PRODUCTION OF CELLULASE BY *PYCNOPORUS SANGUINEUS* IN PALM OIL MILL EFFLUENT: FERMENTATION AND KINETIC STUDIES

by

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LIST OF ABBREVIATIONS

3D	Three dimensional
ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate)
ANOVA	Analysis of variance
BG	Beta-glucosidase (cellobiase)
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
CBH	Cellobiohydrolase
CCD	Central composite design
CMC	Carboxymethyl cellulose
CMCase	Endoglucanase
COD	Chemical oxygen demand
CPO	Crude palm oil
CSTB	Conventional stirred tank bioreactor
DNS	Dinitrosalicylic acid
DO	Dissolved oxygen
DoE	Design of experiment
DF	Degree of freedom
EFB	Empty fruit bunch
Eq.	Equation
FFB	Fresh fruit bunch
Fig.	Figure
FPase	Filter paperase (cellobiohydrolase/exoglucanase)
FPU	Filter paper unit
g	Gram
h	Hour
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HRT	Hydraulic retention time
IU	International unit
L	Liter
LHW	Liquid hot water

LME	Lignin modifying enzyme
LP	Leudeking-Piret
MEA	Malt extract agar
min	Minute
m	Meter
M	Molarity
MSE	Mean squared error
N	Normality
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OFAT	One-factor-at-a-time
Pa	Pascal
p-NPG	p-nitrophenyl-β,D-glucopyranoside
PPF	Palm pressed fibers
pO_2	Partial dissolved oxygen
POME	Palm oil mill effluent
rpm	Revolutions per minute
RSM	Response surface methodology
s	Second
SD	Standard deviation
SmF	Submerged fermentation
SSF	Solid state fermentation
STBEL	Stirred tank bioreactor with external vessel
TSS	Total suspended solids
UASB	Up-flow anaerobic sludge blanket
UASFF	Up-flow anaerobic sludge fixed-film
UML-MFC	Up-flow membrane-less microbial fuel cell
US	Ultrasound
v/v	Volume per volume
vvm	Volume of gas per vessel working volume per minute
w/v	Weight per volume

LIST OF SYMBOLS

		Unit
Δt	Time delay	h
$k_L a$	Volumetric mass transfer coefficient	1/h
K	Consistency coefficient	Pa.s
K_I	Substrate inhibition constant	dimensionless
K_P	Product inhibition constant	dimensionless
K_S	Saturation constant	dimensionless
m_s	Maintenance coefficient	g/g.h
n	Flow behavior index	dimensionless
P_0	Initial product concentration	g/L
P_c	Product concentration at time t_c	g/L
$q\mathrm{O}_2$	Specific oxygen uptake rate	mmol O_2/g_{cell}.h
q_{p1}	Proportionality constant (growth-associated)	dimensionless
q_{p2}	Proportionality constant (non-growth-associated)	dimensionless
r	Inhibition constant	dimensionless
R^2	Correlation coefficient	dimensionless
S_0	Initial substrate concentration	g/L
S_m	Maximum substrate concentration	g/L
t	Time	h
t_c	Time at which cells reach critical concentration	h
X_0	Viable inoculum size	g/L
X_c	Critical cell concentration	g/L
X_m	Maximum cell mass concentration	g/L
$Y_{P/X}$	Product yield per biomass produced	IU/g_{cell}

Greek symbols

		Unit
α	Growth-associated constant	g/g
β	Non-growth-associated constant	g/g.h
γ	Shear rate	1/s
η	Non-Newtonian viscosity	mPa.s
η_a	Apparent viscosity	Pa.s
σ	Variance	dimensionless
τ	Shear stress	Pa
τ_y	Yield stress	Pa
μ	Specific growth rate	g/L.h
μ_0	Initial specific growth rate	g/L.h
μ_m	Maximum specific growth rate	g/L.h

PENGHASILAN ENZIM SELULASE OLEH *PYCNOPORUS SANGUINEUS* DI DALAM KUMBAHAN KILANG KELAPA SAWIT: KAJIAN KINETIK DAN FERMENTASI

ABSTRAK

Biopenguraian kumbahan kilang kelapa sawit (POME) merupakan satu pendekatan baru berasaskan kaedah pengurusan sisa kumbahan secara cekap dan lestari. Kemajuan bioteknologi membolehkan penggunaan semula POME sebagai substratum dalam proses fermentasi bagi penghasilan pelbagai metabolit. Bagi menilai potensi POME sebagai substratum dalam penghasilan enzim selulase, sembilan belas spesis mikrob yang terdiri dari kulat mikro dan makro telah disaring untuk aktiviti selulase. *P. sanguineus* didapati mempunyai kemampuan tinggi untuk menghasilkan enzim selulase dan laccase dengan 2.488, 1.405, 0.232 dan 2.763 IU/mL bagi aktiviti CMCase, FPase, BG dan laccase, masing-masing.

Fermentasi secara mod kelompok menggunakan *P. sanguineus* dan POME sebagai bahan media asas telah dijalankan di dalam kelalang kultur dan bioreaktor. Kajian awal menunjukkan peningkatan 1.5 kali ganda bagi aktiviti enzim selulase menggunakan POME yang dirawat melalui kaedah ultrabunyi. Biojisim sel tertinggi (11.413 g/L) dan aktiviti enzim tertinggi (CMCase, 6.018; FPase, 3.221, BG, 0.644; laccase, 8.149 IU/mL) masing-masing diperoleh pada kepekatan POME 60% (v/v), pH 7 dan menggunakan peptone sebagai sumber nitrogen. Bagi meningkatkan lagi penghasilan selulase, pengoptimuman parameter telah dijalankan menggunakan kaedah sambutan permukaan (RSM) digandingkan dengan rekabentuk rencam berpusat (CCD). Keadaan optima yang didapati melalui kaedah ini ialah kepekatan POME 70% (v/v), saiz inokulum 15% (v/v), kepekatan selulosa (sumber karbon tambahan) 0.8% (w/v) dan kepekatan peptone (sumber nitrogen tambahan) 0.1%

(w/v), di mana aktiviti enzim tertinggi yang diperolehi adalah 10.613, 6.814, 1.04 dan 10.886 IU/mL bagi CMCase, FPase, BG dan laccase masing-masing.

