CHAPTER 6

MORPHOLOGICAL AND CHEMICAL COMPOSITION OF PRETREATED LIGNOCELLULOSIC BIOMASS

6.1 Introduction

The term "Lignocellulosic biomass" refers to higher plants, softwood or hardwood and agricultural residues; *e.g.* sugarcane bagasse, coffee husk and rice husk (Eriksson *et al.*, 1990). Delignification or pretreatment liberates cellulose and hemicellulose from their complex with lignin (Lin and Tanaka, 2006), and is an important step for the biochemical conversion of the biomass into bio-ethanol. Removing the high lignin content is the primary goal of pretreatment processes which make the cellulose more accessible to the enzymes that convert the polysaccharide polymers into fermentation sugars (Chahal, 1991). Pretreatment is one of the most expensive steps in the process of bio-ethenol production and there is a potential to improvement efficiency costs through research (Galbe and Zacchi, 2007). Pretreatment methods can be divided into two main categories; chemical and biological.

The most common chemical pretreatment methods used for lignocellulosic biomass are diluted acid and alkaline to make the biomass more digestible by the enzyme. The acidic pretreatments will hydrolyze the hemicelluloses fraction while leaving the cellulose and lignin intact in the residual solid (Lloyd and Wyman, 2005). Sulfuric acid (H_2SO_4) is the most applied acid used for pretreatment (Xiao and

Clarkson, 1997; Taherzadeh and Karimi, 2007). The alkaline pretreatment refer to the application of alkaline solution to remove lignin and a part of the hemicelluloses, and efficiently increase the accessibility of enzyme to the cellulose (Taherzadeh and Karimi, 2008). The alkaline pretreatment was shown to be more effective on agricultural residues than on wood materials (Xu *et al*., 2007). Sodium hydroxide (NaOH) was repored as the most used as alkaline reagent for lignocellulosic biomass (Gomes *et al*., 2007; Silverstein *et al*., 2007; Zhao *et al*., 2007).

Most pretreatment technologies require expensive instruments or equipment that have high energy requirement, depending on the process. Biological pretreatment refer to wood degrading microorganisms, including brown-, white-, soft-rot fungi, and bacteria to modify the chemical composition and/ or structure of the lignocellulosic biomass so that the modified biomass is more amenable to enzyme digestion (Taherzadeh and Karimi, 2008; Kumar *et al*., 2009). Recently this environmentally friendly approach has received enzymatic saccharification of lignocelluloses in ethanol production process (Alvira *et al*., 2010). The advantages of biological delignification over previous methods are mild reaction conditions, higher product yields and fewer side reactions, and less energy demand and less reactor resistance to pressure and corrosion (Lee, 1997). Fungi have distinct degradation characteristics on lignocellulosic biomass. In general, brown- and soft-rot mainly attack cellulose while inparting minor modifications to lignin component (Schurz, 1978). White-rot fungi present more effectively and more specifically degrade lignin than brown- and soft-rot fungi. Lignin degradation by white-rot fungi is the most effective for biological pretreatment of lignocellulosic biomass, occurs through the action of lignin-degrading enzyme (ligninolytic enzyme) such as peroxidases and laccases (Zheng *et al*., 2009).

In this research, we focus on the morphological characterization and chemical composition of 3 lignocellulosic biomass; sugarcane bagasse, coffee husk and rice husk. Three lignocelluloses were pretreated by chemical and biological pretreatment (fungal and enzymatic pretreatment) in comparison with untreated control.

6.2 Materials and methods

6.2.1 Lignocellulosic biomass preparation

Three lignocellulosic biomass (sugarcane bagasse, coffee husk and rice husk) were combined in ratio 57:6:37 % w/w (presented in chapter 8) and autoclaved at 121°C for 15 minutes.

6.2.2 Lignocellulosic biomass pretreatment

6.2.2.1 Biological pretreatment: enzymatic pretreatment

Crude enzyme produced from *T. polyzona* WR710-1 on orange peel as solid substrates under solid state cultivation was used for enzymatic pretreatment. The solid medium was prepared by adding 10 g of orange peel, 30 ml of basal media (Mikiashvili *et al*., 2006) to test flask and was sterilized at 121°C for 15 minutes. Then active growing white-rot fungal culture was inoculated and incubated for 14 days. The crude enzyme was harvested, extracted with 20 mM potassium phosphate buffer pH 7.0, filtered and clarified by centrifugation at 8000 rpm, 4^{o}C for 10 min to remove mycelia and the clear supernatant was used as crude enzyme source.

Enzymatic pretreatment, mixed substrates 5 g in test flasks were supplemented with crude enzyme 100 ml and incubated for 10 days on a rotary shaker 150 rpm. The cultures were filtered to separate substrates and crude enzyme. Lignocellulosic

substrates were dried at 60^{o}C hot air oven, overnight. Dried substrates were analyzed for morphological characterization and chemical composition.

6.2.2.2 Biological pretreatment: fungal pretreatment

White-rot fungal pretreatment was done by cultured *T. polyzona* WR710-1 directly with lignocellulosic biomass in 2 growth conditions, submerge and solid state fermentation conditions.

