PRODUCTION OF MICROBIAL BIOSURFACTANT USING COST-EFFECTIVE RESOURCES

A THESIS

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by

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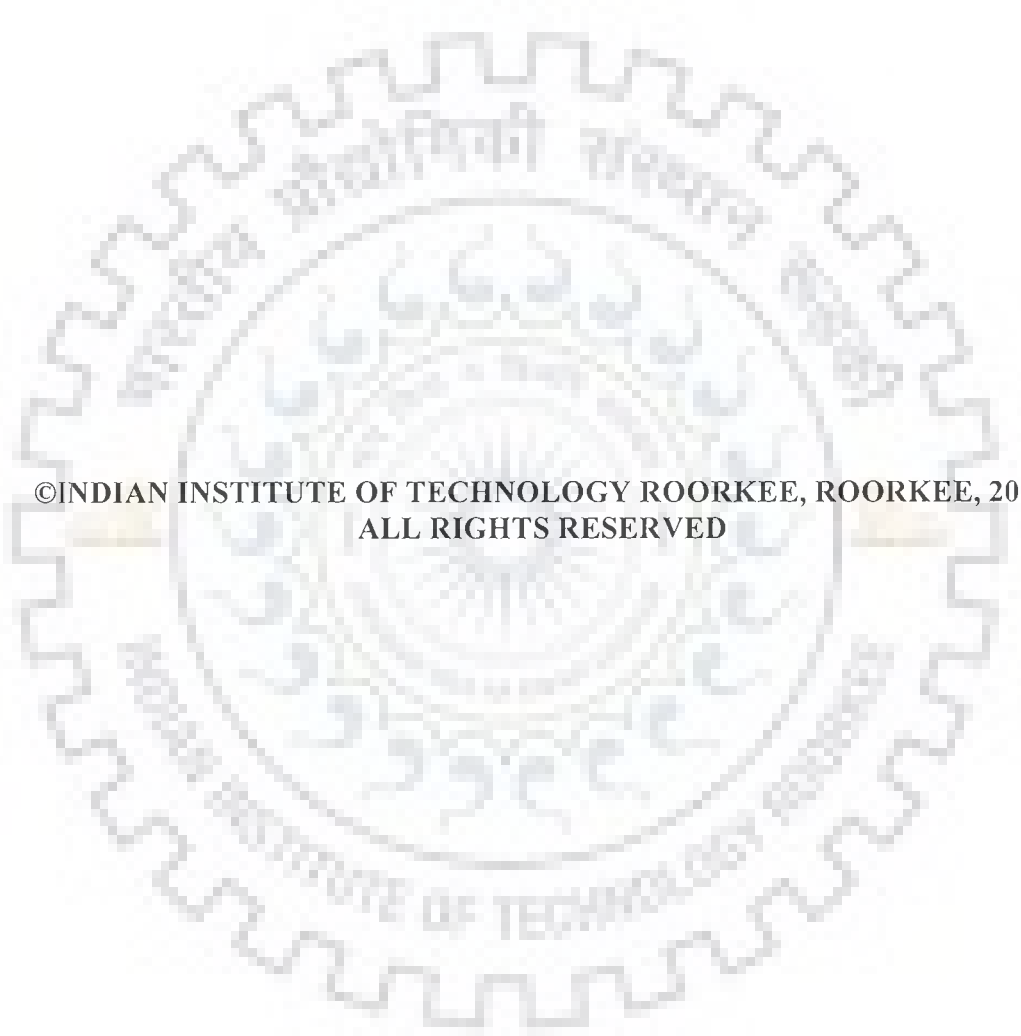

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **Production Of Microbial Biosurfactant Using Cost-Effective Resources** in partial fulfilment of the requirements for the award of degree of Doctor of Philosophy, submitted in the Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January 2005 to October 2009 under the supervision of Dr. Vikas Pruthi, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter embodied in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Deepak Sharma

(DEEPAK SHARMA)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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The Ph.D. Viva-Voce Examination of **Mr. Deepak Sharma**, Research Scholar, has been held on 2nd July 2010

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ABSTRACT

Biosurfactants are structurally diverse group of surface active compounds produced by a wide variety of microorganisms. They have unique amphiphatic properties derived from their complex structures, which include a hydrophilic moiety and a hydrophobic portion.

In the present investigation focus was on the isolation of bacterial strain for utilizing cost-effective substrate for biosurfactant production. A total of 340 bacterial isolates were obtained from hydrocarbon contaminated sites *viz.* oil sludge samples, fuel filling stations, ware houses and phenol waste water of which 26 bacterial isolates (DSVP1- DSVP26) were selected on basis of their ability to grow on hydrocarbons (dodecane, hexadecane, pristane, toluene and fluoranthene). Screening of these bacterial isolates was done for biosurfactant production using drop collapse assay, emulsification assay, hemolytic assay and surface tension reducing ability. Of these five bacterial isolates DSVP2, DSVP9, DSVP11, DSVP18 and DSVP23 were selected as potent biosurfactant producers. Cell surface hydrophobicity tests like hydrocarbon interaction chromatography (HIC), salt aggregation test (SAT), bacterial adherence to hydrocarbons (BATH) and replica plate (RP), confirmed biosurfactant producing ability of these bacterial isolates. These five selected bacterial isolates were further exploited for their ability to produce biosurfactant by utilizing cost effective raw materials (cotton seed hull, tea leaves, wheat bran, corn starch, rice straw, wheat straw, bagasse, ground nut kernel, potato peel, apple peel, cotton seed, molasses, bamboo wood saw dust and gram husk). Amongst above isolates DSVP23 was chosen to be the best candidates for biosurfactant production effectively utilizes cotton seed hull as substrate in minimal salt medium (MSM).

The isolated strain was identified by morphological, biochemical and molecular biology technique using the taxonomic scheme of Bergey's manual of determinative bacteriology and 16S rRNA. Using the Basic Local Alignment Search Tool (BLAST) available in the National Center for Biotechnology Information (NCBI) database, homology of 16S ribosomal RNA (16S rRNA) gene sequence obtained for strain DSVP23 depicted it to be of *B. subtilis* (Gene bank accession no. EU679368).

Biosurfactant from cell free broth of *B. subtilis* DSVP23 isolated using acid precipitation was further extracted by dichloromethane. The surfactant obtained showed reduction in surface-tension value of water from 72mN/m to 28mN/m. The critical micelle concentration of the surfactant was 30 mg/l. The surface tension and emulsification capacity

remained unaltered within a wide pH (2-12), temperature (4-80°C) ranges and under NaCl (2-10%) concentrations.

Biosurfactant was characterized using thin layer chromatography (TLC) and Fourier transforms infrared (FTIR). FTIR spectra showed strong absorption bands of peptides at 3343 cm^{-1}, 1641 cm^{-1}, 1518 cm^{-1} resulting from N-H stretching, C=O stretching and combined C-N stretching mode respectively. 1368 cm^{-1}, 1451 cm^{-1} and 2960 cm^{-1}, bands are predominant and indicate aliphatic chains (CH_2 ,CH_3) of sample. The intense band at 1641 cm^{-1} corresponds to –CO-NH-R group indicated biosurfactant to be lipopeptide in nature. The biosurfactant was further analysed using HPLC. Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and nuclear magnetic resonance (NMR) revealed that the major constituent of lipopeptide being leucine and isoleucine. The presence of a lactone ring in the surfactant was indicated by NMR spectra, which detected an ester carbonyl group, and was supported by the fact that the peptide has a blocked N terminus. The mass spectrum of this sample showed an isolated peak around *m/z* 1044.6.

A high yield of biosurfactant was obtained from a culture of *B. subtilis* DSVP23 using 2% cotton seed hull supplemented with sucrose (2%) as a carbon source in MSM. Ammonium nitrate (4.5g/l) and sodium nitrate (4.0g/l) were among the best nitrogen sources for biosurfactant production when incubated at 37°C temperature with 0.5 vvm and 180 rpm of aeration rate and agitation speed respectively. The addition of various metal supplements (manganese, magnesium, calcium, iron, and trace elements) greatly affected growth and biosurfactant production. A high yield of biosurfactant (4.0-4.5 g/l) was elucidated upon addition of 30 mg/l, $MnSO_4$ and 5mM $FeSO_4$. Amino acids (0.1% w/v), such as aspartic acid, glutamic acid, lysine and valine increased the final yield of biosurfactant.

B. subtilis mutants were obtained using EMS (3%) treatment producing high level of the lipopeptide biosurfactant to that of wild type DSVP23. Mutant was selected using reduction in surface tension value and zone of hemolysis formed compared to that of wild type. EMS treatment resulted in 8 mutant colonies, showing a 2 fold increase in biosurfactant production to that of wild type DSVP23. Comparative analysis of biosurfactant production mutant (*DVM4*) and wild type (DSVP23) to utilize cheap raw materials was assessed using fermentation. The mutant, designated *B. subtilis DVM4*, was capable of producing lipopeptide biosurfactant at concentrations up to 6.0g/l compared to that of 4.0g/l of wild type (DSVP23).

The application of isolated biosurfactant in enhanced oil recovery (EOR) was evaluated using the sand pack technique. Recovery of 76% kerosene oil, 72% motor oil, 68% n-paraffin 70% crude oil and 62% mobile oil was obtained by using 0.5 % of aqueous solution of biosurfactant in sand pack column. Biosurfactant produced by *B. subtilis* DSVP23 was found to be an effective emulsifier when tested against different hydrocarbons kerosene oil (78%), hexadecane (72%), toluene (72%), motor oil (70%), dodecane (68%), tetradecane (64%), and hexane (64%). Thus signifies its potential application in oil spill management.

The biosurfactant obtained using cotton seed hull in MSM as cost effective substrate showed profoundly distinct antibacterial activity toward test organisms namely *S. aureus, E.coli, P. aeruginosa* and *B. cereus* with a zone of inhibition 18mm, 13.8mm, 12.1mm and 11.2mm respectively. Also biosurfactants displayed a maximum antifungal activity against *C. albicans* followed by *R. solani, F. oxysporum* and *T. viride* with a zone of inhibition of 15.2mm, 11.3mm, 10mm and 12.5mm respectively.

The potential of lipopeptide biosurfactant in inhibiting biofilm adhesion of bacteria and fungi was demonstrated by using the MTP assay. In particular, *Candida albicans* and *Staphylococcus aureus* biofilm formation was decreased to 78% and 72% respectively at biosurfactant concentration 10.5 µg/ml and 12 µg/ml. Microscopic studies further explored morphological alterations in biofilms upon biosurfactant treatment. Visualization of biofilms of both yeast and bacterial biofilm ultrastructure by SEM revealed that major damage to the biofilm constituents was caused by lipopeptide biosurfactant. Reduction in viable cell count was also checked using fluorescent viability test using fluorescent dyes (FDA and EtBr). AFM studies revealed topographic images of *S. aureus* and *C. albicans* biofilms. Images revealed that cell disintegration occurred upon lipopeptide biosurfactant treatment.

Biodegradation potential of microbes in degrading mixed substrates (i.e. oily sludge) vis-□-vis pure hydrocarbons like pristane and fluoranthene was also investigated. Degradation of different substrates was assessed by monitoring dry cell biomass. pH, reduction in surface tension, biosurfactant production and gas chromatographic profiles. Our data using capillary gas chromatographic analysis revealed that *B. subtilis* effectively degrade oily sludge components aliphatic hydrocarbons and aromatic hydrocarbons after 7 days of incubation.

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LIST OF PUBLICATION/PAPERS PRESENTED IN CONFERENCES/SYMPOSIA

1. Deepak Sharma, Vikas Pruthi (2009). *Characterization and Bioremediation assessment of biosurfactant producing Bacillus subtilis DSVP23 from petroleum contaminated site*. Microbial Cell Factories (communicated)

2. Priyanka Lal, Deepak Sharma, Parul Pruthi, Vikas Pruthi (2009). *Exopolysaccharide analysis of biofilm forming Candida albicans*. Journal of Applied Microbiology (Accepted)

3. Deepak Sharma, Swaranjit Singh Cameotra and Vikas Pruthi (2008). *Isolation, Identification and Industrial usage of Biosurfactant produced by a hydrocarbon degrading Bacillus Pumillus strain*. Poster presentation at "International Conference on New Horizons in Biotechnology (NHBT-2007)" November 26-29, 2007, Trivandrum, Kerala, India

4. Deepak Sharma, Shuchi Verma, Mani Singh, Renu Bhargava and Vikas Pruthi (2006). '*Microbial Production of surface active agents for oil bioremediation*'. Proceedings of the International Conference on Bio-Nanotechnology, held at Al-Ain, U.A.E. Nov., 18-21. pp. 81-85

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LIST OF ABBREVIATIONS

%	:	Percentage
µl	:	Microlitre
µm	:	Micrometer
°C	:	Degree centigrade
AFM	:	Atomic Force Microscopy
BSA	:	Bovine serum albumin
BATH	:	Bacterial adherence to hydrocarbons
cfu	:	Colony forming unit
CMC	:	Critical micelle concentration
CMD	:	Critical micelle dilution
CDCl3	:	Chloroform Deutrated
Conc.	:	Concentration
CSF	:	Cerebrospinal fluid
Da	:	Dalton
dNTP	:	Deoxy nucleotide Triphosphate
DNA	:	Deoxyribose nucleic acid
EMS	:	Ethyl Methane sulfonate
EtBr	:	Ethidium Bromide
E24	:	Emulsification index
FDA	:	Fluorescein Diacetate
FID	:	Flame ionization detector
FTIR	:	Fourier Transform Infrared spectroscopy
Fig	:	Figure
g	:	Gram
GC	:	Gas chromatograph
h	:	Hour
HPLC	:	High performance liquid chromatography
HIC	:	Hydrophobic interaction chromatography
IIT	:	Indian Institute of Technology
IMTECH	:	Institute of Microbial Technology
IIP	:	Indian Institute of Petroleum
KD	:	Kilo Dalton
IT	:	Interfacial tension
kHz	:	Kilohertz
l	:	Litre
M	:	Molar
mg	:	Milligram
MIC	:	Minimum inhibitory concentration
MALDI-TOF-MS	:	Matrix Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry
MEOR	:	Microbially enhanced oil recovery
min	:	Minute
ml	:	Millilitre
mM	:	Millimolar

mN/m	:	Milli newton/meter
MSM	:	Minimal salt medium
MTCC	:	Microbial Type Culture Collection
MTP	:	Microtitre Plate
N	:	Normal
NCBI	:	The National Center for Biotechnology Information
nm	:	Nanometer
NMR	:	Nuclear Magnetic Resonance
OD	:	Optical density
PBS	:	Phosphate buffer sulphate
PDA	:	Potato dextrose agar
ppm	:	Parts per million
rpm	:	Rotations per minute
RT	:	Room temperature
SAT	:	Salt aggregation test
ST	:	Surface tension
SDA	:	Sabouraud Dextrose agar
SEM	:	Scanning Electron Microscopy
sp.	:	Species
TCA	:	Trichloroacetic acid
v/v	:	Volume/volume
vol	:	Volume
vvm	:	Volume of air per volume of medium per minute
w/v	:	Weight/volume
XTT	:	2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide
YPD	:	Yeast Extract Peptone Dextrose
ZOI	:	Zone of inhibition

CHAPTER 1

INTRODUCTION

Biosurfactants are diverse group compounds, which are amphiphilic molecules with both hydrophilic and hydrophobic domains that reduce surface tension between individual molecules at the surface and interface respectively (Lin *et al.,* 1997, Cunha *et al.*, 2004; Cameotra *et al.,* 2003). Biosurfactants have several advantages over the chemical counterparts, such as lower toxicity; higher biodegradability and tends to retain activity at extreme temperatures, pH, and salinity (Kosaric, 1990; Cameotra and Makkar, 1998, Banat *et al.,* 2000; Amiriyan *et al.,* 2004; Morita *et al.*, 2009).

Parameters which are generally employed to measure the efficiency of the biosurfactant are surface tension (ST), critical micelle concentration (CMC), interfacial tension (IFT), hydrophobic-lipophilic balance (HLB) and cell surface hydrophobicity (Desai and Banat 1997; Zajic and Seffens, 1984; Haferburg *et al.,* 1986; Cooper and Zajic 1980; Parkinson, 1985).

Different types of biosurfactants are reported in the literatures which are produced by a variety of microorganisms (Van Dyke *et al.,* 1991; Kosaric *et al.,* 1987). Biosurfactants are categorised mainly by their chemical composition and their microbial origin. They cover a wide range of chemical types which includes peptides, fatty acid, phospholipids, glycolipids, antibiotics and lipopeptides (Daziel *et al.,* 1996; Kim *et al.,* 2000; Banat *et al.,* 2000; Araji *et al.,* 2007).

The interest in biosurfactant has been steadily increasing in recent years due to the possibility of their production through fermentation and their potential application areas such as environmental protection. Biosurfactants plays an important application in petroleum-related industries which is used in enhanced oil recovery, cleaning oil spills, oil-contaminated tanker cleanup, viscosity control, oil emulsification and removal of crude oil from sludges (Daziel *et al.,* 1996; Bertrand *et al.,* 1994). These industries are known to be the potential target for the application of these compounds.

A major obstacle on the way of wide-scale application of biosurfactant is its high production cost coupled with less production rate as compared to commercially available synthetic surfactants. This result primarily from inefficient bioprocessing methodology but poor strain productivity and the need to use expensive substrates are also responsible for high cost (Fiechter, 1992).

In the present investigation screening, selection and identification of biosurfactant producing microbial strains using cheap raw materials was carried out. All the efforts were directed to obtain high yield of biosurfactant production at limited cost using cost effective substrates as alternatives for biosurfactant production. This was followed by optimization of various physio-chemical parameters contributing to enhanced production of biosurfactant. The chemical structure of isolated biosurfactant from strain *Bacillus subtilis DSVP23* (GenBank accession no. EU679368) was explored with the help of analytic techniques like high performance liquid chromatography (HPLC), Fourier transform infrared (FTIR), Matrix Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) and Nuclear magnetic resonance (NMR) spectroscopy. Mutational studies were carried out for strain improvement thereby resulting in enhanced production of biosurfactant. Combination of mutant strain with cheaper production media and optimized process conditions lead to economical commercial level biosurfactant production. The lipopeptide biosurfactant obtained from cost effective raw material was effective in recovery of entrapped oil from saturated sand pack columns. Environmental (bioremediation), Industrial (emulsifiers, enhanced oil recovery) and Pharmaceutical (antimicrobial, anti-adhesive) applications of isolated lipopeptide biosurfactant using cost effective resources were also evaluated in this study. Isolated biosurfactant was found to reduce the hazardous effects of biofilms, therefore appears to have a great potential for their use in the biomedical field. Thereby, emphasized it to be a potential substitute for chemical surfactants in the future. Briefly, the study highlights the following objectives:

OBJECTIVES OF THE STUDY

1. Isolation, screening and selection of biosurfactant producing microorganism using cheap raw materials.
2. Optimization of various physio-chemical parameters contributing to biosurfactant production from the above selected strains.
3. Attempts for enhanced production of biosurfactant through mutational studies.
4. Purification and characterization of biosurfactant.
5. Studies to check the feasibility of above biosurfactant in controlling environmental pollutants and its applicability in field of oil, food and pharmaceuticals industries.

CHAPTER 2

LITERATURE REVIEW

The microbial world is the under pinning of the global ecosystem and has long been a source of tool organisms and their processes for the use of mankind. Yet, the natural microbial world remains a little known realm. The reason for our limited knowledge is that microbiologists generally have had to cultivate microorganisms in order to study them and indeed even to know of their existence. However, only small fractions of naturally occurring are cultivable. Even then there is no denial in saying life on the planet depends on microbial activities whether it is recycling of major elements that constitute living matter, removal of xenobiotic material from environment or production of important products, it is absolutely true in principle that we always look forward towards microbes for their helping hand (Klekner and Kosaric, 1993).

2.1 Definition of biosurfactants

Biosurfactants, as the name indicates is a term used to describe a large group of structurally diverse molecules produced by microorganisms whose common feature is that they possess surface active properties i.e. they tend to concentrate at the surface and interface between aqueous and gaseous or solids or non aqueous liquid phases (Fiechter, 1992). This property results from the fact that they are amphiphiles containing polar (hydrophobic) and non-polar (hydrophobic) parts which together with their specific chemical identity gives different surfactants their particular properties (Cooper, 1986).

2.2 Distinction between surfactants, detergent and emulsifier

A distinction could be drawn between the term surfactant and more generally used term detergent which refers to a commercial formulation or product that is designed with particular cleaning properties. Most modern detergent formulation contains, besides one third surfactant, larger amount of a builder (to act as a chelating agent) plus small amount of perfumes, colouring agents, whiteners, enzymes and other components. Another term which has been frequently used interchangeably with surface active molecules of microbial origin is emulsifier. Whereas the molecular structure of surfactant is defined i.e. surfactant has both hydrophobic and hydrophilic moieties present within same molecule. The term bioemulsifier is used in an application oriented manner to describe the combination of all the surface active compounds that constitute the emulsion secreted by the cell to facilitate uptake of insoluble substrate.

2.3 Microbial surfactant versus synthetic surfactants

The unique properties of biosurfactant allow their use and possible replacement of chemically synthesized surfactant in a great number of industrial operations. Surfactants are used by many industries and one could easily say that there is almost no modern industrial operation where properties of surface active agents are not exploit.

There are many advantages of biosurfactant as compare to their chemically synthesized counterparts. Some of those are :-

- Biodegradability and low toxicity
- Biochemical and digestibility, which allows their application in cosmetics, pharmaceuticals and as functional additives
- Availability of raw material in large quantities. The carbon source may come from hydrocarbons, carbohydrates and or/lipids, which may be used separately or in combinations with each other
- Acceptable production economics. Depending on the application biosurfactant can also be produced from industrial waste and byproducts and this is of particular interest for bulk production
- Biosurfactant can be tailor made to suit different applications by modifying the growth substrate e.g. when Rhamnose is used as growth substrate Rhamnose lipid is synthesized while a glucose lipid is formed when glucose is supplied as a growth substrate
- Use in environment control: Biosurfactant can be efficiently used in handling industrial emulsion, control of oil spills, biodegradation and detoxification of industrial effluents and in bioremediation of contaminated soil
- Specificity: Biosurfactant, being complex organic molecules with specific functional groups, are often specific in their action. This would be of particular interest in detoxification of specific pollutants, de- emulsification on industrial emulsion, specific cosmetic, pharmaceutical and food application.
- It has been seen that microorganism like *R. erthropolis, P. aeruginosa* and *B.subtilis* reduce the surface tension by 37, 29, and 27 mN/m respectively, as compared to synthetic surfactants like sodium lauryl sulphate that is to only 37 mN/m while a marked decrease in critical micelle concentration is seen in case of biosurfactant which is 15 mg/l as compared to anionic synthetic is seen where there it is only 2023-2890 mg/l.

2.4 Properties for efficient and effective biosurfactants

Biosurfactants can be distinguished as efficient or effective surface active agents by the following methods:

- Efficiency is measured by the concentration of surfactant required to produce some significant reduction in the surface tension of water, while effectiveness is measured by the minimum value of biosurfactant concentration which can lower surface tension.
- Efficiency usually increases with increase in the length of the hydrophobic part of the surfactant and decreases with increased unsaturation or branching. Efficiency also decreases when the terminal hydrophobic group is move to a central position on the other hand effectiveness increases with these changes.
- Efficiency depends upon the concentration of the surfactant at the interface. However, effectiveness depends upon the cohesiveness, the lower the attained surface tension value. Usually branched chain hydrocarbons have lower cohesiveness and hence are more effective surfactant than long chain hydrocarbons.

2.5 Types of biosurfactants

Biosurfactants are classified according to the nature of their polar grouping and categorized mainly by their chemical composition and microbial origin. The major classes of biosurfactant include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants and particulate surfactants.

2.5.1 Glycolipids

Glycolipids are carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. These include the rhamnolipids, sophorolipids, trehalolipids and fructose-lipids.

Rhamnolipid is a glycolipid that contains one or two molecules of rhamnose that are linked to one or two molecules of b-hydroxydecanoic acid. Up to 7 homologues have now been identified (Abalos *et al.,* 2001). L-Rhamnosyl-L-rhamnosyl- b-hydroxydecanoyl-b-hydroxydecanoate and L-rhamnosyl-b- hydroxydecanoyl-b-hydroxydecanoate, referred to as rhamnolipid 1 and 2, respectively, are the principal glycolipids produced by *P. aeruginosa* (Desai and Banat, 1997). Rhamnolipids from *P. aeruginosa* (figure 1) have been

demonstrated to lower the surface tension to 25 to 30 mN/m and the interfacial tension against *n*-hexadecane to 1 mN/m (Lang and Wagner, 1987; Mulligan, 2004).

Different species of the yeast *Candida bombilica* produce extracellular sophorolipids, which consist of dimeric carbohydrate sophorose linked to long chain hydrocarboxylic acids. The lipid portion is connected to the reducing end through a glycosidic linkage (Rosenberg and Ron, 1999). These biosurfactants are a mixture of at least 6-9 different hydrophobic sophorosides.

Sophorolipids has also been reported capable of lowering both surface and interfacial tension, though they are not effective emulsifying agents (Cooper and Paddock, 1984; Kitamoto *et al.*, 2002). Both lactonic and acidic sophorolipids lowered the surface tension to 33 mN/m and the interfacial tension against *n*-hexadecane and water from 40 to 5 mN/m with 10 mg/l of pure sophorolipid. It showed a remarkable stability towards pH and temperature changes. The surface active properties were consistent between pH values of 6-9 and temperature ranging from 20-90°C.

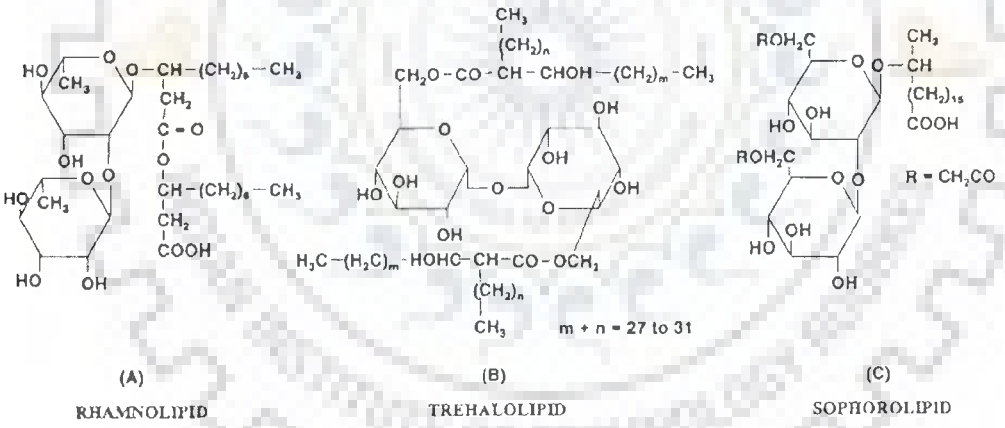


Figure 1: Structure of some common glycolipid of biosurfactants A) Rhamnolipid produced by *P. aeruginosa* B) Trehalose dimycolate produced by *R. erythropolis* and C) Sophorolipid from *T. bombicola*

Biosurfactant types	Microorganisms	Reference
Glycolipids		
Rhamnolipids	*P. aeruginosa* *Pseudomonas* sp.	Guerra-Santos *et al.*, 1986; Robert *et al.*, 1989, Hisatsuka *et al.*, 1971; Lang *et al.*, 1989
Trehalolipids	*R. erythropolis* *N. erythropolis* *Mycobacterium* sp.	Rapp *et al.*, 1979 Margaritis *et al.*, 1980; Margaritis 1979 Cooper *et al.*, 1989
Sophorolipids	*T. bombicola* *T. apicola* *T. petrophilum*	Cooper *et al.*, 1989; Gobbert *et al.*, 1984 Hommel *et al.*, 1987, Cooper and Paddock 1983
Cellobiolipids	*U. zeae*, *U. maydis*	Boothroyd *et al.*, 1956, Syldatk *et al.*, 1985
Lipopeptides and lipoproteins		
Peptide-lipid	*B. licheniformis*	Yakimov *et al.*, 1995, Javaheri *et al.*, 1985
Serrawettin	*S. marcescens*	Matsuyama *et al.*, 1991
Viscosin	*P. fluorescens*	Neu and Poralla 1990
Surfactin	*B. subtilis*	Arima *et al.*, 1968, Bernheimer *et al.*, 1970
Gramicidins	*B. brevis*	Marahiel *et al.*, 1977
Polymyxins	*B. polymyxa*	Suzuki *et al.*, 1965
Subtilisin	*B. subtilis*	Bernheimer *et al.*, 1970
Fatty acids, neutral lipids, and phospholipids		
Fatty acids	*C. lepus*	Cooper *et al.*, 1983, 1978
Neutral lipids	*N. erythropolis*	Lin *et al.*, 1994
Phospholipids	*T. thiooxidans*	Koch *et al.*, 1988
Polymeric surfactants		
Emulsan	*A. calcoaceticus*	Rosenberg *et al.*, 1979; Zosim *et al.*, 1982
Biodispersan	*A. calcoaceticus*	Rosenberg *et al.*, 1988,
Mannan-lipid-protein	*C. tropicalis*	Kappeli *et al.*, 1984
Liposan	*C. lipolytica*	Cirigliano and Carman, 1985, 1984
Carbohydrate-protein-lipid	*P. fluorescens* *D. polymorphis*	Desai *et al.*, 1988; Persson *et al.*, 1988 Singh and Desai, 1989
Protein PA	*P. aeruginosa*	Hisatsuka *et al.*, 1972, 1971
Particulate biosurfactants		
Vesicles and fimbriae	*A. calcoaceticus*	Gutnick *et al.*, 1987; Kappeli *et al.*, 1979

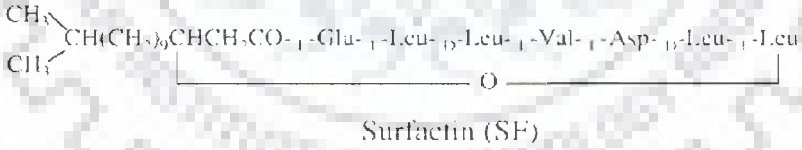
Table 1: Major types of biosurfactant produced by microorganisms.

2.5.2 Lipopeptides

Lipopeptides usually appear as mixtures of closely related compounds which show slight variations in their amino acid composition and/or lipid portion which is mostly a hydroxy fatty acid. A family of cyclic lipopeptides consists of 8 to 17 amino acids and a lipid portion which is composed of 8 to 9 methylene groups and a mixture of linear and branched tails (Desai and Banat, 1997).

Major research on lipopeptides has been concentrated on class of lipopeptides formed by certain strain of *B. subtilis* and *B. pumilis*. Surfactin, a cyclic acidic lipopeptide produced by *B. subtilis*, is one of the most effective biosurfactants known so far (Arima *et al.*, 1968; Mulligan, 2004). It contains seven amino residues and is closed by lactone formation (Carillo *et al.*, 2003). Surfactin is known to be capable of lowering the surface tension from 72 to 27.9 mN/m at a concentration of 0.005% (w/v). An important characteristic of this compound is its ability to lyse red blood cells and may act as an antibiotic, antiviral and hemolytic agent (Arima *et al.*, 1968; Carillo *et al.*, 2003; Cameotra and Makkar, 2004). This property has been used to detect surfactin production through blood agar.

Different strains of *B. licheniformis* produce lipopeptide biosurfactants e.g. lichenysin A (Yakimov *et al.*, 1995), lichenysin B (Lin *et al.*, 1994; McInerney *et al.*, 1990), lichenysin C (Jenny *et al.*, 1991), biosurfactant BL-86 (Satoh *et al.*, 1990).



Surfactin (SF)

Figure 2: Structure of cyclic lipopeptide surfactin produced by *B. subtilis*.

A large number of cyclic lipopetides including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) produced by *B. brevis* (Marahiel *et al.*, 1977) and *B. polymyxa* (Suzuki *et al.*, 1965) respectively, possess remarkable surface active properties. Other types of lipopeptides include orinthine containing lipids from *P. rubescens* (Yamane *et al.*, 1987). Cerilipin an ornithine and taurine containing lipid from *Gluconobacter cerinus IFO 3267* (Tahara *et al.*, 1976).

Other lipopeptides include viscosin produced by *P. fluorescens* (Laycock *et al.*, 1991) and arthrofactin, produced by *Arthrobacter* spp. Strain MIS38 (Morikawa *et al.*, 1993).

Recent structural analysis revealed that it is a mixture of lipopeptides with major components ranging in size from 979 to 1,091 Da. Each molecule contains seven amino acids and a lipid portion which is composed of 8 to 9 methylene groups and a mixture of linear and branched tails. They vary in amino acid composition, position of the lactone ring and lipid portion.

2.5.3 Fatty acids, Phospholipids and Neutral lipids

Fatty acid and phospholipids produced during growth on *n*-alkanes by several bacteria and yeast, have received considerable attention as surfactants (Rosenberg *et al.*, 1999). These biosurfactants are able to produce optically clear microemulsions of alkanes in water (Desai and Desai, 1993). The hydrophilic lipophilic balance (HLB) of fatty acids is found clearly related to the length of the hydrocarbon chain. Example of microorganisms that produced these types of biosurfactants are sulphur-reducing bacteria, *Thio B. thiooxidans* (Beeba and Umbreit, 1971; Desai and Banat,1997) and *Corynebacterium lepus* (Cooper *et al.*, 1979; Rosenberg and Ron, 1999). Extracellular free fatty acids produced by microorganisms grown on alkanes also showed surfactant activity. They include saturated fatty acids in the range of C- 12 to C-14 and the complex fatty acids containing hydroxyl groups and alkyl branches. Phosphatidylethanolamine produced by *Rhodococcus erythropolis* caused a lowering of interfacial tension against hexadecane to less than 1 mN/m and a CMC of 30 mg/L (Kretschmer *et al.*, 1982; Rosenberg and Ron, 1999).

2.5.4 Polymeric biosurfactants

Many bacterial species from different genera produced exocellular polymeric surfactants composed of proteins, polysaccharides, lipopolysaccharides or complex mixture of these biopolymers (Rosenberg *et al.*, 1999). Fatty acids are covalently linked to the polysaccharides through *o*-ester linkages (Zukerberg *et al.*, 1979; Desai and Banat, 1997). The best-studied polymeric biosurfactants are emulsan, liposan and mannoprotein (Desai and Banat, 1997). Emulsan has been characterized as a polyanionic amphipatic heteropolysaccharide (Rosenberg *et al.*, 1979; Rosenberg and Ron 1999). It is a very effective emulsifying agent for hydrocarbons in water even at a concentration of 0.001%. Emulsan also is one of the most powerful emulsion stabilizer known today and resists inversion even at a water-oil ratio of 1:4 (Desai and Banat, 1997).

