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**ENZYMATIC SACCHARIFICATION OF CEDAR AND
SAGO WASTE PULPS WITH AMPHIPATHIC LIGNIN
DERIVATIVES AS CELLULASE-AID AGENT**

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Doctoral Thesis

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ABBREVIATIONS

AGU	: Amyloglucosidase unit
AL	: Acetic acid lignin
AlOH	: Aliphatic hydroxyl
ArOH	: Aromatic hydroxyl
BG	: β -glucosidase
EG	: Endoglucanase
CBH	: Cellobiohydrolase
CBD	: Cellulose binding domain
CD	: Catalytic domain
CMC	: Critical micelle concentration
DAEO	: Dodecyloxy poly(ethylene glycol) glycidyl ether
EPEG	: Ethoxy (2-hydroxy)propoxy poly(ethylene glycol) glycidyl ether
EPLP	: Ethanol pretreated lodgepole pine
FFPRI	: Forestry and Forest Products research institute
FPU	: Filter paper unit
KL	: Klason lignin
LS	: Light scattering

LIPI	: Indonesian Institute of Sciences
MeO	: Methoxyl
$\overline{M}_n$	: Number average molecular mass
$\overline{M}_w$	: Weight average molecular mass
NREL	: National renewable energy laboratory
PEG	: Polyethylene glycol
PEGDE	: Poly(ethylene glycol) diglycidyl ether
<i>p</i> NPG	: <i>p</i> nitrophenyl- β -D-glucopyranoside
<i>p</i> NPL	: <i>p</i> nitrophenyl- β -D-lactoside
RI	: Refractive index
SSL	: Sago soda lignin
SE	: Saccharification efficiency
SELP	: Steam exploded lodgepole pine
SPR	: Surface plasmon resonance
SPS	: Steam pretreated spruce
SSF	: Simultaneous saccharification and fermentation
THF	: Tetrahydrofuran

CHAPTER 1

General introduction

1.1 Lignocellulose as woody biomass

From the end of 19th century, we are highly dependent on various fossil resources, such as petroleum, coal, natural gas, and so on. These are being used for the production of fuel, electricity and chemicals including plastics. Excessive consumption of fossil fuels, particularly in large urban areas, has resulted in generation of CO₂ and high level of pollution during the last few decades. Developing biomass energy is considered to be the main strategy to solve this problem. One of the liquid biofuels receiving the most interest is bioethanol. A bioethanol production from starch, which is so-called as a first-generation of bioethanol, is a very attractive method because of a very simple process. However, it is recently considered to be less desirable because they compete to production of daily food, resulting in raising the price of food feedstock. Therefore, woody biomass or lignocellulosics are drawn much attention as a second-generation feedstock for bioethanol production, because the lignocellulosics have never competed to food production. Bioethanol can be produced from cellulose component in all kinds of plant, especially unused lignocellulosics such as corn stover, rice and wheat straw, and low-quality wood like as timber for thinning cut. Furthermore, it is possible to produce not only the cellulosic ethanol but also acetic acid, lactic acid, acetone and etc., which are called as platform compounds, from cellulose by fermentation. These productions of chemicals will also reduce

the consumption of fossil resources, leading to depression of greenhouse gas emissions (Service, 2007).

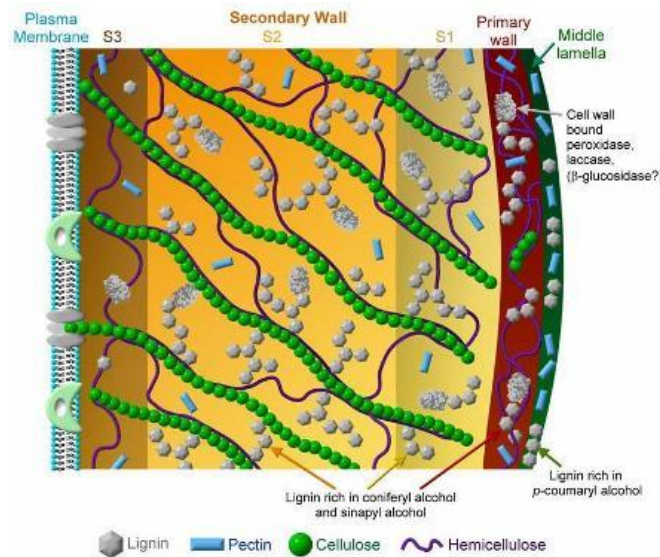


Figure 1-1. A schematic showing cellulose fibrils, laminated with hemicelluloses and lignin polymers.

Source: Achyutan *et al.* (2010).

Lignocellulosics are renewable and abundant, and estimated to be produced in 10-50 billion tons/year as dry matter on the earth, which includes crop residues, grasses, sawdust, wood chips, etc. (Alfaro *et al.*, 2009). The major components of the lignocelluloses are cellulose (about 45%), hemicellulose (ca 20% in softwood, and ca 30% in hardwood) and lignin (ca 30% in softwood, and ca 20% in hardwood) (Monica *et al.* 2009). Cellulose is a fibrous material; therefore, it gives the cell wall mechanical strength and flexibility (Sjöström, 1993). Cellulose fibers are covered by lignin, which plays roles as reinforced material to cellulose and protecting agent against pathogenic attacks of the fungi

and bacteria (Boerjan *et al.* 2003). In addition, the hydrophobic nature of lignin makes cell walls impermeable to allow water transportation through conductive tissues or cells, such as vessels in hardwood and tracheids in softwood in woody stems. Hemicellulose behaves as glue, forming a rigid cell wall upon chemical bonding between cellulose and lignin (Fig. 1-1) (Somerville *et al.*, 2004).

1.2 Hydrolysis of lignocellulose

Fig. 1-2 shows outline of bioethanol production from lignocellulose. There are mainly proposed two kinds of hydrolyses acid and enzymatic hydrolysis or saccharifications. By the methods, cellulose component in lignocelluloses can be converted into glucose as a feedstock for ethanol fermentation. Apparently, acid hydrolysis of lignocellulose with mineral acids, such as sulfuric acid and hydrochloric acid, seemed to be much cheaper than enzymatic hydrolysis with very expensive enzyme, “cellulase”. In the acid hydrolysis, there are proposed two methods, diluted acid method and concentrated acid method. The disadvantages of diluted acid method are to require conditions of high temperature and high pressure, and low glucose yield. Although the concentrated acid method does not require such severe reaction conditions, the corrosion of apparatus is one of disadvantages. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995).

On the other hand, enzymatic hydrolysis of lignocellulose requires delignification of lignocellulose. This fundamental reaction is that cellulase attacks cellulose (Balat *et al.* 2008; Sanchez and Cardona, 2008). However,

cellulase, a larger material than mineral acid, cannot directly attack cellulose, because lignin, which covers cellulose, hampers this enzyme attack to cellulose, although a mineral acid easily attacks cellulose in acid hydrolysis. Delignification is, therefore, one of the most important processes in the enzymatic hydrolysis. When we compared bioethanol production cost between acid saccharification and enzymatic saccharification, the cost of enzymatic saccharification is considered to be comparable to that of acid saccharification, even taking the cost of expensive enzyme into consideration, because enzyme saccharification is usually conducted under mild conditions (pH 4.8 and temperature 45–50 °C) and does not have a corrosion problem (Duff and Murray, 1996).

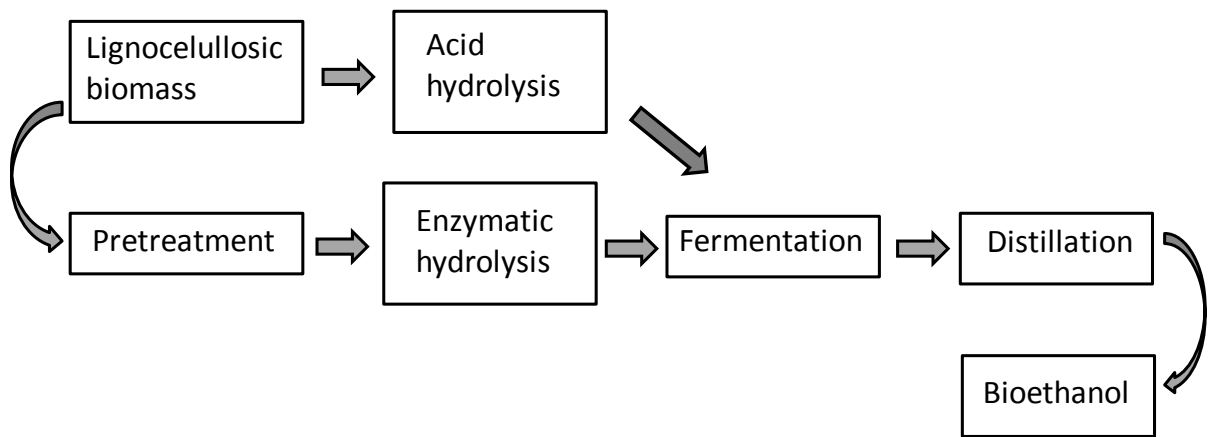


Figure 1-2. Flowsheet for ethanol production from lignocellulosic biomass

In the enzymatic saccharification, a major obstacle is to be reducing the enzymatic activity. This reduction is caused by adsorptions of cellulase on

cellulose and lignin in lignocelluloses as a substrate. Thus, lignin in lignocellulose acts as an inhibitor for enzymatic saccharification (Converse *et al.*, 1990; Errikson *et al.*, 2002a; Pan, 2008). In addition, lignin may also prevent the cell wall from swelling significantly, resulting in restriction of enzyme accessibility (Mooney *et al.*, 1998).

A main subject of my thesis is to propose efficient enzymatic saccharification process of unused woody biomass, timber from forest thinning of Japanese cedar and sago palm waste after starch extraction, by overcoming the problem about the reduction of enzyme activity with novel cellulase-aid agents.

Japanese cedar (*Cryptomeria japonica*)

Japanese cedar or sugi (*Cryptomeria japonica*) is one of the most important conifers in Japanese forestry. An active, nationwide forest-tree breeding program was started in about 1956. At the same time, breeding projects for improving the species tolerance of climatological stress, and resistance to pathogens such as sugi bark borer and sugi bark midge, were initiated (Ohba 1993). Cedar is also distinguished by the interest which has stimulated in many genetic researchers (Ohba, 1980).

In Japan, approximately 64% of the total land area is occupied by forests, and 22% of the total forested area is covered by a single species, the Japanese cedar. In addition, about 13 million cubic meters of woody biomass are generated every year in Japan, mainly from Japanese cedar (Yamashita *et al.*, 2010). To effectively use timber from forest thinning of Japanese cedar, Forestry and Forest

Products Research Institute (FFPRI) in Tsukuba constructed a test plant for bioethanol production from the wood in Akita prefecture.

Sago (*Metroxylon* sp.)

Sago palm (*Metroxylon sagu*) is one of tropical crops that can tolerate wet growing condition, including peat swamps (Jong and Flach, 1995). It is also exploited as a staple and cash crop in Southeast Asia because the trunk contains a large amount of starch (150-250 kg/trunk) (Kuroda *et al*, 2001). Sago is one of Indonesian native plants that can be converted as an energy source and naturally its plantation area about 1.25 million ha in almost every island in Indonesia. Flach (1977) estimated a total of 1.2 millions wild stand of sago palm in Papua (Irian Jaya); 50,000 ha in Maluku and 148,000 ha of semi-cultivated of sago plantation that are distributed in Papua, Maluku, Sulawesi, Kalimantan Sumatera, Riau and Mentawai islands (Fig. 1-3). Papua has been considered as one of the centers for growing location of sago palms, due to the vast stands of natural sago palm and the high genetic variation of sago palm trees (Maturbongs and Luhulima, 2007).

In Indonesia, the starch from sago palm has been used in the cooking of various types of dishes such as jellies, puddings, soups, noodle, biscuit, sago pearls, and many more. In Papua and Maluku islands, they used the sago starch as staple foods in their daily life.

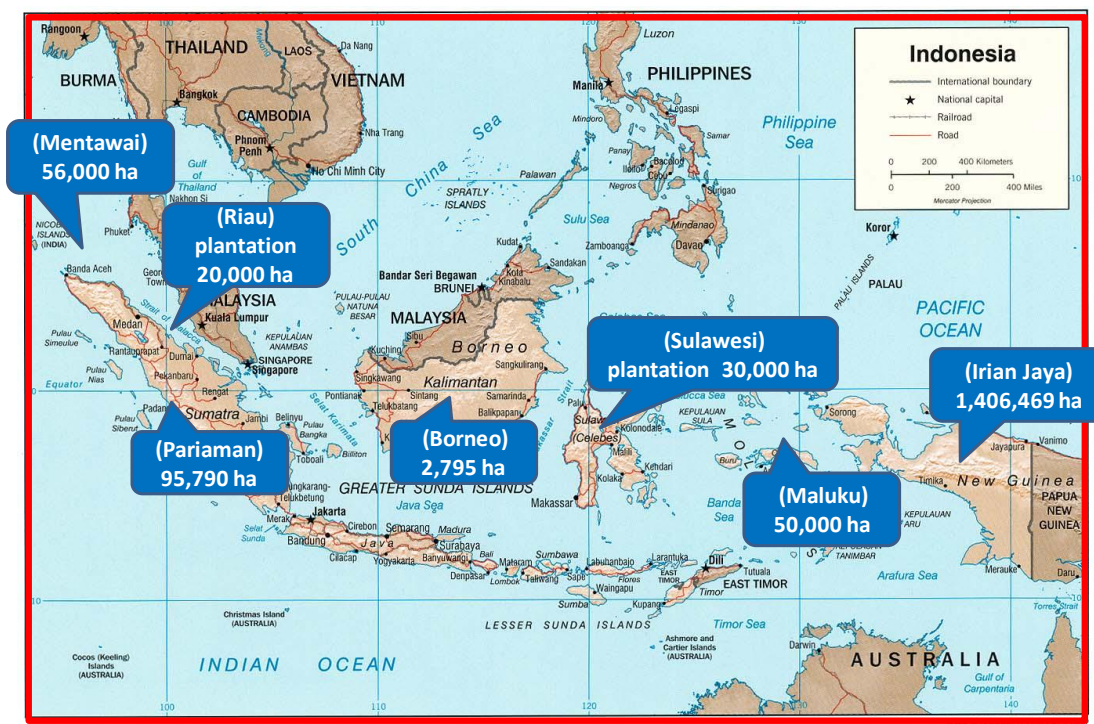


Figure 1-3. Distribution of sago forest in Indonesia

Source : Rostiwati, *et al.* (2008).