Fermentasi secara mod kelompok di dalam bioreaktor tangki teraduk berterusan (CSTB) mendapati penghasilan biojisim dan enzim selulase adalah dipengaruhi oleh kadar pengudaraan dan kelajuan pengadukan. Aktiviti enzim tertinggi didapati pada kadar pengudaraan 1.0 vvm dan kelajuan pengadukan 300 rpm. Pengoptimuman dengan CCD menunjukkan keadaan optimum adalah pada kepekatan POME 70% (v/v), kelajuan pengadukan 350 rpm dan kadar pengudaraan 1.0 vvm di mana aktiviti enzim tertinggi sebanyak 16.073, 10.012, 2.348 dan 12.186 IU/mL diperolehi bagi CMCase, FPase, BG dan laccase masing-masing. Parameter yang teroptimum kemudiannya diadaptasi untuk kajian didalam bioreaktor tangki teraduk bergelung luar (STBEL). Penghasilan enzim didapati meningkat di mana biojisim tertinggi (27.82 g/L) dan aktiviti enzim tertinggi (CMCase, 18.221; FPase, 13.406; BG, 3.37; laccase, 17.481 IU/mL) masing-masing.

Model empirikal telah dicadangkan bagi menerangkan pertumbuhan sel, penghasilan selulase, penggunaan substratum dan penghalangan didalam CSTB. Analisa kinetik pada kepekatan substratum berbeza menunjukkan data eksperimen dan teori sangat bersesuaian dengan nilai R^2 melebihi 0.9. Kajian reologi mendapati cecair media menepati ciri bukan-Newtonian dan telah dijelaskan dengan baik menggunakan model Herschel-Bulkley.

PRODUCTION OF CELLULASE BY *PYCNOPORUS SANGUINEUS* IN PALM OIL MILL EFFLUENT: FERMENTATION AND KINETIC STUDIES

ABSTRACT

Bioconversion of palm oil mill effluent (POME) is a novel approach in view of the increasing emphasis placed on sustainable and efficient industrial wastewater management. Biotechnological advances have seen the reuse of POME as a fermentation substrate for the production of various metabolites. In order to assess the potential of POME as substrate for cellulases production, nineteen microbial strains comprising of micro- and macrofungi were screened for cellulolytic activities. *Pycnoporus sanguineus* was found to be a potent producer of cellulases and laccase with maximum CMCase, FPase, β-glucosidase and laccase activities at 2.488, 1.405, 0.232 and 2.763 IU/mL, respectively.

Batch cultivations of *P. sanguineus* using POME as the base media were subsequently conducted in both shake flask and bioreactor. Preliminary studies in shake flask showed that pre-treatment of POME with ultrasound increased the cellulases activities 1.5-fold. The highest cell biomass (11.413 g/L) and enzymatic activities (CMCase, 6.018; FPase, 3.221; BG, 0.644; laccase, 8.149 IU/mL) were obtained with 60% (v/v) of POME concentration, pH 7 using peptone as a nitrogen source. To further improve the cellulases production, optimization of process parameters were conducted using response surface methodology (RSM) coupled with a central composite design (CCD). The optimum conditions obtained were POME concentration 70% (v/v), inoculums size 15% (v/v), cellulose (carbon) concentration 0.8% (w/v), and peptone (nitrogen) concentration 0.1% (w/v), which resulted in 10.613, 6.814, 1.040 and 10.886 IU/mL of CMCase, FPase, BG and laccase activities, respectively.

Batch culture of *P. sanguineus* in conventional stirred tank bioreactor (CSTB) showed that biomass and cellulase production were influenced by aeration rate and agitation speed. Aeration rate at 1.0 vvm and agitation speed 300 rpm were found to yield the highest cellulases activities and volumetric mass transfer coefficient (k_La). Optimization with CCD indicated an optimum condition at 70% (v/v) POME concentration, 350 rpm agitation speed and 1.0 vvm aeration rate with maximum enzyme activities at 16.073, 10.012, 2.348 and 12.186 IU/ml for CMCase, FPase, BG and laccase, respectively. The optimized parameters were then adapted in the studies using stirred tank bioreactor with external loop (STBEL). Enzyme productions were shown to improve with higher maximum biomass (52.75 g/L) and enzyme activities (CMCase, 18.221, FPase, 13.406, BG, 3.370 and laccase, 17.481 IU/mL) compared to CSTB.

Empirical models were proposed to describe microbial growth, cellulases production, substrate consumption and inhibition in conventional bioreactor. Kinetic analysis at various substrate concentrations showed good agreement between the theoretical and experimental data with R^2 values higher than 0.9. Rheological study showed that the culture broth was consistent with non-Newtonian characteristics and was satisfactorily described by the Herschel-Bulkley model.

CHAPTER 1

INTRODUCTION

1.0 Cellulase: Commercial applications and recent trends in productions

Basic and applied research on microbial cellulase has not only generated significant scientific knowledge, but has also revealed their enormous potential in biotechnology (Bhat, 2000). Among others, cellulase are used in the textile industry for cotton softening and denim finishing; in the detergent market for color care, cleaning and anti-deposition; in the food industry for mashing; and in the pulp and paper industry for de-inking, drainage improvement, and fiber modification (Kirk *et al*., 2002; Cherry and Fidantsef, 2003). Some of the commercial applications of cellulase are tabulated in Table 1.1. In the last two decades, cellulase accounted for approximately 20% of the world enzyme market due to their increasing industrial use especially in textile, food, brewery and wine, and pulp and paper industry (Mathew *et al.*, 2008).

More importantly, the multicomponent enzyme systems of cellulase are extensively investigated because of their ability to decompose cellulosic biomass into glucose, which in turn can be converted to other valuable chemicals and energy (Domingues *et al.*, 2000). The cellulase market is expected to expand dramatically when cellulase are used to hydrolyze pretreated cellulosic materials to sugars, which can be fermented to commodities such as bioethanol and bio-based products on a large scale (Himmel *et al*., 1999; van Beilen and Li, 2002; Cherry and Fidantsef, 2003). The growing concerns about the shortage of fossil fuels, the emission of green house gases and air pollution by incomplete combustion of fossil fuel have also resulted in an increased focus on production of bioethanol from lignocellulosics and

especially the possibility to use cellulase to perform enzymatic hydrolysis of the lignocellulosic materials (Jorgensen *et al.*, 2003).

