

Final Technical report
Project Year (01-04-2013 to 31-03-2016)

Project Title:
**Production and characterization of industrially significant
biomolecules from *Candida tropicalis***



Submitted by
Priji Prakashan

File No: 024/WSD-BLS/2012/CSTE
Order No. 447/2013/KSCSTE dated 27.03.13

Submitted to
**WOMAN SCIENTIST DIVISION
KERALA STATE COUNCIL FOR SCIENCE TECHNOLOGY
AND ENVIRONMENT**

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Submitted by

Priji Prakasan
(Woman Scientist)

Prof. Sailas Benjamin
(Scientist Mentor)



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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 ₂ O	:	double distilled water
FTIR	:	fourier transform infrared spectroscopy
g/l	:	gram per litre
GLBS	:	glycolipid biosurfactant
mg/ml	:	milligram per millilitre
min	:	minutes
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
<i>p</i> NPP	:	<i>para</i> -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
µg	:	microgram
µl	:	microlitre

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Chapter 1

INTRODUCTION

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, pharmaceuticals, *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.

Surfactants are a 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 intensified 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).

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).

Apart from the clinical significance, only a few studies have been reported on the exploration of rumen microflora as a microbial source for the production of industrially significant biomolecules like biosurfactants (Ashish *et al.*, 2011). Under these circumstances, we introduce in this study a new strain of yeast, *Candida tropicalis* BPU1 capable of producing PHB and biosurfactant as well as a bacterium, *Pseudomonas* sp. BUP6 capable of producing biosurfactant and lipase with potentials for industrial exploitation. Specific objectives of the study were the following.

1. Statistical optimization of culture conditions for the production of biosurfactant and poly- β -hydroxybutyrate (PHB) by *C. tropicalis* BPU1.
2. Investigation on the suitability of various substrates for the production of biosurfactant and PHB.
3. Purification and molecular characterization of biosurfactant and PHB from the potent strain.
4. Physico-chemical characterization of the biosurfactant and PHB.

Additional objectives performed

5. Production, purification and characterisation of biosurfactant produced by *Pseudomonas* sp. BUP6.
6. Production, purification and characterization of lipase produced by *Pseudomonas* sp. BUP6

Chapter 2

REVIEW OF LITERATURE

Review of literature

Part I – Polyhydroxybutyrates (PHB)

The increasing environmental concern about the huge accumulation of non-biodegradable chemical pollutants and rapid depletion of non-renewable resources have directed the attention of human kind toward the microbially-derived and eco-friendly compounds, essentially due to their low toxicity and sustainability. Over the past decades, there has been considerable increase in the development and production of biodegradable plastics, which include polyhydroxy alkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides and copolymers and/or their proper blends. It opened up the way for new waste management strategies so as to overcome many undesirable properties of conventional plastics such as durability, resistance to biodegradation, release of toxic gases, concerns about phthalate plasticizers, *etc.* (Pradeep and Benjamin 2012; Pradeep *et al.*, 2014; Sarath Josh *et al.*, 2014). Most of the bioplastics are microbial polyesters, which are potentially biodegradable due to the presence of hydrolyzable ester bonds (Witt *et al.*, 2001). Poly-hydroxybutyrates (PHB) - the predominant member of PHAs - are synthesized and stored in microbial cells as carbon and energy reserves under nutrient-limiting conditions with excess carbon (Belal 2013). Many species of *Bacillus*, *Alcaligenes*, *Ralstonia*, *Pseudomonas*, *Streptomyces*, *Rhodococcus* and *Micrococcus* are well known to produce PHB granules (Davis *et al.*, 2013; Sindhu *et al.*, 2013; Urtuvia *et al.*, 2014).

Generally, production of PHB was well studied in bacteria, but not well documented in eukaryotic cells. However, a few yeasts, such as *Saccharomyces cerevisiae*, *Candida krusei*, *Kloeckera apiculata* and *Kluyveromyces africans* were found to accumulate PHB in their cytoplasm (Leaf *et al.*, 1996; Safak *et al.*, 2002). Since yeasts are well explored physiologically and genetically, production of PHB by them has certain advantages over bacteria. Larger size and physiological flexibility of yeast cells make it a suitable system for the production of industrially significant biomolecules through simple genetic manipulations (Priji *et al.*, 2013).

Commercial exploitation of PHB as biopolymer is mainly hindered by the higher economics of its production and purification. The cost of 1 kg of PHB is about 15 to 30 US\$, whereas the cost of polypropylene is about US\$ 0.70 (Khanna and Srivastava, 2007). Therefore, the investigations on new microbial sources, low cost substrates and cultivation strategies for the production of PHB still remain as a challenge to provide PHB at a cheaper rate; therefore, research on PHB is an emerging area. Though, excess carbon induces PHB accumulation in microbes, the raw and natural starchy substrates like plant tubers and seeds (stored plant foods) were not explored effectively as substrate for the production of PHB.

Part II - Biosurfactants

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.

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.

Part III – Lipases

Lipases are ubiquitous enzymes catalyzing 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 and pH, substrate specificity, enantioselectivity and diversity, lipases have considerable physiological significance with industrial potentials (Benjamin and Pandey 1998; Pandey *et al.*, 1999). In contrast to esterases, lipases are active at the interface of aqueous-nonaqueous system, but not on the dissolved substrates in the reaction medium (Pandey *et al.*, 1999). Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, acidolysis, trans-esterification, alcoholysis and aminolysis (Benjamin and Pandey 1998). Lipases occur widely in biota, but only microbial lipases are commercially significant because of the 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).

Lipases are produced by many microorganisms including bacteria such as *Bacillus* spp., *Lactobacillus* spp., *Pseudomonas* spp., *Staphylococcus aureus*, *S. haemolyticus*; yeasts such as *Candida rugosa* and *C. lipolytica* and fungi such as *Aspergillus* spp., *Rhizopus oryzae*, *Trichosporon laibacchii*, etc. (Chartrain *et al.*, 1993; Benjamin and Pandey 1998; Gao *et al.*, 2000; Namboodiri and Chattopadhyaya 2000). Lipase-producing microorganisms were isolated from diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, oilseeds, and decaying food, compost heaps, coal tips and hot springs (Wang *et al.*, 1995).

The enantio- and regio-selective nature of lipases are exclusively used 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). However, the commercial exploitation of microbial lipases is still in its infancy, due to the high economics of its production and purification. Therefore, the investigation on new lipase sources as well as cultivation strategies of microorganisms for the production of lipase still remains an emerging area of research.

Chapter 3

MATERIALS AND METHODS

Materials and methods

Part I – PHB

Microorganisms

C. tropicalis BPU1 (microbial type culture collection No. 5920) isolated from rumen of Malabari goat and reported from our laboratory [12] was used for this study. The partial 16S rRNA sequence of the isolate identified was submitted to the GenBank nucleotide sequence database with accession number JQ353488. *C. tropicalis* BPU1 was cultured in a medium containing (g/l), 10 soluble commercial starch; 2.5 peptone and 1 NaCl (at pH 6, 37 °C, and agitation 100 rpm) in a temperature controlled orbital shaker (in 100 ml Erlenmeyer flasks) (Scigenics Biotech, India). The culture was analyzed regularly at 12 h intervals for the production of PHB.

Extraction of PHB

The culture was centrifuged at 8000 ×g for 10 min to collect the cell pellet. The pellet, after washing twice in sterile ddH₂O, 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 °C for 1 h. The mixture was then centrifuged so as to get 3 layers - the top aqueous layer, middle layer containing cell debris and the bottom layer of chloroform with dissolved PHB. The heavier bottom layer was collected and evaporated at room temperature (25 °C) to obtain PHB crystals, which were quantified by weighing in a digital balance. PHB yield was defined as the weight of the PHB crystals to the cell dry weight per liter of culture broth.

Use of natural substrates for the production of PHB

The 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) - were supplemented to the medium, instead of commercial starch to assess their influence on the production of PHB. At regular intervals of 12 h, the cell pellets were collected by centrifugation to extract PHB and quantified.

Statistical optimization of PHB production

In order to optimize the production of PHB by *C. tropicalis* BPU1, four different environmental factors (pH, temperature, substrate concentration and incubation time) were selected for the statistical analysis. The interactive effects of these parameters on the production of PHB were evaluated employing Box-Behnken design. Minitab version 14 (Minitab Inc. USA) was used to generate and analyze the experimental design consisting of 27 trials [14]. Analysis at three levels (high, medium and low) represented by +1, 0 and -1, respectively was performed for each parameter. A second order polynomial equation including all interacting terms was used to calculate the predicted response.

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

Where, Y represents the response variable, β_0 , β_i , β_{ii} , β_{ij} are intercept, linear, quadratic and interaction constant coefficients, respectively, and k is the numbers of involved variables. The residual starch was also determined for each trials employing phenol-sulphuric acid method [15].

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.

Characterization of PHB

The crystals of PHB, obtained at the optimized conditions were characterized employing the techniques such as thin layer chromatography (TLC), UV-visible spectrophotometry, Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR) and thermal gravimetric analysis (TGA).

Thin layer chromatography

The PHB crystals (as described above) were esterified with trichloroethylene, HCl and 1-propanol (in the ratio 5:1:4) for 1h at 100 °C. After cooling (24 °C), 1 mL of sterile ddH₂O was added to the propanolyzed sample for phase separation [16], 5 µl of the separated organic phase was spotted on silica gel TLC plates. The TLC run was carried out in ethyl acetate and benzene (1:1) as solvent system, followed by drying in an oven (40 °C). Subsequently, the plates were transferred to iodine chamber for visualization, and the R_f value was calculated.

UV-Visible Spectrophotometry

Scrapes of the observed spot on TLC plates were eluted with hot chloroform (50 °C), and centrifuged at 9400 ×g for 20 min to collect the supernatant, *i.e.*, free of silica; subsequently chloroform was evaporated; conc. H₂SO₄ (3 mL) was added to the residue and heated in a boiling water bath for 15 min. After cooling (25 °C), the absorbance of the sample was measured in the range $\lambda_{190-400}$ against conc. H₂SO₄ as blank.

Fourier transform infrared spectroscopy (FTIR)

The PHB crystals (10 mg) were ground well with 10 mg of spectral grade anhydrous potassium bromide (KBr); and 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⁻¹ using Jasco FTIR spectroscope (4100 series, Japan).

Nuclear magnetic resonance spectroscopy (NMR)

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

Thermal Gravimetric analysis (TGA)

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

Statistics

All experiments were conducted in triplicates and the values were given as 'mean \pm SE'. Minitab version 14, USA was used to create and analyse Box-Behnken design and response surface methodology.

Part II – Biosurfactants

Extraction of biosurfactant

The culture was centrifuged at $6,400 \times g$ 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 °C to precipitate the biosurfactant completely; which was collected by centrifugation at $9,400 \times g$ for 10 min, washed twice with acidified water (pH 2) for obtaining the crude biosurfactant and weighed. The crude biosurfactant was re-dissolved in double distilled water (ddH₂O) having pH 7, extracted with equal volume of chloroform:methanol mixture (2:1); the organic phase was collected, and evaporated to get the yellow-colored and honey-like biosurfactant (Nitschke and Pastore, 2006).

Statistical optimization

The optimization studies of yield of biosurfactant were 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 was performed for each parameter and the result was analyzed using the equation (1).

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

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.

Box-Behnken design and RSM

Analysis at three levels (high, medium and low) represented by +1, 0 and -1 respectively, was performed for each parameter and the results were analyzed by fitting data to the second order polynomial equation (2).

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

Where: Y is lipase activity, X_1, X_2, X_3 are the independent variables, $\beta_1, \beta_2, \beta_3$ are the linear coefficients, $\beta_{12}, \beta_{23}, \beta_{13}$ are the interaction coefficients and $\beta_{11}, \beta_{22}, \beta_{33}$ are the quadratic coefficients.

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 statistically predicted values.

Identification of biosurfactant

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 °C for 30 min, followed by cooling to 25 °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 °C, and the separated spots were visualized using iodine vapour.

FTIR spectroscopy

Biosurfactant (5 mg) was mixed with spectral grade anhydrous 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).

LC-MS spectroscopy

Using an LCQ quadrupole ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA), the biosurfactant was analysed after dissolving in methanol at a concentration of 003 mg/ml. Prepared mobile phase A (acetonitrile) and B (water) and started gradient elution with 30% A in 70% B and which was then raised to 70% mobile phase A after 50 min.

Properties of crude biosurfactant

Physico-chemical properties

The physico-chemical properties of the crude biosurfactant were analyzed, *i.e.*, using the crude biosurfactant prepared by dissolving it (precipitated in acid) in ddH₂O at a concentration of 100 mg/l at pH 7.

Emulsification index (% EI₂₄)

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 (Cooper and Goldenberg, 1987).

Stability

For determining the thermal stability, crude biosurfactant was kept at different temperatures ranging from 4 to 100 °C for 30 min, then cooled to 25 °C and the emulsification index was determined using kerosene. Similarly, pH stability was analyzed 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 %) (Aparna *et al.*, 2014).

Critical micelle concentration (CMC)

Biosurfactant was prepared in ddH₂O 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 particular concentration of the biosurfactant in solution, at which the formation of micelle is initiated, *i.e.*, further increase in the concentration of biosurfactant did not reduce the surface tension of the solution.

Hydrophobicity index

The degree of adherence of *Pseudomonas* sp. BUP6 to various liquid hydrocarbons was determined spectroscopically. A suspension of *Pseudomonas* sp. BUP6 in ddH₂O 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,

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

Where H – Hydrophobicity index

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

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

Biological properties

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

Anti-bacterial activities

Anti-bacterial activities of the biosurfactant produced by *C. tropicalis* BPU1 was determined by micro-dilution method in 96-well flat bottom tissue culture plates against 4 different bacterial cultures, *viz.*, *Acinetobacter baumannii*, *Escherichia coli*, *Proteus mirabilis*, and *Staphylococcus aureus* (Rufino *et al.*, 2011). Briefly, 100 μ l 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 °C for 24 h, and absorbance was measured at λ_{600} . The culture without biosurfactant was maintained as control.

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

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 *C. tropicalis* BPU1 was determined by microdilution method in 96 well flat bottom tissue culture plates against 4 different bacterial cultures, viz., *A. baumannii*, *E. coli*, *P. mirabilis* and *S. aureus* (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 °C, and washed with sterile ddH₂O. Subsequently, 200 µl of bacterial culture (12 h old) was dispensed to each well, and incubated for 12 h at 4 °C. After incubation, the wells were washed with sterile ddH₂O for removing the 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 ddH₂O to remove excess stain. After air drying, the bound dye in each well was re-solubilized by adding 200 µl of 33 % acetic acid to each well, and absorbance was measured at λ_{595} , and ddH₂O without biosurfactant was kept as control.

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

Where: ODs is the optical density of the aqueous layer after treatment with the liquid hydrocarbon at λ_{595} ; and

OD_c is the 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 °C for 2 to 7 days to observe its effects on germination.

Statistics

All experiments were conducted in triplicates and the values were given as 'mean \pm SE'. Minitab version 14, USA was used to create and analyse Box-Behnken design and response surface methodology.

Part III – Lipases

Cultivation strategy and medium for the production of lipase

The pure culture obtained was further cultivated in basal medium, supplied with the required volume of groundnut oil in 'Benjamin flasks' for gradual adaptation to the aerobic system (Priji *et al.*, 2013) and incubated at 37 °C for 24 h in an incubator orbital shaker at 140 rpm (Scigenics Biotech, India). Initially, 0.05% groundnut oil was supplemented to the basal medium, and subsequently sub-cultured into fresh basal medium containing higher concentrations of groundnut oil. By repeated subculture under aerobic system, the oil consumption of the culture was enhanced to 0.5%.

Growth characteristics: During the incubation period, the culture was analyzed for growth and change in pH at regular intervals of 3 h duration. To monitor the bacterial growth, the turbidity measurement was carried out spectrophotometrically at λ_{600} .

Assay for lipase production: Production of lipase by the isolate was estimated quantitatively using *para*-nitrophenyl palmitate (pNPP) as substrate, at regular intervals of 3 h duration. The culture was centrifuged at $9,400 \times g$ for 10 min at 4 °C and the supernatant was assayed for lipase activity. The assay mixture containing 1.8 ml of 0.1M 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 °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 °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.

Vegetable oils as substrates: Suitability of various vegetable oils (0.5%) as inducer for the production of lipase was analyzed 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 optimization 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 1). For the selection of significant factors, Minitab version 14 (Minitab Inc. USA) was used to generate and analyze the experimental design of Plackett–Burman. The main effect of each variable was determined using the equation,

$$E = (\sum M_{i(+1)} - \sum M_{i(-1)}) / N \quad \text{Eq. (1)}$$

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

Box-Behnken model and response surface methodology: The significant parameters suggested by the placket-Burman design were selected for further statistical analysis. The interactive effect of these parameters on production of lipase was 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 result was analyzed by fitting to second order polynomial equation.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{13} X_1 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad \text{Eq. (2)}$$

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.

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.

Purification of lipase

Fractionation by (NH₄)₂SO₄ precipitation

Solid (NH₄)₂SO₄ salt was slowly added to the crude lipase preparation, so as to reach 20% saturation and the precipitate was collected by centrifugation (9,400 × g for 10 min at 4 °C). The supernatant was further precipitated to 20-60% and 60-80% saturation, and the precipitate was collected in each step. Addition of (NH₄)₂SO₄ was carried out with continuous stirring by keeping the solution on ice bath for 1 h in a cold room. The pellets were re-suspended in a minimum volume of 50 mM tris-HCl buffer (pH 8.0) for dialysis. The precipitates (pellets) obtained after (NH₄)₂SO₄ fractionation were dialyzed (cellulose membrane tubes) against 50 mM Tris-HCl buffer (pH 8) for 24 h at 4 °C with continuous stirring with two buffer changes in between. Lipase activity and protein content of each dialysate were determined (Lowry *et al.*, 1951). After that the dialysate was centrifuged (9400 × g for 10 min at 4 °C), and the supernatant so obtained was subjected to gel permeation chromatography.

Gel permeation chromatography

The dialysate fraction, which showed the highest specific activity, was used for gel permeation chromatography, in a column packed with sephadex-G100 (Sigma Aldrich, USA). Using the peristaltic pump (Rivera, India), the flow rate was maintained at 2 ml/20 min.

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.

SDS-PAGE was carried out using 4% stacking gel and 12% separating gel. Broad range protein molecular weight (MW) marker (Genei, Bangalore) 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 visualized using 0.1 % coomassie brilliant blue (CBB) G-250, and photographed.

Confirmation by MALDI-TOF/MS

The prominent lipase band on SDS-PAGE was excised, destained and digested with trypsin. The peptides so obtained were analyzed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker Daltonics, USA) at the mass spectrometry and proteomics core facility at 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.

