SOLID SUBSTRATE FERMENTATION OF RICE STRAW BY *Trichoderma viride* IBRL-TCS06 FOR FERMENTABLE SUGARS PRODUCTION

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SOLID SUBSTRATE FERMENTATION OF RICE STRAW BY *Trichoderma viride* IBRL-TCS06 FOR FERMENTABLE SUGARS PRODUCTION

By

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For My Dearest Family

&

My Best Friends

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LIST OF SYMBOLS

Symbol	Meaning
β	Beta
α	Alpha
p	Para
℃	Degree Celsius
%	Percentage
±	Plus minus
=	Equal
+	Plus
-	Minus

LIST OF ABBREVIATION

ATP	Adenosine 5'-triphosphate
A_w	Water activity
AOAC	Association of Official Analytical Chemists
BERNAS	Padiberas Nasional Berhad
C	carbon
Ca	Calcium
$CaCl_2.2H_2O$	Calcium chloride dehydrate
$C_9H_{11}NO$	*p*-dimetil-amino benzaldehide
cm	Centimeter
CMC	Carboxymethyl cellulase
Co	Corporation
CO_2	Carbon dioxide
$CoCl_2.6H_2O$	Cobaltous chloride hexahydrate
COOH	Carboxyl group
$CuSO_4.5H_2O$	Copper sulphate
Da	Dalton
DOE	Development of Energy
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
E.C.	Enzyme Commision
e.g.	Example

et al.	And all
$FeSO_4.7H_2O$	Ferrous sulfate heptahydrate
g	Gram
g/l	Gram per litre
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
HMF	Hydroxymethylfurfural
i.e.	In other word
IEA	International Energy Agency
KH_2PO_4	Potassium dyhydrogen phosphate
KOH	Potassium hydroxide
$KNaC_4H_4O_6 \cdot 4H_2O$	Potassium Sodium Tartrate
L	Litre
M	Molar
mg/ml	Milligram per milliliter
$MgSO_4$. $7H_2O$	Magnesium sulphate heptahydrate
min	Minute
mM	Millimolar
mm	Millimetre
mU/g	Milliunit per gram
µmol	Micromole
µg	Microgram
N	Nitrogen

NaOH	Sodium hydroxide
NA	Not available
$Na_2C_4H_4O_6$	Sodium tartrate
Na_2CO_3	Sodium carbonate
$Na_2HAsO_4.7H_2O$	Disodium orthoarsenate
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Ammonium dyhydrogen phosphate
Na_2SO_4	Sodium sulphate
$(NH_4)_6Mo_7O_{24}.4H_2O$	Ammonium molibdate
NH_4NO_3	Ammonium nitrate
$(NH_4)_2SO_4$	Ammonium sulfate
nm	Nanometer
OD	Optical density
P	phosphorus
P >	Probability more than
P <	Probability less than
PDA	Potato Dextrose Agar
RH	Relative humidity
rpm	Revolutions per minute
SEM	Scanning electron microscopy
Sdn	Sendirian
sp.	Species
SmF	Submerged fermentation

SSC	Solid substrate cultivation
SSF	Solid substrate fermentation
TAPPI	Technical Association of the Pulp and Paper Industry
TEM	Transmission electron microscopy
U/g	Unit per gram
U. K	United Kingdom
USA	United State of America
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
X	Multiply
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

FERMENTASI SUBSTRAT PEPEJAL OLEH *Trichoderma viride* IBRL-TCS06 MENGGUNAKAN JERAMI PADI UNTUK PENGHASILAN GULA TERFERMENTASI

ABSTRAK

Kajian ini memberi tumpuan kepada penghasilan gula terfermentasi oleh tindakan enzim melalui sistem fermentasi substrat pepejal (SSF). Sekam padi dan jerami padi telah digunakan sebagai substrat untuk menyaringkan mikroorganisma yang berpotensi dalam menghasilkan gula terfermentasi melalui SSF. Sebanyak lima jenis kulat telah diuji iaitu *Aspergillus niger* USM AI-I, *A. niger* USM AI-II, *A. niger* USM AI-F4, *Trichoderma viride* IBRL-TCS06 and *Phanerochaete chrysosporium*, sama ada secara kultur tunggal atau kultur campuran. Keputusan menunjukkan *Trichoderma viride* IBRL-TCS06 berkesan dalam mendegradasi jerami padi untuk menghasilkan gula terfermentasi yang tertinggi iaitu sebanyak 18.13±0.67 mg/g substrat dengan pertumbuhan kulat sebanyak 1.46±0.08 mg glukosamin/g substrat. Aktiviti enzim selulase, xilanase dan mananase pula adalah masing-masing sebanyak 20.35±0.72 U/g substrat, 47.30±0.68 U/g substrate dan 5.47±0.56 U/g substrat dengan keadaan pengkulturan 5 g substrat (0.75 mm saiz substrat), 80% (i/b) kandungan air dan $1x10^6$ spora/ml pada suhu bilik (28±2°C) selama satu minggu. Berbanding dengan pengkulturan tunggal, pengkulturan bercampur menghasilkan kandungan gula terfermentasi yang rendah dengan aktiviti enzim selulase, xilanase dan mannanase yang rendah. Pengolahan substrat adalah penting untuk meningkatkan penghasilan