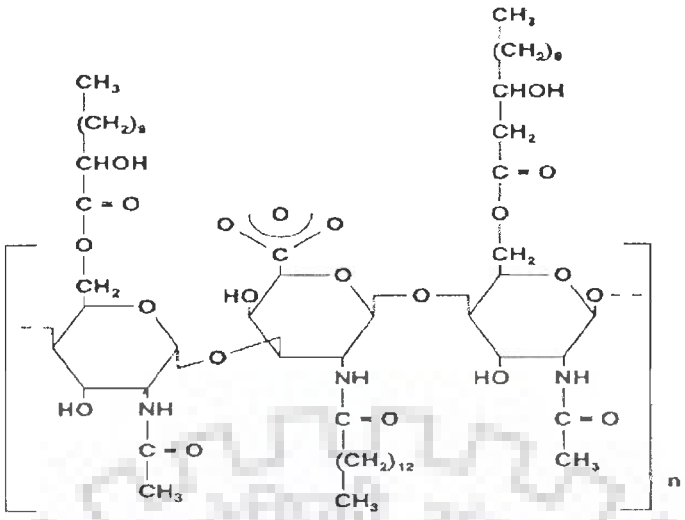


Figure 3: Structure of emulsan, produced by *Acinetobacter calcoaceticus*.

2.5.5 Particulate biosurfactants

This type of biosurfactant includes vesicles and fimbriae produced by *Acinetobacter* sp. (Kappeli and Finnerty, 1979). The purified vesicles are composed of proteins, phospholipids and lipopolisaccharides. This extracellular membrane vesicles partition hydrocarbons form a microemulsion, which plays an important role in alkane uptake by microbial cells (Kappeli and Finnerty, 1979; Desai and Banat, 1997).

Generally, biosurfactant molecules consisted of both hydrophilic and hydrophobic moieties which enable them to accumulate at the interfaces and mediated between phases of different polarity such as oil-in-water or water-in-oil interfaces (Fiechter, 1992). The polar, water soluble part of a biosurfactant may be as simple as a carboxylate or hydroxyl function or a complex mixture of phosphate, amino acids or peptides, anions or cations, or mono-, di- or polysaccharides. Whereas the lipophilic portions are the hydrocarbon tail that usually made of long chain, saturated, unsaturated, hydroxyl or a-alkyl-b-hydroxy fatty acids and may contain cyclic structures (Banat, 1995). This fatty acid is linked to the hydrophilic group by a glycosidic, ester or amide bond (Hommel, 1990; Rosenberg and Ron 1999). Therefore, most of the biosurfactants are lipids, which have the typical amphiphilic structure of a surfactant. Most biosurfactants are either neutral or negatively charged. The negatively charged is an anionic biosurfactants which due to a carboxylate, phosphate or sulphate group. Least number of cationic biosurfactants contains amine functions (Cooper, 1986). A special property of a biosurfactant is their ability to reduce the surface tension of water from 72

mN/m to below 40 mN/m. Furthermore, a good biosurfactant will reduce the surface tension of water to below 35 mN/m (Cooper, 1986; Desai and Banat, 1997). Effective physicochemical properties, which are low interfacial tensions and critical micelle concentration (CMC) and also temperature stability, are the characteristics of these compounds. Their heterogeneous group of surface-active molecules also reduces the CMC and interfacial tension in both aqueous solutions and hydrocarbon mixtures. These properties create micro emulsions in which micelle formation occurs where hydrocarbons can solubilize in water or water-in-hydrocarbons (Banat, 1995).

A biosurfactant must have the ability to improve water loss, which can wet the solid surfaces (Cooper, 1986). Some of the biosurfactants also has the ability to act as an emulsifier. Unfortunately, most of emulsifiers characterized were found to be polymeric, with minimal ability to lower surface tension (Cooper, 1986).

2.6 Evaluation of microbial surface active compounds

Microorganisms have large surface to volume ratios, produce a variety of surface active agents, which alters the conditions prevailing at interfaces. The primary requirement for a surfactant is that it be absorbed, usually from a liquid phase, at one or more interfaces of the system under consideration. The reason that most surfactants concentrate at the interface is that they are amphiphatic i.e. surfactant contains both hydrophobic group usually a hydrocarbon chain which tends to be expelled by water and polar group which is hydrophilic and tends to remain in water.

This give rise to a amphiphatic adsorption in which the hydrophobic groups are oriented away from the water and polar groups towards it. This unique ability of lipids to form micelle (spherical or cylindrical) or bilayer is based mainly on the area of the hydrophilic head and the chain length of the hydrophobic tail. Molecules with small chain length and large head groups generally form spherical micelles. Those with smaller head group tend to associate into cylindrical micelle, while those with long hydrophobic chain from bilayers which in turn, under conditions form vesicles to arrange in different complex forms. The force that hold this structure together in fluid hydrophobic, vanderwall's electrostatic and hydrogen bonding interactions. Since no chemical bonds are formed, these structures are fluid like and are easily transformed from one state to another as conditions such as electrolyte concentration and temperature are changed.

2.6.1 Surface activities

2.6.1.1 Surface tension

Surface tension is a phenomenon involving the cohesive forces between liquid molecules. The molecules at the surface have no neighbouring atoms and adhere more strongly to those directly associated with them on the surface (Garrett, 1972). This would enhance the intermolecular attractive forces at the surface makes it more difficult to move an object through the surface than to move it when it is completely summarized (Attwood and Florence, 1983).

Surface tension can be defined in terms of work, W as follows (Garret, 1972):

Surface tension, $\gamma = W/\Delta A$ where ΔA is the change in surface area.

It can also be defined as the force, F per unit length, L tending to pull the surface back

Surface tension, $\gamma = F/L$

Thus, surface tension is a measurement of the intermolecular attractive forces, which is Van der Waals force in a given liquid. The molecules on the surface of the liquid experience these forces differently to the air than to the liquid. By introducing a substrate into the surface, one with a zero degree contact angle with the liquid, all of the intermolecular forces will pull down on the substrate, thus making the surface tension directly proportional to the balance force of a balance connected to the substrate.

For rapid and accurate measurement of surface tension, Autotensiomat apparatus is generally used. The Du-Nouy surface tension meter is equipped with a sensitive guage and a platinum-iridium ring to be placed just below the surface of the sample. The process involved is the determination of the force required to detach the ring form the surface of the liquid. It is necessary that the wire should be completely wetted by the liquid. The amount of force required to break through the surface is recorded in terms of mN/m.

2.6.1.2 Interfacial tension

At the boundary between two liquids say liquid 'X' & liquid 'Y', a molecule of 'X' is attracted by other 'X' molecule with a force different from that exerted on the same molecule by the molecules of 'Y'. Consequently, a tension exists at this liquid/liquid boundary just as the liquid/vapour interface. Only the nomenclature is slightly different; the terms interfacial tension and interfacial free energy are used instead of surface tension or surface free energy. The dimensions of all four quantities are identical for equilibrium interfacial tensions, a qualitative rule is available. When the interfacial tension is greater, the greater is the

difference between the two phases. This difference decreases on an increase in the mutual solubility when the solubility is greater enough, the two liquids become identical and the interfaces vanishes.

2.6.1.3 Critical micelle concentration

When a surfactant is added to air/water or oil/water system at increasing concentration, reduction of surface tension is observed only up to a critical level beyond which micelle formation takes place. This value is known as critical micelle concentration. In other words, CMC is the concentration of surfactant necessary to initiate micelle formation and is commonly used to measure the surfactant efficiency. At concentration higher than the CMC, it is supposed that additional surfactant molecules aggregate into micelle in the bulk phase and do not contribute to significant further change in the interfaces.

The CMC can be estimated from a plot of surface tension versus the log of percent dilution. The dilution at which surface tension abruptly starts to increase is the CMC of the surfactant. The reciprocal of the CMC (CMC^{-1}) is proportional to the amount of surfactant present and can be used as an approximate measure for biosurfactant concentration. The corresponding dilution of the culture broth is termed as the critical micelle dilution.

2.6.1.4 Hydrophilic lipophilic balance

The hydrophilic-lipophilic balance or HLB value of surfactant is a frequently quoted parameter that can help to predict whether that surfactant will promote w/o or o/w emulsions. The HLB scale is an arbitrary measure of emulsifying properties which assign values of 1.0 to oleic acid and of 20 to sodium oleate. With intermediate value based proportionally on mixture of the two, new surfactants are assessed by comparison with surfactants of known HLB. Surfactants which are more hydrophobic than hydrophilic have HLB value less than 7.0, whereas predominantly hydrophilic surfactants have HLB value greater than 7.0.

2.6.2 Colorimetric and axisymmetric drop shape analysis

Recent advances in this field of surface active agents are largely attributed to the development of quick and reliable method for screening biosurfactant producing microbes and assessing their potential. Detection of biosurfactant by colorimetric estimation was described by Shulga *et al.,* (1993). This test is based on the ability of anionic surfactant to react with the cationic indicator to form a coloured complex. Hansen *et al.,* (1993) and Sigmund and Wanger (1991), also described a colorimetric method for hydrocarbon

degrading and rhamnolipid producing microbial strain, *Pseudomonas spp.* respectively. Vandervegt *et al.,* (1991), assessed the potential of biosurfactant producing bacteria by axisymmetric drop shape analysis (ADSA). In this technique a drop of culture broth is placed on a fluoroethylene propylene surface and the profile of the droplet is determined with a contour monitor.

2.6.3 Drop collapsing test

Biosurfactant is a microbially produced surface-active agent that contains both hydrophobic and hydrophilic groups. Due to their amphipathic nature, surfactants are not uniformly distributed in the solvent but congregate at the solvent surface (Jain *et al.,* 1991). Thus, availability of hydrocarbons and slightly soluble organic compounds can be enhanced by biosurfactants, which can increase aqueous dispersion by many orders of magnitude and reduce the surface and interfacial tensions of aqueous medium.

There are two types of intermolecular attractive forces occurred to molecules liquid (Rao, 1972). Cohesive forces are referred when those forces occur between like molecules. When this cohesive forces at the surface are strong enough, the molecules of a water droplet are held together constitute surface tension. When the attractive forces are between unlike molecules, they are called adhesive forces. Both of the attractive forces between molecules in a liquid can be viewed as residual electrostatic forces and this is called Van der Waals forces (Garret, 1972).

A drop-collapsing technique has been defined as a qualitative assay to screen biosurfactant-producing bacteria. Solutions containing potent biosurfactant will be unable to form stable drops and spread completely over the oily surface, where as solutions without surfactant will retain the drop configuration on the oily surface (Jain *et al.,* 1991). This method is simple, sensitive, easy to perform, reproducible and requires little specialized equipment (Bodour and Miller-Maier, 1998). However, this technique is not correlated to surface tension reduction to confirm its reliability (Youssef *et al.,* 2004).

2.6.4 Emulsification activity

Estimation of emulsification value (E_{24}) to see the emulsification activity of the surfactants was done by vigorously shaking culture broth samples with kerosene oil and measuring the percentage emulsification after 24h (Cooper and Goldenberg 1987). The emulsion index E_{24} is calculated as the ratio of the height of the emulsion layer and the total height of liquid

E_{24} correlates to the surfactant concentration. Evaluating the emulsification capacity is a simple screening method suitable for a first screening of biosurfactant producing microbes. It is applied in many screenings (Willumsen *et al.,* 1997; Schulz *et al.,* 1991; Makkar and Cameotra, 1997; Plaza *et al.,* 2006) whereas the kerosene can be replaced with other hydrophobic compounds, e.g., hexadecane. But surface activity and emulsification capacity do not always correlate (Plaza *et al.,* 2006; Van Dyke *et al.,* 1993). Consequently, this method gives just an indication on the presence of biosurfactant.

2.6.5 Hemolytic activity

Hemolysis on blood agar has been widely used to screen biosurfactant producing bacteria and for preliminary identification of many types of clinically important bacteria (Mulligan *et al.,* 1984). Moran *et al.,* (2002), demonstrated that a cyclic lipopeptide biosurfactant can be easily detected through its ability to cause lyses of mammalian erythrocytes and form spheroplasts. Blood agar is purposely used as an enriched medium for growing of fastidious bacteria and as a differential medium. This technique was first discovered by Bernheimer and Avigad (1970) that reported the production of biosurfactant (surfactin) by *B. subtilis* may cause the red blood cells to lysis. It has been used previously to quantify surfactin (Moran *et al.,* 2002) and rhamnolipids (Johnson and Boese-Marrazzo, 1980). Now-a-days, many researchers have used this technique to screen for biosurfactant production by new isolates (Carrillo *et al.,* 1996; Yonebayashi *et al.,* 2000). However, the method has some limitations (Jain *et al.,* 1991). 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. In addition, Schulz *et al.,* 1991, showed that some biosurfactants do not show any hemolytic activity at all. Youssef *et al.,* (2004) and Plaza *et al.,* (2006) also confirmed the poor specificity of this method. Mulligan *et al.,* (1984) recommend the blood agar method as a preliminary screening method which should be supported by other techniques based on surface activity measurements.

2.6.6 Cell surface hydrophobicity

As reported by Pruthi and Cameotra (1997) this method has been used for rapid evaluation of biosurfactant producing microorganisms which involves cell surface hydrophobicity. Studies on cell surface hydrophobicity such as charges and hydrophobic properties provide valuable insight in understanding the concept of hydrophobicity which is generally associated with a

polar molecule having low surface energy. The simplicity of this technique suggests its implementation in screening of biosurfactant producing microorganisms from the natural sources. Moreover, this technique provides a step further for assaying biosurfactant formation prior to its actual isolation using organic solvents.

Cell surface hydrophobic properties can be measured by:-

2.6.6.1 Hydrophobic interaction chromatography (HIC)

HIC is a chromatographic procedure based on hydrophobic interaction between a non polar group in a gel and non polar region of the solute. The test consists of measuring the amount of cells retained by a hydrophobic gel. The data are expressed in percentage of retention(R): Rp = 100(A1-A0) and Rf= 100(A1-A1)/A1 where A1 is related to the total amount of cells used in the test, A0 is related to the amount of cells which are not retained by gel under conditions favouring hydrophobic interaction i.e. high ionic strength and low water activity. A1 and related to the amount of cells which are not retained by the gel under conditions which do not favour hydrophobic interaction i.e. low ionic strength Rf is the proportion of cells retained by the gel at high ionic strength. Rf is the proportion of cells which remain retained in conditions of lower strength, the latter being imposed by the pH controlling agent.

2.6.6.2 Salt aggregation test (SAT)

The test consists of provoking cell flocculation by increasing salt concentration. The order in which cells are precipitated is the measure of their hydrophobicity, the most hydrophobic being precipitated at low salt concentration that is, the lower the salt concentration required to aggregate the cells, the more hydrophobic is the surface of cells. It is expressed as lowest morality of salt causing aggregation and is observed against a black background.

2.6.6.3 Bacterial adherence to hydrocarbons (BATH)

This assay procedure of evaluating biosurfactant production is based on decrease in absorbance of the lower aqueous phase of microbial suspension when mixed with different hydrocarbons. This simple quantitative procedure suggests that the ability of adhering to hydrocarbon is a characteristic feature of biosurfactant producing microorganism.

2.6.6.4 Adherence to polystyrene: replica plate test (RP)

A simple replica plate method is described for evaluation of the rapid identification of microbial colonies which adhere to polystyrene. In this technique, a flat polystyrene disk is

pressed on the surface of an agar plate containing the colonies to be screened. This replica of the colonies obtained on the polystyrene surface is then washed under a vigorous stream of tap water to remove all cells which were not firmly bound. At this stage translucent area corresponding to the colonies of adherent cells can be observed on the polystyrene surface. Microscopic observation of these spots reveals a dense layer of attached cell and hence is scored positive for their hydrophobic character.

2.7 Biosynthesis of biosurfactants

The metabolic pathways involved in the synthesis of the precursors of biosurfactants are diverse and to some extent dependent on the nature of the principal carbon source. Sugars are required either for the synthesis of structural entities of the cell or for the biosynthesis of amino acids, proteins and nucleic acids. Biosynthetic paths to biosurfactants include the formation of the hydrophilic and the lipophilic moiety of the molecule. These are obviously different and involve partly opposite metabolic routes mainly to carbohydrate containing biosurfactants. The biosynthesis of biosurfactants, in general, and especially glycolipid biosynthesis may fall into one of the following pathways according to Haferburg *et al.* (1986) and Syldatk and Wagner (1987):-

a) Both hydrophilic and lipophilic moieties are synthesized independently of the growth substrate *(de novo* synthesis).

b) The synthesis of lipid moiety depends on the hydrophobic carbon source and is derived from it; the hydrophilic moiety is synthesized *de novo.*

c) The hydrophilic moiety reflects the carbon source used for growth or maintenance, the lipid moiety is synthesized *de novo.*

d) The synthesis of both residues depends on carbon substrate used.

In carbohydrate (fructose, glycerol) containing media, rhamnolipids are produced by *P. aeruginosa* cells at the transition to a stationary growth phase (Hauser and Karnovsky, 1954). The synthetic pathway was elucidated (Hauser and Karnovsky, 1954; Hauser and Karnovsky 1958; Burger *et al.,* 1963) and the optimal conditions for rhamnolipid production by this organism from various radioactive precursors, such as acetate, glycerol, glucose and fructose were established Burger *et al.* (1963). They also described the complete enzymatic synthesis of rhamnolipid by extracts of *P. aeruginosa.* They were able to show that the synthesis of rhamnolipid proceeds by sequential glycosyl transfer reaction, each catalyzed by a specific rhamnosyl transferase and that TDP-rhamnose is an efficient rhamnosyl donor in the synthesis of the rhamnolipid. Whereas RL1 & RL2 are the predominant rhamnolipids

produced in liquid cultures, RL3 and RL4 containing two sugar moieties and one fatty acid moiety and one sugar and one fatty acid moiety, respectively appeared to be produced exclusively by resting cells (Syldatk *et al.,* 1985).

Other examples for the *de novo* synthesis of biosurfactant are the sophorolipids produced by resting and growing cells of *Torulopsis bombicola* from different lipophilic substrates (Gobbert *et al.,* 1984; Hommel *et al.,* 1994; Asmer *et al.,*1988; Cooper and Paddock, 1984; Itoh and Inoue, 1982). Detailed studies concerning excretion and localization of.the whole biosynthetic pathway and its regulation are not yet available. Rilke *et al.* (1992) documented the probable existence of periplasmic vesicle in *T. apicola* derived from the cytoplasmic membranes and which could be separated from the cytoplasmic membrane after cell protoplasting. In *Serratia marcescens,* biosurfactants have been reported to be the main lipid components of excellular vesicles (Matsuyama *et al.,* 1991).

The biosynthetic pathway of the alkane-induced, trehalose mycolates of *R. erythropolis* (Kretschmer *et al.,* 1982; Rapp *et al.,* 1979) differ from the usual sugar linked trehalose mycolated synthesis by the appearance of free intermediates. An influence of the substrate on the sugar moieties of the produced glycolipids is reported by *Arthrobacter paraffineus* KY9303 (Itoh & Suzuki, 1974; Suzuki *et al.,* 1974). This microorganism formed nonionic trehalose lipid when growth on w-alkanes as the sole carbon source. *Arthrobacter paraffineus* KY4303 produced fructose lipids when fructose was used as carbon source but with sucrose, glucose and sucrose lipids were isolated from the culture broth (Suzuki *et al,* 1974). Various studies show that synthesis of number of peptide antibiotics (which display interesting surface active characteristics) in the members of genus *Bacillus is* catalyzed by large multienzyme complexes called peptide synthetase (Kleinkauf and von Dohren, 1990; Lipmann, 1980). The investigation of this mutienzyme contributed basic knowledge on the mechanism of non-ribosomal peptide synthesis. Such multienzyme system which catalyzes these processes activate their substrate aminoacid in two steps involving aminoacyl adenylates and thioesters as reactive intermediates. The first stage involves the activation of amino acids via adenylation (aminoacyl adenylate formation) by ATP.

$$\text{Amino acid} + \text{ATP} \longleftrightarrow \text{Amino acyladenylate} + \text{PPi}$$

The activated amino acids are ultimately transfered and bound to specific sites on the multienzyme complex with reactive sulfhydryl group by thioester linkages. These reaction

centers represent the thiotemplate sites. AMP is formed in this step. This step is also reversible.

Amino acyladenylate + Thioester binding site ⟷ Thioester bound amino acid + AMP

Assembly of the peptide involves a 4' phosphopantetheine cofactor having a reactive-SH group. Multifunctional peptide synthetases of this type are equipped with a 4'-phosphopantetheine arm which functions as an internal swinging arm to mediate the transport of the growing peptide chain between the sites of attachment of the activated amino acids. In other words, it has been postulated (Kleinkauf *et al.*, 1971) that the substrates which are continuously provided at reaction centres are linked together in a series of transpeptidation and transthiolation processes by interaction of the internal central SH group of the phosphopentetheine carrier with the peripheral thiol groups at the reaction centres (Lipmann, 1980; Kleinkauf *et al.*, 1971). This mode of synthesis is called the thiotemplate mechanism. The general scheme for enzymatic peptide formation is as under:

Aminoacyladenylate	**thioester**	**transfer**
$E_1+a_1+MATP^{2-} \rightarrow E(a_1\ AMP) \rightarrow$	$E_1\text{-}S_1\text{-}n_1 \rightarrow$	$E\text{-}S_p\text{-}n_1$
$E_2+a_2+MATP^{2-} \rightarrow E(a_2\ AMP) \rightarrow$	$E_2\text{-}S_2\text{-}a_2 \rightarrow$	$E_2\text{-}S\text{-}a_2\ a_1$
		$E\text{-}S_p\text{-}a_2\ a_1$
$E_3+a_3+MATP^{3-} \rightarrow E(a_3\ AMP) \rightarrow$	$E_3\text{-}S_3\text{-}a_3 \rightarrow$	$E_3\text{-}S\text{-}a_3\ a_2\ a_1$
		$E\text{-}S_p\text{-}a_3\ a_2\ a_1$
$E_n+a_n+MATP^{2-} \rightarrow E(a_n\ AMP) \rightarrow$	$E_n\text{-}S_n\text{-}a_n \rightarrow$	$En\text{-}S_n\text{-}a_n.a_{n1}....a_1$

In this scheme E_n represents an enzyme site for the activation of the nth amino acid a_n of the peptide, the site being either a specific enzyme *per se* or a specific enzyme site within a multienzyme. The amino acid is activated at the expense of ATP (here indicated as $MATP^{2-}$, with M standing for Mg^{2+} or Mn^{2+}), via the formation of enzyme-stabilized aminoacyl-adenylate (a_n AMP). This is then attached to a specific enzyme thiol group S^nH; located in a specific multienzyme site E_n. The transfer of intermediates is mediated by a panthetheine thiol S_pH, located in a specific multienzyme site E. The growing peptides remain bound to the enzyme at their specific multienzyme sites or to the cofactor.

2.8 Regulation of biosurfactant synthesis

For a production of microbial metabolites on a large scale, it is necessary to know the regulatory mechanism of the microorganism used. Only in this way, it will be able to cause an over production of a desired product. Concerning the biosynthesis of biosurfactant, there are some general phenomena, although these substances occur in different structures (Rosenberg, 1986). The production of biosurfactants, in general is regulated by three mechanisms. Viz. (i) induction, (ii) repression and (iii) nitrogen and multivalent ions mediated effects (Desai *et al.*, 1994).

a) Induction

Microbial production of biosurfactant can be achieved by hydrocarbon or other water insoluble substrate (Cooper and Paddock, 1984; Kappeli and Finnerty, 1979). The carbon source is important in influencing the biosurfactant synthesis by induction (Cameotra and Makkar, 1998). In some cases, addition of water immiscible substrates results in induction of biosurfactant production. *Pseudomonas and Candida* sp. (Cirigliano and Carman, 1984; Kawashima *et al.*, 1983) when grown on n-alkanes secrete surface active substrates in the medium. These substances were not detected during the growth on carbohydrates. Rapp *et al.*, (1979) reported that a carbon source induced the formation of non-toxic, cell wall bound corynomycolates by *Rhodoccous erythropolis*. *C. lipolytica* produced an inducible extrcellular biosurfactant (liposan) when grown on water immiscible carbon substrates. Tulloch *et al.*, (1962), have found that the induction of sophorolipid synthesis by addition of long chain fatty acids, hydrocarbons or glycerides to the growth medium of *Torulopsis magnoliae*.

b) Repression

The second phenomenon is the catabolite repression of biosurfactant synthesis by glucose and other primary metabolites. This effect observed in the first step of n-alkane oxidation plays an important role in biosynthesis of many surface active compounds produced by microorganisms. Hauser and Karnosky (1958) have demonstrated a drastic reduction in rhamnolipid synthesis on addition of glucose, acetate and tricarboxylic acids during the growth on glycerol. Conversely, when *B. subtilis* C9 was cultured in a glucose containing medium, it produced a high yield of lipopeptide biosurfactant where as production was inhibited upon addition of hydrocarbon in the medium (Cooper *et al.*, 1981).

c) Effect of nitrogen source and multivalent cations

Nitrogen also plays an important part to the regulation of biosurfactant synthesis. Duvnjak *et al.* (1983), found that urea led to a satisfactory biosurfactant production (Cameotra and Makkar, 1998). Moreover, nitrogen limitation also changed the composition of the biosurfactant production (Syldatk *et al.*, 1985; Desai and Desai, 1993). Phosphate limitation also influences the metabolism of biosurfactant. The change in activity of several intracellular enzymes like alkaline phosphatase, glucose-6-phosphate dehydrogenase and transhydrogenase dependent on phosphate levels indicated a shift in biosurfactant metabolism (Mulligan and Gibbs, 1993).

The limitation of multivalent cations also causes overproduction of biosurfactants (Itoh and Suzuki 1974; Guerra-Santos *et al.*, 1984; Cameotra and Makkar, 1998). Higher yield of rhamnolipid could be achieved in *P. aeruginosa* DSM 2659 by limiting the concentrations of magnesium, calcium, potassium, sodium and trace element salts (Desai and Desai, 1993). Finally, the environmental factors and growth conditions such as temperature, agitation and oxygen availability also affect biosurfactant production through their effect on cellular growth or activity (Cameotra and Makkar, 1998).

2.9 Economics of biosurfactant Production

An important aspects of biosurfactant for a certain field of application is only their technological operation but also the attainable market price. Although many biosurfactants and their production processes have been patented only few biosurfactants like emulsan, a lipopolysaccaride produced by *Acinetobacter spp.* have been commercialized. The reduction cost for all aspects of biosurfactant production involves:-

- Choice of inexpensive raw materials containing carbon, nitrogen, phosphate sources, metals and other components such as sulphur, potassium, calcium and magnesium also required for microbial growth.
- Increase of biosurfactant yield and production rate by biosynthesis control, screening for overproducers, or by genetic manipulation of microorganisms.
- Optimization of fermentation process by closely examining pH, temperature, agitation and aeration rate.
- Reduction of product recover cost which include concentration of produced from fermenter, broth characteristics (pH, viscosity, complexity), product characteristics (molecular wt. Solubility, charge, cell wall bound or extracellular), product yield by

the recovery procedure involve Broth conditioning (pH or heat charge); centrifugation, cell disruption, solvent extraction, adsorption, ultrafiltration etc.

- Production of biosurfactant suitable for specified applications

2.10 Production of biosurfactants

Biosurfactants do not compete economically with synthetic surfactants. Millions of tons of hazardous and non-hazardous wastes are generated each year throughout the world. The treatment and disposal costs for these wastes are a vast financial burden to various industries and may soon outstrip available resources. Thus, there is a great need for better management of these wastes via the concept: reduce, reuse, and recycle. Waste disposal is a growing problem, which explains the increasing interest in use of waste in microbial transformation. To reduce production costs, other carbon sources, such as olive oil mill effluent, whey from cheese making and cassava flour water, used vegetable oils, molasses (by-product) which are industrial waste can be used for biosurfactant production Table 2.

Several studies with plant-derived oils have shown that they can act as effective and cheap raw materials for biosurfactant production. Vollbrecht *et al.,* (1999) investigated the production of biosurfactants using domestic vegetable oils in order to convert renewable resources into higher value products. Their study involved glycolipid production from *Tsukamurella* spec DSM 443, using natural vegetable oils rather than complex media and hydrophobic carbon sources. Of the vegetable oils tested, oleic acid-rich oils and rapeseed oil (C 22:1) gave the best results. On sunflower oil (C18:1), a yield of around 5 g/l glycolipid was obtained. Physiochemical properties of biosurfactants obtained showed that glycolipid could reduce the surface tension of water from 72 mN/m down to 35 mN/m with a critical micelle concentration (cmc) value of 10 mg/l. Sarubbo *et al.,* (1997) evaluated the production of bioemulsifiers by two strains of *Candida lipolytica* (1055 and 1120) using media supplemented with 5% BabaCu oil and 1% glucose as carbon source. Furthermore, various waste oils with their origins at the domestic level, in vegetable oil refineries or the soap industries were found to be suitable for microbial growth and biosurfactant production.

The lactic whey from dairy industries has also been reported as a cheap and viable substrate for biosurfactant production. The effluent from the dairy industry, known as dairy wastewater, supports good microbial growth and is used as a cheap raw material for biosurfactant production. Lactic whey is composed of high levels of lactose (75% of dry matter), 12–14% protein, organic acids and vitamins. Disposal of whey is a major pollution problem for countries depending on dairy economics. Koch *et al.,* (1988) used the lactose

utilizing capability of *Escherichia coli* by cloning the lactose gene Lac ZY from *E. coli* in *P. aeruginosa*, which then grew on whey for biosurfactant production. In addition, Daniel et al. (1998) achieved production of high concentrations of sophorolipids using a two-stage cultivation process: first, deproteinized whey concentrate (DWC) containing 110 g lactose was used for cultivation of the yeast *Cryptococcus curvatus* ATCC 20509; cells were then disrupted by passing the cell suspension directly through a high pressure laboratory homogeniser. After autoclaving, the resulting crude cell extract containing the single-cell oil served as a substrate for growth of *Candida bombicola* ATCC 22214 and for sophorolipid production in a second stage. These studies showed that lactic whey wastes might be comparatively better substrates for biosurfactant production at the commercial scale than synthetic media.

Studies by Fox and Bala, (2000), highlighted the potential environmental threat and economic liability of starch-rich wastes from potato-processing industries. Potato process effluents (wastes from potato processing industries) were used to produce biosurfactant by *B. subtilis*. Thompson *et al.*, (2000) used high solids and low solids potato effluents as substrates for surfactin production. They used effluents diluted 1:10, unamended and amended with trace minerals or corn steep liquor. *B. subtilis* 21332 grew on all three potato substrates regardless of addition of exogenous nutrients. Cassava wastewater, another carbohydrate-rich residue, which is generated in large amounts during the preparation of cassava flour, is also an attractive substrate in biotechnological processes and has been used for surfactin production by *B. subtilis*. Nitschke and Pastore (2006), in their studied obtained that *B. subtilis* decreased the surface tension of the natural manipueria medium (cassava floor waste water) to 28 mN/m whereas for decanted manipueria the lowest value was 26 mN/m. These wastes are obtained at low cost from the respective processing industries and are as potent as low-cost substrates for industrial level biosurfactant production.

Several other waste substrates, such as rice water (effluent from rice processing industry and domestic cooking), cornsteep liquor, and wastewater from the processing of cereals, pulses and molasses, have tremendous potential to support microbial growth and biosurfactant production. Extensive research is needed to establish the suitability of these cost effective substrates in industrial-level biosurfactant production process.

Cost effective raw material	Biosurfactant type	Producer microorganism	Reference
Rapeseed oil	Rhamnolipids	*Pseudomonas sp.*	Trummler *et al.*, 2003
Babasassu oil	Sophorolipids	*Candida lipolytica*	Harrop *et al.*, 2003
Turkish corn oil	Sophorolipids	*Candida bombicola*	Pekin *et al.*, 2005
Sunflower and soybean oil	Rhamnolipid	*P. aeruginosa*	Rahman *et al.*, 2002
Sunflower oil	Lipopeptide	*Serratia marcescens*	Ferraz *et al.*, 2002
Soy bean oil	Mannosylerythritol lipid	*Candida sp.*	Kim *et al.*, 2006
Waste frying oils	Rhamnolipid	*P. aeruginosa*	Haba *et al.*, 2000
Oil refinery waste	Glycolipids	*Candida antarctica*	Bednarski *et al.*, 2004
Potato process effluent	Lipopeptide	*B. subtilis*	Thompson *et al.*, 2000, 2001, Noah *et al.*, 2005, 2002
Curd whey and distillery wastes	Rhamnolipid	*P. aeruginosa*	Dubey and Juwarkar 2001, 2004
Cassava flour wastes	Lipopeptide	*B. subtilis*	Nitschke and Pastore, 2003,2004, 2006
Soapstock waste	Rhamnolipid	*P. aeruginosa*	Nitschke *et al.*, 2005, Benincasa *et al.*, 2002
Peat hydrolysate	Lipopeptide	*B. subtilis*	Sheppard and Mulligan 1987
Olive oil mill effluent	Rhamnolipid	*Pseudomonas sp*	Mercade *et al.*, 1993
Molasses	Lipopeptide, Rhamnolipid	*Bacillus Sp.* *Pseudomonas sp.*	Makkar and Cameotra 1997, Rahman *et al.*, 2006

Table 2: Use of cost effective raw materials for the production of biosurfactants by various Microorganisms

2.11 Recovery of biosurfactants

The recovery and purification of biosurfactants from complex fermentation broth is a major problem in the commercialization of biosurfactants. In many cases, the downstream process increases the cost of biosurfactant production to as high as 60% (Desai *et al.*, 1994; Desai and Banat, 1997). Thus, improving product yield, low material costs and combining the steps of recovery can reduce the recovery costs. Economically biosurfactant recovery processes are mainly depending on its nature location (intracellular, extracellular or cell bound), ionic charge and water solubility (Desai *et al.*, 1994). Most biosurfactants are secreted into the

medium and they are isolated from either culture filtrate or supernatant obtained after removal of cells. The commonly reported techniques or biosurfactant recovery are listed in Table 3.

Precipitation of biosurfactants is also possible in case of protein like compounds such as emulsan (Rosenberg *et al.,* 1979; Shabtai and Gutnick, 1986; Goldman *et al.,* 1982) or a protein emulsifier produced by *Torulopsis petrophilium* (Cooper and Paddock, 1983). The precipitate is obtained by the addition of ammonium sulphate to the supernatants. The recovery of surfactin produced by *B. subtilis* (Arima *et al.,* 1968) and surfactin like biosurfactant from *B. lichenoformis* (Javaheri *et al.,* 1985) have been isolated by acid precipitation. Acetone precipitation techniques have been used to recover emulsifying and solubilizing factor produced by *Pseudomonas* sp. (Cameotra and Singh, 1990; Raddy *et al,* 1983; Goswami and Singh, 1991) and *C. lipolytica* (Roy *et al.,* 1979) and bioemulsifier from *C. tropicalis* and *Debaryomyces polymerphous* (Singh and Desai, 1986).

The most widely used technique is solvent extraction with a variety of solvents at several different ratios. The choice is dependent on cost and effectiveness. Solvents used for this purpose include chloroform-methanol mixture, dichloromethane, ethyl acetate, acetic acid, ether, etc. Some classical example of biosurfactant recovery by solvent extraction procedure include sophorolipids from yeast species (Ristau and Wagner, 1983; Cooper and Paddock, 1984; Gobbert and Wagner, 1984; Cooper and Paddock, 1983). Liposan from *C. lipolytica* and rhamonolipids *of Pseudomonas* sp. (Hisatsuka *et al.,* 1971; Desai *et al.,* 1988). Recently, methyl tertiary-butyl ether was able to extract crude surfactant material produced by *Rhodococcus rubber* IEGM 231 with high product recovery and good functional surfactant characteristics (Kuyukina *et al.,* 2001). However, the use of solvents is time consuming, expensive and not very specific. Further purification must be done by column chromatography, thin-layer chromatography or crystallization (Mulligan and Gibbs, 1993). Stuwer *et al.,* (1987), described an easy and cheaper purification process using liquid chromatography on silica gel for the nonionic glycolipid produced by *T. apicola.* Mannosylerythritol lipids produced by *Candida* sp. are settled down as heavy oils by centrifugation (Kitamoto *et al.,* 1993). Bryant (1990), demonstrated an improved method for the isolation of glycolipid from *Rhodococcus* sp. H13A by using XM-50 diafiltration and isopropanol techniques. These techniques give a purer glycolipid and removes protein impurities.

