

UTILIZATION OF OIL PALM FROND FOR ENZYME AND RENEWABLE
FEEDSTOCK PRODUCTION USING SOLID STATE FERMENTATION

MOHAMED ROSLAN MOHAMAD IKUBAR

UNIVERSITI TEKNOLOGI MALAYSIA

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FEEDSTOCK PRODUCTION USING SOLID STATE FERMENTATION

MOHAMED ROSLAN MOHAMAD IKUBAR

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DEDICATION

Specially dedicated to all my loved ones:
Atta, Ma, Shida, Rozi & the little one, Diana

~

True knowledge exist in knowing that you know nothing
Socrates

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They say life is all about the journey not the destination. This thesis is the accumulation of my four and half year's journey that involved long hours in the lab, countless mistakes and re-writes. But throughout this journey there are plenty of lessons learned; the good ones were good, but the bad ones were my greatest teacher. For that I must praise the lord, Allah SWT, for giving me the strength, persistence and most importantly patience. Looking back, my decision to do Phd was partly out of self-fulfilment and another because Dr. Adibah Yahya convinced me to do so. Knowing her closely for 8-years now, she is the best supervisor a student can ever wish; her humbleness, expertise, and devotion was truly inspiring. I can't remember a time leaving her office and not feeling motivated. Along the way, I had the chance to work with Dr. Musaalbakri Abdul Manan, who was briefly my co-supervisor. I am greatly indebted to his early comments of my proposal that altered the direction of this thesis. My appreciation also goes to the dynamic Professor Dr. Zainura Zainon and Dr. Che Hafizan, my LCA gurus, for without their assistance I would have been lost in uncharted waters. I would also like to thank Dr. Ang Siow Kuang, Dr. Noratiqah Kamsani and Dr. Huszalina Hussin for guiding me during the early stage of my study. My special mention goes to our beloved senior laboratory officer, Pn. Fatimah Harun, who would go the extra mile to help us with our laboratory needs. To my close buddies Dr. Shankar Ramanathan, Dr. Tengku Idzzan Nadzirah Tengku Idris, Ahmad Fawwaz Mohd Raji, Rohaya Mohd Noor, Zulkifli Nordin, Nur Syifaaiyah Abdul Aziz and Norulsazyani Mohd Safri, thank you for making this journey more colourful and bearable. And yes, how would I forget the caring Associate Professor Dr. Madihah Md Salleh and Professor Dr. Noor Aini Abdul Rashid, both who always *belanja makan* and never failed to ask, "*bila nak habis?*" In the end, I don't regret taking this journey, it was a meaningful one indeed. My heartiest thanks to all of you who have been part of it.

ABSTRACT

Oil palm frond (OPF) is the largest agro-residue and lignocellulose biomass in Malaysia amounting to 56 million tons every year. At present, it is mainly utilized as soil fertilizer and animal feed, whereas excess fronds are commonly burned to save up plantation space and to avoid contamination. Such practices undermine the economic value of OPF and contribute to environmental pollution. Tapping into its rich lignocellulose content, the study investigated bioconversion potential of frond petiole for lignocellulolytic enzyme production. Six locally isolated fungi *A. awamori* MMS4, *A. niger* EFB1, *A. fumigatus* SK1, *T. virens* UKM1, *T. viride* MM3, and *T. asperellum* MR1 were screened for their enzyme production capability under solid state fermentation. The fermentation was carried out in petri dish using OPF fibre (0.125 mm) at 60% moisture content and 35°C for 7 days. The fermentation produced enzyme mixture high in lignin peroxidase (20-222 U_g⁻¹), endoxylanase (6-109 U_g⁻¹) and endoglucanase (2-9 U_g⁻¹), alongside exoglucanase, β-glucosidase, manganese peroxidase and laccase. The multi-enzyme mixture was suitable for technical application and when tested for the enzymatic deinking of old newspaper achieved a pulp brightness of up to 51.5% and tensile strength of 34.0 Nm/g. To avoid generation of secondary waste, the remaining fermented OPF (post enzyme extraction) was re-fermented up to three cycles. This further increased enzyme production by 26-79% and fibre utilization by 14-26%. Such repeated fermentation also proved favourable in improving the sustainability and cost of enzyme production at each cycle. Fermented OPF that were no longer fermentable after three cycles was subjected to nutrient regeneration by enzymatic hydrolysis and autolysis. The process generated a nutrient rich hydrolysate containing carbon (1.79 gL⁻¹), nitrogen (0.11 gL⁻¹) and phosphorus (0.36 gL⁻¹) that were useable as growth medium and supported both bacterial and fungal growth. Overall, the study showed the potential bioconversion of OPF into valuable enzymes and the significance of re-fermentation and hydrolysis process to maximise usage of fermented waste as renewable feedstock.

