PRODUCTION OF CELLULASE AND XYLANASE BY INDIGENOUS *ASPERGILLUS NIGER* AI-1 *VIA* SOLID SUBSTRATE FERMENTATION AND ITS APPLICATION IN DEINKING OF MIXED OFFICE WASTE PAPER

PANG PEI KHENG

UNIVERSITI SAINS MALAYSIA

2010

PRODUCTION OF CELLULASE AND XYLANASE BY INDIGENOUS *ASPERGILLUS NIGER* AI-1 *VIA* SOLID SUBSTRATE FERMENTATION AND ITS APPLICATION IN DEINKING OF MIXED OFFICE WASTE PAPER

by

PANG PEI KHENG

Thesis submitted in fulfillment of the requirements

for the degree of Doctor of Philosophy

2010

Acknowledgements

First of all, thanks to ALLAH S.W.T for the courage and blessing that gave to me to complete my thesis finally, after all the challenges and difficulties.

I would like to record my gratitude to Prof Ibrahim Che Omar for his supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences throughout the work. Above all and the most needed, he provided me unflinching encouragement and support in various ways. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student, a researcher and a scientist want to be.

My sincere appreciation also extends to Prof Darah Ibrahim for her unlimited advice, invaluable help, support and guidance throughout my entire project.

I would like to express my heartfelt gratitude to my dear parents and family members for their continuous support and encouragement. Words fail me to express my appreciation to my husband whose dedication, love and persistence confidence in me, has taken the load off my shoulder. His endless support has motivated me to work harder.

I wish to thank all the technicians in the laboratory for providing facilities to carry out experimental works. Last but not least to my fellow lab-mates and my friends who have directly and indirectly given me support, help and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATION

AOAC	Association of Analytical Communities
BG	β-glucosidase
CB	Cellobiase
CBH	Cellobiohydrolase
CD	Cross Machine Direction
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcellulase
CSL	Corn Steep Liquor
CY20S	Czapek Yeast Extract with 20% Sucrose
CYA	Czapek Yeast Agar
CzSA	Czapek Solution Agar
DNS	dinitrosalicylic acid
EG	Endoglucanase
FAO	Food and Agriculture Organization of the United Nations
FPA	Filter Paper Activity
FPU/mL	Filter Paper Unit/millilitre
FWA	Fluorescent Whitening Agents
G25N	Glycerol Nitrate Agar
G	Gravity
GH	Glycoxyl Hydrolase
IUPAC	International Union of Pure and Applied Chemistry
LWC	Lightweight Coated
M	Molar

MD	Machine Direction
MEA	Malt Extract Agar
MOW	Mixed Office Wastes
nm	Nanometre
OBA	Optical Brightening Agents
PDA	Potato Dextrose Agar
PKC	Palm Kernel Cake
PPI	Pulp and Paper International
SC	Supercalendered
SCB	Sugar cane bagasse
SEM	Scanning Electron Microscope
SmF	Submerged fermentation
SSF	Solid substrate fermentation
U	Unit
U/g	Unit/gram
U/g/day	Unit/gram/day

PENGHASILAN ENZIM SELULASE DAN XILANASE DARIPADA *Aspergillus niger* AI-1 TEMPATAN MELALUI FERMENTASI SUBSTRAT PEPEJAL DAN APLIKASINYA DALAM PENYAHDAKWATAN KERTAS BUANGAN PEJABAT

ABSTRAK

Kajian ini memberi tumpuan kepada penghasilan enzim selulase dan xilanase daripada *Aspergillus niger* AI-1 melalui fermentasi substrat pepejal dan penggunaannya dalam proses penyahdakwatan kertas buangan pejabat. Sebanyak 25 pencilan kulat terdiri daripada genera *Aspergillus*, *Penicillium*, *Trichoderma* dan *Mucor* telah dipilih daripada 70 pencilan kulat berdasarkan zon hidrolisis pada agar selulosa dan xylan serta aktiviti enzim yang tinggi. Fermentasi substrat pepejal telah dijalankan menggunakan kultur kulat tersebut dan sisa pertanian tempatan yang terdiri daripada hampas tebu, isirong kelapa sawit, sekam padi dan habuk kayu digunakan sebagai substrat. 3 jenis kulat yang kemudiannya dikenalpasti sebagai *Aspergillus niger* AI-1, *Aspergillus aculeutus* B-1 and *Trichoderma harzianum* C3-2 merupakan penghasil enzim selulase dan xilanase yang terbaik antara jenis kulat lain yang dikaji manakala campuran isirong kelapa sawit dan hampas tebu merupakan substrat yang terbaik. Seterusnya, proses pengoptimuman fizikal dan kimia bagi penghasilan enzim dikaji menggunakan kulat yang terpilih. Keadaan optimum bagi penghasilan enzim selulase (FPAse: 5.33±0.14U/g; CMCase: 41.54±0.15 U/g) dan xilanase (524.12±2.42 U/g) yang tertinggi untuk *Aspergillus niger* AI-1 adalah seperti berikut: 0.5 mm isirong kelapa sawit dan 2 mm hampas tebu (nisbah 1:1), 80% (b/b) lembapan, suhu persekitaran (30±2^{o}C) dan pH 7.0. Tiada sumber tambahan karbon, nitrogen dan aruhan diperlukan. Manakala bagi *Aspergillus*

aculeutus B-1 penghasilan CMCase (52.25±0.24 U/g), FPAse (14.64±0.14 U/g) dan xilanase(564.07±2.35 U/g) yang optimum diperolehi apabila pengfermentasian dilakukan dalam keadaan berikut: hampas tebu (2 mm) dan isirong kelapa sawit (0.5 mm) pada nisbah 1:1, 30^{o}C, pH 8.0, 80% (b/b) lembapan dan 6% (b/b) pepton. Kulat *Trichoderma harzianum* C3-2 menghasilkan enzim CMCase (105.43±0.49 U/g), FPAse (11.32±0.21 U/g), dan xilanase (606.05±0.60U/g) pada tahap maksimum apabila dikulturkan pada keadaan optimum berikut: 70% (b/b) hampas tebu (2mm) dan 30% (b/b) isirong kelapa sawit (0.5mm), 80% (b/b) lembapan, 30^{o}C, pH 6.0, 6% (b/b) dextrin, 6% (b/b) ekstrak yis dan 0.6% (b/b) xylan. Proses pengoptimuman bagi ketiga-tiga kulat yang dikaji berjaya meningkatkan penghasilan enzim selulase antara 22% hingga 138% dan xilanase antara 70% hingga 143% bagi. Didapati bahawa enzim selulase dan xilanase yang diperolehi daripada ketiga-tiga kulat ini hampir mempunyai ciri-ciri yang sama. pH optimum selulase dan xilanase ini ialah antara 3.5-4.5 dan suhu optimum di antara 50^{o}C dan 55^{o}C. Penyahdakwatan secara enzimatik ke atas kertas buangan pejabat dilakukan dengan menggunakan estrak enzim kasar yang dihasilkan oleh *Aspergillus niger* AI-1, *Aspergillus aculeutus* B-1 dan *Trichoderma harzianum* C3-2. Keputusannya, enzim selulase dan xilanase daripada ketiga-tiga kulat ini berjaya menyahdakwatkan kertas buangan pejabat tetapi pada tahap kecekapan yang berlainan. Tahap kecekapan penyahdakwatan yang tertinggi diperolehi menggunakan estrak enzim kasar daripada *Aspergillus niger* AI-1. Oleh sebab itu, kulat tersebut dipilih untuk kajian seterusnya yang melibatkan penghasilan enzim sellulase dan xilanase melalui fermentasi keadaan pepejal menggunakan sistem dulang. Sistem dulang ini berjaya menghasilkan enzim sellulase dan xilanase 2 kali ganda lebih tinggi berbanding dengan sistem kelalang kon dan pada kadar penggunaan substrat yang lebih banyak iaitu 125g. Kajian ini