Table 1.1 Industrial applications of cellulase (Singh *et al.*, 2007)

Industry	Applications
Agriculture	Plant and fungal protoplast; enhanced seed germination; enhanced plant growth and flowering; plant pathogen and disease control
Bioconversion	Conversion of cellulosic materials to ethanol, other solvents, organic acids and single cell protein, lipids
Detergents	Cellulase-based detergents; superior cleaning action without damaging fiber
Environment	Wastewater treatment/purification; biosolids reduction
Fermentation	Macerating enzymes; improve pressing and color extraction of grapes; improve aroma of wines; improve filtration and stability; improve primary fermentation and quality of beer
Food	Improvement of yields in starch and protein extraction; improve maceration; pressing and color extraction of fruits and vegetables; clarification of fruit juices; improve structure and quality of bakery products
Pulp and paper	Co-additive in pulp bleaching; bio-mechanical pulping; improve draining; enzymatic de-inking
Textile	Biostoning and biopolishing of jeans and other fibers; high quality fabrics

Complete cellulase enzyme systems can be produced by a large diversity of microorganisms. On the other hand, commercial productions of cellulase were mostly based on filamentous fungi due to their ability to secrete large amounts of enzyme proteins. Among the best characterized and most widely studied of these systems are the inducible cellulase of the filamentous fungus *Trichoderma reesei* (Domingues *et al.*, 2000). The characteristics and microbial origin of several commercial cellulase preparations are listed in Table 1.2.

Cellulase are relatively costly enzymes, and a significant cost reduction is crucial in order for the cellulase process technology to become commercially practicable. Various biotechnological approaches have been adopted to increase their production and applicability. Reduction in the cost of cellulase can be achieved by concerted efforts which address several key aspects of enzyme production such as the raw material used, microbial strain improvement, and development of suitable bioprocesses (Ahamed and Vermette, 2008; Sukumaran *et al*., 2008; Roslan *et al*., 2009).

One of the most researched approaches is the use of cheap lignocellulosic substrates for production of the enzyme, whereas high cell yields by aerobic cellulolytic bacteria and fungi have led to considerable technological interest in producing microbial cell protein from waste cellulosic biomass (Lynd *et al*., 2002; Sukumaran *et al*., 2008). Various substrates have been evaluated for cellulase production using cellulose, soluble substrates, agricultural residues and other sources. On the other hand, the presence of hemicelluloses and lignin, which rendered the utilization of cellulosic biomass to be more complex, necessitates the development of cost-effective pretreatment technologies that are necessary to separate the polymers (Zhang *et al*., 2006; Brosse *et al*., 2009).

Table 1.2 Commercial cellulase preparations and their enzyme activities (Nieves *et al.*, 1998; Kabel *et al.*, 2006)

Product name	Company	Source	Activity titres		
			FPase[a] (U/ml)	BG[b] (U/ml)	Protein[c] (mg/ml)
Biocellulase TRI	Quest Intl. (USA)	*T. reesei*	68	200	279
Biocellulase A	Quest Intl.	*A. niger*	0.29	32	23
Cellulase TAP10	Amano Enzyme (USA)	*T. viride*	0.42	17	3.3
Cellulase AP30K	Amano Enzyme	*A. niger*	0.17	60	6
Cellulase TRL	Solvay Enzymes (USA)	*T. reesei*	95	170	167
Econase CE	Alko-EDC (USA)	*T. reesei*	40	46	95
Ultra-Low Microbial	Iogen (Canada)	*T. reesei*	88	176	184
Celluclast 1.5L	Novozymes (Denmark)	*T. reesei*	61	26	166
Cellubrix	Novozymes	*A. niger*	56	136	43
Novozymes 188	Novozymes	*A. niger*	<5	1,116	57
Bio-feed Beta L	Novozymes	*A. niger*	<5	12	8
Energex L	Novozymes	*A. niger*	<5	19	28
Ultraflo L	Novozymes	*A. niger*	<5	20	18
Viscozyme L	Novozymes	*A. niger*	<5	23	27
GC 220	Genencor-Danisco (USA)	*T. reesei*	116	215	64
GC 440	Genencor	*T. reesei*	<5	70	29
GC 880	Genencor	*T. reesei*	<5	86	43
Spezyme CP	Genencor	*T. reesei*	49	nd	41

nd, not determined

[a]Determined according to IUPAC method

[b]Determined according to IUPAC method with cellobiose

[c]Determined by using the Bradford method

1.1 Biotechnological potentials of palm oil mill effluent

As one of the world's leading producer and exporter of palm oil, Malaysia boasts an impressive volume of annual crude palm oil (CPO) production. According to the recent report in the Overview of the Malaysian Oil Palm Industry (2009), 17.56 million tons of CPO was produced in 2009 and the number is projected to increase to 18.1 million tons in 2010 (Malaysian Palm Oil Board). On the other hand, intense production of crude palm oil has resulted in even larger amount of wastes being produced. Considerable wastes in varying nature are generated during the palm oil extraction process, including solid waste materials and by-products that include empty fruit bunches (EFB), potash ash, palm kernel, fiber and shells, liquid effluent known as palm oil mill effluent (POME), and gaseous emissions from boilers and incinerators (Industrial Processes and The Environment, 1999).

Treatment and disposal issues are especially prevalent with regard to POME due to its high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), whereas the release of such effluent into receiving water sources posed the risk of severe waterways pollution as a result of oxygen-depletion and other related effects. This adverse effect is further aggravated by the ever increasing volume of POME, estimated at nearly three times the quantity of CPO (Wu *et al.*, 2009). The last decade has seen management of POME evolving from 'treatment of a waste for disposal' to 'beneficial utilization of an asset', with reports abound on biotechnological application of the effluent. Extensive research dedicated to finding solutions for managing POME that are economically and technically feasible has seen the development of biotechnological advances which aimed at transforming POME into value-added products.

The potential utilization of POME through bioconversion into value added products was based on its appreciable amounts of plant nutrients made up by the high compositions and concentrations of carbohydrate, protein, nitrogenous compounds, lipids and minerals (Wu *et al.*, 2009). Among others, biotechnological utilization of POME has resulted in the recovery of bioresources such as antibiotics, bioinsecticides, solvents, polyesters, organic acids, enzymes and biohydrogen, which is summarized in Table 1.3.

