VOT 74262

POLYHYDROXYALKANOATES (PHA) PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) USING MIXED CULTURES IN SEQUENCING BATCH REACTOR (SBR)

(PENGHASILAN POLIHYDROKSIALKANOATES (PHA) DARI EFLUEN KILANG KELAPA SAWIT MENGGUNAKAN KULTUR CAMPURAN DI DALAM REAKTOR JUJUKAN BERKELOMPOK (SBR))

MUZAFFAR ZAINAL ABIDEEN

MOHD. FADHIL BIN MD. DIN

ZAINI UJANG

SALMIATI

RESEARCH VOT NO:
74262

Jabatan Alam Sekitar
Fakulti Kejuruteraan Awam
Universiti Teknologi Malaysia

2007

UNIVERSITI TEKNOLOGI MALAYSIA

BORANG PENGESAHAN
LAPORAN AKHIR PENYELIDIKAN

TAJUK PROJEK **:** **POLYHYDROXYALKANOATES (PHA) PRODUCTION FROM PALM OIL MILL EFFLUENT (POME) USING MIXED CULTURES IN SEQUENCING BATCH REACTOR (SBR)**

Saya **MUZAFFAR ZAINAL ABIDEEN**
(HURUF BESAR)

Mengaku membenarkan **Laporan Akhir Penyelidikan** ini disimpan di Perpustakaan Universiti Teknologi Malaysia dengan syarat-syarat kegunaan seperti berikut :

1. Laporan Akhir Penyelidikan ini adalah hakmilik Universiti Teknologi Malaysia.
2. Perpustakaan Universiti Teknologi Malaysia dibenarkan membuat salinan untuk tujuan rujukan sahaja.
3. Perpustakaan dibenarkan membuat penjualan salinan Laporan Akhir Penyelidikan ini bagi kategori TIDAK TERHAD.
4. * Sila tandakan (/)

☐	SULIT	(Mengandungi maklumat yang berdarjah keselamatan atau Kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972).
☐	TERHAD	(Mengandungi maklumat TERHAD yang telah ditentukan oleh Organisasi/badan di mana penyelidikan dijalankan).
√	TIDAK TERHAD	

TANDATANGAN KETUA PENYELIDIK

MUZAFFAR ZAINAL ABIDEEN

Nama & Cop Ketua Penyelidik

CATATAN : ***** *Jika Laporan Akhir Penyelidikan ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh laporan ini perlu dikelaskan*

ABSTRACT

Polyhydroxyalkanoates (PHAs) are raw materials for production of biodegradable plastics, generated by a range of microbes, cultured under different nutrients and experimental conditions. PHAs usually lipid in nature, are accumulated as storage materials in the form of mobile, amorphous, and liquid granules. Currently, the main limitation for PHAs production is cost of production. Biodegradable plastics from renewable resources, such as PHAs, are alternative to petroleum-based plastic materials, which are non-biodegradable.

The aim of this study was to develop a biological process to produce PHAs from palm oil mill effluent (POME). A fed-batch was utilized for fifteen months for POME particularly to optimize the PHAs production under various experimental conditions.

The POME cultivation was studied under six experimental conditions, such as COD:N:P ratios, HRT=SRT, air flowrates, substrates feeding rates, anoxic/aerobic and microaerophilic-aerobic. The production rate of PHAs under feast-famine regime occurred rapidly between three to four hours during the substrate uptake rate. The results showed that a short chain fatty acid (especially acetic acid) from POME was considered the most optimum carbon source for PHAs production in the study. The optimum experimental condition for high PHAs production from POME recorded in the cycling of microaerophilic-aerobic experiments with a combination of COD/N:COD/P ratio (490:200 g/g), long retention time (6 to 10 h) and slow feeding rate (20 ml/min). This study showed that the increased of PHAs production would not necessarily enhance the removal of total organic carbon (TOC), phosphate (PO_4-P) and nitrate (NO_3-N). TOC removal was recorded at range 18 to 33%, while PO_4-P and NO_3-N removal did not show any consistent trend.

A statistical design of experiment was conducted to optimize the PHAs production and organic removal (TOC, PO_4-P and NO_3-N). Results from response surface method (RSM) analysis, both COD/N:COD/P ratio and air flowrate showed significant influence on PHAs production, TOC, and NO_3-N removal. It can be concluded that the PHAs storage capacity was higher two to three times in aerobic compared to anoxic conditions.

ABSTRAK

Polyhidroksialkanoat (PHAs) merupakan bahan asas plastik terbiorosot yang dihasilkan daripada kepelbagaian mikroorganisma dan dikulturkan menerusi pelbagai keadaan nutrien dan pengaruh eksperimen. PHAs secara semulajadinya adalah lipid dan dikumpulkan sebagai bahan penyimpanan dalam bentuk yang tidak tetap, amorfus dan berbentuk cairan pepejal. Halangan utama penghasilan PHAs ini adalah disebabkan oleh kos operasi yang tinggi. Plastik terbiorosot daripada sumber yang boleh diperbaharui seperti PHAs merupakan alternatif kepada bahan plastik yang berasaskan petroleum.

Matlamat utama kajian ini adalah untuk merekabentuk proses biologi dalam penghasilan PHAS daripada effluent kilang kelapa sawit (EKKS). Kajian secara suapan-kelompok ini telah dilaksanakan selama lima belas bulan bagi meningkatkan penghasilan PHAS dalam pelbagai keadaan eksperimen. Pengkulturan MBM dikaji dengan menggunakan empat keadaan eksperimen iaitu nisbah COD:N, kadar alir udara (*Air*), suhu dan HRT=SRT.

Bagi pengkulturan EKKS, ia telah dijalankan menggunakan enam keadaan eksperimen iaitu nisbah COD:N:P, HRT=SRT, *Air*, kadar suapan substrat, anosik/aerobik dan mikroaerofilik-aerobik. Kadar penghasilan PHAs menggunakan pengaruh *feast-famine* berlaku dengan cepat, di antara tiga hingga empat jam semasa fasa pengambilan substrat. Kajian ini juga mendapati bahawa asid lemak rantaian pendek (terutamanya asid asetik) daripada EKKS adalah sumber karbon yang optimum untuk penghasilan PHAs. Hasil eksperimen yang optimum bagi penghasilan PHAs yang tinggi dicatatkan semasa kajian kitaran mikroaerofilik-aerobik dengan kombinasi nisbah COD/N:COD/P (490:200 g/g), masa tahanan yang lama (6 hingga 10 jam) dan kadar suapan yang perlahan (20 ml/min). Kajian ini mendapati bahawa peningkatan PHAs tidak semestinya akan meningkatkan penyingkiran jumlah organik karbon (TOC), fosfat (PO_4-P) dan nitrat (NO_3-N). Penyingkiran TOC hanya mencapai julat antara 18 hingga 33% sahaja, manakala penyingkiran PO_4-P dan NO_3-N menunjukkan peratusan yang tidak konsisten.

Satu analisis rekabentuk-eksperimen telah digunakan untuk mendapatkan nilai optimum bagi penghasilan PHAs dan penyingkiran organik (TOC, PO_4-P dan NO_3-N). Hasilnya, daripada analisis kaedah tindakbalas permukaan (*RSM*) mendapati bahawa nisbah COD/N:COD/P dan DO merupakan pengaruh utama kepada penghasilan PHAs, penyingkiran TOC dan NO_3-N. Kajian juga mendapati bahawa keupayaan penyimpanan PHAs diperolehi lebih tinggi semasa aerobik berbanding ketika keadaan anosik.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	BORANG PENGESAHAN	i
	TITLE	ii
	ABSTRACT	iii
	ABSTRAK	iv
	TABLE OF CONTENTS	v
	LIST OF TABLES	ix
	LIST OF FIGURES	xiv
	LIST OF SYMBOLS	xviii
	LIST OF ABBREVIATIONS	xx
	LIST OF APPENDICES	xxvi
1	**INTRODUCTION**	1
	1.1 Biodegradable Plastics	1
	1.2 Background of the Study	2
	1.3 POME in Perspective	4
	1.4 Objectives of the Study	5
	1.5 Scope of the Study	6
	1.6 Significance of the Study	7

2	**LITERATURE REVIEW**	8
	2.1 Introduction	8
	2.2 Waste Generation from POME	8
	2.3 PHA as Biodegradable Plastics	12
	2.3.1 Chronological Development of PHA/PHB	13
	2.3.2 Industrial and Commercialization of PHA/PHB	16
	2.3.3 Structure and Biosynthesis of PHAs	20
	2.3.4 Development of the Bioplastics	24
	2.4 Production Cost of PHA	25
	2.5 Applications of Biodegradable Plastics	28
	2.5.1 Medical and Pharmaceutical Applications	28
	2.5.2 Agricultural Applications	29
	2.5.3 Biodegradable Commodity Packaging	29
	2.6 PHA Production in Selected Cultivations	29
	2.6.1 Mixed Cultures and Feast/Famine Regimes	30
	2.6.2 Dynamic Aerobic and Microaerophilic – Aerobic Condition	34
	2.6.3 Nitrification – Denitrification (Aerobic/Anoxic) Condition	37
	2.7 Configuration of PHA Productions	40
	2.7.1 Fed-Batch Cultures (Feed Substrate and Growth Condition)	41
	2.7.2 Bacterial Strain	45
	2.8 Renewable Resources for PHA Production	46
	2.9 Biodegradation of PHA in Waste Environment	48
3	**METHODOLOGY**	52

	3.1	Research Design and Procedure	63
	3.2	The Framework of the Study	53
	3.3	Scope of the Study	54
	3.4	Experimental Set-Up	56
		3.4.1 Methods and Experimental Procedures	57
		3.4.2 Experiment Procedures on SO as Substrate	60
	3.5	Analytical Procedures	61
		3.5.1 Oxygen Uptake Rate/Oxygen Transfer Rate (OUR/OTR) Measurement	63
	3.6	Specific Calculations	65
	3.7	Statistical Optimization Process	69
4	**RESULTS AND DISCUSSION: PHA PRODUCTION, ORGANIC AND NUTRIENT BEHAVIOUR IN PALM OIL MILL EFFLUENT (POME)**		71
	4.1	Introduction to POME Cultivation	71
	4.2	Respirometric Analysis	72
	4.3	Overall Performance in POME Cultivations	79
		4.3.1 PHA Production in Biomass Components	79
		4.3.2 Specific and Kinetic Rates on Substrates, Biomass and PHA	83
		4.3.3 Fatty Acid Uptakes for PHA Constituents	88
		4.3.4 Kinetic Rates of PHA Degradation	93
		4.3.5 Statistical Analysis	95
		4.3.6 Mass Balance of Substrates During	102

Feast-Famine Period

4.4 Development of PHA Productivity (Δf_{PHA}) 106
4.5 Discussion on Specific Findings 108
4.6 Comparative Study 114

5 RESULTS AND DISCUSSION: DESIGN OF PHA PRODUCTION, ORGANIC AND NUTRIENT REMOVAL IN PALM OIL MILL EFFLUENT (POME) USING RESPONSE SURFACE METHOD (RSM) 115

5.1 Introduction to Optimization Process and Response Surface Method (RSM) 115
5.2 Model Diagnostic 121
5.3 Prediction of PHA Production, TOC, NO_3 and PO_4 Removal 126
5.3.1 Response Surface Analysis 127
5.3.2 Optimization Analysis 132
5.3.3 Overall Analysis 136

6 CONCLUSION, SUGGESTION AND RECOMMENDATION 140

6.1 General Observations 139
6.2 Conclusion from This Study 141
6.4 Recommendations and Future Studies 141

REFERENCES 143
Appendices A – C 166 - 183

LIST OF TABLES

TABLE NO.	TITLE	PAGE
1.1	Prevailing effluent discharge standards for crude palm oil (CPO) mills	5
2.1	Typical characteristics of combined raw POME	10
2.2	PHA production from various species of bacteria	17
2.3	Microorganisms and raw materials used for the production of biodegradable plastics along with the names of their manufacturers	18
2.4	Effect of substrate cost and PHB yield on the production cost of PHB	26
2.5	The average PHA productions from selected studies	30
2.7	An overview of waste streams suitable for PHA production	47
2.8	Comparison of emissions for PHA production by mixed cultures, pure culture and the production of PP	48
2.9	Biodegradability of PHB in various environments	50
3.1	Typical value of raw POME	60

3.2	Operating phase with POME as substrates	61
4.1	Comparison concentration of COD fractionation	72
4.2	COD-fractionation after the treatment of POME	74
4.3	Comparison of respirometric analysis on continuous and batch cultures	78
4.4	Biomass components and PHA accumulation during feast period for various experimental works using diluted POME.	80
4.5	Accumulation of PHB content in various operational conditions under acclimatization of biodegradable substrates (S_s and X_s), Y_H and μ.	84
4.6	Summary of PHA constituents produced during feast-famine regime at various experimental works	92
4.7	Statistical analysis for every experimental works	99
4.8	Converted amounts in (C) mmol/cycle for all compounds at selected operating conditions with standard fed-batch system	104
4.9	Comparison of converted amounts for measured compounds in aerobic pulse-fed SBR processes	105
4.10	Comparative study on anoxic/aerobic experiments	111
5.1	The variables and their levels for the CCRD experimental design	118
5.2	Experimental runs conducted in dynamic aerobic study (data shown was not in random order)	119
5.3	ANOVA and regression analysis for selected responses	123
5.4	Results of the regression analysis of the CCRD	127
5.5	Summary of ANOVA in response surface regression	128
5.6	Statistical analysis of different factors used in the optimization study for the PHA production, organic and nutrient removal in the bioreactor	132
5.7	Numerical optimization for factorial design with	133

	the POE	
5.8	Numerical optimization with the overall predicted and desirability obtained from response optimizer with the POE.	133

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	Proposed cycle loop of regenerating waste from POME to biodegradable plastics, end-up with preventing pollution load to environment.	12
2.2	The development of PHA science and technology through the twentieth century	14
2.3	Example of biodegradable plastic manufacturing by several companies	19
2.4	The established biodegradable polyester family	20
2.5	Chemical structure of PHAs produced in bacteria. n^{th} will be available from 100 – 3000	21
2.6	Pathways involved in PHB and PHBV biosynthesis.	26
2.7	PHB production pathways in feast-famine regimes	32
2.8	Cyclic nature of PHA metabolism from synthesis to degradation (during famine period)	34
3.1	Overall studies undertaken to enhance the production rate of PHA	53
3.2	Scope of the study	54
3.3	Experimental set-up for overall processes	56
3.4	Typical cultivation cycle under SBR processes (clockwise sequences)	58
3.5	Schematic diagram for SBR fed-batch	59

	bioreactor	
3.6	SBRs used in this study, (a) 2 liters reactor and, (b) 6 liters reactor that are used in laboratory conditions	59
3.7	Example of a respirogram, where a pulse of organic substrate is added	63
3.8	Schematic representation of the OUR measurement set-up.	64
4.1	Comparison of OUR analysis during PHA accumulation (no nutrient) using diluted POME and, (b) growth phase (nutrient available) using raw POME.	76
4.2	Specific PHA production and substrate uptake rate at different operational and culture experiments. (a) COD:N:P ratio experiments, (b) air flowrate experiments, (c) cycle length experiments, (d) feeding rate experiments, (e) anoxic/aerobic experiments, (f) microaerophilic-aerobic experiments	86
4.3	VFAs concentrations and their percentage being utilized at several experiment works. (a) COD:N:P ratio experiments, (b) air flowrate experiments, (c) cycle length experiments, (d) feeding rate experiments, (e) anoxic/aerobic experiments, (f) microaerophilic-aerobic experiments.	90
4.4	Curve estimated of PHB degradation using differential method. (a) COD:N:P ratio experiments, (b) air flowrate experiments, (c) cycle length experiments, (d) feeding rate experiments, (e) anoxic/aerobic experiments, (f) microaerophilic-aerobic experiments	94
4.5	Degradation of PHB under different fitted	95

	conditions	
4.6	Summary on PHA correlation to other organic and nutrient removal at various experimental works. (a) COD:N:P ratio experiments, (b) air flowrate experiments, (c) cycle length experiments, (d) feeding rate experiments, (e) anoxic/aerobic experiments, (f) microaerophilic-aerobic experiments	101
4.7	PHA produced on COD:N:P ratio, air flowrates, HRT=SRT, feeding rates, anoxic/aerobic and microaerophilic-aerobic	107
4.8	Influences of PHA content on the overall COD:N:P ratio in a standard aerobic experiments	109
4.9	Result for the relative length of the feeding period (FR_{pome} experiment) in fed-batch SBR on the growth rate of bacteria in feast period (bottom line), and on the cellular content of PHA at the end of the feast (dashed line) and famine periods (dotted line)	110
4.10	Changes of PHA production and CDW at 70% and 20% of microaerophilic conditions	113
4.11	Comparative works for specific substrate consumption ($-q_s^{feast}$) and specific (q_p^{feast}) with concerning this study	115
5.1	The flow chart of the modeling and optimization process using MINITAB^{TM}	117
5.2	Residual diagnostics of response model for %PHA: (a) histogram, (b) normal probability, (c) deleted residual vs. observation order, (d) deleted residual vs. fitted value.	122
5.3	Residual model diagnostic for %PHA in four	124

	variables (CODNP ratio, air flowrate, HRT=SRT and feeding rate): (a) normal plot distribution, (b) I-Chart for single observation, (c) histogram pattern, (d) Fitted trend for predicted value.	
5.4	Response surface plot showing variation in prediction PHA production	129
5.5	Response surface plot showing variation in prediction of TOC removal	130
5.6	Response surface plot showing variation in prediction of PO_4 removal	131
5.7	Response surface plot showing variation in prediction of NO_3 removal.	131
5.8	Response optimizer for best factor-response analysis	135
5.9	Pareto chart for PHA production, organic and nutrient removal at different variables (α: 0.1; A: CODNP; B: Air; C: HRT; D: FR). Line of significance is depicted as dotted line and determined by MINITAB™	138
5.10	Main effects plot for PHA production, TOC, PO_4 and NO_3 removal.	139

LIST OF SYMBOLS

CO_2	-	carbon dioxide (mg/l or mmol/l)
C_s	-	substrate concentration (C-mmol/l)
C_x	-	active biomass concentration (C-mmol/l or mg/l or g/l)
$NaOCl_3$	-	sodium hypochlorite
$CHCl_3$	-	chloroform
f_{PHB}	-	fraction of PHB of the total active biomass (C-mol/C-mol)
HAc	-	acetic acid (C-mmol)
HBt	-	butyric acid (C-mmol/l)
HPr	-	propionic acid (C-mmol/l)
k_{fPHA}^{feast}	-	coefficient of PHA production during feast (1/h)
(-) k_{fPHA}^{famine}	-	coefficient of PHA degradation during famine (1/h)
K_{La}	-	oxygen transfer coefficient (1/min)
m_{ATP}	-	maintenance coefficient based on ATP (mol/C-mol. h)
m_p	-	maintenance coefficient for growth on PHB (C-mol/C-mol. h)
m_s	-	maintenance coefficient for growth on acetate (C-mol/C-mol. h)
$M_{W\ (PHB)}$	-	molecular weight of PHB = 21.5 g/Cmol
$M_{W\ (X + ash)}$	-	molecular weight of biomass = 25.1 g/Cmol
NH_4^+-N	-	ammonium-nitrogen (mg/l or mmol/l)
NO_3	-	nitrate (mg/l or mmol/l)
O_2	-	oxygen (mg/l or mmol/l)
PO_4	-	phosphate (mg/l or mmol/l)
$-q_p^{famine}$	-	specific PHB consumption rate in the famine period (C-mmol/l. h)
q_p^{feast}	-	specific PHB synthesis rate in the feast period

		(C-mmol/l. h)
$-q_s^{famine}$	-	specific substrate uptake rate in the famine period (C-mmol/l. h)
$-q_s^{feast}$	-	specific substrate uptake rate in the feast period (C-mmol/l. h)
$-q_s^{feast}$	-	specific substrate uptake rate in the feast period (C-mmol/l. h)
r_p	-	PHA production rate in the feast period (C-mmol/l. h)
$-r_s$	-	substrate uptake rate in the feast period (C-mmol/l. h)
r_x	-	biomass growth rate (C-mmol/l. h)
S_s	-	readily biodegradable matter (g/l)
X	-	active biomass (g/l or C-mmol/l)
X_H	-	heterotrophic organism in concentration
X_s	-	slowly biodegradable matter (g/l)
X_{STO}	-	storage polymer in biomass component
Y_{COD}	-	yield on biomass COD over substrate utilization COD (C-mol/C-mol)
Y_H	-	yield on heterotophs organism (COD/COD)
$Y_{p/s}^{feast}$		Yield of PHA production over substrate uptake during feast period
$Y_{p/x}^{famine}$	-	yield on PHA formation over biomass production in the famine period (C-mol/C-mol)
$Y_{p/x}^{feast}$	-	yield on PHA formation over biomass production in the feast period (C-mol/C-mol)
Y_{sx}^{famine}	-	yield on biomass production for limiting substrate utilization in the famine period (C-mol/C-mol)
Y_{sx}^{feast}	-	yield on biomass production for substrate utilization in the feast period (C-mol/C-mol))
δ	-	$P/2e^-$ ratio = ATP produced per $NADH_2$ oxidized (mol/mol)
Δf_{PHA}	-	fraction of PHA production over biomass (C-mmol/l)
μ^{famine}	-	specific growth rate in famine period (h^{-1})
μ^{feast}	-	specific growth rate in feast period (h^{-1})
$\mu^{overall}$	-	average specific growth rate over one cycle (h^{-1})

LIST OF ABBREVIATIONS

AN	-	ammoniacal nitrogen (mmol/l or mg/l or g/l)
ANOVA	-	analysis of variance
ASM	-	activated sludge model
ATP	-	adenosine triphosphate
ATU	-	allylthiourea
BNR	-	biological nutrient removal
BPR	-	biological phosphorus removal
bsCOD	-	COD biodegradability soluble
CCRD	-	central composite rotatable design
CDW	-	cell dry weight (g/l)
COD	-	chemical oxygen demand (C-mmol/l or mg/l or g/l)
CPO	-	crude palm oil
D	-	overall desirability in POE
d	-	local desirability in POE
DAEs	-	differential algebraic equations
DAPS	-	data acquisition
DO	-	dissolved oxygen (oxygen saturation) (%O2 or mg/l)
DoE	-	Department of Environment
DOE	-	design of experiment
DW	-	Durbin-Watson test
EBPR	-	enhanced biological phosphorus removal
EFB	-	empty fruit bunch
EKKS	-	effluen kilang kelapa sawit
EME	-	Electron Microscope Examination
FFB	-	fresh fruit bunch

GAOs	-	glycogen accumulating organisms
GC	-	gas chromatography
HRT	-	hydraulic retention time (h)
ICI	-	Imperial Chemical Industries
LCFA	-	long-chain-fatty-acid
LOFT	-	lack-of-fit
MBM	-	minyak bunga matahari
MCFA	-	medium-chain-fatty-acid
MPOB	-	Malaysian Palm Oil Board
N & P	-	nitrogen and phosphorus
NA	-	nutrient agar
NADH	-	nicotinamide adenine dinucleotide
NB	-	nutrient broth
NBA	-	Nile Blue A
NO_2^- - N	-	nitrite –nitrogen (mg/l or mmol/l)
NO_3^-- N	-	nitrate-nitrogen (mg/l or mmol/l)
OTR	-	oxygen transfer rate (mg O_2/l. min or mmol/l. h)
OUR	-	oxygen uptake rate (mg O_2/l. min or mmol/l. h)
P(3HB)	-	poly(3-hydroxybutyrate)
P(3HB-co-3HV)	-	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HV)	-	poly(3-hydroxyvalerate)
P(4HB)	-	poly(4-hydroxybutyrate)
PAOs	-	polyphosphate accumulating organisms
PE	-	polyethylene
PGA	-	polygalvanic acid
PHA	-	polyhydroxyalkanoate
PHA_{MCL}	-	polyhydroxyalkanoates (medium-chain-length)
PHA_{SCL}	-	polyhydroxyalkanoates (short-chain-length)
PHB	-	poly-β-hydroxybutyrate
PHH	-	poly-β-hydrohexanoate
PHV	-	poly-β-hydrovaleric
PLA	-	polylactides acid

PO	-	palm oil
PO_4^{3-}/PO_4^-	-	orthophosphate / phosphate (mmol/l or mg/l)
POE	-	propagation of error
POME	-	palm oil mill effluent
PP	-	polypropylene
PS	-	polystyrene
rbCOD	-	readily biodegradable COD
RSD	-	response surface design
RSM	-	response surface method
sbCOD	-	slowly biodegradable COD
SBR	-	sequencing batch reactor
SCFA	-	short-chain-fatty-acid
SED	-	statistical experiment design
SO	-	sunflower oil
SRT	-	sludge retention time (h)
SRT_{true}	-	true sludge retention time occurs in the system (h)
SUR	-	substrate uptake rate (COD mg /g SS. h)
TCA	-	tricarboxylic acid
Temp	-	temperature (^oC)
TOC	-	total organic carbon (C-mmol/l or mg/l or g/l)
VFAs	-	volatile fatty acids
VIF	-	variance inflation factor
VSS	-	volatile suspended solid (g/l)

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
Table A1	General definitions of a biodegradable polymer	167
Table A2	Classification of plastics	168
Table B1	PHA production by microorganisms	170
Figure E1	ANOVA single factor and regression analysis on PHA production for POME cultivation	173
Figure E2	ANOVA single factor and regression analysis on TOC removal for POME cultivation	173
Figure E3	ANOVA single factor and regression analysis on PO_4 removal for POME cultivation	174
Figure E4	ANOVA single factor and regression analysis on NO_3 removal for POME cultivation	175
Table E1	Rank and percentile analysis for PHA production, TOC, PO_4^- and NO_3^- removal at limitation of N and P	175
Table E2	Rank and percentile analysis for PHA production, TOC, PO_4^- and NO_3^- removal at controlling oxygen flowrates	176
Table E3	Rank and percentile analysis for PHA production, TOC, PO_4^- and NO_3^- removal at cycle length behaviour	176
Table E4	Rank and percentile analysis for PHA production,	177

	TOC, PO_4^- and NO_3^- removal at feeding rate mechanisms	
Table E5	Rank and percentile analysis for PHA production, TOC, PO_4^- and NO_3^- removal at anoxic/aerobic conditions	177
Table E6	Rank and percentile analysis for PHA production, TOC, PO_4^- and NO_3^- removal at microaerophilic/aerobic conditions	178
E1	Response Surface Regression: %PHA versus CODNP, Air, HRT, FR	178
E2	Response Surface Regression: %TOC versus CODNP, Air, HRT, FR	179
E3	Response Surface Regression: $\%PO_4$ versus CODNP, Air, HRT, FR	180
E4	Response Surface Regression: $\%NO_3$ versus CODNP, Air, HRT, FR	181
E5	Response optimization	181

CHAPTER I

INTRODUCTION

1.2 Biodegradable Plastics

The current worldwide demand for plastics is in excess of 150 million tonnes per year (Chrissafis *et al.*, 2005; Punrattanasin, 2001). The rapid growth of plastic consumption in recent years has led to concerns from consumers, environmentalists and indeed the plastic industry, regarding the effective management of post consumer waste and greater use of, and dependence on fossil fuels. The present emphasis is on minimizing the unnecessary use of plastics and developing methods of recovery and recycling. Alongside and compatible with these developments, several projects have been carried out searching for alternatives to reduce the environmental impact of plastics. One alternative could be the use of biodegradable plastics. Unfortunately, the term 'biodegradable' has not been applied consistently, resulting in confusion related to its degradation rate and applications. Deterioration or loss in physical integrity of a material is often mistaken for biodegradable. Biodegradable, however, is a natural and complex process of decomposition facilitated by biochemical mechanisms. In the present work the definition of 'biodegradable' as well as 'biodegradability' for plastics according to standardized test methods (Müller *et al*., 2001; Müller, 1994) and DIN 54900-2 (1998) are used, since they are the most stringent ones among the definitions laid down by ASTM, CEN, and ISO (Table A1, Appendix A).

1.3 Background of the Study

Many studies have shown that synthetic plastics (synthetic polymer) are highly resistant to microbial degradation in landfills (Chrissafis *et al.*, 2005; Vivero *et al*., 2004; Braunegg *et al*., 2002). Synthetic polymers, designated as plastics, have become technologically significant since 1940s for replacing glass, wood, masonry and other constructional materials, and even metals in many industrial, domestic, commercial and environmental applications (Liu *et al*., 2005; Cain, 1992). These widespread applications are not only due to their favourable mechanical and thermal properties, but mainly due to stability and durability of plastics. On the other hand, plastics also play an important role for many "short live" applications such as packaging and commodity, as well as hygienic products which represent the major part of plastic wastes (Jerez *et al.*, 2005; Witt *et al*., 1997). Due to persistence character in the environment, the increased costs of solid waste disposal (owing to the reductions in available landfill space), as well as the potential hazards from waste incineration (such as dioxin emission from polyvinyl chloride incineration), plastics has become more and more a waste disposal problem. Slow biodegradation rates of plastic materials have created a need for alternative materials with physical and industrial properties similar to petrochemical derived plastics but are well biodegradable (Table A2, Appendix A).

Consequently, the past two decades have witnessed a growing public and scientific concern regarding the use of biodegradable materials. It is an ecologically potential alternative to conventional plastics offering a solution for the existing grave problem of plastic waste (Ali and Siddiqui, 2005; Bichler *et al*., 1993). Biodegradable plastics do neither contribute to plastic litter nor lead to the depletion of finite resources. The biodegradable plastics from renewable resources origin might be an alternative product for the particular plastic usage today. Current research interest in biodegradable plastics is connected with well-defined areas of application. A number of biodegradable plastics, mostly biodegradable polyesters, have indeed been successfully developed over the last few years to meet the specific demands in various fields and industries (Domenek *et al*., 2004; Gáspár *et al*., 2005). At the end of their useful life, biodegradable plastic derived from

polyhydroxyalkanoates (PHAs) produced in most of microorganisms can be conventionally managed and recycled, landfilled, or incinerated, or they can be returned to nature through biodegradation.

A large amount of organic acids from waste effluent can be used as raw materials for biodegradable material or biopolymer productions (PHAs); thus providing an opportunity to reduce the pollution load. In general, the benefit will be found when these 'cheap' substrates can be used as carbon source for producing bioplastics. Therefore, the production cost can be reduced if 'low quality' substrates are used. Low cost (waste-based) substrates have only recently been recognized for PHAs production (Pozo *et al*., 2002; Wong *et al*., 2002; Carucci *et al*., 2001; Ahn *et al*., 2000 and van Aalst-van Leeuwen *et al*., 1997) because industrial producers are traditionally working towards decreasing the cost price of the biopolymers by increasing the volumetric production capacity of bioreactor systems and improving process technologies. The proposed culture can easily adapt to changes in the composition of the waste materials. In addition, because the processes operate with mixed natural cultures, there is no need for sterilization procedures or expensive sterilizable bioreactor to promote PHAs constituents.

Hassan *et al.* (1996, 1997a, and 2002) found that 7 g/l of organic acids could be obtained from POME. By maintaining pH at 7, mainly acetic and propionic acid can be produced from POME, which is suitable in enhancing the PHAs production in a continuous process. The treated POME and the utilization of these organic acids have been conducted in several experimental studies (Hassan *et al.*, 2002; Nor Aini *et al*., 1999). However, the final PHAs production in these studies was obtained with pure cultures (sterile conditions). Therefore, this study aims to optimize the PHAs production using POME as carbon sources using mixed cultures. The problem statements in this study are as follows**:**

(a) The substances exist in POME could support microbial growth and simultaneously increase the PHAs yield.

(b) The productivity of PHAs (yield rate) can be improved by using mixed culture. This can be achieved by maintaining the biomass growth (the storage activity must be higher than growth mechanisms).

(c) The limiting factors for the feasibility of storage polymer production (PHAs) in POME must be determined at the beginning of accumulation stage.

(d) The optimization of cultures can be formulated by using specific model during starvation periods (feast period).

1.3 POME in Perspective

Malaysia is the world's largest producer of crude palm oil (CPO). Downstream oil palm products in Malaysia account for more than half of the world's output. The palm oil industry has continued to expand for the past 25 years: from the 2.5 million tonnes in 1987 to 13.5 million tonnes in 2003 (Shirkie and Ji, 2004). The palm oil industry is evolving with new technologies, products, processes and markets. Generally, palm oil processing generates many by-products and liquid wastes. For example, 9.9 million tonnes of solid wastes consisting of oil-palm empty bunch, fibre and fruit shell will generate 10 million tonnes of POME (Jaafar and Sukaimi, 2001). POME discharged is always a highly concentrated waste. In a conventional palm oil mill, 600-700 kg of POME is generated for every 1000 kg of processed fresh fruit bunches (FFB) (Hassan *et al.*, 1997b, 2002). In addition more than 30 million tonnes of solid waste is generated in Malaysia in the form of empty fruit bunches (EFB), oil palm fronds and trunks. In Malaysian mills, the Department of Environment (DoE) requires appropriate treatment processes of POME before it can be discharged to watercourse. Table 1.1 presents the required effluent standards for POME in Malaysia according to the Second Schedule of the Environmental Quality (Prescribed Premises) (Crude Palm Oil) Regulation 1977. The effluent discharge level was referred as the typical standard after 1984. However, the

standard will be enforced to the stringent limit (as an example BOD is less than 20 mg/l) under the authorization of DoE.

Table 1.1: Prevailing effluent discharge standards for crude palm oil (CPO) mills

Parameter	Parameter Limits for Crude Palm Oil Mills (Second Schedule)	Remarks
pH	5 – 9	
Biological oxygen demand (BOD)	100	
Chemical oxygen demand (COD)	*	
Total solids (TS)	*	
Suspended solids (SS)	400	
Ammoniacal nitrogen (AN)	150	Value of filtered sample
Total Nitrogen (TN)	200	Value of filtered sample
Oil and grease (O&G)	50	

Source: Second Schedule of the Environmental Quality (Prescribed Premises) (Crude Palm Oil) Regulation 1977

Notes: All in mg/l except pH

* No discharge standard after 1984.

The oil palm fruit processing has been identified as the problem in generating large volume of highly concentrated POME. This problem has generated interest to reduce the pollution loads. In addition, POME could be converted from 'waste' to 'renewable resources'. These renewable raw materials are readily available from replanting and through routine field and mill operations. To date, Malaysia Palm Oil Board (MPOB) puts a significant emphasis in research and development (R&D) for biomass and waste material products that could be potentially produced from oil palm (MPOB, 2005). Integrated technologies in handling waste and resource recovery should be recognized and utilized. It will contribute to position waste management in this sector to be more cost-effective and competitive.

1.4 Objectives of the Study

The aim of this study was to develop a biological process to produce biodegradable plastics (PHAs) from POME. The aim can be achieved by the following specific objectives:

(i) To assess the potential of PHAs production by activated sludge processes cultivated under feast and famine regime using long chain and short chain fatty acids,

(ii) To investigate the operational conditions, which are N and P limitation, DO concentration, cycle length, feeding regime, anoxic and microaerophilic conditions that would maximize PHAs production using similar mixed microbial culture used for activated sludge wastewater treatment,

(iii) To examine the trends of intercellular stored polymers (PHAs), organics (TOC) and nutrient (PO_4-P and NO_3-N) during feast and famine conditions, and

(iv) To optimize and develop model of PHAs production from POME using statistical experiment design and response surface method (RSM).

1.5 Scope of the Study

Activated sludge of POME was cultivated under feast and famine conditions using an activated sludge process, Sequencing Batch Reactor (SBR), to optimize the production of PHAs. POME has been chosen as carbon source because the substrate contains no hazardous or toxic chemical substances and is discharged in large volume from mills (Hassan *et al.,* 2002). Additionally, POME contains high concentration of fatty acids; therefore it is assumed that PHAs can be produced more efficiently compared to sewage. It is well recognized that the contents of POME are essentially biodegradable organics. The biodegradability is influenced by the extent of cellulosic materials present such as the palm fibre residues as well as the residual oil content (volatile fatty acids, VFAs). On the other hand, few investigations have been made under mixed cultures and renewable sources, thus this study was aimed to investigate the potential of PHAs production using lab-scale bioreactor. In order to optimize the material recovery (biodegradable product) from POME, the investigations have been conducted under several conditions. The selected conditions will be explained later in Chapter III. As a comparison, a saponified fatty acid was also studied to examine the effect of fatty acid components. The study was

undertaken to include the main biodegradable components (hydroxybutyrate, HB monomer) including the monitoring of other specific biopolymers (hydroxyvalerate, HV monomer and hydroxyhexanoate, HH monomer).

1.6 Significance of the Study

There are several important aspects to be considered which will be beneficial by achieving the objectives of this study:

(i) Over the past decades, the usage of plastics in packaging and disposal products and generation of solid waste have drastically increased. These non-degradable petrochemical plastics accumulate in environment at the rate of 25 million tonnes per year (Lee *et al.*, 1991). Therefore, reducing non-biodegradable materials will help to prevent environmental problems.

(ii) POME is the major source of water pollutant in Malaysia. For example, in a conventional palm oil mill, 600-700 kg of POME is generated for every 1000 kg of processed FFB (fresh fruit bunches) (Nor Aini *et al.*, 1999). Thus, this study will provide an alternative means to reduce the pollution load (COD basis), due to the carbon uptake to produce PHAs.

(iii) PHAs recovery from POME will assist the industry in managing their wastes to achieve zero emission targets. The processes are essentially to breakdown the organic matter into simpler end-product gases such as methane (CH_4), carbon dioxide (CO_2) and hydrogen sulphide (H_2S) (Hassan *et al.* 2002).

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

In recent years, attempts have been made to develop a process for high polyhydroxyalkanoates or polyhydroxybutyrates (PHA/PHB) yield production using mixed cultures of activated sludge biomass. These have been followed by several studies and investigations, including the species that are responsible for synthesizing the polymers. The conventional approaches using chemostat and batch systems were continuously adapted primarily to validate the mechanism using models, standard mathematical equations and fermentation rates. The discussion in this chapter also covers the properties of PHA, the selected cultivation strategy and the real application of PHA in various applications. Several environmental conditions, aerobic dynamic feeding regime, denitrification-nitrification process and micro limited electron acceptors (microaerophilic condition) in selected wastes have been reviewed.

2.2 Waste Generation from POME

Palm oil mills with wet milling process accounted for the major production of wastes (Kittikun *et al*., 2000). Hence, the increase in number of mills will generate more environmental problem. The major source of pollution comes from fresh fruit bunches (FFB). In fact, every tonne of FFB is composed of 230-250 kg of empty fruit bunches (EFB), 130-150 kg of fibres, 60-65 kg of shell and 55-60 kg of kernels

and 160-200 kg of crude oil. EFB are always in bulk solid residues. However, the EFB, palm fibre and palm shell could also be used for other purposes. The EFB can be used as fuel for boiler, but the constraint is high moisture content and low heating value (dry kg EFB <10 MJ/kg). On the other hand, it can be used as organic fertilizer, mulching materials, mushroom cultivation and production of particle board (Kittikun *et al*., 2000). Palm fibres are used as fuel for boilers (heating value of dry fibres <5 MJ/kg). Other applications of palm fibres include their use as substrate for enzymatic saccharification as animal feed. Finally, palm shell can be used as boiler fuel with heating value of 17 MJ/kg, however, it causes black smoke. The production of activated carbon from palm shell has been also established.

Typically, 0.8 cubic meters of water is required to process one cubic meter of FFB. About 50 percent volume of the waste will be evaporated as steam and boiler blowdown, as well as through piping leakages and wash waters for tankers or others, which are not combined with the effluent line. The processes of EFB will generate liquid waste, called POME, consisting of highly polluted effluent (from sterilizer and oil room) and low polluted effluent (steam condensate, cooling water, boiler discharge and sanitary effluent). POME also refers to the collective term for the liquid wastes discharged from the final stages of palm oil production at the mill. Usually, POME is a combination of wastewater that is generated from major sources:

(a) Sterilizer condensate (about 36% of total POME),
(b) Clarification of the extracted CPO (about 60% of total POME), and
(c) Hydrocyclone wastewater (about 4% of total POME).

Raw POME is discharged to collection pit at a temperature of between 60^{o}C and 65^{o}C. It is an acidic solution with a pH typically between 4 to 5. POME is characterized by its high organic content and biological oxidation demand (BOD) of between 10,250 to 43,750 mg/l (Jaafar and Sukaimi, 2001; Abou Zeid, 2001). It is a viscous brown or grey sludge in appearance and has a total suspended solid (TSS) of about 50,000 mg/l concentration and varies according to the type of process and location of the factory (Abou Zeid, 2001). The TSS of the effluent comprises of dissolved suspended nitrogenous and carbonaceous materials. The nature of these

suspended materials is colloidal slurry, comprising mainly cellulose from the fruits, carbohydrate, residual oil and other organic and inorganic solids. The typical quality characteristics of the raw combined POME are presented in Table 2.1.

Table 2.1: Typical characteristics of combined raw POME

Typical Characteristic	Mean Value	Metals & other Constituents	
		Element	Mean Value
pH	4.2	Phosphorus (P)	180
Oil & Grease	6000	Potassium (K)	2270
BOD_3 @ 30°C	25000	Magnesium (Mg)	615
COD	50000	Calcium (Ca)	440
Total Solids (TS)	40500	Boron (B)	7.6
Suspended Solids (SS)	34000	Iron (Fe)	47
Total Volatile Solids (TVS)	18000	Manganese (Mn)	2.0
Ammoniacal Nitrogen (AN)	35	Copper (Cu)	0.9
Total Nitrogen (TN)	750	Zinc (Zn)	2.3.

Source: Industrial Processes & The Environment (Handbook No. 3) – Crude Palm Oil Industry, 1999

Note: All parameter's unit in mg/l except pH

POME is normally collected in a sludge pit. It is retained in the pit for a day or so, to allow the separation of residual oil. Then, it will be collected before it is pumped to the treatment plant. POME, when discharged untreated or partially treated into a river or stream will damage most of the aquatic life. Most palm oil mills in Malaysia have adopted the ponding system for the treatment of POME (Ma, 1992). It essentially consisting of anaerobic treatment to its optimum stage, and then followed by facultative ponds under aerobic conditions. The system is designed to produce a final discharge with BOD of less than 100 mg/l. In general, there are four types of treatment systems adopted by the palm oil industry, which are:

(a) Waste stabilisation ponds

(b) Activated sludge system

(c) Closed anaerobic digester

(d) Land application system

To minimize overall treatment costs, the different wastewater streams should be collected and treated separately. The oil separation from the wastewater stream by gravity type oil separators is recommended to improve production yield and minimize the organic loading. This will induce the biological treatment system,

afterwards. Unfortunately, the most appropriate secondary treatment for POME is biological digestion with the combination of anaerobic and aerobic ponds. Currently, the management of POME has evolved from treatment of waste for disposal to beneficial utilization of resources (DOE, 1999).

POME contains substantial quantities of valuable plant nutrient that vary according to the degree of treatment to which it is subjected. The potential use of recovery of water and organic matters from POME has been applied for various applications (Hassan *et al.*, 1997b, 2002). Commercial trials and applications of these technologies are currently underway, especially conversion of the solid residual materials into saleable value-added products. Thus, the CPO industry would be much closer to being considered as having 'clean' technology.

The ideal life cycle of eco-friendly exposure for PHA bioplastic making from renewable resources likes POME is a closed-loop process (as depicted in Figure 2.1). The production of bioplastic will subsequently serve as the feed to a microbial fermentation process (at the end of cycle), to promote the environmental friendly effect. Ideally, this process occurs aerobically (in natural and tropical conditions), yielding water (H_2O) and CO_2 in the same proportions that were originally used in photosynthesis. The harmless production of end products could also be generated from microbial fermentation to produce biofuel energy.

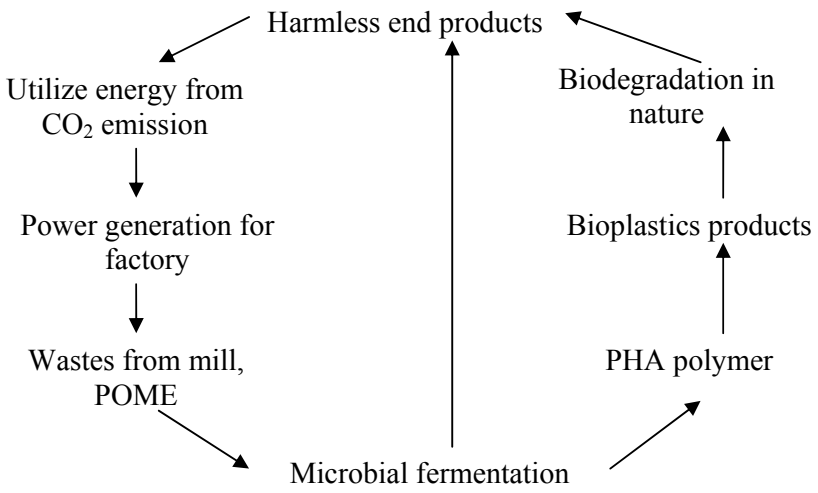


Figure 2.1: Proposed cycle loop of regenerating waste from POME to biodegradable plastics, end-up with preventing pollution load to environment (Source: Angenent *et al.*, 2004)

2.3 PHA as Biodegradable Plastics

Fossil fuel-based polymers (conventional plastics) have numerous benefits including low cost and wide range of physical and mechanical properties (Amass *et al.*, 1998). Thus, it is suitable for many applications (downstream manufacturing). There are two major problems, (i) it is non-biodegradable, therefore, dumped materials at landfills could prolong the biodegradable time of closed landfill, and (ii) the production of this material depletes the natural resources because petroleum-based products is refined into intermediates that serve as feedstock for the major polymer industry (Chua *et al.*, 2003; Calmond-Decriaud *et al.*, 1998). Traditionally, synthetic polymers (conventional plastics) were designed for durability and resistance to the environment and, therefore, tend to accumulate and sustain in landfills. With growing concerns for the environment, synthetic polymers containing hydrolyzable and/or oxidizable groups along the main chain are also being developed. As a result, it is now possible to design PHAs with physical properties that can biodegrade in a predetermined time and manner (Kawai, 2000).

PHAs as known since 1900's are thermoplastics synthesized by bacteria, with the additional advantage of being completely biodegradable, biocompatible, and potentially produced from many renewable resources. PHAs have been developed by industry as a source of biodegradable plastics since the oil crisis in 1970s (Poirier, 2002; Braunegg *et al*., 1998). Additionally, PHA is bio-based product which does not pollute the environment and can reduce the dependence on petrochemicals. These polymers (substrates and recombinant bacteria) will offer opportunities for making PHA materials with consideration of sustainable commodity plastic production. In response to the global environmental problems, PHA is gaining industrial attention as a potential substitute for non-biodegradable polymers.

2.3.1 Chronological Development of PHA/PHB

PHA was first isolated and characterized in 1925 by M. Lemoigne (Lenz and Marchessault, 2005) at the Pasteur Institute in Paris. The first identification of significant bacteria was *Bacillus megaterium*. In 1926, it was also found in other species of bacteria. Since then, it had been studied extensively by biochemists who have generally concluded that bacteria store PHA as an energy reserve similar to human. The potential application of PHAs as biodegradable, renewable and environmental-friendly plastics has been the main driving force in polymer chemistry. It remained an academic curiosity until W. R. Grace in the United States produced small quantities for commercial evaluation in the late 1950s and early 1960s (Lenz and Marchessault, 2005). In 1974, P. Wallen and H. Rohwedder discovered several types of hydroxyalkanoates (HA) in microorganisms (Takabatake *et al.*, 2002). Among the HA units, the major and minor constituents from activated sewage sludge are 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx), respectively (Findlay and White, 1983). In the same report it was shown that the batch-grown *B. megaterium* cells accumulated a polymer consisting of 95% 3HB, 3% 3-hydroxyheptanoate (3HHp), 2% of an 8-carbon HA and trace amounts of three other HA compounds.

Since 1980s, the study was focused on monomer hydroxyalkanoic (HA) identification. The identification of HA units other than 3HB in PHA producers (microorganisms) proved to have major impact on the research and commercial interests (Lenz and Marchessault, 2005; Lee, 1996a). While the homopolymer of P(3HB) is a brittle material with limited applications, the incorporation of a second monomer unit into P(3HB) can significantly enhance its useful properties (Sudesh *et al.*, 2000). This finding is therefore highlighted as a landmark which signifies the beginning of the second developmental stage of research on PHA (Figure 2.2).

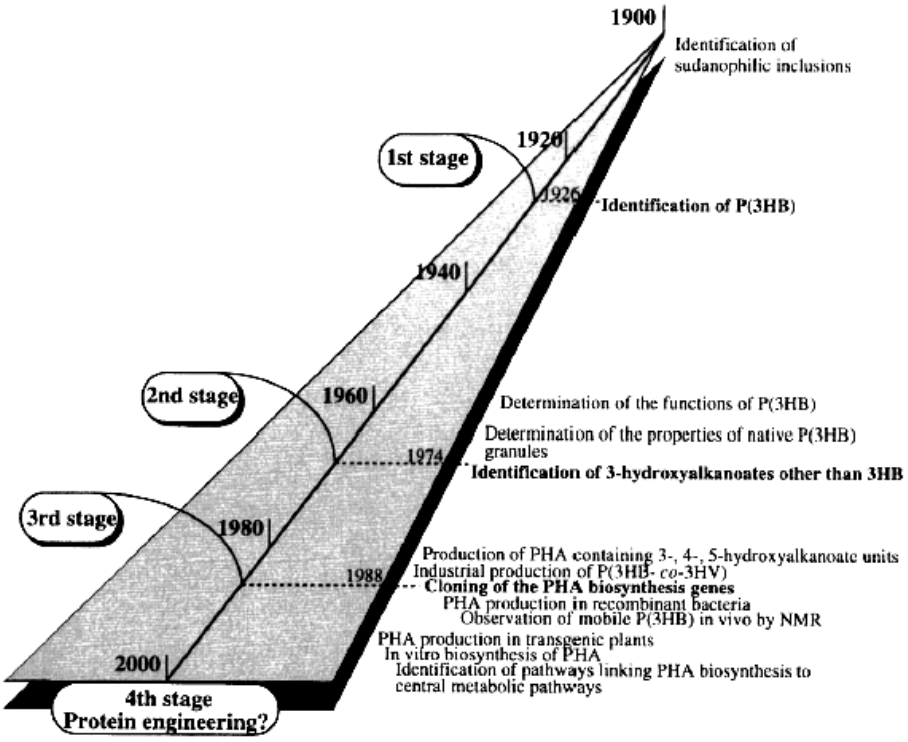


Figure 2.2: The development of PHA science and technology through the twentieth century. (Source: Sudesh *et al.*, 2000)

During the third stage development of PHA (1980s), the research trend was to identify and characterize various potential HA units that could possibly be a constituent of this bacterial polyester. This ultimately resulted in the discovery of

numerous HA constituents (Doi, 1990) including 4HA (Kunioka *et al.*, 1988) and 5HA (Doi *et al*., 1987) by the end of the 1980s. By this time, it was already clear that these storage polymers could be synthesized in Gram-negative and Gram-positive bacteria, aerobic (*cyanobacteria*), anaerobic (non-sulphur and sulphur purple bacteria) photosynthetic bacteria and some in *archaebacteria* (Anderson and Dawes, 1990; Steinbüchel, 1991). The genes coding for enzymes involved in PHA biosynthesis were already cloned from *Ralstonia eutropha, R. eutropha* (formerly known as *Alcaligenes eutrophus, A. eutrophus*) and the genes were also shown to be functionally active in *Escherichia coli (E. coli)* (Doi, 1990; Sudesh *et al*., 2000). Detailed studies on *R. eutropha* had revealed that only three enzymes are involved in the biosynthesis of P(3HB) from acetyl-CoA, and that the regulation of P(3HB) synthesis in this bacterium is achieved at the enzymatic level. The enzyme which carried out the polymerization reaction was identified as the key enzyme and it was designated as PHA synthase. As a result, a number of approximately 300 different HAs are known to occur (Doi, 1990). Therefore, a more general name comprising all these constituents, for example PHA, has been used to designate this family of bacterial reserve polymers.

The active PHA consists of two of sub-units (HV and HB) have been conducted in various processes and cultures. Highly conserved amino acids have been identified based on alignment analysis of the primary structures of these genes and site-specific mutagenesis studies (Gerngross *et al*., 1994). The successful cloning of PHA biosynthetic genes had also enabled the generation of transgenic plants as potential producers of PHA in the future (Poirier *et al*., 1992). What was identified in the beginning of the twentieth century as a sudanophilic bacterial inclusion, was apparently going into the fourth Stage of development (in the year of 2000), such as protein engineering. This Stage would determine whether it is possible to manipulate the PHA production.

2.3.2 Industrial and Commercialization of PHA/PHB

In the late 1980s, the production of bioplastics (PHA) has been commercialized especially in Europe and USA (Luengo *et al*., 2003). The first industrial productions of a copolymer for PHA production are 3HB and 3HV (Ren, 2003; Sudesh and Doi, 2000). Commercial interest lay dormant for over a decade until Imperial Chemical Industries (ICI) began a research and development programme.

More than 40 hydroxyalkanoates were made into polymers in various studies. The project followed their single-cell protein animal feed project. ICI had the skills in place to run large-scale fermentation processes and polymer processing in their plastics division (Holmes, 1985). In the late 1980s, ICI began worldwide commercialization of a family of copolymers; P(3HB-co-4VB). This has been produced with *R. eutropha* strain from various carbon sources such as 1,4-butanediol, 1,6-hexanediol and butyrolactone. The commercial product was recognized as Biopol® (BIOPOL). The first phase of the fermentation involved the growth of *R. eutropha* in glucose-minimal salts medium with an excess of all salts except phosphate (Reddy *et al*., 2003; Byrom, 1987). At the point of phosphate limitation, which induced PHA accumulation, glucose and propionate were supplied in a fed-batch manner.

