PRODUCTION OF CELLULASE ENZYME FROM *Aspergillus terreus* SUK-1 USING SUGAR CANE WASTE: THE EFFECT OF SUBSTRATE CONCENTRATION AND ASSAY TEMPERATURE

MOHD NAZIP BIN KASSEBULLAH

A thesis submitted in fulfillment of the
requirements for the award of degree of
Bachelor of Chemical Engineering

Faculty of Chemical Engineering and Natural Resources
Kolej Universiti Kejuruteraan dan Teknologi Malaysia

NOVEMBER 2006

DECLARATION

‘I declare that thesis is the result of my own research except as cited references.
The thesis has not been accepted for any degree and is concurrently submitted
in candidature of any degree.”

Signature	: ………………………………….
Name of Candidate	: Mohd Nazip bin Kassebullah
Date	: 27 November 2006

To my beloved parent

ACKNOWLEDGEMENT

Thanks to Puan Syarifah and En.Rozaimi for their help in assisting myself to complete the research and this thesis. My companion, Anuar, Nurhayati and others as well who contributes ideas and vital information during the completion of this research and thesis. Hope this research may give benefits everybody in future. May Allah bless you all and help us in the future as well.

ABSTRACT

Natural by product of agricultural waste can be turned to products of commercial interests such as ethanol, glucose and single cell protein. Much effort from scientists and researches all around the world has been put to extend the full use of agricultural waste. Reports of cellulase enzyme production from the bioconversion of lignocellulosics materials has much been made. However there is still much space to find the most suitable condition. In this research, sugar cane bagasse which is used as substrate is subjected to physical treatment using milling and grinding. After that, sugar cane bagasse is use as substrate in the production of cellulase enzyme using *Aspergillus terreus* SUK-1 fungus. Cellulase enzyme production was studied using submerged fermentation under shaking. The production was run in a lab scale using Erlenmeyer flask only. After that the enzyme was assayed at different temperature. The purpose of this research was to investigate the effect of substrate concentration on yield of cellulase and the optimum temperature where the yield of enzyme was the highest. Analysis is based on the activity of carboxymethylcellulase and xylanase enzymes. Result shows that the peak of xylanase activities was 0.011322 U/ml on day 5 using 5g SCB as substrate and fermentation temperature of 50°C while carboxymethylcellulase peak was 0.007985049 U/ml of activity using 3g SCB as substrate and fermentation temperature of 40°C. Xylanase activity was high when assayed at 50°C while carboxymethylcellulase activity was high when assayed at 40°C.

ABSTRAK

Bahan-bahan buangan semulajadi daripada pertanian boleh ditukarkan kepada produk komersial seperti etanol, glukosa dan sel protein tunggal. Banyak usaha daripada saintis dan penyelidik daripada seluruh dunia telah digunapakai untuk memaksimumkan penggunaan buangan pertanian ini. Kajian-kajian tentang penghasilan enzim selulase melalui pertukaran biologi bahan-bahan berlignoselulosa telah banyak dibuat. Walaubagaimanapun, masih ada banyak kemungkinan untuk mencari keadaan yang paling sesuai. Dalam kajian ini, hampas tebu yang digunakan sebagai substrat diproses secara fizikal terlebih dahulu dengan mengisar dan menghancurkannya. Selepas itu, ia digunakan sebagai substrat dalam penghasilan enzim selulase menggunakan kulat *Aspergillus terreus* SUK-1. Penghasilan enzim selulase ini dijalankan secara fermentasi berendam dengan dikacau sepenuh masa. Penghasilan enzim dijalankan dengan skala makmal menggunakan kelalang Erlenmeyer sahaja. Tujuan kajian ini adalah untuk mengkaji efek kepekatan substrat dalam penghasilan enzim selulase dan mencari suhu optimum di mana enzim dihasilakan lebih tinggi. Analisis dijalankan berdasarkan aktiviti enzim karboksimetilselulase dan enzim xilanase. Keputusan menunjukkan bahawa puncak aktiviti xilanase adalah 0.01132 U/ml pada hari ke 5 menggunakan 5g hampas tebu sebagai substrat dengan suhu 50°C manakala puncak aktiviti karboksimetilselulase pula adalah 0.007985049 U/ml menggunakan 3g hampas tebu sebagai substrat dan suhu penapaian 40°C. Aktiviti xilanase pula tinggi apabila diuji ketulenannya pada suhu 50°C sementara karboksimetilselulase pula tinggi pada suhu 40°C.

