

CRANFIELD UNIVERSITY

LAWAL HANO JIBRIL

SUSTAINABLE PRODUCTION OF BIOPLASTICS FROM
VARIOUS LIPID-RICH OIL WASTES USING SELECTED
PSEUDOMONAS STRAINS.

SCHOOL OF WATER, ENERGY, AND ENVIRONMENT
ENERGY AND POWER

PhD

Academic Year: 2022 – 2023

Supervisor: DR STUART WAGLAND

Associate Supervisor: PROFESSOR ANA SOARES

FEBRUARY 2023

CRANFIELD UNIVERSITY

SCHOOL OF WATER, ENERGY, AND ENVIRONMENT
ENERGY AND POWER

PhD

Academic Year 2022 - 2023

LAWAL HANO JIBRIL

SUSTAINABLE PRODUCTION OF BIOPLASTICS FROM
VARIOUS LIPID-RICH CARBON WASTES USING SELECTED
PSEUDOMONAS STRAINS.

Supervisor: DR STUART WAGLAND
Associate Supervisor: PROFESSOR ANA SOARES
FEBRUARY 2023

This thesis is submitted in partial fulfilment of the requirements for
the degree of PhD.

© Cranfield University 2023. All rights reserved. No part of this
publication may be reproduced without the written permission of the
copyright owner.

ABSTRACT

*Bioplastics, also known as polyhydroxyalkanoates (PHAs), are produced by bacteria through aerobic processes using a variety of carbon sources, showing significant potential as a polymer. Despite efforts to produce cost-effective processes, the production of PHA remains expensive, posing challenges in its utilization as a cost-effective material for products. This study aims to demonstrate the potential of these microorganisms as a sustainable solution for producing bioplastics from renewable sources. The suitability of two selected *Pseudomonas oleovorans* (DSMZ1045 and ATCC29347) was investigated to accumulate bioplastic polyhydroxyalkanoates (PHAs) from lipid-rich wastes: Glycerol, fats, oils, and greases (FOGs), soybean oil (SO), and palm oil (PO) were used as carbon sources. Under the conditions of 180 rpm and temperature of 30°C for 48 hours, using 2% (v/w) of each oil. The accumulated PHA was extracted using sodium hydroxide and enzyme-hydrogen peroxide-based methods. The polymers were qualitatively analysed using Fourier transforms infrared (FTIR) and Scanning electron microscopy (SEM). FTIR and SEM analyses revealed that the produced bioplastic polymers are MCL-PHAs types. However, the findings showed that these lipid-rich wastes are efficient carbon feedstocks used in producing MCL-PHAs by the selected *Pseudomonas* strains. The yield accumulated by *P. oleovorans* DSMZ1045 from these waste resources was 43wt%, 39wt%, 34wt%, and 32wt% of dry cell weight, while *P. oleovorans* ATCC29347 was 38%, 37%, 34%, and 33% of cell dry weight, respectively, suggesting the potential for an effective solution for producing bioplastics. The results indicated substantial improvements in PHA accumulation for both strains when pretreatment methods (lipase, microwave, and ultrasound-assisted) were employed. % PHA yield between pretreatment and non-pretreatment conditions with DSMZ1045 and ATCC29347 showing enhancement of 46.88%, 41.18%, 41.03%, and 23.26% of cell dry weight and 40.63%, 38.46%, 26.47%, and 18.60% of cell dry weight, respectively. Glycerol and FOG gave a higher PHA accumulation than the other carbon sources. More polymer yields were achieved using both oil samples from *P.**

oleovorans DSMZ1045 compared to Pseudomonas oleovorans ATCC29347. This research underscores the viability of P. oleovorans DSMZ1045 in producing bioplastics from waste oils, especially those derived from fats, oils, and grease (FOG)

Keywords: Fourier Transform Infrared Spectroscopy, Scanning Electron Microscope, Sodium hydroxide, Hydrogen peroxide, Enzyme.

ACKNOWLEDGEMENTS

All accolades are due to Allah, the Most Gracious and the Most Merciful, for granting this thesis success and providing the strength to do so. I am deeply grateful to my supervisors: Dr. Stuart Wagland, and Professor Ana Soares, for their unwavering support, mentorship, encouragement, and guidance throughout my academic journey. This thesis would not have been possible without their expertise, patience, and dedication. I am honoured to have worked with such a talented and inspiring group. Furthermore, I want to convey my sincere gratitude to Professor Frederic Coulon and Dr. Monica Rivas Casado, who made up my research review team for their tremendous knowledge, suggestions, and support throughout my research review defence meeting. I would also like to thank Professor Sandra Esteves, Professor Jerry Knox, and Dr Yadira Bajon Fernandez for reading my thesis and making my defence fantastic. Also, I would like to thank Diane Johnson, Paul Barton, Leita Rodrigo, Tammy Cowley, and Richard Andrews, who generously devoted their time and expertise during my laboratory work. Their expertise and invaluable insights have helped shape this thesis into what it is today.

Furthermore, I want to extend my heartfelt thanks to my family, especially my LATE Father and Mother. May they rest in peace, AMEEN, my brothers and sisters, my wife, and our kids (Ahmad, Aishat, Yusuf, Fateema, and Ameena), for their emotional support and encouragement during the long and challenging journey of writing this thesis. Their love and belief in me gave me the strength to keep going when it got tough. In addition, thanks go to my colleagues Zahraddeen A. H, Abdul Kabir A., Ayuba K., Bukar A., Zainab I. L., Yahaya D. B., Abdulhakim A., Chindo A., Ajayi A., Shittu A., as well as to everyone else who provided help and encouragement at various stages of my work. Finally, I am grateful to the Nigerian Petroleum Trust Development Fund (PTDF) for supporting this research financially. Their support allowed me to complete this thesis and further my education. More so, I would like to acknowledge the contributors to my study,

who generously gave their time and effort to contribute to this research. Thank you all for your invaluable contributions.

TABLE OF CONTENTS

ABSTRACT	iii
ACKNOWLEDGEMENTS.....	v
LIST OF FIGURES.....	xi
LIST OF TABLES	xiii
LIST OF EQUATIONS.....	xv
LIST OF ABBREVIATIONS	xvi
1 Introduction.....	1
1.1 Background.....	1
1.1 Plastic production.....	2
1.2 Types, Properties, Merit and Demerit of plastics	3
1.3 Justification for study	5
1.4 Problem Statement	5
1.5 Research Questions	6
1.6 Scope of the research.....	6
1.7 Research Hypothesis.....	7
1.8 Research novelty	7
1.9 Research Aim and Objectives.....	8
1.9.1 Aim of the research	8
1.9.2 Objectives of the research.....	8
1.10 Thesis structure	9
2 Literature review.....	11
2.1 Bioplastic	11
2.2 Polyhydroxyalkanoate and its structure	12
2.3 Type and properties of polyhydroxyalkanoate	15
2.4 PHA Biodegradability	17
2.5 Biosynthesis of PHA	18
2.6 PHA extraction (post-processing)	21
2.6.1 Solvent extraction methods.....	22
2.6.2 Chemicals and enzyme methods	23
2.6.3 Extraction Process used.....	24
2.7 Polymer Characterisation.....	26
2.7.1 Thermogravimetric analysis (TGA).....	27
2.7.2 Gas chromatography mass spectrometry (GC-MS)	27
2.7.3 Fourier Transform Infrared	28
2.7.4 Scanning Electron Microscope.....	28
2.8 PHAs applications and their uses	28
2.9 Microorganisms producing PHAs.....	30
2.10 <i>Pseudomonas</i> species producing PHAs.....	34
2.11 Biowaste materials used for PHA production.....	35

2.11.1 Sugars used for PHA production.....	36
2.11.2 Fats and oils used for synthesizing PHAs.....	42
2.12 Analytical methods to determine oil composition.	47
2.13 Fatty Acid Profile of Different Oils	48
2.13.1 Palm oil	48
2.13.2 Glycerol	48
2.13.3 FOG	49
2.13.4 Soybean oil	49
2.14 Pretreatment of Waste Oils for Bioplastic Production	49
2.14.1 Chemical Pretreatment.....	50
2.14.2 Biological Pretreatment	50
2.14.3 Physical Pretreatment	51
2.15 Solutions for emerging bioplastics	51
2.16 Challenges in the production of bioplastics.....	52
3 Sustainable Conversion of Lipid-Rich Wastes to Bioplastics.....	53
Abstract.....	53
3.1 Introduction	54
3.2 Materials and Methods.....	56
3.2.1 Materials.....	56
3.2.1 Equipment.....	56
3.2.2 Methods	57
3.3 Polymer recovery	59
3.3.1 Separation (centrifugation) process.....	59
3.3.2 Microscopic process.....	60
3.3.3 Determination of dry cell weight	60
3.3.4 Polymer extraction	60
3.4 Polymer characterisation	61
3.4.1 SEM analysis	61
3.4.1 FTIR spectroscopy analysis	61
3.5 Results and discussion	62
3.5.1 Growth and production.....	62
3.5.2 Polyhydroxyalkanoate yield.....	65
3.5.3 Effect of carbon sources on CDW and PHA yield	69
3.5.4 SEM analysis	70
3.5.5 FT-IR analysis of polymers.....	71
3.6 Conclusion	73
Acknowledgement	74
References	75
4 Development of pretreatment techniques to improve the synthesis of Polyhydroxyalkanoate (PHA) from waste oils.....	84
Abstract.....	84

4.1 Introduction	86
4.2 Materials and Methods.....	87
4.2.1 Materials used.....	87
4.2.2 Equipment used.	88
4.2.3 Carbon sources.....	88
4.2.4 Methods used.....	89
4.2.5 Statistical analysis.....	91
4.3 Polymer recovery	91
4.3.1 Separation (harvesting).....	91
4.3.2 Determination of dry cell weight	91
4.3.3 Polymer extraction	91
4.3.4 Polymer quantification.....	91
4.4 Polymer characterization	92
4.4.1 SEM analysis	92
4.4.2 FTIR analysis.	92
4.5 Results and Discussion.....	92
4.5.1 Production and growth under pretreatment.....	92
4.5.2 Effect of various pretreatment on PHA content	95
4.5.3 Polyhydroxyalkanoate % Yield	98
4.5.4 Scanning Electron Microscope results	100
4.5.5 FTIR analysis of the extracted polymers	101
4.6 Conclusion	103
Acknowledgement	104
References	105
5 Developing Simple Post-Processing Techniques for Extraction of Bioplastic Produced by <i>Pseudomonas</i> Strains	111
Abstract.....	111
5.1 Introduction	112
5.2 Materials and Methods.....	114
5.2.1 Materials.....	114
5.2.2 Equipment Used.....	114
5.2.3 Carbon Sources	115
5.2.4 Methods	115
5.3 Polymer recovery.....	117
5.3.1 Separation process	117
5.3.2 Determination of cell content.....	117
5.3.3 Polymer extraction	118
5.4 Polymer Characterization.....	119
5.4.1 Scanning electron microscopy	119
5.4.2 Fourier transforms infrared spectroscopy.....	120
5.5 Results and discussion.	120

5.5.1 Growth and production	120
5.5.2 Composition of the FOG.....	122
5.5.3 Percentage yield of the produced polymer	123
5.5.4 Scanning electron microscopy	126
5.5.5 Fourier Transform Infrared Spectroscopy (FTIR)	127
5.6 Conclusion	129
Acknowledgement	129
References	130
6 Discussion	138
6.1 Lipid-rich oil waste	139
6.2 Fermentation Broth Characterization	142
6.2.1 Expected Composition	142
6.2.2 Impact of Process Parameters	143
6.2.3 Impact of Pretreatment.....	144
6.3 Shake flask experiments to evaluate PHA production.	144
6.4 % Yield of PHA from various carbon source	146
6.5 Polymer downstream process.....	148
6.6 Oil Mineral Impact on PHA Quantity	149
6.7 Characterization of the polymer produced.	150
6.7.1 Polymer granules observations	150
6.7.2 Polymer functional groups analysis	151
6.8 Quality of the PHA produced from waste substrates.....	152
6.8.1 Methods of quantifying PHA impurities.....	153
6.8.2 Method of quantifying PHA.....	154
References	156
7 Conclusion, Recommendations and Future Directions.....	167
7.1 Conclusion	167
7.2 Research Future Directions and Contributions	168
7.2.1 Research Future Directions.....	168
7.2.2 Research Contributions to Knowledge	168
7.3 Recommendations.....	172
REFERENCES	173
APPENDICES	211

LIST OF FIGURES

Figure 1-1: Worldwide plastic production from 1950 to 2020.....	2
Figure 2-1: The chemical structural formula of polyhydroxyalkanoate.....	14
Figure 2-2: The three main biosynthesis pathways in PHA.....	20
Figure 2-3: PHA enzyme in <i>C. necator</i> and <i>P. oleovorans</i>	21
Figure 3-1: The schematic procedure of bioplastic production	58
Figure 3-2: TESCAN VEGAN 3 SEM analytical instrument.....	61
Figure 3-3: Bruker (Vertex 70) FTIR analytical instrument.....	62
Figure 3-4: Growth curve of both strains: (a) Soybean oil and (b) Palm oil.....	64
Figure 3-5: Growth curve of both strains: (c) FOG and (d) Glycerol.....	64
Figure 3-6: Effects of various carbon on a) CDW & PHA contents and b) Yield %.....	69
Figure 3-7: SEM granule images from cells: (a) DSMZ1045 & (b) ATCC29347 employed PO.....	71
Figure 3-8: SEM granule images from cells: (a) DSMZ1045 & (b) ATCC29347 employed SO.....	71
Figure 3-9: FTIR analysis of polymer produced: [a] DSMZ1045 utilized (i) GLY, (ii) SO, (iii) FOG; and [b] ATCC29347 utilized (i) GLY, (ii) SO, (iii) FOG.....	73
Figure 4-1: Growth curve of <i>P. oleovorans</i> from pretreatment: a[i-iii] - ATCC29347 and b[i-iii] - DSMZ1045.....	94
Figure 4-2: Impact of each pretreatment on PHA: a) Lipase, b) Ultrasound, and c) Microwave.....	1216
Figure 4-3: Effect of each pretreatment on PHA % yield/CDW	100

Figure 4-4: SEM images of granules observed: a(i-ii) DSMZ1045 and b(i-ii) ATCC29347	101
Figure 4-5: FTIR analysis of the produced PHAs from a) FOG, b) FSO, c) FPO, and d) Reference PHA.....	102
Figure 5-1: Growth and Production curve from <i>Pseudomonas oleovorans</i> (DSMZ1045 and ATCC29347): a) Glycerol and b) FOG.....	121
Figure 5-2: SEM granule images observed in both cells: (a-b) DSMZ1045 & (c-d) ATCC29347	127
Figure 5-3: FTIR analysis from <i>Pseudomonas oleovorans</i> : a[i-ii]-Glycerol, and b(i-ii)-FOG.....	128

LIST OF TABLES

Table 2-1: The comparison of the properties of PHA and conventional plastic...	16
Table 2-2: Uses and applications of polyhydroxyalkanoate in various areas ...	29
Table 2-3: Bacteria and their species are used for PHA production	31-33
Table 2-4: Bacteria used for production of PHA from lignocellulosic waste.....	39
Table 2-5: Different types of bacteria used for production of PHA from Molasse	41
Table 2-6: Pseudomonas and Cupriavidus species producing PHA from lipid-rich wastes.	45
Table 2-7: Fatty acid compositions of the different oils.....	49
Table 3-1: Tests of between-subjects effects	64
Table 3-2: PHA % yield /CDW by the strains from each carbon used	70
Table 3-3: % Yield increased after pretreatment was added.....	72
Table 4-1: Test of between-subject effects.....	95
Table 4-2: Impact of each pretreatment on cell mass and PHA productivity.....	98
Table 5-1: Tests of between-subject effects.....	1212
Table 5-2: Percentage compound composition of FOG.....	12212
Table 5-3: Percentage of moisture, TS, and VS in FOG.....	12212
Table 5-4: Polymer percentage yield from each extraction method.....	1212
Table 7-1: Research contributions impact	195-196
Table 7-2: Grand Means of 95% confidence level.....	213
Table 7-3: Significance level revealed 85.8% from the regression model	213
Table 7-4: Grand Means of 95% confidence level.....	214
Table 7-5: Significance level revealed 91.8% from the regression model.	214
Table 7-6: Grand Means of 95% confidence level.....	214
Table 7-7: Significance level revealed 97.3% from the regression model.	214
Table 7-8: Grand Means of 95% confidence level.....	215

Table 7-9: Significance level revealed 97.3% from the regression model.	243
Table 7-10: Grand Means of 95% confidence level.....	215
Table 7-11:Significance level revealed 93.2% from the regression model.	213
Table 7-12:Grand Means of 95% confidence level.....	213
Table 7-13:Significance level revealed 85.8% from the regression model.	213
Table 7-14:Grand Means of 95% confidence level.....	214
Table 7-15:Significance level revealed 91.8% from the regression model.	214
Table 7-16:Grand Means of 95% confidence level.....	214
Table 7-17: Significance level revealed 97.3% from the regression model. ...	214
Table 7-18: Grand Means of 95% confidence level.....	215
Table 7-19:Significance level revealed 97.3% from the regression model.	215
Table 7-20: Grand Means of 95% confidence level.....	215
Table 7-21: Significance level revealed 97.3% from the regression model. ...	215
Table 7-22:Grand Means of 95% confidence level.....	216
Table 7-23:Significance level revealed 97.3% from the regression model.	216
Table 7-24:Grand Means of 95% confidence level.....	216
Table 7-25:Significance level revealed 97.3% from the regression model.	216

LIST OF EQUATIONS

(3-1) 62

(3-2)..... 62

(3-3)..... 63

(3-4)..... 64

(3-5)..... 67

(3-6)..... 67

(4-1)..... 119

(5-1)..... 118

(5-2)..... 119

(5-3)..... 119

LIST OF ABBREVIATIONS

FT-IR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
SCL-PHA	Short chain length Polyhydroxyalkanoate
MCL-PHA	Medium chain length Polyhydroxyalkanoate
MM	Minimal medium
H ₂ O ₂	Hydrogen peroxide
NaOH	Sodium hydroxide
LCL-PHA	Long chain length Polyhydroxyalkanoate
T _g	Transition temperature
T _m	Melting temperature
NPCM	Non-PHA cell mass
SDS	Sodium dodecyl sulfate
EDTA	Ethylene diamine tetraacetic acid
CDW	Cell dry weight
FOG	Fat, oil, and grease
FSO	Frying soybean oil
FPO	Frying palm oil
PHE	Public Health England Culture Centre
ATCC	American Type Culture Centre
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
VS	Volatile solid
Gly	Glycerol
TS	Total solid
VFAs	Volatile fatty acids
D _f	Differential factor
F	Factor
SE	Standard error
H	Hour
PEs	Polyethylenes
PPs	Polypropylene
PVC	Polyvinyl chloride
PS	Polystyrene
PLA	Polylactic acid
CO ₂	Carbon dioxide
H ₂ O	Water

G+ve	Gram positive
G-ve	Gram negative
Mw	Molecular weight
XRD	X-ray diffraction
DSC	Differential scanning calorimetry
TEM	Transmission electron microscopy
GC-MS	Gas chromatography mass spectroscopy
NMR	Nuclear magnetic resonance
GPC	Gel permeation chromatography
TGA	Thermogravimetric analysis
EDS	Energy dispersive spectroscopy
HPLC	High performance liquid chromatography
GC	Gas chromatography
ANOVA	Analysis of variance
OD	Optical density
WO	Waste oil
MW	Microwave
US	Ultrasound
FAs	Fatty acids
FFAs	Free fatty acids
KOH	Potassium hydroxide
HCL	Hydrochloric acid
H ₂ SO ₄	Sulfuric acid
EM	Electron microscopy
BS	British standard
PTDF	Petroleum technology development fund
RPM	Ram per minute
g/L	Gram per litre
mL	Millilitre
NB	Nutrient broth
PO	<i>Pseudomonas oleovorans</i>

1 Introduction

1.1 Background

The name “plastic” is from the Latin name that means “plasticus”, which originated from the Greek word “plastikos”, meaning capable of being moulded (Ncube *et al.*, 2021; Plastics Europe, 2018). Plastics are synthetic materials made of polymers characterized by their malleability and ability to be moulded into various shapes (Díaz-Mendoza *et al.*, 2020; Iñiguez *et al.*, 2016, 2018). They have become an integral part of our modern society, playing a crucial role in various industries and everyday life (Mohammad *et al.*, 2017). Plastics offer numerous advantages, such as durability, versatility, and cost-effectiveness, making them appropriate for producing many materials (Iñiguez *et al.*, 2016; Thompson *et al.*, 2009; Yates *et al.*, 2021). These plastic materials are widely used in various industries, from packaging and construction to healthcare and electronics, because of their unique qualities like flexibility, quality, and resistance to degradation (Andrady and Neal, 2009; Geyer *et al.*, 2017; Mozejko-Ciesielska *et al.*, 2019; Thompson *et al.*, 2009). They can be used to package perishable commodities, store caustic materials, and create durable products since they are resistant to moisture, chemicals, and degradation. In addition, plastics are transparent, flexible, and heat-resistant, making them suitable for diverse applications such as electrical insulation, plastic films, food packaging, and made every life sector modernized (Geyer *et al.*, 2017; Law and Narayan, 2022; Thompson *et al.*, 2009). However, as the need for plastics continues to soar, their environmental, social and health concerns have gained significant attention. This introduction intends to explore the many aspects of plastics, including their history, classes, benefits, properties, challenges, and the pressing requirement for sustainable practices to mitigate their adverse effects (Mozejko-Ciesielska *et al.*, 2019; Thompson *et al.*, 2009).

1.1 Plastic production

Plastic production has been booming since its arrival, rising from more than 1.5 million tons in the 1950s to almost 380 million tons in 2015, and it is predicted to rise to 2000 million tons by 2050 (Coyle *et al.*, 2020; Geyer *et al.*, 2017; Plastics Europe, 2018; Villarrubia-Gómez *et al.*, 2018). Since the 1950s century, global plastic production has increased enormously (Fig. 1-1), leading to the rise of plastic in the world's modern societies (Villarrubia-Gómez *et al.*, 2018). Most plastic waste is not adequately managed and frequently finds up in landfills, oceans, and other natural areas. According to Jambeck *et al.*, (2021), this build-up of plastic waste negatively affects ecosystems, wildlife, and human health. Approximately 6.3 billion tons of global plastic waste are generated, of which only nine per cent (9%) is predicted to be recycled, with nearly twelve per cent (12%) incinerated (Geyer *et al.*, 2017; Okunola *et al.*, 2019). While the remaining seventy-nine per cent (79%) accumulated in the environment, as detailed in 2015. Hence, in the year 2050, there will be more than twelve billion tons (12bt) of plastic waste in the environment (Geyer *et al.*, 2017). Plastics have many different functional and excellent properties, and numerous products, from domestic items to industrial devices, were made using the plastics, transforming various industries and boosting the economy (Geyer *et al.*, 2017).

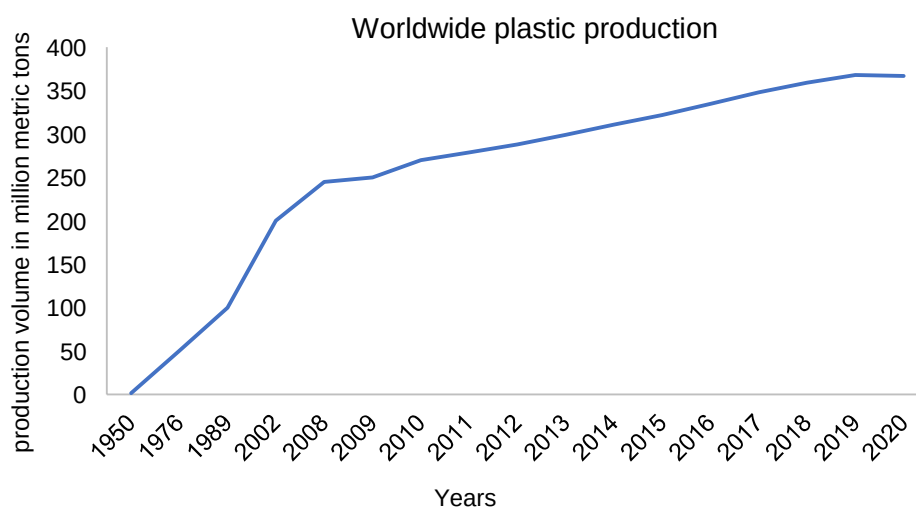


Figure 1-1: Global plastic production from 1950 to 2020. Source: (Statista, 2020)

Since the middle of the 19th century, polymers have been used to create plastics, which are classified as synthetic plastics. However, it was in the early 20th century marked the beginning of modern plastics when a chemist scientist named Leo Baekeland from Belgium created the first plastic known as Bakelite in 1907, which was widely used everywhere, such as in fashion, the electrical, and the automotive industries (Okunola *et al.*, 2019). Since then, advances in polymer science and technology have led to the development of a wide variety of plastics with various properties, including polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), and polystyrene (PS)(Díaz-Mendoza *et al.*, 2020; Jian *et al.*, 2020; Law and Narayan, 2022).

1.2 Types, Properties, Merit and Demerit of plastics

Plastic materials are the most widely used worldwide in packaging, electronics, agriculture, vehicle, and biomedical devices (Costa *et al.*, 2019; Koller and Braunegg, 2018). These plastics have become so standard in modern life that it is hard to manage life without plastic materials. Moreover, many commodities would be much more expensive if not for plastics. This is because they are simple, enduring, flexible, resistant, strong, cheap, and lightweight, to name only a few (Iñiguez *et al.*, 2018; Kaur *et al.*, 2017; Ojumu *et al.*, 2004; Thompson *et al.*, 2009). According to their use, plastics are commonly classified into two main categories: thermoplastics and thermoset plastics (Grigore, 2017). Thermoplastics are a class of plastics that can be melted and reshaped multiple times without undergoing significant chemical changes (Evode *et al.*, 2021). They are widely used in bottles, bags, films, and toys (Andrady., 2003). In contrast, thermoset plastics undergo irreversible physiochemical changes when heated (Evode *et al.*, 2021; Grigore, 2017), resulting in a hardened and rigid structure. They are commonly found in applications requiring high heat resistance, such as electrical insulation and automotive components (Andrady and Neal, 2009).

Plastics are highly in need of various uses, due to their many beneficial characteristics (Evode *et al.*, 2021). They are generally less expensive than other materials and can easily form into various shapes and sizes, offering designs in

product innovation. Moreover, they can be mixed with other materials like fibres or additives to improve their mechanical strength. Also, they are highly durable and resistant to chemicals, moisture, and UV radiation, ensuring the longevity of their products (Andrady, 2017; Andrady and Neal, 2009). Plastics are good electrical insulation, making them valuable in producing electrical and electronic components, in the automobile and aerospace sectors (Andrady, 2017; Andrady and Neal, 2009; Lebreton and Andrady, 2019). Furthermore, the earth cannot stand without plastics due to their numerous applications, shaping the modern economy. Plastics are everywhere and in daily use, especially in the packaging sector (Koushal *et al.*, 2014; Mozejko-Ciesielska *et al.*, 2019; Rouch *et al.*, 2020). Nearly more than 40% are used in packaging (Lebreton and Andrady, 2019; Pinto *et al.*, 2020). Plastics greatly benefited the packaging application sectors, especially in single-use containers and bags for business-to-consumer uses. Building and construction is the second largest sector, such as cladding, gutters, windows, doors, and pipelines, and the third is the automobile (Andrady and Neal, 2009; Plastic Europe, 2019). Plastics make our life simpler and friendlier; life becomes more complex and challenging without them. But, as beautiful as plastics are, making life very sound, the bottom line is that they are not very good for the environment due to their nature of being non-degradable (Ncube *et al.*, 2021). However, it was estimated that around 275 million tons of plastic waste are produced worldwide, with more than eight million tons ending in the ocean yearly, damaging aquatic life and affecting the natural system (Okunola *et al.*, 2019; Smith *et al.*, 2018). According to Hasan *et al.* (2021), if the rapid growth of plastics continues, there will be more plastic in the ocean than fish.

Furthermore, the widespread use of plastics has also led to significant negative environmental concerns (Andrady., 2003). Plastics are typically derived from fossil fuels, primarily crude oil, and their production contributes to greenhouse gas emissions and energy consumption (Geyer *et al.*, 2017). It was estimated that more than four percent (4%) of the world's oil produced is used as raw materials for manufacturing plastic, plus nearly five percent (5%) is used to provide the energy required to produce these plastic materials. Also, about twenty

percent (20%) of the world's total oil consumption is needed to create plastic in 2050 (Hopewell *et al.*, 2009; Lebreton and Andrady, 2019). Improper disposal and inefficient recycling of plastics have resulted in the pollution of land, water bodies, and marine ecosystems, causing harm to wildlife and entering the food chain (Geyer *et al.*, 2017). In addition, plastic waste, particularly single-use items like bags and bottles, has become a major environmental challenge worldwide (Andrady., 2003; Geyer *et al.*, 2017). There is an increasing emphasis on the circular economy idea, which tries to reduce waste output and increase resource efficiency to solve these environmental challenges. However, a potential option to solve the menace of plastic waste in the environment is to develop biodegradable plastics (Geyer *et al.*, 2017).

1.3 Justification for study

The research argues that due to serious waste problems of synthetic plastic in the natural environment, social and human health living on the earth, there is a need to work effectively on sustainable alternatives to synthetic plastics and to address or nullify the negative environmental impact caused by them. However, the prospect of plastics lies in sustainable practices and innovative solutions. Researchers are exploring various ways to develop bioplastics, which are derived from renewable resources and have a reduced environmental footprint. They introduce alternative feedstocks, such as plant-based materials, to reduce dependence on fossil fuels and mitigate environmental impact. Additionally, advances in polymer science and developed technologies aim to improve the recyclability and biodegradability of plastics and develop new materials with enhanced properties. Moreover, advancements in biotechnology may also enable the development of plastics that can be easily degradable.

1.4 Problem Statement

The production of bioplastics as significant potential for addressing the global environmental negative issues related to synthetic plastics. To improve sustainable bioplastic production and exploring alternative cheap and available

carbon sources are needed. *Pseudomonas* strains have shown potential in synthesizing bioplastics from different oil wastes, but there is a need to evaluate their performance to enhance the efficiency and sustainability of bioplastic production. Therefore, this research addresses developing and evaluating a sustainable production for bioplastics using *Pseudomonas* strains from various lipid-rich oil wastes, focusing on improving yield, quality and minimizing environmental impact during the recovery process.

1.5 Research Questions

- I. How effective are the selected strains of *Pseudomonas* in converting various lipid-rich oil wastes into bioplastic?
- II. What is the effectiveness of using these oil wastes (Glycerol, frying soybean oil, FOG, & frying palm oil) on sustainable bioplastic production from *Pseudomonas* strains, compared to high-grade food and oils used in producing bioplastics?
- III. How to improve bioplastic production productivity by *Pseudomonas* strains by employing different pretreatment techniques?

1.6 Scope of the research

Sustainable bioplastic production from waste oils by *Pseudomonas* strains may present some challenges, such as:

1. Cost of production is relatively high compared to traditional petroleum-based plastics. Additional steps, such as oil waste, strain selection, and mild extraction, can reduce the overall cost and explore ways for large-scale production.
2. Availability and consistency of oil wastes can also pose a limitation. Access to a sufficient and consistent supply of waste oils may vary geographically and temporally.

Addressing these limitations will help researchers better understand the feasibility, scalability, and practical implementation. Thus, considering this focusing on addressing sustainability and scalability challenges, optimizing the production process, and conducting a thorough techno-economic analysis of bioplastic production can contribute a lot.

1.7 Research Hypothesis

- i. **Alternative hypothesis (A_1):** Using *Pseudomonas* strains for the sustainable production of bioplastic from waste oils will result in efficient and environmentally friendly bioplastic production, offering a promising solution for waste management and reducing reliance on fossil fuel-based plastics.
- ii. **Null hypothesis (A_0):** No significant difference in the ability of *Pseudomonas* strains for the sustainable production of bioplastic from waste oils will result in efficient and environmentally friendly bioplastic production, offering a promising solution for waste management and reducing reliance on fossil fuel-based plastics.

1.8 Research novelty

Numerous research studies have focused on using waste materials to reduce the production costs of Polyhydroxyalkanoates (PHAs), demonstrating their potential as high-yield PHA synthesis feedstocks (Riedel *et al.*, 2015). Furthermore, various strategies have been proposed in the literature to optimize PHA production while minimizing costs (Koller and Braunegg, 2018; Sabapathy *et al.*, 2020). The exploration of low-cost, sustainable carbon substrates is currently underway on a global scale (Lopar *et al.*, 2013). Waste oils and by-products from oil mills have received the most interest since they provide even more affordable possibilities and have undergone extensive research (Surendran *et al.*, 2020; Tsang *et al.*, 2019)

However, a critical gap exists in the existing literature, as there is no prior research evaluating the sustainability and efficiency of simple, non-invasive pretreatment and post-processing techniques to produce bioplastics by *Pseudomonas* strains, specifically *Pseudomonas oleovorans* (DSMZ1045 and ATCC29347). This study addresses this critical knowledge gap by evaluating the sustainability of bioplastic production from lipid-rich wastes, serving as low-value carbon sources when employed by *Pseudomonas* strains. Additionally, it seeks to compare the yield quantities from each carbon source between non-treated and pretreated techniques. The major goal of the research is to assess how effectively *Pseudomonas* strains [*P. oleovorans* (DSMZ1045 & ATCC29347)] can accumulate high bioplastic (PHA) content in lipid-rich wastes under nitrogen-growth-limited fermentation conditions. Consequently, presenting the empirical data on PHA productivity and sustainability, laying the groundwork for scaling up this process to an industrial level while considering various process-related factors.

1.9 Research Aim and Objectives

1.9.1 Aim of the research

This study aimed to evaluate the production of polyhydroxyalkanoates from waste carbon sources derived from different lipid-rich materials using two (2) selected *Pseudomonas* strain.

1.9.2 Objectives of the research

1. Review and evaluate the production process of polyhydroxyalkanoate (PHA) from waste materials available for bacterial conversion.
2. To select and identify *Pseudomonas* strains effectively utilizing lipid-rich waste oils for bioplastic synthesis.
3. To evaluate the bioplastic production in various lipids-rich oil wastes, including soybean oil, glycerol, FOG, and palm oil utilized by *Pseudomonas oleovorans* DSMZ 1045 and *Pseudomonas oleovorans* ATCC 29347.

4. Investigate the impact of different pre-treatment techniques such as microwave, ultrasound, and lipase on bioplastic production. These have been selected following a thorough literature review.
5. To develop a post-processing technique for bioplastic extraction from *Pseudomonas oleovorans* attending to sustainability principles using simple and mild methods, including sodium hydroxide, hydrogen peroxide, and enzyme.

1.10 Thesis structure

Thesis topic: Sustainable bioplastic production using chosen *Pseudomonas* strains (DSMZ1045 & ATCC29347) using a variety of lipid-rich wastes as carbon substrates, such as glycerol, grease traps, palm oil, fat, oil & grease (FOG), and soybean oil. To foster the production of PHA, new advanced pretreatment techniques for lipid-rich materials are being developed. PHA is also extracted, purified, and characterized from cell biomass to create the best conditions possible for measuring the high PHA yield. These conditions are compared with the conventional approaches of using food-grade carbon substrates with or without pretreatment processes. The format is comprised of the following chapters:

Chapter 1 contains the introduction part of the study. This comprises background of plastics, research novelty, limitations, questions, purpose, significance, hypothesis, aim, objectives, and thesis construction.

Chapter 2 devoted to the conceptual framework and literature review. The conceptual framework deals with production bioplastics from biowastes, polymer characterization, oil analysis and its compositions, polymer extraction, PHA production challenges, and prospects for producing PHA were identified and discussed in detail.

Chapter 3 contains a paper titled: Sustainable Conversion of Lipid-rich Oil Wastes to Bioplastics.

Chapter 4 contains a paper titled: Development of Pretreatment Techniques to Improve the Synthesis of Polyhydroxyalkanoate (PHA) from Oils.

Chapter 5 contains a paper titled: Developing Simple post-treatment Techniques for Extraction of Bioplastic Produced by *Pseudomonas* Strains.

Chapter 6 devoted to discussion, interpretation of results of the thesis and knowledge contributions from the study.

Finally, chapter 7 contains a conclusion and future recommendations.

2 Literature review

This chapter reviews pertinent information concerning various aspects of bioplastic production. First, sections 2.1 and 2.2 discuss the framework of bioplastics and polyhydroxyalkanoate as the way forward to solving synthetic plastic pollution. Section 2.3 outline the polyhydroxyalkanoate types and properties, while section 2.4 contains an overview of polyhydroxyalkanoate biodegradability. Section 2.5 encompasses the biosynthesis of polyhydroxyalkanoate, and section 2.6 covers the polymer extraction process, section 2.7 covers biotechnological applications of PHAs, section 2.8 polymer characterization, and section 2.9 contains microorganisms producing PHAs. Section 2.10 provide detailed on *Pseudomonas* species, and section 2.11 presents an overview of biowastes used to produce PHA. Section 2.12, section 2.13 contains oil analysis, section 2.14 outlook the pretreatment, and section 2.15 describes the challenges associated with the production of PHA. Finally, section 2.16 PHA as promising solution.

2.1 Bioplastic

Bioplastics are defined by most literature as green plastics (Varghese *et al.*, 2022). They are plastic polymers of bio-origin produced from the fermentation process of renewable materials and plant-based materials like agricultural waste, corn, fat and oil, cassava, whey, and sugarcane (Volova *et al.*, 2022). Therefore, these raw materials do not lead to natural resources decline (Chakraborty *et al.*, 2012). Additionally, they are made without fossil oils and have similar properties to fossil fuel-derived plastics with additional advantages of biodegradability, environmentally friendly and biomaterials (Bugnicourt *et al.*, 2014). Therefore, they can significantly contribute to saving the limited fossil fuel resources and reducing the environmental burden caused by oil plastics (Plastics Europe, 2018). Furthermore, bioplastics have widespread advantages over fossil oils-derived plastics in the following areas: biodegradability, security, sustainability, and various environmental benefits. This activates the efforts of producing bioplastics using microorganisms, and the best-known producing ones are

polyhydroxyalkanoates, polylactic acids, polysaccharides, polynucleotides, and polyamides numerous to mention (Chee *et al.*, 2010; Varghese *et al.*, 2022). However, in these types, polyhydroxyalkanoates are the most highly valued due to their low carbon footprint, energy efficiency, environmental protection, and ease of production (Chee *et al.*, 2010; Ezgi *et al.*, 2015).

Bioplastics possess many uses in different areas, including processing food industries, pharmaceuticals, building, agriculture, and biomedicine sectors, to mention a few (Costa *et al.*, 2019; Mannina *et al.*, 2019). Polyhydroxyalkanoate is the most and best example and widely studied bioplastic due to it is biodegradable, non-toxic, and possesses similar characteristics to oil-derived synthetic plastics like polypropylene and polyethylene (Jain and Tiwari, 2015; Keshavarz and Roy, 2010; Mannina *et al.*, 2019; Zhao *et al.*, 2015). Polyhydroxyalkanoate is the twenty-first century's most modern material and is the most outstanding value bioplastic product worldwide. Accordingly, bioplastics are grouped into either biobased or biodegradable; the biobased are of biological origin, while the biodegradable are microbially broken down to carbon dioxide (CO₂), water (H₂O) and organic matter. Bioplastics have similar material properties to oil-based chemically made plastics (Sasidharan and Bhat, 2014).

Moreover, petrochemically synthetic plastics, mainly polypropylene, polyethene, polyvinyl chloride, and polystyrene, are recalcitrant compounds that persist in the environment and create a significant waste problem. These concerns stimulated study efforts towards finding degradable bioplastic alternatives (Tanikkul *et al.*, 2020). Hence, many researchers are trying to develop the best and easy way of producing biodegradable bioplastics, such as polyhydroxyalkanoate. Therefore, using lipid-rich by-products or organic waste to produce bioplastic is helped in making the production of bioplastics very cheap and reduces environmental waste accumulation.

2.2 Polyhydroxyalkanoate and its structure

Polyhydroxyalkanoate is a family of polyester from hydroxyalkanoates, which build up as carbon and energy-reserved materials in many cells of

microorganisms (Almeida *et al.*, 2022; Tanikkul *et al.*, 2020). They are synthesized and accumulated as intracellular granules by some gram-positive and gram-negative bacteria under stress conditions without hazardous effects on the hosts (Castilho *et al.*, 2009). Therefore, polyhydroxyalkanoates are promising for changing polymers of present oil-based plastics to bio-based plastics (Zhou *et al.*, 2022). Furthermore, these PHAs have additional specialized applications over conventional oil-based plastics because they are entirely biodegradable to CO₂ and H₂O and generally manufactured from renewable resources. In addition, PHAs have proven advantageous characteristics over oil-based plastics based on their outstanding properties, including crystallinity, melting temperature, and young's modulus, to mention a few (Muneer *et al.*, 2020). These excellent qualities of bioplastic PHAs made their applications widely used in areas ranging from single-use to hard-use, such as phones and vehicles (Ganesh Saratale *et al.*, 2021; Sharma *et al.*, 2021). However, despite their promising excellent properties, bioplastics PHA do not compete economically with oil-based plastics, with only in 2018 their production raised to 2.11 Mt in the role of marketing value (Mozejko-Ciesielska *et al.*, 2019; Pagliano *et al.*, 2021). This is economically non-competing because their production costs are significantly high, limiting their economic value compared to synthetic plastics (Sharma *et al.*, 2021). Polyhydroxyalkanoates are the most versatile type of biopolymer, with more than 100 monomers obtained from plant sources as a renewable raw material and microorganisms like bacteria, algae, and fungi (Akaraonye *et al.*, 2010). The development of polyhydroxyalkanoate started in the late nineteen century when Beijerinck saw PHA as an inclusion body in bacteria cells. Later in the early twentieth century, Lemoigne first described it after he isolated the first type of PHA called as by the name of polyhydroxybutyrate (PHB) from *Bacillus megaterium*. Since then, many microorganisms capable of synthesizing various polyhydroxyalkanoate, like gram-negative and gram-positive bacteria, have been identified (Bugnicourt *et al.*, 2014; Chee *et al.*, 2010; Thorat *et al.*, 2017). Fig. 2-1 shows the chemical structure formula of polyhydroxyalkanoate.

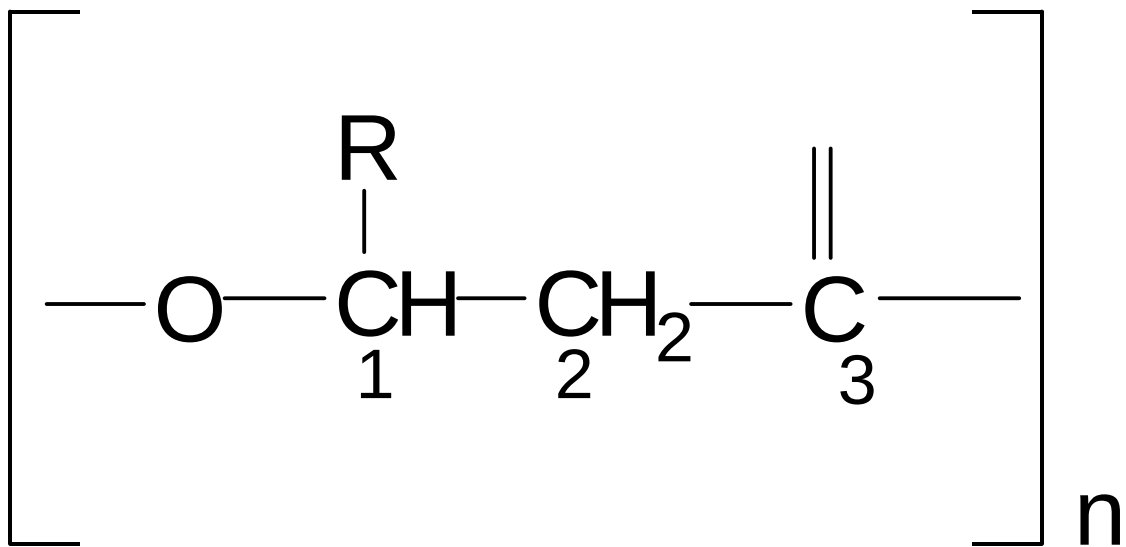


Figure 2-1: The chemical structural formula of polyhydroxyalkanoate.

Furthermore, despite their wide range of applications, polyhydroxyalkanoate uses are still hindered by their high production costs limiting their economic value compared to oil-based plastics (Tan *et al.*, 2014a). This is because the expenses of carbon substrates utilized by the microorganisms are almost up to 50% of the total production costs (Amaro *et al.*, 2019; Koller *et al.*, 2013). Hence, using inexpensive carbon substrates and sources from renewable waste materials like lignocellulosic materials, oily wastes, wastewater, molasses, and whey, but mentioning a few, could reduce the costs of PHAs production (Chee *et al.*, 2010; Du *et al.*, 2012; Kaur *et al.*, 2017; Koller *et al.*, 2017; Surendran *et al.*, 2020). After polyhydroxyalkanoates, the second most important type of bioplastics is polylactic acids (PLAs), and they are produced using renewable resources like sugar, starch etc. (Jambunathan and Zhang, 2016). However, polylactic acids are a family of bioplastics that are biobased/biodegradable polymers produced using the condensation reaction of lactic acid through lactide chain development. Presently, more than 13.9% of polylactic acid bioplastic is globally produced. They are formed via the bacterial fermentation of the L and D isomerization process. as a result, they have the beautiful properties of being biodegradable and thermoplastic, shaping them into various applications, including packaging, printing, medical services, and many more (Varghese *et al.*, 2022). Furthermore,

globally polylactic acids are the most used bioplastics, and it was estimated that by 2020 the capital expenditure would be more than \$5.2 bn (Jambunathan and Zhang, 2016).

2.3 Type and properties of polyhydroxyalkanoate

Polyhydroxyalkanoate is categorized into three groups consist of: The first group is known as poly (SCL-HA) which is a short lengths chain polymer that contains monomers unit of 3 to 5 carbon atoms produced by many microorganisms, e.g., *Alcaligenes latus*, *Ralstonia eutropha* (formerly known as *Cupriavidus necator*) to mention few among them. An excellent example of this short-chain group is polyhydroxybutyrate (PHB). The second group is poly (MCL-HA), a medium-chain length between six to fourteen C-atoms, produced generally by *Pseudomonas* species. Example of this group is polyhydroxyoctanoate, polyhydroxyhexanoate, polyhydroxydecanoate and many too numerous to mention here. At the same time, the third category is poly (LCL-HA), a long-chain length with monomeric units higher than 14 carbon atoms (Grigore *et al.*, 2019; Roja *et al.*, 2019). However, PHA's properties are determined by different monomeric side chain lengths (Liu *et al.*, 2018). As a result, most Scl-PHAs are highly crystalline (55-80%), which makes them stiff and brittle, and they are characterized by complete stereo-regular with high melting temperature (T_m) ranging between 173 – 180°C and low glass transition temperatures (T_g) ranging between 5 and 9°C. These temperatures are essential parameters in-service application of SCL-PHAs.

Furthermore, the properties associated with the copolymers of SCL-PHAs are much better and more desirable than the SCL-PHA homopolymers because they are easy to mould and become hard owing to their low melting point and crystalline (Możejko-Ciesielska and Kiewisz, 2016). On the other side, the mcl-PHAs are more flexible and elastic than the others because of their elastomer's thermal characteristics and low melting point and glass transition properties, with temperatures ranging from 39 to 61°C and -43 to -25°C, respectively. Furthermore, they have a meagre degree of crystalline, about 25%, compared to Scl-PHAs and

conventional plastics (Akaraonye *et al.*, 2010; Możejko-Ciesielska and Kiewisz, 2016) Table 2-1 shows PHA and fossil oil-based plastic (polypropylene) properties.

Table 2-1: The comparison of the properties of PHA and conventional plastic

Properties	scl-PHA	mcl-PHA	Conventional plastic
Melting point (°C)	53-80	30-80	176
Crystalline (%)	40-80	20-40	70
Density (g/cm ³)	1.25	1.05	0.91
Tensile strength (Mpa)	43-04	20	34
Extension to break (%)	6-1000	300-450	400
Glass transition temp (°C)	-148-4	-40-150	-10
Solvent resistant	poor	poor	poor
Biodegradability	good	good	none
UV resistant	good	good	poor

Adopted from (Akaraonye *et al.*, 2010).

This work provides an overview of using lipid-rich oil waste materials as a feedstock for the bacteria to produce and accumulate polyhydroxyalkanoate. It also provided simple and environmental extraction, which is the next step and remains a significant challenge in the production process of PHA. However, developing a cost-effective approach to producing bioplastic PHAs would be of utmost importance. So, it is essential to look for low-cost value carbon substrates which increase the yield and productivity of the PHA polymers or simplify the process of selecting an organism with distinct characteristics of producing high yield and high productivity. For example, *Pseudomonas oleovorans* were chosen here as PHA-producing bacteria because they can produce high yields under many growing conditions. Moreover, they also offer bioprocess advantages that

could increase the low-cost production of PHA compared to *Cupriavidus necator* and other types of bacteria.

Furthermore, because of their potential to produce MCL-PHAs with various carbon substrates and their adaptability to generate a high growth rate, these significantly reduce the production costs and the need for high-grade carbon feedstocks (Borrero-de Acuña *et al.*, 2014; Mahato *et al.*, 2021). Finally, *Pseudomonas* showed promising PHA and are well-known producers of MCL-PHAs using various carbon substrates. Currently, this type of bioplastics has an advantage in terms of applications. Furthermore, medium-chain length groups are more suitable because of their properties that are required for specific uses, as in the biomedicine field over short-chain length polyhydroxyalkanoates (SCL-PHAs) (Rai *et al.*, 2011; Rigouin *et al.*, 2019). However, the main goal here in this study is to develop a method that could be used widely for producing PHA from *Pseudomonas oleovorans* rather than any other microorganisms.

Fatty acids and oils have been effectively used to produce PHAs with a specific composition. Therefore, to continuously produce PHA of the desired quantity and quality, it is vital to understand how the process, the substrates, and the feeding strategy relate to the final polymer content and properties. Though many carbon substrates are explored and obtained, the PHA followed and related to polymer compositions and characteristics. However, fat, oil, and gas (FOG), frying soybean oil, glycerol, and frying palm oil have not been carried out within the context of PHA production and compared their impact in terms of PHAs accumulation yield and productivity by *Pseudomonas oleovorans*.

2.4 PHA Biodegradability

The best differentiating quality of bioplastic PHAs from fossil oil-derived non-degradable plastics is their biodegradability (Sasidharan and Bhat, 2014). Therefore, biodegradation means to convert organic materials into minor compounds such as water, carbon dioxide and organic matter (biomass) by living microorganisms within standard environmental conditions. However, the process

is three-way; the first is the mechanical weakening of the object structure (deterioration), then the microbial breakdown of the object materials (fragmentation), and finally, the forming of the new compound (assimilation). So, the beauty characteristic of bioplastic PHAs is their biodegradability in typical environments. Even though the rate of their degradation depends on various conditions like temperature, pH, nutrient supply, composition, molecular weight, crystallinity, microbial activity, and surface area of the exposure of the polymers themselves (Khanna and Srivastava, 2005; Reddy *et al.*, 2003; Sudesh *et al.*, 2000). Moreover, these conditional properties are classified into two (2) significant groups: Environmental and PHA polymer properties.

However, bioplastic PHA can degrade by itself, taking a sufficient shelf-life time. In addition, microorganisms can degrade bioplastic PHAs by the enzymatic action on high molecular PHAs, which is done by secreting the PHA depolymerase (Reddy *et al.*, 2003; Verlinden *et al.*, 2007). Furthermore, many microorganisms (bacteria and fungi) in the soil, sludge and seawater have been reported to excrete extracellularly PHA degrading enzymes to hydrolysed PHA into water-soluble monomers later into the products as nutrients in the cells (Sudesh *et al.*, 2000). However, their biodegradation is both in aerobic and anaerobic conditions; aerobic conditions result in water and carbon dioxide, while anaerobic produce carbon dioxide and methane (Reddy *et al.*, 2003).

2.5 Biosynthesis of PHA

Polyhydroxyalkanoates are the suitable and best-known bioplastics synthesized and stored by many microorganisms such as fungi, algae, and bacteria from renewable carbon resources. Bacteria have been the potent producers of PHAs, and many types have been discovered and studied. More than 300 gram-positive and gram-negative bacteria (G+ve & G-ve) are identified and efficiently capable of producing bioplastic PHA. Most studies are on *Pseudomonas* species, *Bacillus* species, *Methylobacterium* species, *Cupriavidus necator*, *Enterobacter* species, *Azotobacter vinelandii*, *Rhizobium* species, *Escherichia* species, *Klebsiella* species, *Azomonas macrocytogenes*, *Azotobacter beijerinckii*, *Protomonas*

extorquens and many more too numerous to mention (Chee *et al.*, 2010; Du *et al.*, 2012; Javaid *et al.*, 2020; Varghese *et al.*, 2022). In addition, new and beneficial species that are studied presently comprise *Stenotrophomonas* species, *Xanthomonas* species, and *Haloarchaea* species, among the few listed here (Javaid *et al.*, 2020).

Furthermore, the microbes are categorized into two (2) different kinds based on the conditions of the culture of the fermentation process, which stimulate the accumulation of PHAs. Some need inadequate potential nutrients, but an excess carbon source for various processes of producing PHAs were *P. oleovorans*, *C. necator* and *P. extorquens*. The other groups no longer need to limit nutrients to PHAs' production during their fermentation process and include *A. latus* and *A. vinelandii*. However, these conditions of the fermentation culture of producing PHAs are the requirements within both groups to produce a high PHA accumulation yield. In addition, another class of microorganisms need a standard concentration of salt to produce PHAs, such as Halomonadaceae groups, and an example of this group is *Halomonas* strains (Varghese *et al.*, 2022). Hence, there are various factors to be considered in the process of selection of microorganisms to achieve high and large production of bioplastic PHA, including the ability of the organism to utilize the inexpensive and waste carbon feedstock, the growth rate of the organism, the polymer production rate, and their highest accumulation of the synthesize polymer yield (Lee, 2000; Lee *et al.*, 2000). Therefore, the process of PHA biosynthesis can be grouped into threefold main synthetic pathways: Pathway I contain three critical enzymes in *Ralstonia eutropha*, and pathway II is related to fatty acid used by *Pseudomonas* species and *A. hydrophila*. However, pathway III requires 3-hydroxyacyl-ACP to develop PHA monomer and produce PHA by the act of PHA synthase. The well-known enzymes involved are 3-ketoacyl-CoA reductase, epimerase, and (R)-enoyl-CoA hydratase (Chen, 2010; Laycock *et al.*, 2013; Mannina *et al.*, 2020). Pathways I and III are sugars fermentable, while pathway II uses fatty acids for growth and PHA production, as shown in Fig. 2-2 and 2-3 respectively.

Furthermore, polyhydroxyalkanoate biosynthesis involves various enzymes encoded by many genes responsible for PHAs formation (Reddy *et al.*, 2003). The significant enzyme to produce PHAs is PHA synthase, and it is grouped into four types based on the preferred carbon, sequence, and structure of the amino acid present. Group I and II are dominated by subunit PhaC, while group III has two subunits such as PhaC and PhaE, the group IV is PhaE which resembles group III. Although the first PHA discovered is polyhydroxybutyrate (PHB), the most studied type of polyhydroxyalkanoates (Verlinden *et al.*, 2007), equally, the most studied metabolic pathway is the one found in *Ralstonia eutropha* comprising three enzymes: β -ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase encoded by *phaA*, *phaB* and *phaC* genes in that order (Chen, 2010; Laycock *et al.*, 2013; Mat *et al.*, 2008).

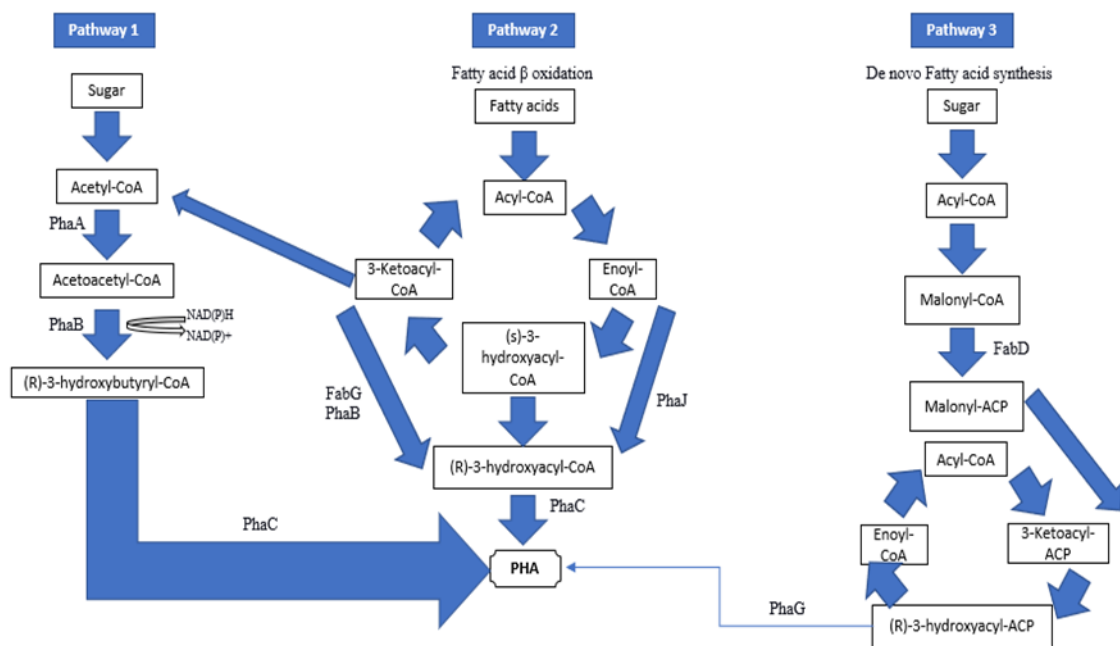


Figure 4-2: The three main biosynthesis pathways in PHA.