Sago starch is produced in 30-60 ton/ha/years as an energy resource for bioethanol as well as food ingredient (Bintoro, 2003). Pith in sago palm is an inner portion of the trunk and starch-accumulated part, and is used as a feedstock for starch industry after removal of the outer bark-like layer. Concomitantly, fibrous sago waste is disposed as a by-product in 4.75 million ton/year by starch mills (Safitri *et al.*, 2009). This waste is usually drained into nearby rivers together with sago effluent without proper treatment, leading to environmental problems (Linggang *et al.*, 2012), they are merely used as animal feed supplement

and chipboard production. The fibrous sago waste contains 58% starch, 23% cellulose, 9.2% hemicelluloses and 3.9% lignin on dry weight basis (Ozawa *et al.*, 1996). Thus, this waste still contains significant amount of starch to be trapped within the lignocellulosic matrix of sago waste as we can see in Figure 1-4 (Adeni *et al.*, 2012).

On the other hand, fibrous sago waste also has the potential to be converted into fermentable sugar through acid and enzymatic hydrolysis. According to Kumoro *et al.* (2008) 0.6234 g of glucose/g of substrate was obtained when it was hydrolyzed with 1.5 M sulphuric acid at 90°C after 120 min, whereas using glucoamylase (6 AGU/ml), 0.5646 g of glucose/g of substrate was obtained within 30 min reaction time. However, it is difficult to achieve complete saccharification, inspite of increasing enzyme dosage, and this might be caused by the remained lignocellulosic part in the waste. To achieve effective saccharification, the residual lignocellulosic part should be hydrolyzed with acid under severe conditions or with cellulolytic enzyme, “cellulase”, under mild conditions.

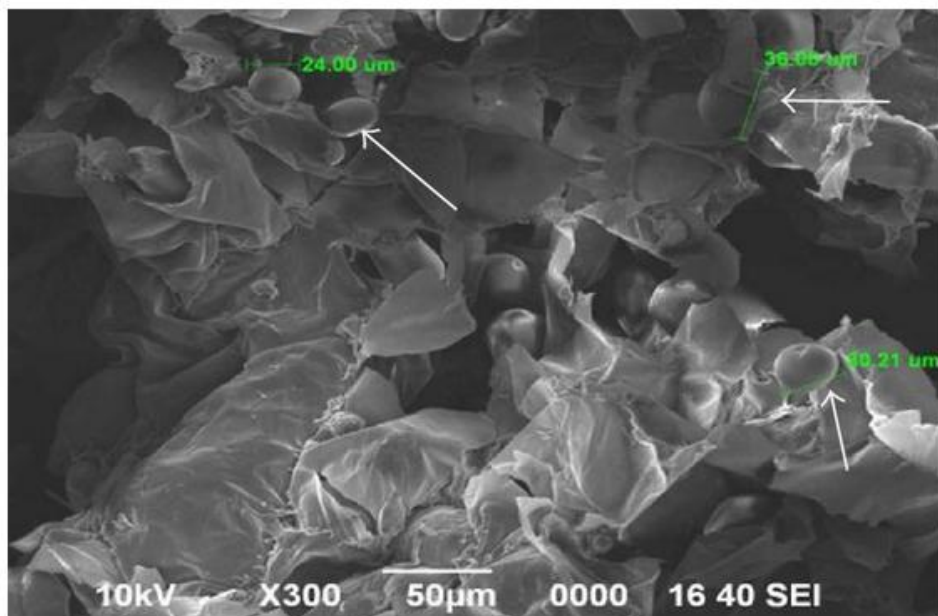


Figure 1-4. Scanning electron micrograph of untreated sago waste. Starch granules (white arrow) remained within the sago waste.
Source : Adeni, *et al.* (2012).

1.3 Pretreatment of lignocelluloses for enzymatic saccharification

For woody biomass, the enzymatic saccharification of the cellulose in the biomass without pretreatment is very low (<20%). The cell wall structure including lignin suppresses accessibility of enzymes to the substrate, cellulose. Therefore, the destruction of cell wall and/or extraction of lignin from woody biomass are one of the methods to improve the saccharification, suggesting that pretreatments are required for the enzymatic saccharification (Lynd *et al.*, 1999). However, no pretreatment suitable for any woody biomass is established. A suitable pretreatment should be developed for each raw material (Hahn-Hägerdahl *et al.*, 2006; Wyman, 2007).

Pretreatment can be classified as physical, biological, chemical or combined methods, and should be conducted with low energy consumption and low chemical input. A representative physical treatment is crushing or milling to yield fine powders. The smaller powders become, the larger saccharification yield is obtained. However, the milling process consumes much energy depending on the particle size (Zhu *et al.* 2009). One of biological pretreatments is delignification with white-rot fungi in the similar way to bio-pulping and bio-bleaching in the pulp production process (Fan *et al.* 1987; Prasad *et al.* 2007). Alkaline pretreatment to remove lignin, such as soda and kraft pulpings is a typically chemical pretreatment. This method conducted under mild conditions is suitable for the saccharification of agricultural waste and herbaceous crops rather than that wood (Kim and Holtzapple, 2005). Enzymatic saccharification of wood requires more severe conditions such as the above pulpings. The use of high concentration of alkali-bases results in partial degradation of hemicelluloses. In this pretreatment, quicklime (CaO) and slaked lime [Ca(OH)₂] are able to be used as a cheap alkali as well as sodium-based alkali (Chang *et al.* 1998; Kaar and Holtzapple, 2000). Lime also removes acetyl groups that have been shown to affect hydrolysis rates. The remove of lignin improves enzymatic breakdown of cellulose through opening up the structure and reducing non-productive cellulase adsorption (Yang and Wyman, 2008). Steam explosion of woody biomass is a representative combined method. The cell wall of woody biomass is destroyed by the explosion process, and then lignin is removed with aqueous alkaline solution to yield the exploded pulp as a substrate (Morjanoff and Gray, 1987; McMillan,

1994; Duff and Murray, 1996). All the pretreatment has pervasive impacts on all other major operation in the overall conversion scheme for ethanol production. But only those that employ chemical currently offer the high yields and low cost also economically feasible (Yang and Wyman, 2008).

1.4 Enzymatic saccharification

Cellulolytic enzyme, cellulase, mainly consists of three types of enzyme: Endoglucanases (EG, known as non-processive type enzyme) (EC 3.2.1.4), which randomly hydrolyses β -1,4-D-glucosidic linkages in the cellulose chain; Cellobiohydrolases (CBH) (EC3.2.1.91; known as processive type of endoglucanase), which moves along the cellulose chain and cleave off cellobiose unit from the end of chain; β -glucosidases (BG, EC 3.2.1.21), which hydrolyse cello-oligosaccharides, cellobiose in particular, to glucose. These three groups of enzymes work synergistically to degrade cellulose so that they create new substrate for each other and preventing product inhibition (Eriksson *et al.*, 2002b; Våjamä *et al.*, 2003). An outline of hydrolysis process by cellulase is shown in Figure 1-5.

This enzymatic saccharification is conducted under mild conditions mentioned above, therefore, it is considered as an environmentally-friendly process. However, the cost-down of this saccharification process is an urgent problem to produce inexpensive bioethanol as an alternative liquid fuel. The cost of cellulase accounts for 25% to 50% of the total cost for ethanol production (Himmel *et al.* 1997).

CELLULASES

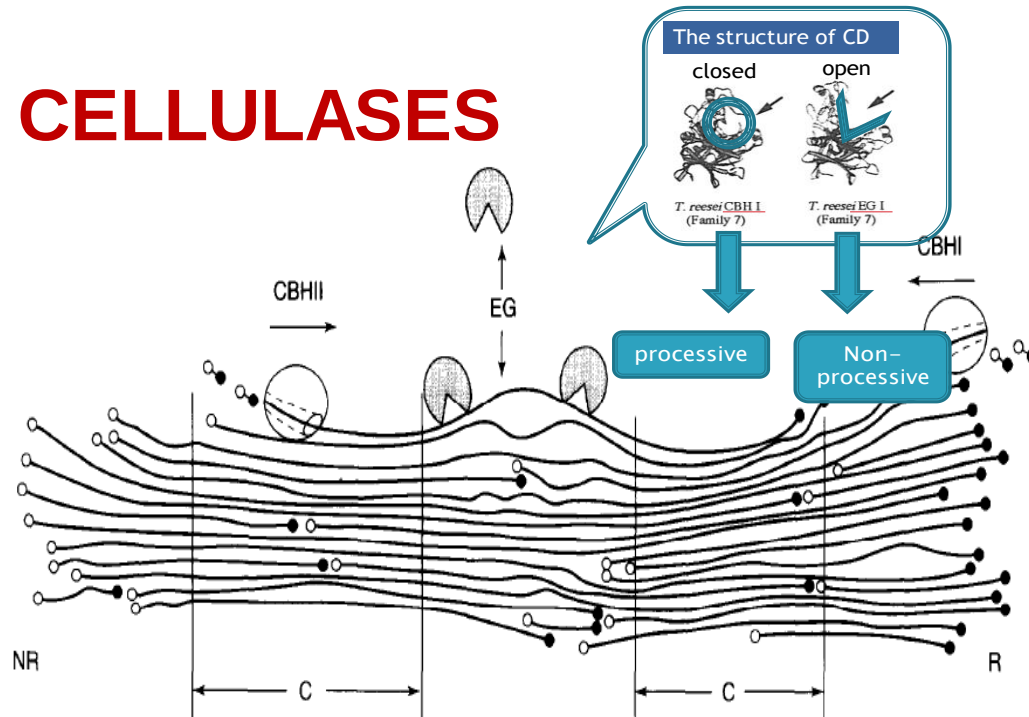


Figure 1-5. Three types of enzyme of cellulase

Source : <http://mmbr.asm.org/content/66/3/506/F1.expansion.html>

Taking enzyme cost and the following fermentation to ethanol into consideration, there are three major subjects to investigate on the improvement of enzymatic hydrolysis: (1) to increase the substrate concentration to get concentrated glucose solution, (2) to suppress the end product inhibition of the enzymes, and (3) to minimize the enzyme loadings. Of course, an increase in the dosage of cellulases can enhance the yield and rate of the hydrolysis, but would

significantly increase the cost of the process (Sun and Cheng, 2002). To overcome the 3rd subject, the following solutions are proposed; (1) development of cheaper enzymes; (2) increasing the cellulase activity by optimization of the enzyme constituent (Sun and Cheng, 2002); (3) the decrease of unproductive enzyme binding (Eriksson *et al.*, 2002b) or (4) re-use enzyme (Lee *et al.* 1995). In addition, charges of hemicellulases also improve the cellulolytic saccharification, because an interaction between cellulose and hemicellulose are cleaved by the enzyme, leading to enhancement of accessibility of cellulase (Berlin *et al.*, 2005). However, it will give additional cost in enzymatic saccharification. Therefore, I considered that the effective saccharification process should be established by re-use of cellulase, and tried to search cellulase-aid agent to enable re-use of cellulase.

A major obstacle to unable re-use of cellulase is a decrease in cellulase activity during saccharification upon two types of cellulase adsorptions onto the substrate as mentioned above (Eriksson *et al.* 2002a; Li *et al.* 2012). One is a specific interaction based on the formation of enzyme-substrate complex. This interaction covers a reversible, productive adsorption of cellulase on the substrate for saccharification and an irreversible, non-productive adsorption that reduces residual cellulase activity (Kristensen *et al.* 2007; Ma *et al.* 2008; Jalak and Väjämäki 2010). The other one is a non-specific interaction between cellulase and residual lignin in the substrate, which is also an irreversible, non-productive adsorption. The interaction is caused by hydrophobic interaction and hydrogen bonding (Kristensen *et al.* 2007; Börjesson *et al.* 2007; Xu *et al.* 2008). Therefore,

the suppression of the non-productive adsorptions is one of the targets to enable re-use of cellulase, leading to that enzymatic saccharification become more economically feasible (Das *et al.* 2011).

1.5 Surfactant and amphipathic lignin derivatives as a cellulase-aid agent

Surfactants are amphiphilic compounds that usually contain a hydrophilic head and a hydrophobic tail. Surfactants are able to self-assemble into micelles and will adsorb onto surface of guest molecule. The assembly and degree of adsorption depends on the surfactant structure and the polarity of the guest surface. Such surfactants have been reported to have a positive effect on enzymatic hydrolysis of lignocellulose, resulting in a faster hydrolysis rate and enabling lower enzyme dosage (Castanon and Wilke, 1981; Ooshima *et al.*, 1986; Park *et al.*, 1992; Helle *et al.*, 1993; Wu and Ju, 1998; Zheng *et al.*, 2008). In addition, surfactants makes re-use of cellulase possible (Otter *et al.*, 1989; Tu *et al.*, 2007). The positive effect of surfactants has been observed not only for enzymatic hydrolysis of cellulose (Helle *et al.*, 1993), but also for a process of simultaneous saccharification and fermentation (SSF) (Alkasrawi *et al.*, 2003). Thereby, I consider surfactants as “cellulase-aid agent, which reinforces cellulolytic activity of cellulase.

Recently, the effort to elucidate the positive effect of surfactants on the improvement of enzymatic hydrolysis is being made intensively (Eriksson *et al.* 2002a; Börjesson *et al.* 2007). The proposed mechanisms are summarized as follows: 1) Surfactants may act as enzyme stabilizers and prevent denaturation. 2)

Surfactants may have an effect on substrate structure, i.e. a surface structure modification or disruption that increases enzyme accessibility. 3) Surfactants may affect enzyme-substrate interactions, in particular by preventing non-productive adsorption of enzymes (Ballesteros *et al.* 1998; Alkasrawi *et al.* 2003; Zhang *et al.* 2009; Helle *et al.* 1993).

Furthermore, surfactants can be used for a process of bioethanol production with various purposes as follows: i) promotion of delignification in physicochemical and biological process as a pretreatment (Kurakake *et al.* 1994) ii) increase in the enzyme production (e.g. cellulase and xylanase), iii) enzyme recovery (Liu *et al.* 2006; Shin *et al.* 2004; Tu and Saddler, 2009), and iv) enhancement of saccharification efficiency (Eriksson *et al.* 2002a; Zheng *et al.* 2008; Zhang *et al.* 2009).