diteruskan dengan pengoptimuman proses penyahdakwatan kertas buangan pejabat bagi meningkatkan tahap kecekapan penyahdakwatan menggunakan enzim kasar sellulase dan xilanase daripada *Aspergillus niger* AI-1. Pengoptimuman ini merangkumi pengoptimuman proses pulpa, hidrolisis enzimatik dan pengoptimuman proces pengapungan. Proses pulpa pada konsistensi 1% selama 1.5 min memberikan tahap kecekapan pengnyahdakwatan yang tertinggi iaitu sebanyak 70%. Pengoptimuman hidrolisis enzimatik pula telah meningkatkan tahap kecekapan penyahdakwatan kepada 93%. Keadaan optimum hidrolisis enzimatik yang diperolehi menggunakan kertas buangan pejabat adalah seperti berikut: 0.10 M HCl, pH 4.5, 50^{o}C, kepekatan pulpa, 4.5% (b/i), jumlah aktiviti enzim, 12.5 U/g dan 60 min masa hidrolisis. Pengoptimuman proses pengapungan seterusnya meningkatkan tahap kecekapan penyahdakwatan ke tahap maksimum iaitu 95%. Keadaan optimumnya adalah seperti berikut: pH 5.0; 0.375% (b/b) Tween 80; kadar pengudaraan, 6L/min; suhu bilik (28±2^{o}C) dan 10 min masa pengapungan. Kajian menggunakan pelbagai jenis kertas terpakai menunjukkan bahawa enzim sellulase dan xilanase ini boleh digunakan untuk menyahdakwatkan kertas terpakai yang lain tetapi pada tahap kecekapan yang rendah. Walaubagaimanapun, apabila campuran kertas terpakai yang merangkumi suratkhabar, majalah dan kertas bercetak komputer digunakan, tahap kecekapan yang memuaskan (65%) diperolehi. Kajian ke atas ciri fisikal kertas penyahdakwatan menunjukkan sedikit peningkatan dalam indeks tensil, indeks koyak dan indeks pecah yang menunjukkan bahawa kekuatan kertas dapat dikekalkan sepanjang process penyahdakwatan secara enzimatik. Hasil daripada kajian ini menunjukkan bahawa enzim sellulase dan xilanase daripada *Aspergillus niger* AI-1 berpotensi digunakan untuk menghasilkan kertas terpakai yang berkualiti.

PRODUCTION OF CELLULASES AND XYLANASE BY INDIGENOUS *Aspergillus niger* AI-1 *VIA* SOLID SUBSTRATE FERMENTATION AND ITS APPLICATION IN DEINKING OF MIXED OFFICE WASTE PAPER

ABSTRACT

The present work focused on the production of cellulases and xylanase using *Aspergillus niger* AI-1 via solid substrate fermentation and its application in enzymatic deinking of mixed office waste paper. A total of 25 out of 70 fungal isolates representing the genera of *Aspergillus*, *Penicillium*, *Trichoderma*, and *Mucor* were selected based on the hydrolysis zone observed in CMC and oat spelt agar plates. They were also selected based on their high cellulases and xylanase activity. Solid substrate fermentation was carried out using these fungal isolates and local agricultural wastes consisting of sugar cane bagasse, palm kernel cake, rice husk and wood dusts were used as substrate. Among them, 3 fungi isolates were selected based on the highest enzymes production on SSF using the mixture of sugar cane bagasse and palm kernel cake as substrate. They were further identified as *Aspergillus niger* AI-1, *Aspergillus aculeutus* B-1 and *Trichoderma harzianum* C3-2. Physical and chemical optimization was carried out to optimize the enzymes production via SSF using these fungal isolates. *Aspergillus niger* AI-1 produced the highest cellulases (FPAse: 5.33±0.14U/g; CMCase: 41.54±0.15 U/g) and xylanase activity (524.12±2.42 U/g) when cultivated under the following optimum conditions: sugar cane bagasse (2mm) and palm kernel cake (0.5mm) at the ratio of 1:1, 80% (w/w) moisture content, pH 7.0 and ambient temperature (30±2^{o}C). No additional carbon, nitrogen and inducers are required. As for *Aspergillus aculeutus* B-1, the production of CMCase (52.25±0.24 U/g), FPAse (14.64±0.14) and xylanase

(564.07±2.35 U/g) reached optimum under the following conditions: 1:1 ratio of sugar cane bagasse (2mm) and palm kernel cake (0.5mm), pH 8.0, 30^{o}C, 80% (w/w) moisture content and 6% (w/w) peptone. The fungus *Trichoderma harzianum* C3-2 produced the highest CMCase (105.43±0.49 U/g), FPAse (11.32±0.21 U/g) and xylanase activity (606.05±0.60U/g) when grown using 30% (w/w) palm kernel cake (0.5mm), 70% (w/w) sugar cane bagasse (2mm) at 30^{o}C, moisture content of 80% (w/w) and pH 6.0. Addition of 6% (w/w) dextrin, 6% (w/w) yeast extract and 0.6% (w/w) xylan to the SSF medium further induced the enzymes production. The optimization study here succesfully increased the enzymes production by the three fungi isolates in the range of 22% to 138% for cellulases and between 70% to 143% in the case of xylanase. Characterization of the cellulases and xylanase produced by *Aspergillus niger* AI-1, *Aspergillus aculeutus* B-1 and *Trichoderma harzianum* C3-2 showed that all the three fungi have almost the same enzymes characteristics. The optimum pH for cellulases and xylanase was between pH 3.5-4.5 and the optimum temperature was between 50^{o}C and 55^{o}C. Enzymatic deinking of mixed office wastes were carried out by using the crude enzymes produced by these fungi isolates. The results showed that the cellulases and xylanase produced by these isolates were able to remove the ink from the waste papers but with different efficiency. The highest deinking efficiency was achieved when the crude enzymes from *Aspergillus niger* AI-1 was used and therefore this isolate was selected for the subsequent study. The production of cellulases and xylanase from *Aspergillus niger* AI-1 was further produced in a laboratory tray system via SSF and it is found out that this system was able to scale up the production of cellulases and xylanase using 125 g of substrates and the enzymes production was increased 2 fold. The study was carried out with the optimization of the deinking process to enhance the ink removal from mixed office

wastes papers. Pulping at 1% consistency for 1.5 min demonstrated the highest deinking efficiency of 70%. Optimization of the enzymatic hydrolysis conditions resulted with further enhancement in the deinking efficiency to 93%. The optimum conditions for enzymatic hydrolysis were: 0.10 M HCl, 50^{o}C, pH 4.5, pulp concentration of 4.5% (w/v), total enzyme concentration of 12.5 U/g and 60 min hydrolysis time. Optimization of the flotation process resulted in the maximum deinking efficiency of 95%. The optimum conditions for the flotation process were pH 5.0, 0.375% (w/w) of Tween 80, airflow rate of 6L/min, room temperature (28±2^{o}C) and 10 min flotation time. Effect of enzymatic deinking using different type of waste papers revealed that the cellulases and xylanase from *Aspergillus niger* AI-1 was able to remove the ink from the waste papers but with low deinking efficiency. However, when mixture of waste papers consisting of newspaper, magazine and computer printouts were used a satisfactory deinking efficiency of 65% was achieved. Physical characteristic of the enzymatic deinked mixed office wastes papers showed a marginal improvement with respect to tensile index, tear index and burst index revealing that the strength of the paper were maintained throughout the entire deinking process. The results obtained in this work suggested the use of cellulases and xylanase obtained from *Aspergillus niger* AI-1 for recycling of mixed office wastes to produce a good quality recycled paper.