gula terfermentasi daripada holoselulosa dengan matlamat untuk menguraikan komponen polimer jerami padi menjadi gula monomer serta meningkatkan penukaran selulosa oleh tindakan enzim. Penghasilan gula terfermentasi yang tinggi diperoleh dengan menggunakan jerami padi yang diolahkan dengan 1.5% KOH (b/i), iaitu sebanyak 30.33±1.04 mg/g substrat gula terfermentasi dan pertumbuhan kulat sebanyak 1.59±0.08 mg glukosamin/g substrat. Pencirian parameter fizikal dan kimia bagi penghasilan gula terfermentasi dijalankan dengan penambahan 0.6% (b/b) $(NH_4)_2SO_4$ dan 0.5% (b/b) $CaCl_2.2H_2O$ dengan $1x10^5$ spora/ml tanpa pengadukan. Sebanyak 43.35±1.87 mg/g gula terfermentasi telah dihasilkan dengan pertumbuhan kulat 2.47±0.10 mg glukosamin/g substrat. Pengkulturan *T. viride* IBRL-TCS06 juga dijalankan dalam sistem dulang cetek dengan $1x10^7$ spora/ml dan pengadukan setiap 72 jam. Penghasilan maksimum gula terfermentasi adalah sebanyak 66.16±1.13 mg/g substrat dengan 2.81±0.09 mg glukosamin/g substrat pertumbuhan kulat pada hari ke-6 pengkulturan. Aktiviti-aktiviti yang didapati adalah aktiviti selulase, xilanase dan mannanase, dengan masing-masing sebanyak 129.92±6.03 U/g substrat, 152.63±5.09 U/g substrat dan 39.36±2.27 U/g substrat. Penghasilan gula terfermentasi adalah rendah berbanding dengan kajian yang dijalankan oleh Zhang dan Cai (2008), di mana mereka melaporkan bahawa 733 mg/g substrat gula terfermentasi telah dihasilkan oleh *T. reesei* ZM4-F3 apabila ditumbuhkan dengan jerami padi yang terolah dengan 2% NaOH. Walau bagaimanapun, penghasilan gula terfermentasi telah berjaya ditingkatkan dalam eksperimen ini, iaitu mencapai peningkatan sebanyak 180% (dari 23.61±0.98 mg/g substrat kepada 66.16±1.13 mg/g substrat).

SOLID SUBSTRATE FERMENTATION OF RICE STRAW BY *Trichoderma viride* IBRL-TCS06 FOR FERMENTABLE SUGARS PRODUCTION

Abstract

The research focused on the production of fermentable sugars in solid substrate fermentation (SSF). The potential use of rice husk and rice straw as substrates were used to screen for potential microorganism that capable of producing fermentable sugars under SSF. Five types of fungi were used, namely *Aspergillus niger* USM AI-I, *A. niger* USM AI-II, *A. niger* USM AI-F4, *Trichoderma viride* IBRL-TCS06 and *Phanerochaete chrysosporium*, either as a single or mixed cultures. Results showed that *Trichoderma viride* IBRL-TCS06 was efficient in degrading rice straw and producing the highest fermentable sugars approximately 18.13 ±0.67 mg/g substrate with the fungal growth of 1.46±0.08 mg glucosamine/g substrate under condition of 5 g of 0.75 mm substrate, 80% (v/w) moisture content and $1x10^6$ spores/ml for 1 week at room temperature (28±2°C). The cellulase, xylanase and mannanase activities were 20.35±0.72 U/g substrate, 47.30±0.68 U/g substrate and 5.47±0.56 U/g substrate, respectively. Mixed culture cultivation resulted in lower cellulase, xylanase and mannanase activities as well as fermentable sugars production compared to individual or mono-culture. Pretreatment was desirable to achieve the highest yield of fermentable sugars from holocellulose with the goals to decompose the polymeric components of the straw and form sugar monomers, enhance enzymatic conversion of the cellulose fraction. The best results of the pretreatment on rice straws were

achieved with 1.5% KOH (w/v), 30.33±1.04 mg fermentable sugars/g substrate and growth of 1.59±0.08 mg/g substrate. Characterization of the physical and chemical parameters for maximum fermentable sugar production were carried out with additional of 0.6% (w/w) of $(NH_4)_2SO_4$ an d 0.5% (w/w) of $CaCl_2.2H_2O$, inoculums size of $1x10^5$ spores/ml without mixing. Results showed that *T. viride* IBRL-TCS06 able to produce about 43.35±1.87 mg/g of fermentable sugars with growth of 2.47±0.10 mg glucosamine/g substrate after 6 days of cultivation. The cultivation of *T. viride* IBRL-TCS06 was then carried out in a tray system with the inoculum size of $1x10^7$ spores/ml and every 72 hours of mixing frequency. The maximum fermentable sugars production was 66.16±1.13 mg/g substrate with the fungal growth of 2.81±0.09 mg glucosamine/g substrate on the 6th-day of cultivation. A great yield of 129.92±6.03 U/g substrate of cellulase activity, 152.63±5.09 U/g substrate of xylanase activity and 39.36±2.27 U/g substrate of mannanase activity were obtained. The yield of fermentable sugars were lower compared to a study by Zhang and Cai (2008), in which they reported that about 733 mg/g substrate of fermentable sugar was produced when *T. reesei* ZM4-F3 was grown on 2% NaOH-pretreated rice straw. However, the fermentable sugar production was greatly enhanced in this experiment in which about 180% of increment in fermentable sugars production (from 23.61±0.98 mg/g substrate to 66.16±1.13 mg/g substrate).

CHAPTER ONE

INTRODUCTION

Agro-industrial residues are the lignocellulosic materials that are one of the most abundant raw materials serving as feedstock for fermentation processes. Many processes have been developed by utilizing these raw materials for the production of bulk chemical and value-added products such as bioethanol, biofertilizers, animal feeds, biopesticides and other biochemical products such as enzymes, organic acid, single cell protein, etc. The main product of lignocellulose degradation is fermentable sugars which can be used as a carbon source by numerous microorganisms (Ibrahim, 2008) before bioconversion to the value-added products. Application of agro-industrial residues in bioprocesses not only provides alternative substrates, and but also helps in solving pollution problems, which otherwise may cause their disposal.

Rice is the most widely grown food grain crop which serves as the staple diet of the population in the world. Rice cultivation yields three by-products, (i) rice straw, the vegetative residue after grain harvested, (ii) rice husks and (iii) rice bran, the residues after grain milling. Rice husk and rice straw are two of the abundant lignocellulosic wastes materials in the world. As per Food and Agricultural (FAO) statistic, world annual rice production in 2007 was about 650 million tons. Every kilogram of grain harvested is accompanied by production of 1-1.5 kg of the straw (Binod *et al*, 2010). Disposing the large amount of straw produced not only wasting resources but also causing environmental impacts. Rice husk, the byproduct of rice processing, is a cheaply

abundant resource in Malaysia, and therefore has a great potential as an industrial fermentation substrate. Rice husk is characterized by low bulk density and high ash content (18–22% by weight).

