SCALING UP OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF MICROWAVE ALKALI PRETREATED EMPTYFRUIT BUNCH FOR LACTIC ACID PRODUCTION

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To my beloved mother and father,
Mr Hassan Muhammad & Mrs Sunniati Adam,
My lovely husband,
Hafizuddin Gunodo,
My Son,
Nazeef Hilman,

With my eternal love and thank you for being the best thing that ever happened to me

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ABSTRACT

Oil palm empty fruit bunches (EFB), a major solid waste in the palm oil industries is a source of lignocellulosic biomass. Cellulose, which is the major component of EFB can be converted to lactic acid. Production of lactic acid is desirable because it can be utilized in industries including bioplastics, chemicals, and cosmetics. The aim of this study is to produce lactic acid on a larger scale from microwave-alkali (Mw-A) pretreated oil palm EFB using simultaneous saccharification and fermentation (SSF) process with *Rhizopus oryzae* fungus. The present work is divided into four different stages; pretreatment of EFB, development of practical and effective procedure for inoculum build up for lactic acid production on a pilot scale, optimization of process to improve the yield by using fed batch mode operation and scale up of lactic acid production in 150 L fermentor. The Mw-A pre-teatment proved to be an effective method for removing lignin, preserving cellulose fraction and enhancing the enzymatic hydrolysis of EFB. The composition changes on the lignin, hemicelluloses and cellulose after pretreatment was used as indicators to represent the effectiveness of the pretreatment. In order to fulfill the requirement of massive inoculum production for large scale fermentation, a study was performed to develop a protocol in preparing inoculum for lactic acid production from EFB. Multi-stage inocula were developed and their fermentation ability was assessed. The procedure performed eliminated the requirement of huge quantity of spore suspension and improved the fermentation consistency. In order to obtain the desired morphological form of *Rhizopus* pellets, several parameters such as concentration of spore suspension, storage time and doses of inoculum were varied. Longer storage time of spore suspension of more than three days led to the formation of free mycelia. Low inoculum concentrations of 10^7 spores/ml are beneficial for formation of pellet. In addition, xylose has a positive effect on pellet formation compared to glucose. To achieve a high lactic acid concentration in the broth, high solids loading was required to allow a higher rate of glucose conversion. However, a decrease in the final lactic acid concentration was observed when running SSF at a massive insoluble solids level. High osmotic pressure in the medium led to poor cellular performance and caused the *Rhizopus oryzae* pellets to break down, affecting the lactic acid production. The process performance was further improved using a fed-batch operation mode. The fed-batch operation was observed to facilitate higher lactic acid concentration of 12 g/L, compared with the SSF batch mode with final lactic acid concentration of 6.8 g/L. For scale-up of the lactic acid fermentation, the strategy was adopted to provide almost equivalent oxygen mass transfer coefficient (k_La) to the different-sized fermentor systems (16 L and 150 L), thus ensuring the same amount of dissolved oxygen supply in each fermentation broth. At k_La value of 0.06 s^{-1}, final lactic acid concentration in both scales were found identical.