Characterization of lipase

The lipase active fraction obtained after gel permeation chromatography was used for its characterization 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 [sodium dodecylsulphate (SDS), triton-X100, tween-80] and modifiers [ethylenediamine tetraacetic acid (EDTA), β -mercaptoethanol] on lipase activity were studied. The effects of these parameters on lipase activity were expressed in percentage of enhanced activity to its initial activity.

Effect of pH on activity and stability of lipase

The optimum pH for lipase activity was determined by measuring the enzymatic activities in 50 mM phosphate buffer (pH 4.0, 5.0, 6.0, 7.0, 7.4, 7.6 and 7.8), 50 mM Tris-HCl buffer (pH 8.0, 8.2, 8.5 and 9.0) and 50 mM carbonate-bicarbonate (pH 10) at 37 °C for 30 min incubation. Stability was measured by pre-incubating lipase at three different pH levels (8, 9 and 10) for 1 to 5 h, and then the activity was measured. Residual activity was determined in relation to the lipase activity at zero h of pre-incubation.

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 °C for 30 min incubation. Stability was measured at by incubating lipase at three different temperatures (40, 45 and 50 °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 SDS, tween-80, triton-X100, EDTA or β -mercaptoethanol at different concentrations of 0.25, 0.5 and 1.0 % at 45 °C and pH 8.2 for 30 min, thereafter the relative activity was determined.

Effect of different metal salts on enzyme 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+} and Cu^{2+} to a final concentration of 0.5, 1.0, and 1.5 mM at 45 °C and pH 8.2 for 30 min incubation.

Calculation of K_m and V_{max}

The enzyme solution was treated with pNPP at a concentration of 10, 20, 40, 50, 60, 80 and 90 mM. The reaction mixture was incubated for 5 min intervals for 1 h at 45°C (pH 8.2). The K_m and V_{max} values were calculated for lipase using the software, Hyper 32.

Chapter 4

RESULTS

Results

Part I – PHB

Production of PHB

C. tropicalis BPU1 was grown in medium containing commercially available soluble starch (1%) at 37 °C for 24 h to collect the biomass, which was subsequently lyophilized. Rapid cooling under vacuum during lyophilization crystallized the accumulated PHB, which appeared as sharp protuberances on yeast cells (Fig. 1 A and B). Lyophilized yeast cells were sonicated to release the cell contents, and sodium hypochlorite dissolved the lipids except PHB, which was extracted using hot chloroform (Fig. 1C). The chloroform extract upon evaporation produced PHB crystals; their topologies and structures were investigated using microscopic analysis. The typical spherulite morphology and radial stacking were observed during crystallization (Fig. 1D and E). The PHB formed crystals with dimensions of around 300×300×3μm (length×breadth×width) (Fig. 1F).

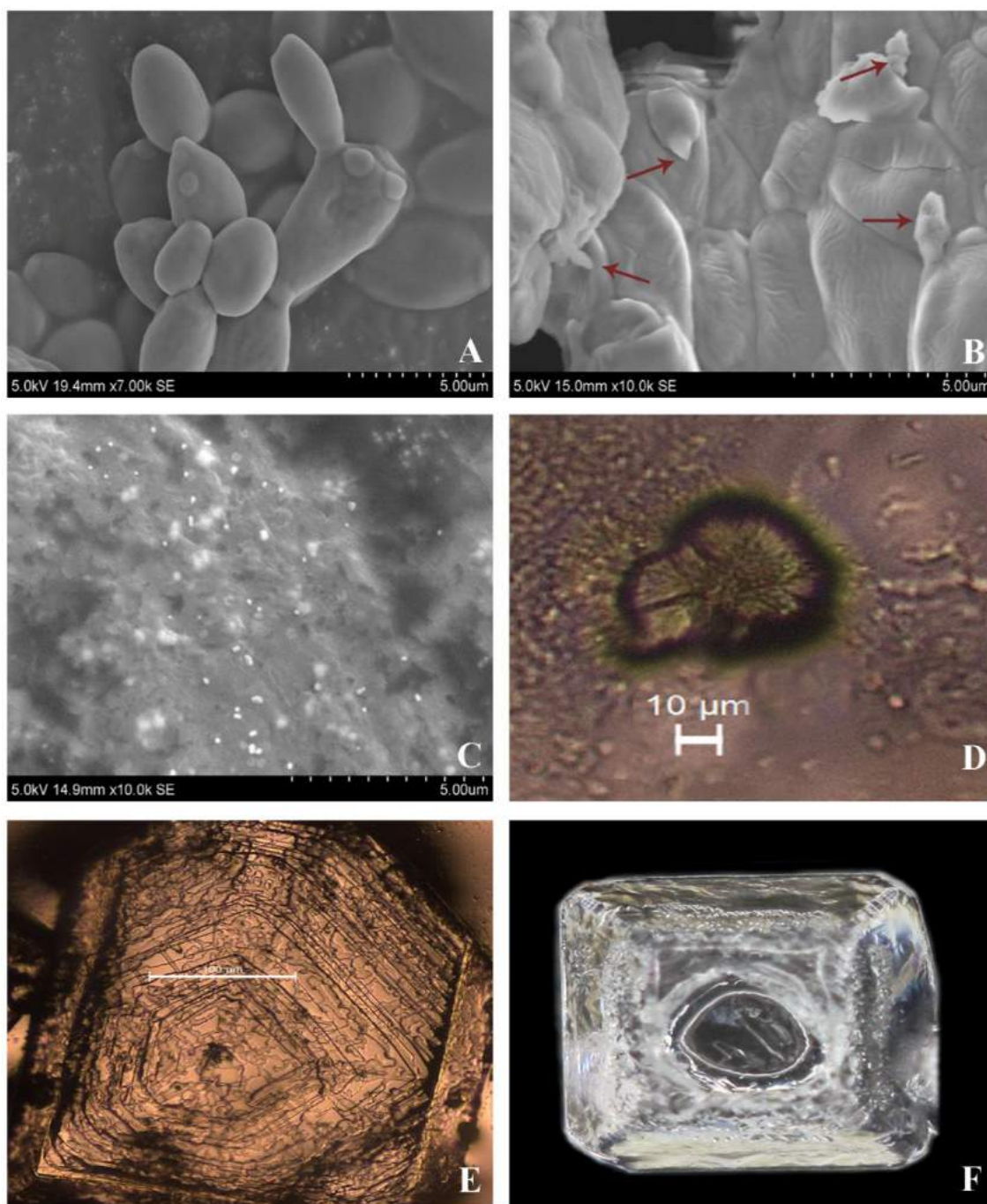


Figure 1. Extraction of PHB from *C. tropicalis* BPU1. A) *C. tropicalis* BPU1 growing in medium supplemented with commercial starch; B) lyophilized cells of *C. tropicalis* BPU1 showing protuberances of crystallized PHB; C) Scanning electron micrograph of extracted PHB in chloroform (Hitachi SU660, Japan); D) spherulite morphology during PHB crystallization (phase contrast microscope; Leica M80, Germany); E) PHB crystals with banding pattern (phase contrast microscope); F) digital image of a magnified PHB crystal (DSLR Canon 450 D, Japan).

Natural raw substrates for PHB production

Natural raw and starchy substrate (flours of potato, tapioca or jack seed) was supplemented in the medium, replacing the commercially available soluble starch. Among the three substrates, potato flour supported the maximum production of PHB (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 (flours of tapioca and jack seed) supported PHB production in lower quantities, 0.24 and 0.28 g/g at 12 and 24 h of incubation, respectively (Fig. 2).

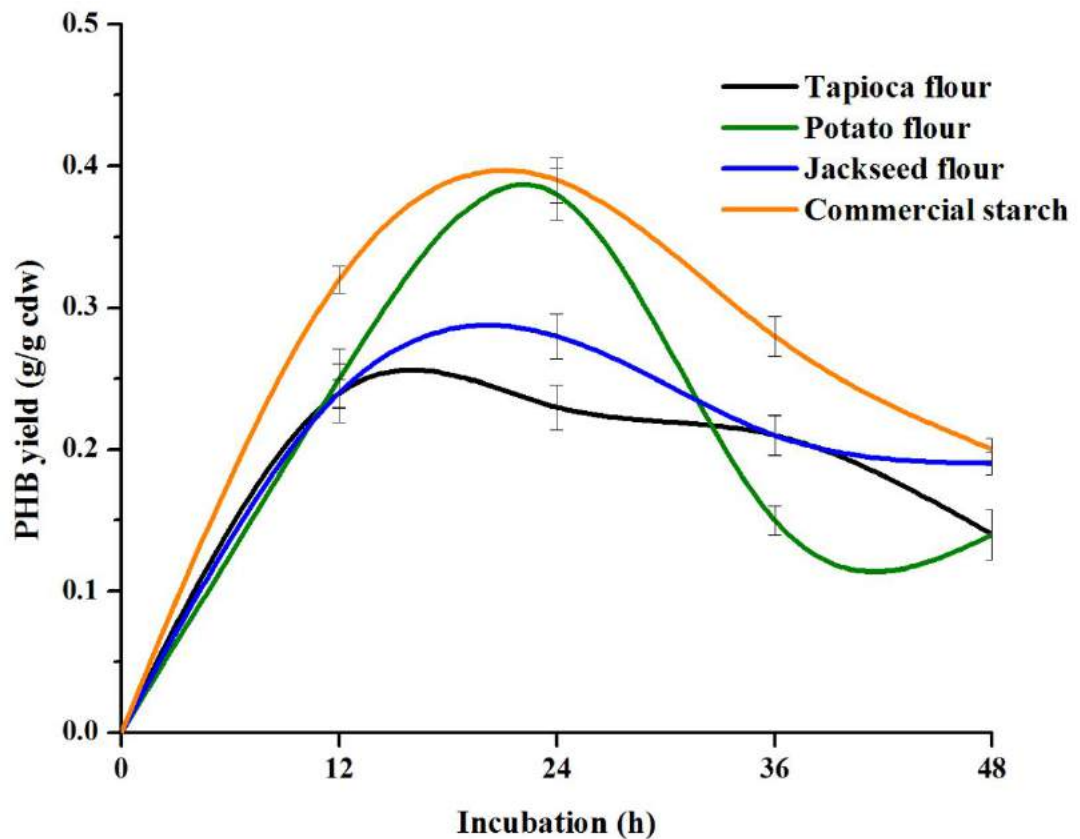


Figure 2. Production of PHB in medium supplemented with starch. Commercial soluble starch or natural starchy substrate was supplemented in the medium to evaluate the production of PHB by *C. tropicalis* BPU1. Among them, commercial starch and potato flour supported the maximum production of PHB at comparable levels of 0.39 and 0.36 g/g cdw respectively, at 24 h of incubation

Statistical optimization of PHB production

Potato flour which supported the maximum yield of PHB was used as substrate for statistical optimization. A set of 27 experiments were designed according to Box-Behnken design (Table 1), and the results showed that the predicted and experimental values did not show significant difference. The results were analyzed by ANOVA (Table 2), and following quadratic regression equation was obtained in terms of PHB production. A second order polynomial function was fitted to the experimental yield of PHB to obtain response surface plots (Fig. 3), which resulted in the following regression equation.

Table 1. Box-Behnken design matrix with the respective responses in terms of PHB yield, biomass yield and carbohydrates used.

Run Order	pH	Temperature (°C)	Incubation (h)	Substrate (%)	Residual starch (%)	Cell dry weight(g)	PHB yield (g/g)
1	6	25	27	2.0	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	2.0	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	2.0	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	2.0	28.677	4.86	0.39
25	5	32.5	27	2.0	67.72	2.79	0.43
26	6	32.5	18	2.0	37.72	3.92	0.22
27	6	32.5	27	1.25	48.24	3.61	0.42

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

Where; X_1 is pH, X_2 is temperature, X_3 is incubation and X_4 is substrate concentration

The R^2 value of 0.98 indicated the aptness of the model, which was in reasonable agreement with the adjusted R^2 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 (Table 3). 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. Correlation coefficient was close to unity, suggesting the significance of the model. The optimum production of PHB was found to be 0.59 g/g cdw (at 38 °C, pH 6.9, substrate 0.5% and incubation 19 h). Thus, the statistical optimization resulted in 0.6 fold increase of PHB accumulation *C. tropicalis* BPU1 over the unoptimized condition..

Table 2. Analysis of Variance (ANOVA) for PHB yield

Source	DF	Seq SS	Adj SS	Adj MS	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		

Table 3. Random combinations of parameters selected for the validation of proposed statistical model with respective predicted responses in terms of PHB yield

pH	Temperature (°C)	Incubation (h)	Substrate (%)	Predicted PHB yield	Observed PHB yield
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

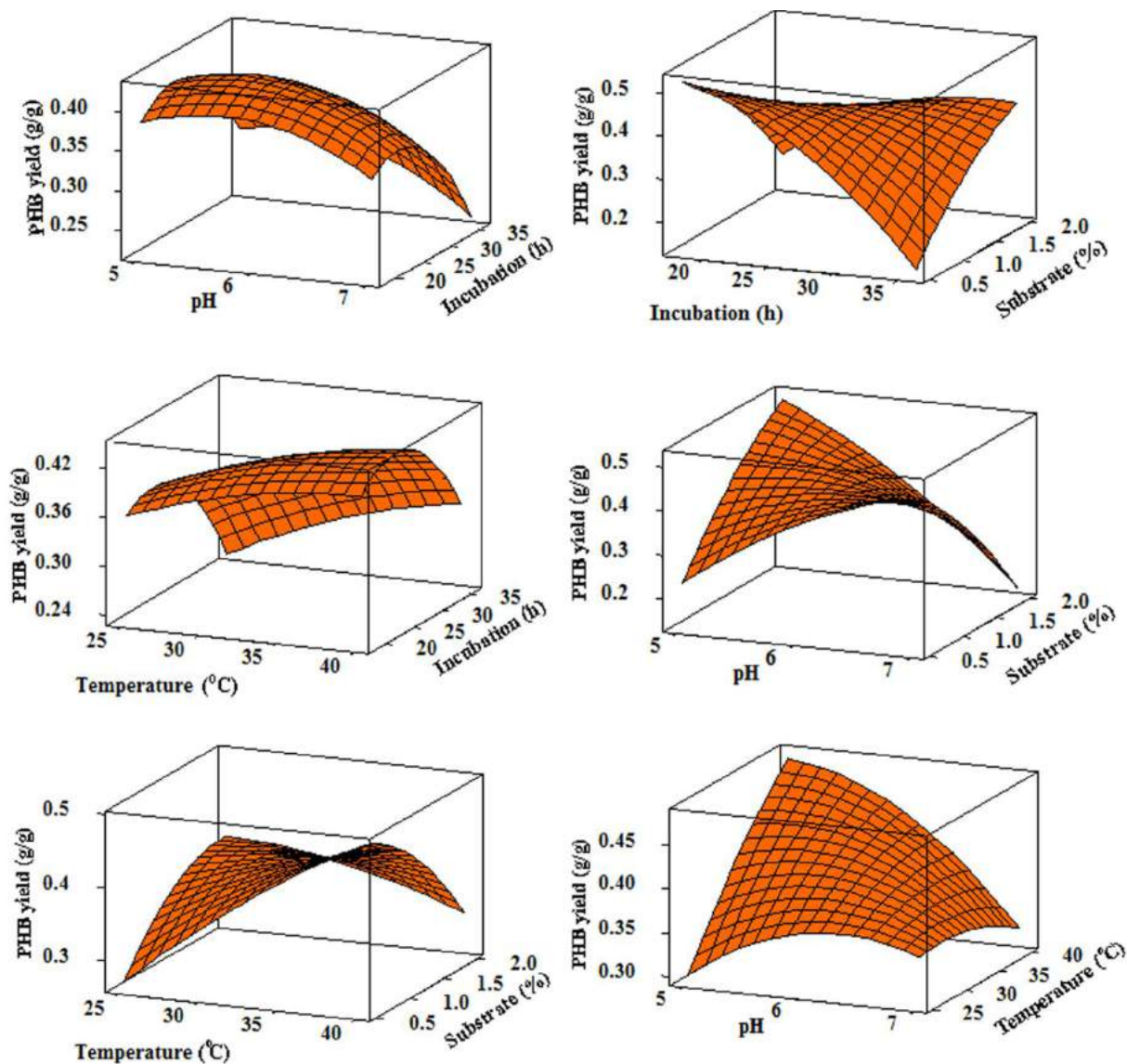


Figure 3. Response surface plots described by the proposed model showing the interactive effects of parameters, temperature (°C), pH, incubation (h) and substrate concentration (%) on the production of PHB by *C. tropicalis* BPU1.

Characterization of PHB

The PHB produced under the optimized conditions (38 °C, pH 6.9, potato flour 0.5% and incubation 19 h) was extracted and used for the characterization studies.

TLC and UV-Visible spectroscopy

PHB was extracted from the production medium containing potato flour under optimized conditions, by solvent extraction method and then subjected to propanolysis to break down the polymer. TLC profile of the extract showed a single spot with R_f value of 0.8 (Fig. 4A). Hot H_2SO_4 converted the PHB in the extract to crotonic acid which showed an absorption maximum at λ_{234} , a clear indication of the predominance of butyric acid moieties in the sample (Fig. 4B).

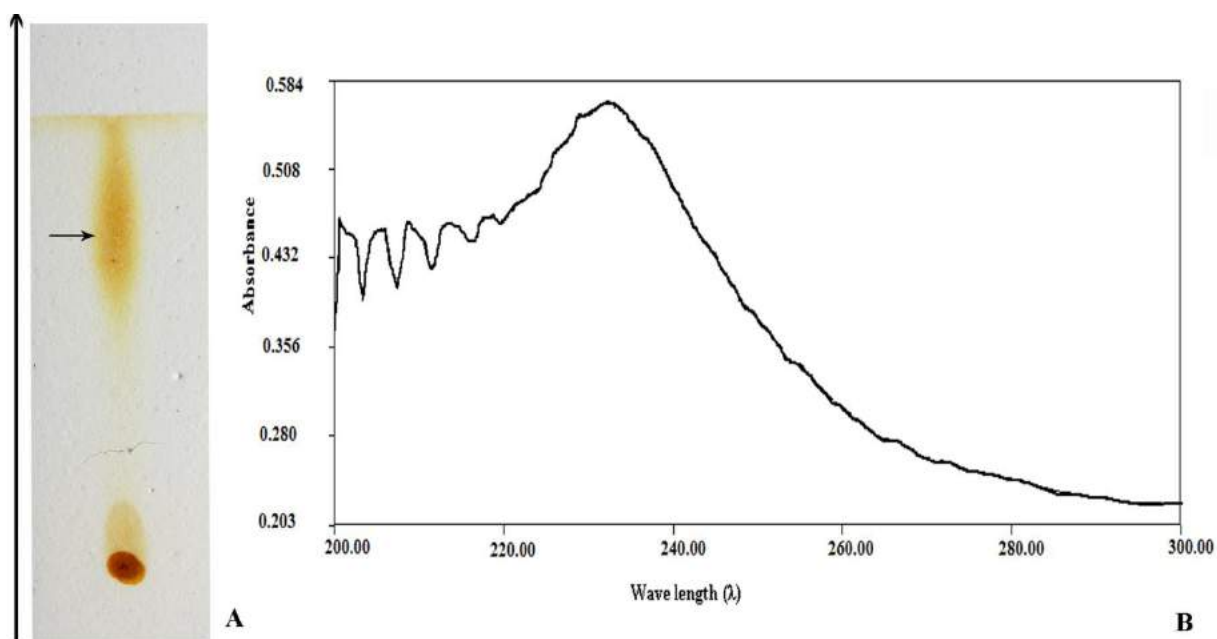


Figure 4. Characterization of PHB by TLC and UV-visible spectrophotometry. A) separated spot on TLC with R_f value 0.8 upon visualization with iodine vapor; B) absorption spectrum of PHB, extracted from the TLC spots, after conversion to crotonic acid with conc. H_2SO_4 showing absorption maximum at λ_{234} .

