CHAPTER VI

EVALUATION OF HIGHLY EFFICIENT MONOMERIC SUGAR YIELD FROM THAI TIGER GRASS (*THYSANOLAENA MAXIMA*)

6.1 Abstract

Tiger grass (*T. maxima*) is considered as an important perennial energy crop in Southeast Asia with a high productivity and a low requirement for fertilizer. The monomeric sugar yield from *T. maxima* by two-stage microwave/chemical pretreatment and enzymatic hydrolysis is evaluated. The optimum conditions of the pretreatment were investigated by varying reaction time, reaction temperature, and chemical concentration to maximize the amount of the obtained monomeric sugar. The *T. maxima* was treated with microwave-assisted NaOH pretreatment using 15:1 liquid-to-solid ratio (LSR), 1 % (w/v) NaOH at 140 °C for 15 min, followed by microwave-assisted H_2SO_4 pretreatment using 15:1 LSR, 0.5 % (w/v) H_2SO_4 at 200 °C for 5 min. The maximum monomeric sugar released was 30.2 g/100g of NaOH-pretreated solids. The enzymatic hydrolysis of the microwave/chemical pretreated *T. maxima* at pH 4.8, 45 °C for 120 h using enzyme amount of 160 µl/g biomass produced an impressive maximum sugar yield of 110.4 g/100g of NaOH-pretreated solids.

(Keywords: Lignocellulosic biomass; *Thysanolaena maxima*; Two-stage pretreatment; *Trichoderma reesei*; *Trichoderma viride*)

6.2 Introduction

The world population has grown rapidly and more countries have been industrial prosperity, energy consumption has thus increased inevitably. Bioethanol has recently become an alternative fuel to solve these problems. Moreover, many technologies in ethanol production have been extensively studied. (Zaldivar *et al.*, 2001). Bioethanol, one of the clean liquid fuels, can be produced through fermentation of sugar or simple starch, such as sugarcane, corn etc. (Kataria and Ghosh, 2011). However, the dramatic increase in bioethanol production using the cornstarch-based technology is not practical due to its competition with human and animal food chains (Sun and Cheng, 2002). Among potential alternative bioresources, lignocellulosic biomass is a promising alternative source for the production of bioethanol because of its relatively low cost, great abundance, and sustainable supply. Lignocellulosic biomass mainly consists of cellulose, hemicelluloses and lignin. These polysaccharides can be depolymerized to monosaccharide then sequentially converted to bioethanol (Hsu *et al.*, 2010).

Bioethanol production using lignocelluloses involves three major steps, viz., pretreatment of substrate, hydrolysis of cellulose to fermentable reducing sugars, and fermentation of sugar to ethanol (Sun and Cheng, 2002). The major problem of bioethanol production from lignocellulosic biomass is the hemicelluloses-lignin matrix and cystallinity of cellulose reducing the efficiency of cellulase during hydrolysis. (Zhu *et al.*, 2008; Laureano-Perez *et al.*, 2005).

Efficient pretreatment and hydrolysis technologies are essential steps in achieving a promising fermentable sugar production. The purposes of pretreatment are to break down the lignin structure, disrupt the crystalline structure of cellulose, and increase surface area for enhancing enzyme accessibility during the hydrolysis step (Mosier *et al.*, 2005). Two-stage microwave/chemical pretreatment was introduced as a potential pretreatment to degrade lignin and hemicelluloses and to release high monomeric sugar yields

using high heating efficiency and short reaction time (Boonmanumsin *et al.*, 2012).

The hydrolysis process using enzymes represents the most effective method to release fermentable sugars from cellulosic materials. The process is usually conducted under mild conditions and does not cause a corrosion problem (Lin *et al.*, 2010). Among various capable enzymes, *Trichoderma* has been extensively studied for cellulase production. Mutant strains of *Trichoderma* sp. (*T.viride*, *T.reesei*, and *T.longibrachiatum*) have been considered to be the most productive and powerful destroyers of the crystalline structure of cellulose (Szczodrak *et al.*, 1988).

Thysanolaena maxima (*T.maxima*), popularly known as 'tiger' or 'broom' grass, is a perennial grass, making it a renewable source that is available in large quantities. The culms of *T.maxima* are solid, smooth, rounded, up to 25 mm in diameter and 3-4 m in height. The leaves of the grass are used as cattle feed. The panicles are terminal, bushy, foxtail-like, and are used for making brooms. The stem portion is usually considered waste (Saikia *et al.*, 1992). Conversion of the stem of *T.maxima* into bioethanol would be a great practice for not only waste management, but also overcoming the energy problem in Thailand. Schematic flowsheet for the conversion of *T. maxima* into bioethanol is shown in Figure 6.1.

The objectives of this work are to optimize two-stage microwave/chemical pretreatment by varying reaction temperatures, reaction times, chemical concentrations, and types of chemicals in order to achieve maximum monomeric sugars from *T.maxima*. The influence of physical parameters, such as temperature, time, pH, enzyme loading, and types of enzymes on enzymatic hydrolysis was investigated to obtain the highest fermentable sugar yields. The structural changes of *T.maxima* were characterized using Fourier transform infrared spectroscopy (FTIR) and Scanning electron microscope (SEM).

6.3 Experimental

6.3.1 Materials and Chemicals

T.maxima was obtained from Lamphun Province, Thailand. Prior to any pretreatment, *T.maxima* was air- and sun-dried before being chopped into small pieces. The dried grass was then milled to obtain 60 mesh-size powders and stored in a sealed plastic bag at room temperature. Sodium hydroxide (NaOH, Labscan Asia Co., Thailand), calcium hydroxide ($Ca(OH)_2$, Sigma Aldrich Chemical Co., USA), sulfuric acid (H_2SO_4, Merck Co., Germany), and phosphoric acid (H_3PO_4, Merck Co., Germany) were utilized in the pretreatment process. Fungal strain *Trichoderma reesei* ATCC 26921 and *Trichoderma viride* C1794 (Sigma Aldrich Chemical Co., USA) were used as sources of cellulase enzyme production. D-(+)-Glucose (G5400), D-(+)-Xylose (X3877), and D-(-)-Arabinose (A6085) standards (Sigma Aldrich Chemicals Co., USA) were used for sugar quantitative analysis. Acetic acid (CH_3COOH), 5-(hydroxymethyl) furfural, and furfural standards (Sigma Aldrich Chemicals Co., USA) were used for inhibitor quantitative analysis.

6.3.2 Chemical Composition Analysis

The concentrations of cellulose, hemicelluloses, lignin, acetic acid, 5-(hydroxymethyl) furfural, furfural, and ash of *T.maxima*, microwave/ NaOH-pretreated *T.maxima*, two-stage microwave/(dilute NaOH followed by dilute H_2SO_4)-pretreated *T.maxima*, and enzymatic hydrolyzed *T.maxima* were quantitatively analyzed using the National Renewable Energy Laboratory (NREL) analytic methods (Sluiter and Sluiter, 2011).