ABSTRAK

Tandan kosong buah kelapa sawit (EFB) adalah salah satu daripada sisa pepejal utama dalam industri minyak sawit dan merupakan sumber biojisim lignoselulosa. Selulosa adalah komponen yang paling tinggi dalam EFB yang boleh ditukar kepada asid laktik. Pengeluaran asid laktik adalah wajar kerana ia telah digunakan dalam industri termasuk bioplastik, bahan kimia, dan kosmetik. Tujuan kajian ini adalah untuk mengeluarkan asid laktik berskala besar daripada EFB terawat gelombang mikro-alkali (Mw-A) melalui proses fermentasi dan pensakaridaan serentak (SSF) menggunakan kulat *Rhizopus oryzae*. Kajian ini dibahagikan kepada empat bahagian yang berbeza iaitu prarawatan EFB untuk mengenal pasti perubahan fizikal dan kimia, pembangunan prosedur yang praktikal dan berkesan untuk menyediakan inokulum bagi penghasilan asid laktik pada skala perintis, pengoptimuman proses untuk meningkatkan hasil asid laktik dengan menggunakan mod operasi suapan berkelompok dan pengskalaan penghasilan asid laktik dalam fermentor 150 L. Prarawatan Mw-A terbukti berkesan untuk mengeluarkan lignin, mengekalkan selulosa dan meningkatkan hidrolisis enzim EFB. Perubahan komposisi pada lignin, hemiselulosa dan selulosa selepas prarawatan digunakan sebagai penunjuk keberkesanan rawatan. Bagi memenuhi keperluan inokulum yang banyak pada fermentasi berskala besar, satu kajian telah dilakukan untuk membangunkan protokol dalam menyediakan inokulum untuk penghasilan asid laktik daripada EFB. Inokula bertingkat telah dibangunkan dan keupayaan fermentasi tersebut dinilai. Prosedur ini dapat menghapuskan keperluan kuantiti spora yang besar dan meningkatkan ketekalan proses fermentasi. Untuk mendapatkan bentuk morfologi yang dikehendaki iaitu pelet *Rhizopus*, beberapa parameter seperti kepekatan spora, masa penyimpanan dan dos inokulum telah dikaji. Penyimpanan spora yang lama iaitu melebihi tiga hari menyebabkan pembentukan miselia. Kepekatan inokulum yang rendah iaitu 10^7 spora/ml bermanfaat untuk pembentukan pelet. Di samping itu, xilosa mempunyai kesan positif ke atas pembentukan pelet berbanding glukosa. Bagi mencapai kepekatan asid laktik yang tinggi, substrat pepejal yang tinggi diperlukan untuk membolehkan kadar penukaran glukosa yang lebih tinggi. Walau bagaimanapun, penurunan hasil asid laktik diperoleh apabila proses SSF dilakukan mengunakan substrat pepejal yang terlalu tinggi. Tekanan osmosis yang tinggi di dalam media membawa kepada prestasi sel yang lemah dan menyebabkan pelet *Rhizopus oryzae* pecah seterusnya menjejaskan pengeluaran asid laktik. Untuk meningkatkan prestasi proses, mod operasi suapan berkelompok telah digunakan. Operasi suapan berkelompok menghasilkan asid laktik yang lebih tinggi iaitu 12 g/L, berbanding dengan mod SSF kelompok dengan kepekatan asid laktik 6.8 g/L. Untuk pengskalaan fermentasi asid laktik, strategi yang diguna pakai telah menyediakan pekali pemindahan jisim oksigen (k_La) yang hampir sama kepada sistem berbeza bersaiz (16 L dan 150 L), dengan itu memastikan jumlah bekalan oksigen terlarut yang sama di dalam setiap brot fermentasi. Pada nilai k_La 0.06 s^{-1}, kepekatan akhir asid laktik di kedua-dua skala didapati serupa.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENT	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	viii
	LIST OF FIGURES	xiv
	LIST OF SYMBOLS	xvii
	LIST OF ABBREVIATIONS	xix
	LIST OF APPENDICES	xx
1	**INTRODUCTION**	**1**
	1.1 Justification and Background	1
	1.2 Problem Statement	6
	1.3 Significant of the Study	7
	1.4 Objectives and Scopes of Study	8
2	**LITERATURE REVIEW**	**11**
	2.1 Lactic acid	11
	2.2 Lactic acid Applications	13

2.3	Lactic acid production		15
2.4	Palm oil empty fruit bunch		17
2.5	Composition in Lignocellulose		19
	2.5.1	Cellulose	19
	2.5.2	Hemicellulose	20
	2.5.3	Lignin	22
2.6	Pre-treatments of lignocelluloses materials		23
2.7	Microwave- assisted Pretreatment of Lignocellulose Biomass		28
2.8	Fermentation approaches for lactic acid production using lignocellulosic material		32
	2.8.1	Separate hydrolysis and fermentation (SHF)	32
	2.8.2	Simultaneous saccharification and fermentation (SSF)	33
2.9	Lactic acid production by *R. Oryzae*		35
2.10	Lactic acid fermentation in fed-batch processes		41
2.11	Production of lactic acid in fermentor systems		42
	2.11.1	Inoculum preparation	45
	2.11.2	Scale up of lactic acid fermentation	46
	2.11.3	Scale up on basis of constant oxygen transfer coefficient k_La	47
	2.11.4	Oxygen transfer coefficient k_La	48
		2.11.4.1 Dynamic gassing out method	49
		2.12.4.1 Static gassing out technique	51
2.12	Summary		54
3	**METHODOLOGY**		**55**
3.1	Materials and Chemicals		55

3.2	Chemicals Reagents and Enzymes	55
3.3	Pretreatment of Empty Fruit Bunches Fibre (Mechanical Grinding)	57
3.4	Microwave pretreatment	58
3.5	Composition Analysis of EFB	59
	3.5.1 Determination of Moisture Content	59
	3.5.2 Determination of Extractive	59
	3.5.3 Determination of Holocellulose	60
	3.5.4 Determination of α-cellulose	61
	3.5.5 Determination of Lignin	61
3.6	Morphology analysisusing Scanning Electron Microscope (FESEM)	62
3.7	Functioning Group Analysis using FT-IR	63
3.8	Enzymatic Hydrolysis	63
3.9	Microorganisms and pre-culture medium	63
3.10	Effect of carbon source and concentration on pellet formation	64
3.11	Effect of spore concentration on pellet formation of pre grown and seed culture	64
3.12	Inoculum preparation for large scale fermentation of lactic acid	65
	3.12.1 Determination of the best inoculum phase to transfer from 250 ml shake flask to 16 l seed culture	66
	3.12.2 Determination of the best inoculum phase to transfer from 16 l seed culture to 150 l fermenter	67
3.13	Specifications of 16 L and 150 L Fermentors	67
3.14	Fermentor start-up	68
3.15	Simultaneous saccharification and fermentation (SSF) of EFB into lactic acid (Batch Fermentation)	68

