Production of high concentration bioethanol by fed-batch type simultaneous saccharification and fermentation of lignocellulosics with amphipathic lignin derivatives

（両親媒性リグニン誘導体を用いたリグノセルロースの同時糖化発酵による高濃度バイオエタノールの製造）

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Doctoral thesis

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ABBREVIATIONS

A-LDs: Amphipathic lignin derivatives

BG: β-glucosidases

CBH: Cellobiohydrolases

CMC: Critical micelle concentration

CSL: Cedar soda lignin

DOPEG: Dodecyloxy-poly (ethylene glycol) glycidyl ether

EG: Endoglucanases

EPEG: Ethoxy-(2-hydroxy)-propoxy-poly (ethylene glycol) glycidyl ether

FB-SSF: Fed-batch type simultaneous saccharification and fermentation

FFPRI: Forestry and Forest Products Research Institute

NREL: National Renewable Energy Laboratory

NUKP: Unbleached softwood kraft pulp

PEG: Polyethylene glycol

PEGDE: Polyethylene glycol diglycidylether

PSA: Pressure swing adsorption

SHF: Separate hydrolysis and fermentation

SSF: Simultaneous saccharification and fermentation

Chapter 1 General Introduction

1.1. Fossil resources and lignocellulosic biomass

Fossil resources, such as coal, petroleum and natural gas, have been used as energy resources or fuel since the middle of 17^{th} century (Belbute et al., 2016), and they have provided more than 80% energy on the total energy consumption for more than 100 years. However, the burning of fossil resources produces approximately 21.3 billion tons of carbon dioxide (CO_2) a year. Carbon dioxide is well-known as a greenhouse gas that causes global warming on the earth (Hoel and Kverndokk, 1996). In addition, the combustion of fossil resources releases gaseous pollutants, such as SO_x and NO_x, which cause acid rain (Hameed and Dignon, 1988). To solve these problems, biomass, phytomass in particular, is focused on as an alternative energy resource from the viewpoint of "Carbon Neutral". Plant biomass or phytomass is photosynthesized from carbon dioxide and water on the ground. When the phytomass is burned or biodegraded under the ground, CO_2 is generated. As a result, the mass balance of atmospheric CO_2 consumption/emission in relation to phytomass is maintained; that is "Carbon Neutral." This concept will be achieved by using only phytomass only. In our daily life, net increase in the atmospheric CO_2 arrives at 10 billion tonnes every year even if the consumption of CO_2 by photosynthesis is taken into consideration. Therefore, the use of fossil resources as a fuel should preferably be at least partly, if not completely, replaces by that of phytomass resources. Since phytomass accounts for over 90% of biomass on the earth, the term 'biomass' will be

used below for simplification even when ‘phytomass’ is thought to be more appropriate.

Among the biomass-derived fuels, bioethanol has been considered as the most promising candidate for an alternative liquid fuel to substitute fossil fuel (Sarkar et al., 2012). When the combustion of bioethanol generates no pollutant, therefore, it can be regarded as a clean fuel (Lin and Tanaka, 2006). Bioethanol is also expected to be a platform compound to produce chemicals and plastics.

In general, bioethanol can be produced by alcohol fermentation of hexoses, mainly glucose, with yeast, *Saccharomyces cerevisiae*. Sugars from sugarcane, sugar beets, molasses and fruits are directly converted into ethanol by the fermentation. Starches from corn, cassava, potatoes and root crops must first be hydrolyzed to fermentable sugars by the action of amylase, and then the sugars are converted into ethanol by fermentation. The bioethanol productions from sugar and starch are very attractive methods because of their relatively simple process and high yield (Baeyens et al., 2015). Thereby, the resultant bioethanol is called a first-generation of bioethanol. However, these types of food-related biomass are recently considered to be undesirable sources, because they compete with the production of daily food, resulting in the rise in the price of food feedstock.

Alternatively, cellulose draws much attention as an alternative source for bioethanol, because it does not compete with food production (Farrell et al., 2006). Cellulose is a linear polysaccharide consisting of β (1→4) glycosidic bond-linked anhydro-D- glucose units with a degree of polymerization of several hundred to thousands (Klemm et al., 2005). Therefore, it can be converted into glucose by

hydrolysis. Cellulose is obtained from lignocellulosic biomass, such as wood, agricultural residues, waste pulp and wastes of paper mills. Therefore, lignocellulosic biomass is called a second-generation feedstock for bioethanol production.

Lignocellulosic biomasses is estimated to be produced in 10-50 billion tons/year as a dry matter on the earth (Alfaro et al., 2009). The major components of wood biomass among the lignocellulosic biomass are cellulose (ca 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). The cellulose fibers are covered with lignin, which plays important roles as a reinforced material for wood cell wall, and a protecting agent against pathogenic attacks by fungi and bacteria (Aysu and Durak, 2015; Boerjan et al., 2003; Himmel et al., 2007). Furthermore, the hydrophobic nature of lignin renders cell walls water-impermeable to allow water transportation through conductive tissues or cells, such as vessel in hardwood and tracheid in softwood. Hemicellulose behaves as glue, forming a rigid cell wall upon chemical bonding between cellulose and lignin (Somerville et al., 2004). The structure of lignocellulose is shown in **Figure 1-1**.

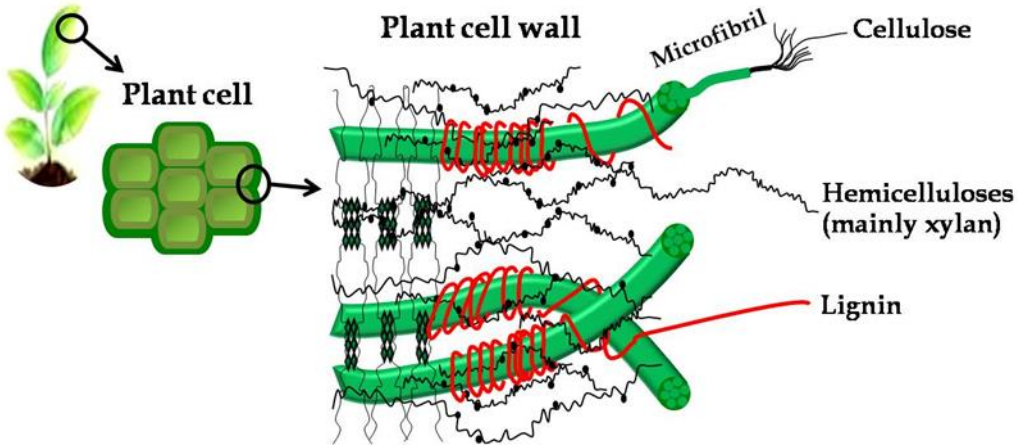


Figure 1-1. A schematic model for Lignocellulosic Components in a Plant Cell Wall (Source: http://www.intechopen.com)

Actually, there have been lots of researches to produce bioethanol successfully from various of lignocellulosic feedstocks such as sugarcane bagasse (Zhang and Zhu, 2017), corn stover (Wang et al., 2014; Zhang et al., 2010), low-quality wood like timber from thinning cut of Japanese cedar (Yamashita et al., 2010), spruce (Hoyer et al., 2013a; Hoyer et al., 2013b), sawdust (Kim et al., 2013), and wastes of paper mill (Elliston et al., 2013). However, 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 tree 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 use timber effectively 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.

1.2. Bioethanol production process based on enzymatic saccharification

Figure 1-2 summarizes the possible bioethanol production processes from lignocellulosics. The whole process is mainly comprised of three steps, saccharification step, fermentation step with yeast, and distillation step. The saccharification step is also proposed to be two kinds of methods, acid and enzymatic hydrolyses, where hydrolysis is the same meaning as saccharification. By those methods, cellulose component in lignocelluloses can be converted into glucose as a feedstock for ethanol fermentation. The acid hydrolysis is further classified into 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 (Sun and Cheng, 2002). In addition, the concentrated acid must be recovered and used again after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995).

Enzymatic saccharification has several advantages over acid hydrolysis. One is its lower utility cost than that of acid hydrolysis, because enzymatic hydrolysis does not require special reaction equipment due to the fact that it is conducted under mild conditions (pH 4.8 and temperature 45–50 °C) and no corrosion problem (Duff and Murray, 1996). Furthermore, the generated monosaccharide is easily subjected to the next step, fermentation, because a similar pH condition is applicable to both saccharification and fermentation.

However, enzymatic hydrolysis has several disadvantages, such as requirement of

pretreatment and high cost of enzyme. Following sections demonstrate the outline of bioethanol production via enzymatic saccharification including the above disadvantages of the saccharification in detail.

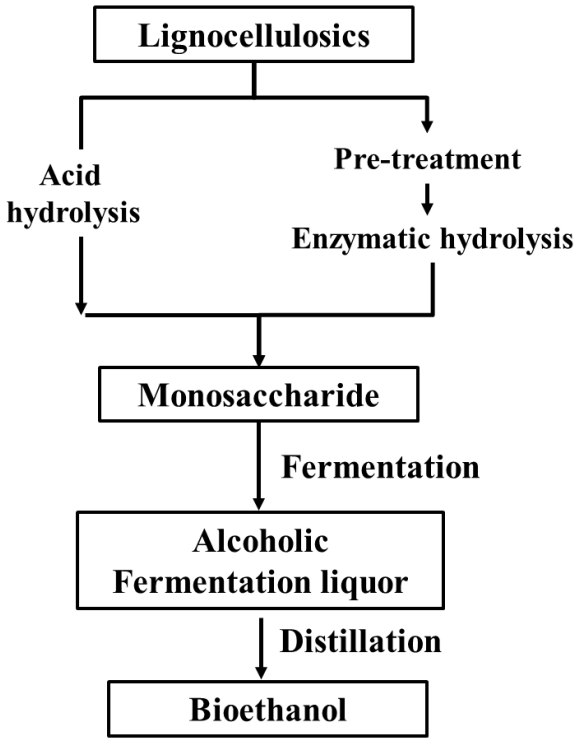


Figure 1-2. Outline of bioethanol production from lignocellulosics

1.2.1. Pretreatment in the enzymatic saccharification of lignocellulosics

In the enzymatic saccharification of lignocellulosics with cellulase, association of cellulase with cellulose, which is referred to as ES (enzyme-substrate) complex formation, is a very important key step (Balat et al., 2008). However, cellulase cannot directly attack cellulose or associate with cellulose, because lignin and hemicelluloses cover cellulose molecules, and hamper the enzymatic attack to cellulose. Therefore, the enzymatic saccharification of lignocellulosics requires a pretreatment step for

delignification of lignocellulose, while the acid saccharification does not require delignification because proton, whose size is much smaller than enzyme, can easily attack cellulose.

