BIODIESEL FROM RUBBER SEED OIL

Thesis submitted to
University of Calicut
in partial fulfillment of requirements
for the award of the degree of

Doctor of Philosophy
in
Botany

by

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CERTIFICATE

Certified that there are no corrections or suggestions recommended by the adjudicators in this thesis titled **"Biodiesel from rubber seed oil"** submitted to University of Calicut by **Mr. Abdul Faisal P.** under our guidance and supervision for the award of Ph.D. degree in Botany and the contents in the thesis and the soft copy are one and the same.

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CERTIFICATE

Certified that this thesis titled "**Biodiesel from rubber seed oil**" embodies the results of a piece of bona fide research work carried out as part fulfillment of requirements for the degree of Doctor of Philosophy in Botany of University of Calicut by **Mr. Abdul Faisal P.** under our guidance and supervision and that no part of the thesis has been submitted for the award of any other degree.

We further certify that such helps or sources of information availed of in this connection have been duly acknowledged.

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DECLARATION

I, Abdul Faisal P., hereby declare that this thesis titled 'Biodiesel from rubber seed oil' being submitted in part fulfillment of requirements for the degree of Doctor of Philosophy in Botany of University of Calicut embodies the results of a bona fide research work done by me under the guidance of Dr. K.V. Mohanan, Professor (Retired), Department of Botany, University of Calicut (Former Head of the Department of Botany, Former Director, School of Biosciences & Former Director, Interuniversity Centre for Plant Biotechnology) and co-guidance of Dr. A. Yusuf (Assistant Professor, Department of Botany & Director, Interuniversity Centre for Plant Biotechnology) and that no part of it has previously formed the basis for the award of any degree, diploma, associateship, fellowship, title or recognition.

Calicut University
10.02.2022

Abdul Faisal P.

Acknowledgement

I first extend my gratitude to the super natural power who is sovereign, merciful and compassionate by whose grace and blessing, a moral being like me has made a small beginning heralding a new chapter in my life.

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. Words cannot adequately express my deep sense of gratitude to my beloved Professor Sailas Benjamin (Late), who opened me the door to the world of scientific venture and with his perpetual energy and enthusiasm in research motivated me with all his advises and to his family for their support, love and prayers. I will cherish his memories in my heart forever.

I accord my profound gratitude to my research supervisor, Dr. K.V. Mohanan, Professor (Rtd.), Department of Botany, University of Calicut (Former Head of the Department of Botany, Former Director, School of Biosciences & Former Director, Interuniversity Centre for Plant Biotechnology), who directed my thoughts, deeds and actions throughout this period of investigation with suggestions, criticism, encouragement and guidance of every possible kind. I extend my deepest gratitude to my research co-guide, Dr. A. Yusuf, Assistant Professor, Plant Biotechnology Division, Department of Botany & Director, Interuniversity Centre for Plant Biotechnology, for the help, motivation and immense support that he showered on me for the successful completion of the present thesis. I express my sincere gratitude to Kerala State Council for Science, Technology and Environment (KSCSTE), Kerala for providing financial support for the successful completion of the major project on alternate fuels in which I worked as project fellow and extended it as my PhD work and also to University of Calicut, for providing research fellowship for the remaining period.

I am deeply indebted to Prof. Jos T. Puthur, Head of the Department and Prof. V.V. Radakrishnan, Prof. Santhosh Nampy, Prof. John E. Thoppil and Prof. K. M. Jayaram former Heads, Department of Botany, University of Calicut 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 sincerely thank Prof. C.C. Harilal, Enivormental Science Laboratory, University of Calicut for providing necessary laboratory facilities and every kind of help through these difficult years. I gratefully extend my sincere thanks to Prof. P. Manimohan, Prof. M. Sabu, Dr. Sunoj Kumar, Dr. A.K. Pradeep, Dr. M. Shamina and all the other teaching and non-teaching staff members of Department of Botany, University of Calicut for providing needful help during the research period. I convey my immense sense of gratitude to my labmates, Mr. Kunhalavi, Dr. Sureka Y. Pawar and Dr. Neethu in the Genetics and Plant Breeding Laboratory for being very helpful and supportive during my research. I also extend my gratitude to my former labmates Dr. V.N. Jisha, Dr. S. Pradeep, Dr. Priji Prakasan, Dr. K.N. Unni, Dr. S. Sajith, Dr. S. Sreedevi and Dr. M.K. Sarath Josh in the Enzyme Technology Laboratory for being very helpful, suggestive and supportive during my research. Mere words of acknowledgment will never express my profound gratitude and indebtedness to my true friends Dr. E.S. Hareesh, Dr. E. Janeeshma and Miss. R. Maya for giving me moral support during my research work. Their understanding, encouraging and personal guidance provided a good basis for the present thesis.

I owe my deepest gratitude to all my teachers who uplifted me to the world of knowledge. I am forever indebted to my loving teachers at GWLPS, AMUPS and GHSS Irimbiliyam for they showed me the way and stressed the importance of diligence and determination. I also wish to express my deep sense of gratitude to all my teachers at SNGS College Pattambi, for their excellent guidance, encouragement and motivation. I express my respect and gratitude to all my teachers for guiding me as a student as well as a person.

I convey my immense sense of gratitude to Prof. N.K. Renuka, Department of Chemistry, University of Calicut and Prof. J. Shibuvardhanan, Department of Zoology, University of Calicut for the helps rendered. I am indebted to the other Departments of Calicut University who provided various instrumentation

facilities. I greatly value the genuine support and encouragement of Dr. N.N. Binitha, Assistant Professor, Research and Post graduate Department of Chemistry, SNGS College Pattambi for GC analysis and interpretation, Dr. Priji Prakasan, Post Doc Fellow, CUSAT, for helping me in RSM analysis and also to Dr. Divya & Dr. Subair, Department of Chemistry, University of Calicut, for helping in FT-IR analysis. Dr. Vinu V. and Dr. Sudha K., Research and Post graduate Department of Chemistry, SNGS College Pattambi helped me with reliable and energetic efforts during this research work. Their encouragement and personal guidance provided a good basis for the present thesis. I also convey my gratitude to CSIF (Central Sophisticated Instrumentation Facility), University of Calicut for SEM & CHNS analysis and Mr. Chandrashekar, Agilent Analytical Labs, Bangalore for GC-MS analysis and interpretation. 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 would like to thank Abijithettan and Jijeshettan for providing chemicals and Binuettan, Remi, Niklesh, Biolinx, Rana Instruments Chennai, Southern Gas Agency, Arun Lab Line and USIC staff for their timely support in overcoming the technical problems associated with instruments. I express my thanks to Dr. P. M. Prakasan, Former Librarian, Department of Botany for the helps rendered. I also express my thanks to Mr. K. Rajesh & co-workers of Bina Photostat, Villunniyal for their tireless support in preparing this manuscript. I express my profound gratitude to all my friends, especially Dr. Syam Radh, Drisya V., Shahina A.S., Swetha K., Nitheesh Roy, Dr. B. Neethu Kannan, Dr. Manu Philip, Dr. Mirshad P.P., Dr. Santhosh Kumar, Sarath G Nair, Dr. Hareesh V.S., Aneesh S., Vishnu, Noble Louis, Akhil, Rajeesh, Dr. Lins Simon, Habeeb, Jishin Prakash, Sameena P.P., Shafeef and Dr. K.V. Ajayan for their loving support, encouragement and care. My special words of thanks to all those who directly or indirectly helped me during this period.

I am deeply indebted to my parents, P. Abdulla and T.U. Ayisha for their unconditional love, affectionate support and great inspiration at all times; I realize the depth of their sacrifices and am forever indebted to them for giving me the freedom to take decisions in my life. I offer my sincere gratitude to my beloved better half Ruhsana P., for her constant love, concern, care and encouragement that gave me the strength and patience to complete my work successfully. My word power fails to express my feeling of gratitude to my loving brothers Muhammed Musthafa and Abdul Rashid, my loved sister Farsana, brother in law Shihabudheen, my nephews Muhammed Bilal, Minha Fathima, Shabana Parvin, Rifa Meharin and Muhammed Mishal for their unconditional love and constant encouragement.

Finally, I express my sincere gratitude to my father-in-law, P. Siddique and mother-in-law, P. Rukkiya for their immense support and care.

Abdul Faisal P.

Dedicated to

Professor Sailas Benjamin (Late)

CONTENTS

PREFACE

Renewable sources of fuels such as biodiesel have become the dream of science since the natural sources of petroleum fuels get depleted rapidly. Moreover, problems of pollution associated with the use of petro-fuels have raised serious concerns. Even when the scientific world is in pursuit of searching for alternate sources of energy like electricity and solar energy for transport purposes, one of the important alternatives is biodiesel produced from non-edible oils obtained from plants. Biodiesel is an eco-friendly; non-toxic, biodegradable and non-inflammable substitute for petro-diesel and chemically these are monoalkyl esters of fatty acids.

Commercial production of biodiesel is still in its early stages of growth and the raw material used and the process involved in transesterification are the major concerns involved. Edible vegetable oils cannot be used for biodiesel production due to their high cost. Moreover, the common method used now is chemically catalyzed transesterification, which has several bottlenecks that can be overcome by using lipase produced by bacteria as the catalyst.

The present experimental programme was planned and executed with a view of using rubber seed oil which is a non edible oil that can be made available at very low cost as the raw material and lipase produced by two strains of *Pseudomonas* as the enzyme. The advantage of using solid state fermentation technique in biodiesel production from rubber seed oil and immobilization of lipase on a substrate and subsequent use of it for repeated cycles of biodiesel production have also been investigated.

Possibility of using decyanated rubber seed cake as a substrate for bacterial production of lipase has been studied. The fuel properties of the biodiesel samples prepared have been analyzed and those were in the range of the values proposed by international standards. The present study has suggested the suitability of it for use as a fuel substitute for petro-diesel.

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LIST OF FIGURES

EQUIPMENTS USED

Item	Brand	Country
Autoclave	Labline	India
CHNS-O analyzer-Flash 2000	Thermo Fisher Scientific	India
Compound microscope	Magnus	India
Cooling centrifuge	Remi	India
Digital SLR camera	Nikon	Japan
Digital pH meter MK-VI	Systronics	India
Double distillation unit	Borosil	India
Environmental shaker	Inlabco	India
Fluorescence microscope	Leica M80	Germany
FT-IR spectrometer	Jasco	Japan
Gas chromatograph	Shimadzu 2010 plus	Japan
Heating mantle	Kemi	India
Image analyser	Nikon Eclipse E400	Japan
Incubator	Inlabco	India
Laboratory oven	Labline	India
Laminar air flow cabinet	Kemi	India
Lyophilizer	Scanvac	Japan
Magnetic stirrer (KMS - 400)	Kemi	India
Micropipettes (0.5 -1000 µl)	Accupipete	India
Minitab 14	Minitab, Inc	USA
Mixer grinder	Philips	India
Mixing block	Bioer	UK
Pressure cooker	Prestige	India
Refrigerated centrifuge	Remi	India
Refrigerator	Godrej	India
Rotary evaporator	Inlabco	India
Scanning electron microscope	Hitachi	Japan
UV-Visible spectrophotometer	Shimadzu	Japan
Viscometer	Borosil	India
Vortex mixture	Kemi	India
Water bath	Rotek	India
Weighing balance	Shimadzu	Japan

ABBREVIATIONS

BSM	:	Basal Salt Medium
CO_2	:	Carbon dioxide
NOx	:	Nitrogen oxides
SO_2	:	Sulphur dioxide
HC	:	Hydrocarbons
SSF	:	Solid State Fermentation
SmF	:	Submerged Fermentation
RSM	:	Response Surface Methodology
BBD	:	Box Behnken Design
RSO	:	Rubber Seed Oil
FAMEs	:	FattyAacid Methyl Esters
GC	:	Gas Chromatography
GC-MS	:	Gas Chromatography-Mass spectrometery
TLC	:	Thin Layer Chromatography
ASTM	:	American Society for Testing and Materials
EN	:	European Norms
IS	:	Indian Standards
MMT	:	Million Metric Tonnes
GW	:	Giga Watt
BECI	:	Bio-Energy Council of India
CH_4	:	Methane
H_2S	:	Hydrogen sulphide
R&D	:	Research and Development
WCO	:	Waste Cooking Oil
RRII	:	Rubber Research Institute of India
Kg	:	Kilogram
Cm	:	Centimeter
g	:	Gram
HCN	:	Hydrogen cyanide
3D	:	Three Dimensional
β	:	Beta
U/ml	:	Units per mililitre
U/gds	:	Units per gram dry substrate
U/g	:	Unit per gram

h	:	Hour
d	:	Day
ddH_2O	:	double distilled water
FTIR	:	fourier transform infrared spectroscopy
g/l	:	gram per litre
TAGs	:	Triacyl glycerides
v/v	:	Volume per Volume
°C	:	Degree Celsius
%	:	Percentage
FFA	:	Free Fatty Acids
HCl	:	Hydrochloric acid
H_2SO_4	:	Sulphuric acid
NaOH	:	Sodium hydroxide
KOH	:	Potassium hydroxide
TCA	:	Trichloroacetic acid
BSA	:	bovine Serum Albumin
CCD	:	Central Composite Design
ANOVA	:	Analysis of variance
DOE	:	Design of experiment
mg/ml	:	milligram per milliliter
M	:	Molar
m	:	Meter
Min	:	Minutes
ml	:	Milliliter
g/kg	:	Gram per kilogram
ml/min	:	Milliliter per minute
mm	:	Millimeter
MPa	:	Mega pascal
°C/min	:	Degree Celsius per minute
MTCC	:	Microbial Type Culture Collection
ATCC	:	American Type Culture Collection
MW	:	Molecular Weight
*p*NPP	:	*para*-nitrophenyl palmitate
rpm	:	Rotation per minute
Sec	:	Seconds
SDS-PAGE	:	Sodium dodecylsulphate-polyacrylamide gel electrophoresis

w/v	:	weight per volume
µg	:	Microgram
N	:	Normal
λ	:	Wave length
RB	:	Round Bottom
kDa	:	Kilo Dalton
wt/wt	:	Weight per weight
wt%	:	Weight percentage
w/v	:	Weight per volume
nm	:	Nano meter
mM	:	Milli molar
µm	:	Micrometer
µl	:	Microlitre
Kg/m^3	:	Kilogram per cubic metre
g/cm^3	:	Gram per cubic centimetre
b.p.	;	Boiling point
ppm	:	Parts per million
KBr	:	Potassium bromide
MSG	:	Monosodium glutamate
TCD	:	Thermal Conductivity Detector
TBA	:	Thiobarbituric acid
NB	:	Nutrient Broth
NA	:	Nutrient Agar
DMSO	:	Dimethyl sulfoxide
DPPH	:	*α,α*-diphenyl-*β*-picrylhydrazyl
MSTFA	:	*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide
RSK	:	Rubber seed kernel
RSS	:	Rubber seed shell
RSP	:	Rubber seed powder
FESEM	:	Field Emission Scanning Electron Microscopy
TEM	:	Transmission Electron Microscopy
R^2	:	Coefficient of determination
CV	:	Coefficient of variation
S/N ratio	:	Signal/noise ratio

Chapter 1 INTRODUCTION

Energy independence is one of the dreams of every nation. Energy demand of the world is mainly satisfied by fossil fuel resources such as petroleum, coal and natural gas. But they are unsustainable, dwindling and depleting in nature [1]. Population explosion has created new challenges for the coming generations and the most major one is tremendous increase in energy demand. The combustion of fossil fuels causes release of hazardous gases like carbon dioxide (CO_2), nitrogen oxides (NOx), hydrocarbons (HC), sulphur dioxide (SO_2), etc. and increases atmospheric CO_2 level which is responsible for global warming. This phenomenon results in climate changes in an unpredictable manner, which causes melting of polar icebergs, rising of sea level, etc. Thus, both energy crisis and environmental concern have focused the world's attention on finding sustainable and renewable energy resources. Biofuels are the finite renewable solutions to replace the depleting and polluting fossil fuels [2].

Bioenergy is increasingly gaining international attention as a source of renewable energy. Tree borne oils are considered as very promising sources of biofuels, particularly biodiesel [3]. India has a long history of using vegetable oils for domestic and industrial purposes before the invention and production of fossil fuels. Traditionally, oils were extracted from different species of edible and non-edible plants and used as a source of energy for lighting and heating. Gradually, these oils were used to produce value added products like soap, candle, cosmetics and pharmaceuticals. In the past, the use of these oils as a substitute for diesel was not encouraging because diesel was available at a much cheaper price. Now, with the increasing price of petroleum products and increasing concern on oil pollution, vegetable oils are likely to have a permanent demand from the automobile industry in India. The use of vegetable oils as alternative fuels has a history of around 100 years when the inventor of the diesel engine Rudolf Diesel first tested peanut oil in his compression ignition engine. He had opined that the use of vegetable oils as engine fuels might seem insignificant at that time but

such oils might become in the course of time, as important as petroleum and the coal tar products. More than 90% of biodiesel production comes from food grade vegetable oils such as coconut oil, soya bean oil, palm oil, sunflower oil, rapeseed oil, linseed oil and sesame oil. As these commodities are of immense use in food, pharmaceutical and cosmetic industries, their use in biodiesel production incurs higher expenditure and it is not economically feasible [4]. These hurdles can be overcome by the use of non-edible oil feed stocks, which possess high adaptability to wastelands and sustained high yield of oil with low cost of production [5]. The use of non-edible oils from the seeds of plants like jatropha, rubber, castor, linseed, moringa, cotton, karanja, neem and tobacco has been proposed to avert the imbalance between human nutrition and fuel sources [6]. Among the non-edible vegetable oils, jatropha oil is the mainly targeted feedstock to meet the rising fuel demands of countries around the world; but its cultivation needs special attention and conversion of agricultural land. In consideration of the cost-effective approach of biodiesel production, the rubber tree has been less investigated on its potentials in Indian context. India Rubber Meet 2016 in Goa reported that the country is the sixth largest producer and fourth largest consumer of natural rubber in the world. In India, rubber plantations are highly concentrated in the southern of Kerala (**Figure 1.1**). A rubber plantation is estimated to produce about 2 tonnes of rubber seeds per hectare per year [7]. Underutilized rubber seeds contain significant amount of oil. Different methods are used to extract the oil from rubber seeds and soxhlet extraction method is usually practiced at industrial level. Oil extraction from rubber seeds employing n-hexane as solvent is also being practiced [8].

Biodiesel is an eco-friendly, non-toxic, biodegradable and non-inflammable substitute for petroleum based diesel and it has significantly lower level of emissions. Biodiesels are monoalkyl esters of fatty acids derived from vegetable oils and animal fats made up of triglycerides which have striking similarity to petroleum derived diesel. It can be used either solely or blended with diesel practically at any proportion without making changes in the existing distribution infrastructure [9].

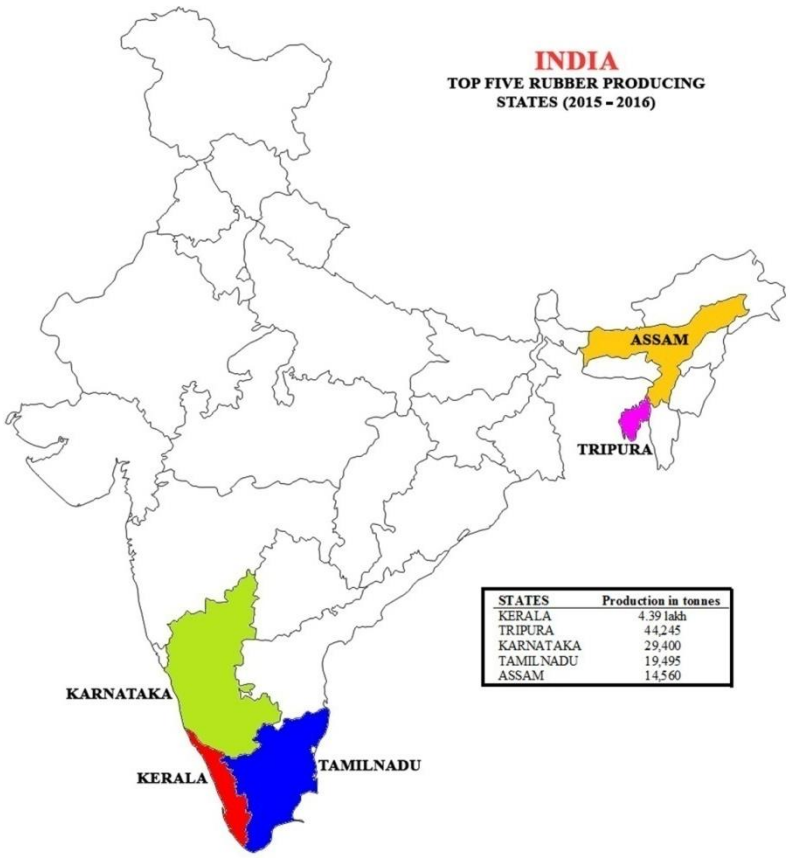

Figure 1.1. Rubber production in India
(Indian Rubber Statistics, Volume 37, 2016)

In view of environmental considerations, biodiesel is considered "carbon neutral" because the carbon dioxide released during consumption has been already sequestered from the atmosphere during the growth of the crop. Conventionally, four methods are followed for the manufacture of biodiesel. They are (a) direct use and blending (b) micro emulsion (c) thermal cracking or pyrolysis (d) transesterification. Among these methods, transesterification seems to be the best and it is the process of exchanging the organic group of an ester with the organic group of an alcohol. Transesterification of triglycerides with methanol can be carried out using both homogeneous (acid or base) and heterogeneous (acid or base) catalysts, but with much limitations. These limitations can be overcome by using green catalysts [10].

Enzymes are biological catalysts and majority of the industrial enzymes are of microbial origin. About 60% of the industrial enzymes are hydrolytic in

nature, among which lipolytic enzymes draw enormous attention because of their immense biotechnological potentials. For environmental and economic reasons, heterogeneous catalysts are better than the homogeneous ones. Enzymatic transesterification has attracted much attention in biodiesel production as it produces high purity product and enables easy separation from the byproduct, glycerol. The enzyme that was found to be capable of catalyzing methanolysis is lipase. Lipases (EC 3.1.1.3) are a subclass of esterase that catalyzes the breakdown of fats and synthesizes esters of glycerol and long chain fatty acids. They have widespread occurrence throughout the flora and fauna, though more abundant in the microflora comprising bacteria, fungi and yeast. Many species of bacterial genera such as *Pseudomonas*, *Bacillus*, *Serratia*, *Alcaligens*, etc. and fungi such as *Aspergillus*, *Penicillium* and *Candida* are the best known producers of lipases [11]. Recent research reports from our laboratory have found out that *Pseudomonas aeruginosa* strain BUP2 and *Pseudomonas* sp. BUP6 isolated from the rumen of Malabari goat are potent producers of lipase [12, 13].

Solid State Fermentation (SSF) is a fermentation technique performed on solid substrate that acts as physical support as well as nutrient source. Many microorganisms secrete lipases during growth on organic residues. By dint of low production cost, greater stability, simplicity and wider availability when compared to Submerged Fermentation (SmF), SSF is considered a novel strategy with higher physiological significance, reliability, cost effectiveness and industrial potentials [14].

The conventional one-by-one optimization strategy for all parameters in obtaining a suitable condition with the maximum yield is a tedious task since large number of combinations would be involved in the process. Therefore, the present study employs Response Surface Methodology (RSM). RSM is a widely used statistical cum mathematical technique, employed for the optimization, modeling and analysis of the production of biodiesel [15]. Box Behnken Design (BBD) and Taguchi method are the most common designs in RSM studies, which in combination with RSM technique is attempted here for the optimization of

different variables so as to attain the maximum catalytic efficiency in the process of the production of biodiesel. To the best of our knowledge, there are no reports on the successful production of biodiesel in reference to rubber seed oil (RSO) with high free fatty acid (FFA), using onsite lipase. Thus, this study is expected to be helpful in the development of a self-sustained system for the production of biodiesel in an industrial perspective from non-edible and untapped RSO, coupled with low cost microbial lipase.

Upon this background, the present study mainly focused on the exploration of unutilized rubber seed for the production of industrially significant biomolecules with special emphasis on lipases and biodiesel. Thus, this study addressed the following objectives:

1. Primary seed characterization and extraction of rubber seed oil by mechanical / soxhlet extraction method.
2. Investigations on the effect of solvents in the extraction of rubber seed oil.
3. Determination and deactivation of cyanogenic glucoside content of rubber seed.
4. Investigations on physico-chemical properties of rubber seed oil.
5. Evaluation of the antibacterial and antioxidant capacity of RSO.
6. Optimization of production, purification and characterization of lipase from decyanated rubber seed cake by SSF method.
7. Immobilization of lipases on celite.
8. Optimization of transesterification of rubber seed oil into biodiesel using onsite lipases by Box-Behnken Design in Response Surface Methodology.
9. Optimization of transesterification of rubber seed oil into biodiesel using immobilized lipases by Taguchi method.
10. Analysis of fatty acid methyl esters (FAME) by TLC, GC, FTIR spectroscopy, GC-MS and CHNS analysis.
11. Analysis of the properties of the biodiesel produced and comparison with ASTM (American Society for Testing and Materials) and EN (European Norms) standards.

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Chapter 2 REVIEW OF LITERATURE

It is a tedious task to give a simple and meaningful definition for energy, which embraces many forms. Generally, it is defined as the capacity of a system to do work. The word energy comes from the Greek epithet ‘energeia’ meaning ‘work’. Energy is stored in the form of potential energy in diverse storehouses in nature. Some of them are renewable or reproducible within a human life span and some are replenished taking millions of years thus making energy resources classified in to renewable and non-renewable.

Energy resources are the primary sources of growth and development of any country [1]. Earth has limited reserves of non-renewable energy resources like fossil fuels, especially coal, natural gas and petroleum. Increasing global connectivity and fast growth of global market especially through industrialization and motorization has led to tremendous increase in the number of vehicles resulting in rapid decline of petroleum resources and dynamic fuel pricing. Devastating emissions caused by petroleum products have forced industrialists and research groups to adopt new technologies aimed at limited environmental pollution and exploitation of renewable resources. Replacement of non-renewable energy sources with renewable resources and the maximum utilization of bioenergy are the major aspects of economic bioprocess. Nowadays, biofuels like bioethanol and biodiesel have significant impact on the global commercial market. Moreover, biofuels offer many advantages including renewability, sustainability, biodegradability, reduction of environmental pollution and increase in regional development and rural manufacturing. The idea of biodiesel was introduced from vegetable oil by Duffy and Patrick in 1853. In 1893, Rudolf Diesel designed the first diesel engine with peanut oil as the fuel. Biodiesel can be used in diesel engines in the pure form or blended with petroleum diesel. If used in the blended form, there is no need for engine modification [2].

2.1. Energy scenario in India

In India, above 60% of the population live in villages/rural areas and they use cheap and domestic energy sources for their day-to-day requirements. Only 80-90% of the rural households are electrified and the remaining 10-20% still lives in dark. Electricity might have reached their village but not yet to their homes [3]. They use solid biomass like dry/decaying wood as the primary source of energy and some are using kerosene; its use in traditional chimney and stoves is a major cause of air pollution. India has a very good background of using non-edible oils obtained from plants as fuel both at domestic and industrial levels.

India is placed the third largest oil consumer in the world behind USA and China. To meet oil demand, India mainly depends on Middle East countries and African countries. India's oil imports increased by about 1.2% in 2017 and reach at 4.37 million barrels per day [4]. Indian consumption of petroleum products also shows an alarming increase from 104.126 Million Metric Tonnes (MMT) in 2002-03 to 184.674 MMT in 2015-16 at a rate of 11.57% as compared to consumption of 165.520 MMT during 2014-15. It reached 193.745 MMT in 2016-17 with a declining of growth rate from 11.57% to 4.91% [5].

Among the petroleum products, diesel is the most important fuel by dint of its wide application almost in all areas of human life like transportation, agricultural, domestic and industrial sectors. Indian industries now have 10-12% of annual growth; but this growth will only be sustainable for the next few years. Securing long-term supply of energy sources is essential to ensure the growth rate by fulfilling the country's future energy requirements. All the countries across the world including the non-petroleum producing countries like India face high scarcity of fossil fuels. In this scenario, considering the importance of energy scarcity and utilization of energy resources the Government of India has taken several steps for the country's sustainable development. Strategies have been developed for the utilization of non-fossil and renewable energy sources such as solar energy, wind energy, hydel energy, tidal energy and biomass energy on a sustainable basis through Bureau of Energy Efficiency (BEE), Ministry of Power,

Government of India. Initiative programmes include Jawaharlal Nehru National Solar Mission (JNNSM), Solar Lantern Programme, Remote Village Lighting Programme, National Biomass Cook stoves Initiative (NBCI), Green Energy Corridor, Repowering of Wind Power Projects, International Solar Alliance and Surya Mitra Scheme [6].

Green revolution has played a pivotal role in Indian agricultural growth and development. About 54.6% of the population is engaged in agriculture and allied sectors. They have to look in to the food and nutritional requirements of the country and also to ensure the sustainable development of the country's wealth [7]. According to the Economy Survey of Government of India (ESGI), India's arable land area of 394.6 million acres is the second largest in the world, behind the United States and the total irrigated crop area of 215.6 million acres is the largest in the world. Given a demand-based market, India can easily fix its potential to cultivate large area of oil-bearing plants. Government of India has initiated a national programme to plant oil-bearing plants on waste/arid lands so that higher amounts of industrial oils will be available in near future. Such oils produced indigenously can be used to produce biofuels so as to partially substitute petroleum fuels and reduce the usage of fossil fuels. Thus, ministries of Rural Development, Environment and Forestry, Petroleum and Natural Gas, Agriculture and Non-conventional Energy Source have taken leading roles in formulating and promoting biodiesel projects. Production and use of biofuels is essential for sustainable development in energy efficiency, increase in productivity and significant reduction in green house gas emissions, solid waste production and thermal pollution [8].

2.2. Non-renewable energy resources

The resources of non-renewable energy which do not replenish within human life time are limited. Basically, they are categorized under the group of carbon derived organic substances. Non-renewable energy sources are stored in nature in the form of coal, crude oil, natural gas and petroleum products. These originated in the 'carboniferous period' that occurred about 300 million years ago

and evolved in the long process of deposition and decomposition of dead organic matter of marine organisms, amphibians, plants and animals settled on the ocean floor or in the porous rocks. Porous rocks on the ocean floor are filled with the energy pockets of coal, oil and natural gas. During earthquakes and tectonic shifts, these get collected in certain particular areas. With the help of modern tools, geologists can locate them [9].

2.2. 1. Coal

Coal is the world's earliest and the most abundant sources of energy - more available than crude oil and natural gas. The energy in coal comes from the energy stored in giant plants and ferns died and preserved probably at the bottom of swamps. Coal chiefly comprises of carbon, hydrogen, oxygen, sulphur, etc. Coal reserves are available ubiquitous, with recoverable reserves in around 70 countries. The biggest reserves are found in the United States, Russia, China, Australia and India [10]. There are six main types of coal that are commonly used by humans namely peat, lignite, bituminous/sub bituminous coal, steam coal, anthracite and graphite [11]. Over ninety percentage of the coal is consumed to generate electricity. Other basics uses of coal power are in steel, cement and paper industries and it is used in numerous other industries as well. A large expansion of coal output makes India the second largest coal producer in the world, but rising demand also indicates that India will become, before 2025, the world's largest coal importer, overtaking Japan, the European Union and China [12].

2.2.2. Petroleum/ crude oil

Petroleum is the general term used to denote crude oil and natural gas and is commonly known as "black gold". It is one of the resources that make the backbone of commodity markets of the world. The word petroleum comes from ancient Greek and medival Latin terms 'petra' meaning 'rock' and 'oleum' meaning 'oil'. Petroleum is a broad group that covers both naturally occurring unprocessed crude oil and petroleum products that are made up of refined crude oil. Crude oil chiefly comprises of hydrocarbons and other organic compounds

made of oxygen, nitrogen and sulfur, and trace amounts of metals such as iron, copper, nickel and vanadium. It can be refined to produce valuable market products like gasoline, diesel and various petrochemical solvents. Saudi Arabia is the world's number one oil exporter and the second largest owner of reserves of naturally occurring oil in the world behind Venezuela [13].

2.3. Renewable energy sources

Renewable energy sources represent the energy produced from sources that do not deplete or can be replenished within our lifetime rapidly from energy sources like solar, wind, geothermal, biomass, hydropower, etc. which are constantly 'renewed' by nature. Today, mankind is very much concerned about the effect of environment on life, and as time passes, renewable energy sources will become the main energy sources on the planet leading to a future that is hundred percent green. Various policy measures have been initiated and special steps taken in addition to providing financial support to various schemes being implemented in this direction in India by the Ministry of New and Renewable Energy for achieving the target of renewable energy capacity to 175 Giga Watt (GW) by the year 2022 [14].

2.3.1. Solar energy

Sun is called 'mother' of all forms of energy. Solar energy is the energy that originates from solar radiations. This energy can be employed in two ways; through thermal route or through the photovoltaic route. It has been used for many processes in industries such as generating electricity, heating, agriculture, transport, biofuels, etc. In India, solar power is a fast developing industry and the country's solar installed capacity reached 20 GW in February 2018. Tamil Nadu is the state with the highest installed solar power capacity in India, while the largest floating solar power plant has been set up on the Banasura Sagar reservoir in Wayanad district of Kerala state [15]. Cochin International Airport of Kerala state is the first airport in the world to run completely on solar power. The growth and increase of cheap solar energy technologies will give huge long-term benefits and it will be helpful to increase the country's economic strength [16].

2.3.2. Hydro energy

Hydro energy is the most accepted ‘green’ technology and it is obtained from water utilizing the basic laws of physics. Water moves and flows downward due to gravity and it is used to rotate turbines so that the kinetic energy of flowing water is converted to mechanical energy. Then through magnetic induction, the generator converts the mechanical energy of the turbines to electricity. It is reliable, cost effective and environment friendly and is a major source of renewable energy used all over the world today to produce electricity. India is the seventh largest producer of hydroelectric power in the world [17].

2.3.3. Wind energy

Wind energy technology is a substitute to conventional energy resources. It is abundant, renewable and environment friendly. In the world, wind energy production showed a growth of 12.5% in 2017. It is predicted that after ten years, the wind power would be contributing up to 19% of the world's electricity thus resulting in considerable reduction in the emission of green house gases. By 2050, 25-30% of global power generation would be from wind. Globally, China has the world's biggest wind energy sector; they share 31% of the world production. Recently, Indian wind energy production has significantly increased. India has the fourth largest installed wind energy capacity in the world. In India, Tamil Nadu has become a leader in the production of wind energy [18].

2.3.4. Nuclear energy

Nuclear energy is the energy that comes from the core of an atom, which is utilized to make electricity. Generally, two methods are followed for the production of this type of energy: nuclear fission and nuclear fusion. Nuclear fusion is the combination of two lighter atoms into a heavier one and nuclear fission is the splitting of a heavy atom. The first commercial nuclear power station was started in the 1950s. Presently, nuclear energy provides about 11% of the world’s electricity from about 450 nuclear power reactors. This development continues and 50 countries utilize nuclear energy produced from about 225 research reactors. In addition, about 60 more reactors are under

construction, while an additional 150-160 are planned. Nuclear energy is the world's second largest source of low carbon power which has been reported as >30% of the total in 2015 [19].

2.3.5. Geothermal energy

Geothermal energy is the thermal energy generated from the core of the earth. Earth's heat is created by the radioactive decay of minerals and that results in high temperature. The high temperature and pressure creates magma (molten rock) in the aesthenosphere which is lighter than solid rock [20, 21]. The first geothermal electric power plant was established in 1904 at Lardarello by the Italian scientist Piero Ginori Conti. Geothermal power is clean, safe, renewable, sustainable, cost effective, reliable and environment friendly; but it releases hazardous hydrogen sulphide with rotten egg smell [22].

2.3.6. Bioenergy

Bioenergy is a form of renewable energy made available from biological sources including living or recently dead organisms and any byproducts of those organisms. It can be used to generate electricity, thermal energy or to produce biofuels and this helps to mitigate climate change by reducing greenhouse gas emissions. Bio-Energy Council of India (BECI) has been established to promote and coordinate the development of the Bioenergy industry in India. BECI is an amalgamation of the renewable energy sector in India comprising various forms of bioenergy including biomass (agri and organic waste) bio-pellets, bio-ethanol, bio-diesel, bio-oil, bio-gas and bio-power as the spectrum of business. It has taken special steps to provide financial support to various schemes being implemented for the development of bioenergy industry in India. BECI focuses to reinventing the clean energy scenario in India by 2022 and it helps to increase regional development and to open up novel opportunities in the employment sector [23].

2.3.6.1. Biomass

The word ‘biomass’ clearly means the organic matter of living plants and animals. It includes wood, leaves, fruits, flowers, grains, seeds and vegetable oils

from plants and animal waste, meat, milk and fats from animals. Main sources of biomass are forests, agricultural land, animal waste, municipal solid waste and industrial waste. Biomass from plants can be broadly classified into two: the first category is the traditional solid mass like cellulose, hemicellulose, lignin, starch, sugar, protein and triglyceride and the second category is the products obtained by the conversion of solid biomass into secondary energy products like ethanol, methanol and biofuels. It is one of the most important renewable sources as it contains energy similar to fossil fuels like petroleum and coal. The biomass fixed in plants by photosynthesis is the most abundant and underutilized biological resource on the planet and is a promising source of material for fuels and raw materials. In its most basic form plant biomass can simply be burned in order to produce heat and electricity. Before the industrial revolution, biomass was the world's most dominant energy source. In the present circumstances, it has great importance in making use of dedicated crops as feedstock for biofuel production [24].

2.3.6.2. Biogas

Biogas is a gas produced from the anaerobic fermentation of organic matters such as carbohydrates in plant material or biodegradable materials like manure, sewage and municipal waste by bacteria [25]. It is mainly methane (CH_4) and carbon dioxide (CO_2) and may have trace amounts of hydrogen sulphide (H_2S), moisture and siloxanes. However, the percentage of methane within biogas can vary between 50% and 80%, depending on the availability of oxygen at the beginning or during the process. When oxygen is required, the bacteria will respire aerobically and will produce a gas with a higher concentration of CO_2 and limited proportion of CH_4. The aerobic digestion residues settle to the bottom of the plant and can be run off and used as manure. These types of biogas generators are most commonly used in the developing world to satisfy the needs of small families. In India, Nepal, Pakistan and Bangladesh biogas produced from the anaerobic digestion of manure in small-scale digestion facilities is called gobar gas and it is estimated that such facilities exist in over 2 million households in India, 50,000 in Bangladesh and thousands

in Pakistan, particularly North Punjab, due to the thriving population of livestock. In India alone, there are over 250 million cattle and if one third of the dung produced annually from these is available for production of biogas, more than 12 million biogas plants can be installed [26]. Within green biorefinery concepts, biogas production is a key technology for the sustainable use of grassland biomass. Algal biomass also can be used to produce biogas through anaerobic digestion [27].

2.3.6.3. Bioethanol

Globalization, industrialization and increasing world population demand high energy requirements. The principal sources of our energy are petroleum fuels and in anticipation of possible shortage, great attention has gone into the production of renewable energy sources including biofuels such as bioethanol. Bioethanol is considered as one of the major alternatives to petroleum fuels. Bioethanol is a form of biological resources that is mainly derived from agricultural feedstocks by sugar fermentation process and also by chemical processes. It can be made from all common energy/food crops such as corn, sugarcane [28], rice [29, 30], potato [31], tapioca [32] and sweet sorghum [33] and also from food processing wastes such as molasses, whey and potato wastes [34]. In 2017, about 117 billion gallons of bioethanol were produced worldwide, of which United States produced the greatest amount in the world (15.8 billion gallons) and with more than seven billion gallons, Brazil ranked second. Biomass mainly is composed of a mixture of carbohydrates such as cellulose, hemicellulose and lignin. Sugarcane and its products are responsible for 40% and grains are responsible for 60% of global ethanol production (primarily in Brazil, Thailand and India) [35]. In order to produce sugar from the biomass, it can be pre-treated with acids or hydrolytic enzymes. The presence of yeast and rapid heating converts the sucrose into fructose and glucose with the help of invertase and these can be further reacted with another enzyme, zymase, to form ethanol and carbon dioxide. Therefore, many studies are going on breeding technologies and genetic engineering to get high bioethanol yield. Bioethanol is commonly used as an additive to petrol in commercial fuel industry at the proportion of 90%

petrol and 10% ethanol. Blending ethanol with petrol helps to burn completely and hence emission rates can be drastically reduced. It is also biodegradable and less toxic than fossil fuels. Carbon dioxide is absorbed by plants and processed via photosynthesis to help the plants to grow. This cycle of synthesis and combustion means bioethanol could potentially be a carbon neutral fuel source. Blending can also lead to reduction in fossil oil use across the globe thereby ensuring higher fuel security and avoiding dependence on oil producing countries to some extent. The rural community will also benefit from the increased demand to grow the crops required for producing bioethanol. However, there are several problems associated with the use of ethanol as an alternative fuel. First, it is costly to produce and use in huge quantities. Most important disadvantage is that some of the ethanol will be partially oxidized and emitted as acetaldehyde, which reacts in air to eventually contribute to the formation of ozone. In addition, the waste product from ethanol production, called swill, though can be used as a soil conditioner on land, is extremely toxic to aquatic life.

2.3.6.4. Biofuels

Biofuels are referred to as a form of liquid or gaseous fuels for the energy sector that are predominantly derived from biomass, that is, plant, algal or animal waste. Biofuels are generally considered to be renewable and are alternative and additive to petroleum fuels. Particularly, in the current scenario of shortage of petroleum, dependence on foreign countries, hike of petroleum prices, shortage of oil refining and increase of environmental concerns, biofuels offer many priorities such as sustainability, cost effectiveness, reduction of exhaust emissions, security of supply and socio-agricultural development [36]. Over the last two decades, the world has turned great attention to biofuels and the industries towards green technologies. In order to promote biofuels in the country, the Indian Government has implemented a “National Policy on Biofuels” which was developed by the Ministry of New and Renewable Energy in 2009. At present, Indian Government has several ongoing programmes such as Make in India, Swachh Bharat Abhiyan, Skill Development, etc. which are aimed to targets of import reduction, cleaner environment, waste to health and wealth

creation, infra structural development in rural areas, employment generation and additional income to farmers. The National Mission has emphasized the scope of feedstock for ethanol production by allowing use of sugar containing resources like sugarcane, sugar beet and sweet sorghum, starch containing crops like corn and cassava and damaged food grains like wheat, broken rice, rotten potatoes and other foods unfit for human consumption. In addition, the policy encourages the production and supply of biodiesel production from non-edible oilseeds, used cooking oil and short gestation crops.

2.3.6.5. Generations of biofuels

Biofuels can be classified in to 1st, 2nd, 3rd and 4th generations depending on the feedstocks and/or the technology involved. First generation biofuels are commonly known as conventional type of biofuels. These are generally produced from food crops by fermentation, distillation and transesterification. Primarily, ethanol is produced by fermentation technology and it contributes one-third of the energy of gasoline. Moreover, it burns cleaner than gasoline and therefore reduces the emission of green house gases. Presently, many countries, including the United States, use this as an additive to gasoline. Another significant product of 1st generation biofuel is biodiesel, which is mainly produced from plant oils or animal fats, which react with alcohol in the presence of catalyst to form a new ester compound. This process is commonly called transesterification. Biodiesel can be used as alternative to petroleum diesel directly or in the form of blends in diesel engines. This technology can be used to overcome fuel scarcity and this also supports agricultural business and the life of rural communities by increasing the demand for the crops used to produce biodiesel. However, there is a chance that this may lead to food crisis and increase in food prices. Already this has caused reduction in the availability of crops in the global food market. Moreover, it has caused a negative impact on biodiversity and also, this is a more expensive option than gasoline, thus making it economically unfavorable.

Generally, 2nd generation biofuels are called advanced biofuels, since they come from distinct biomass like wood, organic waste, food waste and non-edible

food crops. Non-consumable vegetable oil is used as second generation biofuel, because it is not fit for human consumption. The major advantage is that there is no competition with food crops. Second generation biofuels generate higher quantity of energy per unit area from bare and waste land. Second generation biofuels could significantly reduce potential cost and CO_2 production and hence it is desirable to increase their production. When commercialized, the cost of second generation biofuels would be almost equivalent to standard petroleum diesel and more reliable and cost effective [37].

Algae are used as energy source for third generation biofuels which encompass higher quantum of energy when compared to 1^{st} and 2^{nd} generation biofuels. Algae can be grown at low cost using sewage and wastewater and saltwater available in oceans and salt lakes and hence there is no use of fresh water used for human consumption involved. The algae are harvested to extract oil from them and it is converted to biodiesel through a similar process as in the case of 1^{st} generation biofuels. However, further research still needs to be done to make the extraction process simple in order to make it financially competitive to petroleum-based fuels.

Fourth generation biofuels are still with R&D groups and none of them have been practically synthesized and tested. These are expected to be the most promising and are referred to as the most advanced biofuels. The theoretical concepts are explained as the development of genetically modified plants with high oil yield, which are genetic combinations of oil seed plants and algae, which will consume higher amount of carbon dioxide from the atmosphere. Currently only a few companies are working on this and the most popular is Synthetic Genomics of Craig Ventor. They are engaged in prototyping of microorganisms, which can directly produce biofuels from carbon dioxide. It is expected that by 2050 this generation shall become fully developed and shall have a major stake in the power sector of the world.

While biofuels appear to be a more sustainable alternative, there are many problems associated with their production and use. For instance, the use of food

crops for energy production has brought about food shortages and there is a lot of unrest over diverting land and resources to this anvil. Besides, the costs associated with capital outlay for production, use of special material for transport (as in the case of ethanol) and cost of creating new technology do not necessarily make biofuels an attractive financial option. Further, all biofuels are not totally free of environmental impact and certainly do not have the same fuel efficiency to incite interest for total substitution. The answer lies in advancing more investments in research to make biofuels a clean, sustainable and economic fuel resource for the future [38].

2.4. Biodiesel

By dint of energy demand and the deleterious exhaust of hazardous gases from petroleum fuels, investigations on promising alternate fuels have attained good momentum in recent years. As a result, R&D programmes have progressed and the studies are now more focused on the application of vegetable oils as substitutes for fossil fuels. Vegetable oils can be used for the production of biodiesel, which is regarded as an alternative to petro-diesel. It should have important properties of fossil fuels and also should be technically acceptable, economically viable and ecofriendly [39]. Biodiesel has shown its ability to meet the energy demand of the world in transportation, agricultural, commercial, industrial and domestic sectors [40, 41].

Biodiesel refers to the substitutes for conventional diesel fuels that are renewable, non-toxic with low emission profiles of CO_2 and with compatible nature with diesel [42]. Generally, biodiesels are fatty acid alkyl esters like methyl or ethyl esters, produced by the alcoholysis of vegetable oils or animal fat or other lipids and waste cooking oils [43, 44]. Fats and oils are commonly referred to as triglycerides, which are esters of glycerol and made up of one mole of glycerol and three moles of fatty acids [45]. Biodiesel is quite similar to conventional diesel fuel and it can be directly used in existing diesel engines without any modification and can be blended in any ratio with conventional

petroleum and diesel fuel and meets or exceeds the specifications of ASTM D6751 and EN 14214 or any legal successor thereto [46].

2.4.1. History of biodiesel

In the 19th century, the idea of using biodiesel as an alternate fuel to petrodiesel was developed. It started by the use of vegetable oils as alternatives to diesel fuels in emergency situations. In 1940s first trials with vegetable oil methyl and ethyl esters were carried out in France and meanwhile, in Belgium, the famous scientific group working on biodiesel used palm oil ethyl ester as fuel for buses. In the 1990s, France launched the local production of biodiesel fuel from rapeseed oil by transesterification and it became locally popularized as diester [47, 48]. Milestones and various developmental stages of biodiesel are listed in **Table 2.1.**

Table 2.1. Milestone in the development of biodiesel [47]

Year	Events
1830s	Vegetable oils, animal oils and refined turpentine from pine trees were used as lamp oils. Alcohol blends with whale oil were used in most parts of the U.S.
1850s	Blend of alcohol and turpentine called "camphene" or "burning fluid" was used
1853	First scientists E. Duffy and J. Patrick conducted the first transesterification of a vegetable oil
1859	Discovery of petroleum by Edwin Drake in Pennsylvania
1893	Rudolf Diesel's prime diesel engine model, which was fueled by peanut oil, ran for the first time in Augsburg, Germany
1898	The term 'biodiesel' (BD) was first coined by Rudolf Diesel
1900	Rudolf Diesel showed his engine at the world exhibition in Paris; his engine was running on 100% peanut oil
1916	Gutierrez tested castor oil as alternative fuel for the first diesel engine imported into Argentina
1937	The Belgian Scientist, G. Chavanne, was granted a patent for a "procedure for the transformation of vegetable oils". The concept of what is known as "biodiesel" today, was proposed for the first time
1941	Henry Ford built a plastic bodied 'soybean car'
1942	First references to the use of palm oil esters as diesel fuel
1977	A Brazilian scientist, Expedito Parente, applied for the first patent of the industrial process for biodiesel

1979	Research into the use of transesterified sunflower oil, and refining it to diesel fuel standards, was initiated in South Africa
1983	The process for producing fuel-quality, engine-tested biodiesel was completed and published internationally
1987	An Ausrtian company, Gaskoks, established the first biodiesel pilot plant
1989	Gaskoks, established the first industrial scale-plant
1991	Austria's first biodiesel standard was used
1997	German standard, DIN 51606, was formalized
2003	A new Europe-wide biodiesel standard, DIN EN 14214 was published
2005	Minnesota became the US state to mandate that all diesel fuel sold in the state contains part biodiesel, requiring a content of at least 2% biodiesel
2008	ASTM published new Biodiesel Blend Specifications Standards
2008	The current version of the European Standard EN 14214 was published and it supersedes EN 14214:2003

2.4.2. Global biodiesel scenario

Biodiesel is a fast developing alternative fuel in the U.S. and Europe. It was introduced in the early 1990s in United States of America and since then widely commercialized in various countries like Malaysia and Europe. They were utilized to blend a small percentage of biodiesel with diesel for reducing the pollution when it undergoes combustion process. The European countries have implemented 95% tax reduction for using biodiesel in diesel engines. Worldwide, the biodiesel production from vegetable oils during 2004-05 was estimated to be 2.36 million tones. Of this, European countries accounted for 1.93 million tonnes, nearly 89% of all biodiesel production worldwide. U.S. produced 0.14 million tonnes and the rest of the world 0.29 million tonnes. In Europe, U.S., Malaysia and India rapeseed oil, soybean oil, palm oil and jatropha seed oil respectively were used as the major feed stock for biodiesel production [49].

2.4.3. Biodiesel in India

Indian agricultural sector can be considered as the country's economic pillar, with more than 100 million farm holdings. The most widely cultivated crops are cereals, pulses and different types of vegetables. The Indian agriculture sector has been developing steadily during the time of green revolution. This

development was achieved by increasing the area of cultivation and the rate of productivity [50]. Countries like India have taken necessary steps for the production of biodiesel from different feed stocks like non edible oil bearing plants. Government of India constituted Petroleum Conservation Research Association (PCRA) as a registered society under Ministry of Petroleum and Natural Gas in 1978. A committee was also constituted under the Planning Commission and the committee subsequently submitted reports and suggested that biodiesel produced from non-edible oil could be blended at the rate of 20% of biodiesel with diesel [51].

India, after China, Japan, Russia and U.S.A. is ranked 5^{th} in terms of fossil fuel consumption. Reports around 2011 showed that locally produced biodiesel was being used for three wheeler motor rickshaws, and also being used experimentally to run state transport corporation buses in Karnataka state of India and it was reported that biodiesel was a more efficient, cost effective and clean substitute for fossil fuels. Moreover, such a step reduced reliance on fossil fuels thus reducing the emission of atmospheric pollutants and life cycle carbon dioxide emissions and use of biodiesel showed improvements in fuel lubricity and engine wear. One other major economic benefit reported was that production of biodiesel from locally bearing oil plants benefited the local farmers thus making direct impact on a broad range of economic, social and environmental development [52].

2.4.4. Sources of biodiesel

Generally, biodiesel can be produced from triglycerides. Vegetable oils, animal fats, algal oils and used cooking oils are the major feedstocks utilized for the production of biodiesel that runs in diesel engines of car, bus, truck, power generators and construction equipments [53, 54]. For commercial production of biodiesel, researches are more focused on production from vegetable oils than from algae and animal fats [55-58]. Moreover, fats are solid masses in room temperature but oils are liquid in nature, sulphur free, easy to handle, without storage problem, with excellent lubricating properties, environment friendly and greener as feedstock for biodiesel production. Different types of plants are regarded as the sources of vegetable oils. The quantity of CO_2 absorbed from the

atmosphere by plants is higher when compared to the release of CO_2 to the atmosphere during respiration and plants are ecofriendly in this aspect also. Burning of petro-diesel generates higher amount of CO_2 to atmosphere compared to burring of biodiesel. Thus, the use of biodiesel produced from vegetable oils as a fuel will help us to reduce the total CO_2 content of the atmosphere [59].

A large variety of vegetable oils have been used as biofuel. Vegetable oils are triglycerides with unbranched chains of different lengths and degrees of saturation. Molecules of vegetable oils consist of glycerol part and fatty acids part. Glycerol has three carbon atoms and fatty acids have long chains of carbon atoms. Each molecule of fatty acid consists of a carboxyl group attached to a chain of carbon atoms with their associated hydrogen atoms. The number of carbon and hydrogen atoms in the chain determines the quality of those particular fatty acids [60]. The general molecular formula of vegetable oils is shown in **Figure 2.1**. Vegetable oils can be categorized into two: edible oils and non-edible oils. Production of biodiesel from edible and non-edible oils has gathered attention because of the environmental advantages and the renewable nature of the oils. There are more than 350 oil bearing trees and crops identified including the non-edible seed bearing oil plants [61].