Glucose, xylose, fructose, arabinose and galactose are the fermentable sugar. Fermentable sugars can be produced via solid substrate fermentation (SSF) processes, mainly by fungi in its natural habitat. The focus in SSF application will be on screening for host-specific. The selection of a substrate for SSF process depends upon several factors mainly related with cost and availability and thus may involve screening of several agro-industrial residues and microorganisms. In SSF, the substrate not only supplies the nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells.

In all applications the primary requirement is the hydrolysis of lignocellulose into fermentable sugars by lignocellulolytic enzymes, such as cellulases, hemicellulase, pectinases, xylanases, mannanases and also lignin degrading enzymes. Bioconversion of rice straw and rice husk for the production of fermentable sugars and value-added products is flourishing as results of increasing environmental pressure. The utilization of both cellulosic and hemicellulosic sugars present in typical lignocellulosic biomass hydrolyzate is essential. The hydrolysis of polysaccharides is usually catalyzed by hydrolytic enzymes, because enzymatic hydrolysis produces better yields than acid-catalyzed hydrolysis (Pan *et al*., 2005). However, the high cost of cellulase enzymes

often restricts the large scale application of these enzymes in the bioconversion of lignocellulosic biomass.

In plant cell wall, the combination of hemicelluloses and lignin provides a protective barrier around the cellulose, which must be modified or removed before efficient hydrolysis of cellulose can take place in SSF. Besides, the crystalline structure of cellulose makes it highly insoluble and resistant to microbial attack. Therefore, to hydrolyze cellulose and hemicellulose economically in SSF, pretreatment is required to alter the structure of cellulosic biomass making cellulose more accessible to enzyme, that convert the carbohydrate polymers into fermentable sugars in SSF processes. The goal of pretreatment is to break hemicelluloses-lignin matrix and disrupt the crystalline structure of cellulose before the substrates are conducted to SSF processes. As a consequence, the pretreatment must improve the release of sugars (both from the hemicellulose and cellulose fractions) and, at the same time, avoid both the carbohydrates degradation and the formation of products that may inhibit the subsequent hydrolysis and fermentation processes (Sun and Cheng, 2005). It is desirable to increase the conversion yields of polysaccharides into monosaccharide in SSF processes and thus, to enhance fermentable sugar production at the higher productivity.

There are several important factors which affect the enzymatic hydrolysis of rice straw in SSF processes. To improve the yield and rate of the enzymatic hydrolysis, most research has focused on optimization of fermentation process steps and enhancing the enzyme activities, mainly the cellulase, xylanase and mannanase activities. For effective

operation, SSF process must provide suitable conditions for substrates colonization and lignin degradation by the fungi. At the same time, the conditions should minimize carbohydrate consumption by the delignifying fungi and any other organisms present (Reid, 1989; Lee, 1997). Typically, the parameters to be measured and controlled in SSF processes are particle size, moisture content, aeration, pH and temperature, incubation time, nutrient additions, inoculum sizes and mixing frequency.

A thorough understanding of the requirements for the optimal growth conditions for microbial in SSF is required before a bioreactor design can be chosen. Choice of bioreactor will need to be followed by further work optimization of the design and operating conditions (Hardin, 2004). Trays are by far the most common type of bioreactor used in SSF. Trays are a simple design consisting of a flat shallow tray or perforated plates in a moistening chamber, covered with a thin layer of substrate. Tray system provides a good aeration with no heat build-up or no stirring (Bellon-Maurel *et al*., 2003).

Recently, developments in the area of fermentation and bioprocess technology have proved the feasibility of applying solid substrate fermentation for commercial processes development. Lignocellulose bioconversion by SSF processes will have an important role in future biotechnologies mainly due to the favourable economy. Therefore, the need to explore the possibility of using lignocellulolytic materials of rice straw via SSF in producing high yields of fermentable sugars is crucial with the aim to lower the cost of production. In addition, the utilization of these agro industrial wastes,

on the one hand, provides alternative substrates and, on the other, helps in solving pollution problems, which otherwise may cause their disposal (Pandey *et al*., 1999). In conclusion, the potential fungus grow in SSF processes in a simple medium consisting of agricultural waste would be a promising application for fermentable sugars production and thus into other value-added products.

1.1 RESEARCH OBJECTIVES

The objectives of the current research were as follow:

- To screen and isolate potential microorganisms that has the ability to grow and degrade lignocellulosic materials in rice husk or rice straw as substrates.

- To determine effective pretreatment methods and saccharification of lignocellulosic materials into fermentable sugars in SSF.

- To characterize the parameters involved in fermentable sugars production using flask and shallow tray systems.

1.2 RESEARCH SCOPE

Five species of fungal strains obtained from Industrial Biotechnology Research Laboratory (IBRL) consisting of *Trichoderma viride* IBRL-TCS06, *Aspergillus niger* USM AI-I, *Aspergillus niger* USM AI-II, *Aspergillus niger* USM AI-F4 and *Phanerochaete chrysosporium* and two agricultural wastes designated rice husk and rice straw (obtained from Penaga, Butterworth, Malaysia), were elucidated in the production of fermentable sugars and enzyme activities under solid substrate fermentation (SSF). Experiments were conducted to evaluate the effects of individual and combination of fungal cultures on the efficiency of producing fermentable sugars. The fungi and agricultural wastes with the best potential were selected to be used for further studies in SSF. The efficiency of enzymatic hydrolysis is greatly enhanced by pretreatment in which it helps to break down the biomass structure, making it easier for penetration of fungal hyphae and the hydrolysis enzymes. Therefore, to economically hydrolyze (hemi) cellulose in SSF processes, different pretreatments on the agricultural wastes were carried out to evaluate the effects of some hydrolysis parameters in production of fermentable sugars. The improvement of enzymatic hydrolysis of agricultural waste was performed in order to determine the most effective conditions for fermentable sugars production. The conditions that were enhanced using physical parameters and chemical parameters included size of substrates particles, moisture content, mixing frequency, inoculum sizes, time of cultivation, carbon source, nitrogen source and mineral source. Observation of degradation of rice straw by *Trichoderma viride* IBRL-TCS via SSF processes was carried out with microscopic view. Based on these characterization works

in a flask system, production of fermentable sugars using agricultural waste then was improved by further investigation in a larger scale operation using a shallow tray system. In a tray system, improvement of cultural conditions was conducted, which included different thickness of substrates, moisture content, mixing frequency, inoculum sizes and time of cultivation.

