國立臺灣大學生物資源暨農學院森林環境暨資源學系

碩士論文

School of Forestry and Resource Conservation
College of Bioresources and Agriculture
National Taiwan University

Master Thesis

木聚醣酶與生質物性質對吸附與水解現象之影響

Impact of xylanase and biomass properties on adsorption and hydrolysis processes

歐譯陽

Yi-Yang Ou

指導教授：柯淳涵 博士

Advisor: Chun-Han Ko, Ph.D.

中華民國 103 年 7 月

July 2014

謝　誌

本論文承蒙恩師 柯淳涵博士兩年來的用心指導，在各方面都給予寶貴的意見，無論在研究上或是在處理事情的態度上都獲益良多，並且開拓許多學習新事物的機會，以及在實驗過程中的嘗試及摸索皆給予無比的耐心與包容，使本論文得以順利完成，在此表由衷之敬意與謝意。

並感謝淡江大學 徐秀福教授以及 施增廉教授撥冗前來擔任口試委員，鉅細靡遺的指正與審查，並在口試期間給予諸多寶貴的建議，使得論文可以更加的完整與豐富，特此致上最高的謝意。

修業期間，感謝綠色生產研究室的夥伴們，學長秉元、彥廷、芳璟、恆賓、佳興、宇笙、舜文、繼元，學姊慧儀、幸娟，同學愷良、彥廷，學弟宗仁、宗肯，學妹帆純、孟潔，在實驗上的協助與精神上的鼓勵讓我順利完成論文；同時要感謝過去在中研院努力的各位學長姊，讓我能直接獲得許多寶貴的材料；另外，我要感謝臺大森林系羽球隊的各位夥伴，當我在困惑挫折時，能夠讓我有一個地方可以放鬆心情，一起愉快地揮灑汗水。

再來要感謝巧青的陪伴，在我忙碌的實驗生活中多次激勵我，讓我在面對實驗以及論文的時候總是充滿信心，覺得自己有無限的潛能可以完成實驗，也經常一起旅遊、品嘗美食、談論夢想，未來的巨大夢想拼圖希望能與妳一起拼湊。

最後，特別要感謝我最愛的家人，感謝你們一直都很支持我，儘管我們的距離有百里之遙，你們都會經常打電話關心我的近況，分享我的喜怒哀樂，有你們的督促與包容讓我可以順利完成研究所學業，毫無後顧之憂。感謝這些年來關心我以及幫助我的師長、同學與朋友，願和你們分享我此刻所有的快樂與喜悅。

謹誌於臺灣大學森林研究所

2014 年 8 月

摘要

第二代生質酒精的原料主要是木質纖維素，其中半纖維素為地表上含量第二豐富的醣類資源，若能善加利用此資源，可使我們的能源供給能更加穩定，進而降低對石化能源的依賴性。

在本研究中，我們利用木聚醣酶、其變異株以及催化區域和鍵結區域對 4 種不同前處理的基質進行 40ºC 水解和吸附試驗，在試驗過程中，我們透過測定其游離蛋白質含量來確定其已被吸附的程度，再以測定還原糖釋出量來確認酵素的水解效率，在比較酵素及基質吸附水解的結果後，其中含有較多木質素的基質會吸附較多的木聚醣酶且釋出較少的還原糖，使得木聚醣酶的水解效率變低，而含有較少木質素的基質吸附的木聚醣酶量較少，但會釋出比較多的還原糖，因此，酵素水解木質纖維素的效率仍是要視水解出來的還原糖量來確定，而無法純粹以吸附的酵素量多寡來推斷，另一方面，碳水化合物的鍵結區域也是影響水解效率的其中一個因素，具有完整鍵結區域的木聚醣酶還原糖釋出量較多，鍵結區域有缺陷者略差，而不具鍵結區域者幾乎沒有還原糖釋出。

在 4ºC 的吸附試驗中，同樣含有較多木質素的基質具有吸附較多木聚醣酶的能力，但是因此也會影響到親和性的評估，因為其容易產生非專一性的鍵結，不過具有完整鍵結區域的木聚醣酶會有比較強的鍵結力是肯定的。

關鍵字：木質纖維素； 木聚醣酶； 吸附； 水解； 木質素； 碳水化合物鍵結區域

Abstract

Second generation biofuels produced from lignocellulosic feedstock materials and the basic structure of all woody biomass consisted of three basic polymers: cellulose, hemicelluloses and lignin. The hemicellulose was the second abundant carbohydrate resource in the earth. If we made good use of it, we could confirm the energy supply and decrease the dependence on petroleum energy.

In this study, we used 4 kinds of pretreated substrates to be hydrolyzed and adsorbed by xylanase, its mutants, its catalytic domain and binding domain. Comparing with 40ºC adsorption and hydrolysis results, high lignin content substrates adsorbed more xylanase but they released less reducing sugar, which decreased the efficiency of xylanase. Although low lignin content substrates adsorbed less xylanase, they still released reducing sugar more than those containing more lignin. The results showed lignin content had an influence on adsorption and hydrolysis. On the other hand, carbohydrate binding module (CBM) was one of important factors on hydrolysis. With complete CBM, xylanase released more reducing sugar, the one whose CBM had deficiency released less and it would release little reducing sugar without CBM.

On 4ºC adsorption, high lignin content substrates also had higher maximum adsorption and affected the affinity to enzyme because there was more unspecific binding. The xylanase with complete CBM had higher binding strength with substrates.

Keywords: Lignocellulose; Xylanase; Adsorption; Hydroylsis; Lignin; Carbohydrate binding module

Contents

Figure Index

Table Index

1. Introduction

As decreasing resources of fossil carbon sources and the side effects of their usage on natural environments were generated. People all around world paid attention close to renewable energy such as solar energy, wind and bioenergy, etc. Comparing to others energy, bioenergy was more sustainable and stable because the materials come from biomass which was more predictable than other kinds of renewable energy. The first generation bioethanol produced primarily from food crops such as corns, sugar crops and oil seeds had been used in US, Brazil and China. But it was also limited by some issues like competition for land and water used for food and fiber production, high production and processing costs that require government supports in order to compete with petroleum products and widely varying assessments of the net greenhouse gas (GHG) reductions when land-use change was taken into account (Ralph *et al*., 2010).

The impacts of these various concerns had stimulated the development of second generation biofuels produced from non-food biomass. The major components of non-food biomass were lignocellulosic feedstock materials including by-products (corn straw, sugar cane bagasse and forest residues), wastes (organic components of municipal solid wastes), and dedicated feedstocks (purpose-grown vegetative grasses, short rotation forests and other energy crops). Chemical composition of lignocellulosic materials was a key factor affecting efficiency of biofuel production during conversion processes. The

basic structure of all woody biomass consisted of three basic polymers: cellulose, hemicelluloses such as xylan and lignin. Cellulose and hemicellulose, which typically made up two-thirds of cell wall dry matter, were polysaccharides that could be hydrolyzed to sugars and then fermented to bioethanol. Generic block diagram of bioethanol production from lignocellulose materials was given in Fig 1. The basic process steps in producing bioethanol from lignocellulosic materials were: pretreatment, hydrolysis, fermentation and product separation/distillation. (Balat, 2010).

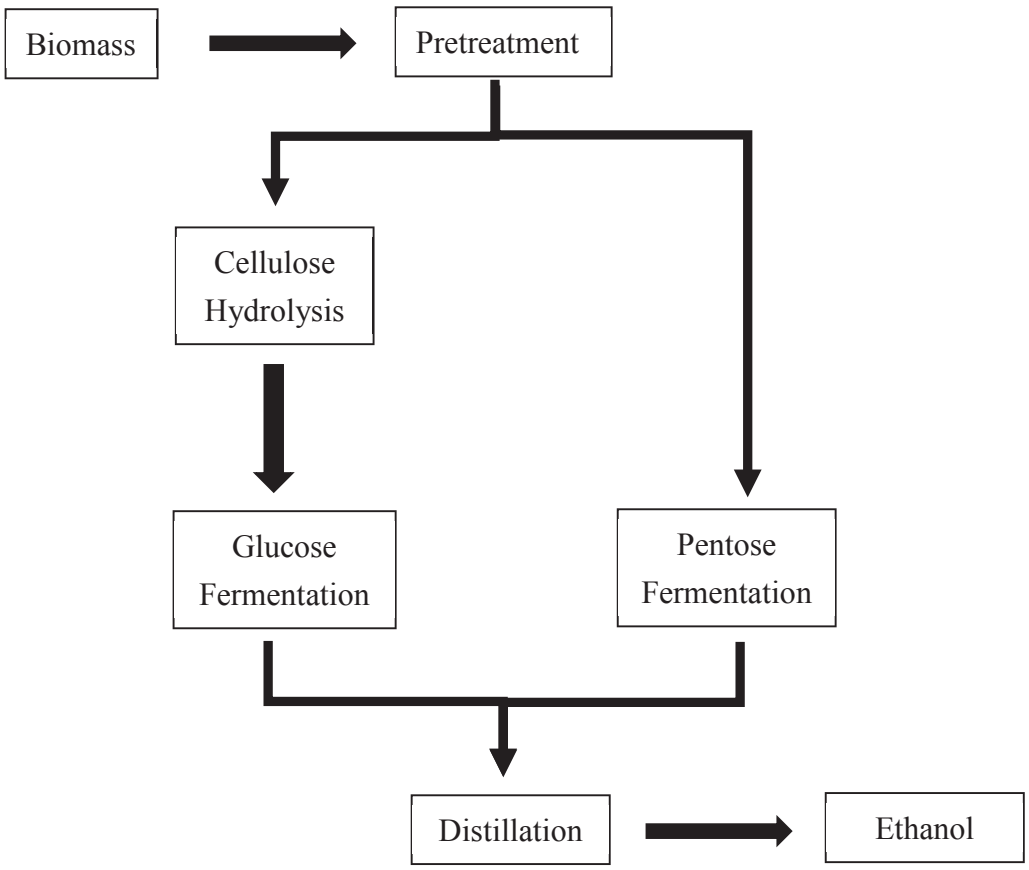


Figure 1. Generic block diagram of bioethanol production from lignocellulose biomass (Balat, 2010).

Although cellulose was the major material to produce bioethanol in lignocellulose, hemicellulose whose backbone was xylan was secondly abundant carbon resource on earth and also played an important role to rise the efficiency in the process of bioethanol conversion. This study focused on enzymatic hydrolysis and adsorption of xylanase on various pretreated biomass to understand relationship between biomass chemical composition, enzymatic hydrolysis and adsorption.