Glycolipids produced by *T. bombicola* (Cooper and Paddock. 1984; Gobbert *et al.,* 1984: Inoue and Itoh 1982), *T. Petrophilum* (Cooper *et al.,* 1988), and *T. apicola* (Hommel *et*

al., 1987; Tulloch *et al.,* 1967) are extracted by chilled ethyl acetate after adsorption on charcoal. Biosurfactant from *P. aeruginosa* has also been recovered in a similar way, except that extraction was carried out in acetone (Neu *et al.,* 1990). Both the glycolipid produced by *Ustilago zeae* and the mannosylerythritol lipid produced by *Candida* sp. (Hauser and Karnovsky, 1958; Spencer *et al.,* 1979) are sedimented as heavy oils upon centrifugation and then extracted in either ethanol or methanol.

The technique of foam fractionation has gained greater significance as it offers an advantage of continuous in situ removal of biosurfactant from the fermentation broth. Neu and Poralla (1990) recovered biosurfactant from *Bacillus sp.* in which foam produced was blown out of the fermentor, collected and centrifuged. Biosurfactant is recovered from the supernatant by cold acetone precipitation. Continuous removal of biosurfactant during the fermentation increases the cell density in the reactor and relieves product inhibition. As a result, bio surfactants yield is increased several fold as in the case of Rhamnolipid production by *Pseudomonas* sp. DSM2874 (Syldatk *et al.,* 1985) and surfactin production by *Bacillus* sp. (Cooper *et al.,* 1981; Neu and Poralla, 1990; Mulligan *et al.,* 1989).

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 biosurfactants such as their surface activity or their ability to form micelles 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 (WAC) (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.

2.12 Mutant and recombinant strains: the hyperproducers

Genetics of microorganism is important factor for production of various biotechnological products because the capacity to produce a metabolite is bestowed by the genes of the organism. Beside the use of cheap raw materials, optimized medium and culture conditions and efficient recovery processes, a production process cannot be made commercially viable and profitable until the yield of the final product by the producer organisms is naturally high (Cooper *et al.,* 1981; Guerra-Santos *et al.,* 1986). Even if high-yielding natural strains are

available, the recombinant hyperproducers are always required, to economize further the production process and to obtain products with better commercially important properties.

Besides the natural biosurfactant producer strains, a few mutant and recombinant varieties with enhanced biosurfactant production characteristics are reported Table 4. Mutants are produced using various agents, for example, transposons (Koch *et al.,* 1991), chemical mutagens such as N-methyl-N-nitro-N-nitrosoguanidine (Tahzibi *et al.,* 2004; Lin *et al.,* 1998: Carrera *et al.,* 1993) radiation (Iqbal *et al.,* 1995; Mulligan *et al.,* 1989). N-methyl- N-nitro-N-nitroso guanidine mutant of this strain, known as *B. subtilis* SD901, has recently been reported by Yoneda *et al.,* (2006), to produce in the range of 8–50 g/l surfactin when grown on flour from soybeans or its extract. This is the highest amount of surfactin production reported to date.

Liu Qingmei *et al.,* (2006), developed a mutant of *B. subtilis* with enhanced surface activity through low energy nitrogen ion beam implantation. There was an interesting finding that after the ion beam implantation the intensities of the components were different from the wild type strain. Similarly UV and gamma mutation have been used for production of mutants. Ultraviolet mutation of *B. subtilis* ATCC 21332 yielded a stable mutant that produced over three times more of the biosurfactant, surfactin, than the parent strain (Mulligan *et al.,* 1989).

Several recombinant strains producing biosurfactants in better yields and showing improved production properties have been developed in recent years. Recombinants of *B. subtilis* have been developed by expressing foreign genes related to surfactin production, resulting in high production of surfactin. A recombinant strain, *B. subtilis* MI 113, was developed with the plasmid pC112 harbouring lpa-14, a gene related to surfactin production. This strain showed enhanced production of surfactin in solid-state fermentation on soybean curd residue (Ohino *et al.,* 1995). Recombinant strains producing high levels of lichenysin have been developed by using whole enzyme module swapping, involving replacement of an entire first L-Glu- and the fifth L-Asp-incorporating modules of surfactin synthetase enzyme (Yakimov *et al.,* 2000). This type of whole enzyme module swapping might find applications in producing novel peptides, redesigning existing peptides, and also for the production of biosurfactants in non-pathogenic industrial strains. The rhamnolipid producer *P. aeruginosa* is naturally unable to use lactose for growth and biosurfactant production; however, lactose using strains of *P. aeruginosa* were developed by inserting *E. coli* lacZY genes into the chromosomes of *P. aeruginosa* (Koch *et al.,* 1988). These recombinant strains were able to

grow and produce biosurfactants on a lactose based media containing cheap materials such as whey.

Thus, recombinant varieties such as these can be used to produce biosurfactants on cheap substrates on which the natural strains are unable to grow. However, *P. aeruginosa* is an opportunistic pathogen in humans and is, therefore, not suitable as an industrial strain. To overcome the problem of the pathogenicity of *P. aeruginosa*, recombinant *P. putida* and *P. fluorescens* were developed that produced *P. aeruginosa* rhamnolipids in good amounts (Ochsner *et al.*, 1995). Recombinant strains such as these can be safely used in biotechnology industries where biosafety is an important consideration. Recently, a recombinant *Gordonia amarae* was developed by insertion, stable maintenance and expression of the Vitreoscilla hemoglobin gene (vgb), resulting in enhanced production of the trehalose lipid biosurfactants in the engineered strain (Dogan *et al.*, 2006). Incorporation of these hyper-producing strains will boost the industrial biosurfactant production process and make it possible to commercialize biosurfactants by making the production process cheaper and safer.

2.13 Factors affecting biosurfactant production

2.13.1 Effect of carbon source

Carbon source is very important in the production of biosurfactant. The carbon sources that had been previously used include carbohydrates, hydrocarbons and vegetable oils. Some organisms produce biosurfactants only in carbohydrates, others only in hydrocarbons, and still others consume several substrates, in combination or separately. In general, optimal yields are obtained with hydrocarbon or carbohydrate and lipids. For carbohydrate, most biosurfactant production has been performed using the more expensive pure forms of the sugar. For example, glucose, fructose and sucrose lipids are produced by several species of *Corynebacterium*, *Nocardia* and *Brevibacterium* during growth on the corresponding sugar (Desai *et al.*, 1994). *B. subtilis* also used glucose both in preliminary experiments, while *P. aeruginosa* used it in pilot plant studies (Mulligan and Gibbs, 1993).

Recovery procedure	Biosurfactants extracted	References
Acid precipitation	Lipopeptide	Sen, 1997;Arima *et al.*, 1968, Javaheri *et al.*, 1985
Ammonium sulfate precipitation	Polymeric biosurfactants, Lipopeptide	Rosenberg *et al.*, 1979; Shabtai and Gutnick 1986
Acetone precipitation	Rhamnolipid	Roy *et al.*, 1979;Raddy *et al.*, 1983
Organic solvent extraction	Rhamnolipid, Trehalose lipids, Cellobiolipids, Sophorolipids, Liposan	Reiling *et al* .,1986 Asselineau and Asselineau 1978;Boothroyd *et al.*, 1956, Cirigliano and Carman, 1985, 1984
Crystallization	Cellobiolipids, Glycolipids	Cirigliano and Carman, 1985, 1984
Continuous mode Centrifugation	Glycolipids	Hauser and Karnovsky, 1958
Adsorption	Rhamnolipid Lipopeptide Glycolipids	Reiling *et al* .,1986
Foam separation and precipitation	Lipopeptide	Cooper *et al.*, 1981; Neu and Poralla ,1990; Davis *et al.*, 2001; Noah *et al.*, 2002
Ultrafiltration	Lipopeptide Glycolipids	Bryant 1980; Mulligan and Gibbs, 1990
Diafiltration and precipitation	Glycolipids	Bryant. 1990;Cameotra and Singh 1990
Tangential flow filtration	Mixed surfactants	Mattei *et al.*, 1985

Table 3: Common methods employed for recovery of biosurfactants.

Mutant and/or recombinant strain	Characteristic feature	References
P. aeruginosa 59C7 Transposon Tn5-GM induced mutant of *P. aeruginosa* PG201	2 times more production	Koch *et al.*, 1991
P. aeruginosa PTCC 1637, Random mutagenesis NTG	10 times more production	Tahzibi *et al.*, 2004
B. licheniformis KGL11, Random mutagenesis NTG	12 times more production	Lin *et al.*, 1991
B. subtilis ATCC 55033, Random mutagenesis NTG	4-5 times more production	Carrera *et al.*, 1991
B. subtilis Suf-1, UV mutant of *B. subtilis* ATCC 21332	3–4 times more production	Mulligan *et al.*, 1989
Recombinant *B. subtilis* strain ATCC 21332 with modified peptide Synthetase enzyme	Production of a lipo hexapeptide with reduced toxicity	Symmank *et al.*, 2002
Recombinant *P. putida* by insertion of *E. coli* lacZY genes into the chromosomes of *P. aeruginosa* strains PAO-1 and PG-201	Use of lactose- and whey-based cheap substrates for biosurfactant production	Koch *et al.*, 1988

Table 4: Mutant and Recombinant strains of microorganisms with enhanced biosurfactant yield and improved product characteristics.

Water-soluble carbon sources, such as mannitol, glycerol and ethanol could be used for rhamnolipid production in *Pseudomonas sp.*, but they are inferior to immiscible substrates like n-alkanes and olive oil (Desai and Banat, 1997). The chain length of the hydrocarbon substrate also has affected biosurfactant production. Optimal production was obtained in *Corynebacterium hydrocarboclastus* with linear alkanes of the chain length C-12 to C-14 (Desai *et al.,* 1994), while *Rhodococcus erythropolis* produces biosurfactants best on C-12 to C-18 *n*-alkanes (Mulligan and Gibbs, 1993). It has been concluded from a number of studies that different carbon sources can influence the composition of the biosurfactant formed and how it is produced. *Arthrobacter* produces 75% extracellular biosurfactant when grown on acetate or ethanol but it is totally extracellular when grown on hydrocarbon (Mulligan and Gibbs, 1993).

2.13.2 Effect of nitrogen source

The nitrogen source in the medium also has a great effect in the production of biosurfactants. They may also contribute to pH control. Organic nitrogen sources include gluten meal, yeast hydrolysates and corn germ, whereas inorganic nitrogen sources include ammonium nitrate, ammonium sulphate, and so on. Among the inorganic salts tested, ammonium salts and urea were preferred for biosurfactant production by *A. paraffineus*, whereas nitrate supported maximum biosurfactant production in *P. aeruginosa* (Desai and Banat, 1997). For surfactin production by *B. subtilis*, ammonium nitrate was a superior nitrogen source than ammonium chloride or sodium nitrate. Doubling the ammonium nitrate from 0.4% to 0.8% increased the surfactin production by a factor of 1.6 (Mulligan and Gibbs, 1993). Yeast extract was found required for glycolipid production by *Torulopsis bombicola*, but was very poor for *P. aeruginosa*. Nitrogen limitation not only causes over production of biosurfactants, but also changes the composition of biosurfactants produced (Syldatk *et al.,* 1985; Desai and Desai, 1993). According to Hommel *et al.,* (1987), it is the absolute quantity of nitrogen and not its relative concentration that is important to give an optimum biomass yield, while the concentration of hydrophobic carbon source determines the conversion of carbon available to the biosurfactant.

2.13.3 Effect of environmental factors

Growth conditions and environmental factors such as temperature, pH, agitation and oxygen availability also affect the production of biosurfactant. Temperature may cause alteration in the composition of the biosurfactant produced by *Pseudomonas sp.* DSM-2874 (Syldatk *et*

al., 1985). A thermophilic *Bacillus sp.* grew and produced biosurfactant at temperature above 40^0C (Banat, 1993). However, heat treatment of some biosurfactants caused no appreciable change in biosurfactant properties such as the surface activity as well as the emulsification efficiency (Abu Ruwaida *et al.,* 1991).

The pH of the medium plays an important role in sophorolipid production by *T. bambicola* (Gobbert *et al.,* 1984). Penta and disaccharide lipid production by *Nocardia corynbacteroides* is however unaffected in the pH range of 6.5 to 8.0 (Powalla *et al.,* 1989). In addition, surface tension and CMC of a biosurfactant remained stable over a wide range of pH values, whereas emulsification had a narrower pH range (Abu Ruwaida *et al.,* 1991).

An increase in agitation speed due to the shear effect results in the reduction of biosurfactant yield produced by *Nocardia erythropolis* (Margaritis *et al.,* 1979; Mulligan and Gibbs, 1993). On the other hand, production of biosurfactant by yeast increases when the agitation and aeration rates increased (Desai and Banat, 1997).

Depending on its effect on cellular activity, salts concentration also found to affect the production of biosurfactant. However, some biosurfactants were not affected by salt concentrations up to 10% (w/v), although slight reductions in the CMC were detected (Abu Ruwaida *et al.,* 1991).

2.13.4 Growth-associated biosurfactant production

A parallel relationship between cell growths, substrate utilization and biosurfactant production exist in growth-associated biosurfactant production. The production of rhamnolipid by some *Pseudomonas* sp., surface-active agent by *B. cereus* IAF 346 and biodispersan by *Bacillus sp.* IAF 343 are all examples of growth-associated biosurfactant production (Desai and Banat, 1997). The carbon source plays important role in biosurfactant production (Itoh and Suzuki, 1974). The chain length of the hydrocarbon used also affects the production of biosurfactant (Syldatk *et al.,* 1985). A mixed growth-associated and non growth-associated process has been reported occurred in the production of cell-free emulsan by *Acinetobacter calcoaceticus* RAG-1. Emulsan-like substance accumulates on the cell surfaces during the exponential growth phase and is released into the medium when protein synthesis decreases (Goldman *et al.,* 1982). Wang and Wang (1990), performed extensive studies on the mechanism of biosurfactant accumulation in *A. calcoaceticus* RAG-1. They revealed that the ratio of cell-bound polymer to dry cell is strongly affected by shear force and as the shear stress increases, the ratio decreases.

2.13.5 Biosurfactant production under growth-limiting conditions

The unique feature of this category of biosurfactant production is the sharp increase in the biosurfactant level as a result of limitation of one or more medium components. In some cases, an overproduction of biosurfactants was dependent on growth-limiting conditions such as N-limitation or limitation of the multivalent cations, so that a rhamnolipid production by *P. aeruginosa* occurred only after reaching the stationary growth phase (Suzuki *et al.*, 1974; Guerra-Santos *et al.*, 1986).

Powalla *et al.*, (1989), has demonstrated that the production of pentasaccharide lipid by *Nocardia corynebacteriods* SM-1 is favoured by inorganic nitrogen sources, and sodium nitrate gave maximum surfactant production. Furthermore, it has been observed that during growth, the initial yield of glycolipid increases rapidly after the exhaustion of the nitrogen source and after attaining the stationary phase of growth.

Hommel *et al.* (1987) have studied extensively the production of water soluble biosurfactant by *Torulopsis apicola.* According to them the absolute quantity of nitrogen and not its relative concentration important to determine the optimum concentration of biomass, whereas the concentration of the hydrophobic carbon source determines the conversion of available carbon into biosurfactants. Moreover, it was shown that the C:N ratio in the medium also plays an important role in biosurfactant production, and a large amount of *n*-hexadecane is found to be incorporated into the surfactant at higher C:N ratio.

Iron concentration has shown to have a dramatic effect on rhamnolipid production by *P. aeruginosa*, which resulting in a threefold increase in the production when cells were shifted from medium containing 36 μM iron to medium containing 18μM iron. However, there was no change in the biomass yield under these conditions (Guerra-Santos *et al.*, 1986). Syldatk and Wagner (1987), stated that the effect of N-limitation or a limitation of the multivalent cations is nonspecific and it is expressed as a change of the physiological state of the microorganisms used for the production of biosurfactant.

2.13.6 Biosurfactant production by resting or immobilized cells

In this category of biosurfactant production, cells do not multiply but continue to utilize carbon source for the synthesis of biosurfactants. The cells used are harvested from the surfactant-producing state of culture broth and maintained in the same state. The wet biomass is washed and used for the production of biosurfactant under specific conditions, so that the effect of possibly disturbing products can be eliminated and the influence of single factors on the synthesis of the compound, such as pH, temperature and salt concentrations can be

examined. Production of rhamnolipid by resting cells could be increased evidently in comparison with growing cells under N-limitation (Syldatk and Wagner, 1987). In contrast to the rhamnolipid production by growing cells, two new rhamnolipids, R3 and R4 were synthesized by the resting cells (Syldatk *et al.,* 1985). The production of these biosurfactants was dependent on the incubation temperature and the carbon source used in the medium. By incubating resting cells in phosphate buffer, repeated use of cells for rhamnolipid production is increased by almost 5 to 6-fold. It is proposed that the effect may be due to relieving the product inhibition. However, biosurfactant production rate was much lower as compared to that with growing cells. Production of biosurfactant by resting cells also has been observed in the production of sophorolipid by *T. bombicola* (Inoue and Itoh, 1982) and trehalose tetraester production by *R. erythropolis* (Syldatk *et al.,* 1985). In this case, the conversion rate of substrate to product was found to be much higher than that observed with growing cell under nitrogen limitation. The production of biosurfactant by resting cells is important for the reduction of cost of product recovery, as in such cases the growth phase and the product formation phases are separated.

2.13.7 Biosurfactant production in addition to precursor

Many reports have showed that the addition of biosurfactant precursors to the growth medium causes both qualitative and quantitative changes in the product. Addition of lipophilic compounds to the medium of *T. apicola* IMET 43747 (Stuwer *et al.,* 1987) and *T. bombicola* (Cooper and Paddock, 1984) resulted in the higher production of biosurfactants. In this case, the carbon source in the medium, particularly the carbohydrate, has great bearing on the type of glycolipid formed. Similarly, increased production of biosurfactants containing different mono, di, or trisaccharides was reported occurred in *Corynebacterium* sp. and *Nocardia* sp. through supplementation of the corresponding sugar in the culture medium (Itoh and Suzuki, 1974). This method of biosurfactant production will probably be of great interest in future because it allows the production of new surface- and interfacially active compounds whereby the chemical and physical properties of these compounds can be influenced by the carbon sources used for biosurfactant formation (Syldatk and Wagner, 1987).

2.14 Potential applications of biosurfactants

Surfactants constitute an important class of industrial chemicals widely used in almost every sector of modern industry. During the last decade demand for surfactants increased about 300% within the US chemical industry (Greek, 1990). About 54% of the total surfactant

output is utilized in household/laundry detergents with only 32% destined for industrial use (Makkar and Cameotra, 1999). Most of the commercially available surfactants are chemical surfactants, mainly petroleum-derived. However, rapid advances in biotechnology and increased environmental awareness among consumers, combined with expected new legislation, has provided further impetus for serious consideration of biological surfactants as possible alternatives to existing products. Biosurfactants have therefore gained considerable interest in recent years due to their low toxicity, biodegradable nature and diversity. Potential industrial applications of biosurfactants include enhanced oil recovery, crude oil drilling, lubricants, surfactant aided bioremediation of water-insoluble pollutants, health care and food processing (Fiechter, 1992; Muller-Hurtig *et al,.* 1993; Velikonja and Kosaric, 1993; Finnerty 1994; Lin, 1996; Desai and Banat, 1997). Despite the advantages demonstrated by biosurfactants, few reports are available regarding their use on food products and food processing.

2.14.1 Biosurfactant and microbial enhanced oil recovery

An area of considerable potential for biosurfactant application is in the field of microbial enhanced oil recovery (MEOR). It is an important tertiary recovery technology, which utilizes microorganisms and their metabolites for residual oil recovery (Banat 1995a). Biosurfactant mediated EOR represents one of the most advanced methods to recover entrapped oil via an aqueous surfactant formulation that is injected into a mature oil reservoir. Where this solution contacts the small pockets of trapped oil, it reduces the interfacial tension (IFT) and mobilizes this trapped oil. Traditional EOR methods have been inconsistent and due to the wide variety of well conditions, there is often excessive chemical loss and gravity segregation renders it less effective. Selection of effective surfactants and a better understanding of the formation of their emulsions with crude oils are important for progress towards Enhanced Oil Recovery (EOR). There are several reports that describe various methods used in laboratory studies of MEOR. Biosurfactants can also aid oil emulsifcation and assist in the detachment of oil films from rocks (Banat 1995; Shennan and Levi, 1987). *In situ* removal of oil is due to multiple effects of the microorganisms on both environment and oil. These effects include gas and acid production, reduction in oil viscosity, plugging by biomass accumulation, reduction in interfacial tension by biosurfactants and degradation of large organic molecules. These are all factors responsible for decreasing the oil viscosity and making its recovery easier. Hayes *et al.,* (1986) in their studies found biosurfactant to be effective in the reduction of the interfacial tension of oil and water in situ, the viscosity of the

oil, the removal of water from the emulsions prior to processing, and in the release of bitumen from tar sands. Emulsan, a high molecular weight has been commercialized for this purpose (Anon, 1984). It contains a polysaccharide with fatty acids and proteins attached. Other high molecular weight biosurfactants are reviewed by Ron and Rosenberg (2002). For the microorganisms to be suitable and useful in MEOR *in situ,* they must be able to grow under the severe environmental conditions encountered in oil reservoirs, such as high temperature, pressure, salinity and low oxygen levels (Cameotra and Makkar 1998). *Bacillus licheniformis* JF-2 is an example, which would be well suited for *in situ* studies for enhanced oil recovery or soil decontamination (Javaheri *et al.,* 1985). Post and Al-Harjan (1988) reported the isolation of a halobacterium capable of producing surface-active agents, while several anaerobic thermophilic bacteria tolerant of pressure and moderate salinity have also been reported to mobilize crude oil in the laboratory (Levi *et al,.* 1985). Makkar and Cameotra 1997; 1998, observed a good sand-pack oil recovery using strains of Bacillus subtilis at 45 °C. Biosurfactant produced by two strains of *B. subtilis* (MTCC 1427 and MTCC 2423) accounted for 56% and 62% oil recovery from oil-saturated sand columns. The two strains grew at 45°C and utilized molasses, a cheap source of nutrient additive. Most of the laboratory studies on MEOR utilize core samples and columns containing the desired substrate. Field studies carried out by Banat (1995), in Czechoslovakia, Hungary, the Netherlands, Poland, Romania, the United States and the USSR, demonstrated effectiveness of biosurfactants in enhanced oil recovery.

2.14.2 Biosurfactants in bioremediation

Microbial remediation of hydrocarbon and crude oil is an emerging technology involving the applications of biosurfactants (Banat 1995; Banat *et al.,* 1991, Bartha 1986; Ghosh *et al.,* 1995; Harvey *et al.,* 1990). Biodegradation of hydrocarbons by native microbial population is the primary mechanism by which hydrocarbon contaminants are removed from the environment (Atlas 1993; Atlas and Bartha 1992). Besides this degradation is dependent on the hydrocarbon composition, oxygen availability, water, temperature, pH and inorganic nutrients. The physical state of the hydrocarbon can also affect biodegradation. Hydrocarbons are hydrophobic and bind firmly to soil particles. The surface area of oil can be increased by adding synthetic or biological surfactants. This results in increased mobility and solubility of hydrocarbons which is essential for microbial degradation (Morgan and Watkinson, 1989). Studies conducted by Dave *et al.,* (1994), demonstrated that the use of mixtures of hydrocarbon degrading microbes for bioagumentation of soil contaminated with slop oil from

a petrochemical industry resulted in the bioreclamation of soil. Van Dyke *et al.,* (1993), showed that biosurfactant produced by *P. aerugenosa* UG2 was helpful in removing hydrophobic compounds from soil. Zhang and Miller (1992), found that the addition of pseudomonas biosurfactant enhanced the dispersion of octadecane. Only limited numbers of microorganisms are capable of degrading polyaromatic hydrocarbons (PAHs). Their biodegradation is limited by their poor availability to the microorganisms which is due to their hydrophobicity, low aqueous solubility and strong adsorptive capacity in soil (Volkering *et al.,* 1995). In an investigation of the capacity of PAH-utilizing bacteria to produce biosurfactants using naphthalene and phenatherene, Dazel *et al.,* (1996), reveled that biosurfactant production was responsible for increase in the aqueous concentration of naphthalene. This indicates the potential role of biosurfactants in increasing the solubility of such compounds.

2.14.3 Biomedical applications

The use and potential commercial application of biosurfactants in the medical field has increased during the past decade. Their antibacterial, antifungal and antiviral activities make them relevant molecules for applications in combating many diseases and as therapeutic agents. In addition, their role as anti-adhesive agents against several pathogens indicates their utility as suitable anti-adhesive coating agents for medical insertional materials leading to a reduction in a large number of hospital infections without the use of synthetic drugs and chemicals. Among the several categories of biosurfactants, lipopeptides are particularly interesting because of their high surface activities and antibiotic potential. Lipopeptides can act as antibiotics, antiviral and antitumour agents, immune modulators or specific toxins and enzyme inhibitors. Ahimou *et al.,* (2001), reported that lipopeptide profile and bacterial hydrophobicity vary greatly with the strains, iturin A being the only lipopeptide type produced by all *B. subtilis* strains. Surfactin was found to be more efficient than iturin A in modifying the *B. subtilis* surface hydrophobic character. This aspect appears essential, in association with the antifungal properties of lipopeptides involved, in the biological control of plant diseases. Morikawa *et al.,* (1993), identified and characterized a biosurfactant, arthrofactin, produced by *Arthrobacter* species, which was found to be seven times more effective than surfactin. Iturin A has been proposed as an effective antifungal agent for profound mycosis Tanaka *et al.,* (1997). Other members of the iturin group, including bacillomycin D and bacillomycin Lc, were also found to have antimicrobial activity against *Aspergillus flavus*, but the different lipid chain length apparently affected the activity

of the lipopeptide against other fungi (Moyne *et al.,* 2001). These biosurfactants help in disruption of the plasma membrane by the formation of small vesicles and the aggregation of intra membranous particles in yeast cells. Kim *et al.* 1998, demonstrated that surfactin is a selective inhibitor for cytosolic PLA2 and aputative anti-inflammatory agent through the inhibitory effect produced by direct interaction with cytosolic PLA2, and that inhibition of cytosolic PLA2 activity may suppress inflammatory responses. Vollenbroich *et al.*, (1997), showed that surfactin treatment improved proliferation rates and led to changes in the morphology of mammalian cells that had been contaminated with mycoplasma. MELs produced by *C. antartica*, (Kitamoto *et al.*, 1993), rhamnolipids produced by *P. aeruginosa* (Lang and Wullbrandt, 1999; Maier and Soberon-Chavez, 2000) and lipopeptides produced by *B. subtilis* (Vollenbroich *et al.*, 1997), and *B. licheniformis* (Fiechter, 1992; Jenny *et al.*, 1991; Yakimov *et al.*, 1995) have been shown to have antimicrobial activities. Jenny *et al.*, (1995), determined the structure and characterized surface activities of biosurfactants produced by *B. licheniformis*, while Lin *et al.*, (1994), described their continuous production. Yakimov *et al.*, (1995), demonstrated the antibacterial activity of lichenysin A, a biosurfactant produced by *B. licheniformis* that compares favourably with other surfactants. More recently, Grangemard *et al.*, (2001), reported the chelating properties of lichenysin, which might explain the membrane-disrupting effect of lipopeptides. In another study, Carrillo *et al.*, (2003), noted a molecular mechanism of membrane permeabilization by surfactin, which may explain surfactin-induced pore formation underlying the antibiotic and haemolytic action of these lipopeptides. This study also suggested that the membrane barrier properties are likely to be damaged in the areas where surfactin oligomers interact with the phospholipids, at concentrations much below the onset for solubilization. Such properties can cause structural fluctuations that may well be the primary mode of the antibiotic action of this lipopeptide. Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or to infection sites; thus, prior adhesion of biosurfactants to solid surfaces might constitute a new and effective means of combating colonization by pathogenic microorganisms. Pre-coating vinyl urethral catheters by running the surfactin solution through them before inoculation with media resulted in a decrease in the amount of biofilm formed by *Salmonella typhimurium*, *Salmonella enterica*, *E.coli* and *Proteus mirabilis* (Singh and Cameotra, 2004).

2.14.4 Other applications of biosurfactants

Increasing interest of potential applications of microbial surface active compounds is based on their broad range of functional properties. Concerns about pesticide pollution have prompted global efforts to find alternative biological control technologies. While Stanghellini *et al.,* (1996) were investigating the effects of synthetic surfactants on controlling the root rot fungal infections of cucumbers and peppers caused by *Pythium aphanidermatum* and *Phytophora capsici,* they observed lysis of fungal zoospores due to some bacterial metabolites in the nutrient solution. The metabolites were thought to be biosurfactants, as their mode of action was similar to the synthetic surfactants. Subsequently the bacterium was identified as *P. aerugenosa* and the biosurfactant as a rhamnolipid (Stanghellini and Miller, 1997). Biosurfactants have also been used in formulating poorly soluble organophosphorus pesticides. Two bacillus strain producing emulsifiers, possibily a glycolipopeptide, were able to for a stable emulsion in the presence of the pesticide fenthion (Patel and Gopinathan, 1986). The compound had some activity against other liquid immiscible organophosphorus pesticides. Biosurfactants may be used for the dispersion of inorganic minerals in mining and manufacturing processes (Rosenberg *et al.,* 1988) described an anionic polysaccharide called biodispersan produced by *Acinetobacter calcoaceticus* A2, was able to prevented flocculation and dispersed a 10% limestone in water mixture. A very attractive feature of biosurfactants if their use in health care and cosmetic industries. Sophorolipids are produced by *C. bombicola* KSM-36 in quantities of 100-150 g/l using palm oil and glucose as carbon source (Itoh, 1987) and by *C. apicola* to about 90 g /l using glucose and sunflower oil as substrates (Stuwer *et al.,* 1987). A product containing one mole sophorolipid and 12 moles propylene glycol has specific compatibility to the skin and has found commercial utility as a skin moisturizer (Yamane, 1987). Sophorolipids is commercially used as humectants by Kao Chemical Corporation for cosmetic makeup brands such as Sofina. In the food industry, biosurfactants are used as emulsifiers for the processing of raw material. Emulsification plays an important role in forming the right consistency and texture as in phase dispersion. A bioemulsifier from *C. utilis* has shown potential use in salad dressing (Shepherd *et al.,* 1995). In addition, biosurfactant such as lecithin and fatty esters also have several promising applications in the food industry as food additives (Edward and Hayashi, 1965). Some other potential commercial applications of biosurfactants are in the pulp and paper industry (Rosenberg *et al.,* 1989), textiles, ceramics (Horowitz and Currie, 1990) and uranium ore processing (McInerney *et al.,* 1990). The usefulness of biosurfactants in other fields is emerging, especially in personal and health care and as therapeutic agents. Enzymatic synthesis of tailor-made surfactants by lipases has given a new dimension to biosurfactant production,

especially in the application of biosurfactants in health care and cosmetics. With increased efforts on developing improved application technologies, strain improvement and production processes, biosurfactants are expected to be among the most versatile process chemicals for use in the near future.

CHAPTER 3

MATERIALS & METHODS

3.1 MATERIALS

3.1.1 Microorganisms

Bacterial strains: *Escherichia coli* (MTCC 443), *Staphlyococcus aureus* (MTCC 96), *Pseudomonas aerugenosa* (MTCC 741), *Bacillus cereus* (MTCC 430) and Fungal strains: *Candida albicans* (MTCC 183), *Rhizoctonia solani* (MTCC 4633), *Fusarium oxysporum* (MTCC 284) and *Trichoderma viride* (MTCC 2047) were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

3.1.2 Culture media

Yeast Extract Peptone Dextrose (YPD) medium, Yeast Extract Phosphate (YEP) medium, Sabouraud Dextrose agar (SDA) medium were purchased from Himedia Chemicals, India.

3.1.3 Chemical Reagents and Diagnostic Kits

Some of the fine chemicals and kits used in the study have been listed below:

- Surfactin,2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide(XTT); Menadione; Ethidium Bromide (EtBr); Ethyl Methanesulfonate (EMS) from Sigma Chemicals, USA.
- Silica gel 60-200 from Merck, Germany.
- Fluoranthene, Pristine, Hexadecane from Acros organics, Belgium.
- Fluorescein Diacetate (FDA); from Himedia Chemicals, India.
- Bovine serum albumin (BSA) from Bangalore Genei, India.
- All culture media and solvents were obtained from Himedia Chemicals and Ranbaxy, India.

3.2 METHODS

3.2.1 Isolation and screening of biosurfactant producer

3.2.1.1 Sampling

A total of 32 soil samples were collected from three contaminated sites viz. oily sludge samples from crude oil storage tank (6 samples), Ankleswar, Gujarat samples contaminated with diesel oil and motor oil from local fuel filling stations (15 samples) and warehouses (12 samples), roorkee. Phenol contaminated water (5 samples) collected in the pre-cleaned glass bottles from a coal gas generator site, BHEL, Haridwar were also used in this study. The samples were stocked in a sterile container and stored at 4^0C until analysed.

3.2.1.2 Media preparation

Minimal salt medium (MSM) containing solution A (0.1% KH_2PO_4 and 0.1% K_2HPO_4 solution B (0.05% $MgSO_4.7H_2O$ and KNO_3), trace metal solution (1.5% nitrilotriacetic acid, 0.5% $MnSO_4.2H_2O$, 0.001% $FeSo_4$, 0.01% $CaCl_2.2H_2O$, 0.1% $CoCl_2.2H_2O$, 0.01% Al K $(SO_4)_2$, 0.001%H_3BO_4, 0.01% $ZnSO_4$ and 0.001% Na_2MoO_4), while the vitamin solution (0.02% biotin, 0.02% folic acid, 0.1% pyridoxine HCl, 0.05% thiamine hcl, 0.05% riboflavin, 0.05% niscotinic acid, 0.05% pantotheic acid, 0.01% cyanacobalamin and 0.05% lipoic acid). The pH of the medium was adjusted to 7.2 (Lal and Khanna, 1996).

3.2.1.3 Bacterial enumeration

Soil (10 g) was shaken with 200mL distilled water for 30 min at 200 rpm and allowed to settle. The supernatant was centrifuged at 3000 rpm for 5 min before plating out. MSM supplemented with crude oil was used for bacterial isolation (Kiyohara *et al.*, 1982). Dilution of the contaminated samples was made to 10^{-3}-10^{-5} times with sterile distilled water; the cultures were then plated on nutrient agar plates to isolate individual colonies. Bacterial populations were detected as cfu (Colony forming unit) per gram of soil. The purified microbial colonies obtained were tested for their ability to grow on 2% hydrocarbon substrates like Crude oil (I.I.P, Dehradun, India), pristane, n-hexadecane, eicosane, toluene, and fluoranthene (Himedia, Acros).

