
# CHARACTERIZATION OF CELLULASE AND XYLANASE FROM *Lentinus polychrous* Lev. LP-PT-1

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# บทคัดย่อ


ชื่อเรื่อง : คุณสมบัติของเอนไซม์เซลลูเลสและไซลาเนสที่ผลิตโดยเชื้อรา *Lentinus polychrous* Lev. LP-PT-1

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ศัพท์สำคัญ : เซลลูเลส ไซลาเนส *Lentinus polychrous* Lev.

เทคโนโลยีชีวภาพเป็นองค์ความรู้ทางด้านวิทยาศาสตร์และเทคโนโลยีที่กำลังได้รับความสนใจเป็นอย่างมากจากนักวิทยาศาสตร์ทั่วโลก วิธีการทางเทคโนโลยีชีวภาพนั้นเป็นการใช้ประโยชน์จากเอนไซม์และจุลินทรีย์เพื่อผลิตผลิตภัณฑ์ในภาคอุตสาหกรรมที่หลากหลาย ผู้วิจัยสนใจศึกษาสภาวะการผลิตเอนไซม์เซลลูเลสและไซลาเนสจากเชื้อรา *Lentinus polychrous* Lev. LP-PT-1 ภายใต้สภาวะการหมักแบบแห้งโดยใช้ฟางข้าวเป็นวัสดุหมัก นอกจากนี้ได้ศึกษาปัจจัยด้านอุณหภูมิ ค่าความเป็นกรดด่างที่มีผลต่อกิจกรรมของเอนไซม์ และคุณสมบัติความเสถียรของเอนไซม์ รวมทั้งการศึกษาเอนไซม์ที่ทำให้บริสุทธิ์บางส่วนและศึกษาน้ำหนักโมเลกุลของเอนไซม์ การศึกษาสภาวะการผลิตเอนไซม์เซลลูเลสและไซลาเนสจากเชื้อรา *L. polychrous* Lev. LP-PT-1 ภายใต้สภาวะการหมักแบบแห้ง พบว่า อุณหภูมิที่เหมาะสมของการผลิตเอนไซม์ไซลาเนสและเซลลูเลสคือ 35 องศาเซลเซียส การปรับความชื้นเริ่มต้นร้อยละ 75 และ 80 ส่งผลต่อการผลิตเอนไซม์เซลลูเลสได้สูงกว่าการปรับความชื้นเริ่มต้นอื่นอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) และการปรับความชื้นเริ่มต้น ร้อยละ 75 และ 80 ส่งผลต่อการผลิตเอนไซม์ไซลาเนสได้สูงกว่าการปรับความชื้นเริ่มต้น ร้อยละ 50 และ 55 อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ส่วนปริมาณเชื้อราเริ่มต้นที่เหมาะสมต่อการผลิตเอนไซม์ไซลาเนสและเซลลูเลส คือ ปริมาณเชื้อราเริ่มต้นที่มีขนาดเส้นผ่าศูนย์กลาง 0.5 เซนติเมตร จำนวน 4 ชิ้นวุ้นต่อวัสดุหมัก 4 กรัม การศึกษาคุณสมบัติความเสถียรของเอนไซม์ไซลาเนสและเซลลูเลสที่ผลิตโดยเชื้อรา *L. polychrous* Lev. LP-PT-1 ด้วยการสกัดเอนไซม์สกัดหยาบโดยใช้ McIlvaine buffer pH 5.0 จากนั้นนำไปบ่มเขย่าที่ความเร็วรอบ 150 รอบต่อนาที เป็นเวลา 30 นาที จากการศึกษาพบว่า เอนไซม์ไซลาเนสและเซลลูเลสมีความเสถียรดีที่อุณหภูมิสูงถึง 60 องศาเซลเซียส ซึ่งเอนไซม์ทั้งสองชนิดเหลือค่ากิจกรรมของ

เอนไซม์ ร้อยละ 89 และ 81 ตามลำดับ การศึกษาค่าความเป็นกรดด่างที่มีผลต่อคุณสมบัติความเสถียรของเอนไซม์นั้น พบว่า เอนไซม์เซลลูเลสมีความเสถียรร้อยละ 94 ในสภาวะ pH 4.0-5.0 ส่วนเอนไซม์ไซลาเนสมีความเสถียรดีในสภาวะค่าความเป็นกรดด่างช่วงกว้างระหว่าง pH 4.0–7.0 การศึกษาปัจจัยด้านอุณหภูมิและค่าความเป็นกรดด่างที่มีผลต่อกิจกรรมของเอนไซม์ไซลาเนสและเซลลูเลส พบว่า เอนไซม์เซลลูเลสมีกิจกรรมดีที่อุณหภูมิ 30-60 องศาเซลเซียส ส่วนกิจกรรมของเอนไซม์ไซลาเนสมีกิจกรรมสูงขึ้นเมื่ออุณหภูมิสูงขึ้นถึง 60 องศาเซลเซียส นอกจากนี้สภาวะค่าความเป็นกรดด่างที่เหมาะสมต่อกิจกรรมของเอนไซม์เซลลูเลสและไซลาเนส คือ pH 4.0-6.0 ส่วนการศึกษาเอนไซม์ที่ทำให้บริสุทธิ์บางส่วนโดยวิธีการตกตะกอนด้วยแอมโมเนียมซัลเฟต ร้อยละ 20-40 และได้ศึกษาน้ำหนักโมเลกุลของเอนไซม์โดยการวิเคราะห์ด้วยวิธี SDS-PAGE พบแถบโปรตีนทั้งหมด 3 หน่วยย่อย คือ 45 kDa, 40 kDa และ 35 kDa ผลการศึกษาในครั้งนี้แสดงให้เห็นว่าคุณสมบัติของเอนไซม์ที่ผลิตจากเชื้อรา *L. polychrous* Lev. LP-PT-1 มีความเสถียรของเอนไซม์ไซลาเนสและเซลลูเลสสูงเหมาะสมในการนำไปประยุกต์ใช้เพื่อประโยชน์ต่อกระบวนการทางเทคโนโลยีชีวภาพในระดับอุตสาหกรรมต่อไป

# ABSTRACT

TITLE : CHARACTERIZATION OF CELLULASE AND XYLANASE FROM *Lentinus polychrous* Lev. LP-PT-1

BY : PANADDA PETCHLUAN

DEGREE : DOCTOR OF PHILOSOPHY

MAJOR : BIOTECHNOLOGY

CHAIR : ASST. PROF. NAREERAT CHAIKONG, Ph.D

KEYWORDS : CELLULASE / XYLANASE / *Lentinus polychrous* Lev.

Biotechnology, a knowledge of science and technology is interesting for the scientists around the world. Biotechnological processing uses enzymes and microorganisms to make products in a wide range of industrial sectors. The culture conditions of cellulase and xylanase production by *Lentinus polychrous* Lev. LP-PT-1, under solid state fermentation (SSF) economical using rice straw as substrates, were investigated. The optimum temperature and pH of enzyme activities and stabilities were examined. Partial purification of enzyme and molecular mass determination were investigated. The xylanase and cellulase production from *Lentinus polychrous* Lev.LP-PT-1 under solid state fermentation indicated that the optimal temperature of xylanase and cellulase production were found to be at 35°C. The 75% and 80% moisture levels showed significantly higher cellulase production than other treatments ($p < 0.05$). The 75% and 80% moisture levels showed significantly higher xylanase production than 50% and 55% moisture levels ($p < 0.05$). The inoculum level at 0.5 cm per disk for the four disks in 4 grams substrate culture was found to be optimum for xylanase and cellulase production. The stability of xylanase and cellulase from *L. polychrous* Lev. LP-PT-1 was determined. Crude enzymes were extracted by McIlvaine buffer pH 5.0 at 150 rpm for 30 minutes. The relatively good thermostability of xylanase and cellulase was maintained at high temperature at 60°C with 89% and 81% of the remaining activity. The cellulase was stable at pH 4.0-5.0 with 94% remaining activity. The xylanase appeared to be stable over a broad pH range of 4.0–7.0. High cellulase activity was achieved at temperatures between 30 and 60°C. The xylanase activity increased with high

temperature to 60°C. The optimum pHs for cellulase and xylanase activity were found in the range of pH 4.0-6.0. The partial purification was achieved using 20-40% ammonium sulfate precipitation. The results from SDS polyacrylamide gel electrophoresis (SDS-PAGE) showed that the partial purified enzyme had 3 subunits with their molecular weight as following 45 kDa, 40 kDa and 35 kDa, respectively. The present findings revealed that the high thermostability of cellulase and xylanase from *Lentinus polychrous* Lev. LP-PT-1 would be potentially more beneficial in the industrial biotechnological applications.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| SSF | Solid state fermentation |
| Smf | Submerged fermentation |
| °C | Degree celsius |
| d | day |
| g | Gram |
| mg | Milligram |
| kg | Kilogram |
| Tg | Teragrams |
| min | minute |
| rpm | Revolutions per minute |
| h | Hour |
| L | Liter |
| ml | Milliliter |
| nm | Nanometer |
| µm | Micrometer |
| mm | Millimeter |
| cm | Centimeter |
| mg/ml | Milligram per milliliter |
| mg/L | Milligram per liter |
| M | Molarity |
| mM | Millimolarity |
| % (w/v) | Percentage weight by volume |
| % (w/w) | Percentage weight by weight |
| % (v/v) | Percentage volume by volume |
| U | Unit |
| µmol | Micromole |
| gds | Gram dry substrate |
| et al | et alia (and others) |

## LIST OF ABBREVIATIONS (CONTINUED)

| | |
|---|---|
| PDA | Potato dextrose agar |
| ANOVA | Analysis of variance |

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Biotechnological processing uses enzymes and microorganisms to make the valuable products in a wide range of industrial sectors. Enzyme production is a growing field of biotechnology.

The organisms able to degrade efficiently the major components of lignocellulosic materials, cellulose, hemicellulose and lignin, are white rot fungi. These fungi possess hydrolytic enzymes like cellulases and xylanases, which typically are induced by their substrates.

The hydrolytic enzymes have attracted considerable research interest, therefore, organisms able to produce them are interesting in view of the potential importance in industrial processes like hydrolysis of lignocellulose to fermentable sugars for fuel ethanol production, bread making and clarification of beer and juices, wine industry, improving the nutritive quality of animal feed, food industries, bioremediation, biobleaching of pulp paper, degradation and detoxification of recalcitrant substances or in the food industry, thus the efficient production of these enzymes in a low-cost medium is interesting for biotechnological applications (Papinutti and Forchiassin, 2007; Katapodis et al., 2007; Sherief et al., 2010).

Rice straw is an rice by-product, the dry stalk of a cereal plant, after the grain or seed has been removed. Rice straw is one of the abundant lignocellulosic waste materials in the world. The annual global production of dry rice is about 526 Tg. Asia is the primary production region with over 90% of global production and the largest harvested area for rice (Kim and Dale, 2004). In many countries, including the Thailand, rice straw is an abundant by-product from rice production. Every kilogram of grain harvested is accompanied by production of 1–1.5 kg of the straw (Maiorella, 1985). Therefore, rice straw shows promise for use as available substrate for the biotechnology application.

Moreover, thermal stability enzymes are better suited for industrial processing of polysaccharides because of their increased activities and stabilities at high temperatures (Katapodis et al., 2007).

The purpose of this work is to investigate the characterization of cellulase and xylanase produced by *Lentinus polychrous* Lev. LP-PT-1. In this research, *Lentinus polychrous* Lev. LP-PT-1 was used to produce enzymes under solid state fermentation. Rice straw, the available and low cost material, was used as substrate. The culture conditions, including temperature, moisture content and inoculum size were studied. The optimum temperature and pH of enzyme activities, stabilities and partial purification enzyme were examined. All the fermentations were carried out in triplicates.

## 1.2 Objectives of the thesis

1.2.1 To investigate the culture condition for cellulase and xylanase production from *Lentinus polychrous* Lev. LP-PT-1

1.2.2 To investigate the cellulase and xylanase characterization from *Lentinus polychrous* Lev. LP-PT-1

## 1.3 Outline of the thesis

Overview on *Lentinus polychrous* Lev. as potential lignocellulolytic producer, industrial applications of enzymes, rice straw as lignocellulosic waste material, enzyme production, factors affect enzyme production process and factors affect enzyme reaction are reviewed in Chapter 2.

The ethanol production from lignocellulosic waste material was determined in Chapter 3.

The culture conditions for the cellulase and xylanase production under solid state fermentation, including temperature, moisture contents and inoculum size were determined. The characterization of enzymes, the effect of pH and temperature on the activity and stability were examined. Partial purification of enzyme and molecular mass determination were investigated in Chapter 4.

The general conclusion and recommendation for further research were described in Chapter 5.

## 1.4 Practical applications

1.4.1 Potential application of thermal stability of enzymes and the characterization for biotechnological processing

1.4.2 Cellulase and xylanase production under solid state fermentation

1.4.3 The economics of cellulase and xylanase production with using agricultural waste, low cost, available, abundant feedstock as substrate and the reduction of enzyme cost via biological processes.

1.4.4 Value added of rice straw.

1.4.5 Environmental friendly processes.

## 1.5 Scope of the study

This thesis is the preliminary study of laboratory scale.

### 1.5.1 The culture conditions

The culture condition for xylanase and cellulase production by the tropical white rot fungus, *Lentinus polychrous* Lev. LP-PT-1 cultured on rice straw as a main substrate under solid state fermentation, the effect of different conditions including the incubation temperature and moisture content and inoculum size.

### 1.5.2 The enzyme characterization

The determination of the optimal temperature and pH for crude enzyme activities, stabilities and partial purification enzyme.

## 1.6 Definition of Terms

1.6.1 Lignocellulosic waste material refer to the by product of plant biomass or residue, that composed of cellulose, hemicellulose and lignin.

1.6.2 Solid state fermentation is process that occurs in the absence or near absence of any fluid in the space between particles. In this system, the substrate is moistened with a thin layer of water on the surface of the substrate.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

Lignocellulosic material, the most abundant renewable biomass on earth, available, and low cost, composed mainly of cellulose fibers embedded tightly within hemicellulose and lignin. Both of cellulose and hemicellulose fractions are polymers of sugars and thereby a potential source of fermentable sugars.

In hydrolysis, the cellulosic part of the materials is converted to sugars. Cellulose can be hydrolyzed to glucose by cellulase enzymes. Cellulases represent a large proportion of the industrial enzymes. They are used in textile, detergent, beverage, juice extraction, animal feed and pulp and paper industries. Currently they are the third largest industrial enzymes being sold worldwide. However, there is also a fast growing market for their use in the biofuel industry and they are predicted to become the largest volume industrial enzyme.

Biotechnological uses and potential applications of xylanase include bioconversion of lignocellulosic material to useful product, clarification of juices, improvement of the consistency of the beer and the digestibility of animal feedstock. One of the major potential applications of xylanase involves the pulp and paper industry.

In this chapter, the overview on *Lentinus polychrous* Lev. as potential lignocellulolytic producer, industrial applications of enzymes, rice straw as lignocellulosic waste material, enzyme production, factors affect enzyme production process and factors affect enzyme reaction are reviewed in Chapter 2.

## 2.2 Overview on *Lentinus polychrous* Lev. as potential lignocellulolytic producer

*Lentinus polychrous* Lev. (also called as *Lentinus praerigidus*, *Panus polychrous*, *Lentinus estriatus*, *Pocillaria eximia*, *Pocillaria estriata*, *Pocillaria polychroa*, *Panus polychrous*, *Lentinus eximius*, *Pocillaria praerigida*) is edible muchroom that their ability to wood degrading capability. It occurs throughout United States, Panama, India, North and North Eastern in Thailand.

*Lentinus polychrous* Lev., known as Kradang mushroom, is recognized as one of the widely commercial cultivated edible mushroom of the North and Northeast of Thailand. It considered being nutritious containing the quantities of protein, fat, and carbohydrate. Moreover, it contains the content of the important elements such as sodium, potassium, calcium, iron, magnesium and zinc.

The white rot fungus is reported about the ability to degrade lignin, hemicellulose and cellulose in nature, due to they can produce and secrete lignocellulolytic enzymes for lignocellulosic degradation and use as a carbon source.

Several studies report that lignocellulolytic enzyme production by the white-rot fungus *Lentinus polychrous* Lev. (Pukahuta et al., 2004; Pukahuta et al., 2005; Budda et al., 2012). The biotechnological potential of ligninolytic enzymes from *L. polychrous* Lev. in the textile dyeing industry, especially in the dye decolorization (Sarnthima et al., 2009; Budda et al., 2012). It has been found that this fungus is capable producer of cellulase and xylanase enzymes (Pukahuta et al., 2004; Pukahuta et al., 2005). Moreover, Thetsrimuang et al. evaluated for their antioxidant properties and cancer cell line cytotoxicity (Thetsrimuang et al., 2011).

