Investigations on industrially significant lipase, rhamnolipid and polyhydroxybutyrate produced by novel rumen microbes: *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6

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Certificate

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Declaration

I, **Priji Prakasan** do hereby declare that this thesis entitled, "**Investigations on industrially significant lipase, rhamnolipid and polyhydroxybutyrate produced by novel rumen microbes: *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6**" is the summary of the research work carried out by me under the supervision of **Dr. Sailas Benjamin**, Professor, Enzyme Technology Laboratory, Biotechnology Division, Department of Botany, University of Calicut, in partial fulfilment of the requirement for the award of Ph.D. degree in Biotechnology of the University of Calicut, and also declare that no part of this thesis has been submitted by me for the award of any other degree or diploma.

University of Calicut **PRIJI PRAKASAN**

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Priji Prakasan

Dedicated to

My Family and Teachers

EQUIPMENTS USED

Item	Brand	Country
Compound microscope	Magnus	India
Cooling centrifuge	Remi	India
Differential scanning calorimeter	Perkin elmer pyris 6	The USA
Digital SLR Camera	Nikon	Japan
Digital pH meter MK-VI	Systronics	India
Double distillation unit	Borosil	India
Electrophoresis unit	Biotech	India
Environmental shaker	Orbitek	India
Fluorescence microscope	Leica M80	Germany
FT- IR	Jasco	Japan
Gel-documentation system	BioRad	Italy
Heating mantle	Kemi	India
Image analyser	Nikon Eclipse E400	Japan
Incubator	Technico	India
Laboratory oven	Labline	India
Laminar air flow cabinet	Kemi	India
NMR Spectrometer	Bruker Avance III	The USA
Magnetic stirrer (KMS - 400)	Kemi	India
Micropipettes (0.5 -1000 µl)	Accupipete	India
Minitab 14	Minitab, Inc	The USA
Refrigerated centrifuge	Remi	India
Refrigerator	Godrej	India
Scanning electron microscope	Hitachi	Japan
Thermogravimetric analysis	Perkin- Elmer TGA	The USA
UV-Visible spectrophotometer	Shimadzu	Japan
Vortex mixture	Kemi	India
Water bath	Scigenics Biotech	India
Weighing balance	Shimadzu	Japan

ABBREVIATIONS

BSM	:	basal salt medium
CMC	:	critical micelle concentration
d	:	day
ddH_2O	:	double distilled water
FTIR	:	fourier transform infrared spectroscopy
g	:	gram
g/l	:	gram per litre
GLBS	:	glycolipid biosurfactant
h	:	hour
l	:	litre
mg	:	milligram
mg/ml	:	milligram per millilitre
min	:	minutes
ml	:	millilitre
mm	:	millimeter
mN/s	:	millinewton per second
MTCC	:	microbial type culture collection
MW	:	molecular weight
P(3HB-co-4HB)	:	poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
PHA	:	polyhydroxyalkanoate
PHB	:	polyhydroxybutyrate
P(HB-co-HH)	:	poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PHV	:	polyhydroxyvalerate
*p*NPP	:	*para*-nitrophenyl palmitate
rpm	:	revolutions per minute
RSM	:	response surface methodology
SDS-PAGE	:	sodium dodecyl sulphate-poly acrylamide gel electrophoresis
sec	:	seconds
SEM	:	scanning electron microscopy
SPS	:	starch-peptone-sodium chloride
TEMED	:	N,N,N',N'-tetra methyl ethylene diamine

TGA	:	thermogravimetric analysis
TLC	:	thin layer chromatography
U/ml	:	units per millilitre
w/v	:	weight per volume
β	:	beta
µg	:	microgram
µl	:	microlitre

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Chapter 1

Introduction

Increasing environmental concern on the huge accumulation of non-biodegradable chemical pollutants and rapid depletion of non-renewable resources has directed the attention of humankind towards microbial-derived compounds, especially due to their low toxicity and eco-compatibility (Benjamin *et al*., 2015; Pradeep *et al*., 2015). Since the beginning of twentieth century, technologies related to the microbial production of biomolecules have progressed to a great extent. Currently, microbes are used for the commercial production of a wide variety of products including industrial enzymes, polymers, pharmaceutics, *etc*. as suitable alternatives to synthetic chemicals. These biomolecules and their producing microorganisms are commercially significant because of the vast variety of biological activities, ease of genetic manipulation and high yield, coupled with exponential growth in inexpensive media and absence of seasonal fluctuations. Enzymes, biopolymers and surface active molecules are classic representatives of the microbial-derived and industrially significant biomolecules.

Enzymes are considered as the catalysts of the nature. Majority of the industrial enzymes are of microbial origin. Until the 1960s, the total sale of enzymes was only a few million dollars annually; since then, the market was broadened up spectacularly. About 60 % of all the industrial enzymes are hydrolytic in nature, among which lipolytic enzymes draw an enormous attention because of their immense biotechnological potentials. Lipids constitute a significant part of the biomass on earth, and lipolytic enzymes play the pivotal role in the turnover of these water-insoluble compounds. Lipases are ubiquitous enzymes catalysing the hydrolysis and synthesis of esters formed by the conjugation of glycerol and long chain fatty acids. Owing to the properties like catalytic activity over a wide range of temperature, pH, substrate specificity, enantioselectivity and diversity; lipases

have considerable physiological significance with industrial potentials (Benjamin and Pandey, 1998). Lipases catalyse a wide range of reactions, including hydrolysis, inter-esterification, acidolysis, trans-esterification, alcoholysis and aminolysis (Pandey *et al*., 1999). Lipases found promising applications in organic synthesis, detergent formulations, nutrition, synthesis of biosurfactants as well as industries related to dairy, agrochemicals, cosmetics, paper, and pharmaceuticals. Because of their wide ranging significance, lipases remain as a subject of intensive study (Pandey *et al*., 1999).

Surfactants are another group of industrially significant molecules that reduce the surface tension or interfacial tensions between the fluid phases. They are widely used in food, pharmaceutical, detergent, agriculture, medicine and textile industries. Most of them are chemically synthesised mainly from the petrochemicals. The recalcitrant and persistent nature of these chemicals raises many environmental and health issues. Due to the low toxicity, diversity, biodegradability, possibility of large scale production, selectivity and performance under extreme conditions, biosurfactants increasingly draw the attention of the scientific community as suitable alternative to the synthetic chemi-surfactants. By 1960s, biosurfactants were considered as hydrocarbon dissolution agents, but later for the last 5 decades, with the emergence of biotechnology and advancement in research extensified its applications in various industries like food, pharmaceuticals, cosmetic, detergent, biomedical and bioremediation (Sreedevi *et al*., 2014).

Use of plastics in everyday human life is nearly boundless. Low cost of production and versatility of plastics have raised its annual production to 250 million tons per year and its growth will continue to increase globally. These synthetic polymers are typically made from non-renewable resources (*i.e*, petroplastics) and are not degradable (Pradeep and Benjamin, 2012). The

hazardous environmental and health issues due to the massive accumulation of plastics and the exponential depletion of non-renewable resources have raised the demand for bioplastics (Sreedevi *et al*., 2014). Over the past decades, there has been considerable increase in the development and production of biodegradable plastics or bioplastics, which include polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides and copolymers and/or proper blends of these (Sreedevi *et al*., 2014). Polyhydroxybutyrate (PHB) is a typical member of the family of PHAs. Emergence of bioplastics opened up novel waste management strategies to overcome many undesirable properties of conventional petroplastics such as durability, resistance to biodegradation, release of toxic gases and phthalate plasticizers to the environment, *etc.* (Pradeep *et al*., 2014; Sarath Josh *et al*., 2014).

One of the thrust areas of current research is the discovery of new resources through extensive and persistent screening of biodiversity rich regions or environments on earth for isolating microbes capable of producing industrially significant biomolecules including enzymes and other metabolites. A large number of natural ecosystems are the sites of impressive diversity of microbial activities. In this study, the rumen, the least explored body part of cattle - apart from its clinical significances - was selected as resource of microbial diversity for the production of industrially significant biomolecules. Rumen, the first chamber of the alimentary canal in cattle, is the primary site of microbial fermentation, where it meets the bulk of natural organic compounds as a part of daily animal feed. Therefore, the rumen provides a suitable site for the isolation of microbes; which are harmless to mankind, compared to the microbes that grow in harsh and extreme environments. It is, therefore, expected that many industrially useful microbes (bacteria, fungi, yeast) have evolved in this environment by continuous exposure to organic substrates.

Upon this background, the present study mainly focused on the exploration of rumen microorganisms for the production of industrially significant biomolecules with special emphasis to lipases, biopolymers and biosurfactants. Thus, this study addresses the following questions:

1. To devise a protocol for the isolation, screening and cultivation of rumen microbes from Malabari goat.

2. To explore whether the rumen microbes can be used for the production of industrially significant biomolecules such as lipase, biosurfactant and biopolymer.

3. Statistical optimisation of the production conditions of the biomolecules such as lipase, biosurfactant and biopolymer to meet the '*low cost and high volume*' strategy of the industries.

4. Purification and characterisation of industrially significant biomolecules such as lipase, biosurfactant and biopolymer.

Chapter 2

Review of literature

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Priji, P., Sajith, S., Unni, K. N., and Benjamin, S. (2015). Glycolipid biosurfactants as candidates for green technologies. *Journal of Detergents and Surfactants*, (under review).

Background and rationale

Increasing concern about the devastating effects of synthetic chemicals on the environment and the prospects of declining petroleum resources have forced the industrialists to adopt new technologies aimed at reduced pollution and exploitation of renewable resources. Replacement of synthetic chemicals with biomolecules and the minimisation of wastes are the major aspects of an economic bioprocess. With the advent of biotechnology, the industries based on microbial bioprocesses are gaining unprecedented momentum. Now-a-days, several thousands of microorganisms and their products are available on the market; still the research world is behind the development of new microbial resources to make the industrial processes economically more feasible.

Rumen is one of the fascinating and complex microbial ecosystems and is termed as '*constant and continuous*' in nature. The study on industrially significant biomolecules from rumen microorganisms opens a new path to the alternative microbial resources and their possible adaptation strategies. Moreover, it provides insights to the possibilities for improving the efficiency of existing production systems. Therefore, the present work is focused on three diverse groups of industrially significant biomolecules; lipase, biosurfactant and polyhydroxyalkanoates (PHAs) produced by the rumen microorganisms.

Considering these facts, the review of literature is presented in four sections: **The first section** provides a general insight into the rumen microflora and the industrially significant molecules produced by them. **The second section** looks critically into the major domains of microbial lipases with an industrial perspective, which include: properties, secretion and industrial applications

with appropriate illustrations. **The third section** elaborates the features of glycolipid biosurfactants with emphasis on their properties and applications, coupled with the current market scenario. **The fourth** section examines the structural diversity and applications of PHAs as a replacement for petroplastics.

SECTION I: RUMEN MICROFLORA

Introduction

Rumen, the preliminary chamber of alimentary canal of ruminants, is a continuous and open ecosystem which provides a suitable environment for the inhabitance of a wide variety of microorganisms especially bacteria, fungi and protozoa, which have evolved through millions of years of natural selection. The rumen microflora play a crucial role in the digestion and fermentation of the major share of the feeds that the animals consume, otherwise proper digestion would be hindered. Thus, there exists a valid and suitable symbiotic relationship between the microflora and ruminant animal (Russell and Rychlik, 2001). Robert Hungate, a pioneering microbial ecologist, is considered as the father of rumen microbiology who initiated the systemic exploration of bovine rumen ecosystem (McSweeney *et al*., 1999). Rumen ecosystem generally accommodates a diverse and specialised population of facultative/obligatory anaerobes including bacteria (species of *Bifidobacterium, Butyvibrio, Lactobacillus, Lactococcus, Propionibacterium, Pseudomonas, Megasphaera, Pediococcus, Ruminococcus, etc.*); fungi (species of *Trichoderma, Mortierella, Pichia, Delacroixia etc*.); and protozoans (*Dasytricha ruminantium, Isotricha prostoma, Eremoplastron dilobum, Entodinium caudatum, Ophryoscolex purkynjei* and *Polyplastron multivesiculatum, etc.*) (McSweeney *et al*., 1999; Kenters *et al*., 2011). Interestingly, it is believed that only 10-20 % of rumen microorganisms are

known so far; the remaining is still unknown in terms of their identity as well as functions (Kobayashi, 2006).

Industrial significance of rumen microflora

The ability of the ruminants to adapt to dietary change is mainly contributed by the microorganisms reside in their gut. Even though, rumen microorganisms are considered as cell factories for the production of enzymes and other metabolites; only a few of them are explored industrially (McSweeney *et al.*, 1999). The rumen microorganisms produce a variety of inducible enzymes like cellulases, lipases, proteases, *etc*. to hydrolyse the feed that the animal consumes - in order to obtain the energy as carbon sources in accessible form (Weimer *et al.*, 2009; Sauer and Mattanovich, 2012). Moreover, they produce a large variety of biochemicals such as antibiotics and organic acids that make the rumen to resemble modern biorefineries. Some of them are listed in **Table 1**.

Table 1. List of some ruminant microorganisms producing industrially significant biomolecules.

Microorganism	**Biomolecule**	**Reference**
Selenomonas ruminantium	Phytase	(Hong *et al.*, 2004)
Ribulococcus albus	Endoglucanase	(Karita *et al.*, 1996)
Eubacterium cellulosolvens	Cellulase	(Yoda, 2006)
Eubacterium cellulosolvens	Epimerase	(Taguchi *et al.*, 2008)
Anaerobiospirillum succiniproducens	Succinic acid	(Sauer *et al.*, 2008)
Megsphaera elsdenii	Butyric, valeric or caproic acids	(Elsden *et al.*, 1951)
Methanobrevibacter	Methane	(Miller and Lin, 2002)
Neocallimastix patriciarum	Xylanase	(Li *et al.*, 1996)
Prevotella ruminicola	Protease	(Wallace and McKain, 1991)
Piromyces sp.	Mannase	(Ali *et al.*, 1995)

Conclusions

The superior activities and specificities of the biomolecules produced by the rumen microorganisms have directed the researchers into their omics-related studies as well as their expressions in other microbe/plant/animal systems. Thus, the emerging technologies foresee and expedite the utilities of these biomolecules in various fields so as to benefit the humankind in a healthy environment.

SECTION 2: MICROBIAL LIPASES

Introduction

Enzymes are considered as the biocatalysts of nature. The increasing concern about the environmental pollutions and stringent government regulations over the world has turned the attention of industries toward green technologies. Majority of the industrial enzymes are of microbial origin. Until the 1960s, the world market of microbial enzymes was only a few million dollars business; but later, the market was broadened up enormously. At present, more than 200 microbial enzymes are used commercially and approximately 20 types are produced on truly industrial scale (Pandey *et al.*, 1999; Li *et al.*, 2012). Most of the industrially significant enzymes are hydrolytic in nature; of which lipolytic enzymes play the key role in the conversion of hydrophobic lipids - that constitute the abundant biomass on earth - into simpler units (Benjamin and Pandey, 1998).

Lipases or triacylglycerol hydrolases (E.C. 3.1.1.3) are ubiquitous enzymes mediating the hydrolysis of ester bond in triglycerides releasing long-chain fatty acids (Hasan *et al.*, 2006). Physiologically lipase hydrolyses triglycerides into diglycerides, monoglycerides, fatty acids and glycerol. They are abundant in animals, plants, bacteria and fungi; where they play the crucial role in lipid metabolism. For the past few decades, lipases have gained

much attention due to their versatile activities toward extreme temperature, pH, organic solvents; and chemo-, regio- and enantio-selectivities (Benjamin and Pandey, 1996). In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface *i.e*., lipase possesses a unique property of catalysing the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase, where the enzyme remains dissolved (Benjamin and Pandey, 2000). For the past two decades, the world-wide production and consumption of microbial lipases have increased considerably, which summarise the lipases as the third largest group of enzymes after proteases and amylases, owing to their fascinating industrial applications. In fact, the panorama of lipase utilisation encompasses many industries like dairy, food, detergents, textile, pharmaceutical, cosmetic, biodiesel, *etc*. (Schmid and Verger, 1998). Upon this background, this review examines the advancements in the various domains of microbial lipases such as production, properties, secretion and industrial applications.

Microbial sources of lipases

Lipases are produced by several microorganisms, namely bacteria, fungi, archea, eucarya; as well as animals and plants - among which bacteria, fungi and yeasts yield the majority of commercial lipases. The commercial significance of microbial lipases is mainly attributed to their vast variety of catalytic activities, ease of genetic manipulation and high yield, coupled with exponential growth of the producing microbes in inexpensive media and absence of seasonal fluctuations (Benjamin and Pandey, 1996). Moreover, most of the microbial lipases do not require cofactors for their activation, and they exhibit broad range of substrate specificity and high enantio-selectivity (Kirk *et al.*, 2002). Many species of bacteria such as *Pseudomonas, Bacillus, Serratia, Alcaligens, etc*., fungi such as *Aspergillus, Penicillium,* and yeast *Candida, etc*. are known to produce lipases (**Table 2**). The potential for the

production of lipases enable these microbes to utilise the non-conventional carbon sources such as lipids that cannot directly pass through the cell membrane and have to hydrolyse partially to release free fatty acids prior to the cellular uptake (Najjar *et al.*, 2011). Even though, a vast variety of microbial species are known for lipase production, only a few are utilised commercially and most of them are extracellular inducible enzymes. *i.e.*, they are synthesised within the cell in the presence of inducers of long chain fatty acids such as vegetable oils, oil industry wastes, surfactants, triglycerides, *etc*. and secreted to the external environment.

Table 2. List of some bacteria and fungi producing lipase with their sources of isolation and lipolytic activities.

	Organism	Source	Substrate	Lipase	Reference
Bacteria	*Pseudomonas aeruginosa*	Wastewater	Olive oil	41.6 U/ml	(Zouaoui and Bouziane, 2011)
	Pseudomonas sp. 3AT	Contaminated soil and water	Olive and sunflower frying oils	2.748 U/ml	(Haba *et al.*, 2000)
	Pseudomonas aeruginosa	Contaminated soil and water	Olive and sunflower frying oils	1.7038 U/ml	(Haba *et al.*, 2000)
	Bacillus sp.	Olive mill wastewater	Tributyrin	168 U/ml	(Ertuğrul *et al.*, 2007)
	Bacillus sp.	Setapak hot spring	Olive oil	4.58 U/ml	(Hamid *et al.*, 2003)
	Pseudomonas aeruginosa KM110	Wastewater of oil processing plant	Olive oil	0.76 U/ml	(Mobarak-Qamsari *et al.*, 2011)
	Staphylococcus warneri	Thai fish sauce	Olive oil	90.12 U/ml	(Kanlayakrit and Boonpan, 2007)
	Staphylococcus saprophyticus	Sea water	Olive oil	42 U/ml	(Fang *et al.*, 2006)
	Burkholderia sp.	Soil	Salad oil	1.720 U/ml	(Matsumiya *et al.*, 2007)
	Bacillus strain THL027	Oil contaminated	Rice bran oil	7.8 U/ml	(Dharmsthiti and Luchai,

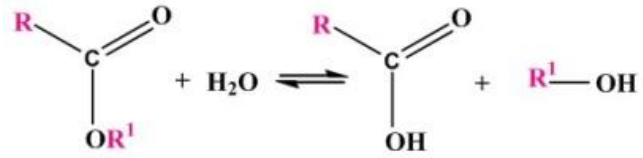
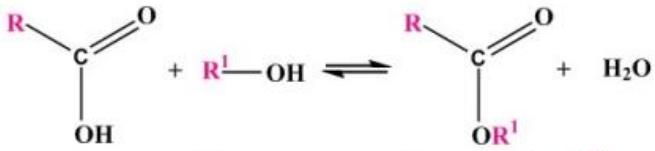
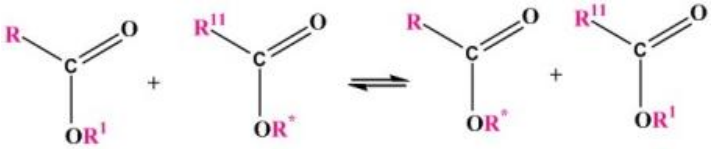
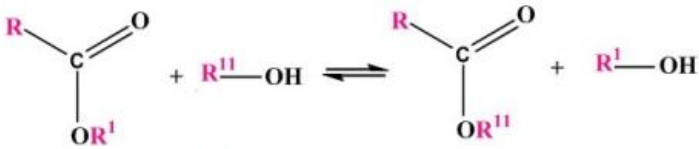
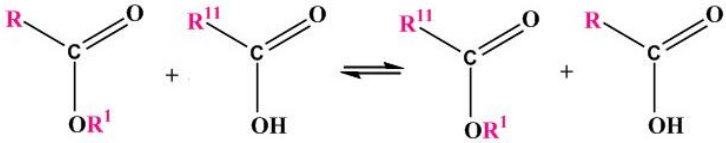
		area			1999)
	Bacillus coagulans BTS3	Kitchen waste	Olive oil	1.16 U/ml	(Kumar *et al.*, 2005)
	Bacillus thermoleovorans CCR11	Hot springs	Olive oil		(Castro-Ochoa *et al.*, 2005)
	Burkholderia multivorans	Oil enriched compost	Palm oil	58 U/ml	(Gupta *et al.*, 2007)
	Bacillus sp. RSJ1	Hot springs	Cotton seed oil	10.5 U/ml	(Sharma *et al.*, 2002)
	Bacillus thermoleovorans IHI-91	Icelandic hot spring	Olive oil	0.300 U/ml	(Markossian *et al.*, 2000)
	Bacillus sphaericus	Soil sample	Olive oil	0.42 U/ml	(Hun *et al.*, 2003)
	Streptomyces rimosus	-	Triolein	19 U/ml	(Abramić *et al.*, 1999)
	Penicillium camembertii Thom PG-3	-	Soybean meal, Jojoba oil	500 U/ml	(Tan *et al.*, 2004)
	Colletotrichum gloesporioides	Brazilian savanna soil	Olive oil	27.7 U/ml	(Colen *et al.*, 2006)
	Rhizopus oryzae KG-5	Contaminated soil	Olive oil	48.66 I.U.	(Shukla and Gupta, 2007)
	Aspevgillus niger	Curd	Gingelly oil cake	236.6 U/g	(Kamini *et al.*, 1998)
	Aspergillus niger NCIM 1207	-	Wheat bran and olive oil	630 IU/g	(Mahadik *et al.*, 2002)
	Candida sp. 99-125 (mutant)	-	Soy bean oil	8060 U/ml	(Tan *et al.*, 2003)
	Penicillium restrictum	Waste of oil industry	Babassu oil cake	30.3 U/g	(Gombert *et al.*, 1999)
Fungi	*Colletotrichum gloeosporioides*	Oil seeds	Pongamia oil cake	983 U/g	(Balaji and Ebenezer, 2008)
	Candida cylindracea		Olive-mill waste water	21.6 U/ml	(Brozzoli *et al.*, 2009)
	Pseudozyma hubeiensis	Phylloplane of *Hibiscus rosa-*	Soy oil	0.386 U/ml	(Bussamara *et al.*, 2010)

	sinensis			
Fusarium oxysporum		Olive oil	16 U/ml	(Rifaat *et al.*, 2010)
Aspergillus niger 11T53A14	Contaminated butter	Soap stock	62.7 U/g	(Damaso *et al.*, 2008)
Penicillium wortmanii	Soil	Olive oil	12.5 U/ml	(Costa and Peralta, 1999)
Rhizopus homothallicus	-	Sugarcane bagasse and olive oil	826 U/g	(Rodriguez *et al.*, 2006)
Mucor sp.	Palm fruit	Palm oil	57 U/ml	(Abbas *et al.*, 2002)
Botryosphaeria ribis	*Eucalyptus citriodora*	Stearic acid	316.7 U/ml	(Messias *et al.*, 2009)
Mucor hiemalis	Palm fruit	Rape seed oil	97 U/ml	(Hiol *et al.*, 1999)
Penicillium simplicissimum	-	Babassu cake	90 U/g	(Gutarra *et al.*, 2009)
Mutant Yarrowia lipolytica		Olive oil	1100 U/ml	(Fickers *et al.*, 2006)
Rhizopus oryzae	Palm fruit	-	120 U/ml	(Hiol *et al.*, 2000)
Aspergillus sp.	Oil cakes and seeds	Wheat raw and olive oil	1934 U/g	(Adinarayana *et al.*, 2004)
Trichoderma viride	Soil	Olive oil	7.3 U/ml	(Kashmiri *et al.*, 2006)

Reactions catalysed by lipase

Lipases catalyse a variety of reactions, which are primarily determined by the availability of water. Principally, they catalyse the hydrolysis of triglycerides at the aqueous-non aqueous interface, and favour the synthesis of esters from alcohols and long chain fatty acids when the water activity is low (Aravindan *et al.*, 2007), *i.e*, lipases can catalyse esterification, inter-esterification, and *trans*-esterification reactions in non-aqueous environments (**Table 3**). The versatility in activities makes lipases a suitable choice of catalyst in many industries.

Table 3. The reactions catalysed by lipase.

Nature of reaction	Name of reaction	Reaction
Hydrolysis	Hydrolysis	
Synthesis	Esterification	
Synthesis	Inter-esterification	
Synthesis	Alcoholysis	
Synthesis	Acidolysis	

Properties of lipase

The *pH*

Most of the microbial lipases are neutral or acidic at their optimum activity. Alkaline lipases, which are stable at pH range 8-10 offer promising applications in many upcoming bio-based industries such as textile, detergent, *etc*. Lipase from *Aspergillus carneus, Bacillus thermoleovorans, Bacillus stearothermophilus, Fusarium oxysporum* were found stable at the pH range 8-10 (Sinchaikul *et al.*, 2001; Castro-Ochoa *et al.*, 2005; Prazeres *et al.*, 2006) whereas lipase produced by *Rhizopus oryzae, Cryptococcus* sp.

showed the maximum activity at the pH range 7-8 (Hiol *et al.*, 2000; Kamini *et al.*, 2000).

Temperature

Generally, most of the microbial lipases are mesophilic in nature, *i.e.*, the optimum temperature for their maximum activity ranges from 25 to 40 ^{o}C, but the increased demand for thermotolerant lipases in industries has led to the exploration of many microbial species producing thermophilic lipases, most of which retained 70-100 % of the activity even at the temperature range 50-70 ^{o}C (Khalil, 2013; Bora and Bora 2012; Gohel *et al.*, 2013); for instance, a lipase from *Bacillus* sp. (an isolate from the hot spring) retained 90 % activity at 60 ^{o}C and 70 % of activity at 70 ^{o}C for 1h (Bora and Bora, 2012); whereas the lipase from *Pseudomonas* sp. strain ZBC1 showed the optimum activity at 80 ^{o}C (Xing *et al.*, 2013). Cold active lipases are active at 10-25 ^{o}C, and they facilitate gentle and efficient industrial applications by significantly reducing energy consumption. Many microbes such as *Rhodococcus cercidiphylli* BZ22, *Penicillium expansum*, *Yarrowia lipolytica*, *Stenotrophomonas maltophilia*, *Pseudoalteromonas* sp. *etc*. isolated from the harsh environments, produced lipases active at low temperatures (Sathish Yadav *et al.*, 2011; Wang *et al.*, 2012; Mohammed *et al.*, 2013; Park *et al.*, 2013; Yu and Margesin, 2014).

Effectors

Usually metal ions can alter the efficiency of an enzyme either by enhancing or by inhibiting the activity. Divalent metal cations such as Ca^{2+}, Cd^{2+}, Fe^{2+}, *etc*. enhance the activity of lipases, among which Ca^{2+} plays a critical role in stabilising the enzyme under detrimental conditions (Khattabi *et al.*, 2003; Verma *et al.*, 2012). Co^{2+}, Zn^{2+}, Mn^{2+}, and Mg^{2+} have mild to strong

inhibitory effect on lipase activity (Kumar *et al.*, 2005). Detergents such as tween 80, tween 20, SDS inhibited the lipase activity whereas triton X-100 supported the enhanced activity (Castro-Ochoa *et al.*, 2005).

Specificity

Specificities of lipases play a crucial role in their possible applications in analytical and industrial purposes, especially in pharmaceutical industry. Majority of the lipases show substrate or region- or enantio-specificites, which are highly determined by the size, shape and hydrophobicities of the binding pockets located in the active site. Some of the lipases, specifically act on tri-, di-, mono-glycerides and other esters. Non-specific lipase completely hydrolyse the triglycereides to fatty acids and glycerol, but most of the extracellular lipases are region-specific especially at 1, 3 positions. Lipase from *Burkholderia cepacia* found applications in organic synthesis due to its enantiospecificities, preferably the (*R*)- enantiomer over the (*S*)- forms (Jaeger *et al.*, 1999).

Interfacial activation

Lipase exhibits a characteristic property, called interfacial activation which makes it a suitable catalyst in water-oil medium, *i.e.*, the activity of the enzyme is significantly increased when the substrates form emulsion in the reaction media. In aqueous medium, the active site of enzyme is covered by a loop of peptide called '*lid*', but contact with the interfacial area induce drastic conformational changes on to the active site, so that the lid moves aside facilitating the enzyme-substrate reaction (Dheeman, 2011).

Active site of lipase

The crystalline structures of many bacterial lipases have been elucidated to date, among which most of them shared a common folding pattern known as

α/β hydrolase (**Figure 1**). Generally, *α/β* hydrolase consists of *α* helices (*α*A - *α*F) packed on either sides of a central beta sheet. The central *β* sheet is made of 8 parallel strands (*β*1- *β*8), except the second strand which is in antiparallel direction. The active site of lipase consists of 3 catalytic residues (the triad), *i.e*., a nucleophilic residue, a catalytic acid residue and a histidine residue. In lipases, the nucleophile is invariably serine, whereas the catalytic acid is either an aspartate or glutamate. The topological position of the nucleophilic residue is often after *β*5 strand, the Asp/Glu residue is after *β*7 strand and the histidine residue is after *β*8 strand (Jaeger *et al.*, 1999) (**Figure 1**). In most of the lipases, the '*lid/hood*' or the flexible fragment is made of one or two alpha helices, which covers the active site at the inactive state of the enzyme. In the presence of hydrophobic substrates, the enzyme undergoes interfacial activation so that the conformational changes at the active site make the lid open, so as to facilitate the entry of substrates to the catalytic residues (Pleiss *et al.*, 1998).

The geometry of the active sites of lipases varies widely and determines the biochemical properties of the enzyme. Generally, it is a deep hydrophobic pocket which exactly fits scissile fatty acids of substrates into it. According to the shapes of the bindings sites, lipase can be categorised into three; lipase with crevice-shaped binding site (*Rhizomucor* and *Rhizopus*), funnel-shaped binding site (Pancreatic lipase, lipase from *C. antarctica* and *Pseudomonas),* and tunnel-shaped binding site (*C. rugosa, Geotrichum candidum*) (Pleiss *et al.*, 1998) (**Figure 2**).

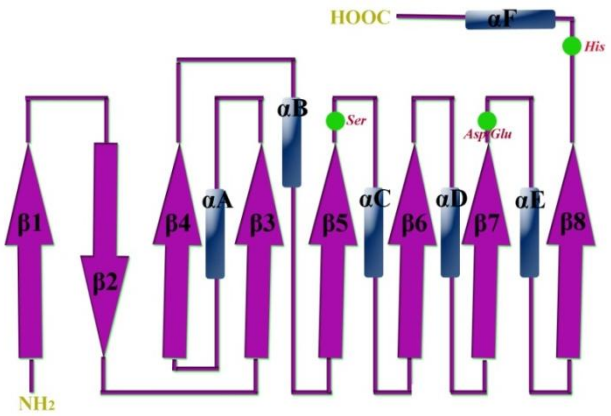


Figure 1. Structure of *α/β* hydrolase

Classification of lipase

Most of the lipases possess a consensus sequence of Gly-x-Ser-x-Gly around the serine residue situated at the active site. But advances on their crystalline studies and sequence analyses revealed the existence of other motifs also. Thus, based on their sequence homology and functional properties, lipases are classified into 8 major groups, among which the first largest family is again classified into 6 subfamilies (Arpigny and Jaeger, 1999; Bornscheuer, 2002) (**Table 4**).

Table 4. Classification of lipases

Family	Subfamily	Microorganism
I	1	*Pseudomonas aeruginosa*
		Pseudomonas fluorescens C9
		Vibrio cholerae
		Acinetobacter calcoaceticus
		Pseudomonas fragi
		Pseudomonas wisconsinensis
		Proteus vulgaris
	2	*Burkholderia glumae*
		Chromobacterium viscosum
		Burkholderia cepacia
		Pseudomonas luteola

	3	*Pseudomonas fluorescens* SIK W1
		Serratia marcescens
	4	*Bacillus subtilis*
		Bacillus pumilus
	5	*Bacillus stearothermophilus*
		Bacillus thermocatenulatus
		Staphylococcus hyicus
		Staphylococcus aureus
		Staphylococcus epidermidis
	6	*Propionibacterium acnes*
		Streptomyces cinnamoneus
II		*Aeromonas hydrophila*
		Streptomyces scabies
		Pseudomonas aeruginosa
		Salmonella typhimurium
		Photorhabdus luminescens
III		*Streptomyces exfoliatus*
		Streptomyces albus
		Moraxella sp.
IV		*Alicyclobacillus acidocaldarius*
		Pseudomonas sp. B11-1
		Archaeoglobus fulgidus
		Alcaligenes eutrophus
		Escherichia coli
		Moraxella sp.
V		*Pseudomonas oleovorans*
		Haemophilus influenzae
		Psychrobacter immobilis
		Moraxella sp.
		Sulfolobus acidocaldarius
		Acetobacter pasteurianus
VI		*Synechocystis* sp.
		Spirulina platensis
		Pseudomonas fluorescens
		Rickettsia prowazekii
		Chlamydia trachomatis

VII	*Arthrobacter oxydans*
	Bacillus subtilis
	Streptomyces coelicolor
VIII	*Arthrobacter globiformis*
	Streptomyces chrysomallus

Source: (Arpigny and Jaeger, 1999)

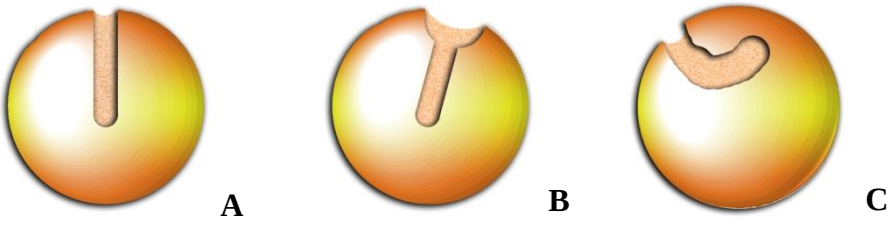


Figure 2. Different shapes of lipase active site (A). tunnel-like (B). funnel-like (C). crevice-like

Lipase secretion pathways

Lipases are extracellular enzymes, and are to be transported across the cell membrane to reach its final destination. In Gram-positive bacteria, secreted enzymes have to cross just a single cytoplasmic membrane whereas in Gram-negative bacteria, it has to be translocated through the periplasm and outer membrane (Jaeger *et al.*, 1999). A series of complex transporter proteins play crucial roles in the transportation of lipase through the membrane. Two major secretory pathways have been identified (represented as type I and II), of which lipases can utilise at least one of them (Jaeger and Eggert, 2002). Type I pathway is mainly mediated by ABC transporters, whereas secreton mediates the type II.

Lipases produced by *P. fluorescence* and *Serratia marcescens* display a C-terminal secretion signal located in the last 60 amino acids, and is not cleaved during secretion. The signal sequence specifically recognises the ABC

protein, triggering the assembly of the functional trans-envelope complex (Delepelaire, 2004). Type I secretory pathway *via* ABC transporters generally consists of three major membrane proteins, *i.e*., the inner membrane ATPase [ATP-binding cassette (ABC) superfamily], the second protein, called membrane-fusion-protein (MFP) which is located in the inner membrane with a large hydrophilic domain and a C-terminal domain interacting with the periplasm and outer membrane respectively. The third protein involved in this pathway is an outer membrane protein (OMP) (**Figure 3**). The assignment of different proteins as these exporters may vary depending on the species. *Serratia marcescens,* the most studied bacterium for type I secretion of lipase possess a signal peptide rich in glycine comprising nine-residue sequence Gly-Gly-X-Gly-X-Asp-X-U-X (where X is any amino acid and U is a large hydrophobic amino acid), and utilise LipB as ABC protein, LipC plays the role of MFP component, and LipD acts as the OMP component (Akatsuka *et al.*, 1995; Jaeger and Eggert, 2002). AprD, AprE and AprF are necessary proteins acting as ABC, MFP and OMP, respectively for the efficient secretion of lipase by *P. fluorescence* (Duong *et al.*, 1994). The exporter constituted by these proteins forms a multiprotein complex across the periplasm for translocating lipase from the cytoplasm to extracellular space without forming any active periplasimic enzyme intermediates. Over expression of the ABC exporter provides a considerable increase in the secretion of the lipase, and therefore an increased yield of extracellular lipase protein (Ahn *et al.*, 2001).

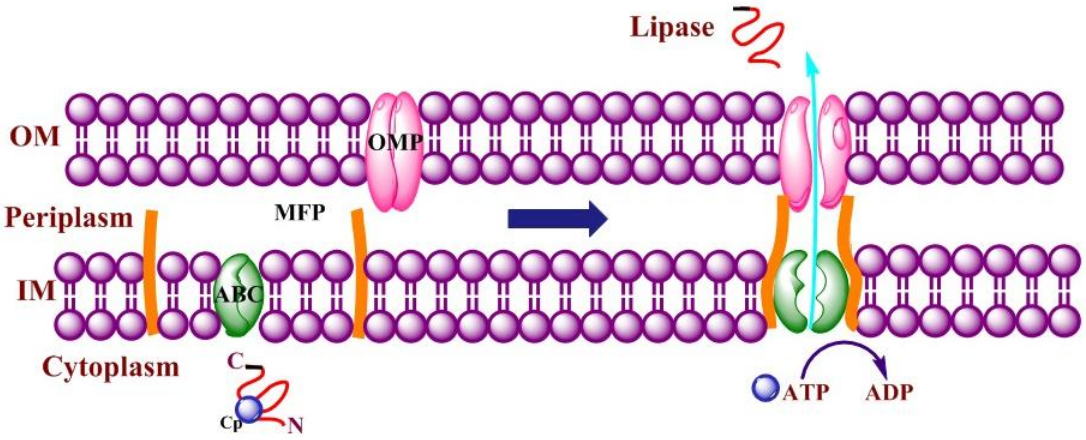


Figure 3. Type I secretory pathway of lipase *via* ABC transporters.

The type II secretory pathway is generally known as sec-dependent pathway, which was found involved in the secretion of lipase in bacteria like *Pseudomonas aeruginosa* and *Bacillus* spp. Many lipases of both Gram-positive and Gram-negative bacteria possess an N-terminal signal sequence and are secreted *via* type II pathway. It consists of two steps: proteins are first translocated across the inner membrane by the general secretory pathway (Cao *et al.*, 2014) or twin-arginine translocation (*Tat*) pathway, and subsequently transported from the periplasm to the exterior by an outer membrane in an extremely short period (**Figure 4**). In Gram-positive bacteria, secreted enzymes have to cross just a single cytoplasmic membrane. Usually, these proteins contain a signal sequence, which directs their translocation *via* the *Sec* or *Tat* translocase, the multi-subunit proteins identified in *Bacillus* spp. However, lipases from Gram-negative bacteria do have to cross a second barrier constituted by the outer membrane. In *P. aeruginosa*, the prolipase exported to the periplasmic space by *Sec* machinery, fold in the periplasm into an enzymatically active conformation with the help of specific intermolecular chaperones called *Lif* proteins (lipase-specific foldases). Subsequently, they are transported through the outer membrane by means of a complex machinery called secreton, consisting of up to 14 different proteins. Similar

multi-component secretons have been identified in lipase producing *P. alcaligenes*, *Aeromonas hydrophila*, *Xanthomonas campestris* and *Vibrio cholera* (Jaeger and Eggert, 2002).

In *P. aeruginosa*, autotranspoters also mediate the transport of enzymes across the outer membrane. These autotransporters form channels spanning in the outer membrane, which is usually made up of a *β*-barrel composed of nearly 14 *β*-sheets and hold the enzyme in close contact to, or even firmly bound to the surface of the cell exposing its catalytic domain (Rosenau and Jaeger, 2000).

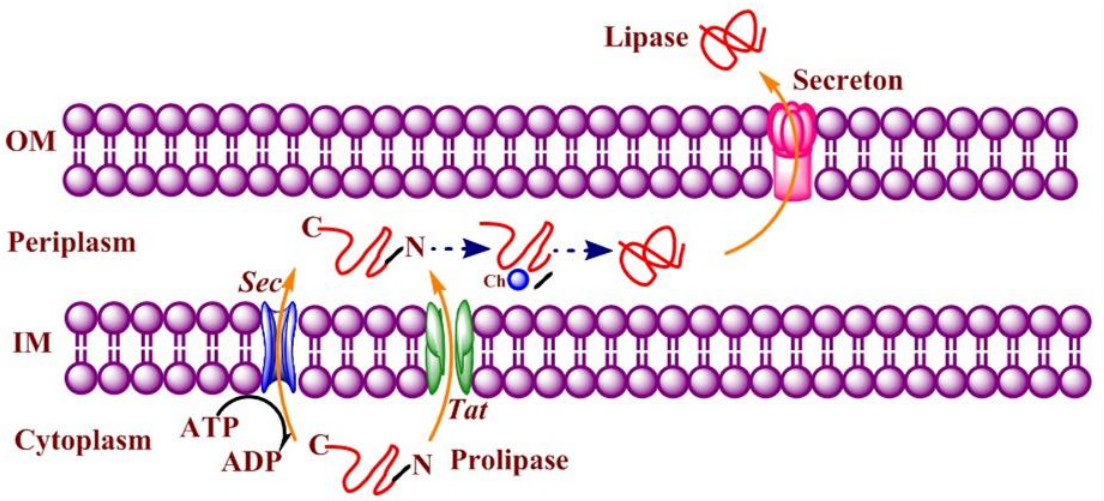


Figure 4. Type II secretory pathway of lipase mediated by *Sec* or *Tat* translocase.

Applications of lipases

Microbial lipases claim a wide variety of industrial applications due to ease for mass production and versatile specificities. Based on total volume of sales, lipases occupy the third largest group of enzymes next to proteases and carbohydrases. The commercial use of lipases is a billion-dollar business and their applications highly dependent on specificities, optimum pH, temperature, tolerance to organic solvents, *etc*. Lipases form an integral part

of the industries ranging from food and fats, dairy, organic chemicals, pharmaceuticals, leather, environmental management, agrochemicals, detergents, oleo-chemicals, tea, cosmetics, and in several bioremediation processes (Verma *et al*., 2012).

Food and Dairy industry

Lipases are widely used in food industries for the hydrolysis or modification of biomaterials. Egg white is an important ingredient of many bakery products such as cakes, bread, *etc*. Contamination of egg white with lipids decreases the desirable foaming ability of a product. Therefore, lipase is used to remove the lipid contamination and to improve the quality of dough and to achieve an even, light-colored crust on the products and a soft texture. Treatment of egg yolk with phospholipase hydrolyses egg lecithin and iso-lecithin, thereby improving emulsifying capacity and heat stability, which can be used to make myonnaise, custards, baby foods, *etc.*(Buxmann *et al.*, 2010). Lipases can enhance the flavor of bakery products by synthesising esters of short-chain fatty acids and alcohols (Macedo *et al.*, 2003). Lipases can also be used in degumming of vegetable oils during the process of refining. The degumming process removes the phospholipid impurities from the crude vegetable oils which may otherwise pose many problems for the storage and processing of vegetable oils from soybean, sunflower and rape seed; for instance, lipase produced by genetically modified *Aspergillus oryzae* (Lecitase® Ultra) was used for the degumming of rapeseed and soybean oils, which removed more than 90 % of phospholipids within 5 h at 50 °C (Yang *et al.*, 2006).

Biolipolysis is being used to make fat free meat and fish. The controlled application of lipase to wheat flour produces different variations of quality; thereby noodles and pasta are given an even and intense color, and their stickiness when overcooked is reduced (Menzi, 1970; Søe *et al.*, 2005). Genetically engineered baker's yeast (*Saccharomyces cereviciae*) with

bacterial lipase gene LIP A resulted in higher productivity of lipase and found application in bread making as a technological additive (Sánchez *et al.*, 2002).

Lipases are extensively used in dairy industry for the hydrolysis of milk fat to improve the flavor of cheese, butter, fat and cream. Enzyme modified cheese technology is now gaining importance for making a variety of cheese with desired flavor and aroma; in this process lipase is generally used under controlled conditions in combination with other hydrolytic enzymes such as protease and amylase. It reduces the bitterness as well as ripening time and modifies flavor intensity (Aminifar and Emam-Djomeh, 2014). Commercial microbial lipase (Piccantase A) enhanced the flavor development during the ripening stage of Talum cheese (Yilmaz *et al.*, 2005). Extracellular lipase produced by *Cryptococcus flavescens* 39-A released short-chain fatty acids (C_4 - C_6) in milk fat during the mozzarella cheese-making process and produced a favorable cheese flavor (Mase *et al.*, 2013). *Penicillium roqueforti*, the lipolytic activities of which contributes to the characteristic flavor and blue-green veined appearance to blue cheese (Cao *et al.*, 2014). Lipases are widely used to produce novel fats through the process of hydrolysis, esterification and inter-esterification. Lipases modify the properties of lipids by altering the positions of fatty acid chains or by adding or removing one or more fatty acids to the glycerides. The position, chain length and degree of unsaturation greatly influence not only the physical properties, but also the nutritional and sensory value of a given triglyceride as well. Thus, high value fats and oils can be synthesised from cheap resources. For example, cocoa butter fat, used in bakery foods, is often in short supply and the price can fluctuate widely. Lipase from *Mucor miehei* can effectively be utilised to produce coco butter like fat from palm olein and distillate from palm oil refinery which contribute to the flavor of chocolate, caramels, toffees and butter creams (Mohamed, 2012). In coffee whiteners, lipases assist in imparting a rich creamy flavor (Godfrey and Reichelt, 1982).

Processing of fats and oil

Processing of fats and oils by the enrichment of specific fatty acids or hydrolysis of triglycerides to release fatty acids or by altering the location of fatty acids is an important application of lipase. It enables the commercial exploitation of naturally produced renewable raw materials such as oils from corn, rapeseed, sunflower, palm, coconut, olives, rice bran, and a wide range of animal fats. Wax esters, esters of long chain fatty acids and alcohols (C ≥12) are widely used in lubricant, pharmaceutical, cosmetic and plasticizer industries, which are usually extracted from expensive spermaceti oil and jojoba oil for commercial applications. Lipase mediates the synthesis of wax esters from cheap oils, for instance, immobilised lipase from *Candida* sp. synthesises the wax ester, cetyl oleate, from oleic acid and cetyl alcohol with over 96 % purity (Li *et al.*, 2011). Lipase catalyses the hydrolysis of salmon oil to increase its omega-3 polyunsaturated fatty acids (PUFA) content. It was observed that lipase from *C. rugosa* increased omega-3 PUFA content by 2.5 folds (Kahveci *et al.*, 2010; Kahveci and Xu, 2011). Similarly, microbial lipases, from *C. rugosa*, *C. cylindracea*, *Mucor javanicus* and *Aspergillus niger* were used for the enzymatic hydrolysis of sardine oil, which increased the content of omega-3 PUFAs by 10-35 % (Okada and Morrissey, 2007). Production of biodiesel consisting of methyl esters (methanolysis) of long chain fatty acids is yet another promising application of lipase which has been widely exploited all over the world. In such cases, immobilised lipase was used, which offers repeated usage of the enzyme without losing its specificity. The enzymatic production of biodiesel by methanolysis of cottonseed oil was studied using immobilised lipase from *C. antarctica* as catalyst in *t*-butanol solvent, in which the ester yield was about 95-97 % (Royon *et al.*, 2007). Lipase producing whole cells of *Rhizopus oryzae* was employed for the production of biodiesel employing the low cost non-edible oil from the seeds of *Jatropha curcas.* A variety of low cost vegetable oils such as sunflower oil,

soybean oil, karanj oil, *etc*. can effectively be used to produce biodiesel by lipase mediated hydrolysis (Kaieda *et al.*, 2001; Noureddini *et al.*, 2005; Modi *et al.*, 2007; Dizge *et al.*, 2009).

Detergents

Now-a-days enzymes have become the key constituents of detergent formulations, of which lipases play an important role for the removal of tough fatty stains such as butter, oil, *etc*. from the fabric that are hard to remove under normal washing. For the last two decades, detergent industry has become one of the biggest markets of microbial lipases, because of their functional importance for the removal of fatty residues in laundry, dishwashers and for cleaning of clogged drains. Addition of these enzymes not only improves the performance of detergents but also offers better ecological acceptance and produce effluent with lower COD and corrosive nature (Nerurkar *et al.*, 2013). Standard wash liquids contain anionic and nonionic surfactants, oxidants, and complexing agents at a pH of about 10 and temperatures around 35-45 ^{o}C, which is a rather hostile environment for enzymes. As a result, massive screening is required to find out suitable enzymes exhibiting low substrate specificity, stability under alkaline pH (8-11), elevated temperature (30-60 ^{o}C), and also compatibility with other ingredients of formulations such as metals, oxidants, surfactants *etc*. Bacterial lipase from *Staphylococcus arlettae* JPBW-1 isolated from the rock salt mine has been assessed for its use in laundry formulations which exhibited good stability towards surfactants and oxidising agents, and removed about 62 % of olive oil from cotton fabrics (Chauhan *et al.*, 2013). A novel alkaline lipase from *Burkholderia cepacia* RGP-10 exhibited better stability towards commercial detergents and oxidising agents than Lipolase® (Rathi *et al.*, 2001). Application of cold active lipase in detergent formulations allows laundering at low temperatures and reduces the energy expenditure. Recently,

many cold-active lipases have been reported in bacteria such as *Pseudoalteromonas* sp. NJ 70 (Wang *et al.*, 2012), *Bacillus sphaericus* (Joseph and Ramteke, 2013), *Microbacterium luteolum* (Joseph *et al.*, 2012), *Pichia lynferdii* (Park *et al.*, 2013) *etc*. Most of them are active at temperature ranging from 0-30 ^{o}C, and showed good tolerance to salt, synthetic surfactants and oxidising agents.