Fourier Transforms Infrared Spectroscopy (FTIR)

The FTIR spectroscopic results (Fig. 5) showed distinct peaks at 3420 cm^{-1} and $3000\text{--}2850\text{ cm}^{-1}$, which correspond to the $-OH$ and $-CH-$ stretchings. The absorption peak at 1741 cm^{-1} was reported to be a PHA marker band, assigned to carbonyl ($-C=O$) ester bond stretching vibration, whereas the absorption bands at $1000\text{--}1200\text{ cm}^{-1}$ were characteristic of $C-O$

stretchings. The IR spectrum of PHA coincides with that of PHB reported earlier (Bayarı and Severcan, 2005; Arun et al., 2009).

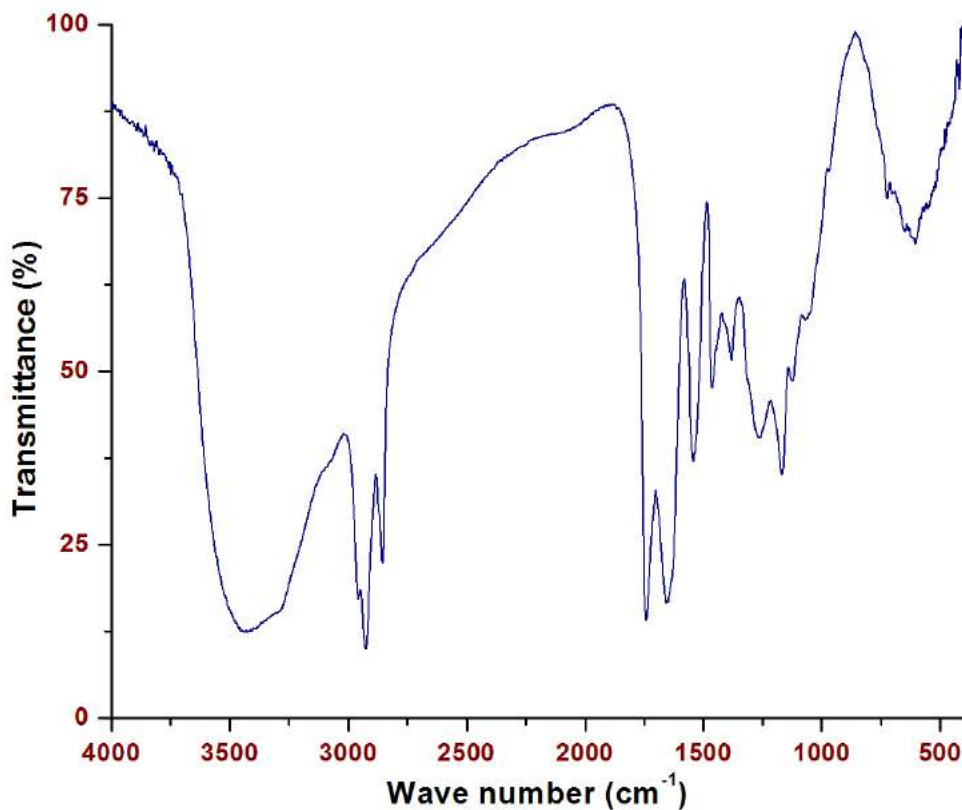


Figure 5. FTIR profile of PHB crystals

Nuclear magnetic resonance spectroscopy (NMR)

The ^1H 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 (Fig. 6A). 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 (Fig. 6B). The other weak resonances on both the spectra may be the impurities or cellular materials trapped during extraction.

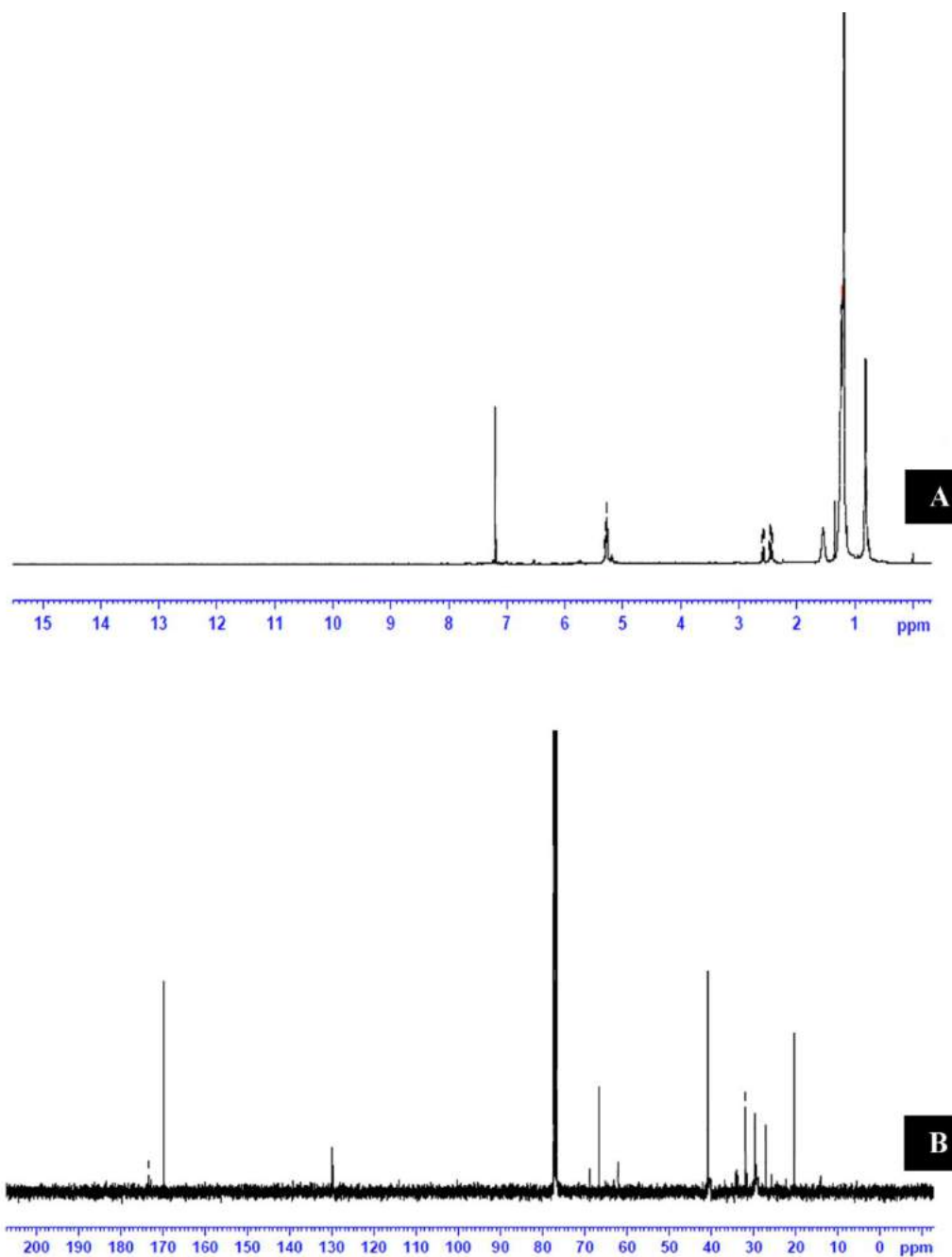


Figure 6. NMR spectra of PHB crystals. A) ^1H NMR spectrum; B) ^{13}C NMR spectrum

Thermal analysis of PHB

From the thermo gravimetric analysis (Fig. 7), the temperature range for rapid thermal degradation of PHB was from 240 to 345 °C with the degradation peaking at 273 °C. The total weight loss within this temperature range was 92 %. The initial drop in weight of

around 6 % might be due to the loss water and other impurities trapped during crystallization.

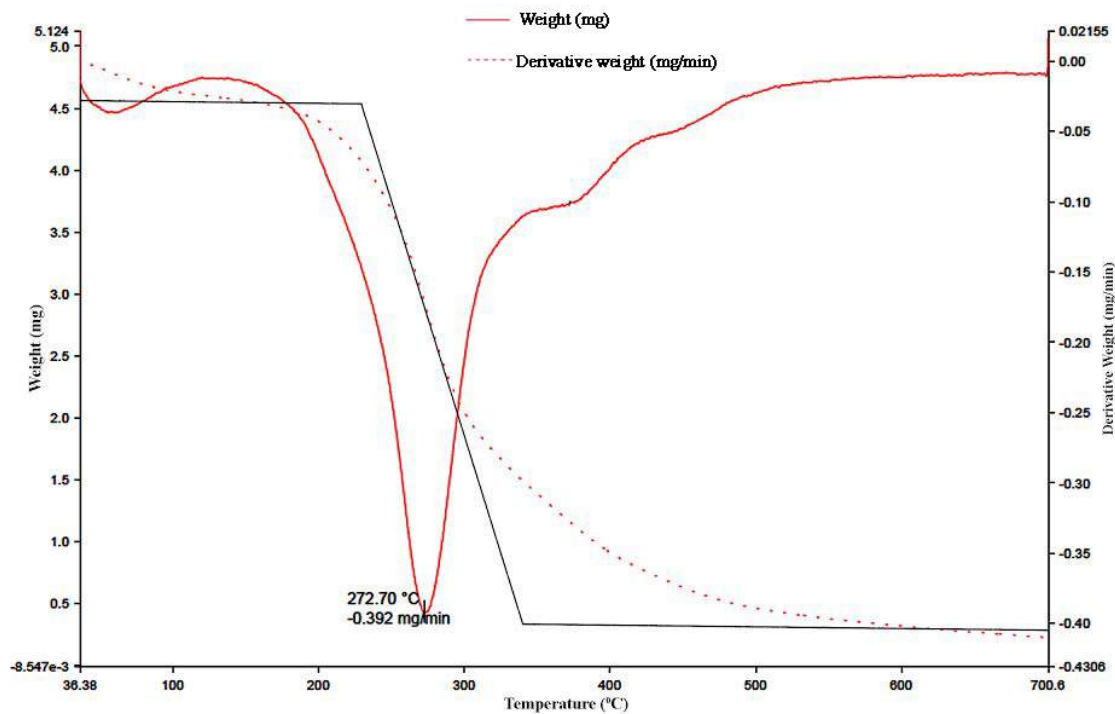


Figure 7. Thermal gravimetric analysis of PHB crystals showing rapid thermal degradation at temperature ranging from 240 to 345 °C with maximum at 273 °C.

Part II – BIOSURFACTANT

1. Production of biosurfactant by *C. tropicalis* BPU1

C. tropicalis BPU1 culture was grown in mineral salt medium supplemented with groundnut oil and the culture parameters for the production of biosurfactant were optimized statistically using response surface methodology (RSM) as pH 6, Temperature 33°C, groundnut oil 0.13%, and incubation 6 days (Fig. 8 & 9). Under optimized conditions, the production of biosurfactant was enhanced from 432 mg/l to 649 mg/l, and 0.5-fold increase had been obtained. The biosurfactant was then purified by double precipitation method and identified as sophorolipids employing FTIR and LC/MS (Fig. 10 & 11).

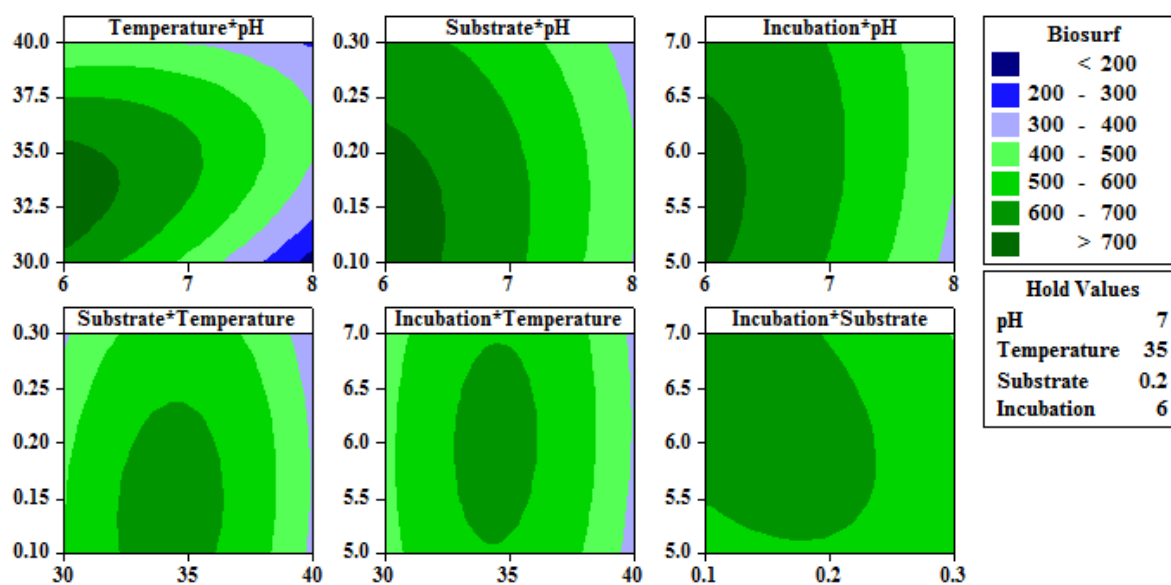


Figure 8. Contour plots described by the proposed model for the production of biosurfactant by *C. tropicalis* BPU1.

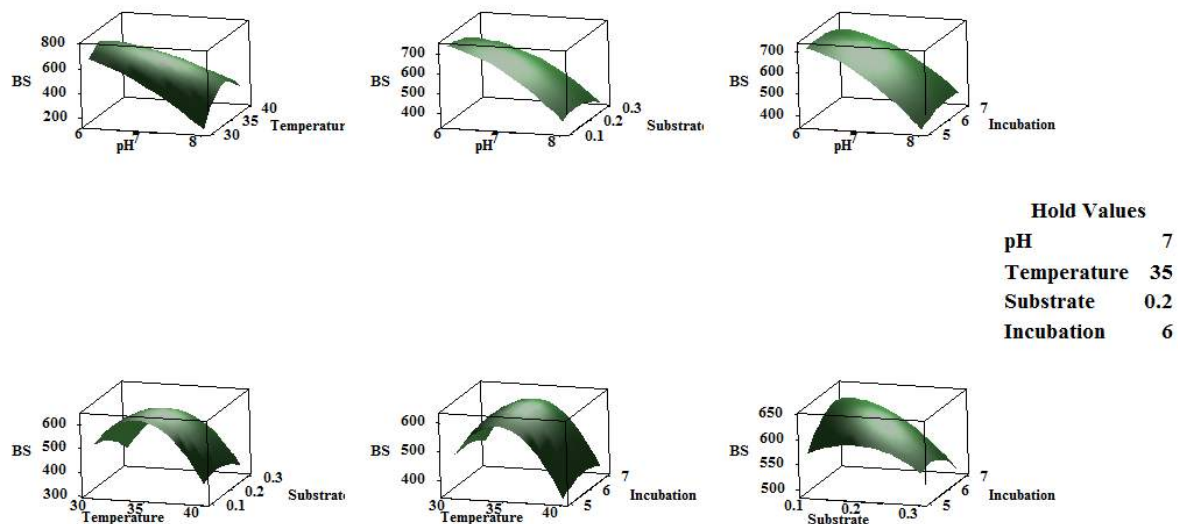


Figure 9. Response surface plots described by the proposed model for the production of biosurfactant by *C. tropicalis* BPU1.