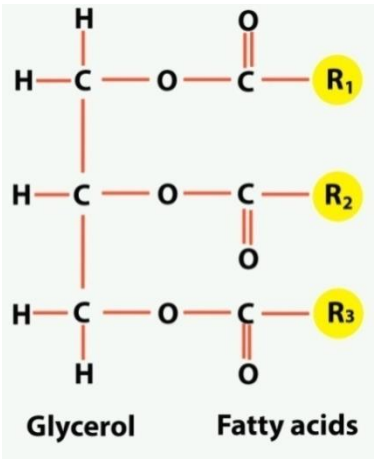

Figure 2.1. General molecular formula of vegetable oil

The major feedstocks for the production of biodiesel include edible oils obtained from plants such as sunflower (*Helianthus annuus*) [62, 63], soybean (*Glycine max*) [64, 65], cottonseed (Gossypium spp.*)* [66-68], rapeseed (*Brassica napus*) [69, 70], groundnut (*Arachis hypogaea*) [71, 72] sesame (*Sesamum*

indicum) [73, 74], oil palm (*Elaeis guineensis*) [75, 76], coconut (*Cocos nucifera*) [77, 78], olive (*Olea europaea*) [79, 80], almond (*Prunus amygdalus*) [81, 82], etc. Among them soybean, sunflower, rapeseed and oil palm are the most studied ones. Recently, studies are being conceded out for biodiesel production from non-edible oil seeds like jatropha (*Jatropha curcas*) [83, 84], castor (*Ricinus communis*) [85, 86], karanja (*Pongamia pinnata*) [87, 88], paradise tree (lakshmi taru) (*Simarouba glauca*) [89, 90], tobacco (*Nicotiana tabacum*) [91, 92], rubber seed (*Hevea brasiliensis*) [93, 94], mahua (*Madhuca indica*) [95, 96] and neem (*Melia azadirachta*) [97, 98].

In recent times, most of the biodiesel studies exploit the edible oil plants as substrates and these vie with food, pharmaceutical and cosmetic uses resulting in high cost of living and high price of the biodiesel generated. The use of non-edible oils from the seeds of plants is being proposed to avert any impending fuel-food crisis. Non-edible oil plants are suitable crops for cultivation on arable land. This is economically feasible when compared to edible crops and this does not drain off the edible vegetable oil supply [99]. Another emerging feedstock used for biodiesel production is micro and macro algae. They have some important properties such as higher biomass production, higher oil yield, faster growth and photosynthetic efficiency when compared to other energy crops. Hence, higher level of attention is being made on oil extraction and production of biodiesel making use of algal oil as the feedstock [100]. Investigations are being made on the utilization of used cooking oil (UCO), chicken fat, lard and tallow for biodiesel production. These materials are inexpensive, readily available and are suitable feedstocks for conversion to biodiesel [101, 102]. Moreover, the use of these substrates as feedstock for biodiesel production provides an alternative way of disposal. Major drawback of animal fat based biodiesel is that it generally has poorer cold temperature properties than vegetable oil-based biodiesel [103].

In India, *Jatropha* is one of the most important feedstock in biodiesel production, whose cultivation demands conversion of agricultural land. Raw materials account for about 70–90% of the production cost of biodiesel and

researchers give special attention to reduce the cost of the raw material [104]. Therefore, the present study has been planed and executed to explore the possibility of producing biodiesel from rubber seed oil since rubber seeds can be easily and cheaply obtained from existing rubber plantations without the utilization of more land and this technology establishes the use of scientific knowledge for the utilization of rubber seeds which otherwise have no economic importance.

2.5. *Hevea brasiliensis* (para rubber tree)

2.5.1. Origin and distribution

Hevea brasiliensis Müll. Arg., the pará rubber tree is the most economically cultivated species of the genus *Hevea* belonging to the spurge family Euphorbiaceae. It is a deciduous perennial plant native to the Amazon forests of South America and cultivated in Asian and African countries also [105]. Rubber cultivations are widely distributed in the South Asian countries namely Thailand, Indonesia, Malaysia, India, China and Vietnam. Thailand is ranked the largest producer of natural rubber in the world; its harvest is estimated to be around a third of the global production. India was the first country in the east to undertake the cultivation of natural rubber as early as in 1880-1890s and commercial cultivation of rubber started in the country in 1902 in Kerala by Periyar Syndicate in the Travancore-Cochin State at Palappilly. On the basis of agro-climatic conditions, rubber growing regions in India can be classified in to two major zones, traditional and non- traditional. Indian rubber cultivation was traditionally confined to Kanyakumari district of Tamil Nadu and Kottayam district of Kerala. When land available for planting in the traditional region declined, Rubber Board searched for other regions suitable for rubber cultivation. Exploratory survey was conducted by the Rubber Board and they found, rubber can be grown as an economically viable crop in the hinterlands of Karnataka and Goa, Konkan region of Maharashtra, coastal regions of Andhra Pradesh and in Orissa, Tripura, Assam and Andaman and Nicobar Islands. India is the sixth largest producer and fourth largest consumer of natural rubber in the world. A rubber plantation is estimated as able to produce about 2 tonnes of rubber seeds per hectare per year [106].

2.5.2. Clones of rubber

Rubber Research Institute of India (RRII) has practiced rubber planting by seed and also using budded and tissue culture plantlets. The quality planting materials approved by Rubber Board of India are classified in to three categories. Category I comprises of materials approved for large scale planting and strongly recommended. Category II clones perform well in medium term periods. Category III planting materials are those which have held out promise of good performance in small scale trials. RRII has further tabulated clones of each category in to specific clones suitable for traditional and non-traditional regions on the basis of agroclimatic conditions and field clonal evaluation trails (**Table 2.2**).

Table 2.2. Specific clones for traditional and non-traditional regions [107]

Region	**Category-I**	**Category-II**	**Category-III**
Traditional Region	RRII 105 PB 260 RRII 414 RRII 417, RRII 422, RRII 430	RRIM 600 GT l RRII 5 RRII 203 PB 28/59 PB 217 PB 312 PB 314 PB 255 PB 280	RRII 118, RRII 208, RRII 300 RRII 429, PR 107, PR 255 PR 261, PB 86, PB 5/51, PB 235 PB 311, PB 330, RRIM 605 RRIM 701, RRIM 703, RRIM 712 RRIC 100, RRIC 102, RRIC 130 KRS 163, IRCA 111, IRCA 109 IRCA 130, SCATC 88-13 BPM 24, Polyclonal seedlings
Non-traditional Region	Clones RRIM 600 and RRII 208	RRII 105 GT1 PB 235 RRII 203 RRII 429 RRII 417 RRII 430	RRII 5, RRII 422, RRII 118 PB 260,PB 310, PB 311 RRIM 703, SCATC 88/13, SCATC 93/114, Haiken1 Polyclonal seedlings

2.5.3. Rubber seed- a potent feed stock for biodiesel

In the current scenario, most of the biodiesel studies exploit the edible oil plants such as soybean, rapeseed, sunflower, safflower, oil palm and canola as substrates and these compete with food, pharmaceutical and cosmetic uses resulting in diminishing of food resources and high cost of edible oils. The use of non-edible oils from the seeds of plants such as rubber, jatropha, castor, linseed, moringa, cotton, karanja, neem and tobacco is being proposed to reduce the fuel-food crisis [108]. In India, *Jatropha* is the targeted feedstock in biodiesel production by using chemical or biological catalyst, whose cultivation demands special attention to conversion of agricultural land. Raw materials account for about 75% of the production cost and the use of non-edible oils will ultimately reduce its price.

Rubber seeds form one potential raw material the least investigated for biodiesel production. The trees shed their leaves in every year during autumn and new leaves develop from March to flowering. Flowers are small but appear in bunches and produce large volume of fruits; initially the fruits are green in colour and turn brown at the time of dehiscence. When the fruits get matured the seed pod breaks and the seeds get scattered on the ground. Rubber trees yield 3-seeded ellipsoidal capsules, each capsule with one seed with variable size (2.5–3 cm long) and weight (2–4 g each); mottled brown and shiny in appearance. The ancillary resources obtained from rubber plantations are wood and seeds and the seeds are with potential use [109].

Indian rubber cultivation has crossed 88,22,000 hectares and India is ranked the sixth largest producer and fourth largest consumer of natural rubber in the world with one of the highest productivity(694,000 tonnes in 2017-18) [110]. The estimated availability of rubber seeds in India is about 150 kg per hectare, which can yield rubber seed oil to the tune of about five thousand four hundred tons per year. Fresh seed contains 65% kernel and 35% shell and contains 40–50% of golden yellow coloured oil [111]. The rubber tree may live for a hundred years or even more. But, its economic life period in plantations, on general

considerations is around 32 years; 7 years of immature phase and 25 years of productive phase [112]. Some studies have already explored the utility of rubber seed oil as a substrate for biodiesel production [108, 113-116].

2.6. General methods for oil extraction

Extraction is the initial phase in the refining processes of oils. Oils and fats are extracted from seeds, fruits or waste fat/lipid of animals utilizing wide range of strategies such as mechanical pressing, solvent extraction, soxhlet extraction, supercritical fluid extraction, etc. In most cases, the oil is extracted directly from the seed or fruit by simple mechanical press and is known as cold pressing. Mechanical pressing is the conventional extraction method and well adapted to rural areas with moderate initial and operating costs. However, low oil yield, turbidity and presence of water and metalic contents are the limitations [117]. For mechanical extraction, either manually operated oil expeller or an engine-operated press can be used. The engine driven option is reported to extract a higher percentage of the available oil, nearly or above 80 percentage compared to 60-65% in the case of manual press. The mechanical expeller can be fed with either whole seeds, kernels or a mix of the two. The seed or fruit is collected, cleaned and dried to crush. Crushing was used to be done between mill stones that later became steel rolls. Seeds with high oil content like rapeseed and sunflower seed are usually mechanically pressed in expellers after a preheating step in indirectly heated conditioners. The oil bearing material is fed to one end of a cylinder where a power-driven worm conveyor forces the material to the other end of the cylinder and out against resistance. The pressure exerted in the process squeezes out the oil [118].

Different modern techniques such as solvent, soxhlet, supercritical CO_2, floculation and microwave assisted extraction methods are employed to accomplish complete extraction of oil from the raw materials, where the amount of oil per ton of seed is increased. For solvent extraction uses, different organic solvents are used depending on the polarity of substances. The most common solvent used in extraction of edible and non-edible oils is n-hexane, which

extracts 95-99% of the oil. However, the use of solvent based oil extraction is only economical in large scale production. The mixture of oil and solvent is separated by rotary evaporator and the solvent is reused in the extraction process. Environmental impacts can be decreased by substitution of solvent based oil extraction with aqueous enzymatic oil extraction, yet that leads to diminishes in the rates of oil extraction [118].

In the case of extraction using supercritical fluid, the oil produced has very high purity but for the high operating and investment cost. Extraction using solvent has a number of advantages, which are higher yield and less turbid oil when compared to mechanical extraction. In the case of using supercritical fluid, the oils are extracted using fluid carbon dioxide as the solvent. Carbon dioxide is changed over to fluid utilizing high pressure making it a safe and effective solvent that allows all the desirable active constituents of a plant to be collected without the risk of heat degradation. Once the extraction is complete, the pressure is released allowing the carbon dioxide to return to its natural gaseous state, leaving behind only the extracted essence of the plant. CO_2 extracted oils are the closest representation of the natural plant product ever achieved. In this case, the extracts include the volatile components as well as the heavier, waxy components that give plants their colour and are therefore thick and waxy in consistency. Some oils undergo a refinement process in order to remove free fatty acids and other undesirable elements naturally present in the raw material, which will result in clear, bright, pale coloured oil with no off flavours or odours and with enhanced shelf life. The oil is reacted with a weak base solution to saponify the free fatty acids into soap, which can be diminished by degumming processes to remove the sticky phospholipids. While the procedures used can vary depending on the type and nature of the particular oil, most of the oils are processed in three stages, neutralization, bleaching and deodorization [119].

Researchers have shown that techniques such as microwave assisted extraction, ultrasound assisted extraction, pressurized liquid extraction and supercritical fluid extraction developed for extraction of valuable components

from plants and seed materials have been successfully used to effectively reduce the major shortcomings of the traditional methods such as soxhlet extraction. These include shorter extraction time, increase in yield of extracted components, decrease in solvent consumption and improvement of the quality of extracts [120].

2.7. Toxicity in rubber

In plants, cyanides are usually bound to sugar molecules in the form of cyanogenic glycosides and are phytotoxins [121]. Chemically, they are L-amino acid derived α-hydroxynitrile constituents of plant secondary metabolites [122, 123]. Such compounds occur in at least 2000 plant species including apricot, peach, apple, mango, cassava, almond, bamboo, rubber, sorghum, Japanese apricot and flax that are used either as food by animals and humans or as herbal medicine. Cyanide is a chemical compound that contains the cyano group (C≡N) and releases cyanide in a process called cyanogenesis [124]. Cyanide is sometimes expressed as HCN, free cyanide, total cyanide, or as linamarin, which is released and degraded by hundreds of species of bacteria, algae, fungi, insects and are found in a number of foods and plants [125, 126]. Such plants produce cyanoglycosides and usually also a corresponding hydrolytic enzyme (beta-glycosidase), which are brought together when the cell structure of the plant is disrupted by a predator, with subsequent breakdown to a sugar and a cyanohydrin, that rapidly decomposes to hydrogen cyanide and an aldehyde or a ketone. This combination of cyanoglycoside and hydrolytic enzyme is the means by which cyanogenic plants are protected against predators [127]. The glycosides, cyanohydrins and hydrogen cyanide are collectively known as cyanogens. About ten cyanogenic glycosides including amygdalin, prunasin, dhurrin, linamarin, lotaustralin and taxiphyllin have been reported in edible plants. The concentration of cyanogenic glycosides is unique to individual plants or among cultivars and shows environmental impacts [122, 128]. The highest concentrations are in the young leaves, newly germinated seedlings and the outer layers of the tuber [129]. Linamarin is located in the plant cell vacuole and the enzyme linamarase is located in the cell wall [130].

Various methods have been used for the qualitative and quantitative analysis of cyanogenic compounds in plants. The colorimetric method via König reaction after acid hydrolysis, picrate paper method and the chromatographic method are the most common ones [131-134]. Significantly, poisonous elements can be removed by boiling, volatilization or solubilization from edible plants and can be eaten after processing [135]. Only free cyanide (CN-) is toxic, and if hydrolysis does not occur, the glycoside remains stable and the food using this product becomes safe. The remaining nonhydrolyzed linamarin present in processed food stuffs cause health problems. For an adult human, consumption of 50 mg to 100 mg of HCN within 24 hours can completely block cellular respiration leading to death. Several symptoms have been related to the consumption of cyanogen containing foodstuff including vomiting, nausea, dizziness, weakness, neuron damage and occasional death. Chronic intake has been linked to goiter especially in iodine deficiency cases [136].

However, many studies carried out in rubber seed indicated that it contains trace amounts of cyanogenous glycosides, which can lead to health problems [137]. It is well known that concentration of poisons may always be found in the seeds of all types of plants such as *Jatropha curcas* whose seed contains the phorbol ester compound [138].

Linamarin is the form of the toxin found in rubber seeds. The molar mass of linamarin ($C_{10}H_{17}NO_6$) is 247.21 g mol^{-1} and the density is 1. 41 g cm^{-1}. The IUPAC name of linamarin is Alpha-hydroxyisobutyonitrile-beta-D-glucopyranoside. Hence, determination of the physicochemical characteristics and toxin compound (linamarin) in the rubber seed are dominantly important to be scientifically researched before the rubber seed oil can be used for both animal and human consumption. The type of toxic component present in the seedcake varies from seed to seed, but for non-edible seed cake, detoxification is highly essential. From several investigations, it is found that deacidification and bleaching could reduce the toxic content [139].

2.8. Enzymes in biodiesel production

Enzymes are complex proteins or conjugated proteins produced by living organisms and they act as catalysts in biochemical reactions. Concern on green protocol among industrial ventures has caused the advancement of alternative greener, safer and sustainable processes. Protein or enzyme engineering is one such initiative, which holds a prominent position in business. Until the 1960s, the total sale of enzymes was only for a few million dollars, annually. Since then, the market has broadened up gradually. At present, more than 200 microbial enzymes are employed commercially and of which approximately 20 types are of industrial origin [140, 141]. Most of the industrially significant enzymes are of microbial origin and lipolytic or hydrolytic in nature that facilitates the cleavage of bonds in hydrophobic molecules into simpler energy units. It plays an important role in renewable energy [142].

2.8.1. Lipases

Lipases or triacylglycerol acylhydrolases (EC 3.1.1.3) are ubiquitous enzymes, which catalyze lipolytic activities such as hydrolysis, alcoholysis, acidolysis, esterification, transesterification, interesterification, stereo selectivity and chiral synthesis [143]. The action of lipase is to catalyze the hydrolysis of triacyl glycerols and the synthesis of esters, free fatty acids, diacyl glycerols, monoacyl glycerols and glycerol [144]. They are mostly seen in plants, animals and microorganisms and microbial lipases are widely used in commercial industries. Microorganisms including bacteria, fungi, yeast and actinomycetes are recognized as preferred sources of extracellular lipases. Many species of bacterial genera such as *Pseudomonas*, *Bacillus*, *Achromobacter*, *Alcaligenes*, *Burkholderia* and *Staphylococcus* and fungi such as *Penicillium*, *Aspergillus*, *Rhizopus*, *Mucor* and *Fusarium* are the common producers of lipases [145-147]. For the last two decades, the world wide production and consumption of microbial lipases has improved significantly, which has made lipases the third largest group of enzymes after proteases and amylases, owing to their fascinating industrial applications due to their versatile activities in extreme temperature, pH, organic solvents and chemo, regio and enantio selectivities [148].

2.8.1.1. Structure of lipase

Lipases or triacylglycerol acyl ester hydrolases belong to the serine hydrolase family [they are called "true" lipases (EC 3.1.1.3)] and carboxyl esterases (EC 3.1.1.1) and are a group of enzymes that are abundantly present in nature [149]. Majority of the lipases are highly variable sized proteins (20-60 kDa) and the consensus sequences of amino acid residues are located on the active site. Lipase has been shown to have an extraordinary competence as a biocatalyst due to the presence of a catalytic triad comprised of nucleophilic serine, histidine and catalytic acid residue of glutamate or aspartate [150]. The active moiety of lipase is possessed by highly conserved sequence of pentapeptide (G-X_1-S-X_2-G), where G is glycine, S is serine, X_1 is histidine and X_2 denotes glutamate or aspartic acid [151].

However, lipases build a common folding motif known as a α/*β*-hydrolase fold which is made of eight parallel strands (*β*1- *β*8) of central *β* sheet and is superhelically twisted by six *α* helices (*α*A -*α*F), surrounded with a catalytic triad [152]. Presence of *α*/*β*-hydrolase fold has been identified in many other distantly or closely related enzymes including acetylcholine esterase from *Torpedo californica* [153], carboxypeptidase II from wheat [154], dienelactone hydrolase from *Pseudomonas* sp. B13 [155], haloalkane dehalogenase from *Xanthobacter autotrophicus* [156], thioesterase from *Vibrio harveyi* [157], cutinase from *Fusarium solani* [158], carboxyl esterase from *Pseudomonas fluorescens* [159] and cholesterol esterase from *Candida cylindracea* [160].

The structures of many different lipases have been determined by X-ray crystallography. Fungal lipases from *Rhizomucor miehei* [161], *Geotrichum candidum* [162], *Candida rugosa* [163], *Humicola lanuginosa* [164], *Penicillium camembertii* [165], *Rhizopus delemar* [166], and *Candida antarctica* [167], mammalian pancreatic lipases [168] and bacterial lipases from *Pseudomonas glumae* [169] and *Chromobacterium viscosum* are identical in terms of amino acid sequences [170, 171]. Aspartic acid may be replaced by glutamic acid in the case of *Geotrichum candidum* lipases which have specificity for hydrolysis of

fatty acids with cis-unsaturated double bonds. A recent study used the bioinformatics tools to detect the common 3D sites of protein with respect to their structural determinants. Some of the latest studies on 3D structures of recombinant lipases include lipase isolated from arctic sea microorganism with improved activity [172], thermally stable mutated lipase from *C. antarctica* [173], thermo, alkali tolerant cloned lipase from *Bacillus licheniformis* [174], etc.

The 3D structure of the enzyme is fundamental tool for enzyme engineering. The significant structural components of lipase include lid, binding pocket, and oxyanion hole and disulfide bond. 3D structure of lipases entail a lid or flap like structure, which is made up of one or more helices of varying length with two hinge segments on both of its ends and it covers the active site at the inactive state of the enzyme. In the presence of hydrophobic substrates, the enzyme undergoes interfacial activation so that the conformational changes at the active site make the lid open, so as to facilitate the entry of substrates to the catalytic residues [175]. The binding pocket of lipases is present on the central β sheet, which can be a hydrophobic, crevice-like binding site located near the protein surface or funnel-like or tunnel-like binding sites. The oxyanion hole is another important component, which largely influences the catalytic efficiency of the enzyme. During hydrolysis, a negatively charged tetrahedral intermediate is generated and the oxygen ion thus formed gets stabilized by hydrogen bonding in which the role of oxyanion hole residues is crucial. The oxyanion hole residues play a pivotal role in stabilizing this oxygen ion by hydrogen bonding. Always, one of the oxyanion hole's residues is positioned next to the nucleophilic serine residue, whereas the second residue is positioned between β_3 strand and a helix. In addition, lipases are cysteine-rich proteins that contain one to four disulfide bonds to maintain their structure. Disulphide bridges formed between cysteine residues significantly contribute to conformational stability by decreasing the entropy of protein [176].

2.8.1.2. Classification of lipases

Lipases promote a broad range of biocatalytic reactions and they act on a variety of substrates. Based on the specificity, functionality and wide applicability, lipases can be categorized in to different classes. On the basis of specificity microbial lipases may be divided into three categories; *viz*., substrate specific, regioselective and enantioselective. Based on the selective reactivity, lipases can be further classified in to non-specific lipases, regio-specific lipases and fatty acid specific lipases.

2.8.1.2.1. Substrate specific lipases

Enzymes are specific biological catalysts; they have specific shape and structure. Each substrates bind to a region on the enzyme called active site, which is composed of a unique combination of amino acid residues. They speed up chemical reactions but are not utilized for the processes. There may be one or more substrates for each type of enzyme, depending on the particular chemical reaction. In some reactions, a single reactant substrate is broken down into multiple products. In others, two substrates may come together to create one larger molecule. There are two theories explaining the enzyme-substrate interaction. In lock-and-key model, only a certain substrate (key) will fit into the active site of the enzyme (lock). In the induced-fit model, the active site and substrate have been distorted very much. Only the proper substrate is capable of inducing their proper shape to form an enzyme-substrate complex. This complex lowers the activation energy and promotes the rapid progression of the reaction process. The enzyme will always return to its original state at the end of the reaction.

Substrate specific lipases can be effectively used in reactions, where they selectively act on a specific substrate in a mixture of crude raw materials, facilitating the desired product synthesis, as demonstrated by lipase use in biodiesel production [177]. Generally, substrates that can be acted upon by substrate specific lipases include fatty acids and alcohol. A recent study reports

the importance of substrate specificity along with enzyme stability in order to exploit lipases in various industrially relevant processes.

2.8.1.2.2. Regioselective lipases

The regioselective lipases tend to steer the lipolytic reaction to produce favorable products of primary (sn-1, 3) and secondary (sn-2) esters, which is importance to chemical and pharmaceutical industries especially in the production of isomeric compounds that exhibit optimal function only under specific configuration. Classifying the regio/stereo selectivity of lipases has traditionally been carried out by the use of hydrolytic assays, where a known substrate is partially hydrolyzed by a lipase and the products of the hydrolysis are characterized [178]. These techniques are often laborious, lengthy and tedious. Sometimes there is a chance of acyl transformation, leading to false interpretation of data. Prior to the exploitation of lipase selectivity in a transesterification reaction, it is desirable to test for the product of triacylglycerols by chromatographic techniques to elucidate the positional character. Some of the recent findings of regioselective lipases include acylation of quercetin with ferulic acid using *Rhizopus oryzae* lipase to synthesize flavonoid derivatives, synthesis of acacetin and resveratrol 3, 5-di-O-beta-glucopyranoside using *Candida antartica* lipase B (Novozym 435) and *Burkholderia cepacia* lipase (Amano PS-IM), etc. [179].

2.8.1.2.3. Enantioselective lipases

Enantioselectivity is the phenomenon of chemical synthesis; it is the synthesis of a specific enantiomer or diastereomer from a racemic mixture over the prochiral precursors [180]. Enantioselective synthesis is a key process in modern chemistry and is particularly important in the field of pharmaceuticals and this includes transesterification of secondary alcohols, hydrolysis of menthol benzoate to cosmetic/food products and hydrolysis of glycidic acid methyl ester to medical/health care products [181, 182].

2.8.1.2.4. Non-specific lipases

This class of lipases is very robust and that acts at random on the triacylglyceride molecule and results in the complete breakdown of triacylglyceride to fatty acid and glycerol. A recent study envisages the use of *Mucor meihei* produced lipases in catalyzing a wide range of reactions from cosmetic industry to biodiesel production. They generally catalyze the hydrolysis of triacylglycerols into free fatty acids and glycerol with mono and diacylglycerols as intermediates. A study is reported on the application of nonspecific *C. antartica* lipase in the acidolysis of canola oil through caprylic acid to produce high purity structured lipids [183].

2.8.1.2.5. Regiospecific lipases

Triacyl glycerides are consisting of three ester bonds between glycerol and three long chain fatty acids. Regio-specific 1, 3 lipases hydrolyze only the primary ester bonds of triacyl glycerol at the positions of C_1 and C_3 atoms of glycerol, and thus release free fatty acids, 1, 2 (2, 3)-diacyl glyceride and 2-monoacyl glyceride. Usually extracellular bacterial lipases are regiospecific, e.g., those from *Bacillus* sp. [184]. Recently, 1, 3-specific immobilized lipases from *Rhizomucor miehei* and *R. delemar* were used in acidolysis of walnut oil with caprylic acid to synthesize long-chain fatty acids [185]. Also, 1, 3-specific immobilized *R. oryzae* lipase was utilized in *Pichia pastoris* for biodiesel production [186].

2.8.1.2.6. Fatty acid specific lipases

The next group comprises of fatty acid specific lipases, which exhibit a pronounced connection to fatty acid molecule. *Achromobacterium lipolyticum* is the only known bacterial source of a lipase showing fatty acid specificity [187]. Lipases perform essential roles in digestion, transport and processing of fat/lipid foodstuff. A detailed study reported on fatty acid specific lipases includes identification of several lipases that exhibit fatty acid specificity towards different substrates with different carbon chain lengths, degree of saturation and different

side chains. These lipases hydrolyze esters having long chain fatty acids with double bonds on C_9 [188].

2.8.1.3. Action of lipase

Insights into the mechanism of catalysis, including the process of interfacial activation have been provided by the crystal structures of lipases, both on their own and in complex with inhibitors. Principally, they act on the oil-water interface between an insoluble substrate phase and the aqueous phase, where the enzyme remains dissolved to release fatty acids and glycerol [189]. At the oil–water interface, the lipase undergoes a change to an activated form by opening the lid, which is termed as "interfacial activation". This interfacial activation is unique to the class of lipases and is also responsible for the versatility of the reactions they catalyze; hydrolysis, esterification, transesterification and interesterification of fats and oils [190].

The conformational changes range from a simple rigid body hinge type motion to complex reorganizations involving changes in the secondary structures. Generally speaking, these structural studies suggest that the hydrophobic lipid binding site is opened up by the rolling back of the lid from the active site at an oil–water interface. However, even in the absence of an oil–water interface, there may be a subtle equilibrium between the two conformations of the enzyme. The geometry of the active sites of lipases varies widely and determines the biochemical properties of the enzyme. Generally, it is a deep hydrophobic pocket that exactly fits scissile fatty acids of substrates into it. According to the shapes of the binding sites, lipase can be categorised into three: lipase with crevice shaped binding site from *Rhizomucor* and *Rhizopus*, funnel shaped binding site (pancreatic lipase) from *C. antarctica* and *Pseudomonas* and tunnel shaped binding site from *C. rugosa* and *Geotrichum candidum* [191].

2.9. Enzyme production by fermentation

Fermentation is a metabolic process in which microorganisms and enzymes are used for the production of compounds which have application in the energy, pharmaceutical, chemical and food industries. Mainly two kinds of

fermentation technologies, Submerged fermentation (SmF) and Solid state fermentation (SSF) are available. Microbial lipases are produced mostly by SmF but SSF can also be used. Generally, enzyme production is organism and substrate dependent and it is released during the late logarithmic or stationary phase of growth. Several species of fungi, yeast and bacteria have been used to produce important industrial enzymes including lipases utilizing the agricultural residues through fermentation techniques due to their ability to grow on particle surfaces using them as sources of carbon and energy. The cultivation period also varies with the microorganism and fast growing bacteria were found to secrete lipase within 24 h. The production of lipase is mostly inducer dependent, and in many cases oils act as good inducers of the enzyme. The organisms are normally grown in a complex nutrient medium containing carbon from oil, sugars, etc. Nitrogen, phosphorous and mineral salts are added as supplements. Other significant factors influencing lipase production include initial pH of the medium, growth temperature, incubation period, agitation and dissolved oxygen concentration. Compounds containing carbon in or on the substrate are busted down by the microorganisms thus producing the enzymes either extracellular or intracellular. The enzymes are isolated by methods such as centrifugation for extracellular produced enzymes and lying of cells for intracellular enzymes. Various industries rely on enzymes for the production of wine making, brewing, cheese making and baking.

2.9.1. Lipase production by submerged fermentation (SmF)

In SmF, enzymes are produced by microorganisms in liquid nutrient media. An example is *Bacillus thermoleovorans* ID-1 producing a thermophilic lipase in a medium containing 1.5% olive oil; whose activity was 520 U/ml at pH of 7.5 and 70°C [192]. *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 [193].

2.9.2. Lipase by solid state fermentation (SSF)

SSF is defined as any fermentation process performed on a solid substrate that acts both as physical support as well as source of nutrients in the absence of free liquid [141]. Low moisture content means that fermentation can only be carried out by a limited number of microorganisms, mainly yeasts and fungi, although some bacteria. SSF is a valuable technique for the utilization of agro-industrial residues to produce value added products of commercial interest. Bioconversion of agricultural residues for lipase production as well as other value added products would hold a prominent position in future biotechnologies, mainly because of its eco-friendliness and flexibility to both developing and developed countries. Utilization and recycling of renewable resources lead to what has been called the '*clean technology'*. Several residues such as straw, bran, oil cakes among others attract increasing attention as abundant and cheap renewable feedstock. SSF represents an interesting alternative to producing industrial enzymes at lower costs due to possibility of using inexpensive agro-industrial residues as culture media. It involves inoculation and incubation of microbes on solid support medium and maintaining low moisture content. The moisture and nutrient contents influence the growth of microorganisms and they secrete useful enzymes [194]. In India, wheat, maize, sugar cane baggase, rice bran, straw and industrial effluents provide sufficient raw materials for SSF [195].

During SSF, temperature critically affects the growth of microorganisms, spore formation, their germination and product formation. The agitation speed proved to improve the enzyme yield effectively in SSF [196]. The agitation forces also depend upon the nature and particle size of the substrate. Aeration has a variable effect on lipase production by different organisms. The degree of aeration appears to be critical in some cases, since thin layer cultures with moderate aeration produced much more lipase than shake cultures with high aeration. It has been reported that SSF supports more stable products, higher productivities, with lower moisture, energy and sterility conditions. Statistical

comparison of the production of lipase between SmF and SSF systems showed that the yield of lipase was quite high in solid state cultivation than in SmF [197].

2.9.3. SSF *vs* SmF

Comparative studies have proved several advantages of SSF over SmF such as higher volumetric productivity, lower energy requirement and easier downstream processing. Agro-industrial residues are generally considered the best substrates for SSF processes, and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce a host of enzymes. Some of the substrates that have been used include sugarcane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soy hull, sago hampas, trimmings dust, saw dust, corn cobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, starch, etc. [194]. Cell cultures in SmF yielded the maximum extracellular lipase activity of 50 U/ml after 22 h of fermentation, and cultivation under SSF strategy yielded a maximum lipase activity of 1,500 U/gds after 12 h of fermentation. Mahant 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 [198].

There are several applications for SSF like the development of bioprocess such as bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial residues, biotransformation of crops and crop residues, nutritional enrichment and biopulping and production of value added products such as biologically active secondary metabolites including antibiotics, alkaloids, plant growth factors, enzymes and organic acids, biopesticides including mycopesticides and bioherbicides, biosurfactants, biofuels and aroma compounds [194].

2.9.4. Microbial production of lipase

Lipases are produced by several microorganisms like bacteria, fungi, archea and eukarya as well as animals and plants, among which bacteria, fungi and yeasts yield the majority of commercial lipases. However, the enzymes from microbial sources currently receive more attention because of their interesting characteristics such as action under mild conditions, stability in organic solvents, high substrate specificity, regioselectivity and enantioselectivity [199, 200]. Moreover, most of the microbial lipases do not require co-factors for their activation and they exhibit broad range of substrate specificity and high enantioselectivity [201]. Microorganisms including bacteria, fungi, yeast and actinomycetes are recognized as preferred sources of extracellular lipases, facilitating the enzyme recovery from the culture broth although species of *Candida, Pseudomonas, Mucor*, *Rhizopus* and *Geotrichum* stand out as the major commercially available strains [202]. Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition besides physicochemical factors such as temperature, pH, and dissolved oxygen. Lipases of microbial origin represent the most widely used class of enzymes in biotechnological applications and organic chemistry [203].

2.9.4.1. Fungal lipase system

Fungal species are preferably cultivated by SSF, while bacteria and yeast are cultivated by SmF. Most of the commercially important lipase producing fungi belong to the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp. and *Rhizomucor* sp. Lipase production by fungi varies according to the strain, the composition of the growth medium, cultivation conditions, pH, temperature, and the kind of carbon and nitrogen sources. Lipase producing microorganisms have been found in different habitats such as industrial wastes, vegetable oil processing factories, dairy plants and soil contaminated with oil and oilseeds among others. Ten best lipase producing species of *Aspergillus* namely *A. alliaceus, A. candidus, A. carneus, A. fischeri, A. niger, A. ochraceus, A. parasiticus, A. sundarbanii, A. terreus and A. versicolor* and nine best lipase producing species of *Penicillium namely P.*

aurantiogriseum, P. brevicompactum, P. camemberti, P. chrysogenum 1, P. coryrnbiferum 1, P. crustosum, P. egyptiacum, P. expansum and *P. spiculisporum* have been identified [204]. Enrichment culture techniques have been used to isolate several lipase producing fungal strains from Brazilian savanna soil. Among the species isolated, a strain of *Colletotrichum gloesporioides* was found to be the best in producing alkaline lipases [205].

A Japanese company used *Candida rugosa* lipase for the production of fatty acids from castor bean. Bussamara et al. (2010) evaluated lipase production from twenty-nine yeast isolates from the phylloplane of *Hibiscus rosa-sinensis* [206]. D'Annibale et al. (2006) used the response surface methodology to optimize culture medium for lipase production by the strain *Candida* sp. After optimization, the authors reported the optimum lipase activity as 6,230 and 9,600 U/ml in shake flasks and in a 5-L bioreactor, respectively [207]. Most of the microorganisms that naturally adapt to agro-residues belong to filamentous fungi because of their ability to spread over and penetrate inside the solid substrate and the fungal mycelia synthesize and release large quantity of extracellular hydrolytic enzymes [208]. A number of *Penicilliium* spp. are good producers of lipases; some of them are already commercialized. In nature, *Penicillum* spp. are versatile and opportunistic fungi, and are mostly saprophytic - as post harvest pathogens, one of the most common causes of fungal spoilage in fruits and vegetables.

In 2000, Castilho et al. presented an economic evaluation of lipase production from *P. restrictum* by SSF and SmF [209]. The total capital investment in SmF was 78% higher than that in SSF. Gombert et al. (1999) reported that babassu cake, the solid waste from babassu oil industry was used as the basal medium for lipase production from *P. restrictum* by SSF. The highest lipase activity (30.3 U/g of initial dry weight) was obtained after 24 h of fermentation with addition of 2% olive oil [210]. Similarly, with babassu cake as basal solid medium, Gutarra et al. (2010) studied the lipase production from *P. simplicissimum* in tray type and packed bed bioreactor. After the optimization of

the culture conditions through response surface methodology, maximum lipase activity obtained in packed bed bioreactor was 30% higher than that in tray type reactor. Soybean meal, an agro-industrial byproduct, was also tested as the basal solid medium for lipase production from *P. simplicissimum* [211].

Among yeasts, *Candida* spp. are the most potential lipase producers. Lipase produced by *C.rugosa* is one of the most used industrial enzymes due to its high activity, both in hydrolysis as well as synthesis. Common species of yeasts that were found to produce lipases are *C. rugosa, C. tropicalis, C. antarctica, C. cylindracea, C. parapsilopsis, C. deformans, C. curvata, C. valida, Yarrowia lipolytica, Rhodotorula glutinis, R. pilimornae, Pichia bispora, P. mexicana, P. sivicola, P. xylosa, P. burtonii, Saccharomycopsis crataegenesis, Torulaspora globosa* and *T. asteroids*. Several strains of yeasts were also identified such as *S. cerevisiae, C. boidinii, C. wickerhamii*, and *Williopsis californica*, of which *S. cerevisiae* and *W. californica* showed good potential to produce lipase [212].

2.9.4.2. Bacterial lipase system

Lipases from several microorganisms have been studied extensively and based on their properties, used in various industries. Bacterial lipases are mostly extracellular and are greatly influenced by nutritional and physico-chemical factors such as temperature, pH, nitrogen and carbon sources, the presence of lipids and inorganic salts, agitation and dissolved oxygen concentration [213, 214]. Initial pH of the growth medium is important for lipase production. Largely, bacteria prefer pH around 7.0 for best growth and lipase production, such as in the case of *Bacillus* sp., *Acinetobacter* sp. and *Burkholderia* sp. [215-217]. However, maximum activity at higher pH (>7.0) has been observed in many cases [218, 219]. Generally, bacterial lipases act in neutral pH, while lipases from *B. stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range (pH 3–12) [220]. Bacterial lipases possess stability over a wide range from pH 4 to pH 11. The optimum temperature for lipase production corresponds with the growth temperature of the

respective microorganism. Incubation periods ranging from few hours to several days have been found to be best suited for maximum lipase production by bacteria. An incubation period of 12 h was found to be optimum for lipase production by *Acinetobacter calcoaceticus* and *Bacillus* sp. RSJ1 [221] and 16 h for *B. thermocatenulatus* [222] while, maximum lipase was produced after 72 h and 96 h of incubation, respectively, in the case of the *Pseudomonas fragi* and *P. fluorescens* BW 96CC [223]. Bacterial lipases generally have temperature optima in the range 30–60°C [224]. A few lipases from *Pseudomonas* spp. have been reported which are stable at 100°C or even beyond to 150°C with a half-life of a few seconds [225].

Another important property of lipases is their enantiomeric or stereoselective nature, wherein they possess the ability to discriminate between the enantiomers of a racemic pair. Such enantiomerically pure or enriched organic compounds are steadily gaining importance in the chemistry of pharmaceutical, agricultural, synthetic organic and natural products [226]. Lipases producing important bacteria are species of *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium* and *Pseudomonas*.

2.9.4.3. *Pseudomonas* lipase

Pseudomonas represents the heterogenous group of gram-negative bacteria such as *Burkholderia cepacia, Burkholderia multivorans, Pseudomonas aeruginosa,* etc. which are 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 or stereoselective nature, in which they have the capability to distinguish between the enantiomers in a racemic mixture and this unique property, is now widely exploited in pharmaceutical and agricultural sectors [227]. Thermostable *Pseudomonas* lipases were reported to withstand 100°C or even beyond to 150°C with a short span of a few seconds [225, 228, 229]. An alkaline lipase from *P. alcaligenes* M-1 was found better for eliminating fatty stains from clothes under

machine wash conditions [230]. Lipase from *Pseudomonas cepacia* acts as a most effective catalytic agent for the ethanolysis and methanolysis of grease [231]. Lumafast and Lipomax are two commercial lipases introduced by Genencor International, AUKBC Research Center, Life Sciences, Anna University, Chennai, India. The lipases from *Pseudomonas* bacteria vary in molecular size with potent application in industrial biotechnology [141, 213] (**Table 2.3**).

Table 2.3. Lipase producing *Pseudomonas* spp.

Organisms	**Molecular weight (kDa)**
P. aeruginosa PAO1	32.7
P. fluorescens	50.24
P. mandelii JR-1	64.47
P. savastanoi	34.42
P. amygdale	34.47
P. weihenstephanensis	82028
P. putida	32.06
P. syringae	36
P. endophytica	67.7
P. helleri	35.18
P. flexibilis	57.5
P. pseudoalcaligenes	30.3
P. antarctica	50.05
P. libanensis	81.02
P. coronafaciens	32.25
P. psychrophila	83.25
P. lundensis	83.11
P. taetrolens	81.1
P. moraviensis R28-S	32.28
P. protegens	32.43
P. avellanae	30.66
P. stutzeri	32.655
P. koreensis	86.96
P. azotoformans	81.11
P. marginalis	59.1
P. orientalis	79.96
P. trivialis	80.85
P. synxantha	81.11
P. poae	81.05

P. lini	32.55
P. kilonensis	81.12
P. mediterranea	35.42
P. cremoricolorata	35.18
P. chlororaphis	82.38
P. taeanensis MS-3	29.49
P. mendocina	30.01
P. simiae	80.96
P. extremaustralis	82.92
p. donghuensis	34.15
P. viridiflava	34.84
P. fragi	32
P. citronellolis	34.8
P. alcaligenes	29.65
P. brassicacearum	36.1
P. balearica	57.77
P. composti	30.1
P. oryzihabitans	35.81
P. tremae	34.47
P. meliae	30.79
P. congelans	34.34
P. cannabina	36.97
P. caricapapayae	34.3
P. xanthomarina	57.18
P. taiwanensis	80.1
P. deceptionensis	12.17
P. frederiksbergensis	32.71
P. rhizosphaerae	35.39
P. rhodesiae	81.03
P. batumici	36.13
P. alkylphenolia	81.37
P. lutea	34.44

2.10. Substrate for lipase production

Microorganisms with potential for producing lipases can be found in different habitats, including wastes of vegetable oils and dairy industries, soils contaminated with oils, seeds and deteriorated food [202]. The nature of the substrate is the most important factor affecting fermentative processes. The choice of the substrate depends upon several factors; mainly related to cost and

availability. Several abundant and cheap agro-residues like brans, oil cakes, bagasse, cotton seed and soybean sludge have been reported as effective for lipase production. Owing to high nutritional content, 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 [198]. Thus, several bacteria, filamentous fungi and yeasts isolated from wastes have been tested for the production of lipases.

Oil cakes of various residues obtained after extraction of oil have been utilized as solid substrate for the fermentative production of lipases and other industrial enzymes. This is because residual oil and other ingredients contained in it serve as inducers for lipase production [232]. Several agricultural residues have been reported to be effective for lipase production and these include brans (wheat, rice, soybean, barley), oil cakes (soybean, coconut, groundnut, cotton, olive, gingelly, babassu), and bagasse (sugarcane). Most of the agricultural residues being utilized for lipase production contain a mixture of both easily consumable and non-consumable substrates that can support the growth of a wide range of microorganisms.

In all the cases, lipids serve as inducers for microbial lipase production. Some of the substrates are to be pre-treated (chemically or mechanically) before using them for enzyme production, such as heat pretreatment, chemical hydrolysis and reduction of particle size by grinding or chopping. It helps for increasing the accessibility of the nutritional components for microbial uptake and growth [141, 233]. Thus, several agro-residues can be utilized as substrate for lipase production, which can be grouped as below.

2.10.1. Oil cakes

Ramachandran et al. (2007) reported that oil cakes (oil meals) stand as the most widely utilized substrate for lipase production. Oil cakes are by-products of oil extraction from seeds [234]. They contain residual nutrients that can serve as both carbon and nitrogen sources. *P. chrysogenum* S1 showed an appreciable level of lipase activity when it was grown on sesame cake and sesame oil (10%)

[235]. There are several reports on the use of different microorganisms such as *P. restrictum, R. verrucosum, A. niger, P. citrinum, P. simplicissimum, Y. lipolytica* and *C. rugosa* for lipase production using babassu oil cake, soybean cake, gingelly oil cake, coconut oil cake, ground oil cake, vegetable oil meal, castor oil cake, niger seed oil cake, olive mill, etc. [236].

Employing *Candida rugosa*, Benjamin and Pandey (1998) reported the use of mixed solid substrate containing wheat bran and coconut oil cake for lipase production [142]. Colla et al. (2010) showed that a mixture of soybean meal and rice husk had synergistic effect on lipase production by *A. niger* [197]. In the case of *R. pussillus*, a mixture of olive cake and sugarcane bagasse enhanced lipase production [237]. Addition of gingelly oil cake to wheat bran (1:3, w/w) increased the lipase activity of *A. niger* MTCC 2594 by 36% (384.3 ± 4.5 U/gds) at 30°C and 72 h compared to that produced by wheat bran alone [238]. Alkan et al. (2007) studied the effect of several agro-industrial residues (wheat bran, rice husk, lentil husk, banana waste, watermelon waste and melon waste) on lipase production by *B. coagulans*. The best result was obtained on solid waste from melon supplemented with NH_4NO_3 and 1% olive oil [239]. An overall 2.4 fold increase in lipase production and 1.8 fold increase in specific activity were obtained from *Burkholderia cepacia* after validation of RSM in shake-flasks [217]. Abdel-Fattah (2002) reported 4 fold increase in lipase production in shake-flask cultures from a thermophilic *Geobacillus* sp. using a Box–Behnken experimental design [240].

2.10.2. Fibrous residues

Fibrous agro-residues are by-products of lignocellulosic origin and classified based on their digestibility. Fibrous residues can be grouped into those with high digestibility (e.g., soybean husk) and those with low digestibility (e.g., sugarcane bagasse) [241]. Each group can serve as a physical support and as a source of carbon and nutrients to sustain microbial growth. In most cases, lipase production using fibrous residues requires nutrient supplementation or a mixture of more than one substrate. Colla et al. (2010) showed that a mixture of soybean

meal and rice husk had synergistic effect on lipase production by *A. niger* [197]. In the case of *R. pussillus*, a mixture of olive cake and sugarcane bagasse enhanced its lipase production [237]. Addition of gingelly oil cake to wheat bran (1:3, w/w) increased the lipase activity of *A. niger* MTCC 2594 by 36% (384.3 ± 4.5 U/g dry substrate) at 30°C and 72 h compared to that produced by wheat bran alone [238].

2.10.3. Industrial effluents

Effluents produced from edible oil refinery, slaughter houses and dairy products industry contain high concentrations (>100 mg/l) of lipids. Effluents such as olive mill wastewater and palm oil mill effluents contain 0.12 to 1.49 g/l and 4–5.7 g/l lipids respectively showing potentials to be used for lipase production [242]. D'Annibale et al. (2006) used olive mill wastewater as a growth medium for lipase production, in which *C. cylindracea* NRRL-Y-17506 showed the highest lipase activity (9.23 U/ml) in the medium supplemented with ammonium chloride and olive oil among the twelve microbial species tested [207]. Brozzoli et al. (2009) assessed lipase production in bench top reactor using olive mill waste water medium and found the maximum production of 20.4 U/ml. Palm oil mill effluent-based medium was developed for lipase production using *C. cylindracea* (ATCC 14830) [243]. Salihu et al. (2011) reported the use of statistical optimization of nutrient components to enhance *C. cylindracea* lipase production and the maximum activity of 20.26 U/ml was realized. Based on these studies, effluents from oil related industries can be used as valuable liquid growth media for production of microbial lipases [244].

2.10.4. Seed lipases

In order to study seed lipases, their physiological functions as well as activity in agricultural products during storage are to be understood. In germinated oilseeds, mobilization of the stored fatty acid is essential to supply energy and carbon for embryonic growth. Lipolytic enzymes catalyze the first step of lipid mobilization [245]. Basically, oilseeds are composed of two fundamental parts: the husk or tegument and the kernel. The husk is the external

layer of the seed covering the kernel. The kernel has two parts: the embryo or germ that will form the new plant when the seed germinates and the albumin or endosperm which stores the reserve nutrients that will feed the plant in the first stages of development. Grains generally contain proteins, and depending on the plant species, starch or triacylglycerols may be present as energy reserve sources. In the mobilization of these three major nutrient reserves during germination, they are hydrolyzed specifically by proteases, amylases and lipases, respectively. Many researchers have investigated seed lipase at the height of its activity during grain germination [180]. In seeds, stored triacylglycerols vary from 20% to 50% of the dry weight. During the germination period, the triacylglycerols stored in oil bodies or oleosomes are quickly used up for the production of energy and synthesis of the sugars, amino acids (mainly asparagine, aspartate, glutamine and glutamate) and carbon chains required for embryonic growth [246].

2.11. Purification strategies for bacterial lipases

Bacterial lipases are mostly secreted in the medium and hence 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 and analytical chemical industries and for elucidating the protein structure high purity enzyme is required [247]. Ideal purification strategies adopted in industries should be low cost, quick, high yielding and agreeable to large-scale operations. In addition, those 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. Recently emerged purification strategies include immune purification, reversed micellar system, membrane processes, hydrophobic interaction chromatography employing an epoxy activated spacer arm as a ligand, column chromatography with polyethylene glycol/ sepharose gel or poly(vinyl alcohol) polymers as stationary phase and aqueous two phase systems [248].

2.12. Properties of bacterial lipases

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. Use of cheap agro-industrial residues as substrates for lipases could reduce the production cost to a considerable level. The properties of bacterial lipases such as pH, temperature, stability, substrate specificity are evaluated under the following sections.

2.12.1. pH

Bacterial lipases exhibit stability over a wide range of pH from 4 to 11 [249-251]. Most of the bacterial lipases show optimum activity at neutral or alkaline pH [194, 252]. Alkaline lipases, which are stable at pH range 8-10 offer promising applications in many upcoming bio-based industries such as textile, detergent, etc. Lipase from *Aspergillus carneus, Bacillus thermoleovorans, Bacillus stearothermophilus* and *Fusarium oxysporum* were found stable at the pH range 8-10 [253-255], whereas lipase produced by *Rhizopus oryzae* and *Cryptococcus* sp. showed the maximum activity at the pH range 7-8 [256, 257] by the exemption of *P. fluorescens* SIK W1 lipase, which has an acidic optimum at pH 4.8 [228].

2.12.2. Temperature

Generally, most of the microbial lipases are mesophilic in nature, i.e., the optimum temperature for their maximum activity ranges from 25 to 40°C, but the increased demand for thermotolerant lipases in industries has led to the exploration of many microbial species producing thermophilic lipases, most of which retained 70-100% of the activity even at the temperature range of 50-70°C [258, 259]. For instance, a lipase from *Bacillus* sp. (an isolate from hot spring) retained 90% activity at 60°C and 70% of activity at 70°C for 1h; whereas a lipase from *Pseudomonas* sp. strain ZBC1 showed the optimum activity at 80°C

[260, 261]. Cold active lipases are active at 10-25°C and they facilitate gentle and efficient industrial applications by significantly reducing energy consumption. Many microbes such as *Rhodococcus cercidiphylli* BZ22, *Penicillium expansum*, *Yarrowia lipolytica*, *Stenotrophomonas maltophilia*, *Pseudoalteromonas* sp., etc. isolated from harsh environments produced lipases active at low temperatures [262-266].

2.12.3. Lipases functional in organic solvents

Prior to mid 1980s, lipases were used most frequently in detergent or clothing applications and in the modification of triglycerides [267]. Therefore, enzyme engineering such as immobilization and entrapment for stabilizing enzymes in the presence of organic solvents has been developed. Since lipases can be used in a variety of reactions like esterification, transesterification, alcoholysis, acidolysis, aminolysis, acylation and resolution of racemic mixtures those have become potentially important for biotechnological and industrial applications [268]. From the processing and economic point of view, high boiling point and low vapor pressure of water result in expensive downstream purification from an aqueous based biotransformation system.

Researchers used many methods to modify enzymes in order to make them suitable to organic solvents, but instead of these expensive modifications, it would be more desirable to screen microbes directly for solvent tolerant [269]. There are several reports on solvent tolerant lipases classified by the degree of solvent tolerance, exploited for industrial use [270]. An organic solvent tolerant lipase from olive oil induced *Aspergillus niger* MYA 135 was purified using series of chromatographic methods [271]. These enzymes are not only stable in these toxic solvents but also capable of catalyzing many synthetic reactions and are considered to be most favorable tools for synthetic reactions in non-aqueous systems [272-275]. The microorganisms belonging to *Bacillus*, *Rhodococcus*, *Staphylococcus* and *Arthrobacter* species are tolerant to very toxic organic solvents [276-278]. The enzymes, which can be utilized in different reaction conditions such as wide pH range, high temperature, salt variant conditions and

in the presence of organic solvents without losing the activities are needed to be explored. Hence, lipases that can act as biocatalysts in anhydrous organic solvents offer new possibilities such as shifting of the thermodynamic equilibrium in favour of synthesis, enable the use of hydrophobic substrates, control or modify enzyme selectivity by solvent engineering, suppress undesirable water dependent side reactions, improve thermal stability of the enzyme and also minimize the chances of contamination. These advantages are often limited by the low stability and/ or activity of biocatalysts in organic systems. Since most lipases easily get denatured in organic solvents and therefore lose their catalytic activities, it becomes pertinent to find lipases that are stable in non-aqueous systems [279, 280]. In order to test solvent toxicity on the microbial cells and organelles, log P, a parameter for solvent hydrophobicity has been established which is helpful to determine the stability of proteins and enzymes [281]. The solvents with log P less than 5 are considered harmful to cell membrane because high degree of partitioning can damage the lipid membrane bilayer [282].

Behaviour of lipase in organic solvents is related to their capacity in both synthetic and hydrolytic reactions. It has been found that *Bacillus* spp. lipases are very stable in hydrophobic organic solvents and their activity is slightly increased in the presence of 10–50% (v/v) of short chain alkanes, benzene and toluene [283, 284]. It has also been found that different lipases behave differently in different organic solvents with different levels of resistance in different reaction systems [285]. It is worth noting that not only the log P alone but cumulative effect of various other parameters such as the dielectric constant, dipole moment, hydrogen bonding and polarizability affect the enzyme activity in organic solvent system [286]. Polarity, denaturation capacity, hydrophobicity and polarity index are also the major factors that decide the stability and catalytic potential of a biocatalyst in an organic medium [287, 288]. However, so far none of them have enabled validation of any serious predictive analysis about catalysis in organic solvents [289].

Lipases differ in their sensitivity towards different organic solvents and are generally more unstable in polar water miscible solvents than in water immiscible solvents [290]. Therefore, efforts are being made to screen the enzymes which can work efficiently with high activity in different types of toxic organic solvents. Some bacterial strains are capable to survive in the presence of short chain alcohols like butanol and also in other toxic solvents like benzene and toluene. Two organic solvent tolerant bacteria *Enterococcus faecalis* and *Clostridium sporogenes* were screened in a medium supplemented with acetone or butanol (5%; v/v) and *S. haemolyticus* was screened in a medium overlaid with 15% benzene and 85% cyclohexane. *S. haemolyticus* was able to grow in the presence of cyclohexane, benzene and toluene [291].