For delignification, physicochemical treatments, such as auto-hydrolysis, steam explosion and hydro-thermolysis, chemical treatments, such as alkali, acid treatments, and several pulping including soda pulping, kraft pulping and organosolv pulping, biological treatment, and electrochemical treatment, have been proposed (Kumar et al., 2009). Recalcitrant cell wall structures of lignocellulosics can be disrupted by those pretreatment methods, resulting in the removal of lignin and hemicelluloses. The pretreatments sometimes cause the reduction of cellulose crystallinity and the increase in the porosity of lignocellulosics (Avci et al., 2013).

A simple physical pretreatment, such as milling and grinding, is also useful to improve efficiency of enzymatic saccharification. By this pretreatment, cellulose is unveiled and from other cell wall components exposed to outside. The physical pretreatment is also sometimes employed in the acid saccharification to accelerate its reaction rate.

1.2.2. Enzymatic saccharification

After pretreatment, the second step is enzymatic saccharification (hydrolysis), by which polysaccharide (cellulose) is convert to monosaccharide (glucose) for fermentation. Cellulose is a β-1,4-D-glucan: namely, it is composed of linear chains of glucose residues jointed by β-1,4-glycosidic linkages. By sectioning these linkages,

glucose can be generated. The enzyme used for saccharification is called cellulolytic enzyme (cellulase). Cellulase mainly consists of three types of enzyme: Endoglucanases (EG, known as a 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 a processive type enzyme), which moves along the cellulose chain and cleaves off cellobiose units from the end of the chain; β-glucosidases (BG, EC 3.2.1.21), which hydrolyse cellobiose to glucose. These three groups of enzymes work synergistically to degrade cellulose. Such different activities minimize production inhibition to produce glucose effectively (Eriksson et al., 2002b; Valjamae et al., 2003). A sketch for the functions of cellulases is shown in **Figure 1-3**. Thus, the term, "cellulase", implies a complex of these enzymes, and therefore commercially available cellulases are cocktail of the enzymes.

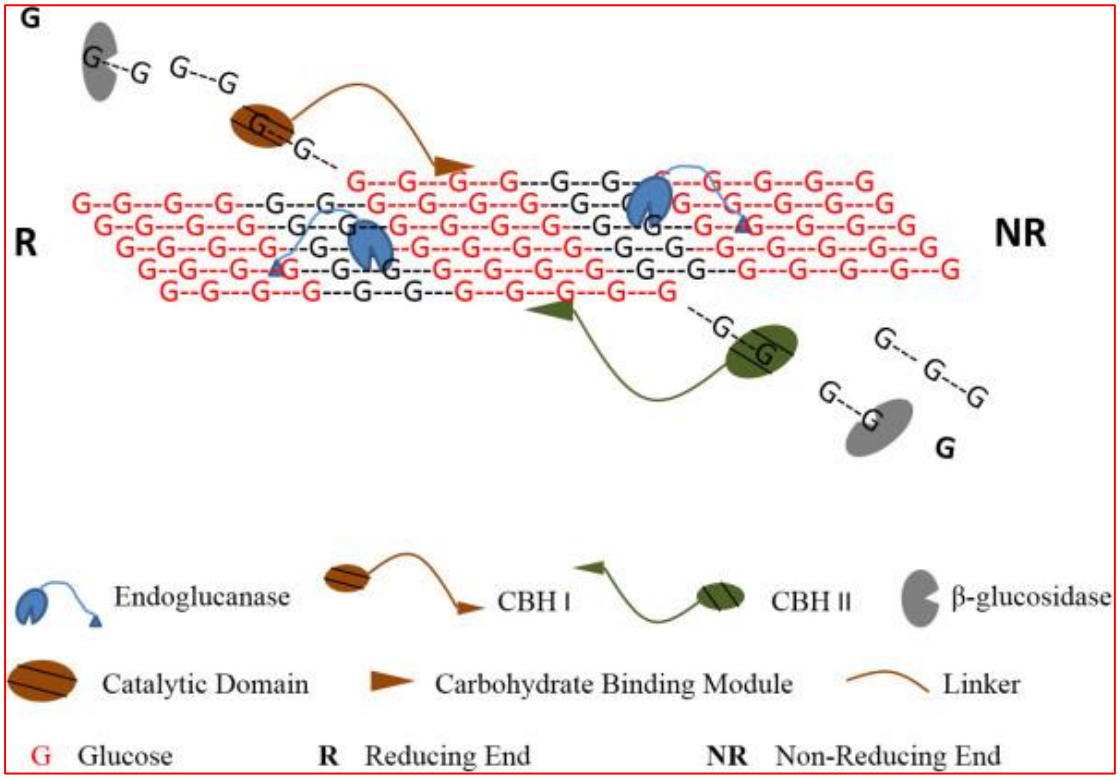


Figure 1-3. Three types of enzyme consisting of cellulase

1.2.3. Ethanol fermentation

The ethanol fermentation process is a conversion of monosaccharide to ethanol with microorganisms, mainly yeast (*Saccharomyces cerevisiae*). In general, only hexose can be converted to ethanol in the yeast fermentation. In the alcohol fermentation, two moles of ethanol and two moles of carbon dioxide besides two moles of ATP are produced from one mole of hexose as follows, where glucose among hexoses is mainly used as a substrate.

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

Saccharomyces cerevisiae has been traditionally used as an industrial yeast to produce ethanol in a brewery and a wine chateau for thousands of years. *S. cerevisiae*

in wine production yields ethanol concentration up to about 14% (Bayrock and Ingledew, 2001; Claassen et al., 1999), while Japanese Sake Yeast produces a little more highly concentrated ethanol. So far, it has also been reported that bioethanol up to 10% can be produced from lignocellulosics (Elliston et al., 2013; Maeda et al., 2013). However, such ethanol concentrations are very low, when they are to be applied to a liquid fuel.

1.2.4. Distillation and production of absolute ethanol

To use the resultant ethanol just after fermentation, its concentration is the key to successful production of absolute ethanol as the fuel. In general, distillation is conducted for the concentration. However, such process gives ethanol up to 95% due to the formation of an azeotropic compound by water and ethanol. To obtain absolute ethanol, several methods have been reported, such as pressure swing adsorption (PSA) process (Kanti et al., 2004; Ling et al., 2010; Ma et al., 2009) and pervaporation (Gaykawad et al., 2013; Huang et al., 2010).

Thus, highly concentrated ethanol can be produced not only from starch but also from lignocellulosics by the combination of distillation and post distillation methods. However, it is more desirable to achieve higher ethanol concentration after fermentation, prior to distillation, for reducing the distillation cost.

1.3. Significance of simultaneous saccharification and fermentation (SSF) in the production of ethanol from lignocellulosics

Two bioethanol productions from lignocellulosics are considered. One is that saccharification and fermentation are conducted individually and consecutively: in other word, fermentation is performed after saccharification. This process is called SHF (separate hydrolysis and fermentation) method. The other is called simultaneous saccharification and fermentation (SSF) method. In this method, both of saccharification and fermentation are conducted in the same reaction vessel at the same time.

In SHF method, saccharification and fermentation can be performed under each optimum condition, such as pH and temperature (Tomas-Pejo et al., 2008). Although a large amount charge of substrate in the SHF seems to be one of the potentially promising methods to produce higher concentrated sugar and ethanol solution (Nakamura et al., 2001), this is not viable because end-product inhibition occurs in the saccharification process (Stenberg et al., 2000; Xiao et al., 2004), which means that glucose and cellobiose generated by saccharification decrease enzyme activity.

To solve this problem, SSF is proposed. In this method, the end-product inhibition is suppressed because glucose, one produced by saccharification process, is spontaneously consumed by the fermentation process (Ballesteros et al., 2004; Olsson et al., 2006). By this advantage, the enzyme loading can be also decreased (Kadar et al., 2004). Moreover, no undesired microbial contamination in SSF occur because contents in the reaction vessels are never transferred into other vessels, and the generated ethanol

acts as an anti-microbial agent. This one-pot process leads to reduction of capital costs and process time (Ask et al., 2012). Therefore, nowadays, SSF is considered to be very promising (Alkasrawi et al., 2013; Boonsawang et al., 2012; Dwiarti et al., 2012; Ishola et al., 2015; Saha et al., 2015; Yoo et al., 2012).

However, SSF also has a few of disadvantages. The enzymatic hydrolysis and the fermentation should be conducted in a vessel under the same conditions, temperature and pH. It is difficult to use yeast repeatedly in SSF, because the yeast exists together with the residual substrate whereas yeast recycling is possible in SHF, when only sugar solution is separated from the saccharification media.

A simple SSF (batch mode SSF) has a serious drawback; substrate charge is limited because the high consistency of substrate hampers agitation of reaction media (Lynd, 1996; Zhao et al., 2013). A method to overcome this problem is fed-batch SSF (FB-SSF), in which the substrate can be charged several times in this system (Cha et al., 2014; Zhang and Zhu, 2017). By changing the modes from simple SSF to FB-SSF, the total substrate loading increased from 15 wt.% (Kang et al., 2015; Lan et al., 2013; Zhang et al., 2010; Zhang and Zhu, 2017) to about 30 wt.% to yield ethanol over 70 % (Hoyer et al., 2013a; Kim et al., 2013; Liu et al., 2010; Maeda et al., 2013; Park et al., 2013; Wang et al., 2014). Furthermore, Gladis et al. (Gladis et al., 2015), reported that an identical ethanol yield was obtained by a smaller enzyme loading in FB-SSF than that in simple SSF.

1.4. Additives to improve enzymatic saccharification

1.4.1. Non-ionic surfactants

In the bioethanol production including enzymatic saccharification, the cost of cellulase accounts for 25% to 50% of the total cost for ethanol production (Himmel et al., 1997). Recently, enzyme price is decreased, but cellulase is still expensive. In addition, cellulase cannot be used repeatedly in general, because the irreversible adsorption (non-productive interaction) of cellulase onto the substrate, mainly cellulose and lignin, occured (Qin et al., 2014; Sun et al., 2016). The interaction between cellulase and cellulose is a specific interaction based on the formation of enzyme-substrate complex (Kristensen et al., 2007; Ma et al., 2008). The other interaction between cellulase and lignin is a non-specific interaction due to the hydrophobic interaction and hydrogen bonding (Borjesson et al., 2007b; Kristensen et al., 2007). To improve enzymatic saccharification, several additives were reported. Among them, some surfactants (Eriksson et al., 2002a; Kim et al., 1982; Pardo, 1996) especially non-ionic surfactants (Park et al., 1992; Wu and Ju, 1998) such as tween-20 (Alkasrawi et al., 2003; Seo et al., 2011), tween-80(Kaar and Holtzapple, 1998; Kristensen et al., 2007), and triton X-100 (Eriksson et al., 2002a) are well known. This function of non-ionic surfactants is caused by the hydrophobic interaction between hydrophobic domain of the surfactants and lignin in the lignocellulosics followed by the inhibition of non-productive binding of cellulase to lignin by hydrophilic domain of surfactants adsorbed on substrate (Eriksson et al., 2002a).