ABSTRAK

Pelepah sawit (OPF) merupakan sisa agro dan biojisim lignoselulosa terbesar Malaysia dengan hasil buangan mencecah 56 juta tan setiap tahun. Pada masa ini, pelepah sawit digunakan sebagai baja tanah dan makanan haiwan, manakala lebihan pelepah dibakar bagi menjimatkan ruang ladang dan mengelakkan kontaminasi. Amalan sebegini merendahkan nilai ekonomi OPF dan menyumbang kepada pencemaran alam sekitar. Meraih kandungannya yang tinggi lignoselulosa, kajian ini menyiasat potensi bio-olahan pelepah sawit untuk penghasilan enzim lignoselulolitik. Enam kulat pencilan tempatan *A. awamori* MMS4, *A. niger* EFB1, *A. fumigatus* SK1, *T. virens* UKM1, *T. viride* MM3, dan *T. asperellum* MR1 telah disaring keupayaan menghasilkan enzim melalui kaedah fermentasi pepejal. Fermentasi telah dilakukan di dalam piring petri menggunakan gentian OPF (0.125 mm) pada kandungan lembapan 60% dan suhu 35°C selama 7 hari. Proses fermentasi tersebut menghasilkan campuran enzim yang tinggi dengan lignin peroksidase (20-222 U_g⁻¹), endoksilanase (6-109 U_g⁻¹) dan endoglukanase (2-9 U_g⁻¹), serta eksoglukanase, β-glukosidase, mangan peroksidase dan lakkase. Campuran pelbagai enzim tersebut sesuai digunakan bagi aplikasi teknikal, dan apabila diuji dalam penyahdakwaatan enzimatik surat khabar lama menghasilkan kecerahan pulpa sehingga 51.5% dan kekuatan tegangan 34.0 Nm/g. Bagi mengelakkan penjanaan sisa sekunder, baki OPF terfermentasi (pasca pengekstrakan enzim) telah difermentasikan semula sehingga tiga kitaran. Ia seterusnya meningkatkan pengeluaran enzim sebanyak 26-79% dan penggunaan serat sebanyak 14-26%. Fermentasi ulangan sebegini juga didapati bersesuaian untuk menambahbaik kemampunan serta kos penghasilan enzim pada setiap kitaran. OPF terpakai yang tidak lagi boleh difermentasi selepas tiga kitaran telah dikenakan penjanaan semula nutrisi melalui proses hidrolisis dan autolisis enzimatik. Proses tersebut menghasilkan hidrolisat bernutrisi tinggi yang mengandungi karbon (1.79 gL⁻¹), nitrogen (0.11 gL⁻¹) dan fosforus (0.36 gL⁻¹) yang boleh digunapakai sebagai medium pertumbuhan dan menyokong pertumbuhan kulat dan bakteria. Secara keseluruhan, kajian ini menunjukkan potensi bio-olahan OPF kepada enzim yang bernilai dan juga kepentingan proses fermentasi ulangan dan hidrolisis bagi memaksimumkan penggunaan bahan yang telah difermentasi sebagai bahan mentah yang boleh diperbaharui.

TABLE OF CONTENTS

| | TITLE | PAGES |
|------------------|--|--------------|
| | DECLARATION | ii |
| | DEDICATION | iii |
| | ACKNOWLEDGEMENT | iv |
| | ABSTRACT | v |
| | ABSTRAK | vi |
| | TABBLE OF CONTENTS | vii |
| | LIST OF TABLES | xv |
| | LIST OF FIGURES | xvii |
| | LIST OF ABBREVIATIONS | xxii |
| | LIST OF APPENDICES | xxiv |
| CHAPTER 1 | INTRODUCTION | 1 |
| | 1.1 Background information | 1 |
| | 1.2 Problem statement | 3 |
| | 1.3 Objectives | 4 |
| | 1.4 Scope of study | 5 |
| CHAPTER 2 | LITERATURE REVIEW | 6 |
| | 2.1 Oil palm biomass waste in Malaysia | 7 |
| | 2.2 Oil palm frond (OPF) | 11 |

| | | |
|------------------|---|-----------|
| 2.3 | Lignocellulosic biomass for enzyme production | 14 |
| 2.3.1 | Cellulose | 16 |
| 2.3.2 | Hemicellulose | 17 |
| 2.3.3 | Lignin | 19 |
| 2.4 | Lignocellulolytic enzyme | 21 |
| 2.5 | Filamentous fungi | 24 |
| 2.5.1 | <i>Aspergillus</i> sp. | 25 |
| 2.5.2 | <i>Trichoderma</i> sp. | 25 |
| 2.5.3 | Fungal growth and enzyme production | 26 |
| 2.6 | Solid state fermentation (SSF) | 29 |
| 2.7 | Utilization of fermented substrate | 35 |
| 2.8 | Industrial enzyme production | 37 |
| 2.9 | Enzyme application for deinking of wastepaper | 39 |
| 2.10 | Life cycle assessment (LCA) | 42 |
| 2.11 | Concluding remark | 49 |
| CHAPTER 3 | MATERIALS AND METHODS | 51 |
| 3.1 | Introduction | 51 |
| 3.2 | Microorganism and cultivation | 53 |
| 3.2.1 | Microorganism | 53 |
| 3.2.2 | Preparation of fungi cultivation medium | 53 |
| 3.2.3 | Fungi cultivation and inoculum preparation | 53 |
| 3.3 | Oil palm frond petiole | 55 |
| 3.3.1 | Preparation of OPF petiole fiber | 55 |
| 3.3.2 | Substrate characterization | 57 |

