STUDY ON THE EFFECT OF SUBSTRATES CONCENTRATION IN THE PRODUCTION OF XYLANASE FROM ASPERGILLUS NIGER USING PALM KERNEL CAKE

ABDUL HAKIM BIN RAHMAD

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

FACULTY OF CHEMICAL ENGINEERING AND NATURAL RESOURCES
UNIVERSITY COLLEGE OF ENGINEERING AND TECHNOLOGY MALAYSIA

NOVEMBER 2006

I declare that this thesis entitled “Study on the Effect of Substrates Concentration in the Production of Xylanase from *Aspergillus niger* Using Palm Kernel Cake” is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : .....

Name : ABDUL HAKIM BIN RAHMAD

Date : 20 NOVEMBER 2006

To my beloved family, lectures and friends

"I/We* hereby declare that I/we* have read this thesis and in my/our* opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering"

Signature : .....

Name of Supervisor I: .....

Date : .....

Signature : .....

Name of Supervisor II: .....

Date : .....

ACKNOWLEDGEMENT

Bismillahirrahmanirrahim

Alhamdulillah, first and foremost I would like to thank to God, without His bless I will not completed this project. Secondly, I wish to express my sincere appreciation to my supervisor, Mr Wan Mohd Hafizuddin B. Wan Mohd Yussof, for constantly guiding and encouraging me throughout this study. Thanks a lot for giving me idea, advice and suggestion to bring this project to its final form. I am also very thankful to my co-supervisor Mr. Rozaimi B. Abu Samah for his critics, advices and motivation. Without their support and interest, this project would not have been the same as presented here.

We are grateful to the staff of Faculty of Chemical Engineering of University College of Engineering and Technology Malaysia for their cheerfulness and professionalism in handling their work. In preparing this project, I was in contact with many people, researches and academicians. They have contributed towards my understanding and thoughts.

In particular, my sincere thankful is also extends to all my colleagues and others who have provided assistance at various occasions. Their views and tips are useful indeed. Unfortunately, it is not possible to list all of them in this limited space. And last, but not least we thank my parents and other family members for their continuous support either moral support, money, time idea and others while completing this project.

ABSTRACT

Xylanase enzymes have many applications in the paper, food and chemical industries. These enzymes are produced by several microorganisms, mainly from fungal species. In this work a selected fungal called *Aspergillus niger* was investigated for production of xylanase in solid state fermentation (SSF). The substrates that used in the production of xylanase enzyme from *Aspergillus niger* were from palm oil waste which called as palm kernel cake. The concentration of the substrates that varied at 1, 3 and 5 g/L was studied in Erlenmeyer flask. The enzyme activity of xylanase was investigated for one week duration that started from the first day until the seventh day of the experiment that based on the activity of xylanase and carboxylmethylcellulase enzyme. Based on the result that obtained through the experiment, it was found that the highest xylanase activity was stated at concentration of 3 g/L on the first day. And for the carboxylmethylcellulase enzyme, the highest of activity was found at the concentration of 3 g/L on the fourth day. By using palm kernel cake as substrates, *Aspergillus niger* was able to grow and then produce xylanase enzyme

ABSTRAK

Enzim xylanase mempunyai banyak aplikasi seperti dalam industri kertas, makanan dan kimia. Enzim ini dihasilkan oleh beberapa mikroorganisma terutamanya daripada spesis kulat. Dalam pengkajian ini kulat terpilih yang dipanggil *Aspergillus niger* dikaji untuk penghasilan enzim xylanase melalui kaedah penapaian. Bahan yang digunakan dalam penghasilan enzim xylanase daripada *Aspergillus niger* adalah dari hasil buangan kelapa sawit yang dipanggil isi rong kelapa sawit . Kepekatan bahan yang berbeza pada 1, 3 dan 5 g/L dikaji di dalam kelalang Erlemeyer. Aktiviti enzim xylanase dikaji untuk jangka masa seminggu yang bermula dari hari pertama sehingga hari ketujuh eksperimen berdasarkan aktiviti enzim xylanase dan enzim carboxylmethylcellulase. Berdasarkan keputusan yang diperolehi melalui eksperimen, didapati bahawa aktiviti enzin xylanase dicatatkan paling tinggi pada kepekatan 3 g/L pada hari pertam. Dan untuk enzim carboxylmethylcellulase, aktiviti paling tinggi didapati pada kepekatan 3g/L iaitu pada hari yang keempat Dengan menggunakan isi rong kelapa sawit sebagai bahan, *Aspergillus niger* boleh membiak dan menghasilkan enzim xylanase.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Introduction

In nature, solid organic substrates such as animal and plant residues, wood, crop residues and fruits, undergo complex microbial degradation and transformation by various microbiological processes. In the industrial sector, this natural process may be utilized in a controlled form and pure culture may be employed if a specific end product is desired. Application of agro industrial residues as substrates is certainly economical and it also reduces environmental pollution.

Several naturally occurring agricultural byproducts such as coconut oil cake, rice bran, wheat and paddy straw, sugar beet pulp, fruit pulps and peels, corn cobs, saw dust, maize bran, rice husk, soy hull, sago hull, grape marc, coconut coir pith, banana waste, tea waste, cassava waste, aspen pulp, sweet sorghum pulp, apple pomace, peanut meal, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch etc. could be used in one or the other industrial bioprocess for the production of value added products through SSF (Pandey et al., 2001).

