GENETIC REGULATION OF BIOSURFACTANT PRODUCTION BY BACTERIAL ISOLATES

Thesis submitted to

THAPAR UNIVERSITY
Deemed University, Patiala, Punjab, India

For the degree of

DOCTOR OF PHILOSOPHY

KAMALJEET KAUR SEKHON

Department of Biotechnology and Environmental Sciences

April 2009

ਸਤਿਨਾਮੁ ਵਾਹਿਗੁਰੂ

ਸੂਹੀ ਮਹਲਾ ੪ ॥
ਕੀਤਾ ਕਰਣਾ ਸਰਬ ਰਜਾਈ ਕਿਛੁ ਕੀਚੈ ਜੇ ਕਰਿ ਸਕੀਐ ॥ ਆਪਣਾ ਕੀਤਾ ਕਿਛੁ ਨ ਹੋਵੈ ਜਿਉ ਹਰਿ ਭਾਵੈ ਤਿਉ ਰਖੀਐ ॥੧॥ ਮੇਰੇ ਹਰਿ ਜੀਉ ਸਭੁ ਕੋ ਤੇਰੈ ਵਸਿ ॥ ਅਸਾ ਜੋਰੁ ਨਾਹੀ ਜੇ ਕਿਛੁ ਕਰਿ ਹਮ ਸਾਕਹ ਜਿਉ ਭਾਵੈ ਤਿਵੈ ਬਖਸਿ ॥੧॥ ਰਹਾਉ ॥ ਸਭੁ ਜੀਉ ਪਿੰਡੁ ਦੀਆ ਤੁਧੁ ਆਪੇ ਤੁਧੁ ਆਪੇ ਕਾਰੈ ਲਾਇਆ ॥ ਜੇਹਾ ਤੂੰ ਹੁਕਮੁ ਕਰਹਿ ਤੇਹੇ ਕੋ ਕਰਮ ਕਮਾਵੈ ਜੇਹਾ ਤੁਧੁ ਧੁਰਿ ਲਿਖਿ ਪਾਇਆ ॥੨॥ ਪੰਚ ਤਤੁ ਕਰਿ ਤੁਧੁ ਸ੍ਰਿਸਟਿ ਸਭ ਸਾਜੀ ਕੋਈ ਛੇਵਾ ਕਰਿਉ ਜੇ ਕਿਛੁ ਕੀਤਾ ਹੋਵੈ ॥ ਇਕਨਾ ਸਤਿਗੁਰੁ ਮੇਲਿ ਤੂੰ ਬੁਝਾਵਹਿ ਇਕਿ ਮਨਮੁਖਿ ਕਰਹਿ ਸਿ ਰੋਵੈ ॥੩॥ ਹਰਿ ਕੀ ਵਡਿਆਈ ਹਉ ਆਖਿ ਨ ਸਾਕਾ ਹਉ ਮੂਰਖੁ ਮੁਗਧੁ ਨੀਚਾਣੁ ॥ ਜਨ ਨਾਨਕ ਕਉ ਹਰਿ ਬਖਸਿ ਲੈ ਮੇਰੇ ਸੁਆਮੀ ਸਰਣਾਗਤਿ ਪਇਆ ਅਜਾਣੁ ॥੪॥੪॥੧੫॥ ੨੪॥

(ਅੰਗ ੭੩੬)

All that happens, and all that will happen, is by His Will. If we could do something by ourselves, we would. By ourselves, we cannot do anything at all. As it pleases the Lord, He preserves us. || 1 || O my Dear Lord, everything is in Your power. I have no power to do anything at all. As it pleases You, You forgive us. || 1 || You Yourself bless us with soul, body and everything. You Yourself cause us to act. As You issue Your Commands, so do we act, according to our pre-ordained destiny. || 2 || You created the entire Universe out of the five elements; if anyone can create a sixth, let him. You unite some with the True Guru, and cause them to understand, while others, the self-willed manmukhs, do their deeds and cry out in pain. || 3 || I cannot describe the glorious greatness of the Lord; I am foolish, thoughtless, idiotic and lowly. Please, forgive servant Nanak, O my Lord and Master; I am ignorant, but I have entered Your Sanctuary. || 4 || 4 || 15 || 24 ||

Shri Guru Granth Sahib, Page 736

This thesis is dedicated to my mom, dad and brother,
who always had been my inspiration and support
throughout this working period

CONTENTS

CERTIFICATE

This is to certify that the research work entitled **"Genetic regulation of biosurfactant production by bacterial isolates"** being submitted to the Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala, in fulfilment of the requirement for the award of degree of **Doctor of Philosophy**, embodies faithful record of original research carried out by **Ms Kamaljeet Kaur** (Regd. no 9021001). She has worked under our guidance and supervision and that this work has not been submitted, in part or full, for any other degree or diploma of Thapar University or any other University.

Dr. Sunil Khanna
(Supervisor)
Professor & Dean, Planning
NIIT Institute of Information Technology (TNI)
NIIT House, Balaji Estate, New Delhi 110 019

Dr N Tejo Prakash
(Supervisor)
Assistant Professor, DBTES
Thapar University, Patiala

Dr N Das
Head, DBTES
Thapar University, Patiala

DECLARATION

I hereby declare that the thesis entitled **"Genetic regulation of biosurfactant production by bacterial isolates"** which is being submitted to the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, in partial fulfillment of the requirement for the degree of **Doctor of Philosophy**, has previously not formed the basis for the award of any degree, diploma, associate ship, fellowship or any other similar title or recognition.

Kamaljeet Kaur
(Regd. no 9021001)

ACKNOWLEDGEMENTS

It is a great opportunity and privilege for me to express words of thanks to all those who have played a very constructive role in completion of my PhD thesis.

"Akaal Sahai"

(God nurtures)

Above all, I am thankful to Waheguru whose spiritual strength made me work throughout this time period. I'm equally thankful to my Alma mater Thapar University for making me what I'm today.

In the outset my reverence goes to Dr. Sunil Khanna, my supervisor and mentor. He has proved to be an asset for me throughout this period. He led me through the research work with a sensitive and guiding hand, demonstrating an insightfulness and compassion that I have rarely encountered. I, from the core of my heart, appreciate his temperament, patience, affection, continuous guidance and criticism.

No words can express my thanks to Dr N Tejo Prakash, my supervisor, for his guidance, support and suggestions. My discussions with him were considerable in shaping my opinions into reality. His skill to transfer knowledge and to put it into application is miraculous. I owe a special thanks to him for facilitating me during the tough and critical times.

I am also gratefully indebted to Dr. Niranjan Das, Professor and Head of Department of Biotechnology and Environmental Sciences, for his suggestions, guidance and thought provoking remarks at crucial period of my research, especially in the molecular biology work.

I acknowledge and appreciate the help provided by Dr. M. S. Reddy, Dr. Dinesh Goyal, Dr. Abhijit Ganguli, Dr. Ranjana Prakash, Dr. Anita Rajor, for their help and guidance during the entire path in an ineffable way.

I'm also grateful to those institutions and centers which aided me in compiling the literature. Special thanks to R&D Center, Thapar University, Institute of Microbial Technology (IMTECH), Chandigarh, University of Delhi, Indian Agriculture Research Institute and Jawahar Lal Nehru University, New Delhi.

I am most appreciative of the love, support and encouragement showered on me by all my friends and colleagues. I would specially thank Purnima, Himani, Aarti Kathpal, Harpreet, Divya, Vijay, Bella and Misha and for being with me during all the ups and downs throughout the course of my investigation.

I would also like to thank God for His blessings in the form of my two lovely nephews, Jodh Singh and Angad Singh.

Last, but by no means least, my deepest gratitude to my parents and my brother, Daljeet Singh who stood by me in both felicitous and difficult times. Their keen insight and support has helped sustain me throughout the years. My strength lies solely in the love and guidance provided to me by my family without which this work would have well remained a dream.

The woods are lovely, dark and deep, but I have miles to go before I sleep…

Kamaljeet Kaur Sekhon

ABBREVIATIONS AND SYMBOLS

The abbreviations for chemicals and symbols follow either the tentative rules of IUPAC-IUB Commission on Biochemical Nomenclature or the instructions to the Authors by the respective journals.

CHEMICALS

dATP	deoxy adenosine 5'-triphosphate
dCTP	deoxy cytosine 5'-triphosphate
dGTP	deoxy guanosine 5'-triphosphate
dTTP	deoxy thymidine 5'-triphosphate
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
EDTA	ethylene diamine tetracetic acid
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
NAD(P)H	nicotinamide adenine dinucleotide, reduced
NaOH	sodium hydroxide
Rnase	ribonuclease
SDS	sodium dodecyl citrate
TAC	triacetin
Tris	tris (hydroxymethyl) amino ethane
TE	tris-EDTA
TBE	tris-borate-EDTA
TEMED	N, N, M, M' tetra methyl ethylene diamine

SYMBOLS

^0C	Degree centigrade
cm	Centimeter
gm	Gram
hrs	Hour (s)
Km	Michaelis Constant
Kbp	Kilobase pair
bp	Base pair
lit	Liter
µl	Microliter
µ	Micro
ml	Milliliter
µg	Microgram
mg	Milligram
mm	Millimeter
M	Molarity
mM	Millimolar
mMoles	Millimoles
min	Minute
nm	Nanometer
N	Normality
OD	Optical density or absorbance
%	per cent
r.p.m	Revolutions per minute
S_{10}	Supernatant fraction obtained after centrifuging homogenate at 10,000g
S_{100}	Supernatant fraction obtained after centrifuging S_{10} at 10,000g
Temp	Temperature
UV	Ultra violet
V_{max}	Maximum velocity
KI	Inhibitor constant

LIST OF INSTRUMENTS:

1. Spectrophotometer (U-2001, Hitachi).
2. Tensiometer (Petro-Diesel Instruments Company, Jencon, Calcutta, India).
3. UV light (Macrovue Uvis-20, Hoefer).
4. Gel-documentation system (Gel Doc Mega, Nucon).
5. Polyacrylamide gel electrophoresis unit (Hoefer Electrophoresis Unit, SE600 series, Amersham Biosciences).
6. Gel dryer (Hoefer, Slab Gel Dryer, Model GD2000, Amersham, Pharmacia Biotech)
7. Milli-Q water purifier (Millipore, Milli-Q Biocel, Molsheim, France).
8. Refrigerated high-speed centrifuge (Himac CR22G, Hitachi).
9. Table top centrifuge (Biofuge, Pico, Heraeus).
10. Gel-filtration and Ion-exchange columns (Pharmacia Biotech).
11. Fraction collector (Frac 100, Pharmacia Biotech).
12. Gradient Mixer (GM-1, Sweden, Pharmacia Biotech).
13. Column pumps (Pump P-1, Pharmacia Biotech).
14. Amicon stirred ultra filtration assembly (Millipore).
15. Lyophilizer (Heto LyoLab 3000 Lyophilizer, Germany).
16. GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA).
17. Weighing balance (Mettler, Toledo).
18. Incubator (Narang Scientific Works, New Delhi, India).
19. Laminar Flow (Thermadyne, Narang Scientific Works, New Delhi, India).
20. Waterbath (Precision, Labcare America).
21. Lab-Shaker (Kuhner, Switzerland).
22. Roller drum (Roll n Grow, Biogentek BGI Pvt Ltd).
23. pH meter (Cyber Scan 510, Eutech Instruments, Singapore).
24. Electrophoresis Power Supply Unit (EPS 601, Amersham Biosciences).
25. Agarose Gel Electrophoresis Unit (Amersham Biosciences).

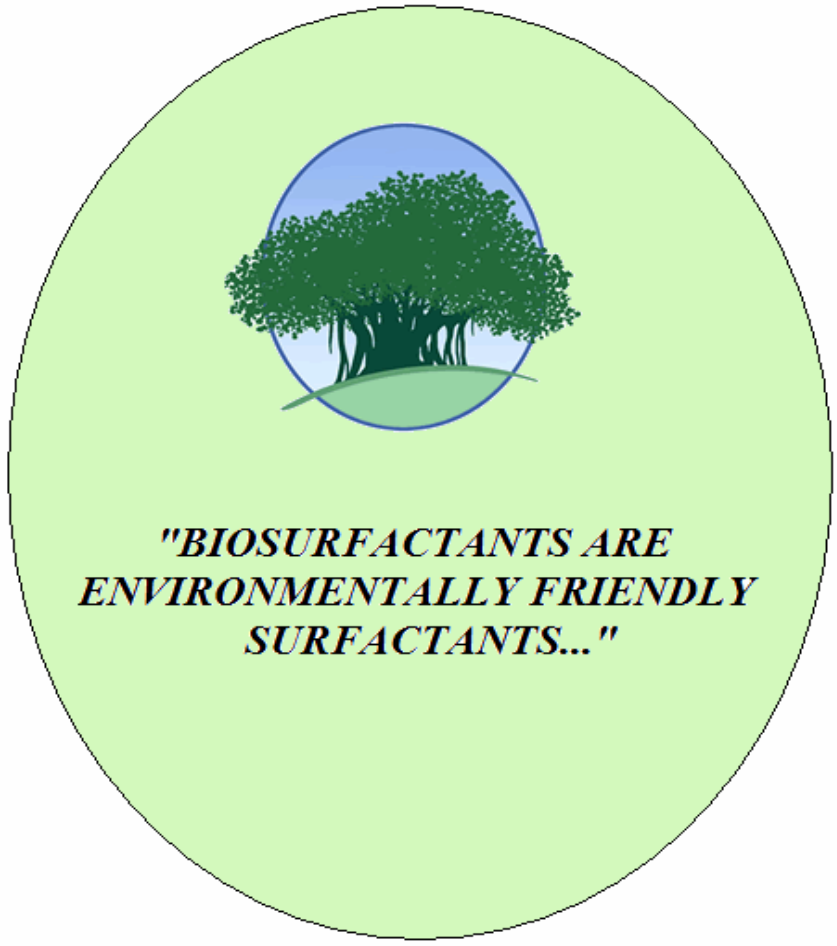
LIST OF TABLES (REVIEW OF LITERATURE)

LIST OF FIGURES (REVIEW OF LITERATURE)

LIST OF FIGURES (RESULTS)

LIST OF TABLES (RESULTS)

LIST OF IMAGES (RESULTS)



Introduction

1. INTRODUCTION

In the new era of global industrialization where many classical industries are being de-emphasized and redirected towards emerging technologies, biotechnology has a challenging edge that is opening several research opportunities.

Bioremediation is a technological process whereby biological systems are harnessed to effect the clean up of environmental pollutants. Microorganisms offer the possibility that organic pollutants can be completely mineralized to inorganic materials.

Microbial systems have a huge repertoire that enables them to degrade panoply of organic pollutants. Improved methods of detection have allowed us to measure or detect organic chemical compounds in the range of parts per billion levels of contaminants in the soil and groundwater. Bioavailability and solubilization of the contaminant is certainly a key factor in determining the feasibility of bioremediation. The contaminants such as hydrophobic compounds, trichloroethylene (TCE), polyaromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's) are poorly soluble thereby complicating the remediation process (Head, 1998). Degradation of organic pollutants by micro-organisms has been studied for many decades and the fruit of this labor is the remarkable understanding of production of biosurfactants which increase the solubility of the contaminant making it bioavailable for its enhanced biodegradation.

Surfactants constitute an important class of industrial chemicals widely used in almost every sector of modern industry. During the last decade demand of surfactants increased about 300% within the US chemical industry. Current worldwide production exceeds 3 million tones per annum (at an estimated value of US $ 4 billion) and is expected to raise over four million tones by the end of the century (Banat *et al.,* 2000). Production of surfactants in the United States and worldwide is estimated at 3.4 X 10^9 kg and 7 X 10^9 kg in 1989, respectively (Kosaric, 2001). Recently, the Jeneil Biosurfactant Corporation in United States (http://www.biosurfactant.com) started the commercial production of biosurfactants such as rhamnolipids. According to Technical Insights, a division of Frost and Sullivan, non-ionic

surfactants, microbial surfactants has begun to enjoy a market (O' Connor, 2002) and Hester 2001, of Technical Insights, a division of Frost and Sullivan has estimated that biosurfactants could capture 10% of the surfactant market by the year 2010 with sales of $200 million US.

Economics is often the bottleneck of all biotechnological processes, especially in the case of biosurfactant production. The economics of producing the biosurfactants has limited its commercial applications (Mulligan, 2005) but the cost can be reduced by improving yields, rates and recovery and using cheap or waste substrates. Biosurfactants can be produced from various substrates mainly renewable sources such as vegetable oils, distillery and dairy wastes, which are economical but have not been reported in detail. Apart form this other cheap non-conventional substrates include crops such as cassava, soybean, sugar beet, sweet potato, straw of wheat and rice, residues from coffee processing, banana waste, water miscible wastes, molasses, whey milk (Cameotra and Makkar, 2002). Enzymatic synthesis of tailor made surfactants by lipases has given a new dimension to biosurfactant production (Cameotra, 2000). Exposure to hydrophobic pollutants in contaminated environments supports the growth of biosurfactant producing microbes. In other words, microbes synthesize biosurfactants, especially during growth on water-immiscible substrates, providing an alternative to chemically prepared conventional surfactants.

Biosurfactants (surfactants of microbial origin) are a heterogeneous group of natural products of interest for biotechnological and industrial applications. The early interest in biosurfactants has arisen with the discovery of their antibiotic activity (Maier, 2003). Gradually, the prospects of biosurfactants have got a great momentum because of their applications in petroleum industry (Banat, 1995; Ron and Rosenberg, 2002; Mulligan, 2005). Moreover, certain obvious advantages of biosurfactants over synthetic surfactants such as mild production condition, lower toxicity, higher biodegradability and environmental compatibility have prompted their tremendous applications in environmental protection as well as in food, cosmetic and pharmaceutical industries (Cameotra and Makkar, 2004; Singh and Cameotra, 2004). Several biosurfactants are also reported to have strong antibacterial, antifungal and antiviral activity. In the biomedical sciences, other relevant uses of biosurfactants include their role as anti-adhesive agents to pathogens, making them useful for treating many diseases and as therapeutic and probiotic agents (Cameotra, 2004).

Research and applications of biosurfactants has expanded significantly in recent years in the areas such as agriculture, oil industry, petrochemistry and paper and pulp industry, amongst others. The development of this line of research is of paramount importance, mainly in view of the present concern with protection of the environment. The biodegradability and lower toxicity of microbial surfactants gives them advantage over their chemical counterparts and therefore make them acceptable and suitable for replacing chemicals (Mukherjee, 2005).

One of the important application being envisaged currently is to exploit the potential of biosurfactants to enhance bioavailability of the major organic chemical wastes such as organic aqueous waste (pesticides), organic liquids (solvents from dry cleaning), oils (lubricating oils, automotive oils, hydraulic oils, fuel oils), organic sludges/solids (painting operations, tars from dyestuffs intermediates) and petroleum hydrocarbons (alkanes, cycloalkanes, aromatics, polycyclic aromatic hydrocarbons, asphaltenes and resins) (Mulligan, 2005). During the two-three decades a wide variety of microorganisms have been reported to produce numerous types of biosurfactants, which degrade these chemical wastes.

"The recent developments in the field of production and recovery of biosurfactants indicate that biosurfactants will be providing promising substitutes to chemically synthesized surfactants in the coming few years. Combined with technologies of large scale fermentation and genetic and metabolic engineering, biosurfactants will be commercially successful chemicals in future".
--- Dr. Niranjan R. Gandhi, President of Jeneil Biosurfactant Inc, Wisconsin, USA.

In the past few years, biosurfactants have gained attention all over the world and there production is expected to rise according to experts.

Review of Literature

2. REVIEW OF LITERATURE

In the recent years, there has been great concern about remediating soil contaminated with hydrophobic chemical compounds. This is particularly important since indiscriminate dumping of materials, insufficient methods for waste storage, treatment and disposal facilities have contributed to the contamination of many sites. Biological methods or bioremediation has been frequently cited as an innovative technology that promises to provide an ecofriendly solution to this menacing problem.

Organic pollutants and metal contaminants in soils and other environments persist because they are either unavailable to degrading microorganisms or products, or due to low solubility of these pollutants, or the degrading microorganisms or microbial consortia are not able to carry out the necessary catabolic reactions, or the physico-chemical environmental conditions are not adequate for degradation or removal. Bioremediation by biosurfactants is currently in vogue as an innovative technology in that the biosurfactants enhance the accessibility and bioavailability of hydrophobic chemicals by forming stable emulsions and lowering the surface tension.

Biosurfactants are surfactants that are produced extracellularly or as part of the cell membrane by bacteria, yeast and fungi. Examples include *Pseudomonas aeruginosa* which produces rhamnolipids, *Candida* (formerly *Torulopsis*) *bombicola* one of the few yeasts to produce biosurfactant, which produces high yields of sophorolipids from vegetable oils and *Bacillus subtilis* which produces a lipopeptide called surfactin (Mulligan, 2005).

This review deals with microbial surfactant research, placing emphasis on the aspects related to biosurfactant production and its promising properties in detail.

2.1. BIOSURFACTANTS

The term biosurfactant refers to any compound from microorganisms, which has some influence on interfaces, i.e. **SURF**ace **ACT**ive **A**ge**NTS**, which brings down the interfacial tension between the two liquids. The term has been used very loosely to describe ‘emulsifying’ and ‘dispersing agents’ that do not significantly lower the surface tension of water but exhibit other properties of a classical surfactant (Desai and Desai, 1993). In literature the terms ‘surfactants’ and ‘emulsifiers’ are often used interchangeably. The term ‘bioemulsifier’ is often 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 the uptake of an insoluble substrate.

Surfactant molecules are able to form either micelles, or reversed micelles, or aggregate to form rod-shaped micelles, bilayers, and vesicles (Fig 1). They accumulate at interfaces and mediate between phases of different polarity such as oil/water, and act as wetting agents on solid surfaces (water/solid) (Fig 2). This dynamic process is based on the ability of the surfactant to reduce the surface tension by governing the arrangement of liquid molecules, thus, influencing the formation of H-bonds and other hydrophobic-hydrophilic interactions. The minimum surface tension value reached and the critical micelle concentration (CMC) needed are the parameters used to measure the efficiency of a surfactant (Rubina *et al.,* 1995). Rosenberg *et al.,* (2002) reported that the hydrocarbon-degrading microorganisms produce biosurfactants of diverse chemical nature and molecular size. These surface-active materials increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability, thereby enhancing the growth of bacteria and the rate of bioremediation.

2.1.1. CLASSIFICATION

Surfactants are amphipathic molecules with both hydrophilic and hydrophobic moieties present within the same molecule. The hydrophobic moiety of a biosurfactant is either a long-chain fatty acid, hydroxy fatty acid or α-alkyl β-hydroxy fatty acid and the hydrophilic moiety can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid, or an alcohol. While, synthetic surfactants are usually classified according to the nature of their polar groups, microbial biosurfactants are generally classified (Table 1) on the basis of their biochemical nature.
The classification of microbial biosurfactants is as follows (Rahman and Gakpe, 2008):

(a) Glycolipids

Glycolipid biosurfactants are usually carbohydrate in combination with long-chain aliphatic acids or hydroxy aliphatic acids. Glycolipids are generally low-molecular-weight biosurfactants (Rosenberg and Ron, 2001). Among the glycolipids, the best known are rhamnolipids, trehalolipids, and sophorolipids.

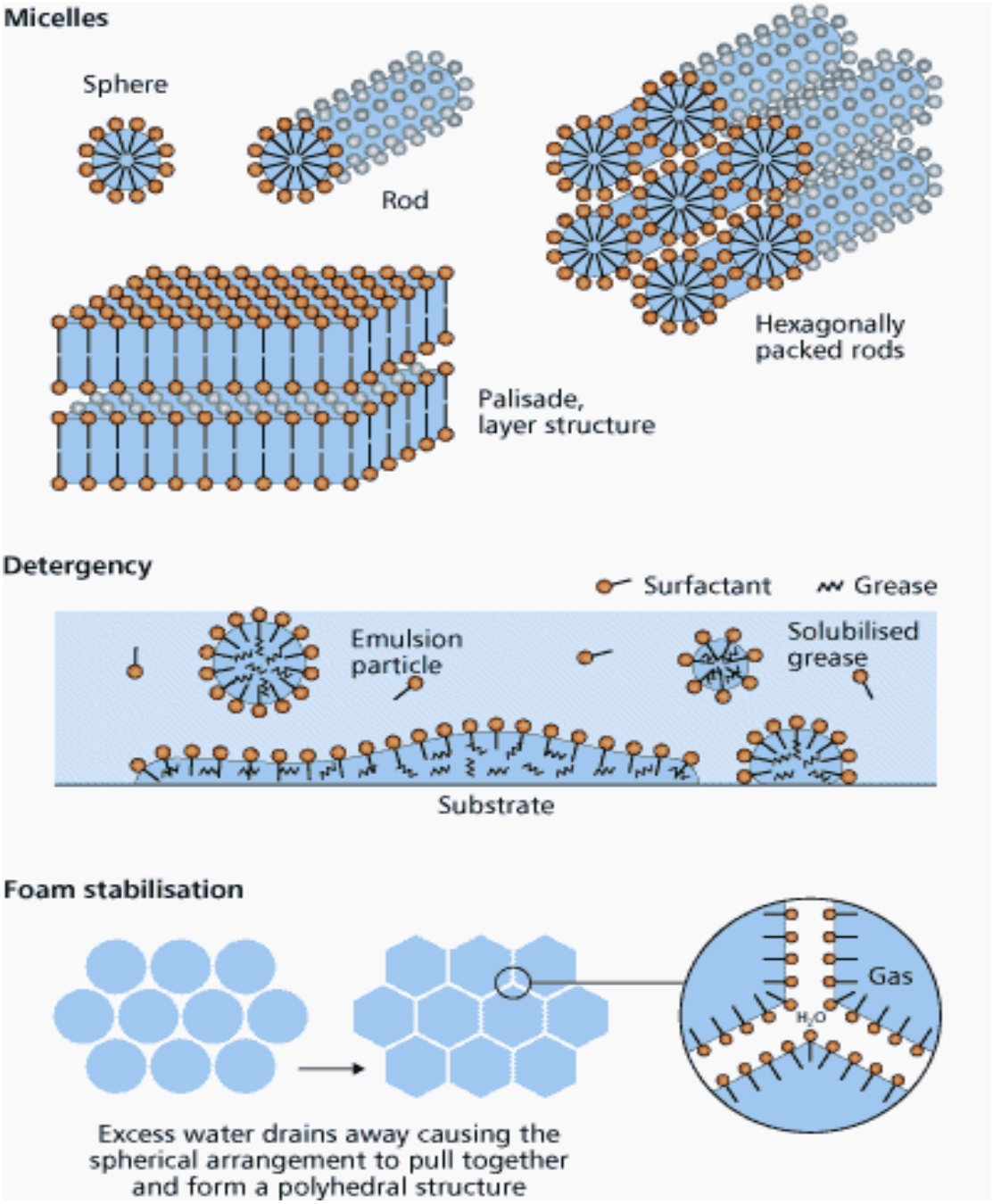


Fig 1: Different types of micelles.
Possible mode of interaction between cell surface and dispersed hydrocarbons.

Courtesy: Desai and Singh, 1986.

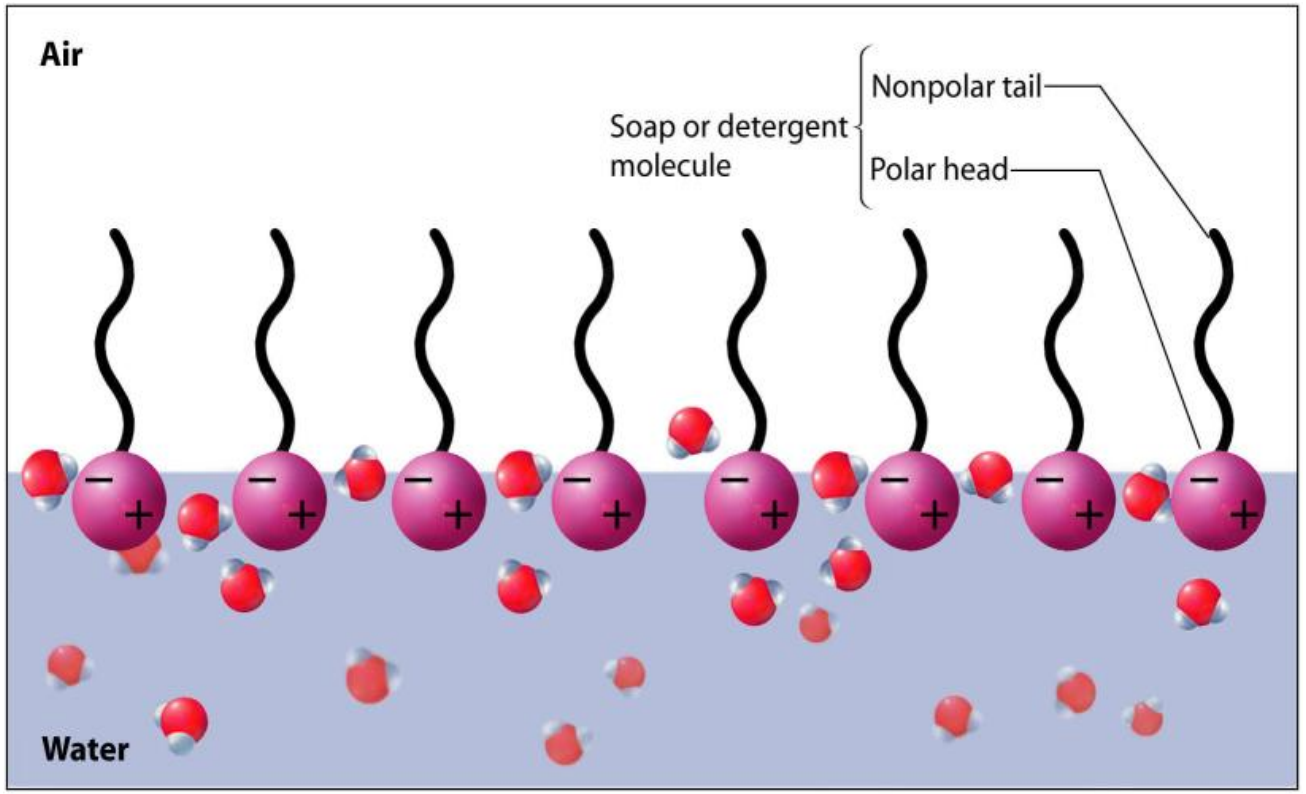


Fig 2: Mediation between phases of different polarity. Interaction between a detergent molecule and the oil/water phase.

Courtesy: John S, Conceptual Chemistry, US. 2004.

Table. 1 (a): Major types of Biosurfactants produced by microorganisms:

Biosurfactant type	Microbial species
Glycolipids	
Trehalose mycolates	*Rhodococcus erythropolis*
	Arthrobacter paraffineus
	Mycobacterium phlei
Trehalose esters	*Mycobacterium fortium*
	Micromonospora spp.
	Mycobacterium smegmatis
	Mycobacterium paraffinicum
	Rhodococcus erythropolis
Mycolates of mono, di	*Corynebacterium diptheriae*
and trisaccharide	*Mycobacterium smegmatis*
	Arthrobacter spp.
Rhamnolipids	*Pseudomonas spp.*
Sophorolipids	*Torulopsis bombicola/ apicola*
	Torulopsis petrophilum, Candida spp.
Rubiwettins	*Serratia rubidaea*
Diglycosyl digyycerides	*Lactobacillus fermenti*
Phospholipid and Fatty acids	
Phospholipid and Fatty acids	*Candida spp., Corynebacterium spp.*
	Micrococcus spp., Acinetobacter spp.
Phospholipids	*Thiobacillus thiooxidans*
	Asperigillus spp.

Desai and Desai, (1993) and Ochsber *et al.,* (1995)

Table. 1(b): Major types of Biosurfactants produced by microorganisms:

Biosurfactant type	Microbial species
Lipopeptides and lipoproteins	
Gramicidens	*Bacillus brevis*
Polymyxins	*Bacillus polymyxa*
Ornithine-lipid	*Pseudomonas rubescens*
	Thiobacillus thiooxidans
Viscosin	*Pseudomonas fluorescens*
Cerilipin	*Glucunobacter cerius*
Lysine-lipid	*Agrobacterium tumefaciens*
Surfactin, subtilysin, subsporin	*Bacillus subtilis*
Lichenysin	*Bacillus licheniformis*
Polymeric surfactants	
Lipo hetero polysaccharide (Emulsan)	*Acinetobacter calcoaceticus RAG-1*
Heteropolysaccharide (Biodispersan)	*Acinetobacter calcoaceticus* A_2
Polysaccharide protein	*Acinetobacter calcoaceticus strains*
Manno-protein	*Saccharomyces cerevisiae*
Carbohydrate-protein	*Candida petrophillum*
	Endomycopsis lipolytica
Mannan-lipid complex	*Candida tropicalis*
Mannose/ erythrose lipid	*Shizonella melanogramma*
	Ustiloga maydis
Carbohydrate-protein-lipid complex	*Pseudomonas fluorescences*
	Debaryomyces polymorphus
Liposan	*Candida lipolytica*
Alasan	*Acinetobacter calcoaceticus*
Particulate biosurfactants	
Membrane vesicles	*Acinetobacter sp. H01-N*
Fimbriae, whole cells	*Acinetobacter calcoaceticus*

Desai and Desai, 1993 and Ochsber *et al.,* (1995)

Rhamnolipid biosurfactants are produced mainly by *Psuedomonas* sp. In *Pseudomonas putida* 21BN biosurfactant produced during growth on glucose and hexadecane lowered the surface tension of the medium to 29 mN m (-1) and formed stable and compact emulsions with emulsifying activity of 69% (Tuleva *et al.,* 2002). *Pseudomonas aeruginosa* PA1 when grown on glycerol and using sodium nitrate as nitrogen source resulted in higher production of the rhamnolipid, with a yield of 3.16 g/L (Anna *et al.,* 2002). In *Pseudomonas aeruginosa* rhamnolipid production is dependent on central metabolic pathways, such as fatty acid synthesis and dTDP-activated sugars, as well as on enzymes participating in the production of the exo-polysaccharide alginate. Synthesis of these surfactants is regulated by a very complex genetic regulatory system that also controls different *Pseudomonas aeruginosa* virulence associated traits (Maier and Chavez, 2000). Santa *et al.* 2001, reported rhamnolipid biosurfactant production by a new strain of *Pseudomonas aeruginosa* PA1 using different carbon sources (n-hexadecane, paraffin oil, glycerol, and babassu oil).

Maier *et al*., (2000) reported that rhamnolipid biosurfactant causes the cell surface of *Pseudomonas aeruginosa* strains to become hydrophobic through release of lipopolysaccharide (LPS). In *Pseudomonas aeruginosa* strains grown on glucose and hexadecane, rhamnolipids caused an overall loss in cellular fatty acid content. Loss of fatty acids was due to release of LPS from the outer membrane, as demonstrated by 2-keto-3-deoxyoctonic acid and SDS-PAGE analysis and further confirmed by scanning electron microscopy (SEM). In *Renibacterium salmoninarum* 27BN during growth on *n*-hexadecane (Christova *et al.,* 2004) biosurfactant produced are of two types, the rhamnolipids RLL and RRLL and the secreted rhamnolipids appeared to be efficient in increasing hexadecane availability for the cells.

Maier *et al.,* (2004) reported a new class of biosurfactants called flavolipids produced by *Flavobacterium* sp. During growth in mineral salts medium, with 2% glucose as the sole carbon source *Flavobacterium* sp. strain MTN11 produced a mixture of 37 flavolipids ranging from 584 to 686 in molecular weight. Partially purified flavolipid mixture exhibited a CMC of 300mg/liter and reduced surface tension to 26.0 mN/m, indicating strong surfactant activity. The mixture was a strong emulsifier, solubilizing agent and a biodegrader.

Sophorolipid are produced in *Torulopsis bombicola* and the types of biosurfactant produced is regulated by the carbon source. *Torulopsis bombicola* when grown on glucose alone produced low levels of biosurfactant consisting mainly of acidic sophorolipid while when grown on glucose with soybean oil, the biosurfactant were complex, containing both lactonic and acidic SL's with saturated and unsaturated C16 and C18 fatty acid moieties (Hu and Ju,

2001). Growth on glucose and hexadecane yielded the high amount of biosurfactant consisting primarily two de-acetylated lactonic isomers with palmitate as the fatty acid moiety.

Thanomsub *et al., (*2004), showed cultural filtrates of thermotolerant yeast strain *Candida ishiwadae,* isolated from plant material in Thailand, to contain relatively large amounts of biosurfactant, which consist of two glycolipids (monoacylglycerols): 1-linoeylglycerol and 1-oleylglycerol. The biosurfactant exhibit higher surface activities tested by drop collapse test than several artificial surfactants such as sodium dodecyl sulphate (SDS), Tween 20, and Tween 80.

Glycolipids were extracted from the supernatant of a culture of *Rhodococcus* sp. strain MS11, grown on n-hexadecane and characterized by ^{1}H- and ^{13}C-NMR-spectroscopy and mass spectrometry. Results demonstrate that *Rhodococcus* sp. strain MS11 was well suited for bioremediation of soils and sediments contaminated for a long time with di-, tri-, and tetrachlorobenzenes as well as alkanes (Rapp and Jurgens, 2003).

In another study by Janssen and Noordman, (2002) it was shown that five biosurfactant producing bacterial strains i.e. *Pseudomonas aeruginosa* UG2, *Acinetobacter calcoaceticus* RAG1, *Rhodococcus erythropolis* DSM 43066, *Rhodococcus erythropolis* ATCC 19558 and strain BCG112 were able to degrade hexadecane in the presence and absence of exogenously added biosurfactants.

(b) Phospholipids and Fatty Acids

Phospholipids are lipids containing one or more phosphate groups. Fatty acids are components of a long chain aliphatic carboxylic acid found in natural fats and oils. Fatty acids also constitute membrane phospholipids and glycolipids. Certain hydrocarbon degrading bacteria and yeasts produce appreciable amounts of phospholipids and fatty acids which act as biosurfactants.

Structured phospholipids (PLs) were produced by lipase-catalyzed acidolysis between soybean PLs and free fatty acids. Lipozyme TL IM catalyzed acidolysis in a solvent free system was optimized using response surface methodology (RSM). Based on the fitted model, the optimal conditions for the production of the structured PLs were framed with the incorporation of acyl donors, including caprylic acid, conjugated linoleic acid, eicosapentaenoic and docosahexaenoic acid, into phospholipids and 39% incorporation of caprylic acid was obtained (Peng *et al*., 2002).

In 2000, Lee *et al.,* purified and characterized the biosurfactants from *Nocardia* sp. L-417, that had strong properties as an emulsifying agent, and surface tension reducing agent. These biosurfactants were purified by ammonium sulphate fractionation, chilled acetone and hexane treatments, silica gel column chromatography and Sephadex LH-20 gel filtration. Purified biosurfactants were stable over a broad range of pHs (2-12) and temperature (100^0 C, 3hrs). The homogeneity was checked and the biosurfactant were named as biosurfactants types I and II by TLC and HPLC.

Several bacteria and yeasts produce large quantities of fatty acid and phospholipid during growth on *n*-alkanes which act as biosurfactants (Desai and Banat, 1997). *Candida tropicalis* and *Debarymyces polymorphus* produce a bioemulsifier during shake cultivation on hexadecane, which consists of at least 40% lipid moiety (Singh and Desai, 1989). A phospholipid biosurfactant is produced by *Asperigillus* sp, grown on hydrocarbons (Miyazima *et al*., 1985). *Arthrobacter* AK-19 (Wayman *et al*., 1984) has been shown to accumulate up to 40-80 % w/w lipids when cultivated on hexadecane.

(c) Peptide and Amino acid containing Lipids

Lipopeptides and lipoproteins are a heterogeneous class of biologically active peptides that serve to carry water-insoluble lipids in blood. The protein component alone is an apolipoprotein. Most of the lipopeptides produced by microorganisms are known for their antibiotic properties, but some have biosurfactant activity also. Rosenberg and Ron (2001) also call the polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers as high-molecular-weight biosurfactants.

'Surfactin', a cyclic lipopeptide, known for its exceptional surfactant power, reported first by Arima *et al.,* (1968) from *Bacillus subtilis* ATCC-21332, is one of the most effective biosurfactants known so far. It lowers the surface tension of water from 72 to 29.9 mM/m at a concentration as low as 0.005%. *Bacillus licheniformis* produces a surfactant called lichenysin, which is similar in structural and physicochemical properties to the 'surfactin' of *Bacillus subtilis* (Javaheri *et al.,* 1985., McInerney *et al.,* 1990). *Pseudomonas fluorescens* 378 produces a surface-active compound called *AP-6*, which consists mainly of carbohydrate and protein (Persson *et al.,* 1988).

In 2004, Bloemberg *et al.,* isolated *Pseudomonas putida* strain PCL1445 from roots of plants grown on site polluted with PAH's. The strain produces biosurfactant at the end of the exponential growth phase. HPLC analysis of supernatant extracts showed two peaks with surface tension reducing activity, assigned as biosurfactants putisolvin I and II. Structural analyses revealed that both are novel cyclic lipodepsipeptides. The difference between the two structures is located in the second amino acid from C-terminus, being valine for putisolvin I and leucine/isoleucine for putisolvin II. These biosurfactants lower the surface tension, influence biofilm development on polyvinyl chloride (PVC) and also break down the existing *Pseudomonas* biofilms. A cyclic lipopeptide surfactant produced by *Pseudomonas fluorescens* isolated from rhizosphere of wheat plants showed the ability to lyse zoospores of *Phytophthora ultimum* var. *sporangiiferum.* The strain SS101, identified as *Pseudomonas fluorescens* biovar II, reduced the surface tension of water from 73 to 30 mNm^{-1}. Five Tn5 mutants of strain SS101 were developed which lacked the abilities to reduce the surface tension of water and to cause lysis of zoospores. A partially purified extract of strain SS101, reduced the surface tension of water to 30 mN m^{-1} and reached the CMC at 25µg ml^{-1}. MS and NMR analysis identified the main constituent as cyclic lipopeptide (1139 Da) containing 9 amino acids and a 10 carbon hydroxy fatty acid (De Souza *et al.,* 2003). Nielsen *et al* in 2002 showed that the cyclic lipopeptide (CLP), amphisin produced by fluorescent *Pseudomonas* species from the sugar beet rhizosphere has antibiotic as well as biosurfactant properties. The reduction of surface activity of the spent growth medium from 71 to 27 mN m^{-1} was indicative of the surfactant activity.

(d) Polymeric Biosurfactants

Polymeric biosurfactants have the ability to alter the rheological properties of aqueous solutions at low concentrations. Therefore, these biopolymers are used as thickeners and to stabilize emulsions, dispersions and suspensions in aqueous systems. Polymeric surfactants are high molecular weight biopolymers with properties like high viscosity, high tensile strength, and resistance to shear. It is because of these properties that polymeric biosurfactants have found a variety of industrial uses in pharmaceutical, cosmetic, food industries etc.

Bacterial strains A2 and HE5 of *Acinetobacter calcoaceticus* isolated by enrichment culture techniques were shown to produce extra cellular, non-dialyzable limestone powders in water, referred to as biodispersans. An optimized medium for the production of biodispersan was developed with ethanol as the substrate (Rosenberg *et al.,* 1988). Oil degrading bacterium *Acinetobacter calcoaceticus* RAG-1 produces a potent extracellular polymeric bioemulsifier called emulsan (Rosenberg *et al.,* 1979). Another example of polymeric biosurfactants is

liposan, which is produced by *Candida lipolytica* and is inducible in nature and is primarily (97-98%) composed of carbohydrates (Cirigliano *et al.,* 1985).

2.1.2. MECHANISM FOR HYDROCARBON UPTAKE

Hydrocarbons include both aliphatic and aromatic compounds and are wide-spread xenobiotics (compounds/group of chemicals which are alien to the existing biological system). Although a great majority of the hydrocarbons are biodegradable, there are a few which can be termed recalcitrant as they persist in the environment for a longer time. Such hydrocarbons are present in crude oil and industrial effluents. Hydrocarbon uptake is essential first step for the bacteria to degrade both straight-chain (labile) and ring (semi-recalcitrant) compounds, with the latter requiring more stages via a series of catalytic reactions. Extracellular surfactants may assist in the uptake process. The first step by which microorganisms utilize liquid hydrocarbons as carbon sources is by the emulsification of the hydrocarbon and water. The hydrocarbons emulsified by microorganisms may then be absorbed easily by the cells.

The main physiological role of biosurfactants is to permit microorganisms to grow on water-immiscible substrates by reducing surface tension at the phase boundary and making the substrate more readily available for uptake and mechanism, but the molecular mechanisms of the uptake processes of these substrates are, however, still not clear.

Nakahara *et al.,* (1977) have proposed 3 pathways for hydrocarbon uptake by microorganisms:

- Direct contact of the cells with large oil drops.
- Direct contact of the cells with fine droplets (sub micron droplets).
- Uptake of dissolved hydrocarbons in the aqueous phase.

During hydrocarbon fermentation, there exists a dynamic equilibrium and so the uptake may be by free cells, cells which may absorb sub micron droplets and cells which are attached to large oil drops, to form flocs. In a study by Singh and Desai (1986), it was shown that initial interaction of hydrocarbons with the microbial cells takes place either by the contact of microorganism with insoluble substrate directly i.e. without any mediator (unmediated interaction in which cell surface properties play important role in the interaction with substrate molecule) or by contact through a mediator (mediated interaction in which the interaction between cell surface and alkane takes place with help of mediators which have emulsifying activities like some biosurfactants). The size of the alkane droplets has been shown to influence the growth rate of the microorganisms.

Among the hydrocarbon-utilizing bacteria, *Pseudomonas aeruginosa* is most frequently isolated from hydrocarbon-impacted environments. To better understand the microbial cell surface adaptation to growth on crude oil, the cell surface properties of two *Pseudomonas aeruginosa* strains, U1 and U3, both isolated from same crude oil degrading microbial community enriched on Bonny Light crude oil (BLC) were compared. The isolates grown on BLC have reduced O-antigen expression compared to that of glucose grown cells and the loss of O-antigen resulted in shorter lipopolysaccharide molecules, increased cell surface hydrophobicity and increased *n*-alkane degradation (Norman, Suau and Morris, 2002).

The studies on the phenomenon of hydrocarbon fermentation and the mechanism of hydrocarbon transport and uptake are still in infancy, and the detailed research is still needed to thoroughly outline the processes involved in the two phenomenons.

2.1.3. BIOSURFACTANT PRODUCTION

Biosurfactants are produced by a variety of microbes, secreted either extracellularly or attached to parts of cells, predominantly during growth on water-immiscible substrates. The production of biosurfactants by microorganisms can be during exponential growth or it may be during the stationary phase of growth when the nutrient limiting conditions start prevailing in the growth medium. In case of growth associated biosurfactant production, there exists a parallel relationship between substrate utilization, growth and biosurfactant production.

Surfactin is a biosurfactant produced by *Bacillus subtilis.* It is a cyclic lipopeptide characterized by a β-hydroxycarbonic acid moiety with strong surface activity as well as antibiotic properties. Production of this lipopeptide antibiotic surfactin was carried out using a recombinant *Bacillus subtilis* (Shoda *et al.,* 1992). Surfactin yield of the recombinant strain was about one and a half times as much as that of *Bacillus subtilis* RB14, the strain in which the surfactin gene originated. Michel *et al.,* (1990) tested 13 strains of *Bacillus subtilis* for the coproduction of the lipopeptide surfactin and the antifungal lipopeptides of the iturin family. Out of 13 strains, only one produced both lipopeptides with a high yield on synthetic medium. Several L-amino acids and various carbon sources were good substrates for the lipopeptide production. The maximum yield of surfactin was about 110 mg/liter and that of iturin A about 39 mg/liter/absorbance unit for the best strain, *Bacillus subtilis* S 499.

Another study by Michel *et al.* in 1992 showed that the surfactin consists of a mixture of two groups of homologous lipopeptides differing by their peptide sequence. Val ^{7} surfactin was characterized as a minor companion of the previously described Leu ^{7} surfactin. The addition of various α-amino acids to the culture media led to variations in the production ratios of the two congeners. *Bacillus subtilis* C-1 isolated from petroleum sludge and producing biosurfactants was identified by using biochemical, physiological and genetic parameters. The rapid sensitive detection and efficient structural characterization of lipopeptide biosurfactants by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry by using whole microbial cells and crude culture filtrates as targets in combination with surface tension measurements, revealed three lipopeptide complexes, the surfactins, the iturins, and the fengycins, characterized by *in situ* structure analysis by using post source decay MALDI-TOF mass spectrometry (Cameotra *et al.,* 2002).

Very little is know about the distribution of biosurfactant producing bacteria in the environment because the present body of knowledge has been developed around a relatively small number of well characterized biosurfactants. Contributing to this is the lack of a concerned effort to perform a comprehensive screening for biosurfactants and the microorganisms that produce them. Therefore Maier *et al* (2003) determined how common culturable surfactant producing bacteria are in undisturbed and contaminated sites. Out of 1,305 colonies screened for biosurfactant production in mineral salts medium containing 2% glucose, from the contaminated and undisturbed soils, 45 isolates were positive for biosurfactant production. These 45 isolates were grouped using repetitive extragenic palindromic (REP)-PCR analysis, which yielded 16 unique isolates. 16s rRNA gene sequence of each isolate, revealed one new biosurfactant producing microbe, a *Flavobacterium* sp. Therefore, it was concluded that the biosurfactant producing microbes are present in most soils even by using a relatively limited screening assay.

Zhang *et al* (2003) performed batch experiments with paraffin as sole carbon source at 30^{0} C and pH of 7 using oil-contaminated soil. Two bacteria identified as *Bacillus* sp. capable of producing biosurfactants were isolated which decreases the surface tension of enrichment medium from 61 to 45 mN/m. The isolates could enhance the dissolved concentration of phenanthrene to above 1mg/ml, which was higher than the saturated solubility of phenanthrene under standard condition.

Bacillus subtilis BBK-1 a halotolerant bacterium produces three kinds of lipopeptides viz Bacillomycin L, plipastatin and surfactin when grown in 8% NaCl. Although the bacterium could

grow in 16% NaCl but was unable to produce biosurfactant (Roongsawang *et al.,* 2002). It was observed that the biosynthetic gene encoding 4'-phosphopantetheinyl transferase was responsible for the biosurfactant activity. The gene existed in a single copy in the genome and the deduced amino acid sequence was almost identical to that of Lpa-14 from *Bacillus subtilis* strain RB14, which co-produces iturin A and surfactin.

Persson *et al* (1988) showed that production of *AP-6,* a glycoprotein type of biosurfactant by *Pseudomonas fluorescens* 378 was growth associated. Similarly, the production of emulsan in *A.calcoaceticus* and the fermentative production of surface-active agents from *Bacillus aereus* IAF-346 and *Bacillus* sp. IAF-343 (Cooper and Goldenberg, 1987) were found to be growth associated. The production of rhamnolipids by *Pseudomonas aeruginosa* is also related to growth (Hisatsuka *et al*., 1971). Extensive studies concluded that in *Acinetobacter calcoaceticus* RAG-1, emulsan or emulsan-like precursors accumulate as capsular material during the exponential growth phase but are released into the medium when the rate of protein synthesis declines i.e. during the stationary phase of growth (Rubinovitz *et al*., 1982; Goldmann *et al*., 1982; and Pine *et al.*, 1983).

Similarly there is very little information about the anaerobic biosurfactant producers. In one study by Javaheri *et al* (1985) *Bacillus licheniformis* JF-2 anaerobically produced a biosurfactant when grown in a glucose mineral salts medium containing yeast extract and $NaNO_3$. Surface tension of the medium was reduced from 70-74 mN/m to as low as 28 mN/m due to the production of an anionic biosurfactant. This biosurfactant termed as lichenysin A, was purified and characterized as a mixture of lipopeptides, with major components ranging from 1,006 to 1,034 Da. Lichenysin A decreases the surface tension of water from 72 to 28 mN/m and achieves the critical micelle concentration (CMC) of 12mg/L (Yakimov, 1995). *Bacillus licheniformis* JF-2 produces a very active lipopeptide under both aerobic and anaerobic conditions (Georgiou *et al.,* 1994). The compound was purified to homogeneity by reverse-phase C_{18} HPLC and it has a molecular weight of 1,035. Amino acid analysis, fast atom mass and infrared spectroscopy and ^{1}H, ^{13}C and two-dimensional nuclear magnetic resonance demonstrated that the biosurfactant consists of a heterogeneous C_{15} fatty acid tail linked to a peptide moiety very similar to that of surfactin. This biosurfactant exhibits a CMC of 10mg/L and reduces the interfacial tension against decane to 6×10^{-3} dyne/cm, which is one of the lowest interfacial tensions ever reported for a microbial surfactant. Mulligan and Roubin (1989), showed that isocitrate dehydrogenase, a tricarboxylic acid (TCA) cycle enzyme, regulated surfactin production in *Bacillus subtilis.* A mutant with 30 times suppressed enzyme activity of

the enzyme 30 times produced three and a half times more surfactin. Increased surfactin yield always correlated with low levels of isocitrate dehydrogenase activity.

2.1.4. MECHANISM OF BIOSYNTHESIS

Growth and biosurfactant production proceed as separate events. In the exponential phase of growth there is often only a very low rate of surfactant production, overproduction of surfactant occurs as the cells cease to grow. Hommel and Ratledge (1993) have proposed a theory that a small amount of biosurfactant is needed for alkane solubilization and uptake. According to them, cells in the uptake process do not consume surfactant. Like the production of other metabolites, once the signal to start surfactant production has been given by the presence of alkane, then surfactant production will continue in an unregulated manner till the signal to stop is received. As that signal i.e. disappearance of the last alkane droplet-never arrives, the cells continue surfactant production even after they have ceased to grow.

High biosurfactant production, which is a phenotypic response, is found to be characteristic under certain qualitative and quantitative environmental conditions. Production of biosurfactants by microbes in general is dependent on nutritional and environmental factors:-

2.1.4.1. Nutritional Factors

Carbon, nitrogen and phosphate sources, metal ions and other additives used in the media formulation may play a crucial role in the production and yield of biosurfactants (Fig 3).

(a) Effect of Carbon Source

In *Pseudomonas* species rhamnolipid production is regulated by the presence of n-alkanes in the medium while in *P aeruginosa* UW-1 higher production rates were achieved when grown on vegetable oil in comparison to liquid hydrocarbon (hexane) (Kosaric and Sukan, 2000). Chayabutra *et al*., (2000) showed that rhamnolipid production in *P aeruginosa* under denitrification conditions with carbon substrates palmitic acid, stearic acid, oleic acid, linoleic acid, glycerol, vegetable oil and glucose was successful and was free from problems like foaming and respiration limitation. *Rhodococcus* sp. H13A produces significant amounts of glycolipids when grown on alkanes and alcohols, but its production is drastically reduced during growth on fatty acids, triglycerides, ethanol, organic acids, and carbohydrates (Singer *et al.*, 1990).

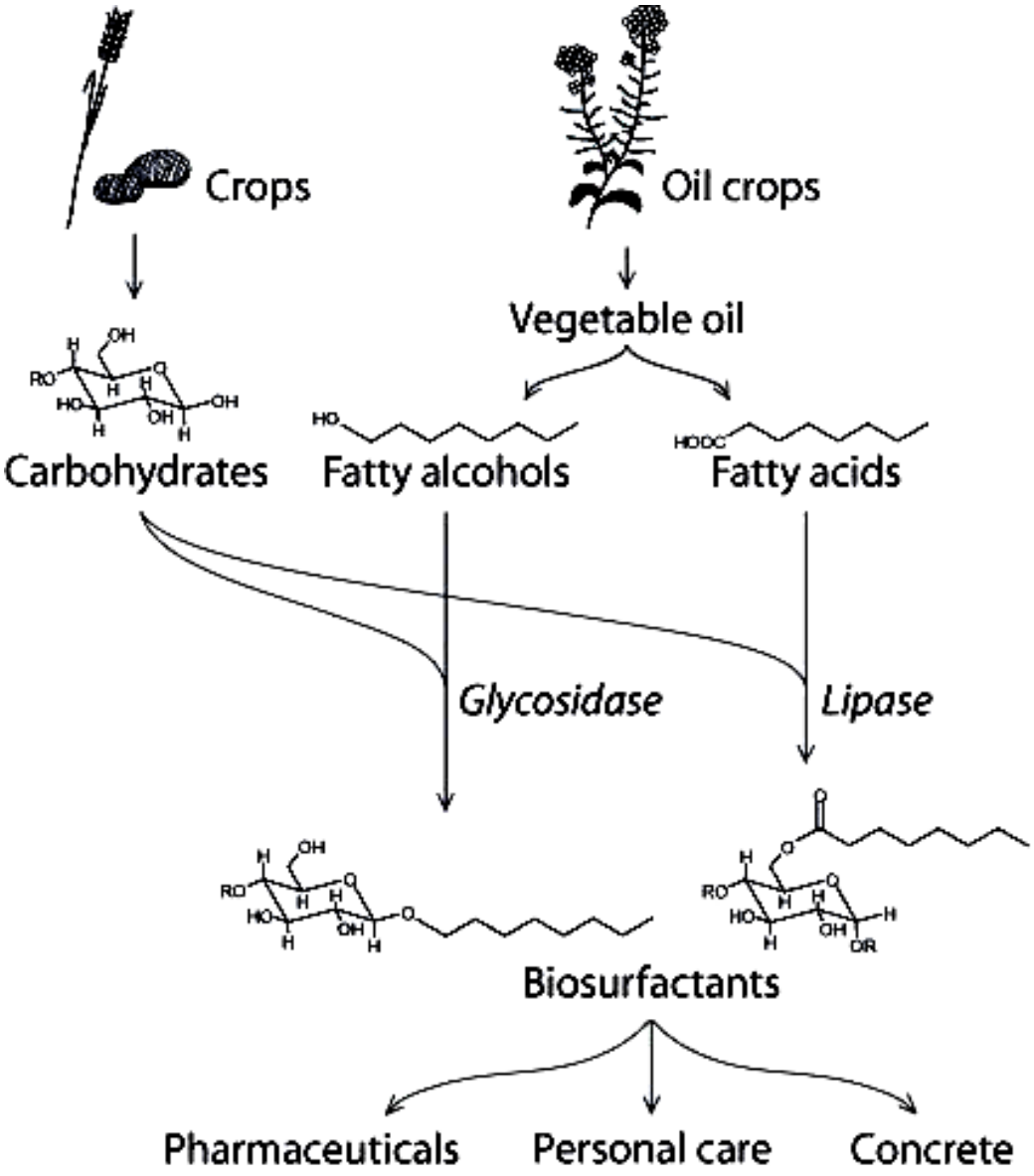


Fig 3 General scheme of possible routes for the synthesis of biosurfactants and the applications.

Courtesy: Sukan and Kosaric, 2000.

Research over the years has shown the preference of microorganisms for hydrocarbon substrates over carbohydrates for biosurfactant production (Table 2). There is sudden burst in the production of biosurfactants whenever microbes grow at the expense of water immiscible substrates. Singh and Desai (1989) have shown hydrocarbon emulsification by *C. trapicalis* and *D. polymorphous* during their cultivation on hexadecane and oily wastes but there was no emulsifier production when both the yeasts were grown in a glucose medium. Little biosurfactant production was observed when cells were grown on a readily available carbon source. Only when all the soluble carbon was consumed and when water-immiscible hydrocarbon was available was biosurfactant production triggered (Banat *et al.,* 1995, Banat and Banarjee, 1991). *Pseudomonas aeruginosa* 44T1 produces rhamnolipids when grown on C_{12} *n*-alkanes or olive oil but not when grown on hexadecane or a mixture of high chain n-alkanes (Robert *et al.*, 1989). *Corynebacterium lepus* produces a large amount of biosurfactant when grown on glucose, but it remains cell bound. The surfactant is released from the cells when treated with hexadecane (Duvnjak and Kosaric, 1985). Oil-degrading strain *Pseudomonas aeruginosa* DS10-129 was used to optimize a substrate for maximum rhamnolipid production. It produced 4.31, 2.98 and 1.77g/L rhamnolipid biosurfactant using soybean oil, safflower oil, and glycerol, respectively. Yield of biosurfactant increased even after the bacterial cultures reached the stationary phase of growth. Characterization of rhamnolipids using mass spectrometry revealed the presence of di-rhamnolpids (Rha-Rha-C_{10}-C_{10}). Emulsification activity of the rhamnolipid biosurfactant produced by *P. aeruginosa* DS10-129 was greater than 70% using all the hydrocarbons tested, including xylene, benzene, hexane, crude oil, kerosene, gasoline, and diesel. *P. aeruginosa* DS10-129 emulsified only hexane and kerosene to that level (Rahman and Banat, 2002).

(b) Effect of Nitrogen source

Like carbon source, nitrogen source in the medium also plays important role. The nature and the concentration of the N-source affect biosurfactant production by *A. paraffineus* ATCC 19558. Rhamnolipid production increases when nitrogen is exhausted in the medium. Nitrogen limitation is also important in the production of sophorose lipids. Production of biosurfactants by *Pseudomonas* sp., *Acinetobacter* sp., and *Torulopsis* sp., can be regulated by the ratio of nitrogen to carbon source (Kosaric and Sukan, 2000). Among the inorganic salts tested, ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* (Cooper *et al*., 1983), whereas nitrate supported maximum surfactant production in *P. aeruginosa* (Kappeli *et al.,* 1984; Macelwee *et al*, 1990; Robert *et al.,* 1989) and *Rhodococcus* spp. (Abu-Ruwaida *et al.,* 1991). Biosurfactant production by *A.paraffineus* is increased by the addition of L-

amino acid, glutamic acid, asparagines, and glycine to the medium (Cooper *et al*., 1983). The structure of surfactin is influenced by the L-amino acid concentration in the medium to produce Val-7 or Leu-7 surfactin (Michel *et al.,* 1992). Similarly, lichenysin-A production is enhanced two-four folds in *Bacillus Licheniformis* BAS50 (Yakimov and Timmis, 1996) by addition of L-glutimic acid and L-aspargine, respectively, to the medium. Robert *et al.,* (1989) and Abu-Ruwaida *et al.,* (1991), observed nitrate to be the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44TI and *Rhodococcus* strain ST-5 growing on olive oil and paraffin, respectively. In *Pseudomonas aeruginosa,* a simultaneous increase in rhamnolipid production and glutamine synthetase activity was observed when growth slowed as the culture became nitrogen limiting (Mulligan and Gibbs, 1989). Similarly, nitrogen limitation caused increased biosurfactant production in *C.tropicalis* IIP-4 (Singh *et al.,* 1990), and *Nocardia* strain SFC-D (Kosaric *et al.,* 1990). Syldatk *et al*., (1985) showed that nitrogen limitation not only causes overproduction of biosurfactant but also changes the composition of the biosurfactant production.

(c) Effect of other factors/sources

Production of biosurfactants is also affected by the phosphate source. The production of lipopeptides biosurfactant by *Bacillus licheniformis* JF-2 was increased from 35 to 110 mg^{-3} by reducing the phosphate concentration from 100 to 50 mmol day^{-1} m^{-3} (Georgiou *et al,* 1994). The addition of iron and manganese salts increases the yield of surfactin by *Bacillus subtilis.* The yield of biosurfactant production is either enhanced or inhibited by addition of antibiotics, such as penicillin or chloramphenicol (Kosaric and Sukan, 2000).

2.1.4.2. Environmental Factors

Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability also influence biosurfactant production through their effects on cellular growth or activity. The temperature is especially important in the case of *A. parraineus* ATTC 19558, *R. erythropolis* and *Pseudomonas* sp. *Bacillus subtilis* C9 has been observed to produce a three fold higher yield of lipopeptides under oxygen limited conditions compared to that under oxygen sufficient conditions (Kosaric and Sukan, 2000). The pH of the medium plays an important role in sophorolipid production by *T.bombicola* (Gobbert *et al.,* 1984). Rhamnolipid production in *Pseudomonas* sp. was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 (Kappeli *et al.,* 1984). In *Pseudomonas* sp. strain DSM-2874 (Syldatk, 1985) temperature causes alteration in the composition of the biosurfactant produced. A thermophilic *Bacillus* sp. grew and produced biosurfactant at temperature above 40^0 C (Banat *et*

al., 1993). Heat treatment of some biosurfactant caused no appreciable change in biosurfactant properties such as the lowering of surface tension and interfacial tension and the emulsification efficiency, all of which remained stable after autoclaving at 120^0 for 15min (Abu-Ruwaida *et al.,* 1991).

Studying the mechanism of biosurfactant production in *A. calcoaceticus* RAG-1, Wang and Wang (1990), showed that the cell-bound polymer/dry-cell ratio decreases as the shear stress increases. Sheppard and Cooper (1990) have concluded that oxygen transfer is one of the key parameters for the process optimization and scale-up of surfactin production in *B. subtilis.* Salt concentration also affected biosurfactant production depending on its effect on cellular activity. Some biosurfactant products, however, were not affected by salt concentrations up to 10% (w/v), although reductions in the CMCs were detected (Abu-Ruwaida *et al.,* 1991). The biodegradation of a wide range of hydrocarbons, including aliphatic, aromatic, halogenated and nitrated compounds, has been shown to occur in various extreme habitats. The biodegradation of many components of petroleum hydrocarbons has been reported in a variety of terrestrial and marine cold ecosystems (Margesin *et al.,* 2001).