Submerged fermentation, lignocelluloses substrates 5 g were submerged with 100 ml of mineral solution (modified from Jianmin *et al*., 2008). Then agar grown white-rot fungal cultures were inoculated and incubated for 15 days on a rotary shaker 150 rpm. The cultures were filtered to remove mycelia. Lignocellulosic substrates were dried at 60^{o}C hot air oven, overnight, and dried substrates were analyzed for morphological characterization and chemical composition.

Solid state fermentation condition, the prepared substrates 5 g were added with 15 ml of mineral solution. Then agar grown white-rot fungal cultures were inoculated and static incubated for 15 days. The cultures were manual remove mycelia and dried at 60^{o}C hot air oven, overnight. Dried substrates were analyzed for morphological characterization and chemical composition.

6.2.2.3 Chemical pretreatment

Dilute sulfuric acid (H_2SO_4), 2% (v/v) and diluted sodium hydroxide (NaOH), 2% (v/v) were used to pretreated 10 g of ground lignocellulosic biomass (sugarcane bagasse, coffee husk and rice husk). Treatments were performed in triplicate at an autoclaved at 121^{o}C with 15 psi (103.4 kPa) for 90 min. Samples were neutralized with hot deionized water and dried at 60^{o}C overnight (Silverstein *et al*., 2007). Dried

substrates were analyzed for morphological characterization and chemical composition.

6.2.3 Biomass surface morphological characterization

Surface morphology of 3 lignocellulosic biomass, sugarcane bagasse, coffee husk and rice husk (pretreated and untreated control) were studied using Scanning Electron Microscopy (SEM). Lignocellulosic samples were dehydrated to critical point dried, mounted onto metal stubs, and coated with gold. Specimens were examined in a Low Vacuum Scanning Electron Microscope: JEOL JSM-5910 LV (Science and Technology Service Center, Faculty of Science, Chiang Mai University, Thailand) with an accelerating voltage of 15 kV.

6.2.4 Composition of lignocellulosic biomass analysis

The chemical analysis of mixed-lignocellulosic biomass were analyzed. Holocellulose was determined using modified acid chlorite method (Browning, 1967). Alpha-cellulose, acid-insoluble lignin and ash in pulp were analyzed using TAPPI test method, T 203 om-88, T222 om-88 and T211 om-85 (TAPPI 1988), respectively (Appendix B).

6.2.5 Total soluble sugar determination

Mixed lignocellulosic biomass (pretreated and untreated control) were hydrolyzed by fungus *Thermoascus aurantiacus* SL16W (Kongbuntad *et al*., 2006) under solid state cultivation, 45^{o}C for 10 days. Total sugar from each experiment was extracted by distilled water and filtrated 10,000 rpm, 4^{o}C for 10 min. Total sugar production was determined by phenol-sulfuric acid method (Dubois *et al*., 1956), using glucose as standard sugar.

6.3 Results and discussion

6.3.1 Biomass surface morphological characterization

Lignocellulosic biomass was treated by biological and chemical pretreatments, the morphological characterization were analyzed. Figure 6.1 showed the scanning electron micrograph of the sugarcane bagasse. Untreated control of bagasse, which the fiber surface, with parallel stripes and rod shape-liked of structure with approximately 150-300 µm in length and 50-100 µm in height was shown in Fig. 6.1a. After biological pretreatment, only pretreated by *T. polyzona* WR710-1 under solid state cultivation condition presented fungal hyphae (FH) and bore holes (H) on the surface of bagasse (Fig. 6.1b), the bore holes used by fungal hyphae to penetrate into pulp cell. However, the destruction of tissues was clearly visible when pretreated by enzymatic pretreatment and fungal pretreatment, *T. polyzona* WR710-1 cultured under submerge condition (Fig. 6.1c and d).

After sugarcane is milled for juice extraction, its bagasse is obtained as a residue, which contains 60-80% of carbohydrates. The fermentation of these carbohydrates could enhance bio-ethanol productivity (Betancur and Pereira, 2010). White-rot fungi possess the capabilities to attack lignin in biological pretreatment process before ethanol fermentation (Dashtban *et al*., 2009). Ramos *et al*. (2004) showed that crude ligninolytic enzyme extracted from *Phanerochaete chrysosporium* fungi was successfully used for biological pretreatment of sugarcane bagasse. Moreover, Sasaki *et al*. (2011) suggested that *Ceriporiopsis subvermispora* pretreatment could be beneficial part of the process to produce ethanol from bagasse.