Xylanase has a wide range of potential biotechnological applications. It is already produced on an industrial scale for use as a food additive for poultry to increase feed efficiency and in wheat flour for improving dough handling and the quality of baked products. Recently the interest in xylanase has markedly increased due to the potential industrial uses, particularly in pulping and bleaching processes, using cellulase-free preparations (Dhillon et al., 2000).

1.2 Objective

The aim of this research is to produce xylanase enzyme from *Aspergillus niger* using palm kernel cake obtained from palm oil residue.

1.3 Research Scope

The scope of this research is to study the effect of different substrate concentration in the production of xylanase enzyme which is 1, 3 and 5 g/L.

1.4 Problem Statement

- To find the best concentration that can produce high amount of xylanase enzyme.
- Reduce cost by looking to biomass that can be use as a substrate.

CHAPTER 2

LITERATURE REVIEW

2.1 Palm Kernel Cake

Palm kernel cake (PKC) is the by products of oil palm industries and is generated after the kernel is crushed to squeeze the oil. It is a useful source of protein and energy which contains (% w/w) dry matter (90); crude protein (16.1); ether extract (0.8); crude fiber (15.2); ash (4); N-free extract (63); calcium (0.29); phosphorous (0.71) and metabolized energy -- N7/kg (6.2) (Marathe et al.,2002).

2.2 Cellulose

Cellulose is the major component of plant biomass and, as such, the most abundant organic polymer on earth. The total amount of cellulose on earth has been estimated at $7x10^{11}$ tons (Coughlan, 1985). It is a linear homo-polysaccharide consisting of anhydrous glucose units that are linked by b-1, 4-glycosidic bonds (Fig. 1). The end of the glucan chain with an anomeric carbon that is not linked to another glucose residue is referred to as the reducing end of the polymer. The other end of the polymer is the non-reducing end. Immediately after synthesis the cellulose chains coalesce into highly crystalline cellulose microfibrils held together by hydrogen bonds, hydrophobic interactions and van der Waals forces. This highly organized packing of the cellulose chains makes it much more resistant to hydrolysis than the a-1,4-linked glucan polymer,

starch. The width of the micro-fibrils depends on the source of the cellulose Nieduszynski and Preston, 1970). In plants the unit micro-fibrils are about 3 nm wide and contain around 35 cellulose chains, but they are often tightly packed in larger, 20–100nm microfibril bundles in the secondary cell wall (Hilden et al., 2003; Persson et al., 2004).

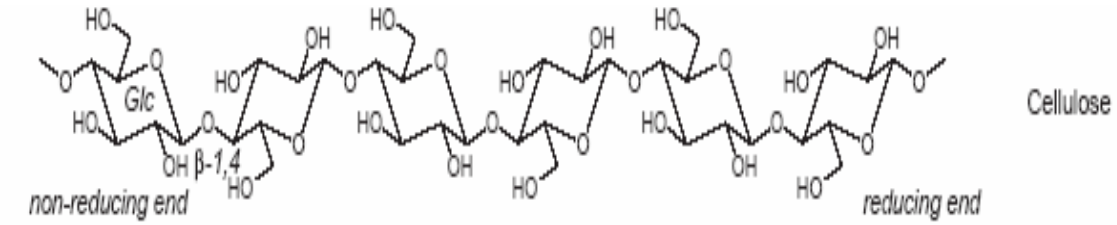

Figure 2.1 The structure for cellulose

2.3 Cellulose In The Plant Cell Wall

Cellulose seldom occurs in pure form, as in cottonseed hairs, but is almost always associated with other components in the complex matrix that constitutes the plant cell wall. Therefore the actual substrate that cellulolytic organisms encounter varies widely in composition and complexity, ranging from easily degradable primary wall material to densely lignified wood. The cellulose microfibrils function as structural reinforcement of the plant cell and they are embedded in a network of other polysaccharides that are collectively called hemicellulose. The plant cells are held together by a middle lamella that mainly consists of lignin and pectin. Firstly, a thin primary wall that is sufficiently flexible to allow growth and expansion is made with a loose structure and short, moderately crystalline, cellulose microfibrils. When the cell has attained its final shape a thicker secondary cell wall is deposited with long and highly crystalline microfibrils.

In most plants the secondary cell walls are subsequently lignified with up to 25–30% lignin. Plant biomass as a whole consists mainly of secondary cell wall material. Wood is built up of elongated plant cells with very thick and densely lignified secondary cell walls. The structure of the wood cell, and the ratio of its different components varies a lot depending on which plant species the cell comes from, cell type, and development stage. The approximate composition of the three most common components in wood is 35–50% cellulose, 20–30% hemicellulose and 20–30% lignin (Sjöström, 1993).

2.4 Hemicellulose

Hemicellulose is defined as the fraction of the cell wall that can be extracted with alkali (Mohr and Schopfer, 1995). It is a heterogeneous mixture of different polysaccharides and the composition varies depending on plant type. The individual chains in hemicellulose are shorter than in cellulose, usually with a DP of 100-200 (Timell, 1967). Xyloglucan is the predominant hemicellulose in the primary walls of dicots and non-graminaceous monocots and may account for up to 20% of the dry weight. It consists of a linear β -1, 4-glucan backbone, as in cellulose, but 3 out of 4 glucose residues are substituted at O6 with β -xylose that, in turn, may carry galactose or arabinose. Xyloglucan coats the surface of the cellulose microfibrils, limiting their aggregation and connecting them via tethers that regulate the mechanical properties of the wall.