Polyethylene glycol (PEG) with a molecular mass of around 4000 Da (PEG 4000)

also improves saccharification efficiency (Borjesson et al., 2007a; Borjesson et al., 2007b; Sipos et al., 2011). In this century, isolated lignin and their derivatives were also found to improve enzymatic saccharification. Some researchers reported usefulness of lignosulfonate (Lou et al., 2013; Nakagame et al., 2011; Wang et al., 2013; Zhou et al., 2013). Lai et al. (Lai et al., 2014) demonstrated the effect of organosolv lignins on saccharification.

1.4.2 Amphipathic lignin derivatives (A-LDs)

As mentioned above, lignin is one of the major components in wood cell wall. Lignin is a by-product in pulping industry and is utilized merely for energy recovery in pulping mill (Goldstein, 1975). Recently, lignin collects much attention because of its global abundance, and a huge potential as a feedstock for polymeric materials, but its utilization as a value-added material is rare. Forest Chemistry Lab. in Hokkaido University has developed amphipathic lignin derivatives (A-LDs) from several kinds of isolated lignins, such as kraft, soda, and organosolv lignins (Aso et al., 2013). A-LDs showed high surface activity like non-ionic surfactant. In addition, A-LDs were found to improve the efficiency of enzymatic saccharification for lignocellulosics. Based on this function, A-LDs can serve as “Celluase-aid agents” (Uraki et al., 2001). The cellulase-aid function of A-LDs were reported much earlier than those of other isolated lignin and their derivatives mentioned above, and PEG 4000.

Winarni et al. (2013) reported the difference in the mechanism of the improved enzymatic saccharification between A-LDs and PEG 4000. PEG 4000 improved

saccharification efficiency or sugar yield better than A-LDs, as shown in **Figure 1-4**. However, A-LDs significantly preserved the residual cellulase activity after the saccharification in a higher level than PEG 4000. They proposed that the difference was caused by the difference in the adsorptions of such additives on guest molecules; PEG 4000 directly associated with substrate (Borjesson et al., 2007a), while A-LDs associated with cellulase (Winarni et al., 2013). This assumption was completely confirmed by Yamamoto et al. (Yamamoto et al., 2017) using a Biacore (GE, Healthcare, Japan), which measured the interaction of two molecules based on the surface plasmon resonance. According to this paper, A-LDs associated with CBH I and II, but not with EG I. But PEG 4000 did not associated with any enzyme components of cellulase cocktail.

The high residual cellulase activity caused by A-LDs would enable repeated use of the enzymes and its successive charge of the substrate to the saccharification media (Winarni et al., 2014).

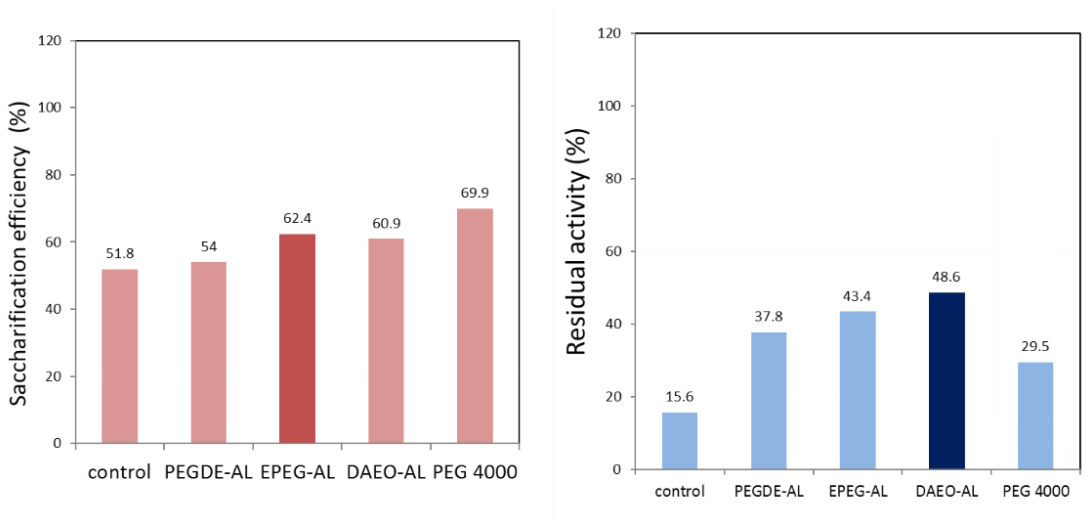


Figure 1-4. Sugar yield and recovery of cellulase activity of unbleached cedar pulp using Meicelase at the initial activity of 10 FPU/g substrate. (PEGDE-AL, EPEG-AL and DAEO-AL is A-LDs made from acetic acid lignin) (Winarni et al., 2013)

1.5. Objectives and research content

Although the effect of A-LDs on enzymatic saccharification has been demonstrated clearly above, their influence on fermentation and SSF including FB-SSF was not investigated. Therefore, a main objective of this thesis is to clarify the effects of A-LDs on fermentation and SSF. The final objective is to produce high concentration bioethanol from lignocellulosics.

To achieve the process of the final objective, I describe the following 4 chapters.

Chapter 2. Preparation of A-LDs and their influences on enzymatic saccharification

In this chapter, firstly, I would like to describe preparation and characteristics of amphipathic lignin derivatives (A-LDs). The lignin (cedar soda lignin; CSL) was

isolated from Japanese cedar chips by soda pulping. A-LDs were prepared by the reaction of three kinds of epoxylated PEG analogs with CSL, and characterized with respect to PEG content and surface activity.

Furthermore, the effect of A-LDs on enzymatic saccharification was investigated, where cedar soda pulp was used as substrate. The reason for using soda pulp was that it was considered as a feedstock for bioethanol production by FFPRI as mentioned above.

Chapter 3. Preliminary fed-batch type SSF with A-LDs

In this chapter, firstly I investigated the effect of A-LDs on ethanol fermentation of glucose with Japanese Sake yeast. Secondly, I investigated batch mode SSF without A-LDs to elucidate the limitation of batch mode. Thirdly, I gave preliminary experiment of FB-SSF with and without A-LDs under one condition as follows. The experiment was carried out with the total substrate loading of 18 g of pulp. The substrate (cedar soda pulp) was subjected to pre-hydrolysis at 50 ºC for 12 h, and then FB-SSF was performed at 38 ºC with an A-LDs loading of 2.5 g/L by horizontal shaking in 100 mL media. Here, only the enzyme loading was varied in the range of 5-10 FPU/g substrate.

Chapter 4. Optimization of fed-batch type SSF conditions to produce highly concentrated bioethanol

I confirmed the positive effect of A-LDs on FB-SSF in chapter 3. In this chapter, I optimized FB-SSF conditions to obtain much higher concentration ethanol as compared with that in preliminary experiment in chapter 3. For optimization of SSF temperature and A-LDs loading, these experiments were carried out in 100-mL scale FB-SSF. For

optimization of stirring conditions, I used a manufactured big jar fermenter equipped with a powerful stirrer, which enabled agitation of substrate suspension at high consistency. In this scale-up FB-SSF experiment, large amount of substrate was required. I did not have enough cedar soda pulp for the experiment, thus, I used unbleached softwood kraft pulp (NUKP), the most popular industrial pulp and commercially available, as substrate in this chapter, which was supplied from Nippon Paper Industries.

Chapter 5. Conclusions

In this chapter, all the results in my doctor experiments are summarized, and significance and social impact of my research are discussed.

Chapter 2 Preparation of A-LDs and their influences on enzymatic saccharification

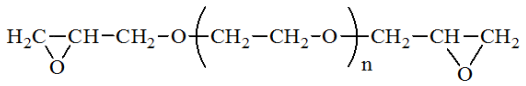
2.1. Introduction

So far, A-LDs have been prepared from isolated lignins, such as softwood and hardwood kraft lignins (Aso et al., 2013), acetic acid lignin (Homma et al., 2008) and sago soda lignin (Winarni et al., 2014). The preparations were carried out by the reaction of isolated lignins with three epoxylated polyethylene glycol (PEG) analoges, polyethylene glycol diglycidylether (PEGDE), ethoxy (2-hydroxy) propoxy poly (ethylene glycol) glycidyl ether (EPEG) and dodecyloxy poly (ethylene glycol) glycidyl ether (DOPEG), under alkali conditions. Chemical structures of the PEG analoges are shown in **Figure 2-1.**

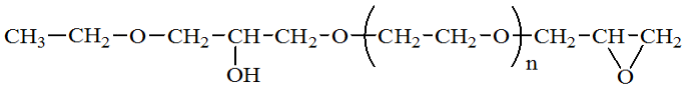
In this study, I prepared A-LDs from CSL and three kinds of PEG analoges. In this chapter, PEG contents and surface activities were measured to understand amphipathic nature of the lignin derivatives.

The positive effects of A-LDs prepared previously on the enzymatic saccharification of several substrates have been proved. Winarni et al. (2013) reported that the A-LD prepared with DOPEG showed the highest sugar yield among A-LDs, and the A-LD prepared with EPEG have the highest residual enzyme activity. Thereby, I investigated the effect of two A-LDs prepared with EPEG and DOPEG on enzymatic saccharification in this chapter.

A) **PEGDE:** Polyethylene glycol diglycidylether



B) **EPEG:** Ethoxy-(2-hydroxy)-propoxy-polyethylene glycol glycidylether



C) **DOPEG:** Dodecyloxy-polyethylene glycol glycidylether

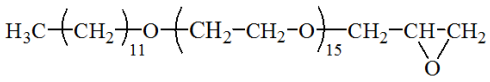


Figure 2-1. Chemical structure of epoxylated PEG derivatives.

2.2. Experimental

2.2.1. Preparation of A-LDs

Two hundred grams of Japanese cedar chip was cooked in an aqueous NaOH solution (52 g of NaOH in 1 L of water) in the presence of 1 g of anthraquinone. The heating time from room temperature to 170 °C was 90 min, and then the final temperature was kept for 90 min. After heating for 180 min, heating was stopped and the cooking pot was cooled down to room temperature. Then, the pulp and black liquor were separated by filtration. Cedar pulp was used as substrate for enzymatic saccharification by washing with distilled water until neutral pH, followed by washing with acetone. The pulp was air-dried for 3 days.