Lipolase, the first industrial lipase was obtained from *Humicola lanuginose*, which was marketed by Novozymes (Denmark). Later on, three genetically modified commercial lipases such as LipoPrime, Lipolase Ultra, Lipex were also marked by expressing the lipase gene of *Humicola lanuginose* in *Aspergillus oryzae*, a fungus. Many other detergent lipases are available on the market and some of them are listed in **Table 5**.

Table 5. Commercial lipases used in detergent industry.

Trade name	Source	Supplier	Expression
Lipolase®	*Humicola lanuginosa*	Novozymes	*Aspergillus oryzae*
LipoPrime™	Protein engineered variant of lipolase	Novozyme	*Aspergillus oryzae*
Lipolase Ultra®	Protein engineered variant of lipolase	Novozymes	*Aspergillus oryzae*
Lipex®	Protein engineered variant of lipolase	Novozymes	*Aspergillus oryzae*
Lipomax™	*Pseudomonas alcaligenes*	Genencor Inc.	*Pseudomonas alcaligenes*
Lipase P	*Pseudomonas fluorescens*	Amano Pharmaceutical Co. Ltd	*Pseudomonas fluorescens*
Luma fast	*Pseudomonas mendocina*	Genencor, USA	*Bacillus* sp.

Leather industry

These days, enzymes are widely used for the processing of hides and skins in leather industry. Lipase and proteases are the most important enzymes which

found applications during bathing, soaking, dehairing and degreasing of skin (Dayanandan *et al.*, 2003; Hasan *et al.*, 2006). During bathing, the enzymes enhance water uptake and loosen the scud and disperse fats and oils together with dirt and other materials present on the skin. Lipase specifically degrades fat but do not damage the leather which is proteinaceous in nature, it hydrolyses the fat on the outside of the hides and skins as well as inside the skin structure. Thus, lipase assisted treatment of leather gives the leather with far better quality and finish with uniform color and cleaner appearance as compared to conventional chemical agents. Lipase also improves the production of hydrophobic waterproof leather, which represents an environment friendly method of leather processing. It was found that the lipase produced by *B. subtilis* can be used for the degreasing process, thereby removing all the fat within 8-12 h of incubation by maintaining natural skin colour (Saran *et al.*, 2013). NovoLime, a protease/lipase blend for enzyme-assisted liming of hides and skins, and NovoCor AD, an acid lipase for degreasing of hides and skins, are some of the commercially available lipases for the leather industry.

Pharmaceutical

Applications of lipase for the synthesis of chiral drugs, kinetic resolution of racemic alcohols, acids, esters or amines are well established. Synthesis of diltiazem hydrochloride, a calcium antagonist (a coronary vasodilator), using lipase mediated asymmetric hydrolysis of *trans*-3-(4-methoxyphenyl) glycidic acid methyl ester [(±)-MPGM] was found to be a more efficient process compared to the conventional chemical synthesis, for which lipase from *Serratia marcescens* was generally employed (Matsumae *et al.*, 1993). Lipase from *C. rugosa,* immobilised on a nylon scaffold, was used to synthesise lovastatin, a drug lowering the serum cholesterol levels, by the region selective acylation of a diol-lactone precursor with 2-methylbutyric

acid in mixtures of organic solvents (Yang *et al.*, 1997). Lipase from *Pseudomonas* sp. AK mediated the kinetic resolution of the chiral silane reagents used for the synthesis of a potent antitumor agent called epothilone A (Zhu and Panek, 2001).

Pulp and paper

The paper industry utilises huge amount of lignocelluloses every year. Historically, the enzymatic applications in paper industry was confined to the treatment of raw starch; but, later in since 1990s, lipase mediated removal of pitch has become an essential process of large scale paper making process. 'pitch' or 'resin stickies' is a term used to collectively describe the hydrophobic components of wood such as triglycerides, waxes, *etc.*(Farrell *et al.*, 1997). Pitch and related substances, which usually create major problems to the machines and cause holes and spots in the final paper, are common in paper mills. It may reduce the production levels and increase equipment maintenance as well as operation costs. Nippon Paper Industries, in Japan, have developed a pitch control method that uses the *C. rugosa* lipase to hydrolyse up to 90 % of the wood triglycerides (Jaeger and Reetz, 1998; Arpigny and Jaeger, 1999). Lipase in paper industry decreases chemical usage thereby reducing pollution level of waste water. It provide prolonged equipment life as lipase removes sticky deposits in the paper machines, save energy and time and reduce composite cost (Farrell *et al.*, 1997). The addition of lipase from *Pseudomonas* sp. (KWI-56) to a deinking composition for ethylene oxide-propylene oxide adduct stearate improved whiteness of paper and reduced residual ink spots (Fukuda *et al.*, 1990). Lipase from *Thermomyces lanuginosus* was immobilised on the resin coated with chitosan along with pectinase, which reduced the pitch deposits in white water by 74 % (Liu *et al.*, 2012). Similarly, alkaline lipase was found to be efficient for removing pitch from the recycled fiber pulping waste water (Liu *et al.*, 2012).

Cosmetics

Recently, lipases found applications in producing many cosmetic ingredients such as retinol, natural dyes, *etc*. For the cosmetic industry, the natural products are always of interest as people demand those products which improve not just the appearance of the skin, but the health of the skin as well. Vitamin A (retinol), vitamin C (ascorbic acid), and derivatives combat many skin disorders including photoaging, psoriasis and acne (Adamczak and Bornscheuer, 2009). Lipase catalysed synthesis of retinyl esters has become popular as the chemical synthesis meets some serious defects and offers mild reaction conditions, high catalytic efficiency, inherent selectivity, and much purer products (Maugard and Legoy, 2000; Maugard *et al.*, 2000a; Moreno-Perez *et al.*, 2013). Lipase from *C. antarctica* efficiently catalyses the *trans*-esterification between glycerides and vitamins to produce retinyl/ascorbic esters (Reyes-Duarte *et al.*, 2011; Lerin *et al.*, 2012; Moreno-Perez *et al.*, 2013; Sun *et al.*, 2013). Immobilised lipase catalyses the synthesis of retinyl L-lactate by the *trans*-esterification reaction between retinol and L-methyl lactate, and the synthesis of ascorbyl L-lactate by the *trans*-esterification of ascorbic acid with L-methyl lactate, with yield over 90 % and 80 %, respectively (Maugard *et al.*, 2000b). Lipase from *C. antartica* also mediated the *trans*-esterification between olive oil and ascorbic acid to produce liposoluble ascorbyl oleate, which is widely used as an antioxidant (Moreno-Perez *et al.*, 2013).

Lipases also found applications in the production of natural dyes such as indigo and its derivatives - the water soluble dyes - to be used in cosmetics. Lipase releases indoxyl from istan B which in combination with isatan C can be processed to produce indigo (Maugard *et al.*, 2002). Aroma esters consisting of short chain fatty acids and alcohol are synthesised by the direct application of lipase, which provides natural fragrance to the cosmetics. In

2001, Gatfield *et al.* (2001) reported a method to produce natural ethyl (E,Z)-2,4-decadienoate, the compound of pear, by lipase mediated *trans*-esterification of stillingia oil with ethanol.

Conclusions

Global enzyme market is expected to rise by 7 % in 2015. Growing trends in the world market of biocatalysts indicate that, developed countries in North America would be the largest consumers followed by Western Europe. Their stringent government rules and regulations made them to adopt green technologies in industries to address the environmental issues as well as to improve the product quality and acceptability. Industrial processes demand enzymes with unique specificities and high performance which attracts the attention of researchers and industrialists to produce novel enzymes to minimise the cost. Application of lipases is broadening up rapidly, due their remarkable potential for accomplishing innumerable novel reactions, both in aqueous and non-aqueous environments. Hence, the demand for the production and characterisation of new lipases is still increasing significantly. Though many microbial lipases have been explored for their mode of actions, the high cost of production and purification hinders its world-wide commercialisation. Moreover, it is necessary to elucidate the reaction mechanism of lipases - both general and type-wise - in tune with the specific need in industry, mode of control and regulated expression to meet the future needs and to hit the anticipated level of commercial demand.

SECTION 3: GLYCOLIPID BIOSURFACTANTS

Introduction

Surfactants are surface active and amphiphilic agents which reduce the surface tension or interfacial tensions between the fluid phases in a mixture. They are widely used in food, pharmaceutical, detergent, agriculture, medicine and textile industries. Most of them are chemically synthesised from raw materials derived mainly from the petrochemicals. The recalcitrant and persistent nature of these raw materials often pose many environmental and health issues. Increasing environmental concern about various chemical pollutants triggers attention toward microbially-derived compounds, essentially due to their low toxicity, diversity, biodegradability, possibility of large scale production, selectivity and performance under extreme conditions (Bustamante *et al.*, 2012; Sarath Josh *et al.*, 2014; Pradeep *et al.*, 2014). The eco-friendly nature and versatile properties of biosurfactants made them a remarkable focus of the scientific community, as suitable alternative to the synthetic chemi-surfactants. In 1960s, biosurfactants were used as hydrocarbon dissolution agents, but with the emergence of biotechnology and advancement in research for the last 6 decades, unprecedented spread in their applications in various fields was witnessed (Kitamoto *et al.*, 2009; Thavasi *et al.*, 2014).

Biosurfactants are produced by living cells, mostly microorganisms, mainly to make their nutrition easier. They are produced either on cell surfaces or excreted extracellularly; most of them have hydrophobic and hydrophilic moieties, *i.e.*, the determining structural components for their physico-chemical properties (Karanth *et al.*, 1999). Mostly, fatty acids or its derivatives constitute the hydrophobic moiety and the hydrophilic part may be a carbohydrate, peptide, amino acid or phosphate (Rahman and Gakpe, 2008). Biosurfactants produce stable emulsions, thereby solubilising or made

miscible hydrocarbons in water or water in hydrocarbons. Thus, they are widely used in many industries like, food, pharmaceutical, cosmetic, detergent, biomedical and bioremediation applications (Reis *et al.*, 2013).

Though biosurfactants are rampant in biota, these secondary metabolites are generally produced by microorganisms - bacteria and fungi in particular; and play crucial role in the survival of the organisms by facilitating the transport of nutrients or interfering with microbe-host interactions or quorum sensing mechanisms for motility (Lahaye *et al.*, 2007; De Dier *et al.*, 2015). Since the spontaneous release of the biosurfactants are often related to aqueous-nonaqueous interfaces in solutions, they are predominantly produced by hydrocarbon degrading microorganisms (Banat, 1995). Insolubility of hydrocarbons severely restricts their consumption/assimilation by microorganisms. Extracellular biosurfactants solubilises these compounds to form emulsion, which makes easier for the microorganisms to interact with the micelle formed (Banat *et al.*, 2010).

Most of the microbial biosurfactants are neutral or anionic in nature (Kapadia and Yagnik, 2013). Based on molecular mass, biosurfactants are generally categorised in to two; low or high molecular mass compounds (Rosenberg and Ron, 1999). Biosurfactants with low molecular mass such as glycolipids, lipopeptides, phospholipids, *etc*., effectively reduce the surface tension or interfacial tensions; whereas biosurfactants with high molecular mass such as polymeric and particulate surfactants bind tightly on to the surfaces producing stable emulsions.

One of the most studied groups of biosurfactants is glycolipids, which comprise of carbohydrates combined with aliphatic fatty acids or hydroxyl aliphatic fatty acids. Now-a-days, glycolipid biosurfactants (GLBS) attract more attention toward commercialisation due to their high emulsification ability and elevated production upon utilising cheap and renewable nutritional

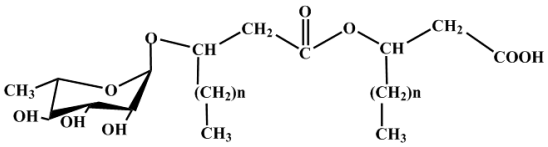
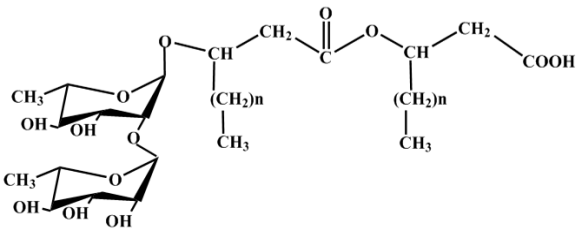
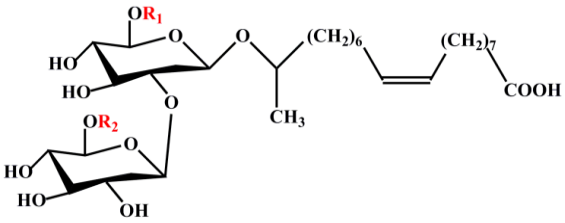
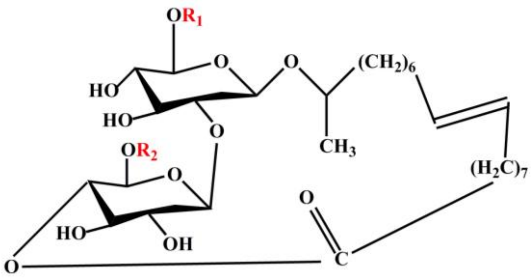
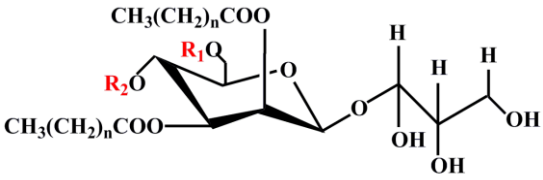
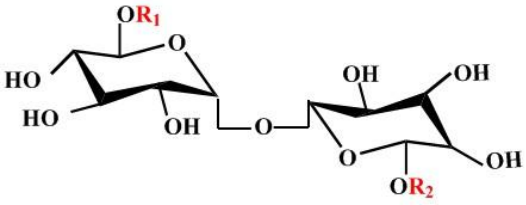
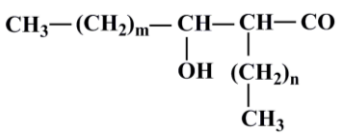
sources. The major candidates of GLBS are rhamnolipids produced mainly by Pseudomonads, sophorolipids produced by yeasts, trehalolipids produced by species of *Mycobacterium*, *Rhodococcus*, *Arthobacter*, *etc*., and mannosylerythritol lipids (MELs) produced by *Candida* spp. (**Table 6 and 7**).

Thus, this focused review addresses the physico-chemical and biological properties, industrial applications as well as the current market scenario of glycolipids, perhaps the most interesting biosurfactants for exploitation to meet the increasing demand.

Table 6. Major GLBS, its constituents and producing microorganisms

Type	Hydrophilic moiety	Hydrophobic moiety	Organisms
Rhamnolipid	Rhamnose	*β*-Hydroxydecanoic acid	*Pseudomonas* sp.
Mannosyl erythritol lipids	Mannose-erythritol disaccharide	Acetylated fatty acid	*Pseudozyma* sp. *Ustilago maydis* *Candida antarctica*
Sophorolipid	Sophorose	Hydroxylated fatty acids	*Candida bombicola* *Starmerella clade*
Trehalolipids	*α*-1,1 Glucose disaccharide	Hydroxylated fatty acids in *α*- and *β*-configurations	*Rhodococcus* sp. *Mycobacterium* sp. *Corynebacterium* sp. *Nocardia* sp.

Table 7. Structure of major GLBS

Trivial name	Structure	Derivatives
Mono-rhamnolipid		*n* = 4 to 8
Di-rhamnolipid		*n* = 4 to 8
Sophorolipid (acidic form)		R_1, R_2 = H/ R_1, R_2 = $COCH_3$/ R_1 = H; R_2 = $COCH_3$/ R_1 = $COCH_3$; R_2 = H
Sophorolipid (lactone form)		R_1, R_2 = H/ R_1, R_2 = $COCH_3$/ R_1 = H; R_2 = $COCH_3$/ R_1 = $COCH_3$; R_2 = H
Mannosyleryth ritol lipids (MEL)		*n* = 6 to 12 MEL A: R_1, R_2 = $COCH_3$ MEL B: R_1= $COCH_3$; R_2 = H MEL C: R_1 = H; R_2 = $COCH_3$ MEL D: R_1, R_2 = H
Trehalolipid		TL1: R_1, R_2 =  *where m+n* = 27 to 31 TL 2: R_1 = R_2 = H

Properties of GLBS

The GLBS exhibit several unique physical, chemical and biological properties that made them superior as compared to their synthetic counterparts.

Physico-chemical properties

Physical properties of biosurfactant include reduction in surface/interfacial tensions, critical micelle concentration, self-assembly, stability, toxicity and biodegradability.

Reduction in surface/interfacial tensions

Due to the amphiphilic nature of GLBS, they aggregate and arrange themselves between the two immiscible phases resulting in the reduction of surface or interfacial tensions. Thus, biosurfactants reduce the repulsive forces in the immiscible liquids and allow them to interact with each other (**Figure 5**). The most efficient biosurfactant can reduce the surface tension of water from 72 to 28 mN/m; and the interfacial tension between hydrocarbon and water will typically be in the range from 30-40 mN/m to below 0.4 mN/m (Desai and Banat, 1997).

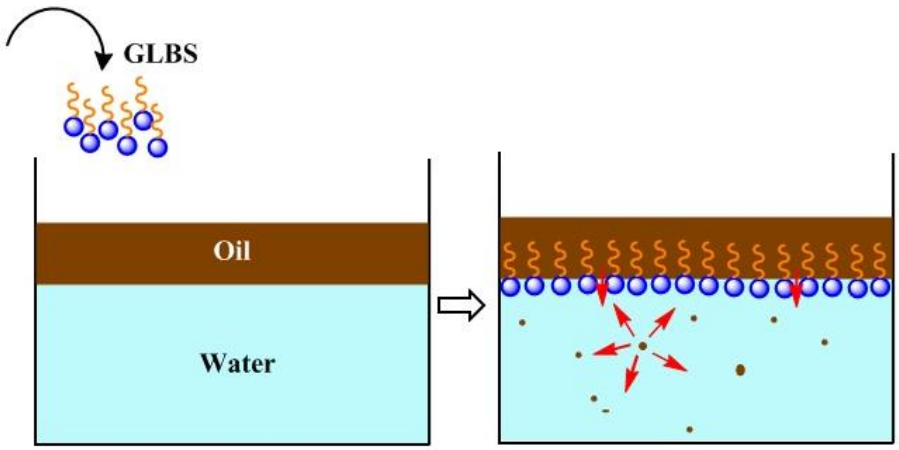


Figure 5. GLBS arrange themselves between two phases, thereby reducing the surface and/or interfacial tensions.

Critical micelle concentration

Biosurfactants at lower concentrations exist as single units. With increasing concentration, it lowers the surface tension of water and at a particular concentration, known as the critical micelle concentration (CMC), surface tension reaches the minimum, after which no decrease in surface tension occurs. Further addition of biosurfactant promotes the aggregation of single units thereby forming micelles, bilayers, vesicles, *etc*., responsible for their emulsifying, dispersing, foaming and detergent activities. Generally, CMC measures the efficiency of the biosurfactant; lower the value of CMC, higher the efficiency. CMC values of GLBS vary from 4 to 350 mg/l (Kitamoto *et al.*, 2009).

Self assembly

GLBS being amphiphilic molecules, exhibit an interesting property of self assembly in aqueous solutions, which makes them promising functional materials for many biological applications. Micelles of GLBS assemble themselves to various ordered structures stabilised by hydrogen, hydrophobic and van der Waal's interactions. GLBS exhibit unique characteristics which are not observed in synthetic surfactants, such as formation of diverse liquid crystals in the hierarchy of vesicle, cubic, hexagonal, sponge or lamellar types (**Figure 6**) - depending on their concentration and nature, as well as the temperature of the surrounding medium in which they are dispersed (Kitamoto *et al.*, 2009; Baccile *et al.*, 2012; Kokubun *et al.*, 2013).

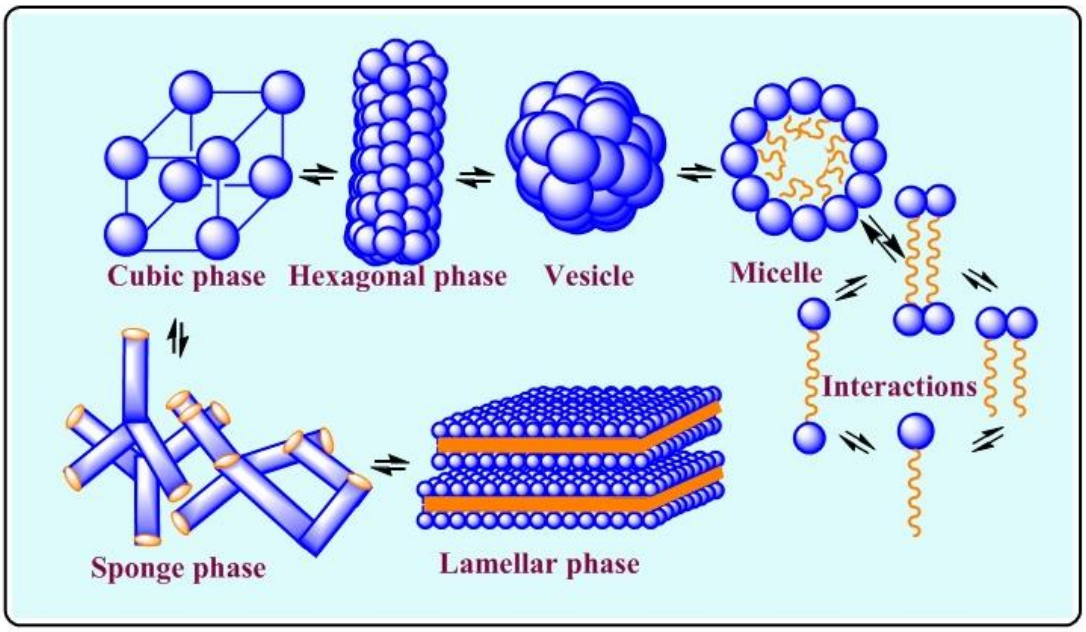


Figure 6. Scheme showing self assembly of GLBS so as to form various ordered structures.

Low toxicity and biodegradability

Owing to their low toxicity and superior biodegradability, biosurfactants are environmentally acceptable. Compared to the synthetic surfactants, biosurfactants are considered as ecologically safe; therefore, they are preferred for the manufacture of 'green' biomolecules of interest, in various fields including bioremediation processes, pharmaceuticals, agriculture, *etc*. (Bustamante *et al.*, 2012). Most of the GLBS are biodegraded by 60-90 % within one week, both aerobically and anaerobically under normal temperature (20-30 ^{o}C), whereas the synthetic surfactants showed no biodegradability even after one week (Kim *et al.*, 2002; Mohan *et al.*, 2006; Hirata *et al.*, 2009; Lima *et al.*, 2011). Despite the limited number of studies (as shown below) devoted to the assessment of toxicity of biosurfactants, they are generally considered as non-toxic: Das and Mukherjee (2005) reported that the GLBS from *P. aeruginosa* did not have any detrimental effects on the tissues of lung, liver, heart and kidney of chicken; and phyto-toxicity studies

also suggested the non-toxicity of biosurfactants; for instance, anionic GLBS from *Candida sphaerica* demonstrated no effect on seed germination of plants including vegetables such as species of *Brassica, Solanum, Chicoria, Lactuca, etc.* (Luna *et al.*, 2013; Sobrinho *et al*., 2013).

Stability

The emulsifying and surface activities of GLBS are generally stable at a wide range of temperature (30 to 80 ^{o}C), pH (2 to 12), salt concentration (2 to 10 %), *etc*. (Imura *et al.*, 2005; Rufino *et al.*, 2007; Lotfabad *et al.*, 2009). Among GLBS, rhamnolipids represent the most prominent group in terms of stability. For instance, rhamnolipid from *P. aeruginosa* MR01 showed no change on its emulsification activity even after autoclaving at 120 ^{o}C (Lotfabad *et al.*, 2009). The higher stability of these biomolecules makes them suitable for application in various industries like oil recovery, bioremediation, *etc*.

Biological properties

The GLBS have attracted the attention of many pharmacological industries as multipurpose bioactive compounds due to their diverse biological activities such as anti-microbial, anti-adhesive, anti-tumor and immunomodulatory activities that are not exhibited by their synthetic analogues (**Figure 7**).

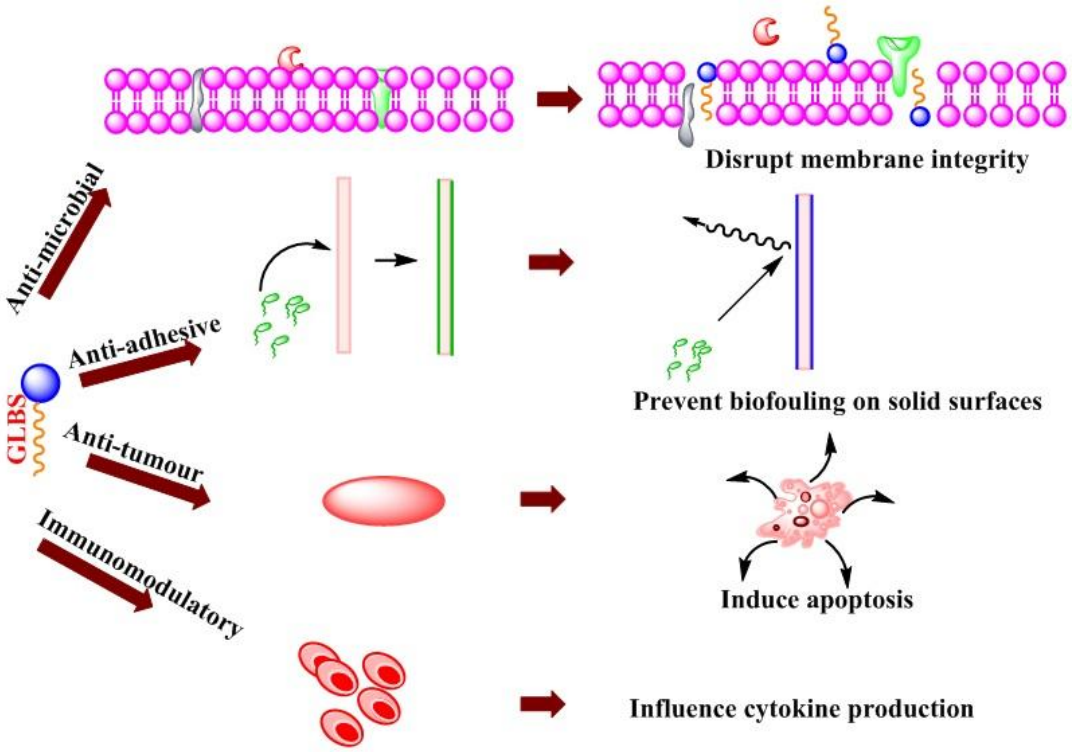


Figure 7. Biological properties of GLBS

Anti-microbial activity

GLBS exhibit potential antimicrobial activities against bacteria, fungi and viruses, mainly due to its ability to interact with the phospholipid components of the microbial cells (particularly cell envelope); thereby affecting the cell permeability and stability (Sotirova *et al.*, 2008). For instance, rhamnolipids produced by *P. aeruginosa* from oil and refinery wastes were found effective against bacteria (*Bacillus, Staphylococcus, Steptomyces, Proteus, Klebsiella, Enterobacter, etc*.) and fungi (*Penicillium, Aspergillus, Chrysogenum, Fusarium, etc.*) at varying concentrations, *i.e*., 8-32 mg/l (Abalos *et al.*, 2001; Haba *et al.*, 2003; Benincasa *et al.*, 2004). MELs produced by *Candida antartica* act as potent inhibitors of Gram-positive bacteria (Kitamoto *et al.*, 1993). Similarly, sophorolipids impaired with the multiplication of several Gram-positive bacteria and viruses (*Bacillus* spp., *Staphylococcus* spp., *Streptococcus* spp., HIV, Herpus virus, *etc*.) at concentrations ranging from

100 to 500 µg/ml (Shah *et al.*, 2005; Gross and Shah, 2007; Sleiman *et al.*, 2009). Generally, trehalolipids showed less/no inhibitory activities against bacteria and yeasts, but found efficient against the fungus, *GlomerelIa cingulata* and influenza virus at concentrations of 300 and 30 mg/l, respectively (Azuma *et al.*, 1987; Shah *et al.*, 2005). Xylolipids produced by the probiotic bacterium, *Lactococcus lactis* showed bactericidal activity against *Escherichia coli* and *Staphylococcus aureus,* which are normally resistant to broad spectrum antibiotics such as methicillin (Saravanakumari and Mani, 2010). Thus, many of the GLBS were found applications in the formulations of pharmaceuticals targeted, for oral and dermal administration.

Anti-adhesive properties

Most of the GLBS exhibit anti-adhesive properties and prevent the formation of biofilms on solid surfaces or sites of infection. Generally, during the initial stages of infections, pathogenic bacteria adhere on to the suitable host surfaces prior to colonisation - leading to the formation of biofilms. Majority of nocosomal infections occur in hospitals through the formation of biofilms on biomedical equipments such as catheters, heart valves, prostheses, intrauterine devices, contact lenses, *etc*., which are difficult to avoid by the use of antibiotics (Davies, 2003). It has been reported that GLBS can efficiently prevent the adherence and biofilm formation of pathogens on solid surfaces and infection sites. For instance, GLBS produced by *Streptococcus thermophilus* inhibited the biofilm formation of bacteria and yeasts on voice prostheses (Rodrigues *et al.*, 2006). Raya *et al*. (2010) investigated the anti-adhesive properties of rhamnolipids against *P. aeruginosa* PAO1 using flow chambers under different shear conditions and found that rhamnolipid (even at a concentration of 13 mg/l) was sufficient enough to prevent the initial attachment of pathogen on glass surfaces at a circulation rate of 22 ml/min (*i.e*., 2.6 mN/m^2 shear). The glycolipid biosurfactant produced by the marine

actinobacterium, *Brevibacterium casei* MSA19 inhibited the biofilm formation of mixed pathogens including *Vibrio* spp., *P. aeruginosa* and *E. coli* (Kiran *et al.*, 2010). Thus, the anti-adhesive property of biosurfactants can effectively be utilised for reducing the biofouling of medical devises due to surface contamination by developing suitable strategies like applying them as coating agents on such medical devices which demand atmost care.

Anti-tumor activity

Now-a-days, biosurfactants are gaining importance as anti-neoplasic agents in biomedicine. They are potent regulators of many cellular functions, trigger apoptosis and differentiation of tumors; thus preventing the processes of progression. Most of them were reported to have less/no effects on normal cells, and hence the specificity in targeting the malignant cells minimise the side effects associated with normal anti-cancer treatments (Fu *et al.*, 2008). Isoda *et al*. (1997) investigated the anti-tumor activity of microbial glycolipids including MELs, polyol lipid, rhamnolipids, sophorolipid, succinoyl trehalose lipids; interestingly, all the candidate biosurfactants induced the differentiation of HL60 human promyelocytic leukemia cell line of human except rhamnolipids. They also inhibited the activity of Ca-dependent protein kinase, which is considered as one of the characteristic properties of anti-tumor agents (Isoda *et al.*, 1997). MELs induced apoptosis in malignant melanoma B16 cells in a dose-dependent manner *i.e.*, at a concentration of 10 μM or higher MELs mediated a series of apoptotic events such as growth arrest, and chromatin condensation and DNA fragmentation in melanoma B16 cells, which usually respond poorly to the treatment with chemotherapeutic reagents (Zhao *et al.*, 1999). Similarly, trehalolipids produced by *Nocardia farcinica* strain BN26 were also found to have potent anti-tumor effects on human malignant cell lines of leukemia and solid tumors of urinary bladder in a dose-dependent manner. Interestingly, they exerted

weak cytotoxic effects on normal human cells, indicating its pharmacological potentials (Christova *et al.*, 2014). It was reported that various derivatives of GLBS induced cytotoxicity at different levels, indicating involvement of specific mechanisms in the process, which need to be evolved by detailed research (Fu *et al.*, 2008). Among various derivatives investigated, methyl ester of sophorolipid mediated more than 60 % of cell death in human pancreatic carcinoma cells at a concentration of 0.5 to 2.0 mg/ml, whereas acidic sophorolipid and lactonic sophorolipid diacetate mediated apoptosis by 40.3 % and 49 % at 0.5 mg/ml, respectively (Fu *et al.*, 2008). Similarly, Shao *et al.* (2012) examined the cyto-toxicity of acidic, mono and diacetylated sophorolipids on human esophageal cancer cell lines; of which di-acetylated lactonic forms (30 µg/ml) exhibited total inhibition of cell growth (Shao *et al.*, 2012).

Eventhough several studies have proposed the possible applications of biosurfactants for preventing the malignancy in mammals, only a few have studied the underlining mechanism of action and specificity. Normally, the integrity and lipid profiles of cell membranes play a crucial role in determining the structure and vital functions of the cells by regulating intercellular interactions, probably through signal transduction (Preetha *et al.*, 2005). It was reported that the surface activities and membrane properties of normal and malignant cells differ widely, which can be identified by the biosurfactants; *i.e.*, from their specific interactions with the target cells followed by the modifications of membrane fluidity and progression of cell cycle leading apoptosis through the mediation of growth arrest, chromatin condensation, fragmentation, cell leakage, *etc*. (Gudiña *et al.*, 2013).

Immunomodulatory properties

GLBS possess potential modulatory effects on immunity. Intra abdominal sepsis is a deadly inflammation of peritoneum, which accounts for 35 %

mortality of the infected patients. Administration of sophorolipids proved effective in the treatment of sepsis *in vivo* in rat models of septic peritonitis and *in vitro* by decreasing the production of nitric oxide and inflammatory cytokine (Bluth *et al.*, 2006; Hardin *et al.*, 2007). Glycolipid biosurfactant produced by an actinobacterium, *Rhodococcus ruber* at a concentration of 10 µg/ml activated the monocytes and induced the production of interleukine (IL-1) and tumor necrosis factor (TNF-α), the major cytokines influencing the polarisation of immune response development (Kuyukina *et al.*, 2007). It was also observed that the glycolipid was found nontoxic to the central nervous system of mice, with no adverse effects on their behavioral activities (Kuyukina *et al.*, 2007).

Applications of GLBS

The basic function of GLBS is to act as surface-active agents (for emulsification, dispersion, moisturising, *etc*.), and compared to synthetic reactants, they are effective even at extremely low concentrations. Interestingly, in addition to its eco-friendly nature, GLBS have unique characteristics such as self assembly, formation of liquid crystals, attractive pharmacological activities *etc*., which are not exhibited by synthetic surfactants; and these unique properties inspire various industries to exploit them as multipurpose biomolecules (Banat *et al.*, 2010). In the past few decades, many investigations were carried out on GLBS, revealing its spectacular applications that made it popular on global market. However, the large scale production of biosurfactants and its commercialisation are hindered by the higher cost of purification and low yield.

In protecting environment

The extensive use of hydrocarbons and chemical pollutants in various fields has resulted in the severe contamination of the environment, owing to their

high toxicity and prolonged persistence. Biosurfactants can be applied to the contaminated sites for the bioremediation of hydrocarbons, pesticides and metals. They enhance the bioavailability of pollutants and increase the hydrophobicity of the cell surface, allowing the hydrocarbon pollutants to interact effectively with the microbial cells (Kuyukina *et al.*, 2005; Thavasi, 2011). GLBS, below the CMC mediated the mobilisation of hydrocarbons by reducing the surface and interfacial tensions between the two immiscible phases; whereas above their CMC enhanced the solubility of hydrocarbons by aggregating the surfactant micelle (Guo, 2009). Saeki *et al.* (2009) studied the hydrocarbon remediation potentials of the GLBS produced by *Gordonia* sp. strain JE-1058, which removed the alkanes and aromatic hydrocarbons from the contaminated sea sand *via* the activity of indigenous marine bacteria. Rhamnolipids were found effective as washing agent for the removal of crude oil, styrene and tetrachloroethylene from soil by mobilisation and solubilisation (Clifford *et al.*, 2007; Mehdi and Giti, 2008). Sophorolipids were also found as suitable soil flushing agents for the bioremediation of crude oil and aromatics, which removed 70 to 97 % of pollutants within 6 days of application (Kang *et al.*, 2010).

GLBS were found effective in the removal of heavy metals like Zn, Cd, Pb, Cu, Ni, *etc*. (Juwarkar *et al.*, 2007; Dahrazma and Mulligan, 2007). During the remediation of heavy metals, the anionic biosurfactants established ionic bonds with the metals, which are stronger than the metal-soil interactions, resulting in the desorption of these metal-biosurfactant complexes by lowering the interfacial tensions (Mulligan *et al.*, 2001; Pacwa-Płociniczak *et al.*, 2011). Wang and Mulligan (2004) employed foam technology for the removal of metals from soil; and reported that compared to the conventional flushing method, the foam of rhamnolipid enhanced the mobility as well as contact of biosurfactants with metals (Wang and Mulligan, 2004).

In microbial enhanced oil-recovery

Biosurfactants found increased applications in the field of microbial enhanced oil recovery (MEOR) to collect the residual oil from the reservoirs. MEOR generally utilises microbes or their metabolites to overcome the poor recovery of crude oil from the rock beds. Generally, three stages of extraction are followed in most of the oil reservoirs. Primary mechanical methods use natural pressure to bring up the oil to the surface; and secondary physical methods include injection of gas and/or water to increase the reservoir pressure to drive the residual crude oil and gas, remaining after the primary oil recovery phase to the surface wells (Banat *et al.*, 2000). MEOR methods are usually devised as tertiary procedures for the enhanced recovery of residual oil left after primary and secondary procedures. Generally, 3 different strategies are employed for the application of biosurfactants in MEOR; (1) injection of microorganisms producing biosurafactants into the reservoir, (2) injection of biosurfactants produced *ex situ* into the reservoir, and (3) biostimulation through the addition of selected nutrients to the reservoir which would enhance the growth of indigenous biosurfactant producing microbes. Selection of strategy depends on pH, salinity, temperature, pressure, geography, *etc*. of the reservoir (Perfumo *et al.*, 2010; Marchant and Banat, 2012a,b). Apart from reducing viscosity and increasing the mobility of oil, biosurfactants also reduce the capillary forces which prevent oil from moving through crevices of the oil rock. Biosurfactants can also bind tightly to the oil-water interface and form emulsion, which stabilises the desorbed oil in water and allows the removal of oil along with the injection water (Sen, 2008; Suthar *et al.*, 2008). Pornsunthorntawee *et al*. (2008a,b) reported that rhamnolipids from *P. aeruginosa* SP4 efficiently recovered oil, in comparison to synthetic counterparts; for this purpose, surfactant solutions was poured onto the sand-packed column, saturated with motor oil. The results showed

that rhamnolipids were more efficient in oil recovery, *i.e*., for the removal of about 57 % of the tested motor oil.

In food processing industries

In food processing, biosurfactants are used as emulsifying and thickening agents so as to maintain the consistency and/or stability of the products. Application of biosurfactants, especially rhamnolipids was found to increase the sortness, texture, volume, aroma and appearance of dough used to make bakery products (Haesendonck and Vanzeveren, 2003). Rhamnolipids are also used in ice creams and butter/decoration creams so as to maintain the thickness, viscocity and also for the solubilisation of flavoring fats and oils (Haesendonck and Vanzeveren, 2003). Rhamnolipids can also be used as a source of rhamnose, which is considered as a potential precursor of high-quality flavor components like furaneol (Linhardt *et al.*, 1989).

In biomedicines and therapeutics

The major GLBS having therapeutic applications include; sophorolipids, MELs, rhamnolipids and trehalolipids (Cameotra and Makkar, 2004). Compared to the glycolipid or antibiotic alone, glycolipids in combination with the conventional antibiotics inhibited the growth of microbes more efficiently - since the self assembly and amphiphilic nature of glycolipids facilitates the entry of drug molecules effectively into the cells. For example, a combination of sophorolipid-tetracycline (20:1) completely inhibited the growth of *S. aereus* within 4 h of exposure, while tetracycline alone could not affect total inhibition till the end of 6 h. similarly, sophorolipid-cefaclor mixture (10:1) was found efficient against *E. coli*, which showed about 50 % of inhibition within 2 h of exposure, as compared to cefaclor alone, showing total inhibition at the end of 6 h exposure (Navare and Prabhune, 2013). Sophorolipids in lactone form found applications in dermatology as it

stimulates fibroblast metabolism, mainly by enhancing collagen neogenesis at a concentration of 0.01 ppm to 5 % of dry matter in formulations (Concaix, 2003). Recently, Peng *et al*. (2014) found that polylactonic sophorolipids (PLS) posses desired characteristics tobe used as biomaterials for tissue engineering. The scaffold made of PLS was found compatible with human mesenchymal stem cells and it promoted the adhesion, spreading and maturation of the lineage similar to tissue culture polystyrene, a common scaffold material. Thus, the study gives fundamental insights to the broad potential applications of GLBS as candidates of green technologies.

Of late, glycolipids are widely used in liposome-mediated gene delivery systems, which overcome the risk factors of using virus vectors for gene transfection. Among the various GLBS, MELs showed excellent orientation property and efficient balance between the hydrophobic and hydrophilic groups that are required to form giant vesicles; hence, they are gaining importance in liposome-mediated drug/gene delivery systems (Kitamoto *et al.*, 2002). Cationic liposomes made of MEL-A were found 50 to 70 % more efficiency than the conventional liposomes for delivering plasmid encoding luciferase to target cells (Inoh *et al.,* 2001; Nakanishi *et al.*, 2013). Recently, it was shown that MEL-A and protamine synergistically accelerated the delivery of foreign gene into the nucleus of target cells (NIH-3T3 cells), and enhanced the gene transfection efficiency (Inoh *et al.*, 2013). These cationic liposomes based on MELs proved to have excellent potentials for applications in gene therapy by successfully delivering small interfering RNA (siRNA) into the cell lines *in vitro* (Inoh *et al.*, 2011). Moreover, MEL-A exhibits good binding affinity towards immunoglobulins and lectins, making them excellent ligands for many immunodiagnostic and therapeutic applications (Teng *et al.*, 2000; Konishi *et al.*, 2007).

Agriculture

In modern agriculture, biopesticides are widely promoted to overcome the adverse environmental effects as well as increased insect resistance of synthetic pesticides. GLBS are found as a suitable measure for the integration into sustainable disease management systems, which may directly be applied on diseased plants or can be employed as substrates for the synthesis of some pesticides. For instance, rhamnolipids were found effective against zoosporogenic plant pathogens which are destructive to the roots, fruits and foliage of crops (Stanghellini *et al*., 1998). Rhamnolipids at a concentration of 500 mg/l protected the crops from oomycete and anthracnose diseases caused by the pathogenic fungi such as *Colletotrichum orbiculare*, *Phytophthore capsici, etc.* (Kim *et al.*, 2000). Moreover, the glycolipidimic composition of rhamnolipids facilitates the penetration of cuticular membrane of aphids (superfamily *Aphidoidea*), a major insect pest of crops (Awada *et al.*, 2011). Complete mortality of *Myzus persicae*, a green peach aphid, was attained by the application of 100 mg/l of ramnolipids within 24 h (Kim *et al.*, 2010). Similarly, potato late blight disease can effectively be controlled by the application of biosurfactants obtained from *Pseudomonas* sp. (Hultberg *et al.*, 2010); and 100 ppm of sophorolipid inhibited 50 % of cell growth of plant pathogenic fungus, *Botrytis cineria* (Kapjung *et al.*, 2002). Sophorolipids alone or in combination with loess or yellow clay inhibited the migration of harmful algal blooms formed due to the species of *Cochlodinium*, *Alexandrium* and *Heterosigma* (Lee *et al.*, 2008; Sun *et al.*, 2004a,b; Wang *et al.*, 2005).

Cosmetics

Surfactants are used in a variety of cosmetics including antidandruff products, skin creams, hair coloring agents, shampoos and conditioners, toothpaste,

mascara, nail polish, lipstick, *etc.* Generally, petrochemical based surfactants such as sodium lauryl sulfate, sodium dodecyl sulfate, and sodium laureth sulfate are used to emulsify the immiscible ingredients used in such cosmetics. They help to spread the material evenly for maintaining the stable viscosity as well as moisture. Increasing concern about the harmful effects of these synthetic substances - particularly on long term use - has raised the demand for natural 'green' products. The basic emulsifying and wetting properties of the biosurfactants enable to maintain the texture and consistency of these products (Haba *et al.*, 2003; Benincasa *et al.*, 2004). Biosurfactants are known to have many advantages over synthetic surfactants such as low irritancy or anti-irritating effects and compatibility with skin. Rhamnolipids, sophorolipids and MELs are the most common GLBS used in cosmetics. Moreover, they are included in the International Nomenclature of Cosmetic Ingredients [INCI] (http://rhamnolipid.com/services-view/cosmetics).

For instance, the unique structure and molecular model of the GLBS - such as rhamnolipid and MELs - showed its similarity with the ceramide molecule. Ceramides are natural lipids present in the intercellular spaces of the stratum corneum, the top layer of epidermis of skin providing the barrier property and helps to maintain the moisture levels (Morita *et al.*, 2009b). Damaged or irritant skin contains lower levels of ceremides (Coderch *et al.*, 2003). Due to the structural similarities, the GLBS will easily penetrate the intercellular spaces in the stratum corneum and form the most ordered lipid bilayer in the intracellular spaces of stratum corneum, which exist in liquid crystalline state at higher temperature and protect the skin from moisture loss (**Figure 8**) (Michiko and Shuhei, 2007; Fukuoka *et al.*, 2011).

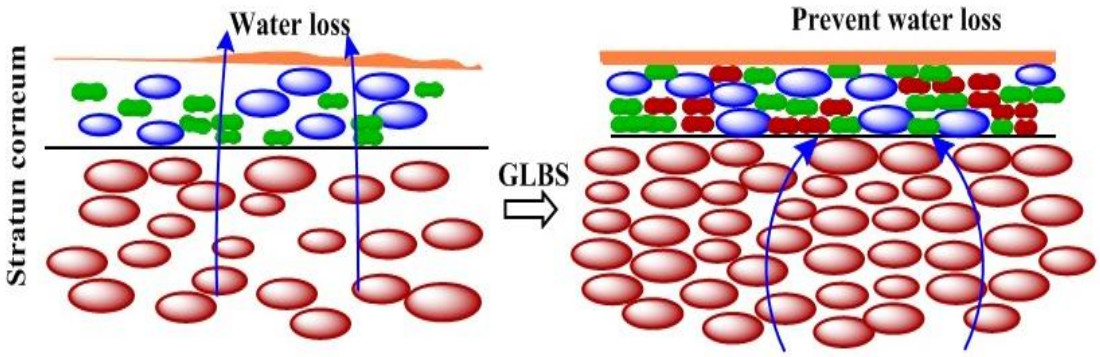


Figure 8. Damaged skin with irregular and loosened stratum corneum is characterised with water loss. GLBS helps to maintain the integrity of skin and provide moisturising effects. ceramide GLBS.

MEL-A produced by the yeast *Pseudozyma antartica* on soybean oil as substrate was found safe and effective as a novel moisturiser for treating the damaged skin (Morita *et al.*, 2009b). SDS damaged cells of commercially available human skin model were treated with different dosages of MEL-A, which showed significant recovery of cell viability in a dose-dependent manner. MEL-A solutions of 5 and 10 % showed the recovery by 73 and 91 %, respectively. Even though, 1 % ceramide showed 100 % recovery of cell viability, the higher cost involved for the production and purification of natural ceramides turned MELs as a potential skin care material (Morita *et al.*, 2009b). Later on, it was found that the aqueous solution of MEL-B also significantly increases the water content of stratum corneum and suppresses the rate of perspiration on human forearm skin, and thus assists the barrier function of the skin (**Figure 8**) (Yamamoto *et al.*, 2012). As the yeast strains of the genus *Psuedozyma* can produce over 100 g/l of MEL utilising vegetable oil as substrate these findings were found pharmaceutically promising (Morita *et al.*, 2009a).

Sophorolipids also found utility as skin moisturiser in commercial formulations (Abe *et al.*, 1981; Hillion *et al.*, 1981). Kao Co Chemical

Corporation in Japan uses sophorolipids in the name of 'Sopholiance S' in make-up cosmetics, *viz*., 'Sofina' and 'Soliance' for applications on skin. Sophorolipids are also present in pencil-shaped lip rouge, lip cream and eye shadow, as well as in compressed powder cosmetics (Kawano *et al*., 1981). 'Ecover', the Belgium based company already launched products such as all-purpose cleaner, interior cleaning spray, window spray, heavy-duty power cleaner, a car wash and wax cleaner containing sophorolipids (Van Bogaert *et al.*, 2007). The tight and long lasting foam making capacity of rhamnolipids attracted its utility in formulating many health care products such as toothpastes, nail care products, deodrants, body washes, conditioners, body lotions, facial creams, facial cleansers, *etc*., (Piljac and Piljac, 2009). Cosmetics containing rhamnolipids have been used as anti-wrinkle and anti-ageing products (Piljac and Piljac, 2009). MELs activate fibroblasts and papilla cells to induce follicle formation and hair growth by *trans*-differentiation of an adult epidermis (Morita *et al.*, 2010b). MELs also repair the damaged hair, making it smooth and flexible (Morita *et al.*, 2010a). Acetylated lactonic sophorolipids were used in formulations for anti-dandruff shampoos, hair gels, deodorant sticks, after-shave lotions and shampoos (Mager *et al.*, 1987). In contract to conventional therapy using corticosteroids, clinical trials have shown that the rhamnolipids were found effective in the treatment of psoriasis, lichen planus, neurodermatitis and human burn/wound healing (Stipcevic *et al.*, 2006). Researchers at Kanebo Cosmetics, and National Institute of Advanced Industrial Science and Technology, Japan found that a broad range of formulations was possible by making the best use of the characteristics of GLBS that can be applied in the development of new skin care products (http://www.kanebo.com/ science/ reports/ 20110601.pdf).

Eventhough these products are more expensive than much cheaper petrochemical-based products around, they are highly acceptable due their

low skin irritability and pleasing skin compatibility (Lourith and Kanlayavattanakul, 2009).