2. Literature Review

2.1. Lignocellulose

Lignocellulosic materials consisted of mainly three types of polymers, namely cellulose, hemicellulose and lignin (Fengel and Wegener, 1984).

2.1.1. Cellulose

Cellulose existed of D -glucose subunits, linked by β-1, 4 glycosidic bonds (Fengel and Wegener, 1984). The cellulose in a plant consisted of parts with a crystalline structure, and parts with an amorphous structure. The cellulose strains were bundled together and formed so called cellulose fibrils or cellulose bundles. These cellulose fibrils were mostly independent and weakly bound through hydrogen bonding (Laureano-Perez *et al*., 2005).

2.1.2. Hemicellulose

Hemicellulose was a complex carbohydrate structure that consisted of different polymers like pentoses (like xylose and arabinose), hexoses (like mannose, glucose and galactose), and sugar acids. The dominant component of hemicellulose for hardwood was xylan and for softwood is glucomannan (Fengel and Wegener, 1984). Hemicellulose had a lower molecular weight than cellulose and branched with short lateral chains that consisted of different sugars, which were easy hydrolyzable polymers (Fengel and

Wegener, 1984). Hemicellulose served as a connection between the lignin and the cellulose fibers and gave the whole cellulose–hemicellulose–lignin network more rigidity (Laureano-Perez *et al*., 2005).

2.1.3. Lignin

Lignin was, after cellulose and hemicellulose, one of the most abundant polymers in nature and was present in the cellular wall. It was an amorphous heteropolymer consisting of three different phenylpropane units (*p*-coumaryl, coniferyl and sinapyl alcohol) that were held together by different kind of linkages. The main purpose of lignin was to give the plant structural support, impermeability, and resistance against microbial attack and oxidative stress. The amorphous heteropolymer was also non-water soluble and optically inactive; all these made the degradation of lignin very tough (Fengel and Wegener, 1984).

2.2. Pretreatment

One of the major barriers for lignocellulose to become the economical production of bioethanol was its recalcitrance. Thus, we had to treat the materials with some physical or chemical methods to break down the structure of biomass feedstock and remove the barriers that made cellulose more accessible to hydrolytic enzymes for conversion to glucose before the hydrolysis process started. The goals of pretreatment on lignocellulosic

material were depicted in Fig 2. Pretreatment had been regarded as one of the most expensive pocessing steps within the conversion of biomass.

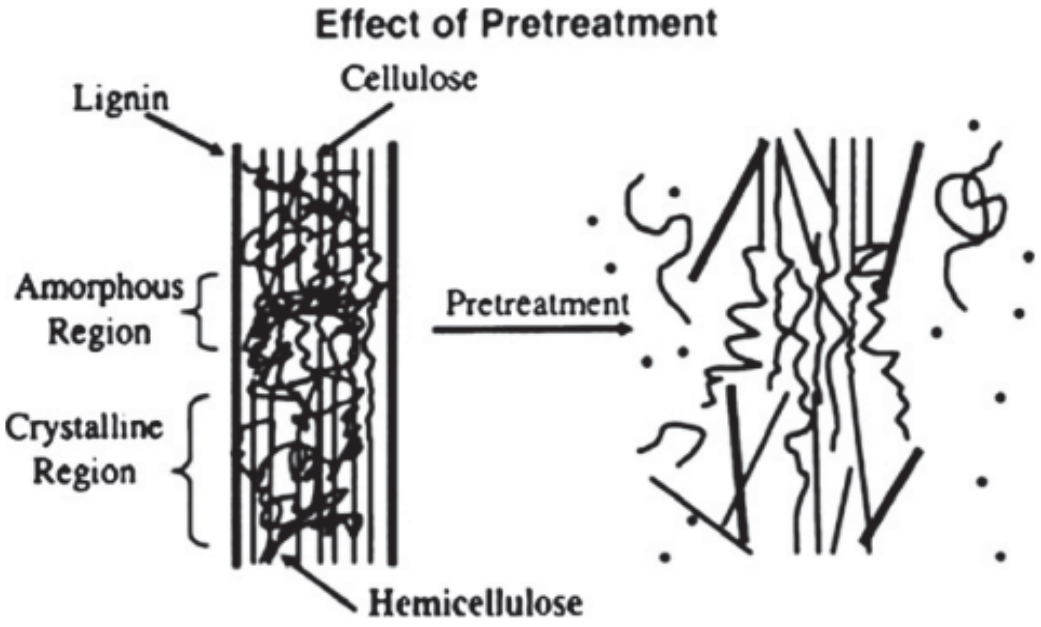


Figure 2. Schematic of goals of pretreatment on lignocellulosic material (Balat, 2010).

Taherzadeh and Karimi (2008) had summarized the goals for an ideal lignocellulosic pretreatment. It should be (1) production of reactive cellulosic fiber for enzymatic attack, (2) avoiding destruction of hemicelluloses and cellulose, (3) avoiding formation of possible inhibitors for hydrolytic enzymes and fermenting microorganisms, (4) minimizing the energy demand, (5) reducing the cost of size reduction for feedstocks, (6) reducing the cost of material for construction of pretreatment reactors, (7) producing less residues, and (8) consumption of little or no chemical and using a cheap chemical.

Pretreatment was crucial for ensuring good ultimate yields of sugars from both

polysaccharides. Hydrolysis without preceding pretreatment yielded typically <20%, whereas yields after pretreatment often exceed 90%. There were physical (milling and grinding), physico-chemical (steam explosion/autohydrolysis, hydrothermolysis, and wet oxidation), chemical (alkali, dilute acid, oxidizing agents, and organic solvents) and biological processes which had been used for pretreatment of lignocellulosic materials.

2.3. Enzyme adsorption and Hydrolysis

The carbohydrate polymers in lignocellulosic materials needed to be converted to simple sugars before fermentation, which was called hydrolysis. The most commonly applied methods could be classified in two groups: chemical hydrolysis (dilute and concentrated acid hydrolysis) and enzymatic hydrolysis. The main characters of the bioethanol were cellulose and hemicellulose and their hydrolytic products as follow (Taherzadeh and Karimi, 2007):

$$\text{Cellulose} \rightarrow \text{Glucan} \rightarrow \text{Gluose} \rightarrow \text{Decomposition products}$$

$$\text{Hemicelluloses} \rightarrow \text{Xylan} \rightarrow \text{Xylose} \rightarrow \text{Furfural}$$

$$\text{Acetyl groups} \rightarrow \text{Acetic acid}$$

Comparing to acid hydrolysis, however, enzymatic hydrolysis had high specificity, mild temperature, less environmental and corrosion problems. The high cost of acid consumption and recovery were major barriers to economic success (Hamelinck *et al*., 2005). But enzymatic hydrolysis of natural lignocellulosic materials was a very slow process because cellulose hydrolysis was hindered by structural parameters of the substrate, such as lignin and hemicellulose content, surface area, and cellulose crystallinity.

Table 1. Comparison of process conditions and performance of three hydrolysis processes (Hamelinck *et al*., 2005).

	Consumables	Temperature (°C)	Time	Glucose yield (%)
Dilute acid	<1% H_2SO_4	215	3 min	50 - 70
Concentrated acid	30-70% H_2SO_4	40	2-6 h	90
Enzymatic	Cellulase	50	1.5 days	75 → 95

Yang and Wyman (2004) reported that lignin removal improved cellulose digestibility, with mechanisms postulated to improve cellulose accessibility, enhancement of cellulase effectiveness by removal of cross linkages to carbohydrates and xylan removal enhance glucan digestibility. Jeoh *et al* (2007) showed that xylan removal enhanced biomass digestibility by increasing cellulose accessibility.

Before the hydrolysis started, enzyme adsorption onto solids was the primary step for enzymatic hydrolysis of pretreated substrate (Kumar and Wyman, 2009) and the hydrolysis rate or yield was claimed to be directly related to the amount of adsorbed enzyme generally (Jeoh *et al*., 2007). Both cellulase and hemicellulase needed to adsorb on the solid surface prior to enzymatic hydrolysis but the complexity of the hemicellulose structure and the array of enzymes involved would result in limited studies of hemicellulolytic enzymes-substrate interactions for biomass (Kumar and Wyman, 2009a).

Kumar and Wyman (2009a) also showed that cellulase adsorption was very rapid for substrate with the maximum in the first 10 min, as adsorption continued with time, the equilibrium will reach slowly in less than 2 hr. They also found a biomass which content higher lignin than others has high affinity to cellulase. Furthermore, they reported that xylanase adsorption not only takes place on xylan but also on glucan and lignin in biomass. This result corresponded with the earlier studies by Ryu and Kim (1998) that used purified Pulpzyme HC to adsorb on lignin and crystalline cellulose. They showed that a significant amount of xylanase was adsorbed onto lignin in alkaline solutions. The binding of xylanase onto lignin was assumed to be caused by a physical interaction such as the van der Waals interaction but the adsorption of purified xylanase onto crystalline cellulose was not significant. Tenkanen *et al*. (1995) who used hemicellulases to adsorb on xylan, mannan and cellulose found that the hemicellulases were also incompletely

bound on mannan and were found to bind readily on cellulose. Thus, the hemicelluloses probably contained not only hemicellulose binding domain but also cellulose binding domain.

Mansfield *et al*. (1999) reviewed the van der Waals contacts and hydrogen bonds were dominant forces in carbohydrate-binding proteins. The CBDs generally have a low content of charged amino acids and a high content of hydroxyl amino acids. Aromatic amino acid residues, tryptophan and tyrosine, were thought to pack onto the sugar rings and increased additional specificity and stability to the enzyme-substrates complexes. The removal of the CBD reduced the hydrolytic efficiency of the enzymes on crystalline cellulose but not on amorphous cellulose.

Palonen *et al*. (2004) used two purified cellulases (cellobiohydrolase and endoglucanase) and their catalytic domains on steam steam pretreated softwood and lignin. They found that both cellobiohydrolase and its catalytic domain exhibited a higher affinity to steam pretreated softwood than endoglucanase or its catalytic domain. They also found that removal of cellulose binding domain decreased the binding efficiency. Their results indicated that the cellulose binding domain had a significant role in the unspecific binding of cellulases to lignin.

