its surface activity (28-29 mN/m) even after heating for 2 hours.

Environmental factors such as pH, salinity and temperature also affects the stability of surfactant synthesized by *Aeromonas sp.* (Illori et al. 2005). The emulsification activity of the biosurfactant was enhanced at pH of 7 and 8. Increase in temperature from 30 to 35ºC had no effect on the emulsification activity of the biosurfactant, the emulsification activity obtained were the same (E24=65%). However, a slight increase in the emulsification activity of the biosurfactant was detected at a temperature of 40ºC (E24=68%). At temperatures of 70°C (E24=56%) and 100ºC (E24=50%), the activities of the biosurfactant decreased. It is however noteworthy to mention that the biosurfactant retained approximately 77% of its original activity (E24=65%) at temperature of 100ºC. The slight stimulation of the emulsification activity of the biosurfactant at NaCl concentration of 5% and pH of 8.0 indicate that those conditions might prevail in the estuarine water where the organism was isolated. Such biosurfactant may be useful for bioremediation of spills in marine environment because of its stability in alkaline condition and in the presence of salt.

The effect of pH, temperature and salinity on the stability of surfactant produced by *Pseudomonas aeruginosa* MR01 was studied by Lotfabad et al. (2009). When the pH was acidic and set to 2, 4 and 6, the surface tension values were 35, 29 and 27 mN/m, respectively. The surface tension reduction was maximum at pH 8 (27mN/m). At pH 10 and 12, the surface tension ranged from 28-29 mN/m. The results obtained from thermal stability analysis of cell-free broth over a wide range of temperature (4–121°C) revealed that the MR01 biosurfactant to be thermostable, there was negligible difference in the surface tension values (26-27 mN/m). With an increase in the NaCl concentration up to 10%, there was no significant effect in surface tension, the surface tension varied from 29-30 mN/m. However, at the highest level of NaCl (12%), the surface activity increased to 32 mN/m.

Gudina et al. (2010) investigated the influence of environmental factors on the stability of surfactant produced by *Lactobacillus paracasei.* In order to study the pH effect on biosurfactant stability, the surface tension of biosurfactant at different pH values (4–10) was determined. The minimum surface tension value was obtained at pH 7 (41.8 mN/m). The surface activity of the biosurfactant remained relatively stable to pH changes between pH 6 and 10, showing higher stability at alkaline (42.4-43.2 mN/m) than acidic conditions (44-46.2 mN/m). The surface tension of the biosurfactant at varied temperature (25, 37 and 60°C) was also measured. The surface tension remained unaltered (42.1 and 41.9 mN/m) at 25 or 37°C and even incubation at 60°C did not cause significant effect on surface tension (43.4 mN/m).

In a study by Khopade et al. (2012), the biosurfactant stability produced by *Nocardia* sp. B4 was tested at different temperature and pH values. The emulsification activity of the biosurfactant was quite stable at the temperatures tested, the emulsification activity (E24) varied from 75-90% over a range of temperature (30-100°C). Heating of the biosurfactant to 100°C caused no significant effect on the biosurfactant stability as indicated by emulsification activity of 75%. The biosurfactant stability was tested over a pH range (4-12), it was observed that the biosurfactant was relatively stable to pH changes between pH 8 and 12 as indicated by emulsification activity (E24=66-90%), showing highest stability at alkaline pH 9 (E24=90%) than acidic conditions (the emulsification activity varied from 10 to 30%). Maximum stability of biosurfactant (E24=90%) was observed at 3% NaCl concentration. Little changes were observed in increased concentration of NaCl up to 8% (E24 varied from 60-70%); at 9% NaCl concentration, the emulsification activity decreased to 50%.

2.21 APPLICATION OF BIOSURFACTANTS

Almost all surfactants currently in use are chemically synthesized. Nevertheless, in recent years, much attention has been directed towards biosurfactants due to their broad range functionality and the diverse capabilities of microbes. Most importantly, they

are environmentally accepted as they are readily biodegradable and have lower toxicity than synthetic surfactants (Desai and Banat 1997). A number of applications of biosurfactants have been researched and reported in literature proving the usefulness of these compounds to mankind in various fields.

2.21.1 APPLICATION OF BIOSURFACTANTS IN BIOREMEDIATION

Bioremediation is currently in vogue as a promising cost-effective and performance-effective technology to address numerous environmental pollution problems. These pollutants range from industrial wastes (e.g. polychlorinated biphenyls, trichloroethylene, pentachlorophenol and dioxin), polyaromatic hydrocarbons, refined petroleum products (e.g. jet fuel, gasoline, diesel fuel and the benzene, toluene, ethylbenzene and xylene cluster), acid mine drainage, pesticides, munitions compounds (e.g. trinitrotoluene), inorganic heavy metals to crude oil (Finnerty 1994). Biosurfactants are emerging as a technology to enhance the accessibility and bioavailability of hydrophobic chemicals, thereby complementing existing (bio) remediation methods (Finnerty 1994).

Maier and Soberon-Chavez (2000) indicated that rhamnolipid biosurfactant addition enhanced the biodegradation of hexadecane, octadecane, n-paraffin, and phenanthrene in liquid systems, in addition to hexadecane, tetradecane, pristine, creosote and hydrocarbon mixtures in soils. Two mechanisms for enhanced biodegradation are possible, enhanced solubility of the substrate for the microbial cells and interaction with the cell surface, which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily and degrade the pollutant (Zhang and Miller 1992; Shreve et al. 1995).

In a study by Hua et al. (2003), the effect of surfactant produced by *Candida antartica* was studied on the biodegradation of n-alkanes that included n-octane (C_8), n-nonane (C_9), n-decane (C_{10}), n-undecane (C_{11}), n-dodecane (C_{12}), n-tridecane (C_{13}), n-tetradecane (C_{14}) and n-hexadecane (C_{16}). The biodegradation rate of the alkanes were as follows: 66% of n-octane, n-nonane (C_9), 76.5% of n-decane (C_{10}), 90.2% of n-undecane (C_{11}), 82.7% of n-dodecane (C_{12}), 60.3% of n-tridecane (C_{13}), 37.7% of n-tetradecane (C_{14}) and 34.7% of n-hexadecane (C_{16}). They also reported the biosurfactant enhanced the degradation of kerosene, 65% of the hydrocarbon was degraded. They concluded that biosurfactant could be a promising choice in application of the practical bioremediation of petroleum pollution.

Obayori et al. (2009) investigated the biodegradative properties of crude oil and diesel using surfactant produced by *Pseudomonas* sp. LP1 strain. The results obtained confirmed the ability of strain LP1 to metabolize the hydrocarbon components of crude and diesel oil. They reported 92.34% degradation of crude oil and 95.29% removal of diesel oil. Biodegradative properties of surfactant producing *Brevibacterium* sp. PDM-3 strain were tested by Reddy et al. (2010). They reported that this strain could degrade 93.92% of the phenanthrene and also had ability to degrade other polyaromatic hydrocarbons such as anthracene and fluorene in the presence of biosurfactant.

Kang et al. (2010) used biosurfactant in studies on biodegradation of aliphatic and aromatic hydrocarbons as well as Iranian light crude oil under laboratory conditions. Addition of this biosurfactant to soil increased biodegradation of tested hydrocarbons with the rate of degradation ranging from 85% to 97% of the total amount of hydrocarbons. Their results indicated that biosurfactant may have potential for facilitating the bioremediation of sites contaminated with hydrocarbons having limited water solubility and increasing the bioavailability of microbial consortia for biodegradation.

Biosurfactants are also useful in bioremediation of sites contaminated with toxic heavy metals like uranium, cadmium, and lead. These observations suggest the usefulness

of natural microbial consortia and their products in solving environmental related problems. A rhamnolipid surfactant has been shown to be capable of removing cadmium, lead and zinc from soil (Herman et al. 1995). Moreover, rhamnolipid surfactant eliminated cadmium toxicity when added at a 10-fold greater concentration than cadmium and reduced toxicity when added at an equimolar concentration (Sandrin et al. 2000). Das et al. (2009) showed cadmium removal from aqueous solution by rhamnolipid surfactant, the addition of surfactant resulted in almost complete removal of 100 ppm of metal ions. The mechanism by which biosurfactant reduces metal toxicity may involve a combination of biosurfactant complexation of cadmium and biosurfactant interaction with the cell surface to alter cadmium uptake. Biosurfactants are efficient in removing bulk arsenic from mine tailings or contaminated soils under alkaline conditions (Wang and Mulligan 2009).

2.21.2 APPLICATION OF BIOSURFACTANT IN FOOD PROCESSING INDUSTRY

Biosurfactants have been used for various food processing application but they usually play a role as food formulation ingredient and anti-adhesive agents. As food formulation ingredient, they promote the formation and stabilization of emulsion due to their ability to decrease the surface and interfacial tension. It is also used to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf -life of starch-containing products, modify rheological properties of wheat dough and improve consistency and texture of fat based products (Krishnaswamy et al. 2008)

2.21.3 APPLICATION OF BIOSURFACTANT IN COSMETIC INDUSTRY

In the cosmetic industry, due to its emulsification, foaming, water binding capacity, spreading and wetting properties effect on viscosity and on product consistency, biosurfactant have been proposed to replace chemically synthesized surfactants. These surfactants are used as emulsifiers, foaming agents, solubilizers, wetting agents,

cleansers, antimicrobial agents, mediators of enzyme action, in insect repellents, antacids, bath products, acne pads, anti-dandruff products, contact lens solutions, baby products, mascara, lipsticks, toothpaste, dentine cleansers, etc (Gharaei-Fathabad 2011).

2.21.4 APPLICATION OF BIOSURFACTANTS IN MEDICINE

Mukherjee et al. (2006) elucidated on the wide range of applications of biosurfactants in medicine they include:

2.21.4.1 ANTIMICROBIAL ACTIVITY

Biosurfactant present an opportunity to be developed as novel antibiotics. They have been reported to have antimicrobial activity against bacteria, fungi, algae and viruses. The high demand for new antimicrobial agents following increased resistance shown by pathogenic microorganisms against existing antimicrobial drugs has drawn attention to biosurfactants as antibacterial agents (Beahl 2006). Some biosurfactants have been reported to be suitable alternatives to synthetic medicines and antimicrobial agents and may therefore be used as effective and safe therapeutic agents, of which the most notable are rhamnolipids and lipopeptides (Singh and Cameotra 2004).

The diverse structures of biosurfactants confer them the ability to display versatile performance. By its structure, biosurfactants exerts its toxicity on the cell membrane permeability bearing the similitude of a detergent like effect (Zhao et al. 2010). Mannosylerythritol lipids (MEL-A and MEL-B) produced by *Candida antarctica* strains have also been reported to exhibit antimicrobial action against Gram-positive bacteria (Kitamoto et al. 1993).

The antifungal activity against phytopathogenic fungi has been demonstrated for glycolipids, such as cellobiose lipids (Kulakovskaya et al. 2010), rhamnolipids (Debode et al. 2007; Varnier et al. 2009) and cyclic lipopeptides (Tran et al. 2007), including

surfactin, iturin and fengycin (Velmurugan et al. 2009; Arguelles-Arias et al. 2009; Snook et al. 2009; Grover et al. 2010). Thimon et al. (1995) described another antifungal biosurfactant, iturin by *Bacillus subtilis*, which affects the morphology and membrane structure of yeast cells.

Antiviral activity of biosurfactants, mainly surfactin and its analogues, has also been described (Naruse et al. 1990). Antibiotic effects and inhibition of growth of human immunodeficiency virus in leukocytes by biosurfactants have been cited in the literature (Kitomato et al. 1993). The succinoyl-trehalose lipid of *Rhodococcus erythropolis* has been reported to inhibit herpes simplex virus and influenza virus with a lethal dose of 10 to 30 μg/ml (Uchida et al. 1989). The more effective inactivation of enveloped viruses, such as retroviruses and herpes viruses, compared to non-enveloped viruses, suggests that this inhibitory action may be mainly due to physico-chemical interactions between the virus envelope and the surfactant (Vollenbroich et al. 1997). Lipopeptides from *Bacillus subtilis* fmbj inactivated cell-free virus of porcine parvovirus, pseudorabies virus, new castle disease virus and bursal disease virus, while it effectively inhibited replication and infectivity of the new castle disease virus and bursal disease virus but had no effect on pseudorabies virus and porcine parvovirus (Huang et al. 2006). Sophorolipids are also claimed to have activity against human immunodeficiency virus (Shah et al. 2005). Similarly, a rhamnolipid and its complex with alginate, both produced by a *Pseudomonas sp.* strain, showed significant antiviral activity against herpes simplex virus types 1 and 2 (Remichkova et al. 2008).

Gharaei-Fathabad (2011) reported that several biosurfactants have strong antibacterial, antifungal and antivirus activity; these surfactants play the role of anti-adhesive agents to pathogens making them useful for treating many diseases as well as its use as therapeutic agent.

2.21.4.2 ANTI-CANCER ACTIVITY

Some microbial extracellular glycolipids induce cell differentiation instead of cell proliferation in the human promyelocytic (PC 12) leukemia cell line, also, exposure of PC 12 cells to biosurfactant enhanced the activity of acetylcholine esterase and interrupted the cell cycle at the G1 phase with resulting overgrowth of neurites and partial cellular differentiation, this suggest that biosurfactant induces neuronal differentiation in PC 12 cells and provides the ground work for the use of microbial extracellular glycolipids as novel reagents for the treatment of cancer cells (Krishnaswamy et al. 2008).

2.21.4.3 ANTI-ADHESIVE AGENTS

Biofilm formation and swarming motility are the key microbial activities in the colonization of a surface and therefore can increase the chance of nosocomial infections on different medical devices (Vinh and Embil 2005). In addition to their direct action against pathogens, biosurfactants can also alter the physical and chemical condition of the environment where biofilms are developing (Mireles et al. 2001). Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites. Rodrigues et al. (2006) demonstrated that pre-coating vinyl urethral catheter by running the biosurfactant solution through them before inoculation with media resulted in the decrease in the amount of biofilm formed by *Salmonella typhimurium, Salmonella enterica, Escherichia coli* and *Proteus mirabilis*. Muthusamy et al. (2008) reported that pretreatment of silicone rubber with *Streptococcus thermophilus* surfactant inhibited 85% adhesion of *Candida albicans* and surfactants from *Lactobacillus fermentum* and *Lactobacillus acidophilus* adsorbed on glass, reduced by 77% the number of adhering uropathogenic cells of *Enterococcus faecalis*.

2.21.4.4 IMMUNOLOGICAL ADJUVANTS

Bacterial lipopeptides constitute potent non-toxic, non-pyrogenic immunological adjuvants when mixed with conventional antigens. An improvement of the humoral

humane response was demonstrated when low molecular mass antigens Iturin AL and herbicolin A were used (Gharaei-Fathabad 2011).

2.21.5 APPLICATION OF BIOSURFACTANTS IN AGRICULTURE

The agricultural industry has also benefited from the production of biosurfactants. Stanghellini and Miller (1997) demonstrated that rhamnolipids are highly effective against three representative genera of zoosporic plant pathogens; *Pythium aphanidermatum*, *Phytophthora capsici* and *Plasmopara lactucea-radicis* and caused cessation of motility and lysis of the entire zoospore population in less than 1 minute. Mosquito larvicidal potency of cyclic lipopeptides (CLPs) secreted by two *Bacillus subtilis* strains DM-03 and DM-04 were studied against third instar larvae of *Culex quinquefasciatus.* These properties of *Bacillus subtilis* CLPs can be exploited for the formulation of a safer, novel biopesticide for effective control of mosquito larvae (Das and Mukherjee 2006). Bioemuslifiers are potentially used in various formulations of herbicides and pesticides (Rosenberg and Ron 1999). Example is the use of glycolipopeptide from *Bacillus sp.* for emulsifying immiscible organophosphorus pesticides (Patel and Gopinath 1986). A cost-effective application of surfactin in mechanical dewatering of peat has been demonstrated (Cooper et al. 1988).

2.21.6 APPLICATIONS OF BIOSURFACTANTS IN COMMERCIAL LAUNDRY DETERGENTS

Almost all surfactants, an important component used in modern day commercial laundry detergents, are chemically synthesized and exert toxicity to fresh water living organisms. Growing public awareness about the environmental hazards and risks associated with chemical surfactants has stimulated the search for ecofriendly, natural substitutes of chemical surfactants in laundry detergents. Biosurfactants such as cyclic lipopeptide (CLP) are stable over a wide pH range (7.0- 12.0) and heating them at high temperature does not result in any loss of their surface-active property (Mukherjee 2007).

They also show good emulsion formation capability with vegetable oils and demonstrate excellent compatibility and stability with commercial laundry detergents favouring their inclusion in laundry detergents formulation (Das and Mukherjee 2007).

2.21.7 APPLICATION OF BIOSURFACTANTS IN NANOTECHNOLOGY

New developments have been made in the area of nanotechnology. Surfactants are emerging as potential nanoparticle stabilizing agents, however, the synthetic surfactants are not economically viable as well as they are not environmentally friendly. Therefore, the biosurfactants are emerging as a green alternate for the synthesis and stabilization of nanoparticles. It was found that the nano-scale silver can be synthesized in reverse micelles using the glycolipid produced by *Brevibacterium casei* MSA19 as stabilizer (Kiran et al. 2010). Rhamnolipids have also been employed to stabilize the silver nanoparticles in liquid phase (Xie et al. 2006; Kumar et al. 2010). Reddy et al. (2009) showed that synthesis of silver nanoparticles could be stabilized by surfactin. Similarly, sophorolipids have been used as reducing and capping agents in synthesis of silver nanoparticles (Kasture et. al. 2008). Nano-rods of NiO were produced by a water-in-oil microemulsion (Palanisamy et. al. 2008). Also, rhamnolipid was evaluated for its effect on the electrokinetic and rheological behavior on nanozirconia particles (Biswas and Raichur 2008). Therefore, the biosurfactant-mediated nanoparticles synthesis can be considered as "green" stabilizer of nanoparticles.

Based on the extensive literature survey on the isolation and screening of surfactant producing microorganisms, medium formulation, studies on the effect of various process parameters on biosurfactant production, partial purification, characterization and application of biosurfactant, it was found that more number of candidate surfactant producers showing high surfactant productivity and pronounced surface tension reduction capacity need to be isolated from potential ecological sources. The identification of potential ecological sources for the isolation of candidate surfactant producers, development of accurate, reliable and rapid screening methods for screening

and the selection of surfactant producers holds the key to the discovery of new surfactant producing strains. The success of the production of biosurfactants is dependent on finding the suitable conditions for their enhanced production. With increase in the number of petroleum hydrocarbon polluted sites and oil spill accidents, biosurfactants are in great demand.

CHAPTER 3

MATERIAL AND METHODS

This chapter describes the experimental details of various methodologies adopted in the present study. The detailed procedure of enrichment and isolation of the surfactant producing microorganisms; selection of the surfactant producers using various screening methods and identification of the candidate surfactant producing bacteria has been presented. The chapter presents the details on the extraction of the extracellular surfactant from the cell-free broth. The procedures employed for the studies on the various factors affecting the production of surfactant, optimization of process variables using statistical method and kinetics of surfactant production have been presented. The experimental procedures followed in partial purification and characterization of surfactant, studies on the effect of various environmental factors affecting surfactant stability and efficiency of surfactant in the biodegradation of crude oil have been presented. In the present study, all the experiments were conducted in triplicates.

3.1 MATERIALS

The materials used in the present study are presented below:

Chemicals	Company and Grade
Beef extract, dextrose, starch, Folin-Ciocalteu, peptone, potassium dihydrogen phosphate, sodium dihydrogen phosphate, sodium nitrate, di-potassium hydrogen phosphate, urea, fructose, potassium chloride, sodium nitrate, ammonium chloride, ferrous sulphate, manganous sulphate, magnesium sulphate, hydrochloric acid, sodium hydroxide, ammonium nitrate, ferric citrate, sodium bicarbonate, ammonium nitrate, disodium hydrogen phosphate, n-hexadecane, chloroform,	Merck

sodium dodecyl sulphate, ethyl acetate, acetone, diethyl ether, disodium hydrogen phosphate, tryptone, dichloromethane, sodium potassium tartarate, paraffin, butanol, acetic acid, Tris HCl, ammonium chloride, copper sulphate, zinc sulphate, ferrous sulphate, ethylene Diamine tetra acetic acid, glycerol, toluene, nickel chloride, cobaltous chloride, potassium iodide, phosphoric acid, silica gel G, potassium borate, sodium thiosulphate, sodium citrate, 3,5-dinitrosalicylic acid, sodium sulphite, potassium sodium tartarate	
Agar, calcium chloride, methylene blue, glycerol, bromothymol blue, ferrous ammonium sulphate, ammonium dihydrogen orthophosphate, potassium nitrate, sodium chloride, potassium hydroxide, sodium carbonate, hydrogen peroxide, ammonium molybdate, magnesium chloride, boric acid, methanol, ferrous chloride, Gram staining kit, phenol, sulphuric acid, iodine, ethanol, ammonium nitrate, sodium molybdate, malachite green, phenol red, alpha naphthol, gelatin, ninhydrin, Kovac's reagent, oxidase discs, phenol	Nice
Nutrient Agar, meat extract, steapsin (lipase)	Hi media
Bovine Serum Albumin	Sigma
Lactose, yeast extract, cetyl triammonium bromide	Central drug house, DH
Sucrose	Thomas baker
Crude oil	Bombay High, India
Sodium silicate	Loba Chemie Pvt. Ltd.
Sodium fluoride	SRL
Coconut oil, olive oil, sunflower oil, Coconut oil cake, peanut oil cake, orange peelings, whey, gasoline, kerosene, motor oil	Locally available
Molasses	Indian Molasses

	Company Limited, Mangalore

3.2 METHODS

The following methodologies were used in the present study:

3.2.1 SAMPLE COLLECTION

The soil and water samples for the isolation of microorganisms were collected from various hydrocarbon contaminated localities in and around Mangalore, Karnataka, India. 100 grams of soil sample was collected from the surface of each site using sterile spatulas and they were transferred into sterile polythene bags. 100 ml of water sample was collected in sterile bottles. The description of the soil and water samples collected is given in table 3.1 and 3.2. The samples were stored at 4ºC till use.

Table 3.1 Description of the soil samples collected

Sl. no.	Name of the sample	Description	Location
1.	S1	Soil around petroleum refining industry	Mangalore Refineries and Petrochemicals Limited, Mangalore, Karnataka, India
2.	S2	Soil around automobile workshop	Mangaladevi, Mangalore, Karnataka, India
3.	S3	Soil around petrol filling station	Mangalore Service Station, Circuit House, Mangalore, Karnataka, India
4.	S4	Soil around diesel filling station	Mangalore Service Station, Circuit House, Mangalore, Karnataka, India

5.	S5	Soil around castrol storage area	Falnir, Mangalore, Karnataka, India
6.	S6	Control	NITK, Surathkal, Karnataka, India

Table 3.2 Description of the water samples collected

Sl. no.	Name of the sample	Description	Location
1.	M1	Water collected from port	New Mangalore Port Trust, Mangalore, Karnataka, India
2.	M2	Water around dock area	Sulthan battery, Mangalore, Karnataka, India
3.	M3	Beach water	Panambur beach, Mangalore, Karnataka, India
4.	M4	Waste water	Mangalore Refineries and Petrochemicals Limited, Mangalore, Karnataka, India
5.	M5	Control	NITK beach, Surathkal, Karnataka, India

3.2.2 ENRICHMENT AND ISOLATION OF MICROORGANISMS

The isolation of microorganisms from hydrocarbon contaminated soil and water samples was carried out by enrichment technique. The basal medium prescribed by Bushnell and Hass (1941) was used for the isolation of the microorganisms from soil. The medium composition is presented in Appendix I.

The soil samples were subjected to enrichment in Bushnell and Hass liquid medium for the isolation of microorganisms. 10 grams of each of the soil sample was

suspended in conical flasks, 100 ml of basal medium containing glucose and 1% (v/v) crude oil was added to the flasks. The media were incubated at 30ºC for 5 days at 150 rpm in an incubator shaker (Scigenics, India). For the enumeration of the total number of microorganisms present in the soil samples, the enriched samples were subjected to serial dilution up to 10^{-5} dilution. 100 µl of the 10^{-5} diluted medium was inoculated on to Bushnell and Hass agar medium using a sterile spreader. The plates were incubated at 30ºC for 2 days. The colonies formed on each plate were counted using a colony counter and the Colony Forming Units (CFU) per ml was calculated by standard plate count method.

The isolation of microorganisms from water samples was carried out in mineral broth medium as prescribed by Maneerat and Phetrong (2007). The composition of the medium is presented in Appendix II.

The water samples collected were subjected enrichment in mineral broth medium. 10 ml of each sample was suspended in conical flasks containing 90 ml of mineral broth medium with glucose and 1% (v/v) crude oil; the media were incubated at 30ºC for 5 days at 150 rpm in an incubator shaker. The total number of microorganisms present in the samples was enumerated by subjecting the enriched samples to serial dilution up to 10^{-5} dilution. 100 µl of the 10^{-5} diluted medium was inoculated on to mineral agar medium using a sterile spreader. The plates were incubated at 30ºC for 2 days. The colonies formed on each plate were counted using a colony counter and the Colony Forming Units (CFU) per ml was calculated by standard plate count method.

3.2.3 SCREENING SURFACTANT PRODUCING MICROORGANISMS

The enriched soil and water samples were subjected to the screening procedures for the selection of surfactant producers. Initially, the microorganisms were screened on the selective Cetyl Trimethyl Ammonium Bromide (CTAB)-methylene blue agar medium. The isolates showing halo formation on the selective media indicating surfactant

production were further confirmed for their surfactant production ability using other screening tests. Drop collapse assay and surface tension measurement were the other screening tests used.

The data obtained in the present investigation was compared with surfactant producing microorganisms that were procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, Maharashtra, India. Bacterial culture, *Pseudomonas aeruginosa* (ATCC 10145) and fungal culture, *Phaenerochaete chrysosporium* (NCIM 1197) were the positive controls used. They were selected based on references in the literature (Matsyuma et al. 1986; Sim et al. 1997).

3.2.3.1 SCREENING ISOLATES ON THE SELECTIVE CETYL TRIMETHYL AMMONIUM BROMIDE (CTAB)-METHYLENE BLUE AGAR MEDIUM

The enriched broth samples were serially diluted up to 10^{-5} dilution and inoculated on to Cetyl Trimethyl Ammonium Bromide (CTAB)-methylene blue agar medium designed by Siegmund and Wagner (1991) in order to screen the surfactant producing microorganisms. The composition of the Cetyl Trimethyl Ammonium Bromide (CTAB)-methylene blue agar medium is given in Appendix III. The plates were incubated at 30ºC for 5-7 days. Colonies formed on the CTAB-methylene blue agar medium were isolated and subsequently subjected to two cycles of enrichment. The media were incubated at 30ºC for 2 days at 150 rpm in an incubator shaker.

3.2.3.2 DROP COLLAPSE ASSAY

The drop-collapse assay was performed on the microtitre plate lids following method described by Bodour and Miller-Maier (1998). The isolates were grown in the enrichment medium and incubated with shaking at 37ºC and 150 rpm for 5 days. The culture broth was subjected to centrifugation at 10,000 rpm for 15 minutes. Both the supernatant and the pellet were screened by drop collapse assay. The assay was performed on a 96 well microtitre plate lids (Hi Media, India). The microtitre plate lids

used were rinsed with hot water followed by ethanol washing. The plate lids were then washed with distilled water and dried. The lids were coated with 2 µl of hydrocarbon. The hydrocarbons evaluated for this test were motor oil, hexadecane, petrol and kerosene oil. The lids were equilibrated for 24 hours to ensure uniform oil coating. A 5 µl aliquot of sample was mixed with 2 µl of methylene blue and then applied onto the center of the oil drops using a micropippetor (Transpette, India). The results were monitored visually within 1 minute. If the drop remained beaded, the result was scored as negative. If the drop collapsed, the result was scored as positive. For comparison, water was used as negative control and sodium dodecyl sulphate (SDS), a synthetic surfactant, was used as positive control. In addition, the diameter of the drop following collapse was also measured.

3.2.3.3 SURFACE TENSION MEASUREMENT

The culture broths of the isolates were centrifuged at 9000 rpm at 4ºC for 30 minutes to separate the culture supernatant and the cell pellet. For the determination of surface tension, the cell-free broth as well as the microorganism pellet was subjected to measurement of surface tension according to the Du Nuoy ring method at room temperature using a tensiometer (Surface tension meter, DST 30 series, Surface and Electro Optics Corporation, Korea) (Willumsen and Karlson 1997). The surface tension values obtained were compared with that of water, which served as the negative control and SDS, which was used as the positive control. All the surface tension value readings were recorded in triplicates.

3.2.4 CULTURE MAINTENANCE AND STORAGE

Nutrient Agar (NA) medium was used for growth and maintenance of bacteria. Nutrient agar medium containing the components-peptone 5.0 g/L, sodium chloride 5.0 g/L, yeast extract 2.0 g/L, beef extract 1.0 g/L and agar 15.0 g/L was used and the initial pH of the medium was maintained at 7.0±0.2. The medium components were suspended in distilled water before autoclaving at 121ºC for 15 minutes at 15 psi. The medium was

then cooled to approximately 50ºC prior before pouring into sterile petri dishes. The molten agar was left to cool and gel at room temperature. The bacteria were streaked on the agar medium and incubated at 30ºC±0.2 for 24 hours and then maintained at 4ºC. The pure cultures of bacteria were sub-cultured at regular intervals.

The selected bacterial cultures were also stored in glycerol suspension at 20ºC. 10% (v/v) glycerol was prepared and autoclaved at 121ºC for 15 minutes at 15 psi. The isolates in their exponential stage were suspended in the sterile glycerol suspension and immediately frozen. The bacterial cultures were recovered when required after thawing the culture vials at 37ºC in a water bath.

3.2.5 IDENTIFICATION OF THE POTENTIAL SURFACTANT PRODUCING BACTERIAL ISOLATE-2B

Based on the results of various screening test results, the bacterial isolate, designated as 2B, was identified as potential surfactant producer and it was selected for further studies. The selected bacterial isolate was identified on the basis of morphological characteristics, different staining techniques, biochemical characteristics (Aneja 2003) and 16 S ribosomal DNA sequencing. The preliminary identification of the bacterial isolate was done based on Bergey's Manual of Systemic Bacteriology *i.e.*, following the dichotomous key of identification and the biochemical tests for the same (Buchanan et al. 1974; Cappuccino and Sherman 2000).

3.2.5.1 COLONY CHARACTERISTICS

Density, form, margin, elevation and colour were the different colony characteristics considered on the basis of which the isolate was characterized.

3.2.5.2 MICROSCOPIC OBSERVATIONS

The various staining techniques employed in the identification of the bacterial isolate were as follows:

3.2.5.2.1 SIMPLE STAINING

A thin bacterial smear was made on a clean glass slide. It was flooded with 2-3 drops of crystal violet for 30 seconds. The slide was washed, air dried and observed through the light microscope.

3.2.5.2.2 GRAM STAINING

Thin smear of the bacterial isolate was prepared and it was heat fixed aseptically. Crystal violet was added on the smear and left for 30 seconds. The slide was washed with distilled water and then flooded with Gram's iodine for 60 seconds. Iodine on the slide was washed with distilled water followed by wash with 95% ethanol. The slide was washed with distilled water. Safranine was added to the slide, kept for 30 seconds and slide was washed with water. The slide was blot dried before observing under microscope.

The presence of purple colour of the isolate indicates that the organism is Gram positive while the presence of pink colour of the isolate indicates that the bacterium is Gram negative. Further, the morphology of the isolate was visualized. The presence of rod shaped bacterium indicates that it is bacilli while round shaped bacterium indicates that it is cocci.

3.2.5.2.3 CAPSULE STAINING

Thin smear of the bacterial isolate was made on a clean glass slide and it was heat fixed. The slide was flooded with crystal violet and incubated for 3 seconds. It was

washed with distilled water and the smear was further treated with 20% copper sulphate. The slide was rinsed with distilled water and observed under microscope.

3.2.5.2.4 ENDOSPORE STAINING

A thin bacterial smear of the bacterial isolate was prepared on a clean slide. The slide was air dried and gently heat fixed aseptically. The slide was covered with a piece of paper towel and placed on a staining rack over the water bath. The paper towel was flooded with Malachite green (primary stain). The slide was steamed for 5 minutes. The slide was allowed to cool and then rinsed with deionized water. Excess water was drained off. Safranine (counter-stain) was added to the slide and kept aside for 2 minutes. Excess safranine was rinsed off with deionized water and the slide was blot dried. The slide was examined with a light microscope for the presence of green coloured endospores.

3.2.5.2.5 MOTILITY TEST

A small drop of liquid bacterial isolate was placed at the centre of a coverslip. A small drop of water was added to the isolate and mixed well. A convex slide was inverted with a central depression over the coverslip and the coverslip was stuck to the slide with paraffin. The slide was inverted so that the drop of bacterial isolate was suspended in the well of the slide. The slide was examined microscopically for motility.

A darting, zig-zag, tumbling or other organized movement indicates that the organism is motile where as no movement or Brownian motion indicates that the organism is non-motile.

3.2.5.3 BIOCHEMICAL CHARACTERISTICS

The following biochemical tests were performed to in order to characterize the isolated bacterium 2B:

3.2.5.3.1 AMYLASE PRODUCTION TEST

Starch agar medium was prepared according to the composition given in the Appendix IV 4.1. The sterilized media was poured into sterile petriplates in aseptic environment. The pure culture of the isolate was aseptically inoculated on to these petriplates. The plates were incubated at 37ºC for 48 hours. After the incubation, the plates were flooded with 5% iodine solution. The plates were observed for the formation of clear zone surrounding the growth for positive test for amylase production.

3.2.5.3.2 CATALASE TEST

The bacterial smear of the isolate was placed on a clean glass slide and 2-3 drops of 3% hydrogen peroxide was added over it. The slide was observed for the production of gas bubbles which indicates a positive reaction for catalase.

3.2.5.3.3 METHYL RED TEST

Glucose broth was prepared (Appendix 1V 4.2) and autoclaved. The pure culture of the isolate was inoculated into the test tubes containing the broth. The tubes were incubated at 37ºC for 48 hours. A few drops of methyl red solution were added to the culture after the incubation. The formation of red colour indicates positive result and yellow colour indicates negative result for methyl red test.

3.2.5.3.4 VOGES- PROSKAUER TEST

Glucose broth was prepared (Appendix IV 4.2), the broth was added into the test tubes and sterilized. The pure culture of the bacterial isolate was inoculated into the broth. The tubes were incubated at 37°C for 48 hours. A few drops of alpha naphthol solution were added, followed by the addition of 1 ml of 40% KOH after the incubation of the culture. The tubes were mixed well and allowed to stand for 30 minutes. The

formation of pink colour indicates positive result and no change in color indicates negative result for Voges test.

3.2.5.3.5 INDOLE PRODUCTION TEST

Tryptone broth (Appendix IV 4.3) was prepared and sterilized. The media was poured into sterile test tubes and the isolate was inoculated. The isolate was incubated at 37ºC for 48 hours. 1 ml of Kovacs reagent was added and observed for colour change. A bright pink colour in the top layer indicates the presence of indole.

3.2.5.3.6 CITRATE UTILIZATION TEST

Simmon's citrate agar medium (Appendix IV 4.4) was prepared, sterilized and poured into sterile test tubes. The isolate was streaked on to the medium. The tubes were incubated at 37ºC for 48 hours. The growth of the isolate on the medium was observed. The change in colour of the medium from its initial green to deep blue indicates positive test for citrate utilization.

3.2.5.3.7 FERMENTATION OF CARBOHYDRATES

In order to test for the formation of fermentation products, tubes were inoculated with the isolate and incubated into the media containing a single carbohydrate (Appendix IV 4.5) (such as glucose, lactose or maltose), a pH indicator (such as phenol red) and a Durham’s tube (a small inverted tube to detect gas production).

If a particular carbohydrate is fermented by the bacterium, acidic end products will be produced which lowers the pH, causing the pH indicator to change colour of the phenol red to yellow. If gas is produced along with the acid, it collects in the Durham tube as a gas bubble. If the carbohydrate is not fermented, no acid or gas will be produced and the phenol red will remain red in colour.

3.2.5.3.8 UREASE TEST

The surface of a urea agar medium (Appendix IV 4.6) slant was streaked with the isolate to be tested and incubated for 8-24 hours at 35ºC. When the isolate utilize urea, ammonia is formed which makes the reaction of the media alkaline, producing a pink-red colour (due to the change in the phenol red indicator). The agar slant and butt of the tube remain light orange in case of a negative test.

3.2.5.3.9 OXIDASE TEST

A piece of the oxidase test paper was held with forceps and touched onto an area of heavy bacterial growth. Colour change to purple within 10 seconds indicates positive test for oxidase where as colour change occurring after 60 seconds indicates negative test for oxidase.

3.2.5.3.10 HYDROGEN SULPHIDE PRODUCTION TEST

The triple sugar iron agar medium (Appendix IV 4.7) was autoclaved and poured into sterile test tubes. The test tubes were inoculated with the bacterial isolate and incubated at 37ºC for 24-48 hours. Blackening of the medium indicates hydrogen sulphide (H_2S) production by the bacterium. No colour change is observed in the case of negative reaction.

3.2.5.3.11 HYDROLYSIS OF GELATIN

The gelatin agar medium (Appendix IV 4.8) was prepared and sterilized. Following sterilization, the medium was inoculated with the isolate and incubated at 37ºC until the gelatin liquefied. The tubes were kept for 48 hours or longer. At the end of the incubation period, the tubes were placed into a 4ºC refrigerator for 30 minutes to 1 hour. The liquified form of the medium implies positive test for gelatin hydrolysis whereas the presence of gel form of the medium indicates a negative reaction.

3.2.5.4 IDENTIFICATION OF BACTERIAL ISOLATE BY PARTIAL 16S RIBOSOMAL DNA SEQUENCING

The bacterial isolate 2B was sent to Agharkar Research Institute, Pune, Maharashtra, India for partial 16S ribosomal DNA sequencing for its identification. The detailed procedure of sequencing is presented in Appendix V. The sequence obtained was compared with National Center for Biotechnology Information Gen Bank entries by using the BLAST algorithm.

3.2.6 SURFACTANT EXTRACTION

Since the surfactant produced by 2B and *Pseudomonas aeruginosa*, respectively, was extracellular in nature as indicated by screening tests, the surfactant was extracted from the whole cell-free culture broth. The cell pellet was removed by centrifugation at 9000 rpm at 4ºC for 30 minutes. Various methods were used for the extraction of the surfactant from the cell-free broth; they were acidification of the cell-free broth followed by extraction using chloroform-methanol mixture (2:1), acidification of the cell-free broth followed by extraction using ethyl acetate, acid precipitation, extraction using acetone and extraction using diethyl ether. The surfactant obtained was then dried at 60ºC till a constant weight in order to quantify the surfactant produced by the bacterial strains (Samadi et al. 2007). The surfactant obtained was weighed using a weighing balance (Sartorius, Germany).

3.2.7 INOCULUM PREPARATION

Inoculum was prepared by growing the bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively, in 50 ml of sterile Nutrient broth (Appendix VI) in 100 ml of Erlenmeyer flasks. The sterile medium was inoculated with one loop full of pure cultures from preserved agar slants. The flasks were incubated at 30°C in an incubator shaker at 150 rpm for 24 hours.

3.2.8 SELECTION OF SURFACTANT PRODUCTION MEDIA

The medium selection for the production of surfactants by the bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively, was carried out in four different surfactant production media (designated as M1, M2, M3 and M4) by shake flask method. The details of the different medium compositions are given in Appendix VII. Twenty four hour cultures of the bacterial strains were inoculated into conical flasks containing the surfactant production media. The flasks were incubated in an incubator shaker at 150 rpm at 30ºC for 7 days. All the flasks were kept in triplicates. The optical density of the medium, bacterial biomass, surfactant concentration and surface tension of the supernatant were evaluated at regular intervals. The growth of the bacterial strains was determined by measuring the optical density of the medium and dry weight of the strains. The procedure of optical density and dry weight measurements are explained in Appendix VIII and IX, respectively.

3.2.9 TIME COURSE STUDY OF SURFACTANT PRODUCTION

250 ml Erlenmeyer flasks containing 100 ml of the PPGAS medium were inoculated with cell cultures of 2B and *Pseudomonas aeruginosa*, respectively. All batch runs were performed in triplicate at 30°C utilizing a rotary shaker at 150 rpm. Samples were collected every 4 hours, transferred to Eppendorfs tubes and then centrifuged at 10,000 rpm for 20 minutes at 4ºC for estimation of bacterial biomass. The total biomass, residual glucose concentration, surface tension measurement and surfactant production were analyzed at regular time intervals. The residual glucose estimation was performed using DNS method presented in appendix X.

3.2.10 STUDIES ON THE EFFECT OF VARIOUS PROCESS PARAMETERS ON SURFACTANT PRODUCTION

Proteose Peptone Glucose Ammonium Salt (PPGAS) medium showing maximum surfactant production by the bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively, was selected to study the effect of various process parameters on surfactant production. Experiments were conducted to determine the effect of process parameters

like inoculum volume, initial pH of the medium, incubation temperature, agitation speed, carbon and nitrogen sources, inducers, buffers and salinity on the surfactant production. All the parameters were performed in shake flasks in triplicates.

3.2.10.1 EFFECT OF INOCULUM VOLUME ON SURFACTANT PRODUCTION

To study the effect of inoculum volume on surfactant production by 2B and *Pseudomonas aeruginosa*, respectively, in PPGAS medium, the media were inoculated with different inoculum volumes [0.5%, 1%, 2%, 3%, 5%, 7% and 10% (v/v)] of the bacterial strains and incubated at 30ºC in an incubator shaker at 150 rpm. All the flasks were kept in triplicates. Determination of growth of the culture, surfactant productivity and surface tension measurements of the supernatant were carried out at regular time intervals.

3.2.10.2 EFFECT OF INITIAL MEDIUM pH ON SURFACTANT PRODUCTION

To study the effect of different pH on the production of surfactant by 2B and *Pseudomonas aeruginosa*, respectively, the initial pH of the PPGAS medium was adjusted to different pH. The range of pH used for the study was 3, 5, 6, 7, 8, 9, 11 and 13. The pH was adjusted with 0.1 M NaOH or HCl using a pH meter. The flasks containing media were autoclaved and then inoculated with the bacterial strains. The flasks were incubated at 30°C in an incubator shaker at 150 rpm. All the flasks were kept in triplicates. The bacterial biomass, surfactant productivity and surface tension measurements of the supernatant were estimated at regular time intervals.

3.2.10.3 EFFECT OF INCUBATION TEMPERATURE ON SURFACTANT PRODUCTION

The effect of incubation temperature was studied on surfactant production by 2B and *Pseudomonas aeruginosa*, respectively, by varying the incubation temperature. The flasks containing sterile PPGAS medium (initial pH 7) were inoculated with the desired culture volumes and incubated at different temperatures such as 10, 20, 30, 37, 45 and 60ºC. All the flasks were kept in triplicates in an incubated shaker at 150 rpm. The bacterial growth, surfactant productivity and surface tension values of the supernatant were estimated at regular time intervals.

3.2.10.4 EFFECT OF AGITATION SPEED ON SURFACTANT PRODUCTION

The effect of agitation speed was studied on surfactant production by 2B and *Pseudomonas aeruginosa*, respectively, by varying the agitation speed. The flasks containing sterile PPGAS medium (initial pH 7) were inoculated with the desired culture volumes and incubated at different agitation speeds such as 50, 100, 150, 200 and 250 rpm in an incubated shaker at 37ºC. The bacterial growth, surfactant productivity and surface tension values of the supernatant were estimated at regular time intervals.

3.2.10.5 EFFECT OF CARBON SOURCE ON SURFACTANT PRODUCTION

The effect of various carbon sources was studied on surfactant production by bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively, by varying the carbon source in the shake flask studies. Different carbon sources evaluated in the study were glucose, sucrose, molasses, coconut oil cake, peanut oil cake, ethanol, glycerol, whey and orange peelings (added at a concentration of 5 g/L w/v for solid carbon source or 5 ml/L v/v for liquid carbon source). The flasks containing sterile PPGAS medium (initial pH 7) were inoculated with the desired culture volumes and incubated at 37ºC in an incubated shaker at 150 rpm. A control flask (without the carbon source) was also maintained. Further, the concentration of the suitable carbon source resulting in maximum surfactant production by the bacterial strains was also studied; the concentration of carbon source was varied from 5-100 g/L (w/v). All the flasks were maintained in triplicates. Bacterial

biomass, surfactant productivity and surface tension measurements of the supernatant were estimated at regular time intervals.

3.2.10.6 EFFECT OF NITROGEN SOURCE ON SURFACTANT PRODUCTION

The effect of different nitrogen source and their concentration on surfactant production by was studied by 2B and *Pseudomonas aeruginosa*, respectively, was studied by varying the nitrogen source in the shake flask experiments. The nitrogen sources tested in the present study included peptone, yeast extract, urea, ammonium nitrate, ammonium chloride and potassium nitrate. The flasks containing sterile PPGAS medium (initial pH 7) were inoculated with the desired culture volumes and incubated at 37ºC in an incubated shaker at 150 rpm. A control flask (without the nitrogen source) was also maintained. Further, the effect of concentration of suitable nitrogen source on surfactant production by the bacterial strains was also studied; the concentration of nitrogen source was varied from 0.5-10 g/L. All the flasks were kept in triplicates. Determination of growth of the isolate, surfactant productivity and surface tension of the supernatant were estimated at regular time intervals.

3.2.10.7 EFFECT OF INDUCER ON SURFACTANT PRODUCTION

The effect of different inducers on surfactant production by 2B and *Pseudomonas aeruginosa*, respectively, was studied by varying the inducer (at 1% (v/v) concentration) in the shake flask experiments. The inducers evaluated in the study were olive oil, n-hexadecane, crude oil, toluene, paraffin, sunflower oil and coconut oil. The flasks containing sterile PPGAS medium (initial pH 7) were inoculated with the desired culture volumes and incubated at 37ºC in an incubated shaker at 150 rpm. A control flask (without the inducer) was also maintained. The concentration of the suitable inducer was further tested to study its effect on surfactant production by the bacterial strains. The concentration of the inducer was varied from 0.5-5% (v/v). All the flasks were kept in triplicates. Determination of growth of the isolate, surfactant productivity and surface tension measurements of the supernatant was performed at regular time intervals.

3.2.10.8 EFFECT OF BUFFER ON SURFACTANT PRODUCTION

The effect of different buffer systems was studied on surfactant production by 2B and *Pseudomonas aeruginosa*, respectively, by varying the buffer in the shake flask experiments. Tris HCl buffer and phosphate buffer were tested for their ability to maximize the productivity of surfactant. The flasks containing sterile PPGAS medium (initial pH 7) were inoculated with the desired culture volumes and incubated at 37ºC in an incubated shaker at 150 rpm. A control flask (without the buffer) was also maintained. All the flasks were kept in triplicates. The bacterial biomass, surfactant productivity and surface tension values of the supernatant were determined at regular time intervals.

3.2.10.9 EFFECT OF SALINITY ON SURFACTANT PRODUCTION

The effect of different concentrations of sodium chloride (NaCl) was studied on surfactant production by varying the concentration of sodium chloride in the shake flask experiments. The concentration of NaCl was varied from 0.5-5% (w/v). The flasks containing sterile PPGAS medium (initial pH 7) were inoculated with the desired culture volumes and incubated at 37ºC in an incubated shaker at 150 rpm. A control flask (without the salt) was also maintained. All the flasks were kept in triplicates. Determination of bacterial growth, surfactant productivity and surface tension values of the supernatant was estimated at regular time intervals.

3.2.11 OPTIMIZATION OF THE MEDIUM COMPONENTS THROUGH STATISTICAL EXPERIMENTAL DESIGNS

In order to study the interaction of different process variables and to obtain the optimal conditions of the significant process variables leading to maximum surfactant production, two statistical approaches were followed:

(i) The process variables involved in surfactant production were screened using Plackett-Burman Design (PBD).

(ii) The optimal concentrations of the selected process variables involved in surfactant production were determined using Response Surface Methodology (RSM).

3.2.11.1 SCREENING PROCESS VARIABLES FOR SURFACTANT PRODUCTION USING PLACKETT-BURMAN DESIGN (PBD)

The main objective of this study was to screen the various parameters and study their interaction effect on surfactant production. Hence, various medium components affecting the surfactant production by 2B and *Pseudomonas aeruginosa*, respectively, were screened using the Plackett-Burman design (PBD).

The Plackett-Burman experimental design, a fractional factorial design, was used in this work to demonstrate the relative importance of medium components on surfactant production by the two bacterial cultures, 2B and *Pseudomonas aeruginosa*, respectively. Eleven independent variables in twelve combinations were organized according to the Plackett-Burman design matrix. For each variable, a high (+1) and low (-1) level was tested. Table 3.3 and 3.4 represents the levels of variables used in surfactant production by the bacterial strains, 2B and *Pseudomonas aeruginosa,* in the present study. The range of each variable was fixed based on the results obtained during shake flask studies. All trials were performed in triplicate and the average of surfactant produced was taken as response. Biosurfactant production was estimated as response for each run. The main effect of each variable was determined according to the following equation:

$$E_{xi} = (\sum M_{i+} - \sum M_{i-}) /N$$

where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the response percentage in trials, in which the independent variable (xi) was present in high and low concentrations, respectively and N is the half number of trials.

Table 3.3 Experimental range and the levels of variables considered in PBD for surfactant production by the bacterial isolate 2B

Variable	Symbol	Unit	Level Low	High Level
			(-)	(+)
Yeast extract	A	g/L	2	10
Peptone	B	g/L	3	30
Potassium nitrate	C	g/L	0.5	2
Ammonium chloride	D	g/L	0.5	2
Glycerol	E	%	0.5	2
Glucose	F	g/L	3	30
Molasses	G	%	0.5	2
Olive oil	H	%	0.5	2
Coconut oil	I	%	0.5	2
Potassium chloride	J	g/L	0.5	5
Sodium chloride	K	g/L	0.5	5

Table 3.4 Experimental range and the levels of variables considered in PBD for surfactant production by the bacterial culture *Pseudomonas aeruginosa*

Variable	Symbol	Unit	Low Level	High Level
			(-)	(+)
Peptone	A	g/L	2	10
Yeast extract	B	g/L	3	10
Ammonium chloride	C	g/L	0.5	2
Ammonium nitrate	D	g/L	0.5	2
Glycerol	E	%	0.5	2
Glucose	F	g/L	3	30
Whey	G	%	0.5	2
n-Hexadecane	H	%	0.5	2
Sunflower oil	I	%	0.5	2
Potassium chloride	J	g/L	0.5	5
Sodium chloride	K	g/L	0.5	5

3.2.11.2 DETERMINATION OF OPTIMAL PROCESS VARIABLE CONDITIONS USING RESPONSE SURFACE METHODOLOGY (RSM)

Based on Plackett-Burman design result, the process variables showing significant effect on surfactant production by the bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively, were selected for further studies. In the present work, Central Composite Design (CCD) module of RSM was adopted for the augmentation of surfactant production by the two bacterial strains. According to this design, the total numbers of treatment combinations were $2^k + 2k + n_0$, where k is the number of independent variables, n_0 is the number of repetitions of experiments at the centre point. For statistical calculations, the variables Xi were coded as xi according to the following equation:

$$xi = (Xi-X_0)/ \delta X$$

where xi is the dimensionless coded value of the variable Xi, X_0 is the value of Xi at the centre point and δX is the step change.

