Isolation and Functional Characterization of Gene Encoding Xylanolytic

Enzyme from Metagenomic Library of Termite Gut

By

Miss Kanyanut Aduljit

A special project submitted to Department of Biotechnology,
Assumption University and National Center for Genetic Engineering and
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Metagenomes of uncultured microorganisms represent an unlimited biodiversity of genetic materials for discovery of novel biocatalysts. In this study, isolation and characterization of gene encoding xylan-degrading enzyme from the already established metagenomic fosmid library of termite gut was completely explored. Briefly, determination of xylanase activity of the selected positive fosmid clone, namely Xyn14.3, on LB agar plate was carried out using functional-based approach at pH ranging from 7.0 to 12.0. The enzyme showed optimal activity at pH 9.0 as it showed the most intense blue-color zone around the colony. In order to identify the gene encoding this enzyme, subcloning and sequencing of Xyn14.3 was performed. Further analysis of the obtained sequence showed an ORF encoding protein of 273 amino acids which contained a conserved domain of glycoside hydrolase family 11 (GH11) with 56% identity to known xylanase in database. The retrieved gene was overexpressed in *E. coli* and the corresponding 30.14 kDa protein was subsequently purified using His-trap chromatography. Biochemical characterization of the recombinant xylanase displayed a wide range of pH activity with a maximum at pH 7.0 and the optimum temperature is 45 °C. The stability of enzyme was highly maintained in pH 7.0 at 40 °C for 2 hours. Enzyme pretreatment of agricultural wastes (e.g. bagasse, rice straw, and rice husk) was investigated using DNS method. The reducing sugar released from these lignocellulosic wastes was increased in a proportion of the amount of enzyme applied. From the overall results, this study provides evidences that metagenome analysis is considered to be powerful tool n order to explanation of novel genes with potential utilization in several industries.

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CHAPTER 1

INTRODUCTION

Humans began to cultivate food crops about 10,000 years ago. As the agricultural production increase, a number of factors need to be in place. Agricultural waste has been left while most of them have been eliminated by incineration. The elimination process creates a lot of pollution such as air and water pollution, which lead to global warming.

The amount of agricultural waste is depended on the efficiency of agricultural production in country. In Thailand, the most agricultural wastes include bagasse, husk, and rice straw as they share 38.78 %, 17 %, and 14 %, respectively in all kinds of plant material wastes found within country (internet: http://www.dede.go.th). These plant materials comprise of lignocellulose, which made up of cellulose and hemicellulose, mainly xylan. Xylans are polysaccharides made from units of xylose (a pentose sugar), which is hydrolyzed by xylanolytic enzyme, so called xylanase. It is mostly used in an industrial scale and commonly applied in animal feeds, paper production, and food production. This enzyme has ability to deconstruct plant structural material by breaking down hemicellulose, a major component of the plant cell wall. Hemicellulose contains many different sugar monomers. Xylose is always the sugar monomer present in the largest amount in hemicellulose. Therefore, xylanase is used to digest this part of hemicellulose. Xylanase is recently used in industrial sector where lignocellulosic material is major source in the process. Previously, xylanase has been isolated from various environmental sources and it has been extracted from many different fungi and bacteria (Sunna and Antranikian, 1997).

Termites are important in the degradation of matter which includes cellulose, hemicellulose, lignin and flavonoid. The gut microorganisms of termites consist of protozoa, bacteria, spirochetes, and fungi. Gut microbiota symbiotically associated with termites are responsible for decomposition of various kinds of organic matter and biomass recycling. The hindgut of higher termite (the family Termitidae) is highly compartmentalized and comprises the midgut (M), first proctodeal segment (P1), main hindgut (P3), colon (P4a and P4b), and rectum (P5) sections. The P1 part of higher termites, especially soil- and wood- feeders belonging to the subfamily Termitidae, generally show high alkalinity (pH9-12). The highly compartmentalized of their hindgut can be expected to be an attractive source of alkaliphilic microorganisms capable to degrade lignocellulosic substances at high pH. Therefore, microorganisms from termite gut probably produce xylanases that function in alkaline condition which are more applicable to use in the industrial sector especially in paper industry. However, most of the microorganisms in termite gut are unculturable in the laboratory environment

that perturbs the isolation of the microorganism from the gut. Hence, metagenome has been applied to screen out the target gene encoded xylanase from unculturable microbes.

Metagenome technique is a high throughput technique that can be applied to screen out of the target xylanase gene from the environmental DNA libraries from termite gut. It is also referred to as "community genomics" or "environmental genomics", metagenomic is the sequencing and analysis of DNA of microorganisms recovered from an environment, without the need for culturing them. A model metagenomic project begins with the isolation of DNA from a mixed microbial population collected from any given environment. Environmental DNA is then sheared into fragments that are used in construction of a DNA clone library. Clone libraries are either small- or medium-insert (2-15 kb insert size) libraries for the further screening for the gene of interested. Therefore, this study aims to isolate novel genes encoding xylanase from termite gut using metagenome and the activity-based approach. The open reading frames that encode xylanases was further subcloned into an expression vector and transformed to a suitable host for over expression of the interested enzyme. The optimum temperature, optimum pH and the stability on pH and temperature of this novel xylanase were analyzed. In addition, the effect of the enzyme on lignocellulosic materials was carried out including rice husk, bagasse, and rice straw.

Objectives of this study

1. To isolate and characterize the full-length gene encoding xylanase of the selected positive clone discovered from metagenomic fosmid library of termite gut.

2. To clone and express the full-length gene in suitable host

3. To characterize the biochemical properties of the obtained recombinant enzyme

4. To determine the ability of xylanase on lignocellulosic materials (rice husk, bagasse, and rice straw) degradation

CHAPTER 2

LITERATURE REVIEW

Thailand has more than 100 million acres used for agriculture. Therefore, Thailand is a major agricultural country where produce a lot of agricultural waste because of various agricultural operations from industrial processing. The plenty of waste produced causes environmental problem. Normally, the main composition of agricultural waste is lignocellulosic material that can be changed to the more value added products. Xylanase have been used in the lignocellulosic application since xylanase can degrade the hemicelluloses compound from lignocellulosic material to be a reducing sugar. Xylanase have been isolated from various environmental sources. Termites are among the most important source of xylanase enzymes due to its ability to degrade lignocellulosic substances. It has a great variety of symbiotic microorganisms in their hindguts, including Bacteria, Archea, and Eukarya. Gut microbiota symbiotically associated with termites are responsible for decomposition of various kinds of organic matter, biomass recycling and lignocellulosic materials (Brune, Emerson, and Breznak, 1995). The highly compartmentalized of their hindgut can be expected to be an attractive source of alkaliphilic microorganisms capable to degrade lignocellulosic substances at high pH. Therefore, microorganisms from termite gut probably produce xylanases that function in alkaline condition which are more applicable to use in the industrial sector. However, most of the microorganisms in termite gut are unculturable in the laboratory environment that perturbs the isolation of the microorganism from the gut. Therefore, metagenome approach has been applied to screen out the target gene from unculturable microbes. This technique is a high throughput technique that can be applied to screen out of the target xylanase gene from the environmental DNA libraries from termite gut (Handelsman et al., 1998).

2.1 Lignocellulosic material

Lignocellulosic materials are a natural, abundant and renewable resource essential to the functioning of industrial societies and critical to the development of a sustainable global economy. Incineration is a waste treatment technology for today that involves the combustion of organic materials and substances (Knok and Andrew, 2005). This process can give rise to polluting wastewaters from runoff and release some greenhouse gas such as CO_2 which is mainly cause of global warming. Therefore, lignocellulosic material has been used to produce value added product because of their structure which have a lot of carbon source. Many processes have been developed that utilize lignocellulose as raw materials for the production of bulk chemicals and value-added fine products such as ethanol, enzymes, single-cell protein. Application of agro-industrial residues in

bioprocesses on the one hand provides alternative substrates, and on the other hand helps in solving pollution problems (Pandey *et al.*, 2000).
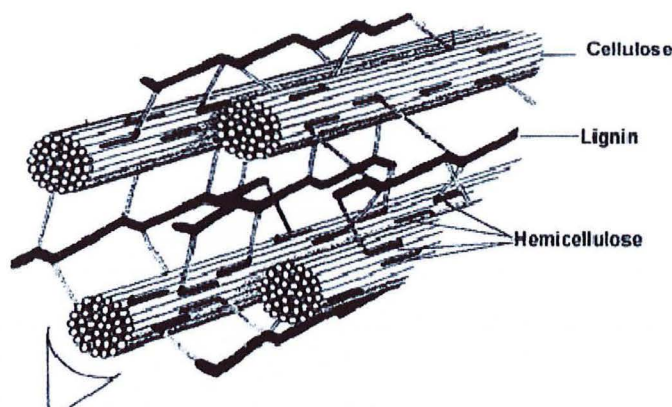
Figure1: Structure of lignocellulose (Internet:http://www.life.ku.dk/forskning/online_artikler/artikler/marken_en_stor_solfanger.aspx)

Lignocellulose is a matrix of cross-linked polysaccharide networks, glycosylated proteins, and lignin as show in figure 1. This matrix has three main components: 38-50 % of cellulose, 17-32 % of hemicellulose, 15-30% of lignin and a little bit flavonoid (Ritter, 2008) as show in figure 2. A major constituent of all plant is cellulose, a $\beta(1–4)$-linked chain of glucose molecules. Hydrogen bonds between different layers of the polysaccharides contribute to the resistance of crystalline cellulose to degradation. While hemicellulose is the matrix polysaccharides of plant cell wall (Nichols and Andrew, 2007). It is composed of various 5- and 6-carbon sugars such as arabinose, galactose, glucose, mannose, and xylose. Lignin is composed of three major phenolic components, namely *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S). Lignin is synthesized by polymerization of these components and their ratio within the polymer varies between different plants, wood tissues, and cell wall layers. Cellulose, hemicellulose, and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall (*Benjamin et al*, 2006).

Figure2: Pie chart of Biomass potential in Thailand 2007

2.1.1 Cellulose

Cellulose is the principal constituent of most plants. It has been accepted for many years that cellulose is a long chain polymer, made up of repeating units of glucose, a simple sugar. Cellulose has been shown to be a long chain polymer with repeating units of D-glucose, a simple sugar as show in figure 3. In the early 1900s, cellulose was further characterized by removing the related plant materials that occur in combination with cellulose by dissolving them in a concentrated sodium hydroxide solution.
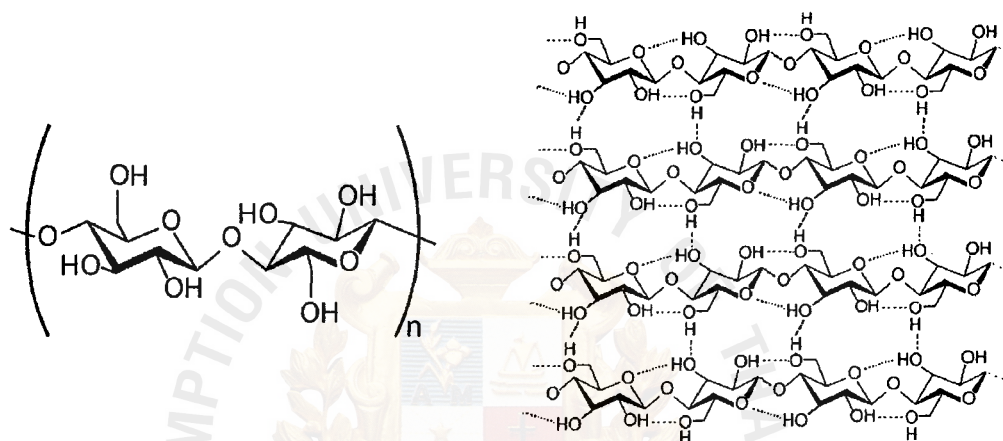
Figure3: Chemical structure of cellulose polymer
(Internet: http://th.wikipedia.org/wiki/Cellulose_strand.jpg)

In the cellulose chain, the glucose units are in 6-membered rings, called pyranoses. They are joined by single oxygen atoms (acetal linkages) between the C-1 of one pyranose ring and the C-4 of the next ring. The pyranose rings of the cellulose molecule have all of the groups larger than hydrogen sticking out from the periphery of the rings. The stereochemistry at carbons 2, 3, 4 and 5 of the glucose molecule are fixed; but when glucose forms a pyranose ring, the hydroxyl at C-4 can approach the carbonyl at C-1 from either side, resulting in two different stereochemistries at C-1. When the hydroxyl group at C-1 is on the same side of the ring as the C-6 carbon, it is said to be in a configuration. In cellulose, the C-1 oxygen is in the opposite, or b configuration. This b configuration, with all functional groups in equatorial positions, causes the molecular chain of cellulose to extend in a more-or-less straight line, making it a good fiber-forming polymer. Amylose, a constituent of starch, is a related polymer of glucose, but with the C-1 oxygen in a configuration. This configuration forces the linkage to the next glucopyranose ring to assume an axial position, and the starch molecules tend to coil, rather than extend. Even though it often has long molecular chains, amylose is not a good fiber-former (Ashmun, 1921). Because of the strong hydrogen bonds that

occur between cellulose chains, cellulose does not melt or dissolve in common solvents. Thus, it is difficult to convert the short fibers from wood pulp into the continuous filaments needed for artificial silk, an early goal of cellulose chemistry. Several different cellulose derivatives were examined as early routes to artificial silk but only two, the acetate and xanthate esters, are of commercial importance for fibers today (see History of Manufactured Fibers).

2.1.2 Lignin

Lignin fills the spaces in the cell wall between cellulose, and hemicellulose components. It is covalently linked to hemicellulose and thereby cross links different plant polysaccharides, conferring mechanical strength to the cell wall and by extension the plant as a whole (Chabannes *et al*, 2001) as show in figure 4 . Lignin is formed by the removal of hydroxyl groups from sugars, creating phenolic compounds and short-chain alcohol ligands. Lignin polymers are heavily cross-linked. There is great variation in lignin, even within the same plant. Therefore, it has evolved to have a somewhat random structure to foil enzymatic attack. Lignin is, in fact, extremely difficult to dissociate. Fortunately, considerable progress has been made since then (Micol and Hake, 2003). The basic monomer of lignin can be thought of as 4-alkylcatechol. The alkyl ligand is a 1 to 3 carbon chain which may be substituted with hydroxy or keto ligands at any or all positions. Both phenol groups and the alkyl substitutions may cross-link to other monomers or their side chains, yielding a light, but strongly cross-linked and randomly ordered mass. Moreover, the chemical structure virtually guarantees that any intermediate degradation product will be a highly toxic phenolic compound. Lignin was a rather early product of plant evolution (Ligrone *et al.* 2000). In the industrial scale, lignins are generated during kraft pulping in alkaline. They contain a small number of aliphatic thiol groups that give the isolated product a characteristic odor especially during heat-treatment. A very small amount of kraft lignin is isolated from pulping liquors. The vast majority is used the vast majority is used as in-house fuel required for the recovery of chemicals (Lora and glasser, 2002). Moreover, Lignin extracted from black liquor - a paper industry waste material, has been characterized and used for the removal of lead and zinc metals to increase the sorption capacity (Srivastava, Singh, and Sharma, 1994).
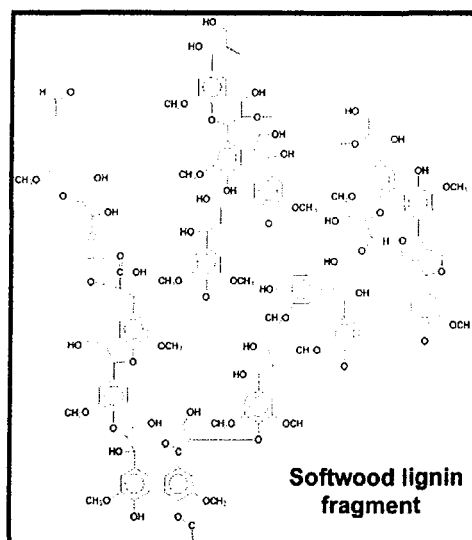
Figure4: Chemical structure of softwood lignin fragment
(Internet:http://sci.waikato.ac.nz/farm/content/plantstructure.html)

2.1.3 Hemicellulose

    Hemicelluloses are a group of plant-derived heteropolysaccharides associated with cellulose and lignin. It is found in the plant cell wall, which consists of sugar units containing 5 or 6 carbons in its backbone and side chains as show in figure 6. Hemicellulose contains 500 to 3,000 sugar units and includes a small amount of pectin, another polysaccharide, with forms a cross-linked network. It mainly composes of carbohydrates based on pentose sugars, mainly xylose, as well as hexose sugars, such as glucose and mannose (Ponder and Richards, 1991). The sugars in the side chains also confer important characteristics on the hemicellulose. The most common hemicelluloses are xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan. In the principal hemicellulose component, xylan, is a polymer of β (1-4)D-xylopyranose. In arabinoxylan, branching occurs at the C2 and C3 positions with α-L-arabinofulanose (Passmore, 1984). The xylan backbone has been found in Glucuronoxylan with 4-0 methylglucuronic acid branching. In addition, arabinose branching as well as acetylating may be present. Gymnosperms contain glucomannans comprised primarily of D-mannosyl and D-glucosyl residues (Enzyme *Nomenclature*). Fermentability of the hemicelluloses by intestinal microflora is also influenced by the sugars and positions. For example, hexose and uronic acid components of hemicellulose are more accessible to bacterial enzymes than the other hemicellulose sugars. Some foods that are relatively high in hemicellulose are bran and whole grains (Marlett and Cheung, 1997).
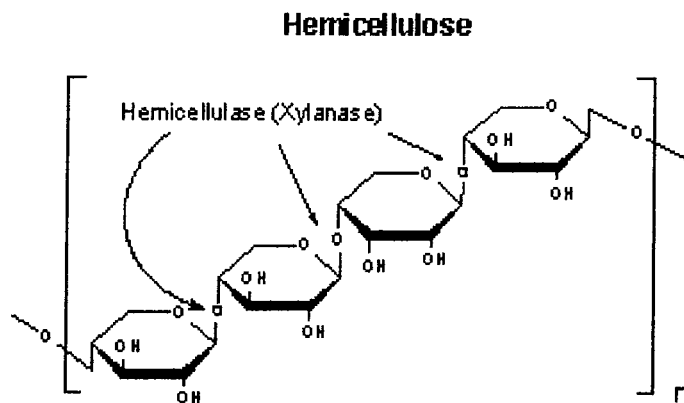
Hemicellulose

Figure5: Chemical structure of hemicellulose
(Internet: http://www.bio.miami.edu/dana/226/226F07_3print.html)