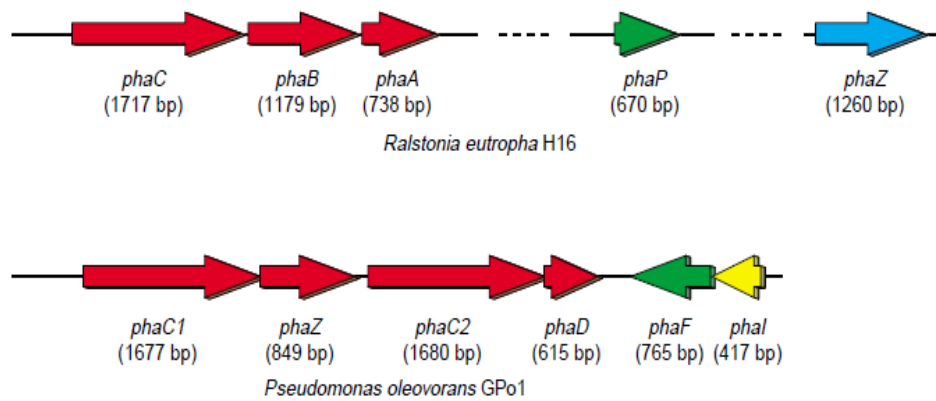


Figure 2-3: PHA enzyme in *C. necator* and *P. oleovorans*: (Luengo *et al.*, 2003)

2.6 PHA extraction (post-processing)

PHA is an intracellularly material. Therefore, its extraction and purification from other cell biomass are complicated and expensive (Chee *et al.*, 2010). Numerous techniques for separating and purifying PHA are reported, such as solvent extraction, chemical or enzymatic action disruption, mechanical methods, or their combination (Koller, 2020; Kunasundari and Sudesh, 2011). Each has its advantages and disadvantages (Kumar *et al.*, 2020). These methods are categorized into two main classical approaches: a) solubilize PHAs using specific solvents, known as the solvent extraction, followed by precipitation, and b) solubilize non-PHAs cellular material and maintain PHAs granules intact, known as the cellular lysis method. The solvent extraction includes halogenated solvents, carbonates, alkanes, esters, alcohols, and ketones, while the cellular lysis comprises an acid and alkaline, surfactants, oxidants, and enzyme (Pagliano *et al.*, 2021). However, the properties and qualities of the polymers depend on the chosen method, especially their molecular weight. Therefore, developing a technique that permits efficient extraction of pure polymer, reduces the cost of production, and minimizes risk to the environment and operators is required (Karine *et al.*, 2021). The most widely applied methods for polymer extraction are described here.

2.6.1 Solvent extraction methods

Solvent extraction is the most effective method for extracting PHA from its biomass components. It involves the modification of the cell membrane permeability, releasing and dissolution of the PHA, followed by the precipitate process (Jiang *et al.*, 2011; Kunasundari and Sudesh, 2011). The most understandable technique in solvent extraction is by using chloroform, then using other halogenated hydrocarbon solvents like dichloromethane, dichloroethane, and chloropropane (Chee *et al.*, 2010; Jiang *et al.*, 2018; Keshavarz and Roy, 2010). Whereas its precipitation is usually induced by non-solvent reagents such as methanol, hexane, acetone, and ethanol for achieving purely high PHA (Koller *et al.*, 2013). This method has some advantages over other PHA polymer extraction methods. For example, the method offers high productivity and removes bacterial endotoxins with minimal damage to the polymer (Kunasundari and Sudesh, 2011; Mannina *et al.*, 2019, 2020). However, large-scale solvent extraction is usually seen as an environmental and human health risk method (Chee *et al.*, 2010; Koller, 2020; Kunasundari and Sudesh, 2011).

Additionally, the method involves a large amount of solvents, and it needs like 20 times per volume of the polymer and about 10 times the antisolvent volume for the precipitation process (Mannina *et al.*, 2020). Therefore, it is a costly and highly operative method (Dutt Tripathi *et al.*, 2021). Furthermore, solvent like chloroform is more irritating to the respiratory tract, eyes, and mucus membrane and is believed to be carcinogenic. In addition, solvent extraction typically destroys the characteristic nature of polymer by decreasing its molecular weight, especially at high temperatures and long extraction periods (Koller, 2020). However, many less toxic non-halogenated solvents such as methyl ethyl ketone, cyclohexanone, cyclic carbonic esters, and many more have been testified to reduce the problem of solvent extraction and production cost (Jiang *et al.*, 2018). Therefore, it is more efficient and cost-effective for the extraction of PHA to use inexpensive and green systems. In addition, the most acceptable and economical extraction method is the one with a high yield and purified extracted PHA with no decrease in molecular weight (Anis *et al.*, 2013b).

2.6.2 Chemicals and enzyme methods

Many chemicals have been reported, including sodium hypochlorite, alkaline, sodium dodecyl sulfate, and many others (Mannina *et al.*, 2019, 2020), to recover PHA from cell biomass. Sodium hypochlorite has been used to separate PHA from cell biomass, and it is rapid and straightforward. In addition, a strong oxidizing chemical is used to dissolve non-PHA cell mass (NPCM) and not the PHA (Dutt Tripathi *et al.*, 2021). Therefore, using sodium hypochlorite has a lot of advantages, such as saving time and energy during the process, and this reduces the costs of the recovery procedure. Another advantage is obtaining high purity of about 96% after extracting the PHA polymer (Mannina *et al.*, 2019, 2020). However, it is related to some degradation issues and a lower molecular weight of about 50% reduction (Chee *et al.*, 2010; Dutt Tripathi *et al.*, 2021). Using sodium dodecyl sulfate (SDS) will reduce the PHA polymer's degradation problem, but it gives lower purity of the polymer.

The advantage of SDS is disrupting the organism's cells and extracting the PHA polymer without degradation. But it also has disadvantages, like a lot of energy is needed due to the high volume of SDS to biomass fraction, which leads to producing a large amount of effluent and a high cost of extraction (Mannina *et al.*, 2019, 2020). However, using sodium hypochlorite or SDS alone does not give as much quality of the achieved PHA. However, good high-quality molecular weight is obtained in combining SDS and sodium hypochlorite compared to different methods of using either sodium hypochlorite or sodium dodecyl sulfate. But, combining these two chemicals has a high recovery yield (Kumar *et al.*, 2020). Using enzymes such as proteases, cellulose, and lysozyme is another technique for removing PHA from cell biomass, which needs gentle separation. This technique is specific to slight PHA degradation, resulting in high recovery and purification of the polymer (Chee *et al.*, 2010; Koller *et al.*, 2013). Furthermore, combining enzymes with several chemicals such as Alcalase, ethylene diamine tetraacetic acid (EDTA), and proteinase K increases PHA extraction purity (Jacquel *et al.*, 2008; Yasotha *et al.*, 2006).

2.6.3 Extraction Process used.

The primary objective of the extraction process is to recover Polyhydroxyalkanoates (PHAs) from the non-PHA cell mass (NPCM) while keeping the recovered material highly pure (Kurian and Das, 2021). The focus is on establishing and applying a method that can efficiently extract PHA from non-PHA cellular constituents, promising that the extracted PHA is very pure. Solvent extraction is a widely employed method in polymer extraction using halogenated solvents like chloroform (Jacquel *et al.*, 2008). Although this technique has high product purity and recovery, its practical application is limited to laboratory-scale. This chemical use poses environmental issues, and further emphasizes the need for scalable and ecologically friendly alternatives (Jacquel *et al.*, 2008; Pagliano *et al.*, 2021).

To address these issues, non-chlorinated solvents have been considered as possible alternatives to the halogenated solvents (Jiang *et al.*, 2018). Other alternative approaches, such as mechanical, alkali, or enzyme, have also been studied as feasible and an economically beneficial method of extracting PHA (Chee *et al.*, 2010; Koller, 2020; Kunasundari and Sudesh, 2011). However, the investigation of sustainable and comparative evaluation methods in extracting Polyhydroxyalkanoates (PHAs) using sodium hydroxide, enzyme, and hydrogen peroxide-based techniques as high-yield PHA recovery and low-cost substitutes has not been extensively carried out.

2.6.3.1 Sodium hydroxide (NaOH)

PHAs are biodegradable polymers that many microorganisms produce and function as energy and carbon storage materials (Ana *et al.*, 2022; Gobi and Vadivelu, 2015; Godbole, 2016). Bacteria can accumulate the PHA in amounts as high as 90% of the cell's dry weight (Du *et al.*, 2012). Removing the NPCM than the PHA can be more valid by dissolving them in a suitable solvent (Kourmentza *et al.*, 2017). As a result, the NPCM was dissolved using various methods to extract the PHA polymer, including sodium hydroxide (Mannina *et al.*, 2020).

Sodium hydroxide (NaOH) is commonly used to extract polyhydroxyalkanoates (PHAs) due to its alkaline nature and ability to break down cell membranes and solubilize the PHAs. Bacterial cells are harvested after fermentation and treated with NaOH during extraction. The harvested biomass is subjected to a cell disruption step to release the intracellular components, including PHA polymer. Sodium hydroxide (NaOH) is added to the biomass suspension to break down the bacterial cell walls and membranes. This can be achieved by incubating the biomass in an alkaline NaOH solution at an appropriate temperature. For instance, 1 M NaOH at 50°C with gentle mixing for 10 minutes may be used as part of a treatment plan for PHA recovery (Gumel *et al.*, 2013). The technique produces high yields and purity of the polymer while reducing recovery costs by about 90% (Madkour *et al.*, 2013a).

The extraction of PHA using sodium hydroxide (NaOH), can be a reliable, affordable, and environmentally friendly alternative to using chlorinated substances. Sodium hydroxide treatment for PHA recovery has moreover been seen as more economically viable than an approach based on organic solvents (Ana *et al.*, 2022; Mannina *et al.*, 2020). The lipids in the bacteria's cell wall are saponified by hydroxides, increasing the membrane permeability and allowing the release of proteins and non-PHA cell components (Mannina *et al.*, 2020; Pérez-Rivero *et al.*, 2019).

After the PHAs have been solubilized and removed from the cell debris, further purification steps may be required to create a purified PHA polymer. These steps start with neutralizing the sodium hydroxide solution to isolate the PHAs in the final state. Moreover, the use of the sodium hydroxide method as an alternative to the chlorinated method for PHA recovery is supported by its effective, easy, non-toxic, and ecologically friendly caused only minor degradation of the polymer molecular weight (Mw) (Ana *et al.*, 2022).

2.6.3.2 Hydrogen peroxide (H₂O₂)

There are many benefits to using hydrogen peroxide in the polymer extraction process. Hydrogen peroxide removes unwanted cellular components and cell

debris during the extraction process and aids in solubilizing the PHA polymer. Furthermore, due to hydrogen peroxide's strong-oxidizing properties, the PHA solution can be purified of contaminants such as proteins, pigments, and other impurities. Even though many well-known and effective extraction methods are available, hydrogen peroxide extraction of polyhydroxyalkanoates (PHAs) from microbial biomass has been considered as a viable technique for effective PHA extraction and recovery. This procedure entails the oxidative breakdown of the cellular constituents, notably proteins and nucleic acids, followed by the isolation of PHA using precipitation methods.

2.6.3.3 Enzyme (Lysozyme)

Enzymes are highly active in hydrolysis processes, using enzymes such as lysozyme, lipases, proteases, nucleases, etc. PHA has been recovered using an enzyme-based method of digestion, which causes no or negligible degradation of PHA by lysis of the bacteria cell and exerting high hydrolytic effects on proteins and other non-polymer materials (Pérez-Rivero *et al.*, 2019). They are attractive candidates for PHA recovery due to their great selectivity and other benefits like low energy requirements, biological specificity, tolerant operating conditions, and minimal capital expenditure requirements (Gonzalez *et al.*, 2021; Kosseva and Rusbandi, 2018; Madkour *et al.*, 2013b). Enzymes can improve the efficacy and purity of the PHA extraction process (Gumel *et al.*, 2013), making it a promising strategy for the large-scale production of PHAs as sustainable bioplastics. However, the cost of using other enzymes generally prohibits their usage in large-scale extraction processes. In addition, cell wall-digesting enzymes are typically microorganism-specific; therefore, a case-based analysis is required (Gonzalez *et al.*, 2021; Gumel *et al.*, 2013).

2.7 Polymer Characterisation

Polymer characterization is based on their physicochemical properties such as molecular weight (Mw), melting temperature (Tm), glass transition (Tg), and molecular number (Mn). The most significant factor influencing the properties of PHAs is molecular weight, as reported by Mozejko and Ciesielski, (2013). REHM,

(2003), reported that factors such as the level of active PHA synthase expression, the type of PHA synthases, the presence of enzymes that hydrolyse PHAs, and the availability of precursors for PHA synthesis are the most influential factors that determine the molecular weight of PHAs (REHM, 2003).

Nuclear magnetic resonance spectroscopy (NMR), gel permeation chromatography (GPC), differential scanning calorimetry (DSC), Fourier transform infrared (FTIR), thermogravimetric analysis (TGA), and tensile strength testing are methods used to characterize the extracted polymers (Wong *et al.*, 2012).

2.7.1 Thermogravimetric analysis (TGA)

This provides information on physicochemical properties, including decomposition temperatures and thermal stability (Redfern, 2008). It is an analysis that measures weight variations in relation to temperature (Liu and Yu, 2006). Thermogravimetric analysis is used to assess detailed properties of the polymers, including thermal degradation and heat stability, and to validate polymer purity (Lorini *et al.*, 2021; Liu and Yu, 2006; Zhang *et al.*, 2009).

2.7.2 Gas chromatography mass spectrometry (GC-MS)

This is an analytical technique that used in analysis the monomeric structure of the biopolyesters with high degree of sensitivity (Samrot *et al.*, 2021). Braunegg *et al.* (1978) found PHA in bacterial cells using GC, in which the cells were treated with a solution comprising concentrated sulfuric acid, methanol, and chloroform to produce methanolysis, which was subsequently identified by GC. However, a modified version of this procedure is presently used to transform the PHA molecule into alkyl esters of PHA monomers, which are then examined using GC-MS analysis (Samrot *et al.*, 2021). It was reported that sulphuric acid and methanol were used to treat the PHA, after which the methanolysis PHAs underwent GC-MS analysis. The two isolates' respective molecular weights were determined to be 242 kDa and 256 kDa (Braunegg *et al.*, 1978). This technique has made it possible to rapidly identify numerous scl-PHAs and mcl-PHAs without

the need for reference standards (Bhuwal *et al.*, 2013). Gas chromatography-mass spectrometry (GC-MS) is an effective and widely used technique for detecting and determining the accuracy of PHA (Samrot *et al.*, 2021).

2.7.3 Fourier Transform Infrared

This is used to identify the functional groups, bonding types, and other components of the PHA (Leong *et al.*, 2017b, 2017a). The crystallinity and the type of PHA were confirmed from the FTIR spectroscopy analysis (Kansiz *et al.*, 2007).

2.7.4 Scanning Electron Microscope

The surface morphology, size, structure, and chemical composition of polymer can all be examined using scanning electron microscopy, which offers high-resolution imaging (Samrot *et al.*, 2021; Barnes *et al.*, 2002).

2.8 PHAs applications and their uses

In addition to their potential replacement for synthetic plastics like polypropylene and polyethylene, PHAs are the most outstanding candidates for many uses because of their excellent properties, including completely biodegradable, thermoplastic, and non-toxicity in living cells. These properties make them a model candidate for a broad spectrum of applications (Luef *et al.*, 2015).

Numerous applications have been described for PHA since the initial production of Biopol by ICI Ltd in 1982. PHAs primarily used in packaging, automobiles, agriculture, and biomedicine (Luef *et al.*, 2015; Możejko-Ciesielska and Kiewisz, 2016; Ray *et al.*, 2016). They are also used in manufacturing and chemical industries in making cosmetic containers like shampoo bottles, moisture barriers, latex paints, herbicides, and insecticides. They are also used in making ultra-strong fibres in fishery industries (Huschner *et al.*, 2015; Możejko-Ciesielska and Kiewisz, 2016). In the medical field, PHAs are used in fracture fixation, implants, drug-delivery systems, wound sutures, dressings, radio-potentiator, and in making disposable medical devices such as syringes, surgical kids, and gloves.

Moreover, they are used in orthopaedic areas like bone plating, spinal cages, and many other uses within this field area. Besides, they are also used in the power generation and textile industries (Możejko-Ciesielska and Kiewisz, 2016; Priyadarshi *et al.*, 2014).

However, numerous companies are currently manufacturing because of the bioplastic PHA's excellent properties and use. Some notable companies include Biocycle PHB industrial SA (Serrano, SP, Brazil), Procter and Gamble Co. Ltd (Cincinnati, OH, USA), Tianjin green bioscience Co. Ltd (Tianjin, China), kanaka (Takasgo, Japan), EHT (Switzerland), biomer (Germany), Tainan biologic Material Co., Ltd. (Ningbo, China) and Metabolix® (Woburn, MA, USA) (Jiang *et al.*, 2016; Możejko-Ciesielska and Kiewisz, 2016) Table 2-2 details the uses and applications of polyhydroxyalkanoate in various areas.

Table 4-2: Uses and applications of polyhydroxyalkanoate in various areas.

Application Field	Uses	References
Agriculture	Controlled release of insecticides, herbicides, and fertilizers (NODAX used as a coating for urea fertilizers that are used in rice fields)	Philip <i>et al.</i> , (2007)
Hygiene Products	Diapers, sanitary napkins, training pants, and bandages	Wang <i>et al.</i> , 2007
Medical	Scaffold material, bone plates, osteosynthetic materials, surgical sutures, swabs, dressing materials for surgery, pericardial patches, artery augments, cardio-logical stents, vascular grafts, heart valves, implants, controlled release of drugs and hormones	Zinn <i>et al.</i> , (2001); Philip <i>et al.</i> , (2007)
Packaging	These include food packaging, plastic beverage bottles, water-resistant paper and cardboard, foils, films, and diaphragms.	Philip <i>et al.</i> , (2007)
Miscellaneous	These are food additives, pressure sensors for keyboards, stretch and acceleration measuring instruments, material testing, shock wave sensors, lighters, gas lighters, microphones, ultrasonic detectors, sound pressure measuring instruments, headphones, loudspeakers, the substrate for denitrification of wastewater, starting materials for other chemicals.	Zinn <i>et al.</i> , (2001). Hiraishi and Khan (2001) Philip <i>et al.</i> , (2007)

2.9 Microorganisms producing PHAs.

The microorganisms used for PHA production cover many bacteria, fungi, and algae. However, have been the most studied and potent producers of bioplastic PHAs. Numerous species of bacteria such as *Ralstonia eutropha*, *Pseudomonas* species, *Alcaligeneslatus*, *Bacillus* species, *Aeromona s* species, *Methylobacterium* species, *Cupriavidusnecator*, *Enterobacter* species, *Escherichia* species, *Rhizobium* species, *Azotobactervinelandii*, *Klebsiella* species, *Azotobacter macrocytogenes*, *Azotobacter beijerinckii*, *Protomonas extorquens*, and many more too numerous to mention have been studied for efficient capacity in producing polyhydroxyalkanoates (Chee *et al.*, 2010; Du *et al.*, 2012; Javaid *et al.*, 2020; Varghese *et al.*, 2022). In addition, new and beneficial species which are studied presently comprise *Stenotrophomonas* species, *Xanthomonas* species, *Staphylococcus* species and *Haloarchaea* species, among the few listed here (Javaid *et al.*, 2020). However, the produced PHA type from these bacteria (i.e., SCL-PHAs or MCL-PHAs) depends on the bacteria used, and the medium constituents, even the carbon resource, are the same (Andler *et al.*, 2021; Raza *et al.*, 2018).

Furthermore, the production rate of PHA also relies on the bacteria's carbon sources, nutrient concentration, and metabolic pathways. Many gram-negative and gram-positive bacteria like *Aeromonas hydrophila*, *Alcaligenes latus*, *Pseudomonas* strains, *Cupriavidus necator*, *Bacillus* species, and *Methylobacterium* species naturally synthesize polyhydroxyalkanoates (Du *et al.*, 2012; Keshavarz and Roy, 2010). The microorganisms are categorized into two groups based on the culture medium conditions that stimulate the PHA accumulation. The first group needs limited nutrients like oxygen, nitrogen and phosphorous, with excess carbon sources for efficient PHA production; such organisms include *Pseudomonas* strains, *Cupriavidus necator* and *Protomonas extorquens*. Whereas the second group does not require the nutrients limitation for them to produce and accumulate the polyhydroxyalkanoates during their fermentation process effectively, such groups include *Alcaligenes latus*, recombinant *Escherichia coli*, *Azotobacter vinelandii* and many other families

(Chee *et al.*, 2010; Choi and Lee, 1999; Khatami *et al.*, 2021). In addition, another class of microorganisms need a standard concentration of salt to produce PHAs, such as Halomonadaceae groups, and an example of this group is *Halomonas* strains (Varghese *et al.*, 2022).

Many living organisms, such as plants, bacteria, fungi, and algae, produce polyhydroxyalkanoates. However, microorganisms are more suitable and sustainable for industrial PHA production since plant cells can only accumulate and give low PHA yields, like less than 10% (w/w), without adversely affecting their growth rate. But microorganisms store polyhydroxyalkanoates at levels as high as 90% (w/w) of their cell dry weight (Mohan Nair *et al.*, 2013; Reddy *et al.*, 2003; Silva *et al.*, 2021; Verlinden *et al.*, 2007). Some significant studied strains are *Alcaligenes species*, *Pseudomonas species*, *Rhodopseudomonas palustris*, *Escherichia coli*, *Bacillus species*, *hydrophila*, as well as *Burkholderia sacchari*, *Aspergillus eutropus* and *Halomonas bolovienis* (Sehgal and Gupta, 2020; Verlinden *et al.*, 2007). More than 300 different microorganisms were identified and efficiently able to synthesize and accumulate PHA, but only a few can produce the polyhydroxyalkanoates on a large scale (Chee *et al.*, 2010). Numerous bacteria, including *Pseudomonas oleovorans*, *Pseudomonas putida*, *Aeromonas hydrophila*, *Cupriavidus necator*, and others, have been observed to produce PHA (Wong *et al.*, 2012), as summarised in Table 2-3.

Table 2-3: Bacteria and their species are used for PHA production.

Genus	Strain	References
<i>Pseudomonas</i>	<i>Pseudomonas</i> sp. AK-3 (MW89432)	(Muneer <i>et al.</i> , 2022)
<i>P. mediterranea</i> 9.1 (CFBP 5447)	<i>Pseudomonas</i> sp. GL06	(Szacherska <i>et al.</i> , 2022)
	<i>Pseudomonas oleovorans</i>	(Aires <i>et al.</i> , 2014) (Aremu, Ishola and Taherzadeh, 2021) (Chmelová <i>et al.</i> , 2022)
	<i>Pseudomonas aeruginosa</i>	(Mahato, Kumar and Singh, 2021) (Saralegui <i>et al.</i> , 2022) (de Oliveira <i>et al.</i> , 2021)
	<i>Pseudomonas resinovorans</i>	(Jeon <i>et al.</i> , 2022) (Follonier <i>et al.</i> , 2014)

	<i>Pseudomonas putida</i>	(Liu <i>et al.</i> , 2022) (Zong <i>et al.</i> , 2022) (Guzik <i>et al.</i> , 2022) (Manoli <i>et al.</i> , 2022) (Poblete-Castro <i>et al.</i> , 2014)
	<i>Pseudomonas entomophila</i>	(Ma <i>et al.</i> , 2022)
	<i>Pseudomonas chlororaphis</i>	(Aloui <i>et al.</i> , 2022) (Muhr <i>et al.</i> , 2013)
	<i>Pseudomonas helmanticensis</i>	(Kondratyev <i>et al.</i> , 2022)
	<i>Pseudomonas sp.</i> I01	(Mozejko and Ciesielski, 2013)
	<i>Pseudomonas mediterranea</i> 9.1 (CFBP5447)	(Pappalardo <i>et al.</i> , 2014)
<i>Cupriavidus</i>	<i>Cupriavidus necator</i> H16	(Loan <i>et al.</i> , 2022) (Jawed <i>et al.</i> , 2022)
	<i>Cupriavidus USMAA2-4</i> <i>malaysiensis</i>	(Wong <i>et al.</i> , 2022)
	<i>Cupriavidus H16CCM3726</i> <i>necator</i>	(Novackova <i>et al.</i> , 2022)
	<i>Ralstonia eutropha</i>	(Gutschmann <i>et al.</i> , 2021)
	<i>Cupriavidus necator</i> DSM545	(Rodríguez-Contreras <i>et al.</i> , 2015) (Brojanigo <i>et al.</i> , 2022)
<i>Bacillus</i>	<i>Bacillus megaterium</i> R11	(Zhang <i>et al.</i> , 2013)
	<i>Bacillus velezensis</i> BV-HSTU-FPP	(Akhter <i>et al.</i> , 2022)
	<i>Bacillus megaterium</i> DSM509	(Shahid <i>et al.</i> , 2013)
	<i>Bacillus megaterium</i> MSB04	(Sathiyarayanan <i>et al.</i> , 2013)
	<i>Bacillus cereus</i>	(Sharma and Bajaj, 2015) (Alemán-Huerta <i>et al.</i> , 2022)
	<i>Bacillus thuringiensis</i>	(Ray and Kalia, 2017)
	<i>Bacillus megaterium</i> ALA2	(Khomlaem <i>et al.</i> , 2023)
	<i>Bacillus subtilis</i> BS-HSTU-F	(Akhter <i>et al.</i> , 2022)
	<i>Bacillus sp.</i> LPPI-18	(Mohammed and Ray, 2022)
	<i>Bacillus cepacia</i> B1-2	(Xu <i>et al.</i> , 2022)
<i>Escherichia coli</i>	<i>Escherichia coli</i> MG1655	(Zhu, Ai and Jia, 2022)
	<i>Escherichia coli</i> DH5α	(Meng <i>et al.</i> , 2022) (Saito <i>et al.</i> , 2022)
	Recombinant <i>E. coli</i>	(Satoh <i>et al.</i> , 2022)
	<i>E. coli</i> BL21 (DE3)	(Shi <i>et al.</i> , 2022)
	<i>Escherichia coli</i> JM109	(Saito <i>et al.</i> , 2022)

<i>Azotobacter</i>	<i>Azotobacter vinelandii</i>	(Mok et al., 2021)
	<i>Azotobacter vinelandii</i> N-15	(Semeniuk et al., 2020)
	<i>Azotobacter vinelandii</i> OP (ATCC13705)	(Millán et al., 2016)
	<i>Azotobacter vinelandii</i> ATCC12837	(Mok et al., 2017)
	<i>Azotobacter vinelandii</i> OPNA	(Castillo and Segura, 2013)
	<i>Azotobacter chroococcum</i> 7B	(Bonartsev et al., 2016)
	<i>Azotobacter</i> sp. FA-8	(Mezzina et al., 2015)
	<i>Azotobacter vinelandii</i> UWD (ATCC53799)	(Priester et al., 2014)
<i>Burkholderia</i>	<i>Burkholderia sacchari</i>	(Cesário et al., 2014)
	<i>Burkholderia thailandensis</i> E264	(Kourmentza et al., 2018)
	<i>Burkholderia sacchari</i> LFM 101	(Mendonça et al., 2014)
	<i>Burkholderia cepacia</i>	(Keenan et al., 2006)
	<i>Burkholderia sacchari</i> DSM 17165	(Ashby et al., 2022)
	<i>Burkholderia cepacia</i> JC-1	(Chin et al., 2022)
	<i>Burkholderia cepacia</i> DSM 5018	(Khatami et al., 2022)
<i>Alcaligenes</i>	<i>Alcaligenes latus</i> DSM 1124	(Gahlawat and Srivastava, 2017)
	<i>Alcaligenes denitrificans</i>	(Yajima et al., 2020)
	<i>Alcaligenes</i> sp. NCIM 5085	(Tripathi et al., 2019)
	<i>Azohydromonas lata</i>	(Penloglou et al., 2017) (Ashby et al., 2022)
<i>Enterobacter</i>	<i>Enterobacter cloacae</i>	(Kingsly et al., 2022)
<i>Halomonas</i>	<i>Halomonas pacifica</i> ASL10	(El-malek et al., 2020)
	<i>Halomonas salifodiane</i> ASL1	(El-malek et al., 2020)
	<i>Halomonas campisalis</i> MCM B-1027	(Kulkarni et al., 2015)
	<i>Halomonas elongata</i> DSM258	(Ilham et al., 2014)
	<i>Halomonas bluephagenesis</i>	(Ma et al., 2020)
	<i>Halomonas ventusta</i> KT832796	(Stanley et al., 2018)

2.10 *Pseudomonas* species producing PHAs.

Pseudomonades are gram-negative (G-ve), non-spore-forming, rod-shaped, and aerobic bacterial groups. In addition, *Pseudomonas* species are widely spread in soil, water, air, and vegetation. *Pseudomonas* play a vital environmental role, and they are the best studied bacterial species in research and industrial applications, comprising different described species (Lalucat *et al.*, 2022; Nikolaidis *et al.*, 2020). Many studies have been done on producing microbial polyhydroxyalkanoate using various microorganisms under lab scale, achieving 70–90% of the biomass weight as PHAs. Many microbes, including *Pseudomonas* species, can accumulate PHAs when grown in different substrates. However, *Pseudomonas* spp. can use many renewable carbon sources such as lipids, hydrocarbons, and agricultural feedstocks to produce mcl-PHAs (De Koning and Witholt, 1997; Sharma *et al.*, 2017b, 2017a).

These bioplastics, like polyhydroxyoctanoate and polyhydroxyhexanoate, are now preferred and advantageous over short chain length (SCL-PHAs) due to their distinctive properties, especially in medical applications (Mozejko-Ciesielska *et al.*, 2019; Tanikkul *et al.*, 2020). Moreover, PHAs show a wide range of properties ranging from stiff elastic to more durable, like synthetic plastics such as polypropylene (Liu *et al.*, 2021).

Due to their multipurpose and ability to use numerous carbon substrates, *Pseudomonas* species have become widely potent and desirable bioplastic PHAs producers use in many industrial biotechnological production processes (Mozejko-Ciesielska *et al.*, 2019). Furthermore, among the diverse types of bioplastics PHA-producing microbes, *Pseudomonas* strains gained much interest because of their potential ability to utilize and cultivate various carbon substrates. They are a versatile candidate for bioremediation and industrial production of biopolymers (Kanavaki *et al.*, 2021; Mozejko-Ciesielska *et al.*, 2019). Additionally, *Pseudomonas* species demonstrate a prevalent and excellent growth rate even at a low value and serve as excellent PHA producers with various carbon substrates to produce mcl-PHAs (Mahato *et al.*, 2021).

2.11 Biowaste materials used for PHA production.

Various factors, such as carbon substrates, PHA productivity, PHA content, PHA yield and recovery methods, enzyme activity and specificity, affect the overall economic production of PHA. Hence, large PHA production's economic viability depends on the efficiency of inexpensive carbon materials, microbial strains, and extraction and purification of PHA (Castilho *et al.*, 2009; Koller *et al.*, 2017). However, using high-grade food-based carbon feeds like food-grade oils, fatty acids, and sugars for polyhydroxyalkanoate production has contributed a lot to the total costs of about 50%, raising many concerns (Koller *et al.*, 2017; Vu *et al.*, 2020). Among these concerns is the sustainability of its production regarding its impact on food prices and food-feed competition since a vast amount needs to be exploited for a kilogram of PHA (Jiang *et al.*, 2016; Koller *et al.*, 2017). Furthermore, their high cost of production is about four to six times more than the costs of producing fossil oil-based synthetic plastics, making them more economically unattractive despite their many advantages (Johnston *et al.*, 2018; Kourmentza *et al.*, 2017). Thus, producing 1 kg of bioplastic PHA using glucose as a carbon source costs \$1.3 compared to \$0.34 when using hemicellulose hydrolysate. Accordingly, this condition of using expensive raw materials successfully became less attractive (Corchado-Lopo *et al.*, 2021). So, it is vital to use non-food-derived feedstocks like biowaste materials as a carbon source, which does not affect the human food chain, to boost their production sustainability (Jiang *et al.*, 2016; Koller *et al.*, 2017).

Furthermore, numerous renewable materials like simple and complex waste residues, including plant oils, fatty acids, and sugars, are available and suitable for microorganisms to utilize and produce polyhydroxyalkanoates (Chee *et al.*, 2010). Generally, these substrates are grouped into sugars, triacylglycerol, and hydrocarbons. Various bacteria utilize sugar and triacylglycerol, while many can utilize hydrocarbons like *Pseudomonas* species (Jiang *et al.*, 2016). A 50% reduction in PHA production cost was reported using biowaste materials as a fermentative carbon feeds (Kumar and Kim, 2018). Additionally, the production of bioplastic PHA using biowaste materials provides the dual advantage of

reducing disposal cost management and creating added valued products, which make them a vital innovation (Castilho *et al.*, 2009). However, using sugar like glucose and sucrose is the most common, and high productivity was achieved when glucose was utilized by *Cupriavidus necator* (Możejko-Ciesielska and Kiewisz, 2016).

Also, for PHAs production, vegetable oils are the most promising carbon feed due to their low-cost, abundance and high productivity of PHA polymer. For example, PHA production was reported using different vegetable oils such as purely oil, heated oil, and waste frying oil (Verlinden *et al.*, 2007), and they have achieved the yield of 1.2 g/g of the polymer from waste frying oil like that of glucose. Another study was reported using cooking oil with 66% PHA from *Pseudomonas aeruginosa* (Fernández *et al.*, 2005). Consequently, these cheap substrate materials must be abundant, qualitative, and adequate without off-season throughout their trends (i.e., consistently). Furthermore, the collection and transport of these substrates to a production or processing site should be accessible and straightforward (Koller *et al.*, 2017). However, the quantity and characteristics of the waste materials generated are vital components in developing robust and cost-effective production of chemical products, including PHA-bioplastic. Therefore, creating an efficient production technology to produce an economically friendly biodegradable polymer would be of utmost importance. Accordingly, looking for low-cost substrates that produce high-yield PHAs as substitutes for conventional feedstocks is essential. Many cheap and abundant carbon sources from agro-industrial residues have produced PHAs. However, hydrocarbon has also been achieved (Jiang *et al.*, 2016; Kaur *et al.*, 2017). Below is a detailed description of some raw materials used for the PHAs production process.

2.11.1 Sugars used for PHA production.

Sugars are classified into monosaccharides (simple sugar), disaccharides (three to six simple sugar) and polysaccharides (many sugars). The first two monosaccharides and disaccharides are directly fermented to obtain PHA.

Examples are glucose and sucrose/lactose, respectively. At the same time, polysaccharides are from the polymerization of simple sugar. Therefore, polysaccharides are not fermented to PHAs directly except to become hydrolysed into simple sugar (Jiang *et al.*, 2016).

2.11.1.1 Lignocellulosic waste materials

Lignocellulose is a complex and heterogeneous material containing about 35-50% cellulose, the most abundant sugar known as glucose. The second large sugar is xylose. It is the content of hemicelluloses which is around 20-35%. The last component is lignin, a complex aromatic structure resistant to the biochemical conversion process, and it is about 10-25% (Brodin *et al.*, 2017). Lignocellulose waste materials are the abundantly available, very cheap and principal source of renewable resources on the earth (Amin *et al.*, 2017; Yang *et al.*, 2019). They are non-food feedstock (i.e., they are not competing with food for humans), potentially producing many industrial and commodity products, including PHAs (Obruca *et al.*, 2015; Yin *et al.*, 2019). However, lignocellulosic materials have emerged as potential feedstock to replace fossil fuel resources due to their capacity to produce various kinds and value products. Lignocellulosic materials need hydrolysis and detoxication to convert into fermentable sugar by removing toxic substances (Khatami *et al.*, 2021; Obruca *et al.*, 2015). Still, these steps make utilizing lignocellulosic materials more complex and expensive. Hence, using the hot water pretreatment technique made it cheap and straightforward with no chemicals required, and it is an environmentally safe procedure. However, the most studied lignocellulosic materials are bagasse, wheat straw, rice straw, wastepaper, and wheat bran, which are too numerous to mention (Khatami *et al.*, 2021). PHA production from *Halomonas campisalis* MCN B-1027 utilized bagasse extract was reported by Kulkarni *et al.* (2015), and they achieved a PHA percentage yield of 47% cell dry weight. Another study revealed that using industrial waste orange peel from *Bacillus subtilis* OK2 can produce a PHA yield of 41% cell dry weight (Sukan *et al.*, 2014). Accordingly, a study was conducted using extruded rice bran to produce PHA from *Haloferax mediterranei* and obtained a high content of about 56% PHA (Huang *et al.*, 2006). Table 2-4 shows

the PHA production by different bacterial strain using lignocellulosic waste materials.

Table 2-4: Bacteria used for production of PHA from lignocellulosic waste.

Bacteria	Carbon source	Type of fermentation	Cultivation proced.	Type of PHA	period (h)	Temp (°C)	%PHA	PHA productivity (g/L/h)	References
<i>Pseudomonas putida</i> strain A514	Kraft lignin	Shaker flask	Batch process	mcl-PHA	72	30	73	0.16	(Lin <i>et al.</i> , 2016)
<i>Bacillus subtilis</i> OK2	Orange peel	Shaker flask	Batch fermentation	P(3H)		30	41	1.24	(Sukan <i>et al.</i> , 2014)
<i>Halomonas campisalis</i> MCM B-1027	Bagasse	Shaker flask	Batch process	PHB-co-PHV	72	37	47	-	(Kulkarni <i>et al.</i> , 2015)
<i>Ralstonia eutropha</i> DSM 545	Steam from wheat straw	Fermentor vessel	Fed-batch	PHB	48	30	83	9.4	(Romero <i>et al.</i> , 2019)
<i>Bacillus firmus</i> NII 0830	Rice straw	Shaker flask	Batch method	PHB	90	30	89	1.697	(Sindhu <i>et al.</i> , 2013)
<i>Haloferax mediterranei</i> ATCC 33500	Rice bran	Shaker flask	Fed-batch	PHBHV	90	37	55.6	0.42	(Huang <i>et al.</i> , 2006)
<i>Lysinibacillus</i> sp	Sugarcane bagasse	Shaker flask	Batch process	PHB	48	37	61.5	5.31	(Ganesh <i>et al.</i> , 2021)
<i>Ralstonia eutropha</i> NCIMB 11599	Wheat bran	Rotary shaker	Batch fermentation	PHB	72	30	62.5	0.255	(Annamalai and Sivakumar, 2016)
<i>Burkholderia sacchari</i>	Wastewater	Shaker	Batch process	PHB	48	30	44.2	3.63	(Al-battashi <i>et al.</i> , 2019)

2.11.1.2 Starch

Starch is obtained from cassava, potato, rice, corn, bread, sorghum etc. Starch has an advantage over glucose in terms of cost as a carbon source because it is less expensive (Koller *et al.*, 2010). According to, using hydrolysed starch (US \$0.22 kg) as substitute of glucose (US \$0.49 kg) would result in manufacturing costs on a scale of 100,000 tonnes of PHA per year dropping from US \$4.91 to US \$3.72 kg. However, starch needs hydrolysis to simple sugar before it uses to produce PHAs. Enzymatic or acid hydrolysis is the most common hydrolysis process in breaking down starch into sugar (Jiang *et al.*, 2016).

2.11.1.3 Molasse

Molasse is an essential waste material from sugar production using sugarcane or sugar beet. It is a potential low-costs carbon substrate for polyhydroxyalkanoate production (Du *et al.*, 2012). Molasse contains high sugar of around 54% (w/w), including sucrose and fructose, which the whole percentage between 62% and 38%, respectively, plus a small quantity of trace elements and vitamins like thiamine, riboflavin, pyridoxine and niacinamide (Gomaa, 2014). However, the annual global molasse output reaches about 50 million tons (Favaro *et al.*, 2019). In addition, it is a very cheap and abundant raw material with great potential to be used as a carbon feed for various industrial products like polyhydroxyalkanoates (Castilho *et al.*, 2009; Du *et al.*, 2012). Hence, using molasses may add value and reduce the cost of producing PHA. Many bacteria use Molasse (sucrose) to produce PHAs without hydrolysis, but some bacteria cannot use them without hydrolysing. These bacteria include *Alcaligenes latus*, *Hydrogenophaga pseudoflava* and *Azotobacter vinelandii* etc. A study by Albuquerque *et al.* (2010) proposed a three-step fermentation strategy using two-stage CSTR for sugarcane molasse to produce PHA under the feast and famine process. The molasses was fermented in the first step, followed by the second step, which is culture selection, and then PHA accumulation in the third step, which produced about 61% cell dry weight (CDW) of PHA content (Albuquerque *et al.*, 2010). . Bacteria used to produce PHA from Molasse waste material, as

seen in Table 2-5

Table2-5: Different types of bacteria used for production of PHA from Molasse

Bacteria used	Carbon source	Type of fermentation	Cultivation procedure	Type of PHA	Operation period (h)	Temp used (°C)	PHA content (%)	PHA productivity (g/L/h)	References
<i>Pseudomonas fluorescens</i> A2a5	Sugarcane	Using bioreactor	Batch process	PHB	96	25	70	0.23	(Jiang <i>et al.</i> , 2008)
<i>Bacillus cereus</i> SPV	Sugarcane	Using shaker flask	Batch process	PHB	72	30	61.07	3.45	(Akaraonye <i>et al.</i> , 2012)
<i>Cupriavidus necator</i> DSM 545	Molasse	Using shaker flask	Batch process	PHB		35	56	11.7	(Dalsasso <i>et al.</i> , 2019)
<i>Bacillus subtilis</i>	Molasse	Shaker flask	Batch process	PHB	72	35	62.21	-	(Gomaa, 2014)
Recombinant <i>E. coli</i> harboring phaC1 of <i>Pseudomonas.s</i> p.LDC-5	Molasse	Using fermentor	Fed-batch process	PHB	72	37	75.5	3.06	Saranya and Shenbagarathai, 2011

2.11.1.4 Whey waste materials.

Whey is a waste product from the cheese and casein production industries comprising proteins, sugar, lipids, vitamins, minerals, and other nourishing nutrients used by microorganisms for growth (Nielsen *et al.*, 2017). Furthermore, whey has numerous valuable uses and is an attractive feedstock. Whey is generated as a liquid after milk is coagulated in massive amounts, about 90% of the milk's whole volume (Favaro *et al.*, 2019). Each year, about 120 M tons of whey are generated worldwide, with only 50% being transformed into human and animal feed products (Amaro *et al.*, 2019; Favaro *et al.*, 2019). Still, the remaining percentage is not adequately treated, creating a serious environmental issue (Amaro *et al.*, 2019). Because of its high sugar content, about 5 to 10% of whole whey is used for pharmaceutical uses and produced as high-grade lactose (Favaro *et al.*, 2019). Moreover, as defined in various studies, whey is a good carbon feed in producing industrial products like ethanol, enzymes and polyhydroxyalkanoates (Castilho *et al.*, 2009; Favaro *et al.*, 2019; Obruca *et al.*,

2011). Therefore, using whey to produce polyhydroxyalkanoates would reduce production costs, environmental contamination, and competition for non-human food production (Amaro *et al.*, 2019). Many bacteria such as *Hydrogenophaga pseudoflava* DSM 1034, *Sinorhizobium meliloti* 41, *Escherichia coli*, *Methylobacterium* sp ZP24, *Thermos thermophiles* HB8, *Bacillus megaterium*, *Paracoccus denitrification* DSM 413 and *Pseudomonas hydrogenovora* are being employed to polyhydroxyalkanoates (Jiang *et al.*, 2016; Kaur *et al.*, 2017; Koller *et al.*, 2008; Obruca *et al.*, 2011; Povolo *et al.*, 2013). A study conducted using whey surplus by *Caulobacter segnis* DSM 29236 that yields and accumulates polyhydroxybutyrate of about 37% cell dry weight was revealed by (Bustamante *et al.*, 2019a). Another study discovered that using *Alcaligenes latus* has achieved a productive yield of up to 0.11 g/L/h from whey substrate utilized as a carbon feed (Berwig *et al.*, 2016). Also, a PHA polymer content of up to 53% cell dry weight using cheese whey by *Haloferax mediterranei* and the productivity of PHA of about 4.04 g/L with a concentration of biomass of up to 7.54 g/L, identified and described as P(3HB-co-3HV) was stated by (Pais *et al.*, 2016).

2.11.2 Fats and oils used for synthesizing PHAs.

Fats and oils are suitable, cheaper raw materials for polyhydroxyalkanoates production. They include animal fats from slaughtering and food industries, plant oils like olive oil, coconut oil, soybean oil, palm oil, and glycerol from biodiesel processing industries (Chee *et al.*, 2010; Moralejo-Gárate *et al.*, 2011). Although fats and oils contain long-chain fatty acyl groups and short fatty acyl groups and are unsaturated, the long chains are saturated and solid. In addition, many bacteria are extensively known to utilize oils to produce PHAs, and the most studied ones are *Pseudomonas* sp., *Cupriavidus nectar*, *Methylobacterium* sp, *Burkholderia* etc. (Aditi *et al.*, 2015; Jiang *et al.*, 2016; Kaur *et al.*, 2017; Obruca *et al.*, 2011; Verlinden *et al.*, 2011).

2.11.2.1 Waste oils.

Many lipids-rich oil wastes, like used oils, have been used as an essential renewable resource in the production of polyhydroxyalkanoates, replacing pure oil and sugar (Nielsen *et al.*, 2017; Verlinden *et al.*, 2007, 2011). Used oils are the lipid-rich surplus generated from the repeated use of vegetable oils in cooking and frying food substances or the lubricating process. However, used oil is inexpensive and universally obtainable feedstock, and it replaces high-grade oils in producing valuable products like bioplastics, biofuels etc. (Bueno-Borges *et al.*, 2019). Generally, waste oil is generated from vegetable oils, and animal fats are discarded in a large amount in the environment daily (Marchetti *et al.*, 2020). About 29 million tons are generated annually (Ruiz *et al.*, 2019a, 2019b; Surendran *et al.*, 2020). However, they create many environmental problems if not correctly disposed of due to insufficient end-of-life management possibilities (Ruiz *et al.*, 2019a, 2019b). For example, about 700,000 to 1000,000 tonnes of waste cooking oil in the UK is generated yearly, while Canada and European countries generate 135,000 and 200,000 yearly, respectively (Awogbemi *et al.*, 2019; Panadare and Rathod, 2015). Countries like China, Japan, and Ireland produce about 4.5, 0.57 and 0.153 million tonnes yearly (Panadare and Rathod, 2015). Also, in 2011 USA generated approximately 0.6 million tonnes of yellow grease (Awogbemi *et al.*, 2019). Thus, disposal of these waste oils causes a lot of obstacles on earth and causes environmental contamination due to illegal throwing away into the soil and water sources (Awogbemi *et al.*, 2019; Mahesh *et al.*, 2015). However, using waste cooking oils to create valuable materials reduces environmental pollution and the cost of maintaining and treating the oil-water risk (Samuel *et al.*, 2019). A study made by using waste oil produced up to 87.5% cell dry weight of polyhydroxyalkanoate using optimal systems, and the PHA extract was identified as P(3HB-co-3HV) in a ratio of 85% mol w of 3HB and 15% of 3HV by *Bacillus thermoamylovorans* as reported by Sangkharak *et al.*, (2020). Another study using waste rapeseed oil and propanol as a precursor achieved 76% PHA with 8% 3-hydroxyvalerate with PHA total yield and productivity yield of 105 g^l-1 and 1.46 g^l-1 from *Cupriavidus necator* H16,

respectively, as reported by (Obruca *et al.*, 2010). Furthermore, a study examined vegetable oils (pure oil, heated oil, and waste frying oil) and a high polyhydroxybutyrate yield in waste frying oil of 1.2 g/L compared to both heated and pure vegetable oils was noted (Verlinden *et al.*, 2011). Pernicova *et al.*, (2019) studied the polyhydroxyalkanoate production from waste frying oil using different *Halomonas* strains. Their study reported that the *Halomonas hydrothermalis* accumulated PHB up to 61.98% of CDW (Pernicova *et al.*, 2019).

2.11.2.2 Glycerol.

Crude glycerol is a low-value by-product obtained from the industrial production of biodiesel. However, massive amounts are generated worldwide. Thus, the global generation of crude glycerol hits around 380 million each year, making it a waste material that poses a disposal concern (Ray *et al.*, 2016). So, there is a need to find a solution at its disposal. One good solution is to use it to produce value-added products like polyhydroxyalkanoate (Gahlawat and Soni, 2017; Ray *et al.*, 2016). It was reported by Volova *et al.* that glycerol is an excellent and suitable carbon feed for generating hydrogen, succinic acid, glycolipid, citric acid, and PHA production (Volova *et al.*, 2019). Besides, glycerol is a good source for producing scl and mcl-PHAs using *Pseudomonas* spp. A good example is that of *Pseudomonas Mediterranea* 9.1 (CFBP 5447), which can produce medium-chain length polyhydroxyalkanoates using crude glycerol, as reported by (Pappalardo *et al.*, 2014). A study by Moita and Co yielded a PHB content of up to 47% from crude glycerol (Moita *et al.*, 2014). Another study described by (Chanasit *et al.*, 2016) reported a high yield of polyhydroxyalkanoate up to 77% cell dry weight from *Pseudomonas mendocina* PSU using liquid biodiesel waste as a rich carbon source. They achieved the PHA monomers of both the short-chain-length PHA (3-hydroxybutyrate) and the medium-chain-length PHA (3-hydroxyoctanoate and 3-hydroxydecanoate) (Chanasit *et al.*, 2016). Table 2-6 shows the species of *Pseudomonas* and *Cupriavidus* produced different types of PHAs from waste oils.

Table 2-6: *Pseudomonas* and *Cupriavidus* species producing PHA from lipid-rich wastes.

Microorganism	Carbon source	Production process	% Yield	Type of PHA produced	References
<i>Pseudomonas oleovorans</i> DSM 1045	Pure glycerol	Shaker flask	46.7	P (3HB-co-3HV)	(Favaro <i>et al.</i> , 2019)
<i>Pseudomonas oleovorans</i> 29347	Jatropha curcas oil	Batch fermentation	26.06	Poly (3HB-co-3HV)	(Allen <i>et al.</i> , 2010)
<i>Pseudomonas</i> sp. GI01	Saponified waste palm oil	Fed-batch	42.69	mcl-PHA	(Mozejko and Ciesielski, 2013)
<i>Pseudomonas oleovorans</i>	Octanoate	pH-stat fed-batch	75	mcl-PHA	(Kim, 2002)
<i>Pseudomonas</i> sp	Olive mill wastewater	Batch experimental	64.4		(Kourmentza <i>et al.</i> , 2015)
<i>Pseudomonas</i> sp. Strain DR2	Waste vegetable oil	Shaker flask	23.54	mcl-PHA	(Song <i>et al.</i> , 2008)
<i>Pseudomonas guezenei</i> TIK669	Coprah oil	Two-step batch	63	P(3-HO-co-3HD).	(Simon-Colin <i>et al.</i> , 2008b)
<i>Ralstonia eutropha</i> (MTCC 1954)	Madhuca Indica flower	Shaker incubator	49.4	PHBV	(Kerketta and Vasanth, 2019)
<i>Cupriavidus necator</i> H16	Palm kernel oil	Shaker flask	90	P(3HB-co-3HV)	(Lumpur <i>et al.</i> , 2008)
<i>Cupriavidus necator</i> B-10646	Palm oil	Shaker flask	72	P(3HB)	(Volova <i>et al.</i> , 2020)
<i>Cupriavidus necator</i>	Crude palm kernel oil	Batch cultivation	68	P(3HB-co-3HHX)	(Anis <i>et al.</i> , 2013b)
<i>Photobacterium</i> sp. TLY01	Soybean oil	Shaker flask	44.42	PHB	(Tian <i>et al.</i> , 2022)
<i>Cupriavidus necator</i> DSM 545	Spent coffee oil	Batch	89.6	P(3HB-co-3HV)	(Ingram and Winterburn, 2021)
<i>Cupriavidus necator</i> H16	Waste cooking oil	Batch cultivation	76.9	PHB	(Loan <i>et al.</i> , 2022)
<i>P. aeruginosa</i> TISTR1287	Palm oil	Shaker incubator	38.2	mcl-PHAs	(Tanikkul <i>et al.</i> , 2020)
<i>Pseudomonas chlororaphis</i>	glycerol	Batch process	32.16	mcl-PHAs	(Aloui <i>et al.</i> , 2022)

2.11.2.3 Hydrocarbons and Wastewaters as substrates

Hydrocarbons include n-alkanes, 1-alkenes, paraffin, methane, aromatic hydrocarbons (styrene and phenylacetate), and those derived from waste plastics, polyolefins, polystyrene etc. Though many bacteria can use hydrocarbons to produce PHAs, the most studied ones include *Pseudomonas* spp., *Acinetobacter*, *Sphingobacterium*, *Brochothrix*, *Methylocystis* species, *Burkholderia*, and many others (Aditi *et al.*, 2015; Jiang *et al.*, 2016; Johnston *et al.*, 2018; Kaur *et al.*, 2017; Pieja *et al.*, 2011; Singh *et al.*, 2014).

Wastewater is a by-product derived from domestic, industrial, commercial, or agricultural activities that need proper environmental management. Depending on the source, the wastewater contains physical, chemical, and biological contaminants. Furthermore, wastewater is an exciting alternative carbon source with varying characteristics from industrial and household practices, predominantly with carbohydrates, proteins, and fats, suitable for producing value-added products. However, because of the complex organic materials attributed to wastewater, it is unclear whether to treat the wastewater via landfill, composting or incineration (Sharma *et al.*, 2020). Therefore, using it to produce valuable products such as hydrogen, bioplastics, and many others is suitable for treating wastewater. Crutchik *et al.* showed that polyhydroxyalkanoates production from wastewater reduces the cost of polymer production by about 15.9 to 19.0% of the total costs (Crutchik *et al.*, 2020). Alsafadi and Al-Mashaqbeh investigated polyhydroxyalkanoates accumulation and stated 43% PHA content with the highest polymer yield of 0.2 g/L and 6.5 mol% of 3HV from olive mill wastewater using olive mill wastewater *Haloferax mediterranei* (Alsafadi and Al-Mashaqbeh, 2017). Another study by Waller *et al.* (2012), they reported a yield of the polyhydroxyalkanoate polymer up to 39% by mixed microbial culture utilizing olive mill wastewater as a carbon source (Waller *et al.*, 2012).

2.12 Analytical methods to determine oil composition.

The composition of oils can be analysed and quantified through various analytical techniques. Common approaches include gas chromatography (GC) or high-performance liquid chromatography (HPLC) (Xu *et al.*, 2013). Additional methods, such as Fourier-transform infrared spectroscopy (FTIR), supercritical fluid chromatography, mass spectrometry, nuclear magnetic resonance spectroscopy, and others, have also been extensively explored and applied (Aubin *et al.*, 2008; Cert *et al.*, 2000). Gas chromatography (GC) is often the preferred method due to its exceptional resolution and sensitivity (Rubab *et al.*, 2020).

The procedure, as outlined by Zainab *et al.* (2018) and Wirasnita *et al.*, (2013), involves the precise weighing of an oil sample, with the quantity used depending on specific analytical requirements. A methanolysis reagent is prepared by mixing methanol with a suitable catalyst, such as sodium hydroxide (NaOH), potassium hydroxide (KOH), or tetramethylguanidine (Wirasnita *et al.*, 2013), which facilitates the conversion of triglycerides into methyl esters. The oil is placed in a vial, and the methanolysis reagent is added before sealing to prevent evaporation. This mixture is then heated to a predetermined temperature, typically within the range of 50-70°C, for a specific duration, usually 1-3 hours (Rueda *et al.*, 2014). To halt the process, hydrochloric acid (HCl), sulfuric acid (H₂SO₄), or boron trifluoride (BF₃) is introduced to neutralize the catalyst and prevent further conversion of fatty acids (Wirasnita *et al.*, 2013).

