DEVELOPMENT AND MICROBIAL CHARACTERIZATION OF AEROBIC GRANULATION USING PALM OIL MILL EFFLUENT (POME)

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Doctor of Philosophy (Bioscience)

Faculty of Biosciences and Bioengineering
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JUNE 2012

...to my lovely sons Adam and Harris

husband Dr. Muhamad Ali Muhammad Yuzir

and our future newborn

with lots of love and shiny sparkles…

ACKNOWLEDGEMENTS

Alhamdulillah, I am thankful to Allah S.W.T., the Almighty, the Most Merciful, for His blessings and guidance throughout my PhD journey.

A world of thanks to my supervisor Dato' Professor Zaini Ujang, for I am indebted to his continuous support and motivation, endless constructive comments and suggestions. It was such an honor to be working under the supervision of the UTM Vice Chancellor himself. I couldn't agree more with Professor Zaini that PhD is a beautiful journey indeed. For all the exciting opportunities, publications and novel findings as well as improvements in technical writings, indeed… *a good teacher educates, a great teacher inspires.*

My heartfelt appreciation to my co-supervisor, Dr. Adibah Yahya, whose motivation and inspiration has enabled me to develop an understanding of the molecular and microbial subjects. Thank you for being there when I mostly needed an overnight advice regarding the phenolic compounds, or whenever I am lost for words to describe the microbes. Your endless support and guidance were priceless! My gratitude and thanks to my external supervisor, Professor Thomas Curtis at Newcastle University, United Kingdom. Thank you for having me in your microbiology laboratory for DGGE and FISH experiments. Thank you for introducing me to the excitements of software and statistical analyses! I couldn't be more excited with the fractal theory and similarity indices. For all the knowledge, meetings, 13 hours flight to UTM and back, you are simply the best!

Special thanks to the Department of Industrial Biotechnology and laboratory technicians at Faculty of Biosciences and Bioengineering, UTM especially Ms. Wan Aznida and Puan Fatimah, for their help and support. I would like to thank the Human Resource Department of UTM for their smooth organization in making my PhD internship at Newcastle University a success. To all my friends at C08 and IPASA UTM, thank you for your motivation, kindness, help and support throughout.

I would also like to thank special people at Cassie Building, Newcastle University namely Mrs Fiona Read, for her help and support during FISH and DGGE experiments. Thank you to Dr. Russell Davenport for his expert advice and guidance on molecular experiments. To Ms. Micol Belluci for her endless help and support on my FISH experiments. Thank you to Dr. Trevor Booth at the School of Medical Sciences, Newcastle University for his help with viewing my FISH slides and aerobic granular samples on the Confocal Laser Scanning Microscope (CLSM). To my postgraduate friends, thank you for your kindness and friendship during my stay in the postgraduate suite (PGR) at Cassie Building.

To my parents Abdullah Sitam and Siti Hajal Haron, this thesis is for both of you. Thank you for your endless prayers, motivation and support. To all my family, thank you for your support and understanding.

To my husband Dr. Muhamad Ali Muhammad Yuzir and my sons Adam Muhamad Ali & Harris Muhamad Ali… this thesis is for you.