The behaviour of the system is explained by the following quadratic equation:

$$Y=\beta_0 + \Sigma \beta_i x_i + \Sigma \beta ii x_i^2 + \Sigma \beta ij x_i x_j$$

where Y is the predicted response, β_0 is offset term, β_i is linear effect, βii is the squared effect and βij is the interactive effect.

The equation was solved using the Minitab 15 software, to estimate the responses of dependent variables. A 2^3 factorial design, with a total number of 20 experiments were conducted.

For the response (surfactant productivity), three independent variables (carbon source, nitrogen source and inducer) were varied simultaneously in relation to the centre point, there being six replicates at the centre point and a single run for each of the other combinations. The minimum and maximum ranges of all the variables considered in the study were in the range of 20-50 g/L for carbon source, the concentration of nitrogen source in the range of 3-8 g/L and inducer concentration in the range of 2-5% (v/v). The level of non-significant variables was set at their corresponding optimal level.

The significant bioprocess variables chosen for the surfactant production by 2B at various levels were as follows: glucose (X_1), olive oil (X_2) and potassium nitrate (X_3), each variable was assessed at three coded levels (-1, 0 and +1), as shown in Table 3.5. For the production of surfactant by *Pseudomonas aeruginosa,* the process variables at various levels considered in the study were glucose (X_1), n-hexadecane (X_2) and ammonium chloride (X_3) (Table 3.6).

Table 3.5 Experimental range and the levels of variables considered in RSM study for surfactant production by the bacterial isolate 2B

Variable	Symbol	Unit	Level		
			-1	0	+1
Glucose	X_1	g/L	20	35	50
Olive oil	X_2	%	2	3.5	5
Potassium nitrate	X_3	g/L	3	5.5	8

Table 3.6 Experimental range and the levels of variables considered in RSM study for surfactant production by *Pseudomonas aeruginosa*

Variable	Symbol	Unit	Level		
			-1	0	+1
Glucose	X_1	g/L	20	35	50

n-Hexadecane	X_2	%	2	3.5	5
Ammonium chloride	X_3	g/L	3	5.5	8

The mathematical representation of the response *Y* and the variables is given as reported by Murugesan et al. (2007):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

where X_1, X_2 and X_3 are the different process variables.

The statistical software package, Minitab 15, was used for the regression analysis of the experimental data and also to plot the response surface graphs and contour plots. The statistical significance of the model equation and the model terms was evaluated by Fisher's test. The quality of fit of the second order polynomial model equation was expressed by the coefficient of determination (R^2). The fitted polynomial equation was then expressed in the form of three-dimensional surface plots, in order to illustrate the relationship between the response and the experimental levels of each of the variables utilized in the present study. Further, shake flask experiments were conducted at optimum levels of carbon source, nitrogen source and the inducer in order to validate the model.

3.2.12 KINETICS OF SURFACTANT PRODUCTION

250 ml Erlenmeyer flasks containing 100 ml of the optimized media were inoculated with cell cultures of 2B and *Pseudomonas aeruginosa*, respectively. All batch runs were performed in triplicate at 37ºC utilizing a rotary shaker at 150 rpm. Samples were collected every 4 hours, transferred to Eppendorfs tubes and then centrifuged at 10,000 rpm for 20 minutes at 4ºC for estimation of bacterial biomass. The total biomass,

residual glucose concentration, surface tension measurement and surfactant production were analyzed at regular time intervals. The residual glucose estimation was performed using DNS method. In addition, the specific growth rate, yield coefficients and substrate affinity were also calculated.

3.2.12.1 DETERMINATION OF SPECIFIC GROWTH RATE

Specific growth rate is the rate at which the microorganism multiplies at given environmental conditions per hour or the number of times the organism undergoes doubling per hour. Specific growth rate was calculated using the formula given by Stanbury et al. (2005) as follows:

$$\text{Specific growth rate } (\mu) = \frac{2.303\ (\log Xt - \log Xo)}{t}$$

where Xt is the concentration of the biomass after incubation time t (g/L),

Xo is the initial concentration of the biomass (g/L)

t is the number of hours of incubation

3.2.12.2 DETERMINATION OF YIELD-COEFFICIENT (Yx/s,Yp/s and Yp/x)

The cellular yield coefficient, Yx/s, is defined as the quantity of cell dry mass produced for the quantity of carbon substrate utilized (Stanbury et al. 2005). It is calculated as:

$$\text{Yield coefficient } (Yx/s) = \frac{X}{(S-s)}$$

where X is the concentration of biomass (g/L)

S is the initial concentration of substrate (g/L)

s is the residual concentration of substrate (g/L)

The product yield coefficient with respect to substrate consumption, Yp/s, is defined as the quantity of cell dry mass produced for the quantity of carbon substrate utilized (Stanbury et al. 2005). It is calculated as:

$$\text{Yield coefficient (Yp/s)} = \frac{P}{(S-s)}$$

where P is the concentration of surfactant (g/L)

S is the initial concentration of substrate (g/L)

s is the residual concentration of substrate (g/L)

The product yield coefficient with respect to biomass production, Yp/x, is defined as the quantity of product synthesized for the quantity of biomass produced (Stanbury et al. 2005). It is calculated as:

$$\text{Yield coefficient (Yp/x)} = \frac{P}{(Xt-Xo)}$$

where P is the concentration of surfactant (g/L)

Xt is the biomass concentration after incubation time t (g/L)

Xo is the initial concentration of biomass (g/L)

3.2.12.3 DETERMINATION OF SUBSTRATE AFFINITY (Ks)

Ks is a measure of the affinity of an organism to its substrate (Stanbury et al. 2005). A graph was plotted by taking the specific growth rate (μ) on the Y-axis and residual substrate concentration (s) on the X-axis. From the graph, maximum specific growth rate (μ_{max}) was calculated. Ks is a residual substrate concentration when (μ)=1/2 (μ_{max}). Hence, Ks value was calculated on the basis of the μ_{max} value from the graph.

3.2.13 EXTRACTION AND PARTIAL PURIFICATION OF THE SURFACTANT

The surfactant produced by the bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively, was extracellular in nature and concentrated in the modified PPGAS broth. During extraction and partial purification, the surfactant concentration and the surface tension measurements were carried out to check the quality of the surfactant during each purification step. Therefore, the following procedures were employed for the extraction and partial purification of the surfactant:

3.2.13.1 ACIDIFICATION

100 ml of the culture broth of the bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively, was subjected to centrifugation at 9000 rpm for 30 minutes at 4ºC. The pH of the cell free broth was set to pH 2 using 1N HCl and kept overnight for precipitation. The precipitate was collected by centrifugation for 30 minutes at 4ºC (Maneerat and Phetrong 2007). The surfactant quantity and surface tension value of the acidified extract were measured.

3.2.13.2 SOLVENT EXTRACTION

The solvent used for extraction of surfactant produced by 2B and *Pseudomonas aeruginosa*, respectively, was chloroform-methanol (2:1). The organic phase was separated. The extraction process was carried out three times. The solvent was evaporated to concentrate the surfactant. The surfactant was then dried at 60ºC to a constant weight prior to get the quantity of surfactant (Samadi et al. 2007). The dried extract was dissolved in 0.1M sodium bicarbonate buffer to measure surface tension value of the surfactant.

3.2.13.3 COLUMN CHROMATOGRAPHY

Following solvent extraction, further purification of the surfactant produced by 2B and *Pseudomonas aeruginosa*, respectively, was done using column chromatography.

A column 26 cm x 3.5 cm (diameter) was prepared using 50 g activated silica gel G (230-400 mesh) chloroform slurry. 5 grams of surfactant was prepared in 10 ml of chloroform. Chloroform: methanol mobile phases were then applied in sequence: 50:3 v/v (200 ml); 50:5 v/v (200 ml); and 50: 50 v/v (200 ml) at a flow rate of 1 ml / minute and 20 ml fractions were collected (Thavasi et al. 2008). The surface tension value and surfactant quantity of each fraction was determined.

3.2.14 CHARACTERIZATION OF THE PARTIALLY PURIFIED SURFACTANT

The study was mainly focused on characterizing the partially purified surfactant produced by the strains, 2B and *Pseudomonas aeruginosa*, respectively. The characterization of the surfactant was performed using the methods described below:-

3.2.14.1 THIN LAYER CHROMATOGRAPHY (TLC)

The surfactant produced by 2B and *Pseudomonas aeruginosa*, respectively, was characterized by thin layer chromatography using silica-gel plates 60 (20 cm x 20 cm, Merck). The development of solvent systems used was differed based on the components tested. The TLC plates were spotted with surfactant extracts and developed with the following: solvent 1-chloroform: methanol: water (65:25:4) for lipids, solvent 2-n-buthanol-acetic acid-water (4:1:1) for amino acids and solvent 3-ethyl acetate-acetic acid-methanol-water (12:3:3:2) for carbohydrate compounds. After developing, the spots were visualized with standard reagents. The lipid components were detected as yellow to brown spots after placing the plates in a closed jar saturated with iodine vapours. Using the ninhydrin solution followed by heating at 90ºC for 5 minutes, generated a purple colour when the compound had an amine function, this confirmed the presence of amino acids. Carbohydrate components were detected as brown spots on the plates after spraying with α-naphthol solution followed by spraying with concentrated sulphuric acid and heating for 5 minutes at 100ºC (Stahl 1969). These components were identified by

comparison with published reports and against lipids, amino acids and carbohydrates standards.

3.2.14.2 BIOCHEMICAL ANALYSIS OF THE SURFACTANT

Following TLC, the biochemical analysis of the various surfactant moieties was performed in order to quantify the individual moieties. Total sugar present in the surfactant was determined by the phenol-sulphuric acid method according to Dubois et al. (1956) as given in Appendix XI. The stand curve was prepared with D-glucose. Total rhamnose content was estimated by orcinol-sulphuric acid method as given in Appendix XII. Total protein content was measured by Lowry's method (1951), standardized with bovine serum albumin as given in Appendix XIII. To determine the lipid content, 0.2 g surfactant sample was blended with chloroform-methanol mixture (2:1). Solvent phase was recovered by centrifuging at 10,000 rpm for 15 min. The extraction process was carried out three times. The whole solvent was collected, evaporated and dried under vacuum. The lipid content was determined by gravimetric estimation (Makkar and Cameotra 1998).

3.2.14.3 FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY

Fourier Transform Infrared (FTIR) spectra of the dried surfactants produced by 2B and *Pseudomonas aeruginosa,* respectively, were recorded on a FTIR spectrometer (Thermo Fisher Scientific, Madison, USA) equipped with a mercury-cadmium-telluride (MCT) detector. About 2 mg of dried surfactant was analyzed by FTIR spectra measurement in wave number range of 4000-400 cm^{-1}. The analysis of IR spectra was carried out by using Thermo software.

3.2.14.4 MASS SPECTROMETRIC ANALYSIS OF THE SURFACTANT

Surfactant produced by the bacterial cultures, 2B and *Pseudomonas aeruginosa,* respectively, was dissolved in methanol and mixed thoroughly. The mass spectrometric analysis of the surfactant was carried out in Shimadzu LC-MS 2010 spectrometer

(Shimadzu, Japan) utilizing electrospray ionization (ESI). The mass spectra were detected using the negative ion mode and scanning was done at 50-1,000 m/z range.

3.2.15 RECONSTIUTIONAL STUDIES OF THE PARTIALLY PURIFIED SURFACTANT

On the basis of chemical composition of the partially purified surfactant of 2B and *Pseudomonas aeruginosa*, respectively; the lipid, protein and carbohydrate moieties were separated by various methods. 1 gram of the partially purified surfactant sample was dissolved in 25 ml of water. Lipid fraction of the partially purified surfactant was separated by incubating the surfactants with 800 µg of steapsin (lipase) (Sekhon et al. 2011). The protein fraction was separated by treating the surfactant samples with 60% ammonium sulphate whereas the carbohydrate moieties were separated by adding 5 ml of hot phenol. In each case, the surface tension of water was measured following the treatment. Control sample consisting of all the fractions of surfactant was also maintained.

3.2.16 STUDIES ON THE EFFECT OF ENVIRONMENTAL FACTORS ON SURFACTANT STABILITY

The surfactant stability studies were based on the determination of temperature, pH and NaCl effects on the surface activity of the surfactant produced by the bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively. To determine the stability of the surfactant at different temperatures, the cell-free broth containing surfactant and the partially purified surfactants were maintained at a temperature in the range 4-121ºC for 30 min. To determine the effect of pH on the surface tension, the pH of the cell-free broth containing surfactant and the partially purified surfactants was adjusted at a value in the range 2.0-12.0 with HCl (6 N) and NaOH (6 N) (Lotfabad et al. 2009). In each case, surface tension values were measured. In order to assess the effect of salinity on surface tension of the cell-free broth containing surfactant and the partially purified surfactants, various concentrations of sodium chloride (2-12%, w/v) were used (Joshi et al. 2008).

3.2.17 STUDIES ON THE SHELF-LIFE OF SURFACTANT

To study the shelf-life of the partially purified surfactant produced by 2B and *Pseudomonas aeruginosa*, respectively, the surfactants were stored at 30ºC for 6 months. The surface tension measurement was carried out at regular time intervals to check the performance of the surfactant.

3.2.18 SURFACTANT ENHANCED BIODEGRADATION OF CRUDE OIL

The partially purified surfactant produced by the bacterial strains, 2B and *Pseudomonas aeruginosa*, respectively, was employed to study the efficiency of surfactant on crude oil degradation. 100 mg of surfactant was added to 50 ml of modified Bushnell Hass Medium containing 1% (v/v) crude oil. The flasks were inoculated with *Nocardia hydrocarboxydans* NCIM 2386. A control flask was maintained in which the surfactant was not added. The flasks were incubated at 37ºC on a rotary shaker (150 rpm). The samples were drawn at regular time intervals to assess the degradation of crude oil.

In order to determine the residual crude oil concentration following degradation at regular time intervals, the crude oil was extracted from the broth after separation of the cells by centrifugation (10,000 rpm for 20 minutes). The residual crude oil in the supernatant was estimated by UV-spectrophotometer method (EPA pt 300 App. C), the procedure is given in Appendix XIV.

CHAPTER 4

RESULTS AND DISCUSSION

The present chapter deals with the experimental results obtained using the methodologies presented in Chapter 3 to meet the stated objectives presented in Chapter 1. The results from enrichment and isolation of microorganisms and screening of the surfactant microorganisms are presented. The results from identification of the potential surfactant producing microorganism using various tests, namely, microscopic observations, biochemical tests as well as partial 16S ribosomal DNA sequencing are presented. This chapter presents results from the studies on extraction of extracellular surfactant from the cell-free broth, various process variables affecting the production of surfactant, optimization of process variables using statistical method and kinetics of surfactant production. The results of the partial purification and characterization of surfactant, effect of various environmental factors affecting surfactant stability and efficiency of surfactant in the biodegradation of crude oil are also presented. The results of various experiments in the present study are presented in the form of tables and figures; the data obtained has been compared with relevant literature. **All the data presented are mean values of 3 readings**.

4.1 ENRICHMENT AND ISOLATION OF MICROORGANISMS

In the literature, various researchers have reported that the density of surfactant producing microorganisms was higher in various petroleum hydrocarbon contaminated localities; they also have confirmed the capability of native microbial population to mineralize hydrocarbons and produce surfactants (Emtiazi and Okerentugba 2003; Shakarami 2004; Balogun and Fagade 2008). Therefore, in the present study, soil samples and water samples for the isolation of surfactant producing microorganisms were collected from various petroleum hydrocarbon contaminated regions in and around

Mangalore, Karnataka, India. The details of the soil and water samples collected are given in Table 3.1 and 3.2.

The principle of enrichment culture is to provide growth conditions that are favorable for the microorganisms of interest and as unfavorable as possible for the competing microorganisms (Walter et al. 2000). In this regard, in the present study, the collected soil and water samples were subjected to enrichment in Bushnell and Hass medium containing glucose and 1% (v/v) crude oil, respectively. In the literature, for the isolation of surfactant producing microorganisms, enriched cultures utilizing hydrophobic compounds were used as the carbon source by a number of authors (Schulz et al. 1991; Mercade et al. 1996; Huy et al. 1999; Rahman et al. 2002; Bento et al. 2005; Ganesh and Lin 2009; Liu et al. 2011). Kappeli and Finnerty (1980) postulated that the function of surfactant produced by microorganisms is related to hydrocarbon uptake and therefore, a spontaneous release of surfactant occurs in the presence of the hydrocarbon substrate.

The isolated microorganisms were enumerated based on Colony Forming Units (CFU). The microbial colonies isolated on Bushnell Hass agar medium from various soil samples were estimated based on the formation of Colony Forming CFU/ gram of the soil sample. Table 4.1 presents the total Colony Forming Units isolated on Bushnell Hass agar medium from various soil samples. The total Colony Forming Units isolated from S1, S2, S3, S4, S5 and S6 soil samples were 59 x 10^5, 42 x 10^5, 96 x 10^5, 83 x 10^5, 24 x 10^5 and 8 x 10^5, respectively. The highest number of Colony Forming Units (96 x 10^5) was isolated from the soil around petrol filling station (S3). The soil samples exposed to petroleum hydrocarbons (S1, S2, S3, S4 and S5) had more number of Colony Forming Units compared to the control soil sample (S6). The reason may be that the microorganisms in the control soil sample were not exposed to petroleum hydrocarbons and hence, were not able to tolerate the same during the enrichment culture. In a similar study, Balogun and Fagade (2008) reported the total Colony Forming Units isolated from various diesel impacted soil samples ranged from 8.6 x 10^4-5.3 x 10^5 CFU/g of soil while

a range of 1.6 x 10^5-4.0 x 10^5 CFU/g of soil was recorded for unimpacted soil samples, respectively.

Table 4.1 Total Colony Forming Units isolated from soil samples following enrichment

Sl. no.	Name of the sample	Colony Forming Units/g of soil
1.	S1	59 x 10^5
2.	S2	42 x 10^5
3.	S3	96 x 10^5
4.	S4	83 x 10^5
5.	S5	24 x 10^5
6.	S6 (Control)	8 x 10^5

Table 4.2 presents the total Colony Forming Units isolated on Mineral agar medium from various water samples. The CFU/ml of water obtained from M1, M2, M3, M4 and M5 water samples were 24 x 10^5, 68 x 10^5, 51 x 10^5, 32 x 10^5 and 3 x 10^5, respectively. The total Colony Forming units was highest (68 x 10^5) in water surrounding the dock yard (M2) that contained petroleum hydrocarbon residues. The water samples (M1, M2, M3 and M4) exposed to petroleum hydrocarbons had more number of microorganisms compared to that of control sample (M5). This is probably because in the control sample, the microorganisms were not exposed to petroleum hydrocarbons and

hence, were not able to tolerate the same during the enrichment procedure. This result is supported by the report of Francy et al. (1991), which indicates that the exposure to hydrophobic pollutants in contaminated environments supports the growth of surfactant producing microorganisms that could sustain their growth by emulsification of the hydrophobic compounds. In a similar study, the total Colony Forming Units/ml isolated from sea water samples at different locations of Tutucorin Harbour, Tamil Nadu, India, by Thavasi et al. (2011) was 1.05×10^3 and 2.13×10^5, respectively.

In the present study, it was observed that the highest number of microorganisms was isolated from soil and water samples which were previously exposed to petroleum hydrocarbons compared to the control samples. The reason might be that the microorganisms isolated from petroleum hydrocarbon regions were acclimatized to petroleum hydrocarbon contaminated environment. Therefore, these microorganisms could grow in the presence of 1% (v/v) crude oil during the enrichment culture. The present study resulted in successful enrichment of microorganisms from different petroleum hydrocarbon contaminated samples that might have the potential to produce surfactant.

Table 4.2 Total Colony Forming Units isolated from water samples following enrichment

Sl. no.	Name of the sample	Colony Forming Units/ml of water
1.	M1	24×10^5
2.	M2	68×10^5
3.	M3	51×10^5
4.	M4	32×10^5
5.	M5 (Control)	3×10^5

SUMMARY

In the present study, soil as well as water samples were collected from petroleum hydrocarbon contaminated localities of Mangalore, Karnataka, for the isolation of microorganisms by enrichment technique based on published reports which suggest that the prior exposure of microbial community to the petroleum hydrocarbon contaminant increases the incidences of the isolation of surfactant producing microorganisms (Rahman et al. 2002; Batista et al. 2006; Samadi et al. 2007; Elouzi et al. 2009). The total Colony Forming Units isolated from petroleum hydrocarbon contaminated regions (S1, S2, S3, S4, S5, M1, M2, M3 and M4) were estimated and compared to that of control samples (S6 and M5). Among the various soil samples (S1, S2, S3, S4, S5 and S6), the maximum number of microorganisms (96×10^5) were isolated from the soil sample around petrol filling station (S3) whereas among the various water samples, the total Colony Forming Units was highest (68×10^5) in the water surrounding the dock yard area (S3). The results of the present study are in agreement with reports in the literature which

describes the isolation of surfactant producing microorganisms from various petroleum hydrocarbon contaminated samples by enrichment technique (Schulz et al. 1991; Mercade et al. 1996; Willumsen and Karlson 1997; Rahman et al. 2002; Bento et al. 2005; Dubey et al. 2012).

4.2 SCREENING SURFACTANT PRODUCING MICROORGANISMS

In the present study, different screening methods such as selective Cetyl Tri Ammonium Bromide (CTAB)-methylene blue agar medium, drop collapse assay and surface tension measurements were used to select surfactant producers from the non-producers. The results of the various screening methods are described below:

4.2.1 SCREENING ISOLATES ON THE SELECTIVE CETYL TRIMETHYL AMMONIUM BROMIDE (CTAB)-METHYLENE BLUE AGAR MEDIUM

Following isolation, the preliminary selection of the surfactant producing microorganisms was carried out by serially diluting the enriched samples up to 10^{-5} dilution. The serially diluted samples were inoculated on to Cetyl Trimethyl Ammonium Bromide (CTAB)-methylene blue agar medium. Colonies formed on CTAB-methylene blue agar medium were enumerated. Table 4.3 represents the total number of microorganisms that were isolated on CTAB-methylene blue agar medium from the soil samples. The total number of microorganisms isolated on CTAB-methylene blue medium from S1, S2, S3, S4, S5 and S6 soil samples were 3×10^5, 18×10^5, 10×10^5, 41×10^5, 18×10^5 and 0, respectively. In the control sample (S6), there was no growth of microorganisms. The results of the present study suggest that the microorganisms isolated from the S1, S2, S3, S4 and S5 soil samples might have the ability to grow in the presence of the detergent, CTAB and produce surfactant which is the characteristic nature

of surfactant producing microorganisms. Similar observation has been reported by Walter et al. (2010). In the literature, the CTAB-methylene blue medium has been used by Tuleva et al. (2002), Tahzibi et al. (2004) and Gunther et al. (2005) as preliminary screening medium for the selection of extracellular surfactant producing microorganisms.

Table 4.3 Total number of microorganisms isolated from soil samples on CTAB-methylene blue agar medium

Sl. no.	Name of the sample	Colony Forming Units/g of soil
1.	S1	3×10^5
2.	S2	18×10^5
3.	S3	10×10^5
4.	S4	41×10^5
5.	S5	18×10^5
6.	S6 (Control)	0

Among the different soil samples, the highest number of microorganisms (6) which showed positive reaction (as indicated by blue halo formation) for surfactant production on CTAB-methylene blue agar medium was isolated from soil around castrol storage area (S5) as shown in Fig. 4.1. The number of microorganisms showing halos on CTAB-methylene blue agar medium plated with S1, S2, S3 and S4 soil samples were 0, 2, 0 and 1, respectively. Totally, 9 Colony Forming Units possessed the ability to produce surfactant as indicated by halo formation on the selective CTAB-methylene blue agar medium. Similar study was carried by Satpute et al. (2008), 21 different bacterial cultures were screened for surfactant production using CTAB-methylene blue agar method. They reported that among the different cultures screened for surfactant production, *Pseudomonas aeruginosa* MTCC 2297 showed halo formation, indicating the bacterium might possess the ability to produce surfactant.

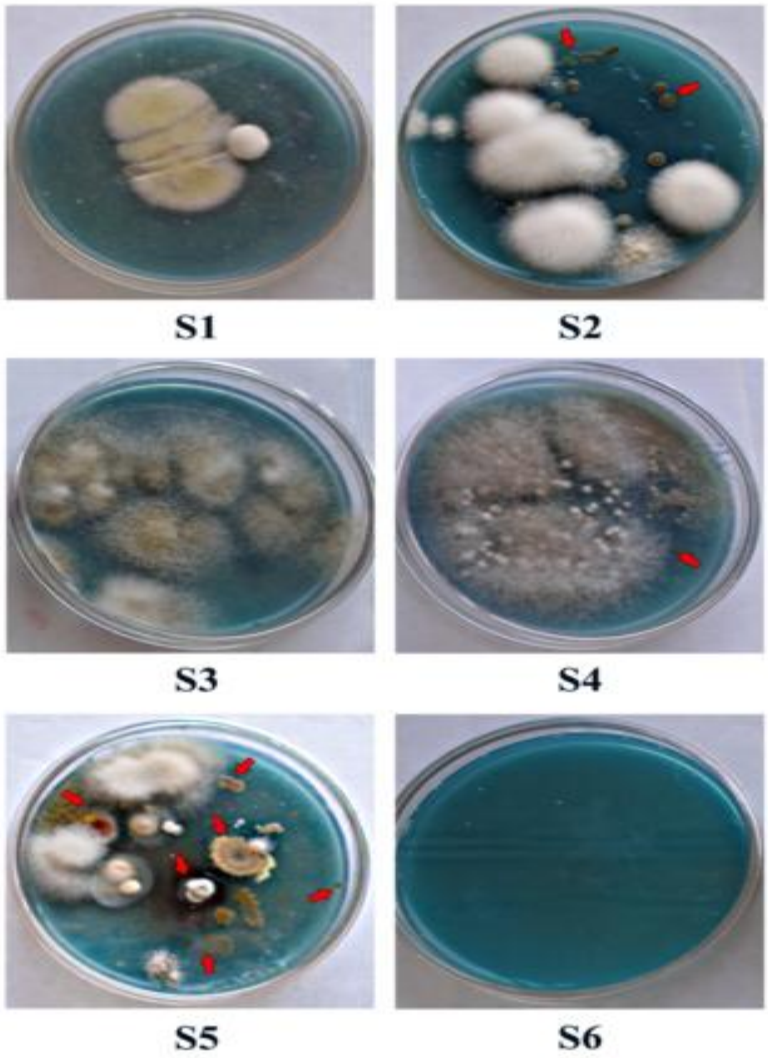


Fig. 4.1 CTAB-methylene blue agar plates showing growth of various microorganisms isolated from soil samples. The red arrows indicate the formation of halos around the microbial colonies.

Further, a study was carried out to identify the microorganisms that formed halo on the CTAB-methylene blue agar medium based on the morphology and microscopic observations. Among the 6 isolates that were isolated from the S5 soil sample, 3 isolates were identified as fungal cultures and 3 isolates were identified as bacterial cultures. The strains showing halos on S2 and S4 soil samples were identified as bacterial isolates. Therefore, in the present study, 3 fungal and 6 bacterial isolates showed halo formation on CTAB-methylene blue agar medium. The fungal isolates were designated as 1F, 2F and 3F whereas the bacterial isolates were designated as 1B, 2B, 3B, 4B, 5B and 6B.

The total number of microorganisms isolated on CTAB-methylene blue agar medium from M1, M2, M3, M4 and M5 water samples were 20 x 10^5, 46 x 10^5, 38 x 10^5, 14 x 10^5 and 0, respectively (Table 4.4). The highest number of microorganisms (46 x 10^3) which showed halo formation on CTAB-methylene blue agar medium was isolated from the sample collected surrounding a dock yard (M2) where there was frequent spillage of petroleum hydrocarbons due to transportation activities. In the control sample, there was no growth of microorganisms. The results of the present study suggest that the microorganisms isolated from M1, M2, M3 and M4 water samples might have the capacity to produce surfactant as these microorganisms could grow in the presence of CTAB. In a similar study by Christova et al. (2004), a bacterial isolate identified as *Renibacterium salmoninarum* 27BN isolated from hydrocarbon contaminated industrial waste water sample showed formation of halo on the selective CTAB-methylene blue agar medium.

Table 4.4 Total number of microorganisms isolated from water samples on CTAB-methylene blue agar medium

Sl. no.	Name of the sample	Colony Forming Units/ml of water
1.	M1	20×10^5
2.	M2	46×10^5
3.	M3	38×10^5
4.	M4	14×10^5
5.	M5 (Control)	0

Among the different water samples, the highest number of microorganisms (3) which showed halo formation on the CTAB-methylene blue agar medium was isolated from water surrounding the dock yard area (M2) as shown in Fig. 4.2. This indicates that these isolates might have the ability to produce surfactant. The number of microorganisms showing halo formation on M1, M3 and M4 samples were 1, 0 and 0, respectively. In the control sample, there was no growth of microorganism. In a study by Hamed et al. (2012) reported that among 16 microorganisms isolated from seawater biofilms (formed in pipelines used for the refrigeration of petroleum derivatives), 8 microorganisms showed halo formation on the CTAB-methylene blue medium indicating that they might possess the capacity to produce surfactant.

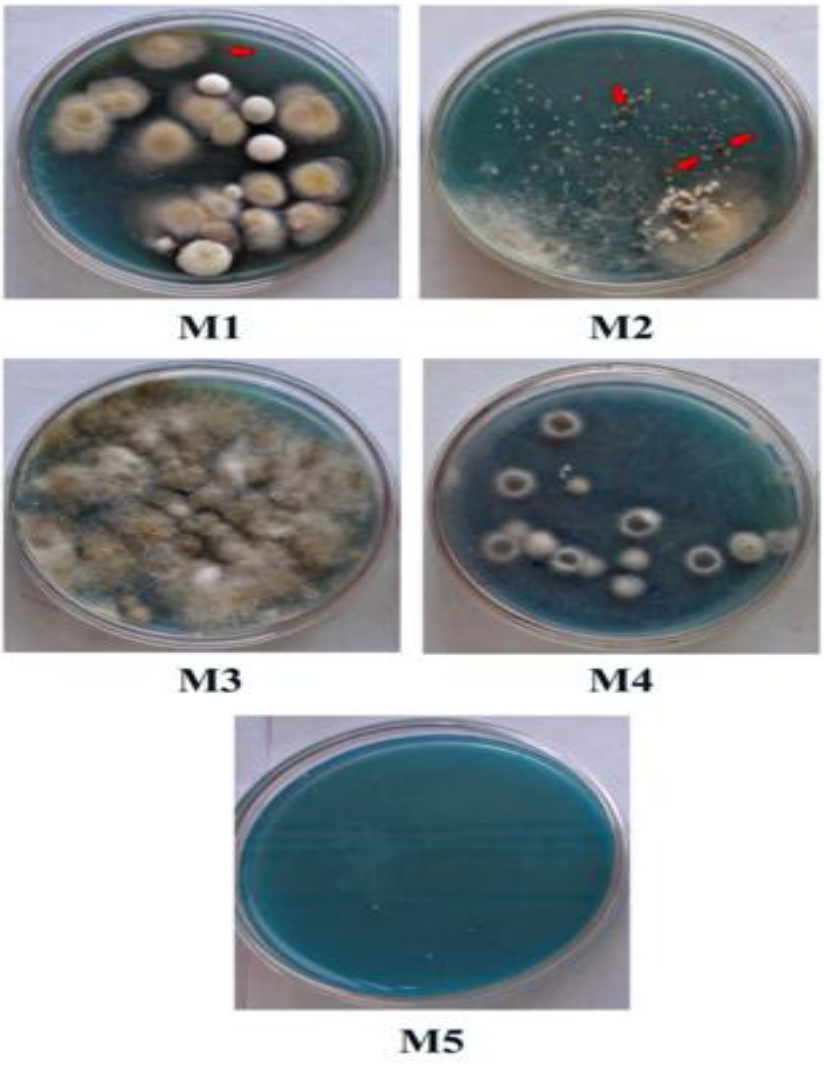


Fig. 4.2 CTAB-methylene blue agar plates showing the growth of various microorganisms isolated from water samples. The red arrows indicate the formation of halos around the microbial colonies.

The isolates showing halo formation on the CTAB-methylene blue agar medium were subjected to identification studies based on the morphology and microscopic observations. Among the 3 isolates (isolated from the M2 soil sample) showing halo formation on the CTAB-methylene blue agar medium, all the 3 isolates were identified as bacterial isolates. The bacterial strains were designated as 1MB, 2MB and 3MB. The isolate showing halo formation on M1 was identified as a fungal isolate and it was designated as 4F. Therefore, in the present study, totally 1 fungal and 3 bacterial isolates showed halo formation on CTAB-methylene blue agar medium.

SUMMARY

In the present study, totally 13 isolates (9 bacterial and 4 fungal isolates) isolated from the soil and water samples showed halo formation on the CTAB-methylene blue agar medium, which indicated that these isolates may have the ability to produce surfactant. The microorganisms showing positive reaction on CTAB-methylene blue agar medium were designated as 1B, 2B, 3B, 4B, 5B, 6B, 1MB, 2MB, 3MB, 1F, 2F, 3F and 4F, respectively. The selective CTAB-methylene blue agar medium has been used by several authors to screen extracellular surfactant production by microorganisms (Tuleva et al. 2001; Tahzibi et al. 2004; Gunther et al. 2005). This method has been widely used since it is a specific agar plate assay which indicates the production of surfactant by colour reaction. It is also reported that CTAB inhibits the growth of most of the microorganisms making it easier for the selection of surfactant producers (Siegmund and Wagner 1991). The present study demonstrated the ability of surfactant production by both bacterial as well as fungal isolates. This is supported by the fact that various strains of prokaryotic and eukaryotic microorganisms are capable of synthesizing surfactants (Lu et al. 2007).

4.2.2 DROP COLLAPSE ASSAY

In the present study, 13 isolates (9 bacterial and 4 fungal isolates) that showed halos on the CTAB-methylene blue medium were screened for surfactant production ability by drop collapse assay. Both the cell-free supernatant as well as the cell pellet was screened for surfactant production. Following drop collapse, the diameter of the drop was also measured. 10 out of 13 isolates showed positive drop collapse assay indicating that these isolates possessed the surfactant production capacity. The supernatants of the isolates coded as 2B, 3B, 5B, 6B, 2MB, 3MB, 1F, 2F and 4F, respectively, and the positive controls (*Pseudomonas aeruginosa* and *Phanerochaete chrysosporium*) showed positive drop collapse test indicating extracellular surfactant production (Table 4.5). The cell-pellet of all the isolates showed negative result for drop collapse assay except for that of the isolate, 4B, which showed slight positive drop collapse activity indicating that the surfactant produced by the isolate might be cell-associated. Water, which served as the negative control, showed no drop collapse. Among all the isolates tested, the supernatant of microorganism 2B, isolated from soil around automobile workshop area (S2), showed rapid drop collapse reaction compared to other isolates used in the study, suggesting extracellular production of the surfactant. The strain, 2B showed maximum droplet diameter of 4 mm following drop collapse activity. The reaction was similar to that observed for Sodium Dodecyl Sulphate (SDS) which was used as the positive control in the experiment. The other isolates, 1B, 1MB and 3F, which showed halo formation on the CTAB-methylene blue agar medium exhibited negative result for drop collapse assay. The reason may be that these isolates might have produced surfactant in very low concentrations which could not be detected by drop collapse assay. Bodour et al. (2003) screened a total of 1,305 isolates by drop collapse method and of these, 45 isolates demonstrated the ability to produce surfactant. Maneerat and Phetrong (2007) isolated 200 marine bacterial strains from oil-spilled seawater collected from harbors and docks in Songkhla Province, Thailand; they found 8 bacterial strains positive for drop collapse assay. Thavasi et al. (2011) isolated 105 strains from Tutucorin Harbour, Tamil Nadu,

India, using enrichment technique. In their study, out of 105 strains screened, 82 strains were positive for drop collapse activity indicating that these isolates might possess the ability to produce surfactant.

Table 4.5 Screening surfactant producing microorganisms using the drop collapse assay

Sl. No.	Microorganism	Drop collapse assay	Droplet diameter (mm)
1.	1B	NA	0 mm
2.	2B	+++	4 mm
3.	3B	++	2 mm
4.	4B	+	1 mm
5.	5B	++	3 mm
6.	6B	++	1 mm
7.	1MB	NA	0 mm
8.	2MB	++	2 mm
9.	3MB	+	3 mm
10.	1F	++	2 mm
11.	2F	++	2 mm
12.	3F	NA	0 mm
13.	4F	+	1 mm
14.	Water	NA	0 mm

15.	Sodium dodecyl sulphate (SDS)	+++	4 mm
16.	*Pseudomonas aeruginosa*	++	3 mm
17.	*Phanerochaete chrysosporium*	++	2 mm

'NA'- no drop collapse activity, '+++'- drop collapse within one minute, '++'- drop collapse after 1 minute, '+'- drop collapse after 3 minutes

Fig. 4.3 shows the drop collapse activities of water, SDS, 2B and *Pseudomonas aeruginosa* in the presence of various hydrocarbons. The microorganisms showing positive drop collapse assay in the present study showed the capacity to collapse the drops in all the hydrocarbons tested. Among the various hydrocarbons used in the drop collapse assay, motor oil (oil from Castrol, SAE 10W40) gave the best qualitative indication of surfactant production compared to gasoline, kerosene and hexadecane. This may be probably because the motor oil has greater viscosity compared to the other hydrocarbons used in the study. This result is in agreement with the reports of Bodour and Maier (1998) as well as Maneerat and Phetrong (2007). Motor oil Penzoil 10W-40 was considered the most effective oil because either the drop collapsed completely in the presence of surfactant or it remained beaded in the absence of surfactant (Zawawi 2005).

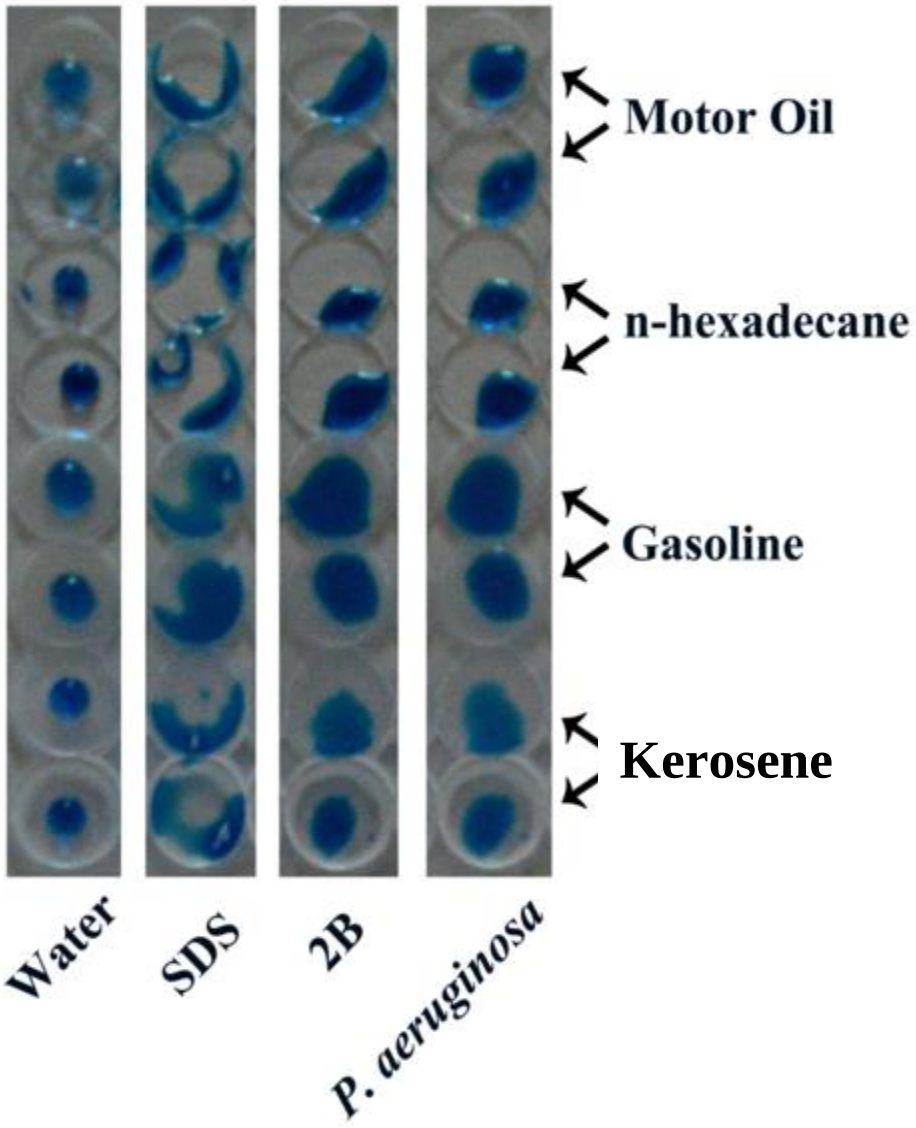


Fig. 4.3 Drop collapse activities of water, SDS, 2B and *Pseudomonas aeruginosa* on different hydrocarbon coated plate

SUMMARY

The 13 isolates showing halo formation on CTAB-methylene blue agar medium were further screened for surfactant production by drop collapse assay. Among the cultures screened, the cell-free supernatants of 9 cultures, designated as 2B, 3B, 5B, 6B, 2MB, 3MB, 1F, 2F and 4F, resulted in the confirmation of extracellular surfactant production capacity whereas the cell pellet of the isolate 4B showed slight positive drop collapse activity indicating the production of cell-associated surfactant. Among all the isolates tested, the supernatant of 2B showed rapid and positive drop collapse assay. Drop collapse assay has been used by various authors to screen potential surfactant producers (Jain et al. 1991; Bodour et al. 2003; Youssef et al. 2004; Thavasi et al. 2011, Khopade et al. 2012).

4.2.3 SURFACE TENSION MEASUREMENT

In the present study, already screened 13 isolates (9 bacterial and 4 fungal isolates) were tested for surfactant production by surface tension measurement. Both the cell-free supernatants as well as the cell pellet were screened for surfactant production. The reduction of surface tension was also calculated with reference to the value of water. Based on the results obtained in the previous screening tests, the supernatants and the pellets of 1B, 2B, 3B, 4B, 5B, 6B, 1MB, 2MB, 3MB, 5MB, 1F, 2F, 3F, 4F isolates and the positive controls (*Pseudomonas aeruginosa* and *Phanerochaete chrysosporium)* were subjected to surface tension measurement.

Table 4.6 summarizes the value of surface tension measured for all the isolates considered in the study. The lowest values of surface tension recorded were 29.33 mN/m obtained from cell-free supernatant of isolate 2B. There was reduction in surface tension of the 2B culture supernatant from 71.39 mN/m to 29.33 mN/m indicating about 58% reduction in the surface tension value. This result is in agreement with those obtained from the drop collapse assay (Section 4.2.2) which gave a significant indication that the

isolate 2B was the most potential extracellular surfactant producer among the isolates screened in this study. Other isolates showed surface tension values ranging from 34.21-48.41 mN/m whereas synthetic surfactant, SDS, lowered the surface tension from 71.39 mN/m to 32.59 mN/m. The positive isolates, *Pseudomonas aeruginosa* and *Phanerochaete chrysosporium*, respectively, reduced the surface tension of the cell-free broth to 34.36 mN/m and 36.44 mN/m, respectively. The surface tension of the cell pellet of various isolates ranged from 48.41-69.46 mN/m. The cell pellet of 4B lowered the surface tension of the broth to 48.41 mN/m, confirming the results obtained in the drop collapse assay described in section 4.2.2. The results of the present study are in agreement with the report of Matsuyama et al. (1991) in which the bacterial strain, *Serratia marcescens*, reduced the surface tension of the cell-free broth to 28 mN/m. The surfactant produced by *Streptomyces tendae*, decreased the surface tension of water from 72 to 39.4 mN/m as reported by Richter et al. (1998). Raza et al. (2006) reported that the surface tension of cell-free culture broth of *Pseudomonas aeruginosa* EBN-8 mutant decreased from 53 to 29 mN/m due to the production of surfactant. From this study, it can be observed that the isolate 2B exhibited significant reduction in the surface tension compared to other strains considered in the study. The surfactant produced by strain 2B compares well with these surfactants mentioned in the literature reports and even surpasses them. Hence, the isolate 2B was used for further studies.

Table 4.6 Surface tension values of the cell-free broth as well as the pellet of the isolates. Results are represented as Mean ± SEM (n=3)

Sl. no.	Microorganism	Surface tension value (mN/m) of cell free broth	Surface tension value (mN/m) of the cell pellet
1.	1B	47.33±0.7	67.89±0.3
2.	2B	29.33 ±2.1	63.41 ±0.8
3.	3B	37.67±0.9	68.13±1.7

4.	4B	48.41±4.1	48.41±0.2
5.	5B	34.21±1.4	64.78±1.7
6.	6B	41.67±3.2	65.12±0.8
7.	1MB	39.64±3.4	69.27±0.6
8.	2MB	47.27±1.6	67.89±1.6
9.	3MB	42.39±1.3	68.09±0.7
10.	1F	43.76±1.6	67.43±1.3
11.	2F	46.52±0.7	66.19±0.2
12.	3F	42.21±3.6	64.24±1.7
13.	4F	46.71±2.2	67.89±1.9
14.	*Pseudomonas aeruginosa*	34.36±1.7	66.62±0.8
15.	*Phanerochaete chrysosporium*	36.44±1.3	69.46±0.4
16.	Sodium Dodecyl Sulphate (SDS)	32.59±0.9	NA
17.	Water	71.39±0.5	NA

NA- Not applicable

SUMMARY

The results obtained in the present study reveal that the isolate 2B lowered the surface tension of the cell-free broth from 71.39 mN/m to 29.33 mN/m since the bacterial isolate produced extracellular surfactant. There was a direct correlation between the results obtained in the present section and the previous sections, wherein the isolate 2B produced halo on the selective CTAB-methylene blue agar medium (Section 4.2.1), showed positive reaction for drop collapse assay (Section 4.2.2) and also reduced the surface tension of the broth to 29.33 mN/m. The isolate 2B exhibited reduction in surface tension compared to other isolates used in the study and also compared to that in the

literature (Cooper and Goldenberg 1987; Das et al. 1998; Plaza et al. 2006). Among the different isolates tested for the ability to produce surfactant, the isolate 2B was identified as potential surfactant producer and used for further studies.

4.3 IDENTIFICATION OF THE POTENTIAL SURFACTANT PRODUCING BACTERIAL ISOLATE-2B

The results of morphological, biochemical tests and sequence analysis of selected bacterial isolate, 2B, isolated from soil around automobile workshop area is presented below:

4.3.1 COLONY CHARACTERISTICS

The bacterial isolate produced glistening, translucent, irregular, entire and flat colonies on the nutrient agar medium. Fig. 4.4 (a) and (b) shows the colonies of the bacterium 2B on nutrient agar medium. During the growth on nutrient agar medium, the diameter of the colonies ranged from 1 to 2 mm within 24 hours of growth at 37ºC. The isolated strain produced creamish coloured colonies within 18 hours of growth [Fig. 4.4 (a)] and after 24 hours, the colonies turned greenish in colour due to production of diffusible green colored fluorescent pigment [Fig. 4.4 (b)]. This indicated that the isolate belonged to the *Pseudomonas* genus since the production of green fluorescent pigment, called pyocyanin, is a feature of this genus (Smriti et al. 2010). However, further confirmatory tests were performed to confirm the identity of the bacteria.

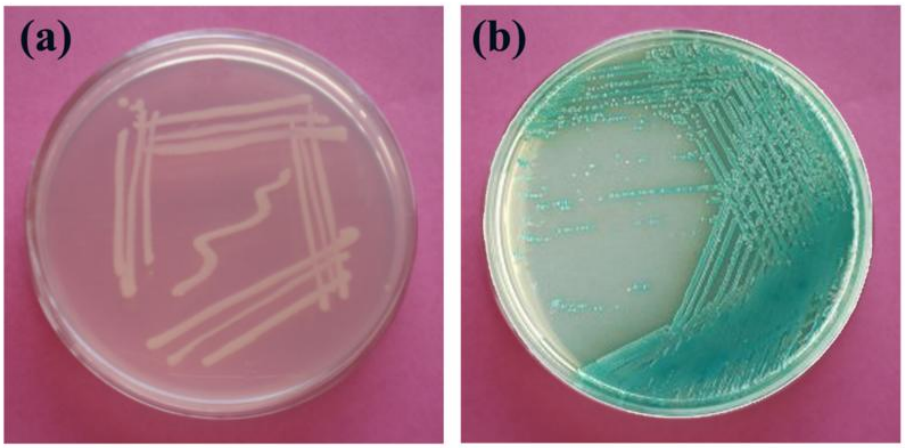


Fig. 4.4 Colony characteristics of the bacterial isolate 2B (a) after 18 hours (b) after 24 hours on the nutrient agar medium

4.3.2 MICROSCOPIC CHARACTERISTICS

Gram staining test revealed that the bacterium 2B was Gram negative and rod shaped. Fig. 4.5 shows the Gram-negative rods of the bacterium 2B. Hanging drop motility test showed that the isolate was motile. The bacterium did not produce endospore. Capsule staining test indicated that the bacterium possessed a capsule layer.

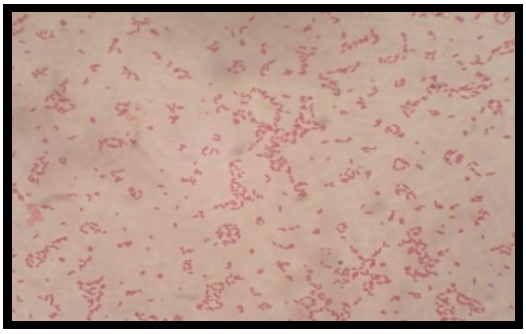


Fig. 4.5 Gram negative rods of the bacterium 2B

The result indicates that the bacterium might be a *Pseudomonas* species as similar result has been observed by Reyes et al. (1981) in the literature. However, biochemical tests were further performed to identify the bacterial isolate, 2B.

4.3.3 BIOCHEMICAL CHARACTERISTICS

The bacterial isolate 2B was subjected to biochemical tests. Since the culture was Gram negative bacterium, it was subjected to Methyl red test, Voges-Proskauer test, Citrate utilization test and Indole test. The culture tested negative for Methyl red test, Voges-Proskauer and Indole test; but tested positive for Citrate utilization test. It showed positive oxidase and catalase test. It showed positive result for glucose fermentation whereas negative results for sucrose and lactose fermentation. The isolate showed negative results for starch hydrolysis, gelatin hydrolysis and urease test. Further, the isolate tested positive for hydrogen sulphide production test. The biochemical tests results match with characteristics of *Pseudomonas sp.* as given in Bergey's Manual of Systematic Bacteriology (Krieg and Holt 1984). On the basis of its morphological, biochemical and cultural characteristics listed in Table 4.7, the isolate 2B matched with the characteristics of *Pseudomonas sp*. The identification of the bacterium 2B was further confirmed using partial 16S ribosomal DNA sequencing.