**Taxonomy**: Kingdom Fungi
Phylum Basidiomycota
Class Agaricomycetes
Order Polyporales
Family Polyporaceae
Genus *Lentinus*
Species *Lentinus polychrous*

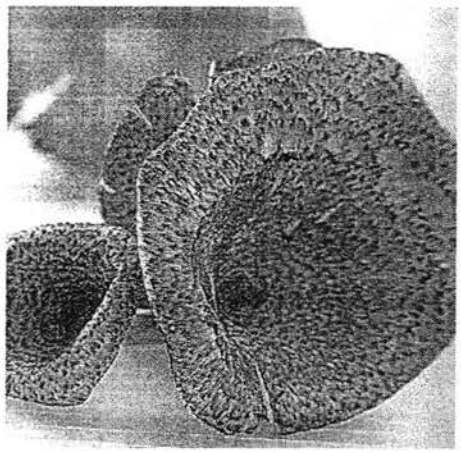


**Figure 2.1** *Lentinus polychrous* Lev. (khaomak.com and Khaomak Mushroom, 2012)

## 2.3 Industrial applications of enzymes produced by solid state fermentation processes

The industrial applications of enzymes produced by solid state fermentation processes as shown in table 2.1

**Table 2.1** Industrial applications of enzymes produced by solid state fermentation processes (Pandey et al, 2000)

| Process | Enzyme |
|---|---|
| Enzyme-assisted ensiling | Fungal cellulase and hemicellulases |
| Bioprocessing of crop and crop residues | Fungal cellulase and hemicellulases |
| Fibre processing (retting) | Fungal pectinases, cellulase and hemicellulases |
| Biopulping | Xylanases |
| Directed composting | Hydrolytic enzymes |
| Soil bioremediation | Laccases, ligninases |
| Post-harvest residue decomposition | *Trichoderma harzianum* cellulase |
| Biopesticide | *T. harzianum* cellulase for helper function |

### 2.3.1 Cellulose degrading enzymes

Cellulases are the group of hydrolytic enzymes that are capable of hydrolysing insoluble cellulose to produce soluble oligosaccharides. At least three fundamental enzyme activities are traditionally found within a cellulase system that demonstrates the degradation of cellulose.

The main technological application of cellulases lies in the conversion of cellulosic fractions from various cellulose containing materials into glucose. Glucose evolved as part of this breakdown can then be further utilized for production of alcohol or for other industrial process.

#### 2.3.1.1 Cellulose hydrolysis

Enzymatic hydrolysis of cellulose is carried out by cellulose enzymes which are highly specific (Beguin and Aubert, 1994). The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions and does not have a corrosion problem (Duff and Murray, 1996). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol (Sun and Cheng, 2002).

The cellulose molecules are composed of long chains of sugar molecules. In the hydrolysis process, these chains are broken down to free the sugar. There are three major cellulose hydrolysis (cellulolysis) processes.

Cellulase is most often employed to hydrolyze cellulose to glucose because of its mild reaction condition and specific action in hydrolysis, producing virtually no glucose degradation products (Kuo and Lee, 2009).

#### 2.3.1.2 Mechanism of cellulose hydrolysis

Cellulose is an insoluble, crystalline polymer of β-1,4-linked cellobiose. In the cell walls of higher plants, it often forms a long bundle or fibril structure embedded into a matrix of other polysaccharides and lignin. Degradation of cellulose is a slow process that requires at least three types of enzymatic activities, the endoglucanase, which randomly breaks the internal β-1,4-glucosidic bonds and creates broken chain ends, and the exoglucanase, which cleaves the

polymer from the ends to produce cellobiose. Finally, the beta-glucosidase hydrolyzes cellobiose to glucose, one of the most fermentable sugars obtainable from cell walls.

Endoglucanase attacks more or less randomly at sites within 1-4-β-D-glucan chains in amorphous regions of cellulose or at the surface of microfibrils. Exoglucanase releases cellobiose from non-reducing ends of β-D-glucan chains. Cellobiase hydrolyzes cellobiose and cellodextrins to glucose.

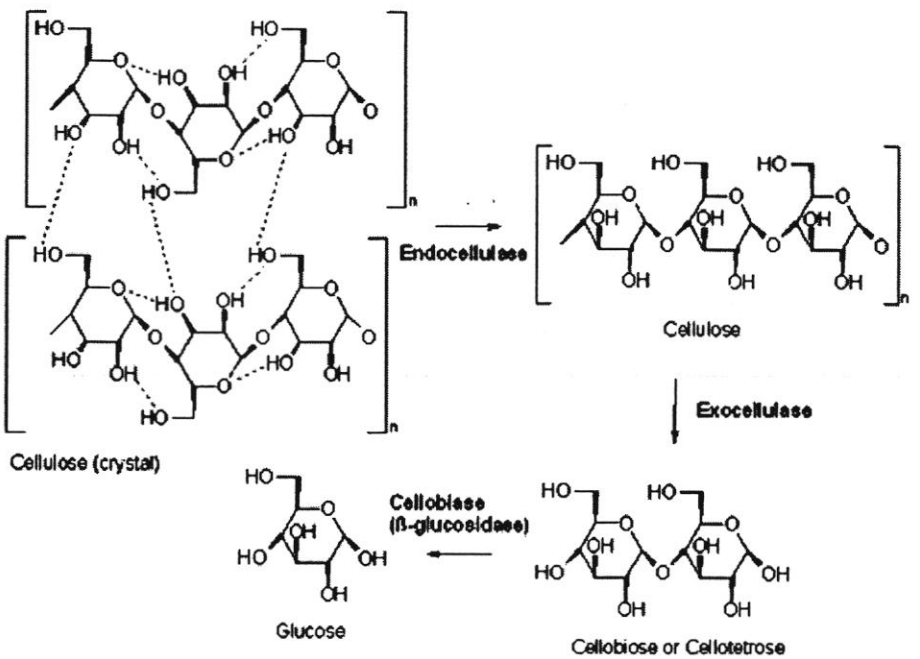



**Figure 2.2** The cellulases reaction (Wikipedia, 2012(b))

### 2.3.2 Xylanase degrading enzymes

Biodegradation of xylan, requires action of several enzymes, among which xylanase plays a key role. Xylanase (E.C. 3.2.1.8.) belong to the pentosanases a group of enzymes that break down components of the cell wall matrix of plants (fiber).

Xylanase (EC 3.2.1.8), an extracellular enzyme is the key enzyme for hemicellulose degradation to break down of xylan, which is a polysaccharide in the plant cell wall. Xylanase cleaves internal glycosidic bonds at random or at specific positions of the xylan backbone into small oligomers and xylose.

The hemicellulytic system involves among others endo-1,4-β-D-xylanase (EC 3.2.1.8), which cleaves internal bonds in the xylan chain; β-xylosidases (EC 3.2.1.37), which cleaves xylooligosaccharides to produce xylose; endo-1,4- β -D-mannanase (EC 3.2.1.78), which cleaves internal bonds; β-mannosidases (EC 3.2.1.25) which cleaves mannooligosaccharides to mannose (Heidorne et al., 2006).

Xylanases were produced by *Pleurotus* strains, which degraded oat-spelt xylan into simple sugars xylose, arabinose etc. Xylan degradation by *Pleurotus* sp. BCCB068 reached 75.1%. The xylan degradation exhibited by these white rot fungi indicates they have potential for use in biotechnological processes related to degradation of hemicellulose sources (Menezes et al., 2009).

## 2.4 Rice straw as lignocellulosic waste material

Lignocellulosic materials consist primarily of three components, namely cellulose, hemicellulose and lignin. Rice straw contains approximately 30–40% w/w cellulose, 20–34% w/w hemicellulose in close association with 9–15% w/w lignin (Kaur et al., 1998; Jin and Chen, 2006; Zhu et al., 2006; Zhang and Cai, 2008). Rice straw is one of the abundant lignocellulosic waste materials in the world. Rice straw is an rice by-product, the dry stalk of a cereal plant, after the grain or seed has been removed. The annual global production of dry rice is about 526 Tg. Asia is the primary production region with over 90% of global production and the largest harvested area for rice (Kim and Dale, 2004). The use of straw as a carbon-neutral energy source is increasing rapidly, especially for bioethanol.

### 2.4.1 Cellulose

Cellulose is made up of long chains of the 6-carbon sugars. It is formed by glucose units, linked by β-1, 4 glycosidic linkages, arranged in bundles (Fig 2.1-2.3). Cellulose consists of 7,000 to 15,000 glucose molecules in the average cellulose polymer. It is the most abundant carbohydrate in nature. The long fibers of cellulose are held together by intermolecular hydrogen bonds (Fig 2.3). Hydrogen bonding continues in the same plane with other chains as well as in planes above and below this plane to form strong, fibrous bundles. In the plant-cell wall, the cellulose molecules are interlinked by another molecule, hemicellulose.

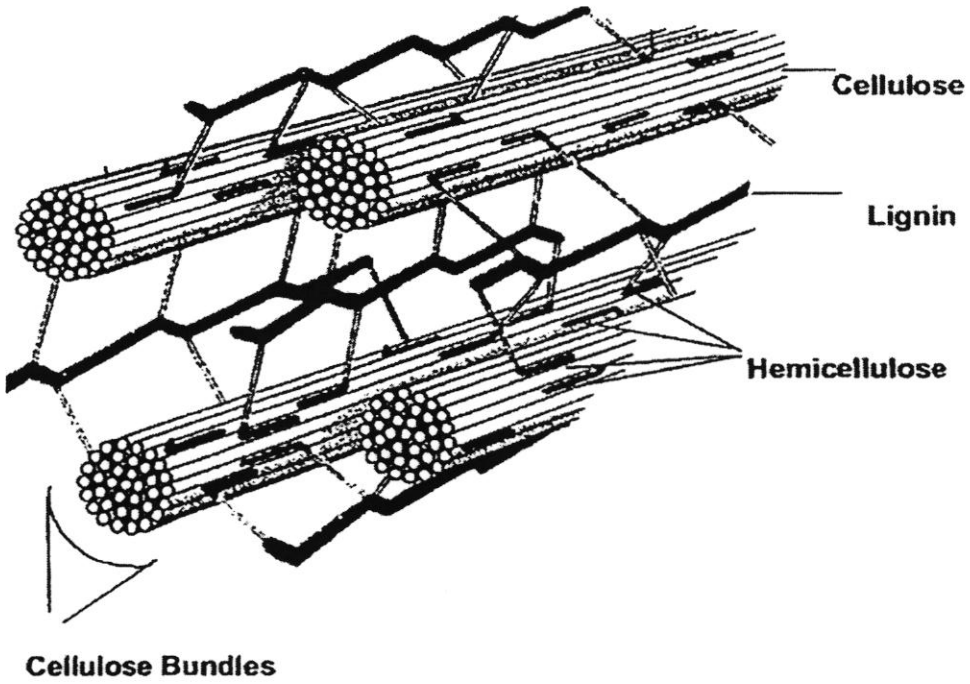



**Figure 2.3** The plant cell wall and compositions compose of cellulose, hemicellulose and lignin. (Modified Khandeparker and Numan, 2008)

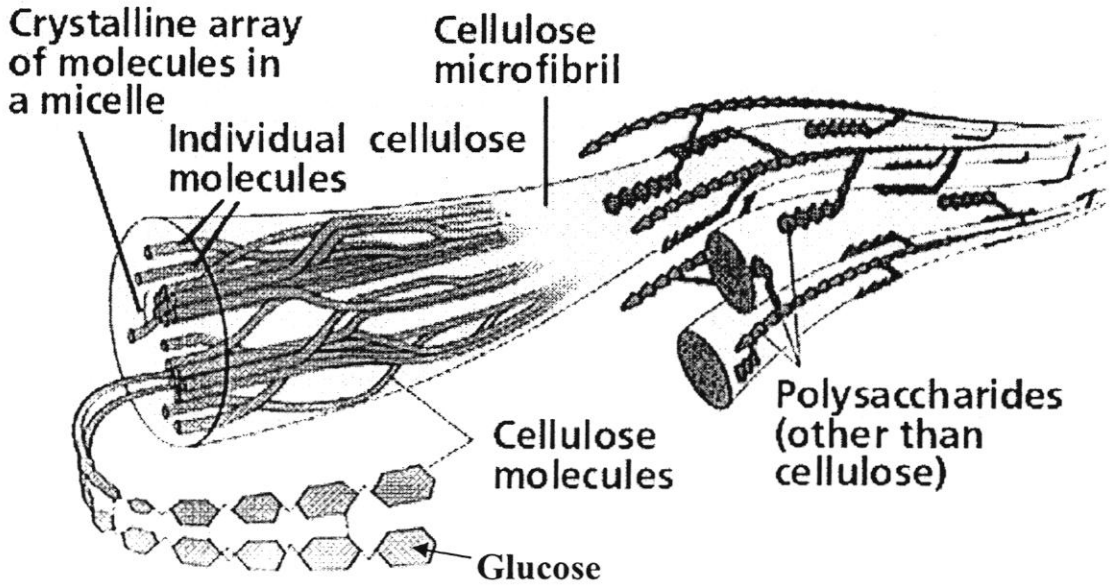



**Figure 2.4** Cellulose microfibril (Farabee, Michael, 2012)

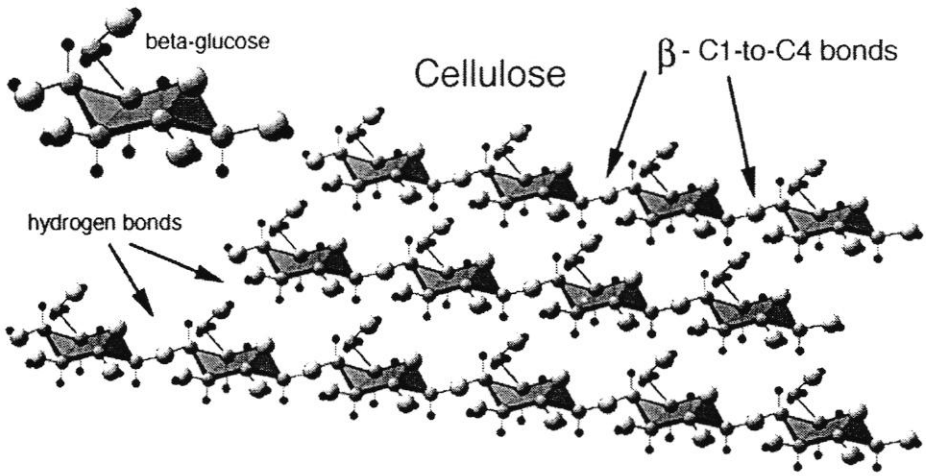



**Figure 2.5** The long unbranched chains of cellulose are held together by hydrogen bonds. (Blamire, John, 2012)

### 2.4.2 Hemicellulose

Hemicelluloses contain most of the pentose sugar and consists of shorter chains - around 500–3,000 sugar units. Xylose is always the sugar monomer present in the largest amount. The hemicellulose is derived from several sugars in addition to glucose, including especially xylose but also mannose, galactose, rhamnose, and arabinose. Furthermore, hemicellulose is branched (Fig 2.6), whereas cellulose is unbranched.

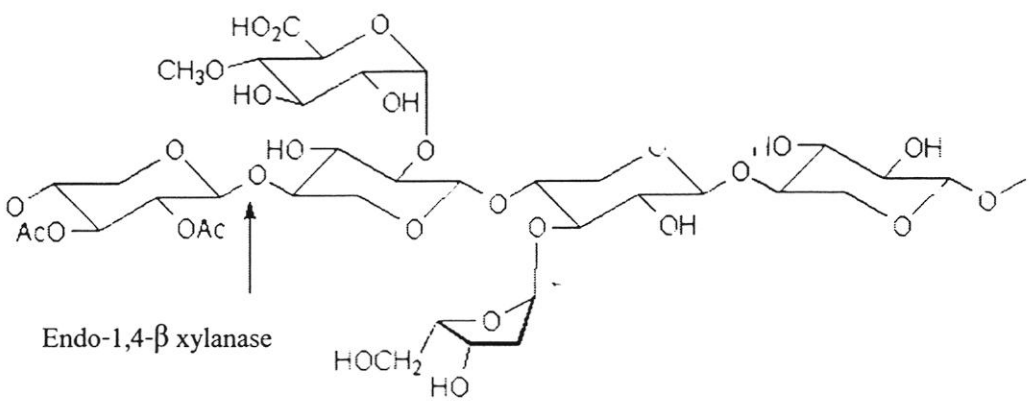



**Figure 2.6** Hemicellulose structure (Department of Chemistry, University of Maine, 2012)

### 2.4.3 Lignin

Another compound called lignin, which is a set of non-sugar molecules acting like a glue to hold the biomass molecules together and gives the plant its structural strength (Murphy and McCarthy, 2005). Lignin fills the spaces in the cell wall between cellulose, hemicellulose, and pectin components, especially in tracheids, sclereids and xylem. The lignin molecule formed by the free radical, oxidative condensation of the three monomers, coniferyl alcohol, sinapyl alcohol and coumaryl alcohol, the formulae for which are shown in Fig. 2.7.

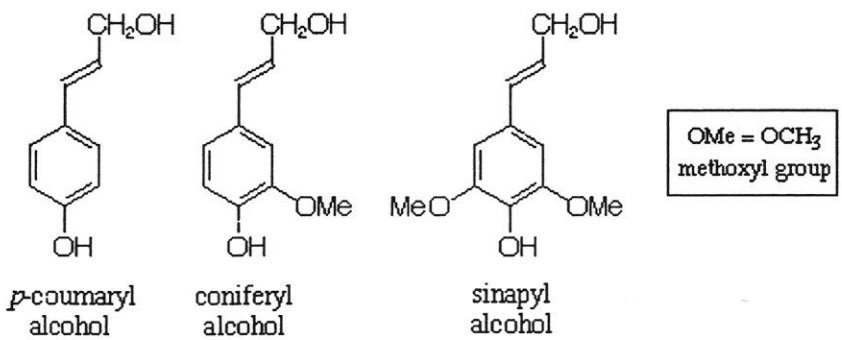



**Figure 2.7** The main components of lignin ; *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. (Helm, Richard, 2012)

## 2.5 Enzyme production

Enzyme production is a growing field of biotechnology. Most enzyme manufacturers produce enzyme using submerged fermentation techniques (Papinutti and Forchiassin, 2007). There is, however, a significant interest in using solid-state fermentation techniques to produce a wide variety of compounds such as antibiotics (Adinarayana et al., 2003), organic acids (Kumar et al., 2003) and a wide variety of enzymes (Pandey et al., 1999; Papinutti and Forchiassin, 2007). A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes. The recent examples of cellulase and xylanase productions under solid state and submerged fermentation as shown in table 2.2.

