

**PRODUCTION OF POLYHYDROXYALKANOATE (PHA) FROM PALM OIL BY
Pseudomonas oleovorans AND AN INDIGENOUS BACTERIAL ISOLATE,
Pseudomonas sp.**

BALAN TERHAD

BY
ZAZALI ALIAS

Perpustakaan Universiti Malaya



A509545291

**A dissertation submitted in partial fulfilment for the Degree of
Master of Biotechnology
at the
Institute of Postgraduate Studies and Research
University of Malaya
Kuala Lumpur**

**PRODUCTION OF POLYHYDROXYALKANOATE (PHA) FROM PALM OIL BY
Pseudomonas oleovorans AND AN INDIGENOUS BACTERIAL ISOLATE,
Pseudomonas sp.**

BALAN TERHAD

BY
ZAZALI ALIAS

Perpustakaan Universiti Malaya



A509545291

**A dissertation submitted in partial fulfilment for the Degree of
Master of Biotechnology
at the
Institute of Postgraduate Studies and Research
University of Malaya
Kuala Lumpur**

ACKNOWLEDGEMENT

Dengan nama ALLAH yang Maha Pemurah lagi Maha Penyayang

Setinggi-tinggi ribuan terimakasih diucapkan kepada Dr. Irene K.P.Tan yang telah memberi kepercayaan, pendedahan serta tunjuk ajar yang berharga disepanjang waktu kursus. Begitu juga, Profesor K.B. Ramachandran, yang telah membantu memudahkan perjalanan penyelidikan dengan sumbangan pandangan yang membina.

Terima kasih juga diucapkan kepada teman sekerja, Pian, Ho dan Shaza; sokongan moral, buah fikiran serta tunjuk ajar anda semua adalah sangat disanjung. Begitu juga, En. Karim, yang telah membantu melicinkan penggunaan peralatan makmal.

Terimakasih juga, kepada pihak Jabatan Kimia, makmal Biologi Molekul, Mikologi, N.P.C. dan Alga, diatas sokongan memberi bantuan penggunaan alatan makmal dan analysis. Begitu juga, Molly, Kak Zaini dan Rafiati yang telah membantu mendapatkan bahan-bahan rujukan.

Keluarga tercinta; emak, Ina, kakak, Emy dan terutamanya arwah ayah, anda semua adalah inspirasi untuk menjadi lebih cecal mengejar cita-cita dan kejayaan. Terimakasih yang mendalam diatas segala pengorbanan yang telah diberikan. Begitu juga buat tunang tersayang, Elly, diatas segala sokongan dan galakan.

Buat akhirnya, terimakasih juga, kepada pihak IRPA, UM, yang membantu dari segi kewangan dalam menjayakan projek ini.

Sekian

Zazali Alias

ABSTRACT

In this study, the yield of biomass and polyhydroxyalkanoate (PHA) of *Pseudomonas oleovorans* grown in batch and fed-batch cultivations were compared. When oleic acid (OA) (0.5% w/v) was used as carbon source, a significant increase in biomass and PHA content were observed through fed-batch cultivation. The biomass and PHA content obtained from batch cultivation were 1.45 ± 0.19 g/l and 2.50 ± 0.77 % cell dry weight (CDW) respectively, while in the fed-batch cultivation, the biomass and PHA content increased significantly to 3.35 ± 0.78 g/l and 32.72 ± 0.30 %CDW respectively.

When saponified palm olein (SPO) (0.5% w/v) was used as a carbon source, the batch cultivation yielded 1.54 ± 0.03 g/l biomass and 3.40 ± 0.76 %CDW of PHA. In the fed-batch cultivation, there was no significant increase in biomass yield (1.9 ± 0.66 g/l) but there was a significant increase in PHA content (14.46 ± 1.18 %CDW).

In the batch cultivation, yield of PHA ($Y_{p/s}$), from OA and SPO were 8.05 mg/g and 10.47 mg/g respectively. The polymer obtained when either OA or SPO was used as a carbon source was a medium chain length PHA (MCL_{PHA}) with hydroxyoctanoic acid (C_8) being the major monomer.

Palm oil-mill effluent (POME) was chosen as the source to isolate and screen bacteria capable of utilising palm oil directly for growth and PHA accumulation. Bacterial isolates were screened by using Sudan Black B and Nile Blue A staining.

Out of 45 isolates, none was capable of utilising palm oil directly for growth. Isolate X4.13 was however, able to grow and accumulate PHA from SPO. When isolation was carried out by an enrichment technique, one isolate, FLP1, was found to be able to grow and accumulate PHA directly from crude palm oil, palm olein, palm stearin, palm kernel oil and oleic acid. The superior isolate was identified as *Pseudomonas* sp. The PHA was then analysed to be polyhydroxybutyrate (PHB), a short chain length PHA (SCL_{PHA}).

Thus, palm oil appears to be a suitable carbon source which could be converted to MCL_{PHA} as well as SCL_{PHA} depending on the bacteria metabolising the oil.