6.3.3 Microwave-assisted: Two-stage Microwave/Chemical Pretreatment Process

6.3.3.1 Microwave-assisted Dilute Sodium Hydroxide Pretreatment

Before any treatment, the mechanical pretreatment was used to reduce the particle size and crystallinity of lignocellulosic biomass in

order to increase surface area and reduce the degree of polymerization (Alvira *et al.*, 2010). The particle size of *T. maxima* after the mechanical pretreatment was analyzed by a particle size analyzer (Malvern/ Mastersizer X) equipped with a 300 mm lens size in the sample detection unit. In this study, the sample was milled to reduce the size and sieved at 60-mesh sieving size. The average sample size of *T. maxima* powders after milling and sieving was found to be about 300 μm.

Alkali pretreatment causes delignification of biomass and makes the lignocelluloses swollen through saponification reactions (Pederson and Meyer, 2010). The *T.maxima* powder was suspended in different concentrations of alkali solution (0.5–2 % (w/v)) for various times (5–60 min) using the optimal 15:1 LSR, as reported elsewhere (Boonmanumsin *et al.*, 2012). The mixture was stirred until homogeneous before being transferred to a Teflon vessel sealed with a Teflon cap. At the optimum alkali concentration and time, the microwave pretreatment was operated under various temperatures (60–180 °C) to obtain the optimum condition for the maximum monomeric sugars released. After the treatment, the mixture was cooled to room temperature and then filtered. The liquid fractions were collected for monomeric sugars analysis. The concentration of the monomeric sugars (glucose, xylose, and arabinose) was determined using high-performance liquid chromatography (HPLC) analysis. The HPLC system (RID-10A, Shimadzu Corp., Kyoto, Japan) was equipped with a refractive index detector, and Aminex-HPX 87H column (300 x 78mm, Bio-Rad Lab, USA) using the following conditions: 0.005 M of H_2SO_4 as the mobile phase, a flow rate of 0.60 ml/min, and 50 μl sample size. Each analysis was made in triplicate. The pretreated solids were neutralized, dried, and stored in a sealed plastic bag for further pretreatment studies.

6.3.3.2 Microwave-assisted Dilute Acid Pretreatment

The objective of the dilute acid pretreatment is to solubilize the hemicellulosic fraction of the biomass and to make the cellulose more accessible to enzymes (Hendriks and Zeeman, 2009). The pretreated solid from the microwave/alkali pretreatment was treated with microwave/dilute H_2SO_4. The alkaline-pretreated *T.maxima* powder was mixed with dilute H_2SO_4

solution (0.5 % (w/v)) using 15:1 LSR. The pretreatment temperature was varied within the range of 120–200 °C, with a reaction time varying from 5 to 60 min to obtain the optimum condition. This optimal condition was then used to study a more suitable acid concentration by varying the H_2SO_4 solution (0.5–3 % (w/v)) to maximize the monomeric sugars released. According to the 2012 study of Boonmanumsin *et al.*, (2012) who obtained the maximum sugars when using H_3PO_4, the microwave/dilute H_3PO_4 pretreatment was thus performed using the optimum conditions obtained from the microwave/dilute H_2SO_4 pretreatment for comparison.

After the two-stage pretreatment process, the liquid fractions were collected for monomeric sugar analysis by HPLC. Each analysis was made in triplicate. The residues were kept in sealed bags for further characterization.

6.3.4 Enzymatic Hydrolysis

The two-stage pretreated hydrolysate was further enzymatically hydrolyzed and the degree of cellulose conversion determined. The hydrolysate from the two-stage pretreatment was left to cool at room temperature and the pH adjusted to 4.8 using 40% (w/v) NaOH. Cellulase, prepared from *T.reesei* ATCC 26921 (160 µl/1 g of pretreated solid), was then added into the hydrolysate. The enzymatic hydrolysis was performed at various temperatures (45–55 °C) for various times (12–168 h) to identify the optimum condition. This optimum condition was used to study the effects of pH (3.0–5.6), enzyme loading (40–280 µl), and strain type of *Trichoderma* sp. (*T.viride* and *T. reesei*) on the efficiency of the enzymatic hydrolysis. The sugar concentration obtained was analyzed by HPLC. Each analysis was made in triplicate.

6.3.5 Total Monomeric Sugar Analysis

Monosaccharides were measured using HPLC system with a pulsed refractive index detector. The standard sugars were used to calibrate the peak area of each component, using the following equations: y = (x+20447)/708234 for glucose, y = (x+2243)/672436 for xylose, and y = (x+1086)/672824 for arabinose

(y = sugar concentrations (g/l), x = peak area). The monomeric sugar yields in form of g/100 g biomass can be calculated from the following equation (Boonmanumsin *et al.*, 2012).

Monomeric sugar yields (g/100 g biomass)

= [Sugar concentration (g/l) x **V** (ml) x 100 g] / [1000 (ml) x **M** (g)]

Where **V** is volume of chemical added (ml) and **M** is the amount of biomass added (g) in the process.

6.3.6 Characterization

The chemical structures of original *T.maxima*, microwave/NaOH-pretreated *T.maxima*, two-stage microwave (dilute NaOH followed by dilute H_2SO_4)-pretreated *T.maxima*, and enzymatic hydrolyzed *T.maxima* were compared by using Fourier transform infrared spectrometer (FTIR, Nicolet nexus 670) in a range of 400-4000 cm^{-1} with a resolution of 1 cm^{-1} and 64 scans per sample. The physical structures of untreated, pretreated, and hydrolyzed *T.maxima* were obtained using Scanning electron microscope (SEM, Hitachi/S-4800) at 2 kV accelerating voltages.

6.4 Results and Discussion

6.4.1 Chemical Composition of *T.maxima*

The chemical composition of *T.maxima* was characterized by the National Renewable Energy Laboratory (NREL) methods (Sluiter and Sluiter, 2011). The result is summarized in Table 6.1, also showing the results obtained from the sample harvested in India (Palni *et al.*, 1994). The main sugars from this biomass contained celluloses (34.3 %) and hemicelluloses (26.9 %). The lignin content accounted for 10.8 %, which is relatively low when compared with *M.sinensis* (harvested from Cha-Chueng-Sao province, Thailand), which contained 17.4 % lignin (Boonmanumsin *et al.*, 2012). These main carbohydrates (cellulose and hemicelluloses) are the potential sugars in the pretreated substrates

(Mosier *et al.*, 2005). *T.maxima* contained less lignin content, which was expected to increase the hydrolysis rate.