CHAPTER TWO

LITERATURE REVIEW

2.1 LIGNOCELLULOLYTIC MATERIALS FROM NATURAL RESOURCES

Lignocellulosic materials include hardwoods, herbaceous crops, forestry waste, municipal solid waste and agricultural residues such as rice straws, rice husk, wheat straws, corn straws and corn stover. Lignocellulosic perennial crops are promising feedstock because of high yield, low costs, good suitability for low-quality land which is more easily available for energy crop, and low environmental impact. It is one of the most abundant natural complex organic carbons in form of plant biomass, which mainly consists of three major components; cellulose, hemicelluloses and lignin (Badhan *et al.*, 2006). These lignocellulolytic materials cannot be easily converted to simple monomeric sugars due to their recalcitrant nature (Adsul *et al.*, 2004).

Plant cell walls are the most abundant and renewable source of fermentable sugars on earth (Himmel *et al*., 1999). In recent years, there has been an increasing trend towards efficiently utilization of agro-industrial residue. Recently, some reviews have been presented on biotechnological potential of several agro-industrial residues for value-addition in SSF (Pandey, 2000b). One of the largest cellulosic agro-industrial by-products is sugarcane bagasse, a fibrous residue of cane stalks left over after the crushing and extraction of the juice from the sugar cane. It is widely used by the sugar factories as fuel for the boilers.

The complex structure of lignocelluloses in plants forms a protective barrier to cell destruction by bacteria and fungi. To make this structure suitable for conversion in fermentative processes, cellulose and hemicelluloses must be hydrolyzed into their corresponding monomers (sugars) for utilization by microorganisms (Iranmahboob *et al.*, 2002). The biological process for converting the lignocelluloses to monomeric sugars requires: (1) delignification to liberate cellulose and hemicelluloses from their complex with lignin; (2) depolymerization of the carbohydrate polymers to produce free sugars.

2.1.1 Plant cells structures

Plant cell walls are mainly composed of cellulose, hemicelluloses and lignin. Cellulose, hemicellulose and lignin are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkages (Pérez *et al*., 2002). Other organic substances (proteins, pectic substances or cutin) and silica are found in the walls and in the middle lamella. The composition and proportions of these compounds vary between plants (Prassad *et al*., 2007) as shown in Table 2.1. Condensed tannins (or proanthocyanidin polymers) may exist in higher plants. They are phenolic compounds of moderately high molecular weight and form effective cross-links with protein and other molecules (Van Soest *et al*., 1987; Saura-Calixto *et al*., 1991).

A growing plant cell is gradually enveloped by a primary wall which contains few non-orientated cellulosic microfibrils, and some non-cellulosic components such as

pectic substances. Pectins are amorphous polysaccharides (mostly galacturonic acid polymers) found in the middle lamella and can be extracted by boiling water, cold dilute acid or boiling solutions containing chelating agents such as ammonium oxalate or ethylenediamine tetraacetic. They are proportionally more common in fruits and pulps than in leaves (Bailey, 1973; Giger-Reverdin, 1995). While ageing, cell walls become thicker and at the same time, cellulose microfibrils embedded in a polysaccharide- lignin matrix settle down along quite well defined axes in order to build the secondary wall (Figure 2.1).

Table 2.1: Composition of some lignocellulosic materials (Sánchez, 2009).

Lignocellulosic residues	Lignin (%)	Hemicelluloses (%)	Cellulose (%)	Ash (%)
Hardwood stems	18-25	24-40	40-55	NA
Softwood stem	25-35	25-35	45-50	NA
Paper	0-15	0	85-99	1.1-3.9
Rice straw	18	24	32.1	NA
Sugarcane bagasse	19-24	27-32	32-44	4.5-9
Wheat straw	16-21	26-32	29-35	NA
Coffee pulp	18.8	46.3	35	8.2
Barley straw	14-15	24-29	31-34	5-7
Oat straw	16-19	27-38	31-37	6-8
Switch grass	12.0	31.4	45	NA
Nut shells	30-40	25-30	25-30	NA
Coffee pulp	18.8	46.3	35	8.2
Banana waste	14	14.8	13.2	11.4