Biocatalysis in organic solvents has several potential advantages over biocatalysis in aqueous media: (i) increased solubility of hydrophobic compounds; (ii) ability to carry out new reactions that are kinetically or thermodynamically restricted in water; (iii) suppression of undesirable side reactions; (iv) control or modification of enzyme selectivity (substrate, enantio-, regio-, prochiral); (v) possibility of recovery of some products by the use of low boiling point organic solvents and (vi) increased enzyme thermostability [292]. In spite of these advantages, enzymes do not always meet desired levels of activity, productivity and, most importantly, stability in organic solvents [293]. Hydrophobic solvents typically lead to higher enzymatic activity and stability than their hydrophilic counterparts, which tend to strip some of the water required for enzymatic function, thereby lowering the catalytic activity [294]. The availability of lipases that were stable in polar solvents would favour new applications in biotechnological processes with polar substrates. Slotema et al. (2003) described oleamide production *via* direct amidation of carboxylic acid with ammonia [295]. For this reaction, the use of hydrophilic organic solvents with increased polarity shifted the thermodynamic equilibrium, leading to: (i) higher enzymatic activity; (ii) higher dissolution of water generated as reaction product while the water activity remains close to the optimum for the enzyme; (iii) higher solubility of substrates and products; and (iv) higher conversions of

substrates into products. Thus, it is still necessary to find out lipases that are stable in polar solvents. It has been reported that the structural properties that confer enzyme thermostability also confer stability in organic solvents [296, 297]. Therefore, thermophilic or thermotolerant organisms can be used as suitable resources to obtain enzymes that are stable in organic solvents, since enzymes from these organisms possess intrinsic thermal stability [298].

2.12.4. Specificity of lipases

Specificities of lipases play a crucial role in their possible applications in analytical and industrial purposes, especially in pharmaceutical industry. Majority of the lipases show substrate/ region/ enantio specificites, which are highly determined by the size, shape and hydrophobicities of the binding pockets located in the active site. Some of the lipases specifically act on tri-, di-, mono-glycerides and other esters. Non-specific lipases completely hydrolyse the triglycereides to fatty acids and glycerol, but most of the extracellular lipases are region specific especially at 1, 3 positions. Lipase from *Burkholderia cepacia* found applications in organic synthesis due to its enantiospecificity, prefering the (*R*) - enantiomer over the (*S*) - forms [299].

2.12.5. Interfacial activation

Lipase exhibits a characteristic property, called interfacial activation, which makes it a suitable catalyst in water-oil medium, i.e., the activity of the enzyme is significantly increased when the substrates form emulsion in the reaction media. In aqueous medium, the active site of enzyme is covered by a loop of peptide called ‘lid’, but contact with the interfacial area induce drastic conformational changes at the active site, so that the lid moves aside facilitating the enzyme-substrate reaction [300].

2.13. Enzyme immobilization

Enzymes are highly potent biocatalysts used for economically important reactions in different industries. Although, its high cost, the lack of long term stability under reaction conditions, difficulty in its separation and impossibility of multiple reuses are major problems faced by these industries. To overcome these

limitations, the immobilization of enzymes onto a suitable support is a plausible solution and thus is becoming a challenging area of research [301]. Immobilization is a technique of anchoring the enzymes in or on an inert support for increasing their functional efficiency, stability and reproducibility. The method of immobilization varies according to the inert support used for it and a dramatic difference was observed in the catalytic efficiency of lipase on different inert supports. More than the nature of solid support, the synthetic activities of lipase depended on the interaction of its hydrophobic 'lid' with the solid support [302].

Binding to a support (carrier), entrapment (encapsulation) and cross-linking are different methods of immobilization. In support binding, the bonding of enzyme on the matrix may be physical, ionic and covalent. Entrapment and cross linking of enzymes in matrix have been introduced to overcome issues of enzyme leakage. For immobilization, different carriers are used as support: modified sepharose, chitosan and chitin, carrageenan, acrylic resin, polypropylin, celite, cellulose, starch, pectin, agarose polyacrylamide, collagen, gelatin, alginate, amino benzyl cellulose, porous glass, silica, zeolites, ceramics, cyanogen bromide, agarose, etc. [303].

The immobilized lipase can provide many advantages over native lipase in the transesterification reaction for biodiesel production, such as ease in catalyst removal from the product, feasible continuous operations and simple product purification [304, 305]. The supports used for lipase immobilization are quite important since their interaction with enzyme molecules may influence the activity and stability of immobilized lipase. Various immobilization techniques have been employed on lipase used for biodiesel production. These include cross linked enzyme aggregates, microwave assisted immobilization, nanotechnology and mesoporous supports [306, 307]. Several new types of carriers and technologies have been implemented in the recent past to improve traditional enzyme immobilization which aimed to enhance enzyme loading, activity and stability to decrease the enzyme biocatalyst cost in industrial biotechnology.

Lipases are immobilized on the matrices by (i) adsorption (ii) covalent bonding (iii) entrapment and (iv) encapsulation. Most recently immobilization of enzymes on nanoparticles has attracted many researchers due to their stability, easy separation from the reaction mixture and efficient reusability [306].

The catalytic potential and sterioselectivity of lipases can be enhanced with the help of immobilization. It was reported that entrapment of lipases in sol gel derived hydrophobic silica enhanced its esterification competence [308]. The lipase of *Candida rugosa* binding on functionalized chitin showed higher hydrolytic activity as compared to its free form at 40^0C in butanol and cuprylic acid system. In a study, lipases showed increased transesterification potential when immobilized on Silica–PVA composite, which was utilized for the production of biodiesel from palm oil and ethanol [309]**.** Covalent immobilization using Eupergit C employed for stabilization of lipase from *Candida rugosa* resulted coupling yields of the enzyme [310]. The recovery of total activity of immobilized lipase on chitosan support by physical adsorption was well exploited in industries [311]. Betigeri and Neau (2002) studied the comparison between the leaching capacity of agarose, alginate and chitosan and proved that alginate had higher leaching capacity as compared to agarose and chitosan [312]. Many industries were using immobilized lipase for the synthesis of aliphatic esters by utilizing its transesterification potential. Yadav and Trivedi (2003) reported that macroporous polyacrylic resin beads of lipase have higher catalytic activity in the reaction of *n*-octanol with vinyl acetate in non-aqueous media, which gives highly fragrant *n*-octayl acetate [313]. Different reactions catalyzed by immobilized enzymes from different organisms are given in **Table 2.4.**

Table 2.4. Different reactions catalyzed by immobilized lipase

Sl. No.	Solid matrix	Reaction	Source of lipase	References
1	Sol gel-derived hydrophobic silica	Esterification	*Aspergillus niger* *Candida antartica* *Rhizomucor miehei*	Reetz et al., 1995 [308]
2	Functionalized chitin	Hydrolysis	*Candida rugosa*	Gomes et al., 2004 [314]
3	Polysiloxane–poly-(vinyl alcohol)	Transesterification	*Thermomyces lanuginosus*, *Pseudomonas fluorescens*, *Burkholderia cepacia*, *Penicillium camembertii*, *Candida antarctica*	Moreira et al., 2007 [309]
4	Hydrophilic acrylate based porous Sepabeads	Esterification	*Humicola lanuginosa*, *Candida antarctica*, *Rhizomucor miehei*	Petkar et al., 2006 [302]
5	Chitosan	Esterification Hydrolysis	*Candida rugosa* Porcine pancreas	Pereira et al., 2003 [311]
6	Agarose Alginate Chitosan	Hydrolysis	*Candida rugosa*	Betigeri and Neau, 2002 [312]
7	Alginate	Glycerolysis	*Pseudomonas sp.*	Cheirsilp et al., 2009 [315]
8	Polyvinyl alcohol (PVA) nanofibrous membranes	Hydrolysis	*Candida rugosa*	Chen et al., 2008 [316]
9	Non porous polystyrene latex and porous Accurel EP400 powder	Hydrolysis	*Candida rugosa*	Murrey et al., 1997 [317]
	Macro-porous polyacrylic resin beads	Transesterification	*Candida rugosa* *Candida antarctica* *Mucor miehei*	Yadav and Trivedi, 2003 [313]
10	Diatoms	Transesterification	*Psedomonas* sp.	

2.14. Application of lipases

Based on total volume of sales, lipases are the third largest group of enzymes next to proteases and carbohydrases. The commercial use of lipases is a billion-dollar business and their applications highly dependent on specificities, selectivity, optimum pH, temperature, tolerance to organic solvents, etc. Microbial lipases are excellent alternatives for classic chemical syntheses with industrial applications due to easiness for production and versatile applications. Bacterial lipases have a wide variety of applications in many industries like diary, food, detergent, fine chemicals, wastewater treatment, cosmetics, pharmaceuticals, leather processing and biomedical assays [318, 319]. Additionally, lipases have an inevitable role in the field of bioenergy, especially in biodiesel production, which is a challenging sector for use of cheap or waste vegetable oils [320].

2.14.1. Detergent industry

Now-a-days enzymes have become one of the major constituents of detergent formulations, of which lipases play an important role for the removal of tough dirty stains such as butter, oil, etc. from the stuff that are hard to eliminate under normal washing. For the last two decades, detergent industry has become one of the biggest markets of microbial lipases, because of their functional importance for the removal of fatty residues in laundry, dishwashers and for cleaning of clogged drains [321]. Standard wash liquids contain anionic and non-ionic surfactants, oxidants, chelating agents, amphoteric agents, citric acid and naturally occurring enzymes that break down stains into smaller pieces to make stains easier to remove. Lipolase, the first industrial lipase was obtained from *Humicola lanuginose*, which was marketed by Novozymes (Denmark). Later on, three genetically modified commercial lipases such as LipoPrime, Lipolase Ultra and Lipex were also marked by expressing the lipase gene of *Humicola lanuginose* in *Aspergillus oryzae*, a fungus. Massive screening is required to find out suitable enzymes exhibiting low substrate specificity, stability under alkaline pH (8-11), elevated temperature (30-60°C) and also compatibility with other ingredients of formulations such as metals, oxidants, surfactants, etc. Bacterial

lipase from *Staphylococcus arlettae* JPBW-1 isolated from rock salt mine has been assessed for its use in laundry formulations which exhibited good stability towards surfactants and oxidising agents, and removed about 62% of olive oil from cotton fabrics [322]. *Pseudomonas alcaligenes* lipase showed elevated activity at washing conditions, such as alkaline pH (7-11) and high temperature up to 60°C [323]. The main advantage of these bio-detergents is high biodegradability, lack of any harmful residues, no adverse effect on sewage treatment processes and absence of risk to aquatic life.

2.14.2. Pulp and paper industry

The paper industry utilizes huge amount of lignocelluloses every year. Historically, the enzymatic applications in paper industry was confined to the treatment of raw starch; but, later since 1990s, lipase mediated removal of 'pitch' has become an essential process of large scale paper making process. Pitch or 'resin stickies' is a term used to collectively describe the hydrophobic components of wood such as triglycerides, waxes, etc. [324]. Pitch and related substances, which usually create major problems to the machines and cause holes and spots in the final paper, are common in paper mills. It may reduce the production levels and increase equipment maintenance as well as operation costs. Nippon Paper Industries, in Japan, have developed a pitch control method that uses the *C. rugosa* lipase to hydrolyse up to 90% of the wood triglycerides [325]. Lipase in paper industry decreases chemical usage thereby reducing pollution level of waste water. It provides prolonged equipment life as lipase removes sticky deposits in the paper machines, saves energy and time and reduced composite cost [324]. The addition of lipase from *Pseudomonas* sp. (KWI-56) to deinking compounds improved whiteness of paper and reduced residual ink spots [326]. Lipase from *Thermomyces lanuginosus* was immobilised on macroporous resin coated with chitosan along with pectinase, which was found to be an efficient treatment for removing pitch deposits in wastewater from papermaking [327].

2.14.3. Textile industry

Use of lipases in textile industries is mainly found in the removal of lubricants from fabric with better absorbency and reducing the chance for frequency of line and break in denim scrape systems. A commercial lipase from Amano Pharmaceutical KK can improve the wetting ability and absorbance in polyester fabrics and improved fabric texture without losing its strength [328]. Moisture trapping ability of polyethylene terephthalate fabrics was achieved by using lipases from *P. cepacia* and *P. fluorescens* [329]. However, lipase from *Pseudomonas* spp. was shown to degrade polymers of aliphatic polyethylene [330].

2.14.4. Food technology

Lipases are widely used in food industries for the hydrolysis or modification of lipid substances. 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 have good market in supermarkets which include bread, nutraceuticals, chocolates, etc. Previously, lipases have been used for cleansing rice flavour, altering soybean milk and for progress in aroma and increase in the fermentation of apple wine, etc. [331].

Lipases modify the properties of lipids by altering the positions of fatty acid chains or by adding or removing one or more fatty acids to the glycerides. The position, chain length and degree of unsaturation greatly influence not only the physical properties, but also the nutritional and sensory value of a given triglyceride as well [200]. Egg white is made up of around 90% water and 10% protein and is an important ingredient of many bakery products such as cakes, bread, etc. Contamination of egg white with lipids is so fast. Therefore, lipase is used to remove the lipid contamination and to improve the quality of dough and texture and to achieve an even, light-colored crust on the products. Treatment of egg yolk with phospholipase hydrolyses egg lecithin and iso-lecithin, thereby used as an emulsifying agent to make myonnaise, custards, baby foods, etc. [332]. Lipases can also be used in degumming of vegetable oils during the

process of biodiesel production. The degumming process removes the phospholipid impurities from the crude vegetable oils which may decrease the free fatty acid content and improve the storage stability of vegetable oils [333]. Another important process of biolipolysis is being used to make fat free meat and fish [334].

2.14.5. Dairy industry

Lipases are widely employed in dairy industry for the hydrolysis of milk fat to produce cheese, butter and cream. Enzyme modified technology has vital role in making of a variety of cheese with desired flavor and aroma; in this process lipase is generally used in combination with other hydrolytic enzymes such as protease and amylase under controlled conditions. It enhances the sweetness as well as softness and modifies flavor intensity [335]. Lipase produced by *Mucor miehei* can effectively be utilized to produce coco butter from palm olein and distillate from palm oil refinery which contributes to the flavor of chocolate, caramels, toffees and butter creams [336]. Thus, high value products can be synthesised from cheap fats and oils.

2.14.6. Leather industry

Nowadays, enzymes are extensively used for the processing of hides and skins in leather industry. Lipase and proteases are the hydrolyzing enzymes with applications in bathing, soaking, dehairing and degreasing of skin [337]. Lipase specifically degrades fat but do not damage leather which is proteinaceous in nature; it hydrolyses the fat on the outside of the hides and skins as well as inside the skin structure. Thus, lipase assisted processes provide leather with good quality and finish with uniform colour and cleaner appearance as compared to conventional chemical agents. Lipase also improves the production of hydrophobic waterproof leather, which represents an environment friendly method of leather processing. It was found that the lipase produced by *B. subtilis* can be used for the degreasing process, removing all the fat and maintaining natural skin colour [338]. NovoLime, a protease/lipase blend for enzyme assisted liming of hides and skins, and NovoCor AD, an acid lipase for degreasing of

hides and skins, are some of the commercially available lipases for the leather industry.

2.14.7. Fine chemistry

Regioselectivity, enantioselectivity and chemoselectivity features of lipases show vast application in fine chemistry. In general, these enzymes demonstrate excellent stability in the presence of organic solvents, in which the substrates are soluble [339]. Lipase from wheat provided higher kinetic resolution to form 2-octanol and 2-butanol. The authors reported the possibility of using wheat lipase in chiral syntheses, in combination with other lipases.

2.14.8. Pharmaceutical industry

Applications of lipase in the synthesis of chiral drugs and kinetic resolution of racemic alcohols, acids, esters and amines are well established. Effective drugs have been synthesized using lipase mediated hydrolysis; such as diltiazem hydrochloride, a calcium antagonist (a coronary vasodilator); lovastatin, a drug lowering the serum cholesterol levels; etc. [340]. Lipase from *Pseudomonas* sp. AK mediated the kinetic resolution of the chiral silane in the synthesis of a potent antitumor agent called epothilone A [341].

2.14.9. Cosmetics

Lipases have got applications in producing many cosmetic ingredients such as retinol, natural dyes, etc. In the cosmetic industry, the demand of natural products which improve the appearance of skin and also the health of the skin increases. Lipases from *Candida antarctica* efficiently catalyzes the transesterification between glycerides and vitamins to produce retinyl/ascorbic esters [342]. Vitamin A (retinol) and vitamin C (ascorbic acid) are vital nutrients for health; its derivatives can cause many skin disorders including photoaging, psoriasis and acne [343]. Lipase mediated transesterification reaction between olive oil and ascorbic acid produces liposoluble ascorbyl oleate, which is widely used as an antioxidant [344]. Lipases also find applications in the production of natural dyes such as indigo used in cosmetics. Lipase releases indoxyl from istan B which can be combined with isatan C and processed to produce indigo [345].

Aroma esters consisting of short chain fatty acids and alcohol are synthesised by the direct application of lipase, which provides natural fragrance to the cosmetics.

2.14.10. Biodiesel industry

Production of biodiesel consisting of methyl esters of long chain fatty acids is yet another promising application of lipase which has been widely exploited all over the world. Research in this field is still in progress due to the flexibility of the enzymes and the high cost of commercialization [346]. In such cases, immobilised lipase is used, which offers repeated usage of the enzyme without losing its specificity. Enzymatic production of biodiesel by methanolysis of cottonseed oil has been studied using immobilised lipase from *C. antartica* as catalyst in *t*-butanol solvent, in which the ester yield was about 95-97% [347]. A variety of low cost vegetable oils such as sunflower oil, soybean oil, karanj oil, jatropha, rubber seed oil, etc. can effectively be used to produce biodiesel by lipase mediated hydrolysis [348].

2.15. Stages of biodiesel production

Biodiesel from fat/vegetable oils proceeds through different procedures such as degumming, pretreatment, transesterification, separation, etc. The key step is the reaction on triglyceride with alcohol in the presence of catalyst under suitable conditions [349]. Before that the vegetable oil/fat is required to be processedto raw material with minimum free fatty acid. In the case of virgin oils, if those contain the least amount of FFA, direct transesterification can be carried out. Thus, in most cases, feedstock requires degumming and pretreatment procedures [350]. The oil impurities such as gum, FFAs and water content present in the oil negatively affect the biodiesel product in terms of quantity as well as quality. Hence, feedstock should be removed from such impurities before subjecting to the transesterification reaction [351].

2.15.1. Degumming of oil

Gums are soluble impurities/substances other than residual metals present in crude vegetable oils. Gums present in oils adversely affect transesterification, in addition to its hindrance to the activity of catalyst and diminishing the

biodiesel yield. Therefore, before degumming the feed stock should be filtered to remove solid impurities. Degumming is the process of removal of gums present in the feedstock by addition of phosphoric acid and water [352].

2.15.2. Pretreatment of oil

The degummed oil will be free from gums and then can be subjected to acid pretreatment to decrease the FFA value. The pretreatment of oil is carried out when the raw material contains high quantity of FFAs. The FFA content of feed stock has to be reduced to less than 1% before chemical catalyzed biodiesel production. Generally, biodiesel production from feedstock with high FFA content is achieved by two steps; acid pretreatment with concentrated HCl or H_2SO_4 followed by transesterification reaction with suitable catalyst by base/acid [353]. As a result of pretreatment, the FFAs are converted to their respective esters by the esterification reaction thereby eliminating the problems of soap formation, emulsification, decrease in biodiesel yield, etc. [354]. However, in the case of enzyme transesterification pretreatment step is additional. These catalysts efficiently esterify free fatty acids and thus can be used for the transesterification of oils or fats having high FFA content [355].

2.15.3. Transesterification

Biodiesel production is a highly prompted and advanced technological area for current researchers. Different methods are currently employed for biodiesel production, such as microemulsion, dilution, pyrolysis and transesterification of oils and fats. However, dilution and microemulsion have got little attention in biodiesel production [356]. Some studies reported that pyrolysis and microemulsion are not economic compared to transesterification method [357]. Similarly, microemulsion and thermal cracking methods would lead to the formation of fuel with undesired properties. Thus, compared to these methods, transesterification is widely accepted and universally established.

At present, transesterification is the most widely studied, accepted and employed method for the production of biodiesel. As we know, oils/fats are triglyerides with different fatty acid chains attached to glycerol. The

transesterification reaction employs lower alcohol and vegetable oils or fats as the feed stock with the presence of catalyst. Transesterification converts these fatty acid triglycerides to the corresponding monoalkyl esters and glycerol. Transesterification reaction is complex; it proceeds through two or three consecutive and reversible steps [358]. Initially, the triglyceride is converted into diglycerides then to monoglycerides and finally to glycerol. In each step one molecule of fatty acid ester is produced. Generally, methanol and ethanol are used for the transesterification reaction [359]. Among these, methanol is employed mosty since it is easily available and cheap. There are reports on ethanol based transesterification reaction in the production of biodiesel, but this technology is limited due to several reasons. The ethyl esters and glycerol are mutually soluble and can lead to tedious task for separation process after the transesterification reaction. The formation of soap and emulsion are the important problems to reduce the biodiesel yield. Ethanol and chemical based transesterification reaction will lead to higher chance of soap formation. This necessitates pretreatment and excess washing and thus further increases the total cost of production [360]. In addition, ethanol is less effective in the conversion of triglycerides when compared to methanol. Hence, methanol is preferentially accepted for biodiesel production sine it is cheap and has high reactivity [361]. Transesterification reactions are aided by different catalysts such as acid, base or enzyme.

2.15.3.1. Acid catalyzed transesterification

Acid catalysts can simultaneously carry out both esterification and transesterification of oil/fat; they could help in easiness of reaction, thereby lowering overall production costs [362]. Generally, acid catalyzed transesterification processes are two types: homogeneous and heterogeneous acid catalyzed transesterification processes.

2.15.3.1.1. Homogeneous acid catalyzed transesterification

In biodiesel production, feedstock containing high FFA and water content could be processed with acid catalysts to avoid saponification and FFA by

esterification while triacylglycerols (TAGs) are converted into ester through transesterification. Commonly used homogenous acid catalysts are sulfuric acid (H_2SO_4), hydrogen chloride (HCl), boron trifluoride (BF_3), nitric acid (HNO_3) and trifluoroacetic acid (CF_3COOH); among these, H_2SO_4 is the most common catalyst by dint of its good catalytic activity and easiness to add directly to methanol. The precautions required for acid catalysis include acid tolerable vessel/reactor, wearing of safety glass and lab coats, etc. This approach is mainly used for waste cooking oil having greater than 20% free fatty acid. It requires a pretreatment step to reduce the free fatty acid content through an esterification reaction with methanol in the presence of sulphuric acid as a catalyst using Taguchi method. The highest FAME production obtained under the optimized condition reported is 98.66% [363].

2.15.3.1.2. Heterogenous acid catalyzed transesterification

Heterogeneous acid catalysts are expected to be the most promising for commercial biodiesel industries in the coming years. The major problems associated with homogenous catalysis can be overcome by the use of heterogenous catalysts [364]. These catalysts have many advantages: non corrosive, environmentally benign, renewable, recycled, reused, cost effective and with fewer disposal problems. Besides, the use of heterogeneous catalysts does not produce soaps through saponification. They are also much easier to separate from the reaction mixture. However, most of the heterogeneous catalysts face some sort of hurdles such as low activity, time consumption, etc. [365]. Therefore, research is being directed towards the development and exploring of environment friendly and cost effective heterogeneous catalysts for biodiesel production.

2.15.3.2. Alkali catalyzed transesterification

In this type of transesterification method we can use different alkaline catalysts as homogeneous and heterogeneous catalysts.

2.15.3.2.1. Homogeneous base catalyzed transesterification

Homogenous catalysis is the one in which the catalyst and the reactants are present in the common physical state [366]. Thus, an effective interaction is possible between the reactant and catalysts; which results in high productivity with excellent product. During the initial works on biodiesel production, common catalysts such as sodium hydroxide (NaOH) and potassium hydroxide (KOH) were employed [367]. Potassium hydroxide has an advantage in that, at the end of reaction, the reaction mixture can be neutralized with phosphoric acid resulting in potassium phosphate which can be used as fertilizer. The reaction mechanism is completed in three steps. The first step in the mechanism of base-catalyzed transesterification of vegetable oils is reaction with base catalyst in the presence of alcohol, producing an alkoxide and the protonated catalyst. The nucleophilic attack of the alkoxide at the carbonyl group of the triglyceride generates a tetrahedral intermediate, from which the alkyl ester and the corresponding anion of the diglyceride are formed. Diglycerides and monoglycerides are converted by the same mechanism to a mixture of alkyl esters and glycerol [368]. Catalysts such as alkaline metal alkoxides, hydroxides and carbonates are most often used [369-371]. Commercially, NaOH and KOH are preferred because of their availability and low cost. The alkoxides are more expensive than the hydroxides and are more difficult to handle because they are hygroscopic. However, biodiesel production using homogenous catalyst faces several problems such as difficulty in separation from the reaction mixture, equipment corrosion, requirement of pretreatment steps and neutralization of reaction there by increasing the total cost of biodiesel production [372]. Homogeneous base catalyzed transesterification is most commonly used for commercial purposes since the process offers high yield in a short time with mild reaction temperature. Homogeneous alkali catalyzed transesterification is much quicker than homogeneous acid catalyzed transesterification [373]. The great thing about this transesterification process is that some of the methanol can be recovered and glycerine is obtained as a byproduct, which is used in pharmaceutical and other applications. However, the general limitation of base catalyzed transesterification

is that it should be carried out in the absence of water in order to avoid soap formation by saponification; which will seriously hinders the production of fuel grade biodiesel [374]. Some researchers investigated the effect of alkaline catalysts on the transesterification of beef tallow [375]. NaOH was found to perform significantly better than NaOMe. Furthermore, a slightly higher concentration of NaOMe with respect to NaOH (0.5 vs. 0.3% w/w) was needed to obtain the maximum conversion of the oil into the corresponding esters. A research studied the reaction of methanol with canola oil at different concentrations of alkaline catalyst (NaOH, KOH, NaOMe and KOMe), reaction temperatures and methanol to oil molar ratios [376].

2.15.3.2.2. Heterogenous base catalyzed transesterification

Heterogenous base catalysts have gained significant position in biodiesel production because the catalyst removal process is simple and does not create waste water during the catalyst removal step. Catalysts such as alkaline earth metal oxides and various alkaline metal compounds like calcium carbonate ($CaCO_3$), magnesium carbonate ($MgCO_3$), sodium carbonate (Na_2CO_3), potassium carbonate (K_2CO_3), sodium oxide (Na_2O), calcium oxide (CaO), magnesium oxide (MgO), zinc oxide (ZnO), lanthanum oxide (La_2O_3), aluminium oxide (Al_2O_3), barium oxide (BaO), cerium dioxide (CeO_2), zirconate (ZrO_3), cobalt(III) oxide (Co_2O_3), tin oxide (SnO_2), oxido-(oxido(oxo)molybdenio)oxy-oxomolybdenum (Mo_2O_5) and barium hydroxide ($Ba(OH)_2$) have been tried for transesterification reaction. These catalysts can be used with good quality feedstock and have several advantages such as catalyst reusability, simplicity in catalyst removal, low reaction temperature requirement, and short reaction time, enticing several researchers to investigate this area. Recently, heterogeneous catalysts derived from both waste industrial and biological resources have attracted interest for biodiesel synthesis [377].

2.15.3.3. Enzyme catalyzed transesterification

Production of biodiesel through enzymatic transesterification has met with explosive expansion recently. This subject was initiated by Cambou and

Klibanov in 1984 by the successful utilization of enzymes in organic solvents. Enzyme catalyzed transesterification has acquired great interest in many industries since the byproduct can be easily recovered, salt and catalyst can be separated, waste water treatment is not required, high production under mild temperature, pressure and pH and ecofriendly nature of the process [378, 379]. One group of such enzymes used in biodiesel production is lipases. Lipases catalyze hydrolysis as well as esterification reactions simultaneously. In China the first industrial scale biodiesel production in the world is in operation [380].

More specifically, it has been recently found that enzymes such as lipases can be used to catalyze transesterification process by immobilizing them in a suitable support [327]. The advantage of immobilization is that the enzyme can be reused without the requirement of complex separation. Also, the processing temperature is below 50°C compared to other processes and it can produce high quality product with less or no downstream operations [381]. Enzymatic production is promising with both extracellular and intracellular lipases. Both types of lipases are able to catalyze efficiently the transesterification of triglycerides in both aqueous and non-aqueous systems. Recent advances in enzymatic biodiesel process are the use of solvent tolerant lipase and immobilization which makes the use of the catalyst effective [382]. The kinetics of the lipase-catalyzed reactions is influenced by a number of factors.

The disadvantages include the inhibition effects observed when methanol is used and the fact that the enzyme is expensive [383]. However, enzymatic production of biodiesel has been extensively reported, although this technology has not yet been commercialized due to high cost of the enzyme. It has been suggested that the cost of lipase can be reduced by the use of recombinant DNA technology [384]. Use of genetically engineered *E. coli* with whole cell biocatalyst expressing lipase (K107) from *Proteus* sp. has been reported. The engineered *E.coli* can produce nearly 100% yield of biodiesel at optimal conditions. Moreover, protein engineering can be utilized to improve enzyme stability, substrate specificity and catalytic efficiency which will facilitate

lowering the cost of the overall process [385]. The cost of biodiesel production can be reduced by using two immobilized lipases (Novozyme 435 and Lipozyme TL-IM) instead of one lipase. These lipases could give a yield of 97.2% of methyl ester from lard oil by using response surface methodology [386]. Enzymatic production is possible using both extracellular and intracellular lipases. Both the lipases are able to effectively catalyze the transesterification of triglycerides in both aqueous and non-aqueous systems. Several lipases from microbial strains, including *Candida antartica, Candida rugosa, Pseudomonas cepacia, Pseudomonas fluorescens, Rhizomucor miehei, Rhizopus chinensis, Rhizopus oryzae, Thermomyces lanuginose, Achromobacter, Alkaligenes, Arthrobacter, Bacillus, Burkholderia* and *Chromobacterium,* have been reported to have transesterification activity. Biocatalysts are gaining higher attention nowadays and have the potential to outperform chemical catalysts for biodiesel production in the future [387].

Biodiesel can be industrially produced by a chemical route using either acidic or alkaline catalysts, which give high conversion levels in a short reaction time. However, the conventional chemical route has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the alkaline catalyst must be removed from the product, alkaline waste water requires treatment, and free fatty acids and water interfere with the reaction. To minimize the above problems, attempts to use enzyme catalyst systems in alcoholysis of triglycerides have been made through enzymatic processes but those are still not commercially developed [388]. Studies related to it consist mainly of optimizing the reaction conditions (alcohol/oil molar ratio, enzyme amount, temperature, time, among others) to establish the characteristics for industrial applications. The key step in enzymatic processes lies in the successful immobilization of the enzyme which will allow for its recovery and reuses [389].

2.16. Optimization by response surface methodology (RSM)

Response surface methodology (RSM) is an effective mathematical and statistical tool for the optimization of reaction conditions. This approach was

developed and introduced by G.E.P. Box and K.B. Wilson in 1951 to explore the relationships between several independent variables (input variables) and one or more response variables (output variable) [390]. The main focus is to design a sequence of experiments to obtain an optimal response by using a second order polynomial equation. The model predicts the value of the unknown output for any desirable input. The results can be compared with the experimental values obtained for the same. The degree of the closeness of the predicted and experimental values will show the excellent fit of the model for the particular experiment.

2.16.1. Response surface designs

The main objective of response surface methods (RSM) is optimization, finding the best set of factor levels to achieve optimum goals that applies an approximation technique called response surface. In response surface designs where the primary aims is factor screening such as two level factorials, fractional factorial methods are being widely used. In general, the design of experiments is used for analysis or experiment point parameter setting, and look for the factor level combinations that give us the maximum yield and minimum costs [391]**.**

2.16.2. Types of experimental designs

The Box-Behnken and Taguchi methods of experiments can also be thoroughly studied for experimental strategy, which changes the values of the input variable for the purpose of studying the response of the system. In this, it is important to identify the experimental factors and their levels. A factor is an input variable that is controlled by the investigator and is manipulated to cause a change in the output. It is also sometimes called independent variable. These are responsible for the output parameters or dependent variables called responses. In the Box-Behnken and Taguchi apparoches the input variables are referred to uncoded variables, as *A, B, C* and *D*. Central Composite and Box-Behnken experimental design of RSM is chosen to find the relationship between the response functions and variables using the statistical software package Design Expert Software. Taguchi method and Box-Behnken design can fit well with

quadric response surface models and offers advantages over other response surface designs. The interaction between the variables and the ANOVA can be studied using RSM. The quality of the fit of this model is expressed by the coefficient of determination (R^2). The fit is confirmed by means of coefficient of correlation [392].

2.16.3. Taguchi method

In the late 1940s, a new standardised version of the DOE method was introduced by Genichi Taguchi and is popularly known as the Taguchi method or Taguchi approach. He spent considerable time and effort to make this experimental technique user friendly and simple. Taguchi method is one of the most effective tools used by researchers and engineers in all types of studies/experiments. To accomplish, Taguchi designed experiments using specially constructed tables known as “Orthogonal Arrays”. The use of these tables makes the design of experiments very easy and consistent. The DOE using the Taguchi approach can economically satisfy the needs of problem solving and product or process design optimization projects. This technique can significantly reduce the time required for experimental investigations. In the Taguchi technique, factors are defined as different variables, which determine the efficiency of any industrial process [393].

2.16.4. Box–Behnken Design (BBD)

There are several experimental designs in RSM. Box-Behnken Design (BBD) for RSM was devised by Box & Behnken in 1960. Box-Behnken design of experiments is used to develop a mathematical correlation between different independent variables such as temperature, pH, moisture, and incubation time on a specific response. The first goal for response surface method is to find the optimum response. When there is more than one response, it is important to find the compromise optimum that does not optimize only one response [394]. In order to understand the surface of a response, graphs are helpful tools. But, when there are more than two independent variables, graphs are difficult or almost impossible to use to illustrate the response surface, since it is beyond 3-

dimension. For this reason, response surface models are essential for analyzing. The Schematic representation of sequence of experiment by RSM is presented in **Figure 2.2.**

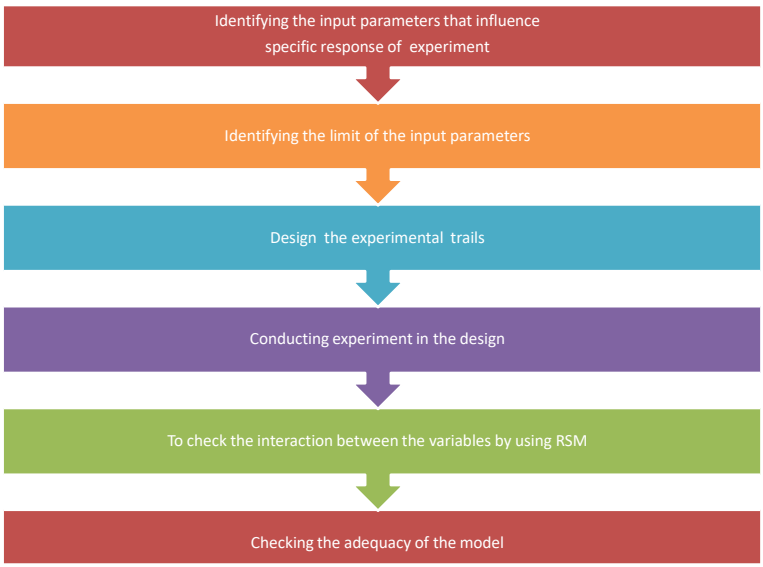

Figure 2.2. The sequence of investigation of experiments by RSM

2.17. Factors influencing transesterification

Transesterification is the bidirectional process, which minimizes the viscosity of the triglyceride oil by converting it into monoalkyl esters [395]. The most important variables that influence transesterification reaction are: molar ratio of alcohol/oil, nature and amount of the catalyst, presence of water/ moisture/ free fatty acid in the feedstocks, reaction temperature, reaction time and agitation speed.

2.17.1. Alcohol/oil molar ratio

The alcohol/vegetable oil molar ratio is one of the main factors that influence acid transesterification. Typically, one molecule of oil is reacted with three molecules of alchol to generate three molecules of corresponding alkyl esters plus one molecule of glycerol as byproduct. In most cases increased ester conversion could be obtained at increased molar ratio of alcohol to oil [396]. Here, excess amount of alcohol can act as a solvent that can ensure proper contact between the reagent molecules and catalysts by enhancing the reaction rate. On the other hand, an excessive amount of alcohol makes the recovery of the

glycerol difficult, so that, the ideal alcohol/oil ratio has to be established empirically, considering each individual process. Further increase in the amount of alcohol will inversely affect the biodiesel yield since this will stop the reversible reaction. Similarly, the alcohol length is positively influenced on biodiesel yield. Lower alcohols like methanol or ethanol are more preferred [397].

2.17.2. Type of catalyst and concentration

Transesterification occurs many folds faster in the presence of catalyst; there is no reaction under normal temperature and pressure without the use of a catalyst. The nature of the catalyst also influences biodiesel yield. Higher concentration of catalyst will result in incomplete conversion. Therefore, there is an optimum amount of catalyst required in all the transesterifcation reactions. However, excess amount of catalysts does not increase the conversion and it may negatively affect the conversion yield by catalyst leaching or emulsion formation [398].

2.17.3. Reaction temperature

The rate of reaction is strongly influenced by the reaction temperature; which promotes the rate of the forward reaction, thereby reducing the reaction time. Especially, the reaction is conducted close to the reflux temperature of the alcohol (ethanol or methanol) at atmospheric pressure [399]. At higher temperature the viscosity of the oil gets reduced and this increases the effective mixing of oil and catalyst, yielding maximum product. A few studies have reported a negative effect of increase in temperature on the conversion. Studies have indicated that given enough time, transesterification can proceed satisfactorily at ambient temperatures in the presence of catalysts. It was observed that biodiesel recovery was affected at very low temperature (cold weather); but conversion was almost unaffected.

2.17.4. Reaction time

Initially, the tarnsesterification reaction wil be very slow till the effective mixing of the components (oil, alcohol and catalyst) and after thet the rate will

get increased with increase in time. Time required for the completion of reaction depends on other reaction parmeters and type of feedstocks, etc. Reaction conducted for a longer time may cause negative impact on production yield since transesterification is a reversible process [400].

2.17.5. Water content

Presence of water in the feedstock oil as well as in the reaction medium adversely affects the transesterification reaction. Moisture presence will lead to hydrolysis reaction and this increases the free fatty acid content in the feedstock. Soap formation from the product ester is high; for better yield the reaction must be water free [401].

2.17.6. Free fatty acid (FFA)

Free fatty acid is a critical factor in biodiesel production. For a base catalyzed transesterification, the FFA content in feedstock must be less than 1%. Otherwise, it will cause some hurdles in the production process. It reacts with the catalyst and soap is formed. Moreover, it is more complicated for layer separation and leads to emulsion formation during the washing proceudure. For feedstocks having high FFA, pretreatment step is essential. But, in the case of enzyme catalyzed reaction; catalysts can efficiently esterify free fatty acids [402].

2.17.7. Agitation speed

Effective mixing of the reactants and catalysts ensures proper contact, thereby playing a significant role in the transesterification. Oil is viscous and immiscible in nature and so the best way for the proper mixing of this under normal condition is continous agitation at optimum level [403].

2.17.8. Quality of reactants

Impurities present in the feedstock (edible and non-edible oil) also affect production levels. Under the same condition, lower production of biodiesel using crude edible and non-edible oils can be obtained, compared to higher production when using refined oil. However, the edible and non edible oils should be properly filtered. Oil quality is very important in this regard. The oil settled at the bottom during storage may give lesser biodiesel recovery because of accumulation of impurities like wax.

2.18. Separation and purification of biodiesel

Once bodiesel is produced the final step for the completion of the reaction is the separation of glycerol from the methyl esters. Due to the low solubility of glycerol in the esters, the separation is simple and easy. The excess methanol tends to act as a solubilizer and can slow down the separation. Hence, hot water is added to the reaction mixture to improve the separation of glycerol [404]. Even after the complete transesterification of triglycerides, small amount of di and mono glycerides is found in the biodiesel product. The mixture is distilled subsequently to remove the unreacted methanol using rotary evaporator. After the glycerol is removed, the ester is washed counter currently and dried. Neutralization before washing reduces the amount of water required for washing and also the emulsion formation tendency [405].

2.19. Specifications of standards for biodiesel

For the commercial utilization of biodiesel as a fuel in diesel engine, it must fulfill some quality requirements. So as to yield better fuel performance and reduced emissions, standard specifications for biodiesel were built up and are additionally being created in every nation so that the engine manufactures and biodiesel producers use similar standards for fuel quality assessment [406]. The most accepted standard fuel specifications for biodiesel are the European standard, EN 14214 and the American standard, ASTM D 6751[407, 408]. Physico-chemical properties of biodiesel may vary with its origin. The biodiesel fuel must fulfill the criteria assigned by the standard specifications for its effective performance. There are specific ranges of values for each of the fuel properties. The slight difference in the standard values in different areas is due to the atmospheric or environmental impacts [409]. The utilization of biodiesel as a component for blending with petro diesel fuel is recommended in India by certain national specifications which are known as Indian standards for biodiesel (IS 15607). It is created by The Bureau of Indian Standards (BIS), an association recognized under the Bureau of Indian

Standards Act, 1986. IS 15607 is primarily based on EN 14214 with some specifications taken from ASTM D 6751 standard. All modern diesel engines are perfect for the use of biodiesel as long as the fuel meets the standard

specifications [410, 411]. The standard values as specified by EN 14214 and ASTM D 6751 for the fuel parameters of biodiesel are listed in **Tables 2.5** to **2.7.**

Table 2.5. American standard fuel specification (ASTM D6751) for biodiesel

Properties	Test method	Limits	Units
Kinematic viscosity, 40°C	D445	1.9-6.0	mm^2/s
Cetane number	D 613	47 min	-
Flash point (closed up)	D 93	93 min	ºC
Cloud point	D 2500	Report	ºC
Water and sediment	D 2709	0.050 max	% (Volume)
Sulfated ash	D 874	0.020 max	% (Mass)
Sulfur	D 5453	0.05 max	% (Mass)
Copper strip corrosion	D 130	No. 3 max	-
Carbonate residue	D 4530	0.050 max	% (Mass)
Acid number	D 664	0.50 max	Mg KOH/g
Free glycerol	D 6584	0.020 max	% (Mass)
Total glycerol	D 6584	0.240 max	% (Mass)
Phosphorus content	D 4591	0.001 max	% (Mass)
Distillation temperature	D 1160	360 max	ºC
Cold soak filterability	D 7501	360 max	Sec

Table 2.6. Indian standard fuel specifications for biodiesel

Properties	Test method	Limits	Units
Density;15ºC	ISO 3675/P 32	860-900	kg/m^3
Viscosity, 40ºC	ISO 3104/P 25	2.5-6.0	mm^2/s
Flash point(closed cup)	P 21	120 min	ºC
Sulfur content	D 5443/P 83	50.0 max	mg/kg
Carbon residue	D 4530	0.05 max	% (m/m)
Cetane number	ISO 5156/P 9	51 min	-
Sulfated ash	ISO 6245/P 4	0.02 max	% (m/m)
Water and sediment	D 2709/ P 40	500 max	mg/kg
Copper strip corrosion (3h, 50 ºC)	ISO 2160/P 15	Class 1	-
Acid value	P 1	0.50 max	mg KOH/g
Earth alkali metals (Ca+Mg)	-	To report	mg/kg

Table 2.7. European standard fuel specifications for biodiesel (EN 14214)

Properties	Test method	Limits	Units
Ester content	EN 14103	96.5 min	% (m/m)
Density; 15 ºC	EN ISO 3675, 12185	860-900	Kg/m^3
Viscosity, 40 ºC	EN ISO 3104, ISO 3105	3.5-5.0	mm^2/s
Total contamination	EN 12662	24 max	mg/kg
Oxidative stability, 110ºC	EN 14112	6.0 min	H
Flash point (closed cup)	EN ISO 2719, 3679	101 min	ºC
Sulfur content	EN ISO 20846, 20884	10.0 max	mg/kg
Carbon residue (10% dist. res)	EN ISO 10370	0.30 max	% (m/m)
Cetane number	EN ISO 5165	51 min	-
Water content	EN ISO 12937	500 max	mg/kg
Total contamination	EN ISO 12662	24 max	mg/kg
Copper strip corrosion (3h, 50ºC)	EN ISO 2160	1	-
Oxidative stability, 110 ºC	EN 14112,15751	6 min	H
Acid value	EN 14104	0.50 max	mg KOH/g
Iodine value	EN 14111	120 max	mg I/100g
Linolenic acid content	EN 14103	12 max	% (m/m)
Methanol content	EN 14110	0.20 max	% (m/m)
Monoglyceride content	EN 14105	0.80 max	% (m/m)
Diglyceride content	EN 14105	0.20 max	% (m/m)
Triglyceride content	EN 14105	0.20 max	% (m/m)
Free glycerine	EN 14105, 14106	0.02	% (m/m)
Total glycerine	EN 14105	0.25	% (m/m)
Alkali metals 1(Na+K)	EN 14108, 14109, 14538	5.0 max	mg/kg
Earth alkali metals (Ca+Mg)	EN 14538	5.0 max	mg/kg
Phosphorus content	EN 14107	4.0 max	mg/kg
Sodium and Potassium combined	EN 14538	5 max	ppm (µg/g)
Calcium and Magnesium combined	EN 14538	5 max	ppm (µg/g)

2.20. Fuel properties of biodiesel

For the long term usage of biodiesel in diesel engines without any modification or technical problems, the physical and chemical characteristics of

the biodiesel fuel should meet the quality requirements. The fuel properties and hence the quality of biodiesel are affected by several factors such as the fatty acid composition of the parent vegetable oil or animal fat, the entire biodiesel process, handling, storage, etc. Changes in these properties strongly affect the performance and emission characteristics of the fuel [412-414]. Some of the major fuel parameters required for the efficient performance of fuel in diesel engine are mentioned here in brief.

2.20.1. FAME content

One of the most important parameters ascribing the use of biodiesel as a fuel is its monoalkyl ester content. It signifies the efficiency of the tranesterification process. Generally, fatty acid methyl esters (FAMEs) are the monoalkyl esters while using methanol as the acyl acceptor or tarnsesterifying agent. According to European standard fuel specification EN 14214, the biodiesel should attain a minimum of 96.5% ester content in it for getting designated as good grade fuel [415, 416].

2.20.2. Free glycerol and total glycerol

Glycerol is the major byproduct of transesterification reaction and is thus separated from the biodiesel samples. It has some value added uses in many industries like paint, soap, etc. Free glycerol refers to the presence of trace amount of glycerol in the purified biodiesel sample during the production process. The sum of unconverted amount of triglycerides and partially esterified mono and diglycerides is collectively referred to as total glycerol. Presence of glycerol content in biodiesel negatively affects the diesel engine and its performance. It can cause injector deposition, clogging and eventually damage. The free glycerol percentage in the sample can be evaluated by gas chromatographic method [417-419].

2.20.3. Acid value

Acid value is the measurement of the amount of free fatty acid (FFA) present in the biodiesel sample. In other terms it is defined as the number of milligrams of KOH required to neutralize the free fatty acids present in 1 gram of

vegetable oils or animal fat. Commonly, all fats and oils are derivatives of triglycerides with high amount of FFA. FFA undergoes oxidation or reduction to form aldehydes, ketones and alcohols. High amount of FFA containing oils have to undergo pretreatment before the transesterification process. Otherwise it will lead to the formation of soap. FFA can cause corrosion of the engine parts [420, 421].

2.20.4. Water content

Washing is an unavoidable procedure in the biodiesel process, especially in the alkali catalyzed route. There will be a chance of trace volume of water content in the purified biodiesel; it adversely affects the characteristics of biodiesel. Presence of water content can result in microbial growth and corrosion. In addition, ester reacts with water to increase the FFA content of the sample and leads to emulsification or hydrolytic oxidation [422].

2.20.5. Density

Density of a fuel directly signifies its mass. Normally the biodiesel fuel has higher density than diesel fuel but lower when compared to vegetable oil or fat. Density of biodiesel will be different with its chemical composition and it has pivotal role in fuel injection system. Higher density of the fuel creates poor fuel atomization resulting in incomplete combustion of the fuel. It causes emission of higher amount of NO_x and particulate matter from the engine [423, 424].

2.20.6. Viscosity

Viscosity is defined as the degree of resistance to the flow of fuel. High viscosity of vegetable oils is to be reduced for use in diesel engines directly. Viscosity of oil or fuel is determined by its chemical structure and composition. Branched structure of oils gives high viscosity than linear structure of biodiesel. Viscosity is also depending on their fatty acid chain length and unstaurations. High value of viscosity leads to combustion problems in engine. Compared to petro diesel, biodiesel has higher value of viscosity by dint of its molecular mass and chemical structure [425-427].

2.20.7. Iodine value

Iodine value of the biodiesel sample determines the presence of double bond in the sample. It indicates high susceptibility to oxidation and negatively affects the stability of biodiesel by polymerization and gum formation [428].

2.20.8. Flash point

Flash point is the lowest temperature at which ignition occurs in a engine by fuel. It is directly related to volatility of the fuel. As compared to diesel, biodiesel has higher flash point which is a benefit to non hazardous to transport. Poor value of flash point indicates the presence of dissolved methanol in the sample and it should be removed to enhance fuel quality and combustion efficiency [429, 430].

2.20.9. Cetane number

The cetane number of a fuel is determined as its capacity to ignite fastly. This property is related with the time delay between the starting of fuel injection and its combustion. Biodiesel from saturated fats has higher cetane number and it is more suitable for use in diesel engines. Fuel with low cetane number reduces the engine performance and combustion efficiency [431].

2.20.10. Calorific value

Total heat energy content of the fuel is referred to as calorific value. It is the measurement of energy produced as a result of combustion of a fuel. A fuel with higher calorific value is mostly preferred for transportation because it improves the engine efficiency by high power generation. Compared to fossil fuels, biodiesel has poor calorific value [432].

2.20.11. Cold flow property

The cold flow properties of a fuel are the sign of its effectiveness at low temperatures. Cloud point, pour point and cold filter plugging point are the cold flow properties of biodiesel. Cloud point is the temperature at which little clumps of wax are seen after cooling of biodiesel. It is related with the filter plugging point, where the fuel starts to clog filters henceforth making obstacle in engine performance. Pour point is the lowest temperature at which the fuel flow is conceivable. All these properties highly affect the engine performance of vehicles at cold condition [433, 434].

2.20.12. Oxidation stability

Oxidation stability is one of the important properties of biodiesel for concerning the storage stability and quality of biodiesel. It demonstrates the level of resistance of a fuel to oxidation as well as its resistance towards water vapor or air for prolonged storage. A few parameters like presence of water, air, light, heat, traces of metals, unsaturation in the fatty acid chain, free fatty acids, etc. can affect the oxidation stability of biodiesel [435]. At typical temperature, the unsaturated part of biodiesel molecule reacts with oxygen to form hydroperoxides. As the oxidation process continues, the hydroperoxide undergoes decomposition to form aldehyde, ketones and acids with pungent smell. The oxidation process results in increase of viscosity and cetane number of biodiesel which have hindering impact. Oxidation stability of biodiesel fuel is determined by copper strip corrosion method. Proper working of the engine strongly depends on these properties [436]. All these properties influence the quality of biodiesel and are determined by standard test methods.

2.20.13. Storage stability

A study on the stability of methyl and ethyl esters of sunflower oil reports that ester fuels (biodiesel) should be stored in airtight containers, the storage temperature should be 30°C [437]. Methyl esters are slightly more stable than ethyl esters. Two parameters, namely temperature and the nature of the storage container, are claimed to have the greatest influence on storage stability [438]. Samples stored in the presence of iron behave differently than those stored in glass. Higher temperature favours degradation of the hydroperoxide at a faster rate than when it is stored at room temperature.

2.21. Blends of biodiesel

Due to the negligible sulfur content, higher lubricity, very high cetane values, etc., blending of biodiesel with petroleum based fuels is preferred. The blending of biodiesel with diesel and petrol in different composition is widely accepted and can be used in the compression ignition engines without any modification. A significant part of the world uses a system known as the B factor to state the amount of biodiesel in any fuel blend. Commonly distributed blends of biodiesel in the retail diesel fuel market place are: 100% biodiesel referred to as B100; 20% biodiesel, 80% petrodiesel labelled B20; 5% biodiesel, 95%

petrodiesel labelled B5; 2% biodiesel, 98% petrodiesel labelled B2. Blends of 20% or lower quantity of biodiesel is common and it can be used in diesel engines with no, or minor modifications. The blending B100 (100% biodiesel) has been assigned as an alternative fuel by the U.S. Department of Energy and the U.S. Department of Transportation. The American Society of Testing and Materials (ASTM) have approved a specification for biodiesel fuel. The addition of biodiesel in the fuel blend diminishes the net carbon emissions, enhances fuel ignition and decreases the toxicity of the air emissions. In addition, biodiesel is secure during storage and transportation as it is biodegradable, less toxic and has higher flash point rather than traditional diesel. Biodiesel has currently not been popularized all over the world. The major bottleneck is the surprisingly high cost of feedstock used for biodiesel production, which prohibits its widespread application [439]. Two ways of reducing the cost of biodiesel production include the utilization of cheap feed stock for production and the utilization of blends of biodiesel in unmodified diesel engines [440, 441]. Some important advantages as well as limitations of biodiesel are presented in **Table 2.8.**

Table 2.8. Advantages and limitations of biodiesel

Advantages	**Limitations**
Produced domestically Economic benefits on national economy No engine modification required Similar fuel mileage to engines running on petroleum diesel Cleaner emission Reduces health hazards More eco-friendly Lesser carbon emissions and greenhouse effect Produces less or no sulfur Higher flash point than diesel fuel Less volatile, safer to store and transport Biodegradable Higher cetane number than petro diesel Excellent lubricity Enhances the life style of farmers Non-toxic Non-flammable	High viscosity Lower energy content Higher cloud point and pour point Higher nitrous oxide emission Higher price High degradation rate Storage for shorter duration

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Chapter 3 MATERIALS AND METHODS

Experiments were designed and carried out to fulfill the objectives of the present study mentioned in the previous chapter. Materials used and experimental methods adapted for the purpose are described below under appropriate heads. Fresh rubber seeds of RRII 105 variety of rubber collected locally and analytical grade chemicals purchased from HiMedia Laboratories (Mumbai, India) and Merck India Ltd. were used for this study. *p*-nitrophenyl palmitate (*p*NPP) was selected as the substrate for lipase assay and it was purchased from Sigma Chemical Co., USA.