**Table 2.2** Recent examples of cellulase and xylanase productions under solid state and submerged fermentation

| Substrate | Microorganism | Enzymes | References |
|---|---|---|---|
| wheat straw | *Phlebia floridensis* | Laccase, Manganese peroxidase CMCase, xylanase (SSF) | Sharma and Arora., 2010 |
| wheat straw | *Aspergillus fumigatus* | crude endoglucanase (SSF and SmF) | Saqib et al., 2010 |
| corncobs and wheat bran | *Pleurotus ostreatus* | Xylanase (Smf) | Qinnghe et al., 2004 |
| Rice bran in basal medium | *Scopulariopsis acremonium* | cellulase and xylanase (Smf) | Sarao et al., 2010 |

Mullai et al. (2010) investigated xylanase production by *Bacillus* species 2129 under submerged cultivation. The result showed 2.388 U/ml. While Virupakshi et al. shows *Bacillus* sp. JB-99 produced xylanase in solid-state fermentation (3644U/g DBB) with bran moistened with mineral salt solution at 1:2.0 (w/v) at 50°C for 72 h. Yeast extract, beef extract and xylan enhanced enzyme production, while glucose, lactose and fructose strongly repressed the production process (Virupakshi et al., 2005). Archana and Satyanarayana reported that the enzyme production of *Bacillus licheniformis* A99 was 22 fold higher in solid state fermentation than in submerged fermentation (Archana and Satyanarayana, 1997). Solid state fermentation was found to be more productive than submerged fermentation interm of exoglucase by *Bacillus subtilis* (Shifique et al., 2004).

All of these hydrolytic enzymes have attracted considerable research interest, therefore, organisms able to produce them are interesting in view of the potential importance in industrial processes like hydrolysis of lignocellulose to fermentable sugars for fuel ethanol production, bread making and clarification of beer and juices, wine industry, improving the nutritive quality of animal feed, food industries, bioremediation, biobleaching of pulp paper,

degradation and detoxification of recalcitrant substances or in the food industry, thus the efficient production of these enzymes in a low-cost medium is interesting for biotechnological applications (Haq et al., 2004; Qinnghe et al., 2004; Papinutti and Forchiassin, 2007; Katapodis et al., 2007).

The white-rot fungi *Lentinus polychrous* is a widely distributed wood rot fungus of considerable interest, a producer of lignocellulolytic enzymes, including xylanase, cellulase (Pukahuta et al., 2004), laccase (Pukahuta et al., 2004; Sarnthima et al., 2009), Manganese peroxidase (MnP) and manganese-independent peroxidase(MIP) (Sarnthima et al., 2009). Enzyme types were secreted by *L. polychrous* that was known for applications in wastewater treatment from the textile industry, which contains synthetic dyes (Pukahuta et al., 2004; Sarnthima et al., 2009).

Pukahuta et al. studied enzyme production from *Lentinus* spp. that could produced combination xylanase, cellulase and laccase (Pukahuta et al., 2004). Sarntima et al. studied the laccase ligninolytic enzyme production by *L. polychrous* Lev. was the highet laccase activity on rice bran was 1449 U/L after 21 days of culture under solid state fermentation (Sarntima et al., 2009). Xylanase and cellulase were produced by *L. polychrous* when grown on potato dextrose broth (PDB) and Fahraeus broth with 2 % rice straw (FRS) (Pukahuta et al., 2004) or rice hust and rice straw (Pukahuta et al., 2005).

Prasertsan et al. investigated optimization of enzyme production from *Aspergillus niger* ATCC 6275 under both submerged and solid-substrate cultivation using palm oil mill as substrate resulted submerged cultivation revealed that pretreatment of ground palm cake did not improve enzyme production. Under solid substrate cultivation, the results indicated that optimal initial moisture contents for xylanase and CMCase activities were 60%, with temperature optima of 30 °C and 35 °C. The optimal inoculum size was 1 x $10^8$ spores/g palm cake with an initial pH of 4.5-5.0. The maximum activities of xylanase and CMCase were 282.9 U/g , 23.8 U/g, respectively. Solid substrate cultivation was a better method for the production of enzyme, particularly xylanase, from *A. niger* ATCC 6275 (Prasertsan et al., 1997).

The fungi, *Thermomyces lanuginosus* strain SSBP produced xylanase activity when cultivated on a medium containing corn cobs as substrate and yeast extract as nitrogen source (Singh et al., 2000.). Xylanase was observed after 96 h at 35°C and pH 6 in 1% oat spelt xylan (35 U/ml) by *Aspergillus terreus* (Chidi et al., 2008). The enzyme produced under solid state

fermentation in which the ratio of solid wheat bran to water (12.5% w/v solid) gave higher enzyme activity than submerged fermentation (1% w/v solid) *A. niger* and *T. reesei*, both fungi preferred solid media than media with too much water for their growth (Widjaja et al., 2009).

Xylanases from thermophilic microorganisms are better suited for industrial processing of polysaccharides because of their increased activities and stabilities at high temperatures (Yang et al., 2006; Katapodis et al., 2007).

The optimum temperature for xylanase activity was between 50-60°C (Carmona et al., 1998; Liu et al., 1998; Georis et al., 2000; Bakir et al., 2001; Ohta et al., 2001; Sá-Pereira et al., 2002; Saha., 2002; Martínez-Trujillo et al., 2003; Wang et al., 2003) as Belfaquih et al. (Belfaquih et al., 2002) who reported that the optimum temperature for xylanase activity in *Streptomyces achromogeness* 5028 (S1), *S. longisporus* ruber 4-167 (S2) and *Streptomyces* sp. 8812 (S3) was between 60°C and 65°C. Similar findings were reported by Rawashdeh *et al.* (Rawashdeh et al., 2005), who studied the optimum temperature for xylanase activity by *Streptomyces* sp. (strain Ib 24D) was 60°C. The most active temperature of xylanase from white-rot fungus *Ceriporiopsis subvermispora* was 60°C (Milagres et al., 2005). And therefore similar to the range observed the optimal temperature for crude xylanase activity by *Aspergillus carneus* M34 was 60°C (Fang et al., 2007). The researchers considered to carry out the enzyme production and characterization from the microorganism for industrial application.


## 2.6. Factors affect enzyme production process

### 2.6.1 Fermentation process

Solid state fermentations (SSF) constitute a convenient way to produce value added products (Pandey et al., 2000; Soccol and Vandenberghe, 2003). In particular, for the production of enzymes, solid-state fermentations present several advantages when compared with traditional submerged fermentation processes (Archana and Satyanarayana, 1997; Widjaja et al., 2009; Saqib et al., 2010). Solid state fermentations produce more stable products and offer higher productivities with lower water, energy and sterility demands. Smaller fermenters and easier downstream processing measures are required (Robinson et al., 2001). Additionally, low-cost agro-industrial by-products and crop residues can be used as substrates (Pandey et al., 1999).

### 2.6.2 Temperature

In general, the fungi can grow well between 15°C and 40°C but high enzyme production rate is generally obtained within an optimal temperature range between 25°C and 35°C (Biswas et al., 1990; Sohail et al., 2009). The metabolism of white rot fungus generates heat and causes temperature gradients in solid state cultivation. The accumulated heat can kill or inhibit the fungal growth and metabolism. Therefore, in the scale-up of solid state cultivation, heat dissipation is one of the key factors that needs to be taken into account in the bioreactor design.

### 2.6.3 Moisture content

In solid-state fermentations, the moisture content plays the most important role because it is strongly related to mass, heat, and gas transfer. For fungi, 30% to 80% moisture is usually used (Singhania et al., 2009; Shi et al., 2008; Taniguchi et al., 2005; Oriol et al., 1988). Outside of this range, the growth of these microorganisms can be strongly limited. High moisture contents usually decrease the substrate porosity and reduce oxygen transfer. Low moisture contents may limit the bioavailability of nutrients. The nature of the solid substrate also plays an important role in enzyme productivity.

Initial moisture content of the substrate is important to the fungal growth in solid state fermentation. Asgher et al. (2006) investigated the solid state cultivation of *P. chrysoporium* on corn cobs with a moisture content ranging from 40% and 90%. The highest ligninase activity was obtained at 70% moisture content. Thus, the optimal moisture content appeared to be 75-85%. In general, high moisture content can provide adequate water for supporting vigorous fungal growth and active metabolic function. However, too much moisture reduces interparticle spaces and substrate porosity in solid state fermentation, which in turn decreases oxygen diffusion and inhibits aerobic cultures (Singhania et al., 2009).

### 2.6.4 Inoculum

Initial microbial load to a medium does affect the growth and in turn metabolite production. Inoculum for solid state fermentation can be prepared by different methods, e.g., mycelium grown in liquid or agar medium, spawn grown in cereal grains, or fungal-precolonized substrate (Reid et al., 1989). *P. chrysosporium* yields spores which is a convenient inoculum and can be mixed evenly with the substrate. In contrast, most white rot basidiomycetes do not produce

spores. Instead, the precolonized lignocellulosic materials in the reactor can also serve as inoculum and the fresh substrate can be fed to partially replace fermented materials.

Qinnghe *et al.* reported xylanase production with the inoculum level at 0.5 cm per disk for the four disks in 50ml liquid culture (corn cob 2.5%+wheat bran 2.5%), by *P. ostreatus* was found to be optimum for xylanase (Qinnghe et al., 2004). Similar, five agar plug (0.8cm) of *P. ostreatus* gave maximum laccase yield 3952 $Ug^{-1}$ using wheat straw as substrate (Patel et al., 2009)

Other Fungi, the enzyme production depend on inoculated by spore. The effect of inoculum size based on the number of spores was examined using the spore concentration on the production of fungal enzymes.

Sarao et al. investigated cellulase and xylanase production under submerged fermentation. It was found that the increase in inoculum size resulted in rapid increase in enzyme production (Sarao et al., 2010). Similarly, Kavya and Padmavathi examined xylanase production by *Aspergillus niger* under solid state fermentation, which indicated at the lower inoculum sizes, it was observed that the time taken to achieve maximum growth or enzyme production was much longer (Kavya and Padmavathi, 2009). While, Reddy and Kumar indicate that further increase in the inoculum level did not increase the tannase production (Reddy and Kumar, 2011).

Higher enzyme production at higher inoculum is related to the rapid growth of the fungus, which resulted in higher degradation of the substrate and increases available of nutrients (Sarao et al., 2010; Kavya and Padmavathi, 2009).

### 2.6.5 Substrate

The nature of the solid substrate also plays an important role in enzyme productivity. The screening of several agro-industrial residues is usually needed to select the most appropriate substrate. Their cost and availability are also factors to be considered when choosing a substrate.

Agro-industrial residues are generally considered the best substrates for the production of enzymes under solid state fermentation processes. A number of such substrates have been employed for the cultivation of microorganisms to produce the variety of enzymes. Some of the substrates that have been used included sugar cane bagasse, wheat bran, rice bran,

wheat straw, rice straw, rice husk, corncobs, palm oil mill waste, aspen pulp, sweet sorghum pulp, apple pomace, starch. However holds the key, and has most commonly been used, in various processes.

### 2.6.6 Microorganisms

A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes. Selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. For example, it has been reported that while a strain of *Aspergillus niger* produced 19 types of enzymes, amylase was being produced by as many as 28 microbial cultures. Thus, the selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions. Generally, hydrolytic enzymes, e.g. cellulases, xylanases, pectinases, etc. are produced by fungal cultures, since such enzymes are used in nature by fungi for their growth. *Trichoderma* spp. and *Aspergillus* spp. have most widely been used for these enzymes. Amylolytic enzymes are commonly produced by filamentous fungi and the preferred strains belong to the species of *Aspergillus* and *Rhizopus*. Although commercial production of amylases is carried out using both fungal and bacterial cultures, bacterial amylase is generally preferred for starch liquefaction due to its high temperature stability. In order to achieve high productivity with less production cost, apparently, genetically modified strains would hold the key to enzyme production.

## 2.7 Factors affect enzyme reaction

The activity of an enzyme is affected by its environmental conditions. Changing these alter the rate of reaction caused by the enzyme. In nature, organisms adjust the conditions of their enzymes to produce an optimum rate of reaction, where necessary, or they may have enzymes which are adapted to function well in extreme conditions where they live.

Enzymatic degradation of plant cell wall has been extensively studied. Temperature, pH and enzyme concentration are important factors for enzyme treatment.

### 2.7.1 Temperature

The activity of enzymes is strongly affected by changes in pH and temperature. Each enzyme works best at a certain pH and temperature, its activity decreasing at values above and below that point.

Increasing temperature increases the kinetic energy that molecules possess. In a fluid, this means that there are more random collisions between molecules. Since enzymes catalyse reactions be randomly colliding with substrate molecules, increasing temperature increases the rate of reaction, forming more product. However, increasing temperature also increases the vibrational energy that molecules have, specifically in this case enzyme molecules, which puts strain on the bonds that hold them together. As temperature increases, more bonds, especially the weaker hydrogen and ionic bonds, will break as a result of this strain. Breaking bonds within the enzyme will cause the active site to change shape. This change in shape means that the active site is less complementary to the shape of the substrate, so that it is less likely to catalyse the reaction. Eventually, the enzyme will become denatured and will no longer function.

As temperature increases, more active site of enzymes molecules shapes will be less complementary to the shape of their substrate, and more enzymes will be denatured. This will decrease the rate of reaction.

In summary, as temperature increases, initially the rate of reaction will increase, because of increased kinetic energy. However, the effect of bond breaking will become greater and greater, and the rate of reaction will begin to decrease. The temperature at which the maximum rate of reaction occurs is called the enzyme's optimum temperature. This is different for different enzymes.

### 2.7.2 pH

pH measures the acidity and basicity of a solution. It is a measure of the hydrogen ion ($H^+$) concentration, and therefore a good indicator of the hydroxide ion ($OH^-$) concentration. It ranges from pH1 to pH14. Lower pH values mean higher $H^+$ concentrations and lower $OH^-$ concentrations. Acid solutions have pH values below 7, and basic solutions (alkalis are bases) have pH values above 7. Deionised water is pH7, which is termed neutral.

$H^+$ and $OH^-$ ions are charged and therefore interfere with hydrogen and ionic bonds that hold together an enzyme, since they will be attracted or repelled by the charges created

by the bonds. This interference causes a change in shape of the enzyme, and importantly, its active site.

Different enzymes have different pH values at which the bonds within them are interfered with in such a way that the shape of their active site is the most complementary to the shape of their substrate. At the pH, the rate of reaction is at an optimum, so this is the optimum pH.

Any change in pH above or below the optimum will quickly cause a decrease in the rate of reaction, since more of the enzyme molecules will have active sites whose shapes are not, or at least less, complementary to the shape of their substrate. Small changes in pH above or below the optimum do not cause a permanent change to the enzyme, since the bonds can be reformed. However, extreme changes in pH can cause enzymes to denature and permanently loose their function. Enzymes in different locations have different optimum pH values since their environmental conditions may be different.

### 2.7.3 Substrate and enzyme concentration

Increasing substrate concentration increases the rate of reaction. This is because more substrate molecules will be colliding with enzyme molecules, so more product will be formed. However, after a certain concentration, any increase will have no effect on the rate of reaction, since substrate concentration will no longer be the limiting factor. The enzymes will effectively become saturated, and will be working at their maximum possible rate.

Increasing enzyme concentration will increase the rate of reaction, as more enzymes will be colliding with substrate molecules.

### 2.7.4 Surfactant

Addition of Tween 20, a non-ionic surfactant significantly improved the hydrolysis of wheat straw by the enzymatic mixture. Glucose recovery reached 34% at 37°C and 46% at 50°C in the presence of cellulase and feruloyl esterase A after addition of Tween 20 (Tabka et al., 2006). The mechanism of surfactant activation is probably due to its adsorption on hydrophobic surfaces mainly composed of lignin fragments. Tween 20 was chosen as the surfactant as it had previously been recognised to be a good enhancer of enzymatic cellulose hydrolysis, non-toxic and suitable for biotechnical use (Tabka et al., 2006).

# CHAPTER 3

# ETHANOL PRODUCTION FROM LIGNOCELLULOSIC WASTE MATERIAL VIA BIOLOGICAL PROCESSES

## 3.1. Introduction

Lignocellulosic materials are the agricultural waste, low cost, available and abundant feedstock. Not only, the need to develop cheaper raw materials but also the reduction of enzyme cost for future ethanol production is considered essential (Guo et al., 2008; Ko et al., 2009; Klein-Marcuschamer et al., 2012).

The contribution of enzyme costs to the economics of lignocellulosic biofuel production continues to be a much debated topic. Whereas some authors argue that the cost of enzymes is a major barrier for biofuel production (Brijwani et al., 2010; Cherry and Fidantsef, 2003; Fang et al., 2009), others implicitly assume that it is not, either because they estimate the cost to be relatively low or because they assume that it will decrease with technological innovation or other advances (Aden and Foust, 2009; Schubert, 2006).