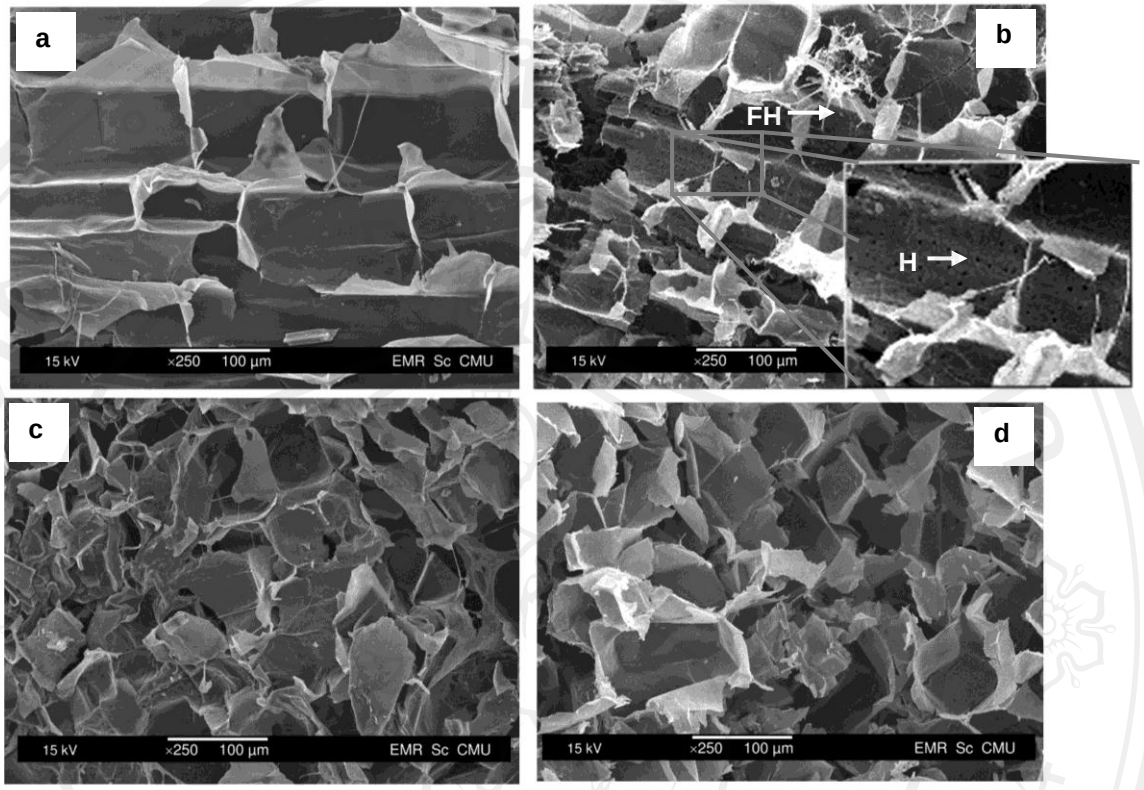

Fig. 6.1 Microstructures of sugarcane bagasse treated by biological pretreatment. **a**: untreated sugarcane bagasse (control), **b**: pretreated by *T. polyzona* WR710-1 under solid state cultivation for 15 days, fungal hyphae (FH) and bore holes (H), **c**: pretreated by crude enzyme laccase produced from *T. polyzona* WR710-1 on orange peel substrate for 9 days and **d**: pretreated by *T. polyzona* WR710-1 under submerge fermentation for 15 days.

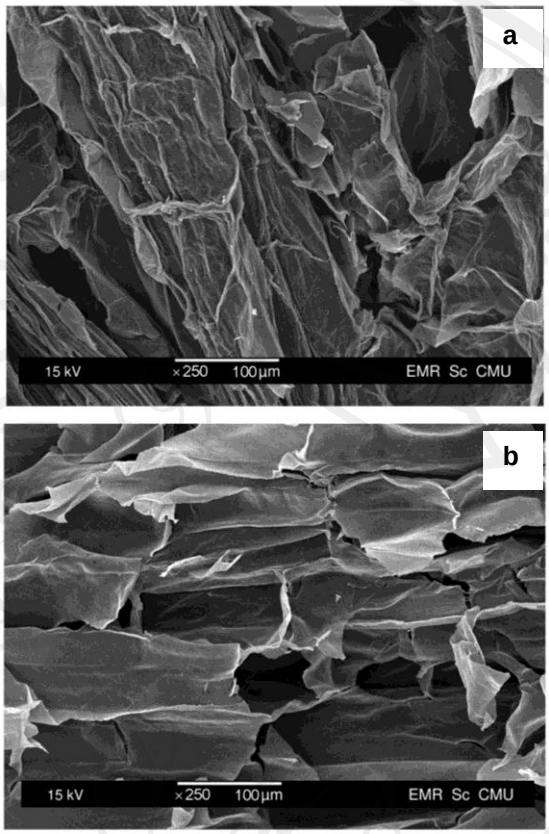

Fig. 7.2 Microstructure of the sugarcane bagasse treated by chemical pretreatment. **a**: pretreated by 2% H_2SO_4 and **b**: pretreated by 2% NaOH, 15 psi, 121^oC for 90 min.

Sugarcane bagasse pretreated by chemical method, 2% of H_2SO_4 and NaOH, the destruction of tissues was clearly visible as showed in Fig 6.2a and b, respectively. These results are in agreement with those of Rezende *et al*. (2011), who have found that the fiber surface of sugarcane bagasse showed more fragile and fragmented structure and the amount of pith was reduced after acid degradation. Many studies suggested that acidic and basic reagents were successfully used for pretreated sugarcane bagasse (Pietrobon *et al*., 2011; Yoon *et al*., 2011).