In primary walls of grasses and secondary walls of all angiosperms the hemicellulose consists mainly of xylans, heteropolymers with a backbone of β -1, 4-linked xylose residues that may be acetylated and substituted with a variety of carbohydrate residues (Clarke, 1997; Sjöström, 1993) (Fig. 1). Arabinoxylan dominates in grasses (20-40% dry weight) whereas *O*-acetyl-(4-*O*-methylglucorono) - xylan is the most common building block in hardwoods (10-35%). Xylans are important also in softwoods (gymnosperms) where arabino-(4-*O*-methylglucorono)-xylan constitutes

about one third of the hemicellulose, but the major component is *O*-acetyl galactoglucomannan that contains a backbone of β-1, 4-linked mannose residues. Softwoods also contain significant amounts of the pectin arabinogalactan (Timell, 1967).

2.5 Hemicellulases

Hemicellulases are generally less complex than pectin, and consist of hetero- or homopolysaccharide main chains, often with short side chains. On the other hand there are several very diverse types of hemicellloses, xylans, glucomannans, xyloglucans, etc. Therefore it is also expected that hemicellulases consist debranching enzymes removing side chains and others modification such as acetylations and endo acting enzymes attacking the main chain. Hemicellulases are generally hydrolytic enzymes, and the most studied is possibly endoxylanases, hydrolyzing β-1, 4 bonds in the main chain of xylan, the main hemicellulose in dicotyledons (Polizeli *et al*., 2005).

2.6 Xylanase Enzyme

Xylanases or endo-1, 4-β-xylanase is the most commonly used feed enzyme (1). Xylanase belongs to the glucanase enzyme family and are characterize by their ability to break down various xylans to produce short-chain xylo-oligosaccharides. Xylanase is supply as sodium/potassium phosphate and glycerol in liquid form. (Shah, 2005).

Xylanase readily crystallizes in ammonium sulfate and sodium/potassium phosphate across pH 3.5 to 9.0. Xylanase can also be crystallizing with others salts, polymers, and organic solvents. Xylanase solubility increases with increasing temperature in moderate concentrations of ammonium sulfate. Xylanase solubility in phosphate buffer pH 9 decreases in the temperature range of 0 to 10^{o}C but remains constant in the range of 10 through 37^{o}C (Shah, 2005).

Xylanase are produced by many bacteria and fungi. The source of xylanase for use in animal feed is mainly fungal, with species of Aspergillus and Trichoderma being among the most commonly used. The catalytic activity of the xylanases differs from source to source, and will depend on the substrate used for the characterization.

As a number of xylanase products are available, there are a number of assays, each with their own definition of a xylanase unit. For product analysis the most common method is the colorimetric dinitrosalicyclic acid (DNS) assay. Within these the pH, temperature and substrate used all differ.

2.7 Extracellular Enzymes

Enzymes play important role and timplement in many fields such as food, feed, brewing, distillery, beverages, fats, oil, textile, pharmaceutical and others. New enzymes and application are being developed constantly. The fraction of global industries consumptions of enzymes are 30% for detergent, 30% for starch, 15% for dairy and the rest is to be distributed between all others application. Almost all commercial enzymes are derived from fungi. Table below show the range of enzymes and the sources derive from.

Table 2.1 Exoenzymes produced by FSSF

Enzyme	**Microorganism**	**Substrate**	**Application**
Pectinases	*A. carbonarius, A. sojae, A. saito A. niger*	Wheat bran	Fruit processing
Glucoamylase	*A. oryzae*	Rice, soybeans	Liquid glucose, Dextrose

	A niger	Wheat bran alone or + corn flour	Brewing, distillery
Proteases	*A. oryzae* *A. oryzae*	Wheat bran Wheat bran + rice Bran + soybean cake	Baking, brewing pharma, protein-hydrolysis, soybean processing
Cellulase	*T. reesei* *Pleurotus sojor-caju*	Wheat bran Agro industrial waste	Digestive aids, animal feed, textile
Xylanase	*A. niger* *A. terreus* *S. pulverulentum*	Wheat bran + rice straw	Conversion of hemicellulose into pentose sugars
Catalase	*R. niveus* *A. oryzae*	Wheat bran Wheat bran	Food industries to remove hydrogen peroxide; for controlled release of oxygen
Phytase	*R. oligosporus*	Rape seed meal	Hydrolysis of phytic acids in feed
Chitinase	*A. niger*	Wheat bran	Food processing and microbial cell lysis
Alpha-galactosidase	*A. awamori*	Wheat bran	sugar refining; soybean milk processing.
Beta-lactose galactosidase	*A. niger, A. oryzae* *Fusarium sp.*	Wheat bran	Digestive aids; hydrolysis

2.8 *Aspergillus sp.*

Aspergillus sp. is a group of moulds, which is found everywhere world-wide. Moulds are also called filamentous fungi. It is a genus of fungi containing approximately 150 recognized species. Members of this genus have been recovered from a variety of habitats, but are especially common as saprophytes on decaying vegetation, soils, and stored food. Some species are parasitic on insects, plants and animals, including man. The most important classes of molds industrially are *Penicilium* and *Aspergillus*. Major useful products of these organisms include antibiotics for example biochemical compounds which kill certain microorganisms or inhibit their growth, organics acid and biological catalysts (Bailey, 1986).

