PRODUCTION, CHARACTERIZATION AND APPLICATIONS OF BIOSURFACTANTS AND EXOPOLYSACCHARIDE FROM *PSEUDOZYMA* SP. NII 08165

THESIS SUBMITTED TO THE UNIVERSITY OF KERALA
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY
UNDER THE FACULTY OF APPLIED SCIENCE

BY

SAJNA KV

BIOTECHNOLOGY DIVISION

CSIR-NATIONAL INSITUTE FOR INTERDISCPLINARY SCIENCE AND TECHNOLOGY,
COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH (CSIR),
THIRUVANANTHAPURAM 695 019,
KERALA, INDIA

2015

DECLARATION

I hereby declare that the Ph.D. thesis entitled, 'Production, Characterization and Applications of Biosurfactants and Exopolysaccharide from *Pseudozyma* sp. NII 08165' is an independent work carried out by me and it has not been submitted anywhere else for any other degree, diploma or title.

Sajna KV

National Institute for Interdisciplinary Science and Technology (NIIST)

Council of Scientific & Industrial Research (CSIR)
(Department of Scientific & Industrial Research, Ministry of S&T, Govt. of India)
Industrial Estate P.O., Trivandrum - 695 019
Kerala, INDIA

Ashok Pandey, *PhD, FBRS, FNASc, FIOBB, FISEES, FAMI*
Head, Centre for Biofuels & Biotechnology Division
Chairman, IBSC

Tel:+91-471-2515 279
Fax:+91-471-24 91 712
Tel/fax:+91-471-24 95 949
E-mail: pandey@niist.res.in; ashokpandey56@yahoo.co.in

3rd December 2015

CERTIFICATE

This is to certify that the work embodied in the thesis entitled "**Production, Characterization and Applications of Biosurfactants and Exopolysaccharide from *Pseudozyma* sp. NII 08165"** has been carried out by Ms Sajna KV under my supervision and guidance.

Sincerely,

Ashok Pandey
Research Supervisor

Acknowledgements

This thesis was a quite challenging and delightful journey for me and would not have been possible without the support of my teachers, friends and colleagues. First and foremost, I would like to express my sincere gratitude to my supervisor, Prof Ashok Pandey for his guidance and support throughout my research. It was an honour to work with such a great scientist and I would also like to thank him for all I have learned from him and for being so patient with me.

I would like to thank Dr Rajeev K Sukumaran, for his guidance and encouragements during my research. His ideas on biosurfactants production from a novel isolate was an inception point for this thesis. No words can express my gratitude to him for his support throughout my research.

It is my privilege to thank Dr A Ajayagosh, Director and Dr Suresh Das, Former Director, CSIR-NIIST, for providing me all the facilities for completing my research.

I owe my gratitude to University Grant Commission (UGC), New Delhi for providing me the Junior Research Fellowship for undertaking this study.

I am thankful to Dr K Madhavan Nampoothiri, Dr Binod P, Dr Sindhu R, Dr Ramesh, Dr Arumugam, Dr Leena and Er. Kiran Kumar for their valuable suggestions and support during my research. I owe my sincere gratitude to Dr A Jayakumaran Nair, Head, Department of Biotechnology, Kerala University and Expert Member of my Doctoral Committee for helping me in improving the thesis quality by his comments on both scientific and technical aspects of the study.

My appreciation goes to Prof D J Lee, National Taiwan University; Prof Christian Larroche, and Prof Claude-Gilles Dussap, Universite Blaise Pascal- Clermont Ferrand II, France for giving an international exposure to my work.

I would like to acknowledge Dr R B N Prasad, Head of the division, and Dr Sanjit Kanjilal, Scientist, Centre for Lipid Research, IICT, Hyderabad, for granting me the opportunity to use their technical facility for the structural elucidation and hydrocarbon analysis.

My sincere thanks to Mr Sankar Sasidaran, Mr Peer Muhammed, Material and Minerals Division; Mr Prakashan, Chemical science and technology, CSIR-NIIST for helping me with technical analysis of the samples.

I am indebted to my seniors Dr Vidya J and Dr Ushasree M V for their encouragement and care. With immense pleasure, I wish to extend my sincere thanks to my former and present colleagues: Kiran, Nishant, Rajasree, Anju, Koyna, Karthik, Vivek, Leya, Kuttiraja, Nimisha, Sabeela, Aravind, Vani, Aymen, Sai Shyam, Sumayya and Rubeena for their support. Special thanks to Varsha, Anusree and Divya for their timely help and support.

My heartfelt gratitude goes to Dr Lalitha Devi Gottumukkala, my best friend for all the emotional support, love and caring. Thank you for being there for me.

My appreciation and friendship goes to Dr Ramya Nikesh for all scientific discussion and technical help for this study. I am happy to place on record my thankfulness to Himani Jayamurthy for all the care and support.

Finally, I am thankful to my all family members for their encouragement and emotional support in the process of accomplishing my Ph D.

Above all, I am grateful to God Almighty for all the blessings, helping me get through the difficult times and making me capable to achieve my wishes and goals.

Sajna K V

CONTENTS

List of Tables

List of Figures

List of Abbreviations

%	Percentage
AFM	Atomic force microscopy
BH	Bushnell Hass
bp	Base pair
CFU	colony forming unit
cHAL	compost humic acid-like matter
CL	cellobiose lipids
CLP	cyclic lipopeptide
CM glucan	Carboxymethyl glucan
CMC	critical micelle concentration
Dab	diaminobutyric acid
DNA	Deoxy ribo nucleic acid
ELSD	evaporative light scattering detector
EOR	Enhanced Oil Recovery
EPA	Environmental Protection Agency
EPS	Exopolysaccahrides
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared
g	gram
g/l	gram per liter
h	hours
i.e	That is
INT	p-iodonitrotetrazolium violet
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
MEL	Mannosylerythritol lipids
mg	milligram
mg/ml	milligram per milli litre
min	minutes
ml	Milli litre

mM	Milli molar
MML	mannosylmannitol lipids
MTCC	Microbial Type Culture Collection
N	Normal
NDH-2	NADH-quinone oxidoreductases
ng	Nanogram
NMR	Nuclear Magnetic Resonance
NPE	Nonylphenol ethoxylate
^{O}C	Degree Celsius
PAH	polyaromatic hydrocarbons
PBS	Phosphate -buffered saline
PFC	Perfluorinated compounds
PFOS	Perfluorooctane sulfonate
pH	Hydrogen ion concentration
POP	Persistent organic pollutant
R&D	Research and Development
RL	Rhamnolipids
rpm	rotation per minute
sec	seconds
SEM	Scanning electron microscopy
SL	Sophorolipids
SLS	Sodium lauryl sulphate
Span 80	Sorbitan monooleate
Td	degradation temperature
TGA	thermogravimetric analysis
TLC	thin layer chromatography
v/v	Volume/volume
w/v	Weight/volume
WHC	Water holding capacity
XRD	X-ray diffraction
β-GlcNAc	β-N-acetyl-D-glucosamine
λ_{max}	Wavelength of maximum absorption
μ	Micro

µg	Microgram
µg/l	Microgram per litre
µg/ml	Microgram per milli litre
µl	Micro litre
µM	Micro molar

Chapter 1: Introduction and Review of Literature

Surfactants are indispensable in our daily life as they are used in large number of products used in day to day life such as detergents, foaming agents, wetting agents, emulsifiers, dispersants, and lubricants. They are amphiphilic molecules that can lower the surface tension of a liquid or interfacial tension between two liquids. Depending on the raw material used for the synthesis, surfactants are categorized into two groups- petro-based and oleo-based surfactants. However, partly due to depleting petroleum resources and negative environmental impacts associated with their chemical nature and production process, surfactants industry has been looking for 'green processes' for their production and eco-friendly applications. This has led the focus of R&D on bio-based surfactants, i.e. biosurfactants. Though oleo-based surfactants are having some ecological benefits due to natural raw material used for their production, the process, products and by-products could be still damaging to the environment.

Chemical surfactants can bio-accumulate and pose significant threat to ecosystem. In addition to this, the process and by-products generated during their production could be hazardous to environment. Surfactants (or products containing them) have usually been disposed on the land or water system. In the rivers and water bodies, they led to form foam mountains and carpets which could affect the aquatic life and fish kills occurred more frequently. Even though they were not the only cause of the increasing environmental pollution, surfactants, such as the branched-chain tetrapropylene-benzenesulfonates, have been recognized as being primarily responsible for this due to their low biodegradability [Hofer et al., 2008]. Furthermore, perfluorinated surfactants, belonging to the group of chemicals called perfluorinated compounds (PFC), put a significant threat to aquatic animals as they destroy the external mucous layer of aquatic animals and cause severe damage to the gills of fishes. Perfluorooctane sulfonate (PFOS), a fluorosurfactant used in impregnation agents for stylish

fashion out-door all-weather clothing and shoes, paper impregnation for food and non-food applications, in metal plating and fire-fighting foams are persistent organic pollutant (POP) and ubiquitously present. Studies have shown that PFOS bioaccumulate and enter into food chain. PFOS can be readily absorbed after oral intake and accumulate in serum, kidney and liver and cause potential developmental and reproductive problems in humans. [http://www.epa.gov/fedfac/pdf/emerging_contaminants_pfos_pfoa.pdf accessed on 09-03-2015].Nonylphenol ethoxylate (NPE) are inexpensive surfactants used in laundry detergents and other products. NPE and its degradation product, alkyl phenol are potent pollutants in fresh water streams and marine source and have endocrine disrupting effect on the organisms [Lozano et al., 2012]. Sodium lauryl sulphate (SLS), a surfactant present nearly in all the personal care items and detergent based cleaners, is produced from the coconut oil through ethoxylation. SLS at high concentration increases the transepidermal water loss and has a degenerative effect on the cell membrane because of its protein denaturing effect, which could lead to skin irritation and cause irritant dermatitis. Because of its moderate hazard nature, recommended concentration of SLS in cosmetic products is less than 1%. Many regulatory agencies such as Food and Drug Administration (FDA) have reported that the occurrence of 1,4-dioxane in cosmetic and other personal care items, which is due to usage of contaminated SLS with 1,4-dioxane. 1, 4-dioxane and nitosamines are formed as by-products during ethoxylation of sulfonated surfactants and are potential carcinogens and mutagens, which cause serious health problems [Black et al., 2001].

Thus, it is desirable to develop and use new bio-based surfactants as possible alternative to chemical surfactants due to environmental and health concerns, stringent regulations and instable and high prices of petroleum and other raw material currently used for their production.

1.1 Biosurfactants

Biosurfactants are microbial amphiphilic compounds with surface active properties. They can effectively reduce the surface tension, decrease the interfacial tension between two immiscible liquids, enhance the emulsification and increase the solubility of compounds. Biosurfactants can be a good alternative to synthetic surfactants due to their structural diversity, selectivity, performance under extreme conditions, environmentally-friendly nature and potentiality of producing at large scale through fermentation. In addition to the above, biosurfactants are safe and display various biological activities, which make them a good candidate for biomedical and pharmaceutical applications. Besides being eco-friendly, biodegradable, non-toxic and hypo allergic, biosurfactants also display various skin care properties, which may lead their application very attractive in cosmetic industry.

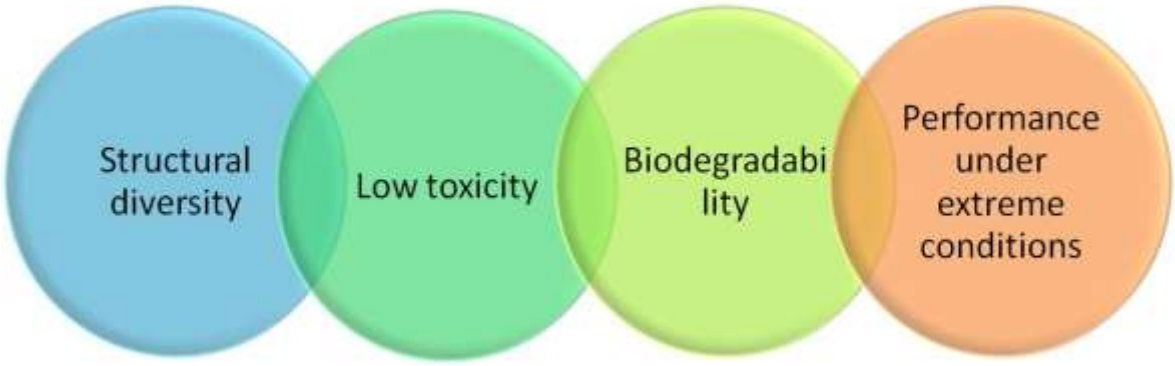

Figure 1.1 Advantages of biosurfactants over chemical surfactants

Structurally, biosurfactants contain a polar moiety, which consists of mono-, di- or polysaccharide or amino acid or peptides and a non-polar moiety consisting of saturated or unsaturated fatty acid. Based on the hydrophilic structure, biosurfactants are classified to the following groups [Desai& Banat, 1997].

1. Glycolipids

2. Lipopeptide & lipoproteins

3. Polymeric biosurfactants

4. Fatty acids, phospholipids & neutral lipids

5. Particulate biosurfactants

1.1.1 Glycolipids Biosurfactants

Most well-studied biosurfactants are glycolipids and are reported from both bacteria and yeast. Glycolipids have monosaccharide, disaccharide or sugar alcohol as polar moiety and long chain aliphatic or hydroxyaliphatic acids as non-polar moiety. Apart from their good surface activity and interesting physico-chemical properties, glycolipids owe their rising commercial viability to their high productivity, ease of recovery and possibility of production from renewable resources [Kitamoto et al., 2002]. Some of the major examples in this regard are given below.

1.1.1.1 Rhamnolipids

Rhamnolipids (RL) are produced through aerobic fermentation using *Pseudomonas aeruginosa*. In rhamolipids, one or two molecules of rhamnose are linked to one or two molecules of β-hydroxy decanoic acids. The structure of rhamnolipid is shown in Figure 1.2. Rhamnolipids lower the surface tension up to 25 to 30 mN/m and interfacial tension against n-hexadecane to 1mN/m [Desai & Banat, 1997].

The biosynthesis of RL comprises three steps, namely: biosynthesis of the lipid moiety, biosynthesis of the sugar moiety, and finally, the enzymatic dimerization and action of rhamnosyl transfers, which yield the final products. Cultivation strategies applied to RL production involve batch, fed-batch, continuous, and integrated microbial/enzymatic processes. Dextrose, glycerol, n-alkanes, and triglycerides have been mostly used as carbon

sources. Reported nitrogen sources include nitrate, ammonium, urea, corn steep liquor, and complex amino acids containing supplements [Abdel-Mawgoud et al., 2011; Blank et al., 2011].Apart from promoting the uptake of water insoluble substrate, the physiological roles of rhamnolipids are also involved in bacterial motility and biofilm formation. Role of rhamnolipids in maintaining the biofilm structure is by promoting the bacterial attachment to surface by creating an open channel surrounding the colonies [Davey et al., 2003].

Rhamnolipids are well reported for the bioremediation due to their remarkable surface activity and emulsification activity. The addition of rhamnolipids changes the cell surface properties of hydrocarbon degrading bacteria, especially the cell surface hydrophobicity and helps to improve the degradation efficiency. However, the effect is dependent on the concentration of rhamnolipids and the strain. The supplementation of rhamnolipids could improve the uptake of phenanthrene by *P. aeruginosa*, but it could also reduce the uptake of phenanthrene by *Bacillus subtilis* and prolong its lag phase of growth. Above the critical micelle concentration (CMC), rhamnolipids enhance the cell hydrophobicity by reducing the lipopolysaccharide content of the bacterial cell, while they change outer membrane composition of the cell below CMC, which contribute to the changes in cell surface morphology [Kaczorek,2012; Zhao et al., 2011; Sotirova et al., 2009]. Simulation studies have shown that rhamnolipids could be used for the bioremediation of marine oil spillage [Chen et al., 2013]. Rhamnolipids have been reported as metal complexing biosurfactants and could reduce organism's toxicity to metals such as cadmium during the biodegradation of naphthalene. Hence, rhamnolipids can be used to treat the soils co-contaminated with metals and hydrocarbons [Sandarin et al., 2009]. Rhamnolipids can be used in the formulation of soil washing agent for the removal of heavy metals and their activity is comparable to that of synthetic metal chelators [Neilson et al., 2003].

The potential application of rhamnolipids in petroleum industry is in the recovery of petroleum and the cleanup of the oil storage tank. Rhamnolipids exhibit high surface and emulsifying activity and are stable in a wide range of temperature, pH and salt concentration. Several studies have concluded that a significant amount of crude oil could be recovered by *ex-situ* application of the rhamnolipids into the oil fields. A commercial Enhanced Oil Recovery (EOR) technology, called Biosurfactants EOR technology from Rhamnolipid Companies Inc. makes use of rhamnolipids for the oil recovery from the mature oil fields. The technology claims a recovery of more than 40% entrapped oil [http://rhamnolipid.com/services-view/oil-sludge-removal/ accessed on 09-03-2015]. Tailor made rhamnolipids can be synthesized for the enhanced mobilization of crude oil. Banat et al. [1991] devised a cost-effective strategy for cleaning the oil storage tank using rhamnolipids containing culture broth. The sludge was effectively removed by the treatment, which also resulted in the recovery of 91% hydrocarbon from the sludge [Perfumo et al., 2010].

Rhamnolipids are potential antimicrobial agents and have been approved by Environmental Protection Agency (EPA) as a biofungicides as they can inhibit the growth of zoosporic plant pathogens such as *Pythium* and *Phytophthora* species. They pose minimal threat to non-target organisms. Their mechanism of action involves the disruption of cell membrane of fungal zoospores [Stanghellini et al., 1997]. Kim et al., [2000] discussed the efficacy of rhamnolipids B in preventing the infection of *Phytophthora capsici* and *Colletotrichum orbiculare* in pepper and cucumber plants, respectively. Zonix^{TM} biofungicide from PropTera^{TM} contains rhamnolipids as active ingredients. Apart from fungicidal activity, rhamnolipids show insecticidal activity also and are effective against green peach aphids, which are major agricultural pests and have a broad host range. They show a dose-dependent mortality against green peach aphids by affecting the cuticle membrane [Kim et al., 2011].

Rhamnolipids have been reported as efficient anti-adhesive and biofilm disrupting agents and could be used to prevent the fouling [Dusane et al., 2010].

Since *P. aeruoginosa* falls under biosafety level 2, proper safety precautions must be followed during the large-scale production of rhamnolipids. Zhu et al., [2012] studied the effect of pH and stirring on the fermentation and developed a pH controlled fed-batch fermentation giving a yield of 70.56 g/L rhamnolipids. Rhamnolipids can be produced as high-value product using various waste substrates such as biodiesel generated crude glycerol. High foaming of the medium is the major bottleneck in the large-scale production of rhamnolipids. To reduce the foaming, a microaerobic denitrifying fermentation technology has been developed by Chen [2006]. The regulatory mechanisms, which hamper the high productivity of rhamnolipids, can be abolished by genetic engineering.

1.1.1.2 Sophorolipids

Sophorolipids (SL) are mainly produced by the yeasts such as *Candida bombicola* and *C. apicola* species, which consist of sophorose linked to long chain hydroxy fatty acids (Figure 1.2). Usually, microorganisms produce a mixture of sophorolipids, consisting of both lactonic (internally esterified) and acidic (free form) sophorolipid [Weber et al., 2012].

SL have critical micelle concentration of around 40-100 mg/l and can be used to stabilize oil in water emulsions as their hydrophilic/lipophilic balance is between 10-13 [Inge et al., 2007].Develter et al.,[2010] found that sophorolipids could be used as potential detergent for hard surface cleaning. Compared to commercial household cleaners, they exhibit superior cleaning performance and low toxicity to aquatic organisms. Detergent action of sophorolipids can be attributed to high surface activity, low foaming ability and fast wetting action. Saraya, a Malaysia based biotech company markets Happy Elephants ™, an eco-friendly household cleaning product, which contains sophorolipids as cleaning agent [http://happy-elephant.info/concept.html accessed on 10-03-2015].

Lactonic sophorolipids exhibit antimicrobial activity and degree of antimicrobial activity is related to the structure of sophorolipids, which is determined by the type of carbon source used for fermentation [Shah&Badia, 2007]. Sophorlipids derivatives such as sophorolipids diacetate ethyl ester derivatives have been reported as having spermicidal and virucidal activity and can be used in the preparation of microbicidal contraceptions [Shah et al., 2005]. Administration of sophorolipids reduces the mortality associated with septic shock in the rats due to their immunomodulatory function [Bluth et al., 2006]. Sophorolipids capped silver nanoparticles are very stable and show strong antimicrobial activity against Gram-positive and Gram-negative bacteria [Singh et al., 2009].

The antimicrobial activity of sophorolipids can be exploited for the treatment of acne vulgaris, as *Propionobacterium acnes*, an acne causing bacteria is very susceptible to sophorolipids. Ashby et al., [2011] developed a polyhydroxy butyrate based scaffold for the optimal delivery of sophorolipids to skin.

High yield of sophorolipids and use of non-pathogenic yeast for fermentation make sophorolipids a commercially competent biosurfactants. High cell density fermentation of *C. bombicola* resulted in remarkably high yield of sophorolipids, as high as 200 g sophorolipids/l/day [Gao et al., 2013]. Kim et al., [2009] developed a fed batch fermentation of *C. bombicola* using glucose and rapeseed oil, which resulted in a yield of 365 g/l sophorolipids during eight days of fermentation.

Feasibility of soy molasses as substrate for the production of sophorolipids was demonstrated by Solaiman et al. [2007], demonstrating that the use of cheaply available substrates for the production of glycolipids could reduce the cost of production. Tailor made sophorolipids could be produced by media engineering strategies. Compared to the conventional sophorolipids, sophorolipids with medium chain fatty acid have high solubility and surface activity with possibly better applicability. Cytochrome P450 monoxygenase, which catalyse

the hydroxylation of fatty acid during sophorolipids biosynthesis, are very specific to fatty acid chain length and result in the incorporation of only long chain fatty acid in sophorolipids structure. Van Bogaert et al., [2011] proposed two strategies to overcome the challenges in producing sophorolipids having fatty acids with chain length of any choice. The addition of already hydroxylated fatty acids is one of the strategies for producing sophorolipids with fatty acids of any chain length in which cytochrome P450 monoxygenase activity is by-passed. The other strategy is the addition of hydrophobic substrate with C16-18 fatty acid structure, which then incorporates into sophorolipids structure and then carrying out post fermentative modification.

Excellent toxicological and eco toxicological profile of the sophorolipids has made it a potential alternative to petrochemical based surfactants in personal and household care cleaning liquids [Kuppert 2014]. One of such intended applications includes the use of sophorolipids as additives in combination with pesticides and thus enhances the efficacy of pesticides for crop protection [Giessler-Blank et al., 2012]. Evonik Industries developed a method for the improved production of sophorolipids by employing genetic modification of *Candida* species and recently has constructed a fermentation plant in United Kingdom that produces sophorolipids for the home-care cleaning market [Schaffer et al., 2013]. Ecover, A Belgium based company that manufactures ecologically sound cleaning products launched a multi-surface spray cleaner containing natural surfactants including sophorolipids. In comparison to the petrochemical based cleaners, this new age cleaning product offers supreme cleaning, complete biodegradability and low toxicity, apart from being skin friendly and active at neutral pH. Their current industrial level production is four tonnes of ecosurfactants per month from the two industrial production unit using 100 l bioreactors [http://naturallysavvy.com/general/ecover-eco-surfactants-the-future-of-cleaning accessed on 10-03-2015]. Ecover has developed a process for the improved production of sophorolactone,

a much sought after isomer of sophorolipids, which has higher surface activity and multiple bioactivity and can be used for skin and hair care formulation and biocide preparation for crop protection [Develter & Fleurackers, 2012; Develter & Renkin, 2012].

1.1.2 Lipopeptide and Lipoprotein Biosurfactants

Lipopeptides are versatile biosurfactants produced by the bacterial and actinomycetes species. A remarkable property of lipopeptides is their antimicrobial activity. Many lipopeptide are well-known antibiotics; some of them are explained below.

1.1.2.1 Surfactin

Surfactin is a cyclic lipopeptide produced by *Bacillus subtilis*. Its chemical structure consists of several variants differing in their fatty acid chain and their peptide moiety. It comprised a peptide loop of seven amino acids (L-asparagine, L-leucine, glutamic acid, L-leucine, L-valine and two D-leucines), and α, β-hydroxy C13-C_{15} fatty acid chain (Figure 1.2). Surfactin has large biomedical applications due to its antimicrobial and biological activity. It exhibits antibacterial, antiviral, antifungal and antimycoplasma activities, which shows its potential as therapeutic agent. It also exhibits antitumor and antihypocholesterolemia properties. Because of its haemolytic property, surfactin can be used as anti-clotting agents [Sen, 2010]. Surfactin has high surface activity with a critical micelle concentration (CMC) of 7.5 μM, but its membrane destabilizing concentration is far less than that of CMC, revealing its high antimicrobial and cytotoxic activities [Heerklotz & Seelig, 2001**]**. Computational simulation studies have revealed that the molecular mechanism behind the antibacterial, ant-viral, anti-mycoplasma and haemolytic activities is mainly due to the detergency effect, which result in the membrane rupture [Deleu et al., 2003]. Due to this mode of action, chances of developing resistance in the microbes are low, which makes it a promising alternative antibiotic for multidrug resistant organism. Like other biosurfactants,

surfactin inhibits biofilm formation and is an effective biocontrol agent against agricultural pests [Sen, 2010].

Kaneka Corp., a Japanese chemical company has established an efficient radioactive decontamination strategy by using their biosurfactant, called Kaneka Surfactin ^{TM}, which is composed of surfactin. They successfully carried out decontamination of the areas affected by the Fukushima No. 1 nuclear power plant disaster. Efficient surface and detergent activity of surfactin make it eliminating the radioactive caesium and other contaminants from the polluted area. Compared to the chemical surfactants, advantages of using surfactin containing cleaning remedy are eco-friendliness, better cleaning, cost-effectiveness and less time duration [http://ajw.asahi.com/article/0311disaster/fukushima/AJ201211200075, http://www.kaneka.co.jp/kaneka-e/csr/pdf/csr_2013_06.pdf, accessed on 11-03-2015]. Apart from bioremediation, surfactin in the form of sodium surfactin is also used in cosmetic preparation such as creams, lotions and face masks.

Since surfactin causes foaming during the fermentation process, foam fractionation can be used for the recovery of surfactants. A bioprocess for the enhanced production of surfactin from a medium containing glucose and metal cations along with continuous removal of product by foam fractionation has been established by Cooper et al., [1981].

Downstream process for the purification of surfactin includes acidifying the culture supernatant followed by the filtration. Neutralization of the trapped liquid with alkaline solution results in the formation of product, sodium surfactin after evaporation [Sun et al., 2013]. Conventional downstream process, which uses organic solvent, is not preferred now by the industries due to environmental and cost concerns.

1.1.2.2 Polymyxin

Polymyxins are cyclic liopeptides produced by *Paenibacillus* species with a general structure consisting of a cyclic peptide with a long hydrophobic tail. Different structural variants of polymyxin are produced by different species of *Paenibacillus* genera. Polymyxin B is produced by *P. polymyxa*, while Polymyxin E ((also known as colistin) is produced by *P. polymyxa* and *P. amylolyticus* [Decrescenzo et al., 2007].

Polymyxins are powerful antibiotics against Gram-negative bacteria. They specifically bind to lipopolysaccharide (LPS) of outer membrane of Gram-negative bacteria and disrupt the cell wall. A secondary mode of action was proposed by Deris et al., [2013] where polymyxins affect the bacterial respiration by inhibiting type II NADH-quinone oxidoreductases (NDH-2) in the bacterial inner membrane. Because of its high endotoxin binding capacities, matrix containing polymyxin is used to remove endotoxin from the fermentation broth of bacterial species such as *E.coli*. Polymyxin exhibits high specificity and binding capacity towards endotoxin and is also feasible at industrial scale [http://www.bio-rad.com/webroot/web/pdf/lsr/literature/1429.pdf accessed on 11-03-2015].

Aerobic fermentation of *P. polymyxa* in the medium containing 3% corn meal and 3% glucose and yeast extract yielded polymyxin at very good concentration [Petty, 1952]. Rangaswamy et al., [2010], proposed a bioprocess model for the large-scale production of polymyxin from oatmeal based medium and purification of polymyxin.

Recombinant DNA technology methods have been adopted for producing polymyxin in *B. subtilis* strain, since molecular tools and expression system are not developed much in *Paenibacillus* species. Objectives of genetic modification have been to improve the yield and to produce new variants of polymyxin, with high activity and low toxicity. Initial attempts to produce polymyxin in *B. subtilis* strain were successful, but only with the addition of L-2, 4-

diaminobutyric acid (Dab). This problem was solved by introducing an ectB gene encoding the diaminobutyrate synthase of *P. polymyxa* into *B. subtilis* strain. Further genetic modification by knocking out abrB gene resulted in the production of high level of polymyxin from *B. subtilis* BSK3S strain [Parky et al., 2012].

1.1.2.3 Lichenysin

Lichensyin are cyclic lipopeptides produced by *B. licheniformis* (Figure 1.2). The structure of lichensyin varies with the species, which make them categorized into A, B, C, D and G. Lichensyin are structurally similar to surfactin, whereas surfactin has a cyclic peptide of seven amino acids including both D- and L-amino acids, Glu-Leu-D-Leu-Val-Asp-D-Leu-Leu, linked from the N-terminus to the C-terminus to form a cyclic moiety by a C12-C 17 β-hydroxy fatty acid, lichenysin have a structure with the primary amino acid sequence L-Gln-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Ile, with minor Leu and Val substitutions at the seventh position.

Lichenysin exhibit high surface activity and chelating activity that explains its antimicrobial functions [Grangemard et al., 2001]. They are stable in a range of temperature, pH and NaCl concentration. Lichenysin are good candidates for microbial enhanced oil recovery, where they are stable at extreme condition of oil wells and improve the recovery of crude oil compared to conventional surfactants [Perfumo et al., 2010]. Biosurfactants composition containing lichenysin and surfactin has been proposed for improved poultry farm practices. Here, bactericidal function of biosurfactants was used to reduce the infections in poultry hens [Bralkowski et al., 2013a]. Lichenysin can be produced at relatively good yield from cheaply available substrate such as vinasse, a byproduct of sugar industry [Bralkowski et al., 2013b].

Though lichenysin have high biological activity, but their yield is very low compared to surfactin. Also, *B. licheniformis* is not a very feasible for genetic modification. Hence, a

novel hydrid synthase concept was proposed by Golyshin et al., [2001]. Surfactin and lichenysin A synthetases, which produce surfactin and lichenysin A, respectively have similar molecular conformation. A hybrid synthetase was constructed to produce a novel lipopeptide with same activity of lichenysin A and high productivity of surfactin. The resultant peptide has same amino acid residues of lichenysin and fatty acid chain of surfactin and expressed at the same level of Surfactin [Golyshin et al., 2001].

1.1.3 Polymeric Biosurfactants

In contrary to the other surface active molecules, polymeric biosurfactants are high molecular weight compounds and generally exhibit high emulsifying activity. Among current polymeric biosurfactants, a more potent one is emulsan.

1.1.3.1 Emulsan

Emulsan is an amphiphilic microbial lipopolysaccharide with covalently attached fatty acids, mainly produced by *Acinetobacter calcoaceticus*, secreted as scaffold material for capsular polysaccharides. It is a high molecular weight biopolymer consisting of amino sugars (D-galactosamine) and D-galactosaminouronic acid groups (Figure 1.2).

The peculiar features of emulsan are its excellent surface activity and emulsifying activity even at low concentration. Emulsan is secreted in to the medium as emulsan-protein complex where protein is covalently attached. Emulsan-protein complex has higher emulsification activity than the apoemulsan, which is emulsan devoid of protein. Emulsan exhibits Newtonian flow behaviour and undergoes a conformational transition at oil-water interfaces [Gutnik&Bach, 2008; Gutnik, 1987].

Emulsan can be used for a range of bioremediation processes from degreasing, oil cleanup to sludge emulsifications. Application of emulsan in enhanced oil recovery has been well

demonstrated as it helps in the recovery and transportation of highly viscous oil and stabilizes oil-water emulsion, which increases the fuel value of the crude oil without the need for removal of water [Gutnik&Bach, 2008; Gutnik, 1987]. Emulsan has been studied to elicit the immunological response by activating the macrophages. A remarkable adjuvant activity had been exhibited by one of the structurally modified emulsan [Panilaitis et al., 2002]. Emulsan can prevent the bacterial adherence to buccal epithelial cells, which makes it an excellent candidate in the preparation of toothpaste or mouthwash that prevents dental caries and plaques [Eigen&Simone, 1988]. Emulsan can be used in the preparation of cosmetics. Apart from emulsifying all cosmetic ingredients and maintaining appropriate consistency, emulsan has moisturizing and film forming activities.

The commercial production of emulsan is well described. A typical production medium containing soybean oil as carbon source and ammonium hydroxide as nitrogen source with a C: N ratio of 7.7g carbon: 1g nitrogen gave a yield of 0.5 g/ litre/hour. Since *Acinetobacter* prefer fatty acid rather than triacyl glycerides, a fed-batch fermentation employing free fatty acids as carbon source was proposed as a feasible technology [Shabati & Wang, 1990]. A number of commercial suppliers use a combination of fatty acids such as palmitic acid, stearic acid and glucose as carbon source for the production of emulsan. Flocculating agents such as kaolin and bentonite can be used for the recovery of emulsan [Gutnik&Rosenberg, 1980]. The choice of carbon source has an impact on the fatty acid composition of the emulsan, predominantly 3-hydroxydecanoic acid, which influences the emulsifying activity of emuslan [Kim et al., 1997]. Physiochemical properties of emulsan can be modified by changing the fatty acid composition, which can lead to emulsan variant with interesting applications.

1.1.3.2 Liposan

Candida lipolytica or *Yarrowia lipolytica* produce a water soluble biopolymer composed of 83% polysaccharide and 17% protein, which is called liposan. Liposan is usually produced by fermentation of the medium containing hydrophobic substrates such as hexadecane. Liposan exhibits high emulsifying activity and emulsion stabilizing properties. [Cirigliano& Carman, 1985]. Because of its inert nature, liposan can be used as smokeable tobacco substitutes [Mua, 2009].

Due to substrate specificity and low productivity of liposan, a thorough media engineering and novel fermentor design are required to develop a cost effective technology for liposan production. Thiamine is very important nutrient for *Y. lipolytica* fermentation for liposan production. Pinchuk et al., [2000] proposed a self cycling fermentor with continuous addition of hydrocarbon, which resulted in a yield of 0.08 g/L. During the final stage of fermentation, cycle time delay was stopped during which the residual hydrocarbon was utilized and resulted in improved yield of 0.095 g/L.

1.1.4 Fatty acids, Phospholipids and Neutral lipids

Several bacteria and yeast produce large quantities of fatty acids and phospholipids during the growth on n-alkanes, for example, the production of phosphatidylethanolamine by *Acinetobacter* and *Rhodococcus* species [Desai&Banat, 1997].

1.1.5 Particulate Biosurfactants

Particulate biosurfactants are of two types- vesicles and whole microbial cells. In the case of *Acinetobacter calcoaceticus*, extracellular membrane vesicles are composed of protein, phospholipid and lipopolysaccharide with a diameter up to 20-50 nm. Extracellular vesicles are usually produced during the growth on n-alkanes [Desai&Banat, 1997]. In some species,

microbial cells itself act as biosurfactants and show high affinity for hydrocarbon-water and air-water interfaces [Karanth et al., 1999].

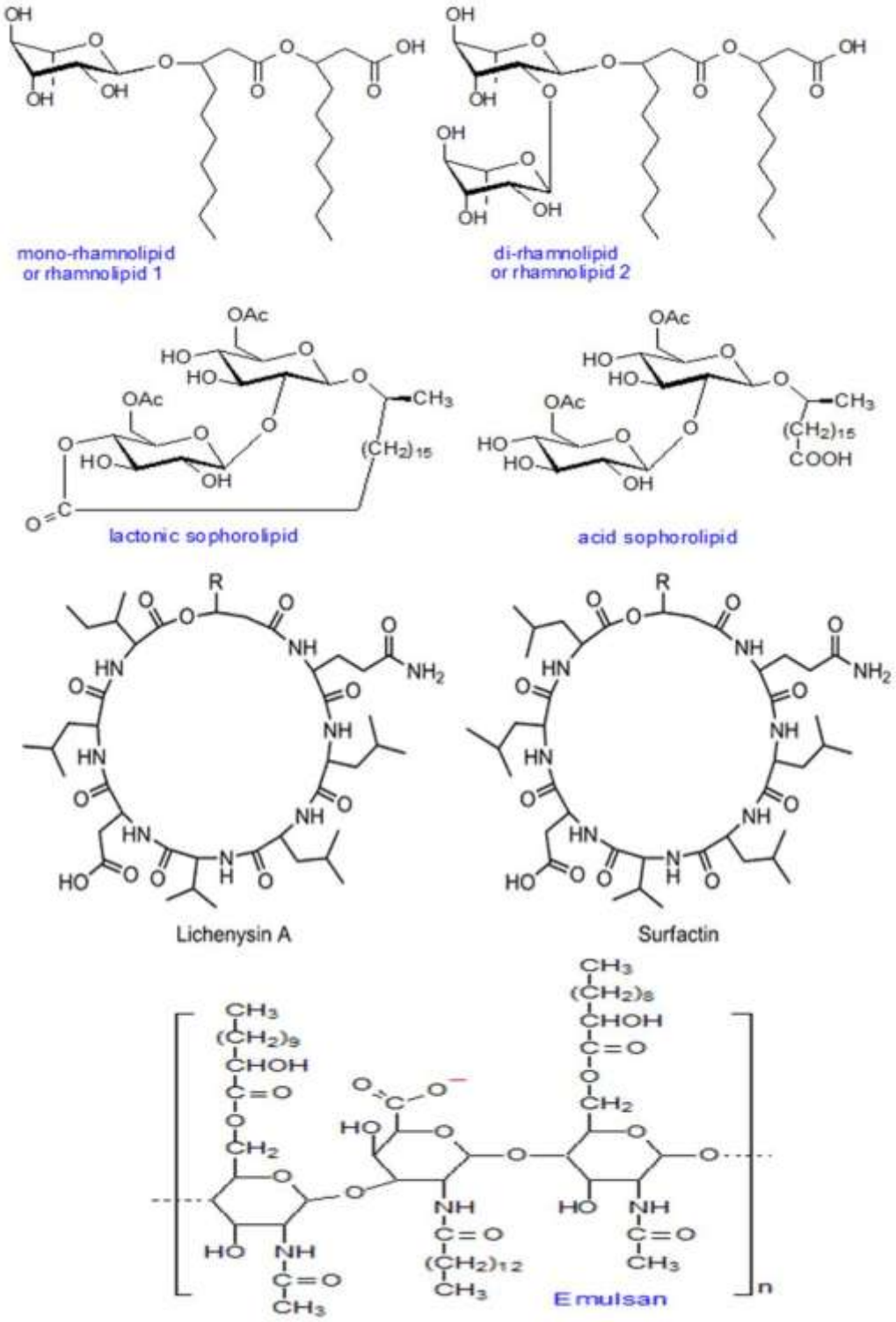

Figure1.2 Structure of different biosurfactants [Desai & Banat, 1997].

1.2 Mannosylerythritol Lipids

Mannosylerythritol lipids (MEL) are a class of glycolipid biosurfactants produced mainly by *Pseudozyma* and some *Ustilago* species. They are considered as one of the most promising biosurfactants due to their excellent surface activity and versatile biochemical functions. MEL contain 4-*O*-*β*-D-mannopyranosyl *meso*- erythritol as the hydrophilic group and a fatty acid and an acetyl group as the hydrophobic moiety [Figure 1.3]. Based on the number of acetyl group and their order of appearance on the thin layer chromatography (TLC), MEL are classified as MEL-A, -B, -C and -D. MEL-A is the di-acetylated compound, while MEL-B and MEL-C are monoacetylated at C6 and C4, respectively. The completely deacetylated structure is known as MEL-D [Kitamoto et al., 2002].