Table 4.7 Microscopic observations and biochemical tests performed for the identification of the bacterial isolate 2B

Characteristics/ Test	Result
Density	Translucent
Form	Irregular
Margin	Entire
Elevation	Flat
Colour	Greenish
Simple staining	Bacilli
Gram staining	Gram negative bacilli
Motility test	Motile
Capsule staining	Presence of capsule
Endospore staining	No endospore
Starch hydrolysis	Negative
Catalase test	Positive
Methyl red test	Negative
Voges-Proskauer test	Negative
Indole test	Negative

Citrate utilization test	Positive
Fermentation of glucose	Positive
Fermentation of sucrose	Negative
Fermentation of lactose	Negative
Urease test	Negative
Hydrogen sulphide production	Positive
Hydrolysis of gelatin	Negative
Oxidase test	Positive

4.3.4 MOLECULAR CHARACTERISTICS

In the present study, the genotypic analysis on the basis of partial 16S ribosomal DNA sequencing was examined to determine the precise taxonomic position of the isolate 2B. The 16S ribosomal DNA sequencing was carried out in Agharkar Research Institute; Pune, Maharashtra, India, which showed 95% maximum identity with the genus *Pseudomonas*. The genome sequence is as shown in Fig. 4.6 which has G- 49, A- 52, T- 41 and C-43. The sequence analysis revealed that the isolate as a novel *Pseudomonas sp.* Hence, the 16S ribosomal DNA sequence of the novel isolated bacterium was submitted in the GenBank database with an accession number JF683582.

Fig 4.6 16S ribosomal DNA sequence of the isolate *Pseudomonas sp.* 2B

TTGGCATGGGCGAAGCCTGTTCCCCCCATGCCGCGTGTGTNTAAGAACGTCTT
CGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTG
CTGTTTTGACGTTACCAACAGAATAAGCACCGGNTAACTTCGTGCCAGCAGC
CGCGGTANTACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG
CGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGA
ACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCC
TGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTCGGCGAAGG
CGACCACCTGGACTGATACTGACACTGAGGTACGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTT
GGGATCCTTGAGATCTTAGTGGCGCACTAACGCGATAAGTCGACCGCCTGGG
GGAGTACNGCCGCNAGGTTAAAACTCAAATGAATTGACNGGGGCCCGCACA
AGCGGTGGAGCATGTGGTTTTAATTCGAAGCACCGCGAANAACCTTACCTGG
CCTTGACATGCTGAGAAANTTTTCCANANATGGGATTGGTGCCTTCGGGAAC
TCANACNCAGGTGCTGCATGGCTGTCNTCCACCTC

Based on the microscopic studies, biochemical tests and 16S ribosomal DNA sequencing results, the selected isolate 2B was characterized as *Pseudomonas sp.* The genus *Pseudomonas* includes metabolically diverse strains thriving in many environmental niches and polluted sites. Among the various microorganisms reported in the literature, *Pseudomonas sp.* is the most common producer of surfactant, isolated from petroleum-contaminated soil samples (Deziel et al. 1996; Pornsunthorntawee et al. 2008; Price et al 2009). Other microorganisms that have been known to produce surfactant include *Arthrobacter paraffineus* (Itoh and Suzuki 1974), *Rhodococcus erythropolis* (Rapp et al. 1979), *Corynebacterium lepus* (Duvnjak and Kosaric 1985), *Bacillus subtilis* HOB 2 (Namir et al. 2009), *Ustilago sp.* (Spencer et al. 1979), *Candida lipolytica* (Cirigliano and Carman 1985), *Nocardia corynebacterioids* (Powalla et al. 1989), *Aspergillus ustus* (Seghal et al. 2009), etc.

SUMMARY

The candidate bacterial isolate 2B was subjected to microscopic studies, biochemical tests and partial 16S ribosomal DNA sequencing for its identification. The results of microscopic studies and biochemical tests matched with the characteristics of the genus *Pseudomonas* as given in Bergey's Manual of Systemic Bacteriology. The sequencing results showed 95% maximum identity with the genus *Pseudomonas* and it was reported to be a novel *Pseudomonas sp.* Pseudomonads are the well known bacteria capable of utilizing hydrocarbons as carbon and energy sources; they produce surfactants which enhance the uptake of immiscible hydrophobic compounds (Al-Tahhan et al. 2000; Beal and Betts 2000; Rahman et al. 2002). The production of surfactant by *Pseudomonas sp.* has been reported by several authors in the literature (Reiling et al. 1986; Zhang and Miller 1992; Wei et al. 2005; Perfumo et al. 2006; Anwanyu and Chukwudi 2010; Dubey et al. 2012). In the present work, we report the extracellular surfactant production by the novel *Pseudomonas sp.* 2B.

4.4 EXTRACTION OF THE SURFACTANT

The positive drop collapse activity of the supernatant and decrease in surface tension the cell free-broth of the bacterial strains *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, indicated the production of extracellular surfactant. Hence, there was a need to extract the surfactant from the broth. Various methods were used for the extraction of the surfactant from the cell-free broth. They were acidification of the cell-free broth followed by extraction using chloroform-methanol mixture (2:1), acidification of the cell-free broth followed by extraction using ethyl acetate, acid precipitation, extraction using acetone and extraction using diethyl ether. Table 4.8 presents the results obtained following the extraction of surfactants.

Among the methods studied in Table 4.8, acidification followed by chloroform: methanol mixture (2:1) extraction was effective in the extraction of the surfactant from

the cell-free broth of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, since both the polar as well as the non-polar components of the surfactant could be extracted. A maximum of 3.40 g/L and 1.40 g/L of surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was obtained during the extraction using the above described method. Acidification of the cell-free broth followed by extraction using ethyl acetate yielded a maximum of 0.91 g/L and 0.42 g/L of surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. 1.32 g/L and 0.76 g/L of surfactant were obtained during the acidification of the cell free broth of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* strains, respectively. Only trace quantity of surfactant could be extracted using diethyl ether. In general, the extraction yield can be improved by an acidification of the sample prior to extraction, as some of the surfactants are present in their protonated form and hence, are less soluble in water (Heyd et al. 2008). Hence, in the present study, the acidification of the broth followed by solvent extraction yielded maximum surfactant synthesized by both the bacterial strains. Surfactant extraction using acidification of the cell-free broth to pH 2 followed by chloroform: methanol (2:1) procedure was used by Tahzibi et al. (2004) and Parveen et al. (2012). Methods used for the surfactant extraction include acid precipitation (Arima et al. 1968), dichloromethane (Cooper et al. 1981), chloroform-methanol mixture (Cirigliano and Carman 1984), ethanol-acetic acid (Cameron et al. 1988), acetone extraction (Neu et al. 1990), chloroform-ethanol mixture (Zhang and Miller 1992), ethyl acetate extraction (Déziel et al. 1999), methyl tertiary-butyl ether (Maria et al. 2001), etc.

Table 4.8 Screening various methods for the extraction of surfactant synthesized by the bacterial strains *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively. Results are represented as Mean ± SEM (n=3)

Sl. No.	Extraction method	Surfactant quantity produced by *Pseudomonas sp.* 2B (g/L)	Surfactant quantity produced by *Pseudomonas aeruginosa* (g/L)
1.	Acidification of the cell-free broth followed by extraction using chloroform-methanol mixture (2:1 ratio)	3.40±0.3	1.40±0.2
2.	Acidification of the cell-free broth followed by extraction using ethyl acetate	0.91±0.1	0.42±0.09
3.	Acid precipitation	1.32±0.07	0.76±0.04
4.	Extraction using acetone	0.34±0.2	0.17±0.3
5.	Extraction using diethyl ether	BDL	BDL

BDL- Below detectable limit (Less than 0.001 g/L)

SUMMARY

In the present study, acidification followed by chloroform: methanol mixture (2:1) extraction was the most effective method in the extraction of the extracellular surfactant from the cell-free broth of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, as compared to other methods. A maximum of 3.4 g/L and 1.4 g/L of surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively was extracted. The present study emphasizes the need to utilize the suitable surfactant extraction method since the extraction of surfactant depends on the nature/structure of the

surfactant produced. This fact is supported by various reports in the literature wherein different methods have been employed for the extraction of surfactants (Hisatsuka et al. 1971; Lang and Wagner 1987; Maneerat and Phetrong 2007; Das et al. 2009).

4.5 SELECTION OF SURFACTANT PRODUCTION MEDIA

In the initial isolation and screening experiments, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, produced a maximum of 3.40 g/L and 1.40 g/L of surfactant quantity on Bushnell-Hass medium amended with 1% (v/v) crude oil. The objective of the present study was to screen different surfactant production media to increase the production of surfactant. The different media used were Proteose Peptone Glucose Ammonium Salt (PPGAS) Medium (designated as M1 medium containing glucose, peptone, Tris HCl, ammonium chloride, potassium chloride and magnesium sulphate), Yeast extract-Peptone-Glucose (YPG) medium (designated as M2 medium containing yeast extract, peptone and glucose), Mineral salt medium (designated as M3 medium containing glucose, potassium nitrate, salts and trace elements) and Mineral medium (designated as M4 medium containing glucose, sodium nitrate, phosphoric acid, salts and trace elements), respectively.

Fig 4.7 presents the results of the effect of media composition on surfactant production by the isolate *Pseudomonas sp.* 2B. Among the four different surfactant production medium considered in the present study, maximum production of surfactant by *Pseudomonas sp.* 2B, occurred in the M1 medium (Proteose Peptone Glucose Ammonium Salts medium). This might be because of the presence of Tris HCl in the medium which might have prevented drastic change in the pH during the surfactant production. Due to the buffering capacity of Tris HCl; the pH remained in the range of 7.0-7.4 during the surfactant production. In addition, the other medium components such as peptone, glucose and ammonium chloride might have triggered the production of surfactant. A maximum of 5.10 g/L and 2.82 g/L of surfactant and biomass concentration was produced by the isolate, *Pseudomonas sp.* 2B in the M1 medium. Least surfactant

production quantity (0.67 g/L) was observed in the M4 medium which was composed of mineral salts and glucose, the pH of the medium varied from 5 to 8 during the growth of the isolate, this might be the reason for least surfactant production. Although the M2 medium (YPG medium) contained yeast extract, glucose as well as peptone), it only triggered the growth of the bacterial isolate *Pseudomonas sp.* 2B. 2.68 g/L of surfactant and 1.25 g/L of biomass quantity was synthesized in the M2 medium by *Pseudomonas sp.* 2B; it was not found to be the suitable one for the production of surfactant. During the growth of the isolate in the M2 medium, the pH dropped to 4, this may be because of the production of acids that affected the production of surfactant. In the medium M3, which mainly contained mineral salts and glucose as carbon source, a maximum of 1.25 g/L of the surfactant and 1.04 g/L of biomass quantity was produced, the pH of the medium varied from 4-9 during the bacterial growth which resulted in decreased surfactant production. Maximum reduction of surface tension of the cell free broth of *Pseudomonas sp.* 2B was attained in the M1 medium, surface tension of the cell-free broth decreased from 69.89 mN/m to 32.23 mN/m. In the M2, M3 and M4 medium, the surface tension values were 38.31 mN/m, 44.67 mN/m and 49.23 mN/m, respectively. The M1 medium (PPGAS medium) was used by Gunther et al. (2005) and they reported a maximum of 1.0 g/L of surfactant production by *Pseudomonas chlororaphis.*

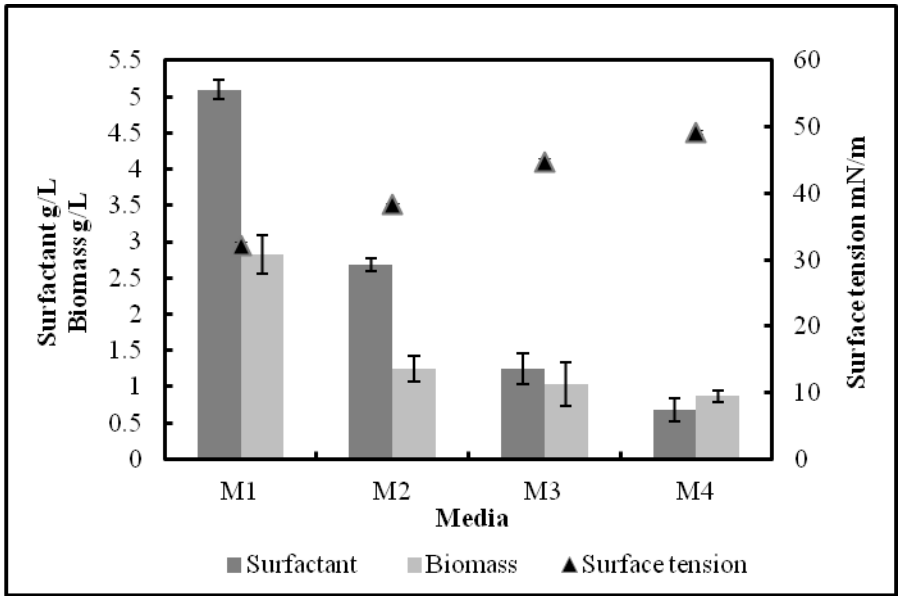


Fig 4.7 Screening various production media for the surfactant production by *Pseudomonas sp.* 2B. The culture flasks were incubated at 30ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig 4.8 presents the results of the effect of media composition on surfactant production by *Pseudomonas aeruginosa*. The bacterium produced a maximum of 4.15 g/L of surfactant and 2.16 g/L of biomass quantity in the M1 medium containing Tris HCl buffer. The surface tension of the cell-free broth lowered from 69.89 mN/m to 34.57 mN/m in the M1 medium. The pH of the broth during surfactant production in the M1 medium ranged between 6.9-7.3. Least surfactant production (0.82 g/L) by *Pseudomonas aeruginosa* was observed in M2 medium as mentioned earlier, the M2 medium supported biomass production (1.07 g/L) rather than surfactant production; the surface tension of the cell-free broth was 47.66 mN/m. In the M3 and M4 medium, a maximum of 0.96 g/L and 0.84 g/L of the surfactant quantity was produced by *Pseudomonas aeruginosa*. The amount of biomass produced in the M3 and M4 was 1.13 g/L and 0.52 g/L, respectively. The surface tension values recorded in the M3 and M4 were 46.24 mN/m and 47.23 mN/m, respectively. It was also observed that during the growth of *Pseudomonas*

aeruginosa in medium M2, M3 and M4, the pH varied from 4-8, which might be the reason for the reduced surfactant production. Similar results were obtained by Mulligan and Gibbs (1989), who reported that *Pseudomonas aeruginosa* ATCC 9027 var. RCII did not produce surfactant in the minimal media mainly due to the presence of phosphate. They further reported the production of surfactant in the PPGAS medium, which was devoid of phosphate, decreased the surface tension of the cell-free broth to 29 mN/m due to the production of surfactant. They also observed that the pH remained in the range of 6.3-7 during the growth of the organism in the PPGAS medium.

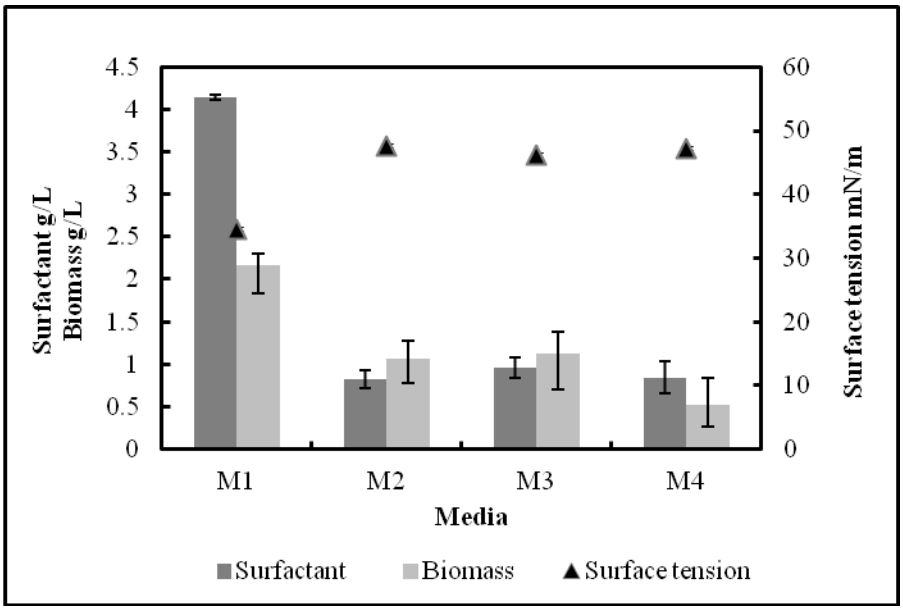


Fig 4.8 Screening various production media for the surfactant production by *Pseudomonas aeruginosa.* The culture flasks were incubated at 30ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

In the present study, the medium M1 (PPGAS medium) was found to be suitable for surfactant production by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, as it supported maximum biomass and surfactant production. A

maximum of 5.10 g/L and 2.82 g/L of surfactant and biomass quantity was recorded for the isolate, *Pseudomonas sp.* 2B in the M1 medium whereas *Pseudomonas aeruginosa* produced a maximum of 4.15 g/L of surfactant and 2.16 g/L of biomass quantity in the M1 medium. Due to the production of the surfactant by the two bacterial strains, the surface tension of the cell-free broth was found to be 32.23 mN/m and 34.57 mN/m, respectively. Hence, the surfactant production medium M1 (Proteose Peptone Glucose Ammonium Salts medium) was selected for further studies. Many researchers have reported a permutation and combination of basic media combined with salts and trace elements for the enhanced production of surfactant. In a study carried out by Ewa and Bogdan (2003), production medium consisting of glucose, potassium salts and other mineral salts was employed for the production of surfactant by *Bacillus coagulans*; a maximum of 0.3 g/L of surfactant quantity was produced. Jiraporn et al. (2006) reported that a maximum of 2.93 g/L of surfactant quantity was secreted by *Pseudomonas aeruginosa* in a production medium consisting of vegetable oil as carbon source, vitamins and trace elements. Mineral salt medium was employed by Victoria et al. (2010) to produce surfactant by *Rhodococcus fascians* and they reported a maximum of 1.8 g/L of surfactant production. The results of the present study are promising since the productivity of surfactant is better compared with the values reported for quantities of surfactant produced by different microbial species in the literature.

4.6 TIME COURSE STUDY OF SURFACTANT PRODUCTION

In order to study the relationship between surfactant production and growth of the bacterial biomass of both the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, were subjected to time course studies in the PPGAS medium.

Fig. 4.9 presents the results of the time course study of surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium. It can be observed that the growth of bacterium, *Pseudomonas sp.* 2B, was accompanied by production of surfactant in PPGAS medium as indicated by increase in the biomass. The production of surfactant coincided

with glucose utilization as indicated by the decrease in the residual glucose concentration during the growth of the organism. There was increase in surfactant production and reduction in surface tension with increase in biomass growth. This suggests that the surfactant produced might be a primary metabolite as the surfactant production occurred in the exponential growth phase. The logarithmic growth phase was observed till 72 hours. Maximum surfactant quantity of 5.13 g/L occurred at 72 hours of growth, the corresponding biomass and surface tension values were 2.89 g/L and 32.18 mN/m, respectively. Further, a slight decrease in the surfactant concentration was observed at 96 hours of incubation, which might have occurred due to the exhaustion of the glucose in the medium with the commencement of stationary phase. The surface tension value of the cell-free broth almost remained constant during the stationary phase as there was no further increase in surfactant quantity at this stage. Similar trend of surfactant production by *Pseudomonas aeruginosa* ATCC 9027 was observed by Mulligan and Gibbs (1989). The results of the present study is in agreement with the trend observed by Abushady et al. (2005) who studied the production of surfactant by *Bacillus subtilis* BBk1 AB01335-1, the strain produced a 0.5 g/L of surfactant quantity at 24 hours. They observed that the surfactant production increased further in course of time, 1.4 g/L of surfactant quantity was produced at 50 hours of incubation. The surfactant production by *Bacillus subtilis* BBk1 AB01335-1 was maximum (1.5 g/L) at 70 hours of incubation and it remained constant till 150 hours of incubation indicating that the bacterial strain was in the stationary phase.

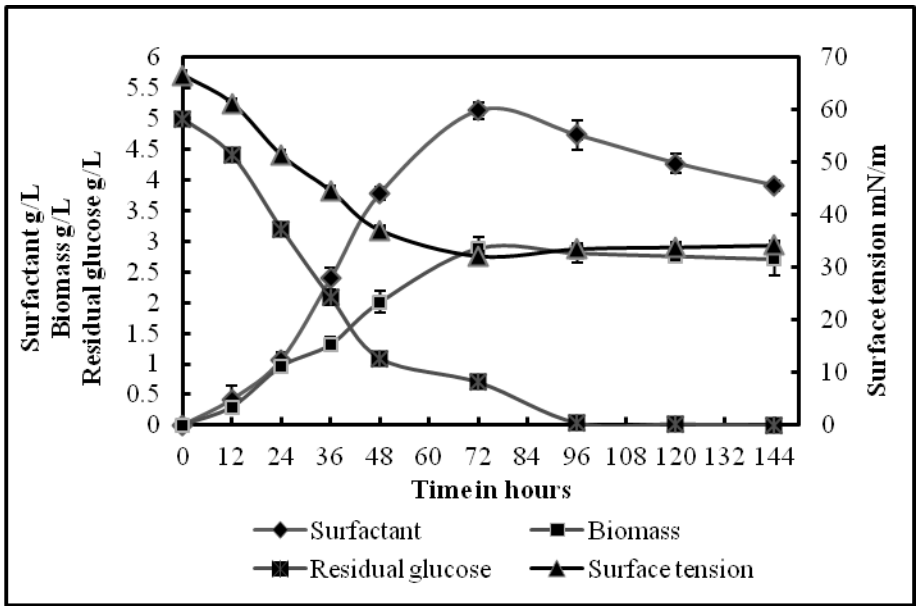


Fig. 4.9 Time course study of surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7). The culture flasks were incubated at 30ºC in incubator shaker at 150 rpm. Results are represented as Mean ± SEM (n=3)

Fig. 4.10 presents the results of the time course study of surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium. The surfactant production was observed in the exponential growth phase, which suggests that surfactant production was associated with microbial growth. Following the growth phase, the bacterial strain entered the stationary phase at 96 hours. Depletion of glucose in the medium was particularly the limiting factor for growth of the bacterial strain as indicated by the residual glucose concentration and this resulted in the stationary phase. A maximum of 4.17 g/L of surfactant and 2.41 g/L of biomass quantity was produced at 96 hours of incubation. The surface tension of the cell-free broth was found to be 34.60 mN/m. This study further reveals that the surfactant produced by the bacterial strain might be a primary metabolite as the surfactant production coincided with bacterial exponential growth phase. Similar observations were made by Cooper et al. (1981) and Lin et al. (1993) where growth associated production of surfactant was reported for *Bacillus*

subtilis and *Bacillus licheniformis* JF-2, respectively. Benincasa et al. (2002) also observed a similar trend during the production of surfactant by *Pseudomonas aeruginosa* LBI.

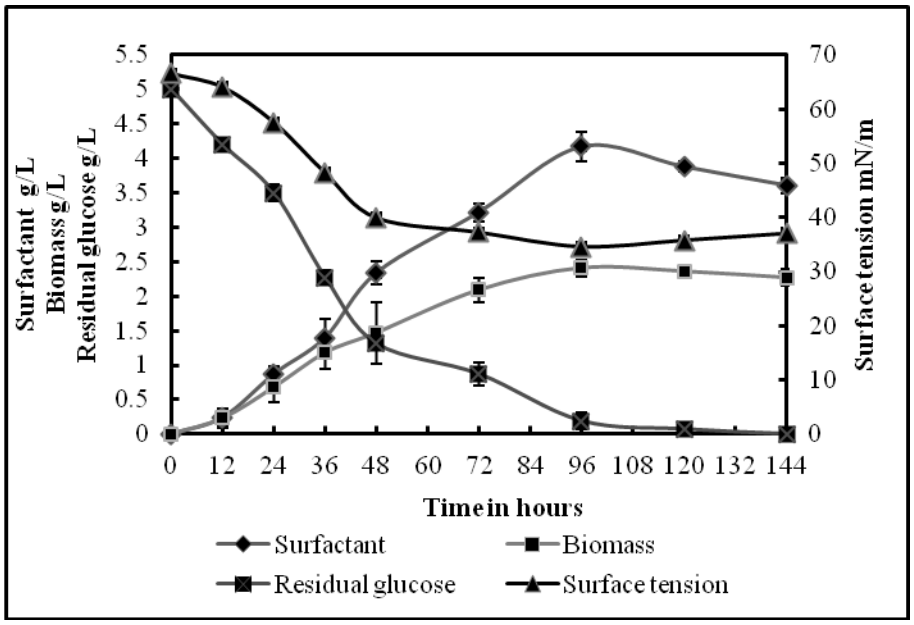


Fig. 4.10 Time course evaluation of surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7). The culture flasks were incubated at 30ºC in incubator shaker at 150 rpm. Results are represented as Mean ± SEM (n=3)

SUMMARY

In the present study, time course study was conducted in order to understand the pattern of biomass growth and surfactant production in the M1 medium (PPGAS medium) by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. A maximum of 5.13 g/L and 2.89 g/L of surfactant and biomass quantity was produced by *Pseudomonas sp.* 2B at 72 hours whereas 4.17 g/L and 2.41 g/L of surfactant and biomass quantity was produced by *Pseudomonas aeruginosa* at 96 hours. The surface tension values of the cell-free broth of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* were 32.18 mN/m and 34.60 mN/m, respectively. *Pseudomonas sp.* 2B and

Pseudomonas aeruginosa, respectively, utilized glucose efficiently during their growth as indicated by the residual glucose concentration. The study further indicates that the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, might be primary metabolite since the surfactant production coincided with exponential growth phase. Similar observations have been made by several authors in the literature where the surfactant produced was confirmed as primary metabolite (Abu-Ruwaida et al. 1991; Tabatabaee et al. 2005; Adebusoye et al. 2008).

4.7 EFFECT OF PROCESS PARAMETERS ON SURFACTANT PRODUCTION

There are a number of operating parameters controlling surfactant production, which are required to be maintained within a certain range of operating conditions in order to maximize the production of surfactant. The quantity of surfactant was strongly dependant on factors, such as pH, temperature, nutrient composition and types of substrate used (Mulligan and Gibbs 1993). In this regard, the effect of process parameters on surfactant production was studied in the present study. The Proteose Peptone Glucose Ammonium Salts medium (PPGAS) was selected to study the effect of process parameters on surfactant production by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. The various process parameters evaluated for surfactant production by the selected bacterial strains were inoculum size, initial pH of the production medium, incubation temperature, different types of carbon and nitrogen sources, concentration of carbon and nitrogen sources, inducer, buffer system and salinity on surfactant production by the two bacterial strains were studied.

4.7.1 EFFECT OF INOCULUM VOLUME ON SURFACTANT PRODUCTION

The effect of inoculum volume on surfactant production was carried out in the PPGAS medium as the density of the microbial cells is an important factor that affects the surfactant production. The medium was inoculated with different inoculum volumes

(0.5%, 1%, 2%, 3%, 5%, 7% and 10% (v/v) (optical density 0.125~10^8 cells) of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively.

Fig. 4.11 presents the results of the effect of inoculum volume on surfactant production by *Pseudomonas sp.* 2B. For 0.5% and 1% (v/v) inoculum volume of *Pseudomonas sp.* 2B cells, the quantity of surfactant produced was 2.45 g/L and 3.96 g/L, respectively; the corresponding biomass quantity was 1.28 g/L and 1.93 g/L, respectively. This might be because lower inoculum size led to production of insufficient number of bacteria, which inturn led to reduced amount of surfactant production. The surface tension values were found to be 37.56 mN/m and 35.76 mN/m for 0.5% and 1% (v/v) of inoculum volume, respectively. With increase in the inoculum size, there was increase in surfactant production till a certain level [2% (v/v) inoculum volume]. Thereafter, increase in the inoculum size led to decrease in the surfactant concentration. It was found that for 2% (v/v) inoculum volume, the bacterial strain showed a maximum of 5.31 g/L of surfactant and 3.29 g/L of biomass production among the different inoculum volumes considered in the study, the corresponding surface tension of the cell-free broth was found to be minimum (32.21 mN/m). When the inoculum volume was 3% (v/v), there was decrease in the surfactant and biomass quantities produced, 4.14 g/L of surfactant and 2.08 g/L of cellular biomass was produced. The corresponding surface tension value was 33.64 mN/m. At higher inoculum volume (10% v/v), the surfactant (2.60 g/L) as well as biomass (1.12 g/L) production was least and also it affected the surface tension of the cell-free broth (38.44 mN/m). This might be because higher inoculum size resulted in reduced dissolved oxygen and increased competition towards nutrients in the culture medium, thus decreasing the growth of the bacterial strain due to exhaustion of nutrients in the medium and in turn, leading to the lowering of surfactant production. Similar observation was reported by Abushady et al. (2005) and they noted that inoculum volume of around 2% (v/v) resulted in a low level of surfactant quantity by *Bacillus subtilis* BBk1 AB01335, indicating that the suitable volume of the inoculum that should be added to the production medium was 2% (v/v). They also observed for 1% (v/v) inoculum

volume, 1.60 g/L of surfactant quantity was produced. A maximum of 2.30 g/L of surfactant quantity was produced for 2% (v/v) by *Bacillus subtilis* BBk1 AB01335, whereas for 3% (v/v) inoculum size, the quantity of surfactant reduced to 1.40 g/L.

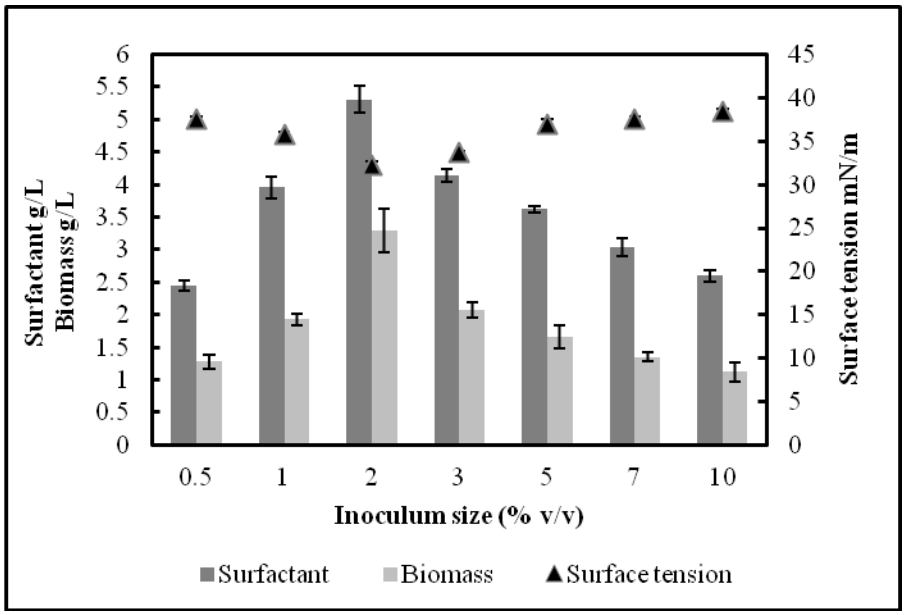


Fig 4.11 Effect of inoculum volume on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7). The culture flasks were incubated at 30ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

The results of the effect of inoculum volume on surfactant production by *Pseudomonas aeruginosa* is presented in Fig 4.12. For 0.5% (v/v) inoculum volume of *Pseudomonas aeruginosa* cells, the amount of surfactant and biomass quantity produced was 2.20 g/L and 1.01 g/L, respectively; the corresponding surface tension of the cell-free broth was found to be 37.43 mN/m. The bacterial strain produced 2.45 g/L of surfactant and 1.28 g/L of biomass quantity for the inoculum volume 1% (v/v); the corresponding surface tension value of cell-free broth was 37.01 mN/m. At 2% (v/v) inoculum volume, the surfactant and biomass quantity was found to be 3.92 g/L and 2.19 g/L, respectively; the surface tension of the cell-free broth was 35.59 mN/m. 3% (v/v) inoculum volume of

the bacterium produced a maximum of 4.25 g/L of surfactant and 2.31 g/L of biomass quantity, minimum value for surface tension obtained was 33.07 mN/m. With further increase in inoculum volume of *Pseudomonas aeruginosa* cells, the surfactant as well as biomass quantities reduced probably due to faster exhaustion of the medium components. For 10% (v/v) inoculum volume, the amount of surfactant and biomass concentration produced was 1.98 g/L and 1.03 g/L, respectively. The surface tension of the cell-free broth (39.47 mN/m) was also affected at 10% (v/v) inoculum size as there was reduction in the surfactant quantity. Increased competition towards nutrients in the culture medium might lead to decrease in the cellular biomass which probably occurs due to exhaustion of nutrients in the medium at higher inoculum volume. The reports in the literature suggest that the maximum production of surfactant was reported when the inoculum volume was in the range of 0.5-2% (v/v) (Makkar and Cameotra 1997; Pornsunthorntawee et al. 2008; Ray 2012).

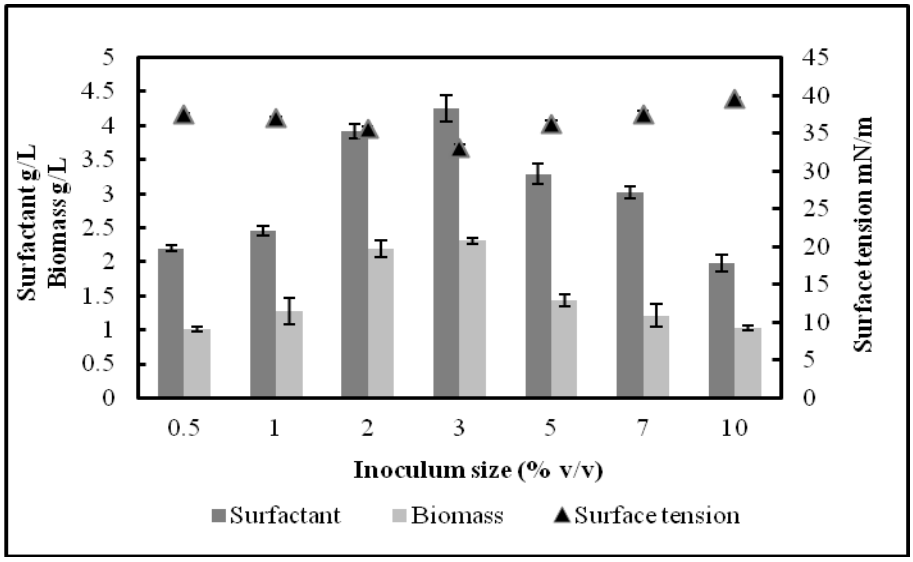


Fig 4.12 Effect of inoculum volume on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7). The culture flasks were incubated at 30ºC in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

The results from the present study indicate that with increase in inoculum volume, there was an increase in the surfactant concentration till a certain inoculum volume, thereafter, there was decrease in the quantity of surfactant produced which in turn affected the surface tension. The results reveal that the suitable inoculum volume for maximum surfactant production by the strains *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, were 2% and 3% (v/v), respectively; a maximum of 5.31 g/L and 4.25 g/L of surfactant quantity was produced. The corresponding surface tension of the cell-free broth was 32.21 mN/m and 33.07 mN/m, respectively. The effect of inoculum volume on surfactant production by various strains has been emphasized in the literature (Dhouha and Semia 2011). In a study by Makkar and Cameotra (2002), 2% (v/v) inoculum volume was added to the medium to achieve maximum surfactant production (1.32 g/L) by *Bacillus subtilis* whereas Onbasli and Aslim (2009) used 1% (v/v) inoculum volume for maximum surfactant production (0.53 g/L) by *Pseudomonas luteola* B17.

4.7.2 EFFECT OF INITIAL MEDIUM pH ON SURFACTANT PRODUCTION

The effect of initial production medium pH on the surfactant production by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively was studied by adjusting the initial pH of the PPGAS medium. The pH range was varied from 3 to 11. Inoculum volume maintained in the present study was 2% and 3% (v/v), for the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively.

Fig. 4.13 presents the results of the effect of initial medium pH on surfactant production by *Pseudomonas sp.* 2B. At acidic (3-5) and alkaline pH (8-11), the surfactant production by *Pseudomonas sp.* 2B was less; the surface tension of the cell-free broth was also affected. At pH 3 and 5, the surfactant quantity produced was 0.82 g/L and 1.78 g/L, respectively; the corresponding biomass values were 1.01 g/L and 1.93 g/L,

respectively. The corresponding surface tension values of the cell-free broth obtained at pH 3 and 5 were 43.82 mN/m and 39.23 mN/m, respectively. At pH 6, 3.95 g/L of surfactant and 2.87 g/L of cellular biomass quantity was produced; the corresponding surface tension of the broth was 36.64 mN/m. Maximum growth and surfactant yield was obtained *Pseudomonas sp.* 2B, at pH 7. The isolate produced a maximum of 5.45 g/L of surfactant and 3.34 g/L of biomass; the lowest surface tension value of 31.04 mN/m was recorded at pH 7. There was decrease in the surfactant and cell biomass concentration in the alkaline pH range (8-11); the quantity of surfactant was in the range of 1.51 to 4.77 g/L and biomass quantity was in the range of 1.05 to 2.55 g/L. The surface tension of the broth ranged from 33.43 to 39.82 mN/m in the alkaline pH condition. The reason for production of lower concentration of surfactant in the highly acidic and alkaline pH range might be because of the production of lower biomass as microorganisms are dependent on pH for their growth as well as synthesis of enzymes involved in the formation of surfactant moieties (Praveesh et al 2011; Sahoo et al. 2011). These results of the present study compare well with those reported by Khopade et al. (2012), the maximum surfactant production by *Nocardiopsis sp.* occurred at pH 7 as indicated by decrease in surface tension (30 mN/m). In their study, the surface tension of the cell-free broth in the acidic pH range (4-5) ranged from 58 to 62 mN/m whereas in the alkaline pH range (8-12), the surface tension values varied from 43 to 50 mN/m. In another study, Powalla et al. (1989) reported that surfactant production by *Nocardia corynebacteroides* was unaffected by the pH in the range of 6.5-8.0.

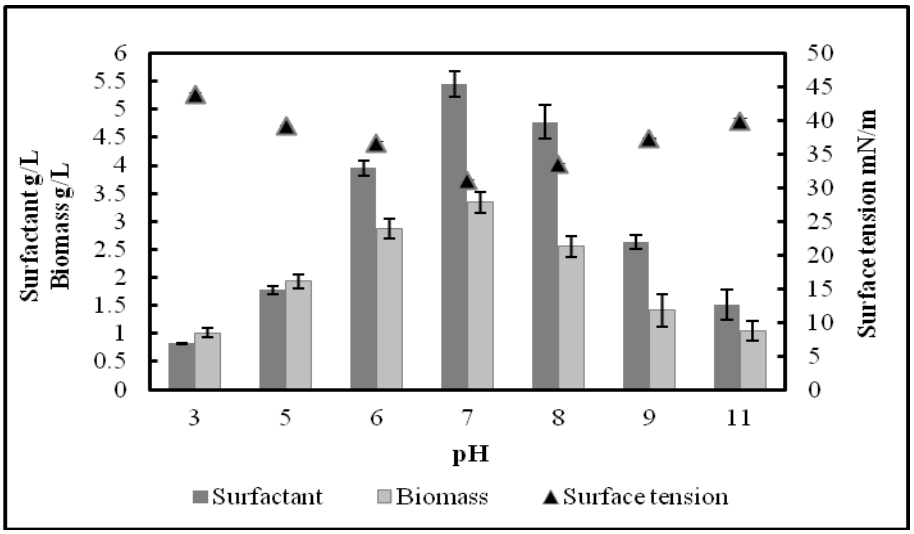


Fig 4.13 Effect of initial medium pH on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium. The culture flasks were incubated at 30ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.14 presents the results of the effect of initial medium pH on surfactant production by *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* also showed maximum surfactant and biomass synthesis at pH 7. At pH 7, the concentration of surfactant and bacterial biomass quantity produced was found to be 4.16 g/L and 2.18 g/L, respectively. The lowest value of surface tension (33.58 mN/m) was attained at pH 7. At acidic pH (3-5), the surfactant produced ranged from 0.53 to 1.32 g/L and the biomass quantity produced ranged from 0.78 to 1.21 g/L, this indicates that the acidic pH was not favourable for maximum surfactant production. Similar observation was made in the alkaline pH range (8-11), where the surfactant quantity ranged from 0.44 to 3.14 g/L, biomass ranged from 0.83 to 1.63 g/L and the surface tension of the cell free broth ranged from 37.49 to 44.18 mN/m. In a similar study carried by Abushady et al. (2005), they reported that at pH 6.5, maximum surfactant production (2.80 g/L) by *Bacillus subtilis* occurred; at pH 5, the surfactant quantity produced was 1.60 g/L whereas at pH 9, the surfactant production reduced to 1.30 g/L. However, Zinjarde and Pant (2002) reported that the maximum production by *Yarrowia lipolytica* occurred when the pH was 8.0, they

further observed that the same pH was prevelant at the place from where the organism was isolated.

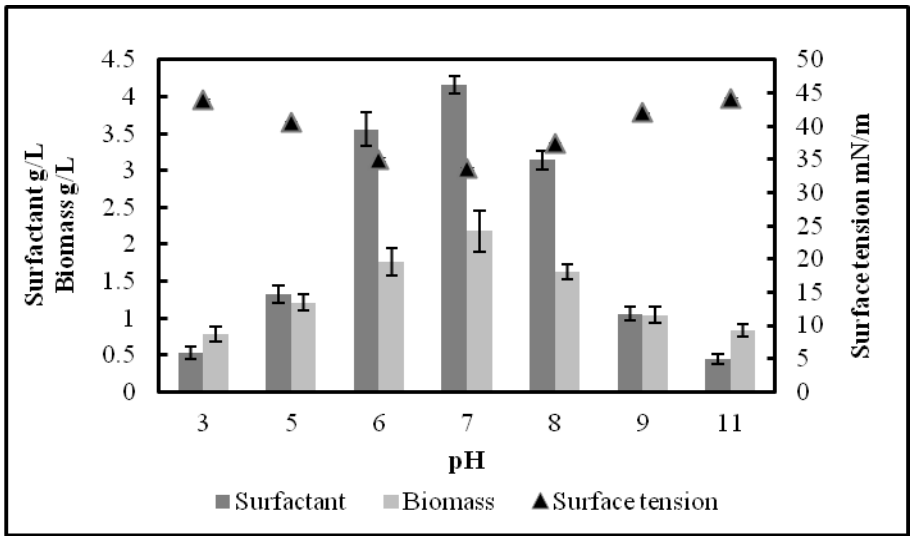


Fig 4.14 Effect of initial medium pH on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium. The culture flasks were incubated at 30ºC in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

Results from the present study showed the bacterial strains were strongly dependent on the pH for their growth and production of surfactants. Any change to both lower or higher pH values caused an appreciable drop in surfactant and biomass production which inturn affected the surface tension reduction potential. The initial pH for maximum surfactant production by the bacterial strains *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, was pH 7; a maximum of 5.45 g/L and 4.16 g/L of surfactant quantity was produced by the strains, which reduced the surface tension of the cell-free broth to 31.04 mN/m and 33.58 mN/m, respectively. Similar studies were reported by various researchers in the literature with the conclusion that initial medium pH ranging from 6-8 favoured maximum surfactant production by various

microorganisms (Guerra-Santos et al. 1984; Powalla et al. 1989, Tabatabee et al. 2005). Initial surfactant production medium pH of 7 was considered for the further studies.

4.7.3 EFFECT OF INCUBATION TEMPERATURE ON SURFACTANT PRODUCTION

The effect of incubation temperature was studied on surfactant production by varying the incubation temperature. The flasks containing PPGAS medium were inoculated with *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* cells and incubated at different temperatures like 10, 20, 30, 37, 45 and 60ºC. The inoculum volume of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* used in the present study was 2% and 3% (v/v), respectively. The initial pH of the PPGAS medium was adjusted to 7 for the surfactant production by both the bacterial strains.

Fig. 4.15 presents the results of the effect of incubation temperature on surfactant production by *Pseudomonas sp.* 2B. The bacterial isolate showed maximum surfactant at 37ºC. A maximum of 5.90 g/L of surfactant and 3.56 g/L of biomass quantity was produced by *Pseudomonas sp.* 2B, the corresponding surface tension of the cell-free broth was found to be 30.04 mN/m. Lower or higher temperatures decreased both growth and surfactant production as detected by lower cellular yields and surfactant concentrations. At lower temperatures, *i.e.*, at 10, 20 and 30ºC, the surfactant quantities obtained were 1.60, 2.82 and 5.52 g/L, and the cellular biomass quantities obtained were 1.09, 1.63 and 3.39 g/L, respectively. The surface tension values at 10, 20 and 30°C obtained were 39.49 mN/m, 36.81 mN/m and 30.97 mN/m, respectively. At the higher temperatures, *i.e.*, at 45ºC to 60ºC; the surfactant quantity produced by *Pseudomonas sp.* 2B was 3.74 and 1.09 g/L, respectively. The corresponding bacterial biomass quantity was found to be 1.92 g/L and 1.13 g/L, respectively and surface tension values were 37.09 mN/m and 42.08 mN/m, respectively. The surfactant concentration was reduced at lower and higher incubation temperatures probably because of the reduced biological activity of the microorganisms (Yakimov et al. 1995). The surfactant structure is made up

of different moieties, the synthesis of each moiety is catalyzed by different enzymes; lower and higher incubation temperatures might have affected the enzyme activities, which inturn led to reduced biomass and surfactant production. The results of the present study are in agreement with the results of the effect of incubation temperature on surfactant production by *Nocardiopsis sp.* (Khopade et al. 2012). They reported that at 4ºC and 15ºC, the surfactant production was reduced as indicated by surface tension values; at these incubation temperatures, the surface tension of the broth was in the range of 70 to 75 mN/m. They further observed that with increase in incubation temperature, there was reduction in the surface tension. At 25ºC, the surface tension of the cell-free broth of *Nocardiopsis sp.* was 38 mN/m. The surface tension of the broth was lowest at 30ºC; a value of 36 mN/m was recorded in their study. At 35, 40, 45 and 60ºC, the surface tension values were 40 mN/m, 42 mN/m, 40 mN/m and 44 mN/m, respectively. Their report indicated that at higher incubation temperature, the surface tension values of the cell-free broth increased which is in agreement with the results of the present study.

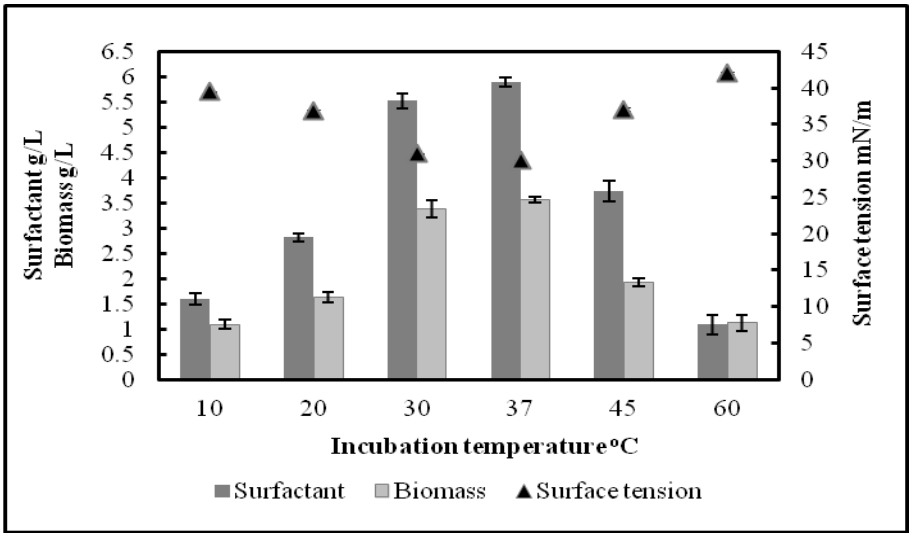


Fig 4.15 Effect of incubation temperature on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7). The culture flasks were incubated at different incubation temperature in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.16 presents the results of the effect of incubation temperature on surfactant production by *Pseudomonas aeruginosa*. Maximum surfactant by *Pseudomonas aeruginosa* was observed at 37ºC. A maximum of 5.41 g/L of surfactant and 3.32 g/L of cell biomass quantities were produced. The surface tension of the cell-free broth was found to be 31.23 mN/m. At lower temperatures, *i.e.*, at 10, 20 and 30ºC, the surfactant quantity obtained was 1.32 g/L, 2.34 g/L and 4.25 g/L, respectively and the cellular biomass quantity obtained was 1.08 g/L, 1.52 g/L and 2.21 g/L, respectively. The surface tension values at 10, 20 and 30ºC observed were 40.61 mN/m, 38.12 mN/m and 33.10 mN/m, respectively. At the higher temperatures, *i.e.*, at 45^{o}C to 60ºC; the surfactant quantity produced by *Pseudomonas sp.* 2B was 4.60 g/L and 1.11 g/L, respectively. The corresponding bacterial biomass quantity was found to be 2.51 g/L and 1.14 g/L, respectively and surface tension values were 32.74 mN/m and 42.02 mN/m, respectively. The results of the present study is in agreement with the reports in literature which suggest that surfactant production by *Pseudomonas aeruginosa* (Guerra-Santos et al. 1984), *Rhodococcus sp.* (Bicca et al. 1999); *Bacillus subtilis* (Abushady et al. 2005) *Pseudomonas sp*. (Raza et al. 2006), *Bacillus megatarium* (Thavasi et al. 2008) was exclusively observed in the range of 30-37°C. It was observed by Husain et al. (1997) that low temperature slowed down surfactant production. However, there are few reports on increased surfactant production by thermophilic bacterial strains, *Bacillus lichenformis* (Yakimov et al. 1995) and *Bacillus subtilis* (Makkar and Cameotra 1997) at 45°C.

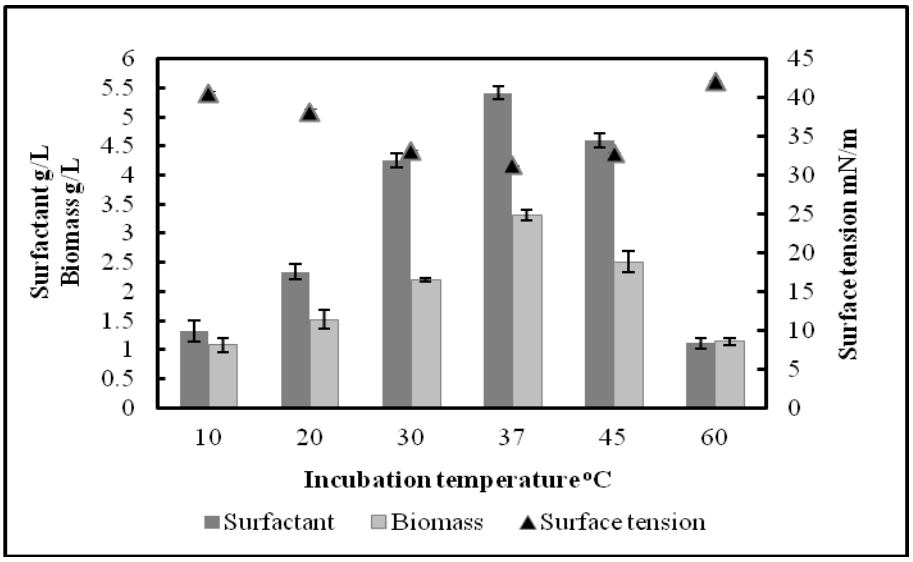


Fig 4.16 Effect of incubation temperature on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7). The culture flasks were incubated at different incubation temperature in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

In the present study, both the bacterial strains showed maximum surfactant at 37°C. It was observed that a slight change in the incubation temperature affected the surfactant synthesis as well microbial growth. Both the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, showed enhanced surfactant production at 37°C. A maximum of 5.90 and 5.41 g/L of surfactant quantity, respectively, was produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, at 37ºC; the corresponding surface tension values of the cell-free broth were 30.04 mN/m and 31.23 mN/m, respectively. The results of the present study are comparable with the reports of Priya and Usharani (2009), the maximum biosynthesis of surfactants by *Bacillus subtilis* and *Pseudomonas aeruginosa*, respectively, occurred at 37°C. Hence, based on the results of the present study, an incubation temperature of 37°C was maintained for future experiments.