Lignocellulosic materials consist of three components, namely cellulose, hemicellulose and lignin, of which the first two can be hydrolysed to monomeric sugars. Rice straw is a widely propagated lignocellulosic waste material in the world. Rice straw contains approximately 35–40% w/w cellulose, 25–30% w/w hemicellulose in close association with 10–15% w/w lignin. The cellulose and hemicellulose can be converted to fermentable sugars by enzymatic hydrolysis process. However, lignocellulose such as rice straw is difficult to hydrolyze by cellulase due to its recalcitrant and heterogenous structure. In the enzymatic method, the lignocellulosic materials are first exposed to a pretreatment, where the cellulose structure is opened up for enzymatic attack, and depending on the hydrolysis method, the hemicellulose might be hydrolyzed to simple sugars. Xylanase and cellulase, the extracellular enzymes are the key enzyme for hemicellulose and

cellulose degradation, respectively. Bioconversion of hard lignocellulosic components by white rot fungi, are due to their ability to produce high cellulases and hemicellulases (Sherift et al., 2010). It is well known that the optimization of pretreatment parameters for each different feedstock is an important issue when enhancing the conversion efficiency during the lignocellulosic biomass-to-ethanol processes (Gupta et al., 2009).

The present research investigated the feasibility of ethanol production from lignocellulosic waste material via biological processes. The simultaneous pretreatment and saccharification by using fungal enzymes and further fermentation process into ethanol was determined.

## 3.2 Materials and methods

### 3.2.1 Lignocellulosic substrate

Rice straw was collected from rice field in Ubon Ratchathani region. The residue was cut, dried in hot air oven at 70°C and milled to pass through 16 Mesh Laboratory test sieve (Endecotts LTD. London, England) in to pieces of 0.2–1.0 cm. and then were dried at 60°C for three days before store in plastic bags.

### 3.2.2 The white rot fungus inoculation preparation

Cultures of the white-rot fungus *L. polychrous* Lev. LP-PT-1 and thermotolerant yeast UBU-1-10 were provided from Asst. Prof. Dr. Charida Pukahuta, Department of Biological Sciences, Faculty of Science, Ubon Ratchathani University. The white rot fungus *L. polychrous* Lev.LP-PT-1 was maintained on a potato dextrose agar medium at 35°C for 5-7 d. The white rot fungus was grown on PDA plate at 35 °C for 5-7 days before cutting inoculating plugs. The thermotolerant yeast UBU-1-10 was grown on YPD agar at 45°C for 24h. And all isolates were stored at 4°C until further processing

### 3.2.3 Enzyme production under solid state fermentation

The enzyme production with *Lentinus polychrous* Lev. LP-PT-1 under solid-state fermentation (SSF) was carried out in 125 ml Erlenmeyer flasks with 4 g ground rice straw and adjusted in 65% moisture content of following synthetic medium (per litre): 1.0g $NH_4NO_3$, 0.8g $KH_2PO_4$, 0.2g $Na_2HPO_4$, 0.5g $MgSO_4$ . $7H_2O$, 4.0g yeast extract (Elisashvili et al., 2008).

Flasks were sterilized in the autoclave for 20 min at 121°C, 15 psi. The samples were allowed to cool to room temperature, and aseptically inoculated with 4 plug cut from the margins of the PDA culture. The cultures were maintained statically at 35°C for 5 days in the incubator. All experiments were performed in triplicate.

The extracellular enzymes were extracted by adding a ten-fold volume of McIlvain buffer pH 5 and transferred to an incubator shaker at 150 rpm at 20±1°C for 30 min. The supernatant containing the fungal crude enzymes were separated from the solids by centrifugation at 10,000 g at 4°C for 10 min. The supernatants containing the fungal enzymes were stored in 1.5 ml microcentrifuge tubes at -20°C prior to enzyme assays.

### 3.2.4 The simultaneous pretreatment and saccharification with using fungal enzymes under submerged fermentation

The pretreatment and saccharification condition was carried out on 4 g of treated rice straw after enzyme production in 125ml Erlenmeyer flask. The 4 g samples were aseptically adding by 60 ml sterilized Mcillvaine buffer pH 5 after the enzyme production process. Cultures were maintained at 50°C, 150 rpm in the incubator shaker. The samples were withdrawn and centrifuged at 10,000 rpm for 10 min at 4°C in a refrigerated bench-top centrifuge (Universal 32, Hettich, Germany) and the supernatants were analysed for enzyme activities, reducing sugar yield and sugar analysis.

### 3.2.5 Fermentation

3.2.5.1 Thermotolerant yeast inoculum preparation

Bioethanol production by the thermotolerant yeast UBU-1-10 was carried out as follow: The yeast inoculums were grown on YPD agar plate at 45°C for 48h. The seed cultures were prepared by inoculating a loopful of cells grown on YPD agar plate into a 250ml cotton-plugged Erlenmayer flask containing 100 ml of YPD medium, followed by incubation at 45±0.5°C, 150 rpm for 24h in the incubator shaker. The yeast cells were harvested by centrifuge 5,000 rpm for 5 min, the supernatant was decanted and cells were washed twice with sterile water. The inoculation concentration of thermotolerant yeast UBU-1-10 was $1.5\times10^{7}$ cells $ml^{-1}$ in 60ml working volume in all the SSF experiments.

3.2.5.2 Simultaneous saccharification and fermentation at 45°C

The rice straw was hydrolysed with crude cellulases and xylanases as previously process and subsequently fermented with thermotolerant yeast UBU-1-10. The hydrolyzate solution was supplemented with other nutrients (Modified Karimi et al., 2006), $(NH_4)_2SO_4$, 7.5; $K_2HPO_4$, 3.5; $MgSO_4 \cdot 7H_2O$, 0.75; $CaCl_2 \cdot 2H_2O$, 1; inoculated with $1.0 \times 10^6$ cells $ml^{-1}$ thermotolerant yeast UBU-1-10., and cultivated at 45°C, 150 rpm for 3 days. All experiments were performed at least in triplicate and results are presented as average values and standard deviation. The cultures were examined for contamination by microscope and plate.

Flasks were sealed with rubber balloons and incubated at 45°C and 150 rpm. Then, samples were centrifuged for 10 min at 10,000 rpm and the supernatant were determined the ethanol content by gas chromatography (GC). Butanol (0.1%) was added in the internal standard.

**3.2.6 Analytical procedures**

3.2.6.1 Enzyme assays

The enzyme assays were determined by the correlation of enzyme activities (U/gds) and cellulose and xylose agar diffusion method. The supernatants of crude enzymes were analysed for enzyme activity. Measurements were made in 50mM citrate buffer (pH 5.0), using the following substrates: 1.0% (w/v) carboxymethylcellulose low viscosity (CMC) for cellulase; 1.0% (w/v) xylan from oat spelt (Sigma) for xylanase. Enzyme activities were determined by mixing 0.5 ml crude enzymes with 1ml of appropriated substrate (1.0% w/v) in McIlvaine buffer (pH 5.0) at the 50 °C for 20 min (Ghose, 1987). Glucose and xylose standard curve were used to calculate the cellulase and xylanase activities. In all assays the release of reducing sugars was measured using the dinitrosalicylic acid reagent method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme, releasing 1 μmol of reducing sugars per minute. The total unit per flask culture was calculated by multiplying IU/ml by the total volume of enzyme extract (Ferraz et al., 2003) and expressed as IU/gram solid of raw material. All measurements were done using a UV-Vis spectrophotometer (Biospec 1601, Shimadzu).

3.2.6.2 Cellulase and xylanase agar diffusion method

The cellulase and xylan were tested for hydrolytic activity using the modified agar diffusion method (Sae-lee, 2007). Ten μl of suspension was dropped onto 0.1%

cellulose and 0.1% xylan agar. Cellulase assay was used as a cellulose-agar medium containing 0.1% carboxymethylcellulose (Sigma) and xylan agar medium added 0.1 % xylan from oat spelt (Sigma) for xylanase activity measurement. After 20 h incubation at 50°C, the plates were stained with Gram's iodine for 3 to 5 minutes (Modified Kasana et al., 2008). The hydrolysis zones of clearance were observed.

3.2.6.3 Sugar analysis

The reducing sugar content was determined using the dinitrosalicylic acid method. The composition of sugars was analyzed by high performance liquid chromatography (HPLC). Collected supernatant was filtered with a membrane filter of 0.45 μm pore size, and released xylose and glucose were analyzed by high performance liquid chromatography using an Inertsil $NH_2$ column (4.6×250 mm) and an RI detector (Shimadzu). The flow rate of the solvent, acetonitrile and water (75:25), was 1 ml/min.

3.2.6.4 Viability assay

Viability assay was used as an measure of the amount of living fungal biomass under submerged fermentation at 50°C. The free yeast cell in hydrolysate during fermentation at 45°C was investigated. The hydrolysate was diluted in appropriate amount of sterilized distilled water. Afterward, it was homogenized with vortex for 30s and proper dilutions were made for these samples. For each sample, at least five dilutions were used for the viability assay. Each dilution was aseptically dropped on PDA plate at three different spots (10 μl/spot) in replicates. The PDA and YPD agar plate were used for fungal and yeast viability assay. The plates were incubated at 35°C for 48 h. The cell viability was expressed as the total number of colony forming units.

3.2.6.5 Ethanol

Ethanol was measured by gas chromatography (GC), using a Shimadzu - GC-17A instrument, with a flame ionization detector (FID) and a Bonded polyethylene glycol (PEG) capillary column (30 m x 0.25 mm, 0.25 μm film thickness) using helium as carrier gas. The GC oven temperature was held at 100°C. The injector and detector temperature was maintained at 200 and 180°C. Peaks were identfied on the basis of sample coincidence with retention times of standards, and quantified using peak areas and the corresponding response factors. All the experiments were carried out by triplicate

### 3.2.7 Statistics

Triplicate determinations of all experiments were performed, and the mean values and standard deviations (S.D.) were reported.

## 3.3 Results and discussions

### 3.3.1 Enzyme production process under solid state fermentation at 35°C

The xylanase and cellulase production from *L. polychrous* Lev. LP-PT-1 under solid state fermentation indicated that xylanase and cellulase were rapidly produced at 35°C in early growth period (Fig 3.1). Xylanase production followed a similar trend to that observed for cellulase production. Activity of cellulase was higher than xylanase. The xylanase and cellulase production during the 4 days was also quite high, and then enzymes were constant later. Preservation of cellulose during pretreatment is expected to improve its availability during hydrolysis.

Xylanase and cellulase were produced during fungus growth. Xylan and cellulose components could induce the production of xylanase and cellulase when used rice straw as a carbon sources for fungus growth. The rice straw could be pretreated by these enzymes. The mycelium of fungus covered rice straw, that became soft, spongy and the color changed to whitish-yellow. Also, Wan (2011) reported fungal enzyme pretreatment changed the structure of corn stover significantly at the pretreatment process (Wan, 2011).

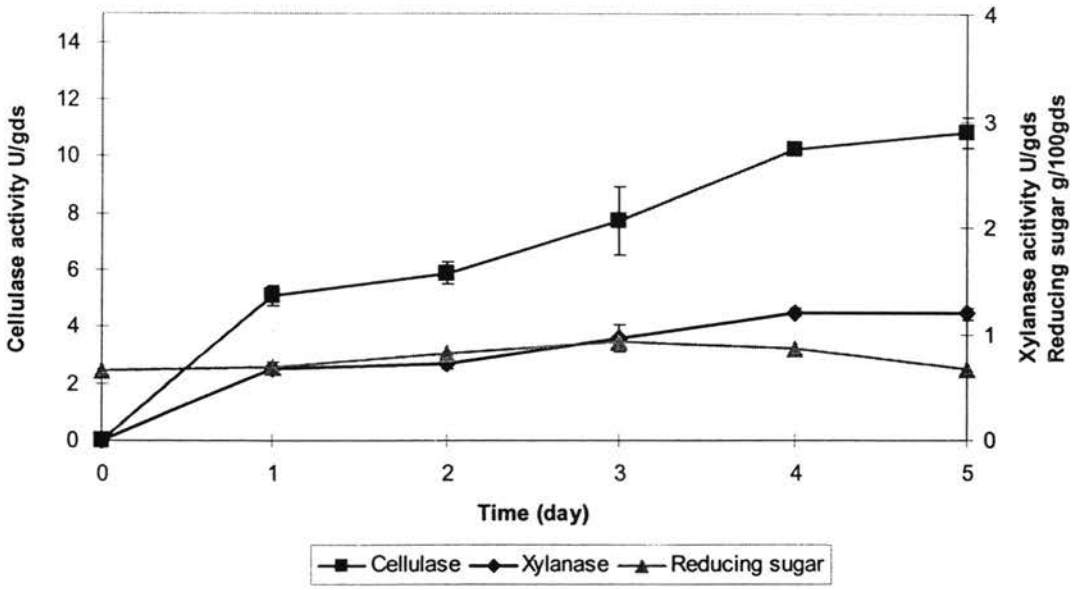



**Figure 3.1** Xylanase and cellulase production of *L. polychrous* Lev.LP-PT-1 at 35°C

### 3.3.2 The simultaneous pretreatment and saccharification with using fungal enzymes under submerged fermentation

The simultaneous pretreatment and saccharification of rice straw using fungal enzymes in pH 5 of Mcillvaine buffer under submerged fermentation at 50°C were evaluated. The reducing sugar yield of pretreated rice straw was rapidly increased for 2 days (2.0g/100gds). Reducing sugar slightly released during hydrolysis of treated rice straw for 6 days (2.7g/100gds) (Fig 3.2). The viability of *L. polychrous* Lev. LP-PT-1 could not detected under submerged fermentation at 50°C since 2 days. The xylanase and cellulase, produced under solid state fermentation, that hydrolyzed the biomass into reducing sugar under submerged fermentation at pH5, 50°C. The optimization conditions of xylanase and cellulase activities were pH 5, 50°C. It was found that the hydrolysis with fungal xylanase and cellulase gave the increasing of xylose and glucose under submerged fermentation at 50°C (Fig 3.3). Xylans, the major portion of the hemicellulose of plant cell walls, are heteropolymers consisting principally of xylose and arabinose (Abdel-Sater and El-Said, 2001). Xylose was slightly increased under submerged fermentation at 50°C. Xylanase degraded xylan from rice straw into xylose. So, rice straw was pretreated by xylanase.

The small amounts of glucose mainly due to the low cellulose conversion. Xylanase could not completely degraded hemicellulose from cellulose. Cellulose could not be convenient for enzyme accessibility. Moreover, lignin, a complex organic compound that binds to cellulose fibers and hardens and strengthens the lignocellulosic material.

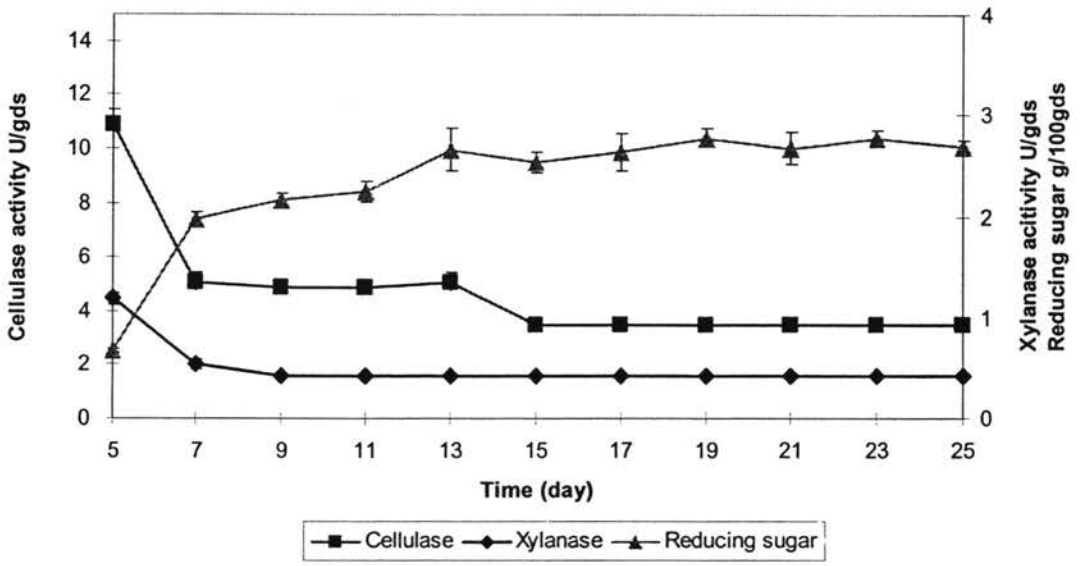



**Figure 3.2** Hydrolysis process under submerged fermentation at 50°C.

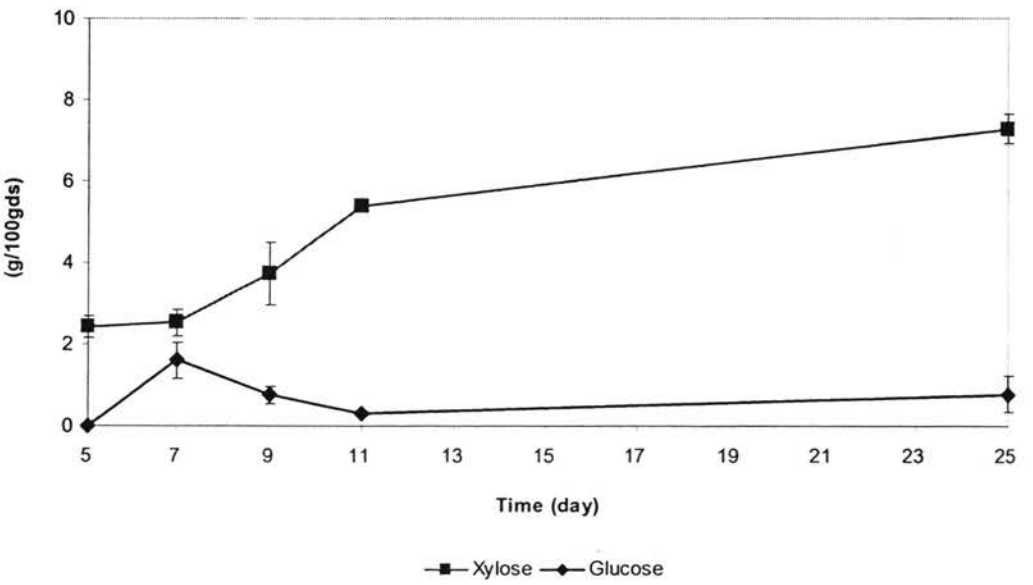



**Figure 3.3** Sugar analysis under submerged fermentation at 50°C.

### 3.3.3 Fermentation process at 45°C

The ethanol fermentation from lignocellulosic waste material via biological processes at 45°C was investigated. The thermotolerant UBU1-10 was inculated into fermentation process. The free yeast cells were highly related to the reducing sugar, xylose and glucose decreased during fermentation process at 45°C. Sugar was used as carbon source for yeast growth. The reducing sugar, xylose and glucose were slow down. The theromotorlerant yeast, UBU-1-10, showed the capable of utilization xylose and glucose as carbon source for yeast growth in liquid media at 45°C (Sutthikhampa et al., 2010). Ethanol (0.046g/gds), was low for pretreated samples mainly due to the low cellulose conversion. Hemicellulose limits enzymatic hydrolysis of lignocellulosic biomass by cross-linking with cellulose fibers. Reducing much more the hemicellulose content of the biomass helps to expose the highly ordered crystalline structure of cellulose and facilitates substrate access by hydrolytic enzymes.