Immobilization of cellulase is also one of the possible concepts to enable its repeated use (Karube *et al.* 1977). However, when cellulase was immobilized onto a solid polymeric support, saccharification efficiency was decreased because of a solid-solid phase reaction between the immobilized cellulase and a lignocellulosic substrate (Wongkhalaung *et al.* 1985). To overcome this drawback, an alternative system was proposed: cellulase was immobilized onto a water-soluble polymeric support, which enabled to improve saccharification efficiency and recovery of cellulase (Woodward 1989). According to Uraki *et al.* (2001) a more useful, water-soluble polymeric supporter, an amphipathic lignin derivative, was developed by the reaction of lignin with poly(ethylene glycol) diglycidyl ether (PEGDE). When cellulase was used together with PEGDE-lignin in a

saccharification system for unbleached pulp, the sugar yield was higher than without the PEGDE-lignin. In addition, 80% of the cellulase activity was recovered after the fifth re-use of the cellulase. In recent research, other two types of amphipathic lignin derivatives with a higher surface activity than PEGDE-lignin been developed by the reaction of several isolated lignins with ethoxy-(2-hydroxy)-propoxy-poly(ethylene glycol) glycidyl ether (EPEG), and dodecyloxy-poly(ethylene glycol) glycidyl ether (DAEO) (Homma, *et al.* 2008). Such lignin derivatives are expected to have superior ability to improve enzymatic saccharification

When the structure of the surfactants was further investigated (Börjesson *et al.*, 2007), it was found that the enhancing effect has increased when the ethylene-oxide (EO) chain (hydrophilic tail) of the surfactant was longer. As the effect was rather dependent of the length of hydrophilic EO tail (Börjesson *et al.*, 2007) instead of the hydrophobic part (Helle *et al.*, 1993), pure poly(ethylene oxide) polymer and poly(ethylene glycol) were also studied and found to increase the hydrolysis of steam-pretreated spruce (SPS). In a study by Kristensen *et al.* (2007) different non-ionic surfactants and PEG 6000 were screened in hydrolysis of wheat straw pretreated with various techniques and PEG was found to be efficient in the increase of cellulose conversion and the free endoglucanase concentration after hydrolysis. PEG 4000 was also tested as an additive in water and dilutes acid pretreatment of corn stover (Qing *et al.*, 2010). Recently, it was found that PEG 4000 was not a surfactant because of the low of its surface activity. In this thesis, I would like to show the different effect of addition of

amphipathic lignin derivatives in enzymatic hydrolysis of Japanese cedar and sago waste pulps.

1.6 Research objectives

Unused cedar wood derived by tree thinning and fibrous sago waste that disposed from sago mills seemed to be promising lignocellulosic for bioethanol production through enzymatic saccharification. A major objective in this thesis is to develop efficient saccharification process for two lignocellulosics with amphipathic lignin derivatives. The enzymatic saccharification for cedar wood, including pretreatment of cedar wood for converting it into substrate, is described in Chapter 2, and the saccharification of sago palm waste after the starch extraction is described in Chapter 3, which includes pretreatment of sago palm waste and preparations of amphipathic lignin derivatives from sago lignin.

In Chapter 2, I configured following sub-objectives and research items:

1. To select a suitable cellulase for the enzymatic saccharification of cedar wood after pretreatment from the commercially available cellulases.
2. To evaluate the effectiveness of amphipathic lignin derivatives on the saccharification, and to compare it with that commercial available surfactant, PEG 4000.
3. To propose the action mechanism of amphipathic lignin derivatives to cellulase.

In Chapter 3, I also configured following sub-objectives and research items:

1. To synthesize amphipathic lignin derivatives from sago lignin

2. To evaluate the effectiveness of the sago lignin derivatives to the saccharification of sago palm waste after pretreatment.
3. To examine the possibility of re-use of cellulase that recovered from the hydrolysate of sago waste.

Finally, I discuss effectiveness of amphipathic lignin derivatives on the both saccharifications, and propose efficient saccharification process as a first process of bioethanol production.

CHAPTER 2

Improvement of enzymatic saccharification of unbleached cedar pulp with amphipathic lignin derivatives

2.1 Introduction

Our dependence on fossil resources and concomitant release of greenhouse gases causing climate change have put an intense focus on exploitation of alternative renewable resources for the production of fuels and chemicals (Horn and Eijsink, 2010). Japanese cedar (*Cryptomeria japonica*) is a major wood species of the softwood plantation in Japan (Baba *et al.* 2011). To effectively use the unused timber from forest thinning, bioethanol production from low-quality wood is one of the major researches in Japan from the viewpoint of biorefinery. The Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Japan, has built a pilot plant in Akita prefecture for bioethanol production from cedar wood through enzymatic saccharification with cellulase. In this pilot plant, soda pulping is used as a delignification process (Magara *et al.* 2010). A dominant obstacle for the saccharification process lies in the high price of cellulase, which accounted for 60% of the total process cost for bioethanol production in the 1980's (Deshpande *et al.* 1984a).

Immobilization of cellulase is one of the possible concepts to enable its repeated use (Karube *et al.* 1977) for cost reduction of cellulase. However, when cellulase was immobilized onto a solid polymeric support, saccharification

efficiency was decreased because of a solid-solid phase reaction between the immobilized cellulase and the lignocellulosic substrate (Wongkhalaung *et al.* 1985). To overcome this drawback, an alternative system was proposed: cellulase was immobilized onto a water-soluble polymeric support to improve efficiency of saccharification and recovery of cellulase (Woodward 1989).

Based on the concept of water-insoluble immobilized enzyme, our research group reported a more useful, water-soluble polymeric support in 2001; as such a polymer, an amphipathic lignin derivatives was prepared by the reaction of lignin with poly(ethylene glycol) diglycidyl ether (PEGDE) (Uraki *et al.* 2001). When cellulase was used together with PEGDE-lignin in a saccharification system for unbleached pulp, the saccharification efficiency was higher than without PEGDE-lignin; in addition, 80% of cellulase activity was recovered after the fifth times re-use of the cellulase from enzymatic saccharification. In 2007, polyethylene glycol (PEG) with its molecular mass of more than 4000 (PEG4000) was reported to improve the cellulolytic saccharification of steam-pretreated spruce: 50% of the substrate was hydrolyzed during saccharification process without PEG4000, while 78% with PEG4000 (Börjesson *et al.* 2007). In addition, PEG4000 increased the residual filter paper activity: only 5% of filter paper activity was found to be maintained after saccharification process without PEG4000, while 56% with PEG4000 (Sipos *et al.* 2010). Another study, using wheat straw lignocelluloses as a substrate, also reports that the cellulose conversion was improved from 51% (without PEG 6000) to 65% (with PEG6000) (Kristensen *et al.* 2007). The usefulness of PEG was reported to be attributable to

prevention of the non-productive association between the cellulase and the substrate caused by hydrogen bonding (Xu *et al.* 2008; Li *et al.* 2012). Similarly, Tween 80, a non-ionic surfactant, was also reported to facilitate hydrolytic enzyme recycling for pretreated lodgepole pine. The result showed that the addition of Tween 80 could save 60% of the total enzyme cost for the saccharification of ethanol-pretreated lodge-pole pine (Tu and Saddler 2009). Thus, non-ionic surfactants are expected to be chemical adjuncts to reduce the hydrolysis cost of lignocellulosics.

Recently, Bardant *et al.* (2010) reported the effect of PEGDE-lignin on the saccharification of unbleached cedar pulp and the pulp derived from empty fruit bunch of oil palm, which were produced as a partially delignified substrate by the aforementioned pilot plant and Indonesian Institute of Sciences (LIPI) (Sudiyani *et al.* 2013). In addition, two other types of amphipathic lignin derivatives with a stronger surface activity than PEGDE-lignin were developed; EPEG- and DAEO-lignin reported by Homma *et al.* (2008 and 2010). The other types of lignin derivatives are expected to improve the efficiency of saccharification more significantly because of a higher surface activity, although the mechanism of how amphipathic lignin derivatives work during the saccharification has not been determined yet. In this chapter, the influence of amphipathic lignin derivatives on the cellulolytic saccharification of unbleached cedar pulp was investigated and compared with the effect of PEG4000. In addition, the interaction between amphipathic lignin derivatives and cellulase was also examined by using the

Biacore[™] System, an analytical instrument based on surface plasmon resonance (SPR), to clarify the function of the lignin derivatives on the saccharification.

2.2 Experimental

2.2.1 Unbleached cedar pulp as a substrate for enzymatic saccharification

Unbleached cedar pulp was prepared by soda pulping of cedar chips (1 kg) with 260 g of NaOH in 6 L of water (Bardant *et al.* 2010). The time to cooking temperature was 90 min (170 °C), and the time at cooking temperature was 150 min. The crude pulp was washed with 1% NaOH and water, successively, and filtered by pressing to a moisture content of 70%. The pulp was lyophilized to remove the residual moisture to yield a dry pulp. The pulp's lignin content was determined using the modified of Klason method (TAPPI T-222 om-83), as insoluble residue through filtration. The concentration of sulphuric acid becomes 4% because the sample was placed in an autoclave at 121 °C for 1 h. The filtrate was subjected to neutral sugar analysis after the neutralization with Ba(OH)₂ followed by removal of precipitate (Slavin and Marlett, 1983).

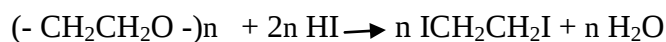
The determination of sugars was carried out on an HPLC device (Shimadzu LC10 System, Kyoto, Japan) equipped with a corona charged aerosol detector (ESA Biosciences Inc., Chelmsford, MA, USA). The column used was Shodex SUGAR SP0810 (7.8-8.0 mm ID x 300 mm) with SUGAR SP-G as a guard column (Showa Denko Co. Ltd., Tokyo, Japan), and the column

temperature was 80 °C. The eluent used was Milli-Q purified water, and its flow rate was set at 0.5 mL/min. The injection volume was 20 µL.

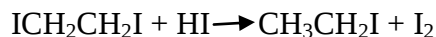
2.2.2 Amphipathic acetic acid derivatives (AL)

Acetic acid lignin (AL) was prepared from the spent liquor of the atmospheric acetic acid pulping of birch wood (Uraki *et al.* 1991). Amphipathic lignin derivatives were prepared by the reaction of AL with poly(ethylene glycol) diglycidyl ether (PEGDE), ethoxy-(2-hydroxy)-propoxy-poly(ethylene glycol) glycidyl ether (EPEG), or dodecyloxy-poly(ethylene glycol) glycidyl ether (DAEO), as shown in Fig. 2-1, in aqueous 1 M NaOH solution according to a previous report (Homma *et al.* 2010). EPEG was prepared according to a previous report (Homma *et al.* 2010). After the derivatization, the reaction solution was acidified to pH 4, and purified by ultrafiltration (cut-off molecular mass, 1000 Da, Advantec). Finally, the residues from ultrafiltration which is amphipathic lignin derivatives were lyophilized to remove the remaining water (Fig. 2-2). PEG contents of PEGDE-, EPEG-, and DAEO-AL were determined by the modified Morgan method (Homma *et al.* 2008; Siggia *et al.* 1958; Morgan 1946). This measurement procedure and theoretical mechanism are described as follows.

The major reaction for calculating PEG content was to cleave the ethylene oxide chain by the action of hydriodic acid (HI). The measurement equipment for PEG content is shown in Fig. 2-3. The reaction in the measurement is below:



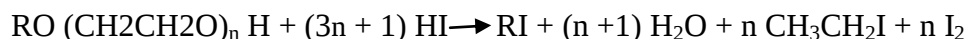
or



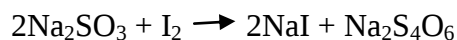
Therefore,



or



On the other hand, reduction of Iodine which is generated



We calculated the percentage of EO chain (%), 1 mol of AL or SSL

$$\text{EO (\%wt)} = \frac{(S - B)}{1000} \times 0.1 \text{ mol/L} \times \frac{44}{2} \times \frac{1}{W_{\text{sample}}} \quad (2-1)$$

EO (%) : Amphipathic lignin derivatives ethylene oxide content (%)

PEG (mol/AL 1 mol) : PEG content

S : titration volume of sample (mL)

B : titration volume of blank (mL)

w : weight of sample (g)

The obtained PEG contents of PEGDE-, EPEG-, and DAEO-AL were 61.2, 72.3, and 68.4%, respectively.

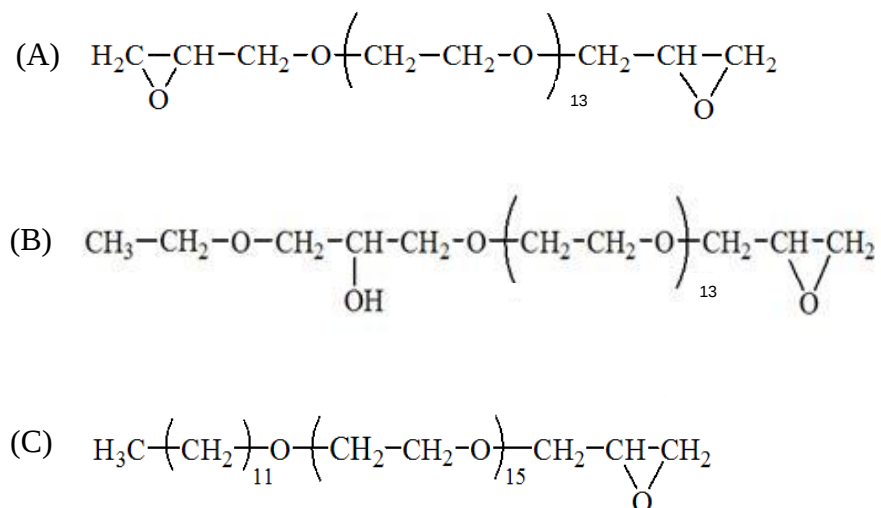


Fig. 2-1. Chemical structure of (A) poly(ethylene glycol) diglycidyl ether (PEGDE), (B) ethoxy-(2-hydroxy)-propoxy-poly(ethylene glycol) glycidyl ether (EPEG) and (C) dodecyloxy-poly(ethylene glycol) glycidyl ether (DAEO)