CONTENTS	PAGE
TITLE	i
ACKNOWLEDGEMENT	ii
ABSTRACT	iii
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF PLATES	xiii
LIST OF COMMON ABBREVIATIONS	xiv
CHAPTER 1 : OBJECTIVE OF THE PROJECT	
1.1 General Introduction	1
1.2 Objective	3
CHAPTER 2 : LITERATURE REVIEW	
2.1 Microbial Polyester	4
2.2 PHA from <i>Pseudomonads</i>	8
2.3 Triglycerides as Carbon Substrate	11
2.4 Fed-Batch Culture Feeding Strategies	15

CHAPTER 3 : MATERIALS AND METHODS

3.1 Sampling Site for Isolating Indigenous Bacteria	19
3.2 Isolation of Bacterial Strains	19
3.3 Screening for PHA-producing Bacteria	19
3.4 Isolation through Enrichment	20
3.5 Identification of Isolate	21
3.6 Bacterial Strain Information	21
3.7 Carbon Substrates	21
3.8 Fermentation	22
3.8.1 Seed Medium	22
3.8.2 Cultivation Conditions	22
3.8.3 Method for Fed-Batch Culture	23
3.9 Saponification of Palm Olein (PO)	24
3.10 Culture Broth Analysis	24
3.10.1 Ammonium	25
3.10.2 Fatty Acids	25
3.10.3 Viable Cell Count	25
3.10.4 Optical Density	26
3.10.5 Cell Dry Weight (CDW) Determination	26
3.11 PHA Extraction	27

3.12 PHA Analysis	28
3.12.1 Nuclear Magnetic Resonance (NMR)	28
3.12.2 Infrared (IR) Spectrometry	28
3.12.3 Gas Chromatography (GC)	28
3.12.3.1 Preparation of Sample	28
3.12.3.2 Quantitative Determination of Polymer Composition	29
CHAPTER 4 : RESULTS AND DISCUSSIONS	
4.1 Comparison Between Batch and Fed-Batch Cultivations of <i>P. oleovorans</i>	32
4.1.1 OA as the Carbon Substrate	32
4.1.2 SPO as the Carbon Substrate	37
4.2 Analysis of PHA Extracted from <i>P. oleovorans</i>	42
4.2.1 IR Analysis	43
4.2.2 ¹ H NMR Analysis	45
4.2.3 ¹³ C NMR Analysis	45
4.2.4 GC Analysis	50
4.3 Isolation and Screening of Isolates from POME	60
4.4 Isolation by Enrichment	64
4.5 Biomass, PHA Content and Monomer Composition of the Isolates	67

CHAPTER 5 : CONCLUSIONS

5.1 Conclusion	78
----------------	----

REFERENCES	83
------------	----

APPENDICES**APPENDIX A-Medium Preparation**

A.1 Nutrient Agar	96
-------------------	----

A.2 Nutrient Rich Medium	96
--------------------------	----

A.3 E2 Medium	96
---------------	----

A.4 Isolation Medium A	96
------------------------	----

A.5 Isolation Medium B	97
------------------------	----

APPENDIX B-Stain Preparation**B.1 Sudan Black B**

B.1.1 Material	97
----------------	----

B.1.2 Procedure	97
-----------------	----

B.2 Nile Blue A

B.2.1 Material	98
----------------	----

B.2.2 Procedure	98
-----------------	----

B.3 Gram Staining

B.3.1 Material 99

B.3.2 Procedure 99

APPENDIX C- Analytical Standard**C.1 Determination of Ammonium Content by Phenolhypochlorite Method**

C.1.1 Material 100

C.1.2 Ammonium Standard Graph 100

C.2 Determination of Fatty Acids Content by Titration

C.2.1 Material 101

C.2.2 Fatty Acids Standard Graph 101

LIST OF TABLES

Table 1 : Fatty Acids composition (%) of Palm Kernel Oil, Palm Oil, Palm Stearin and Palm Olein (Iftikar, PORIM, 1984).	13
Table 2 : Optical Density (660nm) of <i>P. oleovorans</i> grown on OA in batch and fed-batch systems.	34
Table 3 : Viable cell count of <i>P. oleovorans</i> grown on SPO in batch and fed-batch systems.	38
Table 4 : Data on the change of ammonium content of the cultures during 48 hours of cultivation in batch and fed-batch systems when <i>P. oleovorans</i> was cultivated on OA or SPO.	40
Table 5 : Comparison of biomass yield and PHA content obtained from batch and fed-batch systems when <i>P. oleovorans</i> was cultivated on OA or SPO.	41
Table 6 : Monomer composition of PHA extracted from <i>P. oleovorans</i> cultivated on OA or SPO. Results show the average mole % (n=2) as determined by gas chromatography of the β -hydroxyalkanoate methyl esters obtained by acid hydrolysis of the PHA polymers.	59
Table 7 : Biomass yield, PHA content and monomer composition obtained from isolates FLP1 and FLP2 cultured in different carbon sources.	68
Table 8 : Chemical shift assignments for ^{13}C resonance of PHB extracted from isolates FLP1 and FLP2 grown on different carbon sources.	70
Table 9 : Chemical shift assignments for ^1H resonance of PHB extracted from isolates FLP1 and FLP2 grown on different carbon sources.	72
Table 10 : Ammonium concentrations vs optical density (OD) 640nm. Data for standard graph.	102
Table 11 : Palmitic acid concentrations vs volume of titrated NaOH. Data for standard graph.	104