TABLE OF CONTENT

CHAPTER	TITLE	PAGE
TITLE PAGE		**i**
DECLARATION OF ORIGINALITY AND EXCLUSIVENESS		**ii**
ACKNOWLEDGEMENT		**iii**
ABSTRACT		**iv**
ABSTRAK		**v**
TABLE OF CONTENT		**vi**
LIST OF TABLES		**xi**
LIST OF FIGURES		**xii**
LIST OF ABBREVIATIONS		**xiv**
LIST OF APPENDICES		**xv**
CHAPTER 1	**INTRODUCTION**	**1**
	1.1 Introduction	1
	1.2 Research objective	3
	1.3 Research scope	3
CHAPTER 2	**LITERATURE REVIEW**	**4**
	2.1 Sugar cane waste	4
	2.1.1 Sugar cane bagasse composition	5
	2.1.2 Substrate pretreatment	8
	2.2 *Aspergillus terreus* SUK-1	9

CHAPTER	TITLE	PAGE

2.2.1 Macroscopic Features 10
2.2.2 Advantage and disadvantage using *Aspergillus terreus* SUK-1 11

2.3 Cellulose 12

2.4 Enzyme assay 12
2.4.1 Cellulase enzyme 13
2.4.2 Physical and chemical properties 13
2.4.3 Stability and storage 14
2.4.4 Applications 14

2.5 Fermentation 14

CHAPTER 3 MATERIALS AND METHODOLOGY 16

3.1 Substrate preparation 16

3.2 Grinding and milling 16

3.3 Growth substrate 17

3.4 Enzymes assay substrate 17

3.5 Buffer and reagent 17

3.6 Growing Medium 17

3.7 General chemical 18

3.8 *Aspergillus Terreus* SUK-1 Culture Stock 18

3.9 Substrate 18

3.10 Preparation of agar 19

3.11 Sub culturing of *Aspergillus Terreus* SUK-1 19

3.12 Preparation of spore suspension 19

CHAPTER	TITLE	PAGE
	3.13 Preparation of Mandels Media for Inoculums	20
	3.13.1 Preparation of Inoculums	20
	3.14 Preparation of Mandels Media for Cultivation	21
	3.14.1 Cultivating in Enlenmeyer Flask	21
	3.15 Research on growth and yield of cellulose	22
	3.15.1 Cellulase enzyme sources	22
	3.15.2 Substrate preparation	22
	3.15.3 Preparation of DNS Reagent	23
	3.15.4 Preparation of buffer solution	23
	3.15.5 Xylanase assay	23
	3.15.6 Carboxymethylcellulase assay	24
CHAPTER 4	**RESULTS AND DISCUSSION**	**25**
	4.1 Results	25
	4.2 Time course of xylanase enzyme activity	25
	4.3 Time course of CMCase enzyme activity	26
	4.3.1 Phenomenon of enzyme activity	27
	4.4 Effect of varying SCB concentration on xylanase activity	27

CHAPTER	TITLE	PAGE

CHAPTER	TITLE	PAGE
	4.5 Effect of varying SCB concentration on CMCase activity	29
	4.5.1 The influence of varying substrate concentration	29
	4.6 Effect of varying assay temperature on CMCase activity	30
	4.7 Effect of varying assay temperature on xylanase activity	31
	4.7.1 Influence of varying assay temperature on enzyme activities	32
CHAPTER 5	**CONCLUSION AND RECOMMENDATIONS**	**34**
	5.1 Conclusion	34
	5.2 Recommendations	35
REFERENCE		**36**
APPENDIX		**38**

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.0	Sugar cane bagasse waste in ASEAN region year 2004	4
2.1	Oil Palm waste in ASEAN region in year 2004	5
2.2	Rice husk waste in ASEAN region in year 2004	5
2.3	Average Bagasse Composition	6
2.4	*Aspergillus* basics	10
2.5	Microscopic features	10
3.0	Chemicals preparation of Mandels Media for inoculums	20
3.1	Chemicals preparation of Mandels Media for cultivation	21

LIST OF FIGURES

FIGURES NO.	TITLE	PAGE
1.0	Process flow of converting biomass into ethanol	2
2.0	Sugar cane bagasse	6
2.1	Land filling with bagasse	8
2.2	*Aspergillus terreus*	11
2.3	Schematic illustration of the cellulose chain	12
4.0	Time course of xylanase activity from *Aspergillus terreus* SUK-1 using 5g SCB under fermentation at 50°C.	26
4.1	Time course of CMCase enzyme activity from *Aspergillus terreus* SUK-1 using 3g SCB under fermentation at 40°C.	27
4.2	Effect of varying concentration of SCB on Xylanase activity from *Aspergillus terreus* SUK-1 under fermentation at 50°C.	28
4.3	Effect of varying concentration of SCB on CMCase activity from *Aspergillus terreus* SUK-1 under fermentation at 40°C.	29
4.4	CMCase activities at different temperature with substrate concentration of 3g	30

FIGURES NO.	TITLE	PAGE
4.5	Xylanase activities at different temperature with substrate concentration of 5g	31
4.6	Effect of varying assay temperature on CMCase and Xylanase activity	32

LIST OF ABBREVIATIONS

CMCase	- Carboxymethylcellulase
SCB	- Sugar cane waste/bagasse
U/ml	- unit of enzyme activity, μmol/min
Min	- minutes
SmF	- Submerged fermentation
SSF	- Solid State Fermentation