The methyl esters are then extracted from the mixture using a solvent such as n-hexane or diethyl ether, which separates them from the aqueous solution layer (Lee *et al.*, 1998). The extracted methyl esters are diluted to reduce solvent volume and enhance fatty acid concentration through evaporation. Subsequently, further analysis is performed, employing techniques such as gas chromatography (GC) or high-performance liquid chromatography (HPLC) (Cert *et al.*, 2000). These techniques enable the identification and quantification of individual fatty acids in the sample, based on their unique chemical characteristics (Lee *et al.*,

1998). Chromatograms are instrumental in determining the distribution of fatty acids in the oil, facilitating the identification and quantification of various fatty acids based on their retention times (Lee *et al.*, 1998; Rueda *et al.*, 2014). The composition of fatty acids, including saturated, monounsaturated, and polyunsaturated fatty acids, can be elucidated through gas chromatography. This approach serves as a critical tool for oil quality assessment and impurity determination. The objective of oil analysis is to optimize efficiency and achieve the highest separation performance possible, with the results applicable to research or industrial analysis purposes (Marriott *et al.*, 2001).

2.13 Fatty Acid Profile of Different Oils

2.13.1 Palm oil

The composition of palm oil includes various fatty acids and triacylglycerol (Mba *et al.*, 2015), typically consisting of saturated, monounsaturated, and polyunsaturated fatty acids comprising approximately 44% palmitic acid (C16:0), 4% stearic acid (C18:0), as both are saturated while 39% oleic acid (C18:1) is monosaturated and 10% linoleic acid (C18:2) which is polyunsaturated fatty acids (May and Nesaretnam, 2014; Mba *et al.*, 2015; Montoya *et al.*, 2013; Sambanthamurthi *et al.*, 2000), Table 2-7. The oil also contains myristic acid (C14:0) and lauric acid (C12:0) as saturated fatty acids but in minimal amounts as trace contents (Montoya *et al.*, 2013).

2.13.2 Glycerol

Glycerine, fatty acids, and salt were major glycerol components, with percentages average of 33.9%, 10.5%, and 65.2%. While other components were glyceritol 51.4%, ash 13.8%, water 8.9%, and matter organic non-glycerol (MONG) 25.9% (Luo *et al.*, 2016; Ooi *et al.*, 2001).

2.13.3 FOG

Glycerol, fatty acids, and free long-chain fatty acids are the main components of FOGs, which are triglyceride molecules. The most common saturated fatty acids are palmitic acid and stearic acid, while the most common unsaturated fatty acids in FOG wastes are oleic acid and linoleic acid (Jessica *et al.*, 2022), Table 2-7. Also, they have a high carbon-nitrogen ratio due to their high lipid content (Abomohra *et al.*, 2020; Jessica *et al.*, 2022)

2.13.4 Soybean oil

Soybean oil composition contains about 10% palmitic (16:0), 4% stearic (18:0), 20% oleic (18:1), 55% linoleic (18:2), and 10% linolenic (18:3) acids (Song *et al.*, 2023), Table 2-7. Other minor components, such as tocopherols, phytosterols, flavonoids, and carbohydrates, are also present in soybean oil (zaaboul *et al.*, 2022). Soybean is a rich source of numerous primary and secondary metabolites, such as lipids, flavonoids, and sugar metabolites (Zhao and Wang, 2023).

Table 2-7: Fatty acid compositions of different oil

Compositions	Palm oil (%)	Soybean oil (%)	Glycerol (%)	FOG (%)
Palmitic acid (C16:0)	44	10	N/A	38
Stearic acid (C18:0)	4	4	N/A	7.2
Oleic acid (C18:1)	39	20	N/A	36.9
Linoleic acid (C18:2)	10	55	N/A	15.1
Linolenic acid (C18:3)	<0.3	10	N/A	0-2
Others	<0.3	<1	100	0-5

NA = Not applicable

2.14 Pretreatment of Waste Oils for Bioplastic Production

Pretreatment of lipid-rich waste oils plays a crucial role in the production of bioplastics and other value-added products from these feedstocks. Lipid-rich

waste oils, including used cooking oil, animal fats, and other lipid-containing wastes from diverse sources, require thorough pretreatment. The primary objective of the pretreatment is to eliminate impurities and contaminants (Liew *et al.*, 2020), enhance the quality of the feedstock, and render it suitable for further processing. Lipid-rich waste oils inherently contain impurities that necessitate meticulous pretreatment before fermentation (Lam *et al.*, 2016).

This process effectively deconstructs the complex structure of lipid-rich carbon sources, rendering them more amenable to enzymatic activity for the synthesis of polyhydroxyalkanoates (PHAs). This is especially important since lipids require extracellular lipase-mediated hydrolysis within the fermentation culture, a process distinct from that of carbohydrates. Consequently, the activity of lipase plays a critical role in determining the accessibility of the oil substrate to the bacterial culture (Onumaegbu *et al.*, 2018). The quantity and composition of free fatty acids (FFAs) are critical variables in this context. To enhance productivity and efficiency, a variety of pretreatment techniques have been employed, as detailed by Wang *et al.* (2021). Pretreatment techniques are categorized into physical, chemical, biological, mechanical, and combinations of these approaches, as delineated by Chong *et al.* (2021).

2.14.1 Chemical Pretreatment

Chemical pretreatment offers an efficient means to degrade organic materials at a relatively low cost, thereby enhancing the overall production process. This category includes alkaline, acid, and ionic liquid pretreatment methods, as classified by Chong *et al.* (2021).

2.14.2 Biological Pretreatment

Biological pretreatment stands out for its environmental friendliness and minimal energy consumption. It is a cost-effective approach with no adverse environmental impact. Enzymatic and fungal pretreatment represent subsets of biological pretreatment (Chong *et al.*, 2021). Among these, enzyme pretreatment

is noteworthy for its remarkably low energy requirements, as demonstrated by Onumaegbu *et al.* (2018).

2.14.3 Physical Pretreatment

Physical pretreatment methods enhance bioproduct production through the application of techniques such as microwave and ultrasonic pretreatment and others (Onumaegbu *et al.*, 2018). Mechanical pretreatment is another facet of physical pretreatment, often involving the reduction of particle size in organic materials to release the organic fraction into the liquid phase, thereby maximizing bio-production. Various physical pretreatment includes microwave, ultrasonication, and high-pressure homogenization (Chong *et al.*, 2021).

In summary, pretreatment of lipid-rich waste oils encompasses a range of approaches that are pivotal in transforming these feedstocks into valuable bioproducts. The selection of a specific pretreatment method should consider factors such as cost, environmental impact, and energy efficiency, aligning with the desired outcomes of the production process (Onumaegbu *et al.*, 2018).

2.15 Solutions for emerging bioplastics

Fossil-based chemistry polymers persistently accumulate in landfills and oceans, creating severe environmental problems. Therefore, plastic pollution has become perilous to both animals' and humans' lives, causing millions of aquatic lives to die, such as seabirds and other aquatic animals (Nanda *et al.*, 2022). Presently no other task has gotten the globe's interest as much as developing an efficient and sustainable circular economy for polymer materials. However, if the vision is satisfied, it will reduce or solve the burden issues related to fossil-based chemistry polymers (Wang *et al.*, 2011). The best example is the production of polyhydroxyalkanoates (PHAs) that can replace fossil oil-derived monomers that are unsustainable to a sustainable circular economy. PHAs are fast developing and gaining interest in various industry and research areas, and the prospect seems sustainably progressive into the future (Koller *et al.*, 2010).

Nevertheless, the feasibility of producing it from the cheapest substrates is an essential strategy for enhancing the economic standard and reducing the cost of production of these polymers, which limits their applications (Keshavarz and Roy, 2010). Moreover, PHAs are produced using renewable resources through an environmentally friendly synthesis process and can be biodegradable and show sufficient transparency (Su *et al.*, 2018). However, substrates, cultivation method, productivity and recovery process are the most critical parameters that influence the production cost of PHAs. There are many ways of decreasing this production cost of PHA, such as improving a fermentation method that allows a higher production yield of PHA per litre and increasing the processing stability of the PHA polymer in the recovery process (Wei *et al.*, 2013). Lastly, replacing the high great carbon sources with waste materials from the non-food chain. Thus, using waste food and oils or low-value crops to produce value-added products like bioplastics has significant potential in the future circular economy (Porez *et al.*, 2014), where alternatives to costly substrates for the food and chemical industries need to be developed. Furthermore, many bacteria are known to accumulate PHAs as an energy store (including *Pseudomonas* spp.), and various strains are used in industry (Neratko *et al.*, 2018; Song *et al.*, 2008).

2.16 Challenges in the production of bioplastics

The families have the advantages of bio-origin (generated by fermentation of microorganisms), thermoplastic, biodegradability, no fossil resources, and non-toxic and environmentally friendly polymer. Besides having such a good collection, they have certain disadvantages, too, like their compostable nature only under specific conditions due to their brittleness, some show dominant hydrophilic character, and unsatisfactory mechanical properties, particularly in a wet environment (Bhalla, 2015; Chanprateep, 2010). The production of bioplastic polymers plays a vital role in replacing fossil fuel chemistry polymers. However, a few industries are producing PHAs because of the high cost of production due to some production factors like carbon substrates and downstream processes (recovery), which are unreliable measures of their potential future (Khatami *et al.*, 2019).

3 Sustainable Conversion of Lipid-Rich Wastes to Bioplastics.

Authors: Hano, L.J., Soares, A and Wagland, S.

Abstract

*Cheaper oils were used as a carbon source for sustainable bioplastic production. The study evaluated different lipid-rich oil wastes such as glycerol (Gly), fat, oil & grease (FOG), frying soybean oil (FSO), and frying palm oil (FPO) to determine their impact on PHA production. A two-stage production process was designed where the first stage favoured cell growth and the second favoured PHA accumulation. Under the conditions of 180 rpm and temperature of 30°C for 48 hours, using 2% (v/w) of each oil. The yield accumulated by *P. oleovorans* was 43% of CDW, 39% of CDW, 34% of CDW, and 32% of CDW, whereas *P. oleovorans* ATCC29347 was 38% of CDW, 37% of CDW, 34% of CDW, and 33% of CDW, respectively. Glycerol and FOG gave a higher PHA accumulation than the other carbon sources. Fourier transforms infrared (FTIR) and Scanning electron microscopy (SEM) were used in analysing and characterizing the extracted PHA. The FTIR analysis revealed strong bands of the PHA at 1465 cm⁻¹, 1736 cm⁻¹, 2932 cm⁻¹, 2856 cm⁻¹, and 3137 cm⁻¹. FTIR and SEM analyses revealed that the produced PHA was medium chain-length PHA (mcl-PHA) compared with reported PHA in previous studies. The result confirmed the potential of these waste oils utilized by *Pseudomonas* strains to create sustainable bioplastic production.*

Keywords: MCL-PHAs, *Pseudomonas oleovorans*, polyhydroxyalkanoates, FT-IR, SEM.

3.1 Introduction

Bioplastic (PHAs), produced by bacteria through aerobic fermentation of diverse carbon sources, and are promising polymers. They show elastomeric and thermoplastic characteristics that are modified based on their monomer compositions (Samorì *et al.*, 2015). PHAs are gaining a lot of interest as biodegradable alternatives to traditional polymers (Salehizadeh and Van Loosdrecht, 2004). The cost of producing PHA is still very high (5–6 \$/kg) despite efforts to develop inexpensive processes, making it difficult to use them as product materials (Samorì *et al.*, 2015). Microorganisms store them as granules of substance inside of their cells (Luengo *et al.*, 2003; Pei *et al.*, 2022). The most frequent carbon substrates used to make bioplastics are virgin carbon sources, which cause food-feed competition and cost nearly 40% more to produce PHA (Khatami *et al.*, 2021; Rodriguez-Perez *et al.*, 2018; Yin *et al.*, 2019). These virgin carbon sources also add to the burden of land use and food-feed conflicts. To compete with traditional petrochemical-derived plastics, the PHAs' comparatively high production costs must be decreased (Akdoğan and Çelik, 2018; Favaro *et al.*, 2019a; Favaro *et al.*, 2019b; Koller *et al.*, 2017; Rodriguez-Perez *et al.*, 2018). Improvements in bacterial strains and recovery methods can lower the cost of their production. Additionally, employing mixed cultures and inexpensive substrates reduces the cost of making PHAs (Salehizadeh and Van Loosdrecht, 2004; Ana *et al.*, 2022). PHA can be made more affordably by employing inexpensive waste substrates, especially food sector waste, which is a sustainable way to reduce the cost of PHA, especially lipid-rich oil wastes like wasted cooking or frying oil, animal fat, palm kernel oil, etc., (Pernicova *et al.*, 2019). Many bacteria, algae, yeast, and archaea produce them using excess carbon but limited growth nutrients and serve as a source of energy and carbon reserve materials in the form of polyhydroxyalkanoates, which is the most typical and replace the non-degradable conventional plastics (Akdoğan and Çelik, 2018; Chmelová *et al.*, 2022; Loan *et al.*, 2022; Mannina *et al.*, 2020; Min Song *et al.*, 2022; Zher Neoh *et al.*, 2022). Polyhydroxyalkanoate (PHA) is the most attractive type of these biodegradable polymers and a potential replacement for petroleum-

based plastics (Jeon *et al.*, 2014). In addition, PHAs are the most versatile bioplastic, with more than 100 monomers obtained from carbon sources as a renewable raw material and microorganisms like bacteria, algae, and fungi (Akaraonye *et al.*, 2010).

The development of PHAs first started in the late eighteenth century when Beijerinck saw PHAs as inclusion bodies in bacteria cells (Palmeiro-Sánchez *et al.*, 2022). Later in the early twentieth century, Lemoigne first described it after isolating the first type of polyhydroxyalkanoate [PHB (polyhydroxybutyrate)] from *Bacillus megaterium* (Lenz and Marchessault, 2005; Palmeiro-Sánchez *et al.*, 2022). The ability to accumulate PHAs under aerobic and anaerobic circumstances has been found in more than 300 species of bacteria (both gram-negative and gram-positive) and archaea. Furthermore, these microorganisms can store PHA as energy products in their cytoplasm of up to 97% of cell dry weight (Khatami *et al.*, 2021).

PHAs are classified into three categories depending on the number of carbon atoms in their monomer units: 1). Short-chain length (scl) PHAs with 3-5 carbon monomer units, 2). Medium-chain length (mcl) between 6-14 carbon atoms and 3). Long-chain length (lcl) with monomeric units of more than 14 carbon atoms (Roja *et al.*, 2019; Silva *et al.*, 2021). The objective of this study is to evaluate if using waste oils as a low-value carbon substrate for *Pseudomonas* strains to produce bioplastic is sustainable. The viability and practicality of employing *Pseudomonas oleovorans* (DSMZ1045 and ATCC29347) to assess the production of bioplastics from these waste oils have not been extensively studied. The effect on the yield quantity achieved from each carbon used and the sustainability of creating bioplastic using low-value carbon sources was assessed in this study.

3.2 Materials and Methods

3.2.1 Materials

Magnesium sulfate (500 g, Japan, 99%), calcium chloride dihydrate (500 g, Germany, 98%), iron (II) sulfate (250 g, India, 99%), manganese (II) chloride tetrahydrate (500 g, Japan, 99.5%), sodium phosphate monobasic (500 g, Germany, 99%), sodium hydroxide (250 mL, 99.8%, Switzerland), zinc sulfate heptahydrate (500 g, USA, 99%), sodium chloride (250 g, USA, 99.5%), potassium phosphate monobasic (500g, Germany, 99%), calcium chloride hexahydrate (200 g, China, 99%), sodium phosphate dibasic (500 g, Japan, 99%), Nile blue A reagent (100mL, India, 99.9%), nutrient broth powder (500 g, India, 8 g/L) yeast extract (3 g/L), urea (Germany) were all purchased from (Sigma Aldrich), glycerol (500mL, Malaysia, 99.5%), 37% HCL (Australia), 30% H₂O₂ (Spain) (chloroform (2.5 L, Germany, 99.8%), ethanol (2.5 L, UK, 99.8%, acetone (2.5 L, Finland, 99.8%), propan-2-ol (2.5 L, NewZealand, 99.8%), methanol (2.5 L, UK, 99.9%), were all purchased from (Fischer scientific), fat, oil, & grease (FOG) (collected from wastewater treatment plant UK), frying soybean oils, palm oils frying (household), *Pseudomonas oleovorans* DSMZ 1045 and, *Pseudomonas oleovorans* ATCC 29347 (Purchased from ATCC, PHEC, and DSMZ), as lyophilized ampoule culture.

3.2.1 Equipment

Microbiological safety cabinet (Labcare SC-R Recirculating Class II, Labcare System Ltd, England), Centrifuge (SORVALL ST40, ThermoFisher Scientific, Germany), VEGA 3 (Tescan, Czech Republic), Erlenmeyer conical flasks (100 mL, 250 mL, Fisher brand, Germany), Scale (Precisa, UK), Durham bottle (250 mL, 500 mL, Fisher brand, Germany), shaker incubator (Incu-Shaker MIDI, Benchtop Shaking Incubator, Switzerland), Freeze dryer (Christ Alpha 1-2 LDplus, Germany), spectrophotometer (JENWAY 6310, UK), bath ultrasound incubator (Ultrawave, Asset 50A) (Ultrawave Ltd, Cardiff, UK), FTIR techniques (Vertex 70, Bruker), Fume Cupboard (Heraeus, Germany), autoclave, Bench-scale Microwave, Beakers (100 mL, 250 mL, 300 mL, 400 mL, Fisher brand,

Germany), Measuring cylinders (50 mL, 250 mL, 500 mL, and 1000 mL, Technicon, England).

3.2.2 Methods

3.2.2.1 Samples preparation

FOG sample (20 g) was mixed with 600 mL of distilled water and blended. The blended mixture was centrifuged and filtered to produce a filtrate solution to be used as a carbon source. Glycerol, soybean, and palm oils, however, did not require any additional preparation. All the samples were sterilized by autoclaving for 3 h at 121°C before use.

3.2.2.2 Culture Reactivation and Maintenance

The lyophilised ampoule culture of *Pseudomonas oleovorans* (DSMZ1045) and *Pseudomonas oleovorans* (ATCC29347) were revived in nutrient broth medium at 30°C. The nutrient broth media contains nutrient broth powder 8 g/L, yeast extract 3 g/L, and NaCl 2.5 g/L. These ingredients were dissolved in a litre of distilled water and autoclaved at 121°C. The revived cultures were maintained as cryo-conserved at -80°C in the presence of 50% (v/v) glycerol (Nkrumah-Agyeefi and Scholz, 2017).

3.2.2.3 Fermentation stages

The fermentation was carried out using two-stage processes: The first stage favoured microbial growth, while the second stage favoured PHA production. The microbial biomass was obtained and monitored from *Pseudomonas* strains using spectrophotometric analysis. The dry weight of the cells was determined using a JENWAY 6310, as described by (Jiang *et al.*, 2015; Mohammadi *et al.*, 2012b, 2012c, 2012a). The polymer was extracted using sodium hydroxide (NaOH) and hydrogen peroxide (H₂O₂) and purified using cold methanol. The polymer extracted was characterized using Fourier transforms infrared (FTIR) and Scanning electron microscope (SEM) analytical techniques.

3.2.2.4 Growth and production condition

Pseudomonas strains (DSMZ1045 & ATCC29347) were first grown in a 250 mL flask containing 100 mL nutrient broth medium incubated at 30°C for 24 h at 180 rpm with a shaker incubator. The 40 mL of cultures were centrifuged at 3800 rpm for 15 min using a centrifuge technique [(SORVALL ST40 CENTRIFUGE, Thermo Scientific)] to collect the cell pellets. The cell pellets were washed twice with sterilized autoclaved distilled water. The cell pellets were suspended in 100 mL of mineral media for PHA production containing the following: 1.5 g/L KH_2PO_4 , 6.7 g/L Na_2HPO_4 , 4.5 g/L NaH_2PO_4 , 0.06 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.3 g/L MgSO_4 , supplemented with 1 ml/L of a microelement solution. Microelement trace stock contained the following: 1.5 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.03 g/L $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 0.048 g/L CuSO_4 , 0.24 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 1 M of HCl, sterilized using a syringe filter, and added to the medium before inoculation (Zainab *et al.*, 2018) Nitrogen-limiting conditions were created by depriving the medium of 0.54 g/L of urea and 2% (v/v) of oil as carbon feed were added. The pH was adjusted to 7.0, and the medium was autoclaved at 121°C. Experiments were performed under the temperature-controlled shaker conditions set at 30°C for 48 h at 180 rpm. After 48 h of batch fermentation, the fermentation cultures were harvested by centrifugation at 3800 rpm for 15 min, washed twice with distilled water, and then lyophilized to obtain the dry cell weight (CDW g/L). All the experiments were done in triplicate, as seen in Fig (3-1).

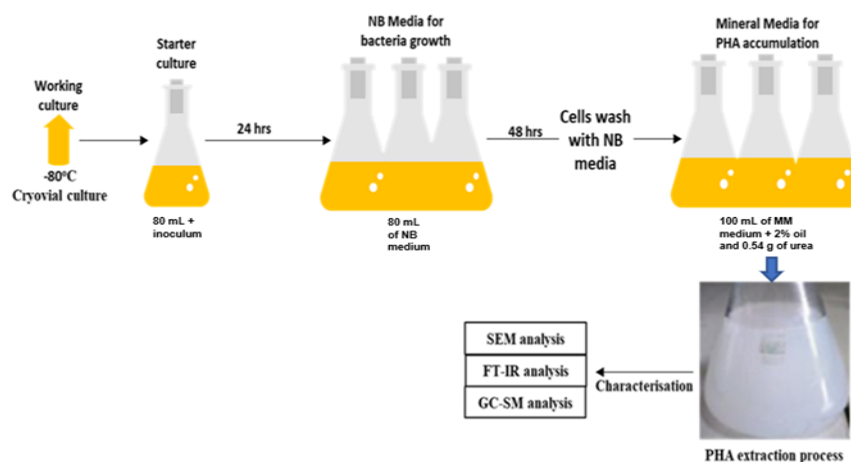


Figure 3-1: The schematic procedure of bioplastic production

3.2.2.5 Optical density

The fermentation culture was monitored and measured by using the optical density at 600 nm (OD_{600nm}) using a JENWAY 6310 spectrophotometer technique. The growth rate (μ) was calculated using Eq. (3-1 & 3-2).

Growth rate (μ) = (ln (final OD) - ln (initial OD)) / time (hours)

$$\mu = \frac{\ln(N_t/N_0)}{(t_2 - t_1)} \quad (3-1)$$

$$\mu = \frac{(\ln(OD_2) - \ln(OD_1))}{(t_2 - t_1)} \quad (3-2)$$

Where OD₁ and OD₂ - are the optical densities of the culture.

(t₁ and t₂) - is the time interval between measurements (in hours).

(μ) - is the growth rate in reciprocal time units (h⁻¹).

3.2.2.6 Statistical analysis

Analysis of variance (ANOVA) was designed to analyse the data obtained using SPSS and Minitab. The effects of the different factors (i.e., lipid-rich oil wastes and *Pseudomonas* strains) were determined by their significant differences between the means. The threshold of P-value = 0.05 was used to evaluate the differences between means. Each experiment was carried out in triplicate and took 48 h.

3.3 Polymer recovery

3.3.1 Separation (centrifugation) process

The fermentation culture (biomass) was centrifuged at 3800 rpm for 15 min to separate the supernatant and the cell pellets differently. After that, the cell pellets were rinsed twice with distilled water to remove other residual debris and dried at 0.010 mbar and -54°C for 24 h using the freeze dryer.

3.3.2 Microscopic process

A microscopic was conducted to verify the PHA accumulation in the cells of the organisms after 48 hours of the fermentation process. Since *Pseudomonas* strains already produce PHA, Nile blue A, a PHA-specific dye, was used to check for the possible polymer granules in their cells, used x100 magnification to identify fluorescing cells. Probes were prepared by mixing 20 µL of the fermentation growth culture with Nile blue dye, allowed for 1 – 2 minutes before being washed using distilled water. After, it was allowed to dry before the fluorescence microscopic viewing process.

3.3.3 Determination of dry cell weight

The cell biomass was harvested by centrifuging the 40 mL of fermentation cultures at 3800 rpm for 15 min, using a centrifuge technique [(SORVALL ST40 CENTRIFUGE, Thermo Scientific)] to assess the biomass and PHA content from the fermentation cultures. The cell pellets were collected and washed twice with distilled water. The cell dry weight was calculated and achieved using the formula below.

$$CDW = W_1 - W_2 \quad (4-3)$$

W_1 is the final cell dry weight of the falcon tube with the pellet, and W_2 is the weight of the falcon tube without the pellet.

3.3.4 Polymer extraction

Then the dried cell pellets were ground using mortar and pestle, and the polymer was extracted with sodium hydroxide and hydrogen peroxide at an incubation temperature of 37°C for 180 min. Finally, the obtained organic phases were evaporated, and the residues were purified using chilled methanol and then dried using in a fume cupboard for one day. Then, the PHA percentage (%) was calculated. Then, the PHA percentage (%) was calculated using the formula below:

$$\%PHA = \frac{\text{Extracted PHA } (\frac{g}{L})}{CDW (\frac{g}{L})} * 100 \quad (4-4)$$

3.4 Polymer characterisation

3.4.1 SEM analysis

The sample materials were mounted into the sample holder and coated with gold (Senthilkumar *et al.*, 2017), and the sample holder was placed onto the TESCAN VEGA 3 (Fig. 3-2), and subjected to SEM examination. The images were taken at different magnifications from 1000-5000x with a used voltage of 10kV (Murugan *et al.*, 2016), or 20kV to examine the morphological structures of each samples.

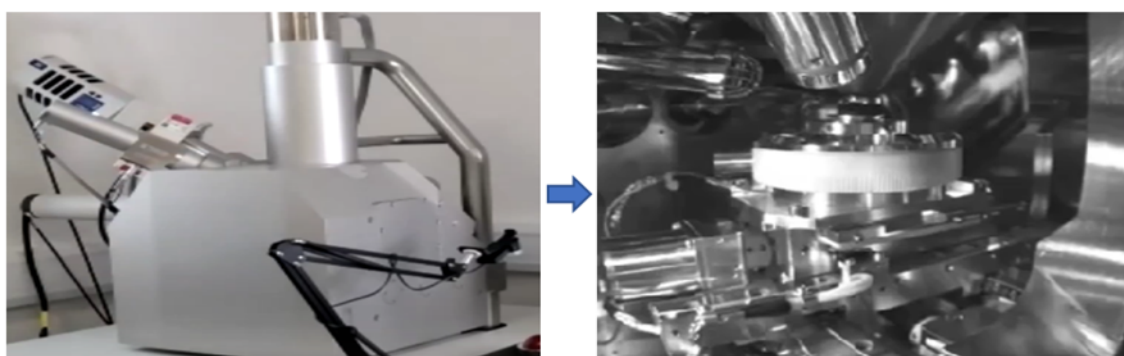


Figure 3-2: TESCAN VEGA 3 SEM analytical instrument

3.4.1 FTIR spectroscopy analysis

FTIR analysis of PHA produced by *Pseudomonas* strains (DSMZ1045 & ATCC29347) was performed using Vertex 70, Bruker, Fig. 3-3. Sample was mixed well with KBr (Rakkan *et al.*, 2022a), and absorbance of each sample was scanned at wavenumbers of 400 cm^{-1} and 4000 cm^{-1} (Kanavaki *et al.*, 2021) waves at a speed of 1 $\mu\text{m}/\text{min}$ with a spectral resolution of 4 cm^{-1} (Vega-Castro *et al.*, 2016). The functional groups in polymer samples were identified.



Figure 3-3: Bruker FTIR analytical instrument

3.5 Results and discussion

3.5.1 Growth and production

The findings from the spectrophotometer-based growth monitoring were averaged, and the $\ln(\text{OD})$ was calculated and used in plotting the growth curve with time [Time (h) on the Y-axis and $\ln(\text{OD})$ on the X-axis]. Software packages SPSS and Minitab were used to analyse standard deviations and average means.

As can be observed in Fig. 3-4 & 3-5, the growth rate gradually increased after 24 hours until it reached 42 hours, at which point it steadily dropped, generating the decline stage. This represents a traditional Monod kinetics growth curve and the decline in optical density after 42 hours can be due to many factors, as reported in various published studies such as the accumulation of toxic substances (volatile acids, ethanol, ammonia, etc.) or the depletion of the essential nutrients (i.e., phosphorous, nitrogen) following their consumption in the exponential phase of growth (Mauerhofer *et al.*, 2019; Muloiwa *et al.*, 2020), which affects the yield of PHA until it reaches 48 hours.

On the other hand, PHA generation is impacted by the bacterial growth and respective factors, as reported in earlier research, such as pH, temperature, nutrient availability, oxygen, phosphorous, and other physical growing conditions have a significant impact on the formation of biomass and consequently PHA. For instance, Mahato *et al.* (2021) describe the optimal growth conditions for biological PHA production, starting from a range of pH (5, 6, 7, and 8), with pH 7 producing the maximum polymer accumulation (58.16%). Different

concentrations of WCO (60, 90, and 120 g/L) were studied by Ruiz *et al.* (2019a) when they employed 60 g/L achieved a biomass of 45 g CDW/L with 19% of CDW as PHA and increased to 1.2 and 1.4-fold when 90 g/L was applied. The biomass remained consistent with 90 g/L when 120 g/L was used, while PHA decreased by 1.2-fold. The absence of nitrogen or phosphate limitations and the abundance of oxygen indicate that some other mechanism was preventing growth when a high concentration was applied. The growth and PHA concentration were estimated by measuring the absorbance of the 1 mL of fermentation culture at OD_{600nm} using a spectrophotometer. The values of growth rate and PHA concentration were recorded as in (g/L), and the specific growth rate at 12 h of incubation between 0.84 h⁻¹ and 0.98 h⁻¹. Additionally, samples of the fermentation cultures were taken during the incubation period at intervals of 0, 3, 6, 9, 12, 24, 27, 30, 42, 45, and 48 hours and the optical density (OD) measured and presented as ln(OD). Fig. 3.4 & 3-5 illustrates the growth curves and the formula used to compute the growth rate:

Growth rate (μ) = (ln (final OD) - ln (initial OD)) / time (hours)

$$\mu = \frac{\ln(N_t/N_0)}{(t_2 - t_1)} \quad (3-5)$$

$$\mu = \frac{(\ln(OD_2) - \ln(OD_1))}{(t_2 - t_1)} \quad (4-6)$$

Where OD₁ and OD₂ are the optical densities of the culture.

(t₁ and t₂) is the time interval between measurements (in hours).

(μ) is the growth rate in reciprocal time units (h⁻¹).

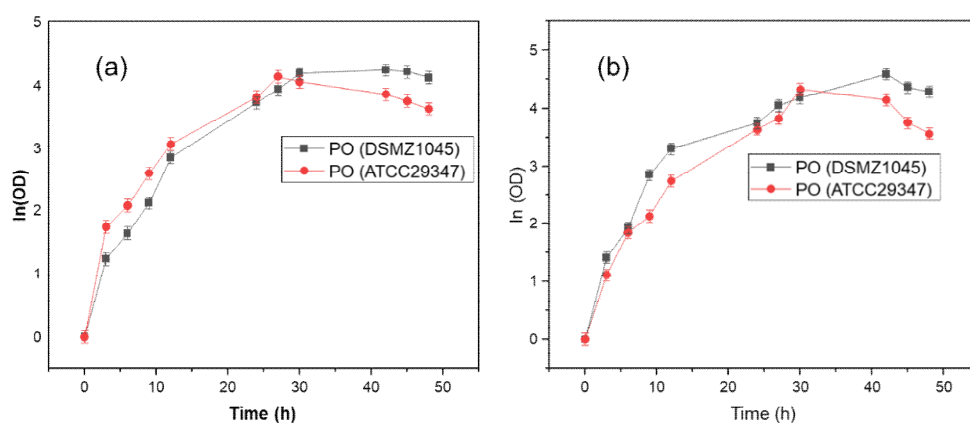


Figure 3-4: Growth curve of both strains: (a) Soybean oil and (b) Palm oil

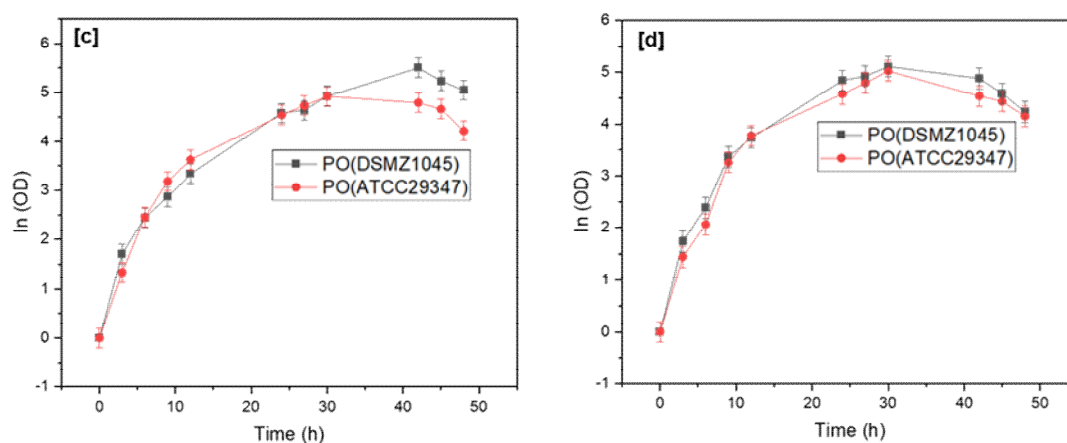


Figure 3-5: Growth curve of both strains: (c) FOG and (d) Glycerol

Table 3-1: Tests of Between-Subjects Effects

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Model	DSMZ1045	442.392	1	442.392	48.173	.000	.828
	ATCC29347	403.308	1	403.308	51.221	.000	.837
Error	DSMZ1045	91.834	10	9.183			
	ATCC29347	78.738	10	7.874			

Both strains begin to grow at 3 h, however, *Pseudomonas oleovorans* DSMZ1045 demonstrated a higher OD_{600nm} value of 5.5 than *Pseudomonas oleovorans* ATCC29347 with an OD_{600nm} of 4.5. Furthermore, the statistical analysis shows the P=0.000 using the threshold of P-value = 0.05, which confirmed that the results were statistically satisfactory, as seen in Table 3-1 above. Also revealed by the outcome is that the regression model accounts for 97.3% of the variability seen in the target variables as the value of R square (R^2) is equal to 0.973%, which means that values are highly correlated, Table 3-1. The efficacy of the predicted model is demonstrated by the proximity of R^2 to 1, and the coefficient of determination (R^2) was used to assess the kinetic growth models (Muloiwa *et al.*, 2020). The optimum bounds with a 95% confidence interval confirmed the consistency of the experimental data, as the statistic of an upper bound on F yields a lower bound on the significance level, as seen in Appendix A (1.1 – 1.4).

3.5.2 Polyhydroxyalkanoate yield.

Flask fermentation processes were carried out to evaluate the effectiveness of each carbon source (Glycerol, frying soybean oil, FOG & frying palm oil) using an environment that credibly served as an essential medium for the bacterial cells. PHA accumulation was observed from both organisms with *Pseudomonas oleovorans* DSMZ1045 gave high yield than *Pseudomonas oleovorans* except in FPO, i.e., 43%/CDW, 39%/CDW, 34%/CDW & 32% of cell dried weight and 38%/CDW, 37%/CDW, 34%/CDW & 33% of cell dried weight, with glycerol, fat, oil and grease, soybean oil and palm oil, respectively. Results show effective production of PHA by these strains from different oil wastes after 48 h of fermentation (Table 3-2), as reported in many published studies (Kim *et al.*, 2007; Kim *et al.*, 2000).

Table 3-2: PHA % yield to cdw by the strains from each carbon used.

Carbon sources	Organism used	%PHA yield/CDW
Gly	<i>Pseudomonas oleovorans</i> DSMZ1045	43
Gly	<i>Pseudomonas oleovorans</i> ATCC29347	38
FSO	<i>Pseudomonas oleovorans</i> DSMZ1045	34
FSO	<i>Pseudomonas oleovorans</i> ATCC29347	34
FPO	<i>Pseudomonas oleovorans</i> DSMZ1045	32
FPO	<i>Pseudomonas oleovorans</i> ATCC29347	33
FOG	<i>Pseudomonas oleovorans</i> DSMZ1045	39
FOG	<i>Pseudomonas oleovorans</i> ATCC29347	38

Furthermore, *Pseudomonas oleovorans* DSMZ1045 had accumulate a higher yield in both pretreatment and non-pretreatment process than *Pseudomonas oleovorans* ATCC29347, Table 3-3. This shows that *Pseudomonas oleovorans* DSMZ1045 could more effectively produce bioplastics from used cooking oils than *Pseudomonas oleovorans* ATCC29347. Although, the precise mechanisms underlying the variance in bioplastic yields between the two strains might be due to their metabolic pathways or other strain-specific properties that enhance their ability to produce bioplastics. Tan *et al.* (2014) stated that PHA accumulation is a greatly dependent metabolic pathway of organisms. The improved yield percentage after pretreatment employed is shown in Table 3-3.

Table 3-3: % Yield increased after pretreatment was employed.

Carbon sources	PHA yield from non-pretreated carbon source (%)	PHA yield from pretreated carbon source (%)	% Change increased between the non and pretreated carbon source
Glycerol	43	53	23.26
Glycerol	43	51	18.60
Glycerol	43	50	16.28
Soybean oil	34	48	41.03
Soybean oil	34	45	26.47
Soybean oil	34	43	26.47
Palm oil	33	47	46.88
Palm oil	33	45	40.63
Palm oil	33	43	34.38
FOG	39	55	41.18
FOG	39	54	38.46
FOG	39	53	35.90

Likewise, a higher yield was obtained in glycerol and FOG than in frying soybean and palm oils from both organisms. The higher production yield observed for glycerol and FOG may be due to the composition of fatty acids present, which are the main precursors of bioplastics (Lee *et al.*, 2019). As reported by Verlinden *et al.* (2011), that the fatty acid components of oil improve polymer accumulation in the bacteria. Accordingly, Kala *et al.* (2010) reported that oils with high levels of fatty acids are more easily converted into bioplastics by bacteria than those with lower levels. A more recent study by Chen *et al.* (2023) revealed that the PHA yield, kind and metabolic are affected by various carbon sources. Therefore, this could be the reason that influences the higher yield in both glycerol and FOG. Furthermore, the other oils (soybean and palm) were frying oil, which probably affects the composition and nutrient contents of the oils due to the incorporation of inhibitory ingredients that might affect the fatty acid levels (Du *et al.*, 2012).

This finding is in line with the studies conducted by Favaro *et al.* (2019), using glycerol as the carbon source, *Pseudomonas oleovorans* DSMZ1045 produced a total percentage yield of 46% cell dry weight PHA. Accordingly, Liu *et al.* (2018) reported that *Pseudomonas mosselii* produced a high PHA content of 48% cell dry weight in 48 h using crude glycerol as a carbon source. Another study by Phukon *et al.* (2014) found that employing glycerol as a carbon source allowed *Pseudomonas aeruginosa* to produce a yield of 22% dry cell weight. *Pseudomonas chlororaphis* was grown on glycerol as a fermentative carbon source and obtained a 32% CDW as a mcl-PHA percentage yield produced (Aloui *et al.*, 2022).

According to one study, palm and soybean oil was used by *Pseudomonas* spp. as carbon sources and achieved a higher yield of 50% mcl-PHA content of cell dry weight (Chen *et al.*, 2014). He *et al.* (1998) reported that *Pseudomonas stutzeri* 1317 produced up to 63% of PHA from 10 g/L of soybean oil. *Pseudomonas oleovorans* ATCC29347 was grown using saponified *Jatropha curcas* oil as the fermentative substrate using a mineral salt medium, and the batch fermentation method resulted in a PHA production yield of 26.06% cell dry weight after 72 h (Allen *et al.*, 2010). A similar study was conducted by Song *et al.* (2008) when *Pseudomonas* sp. cultivated for 72 h in a medium with limited conditions for both nitrogen and phosphate produced 37.34% and 23.52% of mcl-PHAs from waste vegetable oil and maize oil, respectively. More recent work by found that *Pseudomonas putida* produces PHA from hexanoic acid and achieved 19.5% of cell dry mass as mcl-PHA under O₂-limitation conditions (Blunt *et al.*, 2019). Additionally, it was reported in many studies that *Pseudomonas* strains, including *Pseudomonas putida*, *Pseudomonas oleovorans*, *Pseudomonas aeruginosa*, and many others, synthesize significant yields of mcl-PHA with a variety of compositions using plant oils, including vegetable oil, corn oil, and their fatty acids (Gumel *et al.*, 2016 Nitschke *et al.*, 2011; Song, *et al.*, 2008). This finding is consistent with above mentioned published studies that have reported the ability of *Pseudomonas* strains to produce bioplastics from waste oils.

3.5.3 Effect of carbon sources on CDW and PHA yield

The cell dry weight and PHA contents from various carbon sources utilized by these *Pseudomonas* strains and their effects on the quantity of the yields were evaluated and studied after 48 h, as shown in Fig. 3-6 (a & b). The comparison of CDW and PHA contents, yield from palm oil, glycerol, soybean oil, and fat, oil, and grease as the sole carbon source was determined, as shown in Fig. 3-6 (a & b). The PHA contents found during the batch fermentations were between 0.26 and 0.48 g/L of PHA content. While the yield percentages were between 32% of cell dry weight and 43% of cell dry weight (CDW). According to the findings, glycerol and FOG showed the PHA accumulation of 43% of CDW and 39% of CDW from *Pseudomonas oleovorans* DSMZ1045, while 38% of CDW and 37% of CDW from *Pseudomonas oleovorans* ATCC29347, respectively. The other carbon sources (soybean and palm oil) observed lower yields of 34% of CDW and 32% of CDW from *Pseudomonas oleovorans* DSMZ1045 and 34% of CDW and 33% of CDW from *Pseudomonas oleovorans* ATCC29347. Furthermore, this finding is confirmed to be consistent with the previously reported finding that the organism's capacity to multiply and accumulate PHA is specific to the carbon source (Blunt *et al.*, 2019; Chen *et al.*, 2014; Favaro *et al.*, 2019; Kim *et al.*, 2007; Kim, Kim and Rhee, 2000; Pan *et al.*, 2021).

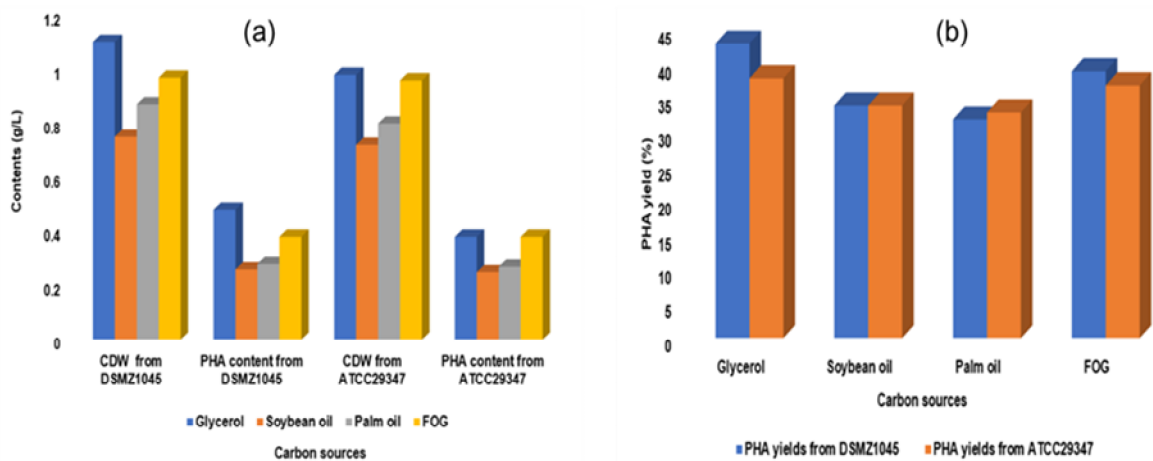


Figure 3-6: Effects of various carbon on a) CDW & PHA contents and b) Yield %

3.5.4 SEM analysis

Scanning electron microscopy (SEM) analysis was performed in order to study the morphology of the granules of the polymer from both organisms. The samples were examined at an accelerating voltage of 10kV or 20kV with imaging at magnifications at 2 μ m and 5 μ m. SEM images obtained showed a consistent look of spherical beads-like structures within the polymer granules produced by some cells, while others exhibited a more homogenous, porous, and denser appearance like a honeycomb structure. This finding suggested that the accumulated polymer displays a malleable quality, as seen in Fig. 3-7 and 3-8. Furthermore, the outcome observed aligns with the observation made by (Ghosh *et al.*, 2019; Nwinyi and Owolabi, 2019; Pan *et al.*, 2021) they reported that SEM analysis showed slightly porous, fine, interconnected particles exhibiting a spherical morphology and remarkably homogeneous distribution within the samples. PHA plays a significant role in the formation and accumulation of granular polymer of the palm oil samples. Figure 3.7 reveals a distinctive appearance from Fig. 3.8, the granular polymer from palm oil was more compacted and malleable with irregular and asymmetric small bead aggregates. This pattern can be attributed to the high fatty acid present in the palm oil as compared to soybean oil (Verlinden *et al.*, 2011). Rebocho *et al.* (2019) revealed that the SEM images discovered were homogenous, rough on the surface, concentrated, and lacked any noticeable pores. The SEM images of the polymers produced (soybean and palm oil) from this study showed slight variations in the surface morphological structures of the polymer produced from the organisms (DSMZ1045 and ATCC29347) used. These consistent findings showed similarity between this research outcome and those reported in the referenced literature. Furthermore, these results are very similar to the ones from this study.

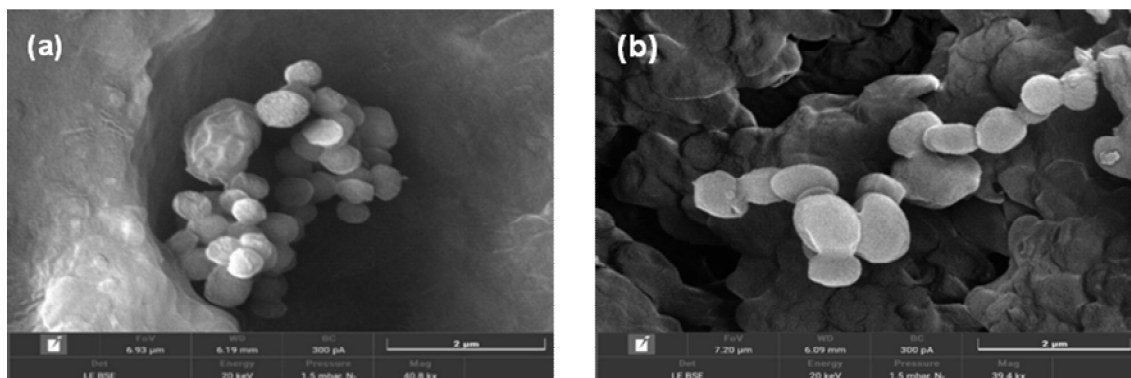


Figure 3-7: SEM granule images from cells: (a) DSMZ1045 & (b) ATCC29347 employed PO.

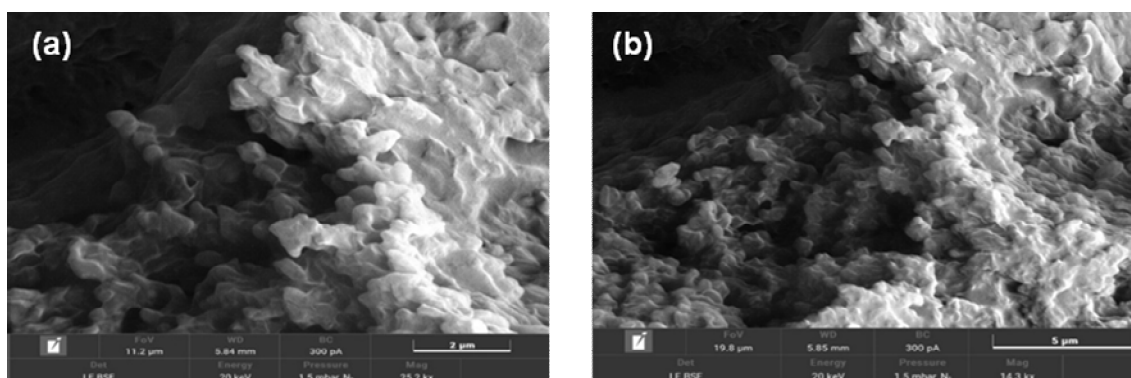


Figure 3-8: SEM granule images from cells: (a) DSMZ1045 & (b) ATCC29347 employed SO

3.5.5 FT-IR analysis of polymers

FT-IR spectroscopy was employed to perform a qualitative analysis of the extracted polymer samples. The analysis was run between the wavenumbers 400 cm^{-1} - 4000 cm^{-1} , and the resulting absorption bands were documented, as illustrated in Figure 3-9. The FTIR spectra of the polymers were categorized based on their respective functional groups. FTIR spectra of PHAs produced by *Pseudomonas oleovorans* DSMZ1045 and *Pseudomonas oleovorans* ATCC29347 grown on different carbon sources as shown in Fig. 3-9a(i-iii) and Fig.3-9b (i-iii) were the standard structure of a polyester with an intense absorption peak corresponding to the stretching band of the ester carbonyl group ($\text{C}=\text{O}$) at $1725\text{--}1740\text{ cm}^{-1}$. The observed absorption bands exhibited distinctive transmittance characteristic peaks at 2989 cm^{-1} , 2959 cm^{-1} , 2932 cm^{-1} , 2922 cm^{-1}

¹, and 2856 cm⁻¹. These bands were attributed to the presence of methyl and methylene groups (CH₃ & CH₂) corresponding strong stretching vibrations, and they are indicative of symmetric and asymmetric side-chain methyl and methylene groups, as typical characteristics bonding groups as reported by (Gumel *et al.*, 2013b). Additionally, Liu *et al.* (2011a) reported that the intensity of these bands during crystallization serves as an indicator of the polymer's crystallinity. Moreover, the functional group bands of CH₃ and CH₂ are typically found within the range of 2800 cm⁻¹ to 3000 cm⁻¹ (Chan Sin *et al.*, 2011; Petpheng *et al.*, 2023). While peaks at 1458 cm⁻¹ and 1368 cm⁻¹ showed the corresponding medium stretching bands of C-H, C-O-C, and C-C as seen in Fig 3-9(ai-aiii). Peaks at 1736 cm⁻¹ indicated the presence of ester carbonyl (C=O) spectra that strongly revealed mcl-PHA from both organisms, as seen in Fig. 3-8. Additionally, the stretching C-O and =CH groups, which typically have strong or medium stretching bands, are associated with peaks at 1156 cm⁻¹, 1078 cm⁻¹, and 907 cm⁻¹. However, the intense peak of C=O found in Fig.3.8 (ai-aii) was minimal and drastic absorbed in Fig. 3.8 (aiii), this can be attributed to the FOG complexity in the PHA polyester. The other absorption bands assigned to C-O and C-C are indications of the amorphous phase of the materials (Aloui *et al.*, 2022).

In addition, Fig. 3.9 (bi - biii) displays similar strong stretching bands and the peak absorption bands followed the same pattern discussed in Fig. 3.8 (ai - aiii), except the presence of a lower stretch of a band of OH at peak 3400 cm⁻¹. This can be attributed to the fact that 3-hydroxy fatty acid in PHA formed bonds between ester and carboxyl groups (Pan *et al.*, 2021). However, both Fig.3.9 (a and b) showed crystalline and amorphous phases in the stretch bands of 1725 cm⁻¹ and 985 cm⁻¹. This observation aligns with FTIR analyses reported in the literature (Aloui *et al.*, 2022; Chen, Huang and Lee, 2014; Rebocho *et al.*, 2019; Sathiyarayanan *et al.*, 2017; Sharma *et al.*, 2017a, 2017b; Simon-Colin *et al.*, 2008b, 2008a) which show typical bands for C=O groups between 1723 cm⁻¹ and 1741 cm⁻¹ and bands for methyl and methylene groups between 2800 cm⁻¹ and 3000 cm⁻¹, while 1285 cm⁻¹ stands for the CH group and a band at 1385 cm⁻¹ for the methyl group. This finding proves that both strains produced MCL-PHA, as

supported by the absorption bands detected and documented in the existing literature.

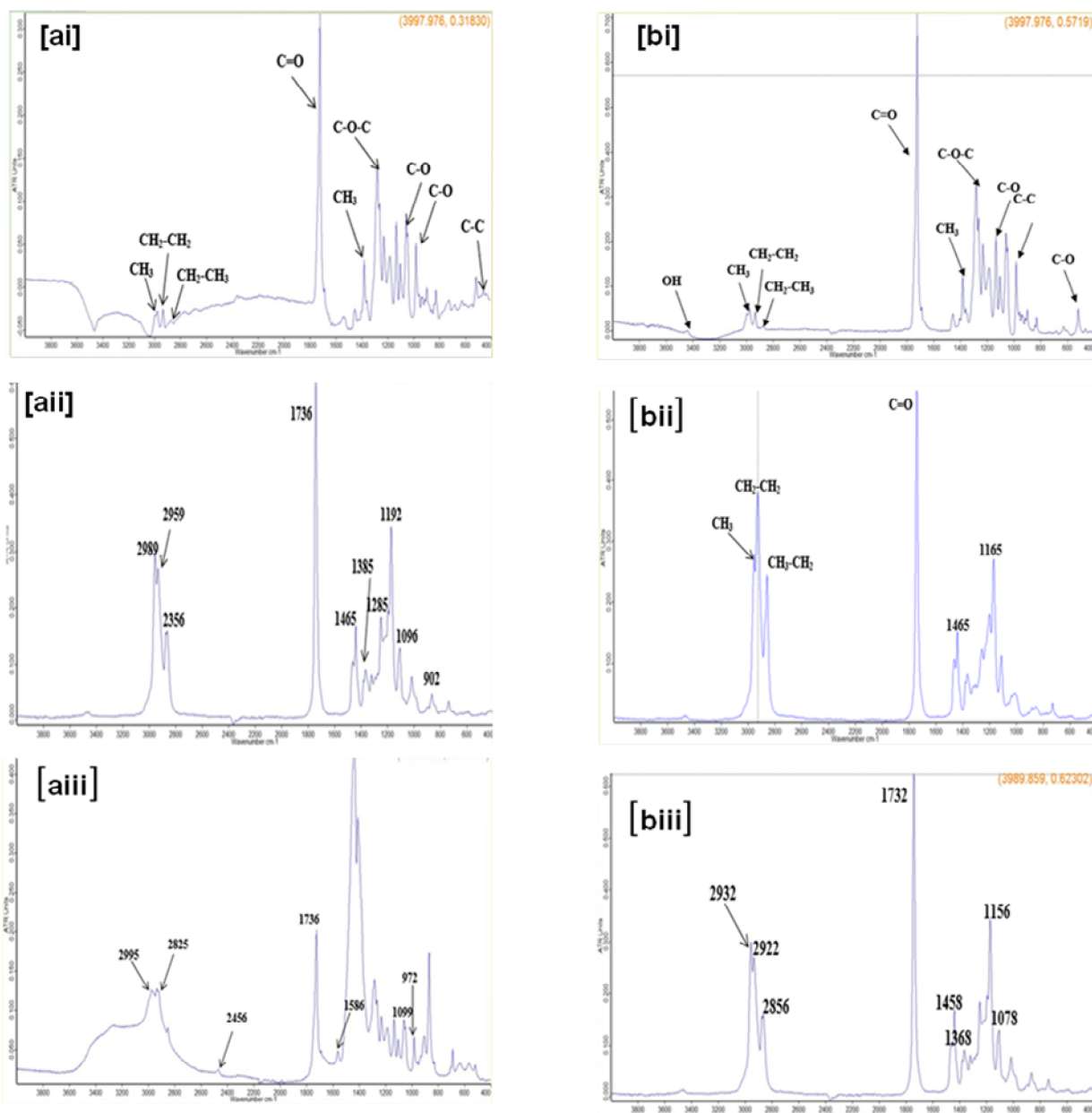


Figure 3-9: FTIR analysis of polymer produced: [a] DSMZ1045 utilized (i) GLY, (ii) SO, (iii) FOG; and [b] ATCC29347 utilized (i) GLY, (ii) SO, (iii) FOG.

3.6 Conclusion

In conclusion, this study aimed to evaluate a sustainable production of bioplastics from various waste oils using selected *Pseudomonas* strains [*Pseudomonas*

oleovorans (DSMZ1045 & ATCC29347)] as the microorganism responsible for conversion and enhancing productivity.