Current market

Biosurfactants have found its own niche on the global market, due to their versatility in industrial applications and eco-friendly nature. The increasing demand for the use of 'green solutions' in bio-based technologies represents the major growth driver of their global market (**Figure 9**), and is well supported by the favorable regulatory scenario in developing countries; but they are not hitting the market as deserved because of the higher economics of production and purifications. In order to sustain the market and to rule over their synthetic counterparts, the price of biosurfactants has to bring several folds down, which itself is one of the major bottlenecks in commercialisation (Marchant and Banat, 2012b). Among the GLBS, sophorolipids and rhamnolipids were emerged as the widely used surfactants on the global market, *i.e.*, they together claim more than 70 % of overall market share. Sophorolipids found major applications in house-hold cleaners and personal care products, while rhamnolipids in bioremediation and oil recovery (http://www.transparencymarketresearch.com/pressrelease/microbial-biosurfactants-market.htm).

According to the survey reports on global market, Europe is reported as the leading region in terms of the consumption of microbial biosurfactants, mainly due to stringent regulations of the government for usage of bio-based products, followed by North America and Asia Pacific; whereas the rest of the world accounts for a smaller share of the market, owing to less consumer awareness and higher prices of products (http://www.persistencemarketresearch.com).

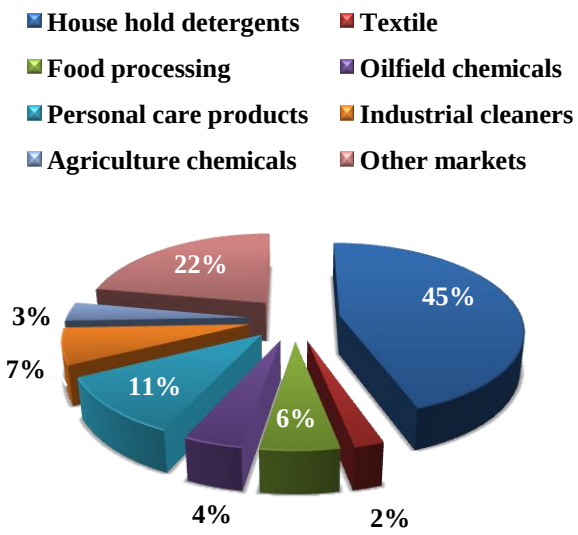


Figure 9. Utilisation of biosurfactants in global market.

Source: www.grandviewresearch.com.

Conclusions

Since current industries are in search for multipurpose natural bioactive compounds, the versatility in the biological properties of biosurfactants make them dominant over the synthetic surfactants. One of the important advantages of GLBS is that, most of them can be produced by growing microbes on renewable substrates or seemingly waste materials. Still the production cost has to be slashed down, so as to compete with synthetic products. Moreover, many of the GLBS play a pivotal role in the pathogenicity of producing micro-organisms. Even though the surface active properties are biotechnologically significant, the possibility for apparent cyto-toxicity hinders its extended applications in various fields. In order to overcome the issues; apart from mechanistic studies, efficacy studies of biosurfactants may be performed on *in vivo* models so as to ensure its ecological acceptance.

SECTION 4: POLYHYDROXYALKANOATES

Introduction

Owing to the inherent flexibility and vulnerability, plastics owe its name to '*plasticos*' (Greek word meaning '*to mold*'). Primarily, the xenobiotic plastics made of petroleum products (*i.e*., the petroplatcs) rules the world market by influencing every nook and corner of modern human life. Of late, bioplastics are seen slowly emerging up on the market of plastics. Petroplastics comprise a heterogeneous group of carbon based versatile polymers that unquestionably top the list of major environmental pollutants. The disposal of plastics is troublesome as it requires around 500 years to degrade normally in the environment, and burning them would produce toxic gases like dioxins (Pradeep *et al*., 2012, 2014; Benjamin *et al*., 2015). The recalcitrant and toxic properties of plastics promote the scientific community to develop biobased eco-friendly plastics (the bioplastics), which are vulnerable to easy biodegradation (Keshavarz and Roy, 2010).

Bioplastics are divided into three groups: (1) completely or partly biobased in origin mainly from bioethanol, but non biodegradable (*e.g*., bio-polyethylene, bio-polypropylene); (2) fossil based in origin but biodegradable due to the presence of hydrolysable backbone (*e.g*., poly-(butylene adipate-*co*-terephthalate); and (3) purely biobased and biodegradable (Vroman and Tighzert, 2009). For the last two decades, the third category (popularly known as bioplastics), has been the focus of green industry primarily due to the increasing concern about the environment and fossil resources (Mülhaupt, 2013). Bioplastics include chemically induced polymers from biologically derived monomers (*e.g*., polylactic acid, polyglycollic acid), microbially produced biopolymers (*e.g*., polyhydroxyalkanoates) or modified natural polymers (starch-polyethylene based) (Sudesh and Iwata, 2008).

Microbial biopolymers can successfully be used as candidates for bioplastics, among which polyhydroxyalkanoates (PHAs) have received key attention as its properties are quite similar to that of synthetic plastics (Chee *et al.,* 2010). The PHAs are microbial polyesters with hydrolysable backbone and are accumulated intracellularly as carbon or energy reserves – produced in presence of excess carbon and/or depletion of nitrogen, phosphorus and magnesium (Sreedevi *et al*., 2014). Microorganisms such as bacteria (species of *Alcaligenes, Azotobacter, Pseudomonas, Bacillus, Aeromonas, Streptomyces, etc*.), Archae (*Haloferax*) (Huang *et al.*, 2006; Du *et al.*, 2012), and a few yeast (*Candida, Saccharomyces*) are known producers of PHAs. Large scale production of PHAs is mainly contributed by bacteria, which can accumulate around 90 % of its cell biomass (Reddy *et al.*, 2003).

Structure and classification of PHAs

PHAs possess hydrolysable polyester backbone, which is responsible for its complete biodegradability to CO_2 and water. Generally, PHAs are made up of hydroxyl fatty acids with alkyl side chains ranging from C_1 to C_{13}. The alkyl group may be aliphatic, aromatic with saturated or unsaturated carbon chains (**Figure 10 and 11**). The versatility of microbial PHAs depends on the carbon source fed to the microorganisms as well as the metabolic pathways triggered in the cells towards production (Loo and Sudesh, 2007). About 100 different types of monomer units have been identified so far that contribute individually or in groups to different types of biopolymers (**Figure 11**). Polyhydroxybutyrate - the predominant member of PHAs - is synthesised microbially as homopolymer or heteroplolymer. However, as illustrated in the (**Figure 12**) the industrially significant PHAs include poly(3-hydroxybutyrate) (PHB)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)], poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(HB-*co*-

4HB)], poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(HB-*co*-HHx)], *etc*. (Suriyamongkol *et al.*, 2007; Sreedevi *et al.*, 2014).

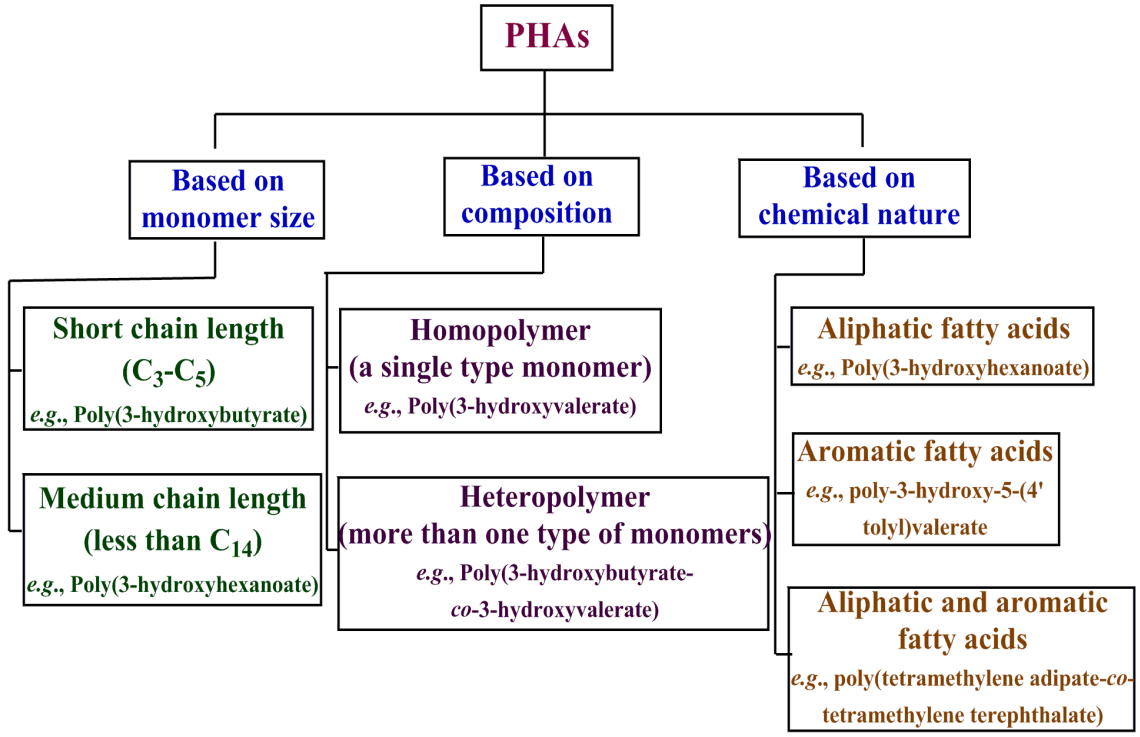


Figure 10. Classification of PHAs.

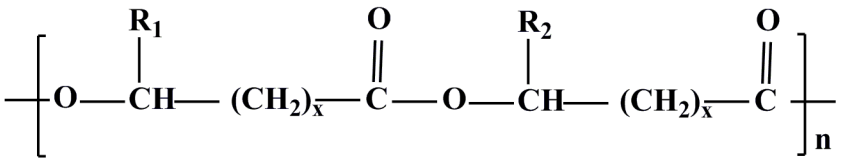


Figure 11. General structure of PHAs. Where, R_1/R_2 = alkyl groups C_1 - C_{13}, x = 1 - 4; n = 100 - 30,000.

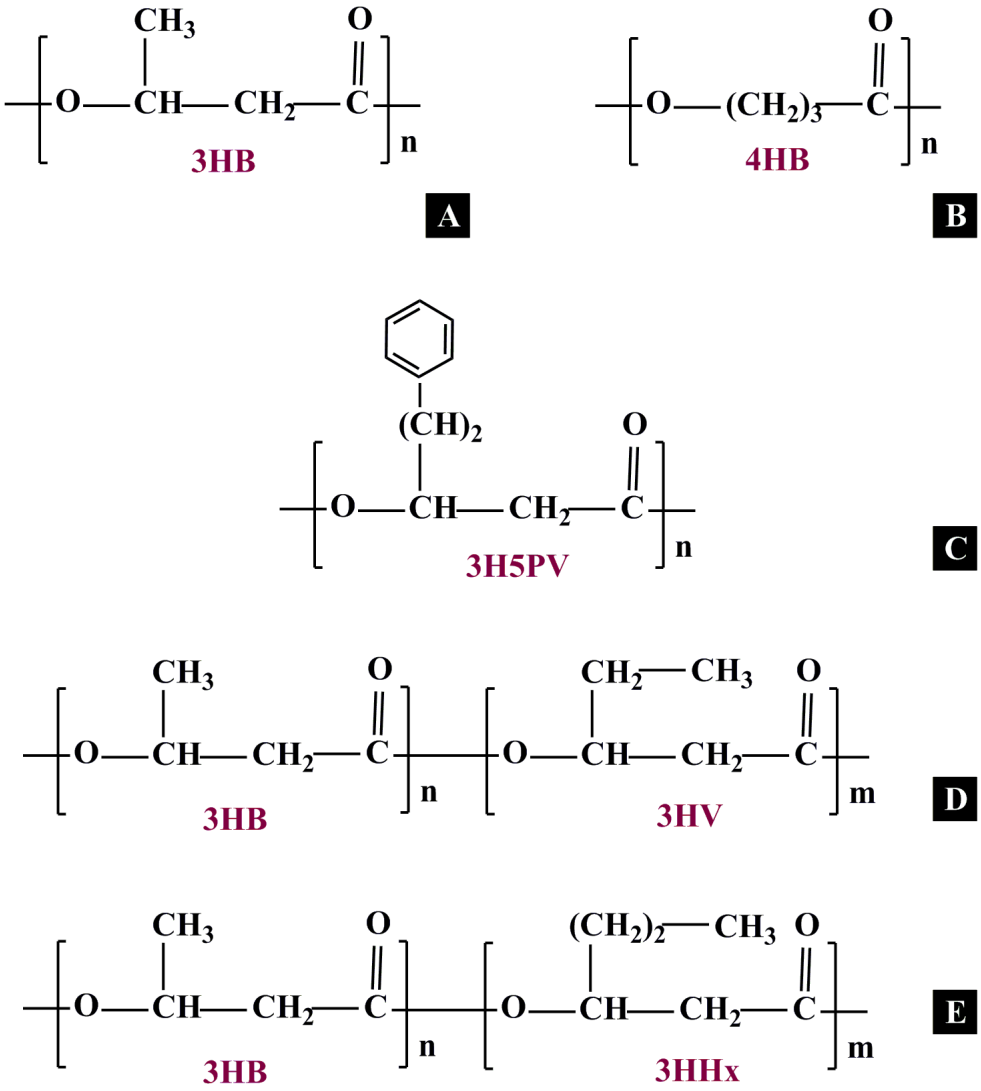


Figure 12. Industrially significant PHAs. (A). Poly-3-hydroxybutyrate (PHB); (B). Poly-3-hydroxyvalerate (PHV); (C). *Poly*(*3-hydroxy-5-phenylvalerate*) [P(BH-*co*-HV)]; (D). Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)]; and (E). Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(HB-*co*-HHx)].

Microbial PHAs from renewable sources

Even though, a variety of Gram positive and negative bacteria, mainly the species of *Bacillus*, *Azetobacter*, *Alcaligens*, *Pseudomonas*, *Aeromonas*, *Methylobacterium* are well known producers of PHAs, its commercialisation is hindered by the high cost of production as compared to the common

thermoplastics (Ciesielski *et al.*, 2014). The development of suitable strategies for the production and recovery of PHAs is deemed necessary for hitting the world market and to compete with the petroplastics as hot candidates for green technologies. According to the economic point of view, 30-40 % of the total cost of bioplastics goes to the substrates used for microbial fermentation. Hence, the scientific community mainly focuses on cheap renewable substrates for the production of PHAs, some of which are listed below.

Molasses

Molasses, the by-product of sugar refineries, is honey-like and dark brown coloured syrup left after the crystallisation of sugar from cane or beet juice. Molasses generally contain 50-70 % of sugars including sucrose, glucose and fructose and the non-sugar fraction comprise of many nitrogenous compounds, amino acids, lipids, sterols *etc*. (Crouse III and Farmer III, 1980; Teclu *et al.*, 2009). For every ton of sugar cane/beet crushed, about 5-6 % of molasses is generated, which can successfully be used as substrate for the industrial production of PHAs (http://www.birla-sugar.com/Our-Products/Molasses). It was sugar beet molasses that used for the first time as carbon source for the production of PHAs by *Azotobacter vinelandii* strain UWD in 1992 (Page, 1992). Later on, several other bacterial cultures were also reported to have intracellular accumulation of PHAs when grown in molasses. For instance, *Bacillus* sp. JMa5 grew well on mineral salt medium supplemented with molasses with an accumulation of 25-35 % of PHB (Wu *et al.*, 2001). *Bacillus cereus* strain SPV showed significantly higher accumulation of PHB (61 %) when grown in medium supplemented with sugar molasses as sole source of carbon (Akaraonye *et al.*, 2012). Similarly, Tripathi *et al*. (2012) investigated the production of PHAs by *Pseudomonases aregunoisa* NCIM No. 2948 on different carbon sources such as cane molasses, glucose, glycerol, sucrose and fructose, among which cane

molassese served as the most suitable substrate supporting the maximum production (5.6 g/l) of PHAs (Tripathi *et al.*, 2012). Interestingly, some studies reported significantly high accumulation of PHAs (approximately 50 %) in the medium containing molasses, employing mixed cultures of activated sludge - both aerobically and anaerobically (Albuquerque *et al.*, 2007, 2010; Bengtsson *et al.*, 2010). Other than beet or cane molasses, soy molasses was also found as promising cheaper substrate for the production of bioplastics, especially PHAs (Full *et al.*, 2006).

Whey

Whey is the liquid by-product left after the curdling or straining of milk for the production of cheese or casein, which is a rich source of proteins, lactose and lipids (Wong and Lee, 1998). *Cupriavidus necator* H16 produced 28.1 g/l of PHB when cultured in liquid medium supplemented with hydrolysed whey as the major nitrogenous source (Obruca *et al.*, 2014). *Hydrogenophaga pseudoflava* DSM1034 produced a co-polymer of 3-hydroxybutyrate, 3-hydroxyvalerate and 4-hydroxybutyrate P(HB-*co*-HV-*co*-HB) in whey (Povolo *et al.*, 2013). *Pseudomonas hydrogenovora* produced the polyester of 3-hydroxybutyrate (1.27 g/l) in hydrolysed whey permeate using fed-batch fermentation process (Koller *et al.*, 2008). Apart from the wild type bacteria, recombinant *E. coli* carrying the recombinant functional biosynthetic genes of PHAs from known producers were successfully employed for the commercial production of PHAs (Pais *et al.*, 2014). Recombinant *E. coli* carrying the genes encoding PHAs from *Alcaligenes latus* showed significantly high productivity of PHB (4.6 g/l) on whey using the pH-static cell recycle fed-batch culture strategy (Park *et al.*, 2002).

Glycerol

Crude glycerol is the major by-product of biodiesel industry. As the production of biodiesel is increased worldwide with the prospects of replacing fossil fuels, crude glycerol has become one of the mainstream industry wastes. Glycerol is also produced as waste from oleo-chemical and bioethanol industries. The crude glycerol contains methanol and salts as impurities and refining of which is quite un-economical for applying in other industries like food, cosmetics, pharmaceutics, and detergency. Hence, the biotechnology is expanding research towards the utilisation of waste glycerol for the production of other industrially significant biomolecules which came out with an interesting result of microbial bioplastics (Ashby *et al.*, 2011; Yang *et al.*, 2012). *Cupriavidus necator* DSM 545 produced copolymers or terpolymers of 3-hydroxybutyrate, 4-hydroxybutyrate and 3-hydroxyvalerate utilising the waste glycerol from the biodiesel plant as carbon source - by employing high-cell density fed-batch culture strategy (Cavalheiro *et al.*, 2012). *Bacillus cereus* accumulated PHAs up to 2.4 % of cell dry weight when cultured in the nitrogen limited medium supplemented with crude glycerol (Shah *et al.*, 2014). Recently, it was found that *Bacillus thuringiensis* EGU45 efficiently convert crude glycerol to PHAs with comparatively good productivity rate of 1.54 - 1.83 g/l under non-limiting nitrogen conditions (Kumar *et al.*, 2015).

Waste water

Industrial waste water is found as another economic and environmentally promosing source of cheap nutrient for the microbial production of PHAs. Waste water from paper mill was used as feed for the production of PHAs by activated sludge of municipal waste treatment plant in a three step process. Initially, acidogenic fermentation of the waste water was carried out anaerobically to concert the organic matter to volatile fatty acids. The second step favoured the enrichment of PHAs accumulating microbes under

feast/famine conditions followed by the enhancement of PHAs accumulation by batch fermentation. The multistep process could achieve PHAs accumulation as 48 % of the sludge dry weight (Bengtsson *et al.*, 2008). Thus, troublesome waste water effluents from several industries like tomato cannery (Liu *et al.*, 2008), oil mills (Pozo *et al.*, 2002; Kourmentza *et al.*, 2015), biodiesel (Dobroth *et al.*, 2011), food industry (Dobroth *et al.*, 2011), brewery (Liu *et al.*, 2011), and municipal waste treatment plants (Chua *et al.*, 2003), *etc*. were also utilised effectively as liquid medium for the production of PHAs by microbial consortium - rather than purified single cultures - for the production of PHAs in order to achieve economic feasibility.

Lignocelluloses and starch

Lignocellulose is considered as one of the most significant non-food biomass available on earth mainly composed of the abundant cellulose, followed by hemicelluloses and lignin (Sajith *et al.*, 2014). The utilisation of lignocelluloses for the production of bioplastics opens a new way for the solid waste management. *Saccharophagus degradans* accumulated PHA granules intracellularly upon growth in medium containing waste from tequila bagasse as carbon source (Munoz *et al.*, 2008). But, in most cases; lignocellulose was hydrolysed to release fermentable sugars - prior to the utilisation as feed for the production of bioplastics (Du *et al.*, 2012). Hydrolysates of sugar maple wood and wheat sraw - after the removal of inhibitory phenolics - were used to produce PHAs by *Burkholderia cepacia* ATCC 17759 and *Burkholderia sacchari* DSM 17165, respectively (Pan *et al.*, 2012; Cesário *et al.*, 2014). Similarly, species of *Pseudomonas* were able to convert the fermentable sugars of hydrolysed perennial ryegrass to intracellular PHAs (20-34 % of cell dry mass) (Davis *et al.*, 2013).

Starch is another important renewable carbon source to be used for the production of microbial PHAS. Microorganisms such as *Bacillus cereus* and

Haloferax mediterranei were reported to produce PHAs utilising starch in its native or hydrolysed forms (Chen *et al.*, 2006; Huang *et al.*, 2006; Halami, 2008; Ali and Jamil, 2014).

Fats and vegetable oils

Application of fats and oils as feed stock for the production of PHAs has been attracted the attention of several industries due to their renewability and relatively low cost. Ashby and foglia (1998) compared the suitability of various triglycerides including animal fats (tallow, lard and butter oil) and vegetable oils (oleic, sunflower, coconut and soybean) for the biosynthesis of PHAs by *Pseudomonas resinovorans*, of which coconut oil supported the maximum yield (1.9 g/l) of PHAs (Ashby and Foglia, 1998). *Comamonas testosterone* produced PHAs with 3-hydroxydecanoate as the major polymer when castor seed oil, olive oil and sesame oil were used as the substrates (Thakor *et al.*, 2005). Vegetable oils such as soybean, palm and corn oils were successfully employed for the production of PHAs (Kahar *et al.*, 2004; Loo *et al.*, 2005; Kek *et al.*, 2008; Shang *et al.*, 2008). In order to make the production process more industrially feasible and economical, Verlinden *et al.* (2011) employed waste frying oil (rape seed) for the production of PHAs by *Cupriavidus necator* and found that the frying oil supported higher production of PHB (0.9 g/l) as compared to the pure oil (0.62 g/l).

Applications

For the last few decades, the extensive research for PHAs has expanded its utility in various fields due to its biocompatibility and biodegradability.

PHAs as packaging material

Initially, Wella AG, Germany, launched everyday articles such as shampoo bottles and packaging materials made of bioplastics (Weiner, 1997). Later on,

PHAs were used widely to make films, bags, disposable items like cups, razor, hygeine products, pens, hygiene products *etc*. PHB found limited applications as the packaging materials due to its high crystallinity and brittleness, nevertheless, incorporation of PHV to PHB reduces its brittleness (Sreedevi *et al*., 2014). The gas barrier property of the copolymer P(HB-*co*-HV) was utilised for making fresh food packaging materials for meat and milk (Philip *et al*., 2007). The piezoelectric properties (*i.e*., permittivity, dielectric constants and electro-mechanical properties) of PHAs were utilised for making various sensors, oscillators, lighters, headphones (Hocking and Marchessault, 1994). Polycaprolactone is another PHA with remarkable good tensile strength which could be employed for making food packaging materials (Siracusa *et al.*, 2008).

Biomedical applications

The biocompatibility of PHAs made them suitable materials for making surgical implants, bone marrow scaffolds and osteosynthetic materials. Galego *et al*. (2000) developed a novel composite material made of P(HB-*co*-HV) (8 %) and hydroxyapatites (30 %) having mechanical strength comparable to that of human bones - that can effectively be used for bone fracture fixation. Later, Shishatkaya *et al*. (2004) investigated the biocompatibility of monofilament sutures made of PHB and copolymer P(HB-*co*-HV) in rats for the healing of facial muscle wounds and found that these intramuscular implants did not induce any adverse immune responses such as inflammations, necrosis or malignancy even after 1 year. P4HB was also developed as a potential absorbable biomaterial with tremendous medical applications. For instance, a composite of P4HB and polyglycolide (PGA) was found as suitable material for heart valve development. The scaffold made of P4HB/PGA allowed prolonged tissue maturation, and degraded completely after 8 weeks of implantation and thus, supported the development

of tissue engineered tri-leaflet heart valve in sheep. It successfully extended its applications for the development of vascular grafts, stents, patches and sutures (Martin and Williams, 2003). Similarly, P(HB-*co*-HHx) was also found as a potent material for bioimplants due its increased biocompatibility and biodegradability, in comparison to PHB (Qu *et al.*, 2006).

Now-a-days, PHAs are widely used in drug delivery systems for the sustainable and targeted release of drug. PHAs are either impregnated along with the drug or used to make biodegradable coats that encapsulate the drug where the dissolution/release of drug into the system depends on the polymer/drug ratio and degradation of the biopolymer rather than diffusion (Philip *et al.*, 2007). Capsules made of P(HB-*co*-HV) was used for the sustainable release of veterinary medicine (Kwan and Steber, 1993). Gentamycin was incorporated into P(HB-*co*-HV),which was found effective for the treatment of orthopaedic infections of due to *Staphylococcus* (Rossi *et al*., 2004). Similarly, another study on mice with ascetic carcinoma, the antitumour drug rubamycin was incorporated into PHB microplarticles, which exhibited pronounced anti-tumour activity (Shishatskaya *et al.*, 2008).

Agricultural applications

The biodegradable films made of PHAs are used as coating for insecticides and fertilizers, so as to enable its slow release in a controlled manner to the field (Philip *et al.*, 2007). Another important application of PHAs in agriculture is in the preparation of commercial inoculants applied in the field for enhancing nitrogen fixation. Incorporation of *Azospirillum* cells with intracellular PHAs showed increased shelf-life, efficiency and reliability of the inoculants (Dobbelaere *et al*., 2001; Philip *et al*., 2007).

Conclusions

PHAs harbour a versatile group of polyesters that can be synthesised from renewable raw materials with the characteristic of complete biodegradability to carbon dioxide and water. Even though, intense research in the field of PHAs has revealed the potential of many microorganisms and renewable resources to support the production of PHAs, their large scale production is not competent enough to replace the petroplastics on the market, principally due to its high cost. Hence, exploration of new resources and biotechnological processes are warranted to expand the utility of PHAs in various field in a sustainable and environment friendly way so as to fulfill the future needs for biopolymers is a sustainable manner-both environmentally and economically.

Chapter 3

Isolation and screening of rumen microbes for the production of industrially significant biomolecules with emphasis on lipase, biosurfactants and polyhydroxyalkanoates

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Aim and Rationale

Rumen microorganisms are highly adapted to the environment where they meet the bulk of hydrocarbons as a part of daily animal feed, and they have the potentials for producing many inducible metabolites - depending on the nutritional composition of the niche in which they grow. Thus, this study focused on the isolation, screening and identification of microbes for the production of industrially significant biomolecules; lipase, biosurfactant and polyhydroxyalkanoates (PHAs) from the rumen content of Malabari goat (*Capra hircus* L.), the least explored natural ecosystem.

Introduction

The rumen is one of the most complicated and most fascinating microbial ecosystems in nature which comprises a diverse population of obligatory and facultative anaerobic bacteria, fungi and protozoa. The rumen microbial population represents a rich and until recently, under-utilised source of biomolecules with large potentials for industrial applications. Apart from some lignocellulolytic microbes, the studies on the ruminal microbial ecosystem were not carried out in detail to date, from an industrial microbiologist's point of view. In order to make use of this rich source of microbes, this study focused on the screening and identification of microbes for the production of three classic representatives of industrially significant biomolecules – lipase, biosurfactants and PHAs. Rumen of Malabari goat was selected for the study. Malabari goat is specifically found in the Malabar region of Kerala State, India.

Materials and methods

Sample preparation

Rumen contents from both male and female Malabari goats were collected aseptically in screw-capped tubes from the slaughter house at Chelari (11.18189600 ^{o}N; 75.82206300 ^{o}E), Malappuram District of Kerala State as described by Privé *et al.* (2010). Briefly, 10 ml sterile double distilled water (ddH_2O) was added to 10 g sample (rumen content) and centrifuged at 800 × *g* for 4 ^{o}C at 5 min. The supernatant (1 ml) obtained as above was serially diluted (up to 10^{-6}) in the pre-sterilised ddH_2O (Unni *et al*., 2014).

Isolation of microbes

The diluted sample (100 µl) was aseptically transferred to semi-synthetic medium – modified de Man-Rogosa-Sharpe (MRS) medium (**Table 8**). Initial cultivation was carried out under anaerobic condition in an anaerobic chamber (KIM Microsystems, India), saturated with mixed gas (80 N_2, 10 CO_2 and 10 H_2, all %). The culture was gradually adapted to the aerobic system by repeated subcultures in a specially designed conical flask, designated as 'Benjamin flask' (**Figure 13**).

An aliquot of the culture was spread onto nutrient agar plates (**Table 9**) for the isolation of bacteria, or on potato-dextrose agar medium (HiMedia, India) for the isolation of fungi, and incubated at 37 °C for 24 h. The culture plates were examined for the presence of individual colonies. The purity of the isolate was confirmed by sub-culturing.

Figure 13. Benjamin flask

Table 8. Composition of de Man-Rogosa-Sharpe (MRS) medium

Ingredient	Weight (g/l)
Peptone	5.0
Beef extract	3.0
NH_4NO_3	5.0
$(NH_4)_2SO_4$	4.0
K_2HPO_4	2.0
NaCl	2.0
$MgSO_4.7H_2O$	0.1
Cysteine-HCl	0.5
pH 6.9 ± 0.1	

Table 9.Composition of nutrient agar

Ingredient	Weight (g/l)
Peptone	5.0
NaCl	5.0
Yeast extract	3.0
Beef extract	3.0
Agar	2.0
pH 7.0 ± 0.1	

Screening for lipase production

The isolated pure cultures were screened for lipase production by agar diffusion methods, as described below.

Tween 80 agar method

The culture was grown on nutrient agar medium supplemented with (in 100 ml) 1 ml tween 80, 0.01 g $CaCl_2$, 1 ml olive oil, and incubated at 37 ^{o}C for 24 h. Appearance of opaque halo around the colonies is the positive inference for the production of lipase.

Chromogenic plate method

Chromogenic agar plates containing (in 100 ml) 1 ml tributyrin, 0.01 g phenol red, 0.001 g $CaCl_2$, 2 g agar were prepared and pH was adjusted between 7.3 and 7.4. The culture (24 h old) supernatant was introduced into the well, which was bored at the centre of chromogenic agar plate, and incubated for 1 h at 37 ^{o}C. Heat inactivated culture supernatant was kept as control. Subsequently, the plates were observed for colour change from red to yellow, an indication for the production of lipase.

Screening for the production of biosurfactant

The isolated pure cultures were screened for the production of biosurfactant as described below.

Blood haemolysis Test

Fresh single colonies were spotted on nutrient agar plates supplemented with 5 % (v/v) human blood, and incubated for 48-72 h at 37 ºC. The colonies were then observed for the presence of haemolytic clear zone around the bacterial growth.

Cetyltrimethylammonium bromide (CTAB) agar plate test

The isolated colonies were spotted on nutrient agar plates, supplemented with (in 100 ml) 1 ml (v/v) olive oil, 0.05 g CTAB, and 0.02 g methylene blue and incubated for 48-72 h at 37 ºC. The plates were then observed for the appearance of bluish halos around the wells to imply the production of biosurfactant.

Drop collapse assay

Pure isolates were cultured in nutrient broth supplemented with 1 % (v/v) olive oil at 37 ^{o}C for 4-5 days. The cultures were then centrifuged at 9,400 × *g* for 10 min and the supernatant was collected. The culture supernatant (25 µl) was added to the surface of parafilm after adding (1 %) bromophenol blue for staining. Distilled water and sodium dodecylsulphate (1 %) were kept as negative and positive controls, respectively. Bromophenol blue had no influence on the shape of the drop (Bodour and Miller-Maier, 1998).

Screening for production of polyhydroxyalkanoates (PHAs)

As PHAs are accumulated in nutrient limited conditions or in the presence of excess carbon, medium containing (g/l) 10 soluble starch; 2.5 peptone and 1 NaCl (pH 7), designated as SPS medium was used for the study.

Microscopic visualisation

Stain was prepared by dissolving Sudan black B (0.3 % w/v) in 60 % ethanol. One day old culture in SPS medium was smeared onto a glass slide and stained with Sudan black B solution for 10 min, rinsed with xylene and counterstained with 0.5 % safranin for 5 sec. Stained samples were observed under oil immersion at 100 X magnification with direct bright field illumination (Burdon *et al.*, 1942).

Nile blue sulphate

Nile blue sulphate solution was prepared by dissolving 0.05 g Nile blue sulphate in l00 ml ethanol. Colonies on SPS agar plates were stained with 5 ml staining solution and shaken gently at 26 ^{o}C. After 20 min, excess stain was drained off, and the plate was air-dried. PHAs producing colonies were detected by irradiating the plate with a short-wave ultraviolet light at a distance of λ_{254} (about 10 cm) from the UV lamp (Kitamura and Doi, 1994).

Sudan black B

A 0.02 % alcoholic solution of Sudan black B was prepared and colonies grown on SPS agar plates were stained with 5 ml of the solution. The plates were kept undisturbed for 30 min. Excess dye was decanted and the plates were gently rinsed by absolute ethanol. PHA producing colonies appeared bluish black, whereas non-producers appeared white (Liu *et al.*, 1998).

Sudan 3

A staining solution was prepared by dissolving 0.2 g Sudan 3 in 25 ml alcohol with gentle warming and then cooled; 25 ml glycerin was added to it. Colonies on SPS agar were stained with the solution for 20 min. Excess stain was removed, followed by washing thrice with dH_2O. PHA producing colonies appeared dark pink.

Identification and characterisation of bacteria

Morphological characterisation (Cappuccino and Sherman, 1983)

The isolate was macromorphologically characterised by observing colony characteristics such as colour, texture and topography of the surface and edges; and micromorphologically by employing conventional staining techniques.

Gram's staining

- The bacterial smear was heat fixed.
- The smear was gently flooded with crystal violet and left it for 1 min.
- Tilted the slide slightly and gently rinsed with tap water or distilled water using a wash bottle.
- Gently flooded the smear with Gram's iodine and left for 1 min.
- Tilted the slide slightly and gently rinsed with tap water.
- Smear was decolorised using 95 % ethyl alcohol and then rinsed with water.
- Gently flooded the smear with safranin to counter-stain and left to stand for 45 sec.
- Tilted the slide slightly and gently rinsed with tap water.
- The slide was observed under binocular microscope (100 X). The photographs were taken by image analyser fitted to a camera (Nikon).

Spore staining by malachite green

- The smear was allowed to air-dry and heat-fixed.
- Smear was flooded with malachite green and placed on a warm hot plate for allowing the preparation to steam for 10 min, then cooled and washed under running tap water.
- Counter stained with safranin for 1 min.
- Washed with running tap water and air-dried.
- The slides were observed under the binocular microscope (100 X).
- The photographs were taken by image analyser fitted with digital camera (Nikon).

Biochemical characterisation (Cappuccino and Sherman, 1983)

The biochemical characterisation included: indole production, methyl red, Voges-Proskauer, citrate utilisation, carbohydrate fermentation (glucose, lactose, sucrose and maltose), starch hydrolysis, and urease tests (**Table 10**).

Indole production test

The cultures were grown in tryptophan broth for 24 to 48 h, subsequently a few drops of Kovac's reagent were added. Formation of a pink indole ring at the surface of culture was recorded as a positive reaction.

Procedure

- One per cent tryptone broth was prepared. It was sterilised by autoclaving at 15 psi, 121 ^{o}C for 15 min.
- Using sterile techniques, the test organism was inoculated into the medium in appropriately labelled conical flasks, and incubated for 4 days in an incubating shaker.
- The culture tubes prepared for indole production test were incubated at 35 ^{o}C for 48 h.
- Kovac's reagent was added to it and the tubes were gently shaken after intervals for 10 to 15 min.
- The culture tubes were subsequently allowed to stand to permit the reagent to come to the top.

Methyl red and Voges-Proskauer (MR-VP) test

Procedure

- MR-VP broth (pH 6.9) was prepared in 10 ml tubes.

- Five ml of the broth was poured in each of the tubes and sterilised by autoclaving at 15 psi, 121 ^{o}C for 20 min.
- MR-VP broth was inoculated and one tube was kept as un-inoculated comparative control.
- All the culture tubes were incubated at 35 ^{o}C for 48 h.
- Half of the tubes were used for methyl red test, and the other half for Voges-Proskauer test.
- In the tubes assigned for methyl red test, 5 drops of methyl red indicator dye was added, the persistence of red colour is an indication for positive test, and change in colour from red to yellow is negative test.
- In the tubes assigned for Voges-Proskauer test, 12 drops of Voges-Proskauer solution A, and three drops of Voges-Proskauer solution B were added.
- The culture tubes were shaken gently for 30 sec with the caps off, to expose the medium to oxygen.
- The reaction was allowed to stand for 15 to 30 min, and observed for a change in colour from yellow to pinkish red.

Citrate utilisation test

To determine the ability of the bacterium to utilise/ferment citrate as the sole source of carbon for metabolism in Simmon's citrate agar slants. Change of colour from green to prussian blue on addition of bromothymol blue, and growth of bacterium was recorded as a positive reaction.

Procedure

- Simmon's citrate agar slants were prepared (pH 6.9).

- All the ingredients, except phosphates - which were to be dissolved separately in 100 ml of water - were dissolved and the volume was made to l litre. The pH was set at 6.9.
- The medium was poured in the culture tubes, and sterilised by autoclaving at 15 psi, 121 ^{o}C, 20 min, and slants were prepared.
- Simmon's citrate agar slants were inoculated by means of a stab inoculation.
- One blank tube (un-inoculated) was kept as control.
- All the slants were incubated at 37 ^{o}C for 48 h.

Carbohydrate fermentation test

To determine the fermentative degradation of various carbohydrates, phenol red carbohydrate broth with respective sugars like glucose, lactose and sucrose were used.

Procedure

- Fermentation medium was prepared. Broth taken into fermentation tubes was autoclaved at 15 psi, 121 ^{o}C, 20 min.
- Four types of sugar fermentation broths (glucose, lactose, sucrose or maltose) were inoculated with each bacterium and one un-inoculated tube was kept as control.
- All the inoculated and un-inoculated tubes were incubated at 35 ^{o}C for 24 to 48 h, and noted for colour change from red to yellow.

Starch hydrolysis test

To determine the ability of microorganisms excreting hydrolytic extracellular enzymes capable of degrading the polysaccharide starch.

Procedure

- Starch-agar medium was melted and cooled to 45 ^{o}C, and poured into sterile petri dishes.
- It was allowed to solidify.
- Using sterile technique, a single streak inoculation was made at the center of the appropriately labeled plate.
- Inoculated plates were incubated for 48 h at 37 ^{o}C in an inverted position.
- The surface of the plates was flooded with iodine solution for 30 sec.
- Excess iodine solution was poured off.

Table 10. Summary of media constituents and reagents used for biochemical analysis.

Biochemical test	Medium used	Reagent	Inference
Indole production test (IPT)	**Tryptone broth** Tryptone - 1.0 % NaCl - 1.0 % pH - 7.0±0.2	**Kovac's reagent** *p*-Dimethyl aminobenzaldehyde (DMAB) - 5 % Amyl alcohol - 75 ml Conc. HCl - 25 ml	Catabolism of tryptophan
Methyl-Red test (MRT)	**MR-VP broth** Glucose - 05 % Peptone - 0.7 % K_2HPO_4 - 0.5 % NaCl - 0.5 % pH - 7.0±0.2	**Methyl red** - 0.02 %	Glucose oxidation
Voges – Proskauer Test (VPT)	**MR-VP broth**	**VP reagent** 12 drops of reagent A and 3 drops of reagent B **Barritt's Reagent A** α- Naphthol - 5.0 % Absolute alcohol - 100 ml **Barritt's Reagent B** KOH - 4.0 % dH_2O - 100 ml	Production of neutral end products
Citrate utilisation test (CUT)	**Simmon's citrate agar** NaCl - 1.0 % $MgSO_4$ - 0.02 % $NH_4H_2PO_4$- 0.1 % KH_2PO_4 - 0.1 % Sodium citrate - 0.2 % Agar - 2.0 % pH - 7.0±0.2	**Bromothymol blue** - 0.008 %	Citrate fermentation
Carbohydrate fermentation *Glucose (GF)* *Lactose (LF)* *Sucrose (SF)*	Peptone - 0.1 % NaCl - 0.05 % Glucose/lactose/ sucrose - 0.1 % pH - 6.9±0.1	**Phenol red** - 0.0012 %	Fermentation of sugars
Starch hydrolysis test	Peptone - 0.1 % NaCl - 0.05 % Starch - 0.1 % pH - 6.9±0.1	**Phenol red** - 0.0012 %	Secretion of extracellular starch hydrolysing enzymes
Urease test (UT)	Peptone - 0.1 % K_2HPO_4 - 0.2 % Urea - 0.1 % pH - 6.9±0.1	**Phenol red** - 0.0012 %	Hydrolysis of urea by urease

Molecular characterisation by 16S rRNA gene sequence analysis

Procedure

- High molecular weight DNA with good quality was isolated from the culture.
- 16S rRNA gene fragment was amplified by PCR to get a single discrete PCR amplicon of 1500 bp and then purified.
- Forward and reverse DNA sequencing reactions of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic analyser.
- Consensus sequence of 16S rRNA gene sequence was generated from forward and reverse sequence data using aligner software.
- The 16S rRNA gene sequence was used to carry out BLAST with the nucleotide database of NCBI. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using Ribosomal Database Project (RDP database) and the phylogenetic tree was constructed by Neighbor-Joining method using MEGA4 (Tamura *et al.*, 2007). The DNA sequencing service of Xcelris Labs, Ahmedabad, India was hired for the purpose.

Identification and characterisation of fungi

Lactophenol cotton blue staining

Fungal isolates were placed on a slide, stained with 1 or 2 drops lactophenol cotton blue, and covered with a cover slip for observing the morphological characteristics. The slides were observed under the binocular microscope (100 X). The photographs were taken using Image Analyser fitted with digital camera (Nikon).

Biochemical characterisation

Fermentation reactions in media containing glucose, lactose, maltose, sucrose, dextrose or cellulose as the sole source of carbon were performed to identify the fungus at species level (Hugh and Leifson, 1953). Nitrogen assimilation test was carried out by the modified auxanographic technique (Kurtzman and Fell, 1999). In brief, agar medium containing 2 % glucose, 0.1 % KH_2PO_4 and 0.05 % $MgSO_4$ was poured into petri dishes, and allowed to solidify. Fungal suspension in sterilised ddH_2O was swabbed onto the surface of the medium. Sterilised filter paper saturated with the nitrogen compounds was placed on the solid surface of the inoculated agar medium and incubated at 37 ^{o}C for 48 h. An area of growth was produced around those compounds that were assimilated.

Molecular identification by 28S rRNA gene sequence analysis

DNA was isolated from the culture. Its quality was evaluated on 1.2 % agarose gel, a single band of high-molecular weight DNA has been observed.

- Fragment of D1/D2 region of LSU (large subunit 28S rRNA gene) gene was amplified by PCR from the above isolated plasmid DNA. A single discrete PCR amplicon band was observed when resolved on agarose gel.
- The PCR amplicon was purified to remove contaminants.
- Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with DF and DR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic analyser.
- Consensus sequence of D2 region of 28S rRNA gene was generated from forward and reverse sequence data using aligner software.
- The D1/D2 region of LSU (Large subunit 28S rDNA) gene sequence was used to carry out BLAST with the nucleotide database of NCBI GenBank database. Based on maximum identity score, first ten sequences were selected the phylogenetic tree was constructed by Neighbor-Joining method using MEGA4 (Tamura *et al.*, 2007).

The DNA sequencing service of Xcelris Labs, Ahmedabad, India was hired for the purpose.

Results

Isolation and screening of microbes

Four bacterial cultures and three fungal cultures were isolated from the rumen content of Malabari goat. All the isolates were screened for the production of lipase, biosurfactant and PHAs. Among the isolates, one bacterium showed positive results for the production of all these three biomolecules (**Figure 13-16**), and one fungus showed positive results for the production of PHAs and biosurfactant (**Figure 17-18**), which were further characterised and identified.

Characterisation of microbes

The pure bacterial isolate formed colonies with smooth, slimy and creamy in appearance. The cells were Gram negative rods with 1.0 to 1.5 μm length and 0.3 to 0.6 μm diameter (**Figure 19**). The pure fungal colonies were smooth, white-to-cream in color and were generally spherical. The cell size was 4 to 9 mm in length and 3 to 5.5 mm in diameter (**Figure 20**). The biochemical characterisation of bacterium showed that it belongs to the genus, *Pseudomonas* whereas the fungal isolate was identified as yeast belonging to the genus *Candida* (**Table 11 and 12**). A consensus sequence of rRNA gene (1409 bp of 16S for the bacterium and 603 bp of the D1/D2 region of the 28S for the fungus) was generated to carry out the BLAST with the non-reduntant (NR) database of NCBI GenBank. The bacterium was identified and designated as *Pseudomonas* sp. BUP6, and yeast as *Candida tropicalis* BPU1. Based on the maximum identity score, first 11 sequences were selected and aligned using Clustal W for constructing the phylogenetic tree using MEGA 4 (**Figure 21**).

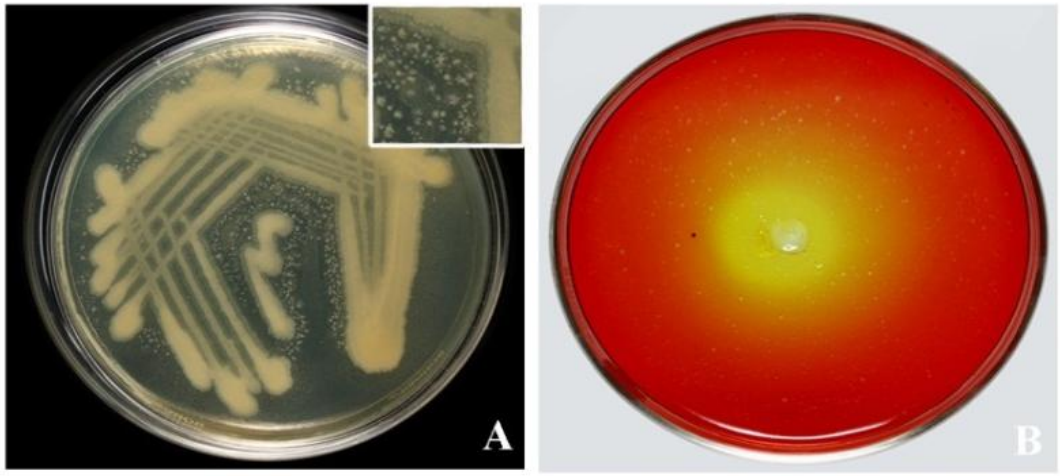

Figure 14. Screening of *Pseudomonas* sp. BUP6 for the production of lipase: (A). tween 80 agar method; production of lipase was indicated by opaque halo around the colonies. Calcium oleate precipitate formed by the hydrolytic activity of lipase is shown at the inset; and (B). chromogenic agar plate method. Production of lipase was indicated by the change in colour from red to yellow around the colonies.

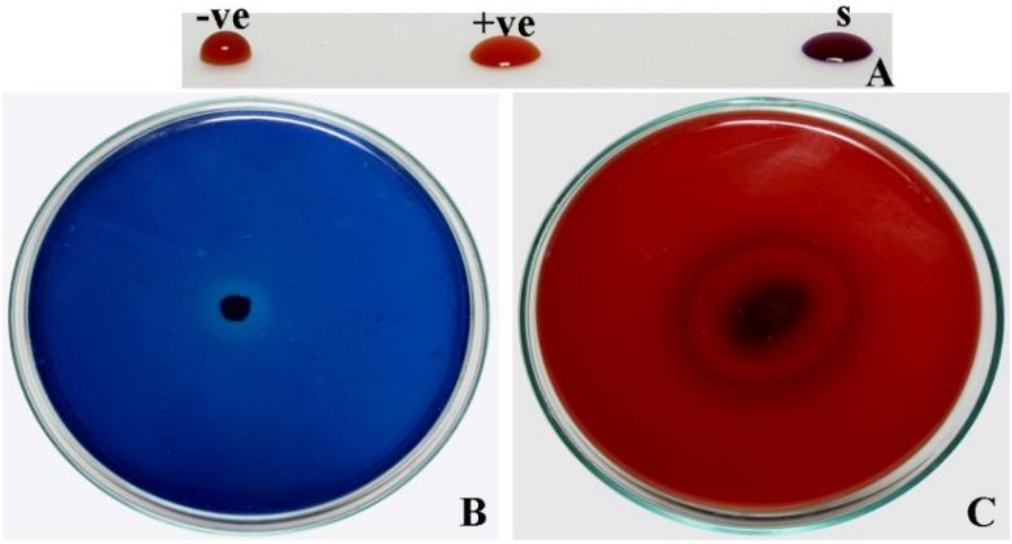

Figure 15. Screening of *Pseudomonas* sp. BUP6 for the production of biosurfactant: (A). drop collapse assay; the collapsed drop of culture supernatant on parafilm indicated the surfactant activity; (B). clear zone around the colony on CTAB agar plate showed the presence of anionic biosurfactant; and (C). zone of haemolysis around the colony on blood agar plate showed the presence of biosurfactant.