ABSTRACT

Palm oil is widely used for food and non-food manufacturing industries and as a biofuel. The production of palm oil generates a large amount of solid and liquid wastes in the form of empty fruit bunch (EFB) and palm oil mill effluent (POME), respectively. As POME contributes to a high pollution process waste, the need to find an efficient and practical approach to preserve the environment is essential. Novel aerobic granular sludge is a compact consortium of self-immobilized bacteria with high rate biological wastewater treatability. This study is aimed at investigating aerobic granular sludge formation in lab-scale sequencing batch reactor (SBR) using POME as substrate. The efficiency of aerobic granular sludge developed for the treatment of POME in corresponds to the structure of bacterial population is monitored. Aerobic granular sludge was developed at volumetric exchange rate (VER) of 50% and cycle duration of 3 hours at flow rate of 3 Lm^{-1} in reactors R1, R2 and R3 operated at OLR of 1.5, 2.5 and 3.5 kgCOD $m^{-3}d^{-1}$, respectively. Shock load conditions were introduced by increasing the OLR in R2 from 2.5 to 19 kgCOD $m^{-3}d^{-1}$. Aerobic granular sludge was successfully formed at an OLR of 2.5 and 3.5 kgCOD $m^{-3}d^{-1}$, respectively while bioflocs remained dominant in R1. Under shock loading rate, aerobic granular sludge was partially disintegrated due to mass transfer limitation within the granule. The average sizes of granules were between 1.0 mm to 4.0 mm. Upon steady state, COD removal efficiency of greater than 80% was recorded for all reactors. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and fluorescence *in situ* hybridisation (FISH) techniques were used to show that the bacterial population changes during aerobic granular sludge formation at different OLRs. The average Raup and Crick similarity indices obtained during the acclimation/granulation and the maturation phases were 0.95 ± 0.02 and 0.72 ± 0.06, respectively. Mature granules maintained good settling property and dominant granular morphology as evidenced by low SVI of 19.9 mL g^{-1} SS at 3.5 kgCOD $m^{-3}d^{-1}$. Visible bands from the DGGE profiles indicated the shift of bacterial population during the aerobic granulation process. *Comamonadaceae* sp., *Leadbetterella* sp. and *Runella* sp. are dominant bacteria that consistently present throughout the granulation process. Distribution of AOB in mature granule was confirmed by FISH technique thus contributing to efficient removal of ammonia in POME of > 80%. Despite the different OLRs, aerobic granular sludge formation was successfully achieved for the treatment of high strength wastewater such as POME.

ABSTRAK

Minyak sawit digunakan secara meluas di dalam industri pembuatan bahan makanan dan bahan bukan makanan dan juga sebagai bio-bahan api. Penghasilan minyak sawit menghasilkan sejumlah besar sisa pejal dan cecair di dalam bentuk tandan kelapa sawit kosong (EFB) dan air sisa minyak sawit atau ringkasnya *palm oil mill effluent* (POME). Memandangkan POME menyumbang kepada sisa cemar pemprosesan yang tinggi, keperluan untuk mendapatkan pendekatan olahan yang praktikal untuk melindungi alam sekitar adalah penting. Butiran enapcemar aerobik merupakan konsortium yang padat dengan bakteria yang mempunyai kadar olahan airsisa biologi yang tinggi. Kajian ini menjurus kepada pembentukan butiran enapcemar aerobik dengan menggunakan POME sebagai substrat di dalam *sequencing batch reactor* (SBR). Keberkesanan butiran enapcemar aerobik yang terbentuk untuk olahan POME bersesuaian dengan struktur populasi bakteria telah di pantau. Butiran enapcemar aerobik terbentuk pada nisbah pertukaran volumetrik (VER) sebanyak 50% dan kitaran selama 3 jam dan kadaralir sebanyak 3 Lm^{-1} di dalam tiga reaktor iaitu R1, R2 dan R3 yang beroperasi pada kadar suapan organik sebanyak 1.5, 2.5 dan 3.5 kgCOD $m^{-3}d^{-1}$. Keadaan beban kejutan dikenakan secara meningkatkan kadar suapan organik di dalam R2 daripada 2.5 ke 19 kgCOD $m^{-3}d^{-1}$. Butiran enapcemar aerobik berjaya dibentuk pada kadar suapan organik sebanyak 2.5 dan 3.5 kgCOD $m^{-3}d^{-1}$, walaubagaimanapun bioflok kekal mendominasi R1. Pada kadar masukan tinggi, butiran enapcemar aerobik berintegrasi disebabkan had pemindahan jisim di dalam butiran. Saiz purata butiran adalah di antara 1.0 mm hingga 4.0 mm. Pada paras stabil, permintaan oksigen biokimia (COD) adalah melebihi 80% bagi kesemua reaktor walaupun pada kadar masukan yang berbeza. Teknik molekular piawai seperti *polymerase chain reaction-denaturing gradient gel electrophoresis* (PCR-DGGE) dan *fluorescence in situ hybridisation* (FISH) telah digunakan bagi menyelidiki struktur microbial butiran enapcemar aerobik. Purata kebersamaan *Raup dan Crick* yang didapati semasa proses aklimasi/pembutiran dan pematangan butiran enapcemar aerobik adalah 0.95 ± 0.02 dan 0.72 ± 0.06. Butiran yang matang mempunyai kemampuan enapcemar dan morfologi butiran yang baik berdasarkan paras SVI 19.9 mL g^{-1} SS pada kadar masukan 3.5 kgCOD $m^{-3}d^{-1}$. Profil DGGE menunjukkan struktur mikrobial berubah secara signifikan semasa proses pembutiran. *Comamonadaceae* sp., *Leadbetterella* sp. dan *Runella* sp. telah diperolehi sepanjang proses pembutiran. Pembahagian spatial AOB di dalam butiran enapcemar aerobik juga terbukti melalui FISH yang menyumbang kepada penyingkiran kandungan ammonia di dalam POME yang melebihi 80%. Walaupun pada kadar masukan berbeza, butiran enapcemar aerobik telah berjaya dibentuk untuk perawatan air sisa berkadaran tinggi seperti POME.