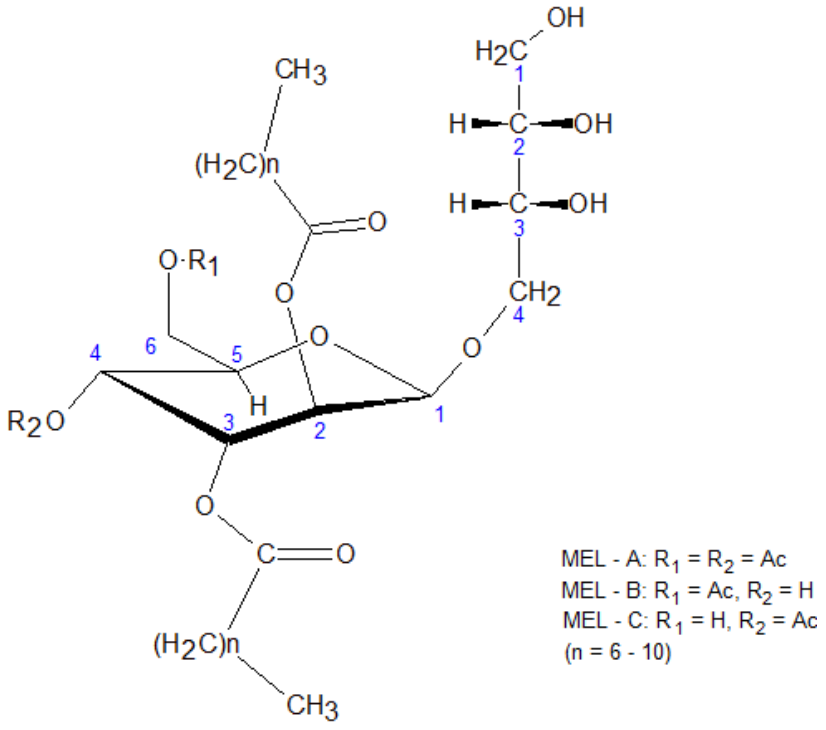

Figure 1.3 Structure of mannosylerythritol lipid

1.2.1 Production of MEL

In 1983, Kawashima et al., [1983] reported the production of unique glycolipids, which was the mixture of 2´,6´-di-*0*-acyl (C8-C14) esters of 4-*0*-β-D-mannosyl-D-erythritol called mannosylerythritol lipids from the yeast *Candida* sp. B-7 during the fermentation of n-alkanes. It has been proposed that MEL production was related to the mechanism involved in alkane uptake by the yeast. In 1990, Kitamoto et al. reported the production of MEL from a yeast isolate designated as T-34 from the medium containing vegetable oil as the carbon source. Strain T-34 was unable to produce MEL from alkanes and glucose or other carbohydrate. Both B-7 and T-34 were identified as *Candida antarctica*. *C. antarctica* T-34 was demonstrated as the best producer of MEL when compared to other strains of *C. antarctica*. Among the vegetable oil such as soybean oil, safflower oil, coconut oil, cottonseed oil, corn oil and palm oil; highest yield was obtained from the soybean oil. Under the optimal medium conditions, with 4% soybean oil as carbon source and 0.1% yeast extract as nitrogen source, the MEL yield was 40 g/l after eight days of fermentation [Kitamoto et al., 1990a, Kitamoto et al., 1990b]. Later, *C. antarctica* was taxonomically reclassified as *Pseudozyma antarctica*.

MEL are produced by *Pseudozyma* species in abundant quantity from the medium containing vegetable oil as carbon source. Since MEL are produced during the stationary phase, resting cells of *Pseudozyma* can be used to produce high amount of mannosylerythritol lipids. The advantages of fermentation with the resting cells over the conventional fermentation with growing cells were cost effectiveness as only carbon source was needed to be supplemented for the production of compound of interest, high yield and facilitated recovery of the compound. Resting cells of *P. antarctica* T-34 yielded of MEL about 46 g/l after six days of fermentation, which was higher than that of growing cells [Kitamoto et al., 1992]. A fed-batch fermentation of resting cells of *P. antarctica* T-34 in a medium containing n-alkane as

carbon source resulted in 140 g/l MEL. This finding also had huge environmental significance from the viewpoint of *in situ* bioremediation of hydrocarbons. [Kitamoto et al., 2001]. Type, concentration of carbon substrate and the stage at which carbon source was added significantly influenced the production of MEL. Feeding of mannose and erythritol at the stationary phase of growth of *P. aphidis* greatly enhanced the production of MEL. The addition of glucose reduced the production of MEL, while the addition of fatty acid methyl ester improved MEL yield. Glycerol supported the growth but not MEL production. Also, initial addition of high concentration of soybean oil was more favourable for the production of MEL by *P. aphidis* rather than batch wise addition of small quantities of soybean oil. Under optimized condition, a yield of 75 g/l was obtained after 10 days long fermentation by *P. aphidis*. [Rau et al., 2005a]. Bioreactor level cultivation of *P.aphidis* DSM 14930 resulted in doubling of the productivity and a threefold increase in the yield of MEL compared to shake flask level cultivation. Here, besides acting as carbon source for the production of MEL, soybean oil also acted as antifoam agent. MEL yield of 165 g/l with a productivity of 13.9 g/l/day was attained with the additional substrate feeding of glucose, sodium nitrate and yeast extract [Rau et al., 2005b]. The production of mannosylerythritol lipid at 95 g/l was achieved with foam-stat, fed-batch fermentation of *Candida* sp. strain SY16 in soybean containing medium. [Kim et al., 2006].

Ustilago maydis, a smut fungus produced the combination of glycolipids such as MEL and cellobiose lipids (CL) at high concentration. The ratio of MEL to CL depends on the type of the carbon substrate and the relative amount of unsaturated fatty acid present in the carbon substrate. The cultivation of *Ustilago maydis* DSM 4500 and ATCC 14826 in vegetable oil with high level of unsaturated fatty acids such as sunflower oil resulted in the production of MEL: CL at 9:1 proportion. The ratio of MEL: CL was shifted to 1:1, by using the vegetable oil with high content of saturated fatty acids such as coconut oil and to 1:9, by using the

hydrophilic substrate such as glucose as carbon source. A yield of 30 g/l glycolipids, which contained MEL predominantly, was obtained with the fermentation of *U.maydis* DSM 4500 in sunflower containing medium [Spockner et al., 1999]. *Kurtzmanomyces* sp. I-11 was also reported to produce MEL-B containing C_8, C_{12} and C_{14} fatty acids [Kakugawa et al., 2002]. Solvent extraction, followed by silica gel column chromatography was usually used for the recovery and purification of mannosylerythritol lipids. A commercially feasible downstream process was proposed by Rau et al., [2005c]. In this method, culture suspension after bioreactor cultivation of *Pseudozyma* was subjected to heating up to 110 ^{O}C for 10 minutes. A solid viscous phase containing MEL was formed, which was recovered by simply pouring off the supernatant. This method was quite environmentally-friendly as it did not generate any solvent waste and resulted in product recovery of 87%.

Mannosylerythritol lipids are commercially available from Biotopia Co., Ltd, a South Korea based Biotech Company and Toyobo/J.

1.2.2 Biosynthesis of MEL

Biosynthetic pathway of extracellular glycolipids is different from than those of intracellular glycolipids. Elaborative studies have been carried out to elucidate the biosynthetic pathway of MEL using different approaches such as microarray techniques, mutational studies and by supplementing different enzyme inhibitors.

Hewald et al., (2006) extensively studied the genes clusters and enzymes involved in the biosynthesis and export of MEL in *U. maydis* using DNA microarray technique. It was shown that the gene cluster involved in MEL biosynthesis consisted of five open reading frames, whose expression was highly induced under nitrogen starvation. In the proposed pathway of MEL biosynthesis, first step was condensation of mannosyl and erythritol catalyzed by the glycosyltransferase Emt1, a stereospecific reaction in which GDP-mannose was directly transferred to C_4 atom of mesoerythritol. Then, enzyme aceyltransferases, Mac1 and Mac2

were involved to transfer the short- and medium-chain fatty acids to positions R-2 and R-3, respectively. The last step, acetylation of deacetylated MEL at positions R-4 and R-6, was catalyzed by a single enzyme-acetyltransferase, Mat1. Mmf1, an exporter protein of major facilitator family was involved in the secretion of MEL.

Regarding the biosynthetic pathway of fatty acids of MEL, Kitamoto et al., (1993a) suggested that fatty acyl structure of MEL was more or less related to the substrate supplemented in the culture medium. According to Tanaka & Fukui (1989), the biosynthetic pathway of fatty acids in the microorganism was divided into three mechanisms.

I. *De novo* synthesis of fatty acids followed by complete β-oxidation.
II. Chain elongation pathway
III. Intact incorporation pathway

When fatty alcohols or acids with chain-length of C_n (C_{12} to C_{18}) were used as substrates, fatty acids in MEL had carbon chain shortened by one or more C_2 units [C_{n-2}, C_{n-4} or C_{n-6} & . . . chains] from that of carbon source used [Kitamoto et al., 1993a]. The addition of cerulenin, inhibitors of *de novo* synthesis had no effect on the production and structure of MEL, which ruled out the involvement of pathway I in MEL biosynthesis. Incorporation of β-oxidation intermediates such as medium chain fatty acids to the MEL showed that pathways II and III had no role in MEL biosynthesis. Hence, it was proposed that a new chain shortening pathway or partial β-oxidation was involved in the biosynthesis of MELs and was supported by further studies in which MEL biosynthesis was inhibited by the addition of 2-Bromooctanoic acids, inhibitor of β-oxidation [Kitamoto et al., 1995, Kitamoto et al., 1998]. When aliphatic hydrocarbons were used as carbon source, a sub-terminal oxidation pathway was involved in the conversion of aliphatic hydrocarbons to fatty acids of MEL, which was catalysed by a secondary alcohol dehyrogenase, Baeyer-Villiger monooxygenase and esterase [Kitamoto et al., 2009].

Morita et al., [2010a] described the importance of gene encoding mitochondrial ADP/ATP carrier in the biosynthesis of MEL as it was highly expressed under MEL-producing conditions. MEL production in *P. antarctica* was enhanced upon the increased gene expression of PaAAC1 by introducing a plasmid containing PaAACI. Mutation studies showed the importance of conserved sequence of PaAACI gene in MEL biosynthesis. Genomic analysis of the 18 Mb genome of *P. antarctica* revealed the highly advanced lipid transport and metabolism pathway controlled by higher number genes than that of the yeast model organism, *Saccharomyces cerevisiae*. Gene expression of *P. antarctica* varied with respect to carbon source. Gene expression of genes involved in fatty acid metabolism was highly expressed under oil supplementation, while gene expression of MEL biosynthesis gene cluster was constant irrespective of the carbon source. Pathway of MEL biosynthesis is shown in Figure 1.4 [Morita et al., 2014].

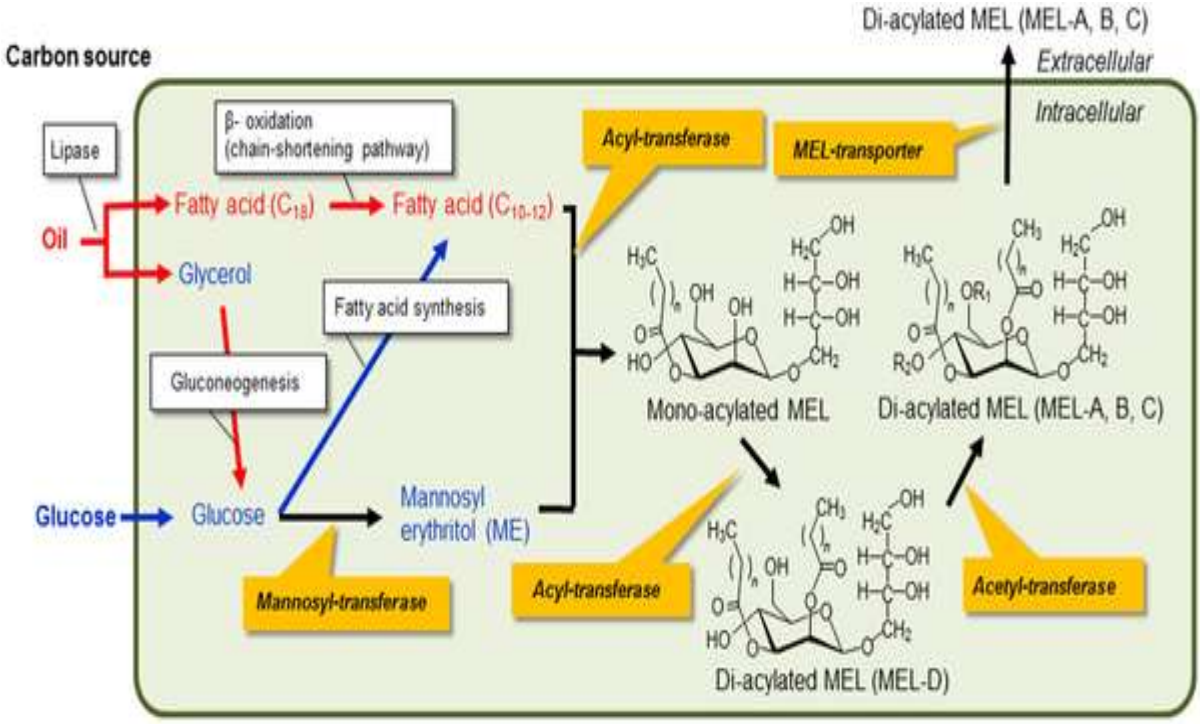

Figure 1.4 Pathway showing biosynthesis of MEL [Morita et al., 2014]

1.2.3 Physiological role of MEL

Initially, MEL were thought to be produced as energy storage materials as MEL were produced on the supplementation of vegetable oil and high concentration of carbohydrates such as glucose and glycerol. While cultivated at 10% glucose concentration, *P. antarctica* T-34 accumulated MEL along with triglycerides which was usually observed as intracellular oil globules. The MEL content made up to more than 10% of the cell dry weight and the pattern of variation in MEL content was similar to that of triglycerides. Hence, it was concluded that MEL were synthesized as storage material along with triglycerides in *Pseudozyma*-like oleaginous yeast [Kitamoto et al., 1992a]. As *Pseudozyma* and other MEL producing fungal species are usually phyllosphere associated yeast, the role of MEL in phyllosphere colonization was studied by Yoshida et al., [2014]. The biological role of MEL towards the *Pseudozyma* species was studied by comparing the phenotypic characteristics of wild type and non MEL producing mutant of *P. antarctica* while growing on the surface of plastic plate, onion peels and fresh leaves of rice and wheat. Wild type strain changed from single cell to elongated ones, when growing on the plastic surface and onion leaves within short period of time and showed bigger colony area on wheat leaf cutting than that of non MEL producing mutant. Further study with HPLC and real-time PCR affirmed the potential role of MEL in fungal morphological development and propagation on plant surface [Yoshida et al., 2014]. Transcriptomic analysis of *P. aphidis* revealed that gene expression involved in cell development was in parallel to MEL synthesis showing the co-regulation between MEL synthesis and cell morphology [Gunther et al., 2015].

1.2.4 Potential Applications of MEL

Mannosylerythritol lipids exhibit diverse functional properties, which reveal their applications in therapeutics, cosmetics, biomedical studies and energy saving technologies.

1.2.4.1 Antimicrobial Activity of MEL

Both MEL-A and B show relatively good antimicrobial activity particularly against Gram-positive bacteria and their minimum inhibitory concentration has been less than that of some glycolipid type synthetic surfactants [Kitamoto et al., 1993b]. The antibacterial activity of MEL can be exploited in food and feed applications. In preparation of feed additives for live stocks, apart from preventing the infectious diseases caused by Gram-positive bacteria, MEL contributed to the improvement in overall health of live stocks [Ito et al., 2011].

1.2.4.2 Cell Differentiation and Apoptosis Inducing Activities of MEL

MEL show antitumor activity against human leukaemia and mouse melanoma cells, and has been proposed as therapeutic agent against cancer. In the case of promyelocytic leukemia cells HL60, MEL A and B at concentration of 5-10 μM resulted in the inhibition of growth and induction of granulocytic differentiation and other morphological changes associated with cell differentiation. Molecular mechanism behind the cell differentiation inducing activity was by affecting the signal transduction pathway such as inhibiting the activity of phospholipid- and Ca^{2+}-dependent protein kinase C and phosphorylation of phosphate cascade system. Growth of mouse melanoma B16 cells was inhibited by MEL-A in dose-dependent manner and biochemical changes associated with apoptosis including the condensation of chromatin and fragmentation of DNA were observed when cells were treated with MEL-A at concentration of 10 μM [Kitamoto et al., 2002]. In the presence of MEL, rat pheochromocytoma PC12 cells underwent partial cellular differentiation characterized by the outgrowth of neurites, increased activity of acetylcholine esterase, cell cycle arrest at the G1 phase and induction of transcription factors involved in up regulation of certain genes such as C-jun [Wakamatsu et al., 2011].

1.2.4.3 MEL for Purification of Glycoprotein

MEL are promising as a tool for the immunodiagnostic and therapeutic application as they show affinity binding to human IgG and their binding constant is four times higher than that of protein-A, the usual candidate in immunoaffinity chromatography of IgG. Use of MEL as an alternate affinity ligand for IgG can address the two major drawbacks associated with the use of protein-A such as high cost and the requirement of high acidic conditions to elute the IgG. They show equal binding affinity as that of gangliosides, another potential glycolipid ligand, which lacks their commercial feasibility due to their limited availability, structural heterogeneity and complicated purification steps in the commercial preparation. However, the feasibility of production of yeast glycolipids by fermentation and ease of recovery can efficiently make up for these commercial challenges. Monolayer of MEL-A show high binding affinity to lectins, which reveals its potential as a probe to study the protein-carbohydrate interactions [Kitamoto et al., 2002: Im et al., 2003; Konishi et al., 2007].

1.2.4.4 MEL as Vehicle for Gene Delivery

Cationic liposomes are the viable alternative molecular cargo to viral vectors for gene transfer due to low toxicity and high transfection efficiency. MEL form thermodynamically stable vesicle and can accelerate membrane fusion between liposome and target cell in a safe and rapid manner. Cationic liposomes prepared with cationic cholesterol and MEL-A effectively delivered the plasmid encoding luciferase gene into the target cells such as HeLa and these newly prepared cationic liposomes showed 50-70 fold increase in the efficiency of gene transfection, when compared to the commercially available cationic liposomes. Additional advantage incurred by the addition of MEL-A was efficient gene transfer with shorter incubation period [Kitamoto et al., 2002; Nakanishi et al., 2013].

1.2.4.5 MEL as Anti-Agglomeration Agent of Ice Particles

Ice slurry system is one of the most advanced and environmental friendly cooling solution providing quality chilling and proper temperature control to the storage unit. Major shortcoming with ice slurry system is ice particle agglomeration, which blocks the piping, requires high power load and reduces efficiency and controllability of the system. Additives, such as surfactants, polymer, alcohol and brine can be added to the system to prevent ice particle agglomeration. As the uses of chemical additives have negative environmental impact, use of biosurfactants could be one of the environmental friendly solutions as an anti-agglomeration agent. MEL showed impressive anti-agglomeration effect on ice slurry with ice packing factor (IPF) of more than 35% at concentration less than of 10 mg/l, while Span 80 (Sorbitan monooleate) exhibited a value of IPF of 30% at a concentration of 1000 mg/l. Ice particle agglomeration is inhibited by the adsorption of surfactants to the ice particle in a regulated manner which results in stable dispersion of ice particle in ice slurry leading to a higher system efficiency. MEL has also been successfully tested on a large scale model with a 300 l ice storage tank [Kitamoto et al., 2002]

1.2.4.6 Skin and Hair Care Applications of MEL

MEL is one of the biosurfactants, which is successfully used in cosmetic industry. Morita et al. [2009b] reported the excellent moisturizing activity of MEL-A which was comparable to that of ceramide. Its mechanism of action could be the efficient penetration of biosurfactants through the epidermis and improvement in moisture retention by forming liquid crystals. MEL-A can be a potential alternative to ceramides with the advantage of economical production through the fermentation technology [Morita et al., 2009b]. MEL-A can be an efficient hair care agent since it can repair the damaged hair and improve the mechanical properties of the hair. MEL-A was proposed to be absorbed onto hair, form a lamellar structure and nourishes hair, compensating the loss of hair surface lipid, methyleicosanoic

acid during the harsh chemical treatment [Morita et al., 2010b]. Kanebo Cosmetics has successfully developed MEL-B based cosmetic products such as skin lotion and cream. Both water in oil emulsion and oil in water emulsion can be prepared by MEL-B [http://www.kanebo.com/research/skincare/biosurfactants.html accessed on 15-03-2015]. Triacylated MEL or mannosylmannitol lipids (MML) produced either by fermentation or by transesterification reaction exhibit remarkable anti-aging effects, which could be correlated to different chirality, self assembling property and liquid crystal forming behaviour with respect to conventional MEL. Apart from being used as activator in cosmetics, these can be incorporated in to the food and drink with anti-aging application as these are safe to take orally [Suzuki et al., 2013].

1.3 Exopolysaccharide

Exopolysaccahrides (EPS) are the high molecular weight sugar polymers, secreted by microbes into the surrounding environment. EPS with impressive physiochemical properties are secreted by microorganisms of different taxonomic groups. EPS may be of homopolymeric or heteropolymeric in sugar composition and may contain lipid or protein moiety, additionally. The physiological roles of EPS are not yet unravelled, but they are implicated in pathogenicity, biofilm formation, quorum sensing etc. Microbial exopolysaccharides possess several industrial applications as they serve as thickening agent, rheology modifier, emulsion stabilizer, film forming agent, suspending agent, slow releasing agent, dispersing agent and stabilizing agent. In most of the cases, polysaccharide exhibit non-Newtonian flow behaviour called shear thinning i.e. the viscosity decreases with increasing shear rate that is beneficial in case of food and cosmetic preparations as it helps in the desired flow and uniform application. So they are the important hydrocolloids in food and cosmetic industries. Certain EPS exhibit biological activity with potential to be used in

therapeutic and pharmaceutical industries [Nwodo et al., 2012; Duboc& Mollet, 2001; Sutherland 1990].

Many yeasts belonging to the genera *Cryptococcus* [Pavlova et al., 2009], *Rhodotorula* [Cho et al., 2001], *Sporobolomyces* [Pavlova et al., 2004] and *Candida utilis* [Chiura et al., 1982] are reported to be EPS producers. EPS production is considered as one of the adaptation strategies for Antarctic yeast [Buzzini et al., 2012]. Yeasts commonly give high yield of EPS that could be easily separated from the culture broth compared to those produced by bacteria, which makes them more suitable for commercial applications [Peterson et al., 1989]. Pullulan, a water soluble glucan gum produced by black yeast *Aureobasidium pullulans*, has commercial applications in both food and oral care industries [Singh et al., 2008]. The exopolysaccharide produced by yeast-like fungus *Tramella* consist of a mannan backbone and has immunoregulatory property [Baets et al., 2001]. *Sporobolomyces Salmonicolor* produces glucomannan having pseudoplastic behaviour and has potential to be used as a thickener in food industry [Pavlova et al., 2004]. The distinct emulsification property, high compatibility with macrophage cell line and protective effect against toxic activity of cytostatic compound such as Avarol makes the *Sporobolomyces* glucomannan an attractive candidate for applications in cosmetic industry [Kuncheva et al., 2007; Poli et al., 2010]. Many psychrophilic yeast strains like *Cryptococcus flavus*, *Cryptococcus laurenti* were found to be good producers of EPS and the EPS from *C. laurenti* showed pronounced emulsification activity along with other hydrocolloids [Pavlova et al., 2009; Pavlova et al., 2011].

Many microbial exopolysaccharides have been approved for food, non-food, cosmetics and biomedical applications. Some well-known examples of EPS are discussed below.

1.3.1 Xanthan Gum

Among microbial polysaccharide, the most sought-after one is xanthan gum. Xanthan gum is an exopolysaccharide of glucose, mannose and glucuronic acid monomers, produced by bacteria *Xanthomonas Campestris*. Xanthan gum is a high molecular weight polymer with the molecular weight varies between $2x10^6$ to $20x10^6$ Da. [Garcia-ochoa et al., 2000]. Kelco Company pioneered the commercial production of xanthan gum in 1960s. Initially corn syrup or molasses were used as the carbon source for production of xanthan gum. Later, a mutant of *Xanthomonas Campestris* was developed, which can utilize lactose in cheese whey and produce xanthan gum at high yield. Recombinant *Xanthomonas campestris* with inserted β-galactosidase gene was developed, which was able to grow in whey containing medium and produce xanthan gum at high yield [Fu& Tseng, 1990]. Xanthan gum production by *Xanthomonas campestris* pv. *mangiferaeindicae* IBSBF 1230 was 46.8 g/l from media containing cheese whey under optimized conditions [Mesomo et al., 2009].

The ability of xanthan gum to form a high viscous solution even at low concentrations makes it an effective gelling agent/ stabilizer. Xanthan gum is excellent hydrocolloids that thicken or stabilize the emulsion, foams and suspensions. Xanthan gum is stable at various temperature and pH. Xanthan gum stabilizes oil-in-water emulsion and compatible with all the ingredients such as salt, metallic cations, surfactants or bioactive compounds that are present in the different commercial formulation. Since xanthan gum improves the flow properties of the formulation, it results in other desirable properties like promoting the formation of rich and creamy lather in shampoos and liquid soaps. In the food and cosmetic preparation, xanthan gum stabilizes the emulsion against coalescence, keeps the product uniform and maintains the consistency.

A major limitation with xanthan gum is their slow dissolution rate. During hydration, partially hydrated gum forms gelatinous layers, which prevents the entry of the water to the

interior of the gum and hence leaves the gum incompletely dissolved. Xanthan gums have been reported to be modified chemically with an objective to prevent the formation of the gelatinous layer and to improve its dissolution [Su et al., 2003]. Genetic engineering of *Xanthomonas campestris* at particular key genes result in secretion of high molecular weight xanthan gum with improved viscosity. Supplementation of high viscous xanthan gum in products reduces the need to add the large quantity of thickener to meet the desired rheology. [Patel et al., 2008].

1.3.2 Dextran

Dextran is an exopolysaccharide produced by lactic acid bacteria, *Leuconostoc mesenteroides.* Structurally it is α-D -glucan, mainly composed of α- 1, 6-glycosidic linkage and a small amount of α-1, 2, α-1, 3 and α-1, 4 branched linkages. Dextran is commercially produced from sucrose containing medium such as molasses and the process parameters affect yield and molecular weight of dextran. A unique high molecular weight dextran of two million Dalton was reported to be produced from the medium containing 15% sucrose under optimized conditions [Sarwat et al., 2008].

Dextran acts as a binding agent that improves dispersion of the components in food formulations. Another important function is thickening by controlling the viscosity of the preparations that helps in maintaining the appropriate consistency. Dextran also acts as a bulking agent to increase the bulk of formulation because of its water binding capacity [Samuel et al., 2002]. Various kinds of dextran and its derivatives have been used in cosmetics depending on the particular requirement. High molecular weight dextrans like dextran-40 *(MW: 40,000 Da),* dextran-70 (>60,000 Da) and dextran derivatives such *as* dextran sulphate and cationic dextran are the most commonly used for industrial purpose. Dextran sulphate is manufactured by sulphonation of dextran while cationic dextran is a salt of dextran with anionic or amphoteric surfactants. Dextran-70 has been reported to be used in

the formulation of bioadhesive patches for wrinkle reduction and facial augmentation in cosmetic preparation [Shiroyo et al., 2013]. Carboxymethyl dextran is prepared by substituting one or more hydroxyl group of dextran with carboxymethyl group by treating with carboxy methylating agent in alkaline solution. Carboxymethyl dextran is used to prepare stable colloidal suspension of magnetic nanoparticles, which is used for various biomedical applications such as magnetic resonance imaging contrast enhancement, tissue repair, cancer treatment and controlled drug delivery [Latorre-Esteves et al., 2009].

1.3.3 Gellan Gum

Gellan gum is an exopolysaccharide of glucose, glucuronic acid and rhamnose with a molecular weight of $5x10^5$ Da produced by *Sphingomonas elodea*. A unique property of gellan gum is its thermostability and viscosity of the solution remains stable over a wide range of temperatures. Gellan gum is popular as Gelrite, the gelling agent used in microbiological and plant tissue culture media. Gellan gum is an effective food and cosmetic additive. Gellan gum works as emulsion stabilizer by preventing the separation of oil and water. Also, gellan gum acts as suspending agent by stabilizing the emulsion and keeping it uniform against temperature fluctuations. Gellan gum is a gelling agent, but not a thickening agent as it does not change the rheology of the solution as efficient as the other gums. Gellan gum is used as an alternative to gelatine in vegan food preparations. Structural modification of gellan gum changes its functional properties and performance. An entirely deacylated gellan gum forms a rigid brittle gel, while gellan gum of low acyl and high glyceryl forms an elastic gel [Bajaj et al., 2007; Chang & Kobzeff, 1993].

1.3.4 Welan Gum

Welan gum is an exopolysaccharide produced by *Alcaligenes* species. It has a similar structure of gellan with the additional side chain of L-rhamnose or mannose. Like gellan, it is stable over a wide range of temperature (up to 150 ^{O}C) and pH (2-12). It exhibits increased

viscosity at low shear rate. By altering the metabolic flux of the pathway of welan production by pH control process, welan production reached a maximum of 25 g/l. [Li et al., 2013]. A hydrocolloid suspension of welan gum is used as stabilizing additive for cement and drilling fluid applications [Skaggs et al., 2001].

1.3.5 Rhamsan

Rhamsan gum is a polysaccharide produced by *Alcaligenes* and *Sphingomonas* species. It has the same backbone structure of gellan with disaccharide side chain of β-D glucose. The advantage of using rhamsan over the other gums is its low concentration that is sufficient to achieve the desired viscosity. Viscosity remains stable over a wide range of temperature and pH. Rhamsan gum exhibits high suspension capability than plant based gums [Mazuel 1991]. Rhamsan could be used as mist suppressant in metal working composition that reduces the misting of the machine and improves the performance during prolonged times of shearing [Collins & Kajhan, 1998].

1.3.6 β-glucan

β-glucans are a heterogeneous group of polysaccharides produced by a variety of sources such as bacteria, fungi and plants. Depending on the source and extraction process, β-glucans vary in their structural and functional properties. β-glucan from yeast contains β-1, 3 and β-1,6 glycosidic linkage and have high biological activity than the plant derived glucans. β-glucans are produced commercially from baker's yeast *Saccharomyces cerevisiae*. Among higher fungi, *Lentinus edodes*, *Ganoderma Incidum* and *Pleurotus ostreatus* are important sources of β-glucan. β-glucans are recognized as nutraceutical compounds due to their significant biological activities such as antioxidant activity, non-specific immune stimulation, hypoglycemic and hypocholesterolemic effect. They could be used as important source of dietary fibre in baked goods, beverages, fruit juices, etc. [Rahar et al., 2011]

Apart from the biological activity; water holding, oil binding and rheological properties of beta glucan make it an effective ingredient in food and cosmetics composition. Petravic tominac et al.,(2010) elaborately discussed cosmetic applications of β-glucan .β-glucan could be used as thickener and emollients in the cosmetic preparation. β-glucan could also be used as one of the photoprotective agents in sunscreen lotion, because of its free radical scavenging activity and ability to induce epidermal macrophage action that results in skin protection. β-glucan is an anti-irritant in the lactic acid based skin creams. β-glucan was reported for its hair growth promoting action by activating hair follicle and promoting hair regeneration. β-glucans act synergistically with anti-aging compounds, which helps in rejuvenating skin and reduces the visible signs of aging. Carboxymethyl glucan (CM glucan) is the modified yeast glucan with improved water solubility. It has broad application than unmodified β-glucans due to their improved biological activity

β-1,3 D-glucan produced mainly by soil-dwelling bacteria include linear β-(1,3) glucan called curdlan, side-chain-branched β-(1-3,1-2) glucans and cyclic β- (1-3,1-6) glucans. Curdlan is mainly produced by *Agrobacterium* species while the rest of the β-1,3 D-glucans were produced by *Bradyrhizobium* species [McIntosh et al., 2005]. Curdlan exhibit unique gelation behaviour and viscoelastic properties on the basis of concentration and temperature of setting. An aqueous dispersion of curdlan forms a thermal irreversible elastic gel by heating above 80 ^{O}C [Funami et al., 1999]. Curdlan could be used as texture modifier in noodles, water holding agent in meat products, fat substitute e.g. vegetable oil containing curdlan could use be used instead of pork fat. [Miwa et al., 1993].

1.3.7 Schizophylllan

Schizophyllan is a nonionic polysaccharide produced by submerged fermentation of basidiomycete *Schizophyllum commune.* Structurally, it is glucan having β-(1-3)-linked backbone with β - (1-6)-linked glucose side chains. Schizophyllan exhibit unique rheological

characteristics depending on the concentration of polymer and shear rate. Schizophyllan show Newtonian behaviour up to certain critical shear rate and then follows a shear thinning behaviour.i.e. critical shear rate decrease with increasing concentration of the polymer [Zhang et al., 2013]. Because of its high temperature and salt tolerance, high mechanical and shear stability and favourable rheological properties, schizophyllan is an ideal biopolymer used for enhanced oil recovery [Briechle et al., 2012]. Schizophyllan was also proposed as gene carrier as it forms complex with single-stranded polynucleotide and protect it from nuclease attack [Sakurai et al., 2001].

1.3.8 Scleroglucan

Fungi, Sclerotium species produce non-ionic exopolysaccharide called scleroglucan that has a structure similar to schizophyllan. High thermal stability, remarkable rheology and compatibility are interesting attributes of scleroglucan. Scleroglucan exhibit shear thinning behaviour with good yield value and are effective suspending and stabilizing agent. Scleroglucan is quite compatible with surfactants, thickening agents and all other ingredients in different formulations. Scleroglucan is also used for oil wells and oil drilling applications [Desbrieres 1984]. Scleroglucan can be used to prepare liquid deicer composition with reduced corrosiveness [Koefod 2011].

1.3.9 Levan

Levan is a heterogeneous class of fructan polymer obtained from plants and microbes. Bacteria, belonging to genera *Bacillus, Streptococcus and Cornybacterium* were reported to produce levan [Yan 1990]. Because of the unique backbone, levan exhibit different functional properties that distinguish from other biopolymers. Levan is marketed as functional food due to their probiotic activity, which also increases the intestinal absorption of minerals [Kang et al., 2009]. As it has low intrinsic viscosity and does not swell in water, levan is not an ideal thickening or gelling agent. Instead, levan could be used to give a smooth finish to the

cosmetic product with a good skin feel

[http://www.polysaccharides.us/levancos_summary.php accessed on 15-03-2015].

1.3.10 Pullulan

Pullulan is a water soluble glucan gum produced by black yeast *Aureobasidium pullulans*. Hayashibara, a major commercial supplier of pullulan, produce pullulan through aerobic fermentation of starch syrup. Pullulan could increase the viscosity of the solution without forming the gel. Pullulan is a widely used food additive, more commonly in oral hygiene products. Pullulan could be used as thickener or binding agent in food and cosmetic compositions. Due to its non-toxic and inert nature, pullulan is a promising biopolymer for various biomedical applications such as gene delivery, targeted drug therapy, tissue engineering and wound healing [Prajapati et al., 2013].

OBJECTIVES OF THE STUDY

With the background as presented on the previous pages, the aim of this study was to explore the culture of *Pseudozyma* sp.NII 08165 for the production of biosurfactants and exopolysaccharides and to study their properties for the possible applications. The specific objectives were to study the:

- Production of glycolipids in submerged fermentation,
- Structural characterization of glycolipids,
- Production of exopolysaccharides in submerged fermentation,
- Structural and physiochemical characterization of exopolysaccharide,
- Application of biosurfactants as laundry additive,
- Application of biosurfactants in bioremediation.

Chapter 2: Microorganism and Screening for Biosurfactants Production

2.1 Introduction

The genera *Pseudozyma* are heterobasidiomycetous yeasts belonging to the family Ustilaginaceae, order Ustilaginales, Class Ustilaginomycetes, Phylum Basidiomycota. The genus comprised of 17 recognized species: *P. antarctica, P.aphidis, P.flucculosa, P. tsukubaensis, P. fusiformata, P. graminicola, P. hubeiensis, P. jujuensis, P. parantarctica, P. prolifica, P. pruni, P. rugulosa, P. shaxiensis, P. thailandica, P. siamensis, P. crassa,* and *P. alboarmeniaca* [Liu 2011, Mekha et al., 2014]. They are epiphytic in nature, and some species of *Pseudozyma* such as *P. aphidis* and *P. flocculosa* are used as the biocontrol agent against plant pathogens [Buxdorf et al., 2013]. *Pseudozyma* species have been reported to produce glycolipids biosurfactants such as mannosylerythritol lipids and cellobiose lipids. Besides glycolipids, some *Pseudozyma* species also produce commercially attractive compounds such as squalene, itaconic acid and erythritol [Chang et al., 2008; Levinson et al., 2006; Jeya et al., 2009].

A strain of *Pseudozyma* designated as *Pseudozyma* sp. NII 08165 was used in the present work. This was isolated as an aerial contaminant on lipase screening plate (triolein-rhodamine agar plates) from the biotechnology lab of NIIST. The culture produced alkali active, thermostable, halotolerant esterase, which was also capable of acting in presence of high methanol concentration [Alex et al., 2014]. Because of ability to grow on media containing lipid as the carbon source and the peculiar media characteristics, the organism was suspected to produce biosurfactants. The stock culture of the isolate was prepared in potato dextrose agar (PDA) slants and stored at 4 ^{O}C and subcultured at every four weeks.

2.2 Materials and Methods

2.2.1 Microscopy

The microbial culture was observed under the phase contrast microscope (Leica DM2000, CMS GmbH) and scanning electron microscope (SEM). For SEM analysis, the stubs containing culture were gold sputtered using SC7620 sputtercoater device and analyzed by SEM at an operating voltage of 30kV (Zeiss Evo-18 Special Edition).

2.2.2 Molecular Identification and Phylogeny

DNA was extracted using yeast genomic DNA isolation protocol [Harju et al. 2004].

The ITS region was amplified using universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC-3') [White et al. 1990]. The PCR amplicon was sequenced, and BLAST analysis was performed using NCBI BLAST tool.

The sequences were aligned using Clustal W software [Thompson et al. 1994]. The phylogenetic analysis was performed with MEGA4 software using Neighbour Joining method [Tamura et al. 2007].

2.2.3 Screening for Biosurfactants Production

The microbial culture was screened for biosurfactants production by classical biosurfactants screening methods, *viz*. drop collapse assay and haemolysis assay. The drop collapse assay is based on the collapse or spreading of the droplets on the hydrophobic surface because of the biosurfactants [Kuiper et al. 2004]. Here 25 µl of 2 days old culture supernatant of *Pseudozyma sp*. NII 08165 was added to the surface of parafilm after adding bromophenol blue for staining. The distilled water was taken as a negative control and 1% SDS was taken as positive control. The dye bromophenol blue has no influence on the shape of the droplets. Haemolysis assay was analyzed by spotting 10 µl of an overnight culture on blood agar plates

(24 g/l potato dextrose, 20 g/l agar, 8g/l NaCl, and 5% goat blood). Haemolysis was monitored after incubation of the plates at 30°C for three days [Hewald et al. 2005].

2.3 Results and Discussion

2.3.1 Microscopy

On microscopy, *Pseudozyma* sp. NII 08165 was observed to have typical elongated spindle-shaped blastoconidia, which grew into pseudohyphae [Figure 2.1]. Fusiform, elongated and spindle-shaped blastoconidia were consistent with *Pseudozyma* species while in related genera *Ustilago*, blastoconidia were spindle-shaped, elongated and irregular [Liu et al., 2013].

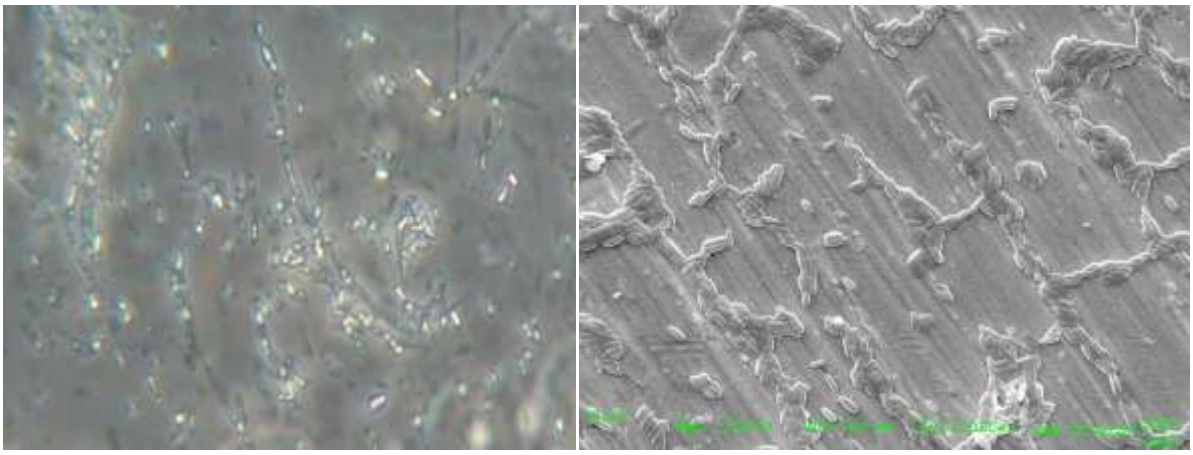

Figure 2.1 (A) Microscopic images of *Pseudozyma* sp. NII 08165; (B) SEM images of *Pseudozyma* sp. NII 08165

2.3.2 Molecular Identification and Phylogeny

The ITS of nuclear DNA has been used as standard marker for fungal DNA barcoding. ITS1 and ITS2 are the internal transcribed spacer found on the either side of 5.8S rRNA gene. While rDNA sequence is highly conserved, ITS region is variable and fast evolving, thus ideal for studying interspecies divergence [Korabecna et al., 2003]. To identify the organism, region of ITS-5.8S rDNA was amplified using the primers ITS1 and ITS4 and sequenced

(Table 2.1). Upon the BLAST analysis, the query sequence showed an identity of 95-99% to the ITS region of different species of *Pseudozyma*. Hence, it was concluded that isolate belonged to *Pseudozyma* genera. The sequence was submitted to NCBI Genbank with accession no JN969989. The strain was deposited in NII Culture Collection and designated as *Pseudozyma* sp. NII 08165.