Table 1.3 Various products or metabolites produced in bioprocesses during the reuse of POME or its derivatives as substrates (Wu *et al*., 2009)

Product	Reference
Penicillin	Suwandi (1991)
Bioinsecticide	Suwandi (1991)
Acetone, butanol, ethanol	Mun *et al*. (1995), Somrutai *et al*. (1996), Kalil *et al*. (2003), Pang *et al*. (2004), Takriff *et al*. (2005), Masngut *et al*. (2006, 2007)
Polyhydroxyalkanoates	Hassan *et al*. (1996, 1997a, 1997b, 1997c, 2002), Md. Din *et al*. (2006)
Organic acids	Hassan *et al*. (1996), Yee *et al*. (2003)
Citric acid	Jamal *et al*. (2005), Alam *et al*. (2008)
Cellulase	Prasertsan *et al.* (1997), Mashitah *et al*. (2002), Alam *et al*. (2006a), Chowdury *et al*. (2006), Laohaprapanon *et al*. (2007)
Lignin peroxidase	Alam *et al*. (2006b), Chowdury *et al*. (2006)
Lipase	Somrutai *et al*. (1996)
Xylanase	Prasertsan *et al*. (1997), Cheng (2006), Laohaprapanon *et al*. (2007)
Protease	Wu *et al*. (2006a)
Hydrogen	Morimoto *et al*. (2004), Atif *et al*. (2005), Vijayaraghavan and Ahmad (2006), O-Thong *et al*. (2007), O-Thong *et al*. (2008a)

1.2 Problem statement

Utilization of POME not only will reduce the polluting effects from the palm oil processing industries, but also provide an alternative solution for liquid waste management system at palm oil mills as well as offering the possibility of creating marketable value-added products. The entirely organic particulates in POME especially provided a potential substrate for cellulase production. The potential of POME as a substrate for cellulase production have been studied previously (Prasertsan *et al*., 1997; Mashitah *et al*., 2002; Alam *et al*., 2006a). However, the possible effects of lignin have not been included in these studies despite the fact that POME was shown to contain lignin. As such, selection of suitable fungi with lignin-modifying properties is envisaged so as to justify the use of POME as substrate.

Microbial cellulase production is influenced by a number of factors including the type of strain used, reaction conditions (temperature, pH etc.) and inducer or substrate type. In turn, identifying the optimum conditions is important as the relationship between these variables has a marked effect on the production of the cellulase enzymes. Cellulase process variables have been studied by many researchers with the aim to optimize the fermentation process variables and improve the cellulase productivities (Lynd *et al.*, 2002). So far, not many systematic studies have been carried out on the production of cellulase in POME using microbial strain with lignin modifying enzymes (LME), specifically using *Pycnoporus sanguineus*. Thus, a detailed study of cellulase production by *P. sanguineus* in POME over a broad range of fermentation variables can provide the much-needed data and act as a mean to assess the performance at selected conditions.

In turn, optimized growth conditions or processes can facilitate the development of better production strategies. Analysis of the fermentation kinetics will provide validated kinetic models capable of predicting saccharification performance over a range of fermentation variables. Meanwhile, the addition of an external recirculation loop is anticipated to address inhibition by end products commonly associated with the conventional stirred tank bioreactor, thereby improving yield of cellulase.

1.3 Research objectives

The main objective of this study is to investigate the potential of palm oil mill effluent (POME) as a low-cost feedstock for cellulase production while providing an alternative strategy for their treatment.

The measurable objectives are:

- To screen a suitable cellulase-producing fungus using POME as substrate
- To study the effect of different culture variables for cellulase production in shake flask cultures and in a bioreactor
- To optimize cellulase production using design of experiment (DoE)
- To propose and validate kinetic models for cellulase production in conventional stirred-tank bioreactor
- To compare cellulase productivity in conventional bioreactor and bioreactor with external loop

1.4 Scope of study

In this study, cellulase production from submerged cultivation of *Pycnoporus sanguineus* in palm oil mill effluent (POME) was studied. *P. sanguineus*, a ubiquitous white rot fungus, is available in large quantities and can be easily grown in basic fermentation medium. The use of POME as fermentation media is an economical way to reduce production cost as well as addressing environmental issue in the palm oil processing industry.

Two series of batch fermentation in shake flask and conventional bioreactor were conducted to determine the effect of selected parameters on cellulase production. Statistical analysis was done using response surface methodology (RSM) to optimize process variables in both series of fermentation. In shake flask fermentation, the input factors that were considered for optimization were initial POME concentration, inoculums size, and initial carbon and nitrogen concentration. Subsequently, the effect of initial POME concentration, aeration rate and agitation speed were studied in a bioreactor. Biomass production, residual cellulose, cellulase and laccase activities were taken as responses of parameter optimization. The optimum results of cellulase production in conventional bioreactor were then compared to a modified bioreactor coupled with an external loop.

Cellulase production kinetics were studied to evaluate cell growth, cellulase formation, substrate consumption and inhibition in conventional bioreactor. Three working models were proposed for each part. For cell growth, the Monod, logistic and modified logistic models were proposed, while cellulase production were proposed to be modeled by the Leudeking-Piret, logistic-incorporated Leudeking-Piret and Leudeking-Piret with time delay equations. Leudeking-Piret-like models incorporating the Monod, logistic and modified logistic were proposed for substrate

consumption. The inhibition by substrate and product were also studied. Several inhibition models such as Aiba, Andrew, Tessier, competitive and noncompetitive substrate were proposed to model the inhibition pattern. The validity of each models were assessed by determining the coefficient of determination (R^2) value and mean square error (MSE).

1.5 Organization of the thesis

The thesis consisted of five chapters that are described systematically in the following order:

Chapter One introduces the biotechnological utilization of palm oil mill effluent for cellulase production and the industrial application of cellulase. This chapter also presents the problem statement, research objectives, and scope of study.

Chapter Two covers an overview of the sources and treatment technologies of POME, the cellulase enzyme system and their sources, fermentation technologies and production kinetics, *Pycnoporus sanguineus* as the organism of interest, as well as method of statistical analysis applied in this study.

Chapter Three describes the material and procedures applied during the experimental stage of the study. This chapter also covers the screening process, fermentation in shake flask and bioreactor, optimization studies, and analytical procedures.