CHAPTER ONE

INTRODUCTION

1.1 Applications of cellulases and xylanases

At present, cellulase is the third largest industrial enzyme in the world (Wilson, 2009) that are sold in large amounts because of its wide range of applications in food industry such as starch processing, brewery and wine industries, animal feed, agriculture, extraction of fruit and vegetables, textiles, detergent, pulp and paper industries, medical/pharmaceutical industries as well as in research and development (Arifoğlu and Őgel, 2000; Jang and Chen, 2003; Latifian *et al*., 2007; Saravanan e*t al*., 2009). Cellulase is a complex of enzymes consisting of endoglucanases (1,4-*β*-D-glucan-4-glucanohydrolase, endocellulases, EC 3.2.1.4), cellobiohydrolases (1,4-*β*-D-glucan-4-cellobiohydrolase, exoglucanases, EC 3.2.1.91) and *β*-glucosidases (*β*-D-glucosido-glucohydrolase, cellobiase, EC 3.2.1.21) that act synergistically to degrade cellulose into low molecular weight oligosaccharides, cellobiose, and eventually glucose (Li *et al*., 2006; Thongekkaew *et al*., 2008).

Cellulases have attracted much interest because of their enormous potential to convert cellulose, a natural abundant and renewable energy resource, to usable products such as soluble oligosaccharides, glucose, alcohol, and other industrially important chemicals (Rocky-Salimi and Hamidi-Esfahani, 2010). Xylanases (1,4-*β*-D-xylan xylanohydrolase; EC 3.2.1.8) are enzyme that hydrolyze xylan, the major structural component of hardwood cell wall. Xylanases are produced on an industrial scale for use in pulp and paper industry, as food additives in poultry products and in wheat

flour for improving dough handling as well as degradation of arabinoxylans in brewing process (Li *et al*., 2007a; Maalej-Achouri *et al*., 2009). Xylanases are also used for the extraction of coffee, plant oils and starch, in the improvement of nutritional properties of agricultural silage and grain feed (Souza *et al.*, 2001) and in combination with pectinases and cellulase for clarification of fruit juices (Li *et al*., 2007b). Filamentous fungi demonstrated to secrete a wide range of cellulases and xylanase, with the genera *Aspergillus* and *Trichoderma* being the most extensively studied and reviewed among the xylanase/cellulase producing fungi (Li *et al*., 2007b).

1.2 Solid substrate fermentation: A promising technology for enzymes production

In view of the potential applications of cellulase and xylanase, the cost of the enzyme production is one of the factors determining the economics of any process. Reducing the costs of enzyme production by optimizing the fermentation medium and conditions are the goal for any industrial application (Shah and Madamwar, 2005a). Solid substrate fermentation (SSF) holds tremendous potential for the production of enzymes. SSF can be used for the production of enzymes using a wide range of low cost agro-industrial residues (sugar cane bagasse, wheat bran, wheat straw, corncobs, rice husk, maize bran etc.), which are generally considered the best substrates for SSF processes (Pandey *et al*., 1999; Singh e*t al*., 2008). SSF is also an attractive and economical process to be used for the production of cellulases and xylanases, due to its low capital investment and low operating expenses (Latifian *et al*., 2007). SSF is characterized by the growth of microorganism in the absence of free water on a solid material which is used as the substrate or the inert support of the microorganism. The solid substrate not only supplies nutrients to microbial cultures, but also serves as an

anchorage for the cells. A large number of fungi are known to grow well on moist substrates in the absence of free-flowing water, whereas many bacteria are unable to grow under these conditions. As a result, the majority of studies involving SSF are conducted using fungi (Singhania e*t al*., 2009).

1.3 Waste paper and paper recycling

Worldwide, the paper production has increased over the last ten years by 4% annually and is projected to further increase by 2% per year. At present, the paper production already reached 383 million tonnes (Food and Agriculture Organization of the United Nations, FAO, 2009). Printing and writing papers constitute 31% of world paper production (Steward *et al*., 2008). The continuously growing paper manufacturing industry not only imposes severe demand on green plants that forms the basic raw material but also created wastepaper which is the largest fraction of solid wastes. Thus, it is obviously not an environmental-friendly approach. Recycling of used paper is an alternative that can alleviate the stress to the environment. The use of recycled paper as secondary fibre has increased greatly over the last two decades. Besides being a low-cost fibre source for paper manufacturing, it preserves forest resources, reduces environmental pollution and conserves water and energy. The three major sources of raw material for such recycling are newsprint, photocopied paper and inkjet-printed papers (Mohandass and Raghukumar, 2005). The significant difficulty in dealing with secondary fibre is the removal of contaminants, particularly ink. The difficulty of ink removal depends primarily on the ink type, printing process and fibre type. Recycling paper requires the removal of printing ink, also called deinking, from the used paper to obtain brighter pulp. Laser and xerographic printed papers or known as mixed office waste (MOW) are fast growing source of waste

paper for recycling due to the increased utilization of office photocopiers and computer print outs. This rate of consumption indicates great potential for reusing high quality fibres such as those used in the laser and xerographic processes. At the same time, it was found out that with MOW, ink removal is most difficult. Indeed, photocopiers and laser printers physically bind the ink (thermosetting toners consisting of non-dispersible synthetic polymers) to the fibres as a result of high heat, making it difficult and expensive to remove by conventional chemical methods (Marques *et al*., 2003).

1.4 Role of cellulases and hemicellulases in enzymatic deinking of MOW

Deinking involves dislodging ink particles from fibre surfaces and then separating the dispersed ink from the fibre suspension by washing or flotation (Marques *et al*., 2003). Enzymatic approaches which involve either ink degradation or fibre surfaces hydrolysis, have shown able to overcome problems encountered by commonly employed chemical deinking techniques using alkaline deinking agents such as sodium hydroxide, sodium carbonate, sodium silicate and hydrogen peroxide. These chemicals not only failed to successfully deink mixed office waste but also created environmental problems (Pala *et al*., 2004; Liu *et al*., 2009). The potential of enzymatic deinking has been assessed and proven successful using different types of enzymes. Lipases and esterases can degrade vegetable-oil based ink. Hemicellulases and cellulases are believed to alter fibre surfaces or bonds in the vicinity of ink particles, thereby freeing the ink for removal by washing or flotation (Kim *et al*., 1991). Results from previous research primarily with cellulases and hemicellulases, indicate that enzymes facilitate ink removal (Lee *et al*., 2007). The brightness of

pulped paper increases in response to enzymatic treatment, often being less or equal to those produced by conventional deinking. Residual ink areas, however, are generally improved relative to those left after chemical deinking treatment. The effect of different hydrolytic enzymes such as cellulases and hemicellulases has been tested for their application in the enzymatic deinking of recycled paper. It has been proven that commercial acidic or alkaline cellulases preparation can effectively remove and disperse laser and xerographic toners, and enzymes appear to be promising for practical application in office paper deinking (Prasad, 1993; Jeffries *et al*., 1994, 1996; Yang *et al*., 1996; Viesteurs *et al*., 1999). Welt and Dinus (1995) reported that cellulases and hemicellulases could also be effective in deinking and improving the brightness of letterpress and colour offset printed newsprint. They found that the best brightness was obtained using the mixture of cellulase and hemicellulases. Similar observations were also reported by many researchers using a combination mixture of celulases and hemicellulases (Mφrkbak and Zimmermann, 1998; Jeffries *et al*., 1994; Heise *et al*., 1996; Jobbins and Franks, 1997). It has also been reported that besides alkaline cellulases, acidic cellulases are also proven to be successfully applied to the deinking of mixed office waste and few advantages were observed (Jobbins and Franks 1997). Using enzymes which are active in acidic environments will lessen the yellowing of derived products. The ratio of endoglucanase and exoglucanase of crude cellulase, known to be an important parameter in determining deinking efficiency and the physical properties of the recycled paper (Han and Koo, 1998).