3.2.1.4 Screening methods

3.2.1.4.1 Surface tension measurement

Surface tension of the culture supernatant was measured using tensiometer (Sigma 703 KSV instruments Ltd., Finland) using Wilhelmy plate measurement technique as described earlier by Pallas and Pethica, 1983. Wilhelmy plate method utilizes a Platinum rectangular plate. The surface tension of MSM medium at 25^0C was used as control. Cell free broth was taken in a 50 ml glass beaker and placed onto the tensiometer platform. The surface tension of each sample was measured with using whilemy plate. The instrument was calibrated using water to a reading of 72 mN/m and all the measurements were taken in triplicate.

3.2.1.4.2 Haemolytic activity

Screening of isolated biosurfactant producing strain was determined by haemolytic activity. The fresh single colony of cultures was streaked on blood agar plates containing 5% (v/v) blood (Himedia, India), respectively and incubated at 37^0C for 48-72 hours. Haemolytic activity was detected by presence of a clear zone around the bacterial colony after incubation for 48 h at 37^0C which is an indicative of biosurfactant production.

3.2.1.4.3 Drop collapse assay

The drop-collapse technique was performed in the polystyrene 96-well Microtitre plate (Bodour and Miller, 1998). Briefly, each well of MTP was coated with a thin layer of oil (2.0 µL). A 5.0 µl aliquot of sample was delivered into the center of the well using a 25 µl glass syringe (Hamilton, USA) by holding the syringe at an angle of 45^0. The coated wells were equilibrated for 24 h to ensure a uniform oil coating. The results were monitored visually after 1 h. If the drop remained beaded, the result was scored as negative where as was scored as positive if drop collapsed.

3.2.1.4.4 Emulsification test

The bacterial broth was centrifuged and was studied for its emulsifying ability by a modified method of Cameron *et al.*, 1998. Cell free broth (2 ml) was pippeted into the screw cap test tube and 3 ml of kerosene was then added. The mixture was vortexed at high speed for 2 min and left at room temperature. The result was observed after 24 h for the stability of emulsion. The total volume of the mixture, volume of emulsified and volume of non-emulsified phase was observed. The emulsification index was calculated by the following equation:

$$E24 = \frac{\text{Height of Emulsion layer X 100}}{\text{Total height}}$$

3.2.1.5 Cell surface hydrophohicity technique

Bacterial strains selected on basis of above screening methods (3.2.1.4), were subjected to cell surface hydrophobicity technique for further assessment of biosurfactant production. In this technique, an array of tests namely Hydrophobic Interaction Chromatography (HIC), Salt Aggregation Test (SAT); Bacterial Adherence to Hydrocarbons (BATH); Adherence is Polystyrene; Replica Plate Test (RP): were performed.

3.2.1.5.1 Hydrophobic interaction chromatography (HIC)

In this method, phenyl Sepharose CL-4B, bed volume approximately 0.6 ml was used as the column packing matrix. The column was equilibrated with a solution of 4.0 M NaCI in 0.5 M citrate buffer. Cell suspension was prepared in the same solution which served for equilibrating the gel and 0.1 ml was introduced on the gel followed by 3 ml of the equilibrating solution. The elute (non retained bacteria) was compared with the original bacterial suspension by measuring the absorbance at 540 nm and the results recorded as a percentage of retained bacteria (Hydrophobic Index, HI).

3.2.1.5.2 Salt aggregation test (SAT)

Sodium phosphate (0.002 M, pH 6.8) was used to dilute a solution of 4 M $(NH_4)_2SO_4$ in 0.002 M sodium phosphate pH 6.8. Serial dilutions were made giving $(NH_4)_2SO_4$ concentration ranging from 4.0 to 0.2 M differing by 0.2 M per dilution. A bacterial suspension of 25 µl (approx. 10^{10} cfu/ml) in 0.002 M sodium phosphate buffer pH 6.8 was mixed with an equal volume of salt solution into 24 well tissue culture tray. The bacterial/salt mixture was gently rocked for 2 min at 25°C and visual reading was performed against a black background. The results were expressed as the lowest molarity of ammonium sulphate causing bacterial aggregation.

3.2.1.5.3 Bacterial adherence to hydrocarbons (BATH)

Bacterial suspensions prepared at intervals in buffer (pH 7.1) containing K_2HPO_4, 22.2 g/l; KH_2PO_4, 7.26 g/l; Urea, 1.8 g/l; $MgSO_4.7H_2O$, 0.2 g/l and distilled water to 1000 ml was dispensed as 1.2 ml into 10 mm diam. test tubes. Hydrocarbons (dodecane, hexadecane, pristane), 0.2 ml, were then added. Following preincubation at 25^0C for 10 min, the mixtures were agitated for 2 min, stood at room temperature for 15 min to allow hydrocarbon separation and the turbidity of the aqueous phase was measured before and after treatment. Results were recorded as the percentage absorbance of the aqueous phase after treatment relative to the initial absorbance of the aqueous phase after treatment relative to the initial absorbance of the bacterial suspension.

3.2.1.5.4 Adherence to polystyrene: replica plate test (RP)

In this technique, 25 mm diameter discs cut from sterile disposable polystyrene petri dishes were used. They were pressed firmly on to confluent agar surface growth and the replica colonies obtained on the polystyrene surface were washed in running water for 2 min. To facilitate visualization and comparison with the original colonies the replica was fixed by dipping in methanol and staining with crystal violet. Greater than 50% coverage of the disc by adherence cells was scored as positive. This procedure was repeated at different time intervals so as to check the age dependent hydrophobicity of the bacterial colonies.

3.2.1.6 Selection of potential biosurfactant producer using cheap raw material

A total of 14 different cost effective feed stocks (cotton seed hull, waste tea, wheat bran, corn starch, rice straw, wheat straw, bagasse, ground nut kernel, potato peel, apple peel, cotton seed, molasses, bamboo wood saw dust and gram husk) with high levels of carbohydrates or lipids, were chosen as raw material for biosurfactant production. Pre-treatment, sterilization and analysis of constituents was done before being used as substrate for biosurfactant production in minimal salt medium.

3.2.1.6.1 Substrate preparation

Pre-treatment was done to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. The purpose meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of by

products inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective. Physical and chemical treatment methods of lignocellulosic materials are described below:

a) Mechanical comminution

Cost effective raw materials were comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding (Millet *et al.*, 1976).

b) Pyrolysis

Pyrolysis was used for pretreatment of lignocellulosic materials. Materials were treated at higher temperatures (300^0C) cellulose rapidly decomposes to produce gaseous products and residual characterization (Kilzer and Broido, 1965).

c) Acid hydrolysis

Mild acid hydrolysis along with heat treatment (1 N H_2SO_4, 97^0C, 2.5 h) was given to the raw materials having high lignin and cellulose content (Esteghlalian *et al.*, 1997).

d) Sterilization of the raw materials

Sterilization of cost effective raw material was done by using autoclaving at121^0C for 15 min at pressure of 15 lbs. These pre-treated samples were stored at 4^0C until needed for further analyses.

e) Compositional analysis of substrates

Total sugars content in the cost effective substrates were determined by the standard dinitrosalicylic acid (DNS) method, total carbohydrates by the standard phenol- sulfuric acid method and proteins by the Bradford microassay using bovine serum albumin as a standard protein.

3.2.1.6.2 Evaluation of biosurfactant production on cost-effective substrates

The ability of isolates to utilize cost effective substrates for biosurfactant production as sole source of carbon and energy was determined. Pre-treated substrate (2% w/v) was added to MSM (100 ml) contained in 250ml of Erlenmeyer flask. A non-inoculated flask was prepared

for each carbon source was used as control. The media after sterilization were inoculated with the test organism respectively.

3.2.2 Characterization of biosurfactant producer

3.2.2.1 Biochemical identification

The selected strain DSVP23 was identified by conventional biochemical tests in accordance with Bergey's Manual of Systematic Bacteriology (Sneath, 1986).

3.2.2.2 Molecular characterization

3.2.2.2.1 Amplification of 16S rRNA gene

Bacterial genomic DNA was extracted for PCR amplification from pure cultures by the method described by Pitcher *et al.*, 1989. Total genomic DNA was purified using a modification of the method described by Gevers *et al.*, 2001, as outlined by De Clerck *et al.*, 2004.

The identification of the selected bacterial isolate DSVP23 was performed using standard 16s rRNA specific universal primers. The Primers set was purchased from Gen script, India and had an oligomer sequence as: forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-AAGGAGGTGATCCAGCCGCA-3'). A reaction mixture containing approximately 50 ng of template DNA, PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$ and 0.001% gelatin), a 0.2 mM concentration of each PCR primer, 0.2 mM conc. of each deoxynucleoside triphosphate and 2.5 units of *Taq* DNA polymerase in a total volume of 50 µL was prepared. PCR conditions involved initial denaturation at 95^0C for 2 min, followed by 30 cycles of denaturation at 95^0C for 1min, annealing for 1 min at 58^0C and extension at 72^0C for 1 min. 5 µl of PCR product was electrophoresed on a 1% agarose gel containing 1mM ethidium bromide for visualization of DNA in the amplified bands on a UV illuminator.

3.2.2.2.2 Phylogenetic analyses

Amplified 16S rDNA fragment of about 1500 bp size obtained from bacterial isolate (750 ng/µl) was partially sequenced commercially (GeneOmbio, India.). The gene sequences were compared with others in the GenBank databases using the NCBI BLAST (www.ncbi.nlm.nih.gov). Gene sequences of 16S rDNA of selected organisms were obtained from GenBank and aligned with gene sequence of our isolates using CLUSTALX software.

The aligned sequences were used to construct a distance matrix (Jukes *et al.*, 1969), after the generation of 1000 bootstrap sets, that was subsequently used to construct a phylogenetic tree using the neighbor-joining method (software MEGA3, Kumar *et al.*, 2004).

3.2.3 Inoculum preparation

A fresh single pure colony of each bacterial isolates was transferred aseptically from agar plate into LB medium using a sterile wire loop. The inoculated medium was then incubated at 37^0C at 180rpm in orbital shaker until the culture reached an optical density (OD600) of between 0.5 to 0.8 prior to use as inoculum.

3.2.4 Culture Maintenance and Storage

All pure isolates were maintained in liquid and solid media. For routine use, the cultures were maintained on nutrient agar (NA) (Himedia, India) plates/slants. Glycerol stocks were prepared and stored at -20^0C, for long-term storage.

3.2.5 Dry biomass

Samples collected at different time intervals were centrifuged (8000 rpm) for 10 min. The pellet was rinsed in distilled water and recentrifuged at room temperature. Biomass was determined by weighing after drying at 105^0C for 24 h.

3.2.6 Extraction of biosurfactant from culture broth

Culture broth of the isolated *Bacillus subtilis* DSVP23 was centrifuged for 10 min at 6000 rpm at 4^0C to obtain cell free supernatants and crude was precipitated from the supernatant by adding 6 N HCl to obtain a final pH of 2.0. The acid precipitate was recovered by centrifugation (8000 rpm for 15 min at 4^0C) and was further extracted with dichloromethane. The precipitate thus formed was collected by centrifugation for 10 min at 1000 rpm at 4^0C and dried under a current of warm air. The product obtained after the extraction procedure was dried at 60^0C to a constant weight prior to get the yield of biosurfactant production.

3.2.7 Adsorption chromatography

After dichloromethane extraction of biosurfactant it was dissolved in methanol. This extract was subjected to column chromatography on silica gel column (30×2.5 cm; Merck, Darmstadt, Germany) pre-packed in $CHCl_3$-MeOH (3:1). The column was eluted stepwise

with mixtures of $CHCl_3$: MeOH (v/v) from 3: 1 to 1:1. Fractions were collected and analysed for surface active properties. Active fractions were pooled and concentrated in vacuum to obtain a crude powder.

3.2.8 Gel filtration chromatography

The crude fraction from silica gel column chromatography were dissolved in a minimum volume of methanol, and chromatographed on a Sephadex LH-20 column (2.6×100 cm) with methanol at a flow rate of 0.1 ml/min (Amersham Biosciences), eluting it with (90%) methanol. Active fractions of 5 ml each were collected and combined using a fraction collector (Amersham Biosciences) and stored at 4^0C.

3.2.9 Properties of biosurfactant

3.2.9.1 Determination of protein content of cell free broth

Protein content of cell free broth was estimated by Lowry *et al.*, 1951 method using bovine serum albumin (BSA) as standard.

3.2.9.2 Determination of residual sugar concentration

Residual substrate concentration (total sugars) was estimated by Anthrone reaction (Roe *et al.*, 1955). In brief 4 ml of Anthrone Reagent (2g of Anthrone dissolved in 1 l of cone, sulfuric acid) was added to 1 ml of sample or standard solution and rapidly mixed in an ice bath. Tubes were incubated in a boiling water bath for 10 min., cooled and read at 620 nm using a reagent blank. Sugar in the sample was determined from calibration curve plotted using sucrose as the standard. (Dubois *et al.*, 1956).

3.2.9.3 Determination of lipid content of the isolated biosurfactant

To determine the lipid content, the biosurfactant was extracted by gently refluxing with diethyl ether for 1 h. This step was repeated thrice. The ether extract was dried with anhydrous Na_2SO_4 and clarified, followed by gravimetric estimation of the crude lipid.

3.2.9.4 Critical micelle concentration (CMC) measurement

Critical micelle concentration (cmc) is the concentration of an amphiphilic component in solution at which the formation of micelles in the solution is initiated. The *cmc* was determined by plotting the surface tension as a function of the logarithm of biosurfactant

concentration. Concentrations ranging from 2.5 to 40 g/l of the crude biosurfactant and isolated fractions were prepared in phosphate buffered saline. The surface tension of each sample was determined by using Wilhelmy plate measurement technique at room temperature (25^0C).

3.2.9.5 Stability studies of isolated biosurfactant

Stability studies were done using cell-free broth obtained by centrifuging cultures at 10000 rpm for 15 min. To study pH stability of the cell-free broth, the pH of the cell-free broth was adjusted to different pH values (4.0-12.0) and change in surface tension value and emulsification activity was measured. The culture liquid pH was adjusted with 1 M NaOH. To study effect of temperature cell free broth was exposed to temperature (4-100^0C) and surface activity and emulsification capacity of the cell-free broth was determined. Similarly, effect by varying NaCl concentrations (2 to 10%) was determined.

3.2.10 Characterization of biosurfactant

3.2.10.1 Thin Layer Chromatography (TLC)

The fractions obtained by elution with methanol were pooled. The biosurfactant was identified by TLC on silica gel 60 (Merck, Darmstadt, Germany) using $CHCl_3/CH_3OH/H_2O$ (65:15:4) as solvent. Components were detected both by spraying with distilled water and heating the plates at 110^0C for 5 min.

3.2.10.2 High Performance Liquid Chromatography (HPLC)

For HPLC analysis, the bacterial culture was withdrawn aseptically and centrifuged at 10000 g for 10 min to pellet the cells. The surfactant in the clarified culture supernatant was measured by reverse phase C_{18} HPLC using Waters HPLC system equipped with a C_{18} column (5 µm, Merck, Germany). The mobile phase used was 50% n-propanol in TFA (trifluoroacetic acid) at 0.5 ml/min with a sample size of 20 µl. The absorbance of the eluent was monitored at 210 nm using UV detector. Surfactin from Sigma (USA) was used as a standard.

3.2.10.3 Fourier Transform Infrared spectroscopy (FTIR)

For FTIR analysis, dried biosurfactant was ground with KBr powder and was dispersed uniformly in a matrix of dry nujol (paraffin) mar, compressed to form an almost transparent

disc for FTIR spectra measurement in the frequency range of 4000-500 cm^{-1}. The biosurfactant sample was characterized using FTIR spectrophotometer (Thermo-Nicolet, USA) equipped with OMNIC software for data analysis.

3.2.10.4 Matrix-assisted laser desorption/ionization Mass spectrometric (MALDI-TOF-MS)

For mass spectrometric analysis of isolated biosurfactant, 0.5 µl of purified biosurfactant were spotted onto an anchor chip positions on a MALDI plate. 0.5 µl of matrix was added to the sample spot. The matrix tried was a saturated solution of 2,5-dihydroxybenzoic acid (DHB) in water and 0.3 mg/ml α-cyano-4-hydroxycinnamic acid in acetone: ethanol (2:1, v/v). A peptide standard was also spotted for external calibration. The spots were left at room temperature to dry and analyzed on Applied Biosystems Voyager System 4402 Mass Spectrometer in delayed mode, with an acceleration voltage 20 kv. The DHB matrix produced the best results therefore; these spots were used for analysis.

3.2.10.5 Nuclear Magnetic resonance spectroscopy (NMR)

One dimensional ^{1}H NMR spectra were recorded at 298K on a 500 MHz NMR spectrophotometer (Bruker, Germany). The samples were prepared as solutions in 100% $CDCl_3$ using approximately 1-3mg of biosurfactant.

3.2.11 Optimization of physio-chemical parameters for biosurfactant production

For optimal production of biosurfactant by *Bacillus subtilis* DSVP23 using cotton seed as substrate, influence of various physio-chemical parameters was studied.

3.2.11.1 Effect of substrate concentration

To investigate the effect of concentration of substrate on biosurfactant production, cotton seed hull was added at conc. 2.0, 4.0, 6.0, 8.0 and 10.0 % (w/v) aseptically into MSM. Effect of cotton seed hull concentrations on biosurfactant yield was determined at different time intervals.

3.2.11.2 Effect of additional carbon source

To study the effect of additional carbon sources on growth and biosurfactant production, MSM was supplemented with 2% (w/v) glucose, starch, sucrose, fructose, maltose and

glycerol individually into the cotton seed hull containing medium. Changes in surface tension value, biosurfactant yield and biomass were measured after 48 h of growth at 37^0C.

3.2.11.3 Effect of organic and inorganic nitrogen sources

0.5% (w/v) inorganic nitrogen sources (ammonium sulphate, ammonium nitrate, ammonium chloride, sodium nitrate, sodium nitrite and potassium nitrate) and organic nitrogen sources (urea, beef extract, yeast extract and tryptone) were supplemented in MSM containing cotton seed hull. Surface activities, biomass and biosurfactant production were measured as described previously.

3.2.11.4 Effect of sodium nitrate and ammonium nitrate concentrations

Biosurfactant production and surface tension, emulsification value (E_{24}) and Biomass were determined by incorporating different concentration (2-10g/l) of sodium nitrate and ammonium nitrate into the MSM.

3.2.11.5 Effect of metal salts

Effect of metal salts: $CaCl_2$, $MnSO_4$, $CoCl_2$, $CuSO_4$, $HgCl_2$, $CaCl_2$, $MgSO_4$ and $FeSO_4$ was determined by incorporating (0.5% w/v) into MSM. Changes in term of growth and biosurfactant yield, surface activities were monitored.

3.2.11.6 Effect of ferrous sulfate and manganese sulfate concentration

Biosurfactant production was evaluated by incorporating different ferrous sulfate concentration (2.0, 3.0, 4.0, 5.0, and 6.0 mM) and magnesium sulfate concentrations (10, 20, 30, 40, and 50 mg/l) in MSM. Results were recorded in terms of changes in surface activities, emulsifying activities and biosurfactant production to that of control without any supplement.

3.2.11.7 Effect of amino acids

Various amino acids at concentration 0.1% (w/v), aspartic acid, alanine, asparagine, glutamic acid, glycine, leucine, lysine and valine were added individually to MSM containing cotton seed hull. Changes in term of growth and biosurfactant yield, surface activities were monitored with respect to control.

3.2.11.8 Effect of inoculum size

B. subtilis DSVP23 at different inoculum size 0.5 to 4.0 % (v/v) from an overnight broth of 10^6 cfu/ml was inoculated into MSM. Changes in surface tension value (mN/m), emulsification value (E_{24}), biomass (g/l) and biosurfactant production (g/l) were estimated.

3.2.11.9 Effect of pH and temperature

Different pH (4.0 to 10.0) and temperature (25-45^{0}C) were used in the study to observe their effect on surface tension value (mN/m), emulsification value (E24), biomass (g/l) and biosurfactant production (g/l) by *B. subtilis* DSVP23.

3.2.11.10 Effect of agitation and aeration

Different agitation speeds (0-250 rpm) and aeration speed (0.25 vvm, 0.50 vvm, 1.0 vvm, 1.5 vvm, 2.0 vvm and 2.5 vvm) were used and their effect on biomass and biosurfactant production was evaluated.

3.2.12 Mutational studies using random mutagenesis approach

To enhance the production of biosurfactant, a random mutagenesis approach was undertaken using a chemical mutagen, Ethyl methane sulfonate. EMS mutagenesis was carried out in the concentration range 1-5% using modified protocol given by Sleep *et al.*, 1991. 100 µl of EMS treated culture was plated evenly onto the LB agar plates and incubated at 37^{0}C for 24 h. Colonies thus formed were picked and grown in LB broth medium and screened for biosurfactant production using haemolytic assay and surface tension reduction. Colony showing maximum reduction in surface tension value was selected for further study.

3.2.12.1 Scale up studies of Wild type DSVP23 and Mutant DVM4 utilizing cheap raw material

Fermentation studies for biosurfactant production by wild type (*B. subtilis* DSVP23) and mutant type (*DVM4*) on a large scale was done using 6.5 L fermentor. Cotton seed hull, wheat bran, rice straw, wheat straw, potato peel, apple peel and molasses were used as cost effective substrate in MSM supplemented with 2% sucrose. The working volume was 2.5 L; the temperature was maintained at 37^{0}C with aeration of 0.5 vvm per minute at 180 rpm. Fermentor was adapted with a collection vessel in the sir exhaust line to collect the foam

overflow. Sterile foam collecting vessels could be changed during the course of fermentation (Biotron, South Korea).

3.2.13 Applications of biosurfactant obtained from cost effective raw material

To study various applications, sand pack test, emulsification index (E_{24}), antimicrobial activity, biofilm removal and role in biodegradation studies for the isolated biosurfactant were performed.

3.2.13.1 Sand pack test

The application of biosurfactant in Enhanced oil recovery (EOR) was evaluated using the sand pack technique (Bordoloi and Konwar, 2008). Briefly, a glass column (30x2.5 cm) was packed with acid washed sand saturated with 100 ml of different oils (kerosene, motor oil, n-paraffin and crude oil). The efficiency of the isolated biosurfactant was tested by adding 100 ml of 0.5% aqueous solution into the column. Recovery of the oil was estimated by measuring the volume of oil released.

3.2.13.2 Determination of emulsification activity/stability

To estimate the emulsification activity 6 ml of hydrocarbon was added to 4 ml of the culture broth in a graduated tube and vortexed at high speed for 2 min. The emulsification index, E_{24} (%) was tested against different hydrocarbons: Kerosene oil, Hexadecane, Toluene, Motor oil, Dodecane, Tetradecane, and Hexane. For measurement of the emulsification activity of the acid precipitated biosurfactant, 4 ml of the biosurfactant solution (1 mg/ml) in water was taken. To this 6 ml hydrocarbon was added and emulsification activity/stability was determined as above. The emulsion stability was determined after 24 h.

3.2.13.3 Antimicrobial studies

The biosurfactant produced by *B. subtilis* DSVP23 was checked against fungal strains: *Candida albicans* MTCC 183, *Rhizoctonia solani* MTCC4633, *Fusarium oxysporum* MTCC 284, *Trichoderma viride* MTCC *2047* and bacterial strains: *Escherichia coli* MTCC443, *Staphlyococcus aureus* MTCC 96, *Pseudomonas aerugenosa* MTCC741, *Bacillus cereus* MTCC430.

3.2.13.3.1 Preparation of biosurfactant stock solutions

1 mg/ml stock solution of crude (solvent extract) active fraction was prepared in methanol. The solution were passed through 0.45 µl syringe filter (Millipore, USA), kept in sterile vials and stored at 4^0C until use.

3.2.13.3.2 Agar well diffusion method

The antimicrobial activity of the biosurfactant was evaluated using the agar well diffusion method (Gerald *et al.*, 1962). For this, the microbial inoculum containing 10^8 cfu/ml were spreaded on the surface of the PDA (for fungal strains) and LB (for bacterial strains) agar plates using a sterile cotton swab. 6 mm wells were dug in the agar media with a sterile borer and different concentrations of biosurfactant (0.5-40 µg/ml) were added to them. The plates incubated for 24 (for bacteria) and 48 h (for fungi) at 37°C. After incubation period, microbial growth was determined by measuring the diameter of zone of inhibition. Controls were maintained in which methanol was used instead of biosurfactant.

3.2.13.3.3 Transmission electron microscopy (TEM)

TEM analysis was performed at the Department of Microscopy, All India Institute of Medical Sciences (AIIMS), New Delhi, India. Samples containing 1 x 10^6 cells/ml of *Staphlyococcus aureus* MTCC 96 were incubated at 37^0C for up to 30 min with 12.0 µg/ml of biosurfactant and were centrifuged at 2000 × g. Controls were run in the presence of 50 mM Tris-HCl buffer pH 8.0. The bacterial cells were fixed by incubation with 2% glutaraldehyde, 2% paraformaldehyde in PBS for 20 min. After washing with 0.1 M sodium phosphate buffer pH 6.8, four times 10 min each, samples were post fixed with osmium tetroxide in the same buffer (1% osmium tetroxide, 0.8% potassium ferrocyanide) for 3 h at room temperature. They were washed three times with buffer and dehydrated in acetone series and embedded in epon resin. Ultrathin sections were mounted on copper grid and stained with 2% (w/v) phosphotungstic acid. The grids were examined by using a transmission electron microscope (Phillips, model CM 10, Holland).

Samples containing *C. albicans* MTCC 183 cells (1 x 10^6 cells/ml) were incubated at 28^0C for up to 30 min with 10.5 µg/ml of biosurfactant and were centrifuged at 2000 x g. Controls were run in the presence of 50 mM Tris-HCl buffer pH 8.0. The cells were fixed by 2% glutaraldehyde, 2% paraformaldehyde in PBS for 20 min. They were post fixed with osmium tetroxide in the same buffer (1% osmium tetroxide, 0.8% potassium ferrocyanide) for 3 h at

room temperature. They were washed three times with buffer and dehydrated in acetone series, and embedded in epon resin. Ultrathin sections were mounted on copper grid and stained with uranyl acetate and lead acetate. The grids were examined using a transmission electron microscope (Phillips, model CM 10, Holland).

3.2.13.4 Effect of biosurfactant on bacterial and fungal biofilm

3.2.13.4.1 *C. albicans* biofilm formation and quantification

For *C. albicans* biofilm formation, 200 µl of 5 x 10^8 cfu/ml of inoculum was suspended in Yeast Peptone Dextrose (YPD) and added to 96-well microtiter plate (MTP) for 90 min of adhesion phase. The wells were washed with sterilized PBS to remove loosely adhered cells. To the washed wells, 10.5 µg/ml biosurfactant was added and MTP was incubated at 37^0C for 24 h. Quantification of biofilm formed was done using XTT reduction assay using microtiter plate reader (Oasys UVM 340) at 492 nm. Testing was performed in triplicate. Fluconazole was used as a positive control in the study.

3.2.13.4.2 *Staphylococcus aureus* biofilm formation and quantification

Effect of isolated biosurfactant on *S. aureus* biofilm was evaluated using protocol given by Christensen *et al.,* (1985). 200 µl of 10^8 cfu/ml of inoculum was suspended in LB medium and added to MTP for 90 min of adhesion phase. The wells were washed with sterilized PBS to remove loosely adhered cells. To the washed wells, 12.0 µg/ml biosurfactant was added and MTP was incubated at 37^0C for 24 h. Biofilms formed by adherent 'sessile' organisms in plate were stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying. Adherent staphylococcal cells usually formed biofilm on all side wells and were uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined using microtiter plate reader 570 nm. These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiment was performed in triplicate and repeated three times, the data was then averaged and standard deviation was calculated.

3.2.13.4.3 Microscopic examination of biosurfactant effect on biofilm

To evaluate the affect of biosurfactant on the bacterial and fungal biofilm, scanning electron microscopy (SEM), fluorescent microscopy and atomic force microscopy studies were performed.

a) Scanning electron microscopy (SEM)

SEM was performed in accordance with the procedure given by Hawser and Douglas, 1994 and Franson *et al.*, 1984. Biofilms formed on biopolymer pieces (1.0 cm^2) were fixed with 2.5% (v/v) glutaraldehyde in PBS (pH 7.2) for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h, and washed with distilled water. The samples were dehydrated in ethanol series (50%, 80%, 90% and 100%). All samples were dried to critical point by Polaron critical point drier, gold coated and viewed under Scanning Electron Microscope (Leo 435, England).

b) Fluorescent microscopy studies

Biopolymer pieces containing biofilms were dipped in suspension containing 1:1 mixture of FDA (1 mg/ml in PBS) and EtBr (0.5 mg/ml in PBS) solution. Samples were incubated at 25^0C for 30 min. After incubation, the samples were examined under fluorescence microscope (Zeiss, Axiovert 25, Japan).

c) Atomic force microscopy (AFM)

Images of biofilms formed on biopolymer surfaces were obtained with a commercial atomic force microscope (NTEGRA NT-MDT, Russia) in semi-contact mode using sharpened silicon nitride cantilevers NSG10S with spring constant about 10 N/m. The cantilevers had an amplitude range 5-15 nm, tip radius 10 nm and cone angle of 22 degree. Height and deflection images were simultaneously acquired at a scan rate of 250 kHz. Data analysis was done using NOVA software.

3.2.13.5 Bioremediation studies

Batch studies under shake flask conditions were carried out to study biodegradation of oily sludge and pure hydrocarbons pristane and fluoranthene under optimal growth conditions for *B. subtilis* DSVP23.

3.2.13.5.1 Growth studies

Erlenmeyer flasks (250ml) containing 50ml of sterilized MSM supplemented with 1% (w/v) oily sludge, Tetra methyl pentadecane or pristane ($C_{19}H_{40}$, a branched alkane compound) and fluoranthene ($C_{16}H_{10}$, a Polycyclic Aromatic Hydrocarbon) were prepared respectively.

Bacterial isolate DSVP23 inoculum 2% (v/v) was added into sterilized flasks containing MSM with oily sludge, fluoranthene and pristane individually. Separate uninoculated experimental flasks containing 50 ml of sterile MSM supplemented with 1% (w/v) hydrocarbon source were taken as controls. The experimental flasks were then incubated at 7 days under shaking conditions (180 rpm) at 37^0C. Sampling was done after every 24 hours for 7 days. Bacterial growth was estimated by determination of cell dry biomass. Cell free broth was analyzed for change in pH and reduction in surface tension along with recovery of biosurfactant.

3.2.14.5.2 Chromatography for residual oily sludge

a) Column chromatography

The residual oily sludge was consecutively extracted twice from the experimental flasks with 50 ml of hexane, methylene chloride and chloroform respectively. All the extracts were pooled and dried at room temperature by evaporation of solvents under a gentle nitrogen stream in a fume hood. After solvent evaporation, the amount of residual oily sludge recovered was estimated gravimetrically and later dissolved in 50 ml of n-pentane and separated into soluble and insoluble fractions (asphaltenes). The soluble fraction was loaded on activated silica gel columns (50×2 cm) and was fractionated into alkane and aromatic fractions using silica gel column chromatography. The silica gel used was of mesh size 60-120 and was activated at a temperature of 70^0C for 2 h. Then it was eluted with 120 ml of hexane first and later with 120 ml toluene at the rate of 30-40 drops per minute to obtain the aliphatic and aromatic fractions respectively in two pre-weighed evaporating dishes. The fractions were then concentrated by evaporation of hexane and toluene under a gentle nitrogen stream in a fume hood.

b) Gas chromatography of residual oily sludge

After all the solvent had evaporated from discs, these were weighed again to gravimetrically estimate the percentage degradation of aliphatic and aromatic fractions with respect to uninoculated controls. Later the aliphatic fraction was dissolved in 10 ml hexane and the aromatic fraction in 5 ml acetone for gas chromatography. These samples were then analyzed in gas chromatograph (Hewlett Packard 5890 Series II). 0.4 ul of these were injected into the respective columns of the gas chromatograph (Hewlett Packard 5890 series II) fitted with Flame Ionization Detector (FID). The aliphatic fraction was injected into a 10 m×0.53 mm

DB 2887 Dimethyl polysiloxane column with 0.25 mm film thickness. The aromatic fraction was injected into a 30 m x 0.25 mm DB 5.625 (5% phenyl) methyl polysiloxane column with 0.25 um film thickness. During analysis, the injector and detector were maintained at 300^0 C and the oven temperature was programmed to rise from 80°C to 240°C at the rate of 5^0C per minute and then held at 240^0 C for 30 min. The carrier gas (nitrogen) was maintained at a flow rate of 1 ml/min.

Various compounds were identified by matching the retention time with authentic standards (Sigma Chemicals, USA; Acros Organics, Belgium. Results were quantified by comparing integrated peak areas of test samples for *Bacillus subtilis*DSVP23 with uninoculated controls.

CHAPTER 4

RESULTS

4.1 Isolation and screening of bacterial isolates for biosurfactant production

Total bacterial population obtained from oil sludge samples, fuel filling stations, ware houses and phenolic waste water were 1.8×10^{7} cfu/g, 2.4×10^{10} cfu/g, 1.6×10^{8} cfu/g and 1.2×10^{6} cfu/ml, respectively after 48h of incubation at 30^{0}C (Table 5).

The bacterial strains so obtained were then purified and checked for their ability to grow on different hydrocarbons *viz.* dodecane, hexadecane, pristane, toluene and fluoranthene as sole carbon source in minimal salt medium (MSM). Among, 340 purified bacterial isolates obtained, 26 isolates (DSVP1-DSVP26) showed the ability to grow on these tested hydrocarbons (Table 6). These 26 bacterial isolates were then tested for hemolytic ability, drop collapse assay, emulsification assay, and surface tension reduction as an index for biosurfactant production (Figure 4). Of these five isolates namely DSVP2, DSVP9, DSVP11, DSVP18 and DSVP23 were found to be potential biosurfactant producer (Table 7).

4.2 Cell surface hydrophobicity studies

A convenient way for confirmation of biosurfactant production by above bacterial isolates was performed using cell surface hydrophobicity studies. This technique includes various assays like hydrophobic interaction chromatography (HIC), salt aggregation test (SAT), bacterial adherence to hydrocarbon (BATH), and adhesion to polystyrene by replica plate test (RP). Results of these tests as shown in (Table 8) confirmed biosurfactant producing ability of isolates: DSVP2, DSVP9, DSVP11, DSVP18 and DSVP23.

4.3 Selection of potential biosurfactant producing strain using cost effective-raw material

The above five selected isolates (DSVP2, DSVP9, DSVP11, DSVP18 and DSVP23) were tested for utilization of 14 different cost-effective raw materials (Figure 5) namely cotton seed hull, tea leaves, wheat bran, corn starch, rice straw, wheat straw, bagasse, ground nut kernel, potato peel, apple peel, cotton seed, molasses, bamboo wood saw dust and gram husk used as substrates for biosurfactant production. The chemical composition of raw materials used in this study is summarized in (Table 9). The maximum surface tension reduction (28.62 mN/m) was achieved by bacterial isolate DSVP23, when the medium was supplemented with 2% cotton seed hull at 37^{0}C and 48 h of incubation (Table 10). This substrate was chosen for further investigation in the study for biosurfactant production.