Table 2.1 Sequence of ITS1-5.8S-ITS4 region of the isolate

AACTTGAGCTACCTTTTTTAACACGGTTGCATCGGTTGGGCCTGTCAAATAGCGCGGACGCGTTCCAAGCTA
CGAGACGGGTTCAACACTTTTACCAAAACACTTTTGATGACTTAGGATTTGAATGATAAAAGTTCATTTTTAA
TAATGAAACCGACTGGTAATGCGGTCGTCTAATTTTAAAAACAACTTTTGGCAACGGATCTCTTGGTTCTCCC
ATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGAAGTGAATCATCGAATCTTTGAACGC
ACCTTGCGCTCCCGGCAGATCTAATCTGGGGAGCATGCCTGTTTGAGGGCCGCGAATTGTTTCGAACAACTG
CTTTTTTTAATTAAGAAGAAGTTGGATCGGTATTGAGGGTTTTGCCATTCACCGTGGCTCCCTCGAAATGCAT
TAGCGCATCCATTTGATAGGCAAGACGGACGAAAGCTCACTTTCGCTCTCTCTTCCCTGCCGGGTTTTGATAA
TATCAGGACTTCGGAGAGGTTGAGTTGGGTACGAGCTGGAGCAACGGCTTGCTGTTTGGAGTGCTTCTGAAA
CCCGCCCATGTCGAGCTTTATTGCTCGAAAGGGA

Figure 2.2 shows the phylogenetic tree constructed with ITS sequences. On the phylogenetic tree, the isolate was positioned near to *P. siamensis*, which was a predominant producer of MEL-C [Morita et al. 2008]. The identity of the *Pseudozyma* strain was important in understanding the production of mannosylerythritol lipids (MEL). Phylogenetic analysis using ITS sequences was undertaken to obtain the relationship of the strain with the ones already reported to produce MEL, so that there could be better understanding of MEL production in relation to the genetic diversity of this culture. The culture was phylogenetically closer to *P. siamensis* by ITS sequence comparison.

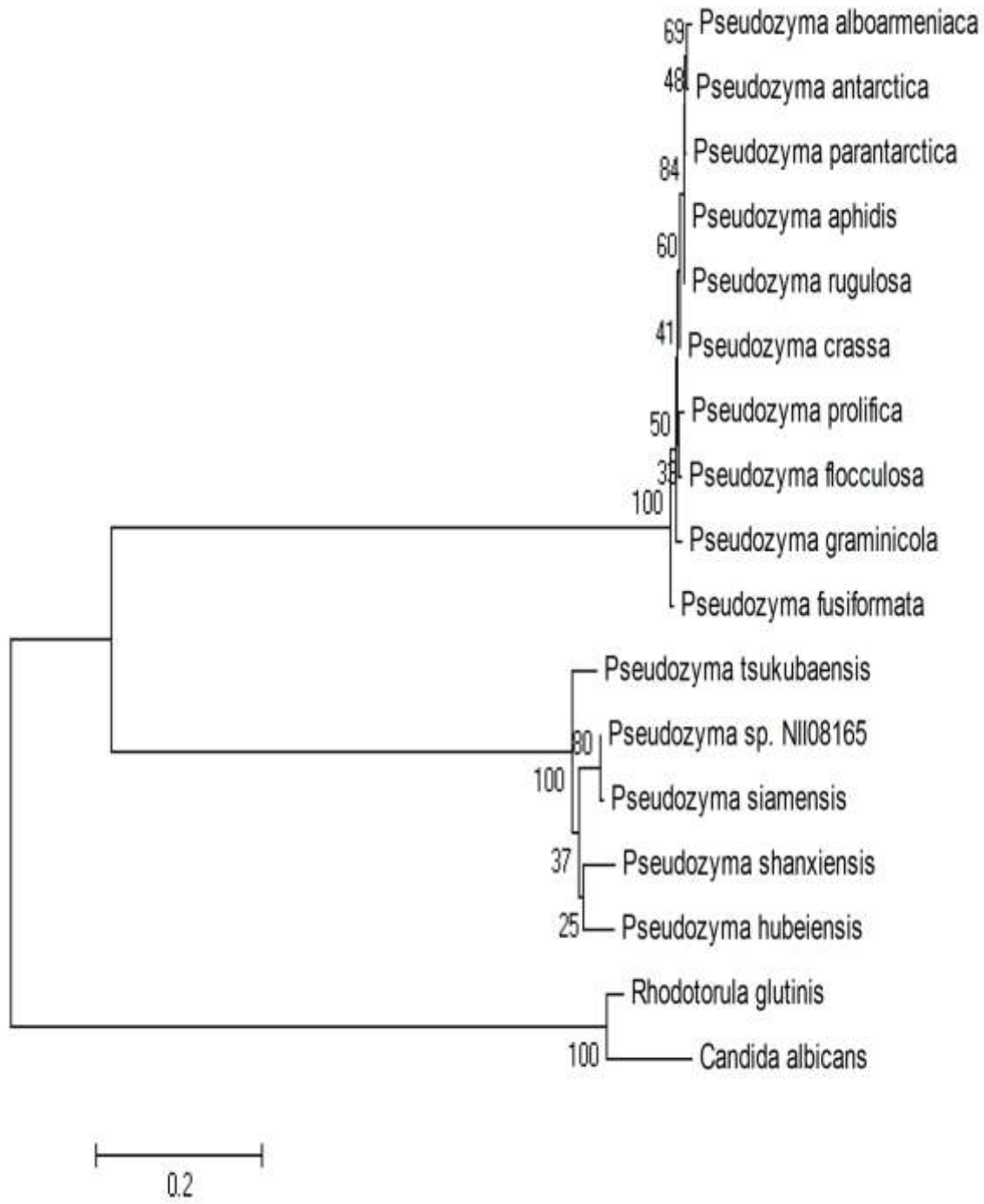

Figure 2.2 Phylogenetic tree of the genus *Pseudozyma* constructed with ITS1-5.8S-ITS4 sequences

Pseudozyma species are classified into four groups based on the production of biosurfactants. The first group comprises of species which produces mainly MEL-A along with other MEL, e.g., *P. antarctica, P.aphidis, P. rugulosa* and *P. parantarctica.* The second group produces MEL or/and other glycolipids such as cellobiose lipids, e.g., *P.flucculosa* and *P. fusiformata*. The third group mainly produces MEL-B or MEL-C, e.g., *P. tsukubaensis, P. hubeiensis, P. shaxiensis* and *P. siamensis*. The fourth group produces no glycolipids at all, e.g., *P. thailandica* and *P. prolifica.* On phylogenetic tree constructed with ITS sequence, MEL-A producers are clustered together and positioned at a distance from MEL-B or MEL-C producers, which are placed independently of each other. Hence, molecular phylogenetic analysis using ITS region sequencing can be used to determine the production pattern of biosurfactants by *Pseudozyma* species [Morita et al., 2008].

2.3.3 Screening for Biosurfactants Production

The strain was positive for drop collapse assay and haemolysis assay, which indicated that the strain could produce some surface active compounds in the culture medium. Drop collapse assay is based on the destabilization of liquid droplets in the presence of surfactants. When the drops of culture supernatant containing biosurfactants are added to the hydrophobic surface, liquid drops spread or collapse because the biosurfactant reduce the interfacial tension between the liquid drops and hydrophobic surface. On drop collapse assay, the culture supernatant of *Pseudozyma* sp. NII 08165 (C) gave collapsed droplet on parafilm just like SDS solution (B). Here distilled water was used as negative control (A) (Figure 2.3). A decline in surface tension causes the lysis of erythrocytes, which is the principle behind the haemolysis assay. On blood agar plate, a prominent clear lytic zone was observed around the growing cells (Figure 2.4). It has been reported that the production of surface active compounds such as mannosylerythritol lipids in the culture medium caused a significant

collapse of the droplet and haemolytic activity by *Ustilago maydis* [Walter et al., 2010, Hewald et al., 2005].

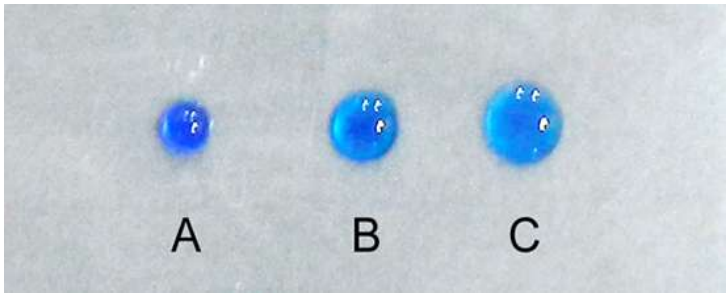

Figure 2.3 Drop collapse assay. The culture supernatant of *Pseudozyma* sp. NII 08165 (C) gave collapsed droplet on parafilm just like SDS solution (B). Here distilled water was used as negative control (A).

Figure 2.4 Haemolysis assay. Image of *Pseudozyma* sp.NII 08165 growing on blood agar plates. Clear lytic zone was observed around the colonies.

2.4 Summary and Conclusions

The aerial contaminant obtained on the lipase screening plate was identified to be a *Pseudozyma* species. The organism was observed to have spindle-shaped blastoconidia and pseudohyphae, consistent with the *Pseudozyma* genera. On the phylogenetic tree, the isolate was positioned near to *P. siamensis*, a known MEL-C producer. The isolate was determined

to be a potential biosurfactants producer based on the classical biosurfactants screening methods such as drop collapse assay and haemolysis assay.

Chapter 3: Production and Characterization of Glycolipids by *Pseudozyma* sp. NII 08165

3. 1 Introduction

Glycolipid production is an important feature of *Pseudozyma* species. Type of glycolipids produced by different *Pseudozyma* species could be different; this feature could even be used as an important taxonomic index in the identification of these yeasts [Morita et al., 2008]. Apart from the conventional MEL, some *Pseudozyma* species produce variants of MEL with distinct physicochemical properties. *P. tsukubaensis* produced a novel diastereomer of MEL-B as the predominant MEL [Fukuoka et al., 2008]. *P. parantarctica* produced a mannosylmannitol lipid – a novel glycolipid with higher hydrophobicity, when mannitol was supplemented into the culture medium [Morita et al., 2009a]. A novel isolate of *P. churashimaensis* produced a novel triacylated MEL variant from the medium containing glucose [Morita et al., 2011]. The strains that produce exclusively one of the MEL isomers have more commercial value, since it facilitates the easy purification of the biosurfactant.

Production of the MEL by certain species of *Pseudozyma* can reach above 100g/l under optimal conditions. A yield of 140 g/l MEL was reported from the fermentation using *P. antarctica* T-34 strain [Kitamoto et al., 2001]. *P. tsukubaensis* 1E5 was reported to produce largest quantities of diastereomer MEL-B (73 g/l) using olive oil and yeast extract as carbon and nitrogen source, respectively [Morita et al., 2010]. *P. hubeiensis* SY-62, a major MEL-C producer gave a maximum yield of 126 g/l MEL in fed-batch fermentation by modifying the concentration of carbon source and yeast extract [Konishi et al., 2011]. Yield and productivity of glycolipids have greatest impact on the practical applications of biosurfactants as they affect the cost of the production in the bioprocesses.

The objective of this study was to explore the production of MEL by a novel *Pseudozyma* sp. NII 08165 to understand the yield, type of MEL produced, its structure and physicochemical properties to find out their potential applications.

3.2 Materials and Methods

3.2.1 Media Preparation and Culture Conditions

Seed culture was prepared by inoculating *Pseudozyma* sp. into growth medium containing (g/l) 40 glucose, 3.0 $NaNO_3$, 0.3 $MgSO_4.7H_2O$, 0.3 KH_2PO_4, 1.0 yeast extract (pH 6.0) and incubated at 30 ^oC, 200 rpm for two days. For MEL production, the seed culture was inoculated in the production medium, which contained (g/l) 40 soybean oil (w/v), 3.0 $NaNO_3$, 0.3 $MgSO_4.7H_2O$, 0.3 KH_2PO_4, 1.0 yeast extract (pH 6.0) and incubated at 30 ^oC, 200 rpm for seven days [Konishi et al., 2007].

3.2.2 Isolation and Analysis of Glycolipids

The glycolipids were extracted from the whole cell culture broth with an equal amount of ethyl acetate. After mixing the contents well, the organic layer was separated and then ethyl acetate was evaporated by placing the flask on a water bath. The crude extract (10 mg) obtained so was dissolved in 1 ml chloroform and subjected to thin layer chromatography (TLC) on silica plates (Silica gel 60F; Merck) using chloroform/methanol/7N ammonium hydroxide (65:15:2, v/v) as solvent system [Konishi et al., 2007]. The spots were visualized by charring the plates at 110^OC by keeping in the oven after Orcinol spray (0.2% Orcinol in 20% H_2SO_4 solution).

3.2.3 HPLC Quantification of Glycolipids

The glycolipids produced were quantified by normal phase HPLC on silica gel column using a low temperature evaporative light scattering detector (ELSD). Agilent-1200 series HPLC was used with the LiChrosorb Si- 60 column (Merck, Germany). Here, a gradient solvent program of chloroform and methanol (from 100:0 to 0:100) was set at a flow rate of 1.0

ml/min [Rau et al., 2005a]. The HPLC analysis was based on the standard curve using the purified MEL, which was prepared according to method 3.2.4.

3.2.4 Purification of Glycolipids

Glycolipids were purified by silica gel column chromatography with silica gel of mesh size 200-400 nm using a gradient elution of chloroform: acetone (10:0 to 0:10, v/v) mixtures as solvent systems [Kitamoto et al., 1990a].

3.2.5 Structural Characterization of Purified Glycolipid

3.2.5.1 FTIR Analysis

The FTIR spectrum of purified glycolipid was recorded by Bruker AlphaT IR spectrophotometer. The sample was prepared by dissolving the purified MEL (10 mg) in 1 ml ethyl acetate and scanned from 500- 4000 cm^{-1}. FTIR analysis of ethyl acetate (99.8% purity) as control was also performed.

3.2.5.2 NMR Spectroscopy

For NMR spectroscopy, the 25 mg purified glycolipid was dissolved in 1 ml $CdCl_3$ and ^{1}H, and ^{13}C NMR analysis was carried out using a Bruker Avance II-500 spectrometer.

3.2.5.3 GC-MS Analysis

GC-MS analysis was performed to determine the fatty acid composition of the purified MEL. The methyl esters of fatty acid were prepared by incubating the purified MEL (10 mg) with 5% HCl- methanol at 60 $^{\circ}C$ for 5 h [Fukuoka et al., 2007b]. The fatty acid methyl esters were extracted with hexane and analyzed by GC-MS. The GC-MS detection was performed with Agilent 6890N Gas Chromatograph connected to Agilent 5973 Mass Selective Detector in the EI mode with a HP-1 ms capillary column. The oven temperature was kept at 60 $^{\circ}C$ for 2 min and programmed from 60$^{\circ}C$ to 170$^{\circ}C$ at 10$^{\circ}C$ /min, kept for 2 min, and finally raised to 300$^{\circ}C$ at 15$^{\circ}C$ /min rate.

3.2.6 Determination of Surface Tension

The surface tension of the aqueous solution of purified MEL-C was determined at different concentration by Wilhelmy plate method using Wilhelmy type automatic tensiometer (Dataphysics DCAT 21) at 25°C. Critical micelle concentration (CMC) was then determined from the break point of the surface tension versus log of bulk concentration curve.

3.3 Results and Discussion

3.3.1 Glycolipid Production

When *Pseudozyma* sp. NII 08165 was grown in the medium containing soybean oil, oil was completely consumed within a week. Microscopic observation of the isolate revealed the presence of oil-like granules within the cell, which might be due to the accumulation of triacylglycerol and glycolipids (Figure 3.1). On seventh day of fermentation, glycolipid production was analyzed by TLC using the solvent system-chloroform: methanol: 7N ammonium hydroxide (65:15:2, v/v); four prominent glycolipid spots were observed (Figure 3.2). Three spots were having R*f* value 0.72. 0.69 and 0.67, which represented MEL-A, MEL-B and MEL-C, respectively. *P. antarctica* T-34 was reported to give four anthrone positive spots in TLC solvent system of chloroform: methanol: water (65:15:2), which were designated as MEL-A (R*f* 0.77), MEL-B (R*f* 0.63), MEL-C (R*f* 0.58) and MEL-D (R*f* 0.52) [Kitamoto et al., 1990a]. The spot with lower R*f* value (0.06) might represent polar glycolipids such as cellobiose lipids. In *P. hubeiensis*, spots of polar glycolipids was shifted to lower R*f* values under alkaline TLC solvent system [chloroform/methanol/7N ammonium hydroxide (65:15:2, v/v)] than neutral TLC solvent system [chloroform/methanol/ water (65:15:2, v/v)] due to the presence of acidic dissociable groups and this kind of acidic glycolipid was speculated to be cellobiose lipids [Konishi et al., 2010]. The faint spots of glycolipids observed above the MELs spots on TLC plate might be triacylated MEL. Triacylated MEL shows higher R*f* vale than diacylated MEL [Morita et al., 2008b]. Some

trace unknown glycolipid spots were also observed below the MEL spots and on the solvent front along with neutral lipids.

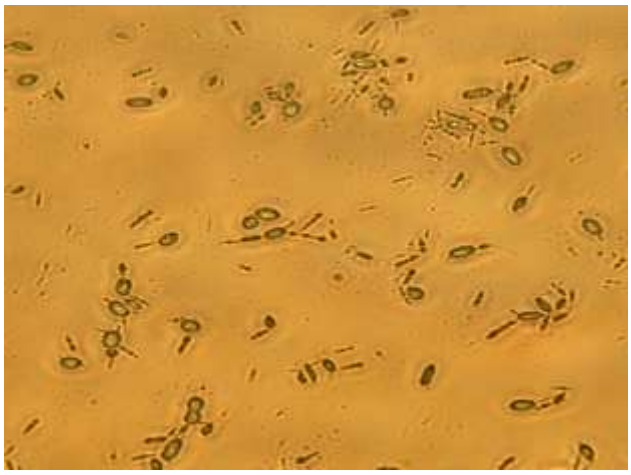

Figure 3.1 Microscopic image of *Pseudozyma* sp. NII 08165 growing on the medium containing vegetable oil. The organisms accumulate MEL along with oil granules.

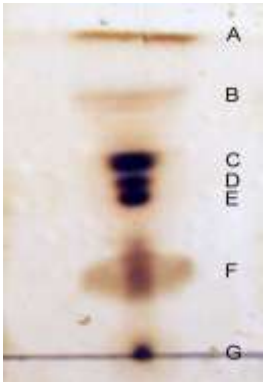

Figure 3.2 TLC picture of glycolipids. Here C, D and E represented MEL-A, MEL-B and MEL-C, respectively. Spots B and G could be triacylated MEL and cellobiose lipids, respectively. Spots of some unknown glycolipids (A and F) were also observed.

3.3.2 Quantification of Glycolipids

To quantify the glycolipids, HPLC analysis of ethyl acetate extract was performed (Figure 3.3). Since the polar column was used, peaks of neutral lipids were followed by that of polar lipids. Peaks of triglycerides and free fatty acids were observed. Separation of the main types

of MEL into four individual peaks has already been demonstrated by Rau et al., [2005a]. Along with fatty acid peak, four peaks of glycolipids were observed. The three peaks with retention time 5.5902, 6.169 and 6.489 corresponded to MEL-A, B and C, respectively. The peaks of MEL isomers were assigned based on the retention time proposed by Morita et al., [2010]. The pure MEL fraction of *P. antarctica* gave three peaks with retention times of 5.89, 6.14 and 6.49, which corresponded to MEL-A, MEL-B and MEL-C [Morita et al., 2010]. *Pseudozyma* sp. NII 08165 produced 12g/l MEL from 40 g/l (w/v) soybean oil. The major glycolipids, MEL-B and MEL-C comprised of 35.7 and 59.6% of all glycolipids. When the concentration of soybean oil was increased up to 80 g/l, the yield was 34 g/l on the ninth day of fermentation. Time course of MEL production is shown in Figure 3.4. The phylogenetically related organism, *P. siamensis* CBS 9960 produced more than 84% MEL-C with MEL-B in the absence of MEL-A. Total MEL production from *P. siamensis* CBS 9960 reached 19 g/l after incubation at 25 ^{O}C for nine days in the medium containing 40 g/l Safflower oil, 40 g/l glucose and 0.2% yeast extract [Morita et al., 2008]. Thus, it was concluded that the *Pseudozyma* sp. NII 08165 was a good producer of MEL, and the productivity could further be improved by optimizing the substrate concentration and media conditions.

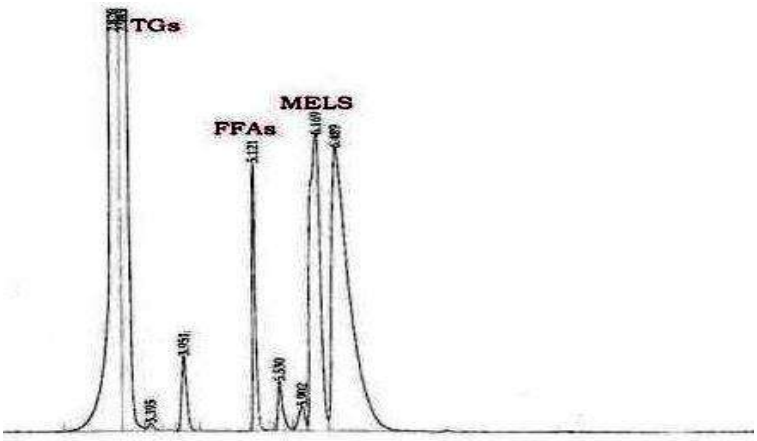

Figure 3.3 HPLC chromatogram of ethyl acetate extract

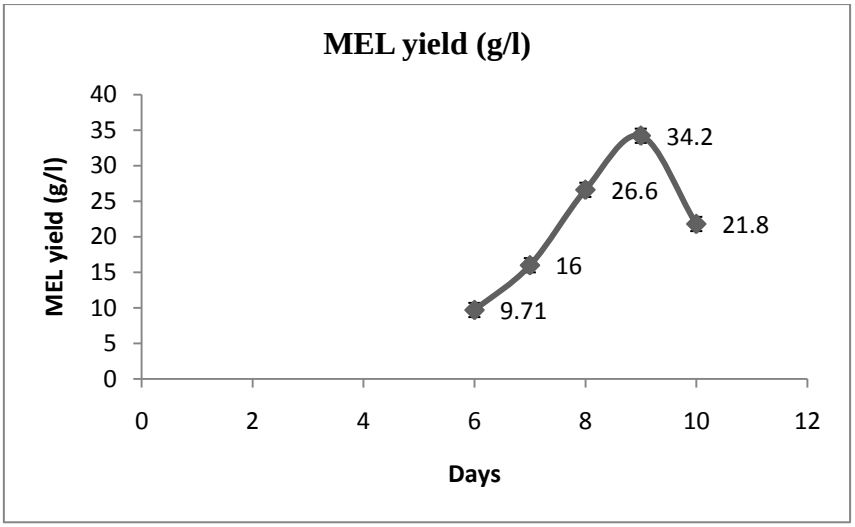

Figure 3.4 Time course of MEL production by *Pseudozyma* sp. NII 08165 in medium containing 80 g/l soybean oil.

3.3.3 Structural Characterization of Purified Glycolipid

The purified compound, which gave single spot on TLC, was subjected to structural determination in detail as described below.

3.3.3.1 FTIR Analysis

The FTIR spectrum is shown in Figure 3.5. On FTIR spectra, a characteristic absorption band appeared at 1747 cm^{-1} was assigned to the stretching vibration of the carboxyl group (-C=0). The absorption band appeared at 2925 cm^{-1} and represented the vibration of the methylene group (-CH_2) present in mannose and erythritol. The absorption band at 3438 cm^{-1} could be attributed to –OH group. A characteristic band at 1079 cm^{-1} revealed the existence of C-O-C linkage. All these absorption bands showed the functional groups of MEL.

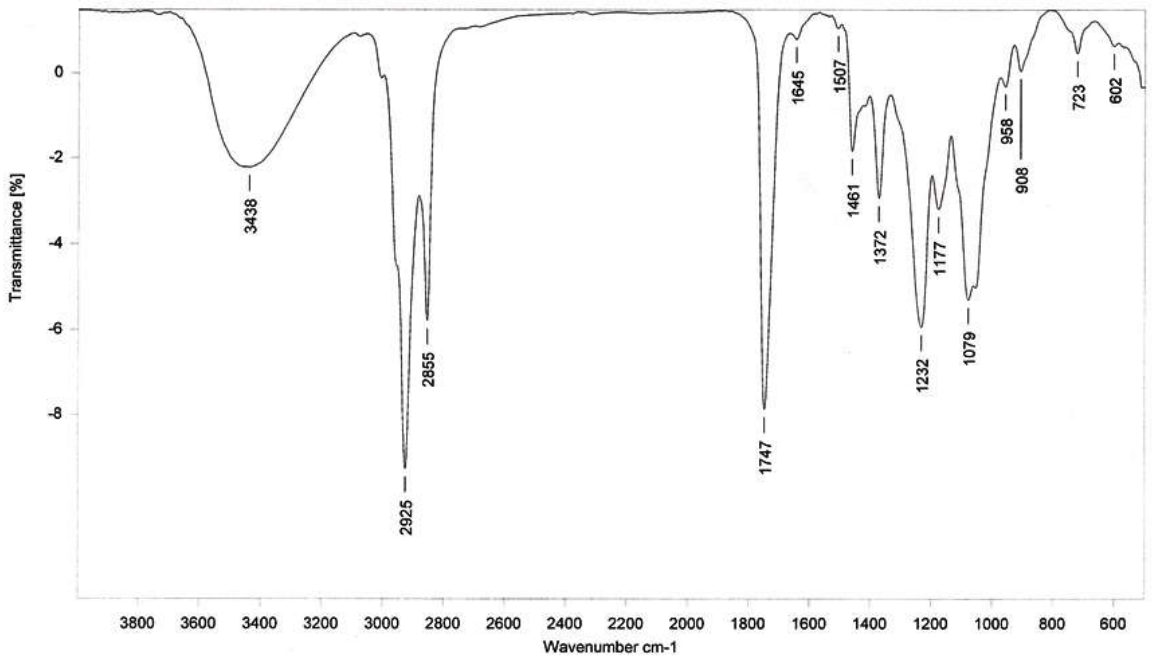

Figure 3.5 FTIR spectrum of purified MEL

3.3.3.2 NMR Spectroscopy

Based on the NMR analysis, the purified compound was confirmed to be 4-O-[4′-O-acetyl-2′, 3′- di-O-alka (e) noil-β-D-mannopyranosyl]-D-erythritol, known as MEL-C (Figure 3.6). The chemical shifts of the compound are summarized in Table 3.1. The chemical shift of the compound matched with those of the previously reported MEL-C of *P. siamensis* [Morita et al., 2008]. On proton NMR spectra, a peak at 2.03 ppm indicated the presence of an acetyl group (-CH3) at C-4' position. A broad peak at 0.88 ppm represented the fatty acid group at C-3' position. Based on a sharp peak at 2.17 ppm and triplet peak at 0.982 ppm, the present MEL-C was determined to have a C2 or C4 acids at the C-2' position of the mannose moiety. In the ^{13}C-NMR spectrum, the peak at 170.2 ppm was assigned to a carbonyl groups (-C=0) bound to C-4′ position on the mannose sugar.

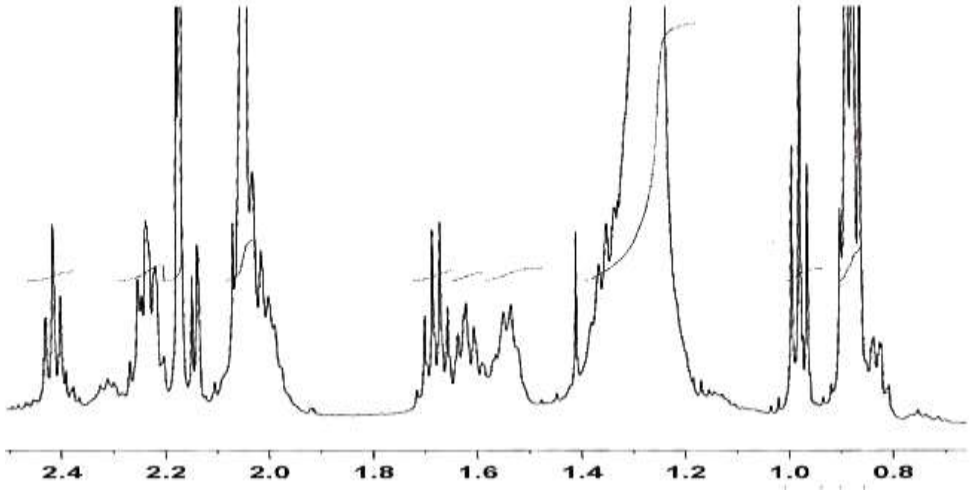

Figure 3.6 ^{1}H NMR spectrum of purified MEL-C

Table 3.1 NMR data for MEL-C (chloroform-*d*, 500 MHz)

Functional group	**^{13}C-NMR $^{\delta}$ (ppm)**	**Functional group**	**^{1}H-NMR $^{\delta}$(ppm)**
D-Mannose			
C-1'	99.05	H-1'	4.79 s
C-2'	69.03	H-2'	5.50 dd
C-3'	71.03	H-3'	5.16 t
C-4'	66.22	H-4'	5.11 dd
C-5'	74.88	H-5'	3.56 m
C-6'	60.43	H-6'	3.6-3.7 m
meso-Erythritol			
C-1	63.40	H-1	3.6-3.7 m
C-2	71.00	H-2	3.6-3.7 m
C-3	72.1	H-3	3.6-3.7 m
C-4	71.89	H-4a	3.77 dd
		H-4b	4.03 dd

Acetyl groups		
-C=0 (C-2')	170.20	
(C-4')	170.81	
-CH_3 (C-2')	20.73	2.05
(C-4')	20.84	2.13
Acyl groups		
-C=0 (C-2')	173.43	
(C-3')	171.22	
-CO-CH_2-(C-2')	35.97	2.41m
(C-3')	33.83	2.23 m
CO-CH_2-CH_2- (C-2')	18.54	1.68 m
(C-3')	24.7-25.6	1.53-1.62 m
$(CH_2)_n$	22.5-31.9	1.25 b
-CH=CH-	127.5-130.5	5.3 m
-CH=CH-CH_2 –CH=CH	26.42	2.75 t
-C=CH-CH_2-	26-27	2.05 b
-CH_3 (C-2')	13.58	0.98 t
(C-3')	14.12	0.86-0.89 m

3.3.3.3 GC-MS Analysis

The fatty acid composition of MEL- C was analyzed by the GC-MS (Table 3.2). It was found that MEL-C mainly comprised of long chain fatty acids, particularly C-14 to C-18 acids (Figure 3.7). The major fatty acid of MEL produced from the soybean oil by *Pseudozyma* sp. NII 08165 was C16 fatty acid (60%). Other than that, MEL-C contained 30% C14 fatty acids, 9% C18 and 0.5% unknown fatty acids. MEL-C from *P. antarctica* contained 23% C8, 57% C10, 10% C12, 6% C14 and 4% unknown fatty acids. MEL-C from *P. shanxiensis* contained 20% C14, >70% C16 and 5% C18, while 40% C14, 51% C16 and 8% C18 made up the fatty acids of MEL-C from *P. siamensis*. The above fatty acid composition was when all the species were cultivated in soybean oil [Fukuoka et al., 2007a; Morita et al., 2008]. This revealed that hydrophobic part of the MEL-C from *Pseudozyma* sp. NII 08165 was different from that of conventional MEL-C, which contained two medium chain fatty acids at mannose moiety and was more similar to that of MEL-C from the phylogenetically closer species *P. siamensis*.

The NMR analysis and fatty acid profiling revealed that MEL-C from *Pseudozyma* sp. NII 08165 contained a unique hydrophobic structure proposed by Morita et al., [2008], as shown in Figure 3.8. The MEL-C produced by NII 08165 had a unique hydrophobic structure similar to *P. siamensis* and *P. shanxiensis* in having a shorter chain like C2 or C4 at the C-2' position and a long chain like C14, C16 or C18 at the C-3' position of the mannose moiety [Fukuoka et al., 2007a; Morita et al., 2008]. On the other hand, conventional MEL have a medium chain acid like C8 to C14 at the C-2' and C-3' positions. This unique hydrophobic structure of MEL-C contributes to higher water solubility and hydrophilicity, the properties that make them highly important in the industry

Table 3.2 Fatty acid composition of MEL-C from *Pseudozyma* sp. NII08165

Fatty acid	Wt (%)
14:0	8.1
14:1	12.7
14:2	8.7
16:0	35.7
16:1	18.9
16:2	6.1
18:0	2.5
18:1	1.9
18:2	3.1
18:3	1.8
Unknown	0.5

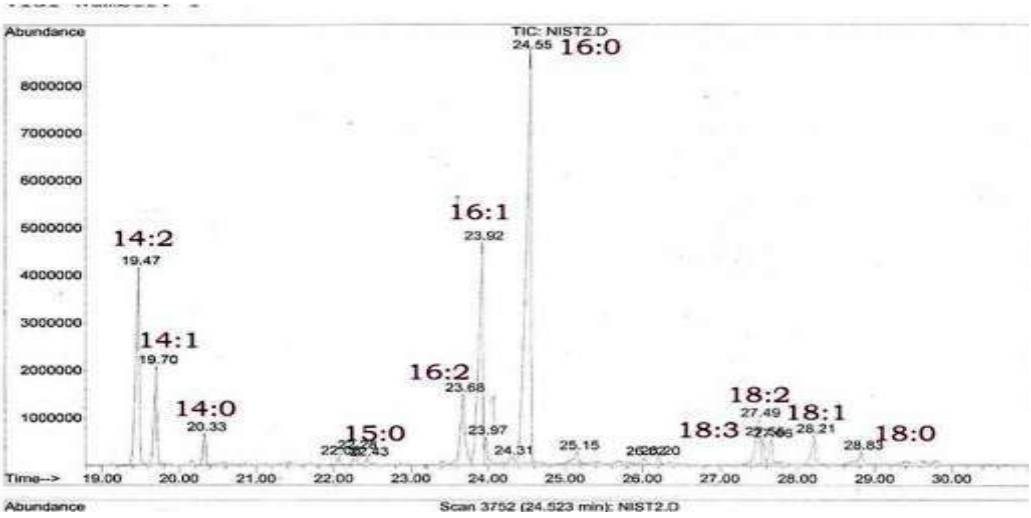

Figure 3.7 GC-MS profile for fatty acid composition of MEL-C

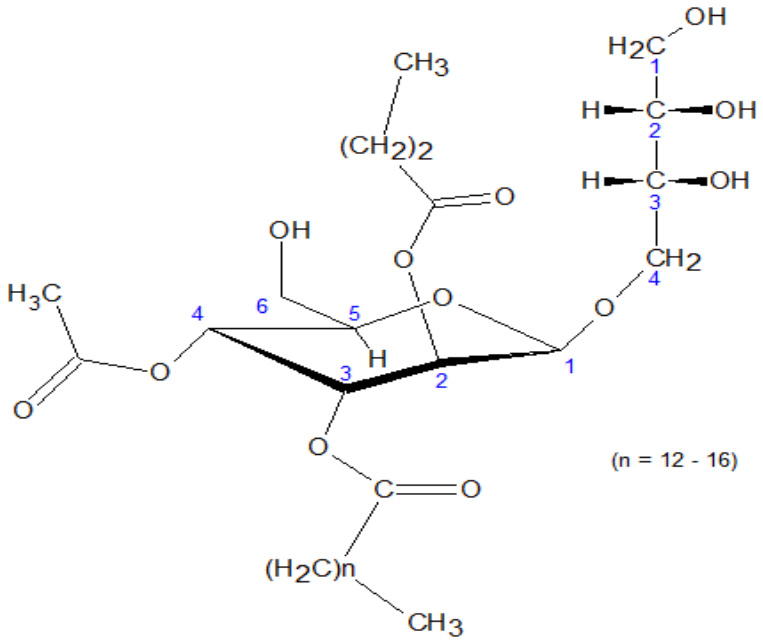

Figure 3.8 The chemical structure of MEL-C from *P. siamensis*. The MEL-C was having a unique hydrophobic structure of short chain fatty acid at the C-2' position and long chain fatty acid at the C-3' position, different from the conventional MEL-C, which mainly possesses two medium chain fatty acids.

3.3.6 Determination of Surface Tension

Surface tension of the production medium dropped drastically from 59.4 mN/m to 30.9 mN/m during the fermentation due to the production of biosurfactants (Figure 3.9). The surface activity of MEL-C produced by *Pseudozyma* sp. NII 08165 was studied by Wilhelmy method (Figure 3.10). The surfactant concentration at which micelle formation begins is known as critical micelle concentration. The CMC of MEL-C was 4.5x 10^{-6} M and the surface tension at the CMC of the MEL was 33mN/m.The purified MEL had good surface activity with the foaming property. This observation was similar to that of MEL-C from *P siamensis* that reported having CMC value of 4.5X10^{-6} with γCMC 30.7 mN/m [Morita et al., 2008]. MEL-A and B from *P. antarctica* were reported to have CMC value of 2.7 x 10^{-6} and 4.5 x 10^{-6} with γCMC of 28.4 mN/m and 28.2 mN/m, respectively [Kitamoto et al., 1993]. CMC of MEL-C was higher than that of MEL-A and MEL-B due to its hydrophilicity. The applicability of MEL varies with the type of MEL. The most hydrophobic MEL, MEL-A was reported to be biologically active and could be used in biomedical and therapeutic applications, while the least hydrophobic MEL, MEL-C could be used in oil-in-water type emulsifier and detergent formulation [Morita et al., 2008].

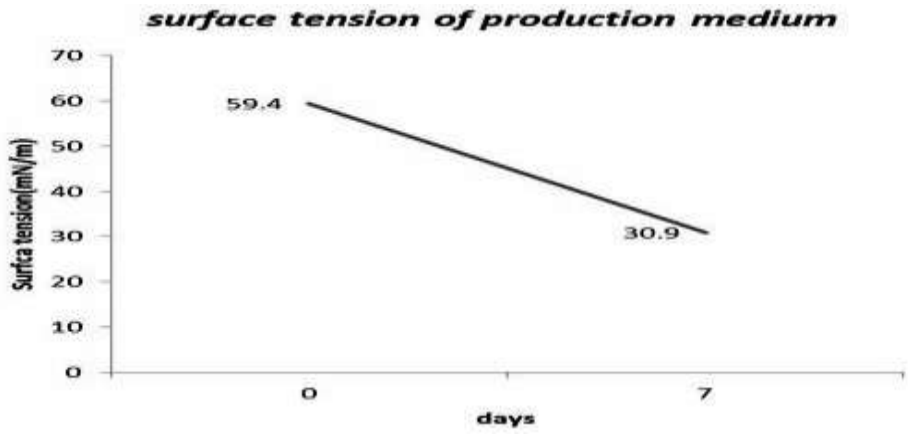

Figure 3.9 Measurement of surface tension of production medium

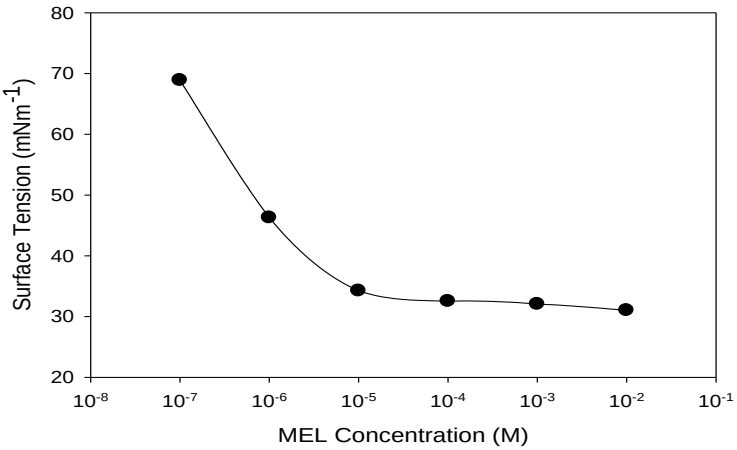

Figure 3.10 CMC determination of MEL-C by surface tension versus concentration plot.

3.4 Summary and Conclusions

Pseudozyma sp. NII 08165 could be considered as potential source of glycolipid biosurfactants as it produced a mixture of MEL A, B and C and some unknown glycolipids when grown on vegetable oil as carbon source. The total production of MEL was 12 g/l and MEL-C was produced in highest quantity than all other MEL. The yield of MEL was increased up to 34 g/l when the concentration of vegetable oil was enhanced. The structure elucidated of MEL-C showed it to have a unique hydrophobic structure as reported for *P. siamensis*. MEL-C exhibited good surface activity with CMC 4.5x 10^{-6} M, and its γCMC was 33 mN/m.

Chapter 4: Production and Structural Characterization of Exopolysaccharide by *Pseudozyma* sp. NII 08165

4.1 Introduction

Exopolysaccharides (EPS) have been recognized as high-value macromolecules due to the peculiarity, functionality and scope of industrial applications. Microbial EPS have several advantages over the plant and animal gums (i.e., polysaccharides), including high productivity and ease of product recovery. Also, atypical molecular structure and conformation conferred them unique, unusual properties with potential industrial applications. Concerning microbial EPS, though there are large number of studies on fungal exopolysaccharide, the information on exopolysaccharide produced by yeasts is still scarce, which arise the need for screening yeast for novel exopolysaccharide [Mahapatra & Banerjee, 2013].

Candida spp., *Pichia* spp., *Aureobasidium pullulans*, *Cryptococcus* spp., *Rhodotorula* spp., *Sporobolomyces* spp. are the major yeast or yeast-like fungi producing exopolysaccharide. There is a large diversity in yeast exopolysaccharide among the same species. For the same species, the strain used, medium composition and culture condition could affect the EPS production, its structure and conformation. Mannans, glucans, phosphomannans, galactomannans, glucomannans and glucuronoxylomannans are the major type of EPS produced by the yeast or yeast-like fungi. Most of EPS possess biological activity, which is determined by the linkage pattern between the sugar monomers and consequent conformation of the EPS. As some of the yeasts are structurally very similar to cell wall polysaccharides, hence it has been speculated that these could be synthesized by the same mechanisms [Van Bogaert et al., 2009].