4.7.4 EFFECT OF AGITATION ON SURFACTANT PRODUCTION

The effect of agitation speed on surfactant production was studied by varying the agitation speed of the flasks containing the PPGAS medium. The flasks were inoculated with the bacterial strains and incubated at 37ºC at different agitation speeds (50, 100, 150, 200 and 250 rpm). A control flask was maintained at static condition.

The results of the effect of agitation speed on surfactant production by *Pseudomonas sp.* 2B is presented in Fig. 4.17. It was observed that with increase in agitation speed, there was an increase in the surfactant production till 150 rpm; further increase in agitation speed caused decrease in surfactant production. At 50 and 100 rpm, the surfactant quantity produced was 2.44 and 3.82 g/L, respectively. The corresponding biomass quantity at 50 and 100 rpm was 1.29 g/L and 1.82 g/L, respectively. The corresponding surface tension values were 37.51 mN/m and 33.67 mN/m, respectively. Lower quantity of surfactant was produced at lower agitation speed due to the scarcity of oxygen experienced by the bacterial culture due to the insufficient mixing. Maximum surfactant production was observed at 150 rpm. The quantity of surfactant and biomass produced was 5.88 g/L and 3.52 g/L, respectively. The corresponding surface tension value was 30.11 mN/m. At agitation speed of 200 rpm and 250 rpm, a decrease in surfactant as well as biomass production was observed. The amount of surfactant produced at 200 rpm and 250 rpm was 4.75 g/L and 3.61 g/L, respectively. The amount of biomass produced at 200 rpm and 250 rpm was 2.62 g/L and 1.95 g/L, respectively. The corresponding surface tension values were 32.53 mN/m and 36.48 mN/m, respectively. In the static condition (control), the quantity of surfactant and biomass produced was 1.58 g/L and 1.19 g/L, respectively. The decrease in the surfactant production at higher agitation speed can be attributed to the harmful effect of the shear forces. The corresponding surface tension value was 39.09 mN/m. This indicates that agitation speed is an important parameter that affects surfactant production. In a similar study by Abushady et al. (2005), the effect of agitation speed on surfactant production

was investigated by varying the agitation speed from 0-200 rpm. They observed that maximum surfactant production by *Bacillus subtilis* AB01335-1 was found to be maximum (1.50 g/L) at 200 rpm. At 50, 100 and 150 rpm, the amount of surfactant produced by the bacterium was 0.52, 0.68 and 1.4 g/L, respectively. They further observed that the least production (0.5 g/L) was observed in the control flask which was kept in static condition.

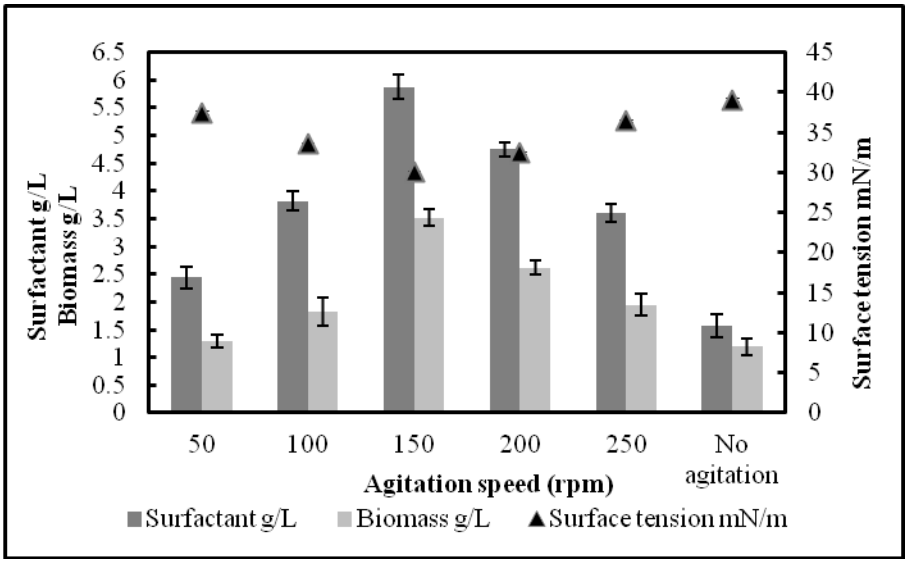


Fig 4.17 Effect of agitation speed on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7). The culture flasks were incubated for 72 hours at 37ºC. Results are represented as Mean ± SEM (n=3)

Fig. 4.18 presents the results of the effect of agitation speed on surfactant production by *Pseudomonas aeruginosa.* The trend was similar to that which was observed in the case of *Pseudomonas sp.* 2B. At 50 rpm and 100 rpm, the amount of surfactant produced was 2.41 g/L and 3.89 g/L, respectively. The corresponding biomass yield was 1.18 g/L and 1.89 g/L, respectively. The surface tension values obtained at 50 rpm and 100 rpm were 37.96 mN/m and 33.94 mN/m, respectively. At 150 rpm, maximum increase in surfactant production was observed. A maximum of 5.37 g/L of biosurfactant and 3.28 g/L of biomass quantity was produced; the corresponding surface

tension value was 31.29 mN/m. Further increase in agitation speed caused a decrease in the yield of surfactant production. At 200 rpm and 250 rpm, the amount of surfactant produced was 3.71 g/L and 2.29 g/L, respectively. The corresponding biomass amount was 1.84 g/L and 1.73 g/L, respectively. The corresponding surface tension values obtained were 36.92 mN/m and 37.61 mN/m at 200 rpm and 250 rpm, respectively. In the control flask (static condition), the amount of surfactant and biomass produced by the bacterial culture was 1.09 g/L and 1.10 g/L, respectively. The corresponding surface tension value was 45.19 mN/m. Loftabad et al. (2009) carried out a similar study, they observed that *Pseudomonas aeruginosa* MR01 produced a maximum of 0.8 g/L of surfactant at 250 rpm when the agitation speed was varied from 0-250 rpm. They further reported that the higher biosurfactant biosynthesis achieved at 250 rpm may be due to the higher biomass concentration reached, because of the beneficial effect of the increased agitation.

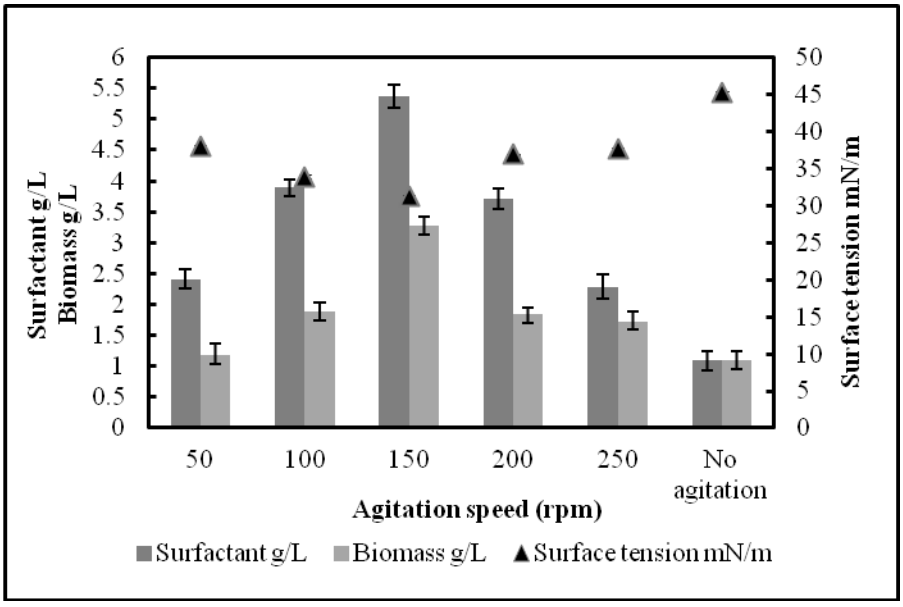


Fig 4.18 Effect of agitation speed on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7). The culture flasks were incubated for 96 hours at 37ºC. Results are represented as Mean ± SEM (n=3)

SUMMARY

In the present study, it was observed that maximum production of surfactant by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was observed at 150 rpm. *Pseudomonas sp.* 2B produced a maximum of 5.88 g/L of surfactant and 3.52 g/L of biomass quantity at agitation speed of 150 rpm; the corresponding surface tension value was 30.11 mN/m. At 150 rpm, a maximum of 5.37 g/L of biosurfactant and 3.28 g/L of biomass quantity was produced by *Pseudomonas aeruginosa*; the corresponding surface tension value was 31.29 mN/m. Nigam et al. (2012) reported that agitation brings about proper mixing of production broth as well as increases the oxygen transfer and thus, has a tremendous effect on the productivity of the system.

4.7.5 EFFECT OF CARBON SOURCE ON SURFACTANT PRODUCTION

Different water miscible and immiscible carbon substrates [at a concentration of 5 g/L w/v for solid carbon source and 5 ml/L (v/v) for liquid carbon source] were tested in the PPGAS medium for their capacity to support growth and surfactant production by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. The different carbon sources evaluated in the present study were glucose, sucrose, molasses, orange peelings, ethanol, glycerol, coconut oil cake, whey and peanut oil cake.

Fig. 4.19 presents the results of the effect of carbon source on surfactant production by *Pseudomonas sp.* 2B. In the present study, it can be observed that the surfactant quantity produced by *Pseudomonas sp.* 2B in medium supplemented with carbon substrates depended on the type of carbon source. All the carbon sources tested favoured extracellular production of surfactant by *Pseudomonas sp.* 2B, which was estimated by the reduction in surface tension of the cell-free broth. The results indicated that maximum surfactant and biomass production occurred when glucose was added to the medium. A maximum of 5.96 g/L of surfactant and 3.49 g/L of biomass quantity was produced at 72 hours and it effectively reduced the surface tension of the cell free broth

to 30.13 mN/m. This could be explained by the fact glucose was utilized easily for the growth of the microorganism due to its high solubility; whereas other carbon sources had varied complexities due to which lower surfactant quantity was produced. Surfactant quantity produced in molasses, glycerol, whey, coconut oil cake, orange peelings, ethanol, sucrose and peanut oil cake containing medium was 4.97 g/L, 4.14 g/L, 4.09 g/L, 4.05 g/L, 3.94 g/L, 3.88 g/L, 3.17 g/L and 2.86 g/L, respectively and their corresponding biomass values were 2.09 g/L, 1.93 g/L, 1.81 g/L, 1.88 g/L, 1.65 g/L, 1.58 g/L, 1.04 g/L and 1.23 g/L, respectively. The corresponding surface tension values were 33.14 mN/m, 33.36 mN/m, 33.48 mN/m, 33.59 mN/m, 34.61 mN/m, 34.72 mN/m, 35.44 mN/m and 35.67 mN/m, respectively. In the absence of the carbon source (control), the amount of surfactant and biomass produced was negligible which emphasizes that the carbon source is one of the important parameters involved in surfactant production. In the literature, different types of carbon sources have been used for surfactant production by different microorganisms. Makkar and Cameotra (1997) reported that *Bacillus subtilis* MTCC 2423 produced 0.652 g/L and 0.744 g/L of surfactant in glucose and sucrose containing medium, these surfactants reduced the surface tension of the cell-free broth to 29 mN/m and 28 mN/m, respectively. Molasses and whey were added in the production medium as carbon source by Patel and Desai (1997) and Dubey and Juwarkar (2001), 0.24 g/L and 0.92 g/L of surfactant quantity was produced by *Pseudomonas aeruginosa* GS3 and *Pseudomonas aeruginosa* BS2, respectively. *Bacillus circulans* produced 2.90 g/L and 2.50 g/L of surfactant quantity when glycerol and sucrose were used as carbon sources (Palashpriya et al. 2009). In a study carried out by George and Jayachandran (2009), a maximum of 9.18 g/L and 2.17 g/L of surfactant quantity was produced by *Pseudomonas aeruginosa* MTCC 2297 in medium containing orange peelings and coconut oil cake, respectively. Hence, it can be concluded from the present study that the strain *Pseudomonas sp.* 2B produced surfactant during its growth on various carbon sources.

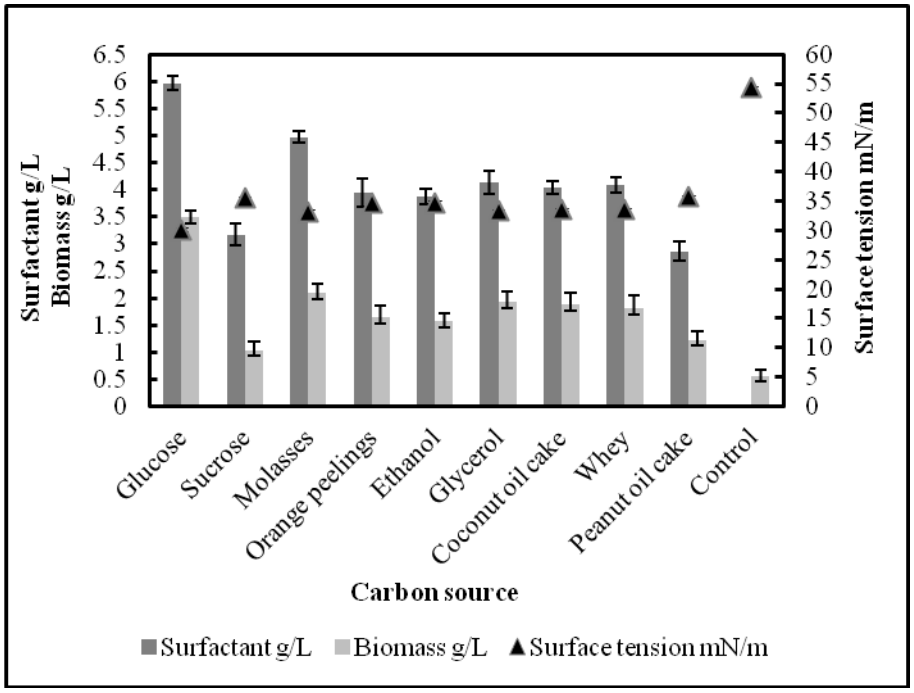


Fig 4.19 Effect of carbon source on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7). The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.20 presents the results of the effect of carbon source on surfactant production by *Pseudomonas aeruginosa*. In the present study, changing the carbon source affected the biomass and surfactant quantity which inturn affected the surface tension of the cell-free broth. A maximum of 5.36 g/L of surfactant and 3.23 g/L of biomass quantity was produced by *Pseudomonas aeruginosa* when glucose was supplemented in the medium. This discrepancy is most probably due to the fermentative substrate that the microorganism utilized during its growth. Growth of the strain *Pseudomonas aeruginosa* in the PPGAS medium containing glucose decreased the surface tension of the cell-free broth to 31.29 mN/m. Surfactant quantity produced in whey, glycerol, molasses, sucrose, ethanol, peanut oil cake, coconut oil cake and orange peelings containing medium were 3.82 g/L, 3.59 g/L, 3.32 g/L, 2.98 g/L, 2.89 g/L, 2.58 g/L, 2.51 g/L and 2.09 g/L, respectively; the corresponding biomass values were 1.42 g/L, 1.27 g/L, 1.16 g/L, 1.09

g/L, 1.08 g/L, 1.06 g/L, 1.03 g/L and 0.93 g/L, respectively. The corresponding surface tension values were 34.48 mN/m, 34.86 mN/m, 35.04 mN/m, 35.51 mN/m, 35.87 mN/m, 36.58 mN/m, 36.51 mN/m and 36.09 mN/m, respectively. In the control flask, the amount of surfactant produced was negligible (0.02 g/L). In a similar study carried by Abushady et al. (2005), maximum surfactant concentration (2.90 g/L) by *Bacillus subtilis* was obtained when glucose was used as the sole carbon source. Palashpriya et al. (2009) reported that marine *Bacillus circulans* produced a maximum of 1.16 g/L of surfactant during its growth on glucose containing medium, which inturn reduced the surface tension of the broth to 28.10 mN/m.

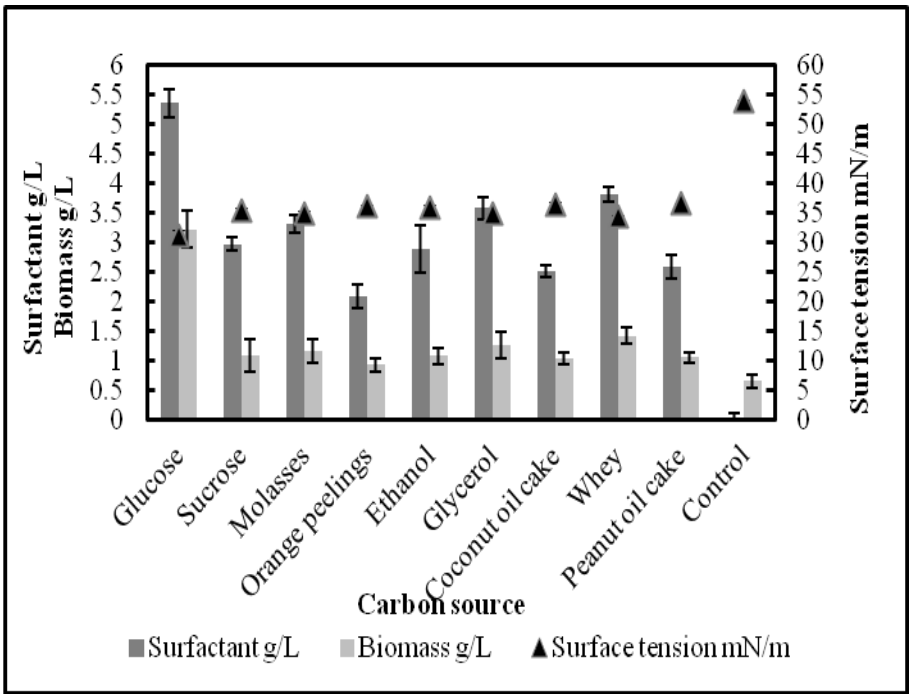


Fig. 4.20 Effect of carbon source on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7). The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

It was observed in the present study that both water-immiscible and miscible carbon sources stimulated the production of significant amount of surfactant by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, with considerable reduction in surface tension values. Among the different carbon sources evaluated, glucose as sole carbon source resulted in maximum surfactant production by the two bacterial strains (5.96 g/L of surfactant quantity by *Pseudomonas sp.* 2B and 5.36 g/L of surfactant quantity by *Pseudomonas aeruginosa*) and biomass production (3.49 g/L by *Pseudomonas sp.* 2B and 3.23 g/L by *Pseudomonas aeruginosa*) leading to significant decrease in surface tension values (30.13 mN/m by *Pseudomonas sp.* 2B and 31.29 mN/m by *Pseudomonas aeruginosa*). Though the bacterial strains produced lesser surfactant quantity during its growth on substrates such as peanut oil cake, glycerol, coconut oil cake, molasses, orange peelings and whey, these carbon sources can be explored as promising low-cost substrates since they contain carbohydrate, protein and lipid-rich residue in addition to micronutrients, which are generated in large amounts in various industries as by-products or wastes. The results of the present study are in agreement with reports of Guerra-Santos et al. (1984), Robert et al. (1989) and Dhouha and Semia (2011) in which glucose as carbon source supported maximum surfactant production by various microorganisms. Further this study will help in understanding the nature of surfactant produced by the strains as it has been postulated that the formation of sugar portion from a carbohydrate regulates the synthesis of a sugar-lipid type of surfactant by glycolytic metabolism (Cameotra and Makkar 1998). Also, it has been reported by Desai and Desai (1993) that glucose as a carbon source is an important key in the regulation of surfactant synthesis. From the present study it can be observed that the surfactant concentration produced by *Pseudomonas sp.* 2B as well as *Pseudomonas aeruginosa* in the presence of glucose is promising compared to the values reported by various researchers. Hence, glucose was chosen as carbon source for further studies.

4.7.6 EFFECT OF INITIAL GLUCOSE CONCENTRATION ON SURFACTANT PRODUCTION

Since the addition of glucose as sole carbon source in the PPGAS production medium showed maximum surfactant production, different concentrations of glucose were evaluated for the production of surfactant by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. Glucose was added to the production medium in concentrations of 10, 20, 30, 50, 70 and 100 g/L (w/v).

Fig. 4.21 presents the results of the effect of glucose concentration on surfactant production by *Pseudomonas sp.* 2B. An increase in surfactant production with increasing initial glucose concentration was observed up to 30 g/L (w/v). At 10 and 20 g/L (w/v) initial glucose concentrations, 6.13 g/L and 6.41 g/L of surfactant as well as 3.61 g/L and 3.99 g/L of biomass quantity, respectively, was produced by the bacterial strain. The corresponding surface tension values of cell-free broth were 29.86 mN/m and 29.14 mN/m, respectively. At 30 g/L (w/v) glucose concentration, the strain produced a maximum of 6.93 g/L of surfactant and 4.23 g/L of biomass quantity; the corresponding surface tension of the cell-free broth obtained was 28.83 mN/m. An increase in surface tension and decrease in surfactant as well as biomass production was observed at higher glucose concentrations, *i.e.* at 50 and 70 g/L (w/v) glucose concentration. 3.04 g/L and 2.41 g/L of surfactant quantity, 1.82 g/L and 1.03 g/L of biomass quantity was produced; the surface tension of the cell-free broth was found to be 35.67 mN/m and 36.08 mN/m, respectively. At 10 g/L (w/v) initial glucose concentration, 0.24 g/L of surfactant quantity and 0.47 g/L of biomass quantity was produced; the surface tension of the cell-free broth was 48.12 mN/m. The reason might be that at higher initial glucose concentrations, the growth of microorganism decreased due to substrate inhibition; which may be because of high osmotic stress imposed on the cells resulting in cell dehydration, ultimately leading to cell death. The results of the present study are in agreement with reports in the literature where the maximum surfactant production was observed at 30 g/L (w/v)

glucose concentration (Abushady et al. 2005; Dhouha and Semia 2011). Abushady et al. (2005) reported that a maximum of 2.1 g/L of surfactant quantity was produced by *Bacillus subtilis* at 30 g/L (w/v) glucose concentration, further increase in glucose concentration led to a decrease in the surfactant quantity (1.9 g/L).

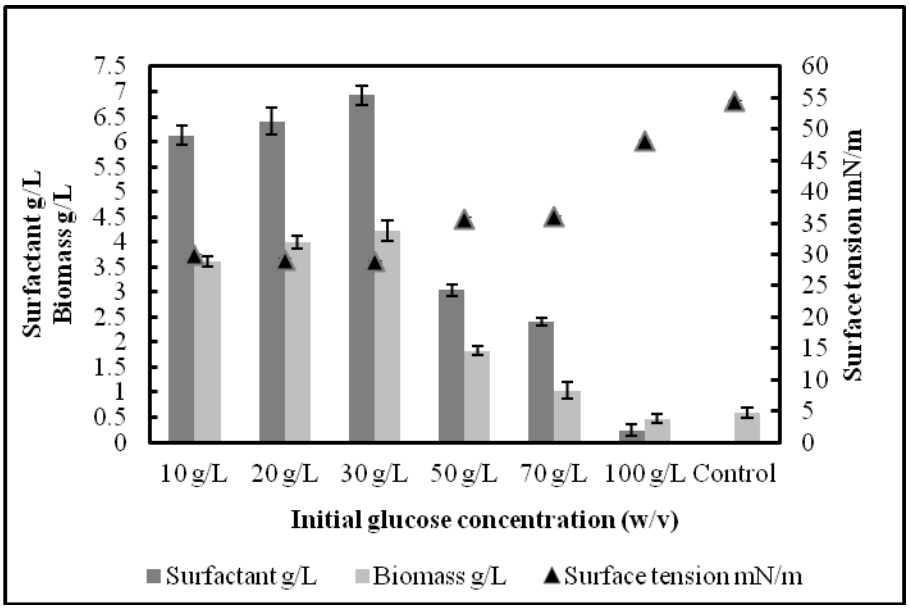


Fig 4.21 Effect of initial glucose concentration on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7). The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.22 presents the results of the effect of concentration of glucose on surfactant production by *Pseudomonas aeruginosa.* In the case of surfactant production by *Pseudomonas aeruginosa*, same trend as *Pseudomonas sp.* 2B was observed. With increase in initial glucose concentration, there was increase surfactant quantity as well as cell biomass quantity till 30 g/l (w/v) glucose concentration. At 10 and 20 g/L (w/v) initial glucose concentrations, the surfactant quantity produced was 5.51 g/L and 5.86 g/L, respectively; the corresponding biomass concentration values were 3.49 g/L and 3.63 g/L. The corresponding surface tension values were found to be 31.04 mN/m and 30.62

mN/m, respectively. Maximum biomass and surfactant production occurred at 30 g/L (w/v) initial glucose concentration; 6.19 g/L of surfactant and 3.81 g/L of biomass quantity was produced and the surface tension value of the broth was found to be 29.51 mN/m. At 50 g/L (w/v) glucose concentration, there was a decrease in surfactant production as well as increase in surface tension value of the cell-free broth. At higher glucose concentrations [50-100 g/L (w/v)], the surfactant quantity produced ranged from 0.16 to 2.65 g/L, the biomass quantity ranged from 0.34 to 1.23 g/L and surface tension values ranged from 37.09 to 48.43 mN/m. In the literature, drastic reduction in the quantity of surfactant produced by *Pseudomonas aeruginosa* (Hauser and Karnovsky 1954; Hayes et al. 1986) and *Candida lipolytica* (Cirigliano and Carman 1984) was observed upon addition of higher concentration of carbon sources such as glucose, acetate, and tricarboxylic acids.

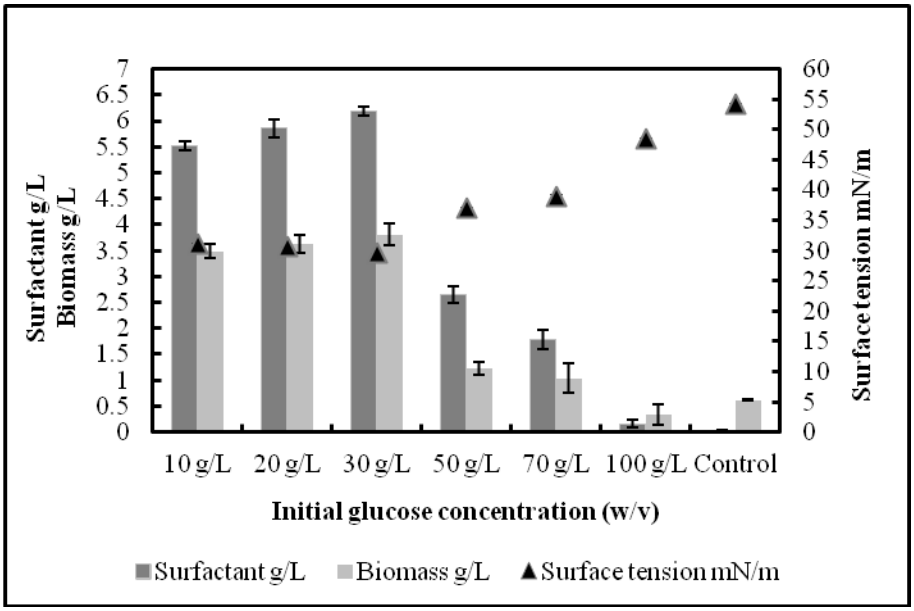


Fig 4.22 Effect of initial glucose concentration on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7). The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

The present study suggests that the initial concentration of glucose is critical for production of the surfactant. Any change in the initial concentration of glucose affected the surfactant production by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. At 30 g/L (w/v) glucose concentration, the *Pseudomonas sp.* 2B produced a maximum of 6.93 g/L of surfactant and 4.23 g/L of biomass quantity, that corresponding surface tension of the cell-free broth obtained was 28.83 mN/m whereas *Pseudomonas aeruginosa* synthesized a maximum of 6.19 g/L of surfactant and 3.81 g/L of biomass quantity, the corresponding surface tension value of the broth was 29.51 mN/m. The addition of higher concentration of glucose in the medium might lead to substrate inhibition, thereby affecting the cell growth and surfactant production. Dhouha and Semia (2011) made a similar observation during the surfactant production by *Bacillus subtilis* SPB1, the organism produced 0.48 g/L and 0.6 g/L of surfactant quantity at 20 and 30 g/L (w/v) initial glucose concentration. They further observed that at 40 g/L (w/v) glucose concentration, the surfactant concentration was maximum (0.72 g/L), further increase in glucose concentration (4.5 g/L) caused decrease in the concentration of surfactant (0.69 g/L) production. In the present study, as maximum concentration of surfactant was produced at 30 g/L (w/v) initial glucose concentration, the same glucose concentration was maintained in the future studies for surfactant production by both the bacterial strains.

4.7.7 EFFECT OF NITROGEN SOURCE ON SURFACTANT PRODUCTION

The bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, were cultivated in the PPGAS medium containing, individual organic or inorganic nitrogen sources for their capacity to support growth and surfactant production. The organic sources evaluated in the study included peptone, yeast extract and urea; the concentration of organic source used in the study was 10 g/L (w/v). The inorganic

sources tested in the present study were ammonium nitrate, ammonium chloride and potassium nitrate; the concentration of inorganic source used was 1 g/L.

Fig. 4.23 presents the results of the effect of nitrogen source on surfactant production by *Pseudomonas sp.* 2B. Maximum surfactant (5.41 g/L) and biomass (3.42 g/L) production by *Pseudomonas sp.* 2B was observed when potassium nitrate was used as the sole nitrogen source, the surface tension of the cell-free broth obtained was 31.09 mN/m. In the presence of ammonium chloride and ammonium nitrate, 4.56 g/L and 4.23 g/L of surfactant quantity was produced. Their corresponding cellular yield was 2.49 g/L and 2.21 g/L, respectively and surface tension values were found to be 32.92 mN/m and 33.25 mN/m, respectively. In the presence of peptone, yeast extract and urea; 3.14 g/L, 2.44 g/L and 1.09 g/L of surfactant quantity; 2.62 g/L, 1.65 g/L and 1.21 g/L of biomass quantity was produced, respectively. The corresponding surface tension values were 35.34 mN/m, 36.03 mN/m and 41.02 mN/m, respectively. In the presence of inorganic nitrogen source, surfactant production was higher compared to that of organic nitrogen source probably because of better diffusibility of inorganic nitrogen source than organic nitrogen source. When there was no nitrogen in the medium, amount of surfactant produced (0.79 g/L) and biomass (1.03 g/L) was low; the surface tension value was 44.15 mN/m. The role of nitrogen in surfactant production has been explained by Amaral et al. (2010), the nitrogen source is an essential source for microbial growth and also it is important in enzyme synthesis that catalyzes the formation of various moieties of the surfactant. Saimmai et al. (2012) reported that maximum amount (0.55 g/L) of surfactant was produced by *Pseudomonas aeruginosa* in sodium nitrate containing medium whereas least quantity of surfactant (0.2 g/L) was produced in potassium nitrate containing medium. In a study conducted by Bharali and Konwar (2011), maximum surfactant (3.14 g/L) synthesis by *Pseudomonas aeruginosa* OBP1 was achieved when ammonium sulphate was amended in the medium. They further reported that in ammonium nitrate, sodium nitrate, urea and yeast extract containing medium, the surfactant quantities obtained were 3.06 g/L, 2.03 g/L, 2.32 g/L and 2.16 g/L, respectively.

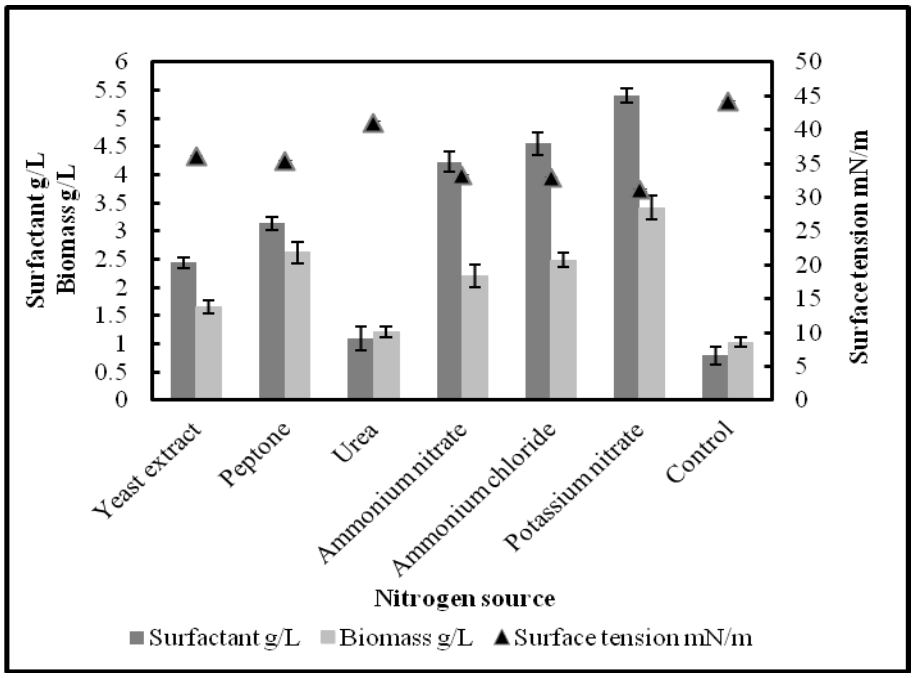


Fig. 4.23 Effect of nitrogen source on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.24 presents the results of the effect of nitrogen source on surfactant production by *Pseudomonas aeruginosa*. Among the various nitrogen sources evaluated, *Pseudomonas aeruginosa* produced a maximum of 4.33 g/L of surfactant and 2.31 g/L of cellular biomass quantity when ammonium chloride was used as the nitrogen source; the surface tension of cell-free broth of *Pseudomonas aeruginosa* was observed to be 33.12 mN/m. 3.94 g/L and 2.76 g/L of surfactant quantity was produced in ammonium nitrate and potassium nitrate containing medium, the corresponding cellular biomass quantity produced was 2.03 g/L and 1.33 g/L, respectively. The corresponding surface tensions of the cell-free broth were found to be 33.68 mN/m and 35.86 mN/m, respectively. In organic nitrogen source containing medium, 2.18 g/L and 2.02 g/L of surfactant quantity, 2.26 g/L and 1.54 g/L of biomass quantity was obtained in yeast extract and peptone

containing medium; the corresponding surface tension values were 36.52 mN/m and 36.94 mN/m, respectively. Least surfactant (1.23 g/L) and biomass (1.01 g/L) production was observed when urea was used as the nitrogen source, showing surface tension value of 40.79 mN/m. In the present study, it was observed that inorganic nitrogen source (ammonium chloride) was the preferred nitrogen source over the organic nitrogen sources tested. These results are in agreement with Dhouha et al. (2011) who reported that ammonium chloride was the preferred nitrogen source for the surfactant production by *Bacillus subtilis* SPB1 strain, a maximum of 0.90 g/L of surfactant was produced, least surfactant synthesis (0.60 g/L) occurred in beef extract (organic nitrogen source) containing medium.

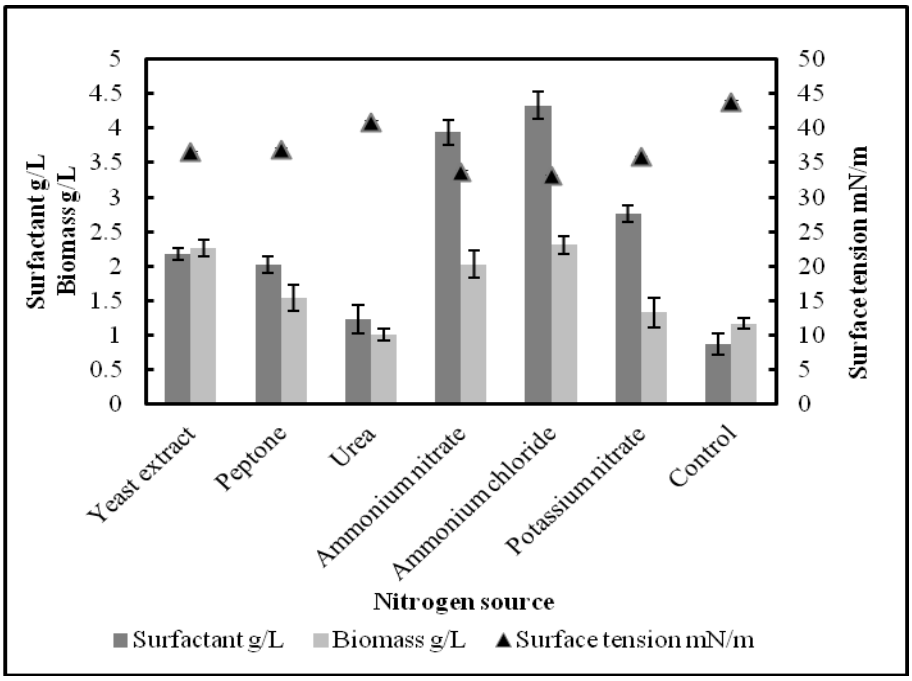


Fig. 4.24 Effect of nitrogen source on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose. The culture flasks were incubated at 37°C in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

In the present study, potassium nitrate and ammonium chloride yielded higher quantity of surfactant by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively; a maximum of 5.41 g/L and 4.33 g/L, respectively, of surfactant quantity was produced. The organic nitrogen sources, urea, yeast extract and peptone, supported bacterial growth in both the strains *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, but the amount of surfactant produced was lesser. The results obtained showed that inorganic nitrogen sources showed maximum production of surfactant than organic nitrogen sources. The organic and inorganic nitrogen sources were used individually which led to lesser surfactant production (5.41 g/L and 4.33 g/L of surfactant production by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively) than when they were combined together (6.93 g/L and 6.19 g/L of surfactant by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively as presented in section 4.7.5 in which a combination of organic and inorganic nitrogen sources were used). In order to improve the quantity of surfactant, organic nitrogen source might be combined with inorganic nitrogen source since the production medium, Proteose Peptone Glucose Ammonium Salts medium (PPGAS), contains peptone and ammonium chloride as nitrogen sources. Hence, in the further studies, a combination of organic and inorganic nitrogen sources was used.

4.7.8 EFFECT OF COMBINATION OF NITROGEN SOURCES ON SURFACTANT PRODUCTION

The bacterial strain *Pseudomonas sp.* 2B was cultivated in the PPGAS medium containing potassium nitrate as inorganic nitrogen source in combination with an organic nitrogen source, for its capacity to support growth and surfactant production. *Pseudomonas aeruginosa* was grown in the PPGAS medium containing ammonium chloride as inorganic nitrogen source in combination with an organic nitrogen source to

maximize bacterial growth and surfactant production. The concentration of organic nitrogen source was 10 g/L (w/v) whereas that of inorganic nitrogen source was 1 g/L.

The results of the effect of combination of nitrogen source on surfactant production by *Pseudomonas sp.* 2B is presented in Fig. 4.25. It was observed that when potassium nitrate was used in combination with peptone, maximum surfactant quantity (7.14 g/L) was produced in the culture medium of *Pseudomonas sp.* 2B; the biomass quantity was 4.49 g/L; the surface tension of the cell-free broth was 27.92 mN/m. When yeast extract was used in combination with potassium nitrate, the surfactant and biomass quantity produced by *Pseudomonas sp.* 2B was 4.54 g/L and 2.43 g/L, respectively; the surface tension of the cell-free broth was 32.83 mN/m. When a combination of urea and potassium nitrate was used, the bacterium produced 1.87 g/L of surfactant and 1.59 g/L of biomass quantity; the surface tension of the cell-free broth was 36.15 mN/m. Similar observation was made by Bharali and Konwar (2011), using a combination of urea and ammonium sulphate, the surfactant yield was 4.57 g/L whereas 4.87 g/L of biomass quantity was produced by *Pseudomonas aeruginosa* strain OBP1, which was much higher compared with the individual nitrogen sources. They observed that when urea was used individually, 2.32 g/L of surfactant quantity was produced whereas in the presence of ammonium sulphate alone, a maximum of 3.14 g/L of surfactant quantity was produced.

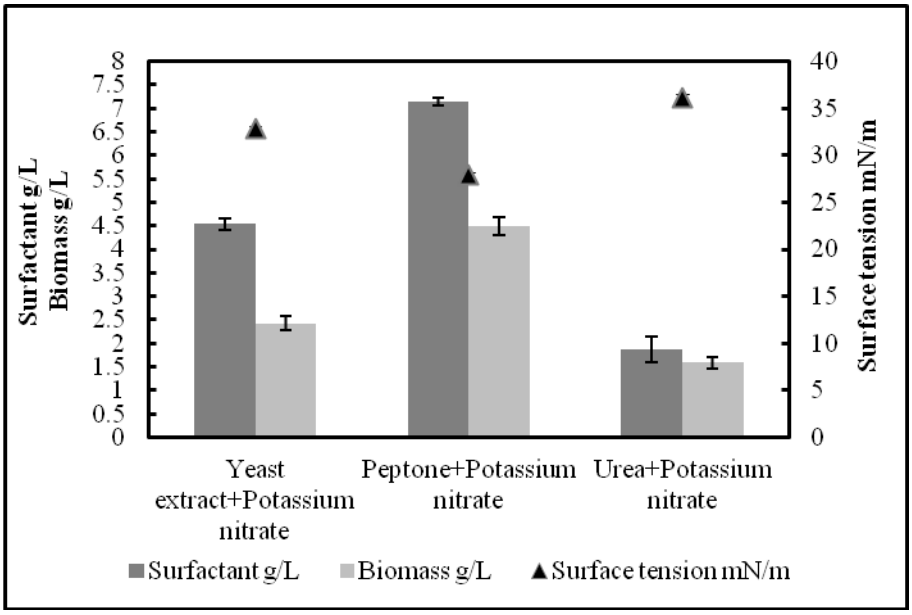


Fig 4.25 Effect of combination of nitrogen source on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

The results of the effect of combination of nitrogen source on surfactant production by *Pseudomonas aeruginosa* is presented in Fig. 4.26. It was observed that when a combination of yeast extract and ammonium chloride was used, maximum surfactant and biomass quantity was produced. 6.31 g/L of surfactant and 3.92 g/L of biomass was produced; the corresponding surface tension value of the cell-free broth was 29.17 mN/m when a combination of yeast extract and ammonium chloride was used. When peptone was used in combination with ammonium chloride, the surfactant and biomass quantity produced was 6.13 g/L and 3.78 g/L, respectively; the corresponding surface tension of the cell-free broth was 29.59 mN/m. When urea and ammonium chloride were combined, the organism produced a maximum of 2.18 g/L of surfactant and 1.79 g/L of biomass; the corresponding surface tension of the cell-free broth was found to be 36.31 mN/m. In a study by Gizele et al. (2010), ammonium sulphate and yeast extract demonstrated to be the suitable nitrogen sources for cell growth and

surfactant synthesis by *Yarrowia lipolytica*, the surfactant production further led to decrease in surface tension value to 20.9 mN/m.

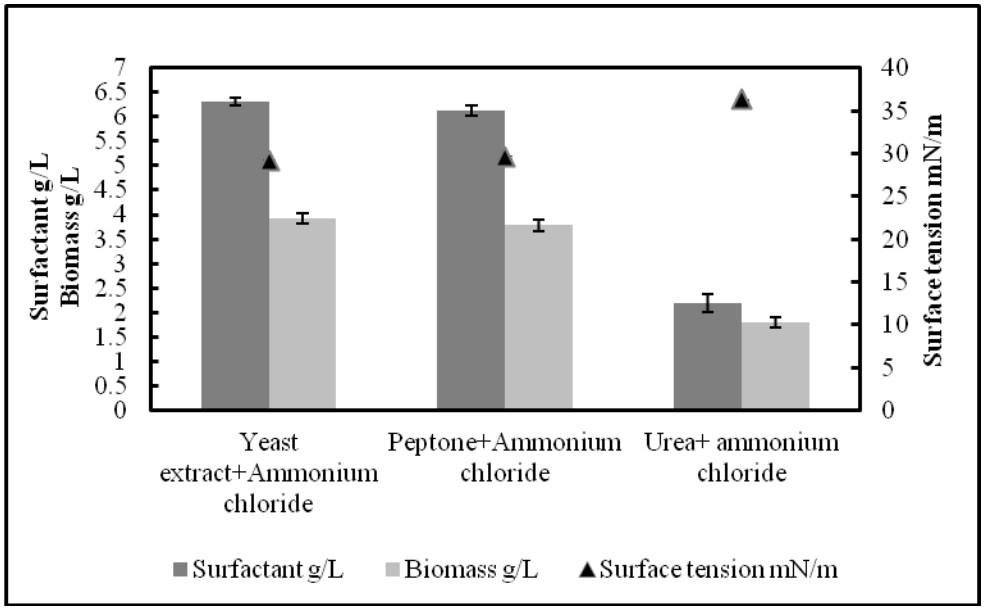


Fig. 4.26 Effect of combination of nitrogen source on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose. The culture flasks were incubated at 37°C in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

The present study emphasizes the importance of addition of both organic as well as inorganic nitrogen source to the production media for enhanced surfactant production. Peptone in combination with potassium nitrate showed maximum production of surfactant (7.14 g/L) by *Pseudomonas sp.* 2B whereas *Pseudomonas aeruginosa* produced a maximum of 6.31 g/L when yeast extract was used in combination with ammonium chloride. This might be because of the presence of synergistic effect when a combination of nitrogen sources was used, that led to maximum surfactant production. Hence, in the further studies, peptone was used in combination with potassium nitrate for

surfactant production by *Pseudomonas sp.* 2B whereas yeast extract was combined with ammonium chloride for surfactant production by *Pseudomonas aeruginosa.*

4.7.9 EFFECT OF INITIAL INORGANIC NITROGEN CONCENTRATION ON SURFACTANT PRODUCTION

Different concentration of inorganic nitrogen source was tested for the maximum yield of surfactant by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. The inorganic nitrogen source concentration was added in the range of 0.5 to 10 g/L (w/v). The organic nitrogen source used in the study were peptone and yeast extract for the production of surfactant by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. The organic nitrogen source concentration maintained in the present study was kept constant (10 g/L w/v).

Fig. 4.27 presents the results of effect of inorganic nitrogen concentration (potassium nitrate) on surfactant production by *Pseudomonas sp.* 2B. It can be observed that there was decrease in the production of surfactant by *Pseudomonas sp.* 2B with increase in potassium nitrate concentration. At 0.5 g/L (w/v) potassium nitrate concentration, the surfactant and biomass quantity produced was 5.51 g/L and 3.13 g/L, respectively, the surface tension of the cell-free broth was 31.09 mN/m. Maximum surfactant production was observed at 1.0 g/L (w/v) potassium nitrate concentration, 7.10 g/L of surfactant and 4.51 g/L of biomass was produced. The surface tension of the cell-free broth was 27.96 mN/m when 1 g/L (w/v) of inorganic nitrate source was added to the medium. The surfactant production was affected at higher concentrations of the nitrogen source. At 1.5, 3, 5, 7 and 10 g/L of potassium nitrate concentration, 6.13 g/L, 4.08 g/L, 2.27 g/L, 0.82 g/L and 0.09 g/L of surfactant quantity was produced; the corresponding biomass values were 3.82 g/L, 2.71 g/L, 2.04 g/L, 1.30 g/L and 0.23 g/L, respectively. The corresponding surface tension values were found to be 29.66 mN/m, 33.43 mN/m, 37.17 mN/m 43.06 mN/m and 47.18 mN/m, respectively. 3.11 g/L of surfactant was produced in the absence of potassium nitrate. The results show that there might be an

inhibitory effect on the bacterial metabolism which might have occurred due to a nutrient transport deficiency with increase in the concentration of nitrogen source. Thaniyavarn et al. (2008), evaluated various nitrogen sources for surfactant production by *Pichia anomala*, maximum surfactant quantity was produced in sodium nitrate supplemented medium. Further, they studied the effect of sodium nitrate concentration on surfactant production by varying the range of sodium nitrate from 0.1-0.5 % (w/v). They reported that with increase in sodium nitrate concentration, there was increase in surfactant production till 0.4% as indicated by surface tension values; there was decrease in surfactant production at 0.5% sodium nitrate concentration. They observed that at 0.1%, 0.2% and 0.3% sodium nitrate concentration, the surface tension values were 34 mN/m, 32 mN/m and 31 mN/m, respectively. They further reported that at 0.4% sodium nitrate concentration, the surface tension reduction was maximum (29 mN/m) and at 0.5% (w/v) sodium nitrate concentration, the surface tension of the broth was 30 mN/m.

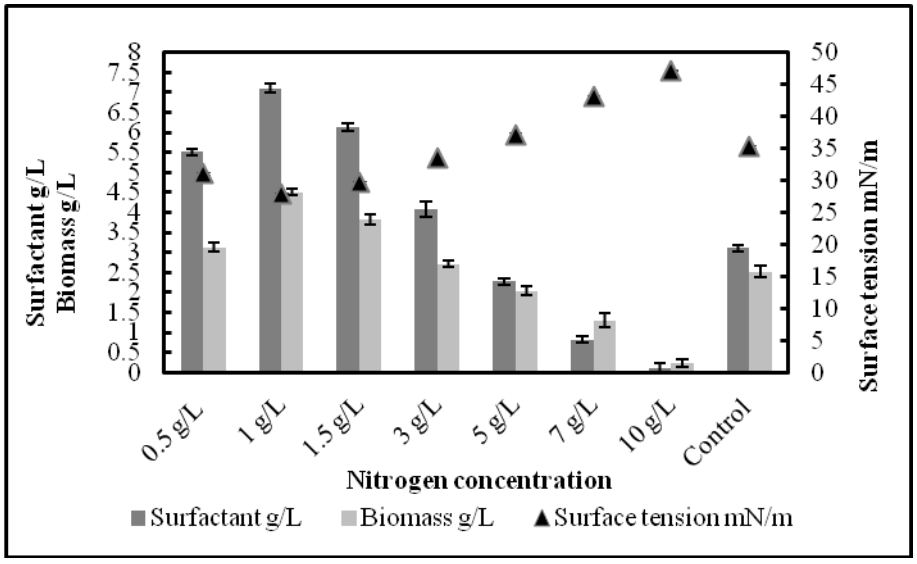


Fig. 4.27 Effect of potassium nitrate concentration on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose. The culture flasks were incubated at 37°C in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.28 presents the results of effect of inorganic nitrogen concentration (ammonium chloride) on surfactant production by *Pseudomonas aeruginosa*. At 0.5 g/L (w/v) of ammonium chloride concentration, the surfactant and biomass quantity produced was 5.59 g/L and 3.08 g/L, respectively, the surface tension of the cell-free broth reduced was 31.93 mN/m. A maximum of 6.29 g/L of surfactant and 3.91 g/L of biomass was synthesized when 1 g/L of ammonium chloride was added to the medium. The corresponding surface tension of broth was 29.20 mN/m. With increase in ammonium chloride concentration, decrease in surfactant concentration and biomass quantity was observed. At 1.5, 3, 5, 7 and 10 g/L of ammonium chloride concentration, 5.08 g/L, 4.02 g/L, 3.09 g/L, 1.32 g/L and 0.14 g/L of surfactant quantity was produced; 3.53 g/L, 2.49 g/L, 2.01 g/L, 1.41 g/L and 0.29 g/L were the corresponding biomass values. The corresponding surface tension values were 32.03 mN/m, 33.52 mN/m, 35.41 mN/m, 41.52 mN/m and 47.04 mN/m, respectively. In a similar study carried by Abushady et al. (2005), at 2 g/L of ammonium nitrate (w/v) concentration, a maximum of 1.5 g/L of surfactant was produced by *Bacillus subtilis* Bbk1. They reported that a maximum of 2.2 g/L of surfactant was produced at 4.6 g/L of ammonium nitrate concentration; there was a decrease in surfactant concentration above 4.6 g/L of ammonium nitrate. At 7.0 g/L of ammonium nitrate concentration, the surfactant quantity produced by *Bacillus subtilis* Bbk1 decreased to 2.0 g/L.