Chapter Four discussed the experimental result together with the data analysis at various operating condition and process parameters. The results are organized into four main sections; preliminary study, batch fermentation in shake flask, batch fermentation in conventional bioreactor and batch fermentation in

bioreactor with external loop. Optimizations of process parameters using response surface methodology were also included for cellulase production in flasks and conventional bioreactor. Kinetic models for cellulase production in conventional bioreactor were proposed and validated. Finally, comparisons were made on enzyme production in conventional and modified bioreactor with external recirculation loop.

Chapter Five gives the overall conclusion based on the findings obtained in Chapter Four. Recommendations for future research are also highlighted.

CHAPTER TWO

LITERATURE REVIEW

2.1 Palm oil mill effluent (POME)

2.1.1 Sources of POME

Based on the milling operations, the palm oil extraction process is divided into dry and wet process. The majority of palm oil mills in Malaysia have adopted the wet process since the dry process is unsuitable for use in large-scale productions (Wu *et al*., 2009, 2010). Large quantities of water are used during the extraction of crude palm oil from the fresh fruit bunch (FFB) using the wet process; typically 1.5 cubic meter of water is used to process one ton of FFB (Sethupathi, 2004). About 50% of the water results in palm oil mill effluents (POME), with the rest of the water lost as steam, mainly through sterilizer exhaust, piping leakages as well as wash waters. Principal sources of POME are the sterilizer condensate (36%), separator sludge/clarification wastewater (60%) and hydrocyclone wastewater (4%) (Industrial Processes and The Environment, 1999).

Figure 2.1 depicted a conventional wet palm oil extraction process and the sources of POME. FFBs received from oil palm plantations are first sterilized by steam-heat treatment at a pressure of 3 bars and temperature of 140°C for 75-90 min. The objectives of this process are to prevent further formation of free fatty acids due to enzyme action, facilitate stripping and prepare the fruit mesocarp for subsequent processing. The steam condensate coming out of the sterilizer is referred to as sterilizer condensate, which constituted the first source of POME (Industrial Processes and The Environment, 1999).

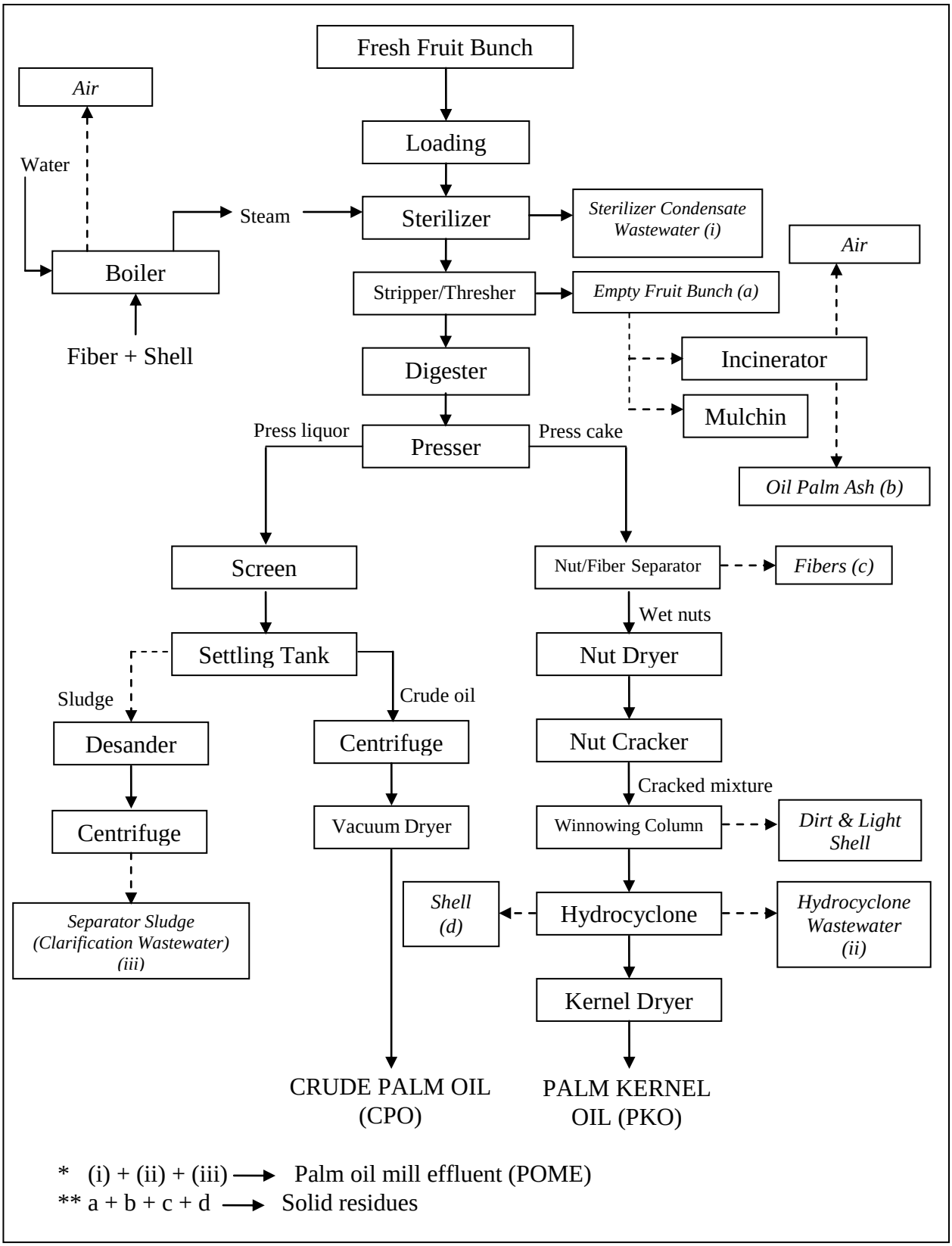

Figure 2.1 Conventional wet palm oil extraction process and sources of waste generation (Industrial Processes and The Environment, 1999; Wu *et al.*, 2010)

Afterwards, the sterilized FFBs are fed to a rotary drum-stripper where the fruits are stripped from their bunch. The detached fruits are then passed into a digester where the fruits are mashed to break the oil-bearing cells of the mesocarp. The digested mash of fruits is then subjected to pressing under high pressure, usually by twin-screw presses, to press out the oil. Following extraction, the crude oil slurry has to go through clarification and purification as they consist of a mixture of palm oil (35-45%), water (45-55%) and fibrous materials in varying proportions. The clarified oil is continuously skimmed off from the top of the clarification tank, while the underflow is passed through a sludge separator to recover the remaining oil. The other stream consisting of water and fibrous debris, generally referred to as separator sludge or clarification wastewater, are discharged as wastewater and constituted the largest portion of POME (Industrial Processes and The Environment, 1999).