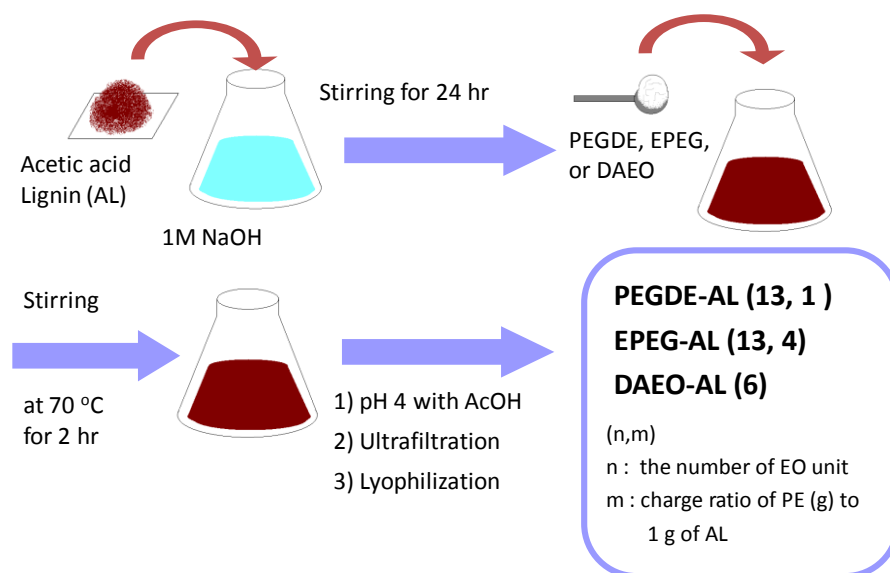


Fig. 2-2. Preparation of amphipathic lignin derivatives

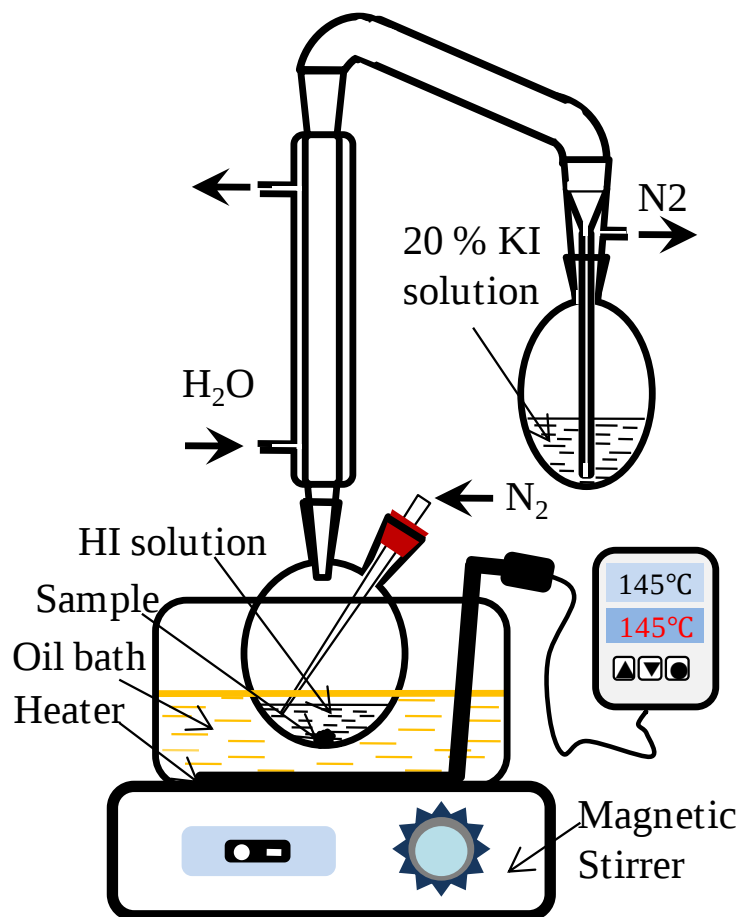


Fig. 2-3. Apparatus for determination of PEG moiety in amphipathic lignin derivatives.

2.2.3 Enzymes and measurement of their activities

Two types of commercially available cellulases, Meicelase (powder form; Meiji Seika Co. Ltd., Tokyo, Japan) and Genencor GC220 (liquid form; Genencor

International Inc., USA; Lot # 4901121718), were used for the enzymatic saccharification. The cellulolytic activity expressed as filter paper unit (FPU) which was measured using the NREL technical report, NREL/TP-510-42628, method (Ghose 1987). The enzyme activity of Meicelase and Genencor GC220 as received were 0.24 FPU/mg and 66.1FPU/mL, respectively.

β -Glucosidase activity of the cellulases was measured according to Berghem and Pettersson method (1974), using the substrate *p*-nitrophenyl β -D-glucopyranoside (*p*NPG). Endoglucanase (EG) activity was measured using 2% carboxymethyl cellulose in 0.05 M sodium citrate buffer (pH 4.8) (Ghose *et al.* 1987). The activity of Cel5A (EG II) and Cel7A (CBH I) was measured using *p*-nitrophenyl β -D-lactoside (*p*NPL) as substrate in accordance with Deshpande *et al.* (1984b).

2.2.4 Saccharification of unbleached cedar pulp

Each amphipathic lignin derivative (10% of substrate on dry weight basis), as well as PEG4000 (purchased from Wako Pure Chemicals (Osaka, Japan)), was dissolved in 150 mL of 50 mM citrate buffer (pH 4.8). Cellulase (10 FPU/g, and 20 FPU/g of substrate) was added to the solution, and the mixture was stirred for 1 h in room temperature. Finally, 1.5 g of unbleached pulp was added to the solution, and the suspension was gently shaken at 50 °C for 48 h. After saccharification, the suspension was filtered through a G4 glass filter. The precipitate was washed three times with the buffer solution, and weighed after

complete drying at 105 °C. The saccharification efficiency (SE) was calculated, using the following equation:

$$\text{Saccharification efficiency (SE) (\%)} = (\text{WS} - \text{WR}) \times 100 / \text{WS} \quad (2-2)$$

where WR is the weight of residue (g) after saccharification, and WS is the initial weight of substrate (g).

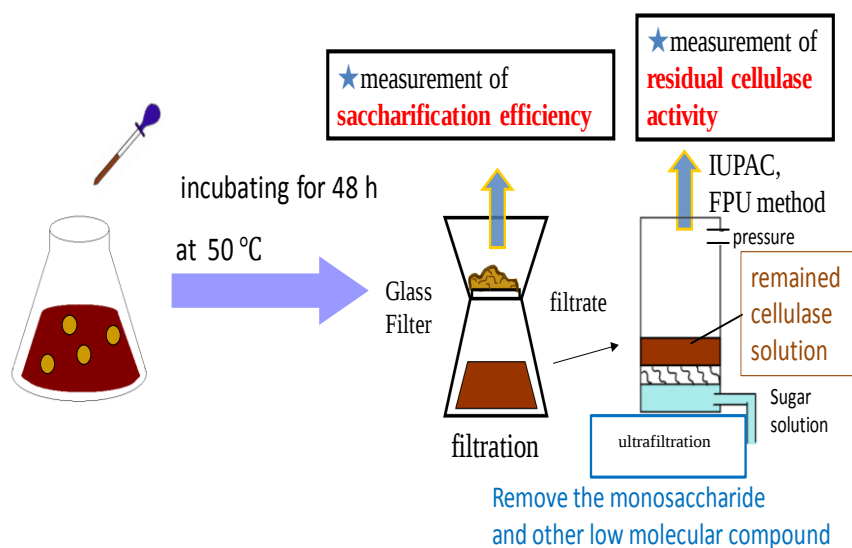


Fig. 2-4. Saccharification of unbleached pulp

The filtrate was subjected to ultrafiltration with a membrane filter (cut-off molecular mass 1000 Da, Advantec). The composition and concentration of neutral sugars in the filtrate were determined by using the HPLC method mentioned above. The residual enzyme solution (*ca.* 30 mL), as an unfiltered fraction concentrate, was diluted with 250 mL of the buffer solution, and subjected again to ultrafiltration up to 30 mL of unfiltered fraction. This process

was repeated three times. Finally, the recovered enzyme solution was further ultrafiltered to the volume of 15 mL (Fig.2-4). The FPU of the concentrated enzyme solution was measured, and a recovered activity of cellulase (%) was calculated as a ratio of remaining activity to initial cellulase activity using the following equation:

$$\text{Recovered activity (\%)} = \frac{\text{FPU after saccharification (unit)}}{\text{FPU of initial cellulase (unit)}} \times 100 \quad (2-3)$$

2.2.5 Interaction between amphipathic lignin derivatives or PEG4000, and CBH II (Cel6A)

CBH II (Cel6A) was isolated from Novozyme NS50013 in accordance to literature reports (Reinikainen *et al.* 1995; Samejima *et al.* 1997). The isolated enzyme was immobilized on a sensor chip CM-5 by amino coupling in a Biacore-X (GE, Healthcare, Japan).

Briefly, the carboxyl group was converted to an active ester form with *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. The enzyme in 1 M citrate buffer solution (pH 4.5) was introduced into a flow cell (flow cell 1), which runs on the surface of the activated sensor chip. After washing with the buffer, ethanolamine hydrochloride solution was introduced into the flow cell to block the unreacted active ester. The other flow cell (flow cell 2) was also blocked with ethanolamine hydrochloride without enzyme immobilization (Fig. 2-5). Finally, each of the amphipathic lignin derivatives and PEG4000 in 50 mM citrate buffer solution were introduced to

both flow cells up to a constant adsorption at a flow rate of 20 $\mu\text{L}/\text{min}$ and 37 $^{\circ}\text{C}$, and then only buffer solution was introduced for desorption. An adsorption-desorption isotherm was obtained by the difference in the adsorbed amount between the flow cell 1 and 2. A binding constant was calculated by using BIA evaluation software ver. 4.1 (GE, Healthcare, Japan).

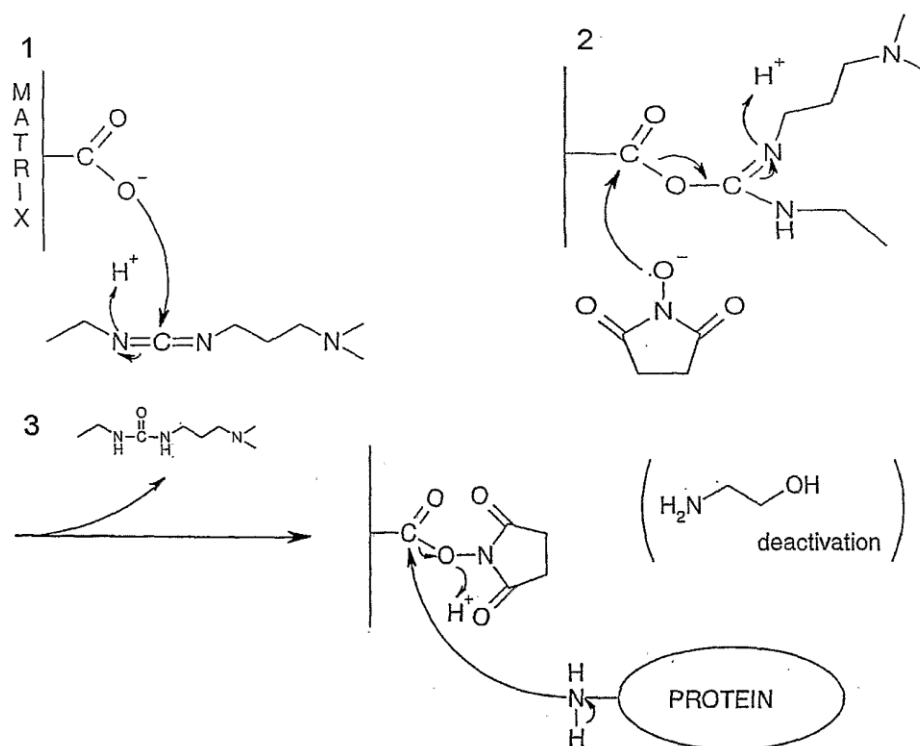


Fig.2-5. The scheme of enzyme immobilization

The complete desorption of adsorbed lignin derivatives or PEG4000 on sensor chip was carried out with an aqueous 0.1 wt.% Triton X-100 solution, and the regenerated sensor chip was used repeatedly for adsorption-desorption experiments.

Molecular masses of amphipathic lignin derivatives were measured on a high-performance size-exclusion chromatography multi-angle laser light scattering (SEC-MALLS; Wyatt, Santa Barbara, CA, USA), with a refractive index detector and a light-scattering detector. Three columns of Shodex K-805L (Showa Denko Co. Ltd., Tokyo, Japan) were connected and used. The column temperature was 40 °C. The eluent was chloroform, and flow rate was 1.0 mL/min.

2.3 Results and discussion

2.3.1 Cellulolytic saccharification of unbleached cedar pulp

Three types of lignin derivatives (EPEG-AL, DAEO-AL, and PEGD-AL) and PEG4000 were employed for the cellulolytic saccharification of unbleached cedar pulp using two types of commercially available cellulases. The unbleached cedar pulp had a Klason lignin content of 6.75%, and a neutral sugar content of 75.1% for glucose and 6.9% for mannose.

Figure 2-6 (A and B) shows the saccharification efficiency (SE) at the cellulase activity of 10 FPU/g of substrate. Comparing SEs of the two cellulases without additive, GC220 showed higher SE than the Meicelase. PEG4000 dramatically improved the SEs of both cellulases, although the amphipathic lignin derivatives also improved SEs significantly. Interestingly, compared to the

controls, PEGDE-AL apparently showed a negative effect on SE at 20 FPU/g (Fig. 2-6 (C and D), while PEGDE-AL gave a positive effect on SE at 10 FPU/g. This negative effect is further discussed in a later section.

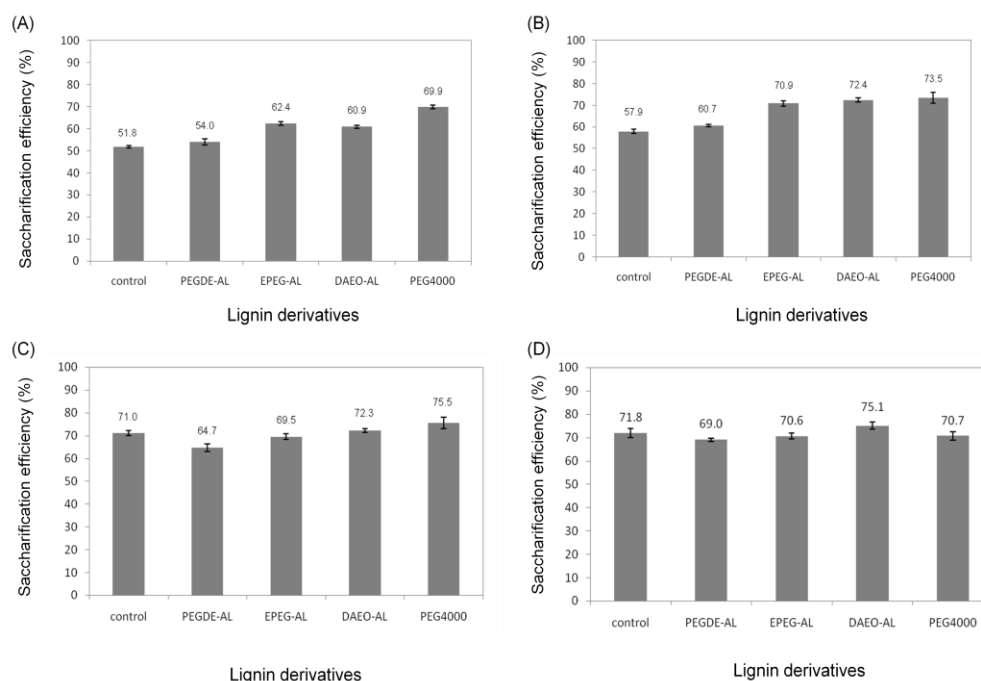


Fig. 2-6. Saccharification efficiency of unbleached cedar pulp, using Meicelase (A) and Genencor GC220 (B) at the initial cellulase activity 10 FPU/g substrate; Meicelase (C) and Genencor GC220 (D) at the initial cellulase activity 20 FPU/g substrate at pH 4.8 and 50 °C.