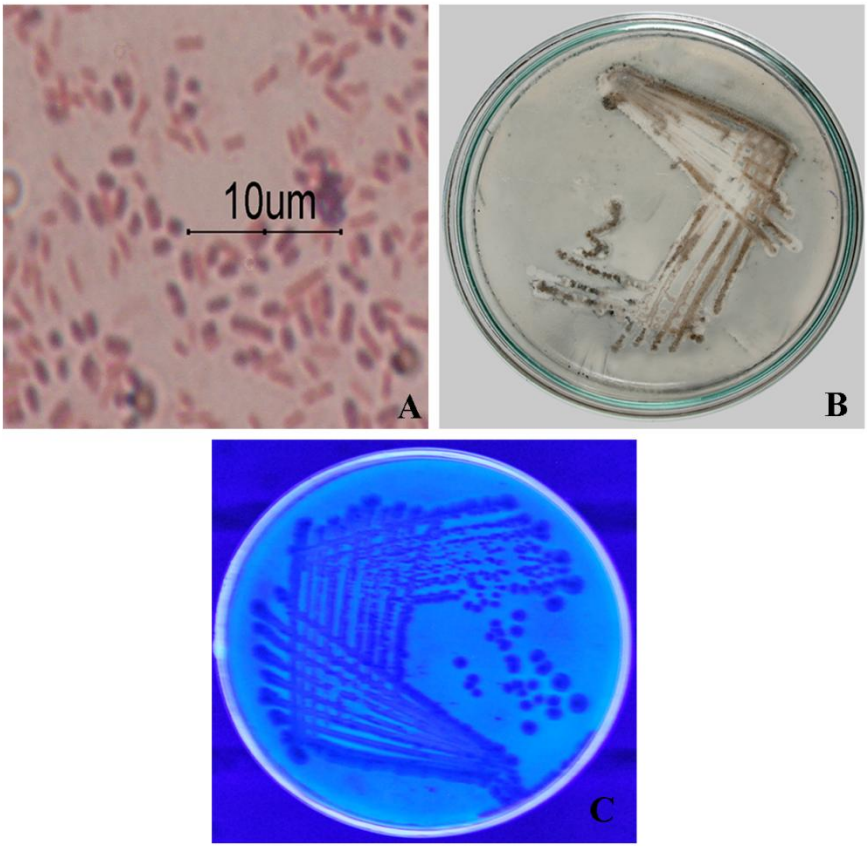

Figure 16. Screening of *Pseudomonas* sp. BUP6 for the production of PHAs: (A). microscopic visualisation of PHA granules on Sudan black B staining. Dark blue black granules are visible in safranin stained cells; (B). blue black colonies on SPS agar plate stained with Sudan black B represents PHA producing cells; and (C). orange fluorescent colonies on SPS agar plate stained with Nile blue sulphate upon UV irradiation represents PHA producing colonies.

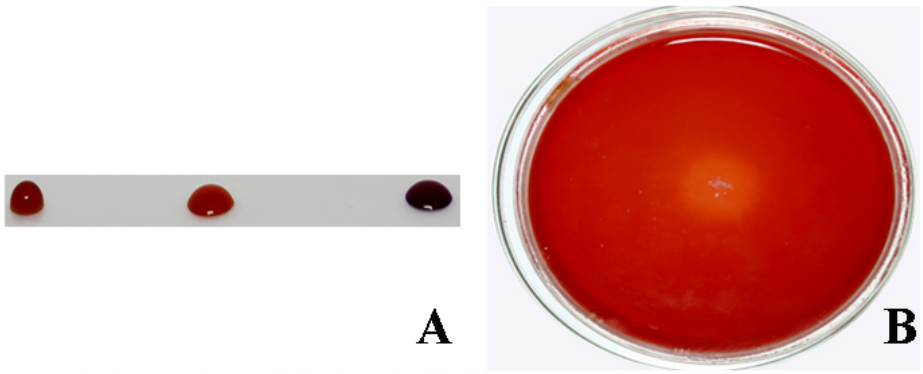

Figure 17. Screening for the production of biosurfactant by *C. tropicalis* BPU1: (A). drop collapse assay; collapsed drop of culture supernatant on parafilm indicated the surfactant activity; and (B). zone of hemolysis around the colony on blood agar plate showed the presence of biosurfactant.

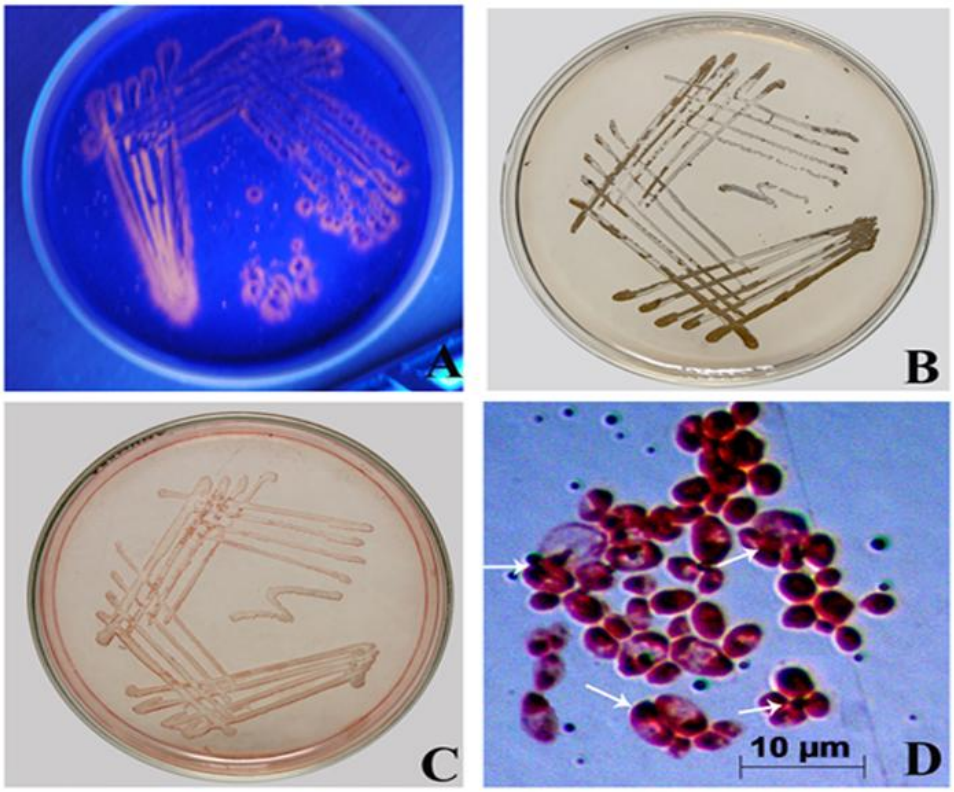

Figure 18. Screening for the production of PHA granules by *C. tropicalis* BPU1: (A). orange fluorescent colonies on PDA plates stained with nile blue sulphate upon UV irradiation represents PHA producing colonies; (B). blue-black colonies on PDA plates stained with Sudan black B represents PHA producing colonies; (C). dark-pink colored colonies on PDA plates stained with Sudan 3 represents PHA producing colonies; (D). microscopic visualisation of PHA granules upon Sudan black B staining.

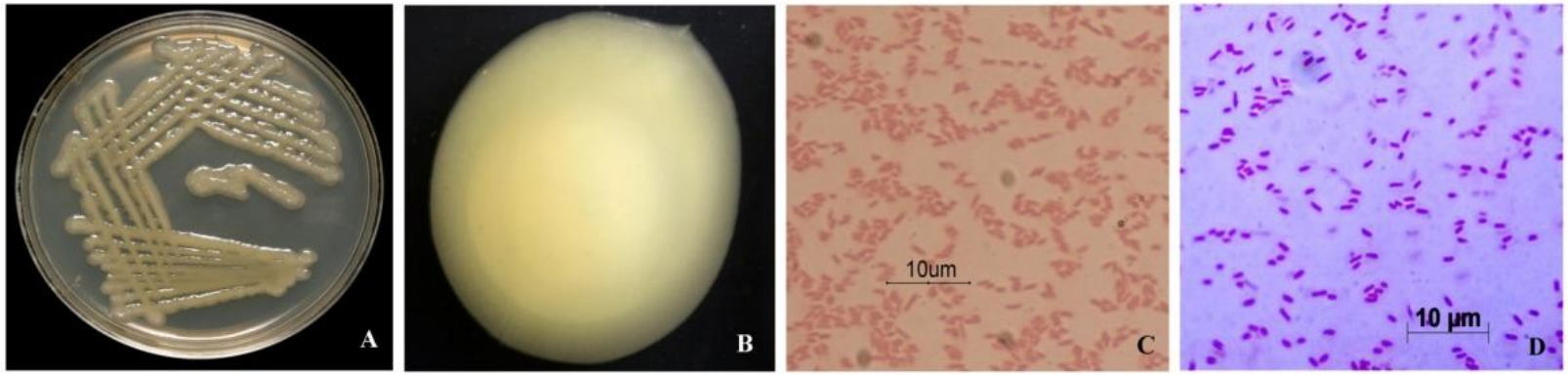

Figure 19. Morphology of *Pseudomonas* sp. BPU6: (A). streak plate on nutrient agar plate; (B). digital image of the single colony appeared slimy and white-to-cream in colour; (C). Gram's staining; and (D). phase contrast microscopic image after spore staining.

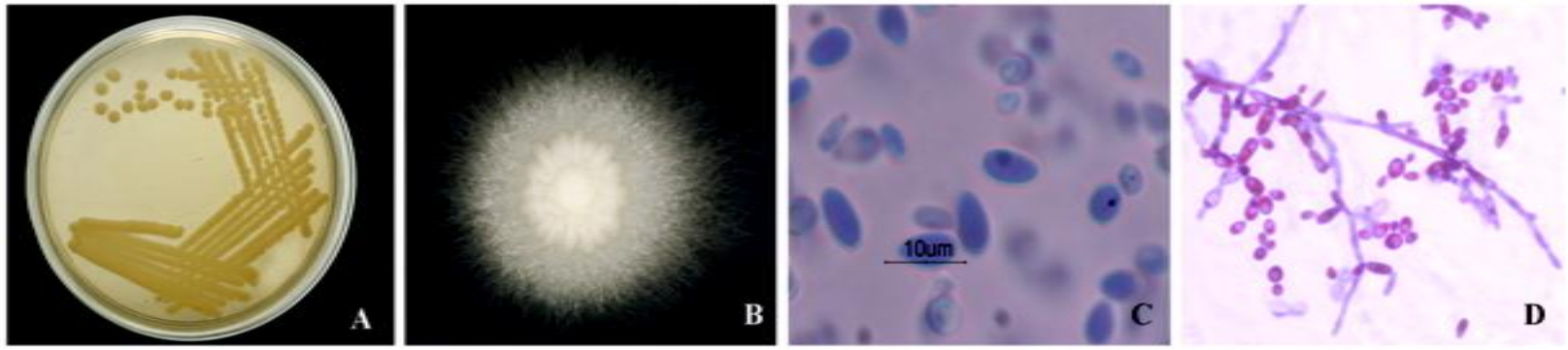

Figure 20. Morphology of *C. tropicalis* BPU1: (A). streak plate on PDA plate; (B). digital image of the magnified view of smooth, white-to-creamy, puffy, and spherical colony; (C). magnified cells after lactophenol cotton blue staining; and (D). image of budding yeast cells in pseudo-filamentous form.

Table 11. Biochemical characteristics of *Pseudomonas* sp. BUP6; citrate utilisation test (CUT), nitrate reduction test (NRT), glucose fermentation, lactose fermentation (LF), sucrose fermentation (SF), starch hydrolysis test, urease test (UT), indole production test (IPT), Voges-Proskauer test (V-PT), methyl red test (MRT).

IPT	MRT	V-PT	CUT	GF	LF	SF	SHT	UT
+ve	–ve	+ve	+ve	+ve	–ve	–ve	+ve	+ve

Table 12. Biochemical characteristics of *C. tropicalis* BPU1

Characteristic	Observation
Fermentation behaviour	
Glucose	+ve (change in colour from red to yellow)
Lactose	–ve (red, colour of reaction mixture did not change)
Sucrose	+ve (change in colour from red to yellow)
Maltose	+ve (change in colour from red to yellow)
Dextrose	+ve (change in colour from red to yellow)
Cellulose	–ve (red, colour of reaction mixture did not change)
Nitrogen assimilation	
$(NH4)_2SO_4$	+ve (showed growth)
$NaNO_3$	–ve (did not show growth)
Peptone	+ve (showed growth)
Glycine	+ve (showed growth)
Arginine	+ve (showed growth)
Utilisation of vegetable oil	+ve (showed growth)

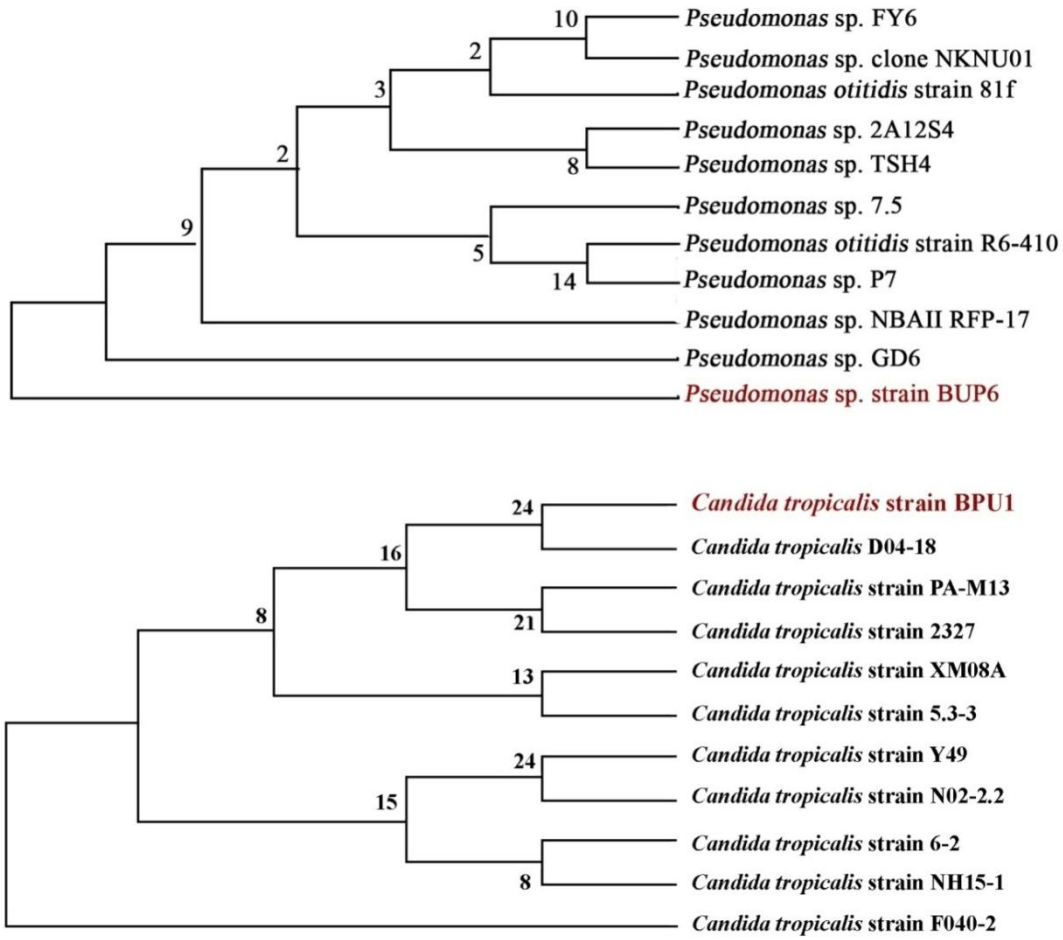

Figure 21. Evolutionary relationships of *Pseudomonas* sp. BUP6 and *C. tropicalis* BPU1 with other 10 related strains

Discussions

The prime objective of this study was to isolate and characterise bacteria and fungi from the rumen of Malabari goat capable of producing certain industrially significant biomolecules; *i.e.*, lipase, biosurfactant and PHAs. Vegetable oil was used as inducer for the production of lipase and biosurfactant, whereas production of PHAs was screened on starch based medium. Among the 6 isolates, one bacterium and fungus each showed significant production of these molecules. By morphological, biochemical and

characterisation of these isolates led to the identification and designation of them as *Pseudomonas sp*. BUP6 and *Candida tropicalis* BPU1, respectively. *Pseudomonas* sp. BUP 6 produced all the three biomolecules, whereas *C. tropicalis* BPU1produced biosurfactant and PHAs.

Many of *Candida* species are not "Generally Regarded as Safe" (GRAS) to be handled as baker's yeast (Benjamin and Pandey, 1998). *C. tropicalis* is a typical inhabitant of the human body, which is not normally considered to cause health issues. It has been noticed that in tropical countries like India; *C. tropicalis* isolates are more prevalent in clinical samples like blood, urine and sputum (Eggimann *et al.*, 2003). Humans are the natural, but not the exclusive habitat of *C. tropicalis*. It has been isolated from animal sources like intestinal contents of marine mammals, birds, bovine, porcine, canine, mice and equine species (Chengappa *et al.*, 1984); alimentary canal of cattle (Foley and Schlafer, 1987); and bovine milk (Lagneau *et al.*, 1996). Moreover, the inhabitance of *Candida* spp. (*C. albicans, C. tropicalis, C. krusei, C. rugosa, C. parapsilosis*) and *Trichosporon* (*T. cutaneum, T. sericeum*) in the guts of ruminants, especially cow and sheep was reported (Lund, 1974). However, no report is available yet in the literature regarding the inhabitance of *C. tropicalis* in the goat (*Capra hircus* L.), an entirely different genus from sheep (*Ovis aries* L.).

Many *Pseudomonas* spp. have been isolated from clinical samples, industrial wastes, petroleum contaminated soils, hot springs, various parts of animals including sheep rumen (Gaoa *et al.*, 2000; Haba *et al.*, 2000). However, no report is available on goat (*Capra hircus* Lin.), a different genus from sheep. Moreover, none of those microbes, isolated from animal/human body parts were exclusively and intensively studied for their industrial significance. In this study, two microbes capable of producing industrially significant

biomolecules were successfully isolated from the rumen of Malabari goat, virtually untapped resource of novel ecosystem.

Conclusions

This study provides clear evidences for the existence of industrially significant microbes in the rumen of goat that have not been exploited resourcefully so far. Two microbes, *Pseudomonas* sp. BUP6 and *Candida tropicalis* BPU1 were successfully isolated, which are capable of producing industrially significant biomolecules from the rumen of Malabari goat.

Chapter 4

Production and statistical optimisation of lipase by *Pseudomonas* sp. BUP6

Published: Priji, P., Unni, K. N., Sajith, S., Binod, P. and Benjamin, S. (2014). Production, optimisation, and partial purification of lipase from *Pseudomonas* sp. strain BUP6, a novel rumen bacterium characterised from Malabari goat. *Biotechnology and Applied Biochemistry*, 62, 71-78.

Aim and rationale

Generally, biotechnology processes are highly dependent on the production parameters and the optimisation of which, not only enhance the yield but also improve the reproducibility of the process. Hence, the formulation of suitable fermentation strategies for the commercial exploitation of the microorganism demands the intensive studies on the effects of physiological and process parameters on the production of enzymes. This study focused on the production of lipase by *Pseudomonas* sp. BUP6 in presence of vegetable oils and statistical optimisation of the culture parameters for the enhancement of lipase production in order to determine their interactive effects.

Introduction

Lipases are ubiquitous enzymes catalysing the hydrolysis and synthesis of esters formed by the combination of glycerol and long-chain fatty acids. The enantio- and regio-selective nature of lipases are exclusively applied in many industries dealing with organic syntheses, hydrolysis of fats and oils, resolution of chiral drugs, modification of fats, flavor enhancement and chemical analyses (Benjamin and Pandey, 1998; Pandey *et al.*, 1999). Since culture conditions influence the properties as well as quantity of the enzyme produced, optimisation of fermentation parameters such as temperature, pH, agitation *etc*. for the production of microbial lipases is of great interest (Wang *et al.*, 1995). The classical method of optimisation generally deals with *one-at-a-time* strategy, which does not depict the combined interactions between the parameters and may lead to misinterpretation of the results (Sim and Kamaruddin, 2008). Now-a-days, modern statistical optimisation systems are used to overcome this problem. RSM is a combination of statistical and mathematical tools, widely used to optimise various biotechnological processes in which linear interaction and quadratic effects of two or more

parameters were estimated to produce the three dimensional contour and surface plots (Burkert *et al.*, 2004; Oliveira et al., 2015). In the present study, principles of RSM were applied for the production of lipase by *Pseudomonas* sp. BUP6.

Materials and methods

Cultivation strategy and medium for the production of lipase

The novel *Pseudomonas* sp. BUP6 isolated from the rumen of Malabari goat was cultivated in basal salt medium (**Table 13**), supplied with groundnut oil at 37 ^{o}C for 24 h in an incubator orbital shaker at 140 rpm. Initially, 0.05 % groundnut oil was supplemented to the basal medium, and subsequently sub-cultured into fresh medium containing higher concentrations of groundnut oil. By repeated subculture, the oil consumption of the culture was enhanced to 0.5 %.

Table 13. Composition of basal salt medium (BSM)

Ingredient	Weight (g/l)
NH_4NO_3	5.0
$(NH_4)_2SO_4$	4.0
Yeast extract	3.0
K_2HPO_4	2.0
NaCl	2.0
$MgSO_4.7H_2O$	0.01
$CaCl_2$	0.01
pH – 7.1±0.1	

Assay for lipase production

Production of lipase by the novel isolate was estimated quantitatively using *para*-nitrophenylpalmitate (*p*NPP) as substrate, by assaying at regular

intervals of 3 h duration. The culture was centrifuged at 9,400 × *g* for 10 min at 4 ^{o}C and the supernatant was assayed for lipase activity. The assay mixture containing 1.8 ml of 0.1 M Tris-HCl buffer with 0.15 M NaCl and 0.5 % triton X-100 was pre-incubated with 200 µl of cell-free culture supernatant at 37 ^{o}C for 10 min. Subsequently, 20 µl of substrate (50 mM *p*NPP in acetonitrile) was added to the reaction mixture and incubated at 37 ^{o}C for 30 min. The amount of *p*-nitrophenol liberated was measured spectrophotometrically at λ_{405}. One unit of lipase corresponds to 1 µmol of *p*-nitrophenol liberated per minute under the standard assay conditions. Control was maintained by adding 200 µl of Tris-HCl buffer instead of enzyme solution. The following formula was used to calculate the lipase activity.

$$\text{Lipase activity (U/ml)} = \frac{\mathrm{A} \times \mathrm{V}f}{\mathcal{E} \times \mathrm{t} \times \mathrm{V}s \times \mathrm{d}} \qquad \text{Eq. (1)}$$

A = Absorbance at λ_{405}

V*f* = Final volume

V*s* = Volume (ml) of lipase used

t = Time of hydrolysis

Ɛ = Extinction coefficient (0.017)

d = Diameter of cuvette (1 cm for standard cuvette)

Vegetable oils as substrates

Suitability of various vegetable oils (0.5 %) as inducer for the production of lipase was analysed by supplementing the basal medium with vegetable oils such as groundnut oil, sunflower oil, olive oil, palm oil and coconut oil, and the cell-free supernatant at 12 h incubation was assayed for lipase activity.

Statistical optimisation of lipase production

Plackett – Burman design

Five factors such as pH, temperature, agitation, inoculum size and incubation time were selected for Plackett-Burman design to identify the physical parameters that influence the production of lipase significantly. Two concentrations (high and low) were evaluated for each parameter and designated as, level +1 and level –1, respectively (**Table 14**). For the selection of significant factors, Minitab version 14 (Minitab Inc. USA) was used to generate and analyse the experimental design of Plackett-Burman. The main effect of each variable was determined using the equation,

$$E = (\Sigma Mi(+1) - \Sigma Mi(-1))/N \qquad \text{Eq. (2)}$$

Where: E is the effect estimate, $M_{i(+1)}$ and $M_{i(-1)}$ are response percentages in trials, in which the independent variable was present in high and low concentrations, respectively, and N is the half number of trials.

Table 14. The levels and code of variables used for Plackett-Burman experimental design.

Variable	Factor	Levels	
		−1	**+1**
X_1	Temperature (^oC)	34	40
X_2	Agitation (rpm)	50	200
X_3	pH	5	7
X_4	Incubation time (h)	12	48
X_5	Groundnut oil (%)	0.1	2

Box-Behnken model and response surface methodology

The significant parameters suggested by the Plackett-Burman design were selected for further statistical analysis. The interactive effects of these parameters on production of lipase were evaluated employing Box-Behnken design. Analysis at three levels (high, medium and low) represented by +1, 0 and −1 respectively, was performed for each parameter and the results were analysed by fitting to second order polynomial equation.

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

Eq. (3)

Where: Y is lipase activity, X_1, X_2, X_3 are the independent variables, β_1, β_2, β_3 are the linear coefficients, β_{12}, β_{23}, β_{13} are the interaction coefficients and β_{11}, β_{22}, β_{33} are the quadratic coefficients.

Validation

To check the validity of quadratic model, 3 experiments - as predicted by the point prediction software Minitab 14 - were performed. Lipase activity was estimated and compared with the predicted values.

Native PAGE

The crude lipase produced at statistically optimised conditions was analysed qualitatively by native polyacrylamide gel electrophoresis (Native-PAGE) using 4 % stacking gel and 8 % resolving gel.

Stacking gel composition

The ingredients used for making stacking gel are given in **Table 15**.

Table 15. Ingredients for stacking gel

Ingredients	Quantity (ml)
ddH_2O	2.1
30 % Acrylamide	0.5
1.5 M Tris buffer (pH, 6.8)	0.38
10 % SDS	0.03
10 % APS	0.03
TEMED	0.003

Composition of resolving gel (8 %)

The ingredients used for making resolving gel are given in **Table 16**.

Table 16. Ingredients required for resolving (8 %) gel

Ingredients	Quantity (ml)
ddH_2O	6.9
30 % Acrylamide	4.0
1.5 M Tris buffer (pH 8.8)	3.8
10 % SDS	0.15
10 % APS	0.15
TEMED	0.009

Samples were allowed to stack at 40 mV and to separate at 70 mV. After run, the gel was washed thoroughly with sterile ddH_2O and equilibrated in 50 mM Tris HCl (pH 8.0) for 30 min at 37 ^oC, subsequently overlaid with molten agar containing 1 % olive oil, 1 % tween 80, 0.01 % $CaCl_2.H_2O$; this preparation was placed undisturbed at 37 ^oC for 24 h and observed for opaque band against a clear background (Haba *et al.*, 2000).

Results

Cultivation strategy and medium for lipase production

Pseudomonas sp. strain BUP6 was tuned to get adapted to higher concentrations of vegetable oil and utilised 0.5 % of groundnut oil within 24 h in a specially designed basal medium. The pH of the medium was gradually decreased from 7.01 to 5.21, and again increased to 6.16 gradually. The maximum lipase activity (96.15 U/ml) was noticed at 12 h of incubation, which gradually decreased (**Figure 22**) upon further incubation. Drop in pH of the medium may be due to the release of free fatty acids from vegetable oil by lipase activity.

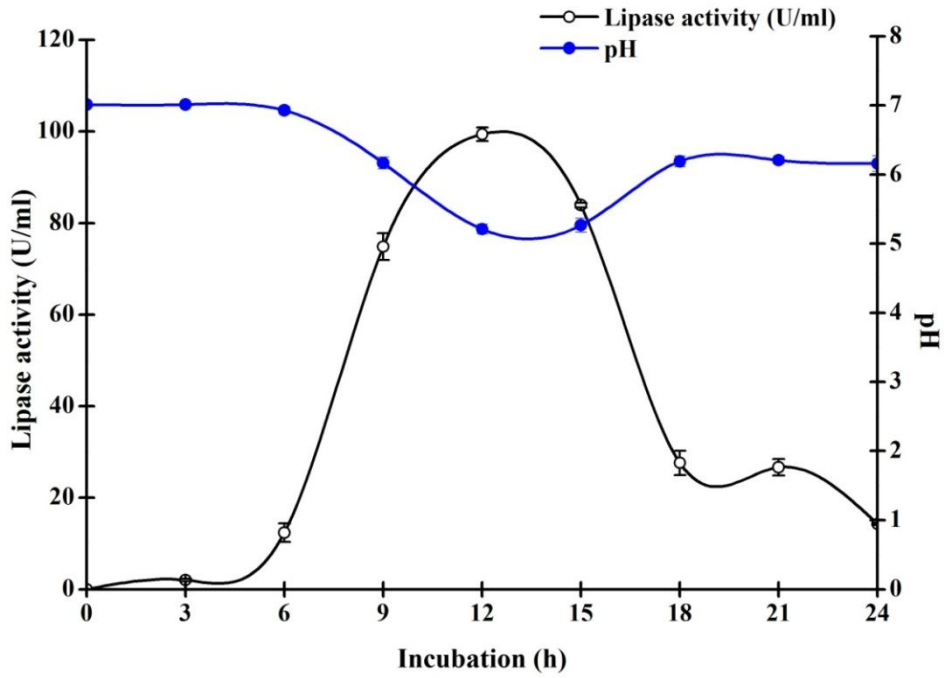

Figure 22. Lipase production by *Pseudomonas* sp. BUP6 was estimated quantitatively using *para*-nitrophenylpalmitate (*p*NPP) as substrate, at regular intervals of 3 h. The maximum lipase (96.15 U/ml) was noticed at 12 h of incubation, which was then gradually decreased. The pH of the medium was gradually decreased from 7.01 to 5.21 and again increased to 6.16.

Suitability of 5 vegetable oils such as groundnut oil, coconut oil, olive oil, sunflower oil and palm oil as inducer for the production of lipase was

analysed (140 rpm, 37 ^{o}C, 12 h), of them, groundnut oil supported the highest level of lipase production (96.15 U/ml). Effects of sunflower oil ((92 U/ml) and olive oil (86.3 U/ml) were comparable to that of groundnut oil, but the other two oils (coconut oil and palm oil) showed much lesser activities, *i.e.*, 64 U/ml and 60 U/ml, respectively (**Figure 23**).

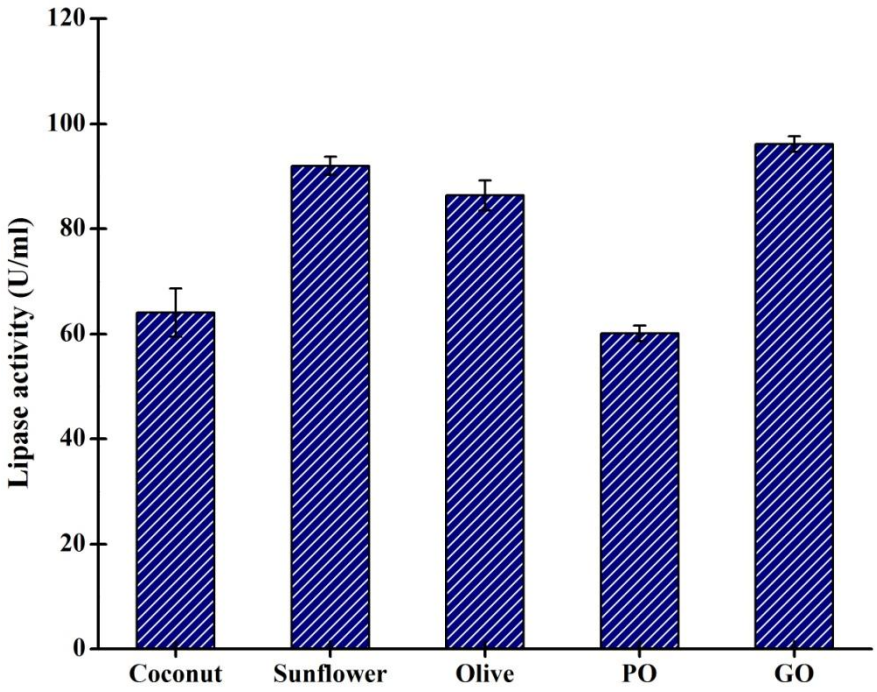

Figure 23. Suitability of various vegetable oils (0.5 %) as inducer for the production of lipase was analysed by supplementing the basal medium with vegetable oils such as groundnut oil, sunflower oil, olive oil, palm oil and coconut oil. At 12 h of incubation, groundnut oil showed the highest production of lipase (96.15 U/ml), compared to other vegetable oils.

Statistical optimisation of lipase production

The Plackett-Burman experimental design - a fractional factorial design - was used in this study to demonstrate the importance of some factors on the production of lipase by *Pseudomonas* sp. BUP6. Initially, 5 different physical parameters, *i.e.*, temperature, pH, substrate concentration, incubation time, agitation were considered (**Table 14**).

Groundnut oil, which supported the highest production of lipase, was supplemented to the medium as inducer for lipase production. A set of 22 experiments were performed and Pareto chart was generated to determine the significant parameters which influenced the production of lipase by *Pseudomonas* sp. BUP6 (**Table 17**). The standardised effects of each parameter showed that temperature, agitation and pH could influence the production of lipase significantly (**Figure 24**).

Table 17. Plackett-Burman matrix with the respective responses in terms of lipase activity.

Run Order	Temperature (^{o}C)	pH	Substrate (%)	Incubation (h)	Agitation (rpm)	Lipase (U/ml)
1	40	5	2.0	48	50	6.04
2	40	7	0.1	48	200	6.14
3	34	7	2.0	12	200	111.2
4	34	5	2.0	48	50	27.2
5	40	5	0.1	48	200	1.86
6	40	7	0.1	12	200	15.58
7	40	7	2.0	12	50	0.85
8	40	7	2.0	48	50	22.91
9	34	7	2.0	48	200	58.64
10	40	5	2.0	48	200	2.67
11	34	7	0.1	48	200	89.47
12	40	5	2.0	12	200	1.14
13	34	7	0.1	48	50	62.96
14	34	5	2.0	12	200	12.89
15	34	5	0.1	48	50	27.09
16	34	5	0.1	12	200	93.9
17	40	5	0.1	12	50	0.08
18	40	7	0.1	12	50	0.57
19	34	7	2.0	12	50	8.38
20	34	5	0.1	12	50	33.19
21	37	6	1.05	30	125	74.23
22	37	6	1.05	30	125	76.66

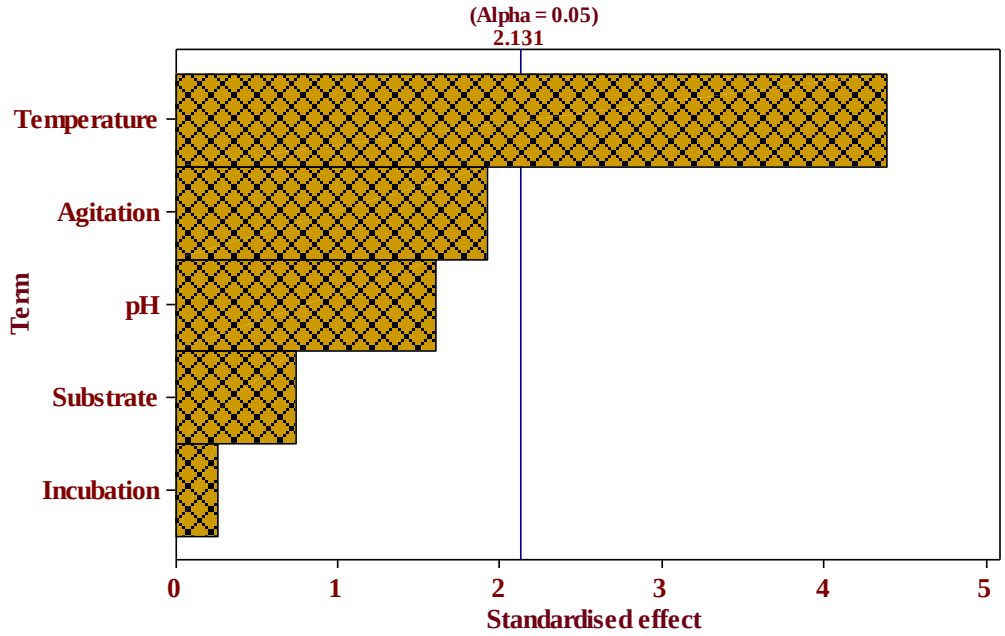

Figure 24. Pareto chart showing the effect estimates of temperature, pH, agitation, substrate concentration and incubation time on the production of lipase (Minitab version 14).

Three significant parameters (temperature, agitation and pH) were subsequently considered for Box-Behnken analysis and response surface methodology to estimate optimum combination of these parameters for maximising the production of lipase. A set of 15 experiments were performed according to Box-Behnken design (**Table 18**), and the results showed that the predicted and experimental values for lipase activities did not show significant difference, that the determination coefficient (R^2) value was 0.9903, *i.e.*, close to unity. A second order polynomial function was fitted to the experimental lipase activity, which resulted in the following regression equation,

$$Y = -10751.5 + 386.27X_1 + 10.3X_2 + 810.43X_3 + 0.02X_1X_2 + 0.08X_2X_3 - 10.22X_1X_3 - 4.31X_1^2 - 0.032X_2^2 - 32.23X_3^2$$

The quadratic regression equation was used to generate two dimensional contour as well as three dimensional surface plots to depict the interactive effects of the selected parameters (**Figure 25-30**).

Table 18. Box-Behnken design matrix with the respective responses in terms of lipase activity.

Run Order	Temperature	Agitation	pH	Observed lipase activity (U/ml)	Predicted lipase activity (U/ml)
1	39.5	150	5	2.02	3.39
2	39.5	175	6	94.30	91.62
3	37	175	7	114.47	121.79
4	42	200	6	16.52	19.19
5	39.5	200	5	0.90	5.54
6	42	150	6	4.37	10.32
7	42	175	5	1.52	1.799
8	39.5	175	6	91.43	91.62
9	37	175	5	0.60	1.91
10	37	150	6	74.43	71.75
11	37	200	6	81.30	75.35
12	42	175	7	13.23	11.91
13	39.5	150	7	72.73	68.09
14	39.5	200	7	79.77	78.41
15	39.5	175	6	89.11	91.62

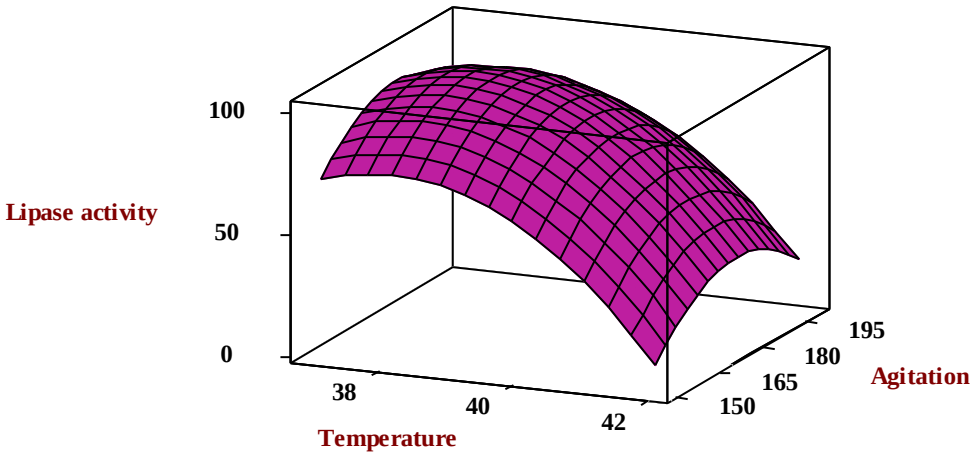

Figure 25. Three dimensional surface plot showing lipase activity (U/ml) *vs*. agitation (rpm) and temperature (^{o}C), at pH 6 (hold value).

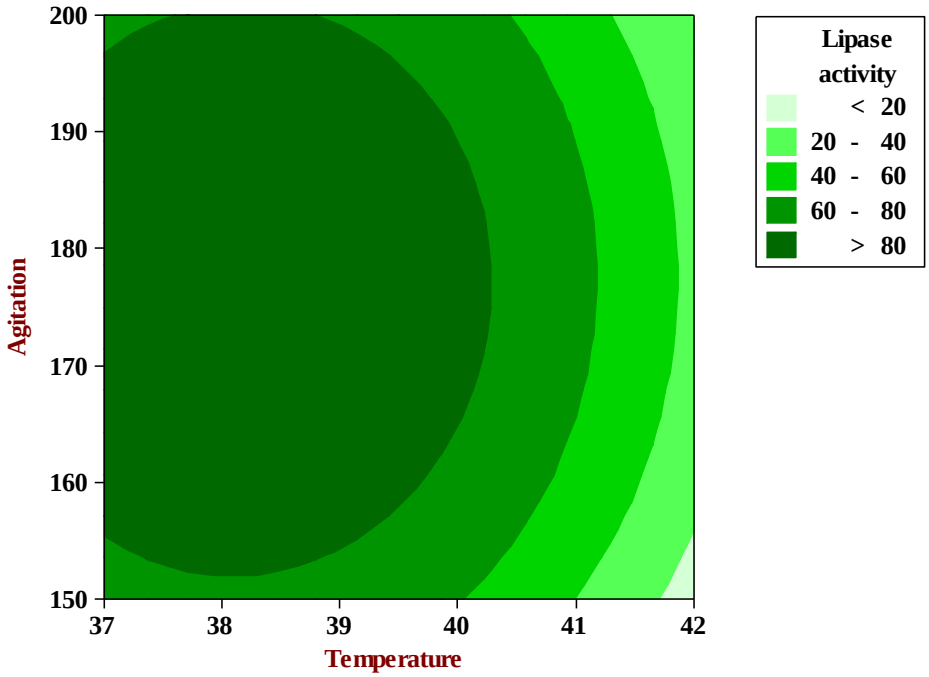

Figure 26. Contour plot showing lipase activity (U/ml) *vs*. agitation (rpm) and temperature (^{o}C), at pH 6 (hold value).

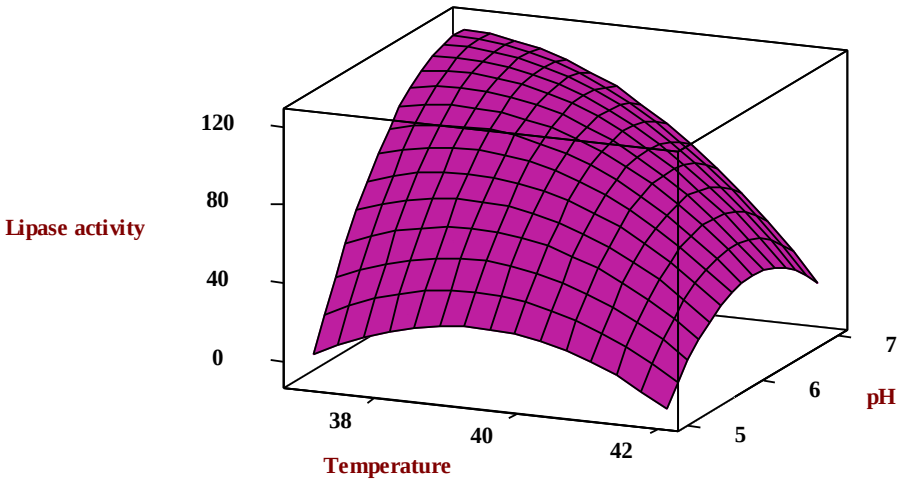

Figure 27. Three dimensional surface plot showing lipase activity (U/ml) *vs*. pH and temperature (^{o}C), at 175 rpm (hold value).

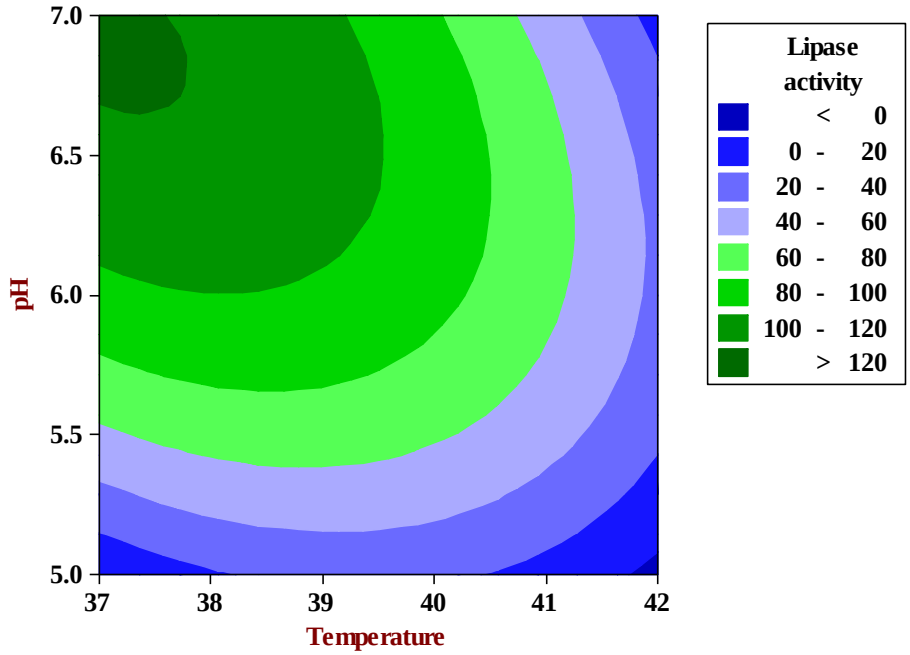

Figure 28. Contour plot showing lipase activity (U/ml) *vs*. pH and temperature (^{o}C), at 175 rpm (hold value).

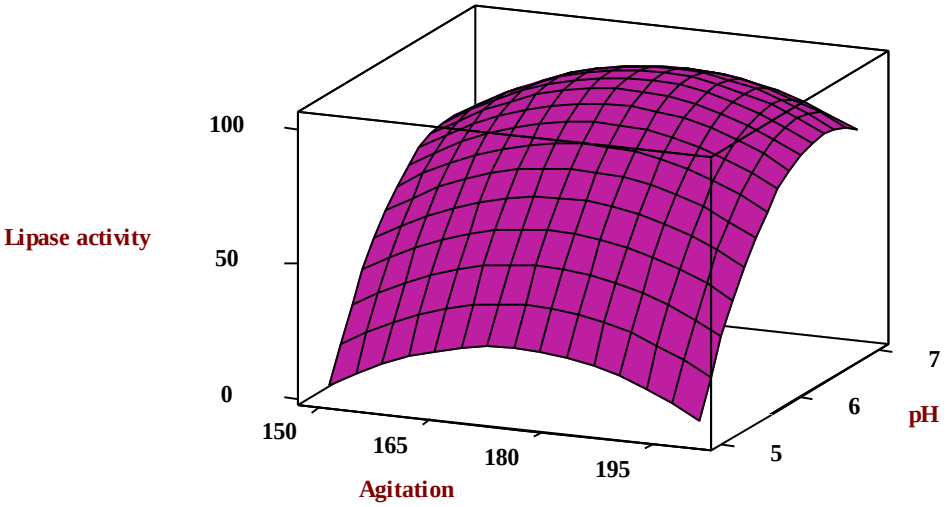

Figure 29. Three dimensional surface plot showing lipase activity (U/ml) *vs*. pH and agitation (rpm) at 39.5 ^{o}C (hold value).

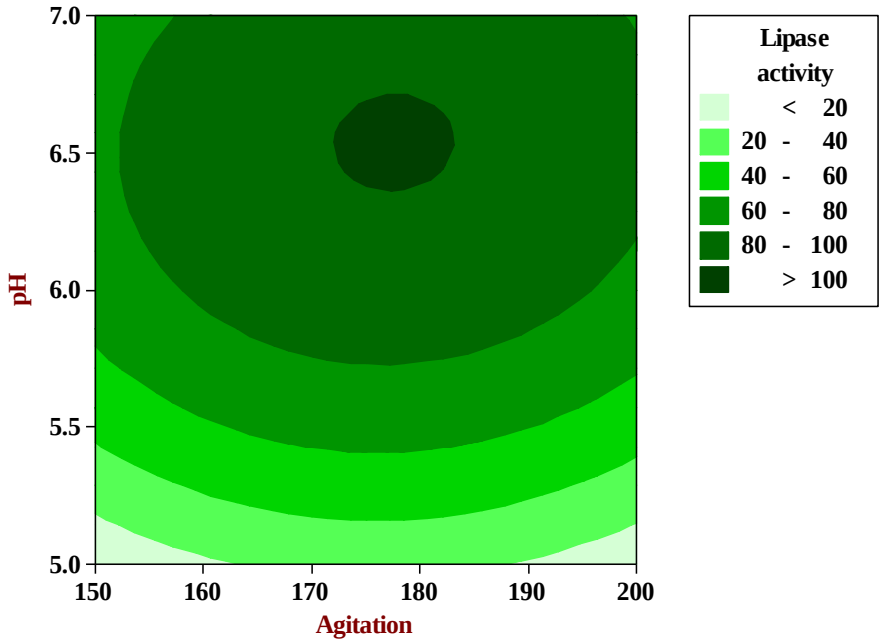

Figure 30. Contour plot showing lipase activity *vs*. pH and agitation (rpm) at 39.5 ^{o}C (hold value).

The summary of the analysis of variance (ANOVA) for the selected quadratic model is shown in **Table 19**. Based on these results, the model was utilised to generate response surfaces for the analysis of the variable effects on the production of lipase.

Table 19. Analysis of variance (ANOVA) for the fitted quadratic model of lipase activity.

Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	9	26137.5	26137.49	2904.17	56.75	0.000
Linear	3	16455.6	7752.97	2584.32	50.50	0.000
Square	3	7049.0	7048.97	2349.66	45.91	0.000
Interaction	3	2632.9	2632.93	877.64	17.15	0.005
Residual Error	5	255.9	255.89	51.18		
Lack-of-Fit	3	242.4	242.37	80.79	11.96	0.078
Pure Error	2	13.5	13.51	6.76		
Total	14	26393.4				

Three random experimental conditions (different from Box-Behnken, but within the range investigated) were evaluated for the validation of the model. In all these instances, model prediction was in good agreement with the experimental data (considering the experimental error), and correlation coefficient was found to be 0.989 (**Table 20**). Correlation coefficient was close to 1.0, suggesting the significance of the model. The optimum production of lipase was found to be 126 U/ml (at 37 °C, pH 6.9 and 200 rpm). Thus, the statistical optimisation resulted in 0.3 fold increase of lipase activity over the unoptimised condition, from *Pseudomonas* sp. strain BUP6.

Table 20. Random combinations of parameters selected for the validation of proposed statistical model with respective predicted responses in terms of lipase activity.