	3.16	Simultaneous saccharification and fermentation (SSF) of EFB into lactic acid (Fed-batch fermentation)	69
	3.17	Dry Weight of Biomass	70
	3.18	Lactic acid concentration	70
	3.19	Dynamic Technique in kLa measurement	71
4	**RESULTS AND DISCUSSION**		73
	4.1	Raw Empty Fruit Bunch (EFB) Composition	73
	4.2	Pretreated EFB Substrates	75
		4.2.1 FESEM Analysis of EFB	77
		4.2.2 Fourier transform infrared (FTIR) Spectroscopy analysis of raw and Mw-alkali treated EFB samples	79
	4.3	Enzymatic Hydrolysis of EFB	81
	4.4	Formation of Pellet *Rhizopus oryzae* NRRL 395 in Bioreactors	83
		4.4.1 Effect of spore concentration on pellet morphology	83
		4.4.2 Effect of storage time of spore suspension on pellet formation	85
		4.4.3 Effect of carbon sources and spore concentration on the fungal morphology	86
	4.5	Inoculum preparation for Scale-up of Production Lactic acid	88
		4.5.1 Fermentation in 16 L seed culture fermenter	89
		4.5.2 Fermentation in the 150 L fermentation	91
	4.6	Simultaneous Saccharification and Fermentation (SSF) of Lactic acid in Airlift Bioreactor (Batch Fermentation)	92

		4.6.1 Effect of enzyme dosage on lactic acid production	95
		4.6.2 Effect of Substrate Concentration on lactic acid production	96
	4.7	Simultaneous Saccharification and Fermentation (SSF) of Lactic acid in Airlift Bioreactor (Fed-Batch Fermentation)	98
	4.8	Effect of Superficial Velocity (V_s) on the Oxygen Transfer Rate Coefficient (k_La) And Lactic Acid Production	101
	4.9	Effect of k_La on Lactic Acid Fermentation in 16 Liter Scale	103
	4.10	Scale-Up Based on Oxygen Mass Transfer Coefficient (k_La)	105
	4.11	Fermentation kinetics of 16 L and 150 L Under same k_La Value	107
5	**CONCLUSION AND RECOMMENDATIONS**		**110**
	5.1	Conclusion	110
	5.2	Recommendations	113
REFERENCES			**114**
Appendices A-G			131-142

LIST OF TABLES

TABLE NO.	TITLE	PAGE
1.1	Composition of various lignocelluloses biomass (Anwar *et al.*, 2014)	3
2.1	Selected physical characteristics of lactic acid	12
2.2	Scenario of oil palm biomass generated in Malaysia from 2001 to 2020 (Hassan *et al*., 1997)	18
2.3	Advantages and limitation of different pre-treatment process	27
2.4	Lactic acid production by fungal fermentation using various type of lignocellulose biomass	37
2.5	Different *Rhizopus* morphologies in lactic acid production	39
3.1	List of chemicals used	52
3.2	Specification of 16 liter and 150 liter fermentors	63
4.1	Chemical composition of native EFB fiber	74
4.2	Chemical composition of palm oil EFB before and after pretreatment	75
4.3	FTIR wavenumber characterization (Nomanbhay *et al.,* 2013)	80
4.4	Effect of spore concentration on pellet formation and lactic acid production	84
4.5	Effect of storage time of pellet on fungal morphological form in 250 ml flask	85
4.6	Effect of different carbon sources on pellet formation	86

4.7	Effect of pre-grown culture conditions on the biomass and lactic acid production in the 16 L seed culture	90
4.8	Effect of inoculum condition on the lag phase duration and biomass concentration in 150 L fermentor	90
4.9	Effect of aeration rate on the production rate of lactic acid and morphology of *R.oryzae* NRRL 395 in 16 L fermentor	103