However, the higher positive effect of PEG4000 was only observed for the SE of Meicelase at 20 FPU/g of substrate, as shown in Fig. 2-6 (C and D). In the case of GC220 at 20 FPU/g of substrate (Fig. 2-6 B), DAEO-AL showed the

highest SE of all the additives examined. In the filtrate, only a water-soluble product detected from the saccharification media by HPLC was glucose. The maximum yield of glucose obtained (75.1%), using DAEO-AL, was almost identical to the glucose content measured in the cedar pulp (75.1%). Thus, cellulose in the unbleached cedar pulp was almost quantitatively hydrolyzed.

The order of increasing surface activity was reported by Homma *et al.* (2010) as PEGDE-AL < EPEG-AL << DAEO-AL. Therefore, these results suggested that SE may be related to surface activity and that PEG4000 was not necessarily the best additive to improve SE for the saccharification of cedar pulp.

2.3.2 Recovery of cellulase activity after one-time saccharification of cedar pulp

Figure 2-7(A-D) show the recovery of cellulase activity after one-time saccharification at the initial activity of 10 and 20 FPU/g of substrate, respectively. Addition of all lignin derivatives (EPEG-AL, DAEO-AL, and PEGDE-AL) obviously improved the recovery of cellulase activity. In particular, in the case of GC220 at 20 FPU/g of substrate, PEGDE-AL and EPEG-AL facilitated the complete recovery of cellulase activity (Fig. 2-7D). In the case of Meicelase at 20 FPU/g of substrate, however, PEGDE-AL and EPEG-AL did not show 100% recovery, though they improved recovery of cellulase activity relative to the control. This difference in recovery of cellulase activity between Meicelase and GC220 is discussed in a later section.

On the other hand, PEG4000 also had a positive effect on the recovery of cellulase activity. However, the increment with PEG4000 was much smaller than with the lignin derivatives. High recovery of cellulase activity by lignin derivatives is expected to enable repeated use of the cellulase, although it is necessary to separate it from the glucose in the saccharification medium.

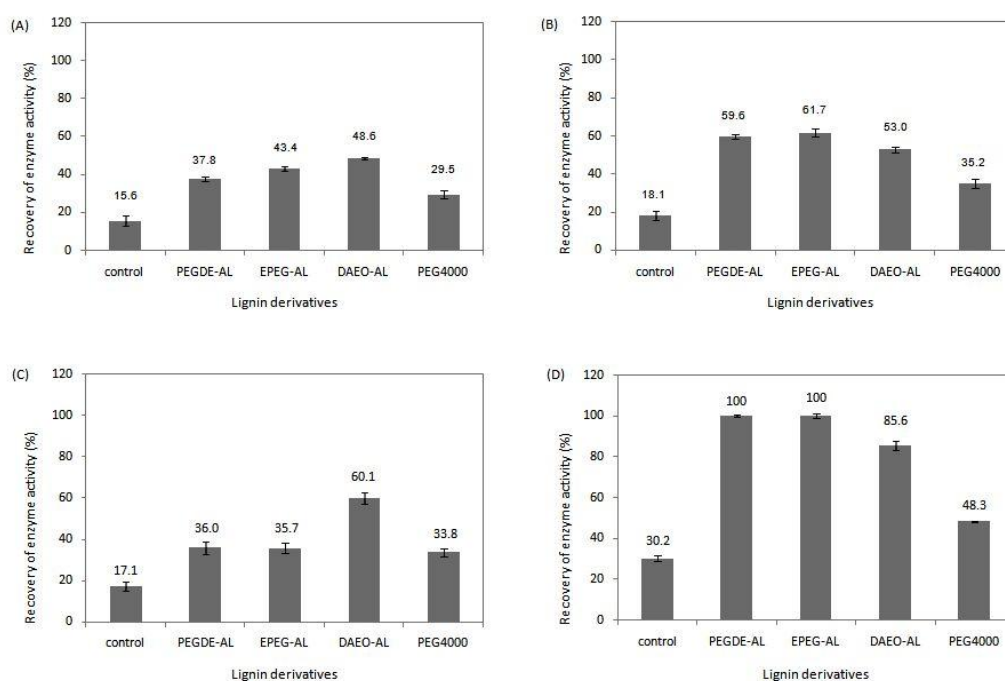


Fig. 2-7. Recovery of cellulase activity of unbleached cedar pulp, using Meicelase (A) and Genencor GC220 (B) at the initial cellulase activity 10 FPU/g substrate; Meicelase (C) and Genencor GC220 (D) at the initial cellulase activity 20 FPU/g substrate at pH 4.8 and 50 °C.

2.3.3 Interaction of amphipathic lignin derivatives and PEG4000 with CBH II

The cellobiohydrolases, Cel6A and Cel7A, which have both catalytic domain and cellulose-binding domain, are major cellulase components secreted from *Trichoderma reesei* (Palonen *et al.* 1999; Watson *et al.* 2009; Igarashi *et al.* 2011). To investigate the direct interaction between the cellobiohydrolases and the additives to improve saccharification of lignocellulose, we successfully isolated pure Cel6A from a commercial cellulase, and immobilized it on a sensor chip to be studied by surface plasmon resonance (SPR). The immobilized amount of the enzyme was 2024 RU (1 RU = 1 pg/mm²). Each citrate buffer solution containing amphipathic lignin derivatives and PEG4000 as analytes was applied to the resultant sensor chip to monitor their adsorption and desorption behaviors.

Figure 2-8 shows the sensorgrams of the adsorption and desorption processes of the analytes. A separate experiment indicated that DAEO-AL adsorbed onto Cel6A much more significantly than the other amphipathic lignin derivatives when measured at the same concentration, which made accurate adsorption-desorption measurements impossible. Thus, the adsorption-desorption behavior of DAEO-AL was measured at one-hundredth concentration range (0.001 to 0.008 g/L).

The amount of desorption of DAEO-AL, EPEG-AL, and PEGDE-AL was 14%, 24%, and 32% of the amount of the adsorption, respectively, at the highest

concentration charged. It is assumed from these results that DAEO- and EPEG-AL have a higher affinity to Cel6A than PEGDE-AL. On the other hand, PEG4000 showed very little adsorption even at the highest order of concentration (1 g/L). Furthermore, 66% of the adsorbed PEG4000 was released. This result suggested that PEG4000 does not directly interact with the active site(s) of the cellulase enzyme.

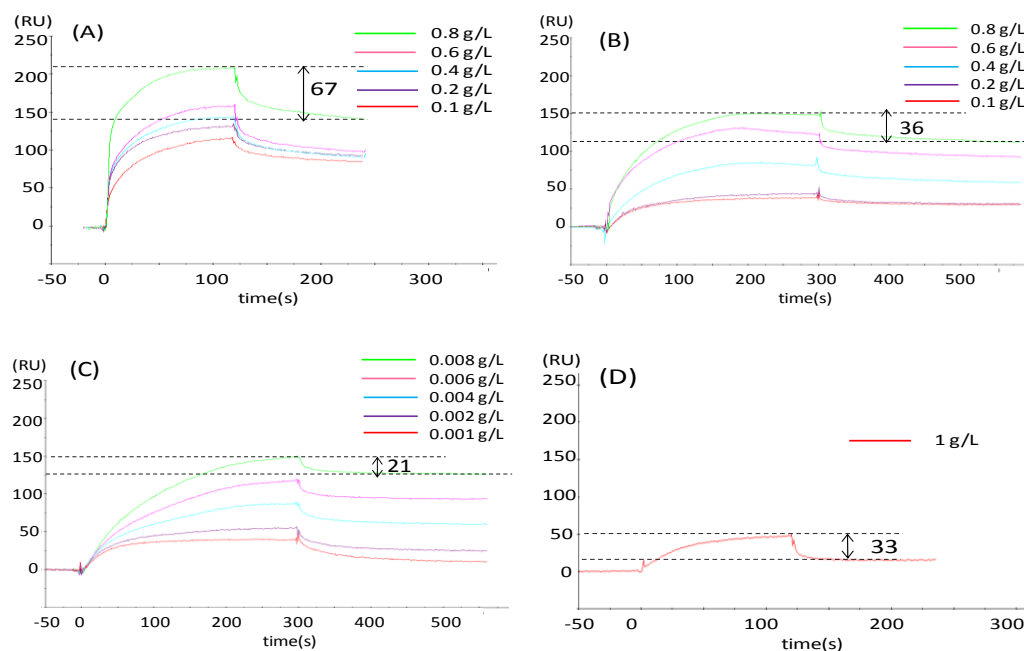


Fig. 2-8. Sensorgram showing the interaction between Cel6A immobilized on a CM5 sensor chip and the amphipathic lignin derivatives as analytes:(A)PEGDE-AL; (B) EPEG-AL; (C) DAEO-AL; and (D) PEG4000.The arrows and values in the figures indicated desorbed amount at the highest concentration of analytes.

In order to compare the binding affinity of the lignin derivatives with cellulase, we calculated a binding constant of the lignin derivatives to Cel6A on the basis of the following assumptions. First, each lignin derivative binds with cellulase in such a manner as one molecule-to-one molecule (one-on-one) interaction. Second, two types of weight-average molecular mass (M_w) for each lignin derivative obtained by SEC-MALLS were used for the calculation; one was obtained by refractive-index detector (RI), while the other by light-scattering detector (LS). The latter value may be overestimated, since this light scattering detector did not have a filter to eliminate self-fluorescence of lignin. The calculated results are shown in Table 2-1. The big difference in M_w between RI and LS might be attributed to lignin conformations in the solution. Isolated lignins showed a very small exponential parameter in Mark-Houwink-Sakurada equation (Goring 1971). In addition, an amphipathic polymeric molecule has a dense structure upon self-aggregation. Therefore, RI showed smaller values. EPEG- and DAEO-AL have four to six digits greater binding constants (K_a -RI and K_a -LS) than PEGDE-AL, as anticipated. The binding constant, K_a , might be related to surface activity. The high affinity affected the saccharification efficiency and recovery of enzyme activity.

Table 2-1. Weight-Average Molecular Mass of the Amphipathic Lignin Derivatives Measured by SEC-MALLS and Binding Constant Calculated on the Basis of One Molecule-to-One Molecule Interaction

Lignin Derivatives	RI (Mw)	K _a -RI (1/M)	LS (Mw)	K _a -LS (1/M)
PEGDE-AL	1260	4.01 x 10 ⁶	2.05 x 10 ⁵	1.74 x 10 ⁴
EPEG-AL	1460	8.38 x 10 ¹⁰	7.79 x 10 ⁶	1.05 x 10 ⁹
DAEO-AL	1900	1.17 x 10 ¹²	1.59 x 10 ⁶	7.04 x 10 ⁹

RI, Weight-average molecular mass was obtained using a refractive-index detector. LS, Weight-average molecular mass was obtained using a light-scattering detector. K_a-RI and K_a-LS are binding constants calculated on the basis of Mw obtained by RI and LS, respectively.

2.3.4 Mechanism on the improvement effect of amphipathic lignin derivatives on the cellulolytic saccharification

Cellulase consists of three major components, cellobiohydrolase, endoglucanase, and β -glucosidase (Becham *et al.* 2012; Jalak *et al.* 2012). Endoglucanases (EG; Cel5A and Cel7B) and cellobiohydrolases (CBH; Cel6A and Cel7A) are the main cellulase components to depolymerize cellulose chain. On the other hand, β -glucosidase cleaves oligosaccharides to yield glucose and does not have a cellulose-binding domain (CBD), which points to a low affinity towards cellulose as a solid substrate (Palonen *et al.* 2004).

Genencor GC220 consists of larger amounts of EG and CBH (163 unit/FPU for CMC and 3.5 unit/FPU for *p*NP-lactoside) than Meicelase, whereas Meicelase is rich in β -glucosidase (Table 2-2). Table 2-3 shows all lignin derivatives had a positive effect on FPU activity of Genencor GC220, but a negative effect on that of Meicelase except for EPEG-AL. These effects of the lignin derivatives on the enzyme activity suggested that the lignin derivatives activated endoglucanase activity upon the direct association, as mentioned above, and suppressed β -glucosidase activity. The difference in the action of lignin derivatives to the cellulases would affect the recovery of the FPU activity after saccharification.

FPU is defined as the amount of enzyme required to liberate 1 μ mol of glucose from cellulose per min. The generated glucose is determined for initial 1h enzymatic hydrolysis in FPU measurement. This value is not an average amount of glucose production per min for 24-h or 48-h enzymatic saccharification. Therefore, an initial production rate of glucose affects FPU value. In the FPU measurement of Meicelase, production of cellobiose and related oligosaccharides must be a rate-limiting step because the glucose conversion quickly proceeds due to the fact that Meicelase is rich in β -glucosidase; endoglucanase is the key enzyme. On the other hand, β -glucosidase activity dramatically influenced the FPU measurement of GC220, because GC220 had only one-third the β -glucosidase activity of Meicelase.

Table 2-2. Hydrolytic Activities of Commercially Available Cellulases

Substrate	Meicelase (unit/FPU)	Genencor GC220 (unit/FPU)
CMC	67.0	163
<i>p</i> NP-lactoside	1.43	3.50
<i>p</i> NP-glucoside	8.20	2.65

CMC, Carboxymethyl cellulose is a substrate for endoglucanase activity measurement; *p*NP-lactoside, *p*-nitrophenyl β -D-lactoside is a substrate for Cel5A and Cel7A activity measurement; *p*NP-glucoside, *p*-nitrophenyl β -D-glucopyranoside is a substrate for β -glucosidase activity measurement. These enzyme activities (unit) were expressed on the amount of cellulase that gives 1 FPU.