2.2 Pretreatment process for the lignocellulosic material

Pretreatment is an important tool for practical cellulose conversion processes. Pretreatment is required to alter the structure of cellulosic biomass to make cellulose more accessible to the enzymes that convert the carbohydrate polymers into fermentable sugars as represented in the schematic diagram (Lynd *et al*, 1996) as see in figure 6. The goal is to break the lignin seal and disrupt the crystalline structure of cellulose in order to enhance the enzymatic digestibility. The carbohydrate components of lignocellulose (cellulose and hemicellulose) are tightly bound to lignin, making the sugars largely inaccessible to enzymes. Before enzymatic hydrolysis, pretreatment with acid or alkali is generally needed to release of sugars from any lignocellulosic material (Hector *et al*, 2008). There are various pretreatment process and conditioning procedures, employing chemical and enzymatic hydrolysis or particle size reduction (physical) of lignocellulosic materials provided some modest improvements to digestion yields and steaming process is a recent technology, which has gained much interest worldwide (Aslam *et al*, 2008). However, these methods spend a lot of investment. The method used in pretreatment step for this research includes base and acid pretreatment using sodium hydroxide and peracetic acid (PAA) respectively. Sodium hydroxide was most effective at removing lignin. Enzymatic hydrolysis is improved by using an alkali to extract a fraction of the lignin from the pretreated material. The removal of the lignin may increase the accessibility of the cellulose to enzymatic attack, and thus improve overall economics of the process (Schell *et al*, 1998). In the use of PAA is a strong oxidant that can remove lignin under mild conditions. PAA pretreatment could greatly enhance the enzymatic digestibility of lignocellulosic material by removing hemicelluloses and lignin. The sulfuric acid in the PAA solution also can cause degradation of hemicelluloses (Zhao *et al*, 2007).
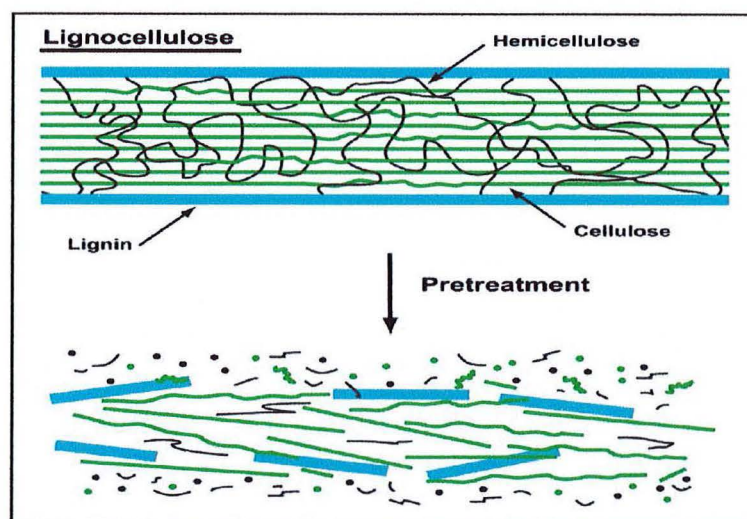
Figure6: Process of pretreatment on Lignocellulose model showing lignin, cellulose and hemicellulose (USDA Agricultural Research Service)

2.3 Some selected agricultural waste for this study

2.3.1   Rice husk

Rice husk is a solid waste from rice mill and sugar cane industry, respectively. Husk (or hull) is the hard protecting coverings of grains of rice. In addition, rice husk can be put to use as building material, fertilizer, insulation material, or fuel. The chemical composition of rice husk composes of 12-16 % of lignin, 26-32 % of hemicellulose, and 45-55 % of cellulose (Fang *et al.*, 2004). The density is about 122 kg/m^3. Rice husk can be used to produce many value added products. Rice hulk can be used to produce mesoporous molecular sieves (e.g., MCM) which are applied as catalysts for various chemical reactions, as a support for drug delivery system and as adsorbent in waste water treatment. Rice husk are the outermost covering of the rice and come as organic rice hulls and natural rice hulls. Rice husk are an inexpensive byproduct of human food processing, serving as a source of fiber that is considered a filler ingredient in cheap pet foods (Chiarakorn *et al*, 2007). The rice itself can be burned and used to power steam engines, used in brewing beer to increase the lautering ability of a mash and used as a "press aid" to improve extraction efficiency of apple pressing (Olivier, 2004). In Thailand and many other Asian countries, where rice harvesting is concentrated, burn-off of rice residues is common. This practice emits large quantities of contaminants into the air and can have serious effects on air quality, public health and the climate (Xu *et al*, 2004). Rice and wheat residues are good sources of cellulose as a base for obtaining resistant and biodegradable fibers that

can replace wood fiber or plastic derived from petroleum. Many products can be developed using pulping processes to obtain fibers and produce compounds that mix fibers with other plastic or inorganic materials (Xu *et al*, 2004; Boonlert, 2005). Moreover, Rice husks had been enzymatically treated in aqueous media to cause the hydrolytic degradation of xylan. The reaction liquors refined by nanofiltration and ion exchange were treated with endoxylanases to reduce the degree of polymerisation of the reaction products (Vegas *et al*, 2008). Therefore, for this research, the activity of xylanase was used to find the efficiency to degrade rice husk and changed to monosaccharide (x y l o s e) .

Figure7: Rice husk, a solid waste from rice mill and sugar cane
(Internet: http://www.citylion.co.th/product/custom/)

2.3.2 Rice straw

Rice straw is an agricultural by-product, the dry stalks of cereal plants, after the grain or seed and chaff has been removed. Rice straw provides an alternative substrate to price useful chemicals such as bioethanol and lactic acid (Rishen *et al*, 2007). The chemical composition of rice straw composes of 30-35% of cellulose, 25-30 % of hemicellulose, and 8-12 % of lignin. The density of rice straw in form of hammer milled is about 40 – 100 kg/m^3 (Summers, 2000). In times gone by, it was regarded as a useful by-product of the harvest, but with the advent of the combine harvester, straw has become more burdensome to agriculture. The use of straw in large-scale biomass power plants is becoming main product. The straw is either used directly in the form of bales, into pellets, which allows the feedstock to be transported over longer distances. Straw is made up about half of the yield of cereal crops such as barley, oats, rice, rye, and wheat. In some research, rice straw had been treated with sulfuric acid to convert lignocellulosic material to sugar. Enzyme treatment of pretreated sample was applied to improve the conversion material to sugar. Combination of acid

pretreatment and subsequent enzyme treatment was increased the sugar yield drastically (Toswathana *et al*, 2010). Therefore, in this research, the xylanase was use to pretreat lignocellulosic material conversed to sugar and to find the suitable material that give highly conversion of lignocellulosic material to sugar.

Figure8: Bundles of rice straw (Xavier Navarro (RSS feed), 2008)

2.3.3 Bagasse

Bagasse is the fibrous residue remaining after sugarcane or sorghum stalks are crushed to extract their juice and is currently used as a renewable resource in the manufacture of pulp and paper products and building materials. The chemical composition of bagasse composes of 46% of cellulose, 27% of pectin, 23% of lignin, and 4% of ash. Hemicellulose and pectin collectively constitute the matrix polysaccharides of the cell wall (Cosgrove and Daniel, 1998). The density of bagasse is low (about 120.1 kg/m3) with a very wide range of particle sizes and high moisture content. These properties are necessary to apply normal design procedures to, for example, pneumatic conveying, fluidization, drying, combustion, etc.(Rasu *et al.*, 1999) Many research efforts have attempted to use bagasse as a renewable feedstock for power generation and for the production of bio-based materials. Bagasse is often used as a primary fuel source for sugar mills; when burned in quantity, it produces sufficient heat energy to supply all the needs of a typical sugar mill, with energy to spare (Rainey, 2009). In the part of bioprocess, bagasse fibers are well suited for tissue, corrugating medium, newsprint, and writing paper (Covey *et al*, 2006). Most chemical bagasse pulp mills concentrate the spent reaction chemicals and combust them to power the paper mills and to recover the reaction chemicals. Moreover, enzyme from a culture of a filamentous fungus can break down the lignin in the fibers of bagasse used as substrate in these trials, transforming this waste

product, after mechanical refining, into paper pulp. As the lignin progressively disappears, the pulp obtained becomes bleached. A manufacturer can use this pulp to make cardboard (ISA: Paper industry growth through biotechnology, 2006). In other research, sugarcane can be used to produce fuel grade ethanol without saccharification. A chemical pretreatment process using alkaline peroxide and hydrolysis was applied to remove lignin which acts as physical barrier to cellulolytic enzyme (Dawson and Boopathy, 2008). Therefore, in this research, the xylanase was use to pretreat bagasse conversed to sugar and to find the suitable material that give highly conversion of lignocellulosic material to sugar.

Figure9: Bagasse, residue of sugar cane
(Internet: http://commons.wikimedia.org/wiki/File:Bagasse_dsc08999.jpg)

2.4 Xylanolytic enzyme

Microbial enzymes have been widely used for the hydrolysis of polysaccharides in lignocellulosic materials to be fermentable sugars for the production of fuel, chemicals, and food and feed. Since xylan is the major component of plant hemicellulose, most investigations of the enzymatic degradation of hemicellulose have focused on xylanolytic enzymes, such as xylanase (Kuhada *et al*, 1998). The enzyme named xylanase deconstructs plant structural material by breaking down hemicellulose, a major component of the plant cell wall which can degrade the linear polysaccharide beta-1, 4-xylan into xylose. This enzyme 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 (Dhillon *et al*, 2000). In nature, some plant consumers or pathogens use xylanase to digest or attack plants. Many microorganisms produce xylanase, but mammals do not. Some herbivorous insects and crustaceans

also produce xylanase. Xylanases have been isolated from various environmental sources for example: xylanases are present in fungi in herbivore for the degradation of plant matter into usable nutrients (Dashek, 1997). Termites are among the most important source of xylanase enzymes due to its ability to degrade lignocellulosic substances at the high alkaline condition.
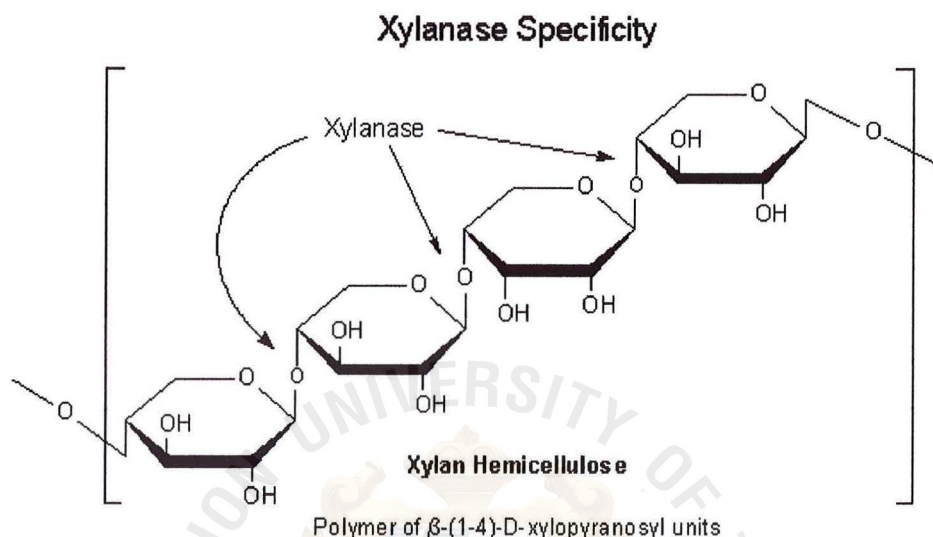
Figure10: Chemical structure of xylan hemicellulose (Internet: http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/2/1/8.html)
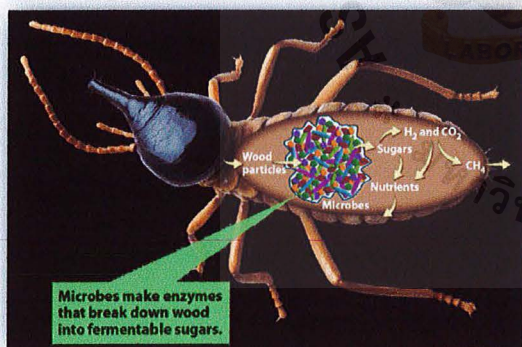
Figure11: Termite Microbes: A Potential Source of Enzymes to Digest Biomass for Bioenergy Production.

Termites are among the most important source of xylanase enzymes due to its ability to degrade lignocellulosic substances at the high pH. It has a great variety of symbiotic microorganisms in their hindguts, including Bacteria, Archea, and Eukarya. Gut microbiota symbiotically associated with termites are responsible for decomposition of various kinds of organic matter, biomass recycling and lignocellulosic materials (Brune, Emerson, and Breznak, 1995). The highly compartmentalized of their hindgut can be expected to be an attractive source of alkaliphilic microorganisms capable to degrade lignocellulosic substances at high pH as show in figure 12. Therefore, microorganisms from termite gut probably produce xylanases that function in alkaline condition which are more applicable to use in the industrial sector. However, most of the microorganisms in termite gut are unculturable in the laboratory environment that perturbs the isolation of the microorganism from the gut. Hence, metagenome has been applied to screen out the target gene from unculturable microbes.
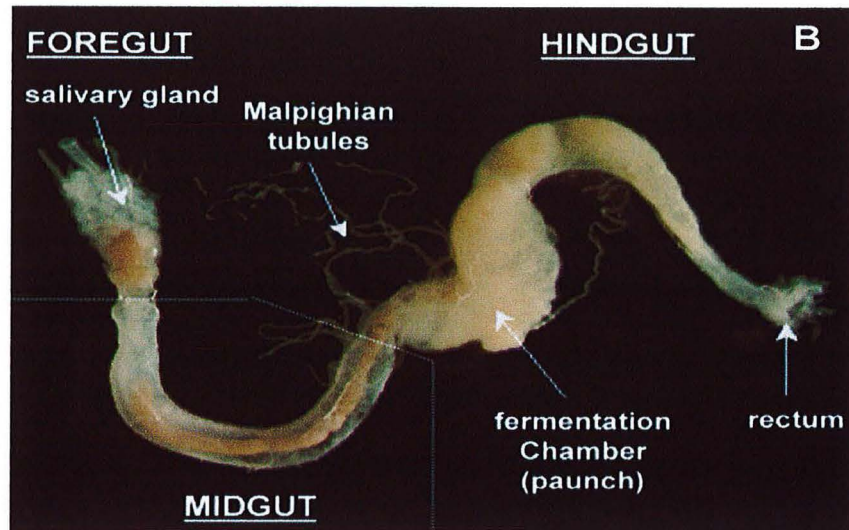
Figure12: Morphology and features of the termite gut. (A & B) Drawing and photo showing different regions of the termite gut. HindGut P1 show the alkaline condition can be expected to be an attractive source of alkaliphilic microorganisms (Scharf and Tartar, 2008)

2.5 Metagenome technique

Microorganisms contain unique properties such as the ability to degrade waste products. The genetic and biological diversity of microorganisms is an important area of scientific research (Handelsman *et al*, 1998). Unfortunately, scientists are able to grow less than 1% of all microorganisms observable in nature under standard laboratory conditions. This leaves scientists unable to study more than 99% of the biological diversity in the environment. Metagenome is the method that scientists can study the environmental system by extracting DNA from organisms in the system and inserting it into a model organism. The model organism then expresses this DNA where it can be studied using standard laboratory techniques by isolation of DNA followed by direct cloning of functional genes from the environmental sample (Tyson and Chapman, 2004). Metagenomic libraries are typically used to search for new forms of a known gene. The key steps of this method involve extraction of metagenomic DNA from arbitrary environmental sources (Rondon *et al*, 2000), transformation of environmental metagenomic libraries into the microbial host of interest, and selection of functional genetic elements conferring the desired phenotype compatible with the chosen host (figure 13). The sequence of DNA can be compared to databases of known DNA to get information regarding the structure and organization of the metagenomic DNA. Information from metagenomic libraries has the ability to enrich the knowledge and applications of many aspects of industry, therapeutics, and environmental sustainability (Vogel and Nalin, 2003).

This information can then be applied to this research to screen out of the target xylanase gene from the environmental DNA libraries from termite gut. Xylanase has proven useful in many ways: biobleaching paper pulp and improving animal feed. Adding xylanase stimulates growth rates by improving digestibility, which also improves the quality of the animal litter. It also changes hemicellulose to sugars so that nutrients formerly trapped within the cell walls are released (Bedford and Morgan, 1996). In addition to converting hemicellulose to nutritive sugar that the cow or other ruminant can digest, xylanase also produces compounds that may be a nutritive source for the ruminal microflora (David *et al*, 1999). Moreover, the research about metagenome method has been applied to discover functional genetic elements encoding chemical tolerance relevant to biomass conversion which can be applied to select for microbial usage and production of specific biomass chemicals. The repertoire of biomass substrates that can be used by a microbial biocatalyst has been expanded by transfer of specific genetic machinery for substrate metabolism from other microbes (Sommer, Church, and Dantas, 2010). The study that aims to isolate novel genes encoding xylanase from termite gut using metagenome is the most advantageous approach.
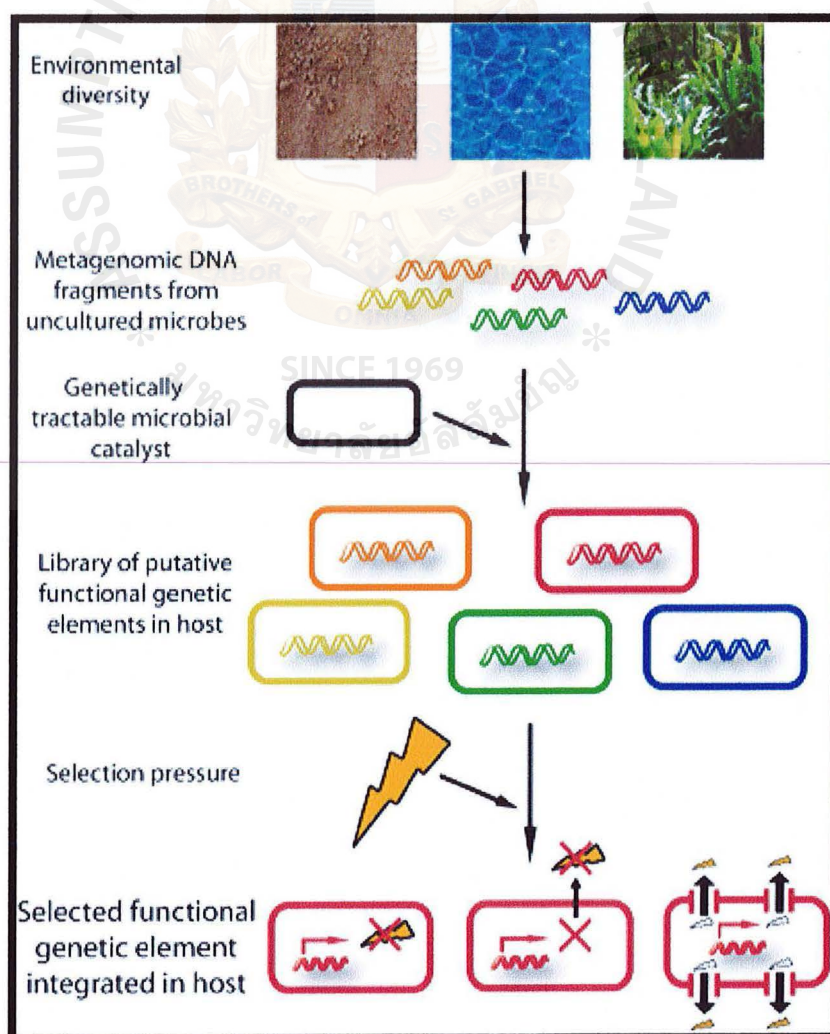
Figure 13: Functional metagenomic platform for discovery of novel functional genetic (Sommer, Church, and Dantas, 2010)

CHAPTER 3

MATERIALS AND METHODS

3.1 The selection of the positive clone

3.1.1 Overlaid technique to select xylanase positive clone

The colonies were overlaid with Enzhance solution (0.5 % buffer A and 1M desired buffer pH7.0 - 12.0) supplemented with AZCL-xylan substrate solution (0.7 % agar and 0.05% AZCL-xylan) and then incubated at 37 °C for a few hours. Blue color released from AZCL-substrates around colonies was represented as a xylanase positive clone.