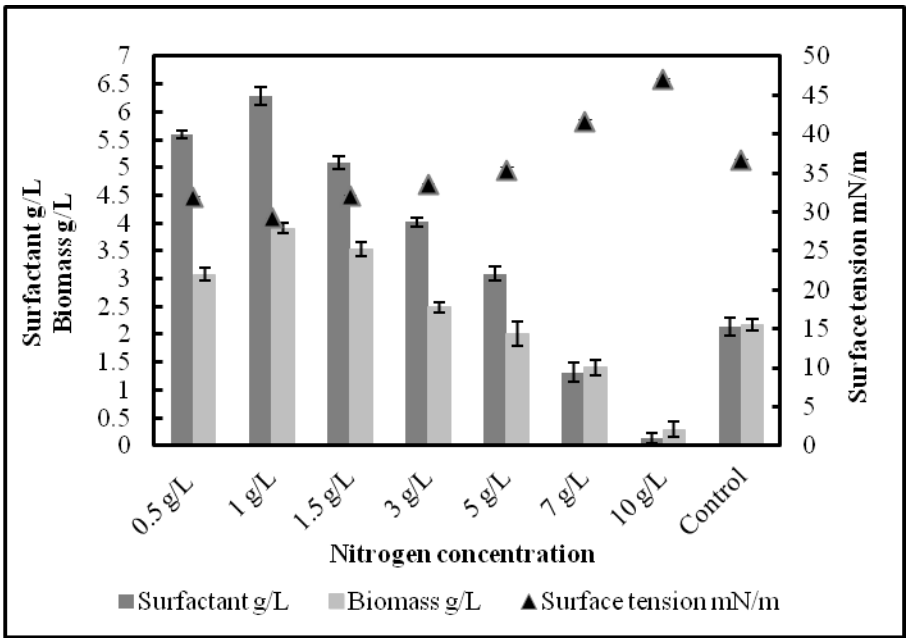


Fig. 4.28 Effect of ammonium chloride concentration on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

From the above study, it can be observed that with increase in inorganic nitrogen source concentration, there was decrease in surfactant and biomass quantity. A maximum of 7.10 g/L of surfactant and 4.51 g/L of bacterial biomass quantity was produced by *Pseudomonas sp.* 2B at 1 g/L of potassium nitrate concentration, the surface tension of the cell free broth was found to be 27.96 mN/m. In the case of *Pseudomonas aeruginosa*, a maximum of 6.29 g/L of surfactant and 3.91 g/L of biomass quantity was synthesized when 1 g/L of ammonium chloride was added to the medium, the surface tension of the cell-free broth was found to be 29.20 mN/m. Similar results have been reported by various authors (Abushady et al. 2005; Thaniyavarn et al. 2008). Hence, 1 g/L (w/v) of inorganic nitrogen source was maintained in the future studies.

4.7.10 EFFECT OF INDUCER ON SURFACTANT PRODUCTION

The effect of inducer on surfactant production by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was studied by the addition of inducers to the production medium as it has been reported in the literature that hydrocarbon inducers added to the fermentation medium induced the production of surfactant (Bento and Gaylarde 1996). Seven hydrocarbon inducers, namely, olive oil, n-hexadecane, crude oil, toluene, paraffin, sunflower oil and coconut oil, were screened for enhanced surfactant production. 1% (v/v) of inducer concentration was supplemented in the PPGAS medium.

Fig. 4.29 presents the results of the effect of inducer on surfactant production by *Pseudomonas sp.* 2B. Among the various inducers evaluated, olive oil enhanced the surfactant production by *Pseudomonas sp.* 2B. A maximum of 8.03 g/L of surfactant and 5.22 g/L of biomass quantity was produced; the surface tension of the cell-free broth obtained was 26.72 mN/m. The induction of surfactant production using olive oil is encouraging since it is a renewable substrate. In the presence of other hydrocarbons, 5.79 g/L (coconut oil), 5.13 g/L (crude oil), 5.08 g/L (sunflower oil), 4.92 g/L (n-hexadecane), 3.36 g/L (toluene), 3.32 g/L (paraffin) of surfactant quantity was produced, which indicates that these hydrocarbons could not sufficiently enhance the surfactant production. The other inducers could not induce surfactant production probably due to the inefficiency of the enzymes to break the different bonds which were present in the hydrocarbons. Rather than acting as inducers, the hydrocarbons served as carbon source resulting in surfactant production. In the absence of the inducer (control), the quantity of surfactant and biomass remained 7.06 g/L and 4.47 g/L, respectively. The corresponding surface tension of the cell-free broth was 27.98 mN/m. Khopade et al. (2012) reported that maximum synthesis of surfactant by *Nocardiopsis sp.* B4 occurred in the presence of olive oil as inducer against other hydrocarbons like castor oil, eucalyptus oil, coconut oil, cod liver oil and clove oil. They reported that an emulsification activity of 200 EU/ml

was observed when olive oil was used; the emulsification activity ranged from 30 to 180 EU/ml when other hydrocarbons were used.

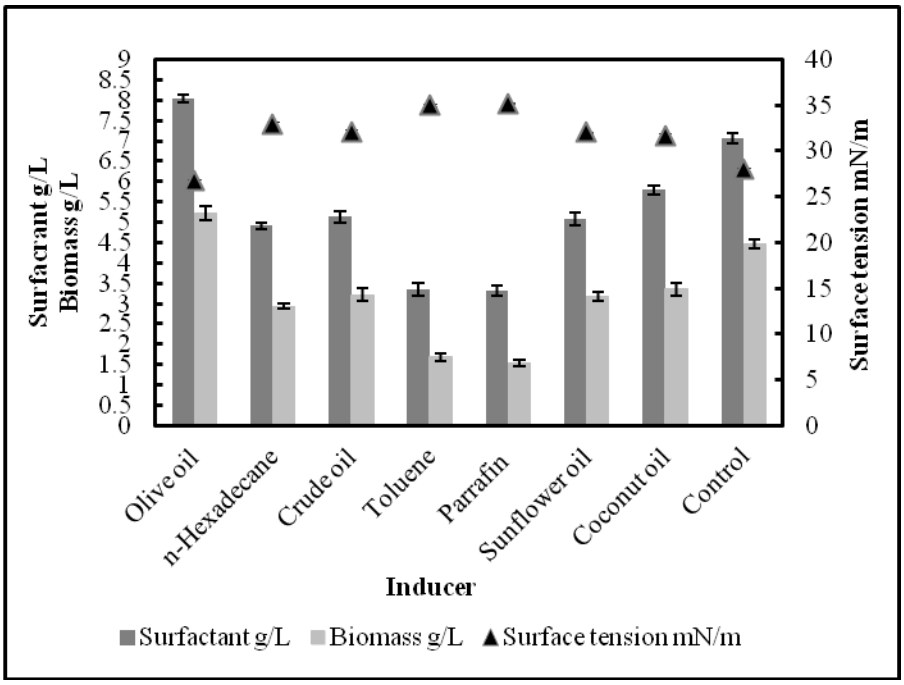


Fig. 4.29 Effect of inducer on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose and 1 g/L of potassium nitrate. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.30 presents the results of the effect of concentration of olive oil (inducer) on surfactant production by *Pseudomonas sp.* 2B. Olive oil concentration was varied from 0.5-5% (v/v). With increase in inducer concentration, there was increase in surfactant till 2% (v/v), the surfactant production decreased thereafter. At 0.5% and 1% (v/v) olive oil concentration, 7.21 g/L and 8.02 g/L of surfactant, 4.63 g/L and 5.18 g/L of biomass quantity was produced; the corresponding surface tension values were found to be 27.54 mN/m and 26.77 mN/m, respectively. However, supplementation of 2% (v/v) olive oil concentration in the production medium yielded maximum surfactant quantity

(8.29 g/L), the biomass quantity obtained was 5.46 g/L and surface tension value of the cell-free broth was 26.34 mN/m. With further increase in the concentration of the inducer, the surfactant quantity produced decreased. At 3% (v/v) olive oil concentration, there was decrease in the surfactant (5.83 g/L) and biomass (3.47 g/L) quantity. The corresponding surface tension value of the cell-free broth was 31.68 mN/m. At 5% (v/v) inducer concentration, the surfactant and bacterial biomass quantity was 1.28 g/L and 1.48 g/L, respectively; the surface tension of the cell-free broth was found to be 40.52 mN/m. In the literature, similar results were observed by Vijay et al. (2006).

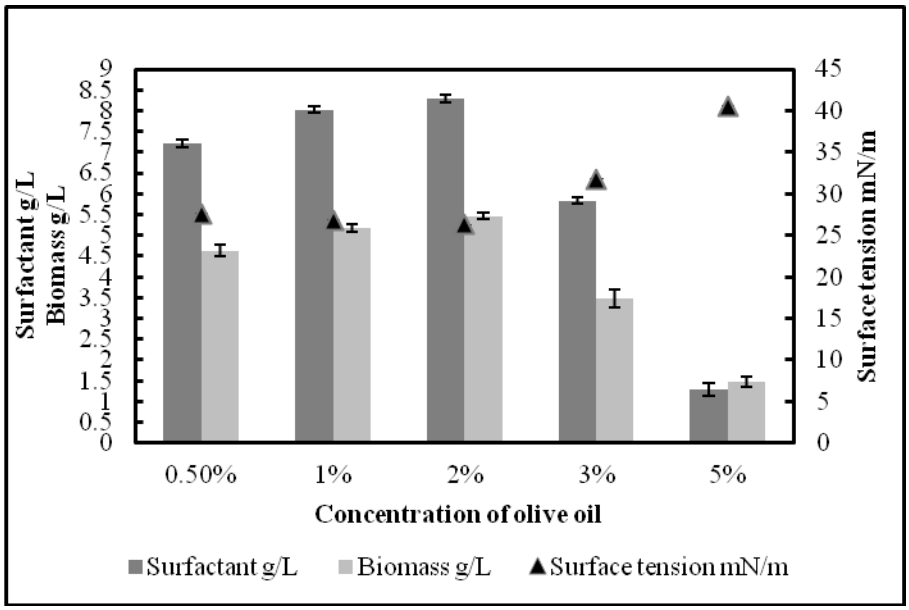


Fig. 4.30 Effect of olive oil concentration on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose and 1 g/L of potassium nitrate. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.31 presents the results of the effect of inducer on surfactant production by *Pseudomonas aeruginosa*. Among the seven inducers evaluated, n-hexadecane enhanced surfactant production by *Pseudomonas aeruginosa.* 7.08 g/L of surfactant and 4.36 g/L of biomass quantity was produced by the bacterial strain; the surface tension value of 28.14

mN/m was achieved. n-hexadecane was preferred over other hydrocarbons probably because of its structure; it is a long chain alkane which can be degraded easily than other hydrocarbons which were complex in nature. In the presence of other hydrocarbons, surfactant production did not increase. A maximum of 5.16 g/L, 4.63 g/L, 4.53 g/L, 4.32 g/L, 3.76 g/L and 3.16 g/L of surfactant quantity was synthesized in sunflower oil, crude oil, olive oil, coconut oil, toluene and paraffin containing medium. The corresponding biomass quantity produced was 2.38 g/L, 1.87 g/L, 2.38 g/L, 2.25 g/L, 1.84 g/L and 1.47 g/L, respectively. The corresponding surface tension values were found to be 32.06 mN/m, 32.83 mN/m, 32.64 mN/m, 33.26 mN/m, 34.09 mN/m and 35.29 mN/m, respectively. In the absence of the inducer (control), the surfactant concentration remained 6.19 g/L. Similar study was carried by Pal et al. (2009). In their study, among the seven inducers screened (namely, n-hexadecane, n-octadecane, dodecane, paraffin, toluene, olive oil and castor oil), toluene enhanced the surfactant production by *Rhodococcus erythropolis* MTCC 2794. They evaluated surfactant production on the basis of emulsification index. They reported that maximum emulsification index (53.84%) was observed when toluene was added to the medium, the emulsification index of the other inducers ranged from 38 to 46 %.

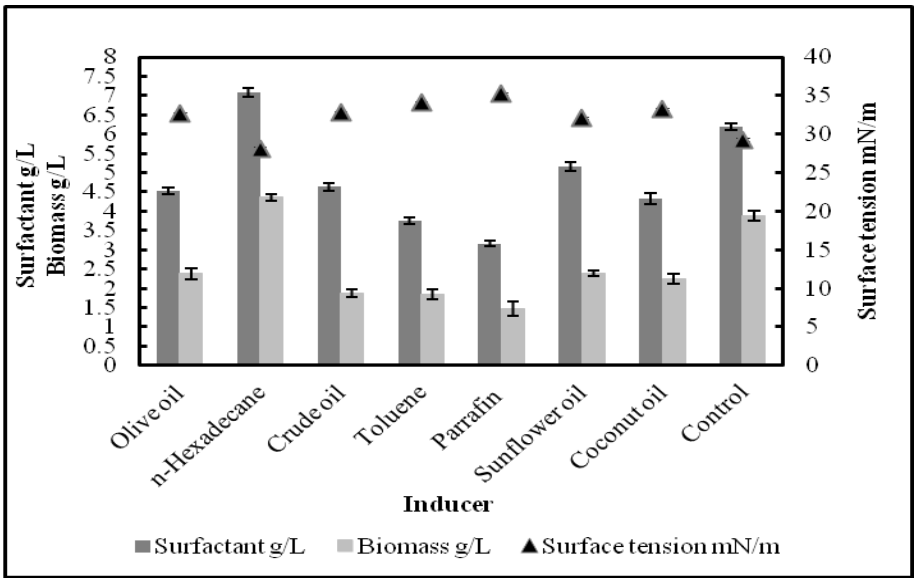


Fig. 4.31 Effect of inducer on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose and 1 g/L of ammonium chloride. The culture flasks were incubated at 37°C in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

Fig. 4.32 presents the results of the effect of concentration of n-hexadecane (inducer) on surfactant production by *Pseudomonas aeruginosa*. The effect of inducer concentration on surfactant production was studied further in order to enhance the production of surfactants. At 0.5% and 1% (v/v) n-hexadecane concentration, the quantity of surfactant produced was 5.17 g/L and 7.11 g/L, respectively. The corresponding biomass yields were 2.42 g/L and 4.41 g/L, respectively. The corresponding surface tension of the cell-free broth was 32.02 mN/m and 28.08 mN/m. It was observed that the maximum surfactant production (7.26 g/L) by *Pseudomonas aeruginosa* occurred at 2% (v/v) n-hexadecane concentration, the corresponding biomass and surface tension values were 4.69 g/L and 27.84 mN/m, respectively. The surfactant production decreased with increase in inducer concentration thereafter. At 5% (v/v) inducer concentration, 2.09 g/L of extracellular surfactant and 2.21 g/L of bacterial biomass was produced; the

corresponding surface tension of cell-free broth was 38.56 mN/m. Mutalik et al. (2008) reported that addition of 6.3% (v/v) n-hexadecane concentration to the surfactant production medium resulted in maximum surfactant concentration by *Rhodococcus* spp. MTCC 2574, a maximum of 10.9 g/L of surfactant was produced whereas Pal et al. (2009) reported that 3% (v/v) n-hexadecane concentration showed an emulsification index of 53.84% by *Rhodococcus erythropolis.*

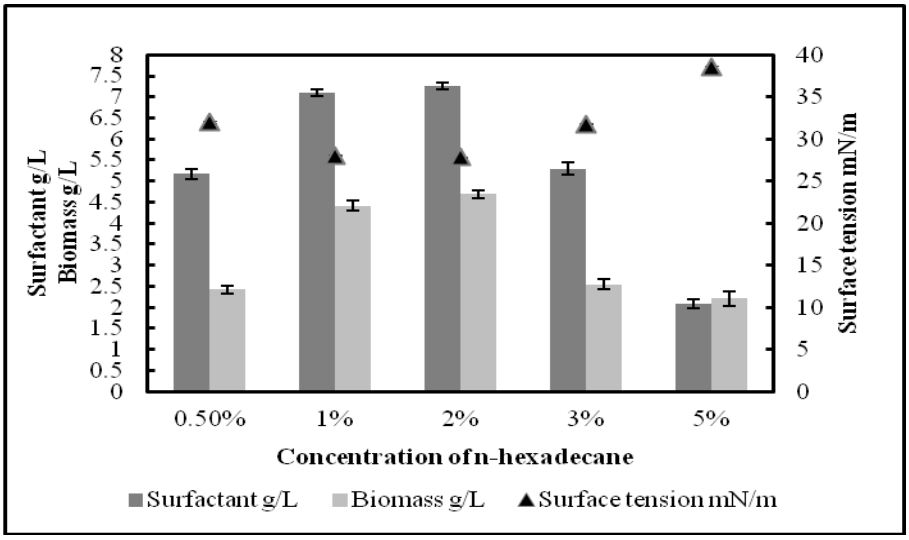


Fig. 4.32 Effect of concentration of n-hexadecane on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose and 1 g/L of ammonium chloride. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3)

SUMMARY

In the present study, it was observed that olive oil and n-hexadecane enhanced surfactant production by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively. A maximum of 8.03 g/L and 7.08 g/L of surfactant quantity weas produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* in olive oil and n-hexadecane supplemented medium; the corresponding surface tension values were 26.72 mN/m and

28.14 mN/m, respectively. Further, 2% (v/v) inducer concentration resulted in maximum synthesis of the surfactant by the bacterial strains. A maximum of 8.29 g/L was produced by *Pseudomonas sp.* 2B in 2% (v/v) olive oil containing medium, the surface tension value of the cell-free broth was 26.34 mN/m. Maximum surfactant production (7.26 g/L) by *Pseudomonas aeruginosa* occurred at 2% (v/v) n-hexadecane concentration, the surface tension of the cell-free broth was 27.84 mN/m. Similar studies have reported the enhancement of surfactant production by various microbial strains in the presence of inducers (Mutalik et al. 2008; Pal et al. 2009). Hence, in the future studies, 2% (v/v) olive oil and n-hexadecane were incorporated as the inducers in the production medium for surfactant production by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively.

4.7.11 EFFECT OF THE BUFFERING SYSTEM ON SURFACTANT PRODUCTION

The effect of the buffering system on surfactant production by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was investigated using two different buffering systems, Tris HCl buffer and phosphate buffer, in the PPGAS medium.

The results of effect of buffering system on surfactant production by *Pseudomonas sp.* 2B is presented in Fig. 4.33. The bacterial strain, *Pseudomonas sp.* 2B produced a maximum of 8.26 g/L of surfactant and 5.52 g/L of biomass quantity in the Tris HCl containing media, the corresponding surface tension value of the cell-free broth was found to be 26.40 mN/m. The pH of the broth remained in the range of 7.0-7.4 during the extracellular surfactant production by the bacterial strain. In the phosphate buffer containing media, 4.87 g/L of surfactant and 2.86 g/L of biomass quantity was produced; the surface tension of the cell-free broth obtained was 32.99 mN/m. In the phosphate buffered medium, the pH varied between 7.1-7.6. A significant difference in the surfactant concentration was found in the presence of buffered and non-buffered media. 2.53 g/L of surfactant and 1.65 g/L of biomass quantity was produced; the

corresponding surface tension of the broth was 38.43 mN/m in the control flask. In the absence of the buffer (control), the pH of the medium dropped to 4.2, hence there was a decrease in the biomass as well as surfactant concentration. During the growth of microorganisms, acids are produced during the logarithmic growth phase, which might lead to decrease in the pH of the medium. The acidic environment of the modified PPGAS medium may be detrimental to cell metabolism, which in turn affected the production of surfactant. In a similar study by Mulligan et al. (1989), they reported that *Pseudomonas aeruginosa* ATCC 9027 var. RCII did not produce surfactant in the phosphate containing minimal media mainly due to the presence of phosphate. They further reported the production of surfactant in the PPGAS medium (containing tris HCl) decreased the surface tension of the cell-free broth to 29 mN/m due to production of surfactant. They also observed that the pH remained in the range of 6.3-7 during the growth of the organism in the PPGAS medium.

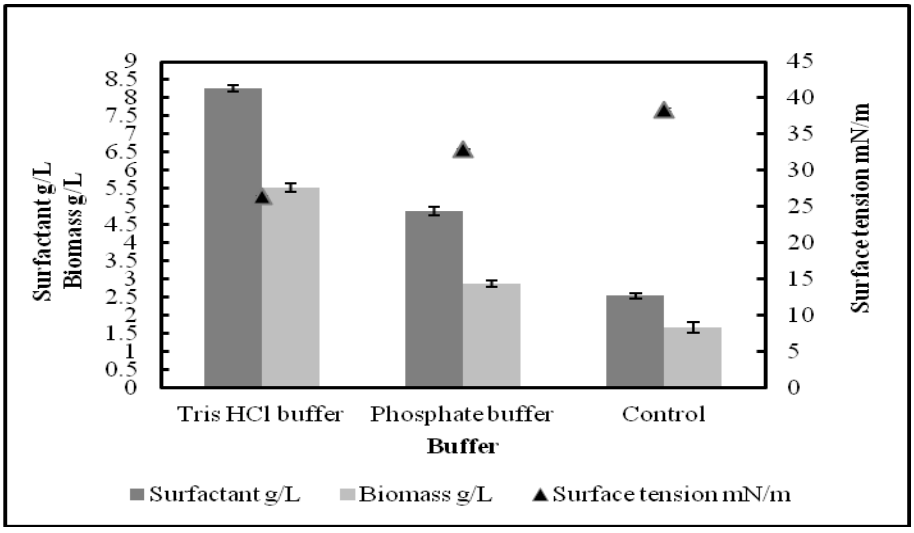


Fig. 4.33 Effect of buffering system on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose, 1 g/L of potassium nitrate and 2% (v/v) olive oil. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3).

The results of the effect of buffering system on surfactant production by *Pseudomonas aeruginosa* is presented in Fig. 4.34. In the presence of Tris HCl in the medium, the surfactant and biomass quantity produced was 7.21 g/L and 4.72 g/L, the corresponding surface tension value of the cell-free broth was 27.92 mN/m. The pH of the medium varied between 6.9 to 7.3 in the Tris HCl containing medium. In the phosphate buffer containing medium, 4.19 g/L of surfactant and 2.15 g/L of biomass quantity was produced, the surface tension of the cell-free broth was 33.18 mN/m. In the non-buffered medium (control), 2.01 g/L of surfactant and 1.23 g/L of biomass quantity was produced; the surface tension of the cell-free broth decreased was found to be 38.72 mN/m. Sim et al. (1997) made a similar observation during surfactant production by *Pseudomonas aeruginosa* UW-1 in the phosphate buffer containing medium, low biomass was produced in the phosphate buffer containing medium.

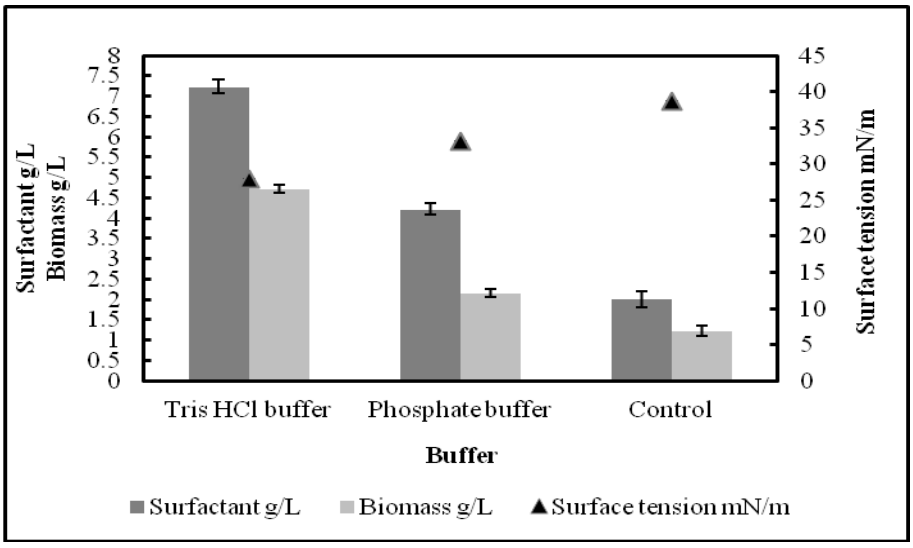


Fig. 4.34 Effect of buffering system on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose, 1 g/L of ammonium chloride and 2% (v/v) n-hexadecane. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3).

SUMMARY

The present study emphasizes the role of buffering system in the production medium for surfactant production. In the presence of the Tris HCl buffer, the production of surfactant by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* was maximum. A maximum of 8.26 g/L of surfactant and 5.52 g/L of cellular biomass was produced by *Pseudomonas sp.* 2B, the surface tension of the cell-free broth was 26.40 mN/m. *Pseudomonas aeruginosa* produced a maximum of 7.21 g/L of surfactant and 4.72 g/L of biomass quantity, the surface tension of the cell-free broth obtained was 27.92 mN/m. Hence, Tris HCl buffer was maintained in the production medium for further studies.

4.7.12 EFFECT OF SALINITY ON SURFACTANT PRODUCTION

Several reports in the literature have indicated that sodium chloride maintains the osmotic pressure in the cells. Hence, to test the effect of medium osmolarity on surfactant production by the bacterial strains *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, in the PPGAS medium, different concentrations of sodium chloride [0.5-5 % (w/v)] was added.

The results of the effect of salinity on surfactant production by *Pseudomonas sp.* 2B is presented in Fig. 4.35. Gradual addition of sodium chloride to glucose-containing PPGAS medium led to an increase in surfactant production by *Pseudomonas sp.* 2B till 1% (w/v) NaCl concentration, thereafter the surfactant concentration decreased. At 0.5% (w/v) NaCl concentration, surfactant and biomass quantity was 8.31 g/L and 5.77 g/L, the surface tension of the cell-free broth was found to be 25.96 mN/m. At 1% (w/v) NaCl concentration, the organism showed maximum surfactant (8.51 g/L) and biomass production (5.90 g/L); the surface tension of the cell-free broth was 25.72 mN/m. At 3% (w/v) NaCl concentration, there was decrease in the production of surfactant, 5.18 g/L of surfactant and 3.49 g/L of bacterial biomass was produced; the surface tension of the cell-free broth obtained was 31.89 mN/m. At 5% (w/v) NaCl concentration, the surfactant

yield reached to as low as 2.73 g/L and the cellular yield was 2.37 g/L; the surface tension of the cell-free broth was 38.36 mN/m. In the control medium, the surfactant concentration was 8.23 g/L and the biomass yield was 5.59 g/L; the surface tension of the cell-free broth was 26.43 mN/m. The presence of sodium chloride affects surfactant production depending on its effect on cellular activity (Yakimov et al. 1995). Similar study was conducted by Khopade et al. (2012), they studied the effect of different NaCl concentrations [ranging from 1-12% (w/v)] on surfactant production by marine *Nocardia sp.* B4. They reported that at 1% (w/v) NaCl concentration, the surface tension of the broth was 38 mN/m; at 2% NaCl concentration, maximum reduction in surface tension value (29 mN/m) was achieved. With increase in NaCl concentration, there was increase in surface tension values indicating the surfactant production by *Nocardia sp.* B4 was affected. They observed that the surface tension of the cell-free broth ranged from 36 to 58 mN/m in the presence of 3-10% NaCl (w/v) concentration.

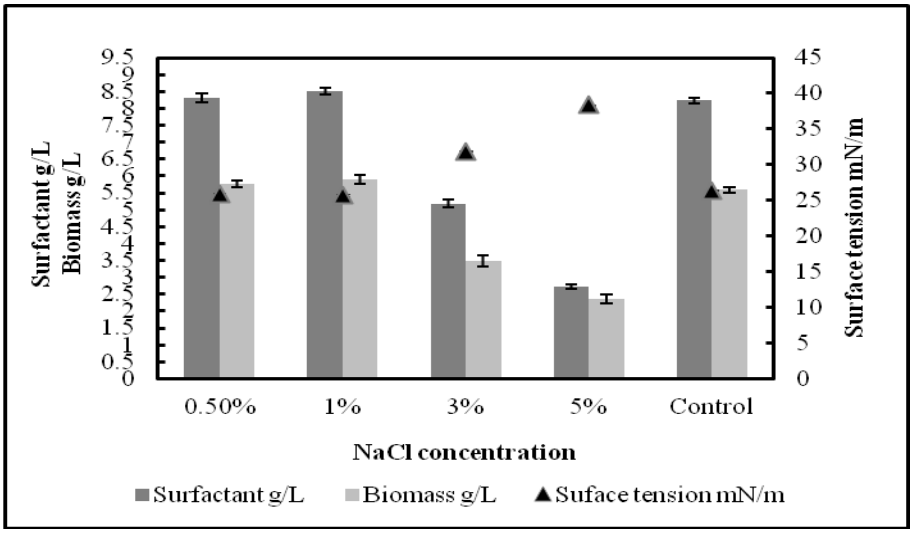


Fig. 4.35 Effect of salinity on surfactant production by *Pseudomonas sp.* 2B in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose, 1 g/L of potassium nitrate and 2% (v/v) olive oil. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 72 hours. Results are represented as Mean ± SEM (n=3).

The results of effect of salinity on surfactant production by *Pseudomonas aeruginosa* is presented in Fig. 4.36. It can be observed that maximum surfactant (7.4 g/L) and biomass (4.94 g/L) was produced at 0.5% (w/v) NaCl concentration, the surface tension of the cell-free broth was 27.41 mN/m. Further increase in NaCl concentration led to decrease in surfactant as well as biomass concentration and also affected surface tension. At 1% and 3% (w/v) NaCl concentration, 5.69 g/L and 3.57 g/L of surfactant as well as 3.21 g/L and 1.78 g/L of biomass quantity was produced. The corresponding surface tension values of the cell-free broth were found to be 31.71 mN/m and 35.10 mN/m, respectively. At 5% (w/v) NaCl concentration, the production of surfactant and biomass was as low as 1.11 g/L and 1.48 g/L, the surface tension of the cell-free broth was 40.91 mN/m. Yakimov et al. (1995) isolated *Bacillus licheniformis* BAS50 from North German oil reservoirs which grew and produced a lipopeptide surfactant at 13% (w/v) NaCl concentration, the surfactant decreased the surface tension of the cell-free broth to 28 mN/m. Tabatabaee et al. (2005) reported that 3% (w/v) NaCl concentration was optimum for maximum surfactant production by *Bacillus sp.*, the surface tension of the cell-free broth was reduced to 37.8 mN/m.

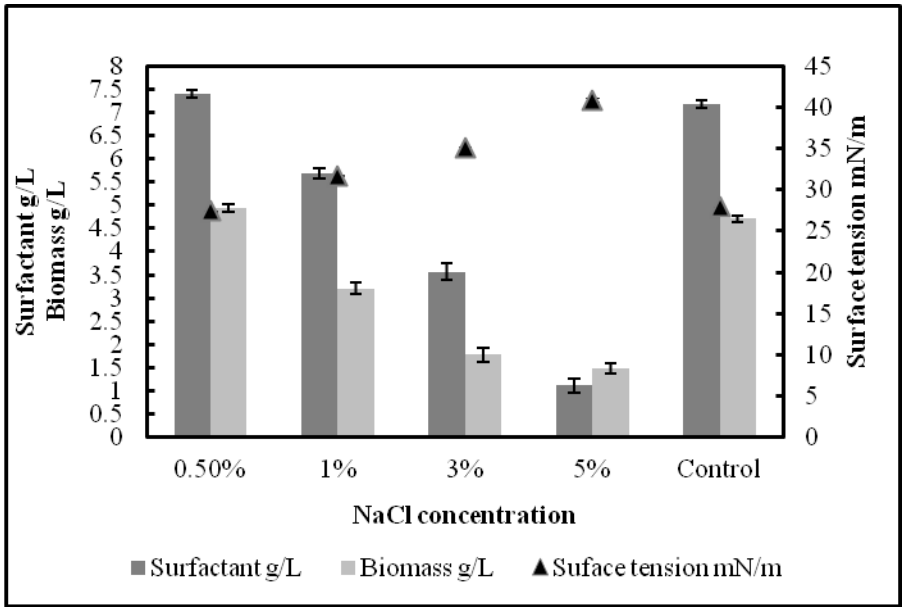


Fig. 4.36 Effect of salinity on surfactant production by *Pseudomonas aeruginosa* in the PPGAS medium (pH 7) containing 30 g/L (w/v) of glucose, 1 g/L of ammonium chloride and 2% (v/v) n-hexadecane. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm for 96 hours. Results are represented as Mean ± SEM (n=3).

SUMMARY

The present study implies that the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, could produce surfactant in saline conditions. *Pseudomonas sp.* 2B produced a maximum of 8.51 g/L of surfactant and 7.40 g/L of surfactant was produced by *Pseudomonas aeruginosa* at 1% and 0.5% (w/v) NaCl concentrations, respectively. The corresponding surface tension values were found to be 25.72 mN/m and 27.41 mN/m, respectively. In the literature, various microorganisms have demonstrated the ability to produce surfactant at different NaCl concentrations (Makkar and Cameotra 2002; Kokare et al. 2007; Khopade et al. 2012).

4.8 OPTIMIZATION OF THE MEDIUM COMPONENTS USING STATISTICAL EXPERIMENTAL DESIGN FOR SURFACTANT PRODUCTION

In the literature, several reports have indicated that the process variables at optimal concentrations have shown synergistic effect through which maximum surfactant production was achieved. In this regard, exploratory shake flask experiments were conducted to obtain the optimal variable concentrations in order to achieve maximum surfactant concentration by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively based on our earlier studies. In the previous section (4.7.5), it was found that increased surfactant production occurred when glucose, molasses and glycerol, were used as the carbon source by *Pseudomonas sp.* 2B whereas *Pseudomonas aeruginosa* produced higher quantity of surfactant in the presence of glycerol, glucose and whey, respectively. Potassium nitrate, ammonium chloride, yeast extract and peptone as nitrogen source increased biomass and surfactant productivity by *Pseudomonas sp.* 2B. Ammonium chloride, ammonium nitrate, peptone and yeast extract showed enhanced surfactant production by *Pseudomonas aeruginosa* (Section 4.7.6). Olive oil and coconut oil as inducers increased surfactant production by *Pseudomonas sp.* 2B whereas n-hexadecane and sunflower oil as inducers resulted in increased surfactant production by *Pseudomonas aeruginosa* (Section 4.7.10). In the presence of sodium chloride, surfactant was synthesized by both the strains (Section 4.7.12). Therefore, these process variables were initially screened in the Placket-Burman desisn studies and further, the optimal concentrations of process variables leading to maximum surfactant production was studied using Response Surface Methodology.

4.8.1 SCREENING VARIOUS PROCESS VARIABLES FOR SURFACTANT PRODUCTION BY PLACKETT-BURMAN DESIGN (PBD)

Based on the previous experimental results, a statistical design, Plackett-Burman method was adopted to find out the effect of various process variables on surfactant production. A design containing 12 experiments was designed for 11 variables using the

"MINITAB 15". For each variable, a high (+1) and low (-1) was tested, the factors chosen and their concentration range is described in Section 3.2.11.1.

The experimental design matrix and the corresponding results obtained for surfactant production by *Pseudomonas sp.* 2B is presented in Table 4.9. Surfactant production was evaluated as response for each run. It was found that maximum surfactant production was achieved in the trial 4 (in which the concentration of yeast extract (A), peptone (B), potassium nitrate (C), ammonium chloride (D), glycerol (E), glucose (F), molasses (G), olive oil (H), coconut oil (I), potassium chloride (J) and sodium chloride (K) was 2 g/L, 30 g/L, 0.5 g/L, 2 g/L, 2 %, 3 g/L, 2 %, 2 %, 2 %, 0.5 g/L and 0.5 g/L, respectively); a maximum of 13.53 g/L of surfactant was produced by the bacterial strain. A 1.58 fold increase in surfactant production was found using Plackett-Burman design (the surfactant quantity obtained in a previous study corresponded to a value of 8.51 g/L as shown in section in 4.7.12). Leonardo et al. (2010) used Plackett-Burman method to understand the interactive effects of various factors influencing surfactant production by *Pseudomonas fluorescens*. In their study, the various factors screened for surfactant production were glucose glycerol, yeast extract, ammonium nitrate, organic nitrogen source: inorganic nitrogen source concentration, soy bean oil, phosphorus, agitation speed, temperature and incubation time. They observed that soybean oil and organic and inorganic nitrogen ratio significantly influenced surfactant production; 1.98 g/L of surfactant was produced in a medium composed of vegetable oil 2% (v/v), K_2HPO_4 0.2% (w/v), yeast extract 0.4 g/L, NH_4NO_3 3.7 g/L, 1 ml of trace elements (20 mg/L $CoCl_3$, 30 mg/L H_3BO_3, 10 mg/L $ZnSO_4$, 1 mg/L $CuSO_4$, 3 mg/L Na_2MoO_4, 10 mg/L $FeSO_4$ and 2.6 mg/L $MnSO_4$) with pH 7.2 and operating conditions of 150 rpm, 6 days and 30ºC.

Table 4.9 Experimental design matrix and the results of experimental runs of Plackett-Burman Design for surfactant production by *Pseudomonas sp.* 2B

Run Order	A	B	C	D	E	F	G	H	I	J	K	Surfactant g/L
1	10	30	0.5	2	2	30	0.5	0.5	0.5	5	0.5	6.02
2	2	30	2	0.5	2	30	2	0.5	0.5	0.5	5	4.08
3	10	3	2	2	0.5	30	2	2	0.5	0.5	0.5	4.4
4	**2**	**30**	**0.5**	**2**	**2**	**3**	**2**	**2**	**2**	**0.5**	**0.5**	**13.53**
5	2	3	2	0.5	2	30	0.5	2	2	5	0.5	6.03
6	2	3	0.5	2	0.5	30	2	0.5	2	5	5	8.02
7	10	3	0.5	0.5	2	3	2	2	0.5	5	5	12.16
8	10	30	0.5	0.5	0.5	30	0.5	2	2	0.5	5	6.79
9	10	30	2	0.5	0.5	3	2	0.5	2	5	0.5	8.15
10	2	30	2	2	0.5	3	0.5	2	0.5	5	5	2.53
11	10	3	2	2	2	3	0.5	0.5	2	0.5	5	5.41
12	2	3	0.5	0.5	0.5	3	0.5	0.5	0.5	0.5	0.5	3.76

where A refers to yeast extract (2-10 g/L), B refers to peptone (3-30 g/L), C refers to potassium nitrate (0.5-2 g/L), D refers to ammonium chloride (0.5-2 g/L), E refers to glycerol (0.5-2%), F refers to glucose (3-30 g/L), G refers to molasses (0.5-2%), H refers to olive oil (0.5-2%), I refers to coconut oil (0.5-2%), J refers to potassium chloride (0.5-2 g/L) and K refers to sodium chloride (0.5-2 g/L).

The main effect of the variables on the responses on the surfactant production by *Pseudomonas sp.* 2B is presented in Fig. 4.37. Potassium nitrate, glucose, olive oil, ammonium chloride, glycerol, coconut oil, yeast extract and molasses, were found to have positive effect on surfactant production; their corresponding main effects were 2.53, 2.18, 2.16, 1.95, 1.54, 1.34, 1.19 and 0.99. Sodium chloride, peptone and potassium chloride had negative effect on surfactant production by *Pseudomonas sp.* 2B; their corresponding effects were found to be -2.87, -2.45 and -0.09. Based on the main effect

values, glucose (carbon source), olive oil (inducer) and potassium nitrate (nitrogen) were found to have significant effect on surfactant production. The results of the present study are in accordance with previous experimental results wherein glucose, potassium nitrate and olive oil have resulted in maximum surfactant production (Section 4.7.5, 4.7.7 and 4.7.10). Therefore, these process variables were considered for further studies.

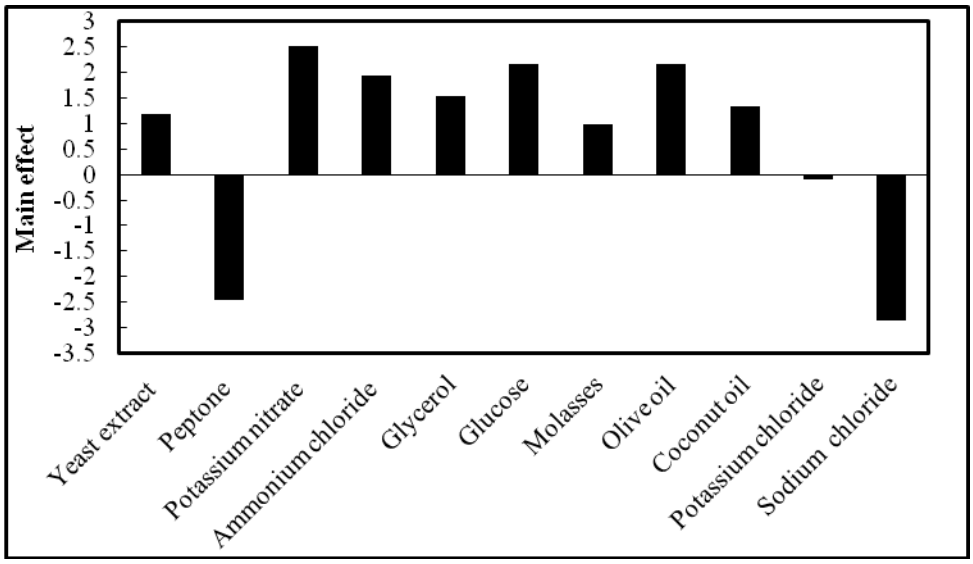


Fig. 4.37 Main effects of the variables on surfactant production by *Pseudomonas sp.* 2B using Plackett-Burman Design. Results are represented as Mean ± SEM (n=3)

Statistical analysis of the responses for surfactant production by *Pseudomonas sp.* 2B is represented in Table 4.10. The model F-value of 112.41 implies that the model is significant. A p-value of less than 0.05 for the variables *viz.*, yeast extract, potassium nitrate, glycerol, glucose, molasses, olive oil and coconut oil indicates that they are significant. The other variables that were not significant were peptone, potassium chloride and sodium chloride. Ammonium chloride was not significant variable though it showed a positive effect. In addition, the predicted R^2 was found to be 0.9290, which is in reasonable agreement with the R^2 of 0.9980 and adjusted R^2 of 0.9891. This revealed that there is good agreement between the experimental and the theoretical values predicted by the model.

Table 4.10 Statistical analysis (ANOVA) for evaluating the significance of variables for surfactant production by *Pseudomonas sp.* 2B

Source	Sum of squares	F-value	p-value
Model	120.82	112.41	0.0088
A- Yeast extract	2.07	17.31	0.0532
B- Peptone	0.98	2.65	0.0896
C-Potassium nitrate	32.28	270.27	0.0037
D-Ammonium chloride	1.29	3.56	0.0956
E- Glycerol	15.37	128.69	0.0135
F- Glucose	8.67	72.60	0.0036
G-Molasses	32.67	273.58	0.0077
H-Olive oil	8.33	69.78	0.0063
I-Coconut oil	18.70	156.59	0.0140
J- Potassium chloride	2.03	17.03	0.0540
K- Sodium chloride	0.70	5.87	0.1364

R^2=0.9980, predicted R^2=0.9290, adjusted R^2=0.9891

The experimental Plackett-Burman design matrix and the corresponding results obtained for surfactant production by *Pseudomonas aeruginosa* is presented in Table 4.11. Surfactant production was evaluated as response for each run. Maximum surfactant production was achieved in the trial 3 (in which the concentrations of peptone (A), yeast extract (B), ammonium chloride (C), ammonium nitrate (D), glycerol (E), glucose (F), whey (G), hexadecane (H), sunflower oil (I), potassium chloride (J) and sodium chloride (K) was 10 g/L, 3 g/L, 2 g/L, 2 g/L, 0.5%, 30 g/L, 2%, 2%, 0.5%, 0.5 g/L and 0.5 g/L,

respectively) a maximum of 9.73 g/L of surfactant quantity was produced. A 1.31 fold increase in surfactant production was found using Plackett-Burman design (the surfactant quantity obtained in the previous study corresponded to a value of 7.4 g/L as shown in section in 4.7.12). In a study carried by Philippe et al. (1999), Plackett-Burman design was used to study the effects of various process variables involved in surfactant production by *Bacillus subtilis* S499; it was observed that agitation, ammonium sulphate, sucrose, trace elements, potassium dihydrogen phosphate, peptone and yeast extract concentrations had positive effect whereas temperature and pH had negative effect on surfactant production. They reported that a maximum of 0.11 g/L of surfactant was produced by *Bacillus subtilis* S499 following Plackett-Burman design.

Table 4.11 Experimental design matrix and the results of experimental runs of Plackett- Burman Design for surfactant production by *Pseudomonas aeruginosa*

Run Order	A	B	C	D	E	F	G	H	I	J	K	Surfactant g/L
1	10	10	0.5	2	2	30	0.5	0.5	0.5	5	0.5	3.4
2	2	10	2	0.5	2	30	2	0.5	0.5	0.5	5	2.24
3	**10**	**3**	**2**	**2**	**0.5**	**30**	**2**	**2**	**0.5**	**0.5**	**0.5**	**9.73**
4	2	10	0.5	2	2	3	2	2	2	0.5	0.5	4.29
5	2	3	2	0.5	2	30	0.5	2	2	5	0.5	8.12
6	2	3	0.5	2	0.5	30	2	0.5	2	5	5	3.03
7	10	3	0.5	0.5	2	3	2	2	0.5	5	5	2.55
8	10	10	0.5	0.5	0.5	30	0.5	2	2	0.5	5	2.02
9	10	10	2	0.5	0.5	3	2	0.5	2	5	0.5	3.04
10	2	10	2	2	0.5	3	0.5	2	0.5	5	5	1.88
11	10	3	2	2	2	3	0.5	0.5	2	0.5	5	4.07
12	2	3	0.5	0.5	0.5	3	0.5	0.5	0.5	0.5	0.5	1.26

where A refers to peptone (2-10 g/L), B refers to yeast extract (3-10 g/L), C refers to ammonium chloride (0.5-2 g/L), D refers to ammonium nitrate (0.5-2 g/L), E refers to glycerol (0.5-2%), F refers to glucose (3-30 g/L), G refers to whey (0.5-2%), H refers to

n-hexadecane (0.5-2%), I refers to sunflower oil (0.5-2%), J refers to potassium chloride (0.5-5 g/L) and K refers to sodium chloride (0.5-5 g/L).

The main effect of the process variables on the responses obtained during the surfactant production studies by *Pseudomonas aeruginosa* is depicted in Fig. 4.38. Ammonium chloride, glucose, n-hexadecane, ammonium nitrate, glycerol, peptone, whey and sunflower oil were found to have positive effect on surfactant production; their corresponding main effects were 2.07, 1.92, 1.91, 1.21, 0.7, 0.67, 0.67 and 0.57. Sodium chloride, yeast extract and potassium chloride had negative effect on surfactant production; their corresponding effects were -2.35, -1.97 and -0.27. The results of the present study are in accordance with previous results where glucose, ammonium chloride and n-hexadecane resulted in maximum surfactant production (Section 4.7.5, 4.7.2.7 and 4.7.2.10). Therefore, glucose (carbon source), n-hexadecane (inducer) and ammonium chloride (nitrogen) were considered for further studies.

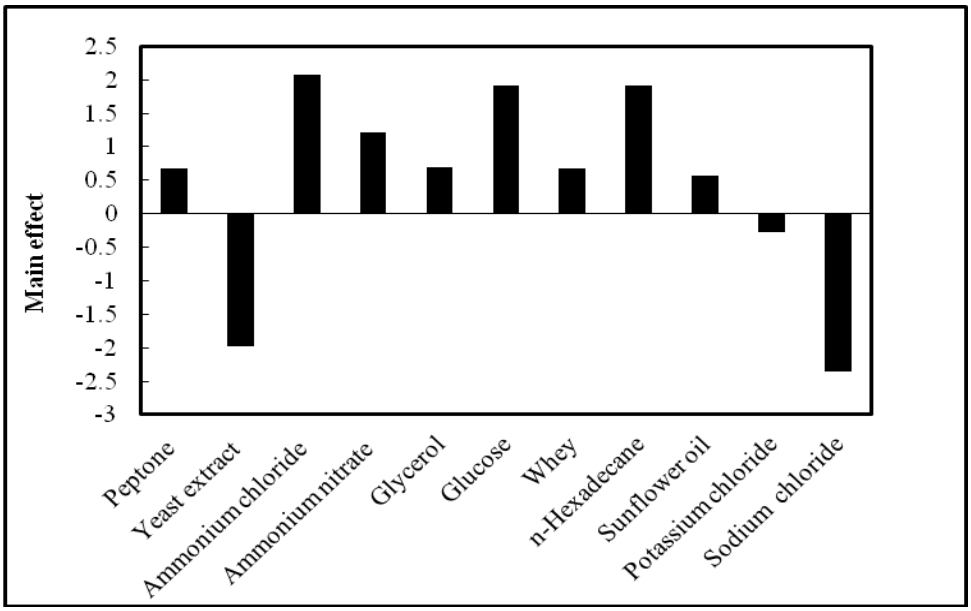


Fig. 4.38 Main effects of the variables on surfactant production by *Pseudomonas aeruginosa* using Plackett-Burman Design. Results are represented as Mean ± SEM (n=3)

Statistical analysis of the responses for surfactant production by *Pseudomonas aeruginosa* is represented in Table 4.12. The model F-value of 70.39 implies that the model is significant. A p-value of less than 0.05 for the variables *viz.*, yeast extract, ammonium chloride, glycerol, glucose, hexadecane, sunflower oil and sodium chloride indicates that they had significant effect on surfactant production. The other variables that were not significant were peptone, ammonium nitrate, whey and potassium chloride. Ammonium chloride was not significant variable, though it showed a positive effect. In addition, the predicted R^2 was found to be 0.8758, which is in reasonable agreement with the R^2 of 0.9672 and adjusted R^2 of 0.8799. This revealed that there is good agreement between the experimental and the theoretical values predicted by the model. The experimental values were fitted into the model and it revealed that the model was significant.

Table 4.12 Statistical analysis (ANOVA) for evaluating the significance of variables for surfactant production by *Pseudomonas aeruginosa*

Source	Sum of squares	F-value	p-value
Model	70.39	11.07	0.0367
A- Peptone	1.33	1.67	0.2869
B- Yeast extract	11.78	14.82	0.0309
C- Ammonium chloride	13.08	16.46	0.0270
D- Ammonium nitrate	4.28	5.39	0.1029
E- Glycerol	5.29	6.42	0.0341
F- Glucose	10.93	13.75	0.0032

G- Whey	1.42	1.79	0.2735
H-n-hexadecane	11.12	13.99	0.0086
I- Sunflower oil	3.49	4.21	0.0333
J- Potassium chloride	1.08	5.14	0.0910
K- Sodium chloride	16.45	20.70	0.0199

R^2=0.9672, predicted R^2=0.8758, adjusted R^2=0.8799

SUMMARY

In the present study, both experimental and predicted values indicated that glucose as carbon source, olive oil as inducer and potassium nitrate as nitrogen source had significant effect on surfactant production by *Pseudomonas sp.* 2B. Both experimental and predicted values indicated that surfactant production by *Pseudomonas aeruginosa* was mainly influenced by glucose as carbon source, n-hexadecane as inducer and ammonium chloride as nitrogen source. The results of the present study were in accordance with results obtained in previous sections wherein the same carbon sources, nitrogen sources and inducers demonstrated enhanced surfactant production ability. Hence, these process variables were chosen for further studies.