The press cake discharged from the screw press, which consisted of oily fiber, nuts and some moisture, are conveyed to a depericarper for nuts and fibers separation. Most of the nuts coming from the nut-fiber separator may have the kernels sticking to the shell. Hydrocyclone is commonly used to separate these kernels and shells. The discharge from this process constituted the last principal source of wastewater stream. Other minor sources of relatively clean wastewater that may be included in POME include turbine cooling water and steam condensates, boiler blowdown, overflows from the vacuum dryers and some floor washings (Industrial Processes and The Environment, 1999).

2.1.2 Characteristics of POME

Raw POME consists of 95-96% water, 0.6-0.7% oil and 4-5% total solids, half of which is suspended solid consisting of debris from the fruit. Fresh POME is a hot (80-90°C) and acidic brownish colloidal suspension, characterized by high amounts of total solids (40,500 mg/L), oil and grease (4000 mg/L), COD (50,000 mg/L) and BOD (25,000 mg/L) (Industrial Processes and The Environment, 1999). Predominantly organic in nature, POME is non-toxic as no chemicals are added during oil extraction (Mashitah *et al*., 2002). Najafpour *et al.* (2005) stated that POME varied in characteristics from one factory to another, depending on the mode or type of processing, the quality of the fresh fruits, the point and time of POME collection and also the method of chemical analysis employed. The characteristics of a typical POME are shown in Table 2.1.

Table 2.1 Characteristics of POME (Industrial Processes and The Environment, 1999)

Parameter*	Range	Average
pH	3.4 – 5.2	4.2
Biochemical oxygen demand (BOD)	10 000 – 44 000	25,000
Chemical oxygen demand (COD)	16 000 – 100 000	50,000
Total solids	11 500 – 79 000	40,500
Suspended solids	5000 – 54 000	18,000
Total volatile solids	9000 – 72 000	34,000
Oil and grease	150 – 18 000	6,000
Total nitrogen	80 - 1400	35
Ammoniacal nitrogen	4 - 80	750

**All parameter's unit in mg/L except pH*

POME contains various suspended components including cell walls, organelles, short fibres, a spectrum of carbohydrates ranging from hemicellulose to simple sugars, a range of nitrogenous compounds from proteins to amino acids, free organic acids and an assembly of minor organic and mineral constituents (Ugoji, 1997). The high compositions and concentrations of carbohydrate, protein,

nitrogenous compounds, lipids and minerals in POME render it possible for reuse through biotechnological means (Wu *et al*., 2009).

2.1.3 Treatment technologies

The end-of-pipe POME treatment strategy generally involved three major steps: pretreatment, biological treatment, followed by land application. As the organic content of POME is generally biodegradable, treatment is based on anaerobic, aerobic and facultative processes. The processes are essentially biochemical and rely on the enhanced growth and metabolic activities of microorganisms to breakdown the organic matter into simple end products such as methane, carbon dioxide and hydrogen sulphide, and water (Industrial Processes and The Environment, 1999). In addition to the traditional biological treatment, recent studies have also focused on physicochemical treatment and membrane filtration process. Some of the advantages and disadvantages of each treatment methods are summarized in Table 2.2.

The various POME treatment schemes currently used in Malaysia are: (a) anaerobic/facultative ponds, (b) tank digestion and mechanical aeration, (c) tank digestion and facultative ponds, (d) decanter and facultative ponds, and (e) physico-chemical and biological treatment (Vijayaraghavan *et al*., 2007). Nevertheless, the conventional treatment technology employed in most of the palm oil mills is the ponding system of biological treatment (Ahmad *et al*., 2009). Biologically treated effluent is then disposed off via land application system, providing essential nutrients for growing plants.

Table 2.2 Advantages and disadvantages of various treatment processes for POME (Wu *et al*., 2010)

Treatment processes	Advantages	Disadvantages
Aerobic digestion	Shorter retention time than anaerobic digestion Filamentous growth and sludge bulking can be prevented	Energy intensive Needs to be incorporated into other treatment systems, preferably anaerobic
Anaerobic digestion	*Suspended growth processes* Generation of biogas in closed digester causes mixing and circulation *Immobilized cell reactor* High COD removal Less sensitive to shock loads with lower HRT *Sludge blanket processes* High organic loadings, short HRT and low energy demand	 Uncontrollable release of methane gas Very dependent on support material Increasing POME load may lead to filter medium blockage Dependent on sludge settleability
Physicochemical treatment	*Sedimentation* - Cheap *Centrifugation* - High TSS removal *Coagulation & flocculation* - Destabilization of POME suspension *Flotation* - Together with chemical flocculation may improve TSS removal *Adsorption* - High removal of residual oil	Long time needed for settling Low COD removal Perform well at low surface loading rate
Membrane filtration	Smaller space requirement Treated water could be used as drinking water High rejection of TSS from POME	Membrane fouling and degradation

More than 85% of palm oil mills in Malaysia use ponding systems solely due to their low costs. However, this method of treatment suffered from several drawbacks such as long hydraulic retention times (HRT), low treatment efficiency, high sludge production, extensive land area requirement, emission of large amount of greenhouse gases (CO_2 and CH_4) and so on. Meanwhile, the formulation of POME discharge standards by phases has brought about catalytic impact in the development of effluent treatment technology in the form of innovative or newly created technology (Yusoff, 2006). Some of the technologies being researched are listed in Table 2.3. Development of cost-effective process for treatment and utilization of POME is essential in order to improve the competitiveness of industrial processing operations and to minimize the environmental hazards.

