Pseudomonas aerugionsa BUP2 – a dual producer of lipase and pyoverdine

Thesis submitted to the University of Calicut in partial fulfilment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY IN

BIOTECHNOLOGY

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April, 2015

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CERTIFICATE

Certified that the Ph.D. thesis entitled ***Pseudomonas aeruginosa*** **BUP2- a dual producer of lipase and pyoverdine** is an authentic record of the original research work accomplished by **Mr. Unni, K.N.** under my supervisionat the Enzyme Technology Laboratory, Biotechnology Division in the Department of Botany, University of Calicut, and that no part thereof has been presented earlier for the award of any other degree or diploma. Also certified that the contents in the thesis is subjected to **Plagiarism Check** using the software, **iThenticate®,** and that no text or data is reproduced from other's work.

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DECLARATION

I, **Unni, K. N.** do hereby declare that this thesis entitled **"*Pseudomonas aeruginosa* BUP2– a dual producer of lipase and pyoverdine"** is the summary of the research work carried out by me under the supervision of **Dr. Sailas Benjamin**, Professor, Department of Botany, University of Calicut in partial fulfilment of the requirement for the award of Ph.D. degree in Biotechnology, and also declare that no part of this thesis has been submitted by me for the award of any other degree or diploma.

Calicut University **UNNI,K. N.**

ACKNOWLEDGEMENT

I am indebted to numerous persons from the beginning of this work and without all those timely help I could not have completed this work. I accord my profound gratitude to my research supervisor ***Dr. Sailas Benjamin,*** *Professor, Enzyme Technology Laboratory, Department of Botany, University of Calicut, for the expert guidance, criticisms, constant support and assiduous efforts to bring out the work to its present zenith.*

I am deeply indebted to ***Professor John E.Thoppil,*** *Head of Department and* ***Professor K. M. Jayaram, Professor M. Sabu, Professor K.V. Mohanan****, former Heads,* Department of Botany, University of Calicut *and for their whole hearted help offered throughout the period and for creating conducive environment in the department for the successful completion of my work.*

I convey my immense sense of gratitude to my labmates ***Dr. Pradeep, Mr. M. K. Sarath Josh,Mr. S. Sajith and Mrs. Priji Prakasan, Mrs. S. Sreedevi, Dr. V. N. Jisha, Mrs. P. Princy, Ms. Neethu Kannan, Mr. Nideesh, Mr. E. S. Hareesh, Mr.A. Faisal*** *in the Enzyme Technology Laboratory for being very helpful, suggestive and supportive during my research.*

I gratefully extend my sincere thanks to ***Professor Abraham Joseph, Dept. of Chemistry AssociateProfessorY. Shibuvardhanan, Dept . of Zoology, Professor P. Manimohan****,* ***AssistantProfessor C. C. Harilal****,* ***AssociateProfessorRadhakrishnan, AssistantProfessor A. K. Pardeep*** *all other* ***Teaching*** and ***Non-teaching Staff*** *,* ***Dept. of Botany*** *for providing needful help during the research period.*

I convey my immense sense of gratitude to ***Professor M. V. Joseph****,****ProfessorManish Kumar*** *and* ***Professor K.K.Elyas****, Dept. of Biotechnology, University of Calicut. I am indebted to other departments of Calicut University for providing various instrumentation facilities.*

I convey my gratitude to **Professor Valerie Geoffroy**, ***Professor*** **Herbert Budzikiewicz** *and* ***Professor D. N. Kamra****for studying in molecular characterization of pyoverdine and for providing me a book on 'Techniques in Rumen Microbiology' which helped me a lot in the early isolation process.*

I express sincere thanks to ***Dr. BinodParameswaran****, Scientist, NIIST, Thiruvananthapuram for helping me in RSM analysis.* ***Dept. of Chemistry****, University of Calicut for FT-IR analysis,* ***Xcelris Labs****, Ahmedabad for molecular identification of bacteria. I also convey gratitude to* ***National Institute of Technology,*** *Kozhikodefor taking fluorescent spectroscopy analyses.*

I extend my thanks to ***Mr. N. B. Shaji, Mr. K. Ajayakumar*** *and* ***Mr. Santhosh Mithra, Art*** *and Photography Unit, University of Calicut for their assistance in the accomplishment of the photographs for the present study.*

I also express my thanks to ***Mr. P. M. Prakashan****, Librarian, Department of Botanyfor the help rendered.*

I also express my thanks to ***Mr. K. Rajesh and co-workers****, Bina Photostat, Villunniyalfor their tireless support in preparing this manuscript.*

I express my heartfelt gratitude to all my ***friends*** *especially* ***Santhosh, Anlu, Sajeesh, Bijesh, Sajith, Noufal, Sanjay, Jithin, Deepesh, Sanith****,* ***Jhon Bosco, Rahul, Rajan, Prabhu, Madhu, Manu, Vimal, Prasad, Shiney, Aparna, Sajith*** *for their loving support and encouragement.*

My special words of thanks to all those who directly and indirectly helped me during this period.

Words cannot adequately express my deep sense gratitude to my family, especially to my wife ***Smitha*** *for her love, personal support and great inspiration at all times. My parents,****Mr. Mohanapanicker*** *and****Mrs.P. J. Shyla,*** *for their unconditional love, support and care throughout my life, especially to achieve*

this milestone in my academic career. My brothers ***Mr. Sreekanth***, **Sanoop** and **Rajeesh** for their love and support. My father-in-law **Mr. Chandran Nair** and mother-in-law **C. Soumini,** for looking after my son **Mahadevan** and for all their support to complete this study.

Above all these, I owe to the ***Almighty*** *for giving me the strength and health for the successful completion of the work.*

Unni, K. N.

Dedicated to

My Family, Teachers and Friends

EQUIPMENTS USED

Item	Brand	Country
Chromatography column	Magnum	India
Compound microscope	Magnus	India
Cooling centrifuge	Remi	India
Digital pH meter MK-VI	Systronics	India
Double distillation Unit	Borosil	India
Electrophoresis Unit	Biotech	India
Environmental shaker	Orbitek	India
FT- IR	Jasco	Japan
Heating Mantle	Kemi	India
Gel-documentation system	BioRad	Italy
Image analyser	Towa Opticals	Japan
UV trans-illuminator	Biotech R&D Laboratories	India
Incubator	Technico	India
Laboratory Oven	Labline	India
Laminar air flow cabinet	Kemi	India
Refrigerated centrifuge	Remi	India
Refrigerator	Godrej	India
Sonicator	QSONICA, LLC, XL-2000	USA
UV-Visible spectrophtometer	Shimadzu	Japan
Spectrofluometer	PerkinElmer LS-45	USA
Magnetic Stirrer (KMS – 400)	Kemi	India
Micropipettes (0.5 -1000 µL)	Accupipete	India
Vortex mixture	Kemi	India
Water bath	Scigenics Biotech	India
Weighing balance	Shimadzu	Japan

ABBREVIATIONS

ddH_2O	:	double distilled water
EDTA	:	Ethylene Diamine Tetraacetic Acid
g	:	gram
g/l	:	:gram per litre
gds	:	dry weight in grams
l	:	litre
mg	:	milligram
mg/ml	:	milligram per millilitre
ml	:	milli litre
MW	:	molecular weight
RSM	:	response surface methodology
SDS-PAGE	:	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
SmF	:	Submerged Fermentation
SSF	:	Solid State Fermentation
TEMED	:	N,N,N',N'-tetra methyl ethylene diamine
U/g	:	Units per gram
U/mg	:	Units per milligram
w/v	:	Weight per volume
α	:	alpha
β	:	beta
ε	:	epsilon
µg	:	microgram
µl	:	microlitre
µM	:	micromolar

Contents

Introduction

Chapter 1
Introduction

Overwhelming demand for a clean and safe environment has led to the discovery of many eco-friendly and biodegradable microbial products to satisfactorily address the public concerns regarding the deleterious effects of various pollutants on nature. Enzymes are the prominent class of microbial products, the use of which can be tracedback to ancient civilisations. The history of enzyme technology began in 1874 when the Danish chemist,Christian Hansen produced the crude rennet by extracting calves' dried stomachs (inner lining of the stomach of newborn or young calves is the rich source of rennet) with saline solution, apparently the first enzyme used for industrial purposes. Today, nearly 4000 enzymes are known to humans; of these, about 200 are in commercial use. Until the 1960s, the total sale of enzymes was only a few million US dollars annually, but by 2015 the global market for industrial enzymes is expected rise to 4.4 billion US dollar business (Binod *et al.*, 2011). Though animals, plants and microbes are the rich source of enzymes, microbial enzymes are preferred to animal and plant sources, because: generally cheaper, easy to control the process parameters, easy to arrange the raw materials and potentially not harmful for use. Of the commercial enzymes, over half is contributed by fungi and yeast, while over a third is from bacteria, and the remaining (10-15%) is from plants and animals (Adrio and Demain, 2014). Carbohydrases (amylase, cellulase, lactase, and pectinase, *etc*.), proteases and lipases are the chief players on enzyme market.

After proteases and carbohydrases, lipases are considered to be the third largest group, based on the total sale of enzymes. Microbial lipases

(triacylglycerol acylhydrolase, E.C. 3.1.1.3) are ubiquitous enzymes in nature that catalyse a broad range of reactions such as hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis (Benjamin and Pandey, 1998; Pandey *et al*., 1999) depending on its aqueous-nonaqueous environment. Commerciallylipases are a billion-dollar business that comprises a wide variety of different applications in detergency, tannery, perfumery, cosmetics, foods, and pharmaceutics (Jaeger *et al*., 1999).

When grown in nutrient medium, microbes usually produce non-ribosomal peptideseither extra- or intra-cellularly which include proteinaceous secondary metabolites such astoxins (*e.g*., *δ*-endotoxin (Smitha *et al*., 2013), siderophores (Saha *et al*., 2013), pigments (Venil *et al*., 2013;Pradeep *et al*., 2014) or biopolymers (Sreedevi *et al*., 2014). They are not vital for the microbial growth, but play crucial role in pathogenesis and as stored energy molecules. Most of them can be utilised ascandidates for improving health, nutrition and economics of humans by serving as immunomodulators, antitumor agents, receptor antagonists and agonists, pesticides, enzyme inhibitors and growth promoters of animals and plants, and colourants (Demain, 1998).

Siderophores are one of the major groups of microbial secondary metabolites produced under iron limiting environment (Meyer, 2000). They are low molecular weight organic compounds embodied with high affinity for Fe (*i.e*., chelating agent); to solubilise them, which is otherwise unavailable to the microbial cells. These heterogeneous molecules such as pyoverdine, ferrichrome, ornibactin, enterobactin, azotobactin, *etc*., are secreted by certain bacteria, fungi and grasses (Saha *et al*., 2013). During the last few decades, siderophores have received much attention owing to their potential roles and

applications in various areas of environmental as well as pharmaceutical research, owing to their non-toxicity, biodegradability and easy for isolation.

Rationale: Microbes show cosmopolitan distribution in air, water, soil, alimentary canal, on skin, leave, *etc.*Rumen is the first and largest part of the alimentary system in cattle, which serves as the rich habitat for innumerable microbiota, represented by bacteria, fungi and protozoa (Jami and Mizrahi, 2012). Most of the microbiota dwelling in rumen are considered as non-pathogenic with no ill effects on humans*i.e.*, generally regarded as safe (GRAS) status, (Cuting 2011); compared to other microorganisms growing under the harsh environments such as polluted soil, effluents, sewage or industrial wastes. In the light of the aforesaid background, this study focuses on the rumen microorganisms for the production of industrially significant biomolecules with emphasis on lipase and siderophore. Thus, the specific objectives of the present study are:

1. To isolateand cultivate useful bacteriainhabiting the rumen of Malabari goat.
2. To investigate whether the isolates could be used for the production of industrially-significant biomolecules such as lipase and pyoverdine, a siderophore.
3. Statistical optimisation of the production conditions for lipase and pyoverdine.
4. Purification and characterisation of lipase and pyoverdine.
5. Application of pyoverdine as *turn-off* biosensor

Review of Literature

Chapter 2
Review of literature

Background andrationale

Rumen of ruminants is a treasury of many bacteria, fungi and protozoa. Species of many bacterial genera: *Bifidobacterium, Butyrivibrio, Lactobacillus, Lactococcus, Propionibacterium* and *Prevetella* (Cotta, 1992; Ogawa *et al*., 2001; Coakley *et al*., 2003; Lin *et al*., 2005; Lin, 2006; Liu *et al*., 2011); fungal genera: *Piromonas, Neocallimastix, Orpinomyces* and *Sphaeromonas* (Wubah and Fuller, 1991; Ando *et al*., 2009); and protozoans like *Dasytricha ruminantium, Isotricha prostoma, Eremoplastron dilobum, Entodinium caudatum*, *Ophryoscolex purkinjei* and *Polyplastron multivesiculatum* (Williams and Coleman, 1997; Or-Rashid *et al*., 2008; Khiaosa-Ard *et al*., 2009, Wright, 2009) were reported from the rumen of various ruminants. Most of the microbial strains have a wide range of genetic and metabolic diversity, which enable them for the production of industrially significant bio-molecules such as enzymes and secondary metabolites. Bacterial strains isolated from the rumen were found thriving on lipids (Hobson and Mann, 1961); cellulose (Stewart*et al.*, 1979); hemicelluloses (Cotta, 1992); starch (Latham *et al.*, 1979); pectin (Paster and Canale-Parola, 1985); sugar (Caldwell and Bryant, 1996) and protein (Hobson and Howard, 1969).

Only a few studies were reported on the pigment producing rumen bacteria; a yellow-red coloured pigment was reported from the cellulose and protein degrading bacterial strains isolated from the rumen (Hungate, 1957;

Blackburn and Hobson, 1960). Duncan *et al*. (1999) reported two pigments: a pyocyanin (blue-green pigment) and pyoverdine (yellowish- green pigment) produced by *Pseudomonas aeruginosa* isolated from sheep rumen. Literature shows that no microflora in the rumen of goat, especially of Malabari goat (*Capra hircus* L.) is explored so far. Since this study explores the rumen bacteria capable of producing lipase and siderophore, literature is reviewed giving emphasis to: Part I. Bacterial lipases, and Part II. Bacterial siderophores.

Part I: Bacterial lipase

Lipases or triacylglycerol acylhydrolases (EC 3.1.1.3) catalise lipolytic activities such as hydrolysis, acidolysis, alcoholysis, esterification, *trans*-esterification, racemic solution, stereo selectivity and chiral synthesis (Reis *et al*., 2009; Khare and Nakajima, 2000). Stability and selectivity of microbial lipases make it a versatile biocatalyst for various industrial applications (Griebeler *et al*., 2009). Most of the commercial lipases are of bacterial or fungal origin. The major fungal lipase producing genera include: *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus* and *Fusarium*. *Bacillus*, *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Burkholderia* and *Staphylococcus* are the predominant bacterial genera producing lipases. Of species of *Pseudomonas*; *aeruginosa, cepacia, fragi* and have commercially been exploited for the production of lipase (Kaieda *et al*., 2001; Alquati *et al*., 2002; Hazaa *et al*., 2009; Cesarin *et al*., 2014).

Owing to the unique properties of lipases like stability, specificity and their action over a wide range of pH and temperature, the scientific community is now focused on the large scale production of lipases for use in food,

pharmaceutical, cosmetics, leather, detergent and textile industries. The major limitation in the commercial use of lipases owes to their high production cost and lack of effective downstream processing. Hence, consumption of cheap agro-industrial residues as substrates for lipases could reduce the production cost to a considerable level. Detailed discussion presented in the succeeding part of this review would communicate an ample insight into the versatilities of microbial lipases.

Structure of Lipases and their mechanism of action

Lipases are serine hydrolases acting on the carboxyl ester bonds present in acylglycerols to release fatty acids and glycerol. Their active site consists of a **Ser-His-Asp/Glu** catalytic triad consisting of a nucleophillic serine located in a highly conserved Gly-X-Ser-X-Gly pentapeptide (Jaeger *et al.*, 1999). The three dimensional structure of lipases revealed a characteristic *α/β* hydrolase fold (Nardini and Dijkstra, 1999). The catalytic core of lipase is composed of a central *β*-sheet consisting of up to eight different *β*-strands connected to six *α*-helices (Jaeger and Reetz, 1998) **(Figure 1)**.

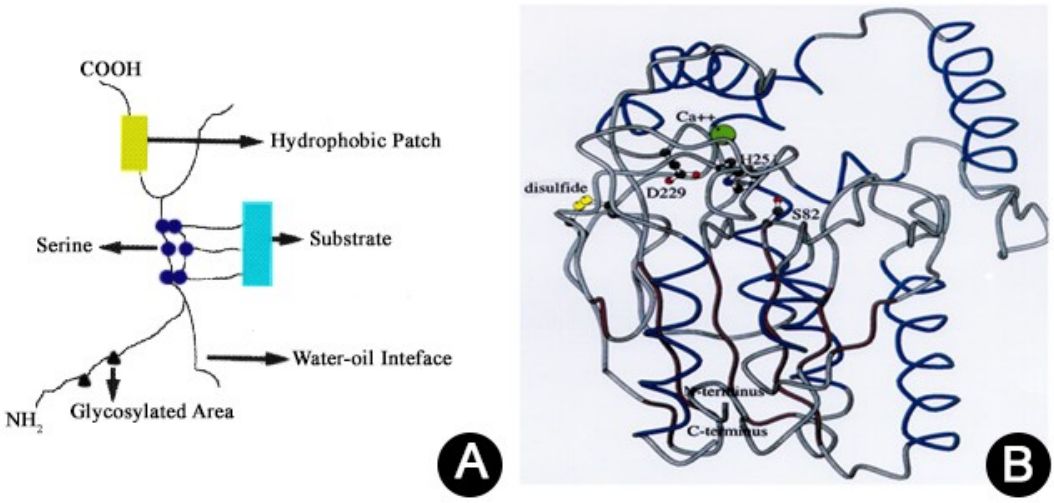


Figure 1. (A) Diagrammatic representation of lipase (Hamam, 2013); **(B)** X-ray structure of *Pseudomonas areuginosa* lipase (Jaeger *et al*., 1999)

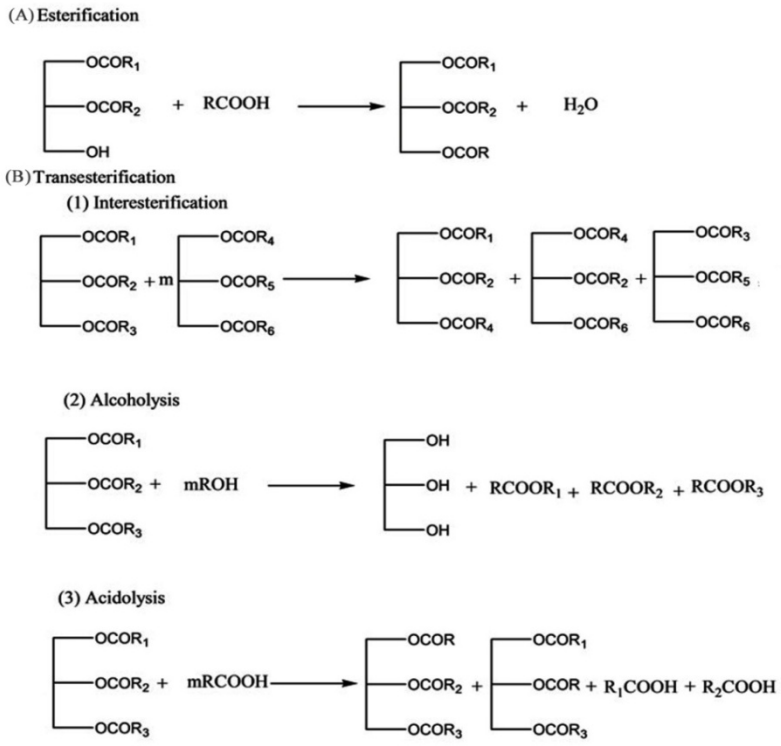


Figure 2. Schematicdiagram representing the mechanism of lipase action

Unlike esterases, lipases are activated only when they are adsorbed to an oil-water interface (Martinelle *et al*., 1995). Lipase initiates hydrolysis of ester *via* an attack by the oxygen atom of the hydroxyl group of the nucleophilic serine residue on the activated carbonyl carbon of the susceptible lipid ester bond. As a result, a transient tetrahedral intermediate is formed with the oxyanion stabilised by two or three hydrogen bonds, the so-called oxyanion hole; The ester bond is cleaved and the alcohol moiety leaves the enzyme. The nucleophilic attack by the catalytic serine is mediated by the catalytic histidine and aspartic (or glutamic) acid (Cygler *et al*., 1994; Schrag *et al*., 1997). A schematic illustration of general action of lipase is given in **Figure 2**.

Bacterial lipase

Production of extracellular bacterial lipases has significant commercial importance, as their bulk production is very easy. Microbial lipases have gained special attention due to their versatile biochemical properties, simple extraction procedures and availability (Macrae and Hammond, 1985; Ghosh *et al*., 1996). Generally, vegetable oil processing factories, dairies, oil contaminated soil, coal tips, decaying food particles, hot springs and compost heaps are the natural habitats of lipase producing microorganisms (Wang *et al*.,1995) **(Table 1)**. Bacterial lipases are classified into 8 different families; of this, family I is the largest, which houses 6 subfamilies (Arpigny and Jaeger, 1999). The lipases from *Pseudomonas* spp. are classified under the Families I.1 and I.2.

***Pseudomonas* lipase**

Pseudomonas represents the heterogenous group of Gram-negative bacteriasuch as *Burkholderia cepacia, Burkholderia multivorans, Pseudomonas aeruginosa, etc*., the well known producers of lipase.*Pseudomonas* lipases exhibit interesting properties such as thermo resistance, coupled with activity at alkaline pH, which make them potential candidates for various biotechnological applications. Another important character of *Pseudomonas* lipases is its enantio-/ stereoselective nature, in which they have the capability to distinguish between the enantiomers in a racemic mixture (Jaeger and Reetz, 2000), and this unique property, is now widely exploited in pharmaceutical and agricultural sectors. Thermostable *Pseudomonas* lipases were reported to withstand 100 ^{o}C or even beyond to 150 ^{o}C with a short span of a few seconds (Andersson *et al*., 1979; Swaisgood and Bozoglul, 1984; Rathi *et al*., 2001). An alkaline lipase from *P. alcaligenes* M-1 was found better for eliminating fatty stains from clothes under machine wash conditions (Gerritse *et al*., 1998). Lipase from *Pseudomonas cepacia* acts as a most effective catalytic agent for the ethanolysis and methanolysis of grease (Hsu *et al*., 2002).

Table 1. Prominent bacteria producing lipases.

Bacteria	Reference
Achromobacter sp.	Mitsuda *et al*., 1990
Acinetobacter sp.	Wakelin and Forster, 1997
Alcaligenes sp.	Ngooi *et al*., 1990
A. denitrificans	Odera *et al*., 1986
Arthrobacter sp.	Hirohara *et al.*, 1985
Bacillus laterosporus	Toyo-Jozo, 1988
B. sphericus	Toyo-Jozo, 1988
B. thermocatenulatus	Rua *et al.*, 1997
B. thiaminolyticus	Toyo-Jozo, 1988
Chromobacterium sp	Mitsuda *et al.*, 1992
C. viscosum	Wu *et al.*, 1996
Cryptococcus laurentii	Yasohara *et al.*, 1995
Flavobacterium ferruginem	Toyo-Jozo, 1988
Geotrichum candidum	Ngooi *et al.*, 1990
Glomus versiforme	Gaspar *et al*., 1997
Hansenula anomala	Ionita *et al*., 1997
Humicola lanuginose	Macris *et al*., 1996
Mycobacterium chelonae	Chen *et al.*, 1997
Neurospora sitophila	Beuchat, 1982
Nocardia amarae	Wakelin and Forster, 1997
Protaminobacter alboflavus	Toyo-Jozo, 1988
Pseudomonas sp.	Buisman *et al*., 1998
P. aeruginosa	Odera *et al.*, 1986
P. cepacia	Ziemann *et al.*, 1994
P. fluorescens	Wu *et al.*, 1996
P. fragi	Santarossa *et al.*, 2005
P. pseudoalcaligenes	Lin *et al.*, 1996
Staphylococcus hyicus	Gotz *et al.*, 1998
S. warneri	Talon *et al.*, 1996
S. xylosus	Talon *et al.*, 1996

Substrates for lipase production

Most of the microbial lipases are extracellular and their production is highly influenced by the composition of the medium, besides physico-chemical factors such as temperature, pH, and dissolved oxygen. Lipases are inducible enzymes, therefore, enhancement in production generally occurs in the presence of a lipids or lipid-like substrates such as hydrolysable esters, oil

industry wastes, vegetable oils, surfactants, fatty acids, triacylglycerols, bile salts, glycerol and tweens (Sharma *et al*., 2001; Damaso *et al*., 2008). Several abundant and cheap agro-residues like brans, oil cakes, bagasse, cottonseed and soybean sludge have been reported as effective for lipase production. Owing to high nutritional content, the agro-industrial residues are considered as very fine substrates for enzyme production; which may help to overcome the agricultural waste management problem, especially *via* solid state fermentation (Mahanta *et al.,* 2008). In addition to this, various nitrogen sources like peptone, yeast extract, tryptone, meat peptone, ammonium sulfate, potassium nitrate, *etc*. were found to enhance lipase production (Lima *et al*., 2003).

Lipase production by fermentation

Submerged fermentation (SmF) has been defined as fermentation in the presence of excess water and it is very easy to monitor. *Bacillus thermoleovorans* ID-1 produced a thermophilic lipase in a medium containing 1.5 % olive oil; whose activity was 520 U/ml at pH of 7.5 and 70 ^{o}C (Lee *et al*., 1999). *Pseudomonas aeruginosa* strain Pse A produced lipase (4580 U/ml) in a medium containing gum arabic as inducer, which was found to be tolerant to organic solvents (Ruchi *et al*., 2008). Solid state fermentation (SSF) is a microbial process in which a solid material is used as the base substrate, on which microorganisms grow well and produce higher quantities of extracellular enzymes and other metabolites than they do in SmF. Mahanta *et al*. (2008) used deoiled jatropha seed cake as carbon source for the cultivation of *Pseudomonas aeruginosa* Pse A, and 625 U/gds lipase activity was reported.

Purification strategies for bacterial lipases

Bacterial lipases are mostly secreted in the medium, purification from the culture medium is the major task faced by industries. In many cases, enzymes used in commercial applications need not require high purity (*e.g*., detergent industry), but in medical, cosmetic, food, analytical chemistry, and for elucidating the protein structure; high purity enzyme is required (Taipa *et al*., 1992; Aires-Barros *et al*., 1994; Saxena *et al*., 2003). Ideal purification strategies adopted in industries should be of low-cost, quick, high-yielding and agreeable to large-scale operations. In addition, it must have the capability for continuous product recovery, with a relatively high selectivity for the desired product. About 80% of the purification schemes attempted thus far have used a precipitation step; followed by gel filtration, and ion exchange chromatography **(Table 2)**. Recently emerged purification strategies include: immune purification, aqueous two-phase systems, reversed micellar system, membrane processes, hydrophobic interaction chromatography employing an epoxy activated spacer arm as a ligand, column chromatography with PEG (polyethylene glycol)/ sepharose gel or poly(vinyl alcohol) polymers as stationary phase, and aqueous two-phase systems (Saxena *et al.*, 2003).

Table 2. Purification strategies for bacterial lipases

Bacterium	Purification strategy	Reference
Acinetobacter calcoaceticus AAC323-1	Triton X-114-based aqueous two-phase partition	Bompensieri *et al*., 1996
A. radioresistens CMC-1	Ammonium sulfate, PD-10 column, Mono Q, phenyl-Sepharose CL-4B column chromatography	Hong and Chang, 1998
Acinetobacter sp. RAG-1	Mono Q, butyl Sepharose column, elution with Triton-X 100	Snellman *et al*., 2002
Bacillus sp.	Ammonium sulfate, acrinol treatment, DEAE-Sephadex A-50,Toyopearl HW-55F, butyl Toyopearl 650 M	Sugihara *et al*., 1991; Palekar *et al*., 2000
B. alcalophilus	50% ammonium sulfate, Sephadex G-100	Ghanem *et al*., 2000
B. pumilus	Ammonium sulfate fractionation, gel filtration on Sephadex G-100	Jose and Kurup, 1999
B.stearothermophilus (recombinant lipase)	CM-Sepharose, DEAE Sepharose	Kim *et al*., 2000
B. thermocatenulatus	Calcium soap, hexane extraction, methanol precipitation, Q-Sepharose (ion exchange)	Schmidt-Dannert *et al*., 1994
Chromobacterium viscosum	Alginate (macroaffinity ligand), elution by NaCl, 0.5 K	Sharma and Gupta, 2001
Pseudomonas sp.	G6 Silicone 21 defoamer, ammonium sulfate (60% saturation) fractionation	Kanwar *et al*., 2002
P. aeruginosa	Ammonium sulfate precipitation, hydroxyapatite column Chromatography	Sharon *et al*., 1998
P. cepacia	Polyoxyethylene detergent C14EO6-based aqueous two-phase partitioning	Terstappen *et al*., 1992
P. fluorescens	Ultrafiltration, ammonium sulfate precipitation, DEAE-Toyopearl 650 M, phenyl Toyopearl 650 M	Kojima *et al*., 1994
P.	Acetone precipitation, Sephadex G-	Lin *et al*.,

Bacterium	Purification strategy	Reference
pseudoalcaligenes F-111	100 chromatography, fractogel phenyl 650 M chromatography, Sephadex G-100 Chromatography	1996
P. pseudomallei	Ammonium sulfate, Sephadex G-150	Kanwar and Goswami, 2002
P. putida 3SK	DEAE-Sephadex A-50, Sephadex G-100	Lee and Rhee, 1993
Serratia marcescens	Ion-exchange chromatography, gel filtration	Abdou, 2003.
Staphylococcus haemolyticus	80% ammonium sulfate, DEAE-Sepharose CL-6B column, CM-Sepharose CL-6B, resource S column (ion-exchange chromatography)	Oh *et al*., 1999
S. warneri 863	Nickel–NTA affinity chromatography, hydroxyapatite column (HIC)	Kampen *et al*.,2001
His6-*S. aureus*	Protamine sulfate, ammonium sulfate, nickel nitrilotriacetate, hydroxyapatite	Simons *et al*., 1998

Properties of bacterial lipases

Desired properties of lipase decide its industrial value. Various properties of bacterial lipases such as pH, temperature, stability, effective of metal ions, substrate specificity are evaluated under the following sections.

pH and temperature

Usually, bacterial lipases shows neutral (Dharmsthiti *et al*., 1998; Lee *et al*., 1999) or alkaline pH optima (Schmidt-Dannert *et al*., 1994; Sidhu *et al*., 1998a, 1998b; Sunna *et al*., 2002), by the exemption of *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8 (Andersson *et al*., 1979). Lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B.*

licheniformis SB-3 show activity in broad pH range (Bradoo *et al*., 1999). Bacterial lipases exhibit stability over a wide range, from pH 4 to 11 (Kojima *et al*., 1994; Wang *et al*., 1995; Khyami-Horani, 1996; Dong *et al*., 1999). In general, bacterial lipases possess temperature optima in the range 30 – 60°C (Lesuisse *et al*., 1993; Wang *et al*., 1995; Dharmsthiti *et al*., 1998; Litthauer *et al*., 2002). But, reports suggest that bacterial lipases exhibit temperature optima in lower as well as higher ranges (Dharmsthiti and Luchai, 1999; Lee *et al*., 1999; Oh *et al*., 1999; Sunna *et al*., 2002). Nawani and Kaur (2000) reported that the thermostability of lipase from *Bacillus* sp was improved by the addition of stabilisers *i.e.*, glycerol, sorbitol, ethylene glycol which retained the enzyme activity at 70 °C even after 150 min incubation. A few species form *Pseudomonas* have been claimed that the enzymes are stable at 100 °C or even beyond 150°C (Andersson *et al*., 1979; Swaisgood and Bozoglu, 1984; Rathi *et al*., 2001). *B. stearothermophilus* lipase was highly thermotolerant with a half-life of 15 - 25 min at 100°C (Bradoo *et al*., 1999).

Stability in organic solvents

Lipase stability in organic solvents is desirable for using them in various chemical reactions. Schmidt-Dannert *et al*. (1994) reported that acetone, ethanol and methanol, *etc*. could the activity of *B. thermocatenulatus* lipase, but acetone and hexane inhibited the action of lipases from*P. aeruginosa* YS-7 and *Bacillus* sp. (Sugihara *et al*., 1991). Lipase from *A. calcoaceticus*LP009 showed unstability with various organic solvents (Dharmsthiti *et al*., 1998).

Effect of metal ions

Cofactors are normally not necessary for lipase activity, but Ca^{2+} (divalent cations) often enhance its activity. Metal ions such as Co, Hg and Sn were

found to inhibit the activity of lipase enzyme (Patkar and Bjorkling, 1994). Calcium-activated lipase were reported from a number of bacteria such as *P. aeruginosa* EF2 (Gilbert *et al*., 1991), *B. thermoleovorans* ID- 1 (Lee *et al*., 1999), *B. subtilis* 168 (Lesuisse *et al*., 1993), *S. aureus* 226 (Muraoka *et al*., 1982). In contrast, activity of lipase from *P. aeruginosa* 10145 was inhibited in the presence of Ca^{2+} (Finkelstein *et al*., 1970); on the other hand, lipase from *A.calcoaceticus* LP009 was stimulated by Fe^{3+} (Dharmsthiti *et al*., 1998).

Application of lipases

Lipases in detergent industry

The lipolytic activity of lipases is mainly used in detergency, especially thermophilic and alkalophilic are preferred in this area. Mostly, it should be capable of performing in the presence of the various components of washing powder formulations (Posorske, 1984; Cheetham, 1995). To increase the action of detergency, a combination of various enzymes such as lipase, amylase, protease and cellulase are used in modern heavy duty powder detergents and automatic dishwasher detergents (Ito *et al.*, 1998). The main

advantage of these bio-detergents is high biodegradability, lack of any harmful residues, no adverse effect on sewage treatment processes and do not cause a risk to aquatic life. *Pseudomonas alcaligenes* lipase showed elevated activity at washing conditions, such as alkaline pH (7 - 11) and at a high temperature up to 60°C (Misset *et al*., 1994). Novo group has introduced an alkaline and positionally non-specific lipase from *Streptomyces* sp., which could be used in a wide range of applications like laundry, dish-washing detergents and industrial cleaners (Pandey *et al.*, 1999).

Lipases in food technology

Modification of fat and oil is one of the major areas in food processing industry, a large potential market in future. Lipases are added to food for improving flavor and taste by the production of esters involving short chain fatty acids and alcohols (Macedo *et al.*, 2003). The lipases play a vital role in the fermentation process of sausage production and to regulate the changes in long-chain fatty acid addition in ripening. Lipase mediated food products in the market include bread, nutraceuticals, chocolates *etc*. Before, lipases of diverse microbial origin have been used for cleansing rice flavor, altering soybean milk and for progress the aroma and increase the fermentation of apple wine, preparation of Koji (fermented cooked rice and/or soya beans), *etc*. (Seitz, 1974).

Pulp and paper industry

Microbial lipases have some crucial role in pulp industries (Bajpai, 1999). Lipases are widely used for increasing the pulping rate of pulp, to augment the whiteness and intensity and deinking of wastepaper. So, it can help the decrease of chemical usage, prolong equipment life, decrease the risk of

pollution level in water and reduce composite cost; and also used to remove hydrophobic components of wood known as ‘pitch’ (Irie *et al*., 1993).

Use of lipase in textile industry

Use of lipases in textile industries is mainly found in the removal of lubricants and to give a fabric with better absorbency for enhanced levelness in dyeing; in addition, it reduces the chance for frequency of line and break in denim scrape systems. Lipase enzymes commercially used for the preparation of the desizing of denim and other cotton fabrics. A commercial lipase from Amano Pharmaceutical KK was dissolved in solution with aliphatic polyester, which improved fabric texture without losing its strength (Dyson *et al*., 2006); lipase can improve the wetting ability and absorbance in polyester fabrics (Hsieh and Cram, 1998). Moisture regaining ability of polyethylene terephthalate fabrics was found to be improved upon using lipases from *P. cepacia* and *P. fluorescens* (Kim and Song, 2006). However, lipase from *Pseudomonas* spp. was shown to degrade polymers of aliphatic polyethylene (Muller *et al*., 2005).

Part II. Bacterial siderophores

Introduction

Iron (Fe) is an essential micronutrient necessary for the growth and survival of bacteria, and it directly controls a wide range of metabolic and signaling functions of the cell (Third *et al*., 2000). In nature, iron exists mainly in two forms *i.e*., the reduced ferrous (Fe^{2+}) and the oxidised ferric (Fe^{3+}) forms, whose redox potentials fluctuate depending on the molecules to which the iron is bound. This special characteristic feature turned iron into a major

redox mediator in biological systems. In living cells, Fe is associated with iron-sulfur clusters or heme, and it plays significant roles in catalytic mechanisms of many enzymes like dehydrogenases, reductases, nitrogenases, *etc*. (Chincholkar*et al*., 2007). Though it is abundant on the earth's crust, iron is not readily accessible in natural environment to many microorganisms owing to its insolubility in water. In order to circumvent this problem, many bacteria secrete chelating molecules called siderophores that solubilse iron to an easily assimilatory form (Meyer *et al*., 2002).