6.4.2 Optimization of Microwave-assisted Alkali Pretreatment

6.4.2.1 Effects of Alkali Concentration and Time

NaOH is known as the appropriate alkaline pretreatment, causing swelling, increasing the internal surface of cellulose, and decreasing the degree of polymerization and crystallinity, thus accelerating lignin structure disruption (Pederson and Meyer, 2010). In this study, the pretreatment was investigated using various alkali concentrations (0.5–2.0 % (w/v)) for 5–60 min, at 120 °C and 15:1 LSR. The maximum yields of monomeric sugars from *T.maxima* were 4.8 g/100 g biomass at 1.0 % (w/v) NaOH for 15 min (Figure 6.2). The monomeric sugar yields increased with increases in NaOH concentration and time because of the higher solubilization of lignin in the NaOH solution. In addition, the microwave irradiation enhanced the solubilization of hemicelluloses in the NaOH solution, which led to a closer contact between lignin and the NaOH solution, resulting in higher lignin solubilization (Zhu *et al.*, 2005). However, the monomeric sugar yields obtained were degraded with increases in NaOH loading and reaction time (Dalgaard *et al.*, 2006). The decreasing trend in the monomeric sugar yields was observed as alkali concentration increased.

6.4.2.2 Effect of Temperature

The effect of pretreatment temperature on *T.maxima* digestibility was studied in a range of 60–180 °C. The maximum yield of monomeric sugars released from *T.maxima* is 6.4 g/100 g biomass at 140 °C using 1.0 % (w/v) NaOH and 15:1 LSR for 15 min as shown in Figure 6.3. At lower temperatures of 60–140 °C, the increment of monomeric sugar yields with increases in the reaction temperature was caused by the disruption of the crystalline structural of cellulose (Hu and Wen, 2008). However, the decrease in monomeric sugar yields at higher temperatures (160–180 °C) is probably due to the degradation process (Lu and Mosier, 2007).

6.4.3 Optimization of Two-stage Pretreatment (Microwave/Dilute NaOH, Followed by Microwave/Dilute H_2SO_4 Pretreatment)

To enhance a more efficient removal of hemicelluloses and lignin and a better biomass digestibility, two-stage pretreatment was employed, as also stated elsewhere (Agbor *et al.*, 2011; Boonmanumsin *et al.*, 2012).

6.4.3.1 Effects of Time and Temperature

Dilute acid pretreatment is known to have the potential to effectively solubilize hemicelluloses and reduce cellulose crystallinity. In addition, dilute acid pretreatment is also a reliable technology on a commercial scale (Hendriks and Zeeman, 2009). High yields of hydrolysis were reported when lignocellulosic biomass was pretreated with dilute H_2SO_4 (Alvira *et al.*, 2010). To study the effects of temperature and time, microwave/ NaOH-pretreated *T.maxima* was treated with microwave/0.5 % (w/v) H_2SO_4 using 15:1 LSR. The temperature range studied was 120–200 °C for various times of 5–60 min. The results exhibited a total sugar yield from the microwave/NaOH-pretreated *T.maxima* of 30.2 g/100 g NaOH-pretreated solids with the optimal conditions of 200 °C for 5 min (Figure 6.4). In Figures 6.4a-b, the xylose yield increases the temperature is raised from 120–160 °C. When the temperature increases to a certain degree, the hemicelluloses begin to solubilize (Garrote *et al.*, 1999). The results confirmed that hemicelluloses are effectively hydrolyzed under mild conditions because hemicelluloses have a random amorphous structure with little strength and are thus easily hydrolyzed to sugars (Lin *et al.*, 2010). However, the reduction of xylose and arabinose occurred at severe temperatures (200 °C) due to sugar degradation. At 200 °C, glucose was released from microwave/ H_2SO_4-pretreated *T.maxima*. The cellulose molecule is a very long polymer of glucose units without branches, causing high crystallinity and a strong resistance to hydrolysis (Lin *et al.*, 2010). Thus, more severe conditions are required for releasing glucose, but an excessive increase in temperature and time can lead to a degradation of glucose, as shown in Figure 6.4c (Hu and Wen, 2008). Too-severe pretreatment conditions especially promote the formation of phenolic and heterocyclic compounds like furfural and HMF (Negro *et al.*, 2003). Figure 6.4d compares all temperatures

studied for various times using the two-stage pretreatment. The optimum conditions of the two-stage microwave/chemical pretreatment of *T.maxima* were obtained by considering the maximum monomeric sugar yields.

6.4.3.2 Effect of Acid Concentration

Microwave/NaOH-pretreated *T.maxima* were pretreated using various concentrations of H_2SO_4 (0.5–3 % (w/v)), 15:1 LSR at 200 °C for 5 min and the results are shown in Figure 6.5a. The monomeric sugar yields decrease with increases in H_2SO_4 concentrations because strong acidic conditions cause the degradation of sugar (Pedersen and Meyer, 2010). Therefore, the maximum monomeric sugar yields obtained from microwave/NaOH-pretreated *T.maxima* were 30.2 g/100g NaOH-pretreated solids using 0.5 % (w/v) H_2SO_4. The amount of glucose released from the microwave/NaOH-pretreated *T.maxima* was 26.9 g/100 g NaOH-pretreated solids. Glucose is the preferred sugar due to the ease of bioethanol conversion (Mosier *et al.*, 2005).

6.4.3.3 Effect of Acid Type

The optimum conditions from the H_2SO_4 pretreatment were used to study the effect of acid type, to compare between H_2SO_4 and H_3PO_4, as shown in Figure 6.5b. Clearly, the total monomeric sugar yield from the H_2SO_4 pretreatment (30.4 g/100 g NaOH-pretreated solids) was much higher than H_3PO_4 pretreatment (19.5 g/100 g NaOH-pretreated solids). The type of acid influenced the monomeric sugar released during the pretreatment. The dilute H_2SO_4 pretreatment could achieve higher reaction rate and significantly improve cellulose hydrolysis (Pedersen and Meyer, 2010). Hsu *et al.* (2010) also used H_2SO_4 (1 %) to treat rice straw at 180 °C for 2 min and obtained the maximum sugar yields of 19.6 g/100 g rice straw.

6.4.4 Optimization of Enzymatic Hydrolysis

To maximize the sugar yield from two-stage pretreated *T. Maxima*, the basic hydrolytic variables of reaction temperature, reaction time, pH, enzyme loading, and type of enzyme were investigated.