LIST OF APPENDIX

APPENDIX	TITLE	PAGE
A	Xylose standard curve	38
B	Glucose standard curve	39
C	CMCase fermentation readings at 30°C	40
D	CMCase fermentation readings at 40°C	42
E	CMCase fermentation readings at 50°C	44
F	Xylanase fermentation readings at 30°C	46
G	Xylanase fermentation readings at 40°C	48
H	Xylanase fermentation readings at 50°C	50
I	Absorbance and enzyme activities reading at various temperatures and concentration	52

CHAPTER 1

INTRODUCTION

1.1 Introduction

Most carbohydrates in plants are in the form of lignocellulose in which is made up of mainly cellulose, hemicellulose, pectin, and lignin. Lignocellulosic material can be any material containing lignocellulose. Lignocellulose is generally found, for example, in the stems, leaves, hulls, husks, and cobs of plants or leaves, branches, and wood of trees. The lignocellulosic materials can also be herbaceous material, agricultural residues, forestry residues, municipal solid wastes, waste paper, and pulp and paper mill residues. Agricultural wastes and in fact all lignocellulosics can be converted into products that are of commercial interest such as ethanol, glucose, and single cell protein.

The most critical part in producing products from lignocellulosic materials is the enzyme production cost. The final target of the whole research is to produce economically acceptable enzymatic conversion of cellulosic biomass to glucose for fermentation to ethanol or other products.

Hydrolysis of these polymers releases a mixture of neutral sugars including glucose, xylose, mannose, galactose, and arabinose. Cost-effective hydrolysis is an important goal in the search for a feasible enzymatic conversion process for lignocellulosic materials. Due to the crystalline structure of cellulose,

as well as its complex structural organization, lignocellulosics are difficult to break down. Process flow in Figure 1 below shows the process of converting biomass into ethanol. Basically it is known that the cost would hike 10 times from initial.

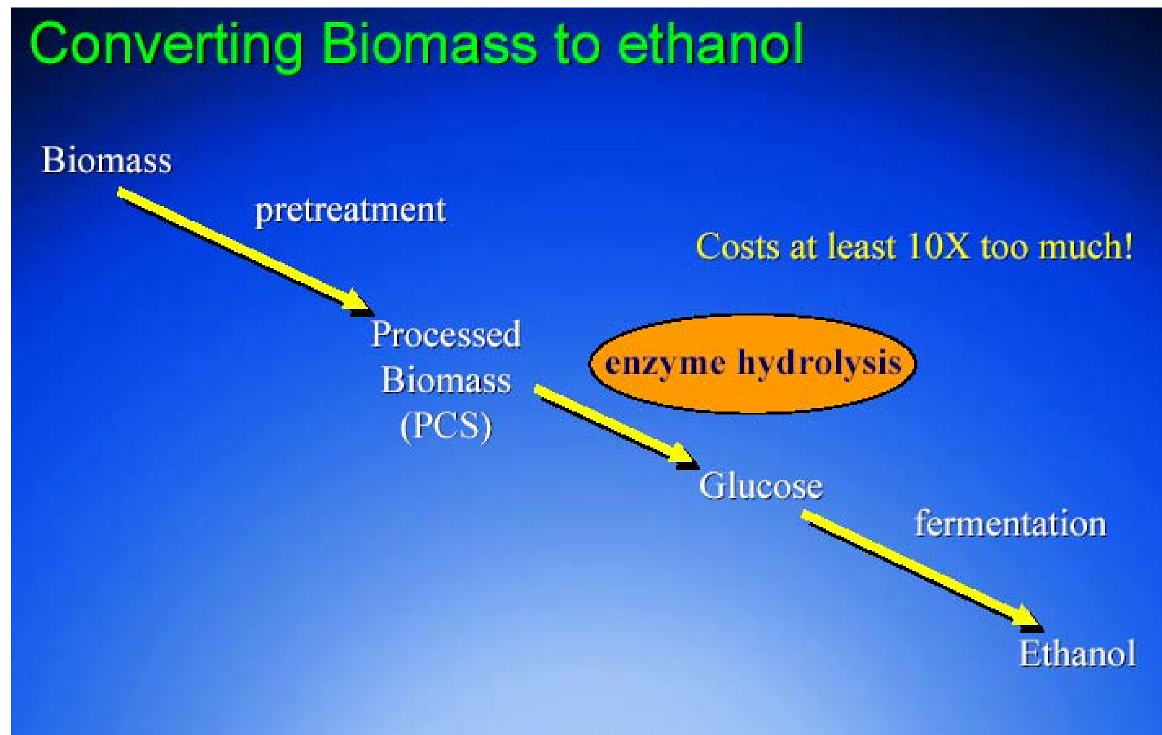

Figure 1.0: Process flow of converting biomass into ethanol.

Cellulase enzyme has been used for the bioconversion of lignocellulosics to these useful products. Many fungi produce enzymes that enable them to break down polysaccharides and proteins into sugars and amino acids that can be assimilated easily. These enzymes are important industry.