However, the strains produced MCL-PHA from these lipid-rich wastes as a carbon source for these bacteria. Both organisms recorded a higher percentage yield of the polymer with glycerol & fat, oil, and grease (FOG) than in soybean oil & palm oil. Higher polymer yields were seen with *P. oleovorans* DSMZ1045 than with *P. oleovorans* ATCC29347, except from palm oil. Varying amounts relative to dry cell weight: 43wt%, 39%wt, 34%wt, and 33%wt were obtained from these waste resources, showcasing promise as an effective solution for bioplastic production. Notably, glycerol and FOG exhibited higher PHA accumulation than other carbon sources. Furthermore, enhanced polymer yields were observed using oil samples from *P. oleovorans* DSMZ1045 in contrast to *P. oleovorans* ATCC29347. The conditions used for the fermentation process was 2% (w/v) of each carbon source, 0.54 g/L of urea, and incubation at 30°C with a shaking speed of 180 rpm for 48 h.

The extracted samples were characterized using FTIR and SEM, confirming their functional groups and morphological structure as mcl-PHA type compared to the previously published literature. The significance of this work contributes to the development of sustainable and environmentally friendly alternatives to traditional petroleum-based plastics and provides a means to manage lipid-rich oil wastes. The study further recommends further research on the bioreactor scale under controlled circumstances using the optimized settings for more enhance the scaling up of the PHA sustainable production and maximize its productivity using batch cultivation. In addition, the ability of *Pseudomonas oleovorans* DSMZ1045 to produce copolymer from waste oils, such as P(3HHx-co-3HO), will also serve as a suggestion.

Acknowledgement

The Nigerian PTDF sponsored this work study, which the author would like to recognise and Cranfield University for creating an enabling learning environment for me to complete my PhD.

References

- Akaraonye, E., Keshavarz, T., & Roy, I. (2010). Production of polyhydroxyalkanoates : the future green materials of choice. **85**(6); pp732–743.
- Akdoğan, M., & Çelik, E. (2018). Purification and characterization of polyhydroxyalkanoate (PHA) from a *Bacillus megaterium* strain using various dehydration techniques. *Journal of Chemical Technology & Biotechnology*, **93**(8); pp2292–2298.
- Allen, A. D., Anderson, W. A., Ayorinde, F. O., & Eribo, B. E. (2010). Biosynthesis and characterization of copolymer poly(3HB-co-3HV) from saponified *Jatropha curcas* oil by *Pseudomonas oleovorans*. *Journal of Industrial Microbiology and Biotechnology*, **37**(8); pp849–856.
- Aloui, H., Khomlaem, C., Torres, C. A. V., Freitas, F., Reis, M. A. M., & Kim, B. S. (2022). Enhanced co-production of medium-chain-length polyhydroxyalkanoates and phenazines from crude glycerol by high cell density cultivation of *Pseudomonas chlororaphis* in membrane bioreactor. *International Journal of Biological Macromolecules*, **211**(5); pp545–555.
- Ana Marta Rodrigues, Rita Dias Guardão Franca, Madalena Dionísio, C. S., & Christian Grandfils, M. A. M. R. and N. D. L. (2022). Polyhydroxyalkanoates from a Mixed Microbial Culture: Extraction Optimization and Polymer Characterization. *Polymers*, **14**(2115); pp1–22.
- Blunt, W., Lagassé, A., Jin, Z., Dartailh, C., Sparling, R., Gapes, D. J., Levin, D. B., & Cicek, N. (2019). Efficacy of medium chain-length polyhydroxyalkanoate biosynthesis from different biochemical pathways under oxygen-limited conditions using *Pseudomonas putida* LS46. *Process Biochemistry*, **82**(4); pp19–31.
- Chan Sin, M., Gan, S. N., Mohd Annuar, M. S., & Ping Tan, I. K. (2011). Chain cleavage mechanism of palm kernel oil derived medium-chain-length poly(3-

- hydroxyalkanoates) during high-temperature decomposition. *Polymer Degradation and Stability*, **96**(9); pp1705–1710.
- Chen, S., Dai, X., Yang, D., Dai, L., & Hua, Y. (2023). Polyhydroxyalkanoate synthesis from primitive components of organic solid waste: Comparison of dominant strains and improvement of metabolic pathways. *Applied Energy*, **344**(2023); pp1–14.
- Chen, Y. J., Huang, Y. C., & Lee, C. Y. (2014). Production and characterization of medium-chain-length polyhydroxyalkanoates by *Pseudomonas mosselii* TO7. *Journal of Bioscience and Bioengineering*, **118**(2); pp145–152.
- Chmelová, D., Legerská, B., Ondrejovič, M., & Miertuš, S. (2022). Optimization of Propagation Medium for Enhanced Polyhydroxyalkanoate Production by *Pseudomonas oleovorans*. *Fermentation*, **8**(1); pp1–12.
- Du, C., Sabirova, J., Soetaert, W., & Ki Carol Lin, S. (2012). Polyhydroxyalkanoates Production From Low-cost Sustainable Raw Materials. *Current Chemical Biology*, **6**(1); pp14–25.
- Favaro, L., Basaglia, M., & Casella, S. (2019). Improving polyhydroxyalkanoate production from inexpensive carbon sources by genetic approaches: a review. *Biofuels, Bioproducts and Biorefining*, **13**(1); pp208–227.
- Favaro, L., Basaglia, M., Rodriguez, J. E. G., Morelli, A., Ibraheem, O., Pizzocchero, V., & Casella, S. (2019). Bacterial production of PHAs from lipid-rich by-products. *Applied Food Biotechnology*, **6**(1); pp45–52.
- Ghosh, S., Gnaim, R., Greiserman, S., Fadeev, L., Gozin, M., & Golberg, A. (2019). Macroalgal biomass subcritical hydrolysates for the production of polyhydroxyalkanoate (PHA) by *Haloferax mediterranei*. *Bioresource Technology*, **271**(2018); pp166–173.
- Gumel, Annuar, Heidelberg, T. (2013). Growth kinetics, effect of carbon substrate in biosynthesis of mcl-PHA by *Pseudomonas putida* Bet001. *Journal of Microbiology*, **45**(2); pp427–438.

- He, W., Tian, W., Zhang, G., Chen, G. Q., & Zhang, Z. (1998). Production of novel polyhydroxyalkanoates by *Pseudomonas stutzeri* 1317 from glucose and soybean oil. *FEMS Microbiology Letters*, **169**(1); pp45–49.
- Idris Zainab-L, Hiroshi Uyama, Cong Li, Yehua Shen, and K. S. (2018). Production of Polyhydroxyalkanoates From Underutilized Plant Oils by *Cupriavidus necator*. *Clean Soil, Air, Water*, **46**(11); pp1–9.
- Jeon, J. M., Brigham, C. J., Kim, Y. H., Kim, H. J., Yi, D. H., Kim, H., Rha, C. K., Sinskey, A. J., & Yang, Y. H. (2014). Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(HB-co-HHx)) from butyrate using engineered *Ralstonia eutropha*. *Applied Microbiology and Biotechnology*, **98**(12); pp5461–5469.
- Kanavaki, I., Drakonaki, A., Geladas, E. D., Spyros, A., Xie, H., & Tsiotis, G. (2021). Polyhydroxyalkanoate (Pha) production in *pseudomonas* sp. phdv1 strain grown on phenol as carbon sources. *Microorganisms*, **9**(8); pp1–11.
- Khatami, K., Perez-Zabaleta, M., Owusu-Agyeman, I., & Cetecioglu, Z. (2021). Waste to bioplastics: How close are we to sustainable polyhydroxyalkanoates production? *Waste Management*, **119**(2021); pp374–388.
- Kim, D. Y., Hyung, W. K., Moon, G. C., & Young, H. R. (2007). Biosynthesis, modification, and biodegradation of bacterial medium-chain-length polyhydroxyalkanoates. *Journal of Microbiology*, **45**(2); pp87–97.
- Kim, D. Y., Kim, Y. B., & Rhee, Y. H. (2000). Evaluation of various carbon substrates for the biosynthesis of polyhydroxyalkanoates bearing functional groups by *Pseudomonas putida*. *International Journal of Biological Macromolecules*, **28**(1); pp23–29.
- Koller, M., Maršálek, L., de Sousa Dias, M. M., & Braunegg, G. (2017). Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New Biotechnology*, **37**(2017), 24–38. <https://doi.org/10.1016/j.nbt.2016.05.001>

- Lenz, R. W., & Marchessault, R. H. (2005). Bacterial polyesters: Biosynthesis, biodegradable plastics and biotechnology. *Biomacromolecules*, **6**(1); pp1–8.
- Liu, Luo, G., Zhou, X. R., & Chen, G. Q. (2011). Biosynthesis of poly(3-hydroxydecanoate) and 3-hydroxydodecanoate dominating polyhydroxyalkanoates by β -oxidation pathway inhibited *Pseudomonas putida*. *Metabolic Engineering*, **13**(1); pp11–17.
- Liu, M. H., Chen, Y. J., & Lee, C. Y. (2018). Characterization of medium-chain-length polyhydroxyalkanoate biosynthesis by *Pseudomonas mosselii* TO7 using crude glycerol. *Bioscience, Biotechnology and Biochemistry*, **82**(3); pp532–539.
- Loan, T. T., Trang, D. T. Q., Huy, P. Q., Ninh, P. X., & Van Thuoc, D. (2022). A fermentation process for the production of poly(3-hydroxybutyrate) using waste cooking oil or waste fish oil as an inexpensive carbon substrate. *Biotechnology Reports*, **33**(2021); pp1–11.
- Luengo, J. M., García, B., Sandoval, A., Naharro, G., & Olivera, E. R. (2003). Bioplastics from microorganisms. *Current Opinion in Microbiology*, **6**(3); pp251–260.
- Mahato, R. P., Kumar, S., & Singh, P. (2021). Optimization of Growth Conditions to Produce Sustainable Polyhydroxyalkanoate Bioplastic by *Pseudomonas aeruginosa* EO1. *Frontiers in Microbiology*, **12**(2021); pp1–9.
- Mannina, G., Presti, D., Montiel-Jarillo, G., Carrera, J., & Suárez-Ojeda, M. E. (2020). Recovery of polyhydroxyalkanoates (PHAs) from wastewater: A review. *Bioresource Technology*, **297**(2019); pp1–12.
- Mauerhofer, L. M., Pappenreiter, P., Paulik, C., Seifert, A. H., Bernacchi, S., & Rittmann, S. K. M. R. (2019). Methods for quantification of growth and productivity in anaerobic microbiology and biotechnology. *Folia Microbiologica*, **64**(3); pp321–360.
- Min Song, H., Chan Joo, J., Hyun Lim, S., Jin Lim, H., Lee, S., & Jae Park, S.

- (2022). Production of polyhydroxyalkanoates containing monomers conferring amorphous and elastomeric properties from renewable resources: Current status and future perspectives. *Bioresource Technology*, **366**(2022); pp1–11.
- Muloiwa, M., Nyende-Byakika, S., & Dinka, M. (2020). Comparison of unstructured kinetic bacterial growth models. *South African Journal of Chemical Engineering*, **33**(2019); pp141–150.
- Murugan, P., Han, L., Gan, C. Y., Maurer, F. H. J., & Sudesh, K. (2016). A new biological recovery approach for PHA using mealworm, *Tenebrio molitor*. *Journal of Biotechnology*, **239**(2016); pp98–105.
- Nitschke, M., Costa, S. G. V. A. O., & Contiero, J. (2011). Rhamnolipids and PHAs: Recent reports on *Pseudomonas*-derived molecules of increasing industrial interest. *Process Biochemistry*, **46**(3); pp621–630.
- Nkrumah-Agyeefi, S., & Scholz, C. (2017). Chemical modification of functionalized polyhydroxyalkanoates via “Click” chemistry: A proof of concept. *International Journal of Biological Macromolecules*, **95**(2017); pp796–808.
- Nwinyi, O. C., & Owolabi, T. A. (2019). Scanning electron microscopy and Fourier transmission analysis of polyhydroxyalkanoates isolated from bacteria species from an abattoir in Ota, Nigeria. *Journal of King Saud University - Science*, **31**(3); pp285–298.
- Palmeiro-Sánchez, T., O’Flaherty, V., & Lens, P. N. L. (2022). Polyhydroxyalkanoate bio-production and its rise as biomaterial of the future. *Journal of Biotechnology*, **348**(2022); pp10–25.
- Pan, L., Li, J., Wang, R., Wang, Y., Lin, Q., Li, C., & Wang, Y. (2021). Biosynthesis of polyhydroxyalkanoate from food waste oil by *Pseudomonas alcaligenes* with simultaneous energy recovery from fermentation wastewater. *Waste Management*, **131**(2021); pp268–276.

- Pei, R., Vicente-Venegas, G., Van Loosdrecht, M. C. M., Kleerebezem, R., & Werker, A. (2022). Quantification of polyhydroxyalkanoate accumulated in waste-activated sludge. *Water Research*, **221**(2022); pp1–12.
- Pernicova, I., Kucera, D., Nebesarova, J., Kalina, M., & Novackova, I. (2019). Production of polyhydroxyalkanoates on waste frying oil employing selected *Halomonas* strains. *Bioresource Technology*, **292**(2019); pp1–4.
- Petpheng, B., Mudtaleb, B., Thongduang, S., Meekhai, T., Pechsiri, J., & Sangkharak, K. (2023). The production of medium-co-long chain length (mcl-co-lcl) polyhydroxyalkanoate from waste soybean oil by *Enterobacter* sp. and its application as an antibacterial agent. *Biomass Conversion and Biorefinery*, **2023**(012); pp1–14.
- Phukon, P., Phukan, M. M., Phukan, S., & Konwar, B. K. (2014). Polyhydroxyalkanoate production by indigenously isolated *Pseudomonas aeruginosa* using glycerol by-product of KCDL biodiesel as an inexpensive carbon source. *Annals of Microbiology*, **64**(4); pp1567–1574.
- Rakkan, T., Chana, N., & Sangkharak, K. (2022). The Integration of Textile Wastewater Treatment with Polyhydroxyalkanoate Production Using Newly Isolated *Enterobacter* Strain TS3. *Waste and Biomass Valorization*, **13**(1); pp571–582.
- Rebocho, A. T., Pereira, J. R., Freitas, F., Neves, L. A., Alves, V. D., Sevrin, C., Grandfils, C., & Reis, M. A. M. (2019). Production of medium chain length polyhydroxyalkanoates by *Pseudomonas citronellolis* grown in apple pulp waste. *Applied Food Biotechnology*, **6**(1); pp71–82.
- Rodriguez-Perez, S., Serrano, A., Panti6n, A. A., & Alonso-Fari6as, B. (2018). Challenges of scaling up PHA production from waste streams. A review. In *Journal of Environmental Management*, **205**(2018); pp215–230.
- Roja, K., Ruben Sudhakar, D., Anto, S., & Mathimani, T. (2019). Extraction and characterization of polyhydroxyalkanoates from marine green alga and cyanobacteria. *Biocatalysis and Agricultural Biotechnology*, **22**(2019); pp1–

11.

- Ruiz, C., Kenny, S. T., Narancic, T., Babu, R., & Connor, K. O. (2019). Conversion of waste cooking oil into medium chain polyhydroxyalkanoates in a high cell density fermentation. *Journal of Biotechnology*, **306**(2019); pp9–15.
- Salehizadeh, H., & Van Loosdrecht, M. C. M. (2004). Production of polyhydroxyalkanoates by mixed culture: Recent trends and biotechnological importance. *Biotechnology Advances*, **22**(3); pp261–279.
- Samorì, C., Abbondanzi, F., Galletti, P., Giorgini, L., Mazzocchetti, L., Torri, C., & Tagliavini, E. (2015). Extraction of polyhydroxyalkanoates from mixed microbial cultures: Impact on polymer quality and recovery. *Bioresource Technology*, **189**(2015); pp195–202.
- Sathiyarayanan, G., Bhatia, S. K., Song, H. S., Jeon, J. M., Kim, J., Lee, Y. K., Kim, Y. G., & Yang, Y. H. (2017). Production and characterization of medium-chain-length polyhydroxyalkanoate copolymer from Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620. *International Journal of Biological Macromolecules*, **97**(2017); pp710–720.
- Senthilkumar, P., Dawn, S. S., Sree Samanvitha, K., Sanjay Kumar, S., Narendra Kumar, G., & Samrot, A. V. (2017). Optimization and characterization of poly[R]hydroxyalkanoate of *Pseudomonas aeruginosa* SU-1 to utilize in nanoparticle synthesis for curcumin delivery. *Biocatalysis and Agricultural Biotechnology*, **12**(2017); pp292–298.
- Sharma, P. K., Munir, R. I., Blunt, W., Dartailh, C., Cheng, J., Charles, T. C., & Levin, D. B. (2017). Synthesis and Physical Properties of Polyhydroxyalkanoate Polymers with Different Monomer Compositions by Recombinant *Pseudomonas putida* LS46 Expressing a Novel PHA SYNTHASE (PhaC1 16) Enzyme. *Applied Sciences*, **7**(242); pp1-13.
- Sharma, P. K., Munir, R. I., de Kievit, T., & Levin, D. B. (2017). Synthesis of polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *pseudomonas chlororaphis*. *Canadian*

- Journal of Microbiology*, **63**(12); pp1009–1024.
- Silva, J. B., Pereira, J. R., Marreiros, B. C., Reis, M. A. M., & Freitas, F. (2021). Microbial production of medium-chain length polyhydroxyalkanoates. *Process Biochemistry*, **102**(2021); pp393–407.
- Simon-Colin, C., Raguénès, G., Costa, B., & Guezennec, J. (2008). Biosynthesis of medium chain length poly-3-hydroxyalkanoates by *Pseudomonas guezennei* from various carbon sources. *Reactive and Functional Polymers*, **68**(11); pp1534–1541.
- Simon-Colin, C., Raguénès, G., Crassous, P., Moppert, X., & Guezennec, J. (2008). A novel mcl-PHA produced on coprah oil by *Pseudomonas guezennei* biovar. tikehau, isolated from a “kopara” mat of French Polynesia. *International Journal of Biological Macromolecules*, **43**(2); pp176–181.
- Song, Jin Hwan, Che Ok Jeon, Mun Hwan Choi, S. C. Y. and W. P. (2008). Polyhydroxyalkanoate (PHA) Production Using Waste Vegetable Oil by *Pseudomonas* sp. Strain DR2. *Journal of Microbiology and Biotechnology*, **18**(8); pp1408–1415.
- Tan, G. Y. A., Chen, C. L., Li, L., Ge, L., Wang, L., Razaad, I. M. N., Li, Y., Zhao, L., Mo, Y., & Wang, J. Y. (2014). Start a research on biopolymer polyhydroxyalkanoate (PHA): A review. *Polymers*, **6**(3); pp706–754.
- Vega-Castro, O., Contreras-Calderon, J., León, E., Segura, A., Arias, M., Pérez, L., & Sobral, P. J. A. (2016). Characterization of a polyhydroxyalkanoate obtained from pineapple peel waste using *Ralstonia eutropha*. *Journal of Biotechnology*, **231**(2016); pp232–238.
- Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D., Piotrowska-Seget, Z., & Radecka, I. K. (2011). Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. *AMB Express*, **1**(1); pp1–8.
- Yin, F., Li, D., Ma, X., & Zhang, C. (2019). Pretreatment of lignocellulosic feedstock to produce fermentable sugars for poly(3-hydroxybutyrate-co-3-

hydroxyvalerate) production using activated sludge. *Bioresource Technology*, **290**(2019); pp1–6.

Zher Neoh, S., Fey Chek, M., Tiang Tan, H., Linares-Pastén, J. A., Nandakumar, A., Hakoshima, T., & Sudesh, K. (2022). Polyhydroxyalkanoate synthase (PhaC): The key enzyme for biopolyester synthesis. *Current Research in Biotechnology*, **4**(1); pp87–101.

4 Development of pretreatment techniques to improve the synthesis of Polyhydroxyalkanoate (PHA) from waste oils.

Authors: Hano, L.J., Soares, A and Wagland, S.

Abstract

*Pretreatment stands a crucial initial phase in transforming waste materials into valuable resources. Exploring lipid-rich waste pretreatment is seldom conducted to promote optimal microbial activity and enhance overall process efficiency. However, the study aimed to investigate the effects of different pretreatment techniques (lipase, microwave, ultrasound-assisted) on the synthesis of bioplastics from two strains of *Pseudomonas oleovorans* (DSMZ1045 and ATCC29347) using lipid-rich oil waste, including glycerol, soybean oil, palm oil and fat, oil & grease (FOG) as a substrate. The effects of different pretreatment methods on the PHA yield [(lipase 53%, 48%, 47%, & 55%/CDW and 51%, 43%, 45% & 54%CDW), (microwave 51%, 45%, 43%, & 52%/CDW, and 49%, 41%, 39% & 50%/CDW), and (ultrasound 50%, 40%, 41%, & 51%/CDW and 49%, 39%, 38% & 49%/CDW) were investigated. Results showed that the pretreatment enhances the production yield of PHA from both organisms. However, enzyme-assisted pretreatment has the highest yield from both carbon sources utilized by *Pseudomonas oleovorans* DSMZ1045 and *Pseudomonas oleovorans* ATCC29347. The finding proved that the pretreatment strategy is promising for high-yield bioplastics using different carbon sources by *Pseudomonas* strains. The produced bioplastic was characterized using Fourier transform infrared spectroscopy (FT-IR) and Scanning electron microscopy (SEM). Results confirmed the analysed functional groups of PHA were medium chain length PHA (mcl-PHA) compared with the reported proven functional groups for mcl-PHA from the related available literature sources. Moreover, the study suggests that enzyme-assisted pretreatment is a promising method for optimizing bioplastic synthesis from lipid-rich oil waste using *Pseudomonas oleovorans* strains.*

Keywords: *Pseudomonas* strains, bioplastics, *Pseudomonas oleovorans*, Lipase, Microwave, Ultrasound.

4.1 Introduction

Synthetic polymer-based materials are one of the most widely used materials in modern life today because of their characteristics (Szacherska *et al.*, 2021). Because of these many uses, most especially in packaging, plastics have become an essential product in modern society. According to reports, the production of plastics hit 348 million tonnes globally in 2017, an increase of 4% yearly (Khatami *et al.*, 2021). Thus, continuously using them causes irreversible changes and significant negative impacts on Earth and natural systems (Szacherska *et al.*, 2021). Furthermore, the two methods of disposing of plastics are landfilling and incineration, which are costly and produce hazardous consequences (Khatami *et al.*, 2021). As a result, the use of sustainable, alternative materials to replace petroleum-based materials is urgently needed. Producing biopolymers to replace traditional plastics is one feasible strategy. Biopolymers are often biodegradable and made from sustainable biomaterials, making the process environmentally friendly (Du *et al.*, 2012).

Bioplastics have attracted a lot of interest because of their sustainability, biodegradability, and ecologically favourable characteristics, and they have many advantages over traditional oil-based plastics (Surendran *et al.*, 2020). Despite their various benefits and uses, PHAs still battle with high production and market costs, which are associated with the carbon sources employed in their production. Since the prior years, its growth has looked at the viability of using organic wastes, particularly waste oils, as low-cost feedstock (Guleria *et al.*, 2022). Hence, the feasible and sustainable method to lower production costs is to use waste oils as carbon sources for PHA biosynthesis. Since they demonstrate a high product-to-substrate yield and are inexpensive, oil wastes have been suggested as carbon sources for PHA production (Cruz *et al.*, 2014). Moreover, using waste oil (WO) as a substrate for the synthesis of PHA can reduce expenses by nearly 50% (Pan *et al.*, 2021). Besides, the production of biopolymers is feasible to become a significant player in the future because of the continued high interest in renewable, environmentally friendly, and bio-based materials. In 2017, up to 2.05 million tonnes of PHAs were produced globally. In

addition, a pre-treatment procedure was added to investigate the possibility of converting these wastes into bioplastics in order to enhance the production yield and make these waste oils accessible to the organisms. Hence, as described by Harris and McCabe (2015), pre-treatment is the treatment of wastes that increases the availability of substrates to microbes, improves the removal of organics, improves reaction kinetics, and increases total production output. Pre-treatment techniques can be broadly categorised as physical, chemical, biological, mechanical, and combinations processes, and different pre-treatment strategies have been thoroughly investigated (Chong *et al.*, 2021; Gao *et al.*, 2010). However, this study focuses on different pre-treatment strategies to effectively use lipid-rich oil wastes as feedstocks to maximize bioplastic production yields. The production of PHA to the greatest possible extent using pre-treatment techniques has not been the subject of many published studies. The main goal of this research is to evaluate the feasibility of this strategy by comparing different pre-treatment techniques that will enhance *Pseudomonas* strains' capacity to manufacture bioplastics from diverse lipid-rich oil waste sources. However, pretreatment of lipid-rich waste, i.e., fat, oil, and grease, is infrequently examined, often in either a microwave or an ultrasound and biological (enzymatic pretreatment). The effects of various pretreatment strategies have not been well-studied. Therefore, this study will provide a pretreatment technique suitable for lipid-rich waste using those methods in bioplastic PHAs production, aiming to enhance microbial activity and increase process efficiency. However, FTIR and SEM tests were used to look into the chemical functional groups and morphological images of the produced PHAs.

4.2 Materials and Methods

4.2.1 Materials used.

Sodium phosphate monobasic (500 g, Germany, 99%), sodium hydroxide (250 mL, 99.8%, Switzerland), magnesium sulfate (500 g, Japan, 99%), calcium chloride dihydrate (500 g, Germany, 98%), iron (II) sulfate (250 g, India, 99%), manganese (II) chloride tetrahydrate (500 g, Japan, 99.5%), zinc sulfate

heptahydrate (500 g, USA, 99%), sodium chloride (250 g, USA, 99.5%), potassium phosphate monobasic (500g, Germany, 99%), calcium chloride hexahydrate (200 g, China, 99%), sodium phosphate dibasic (500 g, Japan, 99%), nutrient broth powder (500 g, India, 8 g/L) yeast extract (3 g/L), urea (Germany) were all purchased from (Sigma Aldrich), glycerol (500mL, Malaysia, 99.5%), 37% HCL (Australia), 30% H₂O₂ (Spain) (chloroform (2.5 L, Germany, 99.8%), ethanol (2.5 L, UK, 99.8%), acetone (2.5 L, Finland, 99.8%), methanol (2.5 L, UK, 99.9%), were all purchased from (Fischer scientific), *Pseudomonas oleovorans* DSMZ 1045 and, *Pseudomonas oleovorans* ATCC 29347 (Purchased from ATCC and PHEC), as lyophilized ampoule culture.

4.2.2 Equipment used.

Shaker incubator (Incu-Shaker MIDI, Benchtop Shaking Incubator, Switzerland), Freeze dryer (Christ Alpha 1-2 LDplus, Germany), spectrophotometer (JENWAY 6310, UK), bath ultrasound incubator (Ultrawave, Asset 50A) (Ultrawave Ltd, Cardiff, UK), microbiological safety cabinet (Labcare SC-R Recirculating Class II, Labcare System Ltd, England), FTIR techniques (Vertex 70, Bruker), Fume Cupboard (Heraeus, Germany), autoclave, Bench-scale Microwave, Beakers (100 mL, 250 mL, 300 mL, 400 mL, Fisher brand, Germany), Measuring cylinders (50 mL, 250 mL, 500 mL, and 1000 mL, Technicon, England), Centrifuge (SORVALL ST40, ThermoFisher Scientific, Germany), VEGA 3 (Tescan, Czech Republic), Erlenmeyer conical flasks (100 mL, 250 mL, Fisher brand, Germany), Scale (Precisa, UK), Durham bottle (250 mL, 500 mL, Fisher brand, Germany).

4.2.3 Carbon sources

This study employed lipid-rich waste such as glycerol, fat, oil, and grease (FOG), frying oils (soybean oil and palm oil). FOG sample was obtained from Severn Trent wastewater treatment plant, UK. Glycerol was ordered from Fisher Scientific, UK. Frying oils were obtained from Cafeteria and household, Cranfield University, UK.

4.2.4 Methods used.

4.2.4.1 Cultures revived and maintenances.

The lyophilized ampoule cultures: *Pseudomonas oleovorans* (DSMZ1045 & ATCC29347), were revived in nutrient medium at 30°C for 24 h at 180 and maintained at -80°C in spirit of 50% (v/v) glycerol (Davis *et al.*, 2013), and used for the whole experiments. The ability of these strains to produce bioplastic, which has been extensively researched (Kanavaki *et al.*, 2021; Mozejko-Ciesielska *et al.*, 2019), was a deciding factor in their selection.

4.2.4.2 Pretreatment methods

Pretreatment is an important stage in the production of PHA. Waste oil acts hostile to microorganisms, and if not treated before being used to produce PHA they become toxic to them and affect the yield and quality of the PHA. To improve PHA production pretreatment techniques were added, including microwave, ultrasonication, and lipase, to increase the viability of using these feedstocks and enable the microbial fermentation process to occur effectively to produce high-quality PHA. The steps for each pretreatment procedure are described below.

4.2.4.2.1 Microwave (MW) pretreatment

Oil sample (10 mL of each) was pretreated in a beaker at high power (450W) at 50°C for 3 min using a bench-scale industrial microwave (MW). After it was allowed to cool to room temperature and aseptically using a filter syringe, impurities were removed after settling down.

4.2.4.2.2 Ultrasound (US) pretreatment

Oil sample (10 mL of each) was placed in a water bath ultrasound incubator (Ultrawave, Asset 50A) at 50°C for 15 min. After that, the sample was removed from the water bath ultrasound and allowed to cool to room temperature, aseptically the oil was separated from the settled impurities using a filter syringe, and it was transferred to a 250 mL flask containing the mineral medium and mixed before the bacterial strain was inoculated.

4.2.4.2.3 Lipase pretreatment

200 μ L of lipase was added to a 10 mL of each oil sample, and the mixture was then shaken at 200 rpm for 10 minutes at 25 °C. Next, using a microbiological safety cabinet (II), the content was gently shaken and aseptically injected into the medium.

4.2.4.3 Bacteria growth and PHA production

Pseudomonas oleovorans (DSMZ1045 & ATCC29347) were grown in 100 mL of nutrient broth (NB) medium (8 g/L of nutrient broth powder, 3 g/L yeast extract, 2.5 g/L NaCl), in 250 mL Erlenmeyer flasks using 5 mL of the revived cultured of *P. oleovorans* (DSMZ1045 & ATCC29347). The cultures were incubated on a shaking at 180 rpm at 30°C for 24 h. The cells were harvested by centrifugation at 3800 rpm for 15 min, and the pellets were transferred to 250 mL flasks containing 100 mL mineral medium (MM) composed as follows: 1.5 g/L KH_2PO_4 , 6.7 g/L Na_2HPO_4 , 4.5 g/L NaH_2PO_4 . This medium was adjusted to a pH of 7.0, autoclaved at 121, and subsequently supplemented with 0.06 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 g/L MgSO_4 , and 1 mL/L of a microelement solution (Ballistreri *et al.*, 2001). Microelement trace stock contained follows 1.5 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.24 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.03 g/L $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 0.048 g/L CuSO_4 , 0.24 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was dissolved in 1 M of HCl, sterilized using a syringe filter (Zainab *et al.*, 2018), 0.54 g/L of urea, and 2% (v/v) of oil as carbon feed, and all were added to the medium before inoculation. After fermentation at 48 h, the fermentation culture broth was harvested by centrifugation (3800 rpm for 15 min), washed up two times with distilled water, and the pellets were lyophilized before polymer extraction.

4.2.4.4 Determination of optical density

Samples were collected for 48 h at an interval of 3 h (i.e., 0,3,6....48 h), after, the sample was properly diluted with milli-Q water, the optical density of the growing cultures was monitored by measuring the absorbance at wavelength 600 nm ($\text{OD}_{600\text{nm}}$) with a spectrophotometer using UV-spectrometer. However, shifting hours were not accounted for due to the fact that experiment readings were not recorded during the period.

4.2.5 Statistical analysis

A one-way analysis of variance (ANOVA) was performed on the data using SPSS and Minitab. The statistical differences between the treatment means were tested for significance using a P-value cut off 0.05. There were three duplicates of each experiment.

4.3 Polymer recovery

4.3.1 Separation (harvesting)

The fermented broth cultures were harvested using a centrifuge (SORVALL ST40 Centrifuge, Thermo Scientific) at 3800 rpm for 15 minutes at room temperature to distinguish the pellet from the supernatant. The pellet was then dried using a freeze-dryer at -54°C for 24 h after being twice cleaned with distilled water to get rid of any remaining debris.

4.3.2 Determination of dry cell weight

The 50 mL centrifuge falcon tubes were filled with 40 mL of the fermentation broth culture sample, which was then centrifuged at 3800 rpm for 15 minutes at room temperature. The supernatants were discarded, and the cell pellets were rinsed twice with distilled water before being freeze-dried at -54°C for 24 hours to achieve the constant weight of the cell biomass. Using dried and pre-weighed falcon tubes, the cell's dry weight was calculated gravimetrically.

4.3.3 Polymer extraction

The polymer was then extracted from the dried powdered pellet using three different extractors: sodium hydroxide, enzyme, and hydrogen peroxide digestion techniques. The polymer was then centrifuged, rinsed with distilled water again, purified using cold methanol, and left to dry for a day in a fume cupboard.

4.3.4 Polymer quantification

After extraction, the quantity of accumulated PHA by the organisms (DSMZ1045 & ATCC29347) was calculated by using the following formula:

$$\%PHA = \frac{PHA \text{ quantity}}{DCW} * 100 \quad (4-1)$$

Where DCW represents dried cell weight.

4.4 Polymer characterization

After polymer quantified, the polymer was analysed and characterized using SEM and FTIR analysis.

4.4.1 SEM analysis

The sample materials were mounted into the sample holder and coated with gold (Senthilkumar *et al.*, 2017), and the sample holder was placed onto the TESCAN VEGA 3 (Fig. 3-2), and subjected to SEM examination. The images were taken at different magnifications from 1000-5000x with a used voltage of 10kV (Murugan *et al.*, 2016), or 20kV to examine the morphological structures of each samples.

4.4.2 FTIR analysis.

FTIR analysis of the samples were performed and scanned using FTIR techniques (VERTEX 70, Bruker). The sample was first prepared, and then it was scanned and observed between wavenumbers of 400 cm⁻¹ and 4000 cm⁻¹ (Kanavaki *et al.*, 2021) waves at a speed of 1 µm/min with a spectral resolution of 4 cm⁻¹ (Vega-Castro *et al.*, 2016). The corresponding absorption bands of the functional groups were identified and recorded.

4.5 Results and Discussion

4.5.1 Production and growth under pretreatment

Pseudomonas oleovorans (DSMZM1045 & ATCC29347) were employed to characterize the production of PHA using various pretreated carbon sources, including glycerol, FOG, soybean oil, and palm oil. 1 mL was withdrawn from each fermentation flask (from zero to 48 h) at intervals of 3 h to measure the optical density and record the values (Hall *et al.*, 2014). The graph curve was

plotted using lnOD (cell/mL) against the time (h), Fig4-1(ai-iii) and (bi-iii). The growth rate of *P. oleovorans* (DSMZ1045 & ATCC29347) at the initial count of 15 h is between 0.97 h⁻¹ and 0.95 h⁻¹, respectively. When the fermentation reaches the stationary stage, the PHA accumulation accelerates to a peak between 38 and 40 h. Adding pretreatment increases cell-dried weight content and PHA accumulation. After 42 h of cultivation, Growth was slightly decreased due to the decrease in nutrients and increase in metabolites, inhibitors, and toxins (Hall et al., 2014). This led to a negative effect on PHA accumulation and reduced PHA production. According to (Kaiser, 2019), growth is only possible in the presence of a suitable environment, enough nutrients, and a mechanism to get rid of waste. Furthermore, the literature reports several models relating to this mechanism. However, the Monod model provides a simple and fundamental explanation of how growth and substrate consumption function (Priyadarshini et al., 2021). The growth curves of these *Pseudomonas* strains (DSMZ1045 & ATCC29347) with different pretreated fermentative substrate as shown in Figure. The result reveals that R² is 0.931 and is used to determine the validity of model as closeness to 1, while efficiency is 93.1% of the variability observed in the target variables, as explained by the regression model (Muloiwa et al., 2020), was statistically significant, Table 4-1. The mean values of the estimated margin confirmed the 95% level of confidence interval within factors (Hall et al., 2014), as they were between the upper and lower bounds of the level of confidence. The mean, standard error, and lower and upper boundaries of the estimated margin are given in Appendix B

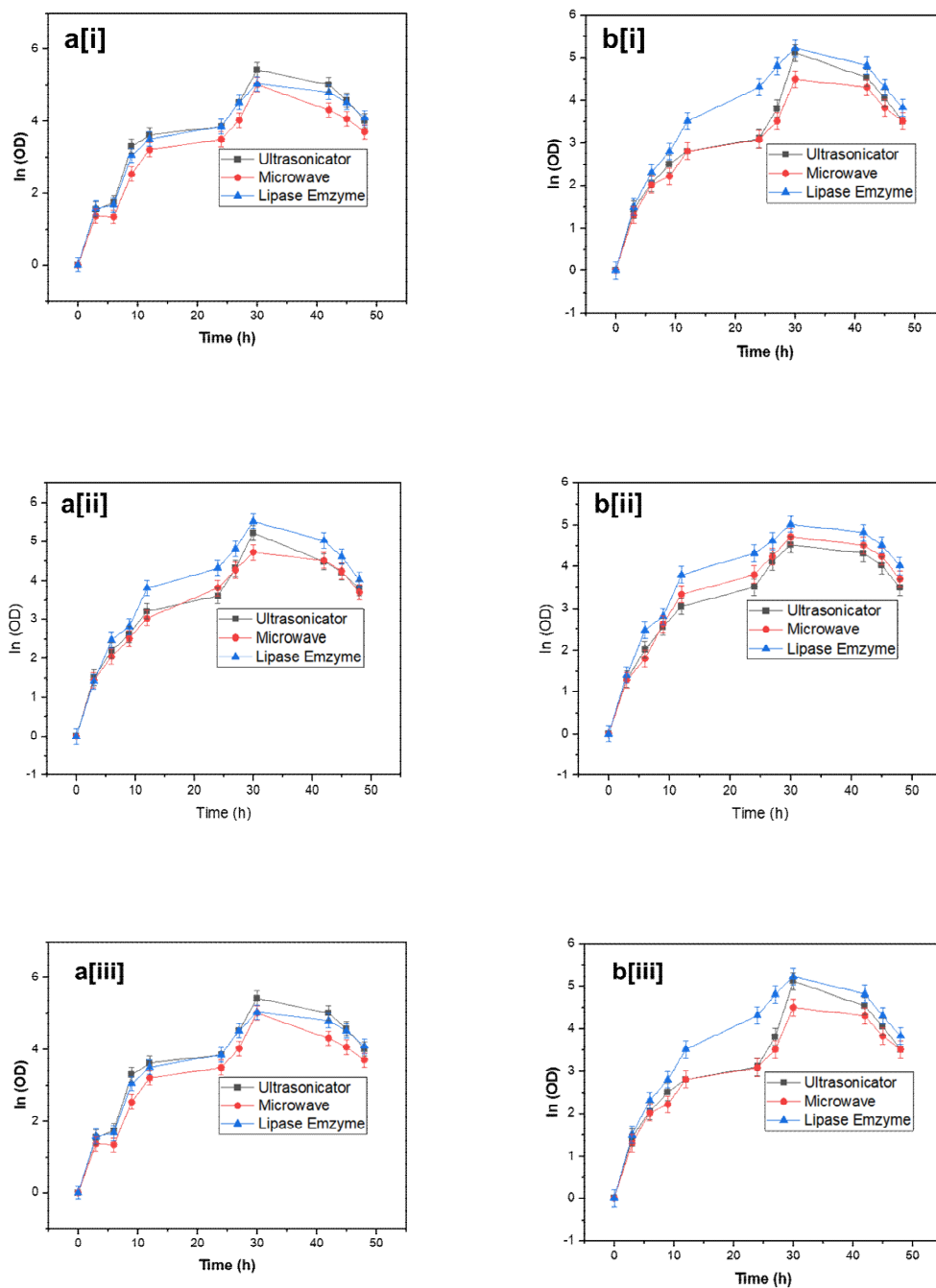


Figure 4-1: Growth curve of *P. oleovorans* from pretreatment: a[i-iii] - ATCC29347 and b[i-iii] - DSMZ1045

Table 4-1: Test of Between-Subject effects

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Model	ULTRASOUND	295.409	1	295.409	45.869	.000	.821
	MICROWAVE	263.192	1	263.192	47.906	.000	.827
	LIPASE	381.480	1	381.480	49.224	.000	.831
Error	ULTRASOUND	64.403	10	6.440			
	MICROWAVE	54.939	10	5.494			
	LIPASE	77.499	10	7.750			

4.5.2 Effect of various pretreatment on PHA content

(Fig. 4-2), revealed and highlights how each pretreatment method affects the percentage PHA yield. Fig.4.2a reveals the performance of the lipase pretreatment method on all the carbon sources. Higher %PHAs were obtained with the glycerol and FOG while soyabean and palm oil showed a decline in their PHAs. Both organisms achieved a high recovery yield from each method, although the findings indicate that *P. oleovorans* DSMZ1045 had a higher percentage recovered polymer yield from each carbon source than *P. oleovorans* ATCC29347. Additionally, the obtained results show that *P. oleovorans* DSMZ1045 and *P. oleovorans* ATCC29347, respectively, have PHA contents of 55% and 54% following lipase pretreatment. However, Fig.4.2b and Fig.4.2c show a similar trend in their PHA yields from FOG as compared to other carbon sources. This can be attributed to the boosting of the surface area to volume ratio, hydrolysing macromolecules, and releasing trapped organic chemicals by *P. oleovorans* DSMZ1045 and *P. oleovorans* ATCC29347 (Harris and McCabe, 2015). Lipase pretreatment enhanced bioplastic production more efficiently than alternative techniques, such as chemical and physical methods. *Pseudomonas*

mediterranea 9.1 was able to accumulate 0.81g/L of PHA when glycerol was used as a carbon source (Palmeri *et al.*, 2012).

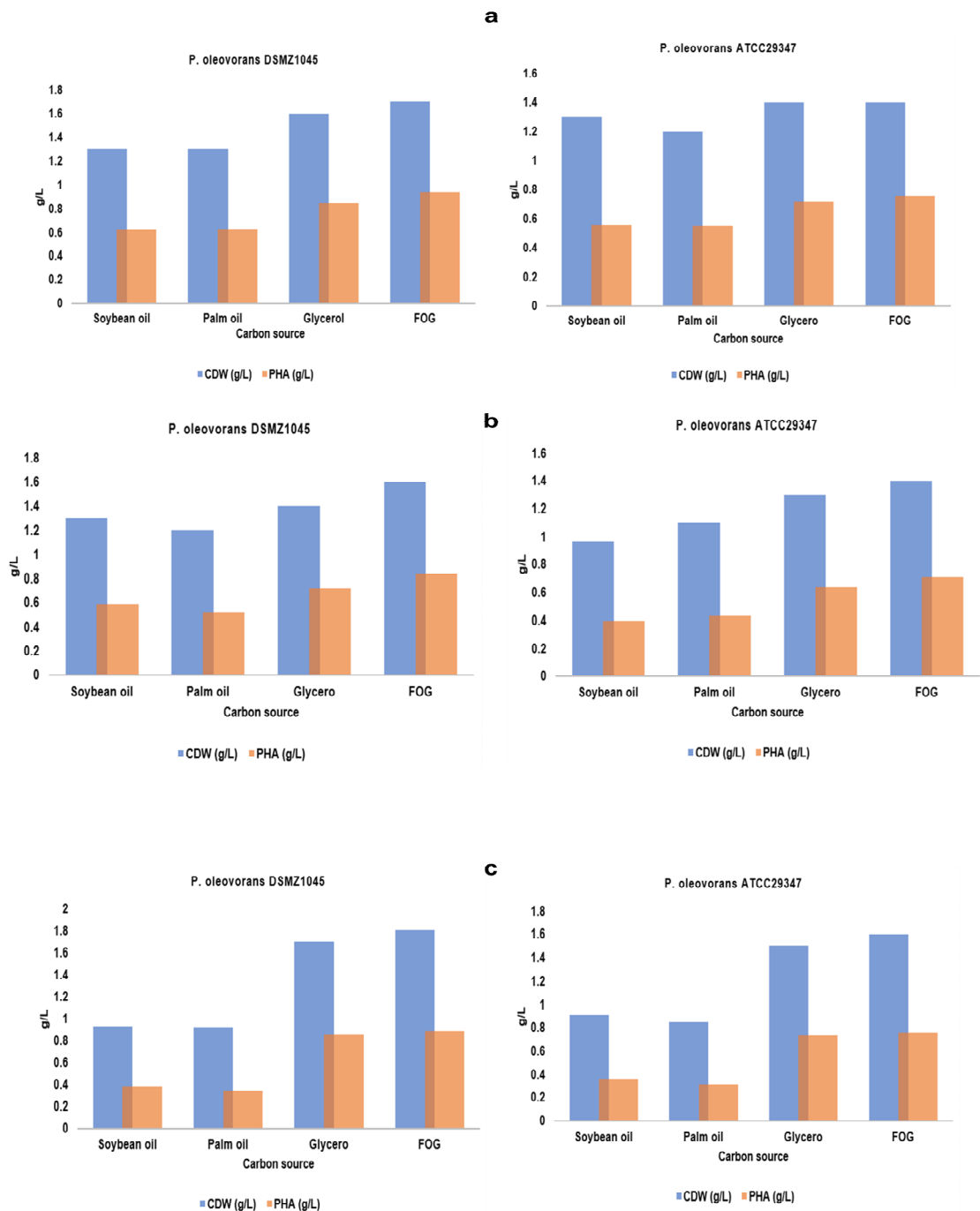


Figure 4-2: Impact of pretreatment techniques on PHA:(a) lipase, (b) ultrasound and (c) microwave.

The distribution of the cell dry and PHA content from each pretreatment strategy is depicted in Table 4-2. The following carbon sources: glycerol, soybean, palm oil, and FOG were mainly compared considering their performance on the premising contents. All carbon source shows a similar pattern in their performance as they favour all the pretreatment techniques. All the bacteria (*Pseudomonas oleovorans* DSMZ1045 and *Pseudomonas oleovorans* ATCC29347) had good cell biomass and PHA content from each carbon source. *P. oleovorans* DSMZ1045 gave the maximum cell mass, and PHA content from each carbon source. However, with glycerol and FOG, the maximum cell biomass of 1.70 g/mL and 1.80 g/mL were achieved, while PHA content (0.89 g/L and 0.94 g/L), respectively. Hence, it was found from this study that the pretreatment approaches led to the highest levels of cell biomass and PHA production. Additionally, the chosen best microbial strains and the substrates may also impact the production and accumulation of PHA, Table 4-2. According to the report from the literature, the demand for bioplastics with a wide range of carbon sources must reach the possible productivity and applications (Sarmiento-Vásquez *et al.*, 2022). This study showed that applying pretreatment to different carbon sources increases cell biomass, PHA content, and productivity.

Table 4-2: Impact of each pretreatment on cell mass and PHA productivity

Organisms	Carbon source	Pretreatment technique applied	CDW (g/L)	PHA (g/L)
<i>P. oleovorans</i> DSMZ1045	Glycerol	Ultrasound	1.70	0.86
<i>P. oleovorans</i> ATCC29347	Glycerol	Ultrasound	1.50	0.74
<i>P. oleovorans</i> DSMZ1045	FOG	Ultrasound	1.80	0.92
<i>P. oleovorans</i> ATCC29347	FOG	Ultrasound	1.60	0.79
<i>P. oleovorans</i> DSMZ1045	Soybean oil	Ultrasound	0.93	0.38
<i>P. oleovorans</i> ATCC29347	Soybean oil	Ultrasound	0.91	0.36
<i>P. oleovorans</i> DSMZ1045	Palm oil	Ultrasound	0.98	0.41
<i>P. oleovorans</i> ATCC29347	Palm oil	Ultrasound	0.92	0.35
<i>P. oleovorans</i> DSMZ1045	Glycerol	Microwave	1.40	0.72
<i>P. oleovorans</i> ATCC29347	Glycerol	Microwave	1.30	0.64
<i>P. oleovorans</i> DSMZ1045	FOG	Microwave	1.60	0.84
<i>P. oleovorans</i> ATCC29347	FOG	Microwave	1.40	0.71
<i>P. oleovorans</i> DSMZ1045	Soybean oil	Microwave	1.30	0.59
<i>P. oleovorans</i> ATCC29347	Soybean oil	Microwave	0.97	0.40
<i>P. oleovorans</i> DSMZ1045	Palm oil	Microwave	1.20	0.52
<i>P. oleovorans</i> ATCC29347	Palm oil	Microwave	1.10	0.43
<i>P. oleovorans</i> DSMZ1045	Glycerol	Lipase	1.60	0.85
<i>P. oleovorans</i> ATCC29347	Glycerol	Lipase	1.40	0.72
<i>P. oleovorans</i> DSMZ1045	FOG	Lipase	1.70	0.94
<i>P. oleovorans</i> ATCC29347	FOG	Lipase	1.40	0.76
<i>P. oleovorans</i> DSMZ1045	Soybean oil	Lipase	1.30	0.63
<i>P. oleovorans</i> ATCC29347	Soybean oil	Lipase	1.30	0.56
<i>P. oleovorans</i> DSMZ1045	Palm oil	Lipase	1.30	0.62
<i>P. oleovorans</i> ATCC29347	Palm oil	Lipase	1.20	0.55

4.5.3 Polyhydroxyalkanoate % Yield

PHA synthesis by *Pseudomonas oleovorans* (DSMZ1045 and ATCC29347) from various pretreatment carbon sources were examined. Both pretreatment

methods enhanced the PHA percentage (%) yield, with lipase giving the highest PHA % yield after 48 h of fermentation, while ultrasound yielding the minimum PHA % from both *Pseudomonas oleovorans* (DSMZ1045 and ATCC29347), Fig4-3(a & b). *Pseudomonas oleovorans* DSMZ1045 produced a 55% yield of PHA/CDW, and *Pseudomonas oleovorans* ATCC29347 produced 54% of PHA/CDW when the lipase technique was employed. While that of microwave and ultrasound (52% & 50% of cell dry weight) and 51% & 49% of cell dry weight) were accumulated by *P. oleovorans* DSMZ1045 and *P. oleovorans* ATCC29347, respectively. Results showed that lipase pretreatment was more effective than ultrasound and microwave pretreatment techniques in maximizing the production of bioplastics. Lipases are extremely versatile enzymes that catalysed various processes, including esterification, interesterification, transesterification, and alcoholysis (Fraga *et al.*, 2021). Additionally, lipases were previously reported in the published literature to enhance the bioproduction of valuable products (Mankar *et al.*, 2021). Accordingly Liu *et al.* (2017), revealed that enzymatic catalysis is an effective technique for processing waste oil in maximizing the production yields of many products, such as biodiesel, bioplastics, and others.

Furthermore, Sarmiento-Vásquez *et al.* (2022) reported a PHA % yield of 39% when pretreated glycerol was used as a carbon source by the *Pseudomonas* strain. PHA % accumulation yield of 54% was obtained by *Pseudomonas* strain H3 utilizing pretreated waste food oil (Pan *et al.*, 2021). Similarly, Zakaria *et al.* (2010) reported a % PHA yield of 59% from pretreated palm oil. Another study revealed that a PHA yield of 43% was accumulated by the strain *Pseudomonas* using pretreated palm oil as a fermentative carbon substrate (Mozejko and Ciesielski, 2013). This study findings shows a great outcome to produce bioplastics when compared it with those in the previous studies. The yield from the two strains of *Pseudomonas* employing different treated carbon sources is comparatively encouraging, and this further suggest that selecting specific *Pseudomonas* strains and pretreatment methods can significantly impact the production yield. For example, *Pseudomonas oleovorans* DSMZ1045 and lipase pretreatment can lead to a more efficient and cost-effective production process.

The study finding agreed with the previously published literature that examined the production of bioplastics using different pretreatment on waste oils, such as glycerol, soybean, and palm oils (Pan *et al.*, 2021; Ravindran *et al.*, 2020; Zakaria *et al.*, 2010; Zhang *et al.*, 2021). Pretreatment has been found to be a promising method for enhancing the synthesis of bioplastics in numerous studies that have been reported (Ebrahim and Ismail, 2019).

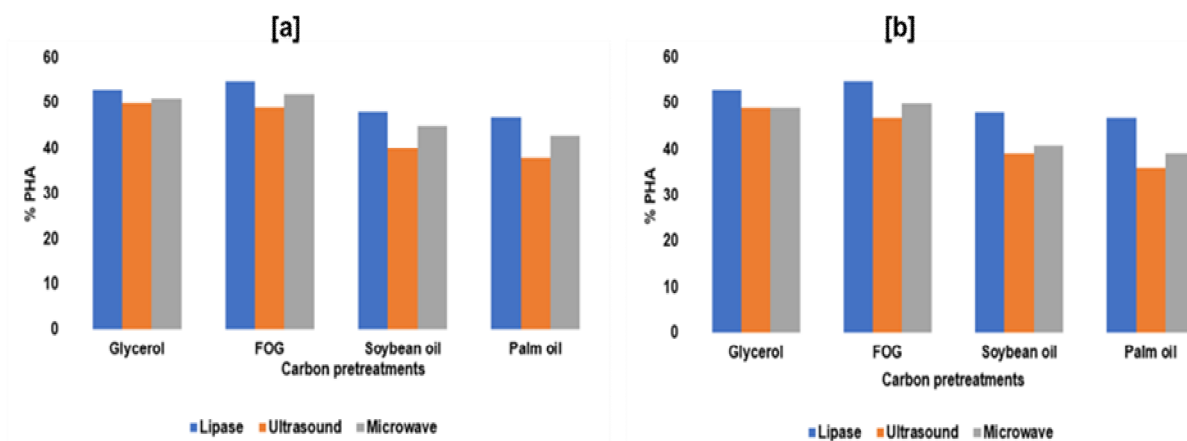


Figure 4-3: Effect of each pretreatment on yield %PHA/CDW

4.5.4 Scanning Electron Microscope results

The morphology of the PHA granules inside the cell was studied using scanning electron microscopy (SEM). The granule images of the polymer obtained from these *Pseudomonas* species [*P. oleovorans* (DSMZ1045 & ATCC29347)] are shown in Fig. 4-4. Both Fig. 4.4 (a and b) revealed compacted polymers with significant porous structures and linked granules. The linked granules were spherical shapes with varying sizes. The morphology exhibits pseudo-spherical granules with an essentially uniform distribution in the two species. However, the presence of a flakes-like whitish formation of PHA was observed in ATCC29347. This produced a result that is identical to other researchers' findings (Liu *et al.*, 2021; Nwinyi and Owolabi, 2019).

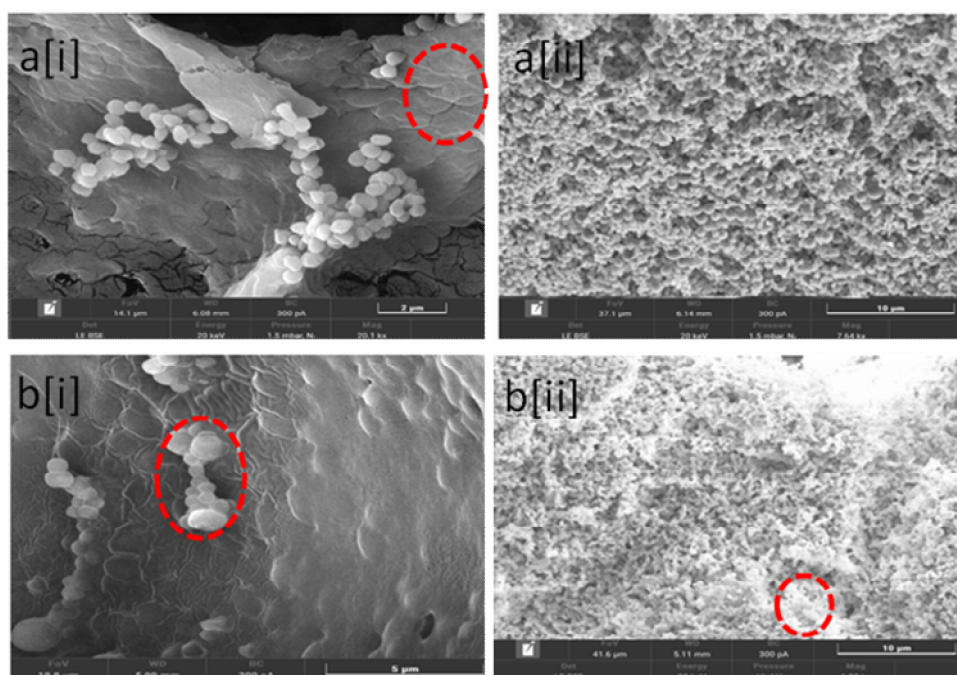
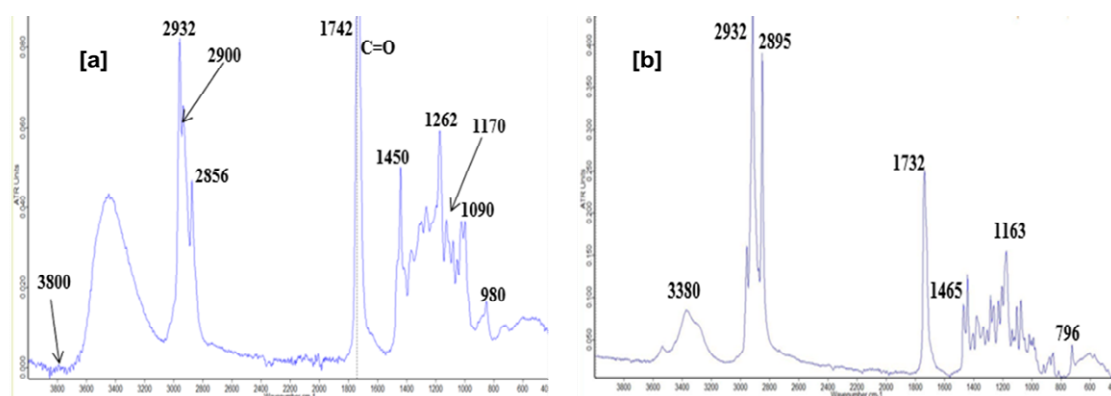


Figure 4-4: SEM images of granules observed: a (i-ii) DSMZ1045 and b(i-ii) ATCC29347.