In 1990, the agricultural and pharmaceutical businesses of ICI, including Biopol® were spun-off as Zeneca Ltd. In 1996, Monsanto acquired the Biopol® business from Zeneca Ltd. Since the acquisition, emphasis at Monsanto has been on producing the polymers in plants and improving their properties for different end-use applications. The content of polyester in dried cells amounted to 80% of weight. Since PHA has high crystallinity and weak mechanical strength and is not suited for practical application to thermoplastics, the bacteria production of a variety of PHAs was examined with many bacteria from various carbon sources such as sugar, alkanoic acids and alcohol (Table 2.2).

Table 2.2: PHA production from various species of bacteria

Polyester produced	Bacteria
P(3HB)	Many strains
P(3HB-*co*-3HV)	*Alcaligenes eutrophus* *Bacilus megaterium* *Chromatium vinosum* *Rhodobacter sphaeroides* *Pseudomonas cepacia* *Methylobacterium extorquens*
P(3HB-*co*-4HB)	*Alcaligenes eutrophus* *Alcaligenes latus* *Comamonas acidovorans*
P(3HA)	*Pseudomonas putida*
P(3HA):C_3-C_{11}	*Pseudomonas aeruginosa* *Psedomonas oleovorans*

(Source: Kawai, 2000)

A range of PHAs with 0 – 24% hydroxyvalerate has been produced under the trade name of Biopol® Zeneca Bio Product and other manufacturers (Table 2.3) and sold in the USA (under the trade name of PHBV), Germany and Japan (Salehizadeh and van Loosdrecht, 2004; Lee, 1996a). However, the PHA production price in Europe is far above the market price of conventional plastics (USD16/kg for Biopol® against USD1/kg for oil-derived plastics). Then, in the year of 1999, the production cost can be lowered by process scale-up, to around USD8/kg at a production rate of 5000 tonnes PHA/year (Salehizadeh and van Loosdrecht, 2004). This price was still unfavourable to compete with conventional plastics. From the literature, the major cost in the PHA production is determined by the cost of substrate and PHA content (Yamane 1992, 1993). PHA content affects the efficiency of the recovery process as well as PHA yield and carbon source. On the other hand, the recovery cost for process with 88% PHB content was only USD0.9/kg PHB (Lee *et al.*, 2000). Therefore, the lower PHB content result in a higher recovery cost mainly due to use of large amount of digesting agents for separating PHB and to the increased cost of waste disposal. By using cheap substrate sources such as agriculture and food industrial wastes (for example; whey, molasses waste, malt and beer waste, and POME), PHA can be produced economically (Luengo *et al.*, 2003; Choi and Lee, 1997; Meesters, 1998). As a conclusion, the material recovery from

POME could be the most appropriate approach since the waste is ready to be collected and suitable for PHA production in mixed cultures.

Table 2.3: Microorganisms and/or raw materials in the production of biodegradable plastics along with the names of their manufacturers

Microorganism/raw material	Manufacturer
A. eutrophus	ZENECA Bio Product, United Kingdom
A. latus	Biotechnologische Forschungs gesellschaft, Austria
Recombinant *E. coli*, starch	Bioventures Alberta, Canada, Warner's Lambert USA; Fertec, Novamont Novara, Italy; Biotech Emmerich, BASF, Ludwigshafen, Bayer Wolf WalsordeLeverkusen, Germany
Cheap substrate	Plyferm, Canada
Bacteria	Biocrop, USA; Asahi Chemical and Institute of Physical and Chemical research, Japan

(Source: Reddy *et al*., 2003)

Metabolix Inc., a start-up company in Cambridge, MA, bought the Biopol® assets from Monsanto and pursued the production of PHAs in plants and recombinant *E. coli*. Their applications focus was on low residue binders for metal and ceramic powder processing. The subsidiary company, Tepha Inc. was using PHAs as a bioabsorbable polymer for higher value-added tissue engineering application (Sudesh *et al*., 2000b). It is also worth nothing that PHAs are just one of many biodegradable polymers that have been commercialized. For example, poly(butylenes-succinate-terephthalate) is sold under the trade names Biomax™ by DuPont, EasterBio™ by Eastman Chemical and Ecoflex™ by BASF. In additions, NatureWorks™ polylactic acid (PLA) is sold by Cargill Dow Polymers (Mohanty *et al*., 2000). All these biodegradable polymers are synthesized chemically except for PLA, in which synthesis involves a biological step to produce lactic acid monomer, followed by chemical synthesis steps to make a polymer (Gruber *et al*., 1993). As a conclusion, many companies are involved to produce affordable biodegradable plastics. The examples of these commercial biodegradable plastics are shown in Figure 2.3.

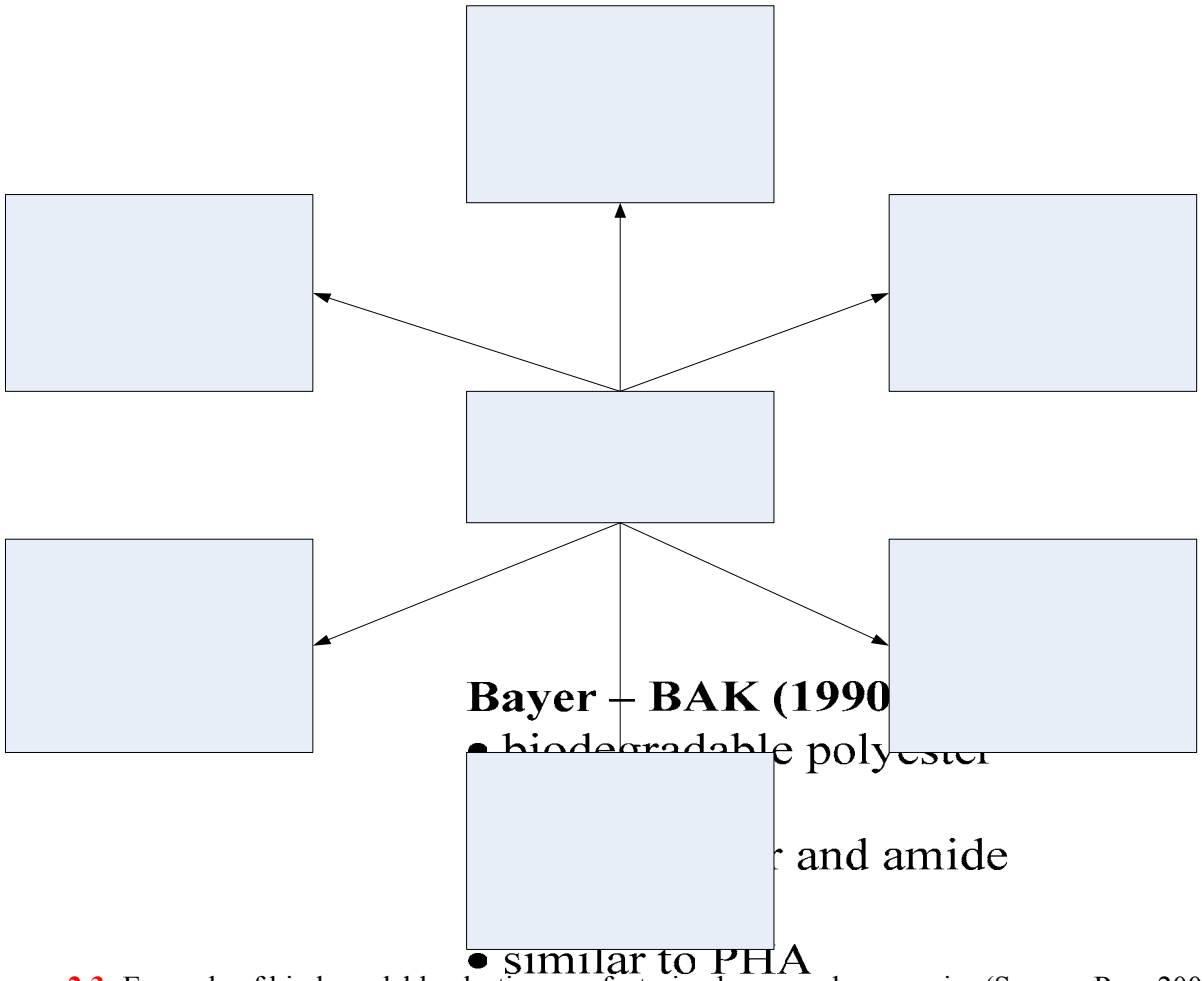


Figure 2.3: Example of biodegradable plastic manufacturing by several companies (Source: Ren, 2003)

BASF - Ecof
• biodegradab
bags
• properties s
• 10000 tonne
plant at Ludw
Germany.

Pioneer Bi
Plastics M

Fasalex, The Institute for

2.3.3 Structure and Biosynthesis of PHAs

PHAs share physical and material properties which recommend them for applications in various areas. They are thermoplastics and/or elastomerics, insoluble in water, enantiomeric pure, non-toxic, biocompatible, piezoelectric and exhibit a high degree of polymerization and molecular weights of up to several million (Steinbüchel and Lütke-Everloh, 2003). The typical weight-average molecular mass of PHAs was in the order of 400 kDa and the melting point (T_m) range was 163 – 174^{o}C. However, the most important feature is the biodegradability of PHAs. PHAs also served as a model to develop various kinds of biodegradable polymers, either by chemical or combination of biotechnological and synthetic processes. There are several types of PHAs which co-exist with aliphatic compounds under the family of polyesters. The detail is shown in Figure 2.4.

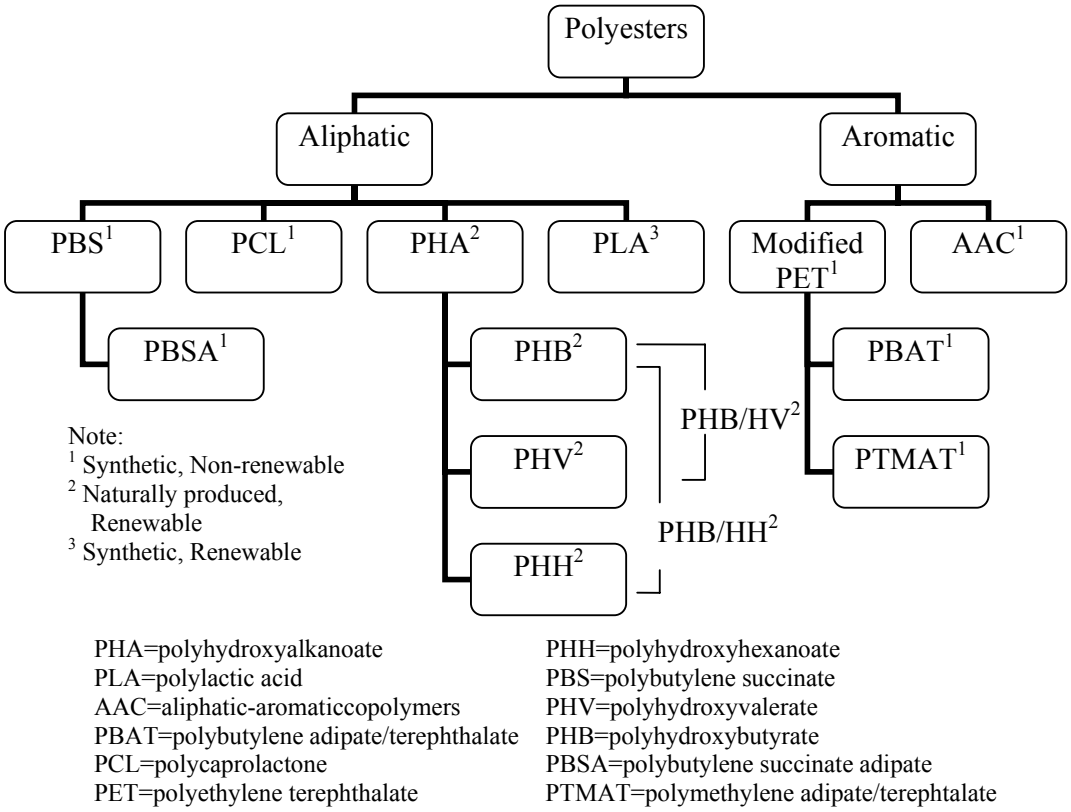
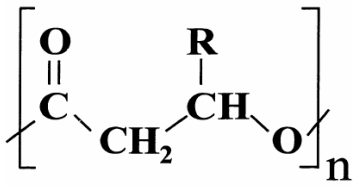


Figure 2.4: The established biodegradable polyester family (Source: Madison and Huisman, 1999)

PHAs are the linear polyester, composed of hydroxy fatty acid monomers in the D configuration (Figure 2.5) that accumulate as carbon/energy or reducing power storage material (Wong, 2001). Majority of the natural PHA producers will be accumulated as intracellular granules (diameter: 0.3-1.0 μm) during the presence of carbon source and nutrients (Yan *et al.*, 2003; Wong, 2001; Lee *et al.*, 1996b; Anderson and Dawes, 1990; Poirier *et al.*, 1995). When growth-limiting conditions are alleviated, the polymer is then catabolized.



n = 1	R = hydrogen	poly(3-hydroxypropionate)	P(3HP)
	R = methyl	poly(3-hydroxybutyrate)	P(3HB)
	R = ethyl	poly(3-hydroxyvalerate)	P(3HV)
	R = propyl	poly(3-hydroxycaproate)	P(3HC)
	R = butyl	poly(3-hydroxyheptanoate)	P(3HH)
	R = pentyl	poly(3-hydroxyoctanoate)	P(3HO)
	R = hexyl	poly(3-hydroxynonanoate)	P(3HN)
	R = heptyl	poly(3-hydroxydecanoate)	P(3HD)
	R = octyl	poly(3-hydroxyundecanoate)	P(3HUD)
	R = nonyl	poly(3-hydroxydodecanoate)	P(3HDD)
n = 2	R = hydrogen	poly(4-hydroxybutyrate)	P(4HB)
n = 3	R = hydrogen	poly(5-hydroxyvalerate)	P(5HV)

Figure 2.5: Chemical structure of PHAs produced in bacteria. n^{th} will be available from 100 – 3000. In the case of PHB, R = CH_3 and in the case of PHBV, R = CH_3 or CH_2CH_3. (Source: Wong, 2001).

Over 130 different monomers have been identified (Steinbüchel and Hein, 2001; Steinbüchel and Valentin, 1995), leading to a large variety of polymers with different properties. Although some of these monomers have been found in natural environment, a larger fraction of monomers have been incorporated into PHA. It was obtained from laboratory conditions in media containing exotic sources of carbon. The number of monomers in a polymer chain 'n' can vary from 4,000 to 20,000 and the resulting number average molecular weight of the polymer is in the range of 2 x 10^5 – 3 x 10^6 g/mol, depending on the monomeric constituents, the growth conditions, and the microorganisms (Lee, 1996a; Byrom, 1994). These are

hydrophobic, lipid structures that are osmotically inactive and can naturally accumulate to greater than 90% of the dry cell weight (DCW) of the organism. Native PHA granules also contain an outer membrane consisting of phospholipids and proteins (Lubove, 1999; Steinbüchel and Valentin, 1995; Steinbüchel, 1991). The proteins include the PHA synthases and PHA depolymerises that are attached to the PHA granules along with small ampiphilic proteins, known as phasins, whose function is still unknown (Steinbüchel and Hein, 2001). Basically, PHAs can be divided into subgroups based on the number of carbon atoms present in its monomer units:-

(a) PHAs with hydroxyalkanoate monomers of less than six carbon atoms (C_3 – C_5 monomers, termed as short-chain-length, SCL of PHA (PHA_{SCL}). *R. eutropha* is a domain bacterium which can produce PHA_{SCL} in their cells.
(b) PHAs with hydroxyalkanoate monomers between 6 to 14 carbon atoms (C_6 – C_{14} monomers, termed as medium-chain-length, MCL of PHA (PHA_{MCL}). *P. oleovorans* is a domain bacterium which can produce PHA_{MCL} in their cells.

PHA_{SCL} possesses higher melting temperature and is stiffer than PHA_{MCL}, while PHA_{MCL} owns the properties of an elastomer with poor tensile strength and higher extension to breakage (Poirier *et al*., 1995). Since the first finding of PHB by Lemoigne in 1926, more than 100 different monomer units have been identified as constituents of PHA in more than 300 different microorganisms (Lee *et al*., 1996b). PHA has been industrially produced by pure cultures including *A. latus*, *A. vinelandii*, *methylotrophs, P. olevorans*, recombinant *A. eutrophus* and recombinant *E. coli*. The first constituent of PHA that was identified more than 70 years ago is [R]-3-hydroxybutyric acid (3HB) (Sudesh and Doi, 2000). Since then, for a long period of time it was thought that 3HB was the only constituent of PHA, hence the term ‘PHB’ was widely used to refer to this bacterial polyester. However, it has been shown that many additional R-3-hydroxyacids can also serve as building blocks for these polyesters. Their physical properties are determined by the nature and sequence of the monomer units present in the polymer chains.

It has been mentioned earlier that PHB is the most common biological polyester produced by various microorganisms in nature. It is also clear that this polyester has a perfectly isotactic structure with only the (R)-configuration. The pendant 'R' group on C_3 varies widely in natural and engineered polymers. PHB isolated from bacteria possesses 55-80% crystallinity, while the molecules within bacteria are amorphous and exist as water insoluble inclusions (Sudesh *et al*., 2000). The exact nature of the enzymes involved in the synthesis (as energy storage) is known to vary between microorganisms. PHB has some properties similar to PP with three unique features: thermoplastic processability, 100% resistance to water, and 100% biodegradability (Hrabak, 1992). Booma *et al.* (1994) stated that PHB is an aliphatic homopolymer with a melting point of 179°C and highly crystalline (80%). It can be degraded at the temperature above its melting point. According to De Koning (1995), the molecular weight of PHB was decreased to approximately half of its original value when it was held at 190°C for 1 hour.

High molecular weight of PHB is recognized as more useful and desirable for industrial applications. The densities of crystalline and amorphous PHB are 1.26 and 1.18 g/cm^3, respectively. Mechanical properties like the Young's modulus (3.5 GPa) and the tensile strength (43 MPa) of PHB material are close to the isotactic PP. The extensions to break (5%) for PHB is however markedly lower than PP (400%). Therefore, PHB appears as a stiffer and more brittle plastic material when compared with PP. Bourque *et al*. (1995) stated that the molecular weight of PHB can be reduced during the polymer processing step. In addition, Lafferty *et al*. (1988) reported that some reduction of PHB molecular weight could occur during the biomass extraction process. Hu *et al*. (2003) and Shimizu *et al*. (1994) found that the average molecular weight of PHB will be decreased when butyric acid increased. The highest average molecular weight of PHB (3.3 x 10^6 g/mol) was obtained with a butyric acid concentration at 0.3 g/l. Meanwhile, the average molecular weight of 2 x 10^6 g/ was obtained at the optimum conditions for PHA production (butyric acid concentration of 3 g/l at pH 8).

PHB, 3HB containing copolyesters and some other PHAs can also be produced from renewable resources. They are all considered as alternative polymers

to non-biodegradable plastics produced from petroleum. The biosynthesis of PHAs starts from the central metabolic intermediate acetyl-CoA with the involvement of three key enzymatic steps (*PhaA, PhaB and PhaC*).

According to Doi (1990), when growth conditions are unbalanced, acetyl-CoA cannot enter the tricarboxylic acid (TCA) cycle to obtain energy for cells due to high concentrations of NADH. The high concentration of NADH is resulted from the cessation of protein synthesis, a process closely coupled to ATP generation by electron transport chain (during nutrient limitation) (Doi *et al.*, 1992a). These high concentrations of NADH inhibit enzyme citrate synthase, one of the key enzymes of the TCA cycle, leading to an increase of acetyl-CoA. Acetyl-CoA is then used as substrate for PHA biosynthesis by a sequence of three enzymatic reactions (e.g. Figure 2.5). In addition, high intracellular concentrations of CoA inhibit enzyme 3-ketothiolase, one of the three enzymes of PHA biosynthesis. When the entry of acetyl-CoA to the TCA cycle is not restricted, free CoA is released as the acetyl moiety from citrate synthase activity. For example, when acetyl-CoA is utilized, intracellular CoA concentration increases, and then PHA synthesis is inhibited. This PHA can serve as a carbon or energy source for microorganisms during starvation periods (Luengo *et al.*, 2003; Lee *et al*., 2000).

2.3.4 Development of the Bioplastics

The resins used to make biodegradable plastics fall into two broad categories: natural and synthetic (Reddy *et al.*, 2003). Natural resins (or biopolymers) are largely based on renewable resources such as starch and cellulose, and PHAs or lactic acid (PLA) produced by microbes. The other polymers such as proteins and pectins may be also potentially developed into biodegradable plastics and polymers. Polylactides (PLA, aliphatic polyesters) formed by polymerization of lactic acid, is usually included in this category since the monomer can be produced by fermentation. The development of biodegradable plastics is an important innovation, because it can contribute to the independency of conventional plastic-material (from petroleum compositions). Plastics produced from PHAs have been reported to be

truly and fully biodegradable as well as PLA and polygalvanic acid (PGA) (Panswad *et al.,* 2003). In addition, the degradation product of PHAs is a common intermediate compound in all higher organisms. Therefore, it is plausible that PHAs is biocompatible to animal tissues and may be used in surgical applications without complexity. Thus, this might reduce a number of non-renewable resources consumption in most of landfills. PHAs are more attract because it can be easily be produced from renewable resources (food processing wastes, palm oil industry, municipal wastes and landfill area) which will help to reduce the pollution load to the natural resources (reservoir, ocean, and rivers). Recently, many researchers tried to produce PHAs by mixed-cultures when exposed to a transient carbon supply. A novel PHA production strategy, which utilizes a mixed bacterial culture in activated sludge for PHA production has been proposed (Hu *et al*. 1997; Satoh *et al*. 1998a; Chua *et al*., 1997; and Takabatake *et al*. 2000). The strategy is achieved by using activated sludge processes, particularly in sequencing batch reactor (SBR) with respect to the feed regime. In a response of mixed cultures, the high productivity of PHA must be conducted in several operating system (limiting N and P, feeding regime, cycle time, etc.). On the other hand, the PHA-producer will be performed in a relatively short period of feast time.

2.4 Production Cost of PHA

The use of PHAs in a wide range of applications has been hampered mainly by their high production cost compared with oil-derived plastics (Byrom *et al*., 1987; Choi and Lee, 1997). With the aim of commercializing PHA, great efforts have been employed to reduce the production cost by the development of bacterial strains and more efficient fermentation/recovery process (Lee *et al*., 2000). The major problem facing commercial production and application of PHA is large investment on bacterial fermentation. Typically, the PHA-bacteria cultivation could be 5 to 10 times more expensive than the petroleum-derived polymers, such as polypropylene (PP) and polyethylene (PE), which will cost approximately USD0.25 to USD0.5/kg (Poirier, 2002). At the same time, the cost of raw material itself accounts for 40%-50% of the total production cost (Purushothaman *et al*., 2001; Yamane *et al*., 1992,

1993). It can be concluded that yields are all in the same range (except the last one that might have a low PHA production). Therefore, the price of substrate has the largest influence on the raw material cost for the production of PHA. The report illustrates that the cheapest substrate cost is 0.214 USD/kg PHA compared with 0.185 USD/kg PP (Kothuis and Schelleman, 1996). A summary survey on various carbon sources from the report of Mosanto Inc. (1995) is given in Table 2.4 (Lee *et al*., 1996b; Madison and Huismon, 1999).

Table 2.4: Effect of substrate cost and PHB yield on the production cost of PHB

Substrate	Price (USD/kg)	Yield (g PHB /g Substrate)	Substrate Cost (USD/kg PHB)
Glucose	0.493	0.38	1.350
Sucrose	0.295	0.40	0.720
Methanol	0.180	0.430	0.420
Acetic acid	0.595	0.380	1.560
Ethanol	0.502	0.500	1.000
Cane molasses	0.220	0.420	0.520
Cheese whey	0.071	0.330	0.220
Hydrolyzed corn starch	0.220	0.185	0.580
Hemicellulose hydrolysate	0.069	0.200	0.340

Source: Lee, (1996b), Madison and Huismon, (1999)

Productivity also has an effect on the production costs (Salehizadeh and van Loosdrecht, 2004). However, this is relative to the substrate, and downstream processing apparently has a weak effect on the final cost. When the PHB productivity increased from 1.98 to 3.2 g/l.h, the PHB cost decreased from USD5.37/kg PHB to USD4.91/kg PHB (Lee *et al*., 2000). Therefore, the cost of the carbon source contributes significantly to the overall production cost of PHA (Yamane, 1992, 1993). As an example, for the process with recombinant *E. coli* with the PHB concentration of 157 g/l (PHB content and productivity was 77%, 3.2 g/l. h, respectively), the cost of the carbon source was 38% (from total operating cost). This estimation was assumed to be 100,000 tonnes/year (Choi and Lee, 1997). In a laboratory fed-batch system using *A. latus*, the highest reported productivity was 4.94 g/l. h which would lead to production costs of USD2.6/kg PHB (Lee *et al*., 2000). PHB content of the produced biomass strongly affects the efficiency of the recovery process. For example, a relatively low PHB content of 50% results in a high recovery cost of USD4.8/kg PHB. A lower PHB content clearly results in a

high recovery cost. Equipment-related costs also increase with decreasing PHA content because a larger amount of cells are needed to be produced to obtain the same amount of PHA.

To reduce the cost, the development of the biodegradable plastics could be obtained from renewable resources. PHB and PHB/HV were also commercialized in a large scale since 1980s. As reported from Lafferty *et al*. (1988), PHB has some essential properties, including tensile strength and flexibility, similar to PE and PS. This copolymer (HB and HV monomer) is a better material for food packaging as this could eliminate the use of antioxidant. Many researchers produced PHB-co-HV productions from an expensive carbon source and using pure culture (Lee, 1996a; Yamane *et al*., 1996; Kim *et al*., 1994). Cultivation strategies, to achieve high biomass PHB-co-HV concentration and high productivity in pure cultures are well defined. Even though the price of PHB is still high, current advances in fermentation and purification technology are recognized to lower the price of PHA (Lee, 1996a). Unfortunately, this is almost impossible to deal in a large scale production. Then, the development of mixed cultures and cheap carbon substrates could be the best implementation method.

The developments of low cost (waste-based) substrates have been recognized for PHA production (e.g. Pozo *et al*., 2002; Wong *et al*., 2002; Carucci *et al*., 2001). A good candidate for economical PHA production would be a mixed culture that can store high PHA content while growing on an inexpensive substrate. Most benefits will be obtained when these cheap substrates can be converted by an open mixed culture because there is no need for sterilization procedures or expensive sterilization fermentors. Waste such as POME can be used for biodegradable plastics production, as well as for the waste reuse. However, current knowledge is very limited for PHA production using activated sludge bacterial cultures.

In conclusion, most of the bacteria can produce PHB from various inexpensive carbon sources. However, in general, the PHB contents and productivity are inconsistent than obtained from purified carbon substrates (Choi *et al*., 1998). The stand-alone process (single polymer) proved unreliable for the PHA productivity;

therefore, many studies have been performed on incorporation of copolymer. For the production of copolymers, co-substrate that serves as the precursor for the co-monomers of PHAs is often needed. Most of the co-substrates are more expensive than the main carbon source and are often harmful for cell growth at high concentrations. Therefore, a better-control of bacterial strains is appropriate to be investigated from mixed carbon sources for cost-effective reasons.

2.5 Applications of Biodegradable Plastics

According to Lafferty *et al.* (1988), the possible applications of bacterial PHA is directly connected with their properties such as biological degradability, thermoplastic characteristics, piezoelectric properties, and depolymerization of PHB to monomeric D(-)-3-hydroxybutyric acid. The applications of bacterial PHAs have concentrated on three principal areas: medical and pharmaceutical, agricultural, and commodity packaging (Holmes, 1985; Huang *et. al*, 1990; Lafferty *et al*., 1988; Lee, 1996b). The most advanced development of bacterial PHAs is in the medical field, especially pharmaceutical applications although they have a considerable potential as consumer goods products.

2.5.1 Medical and Pharmaceutical Applications

The degradation product of P(3HB), D(-)-3-hydroxybutyric acid, is a common intermediate metabolic compound in all higher organisms (Lafferty *et al*., 1988 and Lee, 1996a). Therefore, biocompatible to animal tissues is important and P(3HB) can be implanted in animal tissues without any adverse effect. Some possible applications of bacterial PHAs in the medical and pharmaceutical applications include: biodegradable carrier for long term dosage of drugs inside the body, surgical pins, sutures, and swabs, wound dressing, bone replacements and plates, blood vessel replacements, and stimulation of bone growth and healing by piezoelectric properties. The advantage of using biodegradable plastics during implantation is that it will be biodegraded, thus, the need for surgical removal is not necessary.

2.5.2 Agricultural Applications

PHAs are biodegrading in soil. Therefore, the use of PHAs in agriculture is widely acceptable as biodegradable carrier for long-term dosage of insecticides, herbicides, or fertilizers, seedling containers and plastic sheaths protecting saplings, biodegradable matrix for drug release in veterinary medicine, and tubing for crop irrigation. It is not necessary to remove biodegradable items at the end of harvesting season.

2.5.3 Biodegradable Commodity Packaging

According to Holmes *et al.* (1981), PHAs can be used in extrusion and moulding processes and can be blended with synthetic polymer, e.g., chlorinated PE, to make heteropolymers. Moreover, small additions of PHA improve the property of some conventional polymers, e.g., addition of a small amount of PHA reduces the melting viscosity of acrylonitrile. Tsuchikura (1994) reported that "Biopol®" with high PHV content is more suitable for extrusion blow moulding and extrusion processes, e.g., fabrication of films, sheets, and fibres, while "Biopol®" with low PHV content is more suitable for general injection moulding processes. Also, one particular property of PHB films that make it possible to be used for food packaging is the relatively low oxygen diffusivity. Plastics produced from PHAs have been reported to be biodegraded both in aerobic and anaerobic environments (Choi and Lee, 1997). Shortly, possible applications of PHAs for commodity goods include packaging films, bags and containers, disposal items such as razors, utensils, diapers, and feminine products.

2.6 PHA Production in Selected Cultivations

The disposal of the waste stream can cause considerable environmental problem due to its high biological or chemical oxygen demand. Furthermore, the treatment of waste stream, to make purified effluent needs much effort and is very difficult, because the waste stream often contains various organic compounds. In

conventional activated sludge, PHB and PHB-co-HV were produced from butyrate and/or valerate (Lee *et al*., 1991). When butyrate was used as a sole carbon source, PHB was produced comprising up to 37% of the dry cell weight. When valerate was added to the medium, PHB-co-HV copolymer will be produced. The 3-hydroxyvalerate mole fraction in PHB-co-HV reached a maximum of 54% when valerate was used as sole carbon source. Even though the final PHA concentration was less than 1.0 g/l, the study demonstrated that biodegradable polymer PHA could be produced in an activated sludge.

2.6.1 Mixed Cultures and Feast/Famine Regimes

The idea of PHA production using mixed culture was introduced owing to PHA role as a metabolic intermediate of wastewater treatment and as a biodegradable plastic. The merits of PHA production system by open mixed culture will be economical, simple control process, non-sterilizable, easy to construct and abundance of material recovery from wastes (Satoh *et al.,* 1998b). Considerable efforts have been carried out about PHA production using mixed culture by many researchers (Ueno *et al*., 1993; Saito *et al*., 1995; Hu *et al.,* 1997; Tsunemsa, 1998; Chau *et al*., 1999; Tohyama *et al.,* 2002; Beun *et al*., 2002; van Loosdrecht and Heijnen, 2002; Takabatake *et al*., 2000, 2002). In general, the PHA production of pure and mixed cultures is concluded in Table 2.5.

Table 2.5: The average PHA productions from selected studies

System	Condition applied	PHA production	References
Activated sludge (pure cultures)	Anaerobic	20%	Satoh *et al*., 1998,
	Aerobic	33%	Takabatake *et al*., 2002
	Microaerophilic	62%	
Activated sludge (mixed substrates)	Anaerobic (POME as substrate)	65%	Hassan *et al*. 2002
Activated sludge (mixed cultures)	Fully aerobic	70%	Punrattanasin, 2001
	Feast-famine (aerobic)	62	Beccari *et al.,* 1998
	Feast-famine	67%	Dionisi *et al*., 2001b
	Fed-batch cultures	88%	Lee *et al*., 1996b

Generally, the accumulation of PHAs plays an important role in prokaryotes instead of eukaryotes. Prokaryotes synthesize a wide range of different PHAs and accumulate the polyesters as insoluble inclusions in the cytoplasm for typically carbon and energy consumptions. In order to establish economically feasible for PHA production, CO_2 or residual materials, efforts are undertaken to this engineered novel pathway in recombinant prokaryotic and eukaryotic organisms. This requires transfer of PHA synthase structural genes, expression of suitable substrates at sufficient concentrations.

Under dynamic conditions, the growth of biomass and storage of polymers occur simultaneously when there is an excess of external substrate (feast period). When all the external substrate is consumed, stored polymer can be used as a carbon and energy source (famine period). As known from experiments, the volatile fatty acids (VFAs) form the major soluble substrate. Microorganisms, which are able to quickly store and consume substrate in a more balanced way, have a strong competitive advantage over organisms without capacity of substrate storage (van Loosdrecht *et al*., 1997). Grady *et al.* (1999) noted four basic conditions for the growth of all microorganisms: (1) carbon, (2) inorganic nutrient, (3) energy, and (4) reducing power. Microorganism can derive energy and reduce power (ATP, NADH) from the oxidation reactions, which involve the removal of electrons from the substrate with their ultimate transfer to the terminal electron acceptors. Usually, in wastewater treatment processes, two kinds of terminal electron acceptors (O_2 and NO_3), are utilized by microorganisms for energy derivation purposes. When NO_3 works as the electron acceptor, the environment is called an anoxic condition and the treatment process is called denitrification.

The microorganisms experience rapidly changing conditions of availability of nutrient and can adapt continuously to change in substrate. Sasikala and Ramana (1996) also summarized nutrient limiting conditions that led to PHA accumulation in different microorganisms. In addition to nitrogen, phosphorus, oxygen, and sulfate limitations, there are several compounds that stimulate the accumulation of PHA, e.g. iron, magnesium, manganese, potassium, and sodium. Regularly, it has shown that activated sludge organisms respond to feast/famine regimes by the production of

storage polymers. The reason behind this is that under conditions of periodic carbon-substrate surplus, as under feast-famine conditions, the substrate uptake rate will be larger than required for growth. The synthesis of PHB (kind of storage polymer) involves the condensation of acetyl CoA(s) and reducing power $NADH_2$, as the universal electron carrier of microorganisms. The formation of PHB is particularly useful under conditions of O_2 limitation (microerophilic condition), which supplied the sink of reducing powers. It was observed that activated sludge under transient conditions, mainly caused by discontinuous feeding and variation in electron acceptor presence (O_2, NO_3), is able to store large amount of PHA or PHB. Therefore, PHB reserves may accumulate when cells are limited in O_2 but still have a carbon source available (Dircks *et al*., 2001). Figure 2.7 show the major biosynthetic pathway under transient conditions (feast-famine regimes).

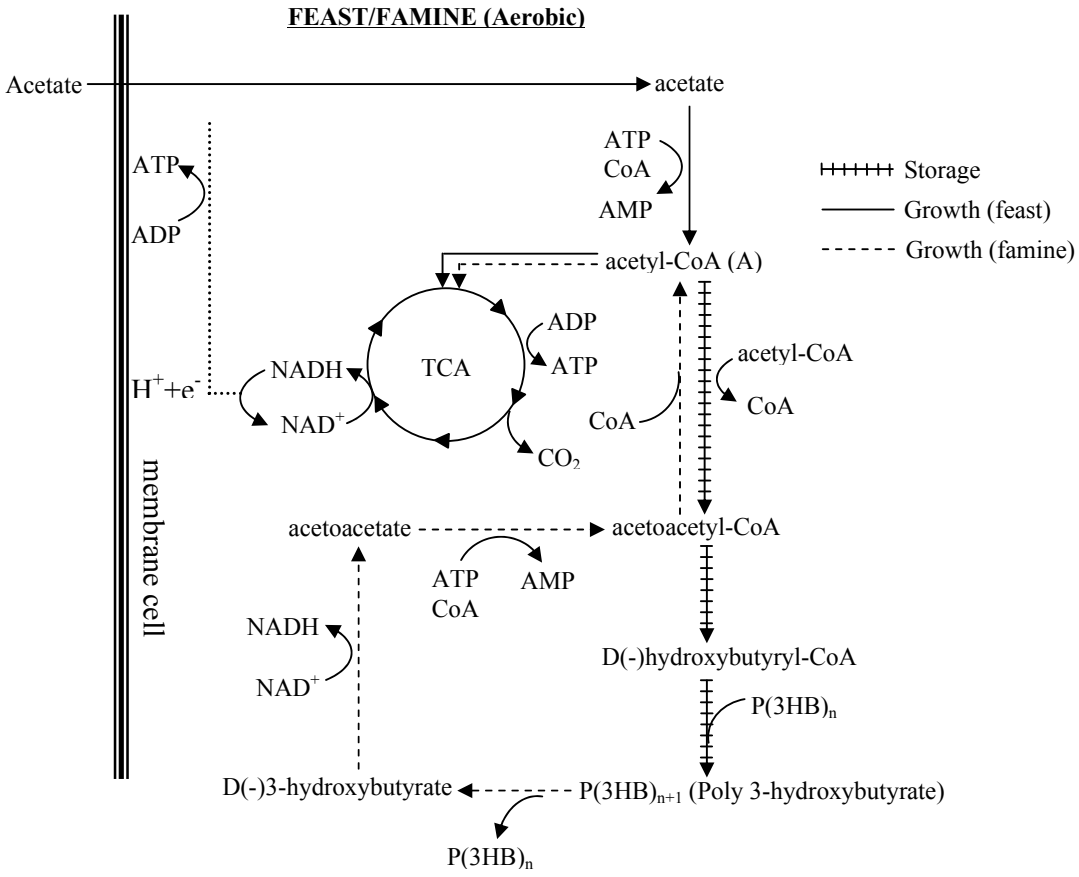


Figure 2.7: PHB production pathways in feast-famine regimes (Source: Reis, *et al*., 2003; Salehizadeh and van Loosdrecht, 2004)

Stoichiometry and kinetics of PHB metabolism in aerobic sludge have been studied (Salehizadeh and van Loosdrecht, 2004, van Loosdrecht *et al.*, 1997; Beccari *et al.*, 2002, Majone *et al.*, 2001). Under feast phase, about 66% - 100% of the substrate is consumed which is used for PHB storage, and the remainder for growth and maintenance. The growth rates in the feast and famine phases are similar, but growth in the feast phase is higher relative to the famine phase. Acetate (one of significant carbon uptake) consumption and PHB production in the feast period both proceed with a zero-order rate in acetate and PHB concentration, respectively. PHB consumption in the famine phase as a carbon and energy source can be described kinetically with *n*th-order degradation equation in PHB concentration. Rate of PHB degradation in famine phase is independent of the type of electron acceptors (Beun *et al.*, 2000a, Beccari *et al.*, 2002). The degradation of PHB results in the production of two acetyl CoA(s) and one $NADH_2$. This degradation occurs when the internal concentration of NAD and free CoA increases while the concentration of acetyl-CoA is low. For example, PHB is degraded in the presence of O_2 when the carbon sources are limited. However, if both O_2 and an external carbon source are present, PHB could not be degraded.

The degradation of PHA by *R. eutropha* can occur simultaneously with its biosynthesis under nitrogen limitation (Doi, 1990). This observation is called "a cyclic nature of PHA metabolism". Doi (1990) also reported that the composition of polymer was changed from PHB homopolymer to PHB-49%PHV copolymer when the substrate was changed from butyric acid to pentanoic acid after 96 hours of nitrogen limitation period. Likewise, when *R. eutropha*, with a PHV fraction of 56% of its PHA content, was fed with butyric acid as a sole substrate under nitrogen limitation, the PHA composition changed markedly, i.e., the fraction of PHV decreased from 56% to 19% after 48 hours. These findings show the simultaneous synthesis and degradation of PHA, i.e., the cyclic nature of PHA metabolism. Figure 2.8 illustrates cyclic metabolism (Punrattanasin, 2001)

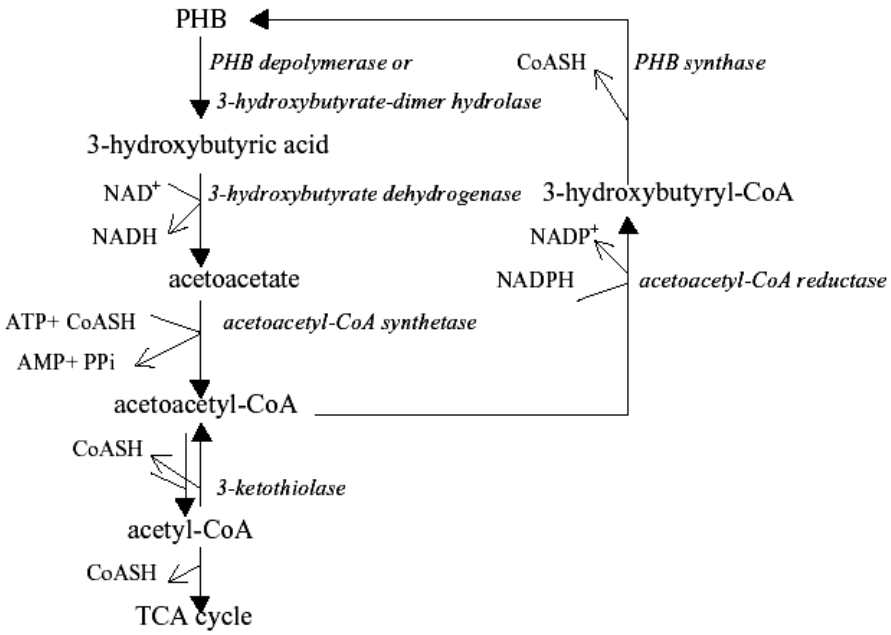


Figure 2.8: Cyclic nature of PHA metabolism from synthesis to degradation (during famine period). (Source: Punrattanasin, 2001).

2.7.2 Dynamic Aerobic and Microaerophilic – Aerobic Condition

Oxygen formed with singlet-oxygen is also called as toxic oxygen. Singlet oxygen is produced both photochemically and biochemically, the latter through the action of various peroxidase enzymes (Wu *et al.*, 2000). Other highly toxic forms of O_2 include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH$), all of which are produced as inadvertent by-products during the reduction of O_2 to H_2O in respiration (Eq. 2.4).

$$O_2 + e^- \rightarrow O_2^- \text{ (superoxide)} \quad (2.1)$$

$$O_2^- + e^- + 2H^+ \rightarrow H_2O_2 \text{ (hydrogen peroxide)} \quad (2.2)$$

$$H_2O_2 + e^- + H^+ \rightarrow H_2O + OH\bullet \text{ (hydroxyl radical)} \quad (2.3)$$

$$OH\bullet + e^- + H^+ \rightarrow H_2O \text{ (water)} \quad (2.4)$$

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \text{ (overall)} \quad (2.5)$$

With such an array of toxic oxygen derivatives, it is perhaps not surprising that organisms have evolved enzymes that destroy toxic oxygen products (Equations 2.6 to 2.9). The most common enzyme in this category is catalase, which attacks H_2O_2; the activity of catalase is illustrated in Eq. 2.6. Another enzyme that destroys H_2O_2 is peroxidase, as shown in Eq 2.7, which differs from catalase in requiring a reductant, usually NADH, producing H_2O_2 as a product. Superoxide is destroyed by the enzyme superoxide dismutase (Eq. 2.8), which combines two molecules of superoxide to form one molecule of H_2O_2 and one molecule of O_2. Superoxide dismutase and catalase working together can thus bring about the conversion of superoxide back to oxygen.

$$H_2O_2 \text{ (by-product)} + H_2O_2 \rightarrow 2H_2O + O_2 \textbf{ (Catalase)} \quad (2.6)$$

$$H_2O_2 + NADH + H^+ \rightarrow 2H_2O + NAD^+ \textbf{ (Peroxidase)} \quad (2.7)$$

$$O_2 + O_2 + 2H^+ \rightarrow H_2O_2 + O_2 \textbf{ (Superoxide dismutase)} \quad (2.8)$$

$$4O_2^- + 4H^+ \rightarrow 2H_2O + 3O_2 \textbf{ (combination eq. 2.8 / catalase)} \quad (2.9)$$

Aerobes and facultative aerobes generally contain both superoxide dismutase and catalase, although a few obligate aerobes lack catalase (Bonting *et al.*, 1992). Superoxide dismutase is indispensable to aerobic cells, and the low levels (or complete absence) of this enzyme in obligate anaerobes is likely the major reason why oxygen is toxic to them. Due to this, most of the experiments must consider the O_2 level as a precursor in biosynthesis reaction (Wong *et al.*, 2000; Satoh *et al.*, 1998b). Therefore, the aerobically processes have been done in most of the configurations in this study.

Activated sludge acclimatized under anaerobic-aerobic conditions accumulates PHA, will have some advantageous that anaerobic-aerobic operation of the activated sludge process is best for the enrichment of PHA accumulating microorganisms (Satoh *et al.*, 1998b). It is known that anaerobic-aerobic activated sludge process usually enriches microorganisms that have a significant metabolism. Typically, under anaerobic/aerobic or anoxic conditions, the PAOs will utilize glycogen and poly-P to convert substrate to PHA. Also, the glycogen is transferred in PHA. After anaerobic phase, bacteria grow on PHA and also produce glycogen

and poly-P again. The regenerated polyphosphate and/or glycogen will be used for the next contact with organic substrates under aerobic conditions. However, the GAOs will only utilize glycogen to convert substrate to PHA. After anaerobic phase, bacteria will grow on PHA and produce glycogen again. In addition, their ability (microorganisms) to accumulate glycogen under aerobic condition adversely affects PHA production, since some part of the organic substrate might be converted to glycogen instead of PHA. As a conclusion, both PAOs and GAOs can be used to produce PHA because they will use fermentation products as a substrate.

The microaerophilic-aerobic activated sludge process is a modification of the anaerobic-aerobic process which will accept a limited amount of oxygen. In the microaerophilic reactor, microorganisms are contacted with organic substrates in the existence of a limited amount of oxygen (Satoh *et al*., 1998a, 1998b). In such conditions, microorganisms can take up organic substrates by consuming energy through oxidative degradation of some part of the organic substrates. If the supply of oxygen is sufficient, the microorganisms may be able to consume enough energy for assimilative activities such as the production of protein, glycogen, and other cellular components simultaneously with taking up of organic substrates. If the supply of oxygen is adequately controlled, it is possible to suppress such assimilative activity while letting microorganisms accumulate PHA. The following aerobic condition, where excess oxygen is supplied is to let microorganisms grow with the consumption of PHA (Marazioti *et al*., 2003).

Ueno *et al*. (1993) and Saito *et al.* (1995) found that activated sludge can be accumulated more PHB under aerobic than under anaerobic conditions. Satoh *et al*. (1998b) introduced the microaerophilic–aerobic process where a limited amount of oxygen is supplied to the anaerobic zone of anaerobic–aerobic operation. In such conditions, microorganisms can take up organic substrates by obtaining energy through oxidative degradation of some part of the organic substrates. PHA will be accumulated more than glycogen under microaerophilic conditions is that PHA production requires less energy than production of glycogen (Satoh *et al*., 1998b). The microorganism will not have the ability to utilize energy reserves of materials such as polyphosphate or glycogen for anaerobic substrate uptake. As a conclusion,

the expected advantages of the enrichment of PHA accumulating microorganisms by microaerophilic-aerobic process are as follow, (a) the PHA accumulators could be selected regardless of whether they have the ability to accumulate polyphosphate or glycogen or not, (b) the selected PHA accumulators will have fewer tendencies to accumulate glycogen (van Loosdrecht *et al*., 1997; Saito *et al.*, 1995).

2.6.3 Nitrification – Denitrification (Aerobic/Anoxic) Condition

Heterotrophic and autotrophic microorganisms take up and assimilate NH_4^+ and NO_3^- after reduction to NH_4, as shown in Eq. 2.10 and 2.11. Assimilation is responsible for some nitrogen removal in wastewater treatment plants. Plant and algal cells uptake nitrogen is preferably in the form of NH_4^+. Cells convert NO_3^- or NH_4^+ to proteins and grow until nitrogen becomes limiting. For each 100 units of carbon assimilated, cells need approximately 10 units of nitrogen (C/N ratio = 10).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \quad (2.10)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (2.11)$$

Conventional biological nitrogen removal from wastewater is performed in a combined process of nitrification (conversion of NH_4^+ into NO_3^-), denitrification (conversion of NO_3^- into N_2-gas) and carbon removal. Nitrogen removal can also be performed in batch process (Bitton, 1994). In all these processes the microorganism experiences alternating aerobic and anoxic (NO_3^- present as electron acceptor) periods: NH_4^+ and NO_3^- are the electron donors and carbon dioxide (CO_2) is the carbon source. NO_3^- and NO_2^- replace oxygen for microbial respiration. Denitrification requires an organic compound as carbon and energy source. This compound is generally the organic substrate present in the raw wastewater. If denitrifiers are subjected to dynamic conditions with respect to the availability of NO_3^- and external substrate, storage polymers like PHB can be formed (van Loosdrecht *et al*., 1997).

Denitrification is classically considered to be a heterotrophic process conducted by microorganisms that require a reduced organic substrate for energy and cell synthesis. The bacteria responsible for denitrification process are both heterotrophic and autotrophic. *Pseudomonas* species are the most common and widely distributed of all denitrifiers and have shown to use a wide array of organic compounds including hydrogen, methanol, carbohydrates, organic acids, alcohols, benzoates, and other aromatic compounds (Metcalf and Eddy, 2003). Generally, denitrification is considered to be an anoxic process, occurring in the presence of NO_3 and the absence of molecular O_2 (Durner *et al.*, 2000). However, several researchers showed that, in certain species, denitrification could occur in the presence of O_2 (Robertson and Kuenen, 1984; Lloyd *et al.*, 1987). Although aerobic denitrification tends to be slower than anoxic denitrification (Robertson and Kuenen, 1992), however if an organism is capable of both, this would presumably enhance its survival ability (Llyod *et al.*, 1987).

Storage of PHB always derived from the high COD/N ratio requirements in nitrification/denitrification processes. In these systems usually the sludge is subjected alternatively to anoxic and aerobic zones. If in the anoxic zone denitrifiers store substrate internally as PHB, it is not efficiently used for denitrification. As a result, more external substrate is needed to reduce all NO_3^- to N_2 (Beun *et al.*, 2002). Microbial storage of substrate as PHB results in higher required COD/N ratios for the denitrification. In addition, PHB is oxidized aerobically in the aerobic zone, which requires extra O_2.

The metabolic model was based on an aerobic model proposed by Van Aalst-van Leeuwen (Van Aalst-van Leeuwen *et al.*, 1997). Seven internal reactions describe the metabolism of the biomass capable of storing PHB and growing with ammonia as sole nitrogen source with either acetate or PHB as carbon and energy source. In these reactions, 0.66 mol ATP is needed for the synthesis of 1 mol biomass precursors from acetyl-CoA (Stouthamer, 1973) and 0.267 C-mol CO_2 is produced (Gommers *et al.*, 1988). 1.5 mol ATP is needed for polymerization of biomass precursors to 1 C-mol of active biomass (Verduyn *et al.*, 1991). The only unknown are the specific ATP consumption due to maintenance processes (m_{ATP})

and the amount of ATP produced per mol of $NADH_2$ oxidized, i.e. the efficiency of the oxidative phosphorylation ($P/2e^-$ ratio of δ)

The oxidative phosphorylation under aerobic conditions can be written as follows:

$$-1\,NADH_2 - 1/2\,O_2 + H_2O + \delta_o\,ATP = 0 \qquad (2.12)$$

and under anoxic conditions as:

$$-1\,NADH_2 - 1.0\,HNO_3 + H_2O + \delta_n\,ATP + 1.0\,HNO_2 = 0 \qquad (2.13)$$

$$-1\,NADH_2 - 0.667\,HNO_2 + H_2O + \delta_n\,ATP + 0.333\,N_2 = 0 \qquad (2.14)$$

It has been demonstrated that the $P/2e^-$ ratios for nitrate and nitrite are approximately the same, and are therefore indicated with one parameter (δ_n) (Beun *et al.*, 2000b). The reason for this is that the energy yield based on electrons is the same. It has also been demonstrated that the efficiencies of oxidative phosphorylation of electron transport to nitrite and nitrate are about 60% of that to oxygen (Sthouthamer, 1988; Kuba *et al.*, 1996).