Run No.	Temperature (^{o}C)	Agitation (rpm)	pH	Observed lipase activity (U/ml)	Predicted lipase activity (U/ml)
1	37	177	6.9	116.06	122.12
2	37	160	6.9	108.33	112.69
3	40	180	6.5	89.29	87.9
4	37	180	6.0	91.6	96.2

Native PAGE

Lipase activity of *Pseudomonas* sp. BUP6 was confirmed by polyacrylamide gel electrophoresis. Lipase reacts with oleic acid esters in the reaction mixture to release free fatty acids which in turn precipitated as calcium oleate. The white zone observed on the gel confirmed the activity of lipase (**Figure 31**).

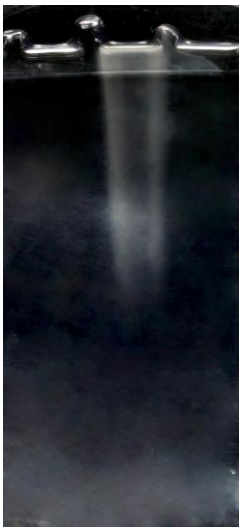

Figure 31. Native PAGE profile of lipase produced by *Pseudomonas* sp. BUP6 on 8 % polyacrylamide gel showing the white colored zone of calcium oleate precipitates.

Discussion

This study investigated the production and statistical optimisation of lipase by *Pseudomonas* sp. BUP6. Initially, the lipase activities of *Pseudomonas* sp. BUP6 was analysed in the presence of various vegetable oils (coconut, olive, palm, sunflower and groundnut) as inducer, among which groundnut oil supported the maximum production of lipase. The process parameters were statistically optimised for enhancing the production of lipase employing the principles of Plackett-Burman and Box-Behnken designs. Lipase produced under the optimised condition (37 ^{o}C, 200 rpm and pH 6.9) was analysed qualitatively by native PAGE.

Pseudomonas sp. strain BUP6 did not show detectable production of lipase in the absence of oils in the semi-synthetic medium used in this study; hence oil was supplemented to it to induce lipase production. The culture was gradually adapted to the oil-containing medium. Most of the published experimental data showed that lipid as carbon sources (especially natural oils) stimulate lipase production (Benjamin and Pandey, 1996; He and Tan, 2006; Kaushik *et al.*, 2006). It is evident from this study that the newly designed basal salt medium is quite suitable for the production of lipase as it supported the maximum lipase activity at early hours of incubation. *P. aeruginosa* KM110, isolated from the wastewater of an oil processing plant produced 0.76 U/ml of lipase at 24 h of incubation, utilising olive oil as substrate (Mobarak-Qamsari *et al.*, 2011). Using palm oil as inducer, *Staphylococcus* sp. isolated from oil contaminated soil produced 25 U/ml of lipase at 48 h of incubation (Sirisha *et al.*, 2010). In 2 % of castor oil supplemented medium, *P. aeruginosa* KKA-5 showed 5 U/ml of lipase activity at 100 h of incubation (Sirisha *et al.*, 2010). Compared to these cultures, *Pseudomonas* sp. described in this study is highly promising and suitable for industrial applications.

Statistical designs, Plackett-Burman followed by Box-Behnken were commonly used for the optimisation of enzyme production parameters as they evaluate the interactive effects of physico-chemical parameters, which are being neglected in the convensional *one-at-a-time* strategy. Moreover, statistical designs are generally less laborious and time consuming as they demand minimum number of experimental runs (Benjamin and Pandey, 1996; Ji *et al.*, 2010); hence such methodologies were adopted in the present study also. Use of Plackett-Burman design for estimating the significant parameters reduced the number of parameters from 5 to 3; thereby reduced the number of experimental trials for RSM analysis. Box-Behnken design with 3 relevant factors was performed for fitting in to second order response surfaces. The R^2 value of 0.99 indicated the accuracy of the model, its closeness to 1 indicates the good correlation between the experimental and predicted values. Thus, the statistical optimisation of process parameters resulted in the increase in the production of lipase by 30 % (126 U/ml at 37 °C, pH 6.9 and 200 rpm) as against the unoptimised condition. Similar strategies *i.e*., Plackett-Burman and Box-Behnken designs were employed to optimise the nutrient sources for maximising the lipolytic activities of *Psychrobacter* sp. ArcL13 which resulted in an enhanced lipase activity of 10.7 U/ml (21 fold increase) (Kim *et al.*, 2015). Likewise, a determination coefficient (R^2) of 0.99 was observed when the process parameters were optimised statistically for the production of lipase by *Burkholderia* sp. using 2 - level factorial design and RSM (Liu *et al.*, 2006). Thus, the study provides a practical basis for the large scale production of lipase by *Pseudomonas* sp. BUP6.

Native or non-denaturing electrophoresis is an important tool for analysing the quality of a functional enzyme. For the detection of lipolytic activities, it is a common practice to impregnate or overlay hydrophobic triglycerides on native polyacrylamide gels. For instance, Rejitha *et al*. (2012) used tributyrin impregnated native gel for visualising the hydrolytic activities of lipase (as

clear zone) produced by *Bacillus* sp. MPTK 912. In this study, a hydrophobic semi-solid substrate containing olive oil and $CaCl_2$ was layered over the gel. Lipase produced by *Pseudomonas* sp. BUP6 migrated through the native gel and produced an opaque zone due to the hydrolysis of olive oil, followed by its precipitation as calcium oleate - which confirmed the lipolytic acivity.

Conclusions

Pseudomonas sp. strain BUP6, isolated from the rumen of Malabari goat produced lipase using ground nut oil as inducer. The extracellular lipase activities of *Pseudomonas* sp. strain BUP6 is comparable to that of many bacteria isolated from harsh environments such as soil or industrial wastes. Moreover, the newly designed basal salt medium is found promising for industrial applications.

Chapter *5*

Purification and characterisation of lipase from *Pseudomonas* sp. BUP6

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Aim and rationale

This study aims at the purification and characterisation of lipase produced by *Pseudomonas* sp. BUP6. Understanding on the kinetics of catalysis of enzymes enables to assess the enzyme physiology as well as its functions *in vivo.*

Introduction

Lipases or triacylglycerol hydrolases (EC 3.1.1.3) are ubiquitous enzymes that hydrolyse the esters of glycerol and long-chain fatty acids. Lipases are produced by several microorganisms belong to bacteria, yeast and fungi, as well as animals and plants. Microbial lipases are widely used in industries owing to its versatile catalytic activities and ease of production/manipulation (Benjamin and Pandey, 1996). In addition to hydrolysis, lipases synthesise esters from glycerol and long chain fatty acids at low water activity, *i.e.*, lipases can catalyse esterification, inter-esterification and *tran*-sesterification reactions in non-aqueous media (Pandey *et al.*, 1999). The versatility in activities and the enantio- and regio- selectivities make lipase a suitable choice of biocatalyst in many industries involved in organic syntheses, hydrolysis of fats and oils, resolution of chiral drugs, modification of fats, flavor enhancement and chemical analyses (Benjamin and Pandey, 1998). In most of the areas, lipases are required as a homogenous preparation with a certain degree of purity (Saxena *et al.*, 2003). Hence, the purification of lipase is a necessary accomplishment prior to industrialisation.

Enzymes are relatively labile molecules to various factors, and most of them are denatured by foaming, heating, drying, and by concentrated acids or bases. Moreover, the target enzyme to be purified encompasses only a small percentage of the total protein in the crude extract. Hence, purification of

enzyme is generally considered as a laborious process. Moreover, the purified enzyme has to be characterised further to explore its possible industrial applications. Upon the aforesaid background, this study focused on the purification and characterisation of lipase from basal salt medium, produced by *Pseudomonas* sp. BUP6.

Materials and methods

Purification of lipase

Ammonium sulphate precipitation

Pseudomonas sp. BUP6 was cultivated in basal salt medium (**Table 13**) supplemented with groundnut oil under optimised conditions (at 37 °C, pH 6.9 and 200 rpm). The culture broth was centrifuged at 9,400 × *g* for 10 min at 4 ^{o}C, and the supernatant was collected. Solid $(NH_4)_2SO_4$ was slowly added to this supernatant so as to reach 20 % saturation initially, and the precipitate was collected by centrifugation (9,400 × *g* for 10 min at 4 ^{o}C). The supernatant was further precipitated to 20-60 % and 60-80 % saturation, and the precipitate was collected separately. Addition of $(NH_4)_2SO_4$ was carried out with continuous stirring in a cold chamber (at 5 °C) for 1 h. The pellets were re-suspended in a minimum volume of 50 mM Tris-HCl buffer (pH 8.0) for dialysis.

Dialysis

The precipitates (pellets) obtained after $(NH_4)_2SO_4$ fractionation were individually enclosed in cellulose membrane tubes, and dialysed against 50 mM Tris-HCl buffer (pH 8.0) for 24 h at 4 ^{o}C with continuous stirring with two buffer changes in between. After that the dialysate was centrifuged (9,400 × *g* for 10 min at 4 ^{o}C), and the supernatant so obtained was subjected to gel permeation chromatography.

Gel permeation chromatography

The dialysate with the highest specific activity was used for gel permeation chromatography. Gel permeation chromatography was performed in column packed with sephadex G100 (Sigma Aldrich, USA), using peristaltic pump (Rivera, India) at a flow rate of 2 ml/20 min.

Protein estimation

The protein content was estimated after every steps of purification using Lowry's (Lowry *et al.*, 1951) method with bovine serum albumin (BSA) as the standard.

Reagents

- Reagent A: 2 % Na_2CO_3 in 0.1 N NaOH
- Reagent B: 500 mg $CuSO_4$ in 1 % Rochelle salt solution
- Reagent C (alkaline copper solution): 50 ml of Reagent A + 1 ml of Reagent B
- Folin-phenol reagent: Commercial Folin-phenol reagent was used after dilution in a 1:1 ratio with ddH_2O.

Procedure

- Pipetted out 0.5 ml of the lipase in the test tube and made up to 1.0 ml with 0.1 N NaOH. Added 5.0 ml of alkaline copper reagent. Vortexed well and allowed to stand for 10 min.
- Added 0.5 ml of Folin's reagent, vortexed well and incubated at 25 ^{o}C for 30 min.
- Read the absorbance at λ_{660} using spectrophotometer.
- Calculations were done using the graph generated from the standard graph of BSA.

Standard BSA graph

- 1 mg/ml stock solution was prepared with BSA.
- Different aliquots of stock solution (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45 and 0.50 µl) were pipetted in to the test tubes and made up to 1.0 ml with ddH_2O, 5.0 ml of alkaline copper reagent was added subsequently; vortexed well and allowed to stand for 10 min.
- 0.5 ml of Folin's reagent was added, vortexed well and incubated at 25 ^oC for 30 min.
- The absorbance was measured at λ_{660} using spectrophotometer.

The optical density was measured at λ_{660} and plotted against the concentration of BSA.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

After each purification step, the purity of lipase was confirmed by SDS-PAGE. SDS-PAGE was performed using a vertical mini gel (8 × 7 cm) slab with notched glass plate system. Gels of 1.5 mm thickness were prepared for the entire study (Laemmli, 1970).

The SDS-PAGE was carried out using 4 % stacking gel (**Table 15**) and 12 % separating gel (**Table 21**). Lipase solution and sample buffer (**Table 22**) were mixed in the 1:1 ratio. The contents were mixed well in a pre-sterilised Eppendorf tube and heated in a boiling water bath for 3 min. Broad range protein molecular weight marker (Genei, Banglore) containing myosin (205 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa) aprotinin (6.5 kDa) and insulin (3.5 kDa) was used for the determination of the MW of protein on the gel. After the electrophoresis, the gel was stained with 0.1 % coomassie brilliant blue G-250 (prepared in a mixture of 50 % methanol and 10 % glacial acetic acid). The destaining

solvent system contained 10 % glacial acetic acid: 45 % methanol: 45 % ddH_2O. The protein bands on the SDS-PAGE gels were visualised and photographed.

Table 21. Ingredients required for resolving (12 %) gel

Ingredients	**Quantity (ml)**
ddH_2O	3.3
30 % acrylamide	4.0
1.5 M Tris buffer (pH, 8.8)	2.5
10 % SDS	0.1
10 % APS	0.1
TEMED	0.004

Table 22. Ingredients for sample buffer

Ingredients	**Quantity (10 ml)**
0.6 M Tris buffer (pH, 6.8)	1.0 ml
SDS	0.1 g
Sucrose	1.0 g
β-mercaptoethanol	0.05 ml
10 mM bromophenol blue	1.0 ml
20 % glycerol	0.4 ml

Made up to 10.0 ml with ddH_2O and stored in a refrigerator.

Confirmation by MALDI-TOF/MS

The prominent lipase band on SDS-PAGE was excised, destained and digested with trypsin. The peptides so obtained were analysed by matrix assisted lazer desorption ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonics, USA) available at the mass spectrometry and proteomics core facility in Rajiv Gandhi Centre for

Biotechnology, Thiruvananthapuram, Kerala. The peptides were identified by searching the peptide mass list against the protein database of National Centre for Biotechnology Information, USA using the software Mascot Search.

Characterisation of lipase

The lipase active fraction obtained after gel permeation chromatography was used for its characterisation studies. In order to identify the characteristics of lipase, *i.e.*, effects of pH, temperature, substrate (*p*-NPP) concentration, different metal ions (Ca^{2+}, K^{+}, Mg^{2+}, Na^{+} and Zn^{2+}), detergents (triton X-100, tween 20, tween 80) and modifiers [ethylenediamine tetra-acetic acid (EDTA), β-mercaptoethanol] on lipase activity were studied. The effects of these parameters on lipase activity were expressed in percentage of activity to its initial activity as the base unit.

Effect of pH on activity and stability of lipase

The optimum pH for lipase activity was determined by measuring its activities in 50 mM phosphate buffer (pH 4.0, 5.0, 6.0, 7.0, 7.4, 7.6 or 7.8), 50 mM Tris-HCl buffer (pH 8.0, 8.2, 8.5 or 9.0) and 50 mM carbonate-bicarbonate (pH 10.0) at 37 ^{o}C for 30 min incubation. Stability was measured by pre-incubating lipase at three different pH levels (7, 8 and 9) for 1 to 5 h, and the activity was measured subsequently. Residual activity was determined in relation to the lipase activity at zero h of pre-incubation at the respective pH.

Effect of temperature on activity and stability of lipase

The optimum temperature for lipase activity was determined by measuring the enzymatic activities in 50 mM Tris-HCl buffer (pH 8.2) at different temperatures, *i.e.*, 25, 30, 35, 40, 43, 45, 48, 50, 55 and 60 ^{o}C for 30 min incubation. Stability was measured at by incubating lipase at three different

temperatures (40, 45 and 50 ^{o}C) for 1 to 5 h; thereafter the residual activity was determined at the respective temperatures.

Effect of different detergents and modifiers on lipase activity

For determining the effect of different detergents and modifiers on lipase activity, the purified lipase was incubated with the reaction mixture containing tween 20, tween 80, triton-X100, EDTA or β-mercaptoethanol at different concentrations (0.25, 0.5 and 1.0 %) at 45 ^{o}C and pH 8.2 for 30 min, thereafter the relative activity was determined.

Effect of different metal salts on lipase activity

Effects of various metal ions on lipase activity was determined by incubating the reaction mixture with different metal salts, *i.e.*, Mg^{2+}, Ni^{2+}, Ca^{2+}, Zn^{2+}, Fe^{3+} or Cu^{2+} to a final concentration of 0.5, 1.0, and 1.5 mM at 45 ^{o}C and pH 8.2 for 30 min incubation.

Calculation of *Km* and *Vmax*

The lipase solution was treated with *p*NPP at a concentration of 10, 20, 40, 50, 60, 80 and 90 mM. The reaction mixture was incubated for 1 h at 45 ^{o}C (pH 8.2) during which the lipase activity was measured at regular intervals of 5 min. The *Km* and *Vmax* values were calculated for lipase using the software, Hyper 32.

Results

Purification of lipase

Of various $(NH_4)_2SO_4$ fractions, 20-60 % fraction showed the maximum lipase activity, which was of 16.9 folds purified with 24.8 % yield (**Table 23**). This fraction (20-60 %) was subjected to gel permeation chromatography. Thirty two fractions were collected at a flow rate of 2 ml/20 min, and the

optical density was measured at λ_{280} which showed a major peak represented by the fraction numbers 10 to 12 (**Figure 32**). The purification fold of sephadex-G100 fraction of lipase was 35.7 with yield of 14.9 % (**Table 23**). The lipase active fraction after each purification step was subjected to SDS-PAGE for purity check. From SDS-PAGE pattern, the apparent MW of the partially purified lipase was estimated as 35 kDa (**Figure 33**).

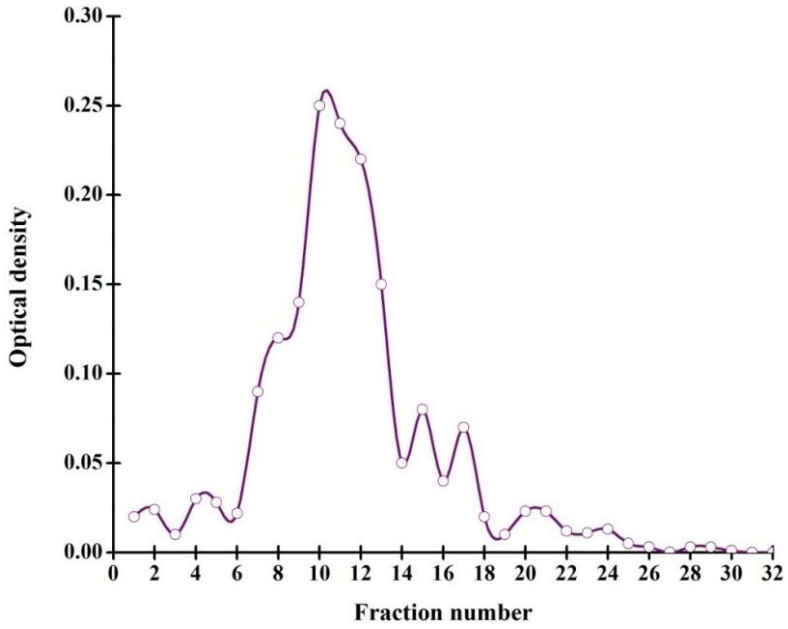


Figure 32. Elution profile of lipase by sephadex G-100 chromatography (2 ml/20 min), in which fraction numbers 10 to12 showed the maximum concentration and purity.

Table 23. Summary of the purification lipase

Purification step	Total protein (mg)	Total lipase activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude	263	27947.3	106.3	100	1
$(NH_4)_2SO_4$ (40-60 %)	3.86	6926.4	1794.4	24.8	16.9
Sephadex-G100	1.098	4166.1	3794.2	14.9	35.7

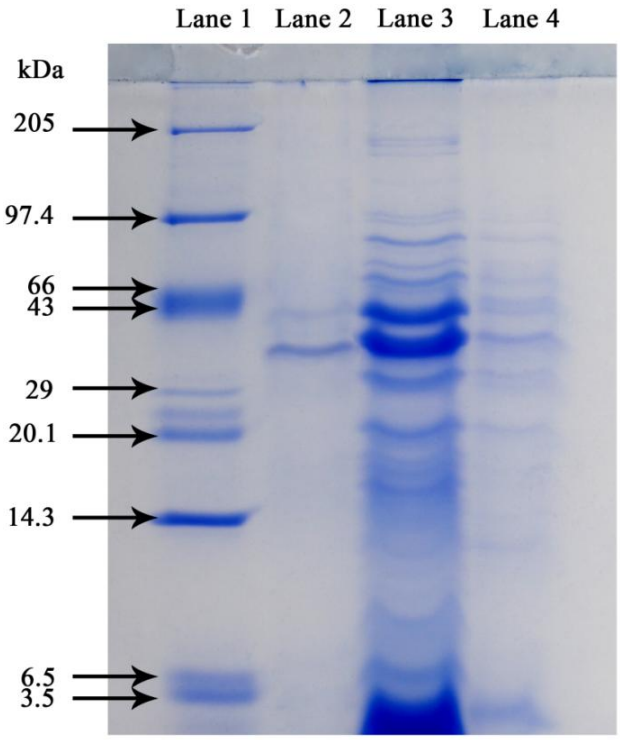


Figure 33. SDS-PAGE profile of lipase from *Pseudomonas* sp. BUP6, showing the purity. Lanes: (1). standard protein molecular weight marker; (2). sepadex G100 column purified lipase showing apparent molecular weight of 35 kDa; (3). 40-60 % $(NH_4)_2SO_4$ fraction; and (4). crude protein harvested (growth conditions: 37 °C, pH 6.9 and 200 rpm).

Confirmation by MALDI-TOF/MS

Protein mass fingerprinting was carried out for the trypsin digested peptides and the resultant list of MWs of peptides (**Figure 34**) was compared with the Swissprot protein database using the software Mascot. The peptide sequences of lipase produced by *Pseudomonas* sp. BUP6 showed similarity of 24 % with that of lipase from *Pseudomonas putida* KT2440 with MW 34.2 kDa (**Figure 35**).

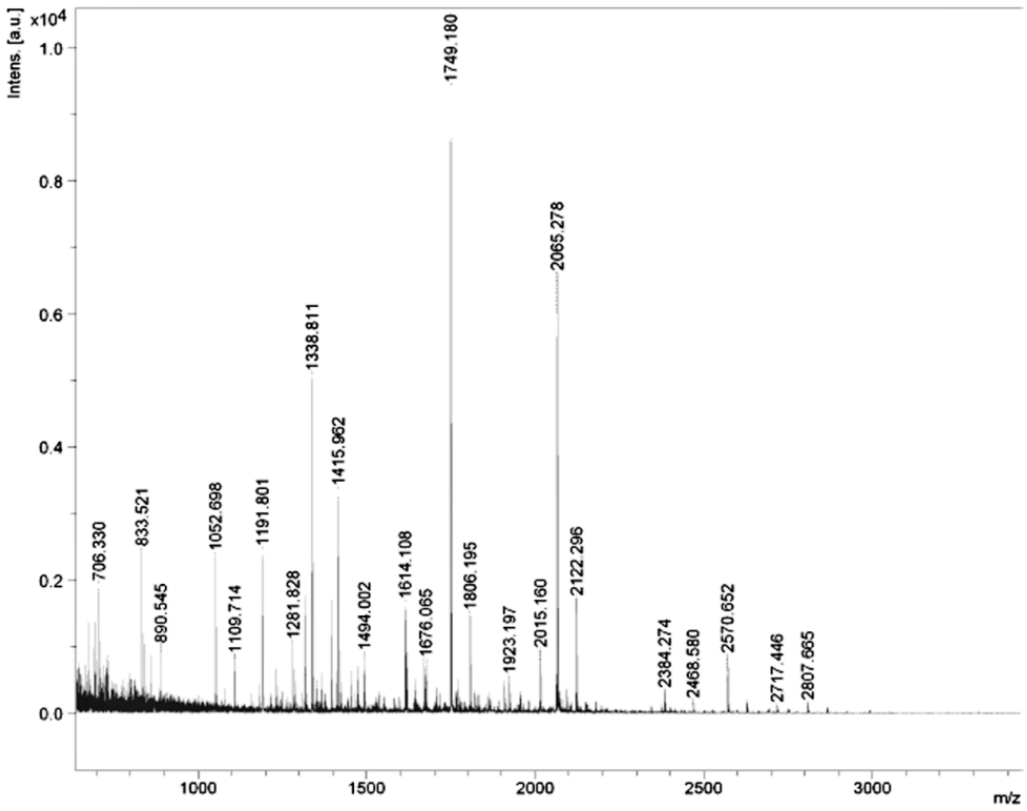


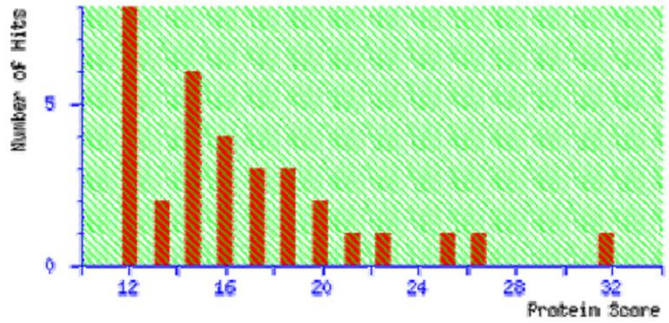
Figure 34. Mass spectrum of trypsin digested peptides of purified lipase from *Pseudomonas* sp. BUP6.

Mascot Search Results

```
User            : baska
Email           : baskar@gmail.com
Search title    : sam
Database        : PSEUDO_LIPASE_NCBI NCBI (15038 sequences; 5636367 residues)
Timestamp       : 17 Nov 2014 at 18:18:39 GMT
Top Score       : 12 for gi|24986618, lipase [Pseudomonas putida KT2440]
```

Mascot Score Histogram

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 54 are significant (p<0.05).



Concise Protein Summary Report

Format As | Concise Protein Summary | Help

Significance threshold p< 0.05 Max. number of hits AUTO

Re-Search All | Search Unmatched

1. gi|24986618 **Mass:** 34207 **Score:** 12
lipase [Pseudomonas putida KT2440]

Search Parameters

```
Type of search          : Peptide Mass Fingerprint
Enzyme                  : Trypsin
Fixed modifications     : Carbamidomethyl (C)
Variable modifications  : Oxidation (M)
Mass values             : Monoisotopic
Protein Mass            : Unrestricted
Peptide Mass Tolerance  : ± 0.7 Da
Peptide Charge State    : 1+
Max Missed Cleavages    : 1
Number of queries       : 54
Selected for scoring    : 12
```

Mascot: http://www.matrixscience.com/

Figure 35. Mascot search result of peptide mass finger print of purified lipase from *Pseudomonas* sp. BUP6.

Characterisation of purified lipase

Lipase active fraction obtained by sephadex G-00 gel permeation chromatography was used for the characterisation studies.

Effect of pH on lipase activity and stability

Effect of pH on lipase activity was measured at normal assay conditions using 50 mM *p*NPP as substrate (37 ^{o}C; 30 min) with varying pH. Purified lipase was active at pH range of 7 to 9 with optimum activity at pH 8.2, which showed a relative activity of 102 % (707.1 ± 9.1 U/ml). However, the activity was inhibited at acidic pH (**Figure 36**). *Pseudomonas* sp. BUP6 lipase maintained more than 90 % of the initial activity at pH 9, which was found stable for 1 h; which clearly indicates the alkaline nature of lipase (**Figure 37**).

Effect of temperature on lipase activity and stability

Purified lipase was active in the 35 to 50 ^{o}C range with optimum activity (891.4 ± 8.7 U/ml) at 45 ^{o}C. At this optimum temperature, lipase showed 128 % of the initial activity with stability for 4 h (**Figure 38**). Even at 50 ^{o}C, lipase found active for 2 h, thereafter the activity was decreased considerably (**Figure 39**).

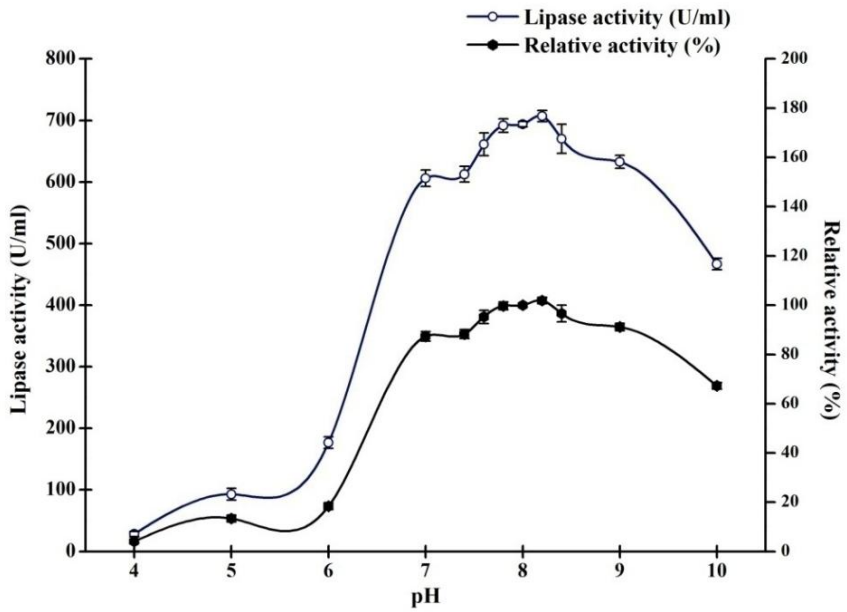


Figure 36. Effect of pH on lipase activity, showing the alkalophilic nature of lipase (pH 7-9). The maximum activity (707.1±9 U/ml) was observed at pH 8.2 with the relative activity of 102 %.

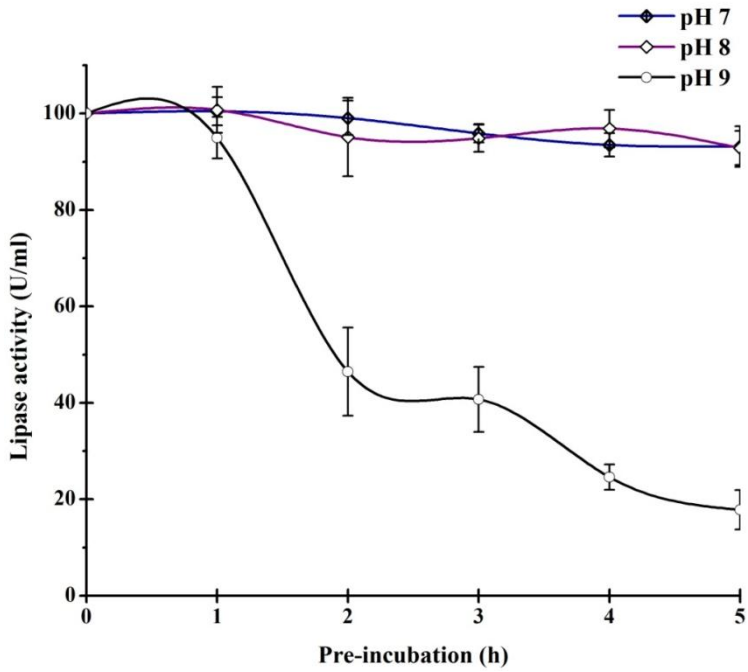


Figure 37. Stability of lipase was analysed at pH 7, 8 and 9. At pH 7 and 8, lipase retained 90 % of activity even after 5 h; whereas at pH 9, 50 % of activity was lost after 2 h of incubation.

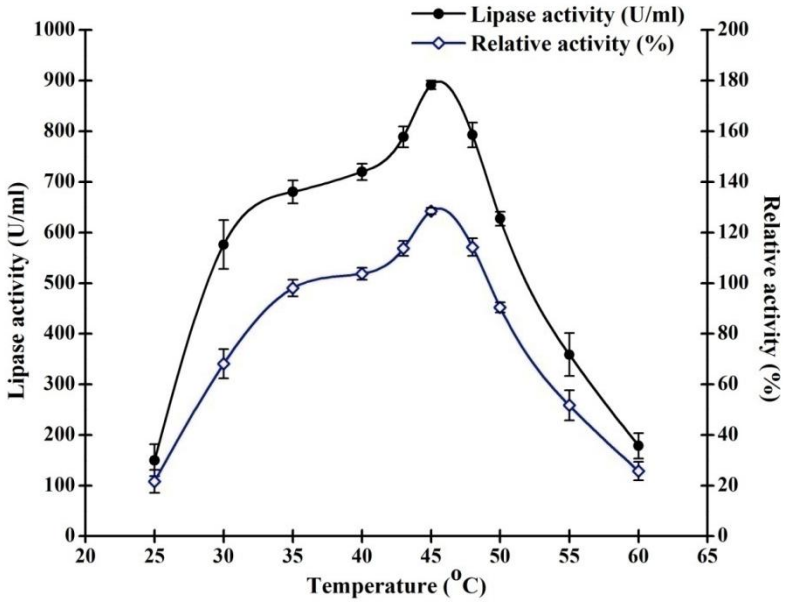


Figure 38. Effect of temperature on the activity of lipase showed its thermotolerent nature. Lipase was found active at temperature ranging from 35 to 50 ^{o}C with optimum activity (891.4±9 U/ml) at 45 ^{o}C and the relative activity of 128 %.

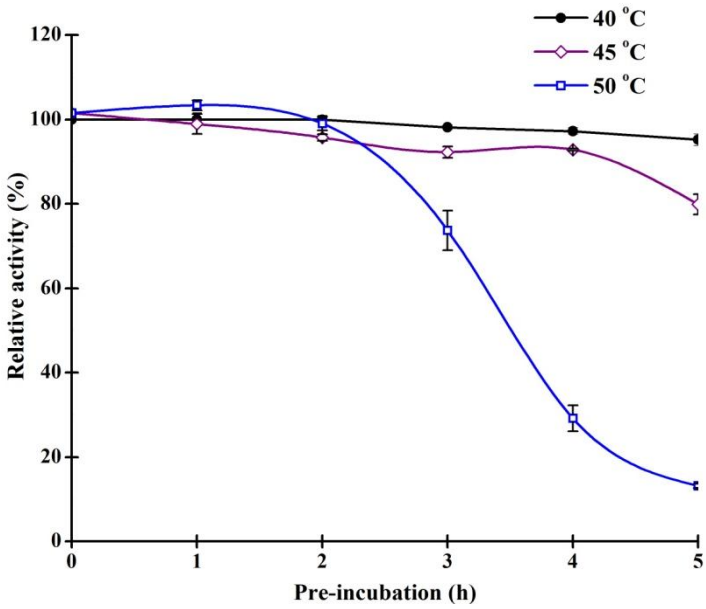


Figure 39. Stability of lipase was analysed at three different temperatures, 40, 45 and 50 ^{o}C.

Effect of detergents and modifiers on lipase activity

Tween 20 and tween 80 inhibited the activity of lipase, *i.e.*, 18.6 % (129 ± 0.7 U/ml) and 15.6 % (109 ± 0.9 U/ml), respectively compared to the relative activities; whereas it was active in the presence of triton X-100 (0.5 %) as under normal assay condition, but lower or higher concentration of triton X-100 (from 0.5 %) inhibited the activity. EDTA exhibited no effect on lipase activity, whereas *β*-mercaptoethanol (ME) inhibited the activity of lipase significantly, *i.e.*, 1.2 % (8 ± 0.8 U/ml) (**Figure 40**).

Effect of metal ions on lipase activity

Lipase activity was enhanced by the addition of Ca^{2+}, Ni^{2+}, and Mg^{2+}; the maximum activity was obtained at 1.0 mM concentration of Ca^{2+} (1428 ± 48.8 U/ml), which was 206 % of the initial activity. However, other three metals, Zn^{2+}, especially, Cu^{2+} and Fe^{2+} were found toxic to lipase, and reduced the activity significantly (**Figure 41**).

Optimised condition

The optimised condition for the activity of lipase produced by *Pseudomonas* sp. BUP6 was pH 8.2, 45 ^{o}C, and 1.0 mM Ca^{2+}, at which the relative activity was enhanced to 206 % against the initial activity under standard conditions (pH 8.0 and 37 ^{o}C) (**Figure 42**).

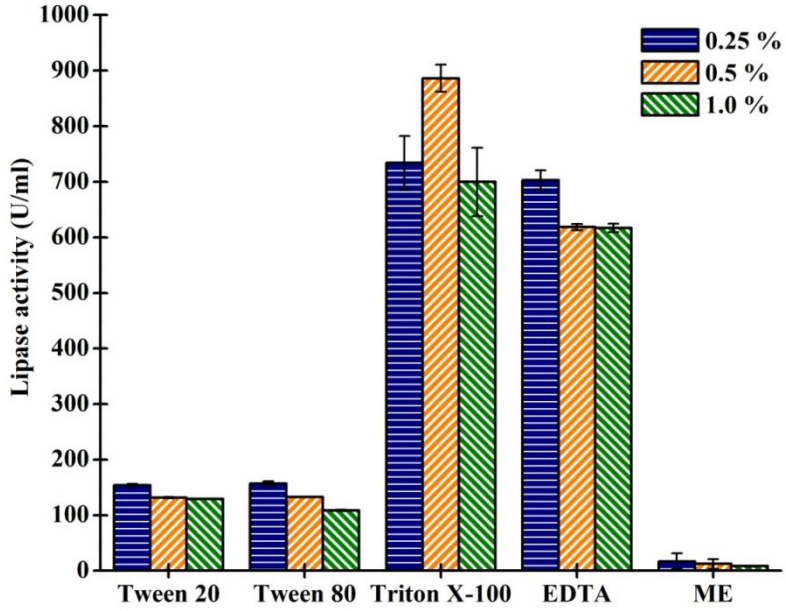


Figure 40. Effect of detergents and modifiers on lipase activity: Triton X-100 (0.5 %) and EDTA (0.25 %) maintained the activity of lipase; whereas tween 20, tween 80 and *β*-mercaptoethanol (ME) inhibited the activity significantly.

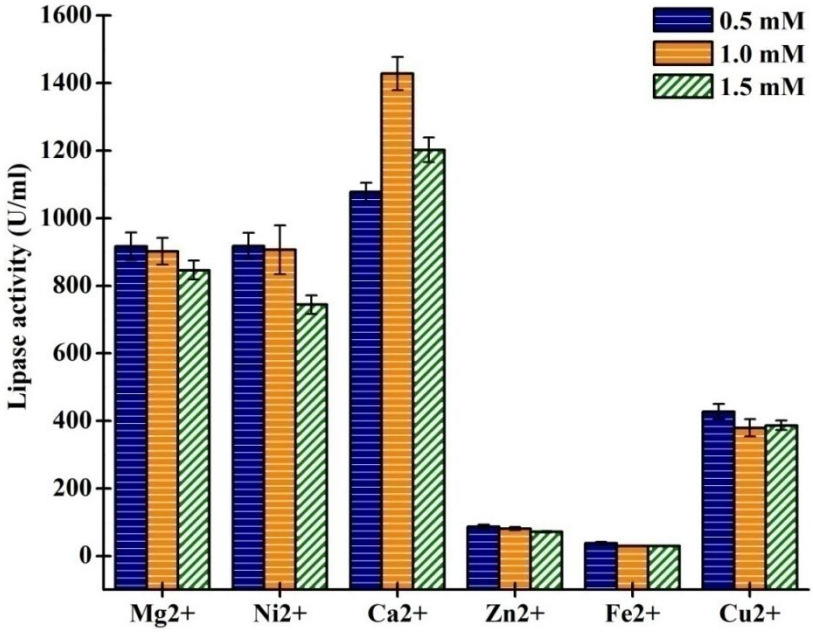


Figure 41. Effect of metal ions on lipase activity: Presence of Ca^{2+} (1.0 mM) in the reaction system enhanced the activity of lipase (1428±48.8 U/ml) by 206 % of the initial activity. Mg^{2+} and Ni^{2+} slightly enhanced the lipase activity; whereas Zn^{2+}, Fe^{2+} and Cu^{2+} reduced the activity significantly.

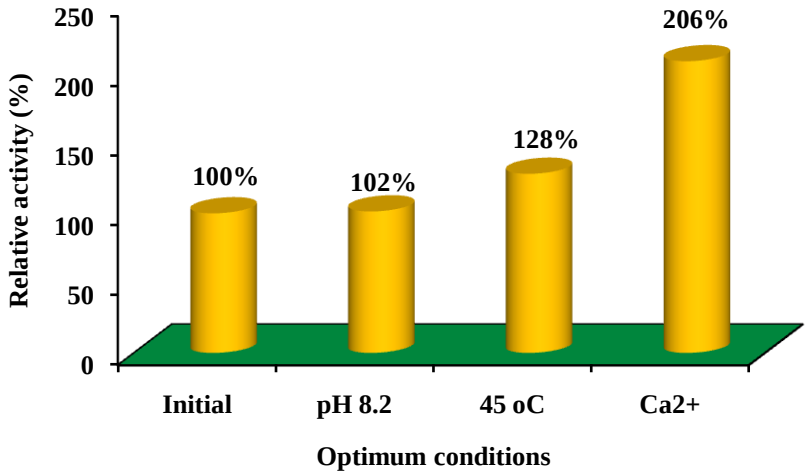


Figure 42. Summary of the activity of lipase produced by *Pseudomonas* sp. BUP6 upon characterisation. Optimum conditions for the maximum activity of lipase were found as pH 8.2, 45 ^{o}C, triton X-100 (0.5 %) and Ca^{2+} (1.0 mM).

Lipase kinetics

The kinetic parametres of the extracellular lipase were determined from Michaelis-Menten (**Figure 43**) and Line-Weaver Burk (**Figure 44**) plots. The *Km* and *Vmax* values of purified lipase were found as 11.6 mM and 668.9 μmol/min/mg, respectively.

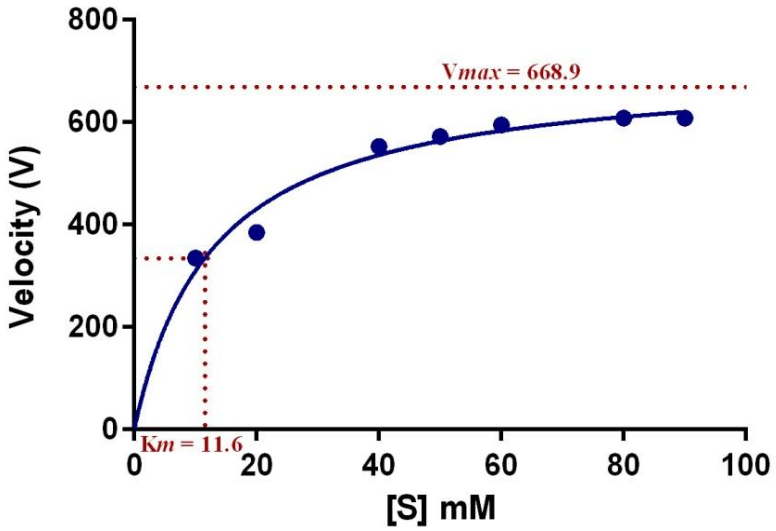


Figure 43. Michaelis-Menton plot of purified lipase indicating its kinetic parameters (*Km* 11.6 mM and *Vmax* 668.9 μmol/min/mg).

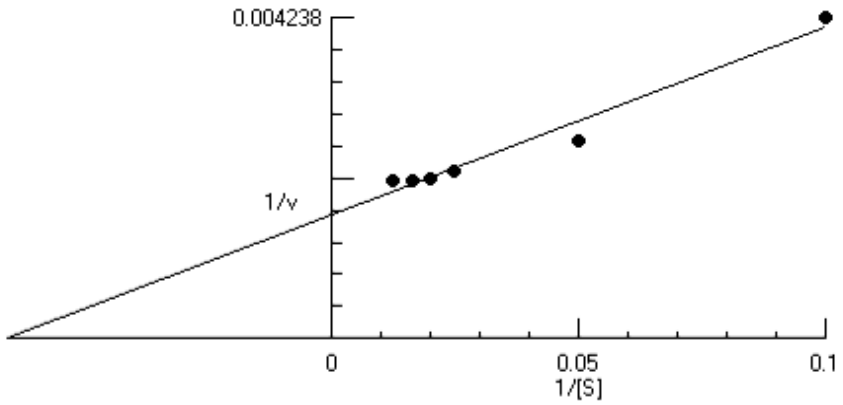


Figure 44. Line-Weaver Burk plot for the extracellular lipase produced by *Pseudomonas* sp. BUP6.

Discussion

The present study was focused on the purification and characterisation of lipase produced by the rumen bacterium, *Pseudomonas* sp. BUP6; which would give an idea on its performance, thereby exploring possible applications in industry. Purification was carried out by $(NH_4)_2SO_4$ salt precipitation, dialysis and sephadex G100 column chromatography. The purified lipase was characterised and subjected to MW determination; followed by stability with pH, temperature, detergents and modifiers.

The first objective of the study was to remove as much as unwanted proteins as possible from the crude extract without much disturbing lipase active fraction. The success of enzyme purification is usually analysed by two critical parameters, *i.e*., the yield and fold of purification. Generally, precipitation, ultrafiltration, gel exclusion chromatography were employed for the purification of extracellular enzymes (Palekar *et al.*, 2000). Employing $(NH_4)_2SO_4$ precipitation and chromatographic techniques, Ogino *et al*. (2007) purified an alkaline thermostable lipase produced by *Pseudomonas*

aeruginosa LST-03 to 34.7 folds with a yield of 12.6 %. An extracellular alkaline lipase from *P. aeruginosa* mutant was purified to homogeneity using acetone precipitation, followed by column chromatography; which resulted in 27 folds purification with 19.6 % final recovery (Bisht *et al.*, 2013). Likewise, $(NH_4)_2SO_4$ precipitation and gel exclusion chromatography were employed to purify lipases from *P. fragi*, *A. pullulans* HN2.3 and *Burkholderia multivorans* with a yield of 18, 12.6 and 0.96 % of the initial activity, respectively (Mencher and Alford, 1967; Liu *et al.*, 2008; Dandavate *et al.*, 2009). Similarly, in the present study, the extracellular lipase produced by *Pseudomonas* sp. BUP6 was efficiently purified by salt precipitation $(NH_4)_2SO_4$, dialysis and sephadex G-100 column chromatography, resulting in a yield of 14.9 % (35.7 folds purification).

From the SDS-PAGE profile, the MW of lipase produced by *Pseudomonas* sp. BUP6 was found as about 35 kDa, which was further confirmed by MALDI-TOF/MS. According to the literature, MW of lipase produced by species of *Pseudomonas* varies from 30 to 95 kDa. For instance, purified lipase produced by *Pseudomonas* sp. strain ATCC 21808 had a MW of 35 kDa, as judged by SDS-PAGE (Kordel *et al.*, 1991), whereas *P. aeruginosa* LX1 produced an extracellular lipase of MW 56 kDa (Ji *et al.*, 2010). The MW of purified lipase produced by *P. aeruginosa* AAU2 was found approximately 81.7 kDa (Bose and Keharia, 2013).

The *para*-nitrophenyl palmitate is one of the common substrates used for lipase assay. The activity of the lipase was analysed in different buffer systems with varying pH, *i.e.*, from 4 to 10, among which pH 8.2 was found as the optimum. Lipase from *Pseudomonas* sp. strain BUP6 remained suggestively active at pH 7 to 9, indicating that the alkaline condition favors the lipase activity. Similarly, the lipase withstood a temperature range of 35 to 50 ^{o}C with an optimum at 45 ^{o}C. Thus, the alkalophilic and thermotolerent

properties of the lipase reveal its possibilities of utilisation in many industries like detergency and tannery.

Of various metal ions tested, presence of Ca^{2+} in the reaction mixture stimulated or stabilised the lipase activity; whereas Mg^{2+} and Ni^{2+} slightly enhanced the activity; however, Zn^{2+}, Fe^{2+} and Cu^{2+} inhibited the activity with strongest inhibition by Fe^{2+}. These effects were at par with that of the lipase from *Pseudomonas* sp. AG-8 (Sharma *et al.*, 2001). The possible explanation for the effect is that some bacterial lipases possess a calcium binding pocket which stabilises its activity (Schrag *et al.*, 1997; Alquati *et al.*, 2002). Among the various detergents and modifiers tested, triton X-100 (0.5 %) favoured the lipase activity, but its higher/lower concentration decreased the activity. Presence of *β*-mercaptoethanol decreased the lipase activity drastically, which indicated the involvement of the disulphide bonds in stabilising the lipase. Moreover, EDTA exhibited no effects on lipase activity. Similar characteristics were exhibited by lipases produced by *P. aeruginosa* and *Geobacillus* sp. TW1 (Li and Zhang, 2005; Gaur *et al.*, 2008).

Km (11.6 mM) and *Vmax* (668.9 µmol/min/mg) values of the lipase produced by *Pseudomonas* sp. strain BUP6 was determined by Michaelis-Menten plot using *p*NPP as substrate. *Km* value is the measure of affinity of enzyme towards a substrate. Low *Km* value represents that the enzyme requires only small quantity of substrate to get saturated. High *Vmax* indicates the higher efficiency of the enzyme. *i.e.*, more substrate molecules are converted to product per unit time when the enzyme is fully saturated with the substrate. In general, the *Km* values of enzymes vary from 10^{-1} to 10^{-5} M (Fullbrook, 1996). Lipase from *P. cepacia* showed the *Km* and *Vmax* of 12 mM and 30 µmol/min, respectively (Pencreac'h and Baratti, 1996); lipase from *P. aeruginosa* PseA showed a *Km* value of 70.4 mM and *Vmax* of 2.24 µmol/min/mg (Gaur *et al.*, 2008), in both studies *p*NPP was used as the

substrate. It shows that lipase from *Pseudomonas* sp. BUP6 is more efficient than many other lipases reported from species of *Pseudomonas*.

Briefly, this study describes the purification and characterisation of alkaline and thermotolerent lipase (35 kDa) from the rumen bacterium, *Pseudomonas* sp. BUP6. The alkaline and thermotolerent nature of lipase offers potential applications in many industries like detergency and tannery. Moreover, it illustrates the industrial potentials of rumen microbes, which can be explored as the candidate of 'green technologies' with GRAS (generally regarded as safe) status in near future.

Conclusions

This study describes the purification of lipase from *Pseudomonas* sp. BUP6 by ammonium sulphate precipitation, dialysis and sephadex G100 column chromatography resulting in a final yield of 14.9 % with 35.7 fold purification. The purified lipase showed an apparent MW of 35 kDa on SDS-PAGE, which was further confirmed by MALDI-TOF/MS analysis. Thus, the alkaline and thermotolerent lipase showed the relative activity of 206 % (optimum activity conditions were: pH 8.2, 45 ^{o}C, 0.5 % triton X-100 and 1.0 mM Ca^{2+}).

Chapter 6

Rumen microbes as producers of biosurfactants with emphasis on *Pseudomonas* sp. BUP6

Aim and rationale

Many species of *Candida* and *Pseudomonas* are well known producers of biosurfactant. Since the culture parameters influence the yield of biosurfactant significantly, its optimisation is required prior to commercialisation so as to maximise the production potentials of the microbes. Thus, the primary primary focus of this study is the quantification of biosurfactant production by the rumen isolates, *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6; followed by the statistical optimisation of culture parameters employing the high yielding microbe among them to develop an assured production process.