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
1.1	Flowchart of research activities	10
2.1	Chemical structure of lactic acid. (a) L(+) lactic acid (b) D(-) lactic acid	11
2.2	Biomass resources in Malaysia (Sulaiman and Abdullah, 2011)	17
2.3	Lignocellulosic structure	19
2.4	The structure of cellulose repeating unit	20
2.5	The structure of hemicelluloses	21
2.6	Lignin structure	22
2.7	Schematic of various pre-treatment method for lignocellulose biomass	24
2.8	Schematic of SSF process	35
2.9	Configuration of a split-cylinder airlift bioreactor (Bai *et al.*, 2001)	44
2.10	Mass balance of oxygen transfer during aerobic fermentation (Charles and Wilson, 1994)	48
2.11	Time course saturation of DO in dynamic gassing out technique	50
2.12	Time course saturation of DO in static gassing out technique	52
3.1	The overall methodology of the experimental work	56
3.2	EFB fiber (a) Shredded EFB (b) Grinded EFB	58
3.2	Inoculation preparation procedure	66

4.1	FESEM images of EFB fiber. (A) Raw EFB (surface 500X) (B) Raw EFB (surface 1000X) (C)Raw EFB (2500X) (D) Mw-alkali treated EFB (surface 500X) (E) Mw-alkali treated EFB (surface 1000X) (F) Mw-alkali treated EFB (surface 2500X)	78
4.2	FT-IR analysis of raw EFB (black line) and Mw-Alkali treated EFB (red line)	79
4.3	Glucose accumulation in enzymatic hydrolysis of EFB:(♦) raw EFB, (■) Mw-alkali treated EFB	82
4.4	Pellet *Rhizopus oryzae* NRRL 395	87
4.5	Growth profile of *R.oryae* NRRL 395 in shake flask experiment	88
4.6	Time courses of glucose and lactic acid concentrations for SSF of Mw-A treated EFB	93
4.7	Effect of enzyme loading on lactic acid yield as a function of time during batch SSF at 35ºC with 15% substrate concentration. (♦) 10 FPU, (■) 30 FPU, (▲) 50 FPU	95
4.8	Effect of substrate loading on the lactic acid yield as a function of time during batch SSF at 35 °C: (♦) 10 g/, (■) 15 g/L, (▲) 20 g/L, and (x) 30 g/L	97
4.9	Profiles of glucose and lactic acid in an operated, fed-batch SSF mode: (♦) lactic acid, (■) glucose. Fermentation conditions: 37 °C, pH: 6.5, initial loading: 15 g/L of EFB, and 10 FPU/g of biomass enzyme. Strategy A: one subsequent substrate feed; Strategy B: two subsequent substrate feeds (feed 1 and feed 2); Strategy C: two subsequent substrate and enzyme feeds (feed 1 and feed 2)	99
4.10	Solid residues of unconverted substrate and pellet, *R. oryzae* (NRRL 395) for strategy C	100

4.11	A correlation between V_s and k_La in the 160 L and 150 L airlift bioreactor. (♦) 16 L bioreactor, (■) 150 L bioreactor	102
4.12	Lactic acid fermentation profiles in 160 L scale at different V_s. (a) V_s = 0.2 cm/s, k_La = 0.011 (b) V_s = 0.5 cm/s, k_La = 0.039 (c) V_s = 0.9 cm/s, k_La =0.061	106
4.13	Time-course profiles of cell growth, sugar consumption, DO concentration and lactic acid production by *R.oryzae* NRRL 395 at same k_La value. (a) in the 16 L fermentor and (b) in the 150 L pilot-scale fermentor	108

IST OF SYMBOLS

%	-	Percentage
C-C	-	Carbon to carbon bond
C-O	-	Stretching modes of hydroxyl
C-O-C	-	Carbon to oxygen bonding
C-OH	-	Carbon to hydroxyl bonding
g/g	-	Mass ratio (gram per gram)
g/L	-	Concentration (gram per litre)
h	-	Time (hour)
L-(+)-	-	Left or sinister
Lb	-	Lactobacillus
M	-	Molarity
mg	-	Miligram
$MgSO_4$	-	Magnesium sulphate
Min	-	Time (Minutes)
NaOH	-	Sodium Hydroxide
O-H	-	Hydroxyl group
^oC	-	Temperature (degree celcius)
R.oryzae	-	*Rhizopus oryzae*
S	-	Syringyl propane units
% T	-	Percentage of transmission
C5	-	Pentose sugar
C6	-	Hexose sugar

$ZnSO_4$	-	Zink sulphuric
$k_L a$	-	Oxygen transfer coefficient
V_s	-	Superficial velocity
M_S	-	Weight of air dried sample
M_{ODS}	-	Weight of oven dried sample
E_e	-	Weight of extractives in extraction thimble
E_{sae}	-	Weight of sample after extraction
H_{ADHS}	-	Weight of air-dried holocellulose sample
C_{ADCS}	-	Weight of air dried cellulose sample
C_{FP}	-	Weight of filter paper
$C_{IntADHS}$	-	Initial weight of air dried holocellulose sample
L_L	-	Weight of lignin
L_{FP}	-	Weight of filter paper