3.1.2 Subcloning of fosmid insert from the selected positive clone with xylanase activity into p-ZEro^{TM}-2vector

The selected representative clones expressing xylanase activity from the fosmid was subcloned in order to identify the gene encoding enzyme of interest. Each fosmid was partially digested by Bsp143I (Sau3AI) and then ligated with T4 DNA ligase into p-ZErO^{TM}-2 vector, which had been isolated and cut with BamHI and purified vector before ligation. The ligated vector was transformed into $E. coli$ Top-10 competent cell by electrophoration in the condition of 25 μf, 600 Ω, and 2kV then incubated at 37 °C for 1 hour. The transformation mixture was plated onto LB agar plate supplemented with 50 μg/m^1 kanamycin (LB-Km) and incubated at 37 °c for 18-24 hours. The transformant expressing xylanolytic was approached using overlay technique and choose the positive subclone showed blue zone around colonies. The obtained positive subclones were subsequently studied for their nucleotide sequencing. The transformation efficiencies were calculated from the use of 5 minute, 10 minute, and 15 minute partial digestion of fosmid.

3.2 Sequencing analysis

The obtained subclone was analyzed for the nucleotide sequence using M13R and M13F primer that hybridize nearby the multiple cloning site of p-ZErO^{TM}-2 vector. The sequence alignment was compared with DNA database search in GenBank using BLAST analysis (http://www.ncbi.nih.gov). The sequence was aligned with the know xylanase genes to identify the consensus sequence of ribosome binding sites and promoter regions by Basic Local Alignment Search Tool, National Center for Biotechnology Information (NCBI). Then BlastX from NCBI was used to identify the homologous sequence and the amino acid sequence was analyzed using ExPaSy Translation tool. The forward primer and reverse primer were designed to amplify the full length gene from the sequencing information.

3.3 Qualification the inserted gene

3.3.1 Amplification the positive subclone and subclone to an expression vector

In order to clone the specific region of predicted gene encoding xylanase, the primer was designed against the 5' end-3' end of the gene with a restriction site to facilitate the cloning in expression vector. The polymer chain reaction (PCR) was performed to amplify the gene for one and a half hour. After PCR product was obtained, purification of PCR product was done by using QIA quick PCR purification kit. Then, the purified product was digested with *Hind*III and *Eco*R I. The digested vector was used to ligate with an expression vector pET-28a(+)and then transformed to *E. coli* DH5α used as a suitable host and then plated on LB agar plate supplemented with 50 μg/ml kanamycin (LB-Km) and incubated at 37 °C for 1 hour.

3.3.2 Checking the existing of inserted gene

3.3.2.1 Size screening

The size screening was used to select the positive subclone which represents inserted gene inside pET-28a (+) vector and then only five positive colonies was used in further step. The colonies recombinants from LB-km agar plate from the previous section were screened out using the size screening method and number 1 to 30 was labeled at every colony. Colonies were mixed with size screening solution and 40 μl to run on 0.8% agarose gel electrophoresis and run the reaction for 30 minute. After that, 5 colonies were chosen, the sequencings were done to confirm the positive result.

3.3.2.2   Colony PCR

The five clones from size screening were rechecked the inserted gene by using colony PCR method with xyn14.3 forward primer and xyn14.3 reverse primer. The positive clones were transformed them to Rosetta^{TM} competent cell. The transformation mixture was plated onto LB agar plate supplemented with 50 μg/ml kanamycin and 34 μg/ml cholamphenical (LB-Km-Cm) and incubated at 37 °c for 18-24 hours. The PCR was done follow by the PCR condition as show in appendix.

3.4   Cloning and Overexpression of xylanase gene in Rosetta^{TM}

After the positive clones from the previous section were transformed to Rosetta^{TM} for an expression of xylanase, the gene encoded for the enzyme was further induced under the optimum condition. The overexpression of the gene encoding xylanolytic activity was carried out using 1 mM of isopropyl-beta-D-thiogalactopyranoside (IPTG) overnight. The protein was isolated out from the cell using the centrifugation (8,000 rpm, 30 minutes) and further conducted by using Sonicator. The isolated protein was run on Sodium Dodicyl Sulfate Polyacrylamide Gel electrophoresis (SDS-PAGE) with the used of 30A to run protein for 1 hour. The protein was further purified using His trap affinity column and the elution buffer (20 mM-Na-phosphate, 0.5 NaCl) was applied with the variation of 100 – 500 mM Imidazole. The enzyme was named as X14.3

3.5. Biochemical Characterization of X 14.3

The crude enzyme was characterized on its biochemical properties including pH optimization, temperature optimization, digestibility on various pH, digestibility on various temperature, pH stability, and temperature stability. Moreover, the concentration of protein including in crude enzyme was investigated as well. The standard curve was constructed by using xylan as a substrate and the concentration of xylose was varied from xylose stock in the unit of μmole/ml. The enzyme was used in the dilution of 2,500 times from the concentration of 0 μg/ml to 0.4 μg/ml.

3.5.1   Concentration of protein

The protein standard curve was constructed by using bovine serum albumin (BSA) in phosphate buffer pH 7.0. Prepare BSA stock solution (1 mg/ml) using phosphate buffer pH 7.0. The range of BSA used in standard curve is between 0 to 1,000 μg and add the reaction tubes the following concentrations of BSA 100, 200, 400, 600, 800, and 1,000 μg/ml. Adjust volume to 1,000 μl with distilled water and add 5 ml of alkaline $CuSO_4$ assay mix. Vortexing and let sit at room temperature for 10 minutes. Add diluted Folin-Ciocalteu reagent. Vortex and leave at room

temperature for 30 minute. Mix by vortex again and read the optical density at 750 nm. The concentration of BSA was varied from 0 μg/ml to 1,000 μg/ml. Then the graph was constructed based on the spectrophotometric technique at the wavelength 540 nm. After the graph was constructed, the linear equation and R-square were calculated. Both of them were used in further step. The concentration of protein in X 14.3 was found comparing with protein standard curve.

3.5.2   Xylose standard curve at various pHs

The xylose standard curve in various pHs were constructed in order to check the amount of xylose in further step. Xylose stock was diluted for 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μmole. The buffer used to prepared pH 5.5, 7.0, 8.0, and 10.0 were Sodium Acetate buffer (NaAC), phosphate buffer, Tris-HCl buffer, and, Glycine-NaOH buffer respectively. The reaction was mixed with 660 μl of DNS solution and boil for 10 minute. The activity was calculated from the optimum density. Graph of each pH was constructed based on the spectrophotometric technique at the wavelength 540 nm.

3.5.3   Optimum pH

NaAC buffer, Phosphate buffer, Tris-HCl buffer and Glycine NaOH buffer was prepared to set up pH 5.5, pH 7.0, pH 8.0, and pH 10.0 respectively. Xylan was used as a substrate and 20 μl of diluted enzyme was added into 320 μl of substrate and incubated at 50 °C for 10 minute. After that the solution was mixed with 660 μl of DNS solution. The activity was calculated referring to the xylose standard curve at each specific which provided the concentration of xylose available in the reaction. The activity of enzyme at each pH was calculated and compared in order to find the optimum pH.

3.5.4   Optimum temperature

The optimum temperature was determined by varied the temperature from 25 °C (room temperature) to 70 °C. The buffer at optimum pH from previous experiment was used to prepare diluted enzyme. Xylan was used as a substrate and 20 μl of diluted enzyme was added into 320 μl of substrate and incubated at 50 °C for 10 minute. After that the solution was mixed with 660 μl of DNS solution. The activity was calculated referring to the xylose standard curve at each specific pH that was used to determine the amount of xylose on the reaction. The activity of enzyme at each temperature was calculated and compared in order t find the optimum pH of the enzyme.

3.5.5 pH stability

The diluted enzyme was determined the pH stability with optimum temperature. Phosphate buffer pH 7.0 and Tris-HCl buffer pH 8.0 and pH 9.0 were used to dilute enzyme. The diluted enzyme in each buffer was incubated at 45 °C for 0 minute, 30 minute, 60 minute, and 90 minute. The total of 20 µl of enzyme solution at each specific time point was mixed with 320 µl of substrate and further incubated at optimum temperature for 10 minute. After that the solution in each condition was mixed with 660 µl of DNS solution. The activity of the enzyme was calculated by referring to the xylose standard curve (as show in appendix). The stability curve of each pH was plotted between relative activity and time for each pH.

3.5.6 Temperature stability

The diluted enzyme was determined the temperature stability at optimum pH of the buffer the total of 20 µl of diluted enzyme was incubated at 40 °C, 45 °C, and 50 °C for 0 minute, 30 minute, 60 minute, and 90 minute. The solution in each period of time was added into 320 µl of substrate and incubated at optimum temperature for 10 minute. After that the solution in each condition was mixed with 660 µl of DNS solution. The stability curve of each temperature was plotted between relative activity and time for each temperature.

3.6. Effect of enzyme activity on lignocellulosic material

The activity of xylanase on lignocellulosic materials was investigated. Agricultural wastes (rice husk, rice straw, and bagasse) were selected to test on the enzymatic pretreatment of the materials. The reducing sugars released from lignocellulosic materials after enzyme treatment was measured using DNS method.

3.6.1 Pretreatment on lignocellulosic material

The method used in pretreatment step includes base and acid pretreatment using sodium hydroxide (NaOH) and peracetic acid (PAA) respectively. PAA was prepared by using acetic acid and 30 % hydrogen peroxide in ratio of 1.5: 1 and mixed with 1.5 % Sulfuric acid then placed it at room temperature for 72 hour. The concentration of NaOH was varied from 0 % to 10% to pretreated lignocellulosic material in order to find out the suitable condition of pretreatment for each type of lignocellulosic material. NaOH was diluted with distilled water to various percentages. Lignocellulosic material was soaked in each concentration of NaOH for overnight. Then, materials were washed with tap water until pH reached the neutral. pH of material was checked to make sure

that material present in neutral pH to avoid the effect of pH with the activity of enzyme. After that, materials were pretreated with peracitic acid by stirring at 70-75 °C until it homogeneous then wash them until pH reached to neutral. Pretreated material was dried at 105 °C for 6 hour.

3.6.2   Effect of enzyme activity in difference concentration of NaOH

After pretreatment the lignocellulosic material, 6.25 µl/ml of 10 times the diluted enzyme with phosphate buffer pH 7.0 was used to degrade 0.3 g of pretreated agricultural waste from the difference pretreatment with the difference concentration of NaOH. The reaction was incubated at the optimum temperature for 0 minute to 100 minute. The sample of 340 µl was taken for every 10 minute to measure the activity of enzyme using DNS method. The amount of xylose (µmol) was compared with the xylose standard curve. The effect of enzyme with the various NaOH pretreatment concentrations was plotted between the amounts of xylose (µmol) and time (minute).

3.6.3   Effect of enzyme activity in suitable condition

The activity of enzyme in the optimum condition for 10 minute was calculated (as show in appendix). The unit of enzyme was varied from 100 units, 500 units and 1,000 units to degrade pretreated agricultural waste by using optimum pH as buffer to dissolve waste and incubated at the optimum temperature for 0 hour, 1 hour, 3 hour, 6 hour, and overnight. The non-pretreated material of each material was used as control. The amount of xylose (µmol) in each condition was measured using DNS method and compared to the standard curve.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Effect of pH on xylanase activity obtained from fosmid positive clone 14.3

4.1.1 Overlaid technique to select xylanase positive clone

In this experiment, the pH of LB agar supplemented with Cholamphenical (Cm) was varied from 7.0 to 12.0 to plate fosmid clone Xyn14.3 and incubated overnight. Then overlay method was used by overlaying the surface with ENZhance overlay solution, and incubated at 37 °C for 1 hour. From the result in figure 13, the darkest blue colonies were obtained at pH 9.0 where the darker the blue colony means the highest the activity of xylanase. This could be roughly estimated that pH 9 was optimum pH for the xylanase activity from subclone named Xyn14.3. The present of blue zone around colonies came from the ability of enzyme in clone Xyn14.3 could digest the substrate in ENZhance overlay solution which showed to be xylanase positive.

pH 7   pH 8   pH 9

pH 10   pH 11   pH 12
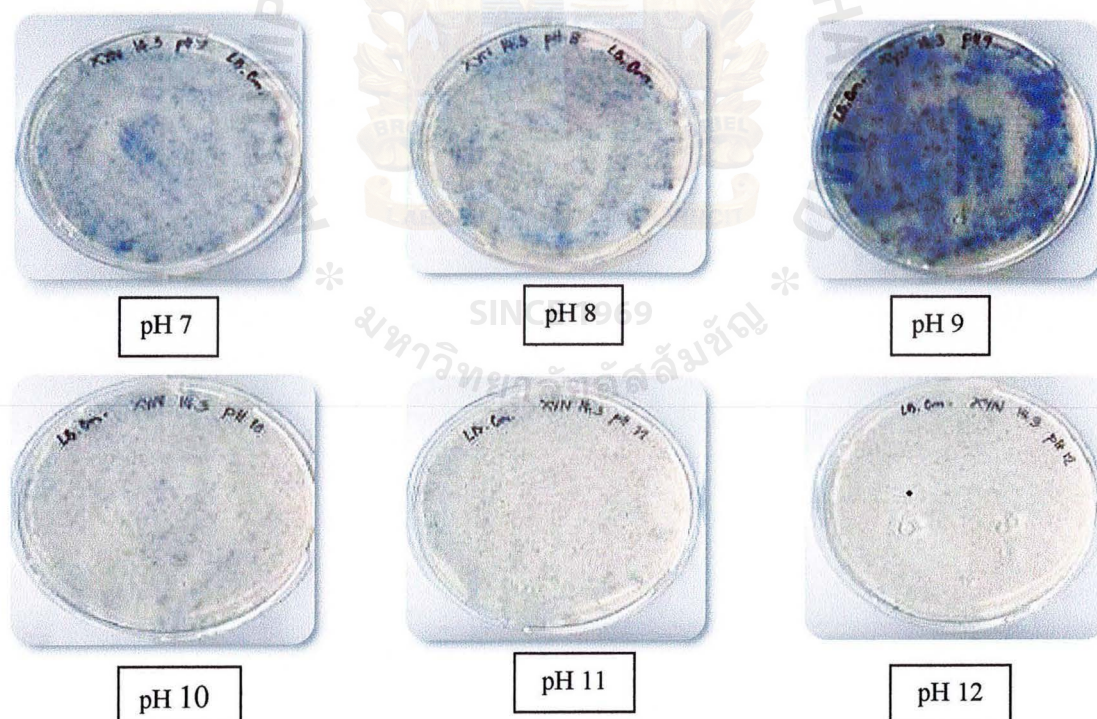
Figure 14: Xylanase activity obtained from library fosmid positive clone 14.3 by using overlaid technique

4.1.2 Subcloning of fosmid insert from the selected positive clone with xylanase activity into p-ZEro™-2vector

4.1.2.1 Partial digestion of extracted fosmid clone Xyn14.3 using Bsp143I (Sau3AI)
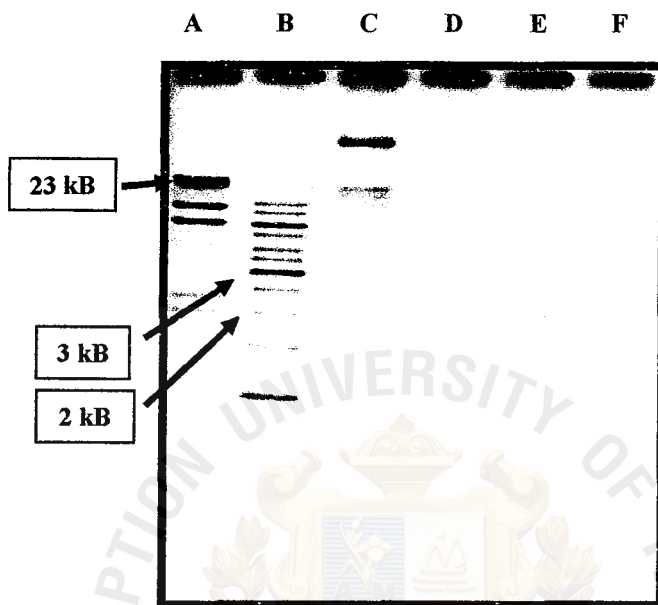
Figure 15: Agarose gel electrophoresis of extracted fosmid digested with Bsp143I ; lane A: λ/HindIII, lane B : 1 kb marker, lane C: Uncut fosmid, lane D: Digested fosmid in 5 min, lane E: Digested fosmid in 10 min, lane F: Digested fosmid in 15 min

The fosmid was extracted from the subclone named Xyn14.3. The partial digestion of extracted fosmid was performed using restriction enzyme called Bsp143I for 5, 10, and 15 minutes. Then, the Agarose gel electrophoresis was run for an hour and compared with λ/HindIII marker (lane A) and 1 kb marker (lane B), undigested fosmid (lane C) and digested fosmid for 5, 10 and 15 minutes (lane D, E and F) respectively. The concentration of DNA in digested fosmid for 5, 10, and 15 minute was measured as 75.5 ng/μl, 86.6 ng/μl, and 67.9 ng/μl respectively. From figure 14, the darker the DNA fragment bands, the higher the concentration of DNA in which 10 minutes partial digestion provided the highest concentration. Therefore, the 10 minute of partial digestion could be the optimum condition to digest fosmid by using Bsp143I , so the products from this process was used for the subclone to p-ZEro™-2 .

4.1.2.2 Digestion of extracted p-ZEro™-2 using BamH1

From this step, the digestion of p-ZErO™-2 vector was performed using BamHI to digest for one and a half hour at 37 °C at the restriction site. Then Agarose gel electrophoresis was run for an hour, compared with λ/HindIII marker (lane A), 1 kb marker (lane B), undigested p-ZErO™-2 vector (lane C), and digested p-ZErO™-2 (lane D) to ensure that the plasmid was completely cut. Since, the concentration of DNA in digested vector was 158.5 ng/μl (refer to the previous experiment).
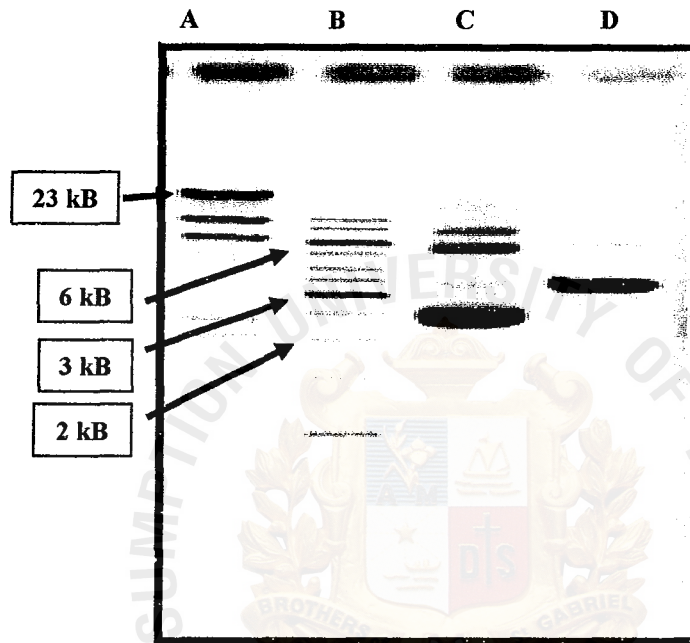
Figure 16: Agarose gel electrophoresis of extracted p-ZErO™-2 digested with BamHI; lane A: λ/HindIII marker, lane B : 1 kb marker, lane C: Uncut p-ZErO™-2, lane D: Digested p-ZErO™-2

From the figure 15, there are a lot of band on lane C, undigested vector was shown the size of vector in form of linear, circular, and supercoil. Since, the size of p-ZErO™-2 is 3.3 kB, lane D shown only one band of digested p-ZErO™-2 vector in the expected size the digested plasmid. After digested p-ZErO™-2 and digested fosmid was prepared, ligated both of them was performed by using T4 DNA ligase at 22 °C for 3 hour and inactivated enzyme at 65 °C for 20 minute. Then, ligated vector was trasfered into E. coli Top 10 as a competent cell and plated on LB agar supplemented with 50 μg/ml Kanamycin and IPTG to select for the recombinants. The transformation efficiency was 16,703 colonies/μg DNA.