On the other hand, Kumar and Wyman (2009a) showed that delignification enhanced cellulase and probably xylanase effectiveness significantly when the substrates produced

by high pH pretreatment. The substrate that contained a significant amount of xylan enhances glucose release and much more xylose release. Várnai *et al.* (2011) found that the cellulase remained mostly bound throughout the hydrolysis of two different types of substrates: Avicel and steam pretreated spruce (SPS). The surface of SPS, rich in lignin, was thus obviously more repulsive towards the β-glucosidase than the crystalline Avicel surface. Catalytically delignified softwood cellulose (COS) which was low-lignin containing substrate had the highest β-glucosidase activity retained at the end of the hydrolysis. The reason might be the less ordered, easily accessible and hydrolysable structure of this substrate and possibly also the altered surface characteristics due to the oxidative pretreatment. The xylanase seemed to depend more on the lignin content than on the xylan content of the substrates. The study showed that the SPS containing the lowest xylan amount adsorbed the xylanase more than the highest xylan-containing COS or the low xylan containing Avicel. The binding behavior of xylanase was obscured and could not be explained by the lignin or xylan contents in the substrate. Heiss-Blanquet *et al*. (2011) found that cellulase adsorption and specific activity were likely to be influenced by structural and compositional characteristics of lignocellulosic substrates. Both were indeed found to be directly proportional to the cellulose content and indirectly proportional to Klason lignin of the substrates. The former was positive correlation and the latter was negative correlation, suggesting that lignin was one of the factors restricting

enzymatic hydrolysis. Ju *et al*. (2013) showed that, apart from its hindrance effect, xylan could facilitate cellulose fibril swelling and thus created more accessible surface area, which improved enzyme and substrate interactions. Surface lignin had a direct impact on enzyme adsorption kinetics and hydrolysis rate. Higher surface lignin content, especially from hydrophobic lignin, led to lower cellulase affinity to the substrate and lower initial hydrolysis rate. Guo *et al*. (2014) found that lignin resources affected enzyme adsorption using structure features such as functional groups and lignin composition. Guaiacyl (G) lignin had a higher adsorption capacity on enzymes than syringyl (S) lignin. The low S/G ratio and high uniform lignin fragment size had good correlations with high adsorption capacity. They found that cellobiohydrolase (CBH) and xylanase were adsorbed the most by all lignins, endoglucanase (EG) showed less inhibition, and β -glucosidase (BG) was the least affected by lignins. The results indicated the important role of carbohydrate-binding module (CBM) in protein adsorption.

3. Materials and methods

3.1. Materials

3.1.1. Biomass

There were four types of biomass in my research, the first of them was unbleached eucalyptus kraft pulp (UEK) and the second one was bleached eucalyptus kraft pulp (BEK). The original kraft pulps samples were produced from Australian Eucalyptus globules chips by using an M/K digester (Peabody, MA, USA), with liquid-to-wood ratio of 1/4. The cooking liquor consisted of NaOH and Na_2S, with 25% sulfidity and 17% active alkali based on chemical charge. Cooking temperature was raised from 25 to 160^{o}C at 1.5^{o}C per minute, then maintained isothermally for 180 min.

Fully bleached pulps were prepared from oxygen bleached pulps by using a common commercial DEDD bleaching sequence (Ko *et al*., 2010). DEDD bleaching sequence was treated with chlorine dioxide (ClO_2) and sodium hydroxide (NaOH) to remove lignin. The dosages of chlorine dioxide were determined by active chlorine multiple and kappa number. In the first stage (D0), there were 10% consistency pulp and chlorine dioxide corresponding to pulp incubating for 1 hr at 70^{o}C. In the next step, the sodium hydroxide was used to alkali extraction to remove lignin (E). Dosage of sodium hydroxide was 1.8% base on gram of pulp sample. Alkali extraction reaction time was 1.5 hr at 65^{o}C. After the alkali extraction, D1 and D2 steps of bleach sequence were carried out. The D1 and

D2 bleach sequence used chlorine dioxide dosage of 0.35% and 0.15% base on pulp sample for each 3.5 hr at 72°C and made the pulp brightness over than 90%.

The third biomass was eucalyptus chips hydrolyzed by 1% sulfuric acid for almost 6 days before steam explosion (ASEP) and the last one was eucalyptus chips treated by steam explosion directly (NSEP). The steam explosion was executed by Institute of Nuclear Energy Research. The condition of steam explosion was to put 1 kg dry treated or not treated eucalyptus chips into reactor and the ratio of liquid/solid was 7 of each feedstock and heated to 190°C with saturated steam for 10-20 minutes.

3.1.2. Materials Sieved

Fiber size fractionation was carried out in a BAUER-McNETT CLASSIFIER (BMC) for 30-45 minutes classifier fitted with 28-, 50-, 100-, 200-mesh screens. The process of manipulation followed the CNS 12428. The fraction retained by the screen was termed RX where X refers to the mesh size. According the results of Tsai (2012), the enzymatic hydrolysis and adsorption of R200 sieved substrate is the most efficient of all because the specific surface area of R200 was more than other sieved substrate.

Although R200 had higher specific surface area, the result of the fiber size distribution showed that there were more R100 pulp retained on mesh screen. Thus, in this study R100 scale materials were used as substrates for further analysis.

3.1.3. Enzymes

Pulpzyme HC was a commercial enzyme with xylanase activity supplied from Novozyme. XylX produced by *Paenibacillus campinanesis* BL11 was found in black liquor and its mutants (H2, L2) were constructed by further research. (Ko *et al*. 2007, 2010). Enzyme could be departed as catalytic domain which made enzyme hydrolyzing a certain polymer and binding domain which made enzyme attaching on polymer stably. The catalytic domain of XylX belonged to GH11 family so it was termed GH 11 and the binding domain also called carbohydrate binding module was abbreviated as CBM (Wang, 2013).

3.1.3.1.Incubation

E. coli (*Escherichia coli*) expressing system was used to produce the enzymes. *E. coli* were routinely cultured in Luria–Bertani (LB) medium. LB medium contained 10 g/L Bacto-tryptone, 5 g/L yeast extract, and 5 g/L NaCl. First, *E. coli* were precultured from plate to liquid broth about 3 mL LB medium in glass tube at 150 rpm overnight. After cultured to certain concentration, *E. coli* were transferred to 500 mL flask and rotating at 150 rpm for 3-4 hours. Then, IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added to final concentration as 0.1 mM for inducing *E. coli* to produce enzyme. Moreover, the enzyme of *E. coli* expressing system was an endo-secreted system. *E. coli*

were disrupted by ultrasonication in ice bath to break the cell wall and let enzyme suspended in buffer. After ultrasonication, the buffer would be centrifuged at 4°C 8500 rpm for 20 minutes to separate the somatic of *E. coli* and enzyme. The supernatant which had enzyme was separated through 0.45 filter as crude enzyme.

3.1.3.2.Purification

The crude enzyme was purified by Ni-NTA column whose motive phase was Tris-HCl buffer with gradient imidazole. The purification made use of affinity between His-tag on expressed enzyme and Ni^{2+}. After binding, we used high concentration imidazole to competitive with His-tag to make enzyme leave Ni-NTA and collected the target enzyme. First, 5 mM imidazole Tris-HCl buffer (binding buffer) was used to stable the condition of all purification system. Second, crude enzyme was added to Ni-NTA column let protein bind with Ni^{2+} as well as there were still some non-specific binding. Thus, 20 mM imidazole Tris-HCl buffer (washing buffer) was used to break the non-specific binding between Ni^{2+} and non-specific protein because the non-specific binding was a weak binding whose binding site would be occupied by imidazole. After washing, 100 mM imidazole Tris-HCl buufer (elute buffer) was used to elute the target enzyme and collected it as purified enzyme. During the process of purification, the spectrophotometer monitored the outlet liquid at 280 nm absorbance.

3.1.3.3.SDS-PAGE

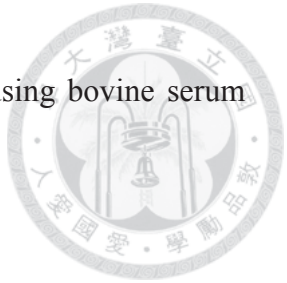
The purified enzymes used SDS-PAGE (Sodium dodecyl sulfate - polyacrylamide gel electrophoresis) to check the degree of purity. 12% polyacrylamide gel was used as running gel and set it on Hoefer electrophoresis equipment. The sample preparation was adding same volume loading dye as sample and put it in boiling water for 10 min. Then, the sample was centrifuged 5000 rpm for 30 min. After loading sample in stacking gel, the volt of power supplement was set at 200 mV for 5 min to stack the protein. Next, the volt of power supplement was changed to 120 mV for 120 min to make the protein separated by the size. Finally, the SDS-PAGE was stained by comassie blue for 30 min and destained by methanol acetate buffer overnight.

3.2. Methods

3.2.1. 40^{o}C enzymatic hydrolysis and adsorption

The pulps were treated with enzyme at 40^{o}C, pH 6 and the consistency was 1%. From the results of Tsai, the enzyme dosage used 5 mg per gram substrate was more suitable for analysis. Pulps were putted in an incubator and incubated for 125 rpm. Each experiment was sampled at 1, 2, 4, 8, 12, 24, 48 hr and then centrifuged 6000 rpm to separate the substrates and supernatant. The supernatant is determined the free enzyme

and released reducing sugar measured by Bradford protein assay using bovine serum albumin as standard and DNSA (dinitrosalicylic acid) respectively.



3.2.2. 4°C enzyme adsorption

The enzyme was added to a 0.1% consistency substrate which was dispersed in pH 6 sodium acetate buffer at 4°C condition to avoid hydrolysis. The buffer was integrated 0.1 M sodium acetate, 20 mM calcium chloride. The enzyme dosages were 5, 10, 15, 20, 30, 40, 50, 60, 70 mg per gram of oven dry substrate. The mixtures were turned on a rotator for 1 hr to be adsorption equilibrium. After equilibrium, the mixtures were centrifuged and the supernatants were measured by the Bradford protein assay using bovine serum albumin as standard. The adsorbed enzyme was calculated by the difference between the amount of protein initially added and the supernatant named free enzyme.