Table 2.3 Laboratory-scale biological POME treatment methods

Methods of treatment	References
Up-flow anaerobic sludge blanket (UASB)	Borja and Banks (1994a)
Up-flow anaerobic filtration	Borja and Banks (1994b)
Fluidized bed reactor	Borja and Banks (1995)
Evaporation method	Ma *et al*. (1997)
Up-flow anaerobic sludge fixed-film reactor (UASFF)	Najafpour *et al*. (2006), Zinatizadeh (2006)
Aerobic activated sludge reactor	Vijayaraghavan *et al*. (2007)
Up-flow membrane-less microbial fuel cell (UML-MFC)	Cheng *et al*. (2010)

2.2 Cellulases

Cellulases are enzymes which hydrolyze the β-1,4-glucosidic linkages of cellulose, producing glucose, cellobiose and cello-oligosaccharides as primary products (Singhania *et al*., 2010). A complete cellulase system consists of three general classes of enzymes: endoglucanases (EG I and II), cellobiohydrolases (CBH I and II) and beta-glucosidase, which together act synergistically to degrade cellulose

to glucose (Reczey *et al*., 1996). As a result of this synergism, cellulase systems exhibit higher collective activity than tphe sum of the activities of individual enzymes (Lynd *et al*., 2002). Cellulase activities are customarily reported in FPU/mL, referring to the filter paper method used to determine overall cellulase activity. Cellulase classes and their mode of action are tabulated in Table 2.4.

Table 2.4 Cellulase classes and mode of action (Ferreira *et al*., 2008)

Class	EC number	Mode of action
Endoglucanases (1,4-β-D-glucan-4-glucanohydrolases)	EC 3.2.1.4	Cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends
Exoglucanases, including:		Act in a processive manner on the reducing or non-reducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products
Cellodextrinases (1,4-β-D-glucan glucanohydrolases)	EC 3.2.1.74	
and cellobiohydrolases (1,4-β-D-glucan cellobiohydrolases)	EC 3.2.1.91	
β-glucosidases or β-glucoside glucohydrolases	EC 3.2.1.21	Hydrolyze soluble cellodextrins and cellobiose to glucose

2.2.1 Cellulase producer

Functionally complete cellulase enzyme systems can be produced by diverse microorganisms such as aerobic and anaerobic bacteria, white rot fungi, soft rot fungi and anaerobic fungi (Kadam, 1996). Table 2.5 lists major groups of microorganisms which have been employed in cellulase production. Members of genera that have received considerable study with respect to their cellulolytic enzymes and/or wood-degrading capability include: a) ascomycetes, e.g. *Bulgaria*, *Chaetomium*, and *Helotium*, b) basidiomycetes, e.g. *Coriolus*, *Phanerochaete*, *Poria*, *Schizophyllum* and *Serpula*, and c) deuteromycetes, e.g. *Aspergillus*, *Cladosporium*, *Fusarium*,

Geotrichum, Myrothecium, Paecilomyces, Penicillium, and *Trichoderma* (Lynd *et al*., 2002).

Table 2.5 Major microorganisms employed in cellulase production (Sukumaran *et al*., 2005)

Major group	Microorganism	
	Genus	Species
Fungi	*Aspergillus*	*A. niger*
		A. nidulans
		A. oryzae (recombinant)
	Fusarium	*F. solani*
		F. oxysporum
	Humicola	*H. insolens*
		H. grisea
	Melanocarpus	*M. albomyces*
	Penicillium	*P. brasilianum*
		P. occitanis
		P. decumbans
	Trichoderma	*T. reesei*
		T. longibrachiatum
		T. harzianum
Bacteria	*Acidothermus*	*A. cellulolyticus*
	Bacillus	*Bacillus sp.*
		B. subtilis
	Clostridium	*C. acetobutylicum*
		C. thermocellum
	Pseudomonas	*P. cellulosa*
Actinomycetes	*Cellulomonas*	*C. fimi*
		C. biazotea
		C. uda
	Streptomyces	*S. drozdowiczii*
		S. sp
		S. lividans
	Thermomonospora	*T. fusca*
		T. curvata

Fungal cellulases have proved to be a better candidate than other microbial cellulases, with their secreted free cellulase complexes comprising all three components of cellulase (Mathew *et al*., 2008). Collectively, more than 14 000 fungal strains have been found to actively degrade cellulose and other insoluble fiber materials (Kadam, 1996). White rot fungi are especially unique in their ability to

degrade all components of lignocellulosic materials, whereas cellulolytic capability is especially well represented among the subdivisions of aerobic fungi (Levin *et al*., 2008; Mathew *et al*., 2008). Currently, most industrial cellulases are produced from aerobic cellulolytic fungi, due to the ability of engineered strains of these organisms to produce extremely large amounts of crude cellulase (Wilson, 2009).

The best characterized and most widely studied cellulase system is that of the soft rot fungus *Trichoderma*, particularly *T. reesei* (Reczey *et al*., 1996). Other *Trichoderma* species such as *T. lignorum*, *T. koningii*, *T. pseudokoningii* and *T. harzianum* also produce active cellulases. Among the advantages of *Trichoderma* are: a) it produces a full complement of cellulases, and b) these enzymes are resistant to chemical inhibitors and are stable under reaction conditions of 50°C and pH 4.8 for 48 h or longer (Kadam, 1996). On the other hand, its drawbacks are: a) the cellulases have low specific activity, b) enzyme synthesis is repressed by soluble sugars, and c) suboptimal level of β-glucosidase is produced (Kadam, 1996; Jørgensen and Olsson, 2006; Mathew *et al*., 2008). Meanwhile, Lynd *et al*. (2002) stated that there are no data to suggest that *Trichoderma* exhibits unusually high rates of growth on cellulosic substrates.

Cellulase systems of microbes are generally grouped into complexed (cell associated) or non-complexed (free) system. The former system is commonly associated with cellulolytic activities of anaerobic bacteria where cellulases are organized into high molecular weight complexes called cellulosomes. On the other hand, cellulases system of filamentous fungi, actinomycetes, and aerobic bacteria are generally non-complexed, i.e. their cellulases are mostly secreted as free molecules (Mathew *et al*., 2008).

2.2.2 Cellulosic substrate

Cellulase preparations have mostly been based on high-purity cellulose as the carbon source, as cellulose has been identified as one of the best inducer for the complete cellulase complex (Linko *et al*., 1996; Reczey *et al*., 1996; Liming and Xueliang, 2004; Ahamed and Vermette, 2008). In spite of the high enzyme concentration and productivity with purified cellulose substrate, the costs are not favorable and too costly for industrial scale fermentations.