The screening for the xylanase positive clone was performed after the transformation using overlaid technique. The experiment was done at the optimum pH of 9 to screen out for the positive clone that expressed the xylanase activity. One positive clone shown with the blue zone around the colony (figure16) was selected for the sequencing to indentify the full-length gene.

Figure 17: The positive clone represented the blue zone around the colony as indicated by the arrow

4.2 Sequence analysis

The sequencing was performed on the positive clone in part 5.1.2 to indentify the full-length gene of this clone. After the clone was analyzed, the sequence was show in the size of 1.4 kb. Then the ExPASy translated tool and ORF finder program were used to analyze in which part of gene that show the open reading flame and used to predict the putative intron within the sequence. Moreover, GT/AT from 5' and 3' splicing site was identify and removed before the sequences were translated into amino acid sequence using Translation tool. From this program, the open reading frame +3 from sequence 3 to sequence 824 represented the longest length of 822 bp. Blastp program was used to compare my sequence with database in NCBI to predict the homologous protein. The Xyn14.3 gene (822 bp) was closely related to a gene encoding xylanase from an uncultured bacterium with 56 % identity and contain highly conserved domain of glycoside hydrolase in family 11 as show below.
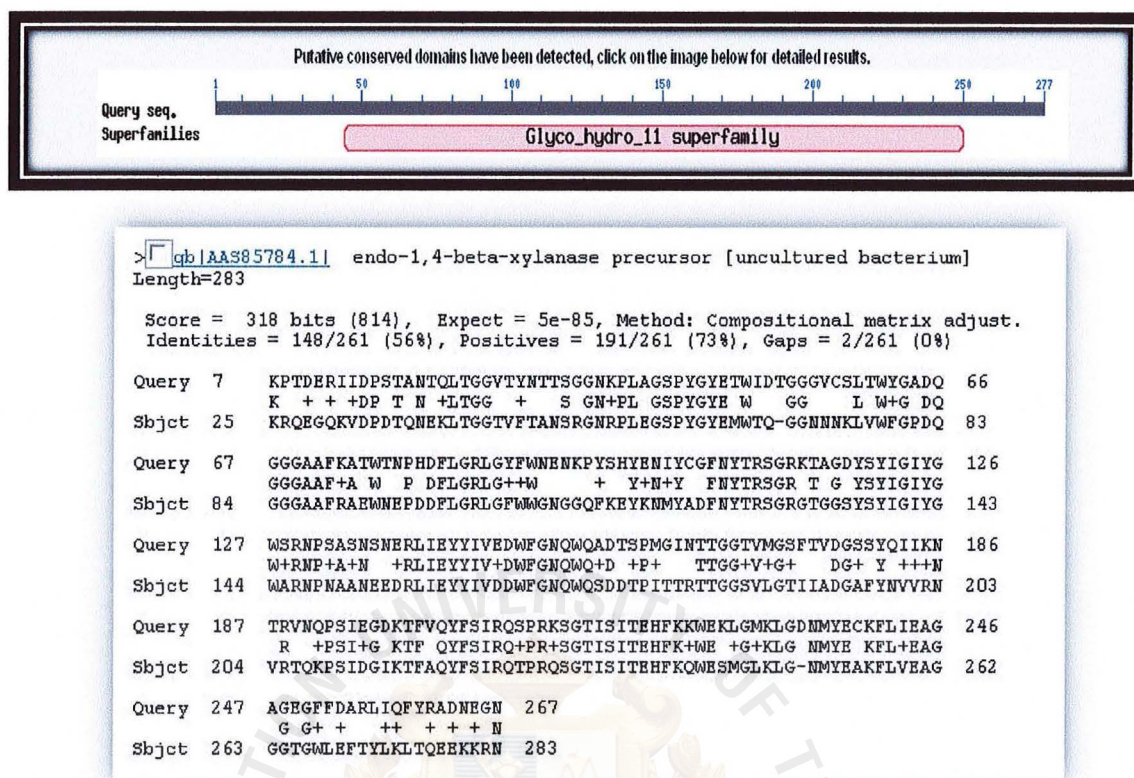
Figure 18: Sequencing analysis on ExPASy translated tool and ORF finder program
Internet: http://blast.ncbi.nlm.nih.gov/Blast.cgi

4.3 Qualification the inserted gene

4.3.1 Amplification the positive subclone and subclone to an expression vector

The forward and reverse primers were designed to amplify the full length gene from the sequencing information using the PCR method. The primers were designed as follow.

Xyn14.3 Forward : GGC GAA TTC CCA TGG GAG AAC AAG TAG TG

Xyn14.3 Reverse : AAG AAG CTT CTT GTA CTA GTT ATA GCT AG

The size of forward primer and reverse primer are 24.7 nmole and 24.1 nmole respectively. The PCR reaction was calculated as show in appendix.
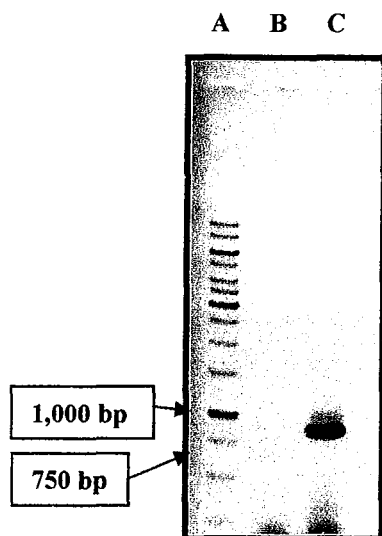
Figure 19: Agarose gel electrophoresis of PCR product ; lane A: 1 kb marker, lane B: negative Xyn14.3, lane C: PCR product of Xyn14.3
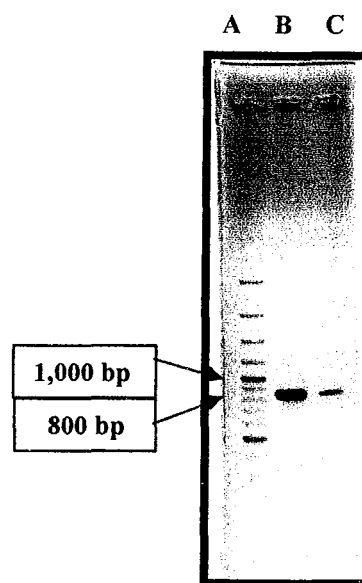
Figure 20: Agarose gel electrophoresis of purification the PCR product ; lane A: 100 bp marker, lane B: 1st purified Xyn14.3 lane C: 2nd purified Xyn14.3

PCR condition was set up at denaturation step at 95 °C for 5 minute, annealing step at 58 °C for 30 second, and extension step at 72 °C for 10 minute. After that, PCR product was run in Agarose gel electrophoresis (figure 18). The PCR product had the expected size around 800 bp which was close to the expected size of 822 bp because of the sequence in full length gene of Xyn14.3. After that, PCR product was purified by using QIA quick PCR purification kit for two times and then run agarose gel electrophoresis again. PBI buffer was added to PCR sample and checked yellow color in order to check proper pH and then inserted to QIA quick spin column at 12,000 rpm for 1 minute and washed with PE buffer. The second purification was done in similar step. As see in the result (figure 19), purified PCR product was compared with 100 bp marker. The first purification was show in lane B that present darker band comparing with the second purification in land C. The size of bands showed between 800 bp and 1000 bp which was close to the expected size of 822 bp. Then the PCR purification was digested by using *Eco* RI and *Hind*III and ligated with pET-28a (+) vector then incubate overnight at 22 °C. Ligated vector was transform into *E. coli* DH5λ by heat shock method and spread them on LB agar supplemented with 50 µg/ml Kanamycin and incubated at 37 °C for 1 hour.

4.3.2 Checking the existing of inserted gene

4.3.2.1 Size screening

The colonies recombinants from LB-km agar plate from the previous section were screened out using the size screening method. Colonies number 1 to 30 were mixed with size screening solution and the total of 40 µl solution was taken to perform size screening on 0.8% agarose gel and run for 30 minute. The result was showed in figure 20. Size screening colonies was compared with 1 kB marker. Most of them shifted up when comparing with pET-28a (+) vector which meant that most of them are recombinant vector containing the inserted DNA. However, the clone number 16 was similar to the size of pET-28a (+) vector that meant this colony was not present the inserted gene inside vector. After that, only five colonies (no. 3, 7, 14, 21, and 28) were transformed into Rosetta™ competent cell to support the gene expression in the further step. The sequencing was done n the positive clones in the later stage.
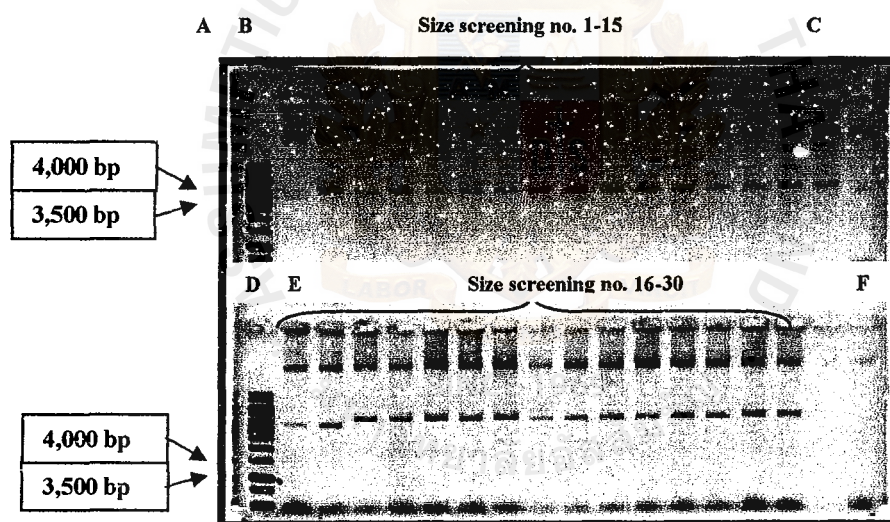
Figure 21: Agarose gel electrophoresis of size screening; lane A and D: 1 kb marker, lane B,C, E, F: pET-28a(+) vector

4.3.2.2   Colony PCR method

The five clones from size screening were rechecked the inserted gene by using colony PCR method. The Xyn14.3 forward primer and Xyn14.3 reverse primer from previous step were used to run PCR reaction. After finish PCR step, PCR product was run on the Agarose gel electrophoresis for an hour compared with 1 kb marker lane A, D, and F and negative pET-28a(+) vector lane B. As the result showed in figure 21, all of them had the expected size approximately 900 bp as expected to be 822 bp.
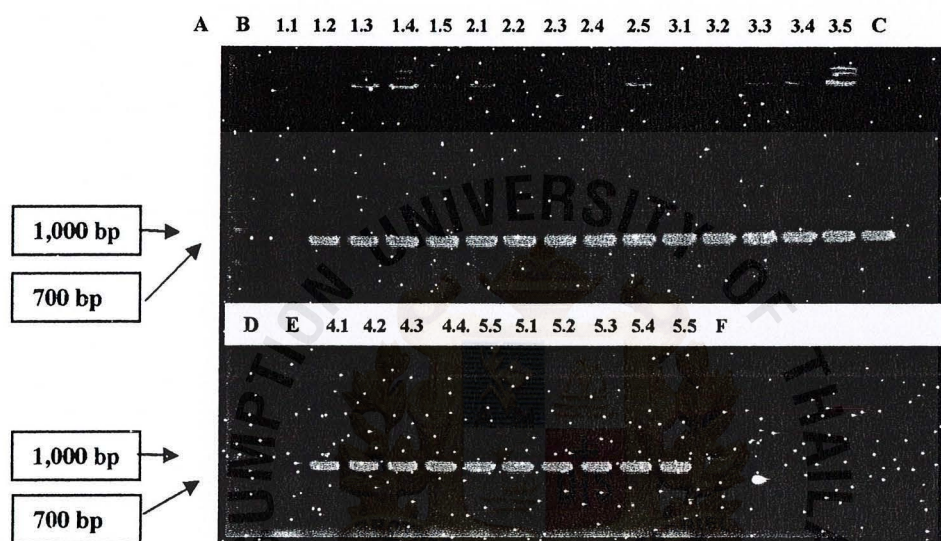
Figure 22: Agarose gel electrophoresis of colony PCR; lane A, D, F : 100 bp marker, lane B,: pET-28a(+) vector, lane C, E: pET-28a(+) vector + template

4.4 Cloning and Overexpression of xylanase gene

After the colony PCR method, 5 recombinant clones in Rosetta[TM] were grow on LB-Km-Cm and incubated overnight. Each tube was induced with 1 mM IPTG (isopropylthiogalactoside) and incubated overnight. The SDS PAGE was used to identify the recombinant protein from each clone. The experiment was performed to compare the protein products from the induced and uninduced conditions for each specific clone. Under the induced condition, protein of the size approximately 30.14 kDa was obtained. Based on the result below (figure22), in yellow box show darker band in inducing of each clone in the size of more than 30 kDa, which was close to the size of the predicted protein from clone Xyn14.3.
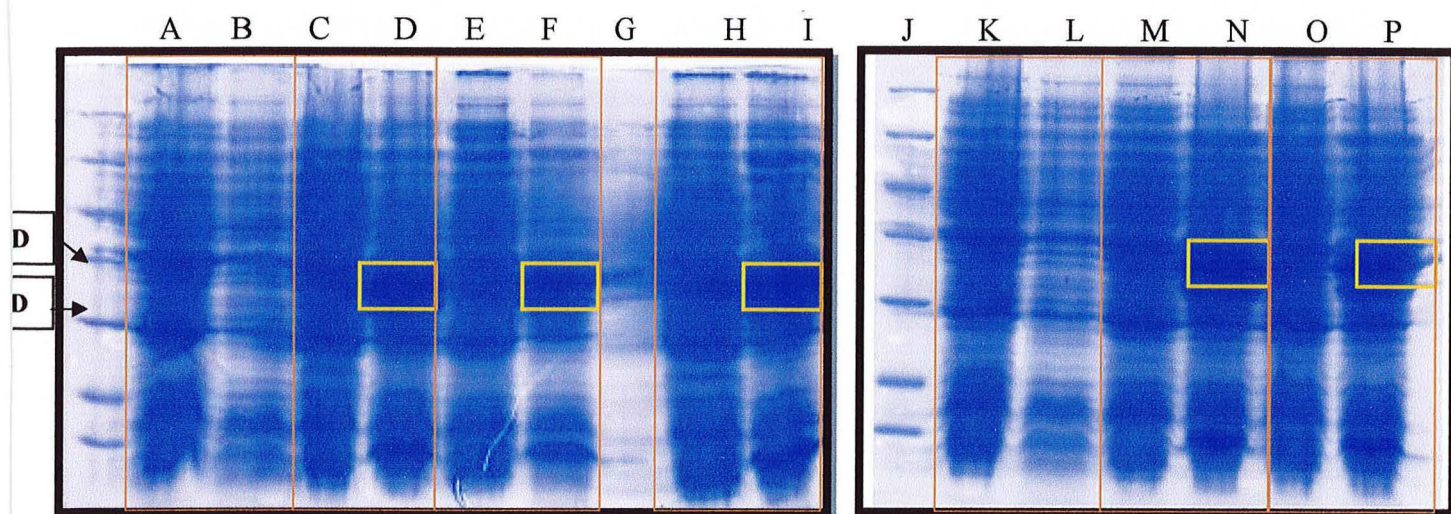
Figure 23: SDS PAGE; Lane A, J : protein marker, Lane B,: uninduced pET-28a(+) vector, lane C, K: induced pET-28a(+) vector, lane D, L: uninduced X14.3(1), lane E: induced X14.3(1), lane D: uninduced X14.3(1), lane E: induced X14.3(1), lane F: uninduced X14.3(2), lane G: induced X14.3(2), lane H: uninduced X14.3(3), lane I: induced X14.3(3), lane K: uninduced X14.3(4), lane L: induced X14.3(4), lane O: uninduced X14.3(5), lane P: induced X14.3(5)

The production of enzyme was done by using the starter culture to add in fresh LB-Km-Cm broth and incubated for three hour which the optimum density reached to 0.5-0.8 and added 1 mM IPTG then incubated overnight at 37 °C. IPTG is inducer in order to open Lac operon by attaches with Lac repressor then Lac repressor was inactivated and then Lac repressor was released from operator. In this research, IPTG was used as inducer to induce enzyme produced from clone Xyn14.3 which was under control of lac promoter in plasmid of Rosetta™ competent cell. Sonicator was used to break the cell. After that the overexpressed enzyme from Xyn14.3 was purified by using His trap affinity column with elusion buffer at various concentrations of Imidazole to find out the optimum concentration. The reaction to purify protein was show in appendix. Based on the result (figure 23), the protein band in lane G (400 mM Imidazole) had shown to be more purified than the other lanes. Therefore, the concentration of Imidazole of 400 mM provided the optimum condition for the protein purification.
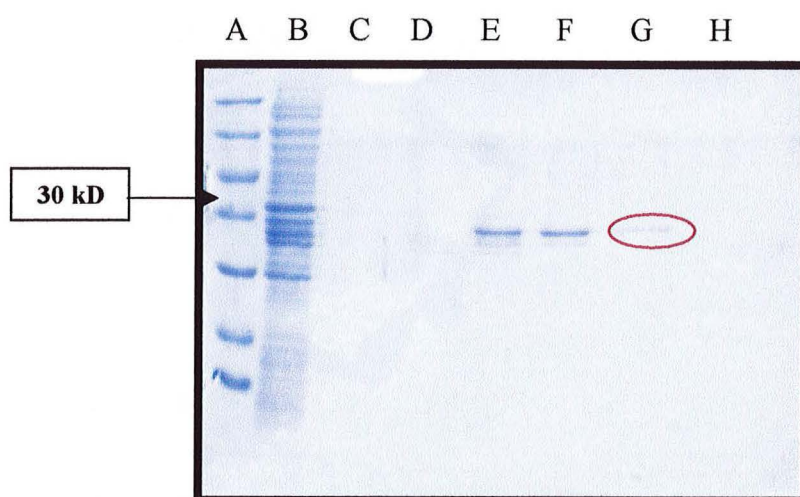
Figure 24: SDS PAGE; lane A: protein marker, lane B: crude enzyme, lane C: flow through, lane D: 100 mM Imidazole, lane E: 200 mM Imidazole, lane F: 300 mM Imidazole, lane G: 400 mM Imidazole, lane H: 500 mM Imidazole,

4.5 Biochemical characterization of X 14.3

4.5.1 Concentration of protein

In this part, brovine serum albumin (BSA) was used for constructed the protein standard curve based on the spectrophotometric technique. The standard curve was showed in the appendix. Linear equation was received which was $y = 0.001x - 0.019$ and R square is 0.984. After that, amount of protein was calculated based on the protein standard curve and the result was show the protein in enzyme for 405 µg/ml. The calculation part was showed in the appendix.