Pseudozyma isolate NII 0865, used in the present work, produced mucous compound when it was grown on glucose-containing medium. Preliminary investigation by phenol-sulfuric acid assay revealed that it was of polysaccharide in nature (Dubois et al., 1956). Detailed structural and physicochemical characterization was carried out to understand the potential industrial applicability of the EPS secreted by *Pseudozyma* isolate. To the best of our knowledge, this has been the first study on the structural and physicochemical characterization of EPS by a *Pseudozyma* species. There was no report on the production of EPS from related species or genera of *Pseudozyma*, and the EPS produced by the *Pseudozyma* isolate exhibited a different production pattern and primary structure from the other reported yeast strains. Hence, *Pseudozyma* EPS seemed to be a novel biopolymer.

4.2 Materials and Methods

4.2.1 Media Preparation and Culture Conditions

Basal medium containing (g/l) 40 (w/w) glucose, 3.0 $NaNO_3$, 0.3 g/l $MgSO_4.7H_2O$, 0.3 KH_2PO_4, 1.0 yeast extract (pH 6.0) was used for the production of EPS [Konishi et al., 2010]. The medium was autoclaved at 121 ^oC for 15 min; after cooling inoculated with seed culture of *Pseudozyma* (4%, v/v) and incubated at 30 ^oC and 200 rpm agitation for four days.

4.2.2 Production of Exopolysaccharide by **Pseudozyma**

Cell growth, EPS yield and viscosity of the culture broth were studied at regular time intervals. Cell growth was determined by taking an aliquot of the medium (10 ml), centrifuging it at 12,000 rpm at 4 ^oC for 20 mins and drying the cell pellet at 80 ^oC for 24 h. To determine the EPS yield, EPS from cell-free culture broth (obtained as above after centrifugation) was precipitated by mixing the supernatant with cold acetone (4 ^oC, 1:2, v/v) for 4 h. The precipitate (EPS) was centrifuged at 12,000 rpm at 4 ^oC for 20 mins and lyophilized (SCAN VAC, Cool Safe 110-4 PRO), and then dry weight was determined. The

apparent viscosity of the cell-free culture broth was measured by visco-rheometer (Rheolab MC1, Model- 749558, Physica).

4.2.3 Isolation of EPS

The culture medium was centrifuged at 12,000 rpm for 20 mins to remove the cells. Two volumes of pre-chilled acetone was added to the supernatant and kept at 4 °C for 4 h for the precipitation of EPS. The precipitate was centrifuged at 12,000 rpm, at 4 ^{o}C for 20 mins. EPS pellet was dissolved in water and was dialyzed against two changes of Milli-Q water. The protein and nucleic acid contents of the EPS were analysed by Bradford assay [Bradford, 1976], and UV spectrophotometry (Nanodrop ND-1000), respectively.

4.2.4 FTIR Analysis

The functional groups of *Pseudozyma* EPS were recorded by Fourier Transform Infrared (FTIR) spectroscopy using Bruker AlphaT IR spectrophotometer. The sample was prepared by grinding EPS (10 mg) with a spatula full of KBr pellets using mortar and pestle and was scanned from 500- 4000 cm^{-1}.

4.2.5 Sugar Analysis

To determine the monosaccharide composition of *Pseudozyma* EPS, 5.0 mg EPS was first hydrolyzed with 2 M H_2SO_4 at 100 °C for three hours. The hydrolysate was then neutralized with calcium carbonate powder, filtered through 0.22 μm syringe filter and the sugar content was determined by HPLC (Shimadzu) using an RI detector on Aminex HPX-87P carbohydrate analysis column (Biorad). The mobile phase was deionised water with a flow rate of 0.6 ml/min. Peak identification was based on the retention times of standard sugars (Glucose, galactose, mannose, xylose, arabinose, N-acetyl-D-glucosamine). The sugars used were HPLC grade (Sigma Aldrich).

4.2.6 Molecular Weight Estimation

Gel permeation chromatography was performed to determine the molecular weight of *Pseudozyma* EPS. EPS sample (10mg/ml) was loaded into gel permeation column of Sepharose 6B (Sigma-Aldrich, USA), and elution of EPS was monitored by phenol- sulphuric acid [Dubois et al., 1956]. The molecular weight was estimated from the standard graph that was plotted using standard dextrans (Sigma-Aldrich, USA).

4.2.7 NMR Analysis

For NMR spectroscopy, the purified EPS was dissolved in D_20 (Merck, India) at a concentration of 1.0 mg/ml and ^{1}H and ^{13}C NMR analysis was carried out using a Bruker Avance II-500 spectrom

4.2.8 Conformation Studies of **Pseudozyma** *EPS by Congo Red Assay*

The conformational structure of *Pseudozyma* EPS solution was determined by measuring the change in the absorption maximum (λ_{max}) of the dye Congo Red in the presence and absence of polysaccharide preparations. *Pseudozyma* EPS solution were prepared at 0.1 % with different NaOH concentrations from 0 to 0.4 M. Congo Red (91 μM) was added to these solutions. The absorption spectra were recorded from 400 to 600 nm at 25 °C with a UV-160A (Shimadzu) spectrophotometer. Xanthan gum was taken as a positive control. As a negative control, solutions of pure dye were used at the same NaOH concentrations [Velasco et al., 2009].

4.2.9 Scanning Electron Microscopic Analysis

Scanning electron microscopy (SEM) was done to study the surface morphology of *Pseudozyma* EPS. EPS solution (1.0 mg/ml) was added to aluminium stubs and dried. The sample was gold sputtered using SC7620 Sputtercoater device and analyzed by Scanning electron microscopy at an operating voltage of 30kV (Zeiss Evo-18 Special Edition).

4.2.10 Atomic Force Microscopy

Aqueous EPS solution (1.0 mg/ml) was added to freshly cleaved mica surface and kept for air-drying overnight. Atomic force microscopic images were recorded under ambient conditions using a NTEGRA system (NT-MDT) operating with a tapping mode regime. Micro-fabricated TiN cantilever tips (NSG10) with a resonance frequency of 299 kHz and a spring constant of 20-80 Nm^{-1} were used.

4.3 Results and Discussion

4.3.1 Production of Exopolysaccharide by Pseudozyma sp. NII 08165

EPS production was compared in two media containing glucose and sucrose as reported by Konishi et al., [2007] and Poli et al., [2010], respectively. Medium used by Poli et al., [2010] contained (g/l) 50 sucrose, 25 $(NH_4)_2SO_4$, 10 KH_2PO_4, 5.0 $MgSO_4 \cdot 7H_2O$, 1.0 NaCl, 1.0 $CaCl_2 \cdot 2H_2O$ and 10 yeast extract (pH 5.3) and generally used for cultivating the yeast species for the production of EPS. When *Pseuodzyma* sp. NII 08165 was grown in sucrose medium, EPS production was much lower than that of glucose containing medium (> 1.0 g/l; results not shown). Thus, glucose was used as the carbon source in further studies. Then EPS production was evaluated using different concentrations of glucose (10-50 g/l). EPS yield increased with increase in the concentration of glucose, and the maximum production of EPS (3.5 g/l) was achieved with 4% glucose concentration.

The cell growth, EPS yield and viscosity of the culture broth were monitored for a period of six days. EPS production reached the maximum on the fourth day of incubation (3.5 g/l); thereafter it decreased. The viscosity data followed the similar pattern with maximum on the fourth day (63 mPa·s) and then declined, showing that the apparent viscosity of the culture broth was directly correlated with EPS production indicating that EPS was the single most important contributor to the viscosity of the culture medium. Results of cell growth, however, showed a different pattern, with increase till fifth day and then decline (Figure 4.1).

Pavlova et al., [2011] reported that EPS yield from *Cryptococcus laurentii*, cultivated in sucrose containing medium was 6.4 g/l on the fourth day of fermentation and pH drop was observed during the course of EPS production, while *Cryptococcus flavus* produced 5.75 g/l EPS [Pavlova et al., 2009]. In the same culture medium, Antarctic yeast isolate, *Sporobolomyces salmonicolor* synthesized 5.6 g/l EPS with the decrease in pH of the culture medium from the initial pH 5.2 to 2.0 [Pavlova et al., 2004]. *Rhodotorula glutinis* produced a novel exopolysaccharide at a concentration of 4 g/l from glucose containing medium under acidic pH range [Cho et al., 2001]. Crude exopolysaccharides at a concentration of 6.2 g/l were obtained by the batch fermentation of *Rhodotorula acheniorum* in sucrose containing medium [Pavlova et al., 2005].

There was no decline in the pH of the culture medium during the course of fermentation by *Pseudozyma*. From this, it could be concluded that EPS biosynthesis by *Pseudozyma* sp. NII 08165 was a unique bioprocess as EPS production was usually accompanied by the decrease in pH value as reported by other yeast strains.

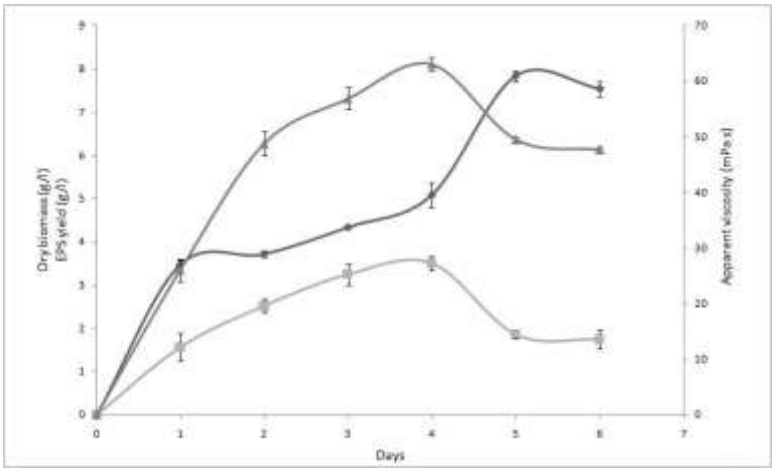

Figure 4.1 Time course study of the growth and EPS production by *Pseudozyma* sp. NII 08165. EPS synthesis (g/l; closed squares), biomass (dry weight, g/l; closed rhombus) and apparent viscosity (mPa s; closed triangles) of *Pseudozyma* sp. NII 08165 in batch culture.

4.3.2 Isolation of EPS and Structural Characterization

The EPS isolated by acetone precipitation was relatively pure and had only negligible amount of protein and nucleic acid.

4.3.3 FTIR Analysis

The FTIR spectrum of *Pseudozyma* EPS was analyzed, and absorption bands were assigned to understand the typical polymeric structure of the carbohydrate (Figure 4.2). A broad stretching in the region 3423 cm^{-1} was observed which represented the stretching vibration of the hydroxyl groups of carbohydrate. This is the characteristic absorption band of carbohydrate ring and is responsible for the water solubility of EPS [Lim et al., 2005, Karbowiak et al., 2011]. The absorption bands at 2922 and 2853 cm^{-1} represented the C-H stretching of methyl and methylene groups [Wang et al., 2010]. The absorption band found in the region 1650- 1540 cm^{-1} usually represents the stretching vibrations of enol and amide groups [Singh et al., 2011; Allen et al., 2004]. The stretching of C=O group was indicated by an absorption band at 1628 cm^{-1}. Similarly, the peaks at 1415 to 1382 cm^{-1} could be assigned to >C=O stretch of the COO^{-}groups and C–O bond from COO^{-}groups [Wang et al., 2010; Haxaire et al., 2002]. A sharp absorption band at 1044 cm^{-1} represents the –C-O stretching vibration [Haxaire et al., 2002]. The wave number region from 1200 to 800 cm^{-1} is the fingerprint region and can be used to characterise different polysaccharides [Copikova et al., 2006, Cern et al., 2003]. The monosaccharide constituents of pectic and hemicellulosic polysaccharides like galactose, mannose and glucose shows the strongest IR bands at 1078 cm^{-1}, 1070 cm^{-1} and 1035 cm^{-1} respectively [Kacurakova et al., 2000]. The absorption bands in the region 1000-1200 cm^{-1} suggested the presence of sugar monomers in the *Pseudozyma* EPS. Siddique et al., [2014] suggested that peaks at 1154 cm^{-1}, 1014 cm^{-1}, 928.90 cm^{-1} represented covalent vibration of glycosidic bridge, chain flexibility around α - (1-6) glycosidic linkage and presence of branched α - (1-3) glycosidic bond, respectively. Peaks at

982-1044 cm^{-1} showed the presence of α -glycosidic linkages in *Pseudozyma* EPS. The absorption bands observed at 734-796 cm^{-1} could be attributed to C-O-C bending vibration [Sekkal & Legrand, 1993].

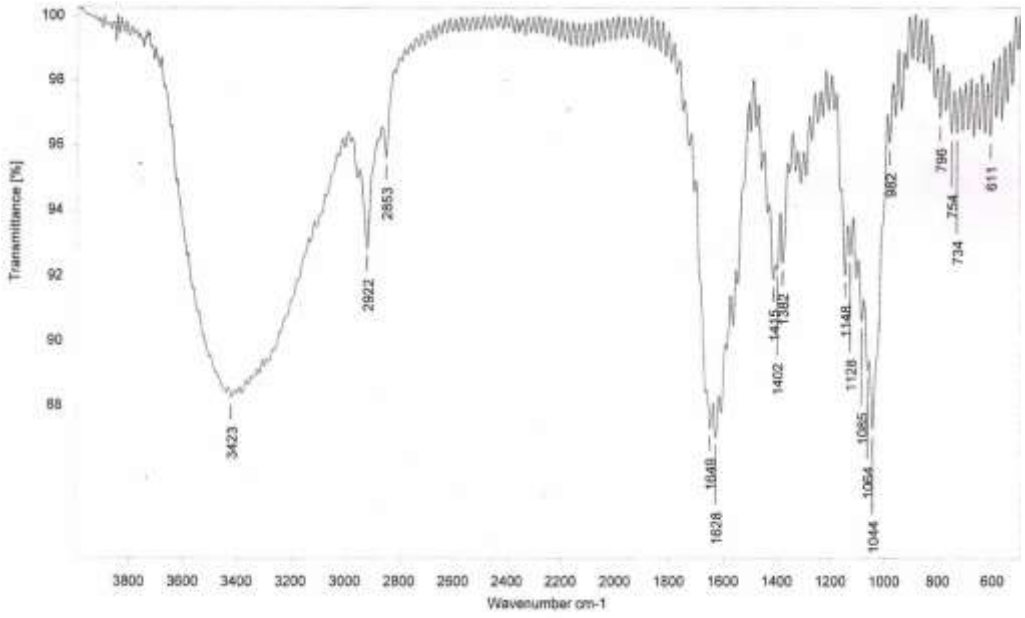

Figure 4.2 FTIR spectrum of *Pseudozyma* EPS showing the typical polymer structure and functional groups of EPS.

4.3.4 Sugar Analysis

Sugar analysis revealed that *Pseudozyma* EPS was a heteropolysaccharide of glucose, galactose and mannose (Figure 4.3). The relative proportion of sugars determined in the EPS was 2.4: 5.0: 2.6 (glucose: galactose: mannose). The quantity and quality of the EPS produced by the microbes are dependent on the carbon source and culture conditions [Laws & Marshall, 2001]. A strain of *Sporobolomyces salmonicolor* produced glucogalacto mannan in the medium containing sucrose as carbon source [Poli et al., 2010]. The EPS of *Cryptococcus flavus* contained mannose, glucose, xylose and galactose [Pavlova et al., 2009]. *Rhodotorula acheniorum* produced two fractions of EPS, which were of mannan while the EPS of *Rhodotorula glutinis* contained neutral sugars and uronic acids [Pavlova et al., 2005, Cho et al., 2001]. The difference in the sugars and their relative proportions contributes to the

unique structures of EPS and their varying properties.

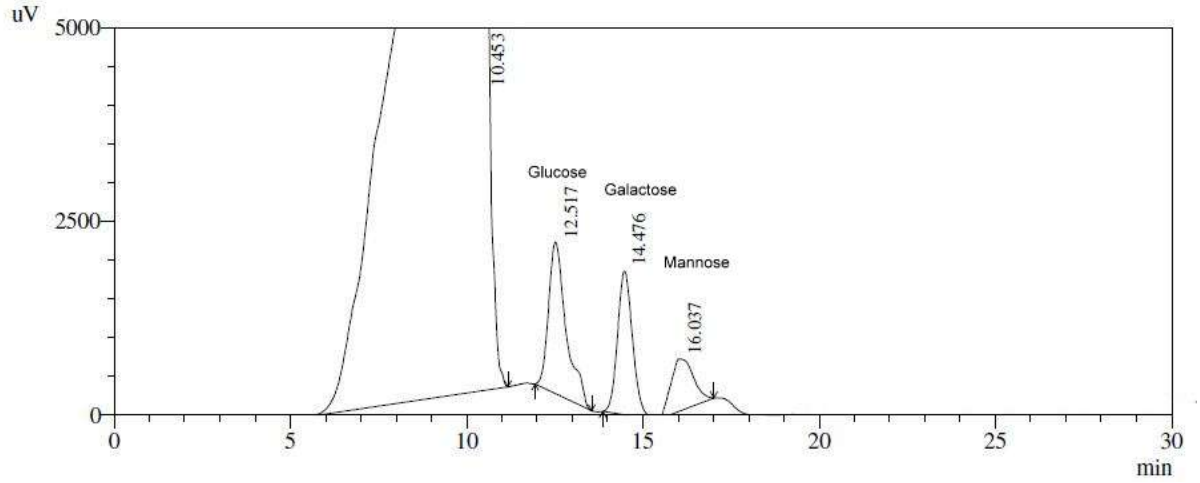

Figure 4.3 HPLC analysis of acid hydrolyzed *Pseudozyma* EPS showing the sugar monomers -glucose, galactose and mannose.

4.3.5 Molecular Weight Estimation

Pseudozyma EPS was determined to have a molecular weight of 1.7 MDa. Generally, yeast EPS are high molecular weight polymers. Exopolysaccharide from a yeast, *Sporobolomyces salmonicolor* was reported to have a molecular weight of >1 MDa, while *Cryptococcus flavus* produced EPS with molecular weight of 1.01 MDa [Poli et al., 2010; Pavlova et al., 2009]. The high molecular weight of *Pseudozyma* EPS could be the reason for its high viscosity and ready-to-precipitate nature in acetone. The high viscosity of *Pseudozyma* EPS can be exploited in various industrial applications and its ready-to-precipitate nature in acetone gives good product recovery thereby making downstream processing easy and efficient.

4.3.6 NMR Analysis

^{1}H NMR analysis of *Pseudozyma* EPS was first performed at 25 ^{o}C. Since no peaks were resolved (results not shown), the analysis was repeated at an elevated temperature such as 50 and 90 ^{o}C. For that, the probe was heated to the respective temperatures and NMR analysis was performed. A better spectrum was resolved when the temperature was 50 ^{o} C (Figure 4.4). At 90 ^{o}C, spectrum was observed as if sample got disintegrated. However, reanalysis of

the same sample at 50 ^{o}C resulted in a better spectrum. NMR peaks were assigned by comparing with the published data in carbohydrate research database (www.glyco.ac.ru).

Figure 4.4 NMR spectrum at 50 ^{o} C (bottom) and 90 ^{o} C (top). A better spectrum was resolved at 50 ^{o} C.

^{1}H NMR spectrum of *Pseudozyma* EPS is shown in Figure 4.5. There are three major regions in ^{1}H NMR spectrum of the carbohydrate polymers: the alkyl region (1.2–2.3 ppm), the ring proton region (3.1–4.5 ppm) and the anomeric region (4.5–5.5 ppm) [Ismail et al., 2010]. Multiple signals at 1.3 ppm represent the H_6 of rhamnose. The peak at 2.063 ppm indicated the presence of N-acetyl group of a β form of N-acetyl-D-glucosamine (β-GlcNAc). Liu et al., [2011] reported that β-GlcNAc and α-GlcNAc gave peaks at 2.06 and 2.18 ppm, respectively on ^{1}H NMR. A sharp solvent peak for acetone was observed at 2.177 ppm. Ring proton region indicates the presence ring protons of sugars, i.e., protons attached to C_2-C_6 of sugars (H_2-H_6) and highlights the presence of pyranose ring. Since ring proton region was overcrowded, it was difficult to assign each and every peak in that area. The anomeric region corresponds to the anomeric protons of the sugars and is the signature area, which is used to differentiate sugar monomers in the carbohydrate structure and the type of glycosidic linkage. The region resonating from 4.5 to 4.8 ppm was α- anomeric region and region from 4.8 to 5.5 ppm was β-anomeric region. There was a broad water peak at 4.69 ppm that was masking a part of the β-anomeric region. A procedure in NMR, called presaturation, attempted to

eliminate the strong water signals in proton NMR failed to due to the high water content of the sample. This could be due to the high hygroscopic nature of *Pseudozyma* EPS. Hence, it was difficult to interpret the presence of all β-anomers from the current proton NMR spectrum. The peak at 4.505 ppm could be assigned to β anomeric proton of the N-acetyl-β-D-glucosamine. Signals 5.216, 5.331, 5.339 and 5.42 ppm corresponded to the α -anomeric protons of the glucose, rhamnose, galactose and mannose (www.glyco.ac.ru). ^{1}H NMR spectrum revealed the presence of α–D-glucopyranose, α-L-rhamnopyranose, α-D-galactopyranose and α-D-mannopyranose in the *Pseudozyma* EPS. The presence of N-acetyl-β-D- glucopyranosamine in the polymer structure was also noted.

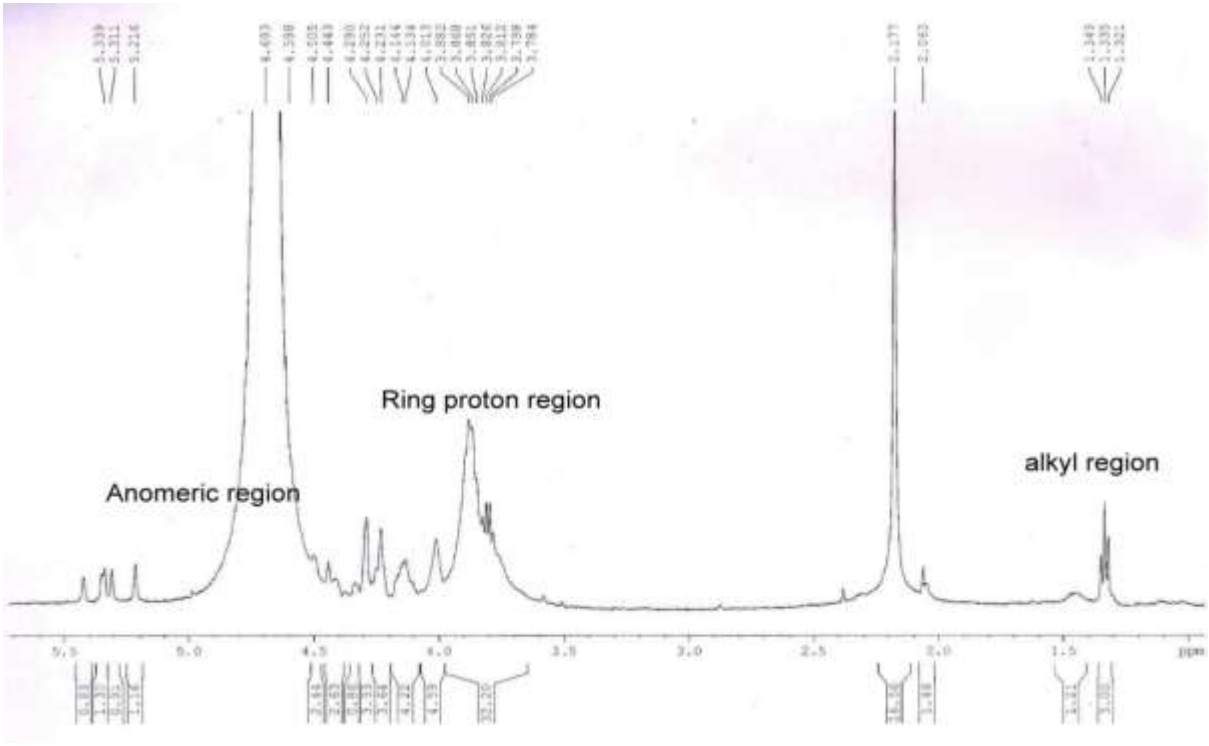

Figure 4.5 ^{1}H NMR spectrum of *Pseudozyma* EPS

Subsequently, ^{13}C NMR was attempted, but no peaks were resolved, even though longer running time and high scanning range were also given (results not shown). A major challenge with *Pseudozyma* EPS is its high viscous nature even at slightly higher concentration.

Further details on the linkage and conformation of the tentative structure needed two-dimensional NMR data, which was not possible in the current scenario.

NMR analysis revealed that emulsion stabilizing polysaccharide from the yeast, *Rhodococcus* strain 33 consisted of rhamnose, galactose, glucose, and glucuronic acid as monomers [Neu et al., 1992]. EPS from *Candida albicans* contained α- and β-D-glucose, α-D-mannose and α-L-rhamnose, which were detected by the peaks at 4.944, 4.995 5.017, 5.041 and 5.196 ppm on ^{1}H NMR. Also, a signal at 2.119 ppm was assigned to β-D-N-acetyl glucosamine [Lal et al., 2009].

4.3.7 Preliminary Conformation Studies of **Pseudozyma** *EPS by Congo Red Assay*

Congo Red assay is a rapid method for detecting the helical conformation in the polysaccharides. It has been reported that polysaccharides with a helical conformation form a complex with Congo Red in dilute alkaline solution while polysaccharides with random coil conformation do not form complexes with Congo Red. The principle of this assay is the formation of polysaccharide-Congo Red complex, which results in the shift of maximum absorption wavelength (λ_{max}) of Congo Red [Velasco et al., 2009].

Changes in λ_{max} of the dye Congo Red in the combination of *Pseudozyma* EPS at different concentrations of NaOH were measured (Figure 4.6). Xanthan gum was taken as a positive control as it was reported to have a five-fold helical structure. At low concentration of NaOH, λ_{max} of Congo Red-EPS solution shifted to longer wavelength (522 nm). Thereafter, λ_{max} dropped gradually reaching the same value as of the control. A similar observation was noted in the case of xanthan gum also. Hydrogen bonds are relevant for the dye-fiber interaction, and λ_{max} displacement is caused by the order-disorder transition that might be due to the breakage of hydrogen bonds. Hence, it could be concluded that *Pseudozyma* EPS adopted an ordered hydrogen bond dependent helical conformation in neutral and slightly alkaline

aqueous solutions. Under strong alkaline condition (> 0.2 M NaOH), the tertiary structure was denatured into random coil conformation.

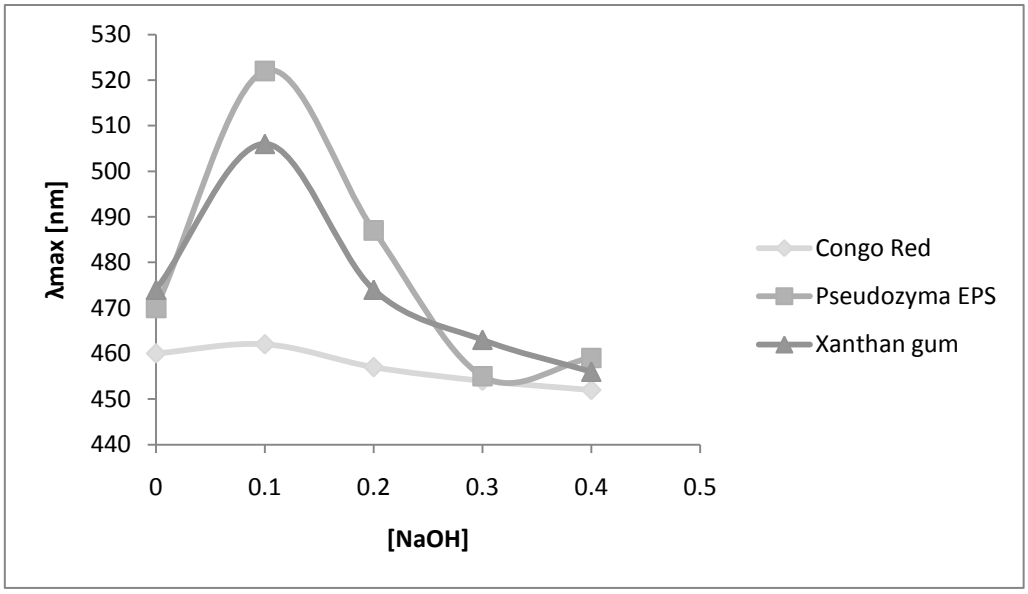

Figure 4.6 The shift in λ_{max} of Congo Red-*Pseudozyma* EPS complex, Congo Red-xanthan gum complex at different NaOH concentrations

Velasco et al., [2009] reported that the tertiary structure of *β*-glucan from *Pediococcus parvulus* was an ordered hydrogen bond dependent helical conformation in neutral and slightly alkaline solution, which got denatured at high concentration of NaOH. A triple helical conformation of immunostimulating polysaccharides from the mushroom, *Lentinus edodes* was studied by helix-coil transitional analysis using Congo Red assay [Lee et al., 2009]. EPS produced by *Botryosphaeria rhodina* exhibited a triple helix conformation, characterized by Congo Red assay [Vasconcelos et al., 2008].

4.3.8 Scanning Electron Microscopic Analysis

The scanning electron micrograph of the *Pseudozyma* EPS is given in Figure 4.7. The *Pseudozyma* EPS formed a film-like structure with smooth and rigid surface. Upon 400X magnification, the EPS appeared to have a compact roof tile like structure. The EPS did not possess any porous nature. All these properties projected it as a good candidate for preparing

the plasticized films. The EPS from *Lactobacillus plantarum* was observed to have a compact structure and was proposed suitable for making plasticized films [Wang et al., 2010]. In a study by Ahmed et al., [2013] SEM scan showed the integral surface structure of EPS from *L. kefiranofaciens,* which was an important feature required for plasticized filmmaking. Exopolysaccharides are one of the promising polymers for manufacturing the bioplastics as it makes the process environmentally-friendly and safe.

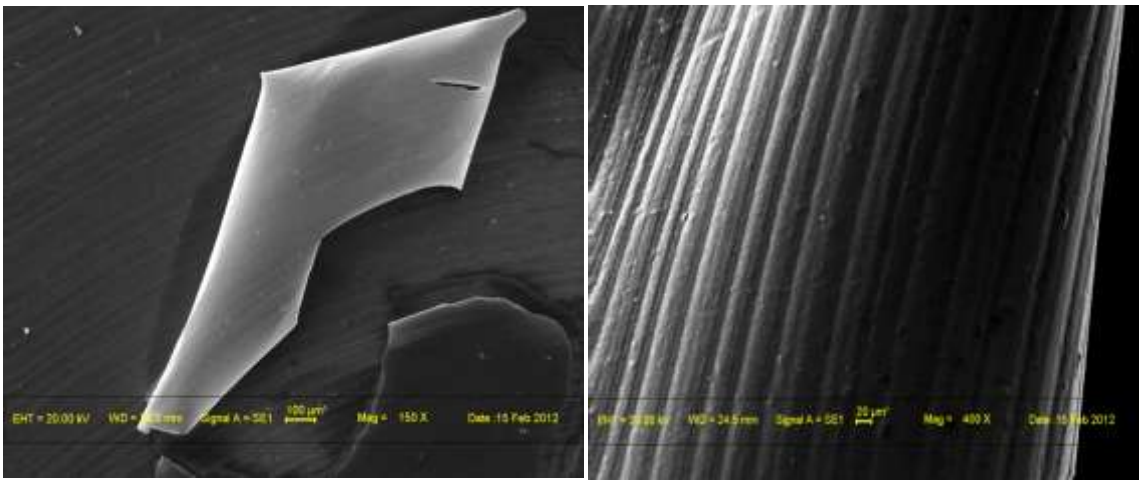

Figure 4.7 Scanning electron micrograph (SEM) revealing the surface morphology of *Pseudozyma* EPS. Compact, non-porous film like structure at 150X magnification (left) and roof-tile like structure at higher magnification - 400X (right).

4.3.9 Atomic Force Microscopy

Atomic force microscopy (AFM) was employed for the three-dimensional analysis of surface structure and surface roughness of *Pseudozyma* EPS. AFM analysis revealed spike-shaped lumps of varying size (Figure 4.8). The lumps were proposed to be formed by inter and intra-molecular aggregation of the polysaccharides [Tao et al., 2008]. The surface structure seemed to be dependent on the EPS concentration. The smaller lumps of 3.0 nm height and comparatively larger ones with 5-6 nm height were observed at lower concentration (10 μg/ml), whereas irregular shaped spikes were observed at higher EPS concentration (100

µg/ml). The surface structure of EPS from *Lactobacillus* species contained round lumps and chains at low concentration and lumps and chains were irregular at higher concentrations [Wang et al., 2010]. Electron microscopy of EPS produced by *Bacillus pumilus* revealed the web-like network structure, which could be due to the intense affinity of EPS molecules for each other, thereby resulting in its excellent viscosifying and thickening properties [Chowdhery et al., 2011]. The molecular structure of *Pseuodzyma* EPS revealed by AFM suggested its strong affinity for water molecules and pseudoplastic nature.

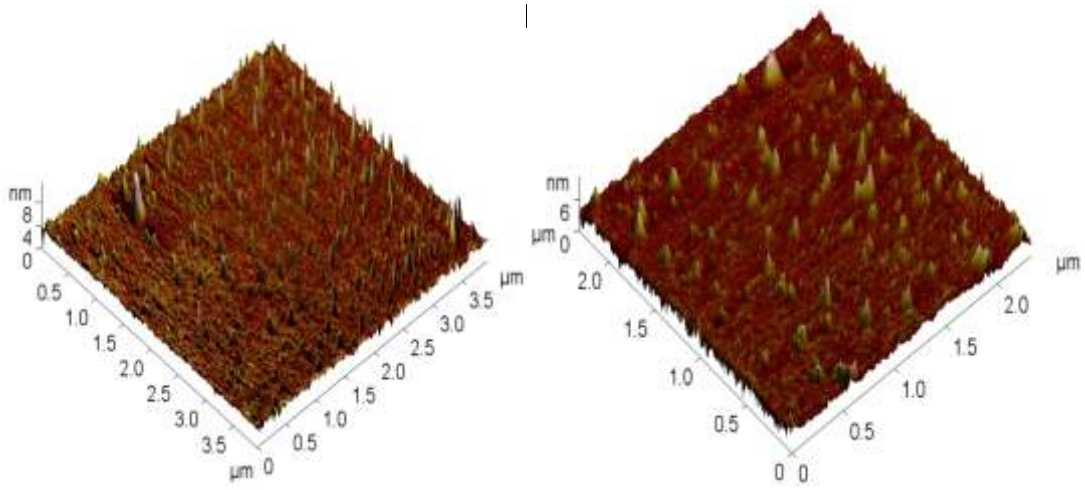

Figure 4.8 Atomic force micrograph (AFM), 3D images of *Pseudozyma* EPS at concentration 10 µg/ml (left) & 100 µg/ml concentration (right). The microstructure of *Pseudozyma* EPS contained spike-shaped lumps that became irregular at higher concentration.

4.4 Summary and Conclusions

Pseudozyma sp. NII 08165 secreted a viscous compound, which turned out to be a novel exopolysaccharide with a yield of 3.5 g/l EPS on fourth day of fermentation. The EPS was a high molecular weight polymer of 1.7 MDa with α–D-glucopyranose, α-D-galactopyranose and α-D-mannopyranose as monomers. FTIR analysis showed the presence of α -glycosidic linkages in the *Pseudozyma* EPS. Detailed structural elucidation by ^{1}H NMR revealed the presence of α-L-rhamnopyranose and N-acetyl-β-D- glucopyranosamine also in the polymer.

Helix-coil transition analysis by Congo Red assay demonstrated a helical conformation of *Pseudozyma* EPS. The compact microstructure suggested the potential application of *Pseudozyma* EPS in preparing plasticized films. Further, the microstructure of *Pseudozyma* EPS was explored by AFM. These findings suggested the necessity of further exploration of ustilaginomycetes yeast for the production of commercially valuable EPS.

Chapter 5: Physicochemical Characterization of *Pseudozyma* Exopolysaccharides

5.1 Introduction

Exopolysaccharide (EPS) can be used as viscosifying, stabilizing, gelling, or emulsifying agents for various applications in food, pharmaceutical, cosmetics, etc. EPS possess unique rheological properties as they form a very viscous formulation at low concentration and exhibit pseudoplastic behaviour. Because of their attractive physicochemical properties, they have the potential to replace the conventionally used hydrocolloids such as starch and pectin. One of the best examples is xanthan gum. Being a food additive, EPS improve the rheology of food preparation. They improve the gelling, and result in the firm texture of food such as jam, jellies, and dessert. EPS also stabilizes the emulsions and are used in food preparation such as mayonnaise, salad dressings, ice-cream, etc. Also, they improve the water retention and prevent syneresis in yogurt preparations, provide protection against bacteriophages in cheese preparations and improve the shelf-life of frozen food preparations. Lactic acid bacteria (LAB) producing exopolysaccharides are used to prepare the functional food. Some EPS especially derived from LAB and *Saccharomyces cerevisiae* with distinctive biological activity such as anti-tumour and cholesterol lowering properties can be used as nutraceuticals [Fanworth et al., 2006].

In cosmetics, EPS are used to stabilize both kinds of emulsions, i.e., oil-in-water emulsion in the case of moisturizing products, and water-in-oil emulsions in the case of night and sun protection creams. EPS also promote flow properties and result in good skin feel and ease of application. In pharmaceutical preparation, EPS are a good stabilizing agent, which suspends insoluble and various bioactive compounds in the pharmaceutical preparations. For example, xanthan gum is used to stabilize the dextromethorphan, a major ingredient in anti-cough

preparation. EPS can be used to manufacture the sustained release tablets because of hydrophilic matrix polymeric structure. Other sectors, where EPS can be potentially useful are agrochemicals, animal feed, carpet printing, cleaning, paint, surface coating and process chemical industries [www.cpkelco.com/xanthangum].

Pseudozyma exopolysaccharide is a high molecular weight polymer with a helical conformation, containing glucose, galactose, mannose, rhamnose and N-acetyl glucosamine as monomers. Detailed physicochemical characterization of this novel EPS is necessary to assess its applicability as hydrocolloids and to predict various potential applications.

5.2 Materials and Methods

5.2.1 Thermogravimetric Analysis

The thermal behaviour of the *Pseudozyma* EPS was studied by the thermogravimetric analysis (TGA) using TG-DTA 6200 (SII Nanotechnology Inc., Japan). The compound was subjected to a temperature range 30-1000 °C under a nitrogen atmosphere at a rate of 10°C/min and the corresponding weight loss was determined.

5.2.2 X-Ray Diffraction (XRD) Analysis

Crystallinity index of EPS was determined by X-ray diffraction methods using X- pert pro diffractometer (PANalytical, Netherlands). The X-ray diffractograms were recorded from 0 to 80° with a step size of 0.03° using a Cu - Kα radiation X- ray (λ= 1.54 Å) generated at a voltage of 40 kV and a current 30 mA.

5.2.3 Rheological Analysis of Aqueous EPS Solution

The dynamic viscosity of aqueous polysaccharide was investigated by visco-rheometer (Rheolab MC1, Model- 749558, Physica). The flow behaviour kinetics was established by Oswald-de-Waele Model (Holdsworth, 1993).

Oswald-de-Waele Model, $\tau = K D_r^n$

Where τ = shear stress (Pa), D_r= shear rate (S^{-1}), K = consistency index ($Pa.s^n$), n = flow index value.

5.2.4 Thickening Efficiency

Thickening efficiency was measured by determining the viscosity at various concentrations of EPS. EPS solution was prepared from 0 to 2% concentration and the viscosity was measured by visco-rheometer (Rheolab MC1, Model- 749558, Physica). Thickening efficiency of xanthan gum was also determined in a similar way.

5.2.5 Suspending Ability

Suspending ability was determined by measuring the yield value. EPS solution (2%) was prepared and the viscosity was measured by visco-rheometer (Rheolab MC1, Model- 749558, Physica). Yield value is calculated by using the following equation [http://www.lubrizol.co.in/Home-Care/Documents/Technical-Data-Sheets/TDS-244-Measurement-Understanding-Yield-Value-Personal-Care-Formulations.pdf].

Yield Value (mPa) = $2*r_1 (\eta_1-\eta_2)$

η_1, η_2 are apparent viscosities obtained at two different spindle speed, r_1 and r_2 only when r_2/r_1= 2. Yield value of 2% xanthan gum was also determined.

5.2.6 Effect of Temperature and pH on Rheology of EPS

An aqueous solution of EPS was prepared at a concentration of 1%.The effect of temperature was studied by incubating EPS solution at 20, 40, 60, 80 and 100 ^oC for three hours, followed by measuring the viscosity of the solution. The pH stability was studied over a pH range of 2.0- 12.0 by dissolving EPS in the appropriate buffers. The stability was investigated by measuring the viscosity by visco-rheometer (Rheolab MC1, Model- 749558, Physica).

5.2.7 Flocculating Activity

The flocculating activity was measured by using the method as described by Lim et al., [2007]. Activated carbon was used as a testing material, which was suspended in distilled water at a concentration of 5 g/l. In a test tube, 10 ml of activated carbon suspension was added and mixed with 0.1ml of $CaCl_2$ solution (6.8 mM). To this mixture, various amounts of EPS were added and vortexed for 30s and allowed to stand at room temperature for 10 min. The turbidity of the upper phase was measured at 550 nm. A control experiment without the EPS was also pursued in the same manner. The flocculating activity (%) was calculated according to the following equation:

$$\text{Flocculating activity} = (B - A)/B \times 100$$ whereas B-absorbance of control, A- absorbance of the sample.