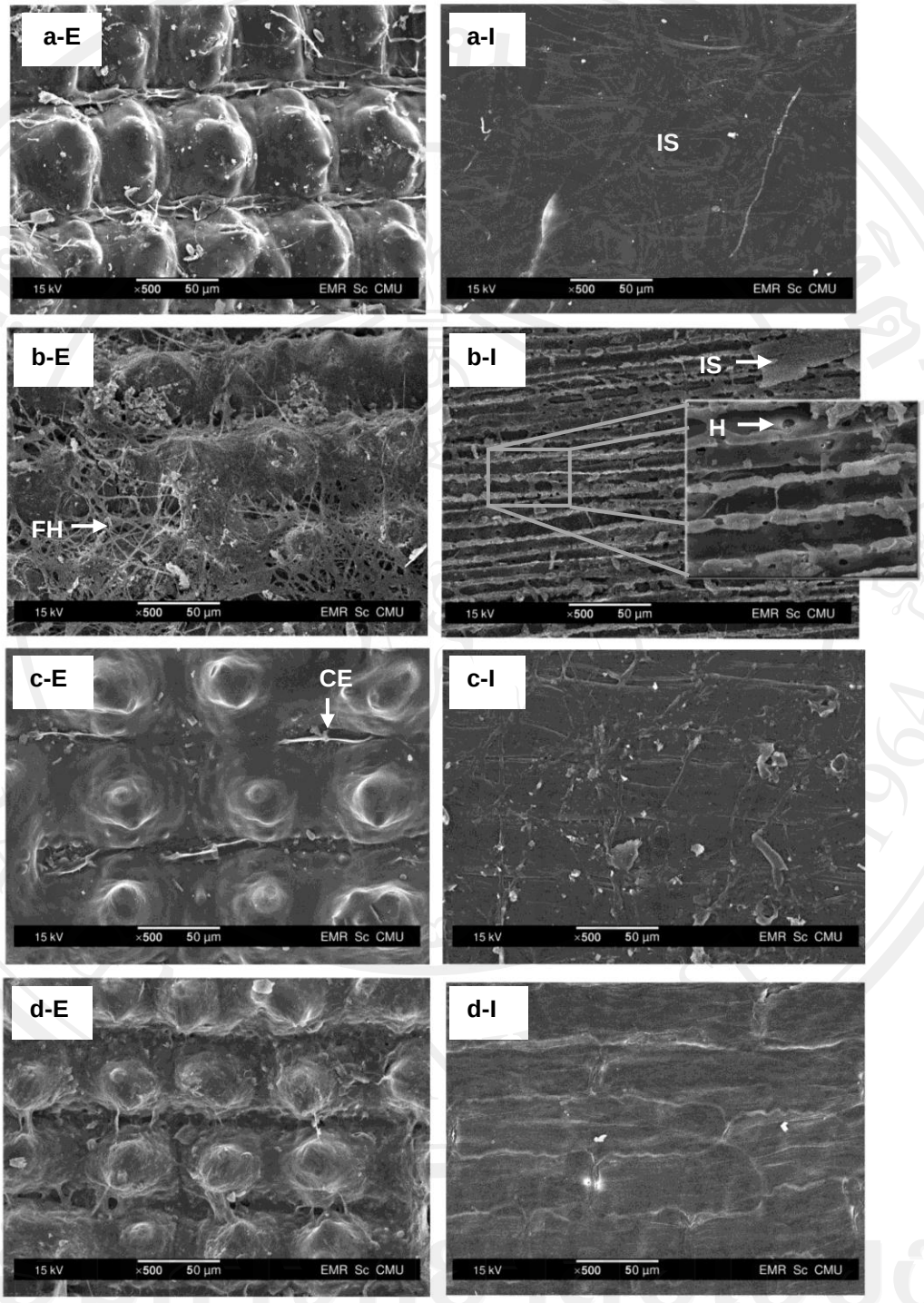

Fig. 6.3 Microstructure of the rice husk treated by biological pretreatment; external (E) and internal (I) surface.

Microstructure with fungal hyphae (FH) and bore holes (H), internal surface (IS) and cell wall etching (CE) , **a**: untreated rice husk (control), **b**: pretreated by *T. polyzona* WR710-1 under solid state cultivation for 15 days, **c**: pretreated by crude enzyme laccase produced from *T. polyzona* WR710-1 on orange peel substrate for 15 days and **d**: pretreated by *T. polyzona* WR710-1 under submerge fermentation for 15 days.