LIST OF FIGURES

Figure 1 : Chemical structure of poly- β -hydroxyalkanoate (Adapted from Doi, Y. 1990).	5
Figure 2 : Biosynthetic pathway of poly- β -hydroxybutyrate (Adapted from Griffin, 1994).	7
Figure 3 : Biosynthetic pathway of poly- β -hydroxyalkanoate through β -oxidation (Adapted from Eggink <i>et al.</i> , 1993).	10
Figure 4 : Optical density and ammonium content of cultures of <i>P. oleovorans</i> grown on OA.	35
Figure 5 : Viable cell count and ammonium content of cultures of <i>P. oleovorans</i> grown on SPO.	39
Figure 6 : IR spectrum of PHA extracted from <i>P. oleovorans</i> grown on (A) OA and (B) SPO.	44
Figure 7 : The ^1H NMR spectrum of PHA extracted from <i>P. oleovorans</i> grown on OA.	46
Figure 8 : The ^1H NMR spectrum of PHA extracted from <i>P. oleovorans</i> grown on SPO.	47
Figure 9 : The ^{13}C NMR spectrum of PHA extracted from <i>P. oleovorans</i> grown on OA	48
Figure 10 : The ^{13}C NMR spectrum of PHA extracted from <i>P. oleovorans</i> grown on SPO.	49
Figure 11 : The GC chromatogram of polyhydroxyalkanoic acid methyl ester Standards. Benzoic acid methyl ester is the internal standard (0.1% v/v).	52
Figure 12 : Standard graph for retention time vs carbon number of 3-hydroxyalkanoate methyl ester.	53

Figure 13 : Standard graph for response factor vs concentration of methyl esters of 3-hydroxyalkanoates (C8,C10,C12,C14,C16).	54
Figure 14 : Standard graph for response factor vs carbon number of 3-hydroxyalkanoate methyl ester of different concentrations.	55
Figure 15 : Standard graph for response factor vs concentration of methyl esters of 3-hydroxyalkanoates (C4,C6).	56
Figure 16 : The GC chromatogram of the PHA composition accumulated by <i>P. oleovorans</i> grown on OA.	57
Figure 17 : The GC chromatogram of the PHA composition accumulated by <i>P. oleovorans</i> grown on SPO.	58
Figure 18 : The ^{13}C NMR spectrum of PHA extracted from isolate FLP1 grown on palm olein.	71
Figure 19 : The ^1H NMR spectrum of PHA extracted from isolate FLP1 grown on palm olein.	73
Figure 20 : The GC chromatogram of the (A) PHA extracted from isolate FLP1 grown on palm olein and (B) standard 3-hydroxybutyric acid methyl ester.	74
Figure 21 : Data from BIOLOG shows the isolate FLP1 resembles <i>Burkholderia cepacia</i> (<i>Pseudomonas cepacia</i>) at 80% Similarity.	77
Figure 22 : Standard graph for ammonium nitrogen.	103
Figure 23 : Standard graph for fatty acid.	105

LIST OF PLATES

- Plate 1 : An isolate (48 hours culture) negatively-stained with Sudan Black B (400X magnification). 62
- Plate 2 : An isolate (48 hours culture) positively-stained with Sudan Black B (400X magnification). 62
- Plate 3 : An isolate (48 hours culture) positively-stained with Nile Blue A (400X magnification). PHA granules appear fluorescent orange. 65
- Plate 4 : Observation of growth based on change in turbidity and colour of culture broth at 48 hours of cultivation in E2 medium (1% w/v PO).
(A) Sterile medium (B) *Pseudomonas oleovorans*
(C) Isolate FLP1 (D) Isolate FLP2. 66

LIST OF COMMON ABBREVIATIONS

ATP	adenosine triphosphate
CDW	cell dry weight
CFU	colony forming unit
CoASH	free coenzyme A
DO	dissolved oxygen
FAO	fatty acid oxidative enzyme
g/l	gram per litre
GC	gas chromatography
IR	infra red
LCFA	long chain fatty acid
LCL	long chain length
LPS	lipopolysaccharide
MCFA	medium chain fatty acid
MCL	medium chain length
NA	nutrient agar
NADP	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
OA	oleic acid

OD	optical density
P(3HB)	poly-3-hydroxybutyric acid
P(3HV)	poly-3-hydroxyvaleric acid
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
PKO	palm kernel oil
PO	palm olein
POME	palm oil-mill effluent
r.p.m.	rotation per minute
RF	response factor
SCFA	short chain fatty acid
SCL	short chain length
SD	standard deviation
SPKO	saponified palm kernel oil
SPO	saponified palm olein
TCA	tricarboxylic acid
v/v	volume per volume
w/v	weight per volume