Approximately 90% of all industrial enzymes are produced in submerged fermentation (SmF), frequently using specifically optimized, and genetically manipulated microorganisms. In this respect SmF processing offers an

insurmountable advantage over Solid state fermentation (SSF). Interestingly, fungi, yeasts and bacteria that were tested in SSF in recent decades exhibited different metabolic strategies under conditions of solid state and submerged fermentation.

In this research, production of cellulase enzyme is made using *Aspergillus terreus* SUK-1 using sugar cane bagasse. (SCB) was subjected to a mixed culture, SmF with *Aspergillus terreus* SUK-1 to produce cellulase and reducing sugars. The optimal concentration and temperature during assay for the production of cellulase enzyme in *Aspergillus terreus* SUK-1 is determined in this experiment.

1.2 Research objective

The objective of this research is to produce cellulase enzyme from *Aspergillus terreus* SUK-1 using sugar cane bagasse.

1.3 Research scope

The scope of this research is to study the effect of substrate concentration in the production of cellulase enzyme from *Aspergillus terreus* SUK-1 using sugar cane bagasse. The other scopes are to determine the effect of temperature during the assay of cellulase enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Sugar cane waste

Sugar cane waste or should we call sugar bagasse is one of the most abundant organic agricultural waste available all-around the world. In ASEAN region only, we can see that the amount of sugar bagasse is higher than any other organic agricultural waste. About 34,220 tonnes of SCB is left upon the production of 118,000 tonnes of sugar. Tables below shows the amount of SCB production in ASEAN region in year 2004 and their counterpart waste.

Table 2.0: Sugar cane bagasse waste in ASEAN region year 2004

Country	**Sugar cane production (1,000 tonnes)**	**Bagasse production (1,000 tonnes)**
Indonesia	31,000	8,990
Philippines	21,000	6,090
Thailand	54,000	15,660
Vietnam	12,000	3,480
Total	**118,000**	**34,220**

Table 2.1: Oil Palm waste in ASEAN region in year 2004

Country	FFB production (1,000 tonnes)	Residue production (1,000 tonnes)
Indonesia	25,000	10,500
Malaysia	42,000	17,640
Philippines	300	126
Thailand	2,300	966
Total	**69,600**	**29,232**

Table 2.2: Rice husk waste in ASEAN region in year 2004

Country	Paddy production (1,000 tonnes)	Rice husk Production (1,000 tonnes)
Indonesia	51,000	11,200
Malaysia	2,000	440
Philippines	11,000	2,420
Thailand	22,000	4,840
Vietnam	28,000	6,160
Total	**114,000**	**25,080**

2.1.1 Sugar Cane Bagasse composition

The sugar cane plants are known to grow best in tropical and subtropical regions.Sugar cane stalk characteristics vary considerably depending on variety. Typical commercial varieties grown under normal field conditions have a height of 1.5 to 3 meters and are 1.8 to 5 cm in diameter. The stalk surface can be greenish, yellowish or reddish in color and is covered with a thin waxy layer (Van Dillewijn, 1952). Sugar cane bagasse is a fibrous residue that remains after crushing the stalks, and contains short fibers. Figure 2.0 below shows the sugar cane bagasse.

Figure 2.0: Sugar cane bagasse.

Basically, it is a waste product that causes mills to incur additional disposal costs. It consists of water, fibers, and small amounts of soluble solids. Percent contribution of each of these components varies according to the variety, maturity, method of harvesting, and the efficiency of the crushing plant. Table 2.2 (Elsunni and Collier, 1996) shows a typical SCB composition.

Table 2.3: Average Bagasse Composition

Item	**Bagasse (%)**
Moisture	49.0
Fiber	48.7
Soluble Solids	2.3

Fibers in bagasse consist mainly of cellulose, pentosans, and lignin. Cellulose is a natural linear polymer and has polymer chains of 2000 to 3000 units

(Paturau, 1989) and a specific gravity about 1.55 (Elsunni and Collier, 1996). Cellulose is highly crystalline regardless of the source. The ordered chains are tightly packed and have strong intermolecular hydrogen bonding because of the preponderance of hydroxyl groups (Romanoschi *et al.*, 1998).

The cellulose is present in three types: α, β and γ. The α cellulose is known as pure cellulose, whereas β and γ cellulose combined are called hemicelluloses (Marthur, 1975). The hemicelluloses are chemically linked with cellulose molecules. The other main compound in sugar cane fiber bundles is lignin which is a high molecular weight substance. Because it is not possible to isolate lignin quantitatively from plant materials without chemical or mechanical degradation, its true molecular weight is not known. The amount of lignin that naturally occurs in sugar cane depends to a great extent on the variety and age of the cane. The amounts of sugar, lignin and lignin-like compounds increase as the plant advances in age until the flowering time, when the plant is considered to be fully mature. Beyond the flowering time, the sugar cane plant tends to consume its stock of sucrose and lignin as a result of physiological changes due to flowering. The depletion of the organic compounds makes the rind and the fiber bundles softer and spongy (Van Dillewijn, 1952).