NA= Not available

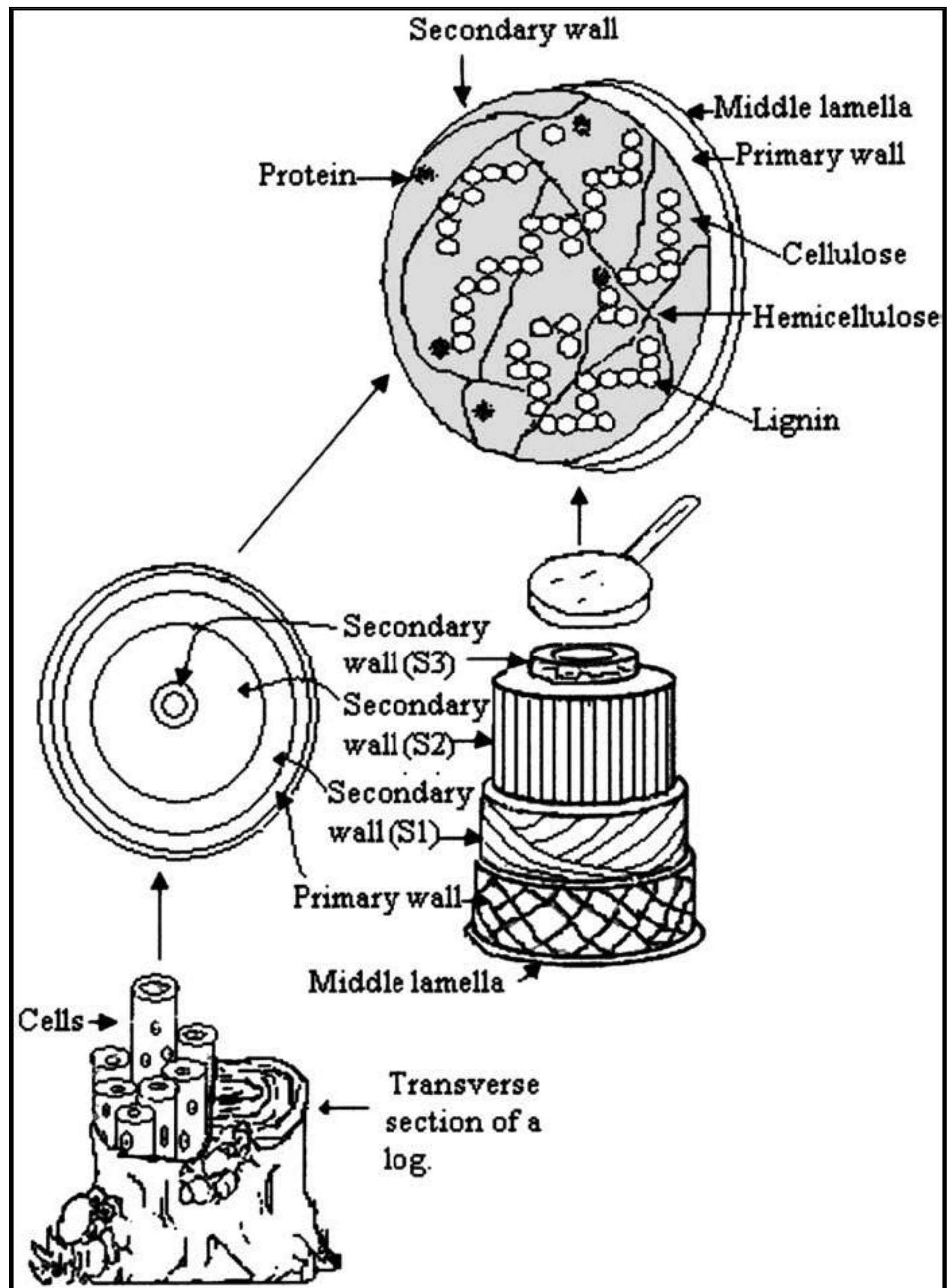

Figure 2.1: Composition of lignocellulosic residues. Cellulose, hemicellulose and lignin (Sánchez, 2009).

2.1.2 Important constituents of lignocellulosic materials

2.1.2.1 Cellulose

Cellulose (40-60% of the dry biomass) occurs predominantly in plants forming their major structural component. It is one of the world's abundant natural compounds and a major waste product from agricultural wastes (Lee, 2005). Cellulose is a high molecular weight linear polymer of D-glucose units (Figure 2.2) linked together by ß-1,4 glucosidic bonds, which can appear as a highly crystalline material (Fan *et al.*, 1982) which gives it strength against disruption by chemical solutions. The hydrogen bonding between cellulose molecules results in the formation of highly ordered crystalline regions that are not ready accessible to water.

The crystalline cellulose can account for approximately 50-90% of the total cellulose, while the remainder being composed of more disorganized amorphous cellulose (Jacobsen and Wyman, 2000). These complex structures of crystalline celluloses are not susceptible to hydrolysis by single enzyme. It is a linear polymer of glucose units, which can be hydrolyzed by the action of ß-glucosidases, cellobiohydrolases and endoglucanases (Coughlan, 1985; Bisaria and Mishra, 1989).

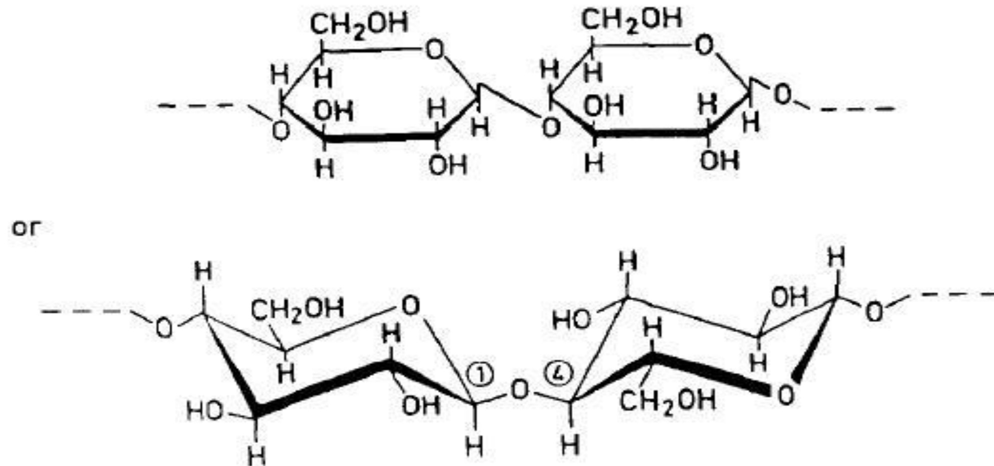

Figure 2.2: Cellobiose, the repeating unit of cellulose (Lutzen *et al.*, 1983).

