DEVELOPMENT OF AN ENZYMATIC DEINKING SYSTEM OF LASER PRINTED WASTE PAPERS

by

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LIST OF SYMBOLS

β	Beta
%	Percentage
&	And
p	Para
°C	Degree Celsius
α	Alpha
±	Plus minus
=	Equal
+	Plus
-	Minus

LIST OF ABBREVIATION

ATCC	American type culture collection
Bhd	Berhad
cm	Centimeter
CMC	Carboxymethyl cellulase
Co	Coorporation
COD	Chemical dissolve oxygen
COOH	Carboxyl group
CSF	Canadian Standard Freeness
Da	Dalton
DNS	dinitrosalicylic acid
E.C.	Enzyme Commision
e.g.	Example
$FeSO_4 \cdot 7H_2O$	Ferrous sulfate heptahydrate
Fig.	Figure
g/cm^3	gram per centimeter cubic
g/m^2	gram per meter square
h	Hour
HCI	Acid hydrochloric
KCI	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
kpa	Kilo pascal
kPa m^2/g	Kilo pascal meter square per gram
kWh	Kilo watt hour

L/min	Liter per minute
LiP	Lignin peroxidase
LTD	Limited
M	Molar
Met	Methionine
mg/ml	milligram per milliliter
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
min	Minute
ml	milliliter
mM	millimolar
MNI	Malaysian Newsprint Industries
mN m^2/g	milli Newton meter square per gram
MnP	Manganese-dependent peroxidase
µmol	micromole
MOW	Mix office waste paper
NaCI	Natrium chloride
NaOH	Sodium hydroxide
nm	nanometer
Nm/g	Newton meter per gram
OD	Optical density
P>	Probability more than
P<	Probability less than
RH	Relative humidity
RM	Ringgit Malaysia
rpm	Rotation per minute

Sdn	Sendirian
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sp.	Species
TAPPI	Technical Association of the Pulp and Paper Industry
Tris-HCI	Trimethylpyridine hydrochloric
U/g	Unit per gram
U.K	United Kingdom
USA	United State of America
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
X	Multiply

PEMBANGUNAN SISTEM PENYAHDAKWATAN SECARA ENZIMATIK KE ATAS KERTAS BUANGAN BERCETAK LASER

ABSTRAK

Kajian ini memberi tumpuan kepada pembangunan proses penyahdakwatan enzimatik ke atas kertas terpakai. Dengan menggunakan kertas terpakai bercetak laser untuk penyahdakwatan enzimatik, pengoptimuman ke atas hidrolisis enzimatik dan penyingkiran ink dijalankan. Aktiviti optimum dan kestabilan terhadap pH dan suhu bagi selulase A " Amano" 3 (C), hemiselulase "Amano"90 (H) dan lipase F-AP 15 (L) dipilih untuk digunakan dalam proses penyahdakwatan. Suhu dan pH optimum bagi enzim C, H dan L adalah 55°C dan pH 2.5, 50°C dan pH 4.5, 40°C dan pH 7.5, masing-masing. Kajian ke atas urutan enzim menunjukkan bahawa, urutan CH memberikan tahap kecekapan penyahdakwatan yang tertinggi (61.7%). Pemerhatian mikroskopi elektron penskanan menunjukkan bahawa enzim menghidrolisis kertas dan mengakibatkan dakwat dikeluarkan dari kertas, hasil daripada tindakan enzim selulase dan hemiselulase. Proses pempulpaan pada konsistensi 1% selama 1 minit memberikan tahap kecekapan penyahdawatan tertinggi (66.2%). Prapengolahan berasid dengan 0.2 M HCI diikuti dengan hidrolisis enzimatik yang optimum (50°C, pH 3.5, tanpa goncangan, kepekatan substrak 4% (b/i), 2.5 U enzim untuk 1g pulpa kering, nisbah C/H, 1:1 dan masa 60 min) meningkatkan penyahdakwatan sebanyak 68.6%. Penyingkiran dakwat melalui pengapungan yang optimum (pH 6.0, 0.5% (b/b) Tween 80, pengudaraan 10 L/min, suhu 35°C dan pengapungan selama 15 min) memberikan tahap kecekapan maksimum (86.6%). Kajian ke atas pelbagai jernis kertas terpakai menunjukkan kertas bercetak laser memberi tahap kecekapan penyahdakwatan yang tertinggi (86.6%) dan suratkhabar menunjukkan tahap kecekapan penyahdakwatan

(12.9%) yang terendah. Pengolahan penyahdakwatan mempengaruhi kadar pengaliran pulpa dan ciri-ciri fizikal (indeks tensil, indeks koyak dan indeks pecah) kertas terpakai yang dikaji. Pengolahan mengakibatkan kadar pengaliran pulpa kertas bercetak laser tertinggi (103.7 L/min). Penyahdakwatan mengakibatkan peningkatan 2.3% indeks tensil kepada kertas majalah tetapi penurunan sebanyak 3.0% kepada kertas bercetak laser. Hidrolisis enzimatik telah menyebabkan penurunan sebanyak 21.1% dalam indeks koyak relatif kepada blank bagi kertas bercetak bubble-jet. Sebaliknya, peningkatan sebanyak 3.1% dalam indeks koyak ditunjukkan oleh kertas bercetak laser. Kajian ke atas kekuatan pecah menunjukkan bahawa hidrolisis enzimatik memberikan peningkatan sebanyak 4.7% dalam indek pecah relatif kepada blank bagi kertas bercetak laser. Dalam masa yang sama, kertas fotokopi menunjukkan penurunan paling tinggi dalam indeks pecah, iaitu sebanyak 8.3%. Hasil yang diperolehi daripada kajian mencadangkan bahawa penyahdakwatan enzimatik merupakan alternatif yang berpotensi kepada kaedah kimia dalam pengitaran semula kertas buangan.