2.8.1 *Aspergillus niger*

Aspergillus niger is a filamentous ascomycete fungus from the order *Eurotiales* and family *Trichocomaceae* which includes all *Aspergillus* and *Penicillium* species. The genus Aspergillus is divided into three sub-genera, *Aspergillus* , *Nidulantes* and *Fumigati* . These subgenera are further divided into several sections. *A. niger* resides in subgenus *Aspergillus* section *Nigri* (Fig. 1). *Aspergillus* is now becoming the genus of choice for large scale comparative evolutionary studies. As multiple Aspergilli genomes become sequenced, there is an opportunity for comparative studies using whole genome alignments.

Unfortunately, the sequenced Aspergilli, *A. fumigatus* , *A. nidulans* and *A. oryzae* (sequenced but not released) are too distantly related to give much useful information about conserved regulatory motifs, speciation and recent genomic rearrangements. However with the impending sequencing of the *A. flavus* genome (Gary Payne , personal communication), the planned release of *A. oryzae* and the prospect of a high quality draft

of *A. niger* , the prospect of a detailed comparative evolutionary study of a filamentous fungus is very realistic. A similar study generated a large amount of novel information about genes and regulatory elements in budding yeast (Kellis et al., 2003; Cliften et al., 2003; Dujon et al., 2004).

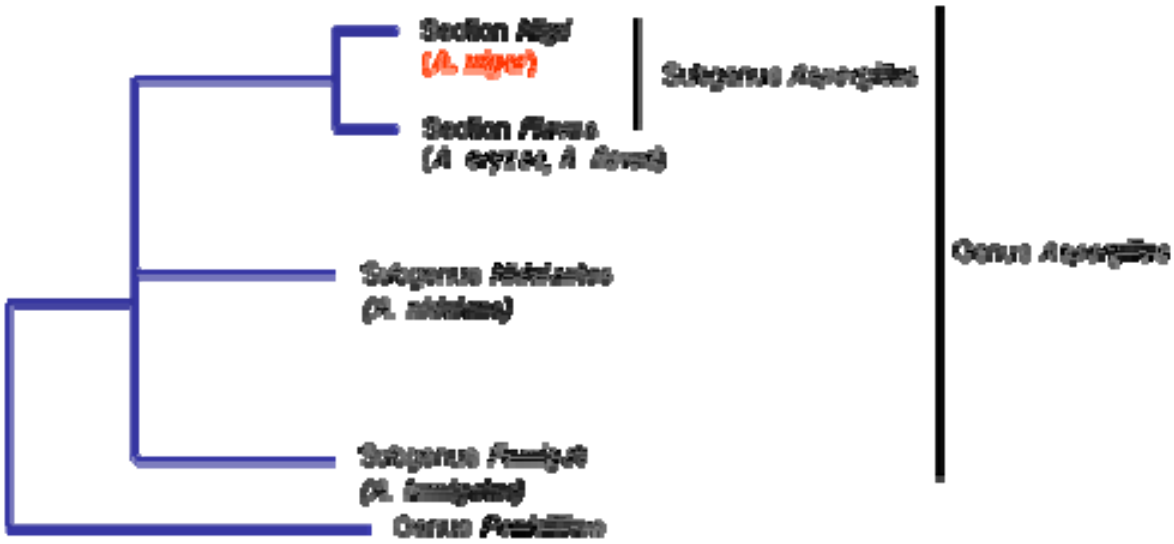

Fig 1. A simplified cladogram representation of *Aspergillus* subgenera and select sections based on based on analyses by Peterson (Peterson, 2000). Currently or planned sequenced genomes are shown in parentheses. Not all sections of subgenera *Aspergillus*, *Nidulantes* or *Fumigati* are shown.

2.9 Fermentation

The basis production of fungal products has largely been by liquid-based Submerged Fermentation (SmF). At present, in interest in Solid State Fermentation (SSF) in the USA is small compared to the oriental countries where fungi are exploited for their natural affinity for moist environments. Japan has always been a trendsetter in the area of SSF, and has some of the largest SSF production plants in the world.

SSF can be defined as the growth of microorganisms on moist, water-insoluble solid substrates in the absence or near-absence of free liquid. SSF has several potential advantages over SmF such as low capital cost, low energy expenditure, less expensive downstream processing, low wastewater output and potential higher volumetric productivity.

2.9.1 Process Protocol for SSF

General guidelines for SSF methodologies have been given by Lonsane *et al*. The step that included in it is first inoculum preparation – generally spores raised on the actual substrate. Then followed by substrate preparation including size reduction, nutrient addition and pH adjustment. Then the step of autoclaving to sterilize/pasteurize and cook the medium for increased amenability to fungal growth takes part in this methodology. Then the step of innoculation of the moist solid medium. Followed by incubation under near optimal conditions in suitable reactor systems. Next step is drying of the solids and extraction of products. Final step is involving in filtration, concentration /purification.

2.9.2 Substrate for SSF

One of the main advantages of SSF is the ease with which fungi can grow on complex natural solid substrates like agro-industrials waste, without much pretreatment.