The analysis of adsorption parameters (maximum adsorption capacity [σ] and equilibrium constant [K_d]) were determined by non-linear regression of the adsorption data to the following Langmuir expression using Sigmaplot software (Lynd *et al*., 2002; Kumar and Wyman, 2008):

$$[\mathrm{CE}] = \frac{\sigma[S_t][E_f]}{K_d + [E_f]}$$

in which [CE] was the amount of adsorbed enzyme in mg/mL, [E_f] the free enzyme concentration in mg/mL, σ the maximum adsorption capacity in mg/mg substrate, [S_t] the substrate concentration in mg/mL, and K_d the equilibrium constant = [C][E]/[CE] in mg of enzyme/mL, where [C] was the concentration of free binding sites on the substrates in mg/mL and [E] and [E_f] were the enzyme concentrations not adsorbed on the substrate in mg/mL.

4. Results and Discussion

4.1. Chemical composition of substrates

The chemical compositions of substrates were shown in Table 2. The Kraft process was effective to remove the lignin of biomass to about 3% and bleach process by DEDD making the lignin content decreasing down to zero. On the other hand, because ASEP was pretreated by dilute sulfuric acid, the percentage of hollocellulose was less than non-acid pretreated and even Kraft pulps. Furthermore, the lignin content of ASEP was about 50% which was similar to hollocellulose. From the Table 2, the biomass pretreated by alkali process (BEK and UEK) had low lignin content and above 90% content of hollocellulose. On the other hand, the biomass pretreated by acid process (ASEP and NSEP) had high lignin content from 21.21 to 56.32 and lower hollocellulose content than Kraft pulps.

Table 2. Chemical composition of lignocellulose substrates

	Chemical composition (%, w/w)			
Substrates	Hollocellulose	Xylan	Lignin	Ash
BEK	96.50	8.34	0	0.87
UEK	94.82	9.88	3.75	0.77
ASEP	58.88	1.10	56.32	1.85
NSEP	76.86	1.41	21.21	1.93

4.2. 40°C hydrolysis and enzyme adsorption

Fig. 3 showed the UEK, BEK, ASEP and NSEP hydrolyzed by Pulpzyme HC at 40°C for 48 hr. UEK hydrolysis by Pulpzyme HC released more reducing sugar at first but it would be overtaken by BEK after 24 hr. The ASEP and NSEP hydrolyzed by Pulpzyme HC released little reducing sugar for 48 hr. The results could confirm from the results of substrate chemical composition. ASEP didn't content xylan and NSEP had a little bit of xylan. UEK had the most xylan than other substrates.

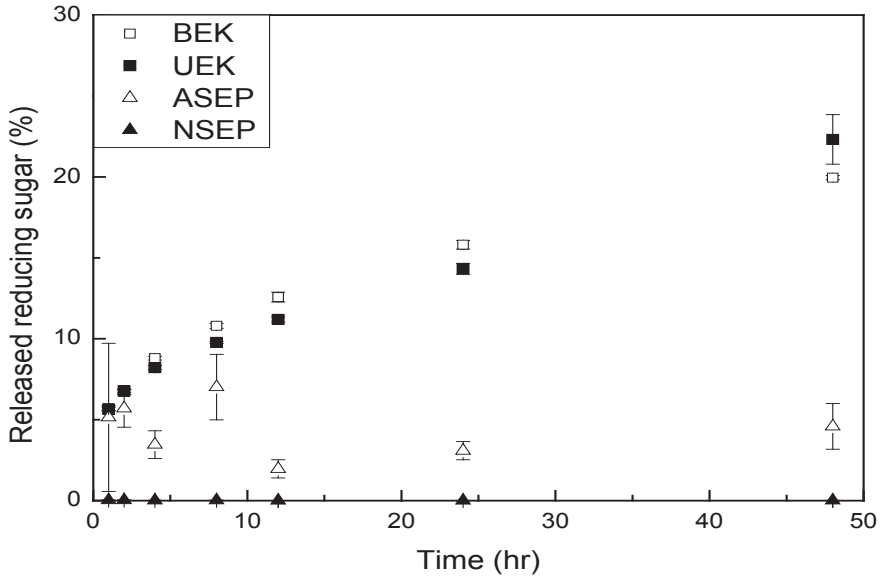


Figure 3. 40°C hydrolysis of Pulpzyme HC

Fig. 4 showed the 40ºC adsorption of Pulpzyme HC. ASEP and NSEP adsorbed almost all Pulpzyme HC from the beginning. BEK adsorbed the least Pulpzyme HC that was half of ASEP and NSEP in the end and the rate of adsorption was slowly. UEK adsorbed 3 mg/g substrate Pulpzyme HC in the beginning and finally adsorbed 4 mg/g substrate. The affinity of UEK 40ºC adsorption was apparently higher than BEK.

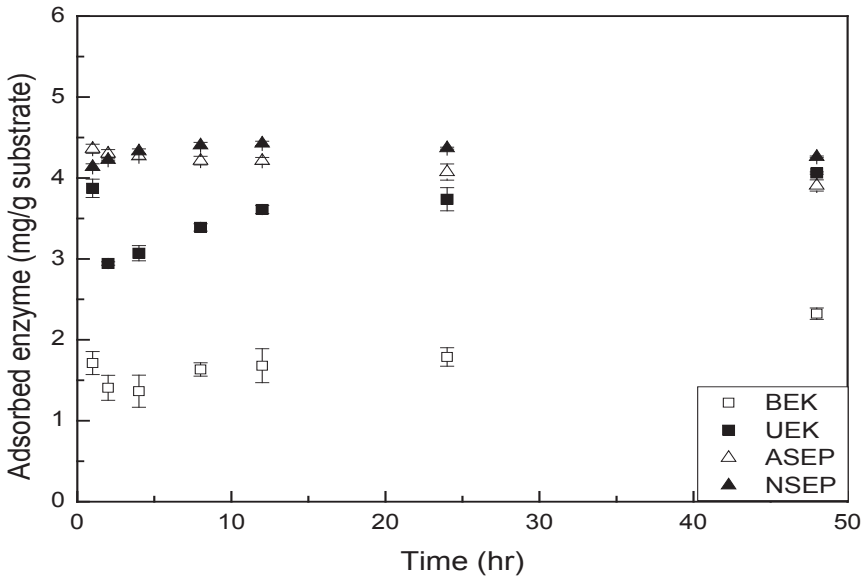


Figure 4. 40ºC adsorption of Pulpzyme HC

Fig. 5 showed the results of XylX was similar with Pulpzyme HC. The reducing sugar of UEK and BEK hydrolysis were higher than the others and after hydrolyzing 24 hr, the reducing sugar of UEK was still higher than that of BEK. ASEP and NSEP also release little reducing sugar for 48 hr.

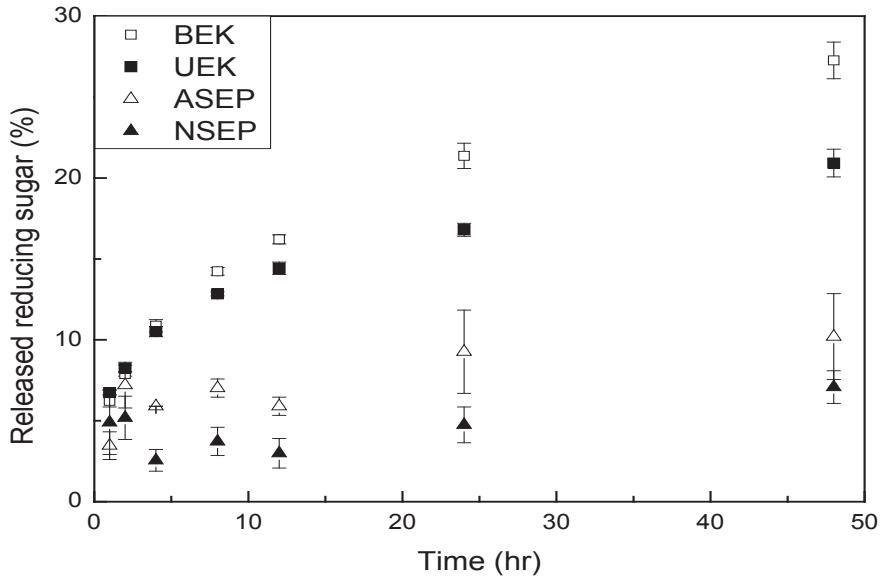


Figure 5. 40ºC hydrolysis of XylX

Fig. 6 showed the adsorption of XylX at 40°C. UEK adsorbed enzyme was equal to ASEP and NSEP and the adsorbed enzyme was stable during 48 hr hydrolysis. BEK adsorption was also less than other lignin content substrates. During 48 hr, BEK didn't adsorbed more XylX significantly. To all substrates, it seemed that XylX reached max adsorption and stable in 1 hr.

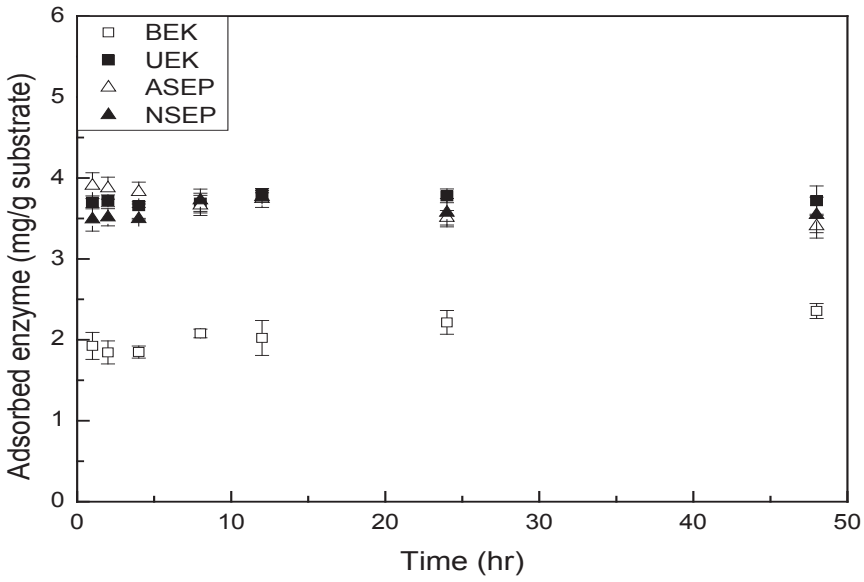


Figure 6. 40°C adsorption of XylX

Figure 7 showed the hydrolysis of XylX-H2. Its results were like above two enzymes that BEK and UEK could release a large amount of reducing sugar than ASEP and NSEP. Furthermore, BEK released more reducing sugar than UEK after hydrolyzing 24 hr. ASEP and NSEP also released little reducing sugar.