Siderophores are low molecular weight, high affinity iron chelating compound comprised with a chromophore, an acyl moiety and variable peptide chain (Unni *et al*., 2004). Siderophores are produced by plants, bacteria, fungi and actinomycetes. Various types of siderophores are identified and characterised from the bacteria such as *Shigella* sp., *Salmonella* sp., *Escherichia* coli, *Yersinia* sp., *Vibrio* sp., *Bordetella* sp., *Pseudomonas* spp., *Mycobacterium tuberculosis*, *Staphylococcus* sp. and *Bacillus anthracis* (**Table 3**). Siderophores are used to trap, mobilise and transport iron to the microbial cells and play a crucial role in the pathogenesis and biofilm formation. Moreover, they tune the microflora of the surrounding environment by regulating the iron availability and protect themselves from heavy metal toxicities (Neilands, 1981). With the advent of novel technologies, many efforts have been accomplished to utilise these extracellular proteinaceous compounds for the welfare of human kind. Predominantly, they found potential applications in environmental as well as pharmaceutical research.

Upon this background, this review critically evaluates the sources, types and applications of microbial siderophores.

Table 3. List of some bacteria producing siderophores

Microorganism	Siderophore	Molecular weight	Ligand type	Reference
Acinetobactor baumanni	Acinetobactin	346.3 Da	Phenolic / Hydroxamate	Yamamoto *et al*., 1994
Agrobacteruium sp.	Agrobactin	403 Da	Catechol 2-hydroxy phenyloxazoline	Sonoda *et al*., 2002
Alteromonas luteoriolaces	Alterobactin	927.9 Da	Phenol/α-hydroxycarboxylate	Reid *et al*., 1993
Azotobactor vinelandii	Azotobactin	1138 Da	Hexadentate hydroxamate / Phenolate	Tindale *et al*., 2000
Bacillus subtilis	Bacillibactin	882.8 Da	Catecholate	May *et al*., 2001
Bordetella sp.	Alcaligin	404.4 g/mol	Hydroxamate	Hou *et al*., 1998
Burkholderia cepacia	Ornibactin	734 g/mol	Hydroxamate/ Hydroxycarboxylate	Skol *et al*., 1999
Erwinia chrysathemi	Chrysobactin	369.4 g/mol	Bidentate	Neema *et al*., 1993
Escherichia coli	Enterobactin	669.6 g/mol	Catechol	Pollack and Neilands, 1970
Halomonas aquamarina strain DS40M3	Aquachelin		Hydroxamte	Martinez *et al*., 2002
Marinobactor hydrocarbonoclasticus	Petrobactin	719.8 Da	Hydroxy carboxylate	Barbeau, *et al*., 2002
Mycobacterium tuberculosis	Mycobactin	828.0 g/mol	Hydroxamate / Phenolate	Snow, 1970
Pseudomonas aeruginosa	Pyoverdine	1,365.4 g/ mol	Hydroxamate / Phenolate	Vossen *et al*., 1999
Pseudomonas cepacia	Cepabactin	155.2 Da	Bidentate	Meyer *etal*., 1989
Rhodococcus erythropolis IGTS8	Heterobactin	598.9 Da	Hexadentate hydroxamate / Phenolate	Carran *et al*., 2001
Salmonella sp.	Salmochelin	993,8 Da	Catecholates and Phenolates	Valdebenito *et al*., 2006
Shigella sp.	Aerobactin	564.5 g/mol	Hexadentate hydroxamate / α-Hydroxycarboxylate	Le Roy *et al*., 1993
Streptomyces pilosus	Desferrioxamine B	560.7 g/mol	Hexadentate hydroxamate	Gordeuk *et al*., 1992
Streptomycocus sp.	Staphylo-	448.1 Da	α-Hydroxy-	Meiwes *et*

Microorganism	Siderophore	Molecular weight	Ligand type	Reference
	ferrin B		carboxylate / α-aminocarboxylate	*al*., 1990
Vibrio cholera	Vibriobactin	705.3 Da	Catechol	Grifith *et al*., 1984
Yersinia pestis	Yersinibactin	479.1 Da	Catecholate	Perry *et al*., 1999

Types of siderophores

Siderophores typically appeared as constant and stable complexes, and exhibit high affinity towards Fe rather than other metal ions present in nature (Hofte, 1993). The efficiency of a siderophore to chelate iron depends on the stability of the metal complex it forms (Wittenwiler, 2007). Generally, siderophores are synthesised by an array of complex multi-enzyme units, and most of them have a peptidic backbone with side chain of amino acid derivatives, which facilitates binding sites for iron. Siderophores are generally classified into three types, based on the moiety that donate oxygen (binding moiety) for coordinating with iron (Miethke, 2007; Saha *et al*., 2013). The binding groups of siderophores may include catecholate (phenolates),hydroxa-mates, carboxylates or mixed ligands **(Figure 3 and 4)**. All the binding sites possess two coordinating sites for iron *via* lone pair of oxygen atoms, thereby forming a pentagonal ring; and most of the siderophores exist as hexadentate or octahedral complexes (Wittenwiler, 2007).

Catecholate siderophores

Catechol type siderophores possess catecholate$C_6H_4(OH)_2$or 2,3-dihydroxybenzoate iron binding groups with two oxygen atoms for chelation. They may be linear or cyclic molecules. Enterobactin produced by *E. coli, Aerobacter aerogenes* and *Salmonella typhimurium* is a typical example for

cyclic catecholate type siderophores, which is made of a cyclic triester of 2,3-dihydroxybenzoylserine (Saha *et al*., 2013, 2015). Agrobactin produced by *Agrobacterium tumifaciens* is a linear catecholate sideriphore with three residues of 2,3-dihydroxybenzoic acids, a spermidine chain and a oxazolin ring (Ong *et al*., 1979). Presence of all the catecholate siderophores could be detected by Neilands spectrophotometric assay, in which the siderophores reacts with $FeCl_3$ to form a wine coloured complex having absorption maximum at λ_{495} (Neilands, 1981).

Hydroxamate siderophores

Gram-positive bacteria like *Pseudomonas fluorescens*, *Neisseria gonorrhoeae*, *N. meningitids*,*etc*., and actinomycetes are the major producers of hydroxamate siderophore, which possesses a characteristic structure [C(=O)N-(OH)-R], where R is an amino acid or its derivative (Saha *et al*., 2015). Ferrichrome is one of the largest family of hydroxamate siderophores mainly produced by fungi such as *Fusarium* and *Trichoderma* spp. Ferribactin is another hydroxamate siderophore reported from *Pseudomonas fluorescens* (Philson and Llinas, 1982). These compounds show the maximum absorbance between $\lambda_{425\text{-}500}$ when bound to iron (Ali and Vidhale, 2013). The ferrioxamines and Desferrioxamine B are yet other important class of siderophores secreted by *Streptomynces pilosus* (Muller and Raymond, 1984).

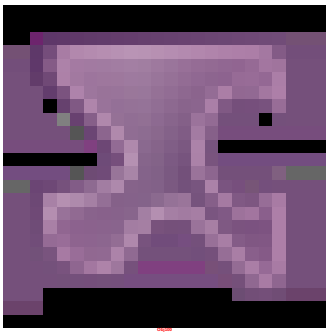
Carboxylate siderophores

Carboxylate siderophores possess carboxyl or hydroxyl donor groups for iron ligation. ‘Rhizobactin’, produced by *Rhizobium meliloti* is one of the best characterised carboxylate types of siderophore(Smith *et al*., 1985). Staphyloferrin A provides two tridentate pendant ligands, comprising of a

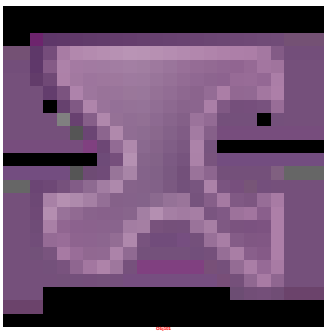
beta-hydroxy and a *beta*-carboxy-substituted carboxylic acid derivative, for octahedral metal chelation (Konetschny-Rapp *et al.*, 1990).

Siderophores with mixed ligands

Pyoverdine (PVD) is one of the best examples of siderophore with mixed ligands. The fluorescent *Pseudomonas* spp. such as *P. aeruginosa*, *P. chlororaphis*, *P. fluorescens*, *P. putida* and *P. syringae* are well-known producers of PVDs (Meyer, 2000). Of them, *P. aeruginosa* is the predominant producer of PVD. Reportedly, three distinct PVD types are being produced by strains of *P. aeruginosa*, *viz*., PVD 1, PVD 2 and PVD 3 - each subtype has characteristic peptide chain (Meyer *et al*., 1997). Of them, PVD 1 and PVD 2 together contribute 84% of PVDs, *i.e*., and the remaining 16% is represented by only PVD 3 subtype (Meyer *et al*., 1997). Mycobactins, produced by Gram-positive *Mycobacterium tuberculosis* is another mixed ligand siderophore, which has two hydroxamates, a phenolate, and oxazoline groups for ligation. The deduced structure of type PVDs are shown in the following **Figure 5** (Visca *et al*., 2007).

Group	Chelation
Hydroxamate	

Catecholate



Hydroxyl-carboxylate

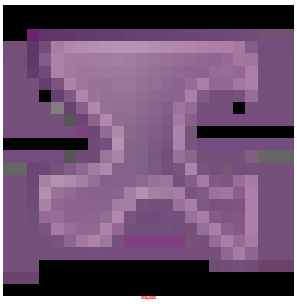


Figure 3. Major types of siderophore ligands and their co-ordination pattern

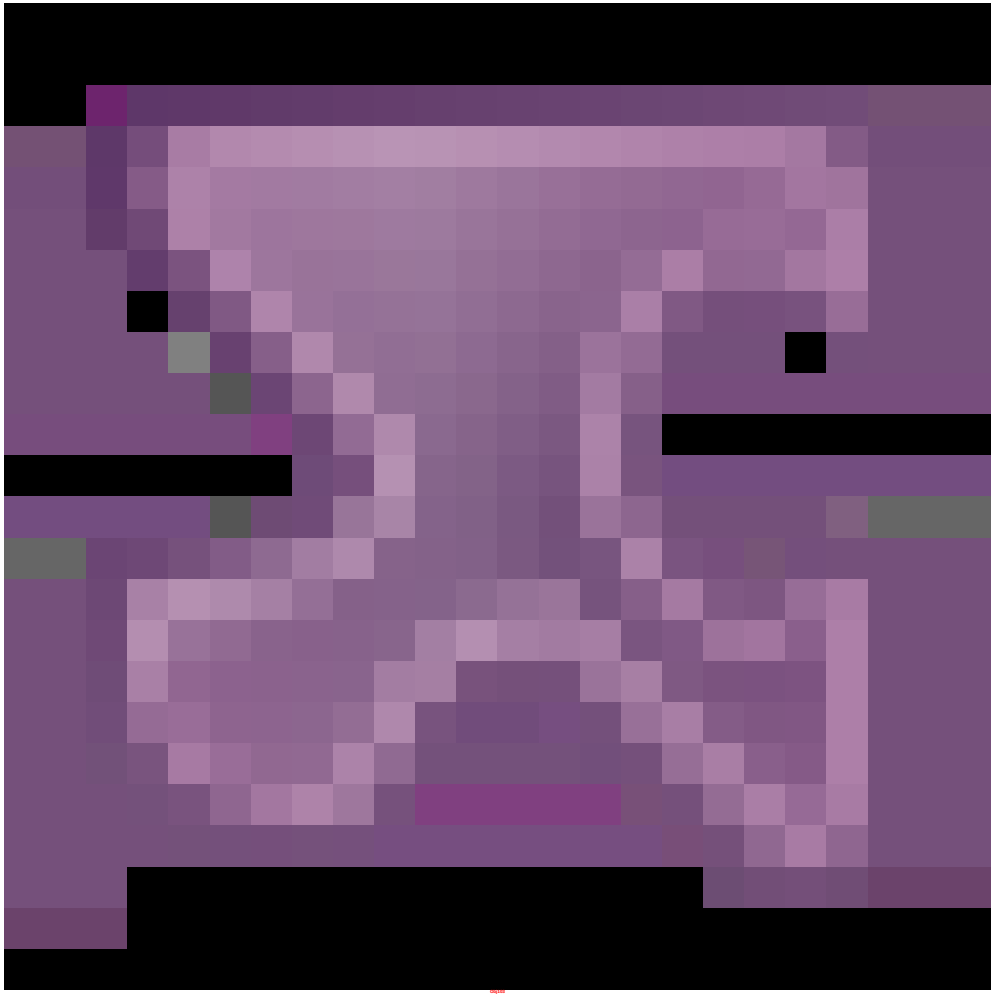


Figure 4. Ligands of bacterial siderophore

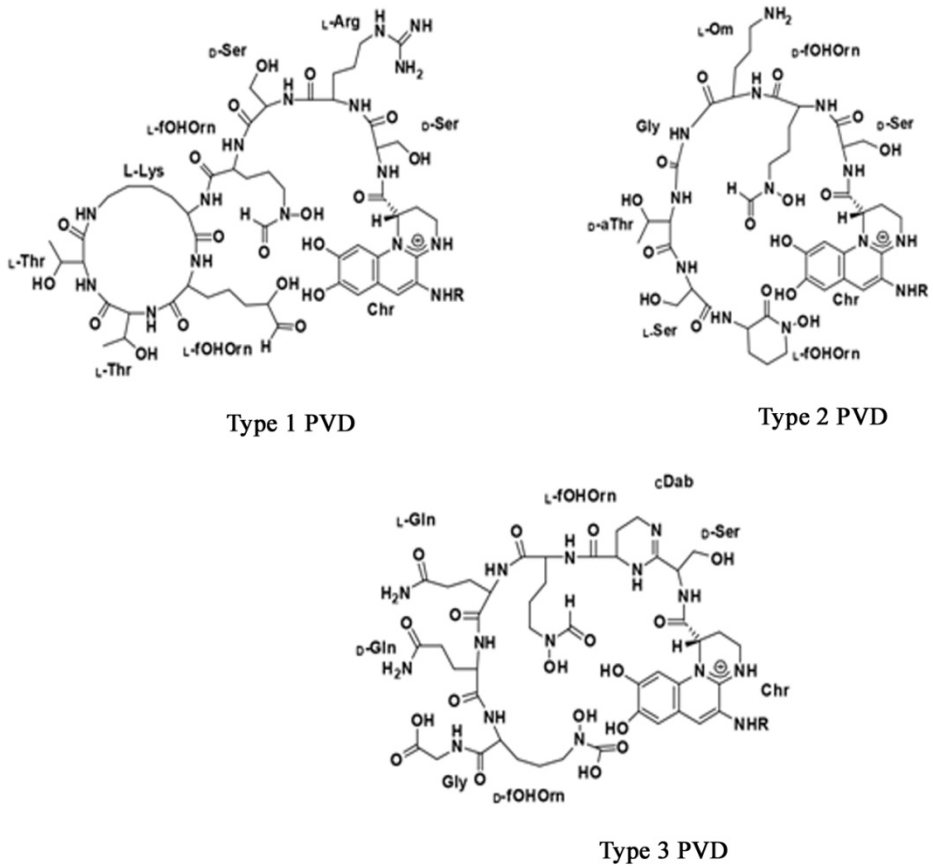


Figure 5. Types of PVD (Visca *et al.,* 2007)

Role of siderophores in bacterial survival

Siderophores play a crucial role in microbial pathogenecity. In humans, iron is bound to many protein complexes present both in intracellular as well as extracellular fluids, haeme in the haemoglobin with central Fe is a typical example. But the strictly regulated homeostasis does not allow the availability of free iron for pathogens in the human body. Hence, the bacterial siderophores contribute significantly to its virulence, *i.e*., by stealing Fe from the host for its survival (Wooldridge and Williams, 1993).

The siderophores act as chelating agents with an extremely high affinity for Fe^{3+} with stability constants of around10^{30}/M (Albrecht-Gary *et al*., 1994).

When the bacterium enters the host where the availability of iron is low, they secrete siderophores to the external environment to compete with the host proteins (like transferrin and hemoglobin) for capturing Fe ion. For instance, *Staphylococcus aureus,* a human pathogen tactically chelates iron for its invasion establishment in the host. During the staphylococcal infection, bacterium secretes the toxin called hemolysins, which rupture the red blood cells and release hemoglobin, followed by degradation into heme and free Fe ion (Tong and Guo, 2009). The siderophore-Fe^{3+} complexes then return back to the specific cell surface receptors, which in turn is dissociated intracellularly to release Fe^{2+} to be incorporated into the bacterial metalloenzymes; and the excess of Fe ions are stored in bacterioferritins and other related proteins (Chiancone *et al*., 2004; Chu *et al*., 2010). Upon assimilating Fe in sufficient quantities, further secretion of siderophore is suppressed by a repressor protein called *Fur* by organising itself into the DNA sequence that regulates the biosynthesis of siderophores (Chu *et al*., 2010).

It is found that metals other than Fe can be sequestered by siderophores in some bacteria. For instance, azotochelin produced by *Azotobacter vinelandii* sequesters molebdate rather than iron due to the high stability of azotobacter-Mo complexes, which in turn reduces the availability of free siderophores to sequester Fe. Thus, the iron deficiency induces the bacterium to produce a more efficient iron chelator, the protochelin (Duheme *et al*., 1998). Similarly, pyoverdine and pyochelin, the two major siderophores produced by *P. aeruginosa* were able to chelate Ag^{+}, Al^{3+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Eu^{3+}, Ga^{3+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, Tb^{3+}, Ti^{+} and Zn^{2+} present in the growth medium at varying intensities, apart from Fe. Interestingly, the siderophores only sequester the elements but not allow them to enter the bacterial cells, and thus

protect the microbes from heavy metal contaminations (Braud *et al*., 2009 a,b). Usually, these heavy metals diffuse into the cells *via* porins present in the cell membrane, but their diffusion into the cell dependent on the size of the sequestered form, i.e., larger ones will be restricted from entry (Schalk *et al*., 2011).

Siderophore iron complex transport mechanism in bacterial cell

Ferri-siderophores, the chelated Fe^{3+} siderophore complexes are transported across the double membrane system of Gram-negative bacteria through the specific receptors on the outer membrane, whereas they are transported by membrane anchored binding proteins in Gram-positive bacteria (Koster, 2001; Braun and Braun, 2002). The transportation of ferri-siderophores is well explored in *E. coli,* which is mediated by three multi-component protein systems of the outer membrane, periplasm and cytoplasmic membrane (Ali and Vidhale, 2013). In *E. coli*, the ferri-siderophores binds to the outer membrane protein called Fep A, which is a barrel shaped protein made up of 22 *beta* strands with a cork or plug like extension made of approximately 160 aminoacid residues. In order to transport the metal conjugate through Fep A, the cork has to be dislocated by utilising the energy from the gradient across the inner membrane with the help of two sets of proteins, Ton B (span the entire periplasm) as well as the cytoplasmic membrane anchor proteins called ExbB and ExbD. In the periplasmic space, the ferri-siderophores are transported to the ABC-transporter system which is composed of 2 proteins, permease that spans the entire inner membrane and ATPase that hydrolyse ATP to release energy, required for the transportation into the cytoplasm. The mechanism by which iron is released from the siderophore is not elucidated completely to date; however, the bound Fe^{3+} of siderophore complexes are

reduced enzymatically to Fe^{2+}; which does not have a high affinity for siderophore, so that it gets dissociated from the complex **(Figure 6)**.

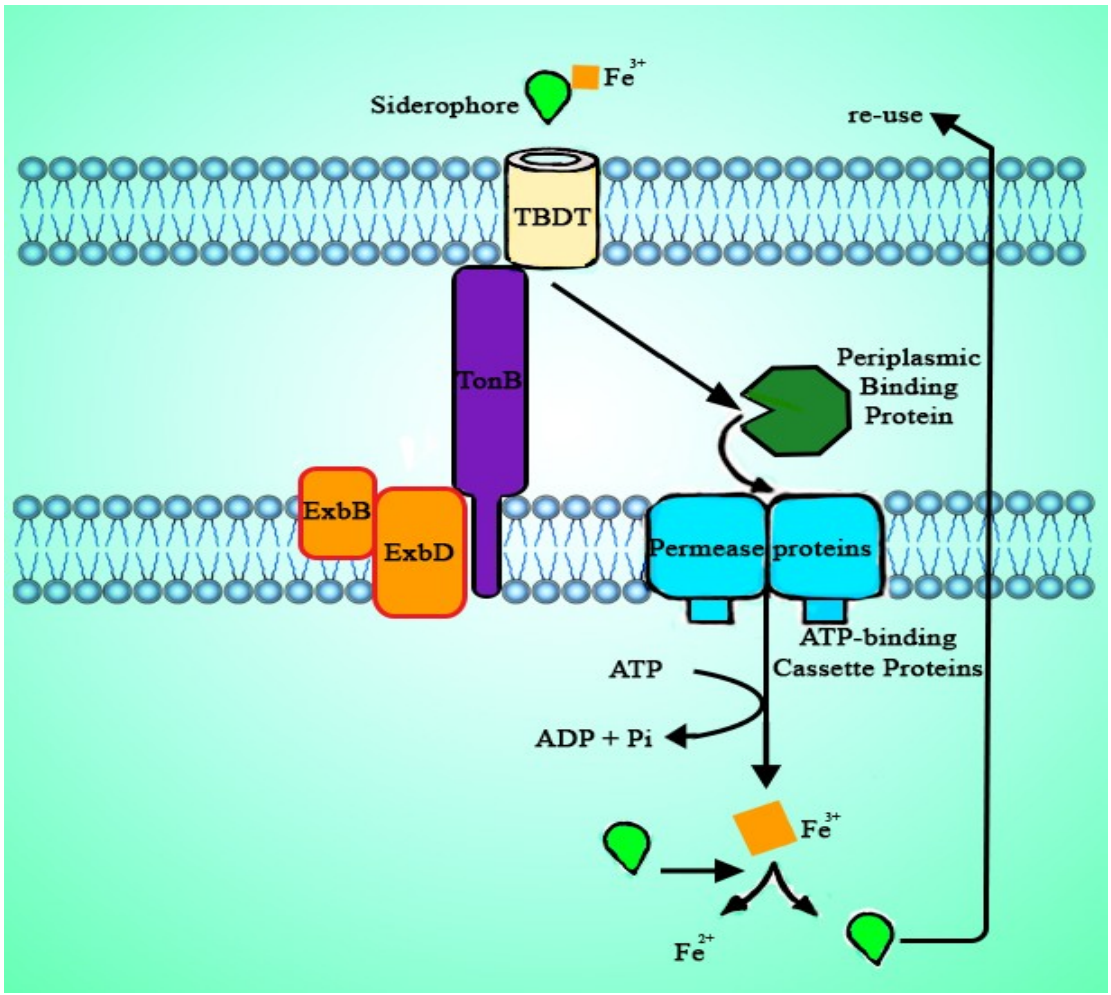


Figure 6. Transport of siderophores into the bacterial cell

In Gram-positive bacteria too, transportation of siderophores is mediated by the ABC-transporter proteins as that of Gram-negative bacteria (Grigg *et al*., 2010).

Biotechnological application of siderophores

Now-a-days, the heavy metal sequestering coupled with the coordinating properties of siderophores is exploited in various field of biotechnology. It is found that the siderophore are successfully used as a drug for the treatment of

iron over loaded therapy, drug delivery systems against multidrug resistant bacteria, bioremediation and biosensor development **(Figure 7).**

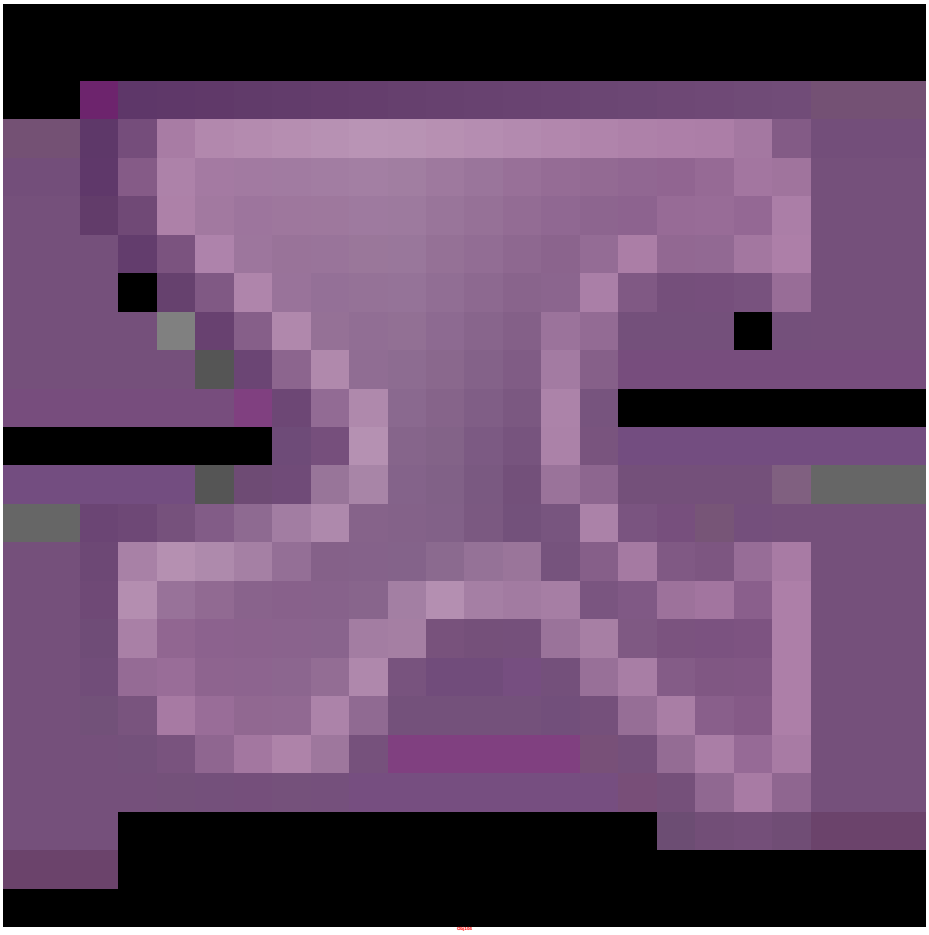


Figure 7. Major applications of siderophores

Medicinal applications

Metal overload toxicity is one of the major deadly conditions, frequently observed in chronically dialysed patients as they have lost the ability to eliminate the metals *via* renal excretion. For instance, *β*-thalassemia is characterised by the overload of iron in the blood stream, which may lead to the damage of liver through hemochromatosis and hemosiderosis; heart and endocrine systems. In such cases, siderophore-mediated treatment is found to be effective. Desferrioxamine, a siderophore produced by *Streptomyces*

pilosus is the only commercially available chelating agent for the treatment of diseases due to Fe and Al overload; though it has got several disadvantages such as low sequestering, lipophilicity and oral inactivity (Gabutti *et al*., 1996; Ackrill *et al*., 1980).

Clinical investigations conducted so far, have confirmed that iron supports the growth of tumor cells in human body. The catalytic effects of iron generate free radicals in an uncontrollable manner, which inactivate the host defense mechanism against neoplastic cells (Lijec Vjesn, 2000). Hence, it is believed that the elimination of free metal iron from the blood stream can prevent the proliferation of malignant cell effectively. Combination therapy of desferrioxamine with recombinant *α*-2-interferon is found effective in the treatment of hepatocellular carcinoma (Kontouras *et al*., 1995). The synergetic effect of deferoxamine and an IgG monoclonal anti-transferrin receptor antibody could be used to control the proliferation of murine lymphoid tumors (Kemp *et al*., 1995). Siderophores such as pyridinone, desferrioxamine, deferiprone were found effective for the removal of non-transferrin bound iron from blood stream after chemotherapy (Miller, 1989).

As siderophores cause iron depletion in cells, it can also be used for the treatment of many parasitic diseases. For instance, desferrioxamine B restricts the growth of *Trypanosoma brucei*, a protozoic parasite causing sleeping sickness (Breidbach *et al*., 2002). Similarly, aerobactin produced by *Klebsiella pneumonia*, acts as antimalarial agent (Gysin *et al*., 1993).

Siderophore mediated drug delivery

Now-a-days bacteria acquire antibiotic resistance at an alarming rate. Reports revealed that almost all approved antibacterial drugs used for clinical

practices as facing bacterial resistance at different intensities; which indicates that no existing antibiotic will be effective in coming one or two decades. The seriousness of this problem is becoming obvious as resistant infections once limited to hospitals are dispersed to the community as well (Marshall, 2008). Siderophore-mediated iron gaining is necessary for the virulence of most pathogenic bacteria, which bear specific cell surface receptors for the purpose (Miethke and Marahiel, 2007). Hence, the siderophore-mediated delivery of antibiotics can selectively target the specific receptors on antibiotic resistant bacteria causing disease and the treatment is now known as Trojan horse strategy. Trojan horse strategy utilises the coordination abilities of siderophores to carry drugs specifically to the target cells. The siderophore-antibiotic conjugate can overcome the membrane associated resistance mechanisms and augment the effectiveness of drugs up to 100-folds relative to passive diffusion (Braun, 1999). In this strategy, the target-specific siderophore is bound to the drug with the help of a spacer, and all together enters the cell where the spacer is hydrolysed enzymatically to release the drug thereby causing death of the cell.

Many natural and synthetic antibiotic conjugates are used for the antimicrobial therapy. The naturally occurring siderophore-antibiotic complexes are known as sideromycins (Braun *et al*., 2009). For instance, albomycin, produced by *Actinomyces subtropicus* is composed of an antibiotic peptide moiety linked to a ferrichrome like siderophore moiety, called the thioribosyl pyrimidine (a nucleoside-analogue) *via* a serine spacer. When albomycin enters the microbial cells *via* ferrichrome uptake system, the serine spacer gets hydrolysed to exert antimicrobial activity (Pramanik and Braun, 2006; Ali and Vidhale, 2013). Salimycin produced by *Streptomyces*

violaceus is another sideromycin, a trishydroxamate siderophore; and the aminoglycoside antibiotic linked by a dicarboxylic spacer. It is found effective against many Gram-positive bacteria especially, *Staphylococci* and *Streptococci*(Miethke and Marahiel, 2007). Ferrimycin A1 is another natural siderophore produced by *Streptomycesgriseoflavus,* which is composed of ferrioxamine B and an antibiotically active group; which is active against Gram-positive bacteria, particularly *Staphylococcus aureus* and *Bacillus* spp. (Ballouche *et al*., 2009; Górska *et al.*, 2014).

With the advent of novel technologies, many synthetic antibiotics have been used as potent candidates for antimicrobial therapies. These synthetic conjugates help to overcome the adaptations of pathogens responsible for antibiotic resistance, such as decreased outer membrane permeability, enzyme inactivation, and diffusion barriers (Górska *et al*., 2014). For instance, the catechol conjugates of the aminopenicillins were found effective against antibiotic resistant strains of *P. aeruginosa* (Page, 2013). When pyoverdine produced by *P. aeruginosa* is coupled with quinolone antibiotic, its effectiveness was found enhanced significantly, in comparison to to quinolone alone used for antibiotic therapy (Hennard *et al*., 2001; Rivault, 2007). A mixed ligand biscatecholate-monohydroxamate siderophore conjugated with carbacephalosporin exhibited remarkable specificity and high bactericidal potency against the Gram-negative pathogen, *Acinetobacter baumannii* (Wencewicz and Miller, 2013). Thus, the utilisation of the chelating property of the siderophores would open up new possibilities in clinical and pharmaceutical applications, when conventional antibiotics are phased out.

Bioremediation

Uncontrollable accumulation of metal wastes in environment is one of the curses of industrialisation in modern world. Majority of the toxic contaminations include heavy (Al, Ni, Pb, Fe, Zn, Cd, Hg, Cu, Cr, *etc*.) and radioactive metals (Co, U, Th, Pu, *etc*.). The appropriate remediation of the toxic metal waste is one of the hectic tasks exposed before the scientific communities. Recent studies have revealed that microbial siderophore mediated metal waste eradication is one of the promising methods for addressing this problem satisfactorily. Siderophores can be used to mobilise metals from metal contaminated soil. For examples, the pyoverdine from *Pseudomonas fluorescens* chelated the metals such as Fe, Ni and Co from uranium mine waste (Edberg *et al*., 2010). Similarly siderophores (pyoverdine and pyochellin) produced by *Pseudomonas aeruginosa* could chelate a wide range of heavy metals such as Ag, Al, Cd, Co, Cr, Cu, Eu, Ga, Hg, Mn, Ni, Pb, Sn, Tb, Ti and Zn (Braud *et al*., 2009 a, b). Desferrioxamine sideophore from *Streptomyces pilosus* could coordinate with plutonium (V), in addition to the iron chelating property (Keberle, 1964); andAzotochelin, a catecholate siderophore secreted by *Azotobacter vinelandii* chelates molybdenum (Duhme *et al*., 1998).

Biosensor

Biological substances (tissues, microorganism, organelles, cell receptors, enzymes, antibodies, nucleic acids) coupled with electronic device is known as biosensors, and are widely used for the detection of toxic chemical substances in natural samples such as soil, water, *etc*. It is based on the fact that biomolecules exhibit better sensitivity and molecular specificity than inorganic and synthesised molecules. Many recent studies revealed that fluorescent siderophore-based biosensor is more suitable for the recognition

of heavy metals in water samples. For example, Barrero *et al*. (1993) immobilised pyoverdine, the fluorescent siderophore from *P. fluorescence* on a porous glass for detecting Fe^{3+} ion from water samples. Pyoverdine secreted by *P. aeruginosa* could chelate Fe^{3+}, Fe^{2+} and Cu^{2+} ions, which quenched the fluorescence intensity (Yodar and Kissalita, 2006, 2011). Parabactin secreted by *Paracoccus denitrificans*could effectively be employed for the detection of iron present in ocean (Chung *et al*., 2006). Pyoverdine could also be used for the identification of furazoidone in water samples, a broad spectrum antibiotic, highly suspected in carcinogenic, genotoxic and mutagenic effects; the detection mechanism was based on the fluorescence quenching occurred due to the electron transfer from pyoverdine to furazolidone (Yin *et al*., 2014).

Conclusion

Thus the latest trend in lipase research is the development of new and improved lipases, which have wide industrial applications and they play a crucial role in the turnover of water insoluble compounds, especially in the catalytic resolution of chemical molecules. Therefore, industry prefers lipases with higher activity available at economical rate.

Bacterial siderophores are considered as a group of versatile biomoecules, which could be exploited in various fields of biotechnology for the sustainability and welfare of humans, animals and plants; and after all, for a healthy environment. To date, only a few studies have revealed the potential benefits of siderophores in highly demanding applications, especially in environmental and medical biotechnology. In tune with emerging technologies, more researches need to be focused to elucidate the specific role

and mechanism of action of each siderophore in various biological systems, *i.e.*, effective utilisation of these natural biomolecules as the candidates for green technologies in biomedicine and in the management of environment.

Isolation and characterisation of microbes from the rumen of Malabari goat

Chapter 3
Isolation and characterisation of microbes from the rumen of Malabari goat

Aim and Rationale

Isolation, screening and identification of microbes for the production of industrially significant biomolecules such as lipase and siderophore from the rumen content of Malabari goat. Rumen is one of the richsources of industrially significant microbial niche. On the basis of these facts, isolation and characterisation of bacteria from the rumen of Malabari goat are envisaged in this study.

Introduction

The inhabitants of the rumen microbial eco-system considered as a multifaceted consortium of diverse microbial genera such as bacteria (10^{10}–10^{11} cells/ml) representing more than 50 genera, anaerobic fungi (10^{3}–10^{5} zoospores/ml) representing five genera, protozoa (10^{4}–10^{5}cells/ml) from 25 genera and bacteriophage (10^{8}–10^{9} cells/ml). Ruminants are usually fed on agricultural residues which contained cellulose, hemicellulose, starch, lignin, protein and a very small quantity of vegetable oils. The symbiotic association of the rumen microbes performs synergistically for the bioconversion of lignocellulosic feeds into volatile fatty acids (Kamra, 2005). The vast microbial diversity can ideally exploit the excellent source of industrially potent bio molecule production by scientific approaches. But so far in India, this is a low-priority area of research for microbiologists, biotechnologists and molecular biologists. Under these point of view, this study particularly

focuses the isolation and selection of rumen microbes which having the tremendous production capability of two classic representatives of industrially significant bio-molecules *i*.e., lipase and siderophore.

Materials and methods

Sample preparation

Rumen contents from both male and female Malabari goats were collected aseptically in screw-capped tubes from the slaughter house at Chelari (11.18189600 ^{o}N; 75.82206300 ^{o}E), Malappuram District of KeralaState, as described by (Prive *et al.*, 2010). Briefly, 10 ml sterile double distilled water (ddH_2O) was added to 10 g sample (rumen content) and centrifuged at 800 × *g* for 4 ^{o}C at 5 min. The supernatant (1 ml) obtained as above was serially diluted (up to 10^{-6}) with pre-sterilised ddH_2O (Priji *et al.*, 2013).