To obtain CSL, the black liquor form soda cooking mentioned above was acidified with concentrated HCl aqueous solution to pH 2 to precipitate lignin. The crude lignin

was collected by filtration, and washed several times with water. Finally, the lignin was lyophilized to yield CSL powder.

Three kinds of A-LDs (PEGDE-SCL, EPEG-SCL and DOPEG-SCL) were prepared by the reactions of CSL with epoxylated PEG analoges, PEGDE, EPEG and DOPEG. Commercially available PEGDE and DOPEG were kindly supplied from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). EPEG was synthesized according to the previous report (Homma et al., 2008). Ten grams of CSL was dissolved in 100 mL of 1 M aqueous NaOH solution, and then 30 g of PEGDE, 30 g of EPEG and 45 g of DOPEG were separately added to the CSL solutions. The solutions were stirred for 2 h at 70 ^{o}C. The reaction solutions were neutralized with glacial acetic acid to pH 4 to quench the reaction, and purified by ultrafiltration through a membrane with a cut-off molecular mass of 1000 Da (Advantec, Tokyo, Japan) (Homma et al., 2010). The ultrafiltration residue was lyophilized to yield A-LDs.

2.2.2. PEG content in A-LDs

PEG content in A-LDs was determined by modified Morgan method (Homma et al., 2008; Siggia et al., 1958)). The instruments for measurement of PEG content in A-LDs was shown in **Figure 2-2**. A mixture of 0.2 g A-LD sample and 5 mL hydroiodic acid solution (57 wt.%) was placed in a two-necked eggplant flask, a N_2 flow was introduced into the flask through a pipette as a flow nozzle. Another eggplant flask containing 25 mL of 20 % potassium iodide solution was set to collect generated iodine. The mixture was first heated at 145 ^{o}C for 90 min. After cooling, all contents in the

glass vessels were washed out with 125 mL of 20% potassium iodide aqueous solution. iodometric titration with 0.1 mol/L sodium thiosulfate solution was conducted, using 5 mL of 1% starch solution as an indicator to the solution, Each sample including the blank was measured at least in duplicate. The PEG content in samples was calculated as follows,

$$\text{PEG (wt. \%)} = [(V_s - V_b) / 1000] \times 0.1 \times (44 / 2) \times (1 / W_s)$$

where: V_s ——titration volume of $Na_2S_2O_3$ for a sample (mL);

V_b ——titration volume of $Na_2S_2O_3$ for blank (mL);

W_s ——sample weight (g);

0.1——concentration of sodium thiosulfate solution (mol L^{-1});

44 ——formula weight of <Ethylene oxide> (g mol^{-1}).

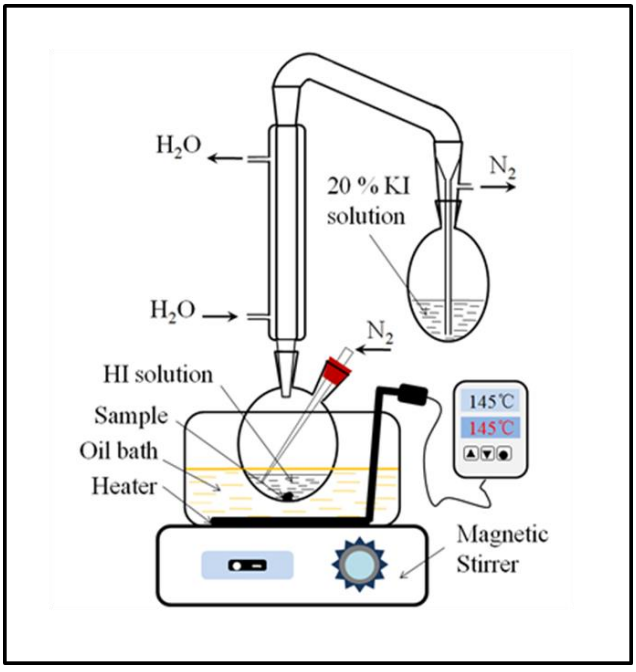


Figure 2-2. Instruments for PEG content measurement

2.2.3. Measurement of water surface tension in the presence of A-LDs

Water surface tension in the presence of A-LDs was measured by Du Noüy ring method (Aso et al., 2013; du Nouy, 1919). A-LDs samples (0.5 g) were separately dissolved in 10 mL milli-Q water with stirring for 1 h. The surface tension was measured with a Du Noüy ring tensiometer (**Figure 2-3**). The sample table of the tensiometer was adjusted to horizontal by using a spirit level. Before measurement, the platinum ring was heated until red, and then it was hung on the front hook. Firstly, zero setting were done by adjusting the pointer on dial to zero mark, and then the rear clamp spring screw were turned until torsion arm nearly touched the supporting stops. Secondly, water or sample solutions were poured into small petri dishes, and the dishes were put on the sample table. Thirdly, the sample table was raised until the platinum ring was immersed into a sample solution. Then, the torsion arm was raised slowly by rotating the screw for tightening the wire until the ring came off from the surface of sample solution. Finally, the dial was read to obtain a value of surface tension. Before sample solution measurement, surface tension of pure water (milli Q water) was measured. When the read of the water surface tension value did not indicate 74.3 mN/m correctly, positions of clamp spring screw and supporting stops were changed, so that the water surface tension was adjusted to 74.3 mN/m.

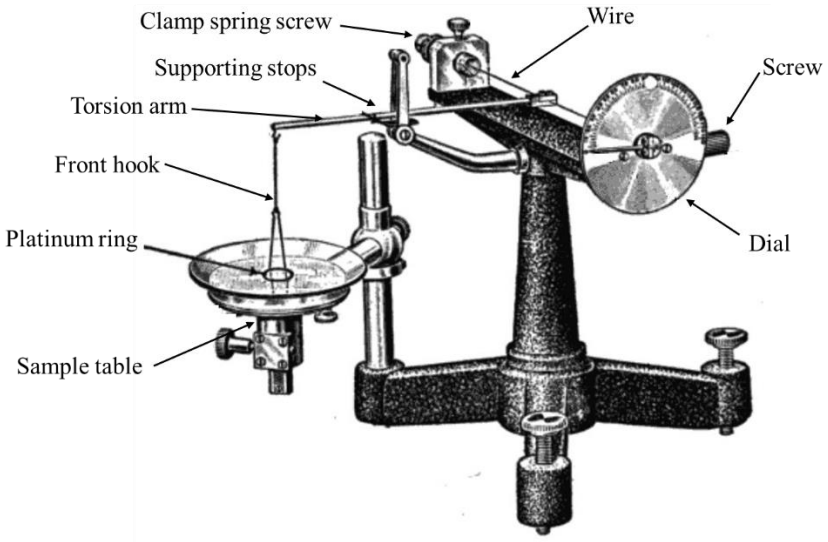


Figure **2-3**. A du Noüy ring tensiometer

2.2.4. Enzymatic saccharification with A-LDs

2.2.4.1. Substrate component measurement

In this study, two kinds of lignocellulosic substrate were used for enzymatic saccharification. One was unbleached cedar soda pulp which was prepared at a test plant of Forestry and Forest Products Research Institute (FFPRI), Japan (Akita, Japan).

The other one was unbleached softwood kraft pulp (NUKP), which was provided by Nippon Paper Industries Co., Ltd. (Tokyo, Japan). The pH of NUKP as received was above 10. Therefore, the pulp was washed with distilled water until neutral pH. The pulp was further washed with acetone, and air-dried for 3 days in a fume hood.

2.2.4.2. Klason lignin content in the pulps

A lignin content in pulps was determined by a modified method of Klason method (TAPPI T-222 om-83) with an autoclave. The first hydrolysis was conducted by the

addition of sulfuric acid (72 wt%, 7.5 mL) into 0.5 g of dry pulp in a 50-mL beaker. The reaction was continued at room temperature for 4 h. Then, the 72% H_2SO_4 slurry was diluted to 4 wt% H_2SO_4 with 207.8 mL of distill water, and transferred to a 500 mL Erlenmeyer flask. The diluted slurry in the flask was heated to 121 ^oC in an autoclave, and the temperature was kept for 1 h. After cooling, the slurry is filtrated with a G4 glass filter to separate the filtrate and the filtration residue. The filtration residue was washed with hot water at 70 ^oC until near pH 7. The weight of empty glass filter before filtration (W_1), which was dried in an oven at 105 ^oC overnight, was measured. After filtration and drying, the used glass filter with the filtration residue (W_2) was also weighed. The klason lignin content of pulp was calculated as follows:

$$\text{Klason lignin content (\%)} = (W_2\text{-}W_1) / W_{\text{dry pulp}} \times 100\ \%$$

2.2.4.3. Sugar constituents

The filtrate in the Klason method was subjected to neutral sugar analysis after the neutralization with $Ba(OH)_2$ until pH 5.5, followed by removal of precipitate by centrifugation (Slavin and Marlett, 1983).

The determination of sugars was carried out on a HPLC (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 water, and its flow rate was set at 0.5 mL/min. The injection volume was 20 μL.

2.2.4.4. Enzymatic hydrolysis

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), was measured according to the NREL technical report, NREL/TP-510-42628, method (Ghose 1987). The enzyme activity of Meicelase and Genencor GC220 as received were 306 FPU/g and 84.1 FPU/mL, respectively.

EPEG-SCL and DOPEG-SCL (10 % of substrate on dry weight basis) were separately dissolved in 100 mL of 50 mM citrate buffer (pH 4.8). Each cellulase with 10 filter paper unit (FPU)/g pulp was added to the solutions, and the mixture was stirred for 1 h at room temperature. Then, to the solutions, 1 g of dry unbleached cedar pulp was added. The suspension was shaken with a Yamato BT23 Digital Shaking Incubator (Tokyo, Japan) for 48 h at 50 ^{o}C and 120 rpm. After saccharification, the suspension was filtered through a G4 glass filter. The precipitate was washed three times with 500 mL of distilled water, and weighed after completely dried at 105 ^{o}C. All the experiments were performed in duplicate. The sugar yield (%) was calculated according to the following equation:

$$\text{Sugar yield \%} = (WS - WR) / WS \times 100$$

Where WS (g) is the initial weight of substrate, and WR (g) is the weight of the filtered residue.

Finally, an enzyme solution was recovered from the filtrate by ultrafiltration (Vivaspin 20, with 10 kDa cut-off membrane: Satorius Co. Göttingen, Germany) in accordance with the previous report (Winarni et al., 2013). FPU of the recovered cellulase solution was measured according to a test method by the National Renewable Energy Laboratory (NREL) technical report, NREL/TP-510-42628, (Ghose 1987) and a recovered activity of cellulase was calculated by the following equation.