Introduction

Surfactants are considered as an important class of industrial chemicals that reduce the surface or interfacial tensions with respect to the interacting phases and play a key role in interfacial chemistry. They are widely used for many domestic as well as industrial applications - as foaming/wetting agents, emulsifiers, collecting agents, plasticisers, *etc*. depending on the field in which they are applied. Due to the increasing awareness on the toxic effects of petroleum based surfactants on the environment; biosurfactants are gaining potential significance as alternatives to the synthetic ones. Biosurfactants are the structurally diverse groups of surface active molecules, produced naturally by microorganisms, and possess 'eco-friendly' properties such as low toxicity, sustainability and biodegradability (Soberón-Chávez and Maier, 2011). They are amphiphilic molecules produced exclusively by microorganisms including bacteria, fungi and yeasts (Saharan *et al.*, 2011). Even though, they are produced from renewable resources, the commercialisation of biosurfactants found stringent environmental niches in its share on the global market due to the low yields, high cost of production and purification as compared to that of synthetic surfactants. Hence, the

mining of better strains and efficient bioprocesses still remain as an interesting topic of research thereby compensating the draw backs.

Materials and methods

Microorganism and production medium

Candida tropicalis BPU1 and *Pseudomonas* sp. BUP6, isolated from the rumen of Malabari goat were cultured in basal salt medium (**Chapter 4; Table 13**) supplemented with 0.5 % groundnut oil (supplied as emulsion in distilled water by sonication) and incubated at 37 ^{o}C at 140 rpm.

Extraction of biosurfactant

The culture was centrifuged at 6,400 × g for 10 min to collect the cell-free supernatant and acidified to pH 2 using 6 N HCl. The acidified supernatant was kept overnight at 4 ^{o}C to precipitate the biosurfactant completely; which was collected by centrifugation at 9,400 × *g* for 10 min, washed twice with acidified water (pH 2) to get the crude biosurfactant and weighed. The crude biosurfactant was re-dissolved in distilled water at pH 7 and then extracted with equal volume of chloroform: methanol mixture (2:1); the organic phase was collected and evaporated to get the yellow-coloured and honey-like biosurfactant.

Statistical optimisation

After quantification, the isolate showed the higher yield of biosurfactant between two microbes (*C. tropicalis* BPU1 and *Pseudomonas* sp. BUP6) was used for the optimisation studies accomplished in three steps.

Screening for significant parameters using Plackett-Burman model

Initially, five different parameters such as pH, temperature, agitation, incubation, and substrate concentration (groundnut oil) were selected for the

statistical analysis using the software Minitab 14. A set of 22 experimental trails were designed according to Plackett-Burman model to find out the significant parameters effecting the production of biosurfactant. Analysis at two levels (high and low) represented by +1 and −1, respectively (**Table 24**) was performed for each parameter and the result was analysed using the equation (1) (Chapter 4).

Table 24. Levels of variables selected for Plackett-Burman design

Variable (X_i)	Parameter	High (+1)	Low (−1)
X_1	pH	5	8
X_2	Temperature (^oC)	20	40
X_3	Agitation (rpm)	100	200
X_4	Incubation (h)	24	72
X_5	Substrate (%)	0.1	1.0

Box-Behnken design and RSM

The interactive effects of the significant parameters suggested by Plackett-Burman design were evaluated using Box-Behnken design. Analysis at three levels (high, medium and low) represented by +1, 0 and −1 respectively, was performed for each parameter and the results were analysed by fitting to second order polynomial equation (2) (Chapter 4).

Validation of the statistical model

To check the validity of quadratic model, 3 experiments - as predicted by the point prediction software Minitab 14 - were performed. Production of biosurfactant was estimated and compared with the predicted values.

Results

Production of biosurfactant

The rumen microbes, *C. tropicalis* BPU1 and *Pseudomonas* sp. BUP6 were grown in the semi-synthetic medium supplemented with groundnut oil and the production profile of biosurfactant was analysed for 7 days at an interval of 24 h. Both the cultures were able to utilise vegetable oil within 6-7 days of incubation. After each day, the biosurfactant was precipitated from the cell free supernatant using 6 N HCl at 4 ^{o}C for 12 h and weighed for the quantification of crude biosurfactant. Eventhough, the culture started to produce biosurfactant after 24 h of incubation, the maximum yield (1912 mg/l) was obtained after 3 d of incubation with *Pseudomonas* sp. BUP6; whereas *C. tropicalis* BPU1 showed delayed production of biosurfactant with the maximum yield (596 mg/l) at 6 d of incubation (**Figure 45**). Among the two cultures, *Pseudomonas* sp. BUP6 supported the maximum production (1912 mg/l) of biosurfactant, in comparison to *C. tropicalis* BPU1 (596 mg/l); therefore, *Pseudomonas* sp. BUP6 was used for further studies.

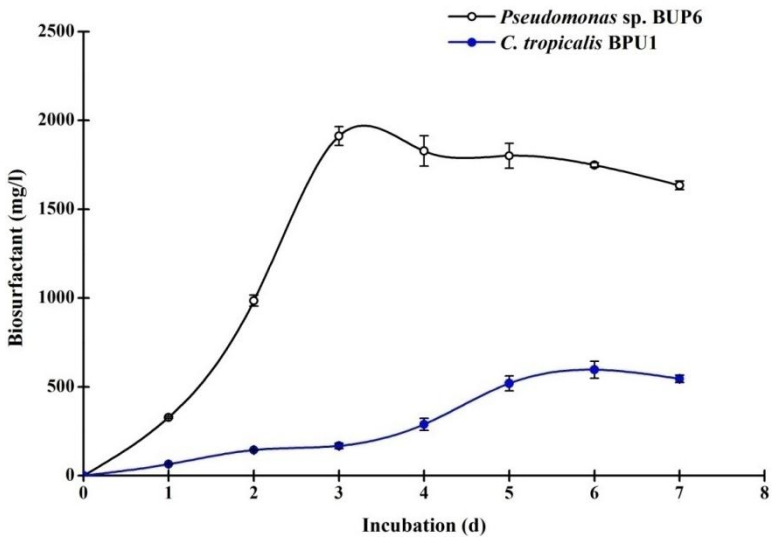


Figure 45. Production profiles of biosurfactant by the rumen isolates. *C. tropicalis* BPU1 showed the maximum production (596 mg/l) of biosurfactant on 6 d of incubation; whereas *Pseudomonas* sp. BUP6 supported the maximum production of biosurfactant (1912 mg/l) on 3 d of incubation.

Statistical optimisation

Screening of independent factors that significantly influence the production of biosurfactant by *Pseudomonas* sp. BUP6 was done according to Plackett-Burman design, and a set of 22 runs were performed at 2 levels; low (−) and high (+) (**Table 24**). The standardised effects of individual parameters on the production of biosurfactant were shown in **Figure 46**. The order of significance indicated by Pareto chart was: incubation>pH>temperature> substrate>agitation. Three parameters (incubation, pH and temperature) out of the 5 selected were found significant for biosurfactant production (**Table 25**), those were focused for further analysis.

Table 25. Plackett-Burman matrix with the respective responses in terms of the production of biosurfactant by *Pseudomonas* sp. BUP6.

Run No.	pH	Temperature (^oC)	Agitation (rpm)	Incubation (h)	Groundnut oil (%)	Observed (mg/l)
1	8	25	200	72	0.1	1443.6
2	8	40	100	72	1.0	1112.7
3	5	40	200	24	1.0	29.83
4	5	25	200	72	0.1	768.0
5	8	25	100	72	1.0	1213.87
6	8	40	100	24	1.0	597.84
7	8	40	200	24	0.1	306.89
8	8	40	200	72	0.1	1091.28
9	5	40	200	72	1.0	592.78
10	8	25	200	72	1.0	1367.53
11	5	40	100	72	1.0	701.1
12	8	25	200	24	1.0	687.6
13	5	40	100	72	0.1	557.8
14	5	25	200	24	1.0	287.3
15	5	25	100	72	0.1	559.45
16	5	25	100	24	1.0	199.24
17	8	25	100	24	0.1	797.8
18	8	40	100	24	0.1	398.9
19	5	40	200	24	0.1	168.0
20	5	25	100	24	0.1	154.2
21	6.5	32.5	150	48	0.55	569.3
22	6.5	32.5	150	48	0.55	587.31

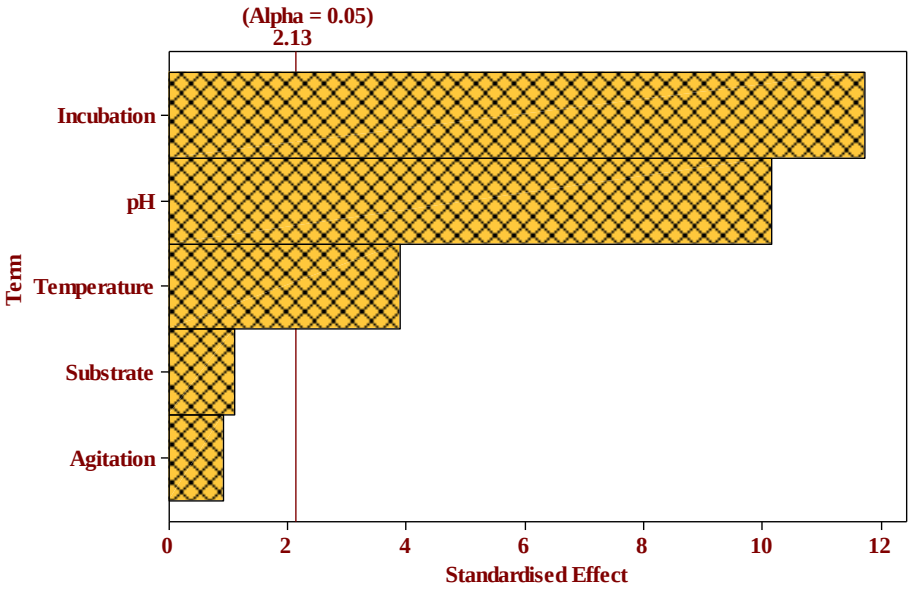


Figure 46. Pareto chart of the standardised effects of parameters on the production of biosurfactant by *Pseudomonas* sp. BUP6.

Table 26. The observed and predicted production of biosurfactant by *Pseudomonas* sp. BUP6, according to Box-Behnken model.

Run No.	Incubation (h)	pH	Temperature (^{o}C)	Observed (mg/l)	Predicted (mg/l)
1	48	6	35	1135	1138
2	96	6	35	620	738
3	48	8	35	965	846
4	96	8	35	1290	1286
5	48	7	30	985	983
6	96	7	30	1445	1328
7	48	7	40	1065	1181
8	96	7	40	875	876
9	72	6	30	1430	1427
10	72	8	30	980	1100
11	72	6	40	965	845
12	72	8	40	1425	1427
13	72	7	35	2005	2051
14	72	7	35	1985	2051
15	72	7	35	2065	2051

Box-Behnken design - composed of 15 experimental runs - was performed to determine the interactive effects of the three significant factors at three levels; low (−1), middle (0) and high (+1). The predicted and observed production of biosurfactant by *Pseudomonas* sp. BUP6 for all the experimental runs were shown in **Table 26** and the results were analysed by ANOVA (**Table 27**). The statistical model for the production of biosurfactant was expressed by the regression equation,

Biosurfactant yield= $-32770.0 + 131.146\ X_1 + 4432.92\ X_2 + 832.583\ X_3 - 1.00405\ X_1^2 - 470.833\ X_2^2 - 15.2333\ X_3^2 + 8.75\ X_1X_2 - 1.35417\ X_1X_3 + 45.5\ X_2X_3$

Where X_1 − incubation;

X_2 − pH;

X_3 − temperature

Table 27. Analysis of variance (ANOVA) for Box-Behnken model.

Source	Degrees of freedom	Sum of squares	Mean squares	F-value	P-value
Regression	9	2814318	312702	17.08	0.003
Linear	3	65825	21942	1.20	0.400
Square	3	2259443	753148	41.14	0.001
Interaction	3	489050	163017	8.90	0.019
Residual Error	5	91542	18308		
Lack-of-Fit	3	84075	28025	7.51	0.120
Pure Error	2	7467	3733		
Total	14	2905860			

Figures 47-52 show the contour plots and response surface plots of the suggested model for the production of biosurfactant. Aptness of the model was determined by the coefficient of determination (R^2) of 0.97, which

indicated that only 3 % of the variation was not explained by the suggested model. Moreover, validation experiments showed a good correlation coefficient of 0.98 - *i.e.*, confirmed as better correlation between the predicted and observed values of biosurfactant production (**Table 28**). Thus, the statistical optimisation resulted in 11 % increase (at pH 7, 35 ^{o}C, incubation 75 h) to get the maximum yield (2070 mg/l) yield of biosurfactant, against the un-optimised condition.

Table 28. Random combinations of parameters selected for the validation of proposed statistical model with respective predicted responses in terms of the production biosurfactant.

Run No.	Incubation (h)	pH	Temperature (^{o}C)	Observed biosurfactant (mg/l)	Predicted biosurfactant (mg/l)
1	48	8	37	971	1010
2	96	6	37	451	471.6
3	96	7	30	1595	1403
4	75	7	35	2070	2127

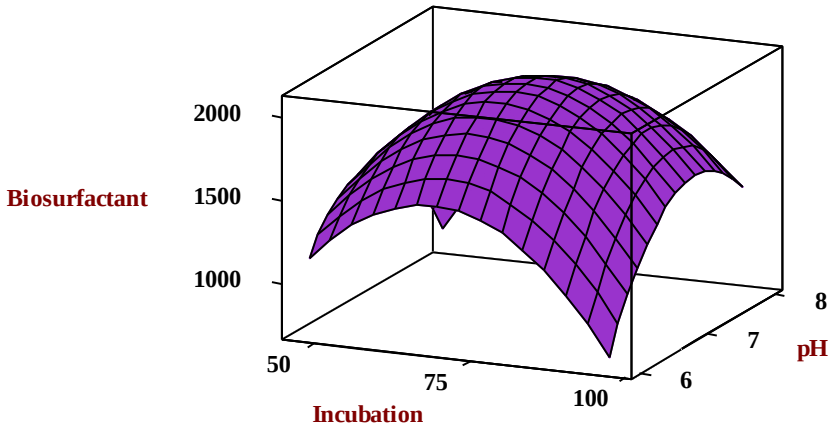


Figure 47. Three dimensional surface plot showing biosurfactant production (mg/l) *vs*. incubation (h) and pH at 35 ^{o}C (hold value of temperature).

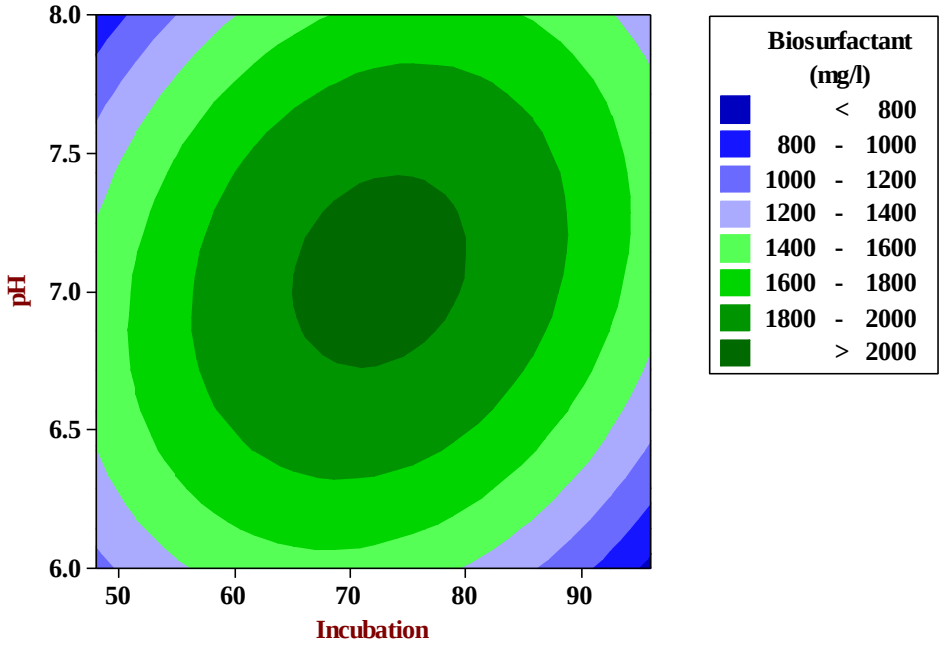


Figure 48. Contour plot showing biosurfactant production (mg/l) *vs*. incubation (h) and pH at hold 35 ^{o}C (hold value of temperature).

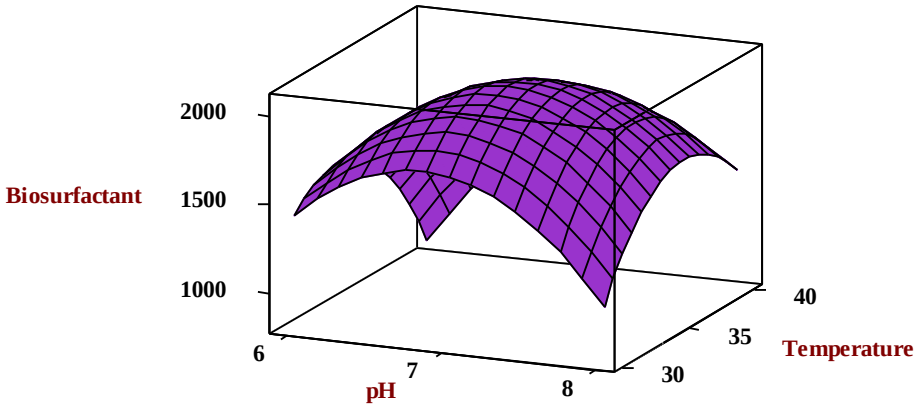


Figure 49. Three dimensional surface plot showing biosurfactant production (mg/l) *vs*. temperature (^{o}C) and pH at 72 h (hold value of incubation).

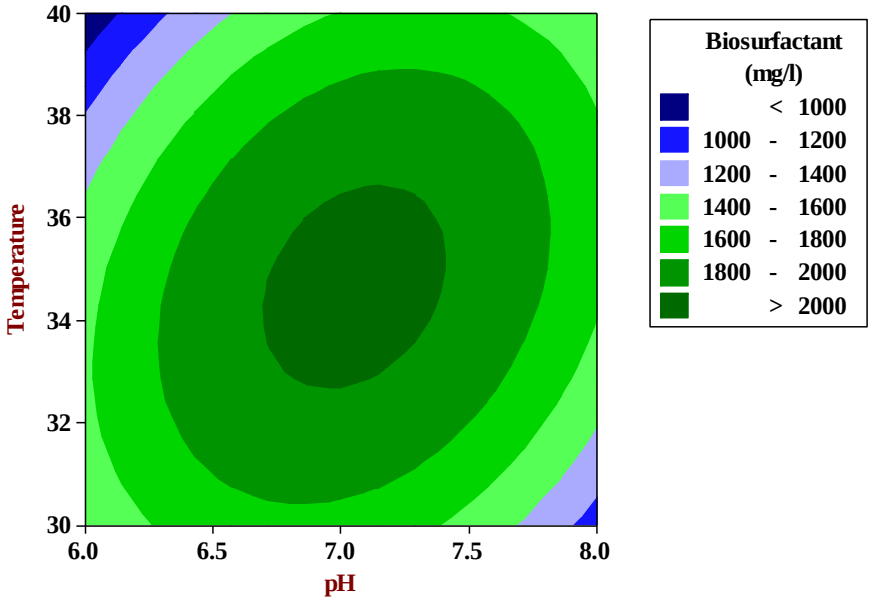


Figure 50. Contour plot showing biosurfactant production (mg/l) *vs*. temperature (^{o}C) and pH at 72 h (hold value of incubation).

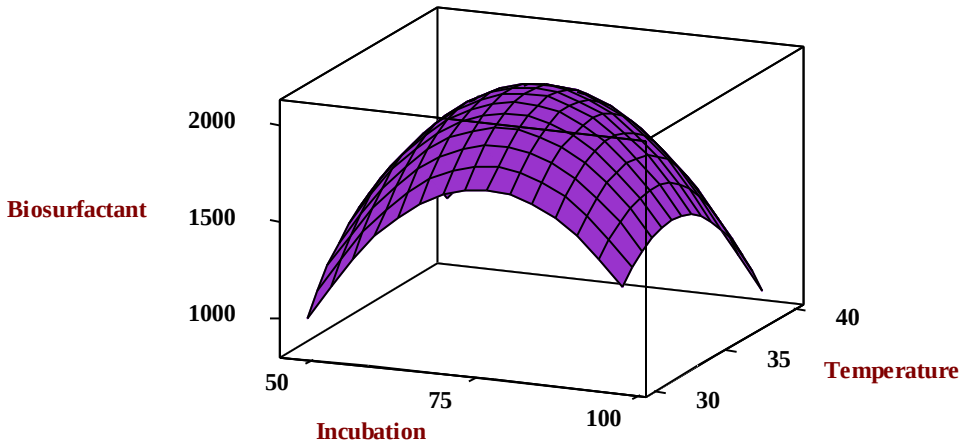


Figure 51. Three dimensional surface plot showing biosurfactant production (mg/l) *vs*. temperature (^{o}C) and incubation (h) at pH 7 (hold value of pH).

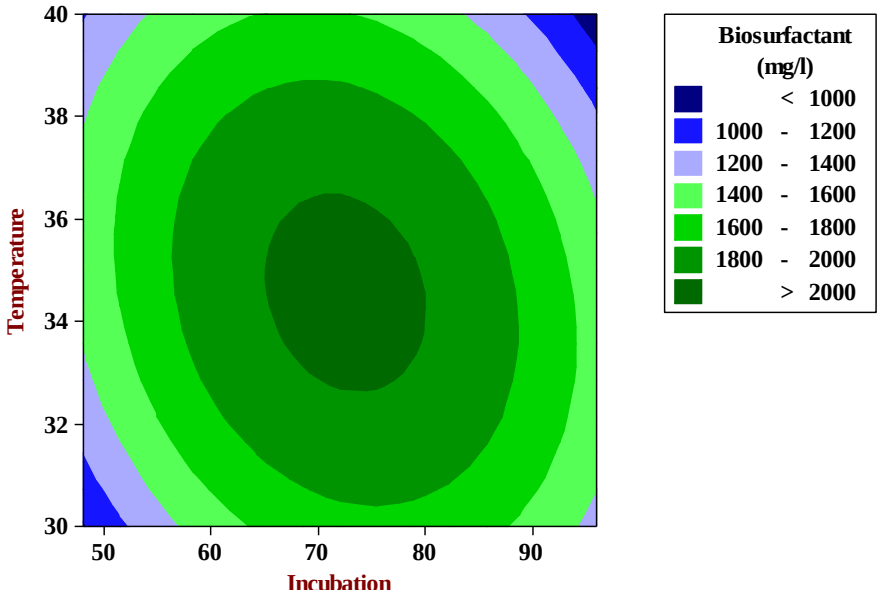


Figure 52. Contour plot showing biosurfactant production (mg/l) *vs*. temperature (^{o}C) and incubation (h) at pH 7 (hold value of pH).

Discussion

The present study focused on the production and statistical optimisation of biosurfactant produced by the rumen yeast (*C. tropicalis* BPU1) and a bacterium (*Pseudomonas* sp. BUP6); of them, *Pseudomonas* sp. BUP6 supported the highest production of biosurfactant against *C. tropicalis* BPU1. Therefore, *Pseudomonas* sp. BUP6 was used for further studies; To obtain the maximum yield (2070 mg/l), significant parameters involved in the production of the biosurfactant were statistically optimised, *i.e*., pH 7, incubation time 75 h and 35 ^{o}C.

Generally, the biosurfactant producing microorganisms are isolated from soil or water samples contaminated with hydrocarbons such as wastes from oil refinery, petroleum, *etc*. (Bodour *et al.*, 2003; Bento *et al.*, 2005). Literature shows that the presence of hydrocarbons in the medium triggers the secretion of biosurfactants in order to improve their solubility and availability. Moreover, vegetable oils efficiently induce the production of biosurfactants by *Pseudomonas* spp. as against other hydrocarbons like glucose and glycerol (Mata-Sandoval *et al.*, 2001; Wei *et al.*, 2005). Hence, in the present study, groundnut oil was used as inducer for the production of biosurfactants by the rumen microorganisms. Species of *Candida* and *Pseudomonas* are known producers of glycolipid type biosurfactants. For instance, *Candida* sp. SY16, isolated from oil contaminated soil produced mannosylerythritol lipid, when grown in a medium supplemented with peptone and soybean oil (Kim *et al.*, 1999). *C. tropicalis* MTCC 230 and *C. lipolytica* UCP 0988 were reported as producing extracellular biosurfactants in the presence of hydrocarbons like petrol, kerosene or soybean oil (Rufino *et al.*, 2013; Verma *et al.*, 2015). Similarly, *C. tropicalis* BPU1 also produced biosurfactant in a medium supplemented with hydrophobic substance, the groundnut oil. Likewise, species of *Pseudomonas* were also reported to produce glycolipid

biosurfactant, especially rhamnolipid in the medium supplemented with coal or glycerol (Saikia *et al.*, 2012; Singh and Tripathi, 2013). In all the aforesaid reports, the microbes were isolated from contaminated soil or water. But, the present study dealt with the discovery of biosurfactant producing microbes from a hostile environment, the goat rumen.

An efficient and economic bioprocess is the bottleneck for any profit-making biotechnology industry, and the nature and type of biosurfactants produced depend upon the environmental conditions like aeration, agitation, energy sources, *etc*. Hence, optimisation of these parameters is important prior to scale-up studies. Response surface methodology is an important statistical tool that exploits both the mathematical as well as experimental methods to explain the effects of various factors or parameters on a particular target process. RSM has certain advantages over the conventional *one-at-a-time* strategy, as it includes minimum number of experimental runs and evaluates the interactive effects of the parameters to find out the most suitable condition to forecast the expected response (Sayyad *et al.*, 2007). The principles of RSM were found applied for the production of biosurfactant from various microbes; for instance, Box-Behnken and central composite designs were employed to increase biosurfactant yield to 146 % (15.68 g/l) from *Pseudomonas aeruginosa* MA01 (Abbasi *et al.*, 2013). In order to optimise the yield of rhamnolipid by *Streptomyces coelicoflavus,* Kalyani *et al*. (2014) used Plackett-Burman design; that resulted in 4 % increase over the unoptimised condition. In the present study, Plackett-Burman and Box-Behnken design were employed effectively to screen as well as to optimise the parameters for maximising the production of biosurfactant. Of the 5 variable selected, only three parameters, *viz*., incubation, pH and temperature showed significant effects on the production of biosurfactant by *Pseudomonas* sp. BUP6. Employing Box-Behnken design, these parameters were further optimised as 75 h, pH 7 and 35 ^{o}C with a maximum biosurfactant yield of

2070 mg/l, *i.e.*, 11 % higher than that obtained under un-optimised conditions. The yield of bio surfactant from the minimal medium supplemented with groundnut oil was quite comparable with the results from many other *Pseudomonas* spp. For instance; *P. aeruginosa* strain BS2 produced 970 mg/l of rhamnolipid in a semi-synthetic medium supplemented with 2 % glucose (Dubey and Juwarkar, 2001). Similarly, the biosurfactant produced by a marine *Bacillus* sp. was quantified as around 2.5 g/l in a synthetic medium (Mukherjee *et al.*, 2008). Thus, *Pseudomonas* sp. BUP6 is found as an efficient producer of biosurfactant.

Conclusions

In comparison to *C. tropicalis* BPU1, *Pseudomonas* sp. BUP6 is found as an efficient producer of biosurfactant. Two stage statistical optimisation processes of environmental parameters (at pH 7, 35 ^{o}C, incubation 75 h) employing Plackett-Burman as well as Box-Behnken model enhanced the biosurfactant yield by 11 % to get the maximum yield (2.070 g/l); an impressive yield, which may be good for industry.

Chapter 7

Characterisation of biosurfactant produced by *Pseudomonas* sp. BUP6

Communicated: Priji, P., Sajith, S., Sreedevi, S., Unni, K. N., and Benjamin, S. (2015). Production, optimisation, and characterisation of biosurfactant produced by *Pseudomonas* sp. strain BUP6, a novel rumen bacterium isolated from Malabari goat. *Journal of Colloids*.

Aim and Rationale

The unique properties of biosurfactants make it suitable candidates of green technologies; to possibly replace the chemically synthesised surfactants in many industrial operations. The understanding of physico-chemical as well as the biological properties of biosurfactants is required to harness them for efficient industrial applications. Hence, the specific objectives of this study were to: (a). analyse the surface hydrophobicity of *Pseudomonas* sp. BUP6; (b). determine the physico-chemical properties such as emulsification index, stability and critical micelle concentration; (c). evaluate the biological properties such as anti-bacterial, anti-adhesive and phytotoxicity; as well as (d). unveil the structural identity of the biosurfactant produced by *Pseudomonas* sp. BUP6 in order to explore its possible industrial applications.

Introduction

Structurally, all the surfactants possess hydrophobic and hydrophilic termini. Based on their chemical constituents such as, glycolipids, phospholipids, lipopeptides, polymeric, fatty acid types, *etc*., the microbial biosurfactants are classified into several categories; among those, glycolipids and lipopeptides are extensively studied and found many applications in food, pharmaceuticals, petroleum industries and environment safety (Okoliegbe and Agarry, 2012; Uzoigwe *et al*., 2015). Rhamnolipid is the predominant group of glycolipid biosurfactant produced by many species of *Pseudomonas*; of which the rhamnose constitutes its hydrophilic terminus, while the hydroxyl fatty acids occupy the hydrophobic terminus. Due to their biological properties such as anti-microbial and anti-adhesive activities, in addition to the higher foaming and emulsification abilities; for the past a few decades, rhamnolipids met

rapid hike in demand in many industries as multifunctional surfactants (Marchant and Banat, 2012a).

Upon the aforesaid background, this chapter examines the surface hydrophobicity of *Pseudomonas* sp. BUP6 cells, as well as the physico-chemical-biological and structural characterisation of biosurfactant produced by it.

Materials and methods

Hydrophobicity index

The degree of adherence of *Pseudomonas* sp. BUP6 to various liquid hydrocarbons was determined spectroscopically. A suspension of *Pseudomonas* sp. BUP6 in ddH_2O was prepared with an absorbance of 0.5 at λ_{600}. The suspension (2 ml) was mixed vigorously with equal volume of the liquid hydrocarbons (petrol, diesel, kerosene, *n*-decane, and groundnut oil), and allowed to stand for 2 min for phase separation. Subsequently, the aqueous layer was collected and absorbance was measured again. The hydrophobicity index was calculated using the formula,

$$H = [1 - \left(\frac{ODs}{ODc}\right)] \times 100$$

Where H – Hydrophobicity index

ODs – Optical density of the aqueous layer after treatment with the liquid hydrocarbon at λ_{600}

ODc – Optical density of the suspension of *Pseudomonas* sp. BUP6 in distilled water at λ_{600} (0.5)

Properties of crude biosurfactant

Physico-chemical properties

The physico-chemical properties were analysed using the crude biosurfactant, prepared by dissolving the biosurfactant (precipitated in acid) in ddH_2O at a concentration of 100 mg/l at pH 7.

Emulsification index (% EI_{24})

The emulsification ability of the biosurfactant was determined using five different hydrocarbons, *viz*., petrol, diesel, kerosene, *n*-decane and groundnut oil. Crude biosurfactant (2 ml) was added to equal volume of hydrocarbon, and vortexed vigorously for 2 min. The mixture was allowed to stand for 24 h. Emulsification index was expressed in percentage of the height of the emulsification layer to the total height of the liquid column.

Stability

For determining the thermal stability, crude biosurfactant was kept at different temperatures ranging from 4 to 100 ^{o}C for 30 min, cooled to 25 ^{o}C; and the emulsification index was determined using kerosene. Similarly, pH stability was analysed by adjusting the pH of crude biosurfactant from 2 to 10, emulsification index was determined subsequently. Effect of NaCl on emulsification index was also determined at different NaCl concentrations (0.1 to 1 %).

Critical miscelle concentration (CMC)

Rhamnolipid was prepared in ddH_2O at varying concentrations (10 to 80 mg/l), and the surface tension of each solution was measured by capillary rise method (Richards and Carver, 1921). CMC is represented by the concentration of the biosurfactant, further increase in concentration did not

reduce the surface tension of the solution, *i.e.*, surface tension remains relatively constant.

$$\gamma = \frac{\rho g h r}{2}$$

Where, γ – surface tension

ρ – liquid density (1000 kg/m^3)

g – acceleration due to gravity (9.8 m/s^2)

r – radius of the capillary tube (0.54×10^{-3} m)

h – height of the water column (m)

Biological properties

Biological properties of the crude biosurfactant such as anti-bacterial, anti-adhesive and phyto-toxic effects were determined to evaluate its possible industrial applications.

Anti-bacterial activities

Anti-bacterial activities of the biosurfactant produced by *Pseudomonas* sp. BUP6 was determined by micro-dilution method in 96-well flat bottom tissue culture plates against 4 different bacterial cultures, *viz.*, *Escherichia coli, Proteus mirabilis, Staphylococcus aureus* and *Acinetobacter baumannii* (Rufino *et al.*, 2011). Briefly, 100 µl of the nutrient broth was placed in the wells of 96 well tissue culture plates, followed by the addition of 100 µl of crude biosurfactant solution (200 mg/l) to the first well, and mixed to make the final concentration of 100 mg/l. From this, 100 µl was transferred serially to the subsequent wells to make the serial dilutions of crude biosurfactant at a concentration of 50, 25, 12.5 or 6.25 mg/l. The 12 h old bacterial culture (2.5

µl) was added to each well, incubated at 37 ^{o}C for 24 h, and absorbance was measured at λ_{600}. The culture without biosurfactant was maintained as control.

$$\text{Anti-bacterial activity (\%)} = [1 - \left(\frac{\text{ODs}}{\text{ODc}}\right)] \times 100$$

ODs – Optical density of the aqueous layer after treatment with the liquid hydrocarbon at λ_{600}

ODc – Optical density of the control wells at λ_{600}

Anti-adhesive properties

Anti-adhesive properties of the biosurfactant produced by *Pseudomonas* sp. BUP6 was determined by microdilution method in 96 well flat bottom tissue culture plates against 4 different bacterial cultures, *viz*., *Escherichia coli, Proteus mirabilis, Staphylococcus aureus,* and *Acinetobacter baumannii* (Rufino *et al.*, 2011). The preparations in the flat-bottom tissue culture plate (96 wells microtitre plate) were incubated with different concentrations of biosurfactant (100, 50, 25, 12.5, 6.25 mg/l) for 24 h at 4 ^{o}C, and washed with sterile ddH_2O. Subsequently, 200 µl of bacterial culture (12 h old) was dispensed to each well, and incubated for 12 h at 4 ^{o}C. After incubation, the wells were washed with sterile ddH_2O to remove unattached bacteria; the adherent microbes were fixed on the surface of wells by washing with 200 µl of methanol and air-dried. Followed by this, to each well, 2 drops of crystal violet was added (incubated for 5 min), and washed with sterile ddH_2O to remove excess stain. After air drying, the bound dye in each well was re-solubilised by adding 200 µl of 33 % acetic acid to each well, and absorbance was measured at λ_{595}. Distilled water without biosurfactant was kept as control.

$$\text{Anti-adhesion (\%)} = [1 - \left(\frac{\text{ODs}}{\text{ODc}}\right)] \times 100$$

ODs – Optical density of the aqueous layer after treatment with the liquid hydrocarbon at λ_{595}

ODc – Optical density of the control wells at λ_{595}

Phyto-toxicity

Phyto-toxicity of the biosurfactant on seed germination was determined employing the protocol as demonstrated by Luna *et al*. (2013). The seeds of rice (*Oryza sativa* L.) and green gram (*Vigna radiate* L.) were washed and placed on cotton and tissue paper wetted with biosurfactant solution in a petri-dish at a concentration of 50 mg/l and 100 mg/l, incubated at 25 ^{o}C for 2 to 7 days to observe its effects on germination.

Characterisation of biosurfactant

The purified biosurfactant (extracted in chloroform) was subjected to structural characterisation by orcinol method, thin layer chromatography and Fourier transform infrared (FTIR) spectroscopy.

Orcinol method

To 1 mg of biosurfactant, 2 ml of orcinol reagent (0.19 % orcinol in 53 % sulphuric acid) was added and heated at 80 ^{o}C for 30 min, followed by cooling to 25 ^{o}C. The chloroform extract of uninoculated medium was used as control (Laabei *et al.*, 2014).

Thin layer chromatography (TLC)

The crude biosurfactant (5 µl) was spotted on to silica G250 TLC plates and developed using chloroform:methanol:acetic acid (65:15:2) as solvent system. After the run, the plate was left to dry at 25 ^{o}C, and the separated spots were visualised using iodine vapour.

Fourier transform infrared spectroscopy (FTIR)

Biosurfactant (5 mg) was mixed with specral grade unhydrous potassium bromide (KBr) and fixed on a sample holder for analysis. FTIR spectroscopic analysis of the sample was carried out at mid infra red region of 400-4000/cm (Jasco FTIR 4100 series, Japan).

Results

Hydrophobicity index

Surface hydrophobicity is an important property of bacteria producing extracellular biosurfactant. It was shown that *Pseudomonas* sp. BUP6 grew attached to the groundnut oil and formed clumps, which were visualised by scanning electron microscopy (SEM) (**Figure 53**). Hydrophobicity index is the measure of surface hydrophobicity; *i.e.*, higher the hydrophobicity index, higher the hydrophobicity of the extracellular matrix is. In this study, the hydrophobicity index of *Pseudomonas* sp. BUP6 was determined using various non-aqueous hydrocarbons such as diesel, kerosene, petrol, *n*-decane, and groundnut oil; and the cells showed the highest hydrophobicity index (37 %) towards groundnut oil followed by petrol, kerosene, *n*-decane and diesel (**Figure 54**). Thus, the results clearly indicated the presence of a characteristic slimy hydrophobic matrix surrounding the cells of *Pseudomonas* sp. BUP6.

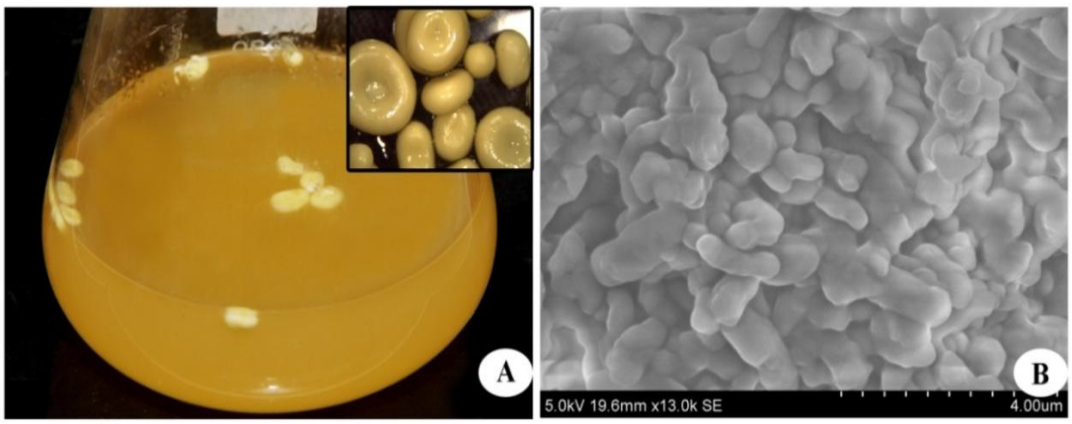


Figure 53. Hydrophobic nature of *Pseudomonas* sp. BUP6: (A). *Pseudomonas* sp. BUP6 growing attached to the oil droplets supplemented in the basal salt medium. The clumps are shown at the inset; and (B). SEM image of clumps (SEM: Hitachi SU660, Japan).

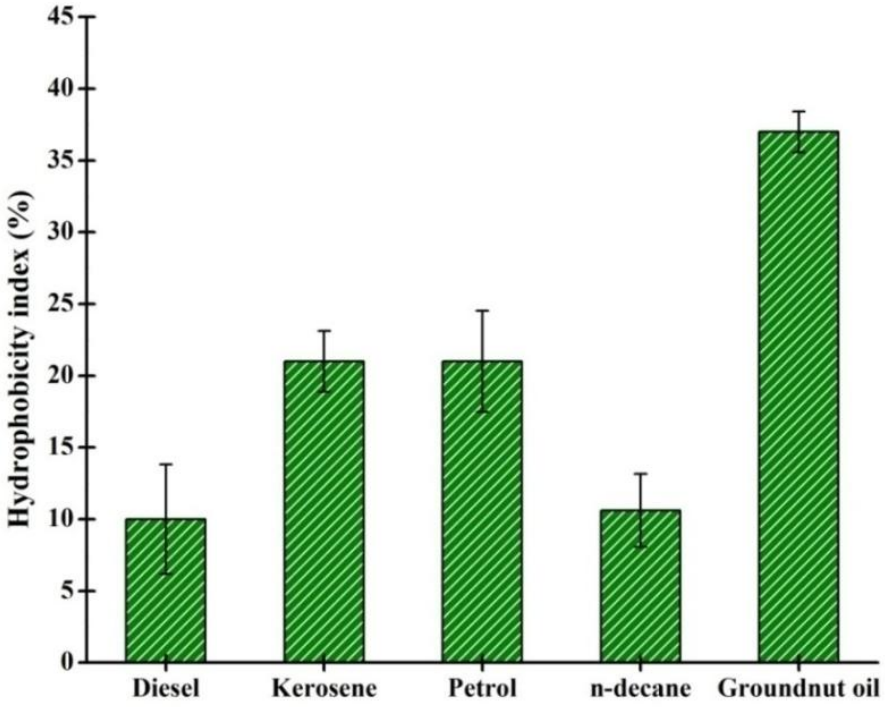


Figure 54. Hydrophobicity index of *Pseudomonas* sp. BUP6 towards various hydrocarbons. The cells showed the maximum hydrophobicity index towards groundnut oil (37 %).

Physico-chemical properties of the biosurfactant

Physico-chemical properties of the biosurfactant such as emulsification index, stability and CMC were investigated. The emulsifying capacity is an important property of biosurfactants, which varies with their chemical nature as well as the microbial source. Emulsification index is the measure of a surfactant to form emulsions. Emulsification index of the biosurfactant produced by *Pseudomonas* sp. BUP6 was determined using five different hydrocarbons, *viz*., petrol, diesel, kerosene, *n*-decane, and groundnut oil. As shown in **Figure 55**, the biosurfactant showed good emulsification indices with all the hydrocarbons tested; among which the maximum emulsification index was observed with kerosene (69 %).

Biosurfactants have potential industrial and environmental applications, which usually involve the exposure to extreme conditions; hence the stability of the biosurfactant under diverse range of temperature, pH and salinity has to be investigated in order to put forward their biotechnological potentials and commercial significance. In this study, the stability of biosurfactant produced by *Pseudomonas* sp. BUP6 was analysed in terms of emulsification index at varying temperature (4 to 100 ^{o}C), pH (3 to 11) and salinity (0.5 to 2.0 %). The biosurfactant was found stable over a wide range of temperature of 20 to 100 ^{o}C, with no significant effects on emulsification even after heating at 100 ^{o}C for 30 min (54 % retained with kerosene), indicating its thermostability. The maximum stable emulsion was obtained at 40 to 60 ^{o}C (68 %) (**Figure 56**). Investigations on pH stability indicated that the biosurfactant produced stable emulsions at a pH range of 3-9 (**Figure 57**). The emulsification index of the biosurfactant was relatively stable (50 %) in the presence of NaCl, upto the concentration of 1.5 %; thereafter it was reduced to 43 % (**Figure 58**). Thus, the stability of biosurfactant produced by *Pseudomonas* sp. BUP6 clearly indicated that it is a good candidate for use in industries related to emulsions.

Critical micelle concentration (CMC) is another important physico-chemical property of a surfactant which measures its efficiency to reduce the surface tension of aqueous layer. Usually, surfactants reduce the surface tension of an aqueous phase until a particular concentration, termed as the CMC; further increase in surfactant concentration does not alter the surface tension of the aqueous solution, but promote the formation of micelles, *i.e*., lower the CMC of surfactant, the higher is its surface activity. The biosurfactant produced by *Pseudomonas* sp. BUP6 reduced the surface tension of ddH_2O from 74 to 34 mN/s. The CMC of the biosurfactant was found as 48 mg/l (**Figure 59**).

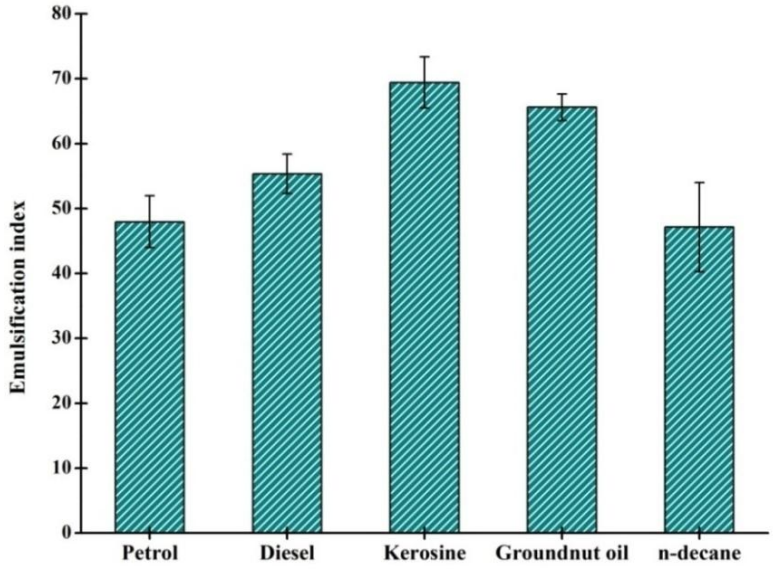


Figure 55. Emulsification index of biosurfactant produced by *Pseudomonas* sp. BUP6 towards various hydrocarbons. The maximum emulsification index was shown towards kerosene (69 %).

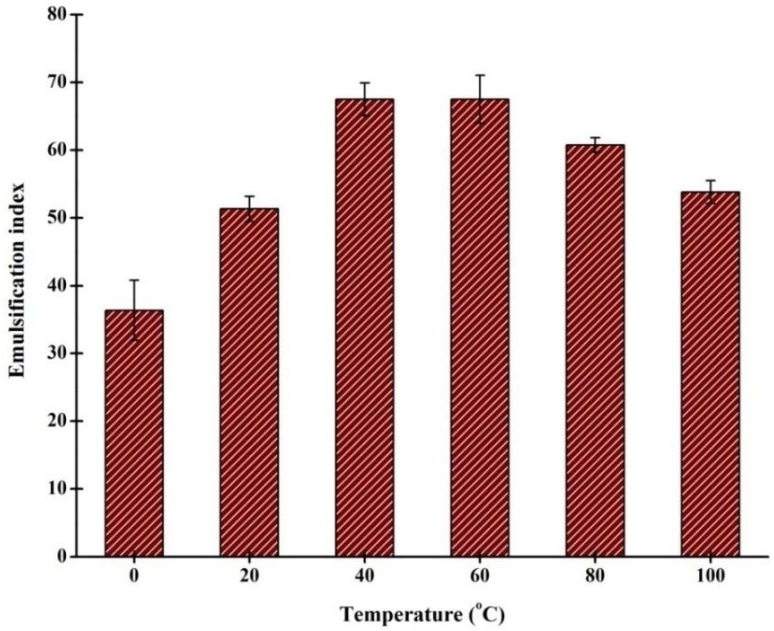


Figure 56. Effects of temperature on the emulsification index of biosurfactant produced by *Pseudomonas* sp. BUP6. The biosurfactant did not show significant variations in emulsification index in a 20 to 100 ^{o}C temperature range.

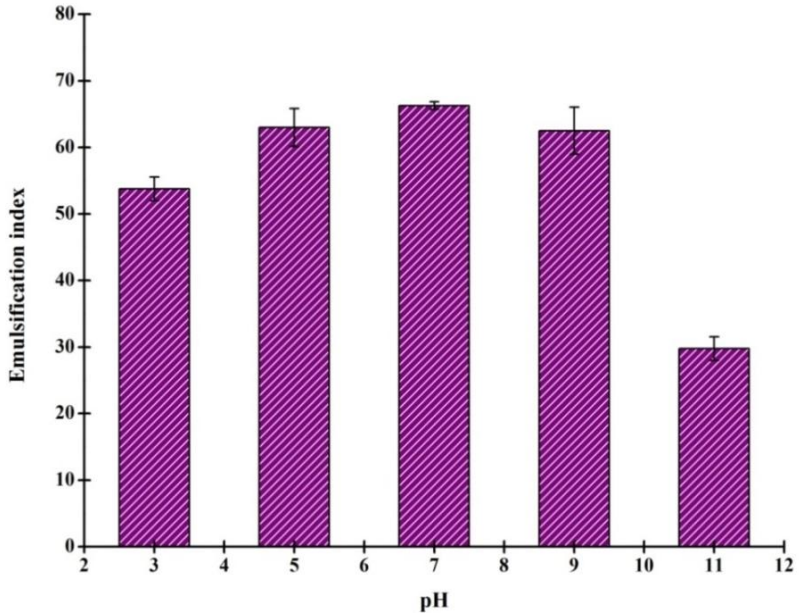


Figure 57. Effects of pH on the emulsification index of biosurfactant produced by *Pseudomonas* sp. BUP6. The biosurfactant did not show significant variations in emulsification index at a pH range of 3 to 9.

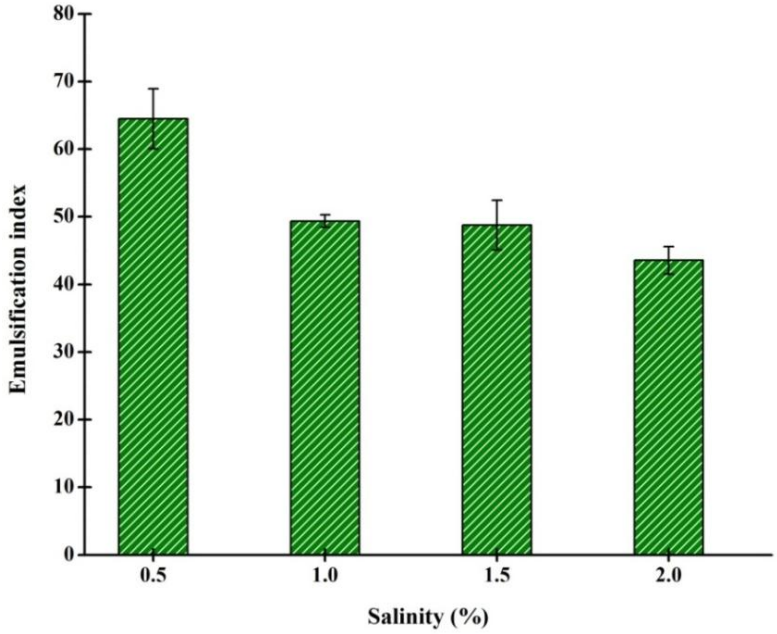


Figure 58. Effect of salinity on the emulsification index of biosurfactant produced by *Pseudomonas* sp. BUP6. The biosurfactant was found relatively stable in terms of emulsification index at salinity of 0.5-1.5 %.