Isolation of microbes

This diluted sample (100 µl) was aseptically transferred to semi-synthetic medium - designated as BUP medium (**Table 4**). The primary stage cultivation was done in anaerobic environment attained by the support of anaerobic chamber (KIM Microsystems, India) saturated with a mixture of gases (80% N_2, 10% $CO_2$and 10% H_2). The microbial strains were steadily adapted to the aerobic system by frequent subcultures in a specially designed conical flask (**Figure 8**) by our laboratory, designated as 'Benjamin flask' (Priji *et al.*, 2013).

Table 4. Composition of BUP medium

Ingredient	Weight (g/l)
Peptone	5
Beef extract	3

NH_4NO_3	5
NH_4SO_4	4
K_2HPO_4	2
NaCl	2
$MgSO_4$	0.1
Cysteine-HCL	0.5
pH 6.7 ± 0.1	

Screening for lipase production

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

Tributyrin agar method*:* Wells were bored on the tributyrin agar plates (1 ml tributyrin, 0.001g $CaCl_2$, 2g agar) using sterile cork borer and 30 µl of the cultural supernatant withdrawn from a overnight (12 h) cultural medium and dispensed on the wells on Tributyrin agar plates. The plates were then incubated at 37 °C for 24 h (Pagu *et al*., 2013). Appearance of opaque halo around the colonies is the positive inference for the production of lipase.

Chromogenic plate method*:*Chromogenic agar plates containing (in 100 ml) 1 ml tributyrin, 0.01g phenol red, 0.001g $CaCl_2$, 2g agar were prepared and pH was adjusted between 7.3 and 7.4. The culture (24 h old) supernatant was then introduced into the well, bored at the centre of chromogenic agar plate, and incubated for 24 h at 37 ^oC. Heat-inactivated culture supernatant was kept as control. Subsequently, the plates were observed for color change from red to yellow, and indication for the production of lipase (Priji *et al*., 2014).

Screening for siderophore production

Chromasurol S (CAS) Assay

CAS assay was employed for the detection of siderophore; both in liquid and on solid-agar media (Schwyn and Neilands, 1987). For the liquid CAS assay, the pure isolatewas grown in minimal medium supplemented with 3.2% piperazine-N, N'- bis (2- ethanesulphonoc acid) (MM9/PIPES), 3% casamino acids and 2% glucose (Schwyn and Neilands, 1987), which was incubated at 37 °C on a rotary shaker at 133 rpm for 48 h. Color change in the medium was scored visually to confirm the production of siderophore, *i.e*., the basic blue colour would change to brown. For solid CAS assay, about 1 mg cell pellet obtained after centrifuging (8000 × *g*) the culture (24 h old) in liquid CAS medium was spotted on to the centre of CAS-agar plates and incubated at 37 °C for 5 d, appearance of a clear orange halo around the spotted cell mass would be an indication for the production of siderophore.

Identification and characterisation of bacteria

Morphological characterisation

The isolates were identified macro-morphologically by observing the colony characteristics such as color, texture and topography of the surface and edges; and also by micro-morphological evaluation on Gram staining. Identification up to species level has been accomplished based on these morpho-cytological as well as biochemical characteristics such as fermentation reactions in the medium containing various sugars (glucose, lactose, sucrose and maltose), IMViC, starch hydrolysis and nitrate reduction tests.

Gram's staining

- The bacterial smear was heat fixed.
- The smear was gently flooded with crystal violet and left it for 1 min.

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

Spore staining by malachite green

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

Biochemical Characterisation

The biochemical characterisation included: indole production, methyl red, Voges-Proskauer, citrate utilisation, carbohydrate fermentation (glucose,

lactose, sucrose and maltose), starch hydrolysis and nitrate reduction tests (Cappuccino and Sherman, 1996) (**Table5**).

Indole production test

To determine the indole production from tryptophan by bacterial catabolism. The cultures were grown in tryptophan broth for 24-48 h, and then a few drops of Kovac's reagent were added. Formation of a pink indole ring at the surface of culture was recorded as a positive reaction.

Procedure

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

Methyl-red and Voges-Proskauer* (MRVP) *test

To determine acid production during microbial metabolism of carbohydrates (Sreedevi *et al.*, 2013)

Procedure

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

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

Citrate utilisation test

To determine the ability of the bacterium to utilise/ferment citrate as the sole carbon source (Sreedevi *et al*., 2013)

Procedure

- Simmon’s citrate agar slants were prepared (pH 6.9).
- All the ingredients, except phosphates, which were to be dissolved separately in 100 ml of water, were dissolved and volume made to 1l. The pH was set at 6.9.

- The medium was poured in the culture tubes, and sterilised by autoclaving at 15 psi, 121 ^{o}C, 20 min and slants were prepared.
- Simmon’s citrate agar slants were inoculated by means of a stab inoculation.
- One blank tube (un-inoculated) was kept as control.

- All the slants were incubated at 37 ^{o}C for 48 h

Carbohydrate fermentation test

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

Starch hydrolysis test

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

Procedure

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

Nitrate reduction test

To determine the ability of the bacteria to reduce nitrates to nitrite.

Procedure

- The nitrate reduction broth supplemented with 0.1% potassium nitrate was prepared.
- The culture was inoculated into the pre-sterilised medium in the conical flask by means of a loop inoculation.
- Incubated all cultures for 24 - 48 h at 37 ^{o}C.
- A piece of starch iodide paper was dipped in each culture, which was precipitated with 1N HCL and kept in hot air oven at 60 ^{o}C for 5 min and observed.

Table 5. Summary of media constituents, reagents and inference in the present study

Biochemical test	Medium used	Reagent	Inference
Indole production test (IPT)	**Tryptone broth** Tryptone - 1.0 % NaCl - 1.0 % pH - 7.0 ±0.2	**Kovac's reagent** p-Dimethyl aminobenzaldehyde (DMAB) – 5% Amyl alcohol - 75 ml Conc. HCL - 25 ml	Catabolism of tryptophan
Methyl-RedTest (MRT)	**MR-VP broth** Glucose – 0.5 % Peptone – 0.7% K_2HPO_4 – 0.5% NaCl – 0.5% pH - 7.0 ±0.2	**Methyl red - 0.02%**	Glucose oxidation
Voges – Proskauer Test (VPT)	**MR-VP broth**	**Voges - Proskauer reagent** 12 drops of reagent A and 3 drops of reagent B **Barritt's Reagent A** α- Naphthol- 5.0% Absolute alcohol- 100 ml **Barritt's Reagent B** KOH –4.0% Distilled water - 100 ml	Production of neutral end products
Citrate utilisation test (CUT)	**Simmon's citrate agar** NaCl - 1.0% $MgSO_4$ - 0.02% $NH_4H_2PO_4$ - 0.1% KH_2PO_4 - 0.1%	**Bromothymol blue - 0.008%**	Citrate fermentation

Biochemical test	Medium used	Reagent	Inference
	Sodium citrate - 0.2% Agar - 2.0% pH - 7.0 ±0.2		
Carbohydrate fermentation test *Glucose fermentation(GF)* *Lactose fermentation(LF)* *Sucrose fermentation(SF)* *Maltose fermentation(MF)*	Peptone - 0.1% NaCl- 0.05% Glucose/lactose/ sucrose/ Maltose - 0.1% pH - 6.9	**Phenol red-0.0012%**	Fermentation of sugars
Starch hydrolysis test (SHT)	Peptone - 0.1% NaCl - 0.05% Starch - 0.1% pH - 6.9	**Phenol red-0.0012%**	Secretion of extracellular starch hydrolysing enzymes
Nitrate reduction test (NRT)	Peptic digest of animal tissue– 0.5% Meet extract– 0.3% KN_O3 - 0.1% Nacl - 3.0 % pH – 7.0	**Sulfanilic acid (Reagent A)** **α-naphthylamine (Reagent B)**	Produced a red precipitate

Molecular characterisation by 16S rDNA sequence analysis

Procedure

- DNA was isolated from the stab-culture. Its quality was evaluated on 1.2 % agarose gel, a single band of high-molecular weight DNA has been observed.
- Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon of 1500 bp was observed when resolved on agarose gel.
- The PCR amplicon was purified.
- Forward and reverse DNA sequencing reactions of PCR amplicon was

carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic analyser.

- Consensus sequence of 16S rDNA sequence was generated from forward and reverse sequence data using aligner software.
- The 16S rDNA gene sequence was used to carry out BLAST with the nucleotide database of NCBI Genbankdatabase. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W (Priji *et al*., 2013). Distance matrix was generated using Ribosomal Datatabase Project (RDP database) and the phylogenetic tree was constructed using MEGA 4 (Tamura *et al*., 2007)

Results

Isolation and screening of microbes

Isolate Five bacterial cultures were isolated from the rumen content of Malabari goat and grown under Benjamin flask **(Figure 8)**. The bacterial cultures were represented as C1, C2, C3, C4 and C5. All the isolates were checked for lipase **(Figure 9)** and siderophore production **(Figure 10)**. Among the isolates, a bacteria (C3) exhibit positive results for the production of all the two bio-molecules.

Characterisation of microbes

Microscopic characters of the pure isolate C3, showed a rod-shaped, asporogenous, Gram-negative cells measured 0.4 to 0.8µm (width) by 1 to 2 µm (length) **(Figure 11)**. The biochemical characterisation as such carbohydrate fermentation, nitrate reduction, starch hydrolysis and IMViC tests, of which methyl red and starch hydrolysis tests were positive, whereas others were negative (**Table 6**). Depends upon the results the pure isolate of C3 showed that it belongs to the genus, *Pseudomonas*. Based on nucleotide homology and phylogenetic analysis,the isolate was further confirmed as a strain of *Pseudomonas aeruginosa*, designated as *P. aeruginosa* strain BUP2. The phylogenetic tree in comparison with related 11 strains of *Pseudomonas aeruginosa* was constructed **(Figure 6)**.

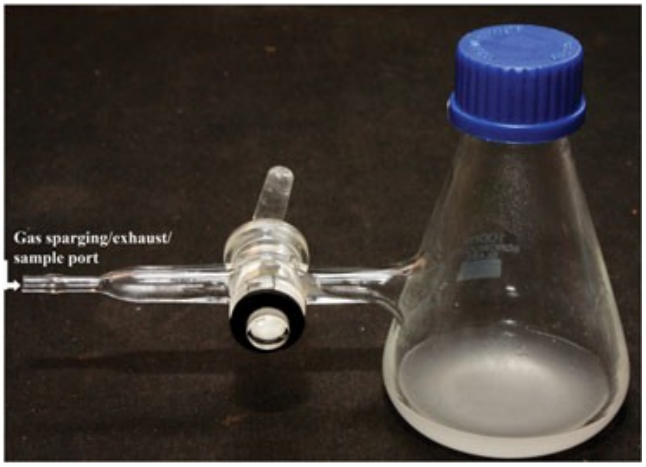


Figure 8. Microbial strains were grown under Benjamin flask containing BUP medium.

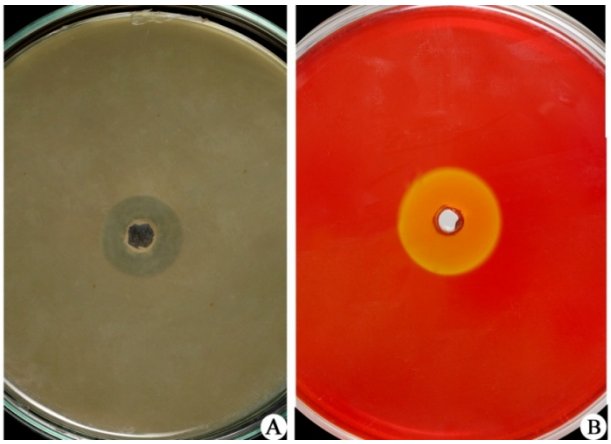


Figure 9. Screening of *P.aeruginosa strain* BUP2 for the production of lipase. **(A)** Tributyrin agar method. Production of lipase was indicated by opaque halo around the well. **(B)** chromogenic agar plate method. Production of lipase was indicated by the change in color from red to yellow around the well.

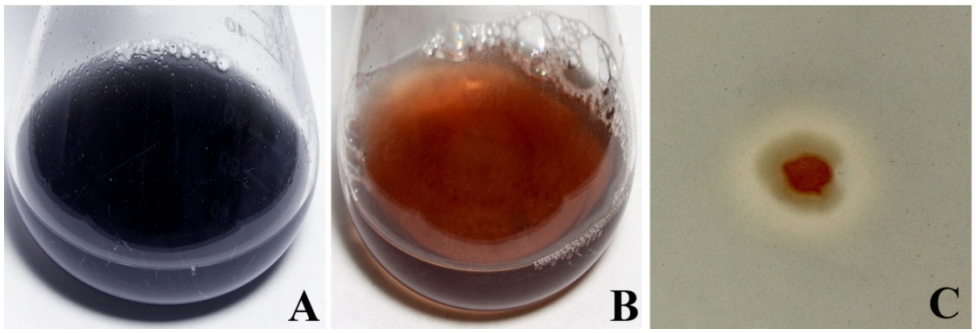


Figure 10. Screening of the siderphore by CAS asay. **(A)** Liquid CAS medium in blue color before inoculation; **(B)** liquid CAS medium changed its color to orange after 48 h incubation indicating siderophore production. **(C)** CAS agar medium shows siderophore production.

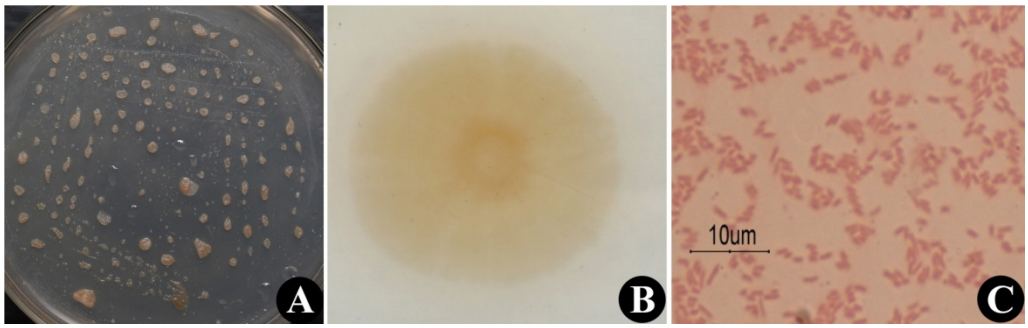


Figure 11. Morphology of *P.aeruginosa* strain BUP2.**(A)** streak plate on nutrient agar plate; **(B)** digital image of the single colony appeared slimy and white-to-cream in colour; **(C)** Gram's stained colonies.

Table 6. Summary of biochemical tests performed on *P. aeruginosa* strain BUP2

Biochemical tests	Results
Citrate utilisation test	-ve
Carbohydrate fermentation test	
Glucose fermrntation	-ve
Maltose fermentation	-ve
Sucrose fermentation	-ve
Lactose fermentation	-ve
Nitrate reduction test	-ve
Indole production test	-ve
Voges - proskauer test	-ve
Starch hydrolysis test	+ve
Methyl red test	+ve

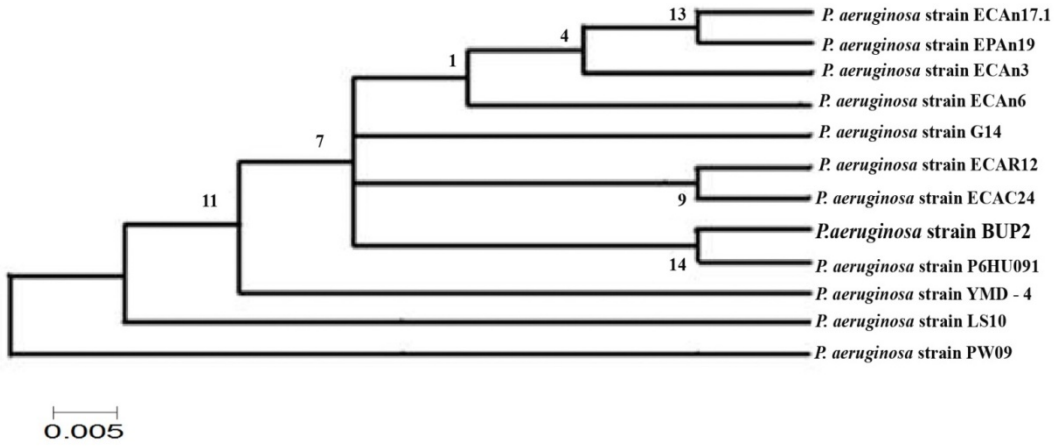


Figure 12. Evolutionary relationships of *Pseudomonas aeruginosa* strain BUP2 with other 11 related strains.

Discussion

The prime objective of the study was to isolate and characterise bacteria capable of producing industrially significant biomolecules; lipase and siderphore from the rumen content of Malabari goat. Among the 5 isolates, one bacterium exhibitsthe potential producer of both the molecules. According to the morphological, biochemical and molecular characterisation result of this bacterium confirmed that it belongs to *Pseudomonas aeruginosa* BUP2.

*Pseudomonas aeruginosa (Pa)*is a Gram-negative, rod-shaped and asporogenous bacterium isolated from various clinical samples (Oyeleke and Okusanmi, 2008). *Pa*inhabits in soil, water, skin flora, and most of the man-made environments throughout the world. Due to its ubiquitous inhabitance in the environment, many animals transiently harbor this bacterium. Apart from humans, various reports are available in literature regarding the inhabitance of *Pa* in the rumen, intestine, milk and fecal matter of cattle and calves including sheep, camel, *etc*. (Mushin and Ziv, 1973; Duncan *et al*., 1999; Leitner and Krifucks, 2007). As far as literature says, this is the first report on the inhabitance and characterisation of a *Pa* strain from the rumen of a goat, especially Malabari goat. In fact, this bacterium was initially a facultative anaerobe, which we tuned to be an aerobe by growing in a specially designed flask, the Benjamin flask (Priji *et al.,* 2013).

There are plenty of reports are available for the isolation of *Pseudomonas* spp. from various sample sources (Dennis and Sokol, 1995). However, no report is available on Malabari goat which is a special breed of domestic goat confined to the Malabar (Northern part) region of Kerala.Thereforethe

microflora inhabiting the rumen of this goat were explored. In this study, *P. aeruginosa* strain BUP2 capable of producing industrially significant lipase and siderphore, were successfully isolated from the rumen of Malabari goat.

Conclusion

This study demonstrated that the *Pseudomonas aeruginosa* starin BUP2 is one of the suitable microorganisms which can produce both industrially significant bio-molecules such as lipase and siderophore successfully. Moreover the goat rumen is considered as one of the fine sources for the isolation of microbes which is responsible for the production of industrially significant bio-molecules. But so far the microbial source of goat rumen is still remain an untapped area of the microbiologists.

Utility of rubber seed as potent solid substrates for the production of lipase by *Pseudomonas aeruginosa* strain BUP2

Chapter 4

Utility of rubber seed as potent solid substrates for the production of lipase by *Pseudomonas areuginosa* strain BUP2

Aim and Rationale

The aim of this study is to check the efficiency of flours of rubber seed, coconut and groundnut or deoiled cakes as solid substrate for the production of lipase by *P. aeruginosa* strain BUP2. India is one of the largest producers of rubber in the world, and every year large quantity of rubber seed emerges as agricultural waste. Studies revealed that higher lipase production could be attained only in the presence of lipidic substrates; since rubber seed oil contains higher levels of fatty acids, which would act as suitable inducer (in addition to providing nutrition) for lipase production. Thus, the flours/cakes of oil seeds utilised as substrate and inducerfor the microbial production of lipase.

Introduction

Being a versatile biocatalyst, microbial lipases (triacylglycerol acylhydrolase, EC3.1.1.3) offer great potentials in biological as well as industrial applications (Pandey *et al*., 1999). Though industry mostly prefers submerged fermentation (SmF) strategy for the production of lipase; solid-state fermentation (SSF) received more attention recently due to its cost effectiveness and higher productivity. Numerous studies attempted for the production of lipase on solid substrate, in which different solid agricultural residues such as cakes from deoiled coconut and groundnut kernels; husks of rice, lentil and wheat; residue from banana, melon, soybean and watermelon;

and brans from wheat and rice have been used (Benjamin and Panday, 1997; Alkan *et al*., 2007; Kempka *et al*., 2008). Oil cake is a cheap byproduct emerged out of oil extraction, which is mainly used in animal and chicken feeds. In this connection, large potential of rubber seed (oil and cake) as substrate for the cultivation of miocroorganisms is seen neglected.

India is one of the largest producers of rubber in the world, and every year huge quantity of rubber seed is treated as agricultural waste. Rubber seed oil is rich in fatty acids (Ramadhas *et al.,* 2005): *i.e.,* 39.6% linoleic acid, 24.6% oleic acid, 16.3% linoleic acid, 8.7% stearic acid 2% palmitic acid, apart from carbohydrate (24.21%) and protein (22.17%). Owing to the high nutritional content of the agro-industrial residues, oil cakes are considered as valuable solid substrates for the production of enzymes by growing suitable microorganisms on it under water-restricted environment, the SSF. Moreover, usage of agricultural residues with an industrial perspective is a best strategy for the better agricultural waste management and abatement of environmental pollution problem due to agricultural residues.

Though manybacteria, yeast, actinomycetes and fungi shown to have potentials for the production of lipase, species of genera *Bacillus*, *Pseudomonas*,*Staphylococcus*, *Candida*, *Geotrichum*,*Aspergillus*, *Mucor*, *Penicillium*, *Rhizopus* and *Rhizomucor*are considered as the best producers of lipase (Aravindan *et al*., 2007). *Pseudomonas aeruginosa* strain BUP2 (MTCC No. 5924), a new bacterial strain reported from this laboratory (Unni *et al*., 2014) was used in this study. Thus, the present study investigates the efficiency of the ground kernels of rubber seed, coconut and groundnut or deoiled cakes as solid substrate for the production of lipase by *P. aeruginosa* strain BUP2.

Materials and Methods

Bacterial culture

Pseudomonas aeruginosa strain BUP2 (MTCC No.5924), a new bacterial strain already reported from this laboratory was used throughout the study, which was isolated from the rumen of Malabari goat (Unni *et al*., 2014).

Chemicals

Analytical grade chemicals from Hi Media Laboratories (Mumbai, India) were used for the study. The *p*-nitro phenyl palmitate (*p*NPP), substrate for lipase assay was purchased from Sigma Chemical Co., USA.

Medium and Inoculum

P. aeruginosa BUP2 was maintained at 4 ^{o}C on mineral salt -oil-agar medium. Seed culture (12 h old) was prepared in the BUP medium by inoculating a loopful of stock culture of bacterium into it (Unni *et al*., 2014), which was incubated at 37 ^{o}C and 130 rpm.

Medium for SSF

For the cultivation of *P. aeruginosa* strain BUP2, kernels of 3 types of nuts/seeds (coconut, groundnut and rubber seed) were used in the ground form or as their deoiled cake for this study. Before grinding, the kernel was chopped into small pieces and dried in an oven for 24 h at 60 ^{o}C. The coconuts (coconut powder and coconut cake) and groundnuts (groundnuts powder and groundnuts cake) were purchased from local market, while rubber seeds were collected from a local plantation. The deoiled cakes were prepared after extracting oil using an expeller manually.

Solid-state fermentation (SSF) strategy

Two grams of ground kernel or their cakes was moistened with 10 ml of BUP medium. All preparations in the flask were autoclaved at 121°C for 15 min, and inoculated with 0.1 ml of inoculum (seed culture) under aseptic condition; and the inoculated media were incubated at respective conditions. In order to check lipase production, fermented samples were assayed at regular intervals of 24 h for 4 days.

Effect of pH

The solid substrate (ground seeds/cake) was moisturised with BUP medium having varying pH concentration (4, 5, 6, 7 and 8) was inoculated with *P.aeruginosa* strain BUP2 and incubated at 25 °C. At regular intervals of 24h, the fermented matter was analysed for estimating the production of lipase.

Effect of temperature

To estimate the role of different temperature (25, 28, 30, 32°C) on lipase production, the ground kernel or cake was inoculated with *P. aeruginosa*strain BUP2 after moisturising it with BUP medium having optimum pH, and assayed for lipase activity at regular intervals of 24 h.

Effect of substrate concentration

Effect of substrate concentration [(10, 20, 30, 40 and 50% (w/v)] on lipase production was checked under the optimised conditions of pH and temperature. The fermented samples were regularly withdrawn at every 24 h interval, and assayed for lipase activity.

Lipase extraction

Lipase was extracted from the fermented solid substrate by the method of (Ramachandran *et al.,* 2004). Crude lipase was extracted by mixing 1g of fermented substrate with 5 ml of 0.1M Tris-HCl buffer (pH 8.0), and mixed well on a vertex mixer. After centrifugation (8000 × *g* for 10 min, 5 ^{o}C), the supernatant was collected for lipase (crude) assay.

Lipase assay

Lipase activity in the cell and debris free supernatant was determined as described by Kilcawley *et al.,* (2002). The reaction mixture (1.8 ml) contained 0.15 M NaCl and 0.5% triton X-100 in 0.1 M Tris-HCl buffer (pH 8.0) and 200 µl supernatant (or ddH_2O in cotrol), which was pre-incubated(10 min) at 37 ^{o}C in a water bath. Subsequently, 20 µl *p*NPP (*p*-nitrophenyl palmitate) in 50 mM acetonitrile was added, and incubated further at 37 ^{o}C for 30 min. The quantity of liberated *p*-nitrophenolwas recorded at λ_{405}. One unit of lipase activity is defined as the quantity of enzyme required for liberating 1 µmol of *p*-nitrophenol under the standard assay conditions. Lipase activity was calculated using the following formula:

$$\text{Lipase activity (U/gds)} = \frac{\Delta E \times Vf}{\varepsilon \times V_S \times Df \times \Delta t \times gds}$$

Where, ΔE is the absorbance at 405 nm; Vf is the final volume of reaction mixture; V_S is the volume of crude supernatant (lipase) used; Df is the dilution factor (i.e., total extracted volume from 1g fermented matter), Δt is the time of incubation in min; ε is the extinction coefficient (0.017); *gds* is the dry weight in grams (*i.e.*, dry matter of the 1 g fermented matter used for extracting lipase.

Statistics

All studies were repeated at least thrice, and an average of 3 values is presented with standard deviation. Microsoft Excel was used to draw the figures.

Results

Effect of pH

The effect of pH on lipase production was investigated at different pH using different ground kernals or their cakes. It showed that slightly acidic or neutral pH supported the maximum production of lipase. Among the different substrates used, rubber seed flour supported the maximum production of lipase (340 U/gds) at pH 6, followed by groundnut flour and its cake (181 and 168 U/gds, respectively) at pH 7; whereas coconut oil flour and its cake supported lipase production at pH 6, but in lesser quantities (130 and 103 U/gds, respectively) (**Figure 13**).

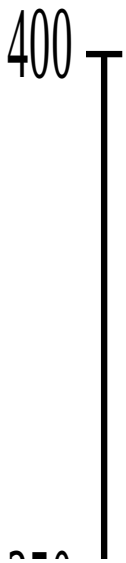


Figure 13. Effect of pH on lipase production by *P. aeruginosa* strain BUP2 on various flours and cakes moisturised with BUP medium.

Effect of culture temperature

The effect of temperature on the production of lipase by *P. aeruginosa* strain BUP2 was determined at various temperature (25 to 32 ^{o}C); of which, all the substrates supported the maximum production of lipase at temperature range between 28 and 30 ^{o}C. Rubber seed flour, coconut flour and groundnut cake supported the maximum lipase production (871, 174 and 203 U/gds, respectively) at 28 ^{o}C; while coconut cake (344 U/gds) and groundnut four (397 U/gds) supported the maximum production of lipase at slightly higher temperature (30 ^{o}C) (**Figure 14**).

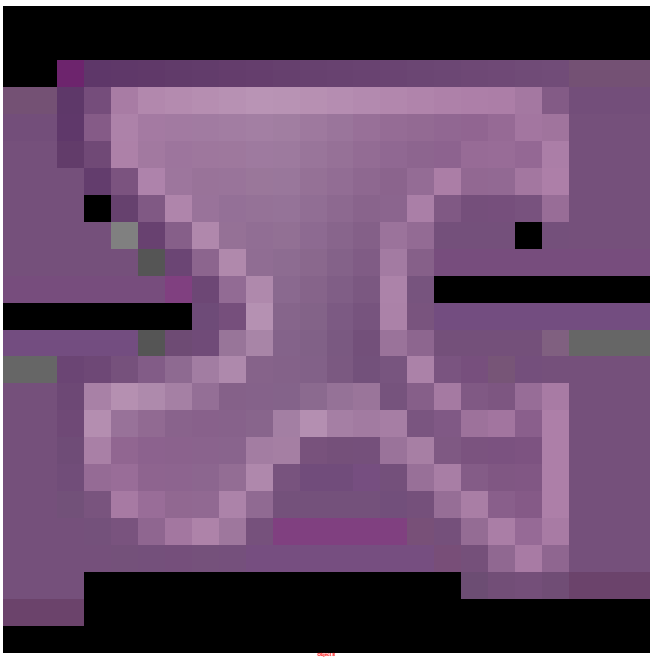


Figure 14. Effect of temperature on lipase production by *P. aeruginosa* strain BUP2 on various flours and cakes moisturised with BUP medium.

Effect of substrate concentration on lipase production

Five solid substrates (coconut flour, coconut cake, groundnut flour, groundnut cake and rubber seed flour) enriched with BUP medium were tested for their effect on lipase production by *P. aeruginosa* strain BUP2. Of them, the maximum lipase production (871 U/gds) was supported by 20 % (w/v) of rubber seed flour under optimised condition (pH 6, 28^{o}C and 48h incubation) (**Figure 15**); and 20 % (w/v) of groundnut flour (pH7, 30 ^{o}C and 48h incubation) supported the production of 398 U/gds lipase (**Figure 16**). Coconut cake (20 %, w/v) and coconut flour (40 %, w/v) supported 327 and 311 U/gds lipase production, respectively (**Figures 17 and 18**). Groundnut cake (30%, w/v) supported comparatively lesser lipase production (244 U/gds), which was at 72 h of incubation (**Figure 19**).

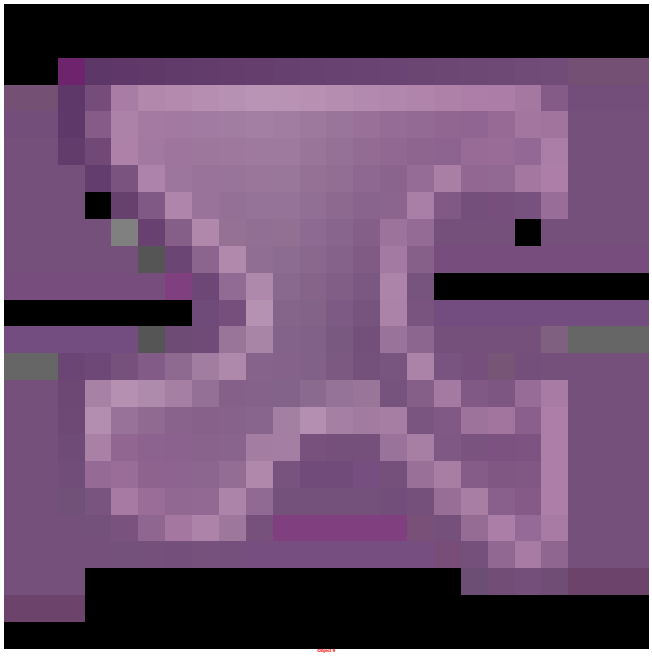


Figure 15. Lipase production profile of *P. aeruginosa* strain BUP2 on 10, 20, 30, 40 or 50 % (w/v) of rubber seed flour enriched with BUP medium.

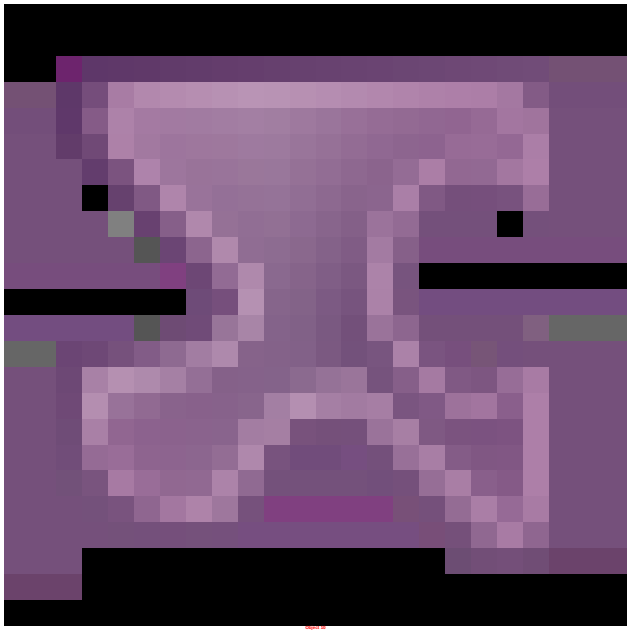


Figure 16. Lipase production profile of *P. aeruginosa* strain BUP2 on 10, 20, 30, 40 or 50% (w/v) of groundnut flour enriched with BUP medium.

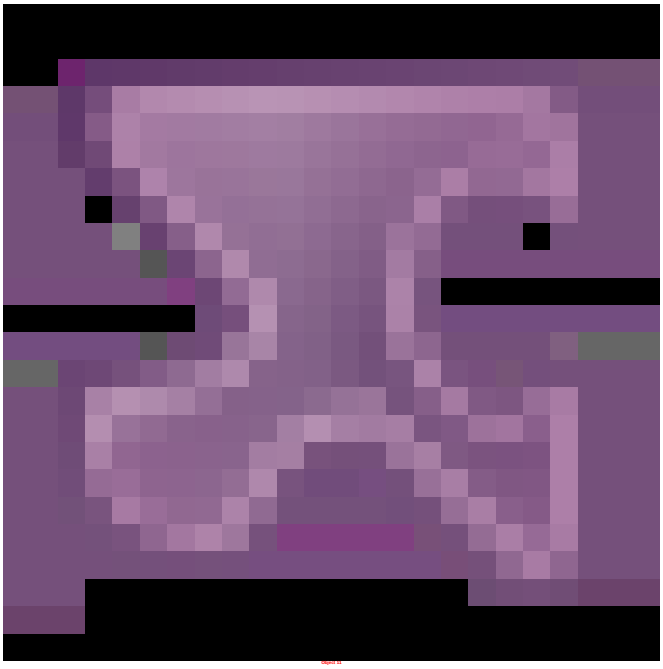


Figure 17. Lipase production profile of *P. aeruginosa* strain BUP2 on 10, 20, 30, 40 or 50 % (w/v) of coconut cake enriched with BUP medium.

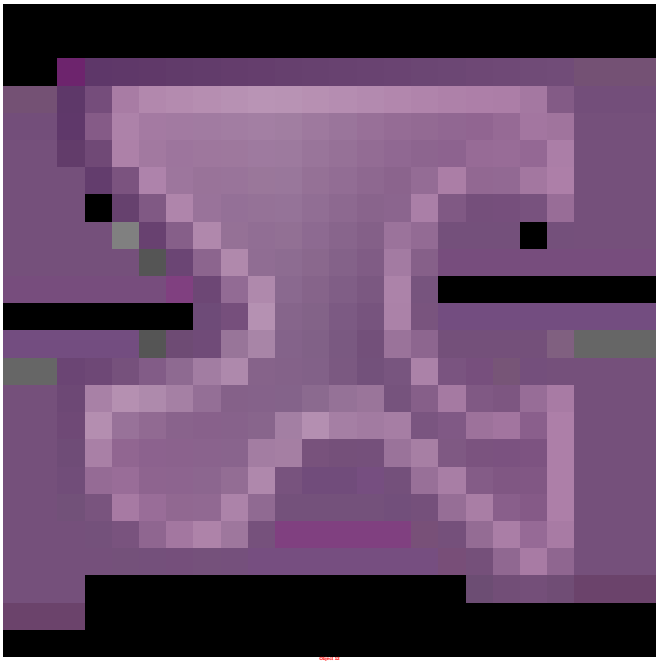


Figure 18. Lipase production profile of *P. aeruginosa* strain BUP2 on 10, 20, 30, 40 or 50 % (w/v) of coconut flour enriched with BUP medium.

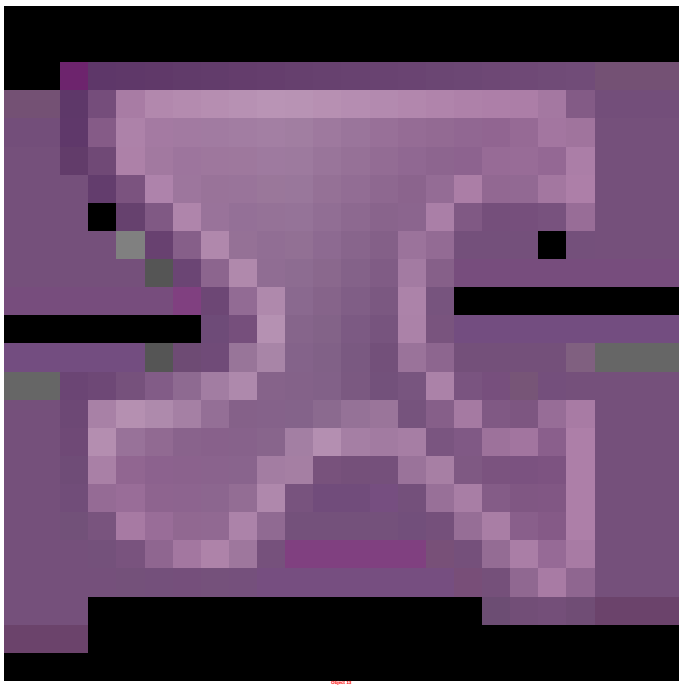


Figure 19. Lipase production profile of *P. aeruginosa* strain BUP2 on 10, 20, 30, 40 or 50% (w/v) of groundnut cake enriched with BUP medium.