LIST OF ABBREVIATIONS

EFB	-	Empty fruit bunch
FPU	-	Filter paper unit
SSF	-	Simultaneous saccharification and fermentation
PLA	-	Poly lactic acid
Mw-A	-	Microwave alkali
DO	-	Dissolved oxygen
OTR	-	Oxygen transfer rate
OUR	-	Oxygen uptake rate
FESEM	-	Field Emission Scanning Microscope
FT-IR	-	Fourier Transform Infrared
GRAS	-	Generally regarded as a safe
MPOB	-	Malaysian Palm oil Board
PDA	-	Potato dextrose agar
SHF	-	Separate hydrolysis and fermentation
sp	-	Species

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A	L(+) Lactic acid Concentration Standard Curve	131
B	Enzymatic Hydrolysis Data	132
C	Fermentation Data (Effect of Enzyme Loading)	133
D	Fermentation Data (Effect of Substrate Loading)	134
E	Fermentation Data (Fed batch)	135
F	Oxygen Solubility	138
G	Dynamic technique calculation	139

CHAPTER 1

INTRODUCTION

1.1 Justification and Background

In Asia and the Pacific region, the consumer demand for non-biodegradable plastics has increased sharply because of urbanization and economic growth. This trend has resulted in an increased of plastic waste which posed a serious threat to the environment and human health. Waste plastics are not biodegradable for hundreds of years. People around the world have begun to realize the magnitude of this problem, and countries are taking steps to reduce the use of non-biodegradable plastics. The negative impact of waste plastics could be addressed or minimized by finding an alternative plastic compound that is truly biodegradable and compostable. After years of research into principle compostable, bio-based plastics, polylactic acid (PLA) is found to be one of the superior choices and has the desired strength, flexibility, and is fully biodegradable. Polylactic acid's physical properties and the degradation rate can be altered by manipulation of the manufacturing process, making it much more versatile.

In the past, the use of PLA as a biodegradable plastic lacked popularity because it was too expensive to produce for large-scale applications because the main component, lactic acid, was too expensive. Today, most commercial lactic acid is produced from the fermentation of starch, glucose, and sucrose (Lunelli *et al*., 2011; Abdel-Rahman et al., 2015; Wakai *et al*., 2014). The fermentation of these sugars into lactic acid is well-established in terms of both the microbiology and the chemical processes.

To reduce the feedstock cost, many studies were focused on the fermentation of agro-industrial wastes, such as lignocellulosic carbohydrates, for lactic acid production (Ye *et al*., 2014; Zhang *et al*., 2016; Miura *et al*., 2004; Pesionne *et al*., 2014). Lignocellulose biomass is viewed as a sustainable lactic acid feedstock. The beauty of this process lies in the opportunity given to the agricultural industry to solve both the economic and environmental problems of waste by turning it into a highly desirable product.

The worldwide production of lignocellulosic biomass is reported to reach about 200×10^{19} metric tons per annum (Saini *et al*., 2015). The lignocellulosic resources included;

(i) agricultural residues (palm trunk and empty fruit bunch (EFB), corncobs, wheat straw, sugarcane bagasse, corn stover, coconut husks, wheat rice, and empty fruit bunches);
(ii) forest residues (hardwood and softwood);
(iii) energy crops (switch grass);
(iv) food wastes
(v) municipal and industrial wastes (waste paper and demolition wood).

The high availability of biomass has made it as one of the most potential resources for transportation fuels and chemicals platform. Previously, most of the lignocellulose wastes were burnt which led to the pollution crisis. Lignocellulosic

biomass consists mainly of three biopolymers: (i) cellulose (~30–50% by weight), (ii) hemicellulose (~19–45% by weight), and (iii) lignin (~15–35% by weight). These polysaccharides are associated with each other in a heteromatrix to different degrees and varying composition depending on the type of biomass, species of plant, and even source of the biomass. The chemical composition of biomass for different types of agriculture, industrial, and forestry wastes is shown in Table 1.1.