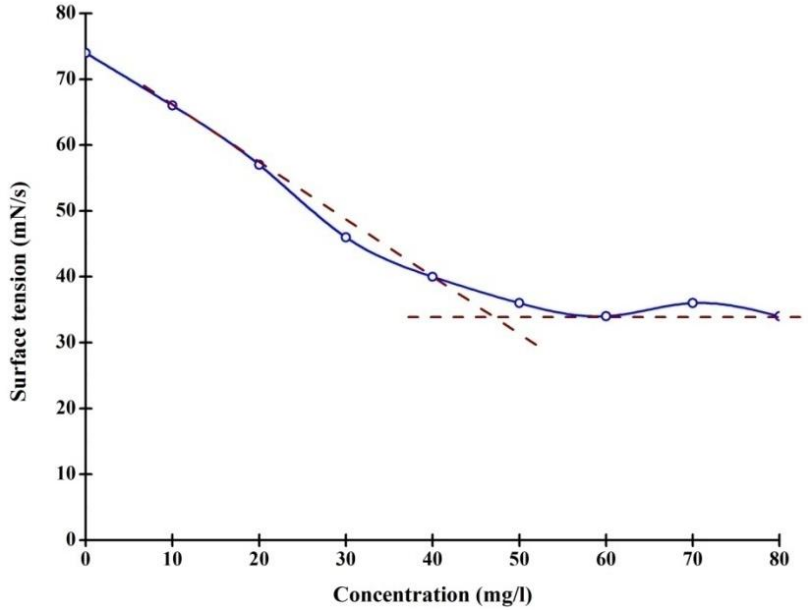


Figure 59. Surface tension *vs*. concentration of biosurfactant produced by *Pseudomonas* sp. BUP6. The biosurfactant showed a critical micelle concentration of 48 mg/l.

Biological properties

Biosurfactants possess interesting biological properties such as anti-bacterial, anti-adhesive and non-toxicity that made them advantageous over the synthetic surfactants. The anti-bacterial activity of the biosurfactant produced by *Pseudomonas* sp. BUP6 was investigated against 4 bacteria (*E. coli*, *P. mirabilis*, *S. aureus* and *A. baumanni*) at varying concentrations (100 to 6.3 mg/l). At a concentration of 100 mg/l (≈2×CMC), the biosurfactant showed 43 and 42 % inhibitory effects against *E. coli* and *S. aureus*, respectively; whereas it did not show significant effect on the growth of *P. mirabilis* and *A. baumannii* (inhibition of growth was approximately 10 %). Even at 50 mg/l (≈CMC), the inhibition concentration was about 31 and 24 % for *E. coli* and *S. aureus* respectively, but further lowering of concentration did not show significant effect on bacterial growth (**Figure 60**). Similarly, the anti-adhesive property of the biosurfactant was the maximum against *E. coli* and *S. aureus*

at 100 mg/l of biosurfactant (49 and 46 %, respectively); but the biosurfactant was found less effective against *P. mirabilis* and *A. baumannii* at all the concentrations investigated (**Figure 61**).

Non-toxicity is another important property of the biosurfactants, which made them suitable candidates for green technologies. In this study, the non-toxicity of the biosurfactant produced by *Pseudomonas* sp. BUP6 was demonstrated *via* investigating the effects on seed germination of green gram and rice. For the phyto-toxic assay, two different concentrations of biosurfactant was used; 50 mg/l (≈CMC) and 100 mg/l (≈2×CMC). It was found that the biosurfactant exerted no significant toxic effects on the germination of the seeds of green gram and rice at both the concentrations investigated, indicating its possible applications in the field of environmental safety (**Figure 62**).

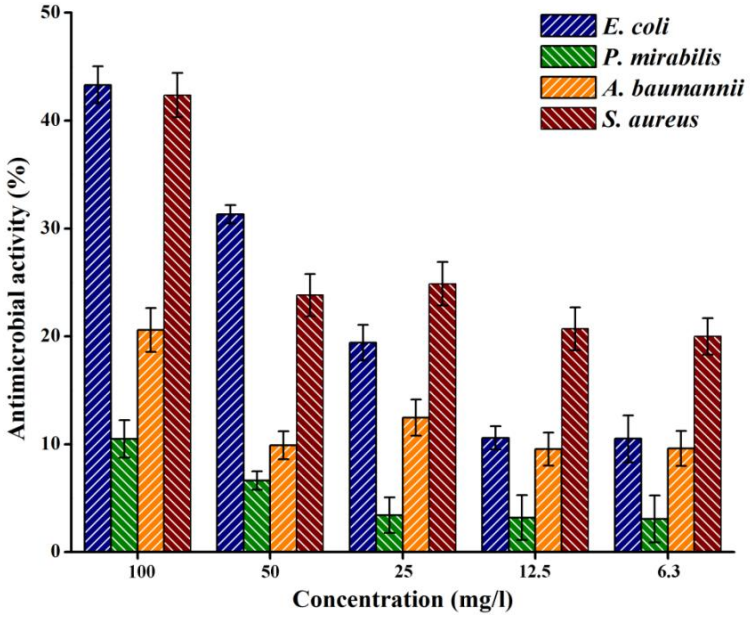


Figure 60. Anti-bacterial activities of biosurfactant produced by *Pseudomonas* sp. BUP6. Anti-bacterial activity was the maximum against *E. coli* (43 %) and *S. aureus* (42 %).

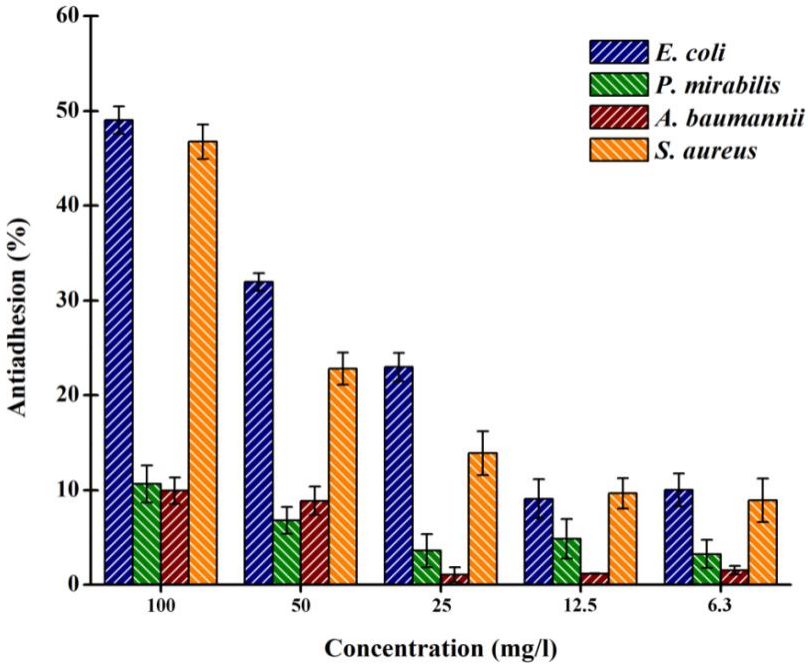


Figure 61. Anti-adhesion property of biosurfactant produced by *Pseudomonas* sp. BUP6. Antiadhesive activity was the maximum against *E. coli* (49 %) and *S. aureus* (46 %).

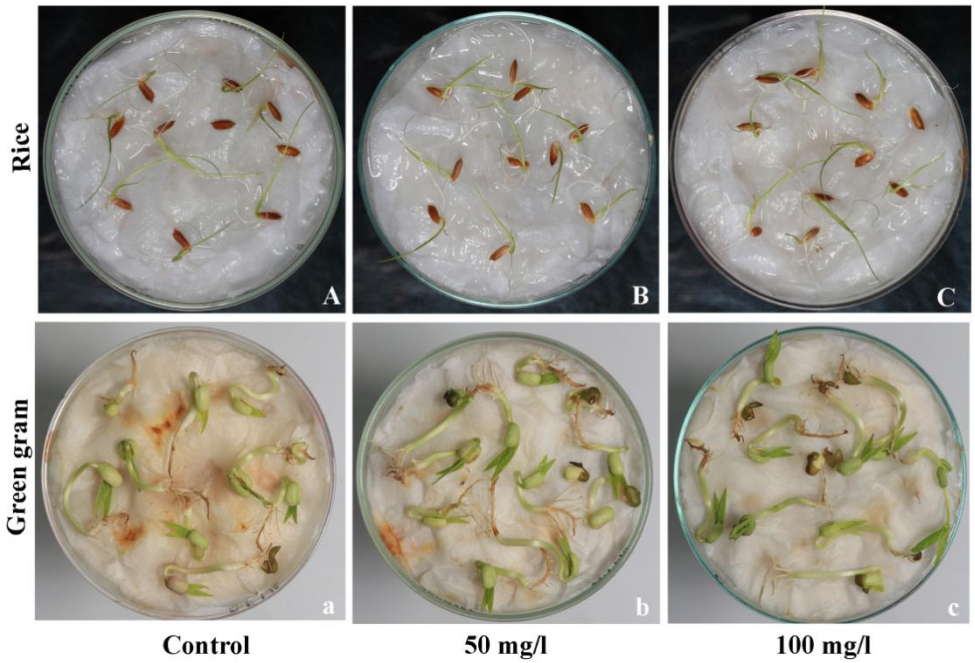


Figure 62. Analysis of phytotoxicity of biosurfactant produced by *Pseudomonas* sp. BUP6. The biosurfactant did not show any toxic effect on seed germination.

Characterisation of biosurfactant

The structural characterisation of purified biosurfactant (chloroform extract) reveals its chemical nature as well as the compositional identity; which was carried out by orcinol method, TLC and FTIR spectroscopy. Upon heating with orcinol reagent, the purified biosurfactant turned muddy brown in colour, a comparable effect to that of standard rhamnose (positive control); which confirmed the presence of sugar moieties in the purified biosurfactant. The chloroform extract of the uninoculated basal salt medium was used as negative control. The TLC plates after treating with iodine vapour produced two spots (0.72 and 0.48 R_f values); which confirmed the presence of mono- and di-rhamnolipids, respectively in the sample (Raza *et al.*, 2014). Further confirmation by FTIR clearly indicated the presence of peaks characteristic to the rhamnolipids. For instance, the broad and significant peak at 3100-3450/cm corresponds to –OH stretching of the glycolipid; whereas multiple peaks at 2900-2800/cm indicate the aliphatic CH_3, CH_2 vibrations. The major peak at 1738/cm is contributed by the C=O group due to the functional ester group, and the vibration at 1637/cm indicates the presence of COO– in the sample. Similarly, the peaks at 1455-1380/cm correspond to the bending vibrations of –OH on carboxylic group; whereas peaks at 1030-1100/cm stand for the C–O–C vibrations in rhamnose (Aparna *et al.*, 2012; Raza *et al.*, 2014). Thus, the FTIR spectrum confirmed that the biosurfactant produced by *Pseudomonas* sp. BUP6 belongs to the category of rhamnolipid (**Figure 63**).

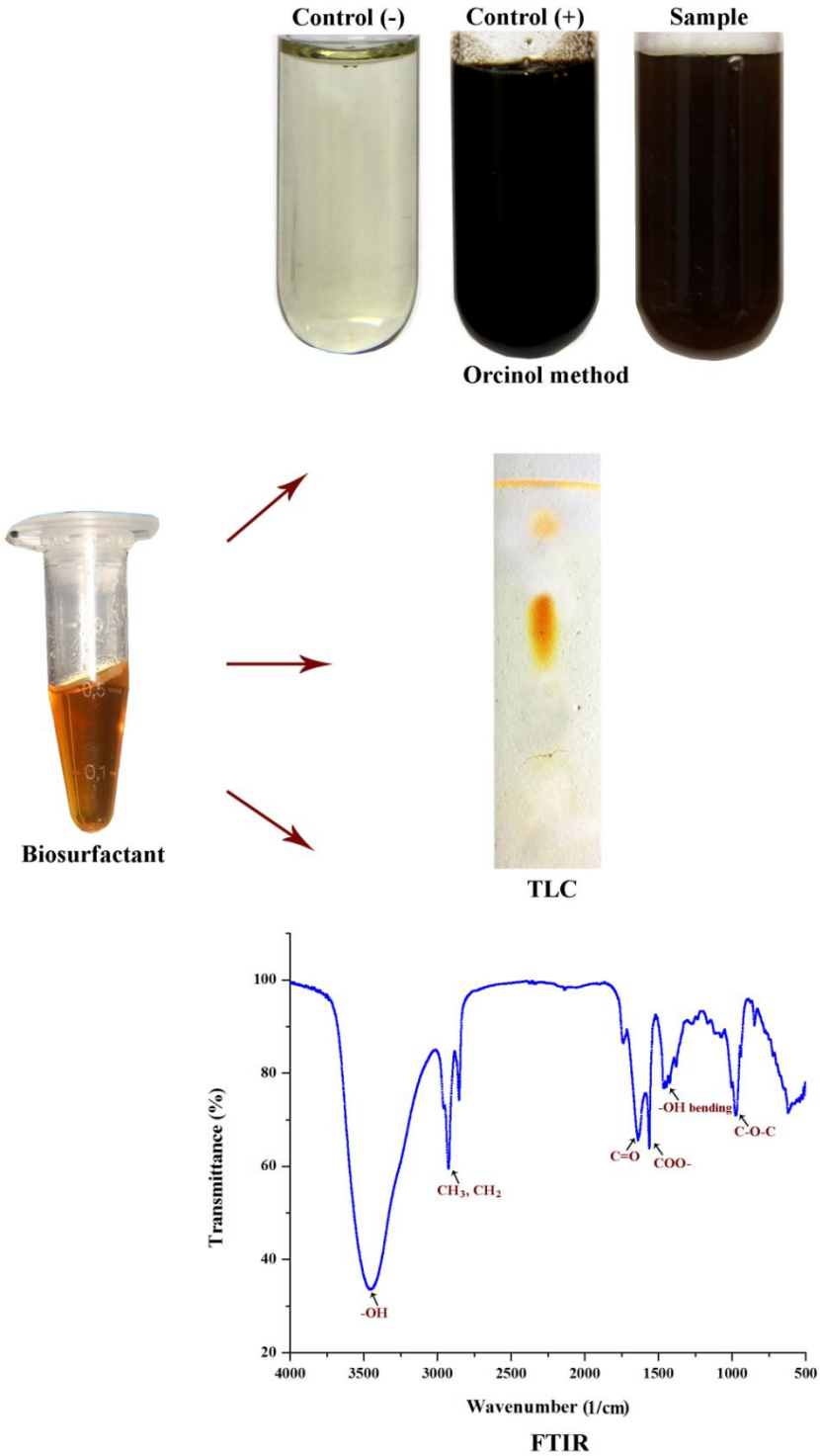


Figure 63. Structural characterisation of biosurfactant produced by *Pseudomonas* sp. BUP6.

Discussion

Recently, biosurfactants have gained overwhelming attention on the world market, because of their characteristic biodegradable, eco-friendly and biocompatible properties with versatile industrial applications. Thus, this study aimed at analysing the surface hydrophobicity of *Pseudomonas* sp. BUP6 cells, coupled with the physico-chemical, biological and structural characterisation of the biosurfactant produced by it.

The first objective of the study was to analyse the surface hydrophobicity of *Pseudomonas* sp. BUP6. Preliminarily, the visual observation itself suggested the hydrophobic nature of *Pseudomonas* sp. BUP6, as it formed clumps in basal salt medium supplemented with groundnut oil by attaching to the oil droplets. The clumps were analysed visually and microscopically to confirm its slimy nature as well as the presence of microbial cells. Moreover, the surface hydrophobicity was confirmed by determining the hydrophobicity index of the bacterial cell suspension towards various non-aqueous hydrocarbons such as groundnut oil, petrol, kerosene, *n*-decane and diesel. When mixed with the non-aqueous layer, *Pseudomonas* sp. BUP6 in aqueous suspension bound to the hydrocarbons and rise up along with it; and thus, showed hydrophobicity index of 10 to 37 % towards all hydrocarbons investigated with a maximum of 37 % towards groundnut oil. This study clearly demonstrated the surface hydrophobicity of *Pseudomonas* sp. BUP6 towards various non-aqueous hydrocarbons, a characteristic property of biosurfactant producing cells (Pruthi and Cameotra, 1997; Bodour and Miller-Maier, 1998; Walter *et al.*, 2010).

Second objective of this study was to analyse the physico-chemical properties of the biosurfactant produced by *Pseudomonas* sp. BUP6, which was accomplished by determining its emulsification index, stability at a wide range of temperature, pH and salinity as well as CMC. Since, the purification

of biosurfactants demands more than 60 % of total cost of its upstream/downstream processes (Silva *et al*., 2010), crude biosurfactant (acid precipitated biosurfactant dissolved in sterile ddH_2O) was used in this study for the physico-chemical analysis, in order to evaluate its possible industrial applications and economic feasibility. Most of the biosurfactants are substrate-specific and emulsify different hydrocarbons at different rates (Silva *et al*., 2010). It was found that the biosurfactant produced by *Pseudomonas* sp. BUP6 showed good emulsification index in kerosene (69 %) as compared to the other hydrocarbons such as petrol, diesel, *n*-decane and groundnut oil. This result was quite comparable with that of the rhamnolipids produced by *P. aeruginosa* J4, which showed the emulsification of index of 78 % with kerosene (Wei *et al.*, 2005). Similarly, Samanta *et al*. (2012) also reported that the biosurfactant produced by *P. aeruginosa* showed the maximum emulsification (64 %) with petrol. Moreover, the biosurfactant produced by *Pseudomonas* sp. BUP6 was found stable in terms of emulsification index at a wide range of pH (3 to 9), temperature (20 to 100 ^{o}C) and salinity (0.5 to 1.0 %). The CMC is another physico-chemical property of surfactants, and is identified as the concentration at which the surfactants start to form micelles and show the lowest surface tension (Banat *et al*., 2000; Nitschke *et al.*, 2005, 2011). Generally, CMC of biosurfactants varies from 10 to 350 mg/l, depending on their composition. For instance, rhamnolipids produced by *Pseudomonas aeruginosa* L21 showed a CMC value of 30 mg/l (Costa *et al.*, 2010), whereas the glycolipid type biosurfactant produced by *P. aeruginosa* LBI showed a CMC of 120 mg/l (Benincasa *et al.*, 2004). The biosurfactant produced by *P. aeruginosa* strain S6 decreased the surface tension of water from 72 to 33.9 mN/s, CMC value of 50 mg/l (Yin *et al.*, 2009). Similarly, in this study, the biosurfactant reduced the surface tension of water from 74 mN/s to 34 mN/s with CMC of 48 mg/l. Thus, the excellent surface activities and stabilities of the biosurfactant produced by *Pseudomonas* sp. BUP6

clearly indicate its possibilities of exploitation to various fields like cosmetics, bioremediation and environmental safety.

The third objective of the study was to explore the biological activities of the biosurfactant such as anti-bacterial, anti-adhesive and non-toxic properties, which critically make them advantageous over the synthetic surfactants. The biosurfactant produced by *Pseudomonas* sp. BUP6 inhibited the growth and adherence of *E. coli* as well as *S. aureus* by 50-30 % at 100 mg/l, indicating its possible applications in medical field. Similar results were observed for the rhamnolipids produced by *P. aeruginosa* L2-1, which was found active against *Bacillus cereus* (32 mg/l), *Micrococcus luteus* (32 mg/l) and *S. aureus* (128 mg/l) (Costa *et al.*, 2010). The increasing environmental pollutions have raised the public concern against synthetic chemicals due to their recalcitrant as well as non-biodegradable nature (Pradeep *et al.*, 2012). It was demonstrated that many of the synthetic surfactants have phyto-toxic effects and retard the germination of rapidly growing plants. For instance, anionic surfactants such as alkylbenzene sulphonates and alkyl ether sulphates severely inhibited the germination of mustard and cress even at a concentration of 10 to 30 mg/l (Liwarska-Bizukojc and Urbaniak, 2007). Hence, the phyto-toxicity of the biosurfactant, rhamnolipids, produced by *Pseudomonas* sp. BUP6 was investigated by observing its effects on the germination of seeds of green gram and rice, and exerted no toxic effects on the germination even at 100 mg/l, revealing its ecological acceptance.

The last objective was to unveil the chemistry of the biosurfactant. Generally, species of *Pseudomonas* are known producers of glycolipid biosurfactants; especially, rhamnolipid (Lotfabad *et al.*, 2009). The structural characterisation of the biosurfactant was carried out employing orcinol method, TLC and FTIR spectroscopy. Orcinol reagent is used to detect the presence of carbohydrate in the biosurfactant, which upon heating produced muddy brown

colour, similar to the standard rhamnose. TLC after visualisation with iodine vapors (it stains lipids) showed the presence of two dark yellow-coloured spots - a lower prominent spot with R_f of 0.48 corresponding to dirhamnolipid and a higher spot at 0.72 which corresponding to monorhamnolipds. Similar pattern of migration on TLC was observed for the rhamnolipids produced by several *Pseudomonas* spp. (Raza *et al.*, 2009; Lotfabad *et al.*, 2010). Moreover, the FTIR spectrum also confirmed that the biosurfactant produced by *Pseudomonas* sp. BUP6 as rhamnolipid. According to Pornsunthorntawee *et al*. (2008b), adsorption bands located at 3468, 2922, 2853, 1743, and 1300 to 1100/cm indicate that the structural characteristics are identical to that of rhamnolipid, which is in accordance with the rhamnolipids produced by several other *Pseudomonas* (Janek *et al.*, 2013). Thus, this study suggests that *Pseudomonas* sp. BUP6 produces rhamnolipid type biosurfactant and the physico-chemical and biological properties clearly indicate its possible applications in the fields of biomedicines, pharmaceutical, agriculture and management of the environment.

Conclusions

Pseudomonas sp. BUP6 is found as an efficient producer of rhamnolipid type biosurfactant with good emulsification and stability indices. The biological properties of this rhamnolipid suggests that it posses anti-bacterial and anti-adhesive activities against *E. coli* and *S. aureus*. Thus, characteristics of rhamnolipid type biosurfactant produced by *Pseudomonas* sp. BUP6 may broaden its applications in various fields as potential candidate for green technologies.

Chapter 8

Rumen microbes as producers of polyhydroxyalkanoates with emphasis on *Candida tropicalis* BPU1

Published: Priji, P., Sajith, S., Sreedevi, S., Unni, K. N., Kumar, S. and Sailas Benjamin. (2015). *Candida tropicalis* BPU1 produces polyhydroxybutyrate on raw starchy substrates. *Starch-Stärke*, 67, 1-10.

Aim and rationale

Polyhydroxyalkanoates (PHAs) are eco-friendly microbial biopolymers, produced as inclusion bodies under the nutrient stress, which can be used as alternatives to synthetic plastics manufactured from petrochemicals. Hence, the production and characterisation of biopolymers from high yielding microorganism is highly recommended to meet its growing demand. Based on the aforesaid background, the present study is focused on the (a). comparative analysis of the potentials of *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6 for the production of PHAs which showed positive results upon preliminary screening; (b). development of suitable fermentation strategy using natural starchy substrates for maximising the yield; and (c). characterisation of the biopolymer produced.

Introduction

Most of the bioplastics are microbial polyesters that are potentially biodegradable due to the presence of hydrolysable ester bonds (Witt *et al.*, 2001). Polyhydroxybutyrate (PHB) − the predominant member of PHAs − is synthesised and stored in microbial cells as carbon and energy reserves under nutrient-limiting conditions with excess carbon (Belal, 2013). Many species of *Bacillus, Alcaligens, Ralstonia, Pseudomonas, Streptomyces, Rhodococcus* and *Micrococcus* are well known to produce PHB granules in the cell (Davis *et al.*, 2013; Sindhu *et al.*, 2013; Urtuvia *et al.*, 2014).

Commercial exploitation of PHB as biopolymer is mainly hindered by the high economics of its production and purification. One kg of PHB costs about 15 to 30 US$, whereas the cost of polypropylene – one of the synthetic polymers - is as low as US$ 0.70 (Khanna and Srivastava, 2007). Therefore, the investigations on new microbial sources, low cost substrates and

cultivation strategies for the production of PHAs still remain as a challenge so as to provide it at cheaper rate; therefore, research on PHAs is an emerging and broadening area. Though, excess carbon induces accumulation on PHAs in microbes, the raw and natural starchy substrates like plant tubers and seeds (stored plant foods) are not explored effectively as substrate for their production. Hence, the present study is aimed at analysing the rumen microbes, *i.e.*, *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6, for the production of biodegradable biopolymers, the PHAs.

Materials and methods

Growth and production profiles in medium supplemented with starch

Two microbial cultures, *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6 were grown in SPS medium (composition is mentioned in chapter 2) and production of PHAs was monitored regularly at 12 h interval. The experiment was initiated with 1 % inoculum (v/v) from 24 h old culture. The cell pellet was collected by centrifugation at 8,000 × *g* for 10 min; from which PHAs were extracted after sonication and quantified.

Extraction of PHAs

The pellet, after washing twice in sterile ddH_2O, was re-suspended in 20 ml of chloroform and sonicated for 10 min at output wattage of 10 W. To this, equal volume of sodium hypochlorite (30 %) was added and incubated at 37 ^{o}C for 1 h. The mixture was then centrifuged to get 3 layers - the top aqueous layer, middle layer containing cell debris and the bottom layer of chloroform with PHAs. The heavier bottom layer was collected and evaporated at 25 ^{o}C to obtain PHAs, which were weighed and quantified. Yield of PHAs is defined as the weight of the PHAs to the cell dry weight of the biomass per liter of culture broth.

Use of natural substrates for the production of PHAs

The fine flours of natural starchy products (stored food of plants) - such as tubers of potato and tapioca or jack seed (dried in oven and powdered using mixer grinder) - was supplemented in the medium replacing commercial starch, to assess its influence on the production of PHAs. At regular intervals of 12 h, the cell pellets were collected by centrifugation (8,000 × *g* for 10 min.) to extract PHAs.

Statistical optimisation of PHAs production

In order to optimise the production of PHAs by *C. tropicalis* BPU1, four different environmental factors such as pH, temperature, substrate (potato flour) concentration and incubation time were selected for the statistical analysis. The interactive effects of these parameters on the production of PHAs were evaluated employing Box-Behnken design. Minitab version 14 (Minitab Inc. USA) was used to generate and analyse the experimental design consisting of 27 trials. Analysis at three levels (high, medium and low) represented by +1, 0 and -1, respectively was performed for each parameter (**Table 29**). The residual starch was also determined for each trials employing phenol-sulphuric acid method. A second order polynomial equation including all interacting terms was used to calculate the predicted response.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j,\ i<j$$

Where, Y represents the response variable, β_0, β_i, β_{ii}, β_{ij} are intercept, linear, quadratic and interaction constant coefficients, respectively when $i < j$, and k is the numbers of involved variables.

Table 29. The levels and code of variables used for Box-Behnken experimental design.

Variable	Factor	Levels		
		-1	**0**	**+1**
X_1	pH	5	6	7
X_2	Temperature (^oC)	25	32.5	40
X_3	Incubation (h)	18	27	36
X_4	Potato flour (%)	0.5	1.25	2.0

To check the validity of quadratic model, four random experimental conditions - as predicted by the point prediction software Minitab 14 - (different from Box-Behnken, but within the range investigated) - were performed.

Characterisation of PHAs

The crystals of PHAs, obtained at the optimised conditions using potato flour as substrate were characterised employing the techniques of thin layer chromatography (TLC), UV-visible spectrophotometry, Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and thermal gravimetric analysis (TGA).

Thin layer chromatography (TLC)

The crystals containing PHAs (as described above) were esterified with trichloroethylene, HCl and 1-propanol (in the ratio 5:1:4) for 1 h at 100 ^oC. After cooling (24 ^oC), 1 ml of sterile ddH_2O was added to the propanolysed sample for phase separation, 5 µl of the separated organic phase was spotted on silica gel TLC plates. The TLC was run with ethyl acetate and benzene (1:1) as solvent system, followed by drying in an oven (40 ^oC). Subsequently, the plates were transferred to iodine chamber for visualisation, and the R_f value was calculated.

UV-visible spectrophotometry

Scrapes of the spots observed on TLC plates were eluted with hot chloroform (50 ^{o}C), and centrifuged at 9,400 × *g* for 20 min to collect the supernatant, *i.e*., free of silica; subsequently chloroform was evaporated off; 3 ml of conc. H_2SO_4 was added to the residue and heated in a boiling water bath for 15 min. After cooling (25 ^{o}C), the absorbance of the sample was measured in the range λ_{190} - λ_{400} against conc. H_2SO_4 as blank.

Fourier transform infrared spectroscopy (FTIR)

The PHA crystals (10 mg) were ground well with 10 mg of spectral grade anhydrous potassium bromide (KBr); the powder was pelleted for IR analysis, and the relative intensity of transmitted light was measured against the wavelength of absorption in the region 400-4000/cm using FTIR spectroscope (Jasco 4100 series, Japan).

Nuclear magnetic resonance (NMR)

^{1}H and ^{13}C NMR spectra of PHA crystals were analysed at 400 MHz using Bruker 400 Avance III spectrophotometer (Bruker BioSpin Corp., Billerica MA). The sample was prepared by dissolving the PHA crystals in deuterated chloroform by mild heating.

Thermal gravimetric analysis (TGA)

TG analysis was conducted using Pyris TGA (Perkin Elmer, Inc., USA) at a temperature scanning rate of 10 ^{o}C/min from 40 to 750 ^{o}C with nitrogen flow rate of 80.0 ml/min, to examine the thermal stability of PHA crystals.

Results

Production of PHAs

The microbial cultures, *C. tropicalis* BPU1 and *Pseudomonas* sp. BUP6 were grown in medium containing commercially available soluble starch (1 %) at 37 ^{o}C for 2 d to collect the cell mass, which was subsequently lyophilised. Rapid cooling under vacuum during lyophillisation crystallised the accumulated PHAs, which appeared as sharp protuberances on the cells. Lyophillised cells were sonicated to release the cell contents, and sodium hypochlorite dissolved the lipids except PHAs, which was extracted using hot chloroform. The topologies and structures of crystals of PHAs were investigated using microscopes (**Figure 64 and 65**). The typical spherulite morphology and radial stacking were observed during crystallisation. The PHAs formed crystals with dimensions of around 300×300×3 μm (length×breadth×width) (**Figure 64D and 65F**). The chloroform extract upon evaporation produced crystals containing PHAs, which were quantified. *C. tropicalis* BUP1 produced PHAs in higher quantities *i.e.*, 0.39 g/g cdw at 24 h of incubation, in comparison to *Pseudomonas* sp. BUP6 which was about 0.27 g/g cdw at 36 h of incubation (**Figure 66**). Hence, *C. tropicalis* BPU1 was selected for further studies.

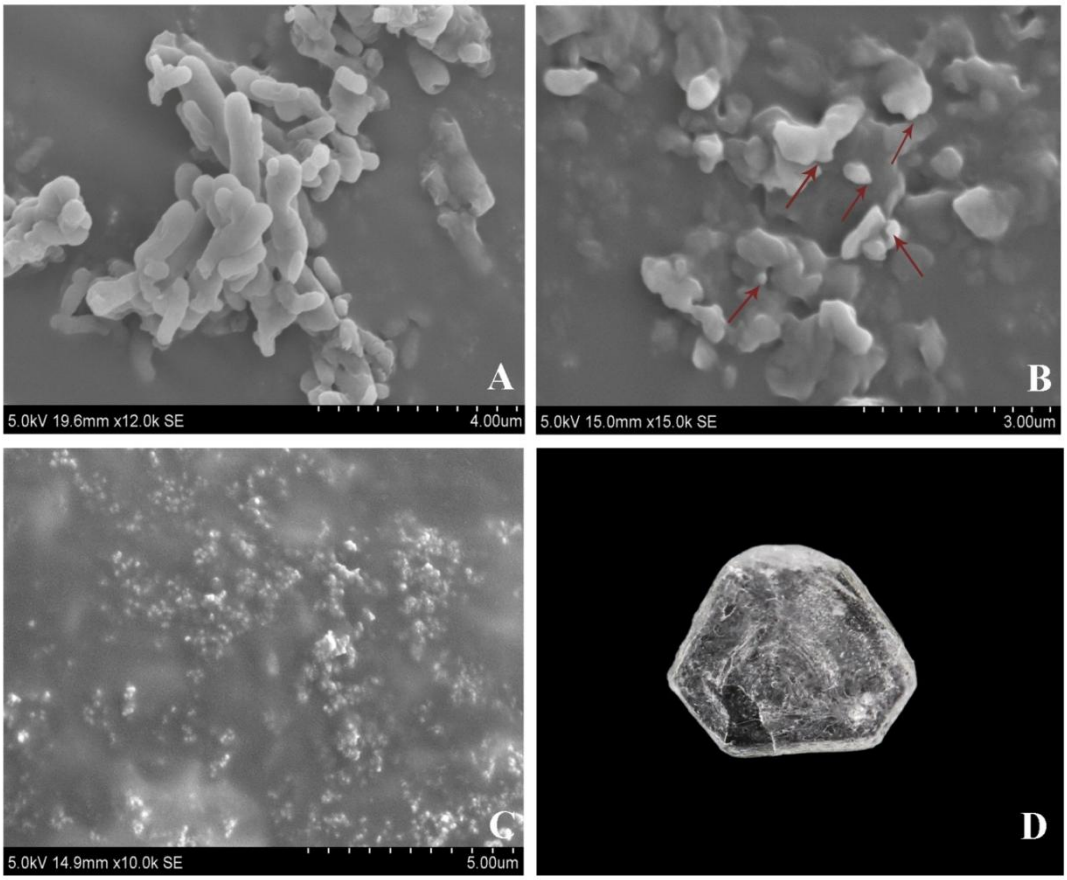


Figure 64. Extraction of PHAs from *Pseudomonas* sp. BUP6: (A). *Pseudomonas* sp. BUP6 growing in medium supplemented with commercial starch; (B). lyophilised cells of *Pseudomonas* sp. BUP6 showing protuberances of crystallised PHAs; (C). scanning electron micrograph of extracted PHAs in chloroform (Hitachi SU660, Japan); and (D) digital image of a magnified crystals of PHAs (DSLR Canon 450 D, Japan).

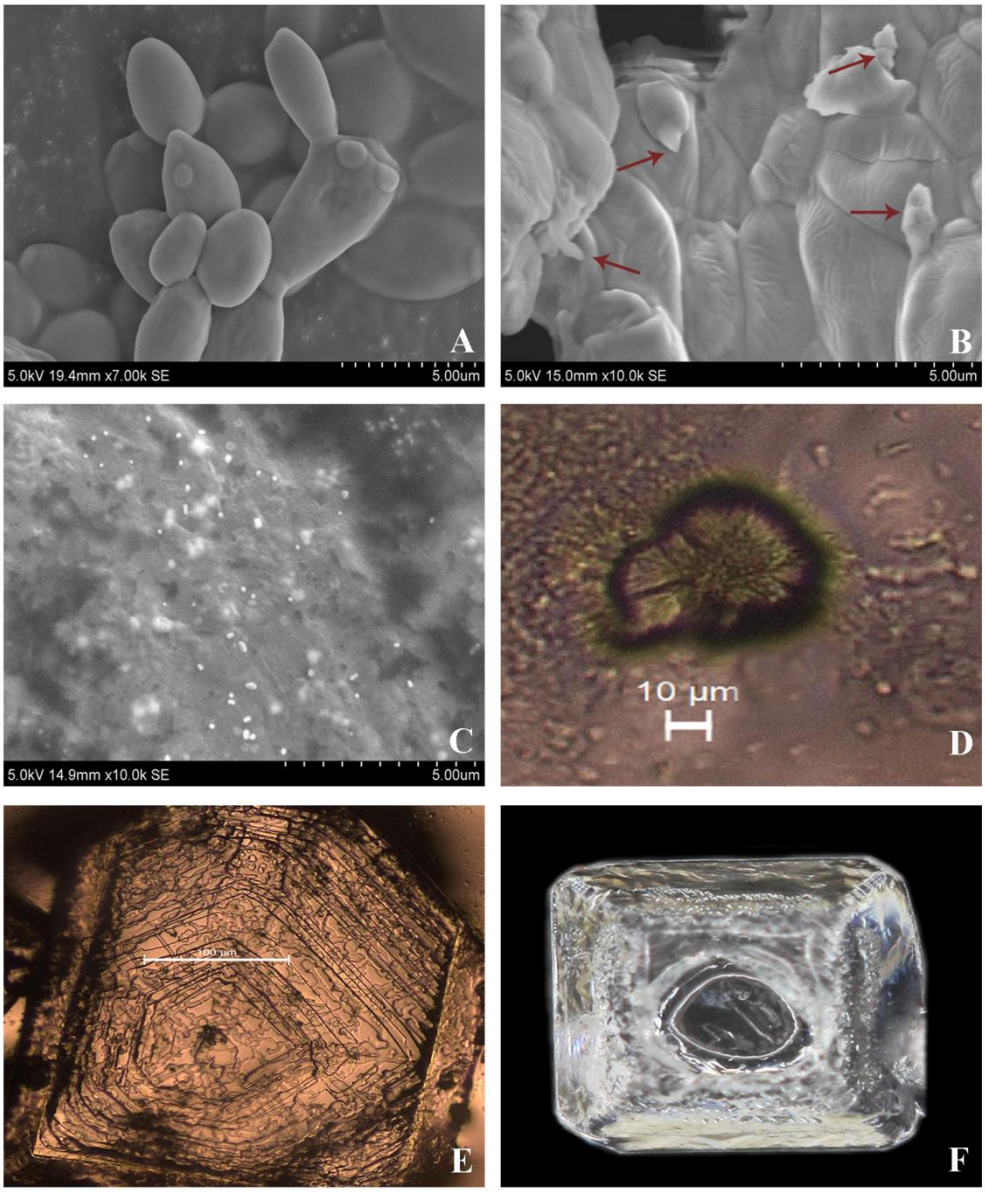


Figure 65. Extraction of PHAs from *C. tropicalis* BPU1: (A). *C. tropicalis* BPU1 growing in medium supplemented with commercial starch; (B). lyophilised cells of *C. tropicalis* BPU1 showing protuberances of crystallised PHAs; (C). scanning electron micrograph of extracted PHAs in chloroform (Hitachi SU660, Japan); (D). spherulite morphology during PHAs crystallisation (phase contrast microscope; Leica M80, Germany); (E). PHAs crystals showing the banding pattern of growth (phase contrast microscope); and (F). digital image of a magnified PHA crystal (DSLR Canon 450 D, Japan).

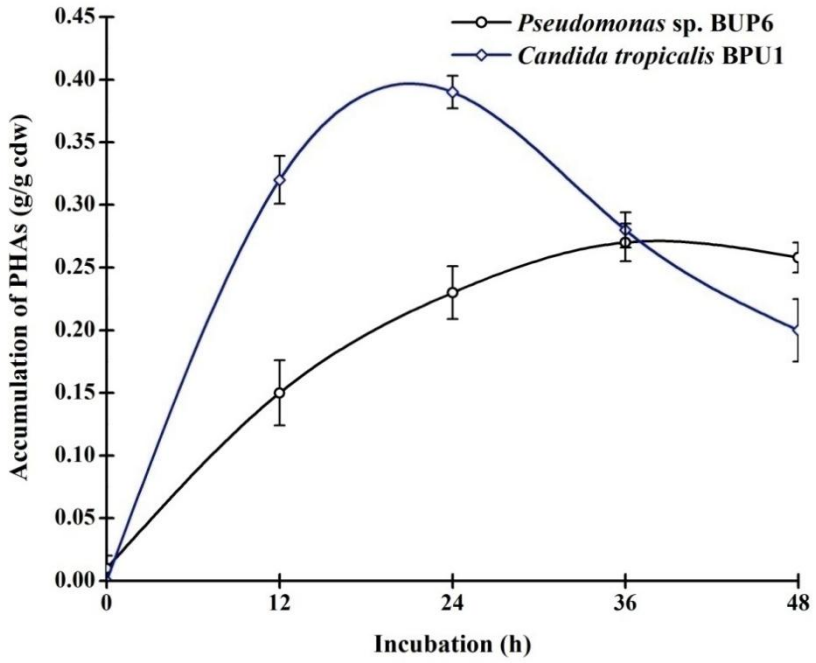


Figure 66. Production profiles of PHAs by the rumen isolates. *C. tropicalis* BPU1 showed the maximum production of PHAs (0.39 g/g cdw) on 24 h of incubation whereas *Pseudomonas* sp. BUP6 supported the maximum production of PHAs of 0.27 g/g cdw on 36 h of incubation.

Natural raw substrates for PHA production

C. tropicalis BPU1 was cultured in medium supplemented with natural raw and starchy substrate (potato flour, tapioca flour or jack seed flour), replacing the commercially available soluble starch and the production of PHAs was estimated. Among the three substrates, potato flour supported the maximum production of PHAs (0.36 g/g cdw) at 24 h of incubation, which was quite comparable with that of commercially available soluble starch (0.39 g/g cdw). The other two natural substrates (tapioca flour and jack seed flour) supported PHAs production at lower quantities, *i.e.*, 0.24 and 0.28 g/g cdw at 12 and 24 h of incubation, respectively (**Figure 67**).

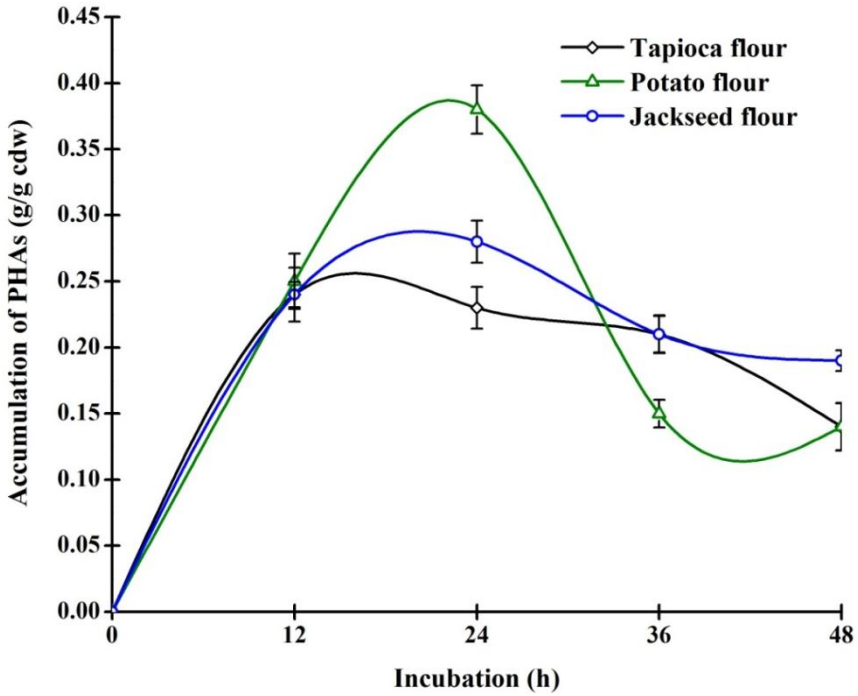


Figure 67. Production of PHAs in medium supplemented with natural substrates by *C. tropicalis* BPU1. Potato flour supported the maximum production of PHAs of 0.36 g/g cdw at 24 h of incubation.

Statistical optimisation of PHA production

Potato flour which supported the maximum yield of PHAs was used as substrate for statistical optimisation. A set of 27 experiments were designed according to Box-Behnken design (**Table 30**), and the regression coefficients were estimated for the production of PHAs *vs.* pH, temperature, incubation and concentration of potato flour. The statistical significance was analysed by ANOVA (**Table 31**). A second order polynomial function was fitted to the experimental yield of PHAs to obtain 2-dimentional contour and 3-dimentional response surface plots (**Figure 68 and 69**), which resulted in the following regression equation.

$$Y=-4.50994+0.922054X_1+0.0754147X_2+0.0117883X_3+1.22411X_4-0.0363505X_1^2-0.0002X_2^2-0.0007X_3^2-0.056X_4^2-0.00707X_1X_2-0.00107X_1X_3-0.205259X_1X_4+0.00014X_2X_3-0.0128924\ X_2X_4+\ 0.0203298\ X_3X_4$$

Table 30. Box-Behnken design matrix with the respective responses in terms of PHAs yield.

Run Order	pH	Temperature (^{o}C)	Incubation (h)	Potato flour (%)	Residual starch (%)	cdw (g)	PHB yield (g/g)
1	6	25	27	2	89.7	0.54	0.39
2	6	40	18	1.25	54.5	3.81	0.43
3	7	32.5	18	1.25	27.13	4.00	0.32
4	6	32.5	27	1.25	45.2	3.49	0.4
5	5	40	27	1.25	57.74	3.09	0.47
6	7	32.5	27	2	56.96	3.71	0.15
7	6	40	36	1.25	20.67	4.22	0.32
8	7	25	27	1.25	53.4	3.20	0.34
9	5	32.5	36	1.25	17.19	5.54	0.34
10	6	25	18	1.25	21.98	4.15	0.38
11	6	32.5	18	0.5	79.65	1.86	0.5
12	6	32.5	36	0.5	63.28	0.5	0.12
13	5	25	27	1.25	42.25	3.76	0.27
14	6	32.5	27	1.25	46.75	3.54	0.41
15	7	40	27	1.25	63.36	2.28	0.32
16	6	40	27	2	47.71	3.46	0.31
17	7	32.5	36	1.25	54.2	3.93	0.24
18	5	32.5	18	1.25	27.72	4.8	0.38
19	6	40	27	0.5	64.92	2.7	0.49
20	7	32.5	27	0.5	55.0	3.36	0.5
21	6	25	36	1.25	46.75	3.54	0.23
22	5	32.5	27	0.5	14.92	5.32	0.25
23	6	25	27	0.5	30.45	4.50	0.29
24	6	32.5	36	2	28.677	4.86	0.39
25	5	32.5	27	2	67.72	2.79	0.43
26	6	32.5	18	2	37.72	3.92	0.22
27	6	32.5	27	1.25	48.24	3.61	0.42

Table 31. Analysis of variance (ANOVA) for PHAs yield

Source	DF	Seq SS	Adj SS	AdjMS	F	P
Regression	14	0.285153	0.285153	0.020368	38.69	0.000
Linear	4	0.059046	0.059046	0.014761	28.04	0.000
Square	4	0.022966	0.022966	0.005741	10.91	0.001
Interaction	6	0.203141	0.203141	0.033857	64.32	0.000
Residual Error	12	0.006317	0.006317	0.000526		

The proposed regression model for the production of PHAs by *C. tropicalis* BPU1 was highly significant at $p<0.05$. The value (0.98) of determination coefficient (R^2) indicated the aptness of the model, which was in reasonable agreement with the adjusted R^2 value of 0.95, and this ensured a satisfactory adjustment of the quadratic model to the experimental data.

Four random experimental conditions (different from Box-Behnken, but within the range investigated) were evaluated for the validation of the model. In all these instances, model prediction was in good agreement with the experimental data (considering the experimental error), and correlation coefficient was found to be 0.98 (**Table 32**). Correlation coefficient was close to unity, suggesting the significance of the model. The optimum production of PHAs was found as 0.59 g/g cdw (at 38 ^{o}C, pH 6.9, potato flour 0.5 % and incubation 19 h). Thus, the statistical optimisation resulted in 0.6 fold (from 0.36 to 0.59 g/g) increase in the accumulation of PHAs by *C. tropicalis* BPU1 over the unoptimised condition.

Table 32. Random combinations of parameters selected for the validation of proposed statistical model with respective predicted responses in terms of the production of PHAs

pH	Temperature (^{o}C)	Incubation (h)	Substrate (%)	Predicted PHAs yield (g/g cdw)	Observed PHAs yield (g/g cdw)
6.9	38	19	0.5	0.63	0.59
6	35	24	1	0.45	0.46
7	40	30	1	0.34	0.31
6	40	30	1.5	0.39	0.34

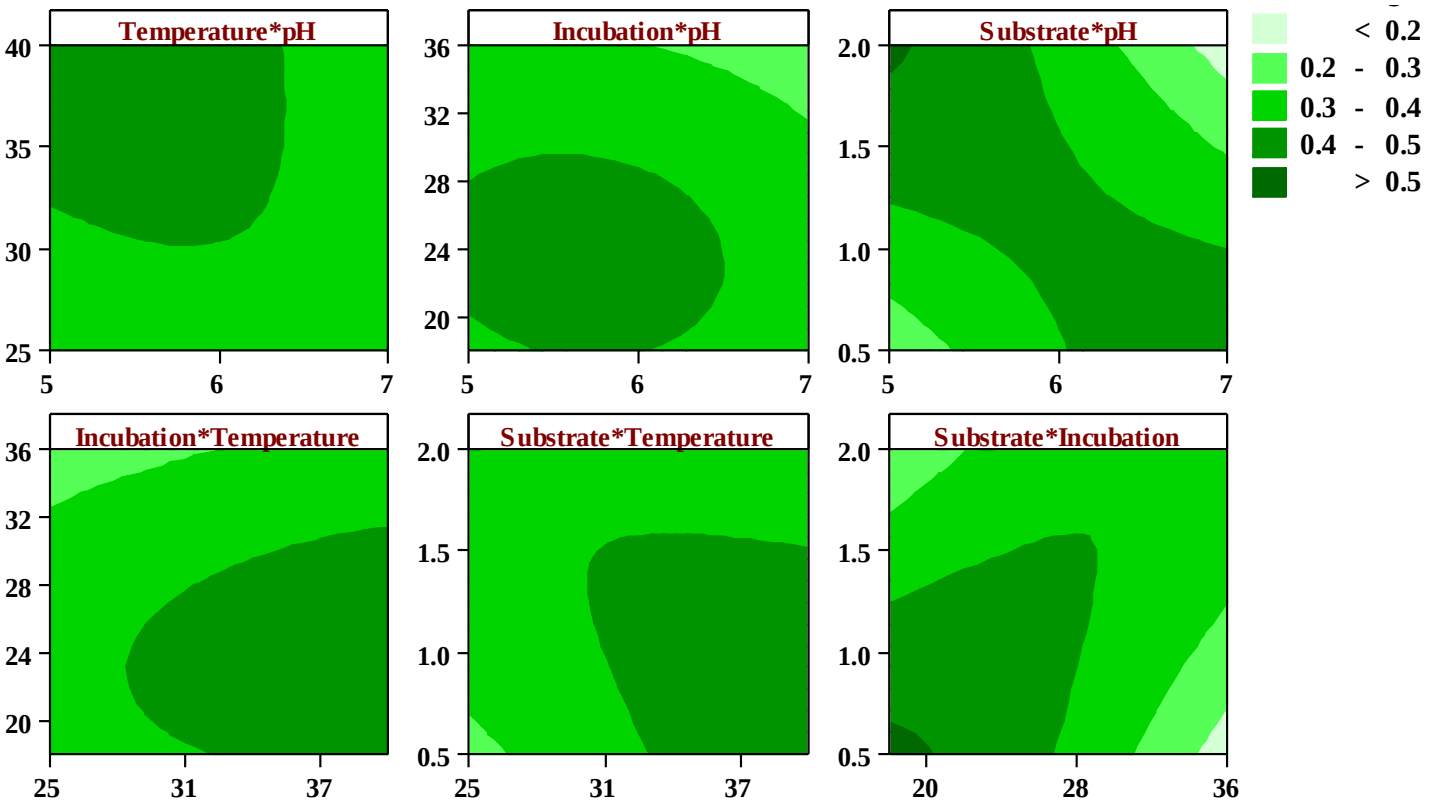


Figure 68. Contour plots described by the proposed model showing the interactive effects of parameters, temperature (^{o}C), pH, incubation (h) and potato flour (%) on the production of PHAs (g/g cdw) by *C. tropicalis* BPU1.