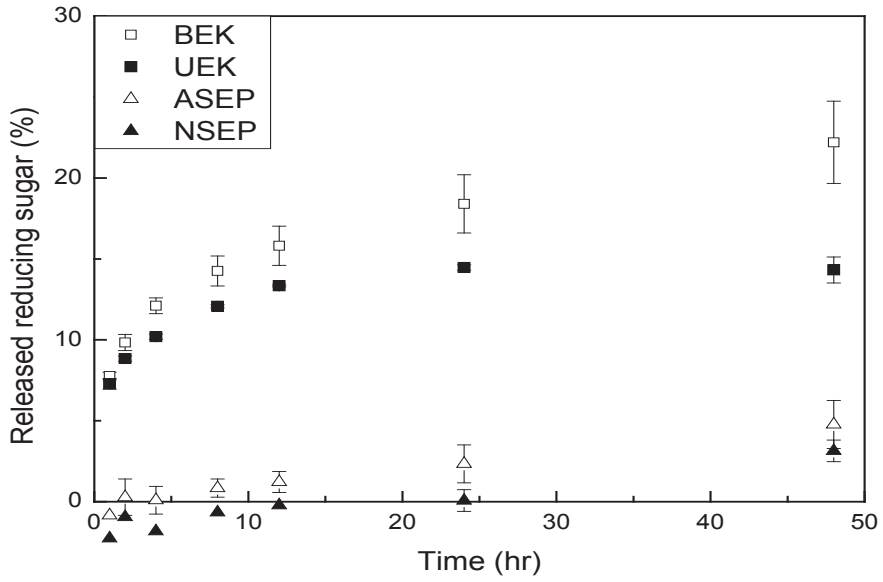


Figure 7. 40ºC hydrolysis of XylX-H2

Fig. 8 showed the 40°C adsorption of XylX-H2. Though BEK still adsorbed the least XylX-H2 in the beginning but its catch up with other substrate after hydrolyzing 12 hr. After the adsorption of BEK caught up with UEK at 12 hr, the reducing sugar of BEK overtook that of UEK at 24 hr. It seemed that the hydrolyzed reducing sugar made more binding site for enzyme to adsorb. To BEK that had no lignin content, it would have no non-specific adsorption and made it releasing more reducing sugar than UEK.

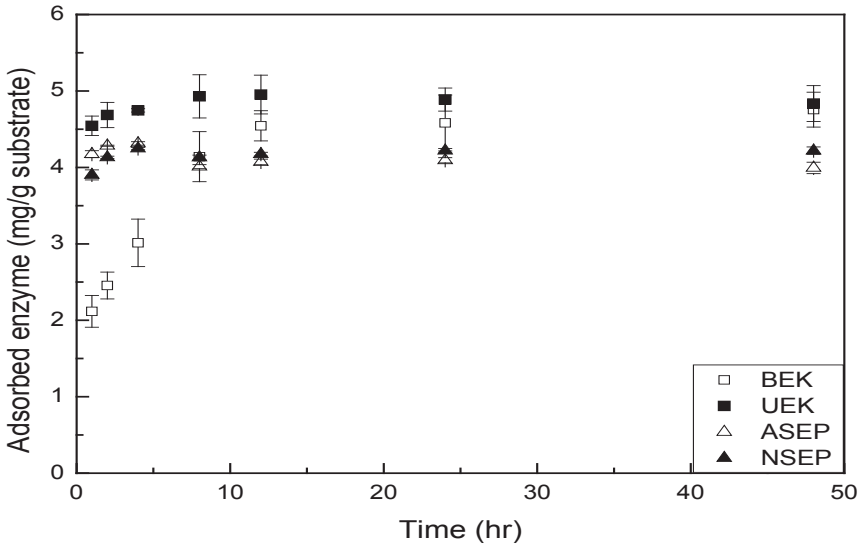


Figure 8. 40°C adsorption of XylX-H2

Fig. 9 was the hydrolysis of XylX-L2. The reducing sugar of BEK and UEK were still higher than ASEP and NSEP but their reducing sugar were less than above enzyme a lot. UEK released about 70 μmole that was half of above enzymes and BEK also released about 50 μmole in 48hr. ASEP and NSEP both still had little reducing sugar released.

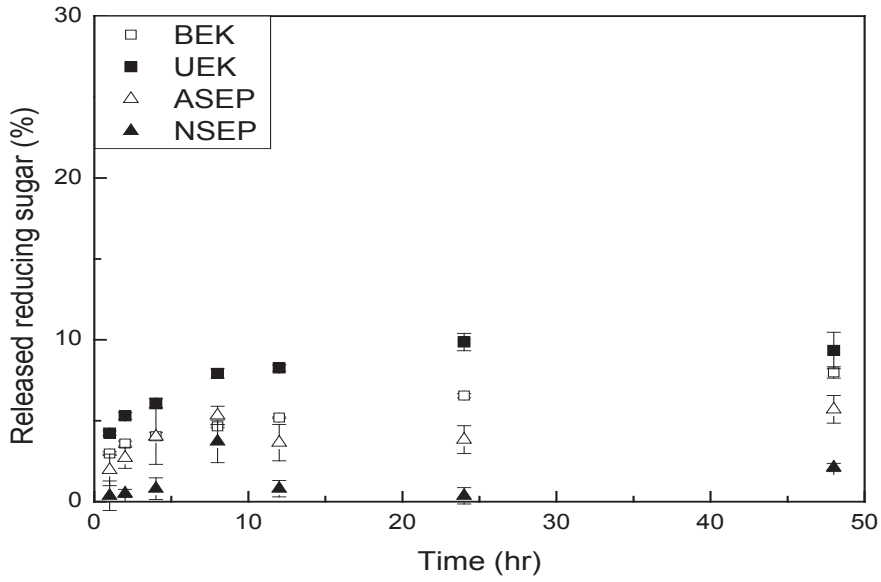


Figure 9. 40ºC hydrolysis of XylX-L2

Fig. 10 showed the 40°C adsorption of XylX-L2. All substrates had max adsorption in 1 hr and the adsorbed enzyme were stable during hydrolyzing 48 hr. The result of adsorption was different from above enzymes which BEK adsorbed more XylX-L2 than other substrates. Because the binding site of XylX-L2 had been deleted, its binding interaction between substrates had to investigate more detail to understand the phenomena.

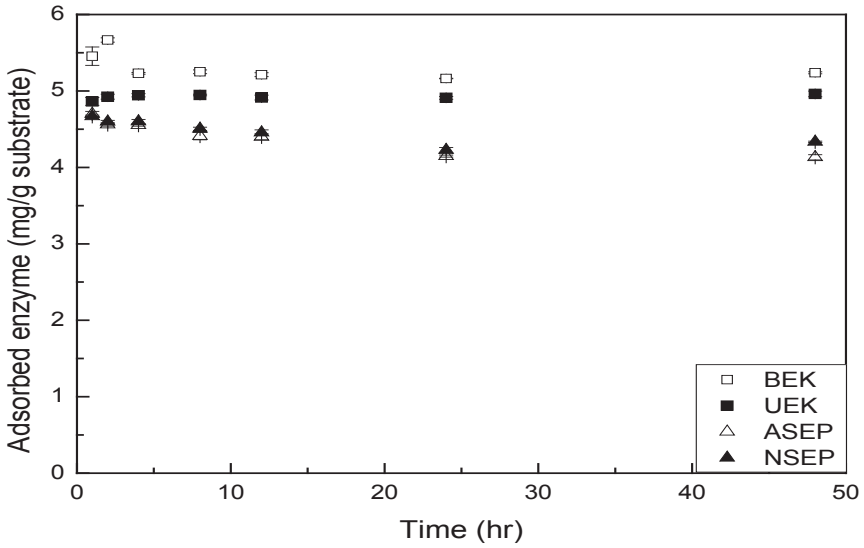


Figure 10. 40°C adsorption of XylX-L2

Figure 11 showed 40°C hydrolysis of CBM. Since CBM just had binding domain, it couldn't hydrolyze any polysaccharide. Its reducing sugar showed very little released or not detectable in most substrates.

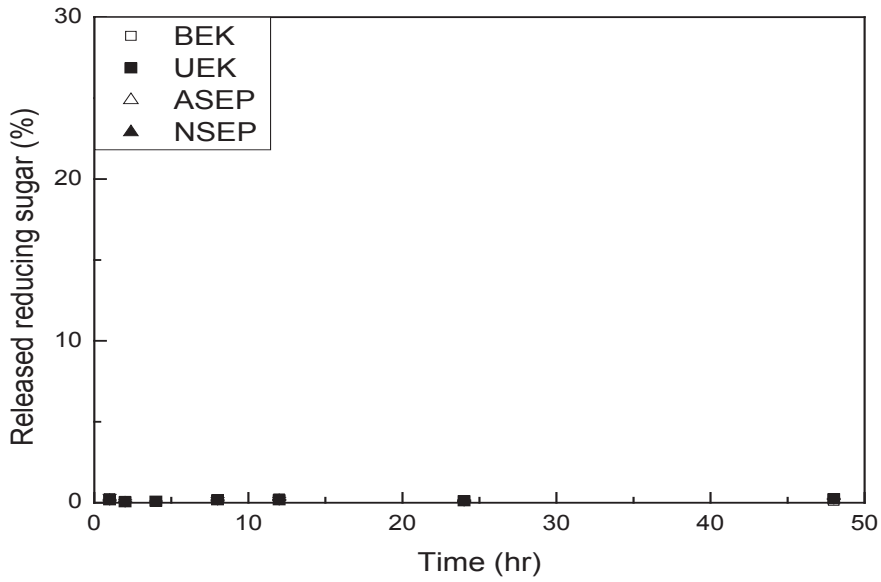


Figure 11. 40°C hydrolysis of CBM

Fig. 12 showed the 40°C adsorption of CBM. All substrates adsorbed CBM and reached maximum adsorption in 1 hr, which showed high affinity of CBM to all substrates. As time went on, BEK and UEK adsorbed more CBM but ASEP and NSEP had slightly desorption of CBM. BEK and UEK might had more crystalline zone for CBM which had more specific force to bind but ASEP and NSEP only had lignin that adsorbed enzyme by van der Waals force.