| | | |
|---------|---|----|
| 3.3.2.1 | Determination of particle size distribution | 57 |
| 3.3.2.2 | Determination of moisture content | 57 |
| 3.3.2.3 | Determination of fibre surface area | 58 |
| 3.3.2.4 | Determination of bulk and particle density | 59 |
| 3.3.2.5 | Determination of porosity and tortuosity | 60 |
| 3.4 | Fermentation | 60 |
| 3.4.1 | Mandel's medium | 60 |
| 3.4.2 | Solid state fermentation | 61 |
| 3.4.3 | Sampling and lignocellulolytic enzyme extraction | 62 |
| 3.5 | Re-fermentation of fermented OPF | 63 |
| 3.5.1 | Lignocellulolytic enzyme extraction | 64 |
| 3.5.2 | Re-fermentation of 100% fermented OPF | 65 |
| 3.5.3 | Re-fermentation of 50% fermented OPF | 65 |
| 3.5.4 | Determination of fungal spore viability | 66 |
| 3.6 | Nutrient regeneration and production of growth medium | 66 |
| 3.6.1 | Pre-treatment of OPF | 67 |
| 3.6.1.1 | Boiling (BL) | 67 |
| 3.6.1.2 | Autoclaving (AC) | 67 |
| 3.6.1.3 | Alkaline (AL) | 67 |
| 3.6.1.4 | Alkaline-autoclaving (ACA) | 68 |
| 3.6.2 | Hydrolysis of non-fermented OPF | 68 |
| 3.6.3 | Hydrolysis and autolysis of fermented OPF | 69 |

| | | |
|--------|---|----|
| 3.6.4 | Preparation of autohydrolysate as growth medium | 69 |
| 3.6.5 | Fungal and bacterial growth on autohydrolysate medium | 70 |
| 3.7 | Bio-deinking of old newspaper | 70 |
| 3.7.1 | Preparation of old newspaper | 70 |
| 3.7.2 | Preparation of pulp | 71 |
| 3.7.3 | Pulp pre-treatment | 71 |
| 3.7.4 | Enzymatic deinking | 72 |
| 3.7.5 | Deinked fiber analysis | 73 |
| 3.8 | Analytical procedures | 74 |
| 3.8.1 | Determination of biomass (colorimetric method) | 74 |
| 3.8.2 | Determination of biomass (N-acetylglucosamine method) | 74 |
| 3.8.3 | Determination of biomass (dry cell weight) | 74 |
| 3.8.4 | Determination of protein content | 75 |
| 3.8.5 | Determination of enzymes | 75 |
| 3.8.6 | Determination of total reducing sugar | 76 |
| 3.8.7 | Determination of polyoses | 76 |
| 3.8.8 | Determination of total phenolic content | 76 |
| 3.8.9 | Determination of hydroxymethyl furfural | 77 |
| 3.8.10 | Determination of nutrient in liquid hydrolysate | 77 |
| 3.8.11 | Determination of lignocellulose content | 78 |
| 3.8.12 | Determination of elemental components (CHNS/O) analyzer | 78 |

| | | | |
|------------------|--------|--|-----------|
| | 3.8.13 | Determination of elemental components | 79 |
| | 3.8.14 | Scanning electron microscopy (SEM) | 79 |
| | 3.8.15 | Fourier transform infrared spectroscopy (FTIR) | 80 |
| 3.9 | | Calculations | 80 |
| | 3.9.1 | Dry cell weight measurement | 80 |
| | 3.9.2 | Sugar yield | 80 |
| | 3.9.3 | Saccharification | 81 |
| | 3.9.4 | Kinetic of growth and yield co-efficient | 81 |
| | 3.9.5 | Statistical analysis | 82 |
| CHAPTER 4 | | PHYSICOCHEMICAL CHARACTERIZATION AND IDENTIFICATION OF OIL PALM FROND AS FERMENTATION SUBSTRATE | 83 |
| | 4.1 | Introduction | 83 |
| | 4.2 | Research methodology | 84 |
| | 4.3 | Results and discussion | 85 |
| | 4.3.1 | Chemical composition of OPF petiole | 85 |
| | 4.3.2 | Physical modification of OPF petiole | 87 |
| | 4.3.3 | Effect of moisture content on fibre surface area, density and porosity | 90 |
| | 4.3.4 | Effect of different inoculum size | 93 |
| | 4.3.5 | Changes in pH during fermentation | 96 |
| | 4.3.6 | Effect of physical modification on OPF fibre | 98 |
| | 4.4 | Summary | 101 |
| CHAPTER 5 | | SCREENING OF ASPERGILLUS AND TRICHODERMA FOR THE PRODUCTION OF | |