Discussion

The primary objective of this work was to investigate the suitability of agricultural product-based fermentation medium for lipase production by *P. aeruginosa* strain BUP2 (MTCC No. 5924). Large quantities of deoiled kernels of seeds/nuts are generated as by-product during the extraction vegetable oil, which would fetch low price to the farmers. Rubber seed (oil or kernel) is not considered as a healthy source of cooking oil or its industrial uses are limited. Thus, a few of them were explored in this study for their utility as substrate for the production of lipase, thereby increasing their market value. SSF is mainly employed with fungi for the production of extracellular enzymes such as lipase, cellulase, amylase, alkaline protease and xylanase. In general, bacterial cultures are not considered suitable for performing SSF due to the higher water activity requirement for their growth. However, numerous

studies showed that bacterial cultures can well be adapted or it can be manipulated for SSF processes (Chakraborty and Srinivasan, 1993; Benjamin and Pandey, 1998; Kaur *et al*., 2001). Bacteria can efficiently produce *α*-amylase, alkaline protease and inulinase by species of *Bacillus*, *Pseudomonas* and *Staphylococcus* (Chakraborty and Srinivasan, 1993; Selvakumar and Pandey, 1999; Prakasham *et al*., 2006; Smitha *et al.*, 2013; Jisha *et al.*, 2014).

In the light of several advantages of SSF, lipase production from the strain of *P. aeruginosa* strain BUP2 was attempted on agricultural products. The rubber seed flour supported the maximum production of lipase. To the best of our knowledge, this is the first report showing rubber seed flour as a potent substrate for the production of lipase *via* SSF. A few studies reported oil cakes as the solid substrate-cum-inducer for the production of lipase by SSF (Benjamin and Pandey, 1996; Ramachandran *et al*., 2007; Singhania *et al*., 2008). Benjamin and Pandey (1998) employing *Candida rugosa*, demonstrated the utility of mixed-solid substrate containing wheat bran and coconut oil cake for lipase production. Recently, efficacy of *Pseudomonas* sp. strain BUP6 for the production of lipase (107 U/gds) on groundnut cake was illustrated by Faisal *et al*., (2014). Deoiled cake from *Jatropha* seed was used as a support for the production of lipase (1084 U/gds) from *P. aeruginosa* PseA through SSF (Mahanta *et al.*, 2008).

Conclusion

This study showed the utility of agricultural products, especially cheaply available rubber seed as a solid medium for the production of valuable lipase. This would enable the rubber farmers to get additional income from their agriculture. Moreover, the ability of *P.aeruginosa* strain BUP2 to grow on rubber seed medium and secrete extracellular lipase is highly promising, as rubber seed is known to embody limarin, a toxin. In this context, efficiency of *P.aeruginosa* strain BUP2 to grow on other cakes and secrete lipase is yet another promising sign of utility of this novel bacterium.

Production, optimisation, purification and Chracterisation of lipase produced by Pesudomonas aeruginosa strain BUP2

Chapter 5

Production, optimisation, purification andcharacterisation of lipase produced by*Pseudomonas aeruginosa* strain BUP2

Aim and Rationale

The aim of this study was to optimise a suitable fermentation medium for lipase production by *Pseudomonas aeruginosa* BUP2, followed by its purification and characterisation. Strains of*P. aeruginosa* are reported as potent producers of lipase on lipidic substrates. Nowa-days, lipase has increasing demand in food, textile and detergents industries; and thus, the relevance of this study.

Introduction

Lipases (E.C. 3.1.1.3), a subclass of the esterases catalyse the hydrolysis of long chain triglycerides (fat) into fatty acid and glycerol. At limited water activity, it can reverse the reaction towards ester synthesis; and this pliable nature of lipases is exploited in a host of bioconversion and catalytic reactions such as hydrolysis of lipids, alcoholysis, acidolysis, aminolysis, esterification, interesterification, transesterification, racemic solution, stereoselective and chiral syntheses (Benjamin and Pandey, 1998; Reis *et al*., 2009). Lipases show cosmopolitan distribution in animals, plants, and microorganisms; of this, microbial lipases (of bacteria, yeast, and fungi) gained much attention, and are widely used for commercial purpose; especially in biotechnological applications (Jaeger and Eggert, 2002). Bacterial lipases are used in dairy, food, detergents, pharmaceuticals, textile, cosmetic and biodiesel industries, apart from the synthesis of fine chemicals, agrochemicals and new polymeric

materials. Stability, selectivity, and broad substrate specificity make microbial lipases more attractive to the bio-industry. Microbial enzymes may either be secretory (extracellular) or cell-bound (intracellular); the former is preferred over the latter due to its ease in downstream processing (Treichel *et al.*, 2010). Commercially, species of *Bacillus* (*e.g., alcalophilus, coagulans, licheniformis, pumilus, stearothermophilus, subtilis, etc.)* and *Pseudomonas* (especially *aeruginosa* and *putida*)claim the major share of bacterial lipases; and *Burkholderia cepacia, Burkholderia multivorans, Staphylococcus caseolyticus* also contribute significantly to the microbial lipases (Lang *et al*., 1998; Treicher, *et al*., 2010).

The Gram-negative *Pseudomonas*, a prolific producer of lipase is demonstrated to have a great deal of metabolic diversity with ability to colonise a wide range of niches (Lang *et al*., 1998; Unni *et al*., 2014). Bacterial lipases as mostly produced by submerged fermentation strategy. Several physical factors such as pH, temperature, agitation, substrate concentration and inoculum size play crucial role in lipase production; normally, optimisation of single variable is adopted as the production strategy. The major drawback of this *one at a time strategy* is that it does not address the interaction effects among the variables, *i.e*., the coexistence of variables. Moreover, it does not describe the net effect of the different medium components on the enzyme production; In addition, is also tedious, and a number of trails required for determining the optimum levels. Unlike this classical method, statistical tool like response surface methodology is applied for optimising the fermentation conditions (Kaushik *et al*., 2010; Liu *et al*., 2006; He and Tan, 2006). Thus, the objectives of the present study are: statistical optimisation (using RSM) of the production conditions for lipase

from *P. aeruginosa* strain BUP2, and purification and characterisation of lipase produced by it.

Materials and Methods

Bacterial strain

Pseudomonas aeruginosa strain BUP2 (*P. aeruginosa* strainBUP2) isolated from the rumen of Malabari goat (Unni *et al*., 2014)was used in this study. This culture is maintained by Institute of Microbial Technology (IMTECH), Chadigarh (MTCC No. 5924).

Growth condition

P. aeruginosa strain BUP2 was grown on BUP medium supplemented with pre-sterilised groundnut oil (1%, v/v). The medium was inoculated with 0.1% (v/v) of overnight (12 h) culture (2.4×10^6 cfu/ml), and incubated on a rotary shaker at 37 ^{o}C and 150 rpm.

Preparation of crude enzyme

The 24 h old culture was centrifuged (9500×*g* for 10 min at 4 ^{o}C), and the supernatant was collected as crude lipase for assay and purification.

Lipase assay

The lipase assay mixture contained NaCl (0.15 M) and triton X-100 (0.5%) in 0.1 M Tris-HCl buffer (pH 8.0); 1.8 ml of this mixer was mixed with 200 µl of culture supernatant (crude lipase solution) or (purified form), pre-incubated at 37 ^{o}C for 10 min. After pre-incubation, 20 µl of substrate [50 mM paranitrophenylpalmitate, (*p*NPP) in acetonitrile] was added to the reaction mixture and mixed well. Subsequently, the mixture was incubated at 37 ^{o}C for 30 min. The quantity of *p*-nitrophenol (*p*NP) librated was measured at λ_{405}. One unit of lipase is defined as the quantity of lipase required for liberating 1 µmol of *p*NP under the standard assay conditions.

Lipase activity (U/ml) = 

ΔE = Absorbance at 405 nm

Vf = Final volume

Vs = Volume (ml) of lipase used

Δt = Time of hydrolysis

ε = Extinction coefficient (0.017)

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

Estimation of protein concentration

The protein concentration was estimated by the method of Lowry *et al.* (1951), wherein bovine serum albumin was sued as standard.

Reagents

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

Procedure

- Pipetted out 0.5 ml of the enzyme in the test tube and made up to 1 ml with 0.1 N NaOH. Add 5.0 ml of alkaline copper reagent. Vortexed well and allowed to stand for 10 min.
- Added 0.5 ml of Folin's reagent, vortexed well and incubated at 25 ^oC for 30 min.
- Read the absorbance at λ_{660} using spectrophotometer.
- Calculations were done using the graph generated from the standard graph of BSA.

Standard BSA graph

- 1 mg/ml stock solution was prepared with BSA.
- Different aliquots of stock solution (0.05, 0.10, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 µl) were pipetted in to the test tubes and made up to 1 ml with 0.1 N NaOH, 5.0 ml of alkaline copper reagent was added subsequently; vortexed well and allowed to stand for 10 min.

- 0.5 ml of Folin's reagent was added, vortexed well and incubated at 25 ^{o}C for 30 min.
- Read the absorbance at λ_{660}using spectrophotometer.
- The values were plotted against concentration *vs* optical density measured at λ_{660}.

The protein concentrations in this experiment were estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Optimisation of lipase production

Physical factors (variables) such as concentration of substrate, inoculum size, pH, temperature, duration of incubation and agitation were optimised for the maximum production of lipase by applying the following statistical approaches. Plackett-Burman design was used for the initial screening of variables; then, suitable variables were subjected to Box-Behnken, followed by response surface methodology (RSM) so as to find out the optimum level of each variable.

Plackett-Burman design

Using this design six variables were screened in 22 experimental runs**(Table 7)**. Each variable was tested at three levels; *i.e*., low (-1), medium (0) and high (+1), and the range of variables was: substrate concentration (0.01, 0.55 and 1 %), inoculum size (0.01, 0.55 and 1.0%), pH (4.0, 6.5 and 9.0), temperature (20, 30 and 40 ^{o}C), duration of incubation (6.0, 39 and 72 h), and agitation (50, 125 and 200 rpm). The experiment was performed in 100 ml conical flasks containing 20 ml medium, and responses were calculated from the lipase activity in the sample.

Table 7. Plackett-Burman experimental design for the production of lipase from *P. aeruginosa* strain BUP2. The parameters selected were pH (4.0, 6.5 and 9.0), temperature (20, 30 and 40°C), Substrate Concentration (0.01, 0.55 and 1.0%), Inoculum size (0.01, 0.55 and 1.0%), agitation (50, 125 and 200 rpm) and incubation time (6.0, 39 and 72h).

Run order	Temperature (°C)	pH	Substrate conc (%)	Incubation (h)	Agitation (rpm)	Inoculum Size (cfu/ml)
1	40	9.0	0.1	72	200	0.1
2	40	4.0	1.0	72	200	1.0
3	40	9.0	0.1	6.0	200	1.0
4	20	4.0	1.0	72	50	1.0
5	30	6.5	0.55	39	125	0.55
6	20	9.0	1.0	6.0	200	1.0
7	20	4.0	0.1	72	50	1.0
8	40	9.0	1.0	6.0	50	1.0
9	20	9.0	1.0	72	200	0.1
10	20	4.0	0.1	6.0	200	0.1
11	20	4.0	1.0	6.0	200	0.1
12	40	4.0	0.1	6.0	50	1.0
13	30	6.5	0.55	39	125	0.55
14	40	9.0	0.1	6.0	50	0.1
15	20	4.0	0.1	6.0	50	0.1
16	40	4.0	1.0	6.0	200	1.0
17	40	9.0	1.0	72	50	0.1
18	20	9.0	0.1	72	200	1.0
19	20	9.0	0.1	72	50	1.0
20	20	9.0	1.0	6.0	50	0.1
21	40	4.0	0.1	72	200	0.1
22	40	4.0	1.0	72	50	0.1

Box-Behnken design and RSM

Significant variables optimised by the Placket-Burman design were selected for Box-Behnken analysis. For the optimisation of lipase production, 15 different combinations of trials were carried out according to the Box–Behnken design, followed by response surface analysis. Each variable was

studied at three levels, *i.e*., low, medium and high**(Table 8)**. The second order polynomial equation applied for the analysis of the results was:

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. To confirm the validity of quadratic model, 3 experiments - as assigned by the point prediction software Minitab 14 - were used; respective lipase activity was calculated and compared with the predicted values**(Table 9)**.

Table 8: Box-Behnken design for the production of lipase from *P. aeruginosa* strain BUP2. The parameters selected were pH concentration (6.0, 7.5 and 9.0); temperature (28, 30, 32 ^{o}C); incubation time of the medium (24, 36 and 48 h).

Run order	**pH**	**Temperature**(^{o}C)	**Incubation (h)**
1	7.5	32	48
2	9.0	28	36
3	7.5	30	36
4	7.5	28	48
5	9.0	30	48
6	7.5	32	24
7	7.5	30	36
8	6.0	28	36
9	6.0	30	24
10	6.0	30	48
11	9.0	32	36
12	7.5	30	36
13	9.0	30	24
14	7.5	28	24
15	6.0	32	36

Table 9. Validation of experimental design for the production of lipase from *P. aeruginosa* strain BUP2.

Sl no	pH	Temperature (°C)	Incubation (h)
1	6.0	28	24
2	8.0	28	28
3	6.0	30	24

Purification of Lipase enzyme

Appropriate quantity of $(NH_4)_2SO_4$ salt (0-20 %, 20-40 %, 40-60 % and 60-80 %) was added to the crude culture supernatant until it reached 80% saturation; which was stirred in a cold room at 4 °C. The precipitated protein was collected by centrifugation (9400 × *g* for 10 min at 4 °C).

Dialysis

The precipitate (pellet) obtained after ammonium sulphate fractionation was dialysed against 50 mM Tris-HCl buffer (pH 8) for 24 h with 2 buffer changes. Cellulose membrane dialysis tubes were used for dialysis. The dialysate was centrifuged (9400 × *g* for 10 min at 4 °C), the precipitate (debris) so obtained was discarded and the supernatant (concentrated protein solution) was used for gel permeation chromatography. The lipase activity and protein content of the dialysate were determined. The protein content was estimated by the method of Lowry *et al.*(1951).

Gel permeation chromatography

The above dialysate was used for gel permeation chromatography. Gel permeation chromatography was done in a column packed with Sephadex G-100 (Sigma Aldrich, USA) using a peristaltic pump (Riviera, India) in a cold chamber.

Calculations

Relative activity = Present activity×100 / Activity of control

Specific activity = Total activity / Total Protein

Fold purification = Specific activity /Crude specific activity

%Yield = Present activity×100 /Total activity of crude extract

Characterisation of purified enzyme

Native PAGE: Native polyacrylamide gel electrophoresis (Native-PAGE) was done using 5 % stacking gel and 8 % resolving gel. Samples were permitted to stack at 40 mV and to separate at 70 mV. The gels were washed thoroughly with sterile ddH_2O and equilibrated in 50 mM Tris HCl (pH 8.0) for 30 min at 30 ^oC; on which, molten agar containing olive oil (1 %), tween 80 (1 %), $CaCl_2.H_2O$ (0.01 %) was applied and incubated at 37 ^oC for 24 h (Haba *et al*., 2000).

Table 10. Components for stacking gel (5 %)

Component	Quantity (5 ml)
ddH_2O	3.4
Acrylamide :bisacrylamide(36 :1)	0.83
1.0 M Tris (pH 6.8)	0.63
10% APS	0.05
TEMED	0.005

Table 11. Components for resolving gel (8 %)

Component	Quantity (10 ml)
ddH_2O	4.6
Acrylamide :bisacrylamide(36 : 1)	2.7
1.5M Tris (pH 8.8)	2.5
10% APS	0.1
TEMED	0.006

SDS-PAGE: To determine the molecular weight (MW), lipase was run on SDS-PAGE using 10% acrylamide gel (Laemmli, 1970), and stained by coomassie brilliant blue (CBB) R-250. The MW protein marker (GeNei, India) was comprised of myosin (205), phosphorylase b (97.4), bovine serum albumin (66), ovalbumin (43), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), lysozyme (14.3), aprotinin (6.5) and insulin (3.5), all in kDa.

Sample buffer composition

The ingredients used for making protein sample buffer are given in **Table 12**

Table 12. Ingredients for protein sample buffer

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

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

Stacking gel composition

The ingredients used for making stacking gel are given in **Table 13.**

Table 13. Ingredients for stacking gel 5 %

Ingredients	Quantity (5ml)
ddH_2O	3.4
Acrylamide : bisacrylamide (36 :1)	0.83
Tris buffer (pH 6.8)	0.63
10% SDS	0.05
10% APS	0.05
TEMED	0.005

Composition of resolving gel (10 %)

The ingredients used for making resolving gel are given in **Table 14.**

Table 14 . Ingredients required for resolving (10 %) gel

Ingredients	Quantity (10 ml)
ddH_2O	4.0
Acrylamide : bisacrylamide (36 :1)	3.3
Tris buffer (pH 8.8)	2.5
10% SDS	0.1
10% APS	0.1
TEMED	0.004

Sample preparation

Enzyme solution and sample buffer were mixed in the ratio of 1:1. The contents were mixed well in a pre-sterilised eppendorf tube and heated in a boiling water bath for 3 min. Broad range protein molecular weight was used for the determination of the MW of protein on the gel.

Staining solution

After the electrophoresis, the gel was stained with 0.1 % coomassie brilliant blue (CBB) G-250 (prepared in a mixture of 10 % glacial acetic acid: 45 % methanol: 45 % ddH_2O).

Destaining

The destaining solvent system contained 10 % glacial acetic acid: 45 % methanol: 45 % ddH_2O. The protein bands on the SDS-PAGE gels were visualised through gel-documentation system and also by canon digital camera and photographed.

Characterisation of lipase enzyme

The lipase active fraction obtained after gel permeation chromatography was used for the characterisation of various properties such as effects of pH, temperature, different metal ions, inhibitors, surfactants, detergents and substrate concentration on enzyme activity.

Effect of pH on the lipase activity

The optimum pH for lipase activity was determined from its activities in citrate (0.1 M, pH 3.0 to 6.0), phosphate (1.0 M, pH 7.0), Tris-HCl (1.0 M, pH 8.0 to 9.0) and carbonate (0.2 M, pH 10) buffers at 37 ^{o}C for 30 min incubation.

Effect of temperature on enzyme activity

The optimum temperature for lipase was calculated from its activities in 0.1 M Tris-HCl buffer (pH 8.0) at different temperatures, *i.e*. 20, 25, 30,35, 37, 40, 45, 50, 55 and 60 ^{o}C for 30 min incubation.

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.*, Ca^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+} to a final concentration of 1, 2.5, 5.0, 7.5 and 10 mM at 45 ^{o}C and pH 8.0 for 30 min incubation.

Effect of different detergents and inhibitors on enzyme activity

Effects of different detergents and inhibitors on lipase activity were determined by incubating the reaction mixture with different detergents (SDS, tween 80 and triton X-100), and inhibitors (EDTA and β-mercaptoethanol) to a final concentration of 0.25, 0.5, 1.0 1.5 or 2 % at 45 ^{o}C and pH 8 for 30 min incubation.

Calculation of *Km* and *Vmax*

The substrate *p*NPP (5, 10, 20, 40, 50, 60 or 80 mM) was added to lipase assay mixture, and incubated for 1 h at 45 ^{o}C and pH 8, and samples were withdrawn at 5 min intervals. The *Km* and *Vmax* values of lipase were calculated using the software, Hyper 32.

Statistics

All studies were repeated at least thrice, and an average of 3 values is presented with standard deviation. Microsoft Excel and Hyper 32 was used to draw the figures.

Results

Optimisation of lipase production

Plackett–Burman experimental analysis - a fractional factorial design - was used for the optimisation of growth conditions of *P. aeruginosa* strainBUP2 for lipase production. Five parameters such as substrate concentration, temperature, pH, incubation time, and agitation were considered initially. Presence of ground nut oil to a final concentration 1 % in the medium was found to be an effective supplement in the medium for lipase production.

Initially, 22 set of experiments (**Table 15**) were designed; accordingly, pareto chart was generated to judge the important parameters responsible for the lipase production at 0.05 significant level **(Figure 20)**. The combined effective of each parameters showed that only temperature, pH and incubation time had significant role in lipase production. These three parameters were subjected to Box–Behnken analysis, followed by RSM for judging the suitable combination of these parameters for enhancing lipase production. Subsequently, a set of 15 experiments (**Table 16**) was done according to Box–Behnken design, and the results (**Figure 21 A-F**) suggested that the predicted and experimental values for lipase production did not show significant difference, *i.e.*, the R^2 value was 0.9 (**Table 17**). Three parameters (temperature, pH and incubation time) selected from the validation experiments fall within the range determined from Box-Behnken were assessed in details for the suitability of the model (**Table 18**). The correlation coefficient of the result was 0.971, which was in good agreement with the predicted and experimental values. The optimum production of lipase was found to be 172 U/ml (at 28 ^{o}C, pH 6.0, and 24 h incubation). Thus, the statistical optimisation resulted in 54 % increase of lipase production over that obtained in un-optimised condition.

Table 15. Result of Plackett-Burman experimental design showing Lipase production by *P.aeruginosa* BUP2

Run order	Temprature (ºC)	pH	Substrate conc (%)	Incuba-tion (h)	Agita-tion (rpm)	Inoculum Size (cfu/ml)	Lipase (U/ml)
1	40	9.0	0.1	72	200	0.1	58.8
2	40	4.0	1.0	72	200	1.0	1.25
3	40	9.0	0.1	6.0	200	1.0	6.8
4	20	4.0	1.0	72	50	1.0	2.1
5	30	6.5	0.55	39	125	0.55	148.7
6	20	9.0	1.0	6.0	200	1.0	4.2
7	20	4.0	0.1	72	50	1.0	1.8
8	40	9.0	1.0	6.0	50	1.0	0.92
9	20	9.0	1.0	72	200	0.1	83
10	20	4.0	0.1	6.0	200	0.1	2.6
11	20	4.0	1.0	6.0	200	0.1	2.2
12	40	4.0	0.1	6.0	50	1.0	0.47
13	30	6.5	0.55	39	125	0.55	142.5
14	40	9.0	0.1	6.0	50	0.1	0.62
15	20	4.0	0.1	6.0	50	0.1	0.24
16	40	4.0	1.0	6.0	200	1.0	1.7
17	40	9.0	1.0	72	50	0.1	1.9
18	20	9.0	0.1	72	200	1.0	106.7
19	20	9.0	0.1	72	50	1.0	101
20	20	9.0	1.0	6.0	50	0.1	2.3
21	40	4.0	0.1	72	200	0.1	0.96
22	40	4.0	1.0	72	50	0.1	0.74

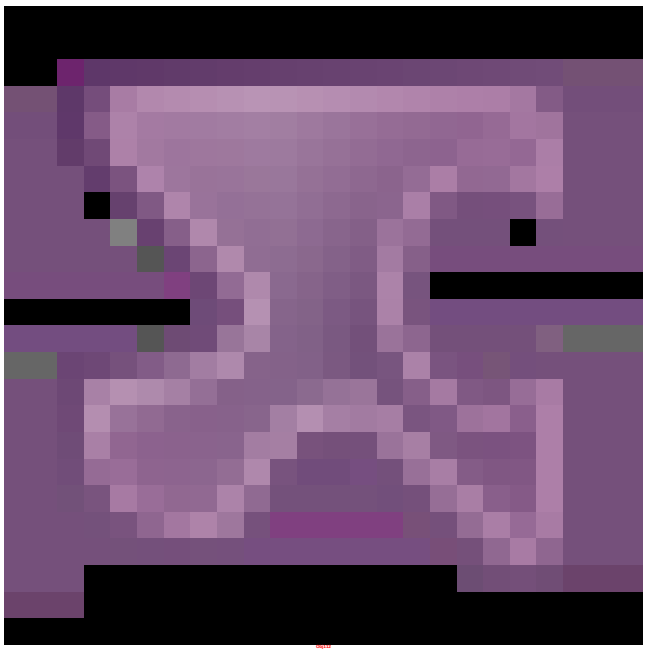


Figure 20. Pareto chart shows the standardised effects on the production of lipase by *P. aeruginosa* strain BUP2. The factors found relevant were in the order: Temperature>pH>incubation time, while agitation, substrate and inoculum concentration were found to have negligible effects

Table 16. Results of Box-Behnken design for the production of lipase by *P. aeruginosa* strain BUP2.

Run order	pH	Temperature (°C)	Incubation (h)	Lipase (U/ml)
1	7.5	32	48	77.05
2	9.0	28	36	124.05
3	7.5	30	36	113.35
4	7.5	28	48	91.14
5	9.0	30	48	64.64
6	7.5	32	24	110
7	7.5	30	36	121.2
8	6.0	28	36	166.35
9	6.0	30	24	155.06
10	6.0	30	48	109.7
11	9.0	32	36	57.45
12	7.5	30	36	120.13
13	9.0	30	24	88.94
14	7.5	28	24	130.94
15	6.0	32	36	99.57

Response surface regression

Regression coefficients were estimated for lipase production (lipase*vs*. temperature, pH and incubation time). The regression model for lipase production was highly significant ($p<0.05$) with an acceptable value of determination coefficient (R^2 = 0.9). The regression equation obtained for lipase production was

$Y = 118.23 – 24.6X_1 – 21X_2 – 17.8X_2X_3 + 0.05X1X2 + 1.78X3 + 0.05X1X2 + 1.7X2X3 + \beta 5.3X1X3 + \beta 2.0X_1^2 + 4.3X_2^2 – 11.6X_3^2$; where X_1, X_2, X_3 are pH, temperature and incubation time, respectively.

Table 17. Analysis of variance of quadratic model *vs*. lipase production

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9.0	11529.9	11529.9	1281.1	4.8	0.050
Linear	3.0	10862.7	10862.7	3620.9	3620.9	0.008
Square	3.0	544.5	544.5	181.5	0.7	0.601
Interaction	3.0	122.6	122.6	40.9	0.2	0.923
Residual Error	5.0	1336.0	1336.0	267.2		
Lack-of- Fit	3.0	1299.8	1299.8	433.3	23.9	0.040
Pure Error	2.0	36.2	36.2	18.1		
Total	14	12865.9				

Table 18. Random combinations of parameters selected for the validation of proposed statistical model with respective observed and predicted responses *vs*. Lipase production by *P.aeruginosa* strain BUP2

Sl No	pH	Temperature (°C)	Incubation (h)	Lipase (U/ml) production Observed	Lipase (U/ml) production Predicted
1	6.0	28	24	172	170.57
2	8.0	28	28	138	133.12
3	6.0	30	24	148	151.99

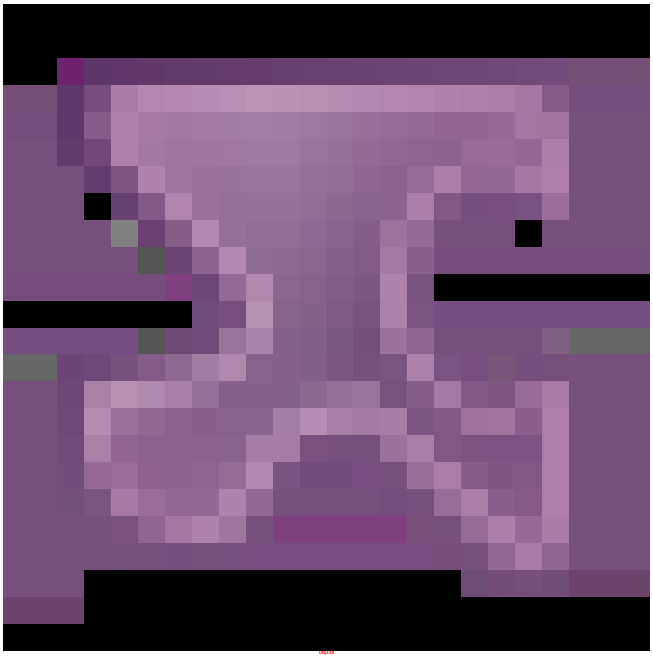


Figure 21.(A) Contour plot showing lipase production *vs* temperature (°C), pH. Here incubation value (36 h) was kept constant**.**

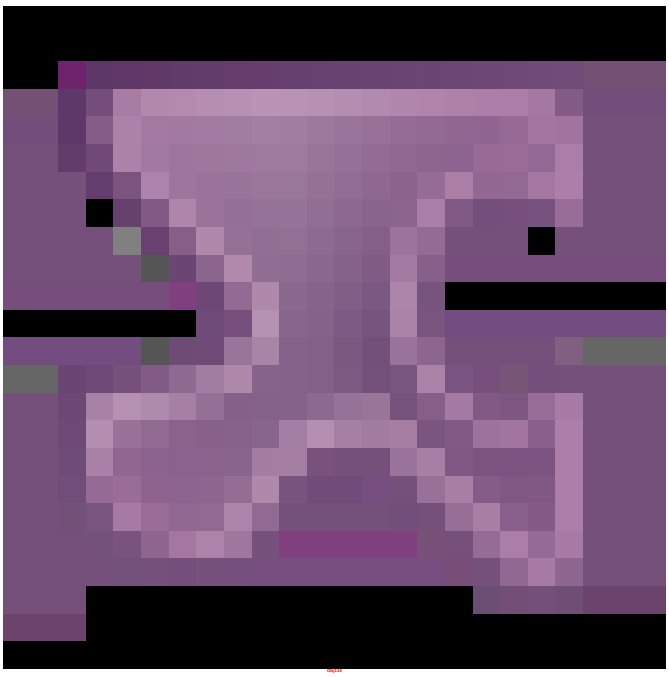


Figure 21.(B) Contour plot showing lipase production *vs* incubation time (h), pH. Here temperature value (30 °C) was kept constant**.**

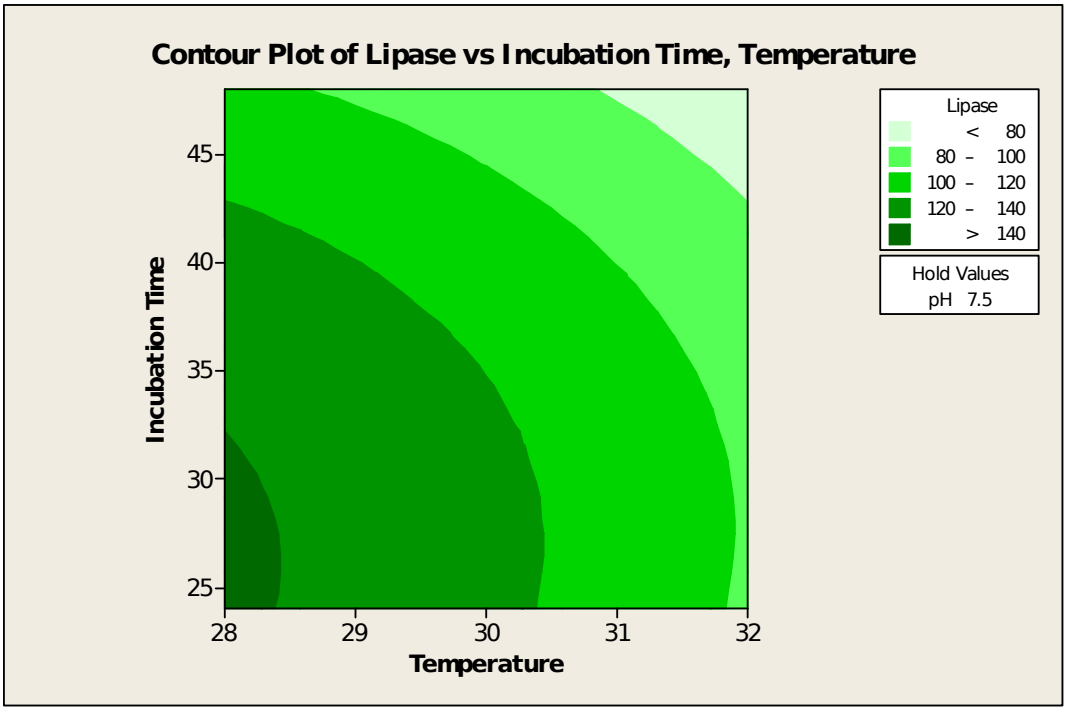


Figure 21.(C) Contour plot showing lipase production *vs* incubation time (h), temperature (°C). Here pH value (7.5) was kept constant**.**

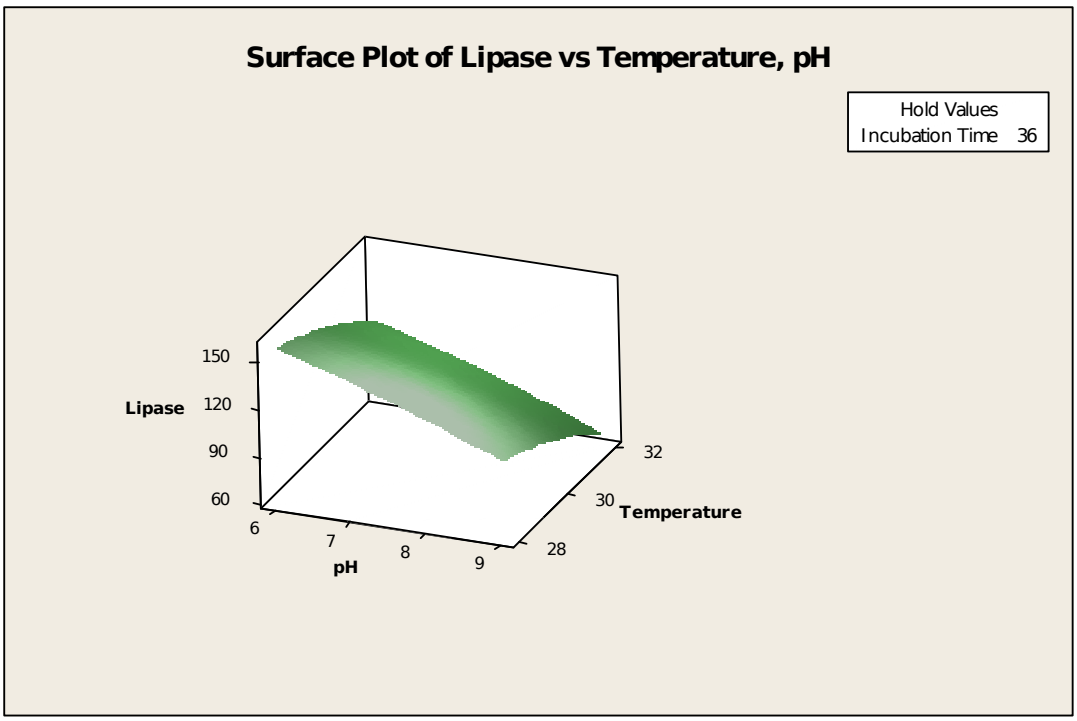


Figure 21.(D) Surface plot showing lipase production *vs* temperature (°C), pH. Here incubation value (36 h) was kept constant**.**

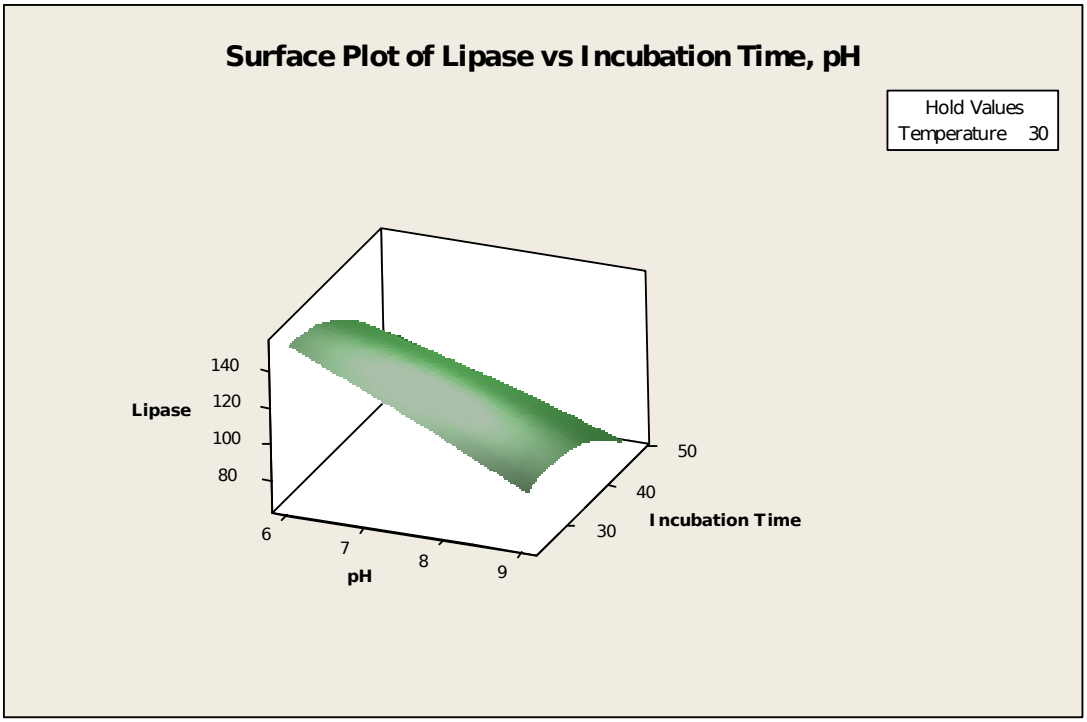


Figure 21.(E) Surface plot showing lipase production *vs* incubation time (h), pH. Here temperature value (30 ^{o}C) was kept constant**.**

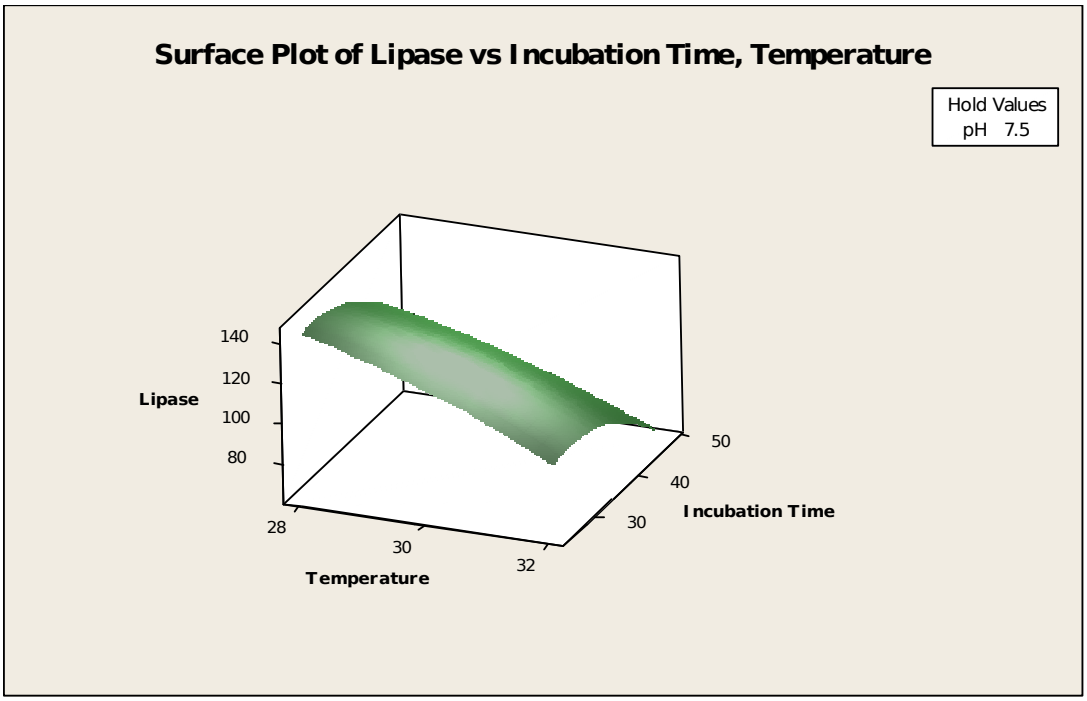


Figure 21.(F) Surface plot showing lipase production *vs* incubation time (h), temperature (^{o}C),. Here pH value (7.5) was kept constant**.**

Purification of lipase

The extracellular lipase of *P. aeruginosa* strain BUP2 in crude supernatant was purified by $(NH_4)_2SO_4$ fractionation and sephadex G-100 gel filtration. Of various $(NH_4)_2SO_4$ fractions, 60-80 % fraction showed the maximum lipase activity (21 folds purified fraction with 27.9 % yield) (**Table 19**); hence this fraction was subjected to gel permeation chromatography. Thirty fractions were collected at a rate of 2ml/20min, and the OD was measured at λ_{280}; which showed a major peak corresponding to fraction numbers 15-16 **(Figure 22**). The purification fold of sephadex G-100 fraction was 36 with a yield of 20%. The lipase after each purification step was subjected to SDS-PAGE and native (non reducing) PAGE was performed for checking the purity of lipase. The apparent MW of partially purified lipase was estimated as 29 kDa by SDS-PAGE. An opaque band showing lipase activity was detected on the gel upon native PAGE (**Figure 23 A and B**).