Recovered activity (%) = FPU after saccharification/ FPU of the initial cellulase ×100

2.3. Results and discussion

2.3.1. Surface activities of A-LDs

The PEG contents in DOPEG-SCL, EPEG-SCL and PEGDE-SCL were 66.1 wt%, 63.5 wt% and 58.5 wt%, respectively. **Figure 2-4** shows water surface tension vs. A-LDs concentrations. DOPEG-SCL clearly showed critical micelle concentration (CMC), which was obtained at an intersection of two regression lines (Homma et al., 2008). The CMC value was 2.0 x 10^{-4} g/mL, and the surface tension at CMC was 37 mN/m. These values were almost identical to those of previously prepared DOPEG-lignin (Homma et al., 2010; Winarni et al., 2013). On the other hand, although EPEG-SCL and PEGDE-SCL also decreased water surface tension, no CMC was observed in **Figure 2-4**. These results revealed that DOPEG-SCL had higher surface activity than EPEG-SCL and PEGDE-SCL. This tendency of A-LDs surface activity was consistent with the previous A-LDs, which were prepared from acetic acid lignin (Homma et al., 2010), kraft lignin (Winarni et al., 2014) and soda sago lignin (Winarni

et al., 2013). Thus, surface activity of A-LDs depends on the structures of the epoxylated PEG analoges, which is independent of lignin origins or isolation methods.

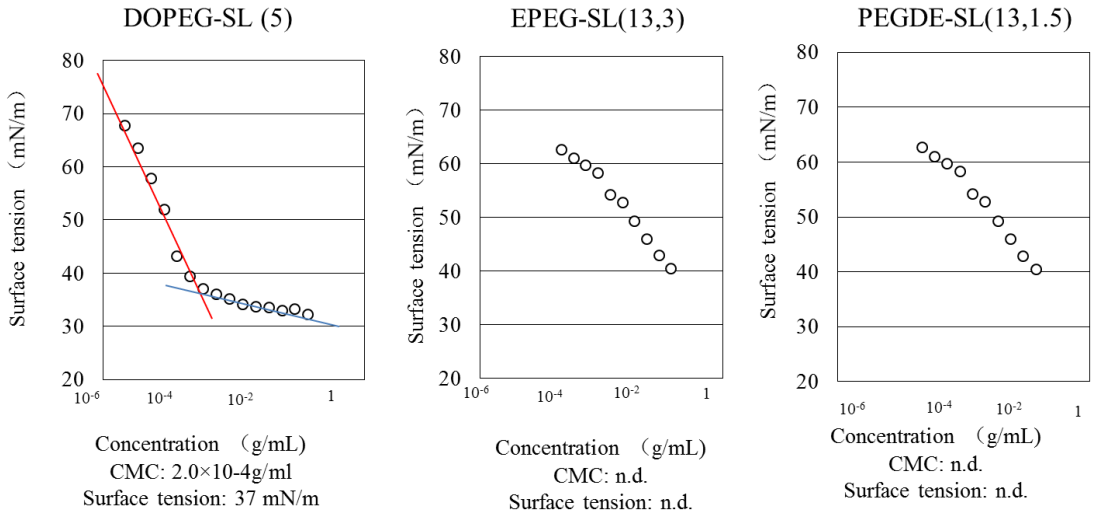


Figure 2-4. Surface tension-concentration isotherm for three A-LDs.

2.3.2. Effect of A-LDs on enzymatic saccharification

For this experiment, two substrates, unbleached cedar pulp and NUKP, were used. The chemical composition of the cedar soda pulp was 75.6 % cellulose, 11.0 % mannan, 5.0 % xylan, 6.9 % Klason lignin and 0.55 % of ash, and that of NUKP was 87.8% cellulose, 3.4% mannan, 5.3% xylan, and 2.1% Klason lignin. The lignin content in cedar soda pulp was much larger than that in NUKP.

Tables 2-1 and **2-2** show the sugar yield and residual enzyme activity after enzymatic saccharification of cedar soda pulp with GC220 and NUKP with Meicelase, respectively, at 10 FPU/g of substrate. **Table 2-3** shows the sugar yield and residual enzyme activity after enzymatic saccharification of NUKP with GC220 of 10 FPU/g of

substrate.

In all Tables, A-LDs (EPEG-SCL and DOPEG-SCL) prepared from CSL increased sugar yields and recovered enzyme activities after the saccharification of two kinds of substrate, as compared with the control experiments. The performance of A-LDs on the saccharification was thought to be similar to those of A-LDs reported previously (Winarni et al., 2014; Winarni et al., 2013).

EPEG-SCL showed higher recovered enzyme activity for the saccharification of cedar soda pulp than DOPEG-SCL (**Table 2-1**), while DOPEG-SCL showed higher recovered enzyme activity for the saccharification of NUKP than EPEG-SCL (**Table 2-2**). These results suggest that EPEG-SCL is useful to improve the saccharification of lignocellulose with high lignin content, and DOPEG-SCL is useful to improve the saccharification of lignocellulose with low lignin content.

If saccharification potential between GC220 and Meicelase is compared, GC220 (control experiment in **Table 2-3**) showed higher sugar yield and recovered enzyme activity than Meicelase (control experiment in **Table 2-2**) in the saccharification of NUKP. This result is consistent with the saccharification of unbleached cedar pulp reported previously (Winarni et al., 2013). According to the paper, the cellobiohydrolase (CBH) activity and CMCase (endoglucanase) activity of GC220 were much larger than those of Meicelase at the same FPU, while Meicelase showed higher β-glucosidase activity than GC220. The larger CBH activity and CMCase activity gave GC220 good performance for enzymatic saccharification.

Table 2-1. Effect of A-LDs on enzymatic saccharification of unbleached cedar soda pulp (lignin 6.9 %) with GC220 as cellulase.

	Sugar yield /%	Recovered enzyme activity /%
Control	60.1	22.5
EPEG-SCL	70.1	55.0
DOPEG-SCL	74.3	53.6

Cellulase charge was 10 FPU/g substrate.

Table 2-2. Effect of A-LDs on enzymatic saccharification of NUKP (lignin 2.1 %) with Meicelase as celulase.

	Sugar yield /%	Recovered enzyme activity %
Control	51.4	17.4
EPEG-SCL	63.0	69.9
DOPEG-SCL	63.9	78.7

Cellulase charge was 10 FPU/g substrate.

Table 2-3. Saccharification of NUKP with GC220 in the presence of DOPEG-SCL.

	Enzyme	**Sugar yield (%)**	**Recovered enzyme activity (%)**
Control	GC220	62.9	32.4
DOPEG-SCL		73.7	86.4

Cellulase charge was 10 FPU/g substrate.

Chapter 3 Preliminary experiment of fed-batch type SSF with A-LDs

3.1. Introduction

It has been proved in chapter 2 that enzymatic saccharification can be remarkably improved by A-LDs. Aims of this chapter is to clarify influences of A-LDs derived from CSL on fermentation and SSF including FB-SSF.

The influence of A-LDs on the fermentation was investigated from the yeast fermentation of glucose, where Japanese Sake yeast was used as a fermentable microorganism. Secondly, batch type SSF was investigated in the absence of A-LDs to elucidate the limitation of batch mode. Finally, the influence of A-LDs on FB-SSF process were investigated in a 100-mL scale of reaction buffer solution under the following conditions. In all FB-SSF experiments, total substrate charge (consistency of pulp in the SSF media), reaction temperature and agitation condition were constant. Only the enzyme charge was changed to elucidate a relationship between enzyme charge and ethanol yield (concentration). Thus, other parameters, such as temperature and agitation condition, were not examined in this chapter. Thereby, the experiments of FB-SSF were preliminarily performed.

3.2. Experimental

3.2.1. Glucose fermentation by Japanese Sake yeast

Japanese Sake yeast, *Saccharomyces cerevisiae*, (2 g of dry powder, Mauri Yeast

Australia Pty Ltd., Queensland, Australia) was dispersed in 100 mL of YP medium [0.05 M of citrate buffer (pH 4.8) including 10 g/L of yeast extract and 20 g/L of peptone] (Dowe and McMillan, 2001) together with 3 g of glucose, and incubated for 2 days at 38 °C with gentle shaking to give a pre-incubated yeast suspension.

Ten grams of glucose was dissolved in 80 mL of YP medium, and 20 mL of pre-incubated yeast suspension and 0.25 g of A-LDs were added to the glucose solution. In the case of control, no A-LDs was added. The mixture was further incubated for 4 days at 38 °C with gentle shaking at 100 rpm. An aliquot was sampled out every day, and ethanol concentration in the aliquot was measured by GC (Shimadzu-GC2010, Shimadzu, Kyoto, Japan) with a capillary column (0.25 mm Φ× 30 m; TC-WAX, GL Science, Tokyo, Japan) and a flame ionization detector. Column temperature was programmed at initial 70 ºC for 5 min, then increased to 150 ºC at a rate of 10 ºC/min, kept at 150 ºC for 5 min. The injector temperature was 180 ºC, and detector temperature was 200 ºC.

3.2.2. Batch type SSF without A-LD

The experiment was carried out in a 300-mL flask with a silicone sponge plug on top. Cellulase (Genencor GC220) with 10 FPU/g of pulp was dissolved in 90 mL, 0.05 M of citrate acid buffer (pH 4.8). One gram of Yeast extract and 2 g of peptone powder were added into the medium. Then, 10 mL of pre-incubated Sake Yeast culture prepared in the above section were poured into the flask. At last, three grams (dry weight) of unbleached cedar soda pulp was added to the medium. This batch type SSF was

performed at 38 ºC with rotational shaking at a rate of 120 rpm for 5 days. The fermentation medium (0.5 mL) was sampled out every day. Glucose and ethanol concentrations in the medium were measured by HPLC (Chapter 2.2.4.3) and GC, respectively.

3.2.3. FB-SSF with and without A-LDs under different enzyme loadings

Three different enzyme (Genencor GC220) loadings were tested, 5 FPU/g, 6.67 FPU/g and 10 FPU/g. Substrate (unbleached cedar pulp) and GC220 were placed in a fermentation vessel according to the time schedule in **Figure 3-1**. **Figure 3-1-(A)** shows a FB-SSF process at a cellulase loading of 5 FPU/g pulp, while **Figure 3-1-(B)** shows them at cellulase loadings of 6.67 and 10 FPU/g pulp.