2.1.5. EFFECT OF BIOSURFACTANTS

Bioremediation is currently in vogue as a promising cost effective and performance effective technology to address numerous environmental pollution problems. Biosurfactants are emerging as a technology to enhance the accessibility and bioavailability of contaminants. Effect of biosurfactants on the biodegradation of petroleum hydrocarbons, polycyclic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs)/ hydrocarbons, oils, heavy metals is of great importance.

The effects of biosurfactants on crude oil desorption and mobilization in a soil system was studied by Kuyukina *et al.,* (2005). It was shown that the biosurfactants from *Rhodococcus ruber* were able to remove the oil from the soil core 1.4-2.3 times greater than that of a synthetic surfactant Tween 60. Biosurfactant enhanced oil mobilization was temperature related and it was slower at 15^0C than at 22-28^0C. A mathematical model was framed using a 1D filtration model to simulate the oil penetration through a soil column. A positive correlation (R^2=0.99) was found between surfactant penetration through oil-contaminated soil and oil removal activity. Biosurfactant was less adsorbed to soil components than synthetic surfactant, thus rapidly penetrating through the soil column and effectively removing 65-82% of crude oil. Thus, *Rhodococcus* biosurfactants can be used for *in situ* remediation of oil contaminated soils. PAHs

degrading bacteria may enhance the bioavailability of PAHs by excreting biosurfactants, by production of extracellular polymeric substances or by forming biofilms. In one such study by Johnsen and Karlson (2004) these hypothesis in the pure cultures of PAHs degrading bacterial strains were tested. Three semi-colloid *Sphingomonas* polysaccharides (Gellan, Welan and Rhamsan) increased the solubility of the PAHs. The increases were most pronounced for the more hydrophobic PAHs. The polysaccharide-sorbed PAH's were bioavailable. Biofilm formation on PAHs crystals may favor the diffusive mass transfer of PAH's from crystals to the bacterial cells.

Chang *et al.,* (2004) showed that trehalose lipid biosurfactants produced by *Rhodococcus erythropolis* enhanced the phenanthrene (Phe) solubilization and biodegradation. The average CMC of trehalose biosurfactants was approx. 16mg total organic carbon (TOC)/L and the addition of biosurfactants at 20-fold the CMC increased the apparent solubility of Phe by more than 30-fold. The addition of biosurfactant (32.2 mg TOC/kg soil) to the soil system also increased both the initial rates (by more than 2 fold) and the extent of Phe mineralization. The study indicates that the trehalose biosurfactants have good solubilization capacity for hydrophobic organic compounds and great potential for applications in bioremediation of sites contaminated with PAHs. Wong *et al* (2004) investigated the effectiveness of nonionic surfactants (Tween 80) and biosurfactants to enhance the solubilization and desorption of phenanthrene (Phe) and pyrene (Pyr) in soil aqueous systems under thermophilic conditions using batch studies. Biosurfactants produced from *Pseudomonas aeruginosa* strain ATCC 9027 and P-CG3 and Tween 80 were used and results showed that biosurfactants could effectively enhance solubility of both Phe and Pyr under thermophilic conditions (55^0C), which was linearly proportional to the conc. of surfactants at conc. above their respective CMC. Degradation of Phe diminished with increasing surfactant concentrations.

In a breakthrough in the applications of biosurfactants for bioremediation, Kosaric *et al.*, (2001) conducted laboratory and pilot plant experiments with herbicide metholachlor, chlorinated aromatics, naphthalene, PAHs and heavy oil. It was observed that the addition of sophorose lipids caused a sharp drop of metholachlor concentration in the methanol extract from soil slurry bioreactors, as well as the degradation rates of both naphthalene and 2,4-dichlorophenol (2,4DCP) were enhanced in the soil slurry with some PAHs were almost completely removed in 22 days. Olivera *et al.,* (2000) used *Bacillus subtilis* O9 biosurfactant (surfactin) and bioaugmentation to enhance the degradation of residual hydrocarbons from ship bilge wastes which consists of oil, grease and seawater. It was observed that surfactin in the form of a crude extract increased hydrocarbon bioavailability and shortened the time required

for its natural biodegradation. It was also noticed that the addition of biosurfactant stimulated hydrocarbon degradation but bioaugmentation did not produce any remarkable effect. Biodegradation of endosulfan isomers in soil-applied and flask-coated conditions was done by an isolated bacterial co culture. Addition of a biosurfactant (400µg/ml), isolated from *Bacillus subtilis* MTCC 1427, enhanced the rate of biodegradation by 30-45% in both the conditions. The biosurfactant mediated enhanced biodegradation of endosulfan isomers has an important bearing in natural environment wherein the pesticide degrading and surfactant-producing microorganisms are continuously interacting (Awasthi *et al.,* 1999, 1997).

2.1.6. PHYSIOLOGY AND GENETICS

(I) REGULATION OF BIOSURFACTANT PRODUCTION

In general, three mechanisms, namely, induction, repression and nitrogen and multivalent ions, operate in the regulation of biosurfactant production (Desai and Banat, 1997). Induction appears to be the general regulation mechanism used to control the onset synthesis of most lipopeptide biosurfactants (Besson *et al.*, 1992, Kluge *et al.,* 1989, Ullrich *et al.*, 1991). Repression of biosurfactant production by *Acinetobacter calcoaceticus* (Forsell *et al.*, 1993) and *Arthrobacter paraffineus* (Duvnjak *et al.*, 1982) on hydrocarbon substrates has been observed with organic acids and D-glucose, respectively. The repression of a protein-like factor responsible for alkane and hexadecane biodegradation in *P.aeruginosa* by glucose, glycerol and palmitic acid has been observed (Hardegger *et al.,* 1994). Rapid biosurfactant production was observed in a *Pseudomonas* strain during growth on glucose and oleic acid, when oleic acid was utilized upon the exhaustion of glucose (Banat *et al.,* 1991).

The synthesis of rhamnolipid in *P.aeruginosa* upon exhaustion of nitrogen and commencement of stationary phase of growth has been observed (Kappeli *et al.,* 1984). The addition of nitrogen source caused an inhibition of rhamnolipid synthesis in resting cells of *Pseudomonas* sp. strain DSM-2874 (Syldatk *et al.,* 1985). Mulligan and Gibbs (1989) showed a direct relationship and glutamine synthetase activity in *P.aeruginosa* RC- II. The concentrations of DL-glutamine, L-glutamic acid, and ammonia in the medium play important role. Ochsner *et al.,* (1995) observed the expression of genes from *P.aeruginosa* for rhamnolipid synthesis in *P.fluorescens* and *P.putida* only under nitrogen limiting conditions.

(II) GENETICS OF BIOSURFACTANT PRODUCTION

The genetics of expression of biosurfactants is still in its infancy but the generation of mutant strains and subsequent isolation of genes involved in the biosynthesis are becoming

increasingly important. Mutants capable of producing several fold higher biosurfactant concentrations than those of parent microbial strains have been isolated (Mulligan and Gibbs, 1989, Ohno *et al.*, 1995).

Genetics of *Bacillus subtilis* and *Pseudomonas aeruginosa* would be discussed in detail in this section:

(a) *Bacillus subtilis*

Surfactin (Fig 4) is the most common lipopeptides including biosurfactants (LPBS) produced by several *bacilli* whose biosynthetic mechanisms have been well studied both genetically and biochemically. Three genetic loci: *srf*A operon, *com*A (previously called *srf*B), and *sfp* are essential for surfactin production in *B. subtilis.* The *srf*A operon (28 kb) consists of four open reading frames that encode three proteins with peptidyl carrier protein (PCP) and /or racemase domain, and a thioesterase like protein. These proteins form a non-ribosomal peptide synthetase complex (surfactin synthetase). Sieber and Marahiel (2003) showed that surfactin synthetase is a large multienzyme complex consisting of three enzymatic subunits (Fig 5), SrfA (402kDa), SrfB (401kDa), and SrfC (144kDa), which consist of seven modules that comprise 24 catalytic domains. On the other hand, *com*A and *sfp* encode a transcriptional activator of the *srf*A gene and a 4'-phosphopantetheinyl transferase (Sfp/PPTase, an activating enzyme of SrfA multienzyme complex), respectively (Roongsawang, 2002). Fuma *et al.,* (1993), also showed that the nucleotide sequence of the 20,535 base pairs of the 5' end of the *srfA* operon, included the *srfA* promoter region, the first open reading frame (ORF), *srfAA,* encoding surfactin synthetase I and part of the second ORF, *srfAB,* encoding surfactin synthetase II. The synthesis of the lipopeptide surfactin by *Bacillus subtilis* is catalyzed by multienzyme thiotemplate mechanism and the genes that encode at least part of the multienzyme complex are contained within the *srfA* operon.

In 1997, Nakayama *et al.,* isolated new variants of surfactin by a recombinant *Bacillus subtilis* MI113 (pC115), carrying a gene responsible for the production of surfactin and iturin A cloned from *Bacillus subtilis* RB14C. De Roubin *et al.,* (1989), isolated a mutant strain of *B. subtilis* (*srf 1*), which produced three times higher levels of 'surfactin' than the wild type. Further studies on the genetics of 'surfactin' production confirmed that there is involvement of three chromosomal genes/loci, *sfp, srfA,* and *comA* (Nakano and Zuber, 1990; 1991; 1992; 1993) for the biosynthesis of lipopeptide surfactin in *Bacillus subtilis.* The *sfp* gene was isolated, its

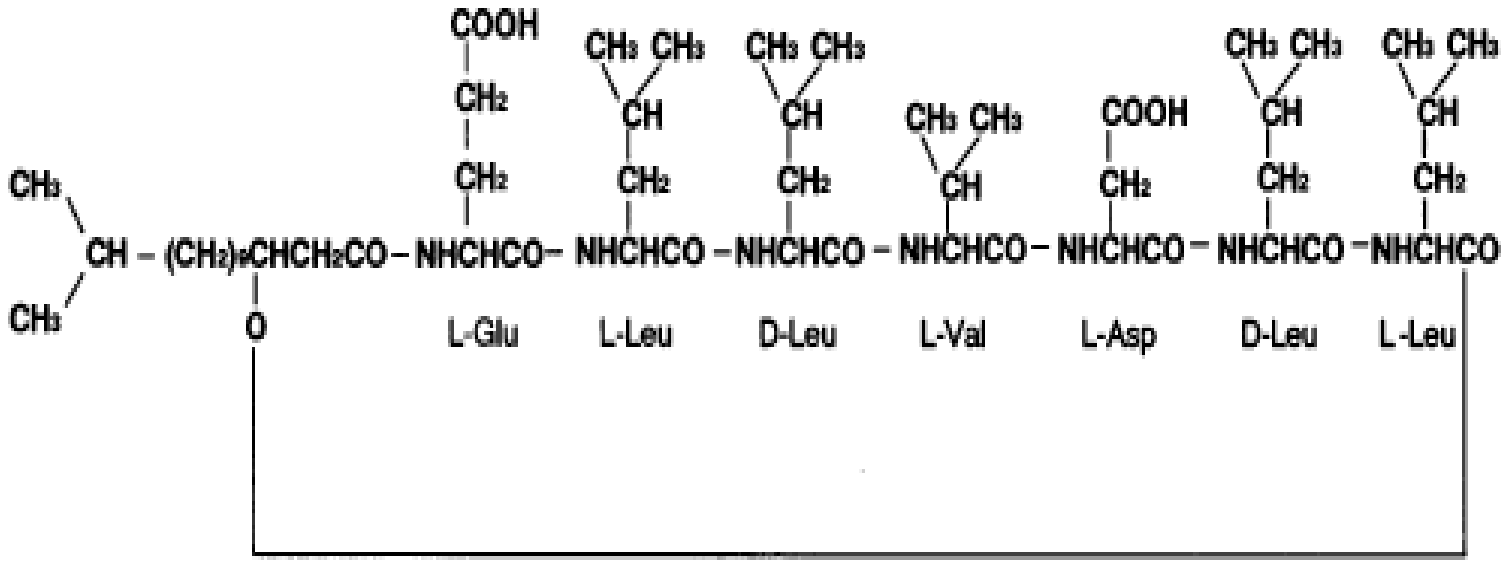


Fig 4: Structure of lipopeptide biosurfactant – surfactin produced by *Bacillus subtilis*.

Courtesy: Shoda *et al,* 1996.

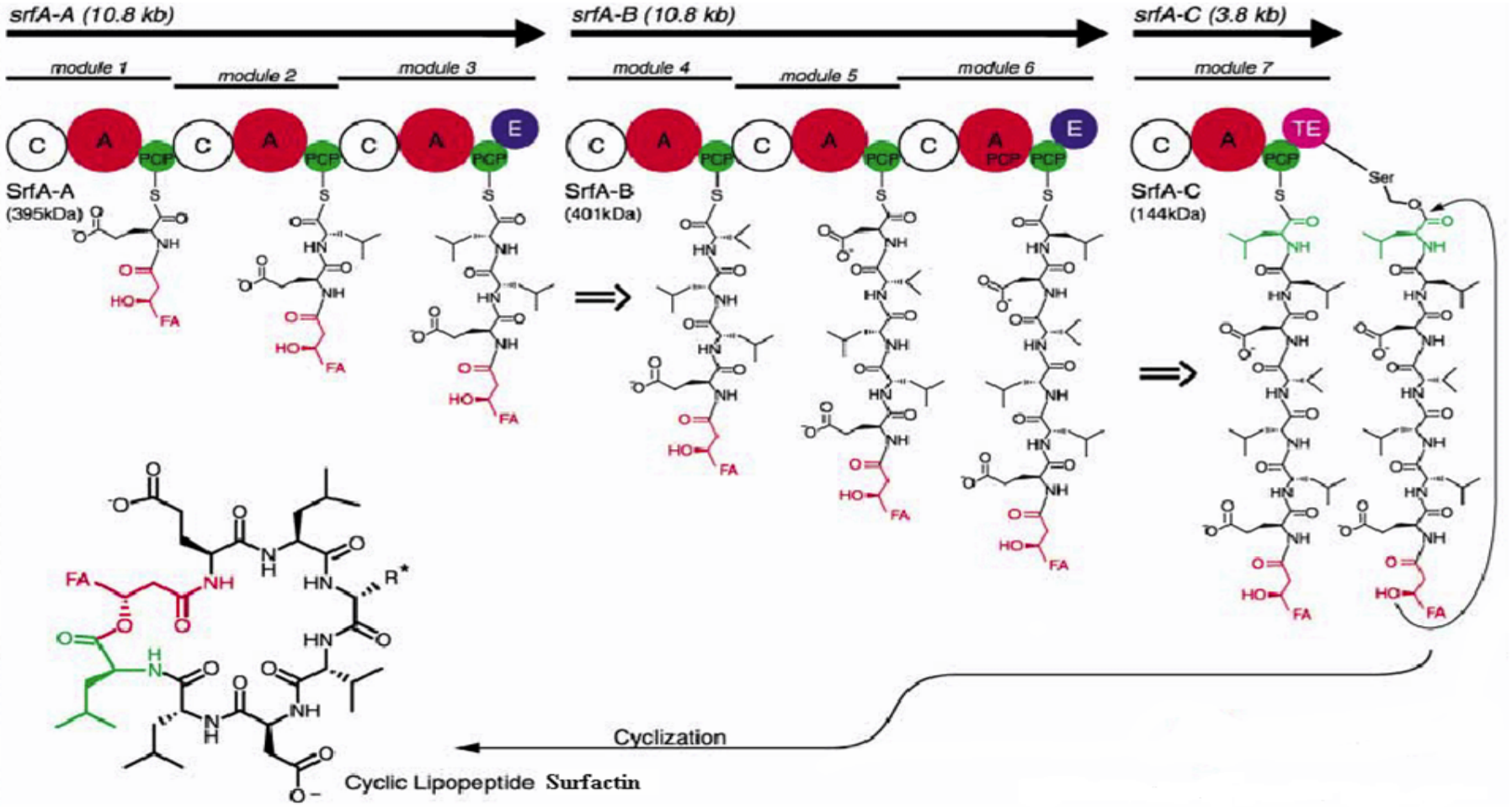


Fig 5: Nonribosomal peptide synthetase (NRPS) assembly of surfactin. The surfactin synthetase consists of 24 individual domains responsible for the catalysis of 24 chemical reactions. These domains catalyze activation (A domain), covalent binding (T domain), elongation (C domain), epimerization (E domain), and release (TE domain) by either cyclization or hydrolysis. These domains are organized in modules, where each module incorporates one dedicated building block into the growing peptide chain.

Courtesy: Sieber and Marahiel (2003).

nucleotide sequence was determined, was expressed in *E. coli* and its putative product was purified for use in antibody production and in amino acid sequence analysis. The gene was plasmid amplified in *Bacillus subtilis* where it conferred a Srf^+ phenotype on sfp^0 (surfactin non-producing) cells. *srfA* is an operon encoding at least some of the enzymes that catalyze surfactin synthesis, *srfB* contains the *comP* and *comA* genes which comprise a signal transduction system involved in the competence development pathway and is required for the transcription of *srfA, sfp* is a gene of unknown function that is required for surfactin production. The expression of *srfA* is induced after the end of exponential growth and is dependent on the products of late-growth regulatory genes *comP, comA, and spo0K* (Nakano and Zuber, 1991). Transcription initiation region of the *srfA* operon is controlled by the *comP-comA* signal transduction system in *Bacillus subtilis.*

The expression of the *srf* operon of *Bacillus subtilis,* encoding surfactin synthetase and the competence regulatory protein ComS, was observed to be reduced when cells were grown in a rich glucose and glutamine containing medium in which pH was 5.0 or lower. The production of the surfactin synthetase subunits and of surfactin itself was also reduced. Raising the pH to near neutrality resulted in dramatic increases in *srf* expression and surfactin production (Cosby *et al.,* 1998). The apparent pH dependent induction of *srf* expression required *spo0K,* which encodes the oligopeptide permease that functions in cell-density-dependent control of sporulation and competence. Nakano *et al.* (1991) indicated that *srfA* locus of *Bacillus subtilis* is defined by a Transposon Tn917 insertion and encodes the enzymes that catalyze surfactin production (a peptide secondary metabolite). The *srfA* locus was isolated by cloning the DNA flanking *srfA::*Tn917 insertions followed by chromosome walking. The cloned region is an operon of over 25kb, which covers the transcription initiation region but not the intact 3' end of *srfA.* Insertion mutations in *srfA,* created by the chromosomal integration of plasmids bearing overlapping *srfA* DNA fragments, were examined for their effects on surfactin production, competence, and sporulation. All three processes were found to require the intact 5' half of the *srfA* operon, whereas the 3' half of *srfA* was found to be required for sporulation and surfactin production but no competence.

Tsuge *et al* (1996) cloned *lpa-8* required for the production of lipopeptide antibiotics surfactin and plipastatin B1 from strain *Bacillus subtilis* YB8. When this gene was inactivated in strain YB8 neither surfactin nor plipastatin B1 was produced. However, the defective strain transformed with an intact *lpa-8* gene had restored ability to produce both peptides. The deduced amino acid sequence of *lpa-8* (224 amino acid residues) showed sequence similarity to that of *sfp* (from surfactin-producing *B. subtilis*), *lpa-14* (from iturin A and surfactin producing *B. subtilis*), *psf-1* (from surfactin-producing *B. pumilus*), gsp (from gramicidin-S-producing *B. brevis*), and *entD* (from siderophore-enterobactin-producing *Escherichia coli*), which are able to complement a defect in the

sfp gene and promote production of the lipopeptide antibiotic surfactin. In 1998, Quadri *et al.,* characterized the *sfp* enzyme, required for the production of the lipoheptapeptide antibiotic surfactin. They showed that this enzyme post translationally phosphopantetheinylates a serine residue in each of the seven peptidyl carrier protein domains of the first three subunits (SrfABC) of surfactin synthetase to yield docking sites for amino acid loading and peptide bond formation.

A 3642-bp genomic region of *Bacillus subtilis* B3 comprising *srfDB3, aspB3, lpaB3,* and *yczEB3* genes that resulted in biosynthesis of surfactin in *B. subtilis* 168 was cloned, sequenced, and characterized by Yao *et al.,* (2003). Among them, the *srfDB3* gene encodes thioesterase, which is required for biosynthesis of surfactin in *B. subtilis,* the *aspB3* gene encodes a putative aspartate amniotransferase-like protein, the *lpaB3* encodes phosphopantetheinyl transferase, which shows high identity to the product of *lpa-14* gene regulating the biosynthesis of iturin A and surfactin in *B. subtilis* RB14; the *yczEB3* encodes a YczE-like protein with significant similarities in signal peptide and part of the ABC transport system. Kim *et al.,* (2000), reported that *Bacillus subtilis* C9 produces a lipopeptide-type biosurfactant, surfactin and rapidly degrades alkanes up to a chain length of C_{19}. The nucleotide sequence of the *sfp* gene cloned from *Bacillus subtilis* C9 was determined. This *sfp* gene was expressed in *Bacillus subtilis* 168 to transform this non-surfactin producer into a surfactin producer. The transformed *Bacillus subtilis* SB103 derivative of the strain 168 was shown to produce surfactin measured by its decrease in surface tension, emulsification activity, and TLC analysis of the surface-active compound isolated from the culture broth. The transformed *Bacillus subtilis* SB103, containing *sfp* gene readily degraded aliphatic hydrocarbons (C_{10-19}) and therefore enhances the bioavailability of hydrophobic hydrocarbons.

Transposon mutagenesis in *Bacillus subtilis* strain 168 generated a surfactin-susceptible mutant strain 801 (Tsuge *et al.,* 2001). Analysis of the region of insertion revealed that *yer*P was the determinant of surfactin self-resistance. When strain 802 was converted to a surfactin producer by introducing a functional *sfp*, which encodes a 4'-phosphopantetheinyl transferase and is mutated in *B. subtilis* strain 168, this *yer*P-deficient strain produced surfactin, although surfactin production was significantly reduced. The expression of *yer*P was at its maximum at the end of the logarithmic growth phase and was not induced by surfactin. *yer*P is the first RND-like gene characterized in gram-positive strains and is supposed to be involved in the efflux of surfactin.

(b) *Pseudomonas aeruginosa*

The genetics of rhamnolipid biosynthesis in *P. aeruginosa* has been extensively studied by Ochsner *et al.* (1994, 1995). The *rhl*ABR gene cluster was found to be responsible for the synthesis of RhlR regulatory protein and a rhamnosyl transferase, both essential for rhamnolipid synthesis. After this, another regulatory gene, *rhlI,* located downstream of the *rhlABR* gene cluster was identified. The expression of the *rhlAB* genes in heterologous hosts has also been studied. In *P. fluorescens* and *P. putida* KT-2442, these genes were expressed and produced rhamnolipid. However, in *E.coli*, active rhamnosyl transferase was synthesized but rhamnolipids were not produced. Transposon Tn5-GM-induced mutant strains of *Pseudomonas aeruginosa* which were unable to produce rhamnolipid biosurfactants and lack rhamnosyltransferase activity were isolated (Ochsner *et al.,*1994). The DNA regions flanking the transposon were cloned and used as specific probes for the isolation of the corresponding wild-type genes from a *P. aeruginosa* wild-type cosmid gene library. Single cosmid clones capable of restoring rhamnolipid synthesis in the mutant strains were isolated and further sub-cloned and sequenced, resulting in the identification of two genes (rhlAB) which are organized as an operon upstream of the previously identified rhlR regulatory gene. The RhlA protein (32.5 kDa) harbors a putative signal sequence, suggesting that this protein is located in the periplasm, while the RhlB protein (47 kDa) contains at least two putative membrane spanning domains. The expression of the rhlAB genes was found to be enhanced 20-fold during the stationary phase of growth under conditions of nitrogen limitation, as measured by using *rhlA::lacZ* fusions. Moreover, the transcriptional activation of the rhlAB genes appears to depend on a functional RhlR regulatory protein. The sequence upstream of the rhlA promoter contains two inverted repeats, which define putative binding sites for the RhlR regulator. The controlled expression of the rhlAB genes in *Escherichia coli* led to the formation of the active rhamnosyltransferase. This provides direct evidence for the fact that the rhamnosyltransferase encoding genes have been identified.

Alteration in substrate requirements of *P.aeruginosa* strains by insertion of a *lac* plasmid from *E.coli* has made it possible to produce rhamnolipid from whey, a waste product from the dairy industry (Koch *et al.,* 1988). Genes responsible for the production of biosurfactants have also been isolated, characterized and cloned in heterogeneous hosts (Ochner *et al.,* 1995, Hardegger *et al.,* 1994). They have also resulted in a several fold increase in the yield of biosurfactants (Ochsner *et al.,* 1995), and a change in the raw material requirement for production (Koch *et al.,* 1988, Ochsner *et al.,* 1995). It was shown that transposan Tn5-GM induced mutants of *P. aeruginosa* PG201 were unable to grow in minimal media with hexadecane as the carbon source and some of these mutants, 6S512 and 59C7, lacked extracellular rhamnolipids (Koch *et al.*, 1991). A gamma ray-

induced mutant of *Pseudomonas aeruginosa* strain S8, capable of hyper production of biosurfactant from hydrocarbons, was isolated and named as EBN-8. The mutant showed 3-4 times more hydrocarbon emulsification/conversion as compared to the parent when grown on Khaskheli crude oil in minimal medium. Enhanced biosurfactant production and hydrocarbon utilization by the mutant was also observed during growth on heptadecane in minimal medium as indicated by emulsion index and surface tension of cell-free culture broth (Iqbal *et al.,* 1995).

Rhamnolipid biosurfactant and the PA bioemulsifying protein produced by *P. aeruginosa*, can improve bioavailability and biodegradation in liquid culture, but their production and roles in soils are unknown. To detect expression of genes for surface-active compounds, the *gfp* reporter gene was fused either to the promoter region of *pra*, which encodes for the emulsifying PA protein, or to the promoter of the transcriptional activator *rh*lR. Green fluorescent protein (GFP) production conferred these gene fusions in *P. aeruginosa* PG201. GFP was produced in sand culture, indicating that the *rh*lR and *pra* genes are both transcribed in unsaturated porous media. Confocal laser scanning microscopy of liquid drops revealed that *gfp* expression was localized at the hexadecane-water interface. Wild-type PG201 and its mutants that are deficient in either PA protein, rhamnolipid synthesis, or both were studied to determine if the genetic potential to make surface-active compounds confers an advantage to *P. aeruginosa* biodegrading hexadecane in sand. Hexadecane depletion rates and carbon utilization efficiency in sand culture were the same for wild type and mutant strains (Holden *et al.,* 2002).

Deziel *et al*., (2003), showed that *Pseudomonas aeruginosa* produces extracellular glycolipids composed of L-rhamnose and 3-hydroxyalkanoic acid called rhamnolipids. Rhamnolipids are synthesized by a rhamnosyltransferase, encoded by the *rhlAB* operon, which catalyses the transfer of TDP-L-rhamnose to 3-(3-hydroxyalkanoyloxy) alkonic acid (HAA) moieties of various lengths. RhlB is the catalytic protein of the rhamnosyltransferase. They also showed that the HAAs produced by *P. aeruginosa* are the actual precursors of rhamnolipid biosynthesis. They presented the evidence, indicating that *rhlA* is required for production of HAAs and that these HAAs display potent surface-active properties. It was shown that the use of ammonium instead of nitrate as source of nitrogen and an excess of available iron both decrease *rhlA* expression and swarming motility. Whereas Chen *et al,* 2005 reported that the quorum-sensing (*las* and *rhl*) systems play critical roles in the pathogenicity of *P. aeruginosa* and in synthesis of rhamnolipids. The two, *rhlI* and *rhlR* null mutants, lacking the ability of synthesizing PA12 or Rh1R protein, produced insignificant amounts of rhamnolipids while having similar growth profiles as the wild-type culture. They showed that the regulatory Rh1R: PAI2 complex is thus essential for rhamnolipid synthesis.

(c) Others

Acinetobacter strain RAG-1 (ATCC 31012) is an industrially important strain that has been extensively characterized with respect to its growth on hydrocarbons and its production of a high molecular mass bioemulsifier, emulsan. The genes encoding the lipase (LipA) and lipase chaperone (LipB) from *Acinetobacter calcoaceticus* RAG-1 were cloned and sequenced. The genes were isolated from a genomic DNA library by complementation of a lipase-deficient transposon mutant of the same strain. Transposon mutagenesis resulted in lipase-negative mutants with deficient bioemulsifier activity. The transposon insertions were in the foldase gene (chaperone) for lipase. These mutants produced the same amount of bioemulsifier as the wild-type RAG-1 based on hexose assays. However, preliminary gas chromatography analyses showed changes in the fatty acid composition of the bioemulsifier produced by the lipase-negative mutants compared to the wild-type bioemulsifier. Therefore, these mutants produce a less efficient bioemulsifier (Sullivan *et al.,* 2002).

The molecular genetics of two structurally diverse biosurfactants, a glycolipid and a lipopeptide, were the first to be characterized. Recent advances include the identification of the structural genes for second lipopeptide and the isolation of a gene responsible for enhanced emulsification activity of a high molecular weight biopolymer. New insight has also developed in the regulatory mechanisms of the originally described surfactants, both of which are controlled by quorum sensing, a mechanism bacteria use to monitor cell density (Sullivan *et al.,* 1998).

2.1.7. BIOSURFACTANT RECOVERY

(A) Mass cultivation for bulk biosurfactant production

Cultivation of bacteria in batch cultures for the production of biosurfactant is not commercially viable when the process is to be used at the industrial scale. Santos *et al*., (1984) have shown that the yield of rhamnolipids was several folds higher when a continuous process was used compared to the batch culture for the mass production of biosurfactants. The production of biosurfactant *AP-6* by *Pseudomonas fluorescens* 378 (Persson *et al.*, 1990) in a microcomputer controlled multibatch fermentation system, which enabled simultaneous running of ten fermenters, greatly cut down the time and equipment costs, compared to traditional laboratory fermenters. Mattei and Bertrand (1985) were able to obtain large amounts (approx. 3 g/l) of biosurfactant continuously by using a tangential flow filtration device. Neu and Poralla (1990) isolated a biosurfactant from *Bacillus* sp. in which the foam produced was blown out of the fermenters, collected and then centrifuged.

Cooper *et al.,* (1981) showed an enhanced production of the lipopeptide, surfactin from *Bacillus subtilis* by continuous product removal by foam fractionation. The surfactin could be easily recovered from the collapsed foam by acid precipitation. The yield was also improved by the addition of either iron or manganese salts. Rhamnolipid biosurfactants were continuously produced with *Pseudomonas aeruginosa* on the pilot plant scale (Reiling *et al.,* 1986). A biosurfactant concentration of approximately 2.25 g liter^{-1} was achieved and a biosurfactant yield on glucose was 77 mg g^{-1} h^{-1}, and the productivity was 147 mg liter^{-1} h^{-1}, corresponding to a daily production of 80 g of biosurfactant. The first enrichment step consisted of an adsorption chromatography, which was followed by an anion-exchange chromatography. The resulting product was 90% pure, and the overall recovery of active material was above 60% with the downstream processing used. Foaming was successfully applied for the concentration of the lipopeptide biosurfactant surfactin from *B. subtilis* ATCC 21332 cell culture broths. Simultaneous high enrichments and recoveries of surfactin could not be obtained (around 70% of the total recovered) at a low concentration during the early stages of foaming. The use of low stirrer speeds was essential in producing foam at a controlled rate. By collecting fractions of the foam produced between 10 and 30 hours, from systems stirred at 166 and 146 rpm, a highly concentrated surfactin extract could be obtained. The surfactin concentration in the foam was 1.22 and 1.67 g l^{-1} respectively, which represented enrichments and recovery of 60%. This study by Davis *et al.,* (2001) points to the utility of foaming as a method for the recovery of surface-active fermentation products, particularly when used in an integrated production/recovery system.

Kuyukina *et al.,* (2001) proposed methyl tertiary-butyl ether (MTBE) as a solvent for extraction of biosurfactants from *Rhodococcus* bacterial cultures. After comparison with other well known solvent systems used for biosurfactant extraction, it was found that MTBE was able to extract crude surfactant material with high product recovery (10 g/l), efficiency (critical micelle concentration (CMC), 130-170 mg/l) and good functional surfactant characteristics (surface and interfacial tensions, 29 and 0.9 mN/m), respectively. The isolated surfactant complex contained 10% polar lipids, mostly glycolipids possessing maximal surface activity. Ultrasonic treatment of the extraction mixture increased the proportion of polar lipids in crude extract, resulting in increasing surfactant efficiency. Due to certain characteristics of MTBE, such as relatively low toxicity, biodegradability, ease of downstream recovery, low flammability and explosion safety, the use of this solvent as an extraction agent in industrial scale biosurfactant production is feasible.

(B) Biosurfactant recovery

The choice of method for recovery of a particular biosurfactant depends on its ionic charge, solubility in water, whether the product is cell bound or extracellular and of course, the cost of recovery. The methods generally used for biosurfactant recovery include solvent extraction, adsorption followed by solvent extraction, precipitation, crystallization, centrifugation, and foam fractionation (Table 3). Most biosurfactants are secreted into the medium and thus are isolated from either culture filtrate or supernatant obtained after removal of cells.

2.1.8. APPLICATIONS OF BIOSURFACTANTS

The interest in the potential applications of microbial surface-active compounds is based on their broad range of functional properties that include emulsification, phase separation, wetting, foaming, solubilization, de-emulsification, corrosion-inhibition, and viscosity reduction (e.g. heavy crude oils). There are, therefore, many areas of industrial application where chemical surfactants could be substituted by biosurfactants in fields as diverse as agriculture, food and beverage industries, industrial cleaning, cosmetics, pharmaceutical industry, petroleum and petrochemical industries, waste treatment, pipeline transportation, additives for rolling oil, as therapeutic agents, in pulp and paper industry, textiles and ceramics (Kosaric *et al*., 1987, 2001, Muthusamy *et al,* 2008) (Table 4).

Potential applications of biosurfactants are in field of soil and marine bioremediation, including polyaromatic hydrocarbon (PAHs) or polychlorinated biphenyls (PCBs) or pesticides bioremediation, bioreclamation of soil, metal contaminated soils bioremediation, and also in oil storage tank cleaning, microbial enhanced oil recovery (Banat, 2000). Sadouk *et al*., 2008 reported the production of biosurfactants from low cost substrates (residual sunflower frying oil) and degradation of diesel oil, clean-up of hydrocarbons contaminated sites, enhanced oil recovery by a *Rhodococcus erythropolis* strain.

Table 2: Use of inexpensive raw materials for the production of biosurfactants by various microbial strains.

Low cost or waste raw material	Biosurfactant type	Producer microbial strain
Rapeseed oil	Rhamnolipids	*Pseudomonas* sp. DSM 2874
Babassu oil	Sophorolipids	*Candida lipolytica* IA 1055
Turkish corn oil	Sophorolipids	*Candida bombicola* ATCC 22214
Sunflower and soybean oil	Rhamnolipid	*Pseudomonas aeruginosa* DS10-129
Sunflower oil	Lipopeptide	*Serratia marcescens*
Soybean oil	Mannosyl erythritol lipid	*Candida* sp. SY16
Waste frying oils (sunflower and olive oil)	Rhamnolipid	*Pseudomonas aeruginosa* 47T2 NCIB 40044
Soybean soap stock waste	Rhamnolipid	*Pseudomonas aeruginosa* LBI
Sunflower oil soap stock waste	Rhamnolipid	*Pseudomonas aeruginosa* LBI
Oil refinery wastes	Glycolipids	*Candida antarctica and/or Candida apicola*
Soybean oil refinery wastes	Rhamnolipids	*Pseudomonas aeruginosa* AT10
Curd whey and distillery wastes	Rhamnolipid	*Pseudomonas aeruginosa* BS2
Potato process effluents	Lipopeptide	*Bacillus subtilis*
Cassava flour wastewater	Lipopeptide	*Bacillus subtilis* ATCC 21332 and *Bacillus subtilis* LB5a

Mukherjee *et al.,* 2006

Table 3: Methods of recovery of various types of biosurfactants from different microbes.

Methods for biosurfactant recovery	Type of biosurfactant extracted	Microbial species
1. Batch recovery		
(a) Solvent extraction		
• Chloroform-methanol	Rhamnolipid bioemulsifier	*Pseudomonas*[A]
• Acetone	Emulsifier	*Pseudomonas* PG1[B]
• Ethyl acetate	Glycolipids	*T.bombicola*
• Hexane	Mycolates	*R. erythropolis*[C]
• Methyl tertiary butyl ether	Glycolipids	*Rhodococcus*[D]
(b) Crystallization	Liposan	*C.lipolytica*[E]
(c) Precipitation		
• Ammonium sulphate	Emulsan, Dispersan	*A.calcoaceticus*[F,G]
• Acid	Surfactin-like-biosurfactant	*B.subtilis*[H],*B.lichniformis*[I]
• Acetone	Emulsifying agent	*Pseudomonas spp.*[J]
2. Continuous recovery		
• Centrifugation	Emulsifying agent	*Pseudomonas sp.*[k]
• Foam separation	Surfactin	*B.subtilis* [L, M]
• Diafilteration/ precipitation	Glycolipid	*Rhodococcus sp.*[N]
• Adsorption	Rhamnolipid	*Pseudomonas*[O]
• Tangential flow filtration	Rhamnolipids	*Pseudomonas sp.*[P]

(A) Hisatsuka *et al.,* (1971), (B) Reddy *et al*., (1983). (C) Rapp *et al.,* (1979). (D) Kuyukina *et al.,* (2001) (E) Cirigliano & Carmn (1985). (F) Rosenberg *et al.,* (1979). (G) Rosenberg *et al*., (1988). (H) Arima *et al.,* (1968). (I) Javaheri et al, (1985). (J) Reddy *et al*., (1985) (K) Reddy *et al.*, (1983) (L) Cooper *et al.*, (1981). (M) Neu and Poralla (1990). (N) Desai & Desai (1993). (O) Cooper & Paddock (1984). (P) Mattei & Bertrand (1985).

Table 4: Applications of biosurfactants (Kosaric, 2001 and Muthusamy *et al,* 2008)

Applications	Role of biosurfactants
Environmental	
Bioremediation	Emulsification of hydrocarbons, lowering of interfacial tension, metal sequestration.
Soil remediation and flushing	Emulsification through adherence to hydrocarbons, dispersion, foaming agent, detergent, soil flushing.
Metals	
Concentration of ores	Wetting and foaming, collectors & frothers
Cutting and forming	Wetting emulsification, lubrication and corrosion inhibition in rolling oils, cutting oils, lubricants etc.
Casting	Mold release additives
Rust and scale removal	In picking and electrolyte cleaning
Plating	Wetting and foaming in electrolyte plating
Paper	
Pulp treatment	Deresinification, washing.
Paper machine	Defoaming, color leveling and dispersing.
Calender	Wetting and leveling, coating and coloring.
Paint and protective coatings	Dispersing and wetting of pigment during grinding
Pigment preparation	Emulsification, dispersion of pigment, stabilize latex, retard sedimentation and pigment separation, rheology
Latex paints	
Waxes and polishes	Emulsify waxes, stabilize emulsions, anti-stat
Petroleum production /products	
Enhanced oil recovery	Improving oil drainage into well bore, stimulating release of oil entrapped by capillaries, wetting of solid surfaces, lowering of interfacial tension, dissolving of oil.
De-emulsification	De-emulsification of oil emulsions, oil solubilization viscosity reduction, wetting agent.
Drilling fluids	Emulsify oil, disperse solids, and modify rheological properties of drilling fluids for oil and gas wells.
Worker of producing wells	Emulsify and disperse sludge and sediment in cleanout of wells

Producing wells	De-emulsify crude petroleum, inhibit corrosion of equipment
Secondary recovery	In flooding operations, preferential wetting
Refined products	Detergent sludge dispersant and corrosion inhibitor in fuel oils crank-case oils and turbine oils
Textiles	Detergent and emulsifier in raw wool scoring, dispersing in viscose
Preparation of fibers	rayon spin bath, lubricant and anti-stat in spinning of hydrophobic filaments
Dyeing and printing	Wetting, penetration, solubilization, emulsification, dye leveling, detergency and dispersion
Finishing of textiles	Wetting and emulsification in finishing formulation, softening, lubricating and anti-stat additive to finishes
Agriculture	
Biocontrol	Facilitation of biocontrol mechanisms of microbes such as parasitism, antibiosis, competition, induced systemic resistance and hypo-virulence.
Phosphate fertilizers	Prevent caking during storage
Spray application	Wetting, dispersing, suspending of powered pesticides and emulsification of pesticide solutions, promote wetting, spreading and penetration of toxicant
Building / construction	
Paving	Improve bond asphalt to gravel and sand
Concrete	Promote air entertainment
Elastomers / plastics	
Emulsion polymerization	Solubilization, emulsification of monomers
Foamed polymers	Introduction of air, control of cell size
Latex adhesive	Promote wetting, improve bond strength
Plastic articles	Antistatic agents
Plastic coating/laminating	Wetting agents
Food and beverages	
Emulsification and de-emulsification	Emulsifier, solubilizer, demulsifier, suspension, wetting, foaming, defoaming, thickener, lubricating agent.
Functional ingredient	Interaction with lipids, proteins and carbohydrates, protecting agent. For cleaning sanitizing
Food processing plants	Improve removal of pesticides and in wax coating
Fruits and vegetables	Solubilize flavor oils, control consistency, retard staling

Bakery and ice cream Crystallization of sugar	Improve washing, reduce processing time Prevent spattering due to super heat and water
Industrial cleaning Janitorial supplies De-scaling Soft goods	 Detergents and sanitizers Wetting agents and corrosion inhibitors in acid cleaning of boiler tubes and heat exchangers Detergents for laundry and dry cleaning
Leather Skins Tanning Hides Dyeing	 Detergent and emulsifier in degreasing Promote wetting and penetration Emulsifiers in fat liquoring Promote wetting and penetration
Biological Microbiological Pharmaceuticals and therapeutics	 Physiological behavior such as cell mobility, cell communication, nutrient accession, cell-cell competition, plant and animal pathogenesis. Antibacterial, antifungal, antiviral agents, adhesive agents, immuno modulatory molecules, vaccines, gene therapy
Bioprocessing Downstream processing	 Biocatalysis in aqueous two-phase systems and micro emulsions, biotransformations, recovery of intracellular products, enhanced production of extracellular enzymes, fermentation products.
Cosmetic Health and beauty products	 Emulsifiers, foaming agents, solubilizers, wetting agents, cleansers, antimicrobial agents, mediators of enzyme action.

2.2. ESTERASES

Esterases are a class of hydrolases, which catalyze the hydrolysis of ester bonds. Esterases and lipases are carboxylic ester hydrolases (EC 3.1.1) that generally have an essential serine residue at their site. The carboxylesterases (EC 3.1.1.1) hydrolyze water-soluble or emulsified esters with relatively short fatty acid chains, whereas lipases (triacyl glycerol acyl hydrolases; EC 3.1.1.3) preferentially attack emulsified substrates with long chain fatty acids. Carboxyl esterases are a group of enzymes widely distributed in nature. They catalyze the hydrolysis of carboxylic acid esters to the free acid anions and alcohol. Short chain esters are cleaved at the highest rate, the optimal length being 3-6 C atoms. Besides, aliphatic carboxyl esters, aromatic esters, and aromatic amides, as well as thioesters are also substrates of these enzymes. A further reaction catalyzed by many carboxyl esterases is the transfer of the ester acyl moiety to nucleophilic acceptors other than water (e.g. alcohol or amino acids). The general reaction being:

Carboxylic ester + H_2O = alcohol + carboxylic acid amine

Regarding the physical state of the substrate, carboxyl esterases act on esters, which are in true or micellar solutions, as well as on those, which are in emulsified form (e.g. tributyrin). This property introduces some problems into the differentiation between carboxyl esterases and lipases. It is obvious that due to the wide and overlapping specificity of carboxyl esterases, an unambiguous classification of these enzymes is hardly possible; therefore, esterases have not been differentiated according to their catalytic properties but mainly on the basis of their behavior towards certain inhibitors.

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids. Lipases occur widely in nature, but only microbial lipases are commercially significant. The many applications of lipases include specialty organic syntheses, hydrolysis of fats and oils, modification of fats, flavor enhancement in food processing, resolution of racemic mixtures, and chemical analyses.

2.2.1. Classification and Mechanism of action

(a) A-esterases (EC 3.1.1.2)

A-esterases are those enzymes, which are not inhibited by organophosphate and can even hydrolyze them. Since A-esterases preferentially cleave aromatic esters (e.g. phenyl acetate) they have been designated as aryl-esterases or aromatic esterases. The general reaction being:

Phenyl acetate + H_2O = phenol + alcohol

(b) B-esterases (EC 3.1.1.7)

In contrast to A-esterases, B-esterases are stoichiometrically inhibited by organophosphate through irreversible phosphorylation of the active site serine. Thus, B-esterases are non-specific esterases or serine hydrolyses. On the basis of their sensitivity towards physostigmine (10^5 mol/L), B-esterases can be further differentiated into choline esterases (EC 3.1.1.8).

Acetyl choline + H_2O = choline + carboxylic acid anion

(c) C-esterases

C-esterases or aliesterases do not hydrolyse DFP (diisopropyl fluoro phosphate) nor are they inhibited by DFP. In contrast to A-esterases, C-esterases are activated rather than inhibited by low concentrations of p-mercurobenzoate or of other agents, which combine selectively with sulfhydryl groups. Most of the fatty acid esterases, simple esterases and lipases, are included under C-esterases.

2.2.2. Physiology of esterase production and its purification

Esterases are enzymes, which are present universally, both in prokaryotes and eukaryotes. In prokaryotes, they are found in fungi as well as in anaerobic and aerobic bacteria, but the present review is restricted only to esterases of the aerobic bacteria.

In Cladoceran *Daphnia magna* exposure to two model organophosphorous and one carbamate pesticides including malathion, chlorpyrifos and carbofuran showed that carboxylesterase (CbE) was more sensitive to organophosphorous than acetylcholinesterase (AChE), whereas both CbE and AChE showed equivalent sensitivities to the carbamate carbofuran (Barata *et al* 2004), Recovery patterns of AChE and CbE activities were similar with 80-100%

recoveries taking place 12 and 96 hrs after exposure to organophosphorous and carbamates pesticides.

During the fermentation of sugars to ethanol, relatively high levels of an undesirable co product, ethyl acetate, are also produced. *Pseudomonas putida* NRRL B-18435 was found to be a source of this activity and a new esterase gene, *estZ* was cloned. Recombinant EstZ esterase was purified to near homogeneity and characterized. It belongs to family IV of lipolytic enzymes and contains the conserved catalytic triad of serine, aspartic acid, and histidine. The native and subunit molecular weights of the recombinant protein were 36,000 indicating that the enzyme exists as a monomer. By using α -naphthyl acetate as a model substrate, optimal activity was observed at pH 7.5 and 40°C. The *Km* and *V*max for α -naphthyl acetate were 18 μM and 48.1 μM min $^{-1}$ mg of protein^{-1}, respectively. Among the aliphatic esters tested, the highest activity was obtained with propyl acetate (96 μmol · min^{-1} · mg of protein^{-1}), followed by ethyl acetate (66 μmol · min^{-1} · mg of protein^{-1}). Expression of *estZ* in *E. coli* KO11 reduced the concentration of ethyl acetate in fermentation broth (4.8% ethanol) to less than 20 mg liter $^{-1}$ (Hasona *et al.,* 2002).

One such study was conducted by Barbaro *et al* in 2001 and the activities of isocitrate lyase, esterase and lipase by the In psychrotrophic *Acinetobacter* sp. strain HH1-1 there was an increased activity of a cell-associated esterase at low temperatures indicating its requirement by the cell at low temperature. Further they observed that in contrast, activities of extracellular lipolytic enzymes and production of an extracellular polysaccharide were negatively affected at the lower temperatures. Also during growth at 25°C, isocitrate lyase activity was detected in cell-free extracts, but at 5°C and after cold shock, activity was measured primarily in the cell culture supernatant. Therefore, it was concluded that the intracellular enzyme, isocitrate lyase, leaked out of the cell after cold shock and during growth at 5°C.

According to Breuil and Kushner (1975), *Acinetobacter* O_{16}, a psychrophilic species, produced extracellular lipase (measured by hydrolysis of olive oil, tributyrin, or β-naphthyl laurate) during the logarithmic phase of growth when grown on a complex medium (peptone plus yeast extract). Very little cell bound lipase was formed. These cells also produced an esterase (measured by hydrolysis of β-naphthyl acetate). At first, all esterase was cell bound; and significant amounts appeared in the external medium late in growth. Adding Tween 80 or ethanol to the normal complex medium, inhibited lipase production. Sodium acetate, oleic acid, olive oil, or Tween 20 added to the synthetic media did affect lipase production. The psychrophilic *Acinetobacter* grew more quickly at 30^0C than at 15 or 20^0 C but produced more lipase at the lower temperature. Esterase production was about the same at 20 and 30 ^{0}C. A mesophilic *Acinetobacter* species produced the same

amount of lipase and esterase at 20 and 30 ^{0}C. The best production of lipase by the psychrophilic *Acinetobacter* occurred in standing cultures. Donnelly and Crawford (1988) showed the production of an extra cellular esterase when the lignocellulose degrading actinomycete *Streptomyces viridosporus* T7A was grown in a mineral salts-yeast extract medium. The extracellular esterase activity was first detected during the late stationary phase and typically followed the appearance of intercellular activity; polyacrlyamide gel electrophoresis showed that several extracellular esterases differing in substrate specificity were produced.

It is believed that the mechanism of esterase excretion is growth dependent and that a higher cell growth of the host is in fact unfavorable for the enzyme production. An automated two component substrate feeding strategy with a pH-stat modal fad-batch culture was developed by Tulin *et al*. (1992). They used a high pH limit to effectively produce esterase from a hyper-protein excreting *Bacillus brevis* HPD31 which in turn harbors a plasmid pHSC131 which carries a *Bacillus stearothermophillus* esterase gene. A two component (polypeptone + glucose) feeding, using different feed rates, showed highest activity of the excreted esterases (34 U/ml) when concentrations of polypeptone and glucose in the nutrient feed solutions were 250 and 41.60 g/L respectively. Both the absence and excessive amount of glucose in the nutrient feed solutions was ineffective for the extracellular esterase formation because without glucose the increase in the cell concentration was minimum while excessive amount of glucose favored growth at the expense of esterase production. The activity of the excreted esterases was increased more then 8 times from 4 U/ml in the conventional batch culture to 34 U/ml obtained in the fed-batch culture.

Wiegel and Shao in 1995, purified two acetyl esterases (EC 3.1.1.6) to gel electrophoretic homogeneity from *Thermoanaerobacterium* sp. strain JW/SL-YS485, an anaerobic, thermophilic endospore former, which is able to utilize various substituted xylans for growth. Both enzymes released acetic acid from chemically acetylated larch xylan. Acetyl xylan esterases I and II had molecular masses of 195 and 106 kDa, respectively, with subunits of 32 kDa (esterase I) and 26 kDa (esterase II). The isoelectric points were 4.2 and 4.3, respectively. As determined by a 2-min assay with 4-methylumbelliferyl acetate as the substrate, the optimal activity of acetyl xylan esterases I and II occurred at pH 7.0 and 80^{0}C and at pH 7.5 and 84^{0}C, respectively. *Km* values of 0.45 and 0.52 mM 4-methylumbelliferyl acetate were observed for acetyl xylan esterases I and II, respectively. At pH 7.0, the temperatures for the 1-h half-lives for acetyl xylan esterases I and II were 75^{0}C and slightly above 100^{0}C, respectively. A novel cephalosporin esterase (EC 3.1.1.41) from *Rhodosporidium toruloides* was purified by Politino *et al.*(1997), to gel electrophoretic homogeneity. The enzyme was a glycoprotein with a molecular mass of 80 kDa. Upon deglycosylation, several forms of the enzyme were observed with a molecular mass range between

60 and 66 kDa. The isoelectric point of the enzyme was 5.6, with the pH optimum of 6.0. The optimal activity of the enzyme occurred at 25°C, with loss of enzyme activity above 25°C. The enzyme deacetylated a variety of cephalosporin derivatives, including cephalosporin C; the *Km* for this substrate was 51.8 mM, and the *V*max was 7.9 mmol/min/mg. In addition to cephalosporins, the enzyme hydrolyzed short-chain *p*-nitro phenyl esters, with the activity decreasing with increasing ester chain length.

Khalameyzer and his coworkers (1999) cloned a 3.2-kb DNA fragment from *Pseudomonas fluorescens* DSM 50106 in an lRESIII phage vector in *Escherichia coli* K-12 showing esterase activity. Esterase EstF1 was encoded by a 999-bp open reading frame (ORF) and exhibited significant amino acid sequence identity with members of the serine hydrolase family. The deduced amino acid sequences of two other C-terminal truncated ORFs exhibited homology to a cyclohexanone monooxygenase and an alkane hydroxylase. However, esterase activity was not induced by growth of *P. fluorescens* DSM 50106 in the presence of several cyclic ketones. The esterase gene was fused to a His tag and expressed in *E. coli*. The gene product was purified by zinc ion affinity chromatography and characterized. The enzyme was membrane bound or membrane associated with an optimum pH of 7.5, and optimum temperature of 43°C. The activity increased from γ-butyrolactone (18.1 U/mg) to ε-caprolactone (21.8 U/mg) to δ-valerolactone (36.5 U/mg). The activities towards the aliphatic esters were significantly lower; the only exception was the activity toward ethyl caprylate, which was the preferred substrate. Similarly, Hong *et al.* (1991) purified an esterase from an *E.coli* clone expressing esterase activity. It was purified by ion exchange and gel-filtration chromatography.

Mathis *et al.* (1998) developed a novel one step purification of esterase from *Bacillus pumilus* by immobilization on Sepharose 6B of pluronic L94, a block copolymer of polyethylene glycol and polypropylene glycol..The enzyme was eluted by stepwise elution with 3 and 0.2 M NaCl with a 140-fold purification of the esterase. An extracellular lipase has been purified to homogeneity from *Pseudomonas aeruginosa* Ef_2 by Gilbert et *al.* (1991). The enzyme was purified from the continuous culture growing on Tween 80, by means of ultra filtration of the culture supernatant followed by anion-exchange (Mono-Q) and gel filtration (Superose) FPLC. This lipase was composed of a single sub unit with Mw of approximately 29kDa and exhibited both lipase and esterase activity, though, lipase activity was eight times higher than esterase activity. The optimum pH and temperature of the purified enzyme for esterase was 9.0 and 50 ^{0}C respectively with p-nitro phenyl acetate and Tween 80, both acting as substrates for esterase activity.

2.2.3. Genetics of Esterases

Several bacterial esterase genes have been identified, cloned, expressed and characterized since they attracted increasingly attention in past few years.

(a) *Bacillus subtilis*

A genomic library of *Bacillus coagulans* strain 81-11 was screened in *Escherichia coli* JM83 for lipolytic activity by using tributyrin agar (TBA) plates by Mnisi *et al.,*(2005). A 2.4 kb DNA fragment was subcloned from a lipolytic positive clone and completely sequenced. Nucleotide sequence analysis predicted a 723 bp open reading frame (ORF), designated *estC1,* encoding a protein of 240 amino acids with an estimated molecular mass of 27,528 Da and a pI of 9.15. The deduced amino acid sequence of the *estC1* gene exhibited significant amino acid sequence identity with carboxylesterases from thermophilic *Geobacillus* sp. and sequence analysis showed that the protein contains the signature G-X-S-X-G included in most esterases and lipases. Enzyme assays using *p*-nitrophenyl (*p*-NP) esters with different acyl chain lengths as the substrate confirmed the esterase activity. *EstC1* exhibited a marked preference for esters of short-chain fatty acids, yielding the highest activity with *p*-NP butyrate. Maximum activity was found at pH 8 and 50^0C, although the enzyme displayed stability at temperatures up to 60^0C.

Diaz *et al* in 2001, isolated a clone producing halos on TBA plates from a genomic library of *Bacillus* sp. BP-7. The insert contained an open reading frame that coded for a protein of 487 amino acids with homology to carboxylesterases. The cloned enzyme showed clear preference for esters of short chain fatty acids. Maximum activity was found at 45^0C and pH 7.5. The enzyme displayed stability in the pH range from 6 to 9.5, and at temperatures from 4^0C to 45^0C. Zymogram analysis of the protein revealed a molecular mass of 53 kDa and a pI of 5.1. The enzyme showed homology to members of the bacterial subclass of type B carboxylesterases, a set of proteins potentially useful for biotechnological applications. A novel gene *lipB,* which encodes an extracellular lipolytic enzyme, was identified in the *Bacillus subtilis* genomic DNA sequence by Eggert and his group in 2000. The group cloned and overexpressed *lipB* in *Bacillus subtilis* and *Escherichia coli* and purified the enzyme from *Bacillus* culture supernatant to electrophoretic homogeneity. LipB hydrolyzed triacylglycerol-esters and p-nitrophenyl esters of fatty acids with short chain lengths of ≤ 10 carbon atoms. Triolein, which is a typical substrate for true lipases, was not hydrolysed suggesting that LipB was an esterase rather than a lipase. The catalytic triad of LipB consists of residues Ser78, Asp134, and His157 as shown by amino acid sequence alignments and site-directed mutagenesis.

Esterase from the thermophilic *Bacillus acidocaldarius* was a thermophilic and thermostable monomeric protein with a molecular mass of 34 KDa. The enzyme characterized as a "B-type" carboxylesterase displayed maximal activity at 65^0 C (D' Auria *et al.* 2000) The enzyme was also quite active at room temperature, an unusual feature for an enzyme isolated from a thermophilic microorganism. The effect of temperature on structural properties of the enzyme and its structural features compared with those of the esterase from hyperthermophilic archeon *Archaeoglobus fulgidus* showed that *Bacillus acidocaldarius* enzyme possesses a secondary structure rich in α-helices as described for esterase isolated from *Archaeoglobus fulgidus*. IR spectra indicated a higher accessibility of the solvent (^2H_2O) to *Bacillus acidocaldarius* esterase than *Archaeoglobus fulgidus* enzyme suggesting in turn, a less compact structure of the former enzyme. Moreover, comparison of *Bacillus acidocaldarius* esterase with the *Archaeoglobus fulgidus* enzyme fluorescence data indicated a higher flexibility of the former enzyme at all temperatures tested, supporting the infrared data and giving a possible explanation of its unusual relative high activity at low temperatures.

Bacillus pumilus gene encoding acetyl xylan esterase (axe) was identified, characterized and expressed and the recombinant enzyme produced in *Escherichia coli* was purified. The recombinant enzyme displayed similar properties to the acetyl xylan esterase (AXE) purified from *B. pumilus.* The AXE primary structure was 76% identical to the cephalosporin C deacetylase of *B. subtilis,* and 40% to two recently identified AXEs from *Thermoanaerobacterium* and *Thermotoga maritima.* These four proteins were of similar size and represent a new family of esterases having broad substrate specificity (Degrassi *et al.,* 2000). In 1975, Abbott and Fukuda partially purified an esterase from *Bacillus subtilis* culture by ammonium sulphate fractionation and ultra filtration. Esterase had a temperature optimum between 40 and 50^0 C and a pH optima of 7.0 with a molecular weight of 190,000. The enzyme was very stable and retained greater than 80% of its activity after storage in solution at 25^0C for 1 month. Because of kinetic properties and excellent stability, this enzyme was proposed to be useful in an immobilized form to prepare large quantities of deacetylated cephalosporin derivatives.

(b) Others

Diaz and his group (2005) identified a carboxylesterase producing *Rhodococcus erythropolis* A band (60 kDa) bearing activity on 4-methylbelliferone-butyrate was detected in *Rhodococcus* CR-53 cells extracts and was the first report on the analysis and visualization of a carboxylesterase from a *Rhodococcus* species.

Ewis *et al.,* (2004) screened the genomic libraries of *Geobacillus stearothermophilus* ATCC12980 and ATCC7954 for esterase/lipase activity and isolated two positive clones. Two genes Est30 and Est55, were identified encoding two different carboxylesterases and genetic rearrangement in the est55 locus. The est30 gene encodes a polypeptide of 248 amino acids, molecular mass of 28,338 Da, while the est55 gene encodes a polypeptide of 499 amino acids, molecular mass of 54,867 Da. Both enzymes were purified to homogeneity from recombinant strains of *Escherichia coli.* Enzyme characterization results showed that while both enzymes possess optimal activities with short chain acyl derivatives, Est55 has a broader pH tolerance (pH 8-9) and optimal temperature range (30-60^{0}C) than Est30. The activation energy of Est55 (35.7 kJ/mol) was found to be significantly lower than that of Est30 (101.9 kJ/mol). Both enzymes were stable at 60^{0}C for more than 2 h; at 70^{0}C, the half-life for thermal inactivation was 40 and 180 min for Est55 and Est30, respectively. With p-nitrophenyl caproate as the substrate and assay at 60^{0}C, Est55 had K(m) and k(cat) values of 0.5 µM and 39,758 s(-1) while Est30 exhibited values of 2.16 µM and 38 s(-1). Inhibition studies indicated that both Est30 and Est55 were strongly inhibited by phenylmethanesulfonyl fluoride, p-hydroxymercuribenzoate, and tosyl-l-phenylalanine, consistent with the proposed presence of Ser-His-Glu catalytic triad of the alpha/beta hydrolase family. The enzymatic properties of Est30 and Est55 revealed that these enzymes have potential applications in biotechnological industries.

Identification and characterization of a GDSL esterase gene located proximal to the *swr* quorum-sensing system of *Serratia liquefaciens* MG1 was coded (Kathrin *et al. 2*003). *S. liquefaciens* MG1 employs the *swr* quorum-sensing system to control various functions, including production of extracellular enzymes and swarming motility. The *estA* gene, located upstream of *swrR* and transcribed in the same direction was identified, which encodes an esterase that belongs to family II of lipolytic enzymes. EstA was heterologously expressed in *Escherichia coli.* The zymograms of EstA on polyacrylamide gels and analysis of a transcriptional fusion of the *estA* promoter to the promoterless *luxAB* genes showed that expression of the esterase is not regulated by the *swr* quorum-sensing system. An *estA* mutant was generated and was found to exhibit growth defects on minimal medium containing Tween 20 or Tween 80 as sole carbon source. The mutant was able to produce greatly reduced amounts of *N*-acyl-homoserine lactone (AHL) signal molecules on Tween-containing medium compared with the wild type, suggesting that under certain growth conditions EstA may be important for providing the cell with precursors required for AHL biosynthesis. In contrast Kim and his group (2003) reported the construction, expression, and one-step purification of a potential esterase in *Escherichia coli* with a hexa histidine tag at its N-terminus. The expression level of the enzyme was more than 20% of the total protein in *E.coli*, resulting in approximately 1.2 mg of the purified proteins by an affinity resin, Ni-NTA, from a 0.2 L of

bacterial culture in a single step. As typical properties, its innate traits that revealed favorable reactions at alkaline pH and high activity to the triglycerides composed of short chain fatty acids (<C6) supported the enzyme to be an esterase. The enzyme was determined to be a monomer with molecular mass of 42 kDa and showed high activity to rac-ketoprofen ethyl ester (27,000 U), with strict selectivity to (S)-enantiomer (>90% ee_p). The small-scale conversion using the recombinant enzyme strongly suggested the enzyme to be useful for enzyme mediated chiral resolution of (S)-ketoprofen.

Kim *et al.,* in 2003, cloned the esterase-encoding gene, estA (a 4.4 kb EcoR1), from *Acinetobacter Iwoffii* 16C-1 genomic DNA into *Escherichia coli* BL21 (DE3) with plasmid vector pET-22b (pEM1). A 2.4 kb *Ava*I-*Sph*I DNA fragment was subcloned (pEM3) and sequenced. *estA* gene encodes a protein of 366 amino acids (40,687 Da) with a pI of 9.17. The EstA signal peptide was 31 amino acids long, and the mature esterase sequence is 335 amino acids long (37.5 kDa). The conserved catalytic serine residue of EstA was in position 210. The EstA sequence was similar to that of the carboxylesterase from *Acinetobacter calcoaceticus* (75% identity, 85% similarity), *Archaeoglobus fulgidus* (37% identity, 59% similarity), and *Mycobacterium tuberculosis* (35% identity, 51% similarity). These enzymes contained the conserved motif G-X_1-S-X_2-G carrying the active-site serine of hydrolytic enzyme. The EstA activity in *A. Iwoffii* 16C-1 remains constant throughout the stationary phase, and the activity in *E. coli* BL21 (DE3) with pEM1 was similar to *A. Iwoffii* 16C-1. Purification of an extracellular lipase, LipA by growing *Acinetobacter* sp. RAG-1 on a carbon source like hexadecane was carried out by Colwell *et al*. 2002. The lipase was harvested from cells grown to stationary phase, and purified with 22% yield and >10-fold purification. It was bound to a butyl sepharose column and eluted in a triton-X-100 gradient. The molecular mass (33 kDa) was determined employing SDS/PAGE. LipA was found to be stable at pH 5.8-9.0 and 55^0–70^0C temperature. LipA was active against a wide range of fatty acid esters of p-nitrophenyl, but preferentially attacks medium length acyl chains (C_6, C_8). The enzyme demonstrated hydrolytic activity in emulsions of both medium and long chain triglycerides, as was shown by zymogram analysis.