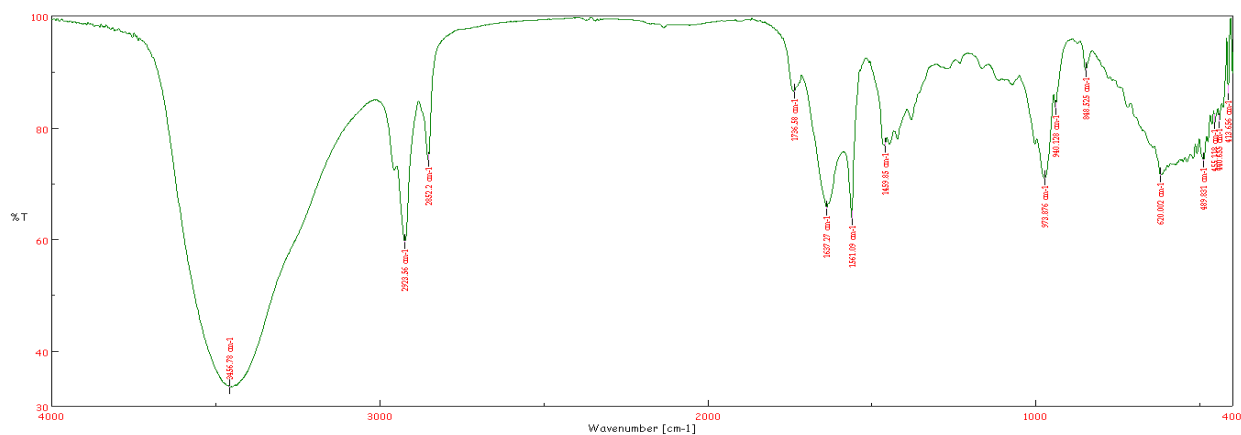


Figure 10. FTIR profile of the biosurfactant produced by *C. tropicalis* BPU1

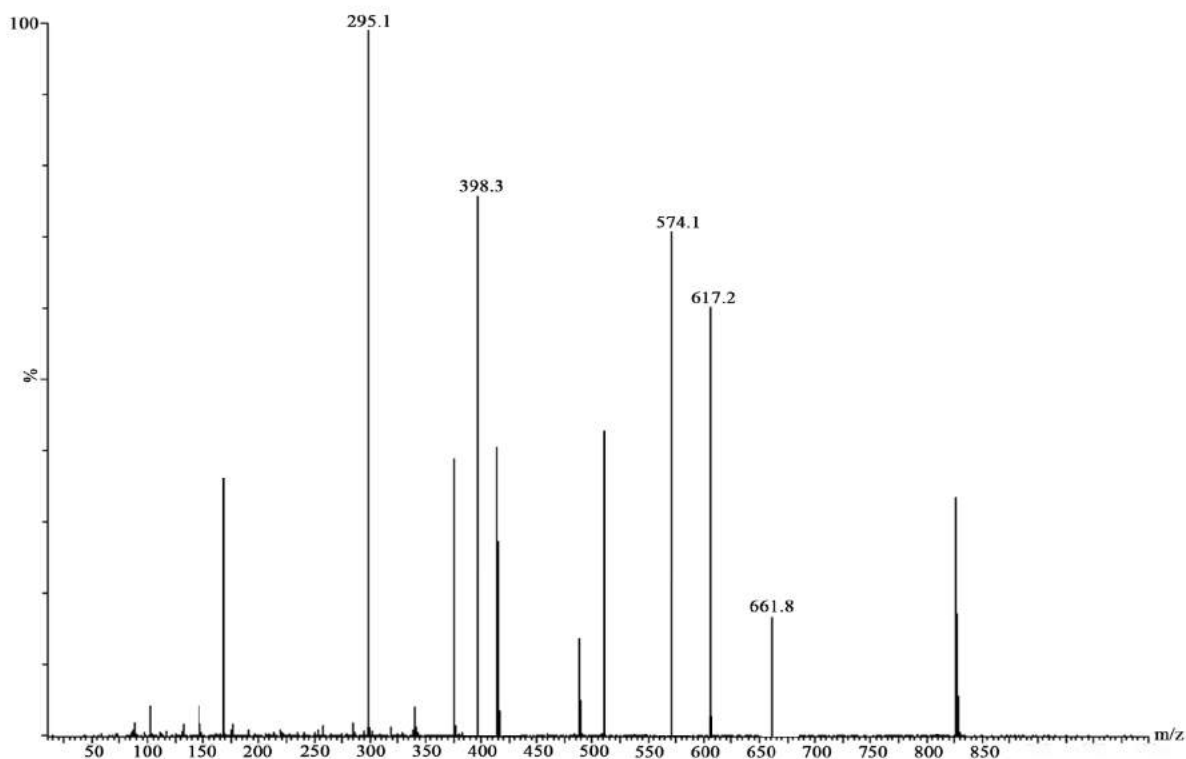


Figure 11. LC/MS profile of the sophorolipid produced by *C. tropicalis* BPU1

Physico-chemical properties of sophorolipids produced by *C. tropicalis* BPU1

Emulsification index of the sophorolipids produced by *C. tropicalis* BPU1 was determined using five different hydrocarbons, viz., coconut oil (CNO), olive oil (OO), groundnut oil (GNO), kerosene, petrol and diesel. As shown in Fig. 12, the biosurfactant showed good emulsification indices with all the hydrocarbons tested; among which the maximum emulsification index was observed with groundnut oil (50 %).

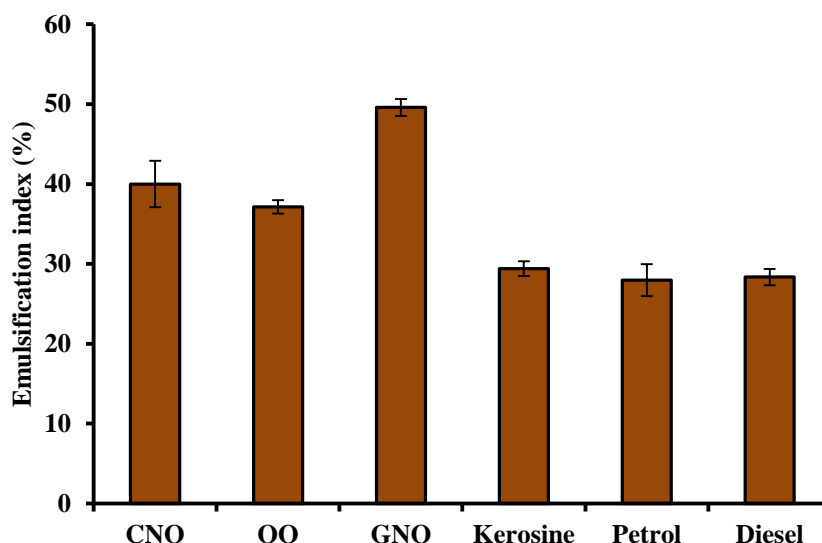


Figure 12. Emulsification index of biosurfactant produced by *C. tropicalis* BPU1 toward various hydrocarbons [the maximum emulsification index was shown toward groundnut oil (50 %)]

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 *C. tropicalis* BPU1 was analyzed in terms of emulsification index at varying temperature (4 to 100 °C), pH (3 to 11) and salinity (0.5 to 2.0 %). The biosurfactant was found stable over a wide range of temperature (20 to 60 °C). The maximum stable emulsion was obtained at 40 to 60 °C (48 %) (Fig. 13A). Investigations on pH stability indicated that the biosurfactant produced stable emulsions at a pH range of 3-7 (Fig. 13B). The emulsification index of the biosurfactant was relatively stable (44 %) in the presence of NaCl, upto concentration of 0.5 %; thereafter it reduced gradually (Fig. 13C). Thus, the stability of biosurfactant produced by *C. tropicalis* BPU1 clearly indicated that it is a good candidate for use in industries related to emulsions.

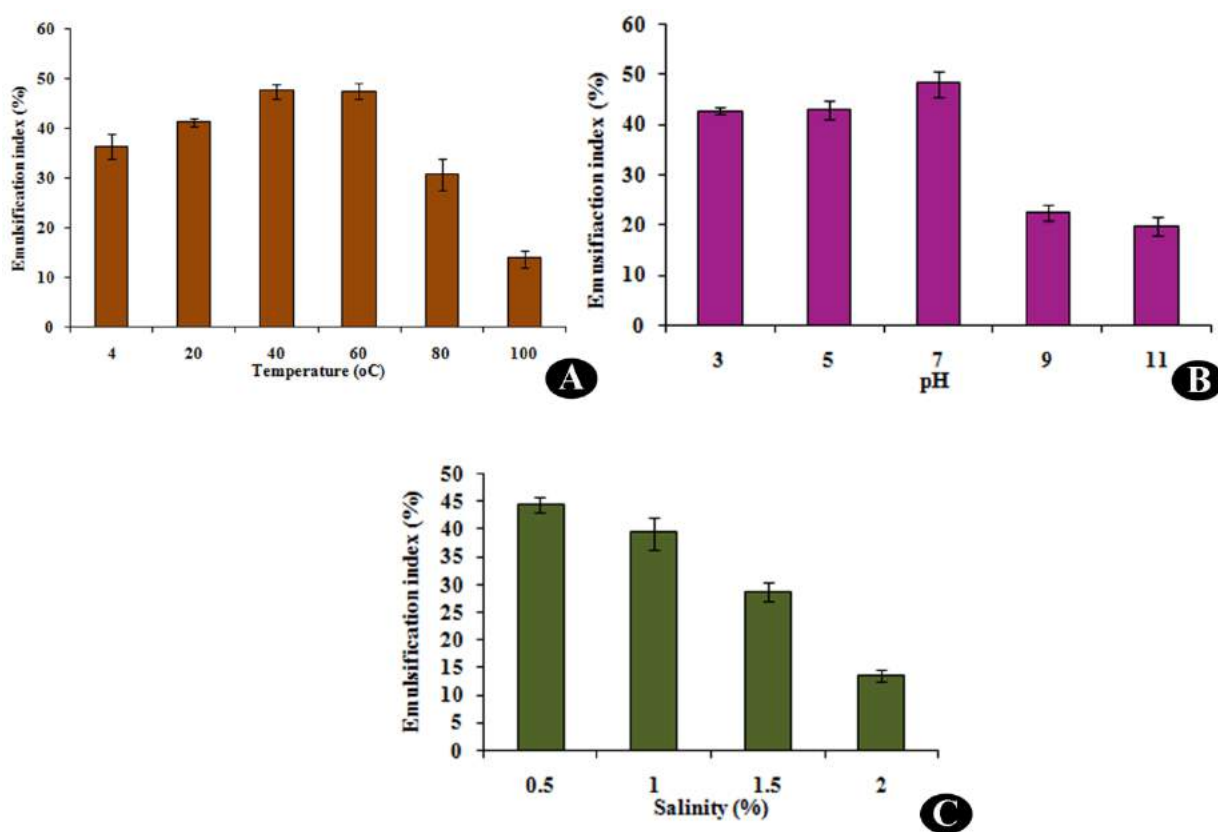


Figure 13. Stability of biosurfactants produced by *C. tropicalis* BPU1 (A) Effects of temperature on the emulsification index of biosurfactant produced by *C. tropicalis* BPU1 (the biosurfactant showed no significant variations in emulsification index in a 4 to 60 °C temperature range); (B) Effects of pH on the emulsification index of biosurfactant produced by *C. tropicalis* BPU1 (the biosurfactant did not show significant variations in emulsification index at a pH range of 3 to 7); (C) Effect of salinity on the emulsification index of biosurfactant produced by *C. tropicalis* BPU1 (the biosurfactant was found relatively stable in terms of emulsification index at salinity of 0 - 0.5 %)

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 it reaches 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 ddH₂O from 74 to 36 mN/m. The CMC of the biosurfactant was found as 200 mg/l (Fig. 14).

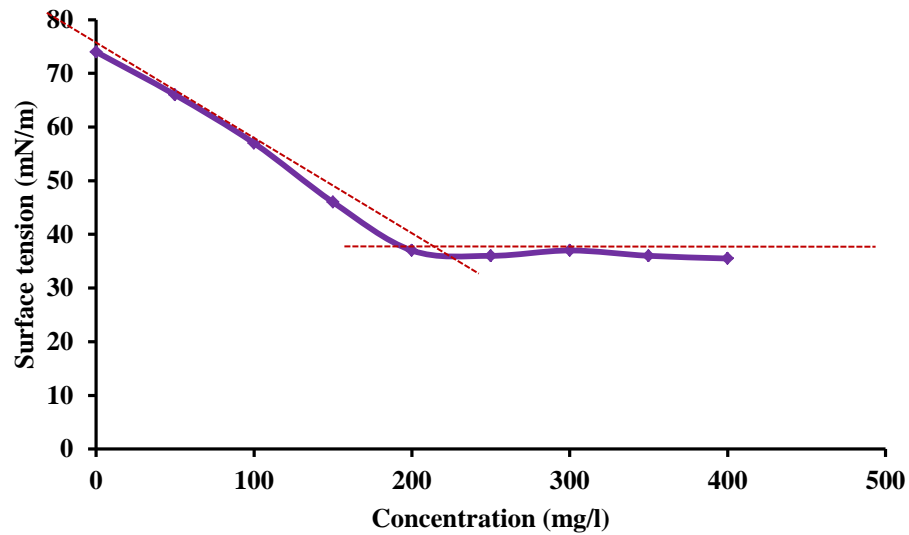


Figure 14. Surface tension vs. concentration of biosurfactant produced by *C. tropicalis* BPU1 (the biosurfactant showed a critical micelle concentration of 200 mg/l).

Biosurfactants possess interesting biological properties such as anti-bacterial, anti-adhesive and non-toxicity that made it advantageous over the synthetic surfactants. The anti-bacterial activity of the biosurfactant produced by *C. tropicalis* BPU1 was investigated against 4 bacteria (*E. coli*, *P. mirabilis*, *S. aureus* and *A. baumannii*) at varying concentrations (200 to 12.5 mg/l). At a concentration of 200 mg/l, the biosurfactant showed 88 and 91 % inhibitory effects against *E. coli* and *S. aureus*, respectively; whereas it not showed significant effect on the growth of *P. mirabilis* and *A. baumannii* (inhibition of growth was approximately 10-15 %)(Fig. 15A). Similarly, the anti-adhesive property of the biosurfactant was the maximum against *E. coli* and *S. aureus* at 200 mg/l of biosurfactant (64 and 77 %, respectively); but the biosurfactant was found less effective against *P. mirabilis* and *A. baumannii* with all the concentrations investigated (Fig. 15B).

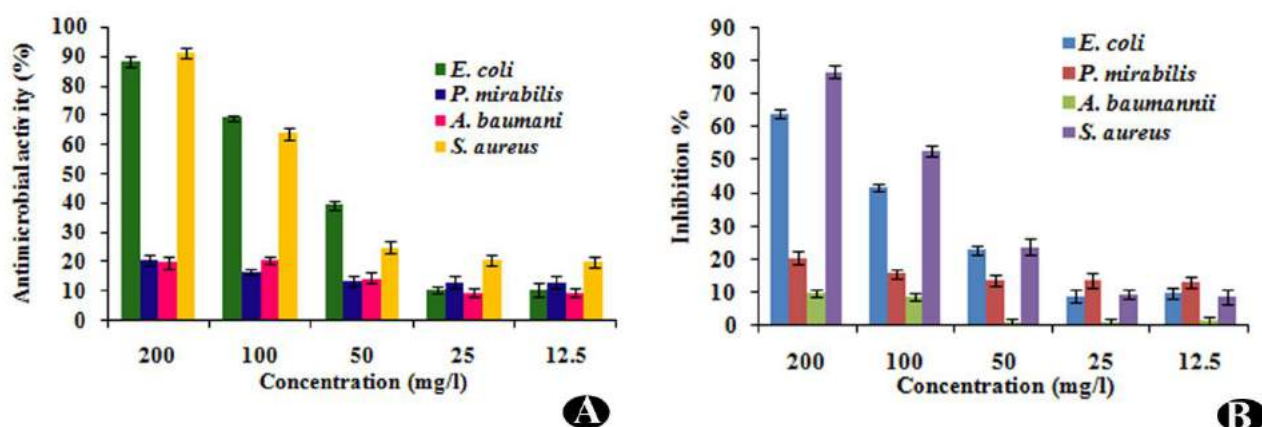


Figure 15. Biological activities of the biosurfactant produced by *C. tropicalis* BPU1: (A) Anti-bacterial activities of biosurfactant produced by *C. tropicalis* BPU1 [anti-bacterial activity was the maximum against *E. coli* (88 %) and *S. aureus* (91 %)]; (B) Anti-adhesion property of biosurfactant produced by *C. tropicalis* BPU1 [antiadhesive activity was the maximum against *E. coli* (64 %) and *S. aureus* (77 %)].

In addition to *C. tropicalis*, we isolated a novel bacterium, *Pseudomonas* sp. BUP6 from the rumen of Malabari goat, which could produce biosurfactant and lipase very efficiently and the details are furnished below.

2. Production of biosurfactant by *Pseudomonas* sp. BUP6

Pseudomonas sp. BUP6, the rumen bacterium, was grown in the semi-synthetic medium supplemented with groundnut oil and the production profile of biosurfactant was analyzed for 7 days at an interval of 24 h. The culture was able to utilize vegetable oil supplied within 6 to 7 days of incubation. After each day, the biosurfactant was precipitated from the cell free supernatant using 6 N HCl at 4 °C for 12 h, and weighed for the quantification of crude biosurfactant. Even though, *Pseudomonas* sp. BUP6 started producing biosurfactant after 24 h of incubation along with a sudden decrease in surface tension of the medium, the maximum yield (1912 mg/l) was obtained after 3 d of incubation (**Fig.16**).

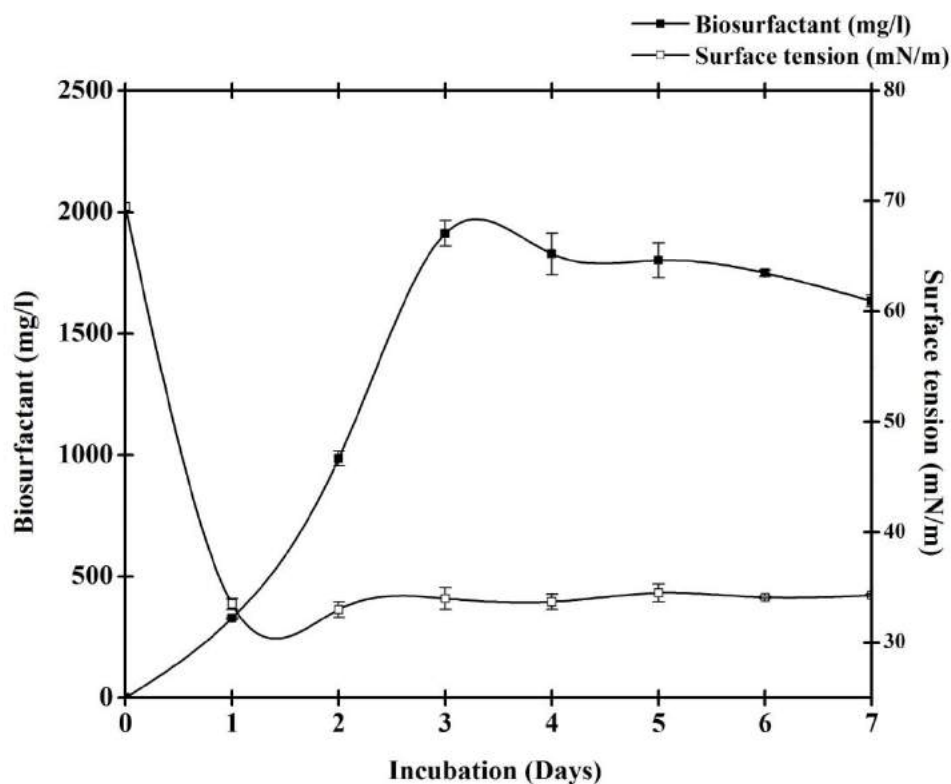


Figure 16. Production profile of biosurfactant in the semi-synthetic medium supplemented with groundnut oil.

Statistical optimization

Screening of independent factors that significantly influenced the production of biosurfactant was performed according to Plackett-Burman design, and a set of 22 runs were performed at 2 levels; low (–) and high (+). The standardized effects of individual parameters on the production of biosurfactant are shown in Fig. 17. The order of significance indicated by Pareto chart was: incubation>pH>temperature>substrate>agitation. Three parameters (incubation, pH and temperature) out of 5 were found significant for the production of biosurfactant, which were selected for Box-Behnken design.

Box-Behnken design comprising 15 experimental runs was performed to determine the interactive effects of three significant factors at three levels; low (–1), middle (0) and high (+1). The predicted and observed production level of biosurfactant by *Pseudomonas* sp. BUP6 for all the experimental runs are shown in Table 4, and the results were analyzed by

ANOVA (Table 5). The statistical model for the production of biosurfactant was expressed by the regression equation,

$$\text{Biosurfactant yield} = -32770.0 + 131.146 X_1 + 4432.92 X_2 + 832.583 X_3 - 1.00405 X_1^2 - 470.833 X_2^2 - 15.2333 X_3^2 + 8.75 X_1 X_2 - 1.35417 X_1 X_3 + 45.5 X_2 X_3$$

Where: X_1 is incubation; X_2 is pH; and X_3 is temperature.