4.5.2 Xylose standard curve at various pHs

Xylose standard curve was constructed in the different condition by using Sodium acetate buffer (NaAc), phosphate buffer, Tris-HCl buffer, and, Glycine-NaOH buffer to prepared pH 5.5, 7.0, 8.0, and 10.0 respectively. Xylose stock was dilute to 0, 0.2, 0.4, 0.6, 0.8, and 1.0 µmole and assayed by using the DNS method. The standard curve was developed by plotting OD_{540} against the concentration of xylose. The graph of each condition was plotted the linear equation and R square were shown in the appendix. The linear equation for pH 7.0 phosphate buffer was $y = 1.456x - 0.084$ and R square was 0.984.

4.5.3 Optimum pH

Birchwood xylan was used as substrate to check the optimum pH of X14.3 enzyme. The pH was varied from 5.0 to 11.0 using 0.2 M NaAc buffer, 0.2 M phosphate buffer, 0.2 M Tris-HCl buffer, and 0.2 M Glycine-NaOH in order to find the optimum pH for X14.3 function. The condition was set up at 50 °C for 10 minute. As xylanase has an activity to hydrolyze xylan become xylose which the amount of xylose (reducing sugar) can be detected by DNS method. The amount of xylose can be used to refer to the enzyme activity. The % relative activity of enzyme was calculated which showed in appendix. Based on the result (figure 24), pH 7.0 provided the highest relative activity of the enzyme. The peak of bell shapes was showed at pH 7.0 and slightly drops in higher pH. It means pH 7.0 was the optimum pH to show for the enzyme activity. The reason why the optimum pH was changed from pH 9.0 from the first overlay technique to be 7.0 from this part might be from the competent cell used in subcloning method is E. coli DH5 λ. The optimum temperature for E. coli growth is around 37 °C at pH 7.0 (Don 2008). Therefore, the higher pH might be effected the lower growth of E. coli which might effect to the activity of enzyme as well.
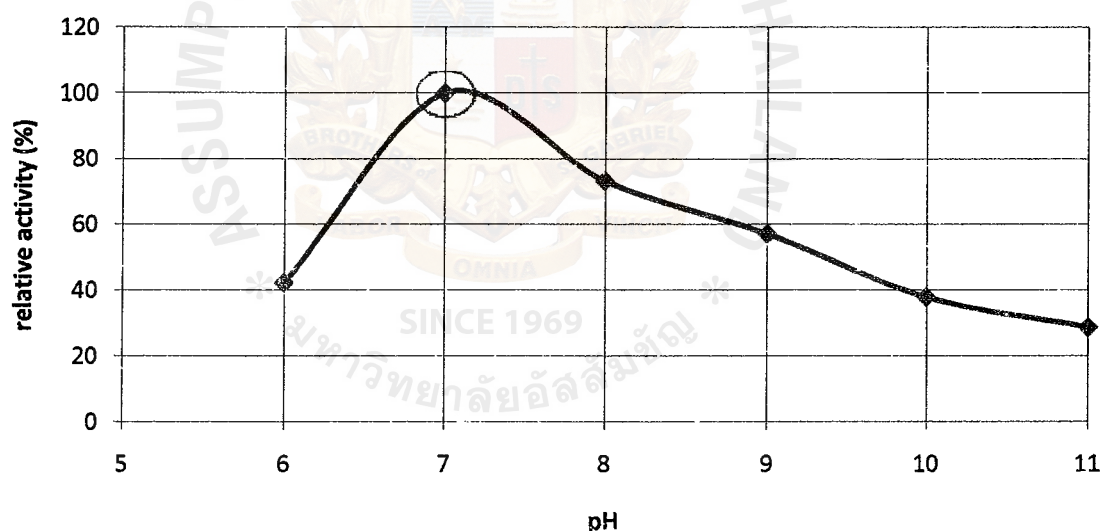
Figure 25: Optimum pH of x 14.3

4.5.4 Optimum temperature

Birchwood xylan was used as substrate to check optimum temperature of X14.3 enzyme. The temperature was varied from 25 °C (room temperature) to 70 °C in order to find the optimum temperature. Graph of the optimum temperature was plotted between the temperature and relative activity. Based on the graph, temperature at 45 °C showed the highest relative activity then slightly drop down to 40% at 60-70 °C. It means 45 °C was the optimum temperature to show highest activity of X14.3 to degrade substrate and change to monosaccharide (xylose). Form previous section

(optimum pH) the optimum temperature and pH of this enzyme was closed to other xylanases produced by strains of CBS 288.54, CAU44, DSM 10635, SSBP, ATCC 46882, DSM 5826 and RT9 that were pH in range of 6.0-6.5_(Alam et al., 1994; Cesar and Mrsa, 1996; Bennett et al., 1998; Singh et al., 2000b; Xiong et al., 2004; Jiang et al., 2005; Li et al., 2005)
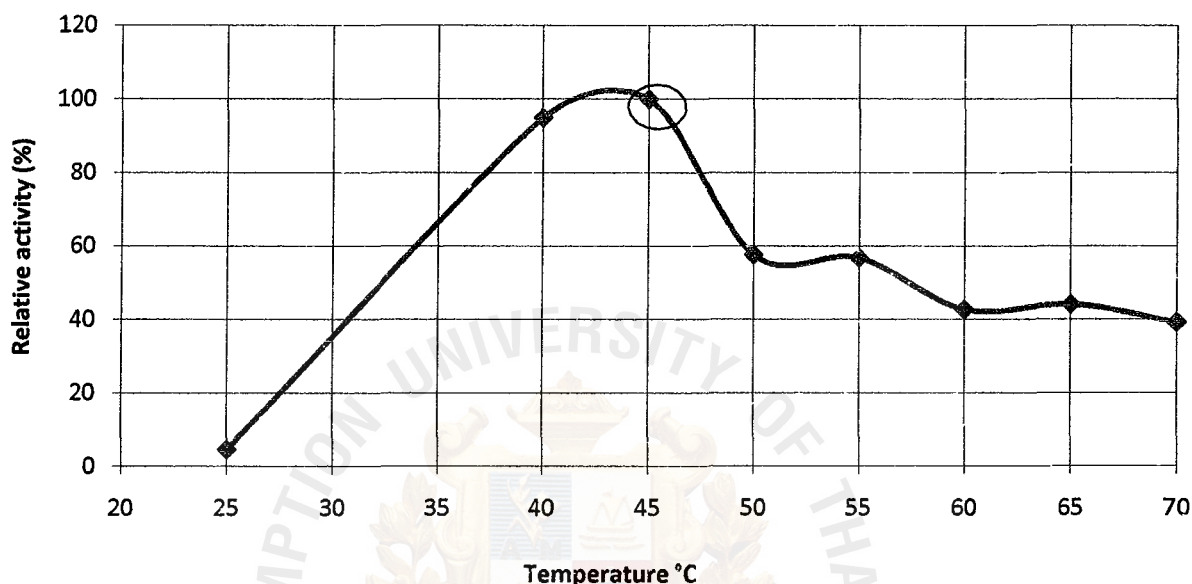
Figure 26: The optimum temperature of x14.3 enzyme

4.5.5 pH Stability

pH stability was used to determined the stability of enzyme at different pH (pH 7.0, pH 8.0, and pH 9.0) during the 2 hour period. Based on result in graph figure 27, the stability of X 14.3 was highly maintained at pH 7.0 for 2 hour. The %relative activity of pH 7.0 was showed at 100% and then slightly drops to 80% and stable for 60 minute. pH 8.0 was showed %relative activity around 70% which drop domestically in first 30 minute before stable for 60 minute. For the graph of pH 9.0, the %relative activity was showed 20% at first 20 minute and then stable until 120 minute. Therefore, pH 7.0 provided the maximum stability or maintained the enzyme stability. Normally, the pH stability of enzyme was most stable between pH 5–7, with an optimum pH of around 6 (Murty and Chandra, 1991). So, pH 7.0 which showed the highly maintained the stability of X14.3 enzyme is closed to the normal stability form the research.
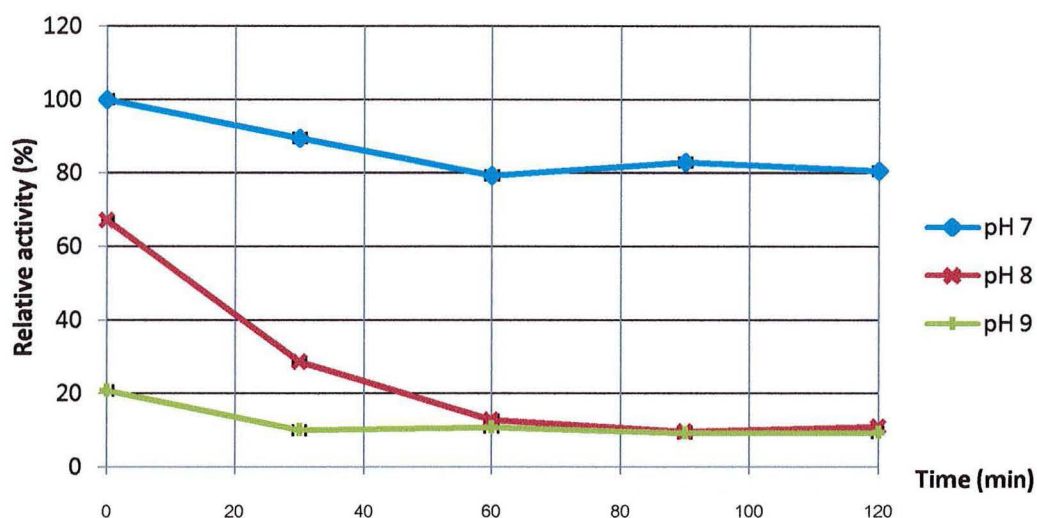
Figure 27: pH stability of X14.3 showed at pH 7.0, 8.0, and 9.0

4.5.6   Temperature stability

The stability of enzyme was determined as the different temperature (40 °C, 45 °C, and 50 °C) for 2 hour period of time. The relative activity of the enzyme could be maintained at the most at 40 °C. At 45 °C, the relative activity was drop down domestically at 60 minute and then stable for 60 minute. At 50 °C, the %relative activity was drop from 30 minute and drop to be as low as about 10%. Normally, the temperature stability of endo-xylanase was stable at 50°C, maintaining over 50% of its activity for 1 h at pH 8 (Chivero, Mutukumira, and Zvauya, 2001). So, 40 °C which showed the highly maintained the stability of X14.3 enzyme is closed to the normal stability form the research.
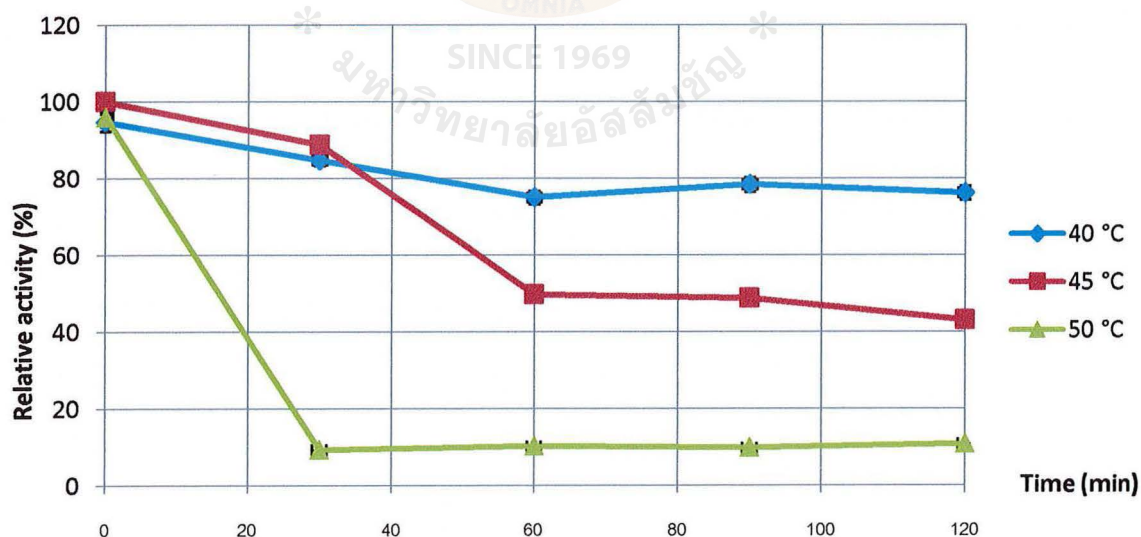
Figure 28: Temperature stability of x 14.3 showed at 40 °C, 45 °C, and 50 °C

4.6   The enzyme activity on lignocellulosic materials

The enzyme activities over the digestion of lignocellulosic materials including rice husk, rice straw, and bagasse were tested at the optimum pH and temperature. Pretreatment of lignocellulosic materials were done by using NaOH and peracitic acid (PAA)

4.6.1 Pretreatment lignocellulosic material

Lignocellulosic material was pretreated with the various concentration of NaOH from 0% to 10% in order to find out the suitable concentration of NaOH that need to be apply prior the enzymatic digestibility. NaOH was used to degrade lignin in the lignocellulosic composition and the neutralization was conducted afterward to ensure that the pH of the treatment would not effect on the enzyme activity. Then PAA was used to open the structure of material that make enzyme degrade easily. The figure 27 was showed 3 kind of agricultural waste after pretreatment.

Figure 29: Structure of Bagasse, Rice husk, and Rice straw after pretreatment with difference concentration of NaOH and PAA

4.6.2 Effect of enzyme activity in difference concentration of NaOH

The pretreated lignocellulosic material at each specific concentration of NaOH was boiled to 80 °C in phosphate buffer pH 7.0 before adding 100 times diluted X14.3. The reaction was incubated at 45 °C. Graph of the activity of enzyme in each pretreatment material was constructed (figure 28, 29, and 30) by plotting the concentration of xylose against the time. The xylose concentration was relevant to the efficiency of the pretreatment process.
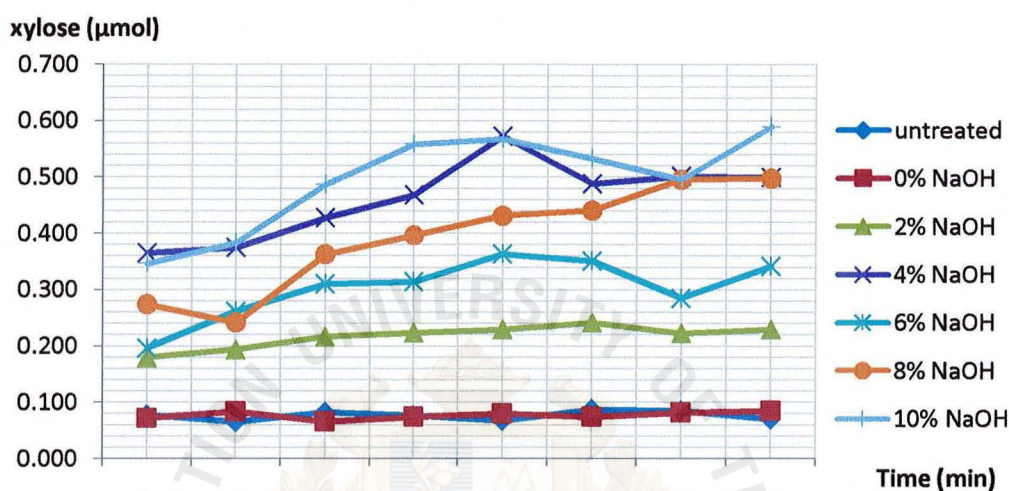
Figure 30: Effect of enzyme with husk in various concentration of NaOH
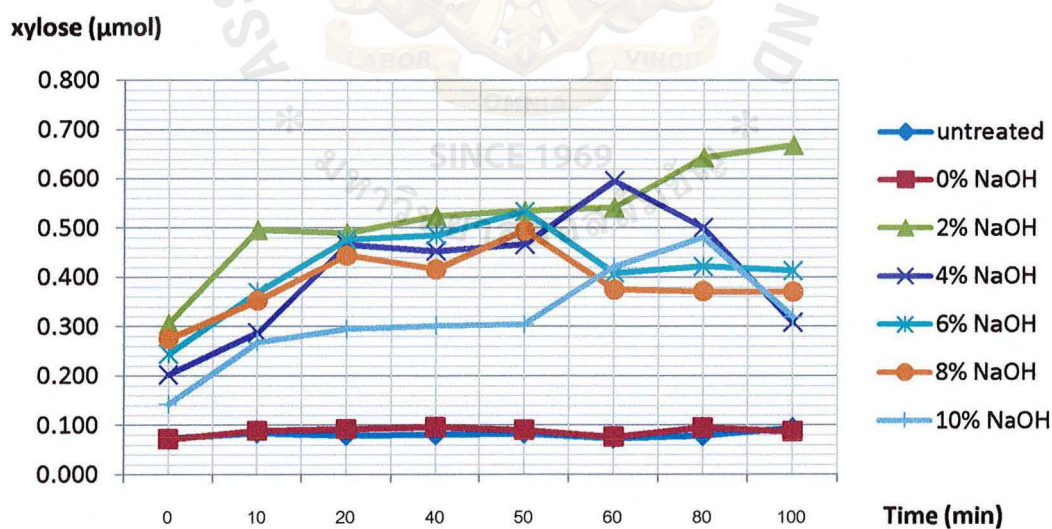
Figure 31: Effect of enzyme with bagasse various concentration of NaOH

xylose (µmol)
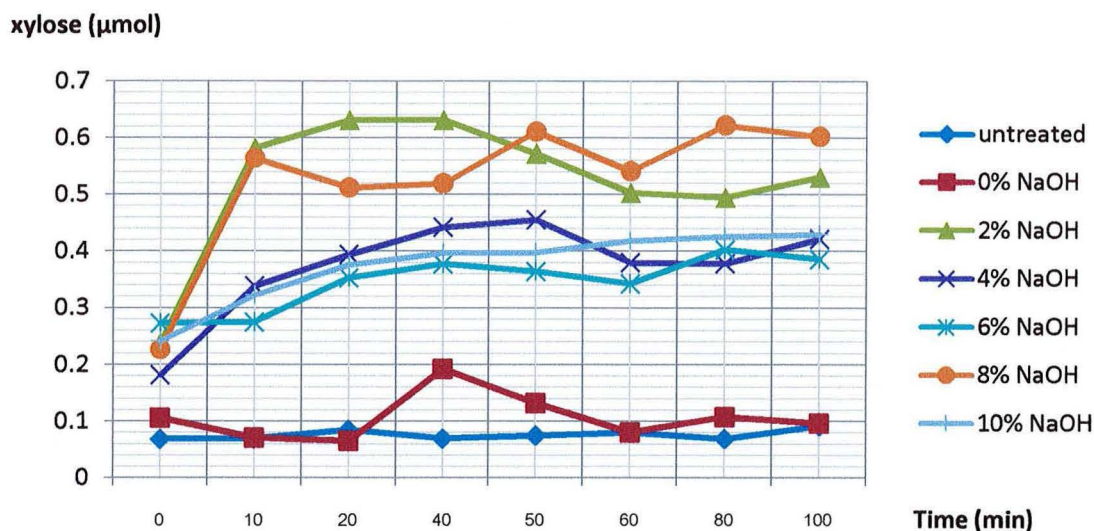
Figure 32: Effect of enzyme with rice straw various concentration of NaOH

According to the result above with 10 times dilution of enzyme, 10 % NaOH was showed the highest amount of xylose (µmol) in husk, 2% NaOH was showed the highest amount of xylose in bagasse and rice straw. Since, the structure of husk has a thicker lignin than bagasse and rice straw, so the highest concentration of NaOH needs to be applied in the pretreatment process (Clieford A. Adams and Novellie, 1974). However, there is the amount of hemicellulose in rice straw and bagasse and the lowest amount in husk. So, the amount of xylose from substrate degraded by X14.3 showed in bagasse and rice straw was slightly higher than the amount of xylose from rice husk. Therefore, this condition was used in further step. Rice husk represented an optimum pretreatment at 10% NaOH for 40 minute. Bagasse and rice straw represented optimum pretreatments at 2% NaOH for 80 and 40 minute respectively. When all pretreated material were compare the efficiency of X 14.3 as show in the graph below, enzymatic pretreatment of lignocellulosic materials showed that the greatest digestion activity was obtained from bagasse, follow by rice straw and rice husk respectively.