During the first two hours of anoxic treatment (applied the transient between anaerobic and aerobic condition), the respiration rate increased due to a high consumption of readily biodegradable organic matters (S_s) resulting in an increase in viable biomass. Generally, in International Water Association (IWA) activated sludge model, it is assumed that denitrification takes place exclusively on S_s (IWA, 2000). Activated Sludge Model 3 (ASM3), however concerns storage and there growth and denitrification occur on storage products. These models are not fully correct in order to keep them simple. They are made to describe a treatment plant not to 100 % explain how things really go. During transformation of S_s, biomass concentration would increase, thus corresponding to significant changes in COD fraction composition at the beginning of experiment. The changing rates were then slowly decreased because S_s were depleted and the slowly biodegradable substrates (X_s) must first be dissolved by extracellular enzymes and thus assimilated at much

slower rates. This process is referred to as hydrolysis. Decay of biomass generated X_s and particulate and dissolved non-biodegradable products. Dissolved products are also formed during degradation of S_s. Storage of PHB occurs when the substrate uptake exceeds the conversion capacity of assimilatory processes. The internally stored PHB can be used for growth later, when there is no external substrate available. Effectively PHB is used to balance the growth rate of the bacteria under dynamic conditions and enables the bacteria to efficiently compete for external substrate (Krishna and van Loosdrecht, 1999a; Majone *et al*., 1999; Beun *et al*., 2000a)

Beun *et al.,* (2000b) concluded that the process of storage and subsequently PHB degradation under anoxic conditions in fed-batch SBR is essentially the same as under aerobic conditions. Under both anoxic and aerobic conditions at similar SRT, about 70% of substrate is used for PHB synthesis, while the rest is used for growth process. The behaviour of microorganisms appears to be very similar under anoxic and anoxic/aerobic conditions. The significant result of the study was that the anoxic specific substrate uptake rate was three to four times lower than aerobic one. It was shown that neither substrate uptake nor PHB degradation nor electron transport was the rate limiting step (Beun *et al.,* 2000a, 2000b).

2.7 Configuration of PHA Productions

Bacteria that are used for the production of PHAs can be divided into two groups based on the culture conditions required for PHA synthesis (Lee, 1996b). The first group of bacteria requires the limitation of an essential nutrient (e.g. N, P, Mg, K, O or S) for the efficient synthesis of PHA from an excess carbon source. The second group of bacteria does not require nutrient limitation for PHA synthesis, and can accumulate polymer during growth. *A. eutropha, Protomonas extorquens, P. oleovorans* and many other bacteria belong to the first group, while some bacteria such as *A. latus*, a mutant strain of *Az. vinelandii*, and recombinant *E. coli* harbouring the *A. eutropha* PHA biosynthesis operand belong to the second group (Choi *et al.,*

1998; Chua *et al*., 1997). Therefore, these characteristics should be considered in developing cultivation methods for the efficient production of PHAs (refers Table B1 in Appendix B). According to this, the fed-batch cultivation will be suitable to promote the PHA accumulation.

2.7.1 Fed-Batch Cultures (Feed Substrate and Growth Condition)

For fed-batch cultures of bacteria belonging to the first group, a two-step cultivation method (but not necessarily requiring two fermentor vessels) is commonly employed. Two stages of cultivation will be essential to biomass growth and maintenance. During the nutrient limitation stage (accumulation period), the residual cell concentration (defined as the cell concentration minus the PHA concentration) remains almost constant, and the cell concentration increases only because of the intracellular accumulation of PHA. However, most of the PHA accumulation always occurs in the first group bacteria, such as *P. extorquens* and *P. oleovorans* (Braunegg *et al.*, 2002; Ma *et al*., 2000). For the cultivation of these bacteria, a mixture of carbon source and a nutrient limited at an optimal ratio should be fed to produce PHA with high productivity. Since the cell concentration at which a nutrient is initially limited significantly affects the final PHA concentration obtainable, it should be optimized with each bacterial strain to be employed. A premature limitation of nutrient will result in low final cell and PHA concentrations, resulting in low PHA productivity, even though high PHA contents may be obtained. If application of nutrient limitation is delayed too long, cells are not able to accumulate much polymer, resulting low PHA content even though high cell concentration can be achieved (Braunegg *et al*., 2002, 1998).

For the fed-batch culture of bacteria belonging to the second group, the development of a nutrient feeding strategy is crucial to the success of the fermentation. In order to reduce cost production from nutrient adaptation, complex nitrogen sources such as corn steep liquor, yeast extract or fish peptone can be supplemented to enhance cell growth as well as polymer accumulation (Lee *et al*., 2000). Cell growth and PHA accumulation needs to be balanced to avoid incomplete

accumulation of PHA or premature termination of fermentation at low cell concentration. There is an interesting relationship between the residual cell concentration and PHA content. Since PHA is accumulated in the cytoplasm, the residual cell concentration will determine how much PHA can potentially be produced. A high PHA content with a low residual cell concentration will result in a low PHA concentration and productivity. A high residual cell concentration with a low PHA content will also reduce the final PHA concentration, productivity and yield. As a conclusion, a high residual cell concentration with a high PHA content will give the best results (Carucci *et al.*, 2001; Su *et al.*, 2000). However, there exists an upper limit of PHA concentration that can be obtained owing to the maximum cell concentration practically achievable in a fermentor. This can be better understood by the following simple equations (Ganduri *et al.*, 2005; Durner *et al.*, 2000):

$$X = R + P \tag{2.15}$$

$$f = P/X \tag{2.16}$$

From equation 2.12 and 2..13;

$$P = \frac{Rf}{(1 - f)} \tag{2.17}$$

Since,

$$X = \frac{P}{f} \leq X_{max} \qquad P_{max} \leq X_{max} f_{max} \tag{2.18}$$

Where, X = cell concentration; P = PHA; R = residual cell, f = PHA content

P_{max}, X_{max}, f_{max} are the maximum attainable PHA concentration, cell concentration and PHA content, respectively

It is important to decide when to stop the cultivation. In most cases, fermentation should be stopped when the productivity is highest. Cells can be cultivated further to obtain a higher PHA concentration, but this may result in a lowered overall productivity. Prolonged cultivation to achieve higher PHA concentration with a slightly lower productivity will be advantageous only if the

PHA content is also increased, thus allowing easier recovery and purification of the polymer.

The concentration of a substrate supplied also affects the amount of polymer produced. For example, when propionate was used as a sole carbon source, the highest PHA content of 56%, produced by *R. eutropha*, was achieved at the propionate concentration of 14 g/l, while the lowest PHA content of 12% was obtained at the substrate concentration of 2 g/l. ICI reported that the copolymer of 3HB-3HV was produced by *R. eutropha* using propionic acid and glucose as a carbon source (Doi *et al*., 1990). The mole percentage of PHV in the copolymer was varied depending on the compositions of the feeding substrate. The PHV content of greater than 95 mol% was obtained when pentanoic and butyric acids were used. Doi *et al.* (1990) stated that the structure and compositions of PHA, as well as its physical and thermal properties can be controlled by composition and concentration of feeding substrates. They did experiments investigating PHA production by *R. eutropha* using various types of substrate. The copolymer of 3-hydroxybutyrate and 3-hydroxypropionate (3HB-3HP) was obtained when 3-hydroxypropionic acid, 1,5-pentanediol, 1,7-heptanediols, or 1,9-nonanediol was used as the carbon source. The copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate (3HB-4HB) was obtained from 4-hydroxybutyric acid, γ-butyrolactone, 1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol, or 1,12-dodecanediol. The copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate (3HB-3HV) was obtained from propionic or pentanoic acid. In addition, the biodegradability of PHA film (initial weights: 4-8 mg and initial film dimensions: 10 x 10 mm. in size and 0.03-0.06 mm. thick) was also studied. It was found that the rate of degradation was enhanced when 3HB and 4HB units were present in the copolymer. The presence of 3HV units reduced the degradation rate of copolymer.

Shimizu *et al*. (1994) investigated the PHA production from *R. eutropha* H16 (ATCC 17699) fed with butyric and valeric acids. Optimum conditions for PHB production using butyric acid by this organism were at the concentration of 3 g/l butyric acid and pH of 8.0. PHV or other PHAs were not reported in this study. PHB content of 75 % was obtained under these conditions, while lower PHB

contents were achieved when pH was kept at 8 and butyric acid concentrations were 0.03, 0.3, 10 g/l, i.e., PHB contents were 44%, 55%, and 63%, respectively.

Steinbüchel and Pieper (1992) studied the production of PHB-PHV copolymer by *R. eutropha* strain R3 under nitrogen limitation. PHA contents were 47%, 35.7%, 29.5%, 21.5% and 43.2% when fructose, gluconate, acetate, succinate and lactate were used as a carbon source, respectively. PHV contents in the copolymer produced from this organism were in the range of 4-7% from all the substrates used. When magnesium or sulphur was a limiting condition and fructose was used as a sole substrate, *R. eutropha* strain R3 could accumulate PHA of 45% or 47% with the PHV fraction of 7% or 6%, respectively.

Suzuki *et al*. (1986) reported the maximum PHB production of 66% of dry weight by *Pseudomonas sp. K* using methanol as a sole carbon and energy source. In order to obtain the high content of PHB, a proper medium composition was utilized. In this study, concentrations of phosphate and ammonium were maintained at low levels. Nitrogen deficiency was found to be the most effective way to stimulate the accumulation of PHB. The limitation of dissolved oxygen (DO) concentration was found to decrease the rate of biomass growth and PHB production. This finding was contradictory to the results reported by others.

Seeking a less costly substrate, Bourque *et al*. (1992) investigated the production of PHA by 118 methylotrophic microorganisms grown on a cheap substrate like methanol. *Methylobacterium extorquens* was found to accumulate a high PHA content when grown on the mixture of methanol and valerate. PHA content of 60-70% with 20%PHV was produced by this organism. Lee and Yu (1997) operated a two-stage bioprocess for PHA production. The first stage was an anaerobic digester. A mixture of volatile fatty acids produced by the first stage was used by *R. eutropha* for PHA production in a subsequent stage. *R. eutropha* was grown under aerobic and nitrogen-limiting conditions. PHA production of about 34% of cell mass was obtained by *R. eutropha* using digested sludge supernatant. The major component of the sludge PHA was C_4 monomers. The sludge PHA had a melting point of 167°C, 9^{o}C lower than PHB homopolymer. *R. eutropha* consumed

approximately 78% of the TOC of the digested sludge supernatant. Acetic acid was the most effective fatty acid used by *R. eutropha* followed by propionic acid, butyric acid, and valeric acid.

2.7.2 Bacterial Strain

Byrom (1992) discussed the industrial production of PHA. *Ralstonia spp.* was an organism of choice because it produced an easily extracted PHA with high molecular weight. PHA productions from *Azobacter* and *methylotrophs* were also investigated. However, PHA with low yield and molecular weight was produced from *methylotrophs* and PHA produced was difficult to extract. *Azobacter* was not an organism of interest because it used carbon substrate for polysaccharide synthesis rather than for PHA production. *R. eutropha* produced 70-80% polymer under phosphate limiting conditions.

Byrom (1990) stated that the problem experienced using the wild-type of *R. eutropha* was that propionate was used ineffectively, i.e., only about one third of proprionate was incorporated into the HV unit of the copolymer. The mutant strain, PS-1, was found to utilize propionate more effectively. A propionate fraction of 80% or greater was incorporated into the HV unit of the copolymer by the mutant strain. The fraction of PHV of 0-30% was obtained when the ratio of the two substrates was varied. *A. latus* can store PHA up to 80% under normal growth condition. Therefore, one-step PHA production process can be used with this organism (Hrabak, 1992).

Yamane *et al.* (1996) studied the production of PHA by *A. latus* using sucrose as a feed substrate. High cell concentration (142 g/l) was obtained in a short culture time (18 hours) and PHB content at the end of the culture time was 50%. They concluded that the innoculum size reduces the culture time. They compared the culture time required for the production of PHB by *R. eutropha* fed with glucose when the same techniques (pH-stat fed-batch) were used. Brandall *et al.* (1998)

stated that *A. vineladii* was not considered for commercial production because it produces PHA with low yield and forms capsules. Strain UWD of this organism, however, is of interest because it is a capsule-negative mutant and produces PHA content of approximately 70-80%.

As a conclusion, PHA contents and its composition are influenced mainly by the strain of microorganisms, the type of substrate employed and its concentration, and environmental growth conditions.

2.8 Renewable Resources for PHA Production

Selection of a suitable substrate is an important factor for optimizing of PHA production and affects on PHA content, composition and polymer properties. Many waste streams from agricultural and agro-industry (e.g. whey, molasses and POME) are potentially useful substrates and possibly may contribute to an economic PHA production. Hassan *et al*. (2002); Nor Aini *et al*. (1999); Hassan *et al.* (1996) have produced organic acids from POME, which were used as fermentation substrates to produce PHA.

The purpose of a zero emission from palm oil (PO) industry incorporating the production of PHA from POME was extensively studied by Hassan and co-workers (Hassan *et al*., 2002). The results showed that by evaporation, the organic acids could be concentrated to about 100 g/l for use as substrates for the fed-batch PHA fermentation. The concentrated organic acids were successfully converted to PHA by *R. eutropha* strain ATCC 17699 under a non-sterile fermentation system when the initial cell density was kept high at 4 g/l. After 150 hours, 20 g/l cells were obtained with more than 50% PHA content. A repeated fed-batch system was also performed to obtain a high cell inoculumn and to mimic the operation of a large PHA production fermentor at C/N ratios of 15 and 30 respectively, with only acetic and propionic acids as carbon sources. It was suggested that the energy for this proposed process could be sufficiently supplied by combustion of the solid wastes from the

palm oil mill. The oil refineries' waste (PO mill) such as cracker condensate and effluent of a partial wet oxidation unit are available as potential sources of VFA (Salehizadeh and van Loosdrecht, 2004). Table 2.7 presents an overview of possible waste streams and their PHA production capacities under the presence of high VFA present in wastes.

Table 2.7: An overview of waste streams suitable for PHA production

Substrate source	Flow (m^3/hr)	Availability (mnth/year)	COD (kg COD/m^3)	Capacity (ton COD/m^3)	Production (ton PHA/year)
Potato starch production	300	2.5	2.5	6750	2431
Innuline production process	600	5	14.0	3066	1134
Sugar beets process	3750	3	1.9	15604	5773
Brewery wastewater	300	12	2.8	7358	2723
Vegetable, fruit and garden	90	12	15	11774	4356
Household garbage	30	12	50	13333	4933

Note: In calculation, the yield *Y* is assumed to be 0.37 kg PHA/kg COD
(Source: Meesters, 1998)

Low–cost production of PHB requires improved fermentation strategies, inexpensive media and easier downstream recovery methods (Luengo *et al.*, 2003). In the past, a different bacterium, *A. eutrophus*, had been the focus of attention as a producer of PHB, but that microorganism requires expensive two-stage cultivation (Marangoni *et al.*, 2001; Byrom, 1990). As for any microbial process, the performance of culture-enrichment is susceptible to many influences, including temperature, pH, carbon-to-nitrogen (C/N) ratio in the feed, concentration of substrates, concentration of trace elements, ionic strength, agitation intensity, and the DO level. Fortunately, many of fermentation processes have been developed to enhance the PHB formation especially for industrialization production.

A PHA production process developed by ICI (now taken over by Mosanto) was evaluated by the Institute for Applied Environmental Economics (Salehizadeh and van Loosdrecht, 2004). In the same report the conventional process for plastic

production (PP) is evaluated. The five (5) most important emissions and the total consumption energy in the full life cycle are presented in Table 2.8 together with the comparison of pure culture and mixed cultures

Table 2.8: Comparison of emissions for PHA production by mixed cultures, pure culture and the production of PP

Elements	PHA (mixed culture) (kg/ton)	PHA (pure culture) (kg/ton)	PP (kg/ton)
Chlorinated compounds	< 20	110	0.24
Heavy metals	0	0.7	5.77
N compounds to wastewater	10	364	0.4
Other emissions to water	5.24	5.24	0.9
CO_2 to air	3000	8920	4257
Energy used (GJ)	39	99.7	6.2

Note: all emissions include production of raw materials
(Source: Salehizadeh and Van Loosdrecht, 2004)

2.9. Biodegradation of PHA in Waste Environment

Biodegradability is defined as the capacity of a substance to be broken down, especially into innocuous products, by the action of microorganisms. Bacteria and fungi are the main participants in the process of biodegradation in the natural world. The breakdown of materials provides them with precursors for cell components and energy for energy-requiring processes.

The three types of biodegradable plastics introduced are photodegradable, semi-biodegradable, and completely biodegradable. Photodegradable plastics have light sensitive groups incorporated directly into the backbone of the polymer as additives. Extensive ultraviolet radiation (several weeks to months) can disintegrate their polymeric structure rendering them open to further bacterial degradation (Kalia *et al.*, 2000). However, landfills lack sunlight and thus they remain non-degraded. Semi-biodegradable plastics are the starch-linked plastics where starch is

incorporated to hold together short fragments of polyethylene. The idea behind starch-linked plastics is that once discarded into landfills, bacteria in the soil will attack the starch and release polymer fragments that can be degraded by other bacteria. Bacteria indeed attack the starch but are turned off by the PE fragments, which thereby remain non-degradable (John and Stephenson, 1996). The third type of biodegradable plastics is rather new and promising because of its actual utilization by bacteria to form a biopolymer, which include the PHA.

PHB is completely degraded by many species of soil bacteria, which use it as an energy source (Luzier, 1992). The polymer is first degraded by extracellular enzymes to monomeric and dimeric hydroxybutyrate, which are then taken up by the cells and metabolized (Lafferty *et al*., 1988). The rate of PHB degradation depends upon surface area, microbial activity, pH temperature, moisture and the presence of other nutrients (Luzier, 1992). Table 2.9 compares the rates of degradation of a one milimeter thick sheet of PHB in various environments.

The degradation rates in moist aerobic soil of a thin film sample of ICI's Biopol® and a similar sample of a blend of corn starch and low density polyethylene (LDPE) have been compared, with the result that the Biopol® was almost completely degraded in 44 days, while the corn starch-LDPE sample showed only 4% decay in that time (Barak *et al*., 1991). Biopol® copolymers usually degraded slightly less rapidly than PHB homopolymer (Miller and Williams, 1987), while copolymers containing 4-hydroxybutyrate degraded more rapidly than PHB homopolymer (Doi *et al*., 1989). This inverse relationship between length of side chains and rates of depolymerization is most likely due to steric hindrances that block degradative enzymes (Doi, 1990). Doi *et al*., (1992a) showed that non-biologically produced PHB, which contained isotactic or atactic chains of R and S isomers, was not degraded because the degrading enzymes are not capable of hydrolyzing S isomers, which are not found in natural PHB. Doi *et al*. (1992b) report that biodegradation of PHB homopolymer and copolymer samples in sea water was independent of monomer composition but strongly related to water temperature, with higher temperatures leading to faster degradation.

Table 2.9: Biodegradability of PHB in various environments

Environment	100% dissolution of 1 mm thick sheet (weeks)	Average rate of corrosion per week (μm)
Anaerobic sewage	6	100
Estuarine sediment	40	10
Aerobic sewage	60	7
Soil (25°C)	75	5
Sea water (15°C)	350	1

(Source: Lafferty *et al*., 1988)

PHA (e.g. P(3HB) and P(3HB-*co*-3HV)s) are degraded in both aerobic and anaerobic environments by the action of extracellular enzymes from microbial populations (Luzier, 1992). Doi and co-workers (Kunioka *et al*., 1989) have further pursued their early studies on the hydrolytic and enzymatic degradation of films of P(3HB), P(3HB-*co*-3HV)s and P(3HB-*co*-4HB)s in various environments. These studies showed that the presence of 4HB units enhances the rates of both types of erosion. Nakamura *et al*. (1992) exposed P(3HB-*co*-4HB) films to extracellular PHA depolymerase isolated from *A. faecalis*. Enzymatic degradation as measured by weight loss was accelerated by 4HB contents up to 28 mol%, but depolymerization was inhibited at 4HB fractions above 85 mol% of the copolyester. In another set of similar experiments (Kang *et al*., 1995), the critical 4HB fraction was 13 mol%. At this point the rate of degradation was about 10 times faster than that of the homopolymer P(3HB). Doi *et al*. (1992b) have speculated that this acceleration could be attributed to the decreased crystallinity of 4HB copolymers relative to P(3HB) and P(3HB-*co*-3HV)s, offering the degradative enzymes better access to the polymer chains. Nishida and Tokiwa (1993) confirmed that crystallinity depressed the microbial degradability of P(3HB). A P(3HB-*co*- 4-mol% 3HP) copolyester was found to enzymatically degrade faster than P(3HB) (Nakamura *et al*., 1992).

There are some general guidelines for the relationship between structure and biodegradation as summarized by Kawai (2000):

(i) Naturally occurring polymers are biodegradable. Chemically modified natural polymers may biodegrade, depending on the extent of modification and the kind of modifying group.

(ii) Synthetic addition polymers with carbon-chain backbones do not biodegrade at molecular weight greater than about 1000. Polyvinyl alcohol is an exception, the biodegradability of which is due to pendant hydroxyl groups which are readily converted to hydrolysable carbonyl groups.

(iii) Synthetic addition polymers with hetero-atoms in their backbones may biodegrade; these include polyacetals and polyesters.

(iv) Synthetic step-growth or condensation polymers are generally biodegradable to a greater or lesser extent, depending on:

- Chain coupling (ester > ether > amide > urethane);
- Molecular weight (lower is faster than higher);
- Morphology (Tm) (amorphous is faster than crystalline);
- Hardness (Tg) (softer is faster than hardner); and
- Hydrophilic versus hydrophobic (hydrophilic is faster than hydrophobic

(v) Water solubility does not guarantee biodegradability

Thus, biodegradability is primarily dependent on hydrolysable and utilizable chemical structure, balance of hydrophobic and molecular weights.

CHAPTER III

RESEARCH METHODOLOGY

3.1 Research Design and Procedure

This study emphasizes two approaches for obtaining bioplastic production by mixed cultures: growth and accumulation processes using a lab-scale SBR. Under serial process, microorganisms grow under non-limiting nutrient conditions with the aim of biomass generation (allowing fast proliferation phase). Then, under parallel process, nutrient available was restricted subsequently to minimize biomass growth (slow-down the biomass production and PHA production periods). In general, the system operated under dynamic aerobic condition at temperature of 30^{o}C. However, PHA accumulation was also studied under microaerophilic-aerobic conditions as well as anoxic process conditions. In this study, five parameters were maintained constant (except when changes are mentioned elsewhere), which are:

(a) The time for the growth phase was operated for at least six hours,
(b) Substrate feeding period was maintained for one hour, considered the best accumulation time for "short period" of PHA-producer,
(c) A two-blade propeller was used in this study without considering the exact influence of the mixing regime,
(d) The rotation per minute (RPM) was maintained from 600 – 1000 in both growth and accumulation periods, and
(e) Single fed-batch was used with fixed working volume and discharge level (volumeexchangeratiois0%)

3.2 The Framework of the Study

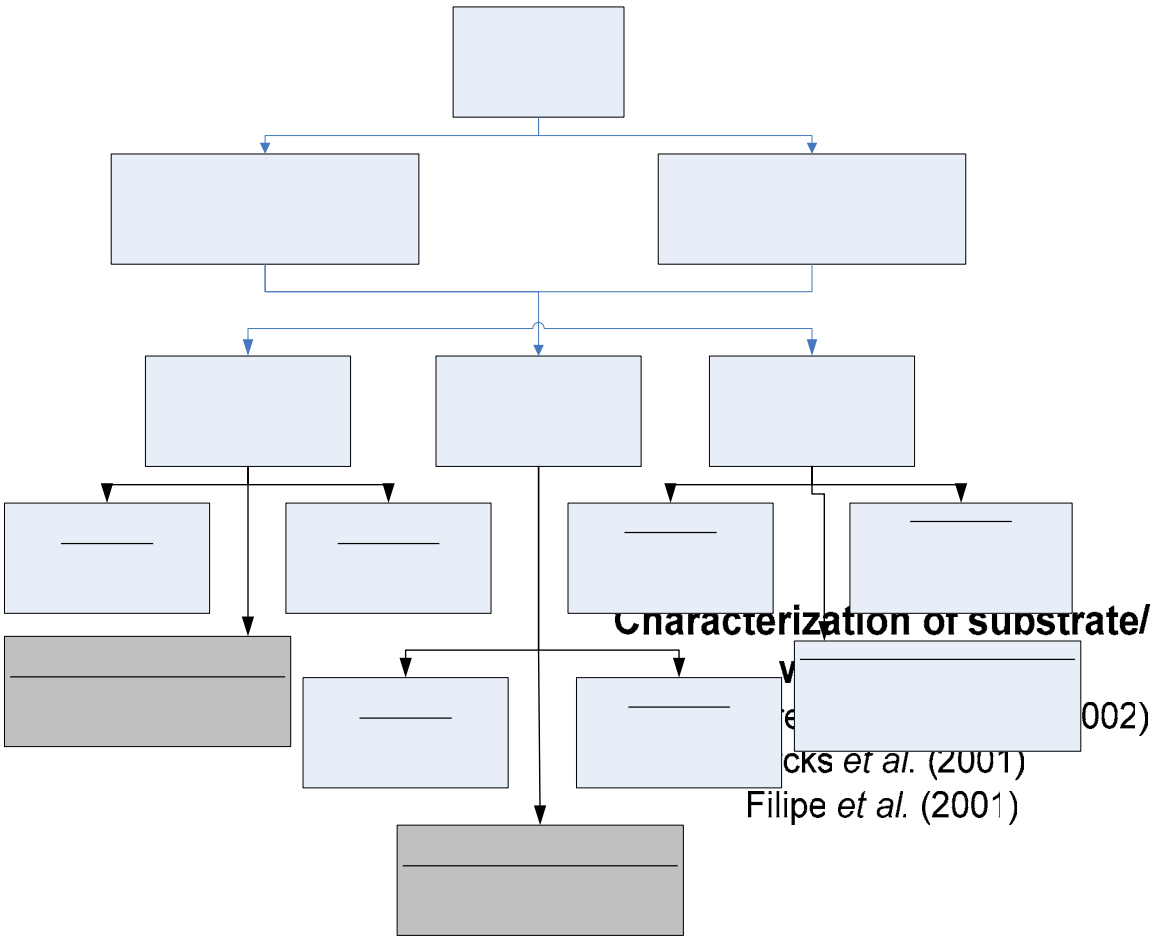


Figure 3.1: Overall studies undertaken to enhance the production rate of PHA

This study was conducted according to the framework as shown in Figure 3.1. There are two processes that have been studied in this work, i.e. aerobic and anoxic processes. The study on anaerobic process was conducted in the past years by other researchers (e.g. Hassan *et al*., 2002, 1997a; Ryu *et al.*, 1999). However, those studies (anaerobic processes) only worked on the selected mixed culture and at low substrate concentrations. Therefore, this study used high substrate concentration, as in POME, and an open mixed culture in order to make a quantify comparison. In addition, the selected operation conditions that have been used in this study were referred to various application and downstream processes (pure culture, mixed culture, synthetic waste, and others.).

Aerobic Process
Pure culture
Carta *et al.* (2001)
Beccari *et al.* (2002)
Mixed culture
Beun *et al.* (2002)
Serafim *et al.* (2004)
Mixed culture + renewable substrate
This study
Pure culture
Majone *et al.* (200
Satoh *et al.* (199

3.3 Scope of the Study

The study on microbial fermentation in SBR system under feast-famine regime was conducted on samples taken from POME (Figure 3.2). The selections of these substrates enable comparison between PHB formations using different types of fatty acids under various experimental factors. POME has long fatty acids, but their carbon chain length is limited to C_{14} atom.

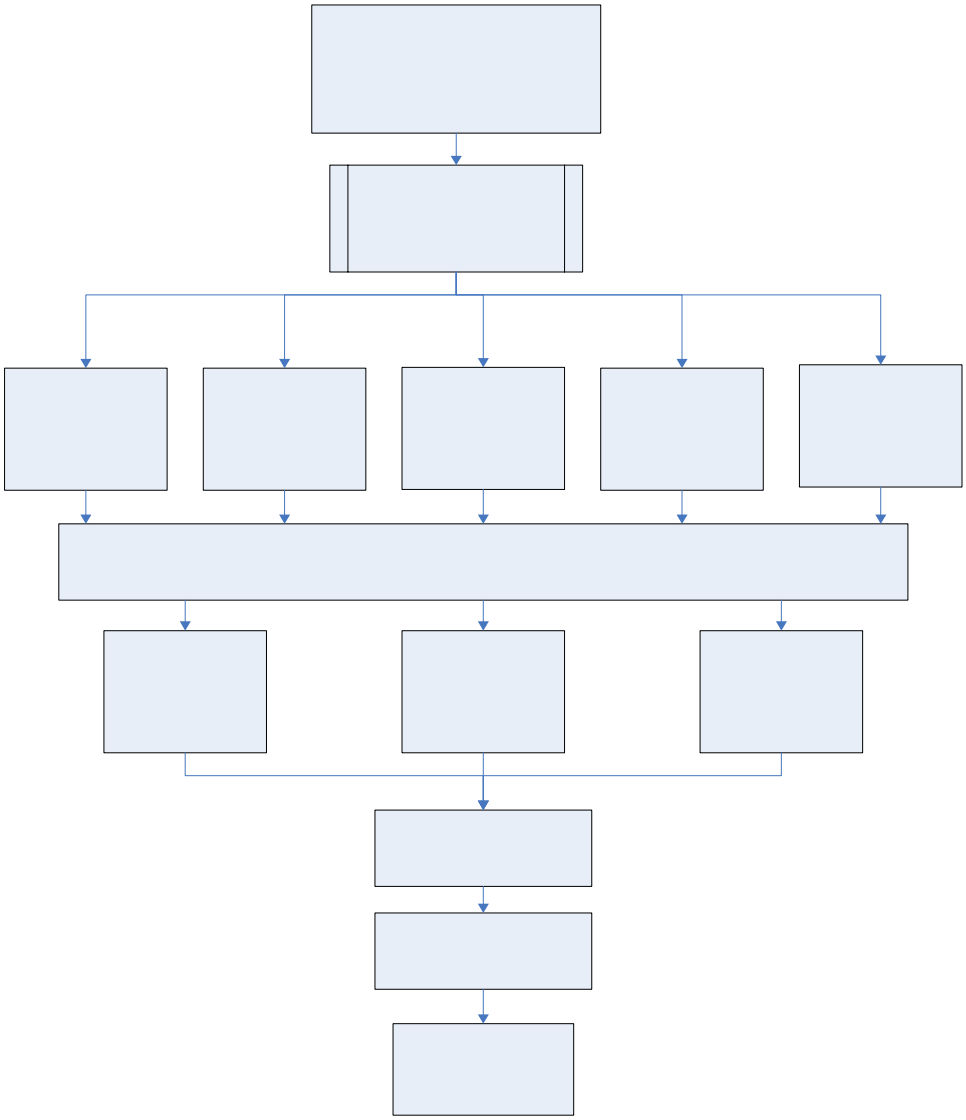


Figure 3.2: Scope of the study

There are experimental conditions that have been chosen as follows:

(a) **Limiting study on N and P**: This study hypothesized that the substrate transported into the cells is first accumulated and then stored as polymer (PHA). The presence of N and P came either from natural decomposition (measure the composition at the end of feeding period) or via adding additional N and P. Therefore, by limiting the ratio of C to N (or COD to N and P), the fraction of substrate derived for cell growth can be controlled.

(b) **Controlling the air flowrate**: This study was conducted to ensure the limiting oxygen saturation (DO saturation) during the feast/famine period. The goal of this study was to maintain the DO saturation at less than 25% per oxygen level, which considered as limiting DO saturation. The DO signal acquisition and control was implemented in a computer equipped with input/output data acquisition board (ISTEK^{TM} with data acquisition, DAPS software).

(c) **Configuration of temperature effects**: This study was operated at four temperature effects (15, 20, 25 and 30^{o}C). The assessment of temperature effects in high fatty acid compositions (SO) was expected to select the preferred experiment (PHA productivity and content) and as a comparison study. Furthermore, it was planned to generate the 'standard' coefficient and stoichiometric rates in the specific substrate composition (with consideration of mixed cultures).

(d) **Long-term study**: This study was conducted over a long-term study to enhance accumulation stages (heterotrophs >> autotrophs). The prolonged system can be done using cycle length experiments.

(e) **Dynamic substrate feeding rate**: This condition was conducted to enhance the PHA storage capacity. The condition must optimize the cell microorganisms in one pulse feeding since the substrate concentration (POME) will slow-down the specific storage rate. The pulse of substrate rate was controlled through peristaltic pump during feeding period.

(f) **Study in anoxic and aerobic condition**: This study was conducted to compare various culture conditions since there was a great impact of electron acceptors (NO_3 and O_2) towards synthesizing the organic components. The anoxic/aerobic was performed in two consecutives period by controlling the

oxygen saturation and nitrification process.

(g) **Study in microaerophilic-aerobic condition**: The limiting of oxygen saturation (< 10%) was conducted for better storage activity (PHA production rate) rather than aerobically condition. Again, two consecutives period have been chosen in this study. Air and NO_3 concentrations were controlled using either gas flowmeter or ATU reagent in certain periods.

3.4 Experimental Set-Up

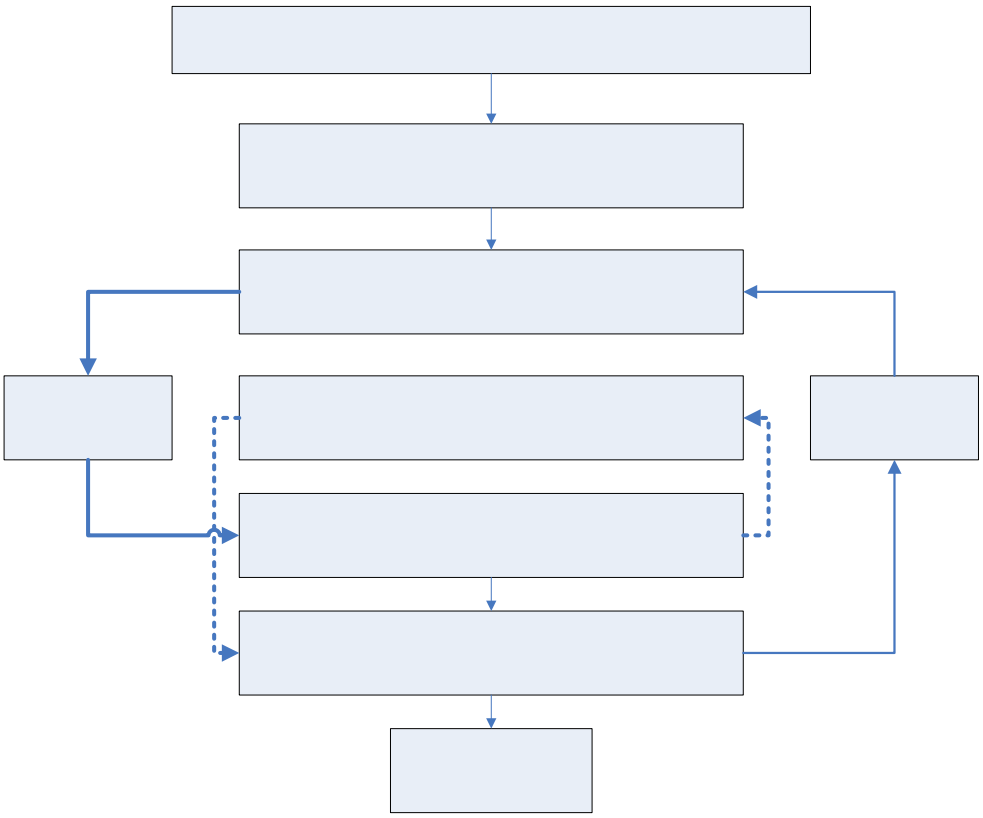


Figure 3.3: Experimental set-up for overall processes

Note: ——— non-limiting nutrient ····· accumulation process
(a) Inoculation for POME experiment, the composition is 30% of mixing POME + 20% of sewage + 40% distilled water
(b) Only for POME cultivations

The fed-batch cultivation will be optimized at different levels and at a different process stage. Initially, the inoculation process was conducted based on

composition of substrate, seeding and distilled water. However, the composition of POME cultivation is slightly different, as shown in Figure 3.3. In general, the proliferation of growth phase was undertaken first to ensure the dynamic population of bacteria growth. Since PHA production typically reached a high value during the end of feast period, the sludge was harvested during that time and the remaining sludge will return to the next cycle.

3.4.1 Methods and Experimental Procedures

The experimental work has performed in two double-jacketed laboratory fermenters with working volume of six litres (POME as a substrate). At least more than 50% of working volume must be designed to be discharged as supernatant. The operating principles of a batch activated sludge system are characterized in just three discrete periods: fill, react and drawing (discharging). In order to maximise the growth rate and fast substrate uptake rate and storage polymer formation, the system was operated in continuous reaction period, which means no settling or allowing the idle phase (HRT = SRT). The length of each phase can be varied independent of the treatment task. The influent is pumped into the tank and mixed with the biomass that settled during the previous cycle until the time for filling is reached. The filling phase can be mixing in aerated (oxygen as electron donor), anoxic (nitrate or nitrite as electron donor) or microaerophilic-aerobic (controlling the oxygen level) conditions. The reaction phase can also be with mixing under the same set of environmental conditions. The cycle of overall processes is shown in Figure 3.4.

The variable parameters selected for study were O_2, N and P. In general, three types of O_2 operating condition were investigated, i.e. the absence of oxygen and presence of nitrate (NO_3^-) (anoxic), O_2 limitation (microaerophilic) and without O_2 limitation. According to the typical composition of POME (high organic contents and low nutrient availability), the experiments were conducted with the limitation of both N and P. Meanwhile, the other operational conditions such as temperature, cycle length and DO have also been investigated. To prevent the possible influence

of nitrification on the measurements, 100 mg of allylthiourea (ATU) was added to the reactor before each sampling cycle.

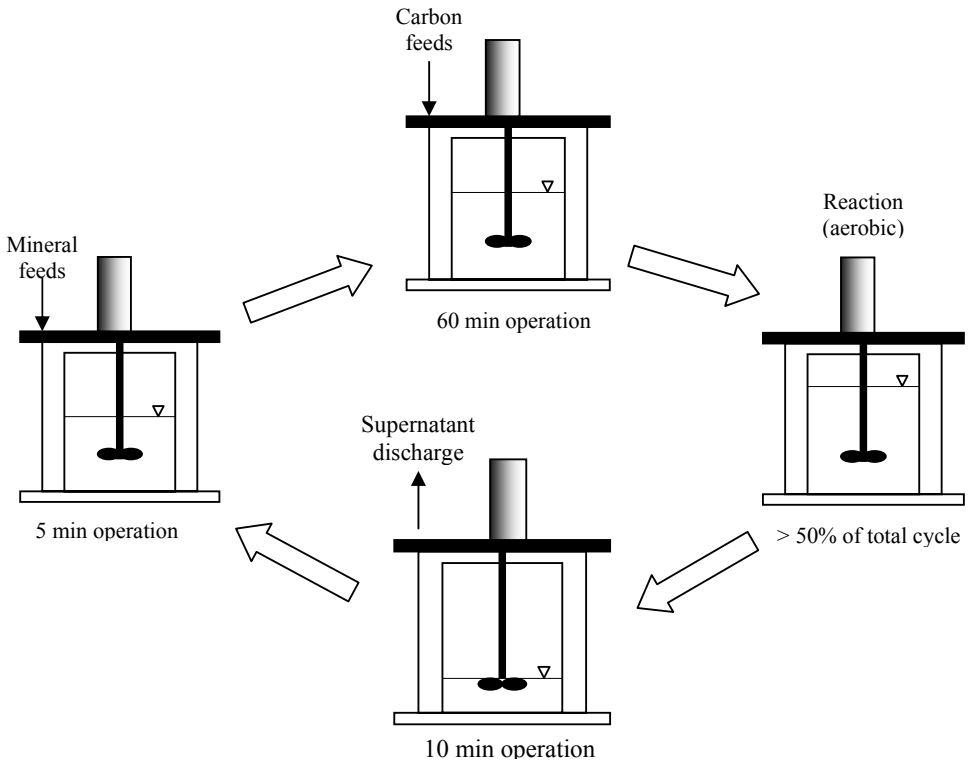


Figure 3.4: Typical cultivation cycle under SBR processes (clockwise sequences)

The condition of steady state condition was determined by using total organic carbon (TOC, Shimadzu, Japan), cell dry weight (CDW) and O_2 profiles. pH (Toledo Mettler, USA) was always maintained at 7.0 ± 0.1 using 2N HCl or 2N NaOH. The temperature in The Netherlands was controlled using TECHNE, model FTE10AD (Germany). At steady state conditions, the process was extensively monitored (pH, DO) and samples were analyzed for VFAs, COD, TOC, NH_4^+, PO_4^{2-}, NO_3^-, PHB, CDW and ash constituents. During growth period, the reactor was well aerated with airflow of at least 10 l/min (POME cultivation). The SBR bioreactor has been controlled by two standard geometry six-blade turbines (stainless steel material). The process was conducted in turbulence regime to ensure completely mixed conditions by using single-stir. The SBR fed-batch cultivations POME are shown in Figures 3.5 and 3.6.

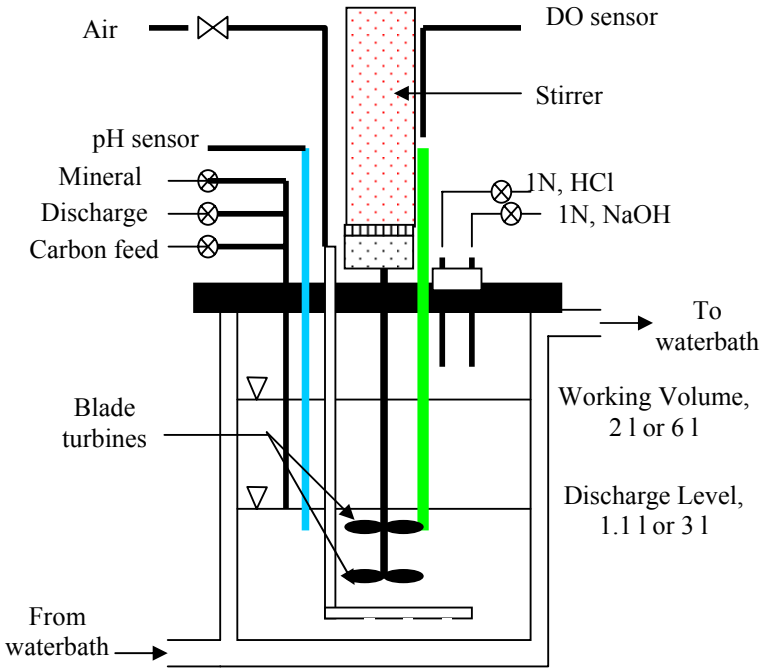


Figure 3.5: Schematic diagram for SBR fed-batch bioreactor

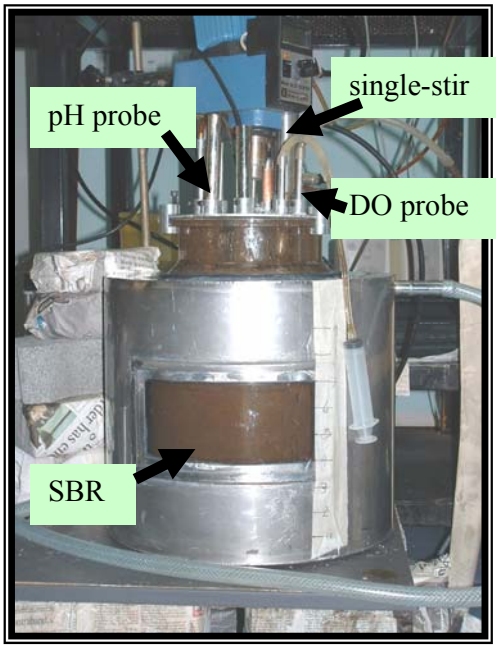


Figure 3.6: SBRs used in this study, 6 liters reactor that is used in laboratory

3.4.2 Experimental Procedures on POME as Substrate

The experimental procedure was performed at Environmental Engineering Laboratory, Universiti Teknologi Malaysia. The study was conducted for 17 months, from July 2003 to December 2004. The substrate was raw POME obtained from Bukit Besar Palm Oil Mill, Kulai, Johor, twice a week. Characterization of POME has monitored by using Gas Chromatography (GC, Chrompack, Germany). Both raw POME and sewage sludge were introduced as inoculums to acclimatize the autotrophic and/or heterotrophic bacteria in the SBR. The experiments were conducted in series (growth and then accumulation phases) for at least six hours of cultivations. Two propellers were used to generate the turbulence regime during the feast condition.

A two consecutive stage bioprocess was developed to obtain high rate of PHA accumulation. This was necessary to grow a bacterial population that contains minimal total phosphorus before starting the subsequent PHA accumulation phase. This approach is similar to the typical commercial PHA production. In addition, PHB accumulation was correlated to the difference in maximum substrate (i.e. VFAs) uptake rate and the substrate required for growth. For an adequate description of activated sludge processes, the dynamics of the production of, and growth on, reserve polymers (PHA) is therefore important. Table 3.1 depicts the typical value of raw POME that was obtained in many treatment plants. It was indicated that lactic and acetic acid are present in a high concentration rather than other fatty acids. Since POME consists of numerous fatty acid components, the expected PHA productions will not only be HB monomer. The other constituents of PHA (e.g. HV and HH monomer) are expected to be occurred.

Table 3.1: Typical value of raw POME compositions

Parameters	Nomenclature	Range (g/l)
Lactic acid	$C_3H_5O_3$	3.85 – 4.0
Formic acid	CH_2O_2	0.2 – 0.3
Acetic acid	$C_2H_4O_2$	3.3 – 4.46
Propionic acid	$C_3H_6O_2$	0 – 0.15
Butyric acid	$C_4H_8O_2$	0 – 0.12

(Source: Malaysia Palm Oil Board, 2005)

During the growth phase, SRT was maintained for at least 10 days to ensure that the biomass grow exponentially in each experiment before starting the next cultivation phase. It was similar in terms of biomass concentration and microbial population. As an example, the biomass was allowed to adjust and grow on the same feed components for about 2-3 SRTs before the PHA accumulation phase. No biomass was discharged from the SBR reactor during the PHA accumulation phase to maximize biomass concentration in the reactor, except that discharged with the supernatant drawn off. In summary, during this cultivation approach the two phases were operated in the same SBR reactor. In general, the overall operation period of POME cultivation is shown in Table 3.2.

Table 3.2: Operating phase with POME as substrate

Experiment(s)	Operating time (min)				
	Aerobic mineral feeding	Aerobic feeding	Aerobic react	Anoxic react	Draw/discharge
Growth	355-360	0-60	60-345	-	345-355
CNP_{pome}	no fill	0-60	60-350	-	350-360
DO_{pome}	no fill	0-60	60-350	-	350-360
HRT_{pome}	no fill	0-60	up to 770	-	up to 780
FR_{pome}	no fill	up to 150	up to 200	-	up to 360
$ANae_{pome}$	no fill	0-60	up to 232	up to 203	up to 360
$MICae_{pome}$	no fill	0-60	60-350	-	350-360

3.5 Analytical Procedures

Samples were taken from the reactor with a 60 ml syringe (Syphon, United Kingdom). The syringe was always rinsed with the content of the reactor before sampling. Part of the sample was stored in the refrigerator for analysis. The remaining supernatant was centrifuged at 10,000 rpm for 10 minutes. The centrifugation for separating the debris and supernatant was performed using Sorval

RC-5B (Hermmicks, Germany) for 15 minutes at 2000 rpm at 4°C and then supernatant filtered by using PVDF-syringe filter. Samples for analysis of NH_4-N, PO_4-P, TOC and COD and VFA were immediately centrifuged and filtered using 0.45 μm filters to separate the bacterial cells from the liquid. The supernatant was stored in refrigerator (for TOC, COD and PHA analysis) and in the freezer (for VFA, VSS, CDW, NH_4^+, NO_3^-, PO_4^{2-} and COD). Analysis of NH_4^+, VSS, PO_4^{2-}, NO_3^- and COD were done in accordance with Standard Methods (APHA, 1995).

Dissolved oxygen concentration in the reactor was measured online using DO electrode, recorded as percentage of air saturation using data acquisition (ISTEK®, Korea). The carbon concentration in the supernatant was measured by gas chromatography (GC), while NH_4^+, NO_3^- and PO_4^{2-} concentrations in the supernatant were measured at 630 nm, 450 nm and 520 nm, respectively with auto analyzers (HACH Spectrophotometer DR-4000U, USA). The supernatant of VFAs were measured according to the type of carbon chains. Acetic acid (HAc), propionic acid (HPr), and butyric acid (HBt) were measured with GC and a flame ionization detector (FID) by direct injection of acidified aqueous samples (pH 2-3) into a Supelco fused-silica capillary column (diameter 0.25 mm x 25 m). The quantification of CDW was performed using the VSS and ash technique according to the Dutch Standard (NNI. NEN).

Samples for the PHA (PHB, PHV and PHH) determination were added to 10 ml tubes containing 2 drops of formaldehyde in order to stop all biological activity immediately. The PHB content was washed with 5 mM phosphate buffer (pH=7) and centrifuged for 10 minutes at 10,000 rpm. The volume of CDW needed should yield at least 50 mg for solids. After that, the solid-free residual was dried using freezer-dryer for almost 24 hours. Solids were weighed as they were placed into 10 ml screw-cap bottle. Before the biomass cell extraction for PHA determination using qualitative (GC method) and quantitative (recovery method) measurements, the saponification process was carried out using the technique proposed by Pavia *et al.* (1988).

3.4.1 Oxygen Uptake Rate/Oxygen Transfer Rate (OUR/OTR) Measurement

The oxygen uptake rate/oxygen transfer rate (OUR/OTR) was measured to ensure the mass transfer and accumulation of organisms achieved in a single experiment. The OUR is also known as "respiration rate". The principle of OUR is based on a series of dissolved oxygen measurements taken on a sample over a period of time. The rate at which microorganism use O_2 is an indicator of the biological activity of the system; high OUR indicate high biological activity; low OUR indicate low biological activity. The O_2 consumption rate as determined in a biological process allows the user to determine the metabolism of the microorganisms. Moreover, the coupling between O_2 and substrate consumption can be used to calculate the amount of the substrate consumed. Empirically, an OUR curve for a batch culture is shown in Figure 3.7.

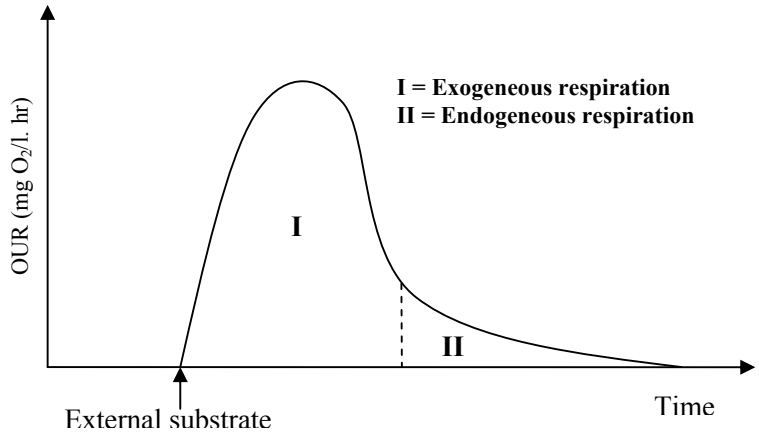


Figure 3.7: Example of a respirogram, where a pulse of organic substrate is added. If certain experimental conditions are met, the peak Phase I and the tail Phase II will be seen.

When interpreting the OUR/OTR curve, it is essential to know the respiration

due to the biomass itself, called endogenous respiration. This respiration is normally assumed to be caused by maintenance of the biomass. The schematic diagram of OUR measurement is shown in Figure 3.8. The OUR vessel was fabricated in just 25 ml to ensure the good monitoring of 'endogenous respiration' of microorganisms. The detail of activity is described in the next paragraph.

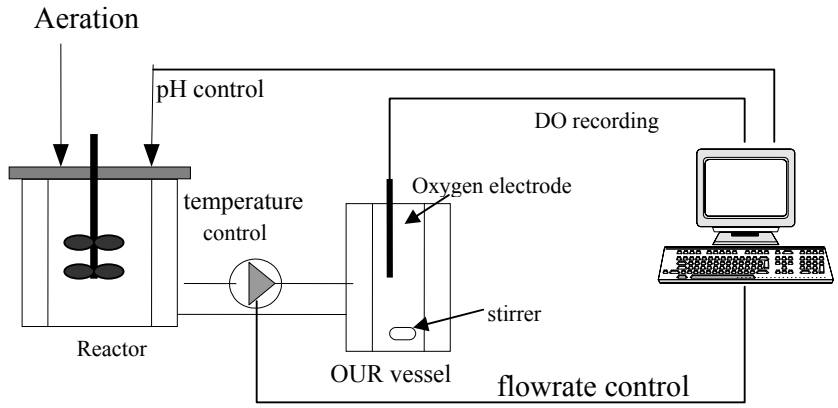


Figure 3.8: Schematic representation of the OUR measurement set-up

I. A fabricated respirometer of 25 ml equipped with a DO probe and a magnetic stirring bar was connected to the reactor and placed on a stirrer. The biomass was pumped directly from the SBR to the vessel and after some time (e.g. three minutes) the recirculation pump was switched off. The decrease in DO concentration during one minute was then measured and electronically recorded using DAPS software (ISTEK®, Korea). In the feast phase the biological activity was high and the DO concentration decreased rapidly. In order to have more measurements in a relatively short feast phase, the DO concentration was measured and recorded for 30 seconds. The dissolved oxygen values against time for each minute were plotted on a graph. A straight line was drawn so that it passes through the greatest number of plotted points (curve of "best fit") and the slope of this line was calculated. The slopes are the OUR of the biomass for a certain time during the cycle. Once OUR is

known the oxygen transfer coefficient (K_La), can be calculated from the following formula:

$$OUR = K_{La} (C_s - C) \quad (3.1)$$

where:
OUR = oxygen uptake rate [mg O_2/l/min]
K_{La} = oxygen transfer coefficient [1/min]
C_s = oxygen saturation concentration in water at 20 ^oC [mg O_2/l]
C = dissolved oxygen concentration in the reactor [mg O_2/l]

II. Sometimes the difference between C_s and C recorded was too small and the calculation of K_{La} with the previous method was not reliable. Another method was then applied. The K_{La} was calculated from the measurement of the oxygen transfer rate (OTR) from the gas to the liquid phase. In order to do this, the reactor was filled with two litres of tap water and nitrogen gas was purged to remove the O_2. Aeration was then applied with the same airflow rate as in the normal operating conditions. The increase of dissolved oxygen concentration (continuously measured on-line) was registered every 30 seconds in a time period of 5 minutes. The oxygen transfer rate can be calculated as:

$$OTR = OUR + \frac{dC}{dt}$$

The purpose for determining OUR and/or OTR is mainly to compute the readily biodegradable (S_s), slowly biodegradable (X_s), inert fractions (S_I and X_I) and yield of hetetrophic organisms. In order to compute the amounts of readily and slowly biodegradable substrate (S_S, X_S), the heterotrophic yield (Y_H) must be known. Hence, Y_H was also determined as referenced in IWA (2000). The concentration of inert soluble organic matter ($S_I + X_I$) was determined as concluded in the IAWQ report (IWA, 2000). Inert fractions ($S_I + X_I$) were calculated from the difference between total COD and the sum of the wastewater components.