**Table 3.1** Fermentation process at 45°C

| Time (day) | Ethanol (g/100gds) | Reducing sugar (g/100gds) | Xylose (g/100gds) | Glucose (g/100gds) | Yeast (CFU/ml) |
|---|---|---|---|---|---|
| 0 | 0.000 | 2.69±0.1 | 7.3 | 0.8 | $4.5x10^5$ |
| 1 | 0.000 | 1.35±0.1 | 3.9 | 0.0 | $83.3 x10^5$ |
| 2 | 0.000 | 1.21±0.1 | 0.7 | * | $101.7 x10^5$ |
| 3 | 0.046 | 1.26±0.1 | * | * | $183.3 x10^5$ |

* denotes that no sugar was detected

## 3.4 Conclusions

The potential of simultaneous pretreatment and saccharification of rice straw by using fungal enzymes, *L. polychrous* Lev. LP-PT-1, to degrade hemicellulose and facilitate biofuel ethanol production was investigated. One of the main challenges of fungal pretreatment is to improve enzymatic hydrolysis, separate hemicellulose from cellulose and thus preserving more cellulose.

In this study, the possibility pretreated rice straw by using *L. polychrous* Lev. LP-PT-1 enzymes under submerged fermentation at 50°C and pH 5. The hemicellulose was degraded by xylanase to xylose. The hemicellulose removal may not be only factor that lead to higher cellulose conversion. The low ethanol yield was obtained for pretreated samples as a result of the limited cellulose conversion.

# CHAPTER 4

# CHARACTERIZATION OF CELLULASE AND XYLANASE FROM *Lentinus polychrous* Lev. LP-PT-1

## 4.1 Introduction

The microbial enzymes are relatively more stable and active with extraordinary properties. Solid state fermentation has more advantages than submerged fermentation due to low capital investment, simplification of the fermentation media, absence of complex machinery, reduced energy requirement and improved product recovery. The metabolites are more thermostable in nature.

Nowadays the interest in cellulases and xylanases have increased due to many potential applications like bioenergy, biofuel production, biodegradation of lignocellulosic residues, pulp and paper industrial process.

Since the production of hydrolytic enzymes is determined by different factors, mainly when performed in natural substrates. Many reports on xylanase and cellulase production are mainly for bacteria and fungi(Archana and Satyanarayana, 1997; Samain et al., 1997; Cristina et al., 1998; Gessesse and Mamo, 1999; Subramaniyan et al., 2001; Techapun et al., 2001; Kumaran et al., 1997; Kim et al., 1997; Qinnghe et al., 2004; Milagres et al., 2005; Niranjane, 2006; Fang et al., 2007; Papinutti and Forchiassin, 2007; Maciel et al., 2008; Sherief et al., 2010; Sharma and Arora, 2010) with only a few reports about *Lentinus polychrous* Lev. (Pukahuta et al., 2004; Pukahuta et al., 2005). However, little has been done on optimization cellulase and xylanase production by *Lentinus polychrous* Lev.LP-PT-1 using rice straw as substrate under solid state fermentation cultivation.

This study examined the culture conditions of solid state fermentation, including temperature, moisture contents and inoculum size for the production of cellulase and xylanase with the selection of cheap and readily available substrate, rice straw. The characterization of enzyme was further made, making the effect of pH and temperature on the activity and stability of enzymes. Partial purification of enzyme and molecular mass determination were investigated.

## 4.2 Materials and methods

### 4.2.1 Lignocellulosic substrate

Rice straw was cut, dried in hot air oven at 70°C and milled to pass through 16 Mesh Laboratory test sieve (Endecotts LTD., London, England). and then were dried at 60°C for three days before store in plastic bags.

### 4.2.2 Fungus and inoculation preparation

The culture of the white-rot fungus *Lentinus polychrous* Lev.LP-PT-1 was kindly provided by Asst.Prof.Dr.Charida Pukahuta, Department of Biological Sciences, Faculty of Science, Ubon Ratchathani University and was maintained on a potato dextrose agar medium (Himedia) at 35°C for 7 days, and stored at 4°C until further processing. The white rot fungus was grown on PDA plate at 35°C for 5-7 days before cutting inoculating plugs.

### 4.2.3 Culture condition under solid state fermentation

The solid-state fermentation (SSF) with *Lentinus polychrous* Lev. LP-PT-1 was carried out in 125 ml Erlenmeyer flasks with 4 g ground rice straw and adjusted the moisture content with the following synthetic medium (per litre): 1.0g $NH_4NO_3$, 0.8g $KH_2PO_4$, 0.2g $Na_2HPO_4$, 0.5g $MgSO_4 . 7H_2O$, 4.0g yeast extract (Elisashvili et al., 2008). The initial pH of the medium was adjusted to 6.0 prior to sterilization by adding 2 M NaOH and 1 M HCl. Flasks were sterilized in the autoclave for 20 min at 121°C and aseptically inoculated with the plugs cut from the margins of the potato dextrose agar culture. The cultures were maintained statically in the incubator. The extracellular enzymes were extracted by adding a ten-fold volume of McIlvaine buffer pH 5.0 and transferred to an incubator shaker at 150 rpm at 30°C for 30 min. The supernatant containing the fungal crude enzymes were separated from the solids by centrifugation at 10,000 rpm at 4°C for 10 min. The supernatants containing the fungal enzymes

were stored in 1.5 ml microcentrifuge tubes at -20 °C prior to enzyme assays. All experiments were performed in triplicate.

**4.2.4 Effect of temperature**

The effect of temperature on the production of enzymes by *L. polychrous* Lev. LP-PT-1 was studied by incubating the inoculated flasks at 20, 25, 27, 30, 35, 40, 45 and 50°C. The enzyme activities were determined as described.

**4.2.5 Effect of moisture contents**

To determine the optimum moisture content of the cultivation, the moisture levels (50%, 55%, 60%, 65%, 70%, 75% and 80% moisture content) were adjusted with the above-mentioned synthetic medium (Elisashvili et al., 2008).

**4.2.6 Effect of inoculum size**

Fungal inoculation plugs (0.5 cm diameter, 5 days old mycelium agar culture) varying from one to twenty plugs were inoculated in a 125ml flask containing 4g substrate adjusted to 65% moisture content with synthetic medium (Elisashvili et al., 2008) for 4 days at 35°C.

**4.2.7 Enzymes characterization**

4.2.7.1 Optimization of temperature and pH for enzyme activity

Crude extracts were assayed for enzyme activity at temperatures ranging from 10 to 80°C. Enzymatic activity was measured by incubating with the appropriated substrate in 50mM citrate buffer, pH 5 for 20 min. The enzymes activity was determined as described.

Similarly to the studies for determination of the temperature, the crude enzymes were assayed at pH values ranging from 3.0 to 8.0 (McIlvaine buffer) with the appropriated substrate at 50°C for 20 min.

4.2.7.2 Stability of enzymes at different temperatures and pHs

The pH stability, the crude enzyme preparation was diluted in McIlvaine buffer (1:2) in a pH range from 3.0 to 8.0 and incubated at 50°C for 60 min.

The thermostability of enzymes was determined by preincubation of the crude enzyme at 35, 40, 45, 50, 55, 60, 65 and 70°C in the absence of substrates. Aliquots were withdrawn at timed intervals and immediately cooled before assaying to determine the residual enzyme activity, using the enzymes assay procedure.

### 4.2.8 Enzyme assays and proteins determination

The supernatants of crude enzymes were analysed for enzyme activity. Cellulase activity was measured using carboxymethylcellulose low viscosity (CMC) as a substrate. Enzyme activities were determined by mixing 0.5 ml crude enzymes with 1ml of carboxymethylcellulose low viscosity (1.0% w/v) in 50mM citrate buffer (pH 5.0) at the 50°C for 20 min (Modified Ghose, 1987). Xylanase activity was assayed with the same method as cellulase assay but xylan from oat spelt (Sigma) was used as a substrate. After incubation of xylanase activity at the 50°C for 20 min, the mixture was centrifuged at 10,000 rpm, 4°C for 10 min. The supernatant was used for the determination of the reducing sugars. Glucose and xylose standard curve was used to calculate the enzymes activities. In all assays, the released of reducing sugars were measured using the dinitrosalicylic acid reagent method (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme, releasing 1 μmol of reducing sugars per minute. The total unit per flask culture was calculated by multiplying U/ml by the total volume of enzyme extract (Ferraz et al., 2003) and expressed as U/gram solid of raw material (Unit/gram dry substrate).

The specific activity was expressed as unit of activity per milligram of protein. The concentration of proteins was determined by Lowry's method, using bovine serum albumin as standard. All measurements were done using a UV-Vis spectrophotometer (Lambda 25, Perkin-Elmer) and evaluated using UV-Winlab software from the same manufacturer.

### 4.2.9 Partial purifiation of enzyme by ammonium sulphate precipitation

The calculated amount of solid ammonium sulphate was added to 50 ml of crude enzyme to achieve 20–40% saturation. After centrifugation at 4,000 rpm at 4°C for 10 min the precipitates were dissolved in 5ml of McIlvaine buffer pH 5.0. The enzyme solution was subjected to concentration and dialysis with Amicon (Millipore, USA). After centrifugation at 4,000 rpm at 4°C for 10 min. Enzyme activity and protein estimation were carried out on the dialysed sample. The partially purified enzyme was used for further studies related to SDS-PAGE and molecular weight determination respectively.

### 4.2.10 SDS-PAGE and molecular mass determination.

For the subunit molecular weight measurement, SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 4% stacking gel and 10% separating gel together

with a mixture of a set of marker proteins. Protein bands were stained with Coomassie Brilliant Blue R-250.

#### 4.2.11 Statistical analysis

All the experiments were conducted in triplicate, and experimental results were represented as the mean ± standard deviation of three identical values. Statistical analysis of variance (ANOVA) was calculated ($p < 0.05$) using in SPSS.

## 4.3. Results and discussion

### 4.3.1 Effect of temperature

This study investigated the productions effect of different incubation temperature on xylanase and cellulase. *Lentinus polychrous* Lev.LP-PT-1 was cultured on rice straw. In order to produce xylanase and cellulase, in solid state fermentation at 20°C, 25°C, 27°C, 30°C, 35°C, 40°C, 45°C and 50°C, while the other parameters kept constant at 65% moisture contents, initial pH 6.0 and 4 pieces of inoculum size. Xylanase, cellulase enzymes activities were determined at the 4 days of incubation time.

The xylanase and cellulase production from *Lentinus polychrous* Lev.LP-PT-1 under solid state fermentation indicated that cellulase and xylanase were rapidly produced at 35°C on 4 days. The production of cellulase was higher than xylanase. Xylanase production followed a similar trend to that observed for cellulase production.

. The results are given in Figure 4.1. It was observed that the temperature at 27°C showed the minimum temperature of xylanase and cellulase production. The maximum temperature showed at 35°C, while all other temperature exhibited lower enzyme activity. Further increase in temperature up to 40°C resulted in sharply decrease in cellulase and xylanase production. The optimum temperature was found to be at 35°C.

The rice straw, lignocellulosic material, had xylan and cellulose components that can induce the production of xylanase and cellulase when used rice straw as a carbon sources for fungus growth. The utilization of insoluble lignocellulosic substrates by mushrooms depends upon the production of enzymes (cellulases, hemicellulases, ligninases) that bring about hydrolysis of the macro molecules of cellulose, hemicellulose and lignin components, respectively thereby,

librating low molecular weight nutrients (Buswell et al., 1993). The low molecular mass degradation products of xylan and cellulose hydrolysis penetrate into the cells and induce the production of hydrolytic enzymes (Haltrich et al., 1996). The optimal temperatures for production of many other fungal cellulase ranged between 30°C and 37°C. For example, the optimal temperature for production of cellullase was set at 35°C under submerged fermentation for *A. niger* (Sohail et al., 2009).

The level of xylanase production at 27°C, 30°C, 35°C and 40°C at 4 days (4.42, 4.50, 5.18, 4.68 U/gds, respectively) in this study was closed to that observed from some other solid cultures of *C. subvermispora*. Xylanase production during solid state microbial pretreatment of corn stover increased rapidly at the early stage and peaked value 4.18 U/g solid around 7 days (Wan, 2011). While, xylanase production during the corn stover biodegradation period of 120 days showed two peaks during a cultivation period and reached a maximum level of 8.9 IU/g solid on 60 days (Xu et al., 2009).

The optimum temperature for enzyme production is similar to the optimum temperature for fungal growth its natural habitat. Pang et al. (2006) studied the optimum temperature for xylanase production by *Trichoderma* spp. FETL c3-2 in solid state fermentation at 25-30°C. Similar observation was also reported by Sarao et al. (2010) in *S. acremonium*, Sudgen and Bhat (1994) in *Sporotrichum thermophile* and by Biswas et al. (1990) in *Aspergillus orchraceus*, who showed that the highest xylanase production were obtained at temperatures that were optimum for the growth of fungi under solid state fermentation.

The xylanase and cellulase production by *L. polychrous* Lev.LP-PT-1 at 27-40°C is in the same range of most other xylanase and cellulase producing fungi. It is noteworthy, that cellulase production by *L. polychrous* Lev.LP-PT-1 was occurred at rather high temperature (40°C). However, overall activities of the xylanase were still very low.

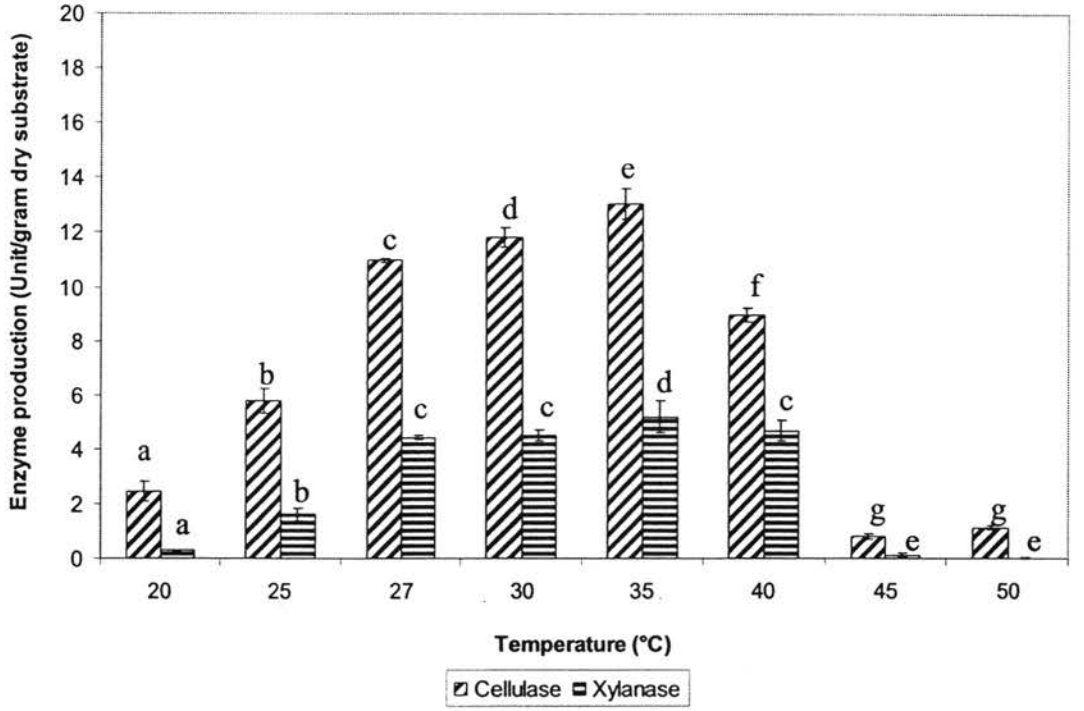



**Figure 4.1** The cellulase and xylanase production affected by temperature (65% moisture contents, 4 pieces of inoculum size).