4.6.3 Effect of enzyme activity over the digestion of pretreated lignocellulosic materials

Since, the optimum concentration of NaOH to pretreat each kind of lignocellulosic material was determined from previous step; the concentration of enzyme X14.3 was varied to be 100 U, 500U, and 1,000 U per grams of pretreated lignocellulosic materials in order to find the digestibility of enzyme in each kind of the lignocellulosic material under the optimum condition (pH, temperature, and optimum concentration of NaOH pretreatment). Pretreated agricultural waste was soaked in 0.2 M phosphate buffer pH 7.0. The activity of the enzyme was received for 937.48 U/ml in 1,000 times dilution.

Since, the relative activity of the enzyme is 937.48 U/ml, the activity of the enzyme was varied to be 100U, 500U, and 1,000 U to digest 0.3 g of pretreated material at each condition was incubated at 45 °C for 0 hour, 1 hour, 3 hour, 6 hour, and overnight. The total of 340 µl from each reaction was taken up and measured the amount of xylose by using DNS method. The graph was plotted between xylose (µmol) and time (hour) as in figure 32.
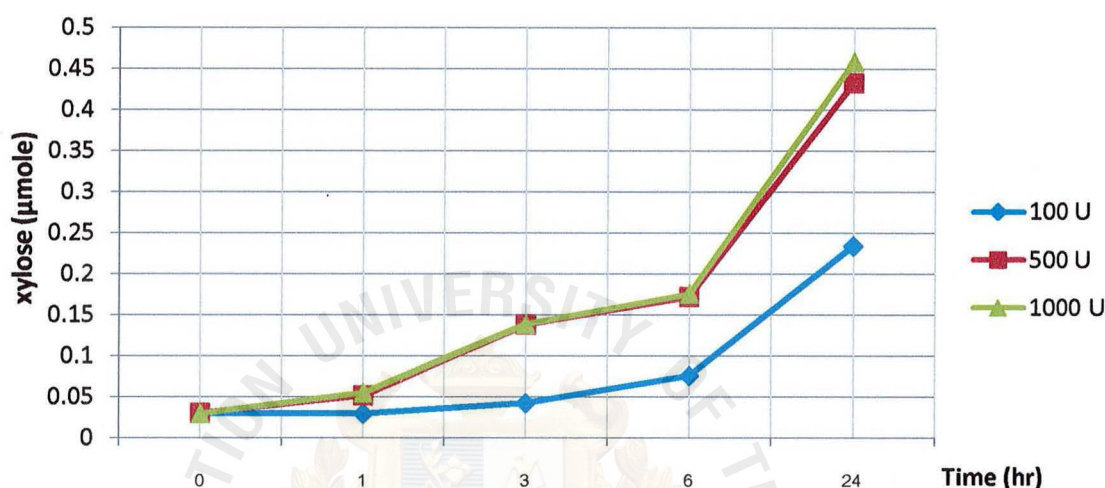
Figure 33: Digestibility of enzyme on bagasse in various enzyme activity (U/g)

From figure 32, the data represented that the enzyme of 500 U/g and 1,000 U/g provided the equal efficiency on the digestibility while the xylose amount at 24 hour was 0.45 µmole. So, the industrial application of X14.3 enzyme on bagasse would be more cost effective by using 500 U/g for the digestion.
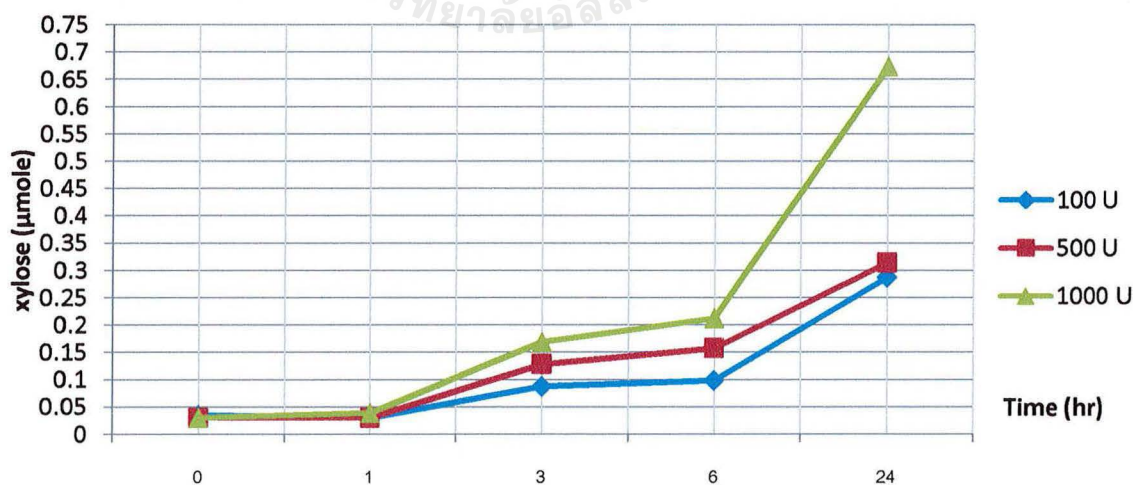
Figure 34: Digestibility of enzyme on rice straw in various enzyme activity (U/g)

In rice straw graph, 1,000 U/g of enzyme digestibility on rice straw provided the highest amount of xylose (µmole) which has approximately0.7 µmol when incubated at 45 °C for 24 hour while the enzyme at the concentration of 100 U/g and 500 U/g provided only 0.3 µmole of xylose.
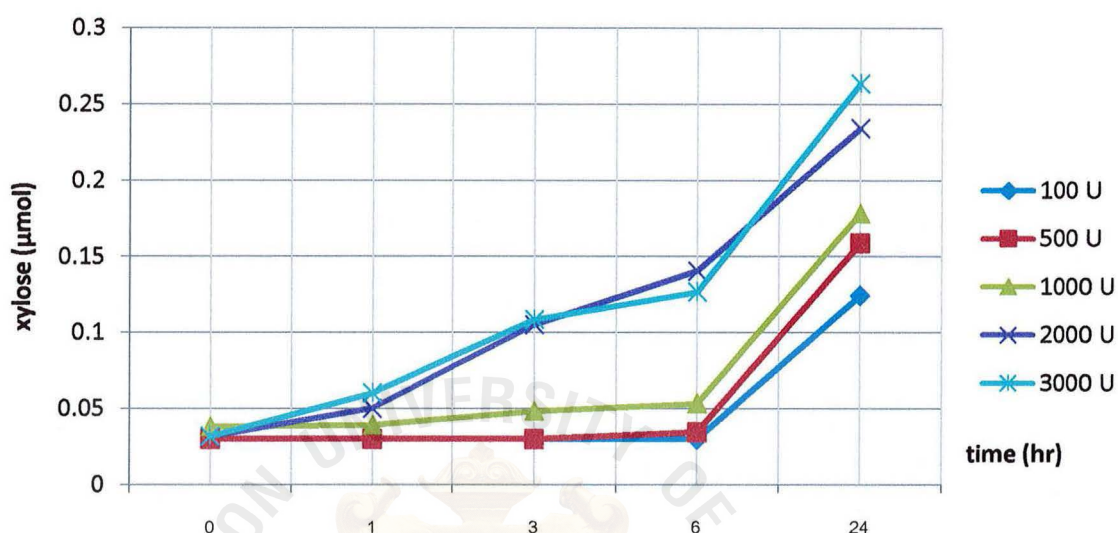
Figure 35: Digestibility of enzyme with rice husk in various enzyme activity (U/g)

In the last figure, 100 U, 500 U, and 1,000 U/g of pretreated rice husk showed very low amount of xylose (µmol) even at the overnight incubation. So, the unit of enzyme was added up to be 2,000 U/g and 3,000 U/g to digest the pretreated rice husk. When the enzyme concentration was increased to be at 3,000 U/g of bagasse, the xylose concentration was increased slightly to 0.25 µmole. Therefore, the activity of X14.3 enzyme over the digestion of rice husk had shown to be lower than bagasse and rice straw. This can be explained by the thicker structure of the rice husk (Clieford A. Adams and Novellie, 1974) or the hemicellulose tightly bounded to the complex at which the other pretreatment rather than NaOH must be performed in order to provide the accessibility for the enzyme. However, this novel enzyme (X14.3) is applicable to use for the digestibility of bagasse and rice straw at the relative activity of 500 U/g and 1,000 U/g respectively.

CHAPTER 6

CONCLUSION

This research can be concluded that fosmid library named Xyn14.3 from metagenomic fosmid library of termite gut was selected the positive clone by using Enzhance overlaid solution. Blue zone released from AZCL-substrates around colonies was indicated the xylanase activity at pH 9.0. The selected representative clones expressing xylanase activity from the fosmid was partial digested by Bsp143I (Sau3AI) and cloned into p-ZErO^{TM}-2 vector which was cut by BamHI and transformed into $E.\ coli$ Top-10. The obtained subclone was analyzed for the nucleotide sequence and the Xyn14.3 gene (822 bp) was closely related to a gene encoding xylanase from an uncultured bacterium with 56 % identity and contain highly conserved domain of glycoside hydrolase in family 11. Xyn14.3 forward and Xyn14.3 reverse primer was designed to amplify subcloned and checked the existing of inserted gene by using size-screening method and colony PCR method by using the same primers. The plasmid was transform to Rosetta^{TM}, which is suitable for overexpression. The expression level and the size of protein obtained were determined using SDS-PAGE. The production of enzyme was performed by inducing with IPTG and purified by using His trap affinity column. The enzyme was named as X 14.3. The crude enzyme was characterized on its biochemical properties. The concentration of protein in enzyme X 14.3 was calculated for 405 µg/ml. The optimum pH and optimum temperature is pH 7.0 at 45 °C. The stability of X14.3 was highly maintained in pH 7.0 at 40 °C for 2 hours. The activity of xylanase on lignocellulosic materials was investigated on pretreated agricultural waste, which was rice husk, rice straw, and bagasse. Rice husk was used in the condition at 10% NaOH for 40 minute. Bagasse was used in the condition at 2% NaOH for 80 minute. Rice straw was used in the condition at 2% NaOH for 40 minute. These three reactions were done in optimum condition of enzyme. The activity was calculated for 937.48 U/ml in 1,000 times dilution, which presented the highest activity in 10 minute. The unit of enzyme was estimated from the optimum dilution of enzyme, which used at 100 U/g, 500 U/g, and 1,000 U/g of pretreated agricultural waste. 32µl, 160 µl, and 640 µl of X 14.3 enzyme was used for industrial scale to degrade bagasse, rice husk, and rice straw overnight to become xylose as the valuable product respectively.

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    Internet: http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi
  - ➢ Nucleotide sequence from NEB cutter program

    Internet: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC168933
  - ➢ pET-28a(+)vector

    Internet: http://www.ecoliwiki.net/colipedia/index.php/pET-28a%28%2B%29
  - ➢ GeneRulerTM 1kb DNA ladder

    Internet: http://www.fermentas.com/en/products/all/dna-electrophoresis/generuler-dna-ladders/sm024-generuler-1
  - ➢ Protein marker

    Internet: http://www.taq-dna.com/protein-marker-us7-print133.html

CHAPTER 7

APPENDIX A

Effect of pH on xylanase activity obtained from fosmid positive clone 14.3

LB broth

Peptone	6	g
Yeast Extract	3	g
NaCl	3	g
Distilled water to	600	ml

LB agar

Peptone	6	g
Yeast Extract	3	g
NaCl	3	g
Purify Agar	9	g
Distilled water to	600	ml

LB-Cm broth

LB broth	18	ml
Chloramphenicol	12.5	µg/ml

LB-Km agar

LB agar	600	ml
Kanamycin	50	µg/ml

LB-Km-IPTG agar

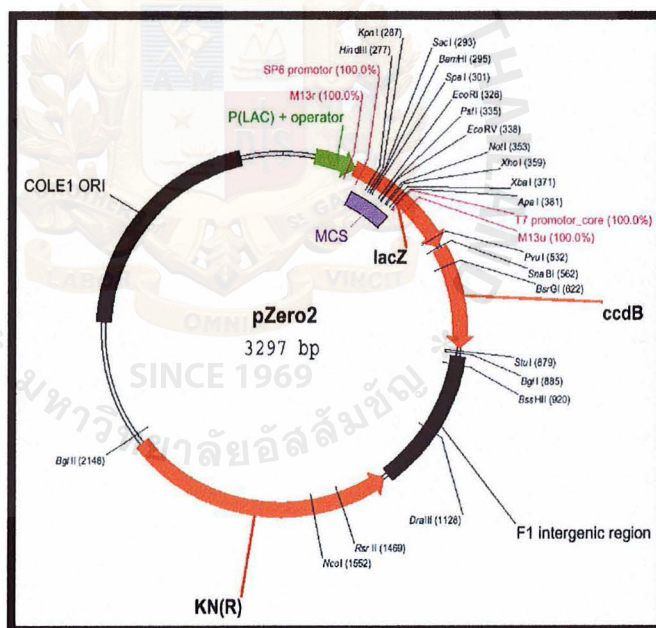|		
LB agar	600	ml
Kanamycin	50	µg/ml
IPTG	0.16	mM

AZCL xylan agar

AZCL-xylan	0.15	g
Purified agar	2.1	g
Distilled water to	300	ml

Enzhance overlay solution

5% buffer A	5	ml
1 M pH buffer	20	ml

Filtered and mixed with 0.2 µm membrane

50 X TAE Buffer Preparation protocol (Tris-Acetate-EDTA)

Tris base	242	g
Acetic Acid	57.1	ml
0.5 M EDTA	100	ml
Distilled water to	1	liters

p-ZEro™-2 vector

Figure 36: p-ZEro™-2 vector

Internet: http://www.imagenes-bio.de/info/vectors/pZero2.gif

Lambda DNA/ *Hind*III marker
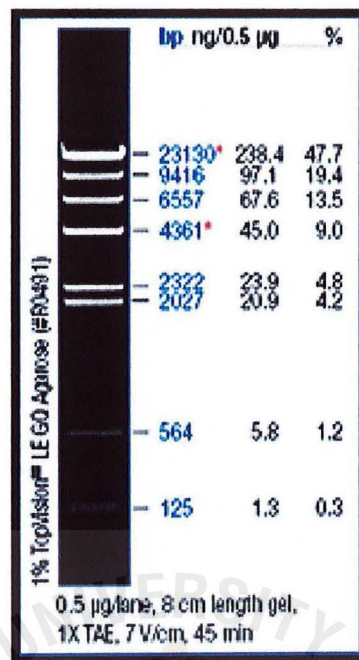
Figure 37: Lambda DNA/*Hind*III marker

Internet: http://www.fermentas.com/en/products/all/dna-electrophoresis/sm010?print
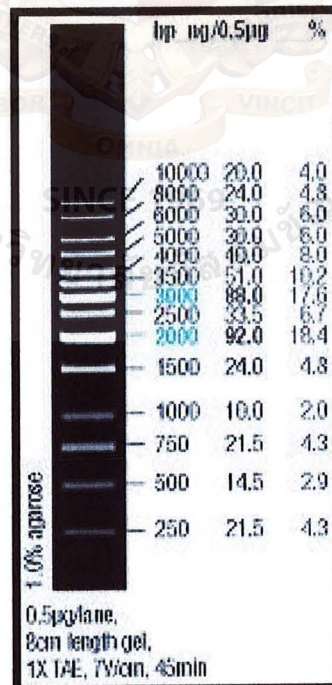
1 kb marker

Figure 38: GeneRuler™ 1kb DNA ladder (Melissa J et al, 2008)

Digestion fosmid reaction with *Bsp*143I (*Sau*3AI)

- Extracted fosmid = 5 µg

    Concentration of DNA of fosmid = 98.3 ng/µl

    $$= \frac{5000}{98.3}$$

    = 52 µl

- 10x buffer *Bsp*143I = 10 µl
- *Bsp*143I (0.2 unit) = 2 µl
- Sterile water = 36 µl

Digestion p-ZEro™-2 vector reaction with *Bam*HI

- Extracted p-ZEro™-2 vector = 5 µg

    Concentration of DNA of p-ZEro™-2 vector = 225.5 ng/µl

    $$= \frac{5000}{225.5}$$

    = 21 µl

- 10x buffer *Bsp*143I = 10 µl
- *Bam*HI = 2 µl
- Sterile water = 67 µl

Ligation reaction

Table 7.1: Ligation reaction of fosmid into p-ZEro™-2 vector

Reaction mixture	Ligation reaction		
	5 minute fosmid digestion	10 minute fosmid digestion	15 minute fosmid digestion
Digested p-ZEro™-2 vector	5	5	5
Digested fosmid	13	12	15
10x ligation buffer	3	3	3
T4 DNA ligase	2	2	2
Sterile water	7	8	5
Total solution	30	30	30

Transformation efficiency

DNA concentration of digested p-ZEro^{TM}-2 vector = 111.6 ng/μl and used 5 μl in ligation reaction. Therefore, there is 558 ng of p-ZEro^{TM}-2 vector in the reaction

> ➤ 5 minute fosmid digestion

Number of colony was counted after plate transformant on LB-km-IPTG agar

Number of colonies   =   2,880 colonies

The concentration of insert in ligation solution is 558 ng

The concentration of insert in 558 ng has number of colonies =   2,880 colonies

So, the concentration of insert in 1,000 ng has number of colonies = 5,162 colonies/μg DNA