Fig. 18 shows the contour 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) as 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 the production of biosurfactant. Thus, the statistical optimization resulted in 11 % increase production (at pH 7, 35 °C, incubation 75 h) so as to get the maximum yield (2070 mg/l) of biosurfactant, against the un-optimized condition.

Table 4. The observed and predicted production of biosurfactant by *Pseudomonas* sp. BUP6, according to Box-Behnken model.

Run No.	Incubation (h)	pH	Temperature (°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

Table 5. 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			

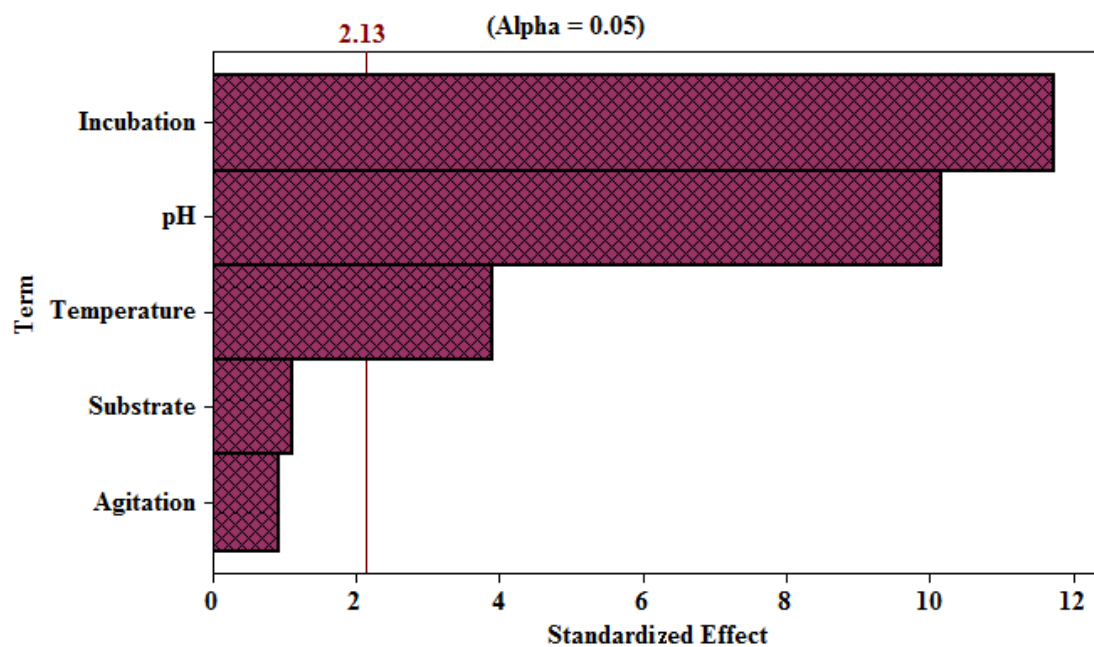


Figure 17. Pareto chart showing the standardized effects of parameters on the production of biosurfactant by *Pseudomonas* sp. BUP6.

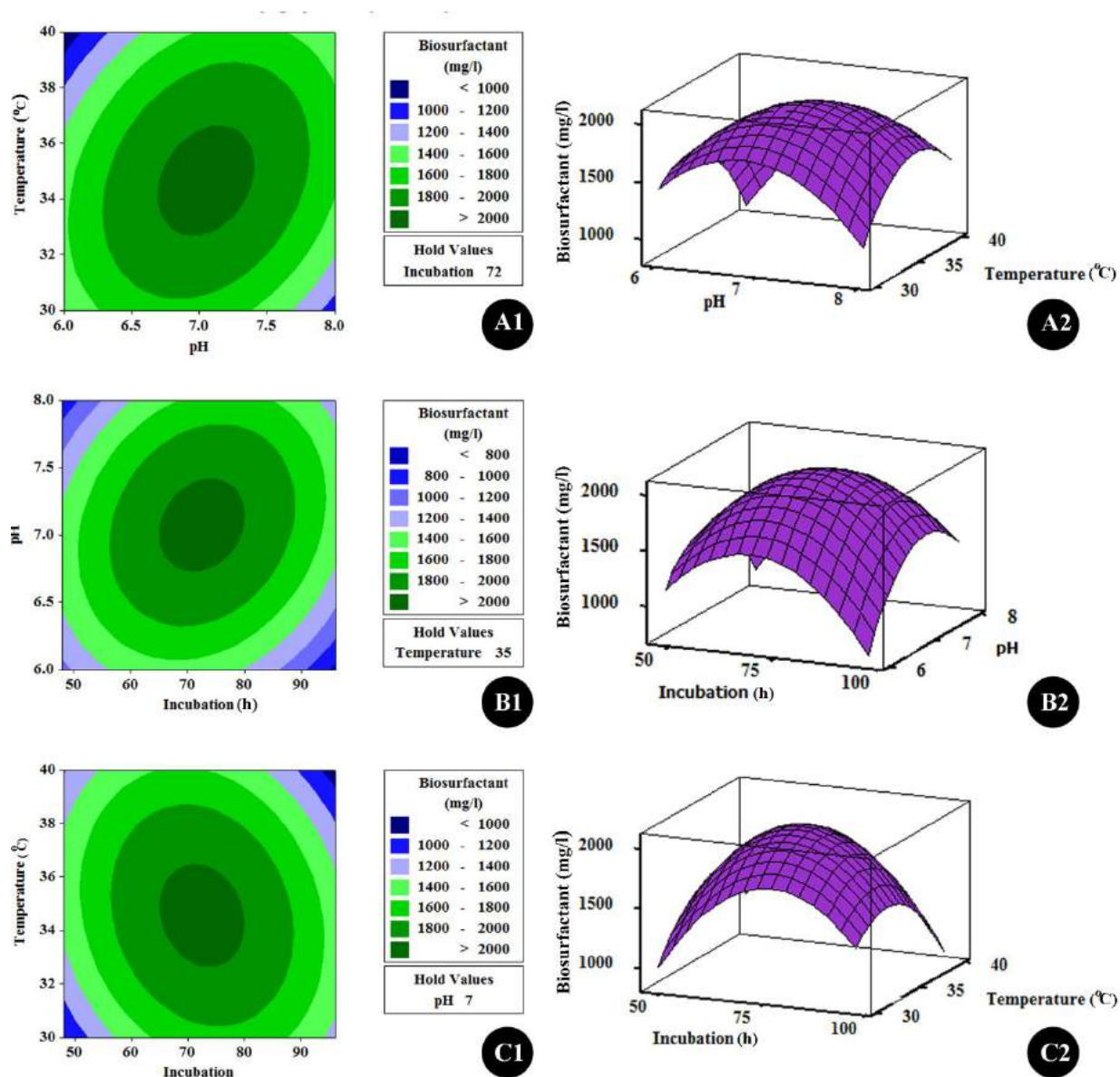


Figure 18. Response surface plots. Surface and contour plots showing the interactive effects of parameters growth parameters like temperature, agitation and pH on production of biosurfactant by *Pseudomonas* sp. strain BUP6.

Hydrophobicity index

Cell surface hydrophobicity is an important property of bacteria producing extracellular biosurfactant; hydrophobicity index is a measure of cell surface hydrophobicity. It was shown that *Pseudomonas* sp. BUP6 grew well attached to the groundnut oil and formed

clumps, which were visualized by scanning electron microscopy (SEM) (Fig. 19A-C). The hydrophobicity index of *Pseudomonas* sp. BUP6 was determined using various non-aqueous hydrocarbons such as diesel, kerosene, petrol, *n*-decane, and groundnut oil. The cells showed the highest hydrophobicity index (37 %) toward groundnut oil followed by petrol, kerosene, *n*-decane and diesel (Fig. 19D). The results clearly indicated the presence of a characteristic slimy hydrophobic matrix surrounding the cells of *Pseudomonas* sp. BUP6.

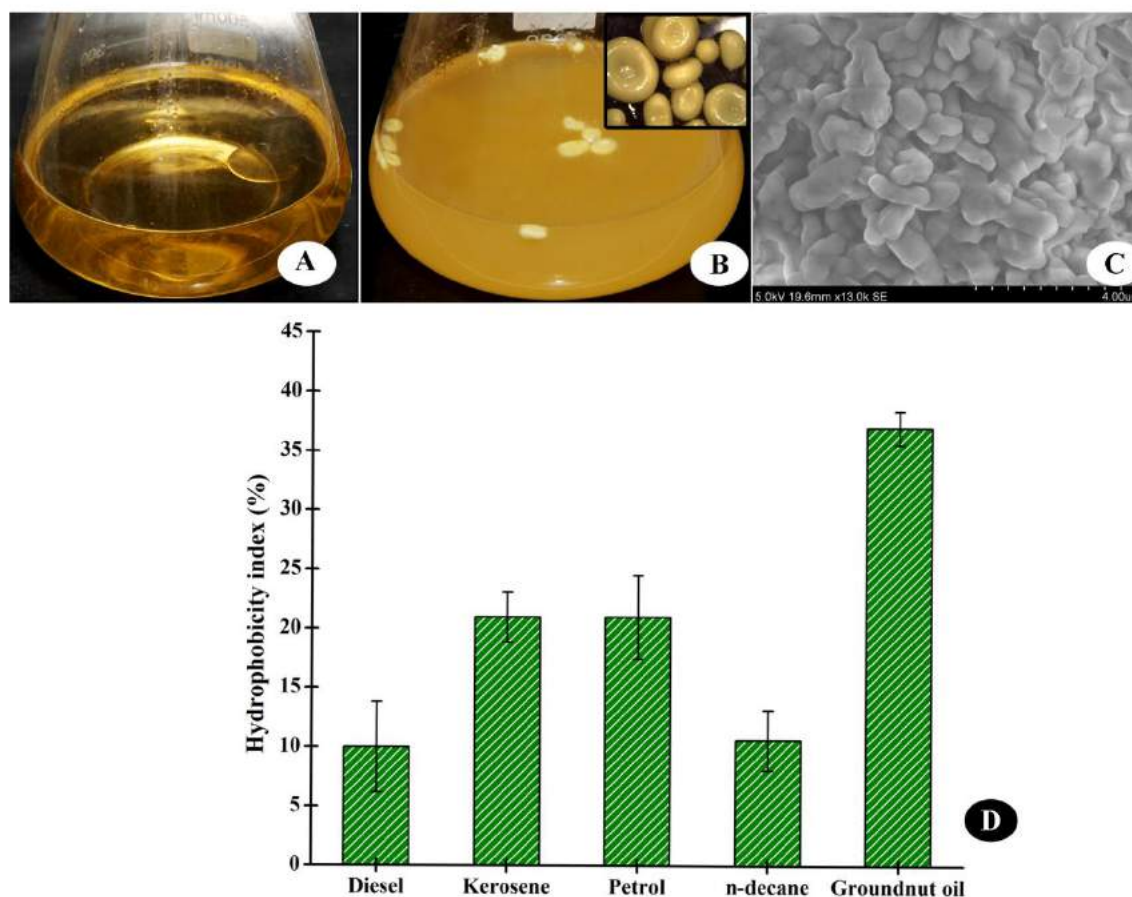


Figure 19. Hydrophobic nature of *Pseudomonas* sp. BUP6: (A) *Pseudomonas* sp. BUP6 in basal salt medium supplemented with groundnut oil at 0 h; (B) After 3d of incubation. *Pseudomonas* sp. BUP6 growing attached to the oil droplets and the clumps are shown at the inset; (C) SEM image of clumps (SEM: Hitachi SU660, Japan); and (D) The cells showed the maximum hydrophobicity index towards groundnut oil (37 %).

Physico-chemical properties of the biosurfactant

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 Fig. 20A, 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 analyzed in terms of emulsification index at varying temperature (4 to 100 °C), pH (3 to 11) and salinity (0.5 to 2.0 %). The biosurfactant was found stable over a wide range of temperature (20 to 100 °C), with no significant effects on emulsification even after heating at 100 °C for 30 min (54 % retained with kerosene), indicating its thermostability. The maximum stable emulsion was obtained at 40 to 60 °C (68 %) (Fig. 20B). Investigations on pH stability indicated that the biosurfactant produced stable emulsions at a pH range of 3-9 (Fig. 20C). The emulsification index of the biosurfactant was relatively stable (50 %) in the presence of NaCl, upto concentration of 1.5 %; thereafter it reduced to 43 % (Fig. 20D). 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 it reaches 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 ddH₂O from 74 to 34 mN/m. The CMC of the biosurfactant was found as 48 mg/l (Fig. 20E).

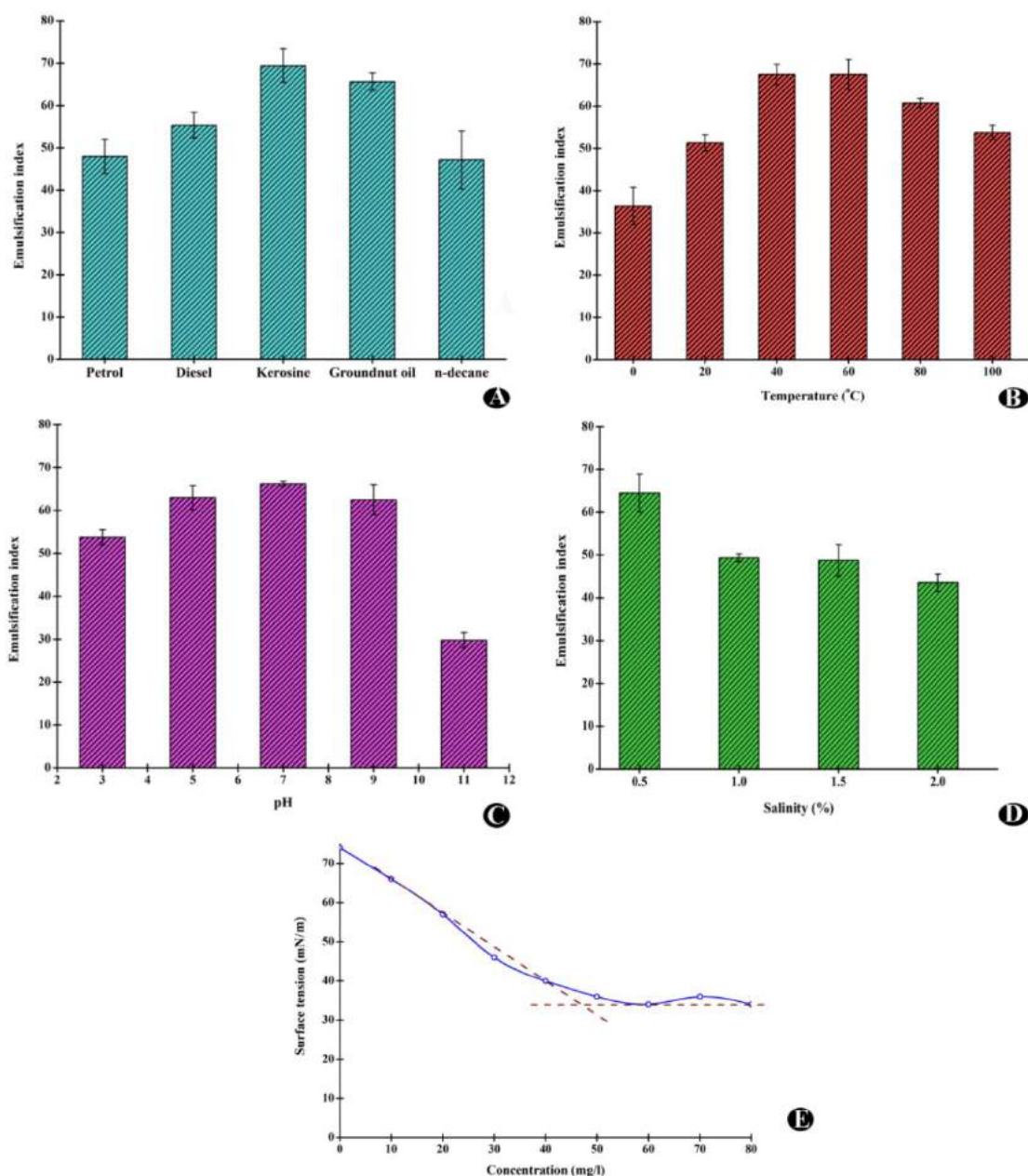


Figure 20. Physico-chemical properties of the biosurfactant produced by *Pseudomonas* sp. BUP6: (A) Emulsification index of biosurfactant produced by *Pseudomonas* sp. BUP6 toward various hydrocarbons [the maximum emulsification index was shown toward kerosene (69 %)]; (B) Effects of temperature on the emulsification index of biosurfactant produced by *Pseudomonas* sp. BUP6 (the biosurfactant showed no significant variations in emulsification index in a 20 to 100 °C temperature range); (C) 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); (D) 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 %); and (E) 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 it 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. baumannii*) at varying concentrations (100 to 6.3 mg/l). At a concentration of 100 mg/l ($\approx 2 \times \text{CMC}$), the biosurfactant showed 43 and 42 % inhibitory effects against *E. coli* and *S. aureus*, respectively; whereas it not showed significant effect on the growth of *P. mirabilis* and *A. baumannii* (inhibition of growth was approximately 10 %) (**Fig. 21A**). 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* with all the concentrations investigated (Fig. 21B).

Non-toxicity is another important property of the biosurfactants, which makes 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 phytotoxic assay, two different concentrations of biosurfactant were used; 50 mg/l ($\approx \text{CMC}$) and 100 mg/l ($\approx 2 \times \text{CMC}$). It was found that the biosurfactant exerted no significant toxic effect 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 (Fig. 21C).