ABSTRACT

The present work deal with the development of the enzymatic deinking process of waste papers. Based on the enzymatic deinking of laser-printed waste papers, studies on the optimization of enzymatic hydrolysis and ink removal were performed using commercial enzyme preparations. The optimum activities and stabilities at different pH and temperatures of the enzymes, cellulase A "Amano"3 (C), hemicellulase "Amano"90 (H) and lipase F-AP 15 (L) were selected for use in deinking process. The optimum temperature and pH of C, H and L were 55°C and pH 2.5, 50°C and pH 4.5, 40°C and pH 7.5, respectively. The use of CH sequentially resulted in highest deinking efficiency (61.7%). Scanning electron micrograph revealed that the enzymatic hydrolysis on paper resulted the toner been ripped off from the paper, which was mainly carried out by enzyme cellulase and hemicellulase. Pulping at 1% consistency for 1 min demonstrated highest deinking efficiency (66.2%). Pre-treatment of paper with 0.25 M HCI followed by enzymatic hydrolysis (at 50°C, pH 3.5, with no agitation, with the pulp concentration of 4% (w/v), 2.5 U of enzymes per gram of air dry weight pulp at C:H ratio of 1:1 and 60 min hydrolysis time) resulted the maximum deinking efficiency of 68.6%. Ink removal under optimum flotation process (pH 6.0, 0.5 % (w/w) of Tween 80, 10 L/min of air flow rate, 35°C and 15 min of flotation time) resulted highest deinking efficiency (86.6%). Effect of enzymatic deinking on different type of waste paper studied revealed that the highest deinking efficiency of 86.6% was detected on laser-printed paper but the lowest deinking efficiency of 12.9% was obtained by newspaper. All enzymatic treatments of waste papers significant influence drainage rate and physical properties of the de-inked paper. Enzymatic treatment resulted highest drainage rate of 103.7 L/min with the laser-printed papers. Enzymatic deinking resulted 2.3% increment in tensile index with magazine paper but 3.0% decreased with laser-

printed paper. Enzymatic hydrolysis caused 21.1% reduction in tear index relative to the blank for bubble jet printed paper. On the other hand, 3.1% improved in tear index was observed for laser printed paper. The study on the burst strength revealed that enzymatic hydrolysis resulted 4.7% increased in burst index compared to the blank for laser-printed paper. Meanwhile, photocopy paper showed the highest reduction in burst index, which was 8.3%. The results obtained in this work suggested that enzymatic deinking can be a potential alternative to the chemical method in the recycling of waste papers.

CHAPTER ONE

INTRODUCTION

1.1 Current status of paper industries and the need for paper recycling

Waste paper is the single largest component of the solid waste materials, which has a big impact on the environment and on human's life. Waste paper is one of the few consumer products that are fairly easy and inexpensive to recycle. Recycling of waste papers will significantly reduce environmental pollution by decreasing the flow of waste to the landfill and reducing associated disposal costs. Nie *et al.*, (1998) reported that based on the Conservatree Paper Company Statistics of 1992, recycling of 1 ton of waste paper can save approximately 17 tress, 7000 gallons of water, 4100 kWh energy, avoid 60 pounds of air pollution and reduce 3 cubic yards of landfill material. Therefore, recycled and reuse of waste papers have become an important and environmentally benign source of raw materials for the pulp and paper industry. Besides being a low cost fiber source for paper and board manufacturing, it also helps to preserve forest resources, reduces disposal of waste, and reduces high-energy consumption.

Malaysia has extensive forest resources with forest covering close to 60% of its land area, but remained to be a major importer of paper. The paper mills in Malaysia are small with only 5 out of 20 mills producing more than 100, 000 metric tones per annum of papers. Although local production has a total capacity of slightly over one million metric tones per annum, however was not able to meet the total consumption of 98 kg per capital or more than 2 million metric tones per annum in year 2000. The production capacities of these mills over the years have not changed. Thus, Malaysia needs to

import newsprint, printing/writing papers and industrial papers. According to Malaysian Timber Council (2003), for the year 2000, Malaysia spent more than RM 2.7 billion importing 1.18 million metric tones of all types of paper. Out of RM 2.7 billion, RM 900 million was spent on importing printing/writing paper. The import is inevitable, since there is only one mill in Malaysia produces printing/writing paper with the capacity of 165 000 metric tones per year.

According to Malaysian Newsprint Industries Sdn Bhd (2002), waste paper constitutes 95% of raw materials in Malaysian paper mills and the waste paper recovery rates in Malaysia hovers around 40%. In year 2000, more than 800, 000 metric tones of waste paper were recovered but none of them was used to produce printing/writing paper. Currently paper mills used chemical approach to de-ink the waste paper and this required the use of large amount of chemical agent and not environmentally friendly.

The use of recycled fibers in newsprint, tissue and currently in higher quality paper grades has significantly increased all over the world for the last two decades, particularly because of consumers' attitude and the successful development of deinking processes. The most common deinking techniques are washing and flotation systems. These two processes can produce high quality of recovered papers.

A significant difficulty in dealing with secondary fiber is the removal of ink through the process of deinking. The deinking is based on two steps. The first step involves the ink detachment from the surface of the disintegrated fibers, which is performed during the pulping process. Secondly the removal of the detached ink particles from the pulp slurry by washing or flotation using the chemical approach. The alkaline deinking process is

widely used and generally considered to be more efficient with respect to the ink detachment. However, it required the used of large amounts of chemical agents such as sodium hydroxide, sodium carbonate, sodium silicate, hydrogen peroxide and surfactants (Prasad *et al.*, 1993, Woodword *et al.*, 1994). This resultant affects the increase in a significant level and concentration of COD in the effluent water caused by the dissolution of carbohydrates and organic additives present in the fibrous materials. Besides that, the effluent will also contain high impurities of chemicals used in the deinking process. Ultimately, this results in a highly environmentally damaging and costly wastewater treatment to meet the environmental regulations (Prasad *et al.*, 1992).

The degree of difficulty in ink removal depends primarily on the ink types, printing process and fiber type. For some ink types, deinking is poorly effective because of the poor ink detachment from the ageing of offset newspaper or insufficient removal of ink from wastepaper printed with water-based flexographic inks. Furthermore, deinking in an alkaline environment induces a smeared pulp or a low brightness.

Mixed office wastepaper (MOW) is a fast growing source of materials for recycling due to increase utilization of office photocopiers and computers print out. It is the most difficult raw material to be de-inked (Prasad, 1993, Gubitz *et al.*, 1998). At the same time it has to be borne in mind that MOW is a large, virtually untapped source of high quality fiber that could be used for high quality grade papers and many other products only if the deinking process could be improved. This is because they are more difficult to de-ink effectively by conventional deinking techniques.