4.8.2 OPTIMIZATION OF THE SIGNIFICANT PROCESS VARIABLES FOR SURFACTANT PRODUCTION BY RESPONSE SURFACE METHODOLOGY (RSM)

On the basis of the results of Plackett-Burman Design, three factors (glucose, olive oil and potassium nitrate for surfactant production by *Pseudomonas sp.* 2B and glucose, n-hexadecane and ammonium chloride for surfactant production by

Pseudomonas aeruginosa) had significant effect on the production of surfactant. To obtain the optimal concentrations of these process variables leading to maximum surfactant production, Response Surface Methodology was used. Experiments were conducted to achieve an optimal combination of the three process variables using Central Composite design. A 2^3 factorial design augmented by 6 axial points ($\alpha = 1.682$) was implemented in 20 experiments wherein the effect of each variable on surfactant production was taken as a response. The levels of the three significant variables: glucose (X_1), olive oil (X_2) and potassium nitrate (X_3) were optimized for surfactant production by *Pseudomonas sp.* 2B whereas glucose (X_1), n-hexadecane (X_2) and ammonium chloride (X_3) were optimized for surfactant production by *Pseudomonas aeruginosa*.

The design matrix and the corresponding results of the RSM experiments for surfactant production by *Pseudomonas sp.* 2B is shown in Table 4.13. Maximum surfactant concentration was obtained in trial 5, corresponding to a concentration of glucose at 35 g/L (w/v), olive oil at 3.5% (v/v) and potassium chloride at 5.5 g/L (w/v); 14.63 g/L of surfactant quantity was produced by the bacterium. Similar study was carried out by Celia et al. (2010), where a maximum of 3.7 g/L of surfactant quantity was produced by *Pseudomonas aeruginosa* LBM10 using RSM; the optimal concentrations of process variables were 30 g/L of glycerol, 12.8 C/N ratio and 80 C/P ratio. 9.20 g/L of surfactant quantity was produced by *Serratia marcescens* DT-1P during the optimization studies using 4% of glycerol, 0.875 g/L of ammonium sulphate, 7.75% of inoculum level, 7 pH and 25ºC temperature as process variables (Rajkumar et al. 2007).

Table 4.13 Design of experiment for Central Composite Design (CCD) matrix and response obtained during surfactant production by *Pseudomonas sp.* 2B

Run Order	Glucose (g/L)	Olive oil (%)	Potassium nitrate (g/L)	Surfactant g/L	
				Experimental	Predicted
1	20	2	8	5.58	5.93
2	50	5	3	6.59	6.37
3	35	3.5	1.2955	7.88	7.78
4	9.7731	3.5	5.5	4.43	3.96
5	**35**	**3.5**	**5.5**	**14.63**	**14.63**
6	60.2268	3.5	5.5	4.81	5.07
7	35	3.5	5.5	14.63	14.63
8	50	2	8	7.14	6.64
9	20	5	3	5.12	5.75
10	35	3.5	5.5	14.63	14.63
11	50	5	8	5.69	6.01
12	35	3.5	5.5	14.63	14.63
13	20	5	8	6.42	6.51
14	35	0.9773	5.5	6.88	7.11
15	35	6.0226	5.5	7.73	7.29
16	35	3.5	5.5	14.63	14.63
17	35	3.5	5.5	14.63	14.63
18	20	2	3	5.09	4.90
19	50	2	3	6.69	6.73
20	35	3.5	9.7044	8.45	8.34

Regression analysis of the transformed data resulted in the following second-order polynomial equation, which describes the surfactant production (Y) by *Pseudomonas sp.* 2B as a function of glucose (X_1), olive oil (X_2) and potassium nitrate (X_3):

$$Y = +14.63+0.33\,X_1+0.055\,X_2+0.17\,X_3-0.30\,X_1X_2-0.28X_1X_3-0.067X_2X_3-3.57\,X_1^2-2.62\,X_2^2-2.32\,X_3^2 \quad \text{(I)}$$

Analysis of variance (ANOVA) was used to evaluate fit adequacy (Table 4.14). The statistical significance of equation was checked by F-test and the analysis of variance

of regression model demonstrates that the model was highly significant as it is evident from the Fischer test with low probability value. The model F-value of 224.13 implies that the model was significant. The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.995 (value > 0.75 indicates the fitness of the model), indicating that 99.5% of the variability in the response could be explained by the model. The 'predicted R^2' was 0.9597 and 'adjusted R^2' was 0.9907, which advocates for high significance of the model. Relatively low coefficient variation (4.43%) confirms the precision and reliability of the experiments performed. It is also seen that the factors with higher significance (p-value < 0.05) were X_1 (glucose) *and* X_1^2, X_2^2 and X_3^2 (squared term of glucose, n-hexadecane and ammonium chloride). Thus, it was a suitable model to predict the surfactant production efficiency using aforementioned experimental conditions.

Table 4.14 Analysis of Variance (ANOVA) for response surface quadratic for surfactant production by *Pseudomonas sp.* 2B

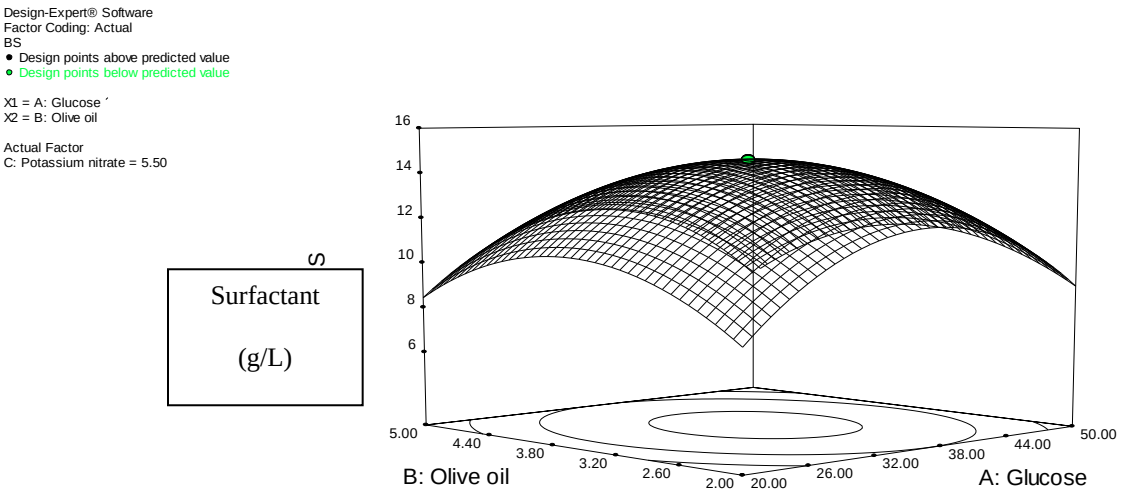
Source	Sum of squares	Degree of freedom	Mean Square	F-value	p-value Prob > F
Model	307.86	9	34.21	224.13	< 0.0001
Glucose (X_1)	1.51	1	1.51	9.88	0.0104
Olive oil (X_2)	0.041	1	0.041	0.27	0.6149
Potassium nitrate (X_3)	0.39	1	0.39	2.53	0.1424
X_1X_2	0.73	1	0.73	4.80	0.0533
X_1X_3	0.63	1	0.63	4.11	0.0701
X_2X_3	0.036	1	0.036	0.24	0.6356

X_1^2	184.06	1	184.06	1206.01	< 0.0001
X_2^2	99.27	1	99.27	650.41	< 0.0001
X_3^2	77.60	1	77.60	508.44	< 0.0001
Residual	1.53	10	0.15		
Lack of Fit	1.52	5	0.30	994.36	0.1063
Pure Error	1.533	5	3.067		

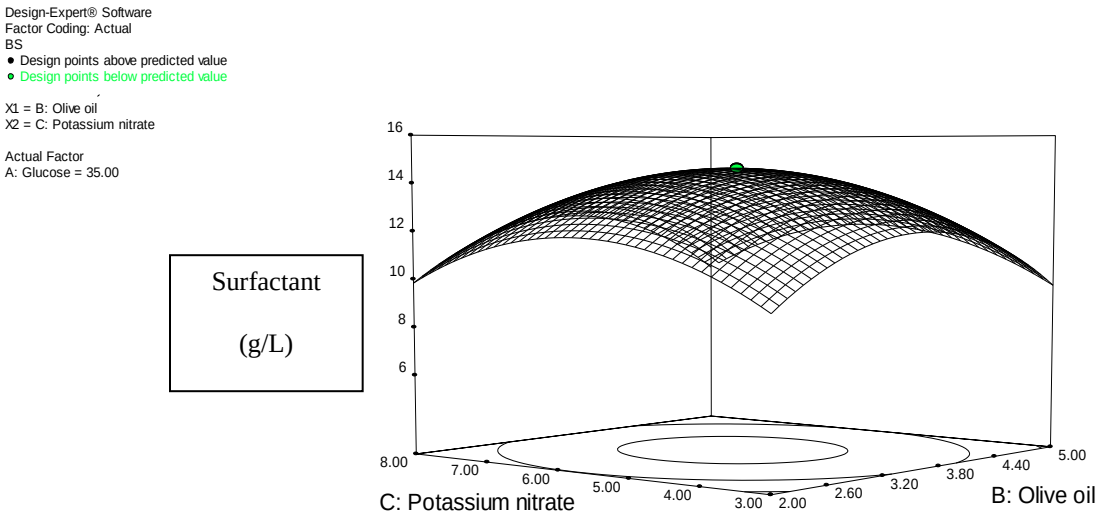
R^2 = 99.51%, predicted R^2 = 95.97%, adjusted R^2= 99.07%,
Coefficient Variation =4.43%

In order to determine the optimum levels of each variable for maximum surfactant production, three-dimensional response surface and contour plots were constructed by plotting the response (surfactant production) on the Z-axis against any two independent variables, while maintaining other variables at their optimum levels. The response at the centre point corresponds to a maximum degree of achievable surfactant production for that factor. As shown in Fig. 4.39 (a), an increase in surfactant production was observed when the concentrations of glucose and olive oil were increased; surfactant production decreased after a certain concentration of the glucose and olive oil. A similar convex shape trend was observed for variation in concentrations of olive oil and potassium nitrate [Fig. 4.39 (b)]; for variation in concentrations of glucose and potassium nitrate [Fig. 4.39 (c)], suggesting that these were well-defined process conditions for surfactant production. The shapes of contour plots indicate the nature and extent of the interactions. Negligible interaction is shown by circular nature of contour plots [Fig. 4.39 (a, b, c)]. Furthermore, the studies of the contour plot also reveal that optimal values of variables lie within the range: glucose 32-38 g/L, olive oil 3.2-3.8% and potassium nitrate 5-6 g/L.

The contour plots show a rather broad plateau region in which the surfactant quantity changes relatively little when the nutrient concentrations were varied. This indicated that the optimal solution can accommodate small errors or variability in the experimental factors. These plots are helpful in studying the effects of the factors variation in the studied field and consequently, in determining the optimal experimental conditions (Kammoun et al. 2008). The experimental data were fitted into the equation (I) and the optimum levels of each variable were determined to be: glucose (35 g/L), olive oil (3.5%) and potassium nitrate (5.5 g/L).



a)



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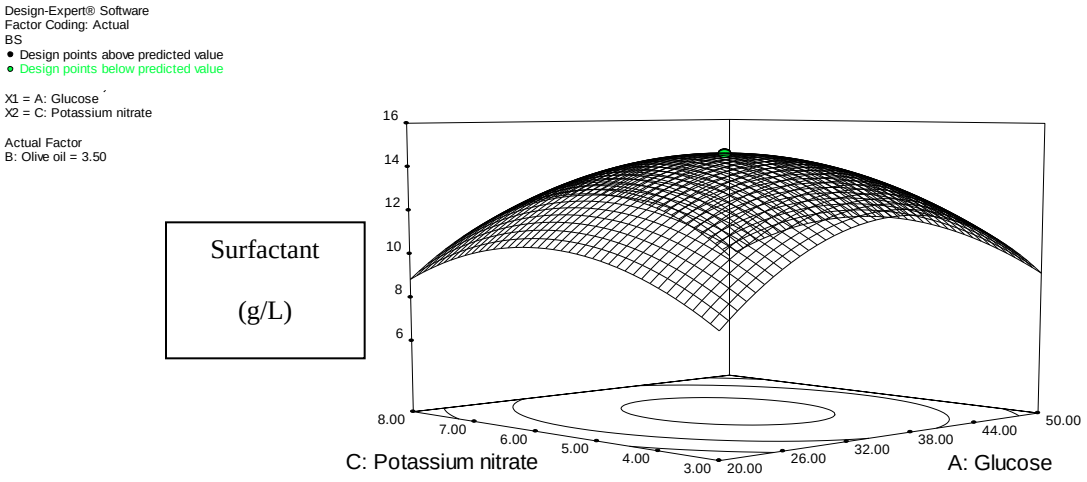


c)

Fig 4.39 Three dimensional response surface plots for surfactant production by *Pseudomonas sp.* 2B as a function of (a) glucose and olive oil (b) olive oil and potassium nitrate (c) glucose and potassium nitrate

In the present study, a maximum of 14.63 g/L of surfactant was produced by *Pseudomonas sp.* 2B at the optimal conditions obtained through the Response Surface Methodology. The optimum concentration of factors leading to maximum surfactant production was found to be 35 g/L of glucose, 3.5% of olive oil and 5.5 g/L of potassium nitrate. A 1.71 fold increase in surfactant production by *Pseudomonas sp.* 2B was observed using this statistical method as compared to yield of surfactant (8.51 g/L) obtained during the previous study described in section 4.7.12.

VALIDATION OF THE MODEL

Experiments were conducted to validate the model using the optimal concentrations of process variables obtained during Response Surface Methodology study. Trials for the validation of the statistical model and regression equation were conducted at concentrations of the variables as shown in the response optimization plot (Fig. 4.40). 35.7645 g/L of glucose, 3.5% of olive oil and 5.5425 g/L of potassium nitrate were the optimal concentrations indicated by the optimization plot for surfactant production by *Pseudomonas sp.* 2B. Under these optimized conditions, the predicted response for surfactant production was 14.6453 g/L and the observed experimental value was found to be 14.63 g/L. The surface tension of the cell-free broth showed lowest value, *i.e.* 21.98 mN/m. The experimental values determined were quite close to the predicted values. These results confirmed the validity of the model with regard to optimum levels of the three chosen variables. Hence, the optimal concentrations of the variables were chosen for further studies.

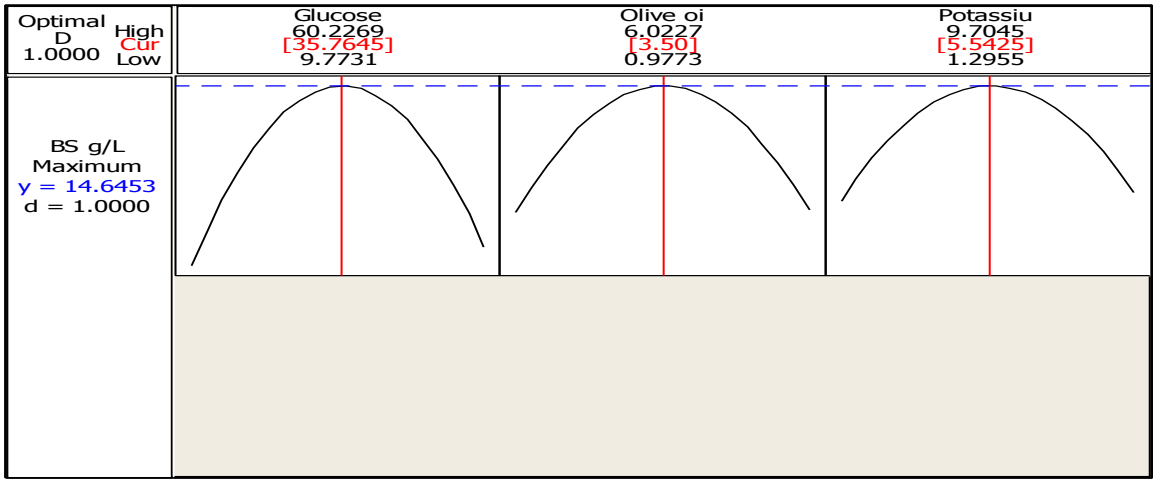


Fig. 4.40 Optimization plot showing the optimum values of the tested variables for surfactant production by *Pseudomonas sp.* 2B

The design of experiments used as input data for developing RSM based model and the corresponding results for surfactant production by *Pseudomonas aeruginosa* is given in Table 4.14. Maximum surfactant concentration was observed in trial 3, corresponding to a concentration of glucose at 35 g/L, n-hexadecane at 3.5% and ammonium chloride at 5.5 g/L; the bacterium produced a maximum of 10.69 g/L of surfactant. Similar results were obtained by Mutalik et al. (2008) where a maximum of 10.9 g/L of surfactant was produced by *Rhodococcus sp.* MTCC 2574 using the medium composition consisting of optimal concentration of mannitol (1.6 g/L), yeast extract (6.92 g/L), meat peptone (19.65 g/L) and n-hexadecane (63.8 g/L).

Table 4.15 Design of experiment for Central Composite Design (CCD) matrix and response obtained during surfactant production by *Pseudomonas aeruginosa*

Run Order	Glucose g/L	n-Hexadecane %	Ammonium chloride g/L	Surfactant g/L Experimental	Predicted
1	35	0.9773	5.5	5.38	5.52
2	9.7731	3.5	5.5	2.93	2.37
3	**35**	**3.5**	**5.5**	**10.68**	**10.69**
4	20	5	8	4.92	5.07
5	60.2268	3.5	5.5	3.31	3.49
6	35	3.5	5.5	10.68	10.69
7	50	2	8	5.64	5.20
8	20	2	8	4.08	4.49
9	35	3.5	5.5	10.68	10.69
10	50	5	3	5.09	4.93
11	20	2	3	3.59	3.46
12	35	3.5	5.5	10.68	10.69
13	35	3.5	5.5	10.68	10.69
14	20	5	3	3.62	4.31
15	35	3.5	9.7044	6.95	6.76
16	35	6.0226	5.5	6.23	5.71
17	35	3.5	5.5	10.68	10.69
18	50	2	3	5.19	5.29
19	35	3.5	1.2955	6.38	6.19
20	50	5	8	4.19	4.57

Regression analysis of the transformed data resulted in the following second-order polynomial equation, which describes the surfactant production (Y) by *Pseudomonas aeruginosa* as a function of glucose (X_1), n-hexadecane (X_2) and ammonium chloride (X_3):

$$Y=+10.65+0.33\ X_1+0.055\ X_2+0.17\ X_3-0.30\ X_1\ X_2-0.28\ X_1\ X_3-0.067\ X_2\ X_3-2.73\ X_1^{\ 2}-1.78\ X_2^{\ 2}-1.47\ X_3^{\ 2} \quad \text{(II)}$$

Variance analysis (ANOVA) was used to evaluate fit adequacy. The statistical significance of equation was checked by F-test and the analysis of variance of regression model demonstrates that the model was highly significant as it is evident from the Fischer test with low probability value. The model F-value of 100.50 implies that the model was significant. The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.9893, indicating that 98.93% of the variability in the response could be explained by the model. The 'predicted R^2' was 0.9130 and the value of the adjusted regression coefficient ('adjusted $R^{2'}$ = 0.979) was also high, which advocates for high significance of the model. Relatively low coefficient variation (6.41%) confirms the precision and reliability of the experiments performed. It is also seen that the factors with higher significance (p-value < 0.05) were X_1 (glucose) *and* X_1^2, X_2^2 and X_3^2 (squared term of glucose, n-hexadecane and ammonium chloride). Thus, it was a suitable model to predict the surfactant production efficiency of *Pseudomonas aeruginosa* using aforementioned experimental conditions.

Table 4.16 Analysis of Variance (ANOVA) for response surface quadratic for surfactant production by *Pseudomonas aeruginosa*

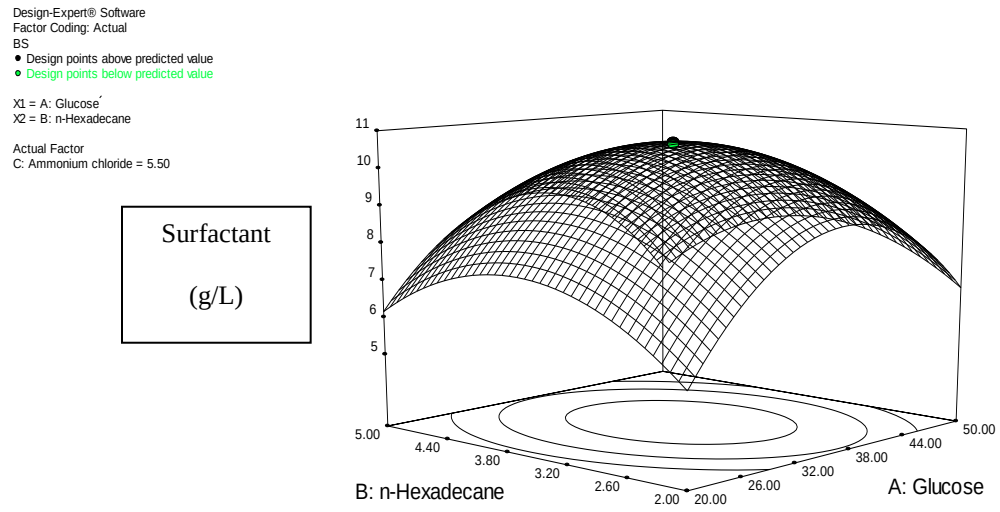
Source	Sum of squares	Degree of freedom	Mean Square	F-value	Prob > F
Model	160.34	9	17.82	100.50	0.0001
Glucose (X_1)	1.51	1	1.51	8.51	0.0154
n-Hexadecane (X_2)	0.041	1	0.041	0.23	0.6404
Ammonium chloride (X_3)	0.39	1	0.39	2.18	0.1704
X_1X_2	0.73	1	0.73	4.13	0.0696
X_1X_3	0.63	1	0.63	3.54	0.0894
X_2X_3	0.036	1	0.036	0.21	0.6599
X_1^2	107.22	1	107.22	604.82	< 0.0001
X_2^2	45.57	1	45.57	257.09	< 0.0001

X_3^2	31.32	1	31.32	176.69	< 0.0001
Residual	1.77	10	0.18		
Lack of Fit	1.77	5	0.35	275.99	0.0121
Pure Error	6.400	5	1.280		

R^2 = 98.93%, predicted R^2 = 91.30%, adjusted R^2= 97.96%, Coefficient Variation= 6.41%

Three-dimensional response surface plots were generated in pairwise combination by plotting the response (surfactant production) on the Z-axis against any two independent variables in order to determine the optimum levels of each variable for maximum surfactant production by *Pseudomonas aeruginosa*, while maintaining other variables at their optimum levels. The response at the centre point corresponds to a maximum degree of achievable surfactant production for that factor. As shown in Fig. 4.41 (a), an increase in surfactant production was observed when the concentrations of glucose and n-hexadecane were increased; surfactant production decreased after a certain concentration of the glucose and n-hexadecane. A similar convex shape trend was observed for variation in concentrations of n-hexadecane and ammonium chloride [Fig. 4.41 (b)]; for variation in glucose and ammonium chloride [Fig. 4.41 (c)], suggesting that these were well-defined process conditions. The shapes of contour plots indicate the nature and extent of the interactions. Negligible interaction is shown by circular nature of contour plots [Fig. 4.41 (a, b, c)]. Furthermore, the studies of the contour plot also reveal that optimal values of variables lie within the range: glucose 32-38 g/L, n-hexadecane 3.2-3.8 % and ammonium chloride 5-6 g/L. The contour plots show a rather broad plateau region in which the surfactant quantity changes relatively little when the nutrient concentrations were varied. This indicated that the optimal solution can accommodate small errors or variability in the experimental factors. The experimental data were fitted into the equation (II) and the optimum levels of each variable were determined to be: glucose (35 g/L), n-hexadecane (3.5%) and ammonium chloride (5.5 g/L). This indicates

that the variables have both individual as well as interaction effects, allowing maximized surfactant production by the organism *Pseudomonas aeruginosa*.



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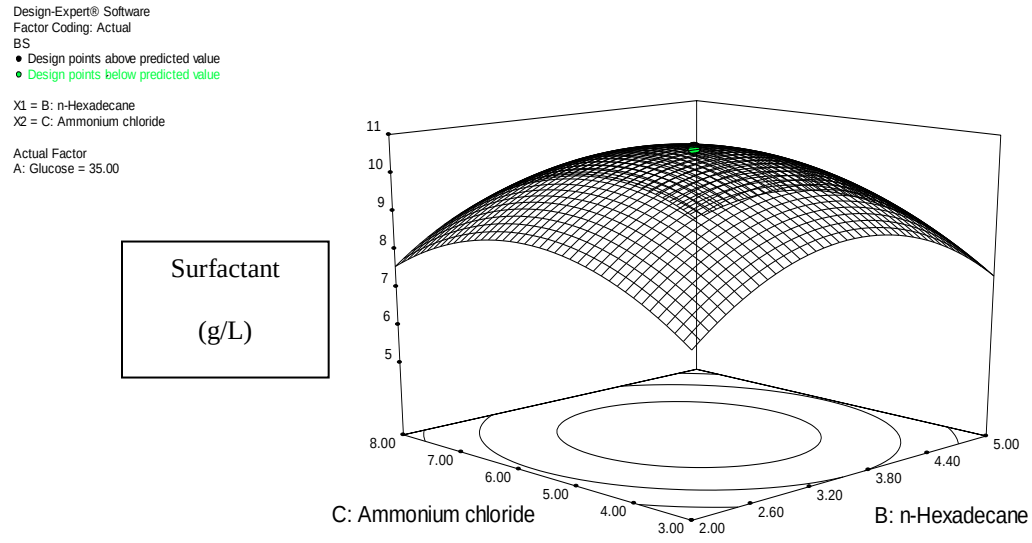

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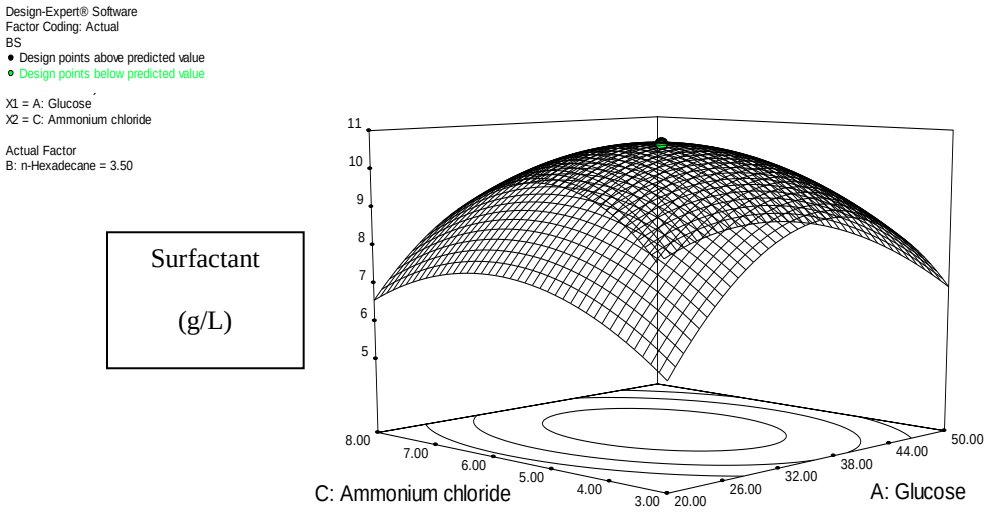


c)

Fig 4.41 Three dimensional response surface plot for surfactant production by *Pseudomonas aeruginosa* as a function of (a) glucose and n-hexadecane (b) olive oil and n-hexadecane (c) glucose and n-hexadecane

In the present study, a maximum of 10.69 g/L of surfactant was produced by *Pseudomonas aeruginosa* using the Response Surface Methodology. The optimum concentration of factors leading to maximum surfactant production was found to be 35 g/L of glucose, 3.5% of n-hexadecane and 5.5 g/L of ammonium chloride. The yield productivity of surfactant before and after optimization was 7.4 g/L (as described in section 4.7.12) and 10.69 g/L, respectively. A 1.44 fold increase in surfactant production by *Pseudomonas aeruginosa* was observed using this statistical method. These results confirmed the validity of the model in regard to optimum levels of the three chosen variables. Hence, the optimal concentrations of the variables were chosen for further studies.

VALIDATION OF THE MODEL

Experiments were conducted to validate the model using the optimal concentrations of process variables at the optimal conditions obtained through the Response Surface Methodology study The optimum values of the tested variables were 35.7645 g/L of glucose, 3.5% of n-hexadecane and 5.6274 g/L of ammonium chloride as shown in the response optimization plot (Fig. 4.42). The maximum experimental response for surfactant production by *Pseudomonas aeruginosa* was 10.68 g/L whereas the predicted value was 10.7044 g/L indicating a strong agreement between them. The surface tension of the cell-free broth showed lowest value, *i.e*. 25.31 mN/m. Hence, these optimal concentrations of the variables were chosen for further studies.

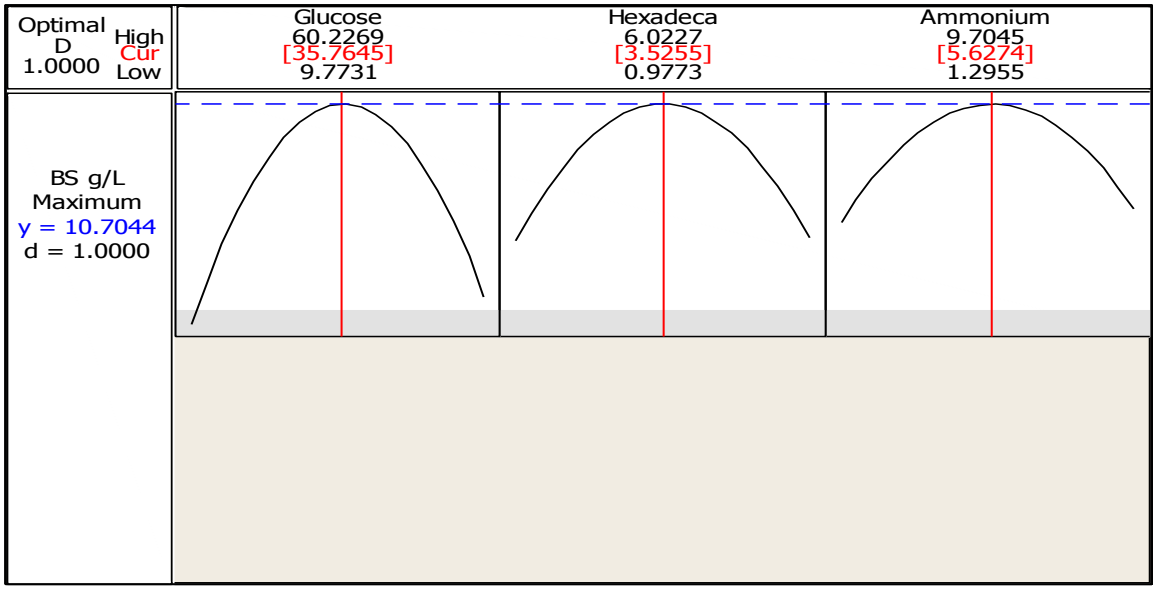


Fig. 4.42 Optimization plot showing the optimum values of the tested variables for surfactant production by *Pseudomonas aeruginosa*

SUMMARY

In the present study, a maximum of 14.63 g/L and 10.69 g/L of surfactant was produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, using the

Response Surface Methodology. The optimum concentration of factors leading to maximum surfactant production by *Pseudomonas sp.* 2B was found to be 35.7645 g/L of glucose, 3.5% of olive oil and 5.5425 g/L of potassium nitrate whereas the optimum concentration of factors leading to maximum surfactant production by *Pseudomonas aeruginosa* was found to be 35.7645 g/L of glucose, 3.5% of n-hexadecane and 5.6274 g/L of ammonium chloride. Hence, these optimal concentrations were maintained in the future studies.

4.9 KINETICS OF SURFACTANT PRODUCTION

Fig. 4.43 shows the results of time course variation of surfactant production, cell growth, surface tension and glucose consumption by *Pseudomonas sp.* 2B. The concentration of glucose was 35.7645 g/L, olive oil was 3.5% and potassium nitrate was 5.5425 g/L in the modified PPGAS medium. The initiation of surfactant production was observed after 8 hours of incubation, the surfactant production increased in the exponential phase along with increase in bacterial biomass and continued till stationary phase of the bacterium. The organism utilized glucose slowly during the incubation time as indicated by decrease in residual glucose concentration. The maximum growth of the bacterium was observed at 60 hours of incubation, where X_{max}=6.81 g/L. However, maximum surfactant production (P_{max}=14.62 g/L) occurred in the stationary phase (at 72 hours), the corresponding biomass was X_{max}=6.47 g/L. The surface tension of the cell-free broth showed lowest value, *i.e.* 21.97 mN/m. Glucose was completely utilized by 36 hours of incubation. Further, the organism might have utilized olive oil as carbon source for growth and surfactant production as surfactant production continued in the stationary phase of the bacterium. A decline in the surfactant production was observed after 96 hours of incubation probably because of the exhaustion of the nutrients. Similar observation was made by Zawawi (2005), where *Actinobacillus sp.* AB-Cr 1 produced maximum surfactant (P_{max}=8.26 g/L) at 72 hours and maximum biomass (X_{max}=3.3 g/L) at 48 hours of incubation when glucose and crude oil were supplemented in the Ramsay

medium. They observed that there was complete exhaustion of glucose within 24 hours of growth, after which the organism utilized crude oil for growth and surfactant production. The present study suggests that the surfactant produced might be a 'primary metabolite' since its production occurred during the exponential growth phase of the bacterium. The results of the present study are in agreement with reports of various researchers that indicate that surfactants are "primary metabolites" and their production coincides with the exponential phase (Abu-Ruwaida et al. 1991; Tabatabaee et al. 2005).

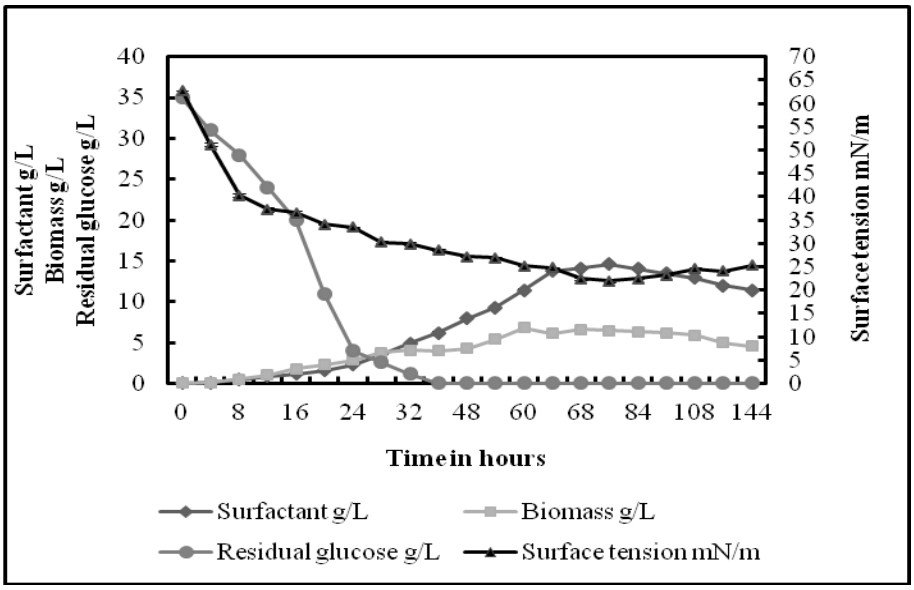


Fig. 4.43 Time course profile of surfactant synthesis, cell growth, surface tension and substrate utilization by *Pseudomonas sp.* 2B in modified PPGAS medium (pH 7) containing 35.7645 g/L of glucose, 3.5% of olive oil and 5.5425 g/L of potassium nitrate. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm. Results are represented as Mean ± SEM (n=3).

The kinetic parameters evaluated in terms of yield factors related to surfactant production by *Pseudomonas sp.* 2B to substrate utilization ($Y_{P/S}$), dry cell biomass to substrate utilization ($Y_{X/S}$) and surfactant production to dry cell biomass ($Y_{P/X}$) are given in Table 4.16. $Y_{P/S}$ = 0.4172 g/g, $Y_{X/S}$ = 0.194 g/g and $Y_{P/X}$ = 2.143 g/g was obtained

during surfactant production by *Pseudomonas sp.* 2B. Specific growth rate (μ) observed was 0.0167/hour whereas the maximum growth rate (μ_{max}) was found to be 0.0288/hour. Ks value of 0.43 g/L suggests that the organism showed moderate affinity towards the substrate. Stanbury et al. (2005) reported that the values of Ks generally ranged between 0-1 g/L. Similar results were obtained by Itoh et al. (1971) during surfactant production by *Pseudomonas aeruginosa* KY 4025 using n-paraffin as carbon source, a yield factor ($Y_{P/S}$) of 0.094 g/g was obtained. In another study, Raza et al. (2006) studied surfactant production by *Pseudomonas aeruginosa* EBN-8 Mutant in minimal medium containing paraffin oil (1%; w/v) as carbon source, they observed that $Y_{P/S}$ = 0.716 g/g; $Y_{P/X}$ = 3.15 g/g at 240 hours of incubation. They reported that a maximum of 6.30 g/L of surfactant was produced by the bacterium.

Table 4.17 Evaluation of kinetic parameters related to surfactant production by *Pseudomonas sp.* 2B

Parameter	Result
Maximum growth rate (μ_{max})	0.0288/hour
Specific growth rate (μ)	0.0167/hour
Yx/s	0.194 g/g
$Y_{P/S}$	0.4172 g/g
$Y_{P/X}$	2.143 g/g
X_{max}	6.81 g/L
P_{max}	14.62 g/L
K_s	0.43 g/L

Fig. 4.44 shows the results of time course variation of surfactant production, cell growth, surface tension and glucose consumption by *Pseudomonas aeruginosa* at the optimized conditions, where the concentrations of glucose was 35.7645 g/L, n-hexadecane was 3.5% and ammonium chloride was 5.6274 g/L in the modified PPGAS medium. Surfactant production was observed after 12 hours of incubation, surfactant synthesis increased in the exponential phase along with increase in bacterial biomass and continued till the stationary phase of the bacterium. The maximum growth of the bacterium was observed at 72 hours of incubation, where X_{max}=6.19 g/L. However, maximum surfactant production (P_{max}=10.67 g/L) occurred in the stationary phase (at 96 hours), the corresponding biomass was X_{max}=6.01 g/L. The surface tension of the cell-free broth showed lowest value, *i.e.* 25.23 mN/m. Glucose was completely utilized by 32 hours of incubation, further the bacterium might have utilized n-hexadecane as carbon source for growth and surfactant production. A decline in the surfactant production was observed after 120 hours of incubation. The present study suggests that the surfactant produced by might be a 'primary metabolite' since its production occurred during the exponential growth phase of the bacterium. The results of the present study are in agreement with reports in the literature that suggest that the production of surfactants by *Pseudomonas aeruginosa* (Hisatsuka et al. 1971), *Bacillus subtilis* (Peypoux et al. 1994), *Acinetobacter calcoaceticus* RAG-1 (Gutnick et al. 2003), etc., were growth associated.

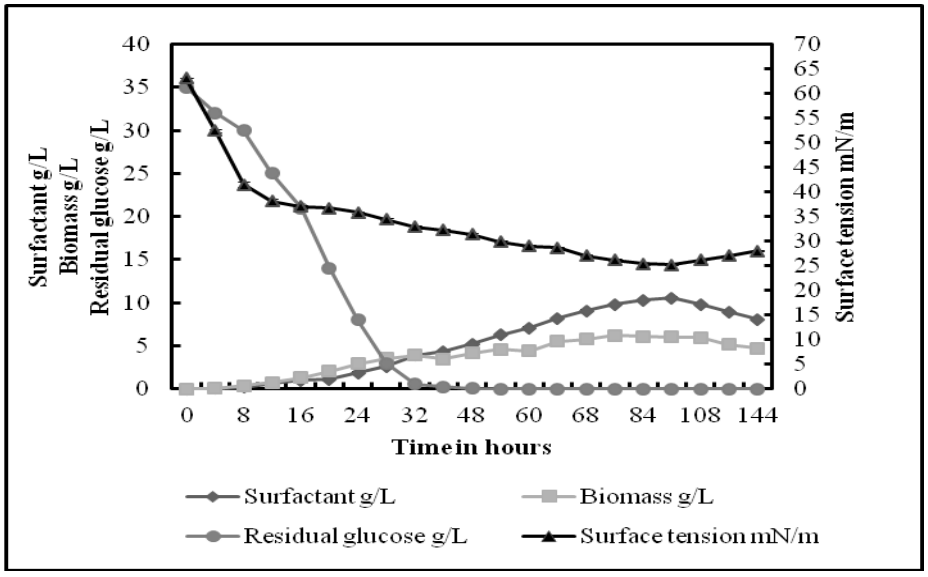


Fig. 4.44 Time course profile of surfactant synthesis, cell growth, surface tension and substrate utilization by *Pseudomonas aeruginosa* in modified PPGAS medium containing 35.7645 g/L of glucose, 3.5% of n-hexadecane and 5.6274 g/L of ammonium chloride. The culture flasks were incubated at 37ºC in incubator shaker at 150 rpm. Results are represented as Mean ± SEM (n=3).

The kinetic parameters evaluated in terms of yield factors related to surfactant production by *Pseudomonas aeruginosa* to substrate utilization ($Y_{P/S}$), dry cell biomass to substrate utilization ($Y_{X/S}$) and surfactant production to dry cell biomass ($Y_{P/X}$) are given in Table 4.17. $Y_{P/S}$ = 0.306 g/g, $Y_{X/S}$ = 0.173 g/g and $Y_{P/X}$ = 1.764 g/g was obtained during the surfactant production by *Pseudomonas aeruginosa*. Specific growth rate (μ) observed was 0.014/hour whereas the maximum growth rate was 0.023/hour. Ks value of 0.59 g/L suggests that the bacterium showed moderate affinity towards the substrate. The results of the present study are in agreement with Yamaguchi et al. (1976) in which value of $Y_{P/S}$ was 0.280 g/g during surfactant production by *Pseudomonas sp*. in n-paraffin (50 g/L) containing medium. The yield factor obtained in the present study ($Y_{P/X}$= 1.764 g/g) with n-hexadecane by *Pseudomonas aeruginosa* was comparable to 1.78 g/g, calculated

from the data reported by Raza et al. (2006) with 1% (w/v) paraffin oil which was used as carbon source by *Pseudomonas aeruginosa* EBN-8 mutant.

Table 4.18 Evaluation of kinetic parameters related to surfactant production by *Pseudomonas aeruginosa*

Parameter	Result
Maximum growth rate (μ_{max})	0.023/hour
Specific growth rate (μ)	0.014/hour
$Y_{X/S}$	0.173 g/g
$Y_{P/S}$	0.306 g/g
$Y_{P/X}$	1.764 g/g
X_{max}	6.19 g/L
P_{max}	10.67 g/L
Ks	0.59 g/L

SUMMARY

In the present study, it was observed that the surfactant produced in the modified PPGAS medium by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, might be primary metabolites since their production coincided with the exponential growth phase of the bacteria. During the surfactant production by *Pseudomonas sp.* 2B, P_{max} and X_{max} values of 14.62 g/L and 6.81 g/L were observed, the surfactant produced lowered the surface tension of the cell free broth to 21.97 mN/m. In case of *Pseudomonas aeruginosa*, P_{max} and X_{max} values were found to be 10.67 g/L and 6.19 g/L, respectively; the surfactant produced by the bacterium reduced the surface

tension of the broth to 25.23 mN/m. It can be concluded that the surfactant production by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* was growth-associated.

4.10 EXTRACTION AND PARTIAL PURIFICATION OF THE SURFACTANT

In the literature, various extraction and partial purification steps have been used by various researchers for the partial purification of surfactants. In this regard the extraction of the surfactant from cell-free broth containing surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was performed followed by partial purification. The surface tension measurement was carried out to check the quality of the surfactant during each purification step. The following procedures were employed for the extraction and partial purification of the surfactant:

4.10.1 ACIDIFICATION

The cell-free broth of the cultures, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* were subjected to acidification using 1N HCl to pH 2. Following acidification, 14.81 g/L and 10.78 g/L of the acidified precipitate were obtained. The surface tension of the acidified surfactant extracts of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* were found to be 21.78 mN/m and 25.02 mN/m, respectively. In the literature, several researchers have employed this technique for partial purification of surfactant (Zhang and Miller 1992; Van Dyke et al. 1993; Haba et al. 2000). Surfactants produced by *Pseudomonas aeruginosa* and *Ustilago zeae,* were extracted from the cell-free broth by acid precipitation (Boothroyd et al. 1956; Spencer et al. 1979).

4.10.2 SOLVENT EXTRACTION

In the present study, the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was extracted using chloroform: methanol mixture in 2:1 ratio after acidification. During solvent extraction, the surfactant was concentrated in the lower organic phase, the solvent was removed using rotary evaporator

and the extract was dried. The dried extract was dissolved in 0.1M sodium bicarbonate buffer to measure surface tension of the surfactant. The surface tension values of extracted surfactants of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, were found to be 21.17 mN/m and 24.96 mN/m, respectively and the surfactant quantity were 14.68 g/L and 10.69 g/L, respectively. In the literature, several solvents have been used to extract surfactants. Surfactant produced by *Pseudomonas aeruginosa* has also been recovered in a similar way, except that extraction was carried out in acetone (Neu et al. 1990). Surfactants produced by *Ustilago zeae* and *Candida sp*. was extracted in either ethanol or methanol (Hauser and Karnovsky 1958; Spencer et al. 1979). Chloroform methanol (2:1) mixture has been to extract and partially purified surfactant according to the reports in the literature (Samadi et al. 2007; Gustafsson et al. 2009) as it is known to extract both hydrophilic and hydrophobic components of the surfactant.

4.10.3 COLUMN CHROMATOGRAPHY

Following solvent extraction, further purification of the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* was performed using column chromatography. The column was packed with silica gel G and the surfactant extract was dissolved in chloroform. The surfactant sample was added to the column and eluted with chloroform: methanol mobile phases in sequence: 50: 3 v/v (200 ml); 50: 5 v/v (200 ml); and 50: 50 v/v (200 ml) at a flow rate of 1 ml/minute and 30 ml fractions were collected. Each fraction was evaporated using a rotary evaporator and dried. The surfactant quantity as well as the surface tension of each fraction were measured.

Out of the 30 fractions collected, fractions 12-25 of surfactant extracts of *Pseudomonas sp.* 2B showed reduction in the surface tension. Fraction 16 (fraction from 50:3 v/v chloroform: methanol phase), 18 (fraction from 50:5 v/v chloroform: methanol phase) and 21 (fraction from 50:5 v/v chloroform: methanol phase) showed surface tension values of 36.12, 31.07 and 35.22 mN/m, respectively. The surfactant concentration obtained in the fractions 16, 18 and 21 were 1.98, 4.87 and 3.48 g/L,

respectively. Fraction 23 (fraction from 50: 50 v/v chloroform: methanol phase) and 25 (fraction from 50:50 v/v chloroform: methanol phase) showed surface tension value 35.93 and 36.01 mN/m, respectively. The surfactant concentrations obtained in the fractions 23 and 25 were 2.11 and 2.01 g/L, respectively. Column chromatography was performed by Raza et al. (2009) to partially purify surfactant produced by *Pseudomonas aeruginosa* EBN mutant; solvents such as hexane, chloroform and chloroform: methanol mixtures were used. They reported that a maximum 0.56 g/L of surfactant was eluted in the chloroform: methanol fraction whereas 0.22 g/L and 0.22 g/L of surfactant were obtained in the hexane and chloroform containing fractions. It can be observed from the surface tension values of different fractions that the surfactant produced by the bacterium might be composed of different moieties.

In the case of *Pseudomonas aeruginosa*, fraction 14 (fraction from 50: 3 v/v chloroform: methanol phase) and 18 (fraction from 50:5 v/v chloroform: methanol phase) showed surface tension values of 36.04 and 31.49 mN/m, respectively. The surfactant concentration obtained in the fractions 14 and 18 were 2.04 and 4.62 g/L, respectively. Fraction 27 (fraction from 50:50 v/v chloroform: methanol phase) showed surface tension value of 35.03 mN/m. The surfactant concentration obtained in the fractions 27 was 3.71 g/L. Similar purification procedure has been followed by Sim et al. (1997) for the partial purification of surfactant produced by *Pseudomonas aeruginosa* UW-1. It has been reported by Itoh et al. (1971) that eluting with chloroform removes pigments and impurities.

SUMMARY

The results of the present study suggest that the surfactant produced by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, were made up of different moieties as suggested by the surface tension values of the different fractions eluted during the column chromatography experiment. The surfactant produced

by the bacterial strains need to be characterized for the identification of different moieties.

4.11 CHARACTERIZATION OF THE SURFACTANT

The present study was carried out to characterize the partially purified surfactant produced by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, by various methods listed below:

4.11.1 THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatography was performed for the identification of different moieties present in the surfactant. Preliminary analysis of the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* strains indicated the presence of lipids as indicated by yellow to brown spots after placing the TLC plate in a closed jar saturated with iodine vapours. Further, the spots were identified on the basis of their Rf values. The Rf values of the standard was 0.82 and 0.66, respectively. The surfactant produced by *Pseudomonas sp.* 2B isolate showed the presence of lipid compounds with the Rf values of 0.84, 0.68, 0.56 and 0.44 respectively (Fig. 4.45). The surfactant produced by *Pseudomonas aeruginosa* showed the presence of lipid compounds with the Rf values of 0.89, 0.67 and 0.46, respectively. Similar results have been obtained by researchers where the Rf value of lipids ranging between 0.44-0.89 indicated that the surfactant was a glycolipid (Itoh et al. 1971; Thanomsub et al. 2007).

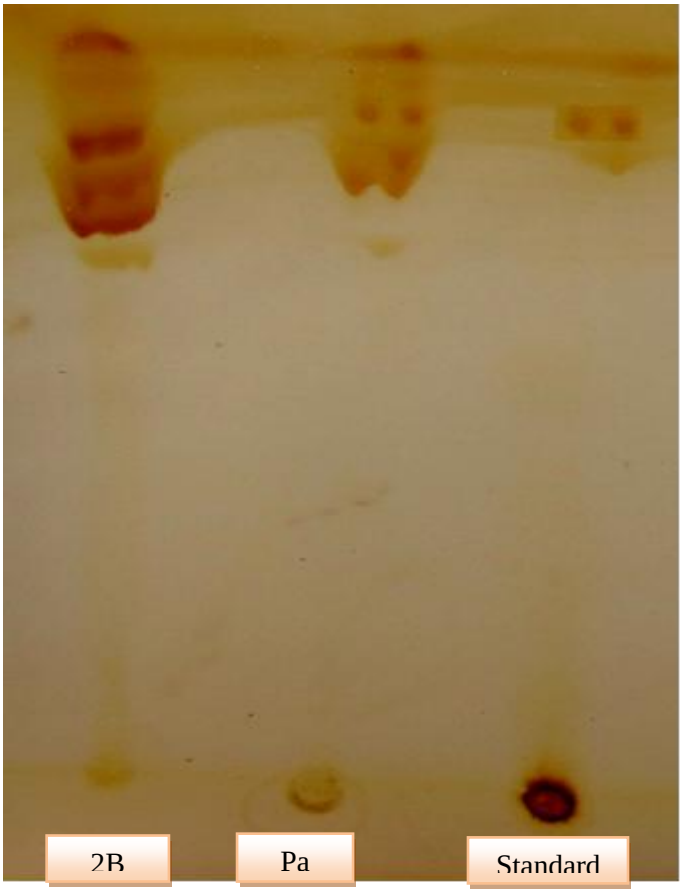


Fig. 4.45 TLC analysis of lipid components of surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*

Purple coloured spots were observed on staining the surfactant with Ninhydrin solution, indicating the presence of amino acids in the surfactant of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. The protein fractions of surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* showed Rf values 0.51 and 0.48, respectively (Fig. 4.46). By comparing the data obtained with the amino acid standard (Rf value of 0.45) and reference from the previous study (Neu et al. 1990), it can be concluded that *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* produced surfactant that contained proteins.