1.5 Objectives of research

Waste materials from agricultural activities can be referred to as “hidden wealth” as they can actually be converted to useful products such as energy, chemicals, and

feedstock as well as for enzymes production (Wilson, 2009). In 2007 alone, Malaysia generates approximately more than 90 million tonnes of agricultural wastes and most of it left unutilized or underutilized (Alam *et al*., 2009). Such agricultural wastes represent vast renewable resources and must be given attention. Agricultural wastes comprises of large pool of proteins and biopolymers which can be utilized by various microorganisms for the production of useful compounds. The role of microorganisms as catalyst for processing of organic renewable resources is reflected in their involvement in several bioconversion processes which have been shown to be feasible. In Malaysia, most of the agricultural wastes such as palm kernel cake, rice husk, rice straw, coconut fibre, wood dust and many other wastes materials are either burned or disposed. Therefore as an environmental friendly approach, these agrowastes can be used as substrates for the growth of fungi to produce useful metabolites. Thus, this research focuses on the use of the agrowastes to isolate a diverse group of indigenous fungi from the natural resources that are capable of producing cellulase and xylanase *via* solid substrate fermentation (SSF). Malaysians consumed almost 3.5 million tonnes of paper annually and almost 35% consists of printing and writing paper (Asean Graphic Paper Forecast, PPI, 2008; FAO, 2009). However, none of these waste papers had been used to produce printing/writing paper (Lee, 2005). The use of recycled fibres has significantly increased all over the world and in order to use these fibres, the removal of ink must be done. Currently, paper mills in Malaysia use chemical approaches to remove the ink from the waste paper which require the use of large amount of chemical agents which resulted in a highly detrimental to the environment. Subsequently, the cost of waste water treatment becomes high in order to meet the environmental regulations and policy. Since the potential of enzymatic deinking has been assessed and proven successful this research

was undertaken to use the cellulases and xylanase from indigenous isolates for the deinking of mixed office wastes papers.

The objectives of the current research are as follow:

i. To isolate potential cellulase/xylanase producing fungi from various sources obtained throughout Northern Region of Peninsular Malaysia.
ii. To utilize agricultural wastes as substrates for solid substrate fermentation (SSF) using the selected fungi isolates for the production of cellulases and xylanase
iii. To determine the optimum conditions for the production of cellulases and xylanase under different cultural conditions *via* SSF
iv. To characterize the cellulases and xylanase for application in enzymatic deinking of mixed office wastes
v. To optimize the enzymes mixtures for use in the hydrolysis of papers for deinking process
vi. To optimize the flotation process for removal of ink and separation
vii. To determine the physical properties of the recycled paper after enzymatic deinking.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 Chemical structure of cellulose and xylan

2.1.1 Cellulose

Cellulose is the most wide spread renewable carbohydrate polymer or carbon source in nature that human being can easily utilized (Jang and Chen, 2003; Wang *et al*., 2004; Liming and Xueliang, 2004). It is the principal constituents of plant materials which forms about half to one third of plant tissues (Dutta *et al*., 2008), component cell wall of algae and its production by non-photosynthetic organisms (certain bacteria, marine invertebrates, fungi, slime moulds and amoeba) has also been documented (Zhang and Lynd, 2004; Zhilina *et al*., 2005). The total amount of cellulose biomass generated worldwide by plant photosynthesis is about 1.14×10^{12} tons annually (Xu *et al*., 2006). Cellulose is found in all naturally occurring organic compounds which accumulate every year in large quantities in the form of municipal solid waste as well as a vast quantities of this materials are produced from activities such as food processing, lumbering operations, paper making, cereal grain harvesting, sugar cane processing (Romanelli *et al*., 1975) and in the form of forest, industrial, residential and other agricultural waste residues (Alam *et al*., 2004). Most of these cellulosic materials generally burnt in the field, resulting in low energy utilization efficiency (10%) (Xu *et al*., 2006) and causes environment and ecological problems (Wang and Yang, 2007). On the other hand, food shortage, energy crisis, fuel and limit of non-renewable resources are now major problems all over the world, which makes the utilization of cellulose is important (Krishna *et al*., 2000; Semádo *et al*.,

2000; Zhang *et al*., 2006; Rocky-Salimi and Hamidi-Esfahani, 2009). Cellulose is an un-branched linear homopolymer of anhydrous D-glucose residues linked together by *β*-1,4-glycosidic bonds (Arifoğlu and Őgel, 2000; Foyle *et al*., 2007; Rakshit, 2004). Depending upon the source, this polymer may extend up to 15,000 residues in length (Okafor, 2007). The glucose moieties exist in the lowest energy conformation of *β*-D-glucopyranose, the chair configuration (Clarke, 1997). Anhydrocellobiose is the repeating unit of cellulose, since the adjacent anhydrous glucose molecules are rotated 180^0 with respect to their neighbour (Fig 2.1). This rotation causes cellulose to be highly symmetrical, since each side chain has an equal number of hydroxyl groups (Gardner and Blackwell, 1974; Clarke, 1997; Glazer and Nikaido, 2007).

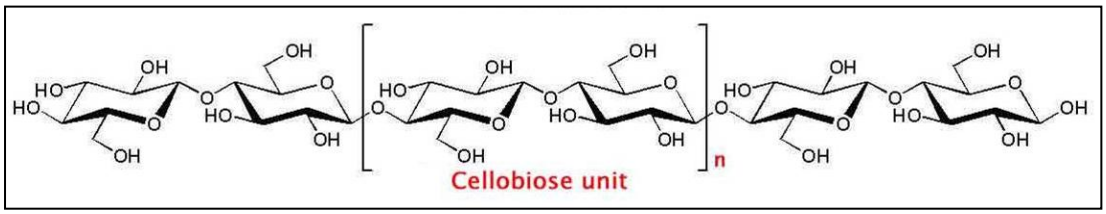

Figure 2.1 Structure of cellulose showing the basic unit is cellobiose (Source: http://sci.waikato.ac.nz)

Despite of its chemical simplicity, the physical and morphological structure of native cellulose is complex and heterogenous. During cellulose biosynthesis, numerous parallel poly-glucose chains form insoluble complexes of crystalline cellulose fibres through hydrogen bonds and van-der-Waals forces (Zimmer, 2005). It been shown that, these parallel poly-glucose chains are oriented with reducing ends at one terminus and non-reducing ends at the others. This substructure results in microfibrils that contain cellulose chains in highly crystalline zones, as well as paracrystalline or amorphous regions, which are more susceptible to degradation than the crystalline region (van Wyk and Mohulatsi, 2003). The secondary and tertiary structures of

native cellulose alone are complicated, and may vary significantly depending on the source and biosynthetic machinery used to produce the biopolymer (i.e. plant or bacteria). Furthermore, cellulose polymers are frequently found in strong association with hemicelluloses and lignin, which resulting in even more complex morphologies and make its bioconversion more difficult (Mansfield and Meder, 2003; Martins *et al*., 2008).

2.1.2 Xylan

After cellulose, hemicelluloses are the second most abundant renewable polysaccharide found on earth with the production rate of 10^{10} tons per year. It is a storage polymer in seeds, structural component of plant cell walls and present in the middle lamella of plant cells (Uffen, 1997; Heck *et al*., 2002; Wainø and Ingvorsen, 2003; Polizeli *et al*., 2005). Xylan is the major group or the primary component of hemicellulose and it is particularly abundant in the tissues that have undergone secondary thickening (Virupakshi *et al*., 2005). It represents up to 30-35% of the total polysaccharides in the cell walls of land plants (Ximenes *et al*., 1996; Saha, 2003; Poorna and Prema, 2007) and accounts for approximately 10-35% and 10-15% of total dry biomass in angiosperms (hardwood) and gymnosperms (softwood), respectively (Beg *et al*., 2001; Sanghi *et al*., 2008). The percentage of xylan varies depending on the type of sources. For example, it can account for more than 90% of the hemicelluloses in kraft pulp of hardwood, and about 50% of hemicellulose of softwood pulps (Bocchini *et al*., 2003).