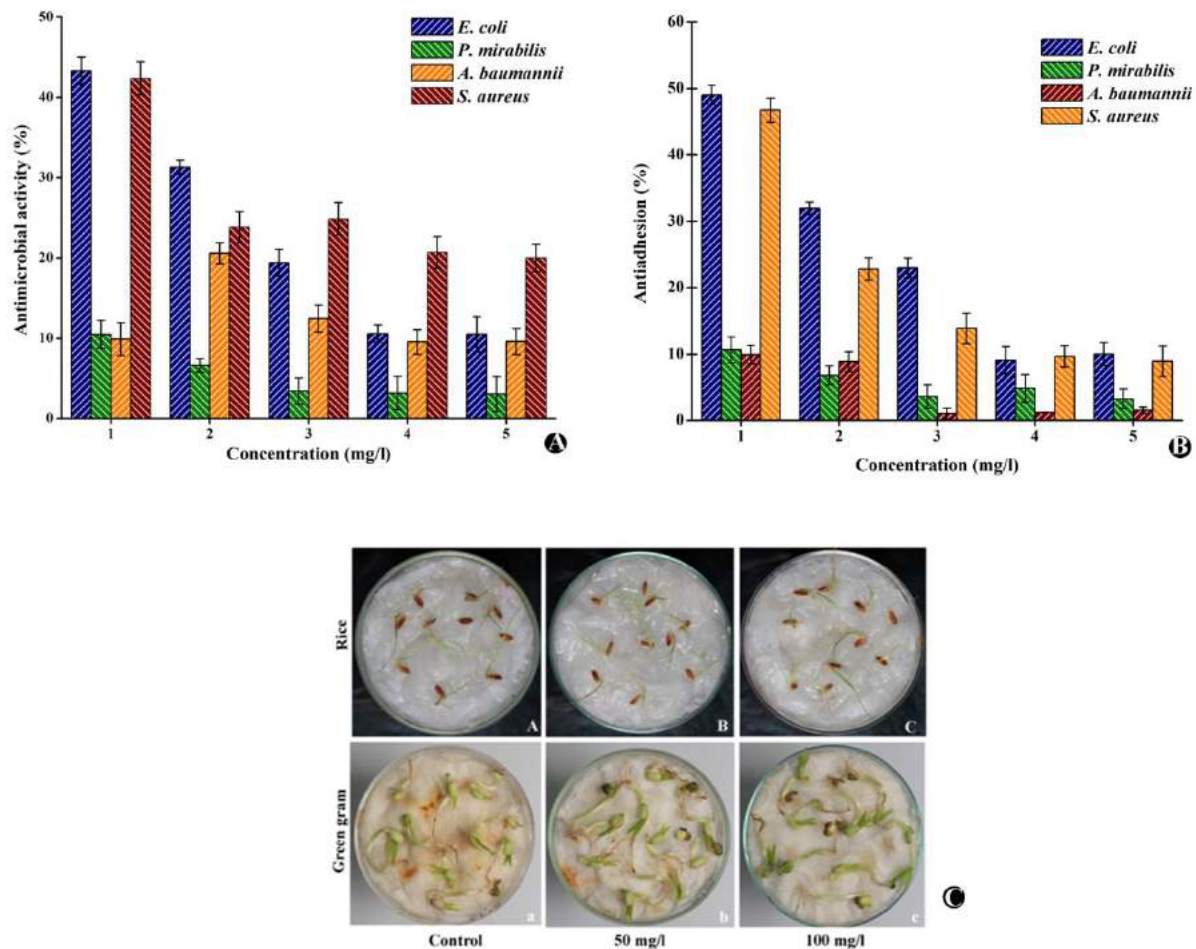


Figure 21. Biological activities of the biosurfactant produced by *Pseudomonas* sp. BUP6: (A) Anti-bacterial activities of biosurfactant produced by *Pseudomonas* sp. BUP6 [anti-bacterial activity was the maximum against *E. coli* (43 %) and *S. aureus* (42 %)]; (B) Anti-adhesion property of biosurfactant produced by *Pseudomonas* sp. BUP6 [antiadhesive activity was the maximum against *E. coli* (49 %) and *S. aureus* (46 %)]; and (C) Analysis of phytotoxicity of biosurfactant produced by *Pseudomonas* sp. BUP6 (the biosurfactant did not show any toxic effect on seed germination).

Characterization of biosurfactant

Upon heating with orcinol reagent, the purified biosurfactant turned muddy brown in color, a comparable effect to that of standard rhamnose (positive control); which confirmed the presence of sugar moieties in the purified biosurfactant and the rhamnose content was quantitatively estimated as 34.2 %. The chloroform extract of the uninoculated medium was used as negative control. The TLC plates after treating with iodine vapor produced two spots (0.72 and 0.48 R_f values); which confirmed the presence of mono- and di-rhamnolipids, respectively in the sample (Fig. 22A). Further confirmation by FTIR clearly indicated the

presence of peaks characteristic to 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 and -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. Thus, the FTIR spectrum confirmed that the biosurfactant produced by *Pseudomonas* sp. BUP6 belongs to the category of rhamnolipid (Fig. 22B).

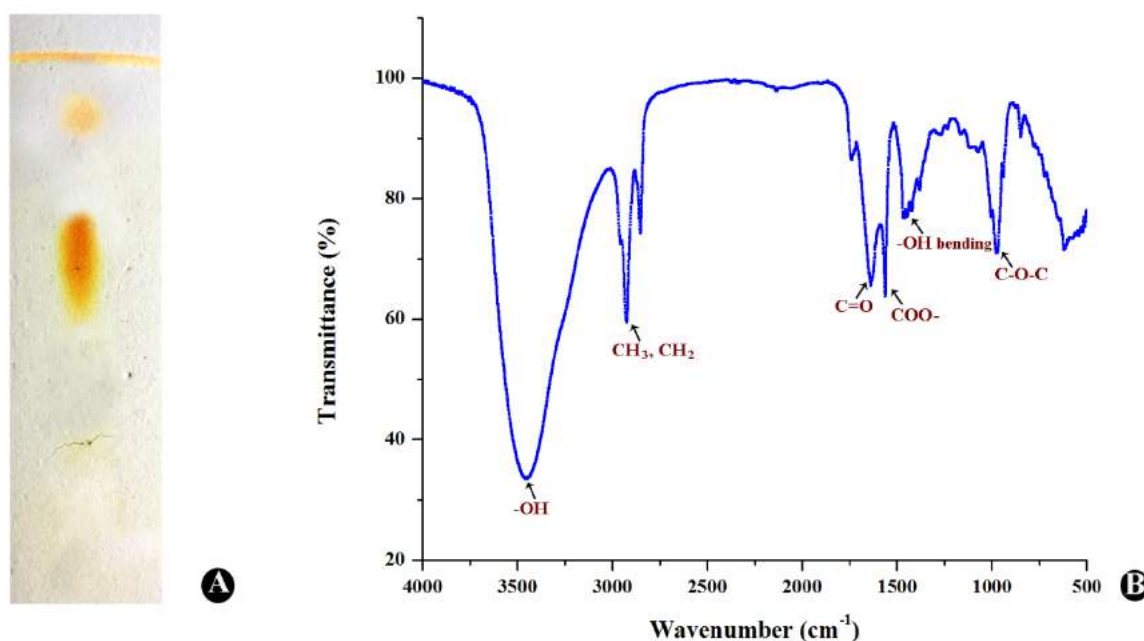


Figure 22. Structural characterization of the biosurfactant produced by *Pseudomonas* sp. BUP6: (A) Thin layer chromatogram showing two spots with R_f values of 0.72 and 0.48 which confirmed the presence of mono- and di-rhamnolipids, respectively; and (B) FTIR profile.

In conclusion, *Pseudomonas* sp. BUP6 is demonstrated as an efficient producer of rhamnolipid type biosurfactant with good surface properties and stability indices. The biological properties of this rhamnolipid suggest that it possesses 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.

Part III – Lipases

Cultivation strategy and medium for lipase production

Pseudomonas sp. strain BUP6 was tuned to get adapted to higher concentrations of vegetable oil and utilized 0.5% of groundnut oil within 24 h in a specially designed basal medium as described. The *Pseudomonas* sp. strain BUP6 showed maximum absorbance at 12 h of incubation. The pH of the medium was gradually decreased from 7.01 to 5.21, and again increased to 6.16 gradually (Fig. 23A). Maximum lipase activity (96.15 U/ml) was noticed at 12 h of incubation, which gradually decreased (Fig. 23B). Drop in pH of the medium may be due to the release of free fatty acids from vegetable oil by lipase activity. 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 analyzed (140 rpm, 37 °C, 12h), 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 activity, i.e., 64 U/ml and 60 U/ml, respectively (Fig. 23C).

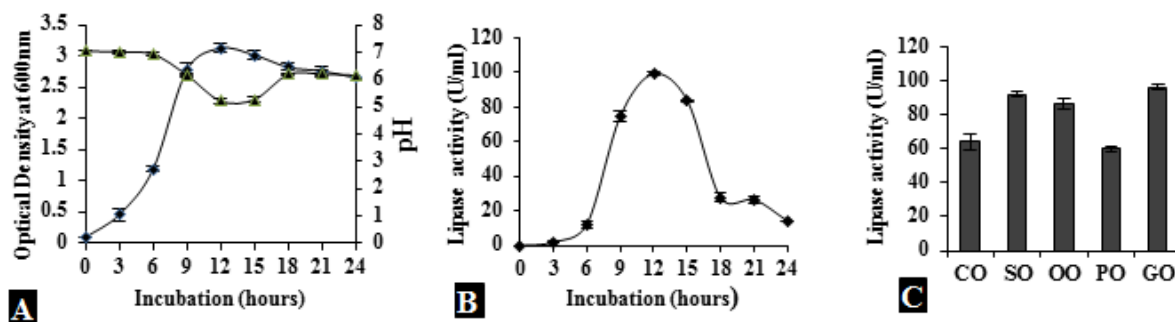


Figure 23. Cultivation strategy and production of lipase (A) growth characteristics of *Pseudomonas* sp. strain BUP6 in basal medium supplemented with 0.5% of groundnut oil. The culture showed maximum absorbance of λ_{600} at 12th h of incubation. pH of the medium was gradually decreased from 7.01 to 5.21 and again increased to 6.16 gradually; (B) lipase production by *Pseudomonas* sp strain BUP6 was estimated quantitatively using *para*-nitrophenyl palmitate (*p*NPP) as substrate, at regular intervals of 3 h. Maximum lipase (96.15 U/ml) was produced at 12 h of incubation which was then gradually decreased; (C) suitability of various vegetable oils (0.5%) as inducer for the production of lipase was analyzed by supplementing the basal medium with vegetable oils such as groundnut oil (GO), sunflower oil (SO), olive oil (OO), palm oil (PO) and coconut oil (CO). At 12 h of incubation, GO showed the highest production of lipase (96.15 U/ml), compared to other vegetable oils.

Statistical optimization 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. strain BUP6. Initially, 5 different physical parameters, *i.e.*, temperature, pH, substrate concentration, incubation time, agitation were considered. 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 at 0.05 significant level. The standardized effects of each parameter showed that temperature, agitation and pH were influenced the production of lipase significantly (Fig. 24). These 3 parameters were subsequently considered for Box-Behnken analysis by response surface methodology to estimate optimum combination of these parameters for maximizing the production of lipase. A set of 15 experiments were conducted according to Box-Behnken design (Table 6) and the results showed that the predicted and experimental values for lipase activities did not show significant difference, that the R^2 value was 0.9903 - close to unity. A second order polynomial function was fitted to the experimental lipase activity, which resulted in the following regression equation,

$$\begin{aligned} \text{Lipase} = & -10751.5 + 386.27X_1 + 10.30X_2 + 810.43X_3 - 4.31X_1^2 - 0.032X_2^2 + 32.23X_3^2 + 0.02X_1X_2 \\ & - 10.22X_1X_3 + 0.08X_2X_3 \end{aligned} \quad \text{Eq. (3)}$$

Table 6 Box-Behnken design matrix with the respective responses in terms of lipase activity

RunOrder	Temperature	Agitation	pH	Lipase activity (U/ml)
1	39.5	150	5	2.02
2	39.5	175	6	94.302
3	37	175	7	114.471
4	42	200	6	16.516
5	39.5	200	5	0.898
6	42	150	6	4.37
7	42	175	5	1.515
8	39.5	175	6	91.431
9	37	175	5	0.594
10	37	150	6	74.428
11	37	200	6	81.301
12	42	175	7	13.229
13	39.5	150	7	72.73
14	39.5	200	7	79.771
15	39.5	175	6	89.113

The summary of the analysis of variance (ANOVA) for the selected quadratic model is shown in Table 7. Based on these results, the model was utilized to generate response surfaces for the analysis of the variable effects on the production of lipase. The response surfaces and corresponding contour plots were obtained using Eq. (3) (Fig. 25).

Three random experimental conditions (different from Box-Behnken, but within the range investigated) were evaluated for 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 8). 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 optimization resulted in 0.3 fold increase of lipase activity over the unoptimized condition, from *Pseudomonas* sp. strain BUP6.

Table 7 Analysis of variance (ANOVA) for the fitted quadratic model of lipase activity.

Term	Coefficient	SE Coefficient	T	P
Constant	91.615	4.130	22.181	0.000
X1	-29.395	2.529	-11.622	0.000
X2	3.117	2.529	1.232	0.273
X3	34.397	2.529	13.599	0.000
X1*X1	-26.932	3.723	-7.234	0.001
X2*X2	-20.530	3.723	-5.514	0.003
X3*X3	-32.231	3.723	-8.657	0.000
X1*X2	1.318	3.577	0.369	0.728
X1*X3	-25.541	3.577	-7.140	0.001
X2*X3	2.041	3.577	0.571	0.593

Table 8 Random combinations of parameters selected for the validation of proposed statistical model with respective predicted responses in terms of lipase activity.

Run No	Temperature	Agitation	pH	Observed activity (U/ml)	Predicted activity (U/ml)
1	37	177	6.9	116.0572	122.116
2	37	160	6.9	108.3262	112.69
3	40	180	6.5	89.28723	87.9
4	37	180	6.0	91.59903	96.2

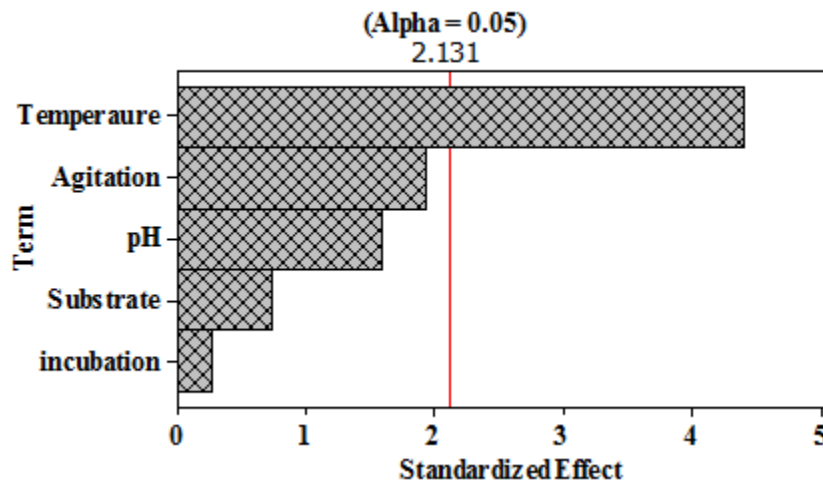


Figure 24. Plackett Burman analysis Pareto chart showing the effect estimates of temperature, pH, agitation, substrate concentration and incubation time on production of lipase (Minitab version 14).

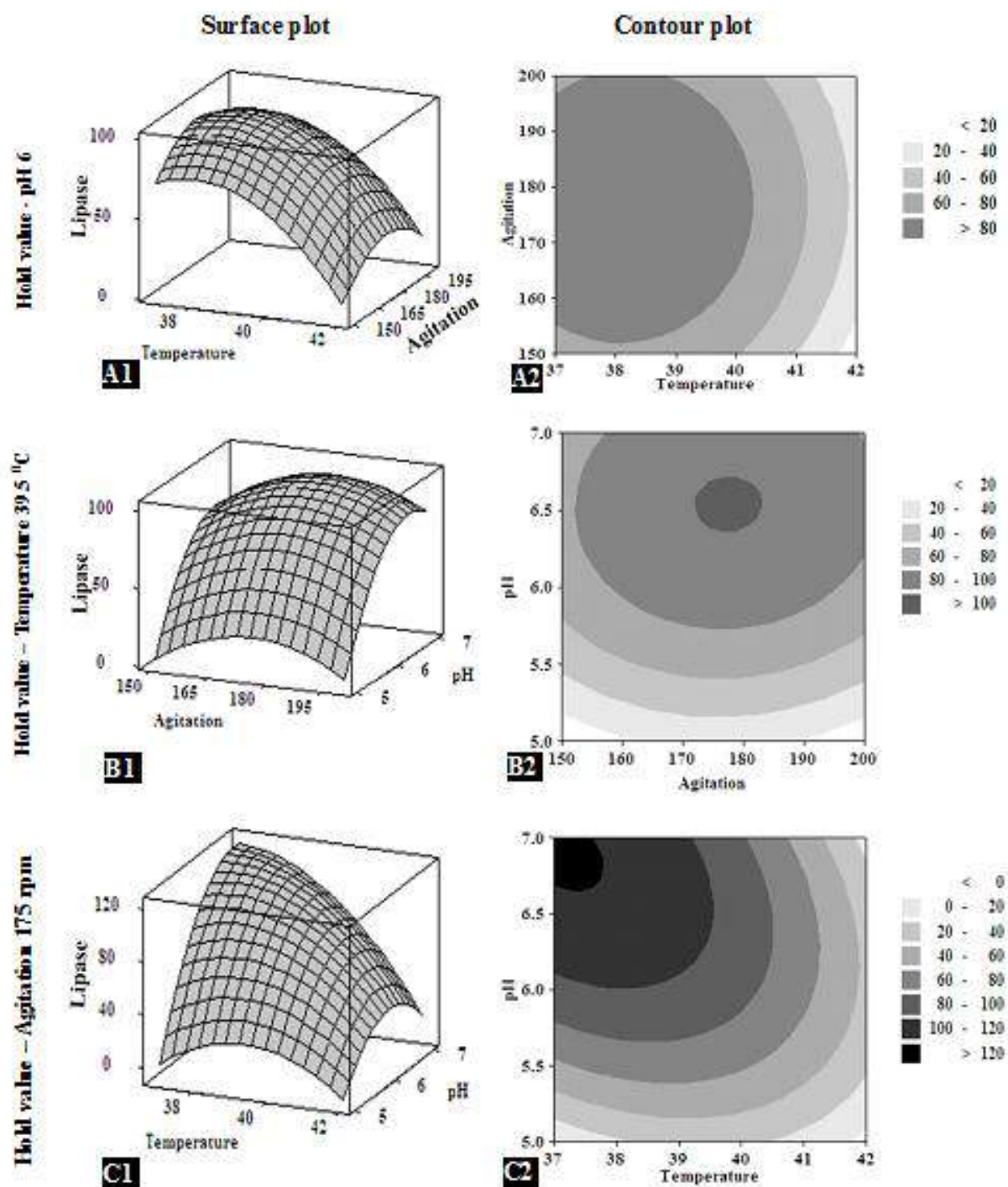


Figure 25. Response surface methodology Surface and contour plots described by the proposed model showing the interactive effects of parameters, temperature, agitation and pH on production of lipase by *Pseudomonas* sp. strain BUP6.

Partial purification and characterization of lipase

Native/non-reducing PAGE and SDS-PAGE were performed to qualitatively characterize the partially purified enzyme. The approximate molecular mass of lipase was estimated as 35 kDa by SDS-PAGE (Fig. 26A). An opaque band showing lipase activity was detected upon native PAGE (Fig. 26B).