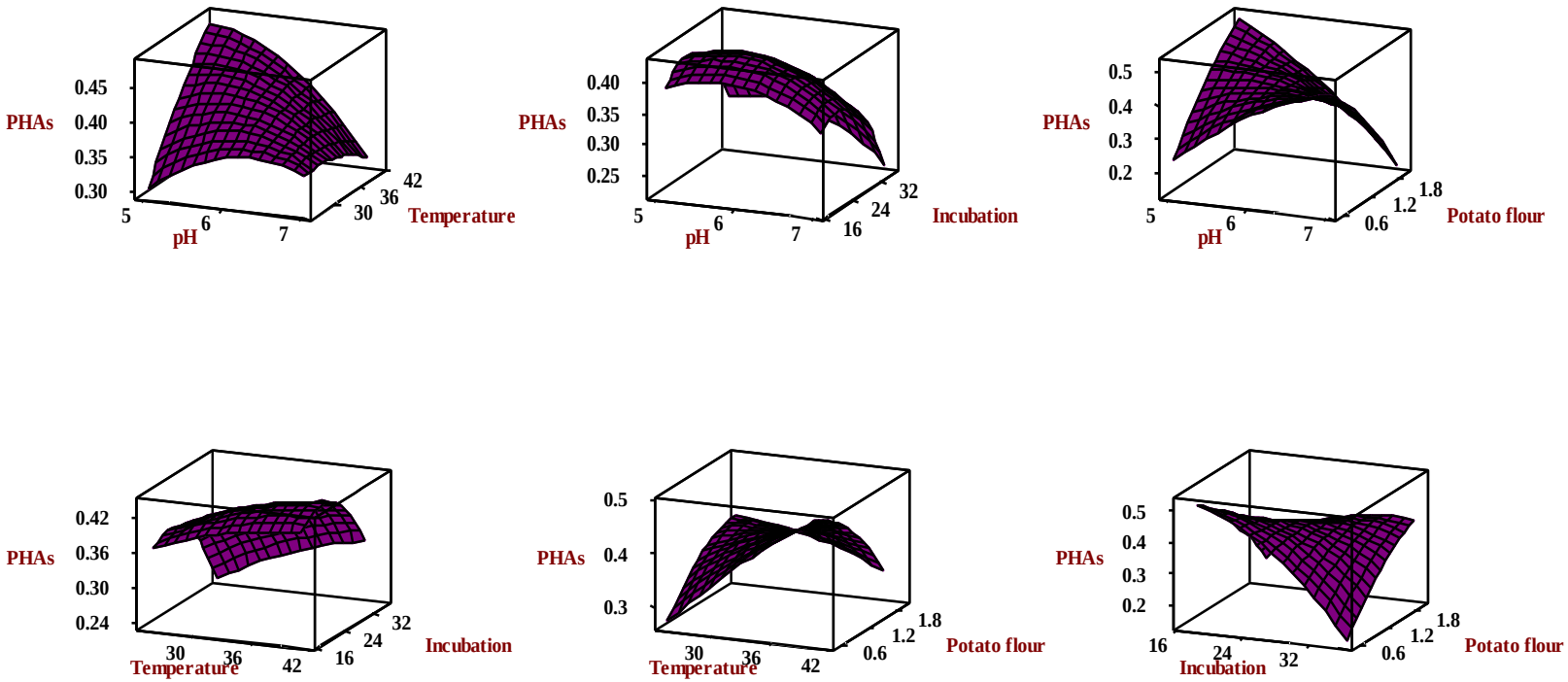


Figure 69. Response surface plots described by the proposed model showing the interactive effects of parameters, temperature (^{o}C), pH, incubation (h) and potato flour (%) on the production of PHAs (g/g cdw) by *C. tropicalis* BPU1.

Characterisation of PHAs

TLC and UV-vis spectroscopy

PHAs were extracted from the production medium by solvent extraction method, subjected to propanolysis to break down the polymer. TLC profile of the extract showed a spot with R_f value of 0.8 (**Figure 70A**). Hot H_2SO_4 converted the PHAs in the extract to crotonic acid, which showed an absorption maximum at $\lambda_{234,}$ a clear indication of the predominance of butyric acid moieties in the sample (**Figure 70B**).

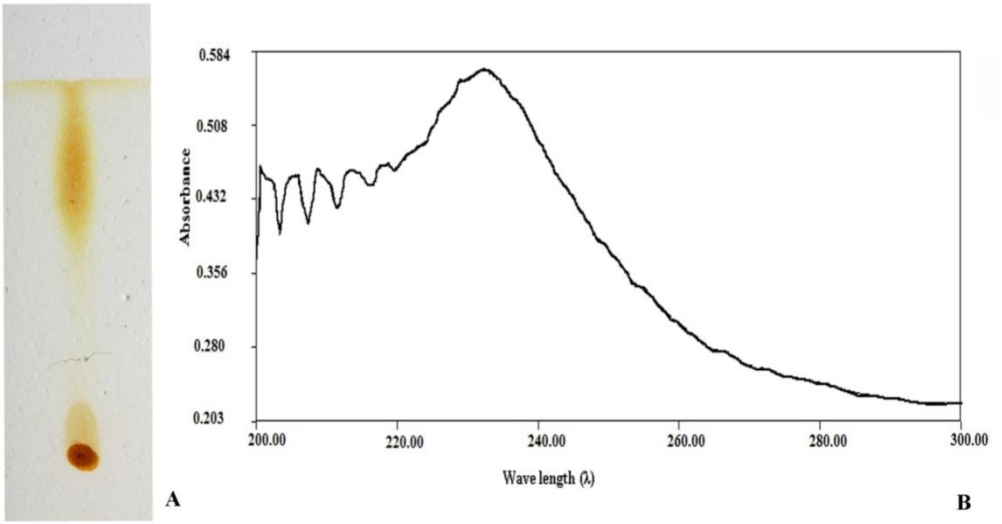


Figure 70. Characterisation of PHAs by TLC and UV-visible spectrophotometry: (A). separated spot on TLC with R_f value 0.8 upon visualisation with iodine vapor; and (B). absorption spectrum of PHA, extracted from the TLC spots, after conversion to crotonic acid with conc. H_2SO_4 showing absorption maximum at λ_{234}.

Fourier transforms infrared spectroscopy (FTIR)

The FTIR spectroscopic result (**Figure 71**), showed distinct peaks at 3420/cm and 3000-2850/cm, which correspond to the -OH and -CH- stretchings. The absorption peak at 1741/cm was reported to be a PHA marker band, assigned to

carbonyl (-C =O) ester bond stretching vibration, whereas the absorption bands at 1000-1200/cm were characteristic of C-O stretchings.

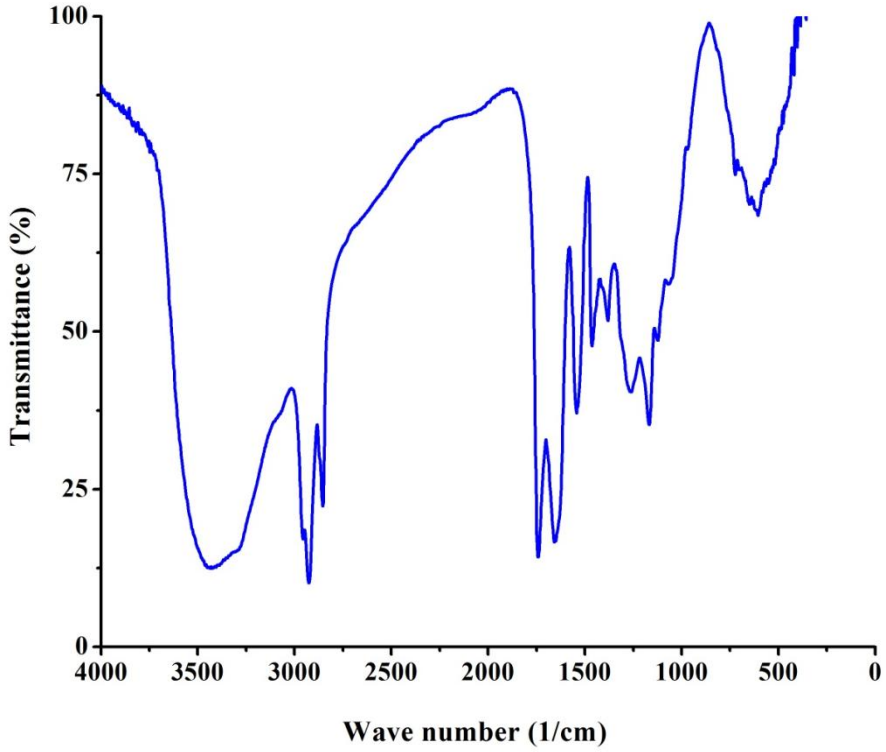


Figure 71. FTIR profile of PHA crystals

Nuclear magnetic resonance (NMR)

The ^{1}H NMR spectrum showed the expected resonances for PHB; *i.e.*, the methyl group at 1.2 ppm, the methylene group at 2.4 and 2.6 ppm, and the methine group at 5.2 ppm (**Figure 72A**). The ^{13}C spectrum showed four sharp peaks of strong intensities, characteristic to the methyl (19.6 ppm), methylene (40.8 ppm); methine (67.5 ppm) and carbonyl (169.7 ppm) groups of PHB (**Figure 72B**). The other weak resonances on both the spectra may be the impurities or cellular materials trapped during extraction.

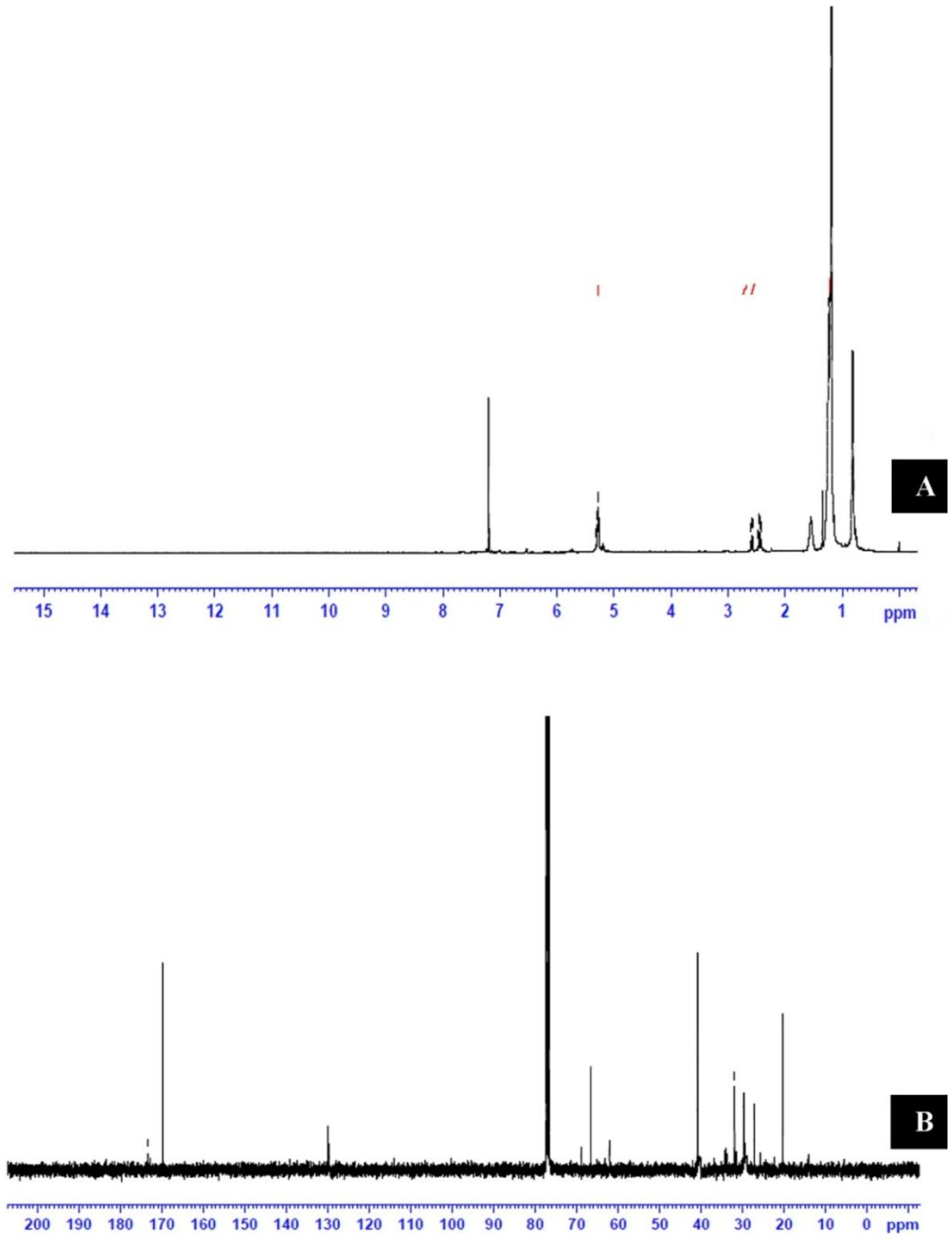


Figure 72. NMR spectra of PHB crystals: (A). ^{1}H NMR spectrum; and (B). ^{13}C NMR spectrum.

Thermal gravimetric analysis (TGA)

From the thermo gravimetric analysis (**Figure 73**), the temperature range for rapid thermal degradation of PHB was from 240 to 345 ^{o}C with the degradation peak at 273 ^{o}C. The total weight loss within this temperature range was 92 %. The initial drop in weight by about 6 % might be due to the loss of water and other impurities trapped during crystallisation.

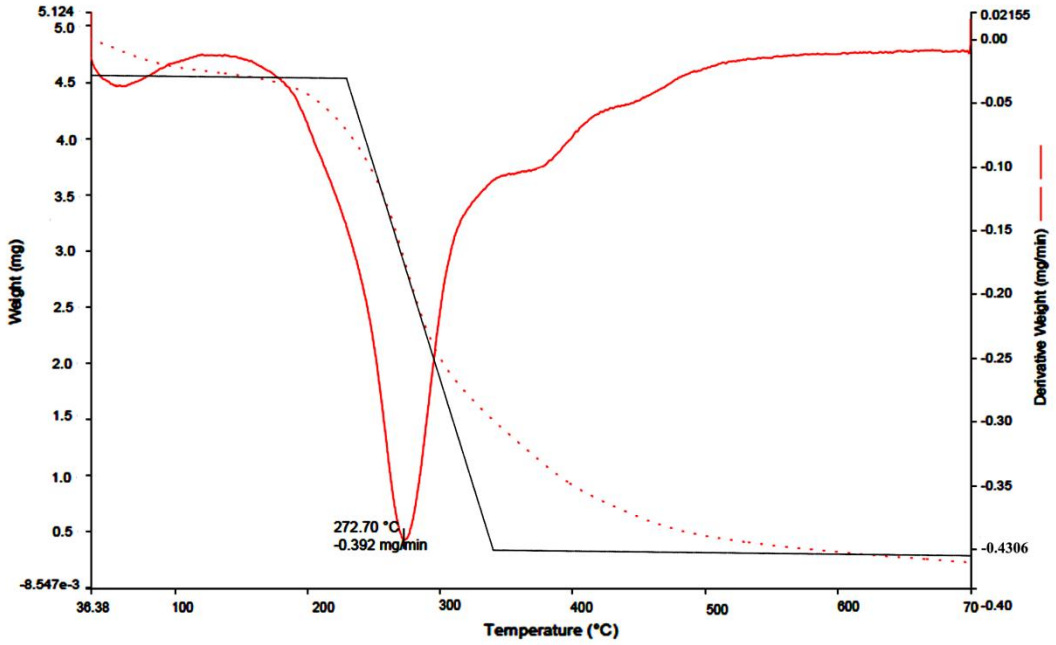


Figure 73. Thermal gravimetric analysis of PHB crystals showed rapid thermal degradation at temperature ranging from 240 to 345 ^{o}C with maximum at 273 ^{o}C.

Discussion

This study reports the potential of rumen microbes *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6 − the novel isolates from the rumen of Malabari goat − to produce PHAs on commercial soluble starch. *C. tropicalis* BPU1 produced PHAs in higher quantities (0.39 g/g cdw), as compared to *Pseudomonas* sp. BUP6, therefore, *C. tropicalis* BPU1 was focused for further studies. Naturally available raw starchy substrates (flours of potato,

tapioca and jack seed) were individually supplemented in the medium replacing the commercial starch to evaluate the efficiency of *C. tropicalis* BPU1 in producing PHAs. Among them, potato powder supported the maximum production of PHAs at comparable levels (0.36 g/g cdw). Using potato flour as substrate, Box-Behnken design and RSM were employed to statistically optimise the culture parameters, which resulted in 0.6 fold increase in production of PHAs over the unoptimised condition (potato powder 0.5 %, pH 6.9, 38 ^{o}C, and 19 h incubation). The crystals of PHAs produced by *C. tropicalis* BPU1 using potato powder as substrate were characterised by TLC, *UV-vis* spectrophotometry, FTIR spectroscopy, NMR and TGA, and the presence of PHB was confirmed in the crystals. Moreover, the crystals showed typical spherulite morphology during its growth, which were thermostable upto 240 ^{o}C. Briefly, this study projects the possibilities of the utilisation of cheap and raw starchy agro-products as substrate for the production of PHAs, especially PHB, and that the eukaryotic unicellular *C. tropicalis* BPU1offers much industrial significance.

The PHB is natural hydrophobic and biodegradable polyester with relatively high melting point and crystallinity. The high cost involved in its production and purification is one of the major bottlenecks toward commercialisation of PHB as a cheaper biopolymer. Hence, the exploration of new strains, substrates and cultivation strategies still remain as a major area of research. *C. tropicalis* BPU1 produced PHB on starchy substrate, which was extracted in crystalline form. The spherulites − semi-crystalline regions associated with crystallisation of polymers from the melt (Barham *et al.*, 1984) − were clearly observed during crystallisation, which grew radially with stacking. It was reported that physical treatments such as centrifugation may readily coalesce the PHB granules into larger masses, which can lead to the apparent acceleration of its crystallisation (Lauzier *et al.*, 1992). The presence of PHB in the crystals was confirmed by TLC, *UV-vis* and FTIR. The thin layer

chromatogram showed a single spot with R_f vaue of 0.8, and uopon sulphuric digestion *UV-vis* spectrum was produced with the maximum absorbance at λ_{234} corresponding to that of crotonic acid (Law and Slepecky, 1961). The FTIR analysis of the crystals showed the presence of ester, -CH stretching, carbonyl group, *etc*., characteristic to PHB and the spectrum resembled closely with the PHB produced by *Vibrio azureus* BTKB33 and *Bacillus* spp. (Sathiyanarayanan *et al.*, 2013b; Sasidharan *et al.*, 2014; Biradar *et al.*, 2015). NMR spectrum also confirmed the presence of PHB in the crystals as reflected by the characteristic resonance of methyl (CH_3), methylene (CH_2) and methane (CH) groups (Sathiyanarayanan *et al.*, 2013a; Dalal *et al.*, 2013). Thermal analysis of the PHB crystals showed rapid degradation at temperature 240 to 345 ^oC (*i.e*., thermostable until 240 ^oC); the initial drop in the thermogram of the PHB crystals may be due to the presence of traces of water and other impurities trapped in it. Upon slow evaporation, water molecules would establish hydrogen bonds with the carbonyl groups of the polyester backbone, resulting in '*pseudo cross-links*' between adjacent polymer chains (De Koning and Lemstra, 1992). It was reported that most of the microbial PHBs are thermostable until 240 to 250 ^oC. PHB produced by *Halomonas* sp. SA8 showed rapid thermal degradation between 245 and 290 °C (de Castro *et al.*, 2014), whereas PHB produced by *A. eutrophus* showed thermal degradation between 250 and 300 ^oC (Hahn *et al.*, 1995).

Irrespective of the great deal of efforts made for the commercialisation of biopolymer, the wide use of PHB remains scarce, because of its high production cost. The cost efficiency in the production of PHB is highly determined by the raw materials used. Generally, substrates rich in sugars such as agro-industrial waste oils, whey from the dairy industry, lignocellulosic wastes, molasses *etc*. were used as substrates for the microbial production of PHB (Purushothaman *et al.*, 2001; Povolo and Casella, 2003; Keenan *et al.*, 2006; Koller *et al.*, 2007). Use of cheap, readily available raw

substrates for the production of PHB still remains as an untapped area of research. This study provided clear evidences for the enhanced production of PHB in raw starchy substrates by *C. tropicalis* BPU1, and the demonstrated productivity was the highest among the known PHB producing yeasts; for instance the yeast, *Saccharomyces diastaticus* produced 0.25 g/g cdw of PHB in a medium containing tryptophan (Şafak *et al.*, 2002). The yield in the present study is quite comparable to that of many bacterial species *Bacillus, Pseudomonas, etc*.; for instance, *Bacillus thuringiensis* IAM 12077 produced 0.44 g/g cdw of PHB in nitrogen deficient medium supplemented with glucose (Pal *et al.*, 2009), and *Pseudomonas cepacia* produced 0.5 g/g cdw of PHB in a medium containing xylose and lactose (Young *et al.*, 1994). Sugar beet juice was also found as suitable source for the production of PHB, which supported an yield of 0.39 g/g cdw PHB by *Alcaligenes latus* (Wang *et al.*, 2013). The advantage of using starchy substrates as carbon source is that, its price is much lower than that of commercial sugars. Since the statistical tools address the linear as well as quadratic interactions of two or more parameters at a time, it could be applied efficiently for evaluating the optimisation of various biotechnological processes (Oliveira *et al.*, 2015). Thus, as demonstrated in this study, statistical optimisation employing Box-Behnken design and RSM enhanced the PHB yield by 1.6 times – an indication for its industrial significance.

Starch is a renewable carbon source available in large quantities, and that most of the plant seeds and tubers are rich in starch. Potato is the third most important food crop of the world after rice and wheat; of which over 50 % is grown in Asian countries like India and China (Horton and Sawyer, 1985). Tapioca and jack seed are also cheap and readily available starchy substrates in these areas. Utilisation of these cheaper crops and seeds for the production of costly biopolymer would reduce the production cost. Moreover, the utility of starchy substrates offers a new strategy for the industrial and economic

exploitation of the under-utilised crops and seeds, especially employing solid- or semisolid- state fermentation systems.

Conclusions

Conventional *C. tropicalis* is known for clinical significance, but this study clearly demonstrates its industrial significance as well. The raw starchy substrates cost around 0.2 to 0.5 US$ per kilogram, whereas the cost of commercial starch is around 30 US$. The high yield of PHB on raw starchy substrates would benefit the biopolymer industry by applying suitable fermentation strategies including submerged fermentation or solid state fermentation. Moreover, application of eukaryotes, especially unicellular microbes such as yeast, for the production of industrially significant molecules is of great significance, possibly with simple genetic manipulations to enhance the production to meet future needs.

Chapter 9

Summary and Conclusions

Principal Goal: The principal goal of the study was to explore the rumen microflora of Malabari goat (*Capra hircus* L.) with a view to produce industrially significant biomolecules employing economically and environmentally feasible strategies. Therefore, three diverse, but prominent representatives of biocatalyst, surface active molecules and bioplastics with emphasis on lipase, biosurfactant and polyhydroxybutyrate were focused in this study. The principal goal is subdivided into four parts: (1) screening, isolation and characterisation of microorganisms capable of producing lipase, biosurfactant and/or polyhydroxybutyrate from the rumen of Malabari goat; (2) production, purification and characterisation of lipase by *Pseudomonas* sp. BUP6; (3) production and characterisation of biosurfactant by *Pseudomonas* sp. BUP6; (4) production and characterisation of polyhydroxyalkanoates (PHAs) by *Candida tropicalis* BPU1.

Introduction

Environmental pollution and the deterioration of the natural non-renewable resources are the major problems that the world is facing today. To balance the environment for living, the global community is now focusing on the natural resources and biomolecules to replace their synthetic counterparts, which are very often harmful to health. The strict Government legislations also add on to the growing demand for the industrially significant biomolecules to establish a sustainable environment globally. Currently, the need for these biomolecules is often met by exploiting microorganisms, due to their rapid multiplication on cheap renewable substrates, high yield, climatic independency, and ease of genetic manipulation. Biocatalysts, biosurfactants and bioplastics are some of the major groups of industrially significant biomolecules produced by microorganisms.

Lipases: or triacylglycerol hydrolases, (EC 3.1.1.3) catalyse the hydrolysis/synthesis of esters formed between glycerol and long-chain fatty

acids. ***Biosurfactants***: are amphiphilic compounds with hydrophobic and hydrophilic termini that reduce the surface and/or interfacial tensions between the two immiscible phases and are mainly produced on microbial cell surfaces or excreted extracellularly. ***Bioplastics***: most of the bioplastics like PHAs are microbial polyesters that are potentially biodegradable, due to the presence of hydrolysable ester bonds. Owing to their low toxicity and biodegradability, they found potential applications in various industries dealing with food, dairy, cosmetics, detergents, bioremediation, oil processing *etc.* as candidates of green technologies to replace their synthetic counterparts.

Rumen is the primary site of fermentation in cattle, wherein a wide variety of microbes including bacteria, fungi and protozoa reside. It is expected that the rumen microflora is highly adapted to a wide variety of hydrocarbons as a part of their diet. Since the bioactive molecules being focused in this study are partly or completely inducible in nature, it is expected that the rumen microbes can perform more or less similar manner as that of any other microbial resources, *i.e.*, to thrive on harsh environments such as contaminated soil, industrial wastes, *etc*. In fact, apart from clinical significances, only a few studies have explored the rumen microflora for the production of industrially significant biomolecules. Thus, such ruminal microbes can safely and effectively be exploited in various industries beneficial to mankind. Upon the foresaid background, this study focused on the production, optimisation and characterisation of lipase, biosurfactant and PHAs produced by the novel isolates of the rumen of Malabari goat (*Capra hircus* L.).

Summary

The major findings emerged out of this study are summarised under the following four parts;

(1) Isolation, screening and characterisation of rumen microorganisms capable of producing lipase, biosurfactant and/or PHAs

Rumen content of both male and female Malabari goats (*Capra hircus* L.) were collected aseptically from the local slaughter house at Chelari, near the University of Calicut, Kerala, India (11.18189600 ^{o}N, 75.82206300 ^{o}E). Initially, the microbial consortium of goat rumen was cultured on modified de Man, Rogosa and Sharpe medium under anaerobic condition in an anaerobic chamber, saturated with mixed gas (80 % N_2, 10 % CO_2 and 10 % H_2). The consortium in liquid medium was gradually adapted to the aerobic system in a conical flask, specially designed for this study, designated a *Benjamin flask* which was then cultivated on nutrient agar and potato dextrose agar media for the isolation of bacteria and fungi, respectively. The purity of the isolates was confirmed by repeated subcultures.

The four bacterial and three fungal isolates thus obtained were then screened for the production of lipase, biosurfactant and PHAs. Screening for the production of lipase (tween 80 agar method and chromogenic plate method) and biosurfactant (blood haemolysis test, drop collapse assay, cetyltrimethylammonium bromide agar plate test) was done on medium containing vegetable oil for induction, whereas production of PHAs was assessed by staining the cultures grown on medium containing soluble starch with specific dyes such as Sudan black B, Sudan 3 and Nile blue sulphate A. Among the seven cultures, one bacterial isolate showed positive results for the production of all the three biomolecules whereas one fungal isolate showed positive for the production of biosurfactant and PHAs. The isolates were then

characterised by morphological, biochemical and molecular techniques (sequencing of 16S rRNA gene for the bacterium and D1/D2 regions of the large subunit 28S rRNA gene for the fungus), and identified as *Pseudomonas* sp. strain BUP6 (GenBank accesion No. KF 550910; MTCC No. 5925) and *Candida tropicalis* strain BPU1 (GenBank accession No. JQ353488; MTCC No. 5920).

(2) Production, purification and characterisation of lipase by *Pseudomonas* sp. BUP6

The novel isolate, *Pseudomonas* sp. BUP6, from the rumen of Malabari goat showed significant production of lipase when grown in a newly designed basal medium [(per litre): 5 g NH_4NO_3, 4 g $(NH_4)_2SO_4$, 3 g yeast extract, 2 g K_2HPO_4, 2 g NaCl, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$] supplemented with vegetable oil. Suitability of 5 vegetable oils such as groundnut oil, coconut oil, olive oil, sunflower oil and palm oil as inducer for the production of lipase was examined, and groundnut oil supported the highest lipase production, *i.e.*, 96.15 U/ml. Various physical parameters required for the maximum production of lipase were optimised statistically. Plackett-Burman design was employed to study the interactive effects of physical parameters and found that temperature, agitation and pH, effected the production of lipase significantly. The optimum conditions for lipase production (37 °C , 200 rpm and pH 6.9) were detected by Box-Behnken design and response surface methodology, which resulted in the 0.3 fold increase (*i.e.*, 126 U/ml) in lipase production over the un-optimised condition.

The extracellular lipase was purified to homogeneity (35.7 times purified with 14.9 % yield) employing $(NH_4)_2SO_4$ salt precipitation and sephadex G-100 chromatography. The purified lipase showed a prominent band of 35 kDa on SDS-PAGE, which was confirmed by MALDI-TOF/MS. Lipase from *Pseudomonas* sp. BUP6 was found stable at a pH range of 7-9, with the

maximum activity (707 U/ml) at pH 8.2 and 102 % relative activity. Lipase was found active at temperature ranging from 35-50 ^{o}C with the maximum activity (891 U/ml) at 45 ^{o}C, 128 % relative activity. Triton X-100 (0.5 %) and EDTA (0.25 %) showed no effect on the activity of lipase, whereas tween 20, tween 80 and β-mercaptoethanol reduced the activity significantly. Moreover, Ca^{2+} (1.0 mM) enhanced the activity of lipase (1428 U/ml) to 206 % of the initial activity. Mg^{2+} and Ni^{2+} slightly enhanced the lipase activity, whereas Zn^{2+}, Fe^{2+} and Cu^{2+} reduced the activity significantly. Using *para*-nitrophenylpalmitate as substrate, the *Km* (11.6 mM) and *Vmax* (668.9 μmol/min/mg) of the purified lipase were also determined. It shows that the extracellular, thermotolerant and alkaline lipase from *Pseudomonas* sp. BUP6 offers potentials for industrial applications.

(3) Production and characterisation of biosurfactant produced by *Pseudomonas* sp. BUP6

The isolates, *Pseudomonas* sp. BUP6 and *C. tropicalis* BPU1 were grown in basal medium (composition is mentioned on the previous part) supplemented with 0.5 % of groundnut oil as microbial biosurfactants are generally produced in the presence of hydrocarbons. The culture supernatants were then acidified to precipitate the crude biosurfactant and weighed. Among the two cultures, *Pseudomonas* sp. BUP6 supported the maximum production of biosurfactant of 1912 mg/l, in comparison to *C. tropicalis* BPU1 (596 mg/l), and used for further studies. The crude biosurfactant was then extracted with chloroform:methanol (2:1) after dissolving in sterile ddH_2O. The organic layer was evaporated to get the yellow colored and honey-like biosurfactant.

The parameters for the production of biosurfactant by *Pseudomonas* sp. BUP6 were statistically optimised using the software MINITAB version 14 to maximise the yield. Initial screening of 5 parameters (pH, temperature, agitation, incubation and substrate concentration) was carried out employing

Plackett-Burman design, which reduced the number of parameters to 3 (pH, temperature and incubation); according to their significance on biosurfactant yield. A suitable statistical model for the production of biosurfactant by *Pseudomonas* sp. BUP6 was established according to Box-Behnken design, employing a set of 22 experiments and the determination coefficient of 0.97 clearly indicated the suitability of the model. A set of four experiments was conducted to analyse the validity of the model using the point prediction tool of MINITAB 14 and the correlation coefficient of the observed and predicted yields was found to be 0.98, a value very close to unity, which indicated the aptness of the model. The biosurfactant produced by *Pseudomonas* sp. BUP6 was then characterised for its physico-chemical, biological and structural properties. The biosurfactant showed the critical micelle concentration (CMC) as 48 mg/l, and was found stable at a wide range of pH (3-9). It maintained more than 90 % of its emulsification ability even after boiling, as well as in presence of sodium chloride (0.5 %). The anti-bacterial and anti-adhesive properties of the biosurfactant were investigated against four pathogenic/opportunistic pathogens such as *Escherichia coli, Proteus mirabilis, Staphylococcus aureus,* and *Acetinobacter baumanni* which showed that at concentrations of 100 mg/l and 50 mg/l, the biosurfactant inhibited the growth and adhesion of *E. coli* and *S. aureus* significantly. The phytotoxicity studies were carried out using the seeds of rice and green gram, and revealed that the biosurfactant did not have any adverse effect on seed germination. The structural characterisation of biosurfactant employing orcinol method, thin layer chromatography and FTIR indicated that the biosurfactant produced by *Pseudomonas* sp. BUP6 belongs to the category the rhamnolipid, a glycolipid. Thus, *Pseudomonas* sp. BUP6 was proved as an efficient producer of rhamnolipid type biosurfactant.

(4) Production and characterisation of PHB by *C. tropicalis* BPU1

The major group of microbial bioplastics - PHAs - was focused in this study. PHAs are accumulated intracellularly in presence of excess carbon and/or limited supply of phosphorus. Initially, commercially available soluble starch (1 %) was supplemented in a medium containing (g/l) 2.5 peptone and 1 NaCl (pH 7), designated as SPS medium, to evaluate the efficiency of *Pseudomonas* sp. BUP6 and *C. tropicalis* BPU1 for the production of PHAs; of them, *C. tropicalis* BPU1 showed better yield of PHAs, and hence, *C. tropicalis* BPU1 was focused in the subsequent studies. Instead of commercial starch, naturally available raw starchy substrates such as flours of potato, tapioca or jack seed was supplemented in the medium, and the PHA produced in crystal-like form was quantified. Among them, potato powder and commercial starch supported the maximum production of PHAs at comparable levels of 0.36 g/g cell dry weight (cdw) and 0.39 g/g cdw, respectively. Subsequently, using potato powder as substrate, Box-Behnken design and response surface methodology were employed to statistically optimise the culture parameters (pH, temperature, incubation and substrate concentration), which resulted in the 0.6 fold increase (*i.e.*, 0.59 g/g cdw) in production of PHAs over the un-optimised condition (potato flour 0.5 %, pH 6.9, 38 ^{o}C, and 19 h incubation). The PHA crystals showed typical spherulite morphology during its growth, and they were characterised as PHB by TLC, UV-visible spectrophotometry, FTIR spectroscopy and NMR. The thermal analysis of PHB crystals showed that they were thermostable upto 240 ^{o}C.

Conclusions

The key message behind the study is that understanding nature opens our view to exploit the wealth of possibilities in the future. On the other hand, the rumen ecosystem provides ongoing enrichment and natural selection of microbes adapted to specific conditions, and represents a virtually untapped

resource of novel products such as enzymes, biopolymers and biosurfactants. Even though the production of these biomolecules have been studied extensively in the last decades, its production on large scale met reality only in a very few cases, and even now it found only limited applications owing to the remarkably high prices, compared to traditional synthetic chemicals. Therefore, the present study reveals an interesting option for the future which is not realised yet and could be used to decrease production costs.

Major outcomes/Deliverables

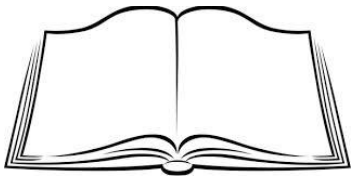
This is the first ever report on the production of industrially significant biomolecules such as lipase, rhamnolipid and polyhydroxybutyrate from the microflora inhabiting the rumen of Malabari goat (*Capra hircus* L.)

- ✓ The rumen of Malabari goat was explored, many bacteria and fungi were identified from the rumen content; of them, *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6 were found significant.
- ✓ *C. tropicalis* BPU1was characterised by morphological, biochemical and molecular techniques; its 28S rRNA gene sequence was identified; culture details were submitted to GenBank (JQ353488).
- ✓ *Pseudomonas* sp. BUP6 was characterised by morphological, biochemical and molecular techniques; its 16S rRNA gene sequence was identified; culture details were submitted to GenBank (KF550910).
- ✓ *C. tropicalis* BPU1 was deposited under Budapest Treaty (patent deposit) at IMTECH with MTCC No. 5920.
- ✓ *Pseudomonas* sp. BUP6 was deposited under Budapest Treaty (patent deposit) at IMTECH with MTCC No. 5925.
- ✓ *Pseudomonas* sp. BUP6 is an efficient producer of lipase; it could produce 126 U/ml of lipase in specially designed basal salt medium using groundnut oil as inducer.

- ✓ Lipase from *Pseudomonas* sp. BUP6 was purified to homogeneity (MW 35 kDa), its maximum activity was 1428 U/ml at 45 ^{o}C and pH 8.2 in presence of Ca^{2+} (1.0 mM).
- ✓ *Pseudomonas* sp. BUP6 is an efficient producer of biosurfactant, it could produce 2070 mg/l of rhamnolipids in a specially designed basal salt medium supplemented with groundnut oil (0.5 %) at optimised conditions of pH 7, 35 ^{o}C and incubation for 75 h.
- ✓ *C. tropicalis* BPU1 produced PHB utilising raw starchy natural substrates as major source of energy.
- ✓ *C. tropicalis* BPU1 produced 0.59 g/g cdw of PHB at optimised conditions of potato flour 0.5 %, pH 6.9, 38 ^{o}C, and 19 h of incubation.
- ✓ Novel strategies for the production of lipase, biosurfactant and PHB were demonstrated.

Leads for further study

- ✓ Protein engineering of lipase produced by *Pseudomonas* sp. BUP6.
- ✓ Preparation of PHB blends for the exploration of its industrial utilities.
- ✓ Utility of rhamnolipids as candidates for green technologies.



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Appendices

Research papers (Published)

1) **Priji, P**., Sajith, S., Sreedevi, S., Unni, K. N., Kumar, S., and Sailas Benjamin. (2015). *Candida tropicalis* BPU1 produces polyhydroxybutyrate on raw starchy substrates. *Starch-Stärke*, 67, 1-10.

2) **Priji, P**., Unni, K.N., Sajith, S., Binod, P. and Benjamin, S. (2014). Production, optimization and partial purification of lipase from *Pseudomonas* sp. strain BUP6, a novel bacterium characterized from Malabari goat. *Biotechnology and Applied Biochemistry*, 62(1), 71−78.

3) **Priji, P**., Unni, K.N., Sajith, S. and Benjamin, S. (2013). *Candida tropicalis* BPU1, a novel isolate from the rumen of the Malabari goat, is a dual producer of biosurfactant and polyhydroxybutyrate. *Yeast*, 30, 103−110.

4) Benjamin, S., **Priji, P**., Sreedharan, S., Wright, A. D. G. and Spener, F. (2015). Pros and cons of CLA consumption: an insight from clinical evidences. *Nutrition and Metabolism*, 12(1), 4.

5) Neethu, K. B., **Priji, P**., Unni, K. N., Sajith, S., Sreedevi, S., Ramani, N., Anitha K., Rosana B., Girish M. B. and Benjamin, S. (2015). New *Bacillus thuringiensis* strain isolated from the gut of Malabari goat is effective against *Tetranychus macfarlanei*. *Journal of Applied Entomology*, DOI: 10.1111/jen.12235.

6) Smitha, R. B., **Priji, P**., Sajith, S., Ramani, N. and Benjamin, S. (2015). *Bacillus thuringiensis* subsp. *kurstaki* in raw solid fermented matter efficiently combats the coconut pest, *Aceria guerreronis* Keifer. *Bt Research*, 6. doi: 10.5376/bt.2015.06.0002.

7) Sajith, S., Sreedevi, S., **Priji, P**., Unni, K. N. and Benjamin, S. (2015). Production and partial purification of cellulase from a new isolate, *Penicillium verruculosum* BS3. *British Microbiology Research Journal*, 9(1), 1-12.

8) Unni, K.N., Abdul Faisal, P., **Priji, P**., Sajith, S., Sreedevi, S., Hareesh, E. S., Nidheesh Roy, T. A. and Benjamin, S. (2015) Rubber seed kernel as potent solid substrate for the production of lipase by *Pseudomonas aeruginosa* strain BUP2. *Advances in Enzyme Research*, 3, 31-38.

9) Smitha, R. B., Sajith, S., **Priji, P**., Unni, K. N., Nidheesh Roy, T. A. and Benjamin, S. (2015). Purification and characterization of amylase from *Bacillus thuringiensis* subsp. *kurstaki*. *Bt Research*, 6, 1−8.

10) Sreedevi, S., Unni, K. N., Sajith, S., **Priji, P**., Sarath Josh, M. K. and Benjamin, S. (2014). Bioplastics: Advances in polyhydroxybutyrate research. *Advances in Polymer Science*, Doi.10.1007/12_2014_297.

11) Jisha, V. N., Smitha, R. B., **Priji, P**., Sajith, S. and Benjamin, S. (2014). Biphasic fermentation is an efficient strategy for the overproduction of δ-endotoxin from *Bacillus thuringiensis*. *Applied Biochemistry and Biotechnology*, 1−17.

12) Unni, K. N., **Priji, P**., Geoffroy, V. A., Doble, M. and Benjamin, S. (2014). *Pseudomonas aeruginosa* BUP2 - A novel strain isolated from Malabari goat produces type 2 pyoverdine. *Advances in Bioscience and Biotechnology*, 5(11), 874.

13) Sajith, S., Sreedevi, S., **Priji, P**., Unni, K. N. and Benjamin, S. (2014). Production and partial purification of cellulase from a novel fungus, *Aspergillus flavus* BS1. *Annals of Microbiology*, 64, 763–771.

14) Abdul Faisal, P., Hareesh, E. S., **Priji, P**., Unni, K. N., Sajith, S., Sreedevi, S., Sarath Josh M. K. and Benjamin, S. (2014). Optimization of parameters for the production of lipase from *Pseudomonas* sp. BUP6 by solid state fermentation. *Advances in Enzyme Research*, 2(04), 125.

15) Jisha, V. N., Smitha, R. B., Pradeep, S., Sreedevi, S., Unni, K. N., Sajith, S., **Priji, P**., Sarath Josh M. K. and Benjamin, S. (2013). Versatility of microbial proteases. *Advances in Enzyme Research*, 1(3), 39−51.

16) Benjamin, S., Smitha R. B., Jisha, V. N., Pradeep, S., Sajith, S., Sreedevi, S., **Priji, P**., Unni, K. N. and Sarath Josh, M. K. (2013). A monograph on amylases from *Bacillus* spp. *Advances in Bioscience and Biotechnology*, 4, 227−241.

17) Rubeena M., Neethu, K. B., Sajith S., Sreedevi S., **Priji, P**., Unni K. N., Sarath Josh M. K., Jisha, V. N., Pradeep S. and Benjamin, S. (2013). Lignocellulolytic activities of a novel strain of *Trichoderma harzianum*. *Advances in Bioscience and Biotechnology*, 4, 214−221.

18) Neethu, K. B., Rubeena M., Sajith S., Sreedevi S., **Priji**, **P**., Unni K. N., Sarath Josh M. K., Jisha, V. N., Pradeep S. and Benjamin, S. (2012). A novel strain of *Trichoderma viride* shows complete lignocellulolytic activities. *Advances in Bioscience and Biotechnology*, 3, 1160−1166.

Research papers (Communicated)

1) **Priji, P**., Sajith, S., Unni, K. N., Abdul Faisal, P. and Benjamin, S. (2015). Microbial lipases − Properties and applications. *Journal of Microbiology, Biotechnology and Food Sciences*, (Under review).

2) **Priji, P**., Sajith, S., Unni, K. N. and Benjamin, S. (2015). Glycolipid biosurfactants as candidates for green technologies. *Journal of Detergents and Surfactants*, (Under review).

3) **Priji, P**., Sajith, S., Unni, K. N. and Benjamin, S. (2015). Purification and characterisation of thermotolerent alkaline lipase from *Pseudomonas* sp. BUP6, a rumen bacterium. *Electronic Journal of Biotechnology*.

4) **Priji, P**., Sajith, S., Sreedevi, S., Unni, K. N. and Benjamin, S. (2015). Production, optimisation, and characterisation of biosurfactant produced by *Pseudomonas* sp. strain BUP6, a novel rumen bacterium isolated from Malabari goat. *Journal of Colloids*.

Seminars/ Proceedings

1) **Priji, P**., Unni, K. N. and Benjamin, S. (2014). A novel strategy for dual production of lipase and PHB by *Pseudomonas* sp. strain BUP6 characterised from rumen of Malabari goat. IJAA, MHS-2014, AABS-Mysore.

2) Unni, K. N., **Priji, P**. and Benjamin, S. (2014). *Pseudomonas aeruginosa* BUP2, a novel strain isolated from Malabari goat produces type 2 pyoverdine. IJAA, MHS-2014, AABS-Mysore.

3) **Priji, P**., Unni, K. N., Shibu Vardhanan, Y. and Benjamin, S. (2011). Rumen bacteria isolated from Indian goat transform vegetable oil into conjugated linoleic acids. Gregor Mendel Foundation, University of Calicut.

GenBank Submissions

1. *Candida tropicalis* strain BPU1 28S ribosomal RNA gene, partial sequence

Accession number: JQ353488

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VERSION     JQ353488.1  GI:384254804
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REFERENCE   1  (bases 1 to 603)
  AUTHORS   Benjamin,S., Prakasan,P. and Unni,K.N.
  TITLE     Conjugated linoleic acid producing yeast from the rumen of Malabari
            goat
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 603)
  AUTHORS   Benjamin,S., Prakasan,P. and Unni,K.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-JAN-2012) Enzyme Technology Laboratory, Biotechnology
            Division, Department of Botany, University of Calicut, Thenhipalam,
            Kozhikode, Kerala 673635, India
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2. *Pseudomonas* sp. BUP6 16S ribosomal RNA gene, partial sequence

Accession number: KF550910

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  ORGANISM  Pseudomonas sp. BUP6
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            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 1409)
  AUTHORS   Benjamin,S., Priji,P. and Unni,K.N.
  TITLE     Direct Submission
  JOURNAL   Submitted (17-JUL-2013) Enzyme Technology Laboratory, Biotechnology
            Division, Department of Botany, University of Calicut, Thenhipalam,
            Kozhikode, Kerala 673635, India
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सूक्ष्मजीव प्ररूप संवर्धन संग्रह एवं जीन बैंक
MICROBIAL TYPE CULTURE COLLECTION & GENE BANK
सूक्ष्मजीव प्रौद्योगिकी संस्थान
सैक्टर 39 ए, चंडीगढ़ 160036 भारत
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CSIR - IMTECH

By Courier

Dr. G.S. Prasad
Senior Principal Scientist

10.03. 2015

To

Dr. Sailas Benjamin,
Director of Research
University of Calicut
Kerala – 673 635

Dear Dr. Sailas Benjamin,

We are here with sending three freeze-dried ampoules of your following cultures sent for deposit in MTCC under Budapest Treaty,

S. No.	Taxonomic Designation	Identfication Reference	MTCC Number Assigned
1.	***Candida tropicalis***	**BPU1**	**MTCC 5920**
2.	***Bacillus amyloliquefaciens***	**BSS5**	**MTCC 5921**
3.	***Bacillus thuringiensis***	**BPU5**	**MTCC 5922**
4.	***Alcaligens faecalis***	**BP11**	**MTCC 5923**
5.	***Pseudomonas aeruginosa***	**BUP2**	**MTCC 5924**
6.	***Pseudomonas* sp.**	**BUP6**	**MTCC 5925**

Examine the viability and characters of the cultures from **one of the ampoules** and inform us whether the cultures are viable and identical in all aspects to the original culture deposited by you or not. Please fill the enclosed IDENTITY CHECK BY DEPOSITOR form and return it to us in 30 days. **Remaining two ampoules are for safe storage at your end.** Official communication related to acceptance of the culture in MTCC shall be sent to you after receiving duly completed Identity Check by Depositor form.

If we do not receive the duly filled IDENTITY CHECK BY DEPOSITOR form in 30 days, it is assumed that the culture is Identical in all aspects to the original culture deposited by you.

Please acknowledge the receipt of the ampoules.

Sincerely yours,

(G. S. PRASAD)

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Information : info.mtcc@imtech.res.in