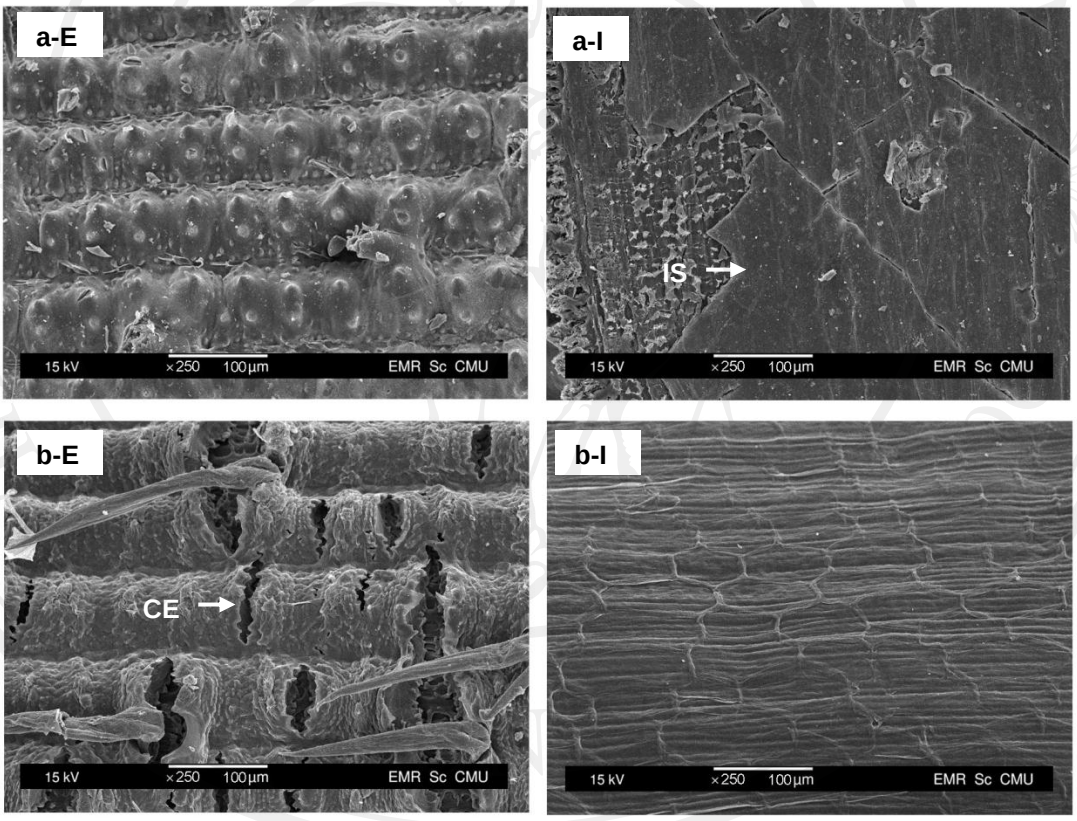

Fig. 6.4 Microstructure of the rice husk treated by chemical pretreatment; external (E) and internal (I) surface.

Microstructure with cell wall etching (CE) and internal surface (IS), **a**: pretreated by 2% H_2SO_4 and **b**: pretreated by 2% NaOH, 15 psi, 121^{o}C for 90 min

The morphology is different for the outer and inner surfaces of rice husk (Liou, 2004). The scanning electron micrograph of untreated rice husk, showed the external surface of rice husk, which was well organized, waxy barrier and a corrugated structure (Fig. 6.3a-E). The exhibits rough surface of rice husk would make the mechanical protecting of cell wall from enzyme hydrolysis. Figure 6.3a-I showed the internal surface of rice husk, which had a lamella structure and contained silica, mainly localized in the tough interlayer of the rice husk. Fungal pretreatment, cultivated with *T. polyzona* WR710-1 under solid state or submerge condition and enzymatic pretreatment by crude enzyme from this fungus. The waxy barrier of external surface of rice husk was decreased and the tough lamella of internal surface also reduced (Fig 6.3c-E, -I and 6.3d-E, -I). In this experiment, only rice husk which pretreated by *T. polyzona* WR710-1 under solid state cultivation for 15 days, external surface of rice husk was covered by fungal hyphae (Fig. 6.3b-E). Micro-pores and striped micro cellulosic fibres of rice husk were shown on internal surface (Fig. 6.3b-I), while untreated (control), with no sign of cell wall degradation. White-rot fungi were used for biological pretreatment in many studies such as, Potumarthi *et al.* (2012) suggested that white-rot fungus *Phanerochete chrysosporium* was used in the biological pretreatment of rice husk for reducing sugars production.

Figure 6.4a-E and -I showed the scanning electron micrograph of rice husk which pretreated by 2% H_2SO_4, the waxy barrier of external surface was decreased and the tough lamella of internal surface was slightly reduced. However rice husk which pretreated by 2% NaOH showed strongly damaged, found many cell wall etching (CE) on external surface (Fig. 6.4b-E). Cellulose micro fibril of rice husk was

exposed due to dissolution of lignin and hemicelluloses and other impurities dissolve in alkaline solution (Syafri *et al*., 2011).

Rice husk is a cellulosic-based fibre, which has been widely application such as used for enzyme production (Masutti *et al*., 2012) and amorphous silica production (Liou, 2004). The main advantages of using rice husk as biodegradable filler are their low cost, low density, high specific strength and modulus, and recyclability (Ismail *et al*., 2001). The main disadvantage of using rice husk as reinforcing filler for composite material is the poor interaction between rice husk particle and polymer matrix (Avella *et al*., 1998). Cooperative of the fibres and non-polar matrices of rice husk surface which are mostly hydrophobic is the main problem due to high polarity and hydrophilicity in nature of cellulosic fibres. Therefore, pretreatment of the rice husk surface is critically important to increase the hydrophobicity of the fibres, improved on minimizing the surface tension and enhances enzyme hydrolysis.