One effective approach to reduce the cost of enzyme production is to replace pure cellulose by relatively cheaper substrates such as lignocellulosic residues. Also, high cell yields by aerobic cellulolytic bacteria and fungi have led to considerable technological interest in producing microbial cell protein from waste cellulosic biomass (Lynd *et al*., 2002). Cellulase production has been attempted on a wide range of agro-residues with varying degree of success. Numerous reports are available on the utilization of various pre-treated lignocellulosic biomass such as corn fiber (Vlaev *et al*., 1997; Xia & Shen, 2003), wheat and rice straw (Romero *et al*., 1999; Kang *et al*., 2004), spruce and willow (Juhász *et al*., 2005) and oil palm residual fibers (Umikalsom, 1997; Mashitah, 1997). The highest enzyme titer and productivity with cellulosic substrate was reported by Watson *et al*. (1984) at 57 FPU/mL and 201 FPU/L/h, respectively, using hardwood sulfite pulp and *T. reesei* Rut C-30.

2.2.2 (a) Nature of cellulosic substrate

Wood, grasses, and most of the plant litter represent the major part of the biomass in nature and are collectively called lignocellulose. The physical structure of lignocellulosic material consists of three separate constituents: cellulose, hemicellulose and lignin. Cellulose and hemicellulose form the main structure of biomass materials, whereas lignin acts as a joining material and binds the fibers together (Champagne and Li, 2009). The three constituents are all present in varying quantities depending on the source and nature of the lignocellulosic material. For instance, lignocellulosic biomass such as agricultural and forestry residues, municipal solid waste and dedicated crops, contain up to 75% of cellulose and hemicelluloses, while lignin constitute 20-35% of wood structure (Gan *et al*., 2003; Adsul *et al*., 2004).

Cellulose present in lignocellulosics is a long chain polymer consisting of D-glucose linked only by β-1,4 bonds, with a high molecular weight of approximately half a million (Gan *et al*., 2003). The structure and hydrogen bonding pattern of cellulose is shown in Figure 2.2. Cellulose is composed of crystalline and amorphous components, each showing different digestibility upon enzymatic attack. Enzymatic access to cellulose is difficult, as in plant tissues it is nearly always encased in hemicelluloses and lignin, which themselves require many enzymes to be degraded (Reilly, 2007). The rate and extent of enzymatic hydrolysis is especially limited by lignin which acts as a shield, preventing the digestible parts of the substrate from being hydrolyzed (Chang and Holtzapple, 2000).

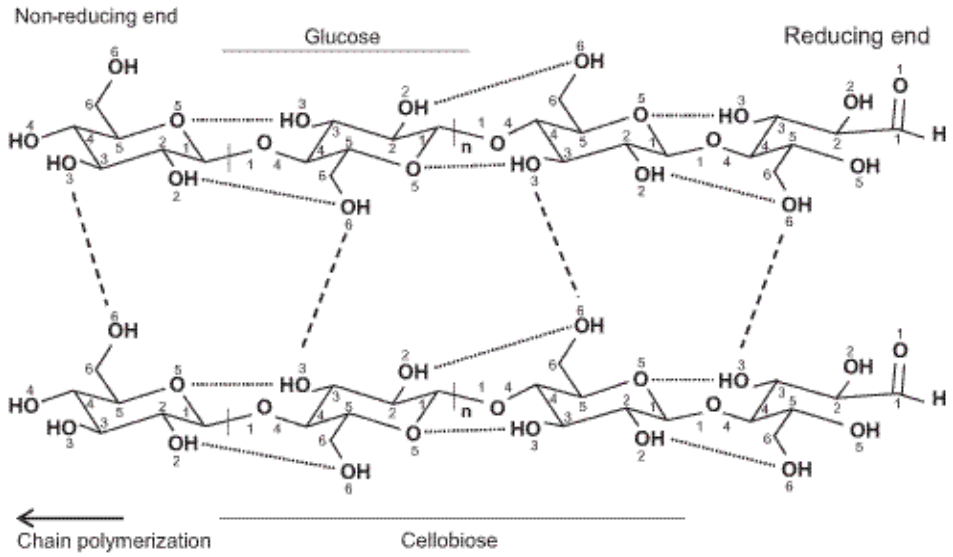

Figure 2.2 The structure and inter- and intra-chain hydrogen bonding pattern in cellulose. Dashed lines: inter-chain hydrogen bonding. Dotted lines: intra-chain hydrogen bonding (Festucci-Bucelli *et al*., 2007)

2.2.2 (b) Pretreatment methods

Effective utilization of lignocellulosic biomass for cellulase production requires the development of cost-effective pretreatment technologies that are necessary to separate and improve access to the cellulose that is intermeshed with hemicellulose and lignin (Brosse *et al*., 2009; Mesa *et al*., 2010). According to Aliyu and Hepher (2000) and Chandra *et al.* (2010), the pretreatment methods should possess two main criteria in order to induce the biosynthesis of cellulose-degrading enzymes: a) facilitate simultaneous disruption of lignin seal to increase the accessibility of cellulose to growing cellulolytic organism, and, b) cause disintegration of the material to create large surface area on which the enzyme complex can act. The effect of pretreatment on the structure of lignocellulosic materials is illustrated in Figure 2.3.

Many pretreatment methods have been employed to enhance the degradation of lignocellulosic materials. Brodeur *et al.* (2011) grouped pretreatment technologies into four classes: physical, chemical, physico-chemical and biological. Physical pretreatment involves breakdown of biomass size and crystallinity by milling or grinding. Improved hydrolysis results from the reduction in crystallinity and improved mass transfer characteristics from reduction in particle size. As the energy requirements for physical pretreatments tend to be higher than the theoretical energy content available in the biomass, this method is seldom used in a full-scale process.

On the other hand, biological pretreatment involves the use of microorganisms (mainly fungi) to degrade lignin and hemicellulose while leaving the cellulose intact (Brodeur *et al.*, 2011). Lignin degradation occurs through the action of lignin degrading enzymes secreted by the fungi. Even though biological pretreatments involve mild conditions and are of low cost, the disadvantages are the low rates of hydrolysis and long pretreatment times required compared to other technologies. Thus, current efforts are focusing on combining biological pretreatments with other pretreatments and in developing novel microorganisms for rapid hydrolysis (Brodeur *et al.*, 2011).

An effective pretreatment is characterized by several criteria: avoid the need to reduce the size of biomass particles, preserves the pentose (hemicelluloses) fractions, limits formation of degradation products that inhibit growth of fermentative microorganism, minimizes energy demands and limits cost. Other basis of comparison for pretreatment options includes low pretreatment catalyst cost or inexpensive catalyst recyle, and generation of higher value of lignin co-product (Mosier *et al*., 2005).