Nowadays bagasse is mainly used as a burning raw material in the sugar cane mill furnaces. The low caloric power of bagasse makes this a low efficiency process. Also, the sugar cane mill management encounters problems regarding regulations of “clean air” from the Environmental Protection Agency, due to the quality of the smoke released in the atmosphere. Presently 85% of sugar cane bagasse production is burnt. Even so, there is an excess of bagasse. Usually this excess is deposited on empty fields altering the landscape.

Figure 2.1: Land filling with bagasse

Approximately 9% of SCB is used in alcohol (ethanol) production. Ethanol is not just a good replacement for the fossil fuels, but it is also an environmentally friendly fuel. Apart from this, ethanol is a very versatile chemical raw material from which a variety of chemicals can be produced. But again, due to the low level of sucrose left in SCB, the efficiency of the ethanol production is quite low (Chiparus, 2004).

2.1.2 Substrate pretreatment

Some features of natural cellulosic materials are known to inhibit their degradation/bioconversion. These are degree of crystallinity and lignification and the capillary structure of cellulose. The crystallinity and lignification limit the accessibility and susceptibility of cellulose to cellulolytic enzymes and other hydrolytic agents (Fan *et al*., 1987). However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported (Kansoh *et al*., 1999). Pre-treatment of cellulose opens up the structure and removes secondary interaction between glucose chains (Fan *et al*., 1987).

For enzymatic processes to be effective, some kind of pretreatment process is thus needed to break the crystalline structure of the lignocellulose and remove the lignin to expose the cellulose and hemicellulose molecules. Depending on the biomass material, either physical or chemical pretreatment methods may be used. Physical methods may use high temperature and pressure, milling, radiation, or freezing; all of which require high-energy consumption. The chemical method uses a solvent to break apart and dissolve the crystalline structure. The lignocellulosic material may be used as is or may be subjected to a pretreatment using conventional method known in the art. For example, physical pretreatment techniques can include various types of milling, irradiation, steaming/steam explosion, and hydrothermolysis; chemical pretreatment techniques can include dilute acid, alkaline, organic solvent, ammonia, sulfur dioxide, carbon dioxide, and pH-controlled hydrothermolysis; and biological pretreatment techniques can involve applying lignin-solubilizing microorganisms.

2.2 *Aspergillus terreus* SUK-1

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. While a teleomorphic state has been described only for some of the *Aspergillus* spp., others are accepted to be mitosporic, without any known sexual spore production. The genus *Aspergillus* includes over 185 species. Around 20 species have so far been reported as causative agents of opportunistic infections in man *Aspergillus terreus* is among the other species less commonly isolated as opportunistic pathogens. *Aspergillus* is a thermophilic fungus, which means that it likes heat and will grow at body temperature or higher. It is xerophilic, which means it can obtain moisture from the air if humidity is at 60% or higher.

2.2.1 Macroscopic Features

The major macroscopic features remarkable in species identification are the growth rate, color of the colony, and thermo tolerance. The color of *Aspergillus terreus* is from Cinnamon to brown on the surface while on the reverse is white to brown. Table 2.3 below shows the basic information about *Aspergillus* while table 2.4 shows the microscopic features of *Aspergillus*. In figure 2.2 we can see *Aspergillus terreus* from microscope.

Table 2.4: *Aspergillus* basics

Temperature	High, 98 degrees or above
Food	Cellulose materials (cloth, cardboard, leather)
Water	Can pluck water vapor from humid air. Will not grow if the relative humidity is under 60%.
Potential allergen	Yes (cause illness when eaten)

Table 2.5: Microscopic features

Species	*Aspergillus terreus*
Conidiophore	Short (<250 µm), smooth, colorless
Philides	Biseriate
Vesicle	Round, loosely radiate head
Hulle cells	solitary, round, produced directly on hyphae

Figure 2.2: *Aspergillus terreus*

2.2.2 Advantages and disadvantages using *Aspergillus terreus* SUK-1

The advantage of *Aspergillus terreus* is its can grown at the lower pH. As a result, this advantage indirectly can minimize the contamination problem. Then, mycotoxins are produced only by a few organisms in stationary phase as secondary metabolisms and they are synthesizing only at a particular stage in the life cycle of the organism. Hence, evening fermentations with such organisms, it may be possible to inhibit the production of toxins by growing the organism at fast growth rates (Gray, 1970). Then the disadvantage of *Aspergillus terreus* is its might be produced mycotoxins during the cultivation process.

2.3 Cellulose

Cellulose is a linear polysaccharide of glucose residues connected by β-1, 4 linkages. Figure 2.3 shows the schematic illustration of cellulose chain.