6.4.4.1 Effect of Temperature and Time

Most cellulase enzymes show an optimum activity at temperatures in the range of 45°–55 °C (Galbe and Zacchi, 2002). In this study, three different temperatures, viz., 45°, 50°, and 55 °C, were carried out. Influence of temperature and time on enzymatic hydrolysis by *T.reesei* ATCC 26921 was shown in Figure 6.6. The results indicate that the maximum monomeric sugar yields at the optimum temperature and time for enzymatic hydrolysis was 103.8 g/100 g NaOH-pretreated solids at 45 °C for 120 h, pH 4.8 using 160 µl of enzyme loading. At 45 °C, the monomeric sugar yields increased gradually from 12 h and reached the maximum after 120 h (see Figure 6.6a). At 50° and 55 °C, the monomeric sugar yield reached maximum at 36 h, as presented in Figures 6.6 b, c. The monomeric sugar yields became better as reaction time increased. At optimum conditions, the monomeric sugar yield liberated was stable (Bura *et al.*, 2009). Many researchers have reported different optimal temperatures for maximum monomeric sugar yields, depending on the strain variation of *Trichoderma* sp. (Kiranmayi *et al.*, 2012).

6.4.4.2 Effect of pH

Commercially, *T.reesei* illustrates the optimum activity over a pH range from 4 to 6 and the enzymes are inactive in the alkali pH range (Wang *et al.*, 2005). The influence of pH on enzymatic hydrolysis was studied at different pHs between 3.0 and 5.6. The maximum monomeric sugar yield in Figure 6.7a of enzymatic hydrolysis was observed at pH 4.8 after 120 h at 45 °C reaction temperature using 160 µl of *T.reesei* ATCC 26921 loading. There was a significant reduction of the monomeric sugar yield when increasing pH from 5.0 to 5.6. Gomes *et al.* (2006) reported the maximum cellulase activity of *Trichoderma* sp SUK-3 at pH between 4.5 and 5.0. In our case, the optimum production using *T.reesei* cellulase is at pH 4.8 (Zhu *et al.*, 2005).

6.4.4.3 Effect of Enzyme Loading

The presence of an adequate amount of enzyme is also an essential to achieve high monomeric sugar yields (Sun and Cheng, 2002). The hydrolysate was hydrolyzed using various enzyme loadings (40–280 µl) at pH 4.8,

45 °C for 120 h. The results in Fig. 6.7b show that the monomeric sugar yield increases with the enzyme loading because increasing the dosage of enzyme in the reaction enhances the yield and rate of the hydrolysis (Sun and Cheng, 2002). However, the enzyme loading above 160 µl slightly affects the monomeric sugar released. Thus, the maximum monomeric sugar yield from hydrolysis of *T.maxima* by *T.reesei* ATCC 26921 was 103.8 g/100g NaOH-pretreated solids at pH 4.8, 45 °C for 120 h using 160 µl of enzyme loading.

6.4.4.4 Effect of Type of Enzyme

To further study whether the enzyme type has influence on the released monomeric sugars, as indicated elsewhere (Jackson *et al.*, 1991), *T.viride* was studied using the optimum conditions from the enzymatic hydrolysis by *T.reesei* ATCC 26921. It was found that (see Figure 6.7c) at pH 4.8, 45 °C for 120 h using 160 µl of enzyme loading the maximum monomeric sugars released by *T.viride* was higher (110.4 g/100 g NaOH-pretreated solids). This result might be due to *T.viride's* tolerance and resistance to inhibitors and other toxic compounds (Jackson *et al.*, 1991). Moreover, *T.viride* T 100-14 has a relatively high specific enzymatic activity and brings progress in the conversion of the cellulose to glucose (Zhou *et al.*, 2008). Therefore, the maximum monomeric sugar yields from enzymatic hydrolysis of *T.maxima* were 110.4 g/100g NaOH-pretreated solids, at pH 4.8, 45 °C for 120 h using 160 µl of enzyme loading. In comparison, Hu and Wen in 2008 reported that the maximum monomeric sugar yield after microwave-assisted alkali pretreatment and enzymatic hydrolysis of switchgrass was 58.7 g/100g biomass using 0.1 g NaOH/g biomass at 190 °C for 30 min. Nevertheless, the maximum yield of monomeric sugars from wheat straw using 0.75 % H_2SO_4 (v/v) pretreatment at 120 °C for 60 min, followed by enzymatic hydrolysis at pH 5.0, 45 °C for 72 h was 56.5 g/100g biomass (Saha *et al.*, 2005).

6.4.5 Effect of Pretreatment and Hydrolysis on Chemical Composition

The change in chemical composition of *T.maxima* is an important factor to determine the efficiency of the microwave/chemical pretreatment and enzymatic hydrolysis. The chemical composition of *T.maxima* treated with the microwave/NaOH, the two-stage microwave/chemical pretreatment, and enzymatic hydrolysis is summarized in Table 6.1.

The microwave-assisted NaOH pretreatment of *T.maxima* significantly improved the concentration of cellulose while the concentration of hemicelluloses and lignin were reduced due to the solubilization of hemicelluloses and lignin in NaOH (Zhu *et al.*, 2005). After the two-stage microwave/chemical pretreatment, the polysaccharide contents in *T.maxima* were dramatically reduced. The cellulose component that remained was 7.25 % because the pretreatment was conducted at 200 °C and the cellulose component was hydrolyzed, degraded, and/or burned.

Mansilla *et al.* in 1998 stated that the hemicelluloses degradation generated acetic acid and furfural at severe pretreatment conditions. Saha *et al.* in 2005 also reported that acetic acid and furfural formation increased with temperature and acid concentration. Surprisingly, in our case, only acetic acid concentration increased (from 0.75 % to 1.76 %) while no HMF and furfural were detected. The residues left from the enzymatic hydrolysis showed that the polysaccharide contents were reduced to 1.81 % and untraceable quantities of acetic acid, 5-(hydroxymethyl) furfural and furfural were produced. These results are consistent with those reported by Kataria and Ghosh (2011) who indicated that the enzymatic hydrolysis gave no inhibitory compounds production. As a result, the two-stage pretreatment and the enzymatic hydrolysis process successfully converted cellulose into monomeric sugars with no inhibitors, which are harmful to fermentative microorganisms.

6.4.6 Fourier Transform Infrared Spectrometer (FTIR) Analysis

FTIR spectroscopy was used to investigate the changes in the composition of *T.maxima* (Figure 6.8). After the microwave/NaOH pretreatment, an absorption peak at 1734 cm^{-1} referring to the ester linkage of C=O decreased because of the partial break of the linkage between hemicelluloses and lignin (Kumar *et al.*, 2009). The peaks around 1248 and 1510 cm^{-1} assigned to aromatic C-O and C=C bonds, respectively, of lignin were also diminished after the microwave/NaOH pretreatment, suggesting partial removal of the lignin. Moreover, other polysaccharide peaks (899, 1112, 1160 cm^{-1}) after the pretreatment process became sharper, as compared to those of the untreated raw material, due to the increase of polysaccharide content after the pretreatment. The spectra of the two-stage pretreatment and enzymatic hydrolysis were significantly changed when compared to the spectra of the untreated and the microwave/NaOH-pretreated samples. Those polysaccharide peaks disappeared due to the polysaccharides hydrolysis and/or degradation (Wang *et al.*, 2010).