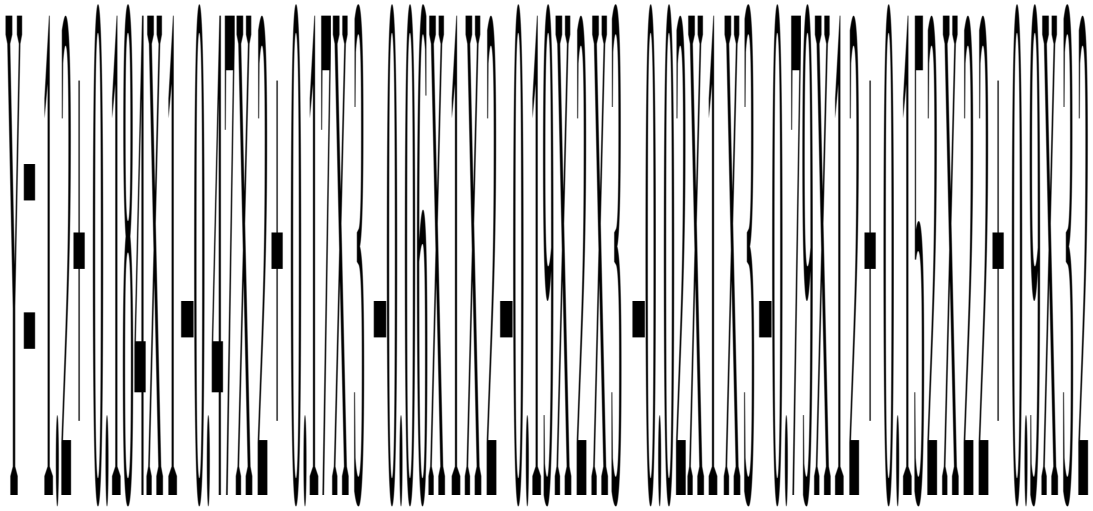


Figure 22. Sephadex G-100 elusion profile of the partially purified lipase. $(NH_4)_2SO_4$ fraction (60-80%) of crude protein harvested from BUP medium. Subsequently dialysis fraction were subjected to sephadex G- 100 which was eluted using Tris-HCl buffer (pH 8.0) with a flow rate of 2 ml per 20 min.

Table 19. Lipase enzyme purification

Volume (ml)	**LipaseActivity(U/ml)**	**Protein(mg/ml)**	**Total protein(mg)**	**Totalactivity(U)**	**Sp. activity(U/mg)**	**Fold**	**Yield(%)**
Culture supernatant	157	2.364	472.8	31480	66.58	1.0	100
60-80% $(NH_4)_2SO_4$ fraction	2535	1.8	6.228	8777	1409.2	21	27.9
Sephadex column	1579	0.66	2.64	6316	2392	36	20

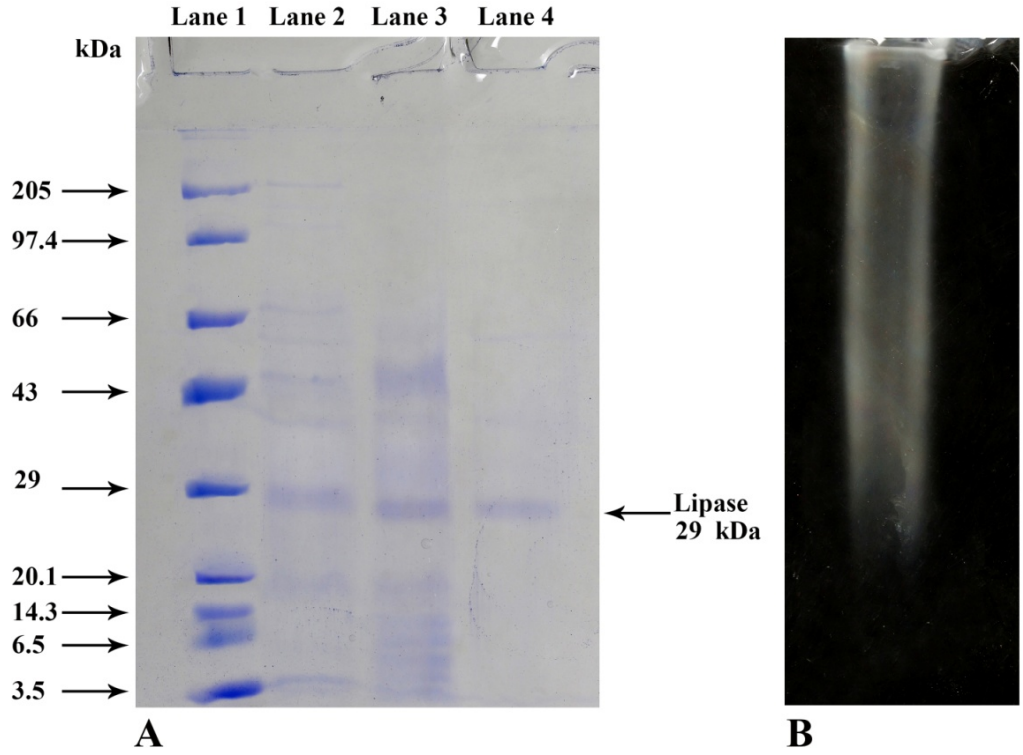


Figure 23. (A) SDS-PAGE (reducing) profile showing lipase active band with approximate MW of 29 kDa. Lane 1 shows the profile of standard protein MW marker. Lane 2 shows crude protein harvested after 24 h incubation. Lane 3 shows the profile of 60–80 % $(NH_4)_2SO_4$ fraction and lane 4 represents the profile of sephadex G-100 gel filtration. **(B)** Native PAGE profile of partially purified lipase on 8 % gel.

Characterisation of purified lipase

Purified lipase obtained by sephadex G-100 gel permeation chromatography was used for the characterisation studies. Compared to the initial activity (crude supernatant), lipase activity was increased in $(NH_4)_2SO_4$ and sephadex G-100 fractions, which was directly proportional to the purity of the lipase used **(Figure 24 A-D)**. In order to check the enhanced activity by a particular factor, the activity of lipase at that condition was compared to that of the normal reaction conditions (pH 8.0, 37 ^oC, 50 mM *p*-NPP and incubation for 30 min), thus fold increase in activity was calculated. It is expressed in relative activity, which is the ratio of the enhanced activity to the initial activity, expressed in percentage.

Effect of pH on lipase activity

Effect of pH on lipase activity was measured at normal assay conditions (37 ^oC, 50 mM *p*-NPP and incubation for 30 min) with varying pH. Purified lipase was active at pH 7.0, 8.0 and 9.0; but the maximum activity (1574 U/ml) was obtained at pH 8, while in acidic pH (5.0) the lipase activity dropped to 177 U/ml. Under optimised pH, lipase exhibited 100 % relative activity (**Figure 24A**). In general, lipaseproduced by *P. aeruginosa* strain BUP2 was better active at alkaline pH.

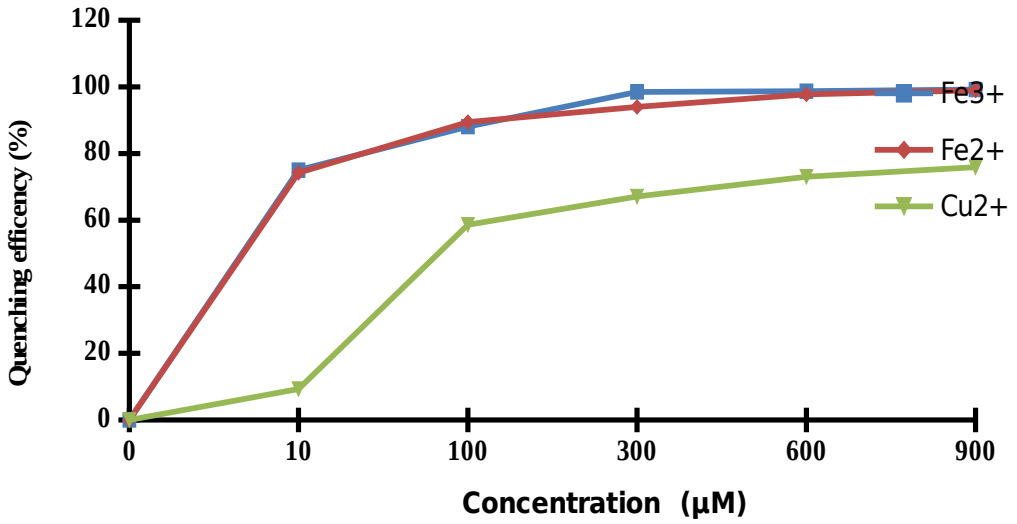


Figure 24. (A) Effect of pH on lipase activity. Maximum lipase activity (1574 U/ml) was noticed at pH 8.0 and the lowest activity (177 U/ml) was at pH 5.0.

Temperature on lipase activity

The assay conditions for assessing the optimum temperature for lipase activity were: fixed pH 8.0, 50 mM *p*-NPP and 30 min., while incubation temperature was varying (20-55 ^{o}C). Purified lipase was active in 30-50 ^{o}C range with the optimum activity at 45 ^{o}C (**Figure 24B**); at this temperature, the lipase activity was 2428 U/ml with 153.8 % relative activity reached, *i.e*., over 50% increase. However, at 55 ^{o}C (1173 U/ml) and above, the activity declined.

Figure 24. (B) Effect of temperature on lipase activity. Maximum lipase activity (2428 U/ml) was noticed at 45 ^{o}C.

Effect of metal ions on lipase active

Presence of the metal ions in the reaction mixture influenced the lipase activity; for which the assay conditions were set at pH 8, 45 ^{o}C, 50 mM *p*-NPP concentration and 30 min incubation, with varying metal ion concentration (1 to 10 mM). Lipase activity significantly enhanced in the presence of Ca^{2+} and the presence of Mn^{2+}; while Mg^{2+} and Fe^{2+} slightly decreased the activity. The maximum lipase activity (2802 U/ml) was obtained at 5 mM concentration of Ca^{2+}; and the relative activity was 177.5% (*i.e*., over 75% increase), compared to unoptimised condition. The remaining two metals, Zn^{2+} and Cu^{2+} inhibited the lipase activity significantly **(Figure 24C**).Thus, pH 8.0, 45 ^{o}C temperature, 50 mM *p*-NPP, 5 mM Ca^{2+} and 30 min incubation were the optimised conditions for lipase assay.

Figure 24. (C) Effect of different metal ions on enzyme activity. Maximum activity for lipase was obtained for 5.0mM Ca^{2+} at 45 ^{o}C and pH 8.0 the activity was (2802 U/ml).

Effect of detergents and inhibitors on lipase activity

In order to check the effect of detergents and inhibitors on lipase activity, the assay conditions were set at pH 8.0, 45 ^{o}C, 5 mM Ca^{2+}, 50 mM *p*-NPP and 30 min incubation time with 0.25, 0.5, 1.0, 1.5 or 2 % SDS, tween 80, triton X-100, EDTA or ME. Triton X-100 (0.5 %) did not show alteration in lipase activity (2802 U/ml), *i.e*., activity was comparable to that of optimised condition; but activity was gradually decreased at its higher concentrations **(Figure 24D).** The presence of tween 80 and SDS in the reaction mixture resulted significant reduction in lipase activity. EDTA almost inhibited the lipase activity, whereas β-mercaptoethanol reduced the relative activity of lipase up to 70% (**Figure 24D).**

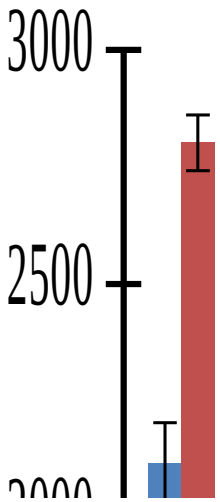


Figure 24. (D) Effect of EDTA and *β*-mercaptoethanol on lipase activity**.** Lipase was active at triton X-100 (0.5 %) as under normal assay condition but gradually reducing the activity at higher concentration of triton X-100. EDTA inhibited the lipase activity whereas *β*-mercaptoethanol reduces the activity of lipase significantly.These activities are higher than that of activities obtained at optimum pH and temperature.

Optimised condition

The optimised conditions for the maximum activity of lipase produced by *P. aeruginosa* strainBUP2: pH 8, 45 ^{o}C temperature, 5.0 mM Ca^{2+} and 0.5% triton X-100. At this condition, the ezyme activity reached at 2802 U/ml, and the relative activity was 177.5% (**Table 20, Figure 25**).

Table 20. Activity of purifiedlipase, under optimised conditions

Factors	Enzyme activity (U/ml)	Relative activity (%)
pH	1574	100
Temperature	2428	153.8
Calcium	2802	177.5

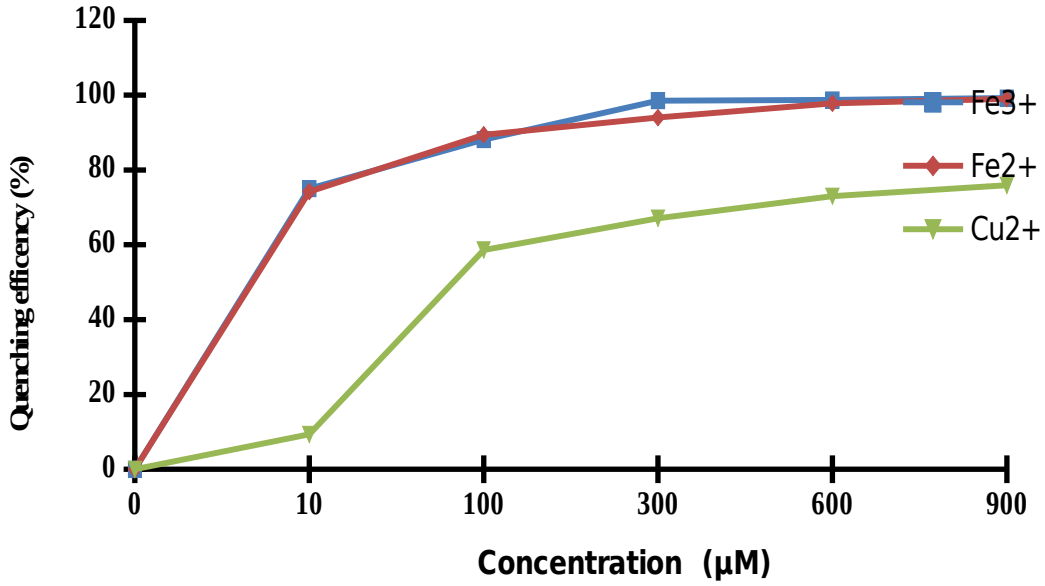


Figure 25. Summary of characterisation of lipase produced by *P. aeruginosa* BUP2. Optimum conditions for maximum activity of lipase was showed in pH 8.0, 45 °C, triton X-100 (0.5 %) and Ca^{2+} (5.0 mM)

Enzyme kinetics

The kinetic parameters of the extracellular lipase were determined from Line Weaver-Burk (**Figure 26**) and Michaelis-Menten (**Figure 27**) plots. The *Km* and *Vmax* values of purified lipase were found to be 4.75 mM and 999.5 µmol/min/mg, respectively.

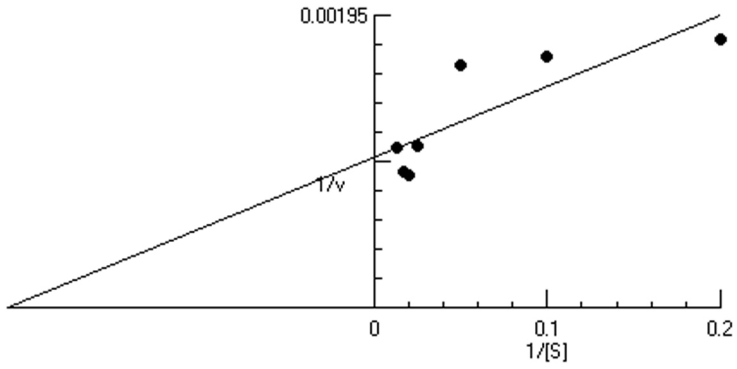


Figure 26. Lineweaver-Burk double reciprocal plot showing the kinetics of purified (sephadex column fraction) lipase.

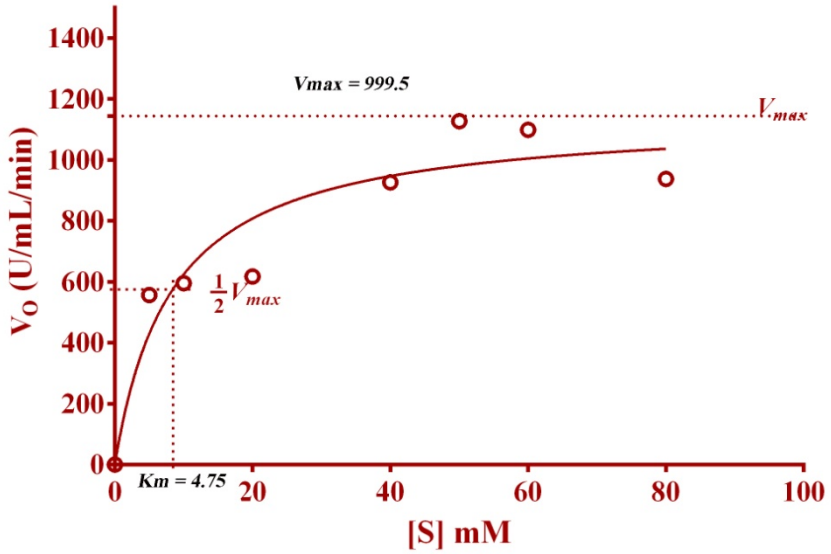


Figure 27. Michaelis Menton plot of purified lipase indicating its kinetic parameters (*Km* 4.75 mM and *Vmax* 999.5 µmol/min/mg).

Discussion

The prime objective of this study was to standardise the lipase production conditions for *P. aeruginosa* strainBUP2, isolated from the rumen of Malabari goat, and to study the characteristics of the lipase produced by it. The traditional classical methods of optimisation have some limitations, when the experiment deals with the combination effect of crucial parameters responsible for lipase production. Moreover *one-at-a time* strategy of the classical approach may often cause wrong selection of parameters in optimisation studies (Sim and Kamaruddin, 2008). Therefore, statistical optimisation methods are used to alleviate this problem. RSM is a combination of statistical and mathematical tools, mainly used for the optimisation studies of various biotechnological processes, in which linear interaction and quadratic effects of two or more parameters are estimated to produce three dimensional contour and surface plots (Burkert *et al*., 2004).

The statistical optimisation strategies - Plackett–Burman and Box–Behnken - were usually adopted for the optimisation of enzyme production (Abdel-Fattah *et al*., 2005; Hasan-Beikdashti *et al*., 2012); therefore, the same methodologies were employed in the present study. Plackett–Burman design with preliminary optimisation predicted the significant parameters for lipase production, and it reduced the number of parameters from five to three, followed by Box–Behnken design with the three relevant factors for fitting the second-order response surfaces. The R^2 value of 0.9 indicated the accuracy of the model. Similar strategy was applied for optimising the parameters for the production of lipase from various bacteria: *P. aeruginosa* (Bisht *et al*., 2012); *P. aeruginosa* PseA (Gaur and Khare, 2011); and *P. aeruginosa* sp. BUP6 MTCC 5925, a rumen bacterium (Priji *et al.*, 2014).

P. aeruginosa strain BUP2 produced 112 U/ml lipase under unoptimsed condition, while it was 172 U/ml under optimised condition (54 % increase); which is much higher than may reported values. Another bacterium (*Pseudomonas* sp. BPU6, a rumen isolate) reported from the same laboratory showed 31% (126 U/ml) increase in lipase production under optimised conditions over unoptimised condition (96 U/ml) (Priji *et al*., 2014). However, Bisht *et al*. (2012) reported 3142 U/ml activity for an alkaline (pH 8) lipase reported from *P. aeruginosa* MTCC-10055; however lipase from *P. aeruginosa* KM-110 showed only 0.76 U/ml activity (Mobarak-Qamsari *et al*., 2011).

Fractionation of crude protein by $(NH_4)_2SO_4$ precipitation, filtration and gel exclusion chromatography are generally used for the purification of lipase enzyme from various bacterial strains (Ghanem *et al*., 2000; Dharmsthiti and

Luchai, 1999). Extracellular lipase from *P. aeruginosa* strain BUP2 was purified (36 folds with 20 % yield) by $(NH_4)_2SO_4$ salt precipitation, dialysis (filtration) and sephadex G-100 column fractionation. Purification of lipase by filtration and sephadex-G100 gel permeation from *P. aeruginosa* increased the specific activity by 8.6 folds (Gaur *et al*., 2008). Palekar *et al*., (2000) purified lipase from *P. aeruginosa* EF2 by filtration and anion-exchange chromatography to 31 folds with 18 % yield showed; likewise, lipase from *P. aeruginosa* LST-03 was purified to 34.7 folds (12.6 yield) by ion exchange and hydrophobic interaction chromatography (Ogino *et al*., 2000).

From the SDS-PAGE profile, the estimated molecular weight of lipase from *P. aeruginosa* strain BUP2 was found as 29 kDa. From literature, the lipases from *Pseudomonas* spp. show a wide range ofMWs, 29 to 95 kDa(Gupta *et al*., 2004; Rahman *et al*., 2005; Karadzic *et al*., 2006). Of this, the MW of purified lipases from *P. aeruginosa* strain SRT9 and *P. aeruginosa* MB5001 were found as 29 kDa by SDS-PAGE (Chartrain *et al*., 1993; Borkar *et al*., 2009) which is comparable to the present report.

Using *p*NPP as substrate, different buffer systems with different pH ranges (5 to 10) were used. Of these, pH 8.0 was found to be efficient for the maximum lipase activity; of this pH 8.0 (at 45 ^{o}C) was found as optimum for *P. aeruginosa* strain BUP2, this alkaline nature offers its potentials in detergency. The *p*NPP is one of the most suitable and generally used substrates for lipase assay (Priji *et al*., 2014). Substrate specificity of lipases depends upon the diversity of the geometry and size of their active sites (Pleiss *et al*., 1998). Generally, lipases from *Pseudomonas* spp. prefer fatty acids triglycerides or their methyl esters with short or average chain length.

On the other hand, various Pseudomonads (*e.g*., *Pseudomonas* sp. S5, *P. alcaligenes* EF2, *P. alcaligenes* 24 and *P. cepacia*,) show preference towards longer chain fatty acids substrates (Pleiss *et al*., 1998; Gupta *et al*., 2004; Rahman *et al*., 2005), which is in conformity to that of *P. aeruginosa* strain BUP2. Temperature and pH optima for lipase are also crucial factors for lipase; because, which determine the commercial value. The lipase produced by *P. aeruginosa* PseA showed optimum pH and temperature as 8 and 40 ^{o}C, respectively (Gaur *et al*., 2008); likewise, Chakraborty and Paulraj (2009) also demonstrated that the lipase produced by *P. fluorescence* MTCC 2421 similar pH and temperature optima. In comparison, the lipase from *P. aeruginosa* strain BUP2 had the same pH optimum with slightly higher temperature preference (45 ^{o}C).

Numerous studies showed that the role of metal ions in stimulating or inhibiting the enzyme activity. Based on this view, effect of different metal ions on the activity of lipase from *P. aeruginosa* strainBUP2 was examined; of this, Ca^{2+} significantly enhanced the activity, and Mn^{2+}, Mg^{2+} and Fe^{2+} were found neutral, whereas Zn^{2+} and Cu^{2+} inhibited the activity. With lipase from *P. aeruginosa* PseA, Gaur *et al*. (2008) showed that the presence of metal ions (Ca^{2+}, Mg^{2+} or Fe^{3+}) in the reaction mixture stabilised or stimulated the activity; on other hand, Cu^{2+} and Zn^{2+} significantly inhibited the enzyme action. The inactivation of lipase action by Cu^{2+} and Zn^{2+} was reported for lipase from *Pseudomonas* sp. (Rahman *et al*., 2005; Kojima and Shimizu, 2003). Among various surfactants tested, triton X-100 was found stabilised the activity of *P. aeruginosa* strain BUP2 lipase, but tween 80 and SDS inhibited the activity. Anbu (2014) also reported that lipase production was stable by the presence of triton X-100. The strong inhibitory effects of tween

80 and SDS were probably due to their competitive binding to lipase with substrates (Peng *et al*., 2010; Borkar *et al*., 2009). The thiol mercaptoethanol and EDTA were found to inhibit the *P. aeruginosa* strainBUP2 lipase even at weak concentration, as shown by the lipase from *P. fluorescens* MTCC 2421 (Chakraborty and Paulraj, 2009) and *Burkholderia multivorans* V2 (Dandavate *et al*., 2009).

The specific activity of the *P. aeruginosa* strainBUP2 was 2392U/mg. In comparison, this value is much higher than those reported for the species of *Pseudomonas* and *Burkholderia*:it was 568 U/mg for *P. koreensis* BK-L07 lipase (Anbu, 2014); 313 U/mg for *P. aeruginosa* CS-2 lipase (Pneg *et al*., 2010); 143 U/mg *P. aeruginosa* PseA (Gaur *et al*., 2008); and 43 U/mg for *Burk. multivorans* V2 lipase (Dandavate *et al*., 2009).

The *Km* (4.75 mM, *i.e*., 4.75 M^{-3}) and *Vmax* (999.5 µmol/min/mg) values of the lipase produced by *P. aeruginosa* strainBUP2 were determined using *p*NPP as substrate. Lipases show wide range of *Km* value and mostof the industrially significant enzymes exhibit the *Km* in a range between 10^{-1} and 10^{-5} M (FulIbrook,1996). The maximum rate of reaction (*V*max) with low *Km* is considered as the affinity of an enzyme for its substrate. Gaur *et al*., (2008) reported that the *Km* value of lipase from *P. aeruginosa* PseA was 70.4 mM with *Vmax* of 2.24 mmol/min/mg against *p*NPP as substrate. The *Km* and *Vmax* of lipase from *P. cepacia* were 12 mM and 30 µmol/min with *p*NPP, respectively when *p*NPP was used as the substrate (Pencreach and Baratti, 1996). From this, it is evident that *P. aeruginosa* strainBUP2 is more efficient than many other lipases from *Pseudomonas* spp.

Conclusion

The alkalophilic and thermostable properties, coupled with stability of lipase from *P. aeruginosa* strain BUP2 in the presence of triton X-100 offer potentials for its use in industries, especially detergency. Compared to many bacterial lipases, 2392 U/mg activity of lipase from *P. aeruginosa* strain BUP2 is highly promising, when industrial applications are concerned.

Pseudomonas aeruginosa strain BUP2 produces type 2pyoverdine

Chapter 6
Pseudomonas aeruginosa strain BUP2 produces type 2 pyoverdine

Aim and Rationale

The specific aim of this study is to statistically optimise the parameters for the production of pyoverdine by *P. aeruginosa* strainBUP2 along with its purification and characterisation. Secretion pyoverdine is one of the common characteristic features of *P. aeruginosa,*when grown under iron limited environment. Reports revealed that fluorescent pyoverdines have some significant roles in the field of medical and environmental biotechnology.

Introduction

Siderophores are a group of iron mobilising molecules produced by certain bacteria, fungi and grasses grow under low iron stress; an adaptation to capture metal ions, especially iron to meet their nutritional and pathogenic requirements (Meyer *et al*., 2002). The siderophores are highly specialised metabolites evolved due to high evolutionary pressure on the microbes to cope up with the deficiency in their iron transport system or to invade the host resulting into pathogenesis. Bacillibactin (*Bacillus subtilis*), enterobactin (*E. coli*), vibriobactin (*Vibrio cholera*), pyoverdine (*Pseudomonas aeruginosa*) are some of the widely studied siderophores produced by bacteria (Saha *et al*., 2013). Fluorescent *Pseudomonads* are known for the characteristic production of yellow-green, water soluble pigments, the pseuobactins or pyoverdines (PVDs) (Visca *et al*., 2007). The peptide-derived PVDs comprise a heterogeneous group of flourescent siderophores with three structural

components: a constant dihydroxyquinoline chromophore conferring colour and fluorescence, an acyl moiety and a peptide moiety with variable number (6-12) of amino acids, including unusual ones (Wendenbaum *et al*., 1983). Studies revealed that PVDs have vital role in the field of environmental (used as a biosensor for Furazolidone detection) and medical biotechnology (preparation for the combination of PVD antibiotic conjugates) (Yin *et al*., 2014; Hennard *et al*., 2001). Therefore, the production, optimisation and characterisation of PVD from novel bacterial strains still continue as an active area of microbial research. The classical method for optimisation of growth factors has some drawback, when it deals with a combination effect of parameters. Statistical optimisation methods such as Response Surface Methodology (RSM) is employed by many authors for the standardisation of bacterial growth with a view to maximise the production of primary and secondary metabolites (Priji *et al.,* 2013). *P. aeruginosa* strainBUP2 is novel bacterium reported from the rumen of Malabari goat with the ability to produce siderophore (Unni *et al*., 2014). Thus, the specific aim of this study, is to apply the principles of RSM for optimising the growth conditions for the production of fluorescent pigment *P. aeruginosa* strainBUP2 in suitable medium and characterisation of the pigment produced.

Materials and Methods

Media

The BUP supplemented with pre-sterilised groundnut oil (1% V/V) or succinate (**Table 21**) medium was used in this study. The media was autoclaved (15 psi, 121 ^{o}C for 15 min) and inoculated with 0.1% (v/v) 12 h old seed cultureof *P. aeruginosa* srain BUP2 as inoculums was incubated in a rotary shaker at 37 ^{o}C and 150 rpm. The cell-free supernatant of succinate and BUP medium containing PVD was extracted with ethyl acetate at regular intervals of 6 and 24 h. Followed by OD value was carried out spectrophotometrically at λ_{404}.

Table 21. Composition of succinate medium

Ingredient	Quantity (g/l)
K_2HPO_4	6.0
KH_2PO_4	3.0
$MgSO_4.7H_2O$	0.2
$(NH_4)_2SO_4$	1.0
Succinic acid	4.0

Growth profile and Biomass

To monitor the bacterial growth, the turbidity (OD at λ_{600}) as well as wet weight of the culture was determined regularly at 6 h interval. The wet weight of the cell pellet was weighed out after centrifugation (8000 × *g* for 10 min).

Change in pH

The pH of medium (whole flask) was measured using a digital pH meter at 24 h interval.

Statistical optimisation of parameters for PVD production

The principles of RSM were used for the optimisation of physical parameters controlling the growth of *P. aeruginosa* srain BUP2 for the maximum production of PVD. Plackett Burman design (PB) was used for the initial screening of variables such as temperature, pH, substrate concentration, duration of incubation, agitation and inoculum size; subsequently, suitable factors were subjected to Box-Behnken design and RSM for finding out the optimum level of each variable.

Plackett-Burman design

Using this design six variables were screened in 22 experimental runs**(Table 22)**. Each variable was tested at three levels; *i.e*., low (-1), medium (0) and high (+1), and the range of variables was: substrate concentration (0.01, 0.55 and 1.0%), inoculum size (0.01, 0.55 and 1.0%), pH (4.0, 6.5 and 9.0), temperature (20, 30 and 40 ^{o}C), and duration of incubation (6.0, 39 and 72 h), agitation (50, 125 and 200 rpm). The experiment was performed in 100 ml conical flasks containing 20 ml medium, and responses were calculated by spectrometrically by frequently withdrawn samples.

Table 22. Plackett-Burman experimental design for the production of PVD from *P. aeruginosa* strainBUP2. The parameters selected were pH (4.0, 6.5 and 9.0), temperature (20, 30 and 40 ^{o}C), substrate Concentration (0.01, 0.55 and 1.0%), inoculum size (0.01, 0.55 and 1.0%), agitation (50, 125 and 200 rpm) and incubation time (6.0, 39 and 72 h).

Run order	Temperature (^{o}C)	pH	Substrate conc (%)	Incubation (h)	Agitation (rpm)	Inoculum Size (cfu/ml)
1	40	9.0	0.1	72	200	0.1
2	40	4.0	1.0	72	200	1.0
3	40	9.0	0.1	6.0	200	1.0
4	20	4.0	1.0	72	50	1.0
5	30	6.5	0.55	39	125	0.55
6	20	9.0	1.0	6.0	200	1.0
7	20	4.0	0.1	72	50	1.0
8	40	9.0	1.0	6.0	50	1.0
9	20	9.0	1.0	72	200	0.1
10	20	4.0	0.1	6.0	200	0.1
11	20	4.0	1.0	6.0	200	0.1
12	40	4.0	0.1	6.0	50	1.0
13	30	6.5	0.55	39	125	0.55
14	40	9.0	0.1	6.0	50	0.1
15	20	4.0	0.1	6.0	50	0.1
16	40	4.0	1.0	6.0	200	1.0
17	40	9.0	1.0	72	50	0.1
18	20	9.0	0.1	72	200	1.0
19	20	9.0	0.1	72	50	1.0
20	20	9.0	1.0	6.0	50	0.1
21	40	4.0	0.1	72	200	0.1
22	40	4.0	1.0	72	50	0.1

Box-Behnken model and response surface methodology

The significant factors optimised by the Placket-Burman design were selected for fitting into Box-Behnken desing**(Table 23)**. Optimisation of PVD production with 15 different combinations of experiments were carried out according to Box–Behnken design, followed by response surface analysis. Each variable was studied at three levels represented by low (-1) medium (0)

and high (+1). The second order polynomial equation applied for the analysis of the results:

$$Y = \beta 0 + \beta 1 X 1 + \beta 2 X 2 + \beta 3 X 3 + \beta 12 X 1 X 2 + \beta 23 X 2 X 3 + \beta 13 X 1 X 3 + \beta 11 X 12 + \beta 22 X 22 + \beta 33$$

Where: Y represents production of PVD, X_1, X_2, $X_3$are the independent variables, β_1, β_2, β_3 are the linear coefficients, β_{12}, β_{23}, $\beta_{13}$are the interaction coefficients and β_{11}, β_{22}, $\beta_{33}$are the quadratic coefficients. To confirm the validity of quadratic model, 3 experiments - as assigned by the point prediction software Minitab 14 - were performed. PVD production was calculated and compared with the predicted values**(Table 24)**.