The structural gene for heroin esterase was cloned from *Rhodococcus* sp. strain H1 and expressed in *E. coli* BL21 (DE3) by Rathbone *et al.,* (1997). The purified enzyme was found to be a tetramer with a Mr of 137,000 and an apparent K*m* of 0.88mM for 6-acetylmorphine. The G-x-S-x-G motif was observed in the deduced amino acid sequence, suggesting that the enzyme is a serine esterase. The gene coding for a novel esterase which stereoselectively hydrolyzes the (+)-trans (1R, 3R) stereoisomer of ethyl chrysanthemate was cloned from *Acinetobacter globiformis* SC-6-98-28 and over expressed in *Escherichia coli* in 1995, by Nishizawa *et al.* The cellular content of

the active enzyme reached 33% of the total soluble protein in the recombinant *E.coli* JM105 cells and 5.6 g/liter of culture by high-density cell cultivation. The hydrolytic activity of the recombinant *E.coli* cells for ethyl chrysanthemate reached 605 µmol of chrysanthemic acid/min/g of dry cells, which is approximately 2500 fold higher than that of *A.globiformis* cells. The stereo selective hydrolysis by the recombinant *E.coli* cells was efficient at substrate concentrations of up to 40% by removing the produced chrysanthemic acid by ultra filtration. The (+)-trans-chrysanthemic acid produced had 100% optical purity. The amino acid sequence of the esterase was found to be similar to that of several class C β-lactamases, D, D-carboxypeptidase, D-aminopeptidase, 6-aminohexanoate-dimer hydrolase, and *Pseudomonas* esterase. The group reported that the Ser-X-X-Lys motif in the esterase was at the active site of the enzyme.

A. calcoaceticus BD413 produces a cell-bound esterase specifically during transition between exponential growth and stationary phase. Kok *et al*. (1993) cloned an esterase gene (tributyrin as the sole carbon source) from the genomic library of *A. calcoaceticus* BD413 DNA in *E.coli*. Assays with model substrates classified the product of the cloned gene as an esterase. Deletion analysis, mapped the functional esterase gene on a 1.8 kb chromosomal DNA fragment. This fragment was sequenced and found to contain one ORF termed as *est* A which encoded a 40 kDa protein. But the deletion of *est* A only partially abolished cell bound esterase activity in *A. calcoaceticus* BD413 cells. The cloning of esterase genes from different microorganisms has made one thing clear that there exists a homology of protein sequences in the *cot* gene. The homology is not restricted to genes or class only, but it extends even in eukaryotes like mouse and man (Kok *et al*., 1993).

Hale *et al*. (1992) cloned an esterase gene from *Streptomyces scabies* FL1 in *Streptomyces lividans* on plasmids pIJ 486 and pIJ 702. The esterase gene from the same organism was cloned into pUC19 plasmid in *E.coli* but in *E.coli* the gene was expressed by using the *lac* promoter sequence of the plasmid. Earlier, in an effort to study the basis for zinc regulation of extracellular esterase production by *Streptomyces scabies*, Schottel *et al*. (1989) cloned the gene for esterase. The cloned esterase gene was used as a probe in northern hybridization to determine the effect of zinc on esterase gene transcription. Chung *et al.* (1991) cloned an esterase gene of *Pseudomonas fluorescens* S1KW1 by generating *Sau* 3A1 DNA fragments of *P. fluorescens* chromosomal DNA and finally inserting them into the *Bam*HI site of pUC19. Twenty clones with esterase activity on tributyrin agar plates were isolated. Only one clone out of the twenty had a lipase activity also. In 1991, Hong *et al*. cloned a carboxyl esterase gene (*est* B), which codes esterase II from *Pseudomonas fluorescens* into *E.coli* JM83. DNA sequencing found a single OFR of 654

nucleotides. The ORF was confirmed by terminal amino acid sequence analysis of the esterase protein.

Cloning and nucleotide sequence of an esterase gene from *Pseudomonas fluorescens* and expression of the gene in *Escherichia coli* was studied by Yoo and his associates (1990). DNA sequencing showed that the ORF is comprised of 708 nucleotides. The coding sequence of the gene was preceded by a potential Shine-Dalgarno sequence and by a promoter like structure. Following the stop codon a structure reminiscent of the *E.coli* rho-independent terminator was present. The enzyme expressed in an *E.coli* clone was mostly in the periplasmic space released to the outside of the cell by osmotic shock and purified to homogeneity by QAE-Sephadex A-50 and DEAE-Sepharose columns. The native form of the enzyme consisted of two identical subunits, each with a MW of 27,000. By studying the properties and substrate specificity, the enzyme was classified as an aryl esterase (EC 3.1.1.2).

In 1989, Reddy *et al.,* cloned a putative esterase gene (*est*) from *Acinetobacter calcoaceticus* RAG-1 into *Escherichia coli.* Esterase positive clones exhibited high levels of esterase activity even in intact cells. In addition, expression of *est* gene conferred on *E.coli* the ability to grow on simple triglycerides such as triacetin (TAC). The original esterase positive plasmid pRA17 carried a 2.2 kb insert from a partial *Mbo*I digest of RAG-1 DNA, which gave a single band with RAG-1 DNA following Southern hybridization. By sub-cloning and sequencing the *est* gene was found to contain a sequence of 870 bp which was responsible for the expression of esterase and could be translated to yield a protein of M_r 32500. In support of the sequencing results was the finding that when pRA17 was expressed in mini cells, a unique peptide of M_r 32500 was identified. This peptide was not found in the *est* gene. The fact that the production of active esterase depended on the orientation of the *est* gene within the vector suggested that transcription proceeded from the *tet* promoter in pBR322.

The ester hydrolyzing enzyme families, including lipase and esterase, mediated a broad range of reactions and thus, were able to act on a variety of ester compounds that are found naturally or exploited industrially. With the increasing demand for pharmacological use, attempts to produce an enantiomer (S)-ketoprofen from the corresponding ethyl ester have recently been proliferating, but information about the structure and function of related enzymes has not been reported to date in detail.

2.3. CORRELATION BETWEEN BIOSURFACTANT PRODUCTION AND ESTERASE ACTIVITY

Limited reports are available in which the esterase-biosurfactant complex has shown any effective emulsification of hydrophobic substrates.

Shabtai and Gutnick (1985) found an esterase activity both in the cell-free growth medium and on the cell surface of *A. calcoaceticus* RAG-1. They showed that enzyme, when it was cell free, released from the cell surface was either emulsan free or associated with the bioemulsifier. Because of this reason they proposed the role for cell bound esterase in the release of emulsan from the surface of *A. calcoaceticus* and in turn suggested that the release process of emulsan involves the cleavage of the ester bond involved in the association of emulsan with the cell surface. The esterase enzyme was partially purified from the cell bound emulsan of *A. calcoaceticus* RAG-1. The partially purified enzyme catalyzed the hydrolysis of acetyl and other acyl groups from triglycerides and alkyl esters. The emulsan itself acted as substrate for the enzyme.

In 2003, Gutnick *et al*, showed a correlation between the activity of a bioemulsifier and an esterase. The extracellular polyanionic, heteropolysaccharide bioemulsifier emulsan produced by oil-degrading microorganism *Acinetobacter venetianus* RAG-1 forms and stabilizes oil-water emulsions with a variety of hydrophobic substrates. Removal of the protein fraction yields a product, apoemulsan, which exhibits much lower emulsifying activity on hydrophobic substrates such as *n*-hexadecane. Gutnick *et al* (2003) showed that the key protein associated with the emulsan complex is a cell surface esterase. The esterase (molecular mass, 34.5 kDa) was cloned, over expressed in *Escherichia coli* BL21 (DE3) behind the phage T7 promoter with the His tag system and after over expression, about 80 to 90% of the protein was found in inclusion bodies. The over expressed esterase was recovered from the inclusion bodies by solubilization with deoxycholate and, after slow dialysis, was purified by metal chelation affinity chromatography. Mixtures containing apoemulsan and the recombinant esterase isolated from cell extracts formed stable oil-water emulsions with very hydrophobic substrates such as hexadecane under conditions in which emulsan itself was ineffective. Similarly, a series of esterase-defective mutants were generated by site-directed mutagenesis, cloned, and over expressed in *E. coli.* Mutant proteins defective in catalytic activity as well as others apparently affected in protein conformation were also active in enhancing the apoemulsan-mediated emulsifying activity.

OBJECTIVES

In the light of this review, the objective of this study is the characterization of biosurfactants from potential bacterial isolates, followed by cloning, expression and regulation of the gene(s) responsible for biosurfactant production and degradation of hydrophobic chemical compounds.

Materials & Methods

3. MATERIALS AND METHODS

3.1. Biological Materials

3.1.1. Bacteria

The bacterial isolate SK320 used in this study was isolated from endosulfan sprayed cashew plantation soil in Kerala, India and was characterized by morphological, physiological, biochemical properties (Table 1) to be *Bacillus* sp. 16s rDNA analysis identified the isolate as *Bacillus subtilis* by MTCC, Chandigarh (India). The bacterial culture was preserved at -80^0C in 50% glycerol throughout this study and was subsequently sub-cultured on luria agar plates. It was regularly maintained on nutrient rich luria broth. The purity of the culture was examined microscopically by gram staining.

3.1.2. Enzymes

Restriction enzymes and other molecular biology reagents were purchased from MBI fermentas and used according to the instructions of manufacturers. Ampicillin, X-Gal, and IPTG were procured from Sigma Chemicals Co.

3.2. Chromatographic material

The matrices, Q-Sepharose and Sephadex G-75, were supplied by Pharmacia (Uppsala, Sweden).

3.3. Chemicals

Chemicals of pure analytical grade were obtained from the following sources: Qualigens, Merck, Himedia, SD-fine, Ranbaxy, SISCO-SRL, Sigma Aldrich.

3.4. Preparation of media

The mediums used for growth were Luria Broth (LB) and Bushnell Hass Broth (BHB). Various carbon sources (triacetin, glucose, sucrose, succinate, glycerol, vegetable oil, mobile oil, olive oil, maltose, n-dodecane, crude oil, tween-40, tween-60, triton-X-100) were used for biosurfactant production. Solid media was prepared by adding 2% (w/v) agar into the liquid media.

3.5. Growth conditions

Bacillus subtilis SK320 was grown in BHB containing 0.5% olive oil (v/v) (Olio di Oliva, Sasso, imported by Nestle India Ltd. from Milano, Italy) as carbon source, at 37^0C and 120rpm and the actively growing culture was used for experimentation. Growth was measured at 600nm, spectrophotometrically.

3.6. Biosurfactant activity (Cooper and Goldenberg, 1987)

A 10 ml aliquot of the bacterial growth was centrifuged at 12,000 $\times$ *g* at 4^0C temperature for 30 min. Supernatant (5 ml) taken in a glass tube and 100μl of mobile oil was added to it. The contents in the tube were vortexed vigorously for one min at full speed and suspension was left undisturbed for 10min. Biosurfactant activity was measured by taking the absorbance at 550nm spectrophotometrically (U-2001, Hitachi) in glass cuvettes against blank of uninoculated medium (5ml) with 100μl oil vortexed similar to the sample. The partially purified biosurfactant was dissolved at a concentration of 1mg/100ml in distilled water and used for surface tension measurements against distilled water as control.

3.7. Surface tension measurement (Du-Nouy tensiometer)

The reduction in surface tension (dynes/cm) was measured by the ring method (Du-Nouy principle) using tensiometer (Petro-Diesel Instruments Company, Jencon, Calcutta, India). The partially purified biosurfactant from *Bacillus subtilis* SK320 and its clones was dissolved at a concentration of 1mg/100 ml i.e. CMC is 10mg/lit in distilled water and used

for surface tension measurements against distilled water as control. Surface tension was measured by dipping the platinum ring in the solution and measuring the force required to detach the ring from the surface of the liquid.

3.8. General Techniques

3.8.1. Gram staining (Christian Gram, 1884)

The morphology of the bacterium was studied using gram staining. Bacterial suspension or smear of 24hr old cells grown in BHB containing olive oil as sole carbon source was air-dried on a glass-slide and covered with crystal violet for 1min. After this 1-2 drops of iodine solution were poured over the bacterial smear and left for 1 min. The smear was then washed with MQ water followed by 2-3 washes with 70% alcohol. Few drops of safranin were then put on the smear and left for 1 min. The smear was washed again with MQ water and air-dried. The smear was then observed under microscope at 45X fold magnification.

3.8.2. Electrophoresis

DNA samples were analyzed by horizontal agarose gel electrophoresis while protein samples were fractionated by polyacrylamide gel electrophoresis.

(a) Agarose gel electrophoresis

Agarose gel electrophoresis was carried out with 0.8% agarose in TBE buffer (Appendix). Agarose was prepared in TBE buffer. After cooling to 50^0C, the agarose solution was poured in gel casting tray with comb. After the gel has completely polymerized, the comb was removed and the gel was placed in an electrophoresis tank containing TBE buffer. DNA sample, mixed with loading/tracking dye (Appendix), was loaded into the well of the submerged gel. The gel was run by applying constant current for the desired time and it was finally stained with ethidium bromide (Appendix) and viewed under UV light (Macrovue Uvis-20, Hoefer). DNA banding pattern pictures were taken on the gel-documentation system (Gel Doc Mega, Nucon).

(b) Polyacrylamide gel electrophoresis

Continuous polyacrylamide gel electrophoresis (PAGE) was carried out with 10% separating gel (Appendix) (Hoefer Electrophoresis Unit, SE600 series, Amersham Biosciences), as described by Laemmli (1970). Separating and stacking gels were prepared and poured into the previously washed and dried glass plates (Plates were washed with soap solution, MQ water followed by chloroform : methanol, 1:1, v/v). After the gel has completely polymerized, the comb was removed and the electrophoresis unit was set. Aliquots of protein samples mixed with loading or tracking dye were now applied to the wells. Prior to loading the protein samples were heated for 5 min, at 90^0C. The gel was run at a current of 25mA for stacking gel and 35mA for separating gel, at 100-110V for 4-5 hrs. Tris glycine (Appendix) was used as buffer for electrophoresis and bromophenol blue (Appendix) was used as the tracking dye. After electrophoretic separation, gel was either silver stained (Merrill, 1990) or activity stained (Liu *et al*, 1992).

3.8.3. Staining of PAGE gels

(a) Silver Staining (Meril, 1990)

Gels were washed for 5 min in Milli-Q (Millipore, Milli-Q Biocel, Molsheim, France) water (MQ) and then placed in a solution containing 5% (v/v) ethanol and 5% (v/v) acetic acid and left over night in this solution with continuous shaking (35 rpm). After 24 hrs, the gels were washed with MQ water for 5 min and then soaked in 10% (w/v) gluteraldehyde solution. After 30 min the unreacted gluteraldehyde was removed by four 30 min washes with MQ water. These gluteraldehyde treated gels were then stained by soaking in ammonical silver nitrate solution (Appendix) for 15 min. The gels were washed 2-3 times with MQ water for an hour after staining and then developed with a solution containing 0.1gm of citric acid and 1ml formaldehyde per liter of MQ water (Appendix). When the required intensity of the band was achieved (usually about 3-5 min), the gel was placed in a solution containing 5% acetic acid to stop further color development. After 15 min, the gels were stored in 30% methanol containing 3% glycerol. For drying, gels were kept

between two folds of cellophane and dried on a gel dryer (Hoefer, Slab Gel Dryer, Model GD2000, Amersham, Pharmacia Biotech).

(b) Activity staining for esterase (Liu *et al,* 1992)

For staining the proteins, which show esterase activity, native PAGE was carried out as described in section 3.8.2 (b). After electrophoretic separation, gels were washed with several changes of MQ water. Thereafter, the gels were soaked in 100ml 0.2M phosphate buffer (pH 6.4) containing 100mg α-naphthyl acetate (alpha-NA), 100mg β-naphthyl acetate (beta-NA) and 100mg Fast Blue RR salt (4-Benzoylamine-2, 5-dimethoxy benzene-diazonium chloride hemi [zinc chloride] salt) or Fast Blue B salt in 3 ml acetone. The native gel was incubated overnight or until the bands appear.

3.9. Preparation of homogenous cell free extract

A 48hr old bacterial culture grown on basal medium with 0.5% (v/v) olive oil was centrifuged at 10,000 X g for 30 min in a refrigerated high-speed centrifuge (Himac CR22G, Hitachi). Pellet was discarded and the supernatant designated as S_{10} was used for enzyme purification purpose. Esterase activity and protein content in the supernatant were measured spectrophotometrically at a wavelength of 405 and 310 nm, respectively.

3.10. Preparation of columns

3.10.1. Q-Sepharose Column

Q-Sepharose column (Pharmacia Biotech) was prepared according to manufacturers instructions. Q-Sepharose column (43 X 3 cm) was equilibrated with 10mM potassium phosphate buffer (pH 7.0) at a flow rate of 30 ml hr^{-1} in the cold room (4^0C). Bacterial supernatant (S_{10}, 30ml) was loaded onto the Q-Sepharose column and allowed to stand for 1 hour. After one hour, the fractions were collected with the help of fraction collector (Frac 100, Pharmacia Biotech) with 10mM potassium phosphate buffer (pH 7.0) at a flow rate of 30 ml hr^{-1}. Protein and esterase activity were determined spectrophotometrically at 310 and

405 nm, respectively. These fractions were used for kinetic studies. The bound proteins were eluted with a linear gradient (Gradient Mixer GM-1, Sweden, Pharmacia Biotech) of 1M NaCl in 10 mM phosphate buffer (pH 7.0) and 5 ml fractions were collected. After use, the matrix was recharged by washing it with 1M NaCl till all the proteins were removed. NaCl was removed by washing the matrix with 10mM potassium phosphate buffer (pH 7.0) and then equilibrating with the same.

3.10.2. Sephadex G-75 Column

Sephadex G-75 Column (Pharmacia Biotech) was also prepared according to manufacturers instructions. The beads were washed with 10mM potassium phosphate buffer (pH 7.0). The column was prepared by pouring the homogeneous slurry into a 72 X 2 cm glass column. The packed dextran column was then equilibrated with 10mM potassium phosphate buffer (pH 7.0) at a flow rate of 30 ml hr^{-1} in the cold room (4^0C). Fractions showing maximum esterase activity were pooled, concentrated by lyophilization and then dialyzed. The enzyme preparation (3 ml) was loaded onto the column and after 15-20 min the fractions of 5ml were collected at a flow rate of 30ml/h. Protein and esterase activities were estimated spectrophotometrically in all the fractions thus obtained. These fractions were used for kinetic studies. After use, the column was regenerated with equilibrating buffer.

3.11. Concentration

Fractions were pooled and concentrated using Amicon stirred ultra filtration assembly (Millipore) in the cold room (4^0C). The assembly was installed following the guidelines of the Millipore booklet. The filter membrane was washed twice with 0.1N NaOH (for 30 min) and placed inside the assembly with shiny/glossy side up. The pooled solution was then poured in the concentrator and it was attached to the nitrogen cylinder (pressure maintained at 20psi). The filter membrane after use was regenerated with 0.1N NaOH and stored in 10% ethanol till further use.

3.12. Dialysis

Dialysis tubing was prepared according to the method of Sambrook *et al* (1982). The tubing was cut into pieces of convenient length (8-10 cm) and boiled for 10 min in large volume of 2% sodium bicarbonate and 1mM EDTA. The diameter of the dialysis tubing was 3.9 or 4 cm. After washing thoroughly in distilled water, the tubings were boiled in distilled water for another 10 min. After cooling, the dialysis tubings were stored in 10% ethanol at 4^0C. Prior to use the tubings were washed thoroughly with distilled water from inside to remove all traces of ethanol. The protein solution (~5ml) was poured in the dialysis tube (8cm) and placed in 10mM potassium phosphate buffer (pH 7) at 4^0C. Buffer was changed after every 1, 4, 16 and 24hrs. The dialysis tube retained most proteins **≥** 12,000 Kda.

3.13. Enzyme (Esterase) activity

3.13.1. Qualitative plate test (Diaz *et al,* 2005)

Esterase activity was observed by direct plate test based on streaking the isolated strain on agar plates supplemented with 0.5% (v/v) olive oil. After 48 to 72 hrs incubation at 37^0C the appearance of hydrolysis haloes visible around the white glistening colonies indicated esterase activity.

3.13.2. Quantitative (Shabtai and Gutnick, 1985)

A sample of enzyme (0.1 ml of S_{10} supernatant fraction) was mixed with phosphate buffer (75mM, pH 7.0) containing 10mM $MgSO_4$ in a final volume of 2ml. The reaction was then started by addition of 100μl of 100mM pNp-acetate (in absolute ethanol). After 30 min of incubation at 30^0C the absorbance at 405 nm was recorded spectrophotometrically. Specific activity was expressed as μmoles/mg protein/min.

3.14. Partial purification of the biosurfactant

For partial purification, *Bacillus subtilis* SK320 was grown in BHB containing 0.5% olive oil (v/v) as carbon source. After 48hrs, when the biosurfactant activity was observed to be maximum in the supernatant, the culture was harvested at 7000X*g* for 30 min at 4^0C. Supernatant was filtered through Whatman No 42 filter paper and 3 volumes of chilled acetone was added to the supernatant and left at -20^0C. After 18-24 hrs the solution was centrifuged at 7000X*g* for 30 min at 4^0 C, air dried and then dissolved in water. This step was repeated 2-3 times. The final precipitate was dissolved in water and the sample was lyophilized (Heto LyoLab 3000 Lyophilizer, Germany). The lyophilized sample was then estimated for biosurfactant activity at 550nm (Section 3.7).

3.15. Chemical determination

3.15.1. Protein (Itzhaki and Gill, 1964)

Protein was determined by biuret method using a standard solution of bovine serum albumin (BSA Fraction V, Himedia). The total volume of protein sample was made up to 2 ml with MQ water and then 1 ml of biuret reagent (Appendix) was added. After 10 min incubation at room temperature, the absorbance was measured at 310nm against a reagent blank in a spectrophotometer. For the fractions eluted from column the protein content was analyzed directly at 280nm against the phosphate buffer as blank in a spectrophotometer.

3.15.2. Ash content

A known weight of lyophilized sample was taken in a pre-weighed glass crucible and kept in the oven for 1 hour at 80^0 C. After evaporating the excessive moisture the charred sample was transferred to the silica crucible and weighed. The sample was then kept in an electric muffle furnace at 550 ± 50^0C for 5 hrs. The cooled crucible was then weighed for residual ash.

3.15.3. Ammonia (Weatherburn, 1967)

The culture was centrifuged at 10000Xg for 30min at 4^0C and the supernatant so obtained was used for ammonia analysis. 5ml of Solution A (Appendix) was taken in a test tube and to it 20µl of the supernatant was added. The mixture was vortexed vigorously and then 5 ml of Solution B (Appendix) was added to it. Samples were then incubated at room temperature for 20 min for color development and absorbance was read at 625nm in a spectrophotometer against a reagent blank.

3.15.4. Inorganic phosphate (Fiske and Row, 1925)

Samples containing inorganic phosphate were diluted to 2ml with distilled water and 0.5ml of acid molybdate (2.5% w/v ammonium molybdate in 2.5 M H_2SO_4) was added followed by addition of 0.1ml of color reagent (Appendix). Color was allowed to develop for 20 min and then absorbance was read at 750nm, spectrophotometrically.

3.15.5. Deoxyribonucleic acid

The quantity of deoxyribonucleic acid (DNA) in the sample was calculated by reading the sample at 260nm and 280nm, against TE, pH 8.0 (Appendix) blank. An optical density of 1 at 260nm corresponds to approx. 50 µg/ml of DNA.

3.15.6. Carbohydrate (Plummer, 1988)

Carbohydrate was measured by anthrone method using glucose as standard. Total volume was made to 2ml with sample and distilled water and then 2ml of anthrone reagent (Appendix) was added. The mouth of the test tubes were covered with glass marbles and kept in boiling water bath. After 10 min, the test tubes were cooled down by incubation on ice for 5 min to stop the reaction and the absorbance was read at 620nm against a reagent blank.

3.15.7. Lipid

Dried partially purified sample was extracted with chloroform: methanol solution (2:1, v/v). The suspension was allowed to stand at room temperature for 5-10min and then centrifuged and supernatant collected. An aliquot (0.2 volume) of distilled water was added to the sample so as to remove any traces of chloroform: methanol, if present. The sample was then vortexed. After giving 2-3 washings with MQ water, the lower layer containing the lipid was transferred to a fresh tube and the sample was lyophilized and weighed to get the total lipid content.

3.16. Isolation of DNA

3.16.1. Chromosomal DNA (Rose *et al.,* 1994)

Bacillus subtilis SK320 was grown in basal medium with 0.5% olive oil (v/v) for 24hrs at 37^0C. Cells (2ml) were harvested at mid-log phase and pellet was washed with 100 µl TE buffer (Appendix). The pellet was suspended in 200 µl of Rose solution (Appendix) and incubated at 90^0C in a preheated water bath. After 20 min, the sample was kept on ice for 5 min. Chloroform : Iso-amyl alcohol (21:1, v/v) was added to the sample and after 30 min at room temperature the sample was centrifuged at 10,000 X g for 10min. The upper layer (approx~200µl) was collected carefully in a fresh eppendorf tube and the supernatant was then treated with RNase (10mg/ml) for 30 min. The sample was then precipitated with chilled 2.5 volume of ethanol (previously kept at -20^0C) and left at -20^0C. After 1 hour incubation the sample was centrifuged for 30 min and the pellet containing the DNA was re-suspended in 50µl TE buffer, pH 8.

3.16.2. Plasmid DNA (Birnboim and Doly, 1979)

Plasmid DNA was isolated using alkaline lysis method. An overnight grown culture (2 ml) on luria broth (LB) containing 100 µg/ml ampicillin was harvested at 7500Xg for 10 min and the pellet was resuspended in 100µl ice-cold Sol-I (Appendix) containing 5mg/ml lysozyme. After 10 min incubation at room temperature, 200µl of Sol-II (Appendix) was

added and the sample was inverted several times to mix the solution. After 15 min incubation on ice, 150µl of chilled Sol-III (Appendix) was added to the sample. After 10 min of incubation on ice the sample was centrifuged at 12,000Xg for 10 min at 4^0C. Supernatant so obtained was transferred into another tube and 600 µl of iso-propanol was added. After 20 min incubation at room temperature, DNA was recovered by centrifugation at 12,000Xg for 15 min. The DNA pellet was dissolved in 100µl TE, pH 8 and stored in refrigerator for further use.

3.17. Cloning of the biosurfactant gene(s)

3.17.1. DNA amplification

Chromosomal DNA of *Bacillus subtilis* SK320 was amplified by polymerase chain reaction using the gene specific primers of lipopeptide biosurfactant, surfactin. The primers were designed using the following website:
http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
The primers were prepared by Operon Biotechnologies, Nattermannallee, Germany (www.operon.com) and received by Genetix Biotec Asia Private Limited, New Delhi, India and supplied to us. The DNA region encoding the four genes viz. *sfp*, *sfp0*, *srfA* and *srfB*, which were required for production of surfactin in *Bacillus subtilis,* were PCR amplified using the following primers:

Gene ID	Sequence 5'- 3'
sfp^0	5'-CTAGAATTCAGATTTACGGAATTTATATG-3' 5'-GGGGAATTCAGGGTGTGCGGCGCATAC--3'
srfA	5'-TCCGTTTTTCCTTGTTCACC-3' 5'-TCTTTCTGCCACTGCATCAC-3'
srfB	5'-GAGGACACTCCCGAAAATCA-3' 5'-TGTACGGCTTTCCGTATTCC-3'
sfp	5'-CGTTCGCTCAGTCATAAGCA-3' 5'-CCTGTATGCACACCCATCTG-3'

The amplification was performed on GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA), using program set to:

Cycle number	Denaturation	Annealing	Extension
30 cycles	5 min at 94^0C 1 min at 94^0C	1 min at 45^0C	1 min at 72^0C
Last cycle	5 min at 94^0C 1 min at 94^0C	1 min at 45^0C	10 min at 72^0C

3.17.2. Ligation

The PCR amplified product of the chromosomal DNA was cloned into the pGEM-T easy vector system (Promega Corporation, Madison, USA) and the ligation reaction was set up at 4^0C according to the instructions provided with pGEM-T easy vector system kit.
The reaction mixture was prepared as follows:

Reagents	Standard reaction	Positive Ctrl	Background Ctrl
2X rapid ligation buffer	5µl	5µl	5µl
pGEM-T vector	1µl	1µl	1µl
PCR product	3µl	---	---
Control Insert DNA (542bp fragment from pGEM-luc vector DNA)	---	2µl	---
T4 DNA ligase	1µl	1µl	1µl
Final volume	10µl	10µl	10µl
The reaction was set and incubated at 4^0C, overnight and was used for transformation			

3.17.3. Transformation (Avery *et al,* 1944)

Transformation was carried out by the $CaCl_2$ method. A single colony of *E.coli* was picked from a freshly grown plate and was transferred into 20 ml of LB broth in a 250 ml flask. The culture was incubated at 37^0C with vigorous shaking (200 cycles/min) in a rotary shaker. After 20 hrs, 200µl (1%) of above saturated culture was transferred aseptically into 20 ml of fresh LB broth in a 250 ml flask. The culture was incubated with vigorous shaking at 37^0C for 2-3 hrs till the culture showed an optical density or absorbance of 0.5 at 550nm. The culture was kept on ice and then centrifuged at 5000 X g for 10 min at 4^0C. Supernatant was discarded and the pellet was then resuspended in 10 ml of ice-cold 0.1M $CaCl_2$ and the tube stored on ice. After 15 min the cells were centrifuged at 5000 X g for 10 min at 4^0C and the pellet was finally resuspended in 1ml of ice-cold 0.1 M $CaCl_2$ and kept on ice for 3 hrs. Aliquot (100µl) of the competent cells were transferred to a sterile prechilled eppendorf tube (1.5ml capacity). Plasmid DNA sample (~100ng in a volume of 5µl) was added to the competent cells in the chilled tube and stored on ice for 30 min. The competent cells were then given a heat-shock at 42^0C for 2 min without shaking. The tubes were rapidly transferred to an ice-bath and the cells were chilled for 1-2 min to stop the reaction. To the tubes, 2 ml of luria broth was added. The culture was then incubated at 37^0C for 60 min to allow the cells/bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. Out of the cells thus transformed, 100µl were taken and plated on each LB antibiotic plate and incubated at 37^0C for 16-24 hrs or until the transformed colonies appeared. Using the blue-white screening method the transformants were further selected on basal media plates amended with olive oil (0.5%, v/v), X-Gal, IPTG and ampicillin. Three positive colonies or clones were chosen for further work and designated as clones *E.coli* pSKP, pSKP0 and pSKA.

3.18. Expression of the biosurfactant gene(s)

The positive transformants obtained were tested for their biosurfactant and esterase activities respectively. *Bacillus subtilis* SK320, *E.coli* DH5α cells (used for transformation), clones *E.coli* pSKP, pSKP0 and pSKA were grown on nutrient agar and basal medium containing 0.5% (v/v) olive oil with and without the addition of ampicillin in the medium at

37^0C for 120 hrs. Growth, biosurfactant (Section 3.7) and esterase activities (Section 3.13.2) were estimated in the culture supernatants at an interval of 24hrs respectively.

3.19. Sequencing of the biosurfactant gene(s)

The biosurfactant genes were sequenced by Bangalore Genei Pvt. Ltd, India (www.bangloregenei.com). These gene sequences were submitted to National Center for Biotechnology Information (USA). The above sequences were also used to analyze inter functional and evolutionary relationships between sequences in the database and to identify members of gene families using National Center for Biotechnology Information's BLAST facility (http://www.ncbi.nlm.nih.gov/BLAST).

Results

4. RESULTS

4.1 Isolation and identification of biosurfactant producing bacteria

Soil samples from endosulfan sprayed cashew plantation fields in Kerala, India were used for the isolation of biosurfactant producing bacteria. Among the various colonies obtained, four isolates i.e. SK96, SK97, SK98 and SK320 were selected for further study. The isolates were grown on various carbon sources and showed maximum growth and biosurfactant activity on olive oil (0.5%, v/v). Among the four isolates, SK320 showed maximum growth when grown with olive oil (0.5%, v/v) as substrate, followed by SK96, SK97 and SK98, respectively (Fig 1). The isolates were screened and tested for their respective biosurfactant activities. Among the various isolates, SK320 showed the highest biosurfactant activity, so it was used for further study (Fig 2).

Isolate SK320 was identified on the basis of morphological, physiological and biochemical characteristics as described below: -

Microscopic examination revealed that the cells of isolate SK320 were gram positive and were either short rods or cocci (Image 1). The irregular, wavy, convex, rough, opaque colonies of this isolate were able to produce oval shaped endospores. The isolate was capable of growing on temperatures ranging from 15^0 to 45^0 C, but the optimum temperature for growth was 37^0C. This motile organism had the ability to grow in a wide range of pH from 5.0 to 11.0. Isolate SK320 also grew on 2.5, 5, 7, 8.5, 10 % NaCl and showed good growth. The isolate did not grow under anaerobic conditions. On the basis of morphological, physiological and biochemical (Table 1, 2, 3) characteristics, the isolate was identified as *Bacillus* sp. Molecular identification of isolate SK320, by analysis of the sequence similarity based on 16S rDNA sequence alignment and database comparison identified that the isolate belongs to *Bacillus* group, with the highest identity (99.0%) to *Bacillus subtilis* in the BLAST hits on query sequence. Therefore isolate SK320 would be referred to as *Bacillus subtilis* SK320.

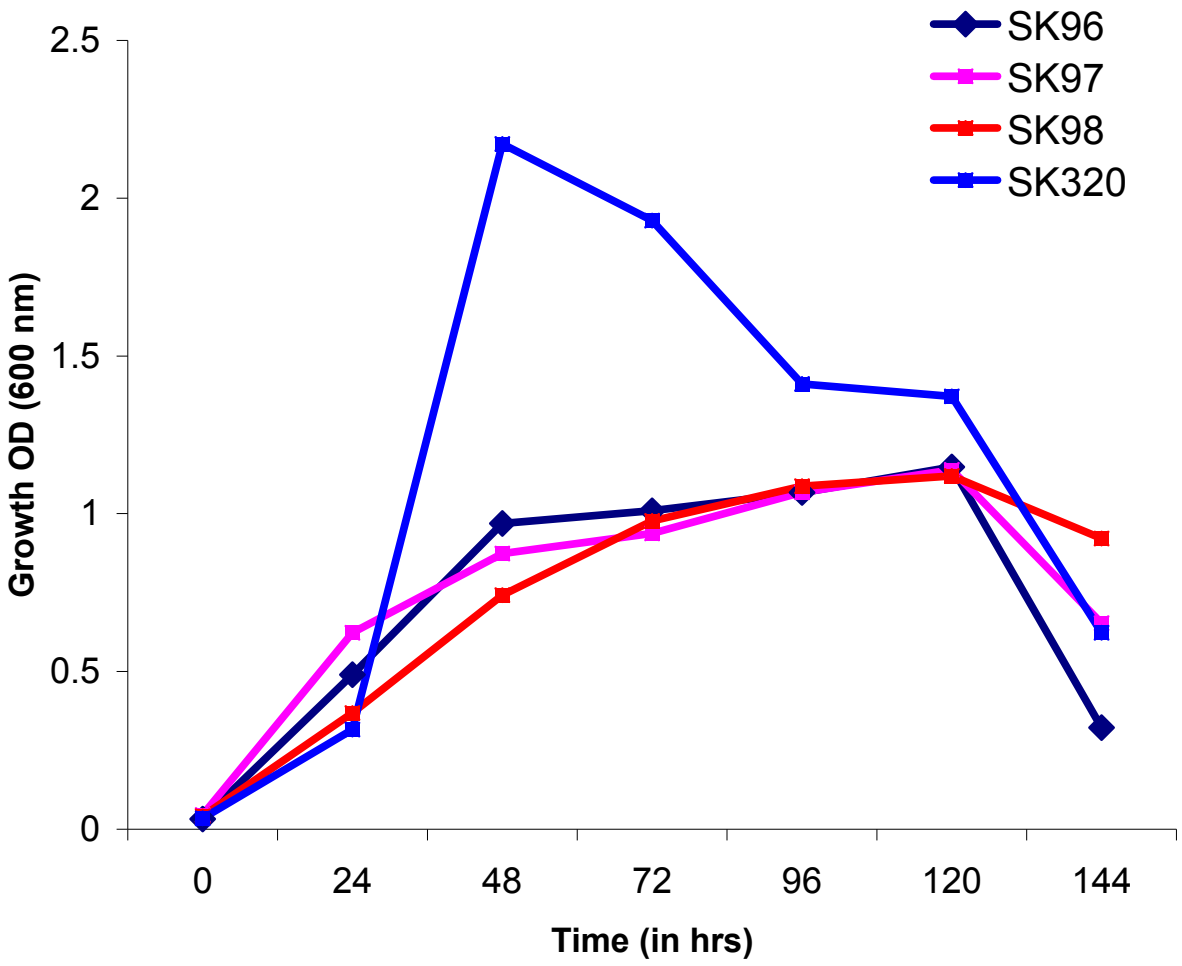


Fig 1: Growth of various isolates.

Isolate SK96, SK97, SK98 and SK320 were grown on basal medium with 0.5% (v/v) olive oil as sole carbon source and growth was measured at 600 nm (section 3.5).

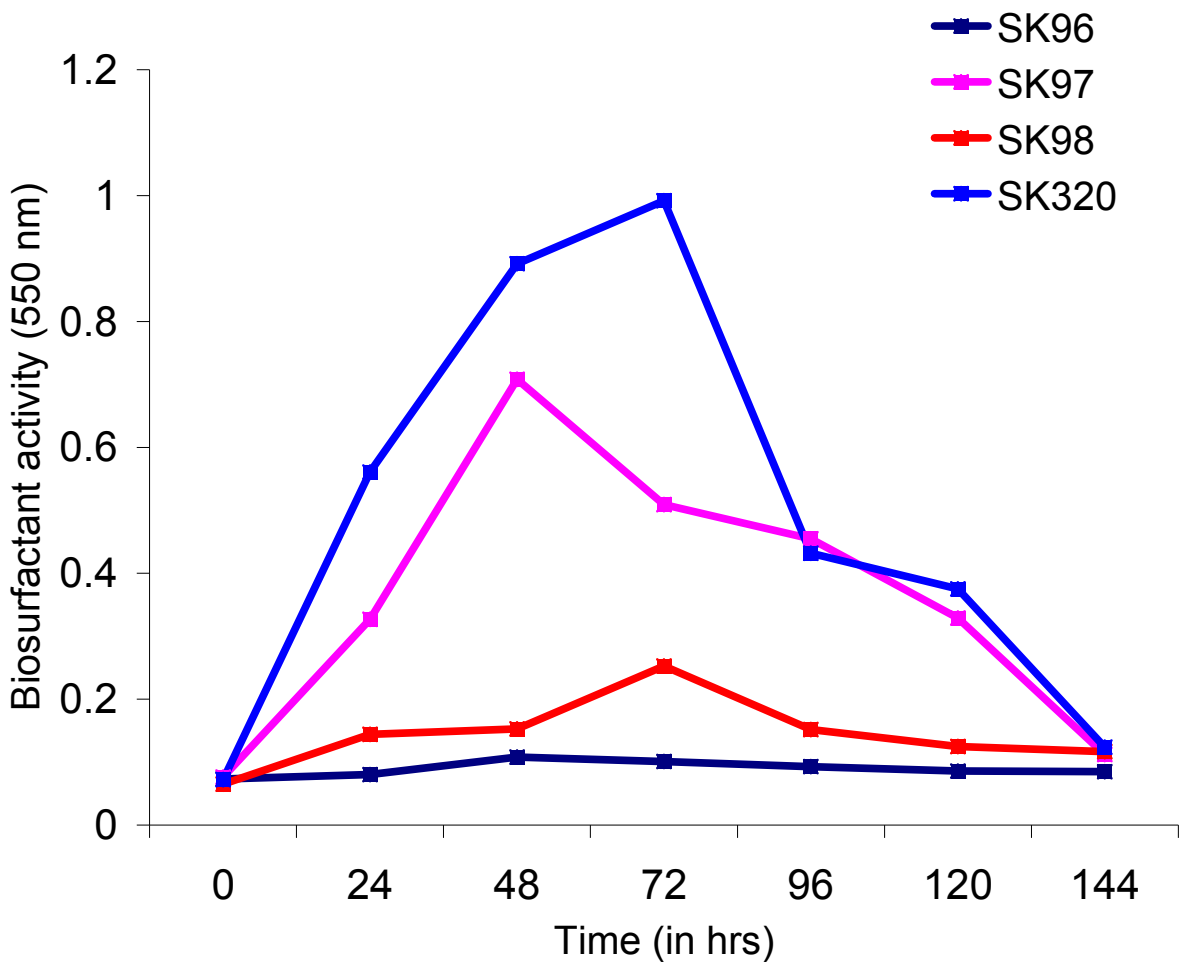


Fig 2: Biosurfactant activity of various isolates.

Isolate SK96, SK97, SK98 and SK320 were grown on basal medium with 0.5% (v/v) olive oil as sole carbon source and the biosurfactant activity was analyzed at 550 nm (section 3.7).

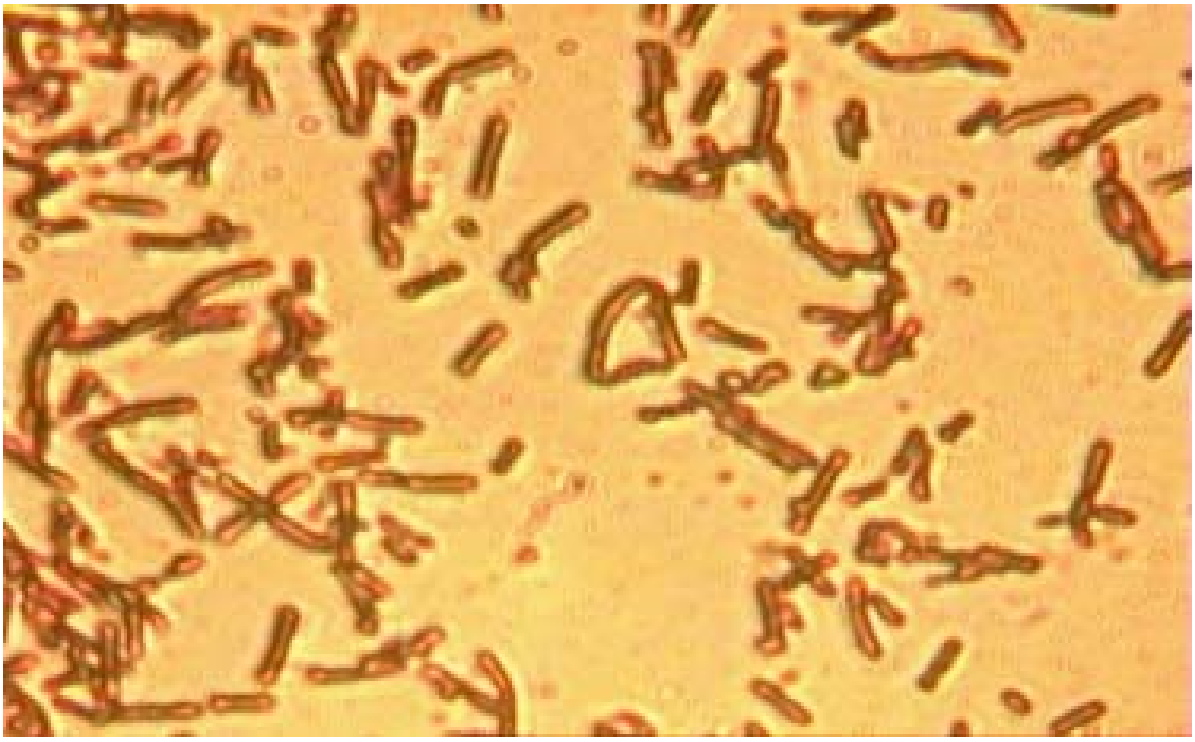


Image 1: Gram staining of *Bacillus subtilis* SK320.

Table 1: Morphological tests for identification of isolate SK320.

Morphological tests	
Colony morphology	
Configuration	Irregular
Margin	Wavy
Elevations	Convex
Surface	Rough
Density	Opaque
Pigments	-
Gram's reaction	+
Cell shape	Rods
Size	Moderate
Arrangement	Single
Spore(s)	
Endospore	+
Position	Central, sub-terminal
Shape	Oval
Sporangia bulging	+
Motility	+
Fluorescence (UV)	-

Table 2: Physiological tests for identification of isolate SK320.

Physiological tests	
Growth at temperatures	
4^0 C	-
10^0 C	-
15^0 C	+
25^0 C	+
30^0 C	+
37^0 C	+
42^0 C	+
45^0 C	+
55^0 C	-
65^0 C	-
Growth at pH	
pH 5.0	+
pH 5.7	+
pH 6.8	+
pH 8.0	+
pH 9.0	+
pH 11.0	+
Growth on NaCl (%)	
2.5	+
5.0	+
7.0	+
8.5	+
10.0	+
Growth under anaerobic condition	-

Table 3: Biochemical tests for identification of isolate SK320.

Biochemical tests	
Growth on Mac Conkey agar	-
Indole and Methly red test	-
Voges Proskauer test	+
Citrate utilization	+
Casein hydrolysis	-
Starch hydrolysis	+
Gelatin hydrolysis	+
Urea hydrolysis and H_2S production	-
Nitrate and Nitrite reduction	-
Cytochrome oxidase	Weak reaction
Catalase test	+
Oxidation/Fermentation/Negative (O/F/-)	-
Acid production from carbohydrates	
Adonitol	-
Arabinose, Galactose, Xylose	±
Cellobiose	+
Glucose/Dextrose	+
Dulcitol	-
Fructose	+
Inositol	-
Inulin	-
Lactose	-
Maltose	-
Mannitol	+
Mannose	+
Melibiose	-
Raffinose	-
Rhamnose	-
Salicin	+
Sorbitol	-
Sucrose	+
Trehalose	+

4.2 Biosurfactant activity during growth of *Bacillus subtilis* SK320

(a) Regulation of biosurfactant activity with various carbon sources

Bacillus subtilis SK320 was grown on basal medium viz. bushnell hass broth (BHB) containing various carbon sources 0.5% (v/v) and monitored for biosurfactant activity. The various carbon sources used were vegetable oil, glycerol, maltose, n-dodecane, mobile oil, crude oil, olive oil, glucose and sucrose. The bacterium was also grown on tween 40, tween 60 and triton X-100 but as these three carbon sources are also effective synthetic surfactants therefore there was formation of emulsion in the medium at 37^0C with the increase in the incubation period. Among the various carbon sources tested for biosurfactant production by *Bacillus subtilis* SK320 in basal medium olive oil, glucose, glycerol and sucrose, gave maximum activity in the range of 1.8 to 1.1 while low levels of biosurfactant activity in the range of 0.098 to 0.68 was detected when grown in the presence of maltose, vegetable oil, mobile oil, n-dodecane, crude oil (Table 4). No biosurfactant activity was observed when *Bacillus subtilis* SK320 was grown in presence of substrates like succinate, tween-40, tween-60, triacetin and triton-X-100. *Bacillus subtilis* SK320 showed maximum biosurfactant activity of 1.8 when the basal medium was supplemented with olive oil 0.5% (v/v) (Fig 3). Olive oil being the best inducer among all the carbon sources was selected for further studies. The biosurfactant activity in the supernatant containing olive oil as substrate increased with time and maximum activity was achieved between 48 to 72 hrs of growth when the bacterium was in its stationary phase (Fig 4). Furthermore, there was a sharp decline (about 50-60%) in the biosurfactant activity during late stationary phase i.e. around 96hr and then it stabilized. The bacterium grew luxuriantly in basal medium and was also capable of emulsifying olive oil to a greater extent. It was ten times of that induced in any of the other three isolates by olive oil in basal medium. The low level of biosurfactant activity at 120 hrs coincided with the complete emulsification of olive oil in the basal medium, visualized as milky appearing growth medium.

The extracellular protein analyzed was maximum when olive oil was used as the substrate with a reading of 6.97 micrograms/ml followed by vegetable oil, glycerol and glucose. The protein content was found to be minimum with mobile oil and crude oil with values as low as 0.18 and 0.23 micrograms/ml (Table 4).

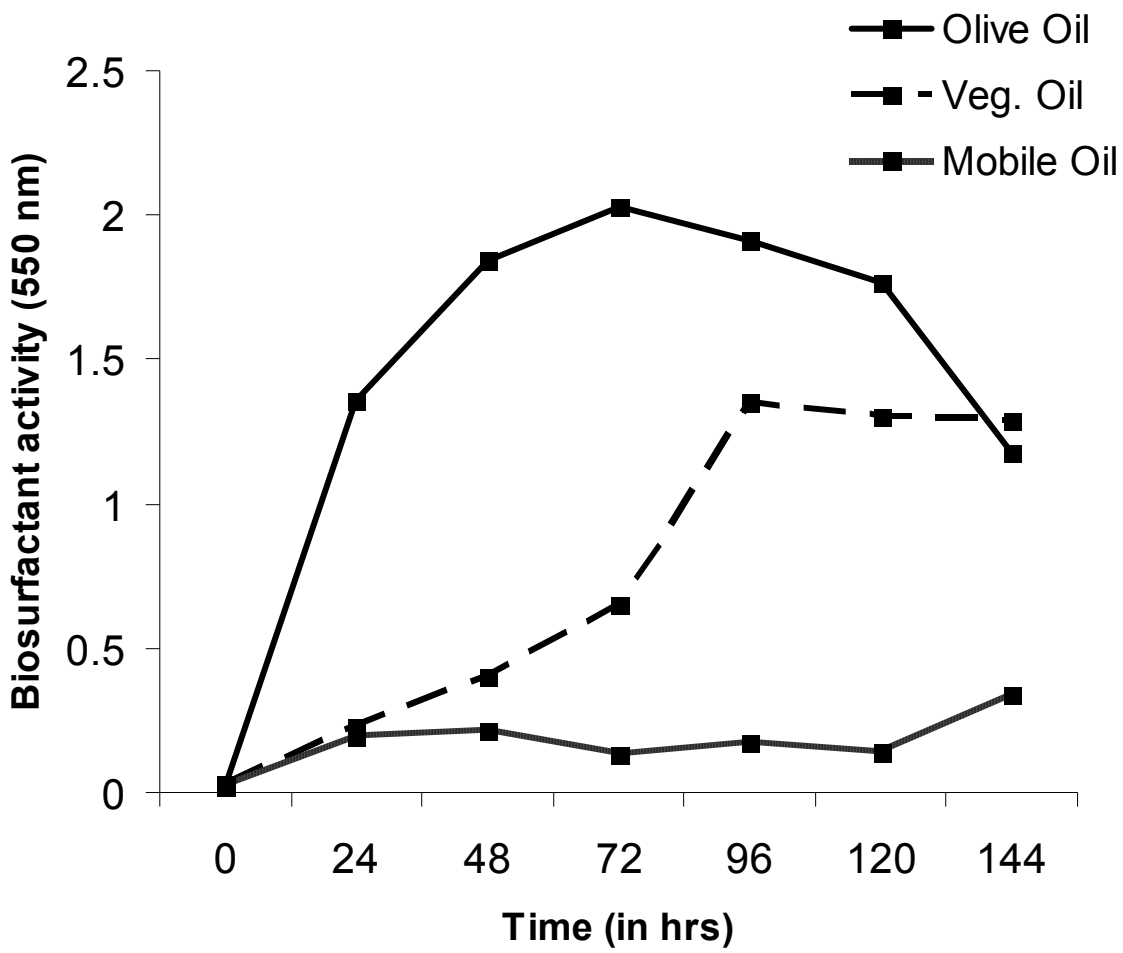


Fig 3: Biosurfactant activity of *Bacillus subtilis* SK320.

***Bacillus subtilis* SK320 was grown on basal medium with various substrates as sole carbon source and biosurfactant activity was analyzed at 550 nm (section 3.7).**

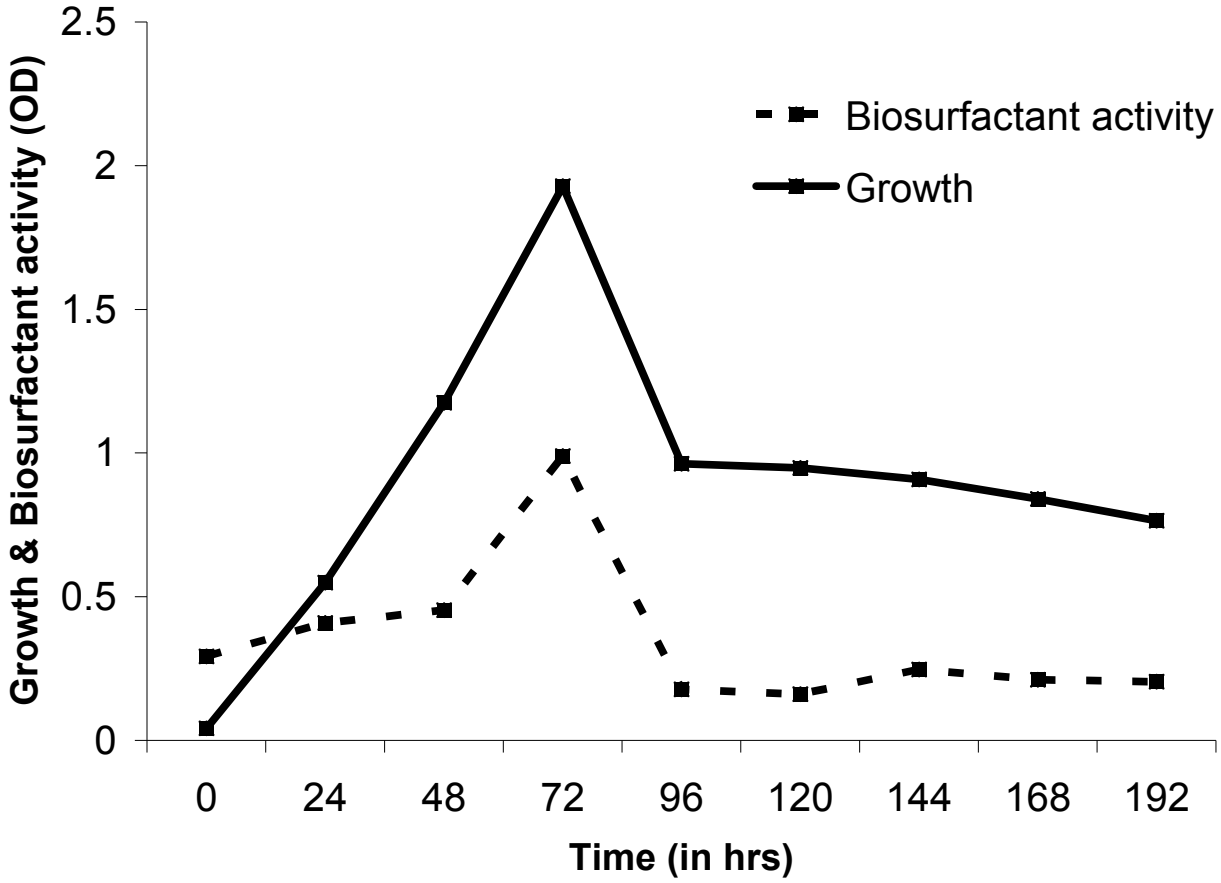


Fig 4: Growth and biosurfactant activity of *Bacillus subtilis* SK320.

***Bacillus subtilis* SK320 was grown on basal medium with 0.5% (v/v) olive oil as sole carbon source. Growth and biosurfactant activity were analyzed at 600nm and 550nm, respectively (sections 3.5 & 3.7).**

Table 4: Biosurfactant activity and extracellular protein of *Bacillus subtilis* SK320 on various substrates after 72 hours.
Biosurfactant activity was analyzed at 550 nm (Section 3.7) and protein was assayed at 310 nm by the biuret method (Section 3.15.1), spectrophotometrically.

Substrate	Biosurfactant activity* (OD 550nm)	Extracellular Protein* (micrograms/ml)
Vegetable oil	0.648	2.66
Glycerol	1.185	2.37
Maltose	0.074	1.23
n-dodecane	0.099	0.32
Mobile oil	0.135	0.18
Crude oil	0.094	0.23
Olive oil	1.841	6.97
Glucose	1.137	2.37
Sucrose	1.321	2.29

(b) Regulation of biosurfactant activity with various nitrogen sources (Ammonia utilization and inorganic phosphate depletion)

Bacillus subtilis SK320 was grown on four different nitrogen sources (0.5%, w/v) i.e. ammonium nitrate (NH_4NO_3), ammonium sulphate ($(NH_4)_2SO_4$), ammonium chloride (NH_4Cl) and sodium nitrate ($NaNO_3$), with five different carbon sources (0.5%, v/v) i.e. sucrose, olive oil, vegetable oil, glucose and glycerol. The isolate grew equally well on all the nitrogen sources, but ammonium nitrate as nitrogen and olive oil as carbon source was found to be most suitable combination (Fig 5), as it supported maximum biosurfactant activity (OD 2.198) given by the isolate, followed by ammonium chloride (OD 0.656) and sodium nitrate (OD 0.643). Olive oil induced maximum biosurfactant activity followed by vegetable oil and sucrose. Esterase activity was also found to be maximum (0.682 µmoles/min) with ammonium nitrate as nitrogen and olive oil as carbon source.

Bacillus subtilis SK320 completely utilized the available ammonia (17.78 mg/ml) in the media by the end of 144 hrs (4.65 mg/ml) of incubation at 37^0C, with ammonium nitrate as nitrogen and olive oil as carbon source (Fig 6). Inorganic phosphate depletion was also observed in the media and the data showed that phosphate was depleted from 28.17 to 7.89 mg/ml, by the bacterial isolate (Fig 7). The analysis reveled that the carbon, nitrogen and phosphorus source supplemented in the media does play some significant role in the physiology and biosynthetic pathway of biosurfactant production.

4.3 Partial purification of biosurfactant

4.3.1 Biochemical characterization

The biosurfactant released by the cells in the supernatant was partially purified as described in section 3.14 and was lyophilized. Biochemical analysis revealed that the partially purified biosurfactant had high lipid (89.4%) content and an ash content of 14.5 %. The protein content was around 7.45% with a carbohydrate content of only 3.15% (Table 5). Owing to the high lipid content of the biosurfactant, we concluded that the biosurfactant belongs to the class of lipopeptides. This also confirms our results as the *Bacillus subtilis* and other *Bacillus* species are known to produce lipopeptide biosurfactants.

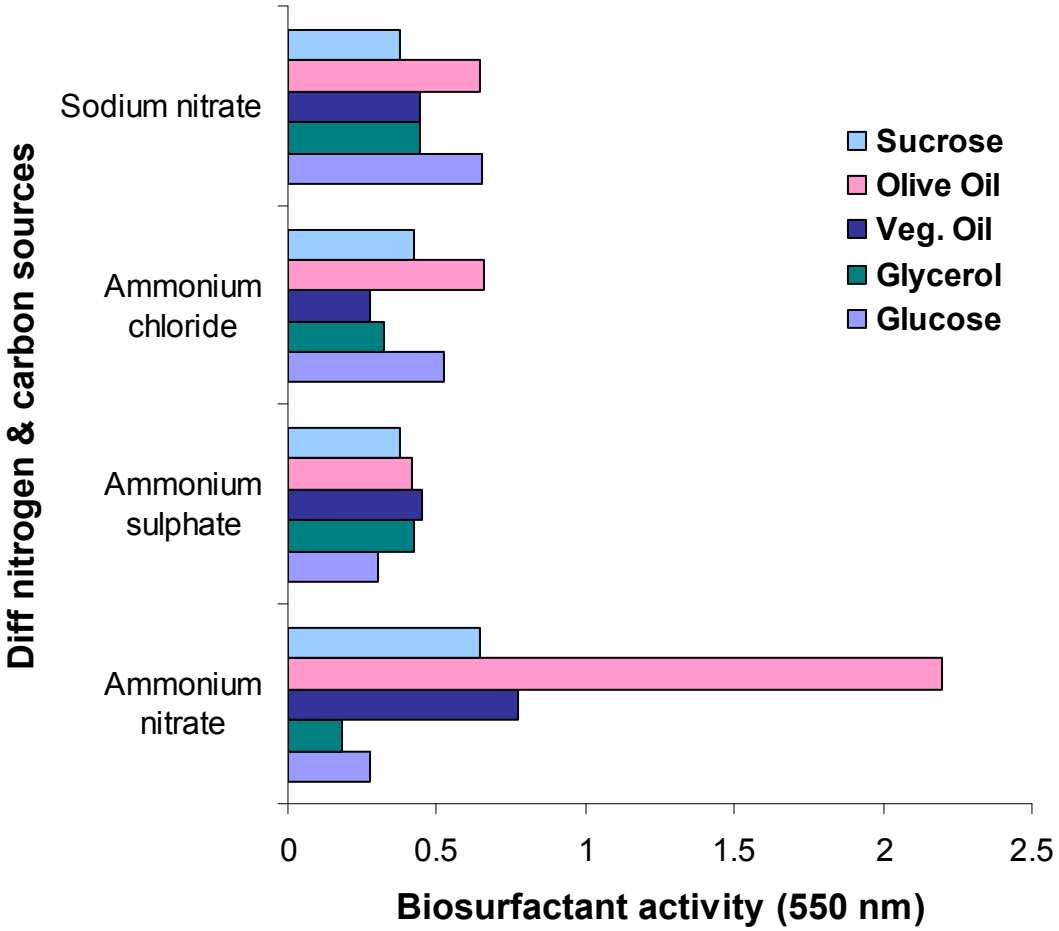


Fig 5: Biosurfactant activity of *Bacillus subtilis* SK320 on various nitrogen and carbon sources.

Biosurfactant activity was measured at 550 nm, spectrophotometrically (section 3.7).

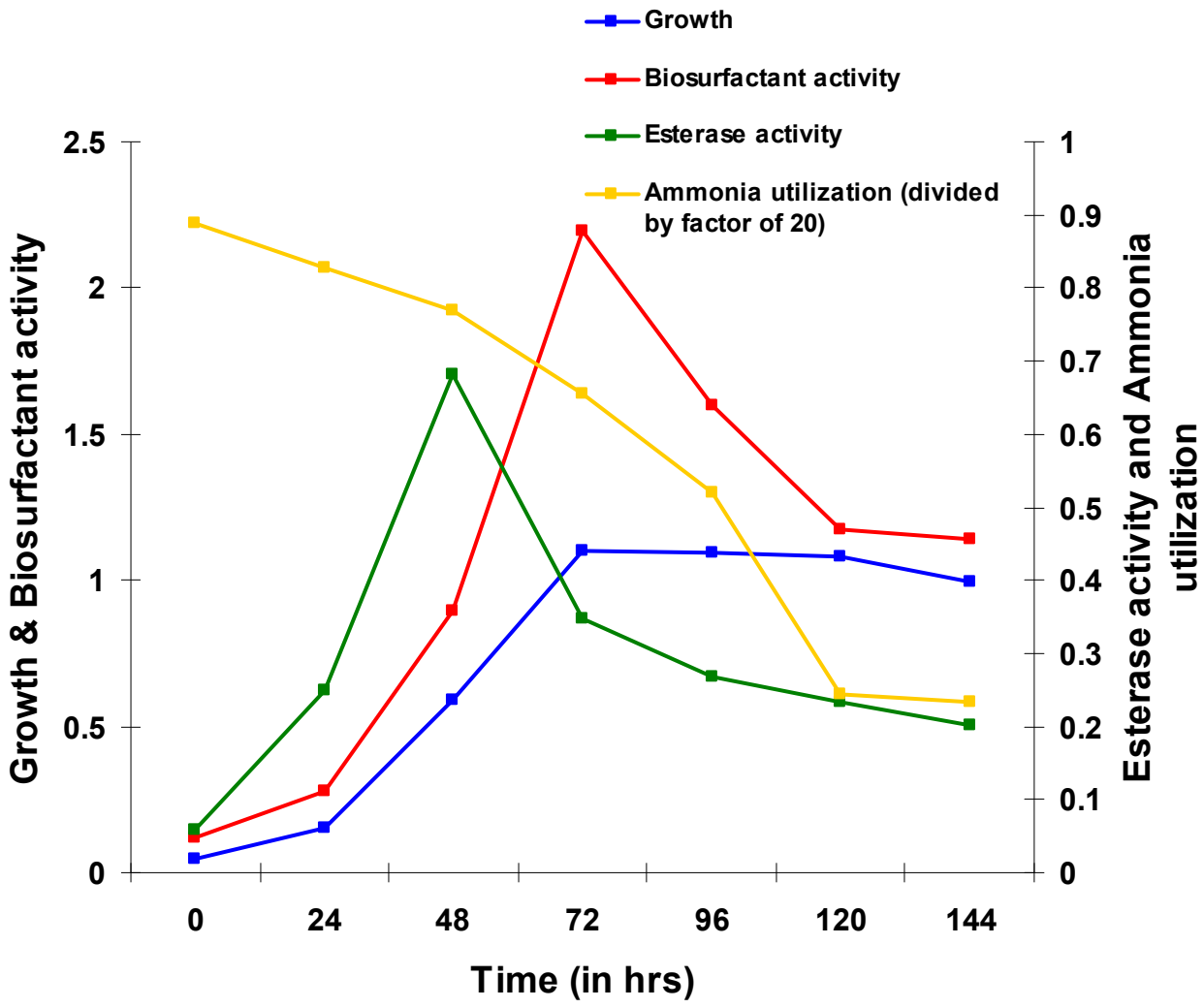


Fig 6: Growth, biosurfactant activity, esterase activity and ammonia utilization of *Bacillus subtilis* SK320.