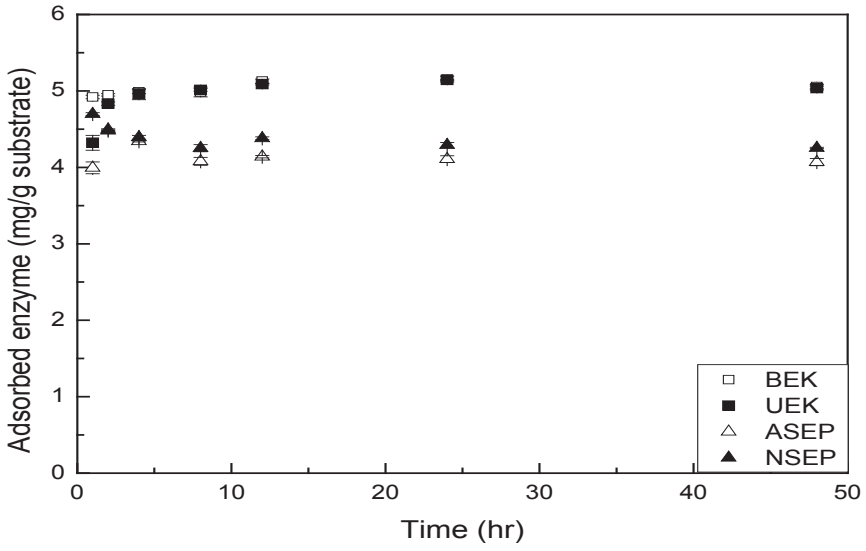


Figure 12. 40°C adsorption of CBM

Fig. 13 showed 40°C hydrolysis of GH 11. BEK and UEK released low reducing sugar about 12 μmole. The reducing sugar of BEK and UEK was less than Pulpzyme HC, XylX and XylX-H2 and came close to zero. The phenomenon was similar with XylX-L2 which binding domain had deficiency and let it released less reducing sugar. Thus, it was reasonably because GH 11 just had catalytic domain without binding domain. ASEP and NSEP still had little reducing sugar released.

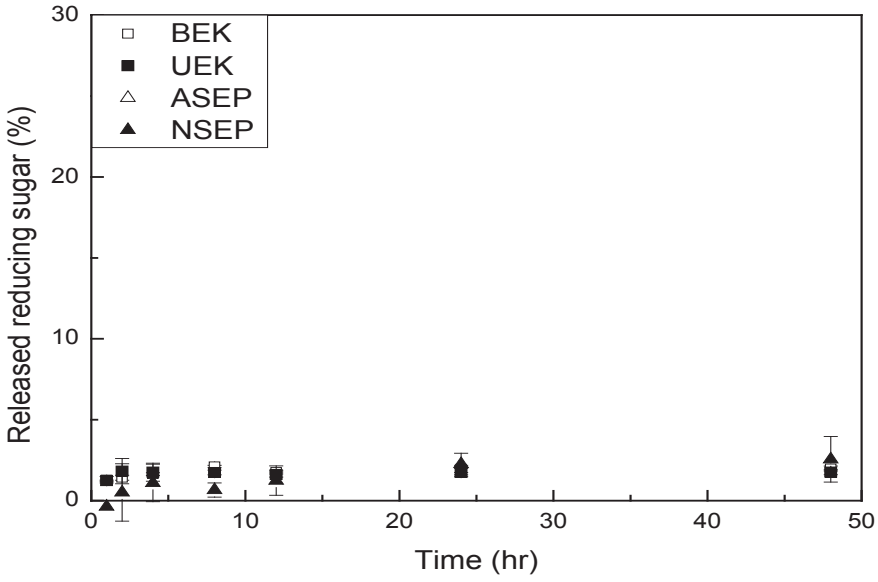


Figure 13. 40°C hydrolysis of GH 11

Fig. 14 showed the 40°C adsorption of GH 11. All substrates had reached maximum adsorption in 1 hr. In addition to UEK, other substrates had slight desorption and the adsorption of BEK increased from 1 to 8 hr and decreased after that. Although all substrates adsorbed more GH 11 than Pulpzyme HC and XylX, the binding of GH 11 without binding domain was unstable for xylanase to hydrolyze xylan.

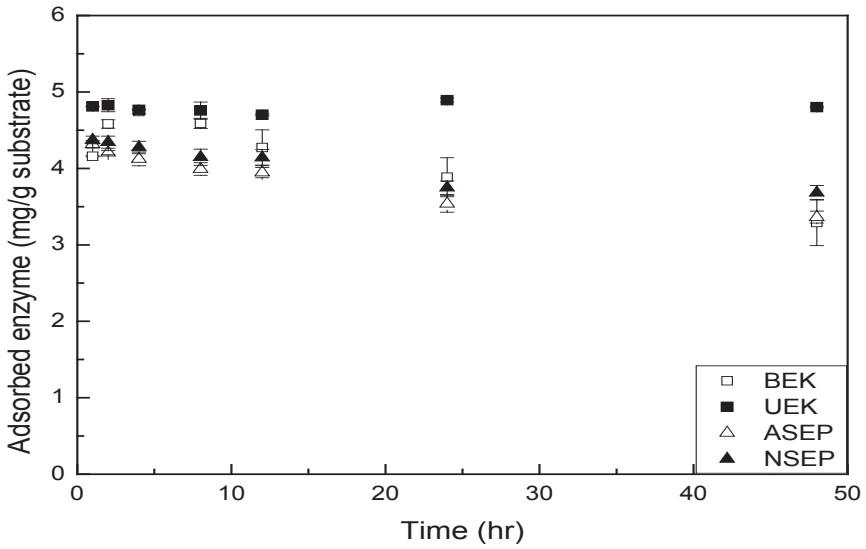


Figure 14. 40°C adsorption of GH 11

According to above results, BEK didn't fully adsorb Pulpzyme HC and XylX but the reducing sugar was equal to full adsorption of UEK. In substrates chemical composition, ASEP didn't have xylan and NSEP just had a little bit of xylan. ASEP and NSEP both adsorbed maximum enzymes and they didn't release reducing sugar. There were some non-specific of lignin which had influenced on enzyme adsorption so there was no direct

relationship between the amount of bound enzyme and the extent of hydrolysis. On the other hand, all substrates adsorbed more XylX-L2 which binding domain was partly deleted than XylX and Pulpzyme HC but the reducing sugar was released less than both of them. Moreover, GH 11 whose structure was only catalytic domain adsorbed on all substrates indeed and it just released a little bit reducing sugar during hydrolyzing 48 hr even less than XylX-L2. Furthermore, they were slightly desorbed from substrates, which might be unstable of enzyme adsorption to cause the low efficiency of enzyme hydrolysis. The binding domain of enzyme played an important role to make enzyme stable adsorption before hydrolysis. On 40^{o}C hydrolysis, alkali pretreated biomass like BEK and UEK released much more reducing sugar because the pretreatment removing lignin increased accessibility for xylanase to hydrolyze xylan.

4.3. 4°C enzyme adsorption

4°C enzyme adsorption was an experiment that enzyme would not hydrolyze substrates where was a more stable environment for enzyme just attaching on substrates because the activation energy was not enough to make reaction start. Fig. 15 showed that Pulpzyme HC was adsorbed by all substrate. BEK adsorbed the least Pulpzyme HC and the others adsorbed similarly from 5 to 20 mg/g substrate of addition enzyme. In the other substrates, ASEP adsorption came to stable first almost at 60 mg/g substrate of addition enzyme. Initially, UEK had an upward trend and was stable in 70 mg/g substrate of addition enzyme. After trying higher enzyme concentration, UEK adsorption came to stable from 70 mg/g substrate of enzyme addition. Three lignin content substrates had adsorbed more lignin that could be explained as lignin had high affinity to xylanase. Moreover, UEK adsorbed more xylanase than ASEP and NSEP at high enzyme addition. It seemed that xylanase would be adsorbed by lignin first and it would adsorbed on xylan with specific bond after saturation of lignin adsorption.

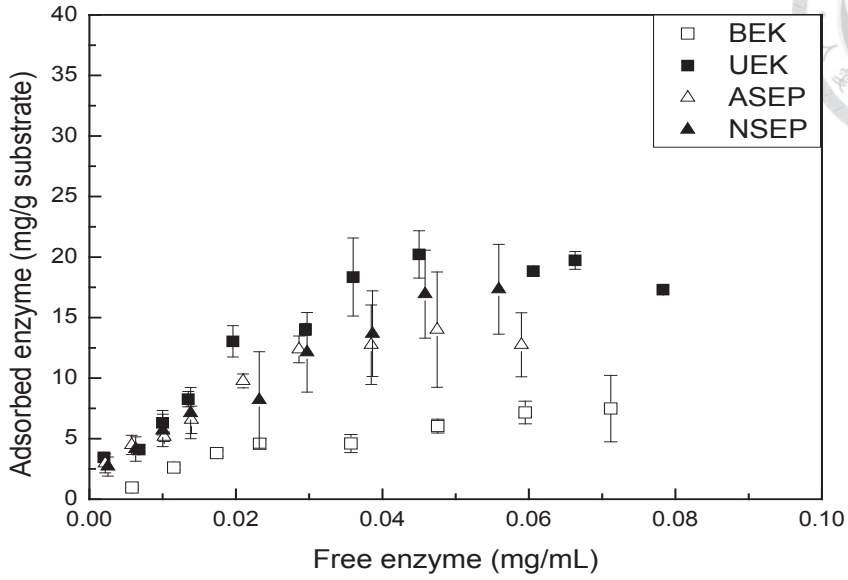


Figure 15. 4ºC adsorption of Pulpzyme HC

Fig. 16 showed the 4ºC adsorption of XylX. After enzyme addition came to 30 mg/g substrate, it was apparently that all substrates divided into two parts. One part included BEK and UEK which were pretreated by pulping process and another included ASEP and NSEP which were pretreated by steam explosion. Look back to the review of enzyme hydrolysis and adsorption, ASEP and NSEP which were high lignin content adsorbed more XylX than BEK and UEK. The adsorption of UEK was slightly higher than BEK but it was not so significant difference. Perhaps, UEK still had 3% lignin content which made it adsorbing enzyme easily. Furthermore, three substrates which had lignin content

adsorbed xylanase faster than BEK. It could be explained as lignin had high affinity with xylanase.