Table 1.1: Composition of various lignocelluloses biomass (Anwar *et al.*, 2014)

Types of biomass	**Lignocellulosic substrate**	**Cellulose (%)**	**Hemicellullose (%)**	**Lignin (%)**
Agriculture waste	Corncob	45	35	15
	Wheat straw	30	50	15
	Barley straw	33-40	20-35	8-17
	Corn stover	39-42	22-28	18-22
	Nut shell	25-30	25-30	30-40
Energy crops	Empty fruit bunch	41	24	21
	Switch grass	45	31.4	12
Forestry waste	Hardwoods stems	40-55	24-40	18-25
	Softwoods stems	45-50	25-30	25-35
	Leaves	15-20	80-85	0
Industrial waste	Waste paper from pulp	60-70	10-20	5-10
	wastewater solid	8-15	0	0

Every year, an estimated more than 200 million dry tons of agricultural residues were generated in the world (Muth *et al.,* 2013). There are many lignocellulosic agricultural waste available for lactic acid production such as EFB, sugarcane baggase, rice straw, corncobs and corn stover. Most of these materials remain potentially accessible and are not effectively utilized. However, the situation

is now changing due to the approaching depletion of fossil oils, and the competitive uses of starch materials as chemical feedstock and food, as well as the increasing public concern about the environment. During the past decades, intensive researches were conducted in this field and has yielded significant progress.

It was reported, about 90 million tonne accumulation of lignocellulose biomass from oil palm milling was generated (Sulaiman *et al*., 2011). The crop residues comprised of trunk, fronds, shells, palm press fibre and empty fruit bunch. The amount of empty fruit bunch that can be sustainably collected in the Malaysia is estimated to be 2.8 million dry tonne/yr. Owing to its sustainable abundance, EFB has been considered to be one of the most promising feedstock for lactic acid production in Malaysia.

However, lignocellulose biomass fractionation is a very complex process as high recovery of polysaccharides (cellulose, hemicellulose, and lignin) is required so that all three components can be fully converted into useful end products (Lee *et al.*, 2014; Jonsson and Martin, 2016). Sometimes, the biomass pretreatment led to over depolymerisation of polysaccharide chains and subsequent sugar ring opening (Lee *et al.*, 2014). Generally, a biomass pre-treatment step is necessary to ensure the separation of cellulose component from the tight bond of polymeric constituents (cellulose, hemicellulose, and lignin) in lignocellulosic biomass. The main intention of this fractionation treatment is to increase the accessibility of cellulose fiber to chemical attack prior to mild hydrolysis of isolated cellulose, by cleaving the ether bonds between glucose chains in order to produce nanosize cellulose intermediate (Lai and Idris, 2013; Ahktar *et al*., 2014).

The process scheme in production of lactic acid from cellulosic biomass has much to share with that of bioethanol production from biomass. It is simultaneous sacchariffication and fermentation (SSF) (Kumneadklang *et al*., 2016; Zhao *et al*., 2015). It is an art in bioprocess engineering that can transform lignocellulosic carbohydrate to end product in single process. There is large volume of research

work has been done to investigate its connection with ethanol production from cellulosic biomass (Narra *et al*., 2015; Elemike *et al*., 2015; Abideen *et al*., 2011). Recently, this technique was also been used in producing lactic acid. It it notably that many of lactic acid producing microorganisms are thermo tolerant. Thus, operating temperatures of SSF are favor to conduct near to the optimum temperature level of the enzymes hydrolysis process. Consequently, the overall process become more efficient, especially in the use of enzymes.

In SSF, the cellulosic substrate, cellulase enzyme and the microorganism are introduced into one reactor. The enzymes breaks the cellulose chains into cellobiose and glocose during the saccharification process. Cellobiose and glucose are known to inhibit enzymes activity. If an enzymatic hydrolysis is carried out separately, the hydrolisis rate is very low due to inhibition of the cellulase by cellobiose and glucose. To overcome this end product inhibition, the SSF process was invented (Ooshima *et al.,* 1985; Spangler and Emert 1986). By introducing microorganism along with cellulase, glucose can be consumed by the microorganism as soon as it formed. As a result, the enzymatic hydrolysis reaction is pulled in the forward direction and the rate of glucose production is significantly enhanced. Therefore, SSF proceeds under glucose limitation and the inhibition of glucose on enzymes is completely eliminated.

The production of lactic acid using the SSF process can be performed by bacteria or fungi cultures. There are several major reasons for using fungi instead of bacteria; among these, fungi is normally used when the substrate is a raw material or a waste material (Soccol *et al*., 1994; Miura *et al*., 2004; Park *et al*., 2004). Under such circumstances, there is no requirement for specific nutrients, and most fungi can tolerate very acidic conditions. Other advantages include the simple and cheap downstream processing, whereby the filamentous or pelleted biomass can easily be removed from the fermentation broth.