Table 23: Box-Behnken design for the production of PVD from *P. aeruginosa* strain BUP2. The parameters selected were pH concentration (6.0, 7.5 and 9.0); temperature (28, 30, 32 ºC); incubation time of the medium (72, 96 and 120 h).

Run Order	pH	Temperature (ºC)	Incubation (h)
1	9.0	30	120
2	7.5	30	96
3	6.0	30	72
4	6.0	32	96
5	9.0	30	72
6	6.0	30	120
7	9.0	28	96
8	6.0	28	96
9	7.5	30	96
10	7.5	30	96
11	7.5	28	72
12	7.5	28	120
13	9.0	32	96
14	7.5	32	72
15	7.5	32	120

Table 24. Validation of experimental design for the production of PVD from *P. aeruginosa* strain BUP2.

Run order	pH	Temperature (°C)	Incubation (h)
1	6.5	28	120
2	7.5	30	72
3	7.5	28	120

Characterisation of purified PVD

Spectrophotometry

In order to check whether the siderophore produced by *P. aeruginosa* strain BUP2 was a PVD, the culture was centrifuged (8000 × g, 4 °C) to collect the supernatant from the cell pellet. Absorption spectrum of the culture supernatant was recorded at λ_{450} to λ_{350}.

Thin Layer Chromatography (TLC)

Purity of the pigments was tested by TLC in several solvent systems. Of which, *n*-butanol, acetic acid and water in a ratio of 3:1:1 was found good. For the detection of fluorescence emitted by the PVD, the TLC plates were air-dried and observed under UV-light at λ_{354} (Bhattacharya, 2010).

Siderosomes

The culture (4 d old) was examined under the fluorescent microscope with specific filter (Ex 425-445HQ, Em 460-510HQ, Dc 450, Nikon, Japan)

Isoelectric Focusing (IEF)

PVDs were analysed by IEF according to the method described earlier (Meyer, 2000), and the visualised bands were compared with those from the representative strains of each PVD type, *viz*., *P. aeruginosa strain* O1 (Pa O1), *P. aeruginosa strain* ATCC 27853 (*Pa* ATCC 27853) and *P. aeruginosa* strain 6 (*Pa* 6).

Purification of pigment and quantification

After 5 d incubation, the culture in BUP medium was centrifuged (8000 × *g*, 4 ^{o}C) to separate the supernatant from the cell pellet. Subsequently, the supernatant was acidified to pH 6 using 12 M HCl; then the supernatant was run through an XAD-4 Amberlite column (15 × 2.5 cm), which retained the PVDs (Meyer *et al*., 1998). After washing with 500 ml ddH_2O, the PVD was eluted with 250 ml 50% methanol, the eluent was vacuum dried and lyophilised. The dried pigment was weighed for quantification.

Results

Growth characteristics of *P. aeruginosa* strain BUP2 in BUP medium

Bacterial growth in BUP medium was monitored spectrometrically, the isolate showed characteristic sigmoid pattern of growth **Figure 28 (A)**. The culture showed exponential growth after 12 h of incubation and reached the plateau by 36 h in the BUP medium, after reaching the maximum at 30 h. Wet weight of the biomass was also in line with the growth pattern mentioned in **Figure 28 (B)**

Change in pH

The pH profile of the culture showed a characteristic increase from initial 6.7 to 7.8, followed by a decrease after 2 d of incubation; this declining pH was associated with the increased secretion of pigment in the medium **Figure 28 (C)**.

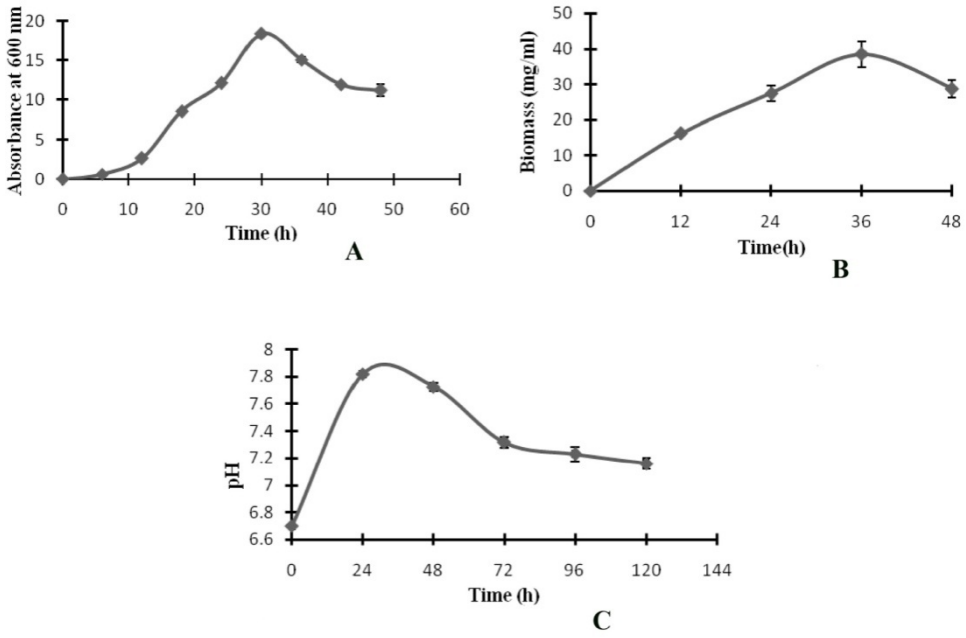


Figure 28. Growth characteristics of *P. aeruginosa* strain BUP2 in BUP medium**. (A)** Growth curve, absorbance at λ_{600}; **(B)** Wet weight of the culture pellet obtained after centrifuging at 8000 × *g* for 10 min; and **(C)** pH change in culture during incubation.

Comparision of PVD production in BUP-groundnut oil andsuccinate media

PVD production was monitored in BUP medium for 5 d with 24h interval. **Figure 29** depicts the pigment production profile of *P. aeruginosa* strain BUP2 in BUP-groundnut oil, as recorded from the absorbance at λ_{404}. The maximum pigment production was observed in the medium on 4 d of incubation with an OD of 1.95; which gradually declined to 1.89 after 5 d.

Likewise, production of PVD in iron limited succinate medium showed that the maximum pigment production was reached in the medium at12 h of incubation with an OD of 1.22, thereafter the production was decreased to 1.01 (**Figure 29 A and B**).

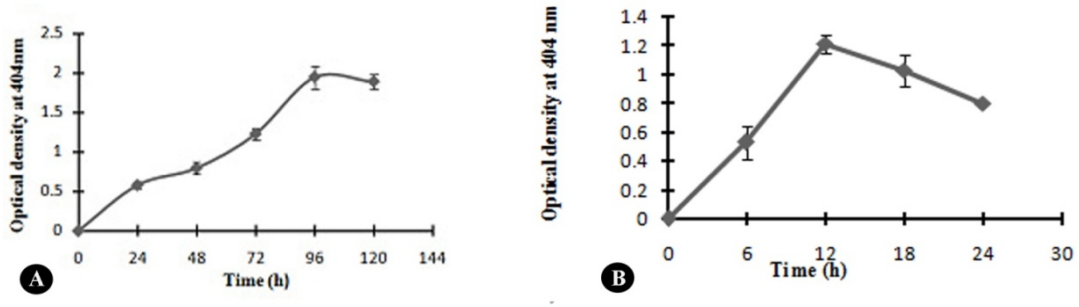


Figure 29. Pigment production profile (OD at λ_{404}) of the *P. aeruginosa* strain BUP2. **(A)** At 24 h intervals, the culture supernatant was monitored for PVD production in BUP medium. **(B)** PVD production in iron limited succinate medium shows that the maximum production of PVD was reached in the medium at 12 h of incubation.

Optimisation of PVD production by Response Surface Methodology (RSM)

RSM was used to optimise the cultural conditions for the PVD production from *P. aeruginosa* strain BUP2. To find the interactive effects of three variables (pH, temperature and incubation time), modelling and statistical analyses were performed using the software MiniTab, version 14. Initially fractional factorial design with Plackett-Burman design was used to reduce the number of runs to a manageable size and to study parameters with main effect **(Table 25)**. Six parameters (temperature, pH, substrate concentration, incubation time, agitation and inoculum size) were included under the trial, and Plackett- Burman analysis was done to find out the effect estimate

(**Figure 30**). From this; pH, temperature and incubation time were selected for further validation and other parameters were fixed.

Table 25. Result of Plackett-Burman experimental design showing pigment production by *P. aeruginosa* strain BUP2.

Run order	Temper-ature(ºC)	pH	Substrate conc (%)	Incuba-tion (h)	Agita-tion(rpm)	Inoculum Size (cfu/ml)	Pigment (Absorbance)
1	40	9.0	0.1	72	200	0.1	0.432
2	40	4.0	1.0	72	200	1.0	0.106
3	40	9.0	0.1	6.0	200	1.0	0.012
4	20	4.0	1.0	72	50	1.0	0.017
5	30	6.5	0.55	39	125	0.55	0.355
6	20	9.0	1.0	6.0	200	1.0	0.036
7	20	4.0	0.1	72	50	1.0	0.0135
8	40	9.0	1.0	6.0	50	1.0	0.099
9	20	9.0	1.0	72	200	0.1	0.143
10	20	4.0	0.1	6.0	200	0.1	0.0165
11	20	4.0	1.0	6.0	200	0.1	0.0185
12	40	4.0	0.1	6.0	50	1.0	0.007
13	30	6.5	0.55	39	125	0.55	0.335
14	40	9.0	0.1	6.0	50	0.1	0.06
15	20	4.0	0.1	6.0	50	0.1	0.007
16	40	4.0	1.0	6.0	200	1.0	0.012
17	40	9.0	1.0	72	50	0.1	0.238
18	20	9.0	0.1	72	200	1.0	0.125
19	20	9.0	0.1	72	50	1.0	0.0715
20	20	9.0	1.0	6.0	50	0.1	0.005
21	40	4.0	0.1	72	200	0.1	0.095
22	40	4.0	1.0	72	50	0.1	0.073

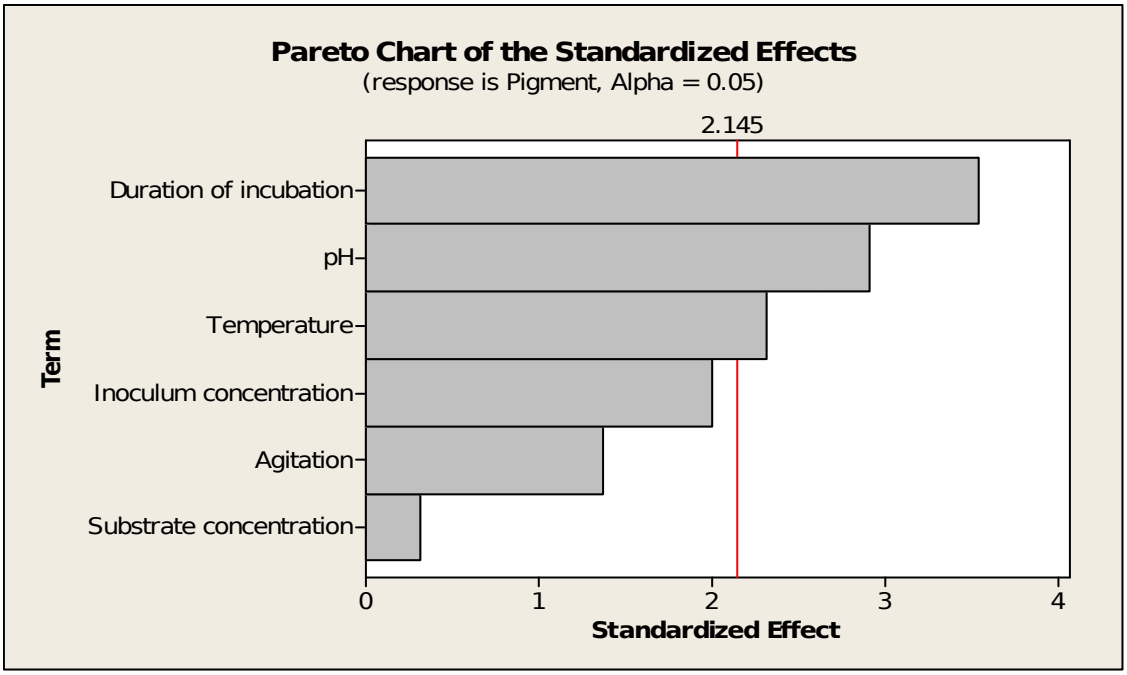


Figure 30. Effect estimates from Plackett-Burman experimental trials: The parameters like, incubation time, pH, temperature showed positive effects, while inoculum size, agitation, substrate concentration showed negative effects on the production of PVD.

Box-Behnken Design

Box-Behnken design was used to study the regression analysis to fit the response function with the experimental data, since all the interactions fall within the operational zone(**Table 26**).

Table 26. Results of Box-Behnken design for the production of PVD by *P.aeruginosa* strain BUP2.

Run order	pH	Temperature (°C)	Incubation (h)	Pigment (absorbance)
1	9.0	30	120	1.083
2	7.5	30	96	1.4385
3	6.0	30	72	0.602
4	6.0	32	96	0.458
5	9.0	30	72	0.962
6	6.0	30	120	0.799
7	9.0	28	96	1.512
8	6.0	28	96	0.979
9	7.5	30	96	1.743
10	7.5	30	96	1.5045
11	7.5	28	72	1.974
12	7.5	28	120	1.634
13	9.0	32	96	0.753
14	7.5	32	72	1.122
15	7.5	32	120	1.255

Response Surface Regression

The regression model for PVD production was highly significant (p<0.012) with a satisfactory value of determination coefficient (R^2 = 0.95) **(Table 27)**. Contour plots and response surface plot for the interactions of temperature and pH; temperature and incubation time; and pH and incubation time on PVD production were constructed **(Figure 31 A-F)**.

The regression equation obtained for the production of PVD production is:

$$Y= 1.2+ 0.184X_1 –0.47X_2+ 0.17X_3 –0.06X_1X_2 –0.19X_2X_3 –0.02X_1X_3 –0.79X_1^2 + 0.152X_2^2 -$$

Where X_1, X_2, X_3 are pH, temperature, incubation time respectively

Table 27: ANOVA for the production of production PVD, from Box-Behnken analysis

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	4.91975	4.91975	0.54664	9.48	0.012
Linear	3	2.23487	2.23487	0.74496	12.92	0.009
Square	3	2.52903	2.52903	0.84301	14.62	0.007
Interaction	3	0.15586	0.15586	0.05195	0.90	0.502
Lack of Fit	3	0.23695	0.23695	0.07898	3.08	0.255

Validation

As per the model, the optimum conditions for PVD production were 28 ^{o}C, pH 6.5 and incubation time 120 h with a the maximum OD value of 2.5. The correlation coefficient of the result was 0.93, which was in good agreement with the predicted and experimental values **(Table 28)**.

Table 28. Validation results for the production of pigment by *P. aeruginosa* strain BUP2. The correlation coefficient between the experimental and predicted values was 0.93.

Run order	pH	Temperature (^{o}C)	Incubation (h)	PVDobserved	PVDpredicted
1	6.5	28	120	2.5	2.12
2	7.5	30	72	1.1	1.816
3	7.5	28	120	1.9	1.914

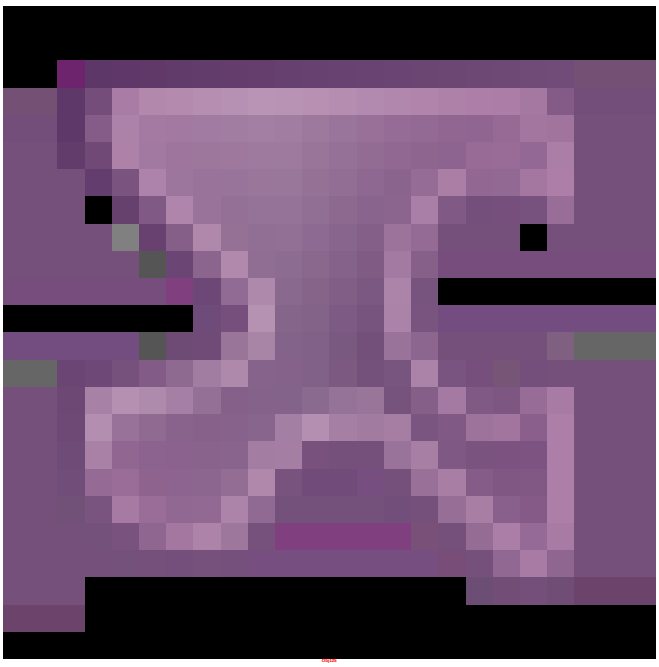


Figure 31. **(A)**Contour plot showing pigment production *vs.* incubation time (h) and temperature. Here pH 7.5 was kept constant.

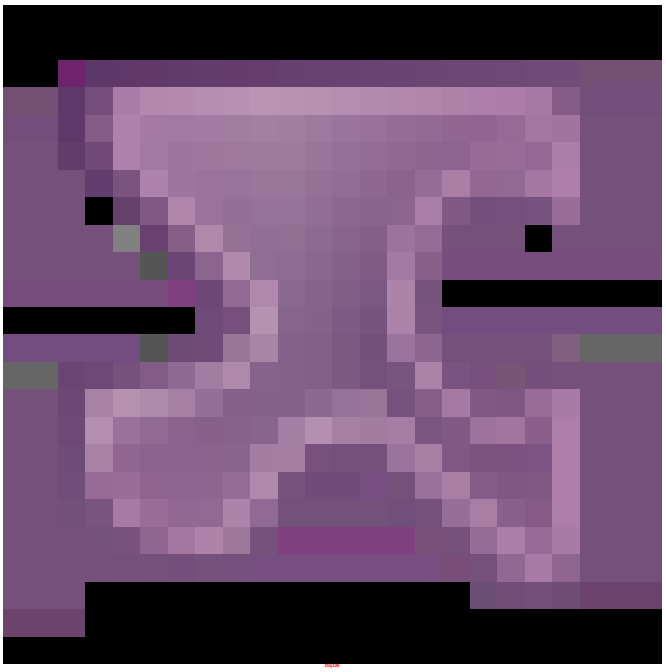


Figure 31. **(B)**Contour plot showing pigment production *vs.* incubation time (h) and pH. Here temperature (30 ºC) was kept constant**.**

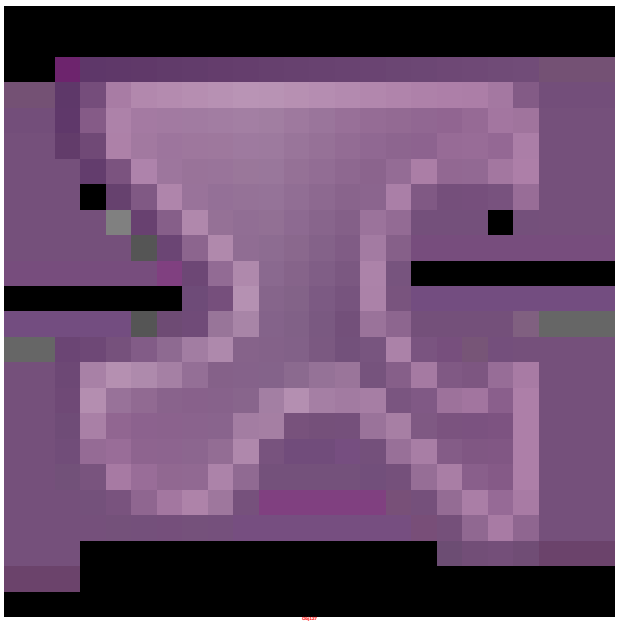


Figure 31.**(C)**Contour plot showing pigment production *vs.* temperature and pH under constant incubation time (96 h)**.**

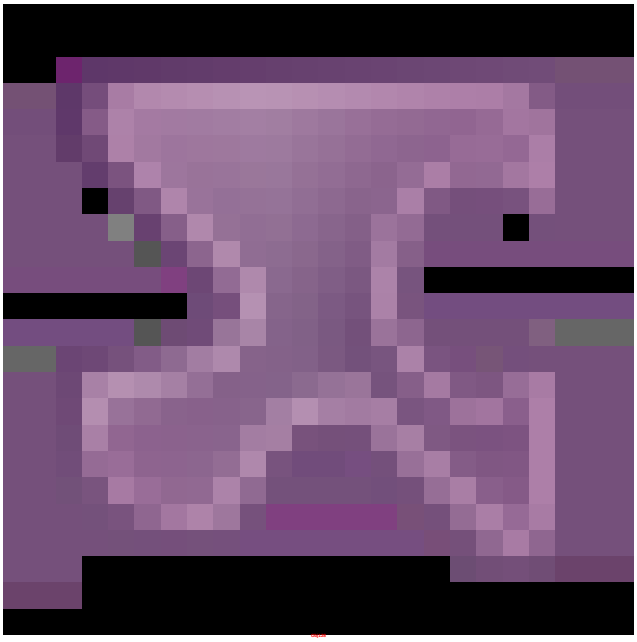


Figure 31.**(D).** Surface plot showing pigment production *vs.* incubation time (h) and temperature. Here pH 7.5 was kept constant.

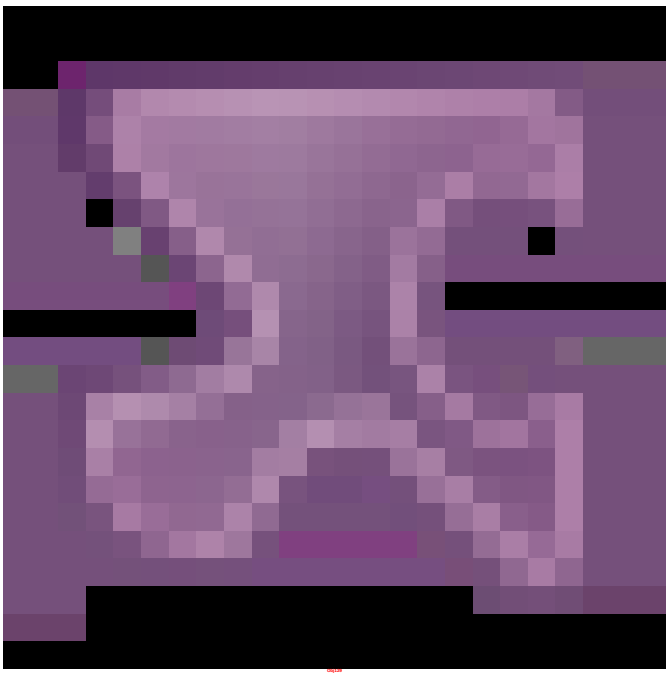


Figure 31.(E) Surface plot showing pigment production *vs.* incubation time (h) and pH. Here temperature value (30 °C) was kept constant**.**

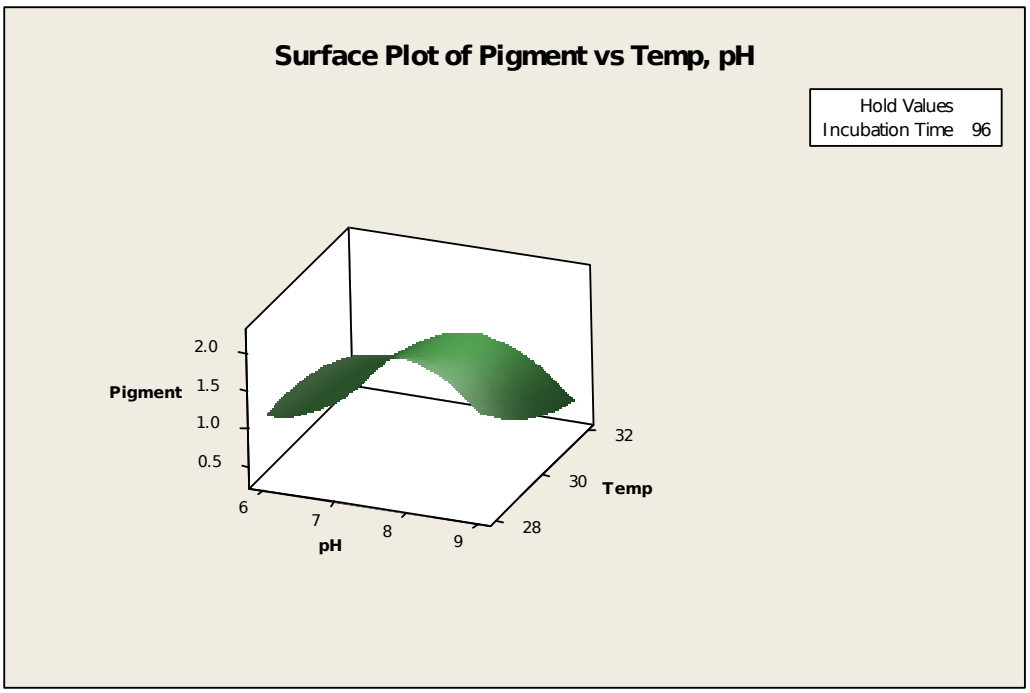


Figure 31.(F) Surface plot showing pigmernt production *vs.* temperature and pH. Here incubation time (96 h) was kept constant.

Spectrophotometry

The preliminary indication of the possible identity of the pigment was generally provided by its absorption spectrum. Upon scanning, the absorption maximum for the PVD was obtained at λ_{404}**Figure 32**. Thus, the characteristic peak obtained at λ_{404} was of PVD (Elliott, 1958)

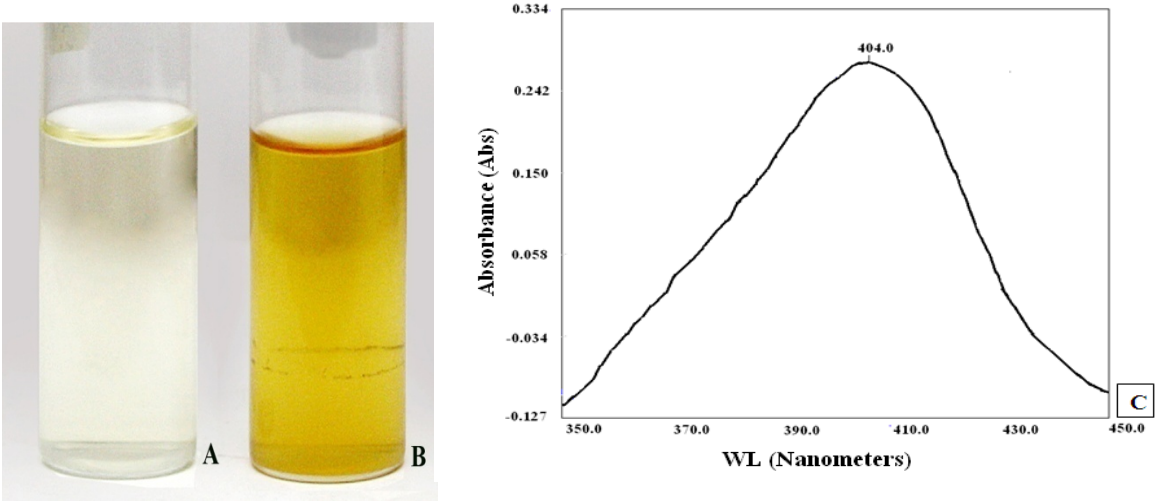


Figure 32. Characterisation of the pigment. **(A)** Control for pigment extraction; **(B)** Extracted yellow-green pigment in ethyl acetate; and **(C)** Characterisatic spectrum of on spectrophotometry. The culture supernatant obtained after centrifuging at 8000 × *g* and 4 °C was monitored for its absorption spectrum from λ_{350} to λ_{450}. The maximum absorption at λ_{404} indicates that the pigment is of PVD.

Thin Layer Chromatography (TLC)

Identification of the siderophore type was performed on TLC. Profile of extracted pigment on silica gel G250 chromatogram showed a single spot with an $R_f$of 0.8 in butanol: acetic acid: water (3:1:1) solvent system. As shown in **Figure 33**, the spot was visible as yellow-brown in visible light and bright orange upon UV (λ_{354} nm) irradiation, which is the characteristic colour property of PVD (Bhattacharya, 2010)

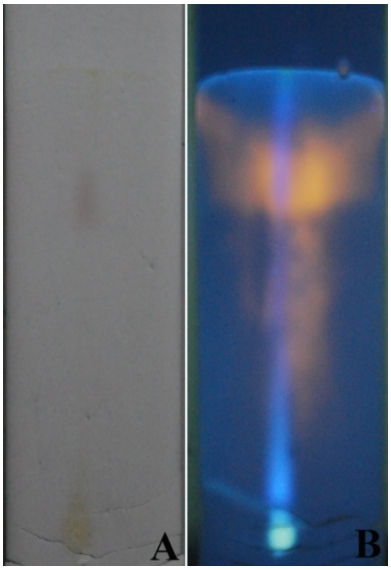


Figure 33.Identification of the type of siderophore by TLC. **(A)**Separation of extracted pigment on silica gel G250 chromatogram showing a single yellow-brown spot R_f of 0.8 in visible light; and **(B)** Purified siderophore spot visible as bright orange upon UV (λ_{354}) irradiation.

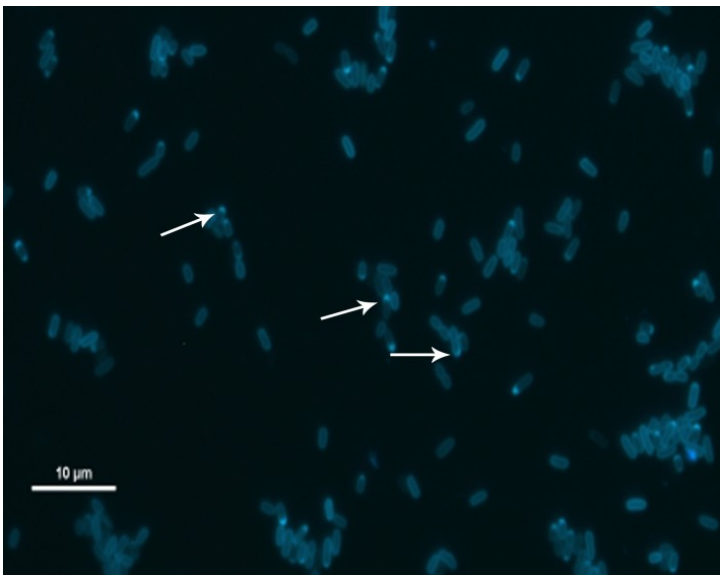


Figure 34. Identification of bacterial siderosomes by fluorescent microscope.

Siderosomes

Conspicuous sidersomes were observed as blue fluorescent granules in the terminal region of the bacterial cells **(Figure 34)**.

Iso-Electric Focusing (IEF)

The IEF analysis was performed for judging the type of PVD produced by *P. aeruginosa* strain BUP2. As depicted in **Figure 35**, the PVD produced by *P. aeruginosa* strain BUP2 was compared with that of 3 known type strains of *P. aeruginosa*, *viz*., *Pa* O1, ATCC 27853 and *Pa*6. From IEF profile, the PVD produced by *P. aeruginosa* strain BUP2 is confirmed as type 2 PVD (Meyer *et al.,* 1997).

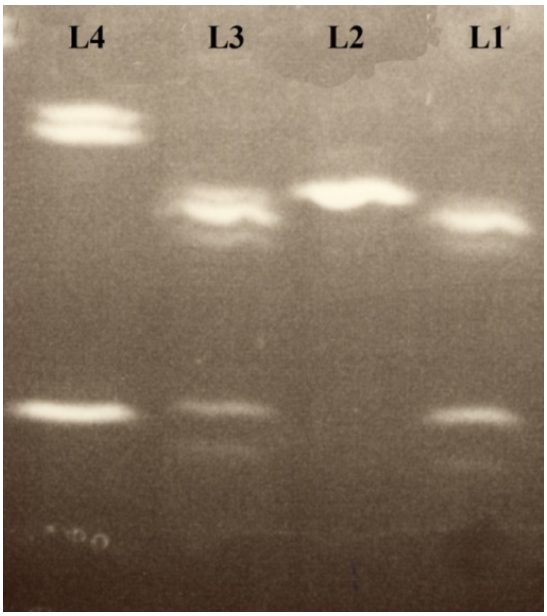


Figure 35. Characterisation of PVD by IEF. PVD produced by *P. aeruginosa* strain BUP2 (Lane 1) was compared with that of 3 known type strain , *viz.*, *Pa*O1 *(Lane 4,* type 1*),Pa* ATCC 27853 (Lane 3, type 2) and *Pa* 6 (Lane 2, type 3), indicating its close similarity to type 2.

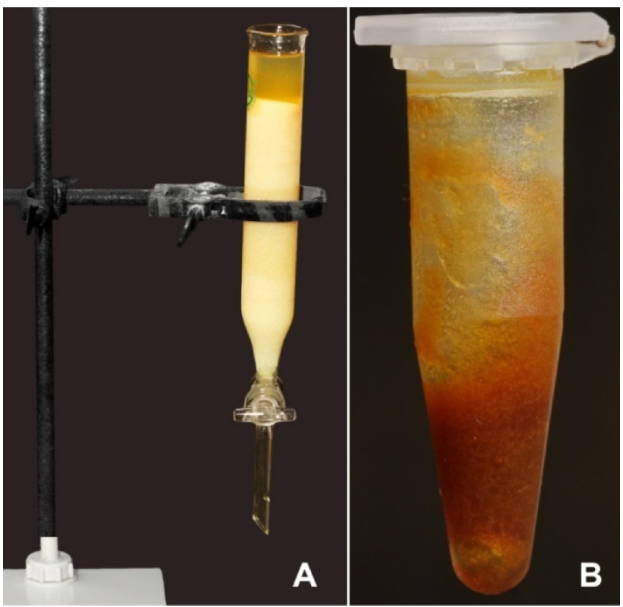


Figure 36.Purification and quantification PVD. **(A)** Concentrated supernatant (pigment) on XAD-4 Amberlite column and **(B)** Purified and dried PVD in eppendorf tube

Purification and Quantification of PVD

PVD production was monitored for 5 d with 24 h interval. **Figure 36** depicts the pigment production profile of *P. aeruginosa* strain BUP2, as recorded from the absorbance at λ_{404}. The maximum pigment production was observed in the medium on 5 d of incubation with an OD of 2.5. It was at this higher OD (2.5), concomitant production of the maximum pigment (dry weight 23.0 mg/ml) was obtained from the culture supernatant in the BUP-groundnut oil medium (**Figure 36**).

Discussion

P. aeruginosa is often seen "growing in distilled water", an evidence for its minimum nutritional requirements (Todar, 2006). *Psuedomonas* spp. were generally cultured in King B medium (King *et al*., 1954), and various other synthetic media (Albesa *et al.*, 1989; Moore *et al.*, 1994) for growth and pigment production. Two media (BUP-groundnut oil and succinate media) were tested for their efficacy in the production of pigment by *P. aeruginosa* strain BUP2. *P. aeruginosa* strain BUP2 was incapable of secreting PVD in the absence of groundnut oil in the semi-synthetic BUP medium. PVD production in succinate medium exhibited PVD production at much earlier,

but with much less yield, *i.e.*, about 50 % less production than in BUP-groundnut oil medium. Therefore, the PVD production conditions were standardised in BUP medium-groundnut oil medium.

The newly designed medium (BUP medium) made the culture well adapted for growth and PVD production, even after drastic change of its ecological niche from the rumen environment (anaerobic state) to artificial liquid conditions (aerobic state). This medium was essential, as the bacterium needs to be tuned to aerobic growth from anaerobic environment, *i.e.*, facultative aerobe. *P. aeruginosa* strain BUP2 showed the characteristic sigmoid growth curve in BUP-groundnut oil medium, and secreted a yellow-green pigment in BUP medium during its late s phase (96 h) of growth (idiophase), which made the culture medium clear and transparent. The pH profile of the culture showed a characteristic increase from 6.7 to 7.9 initially, followed by a decline after 4 d of incubation; this may be due the slightly alkaline nature of the pigment as described by Elliott (1958).