Firstly, 3 g of cedar soda pulp in 70 mL of citrate buffer (0.05 M, pH 4.8) was pre-hydrolyzed at varying loadings of the cellulase (30 FPU for the experiment at 5 FPU/g pulp, 40 FPU at 6.67 FPU/g pulp, and 60 FPU at 10 FPU/g pulp) of Genencor GC220 for 12 h at 50 °C together with or without 0.25 g of EPEG-CSL or DOPEG-CSL. Afterwards, pre-incubated Sake yeast (20 mL) was inoculated into the pre-hydrolyzed pulp suspension, and the mixture was shaken at 114 rpm at 38 °C for 6 days (144 h). During SSF, the pulp and the cellulase were added intermittently, shown in **Figures 3-1-(A)** and **(B)**. In the case of a cellulase loading at 10 FPU/g pulp, a black arrow and a white arrow in **Figure 3-1-(B)** indicate charges of 3 g of pulp and 60 FPU of cellulase, and that of 3 g of pulp, respectively. In the case of 6.67 FPU/g of pulp, a blade arrow indicates 3 g of pulp and 40 FPU of cellulase, and a white arrow indicates 3 g of pulp.

In **Figure 3-1-(A)** at 5 FPU/g pulp, the white arrow shows the charge of 3 g of pulp, and the black arrow shows the charges of 3 g of pulp and 30 FPU. An aliquot of incubated media was taken out every day, and its ethanol concentration was measured by GC. These FB-SSF experiments were carried out in duplicate under the same conditions. Thereby, the ethanol concentration was expressed as the average value. The ethanol conversion (%) on theoretical maximum ethanol production was calculated using the following equation:

$$[\mathrm{EtOH}]_{produced} = [\mathrm{EtOH}]_t - [\mathrm{EtOH}]_o$$

$$\text{Bioethanol conversion (\%)} = \frac{[\mathrm{EtOH}]_{produced}}{0.51\ (\ [\mathrm{W}]\times f\times 1.11)} \times 100\%$$

where $[\mathrm{EtOH}]_{produced}$ is the ethanol concentration generated by the fermentation, $[\mathrm{EtOH}]_t$ is ethanol concentration (g/L) at the time (t) of the fermentation, $[\mathrm{EtOH}]_o$ is ethanol concentration (g/L) at the beginning of the fermentation, [W] is the weight consistency of total dry pulp in SSF, f is a proportion of cellulose fraction of dry biomass (g/g; 0.878 in this study) and 0.51 is a conversion factor for glucose to ethanol based on stoichiometric fermentation of yeast (Hayward et al., 1995).

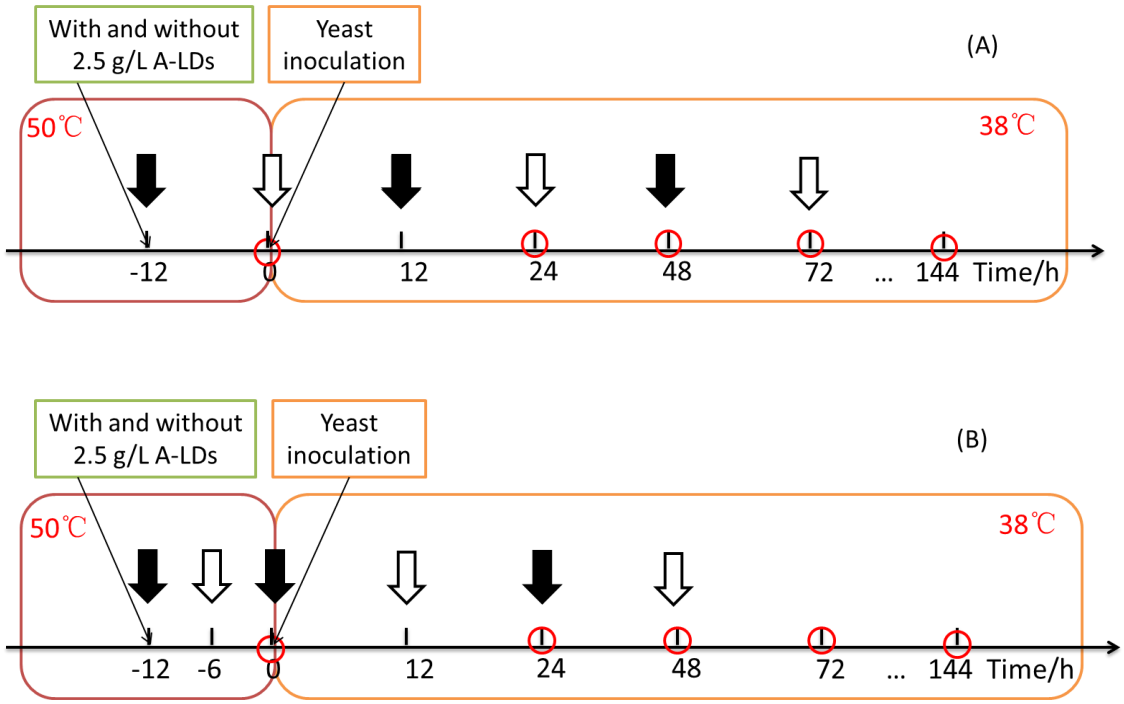


Figure 3-1. Time schedule for reactant charge and sampling during SSF process at a cellulase loading of 5 FPU/g of unbleached cedar soda pulp (A); 6.67 FPU/g and 10 FPU/g (B). (⬇, charge of pulp and cellulase; ⇩, charge of only pulp; ○, sampling for ethanol determination)

3.3. Results and discussion

3.3.1. Influence of A-LDs on ethanol fermentation of glucose with Japanese Sake yeast

Prior to SSF experiment, I investigated the effect of A-LDs (EPEG-SCL and DOPEG-SCL) on glucose fermentation with a commercially available yeast, Japanese Sake Yeast. **Figure 3-2** shows ethanol concentration produced by the fermentation with and without A-LDs. The time of fermentation required to reach the maximum ethanol concentration in the presence of both A-LDs was 2 days, while the time needed in the

absence of A-LDs (as control) was 3-4 days. This result suggested that A-LDs did not suppress the yeast fermentation, but rather accelerated the fermentation. The positive effect of A-LDs on the yeast fermentation may be caused by an increase in the membrane permeability of yeast cell just like other non-ionic surfactants (King et al., 1991; Lee et al., 1996).

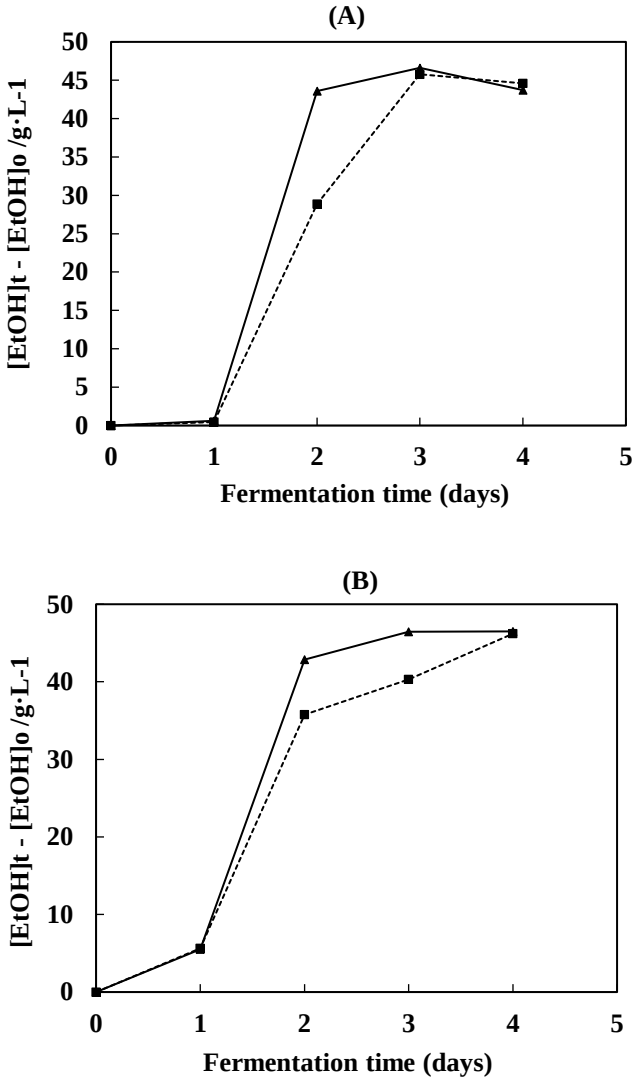


Figure 3-2. Effect of DOPEG-SCL (A) and EPEG-SCL (B) on ethanol fermentation of glucose in the presence of A-LDs (-▲-) and in the absence of A-LDs (-■-).

3.3.2. Batch type SSF without A-LDs

A batch type SSF have been attempted under the following conditions; 3 g of unbleached cedar soda pulp in 100 mL of the citrate buffer at 38 ºC with rotational shaking at 120 rpm. **Figure 3-3** shows concentrations of glucose (dotted line) and ethanol (solid line) generated by batch type SSF. A quick increase in glucose concentration at the first day of the SSF indicated that cellulose saccharification started very quickly. The maximum sugar concentration was 15.9 g/L, which correspond to 70.1 % sugar yield on cellulose in the substrate. On the second day, the glucose concentration was sharply decreased by glucose consumption by yeast to produce ethanol. Afterwards, the ethanol concentration gradually increased until 5 days. The final ethanol concentration was 9.9 g/L, which corresponded to 84.6 % on the theoretical maximum ethanol concentration (11.7 g/L). Although the ethanol conversion was apparently very high, the concentration (about 1%) was much smaller than drinkable alcohol, e.g. beer (5%). This indicates that high distillation cost is required to obtain absolute ethanol under this condition. To obtain higher concentration alcohol, much larger substrate should be charged in the SSF medium. However, only 3 g of charge was found to be a maximum substrate loading in 100 mL of the buffered solution. When more than 3 g of substrate was charged, the substrate could not be soaked well in the buffered solution. To achieve a larger charge of substrate, successive charge of substrate with certain interval is one of the proposed procedures. This modified system in SSF referred to as “fed-batch type SSF (FB-SSF)”.

The result of batch type SSF indicated that the ethanol fermentation started at the highest glucose concentration, which means sufficient amount of glucose is necessary for ethanol production. In the next section, FB-SSF was examined. In this experiment, pre-hydrolysis step of substrate was inserted in the FB-SSF process to obtain high glucose concentration before the yeast addition.