3.1. Rubber seed collection, study of morphology and moisture content

Fresh rubber seeds of RRII 105 clones were collected from the rubber plantation of University of Calicut, Kerala, India (11.1340 °N, 75.8952 °E). Seeds were brought to the laboratory for the determination of their primary properties.

Collected rubber seeds (**Figure 3.1**) were subjected to moisture content analysis and the primary physical properties of the seeds were determined. The seeds were then dried, decorticated and cleaned manually using hammer to free the kernel from the shells. Shell and kernel were weighed separately to find out the seed to kernel ratio.

Figure 3.1. Different growth stages of rubber fruits and seeds; A) premature green fruit, B) mature fruit, C) seed and D) kernel.

The seeds were dried in hot air oven at 70°C for 4 hours. Dried kernels were kept in airtight plastic containers to avoid contamination. The kernels were milled using laboratory blender for preparing rubber seed powder (RSP) and extracting rubber seed oil (RSO).

3.1.1. Study of morphology of the rubber seed

The rubber seeds collected from the fruits were used for the study of morphology and moisture content first. Parameters such as colour of the seed, seed size, seed weight, kernel weight, seed-kernel ratio, shell percentage were determined using standard procedure.

3.1.2. Moisture content determination

100 g of fresh rubber seed kernel was weighed in triplicate using an analytical balance and then placed in a thermostatically controlled hot air oven at 105^{o}C for 24 h. The sample was removed and placed in a desiccator and cooled to room temperature. The samples were weighed after every 3 h interval and proceeded until a constant weight was obtained. Loss in weight of the sample was recorded and percentage of moisture content was calculated by the following equation:

$$\% \text{ of moisture content} = \frac{\text{Loss in weight (g)}}{\text{Weight of the sample}} \times 100$$

3.2. Proximate determination of samples

For the purpose of this study, the parameters such as crude fat content, total lipid content, total protein content, total sugar content, total amino acid content and total phenolics content were analyzed.

3.2.1. Estimation of crude fat content and total lipid content

Moisture determined samples were transferred into clinical tissue paper, bagged, sealed and placed in a thimble with cotton wool to prevent loss of the sample [1]. An anti-bumping granule was added and 150 ml n-hexane poured into a 250 ml round bottom (RB) flask and fluxed for 4 hours at a temperature of 70^{o}C. The flask was removed and evaporated on a steam bath and the oil dried for 30 minutes in an oven at 105°C. The oil was cooled to room temperature and weighed [2].

The total lipid content of rubber seed was determined by modified method of Folch et al., 1957 [3]. 100 g of milled sample was homogenized thoroughly in 25 ml of chilled diethyl ether using a clean glass mortar and pestle. The

homogenate was centrifuge at 10,000 *g* for 10 min. The supernatant was collected in preweighed china dish. The sediment was homogenized again with chilled diethyl ether and the process was repeated 3 or 4 times. Then, the supernatant was added to the same china dish and kept in hot air oven at 60°C for 24 h. The china dish with concentrated aliquots was weighed again and the weight difference was calculated.

3.2.2. Estimation of total protein content

Total protein content was estimated by Lowry's method wherein bovine serum albumin (BSA) was used as the standard [4].

3.2.2.1. Extraction

100 g of chopped tissue sample was homogenized in 50 ml of phosphate buffer using pre-chilled glass mortar and pestle. 1 ml of the homogenate was pipetted to a centrifuge tube and equal volume of 10% trichloroacetic acid (TCA) was added. This mixture was kept in a refrigerator (4^{o}C) for 1 h for flocculation. The protein precipitate was collected by centrifugation at 5000 rpm for 10 min at 4°C. The supernatant was decanted off. The residue was washed twice with chilled 2% TCA followed by washing with 30% perchloric acid to remove starch.

3.2.2.2. Protein assay

Reagents

- Reagent A: 2% Na_2CO_3 in 0.1 N NaOH.
- Reagent B: 500 mg $CuSO_4$ in 1% potassium sodium tartrate.
- 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 sample to the test tube and made up to 1 ml with 0.1 N NaOH. Added 5 ml of alkaline copper reagent and mixed

vigorously in a cyclomixer and incubated at room temperature in dark for 10 min.

- Added 0.5 ml of Folin's reagent, vortexed well and incubated at 25°C for 30 min.
- The colour developed was read at 660 nm in a UV spectrophotometer (Shimadzu, Japan).
- Calculations were done using the graph generated from the standard graph of BSA.

3.2.2.3. Standard BSA graph

- 1 mg/ml stock solution was prepared with BSA.
- Serial dilutions 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°C for 30 min.
- Read the absorbance at λ660 using spectrophotometer.
- Calculation was made with the help of a standard curve plotted against concentration vs optical density and total protein content was expressed as miligram of protein per gram dry weight of plant tissue.

3.2.3. Estimation of total sugar content

3.2.3.1. Extraction

100 g of fresh rubber seed was chopped and homogenized in 25 ml of 80% ethyl alcohol using a clean glass mortar and pestle. The homogenate was transferred to RB flask and boiled for 1 h on a boiling water bath. After cooling it was centrifuged at 7168 *g* for 10 min and the supernatant was collected. The residue was again ground in 10 ml alcohol and again boiled for 30 min. After cooling and centrifugation, the supernatant was added to the homogenate collected earlier. The combined extract was then evaporated to dryness in a clean china dish kept in a hot air oven at 60^{o}C [5].

3.2.3.2. Estimation

From the eluted sample, 0.1 ml aliquot was taken in the test tube and made upto 1 ml. To this, 0.1 ml of 80% (w/v) phenol was added and mixed well. 5 ml of concentrated sulphuric acid was added to the tube carefully from a burette. After cooling, the optical density of the resultant solution was measured at 490 nm using a UV-visible spectrophotometer. D-glucose was used as the standard. The sugar content was calculated by the given equation:

$$\frac{\text{Concentration of standard}}{\text{OD of the standard}} \times \text{Sample OD} \times \frac{\text{Volume of total}}{\text{Volume of aliquot}} \times \frac{1}{\text{Weight}}$$

3.2.4. Estimation of total amino acid content

3.2.4.1. Preparation of reagent solution

Dissolved 20 g of ninhydrin and 3 g of hydrinadantin in 750 ml methyl cellosolve and 250 ml of acetate buffer was added to that mixture.

3.2.4.2. Extraction and estimation

100 g of chopped rubber seed sample was weighed and homogenized with 50 ml of 80 % ethanol and centrifuged at 10,000 *g* for 20 min. 1 ml of supernatant was taken in a 10 ml test tube, to this 1 ml of ninhydrin reagent was added and shaken well. The mixture was placed in boiling water bath for 15 min. On cooling, diluent (1:1, water-propanol) was added to the solution. The final mixture was shaken well and the absorbance was measured at 570 nm. L-leucine was used as the standard. The total amino acid concentration was calculated by the following equation [6].

$$\frac{\text{Concentration of standard}}{\text{OD of the standard}} \times \text{Sample OD} \times \frac{\text{Volume of total}}{\text{Volume of aliquot}} \times \frac{1}{\text{Weight}}$$

3.2.5. Estimation of phenolics

3.2.5.1. Extraction

100 g of fresh tissue was weighed and homogenized in 50 ml of 80% ethanol in a clean mortar and pestle. The homogenate was centrifuged at 10,000 *g* for 20 min and the supernatant was collected. The residue was re-extracted with 80% ethanol. The homogenate was again centrifuged and supernatant was

pooled. The supernatant was evaporated to dryness at 60°C. The residue was dissolved in 5 ml distilled water [7].

3.2.5.2. Estimation

1 ml aliquot was pipetted out and made up to 2 ml with distilled water. Equal volume of Folin Denis reagent was added to it. The content was thoroughly mixed and after 3 min, 2 ml of 1 N sodium carbonate was added. This mixture was kept in water bath for 1 min and the optical density was measured at 650 nm and total phenolics were calculated by the given equation:

$$\frac{\text{Concentration of standard}}{\text{OD of the standard}} \times \text{Sample OD} \times \frac{\text{Volume of total}}{\text{Volume of aliquot}} \times \frac{1}{\text{Weight}}$$

3.2.6. Elemental analysis

Elemental analysis of rubber seed powder to determine carbon, hydrogen, nitrogen and sulphur contents was carried out using elemental analyzer (Thermo Fisher Scientific Elemental Analyzer; Flash 2000). Around 2 mg of the aqueous samples were weighed in soft universal tin containers (O.D: 0.5 mm × H: 0.8 mm) and sealed tightly without air gap. Sample was loaded into the combustion chamber via the auto sampler with the presence of oxygen gas. After combustion, the resulting gases (N_2, O_2, H_2O, and SO_2 or SO_3) were carried by running helium gas, then swept through a GC column that provided separation of the combustion gases, and finally, detected by a thermal conductivity detector (TCD). Total run time was around 12 min. Elemental analysis was carried out in triplicate and average value was reported for each element. The oxygen (O_2) content was calculated by mean difference. Cystine ($C_6H_{12}NO_4S_2$) was used as refernce material. This organic analytical standard consisted of a homogeneous batch of cystine for use as a routine working microanalytical standard for the determination of carbon, hydrogen, nitrogen, oxygen and sulphur. This should be stored between 20°C to 25°C and should be kept tightly sealed, away from light and moisture [8].

3.3. Qualitative analysis of toxicity of rubber seed powder

Picric paper test was conducted to evaluate the presence of linamarin (a cyanogenic glucoside found in some plants including the rubber plant) and it was made using Whatman No.1 filter paper. The filter paper was dipped in alkaline picrate sodium solution prepared from picric acid and sodium carbonate (0.5% (w/v) in 2.5% (w/v) sodium carbonate) and the colourless paper turned immediately to yellow. After 30 min, the filter paper was allowed to air dry, and covered with aluminum foil. A color change from yellow to orange and to brown indicated the presence of linamarin [9].

3.4. Extraction of rubber seed oil (RSO)

To extract rubber seed oil, the kernels were milled using a blender. Extraction using manual oil expeller and extraction using soxhlet apparatus were attempted to extract oil from the dried kernel and to compare the efficacy of the techniques.

3.4.1. Using mini handed oil expeller

Preheated kernels (40 g) were weighed and filled into the feed hopper of the oil expeller (**Figure 3.2**). The expeller shaft was continually heated with spirit lamp. This screw shaft was rotated continuously to crush the seeds. The arrangement of the screw and its shaft was made in the way that the material was progressively compressed as it moved on. Hard and continuous rotation of the press allowed sufficient pressure needed for the extraction of oil. The oil was released from the seeds by gradually raising the pressure. The oil oozed out from the nozzles and was collected in the beaker. Oil yield was expressed as oil content percentage calculated.

Figure 3.2. Mini handed oil expeller

3.4.2. Using soxhlet extraction method

150 ml of chemical grade *n*-hexane was poured into a 250 ml clean dry RB flask. 40 g of the milled sample was bagged in a cellulose thimble and was placed in the centre of the extractor (**Figure 3.3**). The Heating mantle was adjusted to 68^{o}C (boiling point of hexane). When the solvent was boiling the vapour rose through the vertical tube into the condenser at the top. The liquid condensate dripped into the filter paper thimble in the centre which contained the solid sample to be extracted. The extract seeped through the pores of the thimble and filled the siphon tube, where it flowed back down into the RB flask. This was allowed to continue for 50 cycles at 3 h. At the end of the extraction, the oil plus solvent mixture was transferred to sampling flask of rotary evaporator to recover solvent from the oil.

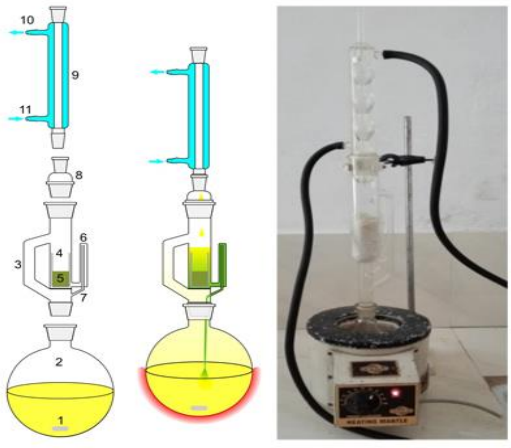

Figure 3.3. Schematic description and pictorial setup of the soxhlet apparatus; 1: stirrer bar, 2: extraction pot, 3: distillation path, 4: soxhlet thimble, 5: extraction sample, 6: siphon arm inlet, 7: siphon arm outlet, 8: expansion adapter, 9: condenser, 10: cooling water in, 11: cooling water out.

3.4.3. Oil recovery using rotary vacuum evaporator

The sampling flask (with the oil mixture collected) was then fitted to a rotary vacuum evaporator (**Figure 3.4**) and dipped in the heating medium (water) until all the mixture was totally immersed. The instrument operation conditions were adjusted and the temperature was set slightly above the boiling point of the solvent (at 70°C). Then, the mixture was rotated slowly (150 rpm) to prevent the mixture getting evaporated out from the instrument. The vacuum pump was switched on to suck up the evaporated volatile solvent. After 30 minutes of running, the heating system was switched off and left to cool down for another 30 minutes. Finally, collected the solvent from collecting flask and oil from sampling unit. Collected oil was measured and stored in airtight screw cap vials in cold room (4^{o}C) and solvent residue was used for further reactions.

Figure 3.4. Separation of oil using rotary evaporator.

3.5. Study of the effect of the solvent and extraction time on oil extraction

In this experiment polar (ethyl acetate & methanol) and non-polar (*n*-hexane & petroleum ether) solvents were used so as to study their effect on soil extraction. 150 ml of each solvent was poured into 250 ml RB flask, and temperature was adjusted on the basis of boiling point of each solvent. The extraction was continued for 2, 4, 6, 8 and 10 hours. At every 2 h cycles extraction mixture was collected. Subsequently the oil was separated using rotary evaporator and the volume was noted.

The extracted RSO was transferred into a dried and cleaned measuring beaker and kept in hot air oven at 110°C for 30 min to ensure the complete removal of solvent. The final volume of the oil was recorded. Collected oil samples were purified using 0.2 µm micro filter. Oil yield was expressed as oil content percentage and calculated using following equation [10].

$$\textbf{RSO yield (wt \%)} = \frac{\textbf{Mass of RSO collected (g)}}{\textbf{Mass of rubber seed kernel used (g)}}\textbf{(at 70 °C)} \times \textbf{100}$$

3.6. Determination of physico-chemical properties of RSO

Several indices were used for the determination of oil quality and for further reactions. Standard ASTM D6751 methods were performed to determine the physicochemical properties like water content, acid value, free fatty acid content, viscosity, density, peroxide value, saponification value and iodine value.

3.6.1. Determination of water content

Water content is determined by weighing the fresh weight and dry weight of the sample. Here, collected RSO was immediately weighted using electronic weighing balance and then it was kept in hot air oven for 5 h at 110°C. After that the weight was again recorded and percentage of water content was calculated using the following formula [2, 11].

$$\textbf{Water content (\%)} = \frac{\textbf{Difference in oil weights (g)}}{\textbf{The mass of the oil before keeping it in the oven (g)}} \times \textbf{100}$$

3.6.2. Determination of acid value and free fatty acid content

1 g of the oil was weighed into a clean dry conical flask. Added 20 ml iso-propyl alcohol and stirrered until it completely dissolved. Two drops of phenolphthalein were added as indicator, and was titrated against 0.1 N standard potassium hydroxide (KOH) solution. The end point of the titration was noted when the pink colour of the indicator appeared. Acid value was calculated using the following equation [11-14].

$$\textbf{Acid value} = \frac{\textbf{N. KOH} \times \textbf{Eq. wt. of KOH} \times \textbf{V. KOH}}{\textbf{Weight of RSO used}}$$

Where, N.KOH= Normality of KOH; equivalent weight of KOH = 56.11; V.KOH = volume of KOH used.

Acid value of the oil or biodiesel sample indicates quantitatively the presence of free fatty acid in it. RSO was compared with different vegetable oils such as rice bran oil, palm oil, sunflower oil, olive oil and ground nut oil.

3.6.3. Determination of viscosity by Ostwald's viscometer

Ostwald's viscometer is a commonly used laboratory instrument, which consists of a U-shaped glass tube held vertically (**Figure 3.5**). The cleaned viscometer was kept in a temperature controlled water bath (30^{o}C). About 50 ml of RSO was allowed to flow through its capillary tube between two etched marks, A and B. The time of flow of the aliquots was noted using a stopwatch. For finding the viscosity of liquids it is important to calibrate the viscometer using a reference liquid. Water is a commonly used reference liquid. The value of viscosity of the sample is calculated from the following relation [2, 12].

$$\eta / \eta^* = dt/d^*t^*$$

Where, η* is viscosity coefficient of the reference sample (0.8007 centipoise (cP) at 30°C and 0.6527 at 40°C for water), d* is the density of the reference sample, and t* is the time flow of the reference sample; d and t are density and time flow of the sample respectively.

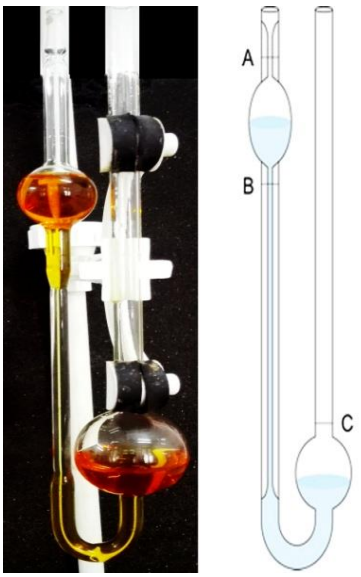

Figure 3.5. Ostwald's viscometer; A, B and C represent the etched marks of capillary tube.

3.6.4. Determination of density

Density of the feedstock was calculated by weighing the sample against different volumes and calculating using the formula:

Density = Mass/Volume

3.6. 5. Determination of peroxide value

For this purose 5 g of rubber seed oil sample was weighed accurately into a 250 ml Erlenmeyer flask. Then 50 ml of acetic acid and chloroform solution (3:2) was added into it and mixed until it completely dissolved. Using a micropipette 0.5 ml of saturated potassium iodide was then added into the mixture and the content was swirled again for a minute. To the solution, 30 ml of distilled water was added and titrated against 0.01 N sodium thiosulfate ($Na_2S_2O_3$) solution using starch as indicator. The end point was recorded when the blue colour suddenly disappeared. A blank was carried out similarly and the peroxide value calculated using following equation [11, 12].

Peroxide value = (S-B) x N x 1000/W

Where, S = Titre value of sample, B = Titre value of blank; N = Concentration of the $Na_2S_2O_3$ solution (0.01 N); W = Weight of sample (g).

3.6.6. Determination of saponification value

About 1 g of the RSO was weighed and dissolved in 12.5 ml of 0.5 N ethanolic solution of KOH. The solution was refluxed using water condenser until the oily layer disappeared. The hot soap solution was titrated against 0.5 N HCl using phenolphthalein indicators when the pink colour disappeared. A blank was performed simultaneously and the saponification value was calculated using the equation:

Saponification value = 56.11 x N x (S - B) /W

Where, N = Concentration of HCl; S = Titre value of the sample; B = Titre value of the blank; W = Weight of sample (g).

3.6.7. Determination of Iodine value

Poured 2.5 ml of rubber seed oil into a 500 ml Erlenmeyer flask. Added 20 ml of cyclohexane and 25 ml of Wijs reagent (Iodine monochloride in acetic acid) into the same flask. Mixed the flask contents thoroughly and stored the

solution at a temperature of 20°C for 30 minutes. After thirty minutes, added 10 ml of 15% potassium iodide solution and 100 ml of water to the solution. Recorded the mass of the solution using an electronic balance. Mixed the contents thoroughly and titrated the solution with 0.1 N solution of sodium thiosulfate until the yellow colour disappeared. Then added 1 ml of starch indicator to the flask and mixed the contents while titrating the solution with 0.1 N of sodium thiosulfate until the blue colour disappeared. Calculated the iodine value (mg of iodine/g of oil) using the following formula [11, 12].

Iodine value = (S) (0.01269) (100)/W

Where, S represents the volume of sodium thiosulfate required to titrate the solution in ml; W represents the weight of the sample titer (g).

3.6.8. Determination of pH Value

One gram of oil sample was poured into a clean dry 25ml beaker and 10 ml of hot distilled water was added to the sample in the beaker and stirred slowly. It was then cooled in a cold-water bath to 25°C. The pH electrode was standardized with buffer solution and the electrode immersed into the sample and the pH value was read and recorded.

3.7. Characterization of RSO

3.7.1. Elemental analysis

Carbon, hydrogen, nitrogen and sulphur contents were determined using elemental analyzer (Thermo Fisher Scientific Elemental Analyzer; Flash 2000). Around 2 mg of the aqueous samples were weighed in soft universal tin containers (O.D: 0.5 mm × H: 0.8 mm) and sealed tightly without air gap. Sample was loaded into the combustion chamber via the auto sampler with the presence of oxygen gas. After combustion, the resulting gases [nitrogen (N_2), oxygen (O_2), water (H_2O), and sulfur dioxide (SO_2) or sulfur trioxide (SO_3) were carried by running helium gas, then swept through a GC column that provided separation of the combustion gases, and finally, detected by a thermal conductivity detector (TCD). Total run time was around 12 min. Elemental analysis was carried out in triplicate and average value was reported for each element. The oxygen (O_2) content was calculated by mean difference. Cystine ($C_6H_{12}NO_4S_2$) was used as

reference material. This organic analytical standard consisted of a homogeneous batch of cystine for use as a routine working microanalytical standard for the determination of carbon, hydrogen, nitrogen, oxygen and sulphur. This should be stored between 20°C to 25°C and should be kept tightly sealed, away from light and moisture [8].

3.7.2. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy is carried out for the structural characterization of a substance so as to find out its chemical nature as well as the compositional identity. FTIR spectroscopy of RSO was carried out using Jasco FTIR 4100 series, Japan. RSO sample (1 g) was mixed with spectral grade of anhydrous potassium bromide (KBr) and fixed on a sample holder for analysis pellete preperation. FTIR analysis of the sample was carried out at mid infra red region (400-4000/cm).

3.7.3. Determination of fatty acid profile

The high FFA containing aliquot (1 µl) was diluted with commercial grade n-hexane (9 µl) and then injected (220°C) in splitless mode of a gas chromatograph (Agilent, 7697A, USA) equipped with a DB-WAX capillary column (Agilent, 30 m × 250 µm, 0.25 µm film thickness) and mass spectrophotometer (Agilent, 5973N). High purity grade helium was used as carrier gas (1 ml/min). The separation was performed on the basis of temperature programme as started from 50°C (5 min), rising to 65°C at a rate of 2°C/ min, and then 200°C (5°C/min, 5 min) and 250°C (10°C/min) that was held for 10 min. The retention time and mass spectra of compounds were identified using the Wiley 7n.1 database. The area percentage method was used to estimate the fatty acid composition of the oil [15].

3.7.4. Evaluation of antibacterial activity

3.7.4.1. Culture and medium

Clinical strains of *Escherichia coli* and *Staphylococcus aureus* were cultured in nutrient broth (NB) medium. The NB contained the following ingredients (%): 0.5 peptone; 0.5 NaCl; 0.3 yeast extract and 0.3 meat extract in

double distilled water. The bacteria were inoculated in the NB medium and incubated in a self-regulating orbital shaker (Inlabco; India) for 12 h at 37°C and 150 rpm. Nutrient agar (NA) dishes were prepared in triplicate. Once the standard culture and dishes were prepared, disc diffusion method was used to study the antibacterial activity [16].

3.7.4.2. Disc diffusion method

Hexane extract of rubber seed oil was resolubilized with 10% DMSO (Dimethyl Sulfoxide) and stored at 4°C in air tight bottles for anti-microbial assay. Antimicrobial activity of RSO were evaluated against standard and clinical strains of the Gram-negative bacterium *E. coli* and Gram-positive bacterium *Staphylococcus aureus* by agar well diffusion method. Briefly, the aliquot of 100 µl from each cell suspension (12 h seed cultures) were swabbed over the surface of NA plate by using sterile cotton and allowed to dry. The DMSO treated as test control, whereas, clinical disc of Tetracycline (30 mcg/disc) was used as positive control and crude RSO was tested against test bacteria. The wells of 8 mm diameter punched into the NA plates and 10−30 µl of RSO was poured on each disc. The plates were then sealed with parafilm to prevent any unwanted contamination. All the plates were incubated at 37°C for 24 h in temperature controlled incubator with presence of light (Flourescent lamp; 18 W) and the diameter of the resulting inhibition zone in each plate was measured. All tests were performed in triplicate.

3.7.5. Study of antioxidant activity

Antioxidants are molecules that are able to scavenge accumulated reactive species, thereby providing cellular protection against DNA damage, membrane destruction and other damages. The oxidative chain reactions initiated by free radicals are often terminated by certain components that are collectively known as antioxidants. Generally, there are two types of antioxidant assays - one is based on the rate of oxidation of lipid content and the other one is based on the rate of radical or electron scavenging ability. The former one includes assays like thiobarbituric acid assay (TBA), malonaldehyde/high-performance liquid

chromatography (MA/HPLC) assay, malonaldehyde/gas chromatography (MA/GC) assay, beta-carotene bleaching assay, and conjugated diene assay. On the other hand, the latter one includes 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, ferric reducing/ antioxidant power (FRAP) assay, ferrous oxidation-xylenol orange (FOX) assay, ferric thiocyanate (FTC) assay, and aldehyde/carboxylic acid (ACA) assay [17]. DPPH assay and Ferric reducing power assay were carried out presently to assess the antioxidant activity of RSO.

3.7.5.1. DPPH assay

DPPH radical scavenging activity was determined using the modified method of Blois [18, 19]. Ethanolic extract of the oil solution (0.1 ml) in a test tube was well mixed with 3.9 ml of methanol and 0.1 ml of α,α-diphenyl-β-picrylhydrazyl (DPPH) solution (1.0 mM in methanol). The mixture was kept at ambient temperature for 30 min. The colour changes from deep violet to light yellow were noted (DPPH reacts with an antioxidant compound which can donate hydrogen; it is reduced). The colour difference was measured by using a UV-VIS spectrophotometer at 517 nm. Ascorbic acid was used as standard reference. The mixture of ethanol (3.3 ml) and sample (0.5 ml) served as blank. The control solution was prepared by mixing ethanol (3.5 ml) and DPPH radical solution (0.3 ml). All measurements were done in triplicate. The DPPH radical scavenging activity percentage was determined by the following equation:

DPPH radical scavenging activity (%) = [(Abs control – Abs sample)/ Abs control] x 100

3.7.5.2. Determination of ferrous reducing power

Reducing power of rubber seed oil was determined according to the method of Takashi [20] with some modifications. Each 250 µl of sample or ascorbic acid (a positive control), 250 µl of 0.1 M phosphate buffer (pH 7.2) and 500 µl of 1% potassium ferricyanide were added to test tube. After incubation at 37°C for 60 min, 250 µl of 10% trichloroacetic acid and 750 µl of deionized water were added. Then, the absorbance was measured at 700 nm (Abs1). Next, 250 ml of 0.1% ferric chloride was added to the mixture and the absorbance was

measured again (Abs2). Ferrous reducing power was calculated by the following equation [15].

Reducing Power = [Abs2 – Abs1] of sample – [Abs2 – Abs1] of control

3.7.5.3. Determination of lipid peroxidation inhibition activity

Increased lipid peroxidation is considered as an important cause of oxidative stress initiation upon various tissue injuries, cell death or the progression of many acute and chronic diseases [21]. Inhibition of lipid peroxidation was determined according to the thiobarbituric acid reactive substances (TBARS) method with some modifications [22]. 100 µl of each extract (EtRSO, MtRSO and HeRSO) was mixed with linoleic acid 900 µl and then incubated the mixture at 100°C for 20 min. 1 ml of buffer solution (pH 3.5) and 1 ml of the mixture of 20 mM of thiobarbituric acid in 10% trichloroacetic acid were added in the resulting mixture and then incubated at 100°C for 30 min to obtain the pink coloured solution. The absorbance was measured at 532 nm using a UV spectrophotometer. All data were run in triplicate and compared with ascorbic acid as positive control and deionized water was used as negative references. The percentage inhibition of lipid peroxidation was calculated by the following equation:

Inhibition of lipid peroxidation (%) = [(Abs control – Abs sample)/ Abs control] x 100

3.8. Lipase production using agrowastes

Deoiled residues of rubber seed flour, cotton seed flour and coconut cake were screened for lipase production by solid state fermentation (SSF) method. The coconut cake and cotton seed flour were purchased from local market, while rubber seed powder was collected from the oil extraction residue obtained as described earlier. The collected agrowaste materials were ground using a laboratory blender and stored in airtight plastic containers.

3.8.1. Microorganisms and maintenance of cultures

Two new bacterial strains, *Pseudomonas aeruginosa* BUP2 (MTCC No.5924) & *Pseudomonas* sp. BUP6 (MTCC No.5925), were employed in this work. These strains were isolated from the rumen of Malabari goat and described

by the Enzyme Technology Laboratory of Department of Botany, University of Calicut, India. These cultures were deposited at Institute of Microbial Technology (IMTECH) Chadigarh. These cultures (*P. aeruginosa* BUP2 and *P.* sp. BUP6) were maintained on semi-synthetic basal salt medium (BSM) and Benjamin, Unni & Priji medium (BUP medium) respectively; which was supplemented with 0.3% of ground nut oil. The inoculated media were incubated in temperature controlled incubator (Biolinx; India) at 37°C for 24 h. The mother cultures were prepared on respective medium plus agar petri-dishes. In between two or three weeks each culture was subcultured. BSM medium & BUP medium comprise the following chemicals as shown in **Table 3.1**. and **Table 3.2.** [23, 24].

Table 3.1. Composition of basal salt medium

Ingredients	**Chemical formula**	**Quantity (g/L)**
Dipotassium hydrogen phosphate	K2HPO4	2.0
Sodium chloride	NaCl	2.0
Ammonium Sulphate	(NH4)2SO4	4.0
Ammonium Nitrate	NH4NO3	5.0
Magnesium Sulphate	MgSO4.7H2O	0.1
Calcium Chloride	CaCl2	0.1
Yeast Extract powder		3.0
pH-7.1±0.1		

Table 3.2. Composition of BUP medium.

Ingredients	**Chemical formula**	**Quantity (g/L)**
Dipotassium hydrogen phosphate	K_2HPO_4	2.0
Sodium chloride	NaCl	2.0
Ammonium Sulphate	$(NH_4)_2SO_4$	4.0
Ammonium Nitrate	NH_4NO_3	5.0
Magnesium Sulphate	$MgSO_4.7H_2O$	0.1
L-Cysteine-HCL		0.5
Beef extract		3.0
Peptone		5.0
pH-6.7±0.1		

3.8.1.1. Preparation of inocula

One loop full of cultures (*P. aeruginosa* BUP2 and *Pseudomonas* sp. BUP6) were inoculated to respective semisynthetic sterilized media at aseptic

conditions and then incubated in fully controlled orbital shaker (Scigenics Biotech, India) at 37°C and 150 rpm for 12 h (in 100 ml Erlenmeyer flasks), so as to reach maximum optical density of 2.5 at 600 nm. The 12 h seed cultures were considered as inocula. Best and active culture was employed for solid-state fermentation techniques.

3.8.2. Solid State Fermentation strategy

Microbial lipases are mostly produced by Submerged fermentation (SmF). Solid state fermentation (SSF) technique is a proven strategy for the effective utilization of agro-industrial residues for producing value added products of commercial interest at low cost. Generally, lipase production is organism specific and it is released during the late logarithmic or stationary phase of its life cycle. Research on lipase progressed very rapidly in the past few decades, giving much emphasis on enzymatic hydrolysis of lipid or fats to biodiesel. Nowadays SSF strategy is increasingly employed as a method for the production of lipase from oil mill effluents because of the several advantages of SSF. Benjamin and Pandey (1998), employing *Candida rugosa*, demonstrated the utility of mixed-solid substrate containing wheat bran and coconut oil cake for lipase production [25]. Another report said that, deoiled cake from *Jatropha* seed was used as a support for the production of lipase (1084 U/gds) from *Pseudomonas aeruginosa* PseA through SSF [26]. Research on lipase progressed very rapidly in the past few decades, giving much emphasis on enzymatic hydrolysis of lipid or fats to biodiesel. Now-a-days SSF strategy is increasingly employed as a method for the production of lipase from oil mill effluents because of its several advantages of SSF. SSF strategy was employed in the present experiment for lipase production from agrowaste. The cutures of *P. aeruginosa* BUP2 and *P.* sp. BUP6) were employed for solid state fermentation (SSF) techniques as suggested by Unni et al. [27].

In the present experiment, fermentation was performed in 100 ml Erlenmeyer flasks. 4 g of substrate (oil cake) was weighed into a conical flask and then moisturised with 50% of the medium. All preparations were made in

triplicate and autoclaved at 121°C, 15 ψ for 15 min and subsequently inoculated with 0.1 ml of *P. aeruginosa* BUP2 and *P.* sp. BUP6 inocula under aseptic condition. All flasks were incubated at 37°C for lipase production. Lipase production was quantitatively assayed at each 24 h interval for 5 days.

3.8.3. Extraction of lipase

After each interval of incubation (24 h), the flasks were withdrawn for lipase assay. Fifty percentage of fermented substrate was taken for the assay and the remaining portion was used for dry weight estimation. Subsequently, the fermented matter was dispersed with 10 ml of 0.1 M tris-HCl buffer (pH 8.0) and stirred for 10 min with magnetic stirrer (Remi; India). Then, the residue was centrifuged at 9400 *g* for 15 min at 4°C and the supernatant was collected. The clear supernatant was used as crude enzyme. Lipase activity and protein content in the crude enzyme were estimated and the best active fractions were used for transesterification reactions. The biomass obtained was dried to a constant weight on a pre-weighed Whatman No.1 filter paper at 60°C in a hot air oven and used for growth determination.

3.8.4. Purification of enzyme

3.8.4.1. Partial purification

The turbid culture was centrifuged at 12,000 *g* at 4°C for 12 min and collected the supernatant as crude lipase. The pellet was decanted and the clear supernatant was used as crude enzyme and subjected to further purification. The purification method consisted of sequential combination of precipitation and dialysis. Weighed quantities of ammonium sulphate $(NH_4)_2SO_4$ were gradually added to the crude lipase, keeping on magnetic stirrer in a cold room (4°C) and stirred for 30 min so as to reach 20% of saturation and the precipitate was collected by centrifugation (9400 *g* for 10 min at 4°C). Subsequently, the process was continued up to 20-40%, 40-60% and 60-80% of salt saturations, and the precipitates were collected separately in each step.

3.8.4.2. Dialysis

The precipitate (pellets) was collected in minimum volume of 0.1 M Tris-HCl buffer (pH 8.0), subsequently dialyzed (dialysis membrane-60, Himedia) against 0.1 M Tris-HCl buffer (pH 8.0) for 24 h at 4°C under continuous stirring with two buffer changes at an interval of 12 h. Then the dialysate was centrifuged (9400 *g* for 10 min at 4°C) and the precipitate (debris) discarded and the supernatant (concentrated protein solution) was stored in the refrigerator for gel permeation chromatography. The lipase activity and protein content of the dialysate were determined.

3.8.4.3. Gel permeation chromatography

The dialysate with the highest specific activity was used for gel permeation chromatography. Gel permeation chromatography was performed in compact and versatile GE AKTA prime plus; one-step purification system. Before the system was switched on, the back pressure was adjusted to less than 1 MPa and checked up for absence of air in the tubings and valves. The column was rinsed twice with 50 ml of eluent buffer at a flow rate of 0.5 ml/min. Before the run, the column was pre-equilibrated with 0.2 M Tris-HCl buffers (pH 8.0) until the UV baseline and pH were stable. Buffer and samples was filtered using 0.22 µm syringe filter. The dialysate was loaded onto pre-packed column of Superose^{TM} 6 (10/300 GL-Tricorn). The fractions were collected on 5 ml borosil glass tubes at a constant flow rate of 0.2 ml/min. The enzyme activity and protein content in best 3 or 4 fractions were estimated. The fractions showing maximum lipase activity were pooled and stored in deep freezer (Haier; Model No: DW-40L262) at -20°C.

3.8.4.4. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

After each purification step, the purity of lipase was confirmed by SDSPAGE. SDS-PAGE was performed using a vertical mini gel (8 cm × 7 cm) slab with notched glass plate system. Gels of 1.5 mm thickness were prepared for the entire study [28]. The SDS-PAGE was carried out using 4% stacking gel (**Table 3.3.**) and 12% separating gel (**Table 3.4.**). Lipase solution and sample

buffer (**Table 3.5.**) were mixed in the 1:1 ratio. The contents were mixed well in a pre-sterilised Eppendorf tube and heated in a boiling water bath for 3 min. Broad range protein molecular weight marker (Genei, Banglore) containing myosin (205 kDa), phosphorylase (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), lysozyme (14.3 kDa) aprotinin (6.5 kDa) and insulin (3.5 kDa) was used for the determination of the MW of protein on the gel. After the electrophoresis, the gel was stained with 0.1% coomassie brilliant blue G-250 (prepared in a mixture of 50% methanol and 10% glacial acetic acid). The destaining solvent system contained 10% glacial acetic acid: 45% methanol: 45% ddH2O. The protein bands on the SDS-PAGE gels were visualised and photographed.

Table 3.3. Ingredients for stacking gel

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

Table 3.4. Ingredients for resolving gel (12%)

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

Table 3.5. Ingredients for sample buffer

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

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

3.8.4.5. Lipase activity assay

Lipase activity of each dialysate fraction was assayed using modified method of Gupta et al. [29]. High grade *para*-nitro phenyl palmitate (*p*NPP) was used as substrate for lipase assay. Briefly, the assay tube containing 1.98 ml of 0.1 M Tris–HCl buffer with 0.15 M NaCl and 0.5% Triton X-100 was pre-incubated with 20 µl of purified lipase at 37°C for 10 min and subsequently, 20 µl of substrate (50 mM *p*NPP in acetonitrile) was added to the reaction mixture at 37°C for 30 min. The intensity of light absorbed by the reaction mixture (chartreuse yellow colour) was monitored using UV–visible spectrophotometer (Shimadzu, Germany) at λ405. The lipase activity was calculated using the following equation:

$$\text{Lipase activity (U/ml)} = \frac{\text{A} \times \text{Vf}}{\text{Vs} \times \varepsilon \times \text{t} \times \text{d}}$$

Where, A represents Absorbance at λ_{405}; Vf- Final volume of assay buffer; Vs- Volume of lipase added (ml); Ɛ- Extinction coefficient of enzyme (0.017); t- Time of hydrolysis; d - Diameter of cuvette (1 cm).

3.8.4.6. Protein estimation

The protein content of the best active fractions was estimated by Lowry's method using bovine serum albumin (BSA) as standard [7] as descried elsewhere.

3.9. Enzyme characterization

The active fraction of lipase obtained by gel permeation chromatography was used for its characterization studies. In order to determine the stability of lipase on different polar solvents, positional specificity of the enzyme and isoelectric point of the protein were analysed. The other significant characteristics of lipase were analysed as described by Unni [30]. These parameters of lipase were expressed in percentage of activity and initial activity was considerd as the base unit.

3.9.1. Lipase stability in organic solvents

The effect of organic solvents on lipase stability was evaluated by measuring the enzyme activity in 50 mM Tris- HCl buffer (pH 8.2) at different time intervals, i.e., 6, 12, 18, and 24 h. The reactions were performed on mixing

block (BIOER, UK) at constant reaction conditions such as 37°C and 1000 rpm. The following organic solvents were selected and classified based on their polarity: non- polar solvent like hexane, petroleum ether, chloroform and toluene and polar solvent like acetonitrile, ethanol, methanol and acetone [31, 32]. All the solvents used were of high laboratory grade. Microcentrifuge tube (2.5 ml) was containing purified enzyme with 1:10 v/v and 1:1 v/v organic solvents for different incubation periods at constant temperature. At the end of the reaction, the tubes were centrifuged and the enzyme part was collected. The assay mixture lacking the solvents was maintained as control and the relative activity was determined [33].

3.9.2. Positional specificity

The positional specificity of lipase was examined by analyzing the hydrolysis products of triolein (99%) using thin layer chromatography (TLC). Briefly, the reaction mixture containing 0.2 ml triolein, 4 ml 50 mM Tris-HCl buffer (pH 8.2) and 0.2 ml lipase (500 U/ml) was incubated at 37°C with shaking at 300 rpm. Samples were taken every 30 min during incubation. Immediately, after the incubation 5 ml petroleum ether was added to each tube and the solution was mixed vigorously. Then, the ether layer was decanted. The products were analyzed by thin layer chromatography (TLC) on silica gel. The solvent system for the TLC analysis consisted of a mixture of petroleum ether, diethyl ether and acetic acid (70:30:1, v/v/v). Spots were detected by spraying with 50% H_2S0_4, followed by heating at 80°C for 30 min [34].

3.9.3. Isoelectric point

The isoelectric point (pI) of a molecule is the pH at which the overall charge of it is zero. In order to determine the isoelectric point of lipase we firstly had to create a series of acetic acid buffers, ranging from 0.3M to 1mM by doubling dilutions method. Once the buffers had been prepared, each of the test tubes was filled with equal volume of (1 ml) acetic acid buffer with different pH (3.6 to 5.6). Then added to this 100 µl enzyme (dissolved in 0.1M sodium acetate) solution and the precipitations were observed. Experiments were performed at 25°C and the pH of the greatest precipitation tube was noted [35].

3.10. Lipase kinetics

Enzyme kinetics deals with the rate of product formation in enzymatic reactions. In the present study, the rate of formation of extracellular lipase was determined from Michaelis-Menten plot and Line-Weaver Burk plots. The *Km* and *Vmax* values of extra cellular purified lipase were found by using *Para*-nitrophenyl palmitate (*p*NPP) as substrate. *Km* value represents the measure of affinity of enzyme towards a substrate. Low *Km* value shows that the enzyme requires only small quantity of substrate to get saturated. *Vmax* value indicates the efficiency of the enzyme.

3.10.1. Calculation of *Km* and *Vmax*

The lipase extract was treated with *p*NPP at concentrations of 10, 20, 30, 40, 50, 60, 70, 80 and 90 mM. The reaction mixture was incubated for 1 h at 45°C (pH 8.2) during which the lipase activity was measured at regular intervals of 5 min. The *Km* and *Vmax* values were calculated for lipase using the software, Hyper 32.

3.11. Lipase immobilization

Enzymes are easily denatured and inactivated in the presence of organic solvents. Therefore, protein engineering and several physical and chemical methods such as immobilization, modification and entrapment for stabilizing enzymes in the presence of organic solvents have been developed [36]. The basic concept for enzyme immobilization is to covalently attach or entrap the protein in support materials; this forms a hard external backbone to lipase molecule, so tht faster reaction rate will occur [37]. Commonly used matrices for entrapment are calcium alignate, and polyacrylamide [38]. The immobilization process was presently done by cell entrapment method using sodium alginate and adsorption method by immobilization on celite. Lipase immobilized through entrapment by celite is more stable than physically adsorbed lipase.

3.11.1. Immobilization by sodium alginate

Sodium alginate entrapment of cells was performed by the method of Bhushan et al. [39]. Firstly, different molar concentrations (0.1, 0.2 and 0.3M) of $CaCl_2$ solutions were prepared and kept in cold condition. Sodium alginate solution was made by 1%-3% in water at room temperature. Both alginate slurry and purified lipase were mixed and stirred for 10 min to get a uniform mixture; the alginate/enzyme mixture was extruded drop by drop into cold sterile 0.2 M $CaCl_2$ solution through a 5 ml pipette from 10 to 20 cm height and kept for curing at 4°C for 1 h. The beads were hardened after some time. Finally these beads were washed with 0.2 M Tris-HCl buffer (pH 8.0) to remove excess calcium ions and unentraped cells. Then the beads were preserved in 1% sodium chloride solution in refrigerator.

3.11.2. Immobilization on celite

Pseudomonas lipase was immobilized on filter aid celite powder, an inert biosupport material; as per the method proposed by Chang et al. [40]. Ammonium salt precipated fraction of lipase (500 U/ml) was dissolved in 3 ml of Tris-HCl buffer solution (pH 8.0) and mixed with 1 g of celite. Cold acetone (15 ml) was added and the mixture was stirred for 30 min at 4°C in cold room. The lipase immobilized particles were collected by vaccum filtration and washed twice with n-hexane to remove the unadsorbed enzyme. Finally, the celite particles bound with lipase; which were lyophilized in a freeze dryer (Scanvac, USA) and stored at 4°C for future use.

3.11.3. Calculation of immobilization efficiency

The protein content of the supernatant was estimated by the method of Lowry *et al*. [4] using bovine serum albumin (BSA) as standard. The immobilization efficiency was calculated by subtracting the amount of free enzyme from the total amount of the lipase used for immobilization. The immobilization efficiency was evaluated by the following equation [41].

q = (Ci- Cf) V1/CiV2 (%)

(q represents the immobilization efficiency (%), Ci and Cf are the initial and final concentration of soluble enzyme in the supernatant (mg/cm^3) and V1 and V2 are the solution volume (cm^3). The activity recovery (%) remaining after immobilization is the ratio between the activity of bound lipase and the total activity of lipase added in the initial immobilization solution).

3.12. Characterization of immobilized lipase

3.12.1. Fourier transform infrared spectroscopy (FTIR)

The filter aid celite powder and vaccum dried immobilized lipase on celite were weighed (5 mg) and mixed with potassium bromide (KBr). FTIR analysis of the samples was carried out at mid-infra-red region of 400–4000/cm (Jasco FTIR 4100 Series, Japan).

3.12.2. Scanning electron microscopy

FESEM (field emission scanning electron microscopy) and transmission electron microscopy (TEM) are used to obtain structural details of the given sample at a resolution of 10^{-9} m. FESEM/SEM are used for collecting structural details at the surface with little information on internal details. SEM is based on collecting a formed image on cathode ray tube by the electron beam reflected from the surface of the given sample. FESEM is an advanced version of SEM technology due to the aspects of improvisation in secondary electron detector technology, producing sample image at ultrahigh resolution [42]. In the case of enzyme immobilization, SEM is used to observe morphology and distribution of enzyme immobilized onto the matrix while FESEM is used in visualizing morphological details at a particular portion of the matrix. Thus, this study employed FESEM and it helped in giving confirmation of enzyme presence on the given matrix. The SEM images of different adsorption materials exhibited highly diverse shapes and structures such as spherical, ball shaped, porous disc or plate with different dimensions. FESEM was used for morphological characterization of the original celite (filter aid powder) and immobilized celite particles. The gold coating was performed by spraying gold powder. Images were obtained at the magnification range from 45 to 30,000 depending on the feature to be examined. The instruments were set at 50 kV and accelerating voltage used was 100 kV.

3.13. Biodiesel production by lipase mediated transesterification

The next step of the study carried out was transesterification of RSO for biodiesel production with both free and immobilized forms of *Pseudomonas* lipase. The optimization of analytical procedures was carried out by using Minitab software where the chosen multivariate statistic technique was Response Surface Methodology (RSM) in order to determine the optimum parameter values so that optimum yield can be achieved. RSM was employed to develop a statistical cum mathematical correlation between different independent parameters such as molar ratio of the reactants, reaction temperature, reaction time and catalyst unit on the production of biodiesel. Three main statistical tools, i.e., analysis of variance (ANOVA), regression analysis (R^2) and 3D plotting of response variables were performed to optimize the optimum conditions for maximum production of biodiesel. The quadratic regression model of BBD (Box–Behnken design), based on the uncoded levels of the independent variables was used for the study. Box-Behnken designs are used to generate higher order response surfaces using fewer required runs than a normal factorial technique. This essentially suppresses selected runs in an attempt to maintain the higher order surface definition. The Box-Behnken design uses the twelve middle edge nodes and three centre nodes to fit a 2nd order equation.

The Box–Behnken design of experiments provides modeling of the response surface. To reduce errors, 27 trials were performed in triplicate and the fatty acid methyl esters (FAMEs) produced was taken as response variable. A second order polynomial equation was fitted to the optimization of biodiesel production, which resulted in the following regression equation:

$$Y = -150.5 + 14.58\ x_1 + 0.3224\ x_2 + 2.859\ x_3 - 6.12\ x_4 - 1.225\ x_1 * x_1 - 0.000307\ x_2 * x_2 - 0.04822\ x_3 * x_3 - 2.692\ x_4 * x_4 + 0.00655\ x_1 * x_2 + 0.0613\ x_1 * x_3 + 0.327\ x_1 * x_4 + 0.000093\ x_2 * x_3 + 0.01813\ x_2 * x_4 + 0.2491\ x_3 * x_4$$

Where, X_1 is RSO to methanol molar ratio, X_2 is lipase unit, X_3 is reaction temperature and X_4 is reaction time.

3.13.1. Purification of feedstocks

For the transesterification reaction, RSO obtained by soxhlet extraction process was subjected to filter to remove solid impurities. Since it contained gums, it was to be removed first and it was done by the process of degumming.

3.13.1.1. Degumming

Degumming of refined RSO was done to eliminate phospholipid and was done by thermal treatment using the degumming agent phosphoric acid in the presence of water [43]. The oil was heated 60°C and then 0.01 wt% of phosphoric acid was added to it with constant stirring (250 rpm). Stirring was continued for 20 min and 2 wt% water was added to the oil followed by raising the temperature to 70–80°C; stirring was continued for 10 mins. The gum in the oil settled at the bottom was removed by filtration. The oil was then dried over anhydrous sodium sulphate.

3.13.2. Experimental setup

The trans-esterification reactions were carried out in a 100 ml three neck RB flask attached to a reflux condenser. The RB flask was fixed on a hot plate magnetic stirrer (2 MLH Remi, India). The reaction mixture contained proper quantity of methanol depending on methanol to oil ratio in the presence of the purified lipase (ammonium sulphate fraction). The magnetic stirrer was adjusted to the defined temperature intervals (30, 45 and 60°C) and constant stirring (200 rpm). The temperature was measured continuously with mercury column thermometer during the process. Diagrammatic representation of the reaction setup is shown in **Figure 3.6.**

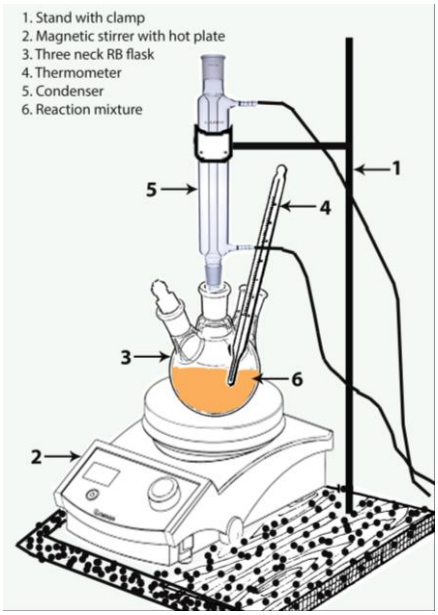

Figure 3.6. Reaction setup; transesterification reaction.

3.13.3. Transesterification of RSO by free lipase and RSM method

Presently, biodiesel production was carried out with onsite lipase using rubber seed oil. The fatty acids in the non-edible RSO were transesterified to their corresponding methyl esters so that the viscosity of the oil would be reduced and it could be used as biodiesel. For transesterification, onsite free lipase produced by *Pseudomonas aeruginosa* BUP2 was used. The important reaction parameters affecting the transesterification reaction are the oil to methanol molar ratio, reaction temperature, reaction time and enzyme concentration [44, 45].

In the present study, the parameters optimized for transesterification process were oil to methanol molar ratio, reaction time, reaction temperature and enzyme concentration (U/ml). Oil to methanol molar ratio was calculated by the following formula:

$$\text{Volume of methanol required} = \frac{\text{Wt. of RSO} \times \text{Molar No. of methanol} \times \text{Eq. wt. of methanol}}{\text{M.Wt. of oil} \times \text{Density of methanol}}$$

3.13.4. Box–Behnken Design (BBD)

The four level three point (high, medium and low) design of BBD was employed. The four independent variables and range have been shown in **Table 3.6.** Significant reaction parameters such as molar ratio of methanol (1:6 to 1:12), temperature (30 to 60°C), time (2 to 6 h), and lipase unit (500 to 1000 U/ml) were selected on the basis of preliminary studies. To reduce errors, the full factorial L27 trials were performed in triplicate as listed in **Table 3.7.** Minitab (version 14, USA) was used for designing and analyzing the experimental trials of Box–Behnken design (BBD) in Response Surface Methodology (RSM) [46].

Table 3.6. Independent variables and levels used in RSM

Independent variables	**Symbols**	**Levels**		
		-1	**0**	**+1**
RSO - methanol ratio (mol/mol)	X_1	1:6	1:9	1:12
Amount of lipase (U/ml)	X_2	500	750	1000
Temperature (°C)	X_3	30	45	60
Reaction time (h)	X_4	2	4	6

Table 3.7. Experimental trials; BBD model for the optimization of biodiesel production

Trial No	Variable levels			
	RSO –to- methanol ratio (mol/mol) X_1	Amount of Lipase (U/ml) X_2	Temperature (°C) X_3	Reaction time (h) X_4
1	1:9	1000	45	6
2	1:6	500	45	4
3	1:9	750	60	6
4	1:6	750	60	4
5	1:9	750	30	2
6	1:9	500	45	2
7	1:12	1000	45	4
8	1:12	750	45	2
9	1:9	750	45	4
10	1:9	500	30	4
11	1:9	1000	30	4
12	1:9	750	45	4
13	1:9	500	45	6
14	1:6	750	45	2
15	1:9	1000	60	4
16	1:9	750	60	2
17	1:12	750	45	6
18	1:6	1000	45	4
19	1:9	500	60	4
20	1:9	750	45	4
21	1:6	750	45	6
22	1:9	750	30	6
23	1:12	750	30	4
24	1:6	750	30	4
25	1:12	750	60	4
26	1:9	1000	45	2
27	1:12	500	45	4

3.13.5. Statistical analysis and validation experiment

The fatty acid methyl ester (FAME) produced was taken as the response variable analyzed using software Minitab version 14 (Minitab USA). Analysis of variance (ANOVA), regression analysis and plotting of response surface were performed to establish optimum conditions for fatty acid methyl ester (FAME) formation. ANOVA was used to test adequacy and fitness of the responses fitting to the second order polynomial equation. A model with *P*-values ($P>F$) less than

0.05 was regarded as significant. The lack-of-fit test was used to compare the residual and pure errors at the replicated design points. If the model fits the data well, lack of fit is not significant. Quadratic model was chosen as a highest-order significant polynomial having a non-significant lack of fit. To check the validity of quadratic model, four trials were predicted by software (**Table 3.8**). Biodiesel produced was calculated and compared with predicted values.

$$Y = \beta 0 + \sum_{i=1}^{k} \beta i Xi + \sum_{i=1}^{k} \beta ii XiXi + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta ij XiXj, \quad i < j$$

(Where, Y represents the response variable, $\beta 0$ is the interception coefficient, βi is the coefficient of the linear effect, βii is the coefficient of quadratic effect, βij is the coefficient of interaction effect when $i < j$, and k is the numbers of involved variables).