4.5.5 FTIR analysis of the extracted polymers

The spectra of the observed polymer are shown in Fig. 4-5 (a-c) using FTIR analysis. Typical sharp bands associated with FOG, frying soybean oil, and frying palm oil, namely $\text{CH}_2\text{-CH}_2$ stretching mode at 2932 cm^{-1} . The stretching band peaks at $1732\text{-}1742\text{ cm}^{-1}$ can be attributed to C=O (carboxylic group), and $2856 - 2932$ can be ascribed to CH_3 and CH_2 , which corresponded to methyl and methylene groups, as can be observed. The stretching bands at $3800\text{-}3380\text{ cm}^{-1}$ present the C-H deformation in Fig. 4.5 (a-c), whereas the peaks at $1450\text{-}980\text{ cm}^{-1}$ can be attributed to the C-O-C asymmetric stretching of FOG, frying palm oil, and frying soybean oil. Furthermore, the chemical structure of the polymer was revealed by the FTIR analysis, which reflects the monomeric units. The absorption bands in 2935 cm^{-1} , 2999 cm^{-1} , 2932 cm^{-1} , and 3170 cm^{-1} were due to C-H , which is stretching vibration bands indicating strong functional groups of methyl and methylene as described by (Abid *et al.*, 2016); Gupta *et al.*, 2017); Mahato *et al.*, 2021). Furthermore, bands in $1730\text{ cm}^{-1} - 740\text{ cm}^{-1}$ were assigned to C=O , as observed in the previous studies(Gumel *et al.*, 2013b). The FTIR

absorption bands obtained were between 2856 cm^{-1} and 2932 cm^{-1} agreeing with those previously reported by (Abid *et al.*, 2016; Gumel *et al.*, 2013b; Mahato *et al.*, 2021). While the absorption bands at $1732 - 1742\text{ cm}^{-1}$ (ester C=O), $1450 - 1639\text{ cm}^{-1}$ (thioester C=O), $1165 - 1193\text{ cm}^{-1}$ (ester C-O) were consistent with stretching groups indicating strong absorption bands of mcl-PHA produced by different published literature (Aloui *et al.*, 2022; Rebocho *et al.*, 2019, 2020; Sathiyarayanan *et al.*, 2017; Sharma *et al.*, 2017b, 2017a; Simon-Colin *et al.*, 2008b, 2008a). The analysed extracted PHA revealed its absorption bands at 1732 cm^{-1} , 1742 cm^{-1} , 1165 cm^{-1} , 1193 cm^{-1} , 1262 cm^{-1} , 1450 cm^{-1} , 3800 cm^{-1} and 1639 cm^{-1} identical to carbonyl functional group, methyl and methylene groups, ester, and methyl terminal groups. The discovered FTIR absorption bands in 1732 cm^{-1} , 1742 cm^{-1} , 2935 cm^{-1} , 2932 cm^{-1} , and 1090 cm^{-1} verified that the analysed and observed PHA was mcl-PHA because of the distinction of the absorption bands similar or closeness to mcl-PHA reported by (Abid *et al.*, 2016; Gumel *et al.*, 2013b; Mahato *et al.*, 2021; Rebocho *et al.*, 2019, 2020; Sharma *et al.*, 2017b, 2017a; Simon-Colin *et al.*, 2008a, 2008b). According to Gupta *et al.* (2017), PHA marker band is located at absorption bands greater than 1730 cm^{-1} , where a carbonyl (C=O) ester bond is stretching. Furthermore, the reported absorption bands obtained agreed with those reported from published studies, as seen in Fig. 4-5 (d).



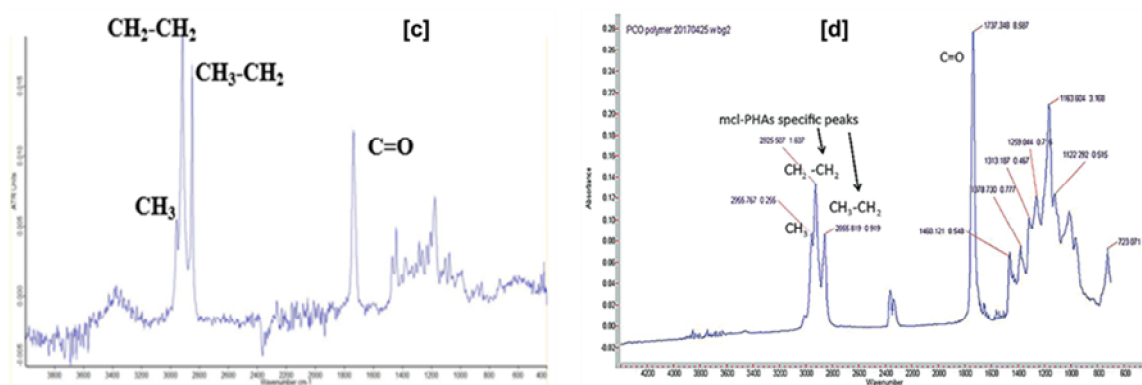


Figure 4-5: FTIR analysis of the produced PHAs from [a] FOG, [b] FSO, [c] FPO, and [d] Reference from literature (Sharma *et al.*, 2017b)

4.6 Conclusion

The results of this study demonstrated the viability of different pretreatment techniques such as enzyme, microwave, and ultrasound and their impact on increasing and enhancing carbon feed accessibility to the strains of the organism for sustainable production of PHA. The pretreated lipid-rich waste oils were feasibly converted to PHA with the highest percentage yield difference of accumulation which reached up to (23.26%, 41.18%, 46.88%, and 41.03%), (13.95%, 32.35%, 33.33%, and 34.38%), and (16.28%, 17.65%, 28.13%, and 30.77%) of cell dry weight (CDW), respectively. The outcome of the study was significant because the obtained PHA shows the same functional chemical groups as mcl-PHA when compared with those reported in the published literature. This study has demonstrated the viability of using lipid-rich waste oils for PHA production.

Finally, to make the procedure practical on a large scale more study is required in using bioreactor systems, strain cultivation, and optimization of additional pretreatment processes that could have an impact, as well as an investigation of their techno-economics and energy use.

Acknowledgement

Thanks to the PTDF and its management for providing me with financial support through the Nigerian PTDF-funded scholarship for this endeavour. Additionally, Cranfield University is acknowledged for providing the facilities that were available and easily accessible for the research.

References

- Abid, S., Ali, Z., & Tanveer, R. (2016). Production kinetics of polyhydroxyalkanoates by using *Pseudomonas aeruginosa* gamma ray mutant strain EBN-8 cultured on soybean oil. *3 Biotech*, **6**(2); pp1–10.
- Aloui, H., Khomlaem, C., Torres, C. A. V., Freitas, F., Reis, M. A. M., & Kim, B. S. (2022). Enhanced co-production of medium-chain-length polyhydroxyalkanoates and phenazines from crude glycerol by high cell density cultivation of *Pseudomonas chlororaphis* in membrane bioreactor. *International Journal of Biological Macromolecules*, **211**(5); pp545–555.
- Ballistreri, A., Giuffrida, M., Guglielmino, S. P. P., Carnazza, S., Ferreri, A., & Impallomeni, G. (2001). Biosynthesis and structural characterization of medium-chain-length poly(3-hydroxyalkanoates) produced by *Pseudomonas aeruginosa* from fatty acids. *International Journal of Biological Macromolecules*, **29**(2); pp107–114.
- Chong, J. W. R., Yew, G. Y., Khoo, K. S., Ho, S. H., & Show, P. L. (2021). Recent advances in food waste pretreatment technology via microalgae for source of polyhydroxyalkanoates. *Journal of Environmental Management*, **293**(2021); pp1–12.
- Cruz, M. V., Paiva, A., Lisboa, P., Freitas, F., Alves, V. D., Simões, P., Barreiros, S., & Reis, M. A. M. (2014). Production of polyhydroxyalkanoates from spent coffee grounds oil obtained by supercritical fluid extraction technology. *Bioresource Technology*, **157**(2014); pp360–363.
- Davis, R., Kataria, R., Cerrone, F., Woods, T., Kenny, S., O'Donovan, A., Guzik, M., Shaikh, H., Duane, G., Gupta, V. K., Tuohy, M. G., Padamatti, R. B., Casey, E., & O'Connor, K. E. (2013). Conversion of grass biomass into fermentable sugars and its utilization for medium chain length polyhydroxyalkanoate (mcl-PHA) production by *Pseudomonas* strains. *Bioresource Technology*, **150**(2013); pp202–209.

- Du, C., Sabirova, J., Soetaert, W., & Ki Carol Lin, S. (2012). Polyhydroxyalkanoates Production From Low-cost Sustainable Raw Materials. *Current Chemical Biology*, **6**(1); pp14–25.
- Fraga, J. L., Pereira, A. da S., Diniz, M. M., Fickers, P., & Amaral, P. F. F. (2021). Valorization of urban waste oil by microbial conversions. *Case Studies in Chemical and Environmental Engineering*, **4**(2021); pp1–7.
- Gao, M., Xu, F., Li, S., Ji, X., Chen, S., & Zhang, D. (2010). Effect of SC-CO₂ pretreatment in increasing rice straw biomass conversion. *Biosystems Engineering*, **106**(4); pp470–475.
- Guleria, S., Singh, H., Sharma, V., Bhardwaj, N., Arya, S. K., Puri, S., & Khatri, M. (2022). Polyhydroxyalkanoates production from domestic waste feedstock: A sustainable approach towards bio-economy. *Journal of Cleaner Production*, **340**(2022); pp1–20.
- Gumel, Annuar, Heidelberg, T. (2013). Growth kinetics, effect of carbon substrate in biosynthesis of mcl-PHA by *Pseudomonas putida* Bet001. *Journal of Microbiology*, **45**(2); pp427–438.
- Gupta, A., Kumar, M., & Thakur, I. S. (2017). Analysis and optimization of process parameters for the production of polyhydroxyalkanoates along with wastewater treatment by *Serratia* sp. ISTVKR1. *Bioresource Technology*, **242**(2017); pp55–59.
- Hall, B. G., Acar, H., Nandipati, A., & Barlow, M. (2014). Growth rates made easy. *Molecular Biology and Evolution*, **31**(1); pp232–238.
- Harris, P. W., & McCabe, B. K. (2015). Review of pre-treatments used in anaerobic digestion and their potential application in high-fat cattle slaughterhouse wastewater. *Applied Energy*, **155**(2015); pp560–575.
- Idris Zainab-L, Hiroshi Uyama, Cong Li, Yehua Shen, and K. S. (2018). Production of Polyhydroxyalkanoates From Underutilized Plant Oils by *Cupriavidus necator*. *Clean Sol, Air, Water*, **46**(11); 1–9.

- Kaiser, G. (2019). Microbiology. *LibreText*, pp1–782.
- Kanavaki, I., Drakonaki, A., Geladas, E. D., Spyros, A., Xie, H., & Tsiotis, G. (2021). Polyhydroxyalkanoate (Pha) production in *pseudomonas* sp. phdv1 strain grown on phenol as carbon sources. *Microorganisms*, **9**(8); pp1–11.
- Khatami, K., Perez-Zabaleta, M., Owusu-Agyeman, I., & Cetecioglu, Z. (2021). Waste to bioplastics: How close are we to sustainable polyhydroxyalkanoates production? *Waste Management*, **119**(2021); pp374–388.
- Liu, C., Wang, X., Yang, H., Liu, C., Zhang, Z., & Chen, G. (2021). Biodegradable polyhydroxyalkanoates production from wheat straw by recombinant *Halomonas elongata* A1. *International Journal of Biological Macromolecules*, **187**(3); pp675–682.
- Mahato, R. P., Kumar, S., & Singh, P. (2021). Optimization of Growth Conditions to Produce Sustainable Polyhydroxyalkanoate Bioplastic by *Pseudomonas aeruginosa* EO1. *Frontiers in Microbiology*, **12**(2021); pp1–9.
- Mankar, A. R., Pandey, A., Modak, A., & Pant, K. K. (2021). Pretreatment of lignocellulosic biomass: A review on recent advances. *Bioresource Technology*, **334**(2021); pp1–12.
- Mozejko-Ciesielska, J., Szacherska, K., & Marciniak, P. (2019). *Pseudomonas* Species as Producers of Eco-friendly Polyhydroxyalkanoates. *Journal of Polymers and the Environment*, **27**(6); pp1151–1166.
- Mozejko, J., & Ciesielski, S. (2013). Saponified waste palm oil is an attractive renewable resource for mcl-polyhydroxyalkanoate synthesis. *Journal of Bioscience and Bioengineering*, **116**(4); pp485–492.
- Muloiwa, M., Nyende-Byakika, S., & Dinka, M. (2020). Comparison of unstructured kinetic bacterial growth models. *South African Journal of Chemical Engineering*, **33**(2019); pp141–150.
- Murugan, P., Han, L., Gan, C. Y., Maurer, F. H. J., & Sudesh, K. (2016). A new

- biological recovery approach for PHA using mealworm, *Tenebrio molitor*. *Journal of Biotechnology*, **239**(2016); pp98–105.
- Nwinyi, O. C., & Owolabi, T. A. (2019). Scanning electron microscopy and Fourier transmission analysis of polyhydroxyalkanoates isolated from bacteria species from an abattoir in Ota, Nigeria. *Journal of King Saud University - Science*, **31**(3); pp285–298.
- Palmeri, R., Pappalardo, F., Fragalà, M., Tomasello, M., Damigella, A., & Catara, A. F. (2012). Polyhydroxyalkanoates (PHAs) production through the conversion of glycerol by selected strains of *Pseudomonas mediterranea* and *Pseudomonas corrugata*. *Chemical Engineering Transactions*, **27**(2012); pp121–126.
- Pan, L., Li, J., Wang, R., Wang, Y., Lin, Q., Li, C., & Wang, Y. (2021). Biosynthesis of polyhydroxyalkanoate from food waste oil by *Pseudomonas alcaligenes* with simultaneous energy recovery from fermentation wastewater. *Waste Management*, **131**(2021); pp268–276.
- Priyadarshini, A., Sahoo, M. M., Raut, P. R., Mahanty, B., & Sahoo, N. K. (2021). Kinetic modeling and process engineering of phenolics microbial and enzymatic biodegradation: A current outlook and challenges. *Journal of Water Process Engineering*, **44**(2021); pp1–23.
- Rebocho, A. T., Pereira, J. R., Freitas, F., Neves, L. A., Alves, V. D., Sevrin, C., Grandfils, C., & Reis, M. A. M. (2019). Production of medium chain length polyhydroxyalkanoates by *Pseudomonas citronellolis* grown in apple pulp waste. *Applied Food Biotechnology*, **6**(1); pp71–82.
- Rebocho, A. T., Pereira, R., Neves, A., Alves, V. D., Sevrin, C., Grandfils, C., Freitas, F., & Reis, M. A. M. (2020). Preparation and Characterization of Films Based on a Natural P (3HB)/mcl-PHA Blend Obtained through the Co-culture of *Cupriavidus Necator* and *Pseudomonas Citronellolis* in Apple Pulp Waste. *Bioengineering*, **7**(34); pp1–18.
- Sarmiento-Vásquez, Z., Vandenberghe, L. P. de S., Karp, S. G., & Soccol, C. R.

- (2022). Production of Polyhydroxyalkanoates through Soybean Hull and Waste Glycerol Valorization: Subsequent Alkaline Pretreatment and Enzymatic Hydrolysis. *Fermentation*, **8**(9); pp1–18.
- Sathiyarayanan, G., Bhatia, S. K., Song, H. S., Jeon, J. M., Kim, J., Lee, Y. K., Kim, Y. G., & Yang, Y. H. (2017). Production and characterization of medium-chain-length polyhydroxyalkanoate copolymer from Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620. *International Journal of Biological Macromolecules*, **97**(2017); pp710–720.
- Senthilkumar, P., Dawn, S. S., Sree Samanvitha, K., Sanjay Kumar, S., Narendra Kumar, G., & Samrot, A. V. (2017). Optimization and characterization of poly[R]hydroxyalkanoate of *Pseudomonas aeruginosa* SU-1 to utilize in nanoparticle synthesis for curcumin delivery. *Biocatalysis and Agricultural Biotechnology*, **12**(2017); pp292–298.
- Sharma, P. K., Munir, R. I., Blunt, W., Dartailh, C., Cheng, J., Charles, T. C., & Levin, D. B. (2017). Synthesis and Physical Properties of Polyhydroxyalkanoate Polymers with Different Monomer Compositions by Recombinant *Pseudomonas putida* LS46 Expressing a Novel PHA SYNTHASE (PhaC1 16) Enzyme. *Applied Sciences*, **7**(242); pp1-13.
- Sharma, P. K., Munir, R. I., de Kievit, T., & Levin, D. B. (2017). Synthesis of polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *pseudomonas chlororaphis*. *Canadian Journal of Microbiology*, **63**(12); pp1009–1024.
- Simon-Colin, C., Raguénès, G., Costa, B., & Guezennec, J. (2008). Biosynthesis of medium chain length poly-3-hydroxyalkanoates by *Pseudomonas guezennei* from various carbon sources. *Reactive and Functional Polymers*, **68**(11); pp1534–1541.
- Simon-Colin, C., Raguénès, G., Crassous, P., Moppert, X., & Guezennec, J. (2008). A novel mcl-PHA produced on coprah oil by *Pseudomonas guezennei* biovar. tikehau, isolated from a “kopara” mat of French Polynesia.

International Journal of Biological Macromolecules, **43**(2), pp176–181.

Surendran, A., Lakshmanan, M., Chee, J. Y., Sulaiman, A. M., Thuoc, D. Van, & Sudesh, K. (2020). Can Polyhydroxyalkanoates Be Produced Efficiently From Waste Plants and Animal Oils? In *Frontiers in Bioengineering and Biotechnology* **8**(2020); pp1–15.

Szacherska, K., Oleskowicz-Popiel, P., Ciesielski, S., & Mozejko-Ciesielska, J. (2021). Volatile fatty acids as carbon sources for polyhydroxyalkanoates production. *Polymers*, **13**(3); pp1–21.

Vega-Castro, O., Contreras-Calderon, J., León, E., Segura, A., Arias, M., Pérez, L., & Sobral, P. J. A. (2016). Characterization of a polyhydroxyalkanoate obtained from pineapple peel waste using *Ralstonia eutropha*. *Journal of Biotechnology*, **231**(2016); pp232–238.

Zakaria, M. R., Tabatabaei, M., Ghazali, F. M., Abd-Aziz, S., Shirai, Y., & Hassan, M. A. (2010). Polyhydroxyalkanoate production from anaerobically treated palm oil mill effluent by new bacterial strain *Comamonas* sp. EB172. *World Journal of Microbiology and Biotechnology*, **26**(5); pp767–774.

5 Developing Simple Post-Processing Techniques for Extraction of Bioplastic Produced by *Pseudomonas* Strains

Authors: Hano, L.J., Soares, A and Wagland, S

Abstract

The advancement of technologies enabling efficient extraction of pure PHA, and the reduction of production expenses is imperative. To simultaneously lower the production costs of bioplastics and minimize waste oil disposal, we explore the potential of employing various Pseudomonas strains, specifically, Pseudomonas oleovorans DSMZ1045 and ATCC29347. Pseudomonas strains' capability was investigated to utilize glycerol (Gly) and fat, oil, and grease (FOG) as carbon substrates for fermentation in the production of polyhydroxyalkanoate (PHA). After 48 h of fermentation with 2% oil (glycerol or FOG) under the optimal temperature of 30°C, the efficiency of simple and mild post-treatment techniques using NaOH, H₂O₂, and lysozyme in extracting the accumulated PHA was examined. The maximum PHA accumulation from these techniques was 43%, 40%, and 40% of cell dry weight (CDW), respectively. The interaction between oil concentration and cultivation time regulated growth and PHA accumulation, resulting in PHA content reaching 43% and 39% of cell dry weight for DSMZ1045 and 38% and 37% for ATCC29347, respectively. Results showed that the extraction techniques used significantly influenced the percentage yield of the PHA, with NaOH giving the highest percentage accumulated yield. FTIR and SEM were further used to analyse the extracted PHA to identify the type of PHA produced. The FTIR and SEM confirmed that the produced PHA from both strains is mc-PHA compared to those found from a reference published literature.

Keywords: *Pseudomonas oleovorans*, Polyhydroxyalkanoates, Lysozyme, FOG, Glycerol.

5.1 Introduction

Plastics are indispensable in daily life activities due to their good qualities. Modern societies are heavily dependent on plastics made from petroleum, and it has become a global problem to manage (Mozejko-Ciesielska *et al.*, 2019; Queirós *et al.*, 2014). Every year, more than 300 million tonnes of plastics are produced worldwide (Ruiz *et al.*, 2019b), with roughly half of this volume going towards products that are discarded after a year from the date of purchase (Singh and Sharma, 2016). Synthetic plastics are difficult to handle because they are persistent, not biodegradable, and accumulate in the environment, severely hampered waste management (Mozejko-Ciesielska *et al.*, 2019). Additionally, it has become clear that fossil fuels are also finite, non-renewable resources, and dependence on them causes supply security failure and contributes to greenhouse gases (GHG) and thus, climate change (Tomás-Pejó *et al.*, 2023).

As a result of the environment's increasing accumulation of synthetic plastic, switching to biodegradable plastics is urgently necessary (Sehgal and Gupta, 2020). Bioplastic made from renewable resources is environmentally friendly, biodegradable, and can replace plastics from petroleum (Roja *et al.*, 2019). Polyhydroxyalkanoates (PHAs) are the most well-known family of biodegradable polymers, which is interesting because of their biodegradability, non-toxicity, and biocompatibility. PHAs also offer many other advantageous characteristics (Mo and Kiewisz, 2020; Mozejko-Ciesielska *et al.*, 2019), with more than 100 different kinds of monomers produced from plants and microorganisms (Akaraonye *et al.*, 2010). They are classified according to the number of carbon atoms in their monomeric side unit chains, short-chain length (scl), <5 carbon atoms, medium-chain length (mcl), 5 to 14 carbon atoms, and long chain length (lcl), >14 carbon atoms (Du *et al.*, 2012). They are synthesis using different enzymes such as - ketothiolase (phaA) to regulate the conversion of acetyl-CoA to acetoacetyl-CoA molecule, acetoacetyl-CoA reductase (phaB) to reduce the acetoacetyl-CoA to 3-hydroxyalkanoic acid, and PHA synthase (Roja *et al.*, 2019).

Numerous studies have shown that various *Pseudomonas* species, including *Pseudomonas pseudoflavas* and *Pseudomonas palleronii*, produce mcl-PHA (Cassuriaga *et al.*, 2018). However, production costs remain a challenge associated with bioplastics (Keshavarz and Roy, 2010). Carbon sources and polymer extraction processes are a significant challenge to overcome in order to make PHA production commercially viable (Kunasundari and Sudesh, 2011; Ruiz *et al.*, 2019b, 2019a). Thus, work has so much done on the upstream process, using low-cost carbon sources and the selection and genetic modification of the organisms used (Du *et al.*, 2012; Pagliano *et al.*, 2021; Surendran *et al.*, 2020). Conversely, downstream processes (i.e., extraction and purification) are some of the least studied sectors of the production process, despite being the most significant in economic comparison (Pagliano *et al.*, 2021). In addition, PHA is an intracellular material, making the extraction procedure much more challenging when compared to extracellular material (Pérez-Rivero *et al.*, 2019). The cost of extraction process of the current bioplastic stands about 30% (Khatami *et al.*, 2021), making them uneconomical to a sustainable solution to synthetic plastics, and there is a need for a solution.

The solvent extraction approach, which uses solvents like chloroform and methanol, has received a lot of attention in the search for PHA recovery techniques (Pérez-Rivero *et al.*, 2019; Yasotha *et al.*, 2008). However, the effects halogenated chemicals have on the environment and human health have made much safer separation techniques necessary (Kheireddine *et al.*, 2008). The PHA obtained by the chemical and enzymatic digestion procedures is the main emphasis of this study. The qualitative analysis of PHA recovered by both digestion methods has not been extensively studied in published literature. This study's primary aim is to evaluate the PHA recovered using both digestive techniques to compare its yield and determine the method's practicality.

5.2 Materials and Methods

5.2.1 Materials

Potassium phosphate monobasic (500 g, Germany, 99%), calcium chloride hexahydrate (200 g, China, 99%), sodium phosphate dibasic (500 g, Japan, 99%), nutrient broth powder (500 g, India, 8 g/L), yeast extract (3 g/L), sodium phosphate monobasic (500 g, Germany, 99%), magnesium sulfate (500 g, Japan, 99%), calcium chloride dihydrate (500 g, Germany, 98%), iron (II) sulfate (250 g, India, 99%), manganese (II) chloride tetrahydrate (500 g, Japan, 99.5%), zinc sulfate heptahydrate (500 g, USA, 99%), sodium chloride (250 g, USA, 99.5%), urea (Germany), sodium hydroxide (250 mL, 99.8%, Switzerland) were all purchased from (Sigma Aldrich), glycerol (500mL, Malaysia, 99.5%), 37% HCL (Australia), 30% H₂O₂ (Spain) (chloroform (2.5 L, Germany, 99.8%), ethanol (2.5 L, UK, 99.8%, acetone (2.5 L, Finland, 99.8%), methanol (2.5 L, UK, 99.9%), were all purchased from (Fischer scientific). The lyophilised ampoule culture: *Pseudomonas oleovorans* DSMZ 1045 and *Pseudomonas oleovorans* ATCC 29347 (Purchased from ATCC, PHE, and DSMZ) were revived in nutrient broth medium at 30°C. These ingredients were dissolved in distilled water and autoclaved at 121°C. The cultures were maintained as cryo-conserved at -80°C in the presence of 50% (v/v) glycerol (Nkrumah-Agyeefi and Scholz, 2017), and were used throughout the experiments. Two selected bacteria (*P. oleovorans* DSMZ1045 & *P. oleovorans* ATCC29347) were used for the growth fermentation and accumulation of PHAs. Nutrient broth (NB) medium was used as a bacteria growth medium, and mineral medium was used as a PHA production medium, while glycerol & fat, oil & grease were used as a fermentative carbon substrate.

5.2.2 Equipment Used

FTIR techniques (Vertex 70, Bruker), shaker incubator (Incu-Shaker MIDI, Benchtop Shaking Incubator, Switzerland), bath ultrasound incubator (Ultrawave, Asset 50A) (Ultrawave Ltd, Cardiff, UK), microbiological safety cabinet (Labcare SC-R Recirculating Class II, Labcare System Ltd, England), Fume Cupboard (Heraeus, Germany), autoclave, Erlenmeyer conical flasks (100 mL, 250 mL,

Fisher brand, Germany) Scale (Precisa, UK), Durham bottle (250 mL, 500 mL, Fisher brand, Germany) Bench-scale Microwave, Beakers (100 mL, 250 mL, 300 mL, 400 mL, Fisher brand, Germany), Measuring cylinders (50 mL, 250 mL, 500 mL, and 1000 mL, Technicon, England) Centrifuge (SORVALL ST40, ThermoFisher Scientific, Germany), VEGA 3 (Tescan, Czech Republic), Freeze dryer (Christ Alpha 1-2 LDplus, Germany), spectrophotometer (JENWAY 6310, UK).

5.2.3 Carbon Sources

This study used carbon sources, such as glycerol, fat, oil, and grease (FOG), frying oils (soybean oil and palm oil). FOG sample was obtained from Severn Trent wastewater treatment plant, UK. Glycerol was ordered from Fisher Scientific, UK.

5.2.4 Methods

5.2.4.1 Reactivation and Maintenance of Cultures

A nutrient broth (NB) medium was used to revive lyophilized cultures at 30°C for 24 h at 180 rpm. The medium is supplemented with nutrient broth powder (8 g/L), yeast extract (3 g/L), and sodium chloride (2.5 g/L), which are dissolved in 1L of distilled water and autoclaved. The reactivated cultures were kept in 50% (v/v) glycerol at -80°C as cryo-preserved cultures and used throughout the experiment.

5.2.4.2 Analysis of FOG

FOG samples were freeze-dried (Christ Alpha 1-2 LD) for 48 h after being thawed at -80°C for 48 h to achieve a balanced weight. The process was carried out in freeze drier rather than an oven to prevent sample losses or changes from heat treatment, and moisture abstraction.

5.2.4.2.1 Total lipid content determination

The total lipid content was determined using Dionex ASE 200 and Thermo-Scientific's accelerated solvent extraction method. Lipids were extracted from the FOG samples using hexane. The hexane was then introduced into a 100 mL

Dionium extraction cell (P/N 068103), which holds the sample, and heated to a high temperature of 125 °C while being under 1500 psi of pressure. After that, the heated extract was poured into a 40 mL collecting vial (P/N 048783) and stored there until the analysis was complete. The fat content of the extract was determined by its weight.

5.2.4.2.2 Nitrogen, Protein and Carbohydrate contents determination

Total nitrogen content in the FOG samples was determined using British Standard BS EN 13652-2:2001 (Dumas) method with an Elementar Vario EL cube analyser. However, protein content was determined by multiplying the total nitrogen and a standard nitrogen conversion factor of 6.25. The carbohydrate in the FOG sample was determined from the percentage differences of lipids, proteins, and ash subtracted from the 100%.

5.2.4.3 Growth and PHA fermentation

Two selected types of *Pseudomonas strains* [*P. oleovorans* (DSMZ1045 & ATCC29347)] were used in this study. Culture strains (DSMZ1045 and ATCC29347) were revived in a nutrient broth medium at 30°C and kept as frozen stocks in 50% glycerol at -80°C. 2 mL from the frozen stock cryovial revived culture was inoculated into a 250 mL shake flask containing 100 mL of nutrient broth medium at 30°C for 24 h at shaker incubation of 180 rpm for the growth conditions. The medium comprised as follows: 8 g/L of nutrient broth powder, 3 g/L of yeast extract, and 2.5 g/L of NaCl. Afterward, the cultures were centrifuged at 3800 rpm for 15 min to collect the pellets, and the collected pellets were inoculated in a 250 mL shake flask containing 100 mL of defined minimal medium: 1.5 KH₂PO₄, 6.7 Na₂HPO₄ (Leong *et al.*, 2017b), 4.5 NaH₂PO₄, 0.06 CaCl₂. 2H₂O, 0.3 MgSO₄. The first three components were dissolved with distilled water and autoclaved at 121°C, and the last two were autoclaved and sterilized separately to avoid precipitate formation (Zainab *et al.*, 2018), under the same conditions. The pH was adjusted to 7.0 using NaOH/HCL before autoclaving. This medium was supplemented with 1 mL trace element solution – comprise (g/L); 1.5 FeSO₄.7H₂O, 0.24 MnCl₂. 4H₂O, 0.03 CoCl₂.2H₂O 0.048 CuSO₄, 0.24

ZnSO₄ · 7H₂O is dissolved in 1 M of HCl and sterilized using a syringe filter. The medium described above is supplemented with 2% oil and 0.54 g/L of urea. The fermentation was conducted triplicate for each bacterium using limiting nutrient conditions at 30 °C in an Incu-shake MIDI Benchtop shaking incubator (Switzerland) set at 180 rpm for 48 h. To evaluate the biomass and PHA synthesis in the *Pseudomonas* strains (DSMZ1045 and ATCC29347) in flask cultures monitored and measured using the optical density at 600nm at 3 hrs intervals.

5.2.4.4 Determination of optical density

Samples were collected at various time intervals (0, 3, 6, 9, 12,48 h). The turbidity of the accumulated cultures was spectrophotometrically monitored by measuring the absorbance at OD_{600nm} with a UV-spectrometer from 0 to 48 h.

5.2.4.5 Analysis of statistics

Analysis of variance (ANOVA) and a test for statistically significant differences was used to analyse the data using the SPSS and Minitab software. The statistical significance of the differences between the means was determined, with a P-value ≤ 0.05 to confirm the statistical significance (SD). Three duplicates of each experiment were run.

5.3 Polymer recovery

5.3.1 Separation process

The fermented broth was harvested by centrifuging at 3800 rpm for 15 minutes at room temperature to separate the pellet from the supernatants. The pellet then was rinsed with distilled water to remove any remaining debris before being freeze-dried at -20°C for 24 hours in a freeze dryer.

5.3.2 Determination of cell content

The cell dry weight was calculated gravimetrically using dried and pre-weighed falcon tubes. Transferring 40 mL of broth culture sample into the tubes, they were centrifuged at 3800 rpm for 15 minutes at room temperature. The supernatant was discarded, and the cell pellet was washed twice with distilled water before

being dried in a freeze dryer at 20°C for 24 hours until a constant weight was achieved.

5.3.3 Polymer extraction

Finally, the polymer was extracted using three techniques: sodium hydroxide, enzyme, or hydrogen peroxide, as described below:

5.3.3.1 Extraction using Sodium hydroxide (NaOH)

300 mg of the sample was ground to powder employed into a 250 mL flask containing 40 mL of 0.5 M of sodium hydroxide solution and incubated at 37°C for 3 h at 250 rpm (Mohammadi *et al.*, 2012c). Then, using Whatman filter paper, the polymer was collected from the aqueous fraction containing cell debris and was allowed to dry for a day in a fume closet. The extracted polymer yield quantity was calculated using Eq. (5-1).

$$\%PHA = \frac{\text{Extracted PHA } (\frac{g}{L})}{CDW (\frac{g}{L})} * 100 \quad (5-1)$$

5.3.3.2 Extraction using Lysozyme.

100 mg of the dried cell was placed into a test tube, 10 mL of 0.05M phosphate buffer (pH 7.2) was added, and then incubated at 30°C for 15 min at 200 rpm. After, the mixture was centrifuged at 5000 x g for 15 min at 25°C and re-suspended again into 5 mL 0.05M phosphate buffer. Then lysozyme solution at enzyme activity of 70000U/mg was added and incubated for 2 hrs at 37 °C for 180 rpm. After washing with 10 mL of 98% acetone, the mixture was centrifuged at 12000 x g for 10 min. The extracted polymer was allowed to dry for 24 h in a fume, and then the yield quantity of the extracted dried polymer was weighed following Eq (5-2).

$$\%PHA = \frac{\text{Extracted PHA } (\frac{g}{L})}{CDW (\frac{g}{L})} * 100 \quad (5-2)$$

5.3.3.3 Extraction using Hydrogen peroxide (H₂O₂)

300 mg of the dried cell was measured and grounded, then added into a 250 mL flask containing 40 mL of 30% hydrogen peroxide at 200 rpm for 4 h at 37°C. After which 20 mL of 0.1 M NaOH was added, then the mixture was centrifuged at 5000 x g for 10 min. The volume of methanol-water (7:3) was added for the precipitation process, and the polymer was collected. After, the precipitate underwent twice acetone washes. Then using Whatman filter paper, the polymer was collected and allowed to dry in a fume closet. The yield of the extracted polymer was calculated as polymer percentage using eq (5-3).

$$\%PHA = \frac{\text{weight of extracted PHA}}{\text{dry cell weight}} * 100 \quad (5-3)$$

5.4 Polymer Characterization

The polymer was characterized by using Scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy techniques.

5.4.1 Scanning electron microscopy

The SEM analysis was conducted using TESCAN VEGA 3 to explore the polymer materials' scanning images. The sample materials were mounted into the sample holder and coated with gold (Senthilkumar *et al.*, 2017), and the sample holder was placed onto the TESCAN VEGA 3 (Fig. 3-2), and subjected to SEM analysis. The images were taken at different magnifications from 1000-5000 x with a used voltage of 10kV (Murugan *et al.*, 2016), or 20kV to examine the morphological structures of each samples.

5.4.2 Fourier transforms infrared spectroscopy.

The extracted PHA sample was first prepared, and then it was scanned and observed between wavenumbers of 400 cm^{-1} and 4000 cm^{-1} (Kanavaki *et al.*, 2021) waves at a speed of $1\text{ }\mu\text{m/min}$ with a spectral resolution of 4 cm^{-1} (Vega-Castro *et al.*, 2016).. Then, the functional groups in the extracted polymer were identified and confirmed using FTIR spectroscopy (VERTEX 70 Bruker). However, the identified functional groups were compared with those found in the previously published literature (Gumel *et al.*, 2013).

5.5 Results and discussion.

5.5.1 Growth and production

Results achieved from monitored grown cultures were summed and averaged, then converted into a natural log (lnOD). The values were used, and the graph curve was plotted using statistical software (SSPS and Minitab) as lnOD against time Fig. 5-1(a & b).

Fig. 5-1(a) depicts the graph curve of the cultures from both bacteria grown with glycerol with the initial lnOD value from *P. oleovorans* DSMZ1045 (1.7) and *P. oleovorans* ATCC29347 (1.4). Both organisms reached their peak values after 40 hours of fermentation at lnOD of 5.3 and 4.9, respectively. However, the organisms had nearly similar growth ($\text{OD}_{600\text{nm}}$) in a medium with glycerol as a carbon source.

Fig. 5-1(b) shows the growth curves of both organisms grown on a medium with FOG as a carbon substrate. The initial values are 1.8 and 1.4, respectively. This was followed by an increase in the values up to their peak values after 30 hours, resulting in 5.5 and 5.0, each. Both organisms reached their maximum value at 42 hours with *P. oleovorans* DSMZ1045 (5.5) and *P. oleovorans* ATCC29347 (5.0).

The yield percentage and concentration of PHA obtained from each carbon source was 43% CDW (0.48 g/L) and 39% CDW (0.38 g/L) accumulated by DSMZ1045. Whereas ATCC29347, accumulated the maximum PHA from each carbon source of 38% CDW (0.38 g/L) and 37.5% CDW (0.36 g/L). The results

evidenced strong statistical significance in final OD across all strains when the growth on all the substrates was examined collectively, showed $p = 0.000$, compared with the correlated $P = 0.005$, as seen in Table 5-1. Furthermore, the $R^2 = .973$ shows 100% confidentiality compared to 95% confidence of the interval, as R^2 closeness to 1 (Hall *et al.*, 2014; Muloiwa *et al.*, 2020), while the estimate mean values were all within the lower and upper bound, which also confirmed 95% confidentiality of the statistically significant, as seen in Appendix A (1.2 & 1.4), respectively.

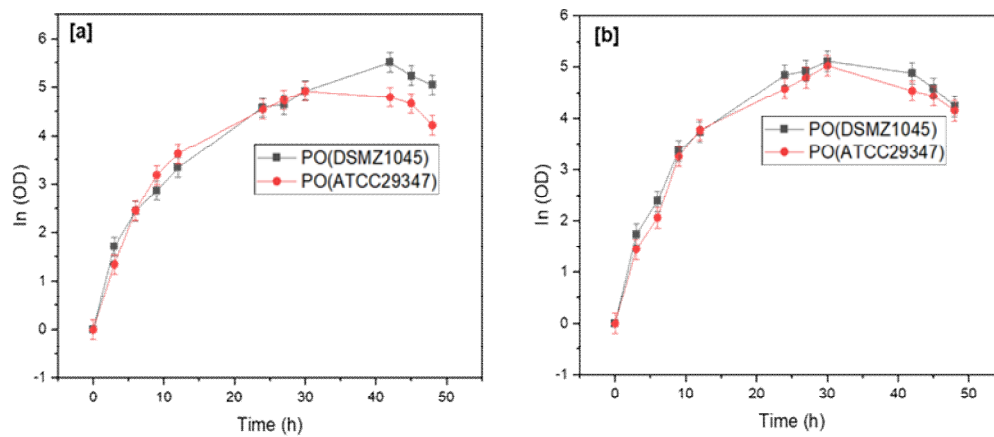


Figure 5-1: Growth and Production curve from *P. oleovorans* (DSMZ1045 and ATCC29347): a] Glycerol & b] FOG

Table 5-1: Tests of Between-Subjects Effects

Source	Measure	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Model	DSMZ1045	431.787	1	431.787	54.121	.000	.844
	ATCC29347	395.001	1	395.001	50.247	.000	.834
Error	DSMZ1045	79.781	10	7.978			
	ATCC29347	78.612	10	7.861			

5.5.2 Composition of the FOG

The chemical constituents of FOG, including lipid, protein, carbohydrates, ash, and moisture contents, were determined and recorded. Table 5-2 suggests a high presence of lipids at 83%, followed by ash, carbohydrates, and protein (7%), (6%), and (4%) of FOG compositions, respectively. However, the percentage moisture content was obtained at an average of 94.5%. This is incredibly high as compared to total solid and volatile solid in the FOG composition as indicated in Table 5-3. Depending on the types of fat, oil, and grease and their collection sources, the physicochemical properties of FOG might vary significantly (Salama *et al.*, 2019). Even though there is a variation, FOG generally has a higher volatile solid to total solids ratio than total solid. The VS in FOG contains saturated (palmitic acid and stearic acid), monounsaturated (oleic acid), and polyunsaturated (linoleic acid) fatty acids (Abomohra *et al.*, 2020). The increased total chemical oxygen demand (tCOD) of FOG is because of the VS and volatile fatty acids (VFAs), which are also advantageous in producing bioproducts (Salama *et al.*, 2019). Furthermore, the cost of bioplastic production can be greatly reduced when made with FOG due to its high oil content. The current study found that FOG had high lipid and carbohydrate contents, which was demonstrated to be a significant substrate for PHA. may be a different option for sustainability.

Table 5-2: Percentage compound compositions.

	Lipids	Proteins	Carbohydrates	Ash
Average	83%	4%	6%	7%
StdEv	4%	0.4%	2%	2%

Table 5-3: Percentage of moisture, TS, and VS

	Moisture content	TS	VS
Average	94.9%	5.1%	4.7%
StdEv	0.7%	0.7%	0.7%

The prospect of using fats, oils, and grease (FOG) to replace high-grade feedstocks to produce bioplastics is an appealing proposition aligned with sustainability goals. The feasibility and impact of this idea are contingent on several factors, including the availability of FOG, the conversion methodology employed, and the demand for PHA. It is worth noting that FOG has become more abundantly available owing to increased global oil consumption, and its cost is comparatively lower than that of virgin oils (Baena *et al.*, 2022). According to published literature, developing countries produce over 50 kg per capita of FOG, while non-developing countries generate around 20 kg per capita (Klaucans and Sams, 2018). In the United Kingdom, FOG production from food service establishments (FSEs) amounts to approximately 78,810 tonnes annually, with households contributing over 14,920 tonnes per year, as reported by (Collin *et al.*, 2020, 2022). While global data is currently unavailable, the global deposition of FOG constitutes a pressing concern that necessitates effective management (Arthur and Blanc, 2013)

One of the promising approaches to address these issues is the development of eco-friendly, lower-environmental-impact, and more sustainable technologies for converting FOG into renewable energy sources, such as biofuels and bioplastics (Biermann *et al.*, 2011). Furthermore, the field of bioplastic production technologies is continuously evolving through ongoing research and development (Sudesh *et al.* 2003), which could influence the feasibility and impact of these processes. It is worth noting that FOGs represent one of the less explored sources of renewable feedstock for bioplastic production. Nevertheless, within the United Kingdom, there is a growing interest in utilizing FOGs for biodiesel production, which has the potential to yield an estimated annual production of approximately 490 gigawatt-hours (GWh) (Biermann *et al.* 2018).

5.5.3 Percentage yield of the produced polymer

The obtained PHA percentage yield from sodium hydroxide, lysozyme, and hydrogen peroxide was recorded as indicated in Table 5-4. According to the results, sodium hydroxide gave the PHA percentage yield using glycerol and FOG

as fermentative substrates. The performance of extraction methods favoured NaOH with 43%/CDW & 39%/CDW, while with lysozyme 40%/CDW & 38%/CDW. Comparatively, the enzyme provided a similar PHA percentage yield to that attained using sodium hydroxide, which was between 40%/CDW and 38%/CDW and between 38%/CDW and 38%/CDW by both strains. However, hydrogen peroxide has a PHA percentage yield of 40%/CDW & 37%/CDW and 36%/CDW & 35%/CDW by both strains, respectively.

Table 5-4: Polymer percentage yield from each extraction method.

Organisms/Extraction method	Fermentation substrate	<i>P. oleovorans</i> DSMZ1045	<i>P. oleovorans</i> ATCC29347
Sodium hydroxide (NaOH)	glycerol	43%	38%
	FOG	39%	37%
Enzyme	glycerol	40%	38%
	FOG	38%	38%
Hydrogen peroxide (H₂O₂)	glycerol	40%	36%
	FOG	37%	35%

The finding showed that each obtained a good percentage yield, with the NaOH having a high recovered yield, followed by the lysozyme and hydrogen peroxide. extraction This might be because the NaOH can effectively break microbial cell walls, making the recovery of PHAs simpler (Park *et al.*, 2011). Also, using NaOH in the PHA extraction increases the end product's purity and quality, as reported by López-Abelairas *et al.* (2015). Gahlawat and Kumar (2019) reported that NaOH is simple and economical and has been widely used to remove and purify PHA from microbial cells. Lam *et al.* (2016), found that NaOH is a strong base that can efficiently break the cell and other impurities and then release the polymer, achieving a high yield and purity.

Numerous studies have been reported and demonstrated that the polymer percentage depends on the concentrations, time, and temperature of incubation (Anis *et al.*, 2013a; Mohammadi *et al.*, 2012b, 2012c, 2012a). (Su *et al.*, 2017), stated that an increase in NaOH enhances the PHA percentage yield and purity. Neves and Müller (2012) reported the yield recovery up to 93.2% and purity of 94% purity from the polymer they extracted. A PHA yield of 41% of cell dry weight was obtained from the extraction process employed with lysozyme (Gutt *et al.*, 2016). Anis *et al.* (2013a) conducted the experimental using 0.1 M NaOH at 30°C for 1-3 h and obtained a yield of 84.9% from less than 30 g/L dried cell. A related study was reported by Mohammadi *et al.* (2012c) using 0.05 M NaOH at 4°C for 1 h which led to achieve a 96.8% polymer recovery yield. Water and NaOH was used to extract PHA from bacterial cells by Mohammadi *et al.* (2012b) and achieved a recovery yield of 96.1 % and 74.9%, respectively. Another study was reported by Mohammadi *et al.* (2012a) using small concentration amount of 0.05 M NaOH produced a polymer by recombinant *C. necator* with percentage of 38.4% of cell dry weight at an incubation temperature of 4°C for 3 h. Choi and Lee (1999) reported more than 70% cell dry weight of polymer content from the digestion of non-PHA biomass using 0.1 M NaOH at 30°C for 1 h. PHA recovery was obtained a yield of 49.1% cell dry weight of PHA content from hydrogen peroxide treatment (Gautam and Gautam, 2018). The findings of this study reported nearly similar results obtained by (Gautam and Gautam, 2018; Gutt *et al.*, 2016; Mohammadi *et al.*, 2012a). However, the yields achieved by (Anis *et al.*, 2013a, 2013b; Choi and Lee, 1999; Mohammadi *et al.*, 2012b, 2012c; Neves and Müller, 2012) are more than the yields seen in this experiment. The polymer yield variation observed in this study may be due to the differences in concentrations, time, and temperature since the yields cited were reported with higher concentrations and temperatures compared to this finding. Studies have revealed that employing simple and mild extraction result with high percentage yields of polymer (Hoseinzadeh *et al.*, 2012).

This study reveals that the NaOH extraction method was good method in extracting the produced bioplastics from waste oils such as glycerol and FOG.

However, enzymes and H₂O₂ may have produced lesser yields due to their potentially limited efficacy in dissolving the complex structure of microbial cell (Hassan *et al.* 2020). However, it's important to note that the results are specific to these tested strains (*P. oleovorans* DSMZ1045 and *P. oleovorans* ATCC29347) in the study, and the findings may not be generalisable to other bacteria species or conditions. Nevertheless, the study proves that the NaOH extraction method is promising for extracting PHA from glycerol and FOG. The employment of the NaOH, H₂O₂, enzyme extraction method may have related to some little disadvantages, such developing undesirable by-products during the extraction process (Kumar *et al.* 2012). The study's findings, however, indicate that the NaOH extraction approach alone was more successful in extraction PHA than either using enzymes alone or in conjunction with NaOH and H₂O₂. However, further studies are necessary to confirm the economic costs of this procedure and optimise bioplastic production conditions.

5.5.4 Scanning electron microscopy

The results of the SEM analysis showed the presence of polymer granules in both organisms (*P. oleovorans* DSMZ1045 and ATCC29347) grown in minimal medium (MM) containing 2% w/v lipid-rich oil wastes (Glycerol or FOG) for 48 hours. In addition, the cells contained polymer granules that varied in size and quantity, as seen in Fig (5-2). The distribution of granules was close and attenuated as seen Fig.5-2 (a & b). The tiny grains and small structure in microbeads form with relatively pore distribution. This indicates widespread deposition of lipid-rich on the *P. oleovorans* DSMZ1045. However, similar patterns in the morphology were obtained as seen Fig. 5-2 (c & d). At different magnification there was distinctive spherical distribution and asymmetrical in their shapes. Also, observed was compacted substrate and less connected high lipid-rich formation. These were all alluded in their performance as indicated in Table 5.3. The SEM result in this investigation is consistent with the findings (Liu *et al.*, 2021; Nwinyi and Owolabi, 2019).

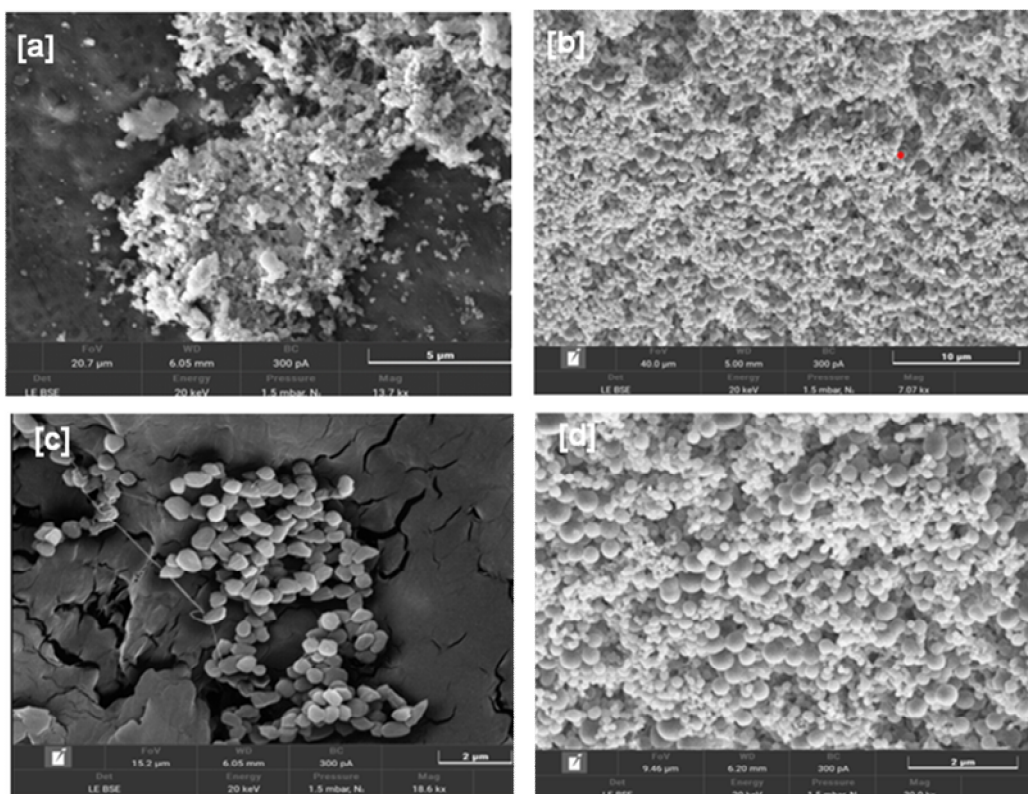


Figure 5-2: SEM images of granules observed in both cells: [a-b] DMSZ1045 and [c-d] ATCC2947.

5.5.5 Fourier Transform Infrared Spectroscopy (FTIR)

The result of FTIR analysis on extracted polymer samples produced PHA exhibit a sharp peak between 1736 cm^{-1} and 1738 cm^{-1} , which is associated with the C=O stretching vibration of the ester function. These ester function bonds are formed between the carboxylic acid and alcohol groups in the monomers used to characterise the MCL-PHA. The peaks on 2856 cm^{-1} – 2936 cm^{-1} were associated with C-H stretching vibrations, indicating the aliphatic chain in the polymer samples. At the same time, peaks between 1400 cm^{-1} – 1460 cm^{-1} are associated with C-O stretching vibrations, which indicated the presence of ether bonds in the samples. They formed between the hydroxyl groups in the monomers also used to characterise the MCL-PHA. Other peaks observed between 1300 cm^{-1} - 1350 cm^{-1} are associated with bending vibrations of the C-H bonds in the alkyl side chains, whereas 1000 cm^{-1} – 1100 cm^{-1} are associated with C-O-C bending

vibrations, which indicated the presence of ester and ether linkages in the sample. A correlation exists between the ratio of peak intensities and the amount of PHA found in intact bacteria, showing that regardless of the lipid-rich material or the organisms used in the PHA fermentation process, both organisms synthesised the same kind of PHAs, attesting that they both produced the same characteristic polymers from each sample examined. Fig. 5-3(ai & aii) and 5-3(bi & bii), respectively.

This produced results that justifiably matched those of other investigations (Aloui et al., 2022; Rebocho et al., 2019; Rebocho et al., 2020; Sathiyarayanan et al., 2017; Sharma et al., 2017b, 2017a; Simon-Colin et al., 2008a, 2008b). Finally, the FTIR analysis helped to understand the extracted PHA's structural composition. The presence of certain groups and bonds that are inherent to mcl-PHA is confirmed by the observed absorption bands and functional groups, which closely match existing literature.

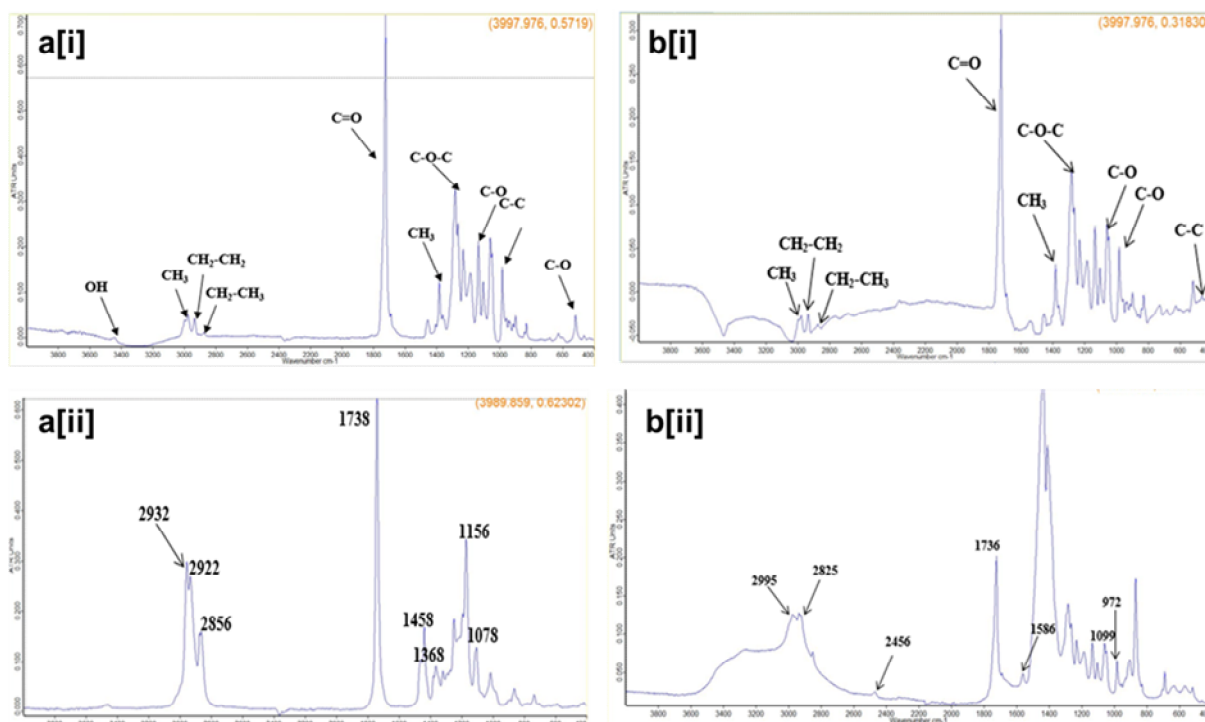


Figure 5-3: FTIR analysis from *P. oleovorans* cell: a [i & ii] - Glycerol and b[i & ii] - FOG.