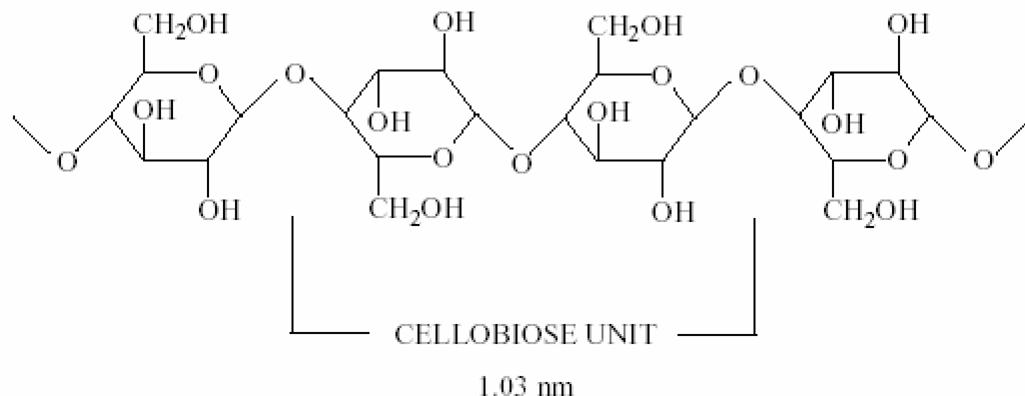

Figure 2.3: Schematic illustration of the cellulose chain

2.4 Enzyme assay

Enzymatic activity is dramatically affected by pH, temperature and substrate concentration. Enzymes are generally active over a specific range of temperature and pH. Model substrates are often employed in place of native substrates in order to assay certain activities.

Enzymes are assayed by measuring the rate at which they produce reducing sugars from their respective substrates. Reducing sugar assays such as the Neslon-Somogyi method or the Dinitrosalicylic acid (DNS) method are used to assay for the product sugars. Reactions are carried out by mixing and incubating a dilution of the enzyme preparation with a known amount of substrate at a buffered pH and set temperature. Xylanase assays are similar to cellulase

assays except that a solution of xylan (oat spelts or birch) is substituted for CMC or filter paper. The DNS assay is easier to use than the Nelson-Somogyi assay. The DNS assay is satisfactory for cellulase activities, but tends to over estimate xylanase activity.

2.4.1 Cellulase enzyme

Cellulase refers to a group of enzymes which, acting together, hydrolyze cellulose. (www.worthington-biochem.com.html). The cellulolytic enzyme may any enzyme involved in the degradation of lignocellulose to glucose, xylose, mannose, galactose, and arabinose. The cellulolytic enzyme may be a multicomponent enzyme preparation, e.g., cellulase, a monocomponent enzyme preparation, e.g., endoglucanase, cellobiohydrolase, glucohydrolase, beta-glucosidase, or a combination of multicomponent and monocomponent enzymes. The cellulolytic enzymes may have activity, i.e., hydrolyze cellulose, either in the acid, neutral, or alkaline pH-range.

Trichoderma reesei and *Aspergillus terreus* has an extensively studied cellulase enzyme complex. This complex converts crystalline, amorphous, and chemically derived celluloses quantitatively to glucose. (Henrissat *et al*, 1999)

2.4.2 Physical and chemical properties

Most cellulase studied has similar pH optima, solubility and amino acid composition. Thermal stability and exact substrate specificity may vary. However, it should be remembered that cellulase preparations generally contain other enzymatic activities besides cellulase, and these may also affect the properties of the preparations. The optimum pH for cellulase preparations is effective between

pH 3 and 7 but the optimum pH generally lies between 4 and 5. Besides that, the optimum temperature is between 40 - 50 °C. (Henrissat *et al*, 1999)

2.4.3 Stability and storage

The activity of cellulase preparations has been found to be completely destroyed after 10-15 minutes at 80 °C. Solutions of cellulase at pH 5-7 are stable for 24 hours at 4 °C. These products should be stored at 4 °C, in a dry place in tightly closed containers. If stored in this manner, lyophilized preparation is stable for several months without significant loss of activity. (Henrissat et al, 1999)

2.4.4 Applications

Cellulase enzyme is used in production of ethanol. The greatest potential for ethanol production from biomass lies in enzymatic hydrolysis of cellulose using cellulase enzymes. Then the other application of cellulase enzyme is various industries such as in alcoholic beverages industries to produce wine. It's also important in chemicals and food industries.

2.5 Fermentation

Fermentation is a chemical reaction in which sugars are broken down into smaller molecules that can be used in living systems. Alcoholic beverages, such as beer, wine, and whiskey, are made from the controlled use of fermentation. Fermentation is an anaerobic process.

Fermentation typically refers to the conversion of sugar to alcohol using yeast. The process is often used to produce wine and beer, but fermentation is also employed in production of cellulase enzyme. The science of fermentation is known as zymology. Fermentation (formerly called zymosis) is the anaerobic metabolic breakdown of a nutrient molecule, such as glucose, without net oxidation. Fermentation does not release all the available energy in a molecule because it merely allows glycolysis (a process that yields two ATP per glucose) to continue by replenishing reduced coenzymes. Fermentation yields lactate, acetic acid, ethanol, or other reduced metabolites. Fermentation is also used much more broadly to refer to the bulk growth of microorganisms on a growth medium. No distinction is made between aerobic and anaerobic metabolism when the word is used in this sense. Fermentation usually implies that the action of the microorganisms is desirable.