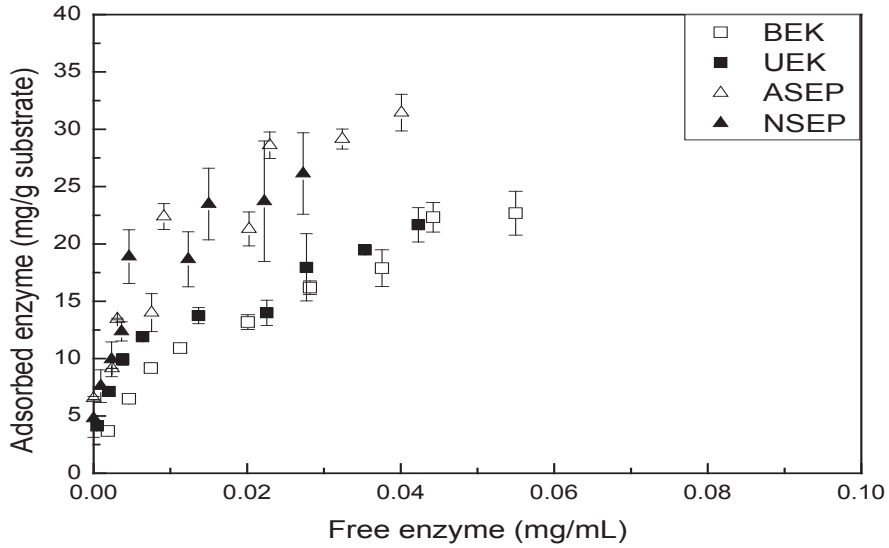


Figure 16. 4ºC adsorption of XylX

Fig. 17 showed 4ºC adsorption of XylX-H2. NSEP could adsorb the most XylX-H2 and it came to stable at 60 mg/g substrate of enzyme addition. The adsorption of BEK and UEK was similar they came to stable at 12mg/g substrate. Furthermore, the affinity of UEK to XylX-H2 was higher than BEK because the adsorption of UEK got higher before 60 mg/g substrate of addition enzyme. This phenomenon was similar with XylX adsorption. Although ASEP didn't adsorbed so much like NSEP, it also could see that adsorption of steam explosion pretreated substrates was higher than that of pulping process.

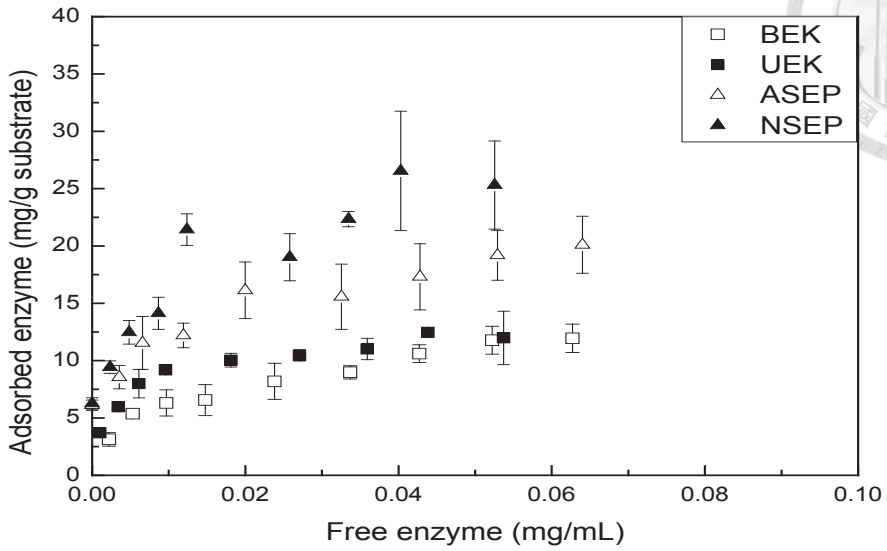


Figure 17. 4ºC adsorption of XylX-H2

Fig. 18 was 4ºC adsorption of XylX-L2. The adsorption of ASEP and NSEP was a part which had a significant rising before 40 mg/g substrate of addition enzyme and still rising after that. This phenomenon showed lignin had high non-specific adsorption with xylanase although its CBM had deficient. BEK adsorbed the least XylX-H2 and didn't more than 5 mg/g substrate. UEK adsorbed about 10 mg/g substrate and still higher than BEK did. BEK and UEK both decreased the adsorption capacity of xylanase because the CBM of XylX-L2 was deficient. Especially BEK, the adsorption of BEK was unstable even if the addition enzyme came to high concentration.

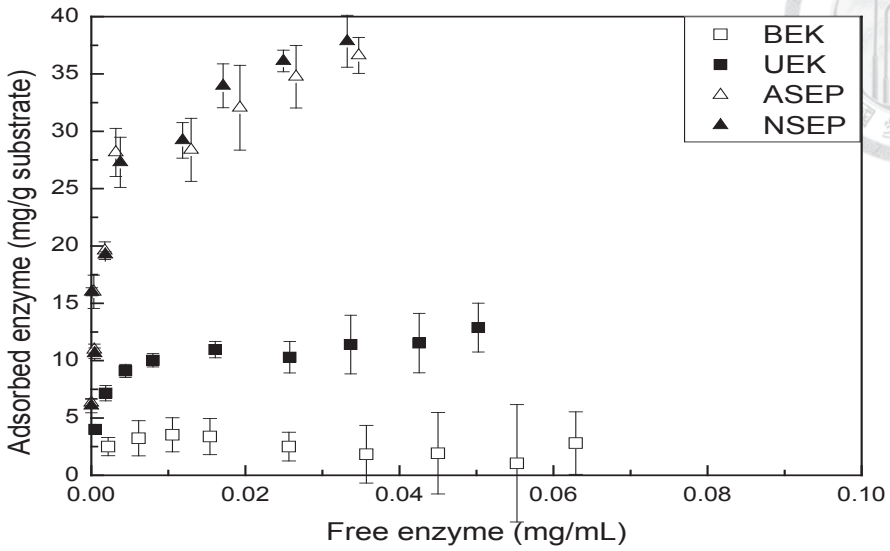


Figure 18. 4ºC adsorption of XylX-L2

Fig. 19 showed 4ºC adsorption of CBM. UEK, ASEP and NSEP adsorbed more CBM than BEK and their adsorption didn't have so much difference. Most of them came to stable after 40 mg/g substrate of addition enzyme. Although BEK adsorption was the least of all, its adsorption was stable when the addition enzyme was high concentration. It showed that CBM played an important role on specific adsorption from the adsorption of BEK.

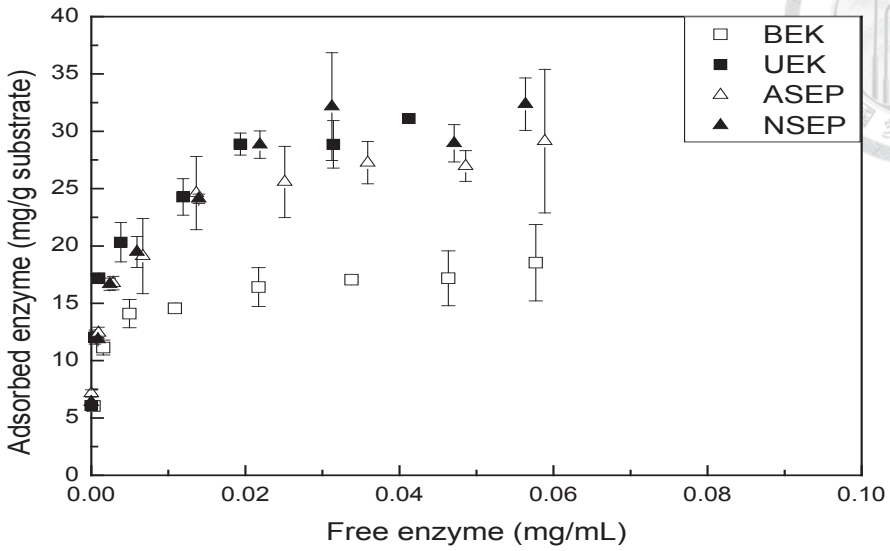


Figure 19. 4ºC adsorption of CBM

Fig. 20 showed that 4ºC adsorption of GH 11. All substrates divided into four parts but it was clearly that ASEP and NSEP adsorption were higher than BEK and UEK. ASEP showed a very rapid adsorption of GH11 and it seemed still rising after 70 mg/g substrate of addition enzyme. The adsorption of ASEP and NSEP seemed that they came to stable after high enzyme addition but that of UEK and BEK still rose linearly without a flat condition to say it was saturation and stable.

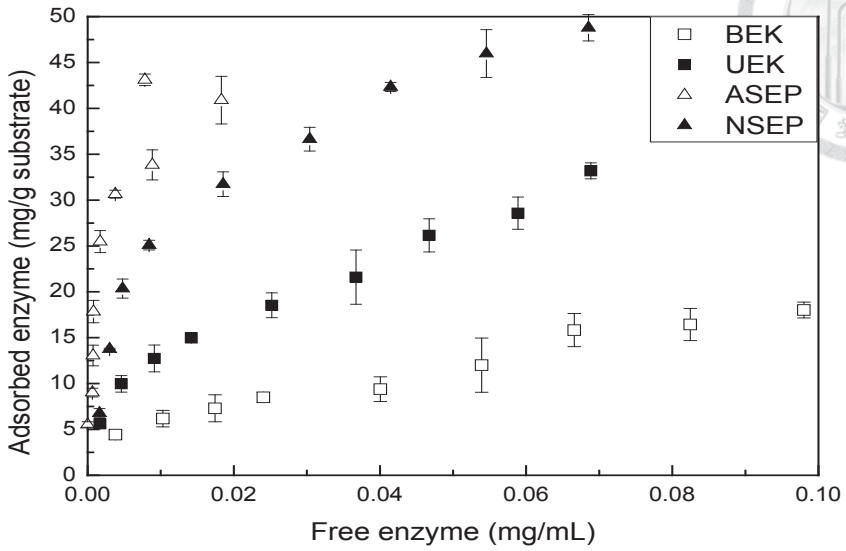


Figure 20. 4°C adsorption of GH 11

4.4. Parameters of 4°C enzyme adsorption

Parameters of 4°C enzyme adsorption were obtained by fitting Langmuir equation and all of them were R> 0.84.