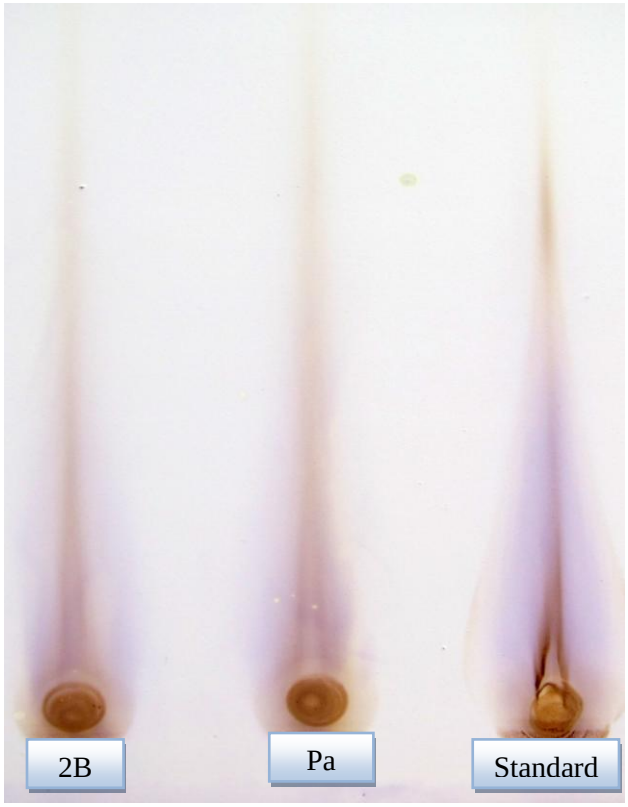


Fig. 4.46 TLC analysis of proteins in surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*

Brown spots were detected on spraying the TLC plates with α-naphthol solution that indicated the presence of carbohydrate in the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* (Fig. 4.47). Further, Rf values of the carbohydrate components of the surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively were found to be 0.83 and 0.81, respectively. Further, the carbohydrate produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* was identified as rhamnose sugar based on the Rf value (0.84). Thaniyavarn et al. (2006) reported that Rf value of 0.83 indicated that the surfactant produced by *Pseudomonas aeruginosa* A41 was a rhamnolipid.

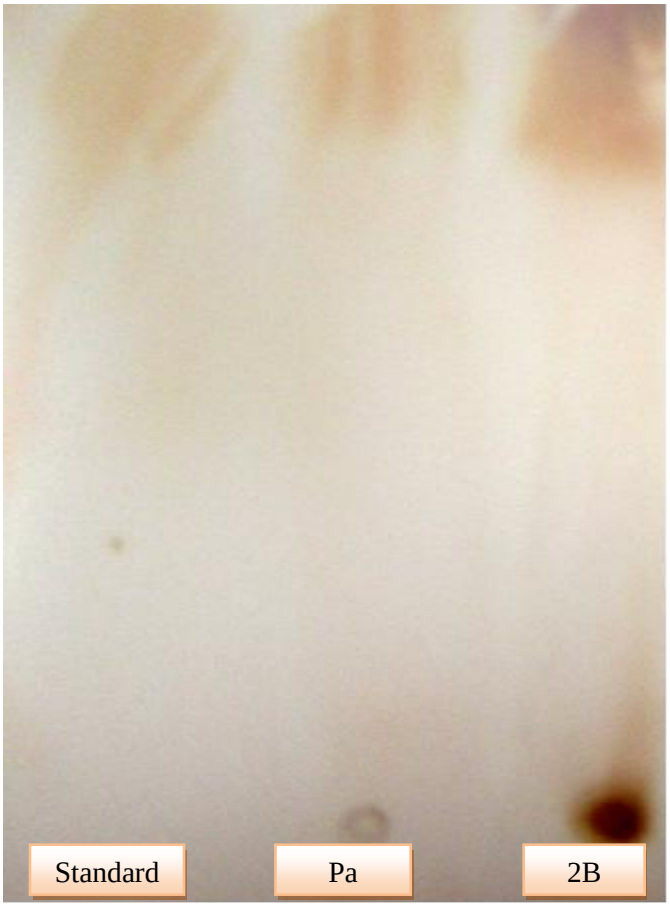


Fig. 4.47 TLC analysis of carbohydrates in surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*

SUMMARY

The present study indicates that the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, might be glycolipoprotein as indicated by the Rf values of different moieties of the surfactant. Further, the carbohydrate moiety of surfactant produced by the bacterial cultures indicated the presence of rhamnose sugar. The surfactant produced by both the bacteria might be rhamnolipopeptide.

Rhamnolipids are the most common glycolipids produced by *Pseudomonas sp,* they are also among the most effective surfactants known today (Kosaric et al. 1987). They are promising surfactants owing to several characteristics such as low minimum surface tension (30-32 mN/m), high emulsifying activity and higher affinity for organic molecules (Mata-Sandoval et al. 1999). However, further characterization of the surfactant produced by the cultures needs to be performed to confirm the nature of surfactant produced by the bacteria.

4.11.2 BIOCHEMICAL ANALYSIS OF THE SURFACTANT

Biochemical analysis of the surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was performed in order to quantify the different moieties of the surfactant. Lowry's test was conducted to estimate protein content in the surfactant whereas the total sugar in the surfactant was quantified using phenol-sulphuric acid method. The lipid content present in the surfactant quantified using gravimetric method. The results revealed that the surfactant produced by *Pseudomonas sp.* 2B was a glycolipoprotein type surfactant, consisting of mixture of lipid, carbohydrate and protein in the ratio of 65%: 31%: 3% (w/w), respectively whereas the ratio of lipid: carbohydrate: protein was 69%: 27%: 1% (w/w) in the surfactant produced by *Pseudomonas aeruginosa.* Further, the total rhamnose in the surfactants was estimated, 78 μg/ml and 41 μg/ml of rhamnose were present in the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively.

The results of the present study are in agreement with Mahmoud et al. (2010), the chemical analysis of *Candida guilliermondii* surfactant revealed that it was a complex of lipids (54%), carbohydrate (34%) and protein (2.3%) while that of *Bacillus subtilis* was found to contain lipid, carbohydrate and protein content in the ratio of 62.3%:7.6%:18.6%, respectively. The chemical compositions and natures of the surfactants are widely divergent and dependent mainly on the fermenting organism (Mahmoud et al. 2010).

4.11.3 FOURIER TRANSFORM INFRARED SPECTRUM (FT-IR) ANALYSIS

The molecular composition of the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was analyzed by FT-IR. Fig. 4.48 and 4.49 represents the FT-IR spectra of the surfactant samples of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*. The characteristic band at 3275.1 cm^{-1}/3280.1 cm^{-1} indicates the presence of -OH bonds. Absorption around 2924.3 cm^{-1}/2928.1 cm^{-1} is assigned to the symmetric stretch (-CH) of $-CH_2$ and $-CH_3$ groups of aliphatic chains. The absorption peak located at 1622.6 cm^{-1}/1625.1 cm^{-1} indicates the presence of ester carbonyl groups (-C=O bond in -COOH). Protein-related weak band at 1524.6 cm^{-1}/ 1529.7 cm^{-1} revealed the presence of NH/-C=O amide bond. The presence of band at 1452.7 cm^{-1} revealed the presence of aromatic compound in the FT-IR spectrum of *Pseudomonas sp.* 2B. The ester carbonyl group was also proved by the presence of the band at 1237.7 cm^{-1} which corresponds to -C=O deformation vibrations. The absorption peak around 1056.3 cm^{-1}/ 1059.1 cm^{-1} can be related to the presence of polysaccharide or polysaccharide-like substances in the surfactant. Similar interpretations with respect to wave numbers have been reported by Tahzibi et al. (2004) and Rodrigues et al. (2006). Based on information with respect to wave numbers in the literature, it can be suggested that the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, might be glycolipoproteins.

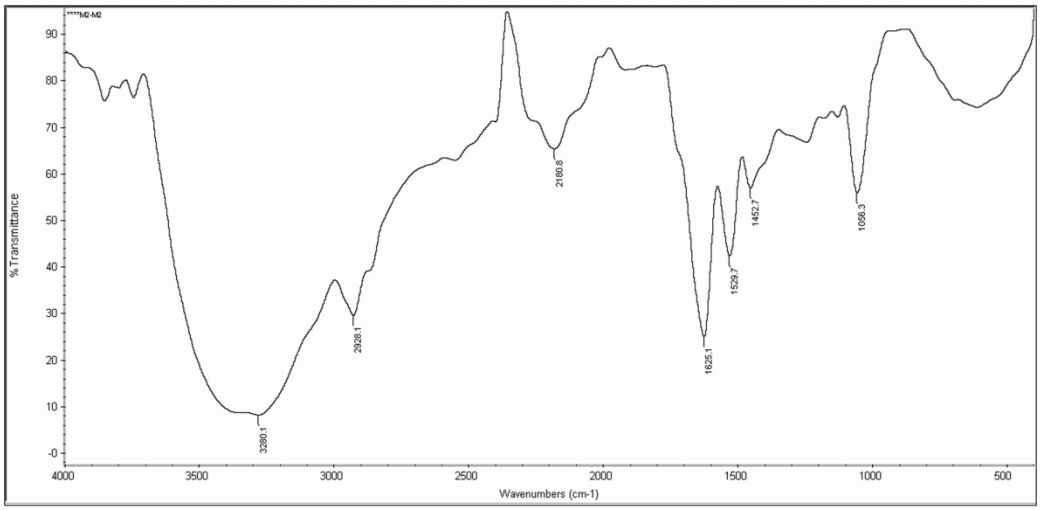


Fig. 4.48 FT-IR spectrum of the surfactant produced by *Pseudomonas sp.* 2B

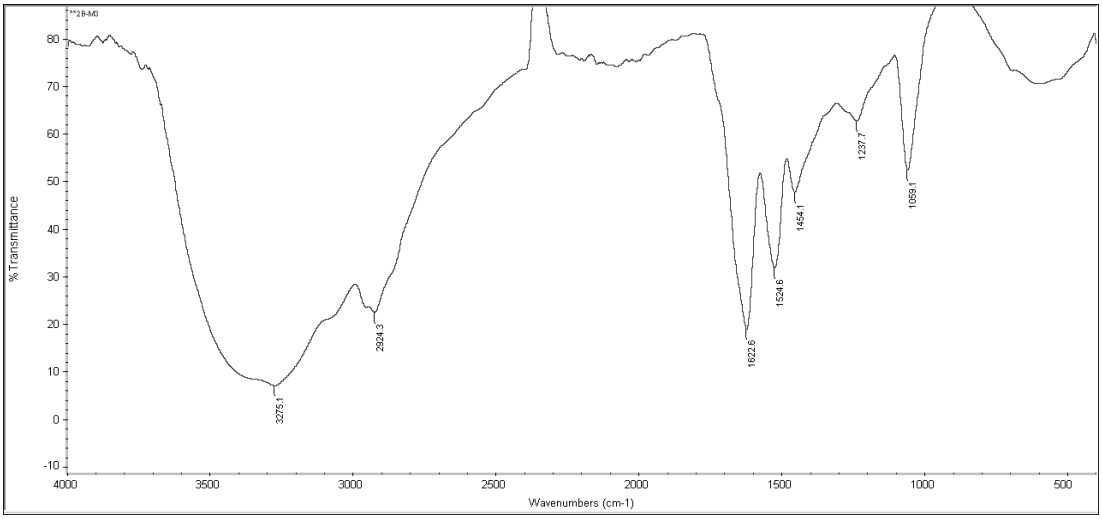


Fig. 4.49 FT-IR spectrum of the surfactant produced by *Pseudomonas aeruginosa*

SUMMARY

The FT-IR analysis of the surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* revealed that the surfactants consisted of different functional groups as indicated by the different wave numbers. The functional groups indicated the presence of lipids, carbohydrates and proteins in the surfactant produced by the bacterial strains which indicates that the surfactant produced may be of glycolipoprotein type.

4.11.4 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRIC (LC-MS) ANALYSIS OF THE SURFACTANT

LC-MS analysis of the surfactants was carried out to characterize the surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively. The mass spectrum of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* surfactants showed a mixture of glycolipoproteins with a molecular weight ranging from 333 and 678 with intense molecular ions at m/z 122, 333, 479, 504, 505, 532, 650 and 678 (Fig. 4.50 and 4.51). The mass spectrometric analysis of the surfactant confirmed the above results with peaks observed at m/z = 333, 479, 504, 505 for lipids, at m/z= 122 for proteins and at 650 and 678 for carbohydrate moieties based on the interpretations made by Deziel et al. (1999) and Rahman et al. (2002).

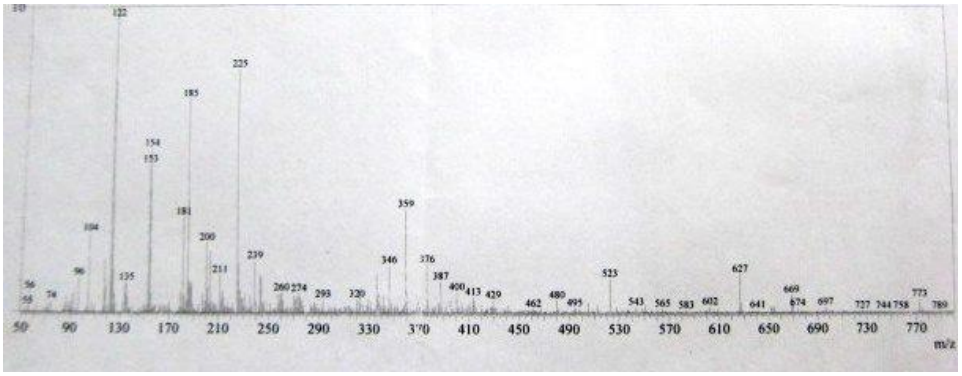


Fig. 4.50 Mass spectrum of surfactant produced by *Pseudomonas sp.* 2B

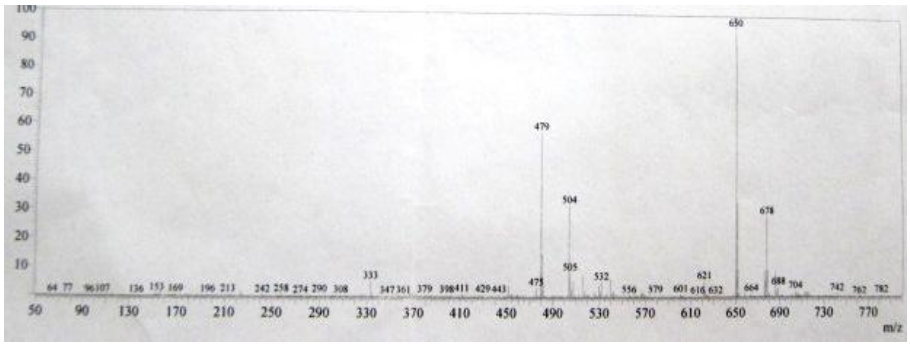


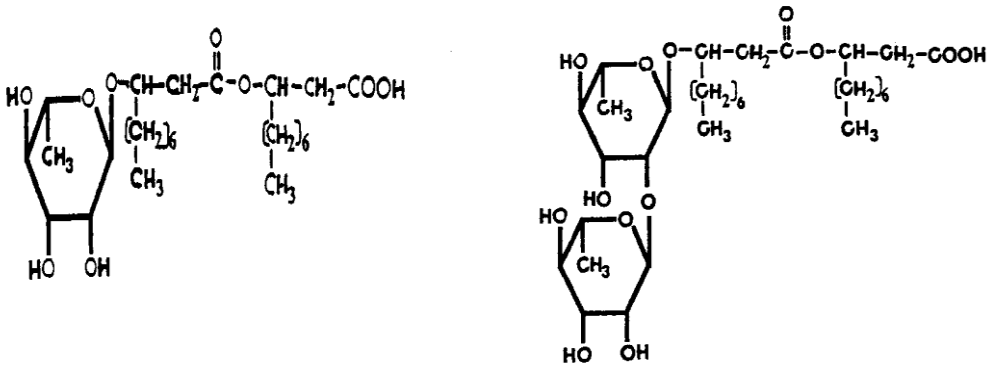
Fig. 4.51 Mass spectrum of surfactant produced by *Pseudomonas aeruginosa*

The m/z values obtained were consistent with the molecular structure of rhamnolipids, *i.e.,* Rha-C_{10}, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12} and Rha-Rha-C_{10}-C_{10}, respectively. Nine rhamnolipid homologues were identified in the present study (Table 4.18). The results presented in Table 4.18 showed the presence of a relatively higher abundance of dirhamnolipid (L-rhamnopyranosyl-L-rhamnopyranosyl-3-hydroxydecanoyl-3 hydroxydecanoate) [Fig. 4.52 (b)] than monorhamnolipid (L-rhamnopyranosyl -3-hydroxydecanoyl-3-hydroxydecanoate) [Fig. 4.52 (a)]. Liu et al. (2011) reported production of dirhamnolipid in abundance by *Pseudomonas sp.* As reported by Déziel et al. (1999), peaks at 333.0 m/z (Rha-C_{10}) and 479.0 m/z (Rha-Rha-C_{10}) indicated fragments produced by cleavage of rhamnolipid molecules.

It has been reported in the literature that the rhamnolipid composition and predominance of a type of congener depends on various factors like type of carbon substrate (Deziel et al. 1999; Rahman et al. 2002), age of the culture (Costa et al. 2006), culture conditions (Hansen et al. 2008) and the strain (Rodrigues et al. 2006). Based on the results obtained, the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, were characterized as rhamnolipoproteins.

Table 4.19 Chemical compositions of rhamnolipid mixture produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* cultures determined by MS analysis

Rhamnolipid congeners	Pseudomolecular ion (m/z)
Rha-Rha-C_8-C_{10}	621.0
Rha-Rha-C_{10}-C_{10}	650.0
Rha-Rha-C_{10}-C_{12}	678.0
Rha-C_{10}-C_{10}	505.0
Rha-C_{10}-C_{12}	532.0
Rha-C_{10}	333.0
Rha-C_{10}-C_{10}	504.0
Rha-Rha-C_{12}-C_{10}	678.0
Rha-Rha-C_{10}	479.0



(a) **(b)**

Fig. 4.52 Structure of a) monorhamnolipid b) dirhamnolipid

SUMMARY

The results of the present study confirmed that the surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, were glycolipoproteins as indicated by the presence of different molecular weight ions. Further, the m/z values obtained were consistent with the molecular structure of rhamnolipoproteins. Hence, the glycolipoproteins were further characterized as rhamnolipoproteins.

4.12 RECONSTITUTIONAL STUDIES OF THE PARTIALLY PURIFIED SURFACTANT

Reconstitutional study of partially purified surfactant of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, was carried out to understand the role of individual moieties of surfactant. Following the removal of the moieties, the surface tension of the other surfactant fractions was recorded. When the protein fraction was removed from the surfactant of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, using 60% ammonium sulphate, the surface tension values were found to be 49.23 mN/m and 51.89 mN/m, respectively against the control samples (containing all the three surfactant fractions) which showed surface tension of 22.09 and 25.67 mN/m, respectively. The increase in the surface tension values was probably observed due to the loss of proteins. This observation is in accordance to the report of Jagtap et al. (2010), they observed that there was decrease in the emulsification activity (273.5 EU/ml) following the removal of protein moiety from the surfactant of *Acinetobacter* species compared to control (400 EU/ml). Sar and Rosenberg (1983) demonstrated that the protein content of the surfactant plays an important role in providing stability to the surfactant. When the carbohydrate moieties were removed from the surfactant of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, using phenol treatment, the surface tension values were found to be 51.62 mN/m and 57.21 mN/m, respectively. The increase in surface tension values was probably due to the instability of the surfactant

due to the loss of the sugar moieties as these are important components of the surfactant structures. Similar observation was reported by Jagtap et al. (2010), they observed that there was decrease in the emulsification activity from 400 EU/ml to 280 EU/ml following the removal of sugar moiety of the surfactant. The incubation of surfactant produced *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, with steapsin (lipase) resulted in appreciable loss in surface activity. The surface tension values increased to 63.29 mN/m 65.15 mN/m, respectively. This result can be supported by the fact that lipids constitute a large percentage of the surfactant and therefore, regulate the surfactant activity in a significant manner (Sekhon et al. 2011). The reconstitutional study revealed that all the fractions of the partially purified surfactant produced by the bacterial cultures, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, when present together displayed maximum reduction in the surface tension.

SUMMARY

The present study emphasizes the role of different surfactant moieties in the maintenance of the stability of surfactant and surface activity. The intact surfactant of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, displayed maximum reduction in the surface tension rather than the individual surfactant components.

4.13 STUDIES ON THE EFFECT OF ENVIRONMENTAL FACTORS ON SURFACTANT STABILITY

The application of surfactant in various fields depends on the environmental conditions. Therefore, their stability of surfactant becomes important over a wide range of environmental conditions. In this regard, the stability of the surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was studied at different temperature, salinities and over a wide range of pH was measured in terms of surface tension. The study was carried out for both the cell-free supernatant containing surfactant

as well as partially purified surfactant. The surface tension values of cell-free supernatant as well as the partially purified surfactant were measured in order to determine the stability.

4.13.1 EFFECT OF pH ON SURFACTANT STABILITY

Fig. 4.53 presents the results of effect of pH on the stability of surfactant produced by *Pseudomonas sp.* 2B. The surface tension values of the cell-free broth containing the surfactant and partially purified surfactant of *Pseudomonas sp.* 2B varied from 29.39-36.24 mN/m and 27.74-35.38 mN/m, respectively, over a pH range of 2 to 12. When the pH was in the acidic range, *i.e.*, 2, 4 and 6; the surface tension values of the cell-free broth containing surfactant were 35.19, 33.48 and 31.39 mN/m, respectively. At pH 7, the surface tension value of the cell-free broth reduced to 29.39 mN/m which was the lowest among the values obtained in the study. With increase in pH from 8 to 12 (alkaline pH), the surface tension of the cell-free broth also increased from 30.14-36.24 mN/m, indicating that the alkaline conditions affected the surface tension values of the surfactant. The partially purified surfactant showed surface tension values of 34.87, 32.61 and 30.87 mN/m at pH 2, 4 and 6. The surface tension value was the lowest (27.74 mN/m) at pH 7. In the alkaline pH (8-12) range, the surface tension values ranged from 28.91-35.38 mN/m. It has been reported in the literature that at acidic and alkaline pH, the surface tension was high, indicating that the surface activity was affected. This may be because at lower or higher pH, the structure of surfactant was modified which in turn affected the surface tension. The results of the present study are in agreement with Eduardo et al. (2010), who reported that at pH 7, maximum reduction in the surface tension (41.8 mN/m) was attained in the cell-free broth containing surfactant produced by *Lactobacillus paracasei.*

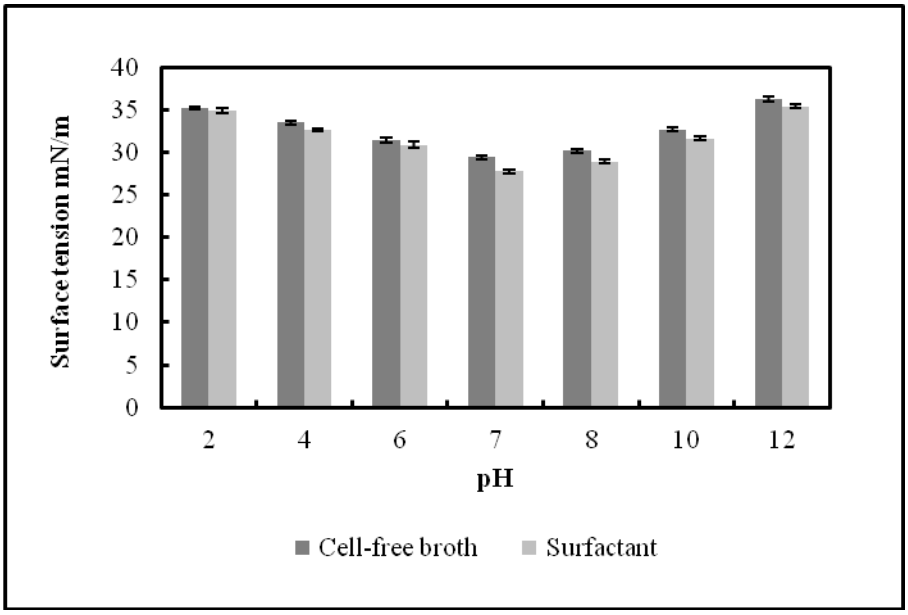


Fig. 4.53 Effect of pH on the stability of surfactant produced by *Pseudomonas sp.* 2B. Results are represented as Mean ± SEM (n=3).

Fig. 4.54 presents the results of effect of pH on the stability of surfactant produced by *Pseudomonas aeruginosa.* In the case of *Pseudomonas aeruginosa*, the surface tension values of the cell-free broth and surfactant varied from 30.71-37.37 mN/m and 28.41-36.59 mN/m, respectively, over a pH range of 2 to 12. When the pH of the cell-free broth was set to 2, 4 and 6, the surface tension values were 36.34, 34.79 and 32.88 mN/m, respectively. At pH 7, the surface tension value reduced to 30.71 mN/m which was the lowest among the values recorded in the study. With increase in pH from 8 to 12, the surface tension also increased from 32.20-37.37 mN/m, indicating that the alkaline conditions affected the surface tension value. The partially purified surfactant showed surface tension values of 35.29, 33.98, 31.76 and 29.13 mN/m at pH 2, 4, 6 and 7. The surface tension value was the lowest (28.41 mN/m) at pH 8. At pH 10 and 12, the surface tension values were 32.87 and 36.59 mN/m, respectively. Similar observation was reported by Lotfabad et al. (2009), at pH 8, the surface tension of the cell-free broth containing surfactant produced by *Pseudomonas aeruginosa* MR01 was reduced; surface

tension value of 27 mN/m was recorded. They also reported that at pH 2, the surface tension value was 35 mN/m whereas at pH 12, the surface tension was found to be 30 mN/m. They further reported that the surface tension remained relatively stable between pH 8-12.

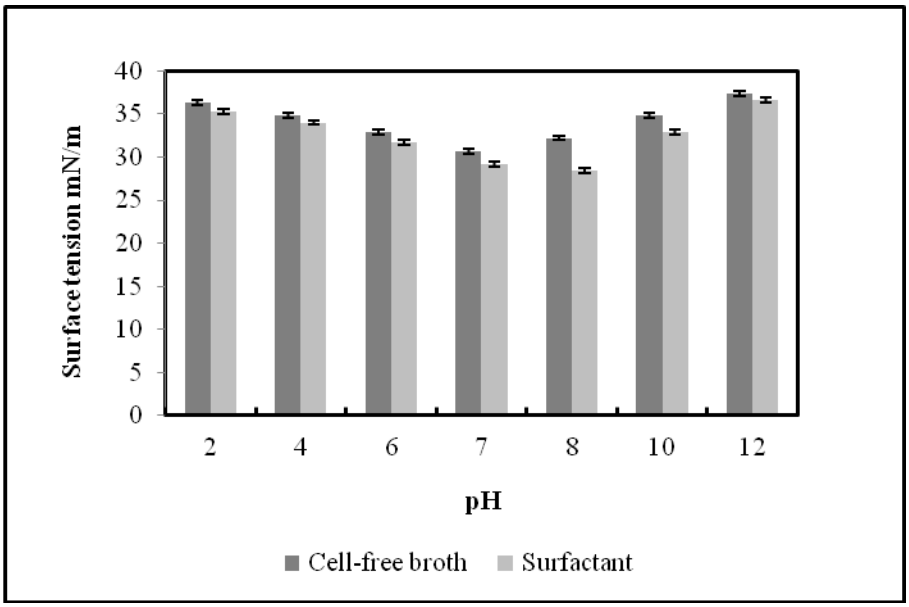


Fig. 4.54 Effect of pH on the stability of surfactant produced by *Pseudomonas aeruginosa.* Results are represented as Mean ± SEM (n=3).

SUMMARY

In the present study, it was observed that the pH of the environment had effect on the surface tension of the cell-free broth containing extracellular surfactant as well as partially purified surfactant produced by the strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively. In the case of *Pseudomonas sp.* 2B, the cell-free broth and partially purified showed reduced surface tension value at pH 7 whereas in the case of *Pseudomonas aeruginosa,* at pH 8, the lowest surface tension values were recorded for the cell-free broth containing surfactant as well as partially purified surfactant.

4.13.2 EFFECT OF TEMPERATURE ON SURFACTANT STABILITY

Fig. 4.55 presents the results of effect of temperature on the stability of surfactant produced by *Pseudomonas sp.* 2B. The stability of the cell-free broth containing surfactant and partially purified surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* was tested over a wide range of temperature (4–121ºC). It was found that the surface tension of the cell-free broth and partially purified surfactant increased when stored at 4ºC; the corresponding surface tension values were 36.56 mN/m and 37.87 mN/m, respectively. When the cell-free broth and partially purified surfactant were stored at 30ºC, there was decrease in surface tension values; 29.17 mN/m and 27.59 mN/m, respectively. With further increase in temperature, *i.e.,* 50 and 75ºC, the surface tension values increased; the surface tension values of cell-free broth containing surfactant and partially purified surfactant ranged from 32.86-35.79 mN/m. Heating of the cell-free supernatant and partially purified surfactant to 100ºC showed surface tension values of 35.89 mN/m and 36.42 mN/m, respectively. Autoclaving the surfactants at 121ºC, showed surface tension values of 36.53 mN/m and 37.09 mN/m, respectively which indicates that the surface tension value was not affected significantly at higher temperature. Though there was not substantial difference in surface tension values at low or high temperature, the surface tension values of the cell-free broth and surfactant showed increased stability. Such extreme thermal stability was reported by Nie et al. (2010) for the *Pseudomonas aeruginosa* strain NY3, the surface tension of the surfactant ranged from 35-36 mN/m at 100 and 121ºC. This suggests that the surfactant was thermally stable over a range of temperature in the present study; it further increased the scope of application in a broader perspective including at conditions where high temperatures prevail as in the microbial enhanced oil recovery (MEOR) processes.

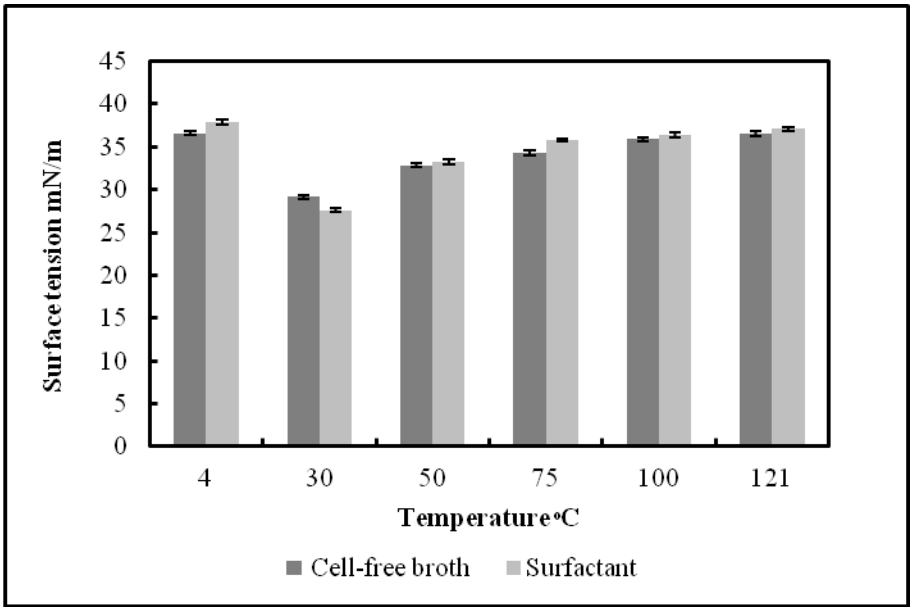


Fig. 4.55 Effect of temperature on the stability of surfactant produced by *Pseudomonas sp.* 2B. Results are represented as Mean ± SEM (n=3).

Fig. 4.56 presents the results of effect of temperature on the stability of surfactant produced by *Pseudomonas aeruginosa*. The surface tension of the cell-free broth and partially purified surfactant was 33.19 mN and 32.55 mN/m, respectively, when stored at 4ºC. At 30ºC, the surface tension was the lowest; the surface tension of the cell-free broth and partially purified surfactant was 30.23 mN/m and 29.41 mN/m, respectively. The surface tension values of cell-free broth and partially purified surfactant at 50 and 75ºC ranged from 32.51-34.70 mN/m. At 100ºC, the cell-free broth and partially purified surfactant showed surface tension values of 36.14 mN/m and 35.03 mN/m, respectively. When the cell-free broth and partially purified surfactant were heated at 121ºC, the surface tension values were 38.39 mN/m and 37.89 mN/m, respectively. Similar observation was reported by Abouseoud et al. (2008), the surface tension value of the surfactant ranged from 35-37 mN/m with increase in temperature from 50-120ºC. The present study might find usefulness of surfactant in food, pharmaceutical and cosmetics industries where heating to achieve sterility is of paramount importance.

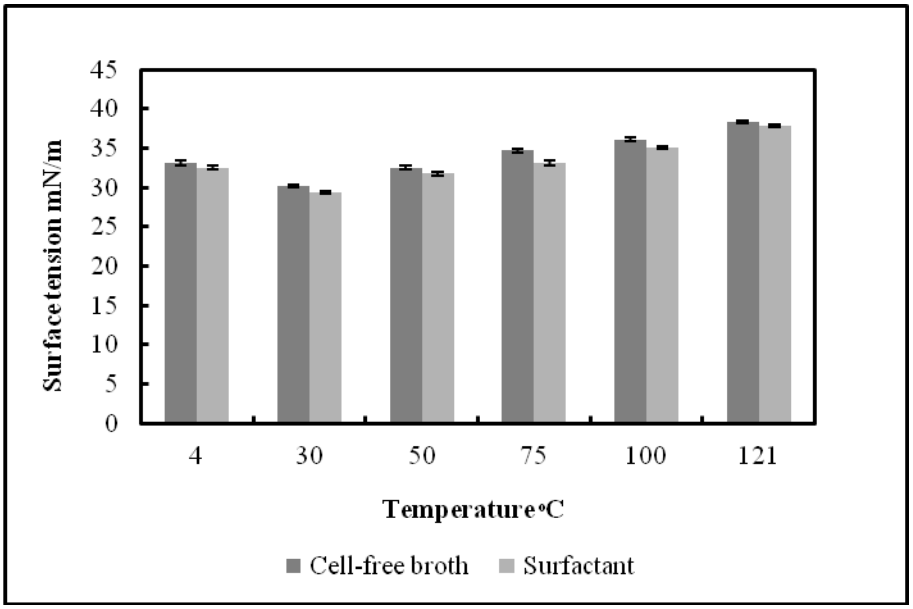


Fig. 4.56 Effect of temperature on the stability of surfactant produced by *Pseudomonas aeruginosa.* Results are represented as Mean ± SEM (n=3).

SUMMARY

The results obtained from temperature stability analysis of cell-free broth and surfactant of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* reveals that the surfactant was thermostable. Irrespective of the operating temperature, the surface tension ranged from 27.59-37.09 mN/m for *Pseudomonas sp.* 2B whereas for *Pseudomonas aeruginosa*, surface tension values ranged from 29.41-38.39 mN/m.

4.13.3 EFFECT OF SALINITY ON SURFACTANT STABILITY

The effect of salinity on the surface tension of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa* was evaluated as it is necessary to develop potential surfactants for their application in bioremediation of contaminated marine environments, especially during oil spills. In the present study, the sodium chloride concentration was varied from 0-12% (w/v).

Fig. 4.57 depicts the effect of addition of sodium chloride on the surface tension of *Pseudomonas sp.* 2B surfactant. The surface tension of the cell-free broth and partially purified surfactant at 0% (w/v) NaCl concentration was 29.03 mN/m and 27.18 mN/m, respectively. With increase in NaCl concentration from 2-10% (w/v), there was slow increase in the surface tension values of the cell-free broth and partially purified surfactant; the values ranged from 29.71-35.88 mN/m for the cell-free broth and 27.89-33.78 mN/m for partially purified surfactant. However, at the highest concentration of NaCl [12% (w/v)], the surface tension of the cell-free broth (38.04 mN/m) and partially purified surfactant (36.66 mN/m) increased as well. The results are in agreement with the findings of Nie et al. (2010), the surface tension of the surfactant at 12% NaCl concentration was found to be 36.60 mN/m. Helvaci et al. (2004) stated that electrolytes directly affect the carboxylate groups of the surfactants. The solution/air interface has a net negative charge due to the ionized carboxylic acid groups at alkaline pH with strong repulsive electrostatic forces between the surfactant molecules. This negative charge is shielded by the Na^+ ions in the electrical double layer in the presence of NaCl, causing the formation of a close-packed monolayer and consequently a decrease in surface tension values.

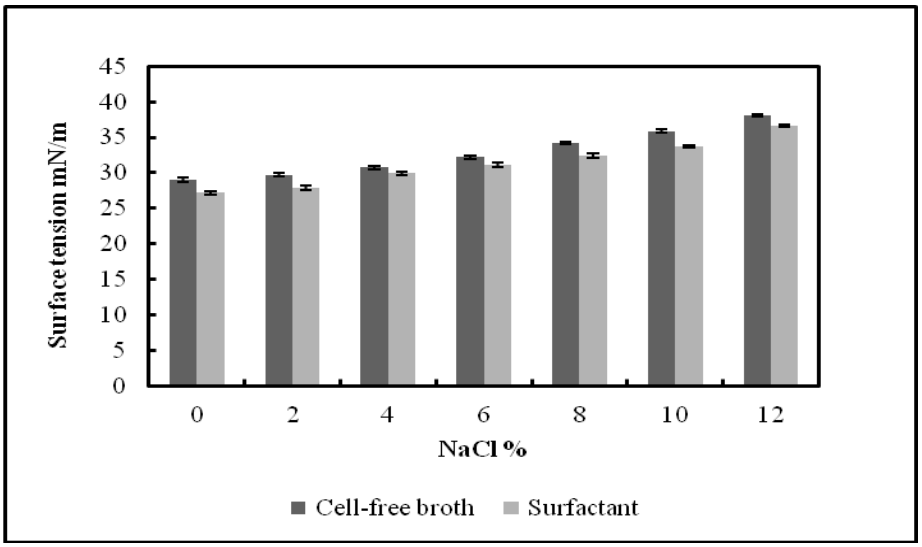


Fig. 4.57 Effect of salinity on the stability of surfactant produced by *Pseudomonas sp.* 2B. Results are represented as Mean ± SEM (n=3).

Fig. 4.58 presents the results of effect of salinity on the stability of surfactant produced by *Pseudomonas aeruginosa.* The surface tension value of cell-free broth and partially purified surfactant at 0% (w/v) NaCl concentration was 30.29 mN/m and 29.48 mN/m, respectively. At 2% (w/v) NaCl concentration, the maximum decrease in surface tension was observed, the cell-free broth showed surface tension value of 31.01 mN/m whereas the partially surfactant showed surface tension value of 29.99 mN/m. With an increase in the NaCl concentration from 4-10% (w/v), the surface tension of cell-free broth and partially purified surfactant varied from 31.73-36.51 mN/m and 30.79-37.67 mN/m, respectively. At 12% (w/v) NaCl concentration, the surface tension of cell-free broth and partially purified surfactant were 39.13 mN/m and 38.70 mN/m, respectively. The results are in accordance with Anyanwu et al. (2011), the stability of surfactant produced by *Serratia marcescens* NSK-1 was tested in a range of 0-20% (w/v), it was observed that at 4% (w/v) NaCl concentration, the surface tension was 38.80 mN/m. At 12% (w/v) NaCl concentrations, the surface tension increased to 40.80 mN/m; the surface tension at 20% (w/v) NaCl concentration was found to be 43.20 mN/m.

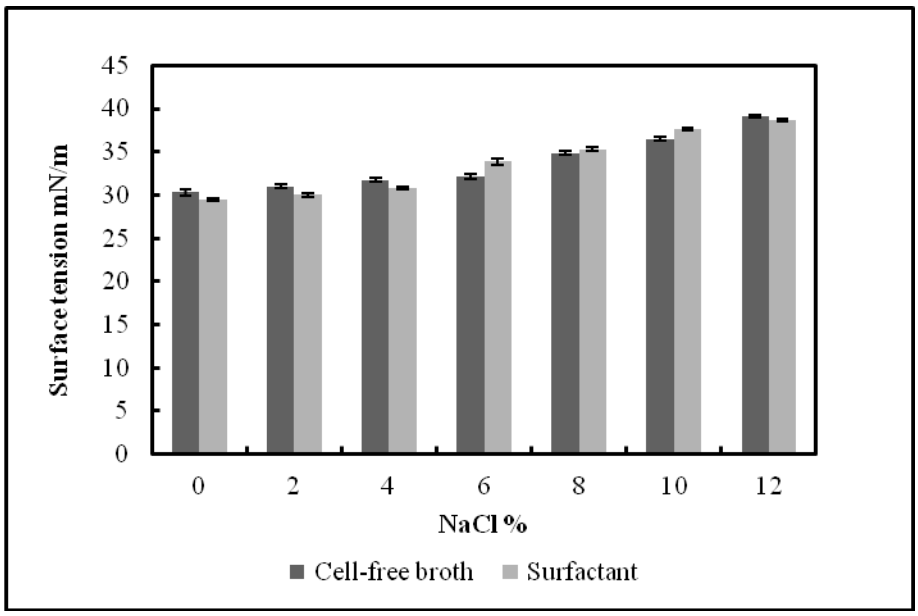


Fig. 4.58 Effect of salinity on the stability of surfactant produced by *Pseudomonas aeruginosa*. Results are represented as Mean ± SEM (n=3).

SUMMARY

The results of the present study suggest that both the cell-free broth as well as the partially purified surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, was stable in saline environmental condition. The cell-free broth of *Pseudomonas sp.* 2B showed surface tension ranging from 29.03-38.04 mN/m whereas that of partially purified surfactant ranged from 27.18 mN/m-36.66 mN/m when sodium chloride concentration was varied from 0-12% (w/v). In the case of *Pseudomonas aeruginosa*, the cell-free broth of *Pseudomonas aeruginosa* showed surface tension ranging from 30.29-39.13 mN/m and the surface tension of partially purified surfactant ranged from 29.48 mN/m-38.70 mN/m. In the range of the experiments studied, there was not significant change in the surface tension values of the cell-free broth containing the surfactant and the partially purified surfactants.

The present findings suggest that the surfactants produced by the bacterial strains *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, has potential application over a wide range of temperature, pH and saline environment. The study also suggests that the cell-free broth can be directly applied without any purification step since the surface tension of the cell-free broth did not vary significantly from those of the partially purified surfactant produced when subjected to different environmental conditions.

4.14 STUDIES ON THE SHELF-LIFE OF SURFACTANT

In order to study the shelf-life of the partially purified surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, the surfactants were stored at 30°C for 6 months. The surface tension measurement was carried out at different time intervals to check the performance of the surfactant.

Fig. 4.59 presents the results of the studies of shelf-life of the partially purified surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. It was observed that the initial surface tension value of the surfactant produced by *Pseudomonas sp.* 2B was 22.08 mN/m. With increase in time, the surface tension values of the surfactant did not show significant change in surface tension values. At the end of 1, 2 and 3 months, the surface tension values were found to be 22.04 mN/m, 22.02 mN/m and 22.00 mN/m, respectively. The surface tension values observed at the end of 4, 5 and 6 months were 21.99 mN/m, 21.95 mN/m and 21.91mN/m, respectively. In the case of the partially purified surfactant produced by *Pseudomonas aeruginosa*, there was a slight increase in the surface tension value after a period of 3 months. The initial surface tension value of the surfactant produced by *Pseudomonas aeruginosa* was 25.59 mN/m. The surface tension values at the end of 1, 2 and 3 months were found to be 25.56 mN/m, 25.51 mN/m and 25.48 mN/m, respectively. At the end of 4, 5 and 6 months, the surface

tension values were 25.56 mN/m, 25.63 mN/m and 25.69 mN/m, respectively. The results suggest that the surface tension values of the surfactant produced by both the bacterial strains did not change significantly when stored for a period of 6 months. There are no reports on the studies on shelf-life of surfactant in the literature.

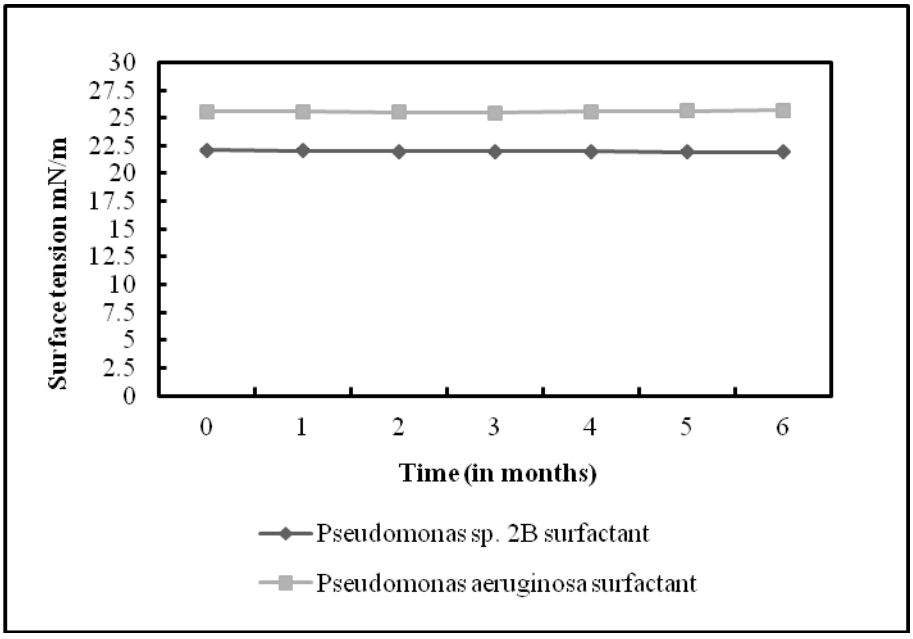


Fig. 4.59 Studies on shelf-life of surfactant produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively. Results are represented as Mean ± SEM (n=3).

SUMMARY

The present work suggests that the surfactant produced by both the bacterial strains did not significantly affect the surface tension values when stored for a period of 6 months. However, further studies have to be carried out for a longer duration of time in different conditions to assess the performance of the surfactant.

4.15 SURFACTANT ENHANCED BIODEGRADATION OF CRUDE OIL

Reports in the literature suggest that surfactants enhance the dispersion and biodegradation of hydrocarbons (Zhang and Miller 1992; Noordman and Janssen 2002; Iwabuchi et al. 2002). In this regard, the efficiency of the surfactant produced by the *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, in the biodegradation of crude oil by *Nocardia hydrocarboxydans* NCIM 2386 was studied. In a report by Kalme et al. (2008), the petroleum hydrocarbon biodegradation potential of *Nocardia hydrocarboxydans* NCIM 2386 has been described. Hence, this bacterial strain was chosen for the present study. 100 mg of surfactant was added to 50 ml of 1% crude oil (corresponding to 800 mg of crude oil/50 ml of media) containing media and biodegradation was assessed at regular intervals. A control flask (without surfactant) was also maintained. The crude oil degradation rate was assessed by estimating the residual crude oil concentration by UV spectrophotometer method.

Fig. 4.60 presents the biodegradation potential of crude oil in the control flask. It was observed that the biodegradation of crude oil by *Nocardia hydrocarboxydans* NCIM 2386 increased slowly in course of time in the control flask. On the 7^{th} and 14^{th} day, 11.23% and 26.63% of crude oil was degraded by the strain. The corresponding biomass values were 3.14 g/L and 5.09 g/L, respectively. An increase in the biomass value was observed in course of time which indicated the microorganism utilized crude oil and degraded it. There was decrease in the residual crude oil concentration; the residual crude oil concentration on the 7^{th} and 14^{th} day was 712 mg and 667 mg/50 ml of media, respectively. On the 21^{st} day and 28^{th} day of incubation, the biodegradation percentage was 34.88% and 65.25%, respectively; the corresponding biomass concentration was 5.11 g/L and 4.88 g/L, respectively. The residual crude oil concentrations were 521 mg and 278 mg/50 ml of media the 21^{st} day and 28^{th} day. The biodegradation rate increased to 68.8% and 70.5% on the 35^{th} and 42^{nd} day of incubation, the corresponding biomass

concentration was 4.70 g/L and 4.38 g/L, respectively. On the 35th and 42nd day, the residual crude oil concentrations were 249 mg and 236 mg/50 ml of media. In a similar work carried out by Kalme et al. (2008), *Nocardia hydrocarboxydans* NCIM 2386 degraded 98% of 10% diesel in 42 days in the absence of surfactant.

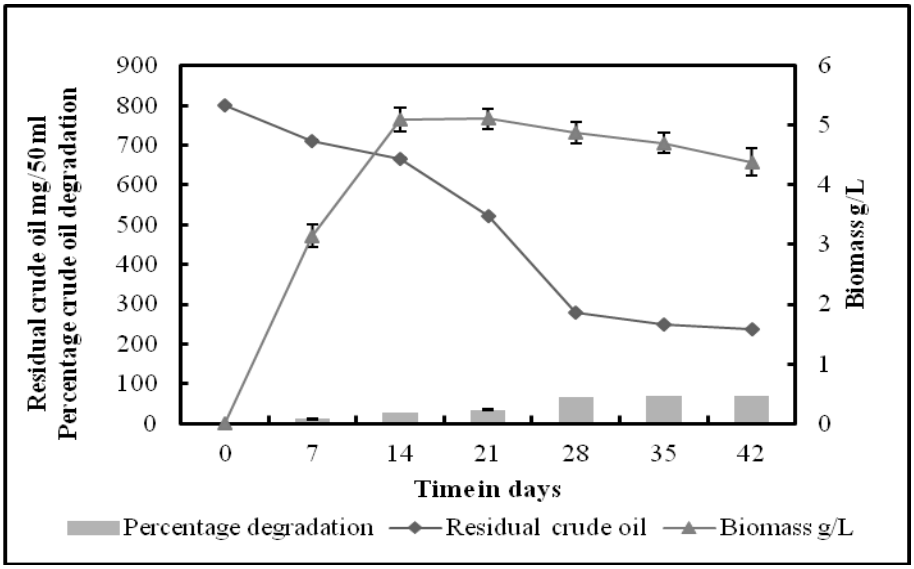


Fig. 4.60 Biodegradation of crude oil by *Nocardia hydrocarboxydans* NCIM 2386 in the absence of surfactant. The culture flasks were incubated at 30^{o}C in incubator shaker at 150 rpm. Results are represented as Mean ± SEM (n=3)

As seen in Fig. 4.61, it is evident that surfactant produced by *Pseudomonas sp.* 2B enhanced the biodegradation of crude oil compared to the control. 34.88 % and 59.25% of crude oil degradation was observed on day 7 and 14, respectively. The corresponding biomass concentration was 6.03 g/L and 7.09 g/L, respectively. The residual crude oil decreased to from 800 mg to 521 mg and 374 mg/50 ml of media, respectively on day 7 and 14. Increase in biomass suggests that the bacterial strain was able to grow in the presence of crude oil and degrade it in the presence of surfactant. On day 21 and 28, the bacterial strain degraded 76.50% and 93.87%, respectively. The residual crude oil

decreased to 188 mg and 49 mg/50 ml of media, respectively. On the 35th and 42nd day of incubation, the biodegradation of crude oil was 94.87% and 95.5%, respectively. The corresponding biomass values were 6.19 g/L and 6.03 g/L, respectively. The residual crude oil values obtained on the 35th and 42nd day were 41 mg and 36 mg/50 ml of media. Compared to the control flask, the biodegradation rate of crude oil was higher in the surfactant containing medium. Higher removal percentage observed in the presence of surfactant was attributed to the interaction of surfactant-water and surfactant-oil (such as interfacial tension reduction), which dominates the interaction of oil-water. Further, the percentage removal was attributed to the reduction of surface and interfacial tensions of surfactant containing solutions; this increases the mobility of oil and consequently, enhances the biodegradation of oil (Haba et al. 2000).