Due to significant number of disadvantages resulted from the conventional chemical methods; an alternative method for deinking process must be sought. Alternatively, the use of enzymes has been reported to be a potentially efficient and less polluting solution to overcome the problem encountered by commonly employed deinking techniques (Prasad *et al.*, 1992, Putz *et al.*, 1994). Furthermore, enzymatic deinking avoids the alkaline environment commonly required in traditional deinking which will cause yellowing of the recycled pulp. Consequently this will cut chemicals costs and significantly reduce COD loaded on the white water system (Putz *et al.*, 1994). In addition to ink removal, enzymatic deinking may contribute to improve strength properties of the paper sheets and freeness, reduces fines content of the recycled fiber pulp, enhance the brightness and cleanliness of the pulp. Additional benefits include the improved operation of thickeners due to better drainage. Improved drainage may results in faster machine speed which yields significant savings in energy and thus overall cost (Heise *et al.*, 1996). In addition, the use of recycled fiber reduces the need for virgin pulp. This result in great savings on the energy required for pulping, bleaching, refining and others, which will also eventually reduce pollution problem (Malaysia Newsprint Industries Sdn Bhd, 2002).

The potential of enzymatic deinking has been assessed and proven successful using a number of different types of enzymes (Gubitz *et al.*, 1998). For example, the removal of oil carrier-based inks can be facilitated by the addition of lipases and esterases. While, hemicellulase and cellulase can be used to release ink from fiber surface by partially hydrolysis of carbohydrate molecules on the fiber surface.

To date, few studies investigating the effect of enzymes on deinking of MOW have been carried out using enzyme preparations containing mixed activities including both cellulase and hemicellulase (Prasad *et al.*, 1993, Jeffries *et al.*, 1994, Pala *et al.*, 2004). However, none of these studies has been performed in Malaysia. Therefore, the need to explore the possibility of using enzymes in the deinking of laser-printed waste paper with the aim to recycle the waste paper must be given priority as an environmental friendly approach in the Malaysia paper industries.

1.2 Research scope

Five commercially available enzymes obtained from Amano Pharmaceutical Co., LTD (Nagoya, Japan) consisting of cellulase A "Amano"3 (C), cellulase T "Amano"4 (T), hemicellulase "Amano"90 (H), lipase F-AP 15 (L) and lipase AY "Amano"30 (AY) and two other enzymes designated cellulase (S) and xylanase (X) were purchased from Sigma (USA) and Fluka, (USA) respectively will be elucidated in the enzymatic deinking process of waste paper. The enzymes will be characterized in term of pH and temperature on the activity and stability. The enzymes with known characteristics with respect to pH and temperature consisting of cellulase (C), hemicellulase "Amano"90 (H) and lipase F-AP 15 (L) were selected to be used for further studies in deinking process. Different enzyme sequences treatment on laser-printed paper was carried out to evaluate the effect of individual and combination enzyme on deinking efficiency. The most effective enzyme sequence will be used in the deinking process to investigate the effect of pulping process, which includes pulping consistency and pulping time on ink removal. The optimization of enzymatic hydrolysis of laser-printed pulp was performed in order to determine the most effective conditions for ink removal. The conditions that were optimized are; treatment with HCI hydrolysis, temperature, pH, agitation, pulp

concentration, enzymes concentration, enzyme ratio and hydrolysis time. The efficiency of the deinking process is greatly influenced by flotation process. Therefore, parameters that were involved in flotation process were optimized in order to obtain a higher deinking efficiency and brightness. The parameters optimized in the flotation process include flotation pH, different surfactants, surfactant concentration, air flow rate, temperatures and flotation time. The properties of the de-inked paper were determined to compare with the darker paper using the exiting methods. The properties examined include not only the brightness of the paper, but also drainage rate and physical properties of the paper like burst strength, tensile strength and tear resistance of paper. Based on these optimization works, enzymatic deinking process of laser printed-paper will be developed. Using the developed enzymatic deinking method, different type of waste papers, which included photocopy papers, bubble jet-printed paper, magazine, newspaper and mixture of all the waste papers were evaluated.

1.3 Research objectives

The objectives of the current research are as follow:

- To evaluate commercial cellulases, hemicellulase, xylanase and lipases preparations for potential use in deinking of laser printed wastepaper.
- To determine and optimize the enzymes mixtures for use in the hydrolysis of papers for deinking process.
- To optimize the flotation process for ink removal and separation.
- To determine physical properties of the recycled paper after the enzymatic deinking and flotation process.
- To evaluate the efficiency of the developed enzymatic deinking process of others types of waste papers.

CHAPTER TWO

LITERATURE SURVEY

2.1 LIGNOCELLULOLYTIC MATERIALS FROM NATURAL RESOURCES

2.1.1 Cellulose

Cellulose occurs predominantly in plants forming their major structural component. It is one of the world's most abundant natural compounds and a major waste product from agricultural wastes. Cotton and wood fibers are the most common source of cellulose and it make up for about 90% of cotton fibers but for only about 45% of the average wood fiber (Eriksson *et al.*, 1990a) . Cellulose is a linear polymer of 1, 4-β-D-glucose units, the number of which varies considerably with an average of about 3000 unit (Priest, 1984). Individual cellulose molecules are associated to form insoluble elementary fibrils that further aggregate to form microfibrils and crystalline cellulose by hydrogen bonding and Van der Waals forces. These complex structures of crystalline cellulose are not susceptible to hydrolysis by single enzyme. Thus, cellulolytic microorganisms mainly fungi secrete various kinds of cellulases to degrade this glucose polymer into glucose which not only supports the growth of the fungus but also supports the growth of other populations in nature (Ghosh & Ghosh,1992).