| | | |
|------------------|--|------------|
| | CRUDE LIGNOCELLULOLYTIC ENZYME | 103 |
| 5.1 | Introduction | 103 |
| 5.2 | Research methodology | 104 |
| 5.3 | Results and discussion | 105 |
| | 5.3.1 Fungal growth kinetics | 105 |
| | 5.3.2 Lignocellulolytic enzyme production | 108 |
| | 5.3.3 Kinetic assessment of fungal growth and enzyme production | 113 |
| | 5.3.4 Sugar production | 114 |
| | 5.3.5 Fibre degradation | 116 |
| | 5.3.6 Comparison of oil palm biomass and enzyme potential | 120 |
| 5.4 | Summary | 123 |
| CHAPTER 6 | EFFECT OF RE-FERMENTATION ON ENZYME PRODUCTION, SUSTAINABILITY, COST AND DEINKING APPLICATION | 125 |
| 6.1 | Introduction | 125 |
| 6.2 | Research methodology | 127 |
| | 6.2.1 Re-fermentation of fermented OPF petiole | 127 |
| | 6.2.2 Environmental impact of enzyme production | 128 |
| | 6.2.2.1 Description of the enzyme production process | 129 |
| | 6.2.2.2 Inventory and modelling | 131 |
| | 6.2.2.3 Life cycle impact assessment | 135 |
| | 6.2.3 Cost of enzyme production | 135 |
| | 6.2.4 Bio-deinking of old newspaper | 136 |

| | | |
|---------|--|-----|
| 6.3 | Results and discussion | 137 |
| 6.3.1 | Effect of re-fermentation on fungal growth and sugar production | 137 |
| 6.3.2 | Effect of re-fermentation on lignocellulolytic enzyme production | 142 |
| 6.3.3 | Effect of re-fermentation on spore viability and concentration | 146 |
| 6.3.4 | Morphological and chemical changes of the fermented OPF production | 149 |
| 6.3.5 | Environmental impact of enzyme production | 154 |
| 6.3.5.1 | Interpretation of LCIA | 154 |
| 6.3.5.2 | Future improvements | 158 |
| 6.3.5.3 | Sensitivity analysis for enzyme production | 158 |
| 6.3.6 | Cost of enzyme production | 160 |
| 6.3.6 | Application of enzyme for deinking of old newspaper (ONP) | 165 |
| 6.4 | Summary | 169 |

| | | |
|------------------|---|------------|
| CHAPTER 7 | HYDROLYSIS OF FERMENTED AND NON-FERMENTED OIL PALM FROND FOR SUGAR AND NUTRIENT REGENERATION | 171 |
| 7.1 | Introduction | 171 |
| 7.2 | Research methodology | 172 |
| 7.3 | Results and discussion | 173 |
| 7.3.1 | Comparison of non-fermented and fermented OPF for hydrolysis | 173 |
| 7.3.2 | Enzymatic hydrolysis of non-fermented OPF | 175 |
| 7.3.3 | Hydrolysis of fermented OPF | 177 |

| | | |
|------------------|--|------------|
| 7.3.4 | Hydrolysate and autolysate nutrient analysis | 181 |
| 7.3.5 | Autohydrolysate as growth medium | 185 |
| 7.3.6 | Solid reduction and fibre analysis | 189 |
| 7.4 | Summary | 195 |
| CHAPTER 8 | CONCLUSION AND FUTURE WORKS | 197 |
| 8.1 | Conclusion | 197 |
| 8.2 | Future works | 199 |
| | REFERENCES | 201 |
| | APPENDICES | 233 |
| | LIST OF PUBLICATION | 267 |

LIST OF TABLES

| TABLE NO. | TITLE | PAGE |
|------------------|---|-------------|
| Table 2.1 | Generation and utilization of oil palm waste | 9 |
| Table 2.2 | Composition of oil palm waste (OPW) | 13 |
| Table 2.3 | Functional group in lignocellulosic biomass | 14 |
| Table 2.4 | Comparison between hardwood and softwood | 18 |
| Table 2.5 | Summary of lignocellulose fiber | 20 |
| Table 2.6 | Types of lignocellulolytic enzymes and their function | 21 |
| Table 2.7 | Different types and examples of wood rotting fungi | 24 |
| Table 2.8 | Growth phases | 27 |
| Table 2.9 | Components of Mandel's medium | 28 |
| Table 2.10 | Comparison of SSF and SmF | 30 |
| Table 2.11 | SSF related factors | 32 |
| Table 2.12 | List of various fungi and substrates used in SSF | 34 |
| Table 2.13 | Re-fermentation of fermented substrate | 36 |
| Table 2.14 | Industrial enzyme cost and market share | 37 |
| Table 2.15 | Comparison of ONP and MOW deinking | 41 |
| Table 2.16 | Types of environmental impact | 45 |
| Table 2.17 | Comparison of environmental impact assessment | 47 |
| Table 3.1 | List of filamentous fungi | 54 |
| Table 3.2 | Composition of modified Mandel's medium | 60 |
| Table 3.3 | Preparation of trace elements | 61 |
| Table 3.4 | SSF set-up | 62 |
| Table 3.5 | Re-fermentation using 100% fermented OPF | 65 |
| Table 3.6 | Re-fermentation using 50% fermented OPF | 66 |
| Table 3.7 | Bio-deinking set-up | 72 |
| Table 3.8 | Deinked fiber analysis | 73 |
| Table 3.9 | List of enzyme analysis | 75 |
| Table 3.10 | Nutrient analysis | 77 |
| Table 3.11 | Fiber analysis | 78 |
| Table 3.12 | Elemental analysis | 79 |