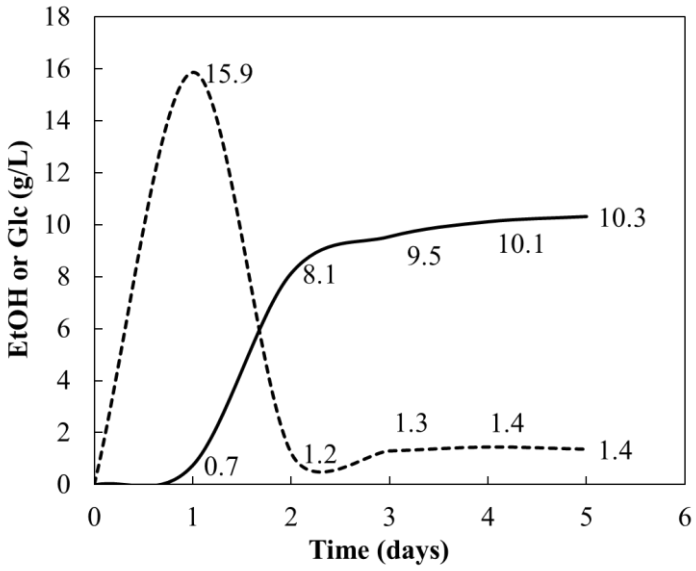


Figure 3-3. Glucose and ethanol concentrations in batch type SSF.

3.3.3. Effect of A-LDs on 100-mL scale of FB-SSF under different enzyme loadings

FB-SSF was also examined using the same enzyme (GC220) and substrate (unbleached cedar soda pulp) as those of previous section. However, three different cellulase loadings, 5, 6.67 and 10 FPU/g substrate, were applied. The disappearance or solubilizing of the substrate upon the saccharification was visibly very slow at the cellulase loading of 5 FPU/g pulp, as compared with other higher cellulase loadings. Thereby, the substrate (3 g x 6 times) was added at 3-day intervals at 5 FPU/g substrate,

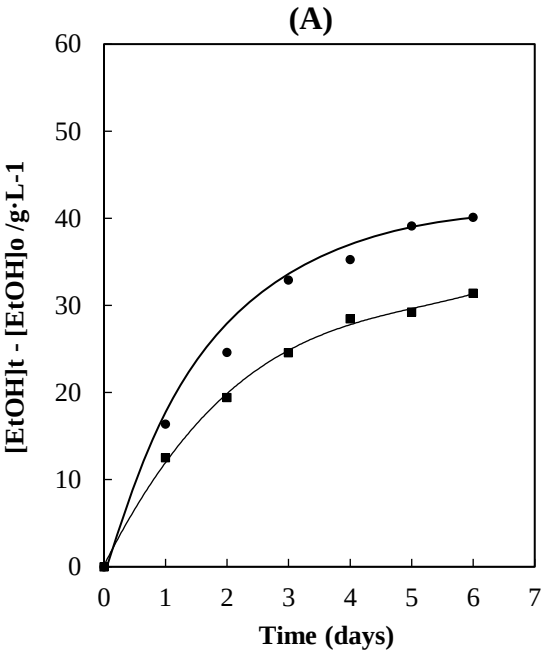
while at 2-day intervals at 6.67 and 10 FPU/g substrate, as shown in **Figure 3-1.**

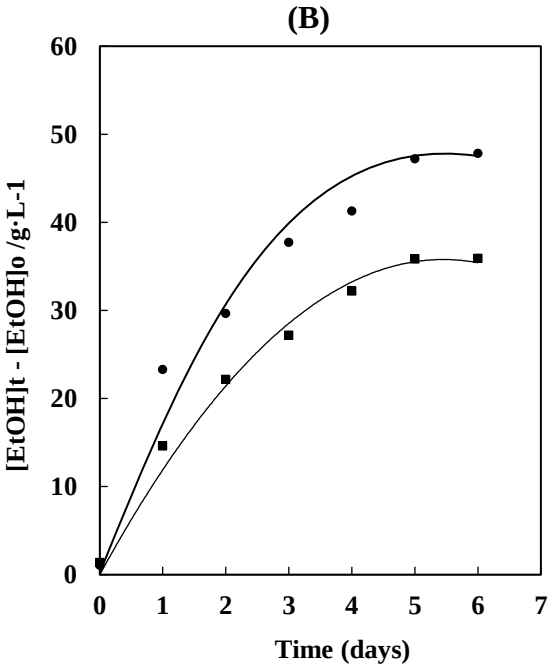
Figure 3-4 shows bioethanol production with and without DOPEG-SCL in FB-SSF. The ethanol concentrations at any cellulase loadings after 1-day fermentation were larger than those without DOPEG-SCL. These results indicated that a larger amount of glucose was produced in the pre-hydrolysis process by the addition of DOPEG-SCL.

The ethanol concentrations under any conditions almost reached the maximum value at around 5th day. **Table 3-1** summarizes the final ethanol concentration, ethanol yield on the theoretical maximum ethanol production for 6-days fermentation, and residual, unfermented glucan after 6-days of fermentation. **Figure 3-4** and **Table 3-1** clearly demonstrated that DOPEG-SCL remarkably improved bioethanol production in the FB-SSF. Although the ethanol yield of 64.1 % at 10 FPU/g of pulp with DOPEG-SCL was the highest among all FB-SSF experiments, 28.1 % of glucan was found to remain in the SSF medium. Therefore, further improvement of FB-SSF efficiency would be possible.

As well, the effect of EPEG-SCL on FB-SSF was also investigated, using cellulase at 5 and 10 FPU/g pulp, the result was shown in **Figure 3-5**. The final bioethanol concentrations and yields on the theoretical maximum production with EPEG-SCL at both cellulase loadings were almost identical to those with DOPEG-SCL. When residual cellulase activities after the 6-days of FB-SSF at 5 FPU/g pulp in the presence of EPEG-SCL and DOPEG-SCL was measured to be 14.3 FPU and 9.9 FPU, respectively, which corresponded to be 16 % and 11 % of total cellulase loading (90

FPU), respectively. Thus, EPEG-SCL showed higher ability for maintaining enzyme activity during SSF than DOPEG-SCL.





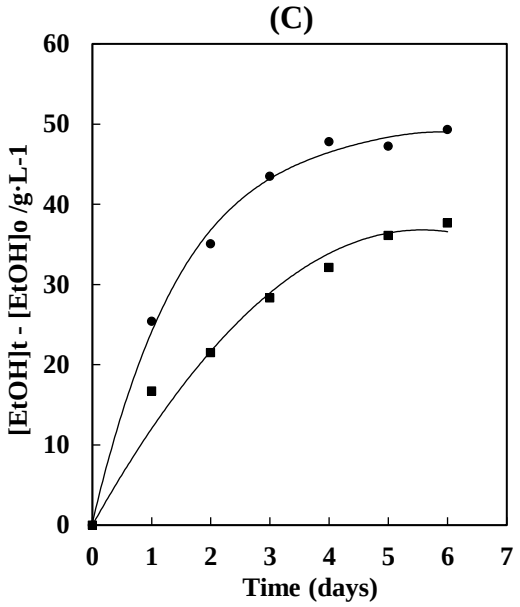


Figure 3-4. Ethanol production in 100 mL scale FB-SSF with GC220 5.0 FPU/g (A); 6.67 FPU/g (B); 10.0 FPU/g (C) of cedar soda pulp in the presence of DOPEG-SCL (-●-) and absence of DOPEG-SCL (-■-).

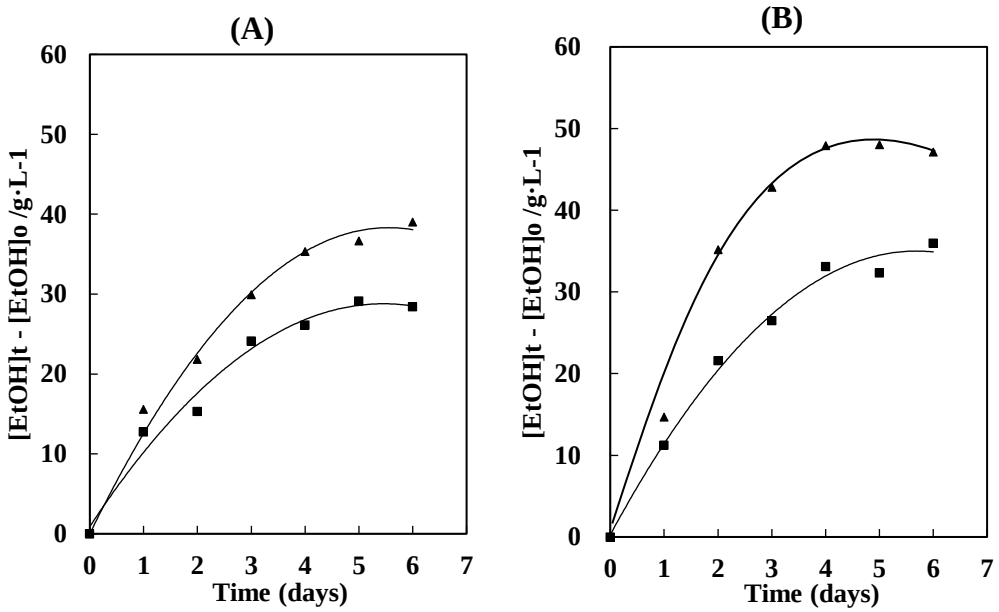


Figure 3-5. Ethanol production in 100 mL scale FB-SSF with GC220 5.0 FPU/g (A); 10.0 FPU/g (B) of cedar soda pulp in the presence of EPEG-SCL (-▲-) and in absence of EPEG-SCL (-■-).

Table 3-1. Bioethanol concentration and yield in 100 mL FB-SSF at different cellulase loadings.

	GC220 loading /FPU·g^{-1}	**Maximum ethanol conc. /g·L^{-1}**	**Ethanol conversion /%**	**Residual glucan content based on initial glucan content /%**
Control	5.0	31.4	40.7	47.6
DOPEG-SCL		40.1	52.0	34.4
Control	6.7	35.9	46.6	47.0
DOPEG-SCL		47.8	62.0	30.6
Control	10.0	37.8	49.0	37.6
DOPEG-SCL		49.4	64.1	28.1
Control	5.0	29.1	37.7	46.5
EPEG-SCL		39.0	50.6	37.2
Control	10.0	36.0	46.7	39.5
EPEG-SCL		48.1	62.4	30.1

In conclusion, this chapter demonstrated that both EPEG-SCL and DOPEG-SCL remarkably increased ethanol production in FB-SSF with various enzyme loadings. Especially, 10 FPU/g enzyme loading achieved approximately 5 % ethanol concentration (50.0 g. L^{-1}). However, the concentration is much lower than that by Japanese Sake. Japanese Sake yeast can ferment glucose to produce ethanol at the concentration of 15-20 % (Ogawa et al., 2000). Therefore, the maximum performance in this system could not overwhelm that by Japanese Sake yeast. In addition, a larger

amount of glucan could not be saccharified and fermented even at 10 FPU/g cellulase. These facts imply that further improvement of FB-SSF efficiency is possible upon the optimization of FB-SSF conditions parameters, such as temperature and agitation conditions.