Plackett-Burman design was effectively applied to diminish the number of runs to a convenient manner; and thus, to study the influential growth parameters in detail. Temperature, pH, substrate concentration (groundnut oil), inoculum size, agitation and incubation time were included in this preliminary experimental design. From the results, temperature, pH and incubation time were selected for further validation and other parameters (substrate concentration, inoculum size and agitation) were made constant. Box-Behnken design was applied to analyse the properties of selected input variables (such as temperature, pH and incubation time); the key factors affecting the production of PVD from *P. aeruginosa* strain BUP2. Thus, the statistical optimisation resulted in enhanced production (30%) of PVD to

compared to the unoptimised condition. The regression model for PVD production was highly significant (p<0.012) with a satisfactory value of determination coefficient (R^2 = 0.95). As illustrated in the contour and three dimensional response surface plots, the optimum conditions such as temperature, pH and incubation time were confined within the design boundary; thus well fit to the model. The validation data were statistically analysed so as to correlate the observed and predicted OD values obtained for the production of PVD.

The pigment was monitored both qualitatively and quantitatively at regular intervals during the growth of *P. aeruginosa* strain BUP2. Strains of *P. aeruginosa* are known to produce a variety of pigments such as pyorubrin, pyomelanin, pyocyanin, PVDs, *etc*., with colour variations from blue to brown (Ogunnariwo *et al.,* 1975). Thus, it was mandatory to confirm the nature of the pigment produced by *P. aeruginosa* strain BUP2. *P. aeruginosa*possesses several virulence factors, one of which is PVD, a yellow-green primary siderophore that steals iron from the host to facilitate its growth; thereby causing iron depletion in the host as reflected through various diseases (Meyer *et al.,* 1996). The absorption spectrum of the extracted siderophore showed a characterestic peak of PVD with absorption maximum at λ_{404}. The peak at λ_{404} is of a typical PVD (Elliott, 1958; Manwar *et al.,* 2004). TLC was used to check the purity of the sample. Single spot obtained on TLC with orange fluorescence upon UV irradiation further illustrated the production of PVD (Bhattacharya, 2010). Subsequently, the subcategory (type) of PVD produced by *P. aeruginosa* strainBUP2 needs to be confirmed. Siderotyping is the most efficient and effective method for the characterisation of novel PVDs. Strains producing structurally different PVDs

are immediately distinguished from each other from typical PVD-IEF patterns. Three distinct PVD types are known to produce by the strains of *P. aeruginosa, viz.*, PVD 1, PVD 2 and PVD 3, which are distinguishable from their peptides at molecular level and IEF patterns (Meyer *et al*., 1997). Generally, PVDs of strains 9 AW, *Pa* 15692, 62.5, 62.6, 71.20, 77.10 and 9 BW are included in group 1, PVDs of strains *Pa* 27853, 63.52, 72.26, 76.110, l W, 1 OCW and *P.fluorescens* ATCC 13525 in group 2, and the group 3 is for the PVDs of strain 51 W and 77.11; all these subtypes can clearly be distinguished on IEF pattern (Meyer *et al*., 1997). The PVD produced by *P. aeruginosa* strain BUP2 was compared with that of three type *P. aeruginosa* strains, *i.e*., *Pa* O1, ATCC 27853 and *Pa*6, the representatives of type 1, type 2 and type 3, respectively. The pattern of bands obtained on IEF for *P. aeruginosa* strain BUP2 clearly corroborates with that of PVD 2. Thus, it is concluded that the PVD produced by *P. aeruginosa* strain BUP2 belongs to type 2 PVD.

Next objective of this study was to quantify the pigment. The column fraction (on XAD-4 Amberlite) of extracted pigment was evaporated to dryness and weighed (Meyer *et al*., 1998). Thus, pure PVD type 2 produced by *P. aeruginosa* strain BUP2 was quantified.

Conclusion

P. aeruginosa strain BUP2 isolated from the rumen of Malabari goat is a novel fluorescent pseudomonad producing type 2 PVD. In order to transform this anaerobe in to a facultative aerobe, a new medium was designed; which enabled easy handling of this bacterium for maximising the PVD production. Moreover, the conspicuous nature of the siderosome produced by *P.*

aeruginosa strain BUP2 is very special to this bacterium. The enhanced production of PVD in the BUP-groundnut oil medium is significant in terms of commercial production, especially when its ferritin like applications is concerned.

Type 2 pyoverdine as *turn-off* biosensor for the rapid detection of iron and copper in contaminated water

Chapter 7

Type 2 pyoverdine as *turn-off* biosensor for the rapid detection of iron and copper in contaminated water

Aim and Rationale

To utilise the fluorescent and metal chelating properties of pyoverdine for the detection of heavy metal contamination in drinking water resources. Heavy metal contamination in drinking water is one of the serious health issues to addressed properly, especially in industrialised regions over the globe. The available methods for the detection of heavy metals are very tedious, costly and also it is insufficient for the rapid analysis of heavy metal contamination in water, especially iron and copper. It is proposed that pyoverdine based *turn-off* biosensor can successfully address these issues.

Introduction

Now-a-days, heavy metal contamination in fresh water resources is one of the major problems, especially in industrialised regions over the world**.** Most of the industrial effluents carry substantial quantity of heavy metals such as iron, copper, zinc, lead, cobalt, cadmium, mercury, manganese and nickel (Singh and Singh Chandal, 2006; Ramola and Singh, 2013). Due to easy availability, removal difficulties and lack of fresh water, the effluents from industries or municipal waste water are widely used for the irrigation purpose (Muchuweti *et al*., 2006)**.** The heavy metals in these improperly treated effluents gradually leach out into the fresh water sources, which ultimately reach in humans. Considerable amount of heavy metals - especially iron and copper - in humans would impact physiological and biochemical functions. Iron-overload

diseases in humans include: hepatitis, hemochromatosis, liver cirrhosis, cancer, and neurodegeneration (Molina-Holgadoand *et al*., 2007); and toxicity of copper is associated with cellular homeostasis, Wilson diseases (Waggoner *et al*., 1999; Bull *et al*., 1993) familial amyotropic lateral sclerosis (Bruijn *et al*., 2004), Alzheimer's disease (Barnham *et al*., 2004), prion diseases (Brown and Kozlowski, 2004), *etc.*

Owing to the adverse effects of these heavy metals, the early stage of their detection in drinking water has much significance, especially in industrialised and thickly populated areas. Various methods are in vogue for the detection of heavy metal contamination in water, for which highly sensitive equipments like atomic absorption spectroscopy**,**inductively coupled plasma mass and atomic emission spectroscopies, *etc.*are used (Aragay *et al*., 2011).Laser-induced breakdown spectroscopy for the detection of various metal like Cr^{3+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} in tap water samples (Zhao *et al*., 2010), and differential surface plasmon resonance with anodic stripping voltammetry capability for detecting heavy metal ions in water (Wang *et al*., 2007), *etc*. have been demonstrated. Apart from these methods, techniques like DNA based on-site (*in situ*) determination of Hg^{2+} detection system (Long *et al*., 2013), protein (Bontidean *et al*., 1998), cell- and protein-based dual (Corbisier *et al*., 1999), phytochelatin (Bontidean *et al*., 2003), conductometric (Chouteau *et al*., 2005) or immobilised enzymes (Malitesta and Guascito, 2005) are also in use.

Pyoverdine (PVD) is a yellowish-green, water soluble fluorescent siderophore, predominantly produced by the species of *Pseudomonas,* and *P. aeruginosa* in particular (Unni *et al*., 2014). Three types of pyoverdines are

reported from *P. aeruginosa* so far, *i.e*., PVD 1, PVD 2 and PVD 3, and each one could be distinguished from the rest based on their peptides at molecular level, and of isoelectric focusing patterns on the gel (Meyer *et al*., 1997; Unni *et al*., 2014). PVD is considered as a strong iron chelator, and also it acts as a signaling molecule for the activation of virulent gene in the pathogenic strains of bacteria, so as to steal ions of iron from the host (Lamont *et al*., 2002). Secretion of PVD is one of the characteristic features of fluorescent pseudomonads during their growth in iron-depleted medium. The structural part of PVD comprised of a chromophore, a variable peptide chain, and an acyl moiety. The chromophore is responsible for imparting color and fluorescence to the PVD (Meyer *et al*., 1998).

The available techniques are not sufficient for the rapid screening of heavy metal contamination in water at low cost. PVD based simple biosensor may help rapid screening of heavy metal contamination in water; therefore, development of anew and inexpensive method for the identification of bio-available heavy metal would be advantageous. The present study envisages to exploring the possibility of type 2 PVD purified from *P. aeruginosa* BUP2 with a focus on: (a) to study the specificity of type 2 PVD with various metal ions; (b) to study the level of detection (LoD) of various metal ions by fluorescence quenching mechanism; and (c) to explore the utility of type 2 PVD for the detection of metal ions from the contaminated water samples.

Materials and methods

Bacterium used

Pseudomonas aeruginosa strain BUP2 described recently from this laboratory was used for the study (Unni *et al*., 2014).

Medium and Chemicals

The synthetic BUP medium (Unni*et al*., 2014) used for the cultivation of *P. aeruginosa* BUP2. Heavy metal ions such as Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Hg^{2+}, Mn^{2+} and Pb^{2+}in the chloride or sulphate form were purchased from Himedia (India). Stock solutions were separately prepared by dissolving each metal salt in sterile double distilled water (ddH_2O).

Production and purification of PVD

P.aeruginosa BUP2 was incubated (5 d) in 250 ml conical flask containing 100 ml of BUP medium (pH 6.7, 37 ^{o}C, at 150 rpm). After 5 d incubation, the culture in BUP medium was centrifuged (8000 × *g*) to collect the supernatant from the cell pellet. The type 2 PVD was purified from the supernatant as previously described (Unni *et al*., 2014).

Specificity of fluorescent biosensor for heavy metal detection

To determine the specificity of type 2 PVD toward heavy metal ions, salt solution of Fe^{3+}, Fe^{2+}, Cu^{2+} as well as Zn^{2+}, Co^{2+}, Cd^{2+}, Hg^{2+}, Mn^{2+} or Pb^{2+} (100 µM) was separately mixed with PVD solution in ddH_2O. The molar concentration of the metal ions was calculated from their respective salt form (sulphate or chloride, excluding water in the crystals).The fluorescence quenching of PVD was monitored using UV trans-illuminator (Biotech R&D Laboratories, India), and quantified by spectrofluorimeter with xenon lamp and 0.5cm quartz cells (PerkinElmer LS-45, Massachusetts, USA).

Analysis of ferric, ferrous and copper ions by using fluorescence quenching method

Different concentration of ferric (Fe^{3+}), ferrous (Fe^{2+}) and copper (Cu^{2+}) ions was individually mixed with purified PVD in ddH_2O to detect whether it could quench the fluorescence intensity emitted by PVD. The purified PVD in ddH_2O without any heavy metal was used as control. For rapid screening, the fluorescence of each solution was detected by a UV trans-illuminator, and quantified/confirmed by spectrofluorimeter.

Fourier-transform infrared spectroscopy (FT-IR)

The air-dried PVD metals complex (Iron and Copper) was pelleted with potassium bromide (KBr), and analysed using FT-IR. The relative intensity of transmitted light was measured against the wave length of absorption in the region of 400-4000/cm.

The reported structure of PVD (Wendenbaum *et al*., 1983) was reproduced using Chem Bio Draw Ultra 12.0 and the binding of the PVD with different metals are derived out of spectroscopic analysis using the same software.

Detection of the sensitivity of fluorescent biosensor

To detect the linear range and LoD by type 2 PVD, different concentrations of Fe^{3+}, Fe^{2+} and Cu^{2+} stock (10 mM) were separately added to the PVD solution. The fluorescence intensity of each sample was measured by the UV trans-illuminator and spectrofluorimeter. The fluorescent quenching efficiency was calculated by the formula, (1-α)/100%; where, α represents the fluorescence intensities upon nonbinding or binding of metal ions to the PVD (Lakowicz, 1999).

Role of temperature and duration of incubations on fluorescence quenching

To observe the role of temperature in fluorescence quenching, pyoverdine solution was mixed with Fe^{3+}, Fe^{2+}or Cu^{2+} (10 µM) at 4, 10, 20, 25, 30, 37and 50^{o}C, and also incubated for different time (10 sec, 20 sec, 50 sec, 1 min, 10 min, 20 min, 30 min, 1 h, 12 h and 24 h).

Detection of heavy metals in contaminated water sample

The drinking water samples were collected from the open wells situated near China clay industry (Madai, Kannur District of Kerala State; 11.8689 ^{o}N; 75.35546 ^{o}E), and copper sulphate manufacturing company (Aluvai, Ernakulum District of KeralaState; 10.00 ^{o}N; 76.33 ^{o}E). The former site is known for Fe contamination, and the latter is known for Cu contamination. To remove particulate matter, the samples were filtered through Whatman filter paper membrane. Both the samples were incubated with PVD solution (50 sec), and fluorescence quenching was monitored using UV trans-illuminator. To confirm the presence of heavy metal ions, the water sample was analysed using atomic absorption spectroscopy (Thermo-make M-650780, USA).

Statistics

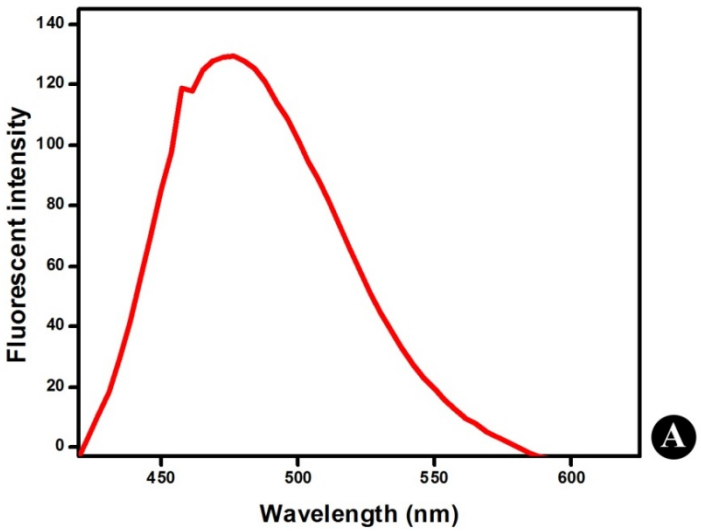
All studies were repeated at least thrice, and an average of 3 values is presented with standard deviation. Microsoft Excel was used to draw the figures and SPSS version 20 was used for calculating the LoD.

Results

The type 2 PVD produced by *P. aeruginosa* BUP2, a novel bacterium described from this laboratory was used for the whole study (Unni *et al*., 2014). The type 2 PVD was purified to homogeniety in the powder from, which was resuspended in double distilled water for various assays. For the rapid screening studies using UV trans-illuminator, visual observations were made, which was reconfirmed by spectrometry. During the screening studies (for checking quenching efficiency), effect of temperature and incubation time were also checked (data not shown), and the optimum temperature and incubation period were worked out as 25 ^{o}C and 50 sec. Further studies were undertaken at this optimum condition.

Quenching efficiency of various metal ions

The fluorescence peak of type 2 PVD was observed at λ_{460}**(Figure 37A)**. Different metals ions (Fe^{3+}, Fe^{2+}, Cu^{2+},Zn^{2+}, Co^{2+}, Cd^{2+}, Hg^{2+}, Mn^{2+} and Pb^{2+}) were tested for their quenching efficiency and out of the nine metal ions studied, only 3 ions, *i.e*., Fe^{3+}, $Fe^{2+}$and Cu^{2+} were found sensitive to type 2 PVD **(Figure 37B and C)**. Most of the metal ions did not show any quenching effect, and hence further detailed studies were limited to Fe^{3+}, $Fe^{2+}$and Cu^{2+}.



Quenching efficency (%)

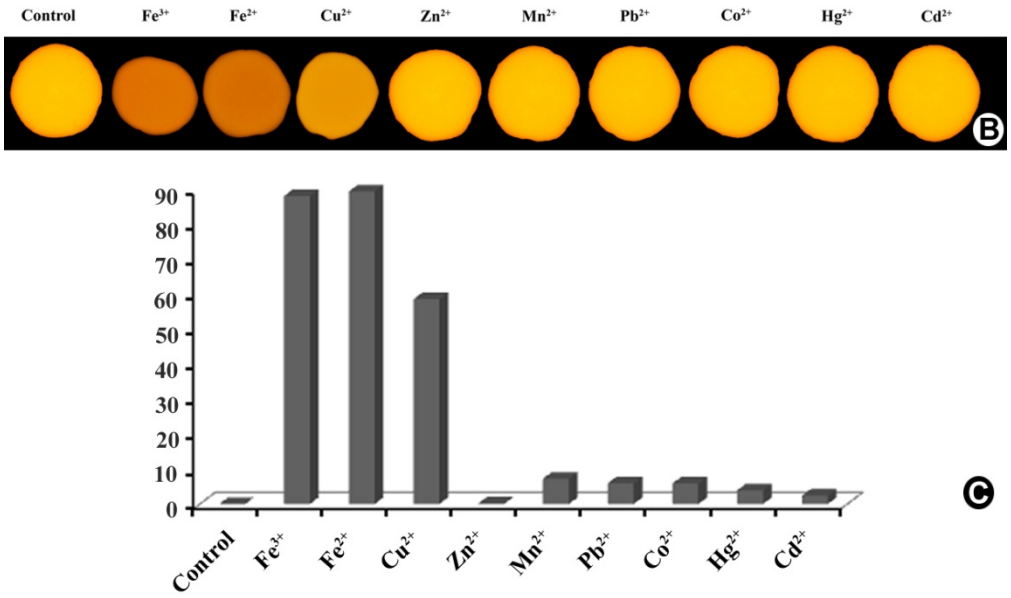


Figure 37. (A) Fluorescent emission spectrum of type 2 PVD purified from *P. aeruginosa* BUP2 without added heavy metal ions. **(B)** Specificity of type 2 PVD biosensor toward heavy metals. Each heavy metal (Fe^{3+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Pb^{2+}, Co^{2+}, Hg^{2+}, Cd^{2+}) was separately mixed with type 2 PVD solution, and the quenching effect was observed with naked eye under with the aid of UV trans-illuminator at λ_{325}; and **(C)** The graphical data show the quantification of fluorescent intensity, as measured by spectrofluorimetry.

Analysis of ferric, ferrous and copper ions using fluorescence quenching method

The type 2 PVD possesses very strong binding affinity with Fe^{3+} and Fe^{2+}, and relatively strong affinity with Cu^{2+}ions, according to the HSAB concept. As shown in **Figure 38A**, the control solution showed higher fluorescence intensity, which gradually reduced depending on the concentration of heavy metal ions.The maximum quenching by Fe^{3+}, Fe^{2+} or Cu^{2+} (10 µM) ion was achieved at 50 sec. The Fe^{3+} and Fe^{2+}ions could quench the fluorescence of PVD up to 75 % in 10 µM; and 98% quenching was achieved with these ions when their concentration was enhanced to 300 µM (**Figure 38B**)**.** However, Cu^{2+} ion showed comparatively weaker quenching efficiency, *i.e.*, 9.5% at its 10 µM concentration, and the maximum quenching (76%) was at 900 µM concentration (**Figure 38B**). Fluorescence quenching imparted by both Fe and Cu ions at 10µm concentration could be detected even by naked eye (**Figure 38A**). The most probable mode of binding of ferric/ ferrous and cupric irons with PVD is shown in **Figure 39AandB**. The IR spectra corresponding to these complexes are given in **Figure 40(A- C)**, and the probable assignments of the IR peaks are given in **Table 29**.

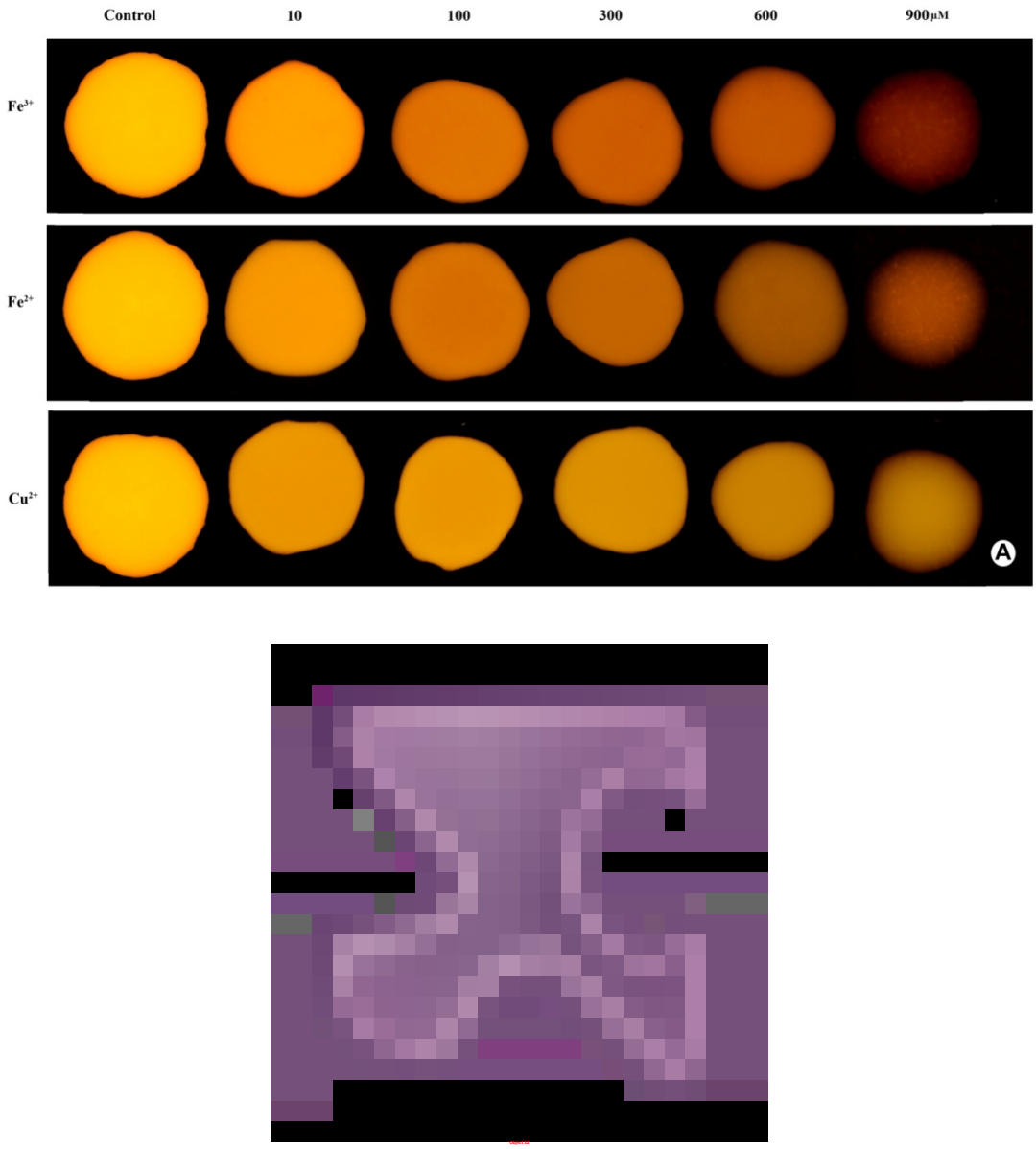


Figure 38. Effect of various concentrations of Fe^{3+}, Fe^{2+} and Cu^{2+} on quenching of fluorescence emitted by PVD. **(A)** Effect of 10, 100, 300, 600 or 900 µM concentration of Fe^{3+}, Fe^{2+} and Cu^{2+} ions on quenching the fluorescence; and **(B)**Graphical representation of the percentage of fluorescent quenching at 10, 100, 300, 600 or 900 µM concentration of Fe^{3+}, Fe^{2+} and Cu^{2+}.

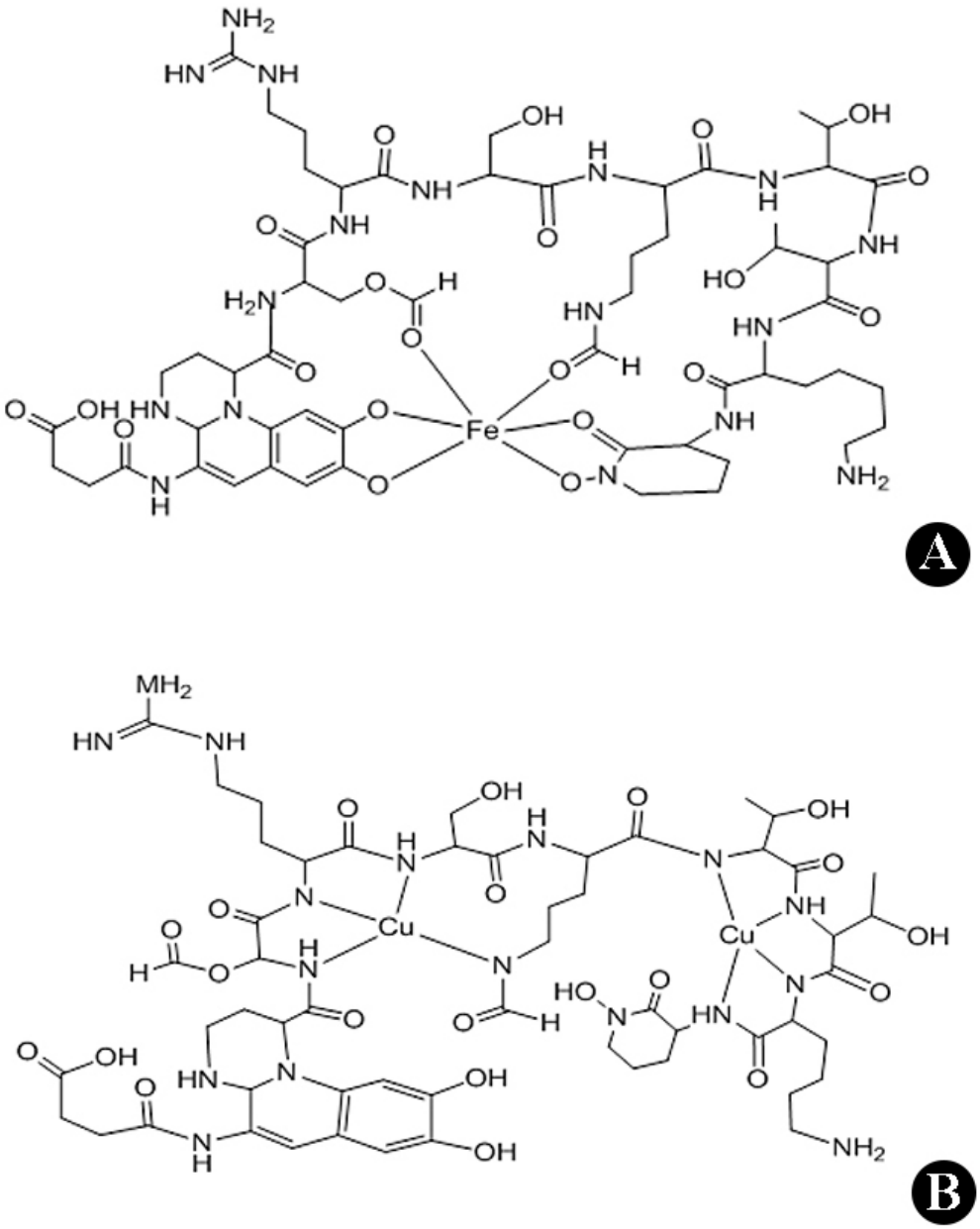


Figure 39. The most probable mode of binding of ferric/ferrous iron and cupric iron. **(A)** Fe-PVD complex, and **(B)** Cu-PVD complex.

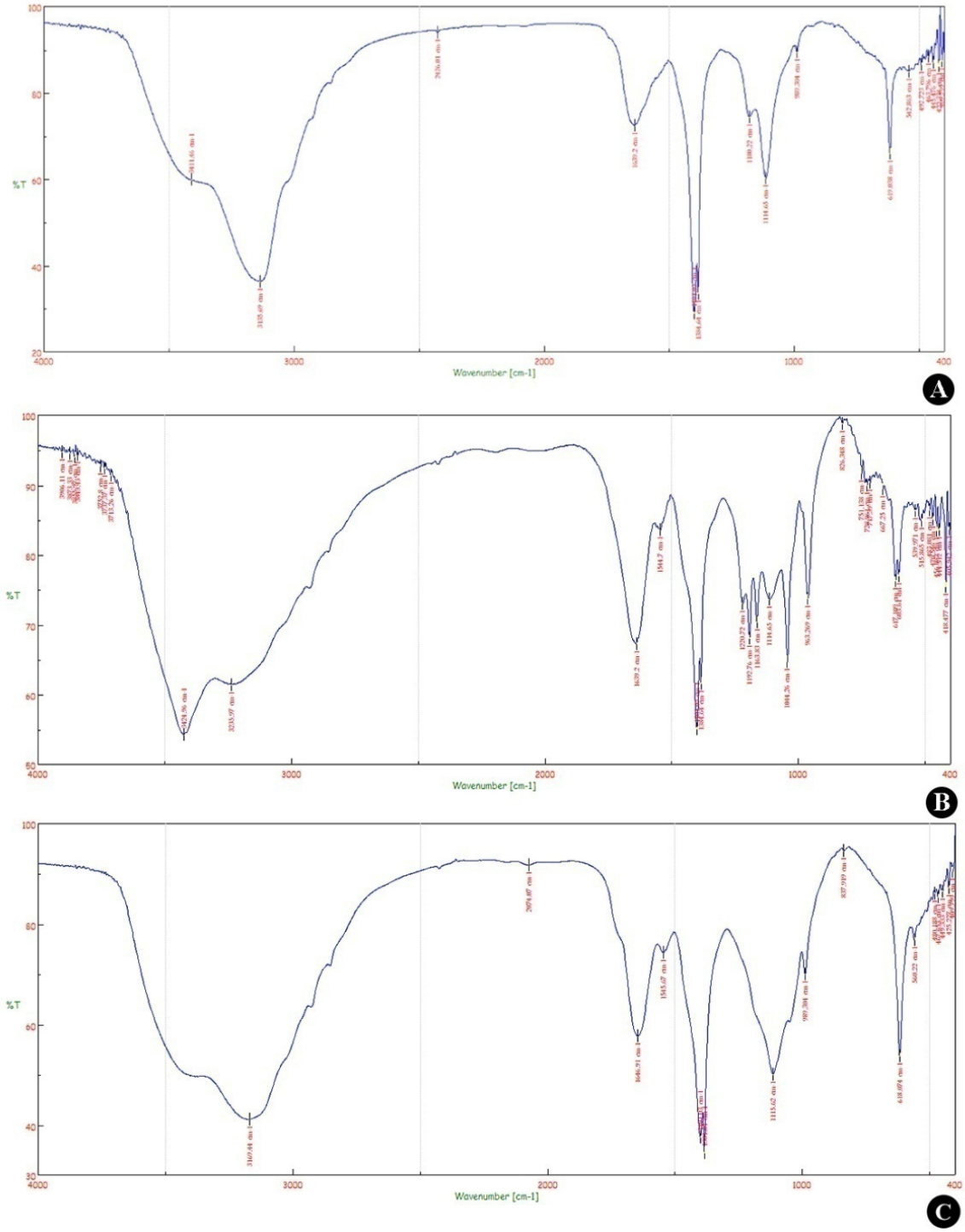
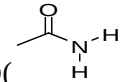
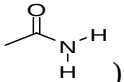
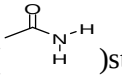


Figure 40. The FTIR spectra of PVD metal complexes. **(A)** PVD, **(B)** Fe-PVD complex, and **(C)** Cu-PVD complex.

Table 29. The FTIR peak assignments of PVD, Fe-PVD and Cu-PVD complexes.

PVD	Fe PVD	Cu PVD
3411 υ (O-H) stretching	3424 υ (O-H) stretching	3411 υ (O-H) stretching
3135 υ (N-H) stretching	3235 υ (N-H) stretching	3169 υ (N-H) stretching
1639 υ() stretching	1639 υ () stretching	1646 υ ()stretching
1400 υ (N-H) bending	1401 υ (N-H) bending	1401 υ (N-H) bending
1384 υ (C=C)	1384 υ (C=C)	1384 υ (C=C)
1114 υ (C-O)	1192 υ (C-O)	1115 υ (C-O)
	617 υ (Fe-O)	618 υ (Cu-N)
	418 υ (Fe-N)	

FT-IR method used for the characterisation of Fe-PVD complex

The major infrared spectral bands for the pyoverdine molecule and its metal complexes especially for Fe (III) and Cu (II) are listed in **Table 29**. A major shift can be observed at 3411/cm in the IR spectrum of pyoverdine molecule; a shifted to 3424/cm in ferripyoverdine indicates that the catachol type OH group is bonded to metal, which results in a bidentate covalent bond with Fe (III). The strong band at 1114/cm, characteristic of C-O stretching vibration of amides remain the same in ferripyoverdine, shows that there is no coordination through amide nitrogen with Fe. The nicotinic hydroxamate group also provides covalent and coordination bands to the metal forming Fe - PVD complex, and is clearly indicated by the band at 1192/cm. The amide I and amide II modes of the peptide bonds are located at 1639 and 1400/cm, without any major change in the stretching frequencies. The appearance of absorption bands at 1384/cm in the spectra recorded before and after coordination is due to C=C stretching of the pyoverdine and its metal

complexes (Dyer, 1965). The C-O stretching frequency for pyoverdine ligand is observed at 1180/cm, but this frequency is shifted to 1044/cm, which further confirmed that the 2,3-diamino 6,7-dihydroxyquinoline group of the pyoverdine molecule binds covalently to the Fe^{+3} ion (Upritchard *et al*., 2007). The C=O stretching and N-H bending modes of the hydroxamate and nicotinic hydroxamate bonds give a major contribution to the formation of a coordinate bond with Fe^{+3} (Nakamoto, 1986). Therefore, the corresponding bands shifted from 3135 to 3235/cm. The band at 617/cm is due to metal-O coordinate bonding and 418/cm is assigned to Fe-N bonding.

FT-IR method used for the characterisation of Cu-PVD complex

The major peak of the pyoverdine ligand (3411/cm for υ (O-H) stretching) remains at the same position in the Cu-PVD molecule indicates that catachol like chromophore do not coordinate to the Cu^{2+} ion. The major absorption bands of amide 1 and amide 11 change from 1639 to 1646/cmwhich reveals that N atoms in amides coordinating as a monodentate ligand to the Cu^{+2} ion. The corresponding stretching vibrations of N-H is observed at 3169/cm. Similarly, the C=O group of the molecule altered as a consequences of adsorption from 1400 to 1401/cm. The band at 617/cm indicates Cu-N monodentate coordinate bond, which is only a possible bond between the amide N and the metal center. There is no additional peak for Cu-O, therefore, the catachol O-H and other oxygen atoms are free from the bonding with Cu^{+2} ion.

From infrared spectra, the change of band at 3411/cm to 3424/cm indicates that a catechol-like group forming part of the quinolone type chromophore together with the hydroxamate groups provided two bi-dentate coordination ligands for the Fe^{3+} ion. The strong band at 1114/cm is characteristic of C-O stretching vibration, and of the catecholate-like groups. It is coordinately bound to metal ions to form Fe -PVD complex, and is clearly indicated by the band at 1192/cm. The prominent absorptions related to the amide I and amide

II modes of the peptides are readily recognised with peaks at 1639 and 1401/cm, but their relative intensities and band shapes were altered as a consequence of the absorption. The appearance of absorption bands at 1491-1503/cm in all spectra is due to C-C ring modes and at 1284-1292/cm due to the C-O stretching modes indicate that the catechol-like 2,3- diamino-6,7-dihydroxyquinoline part of the pyoverdine molecule binds covalently with Fe(III) .

The pyoverdine structure is dominated by an octapeptide chain; therefore, the prominent bands at 3135 and 1400 change to 3235 and1401/cm in the spectrum contain major contributions from amide I (mainly the C=O stretch) and amide II (mainly the N-H bend) modes of the peptide bonds to form a monodentate ligand to Fe. A strong band at 1389/cm most probably arises from the in-plane δ-OH deformation mode of the dihydroxyquinoline ligand. The band at 617 is due to metal-O coordinate bonding.

Sensitivity of fluorescent biosensor by spectrofluorimeter

Variable concentrations (1 to 900 µM) of Fe^{3+}, Fe^{2+} and Cu^{2+} were incubated for 50 sec with the PVD solution at 25 ^{o}C. The fluorescence quenching efficiency of Fe^{3+} and Fe^{2+} showed the linear range from 1 to 60 µM, *i.e.*, 1 to 60 ppm (**Figure 41 and 42**), while the effect of Cu^{2+} was linear within the range between 1 and 20 µM, *i.e.*, 1 to 20 ppm (**Figure 43**). The calculated LoD of fluorescence by quenching method for Fe^{3+}, Fe^{2+} and Cu^{2+} was 0.23, 0.24 and 0.38 µM, respectively.