5.6 Conclusion

This research examined the ability of *Pseudomonas* strains of *Pseudomonas oleovorans* (DSMZ1045 and ATCC29347) to synthesize polyhydroxyalkanoates (PHA) utilizing glycerol and fat, oil, & grease (FOG) as carbon sources. Both strains displayed significant cell dry weight obtained, as evidenced by the cultivation process carried out during 48 h time at a 2% oil concentration. The highest cell dry weight of 1.1 g/L for glycerol and 0.97 g/L for FOG was found in DSMZ1045, while cell dry weights of 0.98 g/L and 0.96 g/L were obtained by ATCC29347, respectively. It was discovered that the interaction between oil concentration and cultivation time regulated both growth and PHA accumulation, resulting in PHA content reaching 43% and 39% of cell dry weight for DSMZ1045 and 38% and 37% for ATCC29347, respectively. In order to find an alternative to chlorinated solvents, which are hazardous to the environment, the study also investigated post-treatment methods for PHA extraction from cell biomass. The influence of these strategies on PHA yield was evaluated using cheap, simple, and mild chemical and biological procedures. The highest outcome was obtained by NaOH and lysozyme of nearly 50% yield. In line with earlier research findings, this result emphasizes the effectiveness of using enzymes and NaOH as extraction strategies.

Finally, this study demonstrates how *Pseudomonas* bacteria can use glycerol and FOG as carbon sources and gives a promising approach for producing and extracting PHA from these carbon sources. Further, an environmentally benign and sustainable method of extracting PHA was provided here by using enzymes and NaOH. To assess the practical viability of these approaches for industrial applications, thorough research on downstream PHA extraction at pilot size is still necessary.

Acknowledgement

Thanks to the Petroleum Trust Development Fund Nigeria for funding this study. Further appreciation is extended to Cranfield University for providing the research lab facilities throughout.

References

- Abomohra, A. E. F., Elsayed, M., Esakkimuthu, S., El-Sheekh, M., & Hanelt, D. (2020). The potential of fat, oil, and grease (FOG) for biodiesel production: A critical review on the recent progress and future perspectives. *Progress in Energy and Combustion Science*, **81**(2020); pp1–33.
- Akaraonye, E., Keshavarz, T., & Roy, I. (2010). Production of polyhydroxyalkanoates : the future green materials of choice. **85**(6); pp732–743.
- Aloui, H., Khomlaem, C., Torres, C. A. V., Freitas, F., Reis, M. A. M., & Kim, B. S. (2022). Enhanced co-production of medium-chain-length polyhydroxyalkanoates and phenazines from crude glycerol by high cell density cultivation of *Pseudomonas chlororaphis* in membrane bioreactor. *International Journal of Biological Macromolecules*, **211**(5), pp545–555.
- Anis, S. N. S., Iqbal, N. M., Kumar, S., & Al-Ashraf, A. (2013). Increased recovery and improved purity of PHA from recombinant *Cupriavidus necator*. *Bioengineered*, **4**(2); pp1–4.
- Anis, S. N. S., Md Iqbal, N., Kumar, S., & Amirul, A. A. (2013). Effect of different recovery strategies of P(3HB-co-3HHx) copolymer from *Cupriavidus necator* recombinant harboring the PHA synthase of *Chromobacterium* sp. USM2. *Separation and Purification Technology*, **102**(2013); pp111–117.
- Baena, A., Orjuela, A., Rakshit, S. K., & Clark, J. H. (2022). Enzymatic hydrolysis of waste fats, oils and greases (FOGs): Status, prospective, and process intensification alternatives. *Chemical Engineering and Processing - Process Intensification*, **175**(4), pp1–21.
- Cassuriaga, A. P. A., Freitas, B. C. B., Morais, M. G., & Costa, J. A. V. (2018). Innovative polyhydroxybutyrate production by *Chlorella fusca* grown with pentoses. *Bioresource Technology*, **265**(2018); pp456–463.
- Choi, J. Il, & Lee, S. Y. (1999). Efficient and economical recovery of poly(3-

- hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. *Biotechnology and Bioengineering*, **62**(5); pp546–553.
- Collin, T., Cunningham, R., Deb, M., Villa, R., MacAdam, J., & Jefferson, B. (2022). Energy potential of household fats, oils, and grease waste. *Water and Environment Journal*, **36**(1); pp132–141.
- Collin, T., Cunningham, R., Jefferson, B., & Villa, R. (2020). Characterization and energy assessment of fats, oils, and grease (FOG) waste at the catchment level. *Waste Management*, **103**(2020); pp399–406.
- Du, C., Sabirova, J., Soetaert, W., & Ki Carol Lin, S. (2012). Polyhydroxyalkanoates Production From Low-cost Sustainable Raw Materials. *Current Chemical Biology*, **6**(1); pp14–25.
- Gahlawat, G., & Kumar Soni, S. (2019). Study on sustainable recovery and extraction of Polyhydroxyalkanoates (PHAs) produced by *Cupriavidus necator* using waste glycerol for medical applications. *Chemical and Biochemical Engineering Quarterly*, **33**(1); pp99–110.
- Gautam, S., & Gautam, A. (2018). Biopolymer - A beginning towards back to nature. *IOP Conference Series: Materials Science and Engineering*, **369**(1); pp1–6.
- Gumel, Annuar, Heidelberg, T. (2013). Growth kinetics, effect of carbon substrate in biosynthesis of mcl-PHA by *Pseudomonas putida* Bet001. *Journal of Microbiology*, **45**(2); pp427–438.
- Gutt, B., Kehl, K., Ren, Q., & Boesel, L. F. (2016). Using ANOVA Models to Compare and Optimize Extraction Protocols of P3HBHV from *Cupriavidus necator*. *Industrial and Engineering Chemistry Research*, **55**(39); pp10355–10365.
- Hall, B. G., Acar, H., Nandipati, A., & Barlow, M. (2014). Growth rates made easy. *Molecular Biology and Evolution*, **31**(1); pp232–238.
- Idris Zainab-L, Hiroshi Uyama, Cong Li, Yehua Shen, and K. S. (2018).

- Production of Polyhydroxyalkanoates From Underutilized Plant Oils by *Cupriavidus necator*. *Clean Soil, Air, Water*, **46**(11); pp1–9.
- Kanavaki, I., Drakonaki, A., Geladas, E. D., Spyros, A., Xie, H., & Tsiotis, G. (2021). Polyhydroxyalkanoate (Pha) production in *pseudomonas* sp. phdv1 strain grown on phenol as carbon sources. *Microorganisms*, **9**(8); pp1–11.
- Keshavarz, T., & Roy, I. (2010). Polyhydroxyalkanoates : bioplastics with a green agenda. *Current Opinion in Microbiology*, **13**(3); pp321–326.
- Klaucans, E., & Sams, K. (2018). Problems with fat, oil, and grease (FOG) in food industry wastewaters and recovered FOG recycling methods using anaerobic co-digestion: A short review. *Key Engineering Materials*, **762**(2018); pp61–68.
- Kunasundari, B., & Sudesh, K. (2011). *Isolation and recovery of microbial polyhydroxyalkanoates*. **5**(7); pp620–634.
- Lam, S. S., Liew, R. K., Jusoh, A., Chong, C. T., Ani, F. N., & Chase, H. A. (2016). Progress in waste oil to sustainable energy, with emphasis on pyrolysis techniques. In *Renewable and Sustainable Energy Reviews*, **53**(2016); pp741–753.
- Leong, Y. K., Show, P. L., Lan, J. C. W., Loh, H. S., Yap, Y. J., & Ling, T. C. (2017). Extraction and purification of Polyhydroxyalkanoates (PHAs): application of Thermoseparating aqueous two-phase extraction. *Journal of Polymer Research*, **24**(10); pp1–10.
- Liu, C., Wang, X., Yang, H., Liu, C., Zhang, Z., & Chen, G. (2021). Biodegradable polyhydroxyalkanoates production from wheat straw by recombinant *Halomonas elongata* A1. *International Journal of Biological Macromolecules*, **187**(3); pp675–682.
- López-Abelairas, M., García-Torreiro, M., Lú-Chau, T., Lema, J. M., & Steinbüchel, A. (2015). Comparison of several methods for the separation of poly(3-hydroxybutyrate) from *Cupriavidus necator* H16 cultures. *Biochemical*

- Engineering Journal*, **93**(2015); pp250–259.
- Mo, J., & Kiewisz, R. (2020). Bacterial polyhydroxyalkanoates : Still fabulous ? **192**(2016); pp271–282.
- Mohammadi, M., Hassan, M. A., Phang, L.-Y., Shirai, Y., Che Man, H., Ariffin, H., Amirul, A. A., & Syairah, S. N. (2012). Efficient Polyhydroxyalkanoate Recovery from Recombinant *Cupriavidus necator* by Using Low Concentration of NaOH. *Environmental Engineering Science*, **29**(8); pp783–789.
- Mohammadi, M., Hassan, M. A., Phang, L. Y., Shirai, Y., Man, H. C., & Ariffin, H. (2012). Intracellular polyhydroxyalkanoates recovery by cleaner halogen-free methods towards zero emission in the palm oil mill. *Journal of Cleaner Production*, **37**(2012); pp353–360.
- Mohammadi, M., Hassan, M. A., Shirai, Y., Man, H. C., Ariffin, H., Yee, L. N., Mumtaz, T., Chong, M. L., & Phang, L. Y. (2012). Separation and Purification of Polyhydroxyalkanoates from Newly Isolated *Comamonas* sp. EB172 by Simple Digestion with Sodium Hydroxide. *Separation Science and Technology*, **47**(3); pp534–541.
- Mozejko-Ciesielska, J., Szacherska, K., & Marciniak, P. (2019). *Pseudomonas* Species as Producers of Eco-friendly Polyhydroxyalkanoates. *Journal of Polymers and the Environment*, **27**(6); pp1151–1166.
- Muloiwa, M., Nyende-Byakika, S., & Dinka, M. (2020). Comparison of unstructured kinetic bacterial growth models. *South African Journal of Chemical Engineering*, **33**(2019); pp141–150.
- Murugan, P., Han, L., Gan, C. Y., Maurer, F. H. J., & Sudesh, K. (2016). A new biological recovery approach for PHA using mealworm, *Tenebrio molitor*. *Journal of Biotechnology*, **239**(2016); pp98–105.
- Neves, A., & Müller, J. (2012). Use of enzymes in the extraction of polyhydroxyalkanoates produced by *Cupriavidus necator*. *Biotechnology*

Progress, **28**(6); pp1575–1580.

- Nkrumah-Agyeefi, S., & Scholz, C. (2017). Chemical modification of functionalized polyhydroxyalkanoates via “Click” chemistry: A proof of concept. *International Journal of Biological Macromolecules*, **95**(2017); pp796–808.
- Nwinyi, O. C., & Owolabi, T. A. (2019). Scanning electron microscopy and Fourier transmission analysis of polyhydroxyalkanoates isolated from bacteria species from an abattoir in Ota, Nigeria. *Journal of King Saud University - Science*, **31**(3); pp285–298.
- Pagliano, G., Galletti, P., Samorì, C., Zaghini, A., & Torri, C. (2021). Recovery of Polyhydroxyalkanoates From Single and Mixed Microbial Cultures: A Review. *Frontiers in Bioengineering and Biotechnology*, **9**(2021); pp1–28.
- Pérez-Rivero, C., López-Gómez, J. P., & Roy, I. (2019). A sustainable approach for the downstream processing of bacterial polyhydroxyalkanoates: State-of-the-art and latest developments. *Biochemical Engineering Journal*, **150**(2019); pp1–13.
- Queirós, D., Rossetti, S., & Serafim, L. S. (2014). PHA production by mixed cultures: A way to valorize wastes from the pulp industry. *Bioresource Technology*, **157**(2014); pp197–205.
- Rebocho, A. T., Pereira, J. R., Freitas, F., Neves, L. A., Alves, V. D., Sevrin, C., Grandfils, C., & Reis, M. A. M. (2019). Production of medium chain length polyhydroxyalkanoates by *Pseudomonas citronellolis* grown in apple pulp waste. *Applied Food Biotechnology*, **6**(1); pp71–82.
- Rebocho, A. T., Pereira, R., Neves, A., Alves, V. D., Sevrin, C., Grandfils, C., Freitas, F., & Reis, M. A. M. (2020). Preparation and Characterization of Films Based on a Natural P (3HB)/mcl-PHA Blend Obtained through the Co-culture of *Cupriavidus Necator* and *Pseudomonas Citronellolis* in Apple Pulp Waste. *Bioengineering*, **7**(34); pp1–18.

- Roja, K., Ruben Sudhakar, D., Anto, S., & Mathimani, T. (2019). Extraction and characterization of polyhydroxyalkanoates from marine green alga and cyanobacteria. *Biocatalysis and Agricultural Biotechnology*, **22**(2019); pp1–11.
- Ruiz, C., Kenny, S. T., Narancic, T., Babu, R., & Connor, K. O. (2019). Conversion of waste cooking oil into medium chain polyhydroxyalkanoates in a high cell density fermentation. *Journal of Biotechnology*, **306**(2019); pp9–15.
- Ruiz, C., Kenny, S. T., Ramesh Babu, P., Walsh, M., Narancic, T., & O'Connor, K. E. (2019). High cell density conversion of hydrolyzed waste cooking oil fatty acids into medium chain length polyhydroxyalkanoate using *pseudomonas putida* KT2440. *Catalysts*, **9**(5); pp1-12.
- Salama, E. S., Saha, S., Kurade, M. B., Dev, S., Chang, S. W., & Jeon, B. H. (2019). Recent trends in anaerobic co-digestion: Fat, oil, and grease (FOG) for enhanced biomethanation. In *Progress in Energy and Combustion Science*, **70**(2019); pp22–42.
- Sathiyarayanan, G., Bhatia, S. K., Song, H. S., Jeon, J. M., Kim, J., Lee, Y. K., Kim, Y. G., & Yang, Y. H. (2017). Production and characterization of medium-chain-length polyhydroxyalkanoate copolymer from Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620. *International Journal of Biological Macromolecules*, **97**(2017); pp710–720.
- Sehgal, R., & Gupta, R. (2020). Polyhydroxyalkanoate and its efficient production: an eco-friendly approach towards development. *3 Biotech*, **10**(12); pp1–14.
- Senthilkumar, P., Dawn, S. S., Sree Samanvitha, K., Sanjay Kumar, S., Narendra Kumar, G., & Samrot, A. V. (2017). Optimization and characterization of poly[R]hydroxyalkanoate of *Pseudomonas aeruginosa* SU-1 to utilize in nanoparticle synthesis for curcumin delivery. *Biocatalysis and Agricultural Biotechnology*, **12**(2017); pp292–298.
- Sharma, P. K., Munir, R. I., Blunt, W., Dartiailh, C., Cheng, J., Charles, T. C., &

- Levin, D. B. (2017). Synthesis and Physical Properties of Polyhydroxyalkanoate Polymers with Different Monomer Compositions by Recombinant *Pseudomonas putida* LS46 Expressing a Novel PHA SYNTHASE (PhaC1 16) Enzyme. *Applied Sciences*, **7**(242); pp1-13.
- Sharma, P. K., Munir, R. I., de Kievit, T., & Levin, D. B. (2017). Synthesis of polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *pseudomonas chlororaphis*. *Canadian Journal of Microbiology*, **63**(12); pp1009–1024.
- Simon-Colin, C., Raguénès, G., Costa, B., & Guezennec, J. (2008). Biosynthesis of medium chain length poly-3-hydroxyalkanoates by *Pseudomonas guezennei* from various carbon sources. *Reactive and Functional Polymers*, **68**(11); pp1534–1541.
- Simon-Colin, C., Raguénès, G., Crassous, P., Moppert, X., & Guezennec, J. (2008). A novel mcl-PHA produced on coprah oil by *Pseudomonas guezennei* biovar. tikehau, isolated from a “kopara” mat of French Polynesia. *International Journal of Biological Macromolecules*, **43**(2); pp176–181.
- Singh, P., & Sharma, V. P. (2016). Integrated Plastic Waste Management: Environmental and Improved Health Approaches. *Procedia Environmental Sciences*, **35**(2016); pp692–700.
- Surendran, A., Lakshmanan, M., Chee, J. Y., Sulaiman, A. M., Thuoc, D. Van, & Sudesh, K. (2020). Can Polyhydroxyalkanoates Be Produced Efficiently From Waste Plants and Animal Oils? In *Frontiers in Bioengineering and Biotechnology* **8**(2020); pp1–15.
- Tomás-Pejó, E., González-Fernández, C., Greses, S., Kennes, C., Otero-Logilde, N., Veiga, M. C., Bolzonella, D., Müller, B., & Passoth, V. (2023). Production of short-chain fatty acids (SCFAs) as chemicals or substrates for microbes to obtain biochemicals. *Biotechnology for Biofuels and Bioproducts*, **16**(1); pp1–17.
- Vega-Castro, O., Contreras-Calderon, J., León, E., Segura, A., Arias, M., Pérez,

L., & Sobral, P. J. A. (2016). Characterization of a polyhydroxyalkanoate obtained from pineapple peel waste using *Ralstonia eutropha*. *Journal of Biotechnology*, **231**(2016); pp232–238.

Yasotha Kathiraser, Mohamed Kheireddine Aroua, K. B. R., & Tan, and I. K. P. (2008). Chemical characterization of medium-chain-length polyhydroxyalkanoates (PHAs) recovered by enzymatic treatment and ultrafiltration. *Journal of Chemical Technology & Biotechnology*, **82**(5); pp847–855.

6 Discussion

As previously discussed in chapters 3, 4, and 5, this chapter provides further discussion, covering the entire topics, including the circular economy and sustainability related to production of bioplastics using lipid-rich wastes, including glycerol, soybean oil, fat, oil, & grease, and palm oil. In today's society, plastics are a necessary material, and many goods made of plastic benefit the general public's health (Halden, 2010; Leong *et al.*, 2016, 2017a). Plastics are everywhere and for everything, with countless applications from face masks and gloves to medical testing kits required to fight COVID-19 (Zuin and Kümmerer, 2022). However, plastics also present substantial environmental waste and global warming associated with plastic use. Therefore, concern over the adverse environmental effects of plastic waste and its global warming is driving the demand for novel and creative material solutions that are economical and environmentally biodegradable. Bioplastics are a new generation of plastic that considerably reduces the environmental impact in terms of global warming and energy use (Bilo *et al.*, 2018). However, the carbon impact of the manufacture of plastics is diminished by renewable feedstock.

Consequently, bioplastics can aid the improved circular economy, biodegradation as a substitute end-of-life preference, and improved material qualities (Rosenboom *et al.*, 2022). Biodegradable bioplastics prevent the material from building up in the environment, which can have negative impacts, such as on marine life. Bioplastics don't generally produce pollutants as they break down, and when they mineralise into water and CO₂ (Min Song *et al.*, 2022), they turn into compounds of hydrogen, carbon, and oxygen. The fascinating among the types of bioplastics is the creation of polyhydroxyalkanoates (PHAs), which can be used to make plastics instead of monomers produced from fossil fuels. They provide an excellent answer to the issue of environmental plastic waste and our reliance on fossil fuels (Andreasi *et al.*, 2021). As a result, their use is expanding. Even though the production of bioplastics is expanding, they still make up less than 0.5 per cent of all plastics. Fernández-Dacosta *et al.* (2015) described

bioplastics production costs as approximately 20–80% higher than oil-based polymers. PHA is said to cost more than between 2.25 and 2.75 US dollars per pound, while petrol-based polymers are reportedly 5–10 times lower (0.60-0.87 US dollars/lb) (Akiyama *et al.*, 2003). Cost and commercial assessment are the two primary factors that could change plastic from oil-based to bio-based. The expenses of feedstock and downstream make up 40–48% and 30% of total costs, respectively (Khatami *et al.*, 2021). In the future circular economy, when alternatives to expensive substrates for the food and chemical industries must be found, the capacity to make the best use of wasted food and oils or low-value crops to produce value-added products has enormous promise. However, waste food, oils, or low-value products are generally a focus of research teams looking for novel methods of producing PHAs to minimise costs and maximise output. This research aims to establish sustainable bioplastic production using various lipid-rich oil wastes.

6.1 Lipid-rich oil waste

An enormous number of waste oils negatively affecting the environment, the economy, and society is developing significant concern worldwide. Over 190 million metric tonnes of waste cooking oils are produced worldwide each year (Mannu *et al.*, 2020), with the European Union (EU) contributing about 1 million tonnes (Mannu *et al.*, 2019, 2020). However, used cooking oil (UCO) is a more harmful and complex hazardous waste stream because it harms aquatic and terrestrial flora and wildlife when dumped in landfills or bodies of water, causing the death of much marine life, and threatening human life too (Bhatia *et al.*, 2021; Hosseinzadeh-Bandbafha *et al.*, 2022). Moreover, WCOs discharge through public sewers necessitates unusual upkeep and raises the expense of water treatment (Hosseinzadeh-Bandbafha *et al.*, 2022; Mannu *et al.*, 2019). It is enormously difficult and expensive to manage these wastes (Bhatia *et al.*, 2021). Therefore, to address this ongoing problem, sustainable solutions utilising green technologies are essential (Dahiya *et al.*, 2018). Making bioplastics from lipid-rich oil waste is a promising sustainable development because it is affordable, readily available, and accessible, with no expenditure necessary. Waste oil no longer

poses a serious environmental threat, and PHAs can now be produced at a far lower cost (Surendran *et al.*, 2020). Additionally, compared to traditional feedstocks, the lipid-rich oil was shown to be more favourable due to its lower cost and higher PHA yield during microbial fermentation (Bhatia *et al.*, 2021). Therefore, lipid-rich oil waste to bioplastics is a viable solution with several benefits, from reducing the risk that this waste poses to the economy, environment, and society.

However, the study is often split into two areas. The possibility of creating bioplastics using lipid-rich wastes and developing a simple, mild, and cheap downstream process. This study revealed that converting lipid-rich oil waste to bioplastics and extracting them with simple chemicals had a less severe impact on the environment and the economy, which can make the process of producing bioplastics less costly when compared with high-grade carbon sources and harsh solvent chemicals. In addition, the findings showed that lipid-rich oil wastes perform better when used as an alternative source for high-grade carbon sources, and there is no conflict with the feed sector involved with them. The results also indicated that biowaste carbon sources are more economically and environmentally sustainable for producing bioplastics because they are inexpensive and cause less/no climate change and waste management costs in converting them to bioplastics. Moreover, after extracting cell content and PHA, the filtrates can be transformed into value-added products like biogas or methane (Pan *et al.*, 2021). For example, Wei *et al.* (2015) employed residual bacterial biomass (RBB) to produce biochar and bio-oil through pyrolysis after gaining the PHA. This zero-value waste filtrate contains proteins, carbohydrates, phenolics, and ash to be easily transformed and pyrolyzed to bio-oil and biochar with a yield of 28% and 48%, respectively. The study demonstrated that co-products can be produced using RBB to increase the economic sustainability of producing PHA from biowastes (Wei *et al.*, 2015). Additionally, pyrolysis oil, which is high in nitrogen, may be employed as a possible carbon substrate for synthesising PHA. However, transforming the waste stream from the PHA production cycle is crucial to reduce environmental waste. This is true both from a financial and

environmental standpoint. Because of this, the conversion of these wastes may play a significant role in current and future PHA production (Yadav *et al.*, 2020). Additionally, Koller, (2015) stated that cell debris from earlier cultivations can be used as a substitute for 29% of the pricey yeast extract that is required as a source of nitrogen and phosphate for the microorganism's efficient cultivation. However, the study offers methods for lowering the cost of PHA production and ecological concerns through reusing the waste streams. The production of bioplastics from biowaste has been the subject of numerous investigations, all with promising findings. Though few studies have gone into deeper detail, bioplastics made from biowaste carbon sources have the potential to be both environmentally and economically sustainable. A sustainable assessment carried out by Fernández-Dacosta *et al.* (2015) showed that the alkali treatment had the best environmental and financial outcomes, with production costs of 1.40 e/kg PHB, a CO₂-equivalent with the lowest global warming of 2.4 kg CO₂-eq/kg PHB, and a non-renewable energy user of 106 MJ/kg PHB. While the solvent-based method results in the procedure with the greatest costs and ecological risks of 1.95 e/kg PHB, 4.30 kg CO₂-eq/kg PHB, and 156 MJ/kg PHB. In another study by Leong *et al.* (2016), they employed several waste materials to manufacture PHAs and found that crude glycerol of 0.362 USD/kg PHA and refined glycerol of 0.484 USD/kg PHA were the most economically viable substrates since they yielded more biopolymers than the alternatives. Dietrich *et al.*, (2017) developed different downstream processing sustainable assessment strategies with results that using sodium hypochlorite produced the highest global warming of 13.728 kg CO₂ eq/kg. As a result, the digestion of sodium hypochlorite had the highest carbon footprint (28.71–29.46 kg CO₂ eq/h). In contrast, treatment with sodium hydroxide or sulfuric acid produced the lowest global warming of 4.08 kg CO₂ eq/h and 6.27 kg CO₂ eq/h, respectively. Furthermore, when compared to sodium hypochlorite, which had recovery costs of 5.23-6.61 €/kg P(3HB), these treatments had the lowest recovery costs of 1.02 €/kg P(3HB) for sodium hydroxide and 1.11 €/kg P(3HB) for sulfuric acid. According to the report, bioplastics are expected to reduce carbon footprint by 1.3–1.4 Gt CO₂/a by 2030,

compared to 3.2–3.7 Gt CO₂/a. of bio-oils (Dietrich *et al.*, 2017). According to findings from Andreasi *et al.* (2021), reported that PHA produced from biowaste materials can surpass PHA from high-grade food (i.e., sugar and starch) and conventional plastics in terms of economic expenses and ecological effects, with four times lower ecological effects and eight times lower economic expenses than conventional plastics. However, it was described that producing bioplastics from biowaste resources attests to their sustainability and simultaneously lowers their contribution to greenhouse gas emissions (Hassan *et al.*, 2013). Agreeing with the literature Talan *et al.* (2020) showed that producing PHAs can significantly reduce carbon footprint by up to 200% while using fossil fuels can be reduced by 95% and can reduce waste following the circular bio-based economy idea. This was demonstrated by various environmental assessments such as life cycle assessments and carbon footprints.

6.2 Fermentation Broth Characterization

6.2.1 Expected Composition

The composition of a fermentation broth can vary significantly depending on the microorganism used, the substrate (Chien Bong *et al.*, 2021), and the specific objectives of the fermentation, as detailed below:

a. VFAs: They are common products of anaerobic fermentation. These include acetic acid, propionic acid, butyric acid, and others (Li and Wilkins, 2020), and they are essential precursors for PHA production (Coats *et al.*, 2016).

b. Minerals: The fermentation medium and the substrate may contribute minerals such as potassium, magnesium, and phosphorus. These minerals are essential for microbial growth and metabolic processes and reflect the composition of the products produced (López-Cuellar *et al.*, 2011).

c. Lipids: The presence of lipids in the broth can vary depending on the microorganism and substrate (Chien Bong *et al.*, 2021). Some microbes are

known for lipid accumulation, which can be harnessed for biodiesel production or biopolymer production (Cinar *et al.*, 2020).

d. Proteins: Proteins can be present in the fermentation broth as a result of microbial biomass production or cell lysis. The concentration of proteins is largely determined by the microorganisms used and the initial substrate composition (Raza *et al.*, 2018; Chien Bong *et al.*, 2021).

6.2.2 Impact of Process Parameters

Various parameters can significantly influence the composition of the fermentation broth:

a. The pH of the fermentation broth can affect the production of VFAs and the solubility of minerals. Microorganisms often have specific pH optima, and deviations from this can impact their metabolic activities. According to Montiel-Jarillo *et al.* (2017), PHA synthesis is significantly influenced by pH, as shown by the enriched biomass's 44% gPHA/g/L PHA content in the absence of regulated pH. Another by Liu *et al.* (2011b), revealed that PHA content was higher at pH 7.0 (64%/CDW) and pH 6.0 (60%/CDW), respectively.

b. Temperature affects the growth rate and metabolic activity of microorganisms. Higher temperatures may result in faster fermentation but could also affect the quality and quantity of the products. As reported by Catherine *et al.* (2022), it was observed that the concentration of 4 g/L acetate resulted in the highest PHA storage rate, accounting for 20% of the cell dry weight. Furthermore, when operating at the optimal temperature of 23°C, the PHA production increased to 24% of the cell dry weight. Regardless of the inoculum, higher temperatures (30 and 37 °C) were found to enhance polyhydroxyalkanoates (PHAs) accumulation by as much as 30%, as documented by Pérez *et al.* (2019).

d. Aeration and Mixing: Oxygen availability, or lack thereof, can affect the metabolism of microorganisms in the broth. Proper aeration and mixing can influence the production of VFAs, lipids, and the overall fermentation process. Liu

et al. (2011b) investigate the PHA accumulation using a continuous aeration approach under sodium acetate concentrations of 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 g/L. The highest PHA content achieved was 46.5%/CDW at 5.5 g/L.

6.2.3 Impact of Pretreatment

Pretreatment methods such as sterilization, pH adjustment, or enzymatic hydrolysis can impact the availability of nutrients in the substrate and influence microbial growth and product formation (Mankar *et al.*, 2021; Moralejo-Gárate *et al.*, 2014).

a. Inhibition or Enhancement: Pre-treatment can also inhibit the growth of undesirable microorganisms or promote the growth of specific strains, thus affecting the final composition of the broth (Mankar *et al.*, 2021).

b. Substrate Availability: Effective pre-treatment can enhance the availability of substrates for microbial growth, potentially increasing product yield (Wen *et al.*, 2020; Rao *et al.*, 2010).

However, the composition of a fermentation broth is highly dependent on the specific fermentation process, microorganisms, and substrates used (Raza *et al.*, 2018). Process parameters and pre-treatment methods can be designed to optimize the desired composition for various applications, including biofuel production, biopolymer synthesis, or waste treatment.

6.3 Shake flask experiments to evaluate PHA production.

Shake flask cultivations using *Pseudomonas oleovorans* strains (DSMZ1045 and ATCC29347) revealed robust cell growth on various lipid-rich waste substrates. Notably, these *Pseudomonas* strains demonstrated a natural emulsification capacity for all feedstocks during shake flask cultivations. The highest cell dry weight (CDW) was achieved with glycerol and fats, oils, and greases (FOG) at 2% oil concentration, yielding 1.1 g/L and 0.97 g/L, respectively. These cultures

also exhibited substantial polyhydroxyalkanoate (PHA) accumulation, reaching 43% and 39% weight percent of PHA per CDW, respectively.

In contrast, cultivations employing 2% frying soybean oil and frying palm oil resulted in lower CDW, measuring 0.75 g/L and 0.87 g/L. However, the PHA yield from these feedstocks remained notable, accounting for 34% and 33% per CDW, respectively. Kahar *et al.* (2004) obtained PHA between the range of 0.72 to 0.76 g/L using soybean oil as a carbon source. Notably, the application of pretreatment techniques resulted in an appreciable enhancement of PHA production, with observed increases of up to 23.26%, 41.03%, 41.18%, and 46.88% per cell dry weight and 18.60%, 38.46%, 26.47%, and 40.63% per CDW, depending on the specific pretreatment method employed.

Generally, a 2% oil concentration proved sufficient to attain substantial CDW and PHA content under the employed limiting nitrogen conditions. Park and Kim (2011) demonstrated that 20g of soybean oil/g of $(\text{NH}_4)_2\text{SO}_4$ was the ideal carbon-to-nitrogen (C/N) ratio that produced the maximum cell and PHA content in flask culture. Moreover, shake flask cultivations indicated that glycerol and FOG as carbon sources led to the highest PHA content, with lipase pretreatment showing the highest weight percent of PHA per CDW when applied in cultures using a 2% oil concentration from each oil substrate. It is worth emphasizing that the highest PHA accumulation was consistently observed with the DSMZ1045 strain across all carbon sources. The fermentation conditions reported in the literature using different oil substrate as seen in (Chien Bong *et al.*, 2021; Verlinden *et al.*, 2011). This study centred on the fermentative production of bioplastic using lipid-rich waste materials as the primary carbon source, with a particular emphasis on assessing its feasibility in relation to environmental impact and sustainability. Moreover, the investigation revealed the economic viability of this approach, attributed to cost-effective procedures that have been developed. These include the utilization of affordable and simple chemicals, such as sodium hydroxide, for the extraction process, the implementation of uncomplicated and energy-efficient pretreatment techniques, and the use of readily available waste lipid-rich substrates.

6.4 % Yield of PHA from various carbon source

Selected *Pseudomonas* strains [*P. oleovorans* (DSMZ1045 & ATCC29347)] were tested for their ability to produce MCL-PHA under various lipid-rich wastes as carbon sources. The lipid-rich wastes that were used to feed the bacteria in a medium include glycerol, soybean oil, FOG, and palm oil. *Pseudomonas oleovorans* DSMZ1045 produced cell contents of 1.1 g/L and 0.97 g/L from glycerol and FOG, and accumulated PHA at percentages of 43% and 39% of CDW, respectively. Then followed by soybean oil and palm oil, accounting for 0.75 and 0.87 g/L, and 34% and 32% of CDW respectively. While *Pseudomonas oleovorans* ATCC29347 had slightly lower (38%, 37%, and 34%) except palm oil with 33% of CDW. However, glycerol showed the highest dry cell content and PHA accumulation from both strains, followed by FOG, soybean oil, and palm oil. As compared to the results of the published studies, this finding is promising, for example (Kang *et al.*, 2017), *Pseudomonas putida* was studied for its ability to produce PHA using palm oil and had the highest production yield of 41% of cell dry weight. (Aloui *et al.*, 2022) reported a related study employing *Pseudomonas oleovorans* ATCC29347 and glycerol, and the highest PHA yield of 32.16% of cell dry weight was achieved. In a study where *Pseudomonas oleovorans* DSMZ1045 was grown on various carbon sources, the organism accumulated 46.7% PHA/CDW from glycerol (Favaro *et al.*, 2019). *P. aeruginosa* EBN-8 was cultured in soybean oil and accumulated a PHA yield of about 50.27% cell dry weight (Abid *et al.*, 2016). Different types of *Pseudomonas* strains have been discovered to produce PHA when grown on oily substrates (Surendran *et al.*, 2020).

In a related study, Leong *et al.* (2016) examined the biosynthesis of PHAs in *C. necator* H16 after 72 hours using four different substrates: soybean oil, used cooking oil (UCO), refined glycerol, and crude glycerol. They found that these four substrates produced PHA yields of 20.73, 11.05, 31.07, and 25.01 g/L, respectively. Another study using food waste oil as an inexpensive material for PHA production by the *Pseudomonas* strain. The PHA yields achieved were CDW of 3.6 g/L, PHA yield of 2.4 g/L, and PHA content of 65 wt% after 72 h of

cultivation with 20 g/L of FWO at the optimal temperature of 25 C and inoculum size of 6% (v/v) (Pan *et al.*, 2021). Bustamante *et al.* (2019) focused on using camelina oil with different *Pseudomonas* strains and obtained a high yield of 40% with *P. resinovorans* than the *P. putida* and *P. oleovorans* that gave a considerable PHA yield of 28.8% CDW with 3.8g/L of PHA concentration. *Pseudomonas* sp. strain DR2 was used to produce the MCL-PHA, which reached up to 23.5%. In terms of yield percentages, PHA was composed of 5.86% of 3HHx, 45.67% of 3HO, 34.88% of 3HD, 8.35% of 3HTD, and 5.24% of 3HHD (Song *et al.*, 2008).

Furthermore, study conducted by Sharma *et al.* (2017) achieved the cell mass between 2.42-5.14 g/L and PHA yield between 11.7% to 32.5% CDW when *P. chlororaphis* was grown on batch culture with fatty acids, fresh and waste vegetable oil, or waste free fatty acids from biodiesel. The monomer composition of the mcl-PHA produced was 43-49% of 3HO, 28-30% of 3HD, 8.6-9.7% of 3HHx, 4-11% of 3HTD and 8.6-9.7% of 3HDD. *Pseudomonas* strain (*P. putida* S12) accumulated MCL-PHA yield of 41% from crude sludge palm oil as a cheap fermentative carbon source (Kang *et al.*, 2017). The synthesis of MCL-PHA by two *Pseudomonas* strains was carried out using waste rapeseed oil as an inexpensive raw material. As a result, the strains (GI01 & GI06) accumulated a significant amount of MCL-PHA, up to 21.0% and 19.3% of CDW, respectively (Mozejko *et al.*, 2011). A more recent study reported the accumulated medium chain length polyhydroxyalkanoate by *Pseudomonas aeruginosa* from lipid-rich oil wastes reach up to 66% of CDW (Talan *et al.*, 2020). Additionally, the inability of the bacteria to create the lipases required for the breakdown of the triacylglycerol constituents may also prevent PHA from being produced from the oil samples' constituent triacylglycerols (Hosseinzadeh-Bandbafha *et al.*, 2022). In this work, MCL-PHA yields were reported to be comparable to those found (Bustamante *et al.*, 2019b; Kang *et al.*, 2017; Pan *et al.*, 2021). However, in contrast to the yields obtained in this work, those from (Mozejko-Ciesielska and Kiewisz, 2016; Mozejko *et al.*, 2011; Sharma *et al.*, 2017b; Song *et al.*, 2008) are all significantly lower.

After PHA production was examined from non-treated lipid-rich wastes, three methods of pretreatment were applied to each carbon source to determine the effect of each pretreatment method on the maximized production yield. When lipase pretreatment was applied, the maximum PHA yield of 55%/CDW and 54% CDW was obtained. The PHA yield of 52%/CDW and 50% of CDW and 51%/CDW and 49% of CDW, respectively, followed by use of microwave and ultrasonic pretreatment procedures. According to the previously published study Mozejko and Ciesielski (2013), *Pseudomonas* Gl01 uses saponified waste palm oil as a carbon source and accumulates PHA yield up to 43% of cell dry weight. The findings show that the formation of bioplastics from lipid-rich wastes can aid in the development of low-cost, sustainable bioplastic production processes and increase the possibility that these eco-friendly polymers will find new industrial applications.

6.5 Polymer downstream process.

The focus of the study was on the efficient extraction of polyhydroxyalkanoates (PHA) from accumulated cell biomass. This is a crucial objective as research endeavours aim to enhance PHA production using methods that are economically viable, simple, and environmentally friendly, thereby overcoming the limitations associated with harsh chemical processes that hinder their industrial feasibility and contribute to ecological issues. In pursuit of this goal, an approach was devised employing NaOH, H₂O₂, and lysozymes to assess their efficacy in extracting PHA from the dried cell biomass. The outcome of this study is of considerable significance, as it successfully demonstrated the extraction of PHA through all three methodologies. Furthermore, all techniques produced a good amount of polymer by the strains, with slight differences observed among them.

In the case of the sodium hydroxide method, the maximum yield achieved was 43% and 39% of cell dry weight (CDW), and 38% and 37% of CDW for *Pseudomonas oleovorans* DSMZ1045 and *Pseudomonas oleovorans* ATCC29347, respectively. Similarly, the lysozyme and hydrogen peroxide

methods yielded a maximum of 40% & 38% of CDW and 40% & 37% of CDW for *P. oleovorans* DSMZ1045, while for *P. oleovorans* ATCC29347, the yields were 38% & 38% of CDW and 36% & 35% of CDW, respectively. This study aligns with previous research by Fernández-Dacosta *et al.* (2015), who utilized alternative methods involving NaOH and SDS, surfactant and NaOCl, and dichloromethane. Interestingly, their study reported comparable yields for all three methods of 73.5%, 75.8%, and 82.2%, with an ultimate purity of 99.9 wt%. Similarly, Irdahayu *et al.* (2017) reported a 97% purity and a recovery yield of 98 wt% through the use of an alkali and surfactant (NaOH & Lysol) technique. These findings collectively emphasize the significance of developing efficient, sustainable, and adaptable methods for PHA extraction from dried cell biomass. In summary, this study shows the efficiency of enhancement of mild and effective approaches for PHA extraction from cell biomass. Furthermore, the finding holds promise for advancing PHA production processes, aligning with the pursuit of sustainable and environmentally conscious industrial practices.

6.6 Oil Mineral Impact on PHA Quantity

The mineral content of oils, including calcium, magnesium, potassium, phosphorus, etc, holds a significant influence on the quality of polyhydroxyalkanoates (PHA). This influence extends to the fermentation process and the chemical and physical properties of the final PHA product. These minerals can serve as essential cofactors for enzymes involved in microbial metabolism, such as magnesium or calcium, and they can catalyse the hydrolysis of ester bonds in triglycerides, the primary constituents of oils (Ganesh Saratale *et al.*, 2021). Such processes have the potential to affect both the yield and the quality of PHA. Hence, the precise control and management of mineral content are imperative in ensuring the desired quality of PHA products. High mineral content may result in the introduction of impurities, thus lowering the quality and properties of the final PHA (Mahato *et al.*, 2023; Koller *et al.*, 2010).

Additionally, minerals play a role in enhancing the formation of stable emulsions during oil hydrolysis. The utilization of oil substrates, including waste oil and plant

oil, has gained substantial attention in PHA production due to their favourable attributes, including mineral content, cost-efficiency, and high product yield (Chien Bong *et al.*, 2021). Factors such as a high carbon content per mass, a high rate of substrate catabolism, and the facile microbial conversion of saturated and unsaturated fats into PHA have been linked to higher PHA yields from oils compared to other carbon substrates like sugars (Mahato *et al.*, 2021; Rao *et al.*, 2010; Surendran *et al.*, 2020). As elucidated by Cruz *et al.* (2016), oil substrates yield substantial PHA production of 0.6–0.8 g/g, with PHA often comprising a substantial proportion (40-90%) of the cell's dry weight (Chien Bong *et al.*, 2021).

Furthermore, there is robust scientific evidence supporting the notion that utilizing oils as carbon feedstocks amplifies PHA production quantities, mainly due to the mineral content inherent to oils (Surendran *et al.*, 2020). Numerous studies have been conducted using oils, such as palm oil, soybean oil, and jatropha oil, as carbon sources for PHA production, consistently achieving high product yields (Chien Bong *et al.*, 2021; Surendran *et al.*, 2020). For instance, Liu *et al.* (2018) reported a substantial PHA content of 48% of cell dry weight, with a PHA productivity of 13.16 mg PHA/L/h, achieved from crude glycerol through the cultivation of *Pseudomonas mosselii* TO7 over a 48-hour fermentation period. In another example (Song *et al.*, 2008), detailed the production of medium-chain-length (MCL)-PHA, reaching up to 37.34% per dried cell weight (DCW) from waste vegetable oil and 23.52% per DCW from waste corn oil using *Pseudomonas* sp. strain DR2. In a comparative study conducted by Verlinden *et al.* (2011), PHA contents of 1.2 g/L were achieved from waste frying oil, with 0.62 g/L and 0.9 g/L from pure oil and heated oil, respectively.

6.7 Characterization of the polymer produced.

6.7.1 Polymer granules observations

The morphologic granules of the obtained polymer were confirmed by scanning electron microscopy (SEM). The analysis was carried out using TESCAN VEGA 3, which gives high-resolution pictures without the need for extensive sample preparation and can be used to examine the morphology of the PHA granules

inside the cell. Images were captured at 10kV and 20kV and magnified between 2000 and x1000 to view the PHA granules in the cells. Some polymer's granules looked like tiny, porous, linked granules, while others were spherical particles with various diameters. The morphology shows relatively uniformly distributed pseudo-spherical granules. This yielded a result that matched the conclusions of many other investigations described by (Liu, Chen and Lee, 2018; Nwinyi and Owolabi, 2019).

6.7.2 Polymer functional groups analysis

The extracted PHA was analysed using FTIR spectroscopy. It was carried out between the wavenumbers of 400 - 4000 cm^{-1} to confirm the functional groups of the PHA produced. The hydroxyl group of PHA was assigned at the absorption bands of 3400 cm^{-1} . The extracted PHA exhibits the characteristic methyl (CH_3) and methylene (CH_2) groups, which are found between the absorption bands of 2800 - 3000 cm^{-1} , as reported in the published studies that the methyl and methylene groups are allocated between 2800 and 3000 cm^{-1} (Petpheng *et al.*, 2023). The stretching of the asymmetrical methyl (CH_3) group results in an absorption band at 2989 cm^{-1} (Gumel *et al.*, 2013). According to published reports, the existence of asymmetric methyl (CH_3) may lead to the formation of hydrogen bonds (Sawant *et al.*, 2017). Additionally, the connections between methyl (CH_3) and carbonyl ($\text{C}=\text{O}$) groups formed due to the stretching in the 2989 cm^{-1} region (Tanikkul *et al.*, 2020). The functional groups responsible for the lateral chains of the monomeric units are the absorption bands at 2932 cm^{-1} and correspond to the stretching of asymmetrical methylene groups (CH_2), as stated in many published studies (Gumel *et al.*, 2013b; Sawant *et al.*, 2017). The symmetrical methyl groups (CH_3) are in the absorption band at 2852 cm^{-1} , and the intensity of this absorption band is because of the conformational change acquired during crystallization (Gumel *et al.*, 2012; Sathiyarayanan *et al.*, 2017). Gupta *et al.* (2017); Liu *et al.* (2018); Tanikkul *et al.* (2020) stated in their studies that the stretching vibrations of the carbonyl ($\text{C}=\text{O}$) ester bond, which are also linked to the polymer's amorphous area and can be a PHA marker band, were assigned to a strong signal band between 1730 – 1740 cm^{-1} . Several solid

bands were observed at absorption bands of 1450 and 1000 cm^{-1} , and it is assigned to C–O stretches bend (Mahato *et al.*, 2021; Pan *et al.*, 2021). At the absorption band of 1385 cm^{-1} , a typical terminal methylene (CH_2) group signal was observed, which is an MCL-PHA characteristic, as reported by (Liu *et al.*, 2018; Simon-Colin *et al.*, 2008a, 2008b). The absorption bands between 1360 to 1000 cm^{-1} exhibit the C-O-C stretching of the ether bonds (Tanikkul *et al.*, 2020). The asymmetric C-O-C and C-O stretching were due to strong vibrations at 1285 and 1165 cm^{-1} (Aloui *et al.*, 2022; Tanikkul *et al.*, 2020). Numerous absorption bands at 1078 to 677 cm^{-1} are attributed to C-O and C-C stretching to the amorphous stage (Sathiyarayanan *et al.*, 2017; Tanikkul *et al.*, 2020). The functional groups of the PHA produced in this work are similar to those previously mentioned above and described for mcl-PHA made by other *Pseudomonas* strains (Aloui *et al.*, 2022; Chandra *et al.*, 2023; Davis *et al.*, 2013; Kourmentza *et al.*, 2019; Liu *et al.*, 2018; Pan *et al.*, 2021). Thus, it was determined that the polymer produced from *Pseudomonas* strains of *Pseudomonas oleovorans* (DSMZ1045 and ATCC29347) is mcl-PHA as described in published literature findings.

6.8 Quality of the PHA produced from waste substrates.

At present, the fermentative production of polyhydroxyalkanoates (PHAs) relies on renewable biomass sources, such as food and starch-based waste, plant oils, organic industrial effluents, and residues from the biodiesel sector (Chien Bong *et al.*, 2021). PHAs derived from these waste substrates may be susceptible to contamination by various constituents, including nucleic acids, endotoxins, proteins, lipids, anti-foaming agents, hypochlorite, as well as other impurities such as bacteria, viruses, and plasmids (Chien Bong *et al.*, 2021; Koller *et al.*, 2013; Mahato *et al.*, 2023). This contamination remains a concern, regardless of the choice of microbial strains or the quantity of PHAs produced, rendering their consumption in other applications, such as medicine, unfeasible (Mahato *et al.*, 2023). This study did not include an evaluation and monitoring of purity during the fermentation and post-extraction processes. Nevertheless, numerous

published studies have undertaken assessments of the purity of the recovered polyhydroxyalkanoate (PHA) content and have elucidated the methodologies employed for quantifying impurities in PHA. For instance, in a study conducted by Lee *et al.* (1999), endotoxin levels in recovered polyhydroxyalkanoates (PHA) were reported for two different extraction methods: chloroform and sodium hydroxide (NaOH). The chloroform extraction method yielded endotoxin levels of less than 10 endotoxin units per gram (EU/g) of PHA, while the NaOH digestion approach proved to be more efficient in extraction but resulted in a significantly higher endotoxin level, exceeding 10^4 EU/g (Lee *et al.*, 1999). Consequently, sodium hydroxide offers several advantages, including polymer integrity preservation, cost-effectiveness, environmental friendliness, and a high purity level of 98% (Koller *et al.*, 2013; Lee *et al.*, 1999). It is essential to remove these contaminants since their presence can render the PHA unsuitable for its intended applications. Various procedures are employed to assess PHA purity and can be categorized into two groups:

6.8.1 Methods of quantifying PHA impurities

6.8.1.1 Protein associate impurities

Approximately 1.9 wt% of the proteins are firmly associated with PHA granules, creating the unique "carbonosomes" structure, which exhibits remarkable resistance to various recovery techniques, including solvents and lytic agents. A potential strategy for mitigating the protein residual content involves the application of proteases subsequent to the extraction process (Jendrossek, 2009; Pagliano *et al.*, 2021)

6.8.1.2 Endotoxins (Lipopolysaccharides)

Lipopolysaccharides (LPS) constitute the cell wall components of bacteria and are released into the environment upon bacterial membrane rupture. These LPS, often referred to as 'endotoxins,' become mixed with the polyhydroxyalkanoate (PHA) during extraction processes (Pagliano *et al.*, 2021). Nevertheless, research indicates that solvent-based extraction methods, such as chloroform,

yield lower levels of endotoxins compared to alkali-based methods like NaOH (Lee *et al.*, 1999). Several potential approaches are available to reduce the residual endotoxin concentration in extracted PHA. These approaches encompass the incorporation of alkaline or oxidizing post-treatments, the execution of multiple cycles of polymer dissolution and precipitation, and the adoption of filtration techniques involving charcoal (Koller *et al.*, 2013; Wampfler *et al.*, 2010).

6.8.1.3 Lipids associated impurities.

The presence of lipids in polyhydroxyalkanoate (PHA) extracts is discernible when solvent-based extraction techniques are employed (Choi and Lee, 1999). Various analytical techniques are used to quantify lipid residues, including thin-layer chromatography (TLC), gas chromatography (GC), liquid chromatography (LC), enzyme-linked immunosorbent assays (ELISA), nuclear magnetic resonance (NMR), and mass spectrometry (MS) (Lee *et al.*, 1998). The MS-based approach is the most effective among all these techniques due to its remarkable attributes, including exceptional accuracy, heightened sensitivity, specificity, and efficiency (Li *et al.*, 2014). For PHA extraction, supercritical carbon dioxide (sCO₂) presents a more practical and secure solvent option (Khosravi-Darani *et al.*, 2004). Its high solubility for numerous lipid-containing and hydrophobic cellular components, combined with the complete evaporation without leaving any residues post-extraction, underscores its suitability (Hejazi *et al.*, 2003; Khosravi-Darani *et al.*, 2004).

6.8.2 Method of quantifying PHA

Several approaches, including methanolysis combined with chromatographic or thermogravimetric (TGA) analyses, have been suggested to evaluate the purity of the extracted polyhydroxyalkanoate (PHA) (Sathiyarayanan *et al.*, 2017). The primary challenge in purity determination methods based on the quantity of PHA in the recovered material is that, even with a modest relative standard deviation (RSD) of the replicates, the purity estimate must closely approach 100%

(Zhang *et al.*, 2021). For instance, gas chromatographic analysis typically requires more than 4-6 replicates to achieve a 95% confidence interval of results within a 1% margin at an RSD of 1-2%. However, TGA analysis offers greater precision (RSD of 1%), facilitating the evaluation of purity levels up to 99% with fewer replicates (Pagliano *et al.*, 2021).

References

- Abid, S., Ali, Z., & Tanveer, R. (2016). Production kinetics of polyhydroxyalkanoates by using *Pseudomonas aeruginosa* gamma ray mutant strain EBN-8 cultured on soybean oil. *3 Biotech*, **6**(2); pp1–10.
- Akiyama, M., Tsuge, T., & Doi, Y. (2003). Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation. *Polymer Degradation and Stability*, **80**(1); pp183–194.
- Aloui, H., Khomlaem, C., Torres, C. A. V., Freitas, F., Reis, M. A. M., & Kim, B. S. (2022). Enhanced co-production of medium-chain-length polyhydroxyalkanoates and phenazines from crude glycerol by high cell density cultivation of *Pseudomonas chlororaphis* in membrane bioreactor. *International Journal of Biological Macromolecules*, **211**(5); pp545–555.
- Andreasi, S., Boldrin, A., Frenna, G., & Astrup, T. F. (2021). An environmental and economic assessment of bioplastic from urban biowaste. The example of polyhydroxyalkanoate. *Bioresource Technology*, **327**(2021); pp1–11.
- Bhatia, S. K., Otari, S. V., Jeon, J. M., Gurav, R., Choi, Y. K., Bhatia, R. K., Pugazhendhi, A., Kumar, V., Rajesh Banu, J., Yoon, J. J., Choi, K. Y., & Yang, Y. H. (2021). Biowaste-to-bioplastic (polyhydroxyalkanoates): Conversion technologies, strategies, challenges, and perspective. *Bioresource Technology*, **326**(1); pp1247-1259.
- Bilo, F., Pandini, S., Sartore, L., Depero, L. E., Gargiulo, G., Bonassi, A., Federici, S., & Bontempi, E. (2018). A sustainable bioplastic obtained from rice straw. *Journal of Cleaner Production*, **200**(2018); pp357–368.
- Bustamante, D., Tortajada, M., Ramon, D., & Rojas, A. (2019). Camelina oil as a promising substrate for mcl-PHA production in *Pseudomonas* sp. cultures. *Applied Food Biotechnology*, **6**(1); pp61–70.
- Catherine, M. C., Guwy, A., & Massanet-Nicolau, J. (2022). Effect of acetate

- concentration, temperature, pH, and nutrient concentration on polyhydroxyalkanoates (PHA) production by glycogen accumulating organisms. *Bioresource Technology Reports*, **20**(9); pp1012-1023.
- Chandra, R., Thakor, A., Mekonnen, T. H., Charles, T. C., & Lee, H. S. (2023). Production of polyhydroxyalkanoate (PHA) copolymer from food waste using mixed culture for carboxylate production and *Pseudomonas putida* for PHA synthesis. *Journal of Environmental Management*, **336**(2023); pp1176-1185.
- Chien Bong, C. P., Alam, M. N. H. Z., Samsudin, S. A., Jamaluddin, J., Adrus, N., Mohd Yusof, A. H., Muis, Z. A., Hashim, H., Salleh, M. M., Abdullah, A. R., & Chuprat, B. R. Bin. (2021). A review of the potential of polyhydroxyalkanoates production from oil-based substrates. *Journal of Environmental Management*, **298**(2021); pp1–11.
- Choi, J. Il, & Lee, S. Y. (1999). Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. *Biotechnology and Bioengineering*, **62**(5); pp546–553.
- Cinar, S. O., Chong, Z. K., Kucuker, M. A., Wieczorek, N., Cengiz, U., & Kuchta, K. (2020). Bioplastic production from microalgae: A review. *International Journal of Environmental Research and Public Health*, **17**(11); pp1–21.
- Coats, E. R., Watson, B. S., & Brinkman, C. K. (2016). Polyhydroxyalkanoate synthesis by mixed microbial consortia cultured on fermented dairy manure: Effect of aeration on process rates/yields and the associated microbial ecology. *Water Research*, **106**(2016); pp26–40.
- Cruz, M. V., Freitas, F., Paiva, A., Mano, F., Dionísio, M., Ramos, A. M., & Reis, M. A. M. (2016). Valorization of fatty acids-containing wastes and byproducts into short- and medium-chain length polyhydroxyalkanoates. *New Biotechnology*, **33**(1); pp206–215.
- Dahiya, S., Kumar, A. N., Shanthi Sravan, J., Chatterjee, S., Sarkar, O., & Mohan, S. V. (2018). Food waste biorefinery: Sustainable strategy for circular bioeconomy. *Bioresource Technology*, **248**(2017); pp2–12.