Isolation source	Type of contamination	Total bacterial population	Number of purified strains isolated	Hydrocarbon utilizing isolates
Oily sludge, Ankleswar, Gujarat	Hydrocarbon contaminated	1.8×10^{7} (CFU/g)	78	12
Fuel filling stations Roorkee, India	Gasoline, Diesel fuel	2.4×10^{10} (CFU/g)	120	8
Warehouses soils Roorkee, India	Motor oil and waste oil	1.6×10^{8} (CFU/g)	96	4
Phenol waste contaminated water, BHEL, Haridwar, India	Phenol contaminated and coal tar contaminated	1.2×10^{6} (CFU/ml)	46	2
	Total		340	26

Table 5: Bacterial population in the original natural habitats and after isolation and selection on 2% hydrocarbon as substrate.

Isolation source	Isolates	Dodecane	Hexadecane	Pristane	Toluene	Fluoranthene
Oily sludge	DSVP1	++	++	-	-	-
	DSVP2	++	++	++	++	++
	DSVP3	++	++	++	++	++
	DSVP4	++	++	-	-	-
	DSVP5	++	++	++	+	-
	DSVP6	++	++	++	-	-
	DSVP7	++	++	++	+	+
	DSVP8	++	++	-	-	-
	DSVP9	++	++	++	+	-
	DSVP10	++	++	++	-	-
	DSVP11	++	++	++	++	++
	DSVP12	++	++	+	+	-
Fuel filling stations	DSVP13	+	+	-	-	-
	DSVP14	++	++	+	+	+
	DSVP15	++	++	++	++	-
	DSVP16	++	++	+	+	-
	DSVP17	++	++	++	++	++
	DSVP18	++	++	++	++	++
	DSVP19	++	++	++	++	-
	DSVP20	++	++	-	-	-
Warehouses soils	DSVP21	++	++	++	++	++
	DSVP22	++	++	++	+	-
	DSVP23	++	++	++	++	++
	DSVP24	++	++	+	-	-
Phenol waste contaminated water	DSVP25	++	++	++	+	-
	DSVP26	++	++	++	+	+

(+) indicates growth and (—) no growth

Table 6: Growth of bacterial isolates on different hydrocarbons.

Isolates	Drop Collapse	Blood Hemolysis	Emulsification Index E_{24} (%)	Surface Tension (mN/m)
DSVP1	—	—	—	—
DSVP2	+	+	+	+
DSVP3	+	—	—	+
DSVP4	—	+	—	—
DSVP5	—	—	+	—
DSVP6	+	—	—	—
DSVP7	+	+	—	—
DSVP8	+	—	—	—
DSVP9	+	+	+	+
DSVP10	+	—	+	+
DSVP11	+	+	+	+
DSVP12	+	—	—	+
DSVP13	+	—	—	—
DSVP14	+	—	—	—
DSVP15	—	—	—	—
DSVP16	—	—	—	—
DSVP17	—	+	+	—
DSVP18	+	+	+	+
DSVP19	+	—	+	+
DSVP20	—	—	—	+
DSVP21	+	—	—	—
DSVP22	+	—	—	—
DSVP23	+	+	+	+
DSVP24	+	—	—	+
DSVP25	—	—	+	—
DSVP26	+	—	+	—

(+) indicates growth and (—) no growth

Table 7: Screening of isolates for biosurfactant production.

Figure 4: Screening methods used for evaluating biosurfactant production:

A. Hemolytic assay
B. Drop collapse assay
C. Emulsification assay
D. Surface tension reduction assay using whelmey plate assay

	ASSAY					
STRAINS	HIC	SAT	RP	BATH		
				DODECANE	HEXADECANE	PRISTANE
DSVP2	91	1.3	—	14	18	19
DSVP9	95	>2.0	+	80	76	79
DSVP11	28	1.2	—	16	15	18
DSVP18	22	>3.0	—	90	87	75
DSVP23	98	1.0	+	14	12	14

Table 8: Cell surface hydrophobicity studies of isolates.

4.4 Characterization and identification of DSVP23

The selected bacterial strain DSVP23 was characterized using microscopic studies, various biochemical tests and molecular analysis using 16S rRNA amplification. On microscopic examination, DSVP23 appeared as gram positive, motile, rod shaped, endospore forming bacteria (Figure 6). On MSM medium, DSVP23 showed large creamish colonies having undulate margin with circular form and flat elevation (Figure 6). Biochemical analysis was performed using Bergey's manual of systematic bacteriology, the strain was tentatively named as *Bacillus subtilis* (Table 11). To confirm the identity of the isolate, PCR amplification and sequencing of 16S rRNA gene was done. The results revealed that the PCR amplified product yielded an amplicon of 1.5 kb (Figure 7) which corresponds to 16S rRNA gene. Alignment of the 16S rRNA gene sequences of bacterial isolate DSVP23 with sequences obtained by doing a BLAST searching revealed 100% similarity to *Bacillus subtilis*. The tree was constructed using the neighbour-joining method (Felsenstein, 1993). Dendrogram showing phylogenetic relationships derived from 16S rRNA gene sequence analysis of strain DSVP23 (GenBank accession no. EU679368) with respect to other *Bacillus* species is shown in Figure 8.

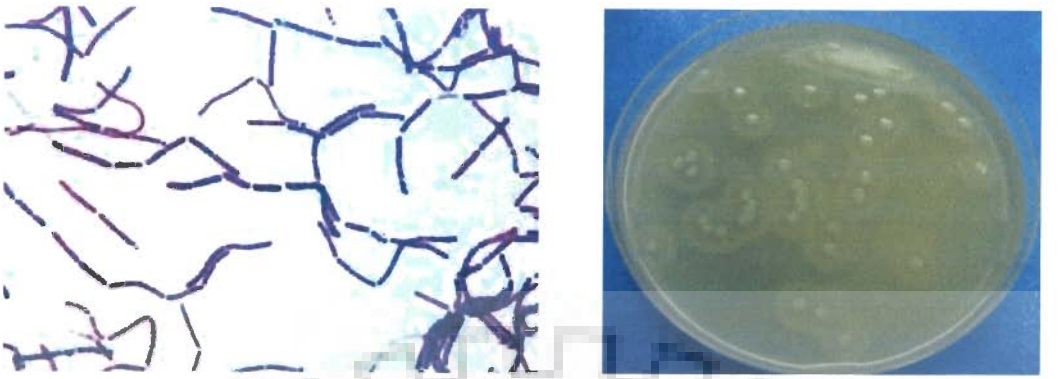
Component (%)	Cotton whole seed	Cotton Hull	Corn Starch	Rice Straw	Wheat Straw	Gram Husk	Wheat Bran	Bamboo saw dust	Tea waste	Bagasse	Molasse	Potato peel	Apple peel	Groundnut kernel
Dry matter	92	92									76.5			
Protein	23	4	0.5	3.68	2.46	4.64	16.73		25		6.3	18.34	2.75	25.2
Ether extract	6.54			1.31	1.52	0.20	4.86		3.53					
Nitrogen free extract	23.60			37.05	40.94	39.79	62.51		57		63			
Rough cellulose	28	53								26-47				
Hemicellulose										19-33				
Lignin										14-23				
Total sugars											46	62.15	21.52	26.1
Soluble sugar														4.5
Fat	20	3-5	0.15								0	0.38	2.66	48.2
Ash	5	2.8	0.15	19.35	9.20	7.12	6.43	4.3	5.87	1-5	16	9.24	1.30	
Calcium	2	15							1.5		0.8			
Carbon				63.86				15.9		90.22				
Nitrogen											1.01			
Iron			0.002											
Phosphorus	6	19							0.53		0.08			
Potassium											4.2			
Sodium											0.2			
Chlorine											1.4			
Magnesium											0.27			
Sulfur											0.5			
Crude fiber	20.8	47.8		38.61	45.88	48.25	9.47		8.60	43-52	0	7.80	7.4	2.1
Ether extract	17.5	1.7												
Moisture			14					11.1		46-52	23.5	9.89	75.25	6
Amylase			25											
Amylopectin			75											
Starch												11.9		11.5
Volatiles								79.8						
Soluble solids										2-6				
Dry solids											55-60			

Table 9: Chemical composition of different raw materials used in study

Raw Material	DSVP2	DSVP9	DSVP11	DSVP18	DSVP23
	Surface Tension value (mN/m)				
Cotton seed hull	34.22	32.24	45.23	42.21	28.62
Tea leaves	32.97	46.48	42.44	49.90	30.02
Wheat bran	34.90	48.23	46.50	44.52	29.28
Corn starch	58.50	54.42	57.24	50.80	34.62
Rice straw	37.80	55.02	50.19	56.91	32.96
Wheat straw	55.91	39.46	42.96	34.44	30.80
Bagasse	36.96	30.48	35.46	36.80	32.90
Ground nut kernel	34.80	40.90	42.90	49.90	32.50
Potato peel	38.63	49.90	68.50	68.50	28.80
Apple peel	32.81	68.50	58.80	38.80	30.91
Cotton seed	50.94	36.80	35.91	55.91	34.82
Molasses	34.05	32.91	30.96	32.96	32.08
Bamboo wood saw dust	37.64	49.96	42.48	43.80	34.20
Gram husk	34.97	34.80	42.20	40.12	35.02

Table 10: Utilization of cheap raw materials by selected isolates.

Figure 5: Cost effective raw materials used in this study.



(a) (b)

Figure 6: (a) Gram staining and (b) Plate showing colony morphology of DSVP23.

Biochemical characterization of *Bacillus subtilis* DSVP23	
Gram Stain	+
Shape	Rod
Motility	+
Spore Formation	+
VP test	+
Indole production	-
Reduction of nitrate	+
Starch hydrolysis	+
Catalase test	+
Oxidase test	+
Utilization of Glucose	+
Utilization of Glactose	+

Table 11: Biochemical analysis of DSVP23.

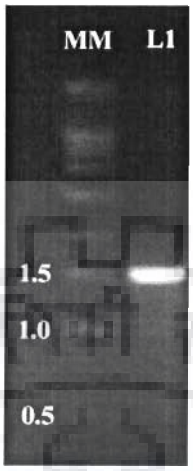


Figure 7: PCR amplified product (1.5 kb) of *Bacillus subtilis* DSVP23, where MM represents molecular weight marker.

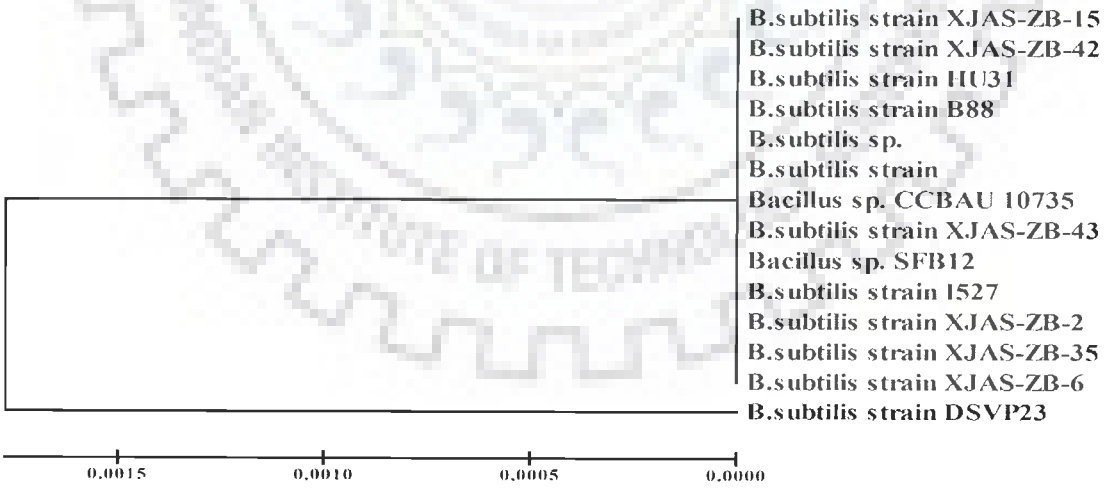


Figure 8: Dendrogram showing phylogenetic relationships derived from 16S rRNA gene sequence analysis of strain DSVP23 with respect to *Bacillus* species.

4.5 Purification of biosurfactant using column chromatography

Acid precipitation was used for extraction of biosurfactant of cell free broth of *B. subtilis* DSVP23 grown on cotton seed hull as substrate. Surface tension value of the crude extract obtained using dichloromethane was found to be 28.6mN/m in an active biosurfactant fraction obtained using silica gel column eluted with $CHCl_3$-MeOH (3:1). These active fractions were pooled, concentrated and further purified using gel filtration chromatography on Sephadex LH-20 column. The biosurfactant elution profile of Sephadex LH-20 column chromatography is shown in Figure. 9. Single peak was obtained when the column was eluted with MeOH and the active fractions were pooled. The organic phase was evaporated to dryness on a rotary evaporator and the pellet was used for further analytical assays.

4.6 Characterization of biosurfactant by TLC

Fraction obtained from gel filtration chromatography was subjected to preliminary characterization using TLC on silica gel 60 plates. Results revealed a white spot on TLC plates when sprayed with distilled water with retention index of 0.56 (Figure 10).

4.7 High performance liquid chromatography (HPLC)

HPLC analysis of the purified biosurfactant showed six major peaks at retention times: 13.65, 19.18, 20.58, 22.61, 25.62, and 23.67 respectively. The sum of the area of six peaks was plotted with a profile of DSVP23 similar to that of standard surfactin (Figure 11).

4.8 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectrum of biosurfactant from *Bacillus subtilis* DSVP23 is shown in Figure 12. The spectra showed strong absorption bands of peptides at 3343 cm^{-1}, 1641 cm^{-1}, 1518 cm^{-1} resulting from N-H stretching, C=O stretching and combined C-N stretching mode. The predominant adsorption bands were 1368 cm^{-1}, 1451 cm^{-1} and 2960 cm^{-1}, which indicate aliphatic chains (CH_2 ,CH_3) of sample. The intense band at 1641 cm^{-1} corresponds to –CO-NH-R group. The absorption region at 1737 cm^{-1} was due to an ester carbonyl band.

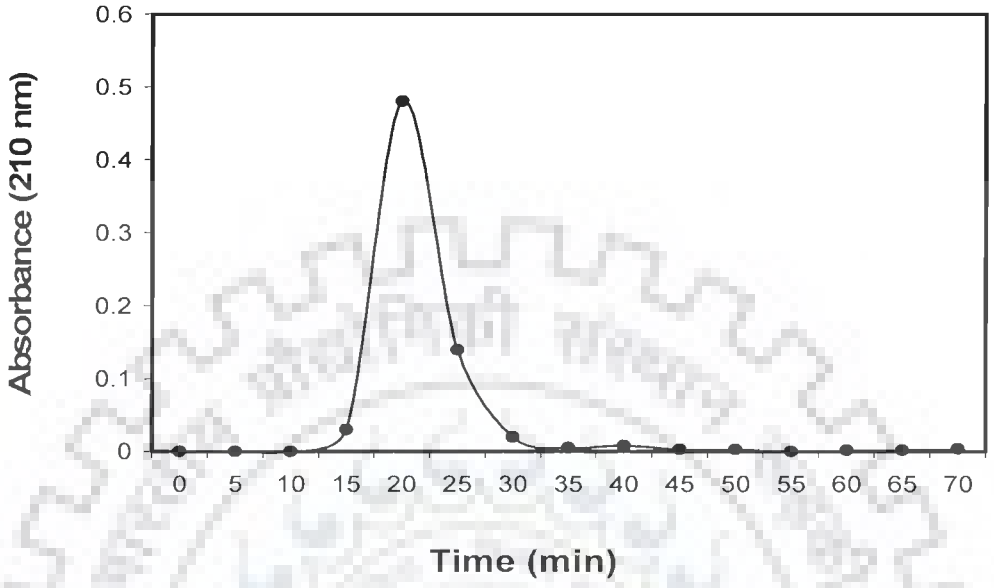


Figure 9: Elution profile of active lipopeptide fraction using Sephadex LH-20 gel filtration chromatography.

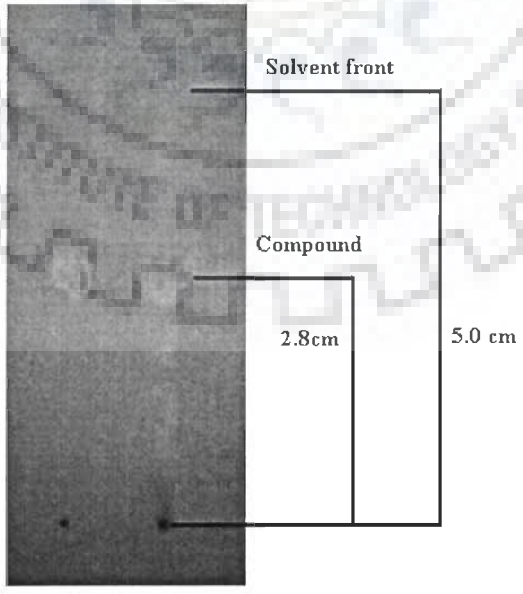


Figure 10: TLC profile of eluted biosurfactant from *Bacillus subtilis* DSVP23.

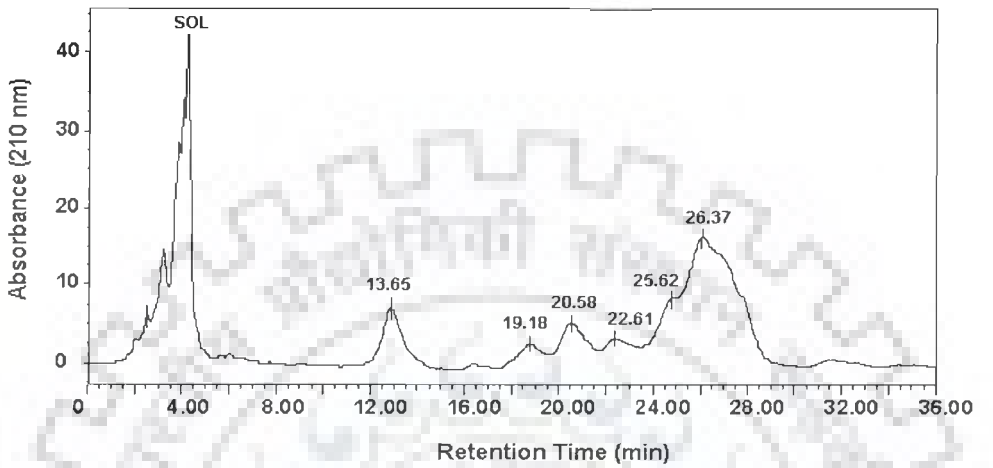


Figure 11: HPLC profile of biosurfactant from *Bacillus subtilis* DSVP23.

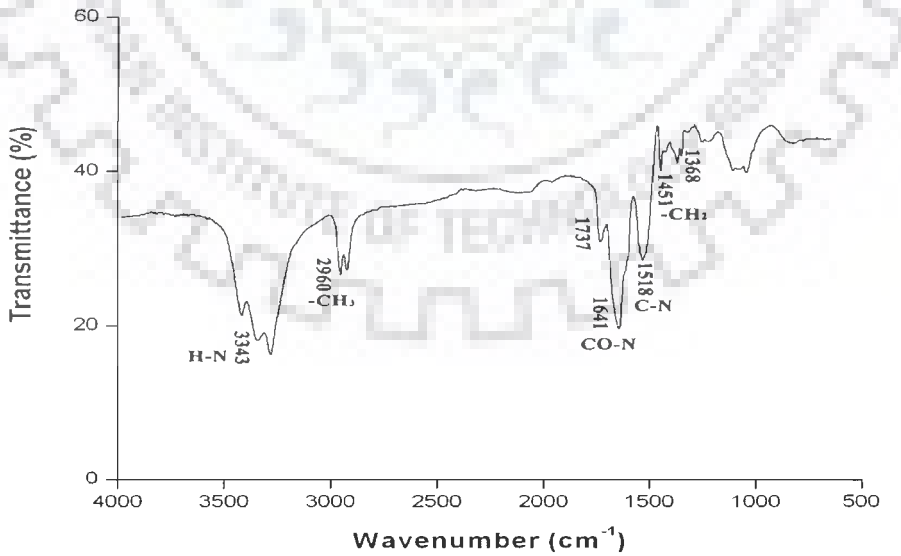


Figure 12: FTIR spectrum of biosurfactant from *Bacillus subtilis* DSVP23.

4.9 Matrix Assisted Laser Desorption Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS)

Data obtained from MALDI-TOF-MS of purified biosurfactant showed well-resolved groups of peaks at *m/z* values between 1000-1060 (Figure 13). The mass peaks at m/z 1165.2, 1022.7, 1044.6, and 1058.6 indicated it to be a lipopeptide with mixture of structural analogs. Mass spectrometry confirmed isomers of surfactin and iturin in purified fractions. The mass peak at 1044.6 was attributed to a surfactin isoform containing a β–hydroxy fatty acid with a chain of 14 carbon atoms. Table 12, shows the assignments of peaks obtained from MALDI-TOF-MS.

4.10 Nuclear Magnetic Resonance (NMR)

Results obtained with 500 MHz for ^{1}H NMR (Figure 14), clearly indicates the molecule being studied is a lipopeptide. The spectrum confirms the presence of a long aliphatic chain (CH_2 at 1.5-1.2 ppm) and a peptide backbone-amide-NH groups are in region from 7.95 to 7.2 ppm. A doublet signal obtained at 0.859 ppm corresponds to $(CH_3)_2$-CH group; specify terminal branching in the fatty acid component. The spectrum indicates the resonance of the following amino acids: valine with peptide CH at 4.6, CH_2 at 1.7, and 2 CH_3 at 0.9 ppm; leucine with peptide CH_2 at 1.7, CH at 1.3 and 2 CH at 0.9 ppm isoleucine with peptide CH at 4.2, CH at 2.2, CH_2 at 1.9 and 2 CH_3 at 0.9 ppm; aspartic acid or asparagines with peptide CH at 4.7 and CH_2 at 2.8 ppm; and glutamic acid or glutaminepeptide CH at 4.3, internal CH 2 at 1.9, and CH 2 α to the carbonyl at 2.1 ppm.

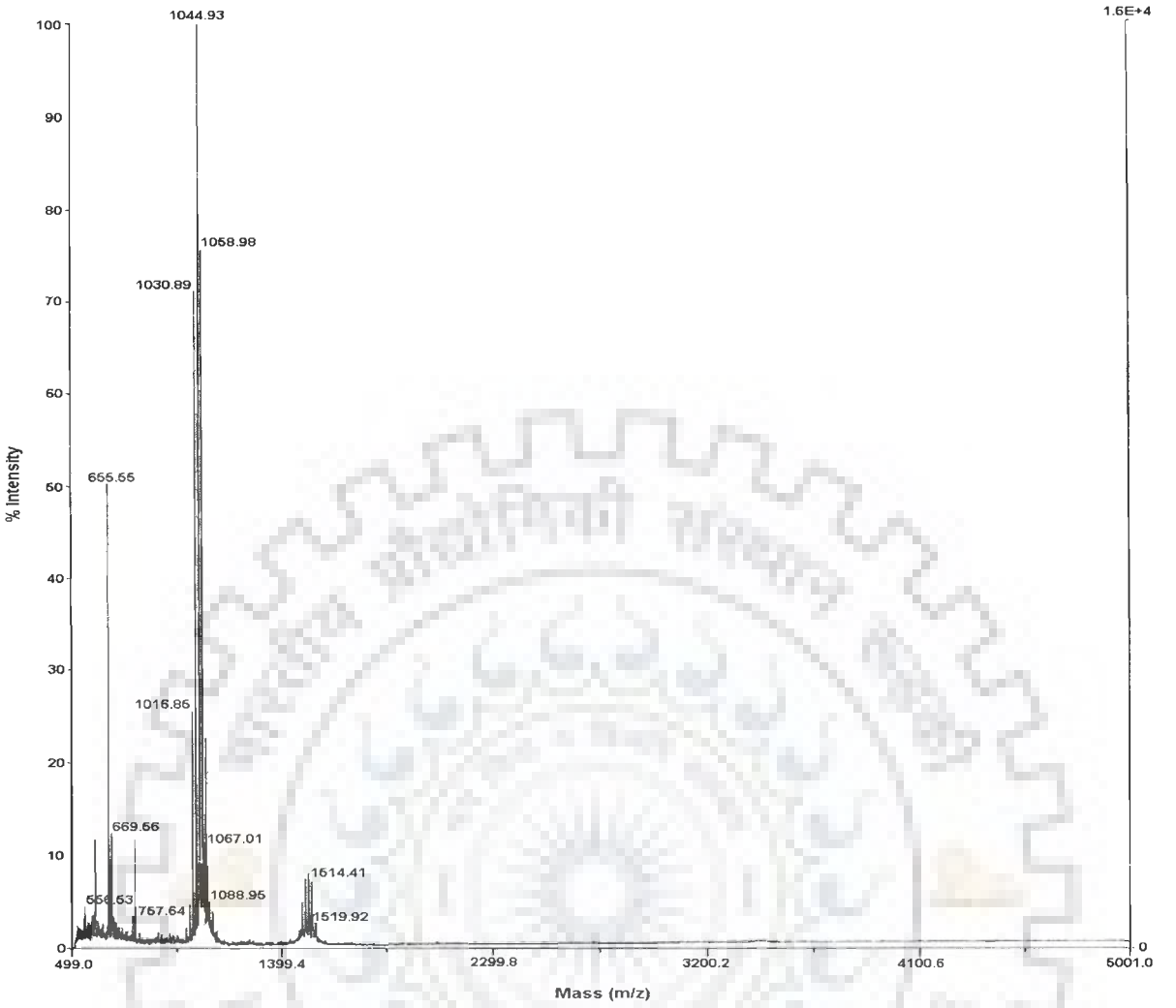


Figure 13: MALDI-TOF-MS profile of biosurfactant from *B. subtilis* DSVP23.

Mass peak (m/z)	Isoforms
1022.78	Leu/Ile-7, C-14 $[M+H]^+$
1030.57	Leu/Ile-7, C-13, $[M+H]^+$
1044.60	Leu/Ile-7, C-14,$[M+Na]^+$
1047.52	Leu/Ile-7, C-13, $[M+K]^+$
1058.64	Leu/Ile-7, C-15,$[M+Na]^+$
1061.60	Leu/Ile-7, C-14, $[M+K]^+$

Table 12: Assignments of mass peaks obtained by MALDI-TOF-MS of purified biosurfactant *B. subtilis* DSVP23.

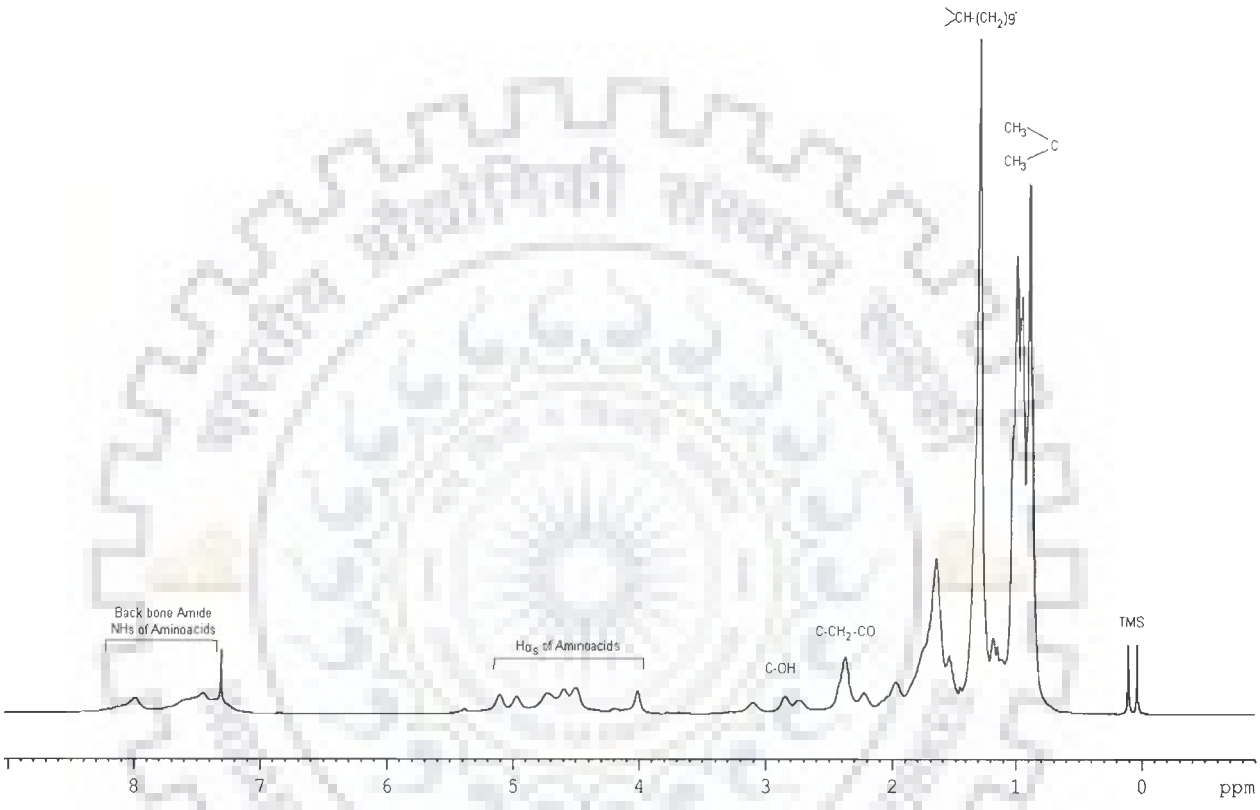


Figure 14: ^{1}H NMR spectrum of biosurfactant from *B. subtilis* DSVP23.

4.11 Physiochemical characterization of biosurfactant

4.11.1 Determination of protein, lipid and residual sugar content of biosurfactant

Biosurfactant isolated from cotton seed hull, was mainly composed of protein and lipid 15.2% protein, while lipid content was 18% respectively. No carbohydrate was detected in the isolated biosurfactant. The remaining constituents 65% were dialysable (inorganic salts) which had precipitated along with the surface active compound. The proportion of the surfactant ratio of protein and lipid remain constant in the acid precipitate at different periods of harvesting.

4.11.2 Stability studies of isolated biosurfactant

The aqueous solution of the *B. subtilis* biosurfactant (0.1%) when incubated for 4 hours at different temperatures (4-100°C) showed little variation in surface tension values. Similarly, a wide range of pH values (4-12) had minimal effect on the surface tension values of the biosurfactant. Data showed no appreciable changes in reduction of the surface tension value of biosurfactant were recorded when the NaCl concentration were varied from 2-10% (Table 13).

4.12 Optimization of biosurfactant production using cost effective resources

4.12.1 Effect of substrate concentration

Optimization of biosurfactant production was carried out in 500 ml flask containing different conc. (2, 4, 6, 8, 10%) of cotton seed hull as a substrate in minimal salt medium. Maximum biosurfactant production (2.42 g/l) by *B. subtilis* DSVP23 took place after 48 h of growth using 2% cotton seed hull (Table 14). To further enhance the production of biosurfactant an additional 2% (w/v) carbon sources namely, glucose, starch, sucrose, fructose, maltose and glycerol, were incorporated individually into the cotton seed hull containing minimal salt medium. Results revealed an increase in biosurfactant yield (3.82 g/l) and biomass (4.0 g/l) with decrease in surface tension value (28.47 mN/m) using sucrose as an additional carbon source in comparison to control (without additional carbon source, Figure 15).

pH	Surface Tension (mN/m)	CMD^{-1} (mN/m)	CMD^{-2} (mN/m)	E24 (%)
4.0	32.8	35.5	37.4	70
6 .0	29.7	36.6	36.5	71
8 .0	30.2	35.1	36.2	70
10 .0	32.2	36.6	40.4	69
12 .0	32.5	38.4	42.7	68
Temperature (^0C)	Surface Tension (mN/m)	CMD^{-1} (mN/m)	CMD^{-2} (mN/m)	E24 (%)
4	30.6	35.4	37.1	71
20	28.6	30.9	32.0	72
40	28.8	30.9	32.6	72
80	30.2	32.3	34.6	70
100	30.3	32.6	34.0	70
NaCl (%)	Surface Tension (mN/m)	CMD^{-1} (mN/m)	CMD^{-2} (mN/m)	E24 (%)
2	29.2	31.46	33.86	70
4	28.6	30.89	32.15	72
6	30.2	32.03	34.26	70
8	30.5	32.02	36.48	68
10	32.2	34.14	38.62	68

Table 13: Stability studies of isolated biosurfactant.

Time (h)	Cotton see hull (concentration)				
	2.0%	4.0%	6.0%	8.0%	10.0%
	Biosurfactant (g/l)				
0	-	-	-	-	-
16	-	-	-	-	-
24	0.54	0.42	0.52	0.62	0.22
36	1.12	0.82	1.14	0.84	0.62
48	**2.42**	2.06	2.02	1.86	1.02
72	2.40	1.94	2.02	1.8	1.33
80	2.41	1.92	1.9	1.78	1.24
96	2.40	1.93	1.92	1.78	1.26

Table 14: Effect of substrate concentration on biosurfactant yield.

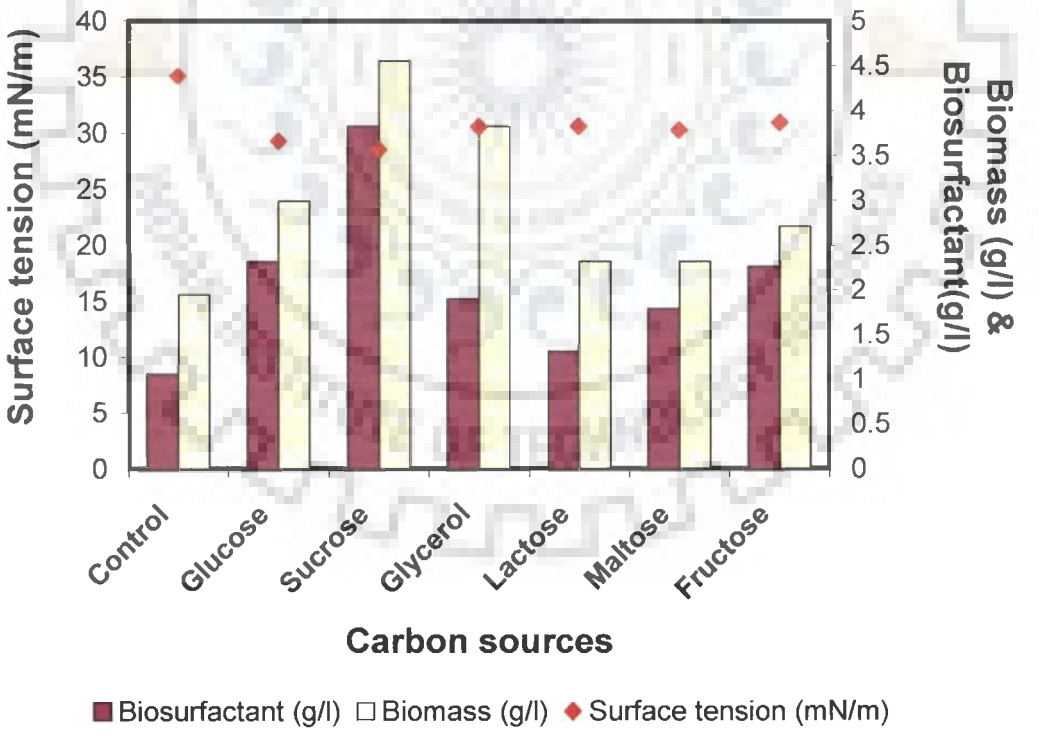


Figure 15: Effect of additional carbon source on surface tension and biosurfactant production.