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LIST OF ABBREVIATIONS

AGS	-	Aerobic granular sludge
AGS_P	-	Photosynthetic aerobic granular sludge
AIM	-	Agensi Inovasi Malaysia
AN	-	Ammoniacal nitrogen
AOB	-	Ammonia oxidizing bacteria
APHA	-	American Public Health Association
BAS	-	Biofilm airlift suspension
BLASTn	-	Basic Local Alignment Search Tool - nucleotide
BOD	-	Biochemical oxygen demand
CDM	-	Clean development mechanism
CER	-	Certified emission reduction
CLSM	-	Confocal laser scanning microscopy
COD	-	Chemical oxygen demand
CSTR	-	Continuous stirred tank reactor
DGGE	-	Denaturing gradient gel electrophoresis
DNA	-	Deoxyribonucleic acid
DO	-	Dissolved oxygen
DOE	-	Department of Environment
EBPR	-	Enhanced biological phosphorus removal
EDM	-	Euclidean distance map
EDTA	-	Ethylenediaminetetraacetic acid
EPS	-	Extracellular polymeric substances
EQA	-	Environmental Quality Act
FA	-	Formamide

FFB	-	Fresh fruit bunches
FISH	-	Fluorescence *in situ* Hybridization
HB	-	Hybridisation buffer
HRT	-	Hydraulic retention time
MLSS	-	Mixed liquor suspended solid
MLVSS	-	Mixed liquor volatile suspended solid
MPOB	-	Malaysian Palm Oil Board
NMDS	-	Non metric multidimensional scaling
OLR	-	Organic loading rate
PAO	-	Polyphosphate-accumulating bacteria
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PFA	-	Paraformaldehyde
POME	-	Palm oil mill effluent
PORE	-	Palm oil refinery effluent
RNA	-	Ribonucleic acid
SBAR	-	Sequencing batch airlift reactor
SBR	-	Sequencing batch reactor
SDS	-	Sodium dodecyl sulphate
SEM	-	Scanning electron microscopy
SRT	-	Sludge retention time
SS	-	Suspended solid
SVI	-	Sludge volume index
TAE	-	Tris-acetate-EDTA
TEMED	-	Tetramethylethylenediamine
TN	-	Total nitrogen
TSS	-	Total suspended solid
UASB	-	Up-flow anaerobic sludge blanket
UASFF	-	Up-flow anaerobic sludge fixed-film
UFF	-	Up-flow fixed film
UPGMA	-	Unweighted pair group method with Arithmetic Mean
UTM	-	Universiti Teknologi Malaysia

UV	-	Ultraviolet
VER	-	Volumetric exchange rate
WB	-	Washing buffer
16s rRNA	-	16 Sequencing Ribosomal Ribonucleic Acid

LIST OF SYMBOLS

b(S)	-	gray-levels distribution
Ca^{2+}	-	calcium
$CaCO_3$	-	calcium carbonate
CO_2	-	carbon dioxide (mgl^{-1} or mmolL^{-1})
COD_{feed}	-	substrate strength in terms of COD concentration (mgL^{-1})
CTO654r	-	reverse AOB specific primer
CTO189f	-	forward AOB specific primer
ddH_2O	-	double sterile water
D_f	-	fractal dimension
H/D	-	column height to diameter ratio (mm)
K_2HPO_4	-	dipotassium phosphate
Mg^{2+}	-	magnesium
$MgCl_2$	-	magnesium chloride
M_w	-	molecular weight
N	-	nitrogen (mgL^{-1})
NaCl	-	sodium chloride (mL)
NaOH	-	sodium hydroxide
NH_4Cl	-	ammonium chloride
NH_4-N	-	ammonia
P	-	phosphorus (mgL^{-1})
PO_4-P	-	phosphate
P/COD	-	phosphorus to chemical oxygen demand ratio
P(S)	-	perimeter
Q	-	substrate flow rate (Ld^{-1})