2.1.2.2 Hemicellulose

Hemicelluloses (20-40% of the dry biomass) are plant heteropolysaccharides widely distributed in nature. It is the second most abundant heteropolymers present in nature (Viikari *et al.*, 1992). It is a branched polysaccharides consisting of the pentoses D-xylose and L-arabinose, and the hexoses D-mannose, D-glucose, D-galactose and uronic acids (Saka, 1991) according to the main sugar components in their backbones. The hemicelluloses branched and linear polysaccharides bound via hydrogen bonds to the cellulose microfibrils and consequently attach to lignin in the plant cell wall. The hemicelluloses form hydrogen bonds with the cellulose microfibrils, increasing the stability of the cellulose-hemicellulose-lignin matrix.

Hemicellulose, because of its branched, amorphous nature and therefore is relatively easy to hydrolyze and not as resistant as cellulose to solubilization and hydrolysis. These polysaccharides are associated with cellulose and lignin, and play an important structurally-supportive role in building up of plant cell walls (Bidlack *et al.*1992; Nakamura, 2003). The two most important and representative hemicelluloses are the hetero-1,4,-ß-D-xylans and the hetero-1,4,-ß-D-mannans (Jiang *et al.,* 2006) .

Xylan backbones in native plant cell walls are extensively acetylated (Holtzapple, 1993). Softwoods (e.g., spruce and pine) and hardwood (e.g., willow, aspen and oak) differ in structure and composition of the hemicelluloses (Saka, 1991). In hardwoods, the O-acetyl groups are combined with the xylose units, whereas in the softwoods, they are combined with the mannose and glucose units of glucomannans (Kim and Holtzapple, 2006). Several studies have shown that removing acetyl groups from xylan greatly enhanced biomass digestibility through increased swell ability, thereby increasing the hydrolysis rate (Grohmann *et al.*1989; Kong *et al.,* 1992; Zhu *et al.*, 2008).

In hardwood xylan, the backbone chain consists of xylose units which are linked by β-(1, 4)-glycosidic bonds and branched by α-(1, 2)-glycosidic bonds with 4-*O*-methylglucuronic acid groups. Mannans are the major polysaccharide of softwood hemicelluloses, accounting for 15-20% in softwood but only 5% in hard wood (Timell, 1967; Lin and Chen, 2004). The main chain of softwood mannans,

galactoglucomannans, is comprised of a linear chain consists of 1, 4-linked β-D-glucopyranose and β-D-mannopyranose units. These variable structure of hemicelluloses required hemicellulase for its complete hydrolysis (Eriksson *et al*., 1990b). Softwood hemicellulose has a higher proportion of mannose and glucose units than hardwood hemicelluloses, which usually contains a higher proportion of xylose units (Palmqvist and Bärbel, 1999).

2.1.2.3 Lignin

Lignin (10-25%) is a structurally complex aromatic biopolymer. It is a highly-branched, three dimensional amorphous heteropolymer and non-water soluble with a wide variety of functional groups providing active centres for chemical and biological interactions. It is substituted of phenylpropane units (benzene ring with a tail of three carbons) joined together by different types of linkages (Ohkuma *et al*., 2001) that hold together cellulose and hemicellulose components of woody biomass. The complex polymer of phenylpropane units cross-linked to each other with a variety of different chemical bonds to form a large molecular structure. The monomeric building unit of lignin is guaiacyl unit and syringyl unit (Marita *et al.*, 2001).

Lignin is an integral cell wall constituent which provides plant strength and resistance to microbial degradation (Tuomela *et al*., 2000). It is present in all lignocellulosic biomass. It is found in the secondary wall and middle lamella of higher plants (Darah and Ibrahim, 2004). Lignin is linked to both hemicellulose and cellulose,

forming a physical seal that acts as an impenetrable barrier in the plant cell wall. It is extremely resistant to chemical and enzymatic degradation in comparison to polysaccharides and other naturally occurring biopolymers. It is present in the cellular wall to give structural support, impermeability and resistance against microbial attack and oxidative stress, thus, must be removed to make the carbohydrates available for further transformation processes. Therefore, biological degradation is achieved mainly by fungi. It is degradable by only few organisms, into higher value products such as organic acids, phenols and vanillin (Hamelinck *et al*., 2005).

2.1.3 Industrial application of lignocellulosic materials

Lignocellulosic biomass such as agricultural and forestry residues, municipal solid waste and dedicated crops provide a low cost feedstock for biological production of fuels and chemicals, which offer economic, environmental and strategic advantages. The high cost of cellulase enzyme production hinders the application of these enzymes to bioethanol production. Therefore, lignocellulosic biomass (energy crops) and wastes (forest, agricultural, and municipal) could offer a huge renewable resource for second generation biofuels production (Tengerdy and Szakacs, 2003; Hahn-Hägerdal *et al*., 2006).

i. Textile Industry

Bio-stoning and bio-polishing are the best-known current textile applications of cellulase. In textile industry, cellulase has been widely exploited such as the stone washing of jeans and finishing of cotton fabrics (Cao and Tan, 2002). During the bio-

stoning process, cellulases act on the cotton fabric and break off the small fibre ends on the yarn surface, thereby loosening the indigo, which is easily removed by mechanical abrasion in the wash cycle. Replacement of pumice stones by a cellulase based treatment reduce wear and tear of washing machines and shorten the treatment times, besides increasing the productivity of the machines because of high loading. Bio-polishing is usually carried out during the textile wet processing stage and includes desizing, scouring, bleaching and finishing. During this process, the cellulases act on small fibre ends that protruded from the fabric surface. Then, the mechanical action will remove the fibres and polish the fabrics (Bhat, 2000)

ii. Food Industry

Hemicellulose is of particular industrial interest since these are readily available bulk source of xylose from which xylitol can be derived. Xylitol is a sweetener similar to sucrose, which is found at low concentrations in fruits and vegetables. It has a broad range of applications in the food industry as well as in healthcare (Náhlík *et al*., 2003). The enzyme hydrolysis of xylan lies in the basis of its utilization as an energy source in animal feed or in different biotechnological processes (Kulkarni *et al.*, 1999). The partial enzyme hydrolysis of xylan changes its physical and chemical properties, which concerns the quality of different products of the food and flavour industry. The growing interest in xylanase production for industrial application is its importance in the bioconversion of agro-industrial residues, as well as food and beverage improvers, in bakery products or for the clarification of wines and fruit juices (Júlio *et al.*, 2005). In brewing, xylanase is applied in filtering improvement (Yin *et al*., 2005). The utilization