4.12.3 Effect of organic and inorganic nitrogen sources

Among organic nitrogen sources used, tryptone gave maximum biosurfactant yield (3.0 g/l) followed by beef extract (2.4 g/l) and yeast extract (2.1 g/l). The inorganic nitrogen sources tested, sodium nitrate and ammonium nitrate served the best nitrogen sources followed by potassium nitrate for biosurfactant production. Minimum surface tension values 28.72 mN/m and 28.82 mN/m were achieved using sodium nitrate and ammonium nitrate respectively (Figure 16). Nitrogen sources like urea, ammonium sulfate and ammonium chloride depicted least reduction in surface tension value compared with nitrogen free medium therefore, were not suitable for biosurfactant production. Figure 17, represents the emulsification activity and biosurfactant production by *B. subtilis* DSVP23 using different concentrations of sodium nitrate and ammonium nitrate at 48 h of growth at 37°C.

4.12.4 Effect of Metal salts

Figure 18, shows effect of metal salts, $CaCl_2$, $MnSO_4$, $CoCl_2$, $CuSO_4$, $HgCl_2$, $CaCl_2$, $MgSO_4$ and $FeSO_4$ on biosurfactant production and growth of *B. subtilis* DSVP23. Among metal salts, an increase in biosurfactant yield (4.2 g/l and 4.5 g/l) and biomass (3.5 g/l and 3.8 g/l) was observed when manganese sulfate and ferrous sulphate were used in the growth medium. Surface tension values were reduced to 27.8 mN/m 28.2 mN/m for ferrous sulfate (5.0mM) and manganese sulphate (30mg/l) respectively. However, copper sulphate, mercuric chloride and cesium chloride showed an inhibitory effect on the growth of *B. subtilis* DSVP23. The biosurfactant production, biomass value, emulsification activity and surface tension values for *B. subtilis* DSVP23 using varying concentrations of ferrous sulfate and manganese sulfate Figure 19.

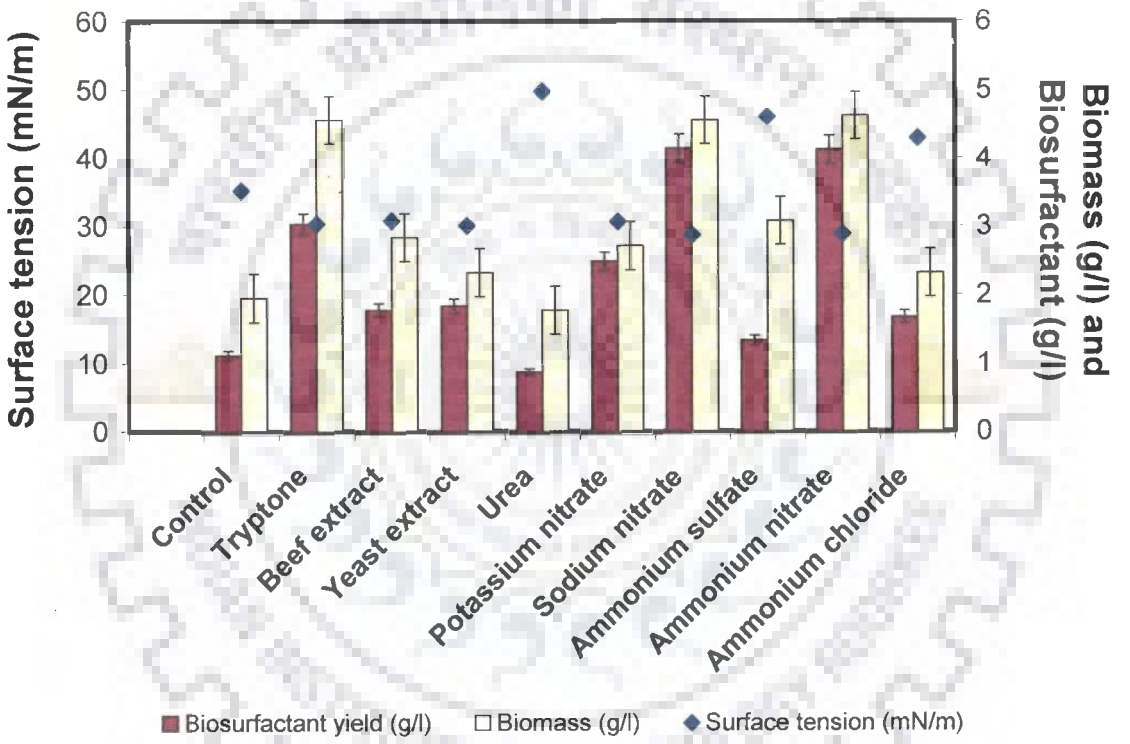


Figure 16: Effect of organic and inorganic nitrogen sources on biosurfactant production

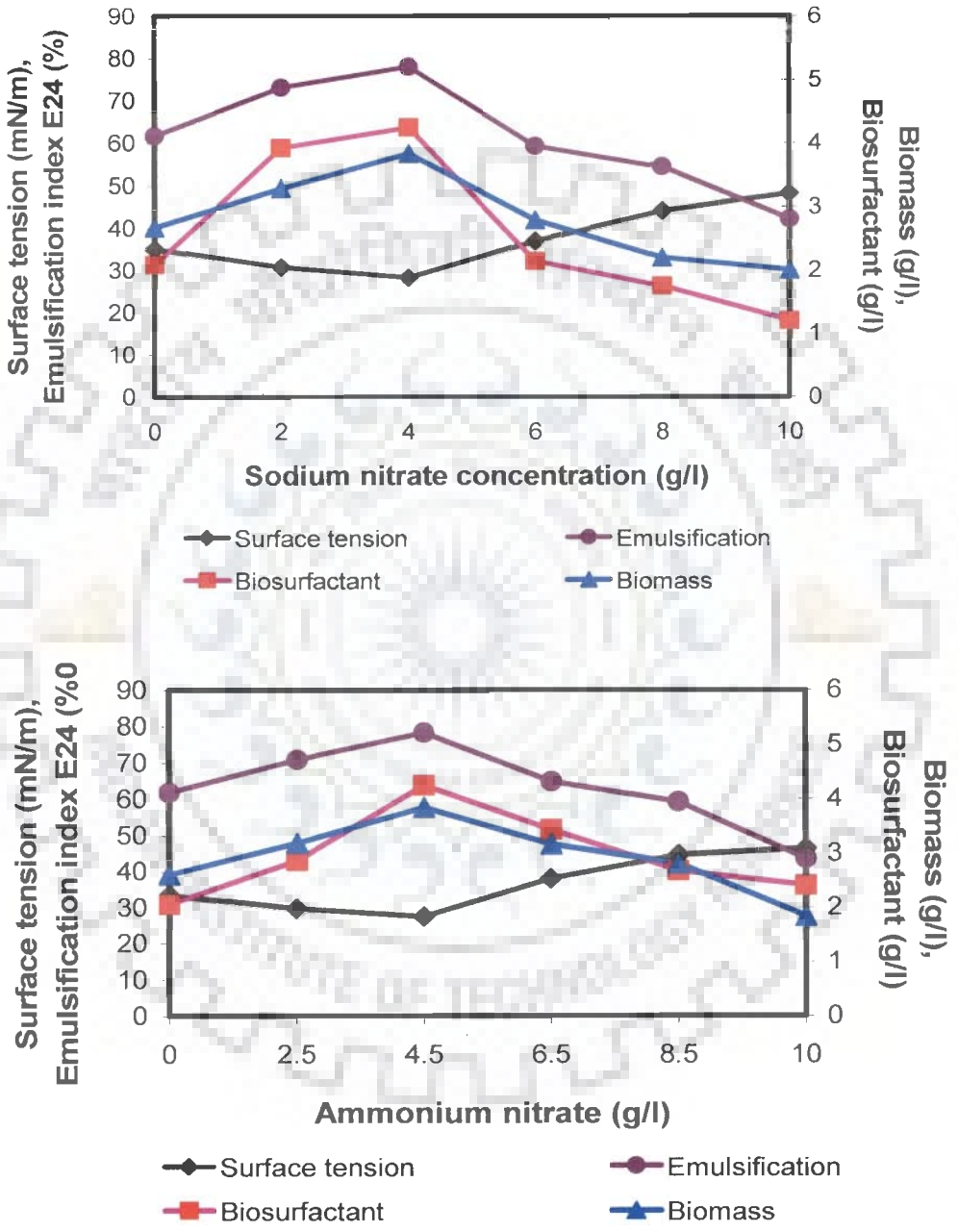


Figure 17: Effect of sodium nitrate and ammonium nitrate concentration on surface tension value, biomass, biosurfactant production and emulsification activity.

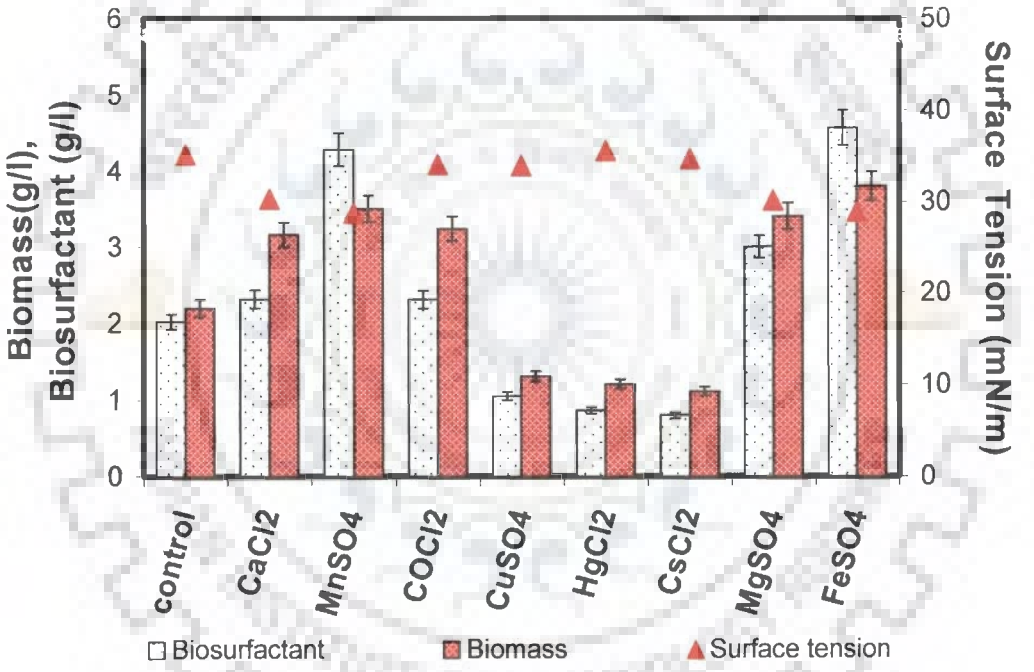


Figure 18: Effect of metal salts on biosurfactant production.

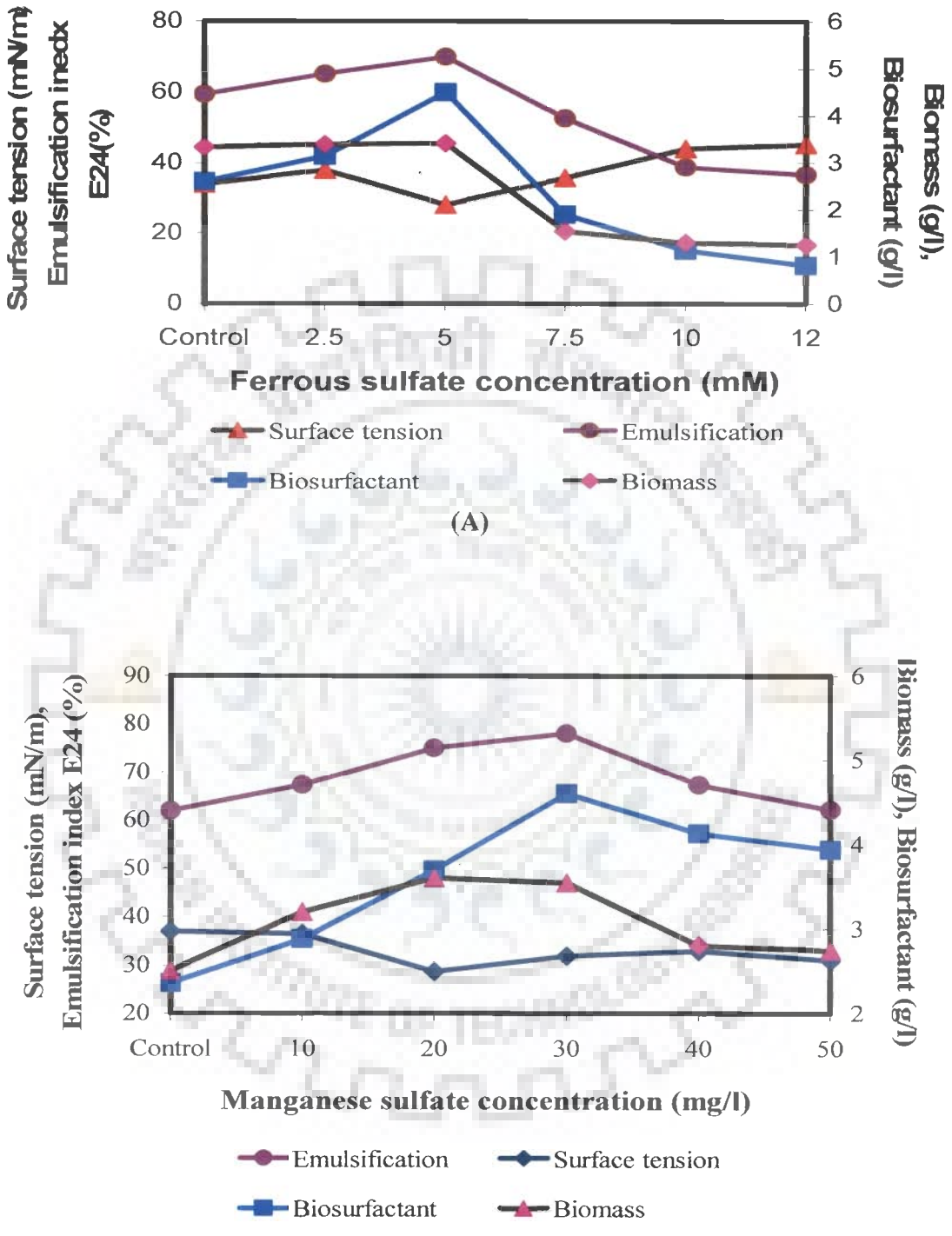


Figure 19: Effect of ferrous sulphate and manganese sulphate concentration on growth and biosurfactant production of *B. subtilis* DSVP23.

4.12.5 Effect of Amino acids

Various amino acids at concentration 0.1% (w/v) like aspartic acid, alanine, asparagine, glutamic acid, glycine, leucine, lysine and valine were added separately and examined to optimize the biosurfactant production by *B. subtilis* DSVP23. Result indicated that amino acids like aspartic acid, glutamic acid, lysine and valine showed stimulating effect on biosurfactant production, while rest of the amino acids did not affect biosurfactant production compared with the control (with amino acid depleted medium, Figure 20).

4.12.6 Effect of Inoculum size

The influence of inoculum size adjusted from 0.5-4.0% (v/v) on growth and biosurfactant production of *B. subtilis* DSVP23 was studied (Figure 21). It was determined that 2% (v/v) was the optimum size of the inoculum to be added to the medium. A steady decline in biosurfactant yield (<4.0 g/l) by *B. subtilis* DSVP23 was observed with an increase in inoculum size beyond 2.0%.

4.12.7 Effect of pH and temperature

The results presented in Table 15, clearly indicate that the pH of the medium and incubation temperature did play a major role in biosurfactant production by *B. subtilis* DSVP23. The organism was able to produce biosurfactant at different pH values and temperature values. Results showed that higher and lower pH values (10.0 and 4.0) resulted in drastic reduction in biosurfactant yield. Similarly it was observed that temperature values above 37°C resulted in lower yield of biosurfactant. Increase in incubation temperature to 45°C decreased biosurfactant yield to 1.41 g/l with negligible emulsification index (26%).

4.12.8 Effect of aeration and agitation

Figure 22 showed that there was an increase in biomass and a decrease in biosurfactant yield with an increase in aeration rate from 0.25 to 1.5 vvm and agitation rate from 0 to 250 rpm. Maximum biosurfactant yield was obtained at 0.5 vvm and 180 rpm respectively.

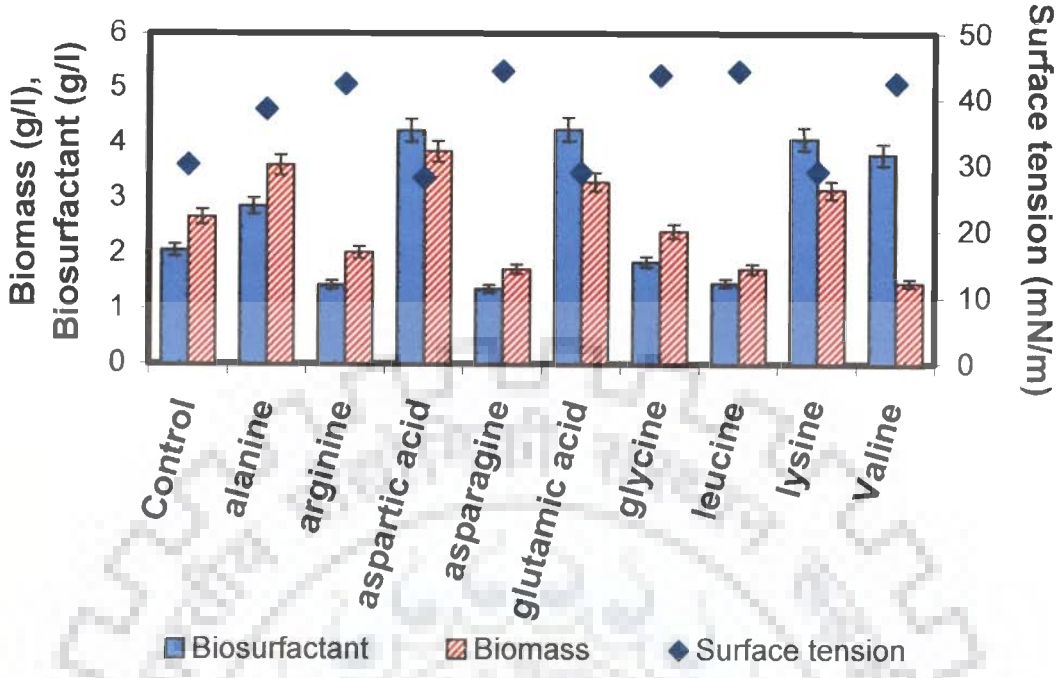


Figure 20: Effect of amino acids on growth and biosurfactant production

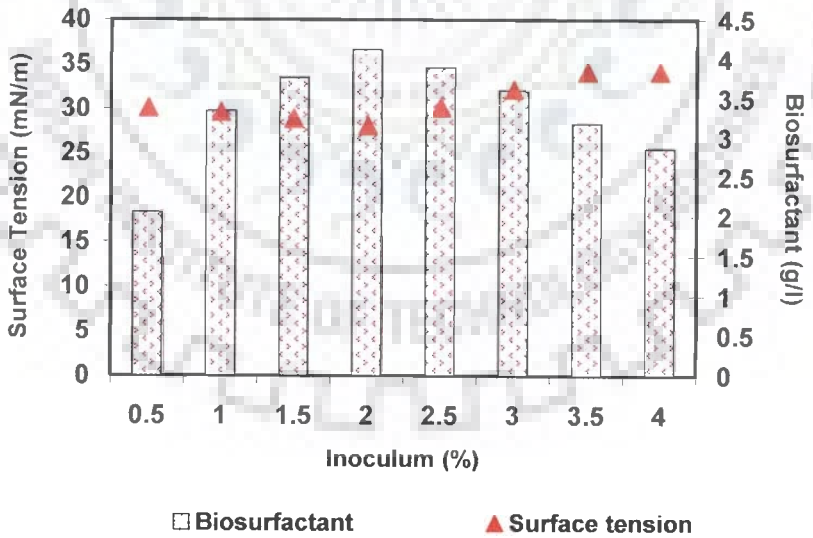


Figure 21: Effect of inoculum size on biosurfactant production.

	Biomass (g/l)	Surface tension (mN/m)	Biosurfactant yield (g/l)	Emulsification Index (E_{24} %)
Temp.				
25	0.82	60.12	1.06	51
30	3.62	38.24	3.2	66
35	3. 82	28.6	3.92	72
37	4.29	28.8	4.02	78
40	3.72	34.2	1.21	46
45	0.85	66.02	1.01	26
pH				
4.0	0.16	56	0.86	32
6.0	3.44	30	3.58	70
7.0	3.48	28.9	4.01	76
8.0	2.48	38	1.06	48
10.0	0.83	46	0.82	59

Table 15: Effect of different pH and temperatures on biosurfactant production.

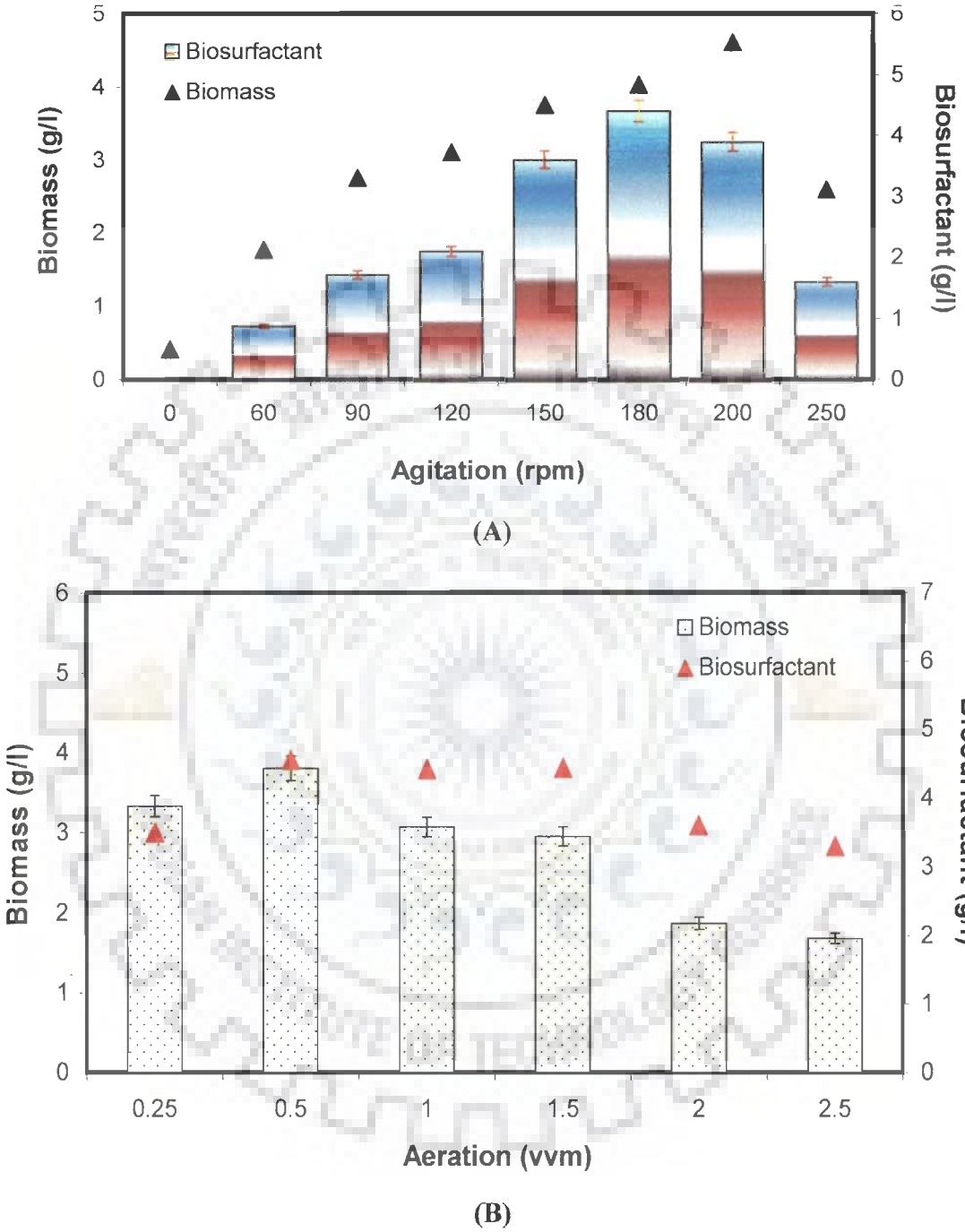


Figure 22. Effect of agitation and aeration on growth and biosurfactant production.

4.13 Mutational studies

Mutational studies done using 3% EMS treatment of *B. subtilis* DSVP23 resulted in 16 mutant colonies which were screened for their efficient biosurfactant producing ability. Results showed that out of 16 colonies screened, 8 colonies namely *DVM1, DVM4, DVM6, DVM7, DVM11, DVM12, DVM13* and *DVM16* gave above 20% increase in biosurfactant production as compared to that of wild type strain (Table 18). Since *DVM4* gave larger zone of hemolysis and maximum reduction in surface tension, it was selected for further studies (Figure 23).

Comparative analysis of wild type (DSVP23) and mutant type (*DVM4*) on nutrient agar medium showed rough morphology of mutant type with the smooth colonies of wild type (Figure 24). Mutant strain *DVM4* effectively utilized selected cheap raw materials for biosurfactant production in comparison to wild type strain DSVP23 (Table 16). Surface tension values of cell free broth were reduced from 28.6 mN/m (for wild type) to 26.8 mN/m (for mutant type) using cotton seed hull as cost effective substrate for growth in earlier optimized MSM (Section 4.13). Results obtained using 6.5 l fermenter indicated an increase in biosurfactant production yield by mutant type (6.0 g/l) compared with wild type (4.0 g/l) as shown in Table 17.

4.14 Applications of biosurfactant obtained from cost effective raw material

4.14.1 Sand pack test

To study the possible commercial applications of the precipitated biosurfactant in microbially enhanced oil recovery (MEOR), a sand pack column test was performed. The *B. subtilis* DSVP23 biosurfactant (0.5% aqueous solution) was effective in recovery of oil as shown in Table 18.

4.14.2 Emulsification index (E_{24})

The *B. subtilis* DSVP23 biosurfactant showed an excellent emulsification capacity in addition to its surface active properties. Emulsification power of the biosurfactant is not only limited to kerosene oil but it is also to emulsify other hydrocarbon also as shown in Table 19. The biosurfactant can therefore, emulsify a wide range of hydrocarbons.

Mutant Colony	Surface Tension (mN/m)	Emulsification Index E_{24} (%)
Control	72	32
DVM1	32.97	72
DVM2	49.90	56
DVM3	68.50	28
DVM4	**27.80**	**80**
DVM5	55.91	30
DVM6	29.96	75
DVM7	30.80	68
DVM8	48.63	60
DVM9	50.81	40
DVM10	50.94	38
DVM11	30.05	76
DVM12	47.64	59
DVM13	32.10	68
DVM14	40.36	55
DVM15	53.40	35
DVM16	32.37	65

Table 16: Surface tension and emulsification index values of mutants.

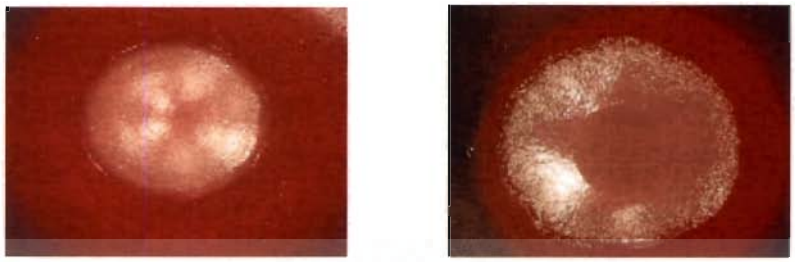


Figure 23: Wild (DSVP23) and Mutant (*DVM4*) type showing hemolytic activity on blood agar plate.

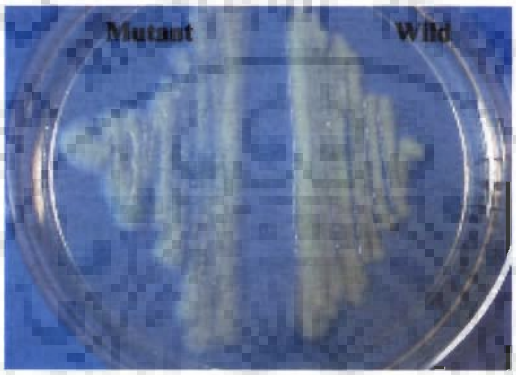


Figure 24: Comparative morphology of *B. subtilis* wild (DSVP23) and mutant (*DVM4*) type on Nutrient agar medium.

Figure 25: Fermentor used in the study.

Type of substrate (2%)	Biomass (g/l)		Surface Tension (mN/m)		Biosurfactant (g/l)		Emulsification Index E_{24} (%)	
	DSVP23	*DVM4*	DSVP23	*DVM4*	DSVP23	*DVM4*	DSVP23	*DVM4*
Cotton seed hull	4.65	4.98	28.8	27.8	4. 18	6.00	78	80
Wheat Bran	3.74	3.84	30.76	30.26	3.13	3.20	70	72
Rice straw	3.62	3.42	32.02	33.02	3.42	3.06	76	74
Wheat straw	3.46	3.76	29.94	29.42	3.68	3.86	76	78
Potato peel	4.21	4.41	29.42	28.86	3.74	4.02	72	79
Apple peel	4.20	4.26	29.82	29.82	3.48	3.48	32	32
Molasses	4.64	4.64	30.48	30.12	3.21	4.71	70	76

Table 17: Comparative study of biosurfactant production by wild type (DSVP23) and mutant type (*DVM4*) on different cost effective raw materials.

Sand Pack column	% Oil release
Kerosene oil	76
Motor oil	72
n-paraffin	68
Crude oil	70
Mobile oil	62

Table 18: Percentage oil release from sand pack column using 0.5 % of aqueous solution.

Carbon source	Emulsification index E_{24} (%)
Hexane	64.0
Toluene	72.0
Dodecane	68.0
Kerosene	78.0
Tetradecane	64.0
Hexadecane	72.0
Motor oil	70.0

Table 19: Emulsification of different hydrocarbons using cell free broth.

4.14.3 Antimicrobial studies of *B. subtilis* DSVP23 biosurfactant

The antimicrobial activity of the *B. subtilis* DSVP23 biosurfactant and its potency was quantitatively assessed by determining the MIC as given in Table 20 and 21. The biosurfactant obtained using cotton seed hull in MSM as cost effective substrate showed profoundly distinct antibacterial activity toward test organisms namely *S. aureus, E.coli, P. aeruginosa* and *B. cereus* with a zone of inhibition 18mm, 13.8mm, 12.1mm and 11.2mm respectively. Also biosurfactants displayed a maximum antifungal activity against *C. albicans* followed by *R. solani*, *F. oxysporum* and *T. viride* with a zone of inhibition of 15.2mm, 11.3mm, 10mm and 12.5mm respectively (Figure 26 and 28).

The transmission electron micrographs of control and biosurfactant treated cells of *S. aureus* are shown in Figure 27. The TEM picture of untreated cells (control) of *S. aureus* showed clear intact cell wall and membrane while the cell wall and membranes of treated cells were altered and clear deformity could be seen both in cell wall and membrane (Figure 27). The shrinkage and damage of cytoplasmic material and membrane could be clearly seen in biosurfactant treated cells compared to control. TEM micrographs indicated that the walls of treated bacterial cells were completely irregular in outline, without showing deposition of membranous material in the cell wall with almost complete solubilization. TEM study clearly indicates that the biosurfactant was able to cause damage of the cell wall and membrane in *S. aureus* which may be resulting in their death.

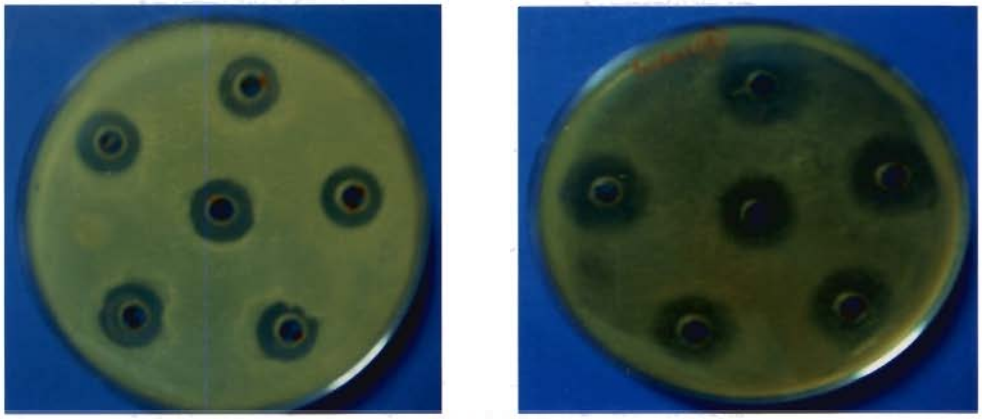
The micrographs of control and biosurfactant treated *C. albicans* cells as shown in Figure 29 indicated that the walls of untreated cells were intact and regular in shape while those of treated with biosurfactant were irregular in outline, without showing deposition of membranous material in the wall as observed in the control cultures. The treated cell interior looked completely necrotic; internal subcellular organelles were barely identified. Large quantities of fat globules were present near the cell periphery. Treatment with biosurfactant caused significant ultrastructural changes in morphology of cell. A pronounced disorganization of the cytoplasm, involution of the vacuole and invaginations of the plasma membranes was detected.

Bacterial Strains	MIC (µg/ml)	ZOI (mm)
Escherichia coli MTCC 443	13.5	13.8
Staphylococcus aureus MTCC 96	12	18
Pseudomonas aeruginosa MTCC 741	16	12.1
Bacillus cereus MTCC 430	18.5	11.2

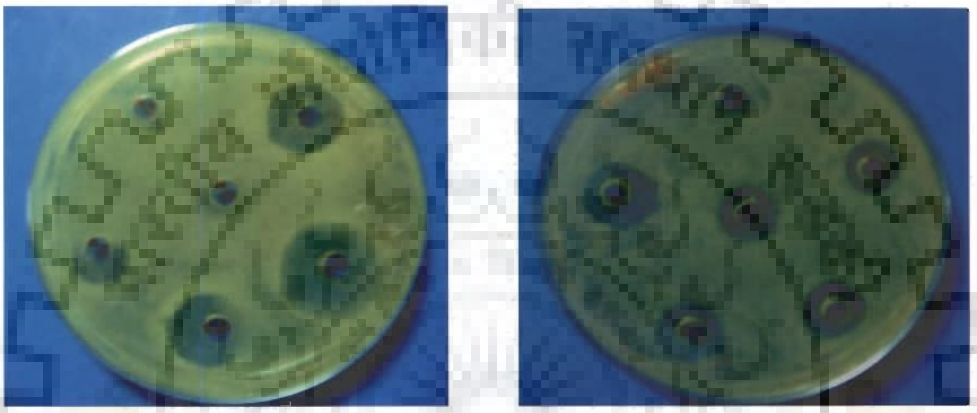
Table 20: MIC and Zone of inhibition values of *B. subtilis* DSVP23 biosurfactant against bacterial species.