### 4.3.2 Effect of moisture contents

This study investigated the effect of moisture contents on xylanase and cellulase production. The data shows the effect of the seven initial moisture levels on xylanase and cellulase production by *L. polychrous* Lev.LP-PT-1 under solid state fermentation within 4 days.

The production of cellulase fairly predominated over xylanase. The 75% and 80% moisture levels showed significantly higher cellulase production than other treatments ($p < 0.05$). There was no significance difference in xylanase production between 75% to 80% moisture levels. However, The 75% and 80% moisture levels showed significantly higher xylanase production than 50% and 55% moisture levels ($p < 0.05$). These results are in agreement with Soliman et al. (2012) showed the optimum initial moisture level for xylanase production by *A. niger* and *T. viride* was 75% (Soliman et al., 2012).

Further increase in the moisture levels supported the fungus growth that may be enhance xylanase and cellulase production. Initial moisture content of the substrate is important for fungal growth and the metabolism in solid state fermentation (Reid, 1989). In a solid-state

fermentation process, the moisture levels influences growth of *Aspergillus niger*, biosynthesis, the secretion of fungal enzymes and protein stability (Pandey et al., 1994). Growth of *Aspergillus niger* and secretion of fungal enzymes correlated well with the moisture levels in solid state fermentation (Pandey et al., 1994).

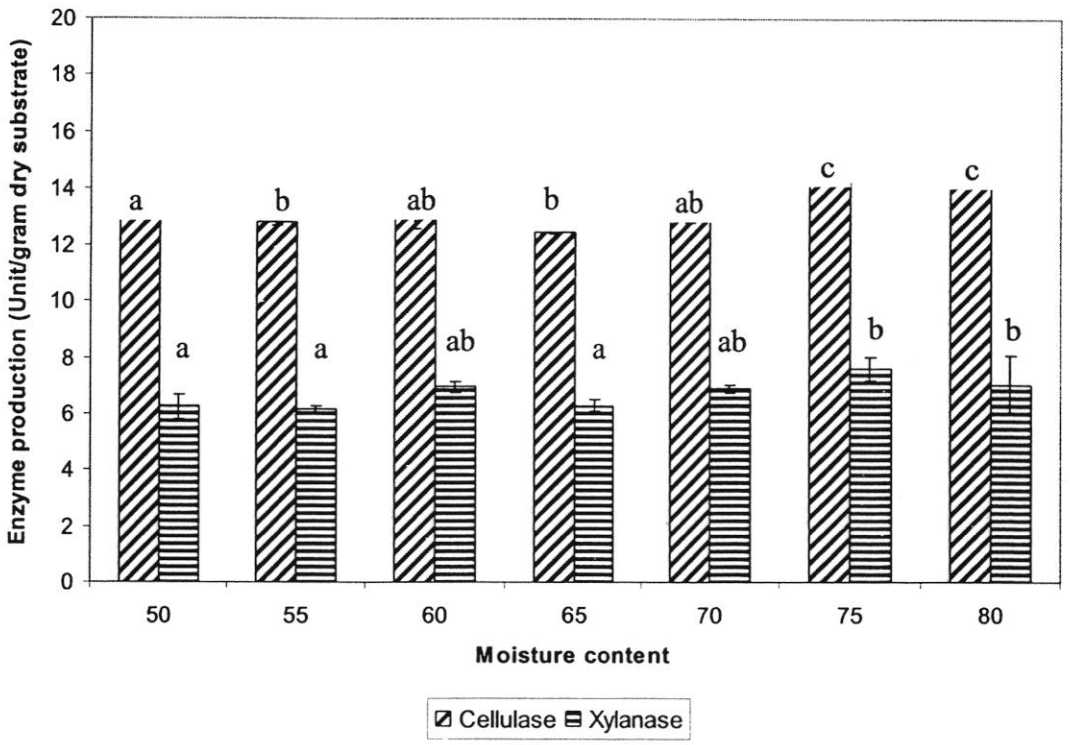



**Figure 4.2** The cellulase and xylanase production effect of moisture contents.

### 4.3.3 Effect of inoculum size

The effect of inoculum size on xylanase and cellulase production is shown in Fig 3.3. Seven levels of inoculum size were tested.

The inoculum size from 1, 2 and 4 pieces cause the cellulase production increased (10.6 $Ug^{-1}$, 11.9 and 13.3 $Ug^{-1}$). But further increase in the inoculum level from 4-20 pieces did not increase the xylanase and cellulase production due to nutrient limitations. Lower levels of inoculum may not be sufficient of initiating growth and enzyme synthesis.

The inoculum level at 0.5 cm per disk for the four disks in 4g substrate culture was found to be optimum for xylanase and cellulase production by *L. polychrous* Lev.LP-PT-1.

Similar to Qinnghe et al (2004) reported xylanase production with the inoculum level at 0.5cm per disk for the four disks in 50 ml liquid culture (corn cob 2.5%+wheat bran 2.5%), by *P. ostreatus* was found to be optimum for xylanase (Qinnghe et al., 2004).

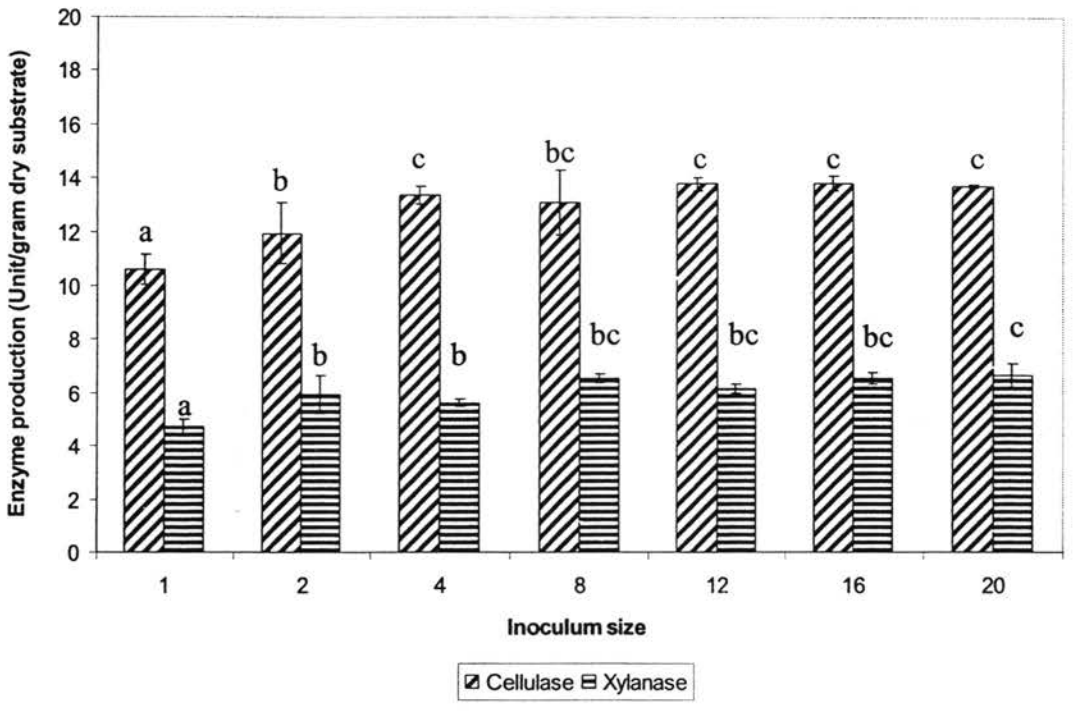



**Figure 4.3** The cellulase and xylanase production effect of inoculum size.

### 4.3.4 Optimization of temperature and pH for enzyme activity

This study investigated the effect of temperature on the activity of crude enzyme extracts at various temperatures ranging from 10°C to 80°C. Figure 3.4 shows the optimization temperature of cellulase and xylanase activities that produced by *L. polychrous* Lev.LP-PT-1 between 10°C to 80°C. The crude xylanase activity increased with high temperature to 60°C. The cellulase showed a good activity between 30°C to 60°C. Both enzyme activities exhibited decreased at values above 60°C.

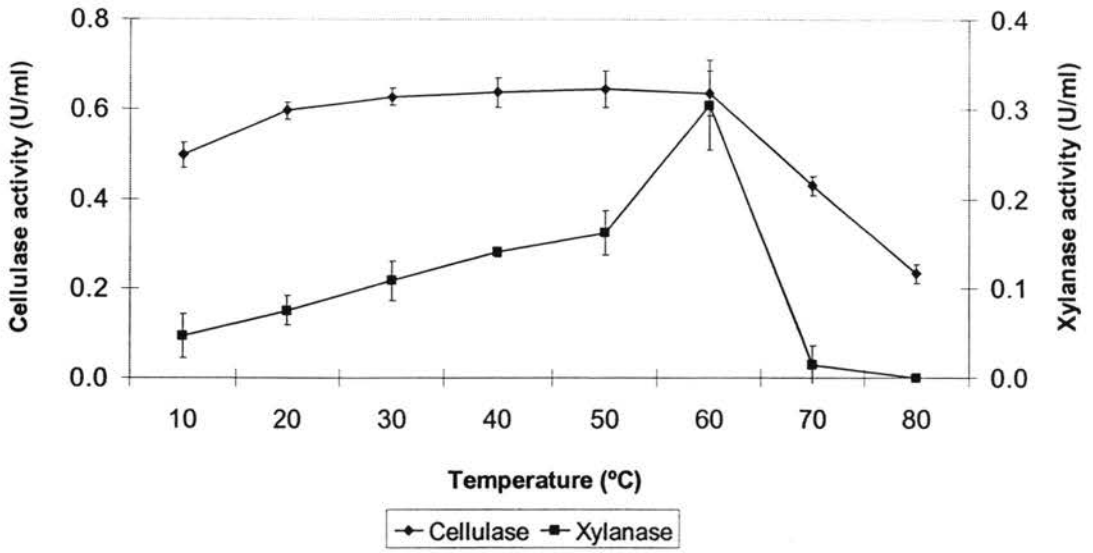



**Figure 4.4** Effect of temperature on xylanase and cellulase activities from *L. polychrous* Lev.LP-PT-1.

The optimization of pH of cellulase activity was investigated. Figure 4.5 shows the effect of the pH on the crude cellulase and xylanase activities of *L. polychrous* Lev. LP-PT-1 was examined at various pHs ranging from pH 3.0 to pH 8.0.

The increasing levels of xylanase were obtained at pH 3.0 to pH 5.0 and continued to decrease slightly with an increase in pH 5 to pH 8.The increasing levels of cellulase were obtained at pH 3.0 to pH 5.0 and decreased drastically with an increase in pH 5 to pH 8.0.

The increasing cellulase and xylanase activities of pH 5.0 resulted higher than pH 3.0, 7.0 and pH 8.0 ($p < 0.05$). The increasing pH to 7.0, their activities were declined. The optimum pHs of cellulase and xylanase activities were at pH range 4.0-6.0. The enzyme has a broad range of pH activity (pH 4.0-6.0). Similar to the pH optimum of cellulase activity was most stable around pH 6.0 (Eriksen and Goksöyr, 1976). And the optimum pH for xylanase and CMCase of *Thermoascus aurantiacus* MIEHE were 5.0-5.5 and 5.0, respectively (Silva et al., 2005).

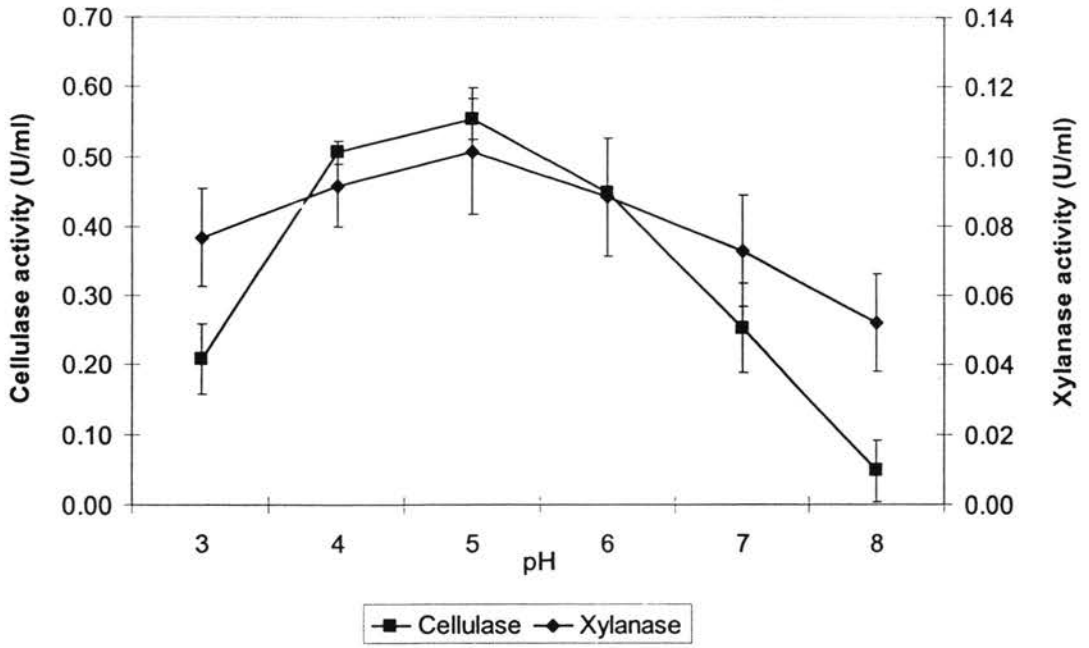



**Figure 4.5** Effect of pH on xylanase and cellulase activities from *L. polychrous* Lev. LP-PT-1.

### 4.3.5 Stability of enzymes at different temperatures and pHs

The crude enzyme was produced by *L. polychrous* Lev.LP-PT-1 at 35°C for 4 days. The extracellular enzymes were extracted by adding a ten-fold volume of McIlvaine buffer pH 5. The enzyme stability analyses were carried out.

The thermostability of enzymes from *L. polychrous* Lev. LP-PT-1 was determined by preincubation of the crude enzyme without substrate at 35, 40, 45, 50, 55, 60, 65 and 70°C. The treated crude enzymes were then assayed for residual enzymatic activities using enzyme assays and expressed as percentage of the appropriate zero time control.

Thermostability assays showed that the crude xylanase and cellulase were highly stable at 35-60°C for 60 min. The xylanase and cellulase stability were high (above 80%) at 35-60°C. The xylanase and cellulase remained 6% and 14% of residual activity after incubation at 70°C for 60 min (Fig. 4.6-4.7).

However, the xylanase thermostability remained more than 81% of activity after incubation at 60°C for 60 min. The xylanase of the present strain was more thermostable than that of the *Pleurotus ostreatus* in which the activity was lost about 97% at 60°C for 15 min (Qinnghe et al., 2004). Also, the *L. polychrous* Lev.LP-PT-1 xylanase exhibited considerable higher

thermostability properties than the partially purified xylanases of *A. foetidus* MTCC 4898. The residual xylanases of *A. foetidus* MTCC 4898 was less than 20% at 60°C for 30 min and completely inactivated within 1 h (Shah and Madamwar, 2005). While, the purified xylanase from *F. proliferatum* NRRL 26517 lost its stability at 60°C with only 22% residual activity for 30 min (Saha, 2002). Moreover, xylanase thermostability of *Cheaetomium thermophilum* retained below 60%of the activity at 60°C after 1 h (Katapodis et al., 2007). The xylanase from *L. polychrous* Lev.LP-PT-1 are thermostable in high temperatures that would rapidly denature most xylanases from other mesophilic fungi. Thus, the xylanase produced during the current study would be advantage over at least some of the other reported xylanase from mesophilic fungi.

The cellulase remained more than 81% of activity after incubation at 60°C. The thermostability of cellulase of the present strain was similar the *P. sanguineus*. Cellulase (CMCase) of *P. sanguineus* resisted up to 60°C showing approximately 80% of the original activity after 1 hr of incubation (Quiroz-Castañeda et al., 2009). While, the cellulase of the present strain was more thermostable than that of the *B. adusta* in which at 50°C half of the activity was lost (Quiroz-Castañeda et al., 2009).

Cellulolytic enzymes that are stable at high temperatures can be used in cellulose saccharification process at elevated temperatures to protect both substrate and products of the enzymatic reaction from microbial contamination and deterioration (Hagerdal et al., 1980).

The xylanase and cellulase produced by *L. polychrous* Lev.LP-PT-1 maintained its stability over a broad of pH evaluated (Fig. 3.8). The xylanase stability showed an increase in activity at pH 3.0 to pH 4.0 and then decreased slightly from pH 4.0 to pH 8.0. The xylanase remained 50% of activity was verified in pH 8.0, while high xylanase stability (above 50 %) was observed from 4.0 to 7.0. However, the favourable pH range for xylanase activity was 4.0 to 7.0. Similarly, the xylanase stability produced from *P. sclerotiorum.* It active high stability (above 80%) was at pH 4 and 6 (Knob and Carmona, 2008). The cellulase stability was high (above 80%) at pH 4.0-6.0. A remarkable drop in cellulase activity was observed below pH 3.0 and above pH 7.0.

The xylanase and cellulase from *L. polychrous* Lev.LP-PT-1 was a novel enzyme, being active at pH 4.0-6.0 and was stable in acid and neutral pH range. The xylanase and cellulase

stability were high 81% of residual activity after incubation at 60°C. These are desirable properties for application in the pulp and paper, as well as in food industries.