Table 3. Maximum enzyme adsorption of all substrates at 4°C

MAX (σ, mg/g substrate)				
Enzymes	Substrates			
	BEK	UEK	ASEP	NSEP
Pulpzyme HC	11.91	26.69	22.54	36.90
XylX	24.31	20.87	30.70	26.53
XylX H2	13.24	12.39	20.50	27.52
XylX L2	3.67	11.80	33.76	36.31
CBM	17.20	30.61	27.95	31.57
GH 11	25.20	39.30	45.18	52.60

From Table 3, BEK adsorption was the lowest to most xylanase except for XylX and XylX-H2. Although BEK maximum adsorption on XylX and XylX-H2 was higher than UEK on them, the maximum adsorption of XylX and XylX-H2 were not significant difference between UEK and BEK. On the other hand, all substrates could also be divided into two parts that acid pretreated and alkali pretreated substrates. Comparing to BEK and UEK, ASEP and NSEP both had higher maximum adsorption to most xylanase and sometimes it even came to almost two times of BEK and UEK. However, the adsorption of Pulpzyme HC and CBM on UEK was similar with ASEP and NSEP. Perhaps, the content of xylan was also one of important factors in these two xylanase.

Table 4. Equilibrium constant of enzyme adsorption at 4°C

Enzymes	Equilibrium constant (K_d, g/L)			
	Substrates			
	BEK	UEK	ASEP	NSEP
Pulpzyme HC	0.0435	0.0242	0.0280	0.0617
XylX	0.0135	0.0046	0.0033	0.0023
XylX H2	0.0110	0.0033	0.0060	0.0064
XylX L2	0.0077	0.0012	0.0007	0.0012
CBM	0.0008	0.0009	0.0017	0.0023
GH 11	0.0460	0.0220	0.0016	0.0096

Table 4 showed equilibrium constant of enzyme adsorption, which meant the affinity of interaction between enzyme and substrate. From the Langmuir equation, the less value K_d was, the easier enzyme was adsorbed. Besides, in order to be more convenient to compare, we showed the reciprocal of K_d as affinity (A) in Table 5.

Table 5. Affinity of enzyme adsorption at 4°C

Enzymes	Affinity (A, L/g)			
	Substrates			
	BEK	UEK	ASEP	NSEP
Pulpzyme HC	22.99	41.32	35.71	16.21
XylX	74.07	217.39	303.03	434.78
XylX H2	90.91	303.03	166.67	156.25
XylX L2	129.87	833.33	1428.57	833.33
CBM	1250.00	1111.11	588.24	434.78
GH 11	21.74	45.45	625.00	104.17

To Pulpzyme HC, UEK was the highest affinity and NSEP was the lowest. To XylX, NSEP had the highest affinity and then was ASEP, UEK and BEK. To XylX-H2, the affinity of UEK was best and that of BEK was lowest. The affinity of ASEP and NSEP were 166.67 and 156.25 that were not significant difference. To XylX-L2, ASEP had the highest affinity and the affinity of UEK was equal to that of NSEP. To CBM, the affinity of BEK was 12500 which was the highest of all substrates and ASEP and NSEP were lower than BEK and UEK. To GH 11, the affinity of ASEP was the highest and BEK was the lowest. Besides, there was a trend that high lignin content substrates was higher affinity to xylanase generally. The trend of CBM affinity was reverse to other enzymes because carbohydrate binding domain had higher affinity to crystalline region. BEK was treated by pulping and bleaching process which made most lignin and xylan removed. Thus, the crystalline region of BEK exposed to surface easily and was attached by CBM. Compared to the maximum adsorption, the substrates which had higher affinity didn't mean it could adsorbed more enzyme. On Pulpzyme HC, NSEP had the highest maximum adsorption but its affinity was lower than other substrates that maximum adsorption were lower than it. There was the same phenomenon on the other substrates where maximum enzyme adsorption was not proportional to its affinity.

Table 6. Binding strength of enzyme adsorption at 4°C

Enzymes	Binding strength (σ × A, mL/g substrate)			
	Substrates			
	BEK	UEK	ASEP	NSEP
Pulpzyme HC	254.17	341.23	805.00	598.06
XylX	1800.74	4536.96	9303.03	11534.78
XylX H2	1203.64	1208.33	3416.67	4300.00
XylX L2	476.62	9833.33	48228.57	30258.33
CBM	21500.00	34011.11	16441.18	13726.09
GH 11	547.83	1786.36	28237.50	5479.17

Table 6 showed the binding strength of all enzymes. Binding strength which was the value of maximum enzyme adsorption multiplying with the value of affinity presented which substrate had more influence on hydrolysis (Kumar and Wyman, 2009b). In the Table 6, the value of ASEP and NSEP were larger than BEK and UEK generally. UEK was larger than BEK a lot and only XylX-H2 without significant difference. The binding strength which was different from other parameters such as maximum adsorption and affinity had a significant trend. It was apparently that acid pretreated substrates had higher binding strength than alkali pulping process substrates.

5. Conclusion

The composition of alkali pretreated substrates removed most of lignin and contained more carbohydrate in substrates. The acid steam explosion pretreated substrates removed most of hemicellulose and contained more lignin in substrates.

From the results of 40ºC adsorption and hydrolysis, high lignin content substrates adsorbed more xylanase and released little reducing sugar. Low lignin content substrates adsorbed less xylanase and released more reducing sugar than them. The lignin content was an important factor on enzyme adsorption and hydrolysis. Furthermore, carbohydrate binding domain had an influence on hydrolysis of substrates. The reducing sugar of XylX-L2 whose carbohydrate binding domain had deficiency was less than XylX and XylX-H2 whose carbohydrate binding domain was complete. The reducing sugar of GH 11 which only had catalytic domain was the least of all.

From 4ºC adsorption, steam explosion pretreated substrates adsorbed more enzyme than alkali pretreated substrates did. In alkali pretreated substrates, UEK maximum adsorption was higher than BEK except that XylX and XylX-H2 had no significant difference.

The parameters of 4ºC adsorption, the maximum adsorption of xylanase didn't correlate with hydrolysis and affinity. Because there was unspecific binding with lignin, steam explosion pretreated substrtaes had better adsorption and affinity of xylanases.

Binding strength of BEK showed that xylanase with carbohydrate binding domain had strong binding. The other substrates showed lignin also had significant influence because the binding strength was the value of maximum adsorption multiplying with affinity.

6. References

Balat M. 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Conversion and Management. 52: 858-875.

Fengel D. and Wegener G. 1984. Wood: Chemistry, Ultrastructure, Reactions. De Gruyter, Berlin. pp 613.

Hamelinck C. N., van Hooijdonk G. and Faaij A. P. C. 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass Bioenergy. 28:384-410.

Heiss-Blanquet S., Zheng D., Ferreira N. L., Lapierre C. and Baumberger S. 2011. Effect of pretreatment and enzymatic hydrolysis of wheat straw on cell wall composition, hydrophobicity and cellulase adsorption. Bioresource Technology. 102: 5938-5946.

Jeoh T., Ishizawa C. I., Davis M. F., Himmel M. E., Adney W. S. and Johnson D. K. 2007. Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. Biotechnology and Bioengineering. 98(1): 112-122.

Ko C. H., Chen W. L., Tsai C. H., Jane W. N., Liu C. C. and Tu J. 2007. *Paenibacillus campinasensis* B11: A wood material – utilizing bacterial strain isolated from black liquor. Bioresourse Technology. 98: 2727-2733.

Ko C. H., Tsai C. H., Tu J., Lee H. Y., Ku L. T., Kuo P. A. and Lai Y. K. 2010. Molecular cloning and characterization of a novel thermostable xylanase from *Paenibacillus campinasensis* B11. Process Biochemistry. 45: 1638-1644.

Kumar R. and Wyman C. E. 2008. An improved method to directly estimate cellulose adsorption on biomass solids. Enzyme and Microbial Technology. 42(5): 426-433.

Kumar R. and Wyman C. E. 2009a. Cellulase adsorption and relationship to features of corn stover solids produced by leading pretreatment. Biotechnology and bioengineering. 103(2): 252-267.

Kumar R. and Wyman C. E. 2009b. Access of Cellulase to Cellulose and Lignin for Poplar Solids Produced by Leading Pretreatment Technologies. Biotechnology Progress. 25(3): 807-819.

Laureano-Perez L., Teymouri F., Alizadeh H. and Dale B.E. 2005. Understanding factors that limit enzymatic hydrolysis of biomass. Applied Biochemistry and Biotechnology. 121-124:1081-1100.

Lynd L. R. Weimer P. J., van Zyl W. H. and Pretorius I. S. 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiology and Molecular Biology Reviews. 66(3): 506-577.

Mansfield S. D., Mooney C. and Saddler J. N. 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnology Progress. 15: 804-

816.

Palonen H., Tjerneld F., Zacchi G. and Tenkanen M. 2004. Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. Journal of Biotechnology. 107: 65-72.

Ryu K. and Kim Y. 1998. Adsorption of a xylanase purified from Pulpzyme HC onto alkali-lignin and crystalline cellulose. Biotechnology Letters. 20(10): 987-990.

Sims R. E. H., Mabee W., Saddler J. N. and Taylor M. 2010. An overview of second generation biofuel technologies. Bioresource Technology. 101: 1570-1580.

Taherzadeh M. J. and Karimi K. 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. International Journal of Molecular Sciences. 9: 1621-51.

Taherzadeh M. J. and Karimi K. 2007. Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review. BioResources. 2: 472-99.

Tenkanen M., Buchert J. and Viikari L. 1995. Binding of hemicellulases on isolated polysaccharide substrates. Enzyme and Microbial Technology. 17: 499-505.

Tsai H. P. 2012. Effect of Diiferent Biomass on Adsorption and Hydrolysis with Complex Enzyme. School of Forestry and Resource Conservation College of Bioresources and Agriculture National Taiwan University. pp 82.

Várnai A., Viikari L., Marjamaa K. and Siika-aho M. 2011. Adsorption of

monocomponent enzymes in enzyme mixture analyzed quantitatively during hydrolysis of lignocellulose substrates. Bioresource Technology. 102: 1220-1227.

Wang Y. S. 2013. Structural investigation for carbohydrate-binding module of Paenibacillus campinasensis BL11 xylanase XylX. School of Forestry and Resource Conservation College of Bioresources and Agriculture National Taiwan University. pp 57.

Yang B. and Wyman C. E. 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnology and Bioengineering. 86(1): 88-95.

Zillox C. and Debeire P. 1998. Hydrolysis of wheat straw by a thermostable endoxylanase L Adsorption and kinetic studies. Enzyme and Microbial Technology.