Fungal Strains	MIC (µg/ml)	ZOI (mm)
Candida albicans MTCC 183	10.5	15.2
Rhizoctonia solani MTCC 4633	20	11.3
Fusarium oxysporum MTCC 284	22	10
Trichoderma viride MTCC 2047	30	12.5

Table 21: MIC and Zone of inhibition values of *B. subtilis* DSVP23 biosurfactant against fungal species.

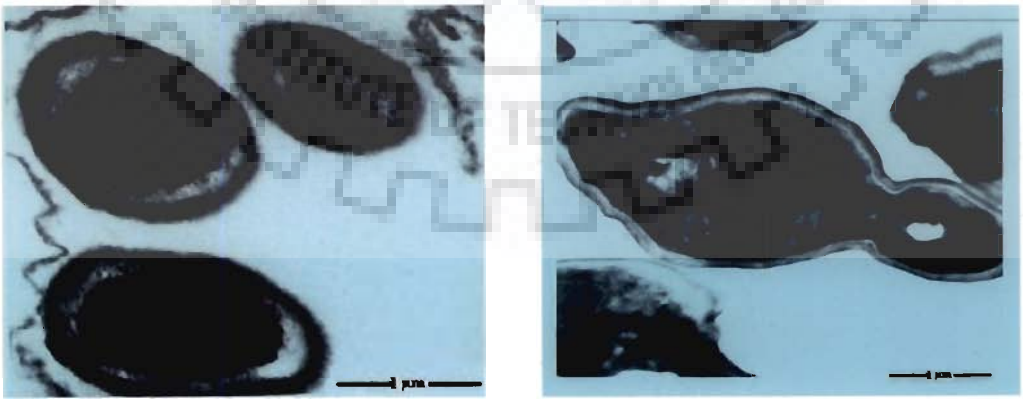


Pseudomonas aeruginosa *Escherichia coli*



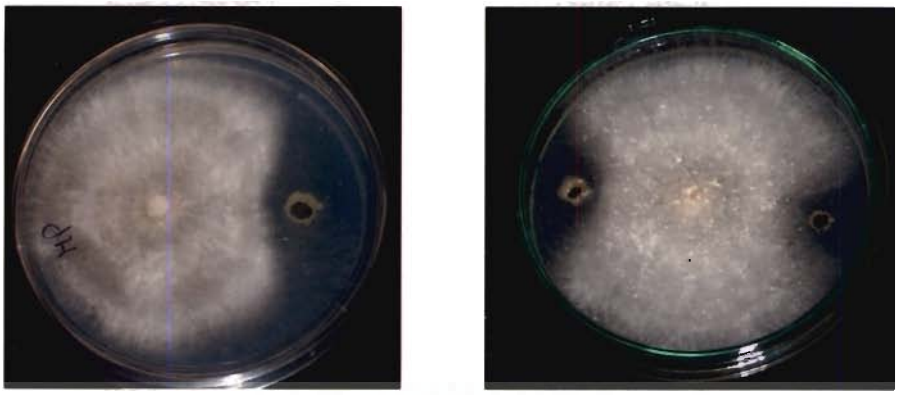
Staphylococcus aureus *Bacillus cereus*

Figure 26: Antibacterial activity of *B. subtilis* DSVP23 biosurfactant against bacterial species.



Control **Treated**

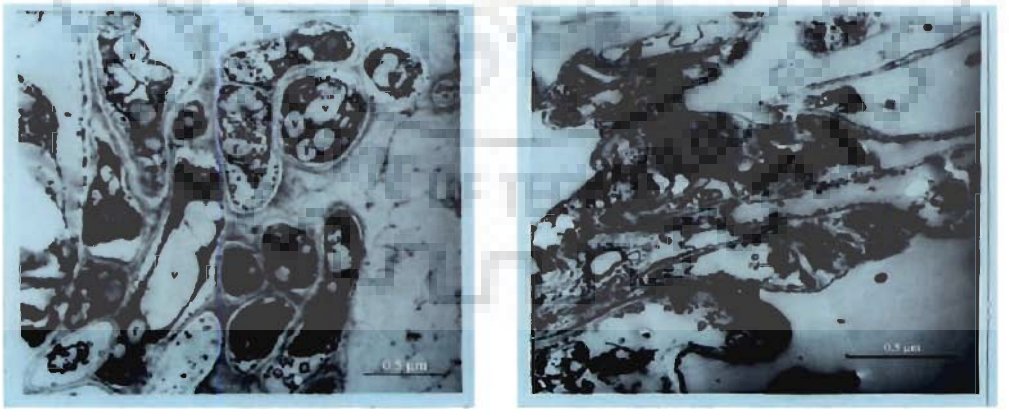
Figure 27: Transmission electron micrographs showing effect of *B. subtilis* DSVP23 biosurfactant on *S. aureus*.



Rhizoctonia solani *Fusarium oxysporum*

Trichoderma viride *Candida albicans*

Figure 28: Antibacterial activity of *B. subtilis* DSVP23 biosurfactant against fungal species.



Control **Treated**

Figure29: Transmission electron micrographs showing effect of *B. subtilis* DSVP23 biosurfactant on *C. albicans*.

4.14.4 Effect of biosurfactant on bacterial and fungal biofilm

Anti-adhesive activity of biosurfactant at concentration 10.5 μg/ml and 12.0 μg/ml assessed using MTP assay resulted in 78% reduction in *C. albicans* biofilm formation respectively and 72% in *S. aureus* biofilm (Figure 30). Microscopic analysis of *C. albicans* and *S. aureus* biofilm on PVC surface was performed using SEM. From electron microscopy it was clear that upon biosurfactant treatment the biofilm matrix was disrupted with an effect on cell morphology in comparison to the control biofilm (Figure 30).

Reduction in viable cell count was also checked using fluorescent viability test using two fluorescent dyes, FDA and EtBr. In control biofilms, only green coloration appeared due to absence of dead cells (Figure 31), viable cells showed well defined intense green fluorescence in areas such as the cell wall and vacuolar region that may be due to acetylesterase activity (Medzon and Brady, 1969). Consequently it was assumed that structures containing these enzymes showed intense green fluorescence. It was also noticed that fluorescence was concentrated at one point inside the viable cells and that the surrounding intracellular components showed a weak fluorescent staining. In case of biosurfactant treated biofilms (Figure 31), reduction in viable cell count (green coloured cell) was clearly observed with dead cells depicting a bright light red fluorescence.

AFM studies revealed topographic images of *S. aureus* and *C. albicans* biofilms shown in (Figure 32). The 2D and 3D image depicted that both *S. aureus* and *C. albicans* cells gives a better image resolution sensing height and texture variations of biofilm on the glass surface. In 3D image, the biofilm cells were seen as ridges that may be formed due to variable production of biofilm matrix, such that matrix is sometimes produced in such a great quantity that it protrudes around the cells and become more pronounced after drying of biofilm. Images obtained before treatment of both *S. aureus* and *C. albicans* biofilms with biosurfactant showed normal smooth surfaces with cell clustering. AFM images of biofilm after lipopeptide biosurfactant treatment infer that after treatment cells were completely disintegrated (Figure 32).

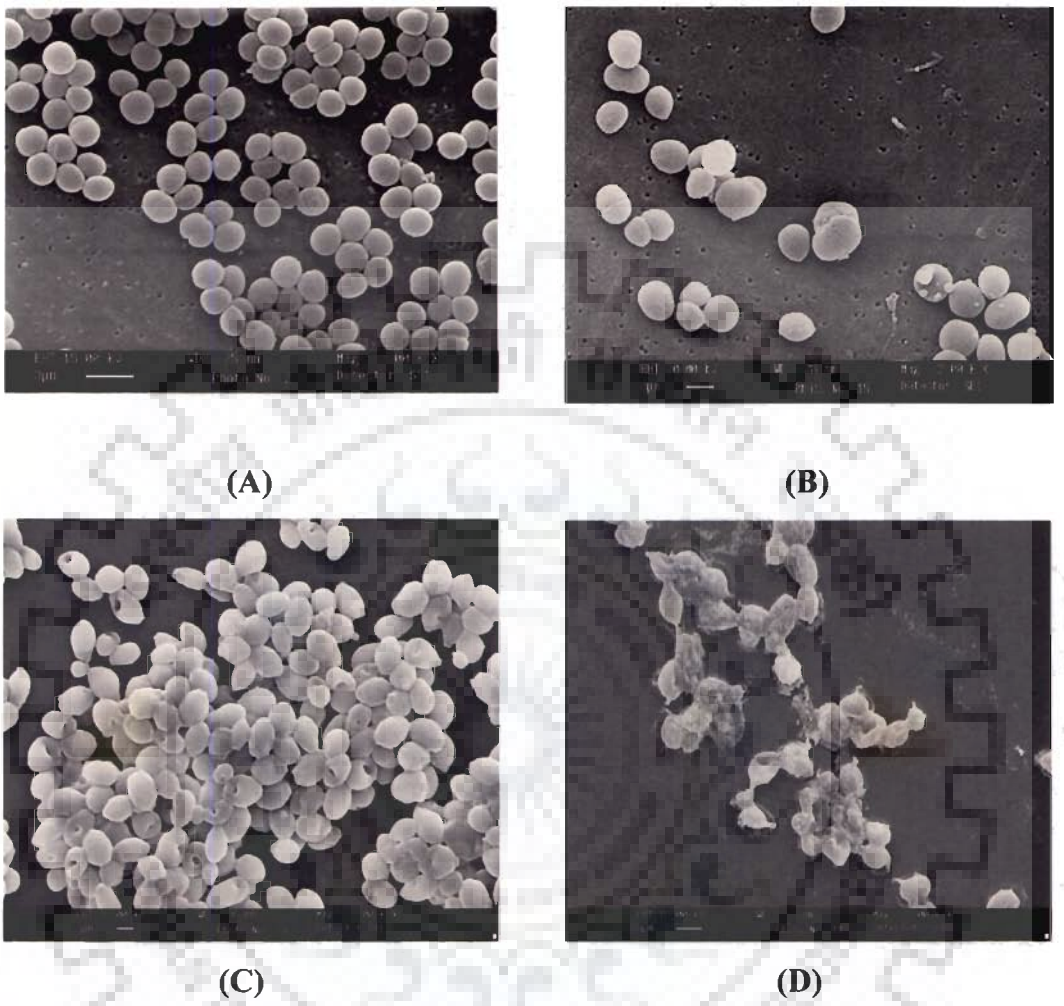


Figure 30: Scanning electron micrographs showing effect of *B. subtilis* DSVP23 biosurfactant. (A) Control *S. aureus* biofilm, (B) Treated *S. aureus* biofilm, (C) Control *C. albicans* biofilm, (D) Treated *C. albicans* biofilm.

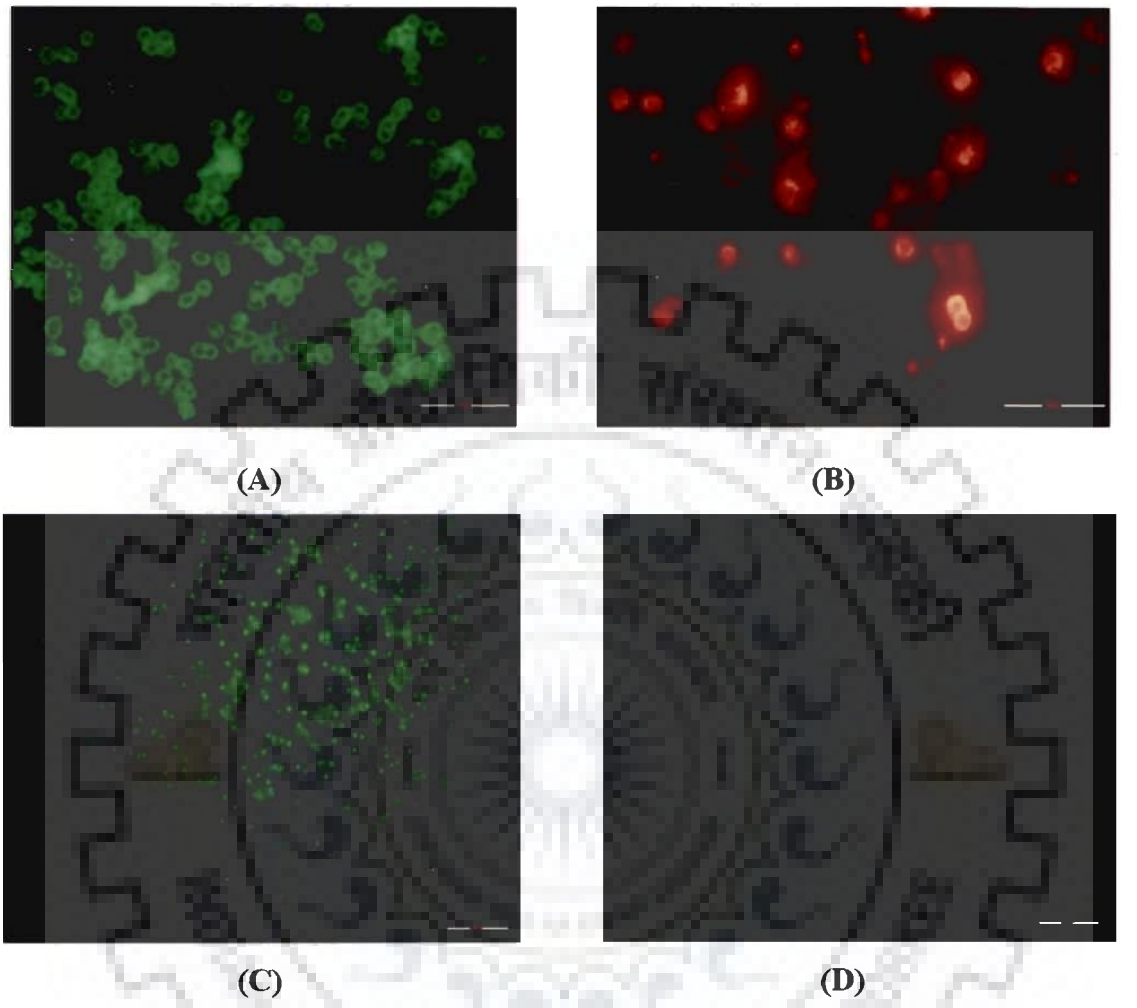


Figure 31: Fluorescent microscopic images showing effect of *B. subtilis* DSVP23 biosurfactant. (A) Control *S. aureus* biofilm, (B) Treated *S. aureus* biofilm, (C) Control *C. albicans* biofilm, (D) Treated *C. albicans* biofilm.

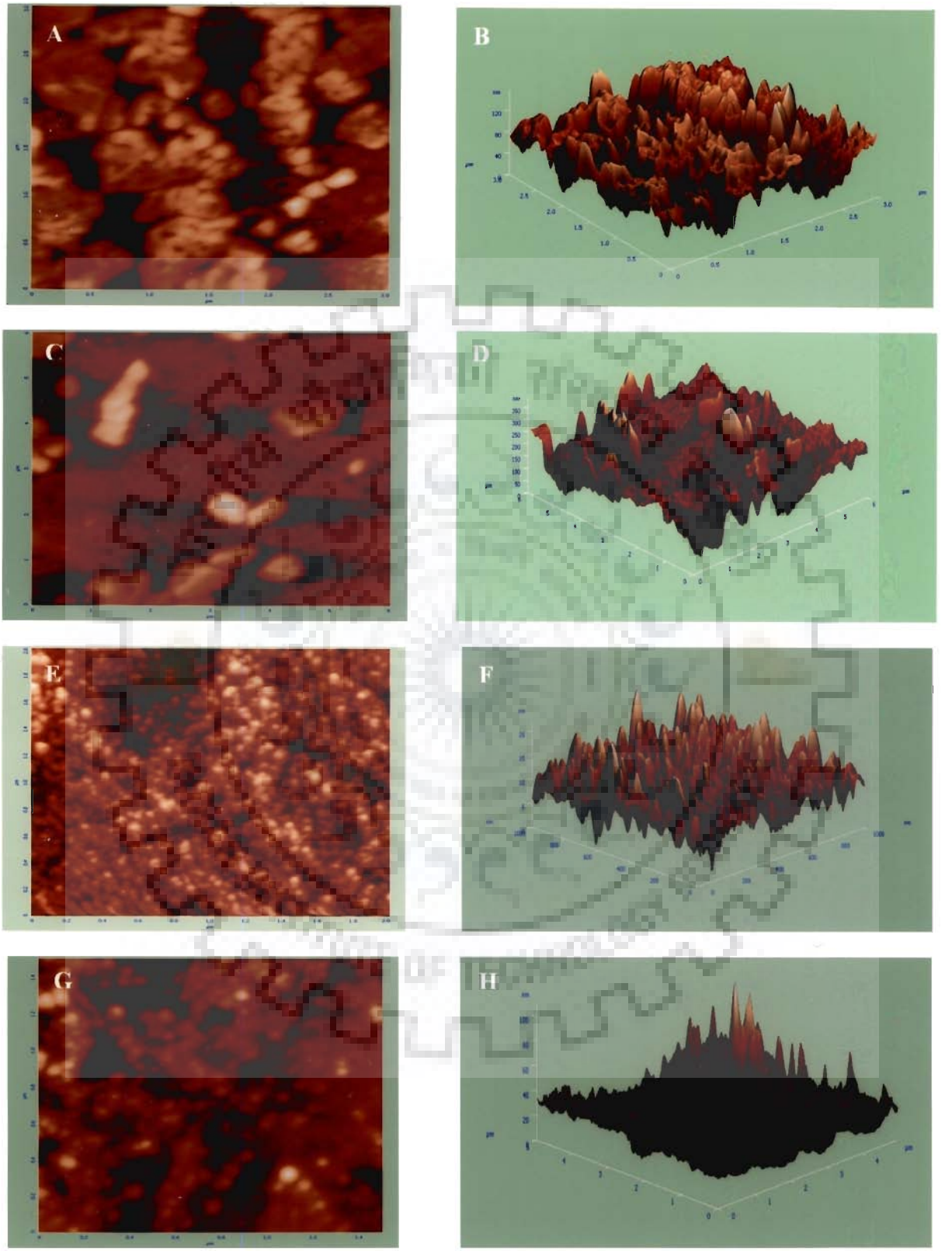


Figure 32: Atomic force micrographs (2D and 3D) showing effect of *B. subtilis* DSVP23 biosurfactant. (A-B) Control *S. aureus* biofilm, (C-D) Treated *S. aureus* biofilm, (E-F) Control *C. albicans* biofilm, (G-H) Treated *C. albicans* biofilm.

4.14.5 Biodegradation studies

The growth pattern of *B. subtilis* DSVP23, on MSM containing 1% oily sludge, pristane and fluoranthene respectively was studied over a period for 7 days. Maximum growth (biomass) was observed after 4 days of incubation on oily sludge (4.9 g/l), after 3rd day for pristane (4.10 g/l) while, it was observed after 5th day for fluoranthene (3.0 g/l) respectively as shown in Figure 33.The growth on substrates was accomplished with simultaneous drop in pH over the five days. The surface tension and biosurfactant production was monitored every 24 h. For pristane maximum biosurfactant was attained after 48 h of growth whereas, it was after 72 h in case of oily sludge and fluoranthene. The maximum reduction in surface tension value was observed (70 mN/m to 28.8 mN/m) on pristane followed by oily sludge (30.2 mN/m) and fluoranthene (35.4 mN/m) after 7 days of incubation.

Gas chromatographic profiles obtained after 5 days of incubation, revealed that *B. subtilis* DSVP23 was able to degrade hydrocarbons of the oily sludge. Figure 34, indicate the visual change in the chromatographic profile of the oily sludge, compared with those of controls.

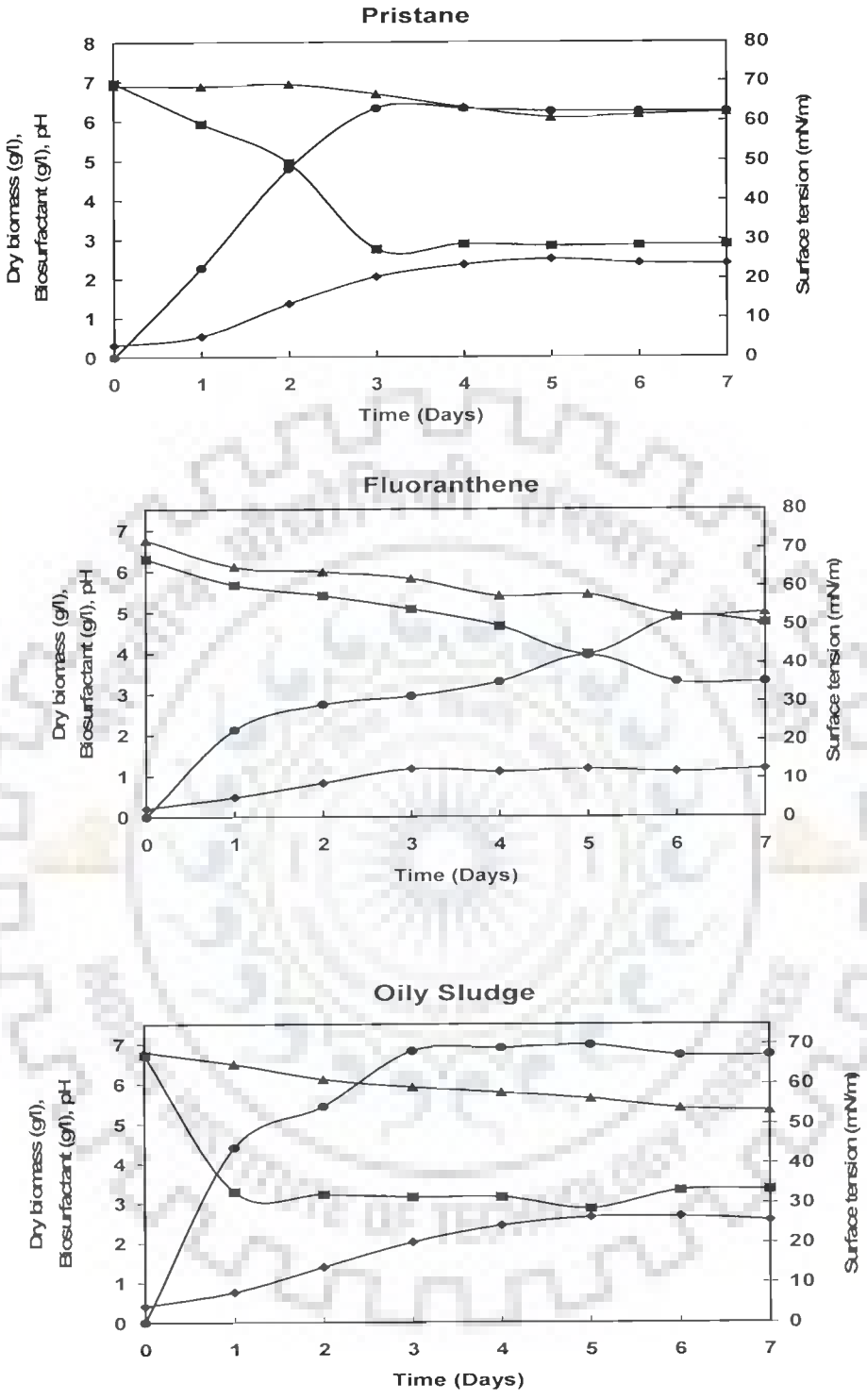


Figure 33: Growth of *Bacillus subtilis* on 1% pristane, 1% fluoranthene & 1% oily sludge: dry biomass (♦), biosurfactant production (●), surface tension (■), pH (▲)

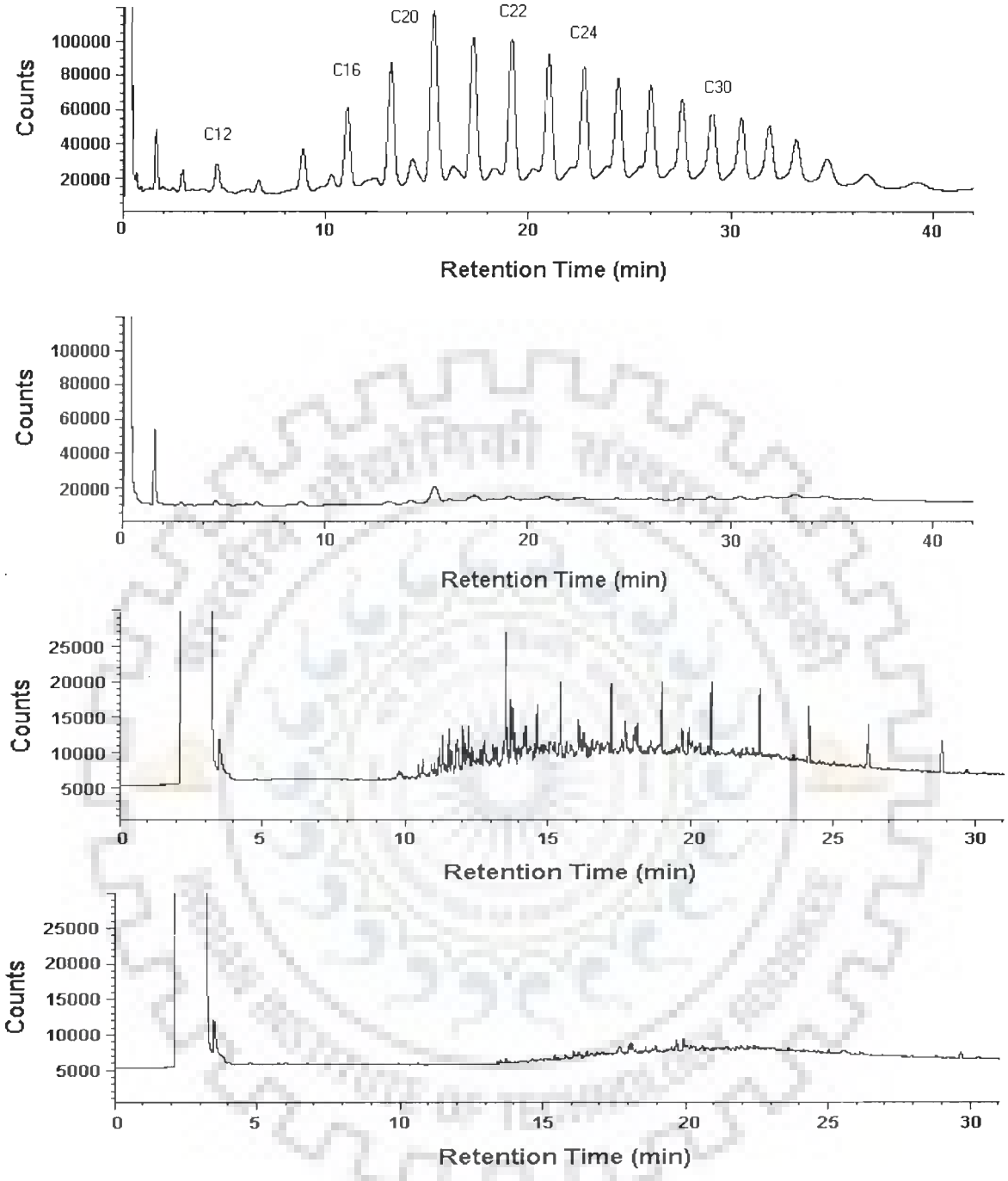


Figure 34: GC profile of hydrocarbon degradation pattern by *B. subtilis* DSVP23 after 5 days: (A) Uninoculated control for aliphatic fraction (B) Oily sludge (Aliphatic fraction) degradation pattern. (C) Uninoculated control for aromatic fraction (D) Oily sludge (Aromatic fraction) degradation pattern.

CHAPTER 5

DISCUSSION

An interest in biosurfactants, the amphiphatic molecules has been steadily increased due to their diversity, environmentally friendly nature, and their potential applications in the crude oil recovery, biomedical field, and food processing industries (Banat, 1995; Fietcher, 1992; Klekner and Kosaric, 1993; Muller-Hurtig *et al.*, 1993; Velikonja and Kosaric 1993). Biosurfactants, have distinct advantages over their chemical counterparts in biodegradability and effectiveness at extreme temperature, pH and in having lower toxicity (Banat *et al.*, 2000).

In present investigation intent was to isolate a novel biosurfactant producing microorganism from petroleum contaminated soil samples, phenolic water and waste oil contaminated soil samples. The samples from these sites were gathered because the capability of native bacterial population to mineralize crude oil hydrocarbons in petroleum oil contaminated sites and probability of finding biosurfactant producing microorganism is higher (Emtiazi and Shakarami, 2004 Okoh, 2003; Kasai, 2002; Okerentugba, 2003). The indigenous population of hydrocarbon degrading bacteria was found to be 2×10^4 CFU/g. Similar results confirming low indigenous population of hydrocarbon degrading bacteria in soil ($10^3\times10^4$ CFU/g) have been obtained during evaluation studies of inoculum addition in oily sludge contaminated soil by Mishra *et al.*, 2001. Enrichment methods are highly selective resulting in the isolation of new microbial species from various natural habitats (Dunbar *et al.*, 1997). A total of 26 morphologically distinct bacterial isolates were selected on the basis of ability to grow on different hydrocarbons. Bacteria growing on petroleum produce potent biosurfactants to come in direct contact with the hydrocarbon substrates therefore are an indirect measure of degradation activity (Makkar and Cameotra, 1998, Bodour *et al.*, 2003; Rahman *et al.*, 2006; Benincasa, 2007).

An efficient screening strategy is the key to success in isolating new and interesting microbes or their variants, because a large number of strains need to be characterized. In the present investigation four parameters haemolytic activity, drop collapse assay, surface tension reduction, and emulsification activity were employed to measure the efficiency for biosurfactant production. All 26 isolates selected in the study were employed to screening methods in order to select the most potential biosurfactant producing bacterium. Screening of biosurfactant producing isolates was performed by using blood agar lysis, emulsification activity, drop collapse test and surface tension reduction assay. A total of five strains namely, namely DSVP2, DSVP9, DSVP11, DSVP18 and DSVP23 were selected on basis of these tests. Several studies have documented these methods as rapid and reliable for screening and

selection of microbes producing biosurfactants (Lin, 1996; Carrillo *et al.,* 1996;, Bicca *et al.,* 1999; Bodour *et al.,* 2004; Banat *et al.,* 2006, Amorim *et al.,* 2006; Banat, 1995; Youssef *et al.,* 2004).

Cell surface hydrophobicity studies were further employed for indirect confirmation of biosurfactant producing isolates. A rapid identification of biosurfactant producing strains can be achieved by assaying this characteristic (Neu & Poralla 1990, Pruthi & Cameotra 1997). HIC, SAT, BATH, RP assay procedures suggests that hydrophobic nature of cell surface is a prerequisite for the identification of biosurfactant producing strain. On comparative analysis the hydrophobic properties of microorganisms, confirmed that the five selected strains were active biosurfactant producers. Reports by Rosenberg *et al.,* (1900) and Pruthi and Cameotra (1997), have recommended the use of cell surface hydrophobicity tests as a characteristic feature of biosurfactant producing microbes.

The majority of known biosurfactants are synthesized from water-immiscible hydrocarbon during biosynthesis (Mulligan, 2004). There were also some biosurfactants that have been reported to be produced using water-soluble compounds such as glucose, sucrose or ethanol as substrate (Desai and Banat, 1997). This opens some new possibilities in utilizing waste and cheap carbohydrate media for biosurfactant production (Kosaric *et al.,* 1987). Biosurfactants are not economically competitive with their synthetic counterparts so different strategies must be devised and explored to reduce production costs. Use of inexpensive substrates can drastically decrease the production cost of biosurfactants. Selection of waste substrates involves the problem of finding a waste with the right balance of carbohydrates and lipids to support optimal growth and production. Agro-industrial wastes, with high levels of carbohydrates or lipids, and urban wastes meet the requirements for use as substrates for biosurfactant production. These agricultural feed stocks are attractive in that they are available in surplus and can be produced in regions with temperate to tropical climates. So far, several renewable substrates from various sources, especially from industrial wastes have been extensively studied for microbial production at an experimental scale. Waste frying oils (Haba *et al.,* 2000), oil refinery wastes (Bednarski *et al.,* 2004), molasses (Gurye *et al.,* 1994; Makkar and Cameotra, 1997; and Patel and Desai, 1997; starch rich wastes (Fox and Bala, 2000) and cassava waste water (Nitchke and Pastore, 2006), oilcakes, cheese whey (Desai *et al.,* 2006), waste soybean oil (Lee *et al.,* 2007), Peat hydrolysate (Mulligan and Sheppard 1987), olive oil mill effluent (Guinea *et al.,* 1993), lactic whey (Kappeli *et al.,* 1988), soybean curd residue, potato process effluent etc. are some of the

examples. In our study five selected isolates were tested for utilization of cost effective raw materials (Cotton seed hull, Tea leaves, Wheat bran, Corn Starch, Rice straw, Wheat straw, Bagasse, Ground nut kernel, Potato peel, Apple peel, Cotton seed, Molasses, Bamboo wood saw dust and Gram Husk) as substrate for biosurfactant production. Amongst selected strains, DSVP23 was found to be the best biosurfactant producer which could grow and produce biosurfactant on all selected 14 different cost-effective raw materials namely cotton seed hull, tea leaves, wheat bran, corn starch, rice straw, wheat straw, bagasse, ground nut kernel, potato peel, apple peel, cotton seed, molasses, bamboo wood saw dust and gram husk as carbon sources. DSVP23 showed maximum reduction in surface tension value from 72 mN/m to 28.7 mN/m when cotton seed hull was used a carbon substrate in MSM.

From the preliminary studies, strain DSVP23 was chosen for the further investigation due to its ability to utilize cost effective raw material and reduce surface tension of the medium. The selected strain DSVP23 was identified microscopically, biochemical analysis and using 16S rRNA technique. The phylogenetic tree was prepared based on neighbour joining analysis of the 16S rRNA gene nucleotide sequences. The sequencing of the 16S rRNA allowed the identification at species level with an identity from 97% to 100 % comparing the sequences to those from the GenBank databases (Ouoba *et al.*, 2004). In our work, on the basis of its morphological, physiological and biochemical features, as well as its 16S rRNA analysis, strain DSVP23 was classified as *B. subtilis* (GenBank accession no. EU679368).