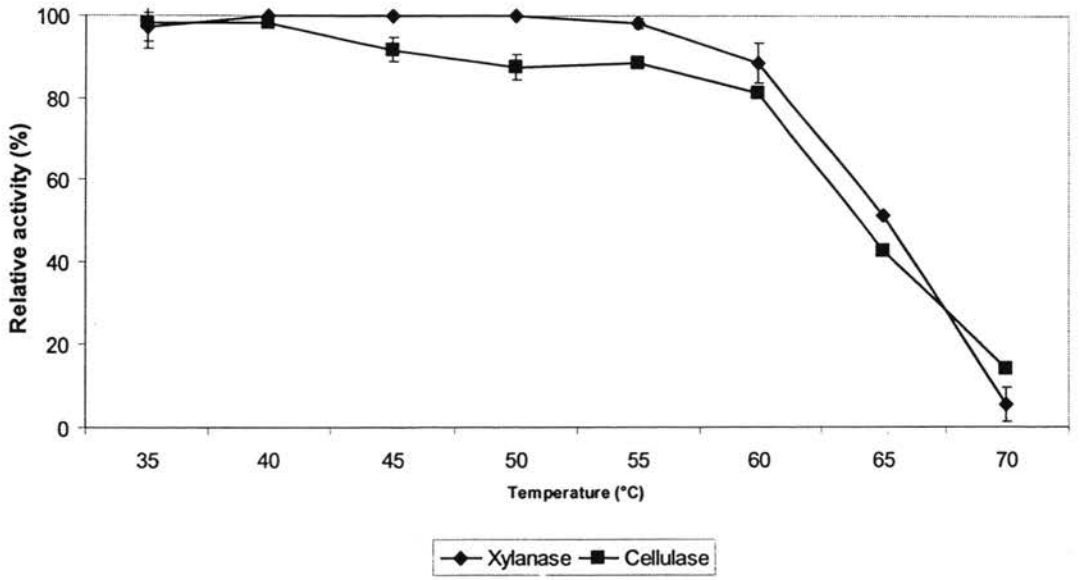
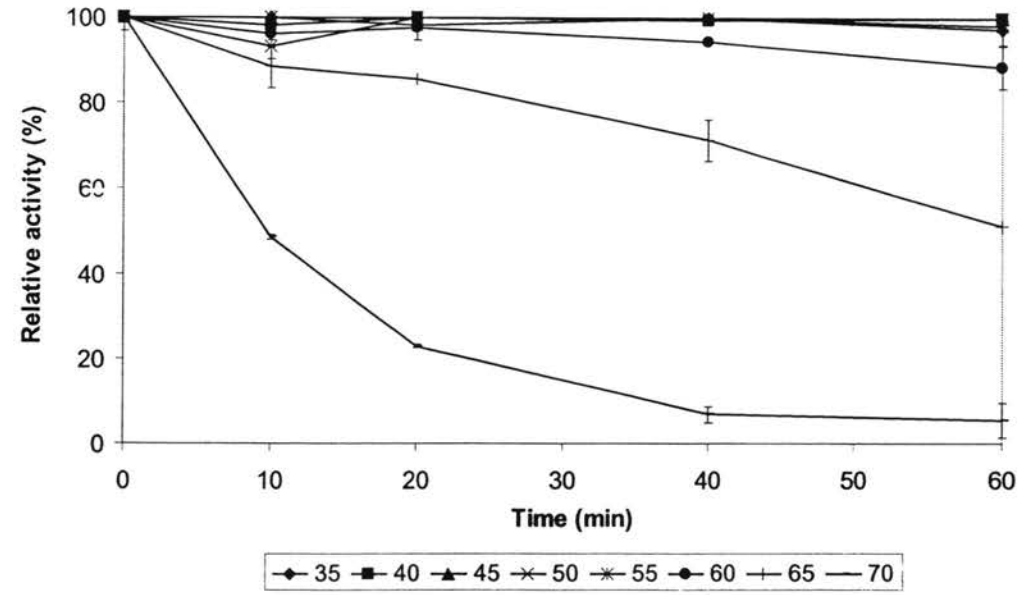
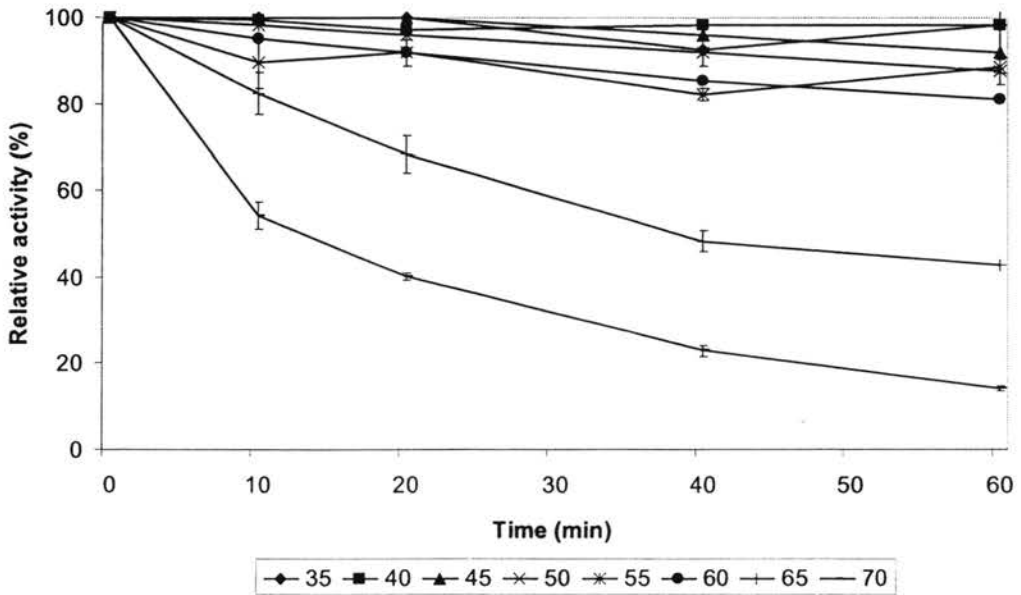



**Figure 4.6** Thermostability of xylanase and cellulase from *L. polychrous* Lev.LP-PT-1 produced under SSF on rice straw. The crude enzyme was preincubated for 60 min and residual enzyme activity was assayed by enzyme assays.




(a)




(b)

**Figure 4.7** Effect of thermo stability on xylanase (a) and cellulase (b) activities from *L. polychrous* Lev.LP-PT-1. The crude enzyme was preincubated for 60 min at various temperatures and residual enzyme activity was assayed by enzyme assays.

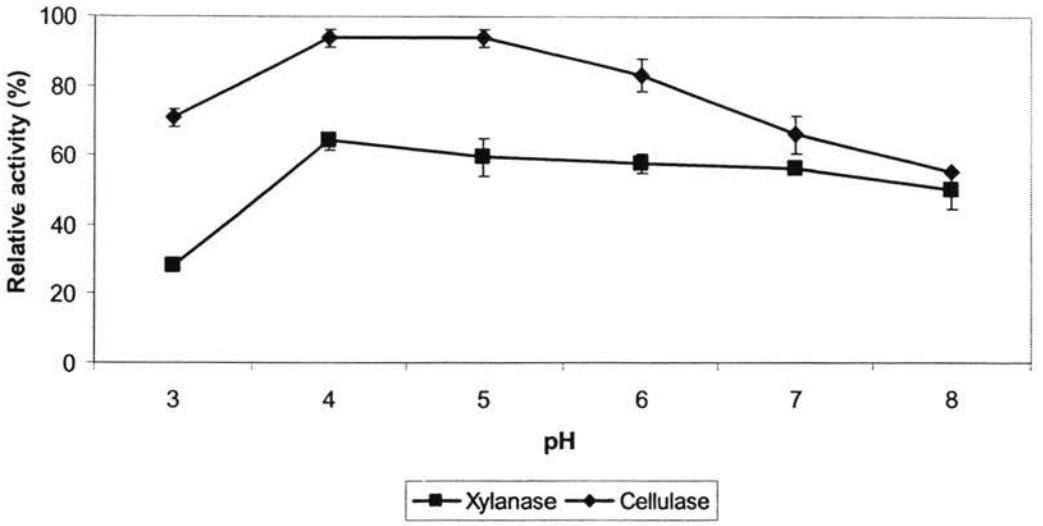



**Figure 4.8** Effect of pH stability on xylanase and cellulase activities from *L. polychrous* Lev.LP-PT-1. The crude enzyme was preincubated for 60 min and residual enzyme activity was assayed by enzyme assays.

### 4.3.6 Partial purifiation of enzyme by ammonium sulphate precipitation

The procedure for the partial purification of extracellular enzyme from *L. polychrous* Lev.LP-PT-1 is shown in Table 4.1. After 20-40% ammonium sulfate precipitation, the partial purified protein appeared as a triple band on SDS-PAGE and had the molecular weight of 45 kDa, 40 kDa and 35 kDa (Fig. 4.9). The enzyme was electrophoretically run at pH 8 on a 10% acrylamide gel and stained with Coomassie Brilliant Blue R-250. This protocol afforded 1.48-fold partial purification of cellulase from the crude enzyme. The specific acitivty of partial purification cellulase was 9.47 U/mg (Table 4.1).

**Table 4.1** Summary of cellulase partial purification from *L. polychrous* Lev. LP-PT-1

| Purification Step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude enzyme | 9.3 | 1.45 | 6.41 | 100 | 1.00 |
| 20-40%$(NH_4)_2SO_4$ | 12.5 | 1.32 | 9.47 | 134.41 | 1.48 |

**Table 4.2** Summary of xylanase partial purification from *L. polychrous* Lev. LP-PT-1

| Purification Step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude enzyme | 9.3 | 1.45 | 6.41 | 100 | 1.00 |
| 20-40%$(NH_4)_2SO_4$ | 7.5 | 1.32 | 5.68 | 80.65 | 0.89 |

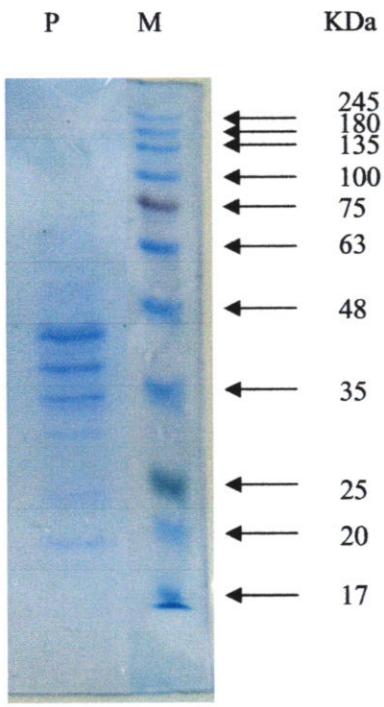



**Figure 4.9** SDS-PAGE of partial purified enzyme from *Lentinus polychrous* Lev.LP-PT-1 (lane P) stained with Coomassie Brilliant Blue R-250. (Lane M) is molecular weight marker.

## 4.4 Conclusions

The results presented in this paper show the importance of studying the characterization of xylanase and cellulase produced by *L. polychrous* Lev.LP-PT-1.

The study of the extracellular hydrolytic enzymes of the *L. polychrous* Lev.LP-PT-1, showed that the fungus is more cellulase production than xylanase. The incubating temperature is one of the most significant factors strongly affected the production of xylanase and cellulase. The optimum temperature for cellulase and xylanase production is similar to the optimum temperature for fungal growth its natural habitat. All the enzymes were stable over a broad pH range and temperature.

The relatively good thermostability of xylanase and cellulase was maintained at high temperature at 60°C with 89% and 81% of the relative activity. The use of higher temperature (60°C) is inhibitory microbial growth, decreasing the possibility microbial contamination. The xylanase appeared to be stable over a broad pH range 4.0–7.0. The cellulase stability was pH 4.0-5.0 with 94% relative activity. The partial purification was achieved using 20-40% ammonium sulfate precipitation. The partial purified enzyme showed a triple band on SDS polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight of 45 kDa, 40 kDa and 35 kDa. These are desirable properties for biotechnological and industrial applications.

# CHAPTER 5

# GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

Biotechnology is the knowledge of science and technology, which use the organisms or products of organisms in various applications. Enzymes are the products of microbial technology. The enzyme applications profit to biotechnology. There is a high demand for thermostable enzymes in the industrial processing.

The purpose of this work is to investigate the characterization of cellulase and xylanase from *Lentinus polychrous* Lev. LP-PT-1. The relatively good thermostability of xylanase and cellulase was maintained at high temperature (60°C). These results considered from effect of pH on xylanase and cellulase activity. The increasing levels of xylanase were obtained at pH 3.0 to pH 5.0 and continued to decrease slightly with an increase in pH 5 to pH 8. The increasing levels of cellulase were obtained at pH 4.0 to pH 5.0 and decreased drastically with an increase in pH 5 to pH 8.0. However, the optimum pHs of cellulase and xylanase activities were at pH range 4.0-6.0. The increasing cellulase and xylanase reaction of pH 5.0 resulted highest trend of their activities.

The xylanase and cellulase production from *Lentinus polychrous* Lev.LP-PT-1 under solid state fermentation indicated that the optimal temperature of xylanase and cellulase production were found to be at 35°C. The 75% and 80% moisture levels showed significantly higher cellulase production than other treatments ($p < 0.05$). The 75% and 80% moisture levels showed significantly higher xylanase production than 50% and 55% moisture levels ($p < 0.05$). The inoculum level at 0.5 cm per disk for the four disks in 4g substrate culture was found to be optimum for xylanase and cellulase production by *L. polychrous* Lev.LP-PT-1.

The thermostability of xylanase and cellulase were produced by *L. polychrous* Lev. LP-PT-1, when grown on rice straw under solid state fermentation. Crude enzymes were extracted by McIlvaine buffer pH 5 at 150 rpm for 30 min. The relatively good thermostability of xylanase and cellulase was maintained at high temperature at 60°C with 89% and 81% of the relative

activity. The use of higher temperature (60°C) is inhibitory microbial growth, decreasing the possibility microbial contamination.

The cellulase was stable at pH 4.0-5.0 with 94% relative activity. The xylanase appeared to be stable over a broad pH range of 4.0–7.0. High cellulase activity was achieved at temperatures between 30 and 60°C. The xylanase activity increased with high temperature to 60°C. The optimum pHs for cellulase and xylanase activity were found in the range of pH 4.0-6.0. The partial purification was achieved using 20-40% ammonium sulfate precipitation. The partial purified enzyme showed a triple band on SDS polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight of 45 kDa, 40 kDa and 35 kDa.

The reduction cost of feedstock and enzyme but also the enzyme stabilities for conversion of lignocellulosic material into fermentable sugar were the alternative way for biotechnology applications.

The enzyme production under solid state fermentation with white rot fungi provide and alternative process due to its energy efficacy and environmental benignity. This study demonstrated that the white rot fungus *L. polychrous* Lev. LP-PT-1 was capable of xylanase, cellulase production. However, xylanase and cellulase production were still very low. Genetic engineering can be used to increasing enzyme production from *L. polychrous* Lev. LP-PT-1.

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# APPENDIX

# APPENDIX

**1. Potato dextrose agar (PDA)**

39 g Potato dextrose agar

1000 ml Distilled water

**2. YPD agar**

10 g Yeast extract

20 g Peptone from casein

20 g Dextrose

20 g Agar

1000 ml distilled water

**3. YPD medium**

10 g Yeast extract

20 g Peptone from casein

20 g Dextrose

1000 ml distilled water

**4. Synthetic media**

1.0g Ammonium nitrate ($NH_4NO_3$)

0.8g Potassium dihydrogen phosphate ($KH_2PO_4$)

0.2g Disodium hydrogenphosphate ($Na_2HPO_4$)

0.5g Magnesium sulphate ($MgSO_4 . 7H_2O$)

4.0g Yeast extract

1000 ml Distilled water

**5. 1% Dinitrosalicylic acid reagent (1% DNS)**

10 g 3, 5-Dinitrosalicylic acid (DNS)

16 g Sodium hydroxide (NaOH)

300 Potassium sodium tartrate ($KnaC_4H_4O_6.4H_2O$)

1000 ml Distilled water

**6. 1% Xylan**

1 g Xylan from oat spelts

100 ml Buffer pH 5.0

**7. 1% Carboxymethylcellulose sodium salt** (CMC)

1 g Carbosymethylcellulose sodium salt

100 ml Buffer pH 5.0

**8. 1 M Citrate buffer**

210 g Citric acid monohydrate ($C_6H_8O_7$. $H_2O$)

50 g Sodium hydroxide (NaOH)

1000 ml Distilled water

**9. Methylene blue solution**

0.3 g of methylene blue

30 mL of 95% ethyl alcohol

0.01 g of potassium hydroxide

100 mL of DI water

**10. Gram's iodine**

2.0 g KI

1.0 g iodine

300 ml distilled water

**11. McIlvaine buffer solution**

11.1 0.2M Disodium hydrogen phosphate

28.392 g Disodium hydrogen phosphate ($Na_2HPO_4$)

1000 ml Distilled water

11.2 0.1M Citric acid

21.014 g Citric acid ($C_6H_8O_7$. $H_2O$ )

1000 ml Distilled water

**Table A.1** McIlvaine buffer solution

(Wikipedia, 2012(a): http://en.wikipedia.org/wiki/Buffer_solution)

| pH | 0.2M $Na_2HPO_4$ (ml) | 0.1M Citric Acid (ml) |
|---|---|---|
| 3.0 | 20.55 | 79.45 |
| 4.0 | 38.55 | 61.45 |
| 5.0 | 51.50 | 48.50 |
| 6.0 | 63.15 | 36.85 |
| 7.0 | 82.35 | 17.65 |
| 8.0 | 97.25 | 2.75 |

**12. 0.5% glucose and 0.5% xylose**

0.5 g Glucose

0.5 g Xylose

100 ml distilled water

**13. 0.1% Ethanol (w/v)**

0.1 g Ethanol

100 ml distilled water

**14. 0.1% Butanol (w/v)**

0.1 g Butanol

100 ml distilled water

**15. Standard ethanol (0.0-0.1% w/v)**

**Table A.2** Standard ethanol (0.0-0.1% w/v)

| No | Standard ethanol (% w/v) | Standard ethanol (0.1% w/v) (µl) | Standard butanol (0.1% w/v) (µl) | Distilled water (µl) |
|---|---|---|---|---|
| 1 | 0.02 | 100 | 500 | 400 |
| 2 | 0.04 | 200 | 500 | 300 |
| 3 | 0.06 | 300 | 500 | 200 |
| 4 | 0.08 | 400 | 500 | 100 |
| 5 | 0.10 | 500 | 500 | - |

**16. Glucose (1 mg/ml)**

0.1 g Glucose

100 ml Distilled water

**17. Xylose (1 mg/ml)**

0.1 g Xylose

100 ml Distilled water

**Table A.3** Standard sugar (0.0-1.0 mg/ml)

| No | Standard sugar 1 mg/ml (ml) | Distilled water (ml) | Standard sugar solution (mg/ml) |
|---|---|---|---|
| 1 | 0.0 | 1.0 | 0 |
| 2 | 0.2 | 0.8 | 0.2 |
| 3 | 0.4 | 0.6 | 0.4 |
| 4 | 0.6 | 0.4 | 0.6 |
| 5 | 0.8 | 0.2 | 0.8 |
| 6 | 1.0 | 0.0 | 1.0 |

**17. Calculation of enzyme activity**

One unit of enzyme activity was defined as the amount of enzyme, releasing 1 μmol of reducing sugars per minute.