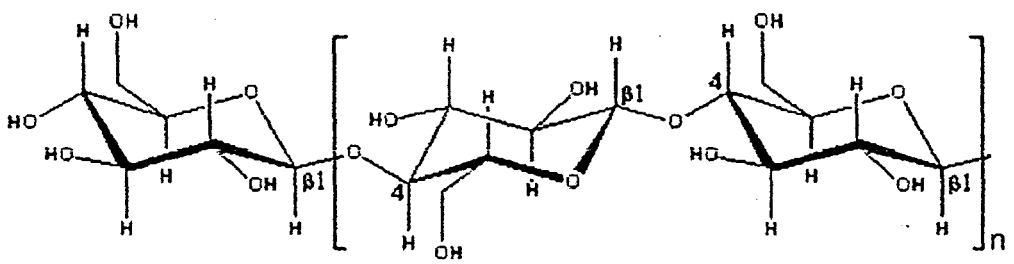
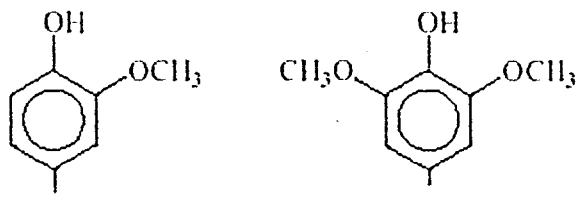


Fig. 2.1 Chemical structure of cellulose. (Chaplin, 2004)

2.1.2 Lignin

Lignin is a complex polymer of phenylpropane units (benzene ring with a tail of three carbons), which are cross-linked to each other with a variety of different chemical bonds to form a large molecular structure. The monomeric building unit of lignin is guaiacyl unit and syringyl unit (Fig. 2.2). The guaiacyl unit is dominant in softwoods. In contrast, the syringyls units are dominant in hardwood. Lignin is found in all vascular plants, between and within the cells and also in the cell walls. It enables trees to grow taller to compete for sunshine and gives mechanical strength to wood by gluing the fibers together. In nature, lignin is very resistant to degradation due to the strong chemical bonds and its complexity. Nonetheless, some organisms, particularly fungi, have developed the necessary enzymes to degrade lignin. The initial reactions are mediated by extracellular lignin and manganese peroxidases, primarily produced by white-rot fungi (Kirk & Farrell, 1987). In natural and unprocessed form, lignins are so complex with molecular weight, which may reach 15, 000 or more and that none of them has ever been completely described. Lignins have been grouped into several types and within each type, there is a lot of variation. Lignin differs from species to species and from one tissue to the other in the same plant. The process of removing them from the plant changes their forms and chemical composition, which makes them ever harder to study (McCrady, 1991).



Guaiacyl **Syringyl**

Fig. 2.2 Monomeric building unit of lignin (Marita *et al.*, 2001)

2.1.3 Hemicelluloses

Hemicelluloses are heterogeneous groups of branched and linear polysaccharides that bound via hydrogen bonds to the cellulose microfibrils and consequently attach to lignin in the plant cell wall. Hemicelluloses together with cellulose, form a highly complex structure, which is one of the most abundant groups of polysaccharide in nature. The hemicelluloses composed of, D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. These individual sugars may be acetylated or methylated. Most of the hemicelluloses contain two to six of these sugars with xylose is the most important sugar component. There are characteristic differences in the composition and structure of the hemicelluloses in hardwood and softwood. The xylose-based hemicelluloses in both hardwoods and softwoods are usually termed xylans. In hardwood xylan, the backbone chain consists of xylose units which are linked by β-(1, 4)-glycosidic bonds and branched by α-(1, 2)-glycosidic bonds with 4-*O*-methylglucuronic acid groups. On the other hand, galactoglucomannans are the principal hemicelluloses in softwood and their backbone is a linear chain consists of 1,4-linked β-D-glucopyranose and β-D-mannopyranose units. In addition, softwood xylan has additional branches consisting of arabinofuranose units linked by α-(1, 3)-glycosidic bonds to the backbone. These variable structure of hemicelluloses required hemicellulase for its complete hydrolysis (Eriksson *et al.*, 1990b).

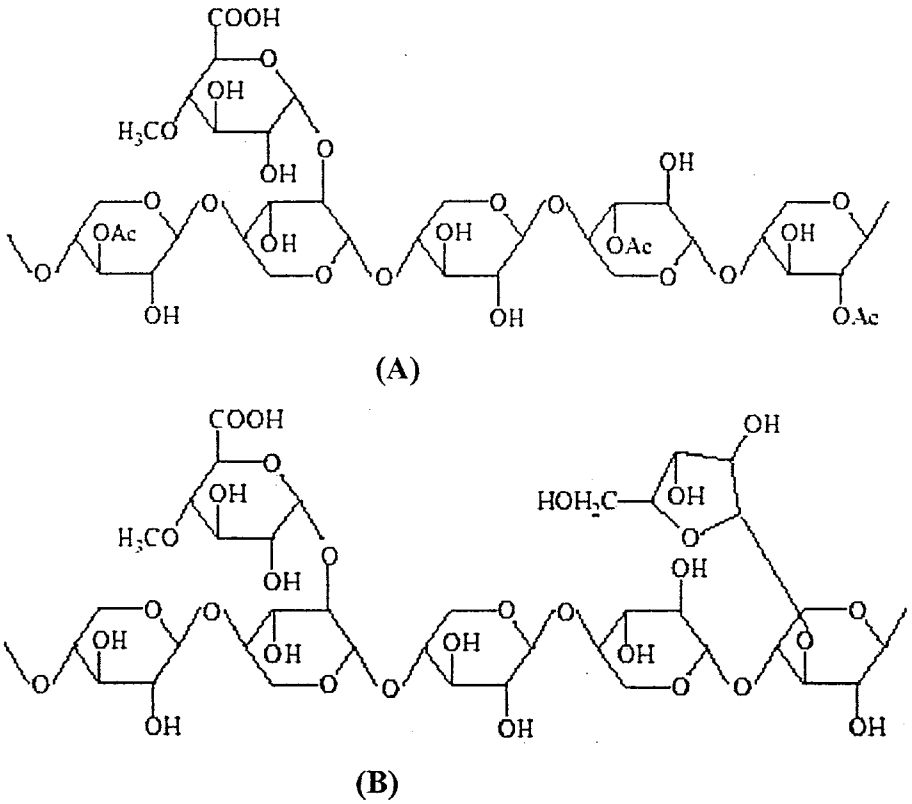


Fig.2.3. Schematic diagram of partial xylan structure. (A) from hardwood and (B) from softwood

2.1.4 Industrial application of lignocellulolytic materials

Lignocellulolytic materials are the major structural components of plants and represent a major source of renewable organic matter. Lignocellulose consists of lignin, hemicellulose and cellulose. The chemical properties of the lignocellulosic components make them a substrate of enormous biotechnological significance (Malherbe & Cloete, 2003). Lignocellulolytic materials can potentially be converted into various different value added products including chemicals, biofuels, cheap energy sources for fermentation, improved animal feeds and human nutrients.