SS_0	-	total amount of granular sludge
SS_t	-	amount of sludge solids in supernatant after *t* min
T	-	temperature (°C)
Tris-HCL	-	tris-sodium hydrochloride
Vr	-	reverse PCR primer
Vf	-	forward PCR primer
V_r	-	working volume of reactor (*L*)
V_{pome}	-	volume of POME (mL)
$V_{tapwater}$	-	volume of tap water (mL)
Vs	-	settling velocity of a particle
§	-	integrity coefficient
d_p	-	diameter of a particle
µ	-	viscosity of a solution
ρ_p	-	density of a particle
ρ	-	density of a solution

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

Oil palm is the most important agro-based crop of Malaysia. Malaysia's palm oil plantations are spread out across both Peninsular and East Malaysia. The total acreage under cultivation grew from 3.38 hectares in 2008 to 4.85 million hectares in 2010 (MPOB, 2011). Palm oil is widely used for food and non-food manufacturing industries and as a biofuel. However, the production of palm oil generates a large amount of solid and liquid wastes in the form of empty fruit bunch (EFB) and palm oil mill effluent (POME), respectively. Malaysia's palm oil industry produced almost 80 million dry tonnes of solid biomass per annum (Agensi Inovasi Malaysia, 2011). This volume is projected to increase to 85 – 110 million dry tonnes by 2020. Similarly, POME volumes are expected to increase from 60 million tonnes today to 70 – 110 million tonnes by 2020. In the year 2008 at least 44 million tonnes of POME was generated in Malaysia and the number is expected to increase in subsequent year (Wu *et al.*, 2010).

Direct discharge of POME into the environment is legally prohibited due to the high chemical oxygen demand (COD) and biological oxygen demand (BOD_5) concentrations of up to 50,000 mgL^{-1} and 25,000 mgL^{-1}, respectively. Furthermore the Deparment of Environment (DOE) Malaysia has enacted the effluent discharge standards which requires POME to be treated prior to discharging into the receiving watercourses. The untreated POME is to comply with legislation limits of BOD_5 of 20 mgL^{-1} for Standard A as outlined in the Fifth Schedule Paragraph 11(1) (a) Environmental Quality (Industrial Effluents) Regulations 2009 (Federal Subsidiary Legislation, 1974). The new regulations also outlined the effluent discharge standard to comply with color discharge of 100 ADMI. Therefore, color removal is fast becoming an important research parameter related to industrial wastewater treatments. Additionally, in recent years, the significance of technological improvements in handling of residues from palm oil production compared to conventional practices are also being increasingly addressed through application of life cycle assessment (LCA) tools (Hansen *et al.*, 2012).

Conventional ponding systems have been the most popular method used for more than 85% of the palm oil mills in Malaysia in 1980s (Ma *et al.,* 1993). However, the introduction of a more compact, mechanical and efficient system has been widespread since 2008 following the implementation of the new effluent discharge standard of BOD_5 of 20 mgL^{-1} as described above. Novel POME treatment methods have since been proposed including anaerobic treatment technologies and high-rate anaerobic digesters (Poh and Chong, 2009; Damayanti *et al.,* 2009; Ujang and Buckley, 2002).

Activated sludge reactor is generally the most common alternative for aerobic system as post-treatment following anaerobic digestions to overcome the area limitation of conventional pond systems. There are several positive outlooks towards the implementation of activated sludge processes in Malaysia particularly its compatibility and technical availability to convert from ponds to activated sludge. The activated sludge reactors are also relatively simple to handle or operate. With the knowledge and skills available in Malaysia, particularly in sewage and industrial wastewater, thus activated sludge became an indigenous technology in Malaysia. Vijayaraghavan *et al.* (2007) investigated the treatment of POME using aerobic oxidation which indicates the effectiveness of aerobic oxidation for diluted POME having corresponding COD of 3925 mgL^{-1}, based on an activated sludge process. Chan *et al.* (2010) further investigated the aerobic treatment of anaerobically digested POME by using a lab-scale sequencing batch reactor (SBR) and found a maximum of COD (95-96%) and BOD (97-98%) removal efficiencies were achieved at optimum OLR and MLVSS concentration ranging from 1.8 to 4.2 kgCOD $m^{-3}d^{-1}$ and 22,000 to 25,000 mgL^{-1}, respectively. However, the present knowledge and expertise in activated sludge process is limited to disperse sludge configurations. Recent development of this technology has been forming a more compact formation of activated sludge which is termed as granulation.