5.2.8 Emulsifying Activity

The emulsifying activity of EPS was determined according to Bramhachari et al., (2007). Briefly, lyophilized EPS (0.5 mg) was dissolved in 0.5 ml deionized water and volume was made up to 2.0ml using Phosphate -buffered saline (PBS). The sample mixtures were vigorously vortexed for 1 min after the addition of hexadecane. The absorbance was read immediately before and after vortexing (A_0) at 540 nm. The decrease in absorbance was recorded after incubation at room temperature for 30 and 60 min (At).A control was run simultaneously with 2.0 ml of PBS and 0.5 ml of hexadecane without EPS. The emulsification activity was expressed as the percentage retention of the emulsion during incubation for the time, t.

$$\text{Emulsifying activity} = At/A0 \times 100$$

The emulsifying activity of xanthan gum was also determined and compared with that of *Pseudozyma* EPS.

5.2.9 Water Holding Capacity

Water holding capacity (WHC) of *Pseudozyma* EPS was determined according to Ahmed et al., [2013]. For this, 0.2 g EPS was suspended in 10 ml of deionised water on a vortex mixer. The dispersed material was centrifuged at 12,000 rpm for 25 min. Unbound water that was not held by EPS material was discarded. All EPS material was dropped on pre-weighed filter paper for complete drainage of water. The weight of precipitated EPS was recorded. The percentage of WHC was calculated by the following equation:

WHC (%) = ([total sample weight after water absorption]/total dry sample weight) x100

5.3 Results and Discussion

5.3.1 Thermogravimetric Analysis

It was clear from the TGA that the *Pseudozyma* EPS underwent a mass loss of approximately 20% up to a temperature of 120 °C, which corresponded to the elimination of the surface bound water molecule (Figure 5.1). This revealed the high water retention ability of this EPS, which could be attributed to the presence of a large number of carboxyl groups as the carboxyl group was bound to water molecule [Kumar et al., 2004]. The exopolysaccharide was thermally stable up to 220 °C, and the degradation temperature (Td) was 250 °C. The thermal degradation happened in two phases, the first being rapid till 300 °C with a mass of 45%, and the second phase starting from 300 °C and proceeding gradually and at slow pace. The slow decomposition might be due to the presence of some thermally stable saccharide moiety. The compound was fully decomposed at 895 °C. This result was in consensus with the EPS obtained from a basidiomycetes fungus, *Trameter vericolor* that followed bi-stage thermal degradation with stability up to 280 °C [Tavares et al., 2005]. The degradation temperature (Td) of EPS from *Sporobolomyces salmonicolor* was also 280 °C [Poli et al., 2010].

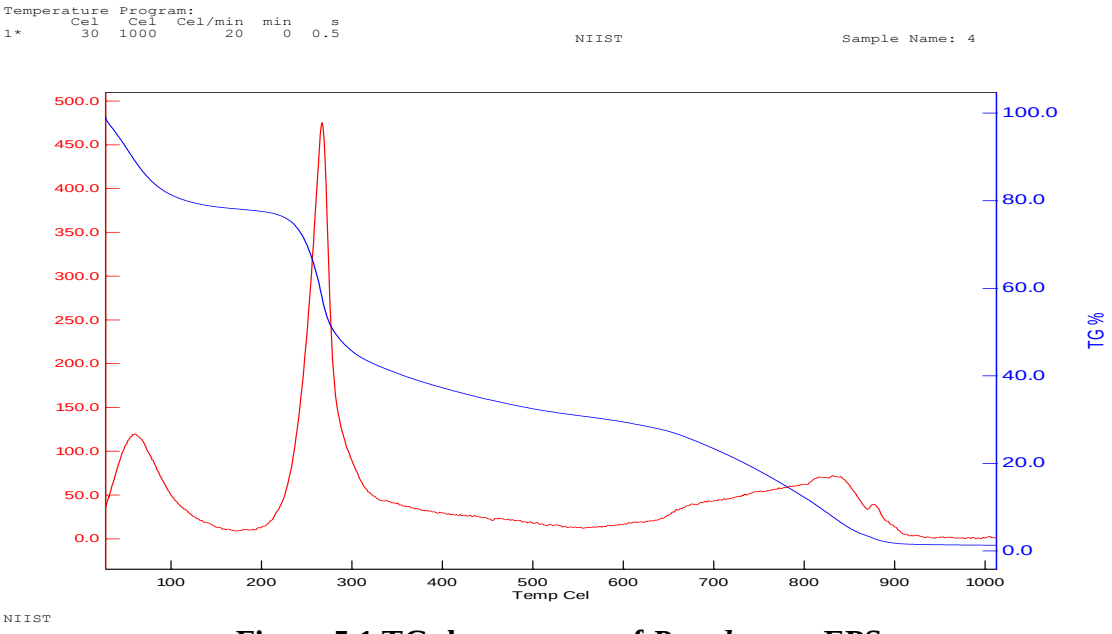

Figure 5.1 TG thermogram of *Pseudozyma* EPS

5.3.2 X-Ray Diffraction (XRD) Analysis

X-ray diffraction is a very powerful tool used for phase identification of the materials. Figure 5.2 illustrates the XRD profile of *Pseudozyma* EPS. No characteristic diffraction peaks were detected in XRD pattern of *Pseudozyma* EPS. Hence, the compound was fully amorphous in nature. Singh et al., [2011] suggested that it was difficult to interpret the broad amorphous peaks of amorphous EPS while easy to interpret narrow crystalline peaks and calculate crystallinity index.

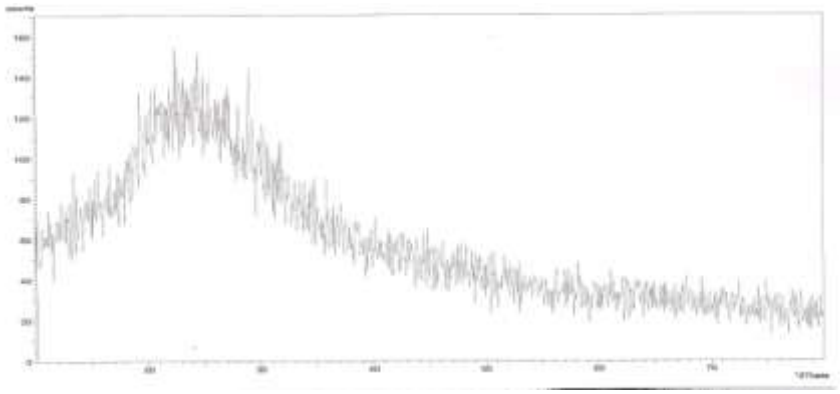

Figure 5.2 XRD profile of *Pseudozyma* EPS

5.3.3 Rheological Analysis of Aqueous EPS Solution

The rheological analyses of EPS solutions are important as it determines the possible applications of EPS as thickeners, stabilizers, emulsifiers and gelling agents, etc. [Pavlova et al., 2005]. The dynamic viscosity of aqueous EPS was measured, and the flow index value was determined by Oswald-de-Waele Model. The flow index value, 'n' for the *Pseudozyma* EPS solution was 0.422, which indicated the pseudoplastic nature of this EPS (Figure 5.3). The apparent viscosity of the solution decreased with increasing shear stress, revealing a non-Newtonian-shear thinning behaviour (Figure 5.4). The pseudoplastic nature of yeast EPS was also demonstrated by Pavlova et al., [2004, 2005]. The rheological analysis, therefore, indicated the potential for use of the *Pseudozyma* EPS as bio-thickener and gelatinizer in various industries.

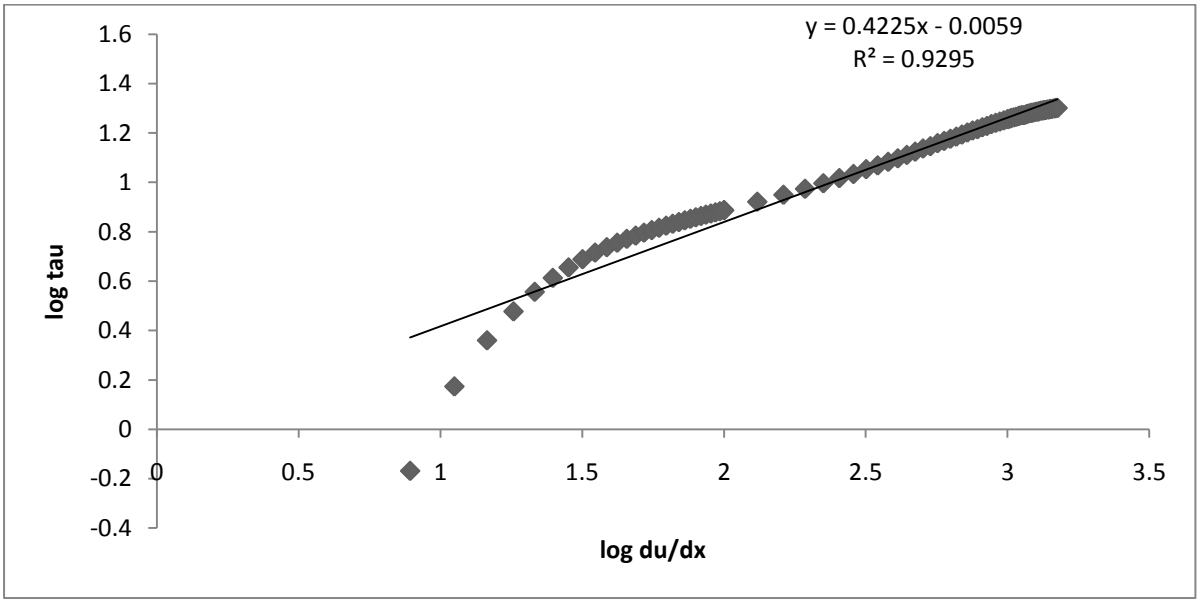

Figure 5.3 Log viscosity versus log shear stress revealing the flow index value n=0.4224, which is less than 1. Hence, the *Pseudozyma* EPS solution is pseudoplastic in behaviour.

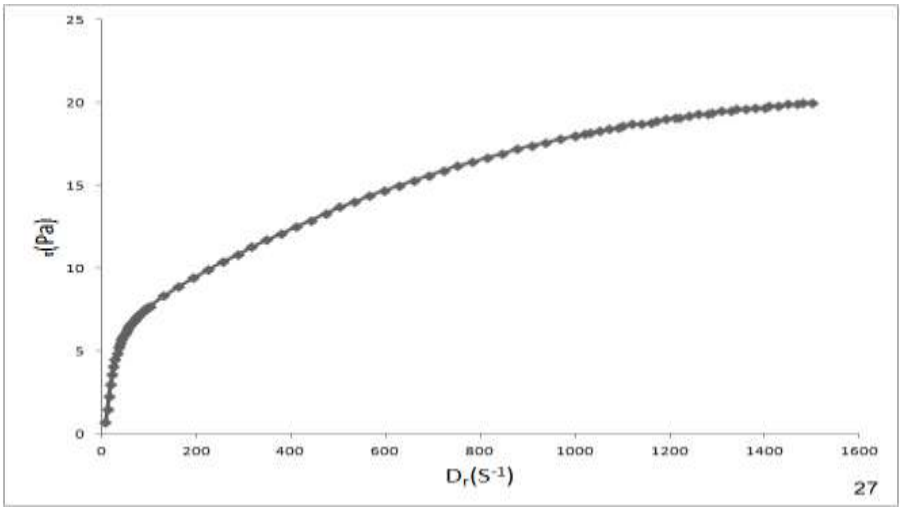

Figure 5.4 Rheological behaviour of aqueous solution of *Pseudozyma* EPS

5.3.4 Thickening Efficiency

Figure 5.5 illustrates the effect of concentration of EPS and xanthan gum upon their viscosity. One of the major applications of EPS is thickening of aqueous system, and thickening efficiency determines the concentration ideal for thickening. At a concentration of 0.25%, *Pseudozyma* EPS resulted in moderate viscosity of the solution. An addition of *Pseudozyma* EPS at a concentration of 0.5% contributed to a significant increase in viscosity, which showed its potential as a thickening agent for the water-based system. *Pseudozyma* EPS at a concentration as high as 2% caused a very high viscosity with a gel-like consistency. Beyond 2%, EPS was not getting dissolved well in the aqueous system. Hence, the concentration ideal for EPS to be used as a thickener could be from 0.5-2%. These findings were similar to that of xanthan gum, an efficient thickening agent used for industrial purposes, and thickening efficiency of *Pseudozyma* EPS was comparable to that of xanthan gum.

A pronounced increase in viscosity at a range of concentration of 0.2- 1% has been reported in the case of Vanzan, a commercial brand of xanthan gum [www.vanderbiltminerals.com/vanzan]. There is a positive correlation between the thickening efficiency of the EPS produced by *Lactococcus lactis* and the viscosity of the

fermented milk [Ruas-Madiedo et al., 2002]. Primary structure, size and composition of EPS play a significant role in thickening properties. Hence, the structural modification of EPS changes the thickening efficiency as side chains increase the stiffness of polymer chains [Tuninier et al., 2001]. Thickening efficiency reveals the performance properties of a polymer in an aqueous system such as high viscosity at higher usage level and the degree of thickening to the ideal viscosity of the final product. Comparing thickening efficiency between Carbopol polymers determines which Carbopol polymers are ideal for thin formulations with maximal stability and minimal viscosity [www.lubrizol.com/flow-and-suspension-properties-of-carbopolpolymers].

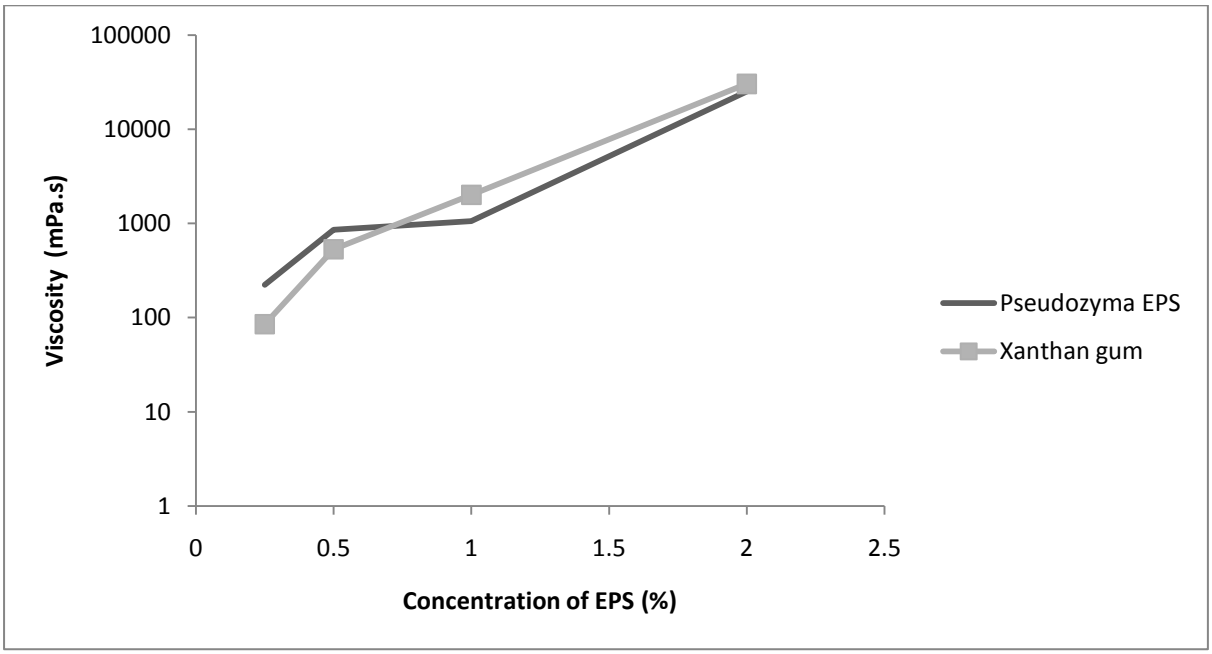

Figure 5.5 Viscosity vs. Concentration of EPS

5.3.5 Suspending Ability

Suspending ability is an important parameter to be considered from application point of view of a polymer and is expressed in terms of yield value. Yield value is the minimum force that must be applied to a fluid to start disrupting the cohesive polymer network imparted by

rheology modifier so that flow can occur. Simply, it is the initial force to initiate the flow. Greater the yield value, more stable suspension would be. Yield value of *Pseudozyma* EPS was 4173 mPa, while xanthan gum showed a value of 9246 mPa at 2% concentration. Xanthan gum has already been reported to have excellent yield value compared to other commercial hydrocolloids. Even though the yield value of *Pseudozyma* EPS was less than that of xanthan gum, it still possessed a significant value, which could make it a possible stabilizing agent in the case of suspension formulation. Polymers with low yield value are not suitable as a stabilizer in the preparation of a suspension. EPS from *Sphingomonas paucimobilis* GS-1 exhibited strong suspending ability and other rheological properties and had potential to be used in oil drilling applications [Shah and Ashtaputre, 1999]. Many EPS lack data on suspending ability.

It has been reported that at a 1% concentration in a 1% KCl solution, yield values of xanthan gum, guar gum, hydroxymethyl cellulose, locust bean gum, carboxymethyl cellulose and sodium alginate are 11300, 4000, 830, 360, 410, 210 mPa, respectively. Xanthan gum also exhibits a notable yield value of 500 mPa even at low concentration of 0.3%, while all the hydrocolloids do not exhibit significant values at this concentration. Properties of xanthan gum such as semirigid conformation and formation of weak network in the solution account for the high yield value of xanthan gum, which contribute to its remarkable ability to stabilize dispersion, such as suspensions and emulsions [Nussinovitch et al., 2013].

5.3.6 Effect of Temperature and pH on Rheology of EPS

Effects of temperature and pH on the rheology of EPS are shown in Tables 5.1 and 5.2. Rheology of *Pseudozyma* EPS was almost stable up to 60 ^{O}C. Compared to ambient temperature, EPS retained 78% activity at 60 ^{O}C, 32% activity at 80 ^{O}C and 4% activity at 100 ^{O}C. When viscosity was monitored online at respective temperature, viscosity was much higher at lower temperature and there was a significant drop in viscosity at higher

temperature, which could be explained by temperature dependence of liquid viscosity (Figure 5.6). Kavita et al., [2014] suggested that either decreased interaction between the molecules led to a looser polymer structure, or different intramolecular arrangement resulted in modified tertiary structure could be the reason for the low viscosity at higher temperature. Viscosity remains constant between pH 4.0-9.0. When compared to the viscosity at neutral pH, EPS exhibited around 84% viscosity at pH 12.0, while viscosity was 45% at pH 2.0. Hydrolysis of glycosidic linkages at high acidic pH could result in lower viscosity [Kavita et al., 2014]. Since it was stable over the pH 4.0-9.0, *Pseudozyma* EPS could be used in both acid and alkaline systems.

At high acidic condition, temperature and pH had a huge impact on the viscosity of capsular EPS produced by *Streptococcus thermophilus.* Hence, flow behaviour of capsular EPS was greatly influenced by the pH and temperature [Purwandari and Vasiljevic, 2010]. The viscosity of EPS produced by *Volcaniella eurihalina* F2-7 strain decreased with temperature, but viscosity values were restored after the cooling. EPS formed a gel-like solution of high viscosity at acidic pH, not at alkaline, or neutral pH [Calvo et al., 1995]. Mauran, an anionic, sulphated heteropolysaccharide of high uronic acid content, synthesized by *Halomonas maura* was highly viscous in nature, and the viscosity was stable over a wide pH range of 3.0-11.0 [Arias et al., 2003].

Table 5.1 The effect of temperature on viscosity

Temperature (^{O}C)	Viscosity (mPa.s)
20	756
40	742
60	586

80	245
100	30

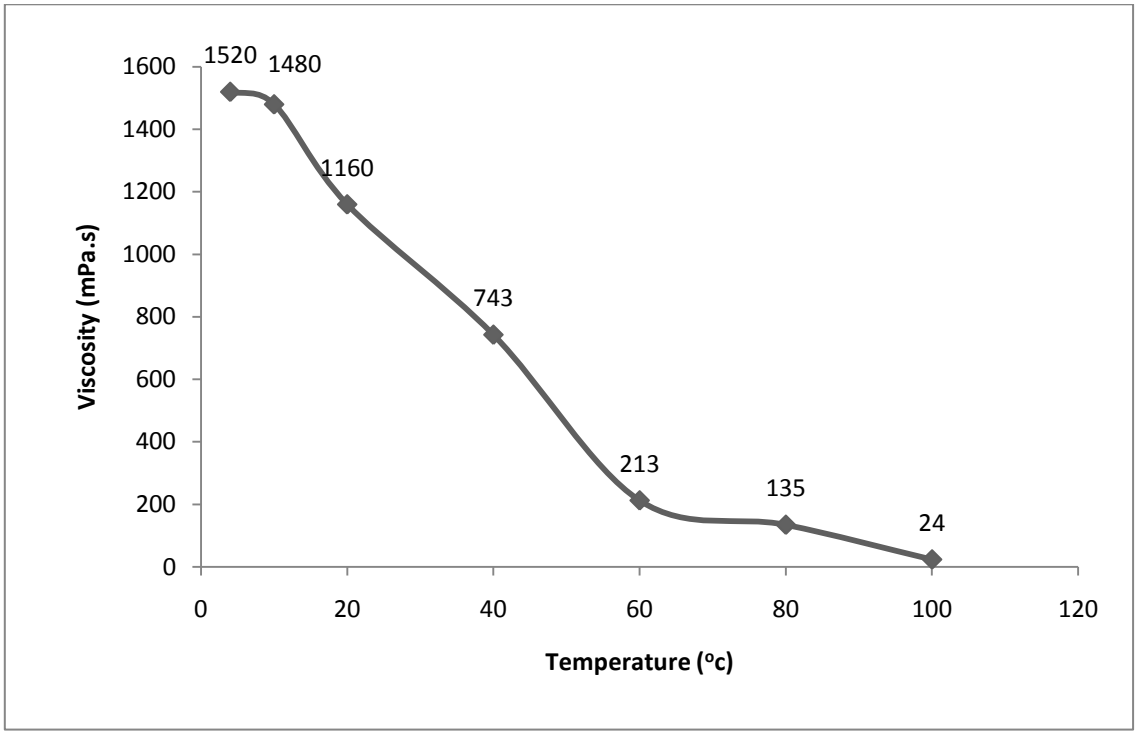

Figure 5.6 Viscosity of *Pseudozyma* EPS at various temperatures.

Table 5.2 Effect of pH on viscosity

pH	Viscosity (mPa.s)
2	332
4	729
7	738
9	742
12	623

5.3.7 Flocculating Activity of EPS

Flocculating activity assay was performed for two different sets of *Pseudozyma* EPS concentration (0.001-0.01, and 0.01-0.1 mg/l) to determine the concentration of EPS at which high flocculating activity was obtained (Figures 5.7 and 5.8). High flocculating activity was in the range of 0.01-0.1 mg/l of EPS. Flocculating activity was compared with that of xanthan gum (Figure 5.9). Optimal EPS concentration for the maximal flocculating activity was 0.01 mg/l and flocculating activity of xanthan gum was maximum in the range of 0.06 to 0.1 mg/l. Hence, *Pseudozyma* EPS exhibited higher flocculating activity than that of xanthan gum and was promising as a potential flocculating agent.

In flocculation, free functional groups present in the EPS act as bridges, which aggregate the suspended particles forming a floc. Concentration of flocculant should be optimized in flocculation as excess flocculants adsorb and destabilize the particle, result in poor flocculation. Flocculating agents are widely used for the waste water treatment, drinking water purification, and various downstream processing. EPSs produced by a *Zoogloea* strain had remarkable flocculating activity, which was better than that of xanthan gun [Lim et al., 2007]. Microbial flocculants are preferred over the chemical flocculants due to their non-toxic and biodegradability nature. Glucan-DM5 exhibited higher flocculating activity than the commercial hydrocolloid guar gum and possessed the potential to be used as bio-flocculent in the dairy industry for making cheese from curd [Das et al., 2014].

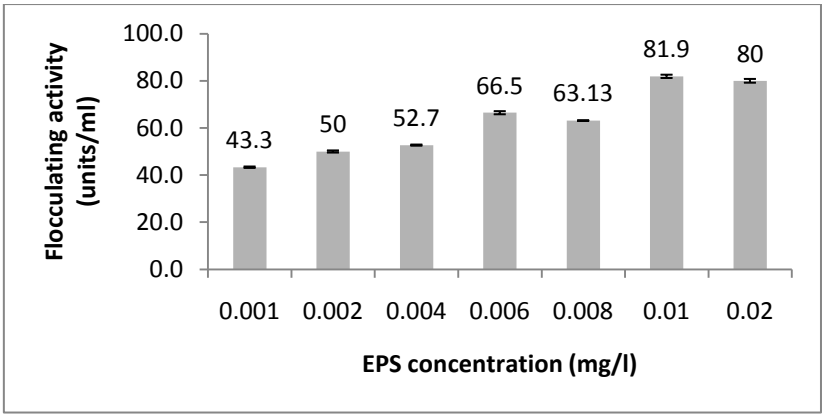

Figure 5.7 Flocculating activity of *Pseudozyma* EPS at a concentration range of 0.001-0.02 mg/l.

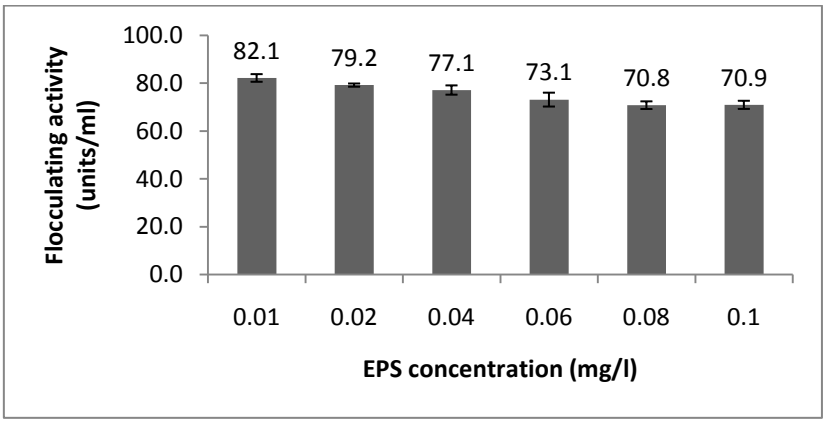

Figure 5.8 Flocculating activity of *Pseudozyma* EPS at a concentration range of 0.01-0.1 mg/l

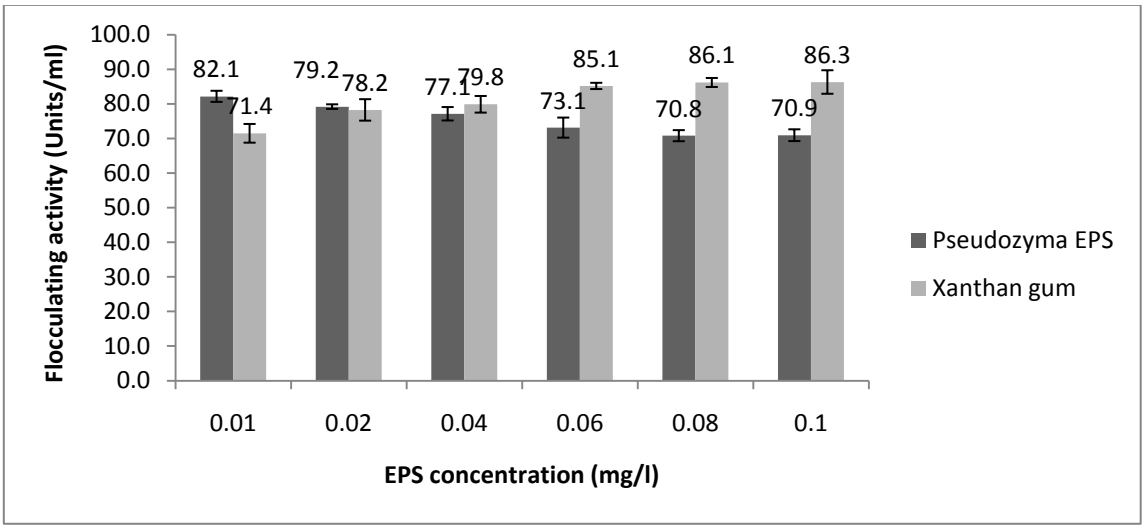

Figure 5.9 Flocculating activity of *Pseudozyma* EPS and xanthan gum

5.3.8 Emulsifying Activity

This assay measures the ability of EPS in retaining the emulsion of hydrocarbons in water. The emulsifying activity of *Pseudozyma* EPS against n-hexadecane was compared with that of xanthan gum (Figure 5.10). *Pseudozyma* EPS retained 54.46% ± 3.6% and 54.23% ± 2.7% activity after 30 and 60 minutes, respectively, whereas xanthan gum retained 53.81% ± 4.0% and 53.45% ± 3.4% after 30 and 60 minutes, respectively. Emulsions produced by *Pseudozyma* EPS were stable like in the case of xanthan gum. *Pseudozyma* EPS exhibited emulsifying activity, which was comparable to that of xanthan gum and could be considered as a potential emulsifying agent for food and cosmetic applications. Polymeric stabilizers such as xanthan gum are necessary for formulating oil-in-water emulsions in skin care products.

Lower toxicity, higher biodegradability and improved performance at high temperature, pH and salinity are some of the advantages of using microbial emulsifiers over the synthetic emulsifiers. The emulsifying activity of EPS is imparted by the functional groups present in the structure [Singh et al., 2011]. Glucan-DM5, an EPS synthesized by *Lactobacillus plantarum* DM5 showed emulsifying activity superior to commercial hydrocolloids such as guar gum and sodium alginate [Das et al., 2014]. Yeast EPS such as mannan and glucomannan exhibit strong emulsifying activity. Because of its distinct emulsion stability when compared to the commercial emulsion stabilizers such as Rofetan and Arlacel, glucomannan can be used for the preparation of emulsion creams [Kuncheva et al., 2007].

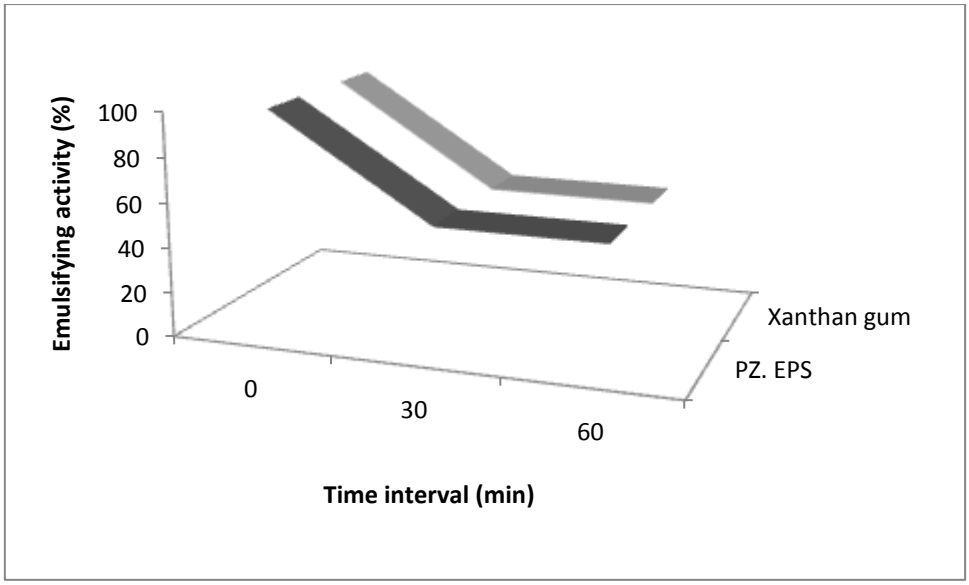

Figure 5.10 Emulsifying activity of *Pseudozyma* EPS and xanthan gum

5.3.9 Water Holding Capacity of Pseudozyma *EPS*

Water holding capacity is one of the hydration properties of hydrocolloids that allow the gum to hold the water. Figure 5.11 illustrates the WHCs of different polymers. WHC of *Pseudozyma* EPS was 23%, whereas guar gum, xanthan gum and cellulose exhibited 17, 31 and 2%, respectively. Though EPS exhibited lower WHC than xanthan gum, its WHC was better than that of guar gum and cellulose. Since the particle size and moisture content of the polymer significantly influenced the hydration properties of the hydrocolloids, crude lyophilized EPS from *Pseudozyma* still possessed good emulsifying activity compared to the commercial grade xanthan gum, which came after a series of quality control.

Water holding capacity of EPS is attributed to the permeable structure of EPS, where it can bind to a large amount of water through hydrogen bonding [Ahmed et al., 2013]. Water holding capacity is one of the important properties of the hydrocolloids considering food and cosmetic industry. Xanthan gum, guar gum and modified cellulose products are commercially used hydrocolloids that enhance the water holding capacity of food, cosmetic and pharmaceutical preparations. High water holding capacity of hydrocolloids causes the

reduction in evaporation rate, alteration in freezing rate and modification in ice crystal formation of food. Hydrocolloids are essential ingredients in the baking industry, which confers various functionalities. One of the primary functions is to serve as anti-staling agent by improving the water holding capacity of the product [Rodge et al., 2012]. Hydrocolloids with high water holding capacity are usually added to the cosmetics such as moisturizing formulation and sun protection creams. One of the prerequisites for a cosmetic ingredient for the facial mask products is its high water holding capacity, where it enhances the moisture uptake by facial skin *[*Phisalaphong et al., 2011].

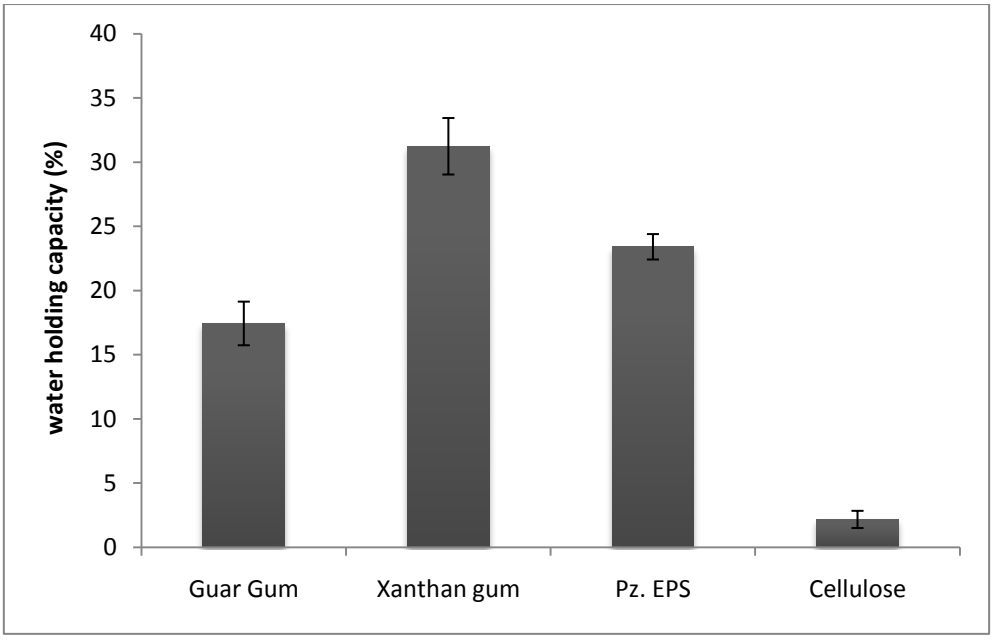

Figure 5.11 Water holding capacity of *Pseudozyma* EPS and different hydrocolloids

5.4 Summary and Conclusions

EPS produced by *Pseudozyma* sp. NII 08165 was investigated with regard to their physicochemical properties to find out the potential applications. EPS exhibited excellent thermal stability making it a potential candidate for various industrial applications. The pseudoplastic behaviour of aqueous EPS indicated the potential applications of *Pseudozyma* EPS as a thickening agent, or gelling agent in food industry. It also possessed significant

thickening efficiency and suspending ability along with good water holding capacity, which further emphasis on its application in food, cosmetic and pharmaceutical industries. Emulsifying and flocculating activity revealed that it could be exploited as potential emulsifying and flocculating agent.

Chapter 6: Application of *Pseudozyma* biosurfactants as laundry additive

6.1 Introduction

The cleansing ability of laundry detergents is partly due to the surfactants. Amphiphilic nature of surfactants facilitates the removal of dirt and grimes from the clothes. During washing with the surfactants, hydrophobic end of the surfactants attach to the grease and solubilize it. The surfactants-dirt micelles, which are suspended in the water, are easily drained away during the rinsing process. Due to 'green' consumer trend, the detergent industry is looking for high-performance, renewable-based ingredients, which ensure the sustainability of the production processes. White biotechnology is a major tool towards the sustainability. Advantages of using biosurfactants over petro-based and oleo-based surfactants are environmental compatibility, renewable origin and high performance [Sajna et al., 2015a].

Several studies have shown that biosurfactants can be good cleaning agents owing to their amphiphilic nature and excellent performance at extreme conditions, which enables them to meet the diverse demands of the detergent industry. Moreover, biosurfactants are readily biodegradable compared to the longer half-life of synthetic surfactants. Other features of biosurfactants that make them attractive in detergent industry are their skin-friendly nature and antimicrobial activity. Using biosurfactants in cleaning agents can pave the way for 'green' cleaning as biosurfactants sustain the environment and preserve the human health. A number of dishwashing, hard surface cleaning and laundry detergents containing sophorolipids as one of the ingredients are out in the market. Develter and Lauryssen, [2010] reported the usefulness of sophorolipid in hard surface cleaning and automatic dishwashing rinse aid formulation due to their outstanding surface activity and low foaming properties.

Sophorolipids can be good laundry detergent additives as they possess good wetting property, emulsification index, antimicrobial activity and high fabric wash power [Joshi-Navare et al., 2013]. When sophorolipid, rhamnolipids, Accell, a biosurfactants derived from the undisclosed yeast strain were tested to see their efficiency in removing beef stain from the cloth, in combination with either bacterial, or yeast lipase enzyme, sophorolipids along with bacterial enzyme gave satisfactory performance [Parry et al., 2012]. Detergent containing a cocktail of both biosurfactants and chemical surfactants utilizing the synergistic action of sophorolipids, rhamnolipids, cellobiose lipids was shown to have enhanced oily soil detergency [Hall et al., 1995]. However, a formulation containing glycolipids biosurfactants in micellar phase and a non-glycolipids biosurfactants in micellar phase showed an improved detergency and was suitable for all the cleaning purpose, ranging from laundry detergent to hard surface cleaning. It has been noted that employing micellar phase sophorolipids is more suitable for hard surface cleaning as they possess an efficient foam breaking activity, since over-foaming of the hard surface cleaner is a disadvantage as it requires a lot of rinsings to remove the foams. Addition of micellar non-glycolipids surfactants along with sophorolipids helps to give suitable foaming properties to the hard surface cleaning formulation, as non-glycolipid biosurfactants helps in the initial foaming and sophorolipids subsequently curb the foaming [Develter et al., 2006]. The low foaming property of sophorolipids as well as their high temperature stability can be exploited for jet-washing, a washing method which uses water pressure to remove dirt from the object and are widely used in dishwashing machine and high-tech washing machines. A mixture of lactone and acidic forms of sophorolipids exhibit better washing performance than that of conventionally used non-ionic surfactants [Furuta et al., 2004].

A formulation containing sophorolipids, cellobiose lipids and a number of bacterial biosurfactants exhibit good flushing performance, dispersibility, foaming power and

dermatological compatibility and are suitable for manual dishwashing applications [Hees et al., 1997]. The crude cyclic lipopeptide (CLP) biosurfactants from *Bacillus subtilis* and rhamnolipids were promising as laundry detergents additives [Mukherjee, 2007; Unilever PLC, 2012].

Due to high production of MEL-C with good surface activity and presence of more than one type of glycolipids, crude glycolipids biosurfactants from *Pseudozyma* sp. NII 08165 was studied for their application as laundry detergents additive.

6.2 Materials and methods

6.2.1 Preparation of Crude Biosurfactants for Wash Analysis

The crude biosurfactants was prepared by washing the ethyl acetate extract with hexane three times to remove the residual oil.

6.2.2 Effect of Temperature and pH on Crude Biosurfactants

An aqueous solution of crude glycolipid was prepared at a concentration of 0.05%.The effect of temperature was studied by incubating the glycolipid solution at various temperature for two hours, followed by measuring the surface tension of the solution. The pH stability was investigated over a pH range of 2.0- 12.0. The stability was studied by measuring the surface tension by Wilhelmy plate method using Wilhelmy type automatic tensionmeter (Dataphysics DCAT 21) at 25 ^{o}C.

6.2.3 Fabric Wash Analysis

Clean white cotton clothes (5cm^2) were stained with goat blood, ketchup and chocolate sauce. 1.0 ml goat blood, ketchup and chocolate sauce were applied separately onto the cloth and cloth was kept for drying overnight. The stained clothes were subjected to wash analysis with

commercial detergent (X brand), glycolipid solution and the mixture of commercial detergent and glycolipid solution. Briefly, the stained cloth was placed put in separate flasks as follows: flask with the tap water only; flask with tap water and commercial detergent (X brand) at a final concentration of 10 mg/ml; flask with the tap water and glycolipids at a final concentration of 10 mg/ml, and flask with the tap water with commercial detergent and glycolipids at final concentration of 5.0mg/ml each. In all the flasks, the final volume was 10 ml. Then, the flasks were kept for agitation at 200 rpm for 20 min at room temperature. After incubation, cloth pieces were taken out, rinsed with the water and dried at room temperature The stain removal was determined by measuring the lightness of cotton clothes using COLORTOUCH Brightnessmeter ISO model (Technidyne Corp, USA), and the percentage of stain removal was calculated using the following equation:

% stain removal= (Lightness of the stained and washed cloth/ Lightness of the clean and unstained cloth) x 100

All the washing experiments were done in three sets, and the mean value was taken.