There are several factors involved in the selection of a suitable substrate for the desired fungi to grow. These are macromolecular structure, particle size and shape, porosity and particle consistency. The complexity of the solid substrate arise from the presence of molecular compounds such as cellulose, starch, lignin and even smaller

sugar molecules. Fungi cannot directly absorb these macromolecules; they are induced by low molecular weight compounds to synthesize and secrete the enzymes to hydrolyze the macromolecules into smaller metabolizable compounds.

As far as particle size is concerned, a large surface area to volume ratio is preferred for high yields especially when the fungi lack sufficient penetrative ability, so that the substrates molecules are more easily accessible for the hydrolytic enzymes. If the area surface is less, then the enzyme diffusion tends to become rate –limiting. The particle size is also important in obtaining favourable physical conditions in the bed. Smaller particles results in bed compaction affecting gas exchange while, larger particles limit substrate accessibility. Different substrate particles have different particles shape, which have a direct bearing on the void fraction when packed into the reactor system. It has been indicated that the spherical and long thin particles support sufficient void spaces than cubical and thin slab-like ones.

The substrate particle would also need to have sufficiently large pore openings for easy mycelial entry and passage. Porosity can be further improved by pretreatment.

2.9.3 Solid State Fermentation

Solid state fermentation (SSF) has impact on the environment. Various natural and industrial carbonaceous wastes can be diverted into the value added products. It is a simple technique in which solid waste substrates such as fibers, bran and others are utilized with a single culture or combination of cellulolytic cultures. Particle size is the most important variable. Smaller particle size of the substrate can increased the productivity and reduce fermentation period considerably (Pallares *et al*., 1996). Medium is sterilized by steam and the fine suspension of the spores is sprayed on the solid substrate. Culture is allowed to grow for a limited period of 10 to 15 days depending upon the degradability or rate of substrate utilization.

2.9.4 Submerged Fermentation

Submerged types fermentation are growing due to the better understanding of fungal metabolism and its positive response. Submerged method has several advantages over surface and solid state cultures such as it requires less man power, gives higher yield and productivity, low cost, less contamination, better temperature control during fermentation, handling with suspended solids.

CHAPTER 3

METHODOLOGY OF RESEARCH

3.0 Materials and methods

3.0.1 Substrates

The palm kernel cake was used as substrates. The palm kernel cake was obtained from palm oil crushing plant at Semambu, Kuantan. Then the substrates were exposed and left to dry under the ray of sun until it was completely dry with no more moisture. Then all the substrates were inserted into the oven for completely drying at temperature of 60^{o}C for the duration of 12 hours. After that, all the dried substrates were milled into very small particle or we can look it more likely in powder form in the grinder. Lastly, all the substrates that we get in powder form were stored in a seal bag at room temperature to be used in vary weight while running the experiment.

3.0.2 Inoculums and enzyme production

Aspergillus niger stock cultures (supplied by the Chemical Engineering Department at the Faculty of Chemical Engineering and Natural Resources, University College of Engineering and Technology Malaysia (KUKTEM)) was maintained on Sabouraud Dextrose Broth that prepared earlier in Schott bottle. Then put in microbiological incubator for 7 day at the temperature of 27^{o}C. At this stage, we tried to

culture the *Aspergillus niger* to be used for next stage of experiment. Then after we successed cultured the *Aspergillus niger*, then the *Aspergillus niger* transferred to the Potato Dextrose agar that prepared earlier and was put in universal bottle by using sterilized loop.

Then put in microbiological incubator for 7 day at the temperature of 27^{o}C. After 7 days at 27^{o}C in microbiological incubator, we can find black spore was harvested and this thing showed that the *Aspergillus niger* was successed to be culture from its original *Aspergillus niger* stock cultures. Then the black spores of *Aspergillus niger* were suspended in 10 ml of deionized water and the suspension was transferred aseptically to 500 ml Erlenmeyer flask that contained 300 ml of sterile Mandel's medium and this step was done in laminar flow hood.

The inoculum was prepared by cultivation in stackable incubator shake (Infors Model AGCH-4103) for 3 days at the temperature of 30^{o}C with the stirrer speed of 150 rpm. After passed the 3 day of inoculum preparation, the amount of 30 ml of mycelium suspension was transferred to 500 ml Erlenmeyer flask that contained 300 ml of sterile Mandel's medium and known amount of substrates to initiate the growth. After the inoculation finished, the Erlenmeyer flask that contained Mandel's medium, substrates and mycelium suspension were incubated again in the stackable incubator shaker (Infors Model AGCH-4103) at the temperature of 30^{o}C with the stirrer speed of 150 rpm and for this time it was run for 7 days. The amounts of the concentration that we used in this experiment were 1, 3 and 5 g/L.

3.0.3 Enzyme Assays

To prepare 0.1 M sodium acetate solution, sodium acetate with an amount of 13.608 g was mixed with 1 liter of distilled water. Then the acetic glacial acid was added

into the 0.1 M sodium acetate solution slowly until get the solution pH is 5.0. Lastly, we put the solution in the labeled bottle and stored at 4^{o}C in a refrigerator.

To obtain 2% xylan solution which act as xylanase assay, an amount of 1 g of xylan powder was diluted in 50 ml of distilled water. This xylanase assay was used to analyze xylanase enzyme activity.

After that, the amount of 0.75 g of Carboxymethylcellulose was dissolved in 50 ml of distilled water to prepared 1.5 % Carboxymethylcellulose solution.