Despite many studies conducted on the bioconversion of lignocellulose biomass using solid substrates, most of the previous studies were focused on the use

of bacteria, *e.g*., *Lactobacillus* spp. (Sreenath *et al*., 2001; Garde *et al*., 2002; Hu *et al*., 2015). Recently, a fungus producing lactic acid, *Rhizopus* spp., was used in the fermentation process and was proven to produce lactic acid from lignocellulose biomass. However, in most of the studies that produced lactic acid *via* SSF using *Rhizopus* spp., the process involved lignocellulose hydrolysate (liquid) as a substrate, which was accomplished in two stages (Miura *et al*. 2004; Zhang *et al*. 2015). Hamzah and Idris (2008) had made an attempt to produce lactic acid using SSF of EFB in a laboratory-scale experiment using *Rhizopus* spp. pellets and cellulolytic enzymes. Unlike other studies (Miura *et al*. 2004; Ye e*t al*. 2014), which used lignocellulose hydrolysate (liquid) as the substrate, Hamzah and Idris (2008) used EFB, as solid substrate which was pretreated using a microwave-alkali method in the one stage SSF and the maximum lactic acid yield was 11 g/l. Besides, Hamzah and Idris (2008), the lactic acid production using a solid substrate and R.*oryzae* has not been widely investigated.

1.2 Problem statement

In Malaysia palm oil tree was cultivated over an area of 5 million hectares. It is predicted that the amount of biomass waste from EFB in year 2020 will reach 2.8 million tons. The huge accumulation of waste lead to serious problem as they do not have any suitable end use and are generally burnt in the fields causing environmental pollution. Therefore EFB as lignocellulose carbohydrate source can be used as a renewable and cheap substrate for lactic acid production. Lactic acid was reported to be produced from microwave alkali pre treated EFB by *Rhizopus oryzae* (Hamzah and Idris, 2008). However, the study was mainly performed at laboratory scale which involved shake flask fermentations. Up to this date, there is no report or data published for large scale production of lactic acid from solid substrate by using *Rhizopus oryzae.* Therefore, the present study aims to scale up the production of lactic acid and optimizes the yield as well. Several issues of cellulosic lactic acid production such as pretreatments and inoculum development were addressed. Scale-

up strategy was also developed that will lead to the advancement of knowledge and provide engineering aspect guidance towards development of commercial industrial-scale of cellulosic lactic acid from EFB.

1.3 Significant of the Study

High production cost and low lactic acid yield are the factors that limit the industrial lactic acid production using lignocellulose biomass. One of the major production cost is the substrate used. In this study, EFB is used as a substrate, which can significantly reduce the feedstock cost for lactic acid production. It is believed that this work would demonstrate the advantages of using low cost substrate derived from agricultural residue for lactic acid production. Process engineering method used in this work, would prove potential application for the future commercial production of lactic acid as a starting material for polylactic acid production. Microbial lactic acid production process unfortunately remains less efficient and its industrialization production process is still in the research stages. Therefore, aim of this project is to determine the possibility of large scale production of lactic acid from EFB at pilot scale. Despite the detailed knowledge of the bio refineries involved in the bio conversion pathway of lactic acid and basic fermentation studies , there is still no published information regarding the engineering aspects of fermentation scale-up of lactic acid from EFB.

1.4 Objectives and Scope of Study

The objectives of this research are:

1) To evaluate the effectiveness of a 30 L microwave alkali pretreatment of empty fruit bunch (EFB) on lactic acid yield and identify the physical and chemical changes on EFB fibre.
2) To develop a preculture technique in a 16 L bioreactor and control strategy to obtain desirable morphological form of *Rhizopus oryzae* NRRL 395 for lactic acid production in airlift bioreactor.
3) To improve the process performance in a 16 L bioreactor by using fed batch mode operation. This stepwise procedure was aimed at achieving high SSF yields, at lower enzyme loading, together with high lactic acid concentration. Effect of addition fresh enzyme during the process was also assessed.
4) To scale up the proposed bench scale SSF procedure and to identify the key issues of cellulosic lactic acid production at larger scales (150 L).

In order to achieve these objectives, the following scope of work should be covered.

1) The raw EFB and microwave-alkali treated EFB composition in terms of cellulose, hemicellulose and lignin content were determined.
2) The chemical and physical changes of EFB fibre using FESEM (Scanning Electron Microscope) and FT-IR (Fourier transform Infrared Spectroscopy were analyzed.
3) Identifying the factors that influence the formation of *Rhizopus oryzae* NRRL 395 pellet in a 16 L bioreactor including spore concentration, storage time of spore suspension and types of carbon sources used.
4) The growth profile of *Rhizopus oryzae* NRRL 395 in bioreactor were determined.
5) The effect of multi-stage inoculum on biomass production and their fermentation ability were assessed.

6) The effect of enzymes dosage, substrate concentration on lactic acid concentration were also investigated.

7) In addition, the effect of fed batch mode operation on lactic acid production using 3 different strategies were included using the following strategies:
   i) Strategy A (substrate and enzymes were added initially. After 24 h of fermentation, an additional 15 g of solid was added).
   ii) Strategy B (substrate and enzymes were added initially. Additional fresh fresh substrate was added twice after 24 h and 48 h).
   iii) Strategy C (substrate and enzymes were added initially. Additional both substrate and enzymes was added at 24 and 48 h of fermentation).