6.4.7 Scanning Electron Microscopy (SEM) Characterization

The SEM micrographs of untreated, microwave-assisted NaOH pretreated, two-stage pretreated, and enzymatic hydrolyzed *T.maxima* identified the change of structural appearance. The untreated *T.maxima* especially showed a fibril structure covered with a flat, smooth, thin film which is normally found in biomass (Hu and Wen, 2008). After the microwave-assisted NaOH pretreatment, the thin film on the surface disappeared, but some fibril structure was still maintained. It is indicated that lignin was removed and cellulose and hemicelluloses were exposed (Hu and Wen, 2008). After the two-stage pretreatment, the fibril structure was disrupted by the acid pretreatment at high temperature. The dilute acid solubilized and broke hemicelluloses. The increased surface area thus enhances efficiency of the enzymatic hydrolysis (Hendriks and Zeeman, 2009). The SEM image of enzymatic hydrolyzed *T.maxima* showed a total destruction of fibril structure and the cellulose crystalline structure was not observed (Prasertwasu *et al.*, 2014), confirming the success of the two-stage

pretreatment and enzymatic hydrolysis on enhancement of the *T.maxima* digestibility.

6.5 Conclusions

T.maxima (Tiger grass) from Lumpoon Province, Thailand, is a potential lignocellulosic feedstock for bioethanol production using two-stage microwave/ chemical pretreatment and enzymatic hydrolysis. Under optimum conditions, the maximum monomeric sugar yields of 110.4 g/ 100 g NaOH-pretreated solids after the complete process. The two-stage pretreatment can be considered as an effective technique for lignin removal and lignocellulosic structure disruption, helping to promote enzymatic hydrolysis efficiency. Furthermore, the hydrolysis process using *T.viride*, is also an incredible process for preparing monomeric sugars, especially glucose.

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6.7 References

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Table 6.1 Chemical compositions of *T.maxima* solid residues from each stage

Composition (% Dry Matter)*	Tiger Grass (*T.maxima*)				
	Untreated (This study)	Untreated (Palni *et al.*, 1994)	Microwave/NaOH	Microwave/NaOH/ H_2SO_4	Microwave/NaOH/ H_2SO_4/Enzyme
Cellulose	34.33±0.44	30.2	77.17±0.82	7.25±0.13	1.81±0.24
Hemicellulose	26.92±0.40	29.6	16.33±1.28	2.41±1.47	1.48±0.45
Lignin	10.75±1.22	9.1	1.85±1.10	0.26±2.10	0.20±0.81
Ash	2.08±0.09	11.8	1.07±0.04	0.65±0.18	0.57±0.02
Acetic acid	-	-	0.75±0.47	1.76±0.11	ND
HMF	-	-	ND	ND	ND
Furfural	-	-	0.64±0.40	ND	ND

* = Composition percentages are on dry weight basis

ND = non-detectable

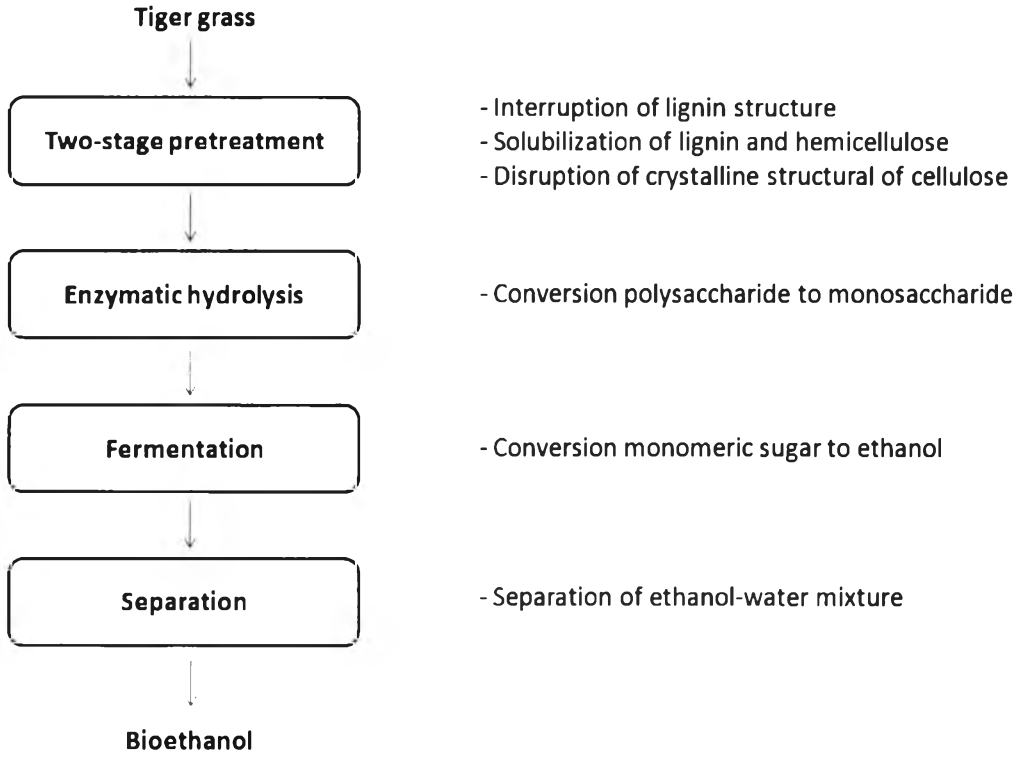

Figure 6.1 Schematic flowsheet for the conversion of *T. maxima* into bioethanol.