***Bacillus subtilis* SK320 was grown on basal medium containing ammonium nitrate (0.5%, w/v) as nitrogen and olive oil (0.5%, v/v) as carbon source. Growth at 600 nm (section 3.5), biosurfactant activity at 550 nm (section 3.7), esterase activity at 405 nm (section 3.13.2) and ammonia was analyzed at 625nm (section 3.15.3), respectively.**

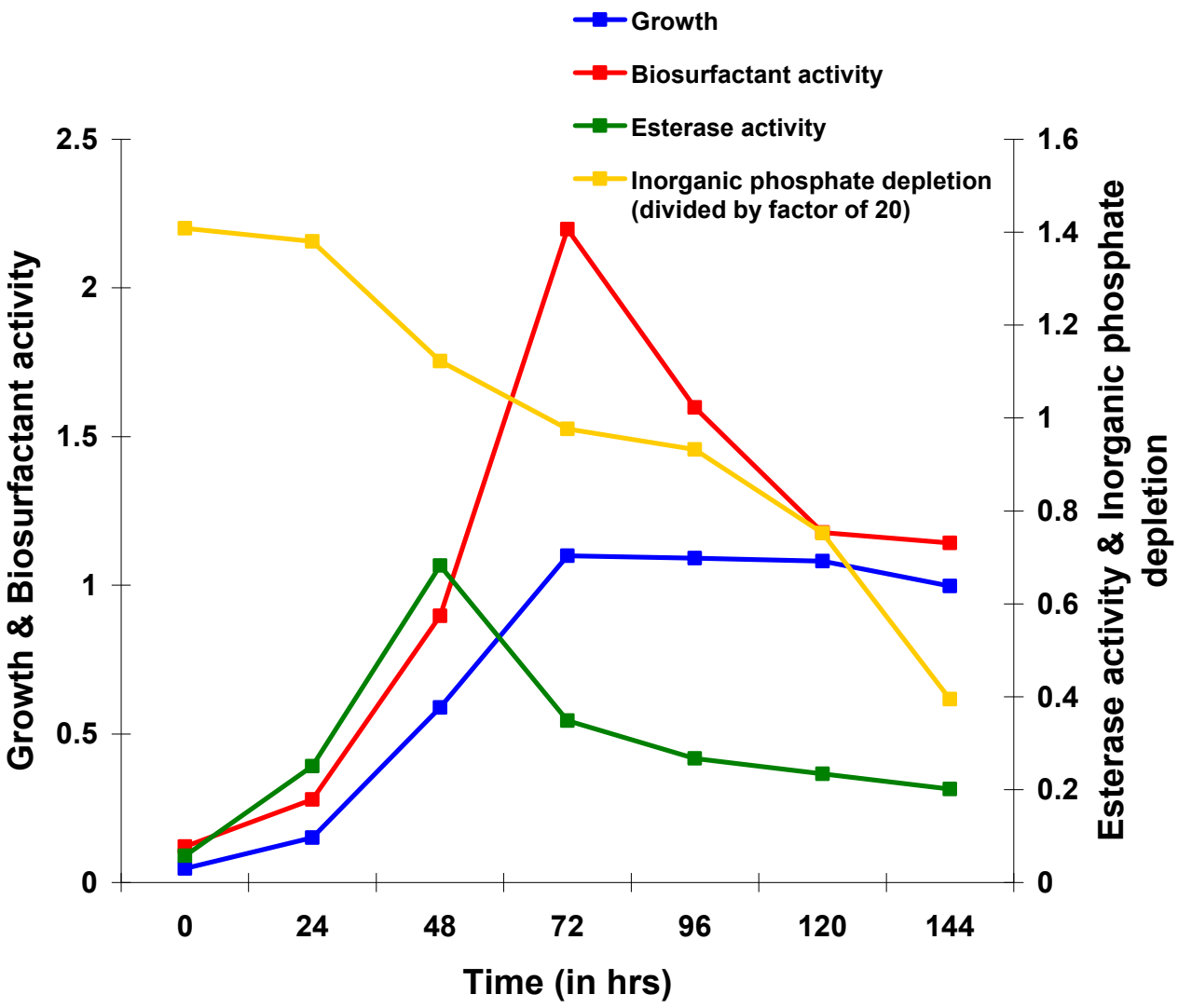
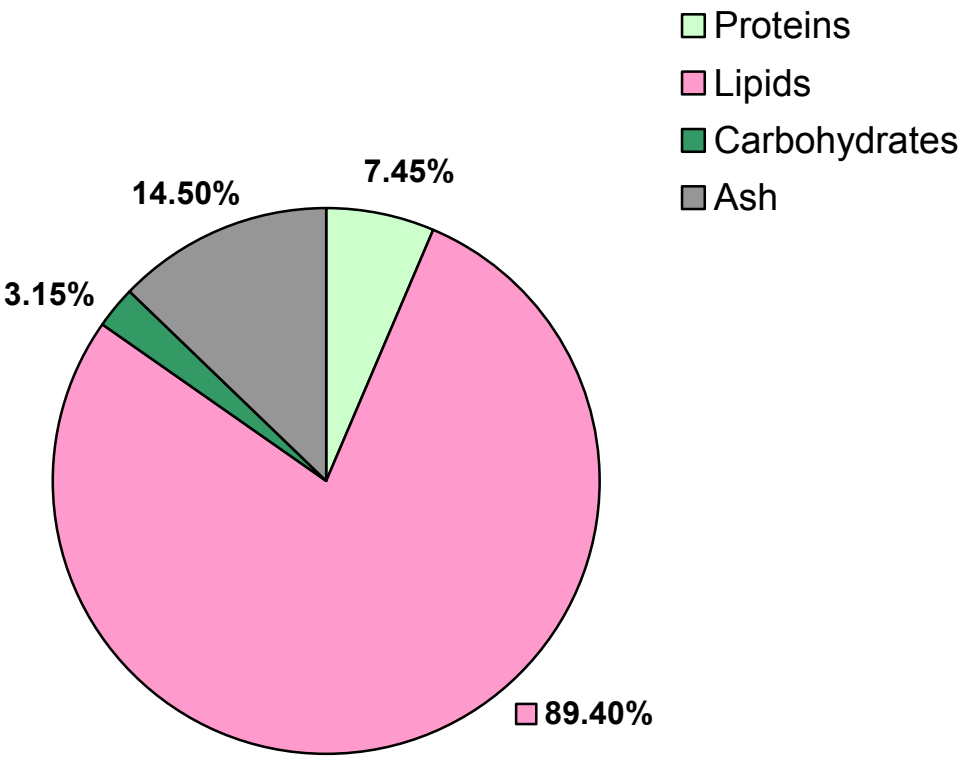


Fig 7: Growth, biosurfactant activity, esterase activity and inorganic phosphate depletion of *Bacillus subtilis* SK320.

***Bacillus subtilis* SK320 was grown on basal medium containing ammonium nitrate (0.5%, w/v) as nitrogen and olive oil (0.5%, v/v) as carbon source. Growth at 600 nm (section 3.5), biosurfactant activity at 550 nm (section 3.7), esterase activity at 405 nm (section 3.13.2) and inorganic phosphate was analyzed at 750 nm (section 3.15.4), respectively.**

Table 5: Biochemical characterization of partially purified biosurfactant of *Bacillus subtilis* SK320.
The biosurfactant was partially purified as described in Section 3.14 and analyzed for protein (Section 3.15.1), lipid (Section 3.15.7), carbohydrate (Section 3.15.6) and ash content (Section 3.15.2), respectively.

Constituent analysis	%
Protein	7.459
Lipids	89.40
Carbohydrates	3.153
Ash content	14.5



4.3.2 Effect of pH, Temperature, NaCl, Proteinase K, and Lipase on partially purified biosurfactant

(a) Effect of pH

The partially purified biosurfactant was dissolved at a concentration of 1mg/ml in Tris Cl buffer of pH range between 3 to 9.5 and the biosurfactant activity was assayed. Because the pKa value of Tris Cl is 8.14 therefore, sodium citrate (pH 3.16, 3.38, 4.40, 5.39, 6.14) and glycine NaOH (pH 8.96, 9.25, 9.93, 10.75) buffers were used in order to estimate the effect of low and high pH on partially purified biosurfactant. As shown in Fig 8, two activity maxima, one around pH 6 and the other around pH 7.5, were observed. There was a 58% decrease in activity as the pH was increased from 6 to 6.5. The activity again increased around 7.5 but increasing the pH further i.e. up to 9.5 resulted in a decrease in biosurfactant activity. In case of sodium citrate and glycine NaOH buffers, as we can observe in Fig 9, there is only one activity peak at pH 8.96 (glycine NaOH). The biosurfactant activity continuously increased with the increase in the pH from 3.16 to 6.14 (sodium citrate), but maximum activity was observed when the partially purified biosurfactant was dissolved in glycine NaOH buffer (pH 8.96). Thereafter, the activity decreased by almost 59% with the increase in the pH upto 10.75.

(b) Effect of temperature

The effect of temperature on the biosurfactant activity of the partially purified biosurfactant was studied by incubating the samples (1mg/ml) at different temperatures (i.e. 20, 30, 40, 50, 60, 100^0C). As shown in Fig 10, when the samples were incubated for 15 mins at different temperatures, there was decrease in the activity observed with the increase in the temperature. There was almost 86% reduction in the activity at 100^0C as compared to the unheated samples. Boiling the partially purified biosurfactant for 30 mins further decreased the activity. When compared with the unheated samples there was almost 100% reduction in the biosurfactant activity when the samples were incubated at 100^0C for 30 minutes (Fig 11).

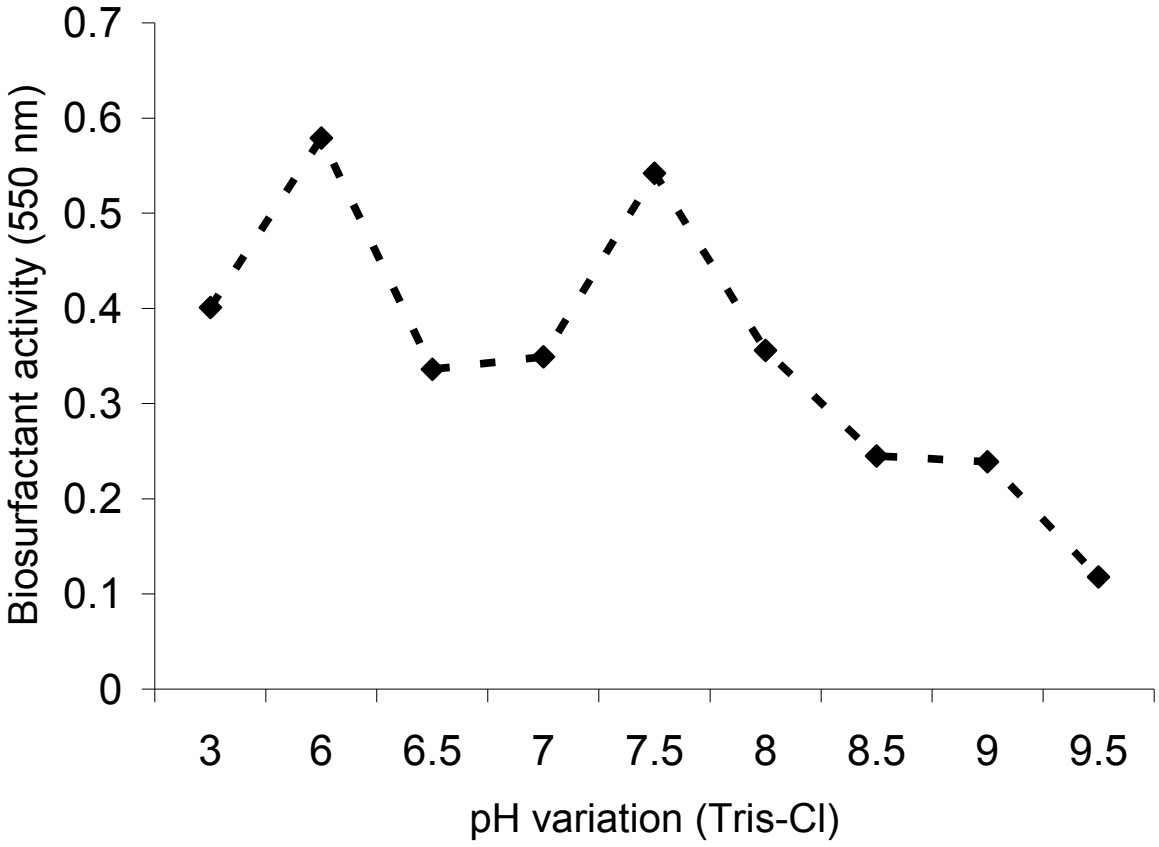


Fig 8: Effect of pH (Tris Cl) on activity of partially purified biosurfactant.

The partially purified biosurfactant was incubated at various pH for 15 min. Biosurfactant activity was analyzed at 550 nm (section 3.7).

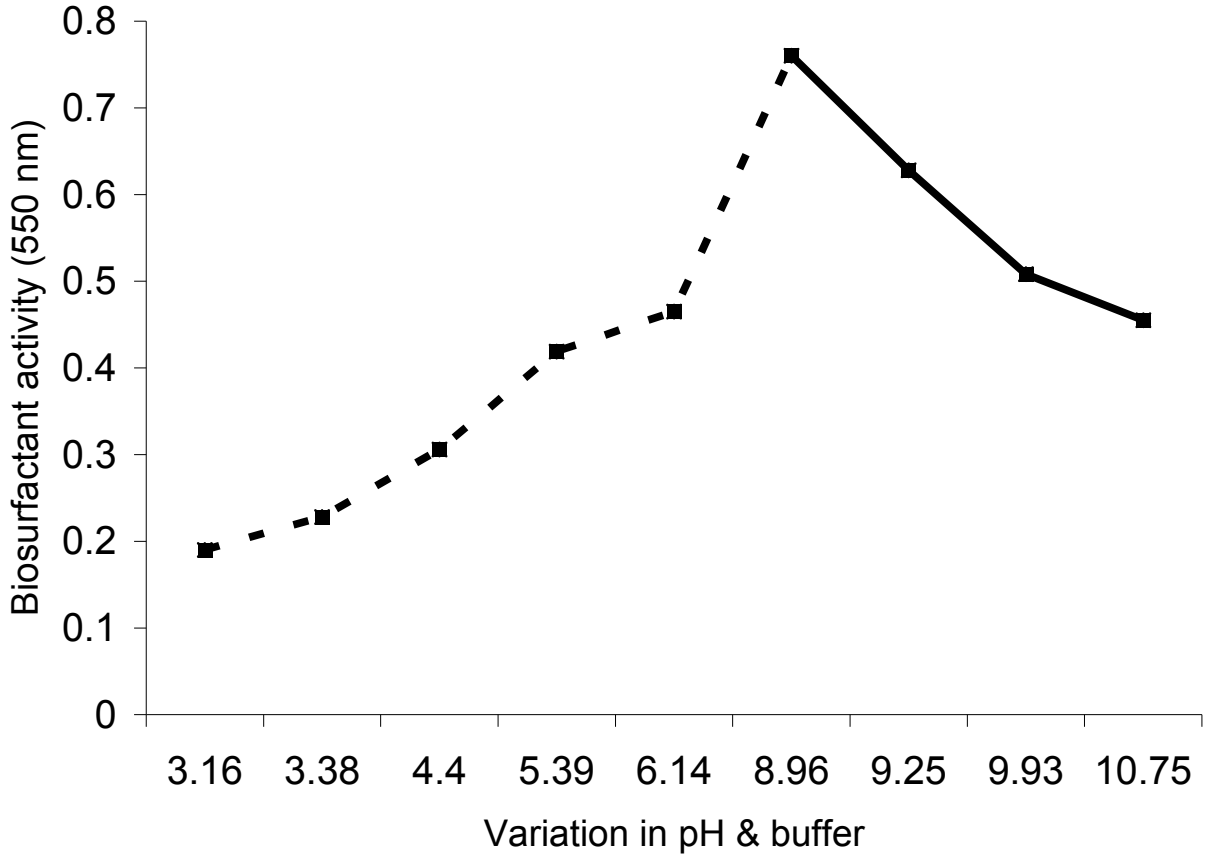


Fig 9: Effect of pH (sodium citrate and glycine NaOH) on activity of partially purified biosurfactant.

The partially purified biosurfactant was incubated at various pH for 15 min. Biosurfactant activity was analyzed at 550 nm (section 3.7).

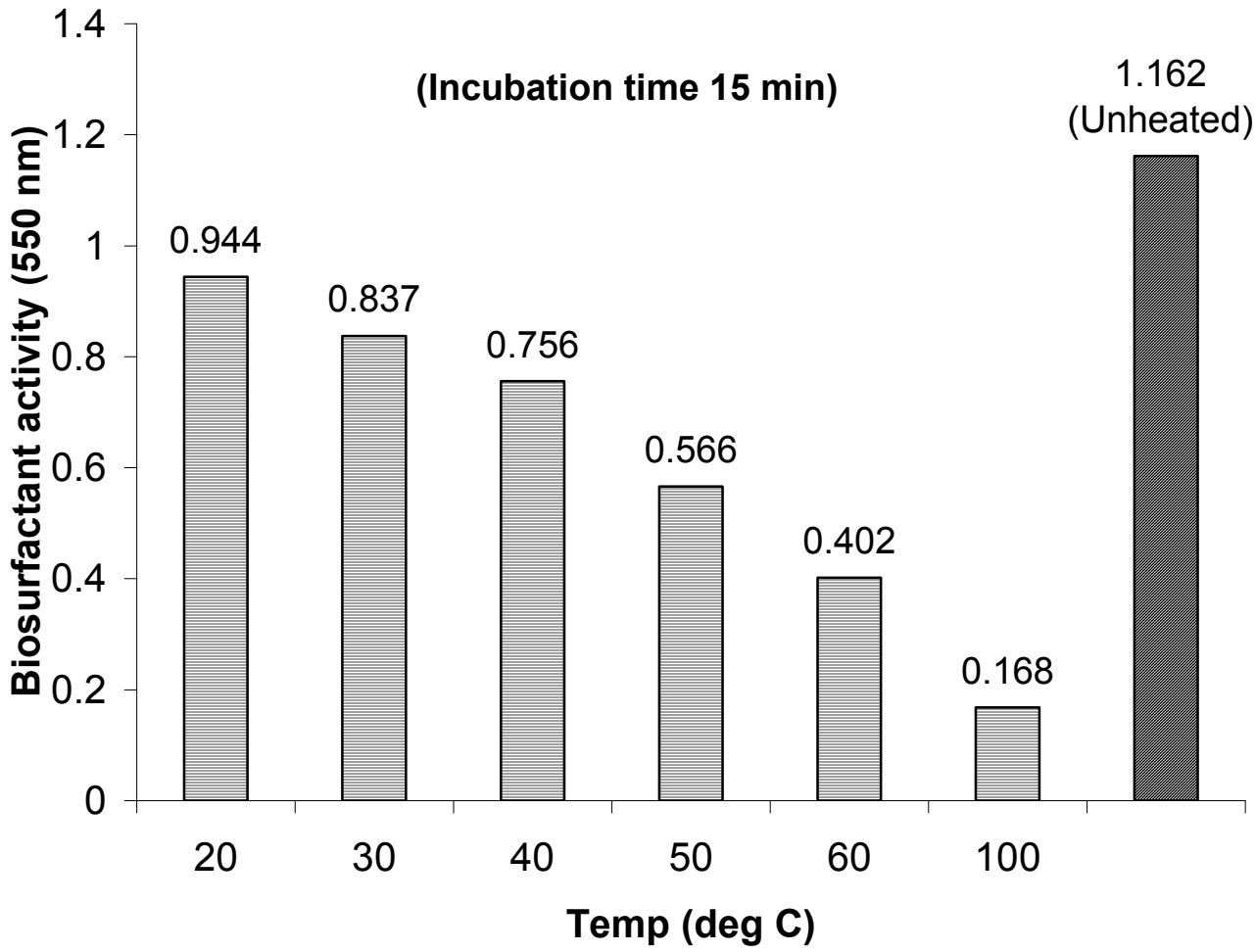


Fig 10: Effect of temperature on partially purified biosurfactant.

The partially purified biosurfactant was incubated at various temperatures for 15 min and then the sample was cooled. Biosurfactant activity was analyzed at 550 nm (section 3.7).

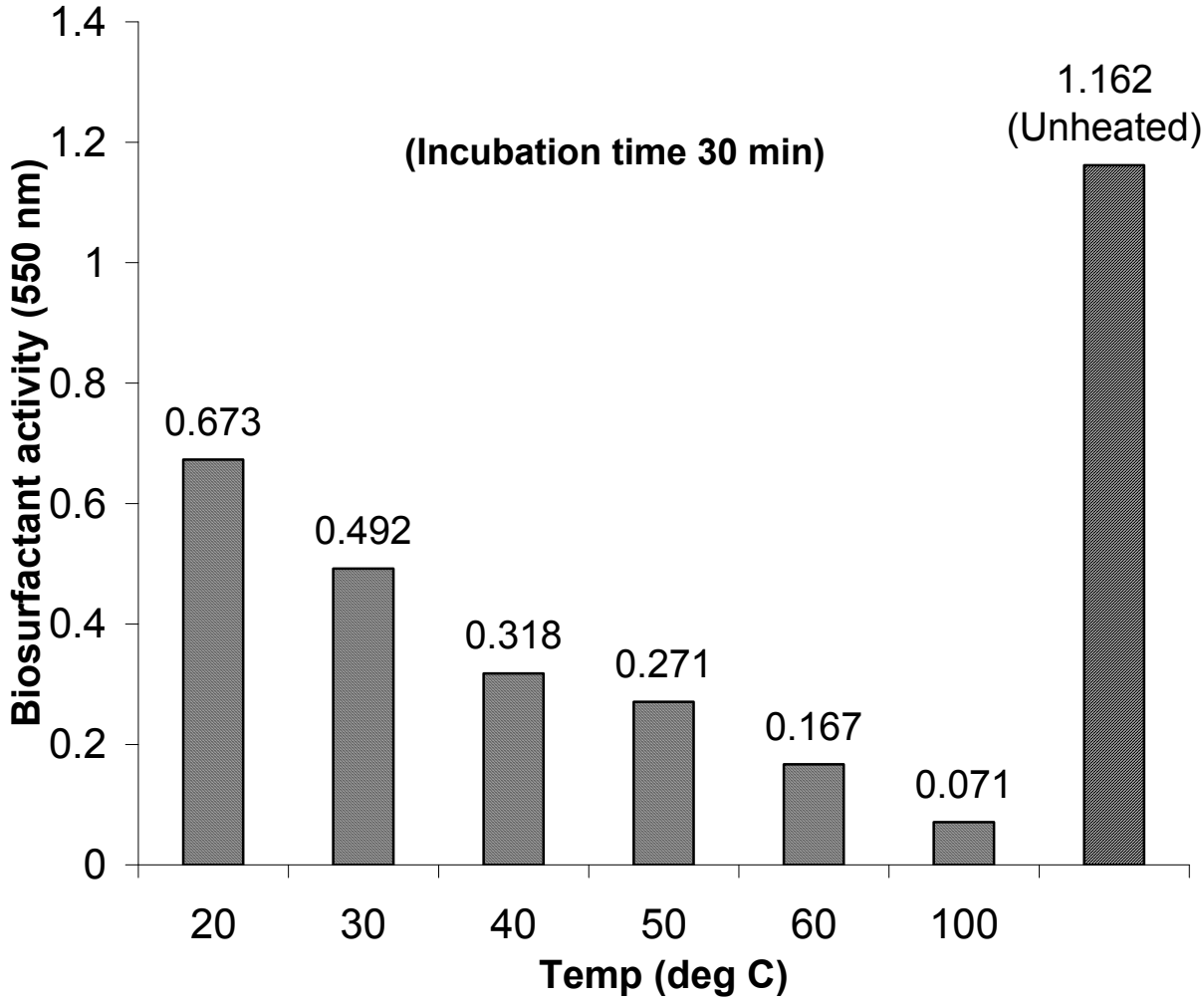


Fig 11: Effect of temperature on partially purified biosurfactant.

The partially purified biosurfactant was incubated at various temperatures for 30 min and then the sample was cooled. Biosurfactant activity was analyzed at 550 nm (section 3.7).

(c) Effect of NaCl

The partially purified biosurfactant was dissolved at a concentration of 1mg/ml and incubated with different concentrations of NaCl (viz. 0.5, 1, 2, 3, 4, 6, 8, 10 gm). Upto 3% NaCl, there was a continuous decrease in the biosurfactant activity, after which it remained constant (Fig 12). Overall it was observed that NaCl addition has a negative effect on the biosurfactant activity of partially purified biosurfactant. The activity reduced by 73% with the addition of 0.5% (v/v) NaCl but it decreased by almost 100% with the addition of NaCl upto 10% (v/v) when compared with the samples in which NaCl was not added.

(d) Effect of Proteinase K

The partially purified biosurfactant (1mg/ml) was incubated with different concentrations of Proteinase K (viz. 0.5, 1, 2, 4mg) at 37^0C. Samples were withdrawn at different time intervals (i.e. 10, 30, 60, 120 minutes) and assayed for biosurfactant activity. As can be seen in Fig 13, there was a reduction in the biosurfactant activity with the addition of Proteinase K. The total loss in the activity was 60 to 75% when 2mg Proteinase K was added and the samples were incubated for 10, 30, 60, 120 min, respectively. With the increase in the concentration of Proteinase K i.e. 4mg, no further loss in the activity was observed.

(e) Effect of lipase (steapsin)

The partially purified biosurfactant was incubated with 100, 200, 500, 700 and 1000µg of lipase and incubated at 37^0C. Samples were withdrawn at regular intervals (30, 60, 90, 120 minutes) and assayed for biosurfactant activity (Fig 14). Biosurfactant activity was found to be 1.202 in the sample without addition of lipase whereas the emulsifying activity of the partially purified biosurfactant was reduced by almost 100% with the addition of lipase in the samples. There was reduction in the biosurfactant activity of the samples as the conc. of lipase increased with the increase in the period of incubation. Addition of 1000µg lipase solution decreased the biosurfactant activity of the samples from 1.202 to as low as 0.032, 0.029, 0.014 and 0.011 with 30, 60, 90 and 120min period of incubation, respectively. The data suggested that the lipid moiety present in the lipopeptide has been completely destroyed by the lipase enzyme further confirming our results.

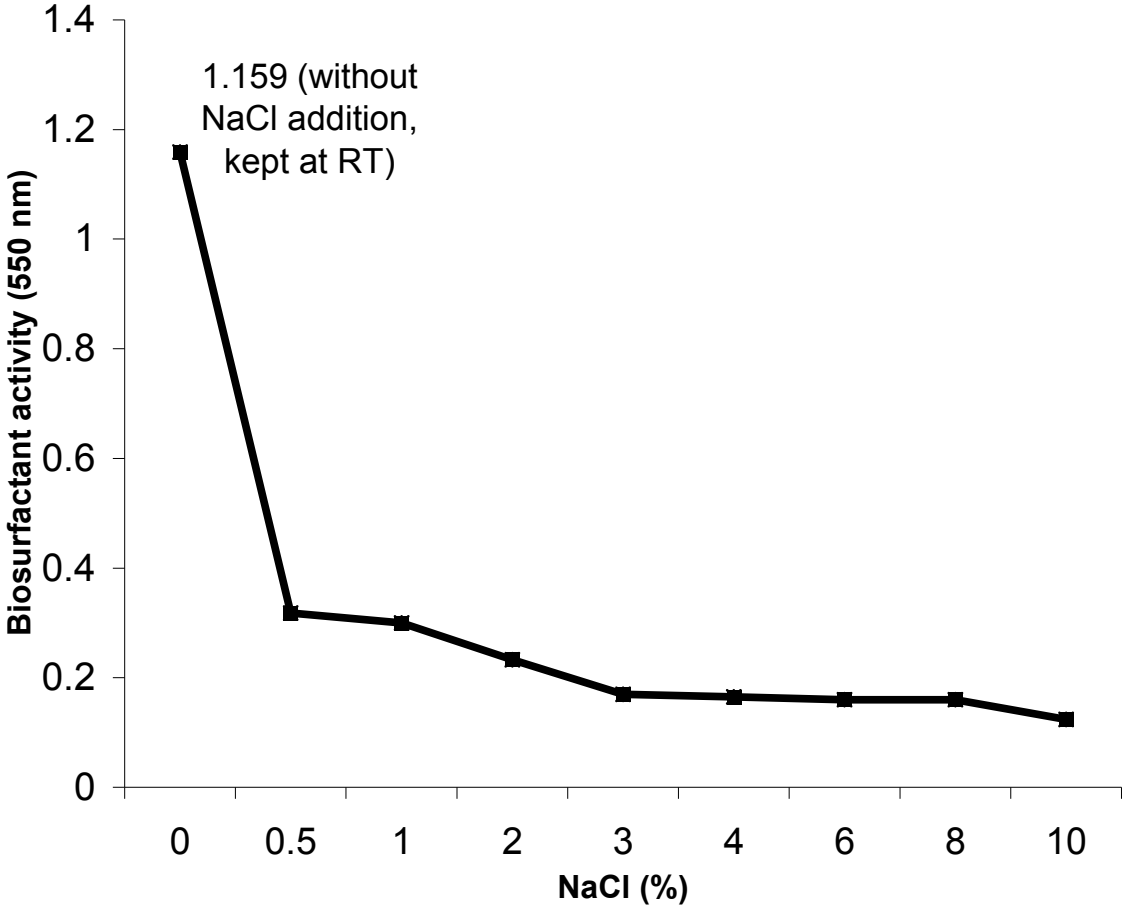


Fig 12: Effect of NaCl addition on partially purified biosurfactant.

The partially purified biosurfactant was incubated at various NaCl concentrations for 15 min and then the sample was analyzed for biosurfactant activity at 550 nm (section 3.7).

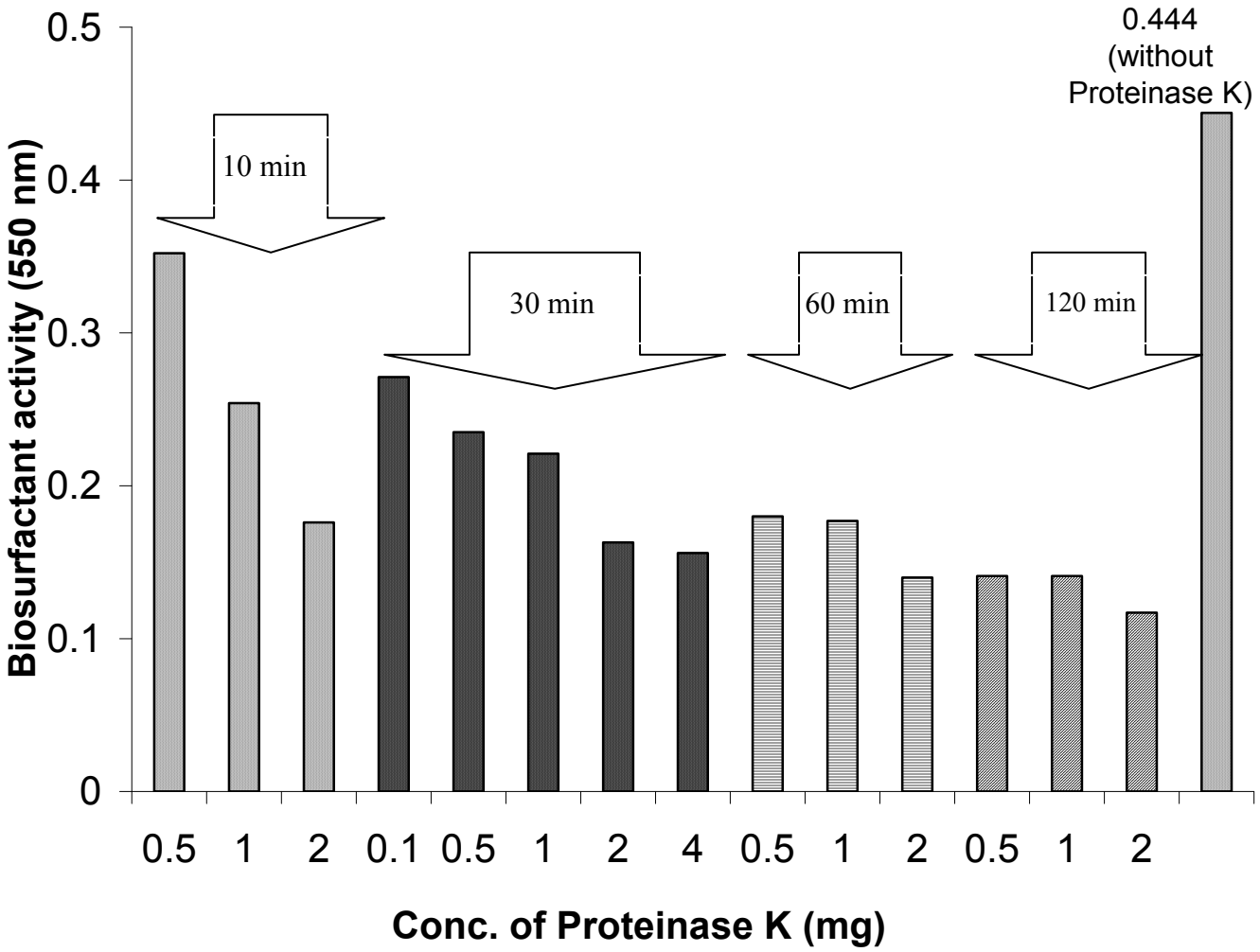


Fig 13: Effect of Proteinase K on partially purified biosurfactant.

The partially purified biosurfactant was incubated at 37^0C with various proteinase concentrations for 10, 30, 60 and 120 min and then the sample was analyzed for biosurfactant activity at 550 nm (section 3.7).

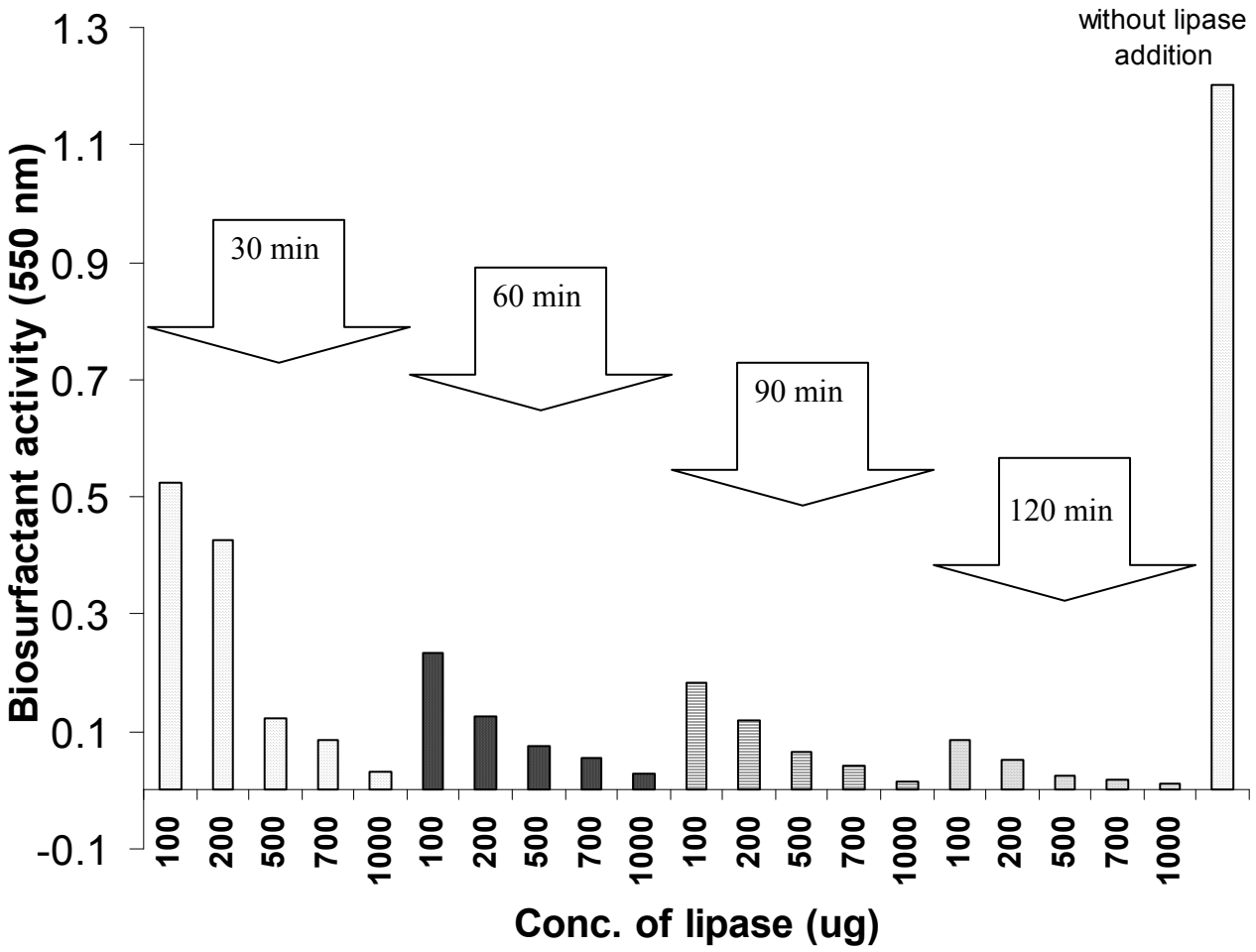


Fig 14: Effect of lipase on partially purified biosurfactant.

The partially purified biosurfactant was incubated at various lipase concentrations for 30, 60, 90 and 120 min and then the sample was analyzed for biosurfactant activity at 550 nm (section 3.7).

4.4 Induction of esterase activity

Induction of esterase activity was studied by growing *Bacillus subtilis* SK320 with different substrates: vegetable oil, glycerol, maltose, n-dodecane, mobile oil, crude oil, olive oil, glucose and sucrose (Table 6). Olive oil was the best inducer of esterase activity amongst all the substrates with the highest activity of 3.143 IU/ml, followed by maltose, glycerol, sucrose and vegetable oil with activities of 0.7929, 0.6616, 0.5097 and 0.4248 IU/ml respectively.

Majority of the esterase was extracellular as the cells after sonication gave a very low esterase activity. To study the pattern of esterase activity three substrates with high, medium and low growth were analyzed (Fig 15). Olive oil supplemented media was successful in inducing maximum esterase activity followed by vegetable oil and mobile oil. Among the four isolates i.e. SK96, SK97, SK98 and SK320, the esterase activity was found to be maximum with isolate SK320 (Fig 16). The production of esterase increased progressively during growth following the same trend and was maximum at 72hr (Fig 17). At 72hrs there was a reduction in the esterase activity after which it remained constant till 192hrs.

4.5 Regulation of biosurfactant and esterase activity with various carbon sources

A comparative analysis of the growth, biosurfactant activity and esterase activity revealed that biosurfactant and esterase activities follow the same trend as that of growth. Both biosurfactant and esterase activities increased in the first 48hrs of growth showing a maxima at 72hrs, after which both the activities declined by almost 50% and becoming negligible by 192hrs (Fig 18). Maximum biosurfactant activity was achieved when the esterase activity was maximum in the culture supernatant i.e. at 72hr, suggesting that there is some possible correlation between the two.

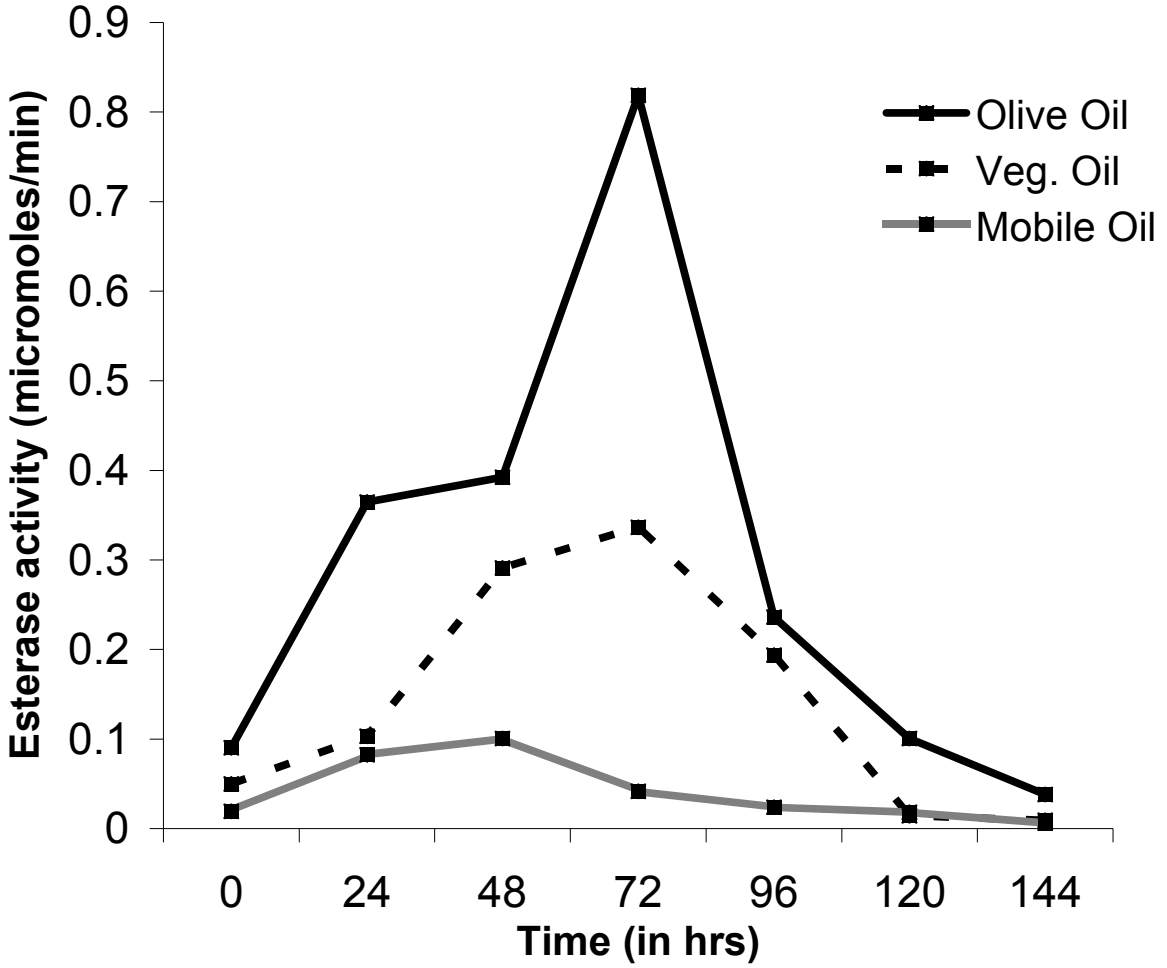


Fig 15: Esterase activity of *Bacillus subtilis* SK320.

***Bacillus subtilis* SK320 was grown on basal medium with various substrates as sole carbon source and esterase activity was analyzed at 405nm (section 3.13.2).**

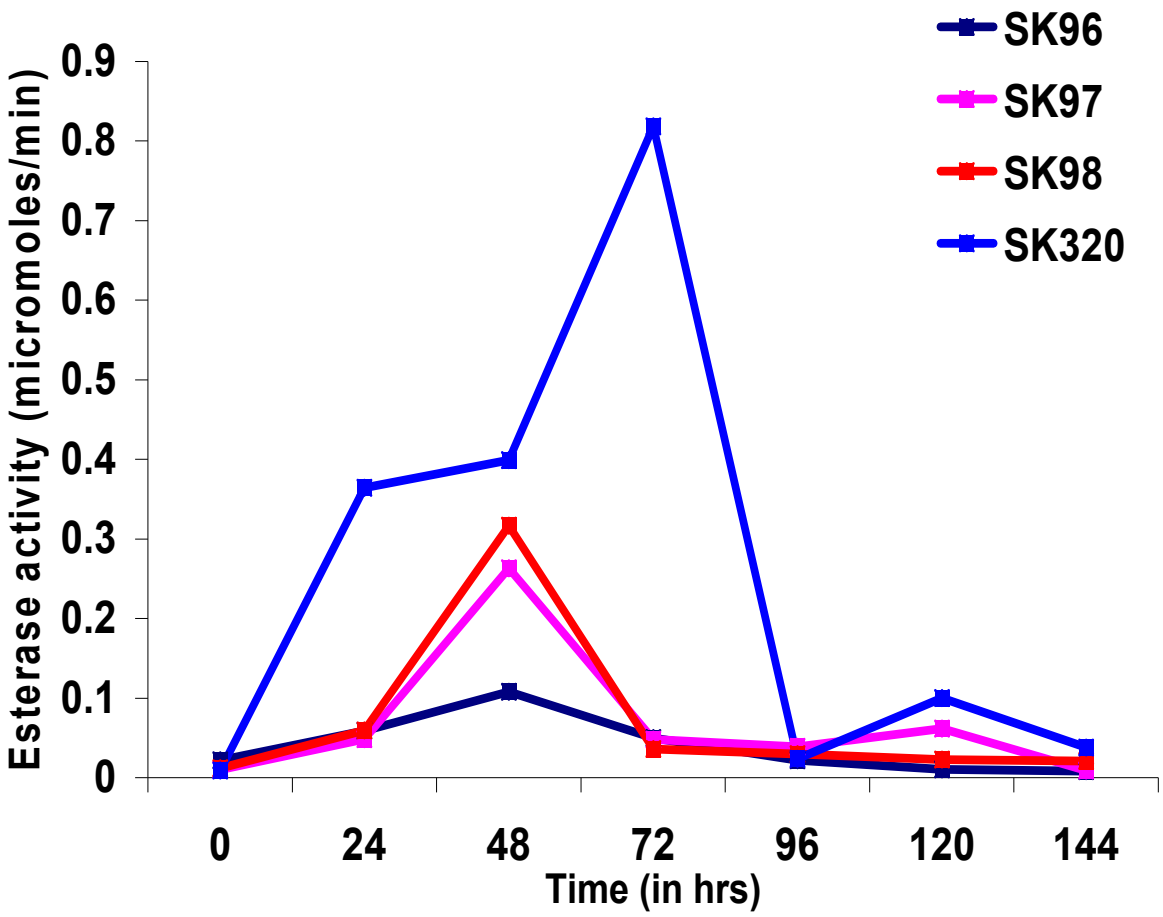


Fig 16: Esterase activity of various isolates.

Isolate SK96, SK97, SK98 and *Bacillus subtilis* SK320 were grown on basal medium with 0.5% (v/v) olive oil as sole carbon source and the esterase activity was analyzed at 405 nm (section 3.13.2).

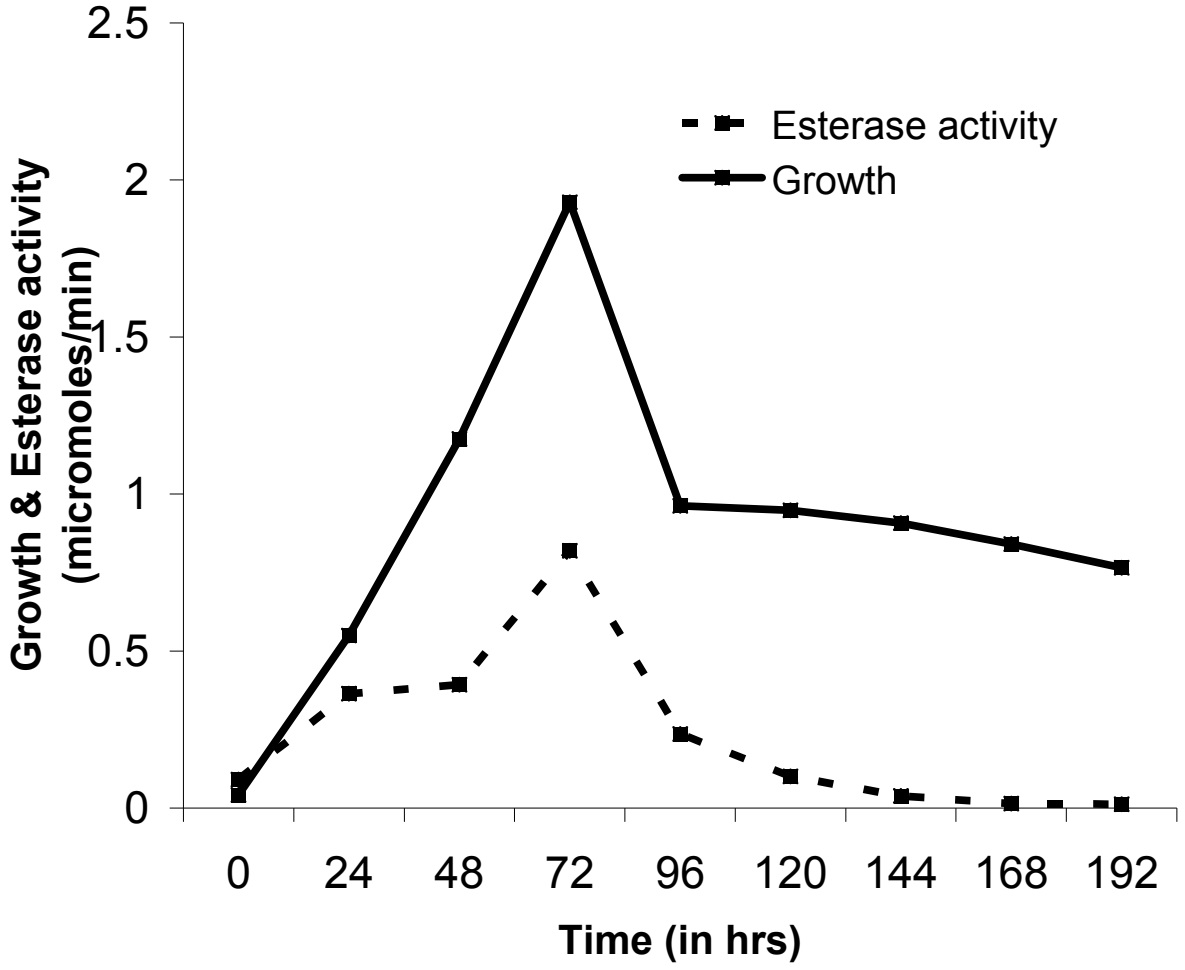


Fig 17: Growth and esterase activity of *Bacillus subtilis* SK320.

***Bacillus subtilis* SK320 was grown on basal medium with 0.5% (v/v) olive oil as sole carbon source. Growth at 600nm and esterase activity was analyzed at 405 nm, spectrophotometrically (sections 3.5 & 3.13.2).**

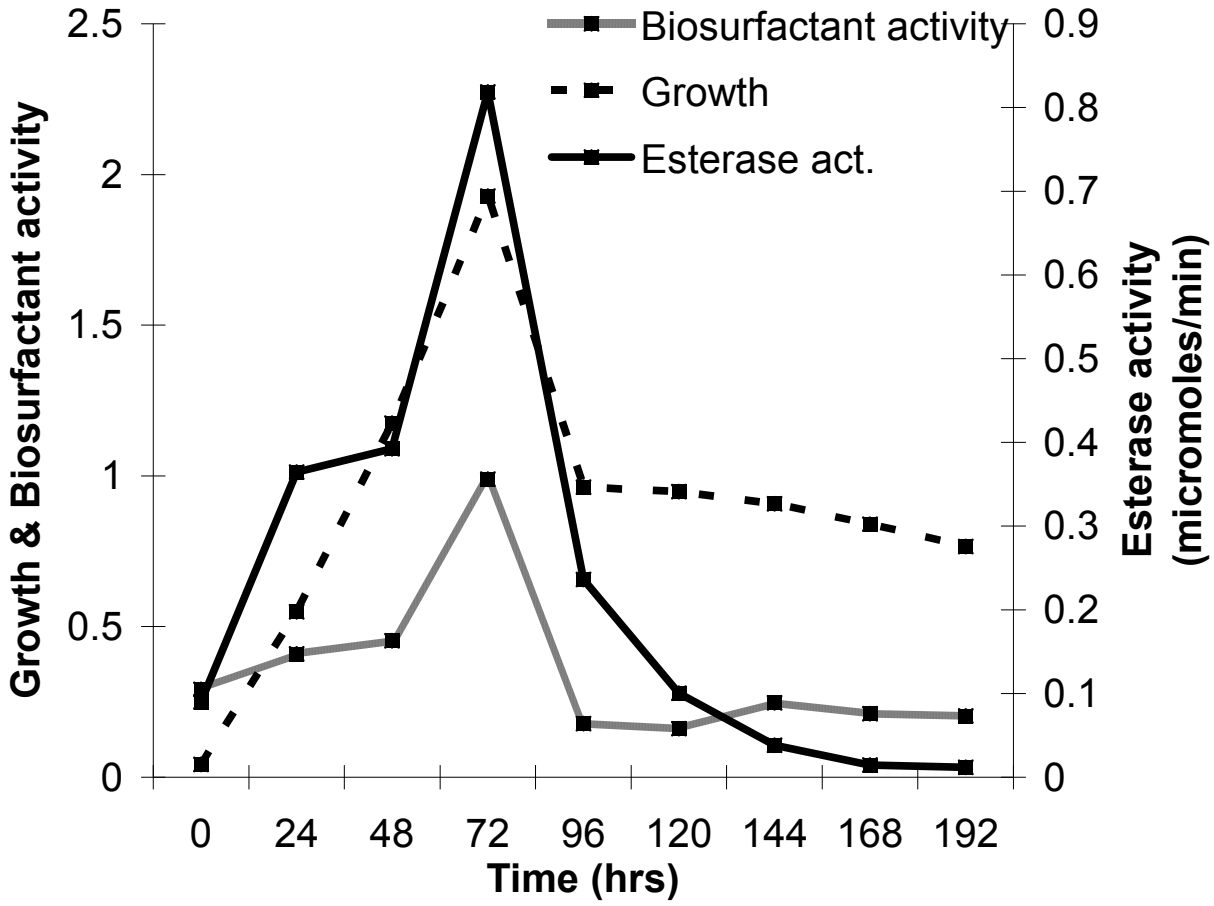


Fig 18: Growth, biosurfactant activity and esterase activity of *Bacillus subtilis* SK320.

***Bacillus subtilis* SK320 was grown on basal medium with 0.5% (v/v) olive oil as sole carbon source. Growth, biosurfactant activity and esterase activity were analyzed at 600nm, 550nm and 405nm, respectively (Section 3.5 & 3.7).**

Table 6: Induction of esterases of *Bacillus subtilis* SK320 grown on various substrates.
***Bacillus subtilis* SK320 was grown on various substrates and supernatant was analyzed for esterase activity (section 3.13.2) and protein (section 3.15.1) after 72hrs of growth.**

Substrate	Esterase activity* (IU/ml)	Extracellular Protein* (micrograms/ml)
Vegetable oil	0.4248	2.66
Glycerol	0.6616	2.37
Maltose	0.7929	1.23
n-dodecane	0.1338	0.32
Mobile oil	0.1853	0.18
Crude oil	0.1081	0.23
Olive oil	3.143	6.97
Glucose	0.3552	2.37
Sucrose	0.5097	2.29

4.6 *Bacillus subtilis* SK320

(a) Purification of esterase

Esterase from *Bacillus subtilis* SK320 was purified from the cell free supernatant of the mid log phase cells grown in basal medium amended with 0.5% (v/v) olive oil as the sole carbon source (Image 2). The initial separation of the extracellular crude enzyme by anion exchange (Sepharose Q) chromatography at pH 7 resolved the protein into three distinct active components giving maximum esterase activity (Fig 19). Component A eluting in the equilibrating buffer constituted about 0.20% of the total esterase activity while the other two components were eluted with NaCl as gradient. The second active protein component (component B) eluting at 480mM NaCl using a 10 to 1000mM NaCl gradient in 10mM sodium phosphate buffer (pH 7.0) constituted 0.14% of the total activity while the third active protein (component C) on Q Sepharose eluted at 780mM NaCl and constituted 0.36% of the total activity loaded.

Components A, B and C were further resolved by gel-filtration chromatography. Component A (specific activity 0.06 IU) fractions from Sepharose Q were concentrated by ultra filtration and loaded unto a Sephadex G 75 column. Two activity peaks designated A1 (specific activity 0.06 IU) and A2 (specific activity 0.06 IU) were obtained (Fig 20). A1 was purified 1.87 fold with a recovery of 5.74 % while A2 was purified 1.76 fold with recovery of 3.11%, respectively. Component B fractions were pooled, dialyzed, concentrated by ultrafiltration and then loaded onto Sephadex G 75. Component B (specific activity 0.02 IU) was resolved into esterase B1 and B2, with specific activity of 0.29 IU, 8.98 fold purification and 16.23% recovery for B1 and with specific activity of 0.12 IU, 3.77 fold purification and 7.85% recovery for B2, respectively (Fig 21). Similarly component C (specific activity 0.02 IU) on Sephadex G 75 was resolved into three active protein components C1, C2 and C3, respectively (Fig 22), with specific activity of 0.31 IU, 9.61 fold purification and 19.27% recovery for C1, with specific activity of 0.09 IU, 2.78 fold purification and 8.10% recovery for C2 and with specific activity of 0.05 IU, 1.52 fold purification and 4.12% recovery for C3 respectively, Table 7. All the esterase sub-components were purified to homogeneity as indicated by native PAGE, followed by activity staining with RR salt (Image 16).

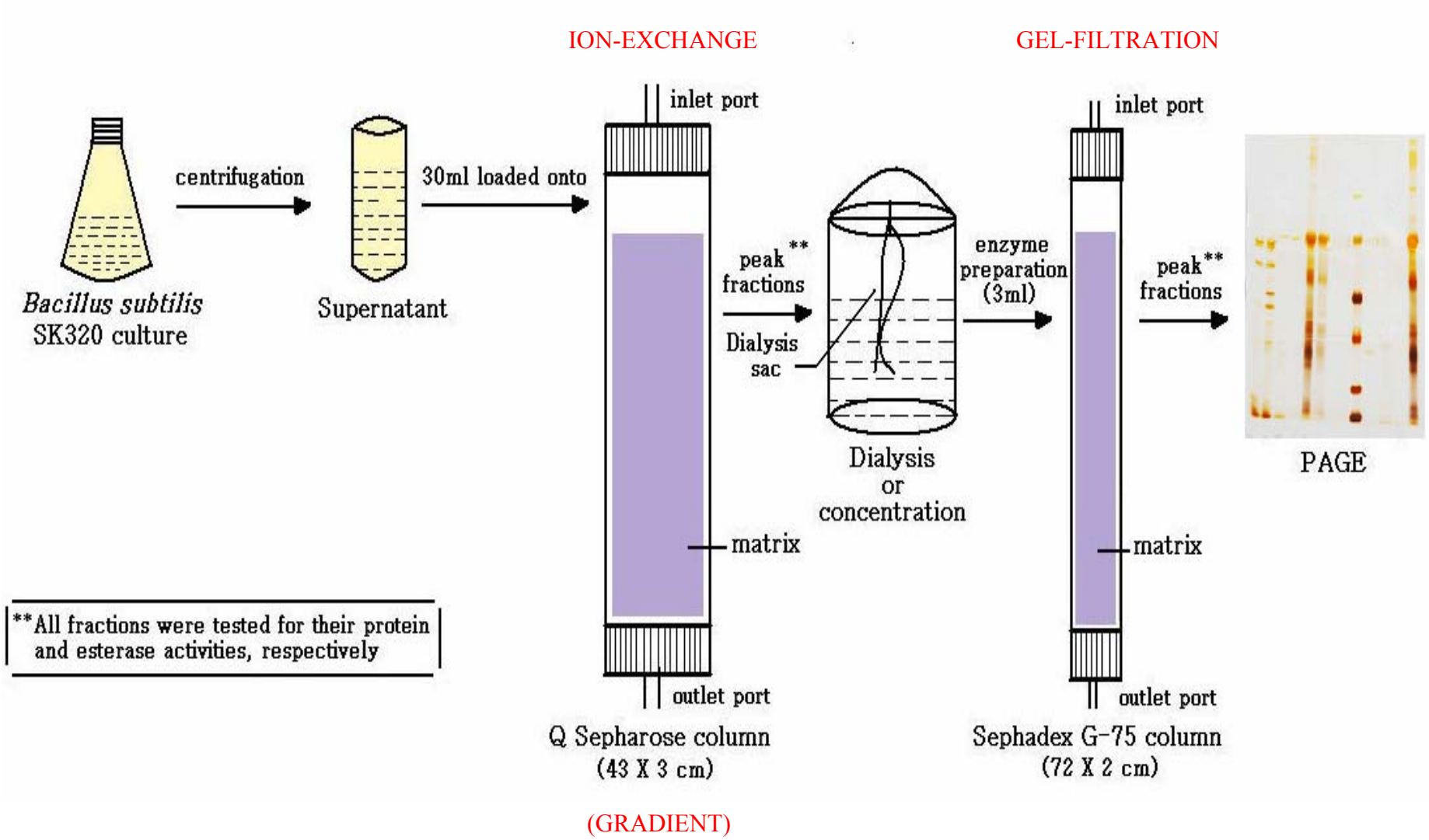


Image 2: Schematic diagram of the methodology adopted for esterase enzyme purification.

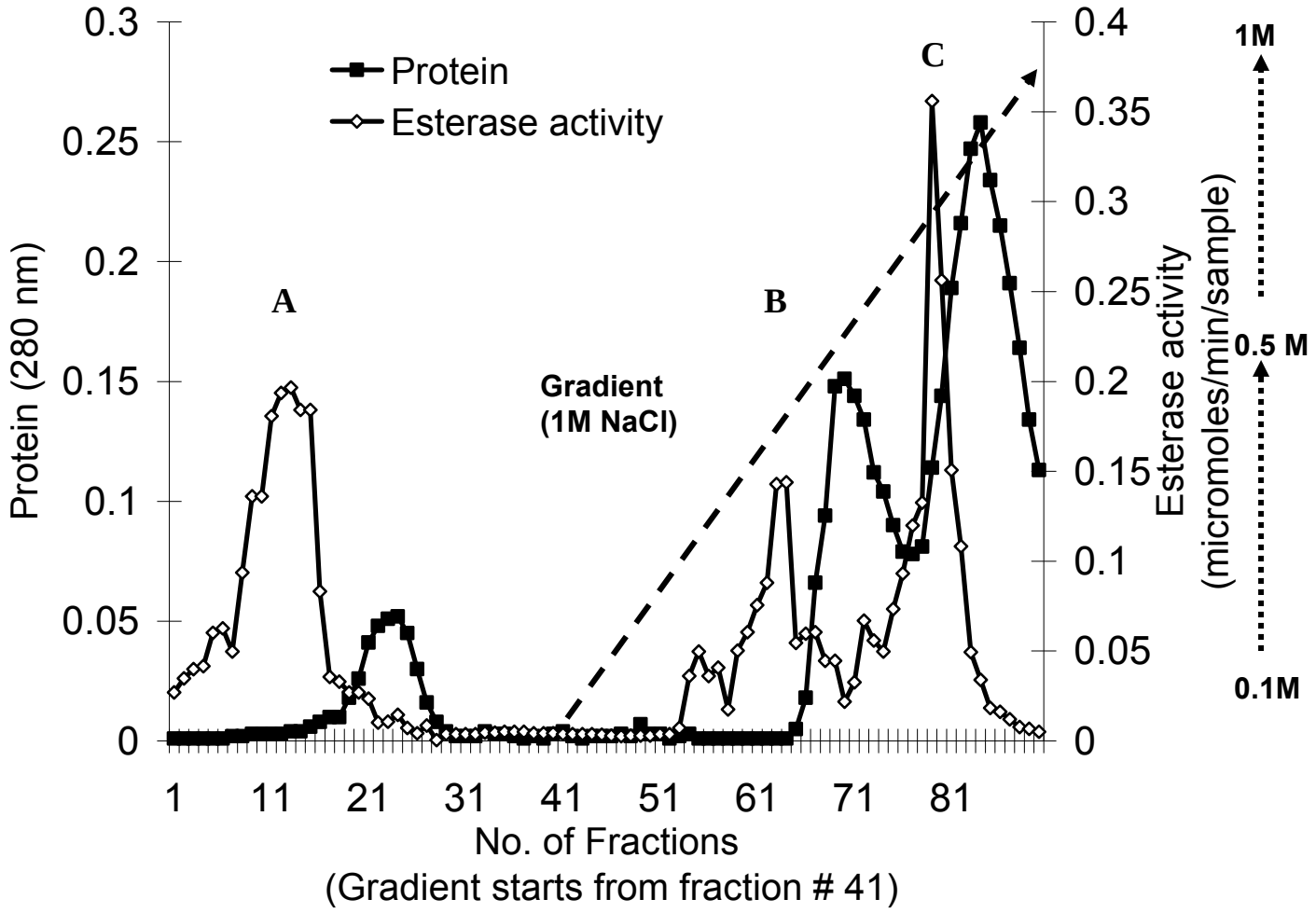


Fig 19: Elution profile of esterase of *Bacillus subtilis* SK320 from ion-exchange column - Sepharose Q.