Unlike cellulose, xylan is a complex and highly branch heteropolysaccharides, and situated between lignin and the collection of cellulose fibres underneath (Beg *et al.,* 2001; Santiago-Hernández *et al*., 2007). It is consisting of a homopolymeric backbone of 1,4 linked *β*-D-xylopyranose units (average of 150-200 in length) and often has short side chain branches of various carbohydrate substituent, most often L-arabinose, glucoronic acid, 4-*O*-methylglucuronic acid, acetic acid, ferulic and *p*-coumaric acid (Saha, 2003; Kamra and Satyanarayana, 2004; Ding *et al*., 2004; Romanowska *et al*., 2006). The xylopyranosyl backbone is substitute at positions C-2, C-3, and C-5 (Fig 2.2) to varying degrees depending upon the type and the stage of the development of the plant (Clarke, 1997; Uffen, 1997; Saha, 2003). The branches of sugar and sugar acids are linked glycosidically to the backbone of xylan whereas acids are attached by ester linkages (Gomes *et al*., 1993). The presence of acetyl groups is responsible for the partial solubility of xylan in water and xylan has better solubility at alkaline pH (Gessesse and Mamo, 1998; Beg *et al*., 2001). However, unsubstituted xylans have also been isolated and not widespread in nature (Beg *et al*., 2001; Saha, 2003). Consistent with their structural chemistry and side-group substitutions, the xylans seem to be interspersed, intertwined, and covalently linked at various points with the overlying „sheath" of lignin, while producing a coat around underlying strands of cellulose via hydrogen bonding (Uffen, 1997).

Worldwide large amount of xylan is release annually in the form of agricultural, municipal, and industrial waste (Gessesse and Mamo, 1998; Saha, 2003; Xu *et al*., 2008). The abundance of xylan wastes represent a potentially renewable energy resource that could be utilized into useful products such as fuels, chemicals, fermentable sugars etc. (Selig *et al*., 2008). Digestion of xylan is also important in

delignification of paper and pulp, digestibility enhancement of animal feed, clarification of juices and improvement of the consistency of beer (Ali *et al*., 2004). Due to the heterogeneity and complex chemical nature of plant xylan, its hydrolysis or conversion requires a diverse suite of synergistic enzymes or drastic chemical reactions (Clarke, 1997; Rizzatti *et al*., 2001).

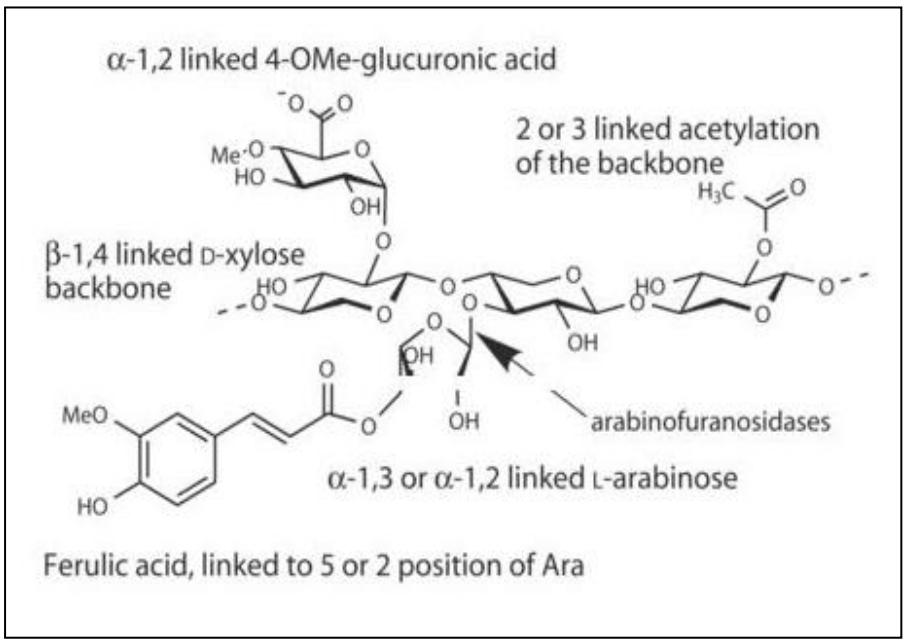

Figure 2.2 Structure of heteroxylan (Source: www.biochemj.org)

2.2 Enzymatic degradation of cellulose and xylan

In the view of complicated nature of cellulose and xylan, it is not surprising that their biodegradation requires a complex system of secreted enzymes with different specificities and mode of action (Clarke, 1997; Yoon *et al*., 2008). A great deal of research on cellulases and xylanases, which involve in the enzymatic degradation of cellulose and xylan has been developed in the last 30 years (Heck *et al*., 2002). These *β*-(1→4) glycoside hydrolases, like all enzymes, are grouped and classified according to their specifications and action patterns.

2.2.1 Cellulases

2.2.1(a) Type of cellulases

Unlike any other enzymes, cellulase is not a single enzyme but a family of at least 3 groups of enzymes (Xiao *et al*., 2004; Okafor, 2007) which is extensively studied because of their ability to decompose cellulosic biomass into glucose by hydrolyzing the *β*-1,4-glycosidic linkages of cellulose (Domingues *et al*., 2001; Yoon *et al*., 2008). Cellulases fall into 13 of the 82-glycoside hydrolase families identified by sequence analysis (Rani *et al*., 2004). The widely accepted mechanism for microbial cellulose hydrolysis involves synergistic actions of three major group of cellulases: 1) endoglucanase (1,4-*β*-D-glucan 4-glucanhydrolase; C_x-cellulase, EC 3.2.1.4); 2) exoglucanase [1,4-*β*-D-glucan cellobiohydrolase (CBH); C_1-cellulase, EC 3.2.1.91)] and 3) *β*-glucosidases (*β*-D-glucoside glucohydrolases; cellobiase; CB, EC 3.2.1.21) (Henrissat, 1994; Jang and Chen, 2003; Seidle *et al*., 2004; Dutta *et al*., 2008).

The cellulose is hydrolysed to glucose recourse by the co-operation of these three cellulases. Endo-and exoglucanases cooperate in cellulose degradation, whereas *β*-glucosidases convert cellobiose to glucose (Zorov *et* al., 2001; Yang *et al*., 2004). The complementary activities of endo-and exo-glucanases lead to synergy, i.e. an enhancement of the activity exhibited by mixture of components over the added activity of the individual enzymes (Srishdsuk *et al*., 1998; Zhang and Lynd, 2004). According to the "endo-exo" synergism model, endoglucanases hydrolyze randomly along the chain by cleaving accessible intramolecular *β*-1,4-glycosidic bonds and thus generate new attack sites (new chain ends) for exoglucanases to act upon (Fig 2.3) (Martins *et al*., 2008; Bansal *et al*., 2009). The action of this group of enzyme

producing various cellooligosaccharides, cellotriose, cellobiose and prefer soluble and amorphous forms of substrate (Okafor, 2007). Its affinity decreases with decreasing degree of polymerization with no activity on cellobiose (Clarke, 1997). Endoglucanases activities are often measure on a soluble high degree of polymerization cellulose derivatives such as carboxymethylcellulose (CMC). The modes of actions of endoglucanases decrease the specific viscosity of CMC significantly with little hydrolysis due to intramolecular cleavages (Irwin *et al*., 1993; Zhang and Lynd, 2004; Thongekkaew *et al*., 2008). Endoglucanase activities can be measure based on a reduction in substrate viscosity and/or increase in reducing ends determined by a reducing sugar assay. Because exoglucanases also increase the number of reducing ends, it is strongly recommends that endoglucanases activities be measure by both methods (Zhang *et al*., 2006).