In alcoholic fermentation, such as occurs in brewer's yeast and some bacteria, the production of lactic acid is bypassed, and the glucose molecule is degraded to two molecules of the two-carbon alcohol, ethanol, and to two molecules of carbon dioxide. Many of the enzymes of lactic acid and alcoholic fermentation are identical to the enzymes that bring about the metabolic conversion known as glycolysis. Alcoholic fermentation is a process that was known to antiquity.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Substrate preparation

The sample first has to be washed to get rid of the dust and other contaminants. An amount of 100 g SCB is being washed with tap water in the sieve tray. SCB is later soak for 3 days in distillation water with 3 changes. After it is washed clean, the sample is then dried in the oven for complete drying at $60^{o}C$ for 10 hours. The sample is then sealed in the seal bag and stored at ambient condition before proceeding to the next step.

3.2 Grinding and milling

The sample that has been washed and dried is taken out from the seal bag and then put inside a dry blender. The sample is grind in the blender until the size of the particles reached the desired size. After the entire sample has been grinded, it is put inside the seal bag weight 1g, 3g and 5g and kept in sealed plastic bag and labeled. The seal bag is stored at ambient condition.

3.3 Growth Substrate

Potato dextrose agar was the agar which is used for the cultivation of *Aspergillus terreus* SUK-1 fungus stock. It was supplied by OXOID Ltd, Basingstoke, Hampshire England.

3.4 Enzymes assay Substrate

Xylan powder and carboxymethylcellose powder was used for assay of xylanase and carboxymethylcellulase enzyme. The purpose of the assay is to check the purity of these enzymes. Sigma-Aldrich Incorporation, Germany, is the supplier for these two substrates.

3.5 Buffer reagent

Buffer that used in this experiment is 0.1 M acetate pH 5.0 buffer. Chemicals used were sodium acetate, glacial acetic acid and 3, 5-dinitrosalicylic acid (DNS). Glacial acetic acid was supplied by Mallinakrodt Baker, Mexico.

3.6 Growing media

Basic mandels media was used as a growing medium for *Aspergillus terreus* SUK-1 fungus. Preparation for this medium of mixed culture was carried out by adding this material: $(NH_4)_2SO_4$, KH_2PO_4, Urea, $CaCl_2.2H_2O$,

$MgSO_4.7H_2O$, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $FeSO_4.7H_2O$, Protease peptone, Tween 80, and substrate which is sugar cane bagasse.

Urea was supplied by HmbG Chemicals from Hamburg, Germany. $CaCl_2.2H_2O$ was supplied by Emory Laboratory Reagents. $FeSO_4.7H_2O$ was supplied by GENE Chemicals and $CoCl_2.6H_2O$ by Progressive Co, HmbG Germany.

3.7 General Chemical

General uses chemicals were like phenol, sodium sulphite and NaOH. Phenol was supplied by Fisher Scientific, USA. Meanwhile NaOH were supplied by R&M Chemicals, UK.

3.8 *Aspergillus terreus* SUK-1 Culture Stock

Aspergillus Terreus SUK-1 culture stock was suppied by the culture stock from Jabatan Kejuruteraan Kimia dan Proces at Fakulti Kejuruteraan, Universiti Kebangsaan Malaysia, UKM.

3.9 Substrate

Substrate that used for this project is sugar cane bagasse which is provided by juice seller for free of charge.

3.10 Preparation of agar

An amount of 7.8g of potato dextrose agar was diluted in 200 mL distilled water. Solution was heated until no colloid is seen to ensure the solution is completely dilute.

Then the solution was poured into universal bottles until the solution is 1/3 height of the bottles. Then the bottles were autoclaved at 121°C and 15 psi for 15 minutes. Lastly, agar was left to cooled and frozen inside freezer at 4°C in a slanting position.

3.11 Sub culturing of *Aspergillus Terreus* SUK-1

Sub cultivation was performed on slanted agars which have been prepared earlier by transferring inoculate from culture stock using loop wire in sterilize condition. This was done in a laminar flow. Loop wire was burnt using Bunsen burner to sterilize it. Spores were harvested after 5 to 6 days old of fungal growth.

3.12 Preparation of spore suspension

This activity was conducted under aseptic condition. Suspension was prepared by adding 6 ml of sterilized distilled water into the universal bottles containing culture stock. Spores were scratched out slowly from the surface of agar using a Pasteur pipette. Method was repeated again by adding 4 ml sterilized distilled water. The 10 ml spore suspension was used to prepare inoculums later.

3.13 Preparation of Mandels Media for Inoculums

Mandels media for inoculums was prepared by mixing these chemicals into 1 L solution. The list of chemicals for preparation of Mandel's media is shown in Table 3.1

Table 3.0: Chemicals preparation of Mandels Media for inoculums.