of xylanase in bread-making significantly improves the desirable texture, loaf volume and shelf life of bread (Courtin and Delcour, 2002; Dutron *et al*., 2004). Mannanases have been useful tested in several industrial processes, such as the extraction of vegetables oils from leguminous seeds and viscosity reduction of the extracts during the manufacture of instant coffee (Tamaru *et al.*, 1995). It can be useful in several processes in the food, feed, as well as in the pulp and paper industries (McCleary, 1988). Tannase is extensively used in the preparation of instant tea, wine, beer and coffee-flavored soft drinks and also as additive for detannification of food (Lokeswari and Raju, 2007).

iii. Biopulping and biobleaching

In the pulp and paper industry, xylanase enzymes enhance the bleaching of pulp, thereby decreasing the amount of chlorine-containing compounds in the process and the subsequent discharge of organochlorines in the effluent (Beg *et al*., 2001). The xylanase from *T. reesei* has been reported to act uniformly on all accessible surfaces of kraft pulp and to be effective during bio-bleaching. Both xylanase and mananase have a synergistic action in the biobleaching of the wood pulp, significantly reducing the amount of chemicals used (Khanongnuch *et al.*, 1998). The aim of cellulase and hemicellulase treatment is either to improve the beatability response or to modify the fibre properties. The addition of cellulase and hemicellulase after beating is to improve the drainage properties of pulps, which determine the speed of paper mills. According to Cao and Tan (2002), cellulase are used together with hemicellulase to improve the drainage and running of paper machines and to enhance the deinking of recycled fibers.

iv. Detergent Industry

A number of enzymes have been evaluated for application in detergent formulation, which was isolated from indigenous sources. In detergent industry, cellulases are added into laundry detergent to improve the colour brightness, hand feel and dirt removal from cotton since cellulase preparation are able to modify the structure of cellulose fibrils. Alkaline lipases and proteases are normally used in the formulation of detergent. The alkaline lipase of *Bacillus* sp. B207 and the lipase of *Pseudomonas paucimobilis* USM A were used as additives in the formulation of detergent (Khoo and Ibrahim, 2003). Apart from lipase, protease of *Cellulomicrobium* sp. with the pH stability in the range of pH 7–10 was also found to be a potential source of alkaline protease which can be used as additive in detergent formulation. Ibrahim (2008) reported that the combination of the lipase and protease resulted in higher performance of debris removal from the cotton fabrics.

2.2 ENZYMES RELATED TO THE DEGRADATION OF LIGNOCELLULOLYTIC MATERIALS

2.2.1 Cellulases

2.2.1.1 Cellulases and basic model of action

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). The cellulase enzyme

complex consists of three types of enzymes that act synergistically in cellulose hydrolysis. The complete system consists of three classes of enzymes: endoglucanase (endo-1, 4-ß-D-glucan 4-glucanohydrolase (EC 3.2.1.4), which cleave internal β-1, 4-glucosidic bonds; exobiohydrolase (1,4-ß-D-glucan cellobiohydrolases (EC 3.2.1.91), which cleave cellobiose from the ends of cellulose chains; and cellobiase (ß-glucosidase, EC 3.2.1.21), which cleaves cellobiose into glucose units.

The first step in the degradation of cellulose is where the endoglucanase attacks more or less randomly at sites within 1,4-ß-D-glucan chains in amorphous regions of cellulose or at the surface of microfibrils. This will disrupt the crystalline structure of cellulose and expose the individual cellulose polysaccharide chains by creating new free ends of the cellulose chain. Subsequently, it was attacked by cellobiohydrolase to release cellobiose from non-reducing ends of 1,4-ß-D-glucan. Cellobiohydrolase is the major component of the fungal cellulase system and can hydrolyze highly crystalline cellulose (Esterbauer *et al*., 1991; Rowell, 1992).

Generally, these two enzymes work synergistically in the hydrolysis of cellulose resulted in soluble oligomer and cellobiose. Cellobiase then hydrolyzes the cellobiose and water-soluble cellodextrins to glucose. β-glucosidase is a key enzyme in the complete hydrolysis of cellulose to glucose molecules. The lack of this enzyme causes an accumulation of cellobiose, which inhibits the action of cellobiohydrolases (CBH) and endoglucanases (EG), thereby decreasing the rate of hydrolysis (Gusakov and Sinitsyn, 1992).

According to Lee (1997), the crystalline structure of cellulose is highly resistant to enzymatic attack and most of the glucan chains in microfibrils are not accessible to enzymes, and any bonds cleaved by endoglucanase action can readily be re-formed owing to the stable orientation of the glucan chains. Thus, degradation of crystalline cellulose requires the synergistic action of both endoglucanase and exoglucanase in which the exoglucanase rapidly removes cellobiose units from the newly created ends formed by endoglucanase action and thus preventing the re-formation of glucosidic bonds.

2.2.1.2 Production of cellulases

In prior publications, various agricultural residues such as corn stover, wheat straw, rice straw, bagasse, etc. were used in cellulase production (Rao *et al.*, 1983; Chahal *et al.*, 1996). Production of cellulase in SSF using various substrates, microorganisms and nutrient solutions has been reported (Yang *et al.*, 2004; Awafo *et al.*, 2000; Jecu, 2000).

Various agricultural substrates and microbial cultures have been used successfully in solid substrate fermentation for cellulase production (Chahal, 1985; Madamwar *et al.*, 1989; Jecu, 2000). Many fungi and bacteria secrete cellulases on the cellulose complex (Rajoka and Malik, 1997; Kalogeris *et al.*, 2003), but fungi get the most research attention because of their aerobic growth conditions and fair production rate (Sun and Cheng, 2002). Bacteria belonging to *Clostridium, Cellulomonas, Bacillus,*

Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora, and *Streptomyces* can produce cellulases (Bisaria, 1991). Cellulase can be produced by fungi under a wide variety of growth conditions.