- Davis, R., Kataria, R., Cerrone, F., Woods, T., Kenny, S., O'Donovan, A., Guzik, M., Shaikh, H., Duane, G., Gupta, V. K., Tuohy, M. G., Padamatti, R. B., Casey, E., & O'Connor, K. E. (2013). Conversion of grass biomass into fermentable sugars and its utilization for medium chain length polyhydroxyalkanoate (mcl-PHA) production by *Pseudomonas strains*. *Bioresource Technology*, **150**(2013); pp202–209.
- Dietrich, K., Dumont, M. J., Del Rio, L. F., & Orsat, V. (2017). Producing PHAs in the bioeconomy — Towards a sustainable bioplastic. *Sustainable Production and Consumption*, **9**(2017); pp58–70.
- Favaro, L., Basaglia, M., Rodriguez, J. E. G., Morelli, A., Ibraheem, O., Pizzocchero, V., & Casella, S. (2019). Bacterial production of PHAs from lipid-rich by-products. *Applied Food Biotechnology*, **6**(1); pp45–52.
- Fernández-Dacosta, C., Posada, J. A., Kleerebezem, R., Cuellar, M. C., & Ramirez, A. (2015). Microbial community-based polyhydroxyalkanoates (PHAs) production from wastewater: Techno-economic analysis and ex-ante environmental assessment. *Bioresource Technology*, **185**(2015); pp368–377.
- Ganesh Saratale, R., Cho, S. K., Dattatraya Saratale, G., Kadam, A. A., Ghodake, G. S., Kumar, M., Naresh Bharagava, R., Kumar, G., Su Kim, D., Mulla, S. I., & Seung Shin, H. (2021). A comprehensive overview and recent advances in polyhydroxyalkanoates (PHA) production using various organic waste streams. In *Bioresource Technology*, 325(2021); pp1-15.
- Gumel, Annuar, Heidelberg, T. (2013). Growth kinetics, effect of carbon substrate in biosynthesis of mcl-PHA by *Pseudomonas putida* Bet001. *Journal of Microbiology*, **45**(2); pp427–438.
- Gumel, A. M., Annuar, M. S. M., & Heidelberg, T. (2012). Biosynthesis and Characterization of Polyhydroxyalkanoates Copolymers Produced by *Pseudomonas putida* Bet001 Isolated from Palm Oil Mill Effluent. *PLoS ONE*, **7**(9); pp1–8.

- Gupta, A., Kumar, M., & Thakur, I. S. (2017). Analysis and optimization of process parameters for the production of polyhydroxyalkanoates along with wastewater treatment by *Serratia* sp. ISTVKR1. *Bioresource Technology*, **242**(2017); pp55–59.
- Halden, R. U. (2010). *Plastics and Health Risks*. <https://doi.org/10.1146/annurev.publhealth.012809.103714>
- Hassan, M. A., Yee, L. N., Yee, P. L., Ariffin, H., Raha, A. R., Shirai, Y., & Sudesh, K. (2013). Sustainable production of polyhydroxyalkanoates from renewable oil palm biomass. *Biomass and Bioenergy*, **50**(2013); pp1–9.
- Hejazi, P., Vasheghani-Farahani, E., & Yamini, Y. (2003). Supercritical Fluid Disruption of *Ralstonia eutropha* for Poly(β -hydroxybutyrate) Recovery. *Biotechnology Progress*, **19**(5); pp1519–1523.
- Hosseinzadeh-Bandbafha, H., Li, C., Chen, X., Peng, W., Aghbashlo, M., Lam, S. S., & Tabatabaei, M. (2022). Managing the hazardous waste cooking oil by conversion into bioenergy through the application of waste-derived green catalysts: A review. *Journal of Hazardous Materials*, **424**(2022); pp1–20.
- Irdahayu, N. M. N. M., Shantini, K., Huong, K.-H., Vigneswari, S., Aziz, N. A., Azizan, M. N. M., & Amirul, A.-A. A. (2017). En route to the economical eco-friendly solvent system in enhancing sustainable recovery of poly(3-hydroxybutyrate- co -4-hydroxybutyrate) copolymer. *Engineering in Life Sciences*, **17**(9); pp1050–1059.
- Jendrossek, D. (2009). Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). *Journal of Bacteriology*, **191**(10); pp3195–3202.
- Kahar, P., Tsuge, T., Taguchi, K., & Doi, Y. (2004). High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. *Polymer Degradation and Stability*, **83**(1); pp79–86.
- Kang, D. K., Lee, C. R., Lee, S. H., Bae, J. H., Park, Y. K., Rhee, Y. H., Sung, B. H., & Sohn, J. H. (2017). Production of polyhydroxyalkanoates from sludge

- palm oil using *pseudomonas putida* S12. *Journal of Microbiology and Biotechnology*, **27**(5); pp990–994.
- Khatami, K., Perez-Zabaleta, M., Owusu-Agyeman, I., & Cetecioglu, Z. (2021). Waste to bioplastics: How close are we to sustainable polyhydroxyalkanoates production? *Waste Management*, **119**(2021); pp374–388.
- Khosravi-Darani, K., Vasheghani-Farahani, E., Shojaosadati, S. A., & Yamini, Y. (2004). Effect of process variables on supercritical fluid disruption of *Ralstonia eutropha* cells for poly(R-hydroxybutyrate) recovery. *Biotechnology Progress*, **20**(6); pp1757–1765.
- Koller, M. (2015). Recycling of Waste Streams of the Biotechnological Poly (hydroxyalkanoate) Production by *Haloferax mediterranei* on Whey. **2015**(3); pp1–9.
- Koller, M., Salerno, A., Muhr, A., Reiterer, A., & Braunegg, G. (2013). Polyhydroxyalkanoates: Biodegradable polymers and plastics from renewable resources. *Materiali in Tehnologije*, **47**(1); pp5–12.
- Kourmentza, C., Araujo, D., Sevrin, C., Roma-Rodrigues, C., Lia Ferreira, J., Freitas, F., Dionisio, M., Baptista, P. V., Fernandes, A. R., Grandfils, C., & Reis, M. A. M. (2019). Occurrence of non-toxic bio emulsifiers during polyhydroxyalkanoate production by *Pseudomonas* strains valorizing crude glycerol by-product. *Bioresource Technology*, **281**(2019); pp31–40.
- Lee, D. S., Noh, B. S., Bae, S. Y., & Kim, K. (1998). Characterization of fatty acids composition in vegetable oils by gas chromatography and chemometrics. *Analytica Chimica Acta*, **358**(2); pp163–175.
- Lee, S. Y., Choi, J. Il, Han, K., & Song, J. Y. (1999). Removal of endotoxin during purification of poly(3-hydroxybutyrate) from gram-negative bacteria. *Applied and Environmental Microbiology*, **65**(6); pp2762–2764.
- Leong, Y. K., Show, P. L., Lan, J. C. W., Loh, H. S., Lam, H. L., & Ling, T. C.

- (2017). Economic and environmental analysis of PHAs production process. *Clean Technologies and Environmental Policy*, **19**(7); pp1941–1953.
- Leong, Y. K., Show, P. L., Lin, H. C., Chang, C. K., Loh, H. S., Lan, J. C. W., & Ling, T. C. (2016). Preliminary integrated economic and environmental analysis of polyhydroxyalkanoates (PHAs) biosynthesis. *Bioresources and Bioprocessing*, **3**(1); pp1–9.
- Li, L., Han, J., Wang, Z., Liu, J., Wei, J., Xiong, S., & Zhao, Z. (2014). Mass spectrometry methodology in lipid analysis. *International Journal of Molecular Sciences*, **15**(6); pp10492–10507.
- Li, M., & Wilkins, M. R. (2020). Recent advances in polyhydroxyalkanoate production: Feedstocks, strains and process developments. *International Journal of Biological Macromolecules*, 156(2020); pp691–703.
- Liu, M. H., Chen, Y. J., & Lee, C. Y. (2018). Characterization of medium-chain-length polyhydroxyalkanoate biosynthesis by *Pseudomonas mosselii* TO7 using crude glycerol. *Bioscience, Biotechnology and Biochemistry*, **82**(3); pp532–539.
- Liu, Z., Wang, Y., He, N., Huang, J., Zhu, K., Shao, W., Wang, H., Yuan, W., & Li, Q. (2011). Optimization of polyhydroxybutyrate (PHB) production by excess activated sludge and microbial community analysis. *Journal of Hazardous Materials*, **185**(1); pp8–16.
- López-Cuellar, M. R., Alba-Flores, J., Rodríguez, J. N. G., & Pérez-Guevara, F. (2011). Production of polyhydroxyalkanoates (PHAs) with canola oil as a carbon source. *International Journal of Biological Macromolecules*, **48**(1); pp74–80.
- Mahato, R. P., Kumar, S., & Singh, P. (2021). Optimization of Growth Conditions to Produce Sustainable Polyhydroxyalkanoate Bioplastic by *Pseudomonas aeruginosa* EO1. *Frontiers in Microbiology*, **12**(2021); pp1–9.
- Mahato, R. P., Kumar, S., & Singh, P. (2023). Production of

- polyhydroxyalkanoates from renewable resources: a review on prospects, challenges, and applications. In *Archives of Microbiology*, **205**(5); pp1-13.
- Mankar, A. R., Pandey, A., Modak, A., & Pant, K. K. (2021). Pretreatment of lignocellulosic biomass: A review on recent advances. *Bioresource Technology*, **334**(2021); pp1–12.
- Mannu, A., Ferro, M., Di Pietro, M. E., & Mele, A. (2019). Innovative applications of waste cooking oil as raw material. *Science Progress*, **102**(2); pp153–160.
- Mannu, A., Garroni, S., Porras, J. I., & Mele, A. (2020). Available technologies and materials for waste cooking oil recycling. In *Processes*, **8**(3); pp. 1–13.
- Martin Koller, Aid Atlić, Miguel Dias, A. R., & Braunegg, and G. (2010). Microbial PHA Production from Waste Raw Materials. In D. G.-Q. Chen (Ed.), *Microbiology Monographs*, **8**(2022); pp85–119.
- Min Song, H., Chan Joo, J., Hyun Lim, S., Jin Lim, H., Lee, S., & Jae Park, S. (2022). Production of polyhydroxyalkanoates containing monomers conferring amorphous and elastomeric properties from renewable resources: Current status and future perspectives. *Bioresource Technology*, **366**(2022); pp1–11.
- Montiel-Jarillo, G., Carrera, J., & Suárez-Ojeda, M. E. (2017). Enrichment of a mixed microbial culture for polyhydroxyalkanoates production: Effect of pH and N and P concentrations. *Science of the Total Environment*, **583**(2017); pp300–307.
- Moralejo-Gárate, H., Kleerebezem, R., Mosquera-Corral, A., Campos, J. L., Palmeiro-Sánchez, T., & van Loosdrecht, M. C. M. (2014). Substrate versatility of polyhydroxyalkanoate producing glycerol grown bacterial enrichment culture. *Water Research*, **66**(2014); pp190–198.
- Możejko-Ciesielska, J., & Kiewisz, R. (2016). Bacterial polyhydroxyalkanoates: Still fabulous? *Microbiological Research*, **192**(2016); pp271–282.
- Mozejko, J., & Ciesielski, S. (2013). Saponified waste palm oil is an attractive

- renewable resource for mcl-polyhydroxyalkanoate synthesis. *Journal of Bioscience and Bioengineering*, **116**(4); pp485–492.
- Mozejko, J., Przybyłek, G., & Ciesielski, S. (2011). Waste rapeseed oil as a substrate for medium-chain-length polyhydroxyalkanoates production. *European Journal of Lipid Science and Technology*, **113**(12); pp1550–1557.
- Nwinyi, O. C., & Owolabi, T. A. (2019). Scanning electron microscopy and Fourier transmission analysis of polyhydroxyalkanoates isolated from bacteria species from an abattoir in Ota, Nigeria. *Journal of King Saud University - Science*, **31**(3); pp285–298.
- Pagliano, G., Galletti, P., Samorì, C., Zaghini, A., & Torri, C. (2021). Recovery of Polyhydroxyalkanoates From Single and Mixed Microbial Cultures: A Review. *Frontiers in Bioengineering and Biotechnology*, **9**(2021); pp1–28.
- Pan, L., Li, J., Wang, R., Wang, Y., Lin, Q., Li, C., & Wang, Y. (2021). Biosynthesis of polyhydroxyalkanoate from food waste oil by *Pseudomonas alcaligenes* with simultaneous energy recovery from fermentation wastewater. *Waste Management*, **131**(2021); pp268–276.
- Park, D. H., & Kim, B. S. (2011). Production of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by *Ralstonia eutropha* from soybean oil. *New Biotechnology*, **28**(6); pp719–724.
- Pérez, R., Cantera, S., Bordel, S., García-Encina, P. A., & Muñoz, R. (2019). The effect of temperature during culture enrichment on methanotrophic polyhydroxyalkanoate production. *International Biodeterioration and Biodegradation*, **140**(1); pp144–151.
- Petpheng, B., Mudtaleb, B., Thongduang, S., Meekhai, T., Pechsiri, J., & Sangkharak, K. (2023). The production of medium-co-long chain length (mcl-co-lcl) polyhydroxyalkanoate from waste soybean oil by *Enterobacter* sp. and its application as an antibacterial agent. *Biomass Conversion and Biorefinery*, **2023**(012); pp1–14.

- Rao, U., Sridhar, R., & Sehgal, P. K. (2010). Biosynthesis and biocompatibility of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) produced by *Cupriavidus necator* from spent palm oil. *Biochemical Engineering Journal*, **49**(1); pp13–20.
- Raza, Z. A., Abid, S., & Banat, I. M. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. In *International Biodeterioration and Biodegradation*, 126(2018); pp45–56.
- Rosenboom, J. G., Langer, R., & Traverso, G. (2022). Bioplastics for a circular economy. *Nature Reviews Materials*, **7**(2); pp117–137.
- Sathiyarayanan, G., Bhatia, S. K., Song, H. S., Jeon, J. M., Kim, J., Lee, Y. K., Kim, Y. G., & Yang, Y. H. (2017). Production and characterization of medium-chain-length polyhydroxyalkanoate copolymer from Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620. *International Journal of Biological Macromolecules*, **97**(2017); pp710–720.
- Sawant, S. S., Salunke, B. K., Taylor, L. E., & Kim, B. S. (2017). Enhanced agarose and xylan degradation for production of polyhydroxyalkanoates by co-culture of marine bacterium, *Saccharophagus degradans* and its contaminant, *Bacillus Cereus*. *Applied Science*, **7**(3); pp1-15.
- Sharma, P. K., Munir, R. I., de Kievit, T., & Levin, D. B. (2017). Synthesis of polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *pseudomonas chlororaphis*. *Canadian Journal of Microbiology*, **63**(12); pp1009–1024.
- Simon-Colin, C., Raguénès, G., Costa, B., & Guezennec, J. (2008). Biosynthesis of medium chain length poly-3-hydroxyalkanoates by *Pseudomonas guezennei* from various carbon sources. *Reactive and Functional Polymers*, **68**(11); pp1534–1541.
- Simon-Colin, C., Raguénès, G., Crassous, P., Moppert, X., & Guezennec, J. (2008). A novel mcl-PHA produced on coprah oil by *Pseudomonas guezennei* biovar. tikehau, isolated from a “kopara” mat of French Polynesia.

- International Journal of Biological Macromolecules*, **43**(2); pp176–181.
- Song, Jin Hwan, Che Ok Jeon, Mun Hwan Choi, S. C. Y. and W. P. (2008). Polyhydroxyalkanoate (PHA) Production Using Waste Vegetable Oil by *Pseudomonas* sp. Strain DR2. *Journal of Microbiology and Biotechnology*, **18**(8); pp1408–1415.
- Surendran, A., Lakshmanan, M., Chee, J. Y., Sulaiman, A. M., Thuoc, D. Van, & Sudesh, K. (2020). Can Polyhydroxyalkanoates Be Produced Efficiently From Waste Plants and Animal Oils? In *Frontiers in Bioengineering and Biotechnology*, **8**(2020); pp1–15.
- Talan, A., Kaur, R., Tyagi, R. D., & Drogui, P. (2020). Bioconversion of oily waste to polyhydroxyalkanoates: Sustainable technology with circular bioeconomy approach and multidimensional impacts. In *Bioresource Technology Reports*, **11**(2020); pp1-10.
- Tanikkul, P., Sullivan, G. L., Sarp, S., & Pisutpaisal, N. (2020). Biosynthesis of medium chain length polyhydroxyalkanoates (mcl-PHAs) from palm oil. *Case Studies in Chemical and Environmental Engineering*, **2**(9); pp1–5.
- Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D., Piotrowska-Seget, Z., & Radecka, I. K. (2011). Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. *AMB Express*, **1**(1); pp1–8.
- Wampfler, B., Ramsauer, T., Kehl, K., Zinn, M., & Thöny-Meyer, L. (2010). Application of activated charcoal in the downstream processing of bacterial olefinic poly(3-hydroxyalkanoates). *Chimia*, **64**(11); pp784–788.
- Wei, L., Liang, S., Guho, N. M., Hanson, A. J., Smith, M. W., Garcia-Perez, M., & McDonald, A. G. (2015). Production and characterization of bio-oil and biochar from the pyrolysis of residual bacterial biomass from a polyhydroxyalkanoate production process. *Journal of Analytical and Applied Pyrolysis*, **115**(2015); pp268–278.
- Wen, Q., Liu, B., Li, F., & Chen, Z. (2020). Substrate strategy optimization for

polyhydroxyalkanoates producing culture enrichment from crude glycerol. *Bioresource Technology*, **311**(3); pp1235-1246.

Yadav, B., Pandey, A., Kumar, L. R., & Tyagi, R. D. (2020). Bioconversion of waste (water)/residues to bioplastics- A circular bioeconomy approach. *Bioresource Technology*, **298**(2020); pp1–15.

Zuin, V. G., & Kümmerer, K. (2022). Chemistry and materials science for a sustainable circular polymeric economy. *Nature Reviews Materials*, **7**(2); pp76–78.

7 Conclusion, Recommendations and Future Directions

7.1 Conclusion

Biodegradable bioplastics like polyhydroxyalkanoates (PHAs) are ideal alternatives to petroleum-based plastics that pollute our environment for a sustainable economy. Due to their thermoplastic characteristics, medium-chain-length (mcl) PHA bioplastics are of great interest. The use of inexpensive resources is a promising approach to reduce the high cost connected with PHA synthesis. This work used nitrogen limit in batch fermentation and operating conditions that encouraged direct PHA accumulation by *Pseudomonas* strains [*Pseudomonas oleovorans* (DSMZ1045 & ATCC29347)]. Various carbon sources were effectively used and investigated for the production of sustainable PHA. The findings showed that, from the viewpoint of carbon sources, glycerol and FOG are the best carbon sources for PHA production. A positive statistical correlation between the organisms and lipid-rich carbon source was shown, allowing up to 43% PHA/total biomass accumulation from non-pretreatment procedures and 55% PHA/total biomass accumulation when the pretreatment technique was employed.

Furthermore, the work evaluated the produced PHA by these *Pseudomonas* strains with different extraction techniques, and the processes led to good outcomes from bioplastic production with the highest accumulated percentage yields among the investigated lipid-rich carbon sources, glycerol, and FOG had the best PHA production contents, indicating the potential for sustainable process scale-up. The polymer characterization analyses show that the produced PHAs exhibit typical morphological characteristics and functional group attributes to MCL-PHAs, corresponding with those described in the published literature. The study proved the sustainable bioplastic production from lipid-rich oil wastes by *pseudomonas* strains. Results show that these *Pseudomonas* strains can efficiently convert lipid-rich oil waste into bioplastics, providing an impactful alternative to petroleum-based plastic

production. This could further lessen the harm that waste oils create to the environment.

7.2 Research Future Directions and Contributions

7.2.1 Research Future Directions

In the future, alternatives to expensive substrates for the food and chemical industries need to be developed. The capacity to make the best use of wasted materials (i.e., food and oils or low-value crops) to produce value-added products has enormous promise for the circular economy. One good example of these valued products is the production of polyhydroxyalkanoates (PHAs), which can replace fossil oil-derived monomers. *Pseudomonas* species and other bacteria are known to store PHAs as an energy source reserve material, and various strains of these bacteria are employed at both laboratory and industrial levels to produce these polymers. However, processes that developed to advance the production process to a cost-effective level, such as using molecular biology and biochemistry to underlie PHA biosynthesis or improve the culture conditions to increase production, are the two main areas of interest for research groups looking for novel PHA producers. This study opens avenues for future research, encouraging further exploration of waste-derived feedstocks for various industrial applications.

7.2.2 Research Contributions to Knowledge

This thesis has made a significant scientific contribution by addressing critical issues in the synthesis of bioplastics using waste oils. This academic discussion highlights the key contributions of this research, emphasizing its importance in the broader context of sustainable materials production.

Novel Approach: This research takes an innovative approach to utilizing waste oils as a feedstock for bioplastic production. The utilization of waste materials in the creation of value-added products like PHAs aligns with the principles of circular economy and resource efficiency.

Exploration of Critical Factors: The research explores various critical factors that impact the synthesis of bioplastics from waste oils. Notably, it delves into the selection of suitable substrates, the choice of microbial strains for polyhydroxyalkanoate (PHA) synthesis, as well as the significance of effective pretreatment and post-processing techniques. These factors play a vital role in determining the quality and yield of bioplastics.

Environmental Considerations: The research evaluates the environmental implications of using waste oils as a raw material for bioplastic production. By using waste oils, which would otherwise pose disposal challenges and environmental risks, the research contributes to reducing environmental pollution and resource depletion.

Sustainability: This research made significant contributions by encouraging the use of high-volume waste oils as resource materials rather than unwanted environmental hazards.

Promotion of Circular Economy: The scientific research aligns with the principles of a circular economy, where waste materials are transformed into valuable products. It promotes the concept of upcycling by converting waste oils into bioplastics, thereby contributing to a more sustainable and resource-efficient industrial system. Hence, the thesis presents notable scientific contributions from the methodological aspect of producing bioplastics, Table 7-1.

Table 7-1: Research contributions to knowledge

	What has been confirmed	What has been developed	What have advanced contributions
Investigational contributions	It has been confirmed that <i>Pseudomonas</i> strains, including <i>Pseudomonas oleovorans</i> produce bioplastics from oily waste resources.	Developed a novel approach of more viable and profitable by using the different lipid-rich substrates as a feedstock to organisms.	As there are limited/no studies on using FOG to make bioplastics, it deepens our understanding of how <i>Pseudomonas oleovorans</i> produce bioplastics from fat, oil, and gas (FOG).
Investigational contributions	According to the studies that have been published, FTIR analysis is the best characterization method that has helped researchers comprehend the functional absorption bands and chemical compositions of the extracted polymer, where these significant absorption bands validated the structural type of PHA produced (Mahato <i>et al.</i> , 2021).	The produced polymer was identified as being mcl-PHA by the characterization investigations using FTIR, which were able to identify the strongest absorption bands of the medium chain type of PHA.	The study used a simple batch method to evaluate various waste materials with different <i>Pseudomonas</i> strains, advancing the sustainable decrease of bioplastic production costs. This can be a reference for future research, resulting in more efficient and large-scale production technology.
Methodological contributions	The sustainable production of bioplastics from lipid-rich oil wastes by <i>pseudomonas</i> strains is demonstrated,	Developed experimental methods to investigate bioplastic production,	The statistical result is statistically significant as $P=0.000$ compared to the threshold of $P\text{-value} = 0.05$ to

which could further lessen the harm that waste oils provide to the environment. ensuring that the results were robust and statistically significant. evaluate the differences between means.

7.3 Recommendations

The following are some recommendations made during this investigation:

- i. Further study is still needed to discover new microorganisms and to modify the PHA-accumulating bacteria that are already known to use a variety of carbon feedstocks and produce copolymers without adding expensive precursors.
- ii. Switching to biorefinery production from batch or fed-batch production may become more viable and profitable.
- iii. It is recommended that the polymer characterization analysis be expanded to include techniques like thermogravimetric analysis, X-ray diffraction, and differential scanning calorimetry, among others, in order to increase the thoroughness of the analysis and provide a more thorough understanding of the polymer's properties.
- iv. Reusing the filtrate from centrifuged culture broth and downstream processing is encouraged since it has the dual benefits of reducing environmental impact and enhancing the viability and efficiency of this material's production.
- v. This research further recommends assessing the bioreactor scale under controlled circumstances using the optimized settings for more enhance the scaling up of the PHA sustainable production and maximize its productivity using batch cultivation.

REFERENCES

- Abomohra, A. E. F., Elsayed, M., Esakkimuthu, S., El-Sheekh, M., & Hanelt, D. (2020). The potential of fat, oil, and grease (FOG) for biodiesel production: A critical review on the recent progress and future perspectives. *Progress in Energy and Combustion Science*, **81**(2020); pp1–33.
- Aditi, S., Shalet, D. S., Manish, N., Pranesh, R., & Katyayini, T. (2015). Microbial production of polyhydroxyalkanoates (PHA) from novel sources : A Review. *International Journal of Research in Biosciences*, **4**(4); pp16–28.
- Aires, D., Antonio, R. V., Rossi, J. M., & Pena, S. (2014). Production of medium-chain-length polyhydroxyalkanoate by *Pseudomonas oleovorans* grown in sugary cassava extract supplemented with andiroba oil. **34**(4); pp738–745.
- Akaraonye, E., Keshavarz, T., & Roy, I. (2010). Production of polyhydroxyalkanoates : the future green materials of choice. **85**(6); pp732–743.
- Akhter, S., Khan, M. A., Mahmud, S., Biki, S. P., Shamsuzzoha, M., Hasan, S. M. K., & Ahmed, M. (2022). Biosynthesis and characterization of bacterial nanocellulose and polyhydroxyalkanoate films using bacterial strains isolated from fermented coconut water. *Process Biochemistry*, **122**(1); pp214–223.
- Al-battashi, H., Annamalai, N., Al-kind, S., & Sadasivan, A. (2019). Production of bioplastic (poly-3-hydroxybutyrate) using waste paper as a feedstock : Optimization of enzymatic hydrolysis and fermentation employing *Burkholderia sacchari*. *Journal of Cleaner Production*, **214**(2019); pp236–247.
- Albuquerque, M. G. E., Concas, S., Bengtsson, S., & Reis, M. A. M. (2010). Mixed culture polyhydroxyalkanoates production from sugar molasses: The use of a 2-stage CSTR system for culture selection. *Bioresource Technology*, **101**(18); pp7112–7122.

- Alemán-Huerta, M. E., Martínez-Herrera, a R. E., Elufisan, b T. O., C, d F. L. G.-P., A, Quintero-Zapata, I., Reyez-López, a M. Á., & Luna-Santillanac, c E. de J. de. (2022). Draft Genome Sequence of a Polyhydroxyalkanoate-Producing. *American Society for Microbiology*, **9**(4); pp15–17.
- Allen, A. D., Anderson, W. A., Ayorinde, F. O., & Eribo, B. E. (2010). Biosynthesis and characterization of copolymer poly(3HB-co-3HV) from saponified *Jatropha curcas* oil by *Pseudomonas oleovorans*. *Journal of Industrial Microbiology and Biotechnology*, **37**(8); pp849–856.
- Almeida, J.R.; Fradinho, J.C.; Carvalho, G.; Oehmen, A.; Reis, M. A. . (2022). Dynamics of Microbial Communities in Phototrophic Polyhydroxyalkanoate Accumulating Cultures. *Microorganisms*, **10**(351); pp1–11.
- Aloui, H., Khomlaem, C., Torres, C. A. V., Freitas, F., Reis, M. A. M., & Kim, B. S. (2022). Enhanced co-production of medium-chain-length polyhydroxyalkanoates and phenazines from crude glycerol by high cell density cultivation of *Pseudomonas chlororaphis* in membrane bioreactor. *International Journal of Biological Macromolecules*, **211**(5); pp545–555.
- Alsafadi, D., & Al-Mashaqbeh, O. (2017). A one-stage cultivation process for the production of poly-3-(hydroxybutyrate-co-hydroxyvalerate) from olive mill wastewater by *Haloferax mediterranei*. *New Biotechnology*, **34**(2017); pp47–53.
- Amaro, T. M. M. M., Rosa, D., Comi, G., Iacumin, L., & Brigham, C. J. (2019). Prospects for the Use of Whey for Polyhydroxyalkanoate (PHA) Production. **10**(2019); pp1–12.
- Amin, F. R., Khalid, H., Zhang, H., Rahman, S., Zhang, R., Liu, G., & Chen, C. (2017). Pretreatment methods of lignocellulosic biomass for anaerobic digestion. *AMB Express*, **7**(1); pp1-12.
- Ana Marta Rodrigues, Rita Dias Guardão Franca, Madalena Dionísio, C. S., & Christian Grandfils, M. A. M. R. and N. D. L. (2022). Polyhydroxyalkanoates from a Mixed Microbial Culture: Extraction Optimization and Polymer

- Characterization. *Polymers*, **14**(2115); pp1–22.
- Andler, R., Vald, C., Urtuvia, V., Andree, C., & Díaz-barrera, A. (2021). Fruit residues as a sustainable feedstock for the production of bacterial polyhydroxyalkanoates. *Journal of Cleaner Production*, **307**(2021); pp1–12.
- Andrady, A. L. (2003). *Plastics and the Environment*. John Wiley & Sons, Inc, **36**(6); pp1–19.
- Andrady, A. L. (2017). The plastic in microplastics: A review. *Marine Pollution Bulletin*, **119**(1); pp12–22.
- Andrady, A. L., & Neal, M. A. (2009). Applications and societal benefits of plastics. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **364**(1526); pp1977–1984.
- Anis, S. N. S., Md Iqbal, N., Kumar, S., & Amirul, A. A. (2013). Effect of different recovery strategies of P(3HB-co-3HHx) copolymer from *Cupriavidus necator* recombinant harboring the PHA synthase of *Chromobacterium* sp. USM2. *Separation and Purification Technology*, **102**(2013); pp111–117.
- Annamalai, N., & Sivakumar, N. (2016). Production of polyhydroxybutyrate from wheat bran hydrolysate using *Ralstonia eutropha* through microbial fermentation. *Journal of Biotechnology*, **237**(2016); pp13–17.
- Aremu, M. O., Ishola, M. M., & Taherzadeh, M. J. (2021). Polyhydroxyalkanoates (Phas) production from volatile fatty acids (vfas) from organic wastes by *pseudomonas oleovorans*. *Fermentation*, **7**(4); pp1–15.
- Ashby, R. D., Qureshi, N., Strahan, G. D., Johnston, D. B., Msanne, J., & Lin, X. (2022). Corn stover hydrolysate and levulinic acid: Mixed substrates for short-chain polyhydroxyalkanoate production. *Biocatalysis and Agricultural Biotechnology*, **43**(100), 1–15.
- Aubin, A. J., Mazza, C. B., Trinite, D. A., Mcconville, P., & Corporation, W. (2008). Analysis of Vegetable Oils by High-Performance Liquid Chromatography Using evaporative Light Scattering Detection and Normal Phase Eluents.

- Waters Application Note, **13**(2008); pp1–5.
- Awogbemi, O., Onuh, E. I., & Inambao, F. L. (2019). Comparative study of properties and fatty acid composition of some neat vegetable oils and waste cooking oils. *International Journal of Low-Carbon Technologies*, **14**(3); pp417–425.
- Barnes, P. R. F., Mulvaney, R., Wolff, E. W., & Robinson, K. (2002). A technique for the examination of polar ice using the scanning electron microscope. *Journal of Microscopy*, **205**(2); pp118–124.
- Berwig, K. H., Baldasso, C., & Dettmer, A. (2016). Production and characterization of poly (3-hydroxybutyrate) generated by *Alcaligenes latus* using lactose and whey after acid protein precipitation process. *Bioresource Technology*, **218**(2016); pp31–37.
- Bhalla, T. C. (2015). Applied Microbiology. *Applied Microbiology*, 1–190. <https://doi.org/10.1007/978-81-322-2259-0>
- Bhuwal, A. K., Singh, G., Aggarwal, N. K., Goyal, V., & Yadav, A. (2013). Isolation and screening of polyhydroxyalkanoates-producing bacteria from pulp, paper, and cardboard industry wastes. *International Journal of Biomaterials*, **2013**(17); pp1–11.
- Bonartsev, A. P., Bonartseva, G. A., Myshkina, V. L., Voinova, V. V., & Mahina, T. K. (2016). Biosynthesis of poly (3-hydroxybutyrate- co-3-hydroxy-4-methylvalerate) by Strain *Azotobacter chroococcum* 7B. **8**(30); pp77–87.
- Boonyawanich, S., Tanikkul, P., Thenchartanan, P., & Pisutpaisal, N. (2021). Productivity of *Pseudomonas putida* TISTR 1522 in polyhydroxyalkanoates (PHAs) production from saponified palm oil. *Applied Biochemistry and Biotechnology*, **193**(4); pp1086–1098.
- Borrero-de Acuña, J. M., Bielecka, A., Häussler, S., Schobert, M., Jahn, M., Wittmann, C., Jahn, D., & Poblete-Castro, I. (2014). Production of medium chain length polyhydroxyalkanoate in metabolic flux optimized *Pseudomonas putida*. *Microbial Cell Factories*, **13**(1); pp88.

- Brodin, M., Vallejos, M., Tanase, M., Cristina, M., & Chinga-carrasco, G. (2017). Lignocellulosic as sustainable resources for the production of bioplastics e A review. *Journal of Cleaner Production*, **162**(2017); pp646–664.
- Brojanigo, S., Alvarado-Morales, M., Basaglia, M., Casella, S., Favaro, L., & Angelidaki, I. (2022). Innovative co-production of polyhydroxyalkanoates and methane from broken rice. *Science of the Total Environment*, **825**(2022); pp1–9.
- Bueno-Borges, L. B., dos Santos, G. C. P., Alencar, S. M., & Regitano-d'Arce, M. A. B. (2019). Improving Waste Cooking Oil Quality for Biodiesel Production with the Ethanollic By-product of Soybean Oil Extraction. *Journal of the American Oil Chemists' Society*, **96**(12); pp1379–1388.
- Bugnicourt, E., Cinelli, P., Lazzeri, A., & Alvarez, V. (2014). Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing, and potential applications in packaging. **8**(11); pp791–808.
- Bustamante, D., Segarra, S., Cerro, C., Tortajada, M., & Ram, D. (2019). In silico prospection of microorganisms to produce polyhydroxyalkanoate from whey : *Caulobacter segnis* DSM 29236 as a suitable industrial strain. *Microb Biotechnol*, **12**(3); pp487–501.
- Castilho, L. R., Mitchell, D. A., & Freire, D. M. G. (2009). Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. *Bioresource Technology*, **100**(23); pp5996–6009.
- Castillo, T. C., & Segura, D. (2013). High production of poly-β-hydroxybutyrate (PHB) by an *Azotobacter vinelandii* mutant altered in PHB regulation using. *Biochemical Engineering Journal*, **82**(2013); pp1–8.
- Cert, A., Moreda, W., & Pérez-Camino, M. C. (2000). Chromatographic analysis of minor constituents in vegetable oils. *Journal of Chromatography A*, **881**(1–2); pp131–148.
- Cesário, M. T., Raposo, R. S., Almeida, M. C. M. D. d., van Keulen, F., Ferreira,

- B. S., Telo, J. P., & Fonseca, M. M. R. d. (2014). Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) by *Burkholderia sacchari* using wheat straw hydrolysates and gamma-butyrolactone. *International Journal of Biological Macromolecules*, **71**(2014); pp59–67.
- Chakraborty, P., Muthukumarappan, K., & Gibbons, W. R. (2012). PHA productivity and yield of *Ralstonia eutropha* when intermittently or continuously fed a mixture of short-chain fatty acids. *Journal of Biomedicine and Biotechnology*, **3**(2012); pp1-13.
- Chanasit, W., Hodgson, B., Sudesh, K., & Umsakul, K. (2016). Efficient production of polyhydroxyalkanoates (PHAs) from *Pseudomonas mendocina* PSU using a biodiesel liquid waste (BLW) as the sole carbon source. *Bioscience, Biotechnology and Biochemistry*, **80**(7); pp1440–1450.
- Chanprateep, S. (2010). Current trends in biodegradable polyhydroxyalkanoates. *Journal of Bioscience and Bioengineering*, **110**(6); pp621–632.
- Chee, J., Yoga, S., Lau, N., Ling, S., & Abed, R. M. M. (2010). Bacterially Produced Polyhydroxyalkanoate (PHA): Converting Renewable Resources into Bioplastics. **2**(2010); pp1395–1404.
- Chen, G.-Q. (2010). Plastics Completely Synthesized by Bacteria: Polyhydroxyalkanoates. *Microbiology Monographs*, **14**(2); pp17–37.
- Chin, J. H. C., Samian, M. R., & Normi, Y. M. (2022). Characterization of polyhydroxyalkanoate production capacity, composition, and weight synthesized by *Burkholderia cepacia* JC-1 from various carbon sources. *Heliyon*, **8**(3); pp1–11.
- Chmelová, D., Legerská, B., Ondrejovič, M., & Miertuš, S. (2022). Optimization of Propagation Medium for Enhanced Polyhydroxyalkanoate Production by *Pseudomonas oleovorans*. *Fermentation*, **8**(1); pp1–12.
- Choi, J. Il, & Lee, S. Y. (1999). Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. *Biotechnology and Bioengineering*, **62**(5); pp546–553.

- Chong, J. W. R., Yew, G. Y., Khoo, K. S., Ho, S. H., & Show, P. L. (2021). Recent advances in food waste pretreatment technology via microalgae for source of polyhydroxyalkanoates. *Journal of Environmental Management*, **293**(2021); pp1–12.
- Corchado-Lopo, C., Martínez-Avila, O., Marti, E., Llimós, J., Busquets, A. M., Kucera, D., Obruca, S., Llenas, L., & Ponsá, S. (2021). Brewer's spent grain as a no-cost substrate for polyhydroxyalkanoates production: Assessment of pretreatment strategies and different bacterial strains. *New Biotechnology*, **62**(2020); pp60–67.
- Costa, S. S., Miranda, A. L., de Moraes, M. G., Costa, J. A. V., & Druzian, J. I. (2019). Microalgae as a source of polyhydroxyalkanoates (PHAs) — A review. *International Journal of Biological Macromolecules*, **131**(2019); pp536–547.
- Coyle, R., Hardiman, G., & Driscoll, K. O. (2020). Microplastics in the marine environment: A review of their sources, distribution processes, uptake and exchange in ecosystems. *Case Studies in Chemical and Environmental Engineering*, **2**(2020); pp1–7.
- Crutchik, D., Franchi, O., Caminos, L., Jeison, D., Belmonte, M., Pedrouso, A., Val del Rio, A., Mosquera-Corral, A., & Campos, J. L. (2020). Polyhydroxyalkanoates (PHAs) production: A feasible economic option for the treatment of sewage sludge in municipal wastewater treatment plants? *Water*, **12**(4); pp1–12.
- De Koning, G. J. M., & Witholt, B. (1997). A process for the recovery of poly(hydroxyalkanoates) from *pseudomonads* part 1: Solubilization. *Bioprocess Engineering*, **17**(1); pp7–13.
- de Oliveira, R. D., Novello, V., da Silva, L. F., Gomez, J. G. C., & Le Roux, G. A. C. (2021). Glucose metabolism in *Pseudomonas aeruginosa* is cyclic when producing Polyhydroxyalkanoates and Rhamnolipids—*Journal of Biotechnology*, **342**(2021); pp54–63.

- Díaz-Mendoza, C., Mouthon-Bello, J., Pérez-Herrera, N. L., & Escobar-Díaz, S. M. (2020). Plastics and microplastics, effects on marine coastal areas: a review. *Environmental Science and Pollution Research*, **27**(32); pp39913–39922.
- Du, C., Sabirova, J., Soetaert, W., & Ki Carol Lin, S. (2012). Polyhydroxyalkanoates Production From Low-cost Sustainable Raw Materials. *Current Chemical Biology*, **6**(1); pp14–25.
- Dutt Tripathi, A., Paul, V., Agarwal, A., Sharma, R., Hashempour-Baltork, F., Rashidi, L., & Khosravi Darani, K. (2021). Production of polyhydroxyalkanoates using dairy processing waste – A review. *Bioresource Technology*, **326**(2020); pp1-12.
- El-malek, F. A., Farag, A., Omar, S., & Khairy, H. (2020). Polyhydroxyalkanoates (PHA) from *Halomonas pacifica* ASL10 and *Halomonas salifodiane* ASL11 were isolated from Mariout salt lakes. *International Journal of Biological Macromolecules*, **161**(2020); pp1318–1328.
- Evode, N., Qamar, S. A., Bilal, M., Barceló, D., & Iqbal, H. M. N. (2021). Plastic waste and its management strategies for environmental sustainability. *Case Studies in Chemical and Environmental Engineering*, **4**(2021); pp1–8.
- Ezgi Bezirhan Arikan, & Havva Duygu Ozsoy. (2015). A Review: Investigation of Bioplastics. *Journal of Civil Engineering and Architecture*, **9**(2); pp188–192.
- Favaro, L., Basaglia, M., & Casella, S. (2019). Improving polyhydroxyalkanoate production from inexpensive carbon sources by genetic approaches: a review. *Biofuels, Bioproducts and Biorefining*, **13**(1); pp208–227.
- Favaro, L., Basaglia, M., Rodriguez, J. E. G., Morelli, A., Ibraheem, O., Pizzocchero, V., & Casella, S. (2019). Bacterial production of PHAs from lipid-rich by-products. *Applied Food Biotechnology*, **6**(1); pp45–52.
- Fernández, D., Rodríguez, E., Bassas, M., Viñas, M., Solanas, A. M., Llorens, J., Marqués, A. M., & Manresa, A. (2005). Agro-industrial oily wastes as substrates for PHA production by the new strain *Pseudomonas aeruginosa*

- NCIB 40045: Effect of culture conditions. *Biochemical Engineering Journal*, **26**(2–3); pp159–167.
- Follonier, S., Goyder, M. S., Silvestri, A. C., Crelier, S., Kalman, F., Riesen, R., & Zinn, M. (2014). Fruit pomace and waste frying oil as sustainable resources for the bioproduction of medium-chain-length polyhydroxyalkanoates. *International Journal of Biological Macromolecules*, **71**(2014); pp42–52.
- G. Braunegg, B. Sonnleimer, and R. M. L., & Tnstitut. (1978). A Rapid Gas Chromatographic Method for the Determination of Poly- γ -hydroxybutyric Acid in Microbial Biomass. *European J. Appl. Microbiol. Biotechnol.*, **6**(2); pp29–37.
- Gahlawat, G., & Soni, S. K. (2017). Bioresource Technology Valorization of waste glycerol for the production of poly copolymer by *Cupriavidus necator* and extraction in a sustainable manner. *Bioresource Technology*, **243**(2017); ppp492–501.
- Gahlawat, G., & Srivastava, A. K. (2017). Model-Based Nutrient Feeding Strategies for the Increased Production of Polyhydroxybutyrate (PHB) by *Alcaligenes latus*. *Applied Biochemistry and Biotechnology*, **183**(2); pp530–542.
- Ganesh, R., Kyung, S., Dattatraya, G., Ghodake, G. S., Naresh, R., Su, D., Nair, S., & Seung, H. (2021). Efficient bioconversion of sugarcane bagasse into polyhydroxybutyrate (PHB) by *Lysinibacillus* sp . and its characterization. *Bioresource Technology*, **324**(2021); pp1–6.
- Ganesh Saratale, R., Cho, S. K., Dattatraya Saratale, G., Kadam, A. A., Ghodake, G. S., Kumar, M., Naresh Bharagava, R., Kumar, G., Su Kim, D., Mulla, S. I., & Seung Shin, H. (2021). A comprehensive overview and recent advances on polyhydroxyalkanoates (PHA) production using various organic waste streams. In *Bioresource Technology* **325**(2021); pp1-15.
- Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, **3**(7); pp25–29.

- Gobi, K., & Vadivelu, V. M. (2015). Polyhydroxyalkanoate recovery and effect of in situ extracellular polymeric substances removal from aerobic granules. *Bioresource Technology*, **189**(2015); pp169–176.
- Godbole, S. (2016). Methods for identification, quantification, and characterization of Polyhydroxyalkanoates - A review. *Research Journal of Biotechnology*, **5**(4); pp4977–4983.
- Gomaa, E. Z. (2014). Production of Polyhydroxyalkanoates (PHAs) By *Bacillus subtilis* and *Escherichia coli* Grown on Cane Molasses Fortified with Ethanol. *Braz. Arch. Biol. Technol.*, **57**(1); pp145–154.
- Gonzalez, K., Navia, R., Liu, S., & Cea, M. (2021). Biological Approaches in Polyhydroxyalkanoates Recovery. *Current Microbiology*, **78**(1); pp1–10.
- Grigore, M. E. (2017). Methods of recycling, properties and applications of recycled thermoplastic polymers. *Recycling*, **2**(4); pp1–11.
- Grigore, M. E., Grigorescu, R. M., Iancu, L., Ion, M., Zaharia, C., Andrei, E. R., Elena, M., Grigorescu, R. M., Iancu, L., Ion, M., Zaharia, C., Ramona, E., & Methods, A. (2019). Methods of synthesis, properties and biomedical applications of polyhydroxyalkanoates : a review. *Journal of Biomaterials Science, Polymer Edition*, **30**(9); pp695–712.
- Gumel, A. M., Annuar, M. S. M., & Chisti, Y. (2013). Recent Advances in the Production, Recovery, and Applications of Polyhydroxyalkanoates. *Journal of Polymers and the Environment*, **21**(2); pp580–605.
- Gutschmann, B., Bock, M. C. E., Jahns, S., Neubauer, P., Brigham, C. J., & Riedel, S. L. (2021). Untargeted metabolomics analysis of *Ralstonia eutropha* during plant oil cultivations reveals the presence of a fucose salvage pathway. *Scientific Reports*, **11**(1); pp1–12.
- Guzik, M. W., Duane, G. F., Kenny, S. T., Casey, E., Mielcarek, P., Wojnarowska, M., & O'Connor, K. E. (2022). A polyhydroxyalkanoates bioprocess improvement case study based on four fed-batch feeding strategies. *Microbial Biotechnology*, **15**(3); pp996–1006.

- Hasan Anik, A., Hossain, S., Alam, M., Binte Sultan, M., Hasnine, M. T., & Rahman, M. M. (2021). Microplastics pollution: A comprehensive review on the sources, fates, effects, and potential remediation. *Environmental Nanotechnology, Monitoring and Management*, **16**(2021); pp1–12.
- Hong, K., Sun, S., Tian, W., Chen, G. Q., & Huang, W. (1999). A rapid method for detecting bacterial polyhydroxyalkanoates in intact cells by Fourier transform infrared spectroscopy. *Applied Microbiology and Biotechnology*, **51**(4); pp523–526.
- Hopewell, J., Dvorak, R., & Kosior, E. (2009). Plastics recycling: Challenges and opportunities. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **364**(1526); pp2115–2126.
- Huang, T. Y., Duan, K. J., Huang, S. Y., & Chen, C. W. (2006). Production of polyhydroxyalkanoates from inexpensive extruded rice bran and starch by *Haloferax mediterranei*. *Journal of Industrial Microbiology and Biotechnology*, **33**(8); pp701–706.
- Huschner, F., Grousseau, E., Brigham, C. J., Plassmeier, J., Popovic, M., Rha, C., & Sinskey, A. J. (2015). Development of a feeding strategy for high cell and PHA density fed-batch fermentation of *Ralstonia eutropha* H16 from organic acids and their salts. *Process Biochemistry*, **50**(2); pp165–172.
- Idris Zainab-L, Hiroshi Uyama, Cong Li, Yehua Shen, and K. S. (2018). Production of Polyhydroxyalkanoates From Underutilized Plant Oils by *Cupriavidus necator*. *Clean Soil, Air, Water*, **46**(11); pp1–9.
- Ilham, M., Nakanomori, S., Kihara, T., Hokamura, A., Matsusaki, H., Tsuge, T., & Mizuno, K. (2014). Characterization of polyhydroxyalkanoate synthases from *Halomonas* sp. O-1 and *Halomonas elongata* DSM2581: Site-directed mutagenesis and recombinant expression. *Polymer Degradation and Stability*, **109**(2014); pp416–423.
- Ingram, H. R., & Winterburn, J. B. (2021). Anabolism of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Cupriavidus necator* DSM 545 from spent coffee

- grounds oil. *New Biotechnology*, **60**(2020); pp12–19.
- Iñiguez, M. E., Conesa, J. A., & Fullana, A. (2016). Marine debris occurrence and treatment: A review. *Renewable and Sustainable Energy Reviews*, **64**(2016); pp394–402.
- Iñiguez, M. E., Conesa, J. A., & Fullana, A. (2018). Recyclability of four types of plastics exposed to UV irradiation in a marine environment. *Waste Management*, **79**(2018); pp339–345.
- Isak, I., Patel, M., Riddell, M., West, M., Bowers, T., Wijeyekoon, S., & Lloyd, J. (2016). Quantification of polyhydroxyalkanoates in mixed and pure cultures biomass by Fourier transform infrared spectroscopy: comparison of different approaches. *Letters in Applied Microbiology*, **63**(2); pp139–146.
- Jacquel, N., Lo, C. W., Wei, Y. H., Wu, H. S., & Wang, S. S. (2008). Isolation and purification of bacterial poly(3-hydroxyalkanoates). *Biochemical Engineering Journal*, **39**(1); pp15–27.
- Jain, R., & Tiwari, A. (2015). Biosynthesis of planet-friendly bioplastics using renewable carbon sources. *Journal of Environmental Health Science and Engineering*, **13**(1); pp3–8.
- Jambeck, J. (2016). Plastic Waste Inputs from Land into the Ocean United Nations Open-ended Informal Consultative Process on Oceans and the Law of the Sea. **6**(2016); pp13–17.
- Jambunathan, P., & Zhang, K. (2016). Engineered biosynthesis of biodegradable polymers. *Journal of Industrial Microbiology and Biotechnology*, **43**(8); pp1037–1058.
- Javaid, H., Nawaz, A., Riaz, N., Mukhtar, H., Ullah, K., Manzoor, R., Kaleem, I., & Murtaza, G. (2020). Valorization of Biomass and Synthetic Waste. *Molecules*, **25**(23); pp5528-5539.
- Jawed, K., Irorere, V. U., Bommareddy, R. R., Minton, N. P., & Kovács, K. (2022). Establishing Mixotrophic Growth of *Cupriavidus necator* H16 on CO₂ and

- Volatile Fatty Acids. *Fermentation*, **8**(3); pp1–15.
- Jeon, J. M., Park, S. J., Son, Y. S., Yang, Y. H., & Yoon, J. J. (2022). Bioconversion of Mixed Alkanes to Polyhydroxyalkanoate by *Pseudomonas resinovornas*: Upcycling of Pyrolysis Oil from Waste-Plastic. *Polymers*, **14**(13); pp1–11.
- Jessica A. Deaver and Sudeep C. Papat. (2022). Fats, Oils, and Grease (FOG): Opportunities, Challenges, and Economic Approaches. In Eduardo_Jacob-Lopes, Leila_Queiroz_Zepka, & Mariany_Costa_Deprá (Eds), *Handbook of Waste Biorefinery* (22nd ed). Springer. <https://doi.org/10.1007/978-3-031-06562-0>
- Jian, J., Xiangbin, Z., & Xianbo, H. (2020). An overview on synthesis, properties, and applications of poly(butylene-adipate-co-terephthalate)–PBAT. *Advanced Industrial and Engineering Polymer Research*, **3**(1); pp19–26.
- Jiang, G., Hill, D. J., Kowalczyk, M., Johnston, B., Adamus, G., Irerere, V., & Radecka, I. (2016). Carbon Sources for Polyhydroxyalkanoates and an Integrated Biorefinery. *International Journal of Molecular Sciences*, **17**(7); pp1–21.
- Jiang, G., Johnston, B., Townrow, D. E., Radecka, I., Koller, M., Chaber, P., Adamus, G., & Kowalczyk, M. (2018). Biomass extraction using non-chlorinated solvents for biocompatibility improvement of polyhydroxyalkanoates. *Polymers*, **10**(7); pp1-14.
- Jiang, Y., Hebly, M., Kleerebezem, R., Muyzer, G., & van Loosdrecht, M. C. M. (2011). Metabolic modeling of mixed substrate uptake for polyhydroxyalkanoate (PHA) production. *Water Research*, **45**(3); pp1309–1321.
- Johnston, B., Radecka, I., Hill, D., Chiellini, E., Ilieva, V. I., Sikorska, W., Musioł, M., Zi, M., Marek, A. A., & Id, D. K. (2018). The Microbial Production of Polyhydroxyalkanoates from Waste Polystyrene Fragments Attained Using Oxidative Degradation. *Polymers*, **10**(9); pp1–22.

- Kanavaki, I., Drakonaki, A., Geladas, E. D., Spyros, A., Xie, H., & Tsiotis, G. (2021). Polyhydroxyalkanoate (Pha) production in *pseudomonas* sp. phdv1 strain grown on phenol as carbon sources. *Microorganisms*, **9**(8); pp1–11.
- Kansiz, M., Domínguez-Vidal, A., McNaughton, D., & Lendl, B. (2007). Fourier-transform infrared (FTIR) spectroscopy for monitoring and determining the degree of crystallisation of polyhydroxyalkanoates (PHAs). *Analytical and Bioanalytical Chemistry*, **388**(5–6); pp1207–1213.
- Karine Laste Macagnan, Mariane Igansi Alves, Sonia Maria Kesserlingh, Claire Tondo Vendruscolo, A. da S. M. (2021). Environmentally friendly method for poly (3-hydroxybutyrate) recovery based on physical adsorption on a solid inorganic inert adsorbent. **256**(2020); pp1–9.
- Kaur, L., Khajuria, R., Parihar, L., & Dimpal Singh, G. (2017). Polyhydroxyalkanoates: Biosynthesis to commercial production- A review. *Journal of Microbiology, Biotechnology and Food Sciences*, **6**(4); pp1098–1106.
- Keenan, T. M., Nakas, J. P., & Tanenbaum, S. W. (2006). Polyhydroxyalkanoate copolymers from forest biomass. *Journal of Industrial Microbiology and Biotechnology*, **33**(7); pp616–626.
- Kerketta, A., & Vasanth, D. (2019). Madhuca indica flower extract as a cheaper carbon source for the production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) using *Ralstonia eutropha*. *Process Biochemistry*, **87**(2019); pp1–9.
- Keshavarz, T., & Roy, I. (2010). Polyhydroxyalkanoates : bioplastics with a green agenda. *Current Opinion in Microbiology*, **13**(3); pp321–326.
- Khanna, S., & Srivastava, A. K. (2005). Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry*, **40**(2); pp607–619.
- Khatami, K., Perez-Zabaleta, M., & Cetecioglu, Z. (2022). Pure cultures for synthetic culture development: Next level municipal waste treatment for polyhydroxyalkanoates production. *Journal of Environmental Management*,

305(2021); pp1–9.

- Khatami, K., Perez-Zabaleta, M., Owusu-Agyeman, I., & Cetecioglu, Z. (2021). Waste to bioplastics: How close are we to sustainable polyhydroxyalkanoates production? *Waste Management*, **119**(2021); pp374–388.
- Khomlaem, C., Aloui, H., Singhvi, M., & Kim, B. S. (2023). Production of polyhydroxyalkanoates and astaxanthin from lignocellulosic biomass in high cell density membrane bioreactor. *Chemical Engineering Journal*, **451**(2); pp1–13.
- Kim, B. S. (2002). Production of medium chain length polyhydroxyalkanoates by fed-batch culture of *Pseudomonas oleovorans*. *Biotechnology Letters*, **24**(2); pp125–130.
- Kingsly, J. S., Chathalingath, N., Parthiban, S. A., Sivakumar, D., Sabtharishi, S., Senniyappan, V., Duraisamy, V. S., H, A. J., & Gunasekar, A. (2022). Utilization of sugarcane molasses as the main carbon source for the production of polyhydroxyalkanoates from *Enterobacter cloacae*. *Energy Nexus*, **6**(2); pp1–6.
- Koller, M. (2020). Established and advanced approaches for the recovery of microbial polyhydroxyalkanoate (PHA) biopolyesters from surrounding microbial biomass. **4**(3); pp113–126.
- Koller, M., Bona, R., Chiellini, E., Fernandes, E. G., Horvat, P., Kutschera, C., Hesse, P., & Braunegg, G. (2008). Polyhydroxyalkanoate production from whey by *Pseudomonas hydrogenovora*. *Bioresource Technology*, **99**(11); pp4854–4863.
- Koller, M., & Braunegg, G. (2018). Advanced approaches to produce polyhydroxyalkanoate (PHA) biopolyesters in a sustainable and economic fashion. *The EuroBiotech Journal*, **2**(2); pp89–103.
- Koller, M., Maršálek, L., de Sousa Dias, M. M., & Braunegg, G. (2017). Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable

- manner. *New Biotechnology*, **37**(2017); pp24–38.
- Koller, M., Niebelschütz, H., & Braunegg, G. (2013). Strategies for recovery and purification of poly[(R)-3-hydroxyalkanoates] (PHA) biopolyesters from surrounding biomass. *Engineering in Life Sciences*, **13**(6); pp549–562.
- Koller, M., Salerno, A., Muhr, A., Reiterer, A., & Braunegg, G. (2013). Polyhydroxyalkanoates: Biodegradable polymers and plastics from renewable resources. *Materiali in Tehnologije*, **47**(1); pp5–12.
- Kondratyev, V., Goryacheva, D., Nepomnyaschiy, A., Zubkov, I., Shishlyannikov, S., & Sorokoumov, P. (2022). Quantitative analysis of medium-chain polyhydroxyalkanoates in bacterial cells via gas chromatography-mass spectrometry: classical method revision and optimization. *International Journal of Polymer Analysis and Characterization*, **27**(1); pp32–42.
- Kosseva, M. R., & Rusbandi, E. (2018). Trends in the biomanufacturing of polyhydroxyalkanoates with a focus on downstream processing. *International Journal of Biological Macromolecules*, **107**(2018); pp762–778.
- Kourmentza, C., Costa, J., Azevedo, Z., Servin, C., Grandfils, C., De Freitas, V., & Reis, M. A. M. (2018). *Burkholderia thailandensis* as a microbial cell factory for the bioconversion of used cooking oil to polyhydroxyalkanoates and rhamnolipids. *Bioresource Technology*, **247**(2017); pp829–837.
- Kourmentza, C., Ntaikou, I., Lyberatos, G., & Kornaros, M. (2015). Polyhydroxyalkanoates from *Pseudomonas* sp. using synthetic and olive mill wastewater under limiting conditions. *International Journal of Biological Macromolecules*, **74**(2015); pp202–210.
- Kourmentza, C., Pl, J., Venetsaneas, N., Burniol-figols, A., Varrone, C., Gavala, H. N., & Reis, M. A. M. (2017). Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production. **4**(55); pp1–43.
- Koushal, V., Sharma, R., Sharma, M., Sharma, R., & Sharma, V. (2014). Plastics : Issues Challenges and Remediation. *International Journal of Waste Resources*, **4**(1); pp1–6.