***Bacillus subtilis* SK320 was grown on basal medium with 0.5% (v/v) olive oil as sole carbon source and after 72 hrs of growth the supernatant was used as the source of crude enzyme. Crude enzyme was loaded onto Sepharose Q column and eluted first with equilibrating buffer and then with 10 mM - 1 M NaCl gradient (section 3.10.1).**

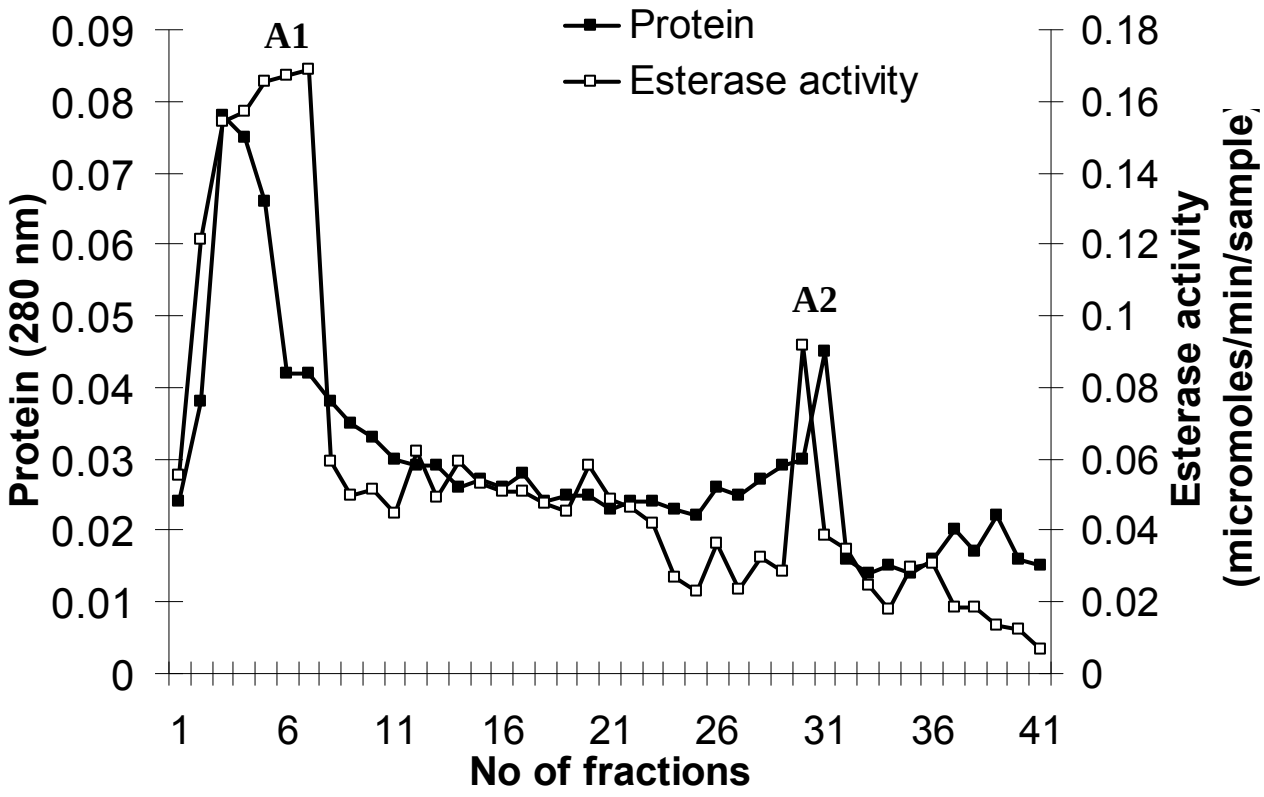


Fig 20: Elution profile of esterase component A from gel-filtration column – Sephadex G75.

Component A (3ml) was loaded onto Sephadex G -75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

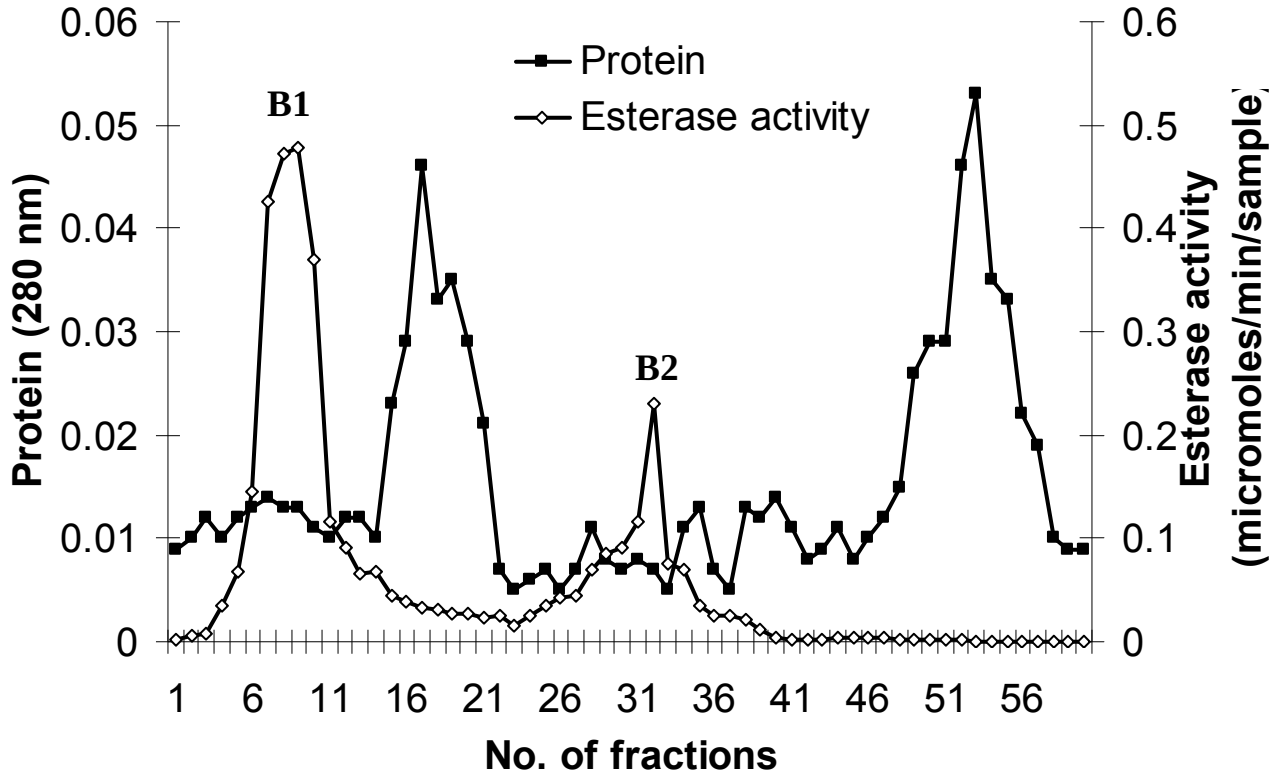


Fig 21: Elution profile of esterase component B from gel-filtration column – Sephadex G -75.

Component B (3ml) was loaded onto Sephadex G -75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

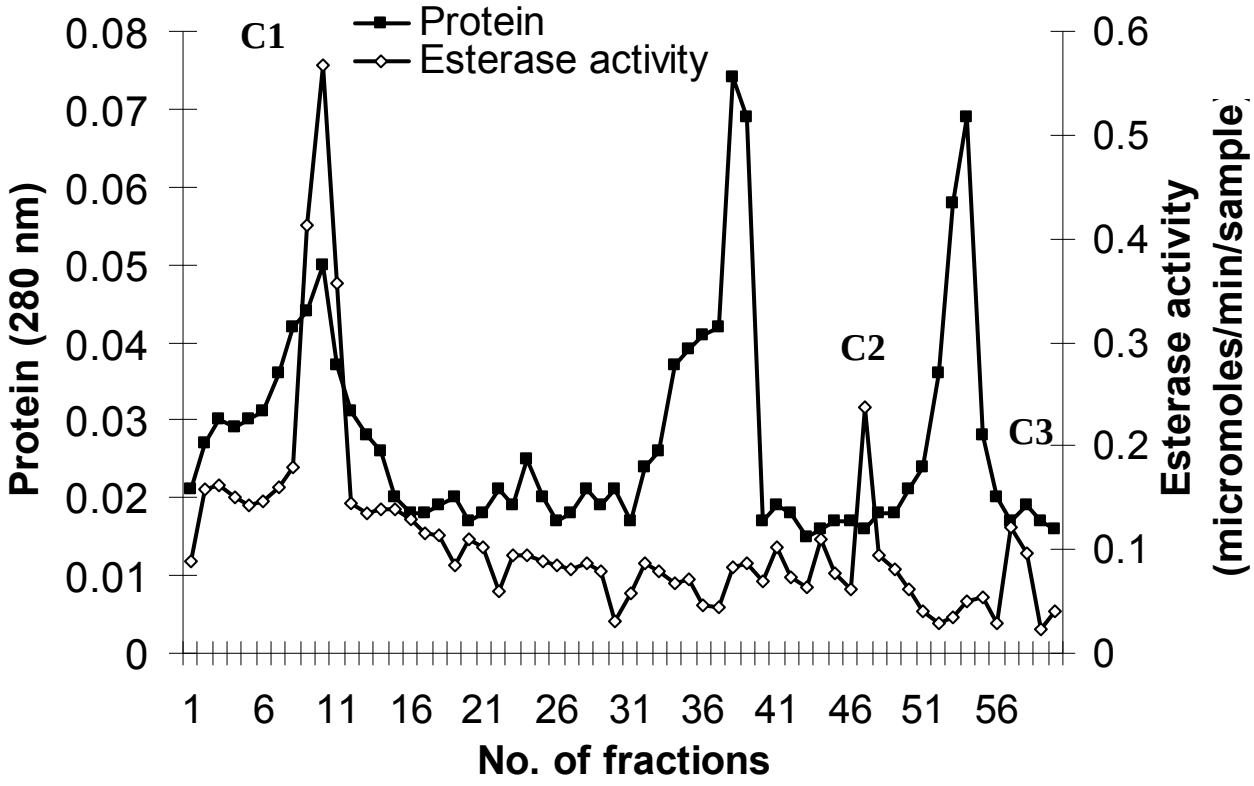


Fig 22: Elution profile of esterase component C from gel-filtration column – Sephadex G75.

Component C (3ml) was loaded onto Sephadex G -75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

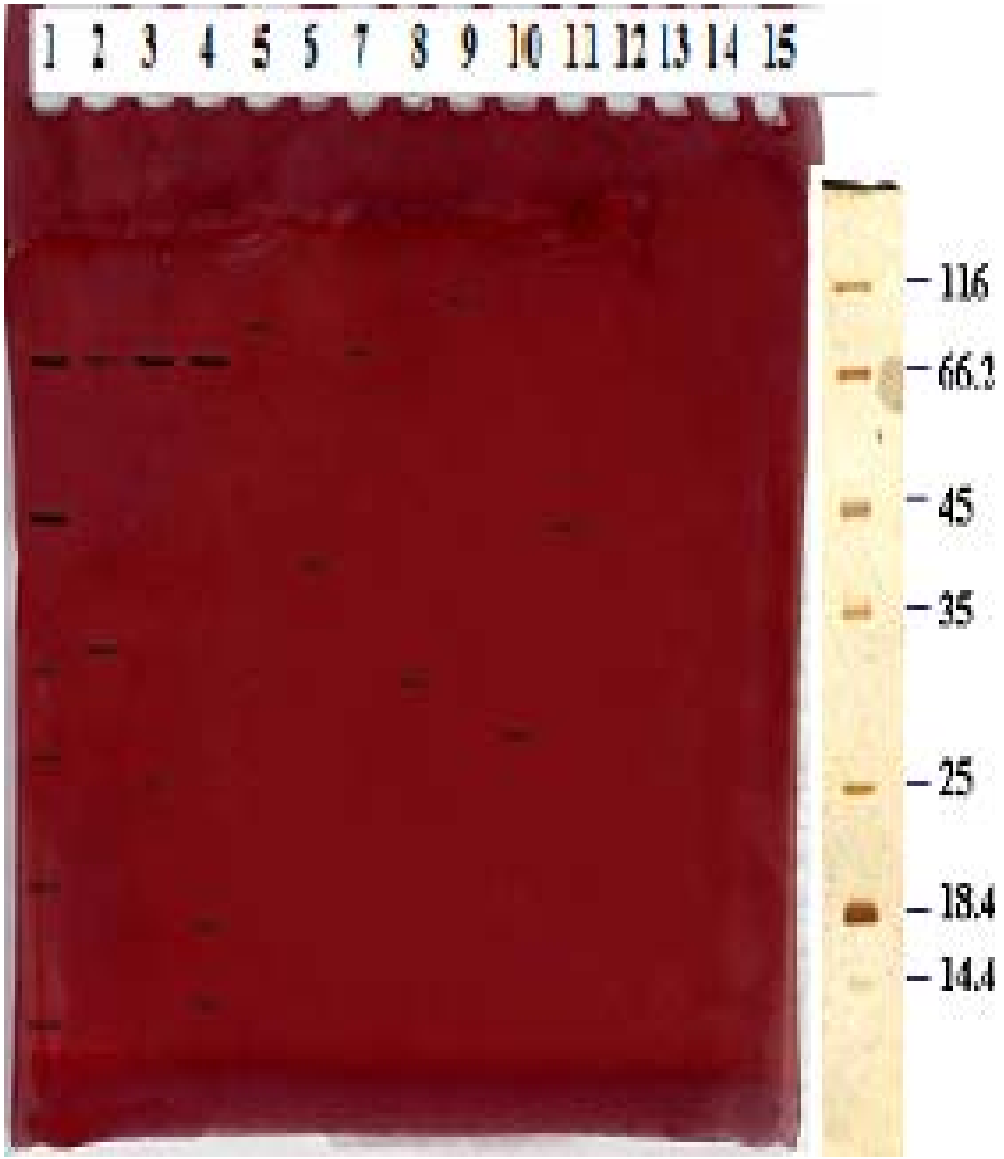


Image 16: Native PAGE analyses and activity staining with Fast Blue RR salt for esterases from *B. subtilis* SK320.

Lane 1: *Bacillus* supernatant,

Lane 2, 3, 4: Q-sepharose activity peaks (A, B, C),

Lanes 5 to 11: G-75 sub-components (A1, A1, B1, B2, C1, C2, C3),

Lane 12 to 15: blank, Last lane: protein molecular weight marker.

(b) Enzyme properties

Effect of pH, temperature, substrate concentration and inhibitor

The properties of purified esterases are shown in Table 8. The enzyme shows strong activity in the pH range of 6 to 8 with an optimal activity around pH 7.0. There was a rapid decline in activity on either side of pH 7.0. All components of esterase were stable at a temperature range of 35 to 50^0C, respectively. The esterase activity of the sub-components increased with increasing concentration of substrate (pNp-acetate) showing saturation kinetics. The michaelis constant (Km) and the maximal velocity (Vmax) of the purified esterases were calculated by plotting the lineweaver-burk plot using different concentrations of substrate. Michaelis (Km) constant for A1, A2, B1, B2, C1, C2, C3 was calculated as 2.94, 2.56, 4.34, 2.5, 9.09, 5.55, 11.1 µmoles whereas the Vmax values were found to be 0.324, 0.334, 0.454, 0.166, 0.833, 0.192, 0.263 µmol min^{-1} mg^{-1}, respectively. Mercuric chloride and lead chloride were the two inhibitors that were used in this study. Effects of addition of these inhibitors in different concentrations were studied and the data revealed that the esterase activity decreases with the addition of the inhibitor in the reaction mixture. The corresponding secondary lineweaver-burk plot was drawn to calculate the inhibitor constant (KI). Esterases components were strongly inhibited by mercury, lead and EDTA with KI (mM) of 0.45, 2.8, 2.8, 0.6, 3.2, 1.2, 6.7 with mercuric chloride, whereas with lead chloride as inhibitor the values observed were 0.30, 2.3, 2.5, 0.8, 3.3, 1.2, 1.4, respectively. The molecular weight (kDa) of the esterase components were 116, 44, 100, 32, 120, 30, 46 for A1, A2, B1, B2, C1, C2, C3 components, respectively.

Table 7: Purification of esterases of *Bacillus subtilis* SK320.
***Bacillus subtilis* SK320 was grown on basal medium containing olive oil (0.5%, v/v). The supernatant was used as source of the enzyme and purified as described in section 3.10.**

	Total Activity (IU)	Total Protein (mg)	Specific Activity (IU/mg)	% Recovery	Fold Purification
Supernatant (Crude)	4.91	153.15	0.03	100	0.00
Q Sepharose					
A	0.20	3.13	0.06	4.00	1.96
B	0.14	9.09	0.02	2.93	0.49
C	0.36	15.53	0.02	7.25	0.71
Sephadex G 75					
A1	0.28	4.70	0.06	5.74	1.87
A2	0.15	2.71	0.06	3.11	1.76
B1	0.80	2.77	0.29	16.23	8.98
B2	0.39	3.19	0.12	7.85	3.77
C1	0.95	3.07	0.31	19.27	9.61
C2	0.40	4.45	0.09	8.10	2.78
C3	0.20	4.15	0.05	4.12	1.52

Table 8: Kinetic properties of esterases of *Bacillus subtilis* SK320

		Components						
		A		B		C		
		A1	A2	B1	B2	C1	C2	C3
Optimum pH		7.0	7.0	7.0	7.0	7.0	7.0	7.0
Optimum temp.		50	35	40	40	40	40	40
Km (µmoles)		2.94	2.56	4.34	2.5	9.09	5.55	11.1
Mw (KDa)		116	44	100	32	120	30	46
Vmax ($\mu molesmg^{-1}min^{-1}$)		0.324	0.334	0.454	0.166	0.833	0.192	0.263
KI (mM)	Mercuric chloride	0.45	2.8	2.8	0.6	3.2	1.2	6.7
	Lead chloride	0.30	2.3	2.5	0.8	3.3	1.2	1.4

4.7 Cloning of biosurfactant gene

The methodology adopted for cloning is outlined in Image 3. The genes encoding the biosurfactant production viz. *sfp*, *sfp0*, *srfA* and *srfB*, of the chromosomal DNA of *Bacillus subtilis* SK320 were PCR amplified (Image 4) and the PCR product was ligated into the pGEM-T easy vector system (Promega Corporation, Madison, USA). No amplification was obtained for *srfB* gene. The ligated mixture was used to transform the *E. coli* DH5α competent cells. The blue-white colonies obtained after overnight incubation at 37^0C were all scrapped off and re-suspended in basal medium with ampicillin (50µg/ml) and incubated overnight at 37^0C. Plasmid DNA was isolated from the broth and the DNA samples were run on 1% agarose gel. The plasmid DNA from the transformants was isolated and was restriction digested with EcoRI enzyme. The pGEM-T easy vector is believed to contain recognition sites for EcoRI flanking the insertion site (Image 5). The plasmid isolated from the positive transformants showed an insert of ~640 to 750bp (Image 6 & 7). PCR amplification of the pGEM-T vector containing the insert was undertaken using the T7-SP6 primers provided with the pGEM-T easy vector kit (Image 8). The gel showed the insert size of ~640 to 750bp for the three clones, respectively thus further confirming the results. The plasmid DNA from the transformants was restriction digested with RsaI (tetracutter) and HindIII (hexacutter) enzymes (Image 9). With RsaI, four bands were observed indicating presence of two restriction sites for RsaI, in both the insert as well as the vector; whereas no HindIII restriction sites were found to be present in the plasmid DNA.

The transformants were plated on basal medium plates with 0.5% olive oil as the carbon source and incubated at 37^0C. A total of three clones i.e. pSKP, pSKP0 and pSKA were selected on the basis of their ability to grow on basal medium with olive oil as the carbon source. All of the three clones were taken for further work.

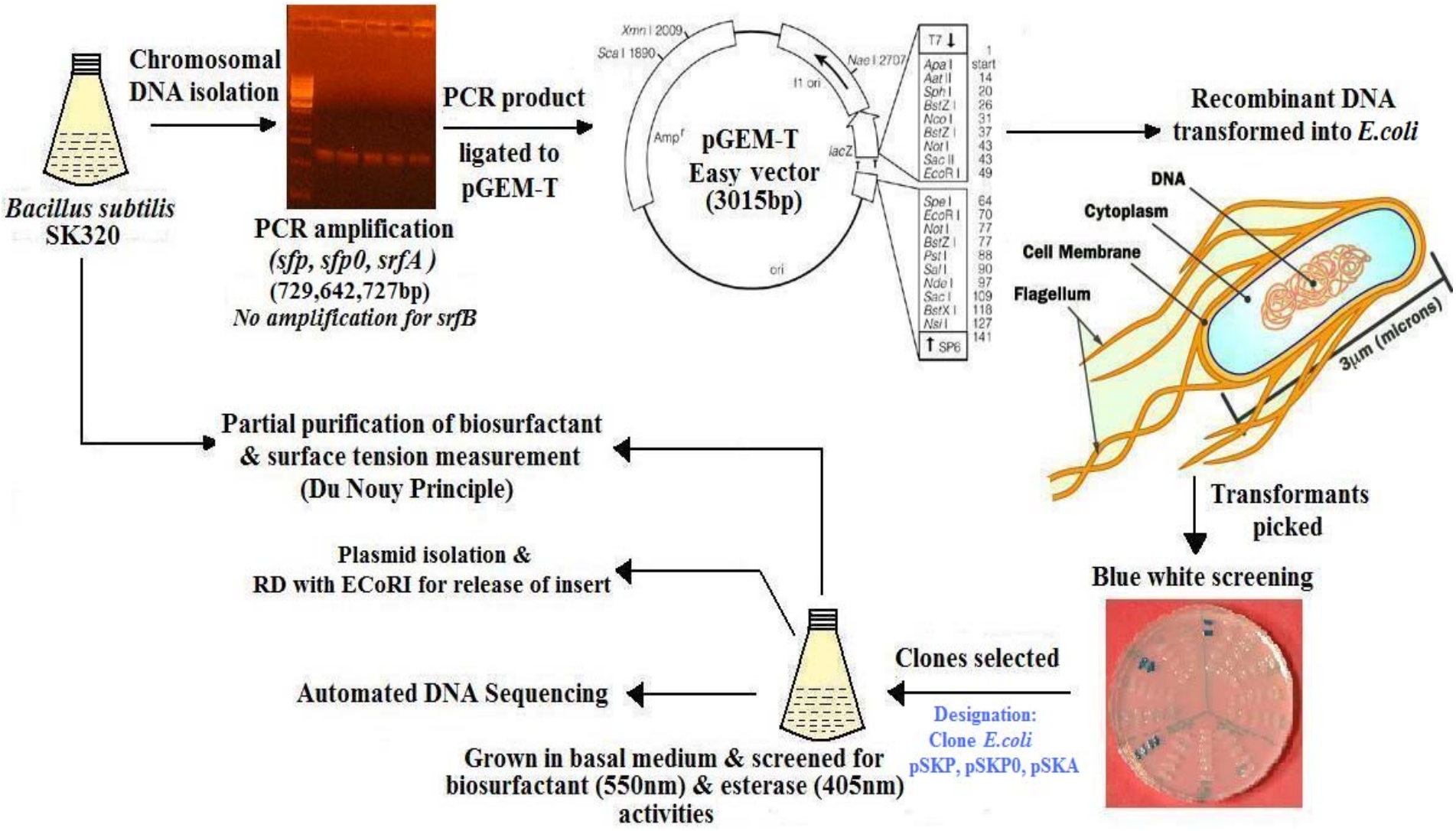


Image 3: Schematic diagram of the methodology adopted for biosurfactant gene cloning

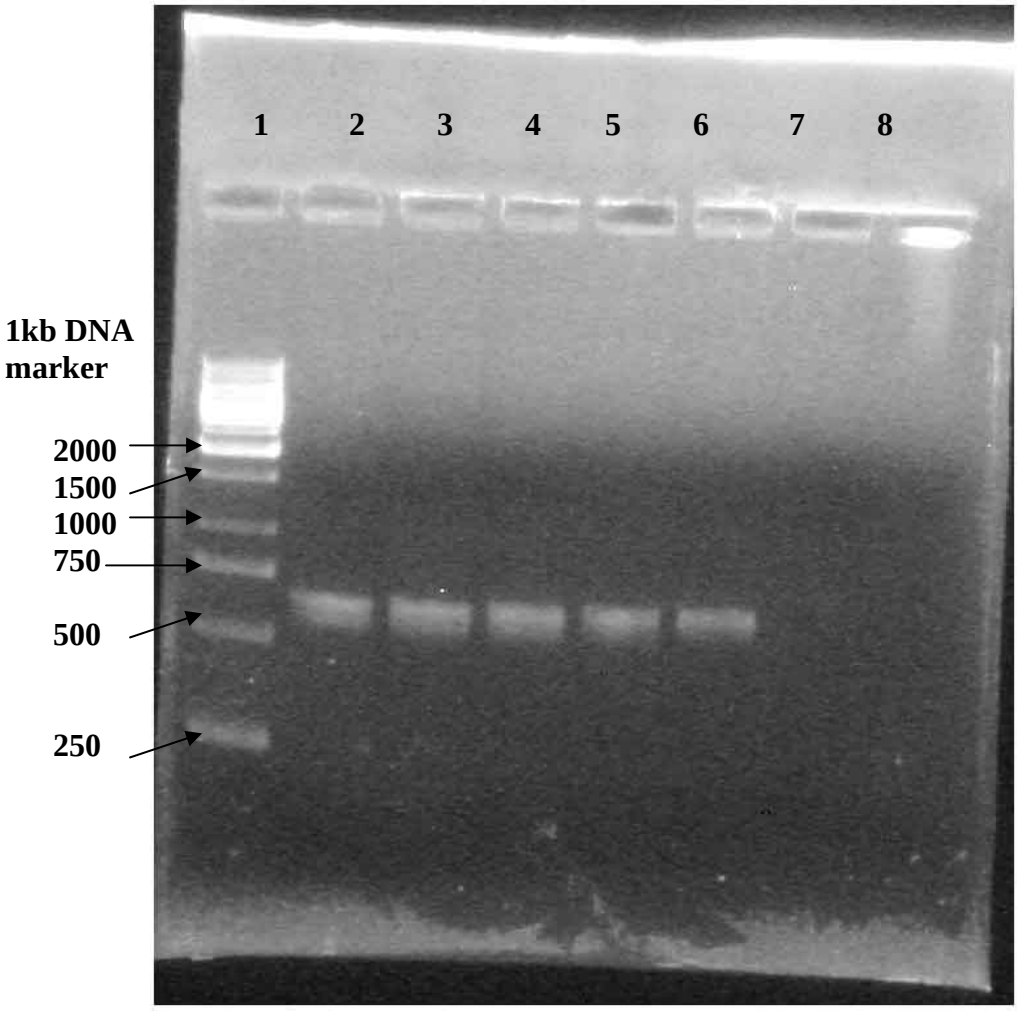


Image 4: PCR amplification of the *Bacillus subtilis* SK320 chromosomal DNA using *Bacillus* gene specific primers.
Lane 1: 1kb DNA marker,
Lane 2: *B. subtilis* SK320 chromosomal DNA amplified using sfp primers,
Lane 3,4: *B. subtilis* SK320 chromosomal DNA amplified using sfp0 primers,
Lane 5,6: *B. subtilis* SK320 chromosomal DNA amplified using srfA primers.

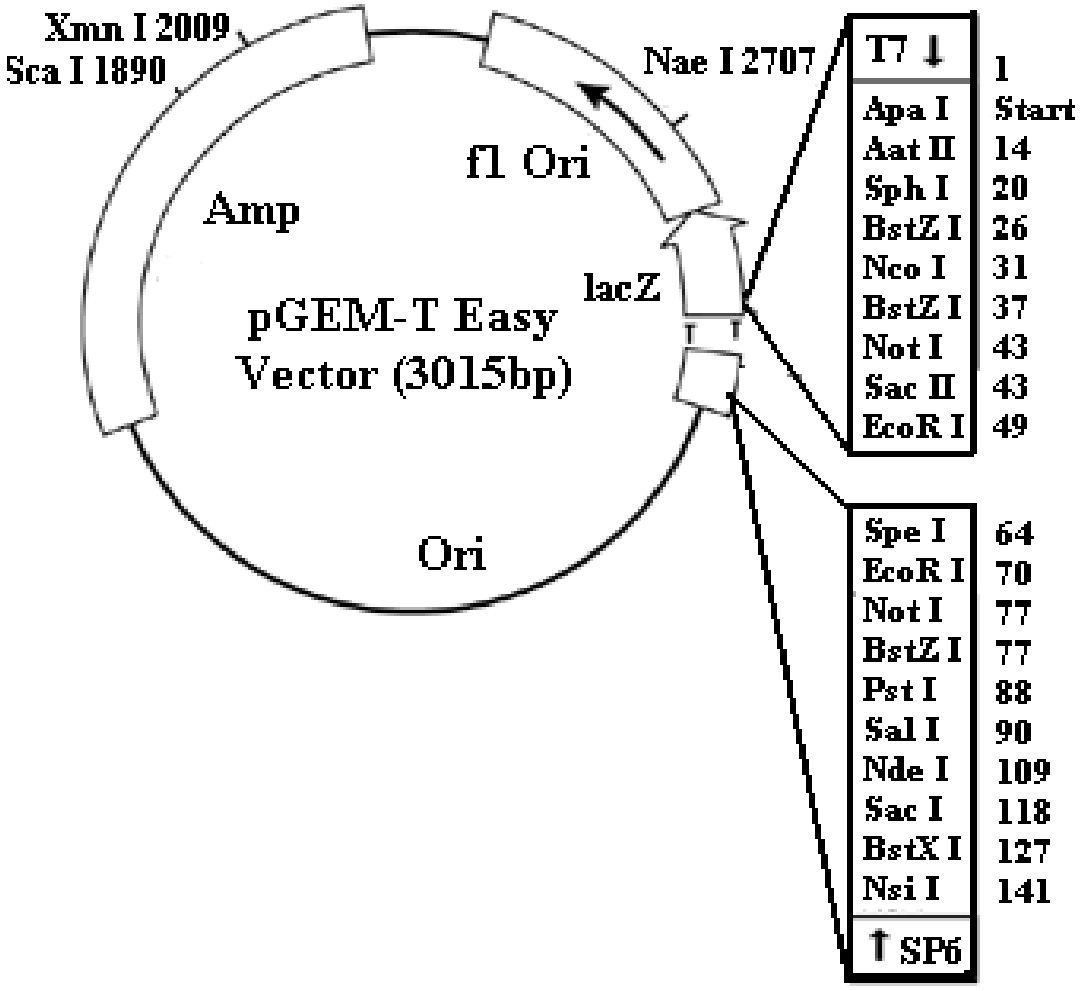


Image 5: pGEM-T easy vector circle map and sequence reference points. The multiple cloning site, is flanked by restriction enzyme sites for BstZ I, Not I, EcoR I, allowing three options for removal of the insert with a single digest.

Courtesy: www.promega.com

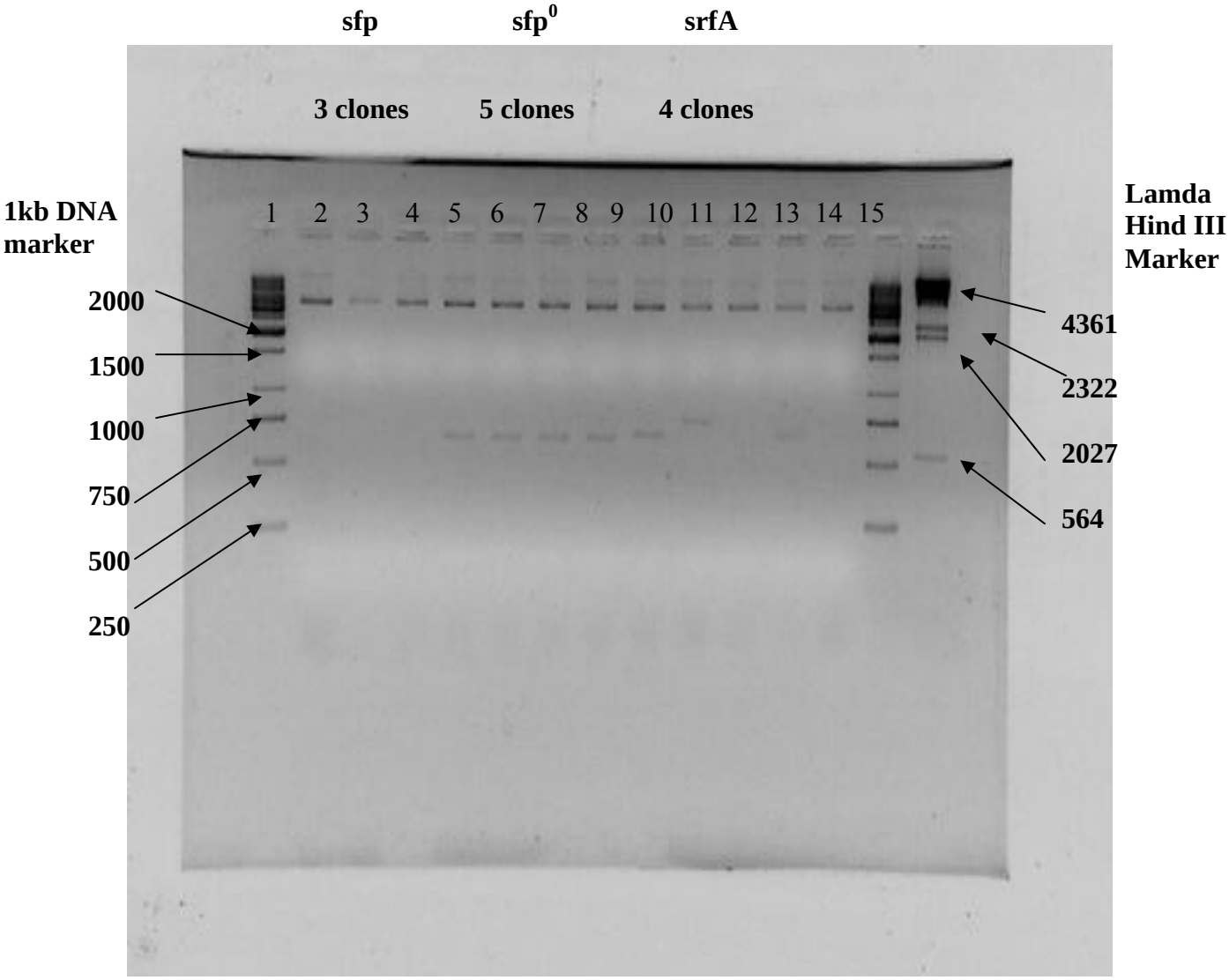


Image 6: Restriction digestion of the isolated plasmid DNA with EcoR1 (MBI Fermentas) enzyme. Lane 1= 1kb DNA marker, lane 2 to 4= sfp clones, lane 5 to 9= sfp^0 clones, lane 10 to 13= srfA clones, lane 14= 1kb DNA marker, lane 15= lambda Hind III marker.

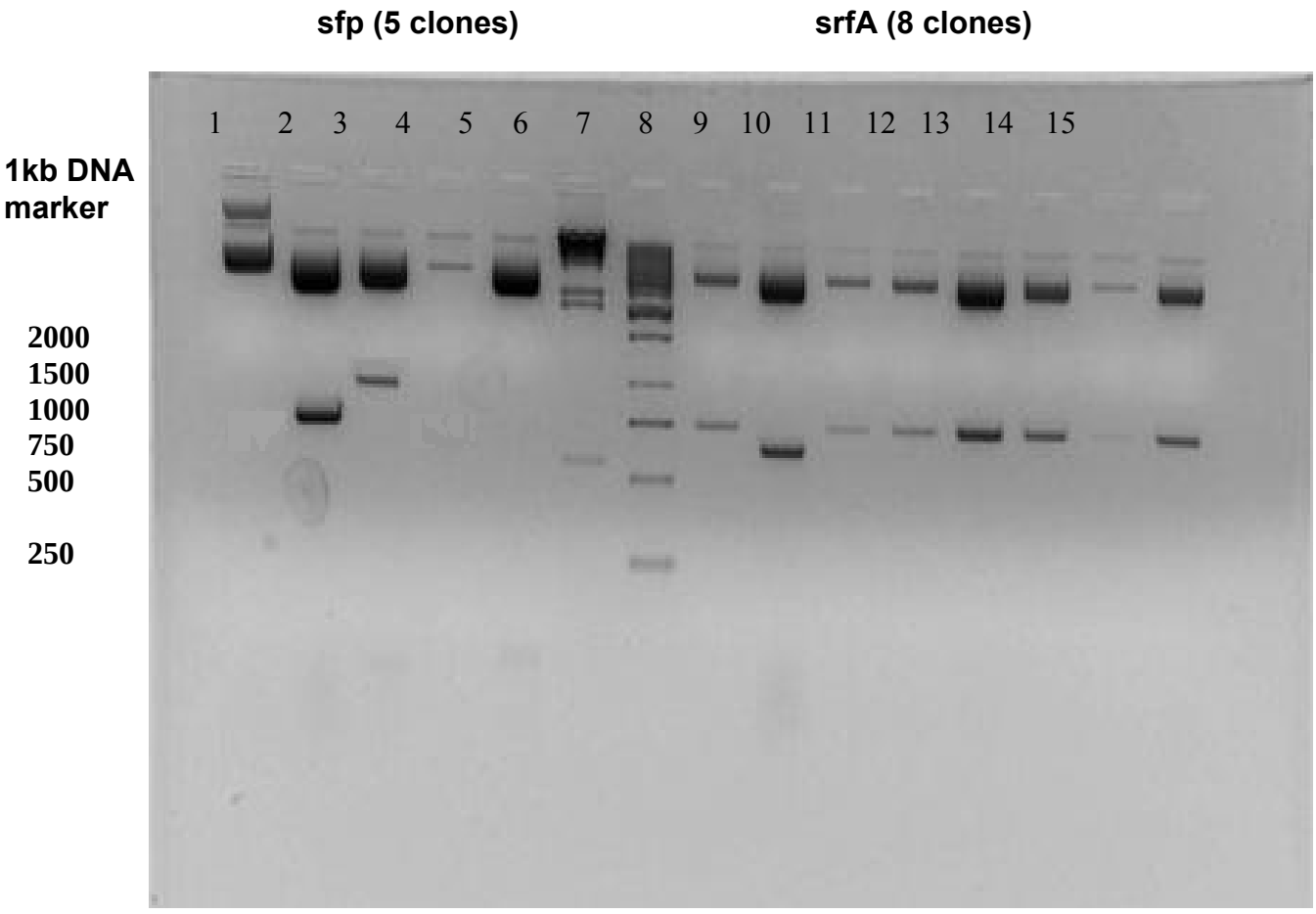


Image 7: Restriction digestion of the isolated plasmid DNA with EcoR1 (MBI Fermentas) enzyme. Lane 1 to 5= sfp clones, lane 6= lambda Hind III marker, lane 7= 1kb DNA marker, lane 8 to 15 = srfA clones. sfp and srfA clones are repeated in this gel as we could not obtain any result in the gel shown in picture.

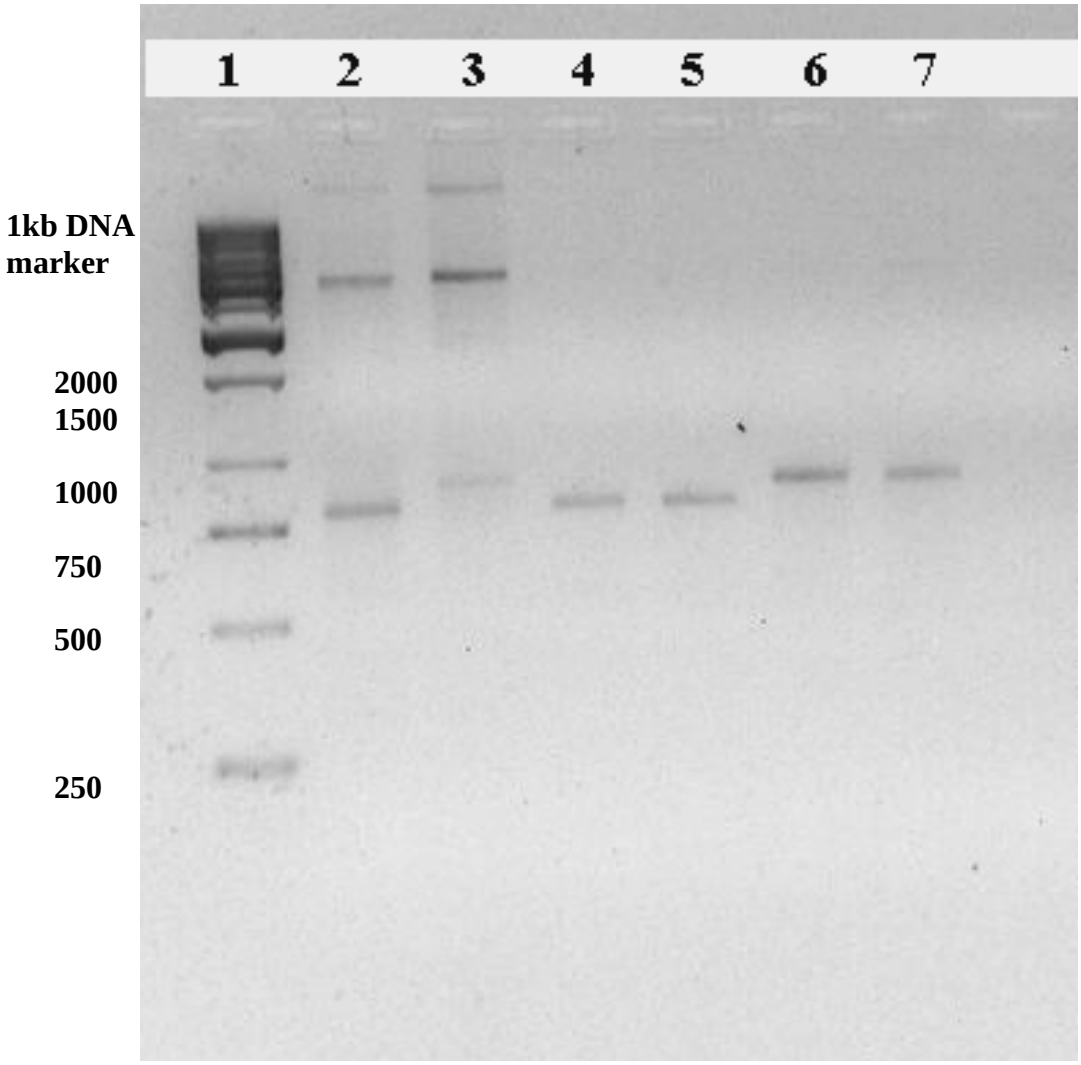


Image 8: PCR amplification of the transformants using T7SP6 primers (provided with pGEM-T easy vector kit). Lane 1: 1kb DNA marker, Lane 2, 3: srfA clones, Lane 4, 5: sfp0 clones, Lane 6, 7: sfp clones.

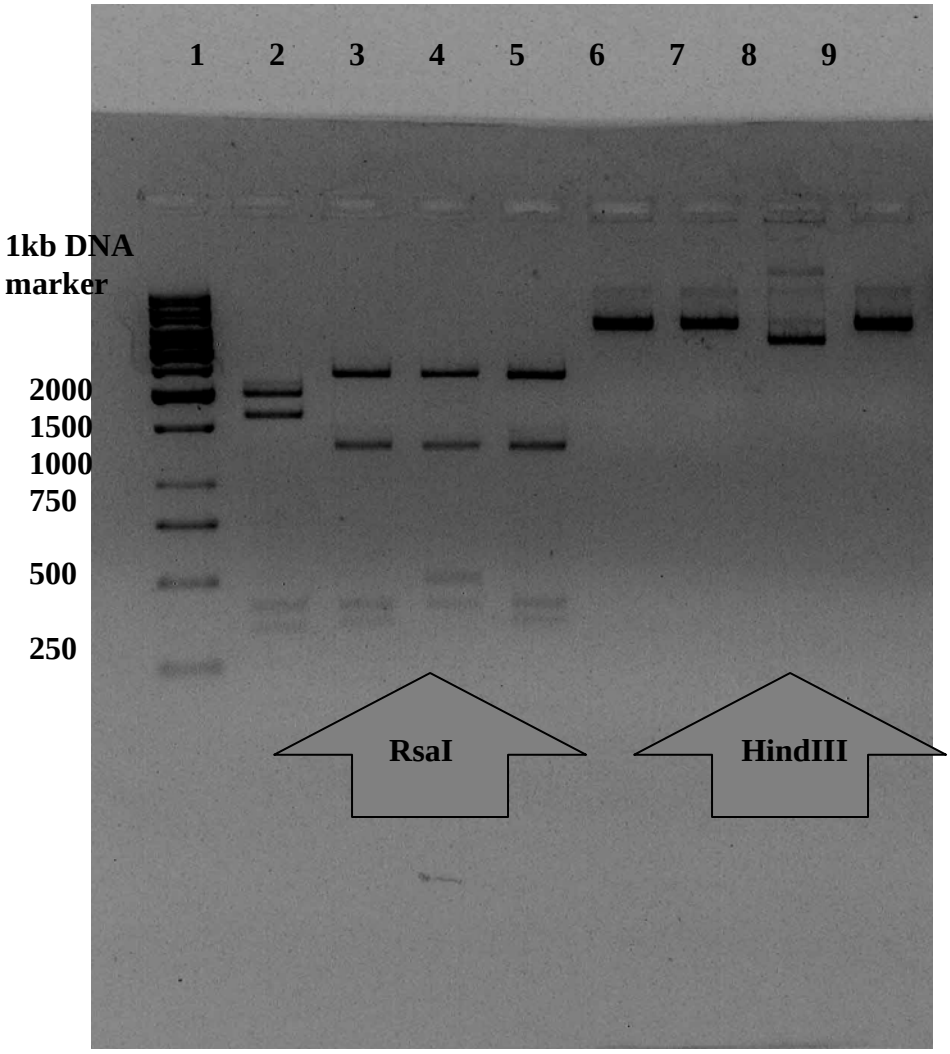


Image 9: Restriction digestion of the isolated plasmid DNA with RsaI (tetracutter) and HindIII (hexacutter) enzymes (MBI Fermentas). Lane 1: 1kb DNA marker, Lane 2: sfp clone, Lane 3: srfA clone, Lane 4,5: sfp0 clones, Lane 6: sfp clone, Lane 7: srfA clone, Lane 8,9: sfp0 clones. RsaI gave four bands for all the clones, respectively; whereas no banding pattern was observed with HindIII enzyme.

4.8 Expression of biosurfactant gene (s)

Gene expression was observed by growing the *E. coli* clones or transformants on basal medium supplemented with 0.5% (v/v) olive oil as the substrate and ampicillin as the selective antibiotic. For making solid media 1.5 to 2% agar was added to the above mentioned liquid broth. The *E. coli* clones pSKP, pSKP0, pSKA, and the parent *Bacillus subtilis* SK320 were grown on the media for 5 days under similar culture conditions of 37^0C and 120 rpm. Growth, biosurfactant activity and esterase activity were estimated in the culture supernatant spectrophotometrically at a regular interval of 24hrs.

As observed in Fig 23, 24 and 25, when the cultures were grown in basal medium the growth was observed to be maximum in the *Bacillus subtilis* SK320 followed by the three clones, respectively. Growth was found to be negligible in the *E.coli* DH5α strain when it was grown on basal medium containing olive oil indicating that *E.coli* DH5α was unable to utilize olive oil as carbon source. The biosurfactant activity in the clones pSKP, pSKP0 and pSKA was found to be more than then the parent *Bacillus subtilis* SK320. From the data it was also clear that the biosurfactant gene was not only successfully expressed but showed over-expression in the respective clones giving almost double the activity shown by *Bacillus subtilis* SK320. The biosurfactant activity increased in the first 24hrs giving the maximum activity between 48 to 72hrs. Maximum biosurfactant activity was observed in case of pSKP0 followed by pSKP. After 72hrs biosurfactant activity started showing a decline reaching a minimum value at 120hrs. Olive oil supplemented in the culture media was found to be completely emulsified by the end of 120hrs. Cloning of the biosurfactant gene from *Bacillus subtilis* SK320 into *E.coli* not only resulted in the expression of the biosurfactant activity but conferred esterase activity in the *E.coli* clones as well. Esterase activity was found to be maximum in all the clones then the parent strain. A correlation between the biosurfactant and esterase activities can also be noticed from the data i.e. both the biosurfactant and esterase activities increased with the increase in the incubation period of the culture medium and were found to be maximum between 48 to 72 hrs, after which there was a reduction in the activities. There was no apparent biosurfactant and esterase activity found in the parent *E.coli* DH5α.

The results suggested that the gene(s) were successfully expressed rather over-expressed in the *E.coli* DH5α cells.

Source	**Biosurfactant activity (OD 550nm)**	**Esterase activity (IU ml^{-1})**	**Growth (OD 600 nm)**
Bacillus subtilis SK320	0.859	4.382	0.514
E.coli Clone pSKP	1.354	8.293	0.351
E.coli Clone pSKP0	1.259	8.521	0.296
E.coli Clone pSKA	1.198	8.465	0.489
E. coli DH5α	0.157	0.101	0.132

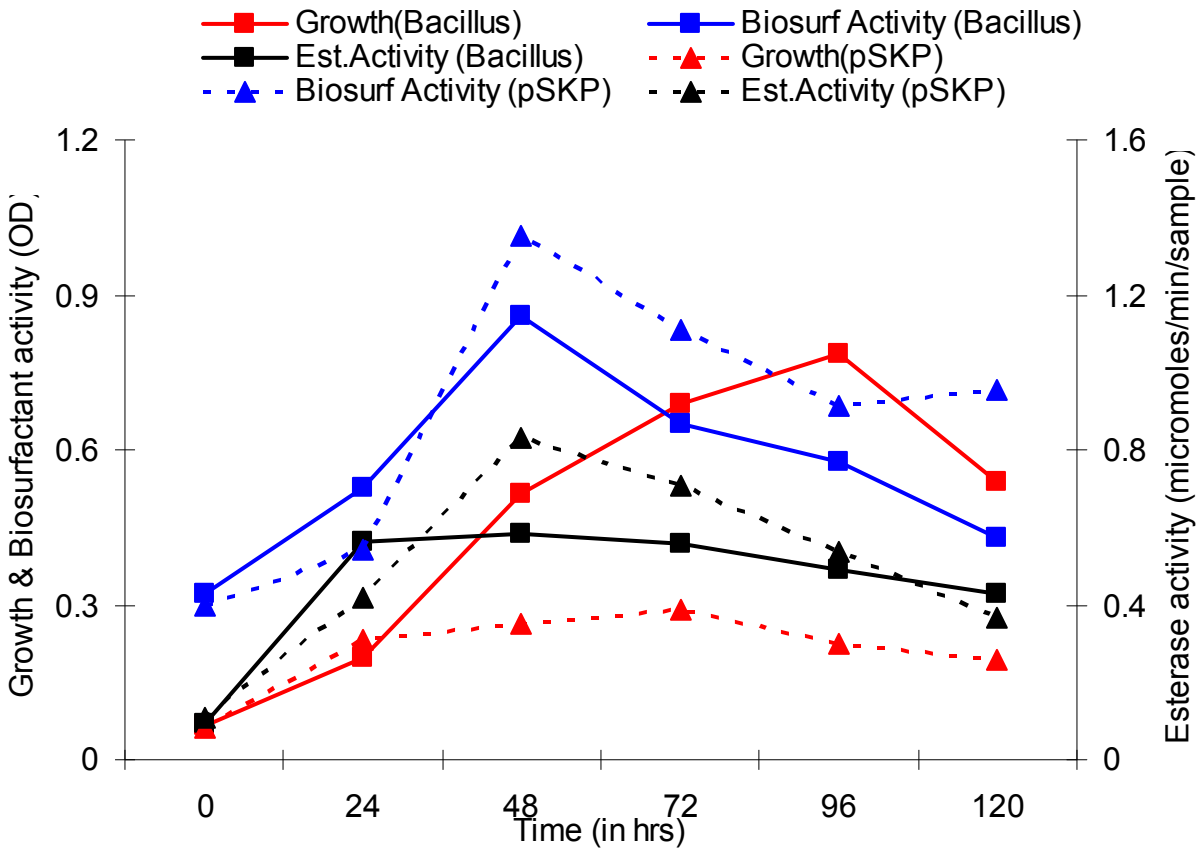


Fig 23: Growth, biosurfactant and esterase activity of *Bacillus subtilis* SK320 and clone *E. coli* pSKP.

***Bacillus subtilis* SK320 and clone *E. coli* pSKP were grown on basal medium with 0.5% (v/v) olive oil as sole carbon source. Growth, biosurfactant activity and esterase activity were analyzed at 600nm, 550nm and 405nm, respectively (Section 3.5 & 3.7).**

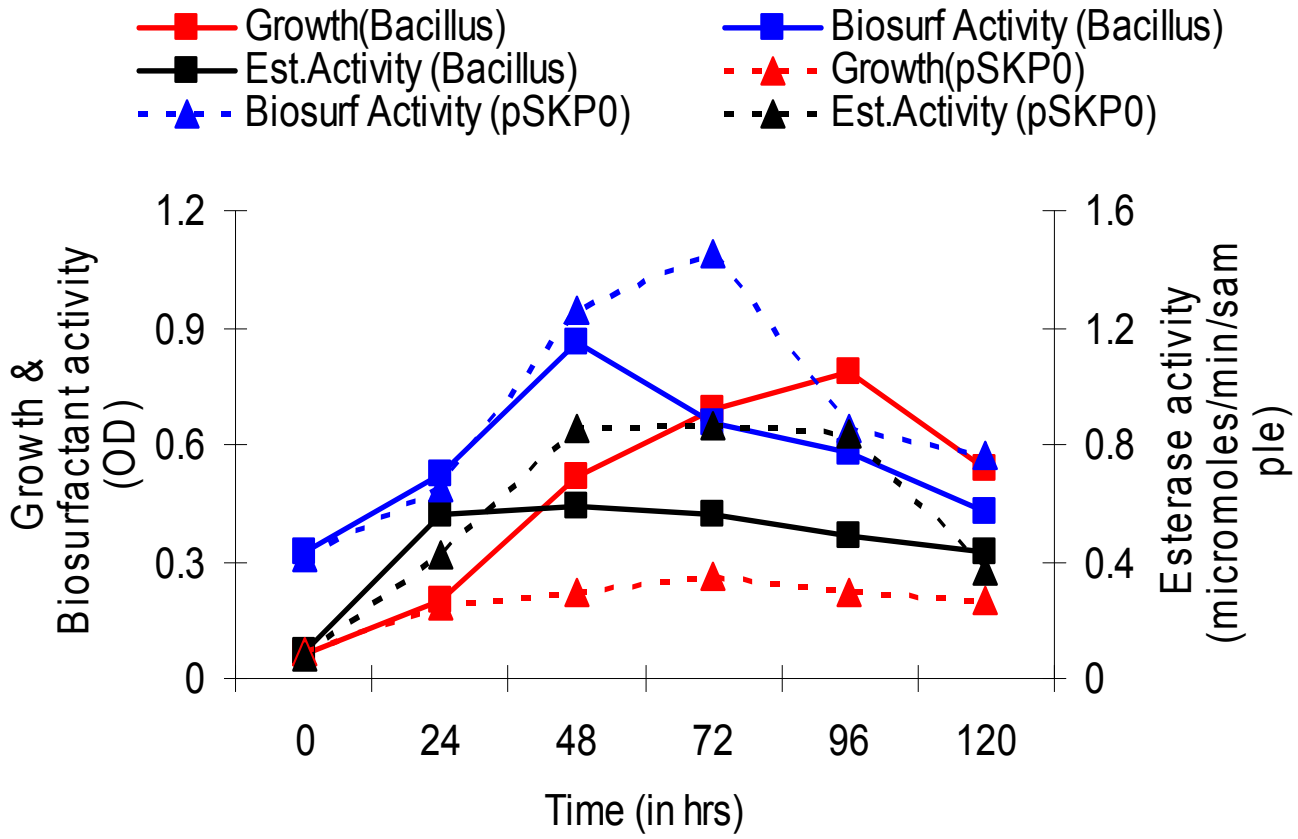


Fig 24: Growth, biosurfactant and esterase activity of *Bacillus subtilis* SK320 and clone *E. coli* pSKP0.

***Bacillus subtilis* SK320 and clone *E. coli* pSKP0 were grown on basal medium with 0.5% (v/v) olive oil as sole carbon source. Growth, biosurfactant activity and esterase activity were analyzed at 600nm, 550nm and 405nm, respectively (Section 3.5 & 3.7).**

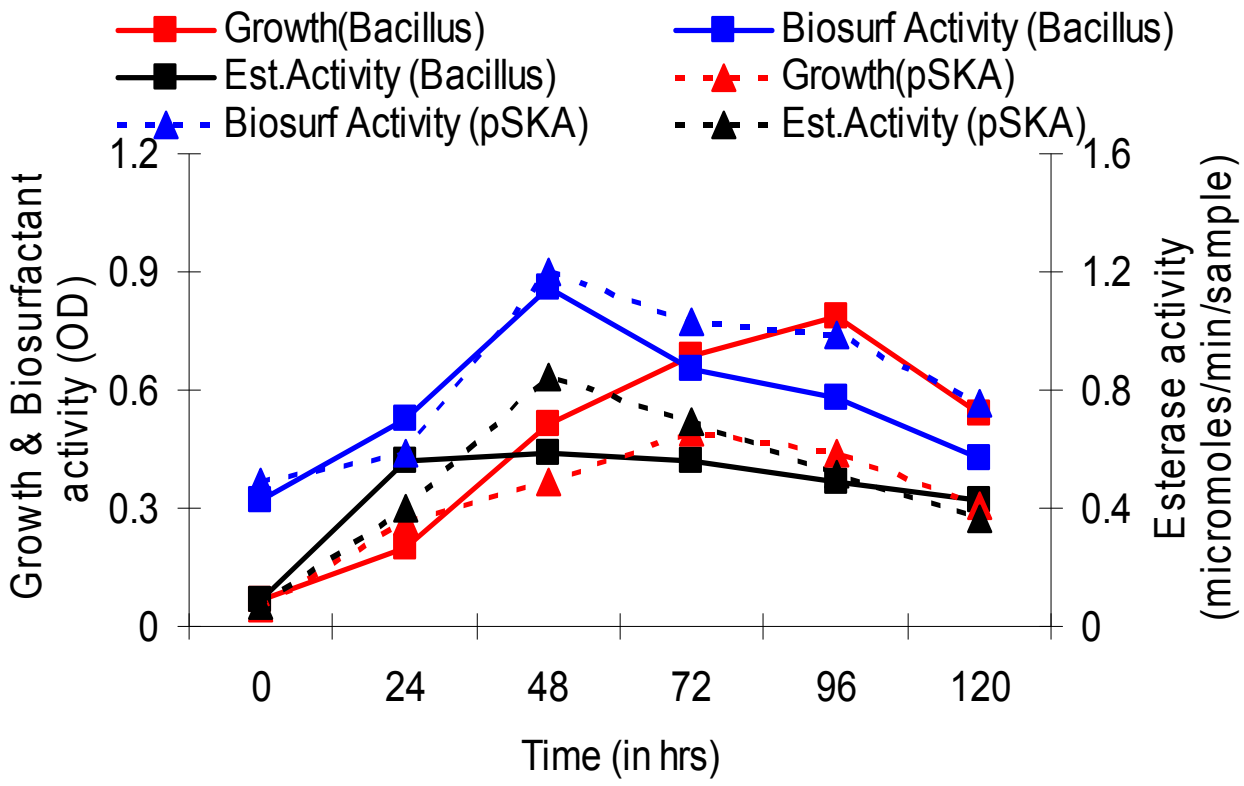


Fig 25: Growth, biosurfactant and esterase activity of *Bacillus subtilis* SK320 and clone *E. coli* pSKA.

***Bacillus subtilis* SK320 and clone *E. coli* pSKA were grown on basal medium with 0.5% (v/v) olive oil as sole carbon source. Growth, biosurfactant activity and esterase activity were analyzed at 600nm, 550nm and 405nm, respectively (Section 3.5 & 3.7).**

4.9 Automated DNA Sequencing

The genes *sfp* (667bp), *sfp0* (642bp) and *srfA* (707bp) were sequenced using Applied Biosystems DNA Sequencer and submitted to National Center for Biotechnology Information (USA). Gene bank accession numbers are: EU822921 (*sfp*), EU822922 (*sfp0*) & EU822923 (*srfA*). The data is available on websites: EMBL in Europe and DNA Data Bank of Japan. The deduced amino acid sequences are shown in Image 10.

The DNA sequences were used to infer functional and evolutionary relationships between sequences in the database and to identify members of gene families using National Center for Biotechnology Information's BLAST (http://www.ncbi.nlm.nih.gov/BLAST) facility. The results revealed sequence similarity of the *sfp, sfp0* and *srfA* genes with *Bacillus subtilis* surfactin synthetase gene, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus megaterium, Bacillus subtilis* srfAA, several lipases from yeast, filamentous fungi to name a few (Image 11, 12, 13). In addition much weaker similarities were also observed. Based on the matching sequences found with BLAST, it was estimated that the greatest overall similarity (99.0%) was with biosurfactant and esterase genes of *Bacillus subtilis.*

Based on the above results the multiple alignment was also performed using MultAlin tool on website: http://prodes.toulouse.inra.fr/multalin/multalin.html in order to access the degree of similarity of the sequenced biosurfactant genes with the esterase gene sequences in the NCBI database. The deduced amino acid sequences from *sfp, sfp0* and *srfA* biosurfactant genes were aligned with two of the esterase gene sequences viz. *Bacillus* sp. NK13 esterase gene (Liu *et al.* 2005, Unpublished, PubMed Accession number: DQ196347) and *Bacillus clausii* KSM-K16 esterase gene (Hakamada *et al.* 1994, PubMed Accession number: AP006627) (Image 14). The results indicated similarity and conserved sequences were observed between the biosurfactant and esterases genes, confirming our results of a possible correlation between the two activities.