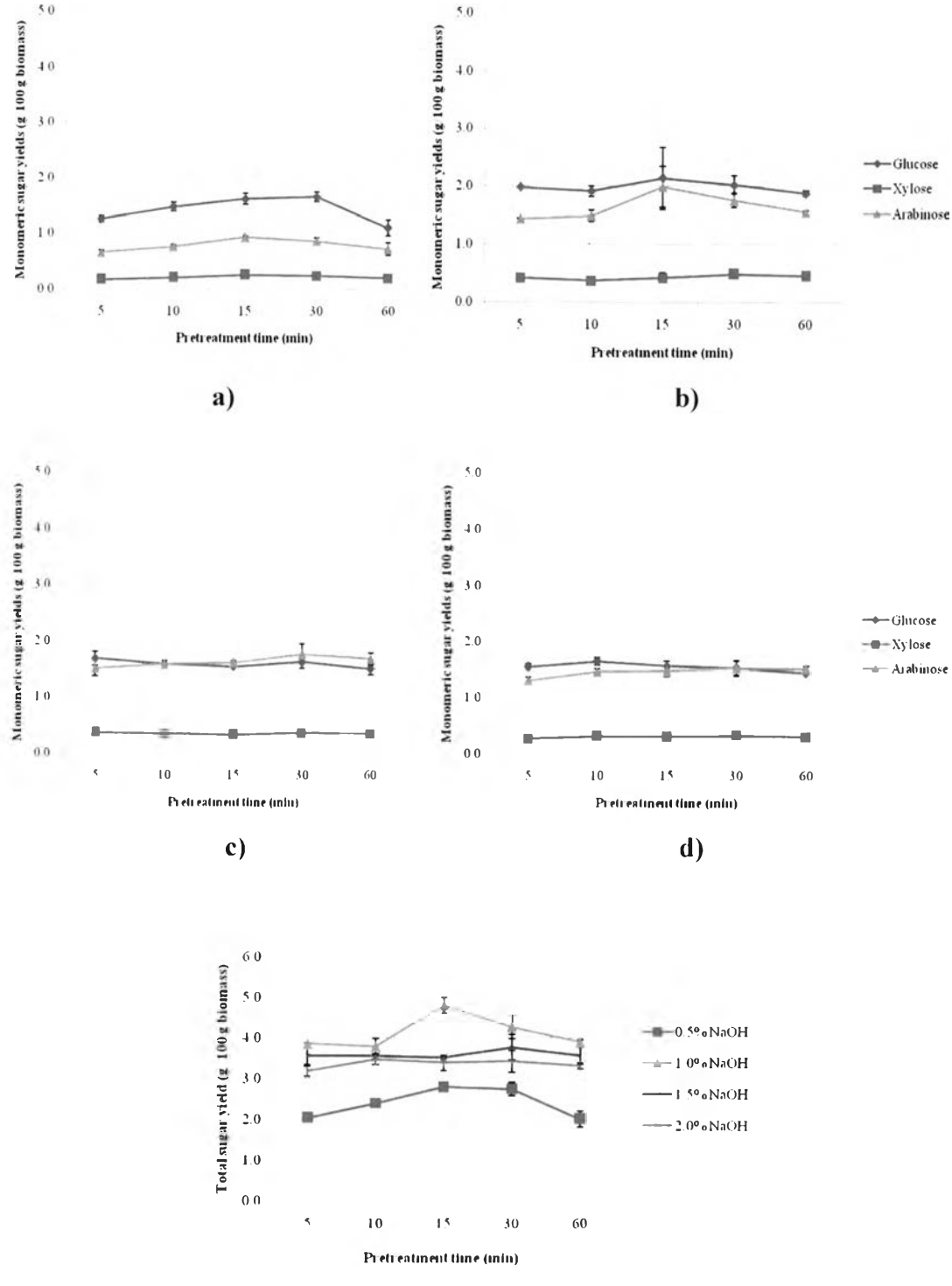

Figure 6.2 The yields of glucose, xylose, and arabinose of untreated *T.maxima* at 120 °C and 15:1 liquid to solid ratio (LSR) at different NaOH concentration (% w/v) and time (5-60 min): a) 0.5 % (w/v), b) 1.0 % (w/v), c) 1.5 % (w/v), d) 2.0 % (w/v) and e) comparison of total monomeric sugar yields.

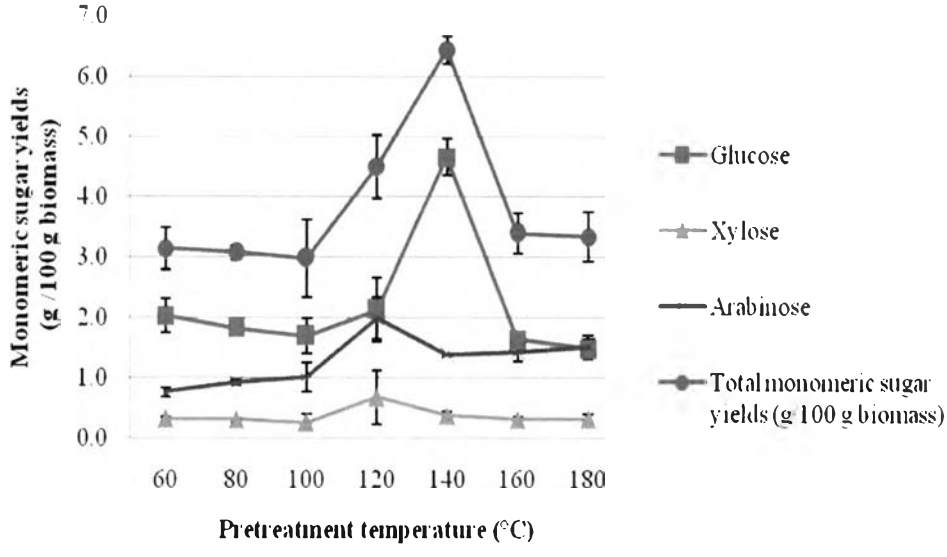

Figure 6.3 Effect of temperature on monomeric sugar yields of *T.maxima* using 0.5 % (w/v) NaOH, 15:1 LSR for 15 min.

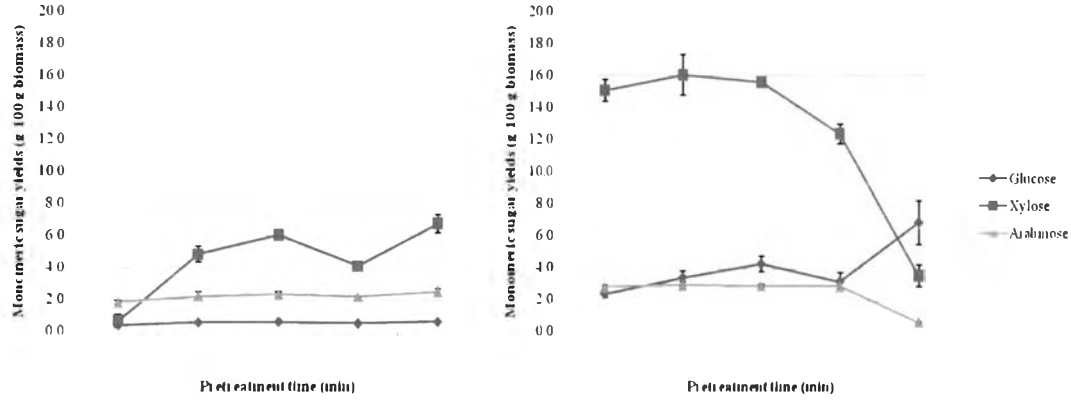

Figure 6.4 The yields of glucose, xylose, and arabinose of pretreated *T.maxima* at 0.5 % (w/v) H_2SO_4 and 15:1 LSR at different temperatures and times: a) 120°, b) 160°, c) 200° microwave-assisted NaOH, and d) comparison of total monomeric sugar yields.