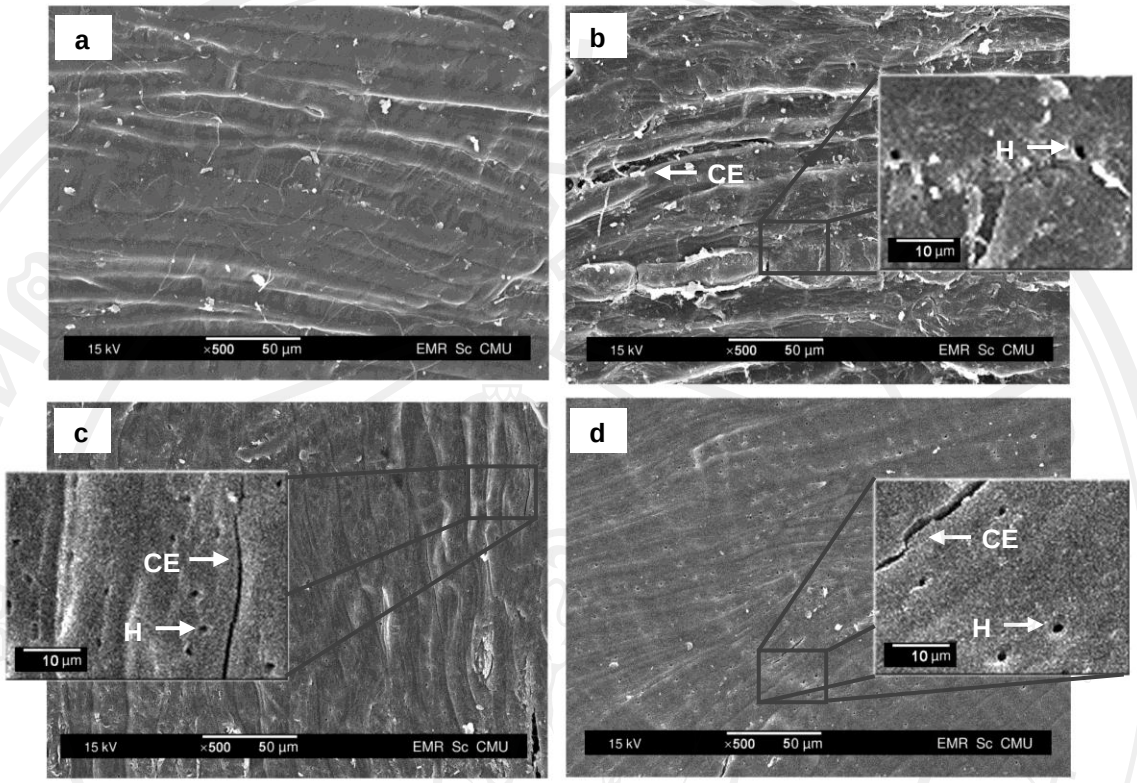

Fig. 6.5 Microstructure of the coffee husk which treated by biological pretreatment. **a**: untreated rice husk (control), **b**: pretreated by *T. polyzona* WR710-1 under solid state cultivation for 15 days, **c**: pretreated by crude enzyme laccase produced from *T. polyzona* WR710-1 on orange peel substrate for 15 days and **d**: pretreated by *T. polyzona* WR710-1 under submerge fermentation for 15 days.

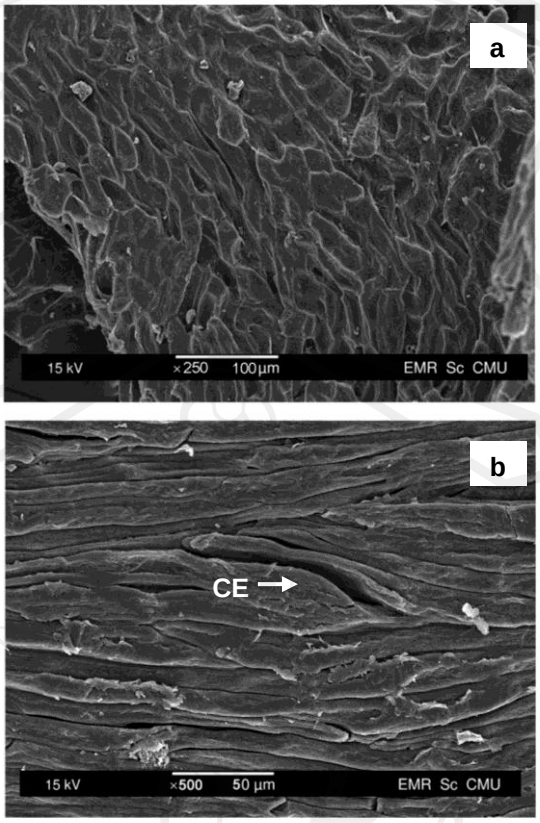

Fig. 6.6 Microstructure of the coffee husk which treated by chemical pretreatment. **a**: pretreated by 2% H_2SO_4 and **b**: pretreated by 2% NaOH, 15 psi, 121^oC for 90 min

Coffee husk is one of the most abundantly available agro-industrial wastes. Coffee husk is a cellulosic-based fibre, which has been widely application such as used for ligninolytic enzyme production (Parani and Eyini, 2012) and used as solid substrates in bio-ethanol production (Navia *et al*., 2011). Pretreatment of the coffee husk is necessary because hydrolysis of non-pretreated biomass is slow, and results in low product yield (Dashtban *et al*., 2009). In this experiment, the morphological characterization of coffee husk surface was studied. The surface structure of the coffee husk without pretreatment was tight, orderly, waxy and the texture was relatively hard (Fig. 6.5a). In contrast, the surface of coffee husk which pretreated by

white-rot fungus (fungal pretreatment) was decreased of wax and contained a lot of micro-pores on its surface, pores size as about 1-2 µm of diameters (Fig. 6.5b, c and d). White-rot fungi showed high ability used for biological pretreatment, decreased the cellulose, hemicellulose content and enhanced the highest amount of reducing sugars by using coffee pulp substrate (Parani and Eyini, 2010).