3.6 Specific Calculations

Measurements of soluble and particulate TOC or COD, CDW, VSS, ash, NH_4^+ - N, NO_3^- and PO_4^3 were performed twice a week (during the acclimatization period); the biomass concentration from the reactor was measured every day. The samples were taken during the reaction phase.

TOC measurements were made to evaluate the biomass concentration and production and to estimate the SRT in the reactor. To determine the quantity of organically bound carbon, the organic molecules must be broken down to single carbon units and converted to a single molecular form that can be quantitatively measured. TOC methods utilize heat and oxygen, ultraviolet irradiation and chemical oxidants to convert organic carbon to carbon dioxide (CO_2). Inorganic carbon (IC) was also measured by HACH analyser (DR-4000U, USA) and the results were used to correct the CO_2 in the offgas.

During the reaction phase two samples of about 7 ml were taken from the reactor, and one of them was filtered in 0.45 μm membrane filters (Millipore, USA). In this way the biomass concentration in the reactor was calculated as:

$$C_X = TOC_{TR} - TOC_{SR} \quad \textbf{(3.3)}$$

where:

C_X = concentration of biomass in the reactor [C-g/l] or [C-mmol/l]

TOC_{TR} = total TOC in the reactor [C- g/l] or [C-mmol/l]

TOC_{SR} = soluble TOC in the reactor [C-g/l] or [C-mmol/l]

The biomass leaves the system with the effluent and that is discharged in each cycle at the end of the reaction phase. Two samples were taken from a mixture of effluent and waste sludge, and one of them was filtered. In this way the biomass production was calculated as:

$$C_X = TOC_{TE} - TOC_{SE} \quad (3.4)$$

where:

C_X = biomass production [C-g/l] or [C-mmol/l]

TOC_{TE} = total TOC in the effluent and waste sludge [C-g/l] or [C-mmol/l]

TOC_{SE} = soluble TOC in the effluent and waste sludge [C-g/l] or [C-mmol/l]

The PHA contents of the biomass were expressed as follow:

(a) Percentage of PHA content

$$\%\,PHA = \frac{PHA}{PHA + X + ash} \times 100\% \qquad [\%/CDW] \quad (3.5)$$

(b) PHA content in mass

$$\frac{\%PHA}{100 - \%\,PHA} = \frac{PHA}{X + ash} \qquad [g/g] \quad (3.6)$$

(c) Fraction of PHA of biomass

$$f_{PHB} = \frac{PHA}{X + ash} = \frac{\%\ PHA}{100 - \%PHA} \times \frac{M_{w(X+ash)}}{M_{w\ (PHA)}} \qquad [C\text{-mol/C-mol}] \qquad (3.7)$$

Note: Calculation for PHB, PHV and PHH can be represented in those equations by replacing the M_w, PHA = PHB + PHV + PHH, X: active biomass concentration (organic material without PHB/PHA) [C-mmol/l]

The amount of PHB present in the reactor was calculated by multiplying f_{PHB} at that time with the amount of X present in the reactor. The C_X in the reactor was assumed to be constant during one cycle of the SBR (between 1% to 4% increase).

The specific PHA or PHV production rate (C-mol/C-mol. h) was calculated by dividing the amount of PHA or PHB produced in the feast period (C-mmol) by the active biomass present in the reactor (C-mmol) and the duration of the feast period (h), assuming a zero order substrate consumption rate and a constant active biomass concentration. The specific fatty acids measurements in the reactor were not used for determination of the substrate uptake rate. These measurements were not reliable due to very fast uptake of substrate in the sampling tubes during preparation of the samples before analysis.

The true sludge retention time (SRT_{true}) was determined as the ratio between the mass of biomass present in the reactor, and the mass flow rate of biomass that leaves the system, which includes the biomass present in waste sludge and effluent;

$$SRT_{true} = \frac{VX}{(Q - Q_w)X_e} \qquad [h]$$

or

$$SRT_{true} = \frac{1}{\mu} \qquad (3.8)$$

where:

V = volume of reactor, [l]; X = biomass concentration, [C-mmol/l]; Q = flowrate, [l/h]; Q_w = waste sludge flowrate, [l/h]; X_e = concentration of biomass in the effluent, [C-mmol/l]

In order to compare the bioconversion measurement between single (acetate as carbon source) and multiple substrate (mixed substrate), the study analyzed the specific determination using specific mass, balanced as proposed by van Aalst van – Leuwen *et al*. (1997). Elemental mass balances on the measured conversions of substrate, biomass, PHB, CO_2, O_2, NH_4^+ and NO_3^- were performed to check the consistency of the data. PHB has been used in all of the balance checks because the concentration could represent as PHA distribution and produced uniformly. There were more conversions measured than needed to define the whole system with elemental balances. The Macrobal software (Beun *et al*., 2000a, 2000b) was used for balancing all the converted amounts and calculating errors. Macrobal can find the best ultimate for all measured data, based on elemental mass balancing principles. By using Macrobal, it was also possible to define the feast and famine period separately in terms of converted compounds. The elemental composition matrix contained the balances over one cycle for the elements C and N for the feast and famine period, the balances for CO_2, O_2, PHB and biomass concentration.

The observed yield, Y_{obs}, corresponding to the amount of VFAs converted into active biomass and HB, was determined using:

$$Y_{obs} = 1 - \frac{\int OURv(t)dt}{\Delta VFAs} [\text{C-mmol/C-mmol VFAs}] \quad (3.9)$$

in which OUR_v stands for the volumetric OUR converted into carbon considering that 1 mmol of O_2 corresponds to 1 mmol of carbon and ΔVFAs is the substrate consumed during the "feast" phase. This parameter, in terms of carbon material balance, can be expressed as:

$$Y_{obs} = Y_{p/s} + Y_{x/s} \text{ [C-mmol/C-mmol VFAs]} \quad (3.10)$$

The material balance for VFAs can be represented by:

$$\Delta Y = Y_{p/s} + Y_{x/s} + \frac{\int OURv(t)dt}{\Delta VFAs} \text{ [C-mmol/C-mmol HAc]} \quad (3.11)$$

3.7 Statistical Optimization Process

Statistical experimental design methods provide a systematic and efficient plan for experimentation to achieve certain goals so that many control factors can be simultaneously studied. A response experimental design called response surface method (RSM) allows us to find the optimal formulation for the experiment. RSM is used to examine the relationship between one or more response variables and a set of quantitative experimental variables or factors. Furthermore, RSM is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. These methods have been employed after the single factor observed from POME cultivations. Statistically designed experiments use a small set of carefully planned experiments. This method is more satisfactory and effective than other methods (e.g. classical one-at-a-time or mathematical methods). Besides, it can model many variables simultaneously with a low number of observation, saving time and costs. Therefore, the RSM is suitable to be used in this study because:

(a) Ability to find factor settings (operating condition) that produce the "best" response of the process dynamics.
(b) Ability to find factor settings that satisfy operating or process specifications.
(c) Ability to identify new operating conditions that could improve the product quality over the quality achieved by current conditions.
(d) Ability to demonstrate relationship between the quantitative factors and the responses.

The process of optimization will be combined with factorial design. Factorial designs allow for the simultaneous study of the effects that several factor may have on a process. When performing an experiment, varying the levels of the factors simultaneously rather than one at a time is efficient. This is true because it will allow the interactions between the factors. The factor and interaction have been chosen from the single factor (COD:N:P ratio, air flowrate, HRT=SRT and feeding rate.).

Design of experiment (DOE) is a systematic approach to problem-solving which is applied to data collection and analysis to obtain information-rich data. DOE is concerned with carrying out experiments under the constraints of minimum expense of time, costs and runs. As a conclusion, the goal of this chapter is to determine the best parameter simultaneously for figuring the formulation on PHA production, organic and nutrient removal during feast period. All of the computational analyses were carried out using statistical software, called MINITAB^{TM}.

CHAPTER IV

RESULTS AND DISCUSSION: PHA PRODUCTION, ORGANIC AND NUTRIENT BEHAVIOUR IN PALM OIL MILL EFFLUENT (POME)

4.1 Introduction to POME Experiments

The main objective of this chapter is to report on the study of use of POME for PHA production under feast-famine conditions. This study also investigated the optimal conditions for PHA yield and recovery processes from activated sludge using chloroform and sodium hypochlorite. In this study, the selected experiment for PHA production inside the biomass polymers has been proposed. Several operating parameters were varied, i.e. (a) COD:N:P ratio, (b) air flowrate and (c) cycle length. Additional aspects studied were variation of (d) feeding rate, (e) operating cycle of anoxic/aerobic sequence and (f) operating cycle of microaerophilic-aerobic sequence. Most of the published works concerning PHA production by mixed-activated sludge focussed on understanding the storage mechanism and not on the optimization of PHA production. Therefore, the optimization of PHA was also evaluated in this study. The quantification of PHA is based on PHB since PHB account for more than 80% of total PHA constituent, with less concentration of polyhydroxyvalerate (PHV) and polyhydroxyhexavelerate (PHH).

4.2 Respirometric Analysis

In this study, OUR measurement was conducted to identify the characterization of COD-fractionation. The COD-value covers a number of organic materials of varying biological qualities. This helps to determine the availability of readily biodegradable (S_S), slowly biodegradable (X_S), storage polymer (X_{STO}), inert organic matters (S_I and X_I) and yield of heterotrophic organisms (Y_H). The calculation formulas are as follows:

For COD-fractionation:

$$C_{COD} = S_S + S_I + X_S + X_I + X_{STO} + X_H \quad \text{(eq. 4.1)}$$

For S_S and X_S:

$$S_s = \frac{1}{1-Y_H}\int_0^{t_1} OUR\,dt\,\frac{V_w + V_b}{V_w} \quad \text{(eq. 4.2)}$$

$$X_s = \frac{1}{1-Y_H}\int_0^{t_2} OUR\,dt\,\frac{V_w + V_b}{V_w} \quad \text{(eq. 4.3)}$$

Table 4.1: Comparison concentration of COD fractionation

Raw Influent	COD total (g/l)	COD-fractionation (g/l)				
		S_S	X_S	X_H	X_{STO}	$S_I + X_I$
Diluted POME[a]	25.34	7.557	17.606	0	0	0.177
Raw POME[b]	54.43	15.345	38.784	0	0	0.300
Sewage[c]	0.57	0.11	0.33	-	-	0.130

Note: (a) in fed-batch reactor, (b) collected from initial discharge (c) data from typical municipal waste; ASM 2002, X_{STO} is additional cell internal storage of PHA excluding X_H in ASM3

Table 4.1 shows the comparison of COD fraction in a typical sewage and sludge from POME. It is obvious that raw POME in fed-batch gave a higher value in all COD fractions compared to the typical sewage. In this study, the S_S is about 28 – 29% of the total COD. As compared to sewage, the S_S would reach to only 19% of total COD. However, Tremier *et al.* (2005) concluded that S_S can be increased up to 29% of total COD if the substrate contains mixture of sludge and bulking agent as shown in this study. For example, inert particulate represents a large part of total COD but is much less biodegradable than the sludge according to the considered

process time scale. Therefore, as observed in this study, X_S was always higher than S_S, which indicates that both fed-batch and raw POME contain high amounts of slowly biodegradable matter. The total inert organic matters (S_I plus X_I) were detected at range 0.5 – 0.7% of the total COD. The correct assessment of the S_S is important because this fraction is conceived as the rate limiting substrate component for heterotrophic growth (X_H). It is also related to OUR measurement as observed in this study. The biodegradable fraction of the present study (S_S and X_S) recorded at more than 90% of the total COD. Thus, this POME is considered as a rich-substrate for PHA production, even larger time is required to utilize it completely. This study was also emphasizing on activated sludge model 3 (ASM3), which includes cell internal storage compounds (X_{STO}). This requires the biomass to be modelled with cell internal structure. Therefore, X_{STO} is provided to compare the degree of fraction inside heterotrophic organisms. No formation of X_{STO} and X_H occurred either in raw POME or sewage influents. The availability fraction of X_{STO} in the medium indicates that the PHA production occurred intracellularly in biomass components as seen in Table 4.2.

Table 4.2 shows the wastewater biological fractionation using a respirometric analysis after the treatment. The goal of this experiment was to identify the biological fractions in fed-batch system. The result obtained from this study was different compared to the typical sewage (14 to 15 times higher than normal raw wastewater). The COD removed from this fed-batch study was recorded at range 35 – 36% from diluted POME. The removal of COD was acceptable since the study conducted in fast period (6 – 12 h). In general, the readily biodegradable COD (S_S) has been removed significantly at range 0.010 to 0.034 g O_2/l. Meanwhile, the slowly biodegradable COD (X_S) ranged from 4.008 to 4.688 g O_2/l. Since the X_S occurred at high concentration compared to S_S, a modification of fed-batch system must be considered to accelerate the biodegradation process. However, since the slowly biodegradable COD concentration (X_S) accumulated as high as 48% than total COD, the targeted substrate (e.g. VFAs, fermentable carbohydrates, etc.) will be degraded in a longer period.

Table 4.2: COD-fractionation after the treatment of POME

Experiments	Total COD (g/l)		COD-fractionation (g/l)				
	Typical raw sewage	Total COD for POME	S_S^+	X_S^+	X_{STO}^+	X_H^+	$S_I + X_I^+$
$CODNP_{pome}$	0.57*	24.425	0.01	13.471	3.120	7.360	0.464
Air_{pome}		24.556	0.012	12.491	3.576	7.930	0.547
HRT_{pome}		24.855	0.023	11.708	4.411	8.320	0.393
FR_{pome}		25.401	0.034	12.270	4.291	8.010	0.13
$ANae_{pome}$		25.343	0.013	14.688	4.735	7.950	0.14
$MICae_{pome}$		26.460	0.024	13.664	4.174	7.950	0.05
Average	-	25.340	0.019	13.932	4.551	7.970	0.305
S. Deviations	-	1.15	0.002	0.553	0.04	0.22	0.06

* data obtained from ASM, 1990, $^+$ data after accumulation stage

The highest level of PHA storage is found in the sludge at low concentrations of O_2 (microaerophilic condition) and NO_3 concentration (anoxic/aerobic experiment) in the medium. This is presumably caused by the presence of high content of COD residuals, which then is causing a selection for microorganisms specialized in metabolizing carbohydrates. However, the specific discussion will be elaborated later. Typically, experiments measure some response of the culture, e.g. spike of the soluble carbon source in batch test or a pre-established cultivation condition. Substrate uptake rate ($-q_s$) and/or OUR are usually determined to monitor the dynamic response of the culture and in general their fast increase is observed. Because such behaviour could also be due to the increase of the μ (the growth response), the presence of storage has been ascertained by also taking into account as stated from Majone *et al*. (1999):

(a) the form of time profiles of OUR and/or OTR;
(b) a high observed yield (often derived from the ratio between oxidized and removed substrate); and
(c) the direct determination of stored polymers and/or growth-associated components.

During limiting condition (known as PHA accumulation) (Figure 4.1a), the OUR curve reached the peak at 42 mg O_2/l. min and then, slightly decreased to only 15 mg O_2/l. min, after 100 min. The peak period of 100 min indicates that the biomass utilized S_S in a short period of time. This resulted in a fast uptake rate since the S_S, such as acetic acid can be removed directly by the microorganisms, similar obtained by Majone *et al.* (1999) and Dionisi *et al.* (2001b). The substrate can also be removed quickly at early accumulation stage for storage and slow response to respiration analysis because lack of nutrients will cause a slow down of metabolic process. This was hindering the present biomass to duplicate their cells. These mechanisms clearly indicated that the substrate is transported into the cell and maintained in an almost unchanged form or transformed into low-molecular weight metabolic intermediates. The type and extent of biomass response depend on its microbial composition and on the physiological state of the different microorganisms, which in turn are defined by previous history of the biomass (i.e. by the operating conditions imposed by the process). Therefore, the PHA production rate was proven higher under limiting nutrient condition rather than in sufficient nutrients.

In contrast, as shown in Figure 4.1(b), the OUR measurement gave a slow response to indicate that the S_s is insufficient for microorganisms at the beginning of cultivations. Five mg of allylthioreum (ATU) was added to the respirometer vessel during the experiments to inhibit the autotrophic microorganisms. In this growth phase, the second increase of the OUR curve occurred after 200 minutes of cultivations. During this period, the increment of OUR curve can be explained by the adaptation of the organic substrate or it could be caused by growth of the biomass. Then, the curve exhibits a sudden drop (after 300 minutes) indicating that the added substrate has been consumed from the medium. The sudden drop of the OUR curve is then followed by continuously decreasing respiration. The respiration in this phase is due to the transformation of a secondary substrate (storage polymer) or it could be due to more difficult access to the remaining primary substrate caused by an accumulation of the substrate in the flocs.

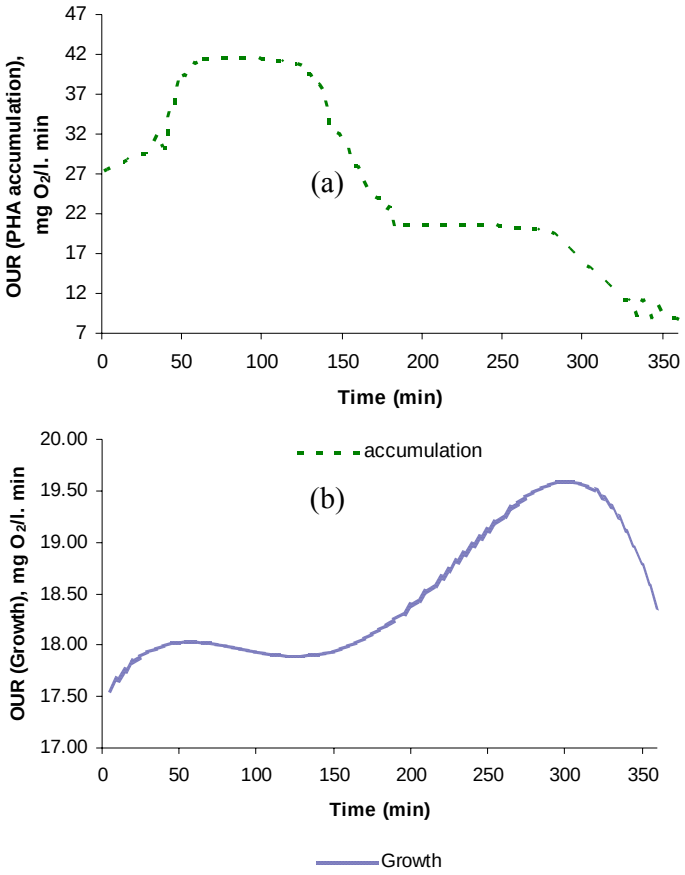


Figure 4.1: (a) Comparison of OUR analysis during PHA accumulation (no nutrient) and, (b) growth phase (nutrient available). Both used diluted POME.

The batch tests with the diluted POME showed a different OUR pattern. Figure 4.1(a) shows a constantly of high and quickly decreasing OUR, while Figure 4.1(b) shows two phases of OUR trend before the microorganism respiration turn back to endogenous stage. The main differences in the OUR profiles could be explained by the availability of high carbon source in the reactor, and the quick decreasing of OUR may affect from the low nutrient present during the feast period. The presence of second OUR phase (Figure 4.1(a)) seems to indicate that a significant fraction of the analytical soluble COD is actually slowly biodegradable, as already reported in previous studies (Carucci *et al.*, 2001; Beccari *et al.*, 2002).

Based on the oxygen consumption, the yields of PHA decreased by considering only the exogenous phase, which are typically much higher than what are expected from the metabolic mechanisms or from pure culture experiments under steady state conditions. The yields in exogeneous phase are typically 0.71 COD/COD (acetate as substrate), 0.76 – 0.88 (glucose) and 0.76 – 0.90 (acetate and glucose) (Majone *et al*., 2001). However, in this study, the fluctuating results ranged from 0.20 – 0.67 depending on the sludge age, temperature and feeding regime. When the slowly biodegradable COD was also present, the interpretation of endogenous phase is much more difficult as what was observed in this study.

Studies with mixed cultures have been performed by comparing dynamic responses of sludges selected from fermentation under continuous feeding (bulking sludges) with that of sludges selected under intermittent feeding or in the presence of a selector (refers to microorganisms and usually known as well-settled sludge). In this study, the effect of various operational conditions, include both a significant change of microbial population and a different physiological state of the different microorganisms.

As shown in Table 4.3, intermittently fed sludge typically exhibit faster substrate uptake and higher observed yields than continuously fed ones. This has usually been explained by a more relevant presence of those microorganisms that are most able to store substrates quickly during the imposed dynamic conditions. A distinction between storage and accumulation was first proposed by Cech and Chudoba (1983) based on the form of substrate profiles versus time. Due to the lower capacity for accumulation with respect to storage, they observed that substrate uptake rate is first quickly decreasing (saturation of the accumulation) and then remains constant (storage still far from saturation). Therefore, it has been suggested that both mechanisms (storage and accumulation) are acting when the sludge is intermittently fed, while only storage is most possible for the continuously fed sludge. The importance of storage response under dynamic conditions for mixed cultures has been confirmed by direct determination of stored polymers in sludge. By using COD balance, Majone *et al*. (1999) have shown that for both intermittently and continuously fed sludge, the storage response is the main mechanism of the

dynamic response. The growth response occurred only to a little extent or not at all, the latter particularly in the case of well settled sludge.

With reference to other previous work, as stated in Table 4.3, the obtained data was compared with several mixed culture experiments. Storage of PHA is an important mechanism when the substrate was enriched with fatty acids. Therefore, the microaerophilic-aerobic condition gave a tremendous effect compared with other studies especially in PHA yield production (ΔY = 0.53 – 0.80 C-mol/C-mol HAc) and the conversion of HAc to active biomass and PHA (Y_{obs} = 0.65 COD/COD). During batch culture, the diluted POME was tested using respirometric vessel and high storage capacity (g/g SS) occurred again under microaerophilic-aerobic condition. This was regulated from the behaviour of sludge utilization rate (SUR) at rate 800 – 1420 mg COD/g SS. h. However, the highest rate of SUR (1730 mg COD/ g SS. h) was recorded in intermittent fed-well settled study, found by Chech and Chudoba (1983). As a conclusion, the usage of POME could also sufficient for generating the PHA production, even the process conducted under low oxygen saturation.

Table 4.3: Comparison of respirometric analysis on continuous and batch cultures

Experiments	Batch Culture / Continous Culture					Reference
	ΔY (C-mol/C-mol HAc)	Y_{obs} (COD/COD)	SUR (COD mg/gSS. h	$Y_{storage}$ (COD/COD)	Storage cap. (g/g SS)	
CODNPpome$_{ave}$	0.66 – 0.69	0.32*	342 – 450	0.46	0.40	this study
Airpome$_{ave}$	0.57 – 0.78	0.44*	300 – 520	0.52	0.33	
HRTpome$_{ave}$	0.42 – 0.65	0.52*	420 – 610	0.33	0.21	
FRpome$_{ave}$	0.59 – 0.72	0.39*	350 – 540	0.34	0.20	
ANaepome$_{ave}$	0.32 – 0.47	0.60*	890 – 1200	0.55	0.53	
MICaepome$_{ave}$	0.53 – 0.80	0.65*	800 – 1420	0.57	0.64	
Continuous-fed bulking	n.a.$^{+}$	n.a.$^{+}$	200(b)	n.a.	n.a.	Cech and Chudoba, 1983
Intermittent – fed-well settling	n.a.$^{+}$	0.56(^{o})$^{+}$	1730(a)	n.a.	0.65(a)	
Continuous-fed bulking	n.a.$^{+}$	0.46^{+}	200 – 260	0.35	-	Majone *et al*., 1999
Intermittent-fed well settling	n.a.$^{+}$	0.33^{+}	800 – 1000	0.75	-	
Intermittent-fed well settling	n.a.$^{+}$	0.52^{+}	740 – 920	0.7	0.47	Beccari *et al*., 2000

Notes: original units of most data converted from authors of the present paper. Y_{obs} from respirometry apart from values indicated with (^{o}). (a) accumulation phase, (b) storage phase, (*) Cmmol/Cmmol VFAs, ($^{+}$) Continous culture

4.3 Overall Performance of POME Cultivations

4.3.1 PHA Production in Biomass Components

In general, the residual biomass value was used to express the cell growth during PHA accumulation. Based on preliminary study (data not shown), the microorganisms grew at a constant specific growth rate until the DO in the culture liquid decreased to almost zero, when the growth became linear. After the cell concentrations exceeded approximately 20 g/l, the growth was gradually suppressed and almost ceased at cell concentration above approximately 18.5 g/l. Therefore, the preliminary showed that the biomass growth could reach a high concentration if nutrients are sufficiently supplied.

The accumulation of PHA was essentially observed after exhaustion of the growth limiting nutrient occurred. Then, all systems were returned to operate under normal growth for 2 – 3 days, before nutrients became limited again for the second time. This will enable the system to produce an appropriate population (especially PHA producers). Liu *et al.* (1998) explained that when the cells contain a high PHA level, it might lose the ability to divide itself further, consequently, lowering its growth. Therefore, maintaining biomass in the system was important for obtaining high PHA concentration and productivity. Nevertheless, the limitation of nutrient period will result in low final cell and PHA concentrations, resulting in low PHA productivity, even though high PHA contents may be obtained. Therefore, the system was only operated in a single period of fed-batch cultures (only 60 min of substrate feeding) to reach a short feeding regime for better activity of PHA storage. In order to determine the pattern of biomass component (PHA content and residual biomass), Table 4.4 was prepared to show the variation value of biomass component.

Table 4.4: Biomass components and PHA accumulation during feast period for various experimental works using diluted POME.

Experiment Code	Total Biomass (g/l)	PHA content (g/l)	Residual biomass (g/l)	PHA productivity (g/l. min)
Variation of N and P limitations				
$CODNP_{pome}$-0.85	7.30±1.27	4.44±1.29	2.86±0.11	0.028±0.05
$CODNP_{pome}$-0.88	8.90±2.08	7.53±0.99	1.37±0.06	0.047±0.05
$CODNP_{pome}$-1.44	7.44±1.06	6.58±1.07	0.86±0.22	0.041±0.09
$CODNP_{pome}$-2.45	8.12±3.44	7.70±1.13	0.42±0.17	0.048±0.17
$CODNP_{pome}$-2.55	9.09±0.98	6.97±0.86	2.12±0.13	0.044±0.01
Variation of DO flowrates				
Air_{pome}-0.5	22.25±2.45	12.25±1.19	1.87±1.08	0.139±0.03
Air_{pome}-1	16.61±3.15	9.48±0.98	3.32±0.86	0.134±0.15
Air_{pome}-1.5	12.10±4.43	8.38±1.09	4.66±0.34	0.097±0.06
Air_{pome}-2	11.83±3.21	5.46±2.07	6.07±1.14	0.129±0.11
Air_{pome}-2.5	10.16±4.07	6.70±1.33	7.43±2.05	0.098±0.05
Variation of cycle lengths				
HRT_{pome}-12	21.71±6.85	14.50±1.12	3.25±0.12	0.121±0.05
HRT_{pome}-14	16.70±7.07	10.38±0.77	3.76±0.76	0.069±0.08
HRT_{pome}-16	28.50±5.45	15.25±1.09	1.98±1.22	0.051±0.09
HRT_{pome}-18	12.64±3.26	9.51±0.93	4.71±0.98	0.052±0.12
HRT_{pome}-26	33.73±8.96	16.84±1.07	1.13±0.34	0.026±0.09
Variation of feeding rates				
FR_{pome}-20	23.59±0.28	18.84±1.12	4.52±0.15	0.199±0.09
FR_{pome}-25	12.44±0.13	7.04±1.09	4.53±0.22	0.086±0.08
FR_{pome}-33	11.26±0.54	6.16±3.22	2.75±0.06	0.150±0.03
FR_{pome}-50	10.75±2.14	7.77±0.45	2.25±0.17	0.094±0.01
FR_{pome}-100	14.13±3.09	7.42±0.98	2.09±1.07	0.158±0.04
Variation of anoxic/aerobic cycles				
$ANae_{pome}$-70%	15.19±3.45	7.29±1.77	3.75±1.25	0.182±0.04
$ANae_{pome}$-60%	17.16±2.11	6.61±0.64	2.51±2.36	0.177±0.07
$ANae_{pome}$-50%	16.22±0.97	9.61±1.13	1.43±1.09	0.147±0.03
$ANae_{pome}$-30%	17.69±3.05	8.08±2.08	2.79±0.98	0.209±0.02
$ANae_{pome}$-20%	12.71±2.09	9.84±1.15	1.46±0.88	0.199±0.01
Variation of microaerophilic-aerobic cycles				
$MICae_{pome}$-70%	17.60±2.45	12.61±1.22	3.19±1.22	0.244±0.09
$MICae_{pome}$-60%	19.31±0.05	13.79±2.03	0.37±2.09	0.461±0.05
$MICae_{pome}$-50%	18.95±2.55	14.47±2.08	0.64±1.02	0.246±0.01
$MICae_{pome}$-30%	15.05±3.35	11.78±1.27	1.11±1.11	0.145±0.02
$MICae_{pome}$-20%	11.51±5.09	6.23±1.05	3.96±0.99	0.082±0.08

Note: Residual biomass=CDW-PHA-Poly-P, PHA content=PHB+PHV+PHH, Biomass composition = PHA content + residual biomass

The results showed that the residual biomass in $CODNP_{pome}$-2.45 decreased significantly to only 0.42 g/l when N and P were limited in the medium. However, when the N and P were exposed to a maximum limiting condition ($CODNP_{pome}$-2.55), the residual biomass increased immediately, resulting low PHA productivity

and content. Therefore, this study suggests that PHA production would not lead to an extraordinary limitation of N and P concentrations. In addition, the indication of lowest residual biomass concentration, 0.42 g/l would possibly improve the PHA content at high concentration, 7.70±1.13 g/l. Similar to this finding, the highest productivity of PHA was attained at $CODNP_{pome}$-2.45 with the rate of 0.048±0.17 g/l. min recorded at the end of feast phase. The reason of decreasing residual biomass from 2.86±0.11 g/l to 0.42±0.17 g/l (from low to high ratio of COD:N:P) could affect from the limiting growth rate. This phenomenon was monitored in metabolic pathways; since the acetyl-CoA cannot enter TCA cycle to obtain energy for cells caused by high concentration of NADH. The behaviour of PHA content and residual biomass was extensively discussed by Panswald *et al.* (2004). They concluded that selected operation conditions, e.g. HRT or SRT had significant impacts on PHA production.

Study on DO concentration effects show that the PHA content and productivity achieved the highest value at Air_{pome}-0.5, which are 12.25 g/l and 0.139 g/l. min, respectively (shown in Table 4.4). A maximum PHA content of 12.25 g/l in this study detected at 200 minutes after the accumulation phase started. Overall experiments found that with low air flowrate, the yield on PHA storage ($Y_{storage}$) can be increased, as depicts previously in Table 4.3. The yield of PHA per unit substrate achieved as high as 0.52 g PHA/ g COD compared to only 0.39 g PHA/g COD during preliminary experiment. At high rate of PHA productivity, low residual biomass occurs (1.87±1.08 g/l), similar to the study of N and P limitation. As a result, the decreasing of air flowrate will reduce the residual biomass, and consequently increase the PHA productivity and content.

The result of cycle length shows that the PHA content varies in the range 9.51 to 16.84 g/l. The study recognized that prolonged cycle length is beneficial for PHA production, as also stated by Guinda *et al.* (2003). However, the contradictory results were obtained for PHA productivity when the retention time increased from HRT_{pome}-12 to HRT_{pome}-26. It decreased from 0.121 to 0.026 g/l. min which could reduce the PHA concentration in a long period. Therefore, no direct conclusion can be made to correlate cycle length and PHA productivity.

Study on feeding rate was examined in order to show the effectiveness of substrate inhibition and PHA production rate, and depicted in Table 4.4. As shown in this feeding regime study, the PHA accumulated was much higher under low flowrate of 20 ml/min compared to the highest rate, 100 ml/min. It was noted that when feeding rate operated at more than 20 ml/min, it can cause a low PHA content in range between 6.16 to 7.77 g/l. Therefore, the well-controlled feeding rate had a significant impact to PHA accumulation.

The cycling phase of anoxic/aerobic was also conducted in this study to determine the effectiveness of PHA enhancement, as shown in Table 4.4. The results show that the PHA content reached to high concentration under long period of aerobic condition such as $ANae_{pome}$-30% and $ANae_{pome}$-20%, with values of 8.08±2.08 and 9.84±1.15 g/l, respectively. The PHA productivity could also increase in the ranged of 0.199 to 0.209 g/l. min. The study also found that prolonged aerobic condition had a slower response of biomass compared to anoxic condition.

The results of cycling phase of microaerophilic-aerobic are shown in Table 4.4. Major PHA content was recorded in long oxygen limitation experiments (e.g. $MICae_{pome}$-70%, $MICae_{pome}$-60% and $MICae_{pome}$-50%) compared to uncontrolled oxygen. It indicated that the PHA content increased from 12 – 14 g/l at the end of microaerophilic phase. In addition, the total productivity of PHA also recorded higher in the range of 0.44 – 0.46 g/l. min compared to other experiments. During the exhaustion of substrates, the residual biomass concentration remained almost constant due to the utilization of PHA. As a result, the mechanism of oxygen limitation performed well at an optimal ratio of organic-to-nutrient, to get high productivity of PHA. However, the study found that when the biomass degrades in the range of 10 – 20% after 10 hours, the PHA will be dropped slightly.

Many studies have reported that some bacteria are capable of converting substrate into intracellular biopolymer under nutrient-deficient conditions and then the PHA will be formed after the growth rate is generally limited (van Loosdrecht and Heijnen, 2002; Herbert *et al.*, 2000). It is postulated that bacteria with such a capability would have a distinctive competititive edge over others in competing for

substrate. These bacteria are also capable of converting excess substrate into intracellular polymers, even under limited nutrient or electron acceptors As reported by Serafim *et al.* (2004), a high substrate concentration (e.g. acetate = 180 C-mmol/l) and low nutrient were favoured for PHA accumulation, even though the specific storage rate decreased due to substrate inhibition. Similar conclusion was obtained from previous study, e.g. Beccari *et al*. (1998), Serafim *et al*. (2004) and Dionissi *et al*. (2001a, 2001b). Those studies stated that during the first substrate pulse addition, substrate was mainly used for storage and biomass became saturated in the polymer content. In order to examine the storage and biomass capability, the study of anoxic/aerobic and microaerophilic-aerobic conditions were conducted (Satoh *et al.*, 1998). The result from this study was similar to those obtained by Beun *et al*. (2000a and 2000b). However, the study of Dionisi *et al.* (2001a) and Kornaros and Lyberatos (1998) indicated that the anoxic condition gave a significant PHA production compared to aerobic condition. On the other hand, Lishman *et al.* (2000) also stated that temperature was not a significant effect. They observed that the yield for the aerobic decreased 4% with decreasing temperature whereas the anoxic yield increased by 8%. The experiments indicated that the observed yield of PHA was 52% higher for anoxic rather than aerobic conditions. However, this study was not giving any specific reason on these behaviours.

4.3.2 Specific and Kinetic Rates on Substrates, Biomass and PHB/PHA

Measurement and calculation of S_s, X_s, Y_H (yield of heterotrophic) and specific growth rates under feast period (μ^{feast}) are shown in Table 4.5. The maximum PHB production (PHB^{max}) is also shown in order to compare the correlation between substrate utilization and storage polymer. The S_s consists low molecular weight compounds, and this study assumed it as VFAs. This S_S is easily degradable and hence contributes to fast respirometric response. In addition, X_s is a slowly biodegradable substrate and consists of high-molecular weight compounds ranging from soluble to colloidal and particulate. The common feature of these compounds is that they cannot pass the cell membrane and undergo hydrolysis to low-molecular compounds (S_s), which are subsequently assimilated and oxidized.

Table 4.5: Accumulation of PHB content for various operational conditions under acclimatization of biodegradable substrates (S_s and X_s), Y_H and μ.

Experiment	Experiment	Biodegradable substrate (g/l)		Y_H (COD/COD) & Specific growth rate, μ (h^{-1})		PHB^{max} (g/l)
		S_s	X_s	Y_H	μ^{feast}	
$CODNP_{pome}$-0.85	COD:N:P ratio	8.38	9.76	0.59	0.330	2.45
$CODNP_{pome}$-0.88		8.12	9.77	0.54	0.191	4.95
$CODNP_{pome}$-1.44		8.10	9.62	0.44	0.200	4.30
$CODNP_{pome}$-2.45		8.19	9.72	0.40	0.031	5.80
$CODNP_{pome}$-2.55		8.19	10.28	0.37	0.082	6.16
Air_{pome}-0.5	DO flowrate	8.53	10.428	0.64	0.027	6.16
Air_{pome}-1		8.43	10.214	0.60	0.010	4.83
Air_{pome}-1.5		8.57	10.892	0.72	0.013	3.44
Air_{pome}-2		8.87	10.840	0.73	0.053	4.60
Air_{pome}-2.5		8.52	10.680	0.70	0.142	5.70
HRT_{pome}-12	Cycle Length	8.54	10.490	0.50	0.200	4.06
HRT_{pome}-14		8.43	10.199	0.63	0.321	5.01
HRT_{pome}-16		8.63	10.201	0.66	0.197	7.02
HRT_{pome}-18		8.61	10.105	0.55	0.114	4.56
HRT_{pome}-26		8.33	10.054	0.50	0.108	8.08
FR_{pome}-20	Feeding rate	8.26	9.701	0.53	0.133	13.80
FR_{pome}-25		8.12	9.570	0.51	0.124	6.42
FR_{pome}-33		8.23	9.892	0.53	0.050	5.05
FR_{pome}-50		8.16	9.805	0.60	0.010	6.42
FR_{pome}-100		8.29	9.700	0.55	0.021	5.60
$ANae_{pome}$-70%	Cycle of anoxic/aerobic	8.31	9.848	0.34	0.154	4.83
$ANae_{pome}$-60%		8.38	9.576	0.27	0.081	4.90
$ANae_{pome}$-50%		8.34	9.633	0.33	0.087	7.50
$ANae_{pome}$-30%		8.25	9.661	0.40	0.181	7.33
$ANae_{pome}$-20%		8.36	9.688	0.41	0.098	9.05
$MICae_{pome}$-70%	Cycle of microaerophilic-aerobic	8.77	9.757	0.41	0.175	10.50
$MICae_{pome}$-60%		8.88	9.530	0.40	0.072	13.24
$MICae_{pome}$-50%		8.88	9.751	0.35	0.032	13.46
$MICae_{pome}$-30%		8.67	9.646	0.42	0.046	11.20
$MICae_{pome}$-20%		8.76	9.698	0.40	0.040	5.40

In general, the Y_H shows higher (> 0.6 g COD/g COD) under air flowrate study and the S_s lower 10% - 50% than X_s in most of the experiments. Y_H is defined as the increase biomass COD per unit COD of totally consumed S_S. The increase in high uptake rate and the specific growth rate (μ) are maintained during the feast period. A high storage rate is expected to occur simultaneously, afterwards. As the bacteria grow on internal substrate during famine period, it can be considered that the μ would not be optimized since there is no competition for internal substrate present

in the medium. Because most of the cultivations occurred under the same SRT system (HRT = SRT), the influence factors of slowly hydrolyzed substrates are nearly negligible. From Table 4.5, it was found that the cycle of microaerophilic-aerobic gave a high PHB^{max} concentration at range 5.40– 13.46 g/l compared to other research works. This was postulated from high S_s (4.67 – 5.88 g/l), which present at low μ^{feast} (0.032 – 0.175 h^{-1}) occurred in the feast period. The dropped growth rate from 0.2 to only 0.032 h^{-1} within 2 hours (as one example), indicate that the inhibition of cell growth occurred during microaerophilic condition. The reduction of μ in this study was similar to that obtained by Van Loosdrecht and Heijnen (2002), Beun *et al*. (2000a) and Dircks *et al*. (2001). They reported that during the storing activity and subsequent growth will lead to a slightly reduced net growth yield. This loss in yield was compensated by the reduced need for RNA and anabolic enzymes.

During high carbon-to-nutrient content, only few bacteria is capable to survive. Most of them are bacteria producing-PHA, as reported by Lafferty *et al.* (1998). The study also found that the combination of N and P limitations applied for PHA production depends on the speciation of the bacteria. If the limitations continue for a long period, cells are not able to accumulate much PHA even if the biomass cells are initiated at high concentration. In a metabolic model, the conversions of components are reduced to a number of internal characteristic reactions (e.g. TOC reduction) of the metabolism (Gernaey *et al.*, 2004; Fillipe *et al.*, 2001).

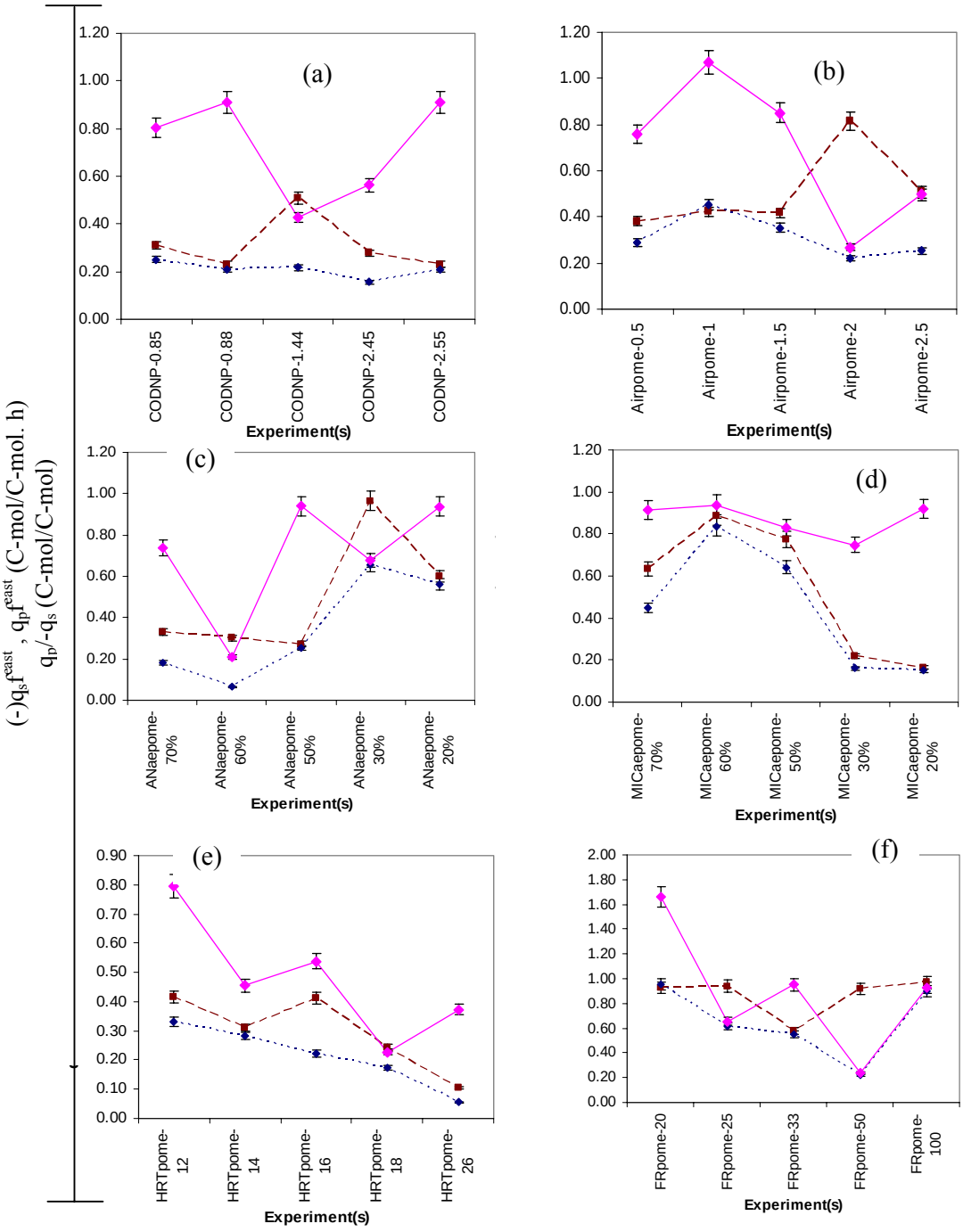


Figure 4.2: Specific PHA production and substrate uptake rate at different operational and culture experiments. (♦) $q_p^{feast}/-q_s^{feast}$ (--■--) $-q_s^{feast}$, (-♦-) q_p^{feast}.

(a) COD:N:P ratio experiments, (b) air flowrate experiments, (c) cycle length experiments, (d) feeding rate experiments, (e) anoxic/aerobic experiments, (f) microaerophilic-aerobic experiments

The correlation between q_p, $-q_s$ and ratio of $q_p/-q_s$ are shown in Figure 5.2 (a – f). From these data, it is clear that the $-q_s$ in the feast period has a rate between 0.4 – 0.9 C-mol/C-mol. h, and hence increase the ratio of $q_p/-q_s$. The data also show that q_p in the feast period is almost constant for different COD:N:P ratio. Meanwhile, during the study of cycle length, the q_p decreased when the period increases. The details are discussed in the next paragraph.

As depicted in Figure 4.2(a), the specific rate of $q_p/-q_s$ fluctuated from $CODNP_{pome}$-0.85 to $CODNP_{pome}$-2.55. The lowest COD:N:P ratio, $CODNP_{pome}$-0.85 ($q_p/-q_s$ = 0.8) has no significant effect compared to the highest COD:N:P ratio ($CODNP_{pome}$-2.55, $q_p/-q_s$ = 0.82).

The results in air flowrate experiments (Figure 4.2(b)) showed that the $-q_s$ accumulated two times higher at high air flowrate, i.e., Air_{pome}-2.5 and Air_{pome}-2 compared in low air flowrate, i.e. Air_{pome}-0.5 and Air_{pome}-1.5. In addition, the q_p was performed four times higher compared to q_p at Air_{pome}-2 and Air_{pome}-2.5.

The results of prolonged-cycle periods (HRT_{pome} experiment) are shown in Figure 4.2(c). The study found that if the cycle period reached to 24 hour, the $-q_s$ and q_p decreased sharply. As a result, the $q_p/-q_s$ ratio also decreased simultaneously. The ratio of $q_p/-q_s$ slowed down from 0.8 to 0.2 C-mol/C-mol (from HRT_{pome}-12 to HRT_{pome}-18) and then, slightly increased to only 0.38 C-mol/C-mol at HRT_{pome}-26. In overall system, the $-q_s^{feast}$ is typically consistent, but this only occurred in the range of 6 – 13 hours.

The results of feeding rates (FR_{pome} experiment) are illustrated in Figure 4.2(d). The $q_p/-q_s$ during feast period reached a high rate at FR_{pome}-20 ($q_p/-q_s$ = 1.6 C-mol/C-mol), followed by FR_{pome}-33 ($q_p/-q_s$ = 0.9 C-mol/C-mol) and FR_{pome}-100

(feast = 0.85 C-mol/C-mol). The lowest value was obtained at FR_{pome}-50 ($q_p/-q_s$ = 0.21 C-mol/C-mol).

Figure 4.2(e)) shows the accumulated substrate will also be oxidized through nitrate reduction under cycle of aerobic/anoxic conditions ($ANae_{pome}$ experiment). This study found that the $-q_s$ will be increased up to 0.91 C-mol/C-mol. h at $ANae_{pome}$-30%. It was observed that the aerobic phase gave a significant effect on substrate utilization. The q_p also increased to 0.7 C-mol/C-mol. h, as a consequent of high $-q_s$.

Figure 4.2(f) shows that the q_p could reached a high rate under long microaerophilic period. For example, $MICae_{pome}$-60% and $MICae_{pome}$-70% depicts the highest q_p, which are 0.7 and 0.6 C-mol/C-mol. h, respectively.

In general, the calculation on this specific rate showed that both cycle of anoxic/aerobic and microaerophilic-aerobic performed high substrate productivity (e.g. $q_p/-q_s$ averagely at 0.9 C-mol/C-mol). However, in general, the ratio of $q_p/-q_s$ will be increased during high carbon fraction (COD:N:P) and cycle length (HRT).

4.3.3 Fatty Acid Uptakes for PHA Constituents

Acetic acid (HAc) was predominantly utilized by the microorganisms because of the quick modification and metabolism in order to store it as a copolymer of intracellular components (Ganduri *et al.*, 2005; Du and Yu, 2001; Du *et al.*, 2001). However, the other specific organic substrates (e.g. butyric, HBt and propionic acids, HPr) were also used by the cells in all of the occurrence periods (e.g. feast phase period).

In the study of N and P limitations (Figure 4.3(a)), the maximum consumption rate (ratio of organic acid and VFAs) of acetic acid appeared at high

COD:N:P ratio up to 80% per total VFAs. However, the butyric and propionic acid show the utilization rate ranging from 10% - 26% per total VFAs.

The result in Air_{pome} experiment (Figure 4.3(b)) shows that oxygen flowrate of 2.5 l/min and 0.5 l/min significantly induced the acetic consumption rate (HAc/VFAs) more than 90%. However, the highest HAc/VFAs occurred at Air_{pome}-1 (99% per total VFAs). The lowest consumption rate of acetic acid occurred at Air_{pome}-0.5, which only reach 49% per total VFAs.

Figure 4.3(c) shows the correlation of fatty acids (HAc, HBt and HPr acids) in HRT_{pome} experiments. Based on this experiment, it can be concluded that the acetic acid will be decreased to only 49% per total VFAs compared with butyric and propionic acid after 10 – 13 hours (HRT_{pome}-26). Lowering the acetic acid consumption rate during long cycle period will consequently, reduce the concentration of PHB constituents.

In the feeding rate study, the converted amount in one cycle measurement reached ten times higher in slow feeding rate (FR_{pome}-20) (Figure 4.3(d)) compared to fast feeding. The high consumption rate of acetic acid occurred at FR_{pome}-33 with almost 100% being utilized as substrate rate (%HAc/VFA). This is followed by FR_{pome}-100 (80%) and FR_{pome}-50 (72%). Therefore, the slower feeding rate is sufficient to allow better acetic uptake rate compared to the faster rate, since the feeding rate has a significant effect to allow quick PHA accumulation into the microorganisms' cell.

As shown in Figure 4.3(e), the average of acetic acid consumption rate was not more than 78.4%. The insignificant consumption of acetic acid concentration could be due to an incomplete adaptation of biomass to substrate removal under transient condition of anoxic/aerobic condition. This may be affected by the duplication of substrate uptake rate under aerobic condition (Dionisi *et al*., 2001b).

As shown in Figure 4.3(f), the percentage of fatty acid over total VFA (e.g. %HAc/VFA) was similar either in prolonged or short period of microaerophilic condition. The study found that the total VFAs increased from 80 to 280 C-mmol/l (from $MICae_{pome}$-70% to $MICae_{pome}$-30%).