Accession # EU822921 (sfp gene)
Size: 1210bp

TATGAGCGCGGGTCAAGCTGTGAGGGCCATTATAAAAGCTTTCGTACGAGCCATGTATATCTGGGGAAATCAG
GGGTCGGGCATCACCATTTGTACCGGGATGCTCATGTTGATATAGCATGGGGAAGGCTGTCCGGAAGCTCAAT
GGATACTTGTCCGTCTGGGCAGGGCTGAAAAGGAATCAAGCGGAAGCGATAAGCCTTGCCTTCCGTTTGATAA
AGCTTTCTTCATGACCATAGATGATAAAAATAGTCTGTTGCTCGTCCTTGTTTTGTAAAAGGTCGCTGTACTC
TGTTTTTGAAAAGAAGCGCTTGGCGATCTCAAGGCTGATCGGTTTGTTTTTCGATATTATGCCGATCGGCTGT
GGATCAAACGCGCAAATGACCCAGCGTCCGGAGTGAGAAATGTGAAATGAGCGTCGGGAAGATCAGGGATGCA
CGGCTTCCCGTATCGTGCGGTAAAGCGGAATCGGATTGTCCATTTGGTTCGTTTCATTATAAGCAGGCCAGTT
TCATTGGGCAAATCTCCTGAATTTGCTTTAGCCACGCAGGGTTACGGGAAACCCGTGCATCCCTGATTTTCCC
CGACGTTCATTTAAACATTTTTCATTCCGGCGCTGGGTCATTTGCGCGTTTGATCCCCAGCCATCGGCATATA
TATTGAAAAAAACGAAACCGATCAGCCTGAGATCGCCAAGCGTTTCTTTTCAAAAACAGAGTACAGCGACCTT
TTAGCAAAAAGACAAGGACGAGCAGACAGACTATTTTATCATTATGGTCAATGAAAGAAAGCTTTATCAAACA
GGAAGGCAAAGGCTTATCGTTCCGCTTGATTCCTTTCAGTGCGCTGCACCAGGACGGACAAGTATCCATTGAG
CTTCCGGACAGCCATTCCCCATGTATATCAAAACGTATGAGGTCGATCCCGGCTACAAAATGGCTGTATGCGC
CGTACACCCTGATTTCCCCGAGGATATCACAATGGTCTCGTACGAAGAGCTTTTATAAATGGCCCATCAACAG
CTTGACACCGCGCTCAATATCTTCCGTTTTCACATTGGAAATATTGATTTTTAATAGATTTTCTTTCGGATAA
TCTGATAAATAATGACGGTCTATCGCCTCAAGGAGAACCCCTTGTTTTTCCAGTCTATGAATCACTCTTGAGG
CGGGCAGATCCTGAGGAAGCACCAGATGGGTGTGCATACAGG

Accession # EU822922 (sfp0 gene)
Size: 642bp

GGGGAATTCAGGGTGTGCGGCGCATACAGCCATTTTGTAGCCGGGATCGACCTCATACGTTTTGATATAGCAT
GGGGAATGGCTGTCCGGAAGCTCAATGGATACTTGTCCGTCCTGGTGCAGGCGCACTGAAAAGGAATCAAGCG
GAAGCGATAAGCCTTTGCCTTCCTGTTTGATAAAGCTTTCTTTCATTGACCATAGATGATAAAAATAGTCTGT
CTGCTCGTCCTTGTCTTTTGCTAAAAGGTCGCTGTACTCTGTTTTTGAAAAGAAGCGCTTGGCGATCTCAAGG
CTGATCGGTTTCGTTTTTTCGATATCTATGCCGATCGGCTGTGAATCAAACGCGCAAATGACCCAGCGTCCGG
AGTGAGAAATGTTGAAATGAGCGTCGGGAAGATCAGGGATGCACGGCTTCCCGTATTCCTGCGTGCTAAAGCG
GATATCGGATTTGTCCAACTGATACTGCCTGCTTATGACTGAGCGAACGAGCACATCTCCCAGCAGGGTGCGG
TGAGCATCTTCTTTATGATAAAATCTCCGGCATTTCTCCCGTTTTTCAGGTGATATGAAAGACATGAACCGTT
CATTTTCTTCCTGTGAAAGCGGGCGGTCCATATAAATTCCGTAAATCTGAATTCTAGA

Accession # EU822923 (srfA gene)
Size: 727bp

TAAAGGAAAATGAAGTGTGGTTTTACGCAAATGTTCATCACGTGATTTCTGATGGTATCTCCATGAATATTCT
CGGGAATGCGATCATGCACATTTATTTAGAATTAGCCAGCGGCTCAGAGACAAAAGAAGGAATCTCGCATTCA
TTTATCGATCATGTTTTATCTGAACAGGAATATGCTCAATCGAAGCGGTTTGAAAAGGACAAGGCGTTTTGGA
ACAAACAATTTGAATCGGTGCCTGAACTTGTTTCCTTGAAACGGAATGCATCCGCAGGGGGAAGTTTAGATGC
TGAGAGGTTCTCTAAAGATGTGCCTGAAGCGCTTCATCAGCAGATTCTGTCGTTTTGTGAGGCGAATAAAGTC
AGTGTTCTTTCGGTATTTCAATCGCTGCTCGCCGCCTATTTGTACAGGGTCAGCGGCCAGAATGATGTTGTGA
CGGGAACATTTATGGGCAACCGGACAAATGCGAAAGAGAAGCAGATGCTTGGCATGTTTGTTTCTACGGTTCC
GCTTCGGACAAACATTGACGGCGGGCAGGCGTTTTCAGAATTTGTCAAAGACCGGATGAAGGGATCTGATGAA
GACACTTCGCCACCAAAAGTATCCGTATAATCTCCTAATCAACGATTTGCGTGAAACAAAGAGCTCTCTGACC
AAGCTGTTCACGGTTTCTCTTGAATATCAAGTGATGCAGTGGCAGAAAGAAATCACTAGTGAATTCGCGG

Image 10: Data showing the three biosurfactant gene sequences that have been submitted to NCBI, USA with the respective accession numbers.

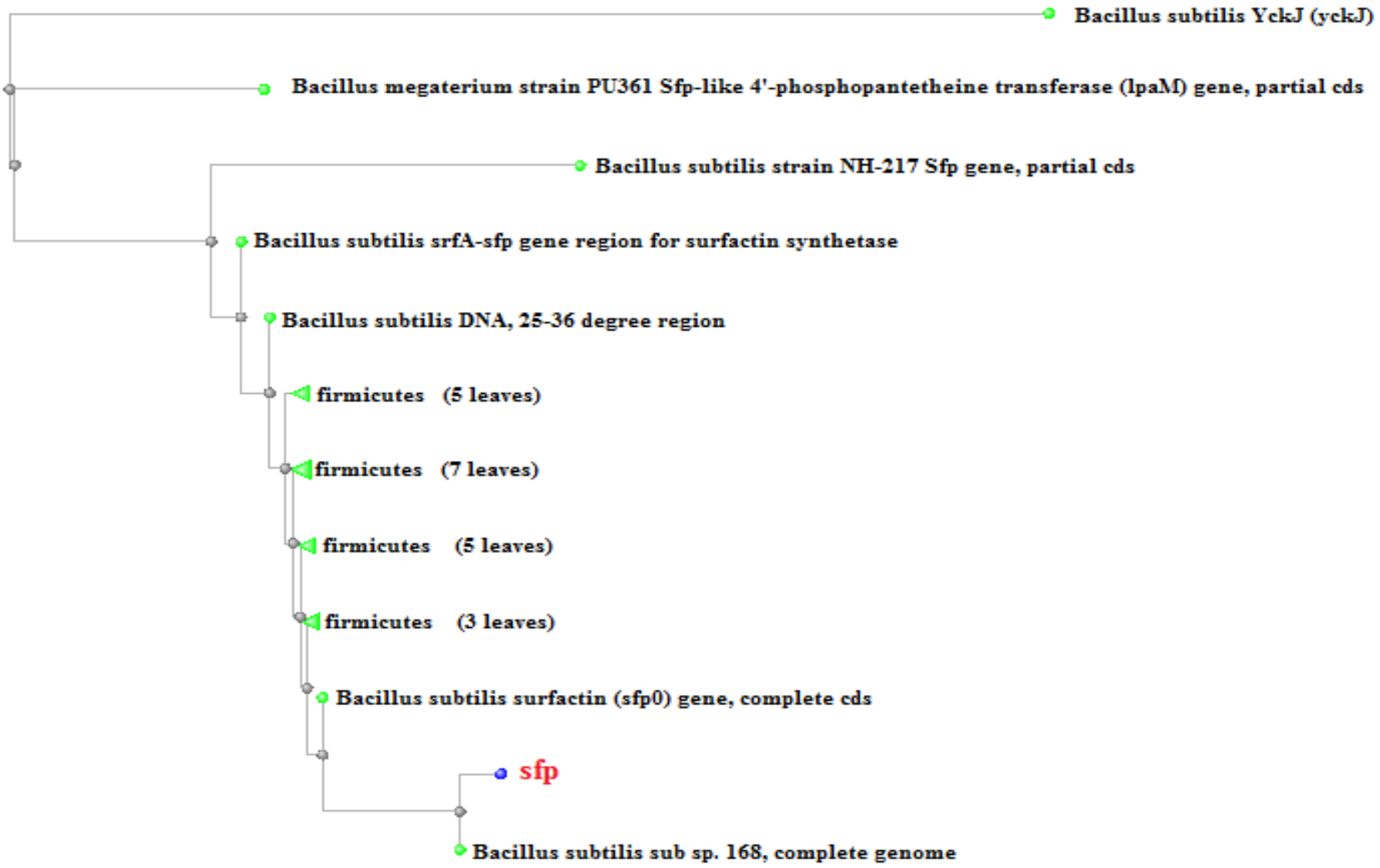


Image 11 (a): Dendogram showing phylogenetic analysis of *sfp* deduced amino acid gene sequence using BLAST.

Sequences producing significant alignment with gene *sfp* (1210bp)	% Identity
Bacillus subtilis subsp. *subtilis* str. 168 complete genome	94%
Bacillus subtilis surfactin (sfpo) gene, complete cds	94%
B.subtilis srfA-sfp gene region for surfactin synthetase	93%
Bacillus subtilis DNA, 25-36 degree region	93%
B.subtilis sfp gene	93%
Bacillus subtilis lpa-8 gene essential for biosynthesis of the lipopeptide antibiotics plipastatin B1 and surfactin, complete cds	93%
Bacillus subtilis sfp-0 gene for inactive surfactin production protein, complete cds	94%
Bacillus subtilis subsp. *subtilis* strain ATCC 6051 YcxD (ycxD) gene, partial cds; and Sfp (sfp) gene, complete cds	93%
Bacillus subtilis subsp. *subtilis* strain NCIB 3610 YcxD (ycxD) gene, partial cds; and Sfp (sfp) gene, complete cds	93%
Bacillus subtilis subsp. *subtilis* strain 166 YcxD (ycxD) gene, partial cds; & Sfp (sfp) gene, sfp0 allele, complete cds	93%
Bacillus subtilis subsp. *subtilis* strain 160 YcxD (ycxD) gene, partial cds; & Sfp (sfp) gene, sfp0 allele, complete cds	93%
Bacillus subtilis subsp. *subtilis* strain 122 YcxD (ycxD) gene, partial cds; & Sfp (sfp) gene, sfp0 allele, complete cds	93%
Bacillus subtilis subsp. *subtilis* strain 168 YcxD (ycxD) gene, partial cds; & phosphopantetheinyl transferase (sfp) gene, sfp0 allele, complete cds	93%
Bacillus subtilis strain 96-41 Sfp (sfp) gene, complete cds	92%
Bacillus subtilis strain NH-160 Sfp gene, partial cds	91%
Bacillus subtilis strain NH-100 Sfp gene, partial cds	92%
Bacillus subtilis subsp. *subtilis* Sfp (sfp) gene, partial cds	89%
Bacillus sphaericus strain ATCC 14577 Sfp-like 4'-phosphopantetheine transferase (lpaSp) gene, partial cds	92%
Bacillus amyloliquefaciens strain BE71 Sfp-like 4'-phosphopantetheine transferase (lpaA) gene, partial cds	91%
Bacillus subtilis strain DSM 2109 Sfp-like 4'-phosphopantetheine transferase (lpaS) gene, partial cds	91%
Bacillus subtilis strain DSM 1088 Sfp-like 4'-phosphopantetheine transferase (lpaS) gene, partial cds	90%
Bacillus subtilis strain NH-217 Sfp gene, partial cds	88%
Bacillus subtilis YckJ (yckJ), YckI (yckI), YczE (yczE), and phosphopantetheinyl transferase (sfp) genes, complete cds	82%
Bacillus megaterium strain PV361 Sfp-like 4'-phosphopantetheine transferase (lpaM) gene, partial cds	89%

Image 11 (b): Sequences showing significant similarity with *sfp* gene.

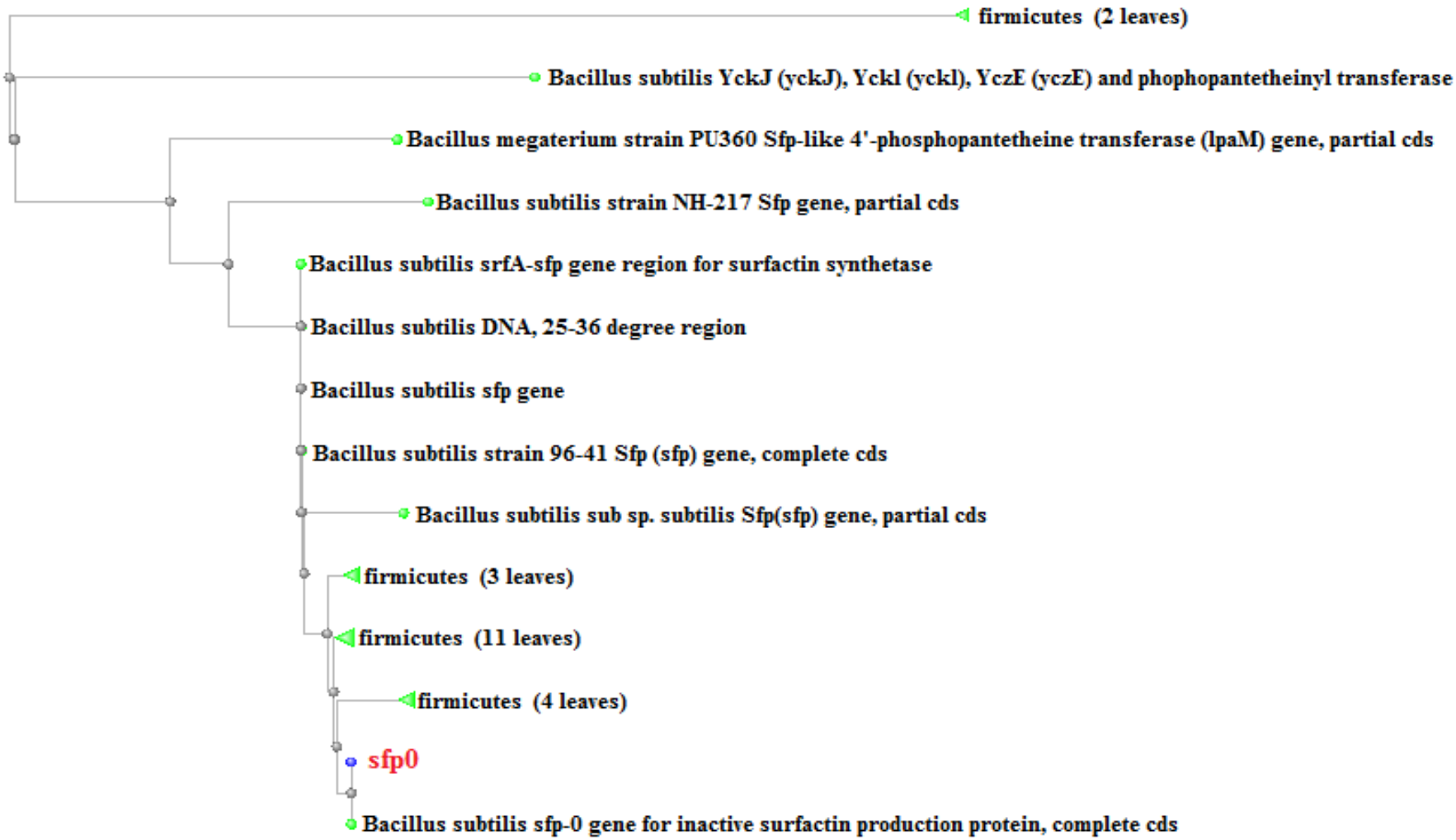


Image 12 (a): Dendogram showing phylogenetic analysis of *sfp0* deduced amino acid gene sequence using BLAST.

Sequences producing significant alignment with gene *sfp0* (642bp)	% Identity
Bacillus subtilis subsp. *subtilis* strain ATCC 6051 YcxD (ycxD) gene, partial cds; and Sfp (sfp) gene, complete cds	99%
Bacillus subtilis subsp. *subtilis* strain NCIB 3610 YcxD (ycxD) gene, partial cds; and Sfp (sfp) gene, complete cds	99%
Bacillus subtilis subsp. *subtilis* str. 168 complete genome	99%
Bacillus subtilis subsp. *subtilis* strain 166 YcxD (ycxD) gene, partial cds; and Sfp (sfp) gene, sfp0 allele, complete cds	99%
Bacillus subtilis subsp. *subtilis* strain 160 YcxD (ycxD) gene, partial cds; and Sfp (sfp) gene, sfp0 allele, complete cds	99%
Bacillus subtilis subsp. *subtilis* strain 122 YcxD (ycxD) gene, partial cds; and Sfp (sfp) gene, sfp0 allele, complete cds	99%
Bacillus subtilis subsp. *subtilis* strain 168 YcxD (ycxD) gene, partial cds; and phosphopantetheinyl transferase (sfp) gene, sfp0 allele, complete cds	99%
Bacillus subtilis surfactin (sfpo) gene, complete cds	99%
Bacillus subtilis sfp-0 gene for inactive surfactin production protein, complete cds	98%
B.subtilis sfp gene	98%
B.subtilis sfp gene	99%
Bacillus subtilis lpa-8 gene essential for biosynthesis of the lipopeptide antibiotics plipastatin B1 and surfactin, complete cds	98%
Bacillus subtilis strain 96-41 Sfp (sfp) gene, complete cds	99%
B.subtilis srfA-sfp gene region for surfactin synthetase	98%
Bacillus subtilis DNA, 25-36 degree region	98%
Bacillus subtilis subsp. *subtilis* Sfp (sfp) gene, partial cds	97%
Bacillus subtilis strain NH-100 Sfp gene, partial cds	99%
Bacillus subtilis strain NH-160 Sfp gene, partial cds	98%
Bacillus sphaericus strain ATCC 14577 Sfp-like 4'-phosphopantetheine transferase (lpaSp) gene, partial cds	97%
Bacillus amyloliquefaciens strain BE71 Sfp-like 4'-phosphopantetheine transferase (lpaA) gene, partial cds	97%
Bacillus subtilis strain DSM 1088 Sfp-like 4'-phosphopantetheine transferase (lpaS) gene, partial cds	97%
Bacillus subtilis strain DSM 2109 Sfp-like 4'-phosphopantetheine transferase (lpaS) gene, partial cds	97%
Bacillus subtilis strain NH-217 Sfp gene, partial cds	94%
Bacillus megaterium strain PV361 Sfp-like 4'-phosphopantetheine transferase (lpaM) gene, partial cds	93%
Bacillus subtilis YckJ (yckJ), YckI (yckI), YczE (yczE), and phosphopantetheinyl transferase (sfp) genes, complete cds	84%
Bacillus amyloliquefaciens strain 96-79 Sfp (sfp) gene, complete cds	74%
Bacillus amyloliquefaciens strain 96-82 Sfp (sfp) gene, complete cds	74%

Image 12 (b): Sequences showing significant similarity with *sfp0* gene.

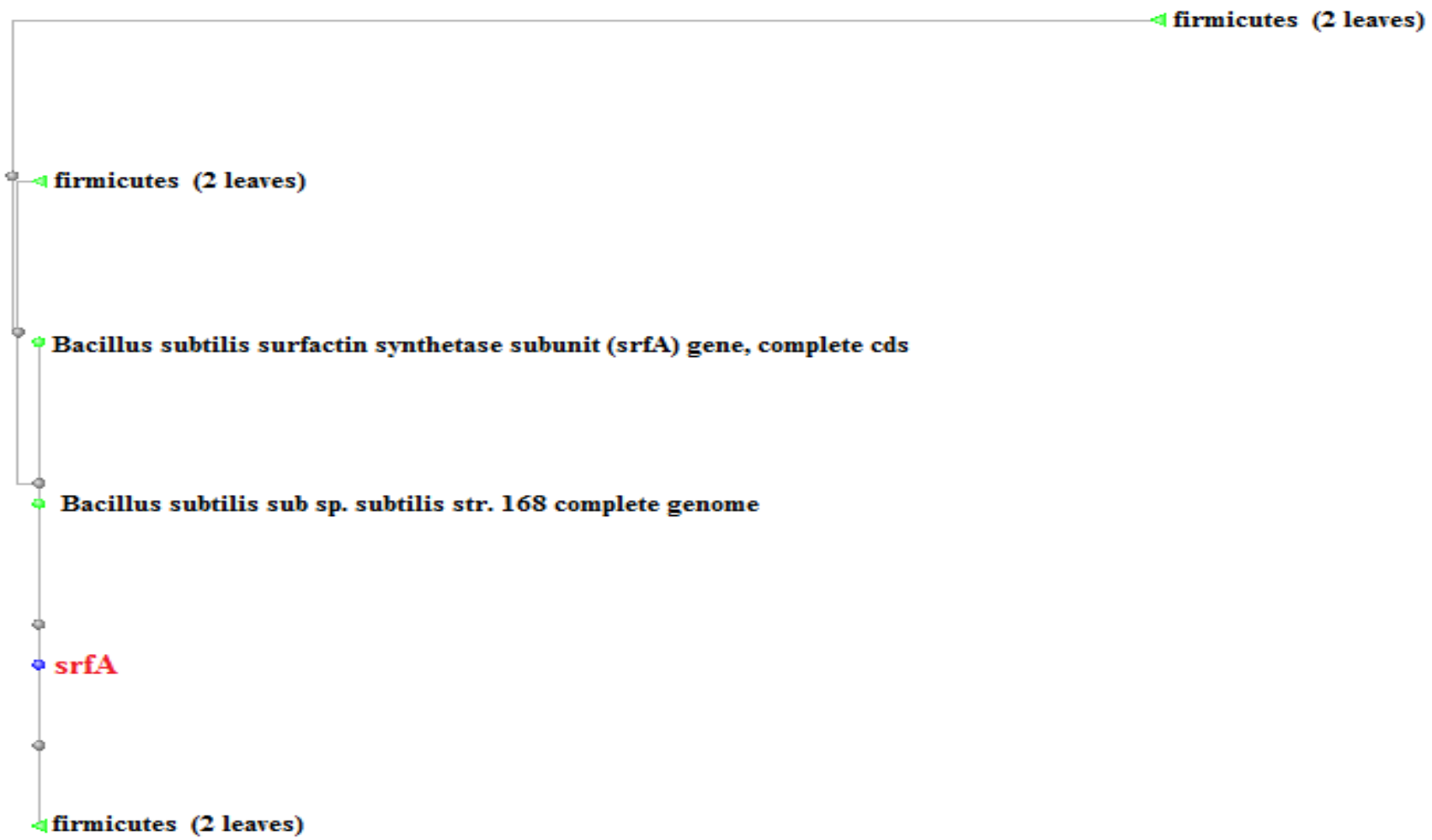


Image 13 (a): Dendogram showing phylogenetic analysis of *srfA* deduced amino acid gene sequence using BLAST.

Sequences producing significant alignment with gene *srfA* (727bp)	% Identity
Bacillus subtilis subsp. subtilis str. 168 complete genome	99%
B.subtilis surfactin synthetase subunit (srfA) gene, complete cds	99%
Bacillus subtilis srfAA and srfAB genes for surfactin synthetase, complete and partial cds	99%
B.subtilis srfA-sfp gene region for surfactin synthetase	99%
Bacillus subtilis DNA, 25-36 degree region	99%
Bacillus subtilis strain 1D1 recombinant surfactin/lichenysin A synthetase (PsrfA-srfAA-lchAA) gene, partial cds	92%
Bacillus subtilis strain 1B1 recombinant surfactin/lichenysin A synthetase (PsrfA-srfAA-lchAA) gene, partial cds	92%

Image 13 (b): Sequences showing significant similarity with *srfA* gene.

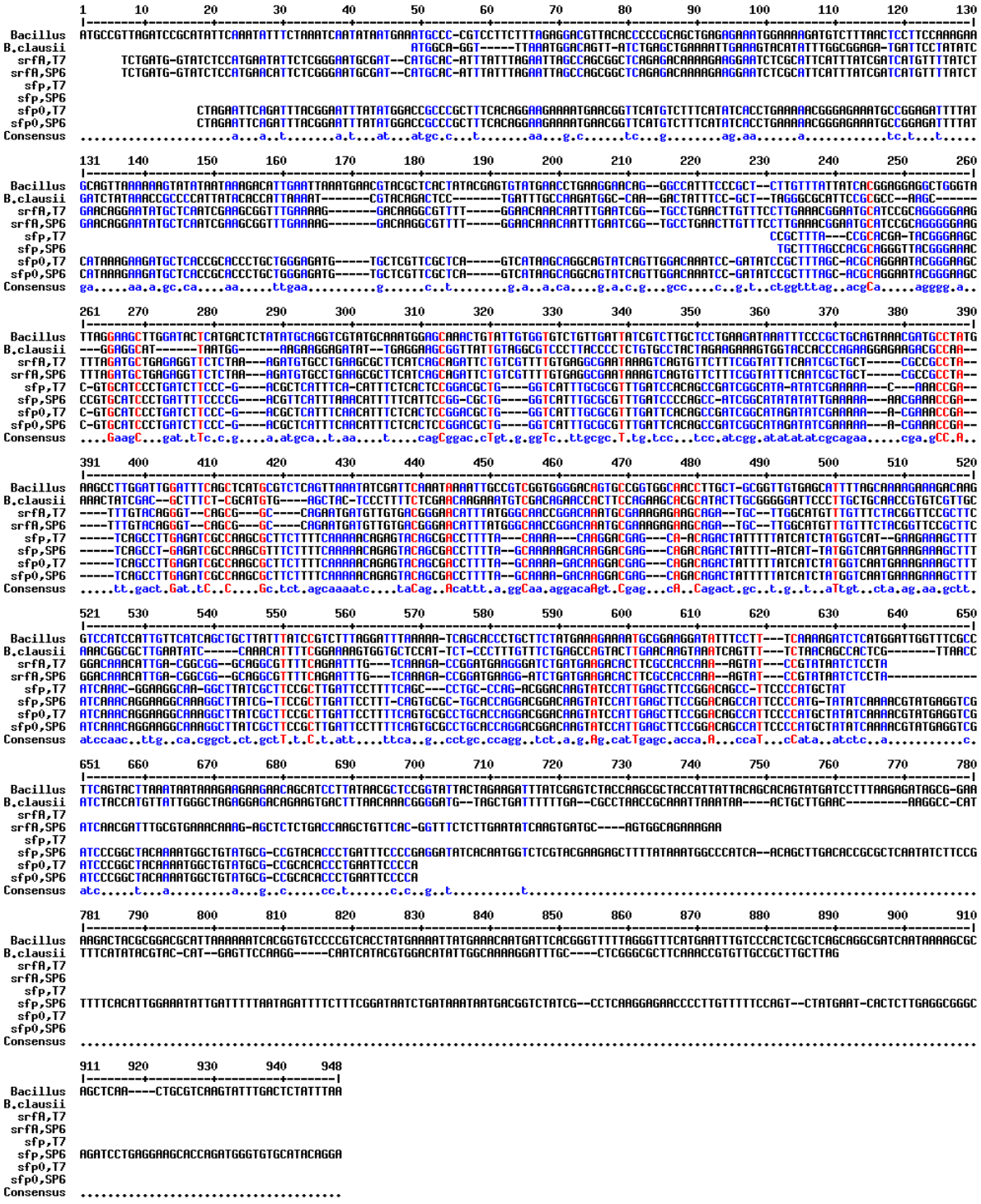


Image 14: MultAlin (Multiple Sequence Alignment) of *sfp*, *sfp0* and *srfA* gene sequences with esterase gene sequences from NCBI database. The conserved sequences are shown in the last line of the alignment table.

***Bacillus* = *Bacillus* sp. NK13 esterase gene (Liu *et al.* 2005, Unpublished, PubMed Accession number: DQ196347). *B. clausii* = *Bacillus clausii* KSM-K16 esterase gene (Hakamada *et al.* 1994, PubMed Accession number: AP006627).**

4.10 Purification of biosurfactant from clones *E.coli* pSKP, pSKP0 and pSKA (Biochemical characterization and surface tension reduction)

For the purification of biosurfactant from pSKP, pSKP0 and pSKA, the clones were grown in basal medium with 0.5% (v/v) olive oil and ampicillin. Biosurfactant released by the cells in the supernatant was partially purified as described in section 3.14. The partially purified biosurfactant was then lyophilized to obtain a clear powder. Biosurfactant yield obtained for pSKP, pSKP0 and pSKA was 2.13, 2.20 and 2.45 gm/lit, respectively. The % yield was much higher than that obtained for parent *Bacillus subtilis* SK320 i.e. 1.2 gm/lit.

Various biochemical and physiological properties of the biosurfactant were studied after its partial purification (Table 9). The biochemical analysis revealed that the partially purified biosurfactant from pSKP had 89.7% lipid, 14.9% ash, 7.21% protein and 3.08% carbohydrate content. Biosurfactant from pSKP0 contained 90.3% lipid, 15.2% ash, 6.73% protein and 2.94% carbohydrate content whereas, biosurfactant obtained from clone pSKA was having 91.0% lipid, 15.38% ash, 6.26% protein and 2.70% carbohydrate content, respectively. The data revealed that the biosurfactant from all the three clones had a high lipid content thereby indicating that the biosurfactant belongs to class of lipopeptides. These results were in accordance with the parent *Bacillus* strain biosurfactant, which had 7.459% protein, 89.40% lipid, 3.153% carbohydrate and 14.5% ash content, respectively.

The partially purified biosurfactant was further taken for the analysis of surface tension values according to DuNouy principle by Ring method (Table 10). The biosurfactant used was 1mg/100ml water i.e. with a CMC of 10mg/liter. Surface tension values were found to be 72.1 dynes/cm for tap water and 70.7 dynes/cm for sterile Milli-Q water. Biosurfactant extracted from parent *Bacillus subtilis* SK320 was able to reduce the surface tension of water to 40.1 dynes/cm, whereas the biosurfactant obtained from clones pSKP, pSKP0 and pSKA reduced the surface tension of tap water to as low as 38.4, 35 and 30.7 dynes/cm, respectively. The results suggested that the expression of biosurfactant gene was responsible for the surface tension reduction shown by the biosurfactant of the clones.

Table 9: Chemical composition of the partially purified biosurfactant from *Bacillus subtilis* SK320 and its clones pSKP, pSKP0 and pSKA.
Biosurfactant was partially purified as described in Section 3.14 and characterized for its chemical constituents viz. protein (Section 3.15.1), lipid (Section 3.15.7), carbohydrate (Section 3.15.6) and ash content (Section 3.15.2), respectively.

Constituent analysis (%)	*Bacillus* sp. SK320	pSKP	pSKP0	pSKA
Protein	7.45	7.21	6.73	6.26
Lipids	89.4	89.7	90.3	91.0
Carbohydrates	3.15	3.08	2.94	2.70
Ash content	14.5	14.9	15.2	15.38

Table 10: Surface tension values and yield of the partially purified biosurfactant from *Bacillus subtilis* SK320 and its clones.
Surface tension was measured according to DuNouy principle by Ring method (section 3.7.1).

	Surface tension (Dynes/cm)	Biosurfactant yield (gm/liter)
***Bacillus subtilis* SK320**	40.1	1.2
pSKP	38.4	2.13
pSKP0	35	2.20
pSKA	30.7	2.45

4.11 Purification of esterase from clones *E.coli* pSKP, pSKP0 and pSKA and the kinetic properties

(a) Clone pSKP

Esterase released in the culture broth of the clone *E. coli* pSKP was purified by ion-exchange and gel-filtration chromatography and the purification steps are shown in Table 11. Ion-exchange chromatography using Q Sepharose resolved the supernatant into three active protein components (P1, P2 and P3) giving maximum esterase activity (Fig 26). Fractions, P1 (specific activity 0.07 IU) pooled from Sepharose Q were concentrated by ultrafiltration and loaded onto Sephadex G 75. Two activity peaks were obtained and designated as P1a (with specific activity of 0.03 IU, 1.19 fold purification and 3.33% recovery) and P1b (with specific activity of 0.05 IU, 1.67 fold purification and 2.83% recovery), respectively (Fig 27). The second protein component (P2, anionic) with specific activity of 0.02 IU on Q Sepharose was eluted at 640mM NaCl using a 10 to 1000mM NaCl gradient in 10mM sodium phosphate buffer (pH 7.0). Active fractions were pooled, dialyzed, concentrated by ultrafiltration and then loaded onto Sephadex G 75. Component P2 was resolved into two components with esterase activity. These were designated as esterase P2a which was purified to 5.30 fold, with 9.66% recovery and 0.16 IU specific activity and P2b which was purified to 2.75 fold, with 5.83% recovery and 0.08 IU specific activity, respectively (Fig 28). The third active protein component (P3, anionic) with specific activity of 0.02 IU on Q Sepharose eluted at 880mM NaCl and on Sephadex G 75 was resolved into three active protein components P3a, P3b and P3c, respectively (Fig 29). Sub-component P3a has a specific activity of 0.19 IU and was purified to 6.46 fold with a recovery of 10.72%, P3b has a specific activity of 0.08 IU and was purified to 2.58 fold with a recovery of 6.81% and P3c has a specific activity of 0.03 IU and was purified to 1.12 fold with a recovery of 2.77%, respectively. These results were in agreement with the elution profile observed for the parent *Bacillus subtilis* SK320 (Fig 19).

The properties of purified esterases from clone pSKP are shown in Table 12. The enzyme shows strong activity in the pH range of 6 to 8. All components of esterases were stable at a temperature range of 40 to 50^0C, respectively. Michaelis (Km) constant for P1a, P1b, P2a, P2b, P3a, P3b, P3c was calculated as 5, 25, 1.66, 1.11, 2.5, 1.58, 2.38 µmoles whereas the Vmax values were found to be 0.238, 1, 0.196, 0.142, 0.243, 0.212, 0.153 µmol min^{-1} mg^{-1}, respectively. Esterases components were strongly inhibited by mercury, lead and EDTA with KI

(mM) of 4, 3.1, 7.5, 0.2, 5, 1.7, 3.1 with mercuric chloride, whereas with lead chloride as inhibitor the values observed were 9.8, 8, 1.3, 0.3, 2.5, 1.3, 1.8, respectively.

(b) Clone pSKP0

Purification of esterase from clone pSKP0 was conducted by growing the culture in basal medium supplemented with 0.5% (v/v) olive oil as the sole substrate. The extracellular crude enzyme/protein was initially resolved by anion exchange (Sepharose Q) chromatography (using phosphate buffer at pH 7) into three distinct active components i.e. P01, P02 and P03 giving maximum esterase activity (Fig 30). Component P01 eluting in the equilibrating buffer constituted about 0.25% of the total esterase activity while the other two components were eluted with NaCl as gradient. The second active protein component (component P02) eluting at 700mM NaCl using a 10 to 1000mM NaCl gradient in 10mM sodium phosphate buffer (pH 7.0) constituted 0.25% of the total activity while the third active protein (component P03) on Q Sepharose eluted at 940mM NaCl and constituted 0.49% of the total activity loaded. Esterase Components P01, P02 and P03 were further separated by gel-filtration chromatography (Sephadex G-75). Component P01 (specific activity 0.05 IU) fractions from Sepharose Q were concentrated by ultra filtration and were loaded unto the Sephadex G 75 column. Two esterase activity peaks designated P01a (specific activity 0.03 IU) and P01b (specific activity 0.04 IU) were obtained (Fig 31). P01a was purified 1.05 fold with a recovery of 3.15% while P01b was purified 1.52 fold with recovery of 3.17%, respectively. Component P02 fractions were pooled, dialyzed, concentrated by ultrafiltration and then loaded onto Sephadex G 75. Component P02 (specific activity 0.01 IU) was resolved into esterase P02a and P02b, with specific activity of 0.14 IU, 4.74 fold purification and 9.56% recovery for P02a and with specific activity of 0.07 IU, 2.46 fold purification and 8.98% recovery for P02b, respectively (Fig 32). Similarly component P03 (specific activity 0.02 IU) on Sephadex G 75 was resolved into three active protein components P03a, P03b and P03c, respectively (Fig 33), with specific activity of 0.13 IU, 4.30 fold purification and 12.04% recovery for P03a, with specific activity of 0.07 IU, 2.40 fold purification and 9.16% recovery for P03b and with specific activity of 0.03 IU, 1.00 fold purification and 3.32% recovery for P03c respectively, Table 13.

The properties of purified esterases from clone pSKP0 are shown in Table 14. The esterase enzyme shows strong activity in the pH range of 6 to 8 with an optimal activity around pH 7.0. There was a rapid decline in activity on either side of pH 7.0. All components of esterase were stable at a temperature range of 35 to 50^0C, respectively. The Michaelis (Km)

constant for P01a, P01b, PO2a, P02b, P03a, P03b and P03c was calculated as 4, 3.90, 2.97, 1.23, 2.93, 1.63 and 2.21 µmoles whereas the Vmax values were found to be 0.412, 0.112, 0.439, 0.529, 0.127, 0.433 and 0.312 µmol min^{-1} mg^{-1}, respectively. Effects of addition of mercuric chloride and lead chloride in different concentrations as inhibitors was also studied and the data suggested that the esterase activity decreases with the addition of the inhibitor in the reaction mixture. Esterases components were strongly inhibited by mercury, lead and EDTA with KI (mM) of 1.2, 3.2, 3.3, 0.8, 4.9, 1.8 and 5.2 with mercuric chloride, whereas with lead chloride as inhibitor the values observed were 2.5, 3.3, 2.0, 0.5, 2.3, 1.5 and 1.7, respectively.

(c) Clone pSKA

Esterase from clone *E. coli* pSKA was purified from the supernatant of the mid log phase cells grown in basal medium amended with 0.5% olive oil as the sole carbon source. Under these conditions esterase accumulates in the medium with no apparent loss of activity. The extracellular protein obtained from the clone *E. coli* pSKA was purified by Q-sepharose followed by Sephadex G-75 (Table 15). Ion-exchange chromatography on Q-sepharose resolved the supernatant into three distinct active protein components (Fig 34) giving maximum esterase activity. The protein eluting in the equilibrating buffer (A1,cationic), constituted only 8% of the total esterase activity. Fractions (A1) pooled from Q Sepharose were concentrated by ultrafiltration and loaded onto Sephadex G 75. Two activity peaks were obtained and designated as A1a and A1b. Esterase A1a and A1b (specific activity 8.4 IU and 9.75 IU) were purified 6.5 and 7.6%, respectively (Fig 35). The second protein component (A2, anionic) on Q Sepharose was eluted at 620mM NaCl using a 10 – 1000mM NaCl gradient in 10mM sodium phosphate buffer (pH 7.0) and constituted 5% of the total activity. Active fractions were pooled, dialyzed, concentrated by ultrafiltration and then loaded onto Sephadex G 75. Component A2 was resolved into two components with esterase activity (Fig 36). These were designated as esterase A2a and A2b respectively and had specific activity of 20.85 and 6.92 IU. The third active protein component (A3, anionic) on Q Sepharose eluted at 820mM NaCl and on Sephadex G 75 was resolved into three active protein components (A3a, A3b and A3c) with specific activity of 30.89, 11.80 and 4.41 IU for A3a, A3b and A3c, respectively (Fig 37). The elution profile of ion exchange and gel-filtration chromatography of clone *E. coli* pSKA was found to be similar to the parent strain i.e. the presence of three esterases A1 (cationic), A2 and A3 (anionic) in the culture supernatant.

The properties of purified esterases are shown in Table 16. The enzyme shows strong activity in the pH range of 6-8 with optimum pH at 7. All components of esterase were stable at $40^{O}C$ except the A3a and A2b component which were stable also at $50^{O}C$ and $55^{O}C$, respectively. Michaelis (Km) constant for A1a, A1b, A2a, A2b, A3a, A3b and A3c was calculated as 2.38, 1.67, 3.06, 2.7, 2.8, 1.60, 1.98 µmoles whereas the Vmax values were found to be 0.168, 0.332, 0.612, 0.167, 0.413, 0.520, 0.608 µmol min^{-1} mg^{-1}, respectively. Esterases components were strongly inhibited by Hg, Pb and EDTA with KI (mM) of 0.55, 2.8, 3.2, 0.8, 3.3, 1.9, and 4.5 for components A1a, A1b, A2a, A2b, A3a, A3b and A3c with mercuric chloride, whereas with lead chloride as inhibitor the values observed were 1.2, 1.9, 5.5, 0.2, 4.1, 2.1 and 1.3, respectively.

4.12 Zymographic analysis and molecular weight determination of purified esterase

Native PAGE was run and activity staining using Fast Blue RR salt (4-Benzoylamine-2, 5-dimethoxy benzene-diazonium chloride hemi [zinc chloride] salt) and Fast Blue B salt revealed that the esterase sub-components were purified to homogeneity. Zymogram analysis showed that the bands were clearly visible upon over night incubation with Fast Blue RR salt whereas it took a longer period of time to get the clear bands with Fast Blue B salt. Also the background was excessively darkened when Fast Blue B salt was used. Therefore Fast Blue RR salt was used in this study.

The results showed wide range of molecular weights for esterase proteins, indicating that *Bacillus* esterases are multimeric in nature. The sub-unit molecular weight (kDa) of the esterase components from pSKP, pSKP0 and pSKA were 66, 44, 116, 55, 30, 28 and 34 for P1a, P1b, P2a, P2b, P3a, P3b, P3c components, 65, 35, 72, 29, 65, 33 and 14 for P01a, P01b, PO2a, P02b, P03a, P03b and P03c components and 53, 45, 53, 33, 55, 16 and 12 for A1a, A1b, A2a, A2b, A3a, A3b and A3c components, respectively (Image 15, 17, 18, 19).

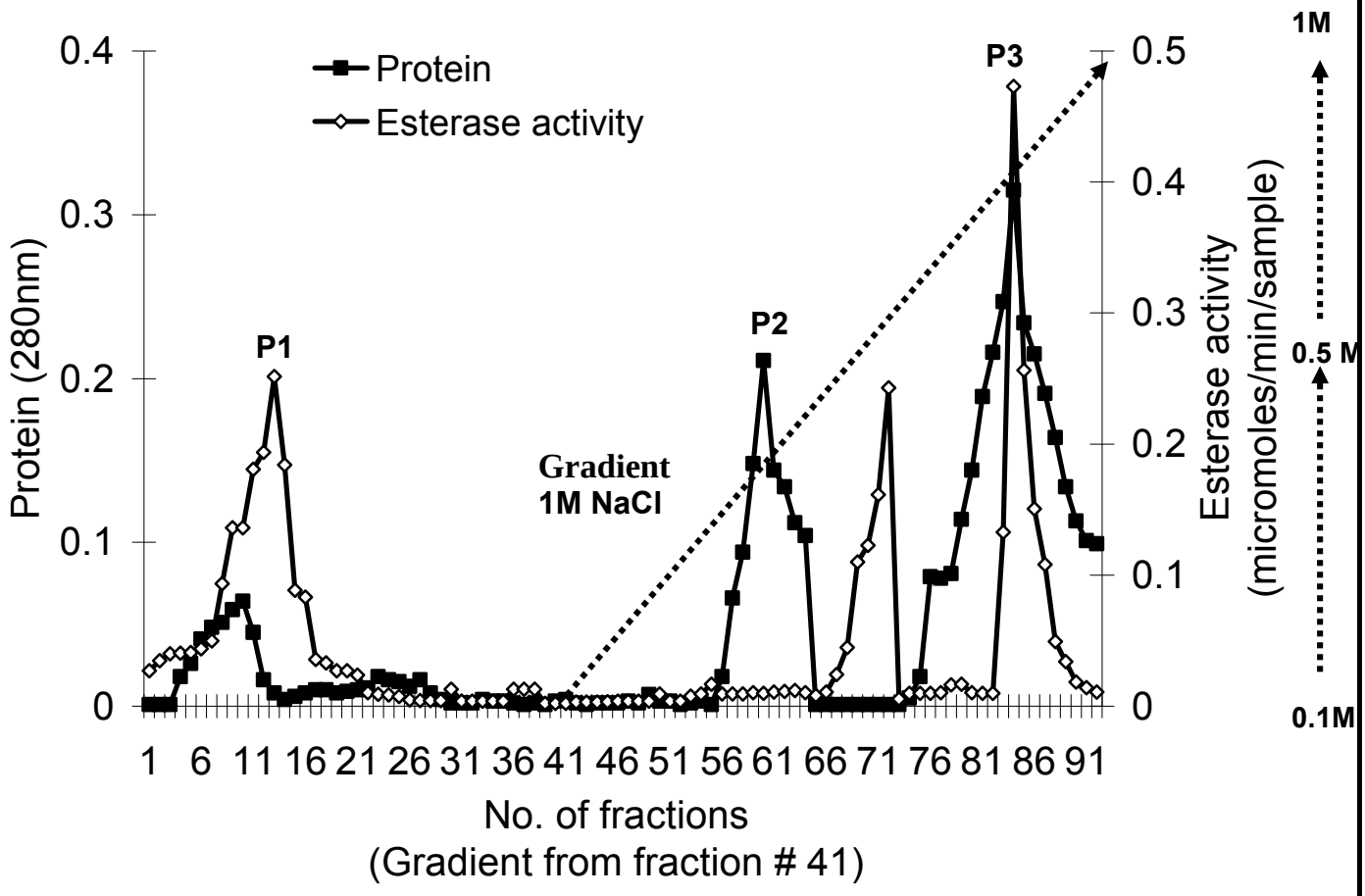


Fig 26: Elution profile of esterase of clone *E.coli* pSKP from ion-exchange column - Sepharose Q. pSKP.

Clone *E.coli* pSKP grown on basal medium with 0.5% (v/v) olive oil as sole carbon source and after 72 hrs of growth the supernatant was used as the source of crude enzyme. Crude enzyme was loaded onto Sepharose Q and eluted first with equilibrating buffer and then with 10 mM - 1 M NaCl gradient (section 3.10.1).

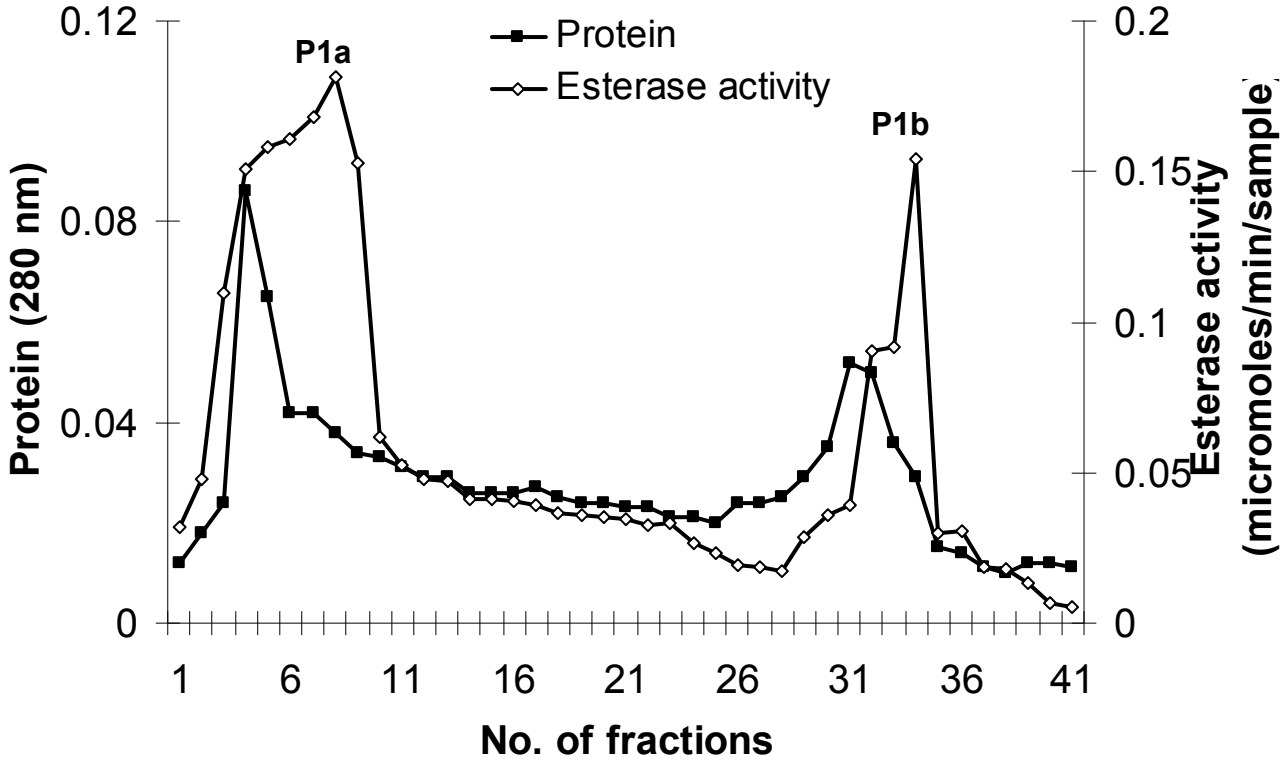


Fig 27: Elution profile of esterase component P1 from gel-filtration column – Sephadex G-75.

Component P1 (3ml) of esterase of clone *E.coli* pSKP was loaded onto Sephadex G75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

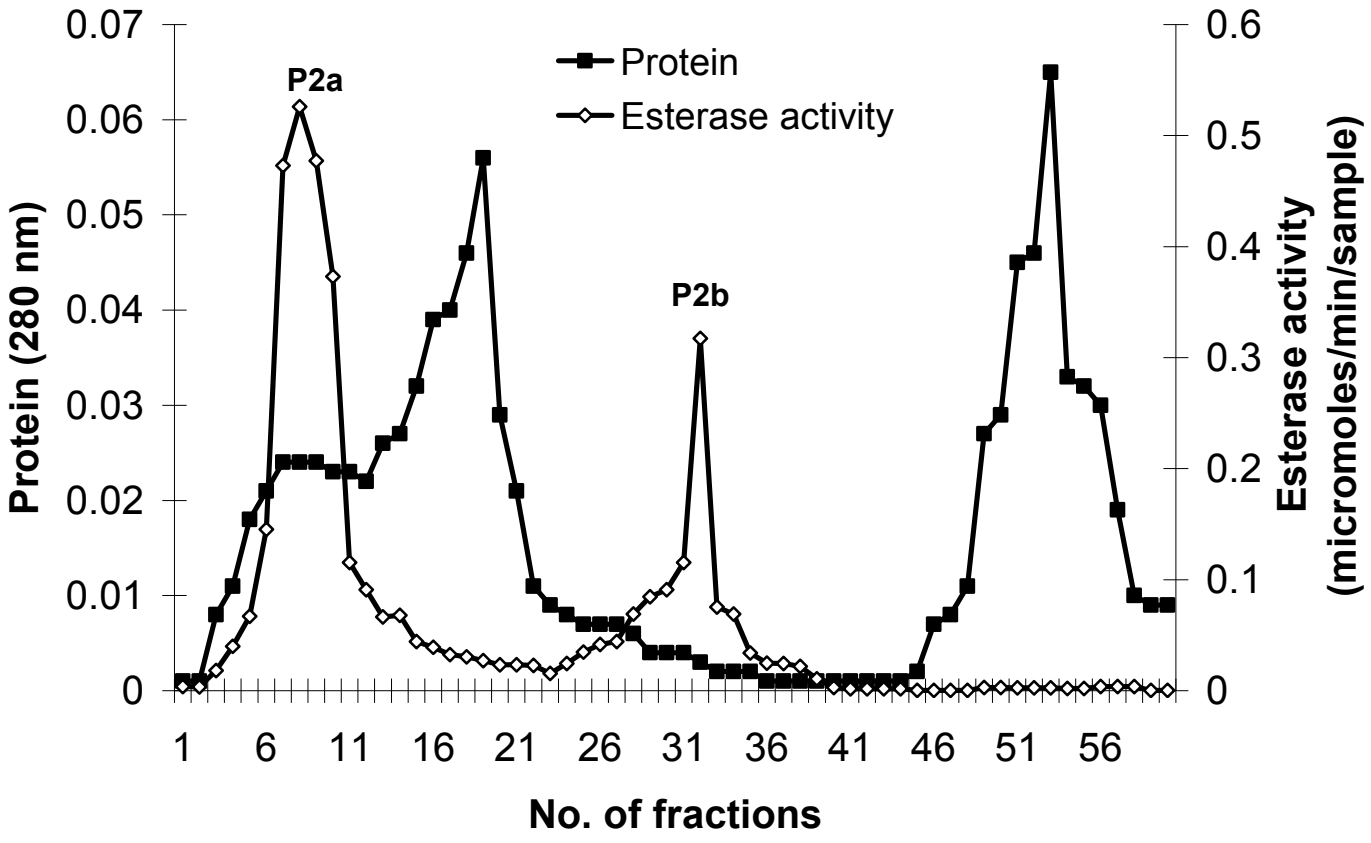


Fig 28: Elution profile of esterase component P2 from gel-filtration column – Sephadex G75.

Component P2 (3ml) of esterase of clone *E.coli* pSKP was loaded onto Sephadex G-75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

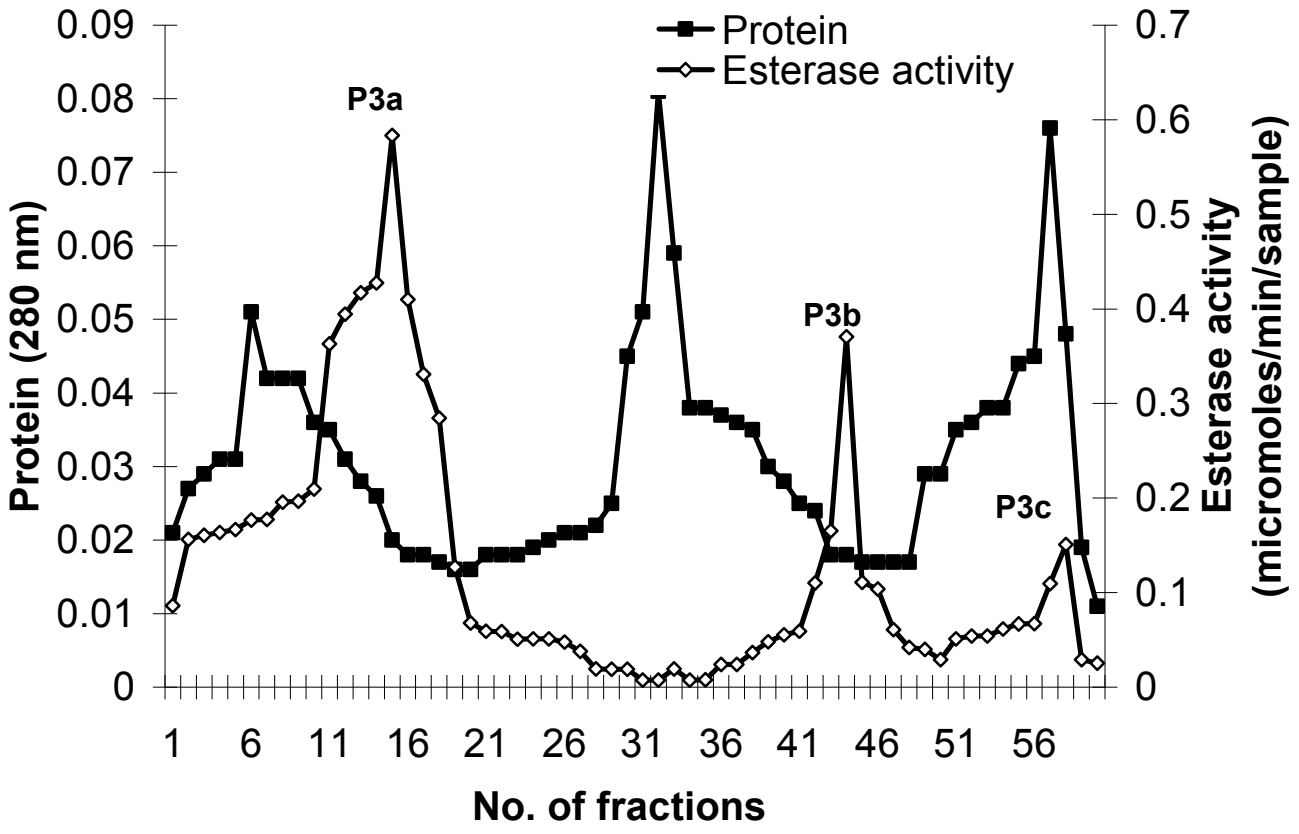


Fig 29: Elution profile of esterase component P3 from gel-filtration chromatography – Sephadex G75.

Component P3 (3ml) of esterase of clone *E.coli* pSKP was loaded onto Sephadex G75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

Table 11: Purification of esterases of clone *E.coli* pSKP.
Clone pSKP was grown on basal medium containing olive oil (0.5%, v/v). The supernatant was used as source of the enzyme and purified as described in section 3.10.

	Total Activity (IU)	Total Protein (mg)	Specific Activity (IU/mg)	% Recovery	Fold Purification
Supernatant (Crude)	5.44	184.93	0.03	100	0
Q Sepharose					
P1	0.25	3.85	0.07	4.62	2.22
P2	0.24	12.70	0.02	4.46	0.65
P3	0.47	18.96	0.02	8.69	0.85
Sephadex G 75					
P1a	0.18	5.18	0.03	3.33	1.19
P1b	0.15	3.13	0.05	2.83	1.67
P2a	0.53	3.37	0.16	9.66	5.30
P2b	0.32	3.91	0.08	5.83	2.75
P3a	0.58	3.07	0.19	10.72	6.46
P3b	0.37	4.88	0.08	6.81	2.58
P3c	0.15	4.58	0.03	2.77	1.12

Table 12: Kinetic properties of esterases of clone *E.coli* pSKP

		Components						
		P1		P2		P3		
		P1a	P1b	P2a	P2b	P3a	P3b	P3c
Optimum pH		7.0	7.0	7.0	7.0	7.0	7.0	7.0
Optimum temp.		40	40	45	50	40	45	40
Km (µmoles)		5	25	1.66	1.11	2.5	1.58	2.38
Mw (KDa)		66	44	116	55	30	28	34
Vmax ($\mu molesmg^{-1}min^{-1}$)		0.238	1	0.196	0.142	0.243	0.212	0.153
KI (mM)	**Mercuric chloride**	4	3.1	7.5	0.2	5	1.7	3.1
	Lead chloride	9.8	8	1.3	0.3	2.5	1.3	1.8

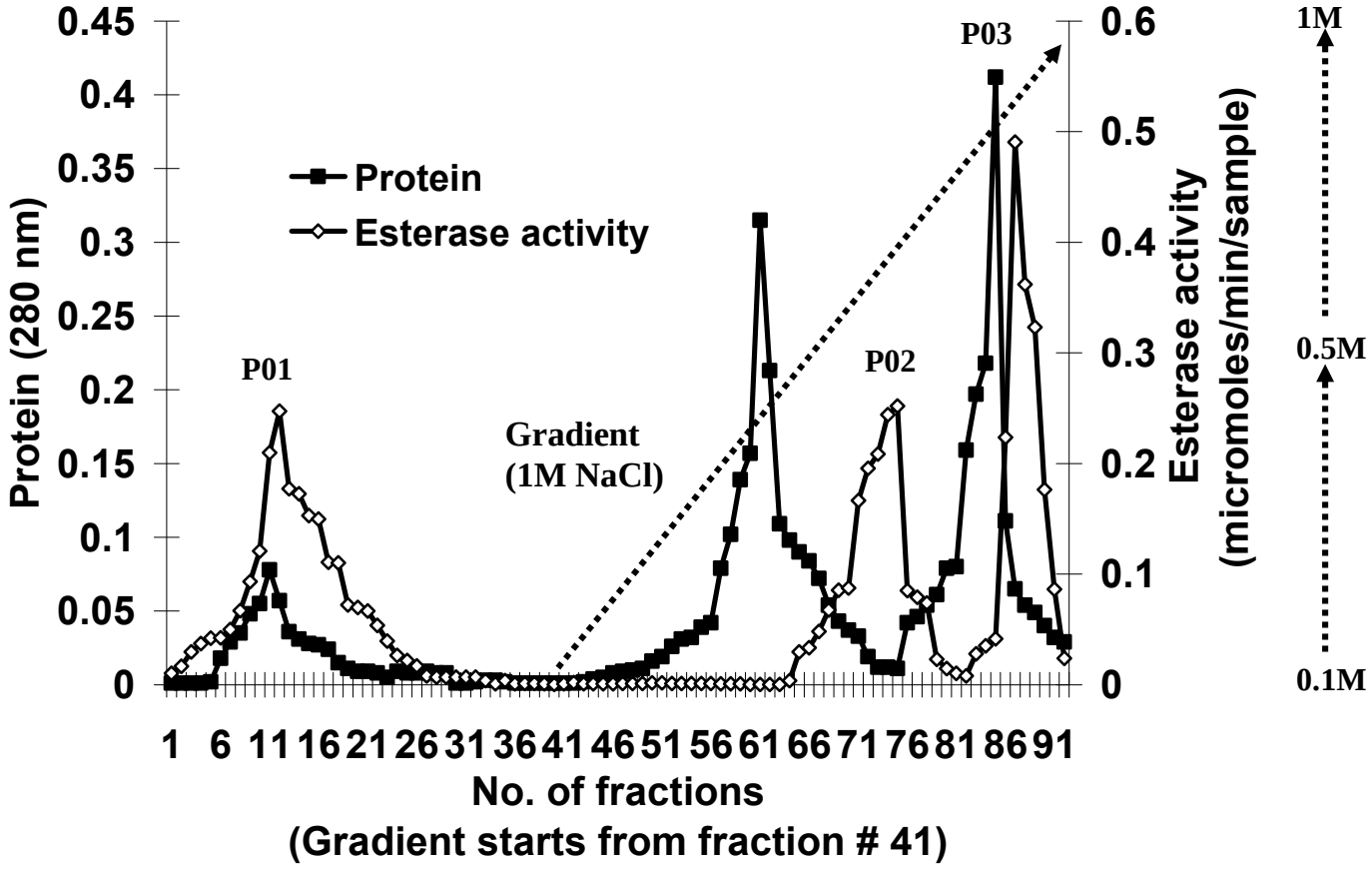


Fig 30: Elution profile of esterase of clone *E.coli* pSKP0 from ion-exchange column - Sepharose Q.

Clone *E.coli* pSKP0 grown on basal medium with 0.5% (v/v) olive oil as sole carbon source and after 72 hrs of growth the supernatant was used as the source of crude enzyme. Crude enzyme was loaded onto Sepharose Q and eluted first with equilibrating buffer and then with 10 mM - 1 M NaCl gradient (section 3.10.1).

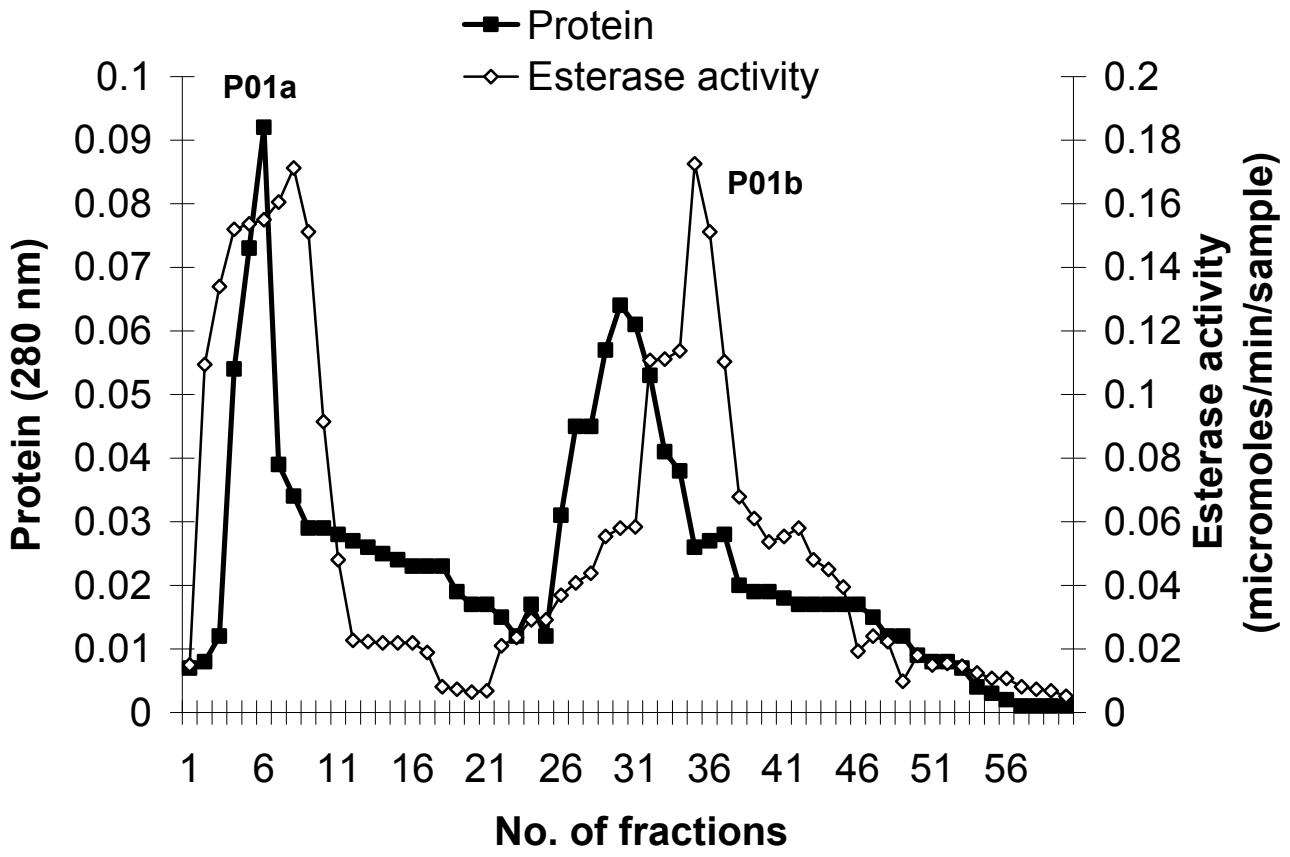


Fig 31: Elution profile of esterase component P01 from gel-filtration column – Sephadex G-75.