Bioconversion of lignocellulosic wastes could make a significant contribution to the production of organic chemicals. Over 75% of organic chemicals are produced from five primary base-chemicals: ethylene, propylene, benzene, toluene and xylene, which are used to synthesis other organic compounds (Coombs, 1987). The aromatic compounds might be produced from lignin whereas the low molecular mass aliphatic compounds can be derived from ethanol produced by fermentation of sugar generated from the cellulose and hemicellulose (Howard *et al.*, 2003).

The demand for ethanol has the most significant market where ethanol is either used as a chemical feedstock or as petrol additive. Brazil produces ethanol from the fermentation of cane juice while corn is used in the USA. In the USA, fuel ethanol has been used in gasohol or oxygenated fuels since the 1980s. It is estimated that 4540 million liters of ethanol is used by the USA transportation sector and the volume will rise phenomenally (Sun & Cheng, 2002). The biological process for converting the lignocellulose to ethanol requires: (1) delignification to liberate cellulose and hemicellulose from their complex with lignin; (2) depolymerization of the carbohydrate polymers to produce free sugars; and (3) fermentation of mixed hexose and pentose sugars to produce ethanol (Jeewon, 1997).

Hemicelluloses are of particular industrial interest since these are readily available bulk source of xylose from which xylitol can be derived. Xylitol is used in food industry as a sweetener. It has odontological applications such as teeth hardening, remineralisation and as an antimicrobial agent. Xylitol also used in chewing gum and toothpaste formulations (Roberto *et al.*, 2003; Parajo *et al.*, 1998). Various bioconversion methods

have been explored for the production of xylitol from hemicellulose using microorganisms or their enzymes (Nigam & Singh, 1995).

Pentose sugars derived from xylose (hemicelluloses) are readily converted to furfural, a raw material for a number of resins, adhesives and a potential intermediate for the manufacture of nylon. Furfural also used in the manufacture of furfural-phenol plastics, varnishes and pesticides (Montane *et al.*, 2002). Over 200 000 tones of furfural with a market price of about $1700 per ton (Montane *et al.*, 2002) is annually produced (Zeitch, 2000).

Lignocellulolytic materials such as grains, cereals and residues are used to produce animal feed. However, it is necessary to add micro-ingredients to improve levels of essential amino acids, vitamins and minerals. New additives such as metabolic modifiers, anti-microbial agents, pro-biotic and special minerals are also incorporated in order to supply essential nutrients, to enhance growth and to avoid diseases (Wenk, 2000). Even if the raw material is of low cost, the addition of these micro-ingredients increases the final prices of feeds. Therefore, microorganisms mainly fungi, have been used to convert agro-industrial wastes in order to obtain products with higher nutritive value, especially with regards to protein and vitamin contents, and with increased digestibility (Dura'n, 1989; Dura'n *et al.*, 1994; Kuhad *et al.*, 1997).

2.2 ENZYMES RELATED TO THE DEGRADATION OF LIGNOCELLULOLYTIC MATERIALS

2.2.1 Cellulases

2.2.1.1 Cellulases and basic model of action

Cellulases are multiple-enzyme complexes, which participate in the stepwise saccharification of native cellulose or cellulose derivatives to glucose. It is a multi-component enzyme system generally composed of 3 enzymes: endoglucanase (endo-1, 4-β-D-glucan 4-glucanohydrolase, E.C.3.2.1.4), exobiohydrolase (1, 4-D-glucan cellobiohydrolase, E.C.3.2.1.91) and cellobiase (β-glucosidase, E.C.3.2.1.21). The hydrolysis of crystalline cellulosic substrates required these enzymes to act synergistically (Eveleigh, 1987). According to Wood & McCrae (1979), the first step in the degradation of cellulose is to attack at multiple internal sites in amorphous regions of the cellulose microfibers by endoglucanases, thereby creating new free ends of the cellulose chain that are subsequently attack by cellobiohydrolase. This latter enzyme removes cellobiose from non-reducing terminal of the polyglucan chain. The action of these two enzymes resulted in soluble oligomer and cellobiose which subsequently being hydrolyzed by cellobiase to yield glucose. This step is extremely important because the accumulation of cellobiose inhibits cellulose degradation by the former two enzymes. Amorphous cellulose is degraded by both endo and exoglucanase separately but each enzyme itself is inactive towards crystalline cellulose. To attack crystalline cellulose, the combined action of the two classes of enzyme seems to be necessary. Cellulases activities are measured using different substrates. Carboxymethyl cellulase activity is measured by carboxymethyl cellulose solution. β-glucosidase and cellobiase

activities are measured by β-nitrophenyl-β-D-glucopyranoside and cellobiose as substrates, respectively (Ghosh & Ghosh, 1992).

2.2.1.2 Production of cellulases

Cellulases can be produced by fungi under a wide variety of growth conditions. For high productivity, cellulose or CMC must be present as one of the carbon sources in the growth medium. According to Eveleigh (1987), the species of *Trichoderma* are by far the best cellulases producers. Significant amount of cellulases was also produced by some species of *Penicillium* and *Aspergillus*. *Aspergillus* is a superior β-glucosidase producer, however β-glucosidase production is low in *Trichoderma* culture. Therefore, for complete depolymerization of cellulose, β-glucosidase from *Aspergillus* is used in conjunction with endo and exoglucanase from *Trichoderma*.