| | | |
|------------|--|-----|
| Table 3.13 | Kinetic parameters | 81 |
| Table 4.1 | Chemical composition (% dry weight) of oil palm biomass | 85 |
| Table 4.2 | Elemental composition of oil palm fibers | 86 |
| Table 4.3 | Effect of different moisture content on physical properties of pressed OPF petiole (0.125mm) | 91 |
| Table 4.4 | FTIR peak and assignments | 100 |
| Table 5.1 | Fungal growth kinetics of cell biomass | 106 |
| Table 5.2 | Lignocellulolytic enzyme activities and total reducing sugar production based on the 7-day average | 109 |
| Table 5.3 | Growth and product yield | 113 |
| Table 5.4 | Comparison of solid state fermentation of oil palm biomass using filamentous fungi | 122 |
| Table 6.1 | Inventory data for lignocellulolytic enzyme production | 133 |
| Table 6.2 | Lignocellulolytic enzyme production from the re-fermentation of OPF using <i>A. awamori</i> MMS4 | 145 |
| Table 6.3 | Percentage degradation of lignocellulose content | 151 |
| Table 6.4 | FTIR band and assignments | 152 |
| Table 6.5 | Lab-scale lignocellulolytic enzyme production cost | 161 |
| Table 6.6 | Chemical costs | 163 |
| Table 6.7 | Fiber analysis of deinked ONP | 167 |
| Table 6.8 | Enzymatic performance during deinking | 167 |
| Table 7.1 | Lignocellulose composition of non-fermented and fermented OPF | 173 |
| Table 7.2 | Effect of pretreatment on sugar yield and saccharification | 175 |
| Table 7.3 | Average enzyme activity of fermented OPF hydrolysis | 178 |
| Table 7.4 | Hydrolysate and autolysate sugar composition (HPLC analysis) | 183 |
| Table 7.5 | Inhibitory compound in OPF hydrolysate | 184 |
| Table 7.6 | Nutrient comparison | 186 |
| Table 7.7 | Comparison of growth medium | 186 |
| Table 7.8 | FTIR band and assignments | 193 |

LIST OF FIGURES

| FIGURE NO. | TITLE | PAGE |
|-------------------|---|-------------|
| Figure 2.1 | Palm oil plantation in Malaysia | 8 |
| Figure 2.2 | Different types of oil palm waste | 8 |
| Figure 2.3 | Number of publications related to oil palm waste | 10 |
| Figure 2.4 | Oil palm frond top and basal comparison | 11 |
| Figure 2.5 | Current research using OPF | 12 |
| Figure 2.6 | Lignocellulose component and structure | 15 |
| Figure 2.7 | Cellulose structure | 16 |
| Figure 2.8 | Chemical monomers of lignin | 19 |
| Figure 2.9 | Synergistic action of cellulolytic enzymes | 22 |
| Figure 2.10 | Enzymatic hydrolysis of xylan | 23 |
| Figure 2.11 | Fungal growth curve | 26 |
| Figure 2.12 | Fungal growth in SSF | 29 |
| Figure 2.13 | Factors affecting solid state fermentation | 31 |
| Figure 2.14 | Market share of enzyme sectors | 37 |
| Figure 2.15 | Mechanism of enzymatic deinking | 40 |
| Figure 2.16 | The cradle-to-grave assessment of biomass-to-fuel production | 42 |
| Figure 2.17 | Framework of performing life cycle assessment | 43 |
| Figure 2.18 | Overview structure of ReCiPe | 44 |
| Figure 3.1 | Experimental design | 52 |
| Figure 3.2 | Images of filamentous fungi | 54 |
| Figure 3.3 | Soil nutrient replenishment using leaflets and stems of OPF | 56 |
| Figure 3.4 | Preparation of OPF fibre | 56 |

| | | |
|-------------|--|-----|
| Figure 3.5 | Fermentation and enzyme preparation | 63 |
| Figure 3.6 | Re-fermentation of fermented OPF | 64 |
| Figure 3.7 | The overall process of bio-deinking of old newspaper | 73 |
| Figure 4.1 | Experimental design | 84 |
| Figure 4.2 | Total reducing sugar in different OPF preparations | 87 |
| Figure 4.3 | Effect of different OPF fibre preparations on endoglucanase production using <i>T. asperellum</i> MR-1 | 88 |
| Figure 4.4 | OPF particle size distribution | 89 |
| Figure 4.5 | Effect of different particle size on biomass and enzyme production using <i>T. asperellum</i> MR-1 | 90 |
| Figure 4.6 | Changes in OPF moisture content during fermentation | 92 |
| Figure 4.7 | Fermentation of OPF petiole fibre | 94 |
| Figure 4.8 | Effect of inoculum size on enzyme production | 95 |
| Figure 4.9 | Changes in pH during the 7-day fermentation of OPF petiole fibre with <i>A. niger</i> EFB1 at 35°C | 96 |
| Figure 4.10 | Biomass (glucosamine) and xylanase production of <i>A. niger</i> EFB1 at 60% moisture and temperature 35°C | 97 |
| Figure 4.11 | Scanning electron microscopy (SEM) images of untreated (A) and treated (B, C and D) OPF fibre | 98 |
| Figure 4.12 | FTIR spectrum of untreated and pre-treated OPF petiole fibre | 100 |
| Figure 5.1 | Experimental design | 104 |
| Figure 5.2 | Fungal growth on pressed OPF petiole | 105 |
| Figure 5.3 | Lignocellulolytic composition for cellulolytic (A) and xylanase/ligninolytic (B) enzymes based on the average of 7-day enzyme production | 111 |
| Figure 5.4 | Production of total reducing sugar | 114 |