Table 3.8. Experimental trials for the validation of the predicted model

Factors	**Levels**		
	-1	0	+1
RSO - methanol ratio (mol/mol)	1:12	1:15	1:18
Catalyst weight (wt%)	4	5	6
Temperature (°C)	45	55	65
Reaction time (min)	90	120	150

3.14. Transesterification of RSO by immobilized lipase and Taguchi method

Genichi Taguchi developed a new method to examine the effect of different parameters of a process for conducting factorial experiments. A large number of experimental investigations are required to identify optimum values of experimental parameters for biodiesel production. Every time full factorial investigation is impossible because of cost and time consideration, a number of experimental investigations need to be reduced significantly. Taguchi method is one of the impressive ways of designing an experiment. Orthogonal array is selected based on number of noise factors, interactions between them and number of signal levels [47]. It could be employed to optimize the performance characteristics of process parameters, which is proved to be a powerful tool that differs from traditional full factorial investigation. The approach could economically satisfy the needs of problem solving and design optimization with less number of experiments. Thus, it is possible to reduce time and cost for the

experimental investigations [48]. Orthogonal array for DOE with four parameters (factors) at three levels (3^4) is examined.

In the present work, L9 orthogonal array was chosen to optimize the four important factors for biodiesel production from refined rubber seed oil. The L9 orthogonal array consists of three level reaction factors as shown in **Table 3.9.** Each reaction factor was allocated in separate column; nine reaction-factor groupings were accessible [49]. Thus, total nine runs were carried out as listed in **Table 3.10.**

Table 3.9. Selected factors and their levels

Trials	RSO to methanol ratio (mol/mol)	Lipase (U/ml)	Reaction temperature (° C)	Reaction time (h)
1	9	800	40	4
2	10	750	45	4
3	7	900	50	6
4	8	600	35	2

Table 3.10. Taguchi L9 orthogonal array

Sl. No	Molar ratio	Catalyst weight (wt%)	Temperature (°C)	Time (min)
1	12	4	45	90
2	12	5	55	120
3	12	6	65	150
4	15	4	55	150
5	15	5	65	90
6	15	6	45	120
7	18	4	65	120
8	18	5	45	150
9	18	6	55	90

3.14.1. Setting and separation

The progress of the reaction was monitored by removing aliquots of reactants at particular time intervals. Instantly, the contents were then transferred to a separating funnel and shaken well. The separating funnel was allowed to stand for 10-12 hours for gravity separation. At this stage, two layers were formed, namely glycerol and biodiesel. The top biodiesel layer was phase separated from the bottom glycerol layer; the glycerol phase was much denser

than biodiesel phase and it settled down while biodiesel floated up. Phase separation occurred by gravity setting into the clear golden liquid biodiesel on the top with the light brown glycerol at the bottom of the separating funnel. On the next day the glycerol at the bottom was carefully decanted into a bottle and the biodiesel layer was collected (**Figure 3.7**).

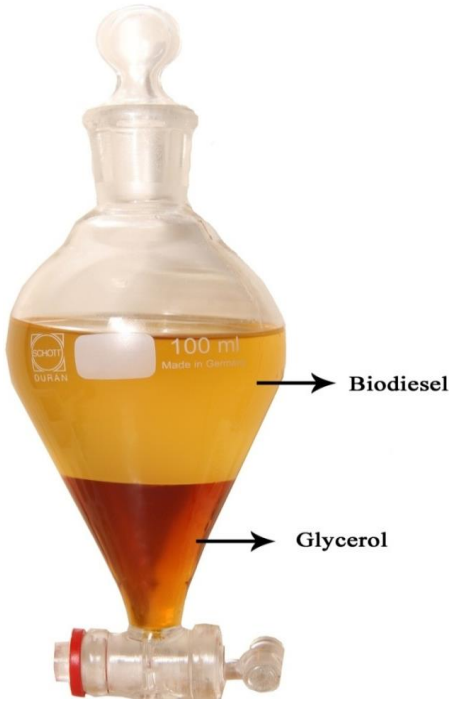

Figure 3.7. Seperating funnel; gravity separation.

3.14.2. Removal of methanol from crude biodiesel

Once the glycerol and biodiesel phase were separated, the excess methanol present in methyl ester (biodiesel) was recovered by hot water treatment and distillation procedure.

3.14.3. Washing of biodiesel

After separating the glycerol layer, biodiesel layer was subjected to hot water treatment. The biodiesel layer was washed with hot water for 4-5 times to remove excess amount of methanol and residual enzyme by air bubbling method. The lower layer containing excess methanol and enzyme was discarded (**Figure 3.8**). This shows that the impurities present in biodiesel were removed completely.

Figure 3.8. Purification of biodiesel with hot water treatment.

3.14.4. Drying of biodiesel

The refined rubber seed biodiesel obtained as above had some impurity in the form of moisture. The next step was to dry it. The ester phase (upper layer) was de-moisturised with anhydrous sodium sulphate and then it was kept in oven at 110°C for 30 min. The clean and dried biodiesel was stored under controlled room temperature (below 28°C) for future use.

3.14.5. Study of reusability of immobilized enzyme

Immobilized enzyme is the right choice to overcome the hurdles of enzyme catalyzed transesterification. It minimizes the cost since it makes reusability of the enzyme in industrial scale enzymatic biodiesel production [50]. Stability and reusability of immobilized lipase from *Pseudomonas aeruginosa* BUP2 was investigated presently. The reaction conditions for optimum production of biodiesel by lipase immobilized on celite were determined by Taguchi method. The amount of enzyme immobilized on celite was measured in terms of enzyme activity expressed as percentage residual activity. Reusability of the enzyme was investigated by repeating the transesterification reaction under the optimum conditions.

To test the stability of immobilized lipase, the lipase immobilized on celite was separated by vaccum filtration and washed with Tris-HCl buffer solution (pH 8.0). The recovered immobilized lipase on celite was used in the next batch of transesterification reaction with fresh substrates. After each cycle, the lipase activity present in the enzyme immobilised matrices was assayed and

the reaction was continued up to 5 cycles. Enzyme activity and stability were calculated and expressed as percentage residual activity.

$$\textbf{Residual activity}\ (\%) = \frac{\textbf{Activity before particular cycle} - \textbf{Activity after particular cycle}}{\textbf{Initial total activity}} \times \mathbf{100}$$

3.15. Analysis of biodiesel

3.15.1. Gas chromatography

For the analysis of biodiesel, Gas chromatography (GC) was employed on the basis of standard ASTM D6584 method. GC determines the amount of glycerol (in derivatized form), mono and diacylglycerols (both also in derivatized form), triacylglycerols and methyl esters in a biodiesel sample. The derivatized glycerol is the first material to elute, followed sequentially by the methyl esters and the derivatized monoacylglycerols, diacylglycerols and triacylglycerols.

In the present experiment, the yield of biodiesel was analyzed by Shimadzu Gas Chromatography System (2010 Plus) (**Figure 3.9**) attached with flame ionization detector and connected with MXT biodiesel column (17 m, 32 mmID and 0.10 μmdf). Sample preparation is a vital step in this analysis. The glycerol, mono-, di- and triglycerides must be derivitized to reduce their polarity and improve the thermal stability of the molecule. The derivatization technique used is silylation. The derivatization reagent used is MSTFA (N-methyl-N (trimethylsilyl) trifluoroacetamide) – the reaction involves the replacement of the active hydrogen of the hydroxyl-group by a trimethylsilyl group. The derivatization procedure for both standards and samples is identical. Weigh approximately 0.1 g of sample or standard into a vial and record the actual weight. Internal standards are added according to the EN or ASTM specification, and 0.1 ml MSTFA (derivatization reagent) is finally added. The samples and standards are allowed to stand for 20 min at room temperature to allow the derivatization reaction to complete. Following derivatization, heptane is added and the vial is capped and shaken. The standards and samples are now ready for analysis (**Figure 3.10**). High purity (99.99%) nitrogen was used as the carrier gas and it had 30 ml/min flow rate. Often standards are used in GC, which are known compounds that will indicate the presence of those compounds in the mixture

based on their retention time in the column. Standards are therefore very useful in establishing the presence of specific compounds in a mixture [51]. The temperature programming used for the present study is given below.

Injector:

Cool on column injection: Programme, oven track Sample size: 1 µl

Mode:	Splitless
Column flow:	30 ml/min
Injection volume:	1 µl

Injector Temperature Programme:

Temp1: 55°C	Temp 2: 180°C	Temp 3: 230°C	Temp 4: 350°C
Time1: 1 min	Time 2: 0 min	Time 3: 0 min	Time 4: 10 min
Rate1: 15°/min	Rate 2: 7°/min	Rate 3: 30°/min	Rate 4: 0°/min

Detector:

Type:	Flame Ionization
Temperature:	350°C
Carrier Gas:	Nitrogen

Oven:

Column: MXT biodiesel TG column (17 m, 32 mmID and 0.10 µmdf)

Oven Temperature Programme:

Temp1: 55°C	Temp2: 180°C	Temp3: 230°C	Temp4: 350°C
Time1: 1min	Time 2: 0 min	Time 3: 0 min	Time 4: 10 min
Rate1: 15°/min	Rate 2: 7°/min	Rate 3: 30°/min	Rate 4: 0°/min

GC oven was conditioned at 55°C for 1 min and heated to 380°C at 15°C/min. Finally, it was maintained at 380°C for 15 minutes and total analytical time was 37 min. FAME conversion was calculated by peak area integration method using the following formula.

$$\%\ \textbf{FAME conversion} = \frac{\textbf{Total peak area of methyl ester (C14: 0 to C24: 0)}}{\textbf{Total peak area from the chromatogram}} \times \mathbf{100}$$

Figure 3.9. Gas chromatograph (Shimadzu 2010 plus) plus purification system with computer

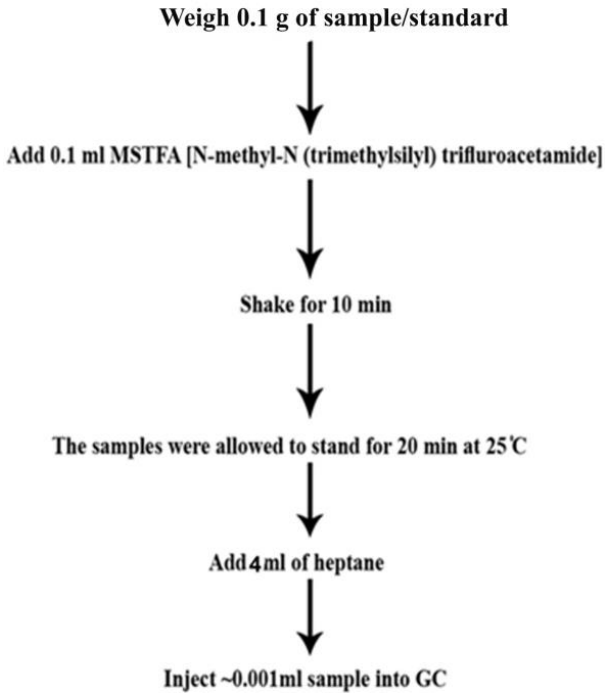

Figure 3.10. Steps involved in sample preparation for gas chromatography.

3.15.2. Effect of acyl acceptor on enzymatic transesterification

For determining the effect of acyl acceptor on the biodiesel production, different alcohols like methanol, 2-propanol and ethanol were selected. Reactions were carried out in optimized conditions predicted by RSM model. RB flask containing RSO to alcohol ratio of 1:10 molar ratio and 750 U/ml lipase was

used. All the experiments were carried out at 45°C for 4 h as duplicates. Production yield of biodiesel was analyzed by GC analysis.

3.16. Characterization of biodiesel

Characterization of the biodiesl produced was carried out by thin layer chromatography, FTIR spectroscopy, elemental analysis and GC-MS.

3.16.1. Thin layer chromatography (TLC)

The formation of methyl ester from non-edible rubber seed oil was analysed by thin layer chromatography (TLC). Silica gel-G coated on glass plate was activated for overnight at 60°C. The samples (0.5 µl) were spotted and placed in solvent saturated closed chamber. The solvent system consisted of mixture of n-hexane: ethylacetate: acetic acid (94:5:1, v/v/v) used as mobile phase [52]. After chromatographic development (~30 min), the plate was allowed to air dry at room temperature (i.e., 30°C) for half an hour, then saturated with iodine chamber (5–10 min) and spots were recorded.

3.16.2. FTIR spectroscopy

Biodiesel aliquots (5 mg) were mixed with IR grade potassium bromide (KBr) and fixed on a sample holder for analysis. FTIR analysis of the samples was carried out at mid infra-red region of 400–4000 cm^{-1} (Jasco FTIR 4100 Series, Japan).

3.16.3. Elemental analysis

Elemental analysis was carried out with the best active aliquots of biodiesel. Analysis was performed in triplicate and average value was reported for each element. The oxygen (O_2) content was calculated by mean difference. After combustion, the resulting gases (N_2, O_2, H_2O, and SO_2 or SO_3) were carried by running helium gas, then swept through a GC column that provided separation of the combustion gases, and finally, detected by a Thermal Conductivity Detector (TCD). Total run time was around 12 min.

3.16.4. Gas Chromatograph-Mass Spectrometer (GC-MS) analysis

Fatty acid methyl esters (FAMEs) formed during the transesterification reaction were analysed in a GC-MS (Agilent, 7697 A, USA) equipped with a DB-WAX capillary column (Agilent, 30 m × 250 μm, 0.25 μm film thickness) and mass spectrophotometer (Agilent, 5973N). High purity grade helium was used as carrier gas (1 ml/min). The separation was performed on the basis of temperature programme. The column temperature programme was started from 50°C (5 min), rising to 65°C at a rate of 2°C/ min, and then 200°C (5°C/min, 5 min) and 250°C (10°C/min) that was held for 10 min. Injector temperature and injection volume were 220°C and 1 μl respectively in the split less mode of a gas chromatograph. The retention time and mass spectra of compounds were compared with Wiley 7n.1 database to identify the compounds.

3.17. Study of fuel properties of biodiesel

Two major fuel specifications establishing the quality requirements for methyl esters as fuels are ASTM D6751 in USA and EN 14214 in Europe [10-13]. Fuel properties of the biodiesel made by lipase mediated tranesterification were determined using standard test procedures. The fuel properties like acid value, water content, density, viscosity, etc., were determined using standard analytical test methods (mentioned earlier) and some of the other parameters such as ester content, mono, di and triglycerides, free and total glycerol content, etc. were analyzed by GC analysis method (ASTM D6584) as described below.

3.17.1. FAME content

FAME content is meant to be a guide to the purity of biodiesel by way of measuring the conversion of triglycerides to methyl esters. In order to commercialize biodiesel as pure biofuel or blending stock for diesel fuels, it must meet the standard specifications for biodiesel fuel (FAME content of 96.5% by EN 14214). Ester content was analyzed using GC [10-13]. Mono, di, and triglycerides were also determined by GC analysis.

3.17.2. Total and free glycerol

The most important criterion for good quality biodiesel is the completion of the transesterification reaction. The incomplete reaction will cause the presence of bound glycerol in the form of un-reacted triglycerides and intermediate mono and diglycerides. Another contaminant found in biodiesel is the free glycerol, which is not removed during the water washing of biodiesel. The combination of the bound and free glycerol is referred to as total glycerol. These data are calculated directly from the gas chromatogram on the basis of the ASTM D6584, which is the prescribed standard method for measuring free and total glycerol. The total glycerol in a biodiesel sample is calculated using the formula given below [10-13]. Besides the acylglycerols, residual alcohol (methanol) can contaminate the final biodiesel product. Amount of methanol can also be analyzed via GC.

Total glyceride= G + (0.25*MG)+(0.15*DG)+(0.10*TG)

Where, G= Free glycerol, MG= Monoglyceride, DG= Diglyceride, TG= Triglyceride.

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Chapter 4 RESULTS AND DISCUSSION

The rubber tree (*Hevea brasiliensis* Müll.Arg.) is a perennial plantation crop cultivated as one of the major cash crops in the southern states of India, especially Kerala. Rubber trees grow at an altitude below 200 m and an average temperature of about 27-30°C. The rubber tree has a height of 25–30 m and has a life of 100 years or more. Rubber tree is monetarily cultivated as a source of latex for the production of different rubber products being used globally for industrial and domestic purposes [1]. The subsidiary resources derived from rubber plantations are rubber wood and rubber seeds. The plants shed their leaves in every year during the month of January and it starts to flowering at four years of age and the flowering season is February–March months. Flowers are small but appear in large clusters. Fruit is a trilobed strucutre and will ripen during July–September. These fruits, after maturing open up and split during sunshine and seeds fall to the ground. The average annual rubber seed production potential in India is about 150-250 kg of seeds per hectare; each tree yields about 800 seeds per year [2]. However, it may depend on the soil nutrients, ecosystem of the located area, type of the clone used, tree age and crop density. Rubber was earlier propagated using seeds, but now seeds are used to raise rootsock seedlings and superior clones are budded/grafted on them [3]. The price of rubber seeds is around five Indian rupee per kilogram. Rubber seeds are produced mostly in Kerala; although the processing of rubber seeds is mostly concentrated in Tamilnadu. Rubber seed oil is produced from rubber seeds on a limited scale now because of its limited end usage. However, this study deliberates research into alternative economic uses of rubber seed and rubber seed oil.

Rubber seed is a vital biomass byproduct which is presently deserted by most of the nations due to lack of appropriate technologies. The volume of rubber seeds produced has a direct relationship with the rubber clone and the age and size of the tree. Canopy cover is another related factor that has positive effects on the yield of rubber seed [4]. Rubber seeds form an important measurable biomass that can be obtained from rubber plantations every year (**Figure 4.1**). Rubber

seed has multiple uses and the most important onles include biodiesel production and industrial oil extraction for lubricants and paints etc. The seeds can be used as a source material for animal feed and fertilizer production.

Figure 4.1. A rubber plantation

4.1. Properties of rubber seed

4.1.1. Morphology of rubber seed

Fresh rubber seeds of RRII 105 clones were collected from the rubber plantation of University of Calicut, Kozhikode, India (11.1340° N, 75.8952° E) for use in the present experiments. Each fruit contains three or four seeds, which fall to the ground when the fruit ripens and splits. Seeds of rubber tree are ellipsoidal capsules, mottled brown, lustrous, slightly compressed, shiny, ovoid, 2 to 3.5 cm x 1.5 to 3.0 cm in shape. Seed weight and seed morphology were determined as described elsewhere. The weight of the seed varies from 2 g to 5 g and it's outer shell is shining grey or pale brown with irregular dark brown dots, blotches and line (**Figure 4.2**). Capsule pressure determines the shape of the seed and it comprises of 58-65% kernel and 35%-42% shell by weight of the seed. However, average weight percentage of the kernel is 61.50% of the seed weight and contains 35% to 50% oil (**Table 4.1**).

Table 4.1. Primary rubber seed properties of the cultivar RRII 105

Properties	Rubber seed
Colour	Mottled Brown
Seed size (cm)	2.5-3.5
Seed weight (g)	3.68-5.03
Kernel weight (g)	2.43-3.41
Seed-kernel ratio (%)	58-65
Shell (%)	35-42
Moisture content (%)	2.5-3.45

Figure 4.2. Rubber seed

4.1.2. Moisture content

Moisture content is the amount of water contained in a sample. Moisture content of rubber seed was determined by measuring the mass of rubber seeds before and after the removal of water by evaporation as described elsewhere. Moisture content of rubber seed has been found to be between 2.5% to 3.45% after oven drying (**Table 4.1**). The result is generally in agreement with the earlier studies that reported moisture content between 3.9% and 4.0% [5, 6, 7]. Low moisture content makes the dried seeds more shelf stable. Rubber seed kernel with higher moisture content will be probably more susceptible to microbial attack and degradation as reported earlier [6]. However, the result differs from the findings of Oyekunle and Omode (9.0%) [8], Oyewusi et al. (14.1%) [9] and Sharma et al. (16%) [10]. Studies have reported that rubber seeds have a high moisture content as high as 29.7% at the time of harvest (about one month after seed fall) [12], and 38% [13]. The moisture content of the seed is in equilibrium with the surrounding air [14]. This varies form clone to clone [15].

Lgeleke and Omorusi have described the moisture content for deterioration free storage of rubber seed to be 7% and below [16]. Moderately high moisture content may cause serious degradation of the fatty materials by hydrolysis, thereby rendering the material poor in stability. Drying the kernel at 60°C for 24 h and storing in almost air tight container has been found to improve storage [17]. The present result implies good indication of longer shelf life for the rubber seed material used in the present study.

4.2. Proximate composition

Study of the proximate composition of rubber seed kernel carried out presently indicated that the seed contains 36.43% fat, 24.61% lipid, 18.65% protein, 13.9% carbohydrate, 1.43% amino acid, 0.19% phenolic content and 4.79% others (**Figure 4.3**). Fat and lipid content were the highest in fresh rubber seed followed by protein, carbohydrate and others respectively. The result shows that rubber seed is a potential source of oil and hence justifies the study on possible industrial uses. A few researchers have already mentioned that rubber seed has significant quantities of nutrients than many other oil seeds and exhibits high essential nutritive value as a better alternative for protein supplements in livestock diets [18, 19].

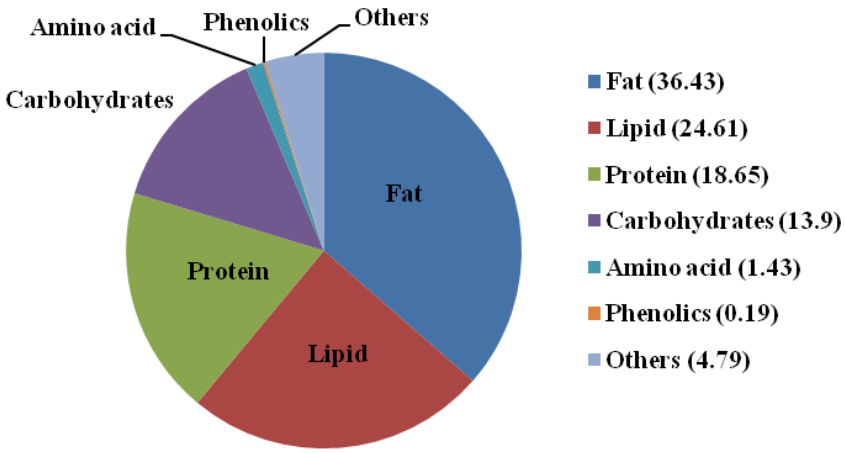

Figure 4.3. Proximate composition of rubber seed

4.2.1. Crude fat & lipid

The crude fat and lipid content of the rubber seeds used in this study was found to be 36.43% and 24.61% respectively. Similarly, previous studies such as Onwurah et al. [7] and Lalabe et al. [20] who have reported it as 42.50% and 49.30% respectively. However, this result is lower when compared to the fat content of 68.53% reported by Eka et al. [21]. Crude fat content tends to improve the digestibility and influence palatability of feed products. The possible reason for this high crude fat content reported by these workers may be due to varietal differences, variation in the proportion of kernel content of the seeds or difference in preparation techniques.

4.2.2. Crude protein

The crude protein content reported in the present study is 18.65% and this report is more likely in unison with earlier the reports of 17.41% [21], 19.40 [20], 21.87 [22] and 22.30% [7]. Crude protein content of rubber seed is considerably high when compared with protein rich crops that are frequently used in food and feed production. The protein content of soybean is in the range from 37 to 40% [23, 24], that of ground nut ranges from 22 to 30% [25] and that of castor bean is 20.11% [26]. Similar to these legumes, rubber seed can be said to be a good source of plant-based protein and can contribute to the daily protein need. The differences in crude protein content as well as other proximate values as reported by earlier studies could be attributed to differences in varieties, climatic conditions, soil composition, agricultural practices or differences in absorption of nitrogenous nutrients and inadequate application of fertilizer to the rubber plantations [18].

4.2.3. Carbohydrates

Plants are autotrophic and serve as the primary producer in the food chain. In plants, transport of carbohydrate from source to sink takes place in the form of sugars [27]. Total carbohydrate content of rubber seeds was found to be 13.9% in the present study. Accumulation of total soluble sugars helps the plants in the production of energy, maintance of membrane integrity, turgor maintenance and

signaling and also protect plants from osmotic and ionic stress [28]. According to earlier workers, total carbohydrate content ranged from 11.58 to 25.19% in rubber seeds. The raw seed meal had the highest percentage of 25.19%, which was reduced to 16.86% by cooking. Similar research was done by Ikanone and Oyekan, which recorded a considerable loss of low molecular weight carbohydrates into the processing water [29]. The fermented seed meal had the lowest value of 11.58%. This is because, activities of microorganisms lead to the breakdown of complex carbohydrates into simpler forms [30]. The carbohydrate content of rubber seed meal reported in the present study compares favourably with 13.80% [31] and 21% [22] reported from earlier studies.

Accumulation of low molecular weight, non-toxic organic compounds, the osmoregulators, in the vacuole and cytosol is an important stress tolerance mechanism operating in plants under various stress conditions and is referred to as osmoregulation. These osmoregulators include soluble sugars, proline, phenolic compounds and other compounds and have an important role in thylakoid membrane stability, which increases the electron transport [32-34].

4.2.4. Amino acids

Amino acid content of rubber seed has been found to be 1.43% in the present study **(Figure 4.3).** Rubber seed contains some essential amino acids, which are more imperative to sustainability, because the body cannot synthesise these amino acids, and they are to be obtained from food/feed or amino acid supplements. Amino acids are the prerequisite substances needed to support metabolic demands which also upholds appropriate body composition and growth rates. Dietary proteins must contain satisfactory and digestible substances of nutritive value [35]. Oyewusi et al. [9] have reported that rubber seeds contain moderate quantitities of glycine, valine, isoleucine, tyrosine and serine, are low in alanine, histidine, threonine, proline, methionine and cysteine and high in glutamic acid, aspartic acid, leucine, arginine, lysine and phenylalanine. Eka et al. [21] have also reported a general pattern closely concurring with the results mentioned above. Aspartic and glutamic acids are the main amino acids in the

processed rubber seed which makes up of 0.20 kg/kg of protein [36]. These amino acids contribute to the palatability and taste characteristics of food, producing a sweet taste from alanine, glycine and threonine; Monosodium glutamate (MSG) like taste from aspartic and glutamic acid, bitter taste from arginine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan and valine and flat taste from lysine and tyrosine [37]. Eka et al. [21] report that rubber seed has a more bitter score of 38.82 from amino acid contents, 27.31 (MSG-like), 19.46 (sweet) and 7.14 (Flat). Glutamic acid, aspartic acid and leucine are the most abundant amino acids in rubber seed while the S-containing amino acids, methionine and cystine, have the lowest concentration.

4.2.5. Phenolics

In the present study, the total amount of phenolic compounds of rubber seed was determined using Folin Ciocalteau method [38] as described earlier. The analysis showed the presence of 0.19% of phenolics in rubber seed **(Figure 4.3)**. Phenolic compounds are known to play significant role in stimulation of protein and ammonia elimination [39]. Grace et al. (1998) have stated that the simple phenolic acids such as cinnamic acid, p-coumaric acid and caffeic acid are common to chlorogenic acid biosynthetic pathway and differ only in the extent of hydroxylation of aromatic ring [40]. There is little evidence that phenolics play a key role in plant growth and development, when directly applied to plant tissues [41]. Some phenolics directly interact with plant hormones or control the activities of enzymes and cause change in hormonal balance [42]. Increased levels of polyphenols are found beneficial to improve antibacterial and antioxidant activity.

4.2.6. Elemental analysis

Elemental analysis is the qualitative and quantitative measurment of elements present in a sample. Elemental analysis of rubber seed powder was carried out using elemental analyzer. From the analysis, it has been revealed that the major components of the samples are carbon and oxygen and it was found that hydrogen, nitrogen, and sulphur are minor components. The percentages of

those components are shown in **Table 4.2**. Rubber seed powder (RSP) contained high percentage of carbon (54.96%), hydrogen (8.24%), nitrogen (1.39%) and sulphur (0.51%). The N content in RSP is the highest in comparison to rubber seed shell (RSS). High nitrogen and ash concentrations reduce hydrocarbon yields during thermochemical conversion. A significant fraction of herbaceous biomass consists of inorganic constituents, commonly referred to ash, and cannot be converted into energy. The inorganic material is commonly associated with oxygen, sulphur and nitrogen containing functional groups [43]. It has been reported that RSP shows optimum S content when compared to other biomass samples. A higher concentration of S can cause sulphation and lead to Cl release. It can cause corrosion by $FeCl_2$ and $ZnCl_2$ in boilers [44]. The H content in RSS is the same with palm kernel shell (PKS) in a study by Jamaluddin et al. (2013) [45]. RSS is the protective covering of the seed and it contains the highest amount of lignocellulosic materials that protect the kernel from termites and other microorganisms. These materials are chemically polar and non-polar in nature and can be used as lignocellulosic biomass for lipase production as well as green chemicals for biofuel production. It can be seen from **Table 4.2** and **Figure 4.4**, that high oxygen and carbon content of RSP can contribute highly to an increase in the conversion process of oil to FAMEs.

Table 4.2. Elemental analysis of RSS *vs* RSP

Element name	**Percentage**	
	RSS	**RSP**
Nitrogen	0	1.39
Carbon	43.98	54.96
Hydrogen	5.40	8.24
Sulphur	0	0.51
Oxygen	50.62	65.10

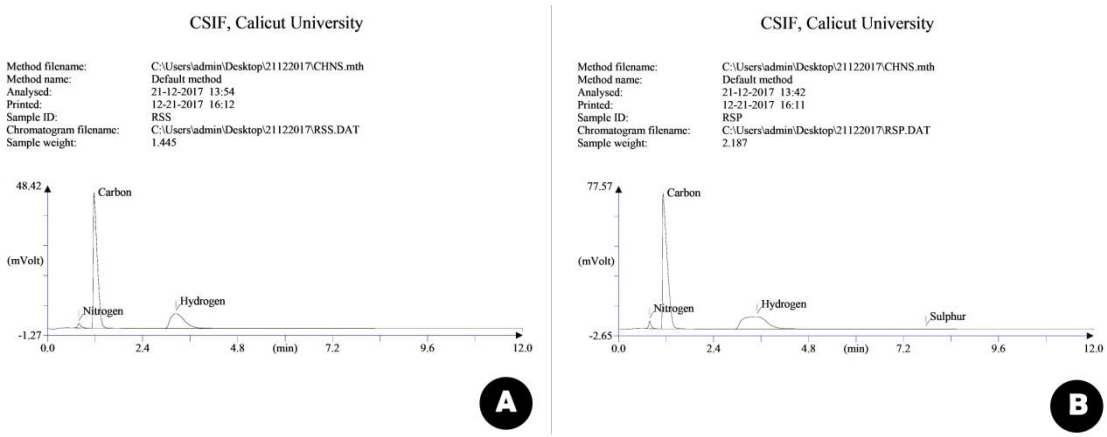

Figure 4.4. Elemental analysis spectrum; A) Rubber seed shell; B) Rubber seed powder

4.3. Toxicity of rubber seed

Anti-nutritional factors are common toxic contents found in any edible plant part. It becomes a threat if the presence is above the safe margin for consumption, which leads to poor nutritive value. Slow growth and improper digestion may be resulted by anti-nutritional factors. Rubber seed has considerably high protein contents and also has anti-nutritional factors such as hydrogen cyanide [46]. In plants, cyanides are usually bound to sugar molecules in the form of cyanogenic glycosides and defend the plant against herbivores like animals and insect feeders [47]. Cyanogenic glycosides are phytotoxins which occur in at least 2000 plant species; they are found in trace amounts in the seeds of apple, peach, mango, almond and rubber [48]. Likely, *Jatropha curcas* seed contains a poisonous phorbol ester compound [49].

In the present study, picrate paper analysis was performed to detect the presence of cyanide content (**Figure 4.5**). This is the most reliable and sensitive method and easy to use in the determination of cyanide presence in rubber seed samples. Presence of cyanide is indicated by a colour change from yellow to orange and to brown. Cyanide is present in the form of a chemical known as linamarin in rubber seeds and several other crop plants like cassava. When the sample was heated for 70^{o}C for, 90 minutes and then subjected to picric paper analysis, there was no brown colouration showing that linamarin was getting considerably reduced when heated.

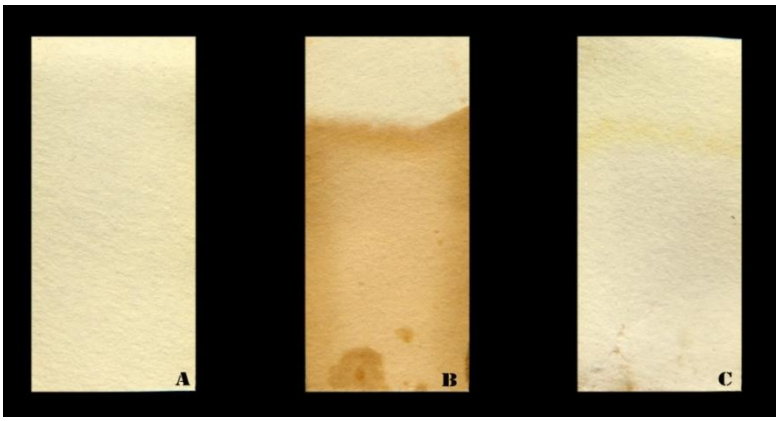

Figure 4.5. Picric paper analysis: (A) Untreated picric paper; (B) Presence of linamarin in fresh seed (brown colouration); and (C) Absence of linamarin after heating (70^{o}C for 90 min).

Rubber seed is an important byproduct of rubber cultivation and its potential as a protein source is well known. Therefore, other approaches have also been been adopted to reduce the anti-nutritional factors to safety margin for consumption. Studies have been carried out to find out the most effective methods and those include storage, heat treatment, soaking, fermentation and enzymatic treatment [50]. Fuller has reported that since rubber seeds slowly lose cyanogenic glycosides, storage for a minimum of four months, detoxification by roasting (350°C for 15 minutes) or soaking in hot water or in a 2.5% ash solution for 12 hours could solve this problem [51].

Cyanogenic glucoside concentration may differ due to hereditary, environmental factors, location, season and soil factors. Numerous methods had been adopted for processing the rubber seed in order to improve the protein content and drastically diminish the levels of cyanogen content and other anti-nutritional factors. Heat treatment, fermentation and adsorbent had proven efficacy in reducing the cyanogens content. Ukpebor et al. [52] and Fortuna et al. [53] affirm that heat treatment and fermentation have tendencies to reduce the level of cyanogen content in rubber seed and improve the nutritional value simultaneously. Ogunka-Nnoka and Mepba [54] state that free and bound cyanide are water soluble and could be leached out through boiling.

4.4. Extraction of oil

Dried, dehulled and milled rubber seed kernels were used for the extraction of rubber seed oil. The oil was extracted using mini handed oil expeller and also by soxhlet extraction methods.

4.4.1. Mini handed oil expeller

In this case, extraction of oil from seeds was carried out by mechanical extraction method as detailed elsewhere. In the oil extraction process using mini handed oil expeller, around 18% yield is obtained. An oil expeller is a horizontally rotating metal screw, which feeds oil-bearing seeds into a barrel shaped outer casing with perforated walls (**Figure 3.2**). The seeds are continuously fed over the expeller, which grinds, crushes and presses the oil out as it passes through the machine. The pressure of the expeller screws ruptures the oil seeds in the product and oil flows through the perforations provided in the casing and it is collected in a trough provided at the bottom of casing.

This oil extraction technology has been in use for a very long time in India and the techniques that are followed for expelling oil are very laborious and relatively inefficient. There has not been any significant improvement in the oil extraction processes and even today a century old technology such as single screw press and hydraulic presses are being used in various oil industries. Mechanical screw presses are ideally preferred for pilot scale studies and they are economical for continuous production. Any improvement in the technique of oil extraction will bridge the technological gap and increase availability of feedstock for extraction of oil [55, 56]. The materials used for manufacturing an expeller press include mild steel. The oil expeller machine uses resistance and constant pressure from the screw drives to move and compress the seed material. In some cases, the expeller pressure created due to pressing, heat is generated in the range of 60–80°C. After oil extraction, it is filtered to remove the impurities. The residue has been used as substarte for solid state fermentation which has high amount of protein and lipid and can be used to feed the cattle. Different methods have been applied for oil extraction, such as mechanical press with and without

solvent and cold percolation as done by Morshed et al. [57]. They optimized solvent to seed ratio (wt./wt.) and the total oil content was determined. They observed that mechanical press with periodic addition of the solvent enhanced the oil yield.

4.4.2. Soxhlet extraction

This is the most efficient method to recover oil from oilseeds, thus soxhlet extraction using n-hexane has been commercialized as a standard practice in today's industry. Here, oil is extracted using Soxhlet apparatus which was invented in 1879 by Franz von Soxhlet (**Figure 3.3**). In the present experiment, the oil plus solvent mixture was collected and transferred to sampling flask of rotary evaporator to recover solvent from the oil **(Figure 4.6).**

The oil content that remain in the waste after mechanical extraction can also be separated by this method. Recent studies have highlighted the process of soxhlet extraction using n-hexane as the commonly practiced solvent because of its inherent advantages. Some of the recent works on oil seed extraction employing n-hexane as solvent included oils from seeds of rubber, yellow horn, *Terminalia catappa* L. and hempseed [58-61]. Recent studies reveal that soxhlet extraction method is more widely practiced at industrial level, by dint of higher oil yield, low operating cost and shorter time [62]. An ideal solvent should be non-polar, nonreactive to the feedstock or oil, as well as the extraction equipments. Ideal solvents should also have high purity to exhibit more uniform operating characteristics, should be immiscible with water for easier separation. Hexane cannot form hydrogen bonds with water and thus hexane is insoluble in water. Ultimately, an ideal solvent should have lower turbidity, should give higher oil yield and should be easily available at low prices.

Figure 4.6. Rubber seed oil collected by Soxhlet apparatus

4.4.3. Rotary evaporator

The next step is solvent recovery from the oil using rotary evaporator. This is the most efficient method to separate oil from solvent. It is also an important step before transesterification reaction. The chances of presence of traces of solvent in the extracted oil are high in soxhlet extraction method and it may inhibit the lipase activity in the reaction mixture during transesterification process.

4.5. Oil yield

4.5.1. Choice of the solvent

The extracted oil was transferred into a clean dry beaker, which was further heated with hot air oven at 80^{o}C for 30 min. to ensure the complete removal of the solvent from the oil. Hexane is a colorless liquid, odorless when pure, with boiling point approximately at 69°C. The oil yield was calculated and expressed as percentage of oil content. The optimum yield was recorded by the usage of n- hexane (39.45%) **(Table 4.3; Figure 4.7**). Previous reports agree with this study; it has been reported as 40-50% by Kyari [63] and Khan and Yusup [64]. It is also reported that oil yield is dependent on drying temperature, drying time, pressing time, and pressing pressure [65].

Figure 4.7 illustrates the trend of oil yield extracted using four different solvents; n-hexane (b.p. 68°C), petroleum ether (b.p. 60-80°C), ethyl acetate

(b.p.77.1°C) and methanol (b.p. 64.7°C). The oil yield obtained with the non-polar solvent hexane was found to be maximum (39.45%) while the lowest value (26.85%) was obtained with the polar solvent methanol. Petroleum ether (34.05%) and ethyl acetate (30.52%) showed second place and third place respectively. The results show that non-polar oil of rubber seed is easier to be extracted by n-hexane. The finding is consistent with the results reported by Sayyar et al. [66]. The effect of non-polar solvent on yield is attributed to the presence of fatty acids of non-polar long chain of hydrocarbon in rubber seeds [50, 67-68]. The choice of solvent used is based on solvent extraction capacity, effects of solvent on oil properties, process safety, solvent volatility and stability, and economic considerations. According to Johnson et al. different properties make a solvent ideal for oil extraction [69]. The most vital property of an ideal solvent is that it must have a high solvent power for triglycerides at elevated temperature which is exhibited by lower alcohols. The solvent should also possess non-reactive selectivity, depending on the desired end product. Currently, n-hexane is extensively used for commodity vegetable oil extraction due to its extraction efficiency and ease of availability. Hexane is categorized as flammable and hazardous air pollutant and is included in the list of toxic chemicals by the US Environmental Protection Agency. As stated in the Prevention of Food Adulteration act 1954, the maximum permissible limit for n-hexane in oil and the meal are only 5ppm and 10ppm respectively. However, despite the disadvantages of using n-hexane as a solvent for extraction, n-hexane is still the major solvent used because of its high solubility, low corrosion, low evaporation loss and greasy residue. Moreover, the product has a better odour and flavour [70].

However, by virtue of the toxicological and ecological concerns of using n-hexane as the extracting solvent, researchers were very much motivated in searching new alternatives. There are also other famous and preferable solvents used, such as petroleum ether, water, alcohols and many more. As reported by Johnson et al. [69], there is quite a number of alternative solvents that can be used, each with its own advantages and disadvantages. In this study, observations have been made to the rubber seed oil extracted the four solvents mrntioned

above. The oils from n-hexane and petroleum ether extractions were golden yellowish, with the original odour of the rubber seed. This is because both the solvents have low polarity and are miscible with the oil. On the other hand, white particles were observed to be extracted together with the oil when using polar solvents of ethyl acetate and methanol. Low polarity solvents like n-hexane and petroleum ether extracted the rubber seed oil with more than 50% yield. This shows that the nature of the extracted lipid is highly dependent on the solvent's polarity, which will determine the selectivity of the solvent used. Futhermore, the differences in dielectric constant of the solvent used is also the main reason for this result. This is because, solubility of the solvent in the oil decreases with increasing dielectric constant value which determines the polarity. The dielectric constants of n-hexane, petroleum ether, ethyl acetate and methanol are 1.88, 2.0, 6.02 and 32.70 respectively. Hron et al. [71] and Ferriera-Dias et al. [72] reported that ethanol is the ideal solvent to co-extract other compounds in oilseeds which are insoluble in non-polar solvents.

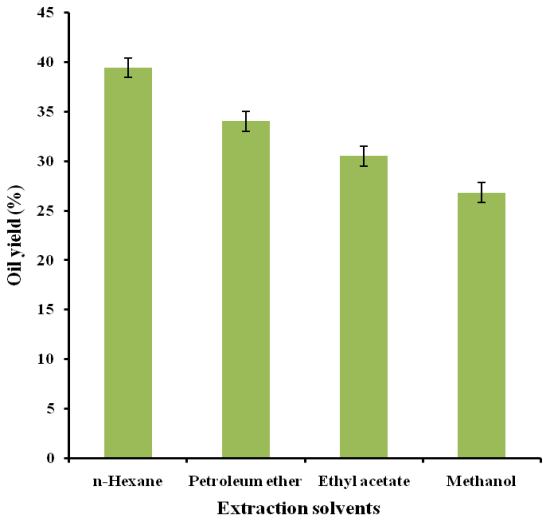

Figure 4.7. Effect of solvents on oil extraction; maximum oil yield was obtained from n-hexane.

4.5.2. Effect of extraction time on oil yield

After every 2 h, the oil was collected and percentage of yield was calculated. **Figure 4.8.** depicts comparative oil yield when extracted using four different solvents at extraction times of 2, 4, 6, 8 and 10 hours. Based on **Figure**

4.8, it can be obviously seen that the maximum oil yield is obtained from the extraction by soxhletation with n-hexane at 6 h (39.45%). From this background, it is implied that oil yield is increased initially with increasing number of circulation and becomes steady with further increase in number of circulations. Further increase in the solvent amount and time does not have any impact on oil yield. The amount of oil yield from the four different solvents did not change significantly after 6 hours. Similar observations have been made by Sayyar et al. (2009) on Jatropha seed extraction [72]. The rubber seed oil can be said to be fully extracted only after 6 hours. This is because, the concentration of oil increases in the solvent resulting in a decrease in the diffusion rate. Therefore, the oil yield level remains consistent even by extending the reaction time when the maximum amount of extractable oil is obtained.

In one cycle of extraction, oil extracted was golden yellowish, but gradually became brownish and had emulsion like appearance. The oil extracted has an unpleasant smell which does not inherit the original odour of the rubber seed. Another observation is that the oil obtained using hexane solvent appeared to be golden yellowish in colour whereas in the case of methanol and ethyl acetate solvents the oil colour was dark brown in colour. The production and utilization of rubber see oil is low at present because of its limited end usage. At present rubber seed oil does not find any major application and hence even the natural production of seeds itself remains underutilized.

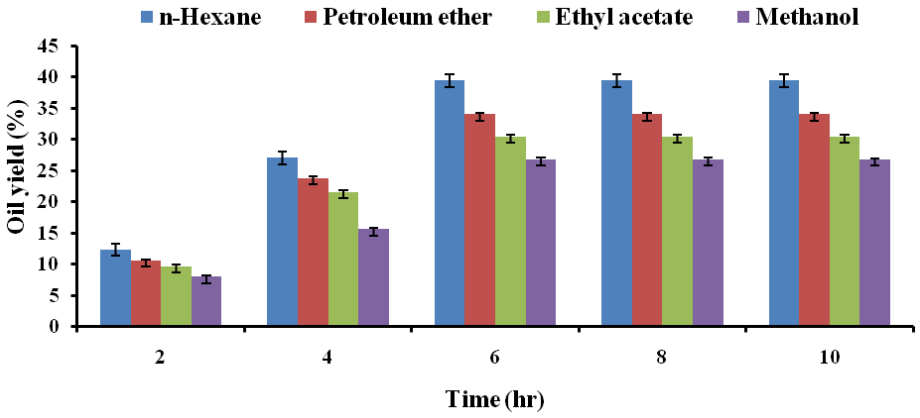

Figure 4.8. Effect of solvent circulation on extraction of RSO; hexane shows optimum yield at 6 hr.

4.6. Physico-chemical properties of RSO

Figure 4.6. shows the extracted rubber seed oil. The colour of the RSO is golden-yellowish with very pleasant peanut smell [73]. Refined RSO was used for determining the physico-chemical properties.

4.6.1. Water content

Refined and filtered RSO showed 6.5% water content **(Table 4.3)**. It has been previously reported by other studies that the optimum range of moisture content in oilseed processing is between 5 to 13%. According to Ebewele et al. the optimum moisture content of rubber seed oil extraction is 7% at 70°C [74]. They also observed that moisture content level fluctuated from 7% up to 10% to 13% with low or higher temperature. This is because, during the extraction process, the moisture in the seeds acts as a heat transfer medium and helps in coagulation of protein for oil yield. Therefore, too high or too low temperature may disturb the function of the moisture in oilseed processing. Moisture content of RSO is found to be somewhat higher than that of palm oil, but it is still low enough to be in the acceptable range. High content of water leads to the formation of soap [75]. Presence of water is one of the internal factors affecting the oxidation stability of biodiesel. It is reported that water content in biodiesel samples can be removed by drying in oven for 4 hr at 105°C. In addition, in some cases calcium chloride is used as adsorbent [76].

Low moisture content is preferable because it will prolong the shelf life of oil thus ensuring storage stability [77, 78]. Low moisture content of oil might be as a result of the effectiveness of the rotary evaporator apparatus used for recovering the oil. In addition, high moisture percentage of biogasoline will decrease the heat of combustion of the fuel and also might leads to corrosion of the automobile parts [79].

Table 4.3. Physiochemical properties of the rubber seed oil

Properties	**This study**	**Reference [**80, 81, 82]
Color	Golden yellow	Golden yellow, light/ dark brown
pH	6.14	6
Oil content (%)	39.45	40-50
Water content (%)	6.5	5-13
Acid value (mg KOH/ g oil)	45.32	1.68-84.41
FFA	22.66	0.84-42.20
Viscosity mm^2/s @ 30°C	5.06	
Density (g/cm^3)	0.874	0.857-0.943
Peroxide value (mg/g)	3.62	1.6-16
Saponification value (mg KOH/g oil)	192.36	183.91-235.28
Iodine value (g I_2/100 g oil)	135.52	113-146

4.6.2. Acid value & FFA

The acid value of the extracted RSO, compared with some commercial oils is presented in **Table 4.4**. The acid value was determined by following standard protocol of EN 14104 titration method. The obtained result was 45.32 mg of KOH/ g of oil which was higher than the acid value of Karanja oil (5.40 mg of KOH/g of oil) [83], Mahua oil (34 mg of KOH/g of oil) [84], Neem oil (32.538 mg of KOH/g of oil) [85] and Jatropha oil (2.24 mg of KOH/g of oil) [86]. Free fatty acid content (22.66%) was calculated based on the acid value from a relationship provided by the AOCS standard and it was greater than common oils. Typically, an acid value of 4 mg KOH/g is set as the maximum for plant oils whereas acid value for RSO between 2 and 81.6 mg KOH/g have been reported. At the initial time it is around 2%; but at room temperature when it is stored for longer time the FFA content gradually increases and after 2-3 months of storing it is found as 25-45%. These high free fatty acid (FFA) values are not necessarily an intrinsic feature of RSO, but will be a function of the processing conditions and technology, as well as the storage conditions of the seeds [15].

The freshness of the oil is related to acid value of the feedstock, while the seeds may generate the free fatty acids during longer storage due to enzyme activities. Hence the acid value becomes one of the important quality targets to

determine the purity of oil. Acid value measures the degree of unsaturation of oil. It corresponds to the amount of potassium hydroxide needed to neutralize the free fatty acids [87]. Comparison of the acid value of RSO with other commercial oils like rice bran oil, palm oil, run flower oil, olive oil and ground nut oil is provided in **Table 4.4**. RSO shows high acid value. This indicates the presence of higher content of unsaturated fatty acids. The acid value of RSO (45.32) obtained in this study is almost similar to that reported by RRIN (43.62) [88]. This is an important parameter to be controlled because highly acidic oil can cause the fuel supply system and the internal combustion engine to corrode severely [89].

Table 4.4. Comparison of acid value of RSO *VS* Commercial oils

Sample oil	**Acid value (mg KOH/g)**	**FFA**
Rice bran oil	1.73	0.865
Palm oil	0.966	0.483
Sunflower oil	7.645	3.822
Olive oil	0.716	0.358
Ground nut oil	0.477	0.238
Rubber seed oil	45.32	22.66

Presence of high amount of free fatty acids (FFA) in the raw oil is a major hurdle in biodiesel production in the base catalyzed transesterification reactions due to the formation of saponified products which causes reduction in biodiesel yield [81]. Such a product is not suited for biodiesel production since low cost feedstocks with high FFA require additional preesterification step with concentrated acids. The uses of corrosive alkali/acid catalysts make the entire process environmentally unfavorable. The best remedy for this situation is enzyme catalyzed biodiesel production, which has the advantage of one step method; additionally it avoids extra efforts for the neutralization and washing steps as well as results in high yield of biodiesel and high purity glycerol byproduct. This makes enzyme catalyzed biodiesel production process eco-friendly and cost effective.

Many pretreatment methods have been proposed for reducing the high FFA content of the oils including extraction by alcohol, steam distillation and

esterification by acid catalysis [90]. Higher acid value indicates the presence of large number of carboxylic acid groups such as fatty acids in an oil caused by oil degradation and combustion [20]. It can also be expressed in terms of the amount of potassium hydroxide required to neutralize the free fatty acids present in 1g of oil sample. The age, quality and degree of oil purity during storage and processing can be known through acid value [91]. Besides it can also be used to monitor the extent of oxidative oil deterioration caused by chemical or enzymatic activity [17, 24]. Moreover, presence of water content also increases the FFA concentration [92].

According to Eka, et al. crude rubber seed oil is rich in unsaturated fatty acids while crude palm oil was rich in saturated fatty acids [21]. Crude oil with high FFA concentration requires tedious refining procedures to convert neutral oil. Catalytic cracking on the other hand is much favoured method because it not only eliminates the need for refining but it also lowers the acid value considerably [19].

4.6.3. Viscosity

Viscosity of RSO at 30°C was measured by Ostwald's viscometer and it was found to be 5.06 mm^2/s **(Table 4.4)** which is higher compared to commercial petroleum-diesel (< 4 mm^2/s) [93]. The viscosity of vegetable oil is generally higher than petrodiesel fuel as a result of its comparatively high molecular mass [94]. The viscosity differs with fatty acid composition and chain length. High viscosity is the major reason for avoiding the use of oils as biofuel in the diesel engines. Therefore it is not advisable to use vegetable oil directly as a fuel. The fatty acids in the viscous vegetable oil are transesterified to their corresponding methyl esters so that the viscosity of the oil will be reduced and it could be used as biodiesel. In contrast, high viscous oils have their own advantages also. They provide extra lubrication of the injector and also avoid leakage and exhaustion generated by fuel injection engines that fits imprecisely resulted by low viscous oil [95].

4.6.4. Density

The density was determined by measuring the volume of RSO accurately and then weighing it in a four digital electronic balance. It is an important oil property, which is directly related to the consistency of the liquid substance. Density of RSO was found to be 874 kg/m^3 **(Table 4.4)**, which is nearly close to jatropha (i.e., 873 kg/m^3) and waste cooking oil (i.e., 868 kg/m^3) [96].

4.6.5. Peroxide value

The peroxide value of RSO obtained is 3.62 mg/g **(Table 4.4)**; which is considerably low. This property is an indication of vegetable oil to deteriorate during storage [97]. Asuquo et al. stated that unsaturated oil is more prone to rancidity because it absorbs more oxygen molecules and develops higher peroxides value [80]. Similarly, crude palm oil also shows higher reading of peroxide value of 5.97 mg/g. These oils appear solid in cooled ambient temperarture [2]. The lower peroxide value of RSO also provides an added advantage as a potential feedstock to produce biodiesel.

4.6.6. Saponification value

Saponification value is an index of average molecular mass of fatty acid content in the oil sample. The saponification value of RSO (183.32 mg KOH/g) **(Table 4.4)** is similar to that of sunflower oil (182.233 mg KOH/g) [98] and higher than that of other vegetable oils such as, corn oil (153.8 mg KOH/g) and mustard oil (125.6 mg KOH/g) [99]. The high saponification value of RSO is a good quality for soap making and shampoos. The high saponification value of the oil could be attributed to the high FFA content and molecular weight of fatty acid.