Fe^{3+}

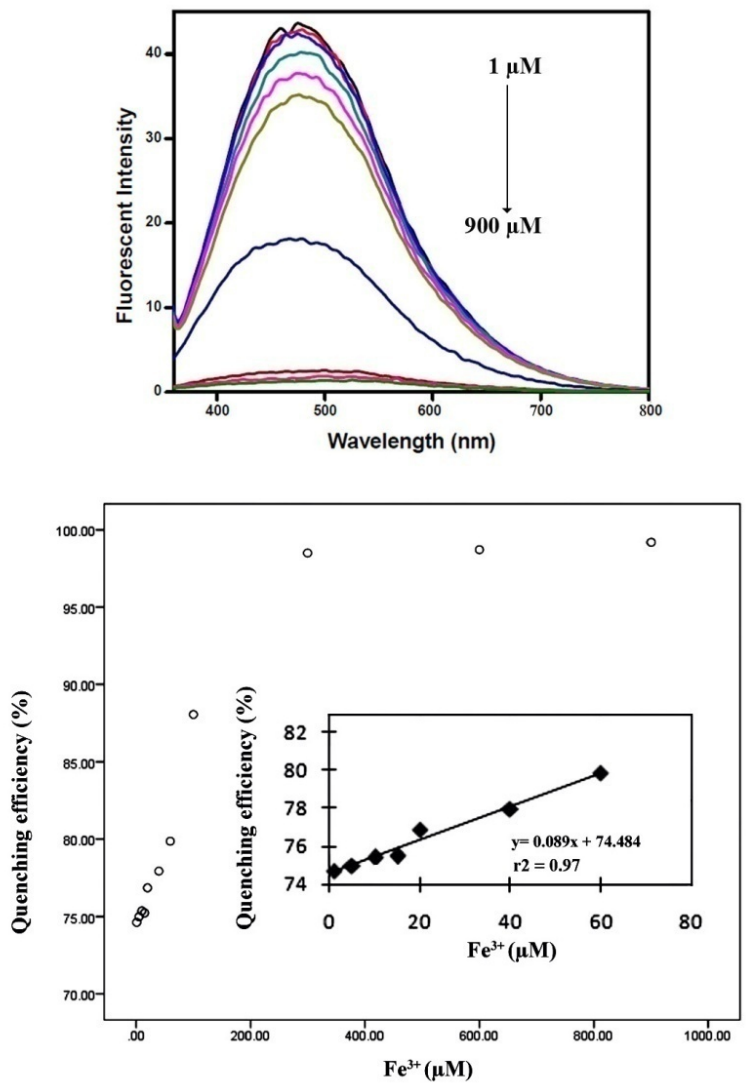


Figure 41. Linear range, and LoD of the type 2 PVD biosensor. **(A)** Fluorescent emission spectra of PVD in the presence of Fe^{3+} at 1, 5, 10, 15, 20, 40, 60, 100, 300, 600, 900 µM concentrations; and **(B)** Detection of Fe^{3+} by PVD biosensor (inset: linearity between 1 and 60 µM with liner regression equation, $y = 0.089x + 74.848$ and $r^2 = 0.97$, where 'y' represents the quenching efficiency and x represents the Fe^{3+} concentrations).

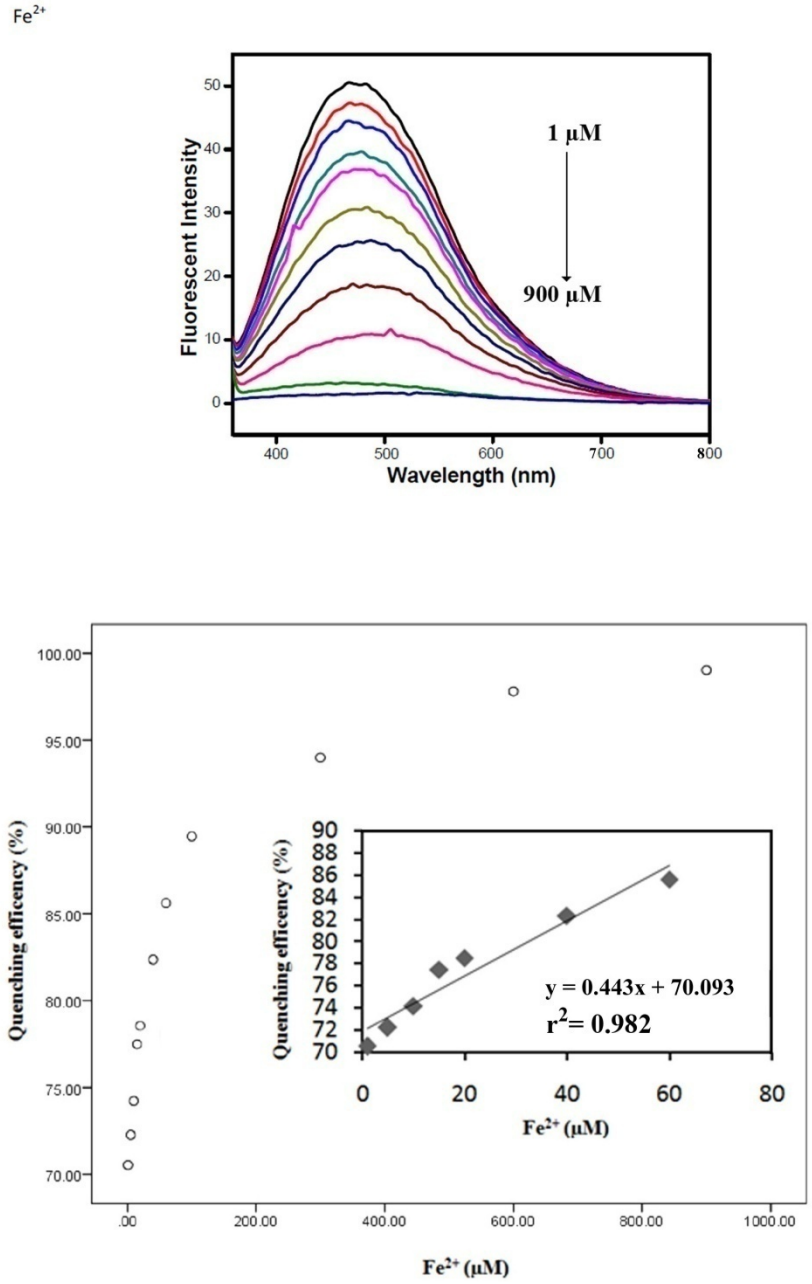


Figure 42. Linear range and LoD of the PVD biosensor. **(A)** Fluorescent emission spectra of PVD in the presence of Fe^{2+} at 1, 5, 10, 15, 20, 40, 60, 100, 300, 600, 900 µM concentrations; and **(B)** Detection of Fe^{2+} by PVD biosensor (inset: linearity between 1 and 60 µM with liner regression equation, $y = 0.443x + 70.093$ and $r^2 = 0.982$, where 'y' represents the quenching efficiency and x represents the Fe^{2+} concentrations).

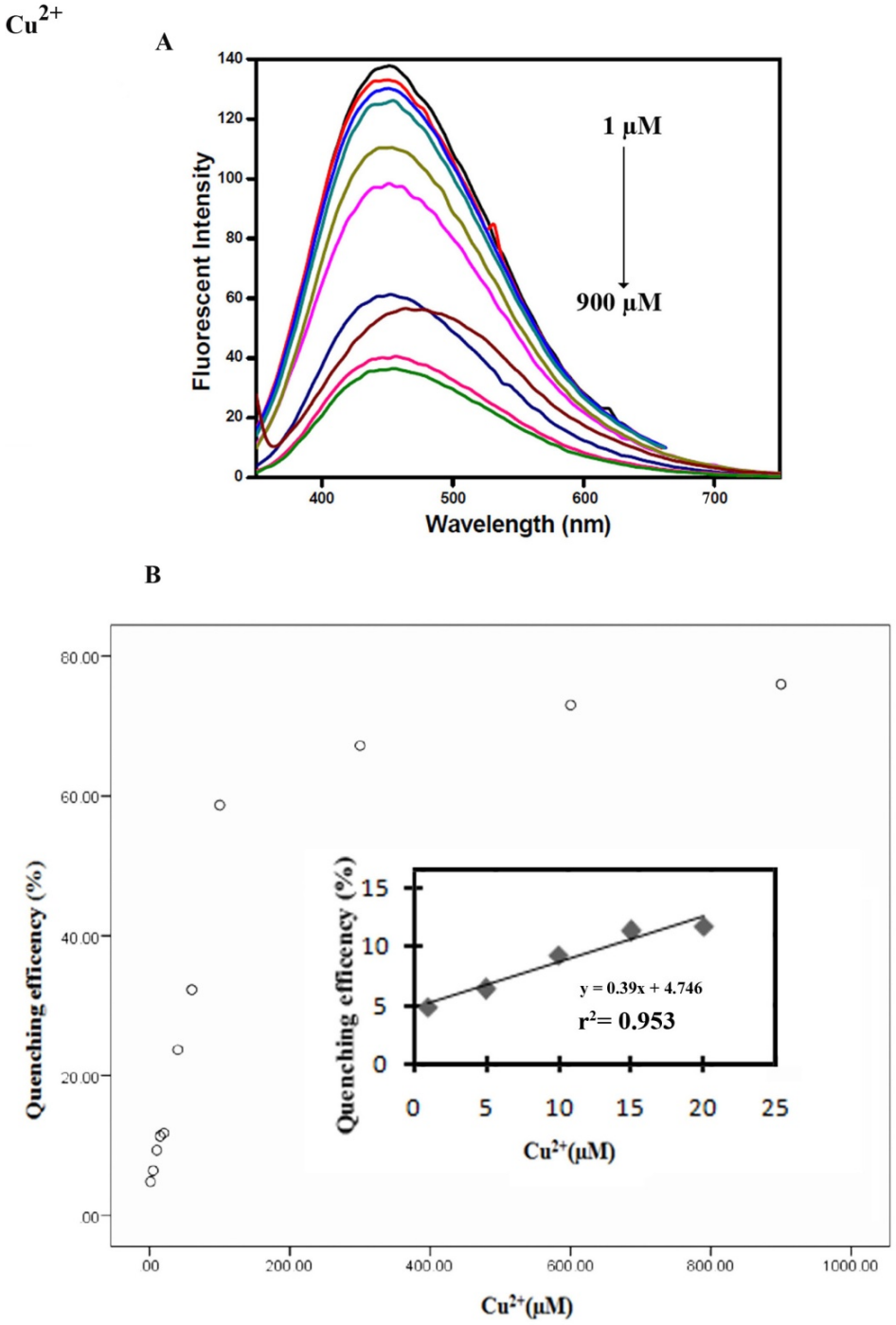


Figure 43. Linear range and LoD of the PVD biosensor. **(A)** Fluorescent emission spectra of PVD in the presence of Cu^{2+} at 1, 5, 10, 15, 20, 40, 60, 100, 300, 600, 900 µM concentrations; and **(B)** Detection of Cu^{2+} by PVD biosensor (inset: linearity between 1 and 20 µM with liner regression equation, $y = 0.39x + 4.746$ and $r^2 = 0.953$, where 'y' represents the quenching efficiency and x represents the Cu^{2+} concentrations).

Detection of heavy metals in contaminated water samples

In order to check the iron or copper contaminations, water samples were collected from well-known Fe (Madai, Kannur, Kerala, India) and Cu (Industrial area Aluvai, Ernakulam, Kerala, India) contaminated sites in Kerala. Analysis showed that the fluorescence of type 2 PVD was rapidly quenched by both the water samples (**Figure 44**). Atomic absorption spectra (**Table 30**) of these water samples showed that the samples contained 327 mg/ l iron and 281 mg/l copper ions.

Table 30. Presence of heavy metal in water sample as measured by Atomic Absorption Spectroscopy.Sample 1: Presence of Fe ions in water sample collected from Madai, Kannur; and Sample 2: Presence of Cu ions in water sample collected from Aluvai, Ernakulum

SI. No	Water Sample	Metal ion	Concentration (mg/l)
1	Well water	Iron	327
2	Well water	Copper	281

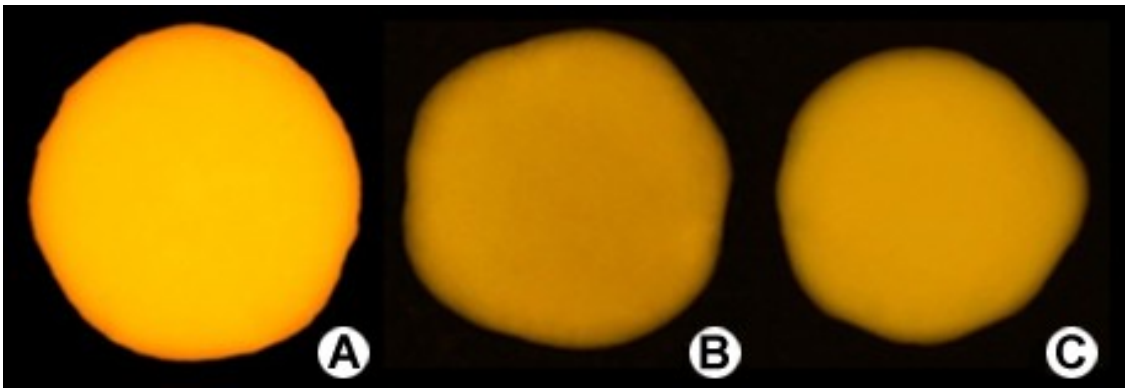


Figure 44. Detection of heavy metal ions in contaminated water sample by type 2 PVD. **(A)** Control; **(B)** Presence of Fe; and **(C)** Presence of Cu.

Discussion

The prime objective of this study was to explore whether the type 2 PVD produced by *P. aeruginosa* BUP2 could directly be used as a *turn-off* biosensor for the rapid detection of iron and copper contaminations in water. *P. aeruginosa* BUP2 was described from this laboratory (isolated from the rumen of Malabari goat); and we showed that it could produce type 2 pyoverdine (purified in the powder form) (Unni *et al.*, 2014).

Determination of iron and copper from water resource is of great interest, since they adversely affect the biota, especially humans. Till date, different costly procedures such as flame photometry, atomic absorption spectroscopy, solid phase extraction, liquid–liquid extraction, co-precipitation and cloud point extraction have been used for the detection of metal ions (Nascentes and Arruda, 2003; Akl *et al*., 2004). But, these techniques pose some natural limitations such as time consuming, requirement of large sample volume, large quantity of organic solvents (which, in turn, are the secondary contaminants), and most of the equipment used for this purpose are highly expensive. Here comes the importance of a cheap, remarkable, non-poisonous and versatile type 2 PVD based *turn-off* biosensor for the rapid detection of iron and copper.

Upon incubation with Fe^{3+}, Fe^{2+}or Cu^{2+}ion, the fluorescent intensity of PVD was decreased, *i.e*., *turn-off* effect. Because of the quenching nature of paramagnetic Fe^{3+}/ Fe^{2+}, development of turn-on fluorescent sensors is not easier. Various types of *turn-off* fluorescent intensity based probes have been developed for the detection of iron and copper from biological samples. These includes, calcein fluorescent biosensor to detect both Fe^{3+} and Fe^{2+} (Lane *et*

al., 2010), 3-hydroxypyridin-4-one fluorescent biosensor to detect Fe^{3+} (Fakih *et al*., 2009) and siderophore based fluorescent biosensor *i.e*., azotobactin*δ* to detect Fe^{3+} (Palanche *et al*., 1999), parabactin to detectFe^{3+} (Lam *et al*., 2006) and PVD to detect Fe/Cu (Barrero *et al*., 1993; Gupta *et al*., 2008; Yoder and Kisaalita, 2006). The 6-dimethylaminobenzothiazole alkyne, a fluorescent biosensor was quenched by Cu^{+} inaqueous solution (Qi *et al*., 2012).

The PVD secreted by *P. aeruginosa* (Barrero *et al*., 1993) or immobilised PVD from *P. fluorescence* on porous glass (Yodar and Kisaalita, 2011) were used for the detection of Fe ions. PVD-doped sol-gel pellets and PVD in solution showed fluorescence quenching capacity by Fe^{3+} andFe^{2+} ions, with a LoD of 0.3 µM for Fe^{3+} (Barrero *et al*., 1993, Yodar and Kisaalita, 2011), *i.e*., a higher LoD than the present report. The PVD secreted by a *P. aeruginosa* strain PA1 was purified through affinity chromatography for the detection of furazolidone from the water sample, which showed an LoD of 0.5 µM (Yin and Zhang, 2014). Apart from this, the culture supernatant of *P. fluorescence* could be used as biosensor for the detection of Fe^{3+} at10 mM concentration; and a fluorescent siderophore from *P. aeruginosa* could chelate Cu^{2+} and Fe^{3+} ions with quenching efficiency of 83% and 66%, respectively at 3.0 µM concentration (Gupta *et al*., 2008; Yoder and Kisaalita, 2006). However, in the present study, 9 metal ions were screened for quenching efficiency; of which, Fe^{2+}, $Fe^{3+}$and Cu^{2+}only showed positive results. From the results, it is clear that the LoD for $Fe^{3+}$was the lowest (0.23 µM), and of Fe^{2+} (0.24 µM) was close to Fe^{3+}, while the LoD of $Cu^{2+}$was 0.38 µM. Moreover, in the present study, the experiments were conducted with the PVD directly without linking to any support or circuit, *i.e*., a small drop of type 2 PVD in double distilled water on a clean glass slide and a UV trans-illuminator. Thus, had it

fabricated into a biosensor for commercial use, the detection limit would be much lower than the commercially available florescent *turn-off* biosensors.

Secondly, we checked the sensitivity of the type 2 PVD. The LoDs demonstrated for Fe and Cu are very promising, and the data are more comparable with His6GFP biosensor for copper, and PVD biosensor (immobilised on sol-gel glass) used for the detection of iron (Isarankura *et al*., 2010;Yoder and Kisaalita, 2006). The regression coefficient values of Fe^{3+} and Fe^{2+} were 97 and 98.2%, respectively, and 95.3 % was for Cu^{2+}. It was observed that the entire reaction with PVD was completed within a few seconds (50 sec.), and no adverse changes were observed in the florescence quenching while incubating the reaction at 25 ^{o}C, which standardised for the purpose by trial and error method.

Finally, it was confirmed that the PVD based *turn-off* biosensor is suitable for the detection of Fe and Cu contamination in drinking water samples with the help of simple equipment, the UV trans-illuminator. Upon incubation with PVD, the suspected water samples exhibited higher fluorescent quenching efficiency, which was at alarming levels.

Conclusion

The type 2 PVD based *turn-off* biosensor exhibited higher sensitivity and specificity with Fe^{3+}, Fe^{2+} and Cu^{2+}. The *turn-off* biosensor proposed in this study is very simple and rapid, which neither requires sophisticated equipment, nor a qualified technician – but only type 2 PVD in water, a glass slide and a UV trans-illuminator. Moreover, the LoD range of the *turn-off* biosensor was highly promising, and that using this technique, Fe and Cu contamination could be detected efficiently at ppm level, at low cost.

Summary and Conclusions

Chapter 8
Summary and Conclusions

The rumen of ruminants is inhabited by a consortium of enormous microflora comprising bacteria, protozoa, fungi and archaea; whose multifaceted activities enable the host with better digestion and absorption. Though microbial community residing the rumen chamber are considered as highly promising organisms for the production of industrially-significant biomolecules with GRAS (generally recognised as safe) status, microbiologists provide less priority for the exploitation of such rumen microbes for the human welfare. Under these circumstances, this study is envisaged to explore the bacteria inhabiting the rumen of Malabari goat for the production valuable biocatalyst like lipase and iron-chelating siderophore.

Compared to plant or animal sources of lipases (triacylglycerol acylhydrolase; EC 3.1.1.3), industry prefers microbial lipases by dint of its versatility of catalysis, stability, ease of genetic manipulation, simple extraction procedures and promising yield. Lipases catalise lipolytic activities such as hydrolysis, acidolysis, alcoholysis, esterification, *trans*-esterification, *inter*-esterification, racemic solution, stereo selectivity and chiral synthesis. Compared to fungal lipases, bacterial lipases offer higher activities with neutral or alkaline pH optima and thermal stability. Among bacterial lipases, those from *Aclaligenes* spp., *Acromobactor* spp., *Arthrobactor* spp., *Bacillus* spp., *Chromobacterium* spp., *Pseudomonas* spp. and *Staphylococcus* spp. are commonly used in industry depending on their specific functions. Mostly, submerged fermentation technique is employed for the production of bacterial lipase

Siderophores are low molecular weight, high affinity iron chelating compounds embodying a chromophore, an acyl moiety and an invariable

peptide chain, mostly produced by bacteria which enable them to capture iron ions to meet their biological requirement. Main function of siderophores is to chelate iron (*i.e*., solubilise) from the surrounding environments and transport into bacterial cell, and this event leads to pathogenesis in the host. Different types of siderophores are reported from the species of the bacterial genera: *Agrobacterium, Acinetobacter, Azotobacter,Bacillus, Bordetella, Erwinia, Escherichia, Mycobacterium, Pseudomonas, Rhodococus, Salmonella, Shigella, Staphylococcus, Streptomyces, Yersinia, Vibrio, etc.*Based on the moiety that donates oxygen (binding moiety) for coordinating with iron, siderophores are generally classified into four major types: catecholate/phenolate,hydroxamate, carboxylate and mixed types. Pyoverdine (PVD) – a mixed type siderophore - is one of the best examples of bacterial siderophore secreted by the fluorescent *Pseudomonas* spp., (*P. aeruginosa*, *P. chlororaphis*, *P. fluorescens*, *P. putida* and *P. syringae*). PVD is mostly produced by *P. aeruginosa,* which are subdivided into three subtypes; *viz.*, PVD 1, PVD 2 and PVD 3 - each subtype has characteristic peptide chain.

Rationale and objectives

Malabari goat is a special breed and no significant studies were conducted on this animal, especially on the microflora inhabiting their rumen. It is expected that the bacteria dwelling in the rumen would produce lipase and siderophores. In the light of this background, the following objectives were set for this unique study: (a) isolation and characterisation of bacteria from the rumen of Malabari goat, (b) Utility of rubber seed kernel as potent solid substrate for the production of lipase by *Pseudomonas aeruginosa* strain BUP2, (c) production, optimisation, purification and chracterisation of lipase produced by*P. aeruginosa* strain BUP2, (d) Production of type 2

pyoverdineby *P. aeruginosa* strain BUP2, and (e) efficacy of type 2 pyoverdine as *turn-off* biosensor for the rapid detection of iron and copper in contaminated water.

Summary

Isolation and characterisation of bacteria from rumen of Malabari goat

Rumen contents from both male and female Malabari goats were collected aseptically in screw-capped tubes from the slaughter house at Chelari (11.18189600 ^{o}N; 75.82206300 ^{o}E), Malappuram District of KeralaState. After suitable serial dilutions of the samples (up to 10^{-6}), the isolates were initially grown on semi-synthetic medium - designated as BUP (abbreviation stands for Benjamin, Unni and Priji) medium. The isolated five bacterial pure cultures were screened for the production of lipase and siderophore by agar diffusion and chromasurolS (CAS) assaymethods. Among the isolates, one isolate exhibit positive results for the production of both lipase and siderophore. Subsequently, the pure isolate was characterised by microbiological, biochemical and molecular methods. Molecular characterisation was done by 16S rDNA sequence analysis. Based on nucleotide homology and phylogenetic analysis, the isolate was further confirmed as a strain of *P. aeruginosa*, designated as *P. aeruginosa* strain BUP2. Then the bacterial sequence and connected details were submitted to GenBank (Accession No. JQ 407054), and the culture is deposited at Institute of Microbial Technology, Chandigarh under Budapest Treaty (*i.e*., patent deposit) with the designation: MTCC No. 5924. The preliminary stage of cultivation was performed under anaerobic environment; subsequently, *P. aeruginosa* strain BUP2 was gradually tuned to the aerobic system by

frequent subcultures in a specially designed conical flask by this laboratory, designated as *Benjamin flask*.

Utility of rubber seed as potent solid substrate for the production of lipase by *P. aeruginosa* strain BUP2

This study explored the utility of flours of rubber seed, coconut and groundnut, and deoiled cakes of coconut and groundnut as solid substrate for the production of lipase by *P. aeruginosa* strain BUP2. Various proportions (10, 20, 30, 40 or 50 %) of flours or cakes were supplemented (w/v) with BUP medium (pH 4.0, 5.0, 6.0, 7.0 or 8.0), and incubated at different temperature (25, 28, 30 or 32 ^{o}C) for 24 to 96 h. The samples were assayed for lipase activity at 24 h intervals. The rubber seed flour (20 %)-BUP medium supported the maximum lipase production (871 U/gds) at 48 h incubation (pH 6.0, 28 ^{o}C), followed by ground nut flour (398 U/gds), while ground nut cake supported the least lipase production (244 U/gds). From this, it is evident that the cheaply available rubber seed kernel is an efficient substrate for the production of lipase, irrespective of its known demerit that it contains a toxin, the limarin; in fact, limarin could not be detected in the fermented matter.

Production, optimisation, purification and characterisation of lipase produced by *P. aeruginosa* strain BUP2

The principal aim of this study was to attain production, optimisation, purification and characterisation of lipase produced by *P. aeruginosa* strain BUP2**.** Lipase production was attained in BUP medium supplemented with 1 % ground nut oil. For maximising the lipase secretion, production parameters were optimised using Plackett–Burmann and Box–Behnken designs, by which

temperature (28 ^{o}C), pH (6.0) and incubation time (24 h) were found as significant factors. At these standardised conditions, the production was enhanced from 112 U/ml initial production to 172 U/ml (*i.e*., ~ 0.5 fold increase). Using $(NH_4)_2SO_4$ fractionation and sephadex G-100 gel filtration, lipase was purified (36 folds with 20 % yield) to homogeneity with 2392 U/mg specific activity, whose apparent MW was 29 kDa, as judged by SDS-PAGE. The maximum activity (2802 U/ml) of the purified lipase was observed at 45 ºC and pH 8.0 in the presence of 5.0 mM Ca^{2+}. The *Km* and *Vmax* of the purified lipase were found as 4.75 mM and 999.5 µmol/min/mg, respectively. Briefly, the alkalophilic lipase produced by *P. aeruginosa* strainBUP2 was thermotolerant with higher activity, which offers potentials for industrial applications.

P. aeruginosa strain BUP2 produces type 2 pyoverdine

This study focuses on the production and characterisation of a siderophore by *P. aeruginosa* BUP2. *P. aeruginosa* BUP2 was grown in BUP medium supplemented with 1% ground nut oil or succinate medium, and incubated in a rotary shaker at 37 ^{o}C and 150 rpm. The cell-free supernatant of BUP and succinate media containing yellowish green pigment (siderophore) was extracted with ethyl acetate at regular intervals of 6 and 24 h; OD measured spectrophotometrically at λ_{404}. The maximum pigment production was observed in the BUP medium on day 4 of incubation (OD of 1.95); which gradually declined to 1.89 after day 5. Likewise, production of siderophore in iron limited succinate medium showed that the maximum pigment production was reached in the medium at12 h of incubation (OD of 1.22), thereafter the production was decreased to 1.01.

For maximising the siderophore secretion, production parameters were optimised using Plackett–Burmann and Box–Behnken designs, by which temperature (28 ^{o}C), pH (6.5) and incubation time (120 h) were found as significant factors. At this statistically optimised condition, the absorption was the maximum at 2.5.; *i.e*., production of siderophore was enhanced by 30 %, compared to that of unoptimised condition. The siderophore was characterised by spectrophotometer, thin layer chromatography and isolectric focusing (IEF). The characteristic orange fluorescence upon UV irradiation on chromatogram and absorption maximum at λ_{404} confirmed that the characteristic siderophore produced by *P.aeruginosa* strain BUP2 was a typical PVD. This PVD was further categorised under type 2 by comparing its profile on the IEF gel with that of the representative strains of each PVD subtypes. Moreover, the type 2 PVD was purified by XAD-4 Amberlite column chromatography and quantified; maximum yield (23.0 mg/ml) was observed on day 5 of incubation.

Type 2 pyoverdine as *turn-off* biosensor for the rapid detection of iron and copper in contaminated water

Employing fluorescent quenching mechanism, type 2 PVD purified from *Pseudomonas aeruginosa* strain BUP2 was used as a simple, convenient and inexpensive tool for the rapid detection of iron and copper in contaminated water. The florescence emitted at $\lambda_{460}$by PVD in sterile water on a glass slide efficiently quenched by heavy metals like Fe and Cu. This quenching efficiency could be assessed by naked eye with the aid of a UV trans-illuminator, upon exposure for about 50 sec at 25 ^{o}C. The linear range of detection for Fe was 1 to 60 µM, while that of Cu was 1 to 20 µM. The limit of detection at µM concentration for Fe^{3+}, $Fe^{2+}$and $Cu^{2+}$was 0.23, 0.24 and

0.38, respectively. Detection of Fe or Cu contamination in water samples, collected from contaminated areas is studied using this method and demonstrated. These metals coordinately bound with the fluorescent material which act as potential ligand (PVD) due to the presence of hetero atoms, and the change in the structure of the molecule was monitored with IR spectral studies. Being a hard acid, Fe^{3+} effectively bound with the O-ligand, and this ability was less for Cu^{2+}, as it is a border line acid. The quenching of the fluorescence is more pronounced in the case of Fe-PVD system than copper-PVD system, and this observation was in accordance with the HSAB concept of Pearson. Thus, it was confirmed that the type 2 PVD produced by *Pseudomonas aeruginosa* strain BUP2 is a low cost *turn-off* biosensor for the rapid screening of heavy metals like iron and copper in drinking water with the help of a simple UV trans-illuminator.

Major outcomes/ deliverables

This is the first ever study on the bacteria inhabiting the rumen of Malabari goat, report on*P. aeruginosa* strain BUP2, coupled with production and purification of lipase, and of pyoverdine produced by *P. aeruginosa* strain BUP2.

- The rumen of Malabari goat (*Capra hircus* L.) was explored, many bacteria were identified from the rumen content; of them *P. aeruginosa* strain BUP2 was found as significant one.
- *P. aeruginosa* strain BUP2 was characterised by microbiological, biochemical and molecular techniques; its rDNA gene sequence was identified; culture details were submitted to GenBank (Accession No JQ 407054).

- *P. aeruginosa* strain BUP2 was deposited under Budapest Treaty (patent deposit) at IMTECH with MTCC No. 5924.
- *P. aeruginosa* strain BUP2 is an efficient producer of lipase, it could produce 871 U/gds lipase on rubber seed under SSF.
- *P. aeruginosa* BUP2 could produce 112 U/ml lipase in specially designed BUP medium, supplemented with 1 % groundnut oil; upon statistical optimisation, production was enhanced to 172 U/ml (54 % increase).
- Lipase produced by *P. aeruginosa* BUP2 was purified to homogeneity, its MW was ~29 kDa.
- Purified lipase from *P. aeruginosa* strain BUP2 was characterised, its optimum activity was 2802 U/ml at 45 ºC and pH 8 in the presence of 5 mM Ca^{2+} (with 2392 U/mg specific activity) with 4.75 Km and Vmax of 999.5 µmol/min/mg
- The water-soluble yellowish green coloured siderophore produced by *P. aeruginosa* strain BUP2 was identified as type 2 pyoverdine.
- *P. aeruginosa* strain BUP2 produced 23.0 mg/ml pyoverdine, after statistical optimisation of production conditions.
- Efficacy of type 2 pyoverdine produced by *P. aeruginosa* strain BUP2 as *turn-off* biosensor for the detection of Fe and Cu contamination was demonstrated.

Leads for further study

- Molecular elucidation of type 2 pyoverdine produced by*P. aeruginosa* strain BUP2 is in progress.
- Utility of type 2 pyoverdine in drugs and drug delivery systems has to be explored**.**
- Since the MW lipase produced by *P. aeruginosa* strain BUP2 is only ~29 kDa, it is a good candidate for protein engineering.

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Appendix I

List of publication

A.Research papers published

1. **Unni, K. N**., Faisal, P. A., Priji, P., Sajith, S., Sreedevi, S., Hareesh, E. S., Nidheesh Roy, T.A., and Benjamin, S. (2015). Rubber seed as potent solid substrate for the production of lipase by *Pseudomonas areuginosa* strain BUP2. *Advances in Enzyme Research*, 3(02), 31.**I. F. 1.1 (Scientific Research Publishing)**.

2. **Unni, K. N**., Priji, P., Geoffroy, V. A., Doble, M., and Benjamin, S. (2014). *Pseudomonas aeruginosa* BUP2– A Novel Strain Isolated from Malabari Goat Produces Type 2 Pyoverdine. *Advances in Bioscience and Biotechnology*, *5*(11), 874. **I.F. 0.63 (Scientific Research Publishing)**.

3. Neethu, K. B., Priji, P., **Unni K. N**.,Sajith S., Sreedevi S., Ramani, N., Anitha, K., Rosana, B., Girish Babu, M., and Benjamin, S. (2015). New *Bacillus thuringiensis* strain isolated from the gut of Malabari goat is effective against *Tetranychus macfarianei*. *Journal of Applied Entomology*. doi: 10.1111/jen 12235. **I.F. 1.7 (Wiley)**.

4. Sreedevi, S., **Unni, K. N**., Sajith, S., Priji, P., Josh, M. S., and Benjamin, S. (2014). Bioplastics: *Advances in Polyhydroxybutyrate Research*. *Advances in Polymer Science*. DOI: 1007/12-2014-297. **I. F. 3.7(Springer)**.

5. Faisal, P. A., Hareesh, E. S., Priji, P., **Unni, K. N**., Sajith, S., Sreedevi, S., Sarath Josh, M.K., and Benjamin, S. (2014). Optimization of Parameters for the Production of Lipase from *Pseudomonas* sp. BUP6 by Solid State Fermentation. *Advances in Enzyme Research*, 2(04), 125. **I. F. 1.1 (Scientific Research Publishing)**.

6. Priji, P., **Unni, K. N**., Sajith, S., Binod, P., and Benjamin, S. (2014). Production, optimization, and partial purification of lipase from *Pseudomonas* sp. strain BUP6, a novel rumen bacterium characterized from Malabari goat. *Biotechnology and Applied Biochemistry*. 62(01), 71-78. **I.F. 1.8 (Wiley)**.

7. Priji, P., **Unni, K. N**., Sajith, S., and Benjamin, S. (2013). *Candida tropicalis* BPU1, a novel isolate from the rumen of the Malabari goat, is a dual producer of biosurfactant and polyhydroxybutyrate. *Yeast*, 30(3), 103-110. **I.F. 1.8 (Wiley)**.

8. Sajith, S., Sreedevi, S., Priji, P., **Unni, K. N**., and Benjamin, S. (2014). Production and partial purification of cellulase from a novel fungus, *Aspergillus flavus* BS1. *Annals of Microbiology*, 64(2), 763-771. **I.F. 1.3 (Springer).**

9. Jisha, V. N., Smitha, R. B., Pradeep, S., Sreedevi, S., **Unni, K. N**., Sajith, S., Priji, P.,Sarath Josh M. K. and Benjamin, S. (2013). Versatility of microbial proteases. *Advances in Enzyme Research*. 1(3), 39-51. **I.F. 1.1 (Scientific Research Publishing)**.

10. Benjamin, S., Smitha R. B., Jisha, V. N., Pradeep, S., Sajith, S., Sreedevi, S., Priji, P., Unni, K. N., and Sarath Josh, M. K. (2013).A monograph on amylases from *Bacillus* spp. *Advances in Bioscience and Biotechnology.* 4, 227-241. **I.F. 0.81 (Scientific Research Publishing)**.

11. Rubeena M., Neethu, K. B., Sajith S., Sreedevi S., Priji, P., Unni K. N., Sarath Josh M. K., Jisha, V. N., Pradeep S. and Benjamin, S. (2013). Lignocellulolytic activities of a novel strain of *Trichoderma harzianum*. *Advances in Bioscience and Biotechnology.* 4, 214-221. **I.F. 0.63 (Scientific Research Publishing)**.

12. Neethu, K. B., Rubeena M., Sajith S., Sreedevi S., Priji, P., Unni K. N., Sarath Josh M. K., Jisha, V. N., Pradeep S. and Benjamin, S. (2012). A novel strain of *Trichoderma viride* shows complete lignocellulolytic activities. *Advances in Bioscience and Biotechnology*. 3, 1160-1166. **I.F. 0.63 (Scientific Research Publishing)**.

B.Seminars/ Proceedings

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

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

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

C. Papers under processing

1. **Unni**,**K .N**.,Priji,P., Nidheesh Roy,T. A., Sajith,S.,Shainy,K. M., Joseph, A., Girish Babu, M., and Benjamin,S.,(2015). Type 2 pyoverdine as *turn-off* biosensor for the rapid detection of iron and copper in contaminated water. ***Environmental monitoring and assessment***, Communicated
2. **Unni**,**K.N**., Priji, P., Sajith, S., Benjamin, S.,(2015). Production, optimization, purification and c haracterization of lipase from *Pseudomonas aeruginosa* BUP2 strain.***Enzyme and microbial Biotechnology***, Communicated

Appendix II

GenBank Submission

Pseudomonas aeruginosa strain BUP2 16S ribosomal RNA gene, partial sequence

GenBank: JQ407054.1

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ORGANISM   Pseudomonas aeruginosa
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            Pseudomonadaceae; Pseudomonas.
REFERENCE   1 (bases 1 to 1209)
  AUTHORS   Benjamin,S., Unni,K.N. and Prakasan,P.
  TITLE   Conjugated linoleic acid producing bacteria from the rumen of
            Malabari goat
  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 1209)
  AUTHORS   Benjamin,S., Unni,K.N. and Prakasan,P.
  TITLE   Direct Submission
  JOURNAL   Submitted (13-JAN-2012) Enzyme Technology Laboratory, Biotechnology
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