6.3 Results and Discussion

6.3.1 Effect of Temperature and pH on Crude Biosurfactants

The crude biosurfactants from *Pseudozyma* sp. NII 08165 were stable at higher temperatures as incubation at these respective temperatures for two hours did not result in any loss of surface activity (Figure 6.1). Temperature stability is an attractive characteristic for a compound to be used as laundry detergent additives as high-temperature washing results in better cleaning. A slight increase in surface activity was noted after incubation at the temperature from 60 to 100^{O}C, which could be due to the higher solubility of biosurfactants at elevated temperature. The crude biosurfactants were stable over the pH range 7.0-12.0, which favoured the scope of application in laundry detergent formulation because pH of

laundry detergent should be usually in the range of 9.0-12.0 (Figure 6.2).Surface tension at pH 12.0 was 29.1, a higher value of surface activity than other pH range. At alkaline pH, ionization of the functional groups of molecules results in higher surface activity [Mitrinova, 2013]. Crude lipopeptide biosurfactants from *Bacillus subtilis* were stable over the pH range of 7.0-12.0, and heating them at 80 ^{o}C did not result in loss of their surface active properties [Mukherjee, 2007].

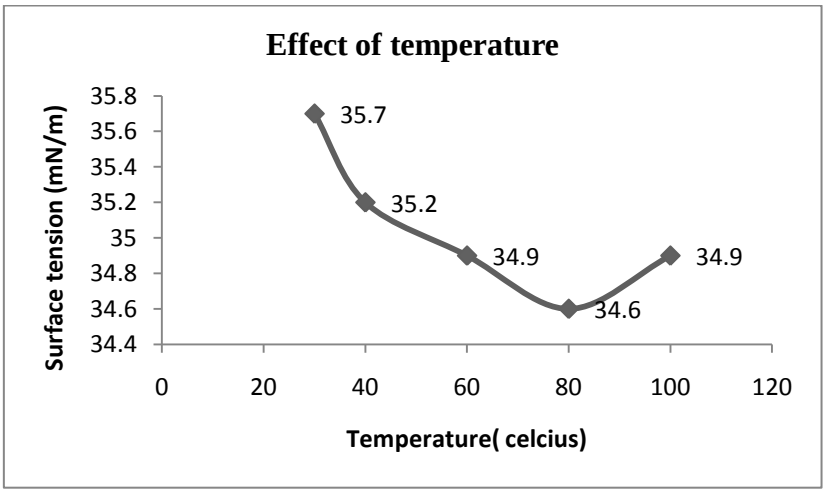

Figure 6.1 Effect of temperature on crude biosurfactants

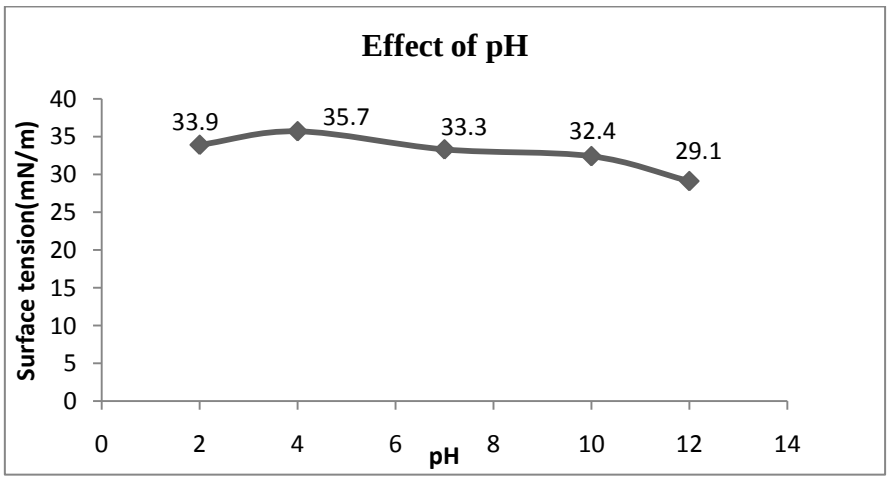

Figure 6.2 Effect of pH on crude biosurfactants

6.3.2 Fabric Wash Analysis

Since lightness is the dimension of the colour of an object by which the object appears to reflect more, or less of the incident light, stain intensity on the cloth can be measured by the lightness. For determining the efficiency of the detergent comprising alpha-amylase variants from alkaliphilic *Bacillus* species, stain removal on clothes was measured by the reflectometry using CIE L*a*b* color space where L was the lightness [Jones et al., 2008].

Fig 6.3 shows percentage stain removal calculated for all the clothes stained with blood, ketchup and chocolate sauce and washed with the tap water, commercial detergent, crude biosurfactants and commercial detergents mixed with the crude biosurfactants from *Pseudozyma* sp. NII 08165 in the proportion of 1:1 (w/w). In this study, commercial detergent mixed with the crude biosurfactants cleaned clothes better than washing with the commercial detergent, or crude biosurfactants alone. Stain removal by the crude biosurfactants alone was efficient and was comparable to that of commercial detergent, although it lacked the additives present in the commercial detergents. The result showed that crude biosurfactants could be used as laundry additive as it improved the wash performance of the detergent. Figure 6.4 shows efficiency of blood stain removal after fabric wash. The bio-washing powder that contains biosurfactants in the presence of chemical surfactants is environmentally friendly, requires less post wash rinsing as glycolipids are non-toxic to the skin. The biosurfactants are very good at loosening the fat due to the structural diversity that results in better cleaning [Mukherjee, 2007]. Because of all these properties, washing with biosurfactants could result in reduced consumption of energy and water. A laundry detergent comprising rhamnolipids and alkyl benzene sulphonate was efficient in removing the fatty soil from the cotton clothes [Unilever PLC, 2012].The wash performance of laundry detergent was improved in the presence of crude lipopeptide biosurfactants, which was

evident from the enhanced removal of oil and blood stain from the cotton fabrics [Mukherjee, 2007].

Biosurfactants such as cHAL (compost humic acid-like matter) obtained from the ground green waste, or aerobically digested compost can be used in the detergent formulations. Studies on cHAL (Compost humic acid-like matter) in detergent formulation revealed a proportion of 1:1 (w/w) biosurfactants-commercial surfactants gave significant synergy on wash performance [Savarino et al., 2010]. Drawbacks of cHAL biosurfactants such as sensitivity to water hardness and fabric yellowing are minimized when biosurfactants are used in combination with the commercial surfactants in detergent formulation [Savarino et al., 2010]. This study revealed the potential application of biosurfactants from *Pseudozyma* sp. NII 08165 as laundry detergent additive, which could add up to the development of sustainable technology for the formulation of laundry detergent using biosurfactants.

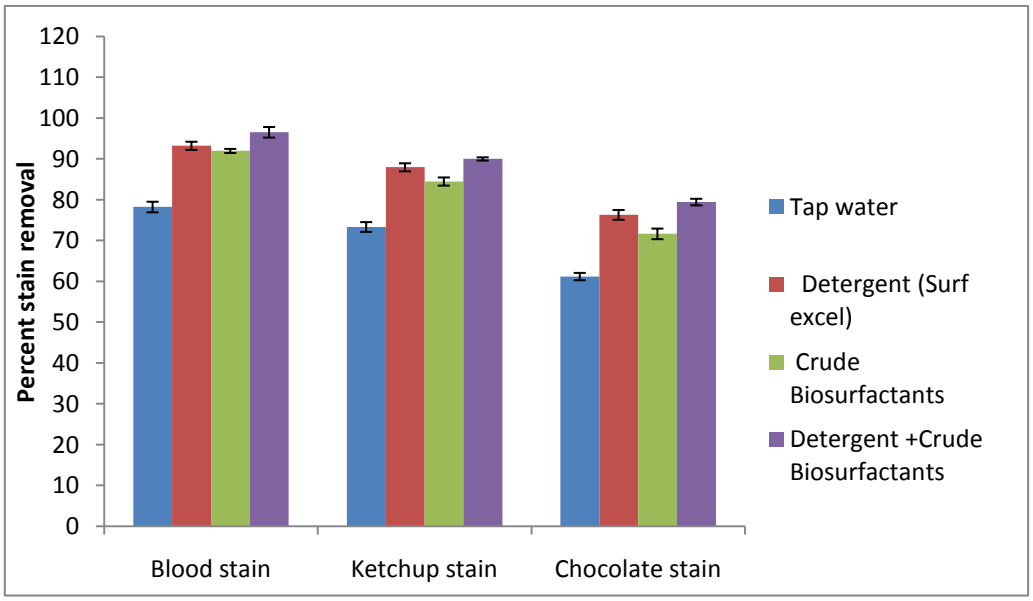

Figure 6.3 Fabric wash analysis. Percent stain removal of stains such as blood, ketchup and chocolate from cotton clothes by commercial detergent, crude biosurfactants from *Pseudozyma* sp. NII 08165 and mixture of commercial detergent and crude biosurfactants from *Pseudozyma* sp. NII 08165. Here tap water was taken as control.

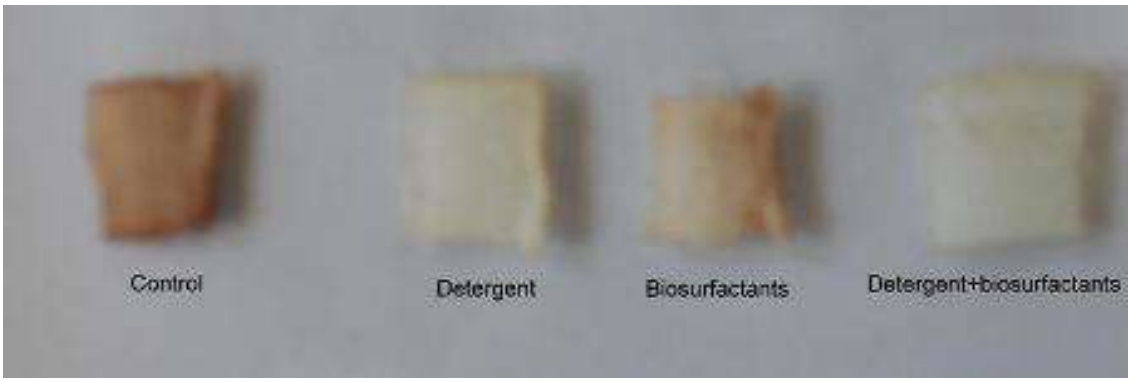

Figure 6.4 Observation of blood stain removal after fabric wash. Clothes were washed with tap water, commercial detergent, *Pseudozyma* biosurfactants, and detergent along with *Pseudozyma* biosurfactants. Addition of *Pseudozyma* biosurfactants along with biosurfactants efficiently removed the stain.

6.4 Summary and Conclusions

Due to the high production of MEL-C with good surface activity and presence of more than one type of glycolipids, crude glycolipids biosurfactants from *Pseudozyma* sp. NII 08165 was studied for their application as laundry detergents additives. The temperature and pH stability of the crude biosurfactants favoured their scope of application as laundry additives. Crude biosurfactants from *Pseudozyma* sp. NII 08165 removed the stains efficiently and could be used in laundry detergent formulations.

Chapter 7: Application of *Pseudozyma* Biosurfactants in Bioremediation

7.1 Introduction

Oil spillage is a major environmental problem with a serious health and environmental consequences and bioremediation is one of the effective clean-up strategies implemented for the restoration of oil polluted environment. Bioremediation makes use of the enzymatic capabilities of hydrocarbon degrading microbes, where the hydrocarbon is degraded to water and CO_2 by mineralization [Atlas, 1991]. Low bioavailability of hydrocarbon is the major challenge in bioremediation of recalcitrant hydrocarbons. Surfactants are the powerful tool for overcoming the low bioavailability of the pollutants. It has been found that the addition of surfactants reduces the interfacial tension, increases the emulsification of hydrophobic pollutants, improves the solubility of hydrocarbon, and thus, enhances the bioavailability of hydrophobic pollutants [Collina et al., 2007]. Kaczorek and Olszanowski [2011] found that natural surfactants such as rhamnolipids and sophorolipids were more effective in promoting hydrocarbon degradation, when compared to chemical surfactants such as Triton X-100.

Several studies have demonstrated the efficacy and performance of biosurfactant assisted bioremediation [Banat, 1991]. Rhamnolipids supplementation improved the bioremediation of crude oil contaminated soil with reduction of total petroleum hydrocarbon up to 86.97% [Zhang et al., 2011]. Apart from the solubilization of hydrocarbons, biosurfactants can also change the surface properties of microbial cells, which leads to the attachment of hydrocarbon to bacteria and a consequent increase in hydrocarbon utilization. The *in-situ* bioremediation of hexadecane in a saturated sand column was promoted by the addition of low level of rhamnolipids [Herman et al., 1997]. Supplementation of rhamnolipids at 15 mg/l concentration improved the efficiency of polyaromatic hydrocarbons (PAH) removal and

soluble COD reduction to 90 and 99%, respectively indicating its potential to treat the waste water abundant in polyaromatic hydrocarbons [Sponza and Gok, 2010]. Rhamnolipids affect the energy-dependent transport of hydrophobic compounds by *Pseudomonas aeruginosa* [Noordman and Janssen, 2002].

Sophorolipids, the glycolipid biosurfactants produced by *Candida bombicola*, have been also reported for enhancing the biodegradation of crude oil where it resulted 80% biodegradation of saturates and 72% of aromatics [Kang et al., 2009]. Challenges in soil bioremediation of phenanthrene such as longer bioprocess and residual pollutants can be avoided by the addition of surfactants such as sophorolipids [Schippers et al., 2000]. Moran et al., [2000] reported that surfactin, produced by *Bacillus subtilis* could be used to promote the biodegradation of hydrocarbon waste by the indigenous microbial consortium. Whang et al., [2008] studied the effectiveness of rhamnolipids and surfactin to improve the diesel solubility, biomass growth and biodegradation efficiency. Similarly, trehalose lipids were proved to enhance the solubility and biodegradation of phenanthrene, which made them useful in the bioremediation of sites contaminated with polyaromatic hydrocarbons [Chang et al., 2004]. Biosurfactants usually have an adverse effect on bioremediation when used at high concentration due to the antimicrobial activity. Hence, for enhancing hydrocarbon degradation, the optimal concentration of biosurfactant needs to be determined.

Pseudozyma sp. NII 08165 was capable of producing biosurfactants, which included mannosylerythritol lipids (MEL) A, B and C. This study was aimed at determining the effect of biosurfactants from this yeast on hydrocarbon degradation by a model microbial culture, *Pseudomonas putida* MTCC 1194. *Pseudozyma* biosurfactants were purified; their surface activity was evaluated and the potential to aid the biodegradation of crude oil was studied.

7.2 Materials and Methods

7.2.1 Culture Medium and Crude Oil

Bushnell Hass (BH) medium was used for all the hydrocarbon degradation studies. It contained (g/l) KH_2PO_4 -1.0, K_2HPO_4 -1.0, NH_4NO_3 -1.0, $MgSO_4.7H_2O$ -0.2, $FeCl_3$-0.05 and $CaCl_2$ -0.02 and an initial pH of 7.0. Yeast extract (1.0 g/l) was supplemented to the medium as the organic nitrogen source. Crude oil (Persian Gulf grade) was procured from Kochi Refineries Ltd, Kochi, India.

7.2.2 Screening of **Pseudomonas putida** *strains*

Five *P. putida* strains procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India were screened to use as the model strain for hydrocarbon degradation studies. These were *P. putida* MTCC 1190, *P. putida* MTCC 1192, *P. putida* MTCC 1194, *P. putida* MTCC 1273, *and P. putida* MTCC 102. Two more strains, *viz. P. putida* NBTC 0822 and *P. putida* NBTC 0823 were also used. Screening was based on microtiter plate based INT (p-iodonitrotetrazolium violet) indicator assay (Haines et al., 1996). Seed cultures of the *P. putida* strains (grown on nutrient broth at 30^{o}C, 200 rpm for 12h) were inoculated into BH medium and incubated at 30 °C for seven days. To a microtitre plate, 200 µl of the culture broth from each strain was added along with 100 µl INT indicator (HiMedia, India, 7.5 g /l prepared in distilled water) and was incubated for 3h. Scoring was performed based on the intensity of the red precipitate formed.

7.2.3 Preparation of Biosurfactants

Pseudozyma sp. NII 08165 was inoculated into production medium containing (g/l) soybean Oil- 40, $NaNO_3$-3.0, $MgSO_4$. $7H_2O$-0.3, KH_2PO_4-0.3, yeast extract- 1, and was incubated at 30 °C, 200 rpm for nine days [Morita et al., 2008]. Biosurfactants were extracted from the

culture broth by adding two volumes ethyl acetate. The ethyl acetate extract was subjected to silica gel column chromatography for purification of glycolipid biosurfactants. During the chromatography, lipids were removed by 100% chloroform elution, followed by 4:1 (v/v) ratio chloroform: ethyl acetate. Finally, the glycolipids were eluted with 100% acetone.

7.2.4 Measurement of Surface Activity of **Pseudozyma** *Biosurfactants*

Surface tension of an aqueous solution of the biosurfactants preparation was determined at different concentrations by Wilhelmy plate method using Wilhelmy type automatic tensiometer (Dataphysics DCAT 21) at 25 °C. Critical micelle concentration (CMC) was then determined from the break point of the surface tension *versus* the log of bulk concentration curve.

7.2.5 Effect of **Pseudozyma** *Biosurfactants on Crude Oil Degradation by* **P. putida**

Seed culture of *P. putida* (5%, v/v) was inoculated into BH medium containing 2% crude oil. Purified biosurfactants was added to the culture medium at the concentrations 1.0, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0 and 100 mg/l. BH medium with the crude oil inoculated with *P. putida* as above but without biosurfactants supplementation was taken as control. All the flasks were incubated at 30 °C for 15 days and the cell growth and utilization of the hydrocarbons were analyzed. Cell growth was measured by determining the colony forming unit (CFU)/ml. After completing the incubation, 100 µl of a 10^{-5} dilution was spread on nutrient agar plates and colonies formed were counted after 12h of incubation at 30^{o}C. Crude oil biodegradation was studied by measuring the peak area of alkane components of crude oil, using GC-MS. and comparing with the control. The alkane fraction in the medium was extracted with the equal amount of n-hexane. BH medium containing 2% crude oil was used as the control. Percent degradation was calculated by following the equation.

$$Percent\ degradation = \left(1 - \frac{peak\ area\ of\ the\ hydrocarbon\ in\ the\ sample}{peak\ area\ of\ the\ hydrocarbon\ in\ the\ control}\right) * 100$$

GC-MS analysis was done with a Shimadzu QP 2010 Gas Chromatograph Mass Spectrometer, fitted with Rxi®-5ms column (Restek) with 30m x 0.25 mm ID. The instrument conditions were the following: helium column flow 1 ml/min, pressure 18.89 psi and split ratio 25:0. Injection volume was 1.0 µl. Initial temperature was kept at 70 °C for 5 min with a temperature ramp of 14 °C min^{-1} and the final temperature of 280 °C was kept for 10 min with a total run time 30 min (Malatova, 2005).

7.2.6 Growth Inhibition of P. putida *by* Pseudozyma *Biosurfactants*

The growth inhibition kinetics of *P. putida* was studied in glucose medium containing varying concentrations of biosurfactants. The specific growth rate was calculated by sampling the cells at regular time intervals. Linearized Haldane growth inhibitory equation was used for representing growth inhibition kinetics and determination of growth inhibition constant (Gottumukkala et al., 2014).

Specific growth rate, $\mu = ((\log_{10}Z - \log_{10}Z_0)\ 2.303)/\ (t - t_0)$

Z and Z_0 corresponded to the cell growth at time t and t_0, respectively.

Linearized Haldane Equation, $1/\mu = 1/\mu_{max} + (I/K_i\mu_{max})$

Linear graph was plotted with 1/µ *versus* initial inhibitor concentrations (S). Maximum specific growth rate (µmax) was obtained from the slope of the curve, and inhibition constant (Ki) was derived from intercept on X axis.

7.2.7 Hydrocarbon Degradation by Pseudozyma

Hydrocarbon degradation by *Pseudozyma* sp. NII 8165 was studied by growing the yeast in BH medium containing diesel, kerosene and petrol as the carbon source at 30 °C, 200 rpm for 35 days. All these were obtained from the local market and were of commercial grade. BH

medium containing no carbon source, inoculated with the *Pseudozyma* was taken as negative control. Sampling was performed at seven days interval to measure the cell dry weight. At the end of incubation, INT assay was performed as indicated above. Hydrocarbon utilization was studied by analysing the hydrocarbon using GC-MS.

7.2.8 Effect of Pseudozyma *Culture Broth on Hydrocarbon Degradation by* P. putida

Seed culture of *P. putida* (5%) was inoculated into BH medium containing 2% crude oil. Culture broth from a nine day old *Pseudozyma* fermentation was supplemented at 2% (v/v) to the above culture medium. Improvement in the degradation was studied by measuring the cell growth and hydrocarbon analysis.

7.2.9 Statistical Analysis

All the values were presented as the mean± standard deviation from at least two experiments. Student's t-test was performed to analyse the statistical significance. A probability level of $P<0.05$ was considered statistically significant [Daniel, 2000].

7.3 Results and Discussion

7.3.1 Screening of P. putida *strains*

For selecting a model hydrocarbon degrading strain, *P. putida* strains were screened based on the INT assay. All of the cultures screened gave a red precipitate in the INT assay. *P. putida* MTCC 1194 was selected as the most potential hydrocarbon degrader as it gave an intense red precipitate in the INT assay. *P. putida* NBTC 0522 and *P. putida* NBTC 0823 gave moderate red precipitate and all other strains gave a slight red precipitate (Table 7.1). INT assay is a routinely used screening method to determine the potential hydrocarbon degrading microbes. Malatova [2005] had employed microtitre based INT assay to investigate the hydrocarbon degradation potential of microbial isolates. In the field studies, INT can be used

as a reliable and efficient method to measure overall microbial activity associated with hydrocarbon degradation [Mathew and Obbard, 2001].

P. putida is a widely used microbe for bioremediation due to its hydrocarbon degradation potential and bio-safety. Successful clean-ups of oil contaminated sites employing *P. putida* along with fertilizers have been reported [Raghavan and Vivekanandan, 1999]. The bacterium possesses both alkane and polyaromatic hydrocarbon degradation pathways, hence can utilize complex substrates such as crude oil.

Table 7.1 INT assay based screening of *P. putida* strains

Organisms	Scoring
P. putida MTCC 1190	+
P. putida MTCC 1192	+
P. putida MTCC 1194	+++
P. putida NBTC 0822	++
P. putida NBTC 0823	++
P. putida MTCC 1273	+
P. putida MTCC 102	+

7.3.2 Pseudozyma *Biosurfactants*

A number of glycolipids are produced by *Pseudozyma*, which have isomeric structure, hence require multi-step purification. Since bioremediation is a cost-sensitive method, bio-augmentation with pure metabolites does not seem economically feasible. Hence, total biosurfactants produced by *Pseudozyma* sp. NII 08165 were extracted and used to study the improvement in biodegradation.

Pseudozyma sp. NII 08165 was a good producer of glycolipid biosurfactants, including all the three isomers of mannosylerythritol lipids (MEL) along with unknown glycolipids. MEL-C,

the major glycolipid produced by *Pseudozyma* sp. NII 08165 contained a non-conventional hydrophobic structure, with shorter chain fatty acids like C2 or C4 at the C-2' position and long chain fatty acids such as C14, C16 and C18 at C-3' position of the mannose moiety [Sajna et al., 2013a]. MEL-C having this particular structure has been proposed to be highly water soluble, hence of application in the formulation of water- in-oil type emulsifiers and detergent formulations [Morita et al., 2008]. The potential of these glycolipid biosurfactants as laundry additives have been reported by fabric wash analysis. In addition to that, they are highly active at high temperature and alkaline pH [Sajna et al., 2013a].

7.3.3 Measurement of Surface Activity of Pseudozyma *biosurfactants*

The critical micelle concentration (CMC) of *Pseudozyma* biosurfactants was 10 mg/l and surface tension at CMC was 33.67 mN/m[Figure 7.1]. CMC is the significant parameter to be considered for the application of surfactants in bioremediation as it indicates the relative concentration of surfactant at which improvement in biodegradation can be observed. Hence, the concentration of biosurfactants to be added for the improving the bioremediation should be studied above and below CMC, which varied in the range of 1 to 100 mg/l. *Pseudozyma* biosurfactants exhibited high surface activity when compared to the reported CMC values of rhamnolipids and surfactin, which were 50 and 45 mg/l, respectively. The application of surfactant above the concentration range of its CMC could lead to improvement in the bioremediation by increasing the bioavailability of hydrocarbons. Solubility of petroleum hydrocarbons was improved remarkably by the addition of biosurfactants such as rhamnolipids and surfactin above their CMC. However, at higher concentration, biosurfactants may have an inhibitory effect on the bioremediation as they exhibit antimicrobial activity [Whang et al., 2008]. Therefore, it is necessary to study the effect of biosurfactants on the bioremediation at different concentration of biosurfactants.

Zeng et al., [2011] studied the effect of monorhamnolipids on the degradation of hexadecane by *Candida tropicalis*, where the CMC of rhamnolipids was determined to be 38.0 mg/l and enhanced biodegradation was observed with the supplementation of 19.0 mg/l of rhamnolipids. Adsorption of rhamnolipids to the cell surface of polyaromatic hydrocarbon (PAH) degrading bacteria depend on the CMC of rhamnolipids. An increase in the concentration of rhamnolipids resulted in increased adsorption until the concentration of rhamnolipids reached CMC, where the adsorption reached a plateau [Zhao et al., 2011]. The addition of biosurfactants from *Bacillus subtilis* below its CMC did not result in any increase in the bioremediation while bioremediation was stimulated by the addition of biosurfactants above CMC [Moran et al., 2000]. Ron & Rosenberg [2002] reported that the addition of low molecular weight biosurfactants with low CMC value resulted in significant improvements in hydrocarbon solubility by incorporating them to the hydrophobic cavities of micelles.

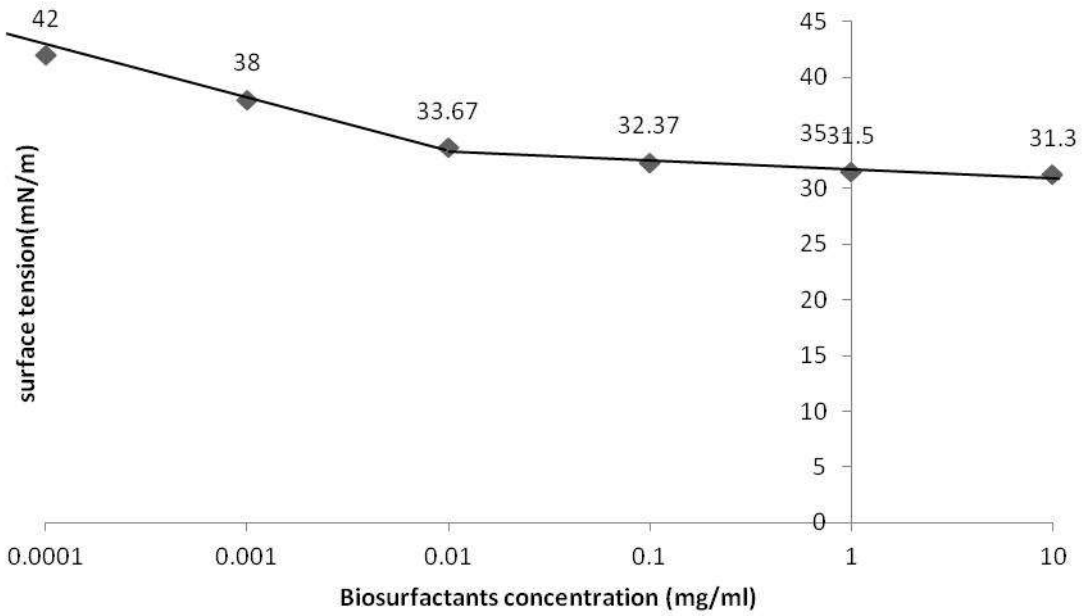

Figure 7.1 Surface activity of *Pseudozyma* biosurfactants

7.3.4 Effect of **Pseudozyma** *Biosurfactants on Crude Oil Degradation by* **P. putida**

Efficiency in the degradation of crude oil was determined by measuring the cell growth and hydrocarbon utilization. Cell growth is indicative of hydrocarbon degradation as complete mineralization results in the formation of biomass, CO_2 and H_2O. The cell count of *P.putida* in control BH medium (without the addition of biosurfactants) was 2.6x 10^6 CFU/ml. Highest cell count (10x10^6 CFU/ml), which was approximately 4X higher than the control, was observed at a biosurfactants concentration of 2.5 mg/l and there was slight decrease in cell count with the increase in biosurfactant supplementation up to 10 mg/l. Cell count at 1, 2.5, 5 and 10 mg/l were 4.1x10^6, 10x10^6, 8x10^6 and 6.5x10^6 CFU/ml respectively. Thereafter, the cell count decreased rapidly with increase in biosurfactant concentration and no colonies were observed at the concentration of 100 mg/l. Cell count observed at the concentrations of 20, 40, 80 and 100 mg/l were 2.1x10^6, 9x$10^{5,}$ 4x10^5 and 0 CFU/ml respectively, indicating the toxicity of biosurfactant at higher concentrations.

Using GC-MS, crude oil utilization by *P. putida* in the presence of *Pseudozyma* biosurfactants was studied. Figure 7.2 shows the percentage degradation of alkanes present in the crude oil by *P. putida* with and without 2.5 mg/l biosurfactants supplementation. A significant level of biodegradation of alkanes was observed on the addition of 2.5 mg/l biosurfactants ($P<0.05$). Supplementation of the biosurfactants improved the degradation of decane (26.7%), undecane (22.5%), dodecane (52.8%), tridecane (23.6%), tetradecane (20.2%), pentadecane (21.4%), hexadecane (10.9%), heptadecane (27.9%), octadecane (21.9%), nonadecane (24%), icosane (19.7%), heneicosane (28.8%), docosane (31.9%), tricosane (13.4%) and tetracosane (6.2%). An average of 23.5 % improvement in the degradation of C10-C24 alkanes was observed. Among the alkanes, dodecane was degraded most while tetracosane, followed by hexadecane was least degraded. The rate of degradation decreased with increase in the chain length of hydrocarbon. The preferential pattern of

hydrocarbon utilization by the microbe when growing in a mixture of complex hydrocarbons suggested that lower chain molecules were utilized in the initial stage and higher chain alkanes towards the later stage of growth. Nevertheless, the rate of hexadecane utilization was low even though it was not a higher chain length alkane. The cleavage of carbon atoms during the degradation of higher chain alkanes might result in the accumulation of medium chain alkanes such as hexadecane, which could possibly explain this observation. Both CFU determination and hydrocarbon analyses revealed that the addition of *Pseudozyma* biosurfactants at a concentration of 2.5 mg/l improved the biodegradation of crude oil by *P. putida.*

Most of the biosurfactants exhibit toxicity at higher concentration, which affects the cell surface properties or metabolic pathways of microbes, leading to the decline in biomass required for degrading the hydrocarbon, and thus hampers the bioremediation. Since the CMC of *Pseudozyma* biosurfactants was 10 mg/l and the surfactant toxicity was exhibited at concentrations above 10 mg/l, it was possible that the formation of hydrocarbon micelles by *Pseudozyma* biosurfactants were the reasons for the decline in hydrocarbon degradation at higher concentrations. Micelle formation may reduce the efficacy of bioremediation, since the aqueous phase concentration of the pollutants is reduced [National Research Council, 1993].

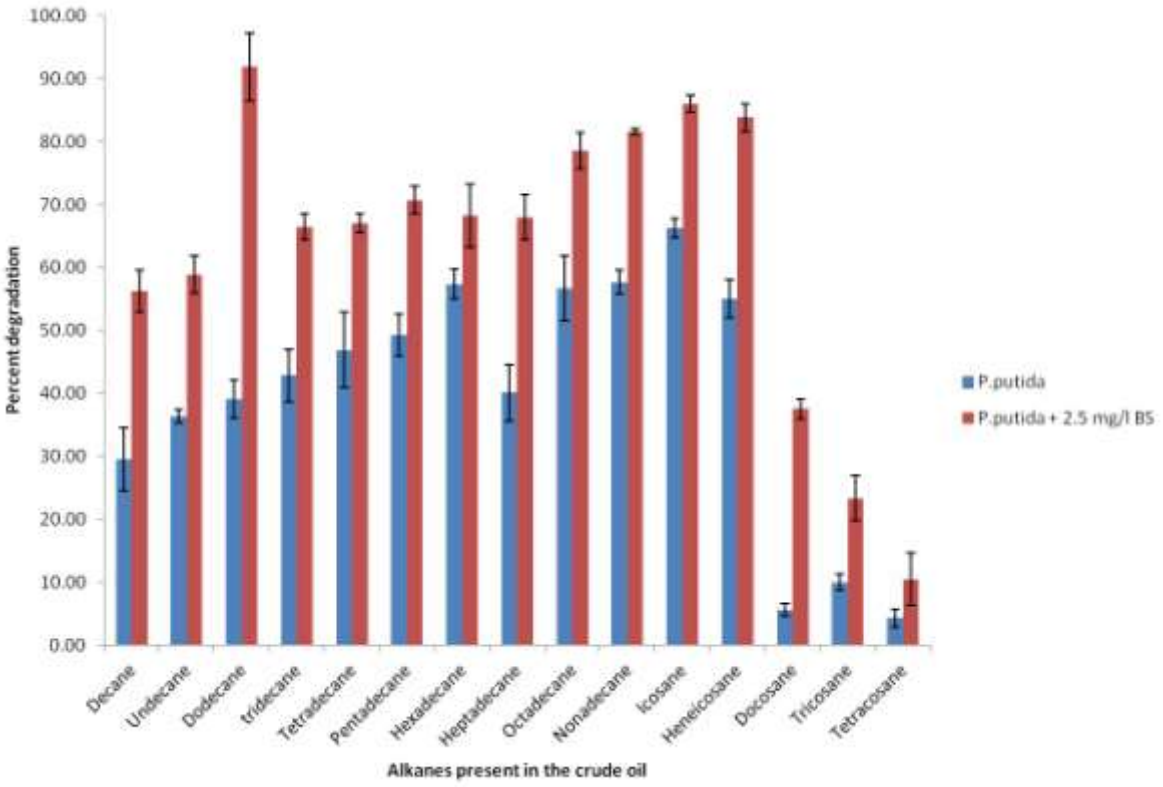

Figure 7.2 Percent degradation of alkanes present in the crude oil by *P. putida* MTCC 1194 on supplementation of 2.5 mg/l biosurfactants

7.3.5 Growth Inhibition Kinetics of Biosurfactants

Growth kinetics of *P. putida* was studied by growing the organism in glucose containing medium at different concentrations of *Pseudozyma* biosurfactants (Figures 7.3 & 7.4). Specific growth rate of *P. putida* at 1.0 or 2.5 mg/l supplementation of biosurfactants was almost similar to that of the bacterium grown without biosurfactants. However, specific growth rate decreased significantly at and above the concentration of 20 mg/l when it became negative. Plotting the linearized Haldane equation gave a line with the regression coefficient of 0.923. Growth inhibition constant (Ki) and maximum specific growth rate (μ max) were 11.07 mg/l and 0.434, respectively. This indicated that the concentration of biosurfactants to be added should be less than 11.07 mg/l and higher concentrations might negatively affect the growth of the organism and its hydrocarbon biodegradation efficiency.

Many biosurfactants exhibit antimicrobial activity and the antimicrobial activity depend on the concentration of biosurfactant and type of strains used for bioremediation. Mannosylerythritol lipids A and B have antimicrobial activity against Gram-positive bacteria and *Pseudomonas* strains are relatively more sensitive to MEL than other Gram-negative bacteria [Kitamoto et al., 1993]. Whang et al., [2008] reported that the addition of surfactin above 80 mg/l had an inhibitory effect on diesel biodegradation by a microbial consortium, which was attributed to cell disruption property of surfactin at higher concentrations. Similarly, the addition of rhamnolipids at a concentration of 240 mg/l had an inhibitory effect on the growth of two hydrocarbon degrading strains, thus preventing phenanthrene degradation by these organisms [Shin et al., 2005]. Nevertheless, it is also known that biosurfactants have significantly lower toxicity than chemical surfactants [Lim et al., 2011].

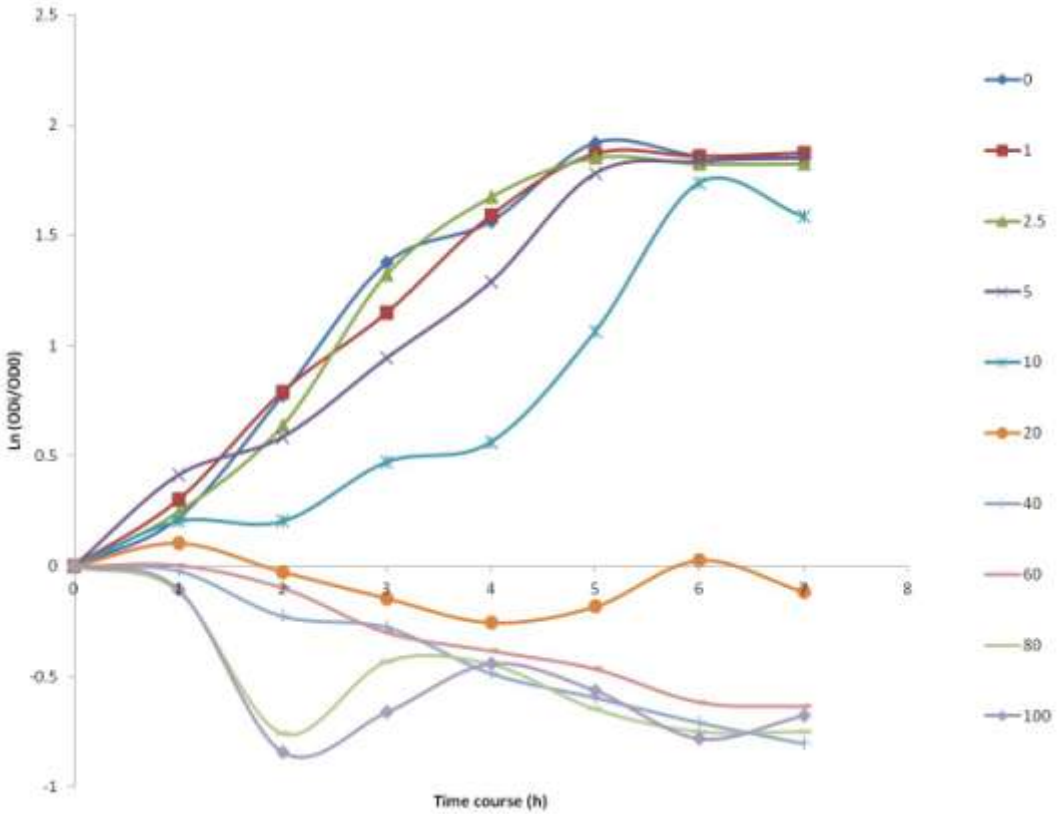

Figure 7.3 Growth kinetics of *P.putida* MTCC 1194 at various concentrations of *Pseudozyma* biosurfactants.

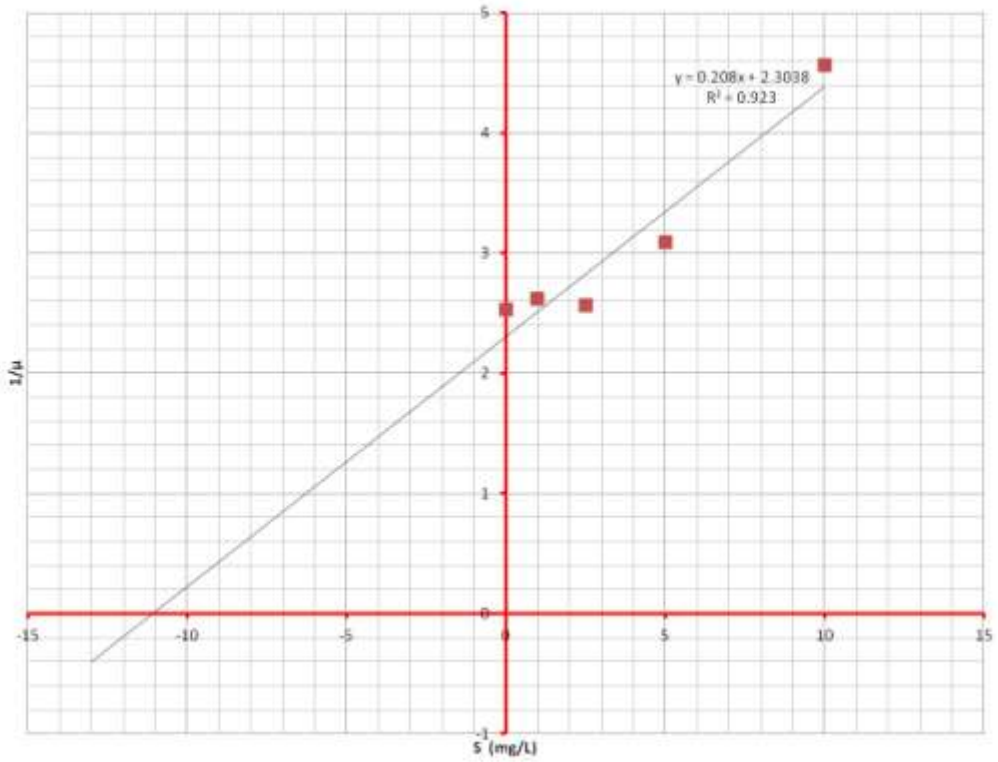

Figure 7.4 Growth inhibition kinetics of *P. putida* MTCC 1194 at various concentrations of *Pseudozyma* biosurfactants.