Exoglucanases cleave cellulose chain/cellooligosaccharide chain at the accessible chain ends to liberate cellobiose, a glucose dimer (Fig 2.3), with preference for crystalline forms of the substrate (Clarke, 1997; Mansfield, 2005; Bansal *et al*., 2009). Some of them can sequentially remove glucose units from the non-reducing end of cellooligosaccharides (Seidle *et al*., 2004; Zhang, 2006). It was reported that *Trichoderma reesei* cellobiohydrolase (CBH) I and II act on the reducing and non-reducing cellulose chain ends (Teeri, 1997; Teeri *et al*., 1998). While the amorphous and soluble cellulose may be degrade through the action of endoglucanases alone, the degradation of crystalline cellulose requires the activity of an exoglucanases, at least in the case of common extracellular fungal cellulases (Zimmer, 2005). Fungal exoglucanases display a broad specificity, hydrolyzing both crystalline and amorphous celluloses, but they are generally to be inactive toward substituted

celluloses such as carboxymethylcellulose (Clarke, 1997; Thongekkaew *et al*., 2008). Avicel has been use for measuring exoglucanases activity but unlike endoglucanases and *β*-glucosidases, there is no substrate specific for exoglucanases within the cellulase mixtures. Many researchers have been working to find an effective substrate for exoglucanases but all the technique used has its own limitations (van Tilbeurgh *et al*., 1982; van Tilbeurgh and Claeyssens, 1985; Deshpande *et al*., 1984; Zhang *et al*., 2006).

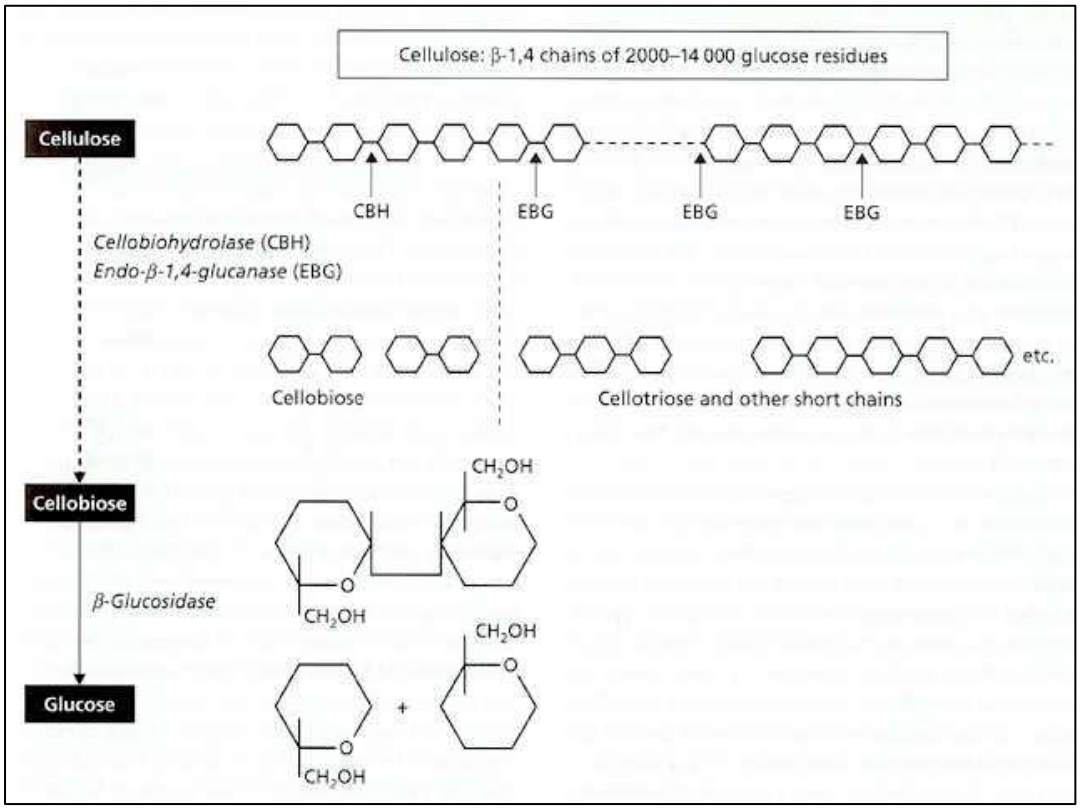

Figure 2.3 Enzymatic degradation of cellulose polymer (Source: www.biology.ed.ac.uk)

β-glucosidases play pivotal roles in cellulose degradation because they are involved in the final step that form glucose (Seidle *et al*., 2004). It is an important component of the cellulase system and acts synergistically with endoglucanase and

cellobiohydrolase for complete degradation of cellulose (Xiao *et al*., 2004). *β*-glucosidases complete the process of cellulose degradation through hydrolysis of cellobiose and short oligosaccharides to two glucose moieties (Fig 2.3) but do not attack cellulose (Okafor, 2007; Martins *et al*., 2008). In addition, *β*-glucosidases also regulate the secretion of cellulases during fungal producer cultivation: the enzyme generates glycosyl transfer products that induce the biosynthesis of celulases (Zorov *et al*., 2001). Cellulase systems with low level of *β*-glucosidases have poor saccharifying power. This is because low *β*-glucosidase activity leads to a build up of cellobiose during hydrolysis, which inhibits the activity of cellobiohydrolases and endoglucanases largely than glucose does. Therefore, extra *β*-glucosidase (BG) needs to be added for an efficient hydrolysis of cellulosic materials (Krogh *et al*., 2004; Xie *et al*., 2004; Bansal *et al*., 2009). *β*-glucosidases activity can easily be detected with simple sensitive assay methods based on coloured or fluorescent products released (Strobel and Russell, 1987; Polacheck *et al*., 1987; Setlow *et al*., 2004). End product inhibition such as glucose, ethanol and cellobiose has demonstrated significant inhibitory effect on the activity of both *β*-glucosidase and cellulase mixtures (Xiao *et al*., 2004). Alternatively, cellobiose can also be degraded oxidatively through the action of a cellobiose-quinone-oxidoreductase (EC 1.1.5.1), which also reduces quinines and phenoxy radicals generated during phenol oxidation, and converts cellobiose to cellobionic acid (Zimmer, 2005).

The whole cellulase activity is often represents by FPA (filter paper activity) which include the synergistic actions of the three groups of cellulases. The single endoglucanase activity is often represents by carboxymethyl cellulase (CMCase) activity (Zhou *et al*., 2004). Although all cellulolytic enzymes have similar bond

specificities (β-1,4), important functional differences are found in their mode of action towards solid substrates (Mansfield and Meder, 2003). Possibly, another exo-acting enzyme called exoglucohydrolase (EC 3.2.1.74) is also involved in cellulose degradation by splitting of glucose instead of cellobiose from the non-reducing end of poly-and-oligosaccharides (Clarke, 1997; Zimmer, 2005). Additionally, it has been suggested that the presence of cellulose binding modules promotes the effective hydrolysis of different cellulose substrates, especially crystalline moieties (Carrard *et al*., 2000). However, synergy has also been observed between two different types of cellobiohydrolases (Irwin *et al*., 1993; Nidetzky *et al*., 1993) as well as between two endoglucanases (Gübítz *et al*., 1998; Mansfield *et al*., 1998).