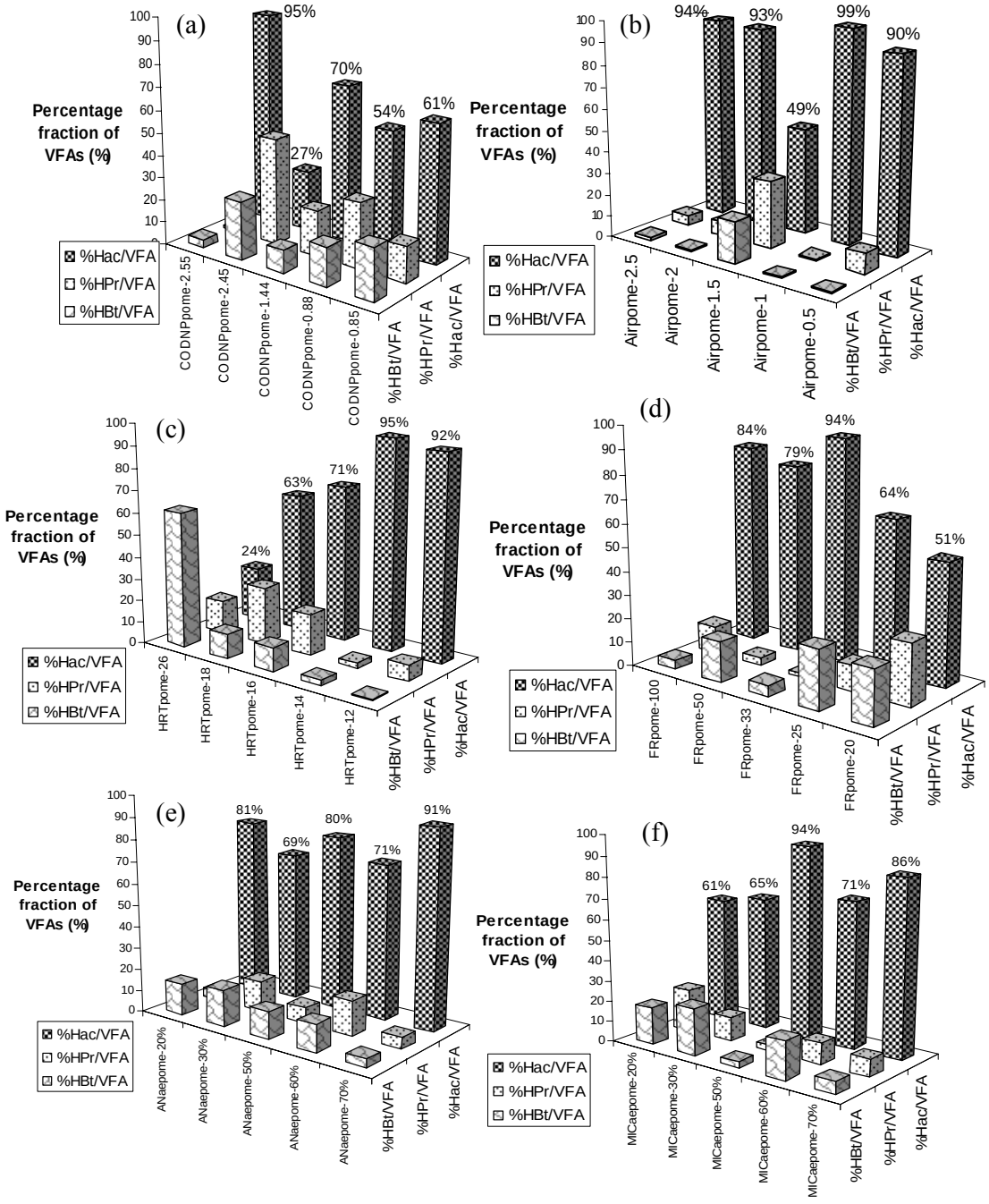


Figure 4.3: VFAs concentrations and their percentage being utilized at several experiment studies. (a) COD:N:P ratio experiments, (b) air flowrate experiments, (c)

cycle length experiments, (d) feeding rate experiments, (e) anoxic/aerobic experiments, (f) microaerophilic-aerobic experiments.

As reported in some literature, only the acetic acid removal was not influenced by the presence of other organic acids, indicating that it utilizes a more specialized pathway. This is coherent with the fact that PHB formation from acetic acid is the most direct pathway, only requiring the direct formation of acetyl-CoA from acetate (Du and Yu, 2002). The storage of PHB and other carbohydrate constituents maintained their importance, both under anoxic and aerobic conditions (Beun *et al.*, 2000b), similar as obtained in this study.

Typically, the concentration of PHA increased directly with VFAs concentration, especially for acetate uptake (Carta *et al.* 2001). However, less attention has been made previously in determining the constituents of PHA in mixed cultures. Most of the published works only focussed on single substrate; therefore, only PHB is possible to be obtained during the experiment (Beun *et al*. 2002, 2000a, 2000b; Majone *et al*. 2001; Dionisi *et al*. 2001a). In reality, not only PHB occurred in the intracellular biomass cell since mixed substrate was introduced to the system (i.e. POME). As reported by Shimizu *at al.* (1999), the effects of carbon-to-nitrogen (COD:N) and concentration of butyric and valeric acids were important to induce the fraction of HB and HV monomers. Therefore, when POME was used as sole carbon source, not only HB units, but also HV and HH units, were produced. The trend of these polymer components are shown in Table 4.6.

In general, the HB monomer increased from low to high COD:N:P ratio (PHB = 62 – 88%). This could be derived from the metabolic mechanisms. When the COD:N:P ratio was increased, the mole flux of acetyl-CoA to the TCA cycle and anabolic reaction pathways decreased and hence, the mole fraction of HB units relatively increased. Nevertheless, the HB constituent could only accumulate as high as 41% per mol, as depicted in DO flowrate study (DO_{pome}-1.5). At the same time, the unit of HV and HH constituents were slightly increase up to 35% and 24% per mol, respectively. It can be concluded that when the DO flowrate increased, the

mole fraction of HB units also significantly increase up to 84%. When the experiments operated in prolong cycle period (HRT_{pome}), the unit of HB constituent increased at rate 28 – 48% per mol. Consequently, the fraction unit of HV and HH constituents were also changed. A low concentration of HB monomer obtained in this study is unexplainable. However, this may be influenced by the initial component of fatty acid fed to the reactor. Under the study of feeding rate (FR_{pome}), there is no specific trend for HB, HV and HH concentrations. Similar trend was also recorded under microaerophilic-aerobic conditions. However, under anoxic/aerobic condition, the HV and HH constituents were detected higher under prolonged anoxic condition (15% and 19%, respectively) compared in aerobic condition. However, the HB constituent could accumulate high, up to 94% per mol at longer aerobic condition.

Table 4.6: Summary of PHA constituents produced during feast-famine regime at various experimental works

Experiment	PHA (g/l)	PHB (% mol)	PHV (% mol)	PHH (% mol)
$CODNP_{pome}$-0.85	4.44	62	25	3
$CODNP_{pome}$-0.88	7.53	65	41	15
$CODNP_{pome}$-1.44	6.58	64	32	3
$CODNP_{pome}$-2.45	7.70	74	22	4
$CODNP_{pome}$-2.55	6.97	88	2	10
Air_{pome}-0.5	12.25	50	44	6
Air_{pome}-1	9.48	51	16	33
Air_{pome}-1.5	8.38	41	35	24
Air_{pome}-2	5.46	84	13	3
Air_{pome}-2.5	6.70	84	4	12
HRT_{pome}-12	14.50	28	42	30
HRT_{pome}-14	10.38	48	33	19
HRT_{pome}-16	15.25	45	26	29
HRT_{pome}-18	9.51	48	32	20
HRT_{pome}-26	16.84	48	30	22
FR_{pome}-20	18.84	73	19	8
FR_{pome}-25	7.04	89	4	7
FR_{pome}-33	6.16	82	12	6
FR_{pome}-50	7.77	82	7	11
FR_{pome}-100	7.42	75	16	9
$ANae_{pome}$-70%	7.29	66	15	19
$ANae_{pome}$-60%	6.61	74	10	16
$ANae_{pome}$-50%	9.61	78	10	12
$ANae_{pome}$-30%	8.08	91	6	3
$ANae_{pome}$-20%	9.84	94	3	3
$MICae_{pome}$-70%	12.61	83	9	9
$MICae_{pome}$-60%	13.79	96	2	2
$MICae_{pome}$-50%	14.47	93	3	4
$MICae_{pome}$-30%	11.78	95	2	4
$MICae_{pome}$-20%	6.23	86	8	6

As for the relevance of this study to industrial production, the usage of POME is realistic to produce a stiff and brittle PHA material (based on the composition of HB and HV concentrations). It was proven by several researchers that the mixed composition of PHA constituents (e.g. HB, HV and HH monomer) can be used as a good biodegradable plastic material (Dionisi *et al*., 2001b; Lee and Yu, 2001; Shimizu *et al*. 1999; Lee, 1996a). However, it is challenging to produce a correlation consistent PHA product from intrinsically heterogeneous wastes likes POME. The results are always hampered by inconsistent PHA production, even after the PHA-producer (biomass populations) occurred in high concentration. This might be regulated from the metabolic pathways. The smaller acids such as butyric and

propionic are first converted to 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA before the microorganism use it to PHA backbones (Du *et al.*, 2001). Large acids such as nonaoic (9:0) and octanoic (8:0) acids have to be broken down to C_4 and C_5 acids via β-oxidation for further polymerization. The mass transfer of organic acids from the acidic slurry (raw POME) to PHA synthesis medium plays an important role in coupled system. It might be the rate-limiting step in the consecutive steps of acidenogenesis, mass transfer and polymerization.

4.3.4 Kinetic Rates of PHA Degradation

The storage of PHB will be distributed over the cells according to a standard distribution (Beun *et al*. 2002), either high or low concentrations. Since most of the studies accumulated high PHB rather than PHV and PHH, the kinetic rate of PHA degradation was referred to HB-monomer. At high PHB levels, it can be assumed that all cells contain PHB and that PHB consumption in all cells can be described with macroscopic approach. A macroscopic description is a simplification of reality in which average parameters may be used to describe the whole system. However, the disadvantage in this approach is no longer valid when the cells contain less of PHB. Therefore, it can not simply be stated that the correlation of PHB degradation is valid at high f_{PHB}-values, should also be valid at low f_{PHB}-values. However, since the cultivation was operated in combination of various types of carbon source (mixed cultures), the production of intracellular polymer is recognized as PHA.

The best fit for all data sets (see Figures 4.4 and 4.5) was calculated based on famine degradation of PHB fraction. This determination has been performed in the same way as SO cultivations. Degradation of PHB in this research obviously occurred at a lower rate than predicted from Kuba *et al.* (1996) and Murnleitner *et al.* (1997) for bio-P cultures. According to the result, the fast PHB degradation rate occurred under COD:N:P conditions with k = -0.032 h^{-1} and n = 1.01. At the same time, the slowest PHB degradation appeared under transient anoxic/aerobic conditions with k = 0.026 h^{-1} and n = 0.189. Due to this, the PHB degradation rate

has been determined from the fastest to slowest reaction which are, $CODNP_{pome}fit$ > $Air_{pome}fit$ > $HRT_{pome}fit$ > $FR_{pome}fit$ > $MICae_{pome}fit$ > $ANae_{pome}fit$.

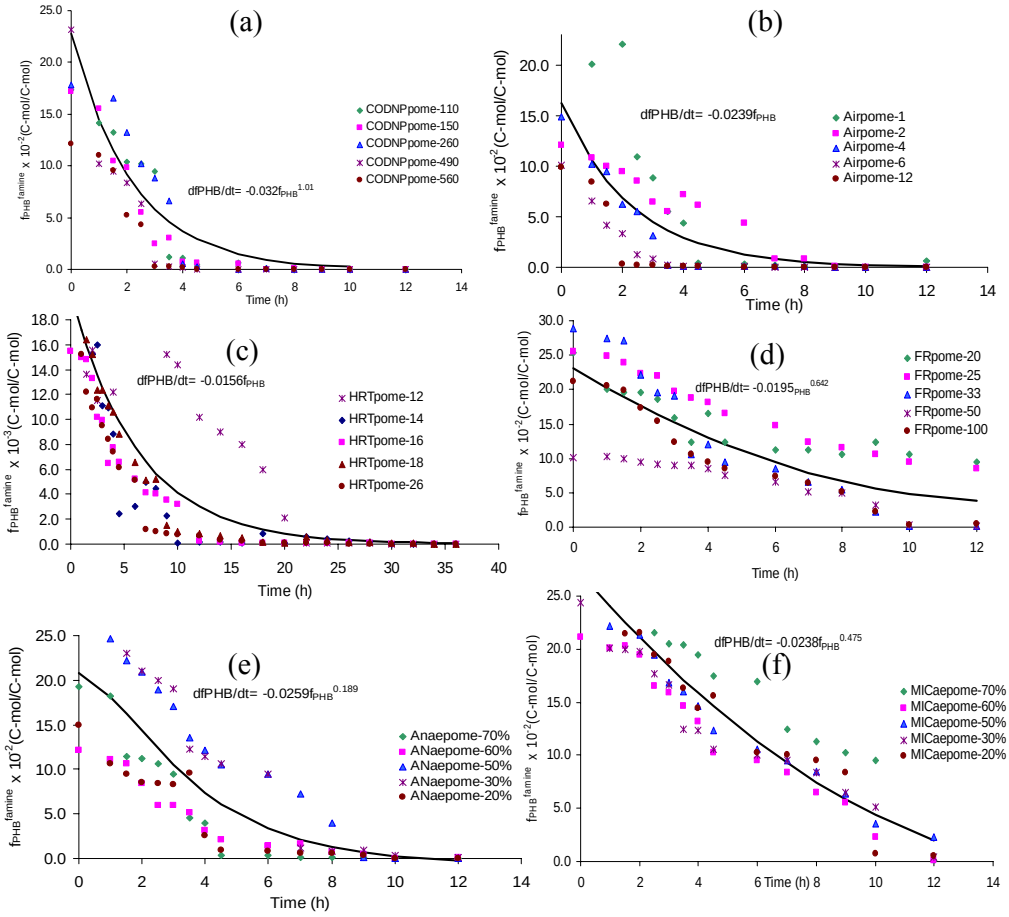


Figure 4.4: Estimation PHB degradation using differential method. (a) COD:N:P ratio experiments, (b) air flowrate experiments, (c) cycle length experiments, (d) feeding rate experiments, (e) anoxic/aerobic experiments, (f) microaerophilic-aerobic experiments.

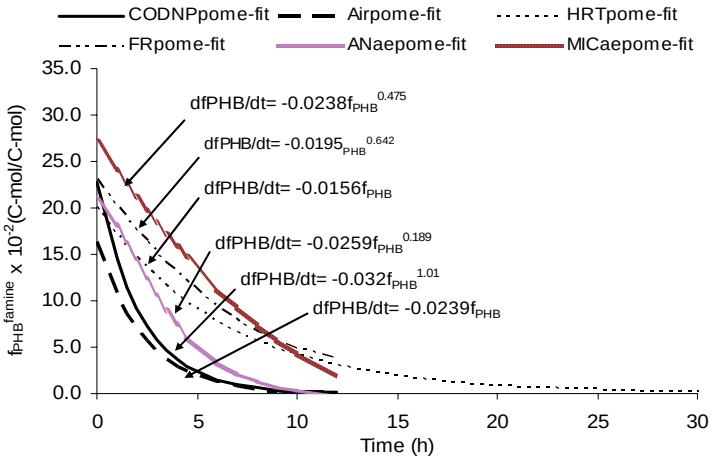


Figure 4.5: Degradation of PHB under different fitted conditions

4.3.5 Statistical Analysis

In experiment of $CODNP_{pome}$, the PHA trends were shown in Table 4.7. Using analysis of variance (ANOVA) single factor, the significant difference (*p*-value) for all of the COD:N:P systems is acceptable (ranged 0.001 – 0.02). The study found that with high COD:N:P ratio (e.g. $CODNP_{pome}$-2.55), the PHA production could increase up to 46.05 ± 6.99%. TOC removal showed the high removal achieved at $CODNP_{pome}$-2.45, which is 19.41 ± 8.12% ($p < 0.0001$ and $r^2 = 0.831$). Meanwhile, the PO_4 removal shows that $CODNP_{pome}$-1.44 can be arose up to 13.81 ± 7.62% ($p < 0.0001$ and $r^2 = 0.58$). The increment of PHA production will result in reduced PO_4 removal, as depicted at $CODNP_{pome}$-2.55. At the same time, the low ratio of COD:N:P showed that the non-removal of NO_3 will occur at $CODNP_{pome}$-0.85, $CODNP_{pome}$-0.88 and $CODNP_{pome}$-1.44.

The study of air flowrates as shown in Table 4.7 indicated that high PHA percentage is depicted at low air flowrate (Air_{pome}-0.5), which accumulated up to 49.72% ± 12.87 ($p = 0.005$ and $r^2 = 0.832$). Then, it was followed by Air_{pome}-1 (45.66 ± 15.98%), Air_{pome}-1.5 (40.99 ± 7.22%), Air_{pome}-2 (38.59 ± 3.58%) and Air_{pome}-2.5 (22.10 ± 5.85%). Meanwhile, the high TOC removal occurred at Air_{pome}-2, which constituted an average of 41.66 ± 2.60% ($p < 0.0001$). In contrast, the

lowest TOC removal appeared at Air_{pome}-2.5 (2.79 ± 9.27%, p = 0.001). In order to perform a suitable range for TOC removal, the flowrate must be conducted at range 1.5 – 2 l/min to reach the removal efficiency between 20 – 40%. The same behaviour was observed under PO_4 removal because the lowest removal depicted at Air_{pome}-2.5 (14.21 ± 5.33%, p < 0.0001). Then, it was followed by Air_{pome}-1.5, Air_{pome}-1, Air_{pome}-0.5 and Air_{pome}-2.5. It was obvious that non-NO_3 removal performed in Air_{pome}-0.5 (-23.28 ± 3.99%, p < 0.0001), Air_{pome}-2.5 (-16.75 ± 12.18%, p = 0.001) and Air_{pome}-1.5 (-2.60 ± 0.92%). However, the high NO_3 removal was found at Air_{pome}-1 (45.80 ± 6.75%) and Air_{pome}-2 (17.85% ± 6.06%).

The study of cycle length (Table 4.7) found that the HRT_{pome}-16 reached a high PHA production, 56.98 ± 2.57%, followed by HRT_{pome}-14 (52.81 ± 2.30%) and the lowest production depicted at HRT_{pome}-26 (28.30 ± 9.69%). TOC removal showed the highest percentage at HRT_{pome}-26 (43.53 ± 4.62%, p < 0.0001), while the lowest removal obtained at HRT_{pome}-12 (1.51 ± 0.12%). The same pattern was also depicted in PO_4 removal, where HRT_{pome}-26 reached the highest removal at 30.90 ± 5.62% (p < 0.0001 and r^2 = 0.715). The lowest removal efficiency of PO_4 showed in HRT_{pome}-16 (10.98 ± 7.56%). Finally, the study also found that all of the HRT studies were observed as non-NO_3 removal.

As shown in Table 4.7 under the feeding rate study (FR_{pome}), the high PHA production showed at FR_{pome}-20, which constitute up to 30.25 ± 17.69% (p=0.019 and r^2=0.67). Under TOC removal, FR_{pome}-20 (21.67 ± 5.43%, p=0.0006) and FR_{pome}-25 (14.75 ± 3.29%, p=0.0002) showed a moderate removal efficiency. A high PO_4 removal depicted at FR_{pome}-25 (34.36 ± 14.01%, p=0.01), followed by FR_{pome}-33 (23.01 ± 10.86%, p < 0.001) and FR_{pome}-50 (27.62 ± 14.19%, p=0.0034). FR_{pome}-25 also performed the high efficiency in NO_3 removal (15.92 ± 2.59%, p=0.0001) followed by FR_{pome}-20, FR_{pome}-50, FR_{pome}-100, and FR_{pome}-33.

Study on anoxic/aerobic condition was also analyzed in Table 4.7. The PHA production varied at range 49 – 62% of PHA production, indicating that the prolonged aerobic has a significant effect than anoxic condition. Under TOC

removal, the prolonged period of aerobic condition could reach up to 33.43±7.47% compared to prolonged anoxic condition. Both PO_4-P and NO_3-N removal showed that the *p*-value is always less than 0.0001, indicating that the system is dependent on the presence of NO_3^- (anoxic condition). The highest PO_4-P removal observed in $ANae_{pome}$-70% (28.45±5.82%, $p < 0.0001$), while for NO_3-N removal obtained at $ANae_{pome}$-50% (32.61±4.81%, $p < 0.0001$).

The condition of microaerophilic-aerobic experiments ($MICae_{pome}$) in Table 4.7 showed that the high PHA production occurred at $MICae_{pome}$-70% (74.06±16.04%, $p = < 0.0001$). It followed by $MICae_{pome}$-60% (68.26±19.69%, $p = 0.005$) and $MICae_{pome}$-50% (57.09±14.25% ($p = 0.031$). In general, the prolonged microaerophilic period (> 3 hours) will exhibit the higher level of PHA storage (more than 40%). The TOC removal efficiency could only reached at average of 18% removal ($p = 0.016$). Both PO_4-P and NO_3-N removal showed the preferred *p*-value (< 0.0001) indicating that the system is dependent on the presence of O_2 or NO_3^-. The highest PO_4-P removal observed in $MICae_{pome}$-20% (20.48±25.13%, $p = 0.014$), while for NO_3-N removal obtained at $MICae_{pome}$-30% (19.19±3.12%, $p < 0.0001$).

In order to optimize the PHA production using POME, it is important to obtain a high cellular PHA content, simultaneously with PHA productivity. It is also recommended that fermented wastewater should be investigated as a potential substrate for PHA production using activated sludge biomass. Therefore, the ranking value has been proposed in all of the studies. The corresponding rank is referred to the best achievement of every variable (PHA, TOC, PO_4 and NO_3), which can summarize as follows:

(a) $CODNP_{pome}$-0.88 > 110 > 490 > 260 > 560
(b) Air_{pome}-1 > 1.5 > 2 > 0.5 > 2.5
(c) HRT_{pome}-26 > 16 > 18 > 14 > 12
(d) FR_{pome}-25 > 20 > 33 > 50 > 100
(e) $ANae_{pome}$-30% > 20% > 50% > 60% > 70%
(f) $MICae_{pome}$-60% > 30% > 50% > 20% > 70%

As a conclusion, the removal of organic carbon (TOC) should be involved in the first step of cyclic metabolic pathway of PHB. The proper cyclic metabolic pathway is proposed by Beun *et al.* (2002). If the period of excess external substrate availability is long enough, the specific growth rate of the biomass will increase to its maximum and PHA synthesis rate will slow down. This effect has been observed by van Aalst-van Leeuwen *et al.* (1997). The second step is to optimize the culture of aerobic PHA-producing species in a bulk production. A dynamic study on PHA formation under washout conditions revealed that most of the cultures left the bioreactor with a quite low PHA content.

In most of the study, a PHA content of 70% was not easily reached. In the present research at high substrate dosage, a possible PHA content may approximately between 40 – 50%. In those cases, a slow down of the PHA production rate due to filling up of the cells with PHA, is not expected. The PHA production rate can be considered constant and not limited by the PHA content. Therefore, the PO_4-P and NO_3-N removal must be controlled in a cycle of feast-famine regime to obtain high PHA productivity. In anoxic/aerobic study, it was obvious that prolonged aerobic condition has more impact on PHA accumulation (typically at more than 50% of aerobic condition) compared with longer anoxic conditions. Marazioti *et al*. (2003) reported that the apparent cell's accumulations under transient anoxic and aerobic conditions were found to be dependent on the time-varying fraction of cultures present in the bioreactor. From the experimental analysis, it can be confirmed that both aerobic phosphorus removal and denitrifying process (PO_4-P and NO_3-N removal) occurred simultaneously.

Table 4.7: Statistical analysis for every experimental works

Experiment	PHA		TOC		PO_4-P		NO_3-N		Ranks
	% PHA Production	SD (*p*)	% TOC removal	SD (*p*)	% PO_4 removal	SD (*p*)	% NO_3 removal	SD (*p*)	
$CODNP_{pome}$-0.85	31.69	7.15 *(0.02)*	0.23	1.34 *(0.25)*	5.73	1.61 *(0.001)*	-1.42	7.61 *(0.02)*	4
$CODNP_{pome}$-0.88	31.44	5.59 *(0.001)*	8.07	5.85 *(0.000)*	0.08	1.38 *(0.199)*	-41.94	9.69 *(0.001)*	5
$CODNP_{pome}$-1.44	33.63	8.44 *(0.06)*	-7.18	0.26 *(0.005)*	13.81	7.62 *(0.000)*	-25.88	20.70 *(0.003)*	2
$CODNP_{pome}$-2.45	37.59	6.95 *(0.001)*	19.41	8.12 *(0.000)*	-11.63	14.72 *(0.003)*	21.60	5.53 *(0.002)*	1
$CODNP_{pome}$-2.55	46.05	6.99 *(0.003)*	15.03	11.67 *(0.63)*	-12.77	4.05 *(0.000)*	23.80	8.14 *(0.01)*	3
Air_{pome}-0.5	49.72	12.87 *(0.005)*	26.88	6.79 *(0.000)*	26.88	6.79 *(0.013)*	-23.28	3.99 *(0.000)*	4
Air_{pome}-1	45.66	15.98 *(0.099)*	23.98	5.45 *(0.000)*	23.98	5.45 *(0.001)*	45.80	6.75 *(0.000)*	1
Air_{pome}-1.5	40.99	7.22 *(0.11)*	24.84	7.43 *(0.001)*	33.73	4.64 *(0.0000)*	-2.60	0.92 *(0.5)*	2
Air_{pome}-2	38.59	5.85 *(0.027)*	41.66	2.60 *(0.001)*	44.40	3.86 *(0.0000)*	17.85	6.06 *(0.004)*	3
Air_{pome}-2.5	22.10	3.58 *(0.003)*	2.79	9.27 *(0.001)*	14.21	5.33 *(0.0002)*	-16.75	12.18 *(0.001)*	5
HRT_{pome}-12	52.81	2.30 *(0.0001)*	1.51	0.12 *(0.01)*	21.80	14.91 *(0.04)*	-35.37	8.44 *(0.0004)*	4
HRT_{pome}-14	53.61	4.28 *(0.0001)*	2.56	4.07 *(0.083)*	13.11	5.20 *(0.013)*	-23.16	3.03 *(0.0001)*	3
HRT_{pome}-16	56.98	2.57 *(0.0001)*	11.45	1.36 *(0.0001)*	10.98	7.56 *(0.744)*	-54.97	5.05 *(0.0001)*	2
HRT_{pome}-18	36.97	2.61 *(0.0001)*	6.52	20.64 *(0.15)*	16.74	4.49 *(0.001)*	-63.13	3.96 *(0.0001)*	5
HRT_{pome}-26	28.30	9.69 *(0.001)*	43.53	4.62 *(0.0001)*	30.90	5.62 *(0.001)*	-17.33	4.55 *(0.0001)*	1
FR_{pome}-20	50.25	17.69 *(0.019)*	21.67	5.43 *(0.0006)*	17.36	5.05 *(0.0004)*	10.92	2.59 *(0.0001)*	2
FR_{pome}-25	45.27	17.07 *(0.055)*	14.75	3.29 *(0.0002)*	34.36	14.01 *(0.01)*	15.95	14.08 *(0.0001)*	1
FR_{pome}-33	47.37	12.77 *(0.117)*	7.97	9.07 *(0.847)*	23.01	10.86 *(0.000)*	-3.58	7.75 *(0.5113)*	3
FR_{pome}-50	35.60	5.85 *(0.027)*	3.68	6.77 *(0.779)*	27.62	14.19 *(0.003)*	4.68	7.62 *(0.039)*	4
FR_{pome}-100	47.99	6.63 *(0.0005)*	2.57	5.08 *(0.811)*	2.73	22.12 *(0.032)*	6.68	6.86 *(0.051)*	5
$ANae_{pome}$-70%	49.01	13.15 *(0.026)*	18.49	5.35 *(0.0001)*	12.21	3.07 *(0.0004)*	28.45	5.82 *(0.0000)*	4
$ANae_{pome}$-60%	56.86	4.34 *(0.013)*	11.93	8.43 *(0.568)*	20.90	3.13 *(0.0000)*	24.77	9.51 *(0.004)*	3
$ANae_{pome}$-50%	50.17	8.09 *(0.003)*	2.33	32.48 *(0.299)*	32.61	4.80 *(0.0000)*	6.07	7.24 *(0.005)*	5
$ANae_{pome}$-30%	63.38	12.83 *(0.175)*	33.43	7.47 *(0.0000)*	35.33	5.10 *(0.0000)*	-13.64	13.25 *(0.0017)*	1
$ANae_{pome}$-20%	62.17	14.19 *(0.024)*	29.87	1.57 *(0.0000)*	17.77	11.25 *(0.0004)*	-8.24	11.06 *(0.945)*	2
$MICae_{pome}$-70%	74.06	16.04 *(0.000)*	18.42	5.16 *(0.001)*	-10.64	2.71 *(0.000)*	8.03	1.53 *(0.000)*	5
$MICae_{pome}$-60%	68.26	19.69 *(0.005)*	25.96	10.43 *(0.001)*	-2.92	2.37 *(0.736)*	13.53	2.73 *(0.000)*	1
$MICae_{pome}$-50%	57.09	14.25 *(0.033)*	18.10	3.32 *(0.000)*	1.63	10.31 *(0.402)*	14.88	3.63 *(0.000)*	4
$MICae_{pome}$-30%	52.55	10.57 *(0.002)*	18.01	10.43 *(0.008)*	2.45	10.07 *(0.340)*	19.19	3.12 *(0.000)*	2
$MICae_{pome}$-20%	49.96	12.30 *(0.005)*	25.42	4.82 *(0.000)*	20.48	25.13 *(0.014)*	9.00	2.48 *(0.0003)*	3

Note: SD = Standard Deviation, *p* = significance different. Negative value indicates non-removal efficiency

Organic wastes are usually in complicated forms that cannot be directly utilized by PHA-producing species (Hsu and Wu, 2002; Herbert *et al*., 2000; Hessekmann *et al*. 2000). Hydrolysis and acidogenesis are the first step to convert the wastes to short-chain-fatty-acid (SCFA) such as acetic, propionic, and butyric acids that can be further utilized by PHA-producing species. In addition, during the accumulation of PHA, the PO_4 and NO_3 removal could also be detected due to the coupled of growth and maintenance. A desired determination has been concluded in the correlation analysis as shown in Figure 4.6.

Results in COD:N:P ratio experiments showed that the amount of polymer stored by cells could not be strongly correlated to nutrient removal (Pearson coefficients less than 0.8). It showed that sludge submitted to aerobic feeding rate could accumulate high amounts of PHA (more 2 – 3 times higher than in COD:N:P conditions alone) by manipulating the air flowrate. During Air_{pome} experiments, no high correlation can be obtained in all of the studies. The long cycle period will benefit TOC and PO_4 removal, while a low cycle period is suitable for inducing the PHA production. The results in feeding regime experiments (FR_{pome}) were also considered as low correlation between PHA production, organic and nutrient removal. In $ANae_{pome}$ and $MICae_{pome}$ experiments, the negative correlation (PHA - PO_4 and PHA – NO_3) was obtained because a high storage activity for PHA production will release the nutrient to balance their energy requirement.

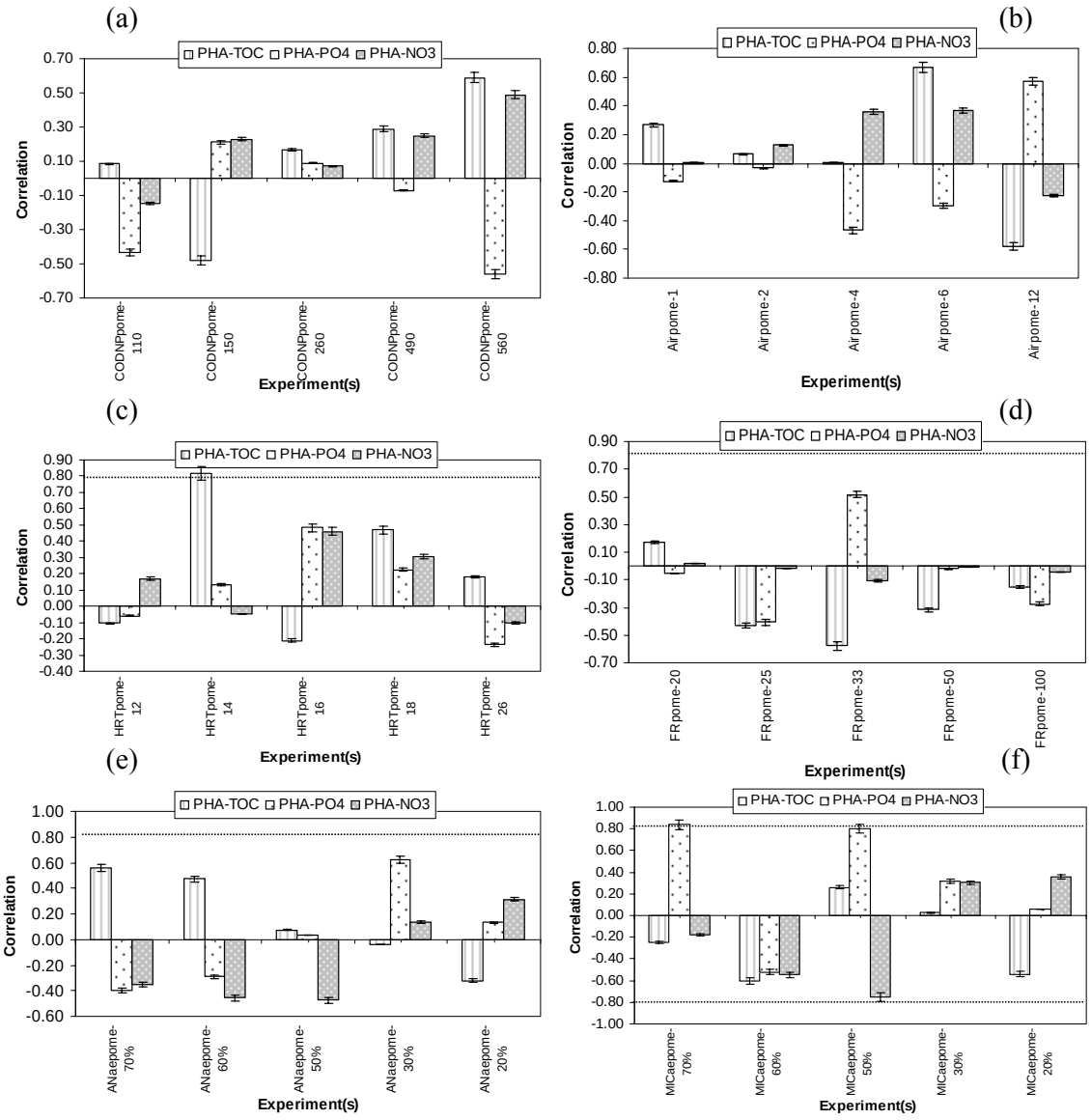


Figure 4.6: Summary on PHA correlation to other organic and nutrient removal at various experiment studies. (a) COD:N:P ratio experiments, (b) air flowrate experiments, (c) cycle length experiments, (d) feeding rate experiments, (e) anoxic/aerobic experiments, (f) microaerophilic-aerobic experiments.

4.3.5 Mass Balance of Substrates During Feast-Famine Period

Table 4.8 presents an overview of the mass balance values during feast, famine and total cycle of selected experiments during a pulse cycle of POME as carbon source. Measurements were repeated three times: the values reported in the table are the average results. The consistency of the mass balance was checked by performing the C-balance over the total cycle; using Macrobal software analysis. The total amount of substrate added in one cycle was consumed during the feast period. An important fraction of substrate was converted and stored as polymer storage compounds.

The results in Table 4.8 showed that a low DO saturation (Air_{pome}-1) will reduce the uptake rate during feast as compared to famine condition. The overall results in the feast phase illustrated that the substrate utilized in growth and accumulation period, response to maximize the substrate uptake. The organisms are probably growing very fast as compared to their average growth rate in the system but due to the very short length of the feast phase, the growth in this phase does not contribute to the overall growth.

The results of the C-balance (Table 4.8) in HRT_{pome} experiment showed that the total cycle were rather good, implicating that the obtained data on substrate, biomass, PHA and CO_2 were quite reliable. However, he residual substrate still present in famine period, even the sludge was remained for over 10 hours. This can be explained by insufficient uptake rate in feast period. As explained previously, the slowly hydrolyzed biodegradable could be the main contributor to this problem.

The converted amount of substrates at FR_{pome}-25 as presented in Table 4.8 indicated that most of the system had similar trends as shown in the previous experiment. It could explain by a low amount of CO_2 production in the feast period, indicating that the PHA production did not produce high biogas concentration. However, the PO_4-released (non-PO_4 removal) to the medium in this study was considered as insufficient removal efficiency.

A cycle of the transient anoxic/aerobic experiment ($ANae_{pome}$-30%) (Table 4.8) is divided into only feast and famine period, excluding the C-balance in feast/famine (under anoxic cycle). This is based on the 'complete' cycle of anoxic/aerobic condition in a single fed-batch experiment. As calculated in the standard anoxic/aerobic condition, a high consumption rate occurred under long aerobic condition compared to the anoxic. Therefore, the conversion to PHA is 10% higher than observed in anoxic condition. However, the anoxic condition could also generate the storage accumulation but the conversion rate of substrate was still lower than aerobic condition. A similar finding was also reported by Beun *et al*. (2002) and Beccari *et al*. (2002). Under normal condition (especially in aerobic process), bacteria utilize the substrate for growth with minor response storage, but during restricted carbon flow (limiting nutrient factor) bacteria may shift its protein synthesis to PHA synthesis for survival (Gernaey *et al.*, 2004; Aulenta *et al*., 2002).

The final comparative study of converted amounts in the complete cycle with POME was depicted in $MICae_{pome}$-60% (Table 4.8). The result showed that PHA production will be affected either by the biomass concentration or O_2 consumption rate in one cycle. In this study, $MICae_{pome}$-60% could only convert to -48.79 C-mmol/cycle. Meanwhile, PHA concentration reached to only 15.68 C-mmol/cycle, which was three times lower than anoxic/aerobic cycling operating phase. The CO_2 production during feast period could only achieved 8.72 C-mmol/cycle compared to HRT_{pome}-26 (89.88 C-mmol/cycle) and DO_{pome}-1 (23.96 C-mmol/cycle) studies.

Table 4.8: Converted amounts in (C) mmol/cycle for all compounds at selected operating conditions with standard fed-batch system

	Compound	Air_{pome}-1 conversion rate ((C) mmol/cycle)	HRT_{pome}-26 conversion rate ((C) mmol/cycle)	FR_{pome}-25 conversion rate ((C) mmol/cycle)	$ANae_{pome}$-30% conversion rate ((C) mmol/cycle)	$MICae_{pome}$-60% conversion rate ((C) mmol/cycle)
Feast	substrate[a]	-68.94±7.45	-295.00±9.25	-69.06±7.45	-99.21±2.32	-48.79±7.45
	biomass	31.43±0.33	166.96±2.03	42.79±0.33	41.43±1.56	24.39±3.33
	PHA	13.55±1.15	38.17±1.22	25.70±1.15	43.77±1.33	15.68±2.15
	CO_2	**23.96±1.64**	**89.88±3.33**	**0.57±0.64**	**14.01±2.96**	**8.72±6.44**
	O_2	-6.33±2.10	-91.20±1.26	-8.52±2.10	-19.80±2.65	-6.70±2.30
	NH_4^+	-10.07±1.03	-13.25±3.40	18.00±1.03	-17.23±2.34	-12.77±2.03
	PO_4^{2-}	-35.65±1.14	-11.82±1.87	-13.34±1.14	-6.80±1.47	-5.13±0.44
Famine(*)	biomass	28.86±2.63	22.41±1.45	17.14±2.63	46.57±1.95	22.44±6.32
	PHA	-13.55±1.15	-18.17±1.22	-25.70±1.15	-43.77±2.12	-15.68±4.88
	CO_2	**15.30±0.56**	**4.24±2.40**	**-8.56±0.55**	**2.80 ±2.15**	**6.76±2.55**
	O_2	-2.35±0.41	-54.50±5.62	-3.50±1.22	-6.40 ±2.63	-4.88±1.41
	NO_3^-	-19.29±0.75	3.14±8.49	1.29±0.99	-0.64 ±1.22	-1.71±2.75
	PO_4^{2-}	-53.47±0.95	-8.06±7.90	-1.72±0.55	-1.74 ±1.63	-1.70±0.95
Total	substrate	-68.94±7.02	-295.00±8.56	-69.06±6.02	-99.21±2.56	-48.79±7.02
	biomass	60.29±4.00	189.37±1.32	59.93±4.20	76.00±2.44	46.83±4.23
	CO_2	**39.26±0.25**	**94.12±1.30**	**-7.99±0.48**	**8.82±0.56**	**15.48±5.55**
	O_2	-8.68±0.56	-145.70±2.20	-12.02±1.54	-12.20±1.99	-10.58±0.58

Note: A minus sign indicates consumption of the compound. Standard deviations after plus/minus signs, while bold values are calculated values. (a) readily available of soluble biodegradable COD, Xs and Ss (*)overall famine period

For comparison, an overview of all converted amounts in C-mmol/cycle and their standard deviations as balanced with Macrobal are shown in Table 4.9. Values for cultures fed with acetate or glucose as single substrate at different SRTs obtained by Beun *et al.* (2000a) and Dircks *et al.* (2001) are summarized in this table as well. However, Carta *et al.* (2001) both acetate and glucose was calculated and identified as mixed substrates. In order to compare those findings, the example of microaerophilic-aerobic result has been analyzed in the last column.

It can be seen that a large fraction of external substrate is stored as PHA or glycogen. In the system with a mixed substrate (except this study), the conversion of acetate and glucose in PHA and glycogen, respectively, is not different. A first comparison indicates that the result for the mixed substrates is appropriate a

weighted average of the conversion of both substrates individually. Since this study was conducted in high substrate concentration (four to eight times higher than other studies), the PHA production had increased up to 15.68±2.15 C-mmol/cycle at feast period. Consequently, this contributed a high CO_2 production compared to other studies.

Table 4.9: Comparison of converted amounts for measured compounds in aerobic pulse-fed SBR processes

	SRT Substrate Compound	Carta *et al.* 2001; 6.1 days; acetate/ glucose ((C) mmol/cycle)	Beun *et al.* 2000a; 3.8 days; acetate ((C) mmol/cycle)	Beun *et al.* 2000a; 9.5 days; acetate ((C) mmol/cycle)	Dircks *et al.* 2001; 3.6 days; glucose ((C) mmol/cycle)	This study; limited oxygen; HRT=SRT; POME ((C) mmol/cycle)
Feast	Substrate[a]	-6.21±0.25	-13.54±0.55	-11.44 ±0.27	-12.46	-48.79±7.45
	Biomass	1.16±0.31	1.36±1.34	0.23±0.77	0.68	24.39±3.33
	Glycogen	-6.18±0.83				-
	PHA	3.66±0.48	5.57±0.75	7.16±0.64	-	15.68±2.15
	CO_2	**3.41±1.02**	**6.61±1.19**	**4.05±0.27**	**0.84**	**8.72±6.44**
	O_2	-3.02±1.04	-	-3.15±0.26	-	-6.70±2.30
	NH_4^+	-0.22±0.06	-0.23±0.23	-0.04±0.13	-0.13	-12.77±2.03
	PO_4^{2-}	-	-	-	-	-5.13±0.44
Famine(*)	Biomass	3.77±0.47	2.61±0.85	2.85±0.73	5.75	22.44±6.32
	Glycogen	-4.16±0.14			-10.96	-
	PHA	-3.66±0.48	-5.57±0.75	-7.16±0.64	-	-15.68±4.88
	CO_2	**4.06±0.49**	**2.96±0.17**	**4.31±0.20**	**6.21**	**6.76±2.55**
	O_2	-4.76±0.51	-3.63±0.15	-5.18±0.19	-	-4.88±1.41
	NO_3^-	-	-	-	-	-1.71±2.75
	PO_4^{2-}	-	-	-	-	-1.70±0.95
Total	Substrate	-	-	-	-	-48.79±7.02
	Biomass	4.93±0.49	3.97±1.04	3.08±0.22	-	46.83±4.23
	CO_2	**7.46±0.10**	**9.57 ±1.18**	**-8.36±0.18**	**-**	**15.48 ±5.55**
	O_2	-	-9.53±1.19	-8.33±0.18	-	-10.58±0.58

Note: A minus sign indicates consumption of the compound. Standard deviations after plus/minus signs, while bold values are calculated values. (a) single substrate or readily available of soluble biodegradable COD, Xs and Ss (*)overall famine period

4.4 Development of PHA Productivity (Δf_{PHA})

In Figure 4.7, the fraction of net polymer produced per unit of active biomass (Δf_{PHA}) during the "feast" phase is presented for all experimental data. Those results are clearly similar with the previous PHA content and concentrations. The experiments were also considered the optimum yield and kinetic rates. The Δf_{PHA} had been used to confirm the productivity of PHA during "standard feast" with minor modification on specific rates (q_p and $-q_s$). The result convincing that ammonia is an important parameter to be controlled in the reactor. Ammonia limitation caused a decrease of the cell growth rate and led to an increase of the polymer storage yield and productivity. The rate of polymer production varied directly with the substrate concentration in the range 150 – 300 C-mmol/l, but decreased sharply for more than 450 C-mmol/l (data not shown). At the same time, the tremendous changing of oxygen saturation will also lead the polymer storage. As depicted in air flowrate experiments, the Δf_{PHA} will slow-down because of the limitation of air supply into the reactor. The limited concentration of oxygen is significant for storage capacity of the cells. Therefore, all of the experiments were conducted in less than 2.5 l/min.

Based on previous study, a high substrate concentration (more than 450 C-mmol/l) favoured PHA accumulation, even though the specific storage rate decreased due to substrate inhibition. In order to overcome inhibition, the same volume of carbon substrate was added to the reactor in five different feeding rates: duration of flowrate from 30 – 120 minutes. In the fast feeding rate (> 75 ml/min), the Δf_{PHA} clearly showed insignificant for polymer storage compared to slow feeding rate (< 55 ml/min). The maximum amount of PHA depicted in this aerobic dynamic feeding was around 50% similar as reported by Beccari *et al.* (1998) and Serafim *et al.* (2004). It was postulated that during initial substrate pulses addition, substrate will be converted for growing or maintenance activities. Then, it slightly increased for storage, while biomass became saturated in polymerization.

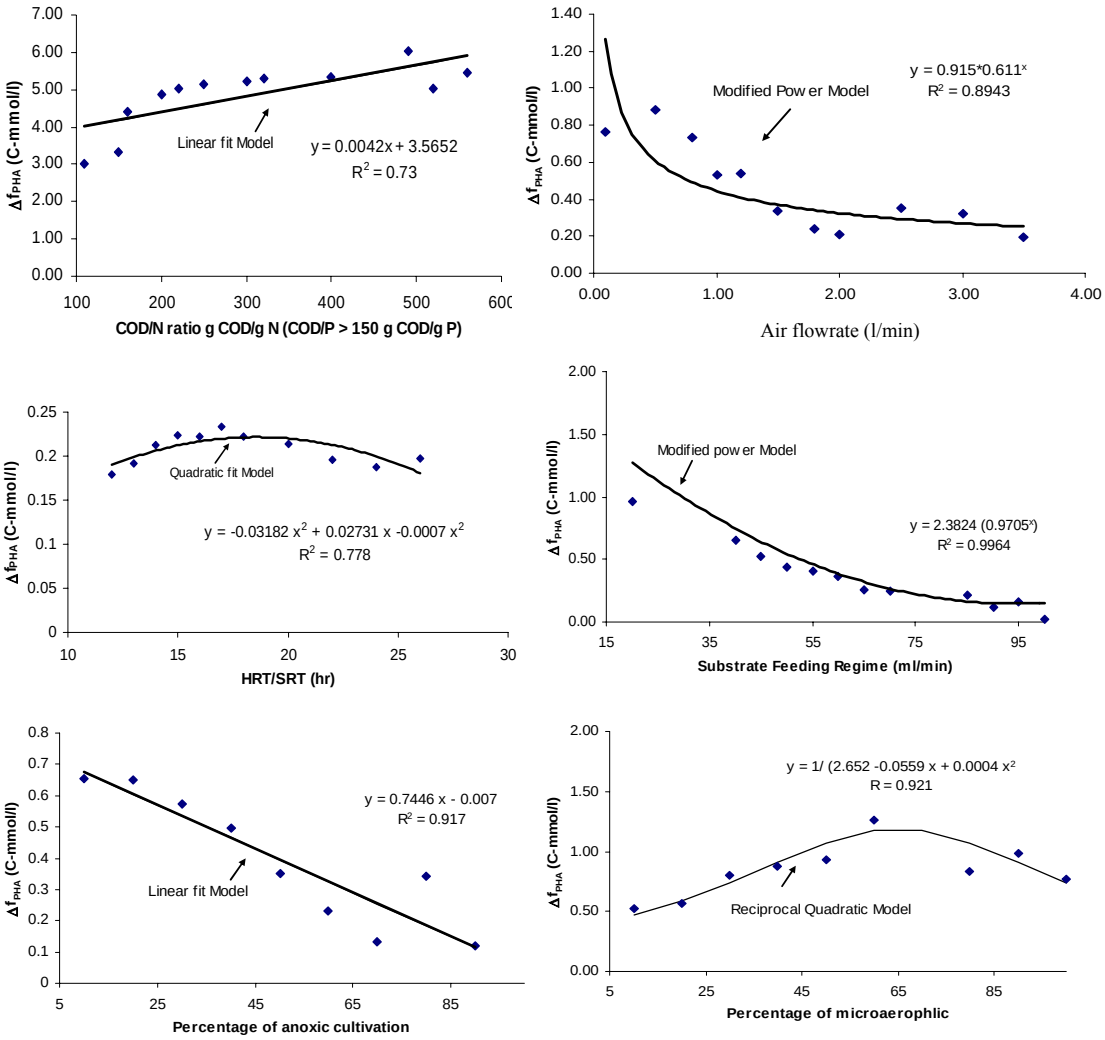


Figure 4.7: PHA produced on COD:N:P ratio, air flowrates, HRT=SRT, feeding rates, anoxic/aerobic and microaerophilic-aerobic. (♦) experiments used for fitting the points, (—) model equation developed from fittings.

In order to understand the behaviour of polymer storage, the anoxic and microaerophilic condition was also conducted in a single fed-batch system. The PHA storage capacity was evaluated higher in "aerobic" condition than in "anoxic". Even though production of polymer occurred for each condition, the Δf_{PHA} decreased from 0.1 – 0.4 C-mmol/l. Therefore, the sludge submitted to aerobic dynamic feeding could accumulate high amounts of PHA by manipulating specific operational condition (COD:N:P, HRT=SRT and oxygen flowrates, substrates feeding) and cultures condition (anoxic/aerobic and microaerophilic-aerobic).

4.5 Discussion on Specific Findings

In the presence of external substrate (S_s), the organisms have a choice to use the substrate for growth or storage processes. Traditionally, it is assumed that competing microorganisms maximized their growth rate, and storage capacity only occurs when some growth related compound (e.g. N and P) gets limited. Many organisms subjected to feast-famine conditions maximize their substrate uptake rate ($-q_s$) (as observed in this study) while growing at a more or less balanced rate. Storing substrate and subsequent growth on it leads to a slightly reduced net growth yield (Beun *et al.*, 2000a; Dircks *et al.*, 2001). This loss in yield could be compensated by the reduced need for RNA and anabolic enzymes where all are been consumed as energy requirement under fast growth on the external substrate and starvation period. The difference between actual $-q_s$ and μ leads to substrate storage. This has not only been observed for heterotrophic organisms, but also for autotrophic organisms (van Loosdrecht and Heijnen, 2002). Table 4.3 previously showed that even when the yield of heterotrophic obtained at range 0.35 – 0.42 g COD/g COD, the PHA production would reach up to 74% ($MICae_{pome}$-70%).

Punrattasin *et al.* (2001a), Chinwetkitvanich *et al.* (2004) and Luengo *et al.* (2003) found that the enrichment of PHA producing bacteria by operating under alternating periods of growth and nutrient limitation conditions was an effective way to achieve high PHA production when the substrate was a mixture of VFAs. Figure 4.8 successfully defines the optimum condition for PHA content in a single fed-batch culture. This study found that when the feed contain N and P limitations, it will enable biomass to store the PHA very fast before the biomass use it for cell growth and anabolic metabolism. Therefore, the optimum storage of PHA content was obtained at approximately 400 COD/N ratio and 200 of COD/P ratio. Several researchers (e.g. Ryu *et al.*, 1999; Du *et al.*, 2001) explained that the PHA accumulated under P limitation is better than N or other essential nutrients. Similar results might have been obtained during these studies, if the biomass was not washed out from the systems because of sludge bulking. However, when the experiment performed under adequate N and P limitations (this study), the biomass lost was only obtained after 4-5 hours (approximately 17% of total cycle). This also indicated by

the peak of PHA accumulation occurring in much shorter time period (fast uptake rate) rather than biomass depletion. Also, it was discovered that not only O_2 will act as electron acceptor to the biomass, but with low concentration of NO_3 the PHA production still can be produced.

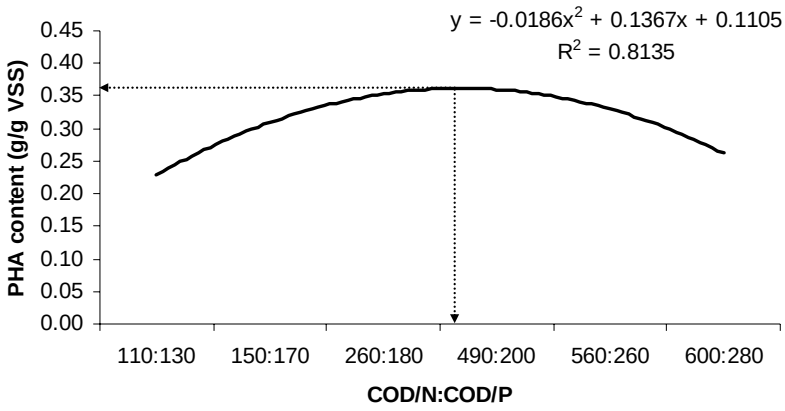


Figure 4.8: Influences of PHA content on the overall COD:N:P ratio in a standard aerobic experiments

As depicted in Figure 4.9, the growth rate consists of two parts, one resulting from growth on S_s and limited by the amount of protein synthesizing system in the biomass, and a second part when the S_s is depleted resulting from growth on PHA. It is notable that the μ increases in the short period of S_s presence. The turn-over of PHA was observed clearly. When the feed rate over cycle length (FR/CL) is increased, the PHA content under feast and famine will reacts opposite. The PHA content at feast period will be decreased, while during famine period it will start to increase. This pattern was also obtained from van Loosdrecht and Heijnen (2002), which indicate that the bacteria always compete on substrate uptake rate and not for growth rate. Therefore, the sufficient feeding rate must be employed in the system to maximize the PHA production rate under short cycle length study. As a conclusion, the growth and PHA production rate can be controlled by manipulating the ratio of FR/CL. The preferred ratio was projected at 0.5 (feeding rate at 20 – 25 ml/min).