| | | |
|-------------|--|-----|
| Figure 5.5 | Polyoses content in crude extract | 115 |
| Figure 5.6 | FTIR transmittance of 7-day fermented OPF | 117 |
| Figure 5.7 | Percentage relative intensity of autoclaved (control) and fermented OPF | 119 |
| Figure 6.1 | Experimental design | 128 |
| Figure 6.2 | Framework for the life cycle assessment (LCA) of lignocellulolytic enzyme production | 129 |
| Figure 6.3 | LCA flow diagram of lignocellulolytic enzyme production | 130 |
| Figure 6.4 | Process model for lignocellulolytic enzyme production in cycle 1 | 134 |
| Figure 6.5 | Process model for lignocellulolytic enzyme production in cycles 2-3 | 134 |
| Figure 6.6 | Bio-deinking process design | 137 |
| Figure 6.7 | Fungal growth and reducing sugar production using 100% fermented OPF on consecutive re-fermentation cycles | 139 |
| Figure 6.8 | Fungal growth and reducing sugar production using 50% fermented OPF on consecutive re-fermentation cycles | 141 |
| Figure 6.9 | Lignocellulolytic enzyme production from the re-fermentation of OPF using <i>A. awamori</i> MMS4 | 143 |
| Figure 6.10 | Total phenolic content | 144 |
| Figure 6.11 | Average spore count | 146 |
| Figure 6.12 | SEM micrographs of 100% fermented OPF cycle 2 and 3 | 147 |
| Figure 6.13 | Morphological changes of the 100% fermented | |

| | | |
|-------------|--|-----|
| | OPF (medium only) | 149 |
| Figure 6.14 | Crude extract comparison | 150 |
| Figure 6.15 | FTIR spectra analysis (4000-800 cm ⁻¹) measured in transmittance (%) | 152 |
| Figure 6.16 | Intensity comparison of fermented samples | 153 |
| Figure 6.17 | Distribution of mid-point impact (in percentage) from the use of chemical, electricity and water in each cycle | 155 |
| Figure 6.18 | Sensitivity result for lignocellulolytic enzyme production | 159 |
| Figure 6.19 | Lignocellulolytic enzyme production process | 160 |
| Figure 6.20 | Cost comparison of enzyme production in cycles 1 & 2 | 162 |
| Figure 7.1 | Experimental design | 172 |
| Figure 7.2 | Comparison of non-fermented and fermented OPF | 174 |
| Figure 7.3 | Average enzyme activity and reducing sugar production of non-fermented OPF hydrolysis | 177 |
| Figure 7.4 | Reducing sugar production during hydrolysis of fermented OPF | 178 |
| Figure 7.5 | Production of protease (A) and free and amino (B) nitrogen during hydrolysis of fermented OPF using water and using enzyme | 180 |
| Figure 7.6 | Hydrolysate of non-fermented OPF | 182 |
| Figure 7.7 | Autolysate of fermented OPF | 182 |
| Figure 7.8 | Comparison of <i>A. awamori</i> MMS4 growth on autohydrolysate and modified Mandel's medium | 182 |

| | | |
|-------------|--|-----|
| Figure 7.9 | Comparison of <i>B. cereus</i> AL2 growth on autohydrolysate and nutrient broth | 188 |
| Figure 7.10 | Solid reduction of hydrolysed non-fermented and fermented OPF | 189 |
| Figure 7.11 | FTIR spectra analysis (4000-530 cm ⁻¹) measured in transmittance (%) | 192 |
| Figure 7.12 | Percentage ratio of intensity of hydrolysed OPF | 194 |