4.6.7. Iodine value

Iodine value determines the level of unsaturation of fatty acids in the form of double bonds and also measures the oxidation stability of oil. The iodine value of RSO is 138 g I_2/g **(Table 4.4)** as estimated by Wij's method, which falls in the range of semi-drying oils. Comparing with the iodine value of palm oil (52); RSO shows a higher value [100]. Higher iodine value indicates the presence of high

percentage of unsaturated fatty acids. However, being highly unsaturated, RSO is also more prone to oxidation and humidity resulting in poor oxidation stability [101]. This attribute benefits in cold region because it reveals excellent cold flow properties. This consequence nonetheless can be improved by adding antioxidants to enhance the oil stabiity. The iodine value indicates the number of double bonds in a vegetable oil. The higher the iodine number, higher will be the amount of iodine needed to break the double bonds and can use it in the paint industry. Similar studies conducted by Salimon et al. and Maliki et al. have reported that iodine value of RSO as 135.79 & 118.8 respectively [50, 68].

4.6.8. Determination of pH

The pH value of 5.8 **(Table 4.4)** obtained for RSO was in favour with previous studies. pH values of some oils reported by Ibeto et al. [101] from sources like *Brachystegia eurycoma* (5.30), *Cucurbita pepo* (5.65), *Luffa cylidrica* (5.59) and *Cucumis melo* (6.07) were also acidic. This value is an indication of the presence of reasonable amount of free fatty acid (FFA) and can be advantageous for soap making. High acidity is not favoured because it will bring difficulties during storage as it will cause corrosion and instability.

General and physicochemical properties of rubber seed and rubber seed oil have been investigated above and the result compares with some literature avalilable, and at par with the findings of Ramadash et al. [89]. However, a range of variation in some properties of the oil may be expected due to gegraphical origin and environmental factors such as rain, soil fertility, agronomic practices, maturation period, and genetic substitution.

4.7. Characterization of RSO

4.7.1. Elemental analysis

Elemental analysis was carried out presently using Elemental Analyser as described elsewhere to find out the percent composition of different elements like carbon, hydrogen, nitrogen and sulpher in the RSO extracted from the rubber seeds. The percent composition of oxygen was estimated by difference method. From the analysis, it has been found that the major elemental components of the

RSO are carbon (78.81%); hydrogen (11.89%), oxygen (9.20%) and sulphur (0.10%) (**Table 4.5** & **Figure 4.9**). No detectable quantity of nitrogen was observed by elemental analysis. According to one earlier report, nitrogen was recorded at very low concentration by elemental analysis in crude RSO [102].

The higher percent of carbon and hydrogen indicates that RSO is a good source of carbohydrates and hydrocarbons. The lower percentage of oxygen, sulphur and nitrogen indicates that is not good for consumption and contains only low amount of proteins and vitamins. Nitrogen is a structural component of proteins and sulphur is also present in proteins and vitamins. The high carbon to nitrogen ratio of an organic material shows it is carbonaceous and has less nitrogen content. High carbon to hydrogen ratio of an organic material shows that it is a source of hydrocarbons and this observation indicates the oily nature of RSO [103].

Table 4.5. Elemental analysis of RSO

Element name	Percentage
Nitrogen	0
Carbon	78.81
Hydrogen	11.89
Sulphur	0.10
Oxygen	9.20

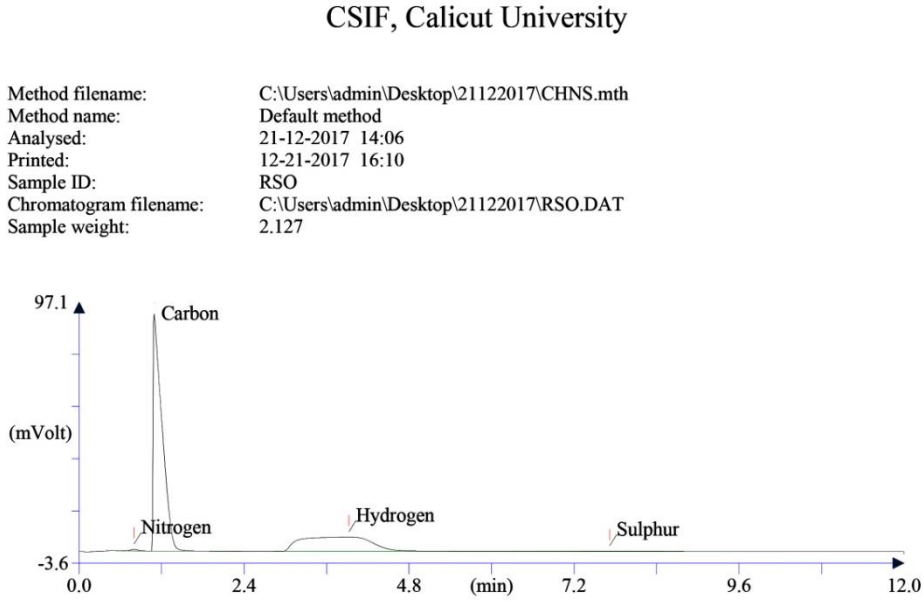

Figure 4.9. Spectrum showing elemental analysis of RSO

4.7.2. FTIR analysis

Structural characterization of the extracted RSO was carried out by FTIR spectroscopy to reveal its chemical nature as well as the compositional identity. FTIR clearly indicated the presence of peaks characteristic to unsaturated fatty acids. For instance, the broad and significant peak at 3010-3427 cm^{-1} corresponds to –OH stretching of the glycolipid, whereas multiple peaks at 2923-2853 cm^{-1} indicate the aliphatic CH_3, CH_2 vibrations. However, the carbonyl band occurs as a doublet, probably indicating two types of C=O. A similar observation has been reported for the IR spectrum of tung oil [104].

The major peak at 1746 cm^{-1} is contributed by the C=O group due to the functional ester group, and the vibration at 1639 cm^{-1} indicates the presence of COO– in the sample. Similarly, the peaks at 1458-1384 cm^{-1} correspond to the bending vibrations of –OH on carboxylic group; whereas peaks at 1050-1118 cm^{-1} stand for the C–O–C vibrations that can be used as an analytical tool to detect RSO adulteration [105, 106]. The functional groups present in RSO are similar to those in other vegetable oils such as *Jatropha curcas* seed oil [107]. The spectrum shows that triglyceride (TG) was the main component of RSO (**Figure 4.10**). The FTIR spectrum represents a fingerprint region of 1458−618 cm^{-1}. The main peaks and their assignment to functional groups of the RSO are given in **Table 4.6.**

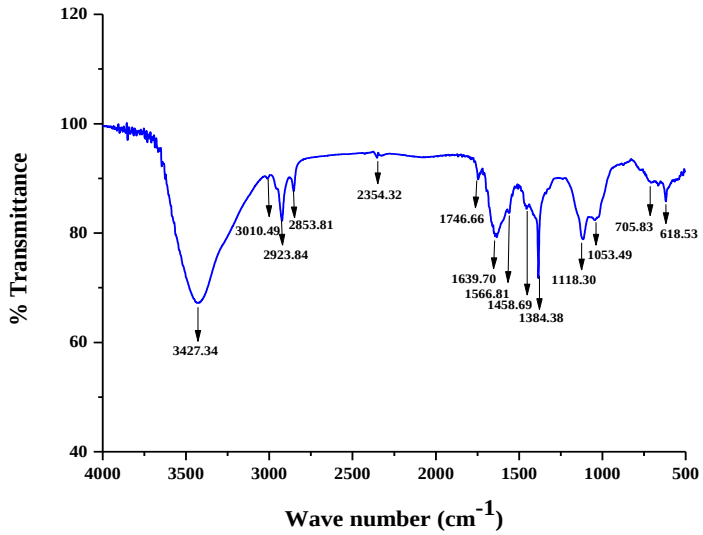

Figure 4.10. FTIR spectrum of rubber seed oil

Table 4.6. The main peakes in the FTIR spectrum of rubber seed oil

Peak (cm^{-1})	Functional group
3010, 2923, 2853	C-H stretching vibration (aliphatic)
1746	C=O stretching vibration (ester)
1458	C=C bending vibration (aliphatic)
1118, 1053	C-O-C stretching vibration (ester)
705, 618	C-H group vibration (aliphatic)

4.7.3. Fatty acid profile of RSO

Vegetable oils are mainly triglycerides of three fatty acid chains with a glycerol backbone. The fatty acid composition of RSO is shown in **Table 4.7.** It shows that refined RSO comprises of linoleic acid (33.43%), oleic acid (29.73%), linolenic acid (15.90), palmitic acid (12.59%), and stearic acid (8.35); which agrees with the results of previous studies. The oil does not contain any unusual fatty acids, and it is a rich source of essential fatty acids. C18:1, C18:2 and C18:3 make up 79.06% of its total fatty acid composition [108]. According to Aigbodion and Bakare, rubber seed oil has good fatty acid constituents with a higher percentage of linolenic acid, an omega 3 fatty acid important in human diet [109]. The type and percentage of fatty acids contained in a vegetable oil depends on the plant species and on the growth conditions of the plant. Though vegetable oils are of very low volatility in nature, they quickly produce volatile combustible compounds upon heating. Compounds like linolenic acid, palmitic acid, caryophyllene, humulene, aromadendrene, have also been identified in vegetable oils by which they are biologically active and possess antimicrobial, antioxidant, anti-inflammatory as well as anti-tumor activity [110].

Table 4.7. Fatty acid composition of rubber seed oil

Fatty acid	Formula	Area (%)		
		This study	Ramadhas et al. [89]	Roschat et al. [111]
Palmitic acid ($C_{16:0}$)	$C_{16}H_{32}O_2$	12.59	10.2	9.1
Stearic acid ($C_{18:0}$)	$C_{18}H_{36}O_2$	8.35	8.7	5.6
Oleic acid ($C_{18:1}$)	$C_{18}H_{34}O_2$	29.73	24.6	24
Linoleic acid ($C_{18:2}$)	$C_{18}H_{32}O_2$	33.43	39.6	46.2
Linolenic acid ($C_{18:3}$)	$C_{18}H_{30}O_2$	15.90	16.3	14.2

4.7.4. Antibacterial activity

Study of antibacterial activity of crude rubber seed oil against the foodborne pathogen *Escherichia coli* and virulent pathogen *Staphylococcus aureus* was carried out by the agar well diffusion method (**Figure 4.11**). The presence of zones of clearance speculates that the extract present in the disc has antimicrobial activity. The antimicrobial activity of RSO was measured in terms of zone of inhibition (ZOI) as shown in **Figure 4.12.** It was observed that the ZOI increased in dose-dependent manner and followed the same trend with respect to different bacterial strains. Tetracycline which was used as positive control showed large size inhibition zone than a RSO extract; while DMSO was used as negative control, which showed no inhibition zone.The highest inhibitory zone (21 mm) was observed in *E. coli* at 30 µl volume, whereas the lowest inhibitory zone (12 mm) was found with *S. aureus* at 10 µl volume.

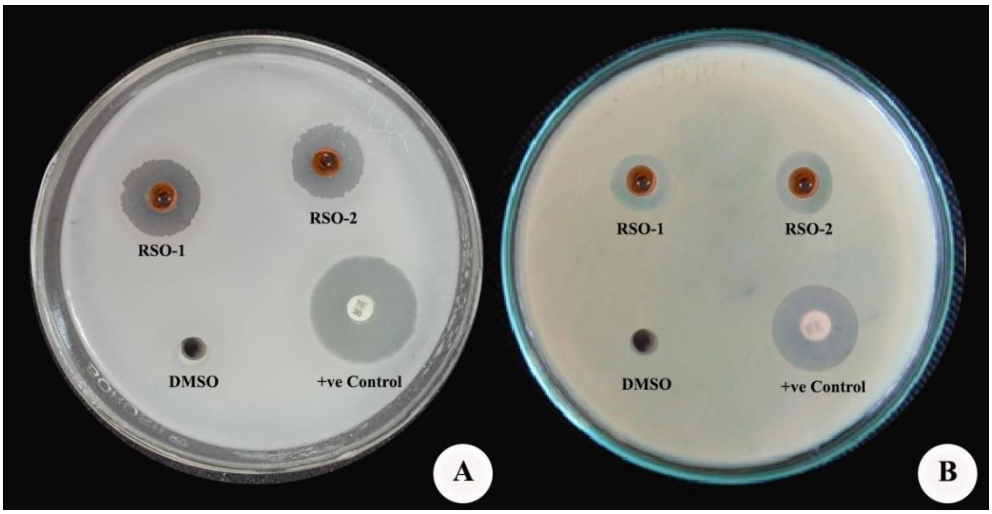

Figure 4.11. Antibacterial activity of RSO against; A) *E. coli.* maximum zone of inhibition at 30 µl (21.0 mm); B) *S. aureus.*, maximum zone of inhibition at 30 µl (16.0 mm)

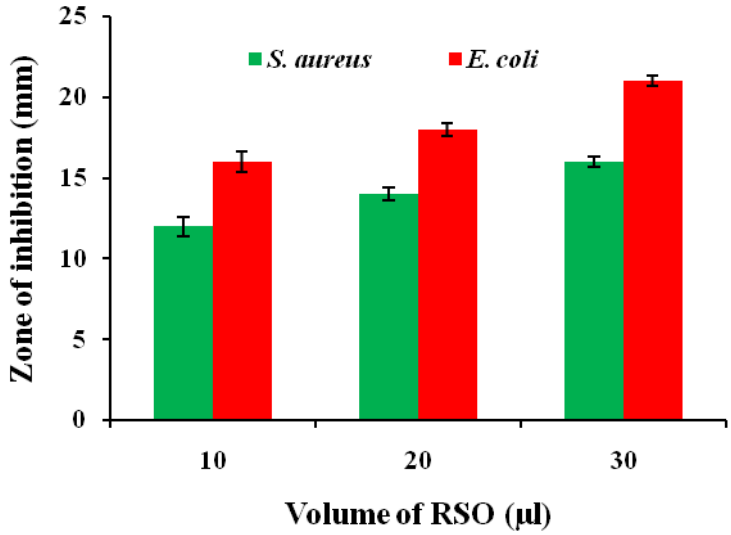

Figure 4.12. Antibacterial activity of RSO; Zone of inhibition (mm) at different volumes (10, 20, and 30 µl)

Higher resistance of gram negative bacteria to external agents has been earlier reported and it is attributed to the presence of lipopolysaccharides in their outer membrane, which make them inherently resistant to antibiotics, detergents and hydrophilic dyes [111]. Similar trend for inhibition of bacterial growth has been observed in earlier studies with other plant extracts [112]. RSO shows high value of fatty acids and are said to exhibit antibacterial activity [113]. By cause of fatty acids can act as anionic surfactants they have antibacterial and antifungal properties at low pH [114]. In addition, they are selective against Gram positive organisms by targeting the structure and function of bacterial cell walls and membranes [115]. The reason for higher sensitivity of the gram positive bacteria than negative bacteria could be ascribed to the differences between their cell wall compositions. The gram positive bacteria contain an outer peptidoglycan layer, which is an ineffective permeability barrier [116]. Here, the inhibition of bacterial growth by RSO takes place by disrupting the bacterial cellwall and destroying the nutrients penetrability leading to death of the cell. RSO incorporated with weather coat paints can be considered ecofriendly with its self cleaning, anti-pollutant and anti-bacterial performances.

4.7.5. Antioxidant activity

The oxidative chain reactions initiated by free radicals in a biological system are often terminated by certain components that are collectively known as

antioxidants. Generally two types of assays are carried out to study the antioxidant activity of a compound. The first type is based on the rate of oxidation of lipid content and the other one is based on the rate of radical or electron scavenging ability. As described elsewhere, two methods coming under the second type were used presently to assess the antioxidant activity of RSO: 2,2-diphenyl-1-picryl hydrazyl (DPPH) assay and ferric reducing power assay.

4.7.5.1. DPPH radical scavenging activity

Being a simple, inexpensive and quick method, DPPH radical scavenging assay was used as one of the techniques to assess the antioxidant activity of RSO. DPPH is a stable free radical even at room temperature, and shows strong absorbance at 517 nm. The DPPH radical accepts an electron or hydrogen radical to become a stable diamagnetic molecule with a different colour. Thus, the degree of its discolouration from purple to yellow is attributed to the hydrogen donating ability of the added compound, which is indicative of its radical scavenging potential [117]. In the present study, the DPPH radical scavenging activity of rubber seed oil was determined by modified method of Blois [118]. The calibration curve was prepared using ascorbic acid as the standard (**Figure 4.13**). **Figure 4.14** demonstrates that, at the concentration examined (1.0 mg/mL), the seed oils from each extract (HeRSO, MtRSO and EtRSO) display different inhibition capabilities on DPPH radical scavenging to ascorbic acid standard (50%, 61.11% and 77.77% inhibition respectively). Significant activity and maximum result were shown by ethanol extract; much difference in the scavenging ability of the same extract might be due to the diversity in the phytoconstituents or composition of oils collected. Seed oils are excellent repositories of fat, oils and phenol that can annihilate the reactive free radicals in a biological system. Rubber seed oil proved to be an effective scavenger of DPPH radicals. Unlike other free radicals, DPPH does not get dimerised; thus remains as a stable free radical. The electrons are delocalized around the molecule in the alcoholic solution and this imparts pink or violet colour to the solution. The odd or unpaired electron in the nitrogen atom of the DPPH radical is reduced by the active antioxidant compounds in the plant extracts by the corresponding release of the hydrogen atom to the hydrazine moiety [119]. On the addition of the ethanolic extract to the alcoholic solution of DPPH, active

antioxidant components in the extracts are released to the system where the free electrons in the reagent (DPPH) are paired off. This subsequently leads to the discoloration of the reaction mixture and the same is measured spectrophotometrically after a definite period. The reaction is so sensitive that it occurs even in the presence of a weak antioxidant [120].

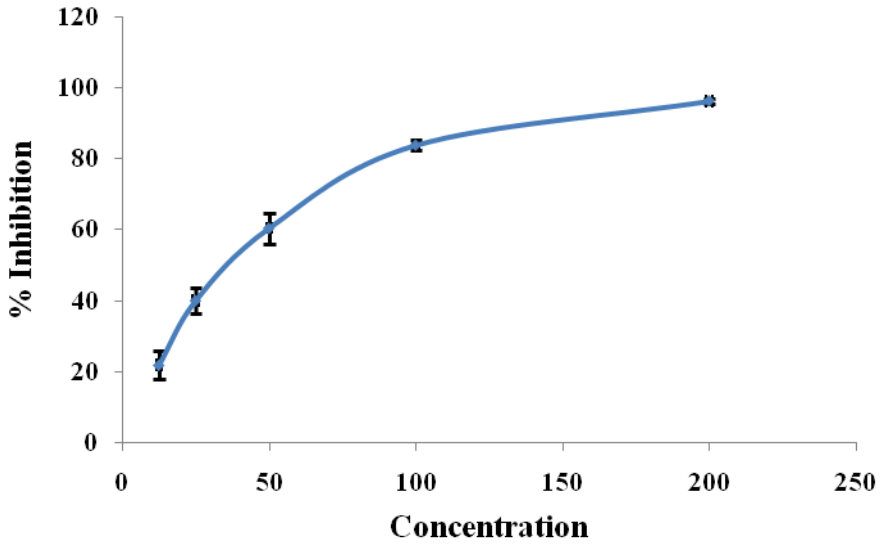

Figure 4.13. Standard calibration curve for DPPH radical scavenging assay

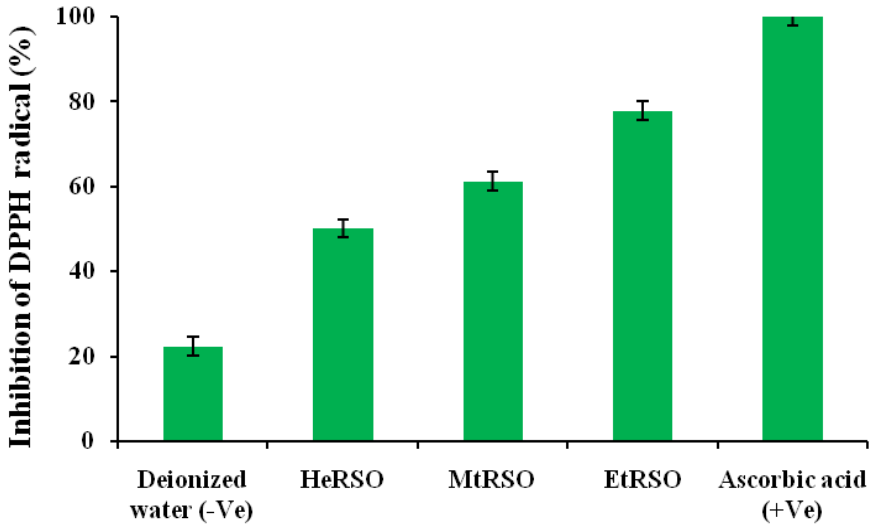

Figure 4.14. DPPH radical scavenging activity exhibited by various solvent extracts of RSO; HeRSO: Hexane RSO; MtRSO: Methanol RSO; EtRSo: Ethanol RSO) Values are expressed as means of three replicated ± standard deviations.

4.7.5.2. Ferrous reducing power

Reducing power assays are usually used to evaluate the capacity of natural antioxidants to donate an electron or hydrogen atom [121]. Natural antioxidants are believed to break free radical chain reactions by donating an electron or hydrogen atom to free radicals and hence antioxidant activities should be reflected in the reducing power. In the present study, the ferrous reducing ability was determined by the method of Takashi [122]. In this assay, the yellow test solution becomes green or blue when the presence of reducers converts the Fe3+/ferricyanide complex to its ferrous form. Thus, higher absorbance at 700 nm indicates greater reducing power [123].

As shown in **Figure 4.15**, reducing power increased progressively in the order of HeRSO, MtRSO and EtRSO.. They have been found to show significant reducing power of 0.25±0.016, 0.434±0.009 and 0.83±0.012 mg of AAE/mL respectively. RSO exhibited much stronger metal chelating activity when compared to the negative control. However, the metal chelating activity of RSO was significantly lower than that of ascorbic acid (positive control). As described above, the ferrous chelating ability may be related to antioxidant activity and may modify the antioxidant activity by influencing other reactions. These results suggest that RSO has a beneficial effect on ferrous chelating ability and may thus exert protection against oxidative damage.

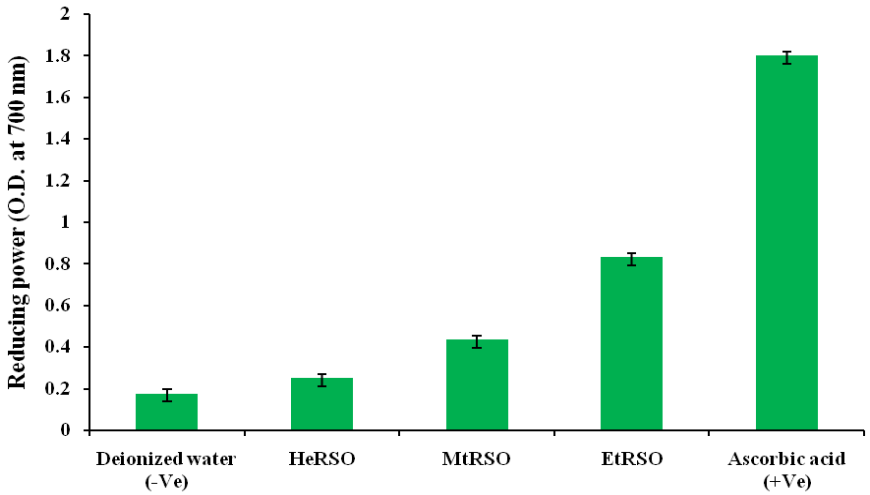

Figure 4.15. Ferrous reducing ability exhibited by various solvent extract of RSO; HeRSO: Hexane RSO; MtRSO: Methanol RSO; EtRSo: Ethanol RSO) Values are expressed as means of three replicated ± standard deviations.

4.7.5.3. Lipid peroxidation inhibition activity

The thiobarbituric acid reactive substances (TBARS) method is often used to measure lipid peroxide inhibition activity. Increased lipid peroxidation is generally believed to be an important underlying cause of oxidative stress initiation upon various tissue injuries, cell death or the progression of many acute and chronic diseases [124]. 100 µl of each extract (EtRSO, MtRSO and HeRSO) was mixed with linoleic acid 900 µl and then incubated the mixture at 100°C for 20 min. 1 ml of buffer solution (pH 3.5) and 1 ml of the mixture of 20 mM of thiobarbituric acid in 10% trichloroacetic acid were added in the resulting mixture and then incubated at 100°C for 30 min to obtain the pink coloured solution. The absorbance was measured at 532 nm using a UV spectrophotometer. The percentage inhibition of lipid peroxidation was calculated by the following equation:

Inhibition of lipid peroxidation (%) = [(Abs control – Abs sample)/ Abs control] x 100

Figure 4.16 shows the results of inhibition of lipid peroxidation in the case of the solvent extracts of RSO such as EtRSO, MtRSO and HeRSO. They showed moderate inhibited lipid peroxidation in the linoleic acid system by 14.87%, 21.38% and 24.59% respectively. The reference compound, ascorbic acid inhibited lipid peroxidation at its full strength (100%). Deionized water (the negative control) showed much lesser lipid peroxide inhibition ability when compared with different extracts of RSO. These results show that RSO shows moderate and significant inhibition of lipid peroxidation and it may be due to the presence of high concentrations of carotenoids, phenols and flavonoids in RSO, which are known to have significant biological activities. Haraguchi (2001) [125] reported that various types of phytochemicals including fatty acids are very effective in preventing lipid peroxidation. RSO, is a rich source of essential fatty acids and C18:1, C18:2 and C18:3 make up 79.06% of its total fatty acid composition. This might be responsible for its significant activity against lipid peroxidation.

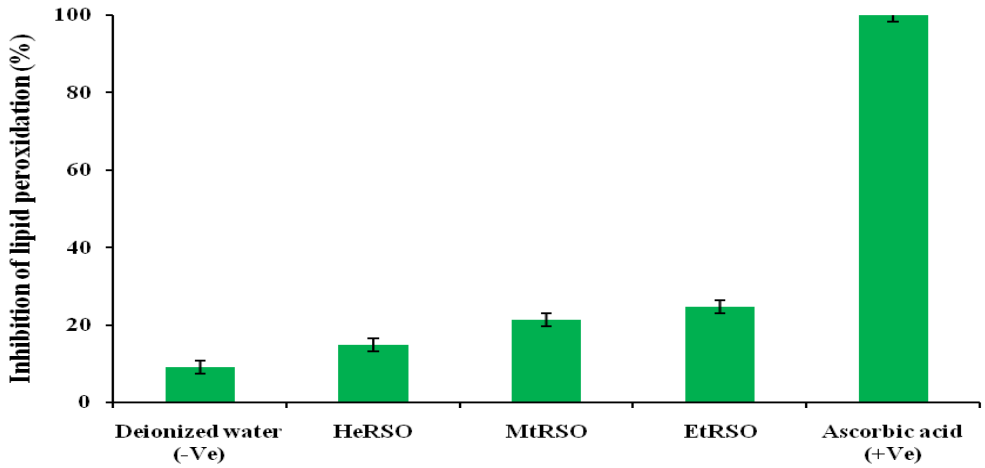

Figure 4.16. Lipid peroxidation inhibition ability exhibited by various solvent extracts of RSO. HeRSO: Hexane RSO; MtRSO: Methanol RSO; EtRSO: Ethanol RSO). Values are expressed as means of three replicated ± standard deviations.

Hron et al. [126] and Ferriera-Dias et al. [127] have reported that ethanol has the capability to co-extract other compounds present in oilseeds which are insoluble in non-polar solvents. Ethanol is a good solvent for extraction compared to the other solvents. The antioxidant activities of the seed oils may be attributed to fatty acid components. Moreover, these activities may have been partly contributed by some other constituents such as phenolic compounds. Plant derived compounds have been documented as excellent free radical scavengers capable of rectifying the damages caused by the oxidative species in the living system. Recently, naturally occurring antioxidants are gaining interest in pharmaceutical, nutraceutical, food and cosmetic industries due to their multiple actions and negligible side effects thereby providing huge scope in replacing synthetic ones. Moreover, natural antioxidants can provide a protective effect against tissue degradation [128]. It is suggested that further studies on the chemical composition of rubber seed oil be carried out in order to use as a natural raw material for cosmetic applications. Linoleic acid and oleic acid are the predominating fatty acids in seed oils and they have currently become increasingly popular in cosmetic industry due to their beneficial effects on skin. Research points to linoleic acid's antioxidant, anti-inflammatory, acne reductive and moisture retentive properties when applied topically on the skin. Since the rubber seeds are by products, development of cosmetic application of rubber seed oil may improve the value of this plant and enhance the agricultural economy.

4.8. Lipase production from agrowastes

Two new bacterial strains, *Pseudomonas aeruginosa* BUP2 (MTCC No.5924) & *Pseudomonas* sp. BUP6 (MTCC No.5925), isolated from the rumen of Malabari goat, described by the Enzyme Technology Laboratory of Department of Botany, University of Calicut, India and deposited at Institute of Microbial Technology (IMTECH) Chadigarh were used for experiments on lipase production from agrowastes including rubber seed cake powder in the present study as describe elsewhere.

4.8.1. Morphological characterization of *P. aeruginosa* BUP2 & *Pseudomonas* sp. BUP6

The clear morphology of the bacteria was observed using binocular microscope (Magnus MLX) and photographs were taken using Image Analyzer (Nikon Eclipse E400, Towa Optical, Japan) fitted with Nikon digital camera (DXM 1200F, Japan). The pure colonies of *Pseudomonas* sp. BUP6 were smooth, slimy and cream in colour **(Figure 4.17 A).** It is Gram-negative and rod shaped with cell range between 1.0 and 1.5 µM in length and 0.3 and 0.6 µM in diameter. *P. aeruginosa* BUP2 with rod-shaped, asporogenous and Gram-negative cells measured 0.4 to 0.8 (width) by 1 to 2 µm (length) **(Figure 4.17 B).** The viable cells of bacteria (3. 24 $\times 10^9$ cfu/ mL) were determined by serial dilution method.

Figure 4.17. Morphology of bacterial cultures; A) *P.* sp. strain BUP6; B) *P. aeruginosa* strain BUP2.

4.8.2. SSF strategy

Microbial lipases are mostly produced by Submerged fermentation (SmF). Solid-state fermentation (SSF) technique is a proven strategy for the effective utilization of agro-industrial residues for producing value added products of commercial interest at low cost. Generally, lipase production is organism specific and it is released during the late logarithmic or stationary phase of its life cycle. Benjamin and Pandey (1998) have demonstrated the utility of mixed-solid substrate containing wheat bran and coconut oil cake for lipase production employing *Candida rugosa* [129]. As per another report deoiled cake from *Jatropha* seed was used as a support for the production of lipase (1084 U/gds) from *P. aeruginosa* PseA through SSF [130]. Research on lipase progressed very rapidly in the past few decades, giving much emphasis on enzymatic hydrolysis of lipid or fats to biodiesel. SSF strategy is increasingly employed now as a method for the production of lipase from oil mill effluents.

4.8.3. Effects of different substrates on lipase activity

Three different de-oiled cakes (rubber seed flour, coconut cake and cotton flour) were employed as solid substrate-cum-inducer for the production of lipase by *P. aeruginosa* BUP2 and *Pseudomonas* sp. BUP6. Of them, rubber seed flour supported the maximum lipase production, i.e., 871 U/gds at 48 h of incubation and 33.98 U/gds at 24 h of incubation by *P. aeruginosa* BUP2 and *Pseudomonas* sp. BUP6 respectively (**Figure 4.18** & **Figure 4.19)**. Lipase production by *P. aeruginosa* BUP2 and *P.* sp. BUP6 on different substrates are presented in **Table 4.8** and **Figure 4.18** to **Figure 4.23**. Coconut cake showed lipase production of 327 U/gds by *P. aeruginosa* BUP2 at 48 h incubation (**Figure 4.20**) and lipase production of 9.97 U/gds (**Figure 4.21**) by *Pseudomonas* sp. BUP6 at 72 h of incubation. Cotton seed flour showed lipase production of 208 U/gds (**Figure 4.22**) by *P. aeruginosa* BUP2 at 48 h incubation and of 5.45 U/gds at 24 h of incubation by *Pseudomonas* sp. BUP6 (Figure **4.23**). When compared to the other two substrates, rubber seed flour showed greater activity in the case of both the species of *Pseudomonas* used and *P. aeruginosa* BUP2 showed higher activity in all the cases. Hence, this was used for further lipase production and purification.

Table 4.8. Maximum lipase activity in different substrates

Substrates	*Pseudomonas aeruginosa* BUP2		*Pseudomonas* sp. BUP6	
	Maximum lipase activity (U/gds)	**Optimum hours of incubation**	**Maximum lipase activity (U/gds)**	**Optimum hours of incubation**
Rubber seed flour	871	48	33.98	24
Coconut oil Cake	327	48	9.97	72
Cotton seed flour	208	48	5.45	24

Large quantities of agricultural residues have been reported to be effective for lipase production and these include brans (wheat, rice, soybean, barley), oil cakes (soya bean, coconut, ground nut, cotton, olive, gingelly, babassu), and bagasse (sugarcane). Oil cakes of various residues obtained after extraction of oils have been utilized for fermentative production of lipases and other industrial enzymes. Most agricultural residues utilized for lipase production contain a mixture of both easily consumable and non-consumable substrates that can support the growth of a wide range of microorganisms. This is because their residual oil contents serve as inducers for lipase production [131].

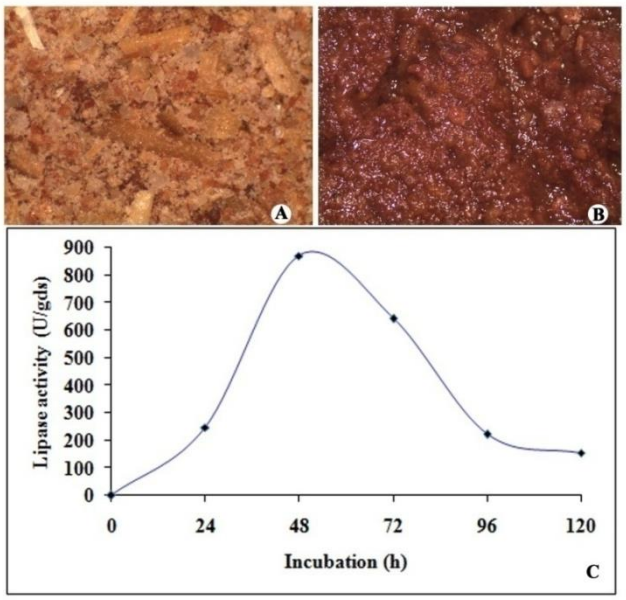

Figure 4.18. Production of lipase by *P. aeruginosa* BUP2 on rubber seed flour. A) rubber seed flour before inoculation. B) rubber seed flour after inoculation. C) lipase activity at different time intervals; maximum lipase activity (871 U/gds) was observed at 48 h interval.

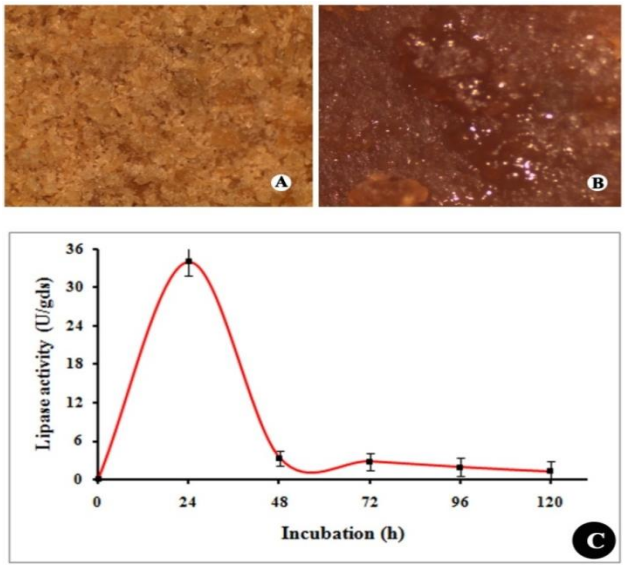

Figure 4.19. Production of lipase by *Pseudomonas* sp. BUP6 on rubber seed flour. A) rubber seed flour before inoculation. B) rubber seed flour after inoculation. C) lipase activity at different time intervals; maximum lipase activity (33.98 U/gds) was observed at 24 h interval.

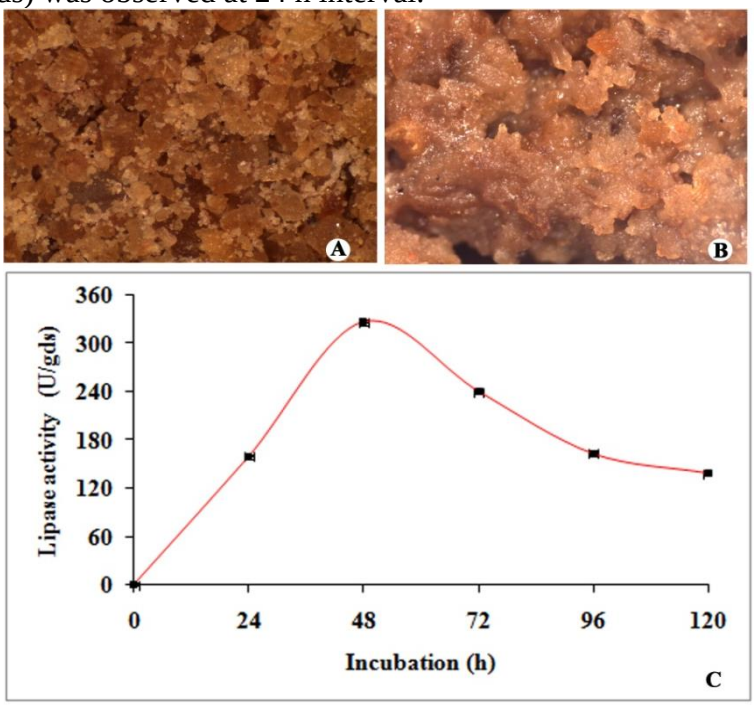

Figure 4.20. Production of lipase by *P. aeruginosa* BUP2 on coconut cake. A) coconut cake before inoculation. B) coconut cake after inoculation. C) lipase activity at different time intervals; maximum lipase activity (327 U/gds) was observed at 48 h interval.

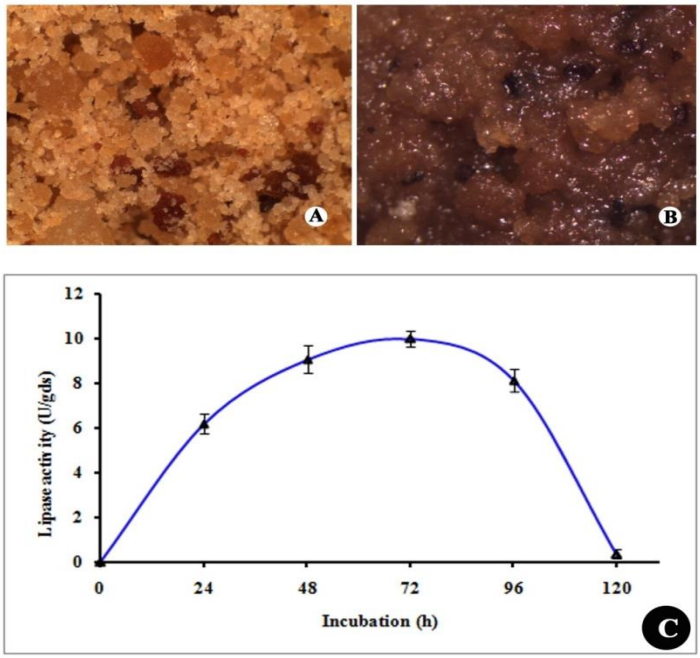

Figure 4.21. Production of lipase by *Pseudomonas* sp. BUP6 on coconut cake. A) coconut cake before inoculation. B) coconut cake after inoculation. C) lipase activity at different time intervals; maximum lipase activity (9.97 U/gds) was observed at 72 h interval.

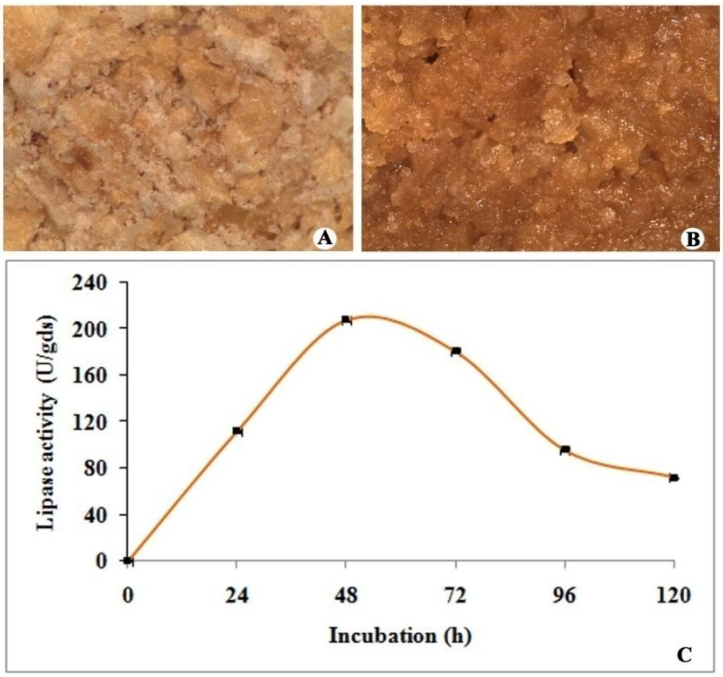

Figure 4.22. Production of lipase by *P. aeruginosa* BUP2 on cotton seed flour. A) cotton seed flour before inoculation. B) cotton seed flour after inoculation. C) lipase activity at different time intervals; maximum lipase activity (208 U/gds) was observed at 48 h interval.

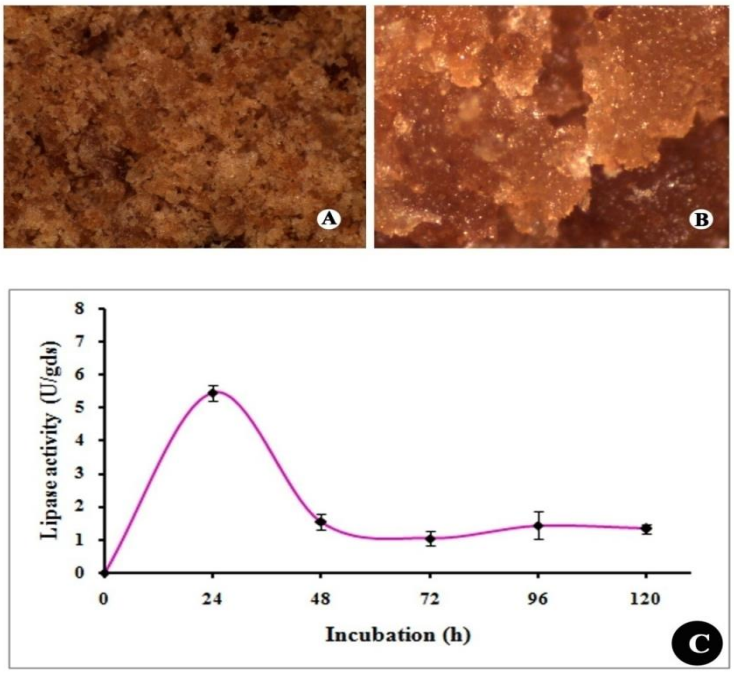

Figure 4.23. Production of lipase by *Pseudomonas* sp. BUP6 on cotton seed flour. A) cotton seed flour before inoculation. B) cotton seed flour after inoculation. C) lipase activity at different time intervals; maximum lipase activity (5.45 U/gds) was observed at 24 h interval.

There are some reports on the use of different microorganisms such as *Penicillium restrictum*, *Aspergillus niger*, *Candida rugosa* and *Yarrowia lipolytica* for lipase production using babassu oil cake, gingelly oil cake, groundnut oil cake and vegetable oil meal [132, 133]. Previous studies have revealed that oil cakes are very good solid substrates cum inducers for the production of lipase by SSF [134]. D'Annibale et al. reported olive mill waste water as a growth medium for lipase production, which showed the highest lipase activity of 9.23 U/ml [135]. Salihu et al. used statistical optimization of nutrient components to enhance lipase production by *Candida cylindracea*, and the maximum activity was 20.26 U/ml [136]. Brozzoli et al. studied lipase production in bench-top reactor using the olive mill waste water medium and obtained the maximum production as 20.4 U/ml [137]. The cultivation period varied with the microorganism, i.e., fast growing bacteria were found to secrete lipase within 24- 48 h [138].

In the present study, the highest activity of lipase was obtained (871 U/gds) at 48 h of incubation using rubber seed as substrate. In India, rubber trees

are grown primarily for its latex and its by products namely the rubber seed oil and cake are not utilized much. Unni et al. have also reported such a high rate of production of lipase in a similar experiment [139]. Under the circumstances, *Pseudomonas aeruginosa* BUP2 has proved to be a highly promising strain for lipase extraction and lipase extracted in this way is used in the forthcoming experiments for further study.

4.8.4. Purification of lipase

Methods to purify lipases generally depend on nonspecific techniques such as salt precipitation, hydrophobic interaction chromatography, gel filtration and ion exchange chromatography. In the present studies, the extracellular lipase produced by *P. aeruginosa* strain BUP2 was carried out by $(NH_4)_2SO_4$ salt precipitation, dialysis and gel permeation chromatography. The prime objective of this step was to remove as much as unwanted proteins as possible from the crude supernant without much disturbing the lipase active fraction. Generally, successive methods of salt precipitation, ultrafiltration, gel exclusion chromatography are employed for the purification of extracellular enzymes [140, 141]. The effectivness of enzyme purification is usually determined by two critical parameters, i.e., the yield and fold of purification. Protein content of crude and purified lipase samples were estimated Lowry's method where the protein react with Folin Ciocalteau reagent to form a dark blue purple colour complex [142].

The present results revealed that *P. aeruginosa* BUP2 had the highest protein content of 2.16 mg/ml, and the result obtained was calculated against the BSA standard curve. Compared to the initial activity (crude supernatant), lipase activity was increased in $(NH_4)_2SO_4$ and column chromatography fractions, which was directly proportional to the purity of the lipase. Of various $(NH_4)_2SO_4$ fractions, 40-80% fraction showed the maximum lipase activity, which was of 19.81 folds purified with 25.42% yield; this fraction was subjected to gel permeation chromatography (**Table 4.9**).

Table 4.9. Summary of the purification of lipase**

Purification Step	Lipase activity (U/ml)	Protein (mg/ml)	Total protein (mg)	Total lipase activity (U)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	142	2.16	432	28400	65.74	1	100
Ammonium sulphate fraction (40-80%)	2188	1.68	5.54	7220.4	1302.38	19.81	25.42
Superose^{TM} column	1384	0.58	1.45	3460	2386.20	36.29	12.18

****Calculations**
Total protein=Protein con. ×Total Vol. of sample
Total lipase activity= LipaseActivity ×Total Vol. of sample
Specific activity = Total activity / Total Protein
Fold purification = Specific activity /Crude specific activity

Earlier workers have reported certain procedures of purification of lipases. Mehta et al. have proposed a method to purify lipase obtained from *Aspergillus fumigatus* using Octyl Sepharose column chromatography [143]. They described that the crude enzyme at 60–70% of ammonium sulphate gave the maximum enzyme activity of 5.8 U/ml and protein content of 1.2 mg/ml with 2.34-fold purification. Employing $(NH_4)_2SO_4$ precipitation and chromatographic techniques, Ogino et al. extracted and purified an alkaline thermostable lipase from *P. aeruginosa* LST-03 to 34.7 folds with a yield of 12.6% [144]. Likewise, an extracellular lipase from *P. aeruginosa* mutant was purified by acetone precipitation, followed by column chromatography; which resulted in 27 folds purification with 19.6% final recovery [145].

Little information has been published on large-scale processes for commercial purification of lipase. Most commercial applications of lipases do not require highly pure enzyme. Further excessive steps of purification are expensive and reduce the overall recovery of the enzyme. Presently, the best dialysate obtained was loaded onto pre-packed Superose^{TM} 6 (10/300 GL-Tricorn) column. Twenty five fractions of 2 ml were collected in 5 ml borosil glass tubes at a constant flow rate of 0.2 ml/min. The enzyme activity and protein content in the best 3 or 4 fractions were estimated; which showed a major peak represented by fraction number 15 (**Figure 4.24**). The fractions showing maximum lipase

activity were pooled and stored in deep freezer (Haier; Model No: DW-40L262) at -20°C. After the preparation of gels, active fractions were subjected to SDS-PAGE for purity check. The purification fold of gel permeation chromatography fraction of lipase was 36.29 with yield of 12.18% (**Table 4.9**). From SDS-PAGE pattern, the apparent molecular weight (MW) of the partially purified lipase was estimated as 29 kDa (**Figure 4.25**).

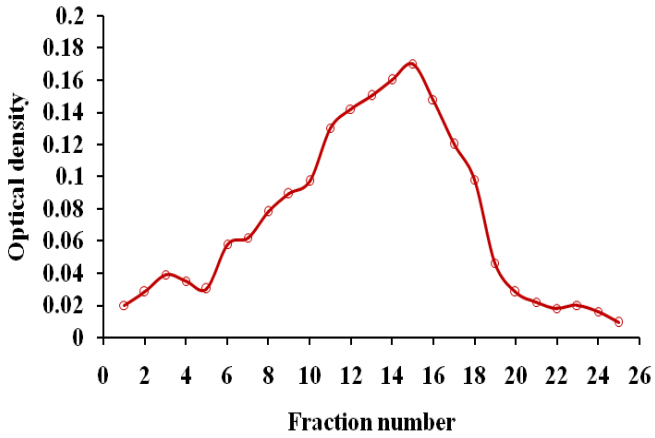

Figure 4.24. Elution profile of lipase by Superose^{TM} column chromatography (2 ml/10 min), in which fraction number 15 showed the maximum concentration and purity.

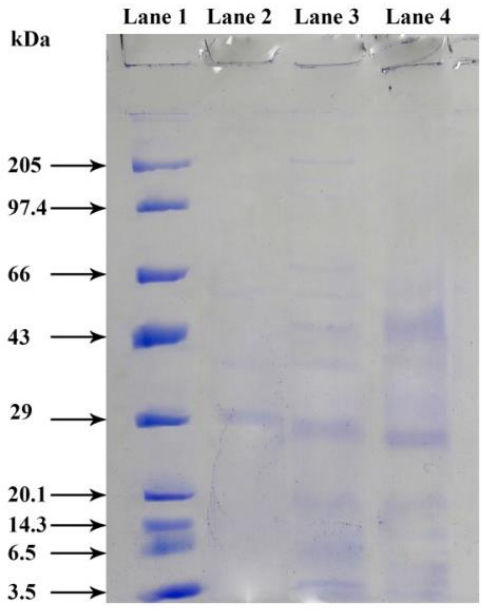

Figure 4.25. SDS-PAGE profile of lipase from *P. aeruginosa* strain BUP2, showing the purity. Lanes: (1) standard protein molecular weight marker; (2) sepadex G100 column purified lipase showing apparent molecular weight of 29 kDa; (3) 40-80% $(NH_4)_2SO_4$ fraction; and (4) crude protein

As per literature, MW of lipase produced by species of *Pseudomonas* varies from 29 to 95 kDa. For instance, an extra cellular lipase isolated and purified from *P. aeruginosa* SRT 9 to apparent homogeneity using ammonium sulfate precipitation followed by chromatographic techniques on Phenyl Sepharose CL- 4B and Mono Q HR 5/5 column, resulting by SDS-PAGE had the MW of 29 kDa [146]. Earlier report of purified lipase from *P. aeruginosa* MB5001 was found as 29 kDa by SDS-PAGE [147]. The MW of purified lipase produced by *P. aeruginosa* LX1 was found to be 56 kDa [148] where as the MW of purified lipase produced by *P. aeruginosa* AAU2 was found approximately as 81.7 kDa [149].

Briefly, the present study describes the purification and characterization of alkaline lipase (29 kDa) which has potential applications in industries like detergency and tannery. Moreover, it illustrates the industrial potentials of rumen microbes, which can be explored as the candidate of 'green technologies' with GRAS (generally regarded as safe) status in near future.

4.9. Characterization of purified lipase

Lipase obtained by SSF process using rubber seed cake as the substrate and *P. aeruginosa* BUP2 as the bacterium for lipase production as described above was used in the forthcoming experiments. The lipase produced was characterized in terms of its activity and stability profiles relative to effectivness of solvent, positional specificity and isoelectric point.

4.9.1. Effect of organic solvents on lipase activity

In the present study, the effect of the enzyme activity against various organic solvents like hexane, petroleum ether, chloroform, toluene, acetonitrile, methanol, ethanol and acetone were studied in different ratios (1:10 and 1:1 v/v). Interestingly, the non-polar solvents like hexane, petroleum ether, chloroform and toluene showed strong stimulatory effect on enzyme activity (**Figure 4.26**); whereas exposures to polar solvents like acetonitrile, methanol, ethanol and acetone showed a drastic reduction in lipase activity (**Figure 4.27**). Stability and activity in organic solvents are important characteristics of protein catalysts used

in organic synthesis reactions. In general, non-polar solvents are more stabilizing than their polar counterparts. The increase in lipase activity in the non-polar solvents could be due to the hydrophobicity of the solvents. The activation of lipase could be due to the fact that organic solvent molecules could interact with hydrophobic amino acid residues present in the lid that covers the catalytic site of the enzyme, thereby maintaining the lipase in its open conformation and promoting catalytic function. There is a tendency for hydrophilic solvents to cause more significant enzyme inactivation than hydrophobic solvents [150]. Conformational structure of enzymes is maintained by many forces such as hydrophobic interactions, electrostatic interactions, Van der Waals forces and hydrogen bonds [151]. Protein unfolding occurs due to the disturbance of the balance. In media containing organic solvents, enzyme deactivation is most probably caused by the disruption of the protein molecule hydrophobic core due to the change of medium hydrophobicity. In particular, polar solvents that can penetrate into the protein are far more capable of inducing structural changes than non-polar solvents and the significant deactivation by polar solvents is due to the stripping-off of crucial bound-water monolayer from the enzyme molecule essential for its activity [152]. In the present study, maximum lipase activity was observed in 1:1 ratio of toluene incubated for 24 h (**Figure 4.26**); whereas, the maximum lipase activity loss was observed at 1:1 ratio in acetone at incubation periods of 24 and 18 h respectively (**Figure 4.27**).