2.2.1(b) Sources of cellulases

A broad range of organisms predominantly molds, fungi and bacteria are capable of producing cellulose-degrading enzymes to solubilise complex components of cellulose to simple molecules for completing the carbon cycle (Badhan *et al*., 2007; Dutta *et al.,* 2008). These organisms, which include aerobes and anaerobs, mesophiles and thermophiles are widespread and abundant in natural environment (Glazer and Nikaido, 2007) (Table 2.1). Most of the earlier studies on cellulases production was carried out on fungi mainly the genus of *Trichoderma* (Kansoh *et al*., 1999). The soft rot fungus *Trichoderma reesei* is one of the efficient producers of cellulases (up to 35g/L) and used industrially for enzyme production (Teeri *et al*., 1983; Labudova and Farkas, 1983; Fagerstam *et al*., 1984; Henrissat *et al*., 1985; Kubicek, 1992; Kubicek *et al*., 1993; Nidetzky *et al*., 1994; Lo *et al*., 2010). It is one of the most successful and extensively studied cellulase systems (Claeyssens and Tomme, 1989; Goyal *et al*., 1991; Teeri *et al*., 1998). It produces two

cellobiohydrolases (CBHI, CBHII), five endo-*β*-1,4-D-glucanases (EGI,EGII,EGIII,EGIV,EGV) and two *β*-D-glucosidases (BGLI,BGLII) that correspond to approximately 80% of the total secreted protein (Xiong *et al*., 2004). One of the main limitations of the cellulolytic systems from *Trichoderma reesei* is the low amount of beta-glucosidase (Krogh *et al*., 2004; Sohail e*t al*., 2009). However, the *Aspergillus* and *Penicillium* sp. are able to produce a complete system of cellulases with relatively high *β*-glucosidase activity (van Wyk, 1999; Castellanos *et al*., 1995; Seidle *et al*., 2004; Jørgensen *et al*., 2005; Sohail *et al*., 2009) but they are not investigated to the same extent and it is still possible to identify new enzymes with interesting features from these species (Panagiotou *et al*., 2006). Some other fungal known for cellulase production include *Chaetomium* sp., *Sporotrichum* sp., *Fusarium* sp., *Humicula* sp., *Neocallimastix* sp., *Phanerochaete* sp., *Myrothecium* and others (Table 2.1) (Thirumale *et al*., 2001; Ahmed *et al*., 2003; Rani *et al*., 2004; Milala *et al*., 2005). Thermophilic and thermotolerant fungi are of central importance as a source of thermostable enzymes, which are important for industrial utilization because of the possible economic benefits of being able to degrade plant residues at elevated temperature. Thermophilic fungi, an exotic group of molds with 33 odd known species, includes some of the prolific producers of thermostable cellulases, i.e., *Thermomyces lanuginosus*, *Thermoascus aurantiacus*, *Humicula insolens* (syn. *Scytalydium thermophilium*), that are potentially important from biotechnological stand point (Maheswari and Chandra, 2000; Brienzo *et al*., 2008). Up to now the most studied group of cellulose-degrading microorganisms is the fungi which are characterized by multicomponent, synergistic cellulolytic enzyme systems. Studies of a few group of cellulolytic soil bacteria provided evidence for the existence of different cellulase systems, which are clustered or bound to the cell walls, whereas filamentous actinomycetes appear to degrade cellulose in similar manner to fungi.

Well known genera of aerobic cellulolytic bacteria are *Bacillus*, *Cellulomonas*, *Streptomyces*, *Cytophaga*, *Cellvibrio* and *Pseudomonas* (Ulrich *et al*., 2007) and strict anaerobs such as *Clostridium* (Heck e*t al*., 2002). Endogenous cellulases have also been found in both terrestrial and aquatic detritivores. They are possibly similar to those of aerobic bacteria. These cellulases in invertebrates can degrade cellulose without microbial assistance, but they appear to be less effective than microbial cellulases (Zimmer, 2005).

Table 2.1 Cellulase producing microorganisms isolated from natural environment

Microorganism	Isolation source	Reference
Fungi		
Absidia corymbifera	Oil mill waste	Gopinath *et al*., 2005
Acremonium stromaticum	Soil from cold-hilly area	Khalid *et al*., 2006
Alternation tenuis	Soil from cold-hilly area	Khalid *et al*., 2006
Aspergillus ellipticus	Decaying wood	Gupte and Madamwar, 1997
Aspergillus flavus	Soil sample	Khalid *et al*., 2006
Aspergillus funiculosus	Soil from cold-hilly area	Khalid *et al*., 2006
Aspergillus fumigatus	Decaying wood	Gupte and Madamwar, 1997
Aspergillus glaucus	Mildewy maize cob	Xu *et al*., 2006
Aspergillus koningi	Soil from hot-plain area	Khalid *et al*., 2006
Aspergillus luchuensis	Soil from hot-plain area	Khalid *et al*., 2006
Aspergillus nidulans	Farm yard manure	Kumar *et al*., 2007
Aspergillus niger	Decomposed jute stacks, decomposing substrate, oil mill waste	Gomes *et al*., 1989; Kumar and Singh, 2001; Gopinath *et al*., 2005
Aspergillus sydowi	Soil from hot-plain area	Khalid *et al*., 2006
Aspergillus terreus	Pulp wastewater and rotten wood; decomposed jute	Gomes *et al.*, 1989; Emtiazi *et al*., 2001
Aspergillus versicolor	Soil sample	Khalid *et al*., 2006
Aureobasidium pulluans	Orange juice residues	Leite *et al*., 2007
Botrytis pilulifera	Soil from hot-plain area	Khalid *et al*., 2006
Chaetomium erraticum	Soil	Soni *et al*., 1999
Cladosporium cladosporioides	Soil sample	Khalid *et al*., 2006
Cladosporium elatum	Soil from hot-plain area	Khalid *et al*., 2006
Cunninghamella echinulata	Oil mill waste	Gopinath *et al*., 2005
Curvularia lunata	Oil mill waste	Gopinath *et al*., 2005

Table 2.1 Continue……

Drechslera hawaiiensis	Soil from hot-plain area	Khalid *et al*., 2006
Fusarium dimenrum	Soil from cold-hilly area	Khalid *et al*., 2006
Fusarium oxysporum	Cumin	Panagiotou *et al*., 2003
Fusarium sambucinum	Soil from hot-plain area	Khalid *et al*., 2006
Fusarium semitectum	Soil from hot-plain area	Khalid *et al*., 2006
Fusarium solani	Oil mill waste	Gopinath *et al*., 2005
Gliocladium deliquescens	Rotten wood chips	Yeoh *et al*., 1984
Gliocladium virens	decomposed jute stacks	Gomes *et al*., 1989
Humicula sp.	Wheat straw compost	Kumar *et al*., 2007
Melanocarpus sp.	Composting soil	Jatinder *et al*., 2006a
Monacrosporium elegans	Soil from hot-plain area	Khalid *et al*., 2006
Monilia geophila	Soil from cold-hilly area	Khalid *et al*., 2006
Monocillium sp.	Soil from hot-plain area	Khalid *et al*., 2006
Mucor geophilus	Soil from cold-hilly area	Khalid *et al*., 2006
Mucor hiemalis	Soil sample	Khalid *et al*., 2006
Mucor pusillus	Soil from cold-hilly area	Khalid *et al*., 2006
Mucor racemosus	Oil mill waste	Gopinath *et al*., 2005
Myceliophthora sp.	Composting soil, India	Badhan *et al*., 2007
Paecilomyces variotii	Oil mill waste	Gopinath *et al*., 2005
Penicillium ahdi (novel)	Soil from hot-plain area	Khalid *et al*., 2006
Penicillium canescens	Soil	Sinitsyna *et al*., 2003
Penicillium citrinum	Soil, decomposed substrate	Kuhad and Singh, 1993; Dutta *et al*., 2008
Penicillium decumbens	Soil from hot-plain area	Khalid *et al*., 2006
Penicillium duclauxi	Soil from hot-plain area	Khalid *et al*., 2006
Penicillium islanidicum	Soil from hot-plain area	Khalid *et al*., 2006
Penicillium lilacinum	Soil from cold-hilly area	Khalid *et al*., 2006
Penicillium notatum	Soil from hot-plain area	Khalid *et al*., 2006
Penicillium puberulum	Soil from hot-plain area	Khalid *et al*., 2006
Penicillium rubrum	Soil from hot-plain area	Khalid *et al*., 2006
Penicillium shaze (novel)	Soil sample	Khalid *et al*., 2006
Penicillium ulaiense	Decayed lemon	Rajal *et al*., 2002
Rhizopus arrhizus	Soil from cold-hilly area	Khalid *et al*., 2006
Rhizopus oryzae	Soil from hot-plain area	Khalid *et al*., 2006
Sclerotinia sclerotiorum	Sunflower	Issam *et al*., 2004
Scytalidium lignicola	Decaying wood	Trivedi and Desai, 1984
Scytalidium thermophilum	Composting soil, soil	Jatinder *et al*., 2006b; Kumar *et al*., 2007
Sporotrichum thermophile	Self-heating wood chip piles	Grajek, 1987
Syncephalastrum racemosum	Oil mill waste	Gopinath *et al*., 2005
Thermoascus aurantiacus	Self-heating wood chip piles	Grajek, 1987
Tiarosporella phaseolina	Diseased jute stem	Gomes *et al*., 1989
Trichoderma harzianum	Decomposed jute stacks; Rotten wood chips	Yeoh *et al.*, 1984; Gomes *et al*., 1989
Trichoderma koningii	Rotten wood chips	Yeoh *et al*., 1984
Trichoderma lignorum	Soil sample	Khalid *et al*., 2006
Trichoderma longibrachiatum	Manure	Trivedi and Desai, 1984
Trichoderma reesei	Decomposing substrate	Kumar and Singh, 2001
Trichoderma viride	Decomposed jute stacks	Gomes *et al*., 1989

Table 2.1 Continue……..