Higher CMC-activity was produced by *Trichoderma* spp when grown on solid substrate fermentation using mixture of beet pulp and wheat bran as substrates compared to mixture of winter barley straw and wheat bran, mixture of beet pulp and sawdust or beet pulp. 270 U/g of CMC-activity was detected when *T. emersonii* UCG 28 was grown at 45°C and pH 6.0 for 4 to 8 days. However, 221 U/g of CMC-activity was obtained when *T. aurantiacus* CMI 67936 was grown at the same conditions (Touhy *et al.*, 1989). *T. reesei* CL-847 mutant gave higher yields of cellulase when cultured on 6% lactose plus cellulosic supplements (Warzywoda *et al.*, 1983).

CMC-activity produced by the *Aspergillus* spp was higher when beet pulp was used as substrate in solid substrate fermentation or mixed with other solid wastes such as wheat bran or sawdust. 449 U/g of CMC-activity was obtained from *A. niger* Z2039 after

growth at 25-30°C, pH 6 for 4 to 8 days. On the other hand, only 15.8 U/g of CMC-activity was detected when *A. fumigatus* PEC was grown at 45°C using mixture of wheat bran and winter barley straw as substrates (Touhy *et al.*, 1989).

2.2.1.3 Hydrolytic properties of cellulases

The optimum temperature for endoglucanase activities of *Trichoderma* and *Aspergillus* range between 50 to 65°C. Endoglucanase obtained from *T. viride* CCMI 84 and *A. terreus* CCMI 498 have an optimum temperature of 55 and 60°C, respectively. The former has an optimum pH of 4.5 and the later has an optimum pH of 5.0 (Marques *et al.*, 2003). Gomes *et al.*, (1992) also reported an optimum pH of 4.5 was obtained for cellulase from *T. viride* isolates. On the other hand, the optimal conditions for actinomycetes cellulase production range between 25 to 55°C. *Streptomyces flavogriseus* have an optimum endoglucanase production at 30°C and pH 7.0. At the same time, the optimum enzyme activity was detected at 55°C and pH 6.0-7.5. Endoglucanase production from *Thermomonospora* species was optimum at 55°C and pH 7.4 while the optimum enzyme activity was detected at 70°C and pH 6.0 (McCarthy, 1987). Cellulases from *Humicola* species have been found and one of these enzymes although exhibited good stability in the alkaline range has an optimum pH of 5.0 at 45°C. On the other hand, cellulases from *T. reesei* are active between pH 3.0 and 7.0 with an optimum temperature around 40°C.

Endoglucanases produced by *T. viride* CCMI 84 and *A. terreus* CCMI 498 were very heat stable since both fungi produced enzymes, which showed 100 and 95%, respectively of the original activities after pre-incubation at 50°C for 2 h (Marques *et al.*, 2003). On the other hand, cellulases obtained from actinomycetes were also very

heat stable. Endoglucanase from *Thermomonospora* species was stable at 65°C for 24 h while endoglucanase obtained from *Thermomonospora curvata* was stable at 70°C for more than 1 h. Endoglucanase obtained from *Streptomyces flavogriseus* was only stable at 40°C for 2 h. Compare to endoglucanase, β-glucosidase are much more heat sensitive. This can be seen when β-glucosidase activity obtained from *S. flavogriseus* and *S. lividans* was decreased after 10 min incubation at 40°C (McCarthy, 1987).

Gel filtration, ion exchange and hydrophobic interaction chromatographies were mainly used to purify cellulases. The molecular weight of exoglucanase (41-68 kDa) is greater than the molecular weight of endoglucanase (12.5-50 kDa). Cellulases obtained from *Trichoderma* species have molecular weight ranging from 12.5 kDa to 65 kDa. 12.5 kDa and 20 kDa of endoglucanase were obtained from *T. reesei* and *T. viride*, respectively. Both have pI value of 4.6 and 7.5, respectively. Exoglucanase obtained from *T. reesei* and *Penicillium fumiculosum* have molecular weight of 65 kDa and 46.3 kDa, respectively (Ghosh & Ghosh, 1992).

Enzyme systems from *T. reesei* have been thoroughly studied. Exobiohydrolase and endoglucanase from *Trichoderma* and *Aspergillus* have molecular mass between 42 kDa to 60 kDa and 12 kDa to 50 kDa, respectively. While the molecular mass of cellobiase is around 35 kDa for *Trichoderma* and 218 kDa for *Aspergillus*. Cellulase from *Aspergillus* is active between pH 4 and 6, the optimal temperature is approximately 55°C. However, cellulase from *Trichoderma* is active between pH 3 and 7 and the optimal temperature is around 40°C. For *Penicillium* the optimal pH is between 5 and 8 (Wolfgang, 1990).

2.2.2 Hemicellulases

2.2.2.1 Hemicellulases and basic model of action

Hemicellulase, a diverse group of enzyme that works synergistically for complete hydrolysis of hemicelluloses. Hemicellulases consisted of 13 different enzymes (Table 2.1). Xylan is the most prevalent hemicellulose composed of D-xylopyranosyl units linked by β-1, 4-glycosidic bonds. Xylanases (E.C. 3.2.1.8) hydrolyze the β-1, 4 bond in the xylan backbone, yielding short xylooligomers which was further hydrolyzed by β-xylosidases (E.C 3.2.1.37) to D-xylose unit (Shallom & Shoham, 2003). In addition to these two enzymes, several accessory enzyme activities are necessary for de-branching of the substituted xylans (Poutanen *et al.*, 1991). For examples, β-mannanases (E.C. 3.2.1.78) hydrolyze mannan-based hemicelluloses and liberate short β-1, 4-mannooligomer that can be further hydrolyzed to mannose by β-mannosidases (E.C.3.2.1.25). While, α-L-arabinofuranosidases (E.C.3.2.1.55) and α-L-arabinonases (E.C.3.2.1.99) hydrolyze arabinofuranosyl containing hemicelluloses.