Ugur et al. (2014) reported that LipSB 25-4 from *Streptomyces bambergiensis* OC 25-4 showed significant solvent-stable characteristics in both hydrophobic and hydrophilic solvents [153]. In contrast, lipase prepared from *Chromobacterium viscosum* showed high activity in acetonitrile where it was employed in transesterification reactions [154]. However, if enzymes are extracellular in origin, they could be naturally stable and exhibit significant activity in the presence of organic solvents. Various organic solvent-tolerant enzymes have been reported in the past decade. Most were lipolytic and proteolytic enzymes. Although impurities often influence the stability of enzymes in the presence of organic solvents, some were investigated without enzyme

purification. In addition, the experimental conditions such as incubation time and ratio of the solvent were studied for the solvent-tolerance of the enzymes. There have been some reports of the purification and characterization of solvent-tolerant lipases. Synthetic reactions with the enzymes are often performed in organic solvents to shift the thermodynamic equilibrium towards synthesis. There have been some reports of lipolytic enzymes with organic solvent-tolerance that are useful for biotechnological applications in detergents, food, dairy, textile, waste water treatment, biodiesel, oil processing, synthesis of pharmaceuticals and production of fine chemicals [155].

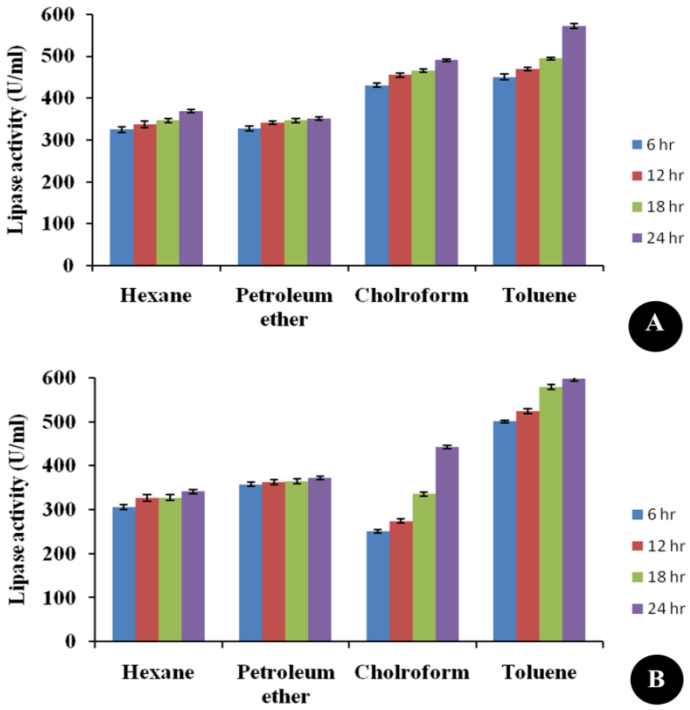

Figure 4.26. Effect of non-polar solvents on lipase activity, A) solvent-enzyme ratio at 1:10 (v/v); B) solvent-enzyme ratio at 1:1 (v/v) observed at 37°C temperature and 1000 rpm.

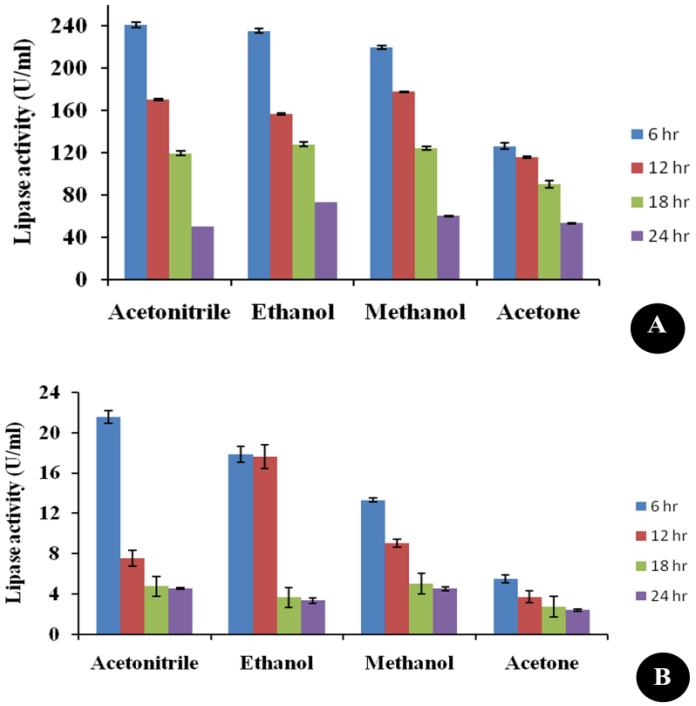

Figure 4.27. Effect of polar solvents on lipase activity, A) solvent-enzyme ratio at 1:10 (v/v); B) solvent-enzyme ratio at 1:1 (v/v) observed at 37°C temperature and 1000 rpm.

4.9.2. Positional specificity of lipase

The positional specificity of purified lipase was examined by thin layer chromatography of the enzymatic hydrolysis products of triolein. Triolein was hydrolyzed by the stirring method, and then the hydrolysis products were extracted using 2.5 ml of diethyl ether. The products were analyzed by thin layer chromatography (TLC) on silica gel. The solvent system for the TLC analysis consisted of a mixture of petroleum ether, diethyl ether, and acetic acid (80:20:1, v/v). Spots were detected by spraying with 50% H_2S0_4, followed by heating at 80°C for 30 min. Microbial lipolytic enzymes such as lipases and esterases have a number of unique characteristics such as substrate specificity, region-specificity and chiral selectivity. Lipases are best known to catalyze the hydrolysis of triglycerides to monoglycerides (MG), diglycerides (DG), free fatty acids (FFA) and glycerol. In the presence of water, hydrolysis is the main reaction; at low water concentration synthesis of esters can occur in the presence of fatty acids and an alcohol. Most lipases are classified into two groups: sn-I,3 regiospecific, which react with the outer positions of the glycerol backbone and non-

regiospecific or random, which act on all three positions. According to the thin-layer chromatography analysis of the hydrolysis products of triolein, *P. aeruginosa* BUP2 lipase cleaved not only the 1,3-positioned ester bonds but also the 2-positioned ester bond (**Figure 4.28**). Therefore, it can be concluded that *P. aeruginosa* BUP2 lipase is very robust and it acts at random on the triacylglyceride molecule and results in the complete breakdown of triacylglyceride to fatty acid and glycerol. Reports have said that lipases from *P. cepaci, P.* sp. S5 and LST03 also cleaved ester bonds non-specifically [156-158]. *Pencillium. simplicissimum* lipase showed nonspecific nature and hydrolysed each of the three bonds of triacylglycerol [159]. However, some *Pseudomonas* lipases cleaved only 1,3-positioned ester bonds, such as those from *P. fragi* and *P.* sp. 2106 [160, 161].

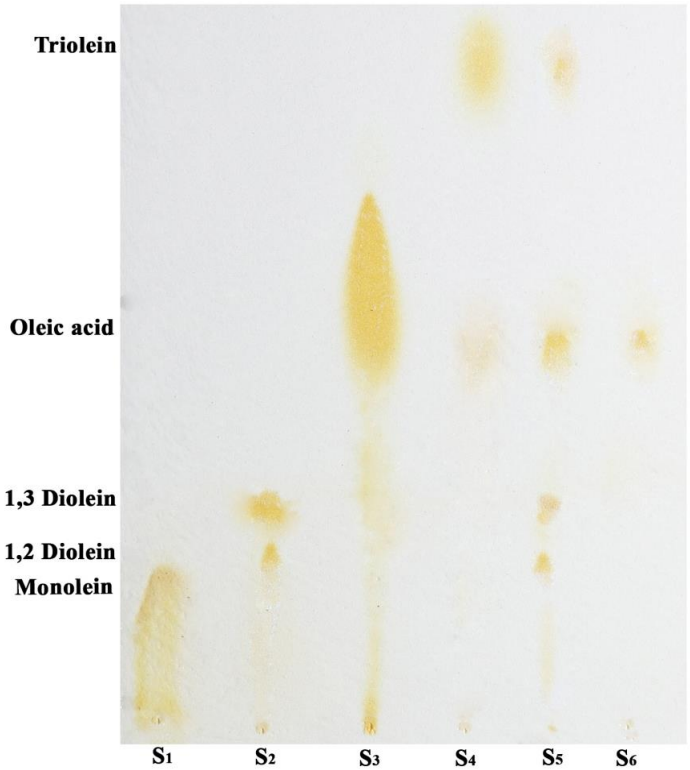

Figure 4.28. TLC chromatograms of the hydrolysis products of triolein catalyzed by *P. aeruginosa* sp. BUP2 lipase. Lanes S1–S4 standards, lane S_1 monolein, lane S_2 diolein, lane S_3 oleic acid; lane S_4 triolein; lane S_5 hydrolysis product; lane S_6 rubber seed oil.

Some lipases can hydrolyze only the 1,3-positioned bonds while others are able to hydrolyze all three ester bonds. A study is reported on the application of nonspecific *Candida antartica* lipase in the acidolysis of canola oil through

caprylic acid to produce high purity structured lipids [162]. So far, only a few lipases show selectivity at the sn-2, or center position of glycerol; where as *Geotrichum candidum* lipase and *C. antarctica* lipase were reported to show sn-2 selectivity [163, 164]. The position 2 specificity of S3 *Penicillium citrinum* lipase is repotedly different from the lipases from *P. cyclopium* and *P. camembertii* Thom PG-3 wich have 1,3-positional specificity [165, 166]. The species and genus dependency of the positional specificity of lipases may provide helpful information for the rapid selection of specific lipases to catalyze certain chemical reactions, thereby avoiding unnecessary selection procedures. They have been widely used for biotechnological applications in detergents, food, dairy, textile, waste water treatment, biodiesel, oil processing, analysis of blood triglycerides, and in the synthesis of pharmaceuticals and fine chemicals [167].

4.9.3. Isoelectric point

The isoelectric point (pI) of a protein is the pH at which the overall charge of the protein is zero. In order to determine the isoelectric point of lipase we firstly had to create a series of acetic acid buffers ranging from 0.3M to 1mM by doubling dilutions method. After adding the lipase to each of the buffer solutions it was instantly visible that a precipitation had formed and that the degree of precipitation varied between the different concentrations of buffer solution used. As a result the lipase precipitated to varying degrees so that the precipitation was the greatest in the buffer with the pH 4.3 closest to the isoelectric point of lipase. It shows that proteins tend to aggregate and precipitate at their pI because there is no electrostatic repulsion keeping them apart.

There is lesser precipitation or turbidity between pH 3.6 and 4.1; i.e., more of the lipase was dissolving in the buffer solution. Precipitation or turbidity was then increased at a pH of 4.3 indicating that at this pH less of the lipase was able to dissolve **(Figure 4.29**). Out of the ten different buffer solutions tested, sample 5 had a pH of 4.3 that was the closest to the isoelectric point of lipase and as a result the greatest degree of precipitation was formed. At it's isoelectric point, the lipase has a net neutral charge of zero and then it is unable to become

dissolved in a buffer solution. At pH values greater than 4.3, the turbidity decreases; this is due to the fact that as the pH increases there are less hydrogen ions in the buffer. At this point the pH is greater than the p*Ka* (acid dissociation constant) and the molecule looses protons to become negatively charged. It is this charge that enabled the enzyme molecules to partially dissolve in the buffer solution and hence less precipitate is formed.

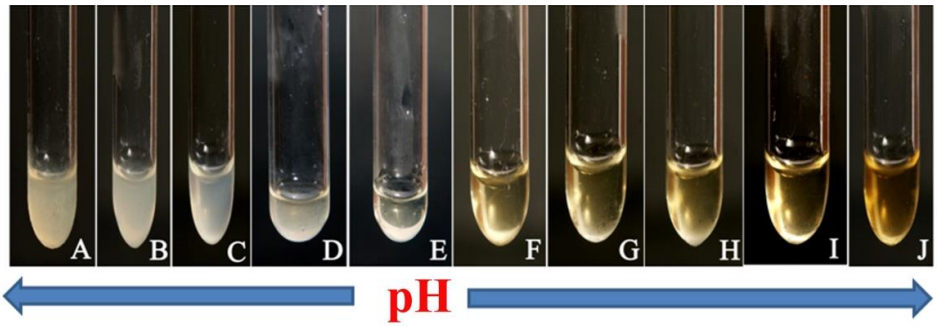

Figure 4.29. Screening for isoelectric point of *P. aeruginosa* sp. BUP2 lipase by serial dilution method: test tube with pH gradient (pH 3.6 to pH 5.3) of acetic acid; A- pH 3.6, B- pH 3.8, C- pH 4.0, D- pH 4.2, E- pH 4.3, F- pH 4.5, G- pH 4.7, H- pH 4.9, I- pH 5.1 and J- pH 5.3. Maximum precipitation is seen in test tube E with pH of 4.3.

Proteins have different pI because of their different amino acid sequences; i.e., relative numbers of anionic and cationic groups. A few reports showed that the alkaline and thermostable lipases from *Pseudomonas pseudoalcaligenes* F-111, *P. fragi* 22.39B, *P. pseudoalcaligenes* M1, *Pseudomonas* sp. KWI-56, *P. aeruginosa* EF2 and *P. fluorescens* AK102 had isoelectric point of 7.3, 7, 5.97, 5, 4.9 and 4 respectively [168-173]. The isoelectric point of the *P. pseudoalcaligenes* F-111alkaline lipase is 7.3, which is close to that of *P. fragi* (7.0), and much higher than those of the other lipases. From this study, lipase from *P. aeruginosa* BUP2 shows the isoelectric point as 4.3, i.e., very close to extracellular lipase from *P. aeruginosa* SRT 9 with isoelectric point of 4.5 [146]. Lipase from a new strain of *Bacillus* sp. ITP-001 shows isoelectric point as 3.12 [174]. Investigations show that the isoelectric point could be used to determine the pH at which a protein is to be crystallized [175]. The isoelectric point determines a protein's minimum solubility level due to protein–protein interactions being favoured over protein–water interactions [176]. Proteins can become more positively or negatively charged by gaining or losing protons due to

the pH of their environment. The isoelectric point (pI) is the pH at which a protein has a net charge of zero and can be used to determine the specific amino acids in the protein sequence.

4.10. Lipase kinetics

The rate of product formation in enzymatic reactions of the extracellular lipase was determined from Michaelis-Menten plot (**Figure 4.30**) and Line-Weaver Burk plots (**Figure 4.31**). The *Km* (14.2 mM) and *Vmax* (699.7 μmol/min/mg) values of extra cellular purified lipase were found by using *p*NPP as substrate. *Km* value represents the measure of affinity of enzyme towards a substrate. Low *Km* value shows that the enzyme requires only small quantity of substrate to get saturated. High *Vmax* indicates the higher efficiency of the enzyme. i.e., more substrate molecules are converted to product per unit time when the enzyme is fully saturated with the substrate.

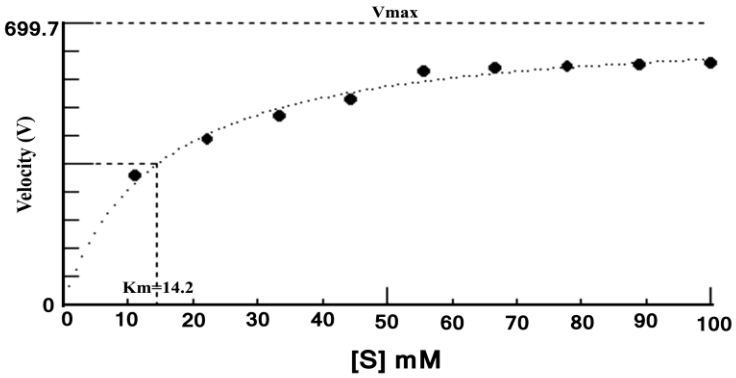

Figure 4.30. Michaelis-Menton plot of purified lipase indicating its kinetic parameters (*Km* 14.2 mM and *Vmax* 699.7 μmol/min/mg).

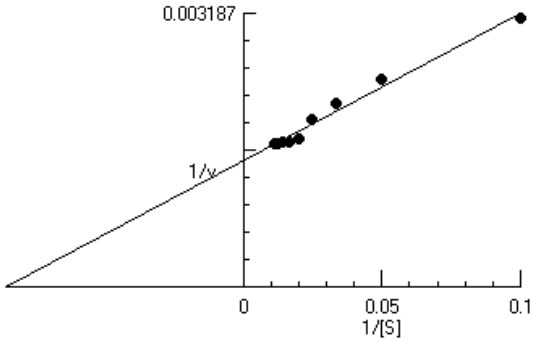

Figure 4.31. Line-Weaver Burk plot for the extracellular lipase produced by *P. aeruginosa* sp. BUP2

In general, the *Km* values of enzymes vary from 10^{-1} to 10^{-5} M [177]. Lipase from *P. aeruginosa* PseA showed a *Km* value of 70.4 mM and *Vmax* of 2.24 μmol/min/mg respectively; lipase from *P. cepacia* showed the *Km* and *Vmax* of 12 mM and 30 μmol/min; in both the studies *p*NPP was used as the substrate [178, 179]. It shows that lipase from *P. aeruginosa* strain BUP2 is more efficient than many other lipases reported from species of *Pseudomonas*.

4.11. Lipase immobilization

Enzymes are easily denatured and inactivated in the presence of organic solvents. Therefore, protein engineering and several physical and chemical methods such as immobilization, modification and entrapment for stabilizing enzymes in the presence of organic solvents have been developed [180]. The basic concept of enzyme immobilization is either to covalently attach or entrap the protein in support materials; this forms a hard external back bone to lipase molecule so that faster reaction rate will occur [181]. Entrapment is recommended by scientists as it is non-toxic, inexpensive, able to retain activity and able to be regenerated [182]. Different matrices, such as agar, alginate and polyacrylamide are employed [183]. Commonly used matrices for entrapment are calcium alignate, and polyacrylamide [184, 185]. The present study screened lipase immobilization on sodium alginate and celite material. However, lipase immobilized through entrapment by celite is more stable than physically adsorbed lipase. Immobilized form of lipase is a suitable catalyst for economic production of biodiesel with enzymatic catalysts because it has higher stability and rate of activity compared to the soluble form.

4.11.1. Immobilization by sodium alginate

In the present study, *P. aeruginosa* BUP2 lipase was immobilized on sodium alginate beads by modified method of Bushan et al. [186]. The lipase produced by *P. aeruginosa* BUP2 has high specific activity, substrate specificity and thermotolerance [187]. In this method purified lipase was mixed and stirred with sodium alginate solution and then extruded drop by drop into cold sterile 0.2 M $CaCl_2$ solution. The hardened beads were washed with 0.2 M Tris-HCl buffer (pH 8.0) to remove excess calcium ions and unentraped materials. The collected beads were preserved in 1% sodium chloride solution in refrigerator.

Effect of alginate, $CaCl_2$ concentrations and enzyme-loading efficiency on immobilization determine the activity of the enzyme. For optimum activity sodium alginate and $CaCl_2$ concentration standardization was done by different concentrations of 1-3% and 0.1 M - 0.3 M respectively. The activity of entrapped lipase was maximum at 0.2M $CaCl_2$ solution and decreased with increase in concentration. The maximum activity was seen in the presence of 1.5% alginate and 0.2M $CaCl_2$ concentration (**Figure 4.32**).

Earlier workers have immobilized lipase from *Candida rugosa* in calcium alginate beads for use in the hydrolysis of oil and grease [188]. Hemachander et al. immobilized lipase in different matrices such as polyacrylamide, agar and alginate for lipase production and found that 4% alginate beads gave an optimal lipase activity of 14 U, compared to free enzyme of 40 U/ml [183]. Similarly, Won et al. reported the effects of immobilization using different ratios, by weight, of enzyme to alginate and bead size for *C. rugosa* lipase entrapment in calcium alginate gel beads [184].

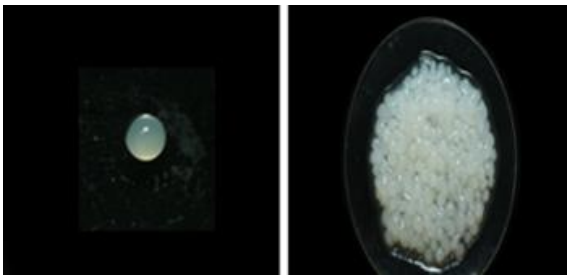

Figure 4.32. Immobilized lipase on sodium alginate.

4.11.2. Immobilization on celite

Pseudomonas lipase was immobilized on filter aid celite powder, by the method of Chang et al. [189]. Freeze dried lipase were stored at 4°C (freezer) for furthure use (**Figure 4.33**). Immobilized lipase-containing filter aid diatomaceous earth (Celite®) supports material; that gave a thin layer covering to the enzyme. Hence, increasing the stability and activity of the enzyme; that would be suitable for production of fatty acid methyl esters. Earlier, Meunier and Legge reported that the immobilization of lipase onto celite by method of entrapment; gave better results in terms of activity, stability and reuse [190]. The immobilized lipase on celite exhibited good hydrolytic activity and more efficient than free enzyme. The

immobilized celite-bound lipase activated much efficiently in water-restricted conditions that resulted in a 2.2-fold increase in the enzyme activity.

Figure 4.33. Immobilized lipase on celite.

4.12. Characterization of immobilized lipase

4.12.1. Fourier transform infrared spectroscopy (FTIR)

FTIR analysis of the samples was performed at the mid-infra-red region of 400–4000/cm (Jasco FTIR 4100 Series, Japan). From spectrum analysis both the samples show same vibrations and bandings (**Figure 4.34**).

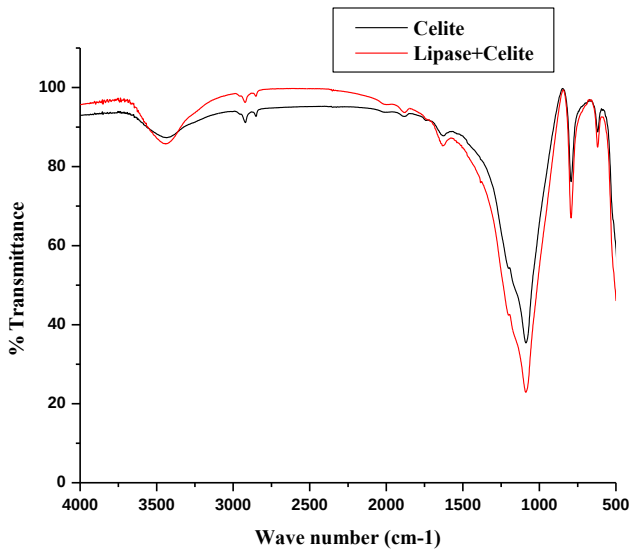

Figure 4.34. FTIR spectrum showing celite *vs* Immobilized lipase on celite

4.12.2. FESEM analysis

Much research has been carried out on the morophological characterization of immobilized materials by field emission scanning electron microscopy (FESEM) analysis. FESEM and transmission electron microscopy (TEM) are used to obtain structural details of the given sample at a resolution of 10^{-9} m. FESEM/SEM are used for collecting structural details at the surface with little information on internal details. SEM is based on collecting a formed image on cathode ray tube by the electron beam reflected from the surface of the given sample. FESEM is an advanced version of SEM technology due to improvisation in secondary electron detector technology, producing sample image at ultrahigh resolution [191]. In the case of enzyme immobilization, SEM is used to observe morphology and distribution of enzyme immobilized onto the matrix while FESEM is used in visualizing morphological details at a particular portion of the matrix. Thus, this study employed FESEM and it helped in giving confirmation of enzyme presence on the given matrix. The SEM images of different adsorption materials exhibited highly diverse shapes and structures such as spherical, ball shaped, porous disc or plate with different dimensions (**Figure 4.35**).

The FESEM images of celite before immobilization and after immobilization are shown in **Figure 4.36**. The SEM image of celite showed a highly porous disc or plate shaped structure on the surface. The presence of a porous structure is suitable for protein attachment and it exposes more surface area. Celite is a good supporting material for lipase because of its high adsorption capacity due to the presence of a large microspore space [192]. Therefore there is no chance of enzyme leaching out and hence it can be employed for reusability studies in transesterification reaction under mild conditions, and could significantly improve the stability of lipase. The immobilized celite materials plus lipase were reported to be effective biological catalysts for the transesterification of oils [193]. SEM micrographs of the celite particles show perfect disc or plate morphology with an approximate size of 969.2 nm. After imobilization, porosity was reduced ranging from 969.2 nm to 118.8 nm. The micrograph clearly reveals

the surface morphology modification due to immobilization, and the pores are well occupied by the enzyme molecules. The enzyme molecules enter deeply into the pores of the celite and are bound to the outer pore surface area which is clearly visible in the picture. The immobilized lipase was consistently more active than the free enzyme and it proved to be stable when subjected to reusability studies.

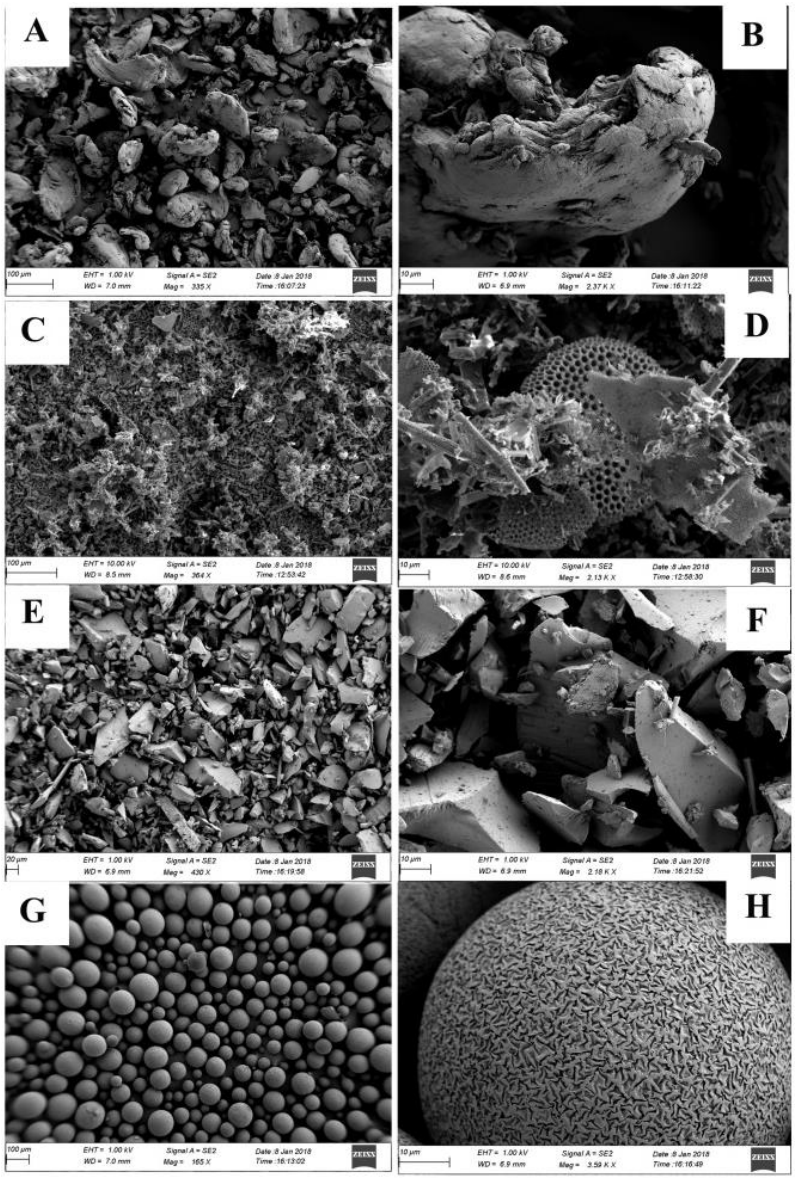

Figure 4.35. FESEM images of different immobilization materials: Agarose (A&B), Celite (C& D), Silica gel (E&F), Sephadex G 100 (G&H).

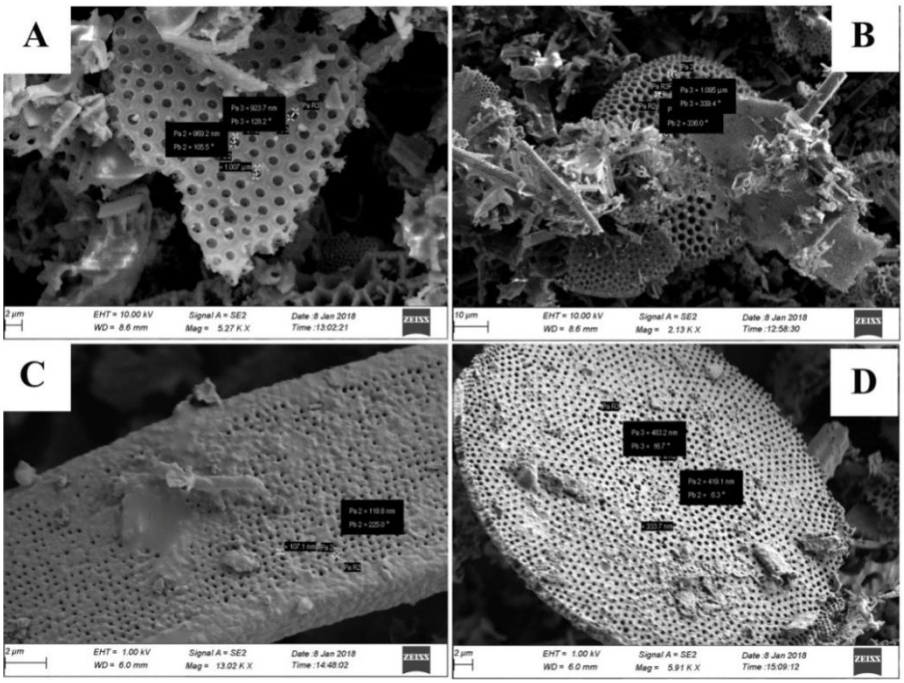

Figure 4.36. FESEM images of celite before immobilization (A&B) after immobilization with *P. aeruginosa* BUP2 lipase (C&D)

4.13. Transesterification of RSO

4.13.1. Transesterification by free lipase and RSM method

The optimization of analytical procedures was carried out by using Minitab software where the chosen multivariate statistic technique is Response Surface Methodology (RSM) in order to determine the values of optimum parameters so that optimum yield can be achieved. RSM was employed to develop a statistical cum mathematical correlation between different independent parameters such as molar ratio of the reactants, reaction temperature, reaction time and catalyst unit on the production of biodiesel. Three main statistical tools i.e., analysis of variance (ANOVA), regression analysis (R^2) and 3D plotting of response variables were performed to optimize the optimum conditions for maximum production of biodiesel. The quadratic regression model of Box–Behnken design (BBD) is based on the uncoded levels of the independent variables. To reduce errors, 27 trials were performed in triplicate and the fatty acid methyl esters (FAMEs) produced was taken as response variable (**Table 4.10**). A second order polynomial equation was fitted to the optimization of biodiesel production, which resulted in the following regression equation:

$$Y = -150.5 + 14.58\,x_1 + 0.3224\,x_2 + 2.859\,x_3 - 6.12\,x_4 - 1.225\,x_1 * x_1 - 0.000307\,x_2 * x_2 - 0.04822\,x_3 * x_3 - 2.692\,x_4 * x_4 + 0.00655\,x_1 * x_2 + 0.0613\,x_1 * x_3 + 0.327\,x_1 * x_4 + 0.000093\,x_2 * x_3 + 0.01813\,x_2 * x_4 + 0.2491\,x_3 * x_4$$

Where, X_1 is RSO to methanol molar ratio, X_2 is lipase unit, X_3 is reaction temperature and X_4 is reaction time.

Table 4.10. Experimental result according to BBD model for the optimization of biodiesel production.

Trial No	Variable levels				Biodiesel production yield	
	RSO to methanol ratio (mol/mol) X_1	Amount of Lipase (U/ml) X_2	Temperature (°C) X_3	Reaction time (h) X_4	Observed	Expected
1	1:9	1000	45	6	74.56 ± 0.23	76.00
2	1:6	500	45	4	68.13 ± 0.11	68.04
3	1:9	750	60	6	85.43 ± 0.16	85.31
4	1:6	750	60	4	73.15 ± 0.54	70.17
5	1:9	750	30	2	81.12 ± 0.61	80.89
6	1:9	500	45	2	78.23 ± 0.24	76.79
7	1:12	1000	45	4	76.12 ± 0.36	75.94
8	1:12	750	45	2	77.28 ± 0.58	77.89
9	1:9	750	45	4	96.46 ± 0.08	97.29
10	1:9	500	30	4	68.24 ± 1.24	66.13
11	1:9	1000	30	4	68.32 ± 1.32	64.25
12	1:9	750	45	4	97.23 ± 0.84	97.29
13	1:9	500	45	6	59.89 ± 0.92	59.04
14	1:6	750	45	2	70.46 ± 1.45	72.72
15	1:9	1000	60	4	66.72 ± 1.23	69.07
16	1:9	750	60	2	72.15 ± 0.68	70.06
17	1:12	750	45	6	84.22 ± 0.54	82.19
18	1:6	1000	45	4	57.46 ± 0.24	57.03
19	1:9	500	60	4	65.24 ± 0.86	69.54
20	1:9	750	45	4	98.18 ± 0.21	97.29
21	1:6	750	45	6	69.56 ± 0.16	69.19
22	1:9	750	30	6	64.51 ± 1.54	66.33
23	1:12	750	30	4	72.16 ± 0.82	75.14
24	1:6	750	30	4	70.00 ± 0.38	71.57
25	1:12	750	60	4	86.34 ± 0.46	84.77
26	1:9	1000	45	2	56.63 ± 0.18	57.48
27	1:12	500	45	4	67.13 ± 0.12	67.29

Analysis of variance (ANOVA) is a method to find out the significance and strength of the experimental result as well as the effects of significant variables and their interaction on the selected responses. The results of ANOVA are tabulated in **Table 4.11**. The quadratic regression model has an F value of 29.68 and p-value is zero, which indicates that the model is significant. The p-value represents the probability of error, and it is used to check the importance of each regression coefficient. The p-value is also pinpointing the interactive effect of each response with independent variables. In general, if the quadratic regression model shows a lack of fit, this indicates that the model does not sufficiently describe the relationship between the independent variables (i.e. methanol-to-oil molar ratio, lipase unit, temperature and time) and the dependent variable. Generally, high R^2 value justifies that there is good correlation between the model and experimental data. Thus, the R^2 value is close to unity (0.9719); which shows that there is good agreement between the model and experimental data. In addition, the coefficient of variation (C.V.) is found to be 0.99, which gives guarantee that the experimental data are accurate as confirmed by Ali et al. 2017 [194].

Table 4.11. Analysis of variance (ANOVA) for Box-Behnken model

Source	**Degree of Freedom**	**Sum of Square**	**Mean Square**	**F Value**	**P Value**
Regression	14	3203.04	228.79	29.68	0.000
Linear	4	302.77	75.69	9.82	0.001
Square	4	2205.13	551.28	71.51	0.000
2 way- Interaction	6	695.13	115.86	15.03	0.000
Residual Error	12	92.52	7.71		
Lack-of-Fit	10	91.03	9.10	12.26	0.078
Pure Error	2	1.48	0.74		
Total	26	3295.55			

Residual is explained as the difference or variation between the experimental and predicted value. Hence, the residuals were normalized and divided with an estimate of their standard deviations, resulting in standardized residuals. Normal distribution versus the standardized residuals obtained from experiments as a straight line were as shown in Figure **4.37.** Moreover, validation

experiments showed a good correlation coefficient of 0.994 and very low P-value, which confirmed that the quadratic polynomial model was highly significant and better correlation occurred between the predicted and observed values of the production of biodiesel. Ali et al. (2015) reported the quadratic response models to be successfully applied for the production of biodiesel by using onsite lipase mediated reaction with central composite design [195]. Similar studies were conducted by Chen et al. (2002) and Shieh et al. (2003) also [196, 197].

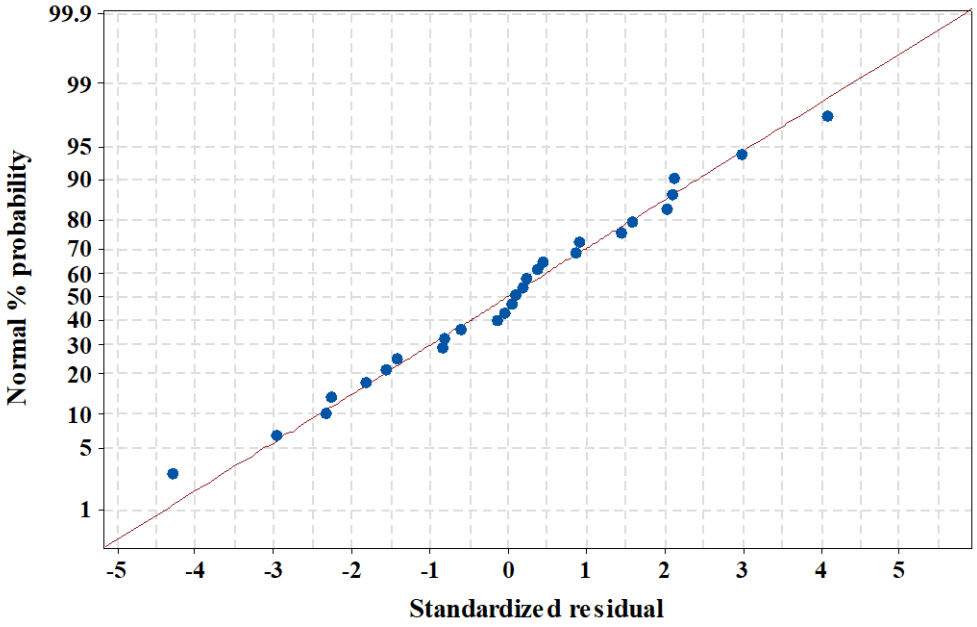

Figure 4.37. Residual plots of BBD design; normal probability plot versus standardized residuals

4.13.2. Effect of parameters

Four variable parameters hold significant impact on the production of biodiesel from rubber seed oil and that encompasses the second-order polynomials. The best way of expressing the effect of all parameters on biodiesel yield within the experimental parameters under investigation is to generate response surface and contour plots of the equation. The 3D response surfaces and 2D contour plots were plotted as shown in **Figure 4.38 & 4.39.** From the shape of contour plots, the significance of the mutual interactions between the independent variables could be estimated. An elliptical profile of the contour plots indicates remarkable interaction between the independent variables. The

responses obtained were of convex nature suggesting that there were well defined optimum operating conditions. Biodiesel yield was sensitive to methanol molar ratio, enzyme concentration, temperature and time.

A reaction with mid range of molar ratio 1:9 (RSO:methanol) and amount of enzyme of 750 U favored maximal yield and conversely it decreased at molar ratio of 12:1 (**Figure 4.38A**). The biodiesel yield was minimal with optimum amount of enzyme and molar ratio; however as the molar ratio was increased while keeping the amount of enzyme at optimum level (750 U), the biodiesel yield reached the maximum value. This indicates the effectiveness of the lipase enzyme being utilized in the minimum amount. Previous studies reported that yield was improved as the amount of enzyme was increased in the reaction system [198-200].

Figure 4.38B shows the interaction of temperature and molar ratio on biodiesel production at the hold values of 750 U of lipase and time of 4 h. The molar ratio of methanol to RSO was one of the most important parameters determining the production of biodiesel. Biodiesel yield initially increased with the increase of methanol-oil molar ratio and further increase in the molar ratio led to a decrease in the yield, in that unresolved methanol inhibited the lipase by destroying the active sites of enzyme [201]. This could be due to the fact that methanol was a denaturing agent of enzymes and insoluble in the oil at high concentration, which made proteins unstable and denatured [202].

Figure 4.39A depicts the response between the amount of enzyme, temperature and trans-esterification reaction percentage yield at constant molar ratio of 9:1 and time of 4 h. Temperature is a key parameter that affects the conformation of lipase and it can initially boost up the yield and then cause a drastic reduction. In the present study, the temperature varied from 30-60°C where the reaction was slow at low temperature. **Figure 4.39B** represents the effect of changing amount of enzyme and time on alcoholysis at 45°C temperature and molar ratio of 9. The highest conversion (99.52%) was obtained at 750 U of enzyme and at 45°C of temperature. The yield is then decreased by the increase in molar ratio of methanol and temperature.

FAME production was more sensitive to both the amount of enzyme and molar ratio compared to temperatures and time. Similar trend was also shown by Azocar et al. [203]. But, the time of 4 h was recorded to give optimum yield near to 80% and at similar temperature range of 45°C as depicted by **Figure 4.39C**. In this study, the maximum yield of 99.52% was obtained at 1:10 molar ratio of oil-to-methanol with the hold values of 45°C of temperature and 4 h of reaction time (**Table 4.12**). The present findings agree with the results obtained by other researchers, who established that at temperature above 50°C, the yield of FAME was decreased due to the deactivation of enzyme at higher temperatures [204-206].

Table 4.12. Experimental trials for the validation of the predicted model

Trials	RSO to methanol ratio (mol/mol)	Lipase (U/ml)	Reaction temperature (° C)	Reaction time (h)	Observed biodiesel (%)	Predicted biodiesel (%)
1	9	800	40	4	96.23	97.36
2	10	750	45	4	99.52	98.12
3	7	900	50	6	78.65	76.32
4	8	600	35	2	83.95	82.31

4.14. Transesterification of RSO by immobilized lipase and Taguchi method

Genichi Taguchi developed a new method to examine the effect of different parameters of a process for conducting factorial experiments. A large number of experimental investigations are required to identify optimum values of experimental parameters for biodiesel production. Every time full factorial investigation is impossible because of cost and time consideration, a number of experimental investigations need to be reduced significantly. Taguchi method is one of the impressive ways of designing an experiment. Orthogonal array is selected based on number of noise factors, interactions between them and number of signal levels [207]. It could be employed to optimize the performance characteristics of process parameters, which is proved to be a powerful tool that differs from traditional full factorial investigation.

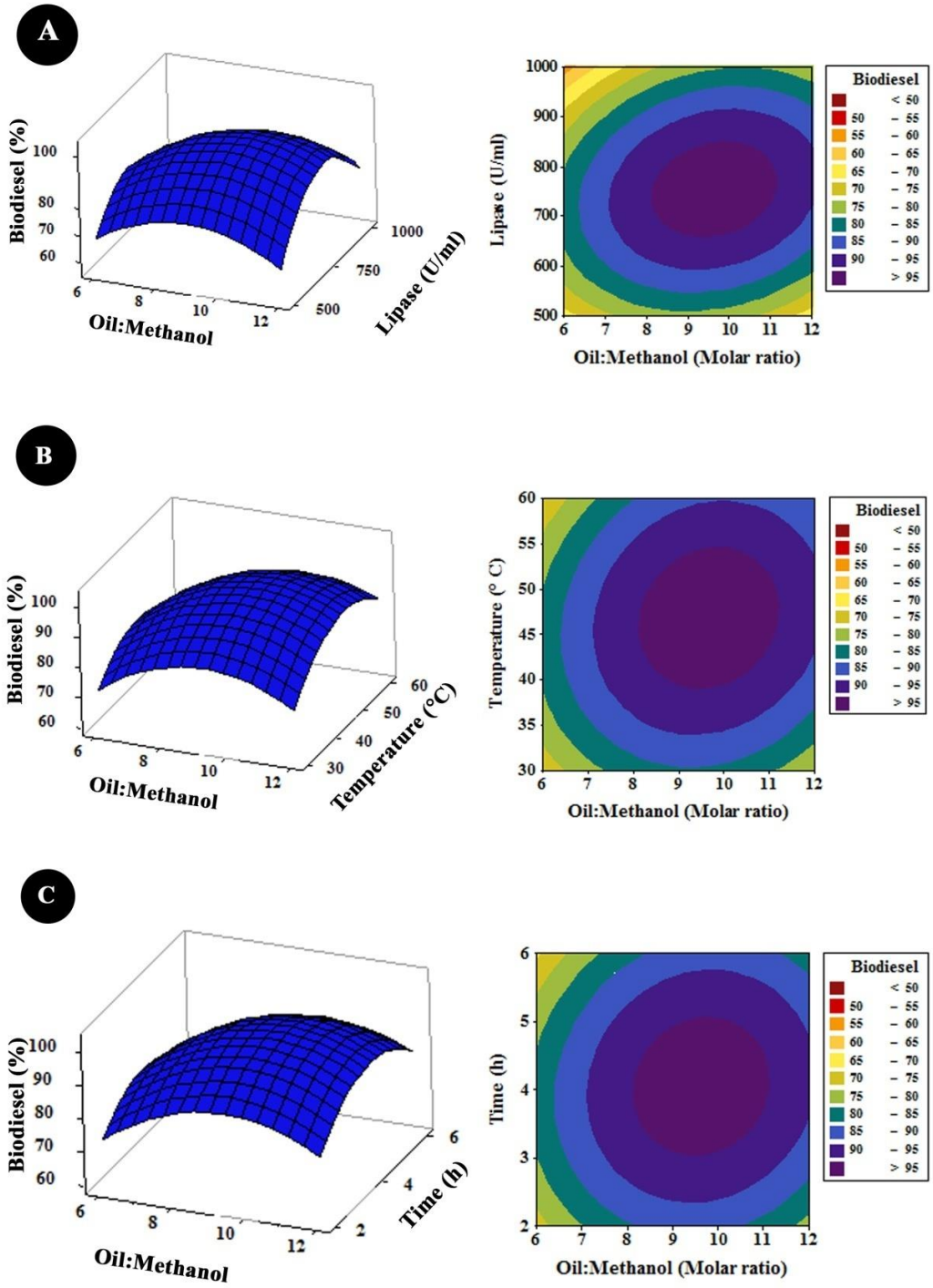

Figure 4.38. Response surface plots (3D) and contour plots (2D) showing the effect of different parameters on production of biodiesel over onsite partial purified lipase from *P. aeruginosa* BUP2. (A; Oil-methanol:lipase; B; Oil-methanol:Temperature; C; Oil-methanol:Time)

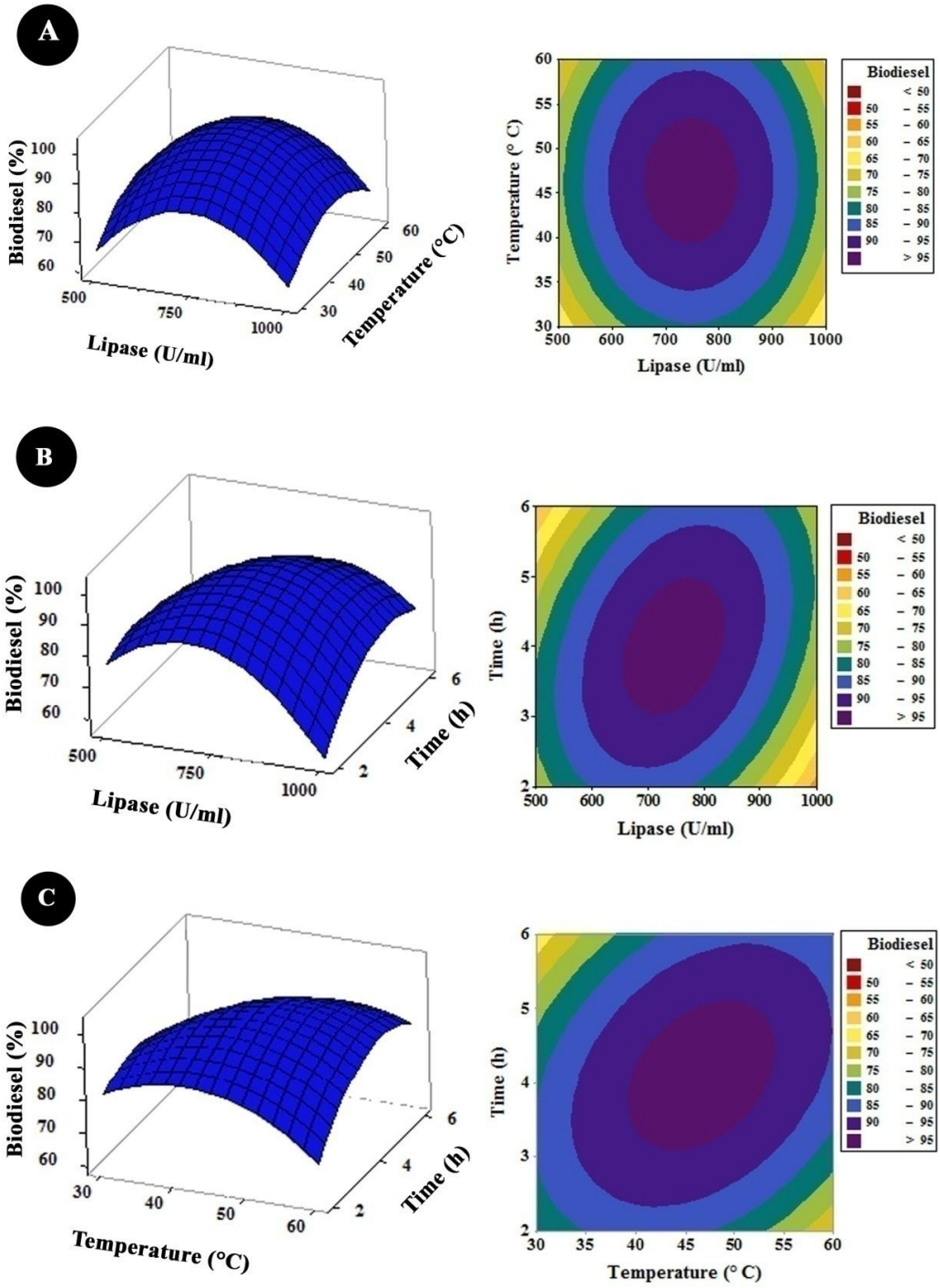

Figure 4.39. Response contour plots (3D) and contour plots (2D) showing the effect of different parameters on production of biodiesel over onsite partial purified lipase from *P. aeruginosa* BUP2. (A; Lipase:Temperature; B; Lipase:Time; C; Temperature:Time)

The approach could economically satisfy the needs of problem solving and design optimization with less number of experiments. Thus, it is possible to reduce time and cost for the experimental investigations [208]. Orthogonal array for DOE with four parameters (factors) at three level (3^4) is examined. Taguchi method is a more refined fractional factorial design of experiments which are based on well defined guidelines. This method uses a special set of arrays of design called orthogonal arrays (OAs). This technique can improve performance of product, process, design, and system with minimal number of experimental time and cost. Taguchi divides the factors into two categories: controllable factors and noise factors. The signal/noise (S/N) ratio provides to precisely measure the quality characteristics of the processing in different levels of processes of both controllable and uncontrollable factors [209]. Among the different parameters influencing the production yield of biodiesel are ratio of alcohol, type of catalyst and its concentration, reaction temperature, time for reaction, agitation or stirring speed, quality of the reactants and moisture content in the oil. Before selecting the orthogonal array, the minimum number of experiments to be conducted as trials and shall be fixed based on the total number of degrees of freedom [210]. Once the independent variables are decided, the number of levels for each variable is decided. The selection of number of levels depends on how the performance parameter is affected due to different level settings. If the performance parameter is a linear function of the independent variable, then the number of level setting shall be two. However, if the independent variable is not linearly related, then one could go for three, four or higher levels depending on whether the relationship is quadratic, cubic or of higher order. If one wants to conduct an experiment to understand the influence of 4 different independent variables with each variable having three levels, then an L9 orthogonal array might be the right choice. The four most influencing parameters such as catalyst concentration, molar ratio of alcohol to oil, reaction temperature, and reaction time and three levels have been considered in this study (**Table 4.13**). Four variables at three levels are used to design the experiment in

MINITAB 15; each experiment has been repeated thrice in order to minimize the errors.

Table 4.13. Experimental result: Taguchi method of biodiesel production

Sl. No	Molar ratio	Catalyst weight (wt%)	Temperature (°C)	Time (min)	FAME (wt%)	S/N Ratio	MEAN
1	12	4	45	90	36.43	27.95	25
2	12	5	55	120	63.17	31.22	36.43
3	12	6	65	150	99.20	36.01	63.17
4	15	4	55	150	72.08	39.93	99.20
5	15	5	65	90	85.89	37.15	72.08
6	15	6	45	120	58.66	38.67	85.89
7	18	4	65	120	82.35	35.36	58.66
8	18	5	45	150	77.17	38.31	82.35
9	18	6	55	90	83.06	37.74	77.17

Kim et al. utilized the taguchi optimization methodology to optimize the control factors such as catalyst concentration, catalyst type, reaction temperature and molar ratio of alcohol to oil, on the transesterification to produce rapeseed methyl ester [211]. Mahamuni and Adewuyi 2010 employed taguchi method to optimize various parameters for the KOH-catalyzed transesterification of soybean oil with methanol [212]. Antolin et al. studied optimization of biodiesel production from sunflower oil employing Taguchi's experimental design [213]. The experiments are conducted as per the level combinations. Since each experiment is the combination of different factor levels, it is essential to segregate the individual effect of independent variables. The mean value of each level of a particular independent variable is calculated as shown in **Figure 4.40** & T**able 4.14.** The sum of square deviation of each mean value of a particular variable indicates whether the performance parameter is sensitive to yield of product. From the above experimental analysis, it is clear that the molar ratio has highly influence on the performance parameter.