It was noticed that biosurfactant production by *B. subtilis* DSVP23 was growth associated because a good correlation is observed between maximum yield of biosurfactant and biomass at 48 h of incubation (at late log phase). A perusal of literature (Sandhya et al., 2005) show that enzyme production by microbes declines with an increase in incubation period, but present study has demonstrated that no significant difference in yield of biosurfactant is noticed between 48 and 96 h of bacterial growth, suggesting that biosurfactant produced by *B. subtilis* DSVP23 was stable over time. Investigation showed that the *B. subtilis* DSVP23 used in this study utilized cotton seed hull as the sole source of carbon and gave maximum biosurfactant yields (2.42 g/l). Maximum biosurfactant production was observed after 48 hours of growth and there was no significant difference in yield over period of time after that, thereby suggesting that isolated surface active compound was stable over time which is in good agreement to findings of Makkar and Cameotra, (1997). Potato substrates were evaluated as a carbon source for surfactant production by *B.*

subtilis ATCC 21332 (Fox and Bala, 2000), Surface tensions dropped from 72 mN/m to 28.3 mN/m and 27.5 mN/m when potato medium and mineral salt medium were used, respectively (Fox and Bala, 2000). Nitschke and Pastore, (2004) used a cassava flour-processing effluent as a substrate for surfactant production by *B. subtilis* LB5a and *B. subtilis* ATCC 21332. This isolate reduced the surface tension of the medium from 49.5 mN/m to 25.9 mN/m and produced a crude biosurfactant at a concentration of 2.2 g/l. For *B. subtilis* LB5, the surface tension of the medium was reduced to 26.6 mN/m, giving a crude biosurfactant concentration of 3.0 g/l. Makkar and Cameotra 1997; in their studies cultivated *B. subtilis* MTCC 2423 and *B. subtilis* MTCC 1427 using molasses as a substrate for biosurfactant production. Ohno *et al.,* (1992 1993, 1995) reported production of iturin and surfactin by a strain of *B. subtilis* NB 22 using wheat bran and okara (soybean curd residue). Ajlani *et al.,* (2007) in their studies have reported pharmamedia (yellow powder from the embryo of cotton seed) as a suitable medium for production of surfactin using *B. subtilis* MZ-7 strain. Clarified cashew apple juice was evaluated as carbon source for surfactin production by *B. subtilis* LAMI005 by Goncalves *et al.,* 2009. They showed that surfactin concentration of 123 mg/l was achieved after 48 h of fermentation, with surface tension value reduction to 29 dynes/cm suggesting clarified cashew apple juice as a renewable and low-cost carbon source.

Methods for isolation of microbial surfactants from culture liquid depend on their biochemical nature, location on the cell surface, solubility, etc. Several conventional extraction methods used for the recovery of biosurfactants have been widely reported in literatures including acid precipitation, solvent extraction, crystallization, ammonium sulfate precipitation and centrifugation (Desai and Banat, 1997). Generally, the solvents used for biosurfactant recovery such as acetone, methanol and chloroform are sometimes toxic in nature and harmful to the environment (Makkar and Cameotra, 1999). Cheap and less toxic solvents have been successfully used in recent years to recover biosurfactants (Kuyukina, *et al.,* 2001). These types of least expensive, less toxic and readily available solvents can be used to reduce the recovery expenses substantially and minimize environmental hazards. In present investigation solvent dichloromethane showed highest recovery of biosurfactant agree which is in agreement with reports of McInerney *et al.,* (1990).

Preliminary chemical characterization of biosurfactant suggested it to be a lipopeptide in nature. Thin-layer chromatography revealed white spot (R_f values 0.56) when the plate was sprayed with water. The standard surfactin presented a retention index of 0.55. No spot was revealed when the plate was sprayed with ninhydrin. However, when the biosurfactant was

submitted to hydrolysis, a spot could be observed when ninhydrin was used, indicating the presence of peptides. Biosurfactant produced by *B. subtilis* DSVP23 had protein and lipid contents 18.0 and 15.2% respectively, similar results were reported by Nitschke and Pastore (2006), for *B. subtilis*. Critical micelle concentration of biosurfactant obtained from DSVP23 was 30 mg/l suggesting it to be an effective surfactant. In comparison to this, CMC value of surfactin (Cooper *et al.*, 1981) and lichenysin (Javaheri *et al.*, 1985) were reported to be 25 mg/l and 20 mg/l, respectively. Isolated biosurfactant was stable at extreme thermal, pH and salt conditions. The surface tension and critical micelle dilution (CMD) values remained stable after exposure to high temperatures (100^0C) even after 4 h. The surface activity was retained over a pH range of 4-12 with minimal deviation in surface tension and CMD^{-1}, whereas CMD^{-2} shown a slight and gradual increase on surface tension with increasing pH values. No appreciable changes were observed on surface tension and CMD values with the addition of NaCl up to 10% in cell free broth thereby demonstrating its potential commercial applications. Results obtained were similar to previous findings by Ilori *et al.*, 2005. In their study they obtained slight stimulation of the activity of the biosurfactant at NaCl concentration of 5% and pH of 8.0 indicating its usefulness for bioremediation of spills in marine environment because of its stability in alkaline condition and in the presence of salt.

FTIR spectrum when compared with previously reported for biosurfactants from *Bacillus* species, bands characteristic of C-N bond, Amide II bond, aliphatic chain and –OH/-NH groups were observed, indicating that this compound is a lipopeptide (Das and Mukherjee, 2007; Joshi *et al.*, 2008; Makkar and Cameotra 1997). Analysis of the spectrum showed typical absorption bands at 3343 cm^{-1} corresponding to N-H stretching of proteins and peptide bonds, concrete evidence that the substance contained a peptide in its structure. Besides this, several reports for lipopeptide biosurfactant production by numerous *Bacillus* spp. are reported in literature (Arima *et al.*, 1968; Cooper *et al.*, 1981; Yakimov 1995; Nitschke, and Pastore, 2006). The nature of the biosurfactant was also confirmed by HPLC analysis. The six peaks obtained in the chromatogram also appeared in standard surfactin, although the relative abundance was not the same for the two samples. The HPLC indicated that the purified biosurfactant was of complex nature similar to standard surfactin. Similar structurally related metabolites of *Bacillus* strains such as surfactin, acylpeptides, and esperin have also been reported (Kakinuma *et al.*, 1969; Hosono *et al.*, 1983).

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry has been used as a novel, efficient method for identification and structural

characterization of microbial secondary metabolites. The detection limit is usually in the upper femtomolar to picomolar range. MALDI-TOF mass spectra of biosurfactant from *B. subtilis* DSVP23 showed prominent groups of mass peaks observed in the mass range between *m/z* 1000 and 1500. The mass spectra of these lipopeptides had peaks, which could be attributed to the protonated forms, as well as to the sodium and potassium adducts. The groups of peaks could be attributed to the isoform ensembles of surfactins, iturins, and fengycins, which represent the well-known biosurfactant families produced by *B. subtilis* (Peypoux, 1978; Kowall, *et al.*, 1998; Vater *et al.*, 2002). The lipopeptide products of *B. subtilis* DSVP23 could be identified as surfactins. These biosurfactants appeared as families of closely related to iturin isoforms which differed in the lengths of their fatty acid side chains, as well as in the amino acid substitutions in their peptide rings. The results were in agreement with the data obtained by Hourdou *et al.*, (1989); Peypoux *et al.*, (1978); Vater *et al.*, (2002, 2003).

Certain nuclei have a magnetic spin, which can be manipulated by an external magnetic field. This is made use of in nuclear magnetic resonance (NMR) spectroscopy. One-dimensional ^{1}H NMR gives a picture of the hydrogens of the sample and proton-proton coupling constants. By using one dimensional NMR techniques the carbon-hydrogen bonding pattern can be elucidated. Our results obtained with ^{1}H NMR clearly indicate that the isolated biosurfactant studied is a lipopeptide. The spectrum represents three main regions corresponding to resonance of amide protons, α-carbon protons and side-chain protons. Analysis of the ^{1}H NMR spectra of isolated biosurfactant has led us to suggest their structural relatedness to lipopeptides from *B. subtilis* (Arima *et al.*, 1968; Hosono and Suzuki, 1983; Makkar and Cameotra, 1999).

One of the important strategies to improve biosurfactant production is to optimize the constituents of growth media. Several studies have demonstrated that the medium composition and growth conditions can influence the type and yield of biosurfactant (Burkert *et al.*, 2008; Ellaiah *et al.*, 2005; Leite *et al.*, 2004; Mercade *et al.*, 2004; Makkar and Cameotra, 2002). With a view to develop an economically feasible technology to improve yield of biosurfactant, a number of factors which include optimization of environmental and fermentation parameters such as pH, temperature, size and age of inoculum, incubation period, effect of addition of some nutrients, trace elements, metal ions and other growth supplements were under taken.

Carbon source influences biosurfactant synthesis by either induction or repression (Makkar and Cameotra, 1997). Induction regulates synthesis of many lipopeptide biosurfactants (Besson *et al.,* 1992; Kludge *et al.,* 1989; Ullrich *et al.,* 1991). The *B. subtilis* strain used in this study was able to utilize cotton seed hull for biosurfactant production. It was observed that when 2% of cotton seed hull was used as sole carbon source an optimal production of biosurfactant took place. It was further observed that upon use of sucrose as an additional carbon source markedly enhanced the biosurfactant production (3.82 g/l) to that of control. Whereas when, sucrose 2% was used as a sole carbon source into MSM without cotton seed hull resulted in lower biosurfactant yield being observed to that of control. Similar, results were obtained by Makkar and Cameotra, (2002), on biosurfactant production by a thermophilic *B. subtilis* MTCC 2423.

After carbon, nitrogen is usually the most plentiful substance in the medium for biosurfactant production. There are several evidences where the nitrogen plays an important role as the medium constituent during the production of surface active compound by microbes (Makkar and Cameotra, 2002). *A. paraffineus* ATCC 19558 preferred ammonium to nitrate for biosurfactant production when inorganic salts were used as nitrogen source (Kosaric *et al.,* 2004). Studies by Robert *et al.,* (1989), of biosurfactant production by *Pseudomonas* 44Ti on olive oil showed that sodium nitrate was the best nitrogen source. In present study among inorganic nitrogen sources tested ammonium nitrate and sodium nitrate potentially effect biosurfactant production by *B. subtilis* DSVP23. Nitrogen requirement was evident from the observation that when there was no nitrogen in the medium i.e control, limited amount of biosurfactant production was observed. At concentration of (4 g/l) sodium nitrate and (4.5 g/l) ammonium nitrate maximum production of biosurfactant took place exhibiting preference of nitrate ions by *B. subtilis* DSVP23. Among organic nitrogen sources tryptone showed enhanced effect on production of biosurfactant (3.0g/l) in comparison to other organic nitrogen sources. Moreover, it was observed that inorganic nitrogen sources ammonium nitrate and sodium nitrate in comparison to organic nitrogen sources tryptone had stimulatory effect on biosurfactant production. The present results are in agreement with those of Abushady *et al.,* 2005; where both ammonium nitrate and sodium nitrate gave higher yield of surfactin concentration. Other reports by Guerra- Santos *et al.,* 1984; Robert *et al.,* 1989; MacElwee *et al.,* 1990; Prieto *et al.,* 2008, on *P. aeruginosa* also supported the finding that nitrate salts supported maximum surfactant production.

Limiting multivalent cation concentrations also causes overproduction of biosurfactants (Guerra-Santos *et al.,* 1984, Itoh & Suzuki, 1974). Guerra-Santos *et al.,* (1986), demonstrated that limiting the concentrations of salts of magnesium, calcium, potassium, sodium and trace elements resulted in a better yield of rhamnolipid in *P. aeruginosa* DSM 2659. Surfactin production by *B. subtilis* was stimulated by addition of either iron or manganese salts to the medium (Cooper *et al.,* 1981). In our study, metal supplements increased biosurfactant production of *B. subtilis* DSVP23 considerably. Higher concentrations, however, inhibited biosurfactant production. The effect of metal cations used together was greater than when they were used individually, as indicated by net yield of biosurfactant when all the metal cations were present in the medium. 5 mM $FeSO_4$ and 30 mg/l $MnSO_4$ in MSM supplemented with 2% cotton seed hull enhanced the production yield of biosurfactant 4.5g/l to that of control 2.0g/l. Sheppard & Cooper, (1991) suggested a possible reason for enhanced production by Mn^{2+} is that they affected nitrogen utilization and K^+ uptake as well as other biochemical functions. Studies by Wei & Chu, 1998, 2002, 2003; Makkar and Cameotra, 2002; and Abushady *et al.,* 2005, also demonstrated overproduction of surfactin by addition of Fe^{2+} and Mn^{2+} ions to the mineral salts medium.

Several amino acids have been found to increase the production of lipopeptides by *B. subtilis* (Chevenet *et al.,* 1986). Effect of addition of amino acids viz. aspartic acid, glutamic acid; lysine and valine in the minimal salt medium resulted in higher yields (4.0-4.5 g/l) of biosurfactant to that of control (2.66 g/l). These amino acids are taken up directly as precursors for the biosurfactant synthesis and thus may result in increased biosurfactant yield (Makkar and Cameotra, 2002). Study on coproduction of surfactin and iturin by Sandrin *et al.,* (1990), showed amino acids were good substrates for production of surfactin.

The inoculum through various stages has a clear-cut effect on the subsequent performance and economics of the process; it is well known that the age and density of inoculum used directly influences the duration of lag phase, specific growth rate, biomass yield and quality of the final product (Abushady *et al.,* 2005). It was observed that MSM when inoculated with 2% *B. subtilis* DSVP23 culture and incubated for different incubation times from 24-120h; there was increase in biosurfactant production up to 48 h which was stable over rest of time period.

Environmental factors and growth conditions as pH, temperature, agitation and oxygen availability also affect biosurfactant production. Among the physical parameters tested pH of the growth medium plays an important role by inducing morphological changes

of the organism thereby biosurfactant production. *B. subtilis* DSVP23 produced biosurfactant in a pH range of 4-10 although a maximum yield of biosurfactant was obtained at pH 7.0. Abushady *et al.,* 2005, showed that surfactin production by *B. subtilis* is unaffected in the pH range of 6.5 to 8. Several other studies have demonstrated role of pH in biosurfactant production Gobbert *et al.,* 1984; Guerra-Santos *et al.,* 1984; Makkar and Cameotra, 2002).

Temperature is directly related to the metabolic activities of the microorganism and it affects proper growth and product formation by the organism (Lonsane *et al.,* 1985). Every organism has its optimum temperature at which it grows best resulting in higher yield of the desired product and hence temperature should be maintained at the optimum of the microorganism (Kundu and Das, 1970, Gautam *et al.,* 1991). Therefore, the influence of incubation at temperatures ranging from 20°C to 45°C was evaluated. It was obtained that *B. subtilis* DSVP23 produced maximum biosurfactant at 37^0C. Temperature was found to be critical parameter for growth and biosurfactant studies (Morikawa *et al.,* 1993; Syldatrk *et al.,* 1985).

The variation in the agitation speed of the cultures (2-250 rpm) influenced the biosurfactant production. In our studies when the medium was agitated at 180 rpm, a lowest value of surface tension was achieved. Agitation speed is a determining factor in the oxygen mass transfer to cultures. Similar results were obtained for biosurfactant production in yeast which increased with increasing the agitation speed (Shepperd & Cooper, 1990). Agitation speed did not showed any shear effect on biosurfactant producing microorganism *B. subtilis* DSVP23 in contrast to results obtained by Abushady *et al.,* 2005.

Aerobic bacteria, like *B. subtilis*, need sufficient oxygen supply for cell growth and metabolite formation. As the oxygen supply acted as a limiting factor for cell growth and production of biosurfactant the oxygen transfer rate should be properly adjusted (Davis *et al.,* 1999; Kim *et al.,* 1997; Lee and Kim, 2004). In our studies it was found that under higher oxygen concentration (1.5 vvm), amount of biomass was maximum but a low level of biosurfactant yield (2-3 g/l) was obtained from MSM containing cheap raw substrate. Previous finding in the literature also pointed out that excessive oxygen supply may not be favourable to surfactin production from *B. subtilis*, because the high rate of aeration and agitation under O_2-sufficient conditions promoted cell growth and foam production in a short time (Davis *et al.,* 1999 and Kim *et al.,* 1997). We observed that under oxygen limiting conditions by *B. subtilis* DSVP23 maximum yield of biosurfactant was attained.

The industrial production process is often dependent on the use of microbial strains with high biosurfactant producing capabilities. With use of cheap raw material, optimized medium and culture conditions, and efficient recovery processes, a production process cannot be made commercially viable and profitable until the yield of the final product by the producer is naturally high (Mukherjee *et al.,* 2006). In our attempt to generate higher biosurfactant yielding mutants of *B. subtilis* DSVP23, upon 3% EMS treatment, we obtained 16 mutants. Screening of mutants was performed using blood agar method besides this surface tension reduction was also performed in order to confirm the surface activity of the mutant. Out of which, a selected mutant strain (*DVM4*) showed distinctly high zone of hemolysis on blood agar plate and reduction in surface activity 26.8 mN/m (*DVM4 mutant* type) to that of DSVP23 wild type (28.6 mN/m). Hemolysis assay using blood agar plate was used for isolation of *B. subtilis* mutant overproducing biosurfactant in accordance to reports by Mulligan *et al.*, (1984, 1989).

Fermentation strategy resulted in a marked enhancement of the biosurfactant production from *B. subtilis DVM4* mutant in comparison to wild type DSVP23. Adjusting the content of each component of MSM to optimal concentrations resulted in a 2 fold increase in biosurfactant production. Comparative studies of biosurfactant production by mutant and wild type were done using different cheap raw materials. Mutant *DVM4* effectively utilized selected cheap raw materials cotton seed hull, wheat bran, rice straw, rice straw, Molasses, apple peel and potato peel to that of wild type. Our studies revealed an increase in biosurfactant yield for *DVM4* mutant type (6.0g/l) compared with DSVP23 wild type (3.0g/l) on MSM with cotton seed hull as substrate. Mutant capable of producing several fold higher biosurfactant concentrations than those of the parent microbial strains have been previously isolated (Kaweshima *et al.,* 1983; Mulligan *et al.,* 1989; Shabtai *et al.,* 1986). Excessively foaming was observed during fermentation run indicated excellent emulsification power of the *B. subtilis DVM4* biosurfactant. Cooper *et al.,* (1981) also showed foaming to be an indicator for enhanced production of biosurfactant during the fermentation studies on *B. subtilis*.

Of the several envisioned industrial applications of the biosurfactants the greatest potential use is in the microbial enhanced oil recovery (Shennai and Levi, 1987). Biosurfactant aid in enhanced oil recovery (EOR) by lowering surface tension/Interfacial tension at oil/water interface. The potential use of biosurfactant in EOR was evaluated using the sand pack column technique. The isolated biosurfactant showed its effectiveness in

producing excellent emulsification against different hydrocarbons: Kerosene oil (78%), Hexadecane (72%), Toluene (72%), Motor oil (70%), Dodecane (68%), Tetradecane (64%), and Hexane (64%) signifying its potential application in oil spill management. Interestingly, emulsion formed was stabilized by the biosurfactant and does not revert to separate oil and water phase even after 90 days. The precipitated biosurfactant (0.5%) was effective in recovery of oil from a sand pack column with known amount of different hydrocarbons. Efficient recovery of 76% kerosene oil, 72% motor oil, 68% n-paraffin 70% crude oil and 62% mobile oil was obtained by using 0.5 % of aqueous solution of biosurfactant in sand pack column. The recovery of oil from saturated sand pack column by isolated biosurfactant in the present study was much higher than that of the biosurfactant from *B. subtilis* strains as reported by Makkar and Cameotra, (1998); Pruthi and Cameotra, (1997), but lower than the biosurfactant from a thermophilic *Bacillus sp.* Banat *et al.*, (1993).

The potential of *Bacillus sp.* to synthesize a wide variety of metabolites with antibacterial or antifungal activity has been intensively exploited in medicine and industry, and is a determinant of their ability to control diseases when applied as a biological control agent (McKeen *et al.*, 1986; Silo-suh *et al.*, 1994; Leifert *et al.*, 1995). *Bacillus* spp. appears to be a relatively abundant source of antimicrobials, since many species of this genus synthesize antimicrobial peptides (Gebhard, 2002; Liu and Hansen, 1990). Surfactin is one such compound produced by *B. subtilis*, it is an cyclic lipopeptide containing seven residues of D- and L-amino acids and one residue of a β-hydroxy fatty acid with an amino acid sequence(Kluge *et al.*, 1988).

In our study antimicrobial properties of biosurfactant obtained using cost effective raw material against various bacterial and fungal strains was evaluated. *B. subtilis* DSVP23 strain was found to produce an antimicrobial substance that inhibited growth of *S. aureus* MTCC 96 and *P. aerugenosa* MTCC 741, among other relevant pathogenic bacteria. In addition, when concentrated by lyophilization, the biosurfactant substance was able to inhibit growth of *E. coli* MTCC443 and *Bacillus cereus* MTCC430. Analysis of antifungal effect of lipopeptide biosurfactant from *B. subtilis* DSVP23 showed its inhibitory activity against various tested pathogenic fungal strains (*C. albicans* MTCC 183, *R. solani* MTCC 4633, *F. oxysporum* MTCC 284, *T. viride* MTCC 2047). The MIC values of the biosurfactant obtained in this study suggested both bactericidal and antifungal properties of biosurfactant. Antimicrobial activity of biosurfactant was highest against strain *S. aureus* and *C. albicans* at 12μg/ml and 10.5 μg/ml concentrations. The different sensitivities are associated to the way

microorganisms respond to different components in the metabolite pool (Rivardo *et al.*, 2009). Several studies have reported antimicrobial action of biosurfactant produced by a *Bacillus* strains to suppress phytopathogenic fungi (Hiradate *et al.*, 2002; Yu *et al.*, 2002 and Cho *et al.*, 2003; Thimon *et al.*, 1999).

In our study an increased magnification and resolution power associated with the TEM technique permitted a more detailed examination of effect of biosurfactants on microbial cells. TEM studies revealed that lipopeptide was capable of affecting the cell surface of both phytopathogenic fungus and bacteria which could be due to their adhering property towards cell surfaces causing deterioration in the integrity of cell membrane and also breakdown in the nutrition cycle (Yalçin and Ergene, 2009). All these cumulative effects can be explained the antimicrobial effects of biosurfactant.

Biofilm formation is a major microbial adaptive strategy to environmental conditions (Emmert and Handelsman, 1999; Davey and Toole, 2000). The need of new antimicrobial agents to overcome bacterial antibiotic tolerance or resistance in biofilms leads to investigations towards novel strategies to fight against bacterial chronic infection. Bacteria demonstrate diverse strategies to protect themselves from environmental assaults and facilitate their own survival. Adhesion is needed for bacterial energy saving and several microorganisms use it to protect their ecological niche. The decreased bacterial adhesion and the reduction of biofilm population can be clinically useful in the removal of bacterial colonization from medical devices surfaces and in urinary tract infections (Rodrigues *et al.*, 2006).

In the present study, the applicability of lipopeptide biosurfactant for removal of biofilm as well as for their antimicrobial activity against cells in biofilms was assessed. Effect of biosurfactant on biofilm formation by a fungal pathogen *Candida albicans* MTCC 183 and a bacterial pathogen *Staphylococcus epidermis* MTCC 435 were studied. MTP assay revealed that biosurfactant at concentration 10.5 µg/ml and 12 µg/ml was able to inhibit biofilm formation by *C. albicans* and *S. epidermis* respectively. Biosurfactant produced by *B. subtilis* was able to disperse biofilm thereby, significantly reducing the viability of these organisms. It was observed that the biosurfactant at 10.5 µg/ml resulted in 78% reduction in *C. albicans* biofilm and 72% in *S. aureus* biofilm at concentration 12 µg/ml. Huang *et al.*, (2008) observed the efficacy of a mixture of surfactin and fengycin (1:1) against planktonic *E. coli* (Irie *et al.*, 2005; Rodrigues *et al.*, 2006; Harshey *et al.*, 2003), demonstrated that lipopeptides were able to inhibit biofilm formation when used to coat surfaces.

SEM microscopic studies revealed that biosurfactant inhibited biofilm formation and cell growth by acting directly on biofilm matrix thereby disrupting and solubilizing its membrane. Images revealed that biosurfactant from *B. subtilis* DSVP23 not only caused considerable decrease in cell aggregation but also demonstrated a degree of changes in the cell morphology. The possible mechanisms of action of biosurfactants could be the binding to cell surface or to components of it, thereby influencing the outer membrane hydrophobicity, as was reported for other biosurfactants (Kuiper et al. 2004; Neu, 1996).

Fluorescent microscopy revealed biosurfactant was more effective to decrease the viability of biofilms by acting directly on the biofilm matrix to disrupt and solubilize its components, perhaps even incorporating the matrix into micelles. Many bacteria like *Bacillus* used in the present studies are well known to secrete biosurfactant which can facilitate surface motility (Deziel *et al.*, 2003; Kohler *et al.*, 2000), affect biofilm architecture (Davey *et al.*, 2003) and alter cell surface polarity (Al-Tahhan *et al.*, 2000; Zhang and Miller, 1994).

Atomic Force Microscopy (AFM) has been used to image film morphologies and probe surface properties like ligand and receptor interactions and viscoelasticity (Ahimou *et al.*, 2007). AFM-based methodology was shown to dissect morphological alterations and subsequent reduction in biofilm formation by *C. albicans* MTCC 183 and *S. epidermis* MTCC 435 using biosurfactant treatment. It can be observed from the images that before treatment of biosurfactant the *S. aureus* and *C. albicans* showed normal smooth surfaces. Meanwhile, clustering of cells was observed. In contrast, cells treated with biosurfactant exhibited dramatic changes in the structure of the cell walls. The outer membrane of the bacteria was extremely rough and some membrane residues were found around the cells. Cells were scattered and few clustering were seen (Cao *et al.*, 2009). Emerson and Camesano, (2004), investigated pathogenic microbial adhesion to biomaterials by measuring the local interaction forces between an immobilized cell and both biomaterial and biofilm surfaces.

Strain *B. subtilis* DSVP23 isolated from the oily sludge contaminated soil was capable of utilizing oily sludge as carbon source. This was shown by an increase of dry cell biomass with a simultaneous pH drop. In general, for the bacterial isolate the dry cell biomass of the culture increased progressively, and then remained constant until the end of the period studied. The pH drop indicated the acidification of the media could be due to release of organic acids as the consequence of degradation of hydrocarbons or to the production of extra cellular polymers (Walker *et al.*, 1975; Kokub *et al.*, 1990). Similar utilization of different

hydrocarbon substrates pristane and fluoranthene was also observed in our investigation of bacterial isolate *B. subtilis* DSVP23. Results obtained from gravimetric analysis and chromatographic techniques also confirmed degradation of substrates. The growth of bacterial isolate *B. subtilis* DSVP23 was accompanied by production of biosurfactant and hence surface tension reduction was also observed. Maximum biosurfactant production was detected during late log growth phase and it was coincident with least surface tension. This correlation was observed earlier by Pruthi and Cameotra, (1995) during the growth of three *Pseudomonas* species on n-dodecane. The findings that biosurfactant produced by *B. subtilis* caused increased hydrocarbon solubilization into the aqueous phase support its uptake in a pseudosolubilized state. Biosurfactants can dissolve hydrocarbons by forming the micelle into aqueous solution (Rosenberg and Rosenberg, 1981). This direct contact between the cell and target hydrocarbon can significantly increase the rate of diffusion into the cell, thereby enhancing growth and increasing the apparent rate of dissolution of the hydrocarbon.

GC analysis has been used to determine the ability of bacterial isolate *B. subtilis* DSVP23 to degrade oily sludge after 5 days of incubation. The chromatographic data indicates that isolate was able to consume almost all saturates and aromatics in the oily sludge when compared with sterile controls. One possible explanation for this phenomenon could be that hydrocarbons, which cannot be used for growth by many soil organisms, can be oxidized if present as co-substrates in a system in which another substrate is available for growth (Sepic *et al.*, 1995). It seems likely that bacteria become modified after attacking lower-molecular mass compounds in a way so that they are able to oxidize even higher, less degradable compounds.

The ability of bacterial isolate *B. subtilis* DSVP23 to degrade hydrocarbons both in mixed (oily sludge) and pure (known hydrocarbons) forms suggests a probable use of such bacteria for the treatment of other oil-wastes.

CHAPTER 6

CONCLUSIONS

The foremost task undertaken in the present investigation deals with the screening of bacterial strains capable of producing maximum biosurfactant. Among 340 isolates from different samples 26 were found to be effective in hydrocarbon utilization of which five strains namely DSVP2, DSVP9, DSVP11, DSVP18 and DSVP23 were screened as biosurfactant production. Hemolytic ability and reduction in surface tension of the medium provided a rapid method for assay of biosurfactant formation by the isolates. Biosurfactant producing ability was further confirmed by assaying cell surface hydrophobicity. Evidence exhibiting direct correlation between biosurfactant production by the isolates and their cell surface hydrophobic properties were obtained. Utilization of cost effective resources such as cotton seed hull, apple peel, potato peel, wheat bran, rice straw and molasses for biosurfactant production by these isolates was further investigated. Of which a bacterial isolate DSVP23 was found to be a potential biosurfactant producer when grown using these substrates reduced the surface tension of water from 72 mN/m to 28.62 mN/m compared to other isolates tested.

Morphological, biochemical and molecular biology tests (16S rRNA) of above strain revealed that the strain DSVP23 belongs to *Bacillus subtilis* (Gene bank accession no. EU679368).

Biochemical characterization (TLC, HPLC and FTIR) of biosurfactant from *B. subtilis* DSVP23 indicated it to be lipopeptide in nature with 15.2% protein content and 18.0 % lipid contents. Analysis by MALDI-TOF-MS and NMR revealed that the major constituent of lipopeptide being leucine and isoleucine. The lipopeptide molecules are detected, in their protonated form or as Na^+ or K^+ adducts, by MALDI-TOF mass spectrometry in the m/z range of 1,044Da.

Nutritional requirements for optimum production of biosurfactant by *Bacillus subtilis* DSVP23 showed 2% cotton seed hull as a suitable carbon source for maximum growth and biosurfactant production. Interestingly enhanced biosurfactant production was observed when 2% sucrose was used as an additional carbon source. After 48 h of growth with shaking at 180 rpm and inoculum size, 2 % (v/v), a high yield of biosurfactant (4.0g/l) was obtained upon addition of 40 mg/l $MnSO_4$ and 5 mM $FeSO_4$ in comparison to control. The optimum incubation temperature and pH for biosurfactant production were 37^0C and 7.0, respectively.

The random mutagenesis approach with 3% EMS was successfully utilized in the study to isolate a *Bacillus subtilis* DSVP23 mutant (*DVM4*). With reduction in surface tension values were from 28.6 mN/m (DSVP23 wild type strain) to 26.8 mN/m (*DVM4*

mutant type) using 2% cotton seed hull as cost effective substrate for growth. Comparative analysis revealed an increase in biosurfactant production for DVM4 mutant type (6.0g/l) to that of DSVP23 wild type (4.0g/l) during fermentation studies.

Biosurfactant formed stable emulsions with a wide variety of hydrocarbon viz. Kerosene oil (78%), Hexadecane (72%), Toluene (72%), Motor oil (70%), Dodecane (68%), Tetradecane (64%), and Hexane (64%). Interestingly by, the emulsion formed was stabilized by the surfactant and does not revert to separate oil and water phase even after 24h. The surface activity remained unaltered within a wide pH (2-12), temperature (4-100°C) ranges and under NaCl concentrations up to 10%.

The precipitated *Bacillus* biosurfactant was effective in recovery of 76% kerosene oil, 72% motor oil, 68% n-paraffin 70% crude oil and 62% mobile oil was obtained by using 0.5% of aqueous solution of biosurfactant in sand pack column.

The biosurfactants showed profound antibacterial activity toward *S. aureus* with 12 µg/ml MIC and 18 mm zone of inhibition (ZOI) followed by *E.coli*, *P. aeruginosa* and *B. cereus*. The biosurfactant displayed antifungal activity against tested fungal strains with the zone of inhibition ranging between 10 and 16 mm. Biosurfactant showed maximum activity against *C. albicans* at 12 µg/ml MIC followed by *R. solani, F. oxysporum* and *T. viride.* These results indicate broad spectrum antimicrobial properties of biosurfactant.

Anti-adhesive activity of biosurfactant at 10.5 µg/ml and 12 µg/ml concentrations resulted in 78% reduction in *Candida albicans* biofilm and 72% in *Staphylococcus aureus* biofilm respectively.

Gas Chromatographic profiles analysis of residual hydrocarbon showed that bacterial isolate DSVP23 was able to degrade (1%) oily sludge under optimal culture conditions.

Thus it could be concluded that this ecofriendly biosurfactant obtained from cost effective raw materials is of considerable interest for product formulations in detergent, cosmetic, and pharmaceutical applications.

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APPENDIX

APPENDIX-I

Yeast Extract Peptone Dextrose medium:

Composition:-

Peptic digest of animal tissue	20.0 g/l
Yeast Extract	10.0 g/l
Dextrose	20.0 g/l
pH	7.0

Distilled water added to make up to 1 litre volume. Sterilize by autoclaving at 15 lbs pressure (121°C) for 30 min.

APPENDIX-II

Yeast Extract Phosphate medium:

Yeast extract	1.0 g/l
Disodium phosphate	0.2 g/l
Monopotassium phosphate	0.3 g/l
Phenol red	0.001 g/l
Agar	20.0 g/l
pH	7.0

Distilled water added to make up to 1 litre volume. Sterilize by autoclaving at 15 lbs pressure (121°C) for 30 min.

APPENDIX-III

Sabouraud Dextrose agar medium:

Composition:-

Peptone	10.0 g/l
Dextrose	40.0 g/l
Agar	15.0 g/l
pH	7.0

Distilled water added to make up to 1 litre volume. Sterilize by autoclaving at 15 lbs pressure (121°C) for 30 min.

APPENDIX-IV

Nutrient agar medium:

Composition:-

Peptone	15.0 g/l
Yeast extract	4.0 g/l
Dipotassium hydrogen phosphate	1.0 g/l
Chromogenic mixture	7.22 g/l
Chloramphenicol	0.5 g/l
Agar	15.0 g/l
pH	7.0

Distilled water added to make up to 1 litre volume. Heat to boiling to dissolve the medium completely. Do not autoclave.

APPENDIX-V

Potato dextrose agar medium

Composition:-

Potato starch	4.0 g/l
Dextrose	20g/l
Agar	15g/l
pH	5.6

Distilled water added to make up to 1 litre volume. Heat to boiling to dissolve the medium completely. Do not autoclave.

APPENDIX-VI

Phenol sulphuric acid assay:

Reagents:-

A) Phenol (5% w/v)

B) Conc. H_2SO_4.

Procedure:-

Mix 200 µl of reagent A to 200 µl of sample solution

⇓

Add 1ml of reagent B rapidly and leave solutions undisturbed

for 10 min before shaking vigorously

⇓

After 30 min determine absorbance at 485 nm

APPENDIX-VII

Lowry's assay:

Reagents:-

A) 2% Na_2CO_3in 0.1N NaOH.

B) 1% Sodium Potassium Tartarate in d/w.

C) 0.5% $CuSO_4.5H_2O$ in d/w.

D) Mix reagents A, B and C in 48:1:1 (v/v) ratio.

E) Folin-Ciocalteau (1:1 v/v) in d/w.

Procedure:-

Add 2 ml of reagent D to 1 ml sample solution

↓

Incubate for 10 min at RT

↓

Add 0.2 ml Folin reagent and incubate for 30 min

↓

Determine absorbance at 600 nm