Table 2.1: Hemicellulases enzymes

Enzyme	Substrate	EC Number
Endo-β-1,4-xylanase	β -1,4-xylan	3.2.1.8
Exo- β-1,4-xylosidase	β -1,4-xylooligomers Xylobiose	3.2.1.37
α-L-Arabinofuranosidase	α-Arabinofuranosyl (1→2) or (1→3) xylooligomers α-1,5-arabinan	3.2.1.55
Endo-α-1,5-arabinanase	α -1,5-arabinan	3.2.1.99
α -Glucuronidase	4-O-methyl-α-glucuronic acid (1→2) xylooligomers	3.2.1.139
Endo-β-1,4-mannanase	β -1,4-mannan	3.2.1.78
Exo-β-1,4-mannanase	β-1,4-mannooligomers Mannobiose	3.2.1.25
α -galactosidase	α -galactopyranose (1→ 6) Mannooligomers	3.2.1.22
β -glucosidase	β -glucopyranose (1→ 4) Mannopyranose	3.2.1.21
Endo-galactanase	β -1,4-galactan	3.2.1.89
Acetyl xylan esterase	2- or 3-o-acetyl xylan	3.2.1.72
Acetyl mannan esterases	2- or 3-o-acetyl mannan	3.1.1.6
Ferulic and *p*-cumaric acid esterases		3.1.1.73

(Source: Shallom & Shoham, 2003)

2.2.2.2 Production of hemicellulases

Hemicellulases not only can be obtained from fungi but also from bacteria. Species of *Trichoderma* have known to produce xylanases (Dekker & Richards, 1976). These enzymes are generally produced along with cellulases during their growth on cellulose and xylan substrates. Senior *et al* (1989a) suggested that in *T. harzianum* 558, the ratio of xylanases and cellulases produced is directly proportional to the ratio of xylan to cellulose in the growth substrate. However, this relationship varies with species because

xylanases and cellulases production are not necessary synchronistic (Biely *et al.*, 1988; Senior *et al.*, 1989b) and can be influenced by incubation time. Furthermore, xylanase production can depend on pH and mineral salt supplementation (Shamala & Sreekantiah, 1986). In *T. reesei*, xylan and xylobiose selectively induce the formation of xylanase whereas sophorose induces production of both cellulase and xylanase. In *A. terreus*, selective induction of xylan degrading enzymes could be achieved by employing xylan, xylobiose or D-xylose as substrates while, in the presence of cellulose or cellobiose both cellulase and xylanase were formed (Hrmova *et al.*, 1989).

High β-xylosidase activity (176 U/g) was produced from *Aspergillus niger* kk2 isolates when grown on solid-state fermentation using rice straw as substrate. However, no β-xylosidase activity was detected when *Aspergillus ustus* was grown on rice straw through solid-state fermentation. On the other hand, low β-xylosidase activity was detected from *Penicillium capsulatum* after growth on solid-state fermentation using beet pulp as substrate (Kang *et al.*, 2004).

2.2.2.3 Hydrolytic properties of hemicellulases

The optimal conditions for activity of *Trichoderma* and *Aspergillus* xylanases range from 45 to 65°C and 28-37°C, respectively. The former has an optimum pH of 3.5 to 6.5 and the latter has an optimum pH of 4 to 6.5. Xylanases with higher optimum temperature were more thermo-stable (Tan *et al.*, 1985; Wood & McCrae, 1986) than those with lower optimum temperature (Beldman *et al.*, 1985; Hashimoto *et al.*, 1971; John & Schmidt, 1988). However, xylanases isolated from *Thermostoga* sp. strain FjSS3-B.1 has an optimum temperature of 105°C at pH 5.5. Furthermore, xylanase

isolated from *Bacillus* sp. have a broad range of pH optimal and stabilities ranging up to pH 10 (Honda *et al.*, 1985; Huang *et al.*, 1991; Okazaki *et al.*, 1985).

β-1, 4-xylosidases produced by *Aspergillus nidulans* and *Thermoanaerobacter ethanolicus* has an optimum temperature of 50 °C and 93°C, respectively. The former has an optimum pH of 5.0 and the latter has an optimum pH of 6.0. On the other hand, exo-β-1, 4-mannosidase produced by *Aspergillus niger* has an optimum temperature of 55°C and pH of 3.5. However, exo-β-1, 4-mannosidase produced by *Pyrococcus furiosus* has an optimum temperature of 105°C and pH of 7.4. α-Glucuronidase produced by *Thermoanaerobacterium saccharolyticum* and *Phanerochaete chrysosporium* shared the same optimum temperature of 50°C. However, the former has an optimum pH of 6 while the latter has an optimum pH of 3.5.

Ion exchange, gel filtration and hydrophobic interactions chromatographies were mainly used to purify xylanase. *Trichoderma* sp. have molecular weight ranging from 15 to 57 kDa. Adsorptive interactions of certain low molecular weight xylanases with resin matrices make these enzymes elute as very small protein that enable their separation from others proteins using ultra-filtration (Dean & Anderson, 1991; Tan *et al.*, 1985). However, some evidence shows that low molecular weight xylanases from *T. viride* are unstable under SDS-PAGE or urea SDS-PAGE (Dean & Andreson, 1991). For potential application of xylanases, the enzymes do not have to be pure as long as cellulases free xylanases was obtained. In order to obtain cellulase free xylanases from *T. harzianum* (Tan *et al.*, 1987), ultra-filtration can be used in conjunction with solvent exchange and through ion exchangers.

2.2.3 Lignin degrading enzymes

2.2.3.1 Lignin degrading enzymes and basic mode of action

Lignins are very resistant to chemical and enzymatic degradation. Biodegradation is achieved mainly by fungi especially white-rot fungi, which are known as lignin degraders in nature, but also by certain actinomycetes. There are three components of the lignin degrading enzyme system: lignin peroxidase, manganese-dependent peroxidase (MnP) and laccase. The lignin degrading enzyme systems are required to efficiently degrades various types of lignins to CO_2 (Hatakka & Uusi-Rauva, 1983). Lignin degradation by these organisms occurs at low levels of nutrient nitrogen and required the presence of carbon source such as glucose or cellulose. Lignin peroxidase (LP) can degrade lignin in the presence of H_2O_2 by oxidizing both phenolic and non-phenolic units in lignin (Jeewon, 1997; Hammel *et al.*, 1993). On the other hand, in the presence of H_2O_2, MnP oxidizes Mn^{2+} to Mn^{3+} and in turn oxidizes phenolic substrates to phenoxy radicals, which undergo subsequent reactions to yield final products. Laccase, a copper enzyme seems to have a similar function as MnP (Jeewon, 1997 Kawai *et al.*, 1988).