In the saccharification at the initial cellulase activity of 10 FPU/g substrate, the activities of both commercial enzymes were insufficient to achieve complete saccharification (Fig. 2-7 A and B). Therefore, it was anticipated that cellulase adsorption onto substrate resulted in the reduction of residual FPU activity. In particular, endoglucanase adsorption in the saccharification with Meicelase would remarkably affect the reduction of residual FPU activity. In fact, the recovery of Meicelase (Fig. 2-7A), having smaller endoglucanase activity than GC220 (Table 2-2), was smaller than that of GC220 (Fig. 2-7B).

Table 2-3. FPU Activity of Commercially Available Cellulases in the Presence of Additives

Additives	Meicelase activity (FPU/mg of enzyme) ^{a)}	Genencor GC220 activity (FPU/mL of enzyme)
PEGDE-AL	0.189	96.9
EPEG-AL	0.238	85.1
DAEO-AL	0.198	76.7
control	0.211	66.1

^{a)} This activity measurement was conducted after one year storage in refrigerator at 4°C since it was received. Thereby, the control activity was slightly reduced than initial activity as received (0.24 FPU/mg of enzyme).

At 20 FPU/g, PEGDE- and EPEG-AL apparently showed the complete recovery of the GC220 activity (Fig. 2-7D). If the same amount of endoglucanase in Meicelase and GC220 adsorbed on the substrate, a significant amount of the residual endoglucanase still remains in GC220, which can supply sufficient amount of cellobiose as a substrate to β -glucosidase. Thus, no reduction of residual FPU activity in GC220 was observed under such conditions. On the other hand, the residual endoglucanase activity in Meicelase must be very small. Therefore, apparent reduction of FPU in Meicelase was clearly observed, even by the addition of such amphipathic lignin derivatives at the initial cellulase activity of 20 FPU/g substrate.

Finally, we discuss the lower SE in the presence of PEGDE-AL at 20 FPU/g than that of control in Fig. 2-6 (C and D). PEGDE, carrying two glycidyl

groups, bridges between lignin macromolecules. PEGDE-AL, then, has a hydrophobic domain more frequently than EPEG- and DAE0-ALs. The hydrophobic domain of PEGDE-AL may sometimes act as an inhibitor of cellulase, covering the active sites of the enzyme and preventing the substrate from insertion into the active sites. But it should be noted that interaction between PEGDE-AL and Cel6A is very weak (Table 2-1), and thus PEGDE-AL can promote the recovery of cellulase activity (Fig. 2-7D).

In this chapter, I confirmed that amphipathic lignin derivatives were found to improve the saccharification efficiency of lignocelluloses saccharification, as well as PEG4000. The amphipathic lignin dramatically improved the recovery of cellulase activity after one-time saccharification as compared with PEG4000. This effect was more significant for Genencor GC220 than Meicelase for enzymatic sccharification of unbleached cedar pulp. These results suggested that the lignin derivatives make it possible to use cellulase repeatedly. And the difference in the mechanism of the improvement of saccharification is the amphipathic lignin derivatives directly interacted with the enzyme, whereas PEG4000 did not.

CHAPTER 3

Enzymatic saccharification of sago pulp from starch extraction waste using sago lignin-based amphipathic derivatives

3.1 Introduction

Sago palm (*Metroxylon* sp.) is considered to be the most important plant species among all starch crops in the world, because of its high starch content and low extraction cost of starch (Ishizaki, 1997). The plantation of sago palm in Indonesia is about 1.2 million ha, and 90% is planted in the provinces of Papua and Maluku (Flach, 1977). Production of sago starch stands at 30-60 ton/ha/year, and the starch is used as an energy resource for bioethanol production as well as a food ingredient (Bintoro, 2003). Pith of sago palm is an inner tissue of the trunk and a storage site for starch. The pith is used as a feedstock for starch industry after the removal of the outer bark-like layer. Concomitantly, fibrous sago waste is exhausted as a byproduct in 4.75 million ton/year by starch mills (Safitri et al., 2009).

A conversion of the fibrous sago waste into fermentable sugars was attempted by a combination with acid and enzymatic hydrolysis, where glucoamylase was used as an enzyme (Kumoro et al. 2008). However, no complete saccharification was performed, since lignocellulosic part in the waste was remained. To achieve effective saccharification, the residual lignocellulosics should be hydrolyzed with acid under severe conditions or with cellulolytic enzyme, cellulase, under mild conditions. In the case of the enzymatic saccharification, a main obstacle is enzyme cost; the cellulase cost still accounts

for 25-50% of the total cost for ethanol production (Himmel et al. 1997). Maintaining cellulolytic activity, along with the reuse of cellulase, is one of the most important targets for reducing the high enzyme cost (Das *et al.* 2011). One of a promising system that makes it possible to reuse cellulase was immobilization of cellulase onto a water-soluble polymeric support (Wongkhalaung et al. 1985).

We have developed three types of amphipathic lignin derivatives, which were prepared by the coupling of acetic acid lignin from birch wood with poly(ethylene glycol) diglycidyl ether (PEGDE), ethoxy-(2-hydroxy)-propoxy-poly(ethylene glycol) glycidyl ether (EPEG), and dodecyloxy-poly(ethylene glycol) glycidyl ether (DAEO), (Homma *et al.* 2008; Homma *et al.* 2010). These amphipathic lignin derivatives dramatically improved the recovery of cellulase activity after one-time saccharification of unbleached cedar pulp, due to the fact that they behaved like as a water-soluble support for immobilization of cellulase; the residual cellulase activity is much larger than that with PEG 4000 (Winarni et al, 2013). Therefore, these results suggest that the lignin derivatives make re-use of cellulase possible.

In this study, such amphipathic lignin derivatives were synthesized from the sago soda- lignin (SSL) derived by soda-anthraquinone pulping of fibrous sago waste after removing starch component. Then, we investigate the influence of these derivatives on the saccharification of the unbleached pulp from fibrous sago waste, in particular re-use of cellulase recovered from the saccharification media by ultrafiltration.

3.2 Experimental

3.2.1 Flowchart of soda sago pulp and amphipathic soda lignin derivatives

An outline of experimental process and sacchrification process for fibrous sago waste with amphipathic lignin derivatives (PEGDE-, EPEG- and DAEO-SSL) is shown in Fig. 3-1 and the experimental procedure is described according to this scheme.

3.2.2 Soda-anthraquinone pulping of fibrous sago waste

Fibrous sago waste was obtained from local sago starch industry in Cimahpar, Bogor, Indonesia. Fibrous sago waste left on the bare ground was washed to remove soil, and dried under sunshine for 2-3 days. This waste was then grounded and collected through 35 mesh screen. The Luff Schoorl method was used to measure the starch content in the material ([ISI 28-1e: http://www.starch.dk/isi/methods/28luff.htm](http://www.starch.dk/isi/methods/28luff.htm), 12th June, 2013). The fibrous sago waste's lignin content was determined using Klason method (TAPPI T-222 om-83), as insoluble residue through filtration. The filtrate was subjected to neutral sugar analysis after neutralization with Ba(OH)₂ followed by removal of precipitate (Slavin and Marlett, 1983). The determination of sugars was carried out using HPLC device (Shimadzu LC10 system, Kyoto, Japan) equipped with a corona charged aerosol detector (ESA Biosciences Inc., Chelmsford, MA, USA). The column used was Shodex SUGAR SP0810 with SUGAR SP-G as a guard column (Showa Denko Co.Ltd., Tokyo, Japan), and the column temperature was 80 °C. The eluent used was Mili-Q purified water, and its flow rate was set at 0.5

mL/min. The injection volume was 20 μ L. The monosaccharide was converted to polysaccharide with divide 1.11 value. Extractive content was measured using 95% ethanol/benzene (1:2 v/v) extraction were made in a soxhlet extractor for 8 hours on 2 g samples in duplicate (TAPPI t6 m-59).

Firstly, fibrous sago waste was hydrolyzed with 4% HCl at 80 $^{\circ}$ C for 60 min as a pretreatment to remove residual starch. This reaction suspension was filtered, and the residue was washed with distilled water. The filtration residue was then rinsed with acetone, and dried. This pretreated sago waste was subjected to the soda-anthraquinone pulping at two alkaline concentrations as follows. The fibrous sago waste (200 g) was pulped, using 30 g or 40 g NaOH in 487 mL of distilled water together with 1 g of anthraquinone. The mixture was heated from room temperature to the cooking temperature (165 $^{\circ}$ C) for 120 min, and the temperature was maintained at 165 $^{\circ}$ C for 90 min. The crude pulp was washed with 1% NaOH solution and distilled water, successively, and filtered by pressing to reduce its moisture content down to 70%. The pulp was lyophilized to yield a dry pulp. The lignin contents of the sago pulps, Pulp-1 and -2, prepared each with 30 g and 40 g NaOH, were 1.8% and 10.2%, respectively, expressed as Klason lignin method.

3.2.3 Preparation of amphipathic sago lignin derivatives

The black liquor from soda pulping process was concentrated under reduced pressure with an evaporator. The concentrate was acidified with HCl to pH 4 to precipitate sago soda-lignin (SSL). This precipitate was collected by

filtration and washed with a small amount of distilled water. The powdered lignin was obtained by lyophilization. Characterization of the lignin, such as determination of aliphatic and aromatic hydroxyl groups, methoxyl groups, and molecular mass, was carried out according to previous reports by Baker (1996) and Goto et al. (2005). A number-average (M_n) and a weight-average (M_w) of molecular masses were measured on a HP-size exclusion chromatography using Shimadzu GPC system with two columns of Shodex KF-803L column at 40 °C. Tetrahydrofuran (THF) was used as an eluent, and the flow rate was 1 mL/min, UV detector was used at 280 nm. The SSL concentration in THF was 1 mg/mL, and the injection volume was 10 μ L. Polystyrene standards were used for the calibration.

Typically, this lignin (10 g) was dissolved in 100 mL of 1 M NaOH aqueous solution, and reacted with an epoxylated PEG derivative at 70 °C for about 2 h, according a previous report (Homma *et al.* 2010). Chemical structures of the PEG derivatives used in this study, PEGDE, EPEG and DAEO, are shown in Fig. 2-1. After the reaction, the solution was acidified to pH 4, and purified by ultrafiltration (cut-off molecular mass, 1000 Da). Finally, an amphipathic lignin derivative obtained was lyophilized. PEG contents of PEGDE-, EPEG-, and DAEO-SSL were found to be 58.6, 61.3 and 63.5%, respectively, which were determined by the modified Morgan method (Homma *et al.* 2008; Siggia *et al.* 1958; Morgan 1946).

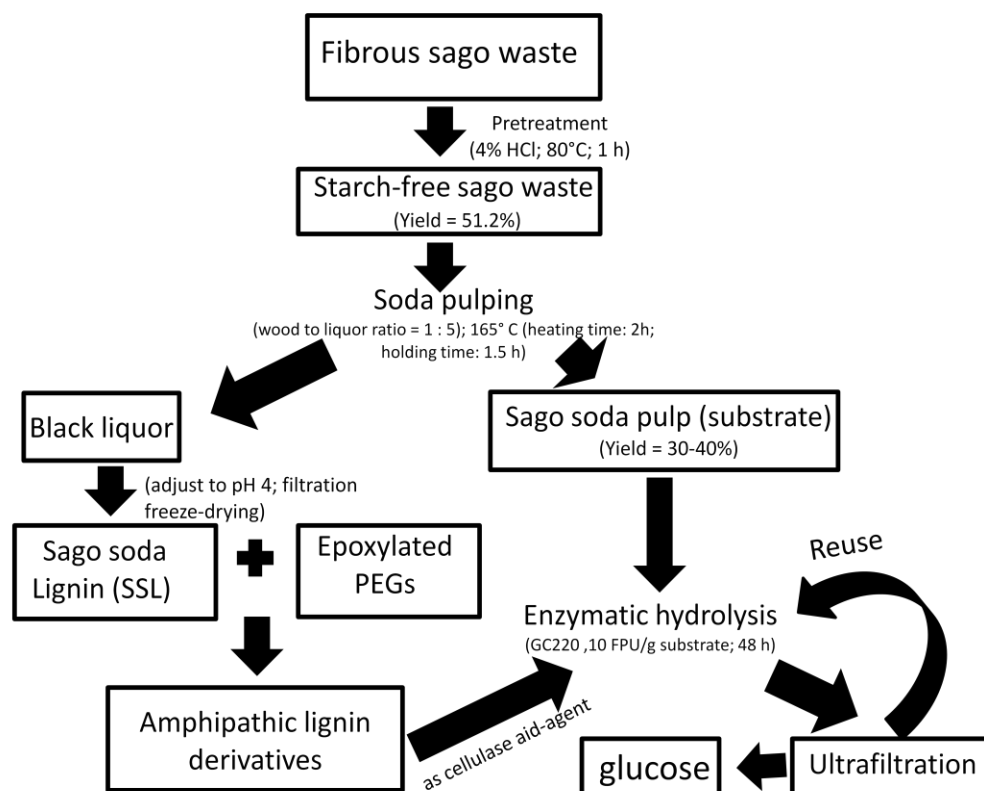


Fig. 3-1. Schematic flow diagram for sago soda pulp and amphipathic lignin derivatives from sago soda lignin

3.2.4 Enzymatic saccharification of the sago unbleached pulp

Genencor GC220 (Genencor International Inc., USA; Lot # 4901121718) was used as a cellulase commercially available. Cellulolytic activity of this enzyme solution as received was 64.9 filter paper unit (FPU) /mL, which was measured, according to the method in NREL technical report, NREL/TP-510-42628 (Ghose 1987).

Each amphipathic lignin derivative (10% of substrate on dry weight basis) was dissolved in 50 mL of 50 mM citrate buffer (pH 4.8). The cellulase at a dosage of 10 FPU/g of substrate was added to the solution, and the mixture was stirred for 1 h. Finally, 0.5 g of unbleached pulp was added to the solution, and the suspension was gently shaken at 50 °C for 48 h. After saccharification, the suspension was filtered through a G4 glass filter. The precipitate was washed three times with the buffer solution, and weighed after complete drying at 105 °C. The saccharification efficiency (SE) was calculated, by using the following equation:

$$SE (\%) = (WS - WR) \times 100 / WS \quad (3-1)$$

where, WS is the initial weight of substrate (g), and WR is the weight of residue (g) after saccharification.