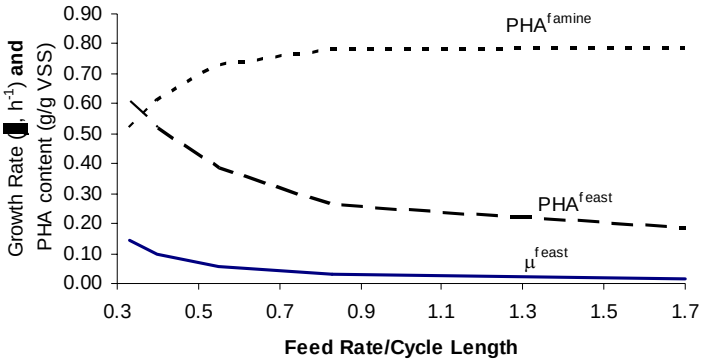


Figure 4.9: Result for the relative length of the feeding period (FR_{pome} experiment) in fed-batch SBR on the growth rate of bacteria in feast period (bottom line), and on the cellular content of PHA at the end of the feast (dashed line) and famine periods (dotted line).

As reported from Dionisi *et al.* (2001), the specific yields and rates on PHA, substrate and biomass were not strongly affected from external electron acceptor (e.g. O_2 or NO_3). However, their findings were contradicted by this study as well as Beun *et al.* (2000b). A remarkable observation was that the anoxic specific substrate uptake rate was 3 – 4 times lower than aerobic. The only explanation could be that nitrate uptake or nitrate/nitrite reduction was rate limiting (Beun *et al.* 2000b).

In contrast with the results obtained by Dionisi *et al.* (2001a, 2001b), it was found that certain microorganisms could perform the aerobic-denitrification as well as anoxic condition. This circumstance has already been reported from a single culture of *T. pantotropha* which can simultaneously utilize oxygen and nitrate during acetate removal under aerobic condition with higher growth rates than under aerobic conditions without nitrate concentration (Dionisi *et al.*, 2001a). It is proven that aerobic denitrification is always present with the mixtures of substrates concentration (Beccari *et al.*, 2002). The behaviour of the microorganisms appeared to be very similar as reported by Beun *et al.* (2000b). The reason of reduction PHA yield under anoxic condition may cause from the microorganisms. The proposed mixed culture in this study is believed to limit their specific growth rate. The results show that substrate uptake, PHA degradation and electron transport were the rate limiting step.

The main difference between completely anoxic and the anoxic/aerobic SBR was the accumulation and subsequent degradation of nitrite in the completely anoxic SBR (Beun *et al*., 2000b). They found that under completely anoxic conditions the nitrite reduction rate falls behind the nitrate reduction rate.

The transient response to substrate spike was investigated for mixed cultures under anoxic/aerobic environment for a range of different operating conditions (COD:N:P ratios and feed length). In comparing the results from Beun *et al.* (2000a and 2000b), this study produce a high rate of PHA content (q_p = 0.343 C-mol/C-mol. h) compared to that obtained previously by Lishman *et al.* (2000). In addition, the difference in specific growth rate between the feast and the famine period is smaller under anoxic than under aerobic condition (Table 4.10). The lower μ the feast period under anoxic conditions can be explained by the lower $-q_s$ in the feast period under anoxic condition. The degradation of PHA during famine period resulting an increase of growth rate under anoxic conditions. The same μ in the famine period under both anoxic and aerobic conditions can be explained by the same average of specific PHA consumption rate. Therefore, it can be concluded that the maintenance mechanism was the same under aerobic and anoxic conditions. As a result, the PHA degradation is influenced by the type of electron acceptor whereas the substrate uptake rate is independent (Saito *et al*., 2004).

Table 4.10: Comparative study on anoxic/aerobic experiments

Parameters	Dimension	Beun *et al.* 2000a	Beun *et al.* 2000b	This study
SRT	days	3.8	6.3	< 1*
C_x	C-mmol/l	45	49.5	660
$-q_s$	C-mol/C-mol.h	0.640	0.170	0.493
q_p	C-mol/C-mol.h	0.270	0.064	0.343
$q_p/-q_s$	C-mol/C-mol	0.410	0.370	0.695
$\mu^{overall}$	h^{-1}	0.011	0.007	0.110
μ^{feast}	h^{-1}	0.065	0.019	0.165
μ^{famine}	h^{-1}	0.008	0.004	0.052
$\mu^{famine, anoxic}$	h^{-1}	-	0.006	0.024
$\mu^{famine, aerobic}$	h^{-1}	-	0.003	0.003
$\mu^{feast}/\mu^{overall}$	-	5.900	2.900	1.499
$\mu^{famine}/\mu^{overall}$	-	0.700	0.600	0.472
$\mu^{famine, anoxic}/\mu^{overall}$	-	-	0.900	0.218
$\mu^{famine, aerobic}/\mu^{overall}$	-	-	0.400	0.031
μ^{feast}/μ^{famine}		8.400	4.600	3.172

Based on HRT (the study used HRT = SRT)

In general, both anoxic and aerobic are appropriate to accumulate PHA inside the cells. On the change from anoxic to aerobic growth conditions, the microorganisms present in the medium have very slow adaptability to aerobic environment especially under long lag phases. This can be understood that DO seemed to act as an inhibitor on the activity of the denitrifying reductases rather than as a repressor of their synthesis (Kornaros *et al.*, 1996). On the other hand, as reported by Kornaros *et al.* (1996) and Kornaros and Lybertos (1996), the changes from aerobic to anoxic conditions could be adapted fast with exhibiting full denitrifying activity and no lag phases. This, however, depends on the cultures and substrates that have been used. The anoxic utilization of substrate (TOC removal) indicated that the constant rate determines the proportion of substrate of electron. Then, it will be passed to the terminal electron acceptor of nitrate (reducing it to dinitrogen gas), and the proportion that are used in the synthesis of new cell mass (Saito *et al*., 2004). Since prolonged anoxic period is determined as 'negative' effect to the system, the proposed ratio anoxic-to-aerobic is 3:7.

Figure 4.10 shows the profiles of PHA and CDW of the microaerophilic reactor with N & P limitations. As shown in the figure, PHA production increased

rapidly the first 30 hours after N & P were eliminated from the feed. The production of PHA was observed during steady-state of microaerophilic phase. It was shown that the PHA accumulation under 70% Microaerophilic continued to increase and reached the maximum content of 70%/CDW, 50 hours after N & P were eliminated. At the same cultivation period, the PHA production declined from 40 to only 30% under 20% Microaerophilic. As mentioned previously, the reduction of PHA content will significantly reduce the efficiency of COD removal from solution. This observation is different from what is observed in typical biological nutrient removal (BNR) systems. PHA production in a BNR system is observed during the anaerobic phase where most of the COD is removed from solution, while PHA consumption occurs in the aerobic phase when it is used as a source of carbon and energy for biomass growth and polyphosphate storage. After PHA content reached the maximum percentage value of 48%/CDW, it then decreased simultaneously with the biomass concentration.

In contrast to typical BNR systems, when PHA production was maximized using microaerophilic/aerobic cycling with N & P limitation, most of the COD was consumed during the aerobic period, i.e., COD consumption during the microaerophilic period was negligible (Punrattanasin *et al*., 2001). Reduction of soluble COD was not observed after 30 hours without N & P addition, and COD during the aerobic period was higher than the influent concentration. This is expected since N & P are essential nutrients required for cellular growth by all living organisms. The results also suggest that the commercially developed PHA production strategy of first developing a culture without nutritional limitations, and then subjecting it to one or more limitations for a short period before harvesting, is most likely to be a successful strategy for PHA production utilizing activated sludge.

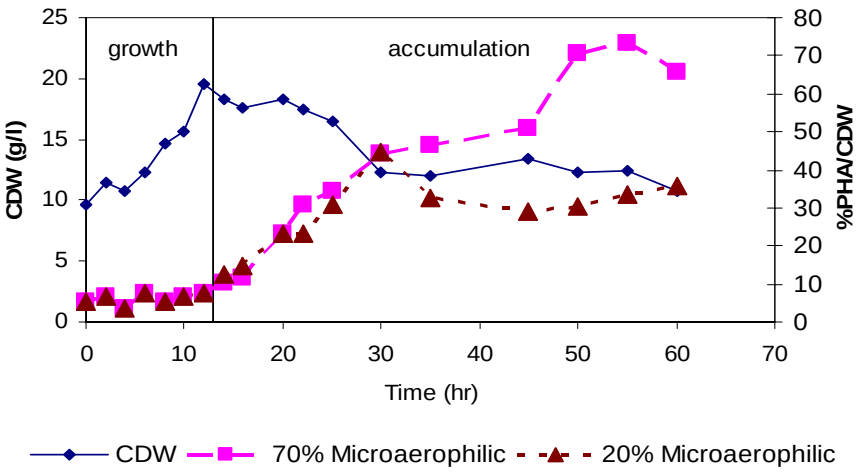


Figure 4.10: Changes of PHA production and CDW at 70% and 20% of microaerophilic conditions

As a conclusion, the study from Du *et al.* (2000) found that the limitation of oxygen could also result in the accumulation of acetyl-CoA and a low intracellular concentration of free CoASH (co-enzymes that are important to built the hydroxybutyric acid polymer). The increase of the acetyl-CoA/CoASH ratio partially relieved the inhibition of β-ketothiolase, which favours the formation of PHA. PHA concentration and content increased rapidly at the early stage of oxygen limitation, then the rising tendency slowly (as shown at 20% of microaerophilic experiment). Comparing with previous works, the PHA production could reach 80% (Du *et al*., 2001; Satoh *et al*., 1998). However, in this study, the PHA productions only reach 74% (see Table 4.7). This indicated that the oxygen limitation is more advantageous in accumulation PHA, but the final PHA production could not reach more than 80% of dried biomass.

4.6 Comparative Study

The statement proved to be the most successful strategy for PHA production using simpler modification of cultivation (i.e. use high organic COD substrate with lower N and P concentrations). The combination of N and P limitations applied for

PHA production must depend on the type of microorganisms. If application of nutrient limitation is extended for a long period, cells are not able to accumulate much polymer even if the cell contents are initiated at high concentration. The comparisons of production rate in some studies are shown in Figure 4.11.

In three of these references, (Majone *et al*., 1998; Krishna and van Loosdrecht, 1999a; Beun *et al*., 2000a) experiments were done with pulse feeding of acetate, resulting in a maximum specific acetate uptake rate. In the experiments of Krishna (Krishna and van Loosdrecht, 1999a) acetate was added continuously during 55 min, followed by a period of 185 min with no external substrate available. In this way, the specific acetate uptake rate was not at maximum rate. The other two references (Carta-Escobar *et al*., 2002; Beun *et al*., 2002) experiments were done with 5 – 8 times higher than a normal pulse, which result in a higher specific acetate uptake rate. Therefore, their specific production rate sharply increased 3 – 4 times higher than normal pulse. This figure also shows that in all these references, the ratio $q_p^{feast}/-q_s^{feast}$ is a constant and has a value of 0.6 C-mol/C-mol. This value (from selected references) is lower than the value found in this study (0.9 C-mol/C-mol), which have been used as comparative study. Even though the specific uptake and production rate are not higher than obtained in the selected references, the overall ratio of $q_p^{feast}/-q_s^{feast}$ indicated that mixed substrate (POME) was more useful than single component (e.g. acetate).

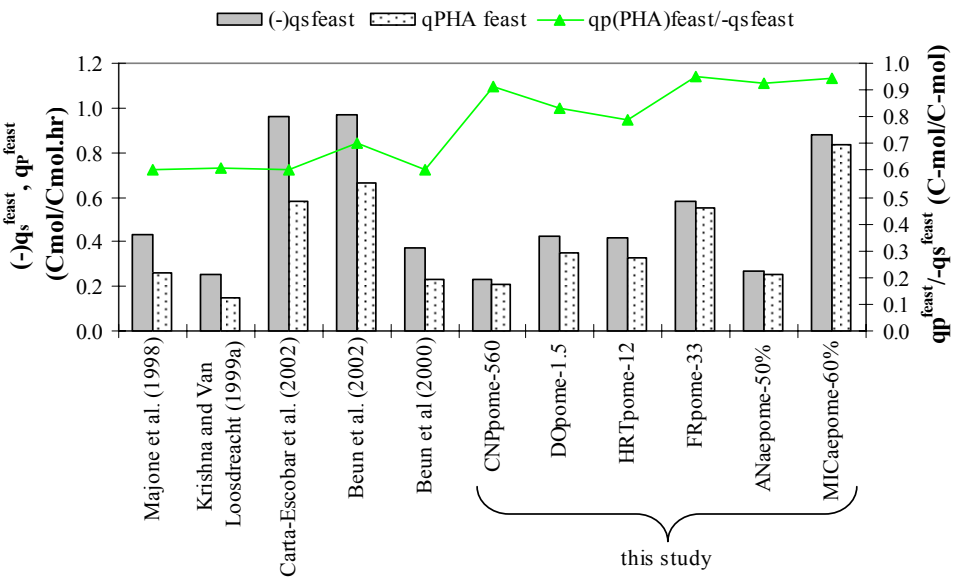


Figure 4.11: Comparison findings for specific substrate consumption ($-q_s^{feast}$) and specific (q_p^{feast}) with concerning to this study

CHAPTER V

RESULTS AND DISCUSSION: DESIGN OF PHA PRODUCTION, ORGANIC AND NUTRIENT REMOVAL IN POME USING RESPONSE SURFACE METHOD (RSM)

5.1 Introduction

The primary goal of designing an experiment statistically is to obtain valid results with minimum of effort, time and resources. In addition, it will help in optimizing the variable parameters (known as scale-up) obtained from single-dependent factor (defined as scale-down) in Chapter IV. The flow behaviour and the optimization of biological reactions in the reactor are issues associated with the scale-up of a bioreactor. Although there have been many studies on the flow behaviour in different types of bioreactors, knowledge about the behaviour of the microorganisms in mixed cultures is limited.

In this study, the experimental design was evaluated using single observation obtained from optimum Δf_{PHA} as shown in Chapter IV. Since the anoxic and microaerophilic conditions are difficult to control in dynamic mixed cultures, the RSM was only performed on four main variables (CODNP ratio, Air, HRT=SRT and feeding rate). Using POME as the model compound, a systematic study which includes operating and utilization of statistical experimental design (SED) have been used. It was conducted to determine the effect of those variables on the PHA process efficiency. The factors of SED were obtained in several ranges, which are:

(a) CODNP ratio experiment (ranged from 400 – 600 g COD/g N, with COD/P range fixed between 150 – 200 g COD/g P),

(b) Air flowrate experiment (ranged from 0.5 – 2.0 l/min),

(c) HRT=SRT experiment (represent for cycle time, ranged from 12 – 26 hours), and

(d) Feeding substrate rate (FR) experiment (ranged from 20 – 50 ml/min).

In addition, the correlations between limiting N and P, Air flowrate, cycle time and feeding substrate rate in influencing the process was explored. The details of each part will be discussed in the following sections followed by results and discussions. In this study, environmental variables such as pH value, temperature, medium composition and the intermittent phase were not included in the experimental variables since it was widely studied (Krishna and van Loosdrecht, 1999a, 1999b; Beun *et al.*, 2002; Aulenta *et al.*, 2002, Angenent *et al.*, 2004; Chinwetkitvanich *et al.*, 2004; Domenek *et al.*, 2004). In many cases, these environmental variables should be considered simultaneously in the fermentation system.

The operations include the determination of the effect of the operational variables and the variation of the variables. In the present study, the response surface is employed to determine the effect of the environmental variables on the cultivation. If the environmental variables have no significant effect on the objective function, such as specific growth rate or productivity, the response surface gives a flat curve with respect to the variables. A wide range of the flat curve implies that the environmental variable can be controlled within the desired range in the large-scale reactor. On the other hand, if the environmental variables significantly affect the cultivation outside of a small range, this means the state variables in the large-scale reactor cannot be manipulated within the required range. It is then necessary to proceed to the second step. The flow chart for this evaluation is shown in Figure 5.1.

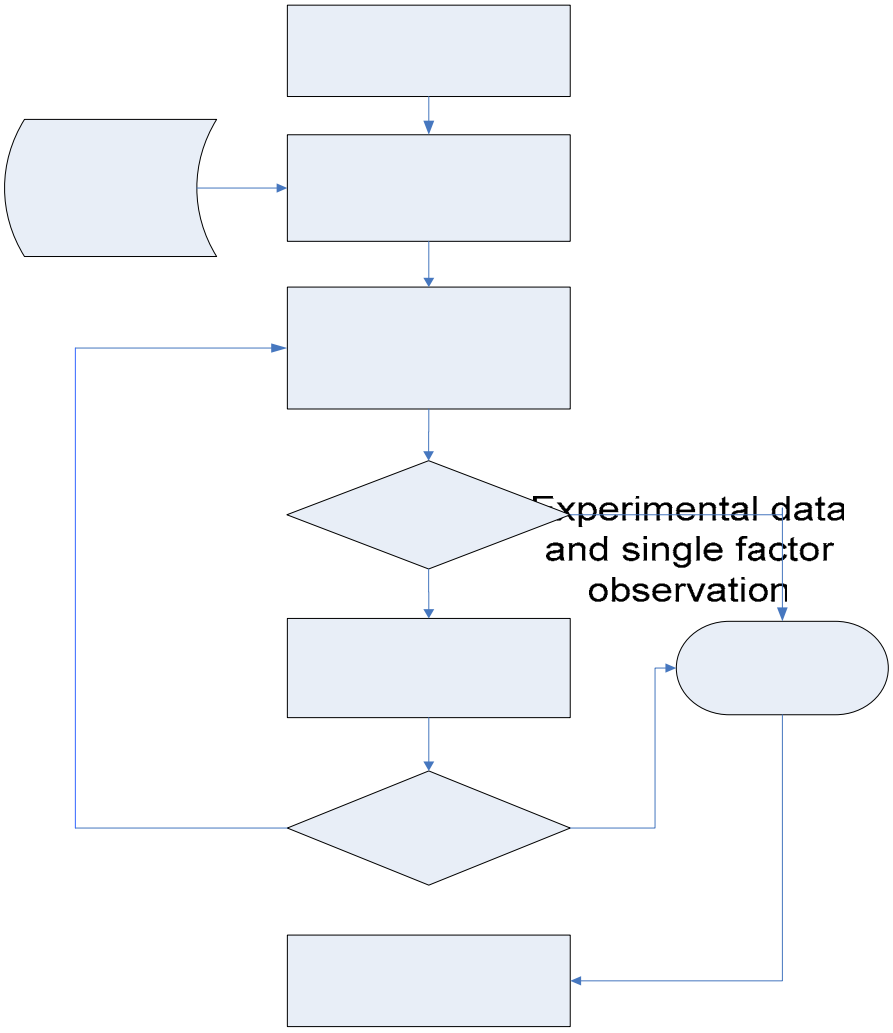


Figure 5.1: The flow chart of the statistical design process using MINITAB™

The experimental work for this study was designed statistically using response surface method (RSM), which is a common and powerful approach for optimizing a multivariate system (Myers and Montgomery, 2002). The RSM has been successfully applied to various bioprocesses (Dey *et al.*, 2001; Lee and Chen, 1997; Waiter *et al.*, 1996; Hsiun, 1995). In particular, Central Composite Rotatable Design (CCRD) was applied using MINITAB™ (version 13.2) statistical software. The CCRD enables the significant factor(s) and interactive factors to be identified quantitatively. CCRDs are often recommended when the design plan calls for sequential experimentation because these designs can incorporate information from a properly planned factorial experiment. Typically, a CCRD is employed for fitting

NO

the second-order polynomial. An empirical equation in the form of a second-order polynomial is obtained with specified and limited data.

The interactions between key factors as well as curvature effects are taken into account and quantified using RSM. In addition, the design facilitates the detection of non-linear behaviour of the effect and the determination of the best setting of the experimental factors that produce the maximum outcome. The advantage of this design over the traditional one-factor-at-a-time (OFAT) approach is that the CCRD enables the significant factor(s) and interactive factors to be identified quantitatively. While it covers the same experimental variables in a matrix design used in the preliminary study, the CCRD requires less number of experiments and hence is more economical. A set of 30 runs were carried out at different CODNP ratio, air flowrate, HRT=SRT and feeding substrate rate (shown in Table 5.1 and Table 5.2).

As shown in Table 5.1, the design of code levels was carried out in an experimental composite design. Since the independent variables were varied in four factors, some of the dependent variables (ranges of code levels) showed a negative value. Therefore, the study was resetting from 'cube' points to the 'axial' points. These circumstances will be elaborated in the next paragraph. As a result, the final dependent variables for COD:N:P ratio, air flowrate, cycle length, and feeding rate are 400 – 600 g COD/g N (150 – 300 g COD/g P), 0.5 – 2 l/min, 12 – 26 h, 20 – 50 ml/min, respectively. All of the experiments were conducted in 8 hours length, except for cycle length study (HRT).

Table 5.1: The variables and their levels for the CCRD experimental design

Independent variables	Symbol	Code levels				
		-ω	-1	0	+1	+ω
(COD:N/COD:P) (wt/wt)	A	400/150	450/200	500/220	550/250	600/300
Air flowrate (l/min)	B	0.500	0.875	1.250	1.625	2.000
Cycle length, HRT (h)	C	12.0	15.5	19.0	22.5	26.0
Feeding rate, FR (ml/min)	D	20.0	27.5	35.0	42.5	50.0

Table 5.2: Experimental runs conducted in dynamic aerobic study (data shown was not in random order)

Run Code[a]	Limiting on N and P ($CODNP_{pome}$) [ ][b]		Controlling Air flowrate, l/min (Air_{pome}) [ ][b]		Cycle length period, hour ($HRT=SRT_{pome}$) [ ][b]		Feeding substrate rate, ml/min (FR_{pome}) [ ][b]	
1	550/250	[+1]	0.875	[-1]	15.5	[-1]	27.5	[-1]
2	450/200	[-1]	1.625	[+1]	15.5	[-1]	27.5	[-1]
3	450/200	[-1]	0.875	[-1]	22.5	[+1]	27.5	[-1]
4	550/250	[+1]	1.625	[+1]	22.5	[+1]	27.5	[-1]
5	450/200	[-1]	0.875	[-1]	15.5	[-1]	42.5	[+1]
6	550/250	[+1]	1.625	[+1]	15.5	[-1]	42.5	[+1]
7	550/250	[+1]	0.875	[-1]	22.5	[+1]	42.5	[+1]
8	450/200	[-1]	1.625	[+1]	22.5	[+1]	42.5	[+1]
9	500/220	[0]	1.250	[0]	19.0	[0]	35.0	[0]
10	500/220	[0]	1.250	[0]	19.0	[0]	35.0	[0]
11	450/200	[-1]	0.875	[-1]	15.5	[-1]	27.5	[-1]
12	550/250	[+1]	1.625	[+1]	15.5	[-1]	27.5	[-1]
13	550/250	[+1]	0.875	[-1]	22.5	[+1]	27.5	[-1]
14	450/200	[-1]	1.625	[+1]	22.5	[+1]	27.5	[-1]
15	550/250	[+1]	0.875	[-1]	15.5	[-1]	42.5	[+1]
16	450/200	[-1]	1.625	[+1]	15.5	[-1]	42.5	[+1]
17	450/200	[-1]	0.875	[-1]	22.5	[+1]	42.5	[+1]
18	550/250	[+1]	1.625	[+1]	22.5	[+1]	42.5	[+1]
19	500/220	[0]	1.250	[0]	19.0	[0]	35.0	[0]
20	500/220	[0]	1.250	[0]	19.0	[0]	35.0	[0]
21	400/150	[-2]	1.250	[0]	19.0	[0]	35.0	[0]
22	600/300	[+2]	1.250	[0]	19.0	[0]	35.0	[0]
23	500/220	[0]	0.500	[-2]	19.0	[0]	35.0	[0]
24	500/220	[0]	2.000	[+2]	19.0	[0]	35.0	[0]
25	500/220	[0]	1.250	[0]	12.0	[-2]	35.0	[0]
26	500/220	[0]	1.250	[0]	26.0	[+2]	35.0	[0]
27	500/220	[0]	1.250	[0]	19.0	[0]	20.0	[-2]
28	500/220	[0]	1.250	[0]	19.0	[0]	50.0	[+2]
29	500/220	[0]	1.250	[0]	19.0	[0]	35.0	[0]
30	500/220	[0]	1.250	[0]	19.0	[0]	35.0	[0]

Note: [a] Based on standard order designed by CCRD

[b][ ] – Coded value as assigned by CCRD

As shown in Table 5.2, the values entered for CCRD factor levels are not the minimum and maximum values in the design (in some cases). They are the low and high settings for the "cube" portion of the design. The axial points are usually outside the "cube" (unless the value is less than or equal to 1). If not, this could lead

to axial points that are not in the region of interest or may be impossible to run. Choosing axial points in the "Factors" subdialog box guarantees all of the design points will fall between the defined minimum and maximum value for the factor(s). In drawing up the experiments, it is a common practice to designate the factor levels by some code instead of writing the actual value of variable (Myers and Montgomery, 2002; Montgomery, 2001). For two-level experiments, they are usually coded as "–1" and "+1" or "-" and "+". When the number of experiments become too large, the number of trials can be reduced to ½, ¼ or so forth of the full number; this design is termed as fractional-factorial. MINITAB™ will then determine the appropriate low and high settings for the "cube" as follows:

$$\text{Low Level Setting} = \frac{(\Psi - 1)\max + (\Psi + 1)\min}{2 * \Psi} \qquad \text{(Eq. 6.1)}$$

$$\text{High Level Setting} = \frac{(\Psi - 1)\min + (\Psi + 1)\max}{2 * \Psi} \qquad \text{(Eq. 6.2)}$$

As shown in Table 5.2, the experiments were divided into three parts: a 2^4 Hadamard matrix factorial run (1 to 8 and 11 to 18), star point runs (21 to 28) and centre point runs (9 to 10, 19 to 20 and 29 to 30). The effect and synergistic effect of the factor(s) were determined based on Hadamard matrix and centre point runs, while the non-linear response behaviour was analyzed using 'star point' and 'centre point' runs. The centre point run was also repeated six times in order to allow better estimate of the experimental error. The runs were carried out in a randomized order. This particularly reduces the effect of time-dependent factors that are not included in the study. Similar to factorial design, the experiments in CCRD need to be conducted in a randomised order to reduce the effect of bias due to the unintended factors.

5.2 Diagnostic Model

Before accepting any model, the adequacy of the adopted model should be analyzed by an appropriate statistical method. The analysis of RSD is to fit a model of the experimental data. Removing observations can affect the variance estimate and also can affect parameter estimates. A large absolute studentized residual (deleted residuals) may indicate that including the observation in the model increases the error variance or that it has a large effect upon the parameter estimates, or both. The major diagnostic method is residual (observed minus predicted) analysis as shown in Figure 5.2, providing diagnostic for residual behaviour. There are several residuals graphs to test the model assumptions. The predictive model used to generate response surface graphs and contour plots contains equation for describing linear and quadratic effects of the process and interaction between process factors and the response (e.g. %PHA, %TOC, %PO_4 and %NO_3). MINITAB^{TM} divides the data into intervals represented as bars in a high-resolution histogram, as asterisks in a character histogram. As depicted in the Figure 5.2(a-d), the response model for PHA production was fixed to the normal distribution. The next analysis is to look at the deleted residuals versus the fitted value (Figure 5.2(b)). There should be no systematic pattern in the plot. The points should fall within a horizontal band centered at zero. Departure from this may suggest a violation of the constant variance assumption. The size of deleted residuals should be independent of its fitted value, which means that the spread should be about the same across all levels of the fitted values. Deleted residuals versus observation order (number) graphs reveal any time-based affects or sequential component (Figure 5.2(c)).

The positive effect of PHA production in four variables was quantified through Figure 6.2 (b – d). A pictorial representation of the effect is shown in Figure 6.2(a) where the highest frequency obtained at -0.5 of deleted residual, which means in the normal ranges (-2 to 2). However, since one of the deleted residual is outside from that range, the ANOVA analysis has been carried out.

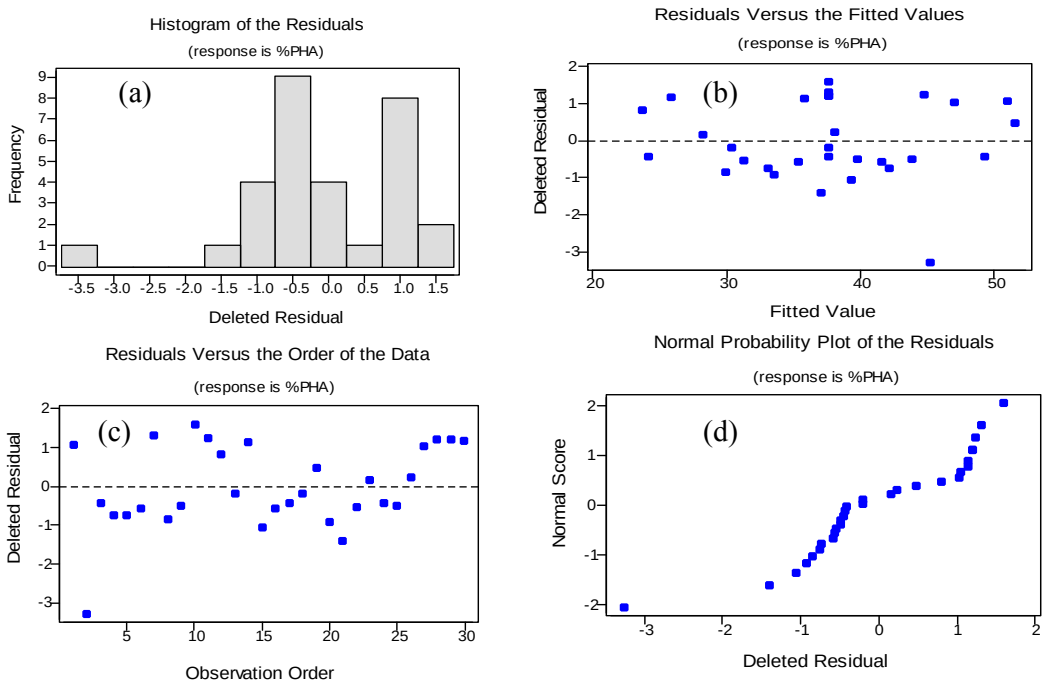


Figure 5.2: Residual diagnostics of response model for %PHA: (a) histogram, (b) normal probability, (c) deleted residual vs. observation order, (d) deleted residual vs. fitted value.

The ANOVA analysis was carried out to obtain the significant correlation. One of the tools is variance inflation factor (VIF). The VIF is used to detect whether one predictor has a strong linear association with the remaining predictors (e.g. PHA production to TOC removal). VIF measures how much the variance of an estimated regression coefficient increases if the predictors (e.g. ratio of carbon, cycle length and etc.) are correlated (multicollinear). If the VIF is equal 1, the predictor indicates no relation; whereas if the VIF calculated more than 1, the response and predictor have a relation (Montgomery and Peck, 1982). The largest VIF among all predictors is often used as an indicator of severe multicollinearity. Montgomery and Peck (1982) suggest that when VIF is greater than 5-10, the regression coefficients are poorly estimated. Since the results from Table 5.3 did not show any relation between predictors, therefore, multiple regressions have been computed, afterwards (model diagnostic). However, the computed VIF value significantly indicated the non-severely multicollinear analysis (without bias results).

Table 5.3: ANOVA and regression analysis for selected responses

Response	Predictor / Analysis	Constant	Factor			
			A	B	C	D
PHA Production	Coefficient	24.66	0.078	-6.294	0.071	0.236
	SE Coeff.	17.90	0.026	3.899	0.557	0.097
	T	1.38	3.00	-1.61	0.13	-2.42
	p	0.18	0.006	0.119	0.900	0.023
	VIF	1.00	1.00	1.00	1.00	1.00
	Durbin-Watson statistic = 1.55, F = 4.37					
	p = 0.008, R-sq = 41.2%, R-sq (adj) = 31.8%					
TOC Removal	Coefficient	-1.74	0.03	-0.34	-0.03	-0.03
	SE Coeff.	8.121	0.012	1.769	0.253	0.044
	T	-0.21	2.42	-0.19	-0.10	-0.62
	p	0.832	0.023	0.849	0.923	0.538
	VIF	1.00	1.00	1.00	1.00	1.00
	Durbin-Watson statistic = 1.72, F = 1.57					
	p = 0.213, R-sq = 20.1%, R-sq (adj) = 7.3%					
NO_3-N Removal	Coefficient	10.00	0.007	-4.068	-0.148	-0.038
	SE Coeff.	8.139	0.012	1.773	0.253	0.044
	T	1.23	0.62	-2.29	-0.58	-0.87
	p	0.231	0.543	0.030	0.566	0.395
	VIF	-	1.00	1.00	1.00	1.00
	Durbin-Watson statistic = 1.75, F = 1.68					
	p = 0.186, R-sq = 21.2%, R-sq (adj) = 8.6%					
PO_4-P Removal	Coefficient	0.244	-0.027	1.549	0.302	0.087
	SE Coeff.	7.987	0.012	1.740	0.249	0.044
	T	0.03	-2.30	0.89	1.21	1.99
	p	0.976	0.030	0.382	0.236	0.057
	VIF	-	1.00	1.00	1.00	1.00
	Durbin-Watson statistic = 2.14, F = 2.88					
	p = 0.043, R-sq = 31.5%, R-sq (adj) = 20.6%					

With regard to PHA production and PO_4-P removal, four effects had *p*-values of less than 0.05 (Table 5.3), indicating that they were significantly different from zero at the 95% confidence level. However, three of those responses (TOC and NO_3) were detected as no significant effect ($p > 0.05$).

Considering the *F*-ratio statistic (Table 5.3), it was concluded that the PHA production (F = 4.37) could cause the major variation in the model analysis. This was because PHA was currently the main contribution in the feast period. The effect of TOC removal was not statistically significant in the study as the degradation of TOC is quite slow in the feast period.

The Durbin-Watson (DW) statistic tests for the presence of autocorrelation in regression residuals by determining whether or not the correlation between two adjacent error terms is zero. The DW statistic tested the residuals to determine if there was any significant correlation based on the order in which they occurred in the data file. Due to the fact that the DW value exceeded 1.4 (Table 5.3), there was probably no significant autocorrelation in the residuals.

The R^2 statistic (as shown in Table 5.3) indicated that the model could only reached as high as 41.3% of the variability in PHA production. Then, the adjusted R^2 statistic, which is more suitable for comparing models with different numbers of independent variables, was only 31.8%. Therefore, the model was unable to predict the interaction effect of carbon ratio, Air flowrate, cycle length and feeding rate was unable to predict. As a conclusion, the study suggested the residual diagnostic to evaluate the predictor of PHA production, TOC removal, NO_3-N removal and PO_4-P removal.

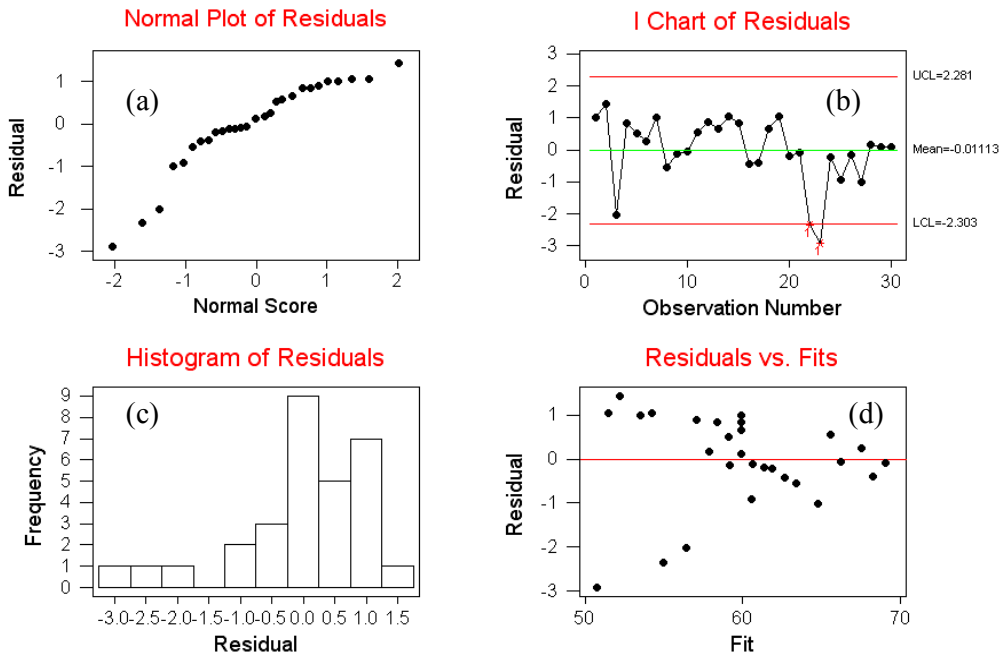


Figure 5.3: Residual diagnostic model for %PHA in four variables (CODNP ratio, Air flowrate, HRT=SRT and feeding rate): (a) normal plot distribution, (b) I-Chart for single observation, (c) histogram pattern, (d) fitted trend for predicted value.

Figure 5.3 showed the residuals plots procedure, which can generate four plots in the best two-predictor model (e.g. PHA production,). This multi regression analysis can provide the best predictive model in one regression analysis. In general, the normal plot (Figure 5.3(a)) shows an approximately linear pattern that is relatively consistent with a normal distribution (-2 to 2). Similarly, the histogram exhibits a pattern that is consistent with a sample from a normal distribution. However, the I Chart, depicted in Figure 5.3(b), illustrates a control chart of individual observations. It reveals that one point labelled with number 1 (at 22 and 23 observation number) is outside the three sigma limits (UCL, Mean and LCL). This flagged point indicated that the values are not significantly used for further analysis. Residual versus fit graph (Figure 5.3(d)) display the real response data plotted against the fitted responses. Points above 2 or below -2 the mean areas of over or under fitted. There were no significant violations of the model assumptions found in this residual analysis except two outlier, as shown previously in Figure 5.3(b). This design point seems to be due to measurement error rather than random experimental error. The plot of residuals versus fits shows that the fit tends to be better for intermediate predicted values (55 to 65). The scattered point means that the trend of fit is nearly optimize in the factor of four variables.

The results suggested that only carbon ratio and AIR flowrate have significant effects to the particular responses (e.g. %PHA, %TOC removal, etc.). From the analysis of the data in Table 5.3 by the least squares method, the following second-order model was fitted. Since the regression analysis did not show a correct evaluation (especially $\%PO_4$ and $\%NO_3$), the RSM was conducted then. The regression equation for this model for %PHA, %TOC removal, $\%PO_4$ removal and $\%NO_3$ removal, are:

$$\%\,\mathrm{PHA} = 67.2 + 0.0489\,\mathrm{CODNP} - 12.2\,\mathrm{Air} \qquad \text{(Eq. 5.3)}$$

$$\%\,\mathrm{TOC}_{\mathrm{removal}} = 16.4 + 0.0224\,\mathrm{CODNP} - 4.54\,\mathrm{Air} \qquad \text{(Eq. 5.4)}$$

$$\%\,\mathrm{PO4}_{\mathrm{removal}} = -29.1 + 7.59\,\mathrm{Air} \qquad \text{(Eq. 5.5)}$$

$$\%\,\mathrm{NO3}_{\mathrm{removal}} = -6.5 + 0.0447\,\mathrm{CODNP} \qquad \text{(Eq. 5.6)}$$

5.3 Prediction of PHA Production, TOC, NO_3 and PO_4 Removal

5.3.1 Response Surface Analysis

Response surface analysis was carried out in this study to determine the second-order behaviour of the factor(s), to model the relationship between the factors and the response, as well as the factor settings that produce the best response (Minitab, 2000). The predictive model used to generate response surface graphs and contour plots contains equations for linear, interaction and quadratic processes of factors. When the problem involves the data that are subjected to experimental errors, statistical methods measure the effects of change in operating variables and their mutual interactions on the process performance through factorial experimental designs.

The data collected from the batch runs were used to develop empirical models describing the experimental results. The models were generated using the method of least squares. The technique involves the estimation of model parameters for the second order models of the form (Montgomery, 2001). By statistical analysis, a mathematical model was obtained, showing the significant importance, which fitted in coded factor space (2, 2) as follow:

$$E(Y) = \beta_i + \sum_{i=1}^{k} \beta_i \, X_i + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} \, X_i \, X_j \qquad \text{(Eq. 5.7)}$$

where $E(Y)$ is the expected value of the response variable, i, j are the model parameters, X_i and X_j are the coded factors being studied and k is the number of factors being studied.

If the environmental variables have no significant effect on the objective function, such as PHA production or productivity level, the response surface has a flat zone with respect to the variables. A wide range of the flat curve implies that the environmental variable can be controlled within the desired range in the large-scale

bioreactor. On the other hand, if the operational variables have no significant interaction in regression analysis (e.g. $p > 0.1$), this means that the state variables in the large-scale bioreactor could not proceed in the selected range.

Table 5.4: Results of the regression analysis of the CCRD

Term	% PHA production		%TOC removal		$\%PO_4$ removal		$\%NO_3$ removal	
	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value
Constant	-861.896	0.000	-356.315	0.000	561.714	0.000	-574.108	0.002
A	1.308	0.063	0.218	0.096	-0.809	0.358	0.627	0.005
B	173.412	0.002	56.182	0.016	-94.604	0.002	-12.176	0.518
C	37.673	0.773	17.121	0.731	-10.836	0.574	40.097	0.275
D	12.340	0.424	10.498	0.282	-17.824	0.169	5.165	0.761
A^2	-0.001	0.099	-0.0002	0.429	0.0003	0.228	-0.0004	0.138
B^2	-12.656	0.140	-1.370	0.749	5.711	0.266	-5.841	0.219
C^2	-0.482	0.679	-0.218	0.717	-0.259	0.714	-0.969	0.151
D^2	0.029	0.877	-0.061	0.529	0.039	0.734	-0.063	0.547
AB	-0.106	0.205	-0.006	0.893	0.002	0.971	-0.029	0.530
AC	-0.007	0.816	0.003	0.867	0.009	0.645	-0.008	0.624
AD	-0.008	0.520	-0.002	0.781	0.012	0.133	0.0001	0.977
BC	-3.968	0.335	-1.850	0.384	3.090	0.222	1.515	0.508
BD	-1.191	0.466	-0.847	0.321	1.256	0.215	0.506	0.579
CD	-0.487	0.428	-0.323	0.313	0.499	0.191	-0.148	0.665
Lack-of-fit*	$p = 0.008$		$p = 0.138$		$p = 0.000$		$p = 0.000$	

Note: 0.01 – 0.04: Highly significant; 0.05 – 0.1: significant; 0.1 – 0.2: less significant; > 0.2: insignificant (Source: Vecchio, 1997), * calculated for Table 6.6

The experimental results of PHA production by a complete four-factor-two-level factorial experiment design with six replications of the central point and eight axial points are shown previously in Table 5.2. The responses of the CCRD design were fitted with a second-order polynomial equation (Eq. (6.8)). Table 5.4 illustrates the final constant values for model analysis. Except for the linear term, either A (variable for COD:N:P ratio) or B (variable for air flowrate) ($p < 0.05$), none of the other (linear, quadratic and interaction terms) were statistically significant. The final second-order polynomial equation, for PHA production, organic and nutrient removals after omitting *p*-value, are:

$$\% \text{ PHA} = -861.896 + 1.308A + 173.412B - 0.001A^2 \quad \text{(Eq. 5.8)}$$

$$\% \text{ TOC} = -356.313 + 218A + 56.182B \quad \text{(Eq. 5.9)}$$

$$\%PO_4 = 561.714 - 94.604B \quad \text{(Eq. 5.10)}$$

$$\%NO_3 = -574.108 + 0.627A \quad \text{(Eq. 5.11)}$$

In order to prove this second-order polynomial equation, the response surface analysis has been shown in Table 5.5 (full results in Appendix E1-E4). The statistical significance of the model equation was evaluated by F-test for analysis of variance (ANOVA), which showed that the regression is statistically significant at 95% ($p < 0.05$) confidence level. The model F-value of 1.50 (in linear regression) for PHA production (taken as one example) implies that the model is statistically significant (DF > F). The *p*-value must be higher than F-value, however, that was not showed in this study. In addition, the coefficient of determination (R^2) was calculated to be 0.769, indicating that the model could only achieve 76.9% of the variability. The ''lack of fit tests'' compares the residual error to the ''Pure Error'' from replicated design points. The ''lack of fit F-value'' of 0.008 (for %PHA) implies the lack of fit is insignificant ($F < \alpha$), since the α is initially set at 2.0. However, with inconsistent variability of F and *p*-value, this implies that the predictive model is not statistically correct and that the process appears insignificant to model. There is only 0.8% (LOFT = 0.008) chance that a ''lack of fit F-value, LOFT'' could occur due to variation of variables. As a result, the estimated models, which fit the experimental data, are only available in small-scale reactions.

Table 5.5: Summary of ANOVA in response surface regression

Response/Source	Regression						Residual error
	linear		square		interaction		LOFT
	F	*p*	F	*p*	F	*p*	
% PHA Production (R^2 = 76.9%)	1.50	0.215	1.30	0.351	0.74	0.624	0.008
% TOC removal (R^2 = 51.4%)	0.74	0.581	0.25	0.905	0.51	0.790	0.138
% NO_3-N removal (R2 = 65.9%)	2.00	0.146	0.76	0.567	1.32	0.308	0.000
% PO_4-P removal (R2 = 56.1%)	0.91	0.483	1.25	0.331	0.27	0.941	0.000

Three-dimensional response surfaces were plotted on the basis of the model equation, to investigate the interaction among the variables and to determine the optimum concentration of each factor for maximum PHA production (together with percentage of TOC, PO_4 and NO_3 removal) by mixed cultures. Since no interaction

between HRT and feeding rates, terms connected with HRT and FR were fixed at 18 hours and 20 ml/min, respectively. The effects of varying the COD:N:P ratio (and other variables) are shown previously in Figures 5.4 to 5.7, which demonstrates that the response surfaces for the three combinations are similar to %PHA, %TOC and $\%NO_3$. However, the response surface for $\%PO_4$ contradicted those three responses. A small trend in the response surface indicates an initial increase in PO_4 removal with the increasing of air flowrate (1.5 – 2.0 l/min), then, a steep decrement occurred from 0 to -15% in PO_4 removal when the COD:N:P ratio increase from 400 to 600 g COD/g N (150 – 300 g COD/g P)

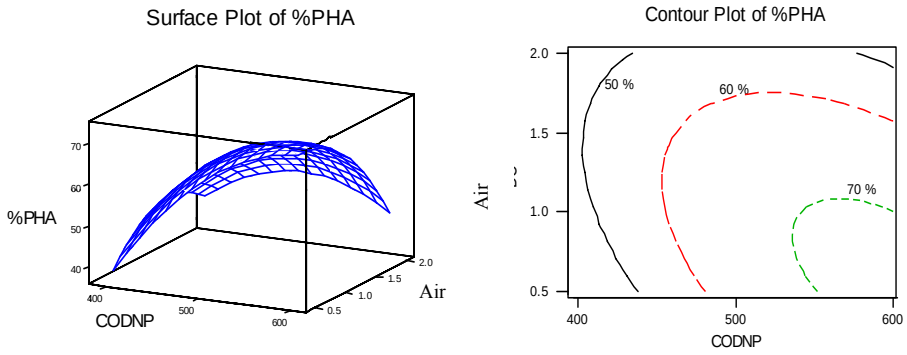


Figure 5.4: Response surface plot showing variation in prediction PHA production

The interactive effect of carbon, nitrogen and phosphorus concentrations on PHA production is clearly revealed in Figure 5.4. At the lowest ratio of COD:N: P (less than 450 g COD/g N and 200 g COD/g P), the increment rate of oxygen supply had little effect on PHA production, organic and nutrient removal for above 1 l/min. Meanwhile, the ratio above 550 g COD/g N (oxygen supply is less than 1 l/min), most of the production (PHA) and reduction (TOC and NO_3) efficiency were performed at high percentage. The variance analysis suggested that there is no significant effect of HRT and substrate feeding rate on the PHA production. The principal factor that influenced PHA accumulation was the concentration of oxygen in the gas phase. The PHA concentration increased with decreasing amounts of oxygen. The results clearly suggested that oxygen limitation has an important role in

PHA production and dissolved oxygen in the cultivation broth should be controlled for attaining a high productivity of PHA.

Figure 5.5 shows the same trend as PHA production. However, the removal efficiency was only reached from 19 - 21% at range of COD:N:P ratio and air flowrate, 500 – 600 g COD/g N (220 – 300 g COD/g P) and 0.5 – 1.5 l/min, respectively. The results show that the increment of neither carbon ratio nor air flowrate did not encourage the organic removal during feast period. A high carbon ratio and air flowrate will slightly reduce the TOC removal. However, the removal efficiency may increase if the cycle length is expanded more than 9 hours. As a result, this will significantly influence the biomass concentration and then, it will cause a significant reduction of PHA

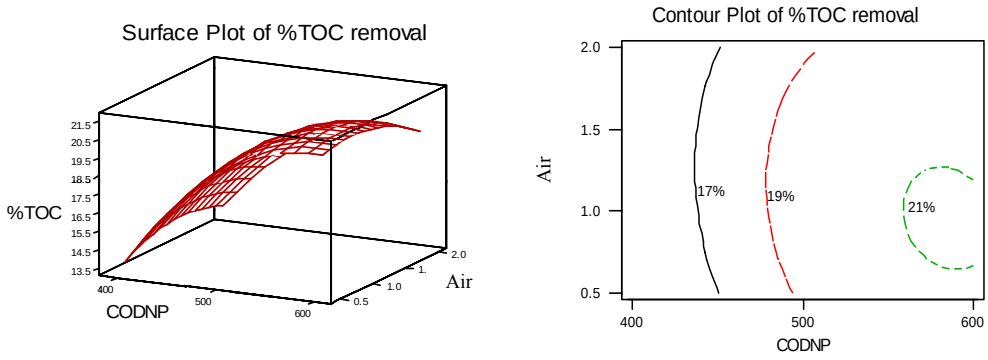


Figure 5.5: Response surface plot showing variation in prediction of TOC removal

As discussed in Chapter V, the PO_4 concentration will be higher than initial value during the peak of PHA production. Therefore, this study was focussed mainly on minimizing these effects. As shown in Figure 5.6, the problem will be eliminated when the carbon ratio is less than 500 g COD/g N (or 220 g COD/g P). On the other hands, the air flowrate was not a major influence to the PO_4 removal. However, when the air flowrate increased from 1.5 – 2 l/min, the non-PO_4 removal efficiency will occur.

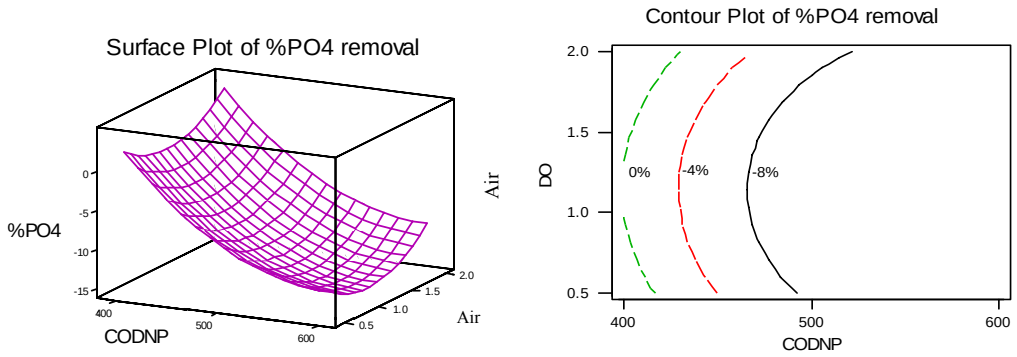


Figure 5.6: Response surface plot showing variation in prediction of PO_4 removal

The analysis of NO_3 removal also contributed significantly to this study. The fundamental studies have revealed that NO_3 could also contribute to PHA production (as elaborated in Chapter V in anoxic/aerobic discussions). Therefore, the response of NO_3 removal must be similar as obtained in PHA production. As shown in Figure 5.7, the optimum NO_3 removal was observed at 550 g COD/g N (250 g COD/g P) and AIR flowrate less than 1 l/min. When the carbon ratio and AIR flowrate increased, the removal efficiency will slightly fall. The same trend will be also observed if these two influence factors operated at low ratio and flowrate.

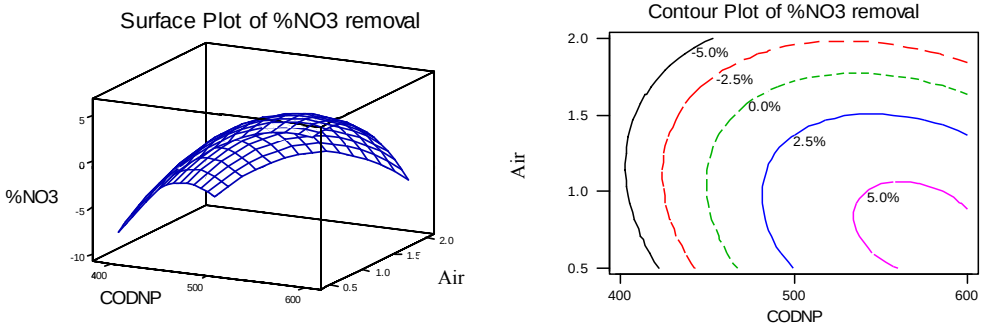


Figure 5.7: Response surface plot showing variation in prediction of NO_3 removal.