7.3.6 *Hydrocarbon Degradation by* Pseudozyma *sp. NII 08165*

Pseudozyma sp. NII 08165 grew by forming smaller emulsions of hydrocarbon in the medium containing diesel and kerosene. Cell growth measurement of *Pseudozyma* growing in different hydrocarbons is shown in Figure 7.5. High biomass yield was observed in the medium containing diesel, followed by kerosene, and the growth was comparatively low in petrol. Since *Pseudozyma* grew in diesel, which contained higher chain alkanes than petrol, the reduced growth in the medium containing petrol could be attributed to the rapid vaporization of petrol at 30 °C. INT assay was positive for *Pseudozyma* grown in the medium containing diesel and kerosene (Figure 7.6). GC-MS analyses revealed that in the medium with diesel, there was a complete utilization of lower chain alkanes (<C9) and a substantial decline in the medium chain alkanes with C9- C31, compared to the control (un-inoculated

BH medium containing diesel). From these results, it was concluded that *Pseudozyma* sp. NII 0815 was capable of degrading the hydrocarbons.

Many yeast species have been reported to degrade hydrocarbon and some by producing biosurfactants. *C. antartica*, during its growth on hydrocarbon produces biosurfactants, which alter the surface properties of microorganism and promotes the biodegradation of n-alkanes [Hua et al., 2003]. However, *Pseudozyma* sp. NII 08165 did not secrete any biosurfactants during its growth on hydrocarbon, as no glycolipids spots were detected by the TLC. Kitamoto et al. [2011] had demonstrated the plastic degrading potential of *Pseudozyma antartica* that could degrade plastic films made from polybutylene succinate.

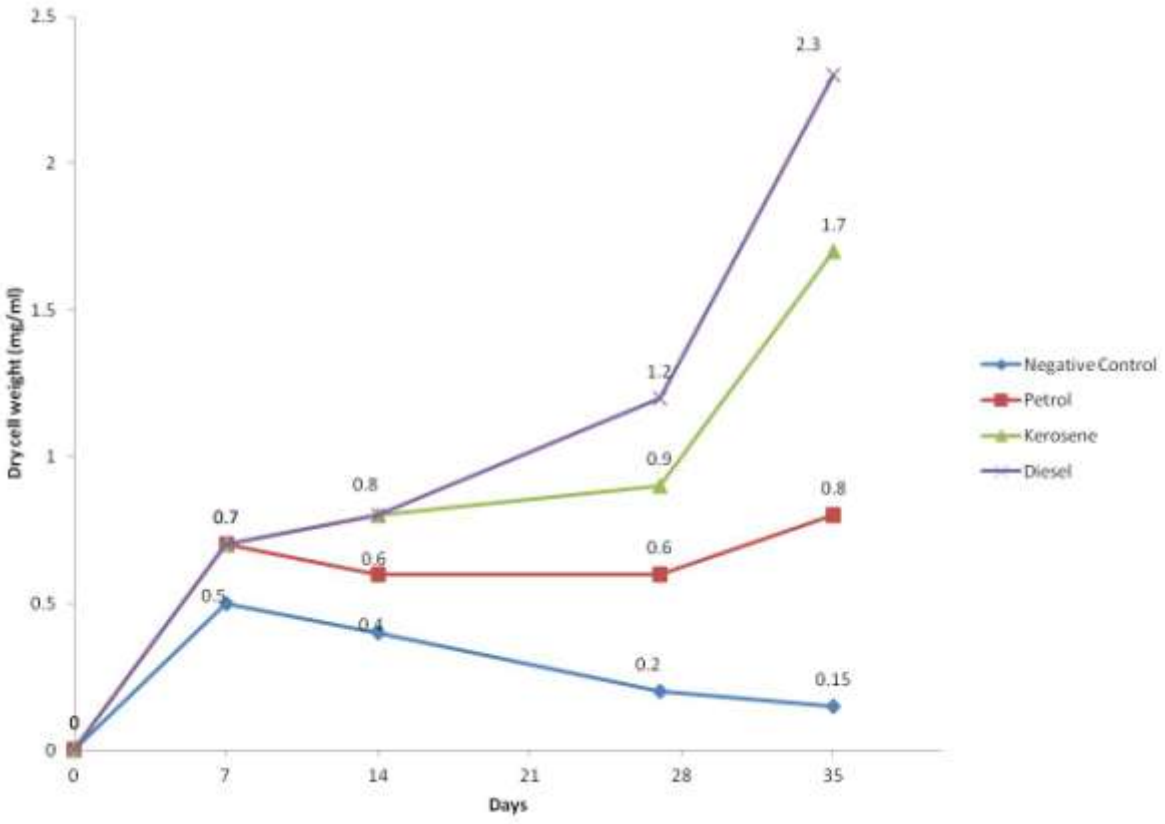

Figure 7.5 Measurement of cell growth of *Pseudozyma* sp. NII 08165, in a medium containing petrol, diesel and kerosene at various time interval. Here negative control is the organism growing in BH medium without any carbon source.

Figure 7.6 INT assay indicating the growth of *Pseudozyma* in medium containing different hydrocarbons such as petrol, diesel and kerosene. Here, A denotes petrol, B denotes diesel, C denotes kerosene and D is the negative control, *Pseudozyma* growing in BH medium without any carbon source.

7.3.7 *Effect of* Pseudozyma *Culture Broth on Hydrocarbon Degradation by* P. putida

Crude oil was efficiently degraded by *P. putida* culture supplemented with the *Pseudozyma* culture broth. The cell count in *P. putida* culture supplemented with the *Pseudozyma* culture broth was 7.80×10^7 CFU/ml, whereas it was only 3×10^6 CFU/ml in the un-supplemented control. Microscopic observation revealed that the developed colonies were *P. putida* only and there were no visible yeast cells. On plating also, the colonies developed were only of *P. putida* and yeast colonies were not detected (results not shown). Percentage degradation of the alkanes in the crude oil by the *P. putida* cultures supplemented with or without *Pseudozyma* culture broth is shown in Figure 7.7. GC-MS analyses revealed that the supplementation of *Pseudozyma* culture broth improved the degradation of decane (48%), undecane (53.9%), dodecane (22.8%), tridecane (37.2%), tetradecane (32.9%), pentadecane (34.7%), hexadecane (27.4%), heptadecane (45%), octadecane (35.4%), nonadecane (34%), icosane (27.4%), heneicosane (34.8%), docosane (91.4%), tricosane (87.5 %) and tetracosane (76.6%). This indicated that the addition of *Pseudozyma* culture broth resulted in improved degradation, even better than that observed for pure biosurfactants supplementation [$P<0.05$].

On an average, 45.9% improvement in the degradation of C10-C24 was observed. Enhancement in the degradation could be attributed to an enhanced growth of *P putida* itself and the presence of biosurfactants in the *Pseudozyma* culture broth. The neutral lipids and fatty acids present in the culture broth could also have supported a higher biomass of *P. putida*. *Pseudozyma* culture broth might contain other metabolites that could aid the bioremediation; the yeast has been previously demonstrated to produce exopolysaccharides (EPS) [Sajna et al., 2013b]. The EPS in the culture broth could also stabilize the hydrocarbon emulsion and improve the bioavailability. The addition of the culture broth could also surpass the inhibitory effect of biosurfactants, which was more prominent when biosurfactants were added in pure form. Biosurfactants assisted hydrocarbon degradation by the supplementation of culture broth containing the biosurfactant(s) could be a cost effective strategy, and thus ideal for large-scale bioremediation as it would avoid the cell removal and multi-step purifications for biosurfactants. The strategy would become more effective when the culture producing the biosurfactant itself is capable of the targeted bioremediation as was the case in this study. This could ensure that even if the cells of the biosurfactant producing microbe were present in the culture broth, this would only aid further the bioremediation. Banat et al., [1991] employed biosurfactant containing culture broth for the cost-effective cleanup of the oil tank and obtained 90% recovery of crude oil from the sludge. The addition of biofertilizers could be avoided to a larger extent as biosurfactant containing broth itself would improve the bioremediation, thus avoiding the environmental impact associated with the addition of fertilizer [Ron & Rosenberg, 2002].

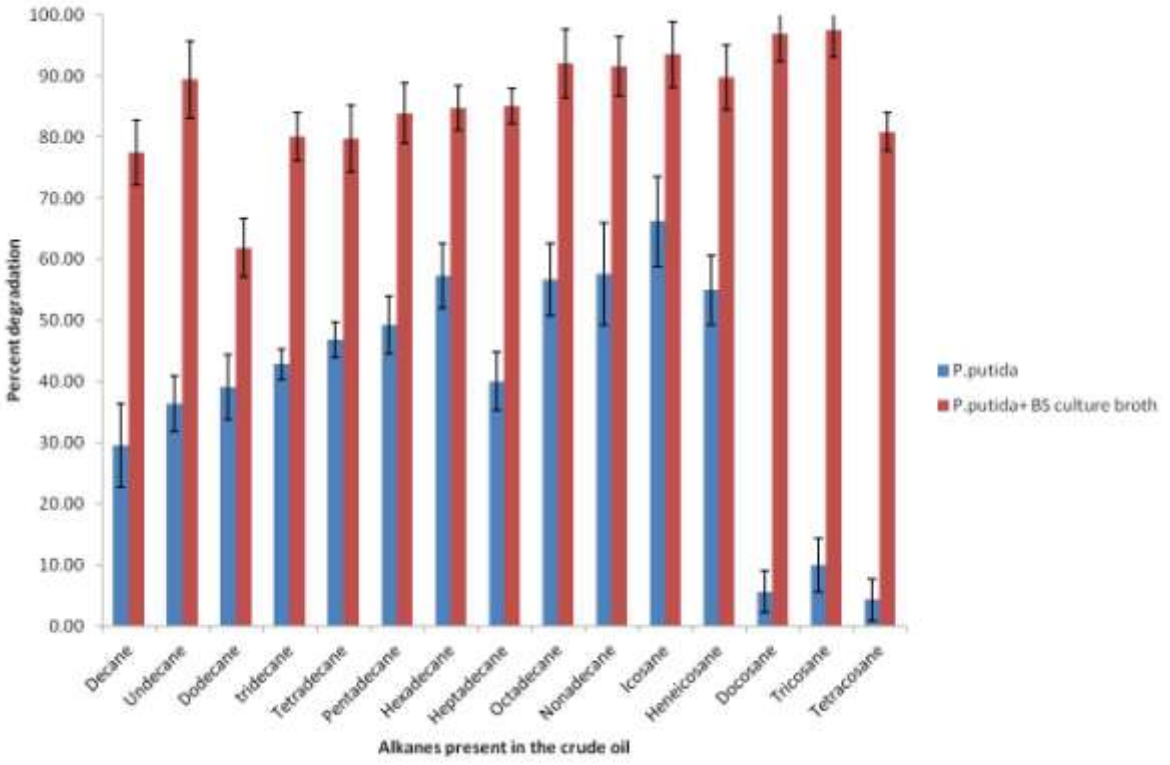

Figure 7.7 Percent degradation of alkanes present in the crude oil by *P. putida* MTCC 1194 and *P. putida* MTCC 1194 on supplementation of *Pseudozyma* culture broth.

7.4 Summary and Conclusions

Biosurfactant-assisted bioremediation using biosurfactants from a *Pseudozyma* isolate was attempted for the first time. *Pseudozyma* sp NII 08165 produced glycolipid biosurfactants including mannosylerythritol lipids. Crude oil degradation by *P. putida* was improved by the supplementation of biosurfactants, but at higher concentrations of biosurfactants, there was an inhibitory effect on the cell growth. Growth inhibitory kinetics revealed that *K*i of *Pseudozyma* biosurfactants on the growth of *P. putida* was 11.07 mg/l. From these results, it was concluded that the supplementation of *P. putida* culture with *Pseudozyma* culture broth could be used as an effective strategy for the improved and cost-effective biodegradation of crude oil.

Chapter 8: Summary and Conclusions

Biosurfactants and exopolysaccharides are the two commercially valuable components of the white biotechnology and a have a broad scope in industrial and medical sectors. Microbial surfactants are potential replacement for the chemicals, or oleo-based surfactants due to their high performance, low ecotoxicity, biodegradability and low carbon footprints and are currently used in for various applications such as petroleum industry, bioremediation, detergent industry, cosmetic industry and therapeutic strategies. Exopolysaccharides (EPS) are high molecular weight compounds secreted by the microorganism to the surroundings and are extensively used in food and feed, pharmaceuticals and agricultural industry. Functionality and prospects of EPS are determined by monomeric composition, structural conformation and molecular weight of the polymer.

Pseudozyma species are ustilaginomycetes anamorphic yeast and some strains are reported to be good producers of biosurfactants. *Pseudozyma* sp NII08165 proved to be a versatile microbe, which produced biosurfactants and EPS by simple modification of fermentation medium, more specifically by changing the carbon source in the medium. In culture medium containing vegetable oil as carbon source, *Pseudozyma* sp NII 08165 produced biosurfactants while the addition of glucose as carbon source resulted in the production of EPS.

Pseudozyma sp. NII 08165 produced glycolipid biosurfactants, which was a combination of all the three Mannosylerythritol lipids (MEL) isomers along with some unknown glycolipids. The total production of MEL was 12 g/l and MEL-C was produced in highest quantity than all other MEL. The yield of MEL was increased up to 34 g/l when the concentration of vegetable oil was enhanced. The structural characterization of purified MEL revealed the hydrophobic structure of MEL-C consisting of short chain fatty acid (C2 or C4) at the C-2’ position and a long chain fatty acid (C14, C16 or C18) at the C-3’ position of the mannose

moiety. The MEL-C showed good surface activity with critical micelle concentration (CMC) of 4.5 x 10^{-6} M and 33 mN/m surface tension at the CMC.

Pseudozyma sp. NII 08165 secreted viscous compound, which was a novel EPS as revealed by performing a detailed structural characterization. A yield of 3.5 g/l EPS was obtained on fourth day of fermentation of *Pseudozyma* sp. NII 08165.The EPS was a high molecular weight polymer of 1.7 MDa with α–D-glucopyranose, α-D-galactopyranose and α-D-mannopyranose as monomers. FTIR analysis showed the presence of α -glycosidic linkages in the *Pseudozyma* EPS. Detailed structural elucidation by ^{1}H NMR revealed the presence of α-L-rhamnopyranose and N-acetyl-β-D- glucopyranosamine also in the polymer. Helix-coil transition analysis by Congo Red assay demonstrated a helical conformation of *Pseudozyma* EPS. The compact microstructure suggested the potential application of *Pseudozyma* EPS in preparing plasticized films. Further, the microstructure of *Pseudozyma* EPS was explored by AFM.

EPS produced by *Pseudozyma* sp. NII 08165 was investigated with regard to their physicochemical properties to find out the potential applications. EPS exhibited excellent thermal stability making it a potential candidate for industrial applications. The pseudoplastic behaviour of aqueous EPS indicated the potential applications of *Pseudozyma* EPS as a thickening agent, or gelling agent in food industry. It also possessed significant thickening efficiency and suspending ability along with good water holding capacity, which further emphasised on its application in food, cosmetic and pharmaceutical industries. Emulsifying and flocculating activity revealed that it could be exploited as potential emulsifying and flocculating agent.

Pseudozyma biosurfactants was explored to be used as laundry additive. Physio-chemical properties of *Pseudozyma* biosurfactants favoured their scope of application as laundry

additive. *Pseudozyma* biosurfactants removed stains efficiently and could be used as additive in next-generation detergents or cleaning formulations.

The implication of *Pseudozyma* biosurfactants in the hydrocarbon degradation showed that crude oil degradation by *Pseudomonas putida* was improved on the supplementation of optimized concentration of biosurfactants, but after a particular concentration, biosurfactants exhibited an inhibitory effect on biodegradation due to toxicity. Growth inhibitory kinetics revealed that inhibition constant of *Pseudozyma* biosurfactants on *P.putida* was 11 mg/l. The results also revealed that *Pseudozyma* sp NII 08165 could be an efficient hydrocarbon degrader, which could be a potential candidate for formulation of consortia n bioremediation of oil spill. Further studies showed that supplementation of *Pseudozyma* culture broth along with *P.putida* might be used as an effective strategy for the improved biodegradation of crude oil.

BIBILIOGRPAHY

Abdel-Mawgoud, A. M., Hausmann, R., Francois Lépine, F., Müller, M. M., Déziel, E.[2011]. Rhamnolipids: Detection, Analysis, Biosynthesis, Genetic Regulation, and Bioengineering of Production, *In* Biosurfactants, Microbiology Monographs 20, *Ed* Soberón-Chávez, G., Berlin Heidelberg, Springer, 13-55.

Ahmed, Z., Wang, Y., Anjum, N., Ahmad, A., Khan, S. T., [2013]. Characterization of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir – Part II. *Food Hydrocolloids*, 30, 343-350.

Alex, D., Shainu, A., Pandey, A., Sukumaran, R.K. [2014]. Esterase Active in Polar Organic Solvents from the yeast *Pseudozyma* sp. NII 08165. *Enzyme Research*, http://dx.doi.org/10.1155/2014/494682

Allen, M. S., Welch, K. T., Prebyl, B. S., Baker, D. C., Meyers, A. J., Sayler, G. S. [2004]. Analysis and glycosyl composition of the exopolysaccharide isolated from the floc-forming wastewater bacterium *Thauera* sp. MZ1T. *Environmental Microbiology*, 6, 780–790.

Arias, S., Moral, A. D., Ferrer, M. R., Tallon, R., Quesada, E., Bejar, V. [2003]. Mauran, an exopolysaccharide produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology. *Extremophles*, 7, 319-326.

Ashby, R. D., Zerkowki, J. A., Solaiman, D. K. Y., Liu, L. S. [2011]. Biopolymer Scaffolds for Use in Delivering Antimicrobial Sophorolipids to the Acne-causing Bacterium *Propionibacterium acnes*. *New Biotechnology*, 28, 24-30.

Atlas, R.M. [1991]. Microbial Hydrocarbon degradation-Bioremediation of Oil Spills, *Journal of Chemical Technology and Biotechnology*, 52, 149-156.

Baets, S.D., Vandamme, E.J. [2001]. Extracellular *Tremella* polysaccharides: structure, properties and applications, *Biotechnology Letters*, 23, 1361–1366.

Bajaj, I.B., Survase, S. A., Saudagar, P. S., Singhal, R. S. [2007]. Gellan Gum: Fermentative Production, Downstream Processing and Applications. *Food Technology and Biotechnology* 45, 341-354.

Banat, I. M. [1995]. Biosurfactants Production and Possible Uses in Microbial Enhanced Oil recovery and Oil Pollution Remediation: A Review, *Bioresource Technology*, 51, 1-12.

Banat, I. M., Samarah, N., Murad, M., Horne, R., Banerjee, S. [1991]. Biosurfactant production and use in oil tank clean-up. *World Journal of Microbial Biotechnology*, 7, 80-88.

Black, R. E., Hurley, F. J., Havery, D. C., [2001], Occurrence of 1,4-Dioxane in Cosmetic Raw Materials and Finished Cosmetic Products. *Journal of Aoac International*, 84, 666-70.

Blank, L., Rosenau, F., Wilhelm, S., Wittgens, A., Tiso, T. [2011]. Means and Methods for Rhamnolipid production, Patent EP 2,573,172.

Bluth, M. H., Kandil, E., Mueller, C. M., Shah, V., Lin, Y. Y, Zhang, H., Dresner, L., Lempert, L., Nowakowski, M., Gross, R., Schulze, R., Zenilman, M. E. [2006]. Sophorolipids Block Lethal Effects of Septic Shock in Rats in a Cecal Ligation and Puncture Model of Experimental Sepsis. *Critical Care Medicine*, 34, 188-195

Bradford, M. M. [1976]. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle protein–dye binding. *Analytical Biochemistry*, 72, 248–254.

Bralkowski, M. P., Brooks, S. A., Hinton, S. M., Wright, D. M., Yang, S. H. [2013a]. Improved poultry farm practices. Patent WO 2013110133.

Bralkowski, M. P., Brooks, S. A., Hinton, S. M., Wright, D. M., Yang, S. H. [2013b]. Method of producing biosurfactants. Patent WO 2013110132.

Bramachari, P. V., Kishore, P. B., Ramadevi, R., Kumar, R., Rao, B. R., Dubey, S. K. [2007]. Isolation and characterization of mucous exopolysaccharide (EPS) produced by *Vibrio furnissii* strain VB0S3. *Journal of Microbial Biotechnology*, 17. 44-51.

Briechle, S., Frever, T. F., Hollmann, R., Kappler, T., Leonhardt, B., Schmidt, J. K., Visser, F., Wenzke, B. [2012]. Method for extracting crude oil from crude oil reservoirs with a high reservoir temperature. Patent WO 2012110539 A1.

Buxdorf, K., Rahat, I., Levy, M. [2013]. *Pseudozyma aphidis* induces ethylene-independent resistance in plants. *Plant Signalling & Behaviour*, 8, doi: 10.4161/psb.26273.

Buzzini,P., Branda, E., Goretti, M., Turchetti, B. [2012]. Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. *FEMS Microbiology Ecology*, 1-25.

Calvo, C., Ferrer, M. R., Martinez-Checa, F., Bejar, V., Quesada, E. [1995]. Some rheological properties of the extracellular polysaccharide produced by *Volcaniella eurihalina* F2-7. *Applied Biochemistry and Biotechnology*, 55, 45-54.

Cern, M., Barros, A. S., Nunes, A., Rocha, S. M., Delgadillo, I., Copikova, J., Coimbra, M. A. [2003]. Use of FT-IR spectroscopy as a tool for the analysis of polysaccharide food additives. *Carbohydrate Polymer*, 51, 383–389.

Chang, H. C. and Kobzeff, J. M. [1993]. High-glyceryl, low-acetyl gellan gum for non-brittle gels. Patent US 5190927 A.

Chang, J. S., Radosevich, M., Jin, Y., Cha, D.K. [2004]. Enhancement of phenanthrene solubilization and biodegradation by trehalose lipid biosurfactants. *Environmental Toxicology and Chemistry*, 23, 2816-2822.

Chang, M. H., Kim, H. J., Jahng, H. K. Y., Hong, S.C. [2008]. The isolation and characterization of *Pseudozyma* sp. JCC 207, a novel producer of squalene. Applied Microbiology & Biotechnology, 78, 963-972.

Chen, Q., Bao, M., Fan, X., Liang, S., Sun, P. [2013]. Rhamnolipids enhance marine oil spill bioremediation in laboratory system. *Marine Pollution Bulletin*, doi: 10.1016/j.marpolbul.2013.01.037.

Chen, C. C., [2006]. Rhamnolipids Production with Denitrying *Pseudomonas aeruginosa*. Ph.D thesis, The University Of Akron, 142 pages.

Chiura, H., Iizuka, M., Yamamoto, T. [1982]. A Glucomannanas an extracellular Product of *Candida utilis*. I. Production and Characterization of a Glucomannan, *Agricultural and Biological Chemistry*, 46, 1723-1731.

Cho,D. H., Chae, H.J., Kim, E.Y. [2001]. Synthesis and Characterization of a Novel Extracellular Polysaccharide by *Rhodotorula glutinis*. *Applied Biochemistry and Biotechnology*, 95,183-193.

Chowdhury, S. R., Basak, R. K., Sen, R., Adhikari, B. [2011]. Characterization and emulsifying property of a carbohydrate polymer produced by *Bacillus pumilus* UW-02 isolated from waste water irrigated agricultural soil. *International Journal of Biological Macromolecules*, 48 , 705-712.

Cirigliano, M. C., Carman, G. M. [1985]. Purification and characterization of Liposan, a bioemulsifier from *Candida lipolytica*. *Applied Environmental Microbiology*, 50, 846-850.

Collina, E., Lasagni, M., Pitea, D., Franzetti, A., Di Gennaro, P., Bestetti, G. [2007]. Bioremediation of Diesel Fuel Contaminated Soil: Effect of Non Ionic Surfactants and Selected Bacteria Addition. *Annali di Chimica*, 97,799-805.

Collins, E.A., Kajhan, S. [1998]. Rhamsan gum as mist suppressant in metal working fluids. Patent CA 2251233 A1.

Cooper, D.G., Macdonlad, C.R., Duff, S. J. B., Kosaric, N. [1981]. Enhanced production of surfactin from *bacillus subtilis* by continuous product removal and metal cation additions. *Applied Environmental Microbiology*, 42, 408-412.

Copikova, J., Barros, A.S., Smidova, I., Cerna, M., Teixeira, D. H., Delgadillo, I. Syystya, A., Coimbra, M. A. [2006]. Influence of hydration of food additive polysaccharides on FT-IR spectra distinction. *Carbohydrate Polymer*, 63, 355–359.

Daniel, W.W. [2000]. Hypothesis testing, *In* Biostatistics-A Foundation for Analysis in the health sciences. Seventh edition, *Eds*. Daniel, W.W., John Wiley and Sons Inc., New York, 166-167.

Das, D., Baruah, R., Goyal, A. [2014]. A food additive with prebiotic properties of an α-d-glucan from *Lactobacillus plantarum* DM5. *International Journal of Biological Macromolecules*, 69, 20–26.

Davey, M. E., Caiazza,N.C. and O'Toole, G.A. [2003]. Rhamnolipid Surfactant Production Affects Biofilm Architecture in *Pseudomonas aeruginosa* PAO1. *Journal of Bacteriology*, 185, 1027-1036.

DeCrescenzo H. E., Phillips, D. R., Peterson, J. B. [2007]. Polymyxin E production by *P. amylolyticus*. *Letters in applied microbiology*, 45, 491-496.

Deleu, M., Bouffioux, O., Razafindralambo, H., Paquot, M., Hbid, C., Thonart, P., Jacques, P., Brasseur, R. [2003]. Interaction of Surfactin with Membranes: A Computational Approach. *Langmuir*, 19, 3377-3385.

Deris, Z. Z., Akter, J., Sivanesan, S., Roberts, K. D., Thompson, P. E., Nation, R. L., Li, J., Velkov, T. [2013]. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. *The journal of Antibiotics*, 67, 147-151.

Desai, J.D., Banat, I. M., [1997], Microbial production of surfactants and their commercial potential. *Microbiology and Molecular Biology Reviews*, 61, 47–64.

Desbrieres, J. [1984]. Novel applications of scleroglucane in the treatment of oil wells and oil drilling installation. Patent EP 0146981 A1.

Develter, D. W. G., Lauryssen, L. M. L. [2010]. Properties and industrial applications of sophorolipids. *European Journal of Lipid Science and Technology*, 112, 628-638.

Develter, D., Fleurackers, S. [2012], Improved sophorolactone production, Patent WO 2012168325

Develter, D., Renkin, M. [2012]. Sophorolactone compositions and uses thereof. Patent WO 2012167815

Develter, D., Renkin, M., Jacobs, I. [2006]. Detergent compositions. Patent EP 1445302 B1.

Duboc, P., Mollet, B. (2001). Applications of exopolysaccharides in the dairy industry, *International Dairy Journal*, 11, 759-768.

Dubois, M., Gilles, K. A., Hamiliton, J. K., Rebers, P. A. Smith, F. [1956]. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry,* 28, 350-356.

Dusane, D. H., Nancharaiah, Y. V., Zinjarde, S. S., Venugopalan, V. P. [2010]. Rhamnolipid mediated disruption of marine *Bacillus pumilus* biofilms. *Colloids and Surface B: Biointerfaces*, 81, 242-248.

Eigen, E., Simone, A. J. [1988]. Control of dental plaque and caries using emulsan. Patent US 4,7373,59.

Fanworth, E.R., Champagne, E.R., Van Calsteren, M. R. [2006]. Exopolysaccharides from Lactic Acid Bacteria: Food Uses, Production, Chemical Structures, and Health Effects. *In* Handbook of Nutraceuticals and Functional Foods, Second Edition, *Eds* Wildman, R. E. C., Wildman, R., Wallace, T. C. CRC Press, USA, 350-359.

Fu, J. F. and Tseng, Y.H. [1990]. Construction of lactose-utilizing Xanthomonas campestris and production of xanthan gum from whey. *Applied Environmental Microbiology*, 56, 919–923.

Fukuoka, T., Morita, T., Konishi, M., Imura, T., Kitamoto, D. [2007a]. Characterisation of new types of mannosylerythritol lipids as biosurfactants produced from soybean oil by a basidiomycetes yeast, *Pseudozyma shanxiensis*. *Journal of Oleo Science*, 56, 435–442.

Fukuoka, T., Morita, T., Konishi, M., Imura, T., Kitamoto, D. [2007b]. Characterization of new glycolipid biosurfactants, tri-acylated mannosylerythritol lipids, produced by *Pseudozyma* yeasts. *Biotechnology Letters*, 29, 1111–1118.

Fukuoka, T., Morita, T., Konishi, M., Imura, T., Kitamoto, D. [2008], A basidiomycetous yeast, *Pseudozyma tsukubaensis*, efficiently produces a novel glycolipid biosurfactant. The identification of a new diastereomer of mannosylerythritol lipid-B. *Carbohydrate Research* 343, 555–560.

Funami, T., Funami,M ., Yada, H. and Nakao, Y. [1999]. Rheological and thermal studies on gelling characteristics of curdlan. *Food Hydrocolloids*, 13, 317-324.

Furuta, T., Iqarashi, K., Hiraya, Y. [2004]. Low-foaming detergent compositions. Patent US 20040171512 A1.

Gao, R., Falkeborg, M., Xu, X., Guo, Z., [2013]. Production of sophorolipids with enhanced volumetric productivity by means of high cell density fermentation. *Applied Microbiology and Biotechnology*, 97, 1103-1111.

García-Ochoa F., Santos, V.E., Casas, J.A. and Gomez, E. [2000]. Xanthan gum: production, recovery, and properties. *Biotechnology Advances* 18, 549-579.

Giessler-Blank, S., Schilling, M., Thum, O., Sieverding, E. [2012]. Use of sophorolipids and derivatives thereof in combination with pesticides as adjuvant/additive for plant protection and the industrial non-crop field. Patent US 20120220464.

Golysin, P., Timmis, K., Yakimy, M., [2001]. Hybrid synthetase, hybrid peptide and composition containing same. Patent EP 1,163,346.

Gottumukkala, L.D., Parameswaran, B., Valappil, S.K., Pandey, A., Sukumaran, R. K. [2014]. Growth and butanol production by *Clostridium sporogenes BE01* in rice straw hydrolysate: kinetics of inhibition by organic acids and the strategies for their removal. *Biomass Conversion and Biorefinery,* DOI 10.1007/s13399-013-0110-6.

Grangemard, I., Wallach, J., Maget-Dana, R., Peypoux, F. [2001]. Lichenysin: a more efficient cation chelator than surfactin. *Applied Biochemistry and Biotechnology,* 90, 199-210.

Gunther, M., Grumaz, C., Lorenz, S., Stevens, P., Lindemann, E., Hirth, T., Sohn, K., Zibek, S., Rupp, S. [2015]. The transcriptomic profile of *Pseudozyma aphidis* during production of mannosylerythritol lipids. *Applied Microbiology and Biotechnology*, 99, 1358-88.

Gutnick, D.L. [1987]. The emulsan polymer: Perspectives on a microbial capsule as an industrial product. *Biopolymers, 26, 223-240.*

Gutnik, D. L., Rosenberg, E. [1980].Production of α-emulsan, Patent US 4,230,801.

Gutnik, D. V., Bach, H. [2008]. Potential applications of *Acinetobacter* in biotechnology. In *Acinetobacter* Molecular biology, *Ed*. Gerischer, U., Caister Academic Press, Germany, 231-263.

Haines, J. R., Wrenn, B. A., Holder, E. L., Strohmeier, K. L., Herrington, R. T., Venosa, A.D. [1996]. Measurement of hydrocarbon-degrading microbial populations by a 96-well plate most-probable-number procedure. *Journal of Industrial Microbiology*, 16, 36-41.

Hall, P. J., Haverkamp, J., Kralingen, C. V., Schmidt, M. [1995]. Synergistic dual-surfactant detergent composition containing sophoroselipid. Patent US 5417879 A.

Harju, S., Fedosyuk, H., Peterson, K. R. [2004]. Rapid isolation of yeast genomic DNA: Bust n' Grab. *BMC Biotechnology,* 4, 8-12.

Haxaire, K., Mare'chal, Y., Milas, M., Rinaudo, M. [2002]. Hydration of Polysaccharide Hyaluronan Observed by IR Spectrometry. I. Preliminary Experiments and Band Assignments. *Biopolymers*. 72, 10-20.

Heerklotz, H., Seelig, J. [2001]. Detergent-Like Action of the Antibiotic Peptide Surfactin on Lipid Membranes. *Biophysical Journal*, 81, 1547–1554.

Hees, U., Fabry, B. [1997]. Use of mixture of glyco-lipid and surfactant in hand dish-washing detergent. Patent DE 19600743 A1.

Herman, D. C., Zhang, Y., Miller, R. M., [1997]. Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. *Applied Environmental Microbiology*, 63, 3622–3627.

Hewald, S., Josephs, K., Bolker, M. [2005]. Genetic analysis of biosurfactants production in *Ustilago maydis*. *Applied and Environmental Microbiology*, 71, 3033–304.

Hewald, S., Linne, U., Scherer, M., Marahiel, M.A., Kamper, J., Bolker, M. [2006]. Identification of a gene cluster for biosynthesis of mannosylerythritol lipids in the basidiomycetous fungus *Ustilago maydis*. *Applied Environmental Microbiology*, 72, 5469-77

Höfer, R., Bigorra, J., [2008], Biomass-based Green Chemistry: Sustainable Solutions for Modern Economies, *Green Chemistry Letters and Reviews*, 1, 79-97.

Holdsworth, S. D. [1993]. Rheological models used for the prediction of the flow properties of food products. *Transactions of the Institute of Chemical Engineers part C*, 71,139–179.

Hua, Z., Chen, J., Lun, S., Wang, X. [2003]. Influence of biosurfactants produced by *Candida antarctica* on surface properties of microorganism and biodegradation of n-alkanes. *Water Research*, 37, 4143-50.

Im, J. H., Yanagishita, H., Ikegami, T., Takeyama, Y., Idemoto, Y., Koura, N., Kitamoto, D. [2003]. Mannosylerythritol lipids, yeast glycolipid biosurfactants, are potential affinity ligand

materials for human immunoglobulin G. *Journal of Biomedical Material Research*, 65, 379–385.

Inge N. A. Bogaert, V., Saerens, K., Muynck, C. D., Develter, D., Soetaert, W., Vandamme, E. J. [2007]. Microbial Production and Applications of sophorolipids. *Applied Microbiology and Biotechnology*, 76, 23-24.

Ismail, B., Nampoothiri, K. M. [2010]. Production, purification and structural characterization of an exopolysaccharide produced by a probiotic *Lactobacillus plantarum* MTCC 9510. *Archives of Microbiology*, 192, 1049–1057.

Ito, S., Suzuki, M., Kobayashi, Y. [2011]. Feed additive and feed. Patent CA 2666674.

Jeya, M., Lee, K. M., Tiwari, M. K., Kim, J.S., Gunasekaran, P., Kim, S.Y., Kim, I.W., Lee, J. K. [2009]. Isolation of a novel high erythritol-producing *Pseudozyma tsukubaensis* and scale-up of erythritol fermentation to industrial level. *Applied Microbiology and Biotechnology*, 83, 225-231.

Jones, B. E., Vroemen, Chang, C., Naab, C., De Nobel, H., Kolkman, M., Weyler, W. [2008]. Alkaliphilic Bacillus Species alpha-Amylase Variants, Compositions Comprising alpha-Amylase Variants, And Methods of Use. Patent USP 20080293607.

Joshi-Navare, K., Prabhune, A. [2013]. A biosurfactant-sophorolipid acts in synergy with antibiotics to enhance their efficiency. *BioMed Research International*, http://dx.doi.org/10.1155/2013/512495

Kacurakova, M., Capek, P., Sasinkova, V., Wellner, N., Ebringerova, A. [2000]. FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. *Carbohydrate Polymer*, 43, 195–203.

Kaczorek, E. [2012]. Effect of External Addition of Rhamnolipids Biosurfactant on the Modification of Gram Positive and Gram Negative Bacteria Cell Surfaces during

Biodegradation of Hydrocarbon Fuel Contamination. *Polish Journal of Environmental Studies,* 21, 901.

Kaczorek, E., Olszanowski, A. [2011]. Uptake of Hydrocarbon by *Pseudomonas fluorescens* (P1) and Pseudomonas putida (K1) Strains in the Presence of Surfactants: A Cell Surface Modification. *Water Air Soil Pollution*, 214, 451–459.

Kakugawa, K., Tamai, M., Imamura, K., Miyamoto, K., Miyoshi, S., Moninaga, Y., Suzuki, O., Miyakawa, T. [2002]. Isolation of yeast *Kurtzmanomyces* sp. I-11, novel producer of mannosylerythritol lipid. Bioscience. *Biotechnology and Biochemistry*, 66, 188-91.

Kang, S.A., Jang, K. H., Seo, J. W., Kim, K. H., Kim, Y.H., et al., [2009]. Levan Applications and Perspectives, *In* Microbial Production of biopolymer and polymer precursors, applications and perspectives, *Ed* Rehm, B.H.A. Caister Academic Press, Caister, 145-158.

Kang, S.W., Kim, Y.B., Shin, J.D., Kim, E.K. [2009]. Enhanced biodegradation of hydrocarbons in soil by microbial biosurfactant, sophorolipid. *Applied Biochemistry and Biotechnology*, 160, 780-790.

Karanth, N. G.K., Deo, P.G., Veenanadig, N.K. [1999]. Microbial production of biosurfactants and their importance. *Current Science*, 77, 116-126.

Karbowiak, T., Ferret, E., Debeaufort, F., Voilley, A., Cayot, C. [2011]. Investigation of water transfer across thin layer biopolymer films by infrared spectroscopy. *Journal of Membrane Science*, 370, 82–90.

Kavita, K., Singh, V. K., Mishra, A., Jha, B. [2014]. Characterisation and anti-biofilm activity of extracellular polymeric substances from *Oceanobacillus iheyensis*. *Carbohydrate polymers, 101*, 29-35.

Kawashima, H., Nakahara, T., Oogaki, M., Tabuchi, T. [1983]. Extracellular production of a mannosylerythritol lipid by a mutant of *Candida* sp. from n-alkanes and triacylglycerols. *Journal of Fermentation Technology*, 61, 143–149.

Kim S. K., Kim, Y. C., Lee, S., Kim, J. C., Yun, M. Y., Kim, I. S. [2011]. Insecticidal activity of rhamnolipid isolated from *Pseudomonas* sp. EP-3 against green peach aphid (*Myzus persicae*). *Journal of Agriculture and Food Chemistry*, 59, 934-8.

Kim, B. S., Lee, J. Y., Hwang, B. K., [2000]. In vivo control and in vitro antifungal activity of rhamnolipid B, a glycolipid antibiotic, against *Phytophthora capsici* and *Colletotrichum orbiculare*. *Pest Management Science*. 56, 1029-1035.

Kim, H. S., Jeon, J. W., Kim, B. H., Ahn, C.Y., Oh, H. M., Yoon, B. D. (2006). Extracellular production of a glycolipid biosurfactant, mannosylerythritol lipid, by *Candida* sp. SY16 using fed-batch fermentation. *Applied Microbiology Biotechnology* 70, 391-6.

Kim, S. Y., Oh, D. K., Kim, J. H. (1997). Biological modification of hydrophobic group in *Acinetobacter calcoaceticus* RAG-1 emulsan. *Journal of Fermentation and Bioengineering* 84, 162-164.

Kim, Y. B., Yun, H. S., Kim, E. K., [2009]. Enhanced sophorolipid production by feeding-rate-controlled fed-batch culture. *Bioresource Technology*, 100, 6028-6032.

Kitamoto, D., Aklba, S., Hiokl, C., and Tabuchi, T. [1990a]. Extracellular accumulation of mannosylerythritol lipids by a strain of *Candida antarctica. Agricultural and Biological Chemistry*, 54, 31-36.

Kitamoto, D., Haneishi, K., Nakahara, T., and Tabuchi, T. [1990b]. Production of mannosylerythritol lipids by *Candida antarctica* from vegetable oils. *Agricultural and Biological Chemistry*, 54, 37-40.

Kitamoto, D., Fujishiro, K., Yanagishita, H., Nakane, T., Nakahara, T. [1992b].Production of mannosylerythritol lipids as biosurfactants by resting cells of *Candida antarctica. Biotechnology Letters*, 14,305-310.

Kitamoto, D., Nakane, T., Nakao, N., Nakahara, T., Tabuchi, T. [1992a]. Intracellular accumulation of mannosylerythritol lipids as storage materials by *Candida antarctica. Applied Microbiology and Biotechnology*, 36, 768-772.

Kitamoto, D., Ikegami, T., Suzuki, G. T., Sasaki, A., Takeyama, Y., Idemoto, Y., Koura, N., Yanagishita, H. [2001]. Microbial conversion of n-alkanes into glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma* (*Candida antarctica*). *Biotechnology Letters*, 23, 1709–1714.

Kitamoto, D., Isoda, H., Nakahara, T., [2002]. Functions and Potential Applications of Glycolipid Biosurfactants -from Energy-Saving Materials to Gene Delivery Carriers. *Journal Of Bioscience and Bioengineering*, 94, 187-201.

Kitamoto, D., Nemoto, T., Yanagishita, H., Nakane, T., Kitamoto, H. K., and Nakahara, T. [1993a]. Fatty acid metabolism of mannosylerythritol lipids as biosurfactants produced by *Candida antarctica. Journal of Japan Oil Chemist Society*, 42, 346-358.

Kitamoto, D., Yanagishita, H., Shinbo, T., Nakane, T., Kamisawa, C., and Nakahara, T. [1993b]. Surface-active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by *Candida antarctica. Journal of Biotechnology*, 29, 91-96.

Kitamoto, D., Yanagishita, H., Haraya, K., Kitamoto, H.K. [1995]. Effect of cerulenin on the production of mannosylerythritol lipids as biosurfactants by *Candida antarctica. Biotechnology Letters*, 17, 25-30.