This filtrate was subjected to ultrafiltration with a polysulfone membrane (cut-off molecular mass, 1000 Da, Advantec). The residual enzyme solution (*ca.* 10 mL) as a concentrate of unfiltered fraction was diluted with 50 mL of the buffer solution, and ultrafiltered again up to 10 mL, and this process was repeated three times. All the ultrafiltrates were combined, and then subjected to sugar analysis on HPLC. The composition and concentration of neutral sugars, mainly glucose, in the filtrate were determined with an HPLC system equipped with Shodex SP0810 (Shimadzu LC10 system, Kyoto, Japan) as mention before. Glucose yield was calculated, according to the following equation, and expressed as glucan yield based on the weight of charged cellulose components as a substrate:

$$\text{Glucose yield (g/g of substrate)} = 0.9 \times \text{glucose (g)} / \text{charged cellulose in the substrate (g)} \quad (3-2)$$

On the other hand, the recovered enzyme solution purified by ultrafiltration was added to a new saccharification media, in which the pulp as the substrate was suspended, and the saccharification was carried out under the same conditions as the first conditions. This saccharification-ultrafiltration process was repeated 4 times. SE measurement and sugar analysis was conducted at each process. After 4 times saccharification, the residual cellulolytic activity (FPU) of the recovered enzyme solution was measured, and calculated according to the following equation;

$$\text{Recovered Activity (\%)} = \frac{\text{FPU after saccharification (unit/g)} \times 100}{\text{FPU of initial cellulase (unit/g)}} \quad (3-3)$$

3.2.5 Measurement of surface activity

Surface tension measurements were conducted by the ring method using a Du Nouy tensiometer (Du, 1919). In this measurements we used milli-Q water (electrical resistivity = 18.2 MΩ. cm⁻¹) and the measurements were done after 6-10 min after the sample addition into a measurement dish. The value of surface tension was obtained as the average value of three different measurements.

3.3 Result and discussion

3.3.1 Fibrous sago waste and its acid hydrolysis

Table 3-1 shows chemical components of the fibrous sago waste as received from the sago starch mill. This result clearly revealed that the waste still contained a significant amount of starch in the fiber (9.3% on dry weight basis). This residual starch is also valuable feedstock, and it may act as an inhibitor for the enzymatic saccharification of lignocellulosics in the waste, because a large number of starch granules were trapped within the lignocellulosic matrix of sago waste (Chew and Shim, 1993). Therefore, mild acid hydrolysis with 4% HCl as a pretreatment was conducted to remove and recover the starch component. Starch component could not be detected, just like mannose, in the treated fibrous sago waste after mild acid hydrolysis (Table 3-1).

Table 3-1. Fibrous sago waste characterization

Compound	Raw material (%)	Pretreated raw material (%)
Starch	9.30	n.d
Glucan	51.0	58.5
Xylan	19.4	5.67
Mannan	2.32	n.d
Lignin	17.5	31.2
Extractive	1.21	1.20
Total	100.7	96.6
n.d., not detected		

In addition, xylan content was decreased, while lignin content was increased. These results suggested that complete removal of starch was carried out together with partial hydrolysis of hemicelluloses by mild acid hydrolysis. On the other hand, sago waste pulp with lignin content 1.8% lignin (pulp-1) consist of glucan (91.8%) and sago waste with 10.2% lignin content (pulp-2) consist of glucan (75.6%) and xylan (4.86%).

3.3.2 Soda-anthraquinone pulping and preparation of amphipathic lignin derivatives

The fibrous sago waste after removing starch still contained lignin, which must hamper cellulase accessibility to substrate and reduce the cellulolytic activity of the enzyme during saccharification, due to enzyme adsorption on lignin (Pan, 2008). The delignification of the pretreated sago waste was attempted by soda-anthraquinone pulping. Unbleached pulps with different residual lignin contents (1.8% and 10.2% as Klason lignin) were obtained by the pulping with 6.7 g and 5 g of NaOH on 1 g of the sago waste, respectively. The pulps with low and high lignin contents were designated as Pulp-1 and Pulp-2, respectively. These unbleached pulps were subjected to the following saccharification.

The lignin (SSL) isolated from the black liquor of the pulping of the sago waste had $\overline{M}_w$ of 3010 and $\overline{M}_w/\overline{M}_n$ of 3.3. The phenolic hydroxyl groups (ArOH), aliphatic hydroxyl groups (AlkOH) and methoxyl groups (MeO) were 3.4, 4.3 and 3.5 (mmol/g of lignin), respectively. SSL was, in turn, converted to amphipathic derivatives by the reaction with 3 types of epoxylated PEG analogues. The

surface activity of the resultant SSL derivatives was evaluated from the decrease in surface tension of water. Fig. 3-2 shows the surface tension vs. logarithm of the derivative concentrations, where the intersection of two regression lines indicates the critical micelle concentration (CMC) (Du, 1919). DAEO-SSL remarkably reduced the surface tension of water, and CMC was found in the figure. EPEG- and PEGDE-SSL also reduced the surface tension, but no CMC was observed. These results implied that the order of surface activity was PEDGE-SSL < EPEG-SSL << DAEO-SSL. This tendency was consistent with the order of amphipathic lignin derivatives prepared previously from not only acetic acid lignin (Homma *et al.* 2010) but also black liquors of kraft pulping and their isolated lignins (Aso *et al.* 2013). Thus, amphipathic lignin derivative with significant surface activity was also prepared from SSL.

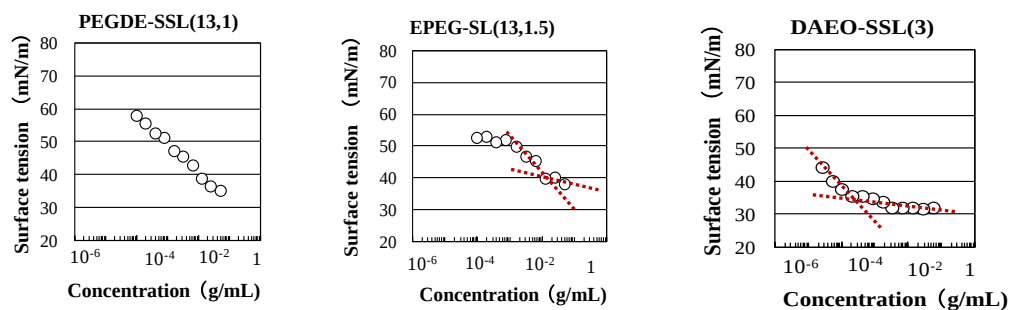


Fig.3-2. Surface tension-concentration isotherm for (A) PEGDE-; (B) EPEG-; and (C) DAEO-SSL

3.3.3 Enzymatic saccharification of unbleached sago pulp

Enzymatic saccharification of the unbleached sago pulp with a commercially available cellulase, Genencor GC220, together with amphipathic SSL derivatives, were investigated to clarify the effect of amphipathic lignin derivatives on the enzymatic saccharification of unbleached sago pulp, especially re-use of cellulase. The cellulase after the saccharification was recovered and purified by ultrafiltration with polysulfone membrane to remove sugars in the saccharification medium. This recovered enzyme was used again for the next saccharification. Figs. 3-3 and 3-4 show saccharification efficiency (SE) for Pulp-1 and Pulp-2 as a substrate, respectively.

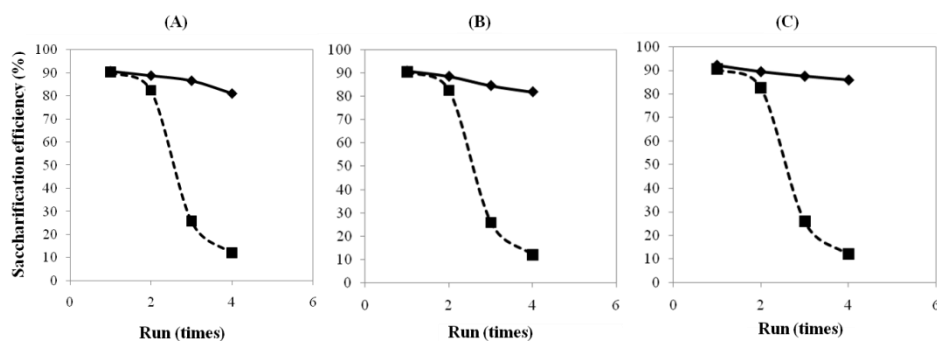


Fig. 3-3. Saccharification efficiency of sago Pulp-1 in several times enzymatic hydrolysis using (A) PEGDE-; (B) EPEG-; and (C) DAEO-SSL; (—■— no additive; —◆— lignin derivatives).

In the first saccharification, SE without additive (as a control experiment) showed about 90% and 77% for the Pulp-1 and Pulp-2, respectively. Taking the lignin content into consideration, Pulp-2 was also hydrolyzed by cellulase as

comparable to Pulp-1. All the SSL-derivatives, DAEO-SSL in particular, showed higher SE than the control experiment. DAEO-SSL remarkably improved its SE (84.9%) for Pulp-2 by 7.5 %, though the other two derivatives (SE, 82.6%) improved by 5.2%. Thus, DAEO-SSL was the most suitable additive for the improvement of SE.

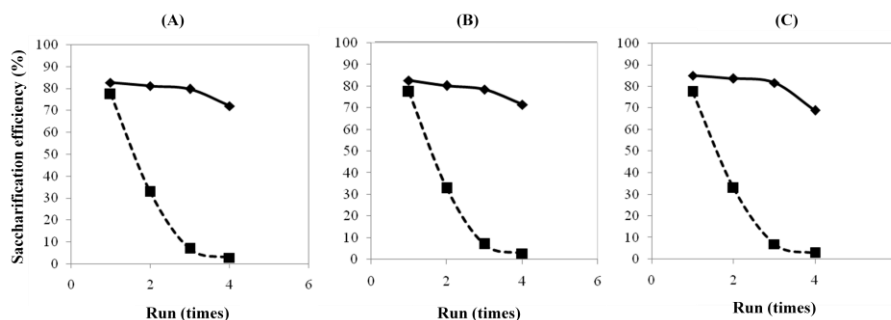


Fig.3-4. Saccharification efficiency of sago Pulp-2 in several times enzymatic hydrolysis using (A) PEGDE-; (B) EPEG-; and (C) DAEO-SSL; (—■— no additive; —◆— lignin derivatives).

3.3.4 Repeated use of the recovered enzyme

SE for Pulp-2 was drastically decreased at one-time saccharification in the repeated-use (re-use) experiment of the recovered cellulase without additive (as a control experiment). SE for Pulp-1 significantly decreased after 2nd saccharification. The SE for Pulp-1 and Pulp-2 at the 4th saccharification was approximately 3% and 12% than those at 1st saccharification. This difference must reflect the cellulase adsorption onto the pulps with different lignin content. Cellulase associates with lignin through non-specific, hydrophobic interaction (Eriksson *et al.* 2002; Li *et al.* 2012). Thereby, it is likely that a large amount of

cellulase adsorbed on Pulp-2 with high lignin content, and this resulted in a quick decrease in SE for Pulp-2. On the other hand, in the case of saccharification of Pulp-1, an initial charge of cellulase at the 1st saccharification must be an excess. Since loss of enzyme upon adsorption on the substrate must also be small because of its low lignin content, a sufficient amount of cellulase was supplied to the 2nd saccharification, resulting in that high SE at the 2nd saccharification was achieved. Afterwards, the remaining cellulolytic activity was insufficient for 3rd saccharification, leading to a remarkable decrease in SE of 3rd saccharification. However, as the decrease rate of cellulase activity in Pulp-1 saccharification was smaller than that in Pulp-2 saccharification, SE at 4th saccharification of Pulp-1 was larger than that of Pulp-2.

By the addition of amphipathic SSL derivatives, SE was maintained at a remarkably high level, even after 4th saccharification. This effect on the preservation of enzyme activity after saccharification must have been caused by direct interaction of lignin derivatives with cellulase, as observed in other amphipathic lignin derivatives reported previously (Winarni et al. 2013).

When SEs between the 1st and the 4th saccharification for Pulp-1 are compared, the SEs with PEGDE- and EPEG-SSL decreased by about 10%, while the SE with DAEO-SSL decreased by only 6%. In the case of the saccharification for Pulp-2, PEGDE- and EPEG-SSL showed a similar tendency in SE reduction to that for Pulp-1. DAEO-SSL indicated higher SEs until the 3rd saccharification than the other SSL derivatives, but the SE with DAEO-SSL at the 4th saccharification was lower than that with the others. Fig. 3-5 shows the residual

cellulase activity after the 4th saccharification. Although the residual activities in the control experiments without additive for both pulps markedly declined, the residual activities in the presence of SSL derivatives still remained significantly. DAEO-SSL showed the highest residual activity among the additives after the 4th saccharification for Pulp-1, but its residual activity after the 4th saccharification of Pulp-2 was the lowest. The lowest residual activity might have caused the lowest SE with DAEO-SSL for Pulp-2 at the 4th saccharification. These results revealed that DAEO-SSL has an ability to preserve the enzyme activity in saccharification of the pulp with low lignin content, but that it does not necessarily maintain the enzyme activity in saccharification of the pulp with high lignin content.

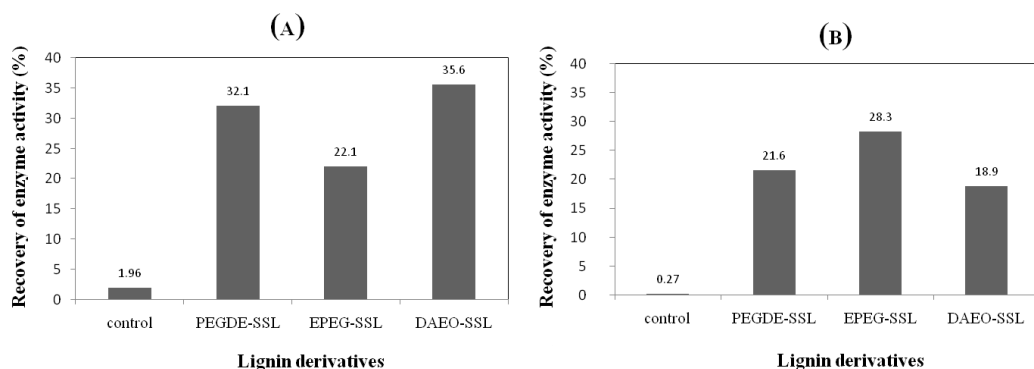


Fig.3-5. Recovery of enzyme activity of sago pulp (A) 1.80% and (B) 10.2% lignin content after fourth-stage saccharification using three kinds of lignin derivatives and control.

Finally, we investigated sugar constituents and their concentration in the saccharification media to confirm the conversion of cellulose to glucose.