Verticillium candelabrum	Soil from hot-plain area	Khalid *et al*., 2006
Verticillium cellulose	Soil from hot-plain area	Khalid *et al*., 2006
Bacteria		
Bacillus circulans	Soil	Kurakake *et al*., 2007
Bacillus subtilis	Soil	Heck *et al*., 2002; Tang *et al*., 2003
Cellulomonas spp	Termite infested soil	Saxena *et al*., 1992
Clostridium absonum	Decomposed plant	Rani and Nand, 2000
Clostridium sp.	Isolated from manure	Lee and Blackburn, 1975
Clostridium thermocellum	Soda-lake sediment	Zvereva *et al*., 2006
Geobacillus thermoleovorans	Sugar refinery wastewater	Tai *et al*., 2004
Sphingomonas paucimobilis	Soil	Kurakake *et al*., 2007
Streptomyces omiyaensis	Goat‟s rumen	Alam e*t al*., 2004
Streptomyces sp.	Soil	Semádo *et al*., 2000; Alani *et al*., 2008

2.2.1(c) Properties of cellulases

As secreted enzymes, the cellulases are relatively resilient to extremes of temperature and pH. With Table 2.2 as evident, most of the well studied cellulases show optimum activity at slightly acidic pH range of 2.5 to 7.0. The temperature optima are quite broad, ranging from 30 to 75^{o}C, but the majority of the enzymes are most active at temperatures equal to 50^{o}C or between 50-60^{o}C (Dutta *et al*., 2008). All of the characterized enzymes from fungi are known to be glycosylated. The pH activity profiles of most cellobiohydrolases show a single sharp optimum between pH 4 and 5, but interestingly those of the enzymes from both *Penicillium funiculosum* and *Trichoderma koningii* are bimodal with optima at pH 2.5 and pH 4.5 (Wood *et al*., 1980). There are a few exceptions among the bacteria, especially species of alkalothermophilic *Bacillus* which show optimum pH at alkaline conditions. For example, the pH activity of profile of the *Bacillus* sp. NK1 cellulase is broad, ranging from 5 to 10.9 (Horikoshi *et al*., 1984; Sashihara *et al*., 1984). These values, as with all of the most established kinetic and enzyme properties have been obtained in vitro

with isolated and purified enzymes, and it is quite possible that cellulases together with the other cellulolytic and heteroxylanolytic enzymes are even more stable when stabilized by the environmental conditions in situ.

2.2.2 Xylan-degrading enzymes

2.2.2(a) Type of xylan-degrading enzymes

Due to the structural heterogeneity of the xylans, the xylan-degrading enzyme system consists of two main families/key of hydrolytic enzymes, the *β*-1,4 endoxylanases (E.C 3.2.1.8), which randomly attack the main chain of xylan backbone internally, and the *β*-xylosidases (E.C 3.2.1.37), which hydrolyze xylooligosaccharides from the non-reducing end to xylose (Ali *et al*., 2004; Cannio *et al*., 2004; Sudan and Bajaj, 2007) (Fig 2.4). Endoxylanase catalyzes the hydrolysis of the xylopyranosyl linkages of *β*-1,4-xylan except for xylobiose releasing xylooligosaccharides of different sizes thus reducing the degree of polymerization of the substrate (Senthilkumar *et al*., 2004; Xu *et al.*, 2005; Yang *et al*., 2005; Nair *et al*., 2008; Rizzatti *et al*., 2008). Endoxylanases can be categorized based on the three-dimensional structure of the catalytic domains that can be deduced from the amino acid sequence. Most endoxylanases are assigned to either glycosyl hydrolase (GH) family 10 or 11. GH10 members are generally larger molecular weight than those of GH11. In addition, GH11 xylanase enzymes usually have higher specific activities, but GH10 xylanases are more efficient at attacking a wider variety of substrates, including xylan decorated with various side-branching chemical moieties (Sá-Pereira *et al*., 2003; Lee *et al*., 2006).

Table 2.2 Properties of some bacteria, yeast and fungal cellulases

Microorganism	pH optimum	pH stability	Optimum temp (°C)	Temp stability (°C)	Reference
Bacteria					
Bacillus strain PDV	4.5-7.0	4.0-10.0	60	70	Sharma *et al*., 1990
Caldibacillus cellulovorans	6.5-7.0	NA	80	70	Huang and Monk, 2004
Cellulomonas uda	5.5-6.5	5.5-8.0	45-50	NA	Clarke, 1997
Clostridium acetobutylicum	4.6	NA	37	NA	Allcock and Woods, 1981
Clostridium cellulolyticum	6.0	NA	48	NA	Fierobe *et al*., 1993
Clostridium papyrosolvens CFR-703	6.5	NA	35	NA	Rani *et al*., 2004.
Clostridium thermocellum	6.1	4.0-8.0	70	60	Johnson *et al*., 1982
Fibrobacter succinogenes	6.4	5.9-7.1	39	<45	McGavin and Forsberg, 1988
Pseudomonas fluorescens	8.0	7.0-8.0	NA	NA	Clarke, 1997
Streptomyces omiyaensis	6.5	NA	45	NA	Alam *et al*., 2004
Streptomyces sp.	5.0	5.0-10.0	50-60	Up to 70	Alani *et al*., 2008
Streptomyces transformant T3-1	6.5	NA	50	40-50	Jang and Chen, 2003
Yeast					
Aureobasidium pullulans	4.5	3.5-7.5	60	50	Leite *et al*., 2007
Saccharomyces cerevisiae	4.0	NA	40-50	NA	Hernández *et al*., 2002
Fungi					
Aspergillus aculeatus	4.5	2.0-9.0	50	45	Murao *et al*., 1988
Aspergillus niger	4.8	NA	60-70	Up to 50	Kang *et al*., 1999
Aspergillus niger No.51	6.0	3.0-9.0	60	0-80	Xie *et al*., 2004
Ceriporiopsis subvermispora (white-rot)	3.5-4.5	NA	60	50	Heidorne *et al*., 2006
Coriolus versicolor	5.0	4.0-6.0	55	55	Idogaki and Kitamoto, 1992
Humicola insolens	5.0	3.5-9.5	50	65	Hayashida *et al*., 1988

Table 2.2 Continue……..

Penicillium canescens	5.0	NA	60-65	40	Sinitsyna *et al*., 2003
Phanerochaete chrysosporium	4.6	NA	60	Up to 60	Khalil, 2002
Sclerotium rolfsii	4.0	4.0-7.0	74	NA	Clarke, 1997
Thermoascus aurantiacus	5.0	5.0-9.0	75	65	Clarke, 1997
Trichoderma reesei QM-9414	4.5	NA	50	NA	Krishna *et al*., 2000
Trichoderma sp. M_7	4.5	NA	50	NA	Petrova *et al*., 2002
Trichoderma viride	4.5	NA	55	NA	Gupta and Gupta, 1979