2.2.3.2 Production of lignin degrading enzymes

Lignin degrading enzymes are mainly produced by white rot fungus since these groups of fungus are known as lignin degraders in nature. Lignin peroxidase (LiP), manganese peroxidase (MnP), laccase and glyoxal oxidase was produced by *Phlebia radiata* 79 (ATCC 64658) during solid-state fermentation of wheat straw with supplemented of 0.05% of glucose (Vares *et al.*, 1995). After purification, three isoforms of LiP and two isoforms of MnP were obtained. Moreover, *P. radiata* 79 has been thoroughly studied in liquid medium (Lundell & Hatakka., 1994; Lundell *et al.*, 1990; Martinez *et al.*, 1994).

According to Lundell & Hatakka (1994), *P. radiata* 79 was found to produce ligninolytic enzyme (LiP, MnP and laccase) when grown in low nitrogen medium containing 56 mM glucose, 0.05% (w/v) Tween 80, low level of manganese and 1 mM of veratryl (3,4-di-methoxybenzyl) alcohol.

Pelaez *et al.*, (1995) screened 68 species of basidiomycetes for lignin degrading enzyme in liquid medium containing 1% of glucose, 0.2% of ammonium tartrate, 0.1% KH_2PO_4 and yeast extract, 0.05% $MgSO_4 \cdot 7H2O$ and KCI and 0.1% of mineral solution. The authors reported that laccase and MnP were found in 50% and 29% of the fungi tested, respectively. No LiP was detected in the different fungi tested. High laccase activity (200 U/L or more) was detected in cultures of *Trametes versicolor*, *Phellinus torulosus*, *Cerrena unicolor* and *Pleurotus eryngii*.

Chaetomium thermophilium isolated from composting municipal solid waste exhibited laccase activity when it was grown at pH 6.0 and 45°C in liquid media containing 0.5% of glucose, 0.06% of L-asparagine, 0.1% of KH_2PO_4, 0.1% of $MgSO_4 \cdot 7H_2O$, 0.05% of KCl and yeast extract, 10 mg/L of $FeSO_4 \cdot 7H_2O$. Laccase activity reached a peak after 24 h in liquid shake culture (Chefetz *et al.*, 1998).

2.2.3.3 Properties of lignin degrading enzymes

The optimum temperature range for fungal laccase is between 30°C and 60°C (Munoz *et al.*, 1997; Slomczynski *et al.*, 1995; Youn *et al.*, 1995). However, laccase produced by thermophilic *C. thermophilium* had an optimum temperature range from 50°C to 60°C and was more stable at higher temperature than laccase purified from mesophilic fungi such as *Pyncoporus cinnabarinus* (Eggert *et al.*, 1996). The laccase was stable for 1 h at

70°C and had half-lives of 24 and 12 h at 40 and 50°C, respectively. The laccase has a molecular weight of 77 kDa and pI value of 5.1 with an optimum activity at pH 6.0 to 8.0 (Chefetz *et al.*, 1998). Another laccase, which was produced by lignin-degrading basidiomycete strain PM1 (CECT 2917) isolated from wastewater of a paper factory, has a molecular weight of 64 kDa and pI value of 3.6. The enzyme retained 90% of its activity for 1 h at pH 3.0 to 9.0 and 24 h between pH 7.0 and 9.0. The laccase has an optimum pH and temperature of 4.5 and 80°C, respectively (Pedro *et al.*, 1993).

The LiP produced by *P. radiata* was clearly different from laccase because of its very acidic pH optimum at 2.5. However, LiP exhibited 25% of its maximum activity at pH 4.5, which was the optimum pH for both laccase and MnP. Unlike LiP and laccase, MnP has a broader pH optimum between pH 4 to 6.0. LiP has a molecular weight of 40-45 kDa and pI value of 4.1-3.2. While MnP and laccase have molecular weight of 48 and 64 kDa, respectively. The former has pI value of 4.7-3.7 while the latter has pI value of 3.5 (Lundell & Hatakka, 1994).

2.3 INDUSTRIAL APPLICATION OF LIGNOCELLULOLYTIC DEGRADING ENZYMES

2.3.1 Application of cellulase in textile industry

Almost all of the natural materials used in fabric manufacturing contained cellulosic fibers, which had a tendency for 'fuzz' formation and 'pilling'. These phenomena are considered as negative features of cellulosic fabrics. Therefore, the prevention and permanent removal of fuzz formation and pilling are necessary in order to increase the commercial value of cellulosic fabrics. This can be accomplished using cellulases in a process called 'bio-polishing' (Galante *et al.*, 1998a). Bio-polishing is usually carried

out during the textile wet processing stage. During this process, the cellulases act on small fiber ends that protrude from the fabric surface, where the mechanical action removes these fibers and polishes the fabrics. The advantages of using cellulases are: (1) removal of short fibers and surface fuzziness; (2) improved color brightness, (3) smooth and glossy appearance, (4) improved finishing and fashionable effects and (5) environmentally friendly process (Bhat, 2000).

2.3.2 Application of hemicellulase in baking industry

Hemicellulases, especially endo-xylanases have been used to improve the quality of biscuits, bread, dough and cakes (Poutanen, 1997). It has been hypothesized that the ability of endo-xylanases to hydrolyze arabinoxylan present in dough facilitates the redistribution of water in both dough and bread, which are responsible for the observed favorable effects on dough handling, bread volume, texture and stability (Maat *et al.*, 1992; Poutanen, 1997). Moreover, the addition of endo-xylanases during dough processing can increase the concentration of arabino xylo-oligosaccharides in bread, which have beneficiary effects on human health (Bhat, 2000).

2.3.3 Application of cellulase and hemicellulase in animal feed industry

Xylanases and β-glucanases have been used in monogastric diets to hydrolyze non-starchy polysaccharides such as barley β-glucans and arabinoxylans (Cowan, 1996; Walsh *et al.*, 1993; Hesselman *et al.*, 1982). The presence of high levels of non-starchy polysaccharides in cereal-based diet resulted in poor feed conversion rate, slow weight gain and sticky droppings by young animals (Galante *et al.*, 1998b; Bedford & Classen, 1992; Chesson, 1987). However, the addition of xylanases and β-glucanases during feed production was found to increase the degradation of non-starchy polysaccharides and