Kitamoto, D., Yanagishita, H., Haraya, K., Kitamoto, H.K. [1998]. Contribution of a chain-shortening pathway to the biosynthesis of the fatty acids of mannosylerythritol lipid (biosurfactant) in the yeast *Candida antarctica*: Effect of b-oxidation inhibitors on biosurfactant synthesis. *Biotechnology Letters*, 20, 813–818.

Kitamoto, D., Yokoshima, T., Yanagishita, H., Haraya, K., Kitamoto, H.K. [2009]. Formation of Glycolipid biosurfactants, Mannosylerythritol Lipid, by *Candida antarctica* from Aliphatic Hydrocarbons via Subterminal Oxidation Pathway. *Journal of Japan Oil Chemists' Society*, 48, 1377-1384.

Kitamoto, H.K., Shinozaki, Y., Cao, X. H., Morita, T., Konishi, M., Tago, K., Kajiwara, H., Koitabashu, M., Yoshida, S., Watanabe, T., Sameshima-Yamashita, Y., Nakajima-Kambe, T., Tsushima, S. [2011]. Phyllosphere yeasts rapidly break down biodegradable plastics. *AMB Express*, 1, 44, doi: 10.1186/2191-0855-1-44.

Koefod, R.S. (2011), Improved deicer composition, CA2776689 A1.

Konishi, M., Fukuoka, T., Nagahama, T., Morita, T., Imura, T., Kitamoto, D., Hatada, Y. [2010]. Biosurfactant-producing yeast isolated from *Calyptogena soyoae* (deep-sea cold-seep clam) in the deep sea. *Journal of Bioscience and Bioengineering*, 110, 169-175.

Konishi, M., Imura, T., Fukuoka, T., Morita, T., Kitamoto, D. [2007]. A yeast glycolipid biosurfactant, mannosylerythritol lipid, shows high binding affinity towards lectins on a self-assembled monolayer system. *Biotechnology Letters*, 29, 473-80.

Konishi, M., Morita, T., Fukuoka, T., Imura, T., Kakugawa, K., Kitamoto, D. [2007]. Production of different types of mannosylerythritol lipids as biosurfactants by the newly isolated yeast strains belonging to the genus *Pseudozyma*. *Applied Microbiology and Biotechnology*, 75, 521–531.

Konishi, M., Nagahama, T., Fukuoka, T., Morita, T., Imura, T., Kitamoto, D., Hatada, Y. [2011]. Yeast extract stimulates production of glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma hubeiensis* SY62. *Journal of Bioscience and Bioengineering*, 111. 702-705.

Korabecna, M., Liska, V., Fajfrlik, K. [2003]. Primers ITS1, ITS2 and ITS4 detect the intraspecies variability in the internal transcribed spacers and 5.8S rRNA gene region in clinical isolates of fungi. *Folia Microbiology*, 48, 233-238.

Kuiper, I., Lagendijk, E.L., Pickford, R., Derrick, J. P., Lamers, G. E., Thomas-Oates, J. E., Lugtenberg, B. J., Bloemberg, G. V. [2004]. Characterization of two *Pseudomonas putida* lipopeptide biosurfactants, putisolvin I and II, which inhibit biofilm formation and break down existing biofilms. *Molecular Microbiology*, 51, 97–113.

Kumar, C. G., Joo, H. S., Kavali, R., Choi, J,W., Chang, C. S. [2004]. Characterization of an extracellular biopolymer flocculant from a haloalkalophilic *Bacillus* isolate. *World Journal of Microbial Biotechnology*, 20, 837-843.

Kuncheva, M., Pavlova, K., Panchev, I., Dobreva, S. [2007]. Emulsifying power of mannan and glucomannan produced by yeasts. *International Journal of Cosmetic Science*, 29, 377–384.

Kuppert, D. [2014].Sophorolipids – The Next Leading Class of Surfactants, 61. SEPAWA Congress, Fulda.

Lal, P., Sharma, D., Pruthi, P., Pruthi, V. [2010]. Exopolysaccharide analysis of biofilm-forming *Candida albicans*. *Journal of Applied Microbiology*, 109, 128-36.

Latorre-Esteves, M., Cortes, A., Torres-lugo, M., Rinaldi, C. [2009]. Synthesis and characterization of carboxymethyl dextran-coated Mn/Zn ferrite for biomedical applications. *Journal of Magnetism and Magnetic Materials*, 321, 3061–3066.

Laws, A., Gu, Y., Marshall, V. [2001]. Biosynthesis, characterisation, and design of bacterial exopolysaccharides from lactic acid bacteria. *Biotechnological Advances*, 19, 597–625.

Lee, H. H., Lee, J. S., Cho, J. Y., Kim, Y. E., Hong, E. K. [2009]. Structural characteristics of immunostimulating polysaccharides from lentinus edodes. *Journal of Microbial Biotechnology*, 19, 455-61.

Levinson, W. E., Kurtzman, C.P., Kuo, T. M. [2006]. Production of itaconic acid by *Pseudozyma antarctica* NRRL Y-7808 under nitrogen-limited growth conditions. *Enzyme and Microbial Technology*, 39, 824-827.

Li, H., Li. S., Xu. H., Chen. X. and Ouyang. P. [2013]. Improvement of welan gum production and redistribution of metabolic flux under pH control process in *Alcaligenes* sp. CGMCC2428. *Biotechnology and Bioprocess Engineering* 18, 399-405.

Lim, D. J., Kim, J. D., Kim, M.Y., Yoo, S. H., Kong, J. Y. [2007]. Physicochemical properties of the exopolysaccharides produced by marine bacterium *Zoogloea* sp. KCCM10036. *Journal of Microbial Biotechnology*, 17, 979-84.

Lim, J. M., Joo, J. H., Kim, H. O., Kim, H. M., Kim, S. W., Hwang, H. J., Yun, J. W. [2005]. Structural analysis and molecular characterization of exopolysaccharides produced by submerged mycelial culture of *Collybia maculata* TG-1. *Carbohydrate Polymer*, 61, 296–303.

Lim, T. M., Procopio, L. C., Brandao, F. D., Leao, B. A., Totola, M. R., Borges, A. C.[2011]. Evaluation of bacterial surfactant toxicity towards petroleum degrading microorganisms. *Bioresource Technology*, 102, 957-64.

Liu, D., [2011], *Ustilago* and *Pseudozyma*, *In* Molecular Detection of Human Fungal Pathogens, *Ed*. Liu D., CRC Press, 687-692.

Liu, F. C., Su, C. R., Wu, T. Y., Su, S. G., Yang, H. L., Lin, J. H. Y., Wu, T. S. [2011]. Efficient ^{1}H-NMR Quantitation and Investigation of*N*-Acetyl-D-glucosamine GlcNAc) and *N*,*N′*-Diacetylchitobiose $(GlcNAc)_2$ from Chitin**.** *International Journal of Molecular Sciences*, 12, 5828–5843.

Lozano, N., Rice, C., Pagano, J., Zintek, L., Barber, L. B., Murphy, E.W., Nettesheim, T., Minarik, T., Schoenfuss, H. L., [2012], Concentration of organic contaminants in fish and their biological effects in a wastewater-dominated urban stream. *Science of the Total Environment*, 420, 191–201.

Mahapatra, S., Banerjee, D. [2013]. Fungal Exopolysaccharide: Production, Composition and Applications. *Microbial Insights*, 6, 1-16.

Malatova, K. [2005]. Isolation and characterization of hydrocarbon degrading bacteria from environmental habitats in western New York State. Institute of Technology, Rochester, 1-93.

Mathew, M., Obbard, J.P. [2001]. Optimisation of the dehydrogenase assay for measurement of indigenous microbial activity in beach sediments contaminated with petroleum. *Biotechnology Letters*, 23, 227-230.

Mazuel, C. [1991]. Pharmaceutical and/or cosmetic composition for local use containing Rhamsan gum. Patent US 4996197 A.

McIntosh, M., Stone, B. A., Stanisich, V.A. [2005]. Curdlan and other bacterial (1→3)-β-D-glucans. *Applied Microbiology & Biotechnology*, 68, 163-73.

Mekha, N., Takashima, M., Boong-Long, J., Cho, O., Sugita, T. [2014], Three new basidiomycetous yeasts, *Pseudozyma alboarmeniaca* sp. nov., *Pseudozyma crassa* sp. nov. and *Pseudozyma siamensis* sp. nov. isolated from Thai patients. *Microbiology and Immunology*, 58, 9-14.

Mesomo, M., Silva, M. F., Boni, G., Padilha, F. F., Mazutti, M., Mossi, A., Oliveira, D. D., Cansian, R. L. Luccio, M. D. and Treichel, H. [2009]. Xanthan gum produced by *Xanthomonas campestris* from cheese whey: production optimisation and rheological characterisation. *Journal of the Science of Food and Agriculture,* 89, 2440-2445.

Miwa, M., Nakao, Y., Nara, K. (1993). Food applications of Curdlan, *In* Food Hydrocolloids, Strcuture, properties and functions, *Eds* Nishinari, K.,Doi, E. Springer, New York. 119-124.

Mitrinova, Z., Tcholakova, S., Golemanov, K., Denkov, N., Vethamuthu, M., Ananthapadmanabhan, K. P. [2013]. Surface and foam properties of SLES + CAPB + fatty acid mixtures: Effect of pH forC12–C16 acids. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 438, 186– 198

Moran, A. C., Olivera, N., Commendatore, M., Esteves, J.L., Sineriz, F. [2000]. Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilis* O9. *Biodegradation*, 11, 65-71.

Morita, T. Konishi, M. Fukuoka, T., Imura, T., Kitamoto, D. [2007]. Physiological difference in the formation of the glycolipid biosurfactants, mannosylerythritol lipids, between *Pseudozyma antarctica* and *Pseudozyma aphidis*. *Applied Microbiology and Biotechnology*, 74, 307–315.

Morita, T., Fukuoka, T., Konishi, M., Imura, T., Yamamoto, S., Kitagawa, M., Sogabe, A., Kitamoto, D. [2009a]. Production of a novel glycolipid biosurfactant, mannosylmannitol lipid, by *Pseudozyma parantarctica* and its interfacial properties. *Applied Microbiology and Biotechnology*, 83, 1017–1025.

Morita, T., Ito, E., Fukuoka, T., Imura, T., Kitamoto, D. [2010a]. The role of PaAAC1 encoding a mitochondrial ADP/ATP carrier in the biosynthesis of extracellular glycolipids, mannosylerythritol lipids, in the basidiomycetous yeast *Pseudozyma antarctica*. *Yeast*, 27, 379-88.

Morita, T., Kitagawa, M., Suzuki, M., Yamamoto, S., Sogabe, A., Yanagidani, S., Imura, T., Fukuoka, T., Kitamoto, D [2009b]. Yeast glycolipid biosurfactant, mannosylerythritol lipid, shows potential moisturizing activity toward cultured human skin cells: the recovery effect of MEL-A on the SDS-damaged human skin cells. *Journal of Oleo Science*, 58, 639–642.

Morita, T., Kitagawa, M., Yamamoto, S., Sogabe, A., Imura, T., Fukuoka, T., Kitamoto, D. [2010b]. Glycolipid biosurfactants, mannosylerythritol lipids, repair the damaged hair. *Journal of Oleo Science* 59, 267–272.

Morita, T., Koike, H., Hagiwara, H., Ito, E., Machida, M., Sato, S., Habe, H., Kitamoto, D. [2014]. Genome and Transcriptome Analysis of the Basidiomycetous Yeast *Pseudozyma antarctica* Producing Extracellular Glycolipids, Mannosylerythritol Lipids. *Plus One*. DOI: 10.1371/journal.pone.0086490

Morita, T., Konishi, M., Fukuoka, T., Imura, T., Kitamoto, D. [2008]. Production of glycolipids biosurfactants, mannosylerythritol lipids, by *Pseudozyma siamensis* CBS 9960 and their interfacial properties. *Journal of n Bioscience and Bioengineering*, 105, 493–502.

Morita, T., Konishi, M., Fukuoka, T., Imura, T., Sakai, H., Kitamoto, D. [2008b]. Efficient production of di- and tri-acylated mannosylerythritol lipids as glycolipid biosurfactants by *Pseudozyma parantarctica* JCM 11752. *Journal of Oleo Science*, 57, 557-565.

Morita, T., Ogura, Y., Takashima, M., Hirose, N., Fukuoka, T., Imura, T., Kondo, Y., Kitamoto, D. [2011]. Isolation of *Pseudozyma churashimaensis* sp. nov., a novel ustilaginomycetous yeast species as a producer of glycolipid biosurfactants, mannosylerythritol lipids. *Journal of Bioscience and Bioengineering*, 112, 137-144.

Morita, T., Takashima, M., Fukuoka, T., Konishi, M., Imura, T., Kitamoto, D. [2010c], Isolation of basidiomycetous yeast *Pseudozyma tsukubaensis* and production glycolipid

biosurfactant, diastereomer type of mannosylerythritol lipid-B. *Applied Microbiology and Biotechnology*, 88, 679–688.

Mua, J. P. [2009]. Smokeable tobacco substitute filler having an increased fill value and method of making same. Patent CA 2574858.

Mukherjee, A. K. [2007]. Potential application of cyclic lipopeptide biosurfactants produced by *Bacillus subtilis* strains in laundry detergent formulations, *Letters in Applied Microbiology*, 45, 330-335.

Nakanishi, M., Inoh, Y., Furuno, T. (2013). New Transfection Agents Based on Liposomes Containing Biosurfactant MEL-A. *Pharmaceutics*, 5, 411-420.

National Research Council, [1993]. Principle of bioremediation. In Situ Bioremediation: When Does it Work?. The National Academies Press. Washington DC, 16-42.

Neilson, J. W., Artiola, J. F., Maier, R.M. [2003]. Characterization of Lead Removal from Contaminated Soils by Nontoxic Soil-Washing Agents. *Journal of Environmental Quality* 32, 899-908.

Neu, T. R., Denglar, T., Jahn, B., Poralla, K. [1992]. Structural studies of an emulsion-stabilizing exopolysaccharide produced by an adhesive, hydrophobic *Rhodococcus* strain. *Journal of General Microbiology*, 138, 2531-2537.

Noordman, W.H., Janssen, D.B. [2002]. Rhamnolipids stimulates uptake of hydrophobic compounds by *Pseudomonas aeruginosa*. *Applied Environmental Microbiology*, 68, 4502-4508.

Nussinovitch, A., Hirashima, M. [2013]. Xantan gum In Cooking Innovations: Using Hydrocolloids for Thickening, Gelling, and Emulsification. CRC Press, USA, 213-242.

Nwodo, U.U., Green, E., Okoh, A.I. [2012]. Bacterial Exopolysaccharides: Functionality and Prospects. *International Journal of Molecular Science*, 13, 14002-14015.

Panilaitis, B., Johri, A., Blank, W., Kaplari, D., Fuhrman, J. [2002]. Adjuvent activity of emuslan, a secreted liopolysaccharide from *Acinetoacter calcoaceticus. Clinical and Diagnostic Laboratory Immunology*, 9, 140-1247.

Parky, S.Y., Choi, S. K., Kim, J., Oh, T.K., Park, S. H. [2012]. Efficient production of polymyxin in the surrogate host *Bacillus subtilis* by introducing a foreign ectB gene and disrupting the abrB gene. *Applied Environmental Microbiology*, 78, 4194-4199.

Parry, A. J., Parry, N. J., Peilow, A. C., Stevenson, P. S. [2012]. Detergent compositions comprising biosurfactants and lipase. Patent WO 2012010407 A1

Patel, Y., Schneider, J. C., Maria, L, L., Lemini, V. [2008]. High viscosity xanthan polymer preparations. Patent US 7439044 B2.

Pavlova, K., Koleva, L., Kratchanova, M., Panchev, I. [2004]. Production and characterization of an exopolysaccharide by yeast. *World Journal of Microbial Biotechnology*, 20,435–439.

Pavlova, K., Panchev, I., Chistozova, T. S. [2005]. Physico-chemical characterization of exomannan from *Rhodoturula achenion* MC. *World Journal of Microbial Biotechnology*, 21, 279–283.

Pavlova, K., Rusinova-Videva, S., Kuncheva, M., Kratchanova, M., Gocheva, M., Dimitrova, S. [2011]. Synthesis and Characterization of an Exopolysaccharide by Antarctic Yeast Strain *Cryptococcus laurentii* AL_{100}. *Applied Biochemistry and Biotechnology*, 163, 1038-1052.

Perfumo, A.., Rancich, I., Banat, I.M. [2010]. Possibilities and Challenges for Biosurfactants Use in Petroleum Industry. *Advances in Experimental Medicine and Biology*, 672,135-145.

Peterson, G. R., Nelson, G.A., Cathey, C.A., Fuller, G.G. [1989]. Rheologically interesting polysaccharides from yeasts. *Applied Biochemistry and Biotechnology*, 20, 845–867.

Petravić-Tominac,V., Zechner-Krpan,V., Grba,S., Srečec,S., Panjkota-Krbavčić,I. and Vidović,L. [2010]. Biological Effects Of Yeast β- Glucans. *Agriculturae Conspectus Scientificus*, 75, 149-158.

Petty, M. A. [1952]. Method of producing polymyxin. Patent US 2595605 A

Phisalaphong, M., Chiaoprakobkij,N. [2012]. Applications and products-Nata de Cocco, *In* Bacterial NanoCellulose: A Sophisticated Multifunctional Material, *Eds*. Gama, M., Gatenholm, P., Klemm,D. CRC Press, USA, 145-155.

Pinchuk, R., [2000]. Liposan Production in the Self-Cycling Fermentor. PhD thesis ,McGill University, Montreal.

Poli, A., Anzelmo, G., Tommonaro, G., Pavlova, K., Casaburi, A., Nicolaus, B. [2010]. Production and chemical characterization of an exopolysaccharide synthesized by psychrophilic yeast strain *Sporobolomyces salmonicolor* AL1 isolated from Livingston Island, Antarctica. *Folia Microbiology*, 55, 576–581.

Prajapati, V.D., Jani, G.K., Khanda, S.M. (2013), Pullulan: an exopolysaccharide and its various applications. *Carbohydrate polymer*, 5, 540-549.

Purwandari, U., Vasiljevic, T. [2010]. Rheological characterization of exopolysaccharides produced by two strains of *Streptococcus thermophilus. International Food Research Journal*, 17, 575-582.

Raghavan, P.U.M., Vivekanandan, M. [1999]. Bioremediation of oil-spilled sites through seeding of naturally adapted *Pseudomonas putida*. *International Biodeterioration and Biodegradation*, 44, 290-32.

Rahar, S., Swami, G., Nagpal, N., Nagpal, M. A., Singh, G.S. [2011]. Preparation, characterization, and biological properties of β-glucans. *Journal of Advanced Pharmaceutical Technology& Research*, 2, 94-103.

Rangaswamy, V., Guduri, B., Anand, H., [2010]. Process for production and purification of polymyxin b sulphate. Patent WO 2010058427 A2

Rau, U., Nguyen, L. A., Roeper, H., Koch, H., Lang, S. [2005c]. Downstream processing of mannosylerythritol lipids produced by *Pseudozyma aphidis. European Journal of Lipid Science and Technology*, 107, 373–380.

Rau, U., Nguyen, L. A., Schulz, S., Wray, V., Nimtz, M., Roeper, H., Koch, H., Lang, S. [2005a]. Formation and analysis of mannosylerythritol lipids secreted by *Pseudozyma aphidis*. *Applied Microbiology and Biotechnology*, 66, 551–593.

Rau, U., Nguyen, L.A., Roeper, H., Koch, H., Lang, S. [2005b]. Fed-batch bioreactor production of mannosylerythritol lipids secreted by *Pseudozyma aphidis*. *Applied Microbiology and Biotechnology*, 68, 607-613.

Rau, U., Nguyen, L.A., Schulz, S., Wray, V., Nimtz, M., Roeper, H., Koch, H. Lang, S. [2005a]. Formation and analysis of mannosylerythritol lipids secreted by *Pseudozyma aphidis*. *Applied Microbiology and Biotechnology*, 66, 551-559.

Ron, E. Z., Rosenberg, E. [2002]. Biosurfactants and oil bioremediation. *Current Opinion in Biotechnology*, 13, 249-52.

Ruas-Madiedo, P., Tuinier, R., Kanning, M., Zoon, P. [2002]. Role of exopolysaccharides produced by *Lactococcus lactis* subsp. *cremoris* on the viscosity of fermented milks. *International Dairy Journal*, 12, 689–695.

Sajna, K. V., Sukumaran, R. K., Gottumukkala, L. D., Pandey, A. [2015a]. White Biotechnology in Biosurfactants, *In* Industrial Biorefineries and White Biotechnology, *Eds*

Taherzadeh, M. J., Pandey, A.,Hofer, R., Nampoothiri,M., Larroche, C. Elsevier, USA.499-517.

Sajna, K. V., Sukumaran, R.K., Gottumukkala, L. D., Jayamurthy, H., Dhar, K. S., Pandey, A. [2013b]. Studies on structural and physical characteristics of a novel exopolysaccharide from *Pseudozyma* sp. NII 08165. *International Journal of Biological Macromolecules*, 59, 84-9.

Sajna, K.V., Sukumaran,R.K., Jayamurthy,H., Reddy, K.K., Kanji-lal,S., Prasad, R.B.N., Pandey, A. [2013a]. Studies on biosurfactants from *Pseudozyma* sp. NII 08165 and their potential application as laundry detergent additives. *Biochemical Engineering Journal*, 78, 85-92.

Sakurai, K., Kimura, T., Koumoto, K., Mizu, M., Kobayashi, R., Shinkai, S. [2001]. Application of schizophyllan as a novel gene carrier. *Nucleic acid Research Supplement*, 1, 223-224.

Samuel, S., Mukherjee, S., Rick, D. and Weinkauf, R. L. [2002]. Skin cosmetic composition containing a dextran or maltodextrin and a weak carboxylic acid. Patent EP 1169015 A2.

Sandrin, T. R., Chech, A. M., Maier, R. M. [2000]. A Rhamnolipids Biosurfactanst Reduces Cadmium Toxicity during Naphthalene Biodegradation. *Applied Environmental Microbiology*, 66, 4585–4588.

Sarwat, F., Qader, S. A.U., Aman, A. and Ahmed, N. [2008]. Production & characterization of a unique dextran from an indigenous *Leuconostoc mesenteroides* CMG713. *International Journal of Biological Science*, 4, 379-86.

Savarino, P., Montoneri, E., Musso, G., Boffa, V. [2010]. Biosurfactants from urban wastes for detergent formulation: surface activity and washing performance. *Journal of surfactants and detergents*, 13, 59-68.

Schaffer, S., Wessel, M., Thiessenhusen, A. [2013]. Cells, nucleic acids, enzymes and use thereof, and methods for the production of sophorolipids. Patent US 20130035403.

Schippers, C., Gessner, K., Muller, T., Scheper, T. [2000]. Microbial degradation of phenanthrene by addition of a sophorolipid mixture. *Journal of Biotechnology*, 83, 189-98.

Sekkal, M., P. Legrand, P. [1993]. A spectroscopic investigation of the carrageenans and agar in the 1500–100 cm^{-1}spectral range. *Spectrochim. Acta. A.* 49, 209–221.

Sen, R. [2010]. Surfactin: biosynthesis, genetics and potential applications. *In* Biosurfactants, Advances in Experimental Medicine and Biology, *Ed* Ramakrishna Sen, Springer, New York, 672, 316-323.

Shabati, Y., Wang, D. I. [1990]. Production of emulsan in a fermentation process using soybean oil (SBO) in a carbon-nitrogen coordinated feed. *Biotechnology and Bioengineering*, 35, 753-765.

Shah, A. K., Ashtaputre, A. A. [1999]. Evaluation of rheological properties of the exopolysaccharide of *Sphingomonas paucimobilis* GS-1 for application in oil exploration. *Journal of Industrial Microbial Biotechnology*, 23, 442-445.

Shah, V., Badia, D. [2007]. Sophorolipids Having Enhanced Antibacterial Activity. *Antimicrobial Agents and Chemotherapy*, 51, 397–400.

Shah, v., Doncel, G. F., Seyoum, T., Eaton, K. M., Zalenskaya, I., Hagver, R., Azim, A., Gross, R. [2005]. Sophorolipids, Microbial Glycolipids with Anti-Human Immunodeficiency Virus and Sperm-Immobilizing Activities. *Antimicrobial Agents and Chemotherapy*, 49, 4093-4100.

Shin, K. H., Ahn, Y., Kim, K. W. [2005]. Toxic effect of biosurfactant addition on the biodegradation of phenanthrene. *Environmental Toxicology and Chemistry*, 24, 2768-74.

Shiroya, T., Bernard, A. L. and Dumousseaux, C. [2013]. Self-standing cosmetic sheet. Patent WO 2013153678 A1.

Siddiqui, N. N., Aman, A., Silipo, A., Ul Qader, S. A., Molinaro, A. [2014]. Structural analysis and characterization of dextran produced by wild and mutant strains of *Leuconostoc mesenteroides*. *Carbohydrate Polymers*, 99, 331– 338.

Singh, R. P., Shukla, M. K., Mishra, A., Kumari, P., Reddy, C.R. K., Jha, B. [2011].Isolation and characterization of exopolysaccharides from seaweed associated bacteria *Bacillus licheniformis*. *Carbohydrate Polymer*, 84, 1019–1026.

Singh, R.S., Sainin, G.K., Kennedy, J.F. [2008]. Pullulan: Microbial sources, production and applications. *Carbohydrate Polymer*, 73, 515-531.

Singh, S., Patel, P., Jaiswal, S., Prabhune, A. A., Ramana, C.V., Prasad, B.L.V. [2009]. A Direct Method for the Preparation of Glycolipid Metal Nanoparticle Conjugates : Sophorolipids as Reducing and Capping Agents for the Synthesis of Water Re-dispersible Silver Nanoparticles and Their Antibacterial Activity. *New Journal of Chemistry*, 33, 646-652.

Skaggs, B., Dial, H., Rakitsky, W. [2001]. Stable suspension of hydrocolloids and superplasticizer. US patent 6309455 B1.

Solaiman, D, K, Y., Ashby, R. D., Zerkowski, J. A., Foglia, T. A. [2007]. Simplified soy molasses-based medium for reduced-cost production of sophorolipids by *Candida bombicola*. *Biotechnology Letters*, 29, 1341-1347.

Sotirova, A., Spasova, D., Vasileva- Tonkova, E., Galabov, D. [2009]. Effects of Rhamnolipid-Biosurfactant on Cell Surface of *Pseudomonas aeruginosa. Microbiological Research*, 164, 297–303.

Spoeckner, S., Wray, V., Nimtz, M., and Lang, S. [1999]. Glycolipids of the smut fungus *Ustilago maydis* from cultivation on renewable resources. *Applied Microbiology and Biotechnology*, 51, 33-39.

Sponza, D.T., Gok, O. [2010]. Effect of rhamnolipids on the aerobic removal of polyaromatic hydrocarbons (PAHs) and COD components from petrochemical wastewater, *Bioresource Technology*, 101, 914–924.

Stanghellini, M. E., Miller, R. M. [1997]. Biosurfactants: Their Identity and Potential Efficacy in the Biological Control of Oosporic Plant Pathogens. *Plant Disease* 81, 4-12.

Su, L., Ji, W. K., Lan, W.Z., Dong, X.Q. [2003]. Chemical modification of xanthan gum to increase dissolution rate. *Carbohydrate Polymer*, 53, 497-499.

Sun, W., Liu, J., Wang, J., Liu, S. [2013]. Method for producing sodium Surfactin. Patent CN 103059108 A.

Sutherland, I. W., [1990]. Medical applications of exopolysaccharides, *In* Biotechnology of Microbial exopolysaccharides, Cambridge University Press, Cambridge, 141-148.

Suzuki, M., Kitagawa, M. Yamamoto, S., Sogabe, A., Kitamoto, D., Morita, T., Fukuoka, T. Imura, T. [2013]. Activator including biosurfactants as active ingredient, mannosylerythritol lipids, and production methods thereof. Patent EP 2074985 B1.

Tamura, K., Dudley, J., Nei, M., Kumar, S. [2007]. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596–1599.

Tanaka, A. and Fukui, S. [1989]. Metabolism of n-alkane. *In* The yeast, Academic Press, London, vol. 3, 261-287.

Tao, F., Biao, G. Z., Yu, J. Z., Ning, Z. H. [2008]. Isolation and characterization of an acidic polysaccharide from *Mesona Blumes* gum. *Carbohydrate Polymer*, 71, 159–169.

Tavares, A. M. P., Agapito, M.S.M., Coelho, M. A. Z., Lopes da Silva, J. A., Barros-Timmons, A., Coutinho, J.A.P., Xavier, A. M.R. B. [2005]. Selection and optimization of culture medium for exopolysaccharide production by *Coriolus* (*Trametes*) *versicolor. World Journal of Microbial Biotechnology*, 21, 1499–1507.

Thompson, J. D., Higgins, D. G., Gibson, T. J. [1994]. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting. Position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680.

Tuinier, R., van Casteren, W. H. M., Looijesteijn, P. J., Schols, H. A., Voragen, A. G. J., & Zoon, P. [2001]. Effects of structural modifications on some physical characteristics of exo-polysaccharides from *Lactococcus lactis*. *Biopolymers*, 59, 160–166.

Unilever PLC, [2012]. Rhamnolipids with improved cleaning, EPA 10170402.1.

Van Bogaert, I. N. A., De Maeseneire, S. L., Vandamme, E. J. [2009]. Extracellular Polysaccharides Produced by Yeasts and Yeast-Like Fungi. *In* Yeast Biotechnology: Diversity and Applications, *Eds* Satyanrayana, T., Konza, G. Springer, Netherland, 651-671.

Van Bogaert, I., Fleurackers, S., Van Kerrebroeck, S., Develter, D., Soetaert, W. [2011]. Production of new-to-nature sophorolipids by cultivating the yeast *Candida bombicola* on unconventional hydrophobic substrates. *Biotechnology and Bioengineering* 108, 734-741.

Vasconcelos, A. F., Monteiro, N. K., Dekker, R. F., Barbosa, A. M., Carbonero, E.R., Silveira, J. L., Sassaki, G. L., da Silva, R. , de Lourdes Corradi da Silva, M. [2008]. Three exopolysaccharides of the beta-(1-->6)-D-glucan type and a beta-(1-->3;1-->6)-D-glucan produced by strains of *Botryosphaeria rhodina* isolated from rotting tropical fruit. *Carbohydrate Research*, 343, 2481-2485.

Velasco, S. E., Areizaga, J., Irastorza, A., Duenas, M. T., Santamaria, A., Munoz, M. E. [2009]. Chemical and rheological properties of the beta-glucan produced by *Pediococcus parvulus*. *Journal of Agricultural Food Chemistry*, 57, 1827-34.

Wakamatsu, Y., Zhao, X., Jin, C., Day, N., Shibahara, M., Nomura, N., Nakahara, T., Murata, T., Yokoyama, K.K. [2001]. Mannosylerythritol lipid induces characteristics of neuronal differentiation in PC12 cells through an ERK-related signal cascade. *European Journal of Biochemistry*, 268, 374-83.

Walter, V., Syldatk, C., Hausmann, R. [2010]. Screening concepts for the isolation of biosurfactant producing microorganisms. *Advances in Experimental Medicine and Biology*, 672, 1-13.

Wang, Y., Li, C., Liu, P., Ahmed, Z., Xiao, P., Bai, X. [2010]. Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KF5 isolated from Tibet Kefir. *Carbohydrate Polymer*, 82, 895–903.

Weber, A., May, A., Zeiner, T., Gorak, A. [2012]. Downstream Processing of Biosurfactants. *Chemical Engineering transaction*. 27. DOI 103303/CET1227020

Whang, L. M., Liu, P. W., Ma, C. C., Cheng, S. S. [2008]. Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. *Journal of Hazardous Materials*, 151, 155-163.

White, T. J., Bruns ,T., Lee, S., Taylor, J. W. [1990]. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* PCR protocols: a guide to methods and applications. *Eds.* Innis, M. A., Gelfand, M. A., Sninsky, J. J., White, T. J., Academic Press, USA, 315-322.

Yan, Y. W. [1990]. Microbial levan, *In* Advances in Applied Microbiology, *Eds* Neidleman, S.L. and Laskin, A. L., Academic Press Inc., San Diego, 35, 170-193.

Yoshida, S., Morita, T., Shinozaki, Y., Watanabe, T., Sameshima-Yamashita, Y., Koitabashi, M., Kitamoto, D., Kitamoto, H. [2014]. Mannosylerythritol lipids secreted by phyllosphere

yeast *Pseudozyma antarctica* is associated with its filamentous growth and propagation on plant surfaces. *Applied Microbiology and Biotechnology*, 98, 6419-29.

Zeng. G., Liu, Z., Zhong, H., Li, J., Yuan, X., Fu, H., Ding, Y., Wang, J., Zhou, M. [2011]. Effect of monorhamnolipid on the degradation of n-hexadecane by *Candida tropicalis* and the association with cell surface properties. *Applied Microbial Biotechnology*, 90, 1155-1161.

Zhang, W., Li, J., Huang, G., Song, W., Huang, Y., [2011]. An experimental study on the bio-surfactant-assisted remediation of crude oil and salt contaminated soils. *Journal of Environmental Science and Health. Part A, Toxic/hazardous Substances & environmental Engineering*, 46, 306-313.

Zhao, Z., Selvam, A., Wong, J. W. [2011]. Effects of Rhamnolipids on Cell Surface Hydrophobicity of PAH Degrading Bacteria and the Biodegradation of Phenanthrene. *Bioresource technology*, 102, 3999-4007.

Zhu, L., Yang, X., Xue, C., Chen, Y., Qu, L., Lu, W. [2012]. Enhanced rhamnolipids production by *Pseudomonas aeruginosa* based on a pH stage-controlled fed-batch fermentation process. *Bioresource Technology*, 117, 208-213.

ANNEXURE I

Table AI-List of Major equipment used

Equipment	Model
Autoclave	HICLAVE HV-50, Hirayama, Japan
Incubator Shaker	Innova 4230, New Brounswick Scientific, USA; Infors HT Ecotron
Incubator	IB-05G JEIO TECH
Balance	Mettler Toledo, India: Sartorius
pH Meter	Systronics, India
Deep Freezer -80 ^{0}C	Sanyo, Japan
Deep Freezer -20^{0}C	Operon, Korea;Voltas, India
Hot Air Oven	Kemi instruments, India
Centrifuge	Hitachi, Japan;Kubota, Japan
Laminar Air Flow	Micro-filt, India
Incubating Water Bath	Julabo, Germany
UV-VIS Spectrophotometer	UV-160A Shimadzu, Japan
Microplate Reader	TECAN Infinite M200 PRO
Electrophoresis Unit	BIORAD
PCR Machine	Ep Gradient Eppendorf, India
Gel Documentation	G-Box Syngene, India
Lyophilizer	Operon, Korea
Nanodrop	Thermoscientific, USA
Vaccum Concentrator	Eppendorf 5301, India

Table A2 Composition of Nutrient Broth

Constituents	Concentration (g/L)
Dextrose	1
Peptone	15
Sodium Chloride	6
Yeast extract	3
Final pH	7.5±0.2

Table A3 Composition of Potato Dextrose Agar

Constituents	Concentration (g/L)
Dextrose	20
Potato extract	4
Agar	20
Final pH	5.6±0.2

ANNEXTURE II

List of Publications

Original Papers

1. Crude oil biodegradation aided by biosurfactants from Pseudozyma sp.NII 08165 or its culture broth**. Kuttuvan Valappil Sajna** , Rajeev Kumar Sukumaran, Lalitha Devi Gottumukkala, Ashok Pandey, *Bioresource Technology*, 191, 133–139 (2015)
2. Studies on biosurfactants from *Pseudozyma* sp. NII 08165 and their potential application as laundry detergent additives. **Kuttuvan Valappil Sajna**, RK. Sukumaran, H Jayamurthy, KK Reddy, S Kanjilal, RBN Prasad & Ashok Pandey, *Biochemical Engineering Journal*, 78, 85-92 (2013).
3. Studies on structural and physical characteristics of a novel exopolysaccharide from *Pseudozyma* sp. NII 08165. **Kuttuvan Valappil Sajna**, RK Sukumaran, LD Guttumukkala, H Jayamurthy, KS Dhar & Ashok Pandey, *International Journal of Biological Macromolecules*, 59, 84-89 (2013).
4. Anti-fungal potentials of extracellular metabolites of Western Ghats isolated Streptomyces sp. NII 1006 against moulds and yeasts. Himani Jayamurthy, **Kuttuvan Valappil Sajna**, Syed G Dastagar & Ashok Pandey. *Indian journal of experimental biology*, 52, 1138-46 (2014).
5. Rice straw hydrolysate to fuel and volatile fatty acid conversion by*Clostridium sporogenes* BE01: bio-electrochemical analysis of the electron transport mediators involved. Lalitha Devi Gottumukkala, Rajeev Kumar Sukumaran, S. Venkata Mohan, **Sajna Kuttuvan Valappil**, Omprakash Sarkar, Ashok Pandey , ***Green Chemistry*, 17**, 3047-3058 (2015).
6. Growth and butanol production by *Clostridium sporogenes BE01*in rice straw hydrolysate: kinetics of inhibition by organic acids and the strategies for their removal. Lalitha Devi Gottumukkala, Binod Parameswaran, **Sajna Kuttavan Valappil**, Ashok Pandey, Rajeev Kumar Sukumaran. *Biomass Conversion and Biorefinery*, DOI: 10.1007/s13399-013-0110-6 (2014).
7. Biobutanol production form rice straw by a non acetone produing Clostridium sporogenes BE01. Lalitha Devi Gottumukkala, Binod Parameswaran**, Sajna Kuttuvan Valappil**, Kuttiraja Mathiyazhakan, Ashok Pandey, Rajeev K Sukumaran, *Bioresource Technology*, 145, 182-187(2013).
8. Formic acid as a potential pretreatment agent for the conversion of sugarcane bagasse to bioethanol. R Sindhu, P Binod, K Satyanagalakshmi, KU Janu**, Kuttuvan Valappil Sajna,** N Kurien, RK Sukumaran & Ashok Pandey. ,*Applied Biochemistry and Biotechnology*, 162(8), 2313-2323 (2010).

Review

1. Lignocellulosic ethanol in India: Prospects, challenges and feedstock availability, RK Sukumaran, VJ Surender, R Sindhu, P Binod, KU Janu, **Kuttuvan Valappil Sajna**, KP Rajasree & Ashok Pandey, *Bioresource Technology*, 101(13), 4826-4833 (2010).

Chapters in the Books/Proceedings

1. White Biotechnology for Speciality Chemicals-Cosmetics. **Kuttuvan Valappil Sajna**, Lalitha Devi Gottumukkala, Rajeev Kumar Sukumaran, Ashok Pandey, *In* Industrial Biorefineries and White Biotechnology (*Eds* Mohammad J Taherzadeh, Ashok Pandey, Rainer Höfer, K. Madhavan Nampoothiri, Christian Larroche),Elsevier, USA; 607-644 [2015].

2. White Biotechnology in Biosurfactants. **Kuttuvan Valappil Sajna**, Rajeev Kumar Sukumaran, Lalitha Devi Gottumukkala, , Ashok Pandey, *In* Industrial Biorefineries and White Biotechnology (*Eds* Mohammad J Taherzadeh, Ashok Pandey, Rainer Höfer, K. Madhavan Nampoothiri, Christian Larroche) , Elsevier, USA; 499-517 [2015].

3. Technological challenges in the production and application of cellulases for lignocellulosic bioethanol production, RK Sukumaran, KP Rajasree, Reeta Rani Singhania, A Mathew, Lalitha Devi G, KU Janu, K Satayanagalakshmi, **Kuttuvan Valappil Sajna**, N Kurian, VJ Surender, P Binod, R Sindhu & Ashok Pandey, *In Proceedings of International Symposium on Bioenergy*, Indian Institute of Technology, Kharagpur, India; 56-62 (2010).

National/International Conferences/Symposia Communications

1. Effect of *Pseudozyma* Biosurfactants on Crude Oil Degradation by *Pseudomonas putida*. **KV Sajna**, RK Sukumaran, LD Gottumukkala & Ashok Pandey, ESBES-IFIBIOP 2014 Symposium, September 7-10, Lille, France (2014).
2. A novel exopolysaccharide production from *Pseudozyma* sp. NII 08165. **KV Sajna**, RK. Sukumaran, LD Gottumukkala, H Jayamurthy, KS Dhar & Ashok Pandey, International Conference on Advances in Biotechnology and Bioinformatics, November 25-27, Pune, India (2013).
3. Biosurfactants: potential microbial oleochemicals with prospective commercial applications. **KV Sajna**, RK Sukumaran, Ashok Pandey, OLEO-2013: 68th Annual Convention of Oleotechnologists' Association of India, August 8-10,(2013).
4. Studies on biosurfactants from *Pseudozyma* NII 08165 and their potential application as laundry additives. **KV Sajna**, RK Sukumaran, H Jayamurthy, KK Reddy, S Kanjilal, RBN Prasad & A Pandey, 5th International Conference on Industrial Bioprocesses, October 7-10, Taipei, Taiwan, 290 (2012)

5. Production of biosurfactants from *Pseudozyma* sp. NII 08165 and its potential application in laundry detergent formulation. **KV Sajna**, RK Sukumaran & Ashok Pandey, International Conference on New Horizons in Biotechnology, Trivandrum, India, November 21-24, (2011).

Gene Submissions

1. Sukumaran,R.K., **Valappil,S.K**., Alex,D. and Pandey,A. *Pseudozyma* sp. NII08165 26S ribosomal RNA gene, Accession number JN969988.
2. Sukumaran,R.K., **Valappil,S.K**., Alex,D. and Pandey,A. *Pseudozyma* sp. NII08165 26S ribosomal RNA gene, Accession number JN969989.