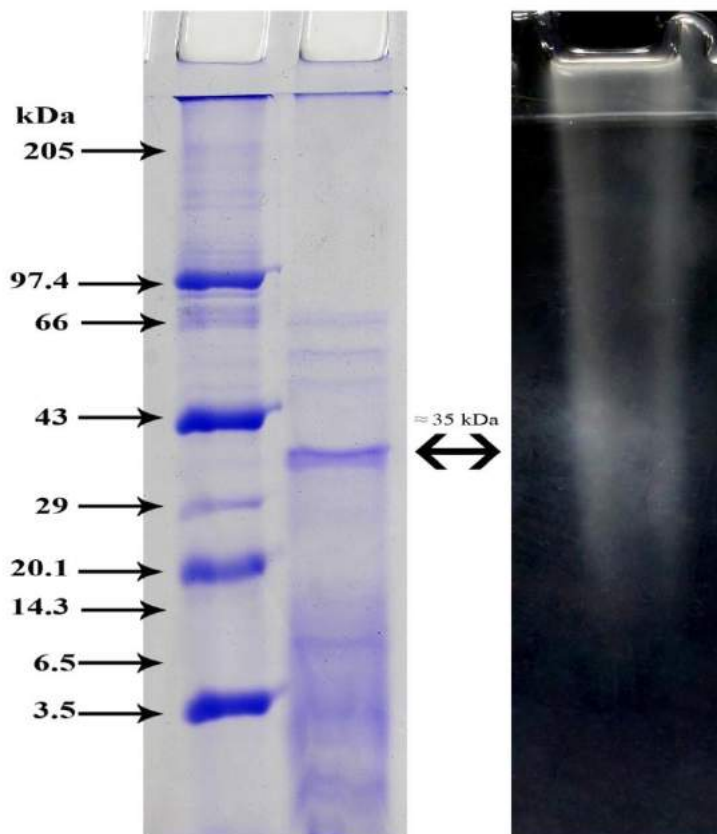


Figure 26. Polyacrylamide gel electrophoresis (PAGE) profile of partially purified lipase (A) SDS-PAGE. Lane 1 is the reference molecular weight marker, lane 2- partially purified lipase on 12% gel (B) native PAGE profile of partially purified lipase on 8% gel.

Purification of lipase

Of various $(\text{NH}_4)_2\text{SO}_4$ fractions, 20-60% fraction showed the maximum lipase activity, which was of 16.9 folds purified with 24.8% yield (Table 9). This fraction (20-60%) was subjected to gel permeation chromatography. Thirty two fractions were collected at a flow rate of 2ml/20min, and the optical density was measured at λ_{280} which showed a major peak

represented by the fraction numbers 10 to 12. The purification fold of sephadex G-100 fraction of lipase was 35.8 with yield of 14.8 % (Table 9). The lipase active fraction after each purification step was subjected to SDS-PAGE for purity check. The apparent MW of the partially purified lipase was estimated as 35 kDa (Fig. 27).

Table 9. Summary of enzyme purification

Step	Lipase (U/ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold	Yield (%)
Crude	139.8	1.3	263.0	27963.1	106.3	1	100
20-60% (NH ₄) ₂ SO ₄	1386.3	0.8	3.9	6931.4	1796.3	16.9	24.8
Sephadex G100	693.9	0.2	1.1	4163.6	3801.4	35.8	14.8

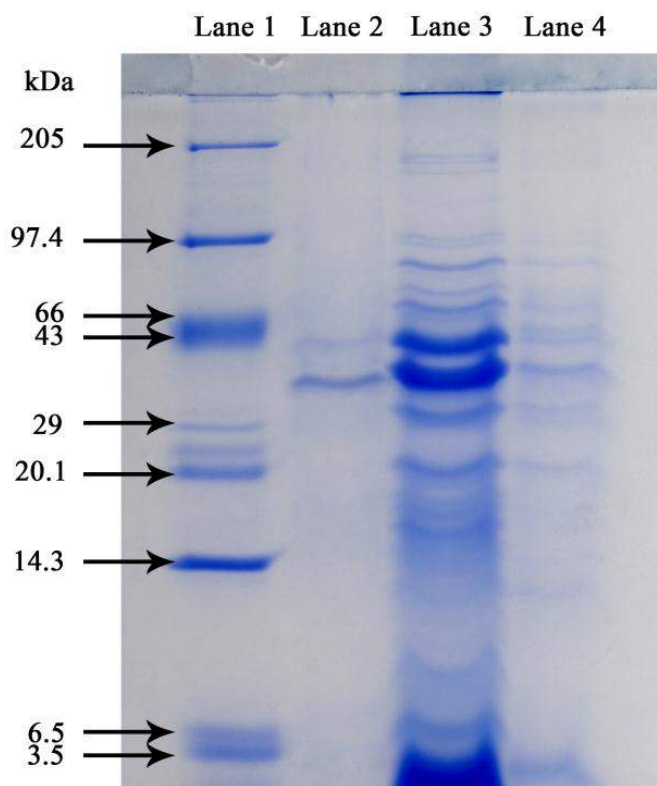


Figure 27. SDS-PAGE profile of lipase from *Pseudomonas* sp. BUP6. Lane 1. standard protein molecular weight marker; Lane 2. sepadex G100 column purified lipase showing apparent molecular weight of 35 kDa; Lane 3. (NH₄)₂SO₄ precipitated fraction; Lane 4. crude enzyme harvested at 37 °C, pH 6.9 and 200 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 was compared to the Swissprot protein database using the software Mascot (Fig. 28). The peptide sequences were matched with that of lipase from *Pseudomonas putida* KT2440 with MW 34.2 kDa.

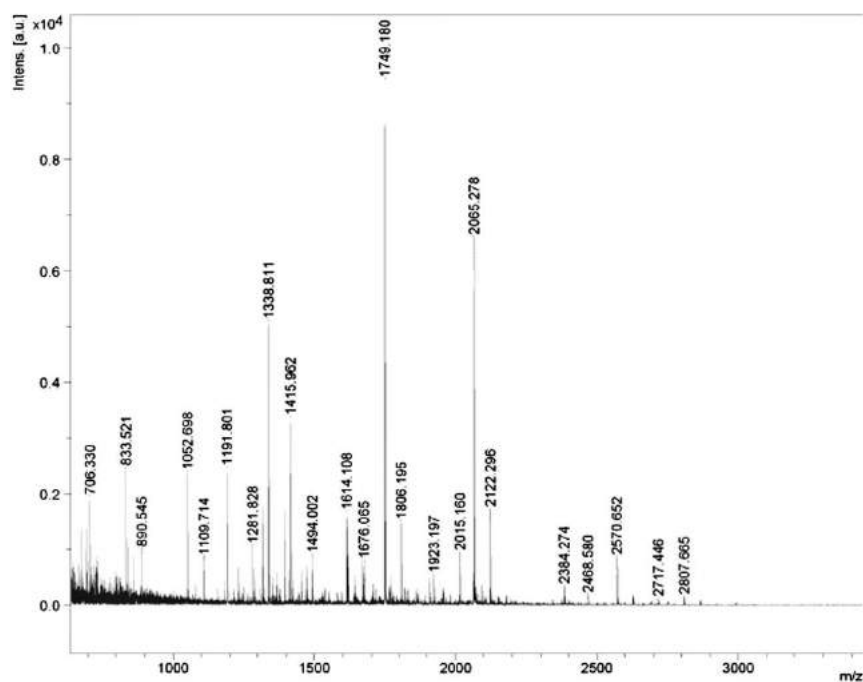


Figure 28. Mass spectrum of trypsin digested peptides of purified lipase from *Pseudomonas* sp. BUP6 with molecular mass 35 kDa.

Characterization of purified lipase

Lipase active fraction obtained by sephadex G-100 gel permeation chromatography was used for the characterization studies.

Effect of pH on lipase activity and stability

Effect of pH on lipase activity was measured at normal assay conditions using 50mM *p*-NPP as substrate (37 °C, and 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). But the enzyme was inhibited at acidic pH. *Pseudomonas* sp. BUP6 lipase maintained more than 90% of the initial activity at pH 9, which was found stable for 1 h - clearly indicating the alkaline nature of lipase (Fig. 29).

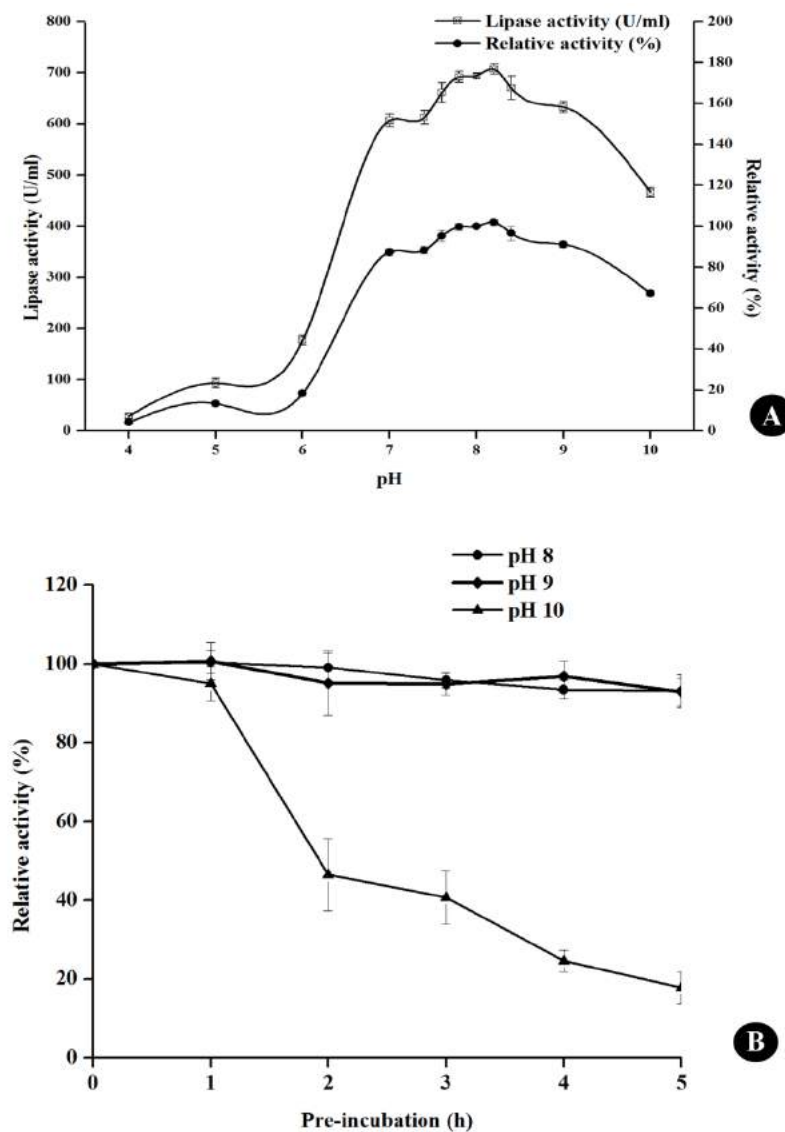


Figure 29. A) Effect of pH on lipase activity showing the alkalophilic nature of lipase (pH 7-9). Maximum activity was observed at pH 8.2 (707.1 ± 9 U/ml) with the relative activity of 102%; B) Stability of lipase was analysed at three pH 8, 9, and 10 among which at the pH 8 and 9, lipase maintained 90% of activity even after 5 h whereas at pH 10, 50 % of activity was lost after 1 h of incubation.

Effect of temperature on lipase activity and stability

Purified lipase was active in the 30 to 50 °C range with optimum activity at 45 °C (891.4 ± 8.7 U/ml). At optimum temperature (45 °C), *Pseudomonas* sp. BUP6 showed 128% of the initial activity with stability for 4 h. Even at 50 °C, lipase found active for two h, thereafter the activity was decreased considerably (Fig. 30).

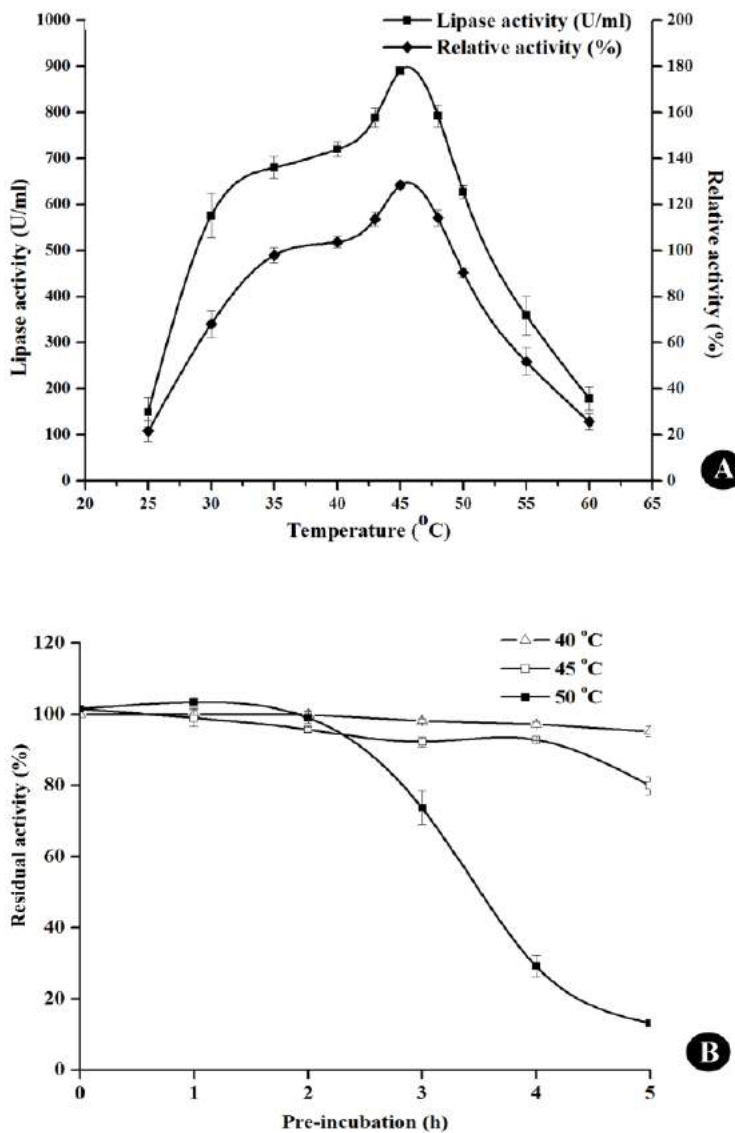


Figure 30. A) Effect of temperature on lipase activity showing its thermophilic nature. Lipase was found active at temperature ranging from 35-50 °C with optimum activity at 45 °C (891.4 ± 9 U/ml) and the relative activity of 128%. B) Stability of lipase was analysed at three different temperatures, 40, 45 and 50 °C among which at the optimum temperature of 45 °C, lipase was found active for 4 h..

Effect of detergents and modifiers on lipase activity

SDS and tween 80 inhibited the activity of lipase to the relative activities of 18.6% and 15.6%, respectively; whereas it was active in the presence of triton X-100 (0.5 %) as under normal assay condition, but lower/higher concentration of triton X100 inhibited the activity. EDTA exhibited no effect on lipase activity, whereas β -mercaptoethanol inhibited the activity of lipase significantly to the relative activity of 1.2% (Fig. 31A).

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 ± 49 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 (Fig. 31B).

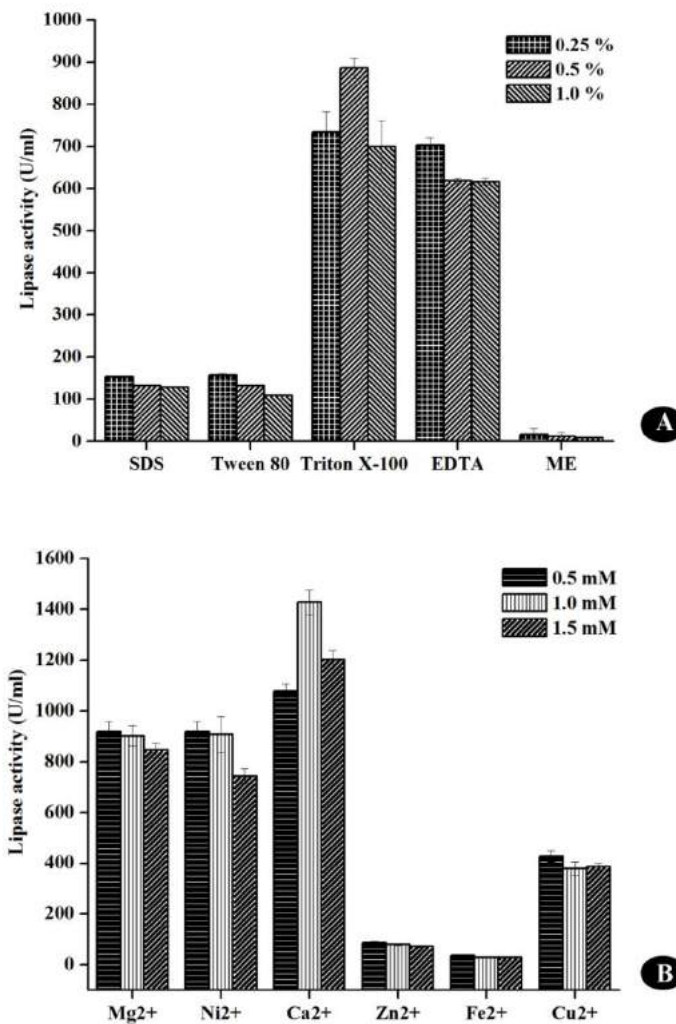


Figure 31. A) Effect of detergents and modifiers on lipase activity. Triton X-100 (0.5%) and EDTA (0.25 %) maintained the activity of lipase whereas SDS, tween 80 and ME reduced the activity significantly; B) Effect of metal ions on lipase activity. Ca²⁺ (1.0 mM) enhanced the activity of lipase (1428±48.8 U/ml) by 206 % of the initial activity. Mg²⁺ and Ni²⁺ slightly enhanced the lipase activity whereas Zn²⁺, Fe²⁺ and Cu²⁺ reduced the activity significantly.

Optimized condition

The optimized condition for the activity of lipase produced by *Pseudomonas* sp. BUP6 was pH 8.2, temperature 45 °C, and 1.0 mM Ca²⁺, at which the relative activity was enhanced to 206% of the initial activity (Table 10).

Table 10. Purification table of lipase from *Pseudomonas* sp. BUP6

Optimum condition	Lipase activity (U/ml)	Relative activity (%)
pH 8.2	707.1±9.1	101.9±1.3
45 °C	891.4±8.7	128.4±1.2
Ca ²⁺	1428±48.8	205.7±10.9

Enzyme kinetics

The kinetic parameters of the extracellular lipase were determined from Michaelis Menten plot (**Figure 32**). The K_m and V_{max} values of purified lipase were found as 11.6 mM and 668.9 $\mu\text{mol}/(\text{min}/\text{mg})$, respectively.