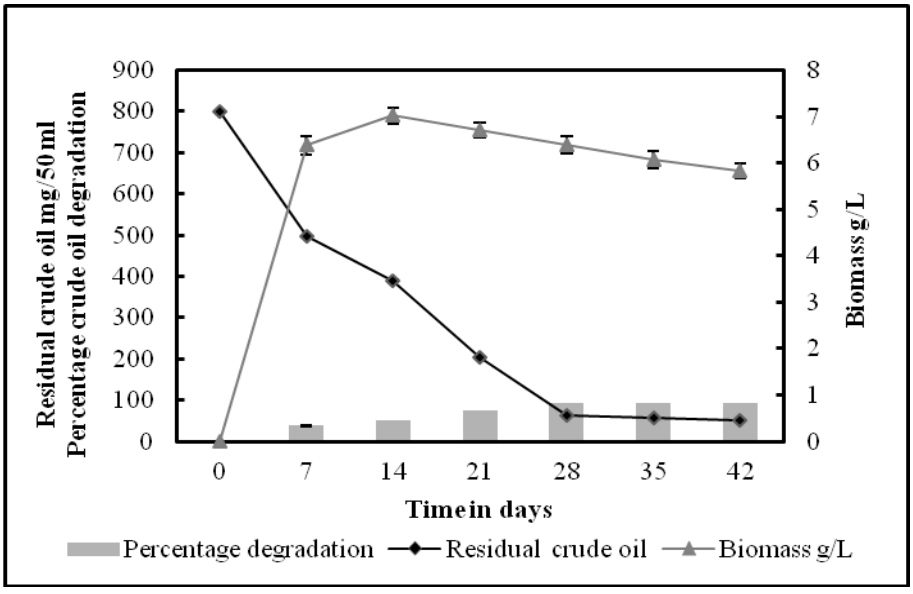


Fig. 4.61 Biodegradation of 1% (v/v) crude oil by *Nocardia hydrocarboxydans* NCIM 2386 in the presence surfactant produced by *Pseudomonas sp.* 2B. The culture flasks were incubated at 30^{o}C in incubator shaker at 150 rpm. Results are represented as Mean ± SEM (n=3)

Fig. 4.62 presents the crude oil biodegradation profile of *Nocardia hydrocarboxydans* in the presence of surfactant produced by *Pseudomonas aeruginosa.*

The surfactant produced by *Pseudomonas aeruginosa* showed 37.75% and 51.13% crude oil biodegradation on day 7 and 14; the corresponding biomass concentrations were 6.38 g/L and 7.02 g/L, respectively. The residual crude oil concentration on 7^{th} and 14^{th} day was 498 mg and 391 mg/50 ml of media, respectively, which indicated that the crude oil concentration decreased in the presence of surfactant. On the 21^{st} and 28^{th} day, the percentage degradation of crude oil was 74.50% and 92%, respectively, suggesting that the addition of surfactant enhanced crude oil degradation in course of time. The residual crude oil concentration reduced to 204 mg and 64 mg/50 ml of media, on the 21^{st} and 28^{th} day of incubation, respectively. On the 35^{th} and 42^{nd} day of incubation, the biodegradation of crude oil was 92.75% and 93.5%, respectively. The corresponding biomass values were 6.08 g/L and 5.84 g/L, respectively. The residual crude oil values obtained on the 35^{th} and 42^{nd} day were 58 mg and 52 mg/50 ml of media. Thus, it was observed in the present study that the biodegradation of crude oil was enhanced in the presence of surfactant. Celik et al. (2008) reported that in the presence of surfactant, *Pseudomonas fluorescens* G6, degraded 56 % of crude oil in 7 days. In a similar study by Darvishi et al. (2011), a consortium of bacteria degraded 50.8% of crude oil in the absence of surfactant; whereas in the presence of surfactant, the degradation percentage of crude oil was 76.3%.

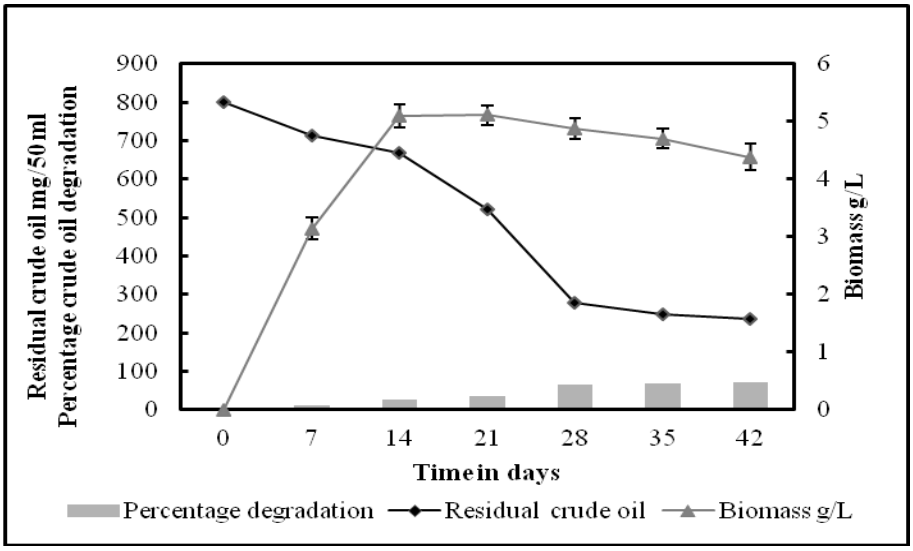


Fig. 4.62 Biodegradation of crude oil by *Nocardia hydrocarboxydans* NCIM 2386 in the presence of surfactant produced by *Pseudomonas aeruginosa*. Results are represented as Mean ± SEM (n=3)

SUMMARY

In the present study, the efficiency of surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, were tested in the biodegradation of crude oil by *Nocardia hydrocarboxydans* NCIM 2386. In the presence of surfactant produced by *Pseudomonas sp.* 2B, *Nocardia hydrocarboxydans* NCIM 2386 degraded 95.5% of crude oil by 42nd day of incubation whereas in the presence of surfactant produced by *Pseudomonas aeruginosa*, 93.5% of crude oil was degraded by *Nocardia hydrocarboxydans* NCIM 2386 by 42nd day. In the absence of surfactant (control), *Nocardia hydrocarboxydans* NCIM 2386 degraded 70.5% of crude oil on the 42nd day.

From the present observations, it can be observed that in the presence of surfactant, the percentage degradation of crude oil was enhanced compared to the control. This property of the obtained surfactants was highly encouraging at the present day concept of oil spills and microbial enhanced oil recovery operations.

CHAPTER 5

RESULTS AND DISCUSSION

The quest for a cheap source of energy coupled with the extensive rate of industrialization has expanded the frontiers of petroleum hydrocarbon exploration with its negative consequence being the contamination of the environment. Leaks and accidental spills occur regularly during exploration, production, refining, transport and storage of petroleum and petroleum products. Due to the serious and long-term damage caused to the ecosystems, terrestrial life, human health and natural resources; there is a need to remediate the petroleum hydrocarbon contaminated sites. Several remediation alternatives have been in use for the restoration of petroleum hydrocarbon contaminated systems. Remediation of petroleum contaminated sites could be achieved by either physicochemical or biological methods. Due to negative consequences of the physicochemical approach, more attention is now given to the exploitation of biological alternatives for the remediation of petroleum hydrocarbon contaminated sites. One of the widely accepted bioremediation methods of petroleum hydrocarbons is biodegradation. Although biodegradation of petroleum hydrocarbon contaminated environments is a promising alternative remedial strategy, the biodegradation rate of these hydrocarbons in the environment is limited by various factors. Traditionally, synthetic surfactants have been used to increase the rate of biodegradation of these hydrocarbons. Synthetic surfactants pose serious threats to the environment as they are synthesized using petroleum hydrocarbon derivatives which are non-biodegradable and toxic to the living organisms. An increase in the concern about environmental protection has recently caused the consideration of alternatives to synthetic surfactants.

In the recent past, surface active molecules of biological origin, referred to as biosurfactants, have gained considerable interest. Biosurfactants have several advantages over their synthetic counterparts, such as lower toxicity, higher biodegradability, better

environmental compatibility, higher selectivity and specificity at extreme temperature, pH and salinity. Moreover, they can be synthesized from renewable feedstock and industrial waste. They are required in small quantities for bringing about reduction in surface tension. These unique properties allow the use of biosurfactants and possible replacement of chemically synthesized surfactants in a great number of applications in various fields.

Although extensive research work has been carried out on the isolation and screening of surfactant producing microorganisms, medium formulation and production of surfactant, partial purification, characterization and application of surfactant, it was found that more number of candidate strains with high surfactant producing capability need to be isolated from potential ecological sources. It was also found that there is a need to optimize various conditions in order to increase surfactant productivity by studying the effect of various process parameters. Also, in the literature, reports on the utilization of biosurfactant in the biodegradation of petroleum hydrocarbons are too scanty. Hence, there is a need for the isolation, screening, and identification of candidate surfactant producing microorganisms. The effect of various process parameters leading to the maximum production of surfactant need to studied so as to bring about significant reduction in surface tension. The surfactant produced by the candidate microorganism can increase the surface area and thereby, reduce the gap between the petroleum hydrocarbon substrate and the microorganism. Therefore, the present study was taken to meet the stated objectives which included isolation, screening and identification studies of potential biosurfactant producer. The effect of various process variables influencing biosurfactant production was studied. Studies were carried out to partially purify and characterize the biosurfactant. In addition, a study was initiated to increase the biodegradation rate of crude oil using the biosurfactant.

In order to meet the stated objectives, soil and water samples were collected from various petroleum hydrocarbon contaminated sites. The microorganisms from various samples collected were isolated by enrichment technique. The soil and water samples

exposed to petroleum hydrocarbons showed greater number of microorganisms compared to the control samples. The isolated microorganisms were screened for their capacity to produce surfactant using various screening tests. Among the several isolates, a bacterial isolate designated as 2B, was identified as a candidate extracellular surfactant producer. The bacterial isolate, 2B, was identified as novel *Pseudomonas sp*. by microscopic, biochemical and molecular characterization. In the present study, we report the extracellular surfactant production by the novel *Pseudomonas sp.* 2B which was deposited in the GenBank database with an accession number JF683582. We have also compared the data of *Pseudomonas sp.* 2B with that of an already reported surfactant producer, *Pseudomonas aeruginosa* (ATCC 10145) based on references in the literature.

Among the different surfactant production media tested, the selection of appropriate surfactant production medium, *i.e.*, Proteose Peptone Glucose Ammonium Salts medium (containing glucose, peptone, Tris HCl, ammonium chloride, potassium chloride and magnesium sulphate) resulted in an increase in the production of surfactant concentration by both the bacterial strains. As a result, there was a significant decrease in the surface tension values.

Results of the effect of various process parameters (such as inoculum size, initial production medium pH, agitation speed, incubation temperature, type and concentration of carbon source, type and concentration of nitrogen source, inducer buffer and salinity) affecting surfactant production by the bacterial strains indicated that these process variables had shown significant effect on surfactant productivity as well as reduction in surface tension. *Pseudomonas sp.* 2B showed maximum surfactant production at 2% (v/v) inoculum size, initial pH 7, incubation temperature of 37^{o}C, agitation speed of 150 rpm, 30 g/L (w/v) glucose as carbon source, a combination of peptone and potassium nitrate as nitrogen source, olive oil as inducer, Tris HCl buffer and 1% (w/v) NaCl concentration. Maximum surfactant was produced by *Pseudomonas aeruginosa* at 3% (v/v) inoculum size, initial production medium pH 7, incubation temperature of 37^{o}C,

agitation speed of 150 rpm, 30 g/L (w/v) glucose as carbon source, a combination of yeast extract and ammonium chloride as nitrogen source, n-hexadecane as inducer, Tris HCl buffer and 0.5% (w/v) NaCl concentration. A maximum of 8.51 g/L of surfactant was produced by *Pseudomonas sp.* 2B, the corresponding surface tension value of the cell-free broth was 25.72 mN/m when the aforementioned process parameters were maintained. *Pseudomonas aeruginosa* produced a maximum of 7.40 g/L of surfactant when the aforementioned process parameters were maintained, the corresponding surface tension value of the cell-free broth was 27.41 mN/m.

Plackett-Burmann design (PBD) was used to find the effect of process variables affecting surfactant production by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively. Glucose as carbon source, olive oil as inducer and potassium nitrate as nitrogen source had significant effect on surfactant production by *Pseudomonas sp.* 2B. Surfactant production by *Pseudomonas aeruginosa* was mainly influenced by glucose as carbon source, n-hexadecane as inducer and ammonium chloride as nitrogen source. Further, Response Surface Methodology (RSM) was used in order to determine the optimal concentrations of the significant process variables leading to maximum surfactant production by the bacterial strains. The optimum medium consisted of 35.7645 g/L of glucose, 3.5% of olive oil and 5.5425 g/L of potassium nitrate for the production of surfactant by *Pseudomonas sp.* 2B whereas for the production of surfactant by *Pseudomonas aeruginosa*, the optimal medium consisted of 35.7645 g/L of glucose, 3.5% of n-hexadecane and 5.6274 g/L of ammonium chloride. Using this statistical design, a maximum of 14.63 g/L and 10.69 g/L of biosurfactant was produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, at optimal concentrations of the medium components.

The time course study experiments indicated that the surfactant produced by the bacterial strains were growth-associated, the study also revealed the surfactants produced might be “primary metabolites” as the production of surfactants coincided with the

exponential growth phase of the bacterial strains. Intrinsic kinetic parameters such as $P_{max,}$ $X_{max,}$ $\mu_{max,}$ μ, $Y_{X/S,}$ $Y_{P/S}$, $Y_{P/X}$ and Ks values were found to be 14.62 g/L, 6.81 g/L, 0.0288/hour, 0.0167/hour, 0.194 g/g, 0.4172 g/g, 2.143 g/g and 0.43 g/L, respectively, for *Pseudomonas sp.* 2B whereas for *Pseudomonas aeruginosa,* the values were 10.67 g/L, 6.19 g/L, 0.023/hour, 0.014/hour, 0.173 g/g, 0.306 g/g, 1.764 g/g and 0.59 g/L, respectively.

The surfactants produced by the bacterial strains were subjected to partial purification. Since the screening tests indicated the extracellular nature of surfactant, the surfactant produced by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, was extracted from the cell-free broth using acidification (to pH 2) followed by solvent extraction using chloroform: methanol mixture in 2:1 ratio. The column chromatography experiment revealed that the surfactants produced by both the bacterial strains were composed of different biomolecule moieties. Therefore, the surfactants produced by both the bacterial strains were subjected to characterization studies. Results of the characterization studies of surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, indicated that the surfactants might be rhamnolipoproteins.

The surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, showed the ability to enhance the biodegradation of crude oil. The present study achieved 95.5% and 93.5% biodegradation of crude oil in the presence of surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, over a span of 42 days. In the control flask, 70.5% of crude oil was degraded by the bacterium.

In the range of experimental investigations carried out under this study, the following conclusions were drawn:

1. The isolation of microorganisms from soil and water samples collected from different ecological sources exposed to petroleum hydrocarbon contaminants was successfully achieved using enrichment technique.

2. The isolation of microorganisms from various soil and water samples previously exposed to petroleum hydrocarbon contaminants resulted in the isolation of greater number of microorganisms compared to control samples which eventually proved to bring about isolation of a candidate surfactant producer, designated as 2B.

3. The screening tests such as selective Cetyl Trimethyl Ammonium Bromide (CTAB)-methylene blue agar medium, drop collapse assay and surface tension measurement were employed for the selection of candidate surfactant producers. This study proved the efficiency of the screening tests that resulted in successful isolation of the candidate surfactant producing bacterial isolate.

4. The candidate bacterial isolate, 2B, was characterized as novel *Pseudomonas sp.* based on microscopic, biochemical and molecular studies. Hence, the 16S ribosomal DNA sequence of the novel isolated bacterium was submitted in the GenBank database with an accession number JF683582. In the present work, we report the extracellular surfactant production by the novel *Pseudomonas sp.* 2B.

5. Acidification followed by chloroform: methanol mixture (2:1) extraction was effective in the extraction of the extracellular surfactant from the cell-free broth of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively as both polar and non-polar components of the surfactant could be extracted. This study was successful in extraction of extracellular surfactant present in the cell-free broth of the bacterial strains.

6. The M1 medium (Proteose Peptone Glucose Ammonium Salts medium) consisting of glucose, peptone, Tris HCl, ammonium chloride, potassium chloride and magnesium sulphate was the best suited surfactant production medium as it supported maximum biomass and surfactant production which inturn caused reduction in the surface tension values.

7. The time course study revealed that the surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, might be "primary metabolite" as the surfactant production coincided with the exponential growth phase of the bacterial strains.

8. The present study also revealed that the various process parameters significantly affected surfactant production by the both the bacterial strains. The maximum surfactant production by *Pseudomonas sp.* 2B was observed at 2% (v/v) inoculum volume, initial medium pH 7, incubation temperature of 37^{o}C, agitation speed of 150 rpm, 30 g/L (w/v) glucose, combination of peptone and potassium nitrate as nitrogen source, 2% (v/v) olive oil, Tris HCl buffer and 1% (w/v) NaCl concentration, a maximum of 8.51 g/L of surfactant was produced by *Pseudomonas sp.* 2B when the aforementioned conditions were maintained. *Pseudomonas aeruginosa* produced a maximum of 7.4 g/L of surfactant at 3% (v/v) inoculum volume, initial medium pH 7, incubation temperature of 37^{o}C, 30 g/L (w/v) glucose, agitation speed of 150 rpm, combination of yeast extract and ammonium chloride as nitrogen source, 2% (v/v) n-hexadecane, Tris HCl buffer and 0.5% (w/v) NaCl concentration.

9. The results of the RSM study were successful in determining the optimal concentrations of the significant process variables leading to maximum surfactant production by both the bacterial strains. The optimum medium consisted of 35.7645 g/L of glucose, 3.5% of olive oil and 5.5425 g/L of potassium nitrate for the production of surfactant by *Pseudomonas sp.* 2B whereas for *Pseudomonas aeruginosa*, the optimal medium consisted of 35.7645 g/L of glucose, 3.5% of n-hexadecane and 5.6274 g/L of ammonium chloride. A maximum of 14.63 g/L and 10.69 g/L of surfactant was produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa*, respectively, at optimal concentrations of the process variables.

10. The results of the column chromatography experiment revealed that the surfactant produced by the bacterial strains was made up of different moieties as indicated by the surface tension values of the different fractions eluted.

11. The surfactants produced by both the bacterial strains were identified as glycolipoproteins (rhamnolipoprotein) based on the characterization studies. This suggests that the surfactant can be easily degraded since it is composed of biomolecules such as carbohydrates, proteins and lipids.

12. The results of the reconstitution study of the partially purified surfactant of *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, was helpful with respect to understanding the role of individual moieties of surfactant in terms of maintenance of stability of the surfactant.

13. The surfactants produced by the bacterial strains, *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, has potential application over a wide range of temperature, pH and saline environment as indicated by the stability of biosurfactant in different environmental conditions. The results suggest that these surfactants may be suitable for use in situations where extreme conditions of temperature, pH and salinity prevail such as enhanced oil recovery operations, marine environments, etc. The study also suggests that the cell-free broth containing the surfactant can be directly applied without any purification step.

14. The surfactants produced by *Pseudomonas sp.* 2B and *Pseudomonas aeruginosa,* respectively, showed enhanced crude oil biodegradation ability. Nearly 95% of the crude oil was degraded in the presence of the surfactant.

15. The present study also reveals that the reduction of surface tension potential by *Pseudomonas sp*. 2B (21.98 mN/m) is more efficient than that of synthetic surfactant, sodium dodecyl sulphate (32.59 mN/m).

The present study has been successful in generating data with respect to local microorganisms involved in the production of surfactant. A novel *Pseudomonas sp*. 2B was isolated from petroleum hydrocarbon environment possessed superior surface

tension reduction capacity (21.98 mN/m) which is significant compared to the surface tension reduction potential shown by synthetic surfactants. The bacterial isolate also produced a maximum of 14.63 g/L of surfactant following medium optimization which is significant compared to many reports in the literature. The surfactant produced by the novel isolate was extracellular in nature and the results of the characterization revealed that the surfactant was composed of carbohydrates, lipids and proteins. As the surfactant produced by the bacterial isolate was composed of biomolecules, the surfactant can be easily degraded. The results of the present study indicate that *Pseudomonas sp*. 2B isolated in the present study is both industrially and environmentally relevant.

FUTURE SCOPE OF THE RESEARCH

- Large scale production of the biosurfactant in fermenters could be carried out.
- Sequencing, engineering and cloning of the gene/s dictating biosurfactant production could be an interesting preposition in an effort to increase biosurfactant production.
- The shelf-life of the biosurfactant may be studied in varied environmental conditions and for prolonged period of time.
- The effect of various process parameters for enhancing the biodegradation of the petroleum hydrocarbons using biosurfactant can be studied.
- Comparative studies can be done with commercially available biosurfactants and synthetic surfactants.

APPENDIX I

BUSHNELL HASS MEDIUM

The basal medium of the following composition was used in the study:

Components	g/L
Magnesium sulphate	0.406
Calcium chloride	0.0265
Potassium dihydrogen phosphate	1.0
Disodium hydrogen phosphate	6.0
Ammonium nitrate	1.0
Ferrous chloride	0.05
Glucose	2.0

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 7.0. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C. The Bushnell and Hass agar medium (pH 7.0) was prepared by adding 10 grams of agar to the basal liquid medium before autoclaving.

APPENDIX II

MINERAL BROTH MEDIUM

The mineral broth medium of the following composition was used in the study:

Components	g/L
Peptone	5.0
Yeast extract	1.0
Ferric citrate	0.1
Sodium chloride	19.45
Magnesium chloride	5.9
Magnesium sulphate	3.24
Calcium chloride	1.8
Potassium chloride	0.55
Sodium bicarbonate	0.16
Boric acid	0.0022
Sodium silicate	0.0004
Sodium fluoride	0.0024
Ammonium nitrate	0.0016
Disodium hydrogen phosphate	0.008

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 7.6. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C. The mineral agar medium of pH 7.6 was prepared by adding 1.5% (w/v) of agar to the liquid medium before sterilization.

APPENDIX III

CETYL TRIMETHYL AMMONIUM BROMIDE (CTAB)-METHYLENE BLUE AGAR MEDIUM

The CTAB-methylene blue agar medium of the following composition was used:

Components	g/L
Solution A : Cetyl trimethyl ammonium bromide	0.2
Calcium chloride	0.1
Potassium dihydrogen phosphate	0.7
Disodium hydrogen phosphate	0.9
Sodium nitrate	2.0
Methylene blue	0.005
Glucose	20.0
Solution B : Ammonium molybdate	0.6
Manganous sulphate	1.2
Magnesium sulphate	0.4
Ferrous sulphate	1.6
Agar	15.0

The components of the medium were dissolved in 1 litre of water and the pH of the medium was set to 5.5. Solution A and solution B of the medium were sterilized separately to avoid precipitation. The components of the solution B were dissolved in 2ml of 37% hydrochloric acid. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C.

APPENDIX IV

MEDIA FOR BIOCHEMICAL TESTS

4.1 AMYLASE PRODUCTION TEST

STARCH AGAR MEDIUM

Components	g/L
Beef extract	3.0
Soluble starch	10.0
Agar	12.0

The components of the medium were dissolved in 1 litre of distilled water and the medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C.

4.2 METHYL RED AND VOGES- PROSKAUER TEST

GLUCOSE BROTH

Components	g/L
Peptone	7.0
Glucose	5.0
Disodium hydrogen phosphate	5.0

The components of the medium were dissolved in 1 litre of distilled water and the pH of the broth was set to 6.9. The medium was autoclaved at 15 psi pressure for 20 minutes at 121°C.

4.3 INDOLE TEST

TRYPTONE BROTH

10.0 grams of tryptone was dissolved in 1 litre of distilled water. The broth was poured into test tubes. The test tubes containing the medium were autoclaved at 15 psi pressure for 20 minutes at 121°C.

4.4 CITRATE UTILIZATION TEST

SIMMON'S CITRATE AGAR MEDIUM

Components	g/L
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.8
Agar	15.0

The components of the medium were dissolved in 1 litre of distilled water and the pH of the broth was set to 6.9. The medium was autoclaved at 15 psi pressure for 20 minutes at 121°C.

4.5 FERMENTATION OF CARBOHYDRATES

CARBOHYDRATE FERMENTATION MEDIUM

Components	g/L
Peptone	10.0
Carbohydrate (glucose/ lactose / sucrose)	5.0
Sodium chloride	15.0
Phenol red	0.018

The components of the medium were dissolved in 1 litre of distilled water; the pH of the medium was set to 7.3. The medium was poured into test tubes and they were autoclaved at 15 psi pressure for 20 minutes at 121°C.

4.6 UREASE TEST

UREA AGAR MEDIUM

Components	g/L
Urea	20.0
Peptone	1.0
Glucose	1.0
Phenol red	0.01
Dipotassium hydrogen phosphate	2.0
Sodium chloride	5.0
Agar	20.0

The components of the medium were dissolved in 1 litre of distilled water. The initial medium of the pH was adjusted to 6.8. The medium was autoclaved at 15 psi pressure for 20 minutes at 121°C.

4.7 HYDROGEN SULPHIDE PRODUCTION TEST

TRIPLE SUGAR IRON AGAR MEDIUM

Components	g/L
Peptone	20.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Sodium chloride	5.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.025
Phenol red	0.025
Agar	30.0

The components of the medium were dissolved in 1 litre of distilled water. The initial pH of the medium was adjusted to 7.4. The medium was autoclaved at 15 psi pressure for 20 minutes at 121°C.

4.8 GELATIN HYDROLYSIS TEST

GELATIN AGAR MEDIUM

Components	g/L
Peptone	10.0
Meat extract	10.0
Sodium chloride	5.0
Gelatin	10.0
Agar	15.0

The components of the medium were dissolved in 1 litre of distilled water; the initial pH of the medium was set to 7.3. The medium was poured into test tubes and the tubes were autoclaved at 15 psi pressure for 20 minutes at 121°C.

APPENDIX V

PARTIAL 16S RIBOSOMAL DNA SEQUENCING PROCEDURE

Each polymerase chain reaction (PCR) mixture contained approximately 10 ng of DNA; 2.5 Mm $MgCl_2$; 1×PCR buffer (Bangalore Genei, Bangalore, India); 200μM each of dCTP, dGTP, dTTP and dATP; 2 pmol of each forward and reverse primer; and 1 U of Taq polymerase (Bangalore Genei, Bangalore, India). The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94°C for 5 min; 30 cycles of 94°C, 60°C and 72°C for 1 min each; and final extension at 72°C for 10 min and the mixture was held at 4°C. The PCR product was precipitated using polyethylene glycol, washed thrice using 70% ethanol and dissolved in Tris-HCl (10 mM, pH 8). The ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) was used for the sequencing of the PCR product. A combination of universal primers was chosen to sequence the gene sequence (Rawlings et al. 1995; Muyzer et al. 1995). Samples were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing output was analyzed using the DNA sequence analyzer computer software (Applied Biosystems). The sequence was compared with National Center for Biotechnology Information Gen Bank entries by using the BLAST algorithm.

APPENDIX VI

NUTRIENT BROTH MEDIUM

Nutrient broth of the following composition was used in the study:

Components	g/L
Peptone	5.0
Sodium chloride	5.0
Yeast extract	2.0
Beef extract	1.0

The components of the nutrient broth medium were weighed and dissolved in 1 litre of water. The initial pH of the medium was set to 7.4±0.2. The medium was sterilized at 15 psi pressure at 121°C for 15 minutes.

APPENDIX VII

BIOSURFACTANT PRODUCTION MEDIA COMPOSITION

7.1 M1 MEDIUM - PROTEOSE PEPTONE GLUCOSE AMMONIUM SALT (PPGAS) MEDIUM

The PPGAS medium of the following composition was used in the study:

Components	g/L
Ammonium chloride	1.0
Potassium chloride	1.5
Tris HCl	19.0
Magnesium sulphate	0.4
Proteose Peptone	10
Glucose	5

The components of the M1 medium were weighed and dissolved in 1 litre of water. The initial pH of the medium was set to 7.0. The medium was sterilized at 15 psi pressure (121°C) for 15 minutes (Gunther et al. 2005).

7.2 M2 MEDIUM -YEAST EXTRACT-PEPTONE-GLUCOSE (YPG) MEDIUM

The YPG medium of the following composition was used in the study:

Components	g/L
Peptone	5.0
Yeast extract	5.0
Glucose	15.0

The components of the medium were dissolved in 1 litre of water. The medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C (Manoj et al. 2008).

7.3 M3 MEDIUM-MINERAL SALT MEDIUM

The mineral salt medium of the following composition was used in the study:

Components	**g/L**
Glucose	5.0
Potassium nitrate	3.0
Disodium hydrogen phosphate	2.2
Potassium dihydrogen phosphate	0.14
Sodium chloride	0.01
Magnesium sulphate	0.6
Calcium chloride	0.04
Ferrous sulphate	0.02
Zinc sulphate	2.32
Manganous sulphate	1.78
Boric acid	0.56
Copper sulphate	1.0
Sodium molybdate	0.39
Cobaltous chloride	0.42
Ethylenediaminetetraacetic acid	1.0
Nickel chloride	0.004
Potassium iodide	0.66

The components were weighed and dissolved in 1 litre of water. The initial pH of the medium was set to 6.8. The medium was sterilized at 15 psi pressure at 121°C for 15 minutes (Makkar and Cameotra 1997).

7.4 M4 MEDIUM-MINERAL MEDIUM

The mineral medium of the following composition was used in the study:

Components	**g/L**
Solution A: Sodium nitrate	2.5
Magnesium sulphate	0.4
Sodium chloride	1.0
Potassium chloride	1.0
Calcium chloride	0.05
Phosphoric acid	10 ml
Solution B: Ferrous sulphate	0.5
Zinc sulphate	1.5
Manganous sulphate	1.5
Potassium borate	0.3
Copper sulphate	0.15
Sodium molybdate	0.1

Solution A and solution B of the medium were weighed and dissolved in 1 litre of water and the initial pH of the medium was set to 7.2. 3% (w/v) glucose was sterilized separately. Solution A and B were sterilized separately at 15 psi pressure at 121°C for 15 minutes.1 ml of solution B was added to 999 ml of solution A after sterilization (Bodour et al. 2003).

APPENDIX VIII

OPTICAL DENSITY MEASUREMENT

The determination of optical density is a method of enumeration of bacterial count (Cappuccino and Natalie 1999). Bacterial growth was determined by measuring the culture optical densities at 600nm (OD600) using spectrophotometer (GBC Scientific Equipment, Australia). 3 ml of the culture broth was added to a glass cuvette and the optical density was measured. The optical densities of the samples removed from cultures were read against uninoculated broth which was used as blank throughout the study for growth determination.

APPENDIX IX

CELL DRY WEIGHT MEASUREMENT

The bacterial cell dry weight was determined as a function of bacterial growth (Cappuccino and Natalie 1999). The empty weight of a sampling vial (Eppendorf, India) was measured. 1 ml of the sample was added to the vial. The vial was centrifuged at 10,000 rpm for 25 minutes at 4ºC. The supernatant was discarded and the vial containing the biomass was dried in an oven at 50ºC to a constant weight. The dry weight of the biomass was determined by subtracting the weight of the vial from the weight of the vial with biomass.

APPENDIX X

GLUCOSE ESTIMATION BY DNS METHOD

The glucose concentration was estimated by 3, 5-dinitrosalicylic acid (DNS) method using UV/VIS spectrophotometer.

Principle

3, 5-DNS (yellow colour) in alkaline solution is reduced to 3-amino, 5-nitro salicylic acid (orange-red).

Reagents

1. Dinitrosalicylic Acid Reagent (DNS Reagent): 1.0 g of dinitrosalicylic acid, 200 mg of crystalline phenol and 50 mg of sodium sulphite were dissolved in 100 ml of 1% NaOH. The reagent was stored at 4°C.

2. 40% Rochelle salt solution (Potassium sodium tartarate).

3. Glucose standard: Aliquots of solution ranging from 0-500 µg/ml were prepared.

Procedure

1. 0.1 ml of test samples were pipetted out into test tubes and the volume was made upto 3 ml with water in all the tubes.

2. 3.0 ml of DNS reagent was added and the contents were heated in a boiling water bath for 5 minutes.

4. When the contents of the tubes were warm, 1.0 ml of 40% Rochelle salt solution was added.

5. The test tubes were cooled and the intensity of dark red colour was read at 510 nm.

6. A calibration curve of concentration of glucose versus optical density was plotted. Concentration range of 0-500 µg/ml gave a linear curve as shown in Fig. 1.

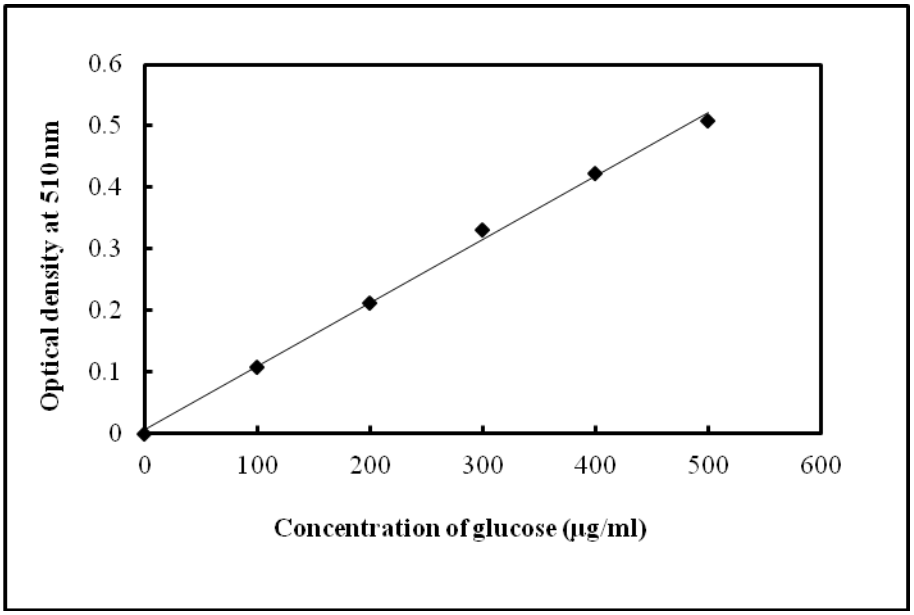


Fig. 1: Calibration curve for the DNS assay for the determination of glucose concentration expressed in µg/ml

APPENDIX XI

ESTIMATION OF TOTAL CARBOHYDRATES BY PHENOL-SULPHURIC ACID METHOD

The total carbohydrate concentration was estimated by phenol-sulphuric acid method using UV/VIS spectrophotometer.

Principle

In hot acidic medium, glucose is dehydrated to form hydroxymethyl furfural. This forms a green coloured product with phenol.

Reagents

5% Phenol: 5.0 g of phenol was dissolved in water and made upto 100 ml.

96% Sulphuric acid

Glucose standard: Aliquots of solution ranging from 0-200 µg/ml were prepared.

Procedure

1. 0.1 ml of the test solutions were pipetted out into test tubes and the volume in each tube was made up to 1 ml with water.

2. 1.0 ml of phenol solution was added to each tube.

3. 5.0 ml of 96% sulphuric acid was added to each tube.

4. After 10 minutes, the contents in the tubes were mixed and placed in a water bath at 25-30°C for 20 minutes.

5. The absorbance of the samples was read at 490 nm.

6. The amount of total carbohydrate present in the sample solution was calculated using the standard graph (as shown in Fig. 2).

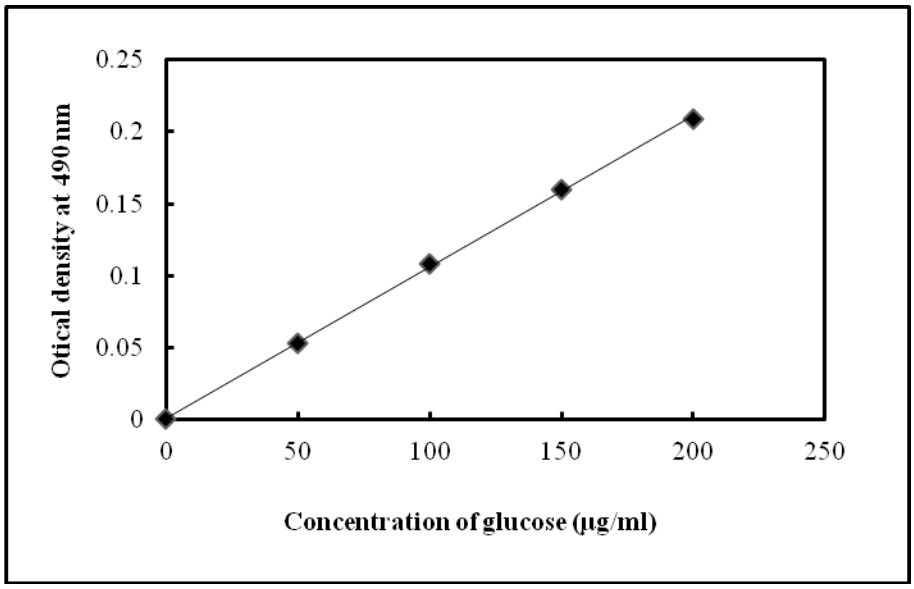


Fig. 2: Calibration curve for the phenol-sulphuric acid method for the determination of total carbohydrate concentration expressed in µg/ml

APPENDIX XII

ESTIMATION OF RHAMNOSE BY ORCINOL-SULPHURIC ACID METHOD

The total rhamnose concentration was estimated by phenol-sulphuric acid method using UV/VIS spectrophotometer.

Principle

In hot acidic medium, rhamonse and orcinol react to give a cherry red coloured product.

Reagents

Orcinol reagent: 0.19 g of orcinol was dissolved 100 ml of water.

53% Sulphuric acid.

Standard rhamnose: Aliquots of solution ranging from 0-200 µg/ml were prepared.

Procedure

1. 0.1 of the test solutions were pipetted out into test tubes and the volume in each tube was made up to 1 ml with water.

2. 1.0 ml of orcinol solution was added to each tube.

3. 8.0 ml of sulphuric acid was added to each tube.

4. After 10 minutes, the contents in the tubes were mixed and placed in a water bath at 80°C for 20 min.

5. The absorbance of the samples was read at 421 nm.

6. The amount of total rhamnose present in the sample solution was calculated using the standard graph (Fig. 3).

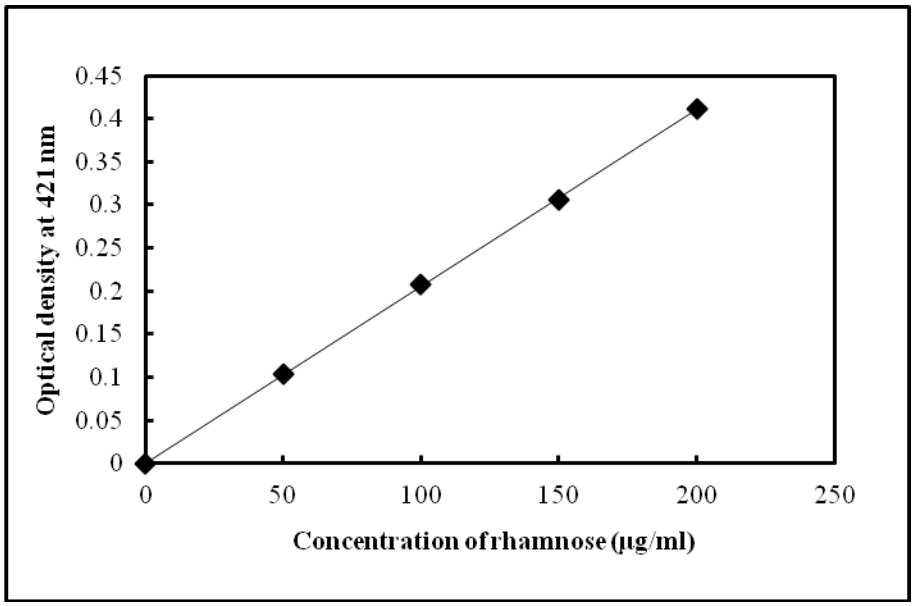


Fig. 3: Calibration curve for the orcinol-sulphuric acid method for the determination of rhamnose concentration expressed in µg/ml

APPENDIX XIII

PROTEIN ESTIMATION BY LOWRY'S METHOD

The concentration of proteins was determined by Lowry's method using UV/VIS spectrophotometer (Thimmaiah 1999).

Principle

The phenolic group of tyrosine and trytophan residues (amino acid) in a protein will produce a blue purple colour complex in an alkaline condition with maximum absorption in the region of 660 nm wavelength, with Folin- Ciocalteau reagent.

Reagents

Reagent A - 2.0 g of sodium carbonate was dissolved in 100 ml of 0.1 N NaOH.

Reagent B - 0.5 g of copper sulphate was dissolved in 100 ml of freshly prepared sodium potassium tartarate (1%).

Reagent C - 50 ml of Reagent A was mixed with 1.0 ml of Reagent B to make the alkaline copper reagent at the time of use.

Reagent D - Folin-Ciocalteau reagent which is commercially available was diluted with water in 1 :1 ratio at the time of use.

Bovine serum albumin (BSA) standard: Aliquots of solution ranging from 0-200 µg/ml were prepared

Procedure

1. 0.5 ml of the test solutions were pipetted out into test tubes and made up to 1.0 ml with sterile distilled water.

2. 5.0 ml of reagent C was added to all the aliquots, mixed well and incubated at room temperature for 10 minutes.

3. 0.5 ml of reagent D was added and incubated in dark for 20 minutes at room temperature.

4. The mixture was mixed well and O.D. was read at 660 nm in an UV/VIS spectrophotometer.

5. A calibration curve of optical density versus concentration of BSA was plotted. Concentration range 0-200 µg/ml gave a linear curve (Fig. 4).

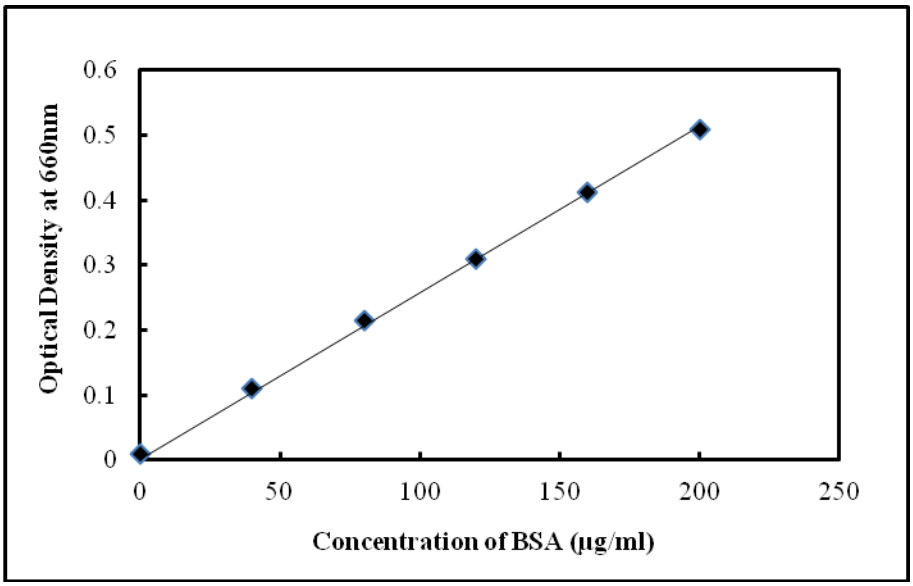


Fig. 4: Calibration curve for Lowry's method for the determination of protein concentration expressed in µg/ml

APPENDIX XIV

CRUDE OIL ESTIMATION

The residual crude oil was estimated by UV-spectrophotometer method as per EPA protocol (EPA Pt. 300, App. C). The biodegradation was estimated by quantification of residual crude oil.

For the standard preparation, known amount of crude oil was added to Bushnell Hass medium (100 ml) and the crude oil was extracted using dichloromethane (DCM). 250 µl, 500 µl, 1000 µl, 1500 µl and 2000 µl of crude oil concentrations 200 mg (w/v), 400 mg, 800 mg and 1000 mg, respectively, was added to conical flasks containing 50 ml of Bushnell Hass medium. The experiments were maintained in triplicates. The medium was mixed with 25 ml of DCM and maintained at 4ºC in a shaker at 150 rpm. The medium was centrifuged at 10,000 rpm for 30 minutes to separate the solution into two layers. It was transferred into separating funnel and the DCM was collected. The DCM was concentrated to 1.0 ml using rotaevaporator. All the samples were made up to 3.0 ml for the UV-spectrophotometer. The optical density was read at wavelengths 340, 370 and 400 nm. The standard concentrations 200 mg, 400 mg, 800 mg and 1000 mg which were extracted and made up to 3.0 ml with DCM were read at above mentioned wavelengths. A calibration plot was drawn at each wavelength.

The response factor (RF) was calculated for each concentration (C) and at each wavelength. The absorbance (A) taken at each wavelength for the known concentration was used to calculate response factor.

$$RFx = \frac{Cx}{Ax}$$

x= corresponding wavelength

The biodegradation was assessed by measuring the residual concentration of crude oil in the sample. The residual crude oil from the samples was extracted with DCM, centrifuged and concentrated to 1.0 ml and made up to 3.0 ml. This was read at 340, 370 and 400 nm and the calibration plots are given in Fig. 5, 6 and 7. The absorbance taken at each wavelength for the known concentration was used to calculate the response factor.

Concentration of oil in unknown was calculated at each wavelength (Cx) by using the respective RFx. The following formula was used to calculate the Cx:

$$\mathbf{Cx= (Ax) \times (RFx) \times (V_{DCM}) \times (Vtw/Vew)}$$

where V_{DCM} is the volume of DCM used, Vtw is the volume of the medium and Vew is the volume of the medium taken for extraction.

The mean of Cx at each wavelength was calculated as follows:

$$\mathbf{C\ mean = \frac{C_{340} + C_{370} + C_{400}}{3}}$$

The percentage degradation was calculated as follows:

$$\mathbf{Percentage\ degradation = \frac{C\ mean}{C\ total} \times 100}$$

where C mean= mean value of oil concentration and C total = total mass of oil initially added to flask.

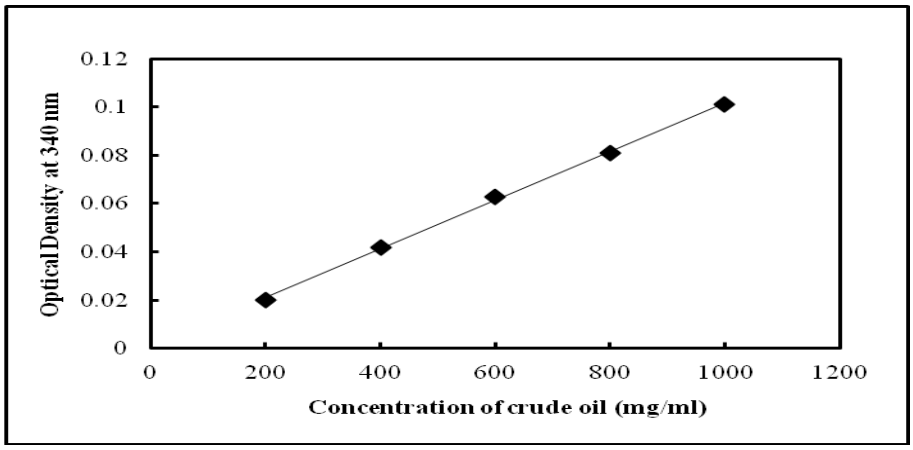


Fig. 5: Calibration curve for crude oil estimation at 340 nm

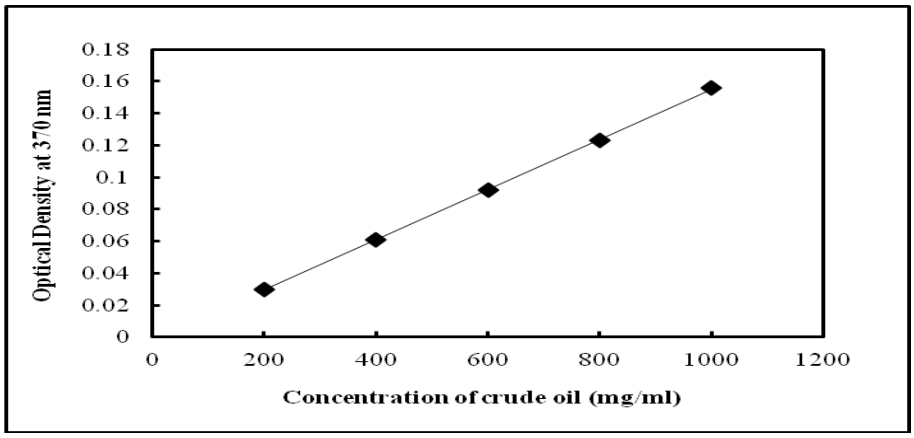


Fig. 6: Calibration curve for crude oil estimation at 370 nm

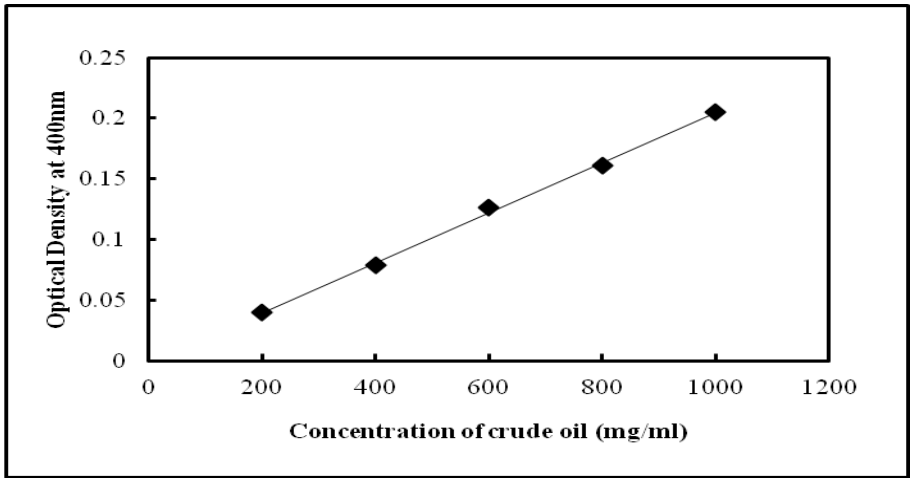


Fig. 7: Calibration curve for crude oil estimation at 400 nm

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