Fungi that have been reported to produce cellulase include *Sclerotium rolfsii, Phanerochaete chrysosporium* and species of *Trichoderma, Aspergillus, Schizophyllum* and *Penicillium* (Sternberg, 1976; Fan *et al*, 1987; Duff and Murray, 1996). According to Eveleigh (1987), the species of *Trichoderma* are by far the best cellulases producers. The cellulolytic fungi *T. viride* and *T. reesei* have been extensively studied for their cellulose production (Domingues *et al*., 2000). To enhance the cellulose titer, various mutants of *Trichoderma* have been developed, among which *T. reesei* RUT C30 is of industrial interest because of its high cellulose production level as well as its ability to grow on waste cellulosic materials (Wen *et al.,* 2005).

Aspergillus is a superior β-glucosidase producer; however the most widely used cellulase from *T. reesei* is poor in β-glucosidase and thus restricts the conversion of β-glucosidase to glucose. The accumulation of β-glucosidase will cause severe feedback inhibition to the cellulase reaction. Therefore, for complete depolymerization of cellulose, ß-glucosidase from *Aspergillus* is used in conjunction with endo and exoglucanase from *Trichoderma* (Lee, 2005).

2.2.2 Hemicellulases

2.2.2.1 Hemicellulases and basic model of action

Hemicellulase is a diverse group of enzyme that works synergistically to break down hemicelluloses for complete hydrolysis of hemicelluloses. Major hemicellulose-degrading enzymes are the xylanases and mannases. Xylanases are the most studied hemicellulases because xylan is the principal constituent of hemicelluloses in plants (Biely, 1985; Okeke, 1995). The molecule of xylan consists of a β-1,4-linked D-xylose backbone and can be substituted by different side groups such as L-arabinose, D-galactose, acetyl, feruloyl, p-coumarouyl and glucuronic acid residues (Izydorczyk and Biliaderis, 1995; Subramanyan and Prema, 2002).

Because of xylan heterogeneity, the xylanolytic enzyme system carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and ρ-coumaric acid) esterase. The best known of these are endo- β-1,4-xylanase (EC 3.2.1.8), which cleaves the internal main chain xylosidic linkages and β-xylosidase (EC 3.2.1.37) which cleaves xylooligosaccharides to produce xylose. In addition to these two enzymes, several accessory enzyme activities are necessary for debranching the substituted xylans (Haltrich *et al*., 1996). The α-arabinofuranosidase and α-glucuronidase remove the arabinose and 4-*O*-methylglucuronic acid substituent, respectively, from the xylan backbone. The esterases hydrolyze the ester linkages between xylose units of the xylan and acetic acid

(acetylxylan esterase) or between arabinose side chain residues and phenolic acids, such as ferulic acid (ferulic acid esterase) and *p*-coumaric acid (*p*-coumaric acid esterase).

Hemicellulolytic microorganisms able to produce specific enzymes to cleave the individual bonds in heteromannans consist of mannose and glucose in the main chain. These enzymes include β- or α-mannanases, mannosidases, glucosidases, galactosidases and acetyl mannan esterases. According to Gübitz *et al.*, (1996), all endo-1,4-β-mannanases (EC 3.2.1.78) cleave the main chain of mannans, yielding mainly mannotriose and mannobiose, while only a few β-mannanases also liberate mannose from mannans. Subsequently, mannooligosaccharides such as mannobiose and mannotriose are further cleaved into mannose by β-mannosidases (EC 3.2.1.25). The side groups are removed by a number of enzyme; α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.6), α-L-arabinofuranosidase (EC 3.2.1.55), and α-galactosidase (EC 3.2.1.22) (Jørgensen *et al.*, 2003)

2.2.2.2 Production of hemicellulases

Generally, hemicellulases are produced along with cellulases during the growth of microorganisms on substrate containing cellulose and xylan. Many microorganisms including bacteria, yeast, actinomycetes and filamentous fungi are reported to produce xylanase (Dekker and Richard, 1976; Fang *et al.*, 2007). There are many reports related to the production of endoxylanase by submerged fermentation (SmF) using bacteria and solid substrate fermentation (SSF) by fungi (Asha and Prema, 2006).

Many bacterial and fungal species are able to utilize xylan as a carbon source because of their synthesis of xylanases. Filamentous fungi such as *Aspergillus* sp. and *Trichoderma* sp. is of particular interest, because they can excrete higher levels of xylanase than yeast and bacteria (Subramanium and Prema, 2002). *T. reesei* is a filamentous mesophilic fungus that has been shown to secrete large amounts of efficient xylan-degrading enzymes (He *et al*., 2009).

Most of the mesophilic xylanase known to date are optimally active at temperature below 50°C and act in acidic or neutral pH ranges. Only few xylanase are reported to be cellulase-free as well as active and stable at alkaline pH and high temperature (Collins *et al*., 2005). Szendefy *et al.* (2003) have demonstrated that SSF can successfully be used for efficient xylanase production by *Thermomyces lanuginosus* using hardwood (eucalyptus) soda-anthraquinone pulp as the sole carbon source and enzyme inducer. High ß-xylosidase activity (176 U/g) was produced from *A. niger* kk2 isolate when grown on solid substrate fermentation using rice straw as substrates. However, no ß-xylosidase activity was detected when *A. ustus* was grown on rice straw through solid substrate fermentation. On the other hand, low ß-xylosidase activity was detected from *Penicillium capsulatum* after growth on solid-state fermentation using beet pulp as substrate (Kang *et al*., 2004).

In Jiang *et al*. (2006) studies, they found that *B. subtilis* WY34 produced a high level of extracellular mannanase during growth on 2% konjac powder at 50°C. Maximum level of mannanase (1105.00 U/ml) was obtained after four days of