Unless the waxy barrier on coffee husk surface was decreased, it was clearly presented of cell wall structure when pretreated by chemical reagent, 2 % H_2SO_4 (Fig. 6.6a). Any decreasing in crystallinity and tightly of coffee husk cell wall was found during pretreatment by 2 % NaOH (Fig. 6.6b). However, no micro-pores produced on coffee husk surface when pretreated by chemical method. In the same way of rice husk, coffee husk presented fibres and non-polar matrices of its surface which are mostly hydrophobic. It is the main problem due to high polarity and hydrophilicity in nature of cellulosic fibres. Therefore, pretreatment of the coffee husk surface is critically important to increase the hydrophobicity of the fibres, improved on minimizing the surface tension and enhances enzyme hydrolysis.

6.3.2 Chemical composition analysis

Three lignocellulosic biomass, sugarcane bagasse, coffee husk and rice husk were combined and the chemical composition of mixed biomass was determined. The chemical composition of untreated biomass (control) and biomass which pretreated by chemical and biological methods were shown in Table 6.1. Untreated lignocellulosic biomass comprised with 35.98 % cellulose, 21.74% of hemicelluloses and 33.41% of lignin.

The main purpose of pretreatment or delignification is to liberate cellulose and hemicelluloses from their complex lignocellulose and remove lignin (Lin and Tanaka,

2006). In this experiment, lignin content showed in Table 6.1 was highly removed by 2% NaOH, lignin remaining about 8.73%. However, lignin was unchanged when treated by 2% H_2SO_4. Taherzadeh and Karimi (2008) explained that the alkaline pretreatment refer to the application of alkaline solution to remove lignin and a part of the hemicelluloses and efficiently increase the accessibility of enzyme to the cellulose. On the other hand, the acidic pretreatments hydrolyzed the hemicelluloses fraction while leaving the cellulose and lignin intact in the residual solid (Lloyd and Wyman, 2005).

In comparison of biological pretreatment and untreated lignocelluloses in this study, the results showed the percentages of lignin were 23.13 and 27.72 when treated by crude enzyme and treated by cultured with white-rot fungus under solid state fermentation, respectively. It was significantly different from untreated lignocellulosic substrates. This result identical to the studied of Zheng *et al*. (2009), white-rot fungi presented more effectively and more specifically degrade lignin than brown- and soft-rot fungi.

As shown in Table 6.1, percentage of cellulose was significant increased to 59.65 % when pretreated by NaOH and 43.89% when pretreated by H_2SO_4. Biological pretreatment by *T. polyzona* WR710-1 showed cellulose was slightly increased in enzymatic pretreatment (38.87 %). While, the cellulose in mixed biomass was not significant different between untreated (control) and pretreated by *T. polyzona* WR710-1 under solid state cultivation. Hemicelluloses was significant increased when mixed lignocelluloses were treated by white-rot fungus, under solid and submerge cultivation and crude enzyme from *T. polyzona* WR710-1, identical to

lignocelluloses which treated by alkaline reagent. While, acidic reagent had no effect on hemicelluloses content (Table 6.1).

Table 6.1 The chemical composition of mixed lignocellulosic biomass (sugarcane bagasse, coffee husk and rice husk)

sample	Chemical composition (%)*		
	cellulose	hemicellulose	lignin
Untreated (control)	35.98± 0.17^{de}	21.74± 0.43^{b}	33.41±1.44^{a}
Fungal pretreatment			
- solid state cultivation; SSC	34.29 ±1.46^{e}	26.17± 0.9^{a}	27.72 ±0.86^{b}
- submerge fermentation; SmF	37.73±0.16^{c}	29.11±0.38^{a}	31.34 ±0.40^{a}
Enzymatic pretreatment	37.87± 2.71^{c}	27.15± 2.82^{a}	23.13±0.64^{c}
Alkali pretreatment	59.65± 0.69^{a}	28.20 ±0.06^{a}	8.73 ± 0.49^{d}
Acidic pretreatment	43.89 ±0.96^{b}	21.08 ± 1.60^{b}	33.15 ± 1.86^{a}

Results are mean of ±SD of three determinations.

*Values with the same letter are not significantly different (p = 0.05) according to Duncan's multiple range test.

After pretreatment method, treated-lignocellulosic biomass was hydrolyzed by *T. aurantiacus* under solid state cultivation for 10 day at high temperature (45 ^{o}C). The total soluble sugar produced from each treated-lignocellulosic biomass was determined by phenol-sulfuric acid method. The results showed in Figure 6.7, the total soluble sugar up to 30 mg/ml was produced by lignocellulosic biomass which pretreated by 2 % NaOH and up to 20 mg/ml was produced by substrates which

pretreated by all biological pretreatment method, respectively. By the way, in the enzyme hydrolysis should be used the commercial enzyme to confirm the total soluble sugar produced from pretreated substrates and compared between fungal (*T. aurantiacus*) hydrolysis.

Although, cellulose in lignocellulosic biomass with biological pretreatment were low (Table 6.1), but the surface structure of these treated-biomass were contained a lot of micro-pores (Fig. 6.1, 6.3 and 6.5), led increasing of enzymatic hydrolysis and total soluble sugar production. On the other hand, lignocellulosic biomass which treated by 2% H_2SO_4 in this study produced total soluble sugar at around 10 mg/ml. Cellulose in these lignocellulic biomass was high which pretreated by acidic reagent (Table 6.1), but the surface structure of these treated-biomass were slightly changed compared between the other pretreatment method. This result supported to Alvira *et al*. (2010), that the creation tissue space and porosity was significant to enzymatic hydrolysis reactions.