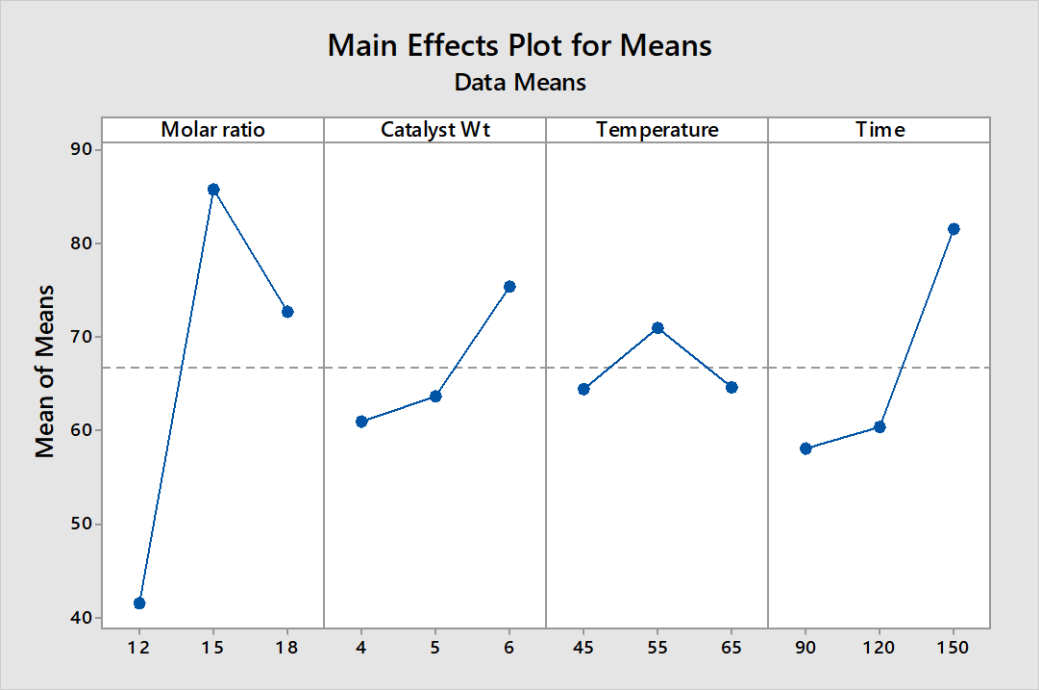

Figure 4.40. The effects of each parameter at different levels on the conversion rate of rubber seed oil to methyl ester

Table 4.14. Response for means by Taguchi

Level	Molar ratio	Time	Catalyst wt	Temperature
1	41.53	58.08	60.95	64.41
2	85.72	60.33	63.62	70.93
3	72.73	81.57	75.41	64.64
Delta	44.19	23.49	14.46	6.52
Rank	1	2	3	4

The main cause of poor yield of biodiesel is the effect of variations in parameters; include temperature or humidity, oil-molar ratio, catalyst concentartion and speed/ agitation. To identify the combined effect of catalyst concentration, molar ratio of alcohol to oil, reaction temperature, and reaction time on yield, the signal-to-noise ratio (S/N ratio) of the Taguchi method is used for data analysis and prediction of optimum parameters. The level having the highest S/N ratio means that it is the optimum level of that parameter. In addition to this, the S/N ratios plays a great role in performing the variance analysis of the conducted experimental studies as well. Usually, there are three categories of the quality characteristics in the analysis of the S/N ratio, i.e., Larger-the-Better

(LTB) for maximization problems, Smaller-the-Better (STB) for minimization problem and Nominal-the-Better (NTB) for normalization problems [207]. In this study, S/N ratio was used to measure the quality characteristics deviating from the desired value. The biodiesel conversion rates are required in high aspect; therefore, in this analysis larger-the-better type of control function was used in calculating the signal-to-noise (S/N) ratio. SNR for each factor level was calculated using the following equation.

$$\eta = -10 \log 10 \sum_{i=1}^{n} \left(\frac{1}{yi}\right) 2$$

Where i is average SNR, *n* is number of experiments conducted at level *i* and y, is measured value.

The S/N ratio (SNR) is to find out the parameter for which noise or variation has a minimal effect on the products. The influence of noise on the production of yield can be found using the ratio; where S is the standard deviation of the performance parameters for each inner array experiment and N is the total number of experiments in the outer orthogonal array. This ratio indicates the functional variation due to noise [214]. The percentage yield of methyl ester from raw RSO under the designed set of nine experiments, their SNRs and overall mean SNR are tabulated in **Table 4.13**. In the present work, the maximization of biodiesel yield is set as objective, hence the larger the better (LTB) SNR model has been used. The results show that the experiment number 3 has the highest FAMEs yield of 99.20% and experiment 1 has the lowest mean yield of 36.43%. Though the set of parameters corresponding to experiment 3 has the highest yield, this would not be the optimum set of parameters. This value implies that the maximum yield of methyl esters of biodiesel production can be achieved when the process parameters are optimized to 1:12 oil-alcohol ratio, 6 wt% of catalyst, 65°C temperature and 150 min reaction time.

Accordingly the optimal level of control or design parameter will be the level with the highest SNR. By using SNR analysis, it is possible to obtain optimum level of each parameter and optimum set of parameters producing the

maximum biodiesel yield; however it is incapable of identifying which factor has influenced the output significantly and how much each factor contributed to the output. The level mean signal to noise ratio (SNR), which is the algebraic mean of all the SNRs of a particular control parameter at a specified level, has been calculated. In this experimental study, SNR has been found to range from 27.95 to 39.93 and so on. The SNRs and rank for all four parameters have been calculated. The rank was given based on the value of DSNR (difference in maximum SNRs to minimum SNRs of particular parameter). Higher DSNR value was assigned rank 1. Based on the rank, the molar ratio of methanol to oil has been identified as the most influencing parameter on the yield of biodiesel. Reaction time and concentration of catalyst are the second and third influencing factors followed by temperature of reaction (**Table 4.15**).

Table 4.15. Response table for Signal to Noise Ratios (Larger is better)

Level	**Molar ratio**	**Time**	**Catalyst wt**	**Temperature**
1	31.73	34.29	34.42	34.98
2	38.59	35.09	35.57	36.30
3	37.14	38.08	37.48	36.18
Delta	6.86	3.80	3.06	1.32
Rank	1	2	3	4

The effects of each parameter at three different levels on biodiesel from rubber seed oil in terms of SNRs are shown in **Figure 4.41.** A higher value of SNRs infers a greater influence of the particular parameter at that level. The maximum value in each graph specifies the optimum level of that particular parameter on the yield of FAMEs. Therefore, the optimum level of each parameter for the maximum yield of biodiesel were Molar ratio of oil to methanol at level 1:12, concentration of catalyst at level 6 wt%, time of reaction at level 150 min and temperature of reaction at level 65°C.

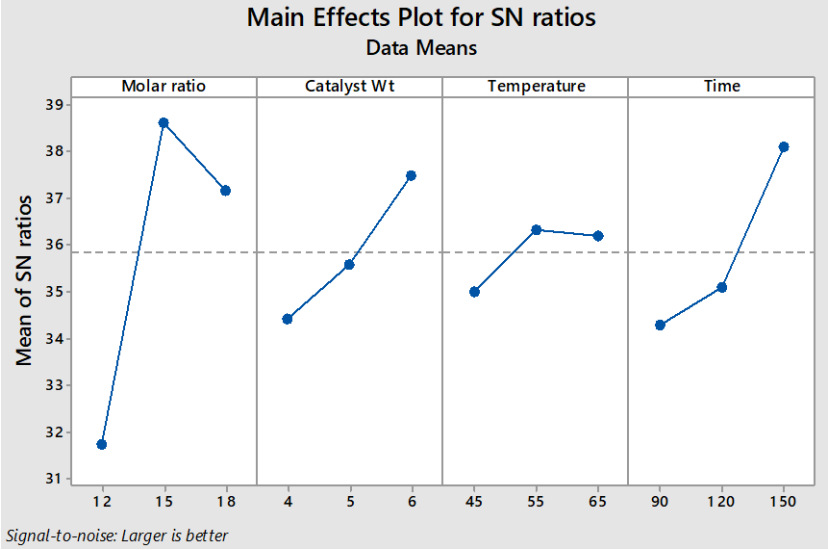

Figure 4.41. The effects of each parameter at different levels on the production of biodiesel

4.14.1. Reusability of immobilized enzyme

Immobilized enzyme is the right choice to overcome the hurdles of enzyme catalyzed transesterification reation. It minimizes the cost, in the sense of reusability of enzyme in industrial scale enzymatic biodiesel production [215]. Stability and reusability of immobilized lipase from *Pseudomonas aeruginosa* sp. BUP2 was investigated in this section. In the present study, the reaction conditions for optimum production of biodiesel by lipase immobilized on celite were determined by Taguchi method. The amount of enzyme immobilized on celite was measured in terms of enzyme activity expressed as percentage residual activity (**Figure 4.42**).

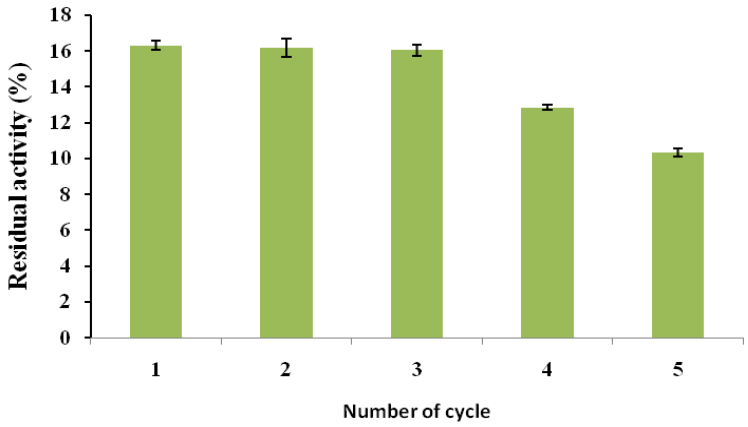

Figure 4.42. Residual activity of lipase after each cycle of reaction.

The reusability of the enzyme was investigated by repeating the transesterification reaction under the optimum condition obtained from the Taguchi method. The enzyme separated from the reaction mixture after the reaction was used and repeated the experiments with the used catalyst till 5 repeated runs. The catalyst could be repeatedly used for three cycles producing fuel grade biodiesel and in the fourth cycle, the FAME content was dropped to 72.08% (**Figure 4.43**) . Thus, the current study indicates that the immobilized lipase can be used for 3 repeated cycles for biodiesel production from rubber seed oil with methanol as an acyl acceptor which will minimize the cost factor in the overall process.

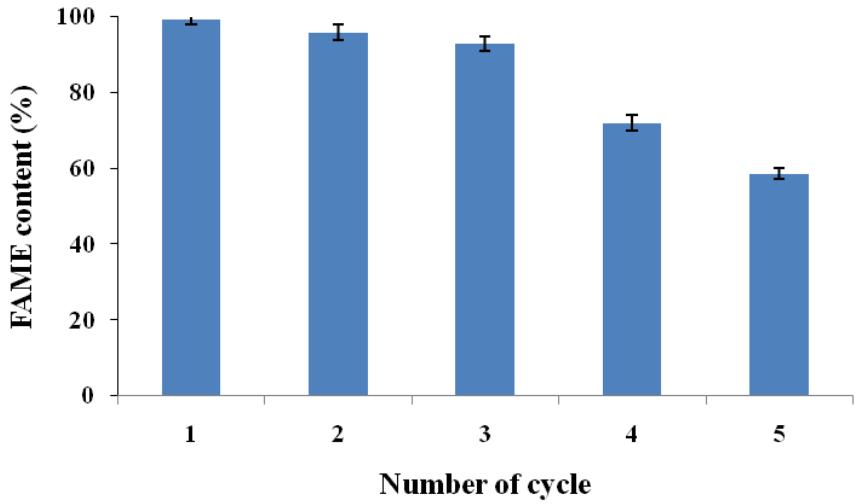

Figure 4.43. Reusability of immobilized lipase results with optimized reaction condition, oil to methanol ratio 1:12; catayst wt% 6; temperature of reaction 65°C and time 150 min.

As previously reported by Du et al. (2005), there was no enzyme loss even after 100 cycles of repeated usage in the presence of methyl acetate [216]. When short chain alcohols like methanol and ethanol were used as acyl acceptor, a large amount of hydrophilic solvents were required for the removal of glycerol from immobilized lipase which was an expensive process and moreover it inhibited lipase activity. Also pH of the reaction mixture is a very influencial parameter for the use of immobilized enzyme. A previous study has seen that immobilized enzyme at pH 8.5 was more stable and could be repetitively used for 5–6 cycles with optimum yield of ester [217]. Similarly, *Chromobacterium*

viscosum lipase was used both as free and immobilized enzyme on Celite-545 for biofuel production from *Jatropha* oil [218]. As reported by Yücel et al. (2014), in a method where the enzyme was immobilized on Celite-545 by adsorption method, the maximum methyl ester yield obtained was 98.3% from transesterification of canola oil [219].

Presently the immobilized lipase after 3rd run was analyzed by FTIR spectral measurements and the FTIR spectra of fresh and the used enzyme are shown in Figure **4.44.** It is clearly observed that the enzyme structure remained unaffected after the repeated catalytic reactions which suggest the stability of the catalyst in the reaction medium.

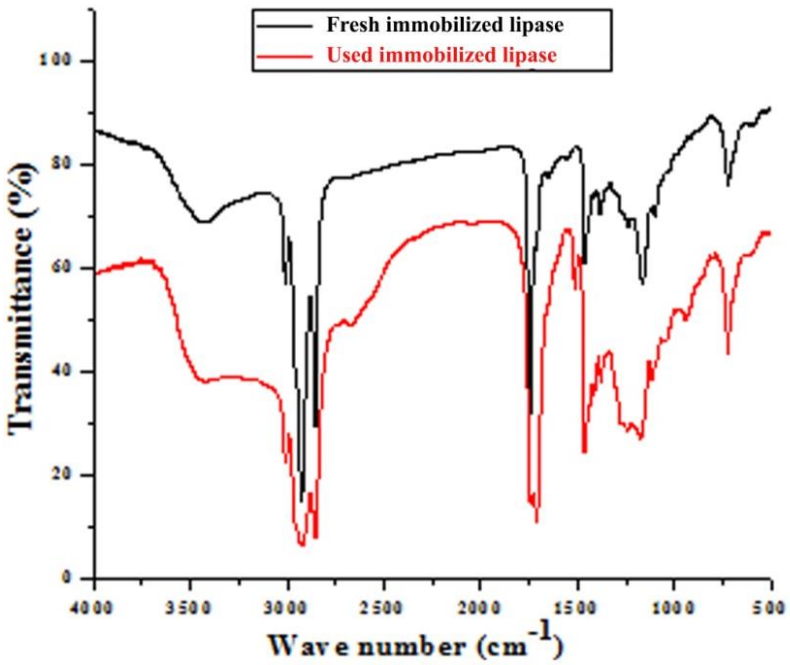

Figure 4.44. FTIR spectra of fresh and used immobilized lipase

Enzyme immobilization represents an interesting tool in enzyme biotechnology that overcomes many of the drawbacks of using soluble enzymes. It exhibits high stability, ease of recovery, enhancement of quality of product, reusability, protection of the purified enzyme from denaturation, etc. Currently, immobilized enzymes are used for many industries such as pharmaceutical, perfumery and synthetic oil production. Therefore, very active enzyme catalysts

can be potentially used in different types of industrial reactors (stirred tank, packed bed, etc.) [220]. The utilization of porous supports confers enzyme immobilization and the hydrolysis of oils by immobilized lipases may be quite useful. Soluble lipases undergo interfacial activation against drops of oils [221]. When using a porous support-immobilized enzyme, it can only act on oil molecules partitioned into the aqueous phase. Previous studies reported that multi-point covalent attachment takes place, rigidifying the enzyme. This reduces spontaneous conformational change and increases the pH stability, thermal stability and reusability of the enzyme [222].

4.15. Analysis of biodiesel

4.15.1. Gas chromatography

Gas chromatography (GC) analysis was employed to determine the conversion of RSO to biodiesel. The standard methyl esters of palmitic, stearic, oleic and linoleic acids were used as standards. Area percentage of RSO to biodiesel was calculated and interpreted with the help of peak integration software GC solution (Shimadzu cooperation).

In GC, the mixture is separated mainly by the boiling point and the structure of the individual compounds. To carry out GC analysis, the sample is usually dissolved in low concentrations in an organic solvent and then injected into the gas chromatograph. In some cases, the sample needs to be derivatized with a specific reagent in order to obtain a useful gas chromatogram. Derivatization with MSTFA (*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide) is required for biodiesel analysis. Glycerol and the mono- and diacylglycerols contain free hydroxyl groups, causing these materials not to perform well in GC. Derivatization improves their performance considerably. Derivatization can provide better resolution between compounds with similar properties. Often standards are used in GC. They are known compounds which will indicate when compounds of a specific nature can be expected to elute. They are therefore very useful in establishing the nature of the compounds in a mixture.

After injection into the gas chromatograph, the sample is separated on a column. A column is a long, thin path (capillary) tube that contains a material with which the sample components interact more or less strongly depending on their structure (polarity) while it is heated. Usually, the solvent used and, if applicable, residual derivatizing agent used for preparing the sample are the first materials to elute from the GC and will be registered by means of a detector. When the detector detects a material eluting from the column at a certain retention time, this will be shown by a peak in the chromatogram. Generally, the integrated value of the peak amplitude over time is proportional to the amount of material causing them. This constitutes the usefulness of GC in quantifying the amounts of components in a mixture. In principle, glycerol and mono-di and triglycerides can be analyzed on highly inert columns coated with polar stationary phases without derivatization. The inertness of the column, required to obtain good peak shapes and satisfactory recovery, cannot be easily maintained in routine analysis. Trimethylsilylation of the free hydroxyl groups of glycerol mono-, di- and triglycerides, however, ensures excellent peak shapes, good recoveries and low detection limits and enormously improves the ruggedness of procedure. For complete silylation of glycerol and partial glyceride, the conditions of the derivatization reaction have to be controlled carefully.

For this study, Shimadzu Gas Chromatography System (2010 Plus) was employed on the basis of standard ASTM D6584 method. The standard methyl esters of palmitic, stearic, oleic and linoleic acids were used as standards and area percentage of RSO to biodiesel was calculated. The chromatograms were drawn and interpreted with the help of peak integration software GC solution (Shimadzu cooperation). GC determines the amount of glycerol (in derivatized form) and mono and diacylglycerols (both also in derivatized form), triacylglycerols and methyl esters in a biodiesel sample. The gas chromatogram depicts that glycerol is the first material to elute, followed sequentially by the methyl esters and the monoacylglycerols, diacylglycerols and triacylglycerols respectively (**Figure 4.45**).

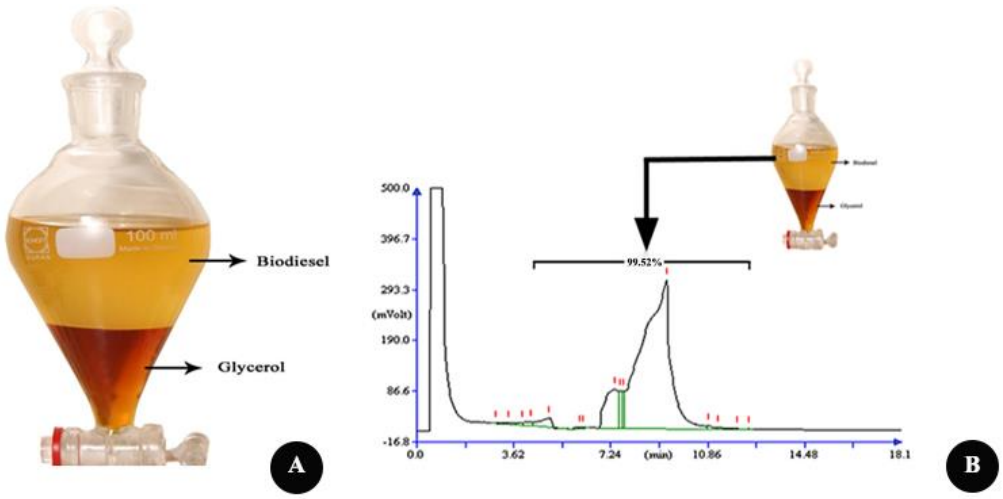

Figure 4.45. Biodiesel by enzymatic trans-esterification. A) Separating funnel with biodiesel sample; B) Gas chromatogram with optimum biodiesel (99.52%) at optimum reaction conditions: RSO to methanol ratio of 1:10 molar ratio, 750 U/ml lipase, 45°C and 4 h

From the above details, rubber seed oil seems to be a promising feedstock for producing biodiesel. The findings are highly important by dint of its low cost and its role in enhancing the economy of rubber farmers. Recently, Amini et al. (2017) demonstrated biodiesel production from *Ocimum basilicum* (sweet basil) seed oil by lipase-catalyzed transesterification [223]. It resulted in 94.58% FAME yield after reaction at 47°C for 68 h in the presence of 6% w/w catalyst and a methanol to oil ratio of 10:1. Charpe and Rathod (2011) compared the transesterification efficiency of lipases from *Aspergillus oryzae*, *P. fluorescence*, *P. cepacea*, and *C. rugosa* using waste frying oil and found that *P. fluorescence* lipase exhibited the highest production of fatty acid methyl esters, 63.8% after optimizing the reaction conditions along with the stepwise addition of methanol [224]. Sebastian et al. (2016) reported comparative studies between chemical and enzymatic transesterification of rubber seed oil for biodiesel production [225]. From this study, they concluded that enzyme catalyzed reaction is superior to chemical method and it has given more pure biodiesel as well as glycerol than that from chemical method.

4.15.2. Effect of acyl acceptor for enzymatic transesterification

For the determination of the best acyl acceptor for biodiesel production, three alcohols were examined. All experiments were conducted at optimum reaction parameters predicted by RSM model. After the reaction, aliquots were collected and GC analyses were done. From the chromatogram, methanol shows high yield with optimum peak (**Figure 4.46**). Alcohol is the inevitable raw material for the production of biodiesel. A number of alcohols have been explored for biodiesel production, the most widely used acyl acceptors are methanol and to a slight extent, ethanol. Other alcohols utilized in producing biodiesel are the short-chain alcohols such as propanol, butanol, isopropanol, tert-butanol, branched alcohols and octanol. However, these alcohols are costly [226]. As earlier mentioned, methanol and ethanol are the most often used alcohols in biodiesel production. Methanol is particularly preferred because of its physical and chemical advantages. Demirbas [227] remarked that methanol, also known as wood alcohol, is usually simpler to find compared to ethanol. Additionally, the short-chain alcohols provide better conversions under the same reaction conditions. On the other hand, long-chain alcohols are usually avoided during transesterification reaction, due to steric hindrance effect.

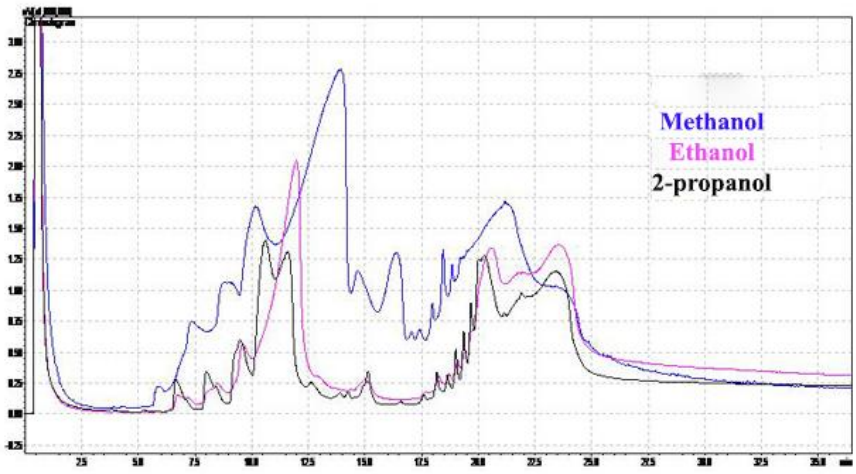

Figure 4.46. Comparison of GC chromatogram of biodiesel product from RSO with various acyl acceptors at optimum reaction conditions; RSO to alcohol ratio 1:10 molar rati), 750 U/ml lipase, 45°C and 4 h

4.16. Characterization of biodiesel

4.16.1. Thin layer chromatography

TLC plate saturated with iodine chamber is an effective tool to identify the appropriate product in transesterification reaction. Spots were corresponding to substrates and products were confirmed with the presence of external standards (Oleic acid, Monolein, Diolein, Triolein and Fatty acid methyl esters) run in parallel and presented in **Figure 4.47**.

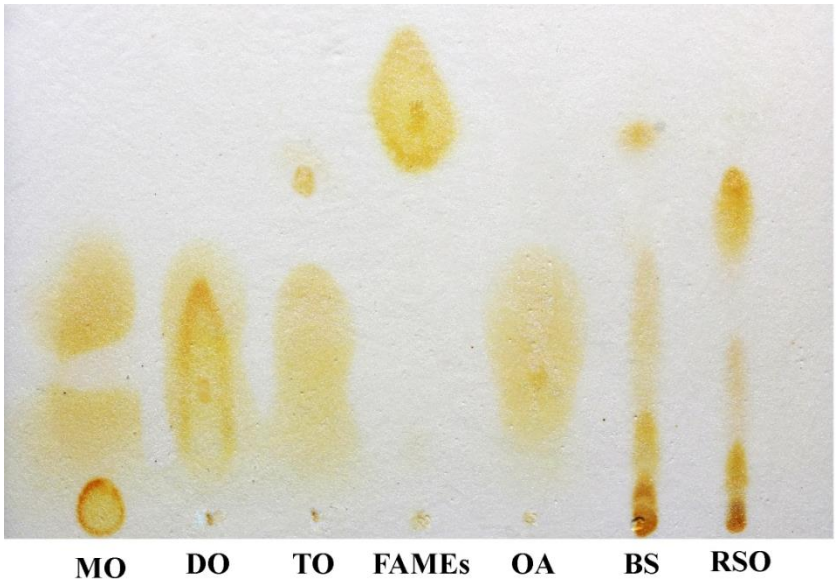

Figure 4.47. TLC plate showing RSO and biodiesel samples with reference standards; MO, Monolein; DO, Diolein; TO, Triolein; FAMEs, Fatty acid methyl esters; OA, oleic acid; BS, Biodiesel, RSO, Rubber seed oil.

4.16.2. FTIR spectroscopy

Fourier-transform infrared spectroscopic (FTIR) analysis can be used to determine the various functional groups present in the sample and it gives high-spectral data over a wide range of absorption. The comparative FTIR spectrum depicts the frequency ranges and their corresponding functional groups and the indicated compounds are represented in **Figure 4.48**. The FTIR spectrum shows definite vibrations of alkanes and poly-aromatic groups and the lack of sulphur. In the biodiesel spectrum a doublet peak in the range of 945.17 cm^{-1} and 1036.56 cm^{-1} due to C-O stretching, is absent for the RSO spectrum. Presence of a broad peak around 945 cm^{-1}, represents the presence of -OH bending for carboxylic dimers in the product, which describes the complete conversion of free fatty acid to its ester. Absorbance in the region 2856-3006 cm^{-1} is due to the stretching of

C-H vibration, and the absorption peak at 1650 cm^{-1} RSO represents C=C stretching vibration. The 1464 cm^{-1} and 1376.91 cm^{-1} absorptions are characteristic of C-H bending vibration for the alkyl groups $–CH_2$- and CH_3- of the hydrocarbon skeleton. The single sharp peak at 720 cm^{-1} due to C-H bending characterizes the presence of C-(CH_2) n-C group. Absence of peaks in the range of 720-945 cm^{-1} and 1750-2800 cm^{-1} confirm the absence of -C-O-O-C stretching and alkynic residues respectively, in the fatty ester composition. From the spectrum, the wide peak of C=O vibration band (in the range 1744−1712 cm^{-1}) of methoxy carbonyl group in biodiesel was observed. Hence, esters were characterized by the strong absorption due to C=O stretching frequency near 1740 cm^{-1} and by the strong absorption involving the stretching of C-O near 1240 cm^{-1} **(Figure 4.48)**. Previous study by Ndana et al. (2013) show the same spectrum of RSO and biodiesel compound produced as methyl ester [228].

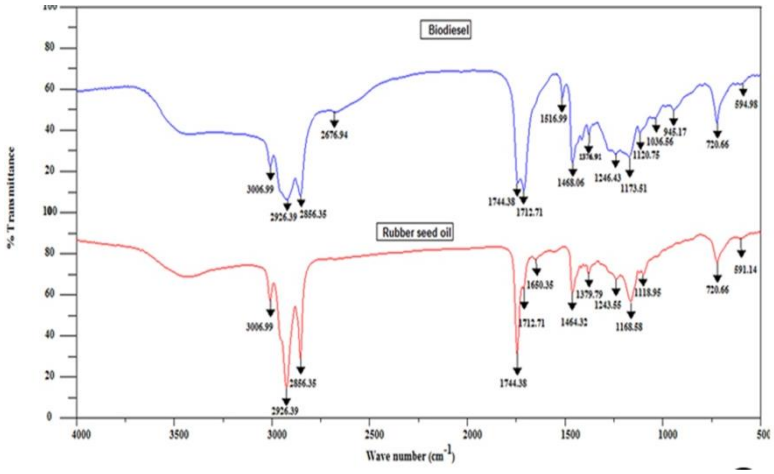

Figure 4.48. FTIR analysis of crude rubber seed oil and biodiesel produced from RSO

4.16.3. Elemental analysis

Elemental analysis is used qualitatively as well as quantitatively for the detection of organic matter based on combustion technique. In this method, sample is burned in an excess of oxygen, and trapped the product as CO_2, H_2O, N_2 and SO_2 or SO_3. From this analysis, the major components of the samples have been found to be carbon, hydrogen and oxygen respectively. The percentages of those components are shown in **Table 4.16**. As compared to petroleum diesel,

biodiesel produces lesser amounts of carbon and unburned hydrocarbons. Absence of sulphur implies that it can be directly used in diesel engines.

Table 4.16. Elemental analysis of rubber seed oil versus biodiesel

Sample	Elemental composition (%)				
	C	H	O*	S	N
Rubber seed oil	78.81	11.89	9.20	0.10	0
Biodiesel	72.67	11.14	16.19	0	0

*calculated by mean difference

4.16.4. GC-MS analysis

Identification of the constituent components of biodiesel by GC-MS, chromatogram and spectra from the analysis GC-MS were compared against a reference standard of the Willey Library. From the analysis chromatogam has known that biodiesel results of the transformation of rubber seed oil have five fatty acid methyl esters. Mainly contained peaks were methyl linoleate, methyl oleate, methyl sterate, methyl palmitate and methyl linolenate (Figure 4.49). These peaks are identified using mass spectroscopy to know the similarities and the fragmentation pattern of the molecular weight of the compounds identified by the fragmentation pattern and molecular weight compounds. The results of the identification of the components making up the biodiesel methyl esters of rubber seed oil transformation results are listed in **Table 4.17**.

Table 4.17. Chemical composition of biodiesel prepared from RSO

Fatty acid methyl ester	Fatty acid type	Molecular formula	Systematic name	Area %
Methyl linoleate	18:2	$C_{19}H_{34}O_2$	9,12- Octadecadienoic acid, methy ester (Z,Z)	33.43
Methyl oleate	18:1	$C_{19}H_{36}O_2$	9- Octadecamonoenoic acid, methyl ester (Z)	29.73
Methyl sterate	18:0	$C_{19}H_{38}O_2$	Octadecanoic acid, methyl ester	8.35
Methyl palmitate	16:0	$C_{17}H_{34}O_2$	Hexadecanoic acid , methyl ester	12.59
methyl linolenate	18:3	$C_{19}H_{32}O_2$	9,12,15-Octadecatrienoic acid, methyl ester (Z,Z,Z)	15.90

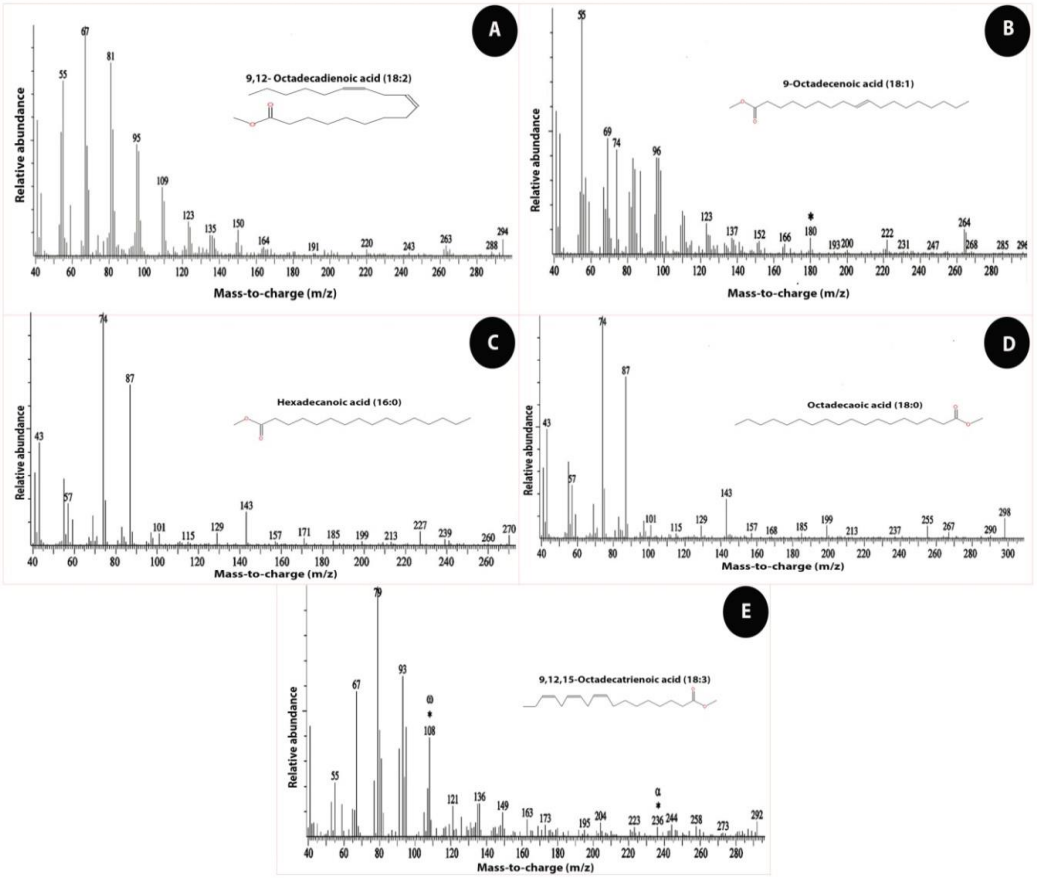

Figure 4.49. Mass spectrum of rubber seed oil. A) linoleic acid methyl ester (33.43%), B) oleic acid methyl ester (29.73%) , C) palmitic acid methyl ester (12.59%), D) stearic acid methyl ester (8.35%) and E) linolenic acid methyl ester (15.90%).

4.17. Fuel properties of biodiesel

The fuel characteristics of the biodiesel produced over onsite lipase under the predicted optimum reaction parameters were determined, and it met the standard specifications of ASTM D 6751 and EN 14214. The results are tabulated (**Table 4.18**). From the results, the ester content was more than 99.52% and the total glycerin was only 0.20%. The FAME content of the biodiesel produced in the lab scale operation indicates the efficiency of the catalyst for the bulk production of biodiesel. Thus, it can be seen that the novel thermotolerant alkaline lipase from *Pseudomonas aeruginosa* BUP2 is found to be a potent candidate for transesterification of RSO and suitable for the industrial scaling up.

Table 4.18. Fuel properties of biodiesel

Properties	**Methods**	**Unit**	**Limits**	**Standards**	**Biodiesel**
Ester content	EN14103	wt.%	96.5 min	EN14214	99.52
Free glycerol	EN14105	wt.%	0.02 max	EN14214	0.00
Monoglyceride	EN 14214	wt.%	0.8	EN14214	0.30
Triglyceride	EN 14214	wt.%	0.20	EN14214	0.18
Total glycerol	EN14105	wt.%	0.25 max	EN14214	0.20
Acid value	EN14104	mg KOH/g	0.50 max	EN14214	0.43
Water content	EN ISO 12937	mg/kg	500 max	EN14214	0.00
Viscosity	D445	mm^2/s	1.96-6.0	ASTMD6751	4.82
Density	EN ISO 3675	g/ m^3	860-900	EN14214	884

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Chapter 5 SUMMARY AND CONCLUSION

Principal Goal

The principal goal of this study was to explore the potential uses of rubber seed with a view to produce industrially significant biomolecules employing economically and environmentally feasible strategies. Rubber seed cake and rubber seed oil are two important products that are obtained from the rubber tree which is usually grown for its latex. The potential use of rubber seed oil in biodiesel production and rubber seed cake for different industrial purposes including lipase production and feed production were the principal goals of investigation in the present study.

Major objectives

1. Primary seed characterization and extraction of rubber seed oil by mechanical / soxhlet extraction method.
2. Investigations on the effect of solvents in the extraction of rubber seed oil.
3. Determination and deactivation of cyanogenic glucoside content of rubber seed.
4. Investigations on physico-chemical properties of rubber seed oil.
5. Evaluation of the antibacterial and antioxidant capacity of RSO.
6. Optimization of production, purification and characterization of lipase from decyanated rubber seed cake by SSF method.
7. Immobilization of lipases on celite.
8. Optimization of transesterification of rubber seed oil into biodiesel using onsite lipases by Box-Behnken Design in Response Surface Methodology.
9. Optimization of transesterification of rubber seed oil into biodiesel using immobilized lipases by Taguchi method.
10. Analysis of fatty acid methyl esters (FAME) by TLC, GC, FTIR spectroscopy, CHNS analysis and GC-MS.
11. Analysis of the properties of the biodiesel produced and comparison with ASTM (American Society for Testing and Materials) and EN (European Norms) standards.

Introduction

Environmental pollution and the deterioration of natural non-renewable resources are the major problems that the world is facing today. To balance the environment for living, the global community is now focusing on natural resources and biomolecules to replace their synthetic counterparts, which are very often harmful to life. Strict Government legislations also add on to the growing demand for industrially significant biomolecules to establish a sustainable environment. Recently a trend has emerged globally in which the need for such biomolecules and bioproducts is often met with by exploiting unutilized, ancillary raw materials. Also, microfloras that live on the globe bring about fruitful changes due to their rapid multiplication, high yield, climate independency and ease of genetic manipulation.

Lipases are triacylglycerol hydrolases, (EC 3.1.1.3) that catalyze the synthesis of esters formed between glycerol and long-chain fatty acids. Biodiesel is an eco-friendly, non-toxic, biodegradable and non-inflammable substitute for petroleum based diesel and chemically these are monoalkyl esters of fatty acids derived from vegetable oils and animal fats.

Commercial biodiesel production is still carried out by chemically catalyzed transesterification process. This process has some bottlenecks such as possibility of operational difficulties like soap formation, washing requirements after the reaction, two step process, high amount of catalyst consumption, difficulty in glycerol recovery and emulsion formation leading to loss of biodiesel yield. It is not suitable for biodiesel production from low cost feedstocks (especially rubber seed oil having high Free Fatty Acid) and requires additional preesterification step with concentrated acids in chemical transesterification. It can be seen that lipase catalyzed biodiesel production process is eco-friendly and cost effective. However, commercial biodiesel production over enzyme route is not well established yet and majority of the research work has been done with commercial lipase. Relatively high cost or extra care required in the enzyme catalyst production process is a problem in the commercial point of view.

Production and extraction of onsite lipase from bacterial strains and production of biodiesel from compounds like rubber seed oil by Solid state fermentation (SSF) or submerged fermentation (SmF) methods have the potential of easy industrial scaling up.

One of the serious issues in biodiesel production is the high cost of the feedstocks. Triglycerides derived from vegetable oils or animal fats are the raw materials for biodiesel production. In the current scenario, vegetable oil price is increasing and it has reached more than double times when compared to conventional diesel fuel, which diminishes the possibility of using biodiesel as a substitute for diesel fuel. Hence, nowadays low cost feedstocks such as rubber seed oil (RSO) are getting more attention. Use of RSO for biodiesel production is beneficial to rubber farmers since it would provide an additional source of income to farmers thus boosting the economy of rubber plantation sector. Over past few years, Indian rubber farmers are facing very critical situations caused by high cost of production, low productivity, land-related issues and dynamic pricing. Rubber seeds are available in abundance, oil from the waste seeds is an ancillary product and it possesses several potential industrial applications in cosmetic, paint and oleochemical sectors. Based on the foresaid background, the present study focused on the extraction and characterization of rubber seed oil, production, optimization and characterization of lipase using rubber seed cake as substrate and biodiesel production from rubber seed oil. The summary of the major experiments carried out and the major findings emerged out of this study are presented below.

Primary seed characterization and extraction of rubber seed oil by mechanical / soxhlet extraction method

The main goal of this study was to carry out preliminary characterization of rubber seeds and assessment of oil extraction from rubber seeds. Fresh rubber seeds of RRII 105 clone were collected from the rubber plantation of University of Calicut, Kerala, India (11.1340 °N, 75.8952 °E). Initially, collected rubber seeds were cleaned, dried and decorticated. The dried kernels were milled using laboratory blender. The proximate composition of rubber seed like moisture

content, fat, total lipid, protein, total sugar and aminoacid and presence of phenolics was evaluated. Moisture content of rubber seed has been found to be between 2.5% to 3.45% after oven drying. Rubber seed kernel contains 36.43% fat, 24.61% lipid, 18.65% protein, 13.9% carbohydrate, 1.43% amino acid, 0.19% phenolic content and 4.79% others. Elemental analysis of rubber seed powder was carried out using elemental analyzer. From the analysis, rubber seed powder (RSP) was found to contain high percentage of carbon (54.96%), hydrogen (8.24%), nitrogen (1.39%) and sulphur (0.51%). In this study, RSO was extracted by mini handed oil expeller and soxhlet extraction. In the oil extraction process using mini handed oil expeller, around 18% yield was obtained. Soxhelt extraction method gave optimum yield of 39.45%.

Investigations on the effect of solvents used for the extraction of rubber seed oil

Effect of solvents on oil yield was examined with four different solvents: n-hexane, petroleum ether, ethyl acetate and methanol. The non-polar solvent hexane was found to give maximum oil yield (39.45%) while the lowest value (26.85%) was obtained with the polar solvent methanol. Petroleum ether (34.05%) and ethyl acetate (30.52%) showed second place and third place respectively. The results show that non-polar oil of rubber seed is easier to be extracted by n-hexane. Currently, n-hexane is extensively used for commodity vegetable oil extraction due to its extraction efficiency and ease of availability. The oils from n-hexane and petroleum ether extractions were golden yellowish, with the original odour of the rubber seed. This is because both the solvents have low polarity and are miscible with the oil. On the other hand, white particles were found extracted together with the oil when using polar solvents like ethyl acetate and methanol. The nature of the extracted lipid is highly dependent on the solvent's polarity, which will determine the selectivity of the solvent used. From the soxhlet extract, the oil was recovered by using rotary evaporator and the same was stored at 4°C for further use.

Determination and deactivation of cyanogenic glucoside content of rubber seed

Presence of cyanide moieties is an important problem associated with many plant products including rubber seeds. Cyanide is present in the form of a chemical known as linamarin in rubber seeds and several other crop plants like cassava. Picrate paper analysis was performed to detect the presence of cyanide content presently. This is the most reliable and sensitive method and easy to use in the determination of cyanide presence in rubber seed samples. Presence of cyanide is indicated by a colour change from yellow to orange and to brown. When the sample was heated for 70 ^{o}C for 90 minutes and then subjected to picric paper analysis, there was no brown colouration showing that linamarin was getting considerably reduced when heated.

Investigations on the physico-chemical properties of rubber seed oil

International Standard of American Society for Testing and Materials (ASTM) method were performed to determine the physico-chemical properties of rubber seed oil. RSO was characterized in terms of moisture content, pH, acid value, free fatty acid, iodine value, saponification value, density and viscosity. RSO was seen with golden yellowish colour and had a pleasant nut-like odour. It contained moisture of 6.5%, had an acid value of 45.32 mg KOH/g oil and FFA content of 23.66. Acid value of RSO was compared with different vegetable oils such as rice bran oil, palm oil, sunflower oil, olive oil and groundnut oil. The high content of unsaturated fatty acids (79.06 %) and moderately low saturated fatty acids (20.94 %) adversely affect the shelf life of the oil compared to other oils. Viscosity of RSO at 30°C was measured by Ostwald's viscometer and it was found to be 5.06 mm^2/s; that was higher than the viscosity of commercial petroleum-diesel (< 4 mm^2/s). Density of RSO was found to be 874 kg/m^3, which was nearly close to jatropha oil (i.e., 873 kg/m^3) and waste cooking oil (i.e., 868 kg/m^3). The peroxide value and saponification value of RSO obtained was 3.62 mg/g and 183.32 mg KOH/g respectively. The iodine value of RSO was 138 g I_2/g as estimated by Wij's method. Elemental analysis has also been carried out

using CHNS/O analyzer. From the analysis, the elemental components of RSO were found to be carbon (78.81%); hydrogen (11.89%), oxygen (9.20%) and sulphur (0.10%). Structural characterization of the extracted RSO was carried out by FTIR spectroscopy. The major peak at 1746 cm^{-1} was found to be contributed by the C=O group due to the functional ester group, and the vibration at 1639 cm^{-1} indicated the presence of COO– in the sample. In addition, fatty acid profiling of RSO was performed by GC-MS analysis and spectrum analysis was done using Wiley 7n.1 database. The result showed the presence of 79.06% of unsaturated fatty acids and 20.94% of saturated fatty acids, which comprised of linoleic acid (33.43%), oleic acid (29.73%), linolenic acid (15.90%), palmitic acid (12.59%) and stearic acid (8.35%). The higher percent of carbon and hydrogen indicates that RSO is a good source of carbohydrates and hydrocarbons.

Evaluation of the antioxidant capacity of RSO

Antibacterial and antioxidant capacity, ferrous reducing power and lipid peroxidation inhibition of RSO were examined. When the antimicrobial activity of RSO was measured in terms of zone of inhibition, the highest inhibitory zone (21 mm) was observed in the case of *Escherichia coli* at 30 µl of volume, whereas the lowest inhibitory zone (12 mm) was found with *Staphylococcus aureus* at 10 µl of volume. Being a simple inexpensive method, DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging assay was used for antioxidant studies. The seed extracts in various solvents like petroleum ether, ethyl acetate and ethanol were subjected to antioxidant assays, *viz*., ferrous reducing power assay and lipid peroxidation inhibition ability, which revealed significant activity and the maximum result, was shown by the ethanol extract.

Optimization of production, purification and characterization of lipase from decyanated rubber seed cake by SSF method

Nowadays, increase in demand for cost effective methods to enhance the production of industrially significant biomolecules, especially microbial enzymes has been observed. Solid State Fermentation (SSF) is a much relevant, cheap and

ecofriendly fermentation strategy compared to Submerged Fermentation (SmF). Microbial lipases are versatile in nature and they have great applications in industries such as food, diary, paper, pulp, cosmetics, detergent, pharmaceutical, etc. Two new bacterial strains, *Pseudomonas aeruginosa* BUP2 (MTCC No.5924) & *Pseudomonas* sp. BUP6 (MTCC No.5925), were employed in this work. The culture was maintained in a newly designed BUP medium and BSM respectively. Here, de-oiled flours of rubber seed, coconut and cotton seed were used as solid substrate for the production of lipase by strains of *P. aeruginosa* BUP2 & *Pseudomonas* sp. BUP6. Fifty percentage of the flours (w/v) were supplemented with basal salt medium (pH 6.8), and incubated at 37°C for 24 h to 120 h. Lipase production was quantitatively assayed at 24 h intervals. Of these, rubber seed flour supported the maximum lipase production, i.e., 871 U/gds at 48 h of incubation and 33.98 U/gds at 24 h of incubation by *P. aeruginosa* BUP2 and *Pseudomonas* sp. BUP6 respectively.

The extracellular lipase was purified to homogeneity employing $(NH4)_2SO_4$ salt precipitation and Superose^{TM} 6 column chromatography. The purified lipase showed a prominent band of 29 kDa on SDS-PAGE. The effect of the enzyme activity against various organic solvents like hexane, petroleum ether, chloroform, toluene, acetonitrile, methanol, ethanol and acetone were studied in different ratios (1:10 and 1:1 v/v). Interestingly, the non-polar solvents like hexane, petroleum ether, chloroform and toluene showed strong stimulatory effect on enzyme; whereas the exposure to polar solvents like acetonitrile, methanol, ethanol and acetone showed a drastic reduction in lipase activity. From this study, maximum lipase activity was observed in 1:1 ratio of toluene incubated for 24 h; whereas, the maximum lipase activity loss was observed at 1:1 ratio in acetone at incubation periods of 24 and 18 h respectively. The positional specificity of lipase was examined by the hydrolysis products of triolein by thin layer chromatography. It is concluded that *P. aeruginosa* BUP2 lipase is very robust and that acts at random on the triacylglyceride molecule and results in the complete breakdown of triacylglyceride to fatty acid and glycerol. Doubling dilution method was followed for determination of isoelectric point. As

a result, the lipase precipitated to varying degrees so that precipitation was the greatest in the buffer with pH 4.3, closest to the isoelectric point of lipase. Using *para*-nitrophenyl palmitate as substrate, the *Km* (14.2 mM) and *Vmax* (699.7 μmol/min/mg) of the purified lipase were also determined.

Immobilization of lipases onto celite

Immobilization was done with sodium alginate and celite as substrate. The immobilized lipase on celite exhibited good hydrolytic activity and was more efficient than free enzyme. Immobilized celite-bound lipase activated much efficiently in water-restricted conditions that resulted in a 2.2-fold increase in enzyme activity. FESEM is employed for visualizing the morphological details and helps in giving confirmation of enzyme presence on the given matrix. SEM micrographs of the celite particles showed perfect disc or plate morphology with an approximate size of 969.2 nm. After immobilization, porosity was reduced ranging from 969.2 nm to 118.8 nm. The micrograph clearly revealed the surface morphology modification due to immobilization, and the pores were well occupied by the enzyme molecules. The results suggest that these biocatalysts could be used for a broad range of applications in different industries including food and beverages, pulp and paper, detergents and textiles, pharmaceutical and chemical, among others.

Optimization of transesterification of rubber seed oil into biodiesel using onsite lipases by Box-Behnken Design in Response Surface Methodology

Box-Behnken Design coupled with Response Surface Methodology method was employed to optimize the process parameters required for the production of biodiesel from RSO over free lipase as catalyst. Response surface methodology (RSM) was employed to correlate between different independent parameters such as molar ratio of the reactants, reaction temperature, reaction time and catalyst unit on the production of biodiesel. From the quadratic regression model, 27 trials were performed in triplicate and the fatty acid methyl ester (FAME) produced was taken as response variable. Three main statistical tools, i.e.; analysis of variance (ANOVA), regression analysis (R^2) and 3D

plotting of response variables were performed to optimize the optimum conditions for maximum production of biodiesel. The R^2 value was close to unity (0.9719) and the coefficient of variation (CV) was found to be 0.99, which showed that there was good agreement between the model and experimental data. The statistical studies confirmed the significance of the model in the optimization of biodiesel production. Biodiesel yield of 99.52% was obtained in the validation experiments at the optimal level of lipase (750 U), methanol ratio (1:10), temperature (45°C) and time (4 h).

Optimization of transesterification of rubber seed oil into biodiesel using immobilized lipase by Taguchi method

This part describes the transesterification of RSO by immobilized lipase and Taguchi method. By using Taguchi method, orthogonal array of DOE with four parameters (factors) at three levels (3^4) is examined; the number of experiments is reduced to nine experiments. The four most influencing parameters such as catalyst concentration, molar ratio of alcohol to oil, reaction temperature and reaction time and three levels have been considered for this study. To identify the combined effect of catalyst concentration, molar ratio of alcohol to oil, reaction temperature and reaction time on yield, the signal/noise ratios (S/N ratios) were determined. From the analysis, the highest FAME yield was 99.20% when the process parameters were optimized to 1:12 oil-alcohol ratio, 6 wt% of catalyst, 65°C temperature and 150 min reaction time. Reusability studies of the catalyst were carried out to assess the stability in the reaction. The reusability of the enzyme was investigated by repeating the transesterification reaction under the optimum condition obtained from the Taguchi method. The enzyme separated from the reaction mixture after the reaction was used and repeated the experiments with the used catalyst till 5 repeated runs. The catalyst could be repeatedly used for three cycles producing fuel grade biodiesel and in the fourth cycle, the FAME content was dropped to 72.08%. Catalyst novality, onsite production of immobilized lipase and statistical optimization are the major

attractions of the present study. To the best of our knowledge, onsite purified lipase was used for biodiesel production for the first time.

Analysis of fatty acid methyl ester (FAME) by TLC, GC, FTIR spectroscopy, CHNS analysis and GC-MS

Biodiesel aliquots were characterized using thin layer chromatography, gas chromatography, fourier transform infra-red spectroscopy, elemental analysis and GC-MS analysis. In TLC plate, spots were corresponding to substrates and products were confirmed with the presence of external standards (Oleic acid, Monolein, Diolein, Triolein and Fatty acid methyl esters). Gas chromatography was employed to determine the conversion of RSO to biodiesel. The standard methyl esters of palmitic, stearic, oleic and linoleic acids were used as standards. Area percentage of RSO to biodiesel was calculated and interpreted with the help of peak integration software GC solution. The gas chromatogram depicted that glycerol was the first material to elute, followed sequentially by the methyl esters and the monoacylglycerols, diacylglycerols and triacylglycerols respectively. The FTIR spectrum showed definite vibrations of alkanes and poly-aromatic groups and the lack of sulphur. Elemental analysis of the biodiesel was also carried out and the results were promising. From this analysis, the major components of the samples have been found to be carbon, hydrogen and oxygen respectively. GC-MS analysis chromatogam has depicted five fatty acid methyl esters. Mainly contained peaks were methyl linoleate, methyl oleate, methyl sterate, methyl palmitate and methyl linolenate.

Property analysis of the biodiesel produced and comparison with ASTM (American Society for Testing and Materials) and EN (European Norms) standards

The fuel properties of the biodiesel obtained from rubber seed oil met the specifications as mentioned in ASTM D6751 and EN 14214 standards. The ester content was more than 99.52% and the total glycerin was only 0.20%. The

FAME content of the biodiesel produced in the lab scale operation indicated the efficiency of the catalyst for the bulk production of biodiesel. Thus, the present study brings out a cost effective method of fuel production from raw feedstocks, which can to a great extent cut short the pressure on conventional fossil fuels.

Conclusion

The key message behind the study is that understanding nature opens new vistas to exploit its wealth in future. Moreover, industrial effluents like oil residues provide natural ecosystems for microbial cultures, and represent a virtually untapped resource of novel products. Even though the production of biomolecules such as biodiesel have been studied in the last decades, their production on large scale met reality only in a very few cases, and even now it has found only limited applications owing to the remarkably high costs involved. The present study reveals an interesting option for the future that could be used to decrease production costs.

In the present investigations, we have produced onsite lipase and its immobilized forms have highly active, reusable and eco-friendly biocatalytic function that can be used to produce biodiesel from unutilized rubber seed oil. The extracted lipase has been characterized by different analytical techniques for investigating the structure, morphology as well as the properties and active phases that are responsible for catalysis. Transesterification reaction was performed for the production of biodiesel from non-edible rubber seed oil. The reaction parameters were optimized with the help of statistical tools. The fuel properties of the prepared biodiesel samples were studied and those were in the range of the values proposed by ASTM and EN international standards. The present study has suggested the suitability of its use as a fuel substitute for petro-diesel.

Major outcomes/Deliverables

- Characterization of seeds and extraction of oil from rubber seeds were attempted.
- The non-polar solvent n-hexane was found to give maximum oil yield (39.45%).
- Presence of traces of cyanogenic glucosides was noticed in fresh seeds, which was removed by heat treatment. This was confirmed by picrate paper analysis.
- Different oil cakes were screened for lipase production by SSF method. Rubber seed flour showed maximum lipase production.
- Checked the efficiency of immobilization of lipase on celite.
- This is the first ever report on the production of biodiesel by onsite lipase from rubber seed oil.

Leads for further study

- To increase kinetic resolution of lipase by enzyme engineering.
- Pilot scale production of biodiesel over low cost feedstocks such as algal oils as well as animal fats.
- Efficiency & engine performance studies with a diesel engine.

GRAPHICAL ABSTRACT