The Di-Nitro Salicyclic Acid (DNS) reagent was prepared by dissolved 10 grams of Di-Nitro Salicyclic Acid (DNS), 2 grams of Phenol and 0.5 gram of Natrium Sulphate into a 500 ml 2 % NaOH solution. After that the solution was added with distilled water until the volume of mixture is 1 liter. Actually, the DNS reagent is sensitive to the light. So as a precaution, the DNS reagent should be wrapped with aluminium foil and has to be stored in a dark place.

3.0.3.1 Xylanase Assay

In this experiment, 1.0 ml of 0.1 M of acetate buffer was added with 0.3 ml of 2 % xylan solution and also an amount of 0.2 ml of sample of enzyme that was already centrifuged earlier (centrifuged in Heraeus High Speed Centrifuge) at speed of 12000 for 30 minutes. All of this mixture was incubated for 30 minutes and maintained at the temperature of 37^{o}C. Then, 3 ml of DNS reagent was added to the mixture and was incubated again for 5 minutes and this time the temperature that we used was 100^{o}C. After 5 minutes of incubation, the mixture was put in the ice or tap water to cold it.

Once the mixture was cold, the deionized water was added until the volume of mixture is 16 ml. By using UV-Vis Single Beam Spectrophotometer (Hitachi U-1800) as analytical equipment, the reduced of sugar were determined at wavelength of 575 nm. For blank purpose, deionized water was used to substitute the sample of enzyme. Then the production of xylose and activity of xylanase enzyme was measured by referred to the standard calibration curve for xylose. One unit of xylanase enzyme was defined as 1 μmol of xylose produced per minute (μmol/min**)** in Internatonal Unit (IU).

3.0.3.2 Carboxymethylcellulase Assay

The same procedure like xylanase assay were applied to find the activity of Carboxymethylcellulase enzyme but by using 1.5 % Carboxymethylcellulase solution to replaced the 2 % xylan solution. Then the glucose produced the activity of Carboxymethylcellulase enzyme was identified by referred to the standard curve of glucose. One unit of Carboxymethylcellulase enzyme was defined as 1 μmol of glucose produced per minute (μmol/min**)** in Internatonal Unit (IU).

3.0.4 Preparation of Agar

The amount of 8.0 gram of potato dextrose agar (PDA) was diluted in 200 ml of distilled water. The solution that we get was heated by using heater to certain condition to ensure no colloid is seen and when this condition achieved, so the solution is completely dilute.

Once the solution ready, the solution was poured into a universal bottle until the solution fulfill 1/3 height of the universal bottle. Then the wool was used to stuff the

universal bottle. The universal bottle was autoclaved at condition of 121^{o}C and 15 psi for the duration of 15 minutes. After the process of autoclave the agar finished the agar was put in the slanting position and left to cool and freeze at room temperature.

3.0.5 Preparation of Mandel's Medium

Mandel's medium was prepared by mixing all of these chemicals into 1L solution. Then the Mandel's elements were autoclaved.

Table 3.1 Chemicals for the preparation of Mandel's medium

Chemicals	g/L
Ammonium Sulfate ($(NH_4)_2SO_4$))	1.4
Pottasium Phosphate (KH_2PO_4)	2.0
Urea	0.3
Calcium Chloride Dehydrate ($CaCl_2$)	0.4
Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	0.6
Manganese (II) Sulfate Monohydrate ($MnSO_4.4H_2O$)	1.0 mg
Zinc Sulfate Heptahydrate ($ZnSO_4.7H_2O$)	1.4 mg
Iron (II) Sulfate heptahydrate ($FeSO_4.7H_2O$)	5.0 mg
Cobalt (II) Chloride Hexahydrate ($CoCl_2.6H_2O$)	3.7 mg
Tween 80	0.75 mg
Protease Peptone	2.0

3.0.6 Research on Growth Rate and Yield of Xylanase.

While the growing period of Xylanase, analysis on the changes activity of enzyme was carried out. An amount of 10 ml of every sample was collected every day for one week for analysis. Sample was then centrifuged at 12000 rpm (centrifuged in Heraeus High Speed Centrifuge) for 30 minutes. Supernatant was analyzed to determine the activity of xylanase enzyme.

CHAPTER 4

RESULT AND DISCUSSION

4.0 Standard Calibration Curve for *Xylanase*

In this experiment, the results that obtained are based on the standard calibration curve of xylose and carboxymethylcellulose as shown in Figure 4.1 and 4.2. The data to produce xylose standard calibration curve is shown in Table 4.1. Different concentrations of xylan were used to get the reading for this xylose standard calibration curve. The concentration used were 0.15, 0.3, 0.5, 0.8 and 1.0 g/L which then were dissolved in 30 ml of deionized water. Then the data that gained were plotted as resulted in Figure 4.1. From the xylose standard calibration curve that we obtained, the enzyme activity of Xylanase for every sample from the experimented was identified. This standard calibration curve was using DNS method.