Materials	mass
$(NH_4)_2SO_4$	1.4g
KH_2PO_4	2.0g
Urea	0.3g
$CaCl_2.2H_20$	0.4g
$MgSO_4.7H_2O$	0.6g
$MnSO_4.H_2O$	1.0 mg
$ZnSO_4.7H_2O$	1.4 mg
$FeSO_4.7H_2O$	5.0 mg
$CoCl_2.6H_2O$	3.7 mg
Protease peptone	0.75g
Tween 80	2.0 mg
A- Cellulose	7.5 g

3.13.1 Preparation of Inoculums

An amount of 250 ml of Mandels media was prepared in a 500 ml Erlenmeyer flask and autoclaved for 15 minutes at 121°C and 15 psi. Then it was cooled down to room temperature and added 10 ml spores suspensions into the flask in sterilize condition. Erlenmeyer flask containing all the chemicals was incubated at 30°C with shaking speed of 150 rpm in incubator for 60 to 72 hours to ensure inoculants in log phase.

3.14 Preparation of Mandels Media for Cultivation

Mandels media for cultivation was prepared by mixing these chemicals into 1 L solution. The list of chemicals for preparation of Mandel's Media is shown in Table 3.2 below.

Table 3.1: Chemicals preparation of Mandels media for cultivation.

Materials	**mass**
$(NH_4)_2SO_4$	1.4
KH_2PO_4	2.0
Urea	0.3
$CaCl_2.2H_20$	0.4
$MgSO_4.7H_2O$	0.6
$MnSO_4.H_2O$	1.0 mg
$ZnSO_4.7H_2O$	1.4 mg
$FeSO_4.7H_2O$	5.0 mg
$CoCl_2.6H_2O$	3.7 mg
Protease peptone	0.75
Tween 80	2.0 mg
Sugar bagasse	1,3,5 g each

3.14.1 Cultivating in Enlenmeyer Flask

An Erlenmeyer flask contained 300 ml basic Mendels media is autoclaved 121°C and 15 psi for 15 minutes. An amount of 30 ml of inocula was inoculated into the flask in sterilize condition. Growth was conducted at 30°C with agitation rate 150 rpm in the stirrer for one week.

3.15 Research on growth and yield of cellulase

When growing, analysis on the changes activity of enzyme was carried out. An amount of 10 ml of sample was collected every day for one week to

analysis. Sample then was centrifuged at 12000 rpm and 4°C for 30 minutes. Supernatant was used in analyzed for carboxymethylcellulase and xylanase enzyme activity.

3.15.1 Cellulase enzyme sources

After seven days growing, the filtrate culture was centrifuged at 12000 rpm and 4°C for 30 minutes. This was to separate the insoluble material and organism cells. Supernatant contain cellulosic was used as the substrate of cellulase enzyme source for research.

3.15.2 Substrate preparation

An amount of 3 g of xylan powder was diluted in 200 ml of distilled water in order to obtain 2% xylan solution. This solution was used in assay of xylanase enzyme.

An amount of 3 g of carboxymethylcellulose powder was diluted in 200 ml of distilled water in order to obtain 1.5% carboxymethylcellulase solution. This solution was used in assay of carboxymethylcellulase enzyme.

3.15.3 Preparation of DNS Reagent

An amount 10 g of 3, 5-dinitrosalicylic acid, 2 g of phenol and 0.5 g of sodium sulphite was diluted in 500 ml of 2% NaOH solution. Distilled water was

added until final volume was 1 liter. Reagent was kept in a black bottle because it was sensitive to light.

3.15.4 Preparation of buffer solution

An amount of 13.608 g sodium acetate was mixed with 1 liter distilled water and gives a 0.1 M sodium acetate solution. After that, glacial acetic acid was added by slowly until the solution pH 5.0 was reached. After all the preparation of buffer solution was finished, then the solution was kept in a labeled bottle at 4°C.

3.15.5 Xylanase assay

Xylanase activity was assayed by measuring the amount of reducing sugar released from xylan. A reaction mixture containing 0.2 ml supernatant (crude enzyme) and 1.3 ml 2% (w/v) xylan in 0.1 M acetate buffer pH 5.0 was incubated at 30°C, 40°C, 50°C for 60 minutes. The reaction was quenched by cooling on ice and the amount of reducing sugar was measured by dinitrosalicylic (DNS) acid procedure with slight modification (Miller, 1965). The mixture was then incubated at 100°C for 5 minutes. Subsequently, the reaction was stopped by cooling on ice. Distilled water was added into the final volume of 16 ml and the color intensity was measured at wavelength of 550 nm. The non enzymatic release of sugar was corrected by setting up a separate blank for each sample. One unit (U or µmol/min) of xylanase activity was defined as the amount of enzyme that produced one µ mol of reducing sugar as xylose per minute under the assay condition.

3.15.6 Carboxymethylcellulase assay

Carboxymethylcellulase activity was determined as a described for the xylanase assay but the incubation was carried out with 2.0 % (w/v) carboxymethylcellulose as a substrate instead of xylan. One unit (U or µmol/min) of of carboxymethylcellulase activity was defined as the amount that produced one µ mol of reducing sugar as sugar as glucose per minute under the assay conditions.