Component P01 (3ml) of esterase of clone *E.coli* pSKP0 was loaded onto Sephadex G-75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

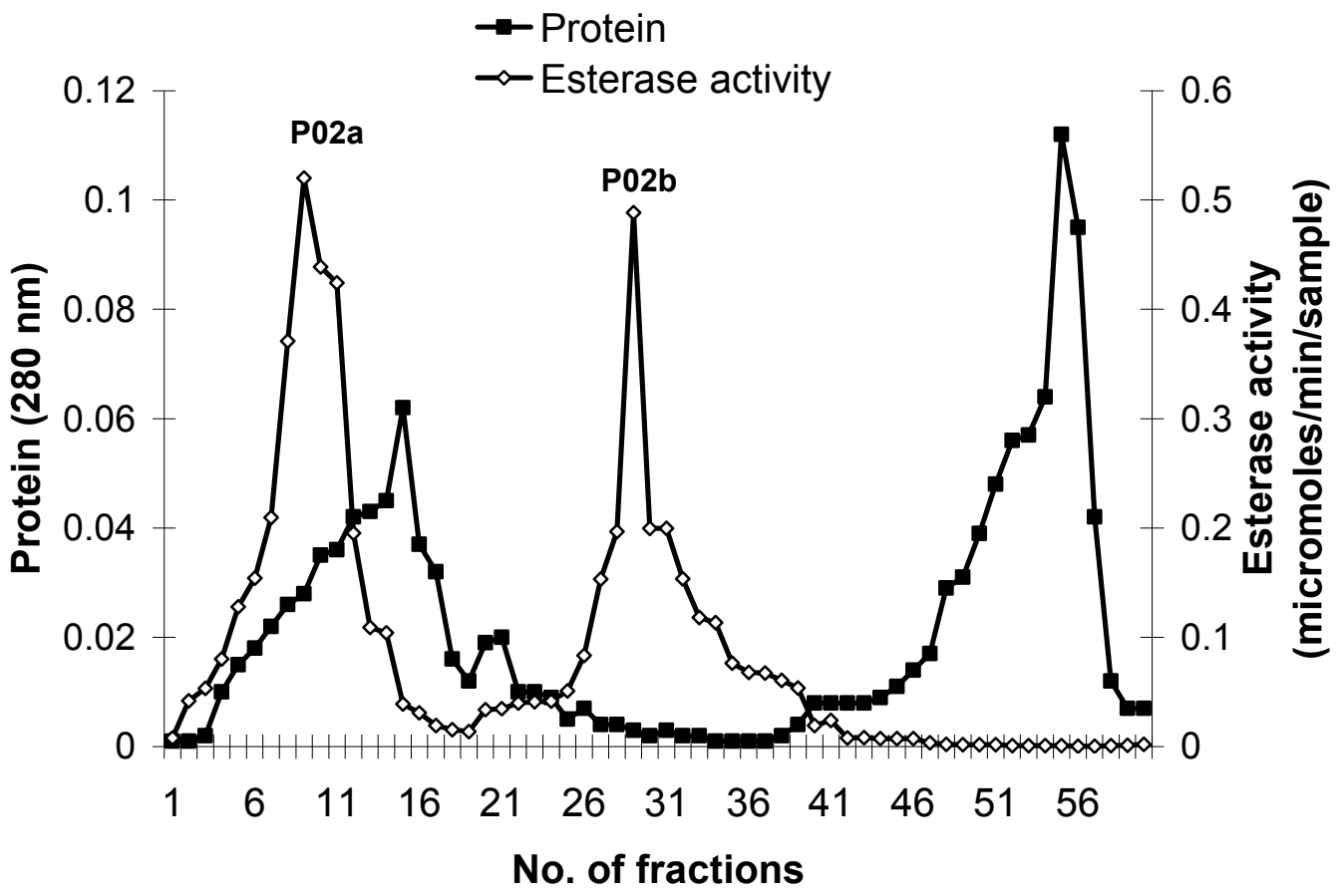


Fig 32: Elution profile of esterase component P02 from gel-filtration column – Sephadex G-75.

Component P02 (3ml) of esterase of clone *E.coli* pSKP0 was loaded onto Sephadex G-75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

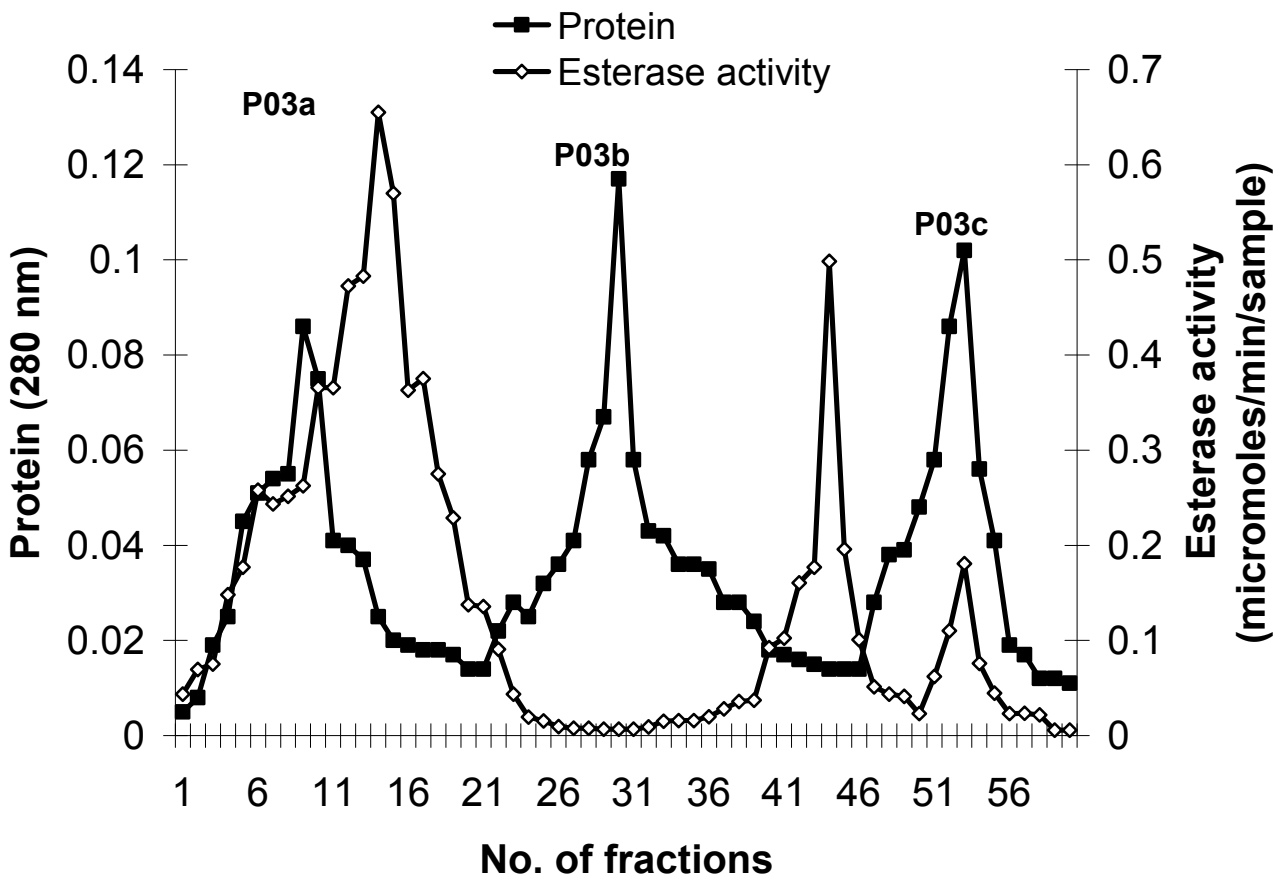


Fig 33: Elution profile of esterase component P03 from gel-filtration column – Sephadex G-75.

Component P03 (3ml) of esterase of clone *E.coli* pSKP0 was loaded onto Sephadex G-75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

Table 13: Purification of esterases of clone pSKP0.
Clone pSKP0 was grown on basal medium containing olive oil (0.5%, v/v). The supernatant was used as source of the enzyme and purified as described in section 3.10.

	Total Activity(IU)	Total Protein(mg)	Specific Activity(IU/mg)	% Recovery	Fold Purification
Supernatant					
Crude	4.92	176.63	0.03	100	0.00
Q Sepharose					
P01	0.25	4.70	0.05	4.54	1.79
P02	0.25	18.96	0.01	4.63	0.45
P03	0.49	24.80	0.02	9.02	0.67
Sephadex G 75					
P01a	0.17	5.54	0.03	3.15	1.05
P01b	0.17	3.85	0.04	3.17	1.52
P02a	0.52	3.73	0.14	9.56	4.74
P02b	0.49	6.74	0.07	8.98	2.46
P03a	0.66	5.18	0.13	12.04	4.30
P03b	0.50	7.04	0.07	9.16	2.40
P03c	0.18	6.14	0.03	3.32	1.00

Table 14: Kinetic properties of esterases of clone pSKP0

		Components						
		P01		P02		P03		
		P01a	P01b	P02a	P02b	P03a	P03b	P03c
Optimum pH		7.0	7.0	7.0	7.0	7.0	7.0	7.0
Optimum temp.		40	35	40	50	45	40	40
Km (µmoles)		4	3.90	2.97	1.23	2.93	1.63	2.21
Mw (KDa)		65	35	72	29	65	33	14
Vmax ($\mu molesmg^{-1}min^{-1}$)		0.412	0.112	0.439	0.529	0.127	0.433	0.312
KI (mM)	**Mercuric chloride**	1.2	3.2	3.3	0.8	4.9	1.8	5.2
	Lead chloride	2.5	3.3	2.0	0.5	2.3	1.5	1.7

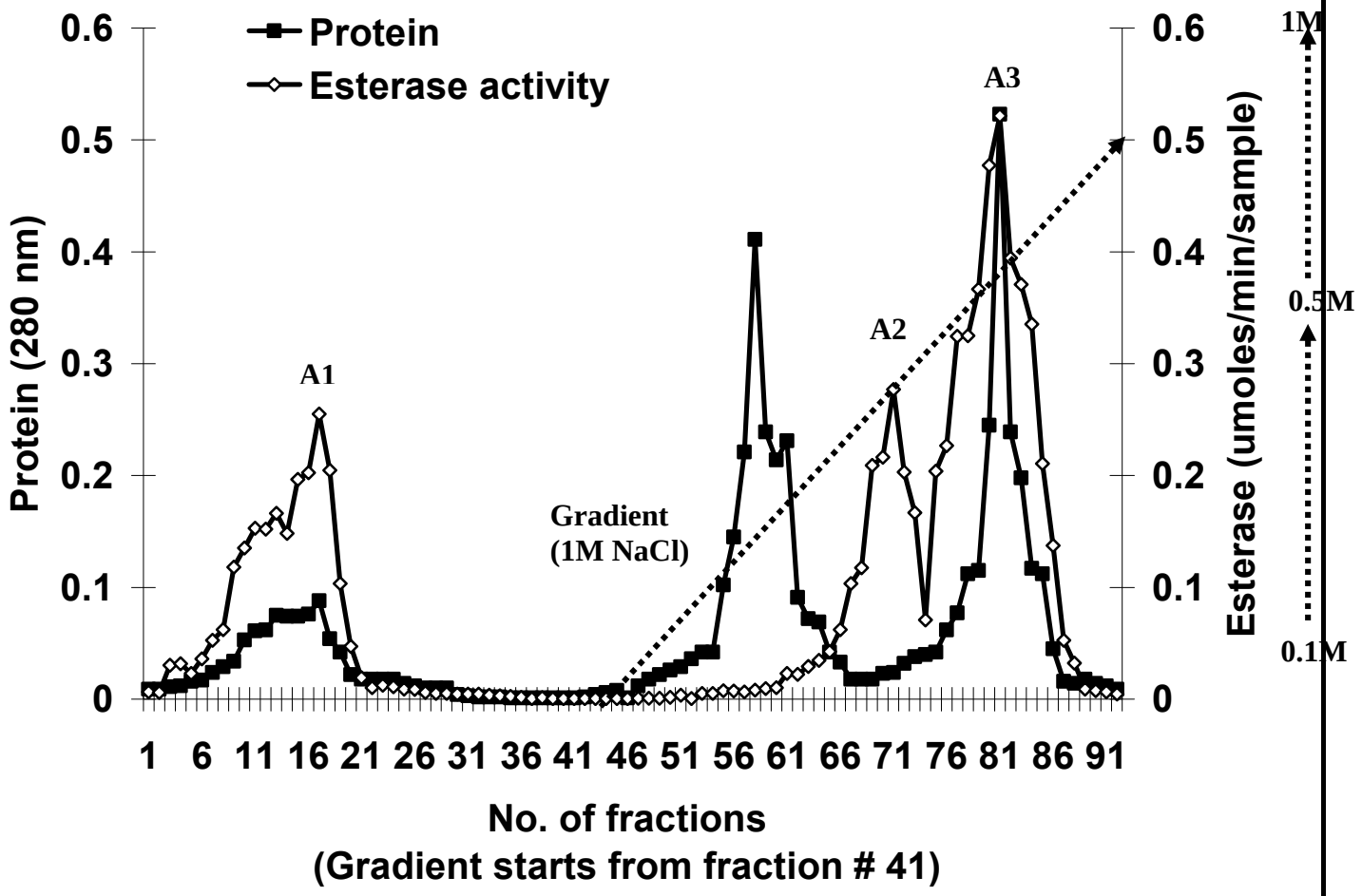


Fig 34: Elution profile of esterase of clone *E.coli* pSKA from ion-exchange column - Sepharose Q.

Clone *E.coli* pSKA grown on basal medium with 0.5% (v/v) olive oil as sole carbon source and after 72 hrs of growth the supernatant was used as the source of crude enzyme. Crude enzyme was loaded onto Sepharose Q and eluted first with equilibrating buffer and then with 10 mM - 1 M NaCl gradient (section 3.10.1).

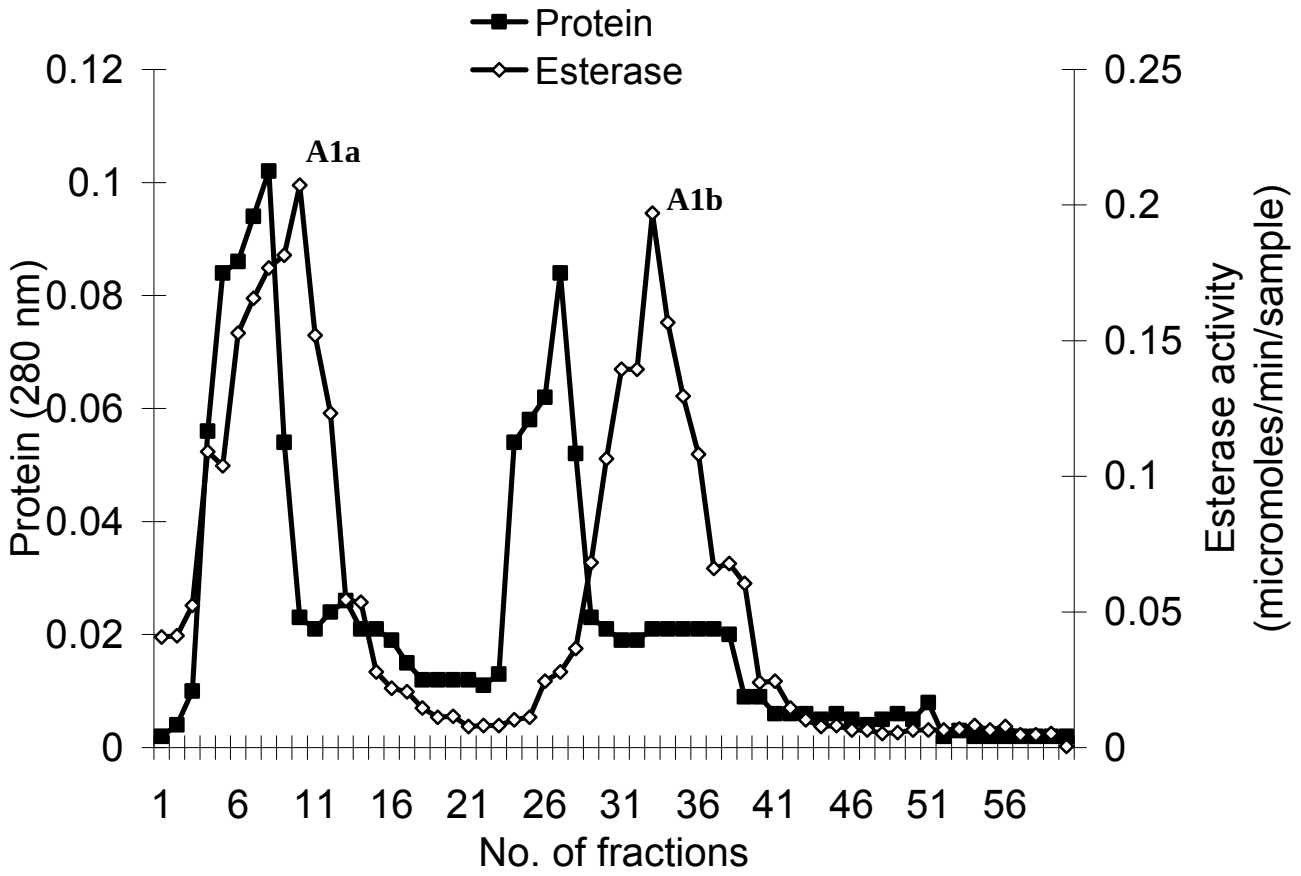


Fig 35: Elution profile of esterase component A1 from gel-filtration chromatography – Sephadex G-75.

Component A1 (3ml) of esterase of clone *E.coli* pSKA was loaded onto Sephadex G-75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

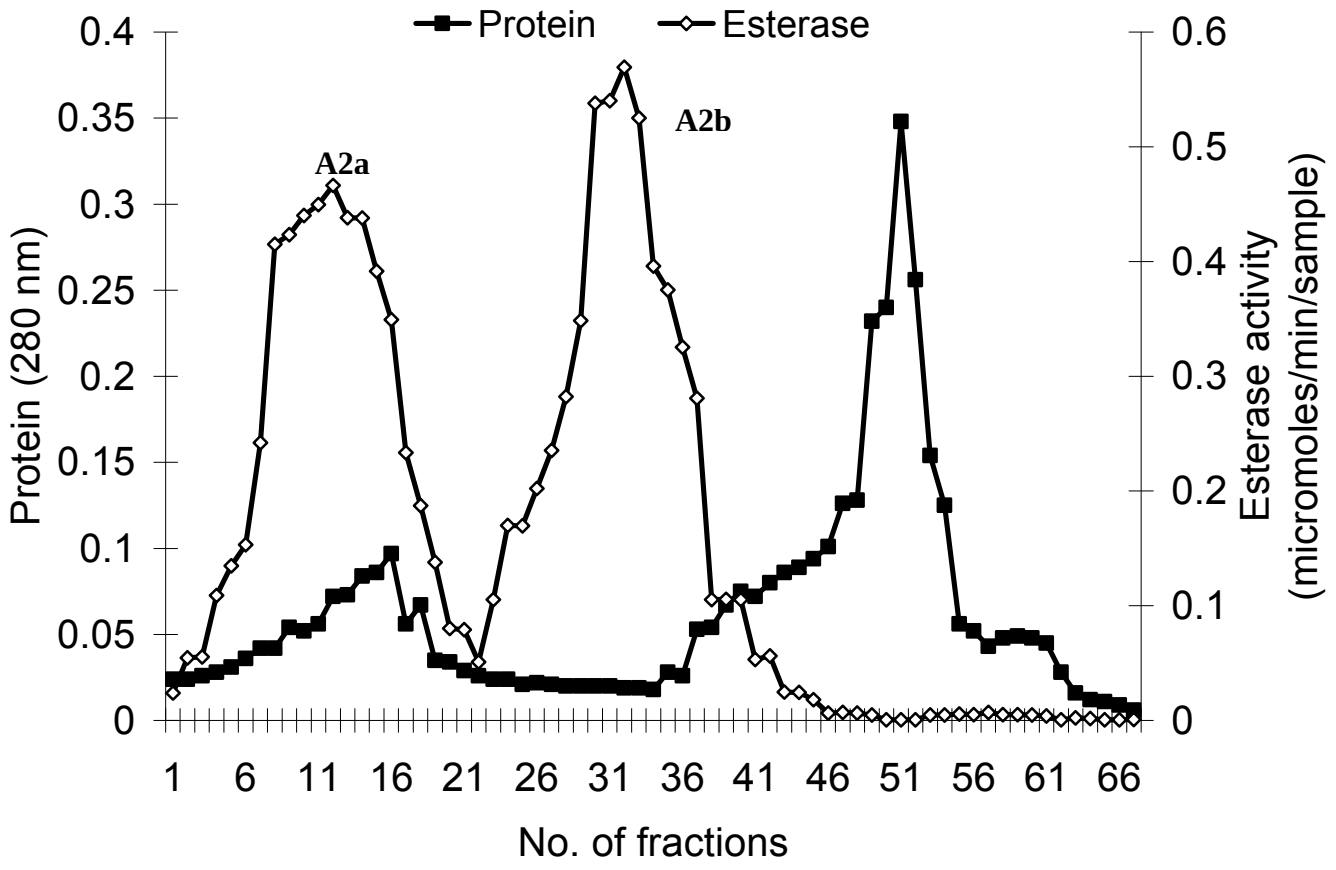


Fig 36: Elution profile of esterase component A2 from gel-filtration chromatography – Sephadex G-75.

Component A2 (3ml) of esterase of clone *E.coli* pSKA was loaded onto Sephadex G-75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

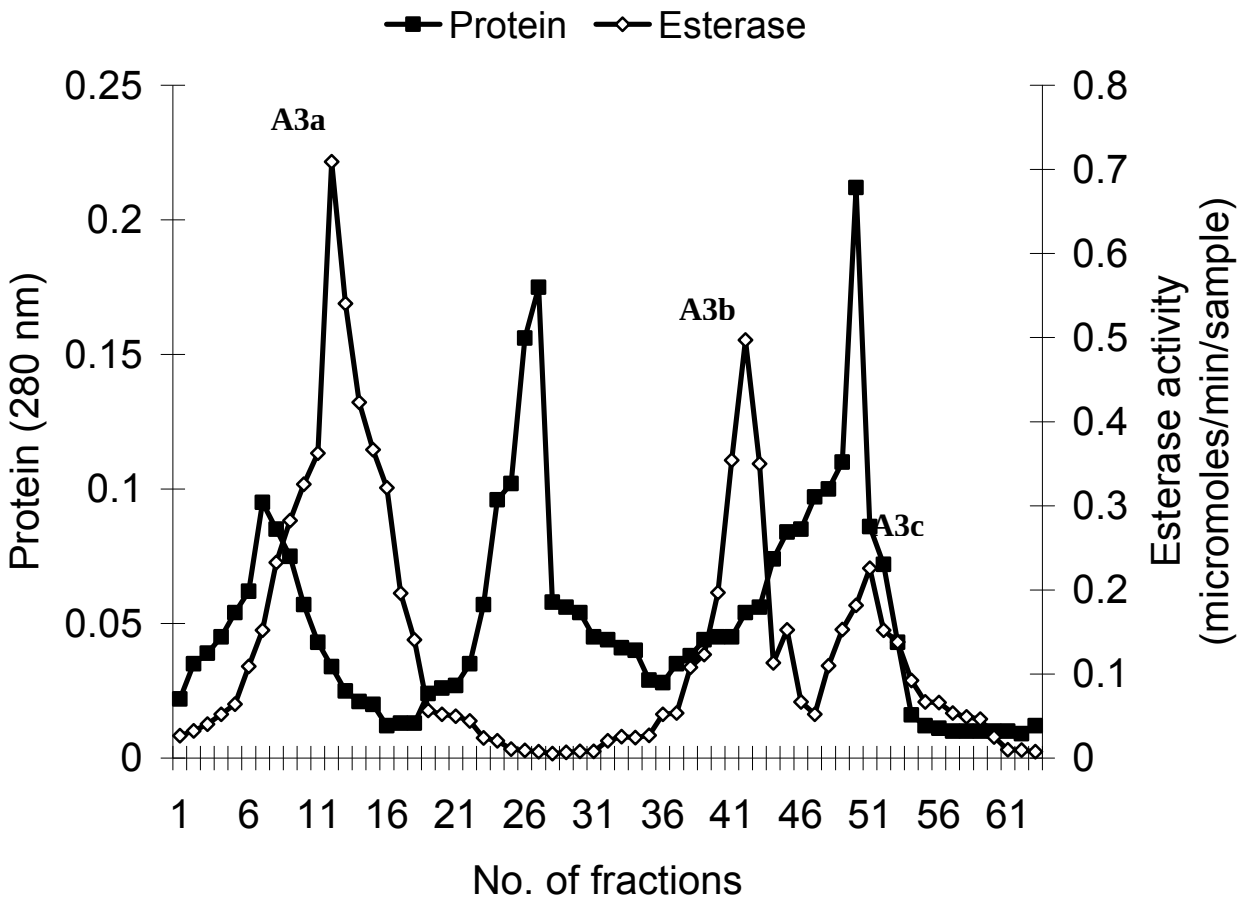


Fig 37: Elution profile of esterase component A3 from gel-filtration chromatography – Sephadex G-75.

Component A3 (3ml) of esterase of clone *E.coli* pSKA was loaded onto Sephadex G-75 column and eluted with equilibrating buffer (section 3.10.2). The protein content at 280nm (section 3.15.1) and esterase activity at 405nm were analyzed spectrophotometrically (section 3.13.2).

Table 15: Purification of esterases of clone *E. coli* pSKA.
Clone pSKA was grown on basal medium containing olive oil (0.5%, v/v). The supernatant was used as source of the enzyme and purified as described in section 3.10.

	Total Activity(IU)	Total Protein(mg)	Specific Activity(IU/mg)	% Recovery	Fold Purification
Crude	172	134	1.28	100	0.00
Q Sephrose					
A1	7.60	10.60	0.72	4.42	0.56
A2	8.30	49.50	0.17	4.83	0.13
A3	15.60	63.07	0.25	9.07	0.19
Sephdex G 75					
A1a	10.35	1.23	8.41	6.02	6.56
A1b	9.85	1.01	9.75	5.73	7.60
A2a	23.35	1.12	20.85	13.58	16.24
A2b	28.50	4.12	6.92	16.57	5.39
A3a	35.52	1.15	30.89	20.65	24.06
A3b	24.89	2.11	11.80	14.47	9.19
A3c	11.30	2.56	4.41	6.57	3.44

Table 16: Kinetic properties of esterases of clone *E.coli* pSKA

		Components						
		A1		A2		A3		
		A1a	A1b	A2a	A2b	A3a	A3b	A3c
Optimum pH		7.0	7.0	7.0	7.0	7.0	7.0	7.0
Optimum temp.		40	40	35	55	50	40	45
Km (µmoles)		2.38	1.67	3.06	2.7	2.8	1.60	1.98
Mw (KDa)		53	45	53	33	55	16	12
Vmax ($\mu molesmg^{-1}min^{-1}$)		0.168	0.332	0.612	0.167	0.413	0.520	0.608
KI (mM)	**Mercuric Chloride**	0.55	2.8	3.2	0.8	3.3	1.9	4.5
	Lead chloride	1.2	1.9	5.5	0.2	4.1	2.1	1.3

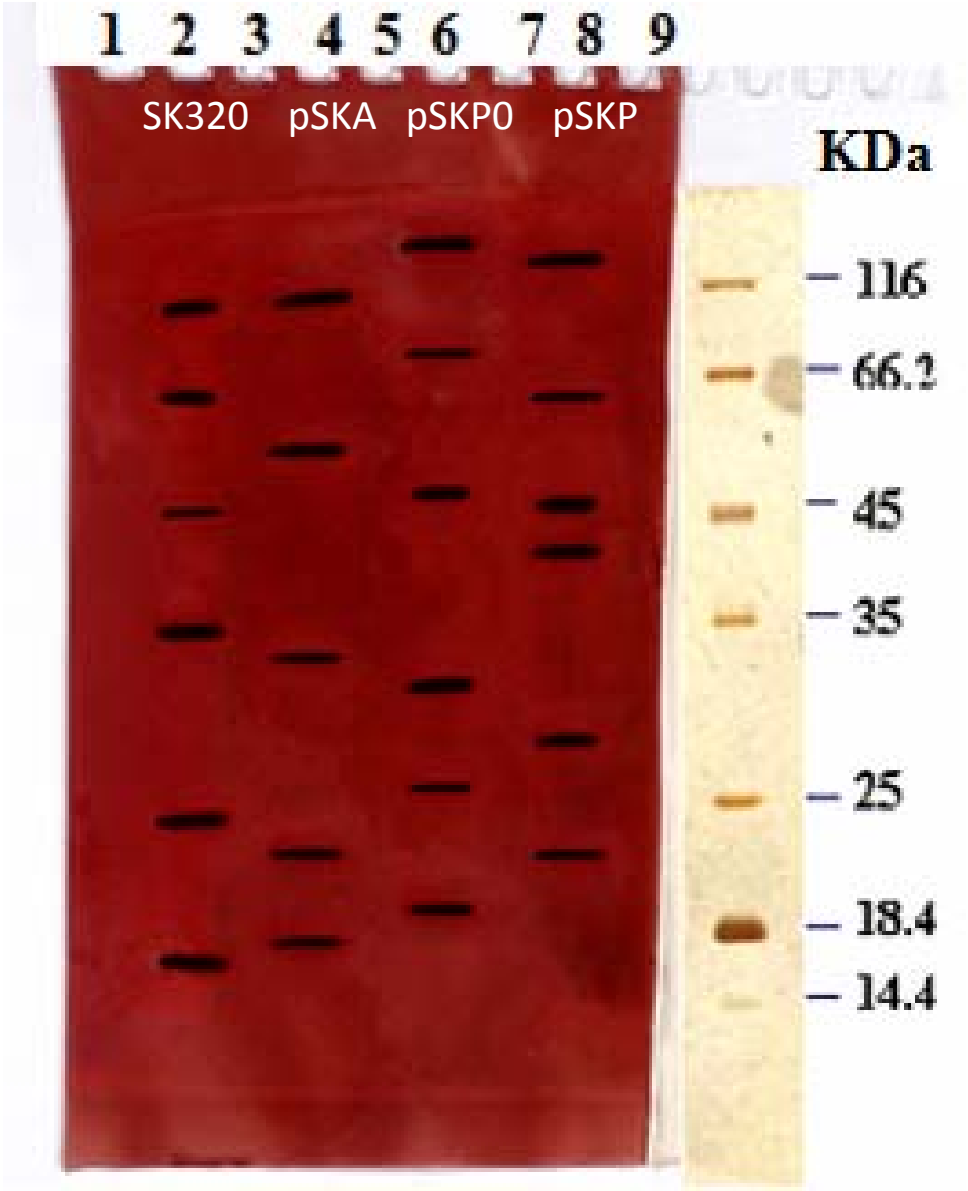


Image 15: Native PAGE analysis of esterases from supernatants.

Lane 2: Supernatant of *Bacillus subtilis* SK320,

Lane 4: Supernatant of pSKP,

Lane 6: Supernatant of pSKP0,

Lane 8: Supernatant of pSKA.

Last lane: protein molecular weight marker.

The protein was analyzed by 7.5% native PAGE followed by activity staining by fast blue RR salt.

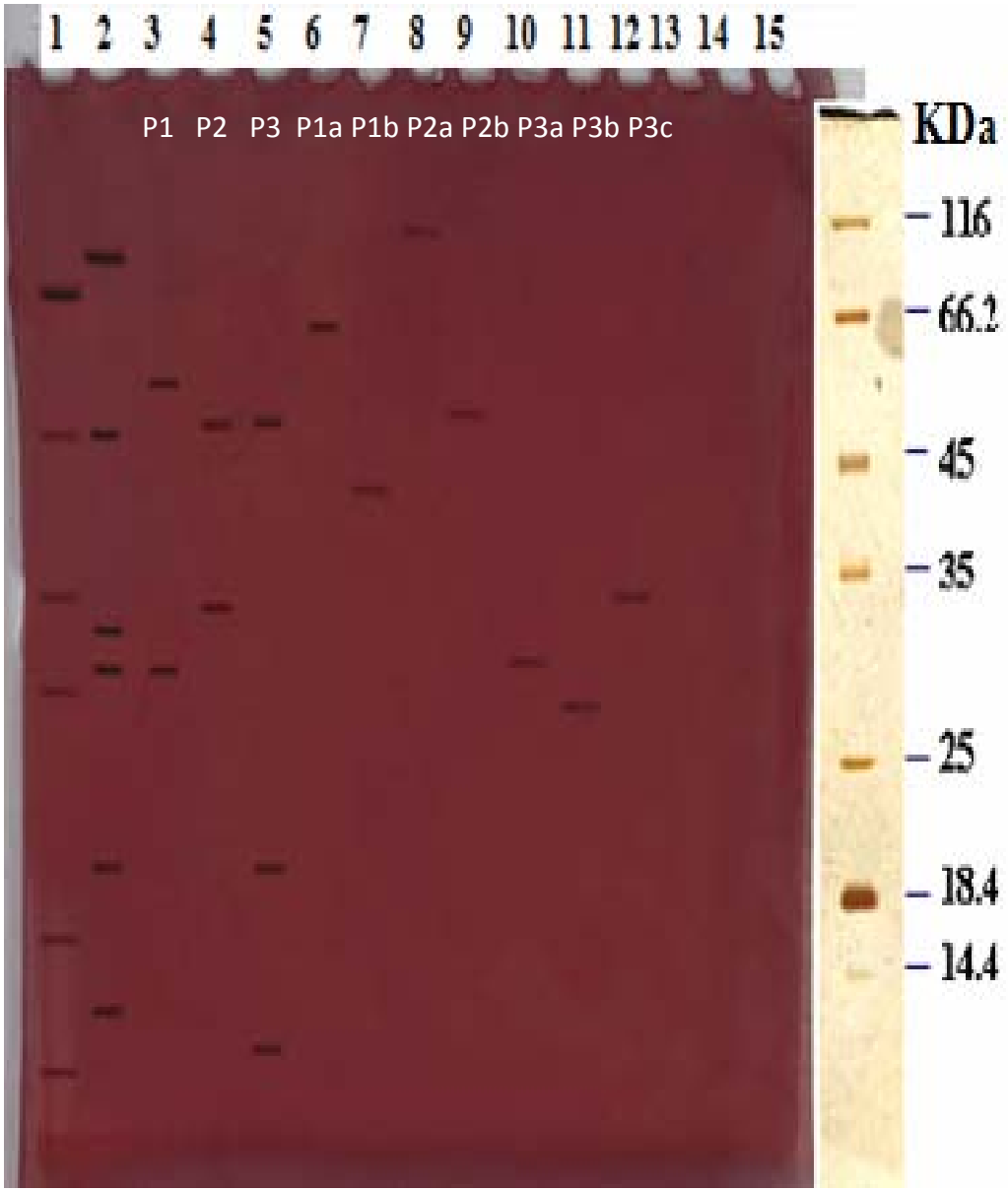


Image 17: Native PAGE analyses and activity staining with Fast Blue RR salt for esterases from clone *E. coli* pSKP.

Lane 1: *Bacillus* supernatant,

Lane 2: pSKP supernatant,

Lane 3, 4, 5: Q-sepharose activity peaks (P1, P2, P3),

Lanes 6 to 12: G-75 sub-components (P1a, P1b, P2a, P2b, P3a, P3b, P3c),

Lane 13 to 15: blank,

Last lane: protein molecular weight marker.

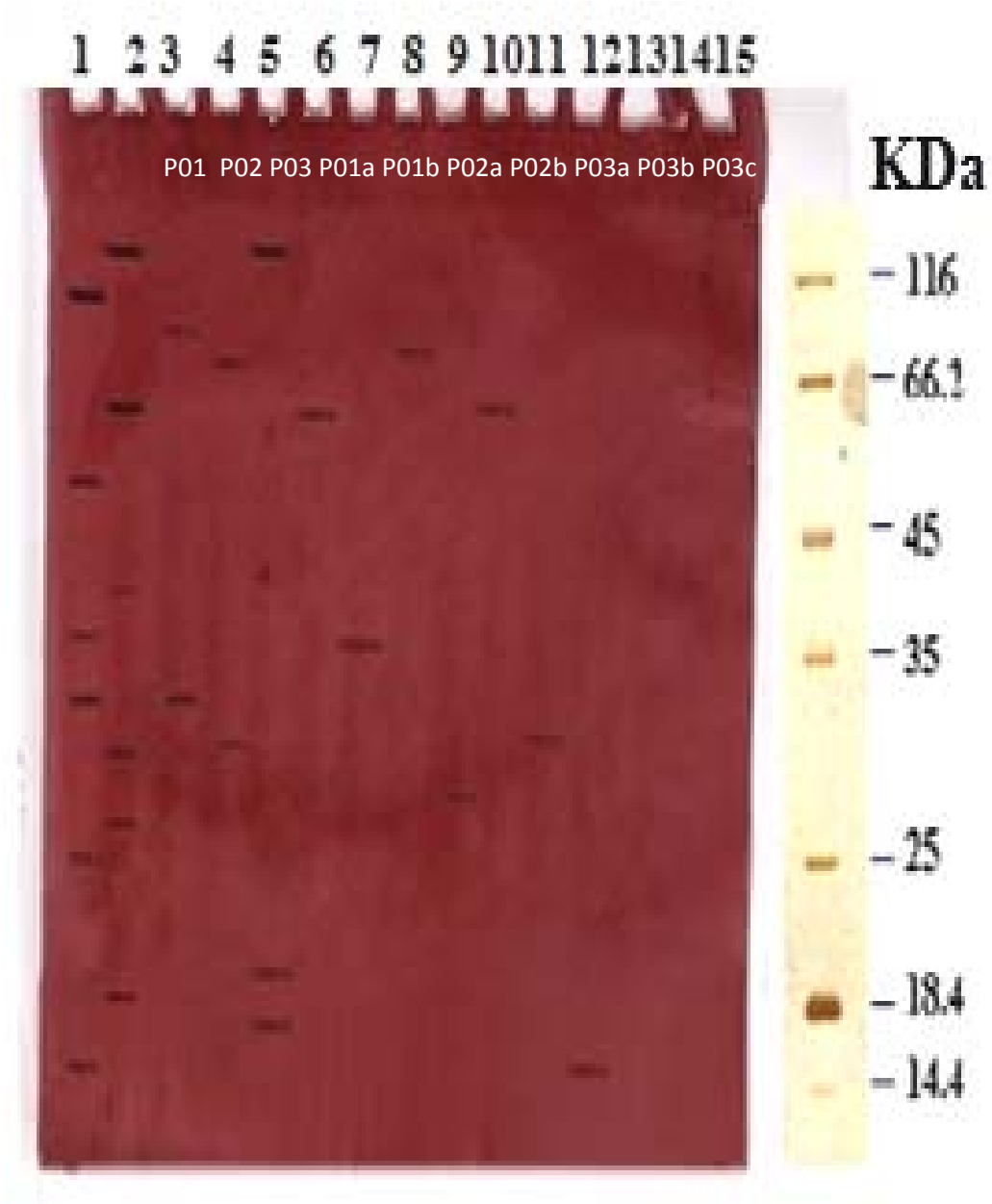


Image 18: Native PAGE analyses and activity staining with Fast Blue RR salt for esterases from clone *E. coli* pSKP0.

Lane 1: *Bacillus* supernatant,

Lane 2: pSKP0 supernatant,

Lane 3, 4, 5: Q-sepharose activity peaks (P01, P02, P03),

Lanes 6 to 12: G-75 sub-components (P01a, P01b, P02a, P02b, P03a, P03b, P03c),

Lane 13 to 15: blank,

Last lane: protein molecular weight marker.

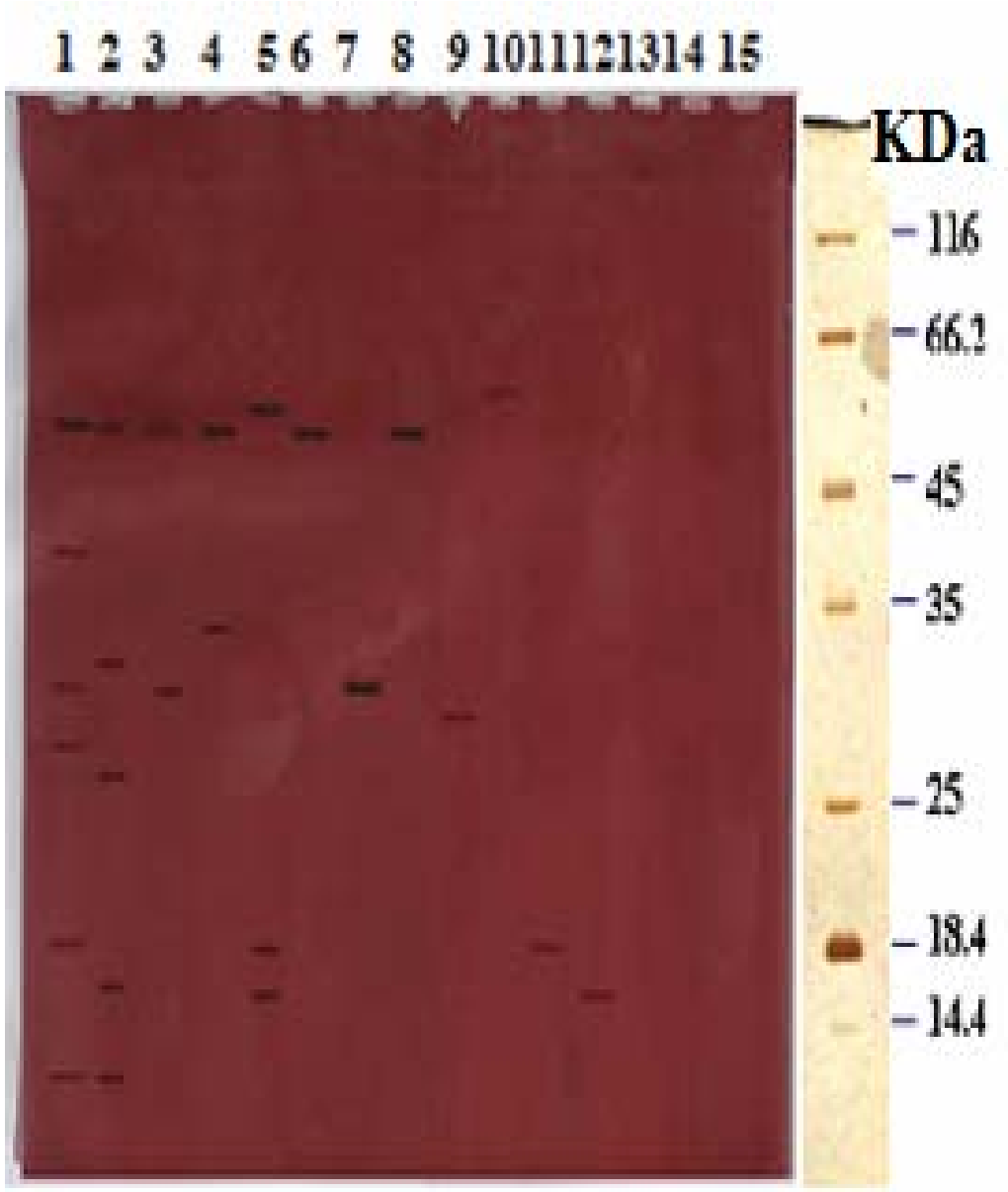


Image 19: Native PAGE analyses and activity staining with Fast Blue RR salt for esterases from clone *E. coli* pSKA.

Lane 1: *Bacillus* supernatant,

Lane 2: pSKA supernatant,

Lane 3, 4, 5: Q-sepharose activity peaks (A1, A2, A3),

Lanes 6 to 12: G-75 sub-components (A1a, A1b, A2a, A2b, A3a, A3b, A3c),

Lane 13 to 15: blank,

Last lane: protein molecular weight marker.

Discussion

5. DISCUSSION

Biomolecules that exhibit particularly high surface activity are classified as biosurfactants (Georgiou *et al*., 1992). Biosurfactants are biological compounds produced by bacteria and fungi that have a broad range of industrial applications as they are being used in a variety of industries ranging from cosmetic, pharmaceutical, food and petroleum industry. They have several advantages over chemical surfactants (Fiechter, 1992). They are amphipathic molecules with both hydrophilic and hydrophobic moieties and have a surface active property of bringing down the surface tension of solutions. They are complex lipids such as glycolipids, lipopeptides, phospholipids, or fatty acids (Cooper and Zajic, 1980). A large number of micro-organisms have been found to be efficient producers of biosurfactants.

In the present research work, we studied the biochemical and genetic regulation of biosurfactant production in *Bacillus subtilis* SK320, an endosulfan degrading bacterium. Endosulfan is very sparingly soluble in water and therefore to enhance the degradability of endosulfan and other hydrophobic compounds it is essential that efficient endosulfan degrading bacteria should produce compounds that could solubilize chemical compounds by reducing the surface tension between solutions. *Bacillus subtilis* SK320 produces biosurfactant and the production of the biosurfactant was regulated by the carbon source. Among the various carbon sources, olive oil (0.5%, v/v) was the best inducer of growth and biosurfactant production. Olive oil was an unconventional carbon source used for the production of this surfactin-type biosurfactant unlike other reports where simple sugar glucose was the carbon source of choice (Kosaric *et al*., 1981, Zuber *et al*., 1998, Javaheri *et al.,* 1985, Yakimov *et al*., 1995). *Candida bombicola* is one of the few yeasts to produce biosurfactants (sophorolipids) from both vegetable oils and sugars (Mulligan, 2005). Highly insoluble carbon source such as n-hexadecane, paraffinic oil, glycerol, babassu oil for *P. aeruginosa* PA1 (Anna *et al*., 2002), soy bean curd residue (okara) for *B. subtilis* YB8 and *B. subtilis* MI113 (Shoda *et al*., 1996, 1997), peat hydrolysate for *B. subtilis* (Mulligan and Sheppard, 1987) and soybean oil, safflower oil, glycerol for *P. aeruginosa* GS9-119 and DS10-129 (Banat *et al.,* 2002) have been reported to induce biosurfactant production. Muthusamy *et al*. (2008) have shown cheap substrates such as vegetable oils and oil wastes, plant-derived oils, lactic whey and distillery wastes, starchy substrates, olive oil mill

effluent, animal fat, soapstock and molasses have the potential for enhancing biosurfactant production.

In *Bacillus subtilis* SK320, the production of biosurfactant was found to be growth associated. Maximum biosurfactant activity was associated with cells at the stationary phase of growth when the nutrient limiting conditions start prevailing in the growth medium. The production of surfactin in culture broth of *Bacillus subtilis* (Peypoux *et al.*, 1999), rhamnolipids by *Pseudomonas aeruginosa* (Hisatsuka *et al.*, 1971), emulsan in *Acinetobacter calcoaceticus* RAG-1 (Gutnick *et al*., 2003), exopolysaccharide in *A. calcoaceticus* BD4 (Kaplan and Rosenberg, 1982) and rhamnolipid AP-6 in *P. fluorescens* 378 (Persson *et al*., 1988) were all found to be growth associated. The rate of growth of all these bacteria varied but maximum biosurfactant activity was only observed when the bacterium entered the stationary phase irrespective of its rate of growth. Hommel and Ratledge (1993) postulated that in bacteria, growth and product formation (biosurfactant) proceed as separate events. In the exponential phase of growth, there is often only a very low rate of surfactant production; over-production of surfactant occurs only when the cells cease to grow. Thus maximum biosurfactant production is generally achieved during stationary phase of growth. This postulate seems to hold true for *Bacillus subtilis* SK320 also.

In our study, the biosurfactant activity in *Bacillus subtilis* SK320 was associated with nitrogen and phosphate depletion in the growth medium. Maximum biosurfactant activity was achieved during the stationary phase of growth of *Bacillus subtilis* SK320 when the nitrogen and inorganic phosphate depletion was the highest i.e. at the lowest level of nitrogen and inorganic phosphate in the growth medium. Robert *et al* (1989), Singer and Finnerty (1990), and Singh *et al* (1990) have shown similar results where the production of biosurfactant in *Pseudomonas* 44T1, *Rhodococcus* sp., and *Candida tropicalis* IIP-4 was linked with nitrogen limiting conditions in the medium. Also the expression of *rhl*AB genes in *Pseudomonas aeruginosa* was found to be enhanced 20-fold during the stationary phase of growth under conditions of nitrogen limitation (Reiser *et al*., 1994). However, in *T. apicola* (Hommel *et al*, 1987), biosurfactant production was not at all related to nitrogen starvation as the maximum biosurfactant production was during the exponential phase of growth. In *P. aeruginosa* (Mulligan *et al.,* 1989), the biosurfactant production was induced

during the shift in phosphate metabolism i.e. towards its depletion and rhamnolipid biosynthesis in *P. aeruginosa* occurred during the late-exponential and stationary phases of growth, typically under conditions of nitrogen or iron limitation (Reiling *et al*., 1986). Therefore, it appears that the growth limiting conditions i.e. depletion of nitrogen and inorganic phosphate was responsible for the regulation of biosurfactant activity. It seems that nitrogen limitation somehow triggers a metabolic shift towards fatty acid synthesis resulting in synthesis of secondary metabolites and thus release of biosurfactant during the stationary phase of growth.

Bacillus subtilis SK320 was capable of growth on variety of nitrogen sources: ammonium nitrate (NH_4NO_3), ammonium sulphate ($(NH_4)_2SO_4$), ammonium chloride (NH_4Cl) and sodium nitrate ($NaNO_3$). However, the bacterium grew luxuriantly on ammonium nitrate and growth on nitrate stimulated maximum production as well as activity of the biosurfactant. *Bacillus subtilis* SK320 showed poor growth on sodium nitrate as nitrogen source. In *P. aeruginosa* (Guerra Santos *et al.,* 1984), and *Nocardia cornyebacteroides* (Powalla *et al.,* 1989), among the inorganic nitrogen sources, nitrate gave the highest biosurfactant production. There seemed to be no correlation between the amount of growth (cell biomass) and level of biosurfactant activity in *Bacillus subtilis* SK320. Ammonium nitrate medium supported the best growth and exhibited a higher biosurfactant activity when compared to other nitrogen sources in which the cell biomass/protein was comparatively quite low.

The purified biosurfactant from *Bacillus subtilis* SK320 was a lipopeptide with 89.4% lipid (w/w) and 7.45% protein (w/w). Boiling or incubating the biosurfactant with proteinase K led to a decrease in biosurfactant activity thereby indicating that the protein moiety was essential for biosurfactant activity. The decrease in activity of the partially purified biosurfactant at pH 8.0 and above also indicated an active role for the protein moiety, as the loss in activity at high pH may be because of denaturation of the protein(s). Lipids constituted a large percentage of the partially purified biosurfactant and therefore regulated the biosurfactant activity in a significant manner. The incubation of biosurfactant with lipase resulted in appreciable loss in biosurfactant activity (up to 100%). In *Pseudomonas* PGI (Reddy *et al.,* 1983) also, the protein moiety was shown to be essential for biosurfactant activity as incubation of the biosurfactant with chymotrypsin reduced both solubilizing and

emulsifying activities to very low levels. Similarly, in *A. calcoaceticus* BD4, the polysaccharide moiety of the biosurfactant alone showed no emulsification activity; but polysaccharide released with protein during growth showed potent emulsification activity (Rosenberg *et al.,* 1983). However, in *A. calcoaceticus* RAG-1, the protein moiety of the emulsan was not at all involved in the activity as the deproteinized emulsan retained all its biosurfactant activity. Emulsan was later characterized to be a lipopolysaccharide (Shabtai and Gutnick, 1985). The biosurfactant of *Bacillus subtilis* SK320 contained 7.45% protein (w/w), which was almost one third when compared to 34% (w/w) in *Pseudomonas* PG-1 (Reddy *et al.,* 1983). The amount of lipid was slightly less (32%, w/w) when compared with the lipid content in *Pseudomonas* PG-1. The difference in the percentages of the chemical constituents could be because of different growth conditions. *Bacillus subtilis* SK320 was grown on basal medium with olive oil as carbon source, while *Pseudomonas* PG-1 was grown in minimal medium with hexadecane/pristane as the substrate (Rosenberg *et al*; 1979, Shabtai and Gutnick, 1985). It has been showed that in *Pseudomonas* spp., the composition of the biosurfactant produced was very much affected by the carbon substrate used for the cultivation (Desai and Desai, 1993).

While studying the regulation of biosurfactant activity in *Bacillus subtilis* SK320 with different carbon sources, we also noticed the induction of esterase activity in the culture medium. *Bacillus subtilis* SK320 was grown with different substrates i.e. vegetable oil, glycerol, maltose, n-dodecane, mobile oil, crude oil, olive oil, glucose and sucrose. Olive oil was the best inducer of esterase activity amongst all the substrates with the highest activity of 3.143 IU/ml, followed by maltose, glycerol, sucrose and vegetable oil with activities of 0.7929, 0.6616, 0.5097 and 0.4248 IU/ml respectively. Olive oil being the best inducer of esterase activity among all the carbon sources was used for further studies. This is in contrast to the observation in *Bacillus* with cephalosporin and acetyl xylan esterase where maximum enzyme activity was observed in nutrient rich medium with glucose, an easily assimilable substrate as the carbon source (Politino *et al.,* 1997, Hasona *et al.,* 2002, Reddy *et al.,* 1989, Khalameyzer *et al.,* 1999).

Esterases belong to the group of hydrolases (carboxylester hydrolases; E.C. 3.1.1.1) which catalyze the formation or cleavage of ester bonds of water-soluble substrates. Esterases are found in plants, animals and microorganisms and show a preference towards short-

chain acetyl esters. Esterase enzymes play a crucial role as catalysts for many biotechnological applications including the biochemical reactions in pharmaceutical, food and chemical industries (Faber, 1997). However the physiological function of bacterial esterases is yet not clear. Some esterases may be involved in plant pathogenicity, carbon source provision and biocide detoxification (Khalameyzer *et al*., 1999) while others catalyze inter-esterification, aminolysis, and peracid formation (Bornscheuer, 2002). Bacteria produce and secrete esterases, which are capable of hydrolyzing fats and oils and typically show highest activity towards the soluble state of its substrate (Fojan *et al*., 2000). The hydrolytic mechanism of most of these esterases resembles the hydrolytic mechanism of lipases and serine proteases. All of these enzymes contain a catalytic triad that usually consists of a serine, a histidine, and an aspartic acid. The serine is embedded in the consensus sequence G-X-S-X-G at the active site, and ester hydrolysis is mediated by a nucleophilic attack of the active serine on the carbonyl of the substrate in a charge-relay system with the two other amino acid residues (Ollis *et al*., 1992).

We observed that in *Bacillus subtilis* SK320 the esterase activity and biosurfactant production worked in perfect synchronization with each other. Esterase activity was found to be maximum when biosurfactant activity was at its peak. This showed that these two phenomena worked in harmony with each other. The results were in accordance with the findings of Shabtai and Gutnick (1985), who also showed the role of esterases in the release of biosurfactant (emulsan), a lipopolysaccharide, in *A. calcoaceticus* RAG-1. The esterified emulsan was active, but the de-esterified emulsan was found to be totally inactive. *Bacillus subtilis* SK320 was found to be a good producer of esterases with the maximum esterase activity being released in the medium during the stationary phase and a very low amount being cell-bound. These results were in contrast to that obtained with *Acenitobacter* spp. (Breuil and Kushner, 1975), where most of the esterase activity was found to be cell associated and only slight amounts appeared in the external medium during late growth. In contrast, in *A. calcoaceticus* RAG-1 (Shabtai and Gutnick, 1985) esterase activity was found both in cell free broth and on the cell surface. With a decrease in the cell-bound activity there was an increase in the cell free esterase activity during growth.

The purification of esterase present in the supernatant of *Bacillus subtilis* SK320 on Sepharose Q showed three major peaks showing highest esterase activity. High activity peaks were further resolved by Sephadex G-75 into their sub-components. The sub-components were active as indicated by the zymogram. The esterase components were purified to homogeneity as revealed by native PAGE followed by activity staining, using RR-salt and B-salt. The elution profile emphasized on the esterase enzyme multiplicity in the supernatant perhaps due to some post-translational modification or some different gene products encoded by the bacterial genome. Multiplicity of esterases has been reported in *Bacillus* species particularly in *B. coagulans*, *B. subtilis* & *B. acidocaldarins, in Pseudomonas fluorescens* (Khalameyzer *et al.,* 1999, Mnisi *et al*., 2005, Higerd *et al*., 1993, Monaco *et al*., 1994, Monaco *et al*., 2000, Choi *et al*., 1990) and in *T. reesei* (Biely *et al.,* 1987) and *P. purpurogenum* (Egana *et al*., 1996). The role of multiplicity can only be explained that despite esterases not being essential for growth in microbes, the multiplicity allows them to function for the hydrolysis of ester compounds for their better assimilation.

The purified esterases showed strong activity in the pH range of 6 to 8. All components of esterases were stable at a temperature range of 35 to 50^0C, respectively. Michaelis (Km) constant for components A1, A2, B1, B2, C1, C2, C3 was found to be between 2.56 to 11.1 µmoles whereas, Vmax values were found to range from 0.166 to 0.833 µmol min^{-1} mg^{-1}, respectively. The native molecular weights of the esterase components from *Bacillus subtilis* SK320 ranged from 30 to 116 kDa. In *B. subtilis* the cephalosporin esterase exhibited Km values of 2.8 X 10^{-3} and 8.3 X 10^{-3} M, for substrates 7-aminocephalosporanic acid (7-ACA) and 7-(thiophene-2-acetamido) cephalosporanic acid (cephalothin) (Abbott and Fukuda,1975). Km and Vmax of 0.88 µmoles and 8.9 U/mg for 6-acetylmorphine was found for the purified heroin esterase from *E. coli* clone of *Rhodococcus* sp. strain H1 (Rathbone *et al.*, 1997), Km values of 0.45 and 0.52 µmoles were observed for acetyl xylan esterases I and II from *Thermoanaerobacterium* sp. strain JW/SL-YS485, when 4-methylumbelliferyl acetate was used as the substrate (Shao and Wiegel,1995) whereas for the esterase from *R. toruloides* the Km and Vmax were found to be 51.8 µmoles and 7.9 µmol min^{-1} mg^{-1} respectively, when cephalosporin C was used as a substrate (Politino *et al*., 1997). None of the enzymes exits as a monomer and this was in accordance with the study of Takimoto *et al.,* 1994 where the *Bacillus subtilis* enzymes are multimeric in nature with the molecular weights ranging from 150 to 280 kDa.

Cloning of the biosurfactant gene(s) i.e. *sfp*, *sfp0* and *srfA* from *Bacillus subtilis* SK320 into *E. coli* was successfully accomplished in this study. The clones of *Bacillus subtilis* SK320 in *E. coli* gave a higher biosurfactant activity compared to the parent strain. Concomitantly we observed that the clones pSKP, pSKP0 and pSKA were not only found to be positive for esterase activity but showed over-expression of the respective gene(s), thereby giving higher esterase activity then the parent strain. The data revealed a correlation between the biosurfactant and esterase activities i.e. both the biosurfactant and esterase activities increases with the increase in the incubation period of the culture medium. Both activities were found to be maximum between 48 to 72 hrs, after which there was a reduction in the activities. *Bacillus subtilis* SK320 when grown on basal medium supplemented with 0.5% (v/v) olive oil showed a rapid growth reaching the maximum at 72 hr after which there was a drop in growth. Esterase production and biosurfactant activity increased gradually up to 24-48 hrs, showed maximum value at 72 hrs and then started declining. In *A calcoaceticus* BD 413 (Kok *et al.,* 1993) high amount of esterase and biosurfactant activity was produced only during the transition from exponential to stationary phase, while in *A calcoaceticus* RAG-1 (Gutnick *et al.,* 2003) esterase activity followed the growth pattern, with the maximum activity being achieved during the stationary phase of growth. Similar increase in lipase, an ester hydrolase activity during transition to stationary phase has been reported in *A calcoaceticus* (Kok *et al.,* 1993). In *Acinetobacter venetianus* RAG-1 the release of emulsan from the bacterial cell surface was mediated by the action of a cell surface esterase, which is one of the key components in the active emulsan-protein complex and itself appears in the growth medium just prior to the appearance of the cell-free emulsifying activity (Gutnick *et al.,* 2003). There was no apparent biosurfactant and esterase activity found in the parent *E.coli* DH5α, whereas the *E.coli* containing the biosurfactant gene showed growth on immiscible substrate olive oil as well as esterase and biosurfactant activity higher than the parent strain. Our results are in accordance with the finding that *sfp* gene is required for cells of *Bacillus subtilis* to become producers of the lipopeptide antibiotic surfactin. The gene was plasmid amplified in *B. subtilis,* where it conferred Srf^{+} phenotype on surfactin non-producing cells, but the clone didn't showed over-production of surfactin (Nakano *et al*., 1992). In contrast to the same report our clones showed over-production of biosurfactant and over-expression of esterase. We also found that the gene(s) responsible for biosurfactant production had positive role to play in the esterase

protein production by the isolate. Similarly, Yao *et al*., (2003) cloned, sequenced and characterized a genomic region of *B. subtilis* B3 comprising *srfDB3, aspB3, lpab3* and *yczEB3* genes. Among them, the *srfDB3* gene encodes thioesterase which is required for biosynthesis of surfactin in *B. subtilis*. A novel gene *lipB* which hydrolyzed triacylglycerol esters and *p*-nitrophenyl esters of fatty acids with short chain lengths of ≤ 10 carbon atoms was cloned from *Bacillus subtilis* and over expressed in *Escherichia coli* and was shown to be an esterase rather than a lipase (Eggert *et al.,* 2000). An esterase from *B. subtilis* (BsubE) cloned, and expressed in *E. coli* showed high homology (75% identity, > 95% homology) to an esterase from thermophilic *B. stearothermophilus* (BsteE) (Henke and Bornscheuer, 2002) but with remarkable differences in their properties. Esterase gene has also been cloned from *Arthrobacter globiformis* (Nishizawa *et al.,* 1995), *Acinetobacter Iwoffi* 16C-1 (Kim *et al.,* 2003), A*cinetobacter calcoaceticus* RAG-1 (Reddy *et al.,* 1989) and *Pseudomonas fluorescens* (Choi *et al.,* 1990, Khalameyzer *et al.,* 1999), respectively.

The genetically engineered construct was then used to confirm the sequences of expressed proteins. The DNA sequences were analyzed in order to determine the nucleotide sequence of the clone. The gene sequences thus obtained were used to infer functional and evolutionary relationships between sequences in the database and to identify members of gene families using National Center for Biotechnology Information's BLAST and FastA facility. The dendogram thus obtained revealed that the deduced amino acid sequence has similarity with *Bacillus subtilis* surfactin synthetase gene, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus megaterium, Bacillus subtilis* srfAA, several lipases from yeast and filamentous fungi. Additionally weaker similarities were also observed with *Clostridium thermocellum*, *Parabacteriodes distasonis*, *Asperigillus* sp and even with mRNA of *Homo sapiens*. Based on the local alignment found with BLAST, it was estimated that the greatest overall similarity (99.0%) was with biosurfactant and esterase genes of *Bacillus subtilis.* Multiple sequence alignment was also performed using MultAlin tool in order to access the degree of similarity or relatedness of the biosurfactant gene sequences to a gene family. The amino acid sequences from *sfp, sfp0* and *srfA* biosurfactant genes were aligned with two of the esterase gene sequences: *Bacillus* sp. NK13 esterase gene, complete cds (Liu *et al.* 2005, Unpublished, PubMed Accession number: DQ196347) and *Bacillus clausii* KSM-K16 DNA, complete genome, esterase gene (Hakamada *et al.* 1994, PubMed Accession number: AP006627). The results revealed

similarity as well as conserved family characteristics between the sequenced genes and the genes taken for alignment. Therefore we conclude that the biosurfactant production gene(s) had some role to play for the release of esterase protein in the culture medium. These findings also suggested the possibility that *Bacillus* species and other related species have esterase enzymes that are evolved from a common ancestor and are responsible for some similar functions.

The clones viz. pSKP, pSKP0 and pSKA were then grown in basal medium supplemented with olive oil and ampicillin for the purification of biosurfactant. The biosurfactant yield obtained was 2.13, 2.20 and 2.45 gm/lit, respectively which was much higher than obtained for parent *Bacillus* strain i.e. 1.2 gm/lit. Surprisingly the gene(s) showed over-expression and thus there was an increase in the % yield of the biosurfactant in the supernatant. The biosurfactant obtained from clones pSKP, pSKP0 and pSKA reduced the surface tension of water to as low as 38.4, 35 and 30.7 dynes/cm, respectively. The surface tension values were in accordance with the values obtained for other species of *Bacillus.* The results suggested that the over expression of biosurfactant gene was responsible for the high surface tension reduction shown by the biosurfactant of the clones. Total production yield of the lipopeptides from *Bacillus subtilis* BBK-1 was about 480 mg/lit at 30^0C for 24hr (Roongsawang *et al*., 2002), whereas *Bacillus* sp. strain IAF 343 gave the yield of 1g/lit on medium containing only water soluble substrates (Cooper and Goldenberg, 1987). *P. aeruginosa* DS10-129 produced 4.31, 2.98 and 1.77 g/lit rhamnolipid biosurfactant using soybean oil, safflower oil, glycerol as substrates (Banat *et al.,* 2002). *Bacillus cereus* IAF 346 produced a monoglyceride biosurfactant that lowered the surface tension of water to 28mN/m with a yield of 1.6 g/lit (pH 6.5) and 1.7 g/lit (pH 7.0) (Cooper and Goldenberg, 1987). *B. subtilis* grown on medium containing 4% glucose gave the yield of 1-2 g/lit of biosurfactant with minimum surface tension of 27mN/m (Kosaric *et al.,* 1981). *B. licheniformis* JF-2 anaerobically produced biosurfactant when grown in glucose rich medium and reduced surface tension of water to 28mN/m (Javaheri *et al.,* 1985). Purified lichenysin A from *B. licheniformis* BAS50 decreased the surface tension of water to 28mN/m with a yield of 70-160 mg/lit (Yakimov *et al*., 1995). The maximum yield of surfactin was about 110 mg/lit by the strain *B. subtilis* S 499 (Michel *et al.,* 1990).