> ➤ 10 minute fosmid digestion

Number of colony was counted after plate transformant on LB-km-IPTG agar

Number of colonies   =   9,320 colonies

The concentration of insert in ligation solution is 558 ng

The concentration of insert in 558 ng has number of colonies =   9,320 colonies

So, the concentration of insert in 1,000 ng has number of colonies = 16,703 colonies/μg DNA

> ➤ 15 minute fosmid digestion

Number of colony was counted after plate transformant on LB-km-IPTG agar

Number of colonies   =   7,280 colonies

The concentration of insert in ligation solution is 558 ng

The concentration of insert in 558 ng has number of colonies =   7,280 colonies

So, the concentration of insert in 1,000 ng has number of colonies = 13,047 colonies/μg DNA

APPENDIX B

Qualification the inserted gene

LB-Km-Cm agar

LB agar	600	ml
Chloramphenicol	34	µg/ml
Kanamycin	50	µg/ml

45 % glycerol

Glycerol	45	g
Distilled water to	100	ml

100 mM CaCl₂

$CaCl_2$	1.1	g
Distilled water to	100	ml

Then take 10ml of this solution and dilute again to 1000ml

PCR condition to amplify the fosmid subclone

Initial denaturation	95 °C	for 5 minute
Denaturation	95 °C	for 30 second
Annealing	58 °C	for 30 second
Extension	72 °C	for 2 minute
Final extension	72 °C	for 10 minute

Denaturation, Annealing, Extension → 30 cycles

PCR condition for colony PCR

Initial denaturation	95 °C	for 5 minute
Denaturation	95 °C	for 30 second
Annealing	55 °C	for 30 second
Extension	72 °C	for 1 minute
Final extension	72 °C	for 5 minute

Denaturation, Annealing, Extension → 30 cycles

PCR reaction to amplify the fosmid subclone

Table 7.2: PCR reaction to amplify the fosmid subclone

Reaction mixture	PCR reaction (μl)
Template DNA (~100 ng)	1.5
10x pfu buffer with $MgSO_4$ (1x)	5
pfu DNA polymerase (2.5U/ μl)	0.5
10 mM dNTP mix (0.2 mM each)	6
Xyn14.3 forward primer	5
Xyn14.3 reverse primer	5
Sterile water	32
Total solution	50 μl

PCR reaction for colony PCR

Table 7.3: PCR reaction for colony PCR

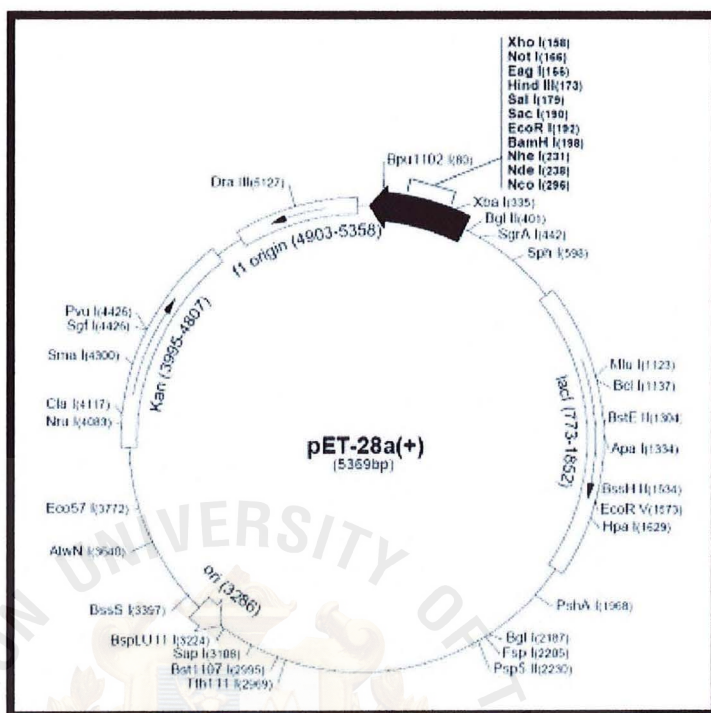| Reaction mixture	PCR reaction (μl)
10x tag buffer with $(NH_4)_2SO_4$ (1x)	2.5
Tag polymerase (1.25 U/ μl)	7.5
2 mM dNTP mix	2.5
Xyn14.3 forward primer	2.5
Xyn14.3 reverse primer	2.5
25 mM $MgCl_2$	2
Sterile water	12.75
Total solution	25 μl

pET-28a(+)vector

Figure 39: pET-28a(+)vector
Internet: http://www.ecoliwiki.net/colipedia/index.php/pET-28a%28%2B%29

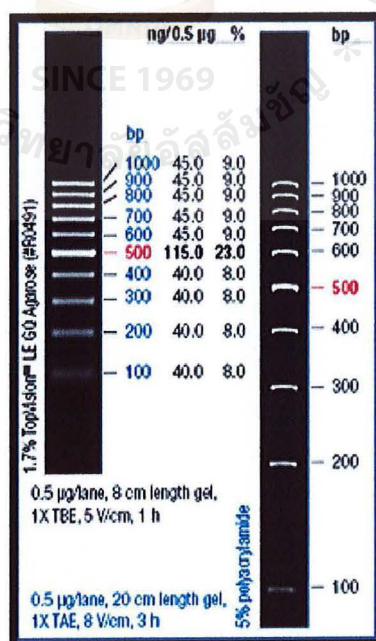**Gene Ruler™ 100bp DNA ladder**

Figure 40: GeneRuler™ 100bp DNA ladder
Internet:http://www.fermentas.com/en/products/all/dna-electrophoresis/generuler-dna-ladders/sm024-generuler-1

Digestion pET-28a(+)vector and Xyn14.3 gene reaction

Table 7.4: Digestion pET-28a(+)vector and Xyn14.3 gene reaction

Reaction mixture	Digestion reaction (μl)	
	pET-28a(+)vector	Xyn14.3 gene
DNA 500 ng	10	20
10x Tango buffer (2x)	8	8
*Eco*RI	0.5	0.5
*Hind*III	0.5	0.5
Sterile water	21	11
Total solution	40	40

Ligation reaction

Table 7.5: ligation reaction of fosmid into pET-28a(+) vector

| Reaction mixture	Ligation reaction
Digested pET-28a(+) vector	20
Digested xyn14.3	10
10x T4 DNA ligase	4
T4 DNA ligase	1
Sterile water	5
Total solution	40

APPENDIX C

The overexpression of the novel gene from clone Xyn14.3

SDS PAGE

12% Separating gel (for 10 ml)

Distilled water	2	ml
30 % Acrylamide mix	2.4	ml
1.5 M Tris-HCl, pH 8.8	1.5	ml
10% SDS	0.06	ml
10% Ammonium persulfate	0.03	ml
TEMED	0.005	ml

5% Stacking gel (for 3 ml)

Distilled water	1.5	ml
30 % Acrylamide mix	0.334	ml
0.5 M Tris-HCl, pH 8.8	0.625	ml
10% SDS	0.025	ml
10% Ammonium persulfate	0.015	ml
TEMED	0.005	ml

Protein marker
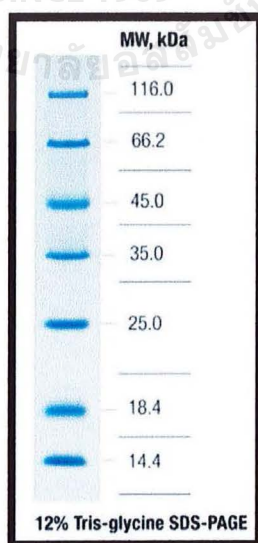
Figure 41: Protein marker

Internet: http://www.taq-dna.com/protein-marker-us7-print133.html

Solution for purify protein

Stripping solution

20 mM Sodium phosphate	2	ml
50 mM EDTA	50	µl

Binding solution

20 mM Sodium phosphate	4	ml
0.5 M NaCl	10	ml
40 mM Imidazole	4	ml

Elution buffer

20 mM Sodium phosphate	0.4	ml
0.5 M NaCl	1	ml
100-500 Imidazole	0.1-0.5	ml

Calculate putative molecular weight

From sequencing analysis, xyn14.3 has 283 amino acid.

$$\text{Putative molecular weight} = \frac{\text{Amino acid} \times 110}{1{,}000}$$

$$= \frac{283 \times 110}{1{,}000}$$

$$= 30.14 \quad \text{kD}$$

APPENDIX D

Biochemical characterization of crude enzyme

Concentration of protein

0.2 M Phosphate buffer pH 7.0

KH_2PO_4	2.632	g
K_2HPO_4	5.341	g
Distilled water to	250	ml

Alkaline CuSO4 assay mix

Copper reagent

    1% $CuSO_4.5H_2O$

Alkaline Reagent

    0.1 M NaOH

    2%   NaCO3

    0.5% Na Tartrate

    0.5% Na Dodecylsulfate (SDS)

Assay Mix (Prepare fresh everyday)

    Mix 50 ml alkaline reagent and 0.5 ml copper reagent

Folin-Ciocalteu reagent

Dilute with an equal volume of water to prepare the desired volume

Raw data of protein standard curve

Table 7.6: Raw data of protein standard curve

| BSA concentration (µg/ml)	OD750			Average	SD
	1	2	3		
0	0	0	0	0	0
100	0.015	0.007	0.011	0.011	0.004
200	0.217	0.185	0.195	0.199	0.016371
300	0.364	0.396	0.378	0.379333	0.016042
400	0.501	0.445	0.463	0.469667	0.028589
500	0.594	0.6	0.594	0.596	0.003464
600	0.752	0.769	0.732	0.751	0.01852
700	0.907	0.814	0.883	0.868	0.04828
800	1.013	0.97	1.121	1.034667	0.077797
900	0.994	-	1.107	1.0505	0.079903
1000	1.115	1.085	1.201	1.133667	0.060211

Graph of protein standard curve

OD750

$y = 0.001x - 0.019$

$R^2 = 0.984$

BSA conc. (µg/ml)
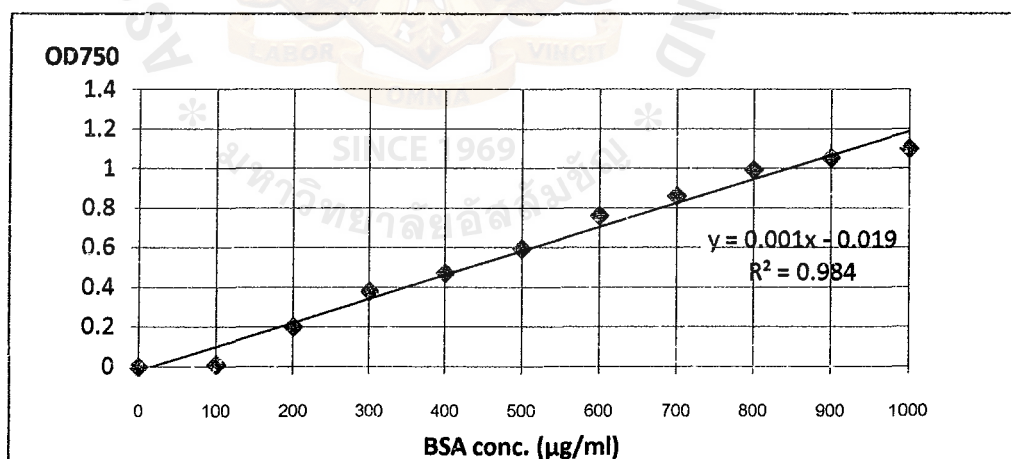
Figure 42: Protein standard curve using BSA stock solution

Calculate protein concentration

Example:

Based on the protein standard curve, 400 µl diluted enzyme was showed the amount of enzyme for 162 µg/ml in range OD_{750} between 0.2-0.7.

Therefore, the total amount of protein in X14.3 $= \dfrac{162\ \mu g/ml \times 1000}{400}$

$$= \quad 405 \quad \mu g/ml$$

Xylose standard curve

DNS solution

3,5- dinitrosalicyclic acid	7.486	g
NaOH	13.348	g
Phenol	0.504	g
Na Metabisulfate	5.86	g
Distilled water to	1,000	ml

0.2 M NaAc buffer pH 5.5

Na acetate	4.1	g
Distilled water to	250	ml

Adjust pH with acetic acid until pH reach to 5.5

0.2 M Tris-HCl buffer pH 8.0

Tris-HCl	6.05	g
Distilled water to	250	ml

Adjust pH with acetic acid until pH reach to 8.0

0.2 M Glycine buffer pH 10.0

Glycine-NaOH	3.75	g
Distilled water to	50	ml

Adjust pH with acetic acid until pH reach to 10.0

Raw data for xylose standard curve

> 0.2M NaAc buffer pH 5.5

Table 7.7: Raw data for xylose standard curve of buffer pH 5.5

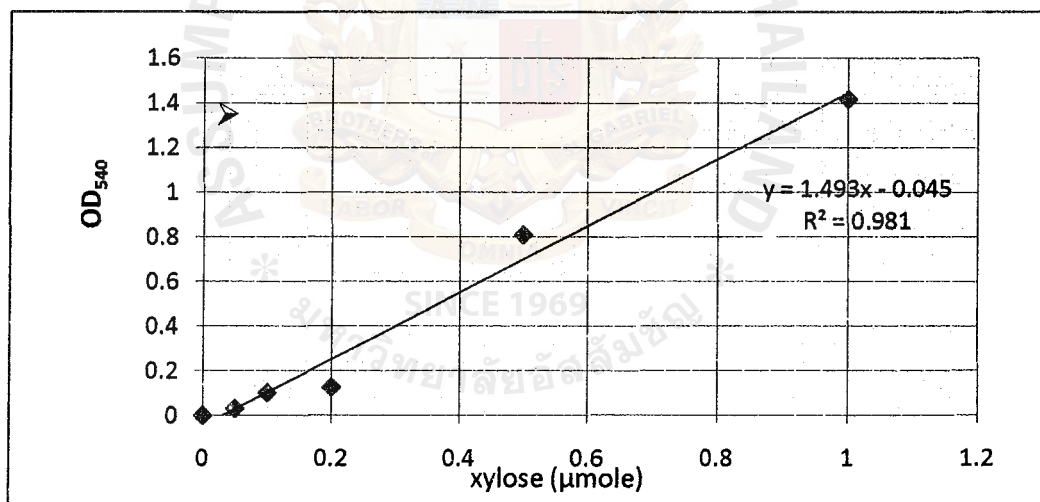| Xylose (μmole)	OD540			Average	SD
	1	2	3		
0	0	0	0	0	0
0.05	0.033	0.037	0.025	0.00611	0.61101
1	0.163	0.063	0.087	0.052205	5.220473
2	0.212	0.65	0.106	0.288391	28.83909
5	0.775	0.83	0.824	0.030172	3.017173
10	1.369	1.318	1.571	0.1338	13.37996

Figure 43: Xylose standard curve at pH 5.5

> 0.2 M Phosphate buffer pH 7.0

Table 7.8: Raw data for xylose standard curve of buffer pH 7.0

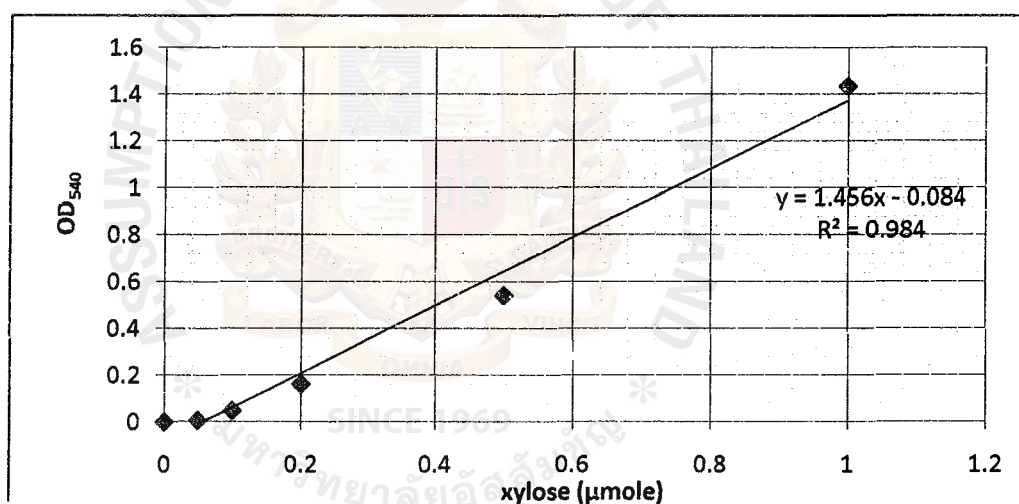| Xylose (μmole)	OD540			Average	SD
	1	2	3		
0	0	0	0	0	0
0.05	0.001	0	0.01	0.005508	0.550757
1	0.023	0.022	0.025	0.001528	0.152753
2	0.133	0.19	0.064	0.063095	6.309517
5	0.591	0.617	0.412	0.111611	11.16109
10	1.423	1.554	1.324	0.11537	11.53704

Figure 44: Xylose standard curve at pH 7.0

> 0.2 M Tris-HCl buffer pH 8.0

Table 7.9: Raw data for xylose standard curve of buffer pH 8.0

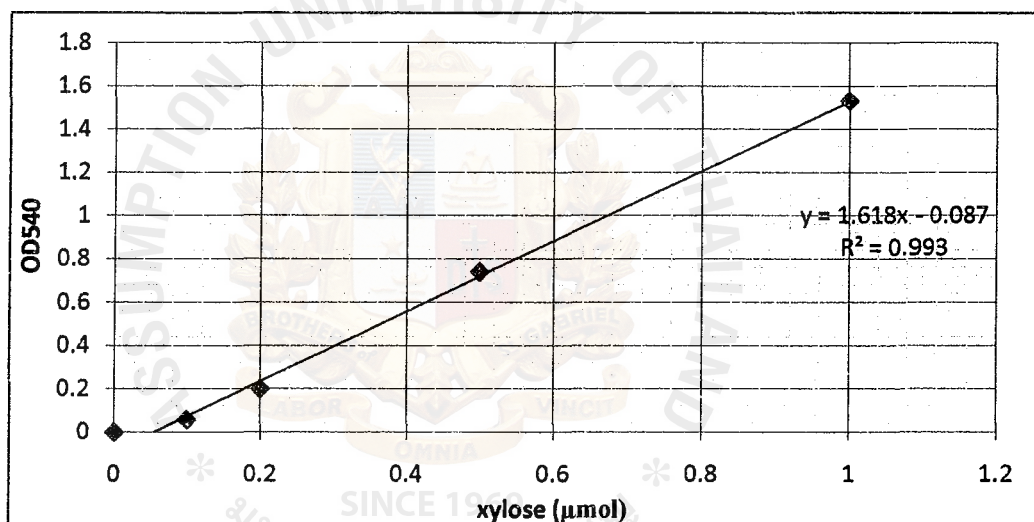| Xylose (μmole)	OD540			Average	SD
	1	2	3		
0	0	0	0	0	0
0.05	0.046	0.059	0.01106	1.106044	0.046
1	0.21	0.147	0.048775	4.877499	0.21
2	0.825	0.8	0.127343	12.73434	0.825
5	1.557	1.468	0.057657	5.765703	1.557
10	0	0	0	0	0