8) Evaluation of oxygen transfer coefficient ($k_L a$) using dynamic gassing out technique.

9) Investigates the effect of aeration rate on volumetric oxygen mass transfer coefficient ($k_L a$) in the 16 L and 150 L fermentor systems.

10) Study effect of aeration rate on the production rate of lactic acid and morphology of *Rhizopus oryzae* NRRL 395.

11) Compare time course profiles of cell growth, glucose consumption, DO concentration and lactic acid production in a 16 L and 150 L bioreactor.

The overall research frame work was presented in flowchart in Figure 1.1

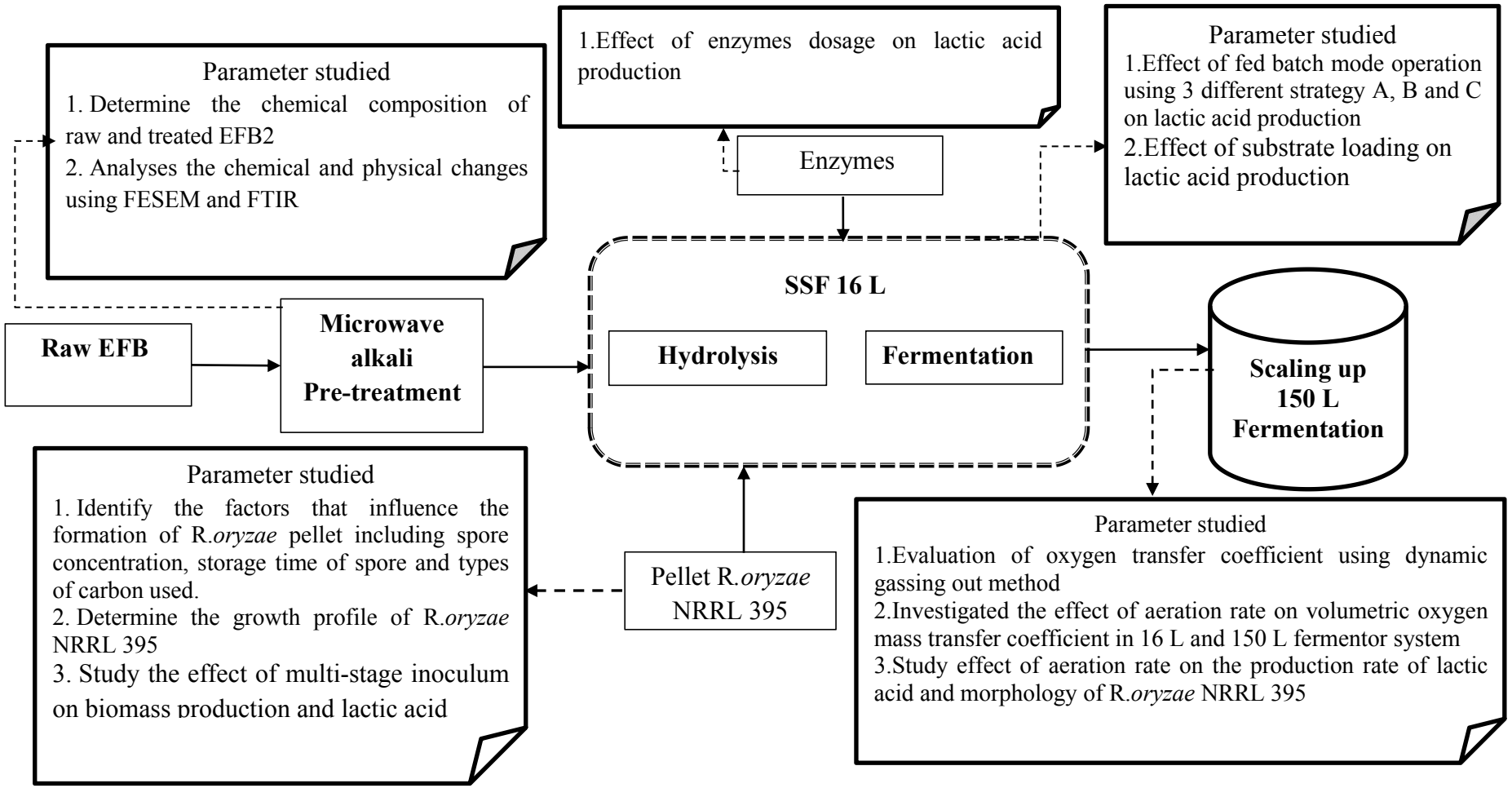

Figure 1.1: Flowchart of research activities

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