Granulation was first achieved for methanogenic sludge in upflow anaerobic sludge blanket (UASB) reactors (Lettinga *et al.*, 1980). Although granulation technology has been applied and investigated in the anaerobic treatment of wastewater over the past decades, aerobic granulation is still a relatively new technology. Aerobic granulation technology has been widely used in various formulations (Arrojo *et al.*, 2004; Wang *et al.*, 2007a; Kishida *et al.*, 2009). Most studies on aerobic granulation were developed in SBR systems (Morgenroth *et al.*, 1997; Beun *et al.*, 1999; Qin *et al.*, 2004; Schwarzenbeck *et al.*, 2005; Wang *et al.*, 2007). Research on aerobic granulation has mainly focused on the mechanism of granule formation using synthetic wastes (Morgenroth *et al.*, 1997; Tay *et al.*, 2001a; Tsuneda *et al.*, 2003; de Kreuk and van Loosdrecht, 2006).

For bacteria to form aerobic granules, the contributions of physical, chemical and biological conditions to the granulation process should be collectively considered. A number of factors such as the type of substrate, the loading rate, aeration intensity, and the hydraulic retention time have been previously reported to have influence on the sludge granulation process (Liu and Tay, 2002; Liu and Tay, 2004). However, several other issues regarding aerobic granulation in real wastewater such as the mechanism, crucial operating factors and evolution of the microbial community remain to be addressed.

1.2 Objectives of the Study

Granules are known as a collection of self-immobilized cells into a spherical form or shape. Aerobic granules are generally acknowledged to have a wide range of beneficial properties when compared to conventional activated sludge flocs i.e. strong structure, good settling property, ability to withstand high organic loading rate. Despite numerous literatures dealing with the development of aerobic granular sludge formation, most of these studies were reported on the microbial aggregation using mainly synthetic wastewater as influent. To date, very little studies reported on the development of aerobic granular sludge formation using industrial wastewater which essentially led to several questions about the formation of aerobic granular sludge using POME.

The present study is regarded as the first study dedicated to the development of aerobic granular sludge with special emphasis on utilization of POME as substrates. The main objectives of this study were to investigate the development and microbial characterizations of aerobic granular sludge using POME. Additionally, this study aimed to provide a solid recommendation towards the implementation of aerobic granulation for sustainable and efficient industrial wastewater treatment technologies.

The specific objectives of the experimental study are as follows:

i. To develop aerobic granular sludge using POME. This will be the first study on aerobic granulation utilizing POME as the main substrate;

ii. To determine the physical and microbial characteristics of aerobic granular sludge developed using POME from the perspective of standard experimental tools i.e. fractal dimension and molecular investigations including FISH and DGGE;

iii. To analyze the diversity and evolutionary shift of bacterial population in POME seed sludge during aerobic granulation process. The role of selected bacterial species in the formation of mature granules is proposed;

iv. To investigate the potential application of aerobic granulation technology as an alternative and/or post treatment options for POME.

1.3 Scope of the Study

The present study focused on the development and microbial characterization of aerobic granular sludge using POME. In general, POME is a liquid by-product from sterilization and milling process of fresh fruit bunch (FFB) and accumulates as a liquid waste at the mills. The granulation process was reported in terms of the morphology and settling ability of the cultivated aerobic granular sludge in POME. Reactor performances were observed based on the COD, ammonia and color removal efficiencies during granulation and maturation phases of the granules. The effects of organic loading rate on the aerobic granular sludge formation were investigated by providing different volumetric organic loading of POME ranging from 1.5 kgCOD $m^{-3}d^{-1}$ to 3.5 kgCOD $m^{-3}d^{-1}$. A shock load condition was tested for reactor performance by sudden increase of OLR from 2.5 to 19 kgCOD $m^{-3}d^{-1}$ upon mature granule formation. The investigations also included laboratory tests on the evolution of bacterial population and characterization of bacterial community by using novel molecular techniques including FISH and DGGE which was conducted at Newcastle University, England. The correlation between the bacterial community structure and aerobic granulation was validated using multivariate statistical analysis.