Figure 45: Xylose standard curve at pH 8.0

> 0.2 M Glycine buffer pH 10.0

Table 7.10: Raw data for xylose standard curve of buffer pH 10.0

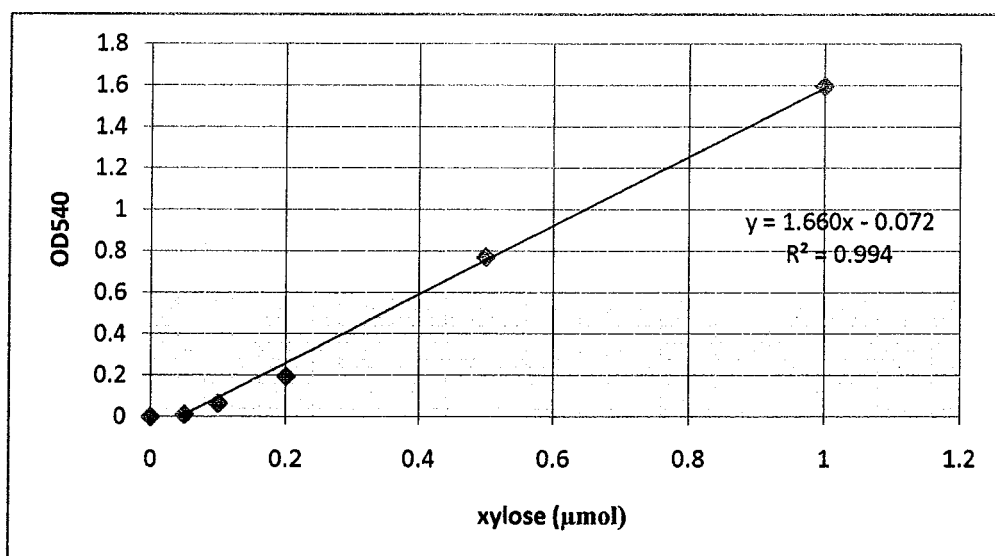| Xylose (μmole)	OD540			Average	SD
	1	2	3		
0	0	0.06	0	0.034641	3.464102
0.05	0.064	0.057	0.071	0.007	0.7
1	0.183	0.201	0.203	0.011015	1.101514
2	0.792	0.753	0.773	0.019502	1.950214
5	1.65	1.674	1.467	0.113221	11.3221
10	0	0.06	0	0.034641	3.464102

Figure 46: Xylose standard curve at pH 10.0

Birchwood xylan substrate

➤ pH 6.0

Birchwood xylan	1	g
0.2M NaAc buffer pH 5.5	50	ml
Distilled water	50	ml

Stir and heat continuously at 80 °C until completely dissolve

➤ pH 7.0

Birchwood xylan	1	g
0.2M phosphate buffer pH 7.0	50	ml
Distilled water	50	ml

Stir and heat continuously at 80 °C until completely dissolve

➤ pH 8.0

Birchwood xylan	1	g
0.2M Tris-HCl pH 8.0	50	ml
Distilled water	50	ml

Stir and heat continuously at 80 °C until completely dissolve

➤ pH 9.0

Birchwood xylan	1	g
0.2M Tris-HCl pH 9.0	50	ml
Distilled water	50	ml

Stir and heat continuously at 80 °C until completely dissolve

➤ pH 10.0

Birchwood xylan	1	g
0.2M Glycine-NaOH buffer pH 11.0	50	ml
Distilled water	50	ml

Stir and heat continuously at 80 °C until completely dissolve

➤ pH 11.0

Birchwood xylan	1	g
0.2M Glycine-NaOH buffer pH 11.0	50	ml
Distilled water	50	ml

Stir and heat continuously at 80 °C until completely dissolve

Enzyme dilution (2,500x)

➤ Phosphate buffer pH 7.0

X 14.3 (pure enzyme)	40	µl
0.2M phosphate buffer pH 7.0	9.96	ml

0.2 M phosphate buffer pH 7.0, 8.0

K_2HPO_4	5.341	g
KH_2PO_4	2.632	g
Distilled water to	250	ml

Adjust pH with HCl until pH reach to 7.0 and 8.0

0.2 M Tris-HCl buffer pH 9.0

Tris-HCl	6.05	g
Distilled water to	250	ml

Adjust pH with HCl until pH reach to 9.0

0.2 M Glycine-NaOH buffer pH 10.0, 11.0

Glycine-NaOH	3.75	g
Distilled water to	250	ml

Adjust pH with NaOH until pH reach to 10.0

Raw data for the optimum pH

Table7.11 : Raw data for the optimum pH

buffer	OD540			SD	Average	X (μmole)	activity (U/ml)	relative activity
	1	2	3					
pH 6.0 NaAc	0.00	0.00	0.03	0.02	0.01	0.04	471.65	24.53
pH 7.0 Phosphate buffer	0.15	0.11	0.15	0.02	0.14	0.15	1923.08	100.00
pH 8.0 Tris-HCl	0.11	0.04	0.15	0.06	0.10	0.11	1411.21	73.38
pH 9.0 Tris-HCl	0.08	0.02	0.06	0.03	0.06	0.09	1099.61	57.18
pH 10.0 Glycine-NaOH	0.03	0.02	0.02	0.00	0.03	0.06	730.42	37.98
pH 11.0 Glycine-NaOH	0.00	0.00	0.00	0.00	0.00	0.04	552.21	28.71

Calculate OD changed to xylose

Example:

Based on the linear equation from xylose standard curve at pH 7.0

$$y = 1.4932x - 0.045 \quad \text{and} \quad \text{average OD}_{540} = 0.01$$

Whereas Y axis is xylose (μmole) X axis is OD_{540}

Therefore, xylose is equal to $(0.01+0.045)/1.493$ $= 0.15$ μmole

Based on the linear equation from xylose standard curve at pH 10.0

$$y = 1.66x - 0.072 \quad \text{and} \quad \text{average OD}_{540} = 0.025$$

Whereas Y axis is xylose (μmole) X axis is OD_{540}

Therefore, xylose is equal to $(0.01+0.072)/1.66$ $= 0.06$ μmole

Calculate activity of enzyme (U/ml)

Example:

Based on the amount of xylose (μmole) from the 20 μl diluted enzyme from pH 7.0 in 2,500 times, this had been incubated for 10 minute

$$\text{The activity of enzyme} = \frac{\text{amount of xylose (μmole)} \times \text{ dilution factor}}{\text{time (minute)} \times \text{volume of enzyme (ml)}}$$

$$= \frac{0.15 \text{ μmole} \times 2,500}{10 \text{ min} \times 0.02 \text{ ml}}$$

$$= \quad 1,923.08 \qquad \text{U/ml}$$

Based on the amount of xylose (μmole) from the 20 μl diluted enzyme from pH 10.0 in 2,500 times which had been incubated for 10 minute

$$\text{The activity of enzyme} = \frac{\text{amount of xylose (μmole)} \times \text{ dilution factor}}{\text{time (minute)} \times \text{volume of enzyme (ml)}}$$

$$= \frac{0.06 \text{ μmole} \times 2,500}{10 \text{ min} \times 0.02 \text{ ml}}$$

$$= \quad 730.42 \text{ U/ml}$$

Calculate % relative activity of enzyme

Example:

Based on the activity of enzyme (U/ml) from pH 7.0 which and pH 10.0 which are 1,923.08 U/ml and 730.42 U/ml and the highest activity presented in pH 7.0.

$$\text{The relative activity of pH 7.0} = \frac{\text{xylose activity(U/ml)}}{\text{the highest xylose activity (U/ml)}} \times 100$$

$$= \frac{1,923.08 \text{ U/ml}}{1,923.08 \text{ U/ml}} \times 100$$

$$= \quad 100$$

$$\text{The relative activity of pH 7.0} = \frac{\text{xylose activity(U/ml)}}{\text{the highest xylose activity (U/ml)}} \times 100$$

$$= \frac{730.42 \text{ U/ml}}{1,923.08 \text{ U/ml}} \times 100$$

$$= \quad 37.98$$

Optimum temperature

Raw data for the optimum temperature

Table 7.12: Raw data for optimum temperature

Temp. °C	OD540				Average	SD	x(μmole)	Activity (U/ml)	Relative activity
	Blank	1	2	3					
(RT)	0.00	0.02	0.00	0.03	0.01	0.00	0.07	84.42	4.61
40	0.00	0.12	0.11	0.12	0.12	0.02	0.14	1739.93	95.00
45	0.00	0.13	0.13	0.13	0.13	0.02	0.15	1831.50	100.00
50	0.00	0.07	0.00	0.04	0.04	0.02	0.08	1055.98	57.66
55	0.00	0.05	0.04	0.02	0.04	0.02	0.08	1041.67	56.88
60	0.00	0.02	0.00	0.00	0.01	0.02	0.06	784.11	42.81
65	0.00	0.02	0.00	0.01	0.01	0.06	0.06	809.87	44.22
70	0.00	0.00	0.00	0.00	0.00	0.03	0.06	721.15	39.38

pH Stability

Raw data for the pH stability

Table 7.13: Raw data for pH stability

pH	Time (min)	OD540			SD	Average	x(μmole)	Activity	Relative activity
		1	2	3					
7	0	0.85	0.70	0.78	0.07	0.78	0.85	0.70	0.78
	30	0.66	0.72	-	0.04	0.69	0.66	0.72	-
	60	0.53	0.60	0.66	0.06	0.60	0.53	0.60	0.66
	90	0.61	0.63	0.65	0.02	0.63	0.61	0.63	0.65
	120	0.66	0.50	0.67	0.10	0.61	0.66	0.50	0.67
8	0	0.58	0.54	0.55	0.02	0.55	0.58	0.54	0.55
	30	0.20	0.17	0.20	0.02	0.19	0.20	0.17	0.20
	60	0.02	0.00	0.08	0.04	0.04	0.02	0.00	0.08
	90	0.00	0.02	0.00	0.01	0.01	0.00	0.02	0.00
	120	0.00	0.03	0.02	0.01	0.02	0.00	0.03	0.02
9	0	0.11	0.12	0.11	0.00	0.11	0.11	0.12	0.11
	30	0.00	0.00	0.02	0.01	0.01	0.00	0.00	0.02
	60	0.00	0.05	0.00	0.03	0.02	0.00	0.05	0.00
	90	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	120	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01

Temperature stability

Raw data for the temperature stability

Table 7.14: Raw data for temperature stability

Temp. (°C)	Time (min)	OD $_{540}$			SD	y=1.456x-0.084	Activity	Relative activity
		1	2	3		x(µmole)		
40	0	0.85	0.70	0.78	2.33	0.59	7383.24	94.71
	30	0.66	0.72	-	1.37	0.53	6610.58	84.80
	60	0.53	0.60	0.66	1.79	0.47	5855.08	75.11
	90	0.61	0.63	0.65	1.89	0.49	6118.36	78.49
	120	0.66	0.50	0.67	1.83	0.48	5949.52	76.32
45	0	0.74	0.84	0.90	2.47	0.62	7795.33	100.00
	30	0.74	0.64	0.79	2.17	0.55	6919.64	88.77
	60	0.46	0.32	0.32	1.10	0.31	3877.63	49.74
	90	0.37	0.32	0.39	1.08	0.30	3800.37	48.75
	120	0.27	0.33	0.33	0.92	0.27	3359.66	43.10
50	0	0.71	0.76	0.89	2.36	0.60	7483.40	96.00
	30	0.00	0.00	-	0.0υ	0.06	721.15	9.25
	90	0.02	0.00	0.00	0.03	0.06	795.56	10.21
	60	0.00	0.00	0.02	0.02	0.06	764.08	9.80
	120	0.00	0.04	0.01	0.04	0.07	841.35	10.79

APPENDIX E

Effect of enzyme activity on lignocellulosic materials

Concentration of NaOH

> 0% NaOH

| NaOH | 0 | g |
| Distilled water to | 100 | ml |

> 2% NaOH

| NaOH | 2 | g |
| Distilled water to | 100 | ml |

> 4% NaOH

| NaOH | 4 | g |
| Distilled water to | 100 | ml |

> 6% NaOH

| NaOH | 6 | g |
| Distilled water to | 100 | ml |

> 8% NaOH

| NaOH | 8 | g |
| Distilled water to | 100 | ml |

> 10% NaOH

| NaOH | 10 | g |
| Distilled water to | 100 | ml |

PAA solution

Acetic acid	1.5	ml
30% Hydrogen peroxide	1	ml
Sulfuric acid	1.5%	w/w

Pretreated lignocellulosic material substrate

Bagasse

Pretreated Bagasse	0.3	g
Distilled water	7.5	ml
Phosphate buffer pH 7.0	2.5	ml

Stir and heat continuously until temperature reached to 80 °C

Rice straw

Pretreated Rice straw	0.3	g
Distilled water	7.5	ml
Phosphate buffer pH 7.0	2.5	ml

Stir and heat continuously until temperature reached to 80 °C

Rice Husk

Pretreated Rice husk	0.3	g
Distilled water	7.5	ml
Phosphate buffer pH 7.0	2.5	ml

Stir and heat continuously until temperature reached to 80 °C

Enzyme dilution 100x

X 14.3 (pure enzyme)	0.1	ml
0.2M phosphate buffer pH 7.0	9.9	ml

Enzyme dilution 500x

X 14.3 (pure enzyme)	0.05	ml
0.2M phosphate buffer pH 7.0	9.95	ml

Enzyme dilution 1,000x

X 14.3 (pure enzyme)	1	µl
0.2M phosphate buffer pH 7.0	9.999	ml

Enzyme dilution 2,000x

X 14.3 (pure enzyme)	2	µl
0.2M phosphate buffer pH 7.0	9.998	ml

Enzyme dilution 3,000x

X 14.3 (pure enzyme)	3	μl
0.2M phosphate buffer pH 7.0	9.997	ml

Calculate enzyme activity (Unit of enzyme)

Example:

According to the 500x dilution enzyme at 10 minute, the activity (U/ml) was used to calculate the amount of enzyme used.

Activity on 500x at 10 minute   =   937.48   U/ml

(1,000 μl of enzyme has the activity for 937.48 U)

If 5 U/g was used to digest the pretreated material,

$$\frac{1,000 \ \mu l \times 5 \ U/g}{973.48 \ U} = 5.136 \ \mu l/g$$

The 0.3 g of pretreated materials was digested with pure enzyme.

Therefore, the amount of enzyme used is $\frac{5.136 \ \mu l \times 0.3 \ g}{1 \ g} = 1.54 \ \mu l$

The amount of enzyme in difference unit

Table 7.15: The amount of enzyme in difference unit

Unit of enzyme (U/g)	Amount of enzyme used (μl)	Amount of enzyme used (μl) for 0.3g lignocellulosic material
100	106.67	32
500	533034	160
1,000	1,066.69	320
2,000	2.13	640
3,000	3.2	960

Raw data of effect of enzyme activity on pretreated lignocellulosic materials

➢ Bagasse

Table7.23: Enzyme activity at 100 U with pretreated bagasse

| Time (hour) | 100 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (μmole)
0	0.000	0.000	0.000	0.000	0.030
1	0.000	0.000	0.000	0.000	0.030
3	0.012	0.027	-	0.020	0.043
6	0.053	0.098	0.061	0.071	0.076
24	0.299	0.309	0.331	0.313	0.234

Table7.24: Enzyme activity at 500 U with pretreated bagasse

| Time (hour) | 500 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (μmole)
0	0.002	0.000	-	0.001	0.031
1	0.012	0.052	0.036	0.033	0.052
3	0.160	0.173	-	0.167	0.139
6	0.199	0.185	0.269	0.218	0.172
24	0.617	0.642	0.593	0.617	0.433

Table7.25: Enzyme activity at 1,000 U with pretreated bagasse

| Time (hour) | 1,000 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (μmole)
0	0.000	0.000	0.000	0.000	0.030
1	0.045	0.017	0.054	0.039	0.055
3	0.185	0.180	0.136	0.167	0.139
6	0.247	0.196	0.227	0.223	0.176
24	0.686	0.615	0.670	0.657	0.458

➤ Rice straw

Table7.26: Enzyme activity at 100 U with pretreated rice straw

| Time (hour) | 100 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (μmole)
0	0.022	0.000	0.000	0.007	0.035
1	0.000	0.000	0.000	0.000	0.030
3	0.083	0.120	0.063	0.089	0.088
6	0.096	0.165	0.055	0.105	0.099
24	0.354	0.421	0.409	0.395	0.287

Table7.27: Enzyme activity at 500 U with pretreated rice straw

| Time (hour) | 500 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (μmole)
0	0.000	0.000	0.000	0.000	0.030
1	0.000	0.000	0.000	0.000	0.030
3	0.068	0.154	0.230	0.151	0.128
6	0.198	0.177	0.212	0.196	0.158
24	-	0.472	0.398	0.435	0.314

Table7.28: Enzyme activity at 1,000 U with pretreated rice straw

| Time (hour) | 1,000 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (µmole)
0	0.000	0.000	0.000	0.000	0.030
1	0.005	0.006	0.029	0.013	0.039
3	0.215	0.248	0.174	0.212	0.168
6	0.283	0.334	0.220	0.279	0.212
24	0.995	1.076	0.888	0.986	0.673

➢ Rice husk

Table7.29: Enzyme activity at 100 U with pretreated rice husk

| Time (hour) | 100 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (µmole)
0	0.000	0.000	0.000	0.000	0.030
1	0.000	0.000	0.000	0.000	0.030
3	0.000	0.000	0.000	0.000	0.030
6	0.000	0.000	0.000	0.000	0.030
24	0.139	0.150	0.145	0.145	0.124

Table7.30: Enzyme activity at 500 U with pretreated rice husk

| Time (hour) | 500 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (µmole)
0	0.000	0.000	0.000	0.000	0.030
1	0.000	0.000	0.000	0.000	0.030
3	0.000	0.000	0.000	0.000	0.030
6	0.000	0.000	0.021	0.007	0.035
24	0.210	0.197	0.185	0.197	0.159

Table7.31: Enzyme activity at 1,000 U with pretreated rice husk

| Time (hour) | 1,000 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (μmole)
0	0.000	0.000	0.038	0.013	0.038
1	0.012	0.030	0.001	0.014	0.039
3	0.042	0.014	0.029	0.028	0.048
6	0.035	0.062	0.011	0.036	0.053
24	0.192	0.257	0.233	0.227	0.178

Table7.32: Enzyme activity at 2,000 U with pretreated rice husk

| Time (hour) | 2,000 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (μmole)
0	0.008	0.000	0.000	0.003	0.032
1	0.056	0.032	0.006	0.031	0.050
3	0.090	0.137	0.119	0.115	0.105
6	0.175	0.177	0.157	0.170	0.141
24	0.315	0.313	0.312	0.313	0.234

Table7.33: Enzyme activity at 3,000 U with pretreated rice husk

| Time (hour) | 3,000 U | | | Average | y = 1.5331x - 0.0459 |
	1	2	3		X (μmole)
0	0.006	0.003	0.000	0.003	0.032
1	0.063	0.035	0.042	0.047	0.060
3	0.138	0.107	0.117	0.121	0.109
6	0.149	0.142	0.155	0.149	0.127
24	0.365	0.388	0.322	0.358	0.264