Generation of cellobiose was confirmed in all cases as shown in Fig. 3-6 through 3-8, suggesting insufficient hydrolysis of cellobiose to glucose. Actually, the β -glucosidase activity of Genencor GC220 was lower than other commercial cellulase such as Meicelase (Winarni et al. 2013). The combined sugar yield (g/ g of substrate) of glucose and cellobiose in the figure are larger than SE. This reason is explained by the fact that the weight of sugar obtained by hydrolysis of polysaccharides are 10% larger than the weight of polysaccharide, due to the introduction of water. Therefore, it can be said that cellulose component in the fibrous sago waste was quantitatively hydrolyzed with cellulase with the assist of amphipathic SSL derivatives.

In this chapter, I confirmed that SE of unbleached sago pulp was improved by the addition of amphipathic lignin derivatives, particularly DAEO-SSL. The recovered cellulase in the presence of the lignin derivatives was able to be used for the next subsequent saccharification, and SE was maintained until the 4th saccharification by re-use of cellulase at a similar level to that at the 1st saccharification. DAEO-SSL showed a superior performance to improve SE and maintain cellulase activity than the other derivatives, but the SE with DAEO-SSL at 4th saccharification of the pulp with high lignin content was found the lowest. This result suggests that the improved performance of DAEO-SSL for the cellulolytic saccharification was suppressed by the residual lignin in the substrate. Thus, we developed amphipathic lignin derivatives from sago palm waste, which made it possible to re-use of cellulase for saccharification.

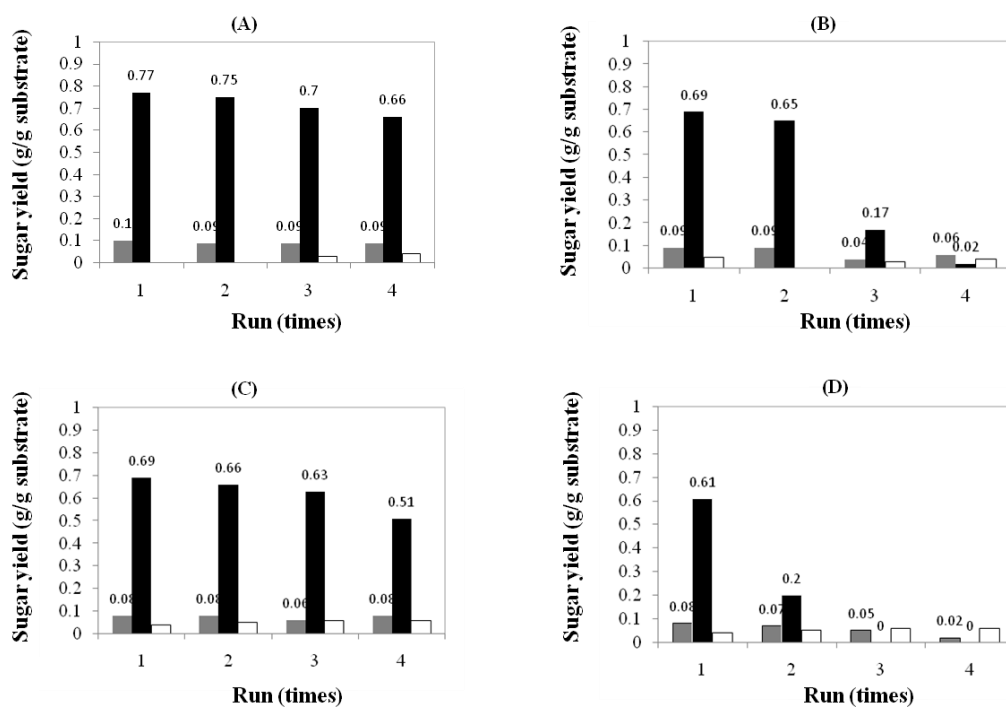


Fig.3-6. Sugar content of sago Pulp-1 (A) using DAEO-SSL and (B) control; (C) Pulp-2 using DAEO-SSL and (D) control, at fourth-stage saccharification; (■ cellobiose; ■ glucose; □ unknown)

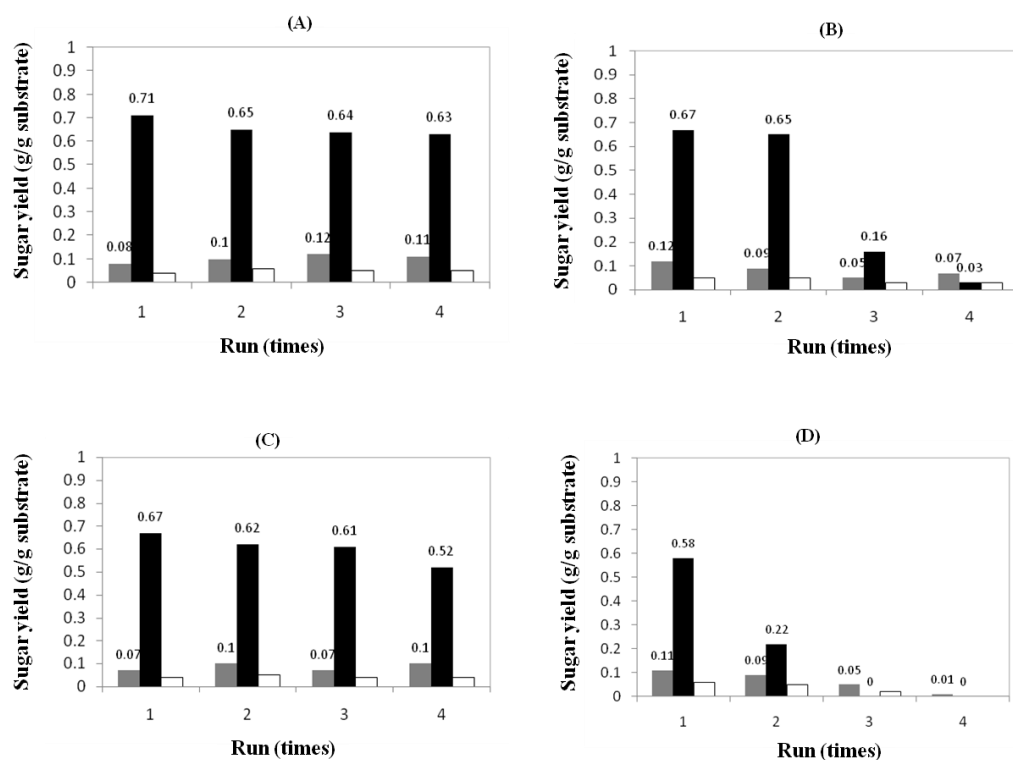


Fig.3-7. Sugar content of sago Pulp-1 (A) using EPEG-SSL and (B) control; (C) Pulp-2 using DAEO-SSL and (D) control, at fourth-stage saccharification; (■ cellobiose; ■ glucose; □ unknown)

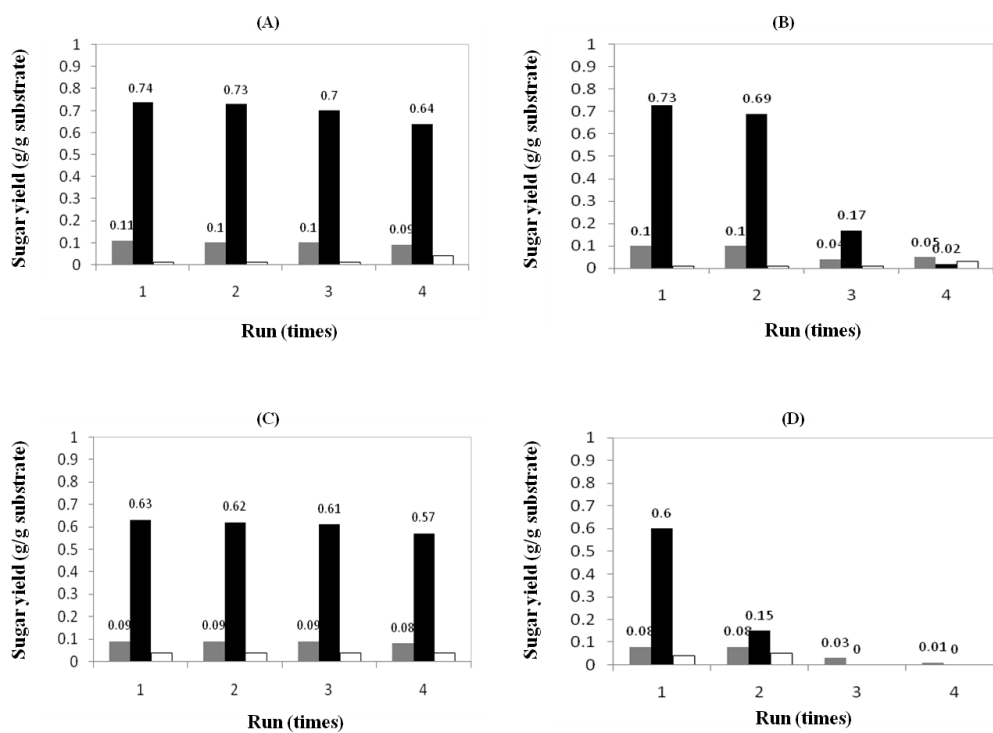


Fig.3-8. Sugar content of sago Pulp-1 (A) using PEGDE-SSL and (B) control; (C) Pulp-2 using DAEO-SSL and (D) control, at fourth-stage saccharification; (■ cellobiose; ■ glucose; □ unknown)

CHAPTER 4

Conclusion

4.1 Concluding remarks

Unbleached cedar and sago waste pulps as the substrate for enzymatic saccharification was prepared by soda pulping. Amphipathic lignin derivatives as a surfactant were prepared by the reaction of acetic acid lignin (organosolv pulping of birch wood) and soda sago lignin with the epoxylated PEG derivatives. The enzymatic saccharification of unbleached cedar and sago waste pulps together with the lignin derivatives was attempted with two types of cellulases commercially available, Meicelase and Genencor GC220.

In Chapter 2, I discussed about:

1. Unused of Japanese cedar from forest thinning production in the forest was utilized became unbleached pulp as a substrate for enzymatic saccharification by soda pulping. And amphipathic lignin derivatives as a surfactant were prepared by the reaction of acetic acid lignin of birch chips with the epoxylated PEG derivatives, i.e PEGDE-AL, EPEG-AL and DAEO-AL.
2. The enzymatic saccharification was attempted with two types of commercially available cellulase, Meicelase and Genencor GC220. Enzymatic saccharification using GC220 showed higher saccharification efficiency (SE) than Meicelase. It was recommended that the GC220 was a suitable cellulase for the saccharification of cedar pulp.

3. The addition of amphipathic lignin derivatives significantly improved the SE compared without lignin derivatives (control). On the other hand, I also compared with available commercially additive PEG4000.
4. Although PEG4000 showed the highest SE at 10 FPU/g of substrate, however at 20 FPU/g of substrate was not the highest one.
5. Amphipathic lignin derivatives showed ability maintained the residual cellulase activity at a higher level than PEG4000 after one-time saccharification. In particular, EPEG- and PEGDE-AL showed complete recovery of cellulase activity at 20 FPU/g of substrate.
6. The different mechanism for improved saccharification using Surface Plasmon Resonance (SPR); amphipathic lignin derivatives showed directly interaction with the enzyme, whereas PEG4000 was not.

In Chapter 3, I explained about:

1. Fibrous sago wastes which were disposed from sago mills still contain significant amount of starch, therefore it was hydrolyzed using mild HCl (aqueous HCl 4%) to remove and recovered the starch as a feedstock for bioethanol.
2. After pretreatment with mild acid, it was pulped using aqueous NaOH (15 and 20%) and anthraquinone addition. The pulp becomes the substrate, on the other hand, sago soda lignin (SSL) was isolated from black liquor of the pulping and converted to amphipathic SSL derivatives by the reaction with the epoxylated PEG derivatives (PEGDE-, EPEG-, and DAEO-SSL)

3. The unbleached sago waste pulp was enzymatically hydrolyzed with Genencor GC220 (10 FPU/g of substrate) mixed together with SSL-derivatives. After one-time saccharification, the cellulase was recovered using ultrafiltration, and the next saccharification was performed with the recovered enzyme. And this process was repeated until 4 times.
4. SE showed dramatically decreased after 2nd saccharification without lignin derivatives. In contrast, addition of SSL-derivatives resulted improved the SE and maintained in a high level even after 4th saccharification. In particular, DAEO-SSL showed the highest SE compared with the others.
5. DAEO-SSL also showed the highest residual activity after 4th saccharification. These results suggest that amphipathic lignin derivatives enable re-use cellulase for enzymatic saccharification of lignocellulosic materials.

4.2 General discussion

The amphipathic lignin derivatives as a surfactant added together with two kinds of commercially available cellulase in enzymatic saccharification of Japanese cedar and sago waste pulp as a substrate. Addition of surfactants during hydrolysis is capable of modifying the cellulose surface prosperity and minimizing the irreversible binding of cellulase on cellulose. The research showed that addition amphipathic lignin derivatives can improve the saccharification efficiency after one-time saccharification of cedar pulp than without lignin derivatives (control). In particular DAEO-AL showed higher SE

than with others AL-derivatives. Although, PEG4000 showed the highest SE at 10 FPU/g substrate of cellulase, such performance was not observed at 20 FPU/g of substrate. In addition AL-derivatives showed can maintain residual cellulase activity after one-time saccharification at a higher level than PEG4000 after one-time saccharification. In particular, EPEG- and PEGDE-AL showed complete recovery at 20 FPU/g of substrate of cellulase. In other hand, DAEO-SSL showed can improve SE than without lignin derivatives for enzymatic saccharification of sago waste pulp. Without lignin derivatives, SE dramatically decreased in 2nd saccharification. Addition of SSL-derivatives can improve the SE and maintain in a high level even after 4th saccharification.

From the previous result, PEG4000 was reported can improve the saccharification efficiency of steam-pretreated spruce (78%) and without PEG4000 only reached about 50% (Börjesson *et al.* 2007). In addition, PEG4000 also increased the residual filter paper activity about 56% (Sipos *et al.* 2010). Similarly, using Tween 80 as a non ionic surfactant resulted glucose yield higher than control. In contrast, anionic (sodium dodecyl sulfate) and cationic (quarternary ammonium salt) showed negative effect in enzymatic saccharification of pretreated wheat straw (Cui *et al.* 2011).

Therefore, I confirmed that amphipathic lignin derivatives from Japanese wood and sago palm wastes as a cellulase aid-agent, which enable re-use of cellulase. This means that the saccharification cost will be remarkably reduced by the use of such lignin derivatives. Thus, this research will provide a useful

method to produce feedstock for 2nd generation bioethanol and an effective utilization of unused lignocellulosic.

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