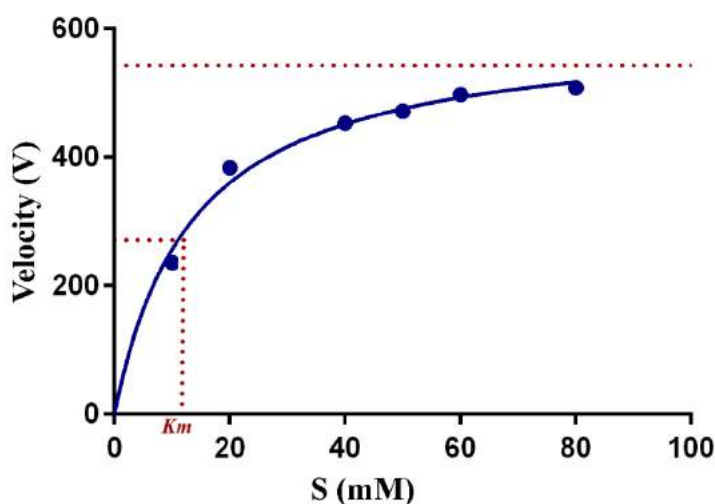


Figure 32. Michaelis Menton plot of purified lipase indicating its kinetic parameters (K_m 11.6 mM and V_{max} 668.9 $\mu\text{mol}/(\text{min}/\text{mg})$).

Thus this study describes the production, purification and characterization of alkaline thermostable lipase (35 kDa) from the rumen bacterium, *Pseudomonas* sp. BUP6. The alkaline and thermotolerant nature of lipase offers potential applications in many industries in detergency, tannery, *etc.* 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.

Chapter 5

DISCUSSION

Discussion

Part I – PHB

The PHB is naturally occurring 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 commercialization of PHB as a cheaper biopolymer. Hence, the exploration of new strains, substrates and cultivation strategies still remain as a major area of research. Very recently, our group found that *C. tropicalis* BPU1, an isolate from the goat rumen, produces PHB significantly (Priji *et al.*, 2013). Apart from the clinical significance, only a few studies have been reported on the exploitation of *C. tropicalis* as a microbial source for the production of industrially significant biomolecules (Ashish *et al.*, 2011). Therefore, the present study focused on the investigation of new substrates for the production of PHB followed by the optimization of culture parameters to enhance the production as well as the characterization of the PHB produced.

C. tropicalis BPU1 produced PHB on starchy substrate, which was extracted in crystalline form. The spherulites - semi-crystalline regions associated with crystallization of polymers from the melt (Barham *et al.*, 1984) - were clearly observed during crystallization, 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 crystallization (Lauzier *et al.*, 1992). The presence of PHB in the crystals was confirmed by TLC, UV-Vis and FTIR. It was reported that most of the microbial PHBs are thermostable until 240-250 °C, after which it shows rapid degradation. For instance, PHB produced by *Halomonas* sp. SA8 showed rapid thermal degradation between 245 and 290°C (Castro *et al.*, 2014) whereas PHB produced by *A. eutrophus* showed thermal degradation between 250-300 °C (Hahn *et al.*, 1995). Similarly, thermal analysis of the PHB crystals produced by *Candida tropicalis* BPU1 showed rapid degradation at temperature 240 to 345 °C (i.e., thermostable until 240 °C); the initial drop in the thermogram of the PHB crystals may be due to the presence of traces of water and other impurities. 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 *et al.*, 1992).

Irrespective of the great deal of efforts made for the commercialization of biopolymer, the wide use of PHB remains scarce, because of its high production cost. The cost efficiency of PHB production 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 and readily available crude substrates for the production of PHB is still an untapped area of research. The study provided clear evidences for the enhanced production of PHB in crude 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 cell dry weight of PHB in medium containing tryptophan (Safak *et al.*, 2002). The yield in the present study is quite comparable to that of many bacterial species *Bacillus*, *Pseudomonas*, *etc.*; *Bacillus thuringiensis* IAM 12077 produced 0.44 g/g cell dry weight of PHB in nitrogen deficient medium supplemented with glucose (Adwitiya *et al.*, 2009), and *Pseudomonas cepacia* produced 0.5 g/g cell mass 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 the yield of 0.39 g/g 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. Moreover, statistical optimization employing Box-Behnken design and RSM enhanced the PHB yield by 1.6 times, indicating its industrial significance; since this statistical tool exhibits linear interaction and quadratic effects of two or more parameters at a time, it could be used efficiently for evaluating the optimization of various biotechnological processes.

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). Starch is the major component of potato, which constitutes about 65-80% of its dry matter; apart from this, lesser proportions of protein (1-5 %), fat (0.1 %), sugars (0.1-0.8 %) and minerals (0.05-0.1 %), which increase its nutritive value

(Kaur and Aggarwal, 2014). Tapioca and jack seed are also cheap and readily available starchy substrates in these areas. Utilization of these crops and seeds for the production of biopolymer would reduce the production cost. Moreover, the utility of starchy substrates offers a new strategy for the industrial and economic exploitation of the underutilized crops and seeds, especially employing solid- or semisolid-state fermentation systems.

In conclusion, conventional *C. tropicalis* is known for clinical significance, but this study clearly demonstrates its industrial significance as well. The crude 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 these substrates would benefit the biopolymer industry by applying suitable fermentation strategies including SmF or SSF. 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.

Part II – Biosurfactants

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 analyzing the surface hydrophobicity of *C. tropicalis* BPU1 and *Pseudomonas* sp. BUP6 cells, coupled with the physico-chemical, biological and structural characterization of the biosurfactant produced by it.

Biosurfactants are a group of natural products of interest for biotechnological and industrial applications. Yeasts are known to produce extracellular emulsifiers when grown on water-immiscible substrates such as alkanes or oils, in order to facilitate their uptake. Among yeasts, *Candida* species have been widely used for hydrocarbon cultivation and production of biosurfactants (Sarubbo *et al.*, 2001).

Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability affect biosurfactant production through their effects on cellular growth or activity. The pH of the medium plays an important role in biosurfactant production. The optimum production of biosurfactant by *Candida* sp. varies widely for different isolates. For instance, Jagtap *et al.*, 2010 showed that maximum production of bioemulsifier was at pH 7 though less activity was at pHs 6, 8 and 9. In contrary, Sudha *et al.*, 2010 observed that pH 3.5 was the optimum for the production of sophorolipid from *C. tropicalis*. Our study showed that the sophorolipid production by *C. tropicalis* BPU1 was optimum at pH 6. Hesham *et al.*, 2012 indicated that the optimum temperature for production of biosurfactant by all *Candida* isolates was mostly 20°C. In contrary, Sudha *et al.*, 2010 observed that temperature 30 °C was optimum for production of sophorolipids from *C. tropicalis*. Also, Jagtap *et al.*, 2010 found that optimum temperature for bioemulsifier production was 37 °C which can be attributed to the habitat of the organism. Our data is also in agreement with the aforesaid results where the optimum temperature was found as 33 °C. *Candida* strains showed a significant and optimum production of biosurfactant at an incubation period 8 days (Hesham *et al.*, 2012). But, the strain, *C. tropicalis* BPU1 reached the optimum production of biosurfactant quite early. i.e., at 6 days of incubation.

The critical micelle concentration (CMC) is the minimum biosurfactant concentration necessary to reduce the surface tension to the maximum extent. The biosurfactant from *C. sphaerica* showed a great surface tension reduction capacity since the water surface tension was reduced from 70 to 25 mN/m with the increase of the biosurfactant concentration up to CMC of 0.25 mg/ml (Luna *et al.*, 2012). Similarly, *C. tropicalis* BPU1 showed a CMC value of 0.2 mg/ml. Stability of the cell free broth containing the biosurfactant was investigated over a wide range of pH, temperature and salinity ; the results indicated the suitability of the surfactant to be used for pharmaceutical and environmental applications which was further confirmed by its antiadhesive/inhibitory properties against the common pathogens, *E. coli* and *S. aureus*.

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 optimize the yield of rhamnolipid by *Streptomyces coelicoflavus*, Kalyani *et al.* (2014) used Plackett-Burman design; that resulted in 4 % increase over the unoptimized condition. In the present study, Plackett-Burman and Box-Behnken design were employed effectively to screen as well as to optimize the parameters for maximizing 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 optimized as 75 h, pH 7 and 35 °C with a maximum biosurfactant yield of 2070 mg/l, *i.e.*, 11 % higher than that obtained under un-optimized conditions. The yield of biosurfactant 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.

The study was to analyze 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 analyzed 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).

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 ddH₂O) 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 °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 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.

Generally, species of *Pseudomonas* are known producers of glycolipid biosurfactants; especially, rhamnolipid (Lotfabad *et al.*, 2009). The structural characterization 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 visualization 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 monorhamnolipids. 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 Pornsunthorntaweew *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.

In conclusion, *Pseudomonas* sp. BUP6 is found as an efficient producer of biosurfactant with good emulsification and stability indices as compared to that of *C. tropicalis* BPU1. 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.

Part III – Lipases

Most published experimental data have shown that lipid 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 medium is quite suitable for the production of lipase as it supported 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, utilizing olive oil as substrate (Mobarak-Qamsaari *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 (Sharon *et al.*, 1998). Compared to these cultures, *Pseudomonas* sp. strain BUP6 described in this study is highly promising and suitable for industrial applications.

Since culture conditions influence the properties as well as quantity of the enzyme produced, optimization of fermentation parameters such as temperature, pH and agitation for the production of microbial lipases is of great interest (Wang *et al.*, 1995). The classical method of optimization 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 optimization systems are used to overcome this problem. RSM is a combination of statistical and mathematical tool, widely used to optimize various biotechnological processes in which linear interaction and quadratic effects of two or more parameters were estimated to produce three dimensional contour and surface plots (Burkert *et al.*, 2004). Usage of Plackett-Burman design for estimating the significant parameters reduced the number of parameters from 5 to 3, and thereby reducing the experimental trials for RSM analysis. In this study, Box-Behnken design with 3 relevant factors was performed for fitting a 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.

Having optimized the physical parameters, the apparent molecular mass of partially purified lipase produced *Pseudomonas* sp. strain BUP6 was found to be 35 kDa by SDS-PAGE. According to literature, molecular mass of lipases from *Pseudomonas* spp. varies from 30 to 95 kDa. The purified lipase produced by *P. aeruginosa* MB5001 had a molecular mass of 29 kDa, as judged by SDS-PAGE (Chartrain *et al.*, 1993); whereas *P. aeruginosa* LX1 produced an extracellular lipase of molecular mass 56 kDa (Ji *et al.*, 2010). An extracellular lipase produced by *P. gessardii* showed an apparent molecular mass of 94 kDa (Kandasamy *et al.*, 2010). The opaque zone observed in native PAGE gel also confirmed the enzyme produced by *Pseudomonas* sp. strain BUP6 as lipase.

The first objective of the study was to remove as much as unwanted proteins as possible retaining the lipase activity. The progress of enzyme purification is usually analyzed by two critical parameters the yield and fold of purification. Generally, precipitation, ultrafiltration, gel exclusion chromatography were used for the purification of extracellular enzymes (Palekar *et al.*, 2000). Ogino *et al.*, (2007) purified an alkaline thermostable lipase produced by *Pseudomonas aeruginosa* LST-03 to 34.7 fold with a yield of 12.6 % employing $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatographic techniques. An extracellular alkaline lipase from *P. aeruginosa* mutant has been purified to homogeneity using acetone precipitation

followed by column chromatography which resulted in 27-fold purification with 19.6% final recovery (Bisht *et al.*, 2013). Likewise, $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel exclusion chromatography were employed to purify lipase from *P. fragi*, *A. pullulans* HN2.3 and *Burkholderia multivorans* with a yield of 18%, 12.6% and 0.96% of the initial activity (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 $(\text{NH}_4)_2\text{SO}_4$, dialysis and sephadex G 100 column, resulting in a yield of 14.8% (35.8 folds purification).

From the SDS-PAGE profile, the MW of lipase produced by *Pseudomonas* sp. BUP6 was found to be 35kDa, which was confirmed by MALDI-TOF/MS. According to the literature, MW of lipase produced by *Pseudomonas* spp. varied from 30 to 95 kDa (Priji *et al.*, 2014). 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 Kehria 2013).

The *para*-nitrophenyl palmitate is one of the common substrates used for lipase assay (Priji *et al.*, 2014). The activity of the enzyme was analyzed in different buffer systems with varying pH 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 enzyme activity. Similarly, the lipase withstands a temperature range of 35 to 50 °C with an optimum at 45 °C. Thus, the alkalophilic and thermostable properties of the enzyme reveal its possibilities of utilization in many industries like detergency and tannery.

Of various metal ions tested, Ca^{2+} in the reaction mixture stimulated or stabilized the lipase activity by 206%, whereas Mg^{2+} and Ni^{2+} slightly enhanced the activity; however, Zn^{2+} , Fe^{2+} and Cu^{2+} inhibited the activity, of which Fe^{2+} showed the strongest inhibitory activity. The 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 stabilizes its activity (Schrag *et al.*, 1997; Alquati *et al.*, 2002). Among the various detergents and modifiers, 0.5% of the triton X100 supported the enzyme activity, but its higher/lower concentration decreased the activity. Presence of β -mercaptoethanol drastically decreased the lipase activity, which indicated the involvement of the disulphide bonds in stabilizing the enzyme. Moreover, EDTA exhibited no effects on lipase activity. Similar characteristics were shown by lipases produced by *P. aeruginosa* and *Geobacillus* sp. TW1 (Li and Zhang 2005; Gaur *et al.*, 2008).

K_m (11.6 mM) and V_{max} (668.9 $\mu\text{mol}/\text{min}/\text{mg}$) values of the lipase produced by *Pseudomonas* sp. strain BUP6 was determined by Michaelis Menten plot using *p*NPP as substrate. K_m value is the measure of affinity of enzyme towards a substrate. Low K_m value represents that the enzyme requires only small amount of substrate to get saturated. High V_{max} indicates the high 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 K_m values of enzymes vary from 10^{-1} to 10^{-5} M (Fullbrook 1996). Lipase from *P. cepacia* showed K_m and V_{max} of 12 mM and 30 $\mu\text{mol}/\text{min}$, respectively using *p*NPP as substrate (Pencrac'h and Baratti 1996). *P. aeruginosa* PseA lipase showed a K_m value of 70.4 mM and V_{max} of 2.24 mmol/(min mg) with *p*NPP as substrate (Gaur *et al.*, 2008). It shows that lipase from *Pseudomonas* sp. BUP6 is more efficient than many other lipases from *Pseudomonas* spp.

Thus, this study describes the purification and characterization of alkaline thermostable lipase (35 kDa) from the rumen bacterium, *Pseudomonas* sp. BUP6. The alkaline and thermotolerant nature of lipase offers potential applications in many industries in detergency, tannery, *etc.* 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.

Chapter 6

SUMMARY AND CONCLUSIONS

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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. Biosurfactants and bioplastics are some of the major groups of industrially significant biomolecules produced by microorganisms.

The rumen yeast, *C. tropicalis* BPU1 was 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 parameters for the production of biosurfactant were optimized statistically using response surface methodology (RSM) as pH 6, 33°C, substrate 0.13%, and incubation 6 days. Under optimized conditions, the production of biosurfactant was enhanced from 432 mg/l to 649 mg/l, and 0.5-fold increase had been obtained. The biosurfactant was then purified by double precipitation method and identified as sophorolipids employing FTIR and LC/MS. The sophorolipids produced by *C. tropicalis* BPU1 was then characterised for its physico-chemical and biological properties. The biosurfactant showed the critical micelle concentration (CMC) as 200 mg/l, and was found stable at a range of pH 3-7, temperature 40-60 °C 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 *Acetobacter baumannii* which showed that at concentrations of 200 mg/l and 100 mg/l, the biosurfactant inhibited the growth and adhesion of *E. coli* and *S. aureus* significantly. Thus, *C. tropicalis* BPU1 was proved as an efficient producer of sophorolipid type biosurfactant.

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) to evaluate the efficiency of *C. tropicalis* BPU1 for the production of PHAs. 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 °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 °C.

In addition to *C. tropicalis*, we isolated a novel bacterium, *Pseudomonas* sp. BUP6 from the rumen of Malabari goat, which could produce lipase and biosurfactant effectively. The culture parameters for the production of lipase was statistically optimized as 37 °C , 200 rpm and pH 6.9 (129 U/ml). The r lipase was purified to homogeneity and then characterized which showed that the extracellular, thermotolerant and alkaline lipase from *Pseudomonas* sp. BUP6 offers potentials for industrial applications. Moreover, *Pseudomonas* sp. BUP6 is demonstrated as an efficient producer of rhamnolipid type biosurfactant with good surface properties and stability indices. The biological properties of this rhamnolipid suggest that it possesses 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.

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Appendix I

Salient outcome of the project

- ✓ *C. tropicalis* BPU1 is an efficient producer of biosurfactant, it could produce 649 mg/l of sophorolipids in a specially designed basal salt medium supplemented with groundnut oil (0.5 %) at optimised conditions of pH 6, 33°C, groundnut oil 0.13%, and incubation 6 days
- ✓ *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 °C, and 19 h of incubation.
- ✓ *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 °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 °C and incubation for 75 h.
- ✓ Novel strategies for the production of lipase, biosurfactant and PHB were demonstrated.

Scope for future line of work

- ✓ 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.

Appendix II

Publications

1. Priji, P., Sajith, S., Unni, K. N., Anderson, R. C., & Benjamin, S. (2016). *Pseudomonas* sp. BUP6, a novel isolate from Malabari goat produces an efficient rhamnolipid type biosurfactant. *Journal of Basic Microbiology*, 56, 1-13.
2. Priji, P., Sajith, S., Sreedevi, S., Unni, K. N., Kumar, S., & Benjamin, S. (2016). *Candida tropicalis* BPU1 produces polyhydroxybutyrate on raw starchy substrates. *Starch-Stärke*, 68(1-2), 57-66.
3. Priji, P., Unni, K. N., Sajith, S., Binod, P., & Benjamin, S. (2015). Production, optimization, and partial purification of lipase from *Pseudomonas* sp. strain BUP6, a novel rumen bacterium characterized from Malabari goat. *Biotechnology and applied biochemistry*, 62(1), 71-78.
4. Priji, P., Unni, K. N., Sajith, S., & 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(3), 103-110.
5. Priji, P., Sajith, S., Abdul Faisal, P. and Benjamin, S., Microbial lipases – Properties and applications. *Journal of Microbiology, Biotechnology and Food Sciences*. (Accepted).
6. Priji, P., Sajith, S., Unni, K. N. and Benjamin, S. Glycolipid biosurfactants as candidates for green technologies. *Journal of Detergents and Surfactants*. (Communicated).
7. Priji, P., Sajith, S., Unni, K. N. and Benjamin, S. Purification and characterisation of thermotolerant alkaline lipase from *Pseudomonas* sp. BUP6, a rumen bacterium. *Electronic Journal of Biotechnology*. (Communicated).

Ph.D Thesis

Priji, P. 2016. Investigations on industrially significant lipase, rhamnolipid, and polyhydroxybutyrate produced by novel rumen microbes *Candida tropicalis* BPU1 and *Pseudomonas* sp. BUP6. University of Calicut.

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