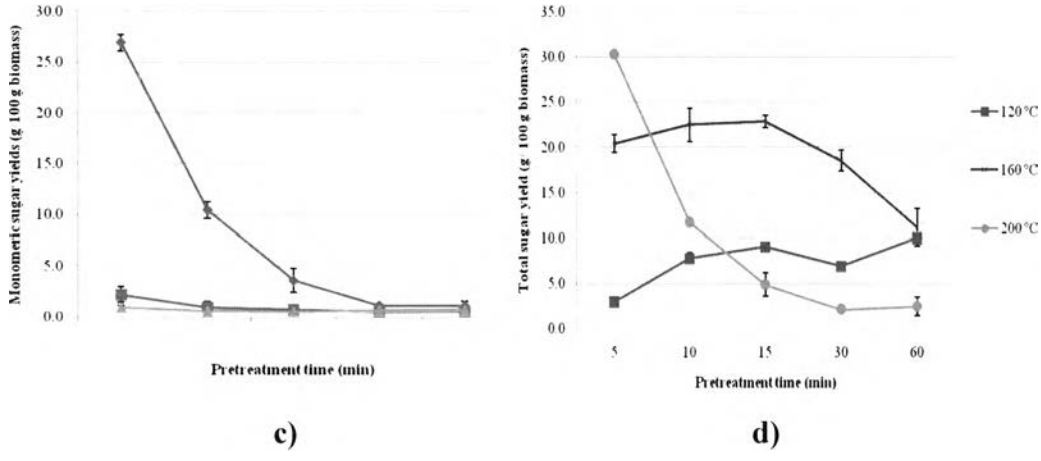

Figure 6.4 The yields of glucose, xylose, and arabinose of pretreated *T.maxima* at 0.5 % (w/v) H_2SO_4 and 15:1 LSR at different temperatures and times: a) 120°, b) 160°, c) 200° microwave-assisted NaOH, and d) comparison of total monomeric sugar yields. (Con't.)

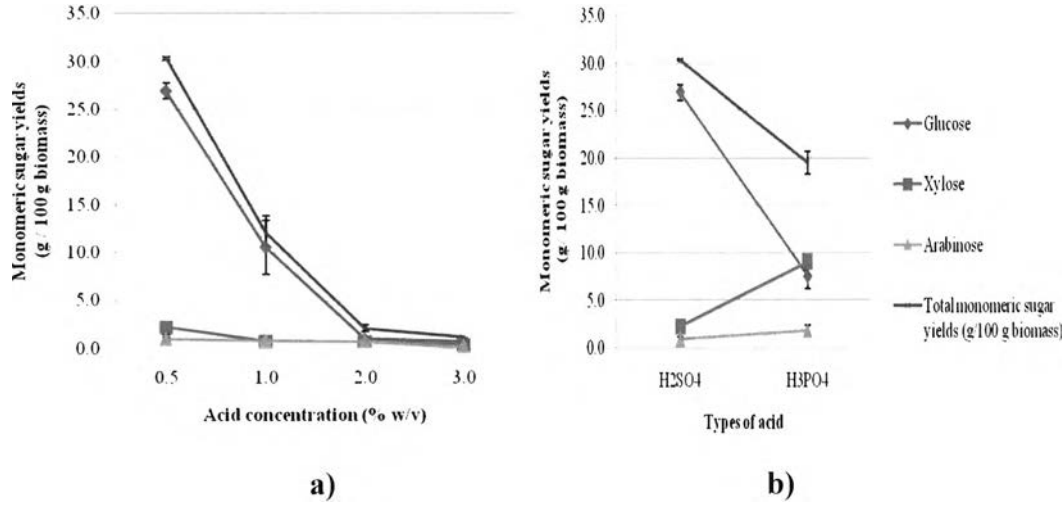

Figure 6.5 The monomeric sugar yields of *T.maxima* at 200 °C/5 min and 15:1 LSR: a) effect of acid concentration and b) effect of acid type using 0.5 % (w/v) acid concentration.