LIST OF ABBREVIATIONS

| | | |
|--------|---|--|
| ABTS | - | Azinobis ethyl benzothiazoline sulfonate |
| AC | - | Autoclaved pre-treatment |
| ACA | - | Alkaline and autoclaved pre-treatment |
| AL | - | Alkaline pre-treatment |
| BSA | - | Bovine serum albumin |
| BL | - | Boiling |
| CMC | - | Carboxymethyl cellulose |
| CMCase | - | Carboxymethyl cellulase |
| DNS | - | Dinitrosalicylic acid |
| EFB | - | Empty fruit bunch |
| FAN | - | Free amino nitrogen |
| Fpase | - | Filter paperase |
| FTIR | - | Fourier-transform infrared spectroscopy |
| OPF | - | Oil palm frond |
| g | - | gram |
| HCL | - | Hydrochloric acid |
| HPLC | - | High performance liquid chromatography |
| ISO | - | International organization for standardization |
| L | - | Liter |
| LCA | - | Life cycle assessment |
| LiP | - | Lignin peroxidase |
| MF | - | Mesocarp fibre |
| Min | - | Minutes |
| Mg | - | Miligram |
| mL | - | Mililiter |
| MnP | - | Manganese peroxidase |
| MOW | - | Mixed office waste |
| MW | - | Molecular weight |
| NaOH | - | Sodium hydroxide |
| °C | - | Degree celsius |

| | | |
|------|---|--------------------------------------|
| ONP | - | Oil news paper |
| OPT | - | Oil palm trunk |
| OPW | - | Old palm waste |
| PDA | - | Potato Dextrose Agar |
| PKF | - | Palm kernel shell |
| POME | - | Palm oil mill effluent |
| pNPG | - | p-nitrophenyl β -D-glucosidase |
| rpm | - | Rotation per minute |
| SEM | - | Scanning electron microscopy |
| SmF | - | Submerged fermentation |
| SSF | - | Solid state fermentation |
| TOC | - | Total organic carbon |
| TPC | - | Total phenolic concentration |
| TN | - | Total nitrogen |
| TP | - | Total phosphorus |
| U | - | Unit activity enzyme |
| UT | - | Untreated |
| v/v | - | Volume per volume |
| w/v | - | Weight per volume |

LIST OF APPENDICES

| APPENDIX | TITLE | PAGE |
|----------|---|------|
| A | Buffer preparation | 222 |
| B | Spore count using haemocytometer | 223 |
| C | Fungal biomass (N-acetylglucosamine) | 224 |
| D | 3,5-dinitrosalicylic acid (DNS) preparation | 226 |
| E | Endoglucanase assay | 228 |
| F | β -glucosidase assay | 230 |
| G | Exoglucanase assay | 232 |
| H | Endoxylanase assay | 234 |
| I | Lignin peroxidase assay | 236 |
| J | Manganese peroxidase assay | 237 |
| K | Laccase assay | 238 |
| L | Protease assay | 239 |
| M | Free amino nitrogen assay | 241 |
| N | Total phenolic content assay | 243 |
| O | Protein content (Lowry method) | 245 |
| P | Total organic carbon | 247 |
| Q | Total nitrogen | 248 |
| R | Total phosphorus | 249 |
| S | Determination of polyoses (HPLC) | 250 |
| T | Environmental impact of enzyme production | 252 |

CHAPTER 1

INTRODUCTION

1.1 Background information

About 56 million tonnes of oil palm frond (OPF) are generated every year and is possibly the largest agricultural waste in Malaysia (Abdullah *et al.*, 2016). However, the utilization of OPF and oil palm waste in general are quite limited. At present, OPF wastes are used for soil replenishment and as animal feed, while a larger portion are burned to save up plantation space and to avoid contamination (Ishida & Abu Hassan, 1997; Islam *et al.*, 2000; Lee *et al.*, 2016). Realising the potential of oil palm biomass, the Malaysian government initiated the *National Biomass Strategy 2020* to convert the largely underutilized biomass waste into value added bio-products such as enzymes, biofuel, bio-fertilizer, and bio-based chemicals (Malaysia, 2011).

Biomass waste with potentially high lignocellulose content such as OPF can be exploited for lignocellulolytic enzyme production (Saini *et al.*, 2015). A lignocellulolytic enzyme contain a mixture of cellulase, xylanase, and ligninolytic enzymes (Saini *et al.*, 2015). These enzymes are in demand today due to their widespread application in various industries (Anwar *et al.*, 2014). One such industry that can benefit from enzyme application is the pulp and paper industry. To date, they are one of the largest environmental polluters due to their over reliance on harmful high alkaline chemicals (Ashrafi *et al.*, 2015). Lignocellulolytic enzyme can be applied for wastepaper processing especially in the deinking and bleaching process (Davison & Finkelstein, 2012; Lee *et al.*, 2007). However, the biggest bottleneck for enzymatic application in industries is the high cost of enzyme production itself (Liu *et al.*, 2016).

Commercial enzymes are conventionally produced by submerged fermentation (SmF). Today, solid state fermentation (SSF) are gaining significant interest for enzyme production (Singhania *et al.*, 2010). The combination of SSF and filamentous fungi have yield promising results in the production of hydrolytic enzymes such as cellulase and xylanase. In addition, fungal fermentation under SSF is capable of producing higher volumetric extracellular enzymes than SmF (di Cologna *et al.*, 2017; Viniegra-González *et al.*, 2003). This is because filamentous fungi can thrive under low water environment and could consume nutrient directly by degrading the solid substrate (Manpreet *et al.*, 2005). Another advantage of SSF is that, it is simple and cheaper to operate. It uses low amount of water and the fermenter set-up does not require agitator and complex stirring mechanism (Pandey *et al.*, 2008).