The analysis from response surface analysis was initially carried out using full quadratic terms (i.e. linear, interaction and squares). The results which consist of estimated regression coefficients and ANOVA tables are calculated in Table 5.6. Based on the *p*-value (less than 0.2) for PHA production, the results indicate that only linear terms are significant, while the square terms are considered insignificant. The R-squared value of the model is acceptable (79.6%) but the *p*-value of the lack of fit test (LOFT) (0.008) indicates its significance. This implies that the predictive knowledge of the model could not model in linear interactions.

Table 5.6: Statistical analysis of different factors used in the optimization study for the PHA production, organic and nutrient removal in the reactor

Responses	Adjusted Sum of square	R-squared	Adjusted Mean square	F-value	*p*-value
% PHA production	214.86	79.6%	53.715	1.50	0.251
% TOC removal	28.207	59.4%	7.052	0.74	0.581
% PO_4 removal	106.130	61.2%	26.532	2.00	0.146
% NO_3 removal	40.886	64.3%	10.222	0.91	0.483

5.3.2 Optimization Analysis

Many designed experiments have been applied to optimize the conditions that will produce the 'best' value for 'response-variables' analysis. Depending on the design type (e.g. mathematical design), the operating conditions that could control may include one or more of the following design variables: factor, component, process variables, or amount variables. Optimal settings of the design variables for one response may be far from optimal or even physically impossible for another response. Response optimization is a method that allows for compromise among the various responses. This will allow to show statistical effects and the dynamic nature of the process simultaneously from the single design of experiment.

As shown in Tables 5.7 and 5.8, the numerical optimization can be represented by a general non-linear algorithm with constraints applied to the main objective function. This is a desirability function for multiple responses. In numerical optimization, the desired goals (constraints) for each response and factor, such as maximize, minimize, target, within range, etc. were selected along with weight and importance that can be assigned to each goal. A weight for each goal can adjust the shape of the desirability. The importance of each goal can change the relation to other goal. The goals are combined into an overall desirability function, which is an objective function of optimization with its outcome ranging from zero (beyond the goal limits) to one (matching to the exact goal).

Tables 5.7 and 5.8 introduce constraints for the responses and factors, and optimal points based on the desirability function. The propagation of error (POE) method makes the production process insensitive to variations in input factors. Point prediction is used to make predictions for responses at any factor combination (COD:N:P ratio, AIR, HRT, FR). As shown in Table 5.8, the desirability value indicated that the predicted point is accepted. The overall desirability (D) is a measure of how well the variable is satisfied for the combined goals (in all of the responses). Overall desirability has a range of zero to one. One represents the ideal case; zero indicates that one or more responses are outside their acceptable limits.

Table 5.7: Numerical optimization for factorial design with the POE

Responses	Goal	Lower limit	Target	Upper limit	Weight	Importance
% PHA production	Target	60	66	70	1	3
% TOC removal	Target	15	19	22	1	3
% PO_4 removal	Minimize	-10	-10	3	1	3
% NO_3 removal	Target	-10	3.5	5	1	3

Table 5.8: Numerical optimization with the overall predicted and desirability obtained from response optimizer with the POE.

Responses	Predicted	Desirability	Starting point	Global solution	Composite desirability
% PHA production	66.29	0.927	CODNP ratio = 600 g COD/g N and 200 g COD/g P, air = 0.5 l/min, HRT = 18 hours, FR = 20 ml/min	CODNP ratio = 509 g COD/g N and 200 g COD/g P, air = 0.59 l/min, HRT = 20 hours, FR = 20 ml/min	0.921
% TOC removal	19.39	0.870			
% PO_4 removal	-9.24	0.942			
% NO_3 removal	2.80	0.948			

From the response optimizer (with POE), the calculated maximum (or optimum) value of PHA production (with 19.39% of TOC and 2.80% of NO_3 removal) was 66.29 ± 0.35% (95% confidence interval) for the following culture conditions: 0.59 l/min air flowrate, with HRT = 20 hours and substrate feeding rate is 20 ml/min. The maximum calculated value of PHA production (Eq. 6.8) was closed to the experimental maximum obtained in run 30, as shown previously in Table 5.2. The optimal response factor levels for PHA differed from those for the organic removal, as is generally the case for secondary metabolites.

This application of the POE method to the process modeling searches for the compositions of mixtures that minimize variation in the response, creating formulae robust to variation in the input values. Uses of the POE begin with construction of the RSM, and information about the standard deviation should be prepared. Then, a RSM graph of the factor variation transmitted to the selected response. Ultimately, optimal factor settings can be detected that get the selected response on target with minimal variation by employing multiple-response numerical optimization, with setting the goal for POE to minimize, as shown in Tables 5.7 and 5.8.

In order to analyze the main interaction effect, the graphic analysis on this optimization is shown in Figure 5.8. This optimization plot allows user to interactively change the input variable settings to perform sensitivity analyses and possibly improve the initial solution (e.g. COD:N:P ratio, Air flowrate, etc.). The

local desirability (d) in this study was obtained at range 0.86 – 0.95, which indicates that the prediction values of PHA, TOC, NO_3 and PO_4 acceptable. The figure also shows that the carbon ratio was the preAirminant effect to achieve the main objective of this study. Therefore, the curve line has a sharp shape compared to other influence factors. As a conclusion, carbon ratio must be well controlled in order to reach both PHA production and nutrient removal. At the same time, the study also suggests that the air flowrate, HRT and FR should be operated at 0.59 l/min, 20 h and 22 ml/min, respectively.

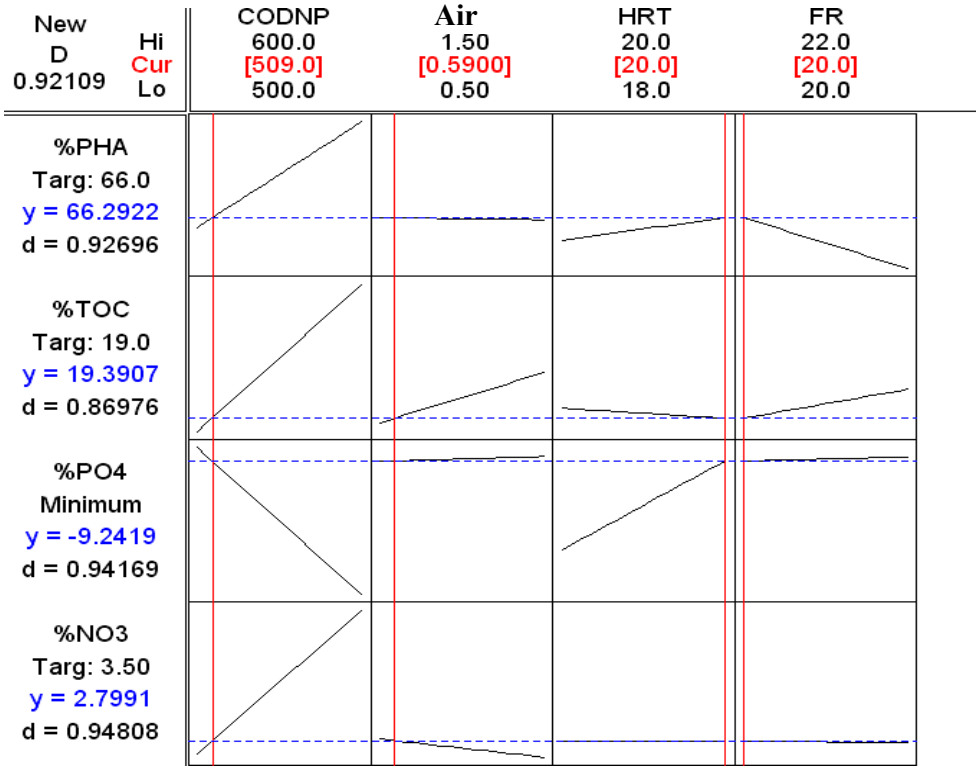


Figure 5.8: Response optimizer for best factor-response analysis

Further verification experiment with this optimal formulation is not performed since this formulation was for the robust process under the assumed optimized condition and no higher results were expected from further experimental design. The POE method makes the production process insensitive to the variation in inputs factors. The POE method needs calculations of partial derivatives to find broad flat areas (high or low plateau) on the response surface and to generate POE plots that show how that error is transmitted to the response as introduced in Figures

5.4 to 5.7. Then, conditions (factor settings) were searched to minimize the transmitted variation, which produce a process robust to control these factors. However, POE will be varied to the measured response to differing degrees only when the response surface is non-linear. Therefore, POE is available only for second order or higher model.

The goal of robust process is to find the most stable region of product and quality, with consideration of efficient process range (Cornell, 2002; Myers and Montgomery, 2002). Such a desirable operating region can be obtained by searching for response surface or more accurately by using a mathematical method (calculus) to minimize propagation of error (POE or response variation) from varying input. However, this study will only performed using MINITAB^{TM} as tool for minimizing the POE.

5.3.3 Overall Analysis

Based on a statistical design, an optimal medium has been developed for maximizing the production of PHA with an efficient organic and nutrient removal. Since the cultivation was irrelevant for PO_4 removal (non-removal efficiency), the study focussed mainly in minimizing the effects.

The result from Pareto chart (Figure 5.9) suggested that attaining a high production of PHA required a limiting air flowrate and high COD:N:P ratio. Considering this, a second set of experiments was carried out under selected range of air (0.5 – 1.5 l/min). The concentrations of carbon, nitrogen and phosphate sources were varied in a narrow interval around the central composite optimum in a 16 factorial experimental design. The PHA production (%PHA) were sharply reduced at limiting COD:N:P and/or air flowrate in comparison with the 2-level factorial optimal process. The effect of oxygen limitation on morphology, growth and product formation by microorganisms has been reported in chapter IV and V. When harvesting a high PHA production during feast period, COD:N:P ratio and air

flowrate gave significant effect to TOC and NO_3 removal. Therefore, it can be concluded that NO_3 concentration was also influential on PHA production instead of the degradation of organic constituents. Nitrate and nitrite will replace oxygen for microbial respiration. Denitrification requires an organic compound as carbon and energy source. If denitrifiers are subjected to dynamic conditions with respect to the availability of nitrate and substrate, storage polymers like PHB can be formed (Van Loosdrecht *et al.,* 1997)

In view of these results, as no increase in CODNP ratio in the new experimental range of variables, the conditions found for the maximum production and removal (%PHA, %TOC, $\%PO_4$ and $\%NO_3$) in the 2-level factorial design experiment were optimal. Thus, a culture medium for maximizing PHA production should contain 450 – 500 g COD/g N, 180-200 as g COD/g P and air = 0.5 – 0.6 l/min or 5 – 6.2% DO saturation. A high transfer rate of oxygen should be maintained in the bioreactor during feast period for better storage capacity. An independent experiment was used to verify the optimum conditions identified in the 2-level factorial experiment. The verification consisted of triplicate runs comparing the control bioprocess (i.e. original non-optimal medium) and the optimized growth medium with 80% air in the gas phase (at least 10 l/min). After seven days of culture the biomass concentrations obtained were 10% higher than single factor in the optimized conditions. The biomass concentrations successfully enhanced the PHA content in their cells (increase up to 60% of total dry weight) after reconfiguring the operational condition.

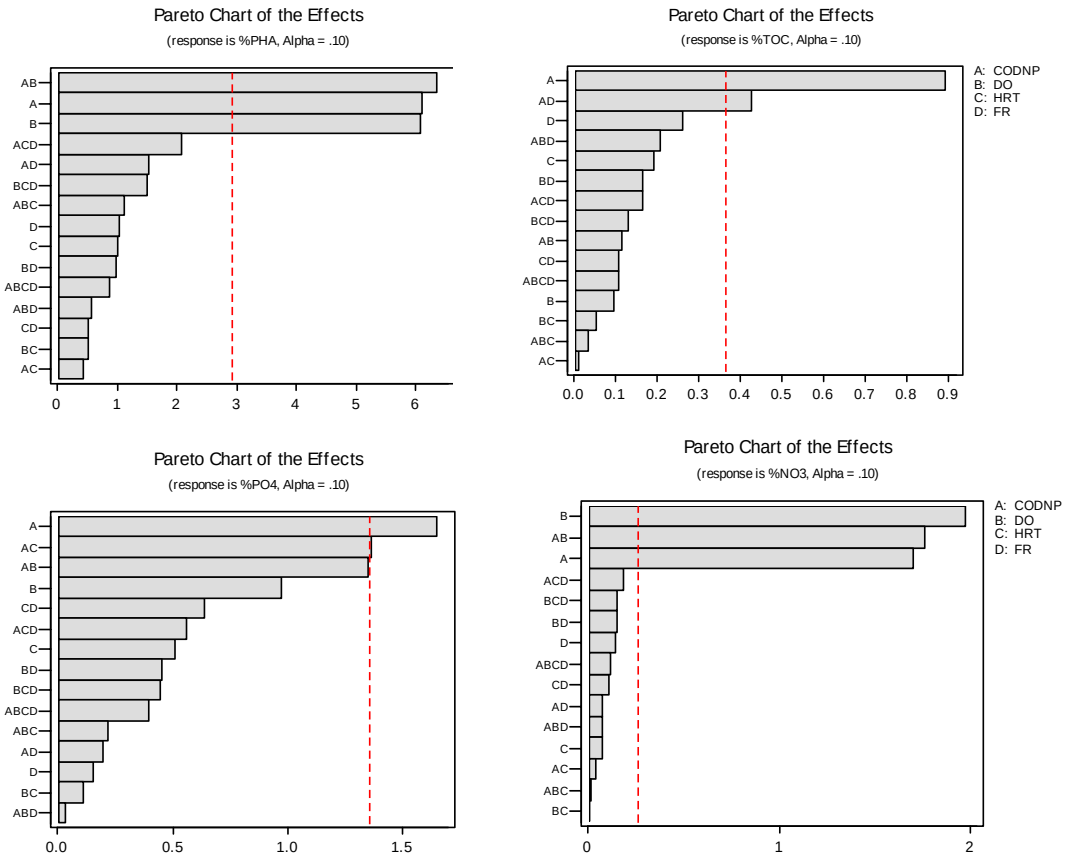


Figure 5.9: Pareto chart for PHA production, organic and nutrient removal at different variables (α: 0.1; A: CODNP; B: Air; C: HRT; D: FR). Line of significance is depicted as dotted line and determined by MINITAB™

The best correlation between variables were used in this study is depicted in Figure 5.10. HRT and feeding substrate rate (FR) were confirmed not the main effect for the cultivation. Since the inhibition of substrate has been minimized in the beginning of the experiment (e.g. dilution factor), the feeding regime becomes less significant. Meanwhile, since the HRT configuration allows fast uptake rate of substrate, the cultivation period must be operated at least in 10 hour length. The period must be well controlled because most of the cultures tend to "lose" their storage ability (PHA production and reduction of organic components) above 10 hours.

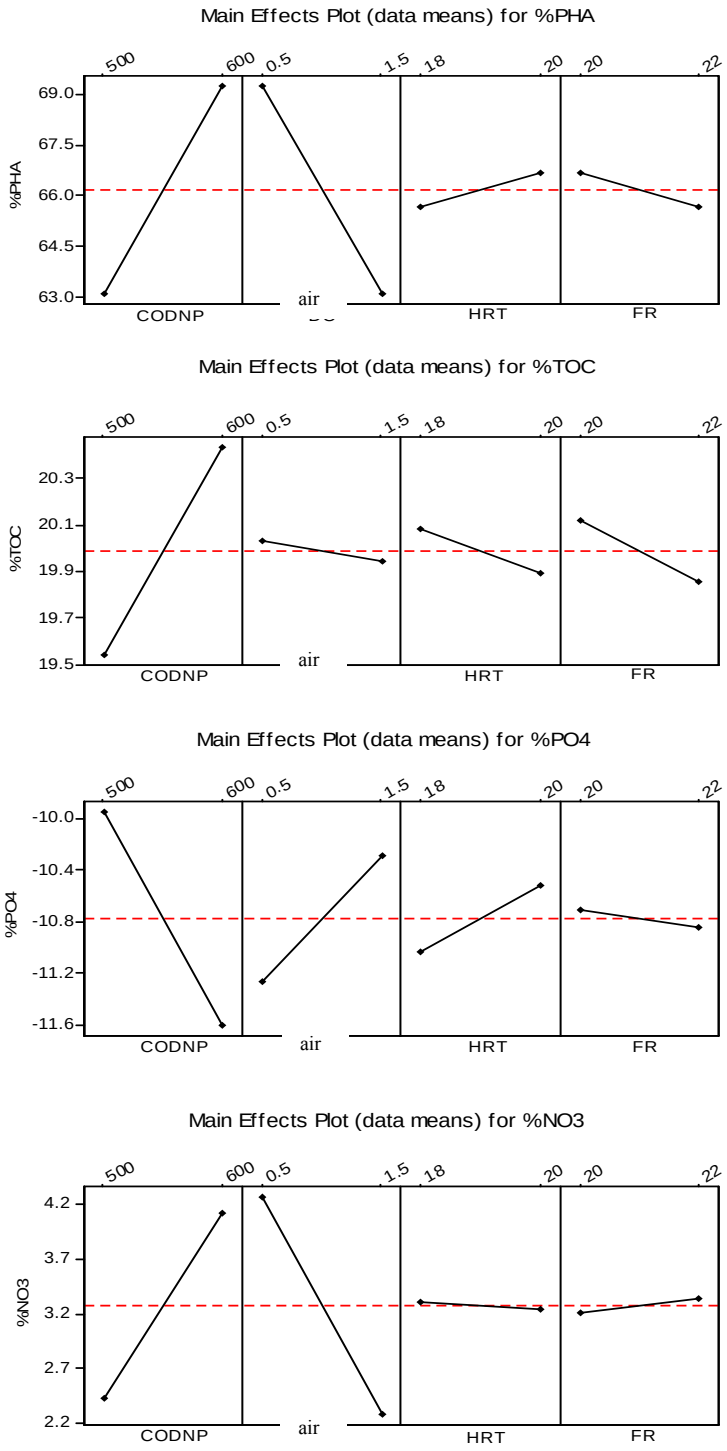


Figure 5.10: Main effects plot for PHA production, TOC, PO_4 and NO_3 removal.

CHAPTER VI

CONCLUSION, SUGGESTION AND RECOMMENDATION

6.1 General Observations

Study on fatty acid components from agricultural waste (POME), the storage capacity (PHA accumulation) was discussed as well in different favourable conditions. Typically storage of PHA has been conducted in single fed-batch experiments, where a limitation of electron acceptor (O_2,) and also nutrient (N,P) concentrations are exposed to the system. The microorganisms living in the system experienced famine situations, where practically no exogenous substrate (rbCOD or S_s) is present for growth or even energy-maintenance. However, the activated sludge population also experienced the opposite condition, known as feast periods with plenty of substrates. This dynamic substrate conditions found in wastewater will lead to a situation where bacteria capable of substrate storage are the fittest for survival. This is based on the assumption that the stored material will allow the bacteria to obtain a more balanced growth when living under both feast and famine conditions.

All of the systems were conducted under high concentration of substrate with low amounts of nutrients present in the medium. The biomass will be adapted to the variation of influent COD-load during feast and famine conditions. This will lead to a selection of PHA-producer. The initial concentration of PHA in activated sludge in systems feed was 0.2 – 5% under normal operation conditions for POME experiments. The concentration of PHA in the latter system was lowered by five factors including the dilution of biomass by inert material incorporated into the activatedsludgefloc.Several studies supported the theory of the strong influence of growth environment on the

kinetics and on the population dynamics of activated sludge. There are some findings to be concerned, which are:

(i) PHB is the most dominant storage polymer as it is directly formed from the central metabolite acetyl-CoA. Only it is recently that it became generally accepted that PHB plays an important role in especially sequentially fed activated sludge processes. The recovery of PHB from activated sludge was also conducted to harvest a quality of bioplastic material from sludge. However, the purity of PHB obtained from the sludge only achieve as high as 80%. Therefore, further improvement must be developed to obtain PHB at high concentration (> 80%) without serious degradation of the PHB (Section 4.7, Chapter IV).

(ii) The main energy and carbon source comes from readily biodegradable substrate (S_s), slowly biodegradable substrates (X_s) and their hydrolysis products. However, the inconsistent PHA storage will strictly inhibit the ability of storage capacity (X_{STO}) (e.g. Section 4.1 and 4.3.2, Chapter IV).

(iii) In cultivation systems with high concentration gradients of soluble substrates (e.g. VFAs), a high oxygen uptake rate does not support the high growth and substrate consumption, as should happen in balanced growth conditions. The unbalanced growth will be beneficial for PHB/PHA production but the crucial time is their harvesting period (Section 4.3.3, Chapter IV).

(iv) Biodegradable plastics, especially PHB, continue to make progress in both commercial and scientific field. However, the problems of brittleness, low mechanical strength and high production have been improved by using other constituents such as HV and HH monomers. These constituents are easily converted from raw organic waste especially from LCFA or SCFA source (Table 4.5, Chapter IV).

6.2 Conclusion of This Study

(a) The production rate of PHA under feast-famine regime occurred in a fast period between 3 – 4 hours during the phase of uptake rate. Therefore, a short chain fatty acid (especially acetic acid) is always the first to be utilized for PHA production. PHA production is growth associated mechanisms. Degradation of the formed PHA commences with the onset of the stationary phase; hence, timing the harvest is essential to prevent loss of the produced PHA.

(b) Cycling of microaerophilic-aerobic condition influenced the PHA production. Meanwhile, the cycle of anoxic/aerobic condition could also increase the PHA productivity and content. However, since both operations are difficult to control, the combination of N, P, DO flowrate and cycle length could also contribute to enhance PHA production.

(c) PHA production would not necessarily improve TOC, PO_4-P and NO_3-N removal. The study found that TOC removal varied between 18 – 33% under high PHA production. The inconsistent removal and non-removal efficiencies (in PO_4-P and NO_3-N) were detected in some experiments, but it can be improved under anoxic/aerobic condition.

(d) Both COD:N:P ratio and DO flowrate have significant influence on PHA production, TOC, and NO_3-N removal. This was confirmed by advanced statistical analysis, called response surface method (RSM).

6.3 Recommendations and Future Studies

Biodegradable plastics, such as PHB, continue to make progress in both the commercial and scientific fields. However, their use as a replacement for conventional plastics for a wide range of applications has been hindered by their brittleness, low mechanical strength and high production cost. There seems to be two challenging

subjects for industrial production of microbial PHA, especially copolymers containing 3HB as major constituent. One is to gain cost competitiveness of PHA against petrochemical- based common polymers such as polyethylene (PE), polypropylene (PP) and polystyrene (PS). Further studies should be made in order to increase the robust process of PHA production, which are:

(a) The current advances in metabolic engineering supported by the genome information and bioinformatics have opened a cascade of opportunities to introduce new metabolic pathways. This would help not only to broaden the utilizable substrate range and produce tailor-made PHA but also enhance the current PHA yields.

(b) Much more effort is required in this area (cultivation conditions) to increase the production of bioplastics to successfully replace the non-degradable plastics. Thus, the future of bioplastics depends on the efforts towards fulfilling requirements of price and performance. More importantly, this high bioplastics is comparable with the polymer content obtained in pure cultures, which demonstrates that the organic wastes can be utilized and recycled for valuable products.

(c) A desired system may consist of two separated bioreactors to meet the needs of different physiologies and metabolic activities of two types of microbial populations, one for anaerobic acidogenesis of organic wastes and another for enriched culture of PHA-producing species. Two types of system (hybrid) should consider as well in order to meet the effluent quality (organic and nutrient removal). The favourable condition could be continuously adapted in between of the system and this will reduce the other influence factors (for example, HRT/SRT, diffusion rate and harvesting time).

REFERENCE

Abou-Zeid, D.M., Müller, R.J. and Deckwer, W.-D. (2001). Degradation of Natural and Synthetic Polyesters under Anaerobic Conditions. *J. Biotechnol.* **86**(2): 113-126.

Activated sludge models ASM1, ASM2, ASM2d and ASM3. IWA Task Group on Mathematical Modeling for Design and Operation of Biological Wastewater Treatment. London, UK: IWA Publishing; 2000.

Ahn, W.S., Park, S.J. and Lee, S.Y. (2000). Production Of Poly(3-Hydroxybutyrate) by Fed-Batch Culture of Recombinant *Escherichia Coli* with a Highly Concentrated Whey Solution. *Appl. Env. Microbiol*. **66**(8): 3624 – 3627

Ahn., J., Daidou, T., Tsuneda, S. and Hirata, A. (2001). Metabolic Behavior of Denitrifying Phosphate-Accumulating Organisms under Nitrate and Nitrite Electron Acceptor Conditions. *J. Biosci. Bioeng*. **92**(5): 442-446.

Al-Awadhi, N., Egli, T., Hmer, G. and Mason, C.A. (1990). The Process Utility of Thermotolerant Methylotrophic Bacteria: An Evaluation in Chemostat Culture. *Biotechnol. Bioeng*. **38**: 816-820.

Ali, M. F and Siddiqui, M. N (2005). Thermal and Catalytic Decomposition Behavior of PVC Mixed Plastic Waste with Petroleum Residue. *Journal of Analytical and Pyrolysis*. **74**(1-2): 282-289

Amass, W., Amass, A. and Tighe, B. (1998). Featured Article: A Review Of Biodegradable Polymers: Uses, Current Developments In The Synthesis And Characterization Of Biodegradable Polyesters, Blends Of Biodegradable Polymers And Recent Advances In Biodegradation Studies. *Polym. Intern*. **47**: 89-144.

Anderson, A.J., Dawes, E.A. (1990). Occurence, Metabolism, Metabolic Rate, and Industrial Uses of Bacterial Polyhydroxyalkanoates. *Microbiol Rev*. **54**(4): 450 - 472.

Anderson, A. J., Williams, D. R., Taidi, B., Dawes, E.A., and Ewing, D. F. (1992). Studies on Copolyester Synthesis By *Rhodococcus Ruber* and Factors Influencing The Molecular Mass of Polyhydroxybutyrate Accumulated by *Methylobacterium Extorquens* And *Alcaligenes Eutrophus*. *FEMS Microbiology Reviews*. **130**: 93-102

Angenent, L. T., Karim, K., Al-Dahhan, M. H., Wrenn, B. A. and Domı´guez-Espinosa, D. (2004). Production of Biochemicals and Bioenergy from Industrial Agricultural Wastewater. *Trends in Biotechnol*. **22**(9): 477 - 485

APHA, AWWA and WPCF (1995) *Standard Methods of the Examination of Water and Wastewater*. 19th ed. American Public Health Association, Washington DC.

Aulenta, F., Bassani, C., Ligthart, J., Majone, M. and Tilche, A. (2002). Calorimetry: A Tool Assessing Microbial Activity under Aerobic and Anoxic Conditions. *Wat. Res*. **36**: 1297 – 1305.

Barak, P., Coquet, Y., Halbach, T.R. and Moline, J.A.E. (1991). Organic Chemicals in the Environment: Biodegradability of Polyhydroxybutyrate(Co-Hydroxyvalerate) and Starch-Incorporated Polyethylene Plastic Films in Soils. *J. Environ. Qual*. **20**: 173 – 179.

Beatons, D., Vanrolleghem, P.A., van Loosdrecht, M.C.M. and Hosten, L.H. (1999). Temperature Effects in Bio-P Removal. *Wat. Sci. Tech.* **39**(1): 215 – 225.

Beccari, M., D. Dionisi, Giuliani, A., Majone, M. and Ramadori, R. (2002). Effect of Different Carbon Sources on Aerobic Storage by Activated Sludge. *Wat. Sci. Tech.* **45**(6): 157-168.

Beccari, M., Majone, M., Massanisso, P. and Ramadori, R. (1998). A Bulking Sludge with High Storage Response Selected under Intermittent Feeding. *Wat. Res.* **32**: 3403-3413.

Beun, J.J., Dirks, K., van Loosdrecht, M.C.M. and Heijnen, J.J. (2002). Poly-(β–Hydroxybutyrate) Metabolism in Dynamically Fed Mixed Microbial Cultures. *Wat Res*. **36**: 1167-1180.

Beun, J.J., Paletta, F., van Loosdrecht, M.C.M. and Heijnen, J.J. (2000a) Stoichiometry and Kinetics of Polyhydroxybutyrate Metabolism in Aerobic, Slow Growing, Activated Sludge Cultures. *Biotechnol. Bioeng*. **67**: 379-389.

Beun, J.J., Paletta, F., van Loosdrecht, M.C.M. and Heijnen, J.J. (2000b) Stoichiometry and Kinetics of Polyhydroxybutyrate Metabolism under Denitrifying Conditions in Activated Sludge Cultures. *Biotech. Bioeng*. **68**: 496-507.

Bichler, C., Drittler, R., Langowski, H.-C. Starnecker, A. and Utz, H. (1993). Biologisch Abbaubare Verpackungsmaterialien- Die Lösung Des Müllproblems? *Bioengineering*. **9**(6): 9-17.

Bitton, G. (1994). *Wastewater Microbiology*. Third Avenue, New York: Willey-Liss Inc..

Blaylock, M. (2002). *Bioconversion of Nonylphenol Ethoxylate to Poly(3-Hydroxybutyrate) by Ralstonia eutropha and Others PHA-producing Bacteria*. Howard University: PhD Thesis.

Bond, P.L., Erhart, R., Wagner, M., Keller, J. and Blackall, L.L. (1999). Identification of Some of the Major Groups of Bacteria in Efficient and Nonefficient Biological Phosphorous Removal. *App. Environ. Microbiol*. **65**: 4077-4084.

Bonting, C., van Veen, H., Taverne, A., Kortstee, G., and Zehnder, A.J.B. (1992). Regulation of Polyphosphate Metabolism in *Acinetobacter johnsonii* 210a Grown in Carbon- and Phosphate-Limited Continuous Culture. *Arc. Microbiol*. **158**: 139-144.

Booma, M., Selke, S. E., and Giacin, J. R. (1994). Degradable Plastics. *Journal of Elastomers and Plastics*. **26**: 104-142.

Bourque, D., Pomerleau, Y., and Groleau, D. (1995). High-Cell-Density Production of Poly-β-hydroxybutyrate (PHB) from Methanol by *Methylobacterium extorquens*: Production of High-Molecular-Mass PHB. *Appl. Microbiol. Biotechnol*. **44**: 367-376.

Bourque, Ouellette, B., Andre, G., and Groleau, D. (1992). Production of Poly-β-hydroxybutyrate from Methanol: Characterization of a New Isolate of *Methylobacterium extorquens*. *Appl. Microbiol. Biotechnol*. **37**: 7-12.

Brandl, H., Gross, R.A., Lenz, R.W. and Fuller, R.C. (1988). *Pseudomonas Oleovorans* as a Source of Poly(β-Hydroxyalkanoates) for Potential Applications as Biodegradable Plastics. *Appl. Environ. Microbiol*. **54**(8): 1977 – 1982

Braunegg, G., Bona, R., Schellauf, F., Wallner, E. (2002). Polyhydroxyalkanoates (PHAs): Sustainable Biopolyester Production. *Polimery*. **47**(7-8): 479-484.

Braunegg, G., Lefebvre, G. and Genser, K.F. (1998). Polyhydroxyalkanoates, Biopolyesters From Renewable Resources: Physiological And Engineering Aspects. *Journal of Biotechnology*. **65**: 127-161.

Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, C.M., Alaerts, G.J. and Heijnen, J.J. (1997). Temperature Effects on Physiology of Biological Phosphorus Removal. *J. Env. Eng*. **123**(2): 144-153.

Brdjanovic, D., Logemann, S., van Loosdrecht, M. C. M., Hooijmans, C. M., Alaerts, G. J. and Heijnen, J. J. (1998). Influence of Temperature on Biological Phosphorus Removal: Process and Molecular Ecological Studies. *Water Res.* **32**(4): 1035-1048.

Bylund, F., Castan, A., Mikkola, R., Veide, A., Larsson, G. (2000). Influence of Scale-Up on the Quality of Recombinant Human Growth Hormone. *Biotechnol. Bioeng*. **69**(2): 119–128.

Byrom, D. (1987). Polymer Synthesis By Microorganisms: Technology And Economics. *Trends Biotechnol*. **5**: 246-250.

Byrom, D. (1990). Industrial production of Copolymer from *Alcaligenes eutrophus*. In: *Novel Biodegradable Microbial Polymers*, Dawes, E. A. (ed.), Kluwer Academic Publishers: The Netherlands. 113-117.

Byrom, D. (1992). Production of PHB: Poly-β-hydroxyvalerate Copolymers. *FEMS Microbiology Reviews*. **103**: 247-250.

Byrom, D. (1994). Polyhydroxyalkanoates, In D.P. Mobley (ed.), *Plastics from Microbes: Microbial Synthesis Synthesis of Polymers and Polymer Precursors*. Hanser/Gardner Publication: New York. 5-33

Caballero, K.P., Karel, S.F., Register, R.A. (1995). Biosynthesis and Characterization of Hyroxybutyrate – Hydroxycaproate Copolymers. *Int. J. Biol. Maromol*. **17**(2): 86 – 92.

Cain, R.B. (1992). Microbial Degradation Of Synthetic Polymers. In: Frey, J.C. (eds). *Microbial control of pollution. 48th symposium of the society for general microbiology held at the University of Cardiff.* Bath Press, Bath: Avon, UK. 293-338.

Calmond-Decriaud, A., Bellon-Maurel, V. and Silvestre, F. (1998). Standard Methods for Testing the Aerobic Biodegradation of Polymeric Materials. Review and Perspectives. *Adv. Polym. Sci*. **135**: 207-226.

Carta, F., Beun, J. J., van Loosdrecht, M. C. M. and Heijnen, J. J. (2001). Simultaneous Storage and Degradation of PHB and Glycogen in Activated Sludge Cultures. *Water Res.* **35**(11): 2693-2701.

Carucci, A., Dionisi, D., Majone, M., Rolle, E. and Smurra, P. (2001). Aerobic Storage by Activated Sludge on Real Wastewater. *Wat. Res*. **35**(16): 3833-3844.

Chau H and Yu P.H.F. (1999). Production of Biodegradable Plastics from Chemical Wastewater: A Treatment. *Wat Sci Tech*. **39**: 273-280.

Chech, J.S. and Hartman, P. (1993). Competition between Polyphosphate and Polysaccharide Accumulating Bacteria in Enhanced Biological Phosphate Removal Systems. *Wat. Res*. **27**: 1219-1225.

Choi, E., Rhu, D., Yun, Z. and Lee, E. (1998). Temperature Effects on Biological Nutrient Removal System with Municipal Wastewater. *Wat. Sci. Technol*. **37**(9): 219 - 226.

Choi, J. I. and Lee, S. Y. (1999). Production of Poly(3-Hydroxybutyrate) P(3HB) with High P(3HB) Content by Recombinant *Escherichia coli* Harboring the *Alcaligenes latus* P(3HB) Biosynthesis Genes and the E-Coli Ftsz Gene. *J. Microbiol. Biotechnol.* **9**(6): 722-725.

Choi, J and Lee, S.Y. (1997). Process Analysis and Economic Evaluation for Poly (3-Hydroxybutyrate) Production by Fermentation. *Bioprocess Eng*. **17**: 335-342.

Chrissafis, K., Paraskevopoulos, K.M. and Bikiaris, D.N. (2005). Thermal Degradation Mechanism of Poly(Ethylene Succinate) and Poly(Butylene Succinate): Comparative Study. *Thermochimica Acta.* **435**(2): 142-150

Chua, A.S.M., Takabatake, H., Satoh, H. and Mino, T. (2002). Production of Polyhydroxyalkanoates (PHAs) by Activated Sludge Treating Municipal Wastewater: Effect of pH, Sludge Retention Time (SRT) and Acetate Concentration in Influent. *Water Research*. **37**: 3602-3611.

Chung, Y.J., Cha, H.J., Yeo, J.S. and Yoo, Y.J. (1997) Production of Poly(3-Hydroxybutyric-Co-3-Hydroxyvaleric) Acid Using Propionic Acid by pH Regulation. *J. Ferment. Bioeng*. **83**: 492-495.

Cornell, J.A. (2002). *Experiments with Mixtures*. 3rd ed. New York. N.Y.: John Wiley and Sons.

Daniel, M., Choi, J. H., Kim, J. H., and Lebeault, J. M. (1992). Effect of Nutrient Deficiency on Accumulation and Relative Molecular Weight of Poly-β-Hydroxybutyric Acid by *Methylotrophic* bacterium, *Pseudomonas* 135. *Appl. Microbiol. Biotechnol*. **37**. 702-706.

De Koning, G. (1995). Physical Properties of Bacterial Poly((R)-3 hydroxyalkanoates). *Can. J. Microbiol*. **41**(suppl. 1): 303-309.

Department of Environment (1999). Industrial processes & the environmental – Crude Palm Oil Industry. Handbook No. 3. Ministry of Sciences, Technology and the Environment, Malaysia.

Dey, G., Mitra, A., Banerjee, R. and Maiti, B.R. (2001). Enhanced Production of Amylase by Optimization of Nutritional Constituents Using Response Surface Methodology. *Biochem. Eng. J.* **7**(3): 227–231.

DIN 54900-2 (1998). Prüfung der Kompostierbarkeit von Kunststoffen. Deutsches Institut für Normung e.V., Beuth Vrlag GmbH, Berlin.

Dionisi, D., Majone, M., Ramadori, R. and Beccari, M (2001a). The Storage of Acetate under Anoxic Conditions. *Wat Res*. **35**: 2661-2668.

Dionisi, D., Majone, M., Tandoi, V. and Beccari, M. (2001b). Sequencing Batch Reactor: Influence of Periodic Operation on Performance of Activated Sludge in Biological Wastewater Treatment. *Ind. Eng. Chem. Res*. **40**: 5110-5119.

Dircks, K., Beun, J.J., van Loosdrecht, M.C.M., Heijnen, J.J. and Henze, M. (2001) Glyogen Metabolism In Aerobic Mixed Culture. *Biotech. Bioeng*. **73**: 85-94.

Dircks, K., Beun, J. J., van Loosdrecht, M., Heijnen, J. J. and Henze, M. (2000). Glycogen Metabolism in Aerobic Mixed Cultures. *Biotechnology and Bioengineering*. **73**(2): 85-94.

Doi, Y. (1990). *Microbial Polyesters*. New York: VCH Publishers,

Doi, Y., Kanesawa, Y., Kawaguchi, Y. and Kunioka, M. (1989). Hydrolytic Degradation Of Microbial Poly(Hydroxyalkanoates). *Makromol. Chem. Rapid Commun*. **10**: 227 – 230.

Doi, Y., Kumagai, Y., Tanahashi, N. and Mukai, K. (1992a) Structural effects on biodegradation of microbial and synthetic poly(hydroxyalkanoates). In: Vert, M. (ed.). *Biodegradable Polymers and Plastics*. England: Royal Society of Chemistry, Cambridge. 139 – 148.

Doi, Y., Kanesawa, Y., Tanahashi, N. and Kumagai, Y. (1992b). Biodegradation of Microbial Polyesters in the Marine Environment. *Polymer Degradation and Stability*. **36**: 173 – 177.

Doi, Y., Kunioka, M., Nakamura, Y., and Soga, K. (1986). Nuclear Magnetic Resonance Studies on Poly(β-hydroxybutyrate) and a Copolymer of β-hydroxybutyrate and β-hydroxyvalerate Isolated from *Alcaligenes eutrophus* H16. *Macromolecules*. 19: 2860-2864.

Doi, Y., Tamaki, A., Kunioka, M., and Soga, K. (1987). Biosynthesis Of Terpolyesters of 3-Hydroxybutyrate, 3-Hydroxyvalerate, and 5-Hydroxyvalerate in *Alcaligenes eutrophus* from 5-chloropentanoic and Pentanoic Acids. *Makromol Chem. Rapid Commun*. **8**: 631 - 635.

Du, G. C. C., Chen, J., Yu, J. and Lun, S. Y. (2001). Feeding Strategy of Propionic Acid for Production of Poly(3- Hydroxybutyrate-Co-3-Hydroxyvalerate) with *Ralstonia eutropha*. *Biochem. Eng. J.* **8**(2): 103-110.

Du, G., Si, Y. and Yu, J. (2001). Inhibitory Effect of Medium-Chain-Length Fatty Acids on Synthesis of Polyhydroxyalkanoates from Volatile Fatty Acids by *Ralstonia eutropha. Biotechnol. Lett.* **23**(19): 1613-1617.

Du, G. C., Chen, J. A., Gao, H. J., Chen, Y. G. and Lun, S. Y. (2000). Effects of Environmental Conditions on Cell Growth and Poly- Beta-Hydroxybutyrate Accumulation in *Alcaligenes eutrophus. World Journal of Microbiology & Biotechnology*. **16**(1): 9-13.

Durner, R., Witholt, B. and Egli, T. (2000). Accumulation for poly[(R)-3-hydroxyalkanoates] in *Pseudomonas oleovorans* During Growth With Octanoate in Continuous Culture At Different Dilution Rates. *Appl. Environ. Microbiol*. **66**: 3408-3414.

Durner, R.A. (1998). Feast and Starvation: Accumulation of Bioplastic in *Pseudomona oleovorans*. PhD thesis No. 12591, Swiss Federal Institute of Technology, Zurich, Switzerland.

Egli, T. and Quayle, J.R. (1986). Influence Of Carbon:Nitrogen Ratio Of Growth Medium On The Cellular Composition And The Ability Of The Methylotrophic Yeast *Hansenula Polymrpha* To Utilize Mixed Carbon Sources. *J. Gen Microbiol*. **132**: 1779-1788.

Filipe, C.D.M., Daigger, G.T., Leslie, J. and Grady, C.P. (2001). A Metabolic Model for Acetate Uptake Under Anaerobic Condition by Glycogen Accumulating Organisms: Stoichiometry, Kinetics, and the Effect of pH. *Biotechnol. Bioeng*. **76**: 17-31.

Findlay, R.H., and White, D.C. (1983). Polymeric β-Hydroxyalkanoates from Environmental Samples and *Bacillus megaterium*. *Appl. Environ. Microbiol*. **45**: 71 - 78.

George, S., Larsson, G. and Enfors, S.O. (1993). A Scale-Down Two-Compartment Reactor with Controlled Substrate Oscillations: Metabolic Response of *Saccharomyces Cerevisiae*. *Bioprocess Eng*. **9**: 249–257.

Gerngross, T.U., Snell, K.D., Peoples, O.P., Sinskey, A.J., Csuhai, E., Masamune, S., and Stubbe, J. (1994). Over Expression And Purification of the Soluble Polyhydroxyalkanoate Synthase from *Alcaligenes eutrophus:* Evidence For a Required Posttranslational Modification for Catalytic Activity. *Biochemistry*. **33**(31): 9311 - 9320.

Ginestet, P., Maisonnier, A. and Spérandio, M. (2002). Wastewater COD Characterization: Biodegradability of Physico-Chemical Fractions. *Wat. Sci. Technol.* **45**(6): 89 – 97.

Gommers, P.J.F., van Schie, B.J., van Dijken, J.P. and Kuenen, J.G. (1988). Biochemical Limits to Microbial Growth Yield: An Analysis of Mixed Substrate Utilization. *Biotechnol. Bioeng*. **45**: 258 – 269

Grace, W.R. & Co. (1962) US Pat. 3,036,959; US Pat. 3,044,942 (1962); US Pat. 3,225,766 (1965).

Grady, C.P.L. Jr., Daigger, G.T. and Lim, H.C. (1999). *Biological Wastewater Treatment* (2nd edition). United Kingdom: Marcel Dekker, INC.

Gruber, O.R., Hall, E.S., Kolstad, J.J., Iwen, M.L., Benson, R.D. and Borchardt, R.L. (1993) Patent 5,247,059.

Hassan, M.A., Nawata, O., Shirai, Y., Nor Aini, A.R., Yee, P.L., Arif, A., and Abdul Karim, M.I. (2002). A Proposal for Zero Emission from Palm Oil Industry Incorporating the Production of Polyhydroxyalkanoates from Palm Oil Mill Effluent. *J. Chem. Eng. of Japan*. **35**(1): 9 – 14.

Hassan, M.A., Shirai, N., Kusubayashi, N., Abdul Karim, M.I., Nakanishi, K., Hashimoto, K. (1997a). The Production Of Polyhydroxyalkanoates From Palm Oil Mill Effluent by *Rhodobacter spheroides*. *J. Ferment. Bioeng*. **83:** 485–488.

Hassan, M.A., Shirai, N., Umeki, H., Abdul Karim, M.I., Nakanishi, K., Hashimoto, K. (1997b). Acetic Acid Separation From Anaerobically Treated Palm Oil Mill Effluent for the Production of Polyhydroxyalkanoate by *Alcaligenes eutrophus*. ***Biosci. Biotechnol. Biochem***. **61**: 1465 –1468.

Hassan, M.A., Shirai, Y., Kusubayashi, N., Abdul Karim, M.I., Nakanishi, and Hashimoto, K. (1996). Effect of Organic Acid Profiles during Anaerobic Treatment of Palm Oil Mill Effluent on the Production of Polyhydroxyalkanoates by *Rhodobacter sphaeroides*. ***J. Ferment. Bioeng***. **82**:151-156.

Haywood, G. W., Anderson, A. J., and Dawes, E. A. (1989). A Survey of the Accumulation of Novel Polyhydroxyalkanoates by Bacteria. *Biotechnology Letters*. **11**(7): 471-476.

Haywood, G. W., Anderson, A. J., Williams, D. R., and Dawes, E. A. (1991). Accumulation of a poly (hydroxyalkanoate) copolymer containing primarily 3-hydroxyvarate from simple carbohydrate substrates by *Rhodococcus sp*. NCIMB 40126. *International Journal of Biological Macromolecules*. **13**: 83-88.

Herbert, H., Fang, P and Liu, Y. (2000). Intracellular Polymers in Aerobic Sludge of Sequencing Batch Reactors. ***J. Environ. Eng***. **126**(8): 732 – 738.

Hessekmann, R.P.X., van Rammell, R., Resnick, S.M., Hany, R. and Zehnder, A.J.B. (2000). Anaerobic Metabolism of Bacteria Performing Enhanced Biological Phosphate Removal. ***Wat. Res***. **34**: 3487-3897.

Holmes, P.A. (1988). Biologically produced PHA Polymers and Copolymers. In:Bassett D.C. (ed.). *Development in Crystalline Polymers*. London: Elsevier. 1-65.

Howells, E.R. (1982). Opportunities in Biotechnology for the Chemical Industry. *Chem. Industry*. **8**: 508-511.

Hrabak, O. (1992). Industrial Production of Poly-β-hydroxybutyrate. *FEMS Microbiology Reviews*. **103**: 251-256.

Hsiun, D.Y. (1995). *Design of an Airlift Reactor with Net Draft Tubes and Its Application in Fermentation of Bacillus thuringiensis*. National Tsing Hua University, Singapore: Ph.D. Dissertation.

Hsu, Y.L. and Wu, W.T. (2002). A Novel Approach for Scaling-Up a Fermentation System. *Biochem. Eng. Journ*. **11**: 123–130

Hu, W.F., Chua, H., Yu, P.H.F. (1997). Synthesis of Poly-(hydroxy butyrate-co-hydroxyvalerate) from Activated Sludge. *Biotechnol Lett.* **9**: 695-698.

Huang, J., Shetty, A. S., and Wang, M. (1990). Biodegradable Plastics: A Review. *Advances in Polymer Technology*. **10**(1): 23-30.

Ishizaki, A., Lee, T.M., Todd, J. I. and Yoshino, S. (1997). Solvent production from palm oil waste. *Biotechnology for Sustainable Utilization of Biological Resources in the Tropics*. **11**: 1-8.

Ishizaki, A. and Tanaka, K. (1991) Production of Poly-β-hydroxybutyric acid from carbon dioxide by Alkaligenes eutrophus, *Bioeng*.71, 254-257.

Jaafar, M. and Sukaimi, J. (2001). The Future of Palm Oil in the New Millennium in Malaysia. Kuala Lumpur, Malaysian Palm Oil Board**:** 10-13.

Jones, M. and Stephenson, T. (1996). The Effect of Temperature on Enhanced Biological Phosphorus Removal. *Env. Technol*. **17**: 965 – 979.

Kalia, V.C., Sonakya, V., Raizada, N. (2000). Anaerobic digestion of Banana Stem Waste. *Bioresour. Technol*. **73**: 191–193.

Kang, C.-K., Kusaka, S., and Doi, Y. (1995). Structure and Properties of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) Produced by *Alcaligenes eutrophus*. *Biotechnol. Lett*. **17**: 583–588.

Kawai, F (2000) Breakdown of Plastics and Polymers by Microorganism. *Adv. Biochem. Eng. Biotechnol.* **52**: 154-194

Kim, B.S., Lee, S.C., Lee, S.Y., Chang, H.N., Chang, Y.K. and Woo, S.I. (1994). Production of Poly(3-hydroxybutyric acid) by Recombinant *Escherichia coli* in Flask and Fed Batch Cultures. *J. Environ. Polymer Degrad*. **2**: 169-176.

Kittikun, A. H., Prasertsan, P., Srisuwan, G. and Krause, A. (2000). Environmental Management for Palm Oil Mill. *Material Flow Analysis of Integrated Bio-Systems*. March - October 2000. University of Tokyo: IBSN, 1-12.

Kleijntjens, R.H. (1991). *Biotechnological Slurry Process for the Decontamination of Excavated Polluted Soils*. Delft University of Technology, The Netherlands: Ph.D. Thesis

Kornaros, M., and Lybertos, G. (1996). Kinetic Modelling Of *Pseudomonas Denitrificans* Growth and Denitrificans under Aerobic, Anoxic and Transient Operating Conditions. *Wat Res*. **32**(6): 1912 – 1922.

Kornaros, M., Zafiri, C. and Lybertos, G. (1996). Kinetics of Denitrification by *Pseudomonas Denitrificans* under Growth Conditions Limited by Carbon and/or Nitrate or Nitrite. *Wat. Environ. Res*. **68**: 934 – 945.

Krishna, C., van Loosdrecht, M.C.M. (1999a). Effect of Temperature on Storage Polymers and Settleability of Activated Sludge. *Wat. Res*. **33**(10): 2374-2382.

Krishna, C. and van Loosdrecht, M. C. M. (1999b). Substrate Flux into Storage and Growth In Relation To Activated Sludge Modeling. *Water Res.* **33**(14): 3149-3161.

Kuba, T., van Loosdrecht, M.C.M. and Heijnen, J.J. (1996). Phosphorus and Nitrogen Removal with Minimal COD Requirement by Integration of Denitrifying Dephosphatation and Nitrification in a Two-Sludge System. *Wat. Res*. **30**(7): 1702-1710.

Kunioka, M., Kawagushi, Y., Doi, Y., (1989). Production of Biodegradable Copolyesters of 3-Hydroxybutyrate and 4-Hydroxybutyrate by *Alcaligenes Eutrophus*. *Appl. Microbiol. Biotechnol*. **30**: 569–573.

Kunioka, M., Nakamura, Y., and Doi, Y. (1988). New Bacterial Copolyesters Produced in *Alcaligenes Eutrophus* from Organic Acids. *Polym. Commun*. **29**: 174 - 176.

Lafferty, R. M., Korsatko, B., and Korsatko, W. (1988). Microbial Production of Poly-β-hydroxybutyric Acid. In: *Biotechnology*, Rehm, H. J. and Reed, G. (eds.) VCH Publishers: New York. 135-176.

Lee, H. S., Oh, H. D., Ahn, S. W., Lee Y., Choi, J. and Lee, Y. S. (2000). Production of poly(3-hydroxybutyrate-co-hydroxyhexanoate) by High-Cell-Density Cultivation of *Aeromonas hydrophila*. *Biotech. Bioeng*. **67**(2): 240-244

Lee, S. and Yu, J. (1997). Production of Biodegradable Thermoplastics from Municipal Sludge by a Two-Stage Bioprocess. *Resources, Conservation and Recycling*. **19**: 151-164.

Lee, S. Y., Choi, J. I. and Lee, S. H. (2000). Production of Polyhydroxyalkanoates by Fermentation of Bacteria. *Macromol. Symp.* **159**: 259-266.

Lee, S.Y. (1996a). Bacterial Polyhydroxyalkanoates. *Biotechnol Bioeng*. **49**: 1-14.

Lee, S.Y. (1996b). Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. *Trends Biotechnol*. **14**. 431-438.

Lee, S.L. and Chen, W.C. (1997). Optimization of Medium Composition for the Production of Glucosyltransferase by *Aspergillus Niger* with Response Surface Methodology. *Enzyme Microb. Technol.* **21**: 436–440.

Lemos, C., Viana, C., Sagueiro, E.N., Rmas, A.M. Crespo SG, Reis, M.A.M. (1998) Effect of Carbon Source on the Formation of Polyhydroxyalkanoates by a Phosphate Accumulating Mixed Culture. *Enz. Microbiol. Technol*. **22**: 662-671.

Lishman, L.A., Legge, R.L. and Farquhar, G.J. (2000) Temperature Effect on Wastewater Treatment under Aerobic and Anoxic Condition. *Wat. Res*. **34**(8): 2263 – 2276

Liu, Y. H., Geiger, C. and Randall, A.A. (2000). The Role of PHA Form in Determining the Response of Enhanced Biological Phosphorus Removal Biomass to Volatile Fatty Acids, Proceedings, WEFTEC 2000, 73rd Annual Conference & Exposition on Water Quality and Wastewater Treatment, Anaheim, CA, USA, 14-18 October 2000.

Liu, F., Li, W., Ridgway, D. and Gu, T. (1998). Production of Poly-β-hydroxybutyrate on Molasses by Recommbinant *Escherichia coli*. *Biotechnology Letters*. **20**(4): 345-348.