- Kulkarni, S. O., Kanekar, P. P., Jog, J. P., Sarnaik, S. S., & Nilegaonkar, S. S. (2015). Production of copolymer, poly (hydroxybutyrate-co-hydroxyvalerate) by *Halomonas campisalis* MCM B-1027 using agro-wastes. *International Journal of Biological Macromolecules*, **72**(2015); pp784–789.
- Kumar, M., Rathour, R., Singh, R., Sun, Y., Pandey, A., Gnansounou, E., Andrew Lin, K. Y., Tsang, D. C. W., & Thakur, I. S. (2020). Bacterial polyhydroxyalkanoates: Opportunities, challenges, and prospects. *Journal of Cleaner Production*, **263**(2020); pp1-15.
- Kumar, P., & Kim, B. S. (2018). Valorization of polyhydroxyalkanoates production process by co-synthesis of value-added products. *Bioresource Technology*, **269**(2018); pp544–556.
- Kunasundari, B., & Sudesh, K. (2011). Isolation and recovery of microbial polyhydroxyalkanoates. **5**(7); pp620–634.
- Lalucat, J., Gomila, M., Mulet, M., Zaruma, A., & García-Valdés, E. (2022). Past, present and future of the boundaries of the *Pseudomonas* genus: Proposal of Stutzerimonas gen. Nov. *Systematic and Applied Microbiology*, **45**(1); pp1–16.
- Lam, S. S., Liew, R. K., Jusoh, A., Chong, C. T., Ani, F. N., & Chase, H. A. (2016). Progress in waste oil to sustainable energy, with emphasis on pyrolysis techniques. In *Renewable and Sustainable Energy Reviews*. **53**(2016); pp741–753.
- Law, K. L., & Narayan, R. (2022). Reducing environmental plastic pollution by designing polymer materials for managed end-of-life. *Nature Reviews Materials*, **7**(2); pp104–116.
- Laycock, B., Halley, P., Pratt, S., Werker, A., & Lant, P. (2013). The chemomechanical properties of microbial polyhydroxyalkanoates. *Progress in Polymer Science*, **38**(3–4); pp536–583.
- Lebreton, L., & Andrady, A. (2019). Future scenarios of global plastic waste generation and disposal. *Palgrave Communications*, **5**(1); pp1–11.

- Lee, D. S., Noh, B. S., Bae, S. Y., & Kim, K. (1998). Characterization of fatty acids composition in vegetable oils by gas chromatography and chemometrics. *Analytica Chimica Acta*, **358**(2); pp163–175.
- Lee, S. Y. (2000). Bacterial polyhydroxyalkanoates. *Biotechnology and Bioengineering*, **49**(1); pp1–14.
- Lee, S. Y., Wong, H. H., Choi, J. Il, Lee, S. H., Lee, S. C., & Han, C. S. (2000). Production of medium-chain-length polyhydroxyalkanoates by high-cell-density cultivation *Pseudomonas putida* under phosphorus limitation. *Biotechnology and Bioengineering*, **68**(4); pp466–470.
- Leong, Y. K., Show, P. L., Lan, J. C. W., Loh, H. S., Lam, H. L., & Ling, T. C. (2017). Economic and environmental analysis of PHAs production process. *Clean Technologies and Environmental Policy*, **19**(7); pp1941–1953.
- Leong, Y. K., Show, P. L., Lan, J. C. W., Loh, H. S., Yap, Y. J., & Ling, T. C. (2017). Extraction and purification of Polyhydroxyalkanoates (PHAs): application of Thermoseparating aqueous two-phase extraction. *Journal of Polymer Research*, **24**(10); pp1–10.
- Liew, Y. X., Chan, Y. J., Manickam, S., Chong, M. F., Chong, S., Tiong, T. J., Lim, J. W., & Pan, G. T. (2020). Enzymatic pretreatment to enhance anaerobic bioconversion of high strength wastewater to biogas: A review. *Science of the Total Environment*, **713**(2020); pp1363-1373.
- Lin, L., Cheng, Y., Pu, Y., Sun, S., Li, X., Jin, M., Pierson, E. A., Gross, D. C., Dale, B. E., Dai, S. Y., Ragauskas, A. J., & Yuan, J. S. (2016). Systems biology-guided biodesign of consolidated lignin conversion. *Green Chemistry*, **18**(20); pp5536–5547.
- Liu, C., Wang, X., Yang, H., Liu, C., Zhang, Z., & Chen, G. (2021). Biodegradable polyhydroxyalkanoates production from wheat straw by recombinant *Halomonas elongata* A1. *International Journal of Biological Macromolecules*, **187**(3); pp675–682.
- Liu, H., Chen, Y., Zhang, Y., Zhao, W., Guo, H., Wang, S., Xia, W., Wang, S.,

- Liu, R., & Yang, C. (2022). Enhanced production of polyhydroxyalkanoates in *Pseudomonas putida* KT2440 by a combination of genome streamlining and promoter engineering. *International Journal of Biological Macromolecules*, **209**(2022); pp117–124.
- Liu, M. H., Chen, Y. J., & Lee, C. Y. (2018). Characterization of medium-chain-length polyhydroxyalkanoate biosynthesis by *Pseudomonas mosselii* TO7 using crude glycerol. *Bioscience, Biotechnology and Biochemistry*, **82**(3); pp532–539.
- Liu, X., & Yu, W. (2006). Evaluating the thermal stability of high-performance fibers by TGA. *Journal of Applied Polymer Science*, **99**(3); pp937–944.
- Loan, T. T., Trang, D. T. Q., Huy, P. Q., Ninh, P. X., & Van Thuoc, D. (2022). A fermentation process for the production of poly(3-hydroxybutyrate) using waste cooking oil or waste fish oil as an inexpensive carbon substrate. *Biotechnology Reports*, **33**(2021); pp1–11.
- Lopar, M., Vrana Špoljarić, I., Atlić, A., Koller, M., Braunegg, G., & Horvat, P. (2013). Five-step continuous production of PHB analyzed by elementary flux, modes, yield space analysis, and highly structured metabolic model. *Biochemical Engineering Journal*, **79**(2013); pp57–70.
- Lorini, L., Martinelli, A., Pavan, P., Majone, M., & Valentino, F. (2021). Downstream processing and characterization of polyhydroxyalkanoates (PHAs) produced by mixed microbial culture (MMC) and organic urban waste as substrate. *Biomass Conversion and Biorefinery*, **11**(2021); pp693–703.
- Luef, K. P., Stelzer, F., & Wiesbrock, F. (2015). Poly (hydroxy alkanoate) in Medical Applications. **29**(2); pp287–297.
- Luengo, J. M., García, B., Sandoval, A., Naharro, G., & Olivera, E. R. (2003). Bioplastics from microorganisms. *Current Opinion in Microbiology*, **6**(3); pp251–260.
- Lumpur, U. K., Loo, C. Y., & Nomura, C. T. (2008). Biosynthesis of polyhydroxyalkanoate copolymers from mixtures of plant oils and 3-

- hydroxyvalerate precursors. *Bioresource Technology*, **99**(15); pp6844–6851.
- Luo, X., Ge, X., Cui, S., & Li, Y. (2016). Value-added processing of crude glycerol into chemicals and polymers. *Bioresource Technology*, **215**(2016); pp144–154.
- Ma, H., Zhao, Y., Huang, W., Zhang, L., Wu, F., Ye, J., & Chen, G. Q. (2020). Rational flux-tuning of *Halomonas bluephagenesis* for co-production of bioplastic PHB and ectoine. *Nature Communications*, **11**(1); pp1–12.
- Ma, Y., Zheng, X., Lin, Y., Zhang, L., Yuan, Y., Wang, H., Winterburn, J., Wu, F., Wu, Q., Ye, J. W., & Chen, G. Q. (2022). Engineering an oleic acid-induced system for *Halomonas*, *E. coli*, and *Pseudomonas*. *Metabolic Engineering*, **72**(2022); pp325–336.
- Madkour, M. H., Heinrich, D., Alghamdi, M. A., Shabbaj, I. I., & Steinbüchel A. (2013). PHA Recovery from Biomass. *Biomacromolecules*, **14**(9); pp2963–2972.
- Madkour, M. H., Heinrich, D., Alghamdi, M. A., Shabbaj, I. I., & Steinbüchel, A. (2013). PHA recovery from biomass. *Biomacromolecules*, **14**(9); pp2963–2972.
- Mahato, R. P., Kumar, S., & Singh, P. (2021). Optimization of Growth Conditions to Produce Sustainable Polyhydroxyalkanoate Bioplastic by *Pseudomonas aeruginosa* EO1. *Frontiers in Microbiology*, **12**(2021); 1–9.
- Mahesh, S. E., Ramanathan, A., Begum, K. M. M. S., & Narayanan, A. (2015). Biodiesel production from waste cooking oil using KBr-impregnated CaO as a catalyst. *Energy Conversion and Management*, **91**(2015); pp442–450.
- Mannina, G., Presti, D., Montiel-Jarillo, G., Carrera, J., & Suárez-Ojeda, M. E. (2020). Recovery of polyhydroxyalkanoates (PHAs) from wastewater: A review. *Bioresource Technology*, **297**(2019); pp1–12.
- Mannina, G., Presti, D., Montiel-Jarillo, G., & Suárez-Ojeda, M. E. (2019).

- Bioplastic recovery from wastewater: A new protocol for polyhydroxyalkanoates (PHA) extraction from mixed microbial cultures. *Bioresource Technology*, **282**(2019); pp361–369.
- Manoli, M. T., Nogales, J., & Prieto, A. (2022). Synthetic Control of Metabolic States in *Pseudomonas putida* by Tuning Polyhydroxyalkanoate Cycle. *American Society for Microbiology*, **13**(1); pp1–19.
- Marchetti, R., Vasmara, C., Bertin, L., & Fiume, F. (2020). Conversion of waste cooking oil into biogas: perspectives and limits. *Applied Microbiology and Biotechnology*, **104**(7); pp2833–2856.
- Marriott, P. J., Shellie, R., & Cornwell, C. (2001). Gas chromatographic technologies for the analysis of essential oils. *Journal of Chromatography A*, **936**(1–2); pp1–22.
- Martin Koller, Aid Atlić, Miguel Dias, A. R., & Braunegg, and G. (2010). Microbial PHA Production from Waste Raw Materials. In D. G.-Q. Chen (Ed.), *Microbiology Monographs*, **13**(2010); pp85–119.
- Mat Yunus, A. M., Ahmad Parveez, G. K., & Ho, C. L. (2008). Review: Transgenic plants producing polyhydroxyalkanoates. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, **16**(1); pp1–10.
- May, C. Y., & Nesaretnam, K. (2014). Research advancements in palm oil nutrition. *European Journal of Lipid Science and Technology*, **116**(10); pp1301–1315.
- Mba, O. I., Dumont, M. J., & Ngadi, M. (2015). Palm oil: Processing, characterization, and utilization in the food industry - A review. *Food Bioscience*, **10**(2015); pp26–41.
- Mendonça, T. T., Gomez, J. G. C., Buffoni, E., Sánchez Rodriguez, R. J., Schripsema, J., Lopes, M. S. G., & Silva, L. F. (2014). Exploring the potential of *Burkholderia sacchari* to produce polyhydroxyalkanoates. *Journal of Applied Microbiology*, **116**(4); pp815–829.

- Meng, D., Miao, C., Liu, Y., Wang, F., Chen, L., Huang, Z., Fan, X., Gu, P., & Li, Q. (2022). Metabolic engineering for the biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from glucose and propionic acid in recombinant *Escherichia coli*. *Bioresource Technology*, **348**(2); pp1–9.
- Mezzina, M. P., Wetzler, D. E., de Almeida, A., Dinjaski, N., Prieto, M. A., & Pettinari, M. J. (2015). A phasing with extra talents: A polyhydroxyalkanoate granule-associated protein has chaperone activity. *Environmental Microbiology*, **17**(5); pp1765–1776.
- Mielanczyk, L., Matysiak, N., Michalski, M., Buldak, R., & Wojnicz, R. (2014). Closer to the native state. Critical evaluation of cryo-techniques for Transmission Electron Microscopy: Preparation of biological samples. *Folia Histochemica et Cytobiologica*, **52**(1); pp1–17.
- Millán, M., Segura, D., Galindo, E., & Peña, C. (2016). Molecular mass of poly-3-hydroxybutyrate (P3HB) produced by *Azotobacter vinelandii* is determined by the ratio of synthesis and degradation under fixed dissolved oxygen tension. *Process Biochemistry*, **51**(8); pp950–958.
- Mohammad Bakri Alaa Hammami, Eman Qasem Mohammed, Anas Mohammad Hashem, Mina Amer Al-Khafaji, Fatima Alqahtani, S. A. & N. D. (2017). Survey on awareness and attitudes of secondary school students regarding plastic pollution: implications for environmental education and public health in Sharjah city, UAE. *Environmental Science and Pollution Research*, **24**(25); pp20626–20633.
- Mohammed, S., & Ray, L. (2022). Polyhydroxyalkanoate recovery from newly screened *Bacillus* sp. LPPI-18 using various methods of extraction from the Loktak Lake sediment sample. *Journal of Genetic Engineering and Biotechnology*, **20**(1), 1–20.
- Mohan Nair, A., Annamalai, K., Kamala Kannan, S., & Kuppusamy, S. (2013). Utilization of sugarcane molasses for the production of polyhydroxyalkanoates using *Bacillus subtilis*. *Malaya Journal of*

- Biosciences*, **2014**(1); pp24–30.
- Moita, R., Freches, A., & Lemos, P. C. (2014). ScienceDirect Crude glycerol as feedstock for polyhydroxyalkanoates production by mixed microbial cultures. *Water Research*, **58**(2014); pp9–20.
- Mok, P. S., Chuah, J. A., Najimudin, N., Liew, P. W. Y., Jong, B. C., & Sudesh, K. (2021). In vivo characterization and application of the pha synthase from *azotobacter vinelandii* for the biosynthesis of polyhydroxyalkanoate containing 4-hydroxybutyrate. *Polymers*, **13**(10); pp1-11.
- Mok, P. S., Sudesh, K., Liew, P. W. Y., Jong, B. C., & Najimudin, N. (2017). Characterization of polyhydroxyalkanoate production by mutant *Azotobacter vinelandii*. *Malaysian Applied Biology*, **46**(1); pp93–100.
- Montoya, C., Lopes, R., Flori, A., Cros, D., Cuellar, T., Summo, M., Espeout, S., Rivallan, R., Risterucci, A. M., Bittencourt, D., Zambrano, J. R., Alarcón G, W. H., Villeneuve, P., Pina, M., Nouy, B., Amblard, P., Ritter, E., Leroy, T., & Billotte, N. (2013). Quantitative trait loci (QTLs) analysis of palm oil fatty acid composition in an interspecific pseudo-backcross from *Elaeis oleifera* (H.B.K.) Cortés and oil palm (*Elaeis guineensis* Jacq.). *Tree Genetics and Genomes*, **9**(5); pp1207–1225.
- Moralejo-Gárate, H., Mar'Atusalihat, E., Kleerebezem, R., & Van Loosdrecht, M. C. M. (2011). Microbial community engineering for biopolymer production from glycerol. *Applied Microbiology and Biotechnology*, **92**(3); pp631–639.
- Możejko-Ciesielska, J., & Kiewisz, R. (2016). Bacterial polyhydroxyalkanoates: Still fabulous? *Microbiological Research*, **192**(2016); pp271–282.
- Możejko-Ciesielska, J., Szacherska, K., & Marciniak, P. (2019). *Pseudomonas* Species as Producers of Eco-friendly Polyhydroxyalkanoates. *Journal of Polymers and the Environment*, **27**(6); pp1151–1166.
- Możejko, J., & Ciesielski, S. (2013). Saponified waste palm oil is an attractive renewable resource for mcl-polyhydroxyalkanoate synthesis. *Journal of Bioscience and Bioengineering*, **116**(4); pp485–492.

- Muhr, A., Rechberger, E. M., Salerno, A., Reiterer, A., Malli, K., Strohmeier, K., Schober, S., Mittelbach, M., & Koller, M. (2013). Novel Description of mcl-PHA Biosynthesis by *Pseudomonas chlororaphis* from Animal-Derived Waste. *Journal of Biotechnology*, **165**(1); pp45–51.
- Muneer, F., Rasul, I., Azeem, F., Siddique, M. H., Zubair, M., & Nadeem, H. (2020). Microbial Polyhydroxyalkanoates (PHAs): Efficient Replacement of Synthetic Polymers. *Journal of Polymers and the Environment*, **28**(9); pp2301–2323.
- Muneer, F., Rasul, I., Qasim, M., Sajid, A., & Nadeem, H. (2022). Optimization, Production, and Characterization of Polyhydroxyalkanoate (PHA) from Indigenously Isolated Novel Bacteria. *Journal of Polymers and the Environment*, **30**(8); pp3523–3533.
- Nanda, S., Patra, B. R., Patel, R., Bakos, J., & Dalai, A. K. (2022). Innovations in applications and prospects of bioplastics and biopolymers: a review. *Environmental Chemistry Letters*, **20**(1); pp379–395.
- Ncube, L. K., Ude, A. U., Ogunmuyiwa, E. N., Zulkifli, R., & Beas, I. N. (2021). An overview of plastic waste generation and management in food packaging industries. *Recycling*, **6**(1); pp1–25.
- Nielsen, C., Rahman, A., Rehman, A. U., Walsh, M. K., & Miller, C. D. (2017). Food waste conversion to microbial polyhydroxyalkanoates. *Microb Biotechnol*, **10**(6); pp1338–1352.
- Nikolaidis, M., Mossialos, D., Oliver, S. G., & Amoutzias, G. D. (2020). Comparative analysis of the core proteomes among the *Pseudomonas* major evolutionary groups reveals species-specific adaptations for *Pseudomonas aeruginosa* and *Pseudomonas chlororaphis*. *Diversity*, **12**(8); pp1–21.
- Novackova, I., Hrabalova, V., Slaninova, E., Sedlacek, P., Samek, O., Koller, M., Krzyzanek, V., Hrubanova, K., Mrazova, K., Nebesarova, J., & Obruca, S. (2022). The role of polyhydroxyalkanoates in the adaptation of *Cupriavidus necator* to osmotic pressure and high concentration of copper ions.

International Journal of Biological Macromolecules, **206**(2); pp977–989.

- Obruca, S., Benesova, P., Marsalek, L., & Marova, I. (2015). Use of lignocellulosic materials for PHA production. *Chemical and Biochemical Engineering Quarterly*, **29**(2); pp135–144.
- Obruca, S., Marova, I., Melusova, S., & Mravcova, L. (2011). Production of polyhydroxyalkanoates from cheese whey employing *Bacillus megaterium* CCM 2037. *Annals of Microbiology*, **61**(4); pp947–953.
- Obruca, S., Marova, I., Snajdar, O., Mravcova, L., & Svoboda, Z. (2010). Production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Cupriavidus necator* from waste rapeseed oil using propanol as a precursor of 3-hydroxyvalerate. *Biotechnology Letters*, **32**(12); pp1925–1932.
- Ojumu, T.V, Yu, J and Solomon, B. . (2004). Production of Polyhydroxyalkanoates, a bacterial biodegradable polymer. *African Journal of Biotechnology*, **3**(1); pp18–24.
- Okunola A, A., Kehinde I, O., Oluwaseun, A., & Olufiropo E, A. (2019). Public and Environmental Health Effects of Plastic Wastes Disposal: A Review. *Journal of Toxicology and Risk Assessment*, **5**(1); pp1–13.
- Onumaegbu, C., Mooney, J., Alaswad, A., & Olabi, A. G. (2018). Pre-treatment methods for the production of biofuel from microalgae biomass. *Renewable and Sustainable Energy Reviews*, **93**(2018); pp16–26.
- Ooi, T. L., Yong, K. C., Dzulkefly, K., Wan Yunus, W. M. Z., & Hazimah, A. H. (2001). Crude Glycerine Recovery From Glycerol Residue Waste From a Palm Kernel Oil Methyl Ester Plant. *Journal of Oil Palm Research*, **13**(2); pp16–22.
- Pagliano, G., Galletti, P., Samorì, C., Zaghini, A., & Torri, C. (2021). Recovery of Polyhydroxyalkanoates From Single and Mixed Microbial Cultures: A Review. *Frontiers in Bioengineering and Biotechnology*, **9**(2021); pp1–28.
- Pais, J., Serafim, S., Freitas, F., & Reis, M. A. M. (2016). Conversion of cheese

- whey into poly (3- hydroxybutyrate- co -3-hydroxyvalerate) by *Haloferax mediterranei*. *New Biotechnology*, **33**(1); pp224–239.
- Panadare, D. C., & Rathod, V. K. (2015). Applications of Waste Cooking Oil Other Than Biodiesel : A Review. *Journal of Chemical Engineering*, **12**(3); pp55–76.
- Pappalardo, F., Fragalà, M., Mineo, P. G., Damigella, A., Catara, A. F., Palmeri, R., & Rescifina, A. (2014). Production of filmable medium-chain-length polyhydroxyalkanoates produced from glycerol by *Pseudomonas mediterranea*. *International Journal of Biological Macromolecules*, **65**(2014); pp89–96.
- Penloglou, G., Vasileiadou, A., Chatzidoukas, C., & Kiparissides, C. (2017). Model-based intensification of a fed-batch microbial process for the maximization of polyhydroxybutyrate (PHB) production rate. *Bioprocess and Biosystems Engineering*, **40**(8); pp1247–1260.
- Pérez-Rivero, C., López-Gómez, J. P., & Roy, I. (2019). A sustainable approach for the downstream processing of bacterial polyhydroxyalkanoates: State-of-the-art and latest developments. *Biochemical Engineering Journal*, **150**(2019); pp1–13.
- Pernicova, I., Kucera, D., Nebesarova, J., Kalina, M., & Novackova, I. (2019). Production of polyhydroxyalkanoates on waste frying oil employing selected *Halomonas* strains. *Bioresource Technology*, **292**(2019); pp1–4.
- Pieja, A. J., Sundstrom, E. R., & Criddle, C. S. (2011). Poly-3-hydroxybutyrate metabolism in the type II Methanotroph *Methylocystis parvus* OBBP. *Applied and Environmental Microbiology*, **77**(17); pp6012–6019.
- Pinto Da Costa, J., Rocha Santos, T., & Duarte, A. (2020). The environmental impacts of plastics and micro-plastics use, waste and pollution: EU and national measures. *European Union*, **10**(2020); pp10–62.
- Plastic Europe, P. (2019). Plastics - the Facts 2019. *Plastics Europe, Group Market Research Conversio Market & Strategy GmbH*, **14**(2019); pp35-39.

Plastics Europe. (2018). Plastics – the Facts. *Plastics – the Facts 2018*, 38.

Poblete-Castro, I., Rodriguez, A. L., Chi Lam, C. M., & Kessler, W. (2014). Improved production of medium-chain-length polyhydroxyalkanoates in glucose-based fed-batch cultivations of metabolically engineered *Pseudomonas putida* strains. *Journal of Microbiology and Biotechnology*, **24**(1); pp59–69.

Povolo, S., & Casella, S. (2003). Bacterial production of PHA from lactose and cheese whey permeate. *Macromolecular Symposia*, **197**(1); pp1–9.

Povolo, S., Romanelli, M. G., Basaglia, M., Ilieva, V. I., Corti, A., Morelli, A., Chiellini, E., & Casella, S. (2013). Polyhydroxyalkanoate biosynthesis by *Hydrogenophaga pseudoflava* DSM1034 from structurally unrelated carbon sources. *New Biotechnology*, **30**(6); pp629–634.

Priester, J. H., Van De Werfhorst, L. C., Ge, Y., Adeleye, A. S., Tomar, S., Tom, L. M., Piceno, Y. M., Andersen, G. L., & Holden, P. A. (2014). Effects of TiO₂ and Ag nanoparticles on polyhydroxybutyrate biosynthesis by activated sludge bacteria. *Environmental Science and Technology*, **48**(24); pp14712–14720.

Priyadarshi, S., Shukla, A., & Borse, B. B. (2014). Review Article Polyhydroxyalkanoates : Role of *Ralstonia eutropha*. *International Journal of Biomedical And Advance Research*, **5**(2); pp68–76.

Rai, R., Keshavarz, T., Roether, J. A., Boccaccini, A. R., & Roy, I. (2011). Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future. In *Materials Science and Engineering R: Reports*, **72**(3); pp29–47.

Rakkan, T., Chana, N., & Sangkharak, K. (2022a). Nitrogen Reduction in Conjunction with Polyhydroxyalkanoates Production Using Mixed *Enterobacter* Strains. *Waste and Biomass Valorization*, **13**(3); pp1515–1522.

Rakkan, T., Chana, N., & Sangkharak, K. (2022b). The Integration of Textile Wastewater Treatment with Polyhydroxyalkanoate Production Using Newly

- Isolated *Enterobacter* Strain TS3. *Waste and Biomass Valorization*, **13**(1); pp571–582.
- Ray, S., & Kalia, V. C. (2017). Co-metabolism of substrates by *Bacillus thuringiensis* regulates polyhydroxyalkanoate copolymer composition. *Bioresource Technology*, **224**(2017); pp743–747.
- Ray, S., Prajapati, V., Patel, K., & Trivedi, U. (2016). Optimization and characterization of PHA from isolate *Pannonibacter phragmitetus* ERC8 using glycerol waste. *International Journal of Biological Macromolecules*, **86**(2016); pp741–749.
- Raza, Z. A., Abid, S., & Banat, I. M. (2018). Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. In *International Biodeterioration and Biodegradation*, **126**(2016); pp45–56.
- Reddy, C. S. K., Ghai, R., & Kalia, V. C. (2003). Polyhydroxyalkanoates : an overview. *Bioresource Technology*, **87**(2003), 137–146.
- Redfern, A. W. and C. J. P. (2008). Thermogravimetric Analysis. *Principles and Applications of Thermal Analysis*, **88**(1053); pp906–924.
- REHM, B. H. A. (2003). Polyester synthases: natural catalysts for plastics Bernd. *Biochem Journal*, **376**(1); pp15–33.
- Riedel, S. L., Jahns, S., Koenig, S., Bock, M. C. E., Brigham, C. J., Bader, J., & Stahl, U. (2015). Polyhydroxyalkanoates production with *Ralstonia eutropha* from low-quality waste animal fats. *Journal of Biotechnology*, **214**(2015); pp119–127.
- Rigouin, C., Lajus, S., Ocando, C., Borsenberger, V., Nicaud, J. M., Marty, A., Avérous, L., & Bordes, F. (2019). Production and characterization of two medium-chain-length polyhydroxyalkanoates by engineered strains of *Yarrowia lipolytica*. *Microbial Cell Factories*, **18**(99); pp1–9.
- Rodríguez-Contreras, A., Koller, M., Miranda-de Sousa Dias, M., Calafell-Monfort, M., Braunegg, G., & Marqués-Calvo, M. S. (2015). Influence of

- glycerol on poly(3-hydroxybutyrate) production by *Cupriavidus necator* and *Burkholderia sacchari*. *Biochemical Engineering Journal*, **94**(2015); pp50–57.
- Roja, K., Ruben Sudhakar, D., Anto, S., & Mathimani, T. (2019). Extraction and characterization of polyhydroxyalkanoates from marine green alga and cyanobacteria. *Biocatalysis and Agricultural Biotechnology*, **22**(2019); pp1–11.
- Romero, L., Byrne, E., Niel, E. W. J. Van, & Sayed, M. (2019). Bioresource Technology Hydrogen and polyhydroxybutyrate production from wheat straw hydrolysate using *Caldicellulosiruptor* species and *Ralstonia eutropha* in a coupled process. *Bioresource Technology*, **272**(2018); pp259–266.
- Rouch, D. A., Policy, C., Group, S., & Policy, C. (2020). *Clarendon Policy & Strategy Group Plastic future : November 2019*.
- Rubab, S., Rizwani, G. H., Bahadur, S., Shah, M., Alsamadany, H., Alzahrani, Y., Shuaib, M., Hershan, A., Hobani, Y. H., & Shah, A. A. (2020). Determination of the GC–MS analysis of seed oil and assessment of pharmacokinetics of leaf extract of *Camellia sinensis* L. *Journal of King Saud University - Science*, **32**(7); pp3138–3144.
- Rueda, A., Seiquer, I., Olalla, M., Giménez, R., Lara, L., & Cabrera-Vique, C. (2014). Characterization of the fatty acid profile of argan oil and other edible vegetable oils by gas chromatography and discriminant analysis. *Journal of Chemistry*, **2014**(2014); pp1–9.
- Ruiz, C., Kenny, S. T., Narancic, T., Babu, R., & Connor, K. O. (2019). Conversion of waste cooking oil into medium chain polyhydroxyalkanoates in a high cell density fermentation. *Journal of Biotechnology*, **306**(2019); pp9–15.
- Ruiz, C., Kenny, S. T., Ramesh Babu, P., Walsh, M., Narancic, T., & O'Connor, K. E. (2019). High cell density conversion of hydrolyzed waste cooking oil fatty acids into medium chain length polyhydroxyalkanoate using *pseudomonas putida* KT2440. *Catalysts*, **9**(5); pp1-15.

- Sabapathy, P. C., Devaraj, S., Meixner, K., Anburajan, P., Kathirvel, P., Ravikumar, Y., Zabeed, H. M., & Qi, X. (2020). Recent developments in Polyhydroxyalkanoates (PHAs) production-A review. *Bioresource Technology*, **306**(2020); pp1–14.
- Saito, S., Imai, R., Miyahara, Y., Nakagawa, M., Orita, I., Tsuge, T., & Fukui, T. (2022). Biosynthesis of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) From Glucose by *Escherichia coli* Through Butyryl-CoA Formation Driven by Ccr-Emd Combination. *Frontiers in Bioengineering and Biotechnology*, **10**(5); pp1–10.
- Sambanthamurthi, R., Sundram, K., & Tan, Y. A. (2000). Chemistry and biochemistry of palm oil. In *Progress in Lipid Research*, 39(6); pp1-10.
- Samrot, A. V., Samanvitha, S. K., Shobana, N., Renitta, E. R., Kumar, P. S., Kumar, S. S., Abirami, S., Dhiva, S., Bavanilatha, M., Prakash, P., Saigeetha, S., Shree, K. S., & Thirumurugan, R. (2021). The synthesis, characterization, and applications of polyhydroxyalkanoates (Phas) and pha-based nanoparticles. *Polymers*, **13**(19); pp1–29.
- Samuel, M. M., Betty, M. N., Kasili, R., Edward, K. M., & Naumih, N. M. (2019). Production of polyhydroxyalkanoates by hydrocarbonaclastic bacteria. *African Journal of Biotechnology*, **18**(17); pp352–364.
- Sangkharak, K., Khaithongkaeo, P., Chuaikhunupakarn, T., Choonut, A., & Prasertsan, P. (2020). The production of polyhydroxyalkanoate from waste cooking oil and its application in biofuel production. *Biomass Conversion and Biorefinery*, **11**(2020); pp1–14.
- Saralegui, C., Herencias, C., Halperin, A. V., de Dios-Caballero, J., Pérez-Viso, B., Salgado, S., Lanza, V. F., Cantón, R., Baquero, F., Prieto, M. A., & del Campo, R. (2022). Strain-specific predation of *Bdellovibrio bacteriovorus* on *Pseudomonas aeruginosa* with a higher range for cystic fibrosis than for bacteremia isolates. *Scientific Reports*, **12**(1); pp1–7.
- Sasidharan, R. S., & Bhat, S. G. (2014). Bacterial Polyhydroxyalkanoates

- Production and its Applications. **8**(2014); pp2341-2351.
- Sathiyarayanan, G., Kiran, G. S., Selvin, J., & Saibaba, G. (2013). Optimization of polyhydroxybutyrate production by marine *Bacillus megaterium* MSBN04 under solid-state culture. *International Journal of Biological Macromolecules*, **60**(2013); pp253–261.
- Satoh, K., Kawakami, T., Isobe, N., Pasquier, L., Tomita, H., Zinn, M., & Matsumoto, K. (2022). Versatile aliphatic polyester biosynthesis system for producing random and block copolymers composed of 2-, 3-, 4-, 5-, and 6-hydroxyalkanoates using the sequence-regulating polyhydroxyalkanoate synthase PhaCAR. *Microbial Cell Factories*, **21**(1); pp1–14.
- Sehgal, R., & Gupta, R. (2020). Polyhydroxyalkanoate and its efficient production: an eco-friendly approach towards development. *3 Biotech*, **10**(12); pp1–14.
- Semeniuk, I., Pokynbroda, T., Kochubei, V., Midyana, H., Karpenko, O., & Skorokhoda, V. (2020). Biosynthesis and characteristics of polyhydroxyalkanoates. 1. polyhydroxybutyrates of azotobacter vinelandii n-15. *Chemistry and Chemical Technology*, **14**(4); pp463–467.
- Shahid, S., Mosrati, R., Ledauphin, J. Ô., Amiel, C., Fontaine, P., Gaillard, J. L., & Corroler, D. (2013). Impact of carbon source and variable nitrogen conditions on bacterial biosynthesis of polyhydroxyalkanoates: Evidence of an atypical metabolism in *Bacillus megaterium* DSM 509. *Journal of Bioscience and Bioengineering*, **116**(3); pp302–308.
- Sharma, P., & Bajaj, B. K. (2015). Production and characterization of poly-3-hydroxybutyrate from *Bacillus cereus* PS 10. *International Journal of Biological Macromolecules*, **81**(2015); pp241–248.
- Sharma, P., Gaur, V. K., Kim, S., & Pandey, A. (2020). Microbial strategies for bio-transforming food waste into resources. *Bioresource Technology*, **299**(2019); pp1–11.
- Sharma, P. K., Munir, R. I., Blunt, W., Dartailh, C., Cheng, J., Charles, T. C., &

- Levin, D. B. (2017). Synthesis and Physical Properties of Polyhydroxyalkanoate Polymers with Different Monomer Compositions by Recombinant *Pseudomonas putida* LS46 Expressing a Novel PHA SYNTHASE (PhaC1 16) Enzyme. *Applied Sciences*, **7**(242); pp1-13.
- Sharma, P. K., Munir, R. I., de Kievit, T., & Levin, D. B. (2017). Synthesis of polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *pseudomonas chlororaphis*. *Canadian Journal of Microbiology*, **63**(12); pp1009–1024.
- Sharma, V., Sehgal, R., & Gupta, R. (2021). Polyhydroxyalkanoate (PHA): Properties and Modifications. *Polymer*, **212**(2020); pp1231-1241.
- Shi, M., Li, M., Yang, A., Miao, X., Yang, L., Pandhal, J., & Zou, H. (2022). Class I Polyhydroxyalkanoate (PHA) Synthase Increased Polylactic Acid Production in Engineered Escherichia Coli. *Frontiers in Bioengineering and Biotechnology*, **10**(6); pp1–7.
- Silva, J. B., Pereira, J. R., Marreiros, B. C., Reis, M. A. M., & Freitas, F. (2021). Microbial production of medium-chain length polyhydroxyalkanoates. *Process Biochemistry*, **102**(2021); pp393–407.
- Simon-Colin, C., Raguénès, G., Crassous, P., Moppert, X., & Guezennec, J. (2008). A novel mcl-PHA produced on coprah oil by *Pseudomonas guezennei* biovar. tikehau, isolated from a “kopara” mat of French Polynesia. *International Journal of Biological Macromolecules*, **43**(2); pp176–181.
- Sindhu, R., Silviya, N., Binod, P., & Pandey, A. (2013). Pentose-rich hydrolysate from acid-pretreated rice straw as a carbon source for the production of poly-3-hydroxybutyrate. *Biochemical Engineering Journal*, **78**(2013); pp67–72.
- Singh Saharan, B., Grewal, A., & Kumar, P. (2014). Biotechnological Production of Polyhydroxyalkanoates: A Review on Trends and Latest Developments. *Chinese Journal of Biology*, **2014**(1); pp1–18.
- Smith, M., Love, D. C., Rochman, C. M., & Neff, R. A. (2018). Microplastics in Seafood and the Implications for Human Health. *Current Environmental*

Health Reports, **5**(3); pp375–386.

- Song, Jin Hwan, Che Ok Jeon, Mun Hwan Choi, S. C. Y. and W. P. (2008). Polyhydroxyalkanoate (PHA) Production Using Waste Vegetable Oil by *Pseudomonas* sp. Strain DR2. *Journal of Microbiology and Biotechnology*, **18**(8); pp1408–1415.
- Song, H., Taylor, D. C., & Zhang, M. (2023). Bioengineering of Soybean Oil and Its Impact on Agronomic Traits. *International Journal of Molecular Sciences*, **24**(3); pp1–23.
- Stanley, A., Punil Kumar, H. N., Mutturi, S., & Vijayendra, S. V. N. (2018). Fed-Batch Strategies for Production of PHA Using a Native Isolate of *Halomonas venusta* KT832796 Strain. *Applied Biochemistry and Biotechnology*, **184**(3); pp935–952.
- Sudesh, K., Abe, H., & Doi, Y. (2000). Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. *Progress in Polymer Science (Oxford)*, **25**(10); pp1503–1555.
- Sukan, A., Roy, I., & Keshavarz, T. (2014). Agro-Industrial Waste Materials as Substrates for the Production of Poly(3-Hydroxybutyric Acid). *Journal of Biomaterials and Nanobiotechnology*, **05**(04); pp229–240.
- Surendran, A., Lakshmanan, M., Chee, J. Y., Sulaiman, A. M., Thuoc, D. Van, & Sudesh, K. (2020). Can Polyhydroxyalkanoates Be Produced Efficiently From Waste Plants and Animal Oils? In *Frontiers in Bioengineering and Biotechnology* 8(2020); pp1–15.
- Szacherska, K., Moraczewski, K., Czaplicki, S., Oleskowicz-Popiel, P., & Mozejko-Ciesielska, J. (2022). Effect of short- and medium-chain fatty acid mixture on polyhydroxyalkanoate production by *Pseudomonas* strains grown under different culture conditions. *Frontiers in Bioengineering and Biotechnology*, **10**(2022); pp1–10.
- Tan, G. Y. A., Chen, C. L., Ge, L., Li, L., Wang, L., Zhao, L., Mo, Y., Tan, S. N., & Wang, J. Y. (2014). Enhanced gas chromatography-mass spectrometry

- method for bacterial polyhydroxyalkanoates analysis. *Journal of Bioscience and Bioengineering*, **117**(3); pp379–382.
- Tanikkul, P., Sullivan, G. L., Sarp, S., & Pisutpaisal, N. (2020). Biosynthesis of medium chain length polyhydroxyalkanoates (mcl-PHAs) from palm oil. *Case Studies in Chemical and Environmental Engineering*, **2**(9); pp1–5.
- Thompson, R. C., Moore, C. J., Saal, F. S. V., & Swan, S. H. (2009). Plastics, the environment, and human health: Current consensus and future trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **364**(1526); pp2153–2166.
- Thorat Gadgil, B. S., Killi, N., & Rathna, G. V. N. (2017). Polyhydroxyalkanoates as biomaterials. *MedChemComm*, **8**(9); pp1774–1787.
- Tian, J., Sinskey, A. J., & Stubbe, J. A. (2005). Kinetic studies of polyhydroxybutyrate granule formation in *Wautersia eutropha* H16 by transmission electron microscopy. *Journal of Bacteriology*, **187**(11); pp3814–3824.
- Tian, L., Li, H., Song, X., Ma, L., & Li, Z. J. (2022). Production of polyhydroxyalkanoates by a novel strain of *Photobacterium* using soybean oil and corn starch. *Journal of Environmental Chemical Engineering*, **10**(5); pp1-12.
- Tripathi, A. D., Raj Joshi, T., Kumar Srivastava, S., Darani, K. K., Khade, S., & Srivastava, J. (2019). Effect of nutritional supplements on bio-plastics (PHB) production utilizing sugar refinery waste with potential application in food packaging. *Preparative Biochemistry and Biotechnology*, **49**(6); pp567–577.
- Tsang, Y. F., Kumar, V., Samadar, P., Yang, Y., Lee, J., Ok, Y. S., Song, H., Kim, K. H., Kwon, E. E., & Jeon, Y. J. (2019). Production of bioplastic through food waste valorization. *Environment International*, **127**(3); pp625–644.
- Varghese, S., Dhanraj, N. D., Rebello, S., Sindhu, R., Binod, P., Pandey, A., Jisha, M. S., & Awasthi, M. K. (2022). Leads and hurdles to sustainable microbial bioplastic production. *Chemosphere*, **305**(2022); pp1–15.

- Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D., Piotrowska-Seget, Z., & Radecka, I. K. (2011). Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. *AMB Express*, **1**(1); pp1–8.
- Verlinden, R. A. J., Hill, D. J., Kenward, M. A., Williams, C. D., & Radecka, I. (2007). Bacterial synthesis of biodegradable polyhydroxyalkanoates. *Appl Microbiol*, **102**(6); pp1437–1449.
- Villarrubia-Gómez, P., Cornell, S. E., & Fabres, J. (2018). Marine plastic pollution as a planetary boundary threat – The drifting piece in the sustainability puzzle. *Marine Policy*, 96(December 2017), 213–220. <https://doi.org/10.1016/j.marpol.2017.11.035>
- Volova, T., Demidenko, A., Kiselev, E., Baranovskiy, S., & Shishatskaya, E. (2019). Polyhydroxyalkanoate synthesis based on glycerol and implementation of the process under conditions of pilot production. *Applied Microbiology and Biotechnology*, **103**(1); pp225–237.
- Volova, T. G., Kiselev, E. G., Demidenko, A. V., Zhila, N. O., Nemtsev, I. V., & Lukyanenko, A. V. (2022). Production and properties of microbial polyhydroxyalkanoates synthesized from hydrolysates of Jerusalem artichoke tubers and vegetative biomass. *Polymers*, **14**(1); pp1–25.
- Volova, T., Sapozhnikova, K., & Zhila, N. (2020). *Cupriavidus necator* B-10646 growth and polyhydroxyalkanoates production on different plant oils. *International Journal of Biological Macromolecules*, **164**(1); pp121–130.
- Vu, D. H., Åkesson, D., Taherzadeh, M. J., & Ferreira, J. A. (2020). Recycling strategies for polyhydroxyalkanoate-based waste materials: An overview. *Bioresource Technology*, **298**(2019); pp1–9.
- Waller, J. L., Green, P. G., & Loge, F. J. (2012). Mixed-culture polyhydroxyalkanoate production from olive oil mill pomace. *Bioresource Technology*, **120**(2012); pp285–289.
- Wang, J., Liu, S., Huang, J., & Qu, Z. (2021). A review on polyhydroxyalkanoate production from agricultural waste Biomass: Development, Advances,

- circular Approach, and challenges. *Bioresource Technology*, **342**(9); pp12600-1268.
- Wirasnita, R., Hadibarata, T., Novelina, Y. M., Yusoff, A. R. M., & Yusop, Z. (2013). A modified methylation method to determine fatty acid content by gas chromatography. *Bulletin of the Korean Chemical Society*, **34**(11); pp3239–3242.
- Wong, H. S. J., Azami, N. A., & Amirul, A. A. A. (2022). Enhanced production of polyhydroxyalkanoate with manipulable and reproducible 3-hydroxy valerate fraction by high alcohol tolerant *Cupriavidus malaysiensis* USMAA2-4 transformant. *Bioprocess and Biosystems Engineering*, **45**(8); pp1331–1347.
- Wong, Y. M., Brigham, C. J., Rha, C. K., Sinskey, A. J., & Sudesh, K. (2012). Biosynthesis and characterization of polyhydroxyalkanoate containing high 3-hydroxyhexanoate monomer fraction from crude palm kernel oil by recombinant *Cupriavidus necator*. *Bioresource Technology*, **121**, 320–327.
- Xu, B., Zhang, M., Xing, C., Mothibe, K. J., & Zhu, C. (2013). Composition, characterization and analysis of seed oil of *Suaeda salsa* L. *International Journal of Food Science and Technology*, **48**(4); pp879–885.
- Xu, T., Zong, Q. J., Liu, H., Wang, L., Liu, Z. H., Li, B. Z., & Yuan, Y. J. (2022). Identifying ligninolytic bacteria for lignin valorization to bioplastics. *Bioresource Technology*, **358**(5); pp1273-1283.
- Yajima, T., Nagatomo, M., Wakabayashi, A., Sato, M., Taguchi, S., & Maeda, M. (2020). Bioconversion of biphenyl to a polyhydroxyalkanoate copolymer by *Alcaligenes denitrificans* A41. *AMB Express*, **10**(1); pp1–9.
- Yang, J., Ching, Y. C., & Chuah, C. H. (2019). Applications of Lignocellulosic Fibers and Lignin in. *Polymers*, **11**(751); pp1–26.
- Yasothea, K., Aroua, M. K., Ramachandran, K. B., & Tan, I. K. P. (2006). Recovery of medium-chain-length polyhydroxyalkanoates (PHAs) through enzymatic digestion treatments and ultrafiltration. *Biochemical Engineering Journal*,

30(3); pp260–268.

- Yin, F., Li, D., Ma, X., & Zhang, C. (2019). Pretreatment of lignocellulosic feedstock to produce fermentable sugars for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) production using activated sludge. *Bioresource Technology*, **290**(2019); pp1–6.
- zaaboul, F., Zhao, Q., Xu, Y. J., & Liu, Y. F. (2022). Soybean oil bodies: A review on composition, properties, food applications, and future research aspects. *Food Hydrocolloids*, **124**(2022); pp1–13.
- Zhang, T., Jin, J., Yang, S., Hu, D., Li, G., & Jiang, J. (2009). Synthesis and characterization of fluorinated PBO with high thermal stability and low dielectric constant. *Journal of Macromolecular Science, Part B: Physics*, **48**(6); pp1114–1124.
- Zhang, Y., Sun, W., Wang, H., & Geng, A. (2013). Polyhydroxybutyrate production from oil palm empty fruit bunch using *Bacillus megaterium* R11. *Bioresource Technology*, **147**(2013); pp307–314.
- Zhao, X., & Wang, J. (2023). Combined analysis of the metabolome and transcriptome provides insight into seed oil accumulation in soybeans. *Biotechnology for Biofuels and Bioproducts*, **16**(70); pp1–23.
- Zhao, Y., Rao, Z., Xue, Y., Gong, P., Ji, Y., & Ma, Y. (2015). Biosynthesis, property comparison, and hemocompatibility of bacterial and haloarchaeal poly(3-hydroxybutyrate-co-3-hydroxyvalerate). *Science Bulletin*, **60**(22); pp1901–1910.
- Zhou, W., Colpa, D. I., Geurkink, B., Euverink, G. J. W., & Krooneman, J. (2022). The impact of carbon to nitrogen ratios and pH on the microbial prevalence and polyhydroxybutyrate production levels using a mixed microbial starter culture. *Science of the Total Environment*, **811**(2022); pp1–10.
- Zhu, Y., Ai, M., & Jia, X. (2022). Optimization of a Two-Species Microbial Consortium for Improved Mcl-PHA Production From Glucose–Xylose Mixtures. *Frontiers in Bioengineering and Biotechnology*, **9**(1); pp1–15.

Zong, Q. J., Xu, T., Liu, H., Xu, L., Zhang, R. K., Li, B. Z., Liu, Z. H., & Yuan, Y. J. (2022). Microbial Valorization of Lignin to Bioplastic by Genome-Reduced *Pseudomonas putida*. *Frontiers in Microbiology*, **13**(2022); pp1–13.

APPENDICES

Appendix A Non-Pretreatment

A.1 Estimated Marginal Mean

A.1.1 Frying Palm Oil utilized by both strains.

Table 7-2: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DSMZ1045	3.155	.443	2.167	4.143
ATCC29347	2.825	.421	1.887	3.762

Table 4-3: Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DSMZ1045	3.151	.443	2.165	4.137
	3.159	.444	2.170	4.148
	3.154	.444	2.165	4.143
ATCC29347	2.819	.418	1.887	3.750
	2.828	.422	1.887	3.768
	2.828	.422	1.889	3.768

R Squared = .973 (Adjusted R Squared = .914)

A.1.2 Glycerol utilized by both strains.

Table 7-4: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DSMZ1045	3.661	.528	2.486	4.837
ATCC29347	3.496	.488	2.408	4.584

Table 7-5: Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DSMZ1045	3.652	.529	2.474	4.830
	3.669	.527	2.494	4.844
	3.663	.526	2.490	4.836
ATCC29347	3.495	.493	2.397	4.593
	3.507	.492	2.411	4.604
	3.486	.481	2.414	4.557

R Squared = .973 (Adjusted R Squared = .981)

A.1.3 Frying Soybean oil utilized by both strains.

Table 7-6: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DSMZ1045	2.932	.445	1.940	3.923
ATCC29347	2.974	.386	2.115	3.834

Table 7-7: Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DSMZ1045	2.920	.445	1.928	3.912
	2.945	.443	1.958	3.932
	2.930	.446	1.935	3.924
ATCC29347	2.960	.386	2.100	3.819
	2.988	.385	2.130	3.845
	2.976	.386	2.116	3.836

R Squared = .973 (Adjusted R Squared = .906)

A.1.4 FOG utilized by both strains.

Table 7-8: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DSMZ1045	2.932	.445	1.940	3.923
ATCC29347	2.974	.386	2.115	3.834

Table 7-9: Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DSMZ1045	3.627	.492	2.532	4.723
	3.626	.492	2.529	4.724
	3.598	.492	2.503	4.693
ATCC29347	3.453	.489	2.365	4.542
	3.461	.488	2.373	4.548
	3.465	.488	2.378	4.552

R Squared = .973 (Adjusted R Squared = .856)

Appendix B Pretreatment

B.1 Estimated Marginal means.

B.1.1 Glycerol + DSMZ1045

Table 7-2: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASONICATOR	3.227	.470	2.180	4.274
MICROWAVE	2.999	.453	1.990	4.009
LIPASE	3.418	.499	2.306	4.529

Table 7-3:Significance level revealed 93.2% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASONICATOR	3.086	.538	1.888	4.283
	3.304	.447	2.308	4.300
	3.292	.451	2.287	4.296
MICROWAVE	2.826	.498	1.716	3.937
	3.086	.444	2.096	4.076
	3.085	.442	2.101	4.069
LIPASE	3.253	.569	1.985	4.522
	3.502	.479	2.435	4.569
	3.498	.477	2.436	4.560

R Squared = .932 (Adjusted R Squared = .901)

B.1.2 Glycerol + ATCC29347

Table 7-4:Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASOUND	2.992	.442	2.008	3.976
MICROWAVE	2.824	.408	1.915	3.733
LIPASE	3.400	.485	2.320	4.480

Table 7-5:Significance level revealed 85.8% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASOUND	2.990	.440	2.010	3.971
	2.985	.440	2.005	3.965
	3.000	.446	2.007	3.994
MICROWAVE	2.829	.407	1.923	3.735
	2.833	.407	1.927	3.740
	2.810	.411	1.893	3.726
LIPASE	3.397	.485	2.317	4.476
	3.403	.485	2.323	4.483
	3.400	.485	2.320	4.480

R Squared = .858 (Adjusted R Squared = .858)

B.1.3 Frying Soybean oil + DSMZ1045

Table 7-6: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASONICATOR	3.421	.503	2.301	4.542
MICROWAVE	3.009	.461	1.983	4.036
LIPASE	3.333	.485	2.254	4.413

Table 7-7: Significance level revealed 91.8% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASONICATOR	3.271	.576	1.988	4.554
	3.520	.487	2.436	4.604
	3.473	.478	2.407	4.539
MICROWAVE	2.884	.516	1.734	4.034
	3.085	.442	2.101	4.070
	3.058	.445	2.067	4.049
LIPASE	3.181	.553	1.948	4.414
	3.414	.471	2.364	4.463
	3.405	.460	2.380	4.430

R Squared = .918 (Adjusted R Squared = .918)

B.1.4 Frying Soybean oil + ATCC29347

Table 7-16: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASOUND	2.992	.425	2.044	3.939
MICROWAVE	3.114	.457	2.097	4.132
LIPASE	3.433	.481	2.362	4.504

Table 7-87: Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASOUND	2.974	.423	2.030	3.917
	3.014	.431	2.053	3.974
	2.987	.422	2.046	3.928
MICROWAVE	3.113	.459	2.090	4.135
	3.124	.457	2.105	4.143
	3.105	.454	2.094	4.116
LIPASE	3.437	.481	2.366	4.508
	3.436	.484	2.359	4.514
	3.426	.478	2.361	4.490

R Squared = .973 (Adjusted R Squared = .856)

B.1.5 FOG + DSMZ1045

Table 7-18: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASONICATOR	3.549	.523	2.384	4.713
MICROWAVE	3.247	.475	2.190	4.305
LIPASE	3.598	.511	2.460	4.736

Table 1-18: Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASONICATOR	3.390	.595	2.064	4.715
	3.614	.501	2.498	4.730
	3.643	.501	2.526	4.760
MICROWAVE	3.089	.544	1.877	4.301
	3.330	.451	2.326	4.335
	3.323	.460	2.298	4.349
LIPASE	3.417	.590	2.103	4.732
	3.692	.490	2.600	4.784
	3.684	.488	2.598	4.771

R Squared = .973 (Adjusted R Squared = .871)

B.1.6 FOG + ATCC29347

Table 7-19: Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASOUND	3.195	.456	2.178	4.212
MICROWAVE	3.115	.447	2.119	4.110
LIPASE	3.524	.509	2.390	4.658

Table 7-20: Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASOUND	3.224	.466	2.187	4.261
	3.169	.453	2.159	4.178
	3.192	.453	2.184	4.200
MICROWAVE	3.106	.448	2.108	4.105
	3.120	.447	2.124	4.117
	3.117	.445	2.126	4.109
LIPASE	3.524	.508	2.392	4.657
	3.521	.510	2.384	4.658
	3.528	.508	2.395	4.660

R Squared = .973 (Adjusted R Squared = .982)

B.1.7 Frying Palm oil + DSMZ1045

Table 7-9:Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASONICATOR	3.360	.494	2.259	4.461
MICROWAVE	3.195	.470	2.148	4.242
LIPASE	3.386	.496	2.281	4.491

Table 7-10:Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASONICATOR	3.207	.565	1.947	4.467
	3.446	.468	2.404	4.487
	3.428	.480	2.357	4.498
MICROWAVE	3.056	.539	1.856	4.257
	3.244	.449	2.244	4.245
	3.285	.450	2.282	4.288
LIPASE	3.238	.565	1.979	4.498
	3.461	.477	2.398	4.523
	3.459	.476	2.399	4.519

R Squared = .973 (Adjusted R Squared = .917)

B.1.8 Frying Palm oil + ATCC29347

Table 7-11:Grand Means of 95% confidence level

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASOUND	3.184	.447	2.187	4.180
MICROWAVE	2.999	.431	2.039	3.960
LIPASE	3.387	.472	2.334	4.439

Table 7-12:Significance level revealed 97.3% from the regression model.

Measure	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
ULTRASOUND	3.170	.443	2.182	4.158
	3.212	.454	2.201	4.224
	3.169	.445	2.177	4.161
MICROWAVE	2.994	.432	2.031	3.956
	3.024	.431	2.063	3.984
	2.981	.431	2.020	3.941
LIPASE	3.374	.472	2.323	4.426
	3.383	.473	2.329	4.437
	3.403	.473	2.349	4.457

R Squared = .973 (Adjusted R Squared = .972)