1 mol Glucose = 180 g glucose

1 μmol Glucose = 180 μg glucose = 0.18 mg glucose

$$\text{U/ml} = \frac{\text{reducing sugars mg of 1 ml crude enzyme (mg/ml)}}{(0.18\text{ mg}) \times \text{min}}$$

$$\text{U/gds} = \frac{\text{U/ml} \times \text{total volume of enzyme extract (ml)}}{\text{gram solid of raw material (g)}}$$

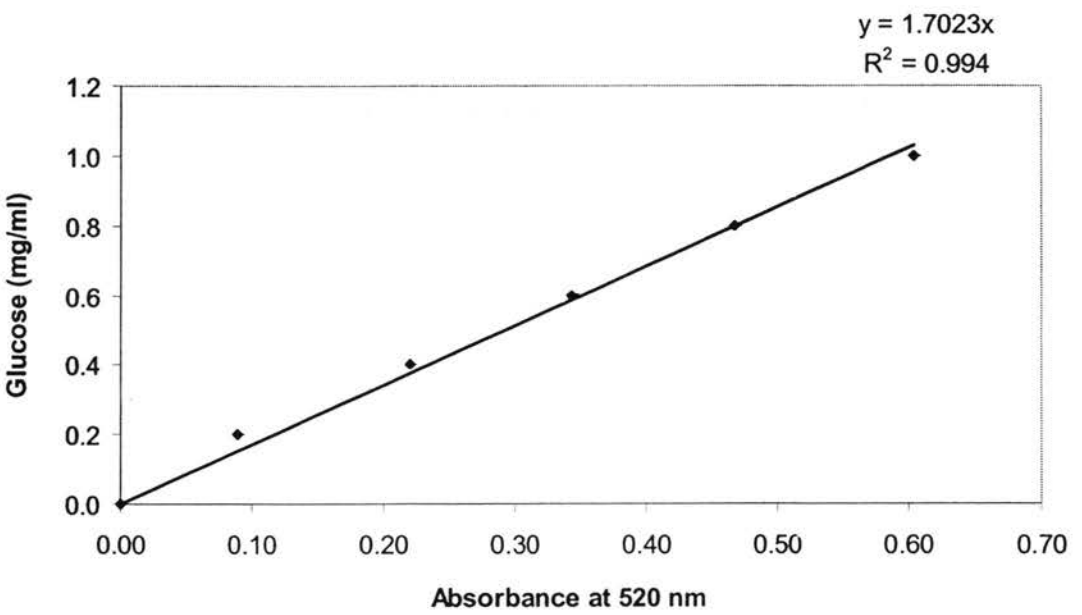


**Figure A.1** Calibration curves for glucose standard.

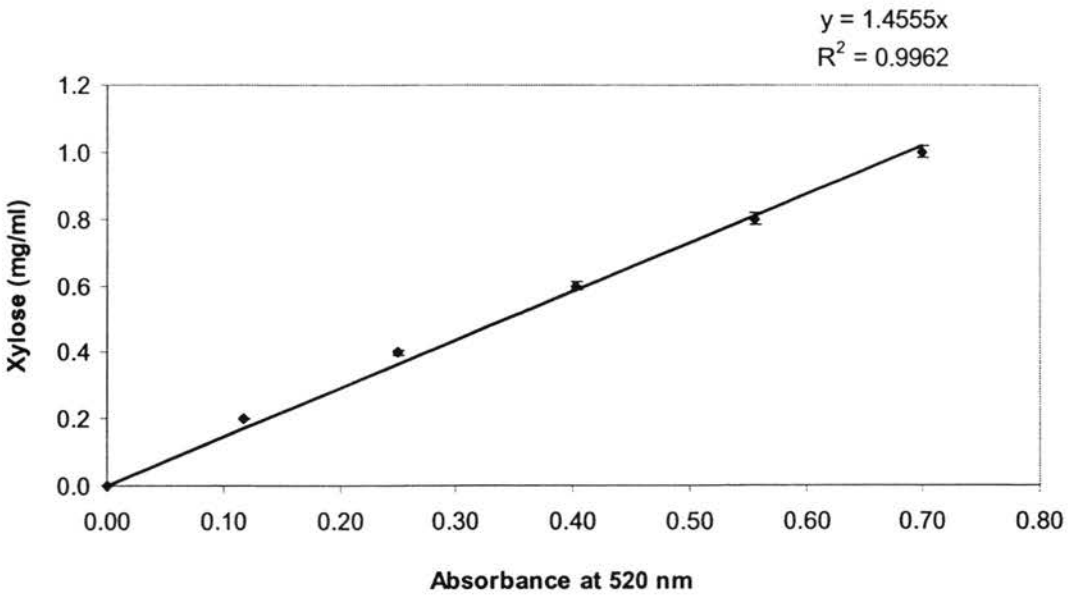


**Figure A.2** Calibration curves for xylose standard.

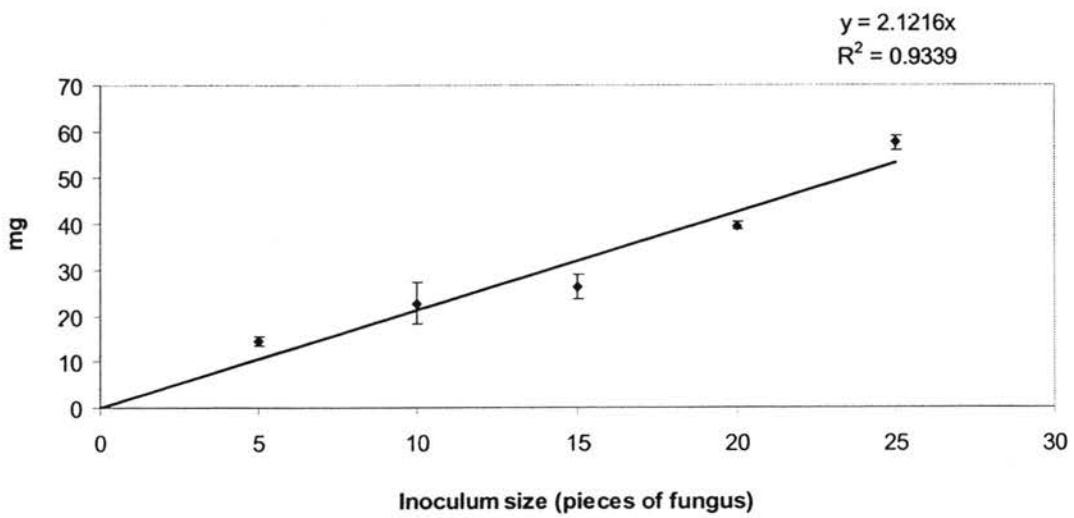


**Figure A.3** Calibration curves for inoculum size of fungus.

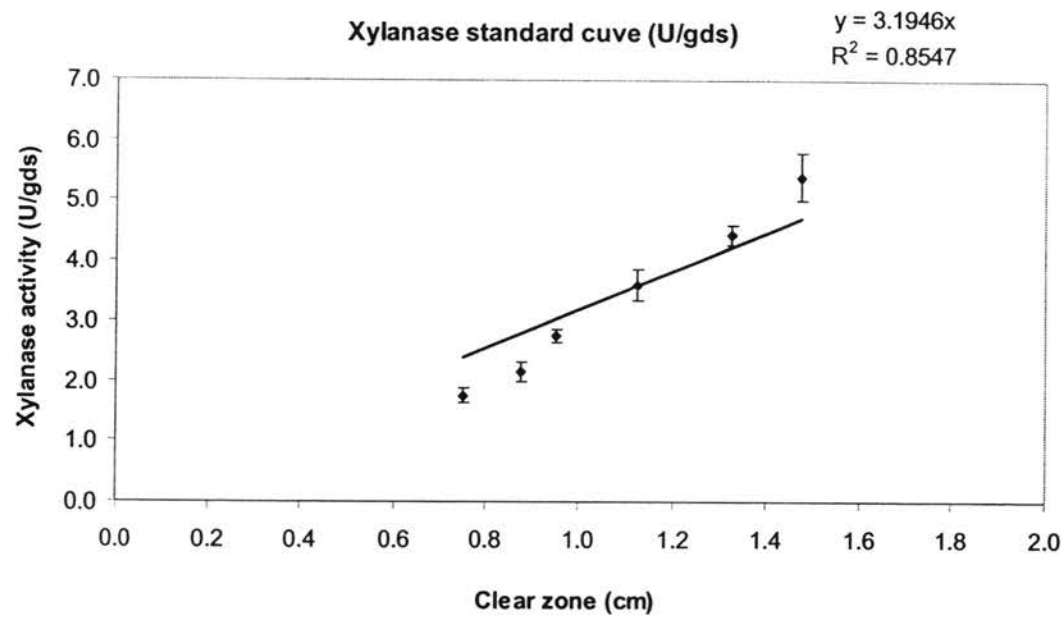


**Figure A.4** Calibration curves for xylanase standard

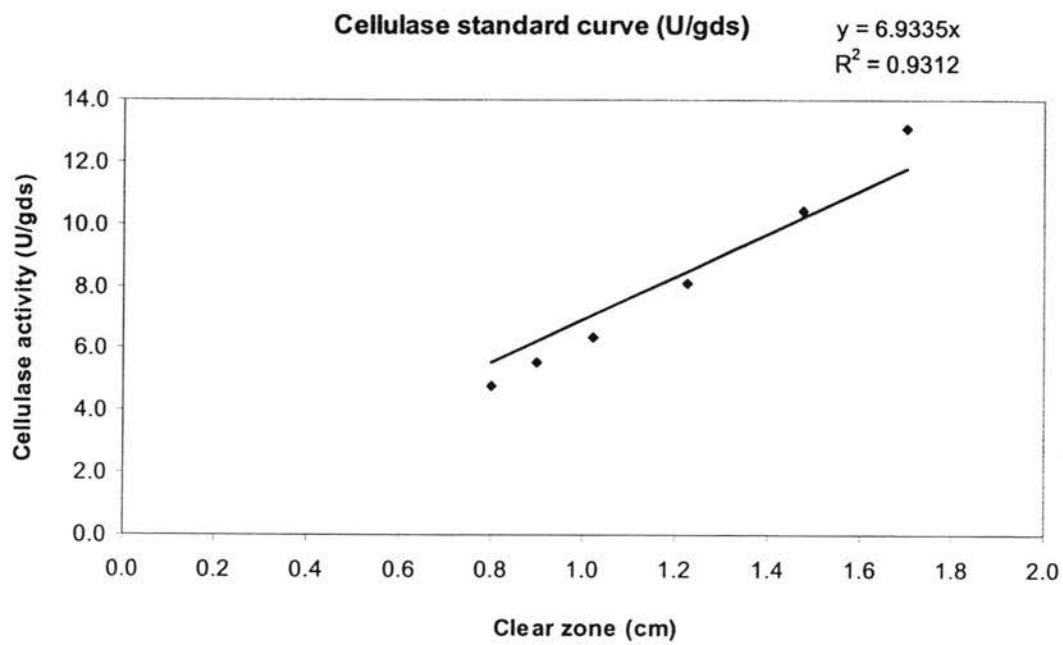


**Figure A.5** Calibration curves for cellulase standard

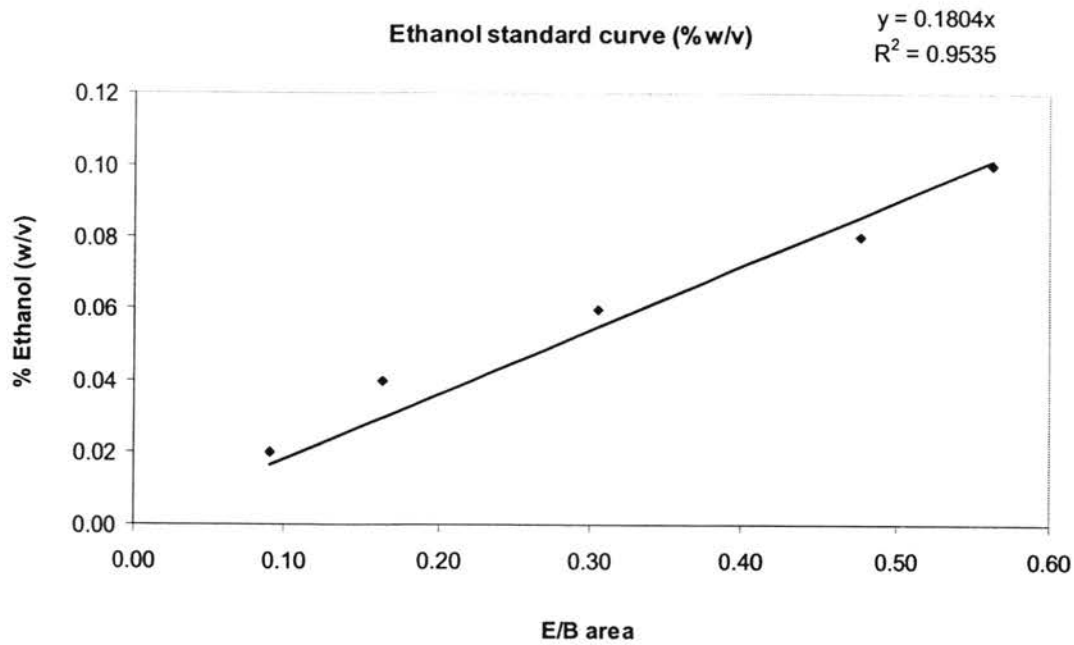


**Figure A.6** Calibration curves for ethanol standard

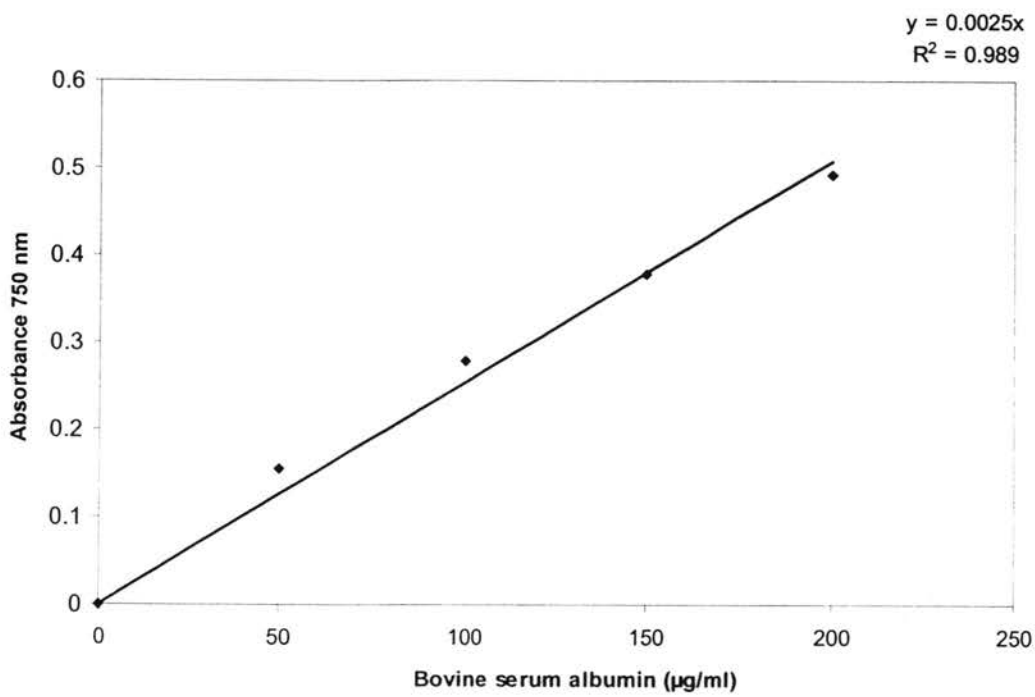


**Figure A.7** Calibration curves for protein