Liu, W.T., Mino, T., Nakamura, K. and Matsuo, T. (1996). Glycogen Accumulating Population and its Anaerobic Substrate Uptake in Anaerobic-Aerobic Activated Sludge without Biological Phosphate Removal. *Wat Res*. **30**: 75-82.

Llyod, D., Boddy, L. and Davies, K.J.P. (1987). Persistence of Bacterial Denitrification Capacity under Aerobic Conditions: The Rule Rather Than Exception. *FEMS Microbiol. Ecol.* **45**: 185-190.

Luzier, W.D. (1992). Material Derived from Biomass/Biodegradable Materials. *Proc. Natl. Acad. Sci. USA*. **89**: 839 – 842

Ma, C. K., Chua, H., Yu, P. H. F., and Hong, K. (2000). Optimal Production of Polyhydroxyalkanoates in Activated Sludge Biomass. *Appl. Biochem. and Biotech.* **84-86**: 981-989.

Ma, A.N. (1992). ENSEARCH monograph on environmental protection technology: water pollution control in palm oil industries in Malaysia, Environmental Management and Research Association of Malaysia.

Madison, L.L. and Huisman, G.W. (1999). Metabolic Engineering of Poly-(3-hydroxyalkanoates): from DNA to Plastic. *Microbiol. Mol. Biol. Rev*. **63**: 21-53.

Majone, M., Beccari, M., Dionisi, D., Levantesi, C. and Renzi, V. (2001). Role of Storage Phenomena on Removal of Different Substrates During Pre-denitrification. *Wat. Res*. **42**: 151-158.

Majone, M., Dircks, K. and Beun, J. J. (1999). Aerobic Storage under Dynamic Conditions in Activated Sludge Processes. The State of the Art. *Water Sci. Technol.* **39**(1): 61-73.

Malaysia Palm Oil Board (2005). Palm Oil Update – for the Latest Information on Palm Oil. 25(09/05). Ministry of Plantation Industries and Commodities Malaysia.

Mamais, D. and Jenkins, D. (1992). The Effects of MCRT and Temperature on Enhanced Biological Phosphorus Removal. *Wat. Sci. Technol*. **26**(5-6): 955 – 965.

Marangoni, C., Furigo, A. and Aragao, G. M. F. (2001). The Influence of Substrate Source on the Growth of *Ralstonia Eutropha,* Aiming At the Production of Polyhydroxyalkanoate. *Braz. J. Chem. Eng.* **18**(2): 175-180.

Marazioti, C., Kornaros, M. and Lyberatos, G. (2003). Kinetic Modelling of a Mixed Culture of *Pseudomonas denitrificans* and *Basillus subtilis* under Aerobic and Anoxic Operating Conditions. *Wat. Res*. **37**: 1239 – 1251.

McClintock, S., Randall, C.W. and Pattarkine, V. (1992). The Effects of Temperature and Mean Cell Residence Time on Enhanced Biological Phosphorus Removal. Environmental Engineering, the Proceeding of the 1991 Specialty Conference on Environmental Engineering, ASCE. 319 – 324.

Md Din, M. F., Ujang, Z. and van Loosdrecht, M.C.M. (2004). Polyhydroxybutyrate (PHB) Production from Mixed Cultures of Sewage Sludge and Palm Oil Mill Effluent (POME): The Influence of C/N Ratio and Slow Accumulation Factor. *Wat. Env. Manag. Series*. **ISBN: 1 84339 503 7**: 115-112.

Meesters, K. P. H., van Loosdrecht, M.C.M. and Heijnen, J.J. (1998). Production of poly-3-hydroxyalkanoates from waste streams. Report of Technical University of Delft, The Netherlands**:** 88.

Miller, N.D. and Williams, D.F. (1987). On The Biodegradation of Poly-β-Hydroxybutyrate (PHB) Homopolymer and Poly-β-Hydroxybutyrate-Hydroxyvalerate Copolymers. *Biomaterials*. **8**: 129 – 138.

MINITAB. (2000) MINITAB User's Guide 2: Data Analysis and Quality Tools, Minitab Inc.

Montgomery D.C. (2001). Design and Analysis of Experiments. 5th ed., New York, N.Y.: John Wiley and Sons.

Müller, R.J., Augusta, J., Walter, T. and Widdecke, H. (1994). The Development and Modification of Some Special Test Methods and the Progress in Standardisation of Test Methods in Germany. *In*: Doi, Y. and Fukuda, K. (eds.) *Biodegradable Plastics and Polymers*. Elsevier: New York. 237-249.

Müller, R.J., Kleeberg, H. and Deckwer, W.D. (2001). Biodegradation of Polyesters Containing Aromatic Constituents. *J. Biotech*. **86**: 87 – 95.

Murnleitner, E., Kuba, T., van Loosdrecht, M.C.M. and Heijnen, J.J. (1997). An Integrated Metabolic Model for the Aerobic and Denitrifying Biological Phosphorus Removal. *Biotechnology and Bioengineering*. **54**: 434-450.

Myers, R.H. and Montgomery, D.C. (2002). *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*. 2nd ed., New York, N.Y.: John Wiley and Sons

Nakamura, S., Doi, Y., and Scandola, M. (1992). Microbial Synthesis and Characterization of Poly(3-Hydroxybutyrate-*Co*-4-Hydroxybutyrate). *Macromolecules* **25**: 4237–4241.

Nishida, H., and Tokiwa, Y. (1993). Effects of Higher-Order Structure Of Poly(3-Hydroxybutyrate) on Its Biodegradation: Effects of Crystal Structure on Microbial Degradation. *J. Environ. Polym. Degrad*. **1**: 65–80.

NNI. NEN 6621 (1982). Bepaling van de asrest. Nederlands Normalisatie Instituut, Delft

Nor Aini, A.R., Hassan, M.A., Shirai, Y., Abdul Karim, M.I., and Arif, A.B. (1999). Production of Organic Acid from Palm Oil Mill Effluent during Continuous Anaerobic Treatment. *Asia Pacific J. Mol. Biol. Biotechnol*. **7**: 179-184.

Orhon, D., Ateş, E., Sözen, S. and Çokgör, E. U. (1997). Characterization and COD Fractionation of Domestic Wastewaters. *Environ. Poll*. **95**(2): 191 – 204.

Pereira, H., Lemos, P. C., and Reis, M. A. M. (1996). Model for Carbon Metabolism in Biological Phosphorus Removal Processes Based on *In Vivo* 13C-NMR Labeling Experiments. *Wat. Res.*. **30** (9): 2128-2138.

Poirier, Y., Dennis, D.E., Klomparens, K., and Somerville, C. (1992). Polyhydroxybutyrate, A Biodegradable Thermoplastic, Produced In Transgenic Plants. *Science*. **256**: 520 - 523.

Poirier, Y. (2002). Polyhydroxyalknoate Synthesis in Plants as a Tool for Biotechnology and Basic Studies of Lipid Metabolism. *Progress in Lipid Research* **41**(2): 131-155.

Poirier, Y., Nawrath, C. and Somerville, C. (1995). Production of Polyhydroxyalkanoates, a Family of Biodegradable Plastics and Elastomers, In Bacteria and Plants. *Biotechnol* **13**: 142-150.

Preusting, H., Hazenberg, W., and Witholt, B. (1993). Continuous Production of Poly(3-hydroxyalkanoates) by *Pseudomonas oleovorans* in a High-Cell-Density, Two-Liquid-Phase Chemostat. *Enzyme Microb. Technol*. **15**: 311-316.

Punrattanasin, W. (2001). *The utilization of activated sludge polyhydroxyalkanoates for the production of biodegradable plastics*. Faculty of the Virginia Polytechnic Institute and State University: Ph.D, Thesis

Punrattanasin, W. and Randall, C.W. (1998). The Effect of the Influent COD/TP Ratio upon the Performance of Biological Nutrient Removal Processes. Proc. Of Water Quality International. IAWQ 19th Biennial International Conference, Vancouver, B.C., Canada. 292 – 298.

Purushothaman, M., Anderson, R.K.I., Narayana, S. and Jayaraman, V.K. (2001). Industrial Byproducts as Cheaper Medium Components Influencing the Production of Polyhydroxyalkanoates (PHA) – Biodegradable Plastics. *Biopro. Biosyst. Eng*. **24**(3): 131-136.

Randall, C.W., Barnard, J.L. and Stensel, H.D. (1992). Design and Retrofit of Wastewater Treatment Plants for Biological Nutrient Removal. Water Quality Management Library, Lancaster, Pensylvania, USA.

Reddy, C. S. K., Ghai, R., Rashmi and Kalia, V. C. (2003). Polyhydroxyalkanoates: an Overview. *Bioresour. Technol.* **87**(2): 137-146.

Ren, X. (2003). Biodegradable plastics: a solution or a challenge? *Journal of Cleaner Production*. **11**(1): 27-40.

Robertson, L.A. and Kuenen, J.G. (1984). Aerobic Denitrification: a Controversy Revived. A*rch Microbiol*. **139**: 351-354.

Robertson, L.A. and Kuenen, J.G. (1992). Nitrogen removal from water and waste. Microbial control of pollution. Fry, J.C., Gadd, G.M., Herbert, R., Jones, C.W. and Watson – eds.). *Society for General Microbiology Symposium*. **48**: 227-267.

Rusendi, D. and Sheppard, J.D. (1995). Hydrolysis of Potato Processing Waste for the Production of Poly-Beta-Hydroxybutyrate. *Bioresour. Technol*. **42**: 351-356.

Ryu, H. W., Cho, K. S., Kim, B. S., Chang, Y. K., Chang, H. N. and Shim, H. J. (1999). Mass Production of Poly (3-Hydroxybutyrate) By Fed-Batch Cultures of Ralstonia eutropha With Nitrogen and Phosphate Limitation. *J. Microbiol. Biotechnol.* **9**(6): 751-756.

Saito, T., Brdjanovic, D. and van Loosdrecht, M.C.M. (2004). Effect of Nitrite on Phosphate Uptake by Phosphate Accumulating Organism. *Wat. Res*. **38**: 3760-3768.

Saito, Y., Soejima, T., Tomozawa, T., Doi, Y. and Kiya, F. (1995). Production of Biodegradable Plastics From Volatile Acids Using Activated Sludge. *Environ Systems Eng*. 52: 145-154

Salehizadeh, H. and van Loosdrecht, M.C.M. (2004). Production of Polyhydroxyalkanoates by Mixed Culture: Recent Trends and Biotechnical Importance. *Biotech. Adv*. 22: 261-279.

Sasikala, CH. and Ramana, CH. V. (1996). Biodegradable Polyesters. In: *Advances in Applied Microbiology, Volume 42*, Neidleman, S. L. and Laskin, A. I. (eds.), Academic Press, California. 97-218.

Satoh, H., Mino, T. and Matsuo, T. (1998a). Anaerobic Uptake of Glutamate and Aspartate by Enhanced Biological Phosphorus Removal Activated Sludge. *Wat Sci Tech.* 37: 579-582.

Satoh, H., Iwamoto, Y., Mino, T. and Matsuo, T. (1998b). Activated Sludge as a Possible Source of Biodegradable Plastic. *Wat Sci Tech*. **38**: 103-109.

Satoh, H., Mino, T. and Matsuo, T. (1994). Deterioration of Enhanced Biological Phosphorus Removal by the Domination of Microorganisms without Polyphosphate Accumulation. *Wat. Sci. Tech*. **3**: 203-211.

Senior, P. J., Beech, G. A., Ritchie, G. A. F., and Dawes, E. A. (1972). The Role of Oxygen Limitation in the Formation of Poly-β-Hydroxybutyrate during Batch and Continuous Culture of *Azotobacter beijerinckii*. *The Biochemistry Journal*. **128**: 1193-1201.

Shi, H., Shiraishi, M., and Shimizu, K. (1997). Metabolic Flux Analysis For Biosynthesis of Poly(β-Hydroxybutyric Acid) in *Alcaligenes Eutrophus* from Various Carbon Sources. *J. Ferment. Bioeng*. **80**(6): 579-587.

Shimizu, H., Tamura, S., Ishihara, Y., Shioya, S., and Suga, K. (1994). Control of Molecular Weight Distribution and Mole Fraction In Poly(-D(-)-3-Hydroxyalkanoates) (PHA) Production by *Alcaligenes Eutrophus.* In: *Biodegradable Plastics and Polymers*, Doi, Y. and Fukuda, K. (eds.), Elsevier Science B.V., New York, 365-372.

Smolders, G. J. F., van Loosdrecht, M. C. M. and Heijnen, J. J. (1996). Steady State Analysis to Evaluate the Phosphate Removal Capacity and Acetate Requirement of Biological Phosphorus Removing Mainstream and Sidestream Process Configurations. *Wat. Res*. **30**: 2748-2760.

Stein, R.S. (1992). Polymer Recycling: Opportunities and Limitations. *Proceedings of the National Academy of Sciences*. **89**. 835 – 838.

Steinbüchel, A, (1991). Polyhydroxyalkanoic Acids. In: Byrom, D. (ed.) Biomaterial. *Novel Materials from Biological Sources*. Stockton Press. New York. 125 – 213

Steinbüchel, A. and Pieper, U. (1992). Production of a Copolyester of 3-Hydroxybutyric Acid and 3-Hydroxyvaleric Acid from Single Unrelated Carbon Sources by a Mutant of *Alcaligenes eutrophus*. *Appl. Microbiol. Biotechnol*. **37**: 1-6.

Steinbüchel, A., Aerts, K., Babel, W., Follner, C., Liebergesell, M., Madkour, M.H., Mayer, F., Pieper-Frust, U., Pries, A., Valentin, H.E. and Wieezorek, R. (1995). Considerations on the Structure and Biochemistry Of Bacterial Polyhydroxyalkanoic Acid Inclusions. *Can. Journ. Microbiol*. **41** (Suppl. 1). 94 – 105.

Steinbüchel, A., and Hein, S. (2001). Biochemical and Molecular Basis of Microbial Synthesis of Polyhydroxyalkanoates in Microorganisms. *Biopolyesters*. **71**. 82 – 123.

Steinbüchel, A., and Valentin, H.E. (1995). Diversity of Bacterial Polyhydroxyalkanoic Acids. *FEMS Microbiol. Lett*. **128**: 219-228

Stouthamer, A.H. (1988). Dissimilatory Reduction of Oxidized Nitrogen Compounds. In: AJB Zehnder. Biology of Anaerobic Microorganisms. New York: Wiley & Sons. 245 – 303.

Su, L., Lenz, R.W., Takagi, Y, Zhang, S., Goodwin, S., Zhong, L. and Martin, D.P. (2000). Enzymatic Polymerization of (*R*)-3-Hydroxyalkanoates by a Bacterial Polymerase. *Macromolecules*. **33**: 229-231

Sudesh, K. and Doi, Y. (2000). Molecular Design and Biosynthesis of Biodegradable Polyesters. *Polymers for Adv. Technol*. **11**: 8-12. 865-872.

Sudesh, K., H. Abe, H and Doi, Y. (2000). Synthesis, Structure and Properties of Polyhydroxyalkanoates: Biological Polyesters. *Prog. Polym. Sci.* **25**(10): 1503-1555.

Suzuki, T., Yamane, T., and Shimizu, S. (1986). Mass Production of Poly-β-Hydroxybutyric Acid by Fully Automatic Fed-Batch Culture of Methylotroph. *Appl. Microbiol. Biotechnol.* **23**: 322-329.

Takabatake, H., Satoh, H., Mino, T. and Matsuo, T. (2002). PHA Production Potential Of Activated Sludge Treating Wastewater. *Wat. Sci. Tech.* **45**: 119-126.

Tian, W., Hong, K., Chen, G.Q., Wu, Q., Zhang, R. and Huang, W. (2000). Production of Polyesters Consisting of Medium Chain Length 3-Hydroxyalkanoic Acids by *Pseudomonas Mendocina* 0806 from Various Carbon Sources. *Antonie van Leeuwenhoek*. **77**: 31-36.

Tohyama, M., Patarinska, T., Qiang, Z. and Shimizu, K. (2002). Modeling of the Mixed Culture and Periodic Control for PHB Production. *Biochem. Eng. Jour*. **10**: 157 – 173.

Tsunemasa N. (1988). Utilization of Excess Sludge for the Production of Biodegradable Plastics. *Wat Waste*. **40**: 981-986 (In Japanese).

Ueno, T., Satoh, H., Mino, T. and Matsuo, T. (1993). Production of Biodegradable Plastics. *Polymer Preprint*. **42:** 981-986 (In Japanese).

van Aalst-van Leeuwen, M. A., Pot, M. A., Van Loosdrecht, M. C. M. and Heijnen, J. J. (1997). Kinetic Modeling of Poly (β̃-Hydroxybutyrate): Production and Consumption by *Paraccus Pantotrophus* under Dynamic Substrate Supply. *Biotechnol. Bioeng*. **55**. 773-782.

van den Eynde, E., Vriens, I., Wynats, M. and Verachtert, H. (1984). Transient Behaviour and Time Aspects of Intermittently and Continously Fed Bacterial Cultures with Regards to Filamentous Bulking of Activated Sludge. *Eur. J. Appl. Microbiol. Biotechnol*. **19**. 44-52.

van Loosdrecht, M. C. M. and Heijnen, J. J. (2002). Modeling of Activated Sludge Processes with Structured Biomass. *Wat. Sci. Tech*. **45**: 13-23.

Verduyn, C., Stouthamer, A.H., Scheffers, W.A. and Van Dijken, J.P. (1991). A Theoretical Evaluation of Growth Yield of Yeasts. *Ant. Leeuwenh*. **59**: 49 – 63.

Wallen, L.L., and Rohwedder, W.K. (1974). Poly-β-hydroxyalkanoate from Activated Sludge. *Environ. Sci. Technol*. **8**: 576 - 579.

Wang, Y., Yamada, S., Asakawa, N., Yamane, T., Yoshie, N. and Inoue, Y. (2001). Comonomer Compositional Distribution and Thermal and Morphological Characteristics of Bacterial Poly (3- Hydroxybutyrate-Co-3-Hydroxyvalerate) with High 3- Hydroxyvalerate Content. *Biomacromolecules*. **2**(4): 1315-1323.

Waitier, D., Dubourguier, H.C., Leguerinel, I. and Hornez, J.P. (1996). Response Surface Models to Describe the Effects of Temperature, pH, And Ethanol Concentration on Growth Kinetics and End Products of A *Pectinatus* Sp. *Appl. Environ. Microbiol.* **62**. 1233–1237.

Witt. U.; Müller, R.-J. and Klein, J. (1997). Biologisch Abbaubare Polymere- Status und Perspektiven. Report of the Franz-Patat-Zentrum, Braunschweig, ISBN 3-00-001529-9.

Wong, A. L., Chua, H., Lo, W. H. and Yu, P. H. F. (2000). Synthesis of Bioplastics from Food Industry Wastes with Activated Sludge Biomass. *Wat. Sci. Tech.* **41**(12): 55-59.

Wu, Q., Sun, S. Q., Yu, P. H. F., Chan, A. X. Z. and Chen, G. Q. (2000). Environmental Dependence of Microbial Synthesis of Polyhydroxyalkanoates. *Acta Polym. Sin.* **6**: 751-756.

Yamane, T (1992). Cultivation Engineering of Microbial Bioplastics Production. *FEMS Microbiol. Rev*. **103**: 257-264.

Yamane, T (1993). Yield of Poly D(-)-3-hydroxybutyrate from Various Carbon Sources: A Theoritical Study. *Biotechnol. Bioeng*. **41**: 165-170.

Yamane, T., Fukunage, M. and Lee, Y.W. (1996). Increased PHA Productivity by High-Cell Density Fed-Batch Culture of *Alcaligenes Latus*, A Growth-Associated PHA Producer. *Biotechnol. Bioeng*. **50**: 197-202.

Yu, J. and Wang, J. (2001) Metabolic Flux Modelling of Detoxification of Acetic Acid by *Ralstonia eutropha* at Slightly Alkanline pH levels. *Biotech. Bioeng*. **73**: 458-464.

APPENDIX A

Table A1: General definitions of a biodegradable polymer (or plastic) proposed by Standard Authorities and summarized by Calmen-Decriaud *et al.*,1998.

Standard Authorities	Biodegradable plastics
ISO 472-1988	A plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastic and the application in a period of time that determines its classification. The change in the chemical structure results from the action of naturally occurring microorganisms.
ASTM sub-committee D20-96	A degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria and fungi.
DIN 103.2-1993 German working group	A plastic material is called biodegradable if all its organic compounds undergo a complete biodegradation process. Environmental conditions and the rates of biodegradation are to be determined by standardized methods.
CEN (May 1993)	A degradable material in which degradation results from the action of microorganisms and ultimately the material is converted to water, carbon dioxide and/or methane and new cell biomass.
Japanese Biodegradable Plastic Society (1994)	Polymeric materials which are changed into lower weight compounds where at least one step in the degradation process is through metabolism in the presence of naturally occurring organisms.

Table A2: Classification of plastics

Types of Plastic					
PET (Poly Ethylene Terephthalate)	**HDPE (High Density Polyethylene)**	**PVC or V (Vinyl polyvinyl chloride)**	**LDPE (Low Density Polyethylene)**	**PP (Poly propylene)**	**PS (Polystyrene)**
Accounts of 20 - 30% of the bottle market and also is the most commonly recycled plastic in the US. PET formed in a variety of food stuff package and is used mainly for its clarity, toughiness and ability to resist permeation by carbon dioxide. Some examples of products possible from recycled PET are carpets, auto parts and geotextiles.	Accounts for 50 - 60% of the bottle market. HDPE is used to make milk jugs, butter tubs, detergent bottles, motor oil containers and bleach bottle to name a few. Recycled HDPE can be used to make flowerpots, trash cans, traffic borders, industrial pallets and other related items.	Accounts for 5 - 10% of all plastic packaging. It is used to make bottles (water, shampoo, cooking oil), garden hoses, flooring, credit cards, shower curtains and many more related items. The main problem with PVCs is that when it is incinerated it contributes to the production of HCl. Recycled PVC is used to make drainage pipes, handrails and sewer pipes among others	Accounts for 5 - 10% of all plastic produced. Its uses include shrink wrap packaging, plastic sandwich bag, and clothing wrap. Recycled LDPE can be used to make almost everything that the virgin resin is used for.	Accounts for 5 - 10% of all plastic produced. It is used to make plastic bottle caps, plastic lids, drinking straws, broom fibers, rope, twine, yogurt containers and carpets. Recycled PP can be used to produce or has the potential to be used for auto parts, bird feeders and battery cases.	Accounts of 5 - 10% of all plastic produced. It is used to make stryroform cups, egg cartons and fast food packing. Recycled PS can be used to make light switch plates, note pad holders, cassette tape cases, reusable cafeteria trays and waste baskets.

Source: http://dirac.py.iup.edu/college/chemistry/chem-course/plastic.html

APPENDIX B

Table B1: PHA production by microorganisms

Organism	Substrate, conc.	Limiting condition	PHA (% w/w)	Composition of PHA PHB(%)	PHV(%)	Reference
Azobacter beijerinckii	G, 5g/L	O_2	35	NR	NR	Senior, 1972
	G, 20 g/L		75	NR	NR	
Azotobacter vinelandii UWD	V, 10mM + G, NR V,	NR	94	72	18	Page and Manchak, 1995
	20mM + G, NR		64	78	22	
	V, 30mM + G, NR		74	75	25	
	V, 10mM		67	84	16	
	V, 20mM		36	79	21	
	V, 30mM		29	72	28	
Rhodococcus sp. NCIMB 40126	A, 10 g/L	NH_4^+	29	31	69	Haywood *et al.*, 1991
	L, 10 g/L		25	22	78	
	G, 10 g/L		21	25	75	
Methylobacterium extorquens	M, 1 g/L + V, 0.5g/L	None	60-70	80	20	Bourque *et al.*, 1992
Methylobacterium extorquens	M, 1.4 g/L	NH_4^+	40-60	100	0	Bourque *et al.*, 1995
Rhodococcus sp. ATCC 19070	A, 10 g/L	NH_4^+	4	27	73	Haywood *et al.*, 1991
	L, 10 g/L		2	28	72	
	G, 10 g/L		14	9	91	
Rhodococcus ruber NCIMB 40126	G, NR	SO_4^{2-}	16.2	30.3	69.7	Anderson *et al.*, 1992
	VA, NR		26.2	18	82	
	G+VA, NR		27.7	21.9	78.1	
Corynebacterium hydrocarboxydans ATCC 21767	A, 10 g/L	NH_4^+	21	50	50	Haywood *et al.*, 1991
	L, 10 g/L		2	61	39	
	G, 10 g/L		8	28	72	
Alcaligenes latus	Sucrose, 20 g/L	None	50	100	0	Yamane *et al.*, 1996
R. eutropha Strain R3	F, 0.5 g/L	NH_4^+	47	93	7	Steinbuchel and Pieper, 1992
	GL, 0.5 g/L	NH_4^+	35.7	94	6	
	A, 0.5 g/L	NH_4^+	29.5	96	4	
	S, 0.8 g/L	NH_4^+	21.5	93	7	
	L, 0.5 g/L	NH_4^+	43.2	96	4	
	F, NR	Mg^{2+}	45	93	7	
	F, NR	SO_4^{2-}	47	94	6	
	GL,NR	Mg^{2+}	33	95	5	
	GL,NR	SO_4^{2-}	38	96	4	
R. eutropha	Digested sludge supernatant	NH_4^+	34	NR (majority)	NR	Lee and Yu, 1997
R. eutropha H16	A, 22 g/L	NH_4^+	53	100	0	Doi *et al.*, 1986
	A,22 g/L+P,10 g/L		51	81	19	
	P, 22 g/L		35	57	43	
R. eutropha H16 (ATCC 17699)	B, 0.03g/L	NH_4^+	44	100	0	Shimizu, *et al.*, 1994
	B, 0.3 g/L		55	100	0	
	B, 3 g/L		75	100	0	
	B,10 g/L		63	100	0	
	B,1.8g/L+V,1.2g/L		48	NR	NR	
	V, 3 g/L		38	NR	NR	

continued Table B1

Organism	Substrate, conc.	Limiting condition	PHA (% w/w)	Composition of PHA PHB(%)	PHV(%)	Reference
R. eutropha H16	A, 20 g/L	NH_4^+	51	100	0	Doi *et al.*, 1987
	A, 5 g/L		13	100	0	
	A, 20 g/L + P, 1 g/L		46	98	2	
	A, 20 g/L + P, 2 g/L		52	95	5	
	A, 20 g/L + P, 4 g/L		51	91	9	
	A, 5 g/L + P, 5 g/L		22	79	21	
	A, 5 g/L + P, 10 g/L		38	74	26	
	A, 5 g/L +P,20g/L		45	72	28	
	P, 2 g/L		12	78	22	
	P, 6 g/L		18	76	24	
	P, 10 g/L		28	72	28	
	P, 14 g/L		42	69	31	
	P, 18 g/L		56	73	27	
	P, 22 g/L		31	70	30	
	P, 26 g/L		40	56	44	
	P, 30 g/L		35	55	45	
R. eutropha NCIB 11599	G, 10 g/L+P, 1 g/L G, 10 g/L+V, 1 g/L	NH_4^+	59 60	85 78	15 22	Haywood *et al.*, 1989
Alcaligenes feacalis NCIB 8156	A, 10 g/L+P, 1 g/L A, 10 g/L+V, 1 g/L	NH_4^+	14 5	78 52	22 48	Haywood *et al.*, 1989
Pseudomonas extorquens MP4	M, 10 g/L+P, 1 g/L M, 10g/L+V, 1 g/L	NH_4^+	26 5	95 46	5 54	Haywood *et al.*, 1989
Pseudomonas sp. K	M, 1% (v/v)	NH_4^+	52-57	100	0	*Suzuki et al.*, 1986
		SO_4^{2-}	48-53	100	0	
		Mg^{2+}	45-50	100	0	
		Fe^{2+}	43-48	100	0	
		Mn^{2+}	50-55	100	0	
		Ca^{2+}	0-10	100	0	
		Zn^{2+}	0-10	100	0	
		Co^{2+}	0-10	100	0	
		Cu^{2+}	0-10	100	0	
		Mo^{6+}	0-10	100	0	
		Na^+	0-10	100	0	
Pseudomonas 135	M, 0.5% (v/v)	NH_4^+	37	100	0	Daniel *et al.*, 1992
		Mg^{2+}	42.5	100	0	
		PO_4^{3-}	34.5	100	0	

A = acetate, B = butyric acid, F = fructose, G = glucose, GL = gluconate, M = methanol, L = lactate, P = propionate, S = succinate, V = valerate, VA = valine, NR = not reported

APPENDIX C

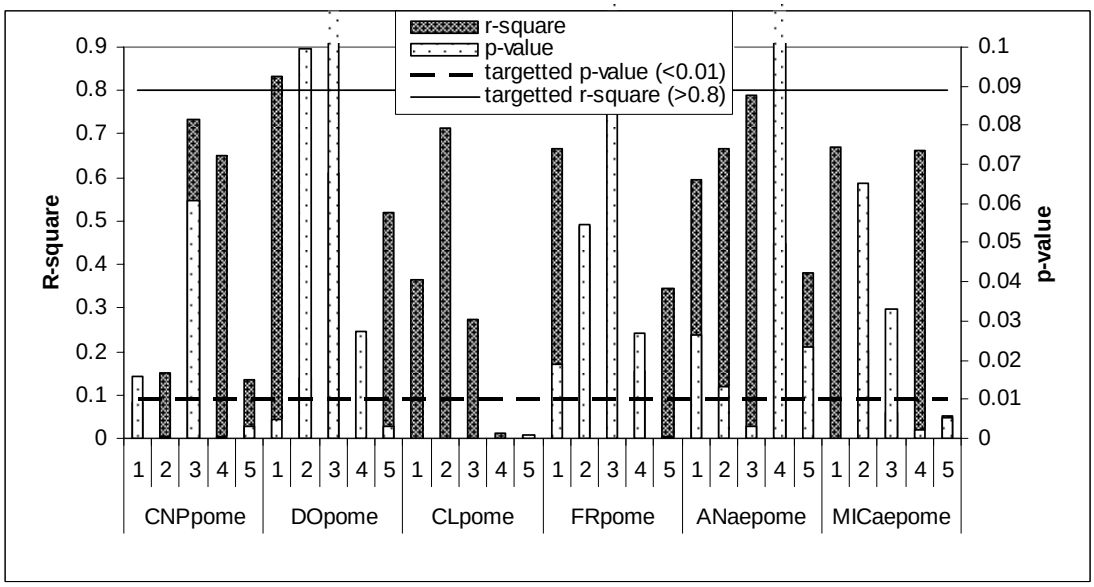


Figure E1: ANOVA single factor and regression analysis on PHA production for POME cultivation

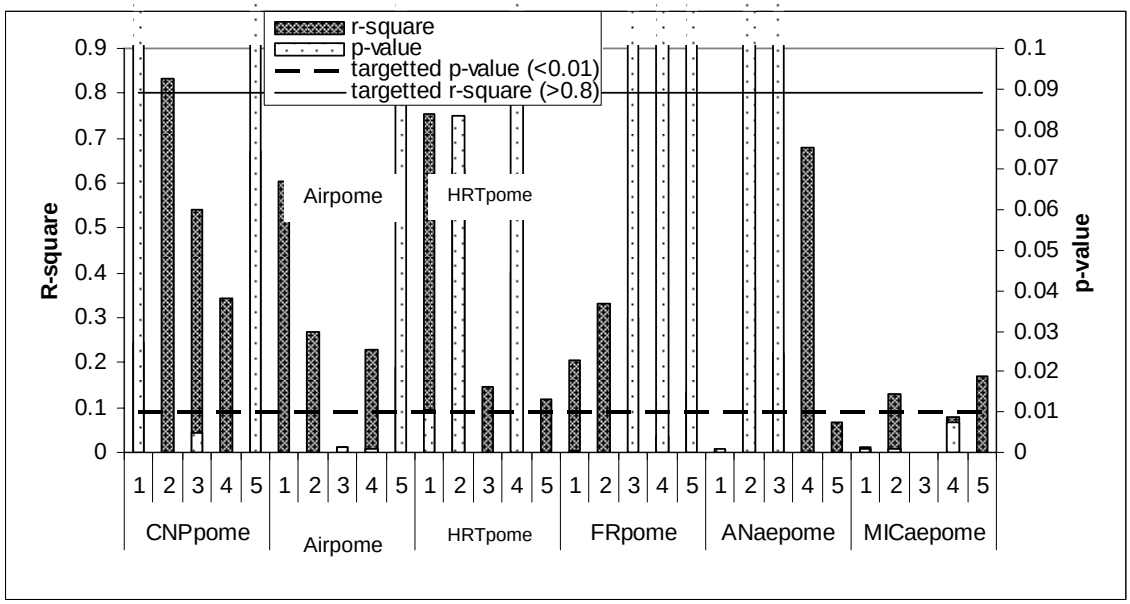


Figure E2: ANOVA single factor and regression analysis on TOC removal for POME cultivation

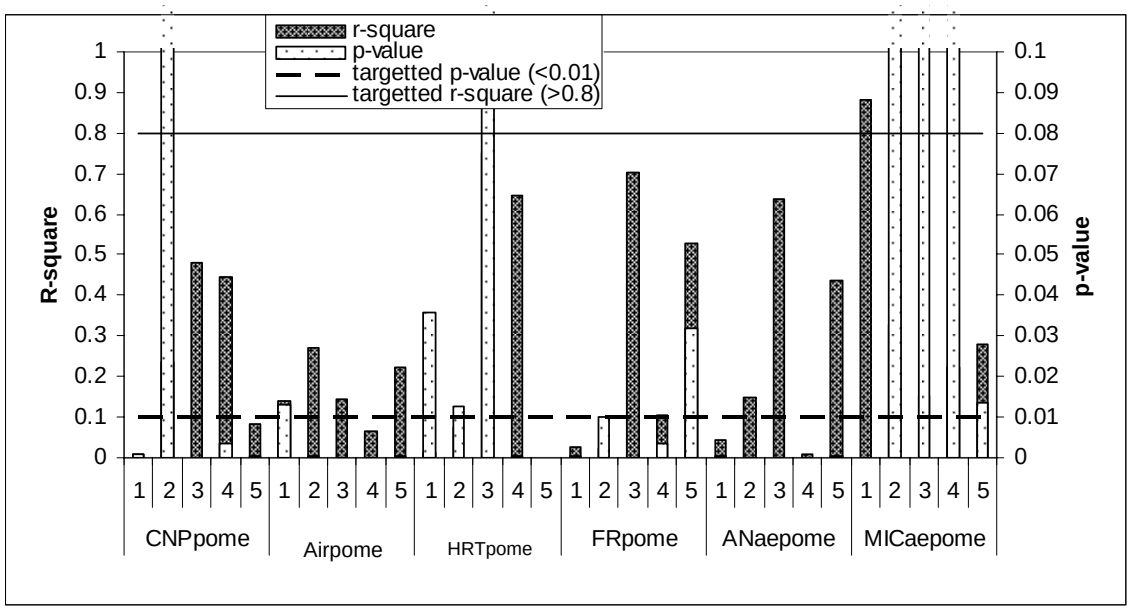


Figure E3: ANOVA single factor and regression analysis on PO_4 removal for POME cultivation

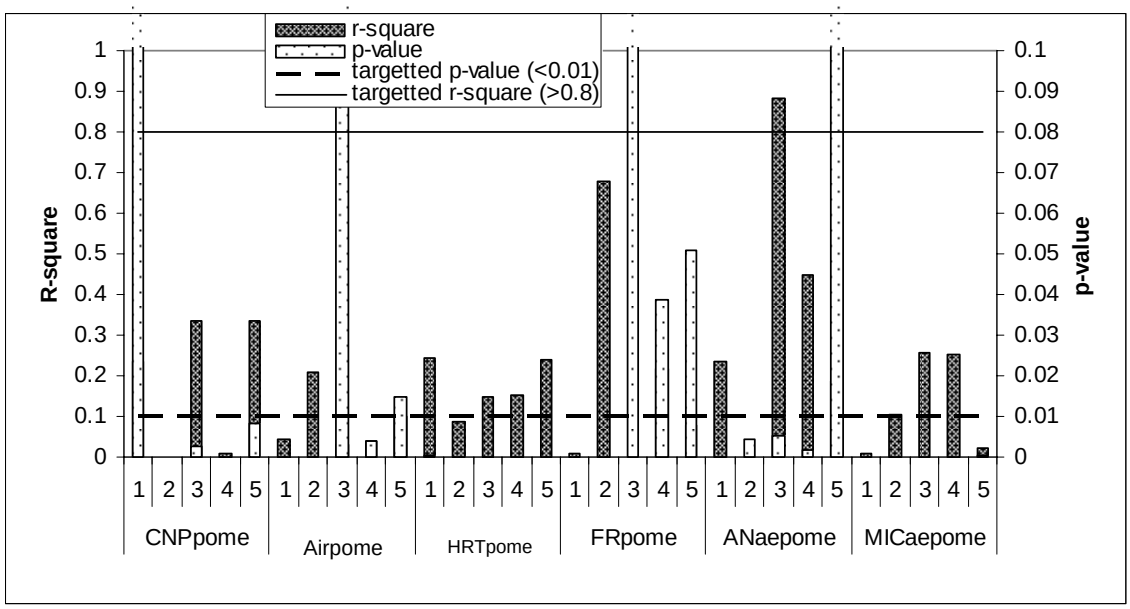


Figure E4: ANOVA single factor and regression analysis on NO_3 removal for POME cultivation

Table E1: Rank and percentile analysis for PHA production, TOC, PO_4 and NO_3 removal at limitation of N and P

Exp.	*%PHA*	*Rank*	*Percent*	*Exp.*	*%TOC*	*Rank*	*Percent*
CNPpome-3	24.54	1	100.00%	CNPpome-4	19.41	1	100.00%
CNPpome-4	23.04	2	75.00%	CNPpome-5	15.03	2	75.00%
CNPpome-1	22.60	3	50.00%	CNPpome-2	8.07	3	50.00%
CNPpome-5	22.41	4	25.00%	CNPpome-1	0.23	4	25.00%
CNPpome-2	22.35	5	0.00%	CNPpome-3	-7.18	5	0.00%

Exp	*%PO_4*	*Rank*	*Percent*	*Exp.*	*%NO_3*	*Rank*	*Percent*
CNPpome-3	13.81	1	100.00%	CNPpome-5	23.80	1	100.00%
CNPpome-1	5.73	2	75.00%	CNPpome-4	21.60	2	75.00%
CNPpome-2	0.08	3	50.00%	CNPpome-1	-1.42	3	50.00%
CNPpome-4	-11.63	4	25.00%	CNPpome-3	-25.88	4	25.00%
CNPpome-5	-12.77	5	0.00%	CNPpome-2	-41.94	5	0.00%

Table E2: Rank and percentile analysis for PHA production, TOC, PO_4 and NO_3 removal at controlling oxygen flowrates

Exp.	*%PHA*	*Rank*	*Percent*	*Exp.*	*%TOC*	*Rank*	*Percent*
Airpome-2	42.16	1	100.00%	Airpome-4	38.67	1	100.00%
Airpome-1	39.72	2	75.00%	Airpome-1	26.88	2	75.00%
Airpome-3	30.01	3	50.00%	Airpome-3	24.84	3	50.00%
Airpome-4	21.32	4	25.00%	Airpome-2	23.98	4	25.00%
Airpome-5	12.14	5	0.00%	Airpome-5	2.79	5	0.00%

Exp.	*%PO_4*	*Rank*	*Percent*	*Exp.*	*%NO_3*	*Rank*	*Percent*
Airpome-4	44.40	1	100.00%	Airpome-2	45.80	1	100.00%
Airpome-3	33.73	2	75.00%	Airpome-3	-2.60	2	75.00%
Airpome-2	14.81	3	50.00%	Airpome-4	-16.75	3	25.00%
Airpome-5	14.21	4	25.00%	Airpome-5	-16.75	3	25.00%
Airpome-1	2.09	5	0.00%	Airpome-1	-23.28	5	0.00%

Table E3: Rank and percentile analysis for PHA production, TOC, PO_4 and NO_3 removal at cycle length behaviour

Exp.	*%PHA*	*Rank*	*Percent*	*Exp.*	*%TOC*	*Rank*	*Percent*
Airpome-3	56.98	1	100.00%	Airpome-5	43.87	1	100.00%
Airpome-2	53.60	2	75.00%	Airpome-3	11.35	2	75.00%
Airpome-1	52.81	3	50.00%	Airpome-4	6.52	3	50.00%
Airpome-4	36.97	4	25.00%	Airpome-2	2.56	4	25.00%
Airpome-5	28.30	5	0.00%	Airpome-1	1.51	5	0.00%

Exp.	*%PO_4*	*Rank*	*Percent*	*Exp.*	*%NO_3*	*Rank*	*Percent*
Airpome-5	30.90	1	100.00%	Airpome-5	-17.33	1	100.00%
Airpome-1	21.80	2	75.00%	Airpome-2	-23.16	2	75.00%
Airpome-4	16.74	3	50.00%	Airpome-1	-35.37	3	50.00%
Airpome-2	13.11	4	25.00%	Airpome-3	-55.44	4	25.00%
Airpome-3	9.93	5	0.00%	Airpome-4	-63.13	5	0.00%

Table E4: Rank and percentile analysis for PHA production, TOC, PO_4 and NO_3 removal at feeding rate mechanisms

Exp.	*%PHA*	*Rank*	*Percent*	*Exp.*	*%TOC*	*Rank*	*Percent*
FRpome-1	40.07	1	100.00%	FRpome-1	21.67	1	100.00%
FRpome-2	27.26	2	75.00%	FRpome-2	14.75	2	75.00%
FRpome-5	23.48	3	50.00%	FRpome-3	7.81	3	50.00%
FRpome-3	21.99	4	25.00%	FRpome-4	3.68	4	25.00%
FRpome-4	12.31	5	0.00%	FRpome-5	1.91	5	0.00%

Exp.	*%PO_4*	*Rank*	*Percent*	*Exp.*	*%NO_3*	*Rank*	*Percent*
FRpome-2	34.36	1	100.00%	FRpome-2	15.95	1	100.00%
FRpome-4	27.62	2	75.00%	FRpome-1	10.92	2	75.00%
FRpome-3	23.68	3	50.00%	FRpome-5	6.68	3	50.00%
FRpome-1	17.36	4	25.00%	FRpome-4	4.68	4	25.00%
FRpome-5	2.73	5	0.00%	FRpome-3	-3.80	5	0.00%

Table E5: Rank and percentile analysis for PHA production, TOC, PO_4 and NO_3 removal at anoxic/aerobic conditions

Exp.	*%PHA*	*Rank*	*Percent*	*Exp.*	*%TOC*	*Rank*	*Percent*
ANaepome-4	63.38	1	100.00%	ANaepome-4	33.43	1	100.00%
ANaepome-5	62.17	2	75.00%	ANaepome-5	29.64	2	75.00%
ANaepome-2	56.86	3	50.00%	ANaepome-1	18.49	3	50.00%
ANaepome-3	50.17	4	25.00%	ANaepome-2	11.93	4	25.00%
ANaepome-1	49.01	5	0.00%	ANaepome-3	1.53	5	0.00%

Exp.	*%PO4*	*Rank*	*Percent*	*Exp.*	*%NO3*	*Rank*	*Percent*
ANaepome-4	35.33	1	100.00%	ANaepome-1	28.45	1	100.00%
ANaepome-3	32.91	2	75.00%	ANaepome-2	24.77	2	75.00%
ANaepome-2	20.90	3	50.00%	ANaepome-3	4.75	3	50.00%
ANaepome-5	17.77	4	25.00%	ANaepome-5	-8.24	4	25.00%
ANaepome-1	12.21	5	0.00%	ANaepome-4	-13.64	5	0.00%

Table E6: Rank and percentile analysis for PHA production, TOC, PO_4 and NO_3 removal at microaerophilic/aerobic conditions

Exp.	*%PHA*	*Rank*	*Percent*	*Exp.*	*%TOC*	*Rank*	*Percent*
MICaepome-1	74.06	1	100.00%	MICaepome-2	25.96	1	100.00%
MICaepome-2	68.26	2	75.00%	MICaepome-5	25.95	2	75.00%
MICaepome-3	57.09	3	50.00%	MICaepome-1	18.42	3	50.00%
MICaepome-4	52.55	4	25.00%	MICaepome-4	18.01	4	25.00%
MICaepome-5	49.98	5	0.00%	MICaepome-3	17.94	5	0.00%

Exp.	*%PO4*	*Rank*	*Percent*	*Exp.*	*%NO3*	*Rank*	*Percent*
MICaepome-5	20.48	1	100.00%	MICaepome-4	19.19	1	100.00%
MICaepome-4	2.45	2	75.00%	MICaepome-3	15.12	2	75.00%
MICaepome-3	1.63	3	50.00%	MICaepome-2	13.53	3	50.00%
MICaepome-2	-2.92	4	25.00%	MICaepome-5	9.00	4	25.00%
MICaepome-1	-10.64	5	0.00%	MICaepome-1	8.03	5	0.00%

E1: Response Surface Regression: %PHA versus CODNP, Air, HRT, FR

```
The analysis was done using uncoded units.
```

Estimated Regression Coefficients for %PHA

```
Term                Coef     SE Coef          T      P
Constant         -861.9     609.895     -1.413  0.178
CODNP               1.3       0.773      1.692  0.111
Air               173.4      93.475      1.855  0.083
HRT                37.7      46.473      0.811  0.430
FR                 12.3      15.457      0.798  0.437
CODNP*CODNP        -0.0       0.000     -1.759  0.099
Air*Air           -12.7       8.114     -1.560  0.140
HRT*HRT            -0.5       1.141     -0.423  0.679
FR*FR               0.0       0.183      0.158  0.877
CODNP*Air          -0.1       0.080     -1.325  0.205
CODNP*HRT          -0.0       0.030     -0.237  0.816
CODNP*FR           -0.0       0.012     -0.658  0.520
Air*HRT            -4.0       3.984     -0.996  0.335
Air*FR             -1.2       1.593     -0.748  0.466
HRT*FR             -0.5       0.598     -0.815  0.428

S = 5.975       R-Sq = 79.6%     R-Sq(adj) = 63.4%
```

Analysis of Variance for %PHA

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	1019.71	1019.71	72.836	2.04	0.092
Linear	4	675.20	214.86	53.715	1.50	0.251
Square	4	185.26	185.26	46.315	1.30	0.315
Interaction	6	159.24	159.24	26.541	0.74	0.624
Residual Error	15	535.60	535.60	35.707		
Lack-of-Fit	10	512.04	512.04	51.204	10.87	0.008
Pure Error	5	23.55	23.55	4.711		
Total	29	1555.30				

Unusual Observations for %PHA

Observation	%PHA	Fit	SE Fit	Residual	St Resid
23	35.220	46.847	4.564	-11.627	-3.01R

R denotes an observation with a large standardized residual.

E2: Response Surface Regression: %TOC versus CODNP, Air, HRT, FR

The analysis was done using uncoded units.

Estimated Regression Coefficients for %TOC

Term	Coef	SE Coef	T	P
Constant	-356.3	315.611	-1.129	0.277
CODNP	0.2	0.400	0.545	0.594
Air	56.2	48.372	1.161	0.264
HRT	17.1	24.049	0.712	0.487
FR	10.5	7.999	1.313	0.209
CODNP*CODNP	-0.0	0.000	-0.813	0.429
Air*Air	-1.4	4.199	-0.326	0.749
HRT*HRT	-0.2	0.590	-0.369	0.717
FR*FR	-0.1	0.094	-0.644	0.529
CODNP*Air	-0.0	0.041	-0.137	0.893
CODNP*HRT	0.0	0.015	0.170	0.867
CODNP*FR	-0.0	0.006	-0.283	0.781
Air*HRT	-1.8	2.061	-0.897	0.384
Air*FR	-0.8	0.825	-1.027	0.321
HRT*FR	-0.3	0.309	-1.043	0.313

S = 3.092 R-Sq = 51.4% R-Sq(adj) = 6.1%

Analysis of Variance for %TOC

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	151.867	151.867	10.848	1.13	0.404
Linear	4	112.922	28.207	7.052	0.74	0.581
Square	4	9.542	9.542	2.386	0.25	0.905
Interaction	6	29.404	29.404	4.901	0.51	0.790
Residual Error	15	143.428	143.428	9.562		
Lack-of-Fit	10	121.374	121.374	12.137	2.75	0.138
Pure Error	5	22.053	22.053	4.411		
Total	29	295.295				

Unusual Observations for %TOC

Observation	%TOC	Fit	SE Fit	Residual	St Resid
23	10.600	15.688	2.362	-5.088	-2.55R
25	14.300	18.554	2.362	-4.254	-2.13R

R denotes an observation with a large standardized residual.

E3: Response Surface Regression: %PO4 versus CODNP, Air, HRT, FR

The analysis was done using uncoded units.

Estimated Regression Coefficients for %PO4

Term	Coef	SE Coef	T	P
Constant	561.71	371.495	1.512	0.151
CODNP	-0.81	0.471	-1.719	0.106
Air	-94.60	56.937	-1.662	0.117
HRT	-10.84	28.307	-0.383	0.707
FR	-17.82	9.415	-1.893	0.078
CODNP*CODNP	0.00	0.000	1.258	0.228
Air*Air	5.71	4.942	1.156	0.266
HRT*HRT	-0.26	0.695	-0.373	0.714
FR*FR	0.04	0.111	0.346	0.734
CODNP*Air	0.00	0.049	0.037	0.971
CODNP*HRT	0.01	0.018	0.470	0.645
CODNP*FR	0.01	0.007	1.589	0.133
Air*HRT	3.09	2.426	1.273	0.222
Air*FR	1.26	0.971	1.294	0.215
HRT*FR	0.50	0.364	1.370	0.191

S = 3.640 R-Sq = 65.9% R-Sq(adj) = 34.0%

Analysis of Variance for %PO4

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	383.706	383.706	27.4076	2.07	0.087
Linear	4	238.474	106.130	26.5324	2.00	0.146
Square	4	40.306	40.306	10.0766	0.76	0.567
Interaction	6	104.926	104.926	17.4877	1.32	0.308
Residual Error	15	198.716	198.716	13.2477		
Lack-of-Fit	10	197.953	197.953	19.7953	129.66	0.000
Pure Error	5	0.763	0.763	0.1527		
Total	29	582.422				

Unusual Observations for %PO4

Observation	%PO4	Fit	SE Fit	Residual	St Resid
3	6.540	1.175	2.780	5.365	2.28R
23	4.200	-0.709	2.780	4.909	2.09R

R denotes an observation with a large standardized residual.

E4: Response Surface Regression: %NO3 versus CODNP, Air, HRT, FR

The analysis was done using uncoded units.

Estimated Regression Coefficients for %NO3

Term	Coef	SE Coef	T	P
Constant	-574.1	341.847	-1.679	0.114
CODNP	0.6	0.433	1.448	0.168
Air	-12.2	52.393	-0.232	0.819
HRT	40.1	26.048	1.539	0.145
FR	5.2	8.664	0.596	0.560
CODNP*CODNP	-0.0	0.000	-1.566	0.138
Air*Air	-5.8	4.548	-1.284	0.219
HRT*HRT	-1.0	0.640	-1.515	0.151
FR*FR	-0.1	0.102	-0.616	0.547
CODNP*Air	-0.0	0.045	-0.643	0.530
CODNP*HRT	-0.0	0.017	-0.501	0.624
CODNP*FR	0.0	0.007	0.029	0.977
Air*HRT	1.5	2.233	0.679	0.508
Air*FR	0.5	0.893	0.567	0.579
HRT*FR	-0.1	0.335	-0.441	0.665

S = 3.349 R-Sq = 56.1% R-Sq(adj) = 15.1%

Analysis of Variance for %NO3

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	214.795	214.795	15.3425	1.37	0.277
Linear	4	140.185	40.886	10.2216	0.91	0.483
Square	4	56.206	56.206	14.0516	1.25	0.331
Interaction	6	18.405	18.405	3.0674	0.27	0.941
Residual Error	15	168.264	168.264	11.2176		
Lack-of-Fit	10	166.366	166.366	16.6366	43.83	0.000
Pure Error	5	1.898	1.898	0.3796		
Total	29	383.059				

Unusual Observations for %NO3

Observation	%NO3	Fit	SE Fit	Residual	St Resid
11	7.500	2.684	2.558	4.816	2.23R
22	2.140	-3.458	2.558	5.598	2.59R
27	-0.200	5.477	2.558	-5.677	-2.63R

R denotes an observation with a large standardized residual.

E5: Response Optimization

Parameters

	Goal	Lower	Target	Upper	Weight	Import
%PHA	Target	60	66.0	70	1	3
%TOC	Target	15	19.0	22	1	3
%PO4	Minimum	-10	-10.0	3	1	3
%NO3	Target	-10	3.5	5	1	3

Starting Point

CODNP = 600.0
Air = 0.5
HRT = 18.0
FR = 20.0

Local Solution

CODNP = 500.125
Air = 0.500
HRT = 20.000
FR = 20.000

Predicted Responses

%PHA = 65.4126, desirability = 0.90210

```
%TOC         =  19.2020, desirability =  0.93267
%PO4         =  -8.9443, desirability =  0.91879
%NO3         =   2.5342, desirability =  0.92846

Composite Desirability =  0.92043

Global Solution

CODNP        =  500.125
Air           =    0.500
HRT          =   20.000
FR           =   20.000

Predicted Responses

%PHA         =  65.4126, desirability =  0.90210
%TOC         =  19.2020, desirability =  0.93267
%PO4         =  -8.9443, desirability =  0.91879
%NO3         =   2.5342, desirability =  0.92846

Composite Desirability =  0.92043
```