1.4 Significance of the Study

As available space for existing palm oil treatment plant is often limited, more compact treatment systems have been developed to avoid large footprints such as membrane bioreactors (Ujang and Anderson, 1996; Ujang and Anderson, 2000; Ujang *et al.* 2007), biofilm systems (Hall, 1987; Gebara, 1999; Kargi and Eker, 2003; Mohan *et al.*, 2007; Mohan *et al.*, 2008; Lai *et al.*, 2009) and aerobic granular sludge technology (Beun *et al,* 2000; Tay *et al.*, 2004; de Kreuk and van Loosdrecht, 2006; Anuar *et al.*, 2007; Sunil *et al.*, 2008). Several significant issues are identified to give benefits to this study as follows:

i. POME is a highly polluted source of wastewater to water body. An efficient and practical approach for POME treatment is essential to fulfil the requirements for zero discharge and cleaner production as outlined in the National Biomass Strategy Report 2020 (Agensi Inovasi Malaysia, 2011).

ii. As palm oil represents the second largest export of Malaysia, the large amount of palm oil residues resulting from the harvest particularly POME must be managed in a manner that also addresses environmental concerns related to the current POME treatment methods. Aerobic granulation is therefore proposed as an alternative and/or post-treatment method for sustainable and efficient POME treatment strategy.

iii. Aerobic granular sludge is widely known to have several beneficial engineering properties i.e. strong and dense microstructure, ability to withstand high organic loading rates, stable and compact shape making it an increasingly popular choice for recent wastewater treatment methods over the conventional activated sludge methods. This study is regarded as the first study constituting aerobic granular sludge development using POME activated sludge as seeding and raw POME as the main substrate.

iv. Since aerobic granular sludge has been extensively developed using SBR, similar technical approach is used for developing aerobic granular sludge using POME. Several modifications were made to tailor the mechanical acquisition of reactors applicable for POME treatment. From Malaysian perspective as the second largest producer of palm oil in the world, aerobic granular sludge is a promising technology for dealing with the continuous production of liquid wastes at the mills due to its space saving and compactness of reactor specification.

v. This study brings forward an additional concept of cleaner production in activated sludge system by promoting adaptation of aerobic granulation for better effluent quality in POME treatments. By using the SBR, intermittent feast-famine operation will provide the most suitable environment for the growth of microorganisms promoting aerobic granular sludge formation.

vi. The molecular investigations of aerobic granular sludge provides basic understanding in the microbial evolution of the granules developed using POME to further strengthen aerobic granulation technique specifically designed for efficient and sustainable POME treatments.

1.5 Thesis Organization

This thesis is organized into seven chapters. **Chapter 2** provides an overview of relevant literature and covers basic principles of characteristics and treatment of POME, granulation technology in comparison to a conventional activated sludge system and aerobic granulation technology. The granulation process in SBR reactors is also described. This chapter gives the characteristics and properties of aerobic granular sludge and the relation to its chemical, physico-chemical, biological properties, and various applications. Several parameters governing aerobic granular sludge formation were briefly explained. The sequencing batch reactor (SBR) technology was used in order to achieve successful aerobic granular sludge formation.

Chapter 3 describes the laboratory equipments and general experimental procedures including wastewater feed, nutrients and sludge used in the present study. The reactor start-up procedures, operational conditions and molecular analyses used in the present study are also described in this chapter.

Chapters 4, **5** and **6** present the results and discussions of this study. The results are divided into three main chapters; (a) the development of aerobic granular sludge using POME; (b) the effects of organic loading rates on aerobic granulation and (c) bacterial community and structure analyses of aerobic granular sludge developed using POME.

The conclusions from this research are given in **Chapter 7**, stating specific achievement, problems and recommendations. General conclusions are also drawn from the experiences gained during this study, the wider implications of the results and some recommendations for future work.

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