Although lignocellulose biomass such as oil palm frond has great potential to be converted into value-added product such as lignocellulolytic enzyme, certain challenges related to its production such as cost and environmental impact has to be addressed. These crucial factors if left unchecked at lab-scale could become a hindrance in future up-scaling. For that reason, incorporating certain bio-refinery techniques such as re-fermentation and nutrient regeneration may be useful (Chang & Webb, 2017). Re-fermentation involves the re-use of fermented substrate for the production of same or other related product, while nutrient regeneration involves the retrieval of essential nutrient from fermented material (Koutinas *et al.*, 2005). Adopting such techniques in the production scheme could maximize substrate utilization, improve enzyme production and possibly generate other value added product along the way.

Finally, enzyme production has to be sustainable for it to be beneficial. Most claims on enzymes being a greener alternative to chemicals are not substantiated with quantitative findings (Jegannathan & Nielsen, 2013). Thus, environmental assessment of enzyme production is necessary for it to be truly sustainable. Today, life-cycle assessment (LCA) has become an indispensable tool to ascertain the sustainability of such process in a cheap and easy way.

1.2 Problem statements

In recent years, oil palm frond (OPF) has gained prominence due to its diverse usage potential. For example, OPF's leaflets and stems sections are rich in soil nutrient and can be utilized as natural soil fertilizers. However, other larger section of the frond such as the petiole are commonly burned, and this contributed to pollution and wastage of lignocellulose content. The study proposes to investigate the suitability and potential of OPF petiole as a substrate for solid state fermentation.

Fungal fermentation of heterogeneous lignocellulose substrate such as OPF are likely to produce a diverse enzyme mixture (Anwar *et al.*, 2014). At present, many SSF studies are mainly focused on hydrolytic enzymes such as cellulase and xylanase while disregarding enzymes such as lignin peroxidase, manganese peroxidase and laccase that might be present in the mixture. Furthermore, these oxidative enzymes are often exclusively produced and researched using white-rot basidiomycetes fungi (e.g., *Phanerochaete* sp.) (Yoon *et al.*, 2014). This study intends to investigate all seven enzymes associated to the lignocellulolytic mixture using soft-rot ascomycetes fungi (e.g., *Aspergillus* and *Trichoderma* sp.) to better understand its enzymatic activity, composition and application potential.

Most fermentation studies today are too product oriented with little attention given to the fermented residue/substrate. At the end of fermentation or post-extraction process, fermented residue may still contain undigested fibre components, protein and microbial cells (Chang & Webb, 2017; Koutinas *et al.*, 2005). Discarding the solid residue would result in wastage of useful organic matter and contribute to the generation of secondary waste. Therefore, this study propose to re-ferment the fermented residue to increase its level of utilization and also to further hydrolyse it for the purpose of nutrient regeneration.

Lab-scale enzyme studies are often focused on process optimization and purification to increase enzymatic yield. Although this is important, other practical aspect such as cost, environmental impact and final applicability of the enzyme produced should also be given weight (da Gama Ferreira *et al.*, 2018; Olofsson *et al.*, 2017). Commercial enzymes are not only expensive to produce, its production process may not be as environmentally friendly as expected (Klein-Marcuschamer *et al.*, 2012). For that reason, the enzyme produced in this study are evaluated for its potential impact to environment and its enzymatic hydrolytic application on raw lignocellulose biomass and wastepaper while estimating the cost of enzyme produced at small scale.

1.3 Objectives

1. To analyse the physicochemical characteristic of oil palm frond petiole as lignocellulose substrate for solid state fermentation
2. To select the best lignocellulolytic enzyme producer among *Aspergillus* and *Trichoderma* species
3. To analyse the re-fermentation potential of fermented oil palm frond petiole and its effect on substrate utilization and lignocellulolytic enzyme production
4. To compare the enzymatic hydrolysis of fermented and non-fermented oil palm frond petiole for the production of nutrient rich medium
5. To evaluate the environmental impact and application potential of crude lignocellulolytic enzyme

1.4 Scope of study

The study only focuses on the petiole section of the oil palm frond and are subjected to physical modifications. The characterization of the frond petiole covered aspects of its chemical and elemental composition, size distribution, moisture content, surface area and porosity. Six filamentous fungi from *Aspergillus* and *Trichoderma* species were screened to determine the best lignocellulolytic enzyme producer and the selected strain were used throughout the study. Solid state fermentation was carried out using glass petri dish for the benefit of space and practicality. The re-fermentation of fermented frond petiole was studied in several cycles until the fermented substrate was no longer efficient in producing lignocellulolytic enzyme. The crude enzyme produced were applied for the bio-deinking of pre-treated old newspaper. The crude enzyme was also used for the enzymatic hydrolysis of non-fermented and fermented frond petiole for the production nutrient rich hydrolysate and autolysate. Both nutrient rich lysates were combined as autohydrolysate that would serve as a growth medium and tested on bacteria and fungi. The overall cost and environmental impact of crude lignocellulolytic enzyme production during initial fermentation and re-fermentation processes were assessed. Both assessments took into account the raw materials, chemicals and utilities used during the enzyme production process.

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