Table 4.1 Data for xylose standard calibration curve

Xylose concentration, µmol/mL	Absorbance (575 nm)
0	0
0.15	0.339
0.3	0.186
0.5	0.112
0.8	0.077
1.0	0.016

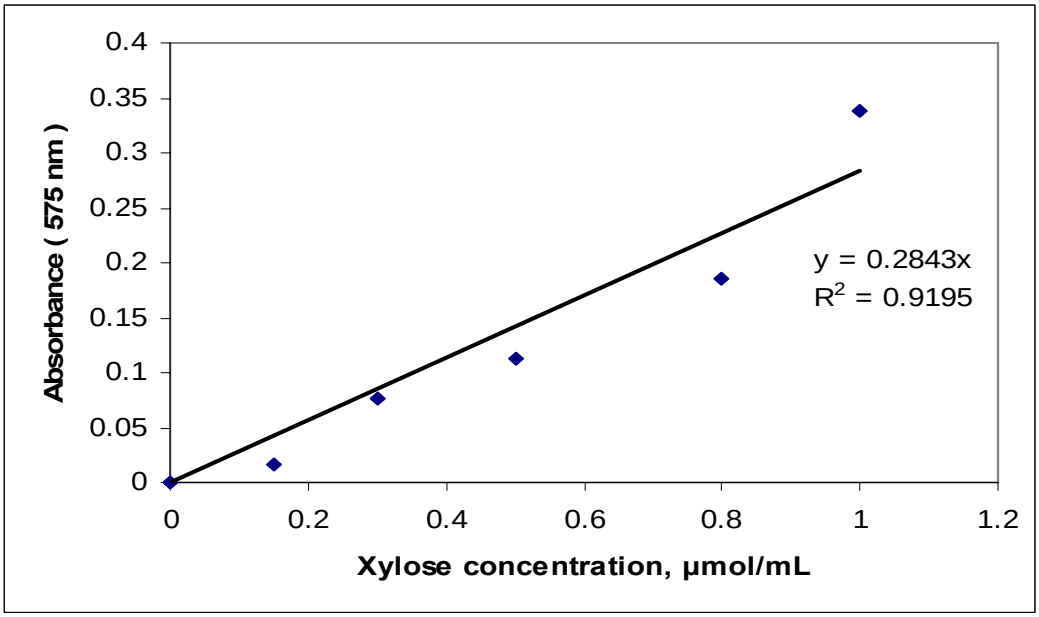

Figure 4.1 Xylose standard calibration curve

4.1 Standard Calibration Curve for *Carboxymethylcellulase*

To produce standard calibration curve for carboxymethylcellulose enzyme, the same step were implemented just like to produce standard calibration curve for xylanase enzyme. Different concentrations of glucose were used to get the reading for this carboxymethylcellulose standard calibration curve. The concentrations used were 0.15, 0.3, 0.5, 0.8 and 1.0 g/L which then were dissolved in 30 ml of deionized water. Then the data that gained were plotted as resulted in Figure 4.2. From the glucose standard calibration curve that we obtained, the enzyme activity of carboxymethylcellulose for every sample from the experimented was gained and identified. This standard calibration curve was using DNS method.

Table 4.2 Data for glucose standard calibration curve

Glucose concentration, µmol/mL	Absorbance (575 nm)
0	0
0.15	0.028
0.3	0.055
0.5	0.177
0.8	0.284
1.0	0.399

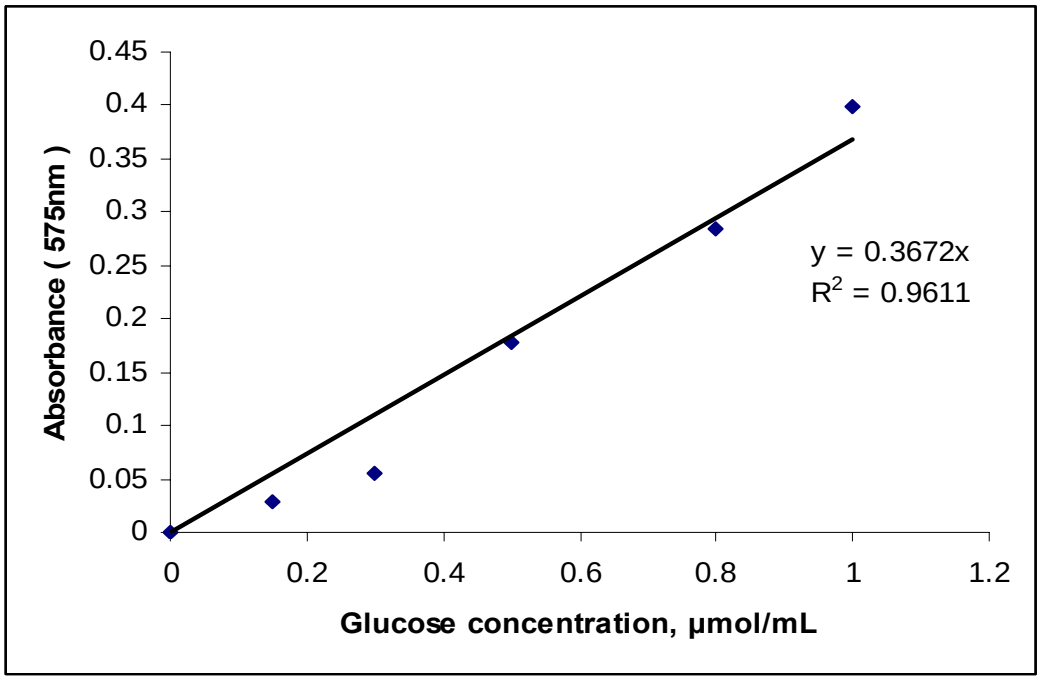

Figure 4.2 Glucose standard calibration curve

4.2 Effect of Different Concentration

4.2.1 Enzyme Activity of *Xylanase*

In this experiment, different concentrations of substrates were used like 1, 3 and 5 g/L. And Table 4.3 and Figure 4.3 showed the *Xylanase* enzyme activity at varied of substrate concentration.