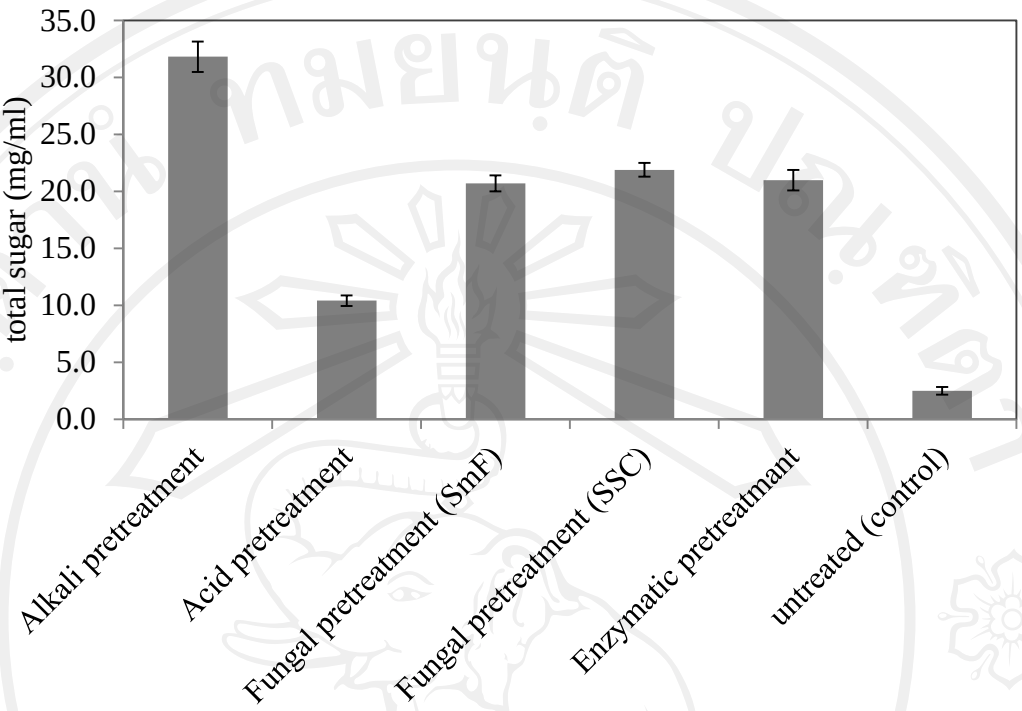

Fig. 6.7 Comparison of total soluble sugar produced from fungal hydrolysis (*T. aurantiacus* SL16W) by using chemical, biological pretreated and control (without pretreatment) substrates.

The encouraging results described above show that all pretreatment processes, biological and chemical method can significantly improve enzyme hydrolysis. The surface morphology study showed a decrease in the lignin barrier, cell wall thickness (waxy barrier), crystallinity and an increase in available surface area (pore volume) of lignocellulosic biomass. The creation tissue space and porosity was very helpful in the saccharification process, which increases the rate of enzymatic hydrolysis reactions (Alvira *et al*., 2010).

When cellulose and hemicelluloses are hydrolyzed, a mixture of monosaccharide such as glucose, xylose, galactose and mannose were produced. Fermentation involves microorganisms which consume sugar as a carbon source. Thus, the chemical composition, cellulose and hemicelluloses content are significant for the production of bio-ethanol. Many lignocellulosic biomasses successfully use acidic reagents in pretreatment. However, acidic pretreatments result in high concentrations of furfurals in the liquid phase (Eggeman and Elander, 2005). Furfural has a negative effect on the specific growth rate. Moreover, furfural and catechol showed a synergistic effect on toxicity with respect to ethanol yield and biomass yield (José *et al*, 2006). Alkali reagents were wildly used for chemical pretreatment. For example, Han *et al*. (2012) treated wheat straw with 1% NaOH for 1.5 h (121°C/15psi) and found that the cellulose content of wheat straw increased by 44.52%. The alkaline methods may result in high concentrations of ferulate and acetate in the hydrolysate. These compounds are present in the sugar solution and have deleterious effects on the fermentative microorganism (Eggeman and Elander, 2005).

The delignification rate of using fungal pretreatment is often low, the required growth conditions and the large amount of space needed to perform biological pretreatment are all disadvantages that make this method less attractive on an industrial scale (Agbor *et al*., 2011). On the other hand, biological pretreatment promotes many advantages over previous methods including mild reaction conditions, higher product yields, fewer side reactions, less energy demands and less reactor resistance to pressure and corrosion (Lee, 1997). From this study, white-rot fungus, *T. polyzona* WR710-1 had potential to use for biological pretreatment of sugarcane

bagasse, coffee husk and rice husk. Many white-rot fungi also showed the potential to treat in many lignocellulosic biomasses, such as bamboo residues (Zhang *et al*., 2007), wheat straw (Salvachúa *et al*., 2011), cotton stalk (Shi *et al*., 2009) and corn stover (Gao *et al*., 2012).