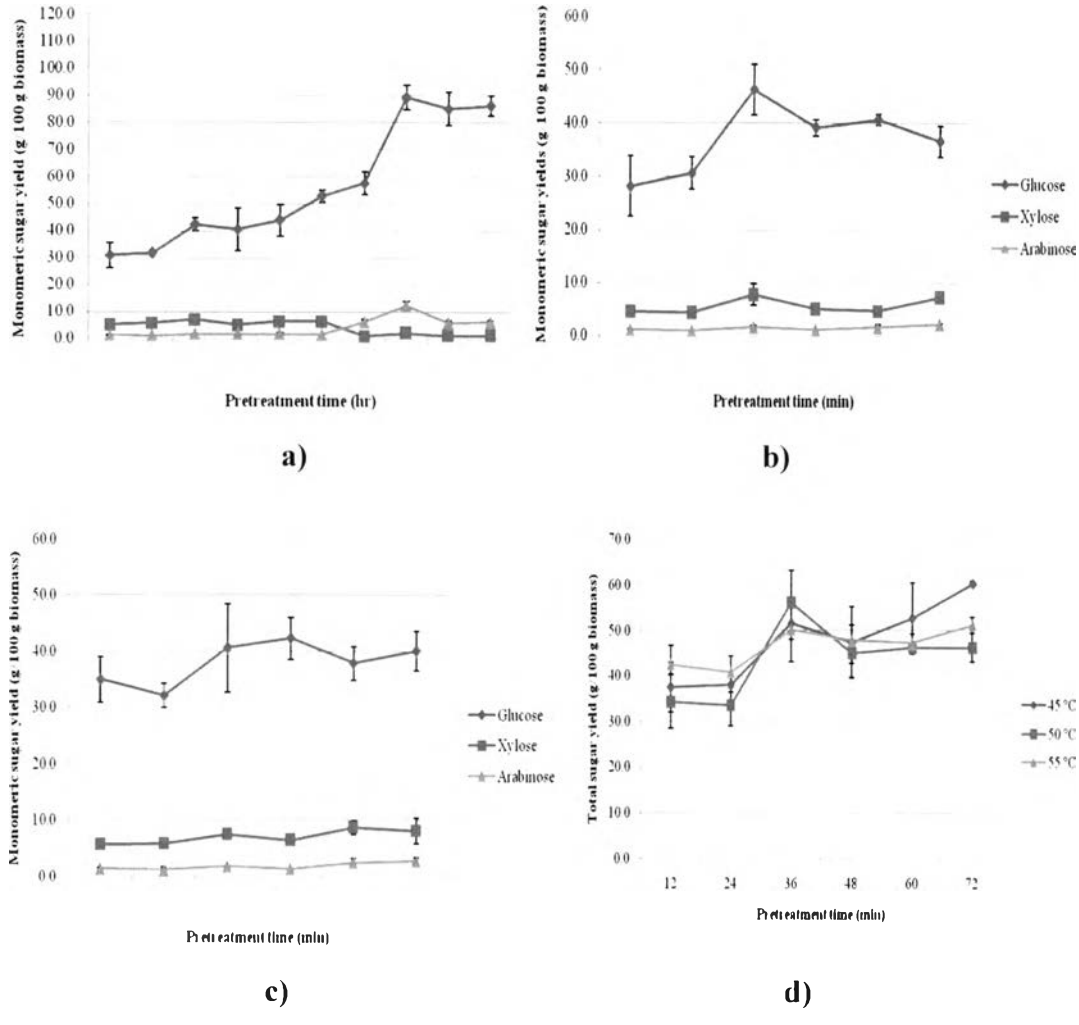

Figure 6.6 The yields of glucose, xylose, and arabinose of enzymatic hydrolyzed *T.maxima* at pH 4.8, 160 µl of *T.reesei* ATCC 26921 loading at different temperatures and times: a) 45°, b) 50°, c) 55°, and d) comparison of total monomeric sugar yields.

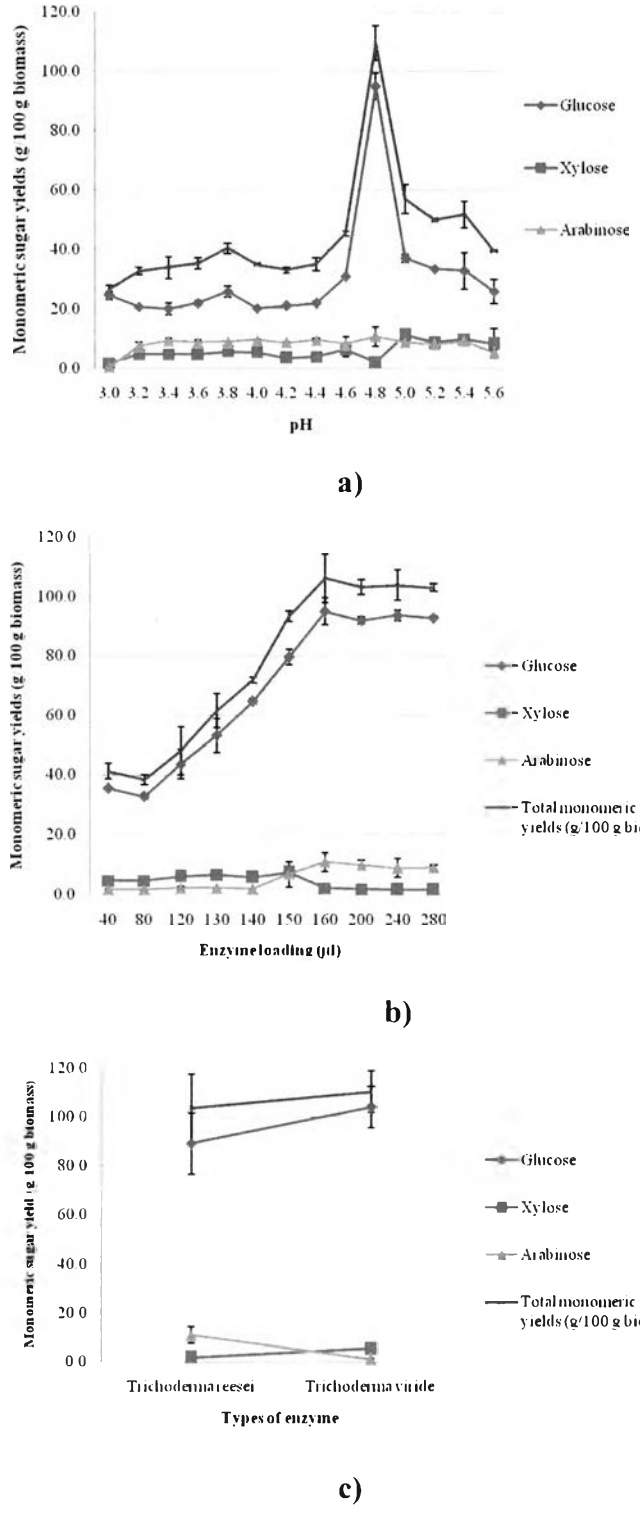

Figure 6.7 The monomeric sugar yields a) effect of varying pH (3.0-5.6) by *Trichoderma reesei* ATCC 26921, b) effect of enzyme loading (40-280 µl) by *Trichoderma reesei* ATCC 26921, and c) effect of enzyme type, using 160 µl of enzyme loading at 45 °C/ 120 h.

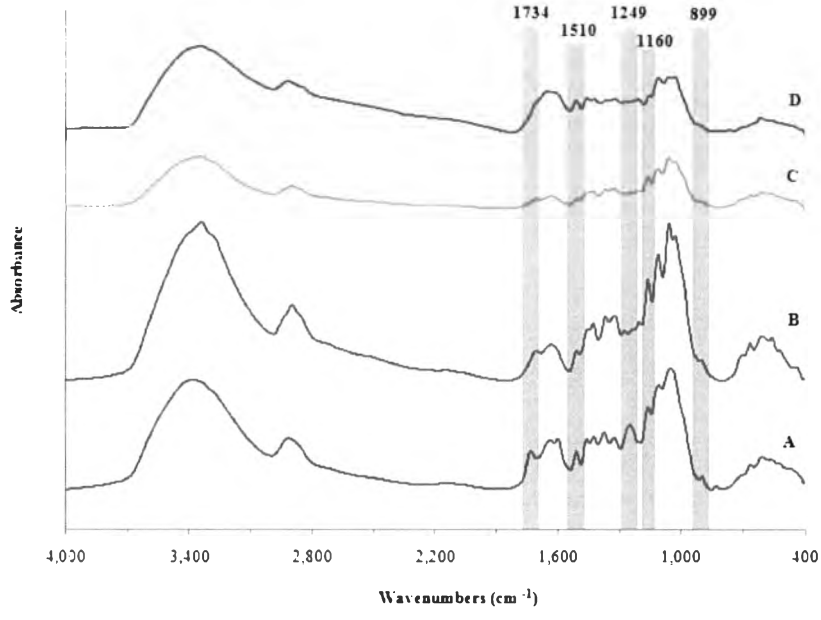

Figure 6.8 FTIR spectra of (A) raw, (B) microwave-assisted NaOH pretreated, (C) two-stage pretreated, and (D) enzymatic hydrolyzed *T.maxima.*