Table 4.3 *Xylanase* enzyme activity for different concentration

		Concentration (g/L)		
Time (hr)	**Day**	**1**	**3**	**5**
Control	0	0	0	0
24	1	0.1055	0.1477	0.102
48	2	0.0546	0.1092	0.0787
72	3	0.1196	0.1301	0.109
96	4	0.0528	0.0598	0.0739
120	5	0.0457	0.0668	0.097
144	6	0.0458	0.0757	0.0901
168	7	0.0281	0.0675	0.0785

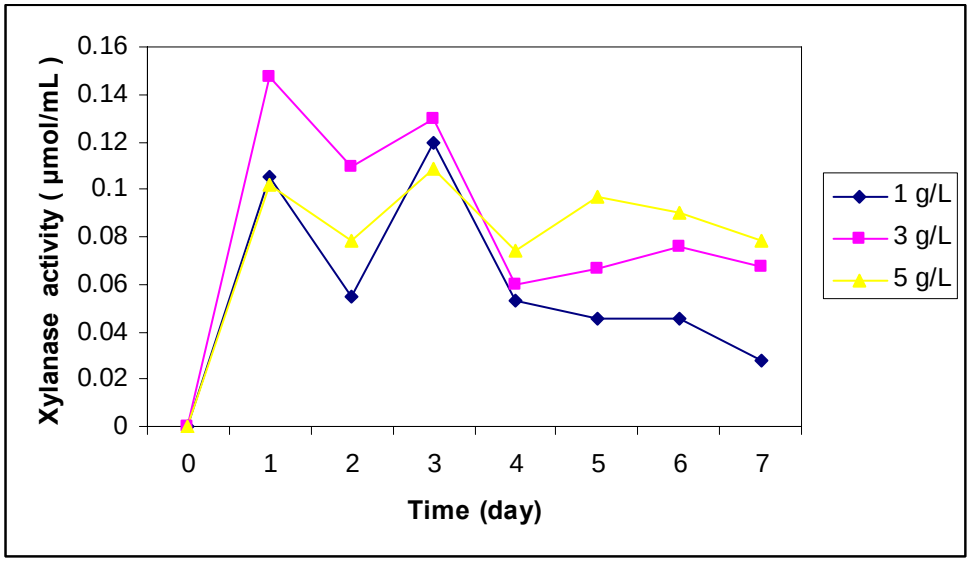

Figure 4.3 *Xylanase* Enzyme activity at different concentration of substrates.

Based on Figure 4.3, the concentration of 3 g/L produced the highest xylanase activity followed by 5 g/L and 1 g/L. The findings also stated that the maximum

xylanase activity was 0.1477 IU/mL on the first day. The gained result showed that the activity of enzyme was fluctuated for every concentration of samples (1, 3 and 5 g/L).

This thing may be happened because the *Aspergillus niger* just need the moderate amount of substrates concentration to produce enzyme especially when dealed with palm kernel cake as substrates. This may be related with the suitability of palm kernel cake as substrates that related to the nutritional content of the substrates. Palm kernel cake has higher protein that content of 16 % compared to other substrates like sugar cane bagasse, paddy straws or rubber wood saw dusts which are in the range of 1-3% as determined by the method of Macro Kjeldahl (AOAC, 1997).

Besides that, palm kernel cake was also found to contain a higher moisture content of about 6-7 % compared to other substrates. At the same time, we can found that the particle size of palm kernel cake was relatively smaller than other substrates that already were being used before and this thing giving higher surface area which would give advantage in ease of oxygen diffusion and nutrient absorption and assimilation by the mycelia.

In a study carried out by Takwai et al., (2005) it was concluded that the rate of glucose accumulation increases with the enzyme concentration while studied on the quantification of cellulose activity using cellulose azure and *Aspergillus niger* as substrates and microorganism.

But the experimental results revealed that the moderate concentration also can produce high of enzyme activity. However, although there are same strains which showed higher productivity, the comparison remains inconclusive since in the study of those strains, the substrates and cultural conditions used were different.

From the graph (Figure 4.3), after day four, the activity of the xylanase enzyme started to decrease. This may be because the *Aspergillus niger* started to enter the death phase or decline phase after the nutrient depletion or toxic product accumulation happened and this thing caused disability to continue the process of fermentation.

4.2.2 Enzyme Activity of *Carboxymethylcellulase*

In this experiment, different concentrations of substrates were used like 1, 3 and 5 g/L. And Table 4.3 and Figure 4.3 showed the *Carboxymethylcellulase* enzyme activity at varied substrate concentration.

Table 4.4 *Carboxymethylcellulase* enzyme activity for different concentration

		Concentration (g/L)		
Time (hr)	**Day**	**1**	**3**	**5**
Control	0	0	0	0
24	1	0.0136	0.0082	0.0163
48	2	0.049	0.049	0.0463
72	3	0.049	0.0381	0.0545
96	4	0.0654	0.0735	0.0681
120	5	0.0458	0.0163	0.0272
144	6	0.0163	0.0191	0.0354
168	7	0.0027	0.0054	0.0218