Esterases from clones *E. coli* pSKP, pSKP0 and pSKA on purification with Sepharose Q showed three distinct active protein components, respectively, giving maximum esterase activity similar to the parent strain. Purified esterases from all the three clones pSKP, pSKP0 and pSKA showed strong activity in the pH range of 6 to 8 with an optimal activity around pH 7.0. All components of esterase for clone pSKP were stable at a temperature range of 40 to 50^0C, respectively, whereas a temperature range of 35 to 50^0C was found to be suitable for esterase components of clone pSKP0, respectively. All components of clone pSKA esterase were stable at 40^OC except the A3a and A2b component which were stable also at 50^OC and 55^OC, respectively. This is in contrary to esterase enzymes from the thermophilic *Geobacillus* spp. which are active at temperatures in excess of 65^0C (Henke and Bornscheuer, 2002). Michaelis (Km) constant for the esterase components of clone pSKP, pSKP0 and pSKA was observed to be in range of 1.11 to 25 µmoles whereas the Vmax values were in range of 0.112 to 1 µmol min^{-1} mg^{-1}, respectively. Like the parent strain clone pSKP, pSKP0 and pSKA esterase components were strongly inhibited by mercury, lead and EDTA, whereas metal ions such as Fe^{2+}, Ca^{2+} and Mg^{2+} greatly increased the xylanase activity and Mn^{2+} strongly inhibited the activity of acetyl esterase from *Bacillus* sp. strain k-1 (Ratanakhanokchai *et al.,* 1999). The temperature stability profiles of LipA and LipB showed that these wild-type enzymes remained stable up to a temperature of 45^0C and showed 80-95% residual activity after 48 h incubation at room temperature with an alkaline pH range of 11-12 (Eggert *et al*., 2000). In contrast the novel esterase from *B. sublitis* BsubE was almost inactivated at 50^0C and that from *B. stearothermophilus* showed highest activity at 65-70^0C (Henke and Bornscheuer, 2002). The recombinant esterase from *A. globiformis* showed a Vmax and Km for (+)- *trans*-ethyl chrysanthemate as 8.7 µmol/min/mg and 3.3 mM, whereas the optimum pH and temperature were 9.5 and 50^0C, respectively (Nishizawa *et al*., 1995). The optimum pH and temperature of the purified esterase EstF1 from *P. fluorescens* were 7.5 and 43^0C, respectively. The Vmax and Km values from hydrolysis of δ-valerolactone, γ-butyrolactone, ε- caprolactone and ethyl acetate were found to be 39, 25, 22, 17 U/mg and 7.5, 12.6, 23.7, 45.2 mM, respectively (Khalameyzer *et al*., 1999) whereas the Km and Vmax using α-naphthyl acetate were found to be 1.54 mM and 360 µmol/min/mg for acetyl xylan esterase from *B. pumilus* PS213 (Degrassi *et al.,* 1998). The recombinant *Bacillus subtilis* enzyme sfp, excised from srfB1 and srfB2 modules showed Km values of 1.3-1.8µM (Quadri *et al*., 1998). Carboxylesterase (EstC1) from *Bacillus coagulans* 81-11 showed maximum activity

at pH 8 and 50^0 C, although the enzyme displayed stability at 60^0C also (Mnisi *et al.,* 2005) whereas the carboxylesterase from *Bacillus* sp. BP-7 showed maximum activity at 45^0 C and pH 7.5 (Diaz *et al.,* 2001).

The purified esterase components on native PAGE showed subunit molecular weight of the esterases from *Bacillus subtilis* SK320 and its clones in the range from 12 to 114 kDa, respectively. These results are in accordance with the reported molecular weights of esterases from *B. subtilis* TEB 1003 (Eggert *et al*., 2000), *Pseudomonas fluorescens* (Khalameyzer *et al*., 1999), arylesterase cloned from *Pseudomonas fluorescens* and expressed in *E. coli* (Choi *et al*., 1990) and the recombinant esterase of *A. globiformis* from *E. coli* JM105 (Nishizawa *et al*., 1995). The results clearly indicate that the esterase proteins have a wide range of molecular weights which is also observed in the zymogram of endosulfan degrading *Bacillus subtilis* SK320. The difference in the molecular weights as seen in *Bacillus subtilis* SK320 and its clones could be due to some post translational modifications of *Bacillus* proteins in *E. coli.*

In summary, our results have demonstrated that the extracellular enzymes from *Bacillus subtilis* SK320 and its clones show different biochemical and kinetic characteristics. The substrate specificities determined for the enzymes led us to classify these enzymes as extracellular esterases. Furthermore, we have obtained clear experimental evidence that there is some possible correlation between the release of esterase protein in the culture medium and biosurfactant production. We have successfully cloned three genes responsible for biosurfactant and esterase production from endosulfan degrading *Bacillus subtilis* SK320 and over-expressed them in *Escherichia coli.* We have observed multiplicity in the esterase activity. The clones clearly showed over-expression of the respective genes. Esterases encoded by *Bacillus subtilis* SK320 and its clones had emulsification property. We also conclude that the biosurfactant extracted/purified from *Bacillus subtilis* SK320 and its clones was capable of solubilizing and degrading hydrophobic chemical compounds to a greater extent and can be widely used in the food, detergent, pharmaceutical and chemical industries. This is the first report of an esterase with biosurfactant activity in any *Bacillus* species.

Summary

6. SUMMARY

1. Among the four isolates SK96, SK97, SK98 and SK320 selected from endosulfan sprayed soil of cashew plantation, Kerala, India for biosurfactant activity, isolate SK320 showed maximum growth and biosurfactant activity when grown on 0.5% olive oil (v/v) as sole carbon source. Isolate SK320 was identified as *Bacillus subtilis* by 16s rDNA analysis.
2. Among the various carbon sources tested for biosurfactant production by *Bacillus subtilis* SK320, glucose, glycerol, sucrose and olive oil gave maximum biosurfactant activity in the range of 1.1 to 1.8, while low levels of biosurfactant activity in the range of 0.074 to 0.094 was detected when the isolate was grown in the presence of maltose, vegetable oil, mobile oil, n-dodecane, crude oil. No biosurfactant activity was observed when *Bacillus subtilis* SK320 was grown in presence of substrates like succinate, tween-40, tween-60, triacetin, triton-X-100. Olive oil (0.5%, v/v) was found to be the substrate of choice, with maximum biosurfactant activity being observed between 48 to 72 hrs. It was observed that olive oil was completely emulsified by the end of 120 hrs, indicating that the biosurfactant had good emulsification properties.
3. Partial purification of biosurfactant from *Bacillus subtilis* SK320 showed that the biosurfactant had high lipid content (89.4%) and very low carbohydrate content (3.15%). Protein content was found to be 7.45% whereas the ash content was around 14.5%. These results indicated that the biosurfactant belong to the category of lipopeptides.
4. The optimum pH for maximum biosurfactant activity was pH 6 and 7.5. The biosurfactant was found to be heat labile and increase in temperature resulted in loss of activity with 57% reduction at 20^0C and 86% reduction activity at 100^0C as compared to the unheated samples. Similarly NaCl inhibited biosurfactant activity with complete loss of activity at 10% NaCl (v/v). Owing to the lipopeptide properties of the biosurfactant, it was observed that addition of proteinase K and lipase reduced the biosurfactant activity by almost 60-70% indicating that both protein and lipid were essential for its activity.

5. The partially purified biosurfactant was able to reduce the surface tension of water from 72 to 40.1 dyne/cm. The total yield of partially purified biosurfactant from *Bacillus subtilis* SK320 was 1.2 gm/lit.
6. Esterase activity in *Bacillus subtilis* SK320 was growth dependent, showing highest esterase activity (3.143 IU/ml) between 48 to 72 hrs of growth. Maximum biosurfactant activity (OD 1.841) was also observed during this phase of growth. This proposed a possible correlation between the two activities.
7. Purification of esterase from *Bacillus subtilis* SK320 resolved the enzyme into three active components i.e. A, B and C on ion exchange Sepharose Q column. Component A (specific activity 0.06 IU) was further resolved into two active components A1 and A2 on molecular sieve Sephadex G-75 column, component B (specific activity 0.02 IU) was further resolved into two active components B1 and B2 on Sephadex G-75 column while component C (specific activity 0.02 IU) was resolved into three active components C1, C2 and C3, respectively. The enzyme components showed strong activity in the pH range of 6 to 8 with an optimal activity around pH 7.0. All components of esterase were stable at a temperature range of 35 to 50^0C, respectively. Michaelis (Km) constant for A1, A2, B1, B2, C1, C2, C3 was calculated as 2.94, 2.56, 4.34, 2.5, 9.09, 5.55, 11.1 µmoles whereas the Vmax values were found to be 0.324, 0.334, 0.454, 0.166, 0.833, 0.192, 0.263 µmol min^{-1} mg^{-1}, respectively. Esterases components were strongly inhibited by mercury, lead and EDTA with KI (mM) of 0.45, 2.8, 2.8, 0.6, 3.2, 1.2, 6.7 with mercuric chloride, whereas with lead chloride as inhibitor the values observed were 0.30, 2.3, 2.5, 0.8, 3.3, 1.2, 1.4, respectively.
8. Three genes viz. *sfp*, *sfp0* and *srfA* responsible for biosurfactant production in *Bacillus subtilis* SK320 were cloned in *E.coli* using pGEM-T easy vector system. The clones *E.coli* pSKP, *E.coli* pSKP0 and *E.coli* pSKA harboring the three biosurfactant genes respectively, had a higher biosurfactant activity compared to the parent *Bacillus subtilis* SK320.
9. Cloning of the biosurfactant gene also conferred esterase activity in the clones, further indicating a possible correlation between biosurfactant and esterase activities.
10. Biosurfactant and esterase activities in the three clones were found to be maximum between 48 to 72 hrs. This observation was in accordance with the parent *Bacillus subtilis* SK320. Biosurfactant activity was observed to be maximum in pSKP0 followed

by pSKP and pSKA, whereas esterase activity was maximum in pSKP0 and pSKA followed by pSKP.

11. Based on the matching sequences found with BLAST, it was estimated that the greatest overall similarity (99.0%) of the deduced sequences of pSKP, pSKP0 and pSKA was with biosurfactant and esterase genes of *Bacillus subtilis.*
12. Partial purification of biosurfactant from clone pSKP showed high lipid content of 89.7%, whereas the ash, protein and carbohydrate content were found to be 14.9, 7.21 and 3.08% respectively. *E.coli* clone pSKP was able to reduce surface tension of water from 72 to 38.4 dyne/cm. The biosurfactant yield from pSKP was 2.13 gm/liter.
13. Purification of esterase from pSKP resolved the enzyme into three active components i.e. P1, P2 and P3 on ion exchange Sepharose Q column. Component P1 (specific activity 0.07 IU) was further resolved into two active components P1a & P1b on molecular sieve Sephadex G-75 column, component P2 (specific activity 0.02 IU) was resolved into two active components P2a & P2b while component P3 (specific activity 0.02 IU) was resolved into three active components P3a, P3b and P3c, respectively. The purified esterases from clone pSKP showed strong activity in the pH range of 6 to 8. All components of esterase were stable at a temperature range of 40 to 50^0C, respectively. Michaelis (Km) constant for P1a, P1b, P2a, P2b, P3a, P3b, P3c was calculated as 5, 25, 1.66, 1.11, 2.5, 1.58, 2.38 µmoles whereas the Vmax values were found to be 0.238, 1, 0.196, 0.142, 0.243, 0.212, 0.153 µmol min^{-1} mg^{-1}, respectively. Esterases components were strongly inhibited by mercury, lead and EDTA with KI (mM) of 4, 3.1, 7.5, 0.2, 5, 1.7, 3.1 with mercuric chloride, whereas with lead chloride as inhibitor the values observed were 9.8, 8, 1.3, 0.3, 2.5, 1.3, 1.8, respectively.
14. Partially purified biosurfactant from pSKP0 showed a lipid content of 90.3%, whereas the ash, protein and carbohydrate content were found to be 15.2, 6.73 and 2.94%. pSKP0 was able to reduce surface tension of water from 72 to 35 dyne/cm. The biosurfactant yield from pSKP0 was 2.20 gm/liter.
15. Purification of esterase from pSKP0 resolved the enzyme into three active components i.e. P01, P02 and P03 on ion exchange column. Component P01 (specific activity 0.05 IU) was further resolved into two active components P01a & P01b on Sephadex G-75, component P02 (specific activity 0.01 IU) was resolved into two active components P02a & P02b and component P03 (specific activity 0.02 IU) was resolved into three active components P03a, P03b and P03c, respectively on

Sephadex G-75 column. The purified esterases from clone pSKP0 showed strong activity in the pH range of 6 to 8 with an optimal activity around pH 7.0. There was a rapid decline in activity on either side of pH 7.0. All components of esterase were stable at a temperature range of 35 to 50^0C, respectively. The Michaelis (Km) constant for P01a, P01b, PO2a, P02b, P03a, P03b and P03c was calculated as 4, 3.90, 2.97, 1.23, 2.93, 1.63 and 2.21 µmoles whereas the Vmax values were found to be 0.412, 0.112, 0.439, 0.529, 0.127, 0.433 and 0.312 µmol min^{-1} mg^{-1}, respectively. Esterase components were strongly inhibited by mercury, lead and EDTA with KI (mM) of 1.2, 3.2, 3.3, 0.8, 4.9, 1.8 and 5.2 with mercuric chloride, whereas with lead chloride as inhibitor the values observed were 2.5, 3.3, 2.0, 0.5, 2.3, 1.5 and 1.7, respectively.

16. Partial purification of biosurfactant from clone pSKA showed high lipid content of 91.0%, whereas the ash, protein and carbohydrate content were found to be 15.38, 6.26 and 2.70%. Clone pSKA was able to reduce surface tension of water from 72 to 30.7 dyne/cm. The biosurfactant yield from pSKA was 2.45 gm/liter.
17. Purification of esterase from pSKA resolved the enzyme into three active components i.e. A1, A2 and A3 on Sepharose Q. Component A1 (specific activity 0.72 IU) was further resolved into two active components A1a & A1b on molecular sieve column, component A2 (specific activity 0.17 IU) was resolved into two active components A2a & A2b and component A3 (specific activity 0.25 IU) was resolved into three active components A3a, A3b and A3c, respectively on Sephadex G-75. The purified esterases of pSKA showed strong activity in the pH range of 6-8 with optimum pH at 7. All components of esterase were stable at 40^OC except the A3a and A2b component which were stable also at 50^OC and 55^OC, respectively. Michaelis (Km) constant for A1a, A1b, A2a, A2b, A3a, A3b and A3c was calculated as 2.38, 1.67, 3.06, 2.7, 2.8, 1.60, 1.98 µmoles whereas the Vmax values were found to be 0.168, 0.332, 0.612, 0.167, 0.413, 0.520, 0.608 µmol min^{-1} mg^{-1}, respectively. Esterases components were strongly inhibited by Hg, Pb and EDTA with KI (mM) of 0.55, 2.8, 3.2, 0.8, 3.3, 1.9, and 4.5 for components A1a, A1b, A2a, A2b, A3a, A3b and A3c with mercuric chloride, whereas with lead chloride as inhibitor the values observed were 1.2, 1.9, 5.5, 0.2, 4.1, 2.1 and 1.3, respectively.
18. Purified esterase protein fractions were analyzed by native PAGE. Activity staining for esterases further showed single bands for the protein indicating that gel permeation

chromatography was able to purify the esterase protein to homogeneity. None of the esterase sub-components exist as monomer. The results showed wide range of molecular weights for esterase proteins, indicating that *Bacillus* esterases are multimeric in nature.

19. There was multiplicity in the esterase activity in the endosulfan degrading *Bacillus subtilis* SK320 and there was an enhancement in esterase production and activity when the gene was cloned in *E. coli*. Endosulfan degrading *Bacillus subtilis* SK320 and its clones produce esterases which have the emulsification property. Data also revealed higher biosurfactant yield in the clones.
20. Biosurfactant production and esterase activity work in perfect synchronization in *Bacillus subtilis* SK320 and its clones. Esterase is the key protein associated with the lipopeptide complex. This observation is first to be reported for any *Bacillus* species.

Bibliography

BIBLIOGRAPHY

1. Abbott B. J., Fukuda D. (1975). Physical properties and kinetic behavior of a cephalosporin acetyl esterase produced by *Bacillus subtilis.* Appl. Microbiol. 30:413-419.
2. Abu-Ruwaida A. S., Banat. I. M., Khamis. A. (1991). Nutritional requirements and growth characteristics of a biosurfactant producing *Rhodococcus* bacterium. World J. Microbiol. Biotechnol. 7:53-61.
3. Anna L. M. S., Sebastain G. V., Menezes E.P., Alves T. L. M., Santos A. S., Pereira N., Freire D. M. G. (2002). Production of biosurfactants from *Pseudomonas aeruginosa* PA1 isolated in oil environments. Brazilian J. Chem. Engg. 19:159-166.
4. Arima K., Kakinuma A., Tamura G. (1968). Surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem. Biophys. Res. Commun. 31:488-494.
5. Auria S. D., Herman P., Lakowicz J. R., Tanfani F., Bertoli E., Manco G., Rossi M. (2000). The esterase from the thermophilic eubacterium *Bacillus acidocaldarius:* structural-functional relationship and caparison with the esterase from the hyperthermophilic archeon *Archaeoglobus fulgidus*. Proteins: Structure, Function and Genetics. 40:473-481.
6. Avery O.T., Macleod C. M., McCarty M. (1944-1979). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. J. Exp. Med. 149:297-326.
7. Awasthi N., Manickam N., Kumar A. (1997). Biodegradation of endosulfan by a bacterial coculture. Bull Environ. Contam. Toxicol. 59:928-934.
8. Banat I. M. (1993). The isolation of a thermophilic biosurfactant producing *Bacillus* sp. Biotechnol. Lett. 15:591-594.
9. Banat I. M. (1995). Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. Biores Technol. 51:1-12.
10. Banat I. M., Makkar R. S., Cameotra. S. S. (2000). Potential commercial Applications of microbial surfactants. Appl. Microbiol. Biotechnol. 53:495-508.
11. Banat I. M., Samarah N., Murad M., Horne R., Banarjee S. (1991). Biosurfactant production and used in oil tank clean up. World J. Microbiol. Biotechnol. 7:80-88.
12. Barata C., Solayan A., Porte C. (2004). Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. Aqua Toxicol. 66:125-139.
13. Barbaro S.E., Trevors J.T., Inniss W.E. (2001). Effects of low temperature, cold shock, and various carbon sources on esterase and lipase activities and exopolysaccharide production by a psychrotrophic *Acinetobacter* sp. Can. J. Microbiol. 47:194-205

14. Belsky I., Gutnick D. L., Rosenberg E. (1979). Emulsifier of *Arthrobacter RAG1:* determination of emulsifier bound fatty acids. FEMS Lett. 101:175-178.
15. Besson F., Michel G. (1992). Biosynthesis of surfactin synthesis by *Bacillus subtilis.* Evidence for amino acid activating enzymes. Biotechnol. Lett. 14:1013-1018.
16. Birnboim H., Doly J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7:1513-1523.
17. Bloemberg G. V., Kuiper I., Lagendijk E. L., Pickford R., Derrick J. P., Lamers G. E. M., Thomas-Oates J. E., Lugtenberg B. J. J. (2004). Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. Mol. Microbiol. 51:97-113.
18. Bornscheuer U. T. (2002). Microbial carboxyl esterases: Classification, properties and application in biocatalysis. FEMS Microbiol Rev. 733:1-9.
19. Breuil C., Kushner D. J. (1975). Lipase and esterase formation by psycrophilic and mesophilic *Acinetbacter* species. Can. J. Micribiol. 21:423-33.
20. Cameotra S. S., Vater J., Kablitz B., Wilde C., Franke P., Mehta N. (2002). Matrix assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. App. Environ. Microbiol. 68:6210-6219.
21. Cameotra S. S., Makkar, R.S. (2004). Recent applications of biosurfactants as biological and immunological molecules. Curr. Opin. Microbiol. 7:262-266.
22. Chang J. S., Radosevich M., Jin Y., Cha D. K. (2004). Enhancement of phenanthrene solubilization and biodegradation by trehalose lipid biosurfactants. Environ Toxicol Chem. 23:2816-2822.
23. Chayabutra C., Wu J., Ju L. W. (2000). Rhamnolipid production by *Pseudomonas aeruginosa* under denitrification: effect of limiting nutrients and carbon sources. Biotechnol. Bioengg. 72:25-33.
24. Chen C., Riadi L., Suh S-J., Ohman D. E., Ju L-K. (2005). Degradation and synthesis kinetics of quorum-sensing autoinducer in *Pseudomonas aeruginosa* cultivation. J Bacteriol. 117:1-10.
25. Choi K. D., Jeohn G. H., Rhee J. S., Yoo O. J. (1990). Cloning and nucleotide sequence of an esterase gene from *Pseudomonas fluorescens* and expression of the gene in *Escherichia coli.* Agric. Biol. Chem. 54:2039-2045.
26. Christova N., Tuleva B., Lalchev Z., Jordanova A., Jordanov. (2004). Rhamnolipid biosurfactants produced by *Renibacterium salmoninarum 27BN* during growth on *n*-hexadecane. Z. Naturforsch. 59:70-74.

27. Chung G. H., Lee Y. P., Joehn G. H., Yoo, O. J., Rhae, J. S. (1991). Cloning and nucleotide sequence of thermostable lipase gene from *Pseudomonas fluorescens* S1KW1. Agric. Biol. Chem. 9:2359-2365.
28. Cirigliano M., Carman G. M. (1984). Isolation of a bioemulsifier from *Candida lipolytica.* Appl. Environ. Microbiol. 48:747-750.
29. Cirigliano M., Carman G.M. (1985). Purification and characterization of Liposan, a bioemulsifier from *Candida lipolytica.* Appl. Environ. Microbiol. 50:846-850.
30. Colwell R. R., Snellman E. A., Sullivan E. R. (2002). Purification and properties of the extracellular lipase, LipA of *Acinetobacter* sp. RAG-1. Eur. J. Biochem. 269:5771-5779.
31. Cooper D.G., Zajic J.E. (1980). Surface-active compounds from microorganism. Adv. Appl. Microbiol. 26:229-256.
32. Cooper D.G., Goldenberg B.G. (1987). Surface-active agents from two *Bacillus* species. Appl. Environ. Microbiol. 53:224-229.
33. Cooper D.G., Paddock D.A. (1983). *Torulopsis petrophilum* and surface activity. Appl. Environ. Microbiol. 46:1426-1429.
34. Cooper D.G., Paddock D.A. (1984). Production of a biosurfactant from *Torulopsis bombicola.* Appl. Environ. Microbiol. 47:173-176.
35. Cooper D.G., Macdonald C.R., Duff J.B., Kosaric N. (1981). Enhanced production of Surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl. Environ. Microbiol. 42:408-412.
36. Cosby W. M., Vollenbroich Lee O. H., Zuber P. (1998). Altered *srf* expression in *Bacillus subtilis* resulting from changes in culture pH is dependent on the Spo0K oligopeptide permease and the ComQX system of extracellular control. J Bacteriol. 180:1438-1445.
37. Das K., Mukherjee A. K. (2005) Characterization of biochemical properties and biological activities of biosurfactants produced by *Pseudomonas aeruginosa* mucoid and non-mucoid strains isolated from hydrocarbon-contaminated soil samples. Appl. Microbiol. Cell Physiol. 192-199.
38. Davis D. A., Lynch H. C., Varley J. (2001). The application of foaming for the recovery of Surfactin from *B. subtilis* ATCC 21332 cultures. Enzyme Microbiol Technol. 28:346-354.
39. De Roubin M.R., Mulligan C.N., Gibbs B. F. (1989). Correlation of enhanced surfactin production with decreased isocitrate dehydrogenase activity. Can. J. Microbiol. 35:854-859.
40. De Souza J. T., Boer M. D., Waard P. D., Beek T. A. V., Raaijmakers J. M. (2003). Biochemical, genetic and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens.* Appl. Environ. Microbiol. 69:7161-7172.

41. Degrassi G., Kojic M., Ljubijankic G., Venturi V. (2000). The acetyl xylan esterase of *Bacillus pumilus* belongs to a family of esterases with broad substrate specificity. Microbiology. 146:1585-1591.
42. Degrassi G., Okeke B. C., Bruschi C. V., Venturi V. (1998). Purification and characterization of an acetyl xylan esterase from *Bacillus pumilus.* Appl. Environ. Microbiol. 64:789-792.
43. Desai J.D., Banat I.M. (1997). Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biology Reviews. 61:47-64.
44. Deziel E., Lepine F., Milot S., Villemur R. (2003). *rhlA* is required for the production of a noval biosurfactant promoting swarming motility in *Pseudomonas aeruginosa:* 3-(3-hydroxyalkanoyloxy) alkonic acids (HAAs), the precursors of rhamnolipids. Microbiology. 149:2005-2013.
45. Diaz P., Falcocchio S., Ruiz C., Pastor F. I. J., Saso L. (2005). Identification of a carboxylesterase producing *Rhodococcus* soil isolate. Can. J. Microbiol. 51:753-758.
46. Donnelly P.K., Crawford D.L. (1988). Production of a *Streptococcus viridosporous* T7A of an enzyme which cleave aromatic acids from lignocellulose. Appl. Environ. Microbiol. 54:2237-2244.
47. Duvnjak Z., Kosaric N. (1985). Production and release of surfactant by *Corynebacerium lepus* in hydrocarbon and glucose media. Biotechnol. Lett. 7:793-796.
48. Duvnjak Z., Cooper D.G., Kosaric N. (1982). Production of surfactant by *Arthrobacter paraffineus* ATCC 19558. Biotechnol. Bioengg. 24:165-175.
49. Egana L., Gutierrez R., Caputo V., Peirano A., Steiner J., Eyzaguirre J. (1996). Purification and characterization of two acetyl xylan esterases from *Penicillium purpurogenum.* Biotechnol. Appl. Biochem. 24:33-39.
50. Eggert T., Pencreach G., Douchet I., Verger R., Jaeger K-E. (2000). A novel extracellular esterase from *Bacillus subtilis* and its conversion to a monoacylglycerol hydrolase. Eur. J. Biochem. 267:6459-6469.
51. Ewis H. E., Abdelal A. T., Lu C. D. (2004) Molecular cloning and characterization of two thermostable carboxyl esterases from *Geobacillus stearothermophilus*. Gene. 31:187-95.
52. Faber K. (1997). Biotransformations of non-natural compounds: State of art and future development. Pure Appl Chem. 69:1613-1633.
53. Fiechter A. (1992). Integrated systems for biosurfactant synthesis. Pure Appl. Chem. 64:1739-1743.
54. Fiske C.H., Subba Row Y. (1925). The calorimetric determination of phosphorous. J. Biol. Chem. 66:375-400.
55. Fojan P., Jonson P.H., Petersen M.T., Petersen S.B. (2000). What distinguishes an esterase from a lipase: a novel structural approach. Biochimie. 82:1033-1041.

56. Forsell P., Parovuori P., Linko., Poutanen. (1993). Enzymatic trans-esterification of rapeseed oil and lauric acid in a continuous reactor. J. Am. Oil Chem. Soc. 70:1105-1109.
57. Fuma S., Fujishima Y., Corbell N., D'Souza C., Nakano M. M., Zuber P., Yamane K. (1993). Nucleotide sequence of 5' portion of *srfA* that contains the region required for competence establishment in *Bacillus subtilis.* Nucleic Acids Research. 21:93-97.
58. Georgiou G., Lin S. C., Carswell K., Sharma S. (1994). Continuous production of the lipopeptide biosurfactant of *Bacillus licheniformis* JF-2. App. Microbiol. Biotechnol. 41:281-285.
59. Georgiou G., Lin S. C., Sharma M. M. (1992). Surface active compounds from microorganisms. Bio/Technol. 10:60-65.
60. Gilbert E. J., Cornish A., Jones C. W. (1991). Purification and properties of extra cellular lipase from *Pseudomonas aeruginosa* EF_2. J. Microbiol. 137:2223-2229.
61. Gobbert U., Lang S., Waghner F. (1984). Sophorose lipid formation by resting cells of *Torulopsis bombicola*. Biotechnol. Lett. 6:225-230.
62. Goldmann S., Shabtai Y., Rubinovitz C., Rosenberg E., Gutnick D.L. (1982). Emulsan production in *Acinetobacter calcoaceticus*: distribution of cell free and cell associated cross-reacting material. Appl. Environ. Microbiol. 44:165-170.
63. Guerra-Santos L. H., Kappeli O., Fiechter A. (1984). *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl. Environ. Microbiol. 48:301-305.
64. Gutnick D., Bach H., Berdichevsky Y. (2003). An exocellular protein from the oil-degrading microbe *Acinetobacter venetianus* RAG-1 enhances the emulsifying activity of the polymeric bioemulsifier emulsan. Appl Environ Microbiol. 69:2608–2615.
65. Hakamada Y., Kobayashi T., Hitomi J., Kawai S., Ito S. (1994). Molecular cloning and nucleotide sequence of the gene for an alkaline protease from the alkalophilic *Bacillus* sp. KSM-K16. J. Ferment. Bioeng. 78:105-108.
66. Hale V., McGrew M., Carlson B., Schottel, J. L. (1992). Heterologous expression and secretion of *Streptococcus scabies* esterase in *Streptomyces lividans* and *Escherichia coli.* J. Bacteriol. 174:2431-2439.
67. Hardegger M., Koch A. K., Ochsner U. A., Fiechter A., Reiser J. (1994). Cloning and heterologous expression of a gene encoding an alkane induced extracellular protein involved in alkane assimilation from *Pseudomonas aeruginosa.* Appl. Environ. Microbiol. 60:3679-3687.
68. Hasona A., York S. W., Yomano L. P., Ingram L. O., Shanmugam K. T. (2002). Decreasing the level of ethyl acetate in ethanolic fermentation broths of *Escherichia coli* KO11 by expression of *Pseudomonas putida estZ* esterase. Appl. Environ. Microbiol. 68:2651-2659.

69. Head M. I. (1998). Bioremediation: towards a credible technology. Microbiology. 144:599-608.
70. Henke E, Bornscheuer (2002) Esterases from *Bacillus subtilis* and *B. stearothermophilus* share high sequence homology but differ substantially in their properties. Appl. Microbiol. Biotechnol. 60:320-326.
71. Hester A. (2001). IB Market forecast. Industrial Bioprocessing. 25(5):3.
72. Higerd T.B., Spizizen J. (1973). Isolation of two acetyl esterases from extracts of Bacillus subtilis. J. Bacteriol. 114:1184-1192.
73. Hisatsuka K., Nakahara T., Minoda Y., Yamada K. (1977). Formation of protein-like activator for n-alkane oxidation and its properties. Agric. Biol. Chem. 41:445-450.
74. Hisatsuka K., Nakahara T., Sano N., Yamada K. (1971). Formation of rhamnolipid by *P.aeruginosa* and its function in hydrocarbon fermentation. Agric. Biol. Chem. 35:686-692.
75. Holden P. A., La Montagne M. G., Bruce A. K., Miller W. G., Lindow, S. E. (2002). Assessing the role of *Pseudomonas aeruginosa* surface-active gene expression in hexadecane biodegradation in sand. Appl. Environ. Microbiol. 68:2509-18.
76. Hommel R. K., Stower O., Stuber W., Haferburg D., Kleber H. P. (1987). Production of water soluble surface active exolipids by *Torulopsis apicola.* Appl. Microbiol. Biotechnol. 26:199-205.
77. Hong K. H., Jang W. H., Choi K. D., Yoo O. J. (1991). Characterization of *Pseudomonas fluorescens* carboxylesterase: cloning and expression of the esterase gene in *Escherichia coli.* Agric. Biol. Chem. 55:2839-2845.
78. Hu Y., Ju L. K. (2001) Sophorolipid production from different lipid precursors observed with LC-MS. Enzym. Microbial. Tech. 29:593-601.
79. Iqbal S., Khalid Z. M., Malik K. A. (1995). Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactants by a gamma ray-induced mutant of *Pseudomonas aeruginosa*. Lett. Appl. Microbiol. 21:176-179.
80. Itzhaki R. F., Gill D. M. (1964). A micro Biuret method for estimating proteins. Anal. Biochem. 9:401-410.
81. Janssen D. B., Noordman W. H. (2002). Rhamnolipid stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa.* Appl. Environ. Microbiol. 4502-4508.
82. Javaheri M., Jenneman G. E., McInerney M. J., Knapp R. M. (1985) Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. App. Environ. Microbiol. 50:698-700.
83. Johnsen A. R., Karlson U. (2004). Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. Appl Microbiol Biotechnol. 63:452-459.

84. Kaplan N., Rosenberg E. (1982). Exopolysaccharide distribution and bioemulsifier production in *Acinetobacter calcoaceticus* BD-4 and BD413. Appl. Environ. Microbiol. 44:1335-1341.
85. Kappeli D., Walther P. M., Fiechter A. (1984). Structure of the cell surface of yeast *Candida tropicalis* and its relation to hydrocarbon transport. Arch. Microbiol. 138:279-282.
86. Kathrin R, Daniela T., Michael G., Helmut S., Leo E. (2003). Identification and characterization of a GDSL esterase gene located proximal to the *swr* quorum-sensing system of *Serratia liquefaciens* MG1. Appl. Environ. Microbiol. 69:3901–3910.
87. Khalameyzer V., Fischer I., Bornscheuer U. T., Altenbuchner J. (1999). Screening, nucleotide sequence, and biochemical characterization of an esterase from *Pseudomonas fluorescens* with high activity towards lactones. Appl. Environ. Microbiol. 65:477-482.
88. Kim H. E., Lee I. So., Kim J. H., Hahn K. H., Park U. J., Han H. S., Park K. R. (2003). Gene cloning, sequencing, and expression of an esterase from *Acinetobacter Iwoffii* 16C-1. Current Microbiol. 46:291-295.
89. Kim H-S., Kim S-B., Park S-H., Oh H-M., Park Y., Kim C., Katsuragi T., Tani Y., Yoon B-D. (2000). Expression of *sfp* gene and hydrocarbon degradation by *Bacillus subtilis.* Biotech. Lett. 22:1431-1436.
90. Kluge B., Vater J., Sainikow J., Eckart K. (1989). Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332. FEBS Lett. 234:107-110.
91. Koch A. K., Reiser J., Kappeli O., Fiechter A. (1988). Genetic construction of lactose-utilizing strains of *Pseudomonas aeruginosa* and their application in biosurfactant production. Nat BioTechnol. 6:1335-1339.
92. Koch A. K., Reiser J., Kappeli O., Fiechter A. (1991). Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J. Bacteriol. 173:4212-4219.
93. Kok R. G., Christoffels V. M., Vosman B., Hellingwerf K. J. (1993). Growth-phase-dependent expression of the lipolytic system of *Acinetobacter calcoaceticus* BD413: cloning of a gene encoding one of the esterases. J Gen Microbiol. 139:2329-2342.
94. Kosaric N. (2001). Biosurfactants and their application for soil bioremediation. Food Technol. Biotechnol. 39:295-304.
95. Kosaric N., Choi H.Y., Bhaszezyk R. (1990). Biosurfactant production from *Nocardia* SFC-D, Tenside Surf. Det. 27:294-297.
96. Kuyukina M. S., Ivshina I. B., Makarov S. O., Litvinenko L. V., Cunningham C. J., Philp J. C. (2005). Effect of biosurfactants on crude oil desorption and mobilization in a soil system. Environ International. 31:155-161.

97. Kuyukina M. S., Ivshina I. B., Philp J. C., Christofi N., Dunbar S. A., Ritchkova M. I. (2001). Recovery of *Rhodococcus* biosurfactants using methyl tertiary-butyl ether extraction. J Microbiol Methods. 46:149-156.
98. Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227:680-685.
99. Lee T. H., Lee D.J., Lee S. O., Lim E. J., Kim S. H. (2000). Purification and characterization of biosurfactants from Nocardia sp. L-417, Biotechnol. Appl. Biochem. 31:249-253.
100. Lin J., Minton M. A., Sharma M. M., Georgiou G. (1994) Structural and immunological characterization of a biosurfactant produced by *Bacillus licheniformis* JF-2. App. Environ. Microbiol. 60:31-38.
101. Liu D., Li Z., Guo X., Cao X. (1992). Relative staining strength of esterase bands changing according to staining agents. Rice Genet. Newslett. (Gramene). 9:110.
102. Liu G., Tan Z., Zhang J. (2005). Cloning of esterase gene from *Bacillus* sp. NK13. Unpublished.
103. MacElwee C. G., Lee H., Trevors, J. T. (1990). Production of extracellular emulsifying agent by *Pseudomonas aeruginosa* UG-1. J. Ind. Microbiol. 5:25-52.
104. Maier R. M., Bodour A. A., Barajas-Guerrero C., Jiorle B. V., Malcomson M. E., Paull A. K., Somogyi A., Trinh L. N., Bates R. B. (2004). Structure and characterization of flavolipids, a novel class of biosurfactants produced by *Flavobacterium* sp. strain MTN11. Appl. Environ. Microbiol. 70:114-120.
105. Maier R. M., Bodour A. A., Drees K. P. (2003). Distribution of biosurfactant producing bacteria in undisturbed and contaminated arid southwestern soils. Appl. Environ. Microbiol. 69:3280-3287.
106. Maier R. M., Chavez G. S. (2000). *Pseudomonas aeruginosa* rhamnolipids" biosynthesis and potential applications. Appl. Microbiol. Biotechnol. 54:625-633.
107. Makkar R. S., Cameotra S.S. (2002). An update on the use of unconventional substrates for biosurfactant production and their new applications. Mini Review. Appl. Microbiol. Biotechnol. 58:428-434.
108. Manco G., Di Gennaro S., De Rosa M., Rossi M. (1994). Purification and characterization of the thermostable carboxylesterase from the thermoacidophilic eubacterium *Bacillus acidocaldarius*. Eur J Biochem. 221:965-972.
109. Manco G., Giosue E., D'Auri S., Herman P., Carrea G., Rossi M. (2000). Cloning, overexpression and properties of a new thermophilic and thermostable esterase with sequence similarity to hormone sensitive lipase subfamily from the archeon, *Archaeoglobus fulgidus.* Arch Biochem Biophys. 373:182-192.

110. Margesin R., Schinner F. (2001) Biodegradation and bioremediation of hydrocarbons in extreme environments.. Appl Microbiol Biotechnol. 56:650-663.
111. Mathis R., Mourey A., Hubert P. (1998). One-step purification of a lipolytic enzyme from *Bacillus pumilus* by mild hydrophobic interaction chromatography on polyoxyalkylene glycol-bound sepharose 6B. App. Environ. Microbiol. 54:1307-1308.
112. Mattei G., Bertrand J. C. (1985). Production of biosurfactants by a mixed bacteria population grown in continuous culture on crude oil. Biotechnol. Lett. 7:217-222.
113. McInerney M. J., Javaheri M., Nagle D. P. (1990). Properties of the biosurfactant produced by *Bacillus licheniformis* JF-2. J. Ind. Microbiol. 5:95-102.
114. Merrill C.R. (1990). Silver staining of proteins and DNA. Nature. 343:779-780.
115. Michel G., Peypoux F. (1992). Controlled biosynthesis of Val^7 and Leu^7 surfactins. App. Microbiol. Biotechnol. 36:515-517.
116. Michel G., Sandrin C., Peypoux F. (1990). Coproduction of surfactin and Iturin A, lipopeptides with surfactant and antifungal properties, by *Bacillus subtilis.* Biotechnol. App. Biochem. 12:370-375.
117. Miyazima D., Lida M. R., Iizuka H. (1985). Phospholipid derived from hydrocarbons by fungi. J. Fermant. Technol. 63:219-224.
118. Mnisi S. M., Louw M. E., Theron J. (2005). Cloning and characterization of a carboxyl esterase from *Bacillus coagulans* 81-11. Curr. Microbiol. 50:196-201.
119. Mukherjee S, Das P, Sen R. (2006). Towards commercial production of microbial surfactants. Trend Biotech. 24:509:515.
120. Mulligan C. N., Sheppard J. D. (1987). The production of surfactin by *Bacillus subtilis* grown on peat hydrolysate. App. Microbiol. Biotechnol. 27:110-116.
121. Mulligan C. N. (2005). Environmental applications for biosurfactants. Environ. Pollution. 133:183-198.
122. Mulligan C. N., Gibbs B. F. (1989). Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa.* Appl. Environ. Microbiol. 55:3016-3019.
123. Mulligan C. N., Chow T. Y. K., Gibbs B. F. (1989a). Enhanced biosurfactant production by a *Bacillus subtilis* mutant. Appl. Microbiol. Biotechnol. 31:486-489.
124. Mulligan C. N., Mahmourides G., Gibbs B. F. (1989b). The influence of phosphate metabolism on biosurfactant by *Pseudomonas aeruginosa*. J. Biotechnol. 12:199-210.
125. Mulligan C. N., Roubin M. D., Gibbs B. F. (1989). Correlation of enhanced surfactin production with decreased isocitrate dehydrogenase activity. Can J. Microbiol. 35:854-859.
126. Muthusamy K., Gopalakrishnan S., Ravi T. K, Sivachidambaram P. (2008). Biosurfactants: Properties, commercial production and application. Curr Science. 94:736-747.

127. Nakahara T., Erickson L.E., Gutierrez J.R. (1977). Characteristic of hydrocarbon uptake in culture with two liquid phases. Biotechnol. Bioengg. 19: 9-25.
128. Nakano M. M., Magnuson R., Myers A., Curry J., Grossman A., Zuber P. (1991). s*rf*A: Is an operon required for surfactin production, competence development, and efficient sporulation in *Bacillus subtilis.* J Bacteriol. 173:1770-1778.
129. Nakano M., Xia L., Zuber P. (1991). Transcription initiation region of the *srfA* operon, which is controlled by the *comP -comA* signal transduction system in *Bacillus subtilis.* J Bacteriol. 173:5487-5493.
130. Nakano M., Zuber P. (1989). Cloning and characterization of *srfB,* a regulatory gene involved in surfactin production and competence in *Bacillus subtilis.* J Bacteriol. 171:5347-5353.
131. Nakano M., Zuber P. (1993). Mutational analysis of the regulatory region of the *srfA* operon in *Bacillus subtilis.* J. Bacteriol. 175:3188-3191.
132. Nakano M., Zuber P., Corbell N., Besson J. (1992). Isolation and characterization of *sfp:* a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis.* Mol. Gen. Genet. 232:313-321.
133. Nakano M. M., Zuber P. (1990). Molecular biology of antibiotic production in *Bacillus*. Crit. Rev. Biotechnol. 10:223-240.
134. Nakayama S., Takahashi S., Hirai M., Shoda M. (1997). Isolation of new variants of surfactin by a recombinant *Bacillus subtilis.* App. Microbiol. Biotechnol. 48:80-82.
135. Neu T. R., Poralla K. (1990). Emulsifying agents from bacteria isolated during screening for cells with hydrophobic surfaces. Appl. Microbiol. Biotechnol. 32:521-525.
136. Neu T. R., Hartner T., Poralla K. (1990). Surface active properties of viscosin: a peptidolipid antibiotic. Appl. Microbiol. Biotechnol. 32:518-520.
137. Nielsen T. H., Koch B., Sorensen D., Anderson J. B., Christophersen C., Moli S., Givskov M., Sorensen J., Nybroe O. (2002) Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudates via the Gac two-component regulatory system. Appl. and Environ. Microbiol. 68:4509-4516.
138. Nielsen T.H., Sorensen D., Tobiasen. C., Anderson J.B., Christophersen C. (2002). Antibiotic and Biosurfactant Properties of Cyclic Lipopeptides produced by Fluorescent *Pseudomonas* spp. From the sugar beet Rhizosphere. Appl. Environ. Microbiol. 3416-3423.
139. Nishizawa M., Shimizu M., Ohkawa H., Kanaoka M. (1995). Stereo selective production of (+)-trans-chrysanthemic acid by a microbial esterase: cloning, nucleotide sequence, and over expression of the esterase gene of *Arthrobacter globiformis* in *Escherichia coli.* App. Environ. Microbiol. 61:3208-3215.

140. Norman R. S., Suau R. F., Morris P. J. (2002). Variability in *Pseudomonas aeruginosa* lipopolysaccharide expression during crude oil degradation. Appl. Environ. Microbiol. 68:5096-5103.
141. O'Connor L. (2002). Market forecast: microbial biosurfactants. Industrial Bioprocessing. 24:10-11.
142. Ochsner U. A., Fiechter A., Reiser J. (1994). Isolation, characterization, and expression in *Escherichia coli* of the *Pseudomonas aeruginosa* rhlAB genes encoding a rhamnosyltransferase involved in rhamnolipid biosurfactant synthesis. J. Biol. Chem. 269:19787-19795.
143. Ochsner U. A., Hembach T., Fiechter A. (1996). Production of Rhamnolipid Biosurfactants. Adv. Biochem. Engg. / Biotechnol. 53:89-116.
144. Ohno A., Ano T., Shoda M. (1995). Production of a lipopeptide antibiotic, surfactin, by recombinant *Bacillus subtilis* in solid state fermentation. Biotechnol. Bioengg. 47:209-214.
145. Olivera N. L., Commendatore M. G., Moran A. C., Esteves J. L. (2000). Biosurfactant enhanced degradation of residual hydrocarbons from ship bilge wastes. J Ind. Microbiol Biotechnol. 25:70-73.
146. Ollis D. L., Cheah E., Dijkstra B., Frolow F., Franken S., Harel M., Remington S.J., Silman I. (1992). The α/β hydrolase fold. Protein Eng. 5:197-211.
147. Peng L., Xu X., Mu H., Nissen, J A. (2002). Production of structured phospholipids by lipase-catalyzed acidolysis: optimization using response surface methodology. Enzyme Microbial Tech. 31:523-532.
148. Persson A., Molin G., Andersson N., Sjoholm J. (1990). Biosurfactant yields and nutrient consumption of *Pseudomonas flurescens* 378 studied in a microcomputer controlled mutifermentation system. Biotechnol. Bioengg. 36:252-255.
149. Persson A., Osterberg E., Dostalek M. (1988). Biosurfactant production by *Pseudomonas fluorescens* 378: growth and product characteristics. Appl. Microbiol. Biotechnol. 29:1-4.
150. Peypoux F., Bonmatin J. M., Wallach J. (1999). Recent trends in the biochemistry of surfactin. App. Microbiol. Biotechnol. 51:553-563.
151. Pines O., Bayer E. A., Gutnick D. L. (1983). Localization of emulsan-like polymers associated with cell surface of *Acinetobacter calcoaceticus.* J. Bacteriol. 154:893-905.
152. Politino M., Tonzi S. M., Burnett W. V., Romancik G., Usher J. J. (1997). Purification and characterization of a cephalosporin esterase from *Rhodosporidium toruloides.* Appl. Environ. Microbiol. 63:4807-4811.
153. Powalla M., Lang S., Wray V. (1989). Penta- and disaccharide lipid formation by *Nocardia corynebacteroides* grown on n-alkanes. Appl. Microbiol. Biotechnol 31:473-479.

154. Prim N., Pastor F. I. J., Diaz P. (2001). Cloning and characterization of a bacterial cell-bound type B carboxyl esterase from *Bacillus* sp. BP-7. Curr. Microbiol. 42:237-240.
155. Quadri L. E. N., Weinreb P. H., Lei M., Nakano M., Zuber P., Walsh C. T. (1998). Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. Biochem. 37:1585-1595.
156. Rahman K. S. M., Rahman T. J., McClean S., Marchant R., Banat I. M. (2002). Rhamnolipid biosurfactant production by strains of *Pseudomonas aeruginosa* using low-cost raw materials. Biotechnol. Prog. 18:1277-1281.
157. Rahman P. K. S. M., Gakpe E. (2008). Production, characterization and applications of biosurfactants – Review. Biotechnol. 7:360-370.
158. Rapp P., Jurgens-Gabriel L. H. E. (2003). Degradation of alkanes and highly chlorinated benzenes and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. and genetic characterization of its chlorobenzene dioxygenase. Microbiology. 149:2879-2890.
159. Rapp P., Bock H., Wray V., Wagner F. (1979). Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erythropolis* grown on n-alkanes. J. Gen. Microbiol. 115:491-503.
160. Ratanakhanokchai K., Kyu K. L., Tanticharoen M. (1999). Purification and properties of a xylan binding endoxylanase from alkaliphilic *Bacillus* sp. strain K-1. App. Environ. Microbiol. 65:694-697.
161. Rathbone D. A., Holt P., Lowe C. R., Bruce N. C. (1997). Molecular analysis of the *Rhodococcus* sp. strain H1 *her* gene and characterization of its product, a heroin esterase expressed in *Escherichia coli.* App. Environ. Microbiol. 63:2062-2066.
162. Reddy P. G., Allon R., Mevarech M., Mendelovitz S., Sato Y., Gutnick D. L. (1989). Cloning and expression in *Escherichia coli* of an esterase coding gene from the oil-degrading bacterium *Acinetobacter calcoaceticus* RAG-1. Gene. 76:145-152.
163. Raddy P. G., Singh H. D., Pathak M. G., Bhagat S. D., Baruah J. N. (1983). Isolation and functional characterization of hydrocarbon emulsifying and solubilizing factors produced by a *Pseudomonas* species. Biotech. Bioengg. 25:387-401.
164. Reiling H. E., Thanei-Wyss U., Guerra-Santos L. H., Hirt R., Kappeli O., Fiechter A. (1986). Pilot plant production of rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. Appl Environ Microbiol. 51:985-989.
165. Robert M., Mercade M. E, Bosch M. P., Parra J. L., Espuny M. J., Manresa M. A., Guinea J. (1989). Effect of carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1. Biotechnol. Lett. 11:871-874.
166. Ron E. Z., Rosenberg E. (2001). Natural roles of biosurfactants - Mini Review. Environ. Microbiol. 3:229-236.

167. Ron E. Z., Rosenberg, E. (2002). Biosurfactants and Oil bioremediation. Currr Opin Biotechnol. 13:249-252.
168. Roongsawang N., Thaniyavarn J., Thaniyavarn S., Kameyama T., Haruki M., Imanaka T., Morikawa M., Kanaya S. (2002) Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. Extremophiles. 6:499-506.
169. Rose O. C., Brookes M. I., Mallet J. L. B. (1994). A quick and simple non-lethal method for extracting DNA from butterfly wings. Mol. Eco. 3:275.
170. Rosenberg E., Rubinovitz C., Gottlieb A., Rosenhak S., Ron E. Z. (1988a). Production of biodispersan by *Acinetobacter calcoaceticus* A2. Appl. Environ. Microbiol. 54:317-322.
171. Rosenberg E., Zuckerberg A., Rubinovitz C., Gutnick D. L. (1979). Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. 37:402-408.
172. Rosenberg E. G. A., Rosenberg M. (1983). Inhibition of bacterial adherence to hydrocarbons and epithelial cells by emulsan. Infect. Immun. 39:1024-1028.
173. Rubina S., Khanna, S. (1995). Biosurfactants. Ind. J. Microbiol. 35:165-184.
174. Rubinovitz C., Gutnick D.I., Rosenberg E. (1982). Emulsan production by *Acinetbacter calcoaceticus* in the presence of chloamphenicol. J. Bacteriol. 152:126-132.
175. Rubinovitz C., Rosenberg E., Legmann R., Ron E. Z. (1988b). Purification and chemical properties of *Acinetobacter calcoaceticus* A2 biodispersan. Appl. Environ. Microbiol. 54:323-326.
176. Ruiz C., Pastor F. I. J., Diaz P. (2005). Isolation of lipid-and polysaccharide degrading microorganisms from subtropical forest soil, and analysis of lipolytic strain *Bacillus* sp. CR-179. Lett. App. Microbiol. 40:218-227.
177. Sadouk Z., Hacene H., Tazerouti. (2008). Biosurfactants production from low cost substrate and degradation of dieseal oil by a *Rhodococcus* strain. Oil & Gas Sci. Tech – Rev. 63:747-753.
178. Santa L. M., Sebastian G. V., Pereira N. Jr., Alves T. L., Menezes E. P., Freire D. M. (2001) Production of biosurfactant from a new and promising strain of *Pseudomonas aeruginosa* PA1. Appl. Biochem. Biotechnol. 93:459-467
179. Shabtai Y., Gutnick D. L. (1985). Exocellular esterase and emulsan release from the cell surface of *Acinetobacter calcoaceticus.* J Bacteriol. 161:1176-1181.
180. Shao W., Wiegel J. (1995). Purification and characterization of two thermostable acetyl xylan esterases *Thermoanaerobacterium* sp. strain JW/SL-YS485. Appl. Environ. Microbiol. 61:729-733.

181. Sheppard J. D., Cooper D. G. (1990). The effect of Biosurfactant on oxygen transfer in a cyclone reactor. J. Chem. Technol. Biotechnol. 48:325-336.
182. Shoda M., Ohno A., Ano T. (1992). Production of a lipopeptide antibiotic surfactin with recombinant *Bacillus subtilis.* Biotech. Lett. 14:1165-1168.
183. Sieber S. A., Marahiel M. A. (2003). Learning from nature's drug factories: nonribosomal synthesis of macrocyclic peptides. (Mini Review). J Bacteriol. 185:7036-7043.
184. Singer M. E., Finnerty W. R. (1990). Physiology of biosurfactant synthesis by *Rhodococcus* sp. H13-A. Can. J. Microbiol. 36:741-745.
185. Singh M., Desai J. D. (1986) Uptake of water insoluble substrates by microorganisms. J Sci. & Ind. Research. 45:413-417.
186. Singh M., Desai J. D. (1989). Hydrocarbon emulsification by *Candida tropicalis* and *Deboryomyces polymorphus*. Ind. J. Exp. Biol. 27:224-226.
187. Singh M., Saini V., Adhikari D. K., Desai J. D., Sista V. R. (1990). Production of bioemulsifier by a SCP-producing strain of *Candida tropicalis* during hydrocarbon fermentation. Biotechnol. Lett. 12:743-746.
188. Singh P., Cameotra S. S. (2004). Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol. 22:142-146.
189. Sullivan E. R. (1998). Molecular genetics of biosurfactant production. Curr Opin Biotechnol. 9:263-269.
190. Syldatk C., Lang S., Wagner F. (1985). Chemical and physical characterization of four interfacial-active rhamnolipids from *Pseudomonas* sp. DSM 2874 grown on n-alkanes. Z. Natureforsch. 40:51-60.
191. Takimoto A. K., Mitsushima S., Yagi S., Sonoyama T. (1994). Purification, characterization and partial amino acid sequence of a novel cephalosporin-C deacetylase from *Bacillus subtilis.* J. Ferment. Bioeng. 77:17-22.
192. Thanomsub B., Watcharachaipong T., Chotelersak K., Arunrattiyokorn P., Nitoda T., Kanzaki H. (2004). Monoacylglycerols: glycolipid biosurfactants produced by a thermotolerant yeast, *Candida ishiwadae.* J. Appl. Microbiol. 96:588-592.
193. Tsuge K., Ano T., Shoda M. (1996). Isolation of a gene essential for biosynthesis of the lipopeptide antibiotic plipastatin B1 and surfactin in *Bacillus subtilis* YB8. Arch. Microbiol. 165:243-251.
194. Tsuge K., Ohata Y., Shoda M. (2001). Gene *yer*P, involved in surfactin self-resistance in *Bacillus subtilis.* Antimicrob. Agents Chemo. 45:3566-3573.
195. Tuleva B. K., Ivanov G. R., Christova N. E. (2002). Biosurfactant production by a new *Pseudomonas putida* strain. Z Naturforsch. 57:356-360.

196. Tulin E. E., Uede S., Yamagata H., Udaka S., Yamane T. (1992). Effective extracellular production of *Bacillus stearothermophilus* esterase by pH-stat model fed-batch culture of recombinant *Bacillus brevis.* Biotechnol. Bioeng. 40:844-850.
197. Ullrich C., Kluge B., Palacz Z., Vater J. (1991). Cell free biosynthesis of surfactin, a cyclin lipopeptide produced by *Bacillus subtilis.* Biochemistry. 30:6503-6508.
198. Wang S. D., Wang D. I. C. (1990). Mechanisms for biopolymer accumulation in immobilized *Acinetobacter calcoaceticus* system. Biotechnol. Bioengg. 36:402-410.
199. Weatherburn M. W. (1967). Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39:971-974.
200. Wong J. W., Fang M., Zhao Z., Xing B. (2004). Effect of surfactants on solubilization and degradation of phenanthrene under thermophilic conditions. J. Environ Qual. 33:2015-2025.
201. Yakimov M. M., Timmis K. N., Wray V., Fredrickson H. L. (1995). Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. Appl. Environ. Microbiol. 61:1705-1713.
202. Yakimov M. M., Fredrickson H. L., Timmis K. N. (1996). Effect of heterogenecity of hydrophobic moieties on surface activity of lichenysin A, a lipopeptide biosurfactant from *Bacillus licheniformis* BAS50. Biotechnol. Appl. Biochem. 23:13-18.
203. Yao S., Gao X., Fuchsbauer, Hillen W., Vater J., Wang J. (2003). Cloning, sequencing, and characterization of the genetic region relevant to biosynthesis of the lipopeptides Iturin A and surfactin in *Bacillus subtilis.* Curr. Microbiol. 47:272-277.
204. Yoo O. J., Choi K. D., Jeohn G. H., Rhee J. S. (1990). Cloning and nucleotide sequence of an esterase gene from *Pseudomonas fluorescens* and expression of the gene in *Escherichia coli.* Agric. Biol. Chem. 54:2039-2045.
205. Zhang W., Lu X., Zhang X., Li G. (2003) Production of biosurfactant and its role in the biodegradation of oil hydrocarbons. J. Environ. Sci. and Health. 38:483-492.

BOOKS / MANUALS:

1. Awasthi N., Kumar A., Makkar R., Cameotra S. S. (1999). Biodegradation of soil applied endosulfan in the presence of a Biosurfactant. Marcel Dekker, Inc. 793-803.
2. Desai J. D., Desai A.J. (1993). Production of biosurfactants In Surfactant Science Series. (Kosaric, N. Eds), Marcell Dekker, Inc., New York, 48:62-97.
3. Gram C. (1884). Ref: Pelczar M.J, Chan E.C.S, Krieg N.R. Microbiology. Tata McGraw-Hill Publishing Company Ltd. Ed.1993.
4. Hommel R.K., Ratledge C (1993). Biosynthetic Mechanisms of low Molecular weight surfactants and their precursor molecules. In Biosurfactant, Surfactant Science Series, (Kosaric, N., Eds.), Marcel Dekker, Inc., New York. Vol.48:64.
5. John S. (2004). Conceptual Chemistry. Allyn and Bacon Publishers. ISBN: 0321236440. Santa Clara, CA, United States.
6. Kosaric N., Sukan F. V., (2000) Biosurfactants. Encyclopedia of Microbiology. Second edition. 1:618-635.
7. Kosaric N., Cairn W. L., Gray N. C. C. (Eds). (1987). In Biosurfactant and Biotechnology, Surfactant Science Series, Marcel Dekker, Inc., New York. Vol.25.
8. Page C. A., Bonner J. S., Thomas J. M., Robin L. A. Enhanced solubility of petroleum hydrocarbons using various biosurfactants. In-Situ and On-Situ Bioremediation, Bioremediation Proceedings. Vol.2.
9. Plummer D. T. (1988). An introduction to Practical Biochemistry. Third Edition. Tata McGraw-Hill Publishing Company Ltd.
10. Sambrook J., Maniatis T., Fritsch E. F. (1982). Molecular cloning, A laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
11. Schottel J. L., Willard J. M. A., Raymev G. (1989). Regulation of extracellular esterase production by zinc in *Streptococcus scabies.* In Genetics and Molecular Biology of industrial microorganisms. Eds. 113.
12. Wayman M., Jenkins A. D., Kurmady A. G. (1984). In Biotechnology for the oil and fat Industry (Ratledge C., Dawson P.S.S., Rattaray, J.B.M. Eds), American Oil Chemistry Society, IL, 129.

APPENDIX

Solutions and buffers used for agarose gel electrophoresis

TBE Buffer (5X)

Tris-base	27g
Boric acid	14g
EDTA (0.5M, pH 8.0)	10ml
MQ water	500ml

Store at room temp.

Gel loading dye

Bromophenol blue	0.25%
Xylene cynol	0.25%
Ficoll type 400	15%

Store at room temp.

Ethidium bromide

Ethidium bromide (1gm) was dissolved in 100ml MQ water and stored in amber colored bottle at 4^0C.

Solutions and buffers used for polyacrylamide gel electrophoresis

Running Gel (10%)	**35ml**
1.875 M Tris-HCl pH 8.8	7ml
Acrylamide*	11.76ml
MQ water	15.82ml
SDS (10%, w/v)	350µl
APS (10%, w/v)	116.9µl
TEMED	17.5µl

Add the components in the same sequence as mentioned.

Stacking Gel (6%)	**10ml**
0.5 M Tris-HCl pH 6.8	2.5ml
Acrylamide*	1.3ml

MQ water	6.1ml
SDS (10%, w/v)	100µl
APS (10%, w/v)	50µl
TEMED	10µl

Add the components in the same sequence as mentioned.

***Acrylamide Stock Solution (30%)**

Acrylamide	29.2%
N, N- methylene Bis-Acrylamide	0.8%

Light sensitive solution. Stored in amber colored bottle at 4 degree Celsius.

Reservoir Buffer

Tris	3.02g
Glycine	14.41g
SDS	1.0g
Milli Q water	1 liter

Loading buffer or Tracking dye

1.25M Tris- HCl buffer (pH 6.8)	2.5ml
SDS	1.0g
β-mercaptoethanol	2.5ml
Glycerol (87%)	5.8ml
Bromophenol Blue	5mg

Make up the volume to 50ml with MQ water. The solution was stored at room temperature. The SDS reducing buffer was prepared by adding 50µl of β -mercaptoethanol to a 950µl of stock sample buffer just before use.

Silver Staining

Staining Solution

MQ water	100ml
7.6% Sodium Hydroxide	1.0ml
Silver nitrate	0.8g in 4ml MQ water
14.8M Ammonium hydroxide	1.4ml

Light sensitive solution. Use this solution within 5 minutes after preparation.

Developing Solution

1% Citric Acid	2.5ml
Formaldehyde	0.25ml

Use this solution within 2 hours after preparation.

Biochemical reagents

Anthrone reagent

0.2g anthrone in 100ml concentrated H_2SO_4. Store in amber colored bottle.

Biuret reagent

NaOH	150g
$CuSO_4.5H_2O$	1.05g
MQ water	500ml

Dissolve NaOH in ice-bath and add cupric sulphate solution slowly with continuous stirring. Make up the final volume. Reagent discarded if black or reddish precipitate persist.

Bovine Serum Albumin fraction V stock

1mg/ml stock was prepared to make the protein standard curve.

Solution A

Phenol	5g
Sodium nitroprusside	25mg
MQ water	500ml

Store in amber colored bottle.

Solution B

NaOH	2.5g
Sodium hypochlorite	4.2ml
MQ water	500ml

Store in amber colored bottle.

Color reagent

1-amino 2-naphthol 4-sulphonic acid	1g
Sodium sulphite (anhydrous)	3g
Sodium metabisulphite	6g

Mixed and stored at 4^0C in dark.

Reagent prepared fresh before use by dissolving 0.25g of mixture in 10ml MQ water.

Solutions and buffers used for molecular biology work

All solutions were either autoclaved or filter sterilized and stored at 4^0C or 20^0C.

Ampicillin stock

50mg/ml stock was used after filter sterilization. Working concentration was 50µg/ml.

TE (Tris-EDTA) buffer (pH 8.0)

Tris	10mM
EDTA	1mM

Rose solution

EDTA (pH 8.0)	0.37g
Tris-HCl (pH 8.0)	0.12g
Sodium lauryl Sulphate	1g
Polyvinylpolyrrolidone	1g
MQ water	100ml

Warm slightly if precipitates start appearing.

Solution I

Glucose	50mM
Tris-HCl (pH 8.0)	25mM
EDTA	10mM

Solution II

NaOH	0.2N
SDS	1%

Solution III

Potassium acetate (5M)	60ml
Glacial acetic acid	11.5ml
MQ water	28.5ml

****