Chapter 4 Optimization of fed-batch type SSF conditions to produce highly concentrated bioethanol

4.1. Introduction

In chapter 3, I demonstrated that two A-LDs derived from Japanese cedar, DOPEG-SCL and EPEG-SCL, improved not only the yeast fermentation of glucose but also ethanol production efficiency in FB-SSF. The ethanol concentration of 5% produced by preliminary FB-SSF experiments was not high, and a larger amount of glucan (28%) remained. To reduce distillation cost, high concentration ethanol should be produced (Dias et al., 2011). Therefore, in this chapter, optimization of FB-SSF conditions was attempted to achieve it. The conditions were optimized with respect to temperature at SSF process, loading amount of A-LDs, and stirring program.

The reasons why SSF temperature is optimized are as follows. The optimum temperature for cellulase activity is 50-60 ºC (Zambare et al., 2011), whereas the suitable temperature for ethanol fermentation is usually below 35 ºC (Torija et al., 2003). The optimized temperatures for enzymatic saccharification and fermentation are quite different. Thus, it is necessary to search a suitable temperature, or temperature program, for SSF process.

Several researches pointed out that the agitation conditions, such as rate and power, could remarkably influence saccharification rate and yield of lignocellulosics (Gunjikar et al., 2001; Guo et al., 2015; Taneda et al., 2012). Therefore, a big jar fermenter (3 L) equipped with a strong mechanical stirrer and a sensitive agitation speed controller was

designed and manufactured in this study.

To conduct FB-SSF using the big jar fermenter, much pulp of about 10 kg was required. However, it was very difficult to prepare much cedar soda pulp in my situation because Forest Chemistry Lab. did not have a big digester or an autoclave as a pulping vessel. Instead of unbleached cedar soda pulp, commercial unbleached softwood kraft pulp (NUKP) was used as lignocellulosic substrate in this chapter, because it was commercially available easily obtained in a large amount from a paper mill. Actually, it was kindly supplied from Nippon Paper Industries.

4.2. Experimental

4.2.1. Fermentation temperature test for Japanese Sake yeast

Yeast cells in pre-incubated medium prepared from 0.4 g of dry Japanese Sake yeast (chapter 3.2.1) were collected by centrifugation for 10 min at 2500 rpm. The obtained yeast cells were mixed with 40 g of glucose in 100 mL of YP medium. The mixture in YP medium was gently shaken for 144 h at 20, 25, 28, 30, 35, 38 and 42 ^{o}C. An aliquot was sampled out every day, and its ethanol concentration was measured by GC (chapter 3.2.1.).

4.2.2. Conditions optimization of 100-mL scale FB-SSF

4.2.2.1. FB-SSF for optimization of FB-SSF temperature

NUKP was used as lignocellulosic substrate in this chapter, of which sugar constituents were shown in chapter 2.3.2. The cellulase used in this experiment was

Meicelase. After pre-hydrolysis at 50 °C in 100 mL of citrate buffered solution, the medium was cooled to 25, 35, 36.5, 38 or 40 °C, and 10 mL of pre-incubated yeast in 10X YP medium were inoculated in the pre-hydrolysis medium. The substrate and cellulase were charged according to **Figure 3-1 (B)**. In this experiment, DOPEG-SCL concentration was 2.5 g/L.

4.2.2.2. FB-SSF for optimization of A-LDs loading

Both DOPEG-SCL and EPEG-SCL were separately added to the FB-SSF medium to adjust their concentrations at 2.5, 3.0 and 5.0 g/L. Fermentation temperature was 38 °C. Other conditions were identical to the above conditions.

4.2.3. 1.5-L scale FB-SSF

1.5-L scale FB-SSF was performed using a custom-made jar fermenter. Its volume was 3.5 L, and it was equipped with a strong mechanical stirrer and two impellers (**Figure 4-1**). The speed of the stirrer was controlled by the combination with a gear head motor (GFV4G15, Oriental Motor Co., Ltd., Japan) and a controller (BMUD60-A2, Oriental Motor Co., Ltd., Tokyo, Japan). The tank was heated by a rectangle silicone rubber heater, which covered outside the tank body [the red one shown in **Figure 4-1-(C)**]. The heater was connected to a thermometer to maintain the constant temperature of the tank body. The temperature inside the jar fermenter was measured on a thermometer inserted into the tank from top **[Figure 4-1-(B)]**. Materials, such as substrate and enzyme, were inserted into the tank through an inlet on the top of tank

[**Figure 4-1-(B)]**. After the insertion, the inlet was covered with a lid. The jar fermenter was sterilized with 75 % (v/v) ethanol before use.

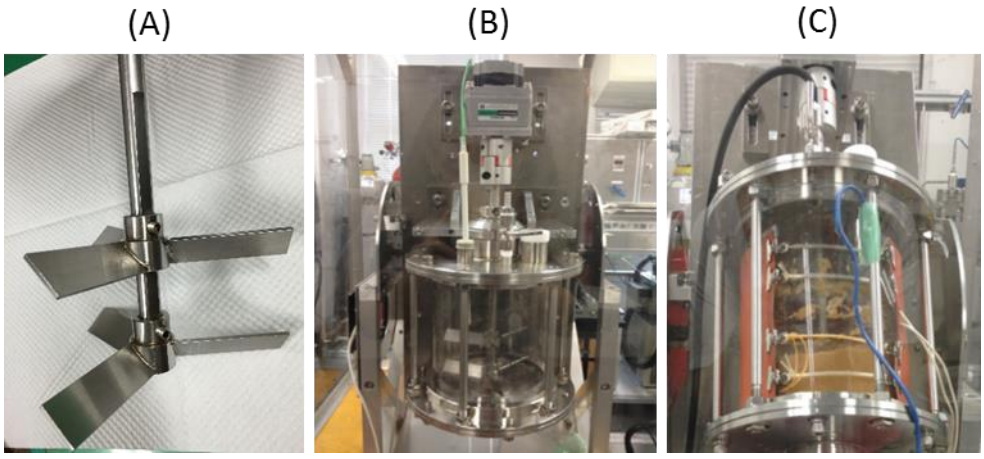


Figure 4-1. Photo of custom-made jar fermenter. (A), impeller inside the tank; (B), front photo of the jar fermenter; (C), operation photo.

Cellulase (Meicelase or GC220; 1200 or 1500 FPU) in 1.35 L of the citrate buffered solution was poured into the jar fermenter together with or without A-LDs. In the case of A-LDs addition, the medium in the tank was intensively mixed for 1 hour for complete dissolving A-LDs. Then, the jar fermenter was heated to 50 ºC. Forty grams of substrate was added to this medium, and pre-hydrolysis process began. After 4 h of the first substrate addition, another 40-g substrate was added to the tank. At the time of 8 h pre-hydrolysis, the third charge (40 g) was done. At 12 h of pre-hydrolysis, the jar fermenter was cooled to 38ºC, and pre-incubated yeast prepared from 6 g dry yeast in 150 mL of 10XYP medium was poured into the fermenter. As a result, total volume of the medium was 1.5 L.

During SSF process, substrate and cellulase (Mecelase or GC220) were

intermittently added to the SSF media according to the charging program indicated as a dashed line and an arrow, respectively, in **Figures 4-3** to **Figure 4-6**. The ratio of the enzyme to substrate was maintained at 10 FPU/g throughout the process. The stirring conditions was tested in the next section. Slurry samples (1 mL) were sampled out at 24, 48, 72, 96, 120 and 144 h of SSF process. After centrifugation, the ethanol concentration in supernatant was monitored by GC as mentioned in chapter 3.2.1.

4.2.4. Stirring program optimization for 1.5-L scale FB-SSF

Four kinds of stirring program were designed for FB-SSF with pre-hydrolysis (**Table 4-1**). In the case of program 1, the stirring frequency controlled at an invariant low speed of 10 rpm during the whole process. And in program 2 oppositely, it keeps a high invariant speed of 40 rpm. However, the Program 3 adopts a variant speed of 40 rpm during pre-hydrolysis, 20 rpm during substrate charging in first 3 days and then 10 rpm during left days. The only difference of program 4 from program 3 is that, the last 3 days are under an intermittent stirring of 5 min at 40 rpm and 55 min rest in each hour controlled by PT50DW digital timer (REVEX, Kawaguchi, Japan). The SSF processes with four stirring programs were carried out under the same conditions as those mentioned above, but no A-LDs addition. Cellulase in this experiment were Meicelase, and the charging time curve of cellulase and NUKP were indicated as an arrow and a dashed line, respectively, in **Figure 4-3.**

Table 4-1. Four different stirring programs for optimization of FB-SSF.

	Stirring speed (rpm)		
Time	-12 ~ 0 h	0 ~ 72 h	72 ~ 144 h
Program 1	10	10	10
Program 2	40	40	40
Program 3	40	20	10
Program 4	40	20	The following program was repeated: 40 rpm for initial 5 min, and then 0 rpm for the remaining 55 min.

4.2.5. A-LDs concentration optimization for 1.5 L FB-SSF

Two kinds of A-LDs, DOPEG-SCL and EPEG-SCL were used as additives in this experiment, the A-LDs concentration was project as 0.0, 2.0, 3.0 and 5.0 g/L of medium volume. The substrate and cellulase used in this experiment was NUKP and Meicelase, respectively. The charged amount of NUKP and the addition of Meicelase are also indicated by dashed lines and arrows, respectively in **Figure 4-4.** The total substrate loading was 360 g (40 g x 9 times) including the loading in the pre-hydrolysis process, and the total enzyme loading was 3600 FPU (1200 FPU x 3 times). The stirring strategy used in this experiment was the same as agitation program 4 mentioned in the previous section.

4.2.6. A 1.5-L scale FB-SSF with high substrate loading

In this experiment, Genecor GC220 was used instead of Meicelase. Two kinds of 1.5-L scale FB-SSF at high substrate loading of 24% and 30% were conducted. In both experiments, the ratio of enzyme to substrate was identical to 10 FPU/g substrate; 3600 FPU of cellulase was applied for 24% of substrate loading, and 4500 FPU for 30% of the loading. The timings of the charge of substrate and enzyme are shown as dashed lines and arrows, respectively, in **Figures 4-5 and 4-6**. The stirring strategy used in this section was the same as agitation program 4.

4.3. Results and discussion

4.3.1. Determination of optimum temperature for 100-mL scale FB-SSF with DOPEG-SCL

For yeast, the growth temperature and ethanol generation temperature is normally different. In addition, optimal yeast fermentation temperature to produce ethanol generally depends on the yeast stains. Firstly, the optimum fermentation temperature of Japanese Sake yeast was investigated using glucose as a substrate at temperature ranging from 20 to 42 ºC. The maximum ethanol concentration at 20, 25, 28, 30, 35, 38 and 42 ºC was 56, 102, 111, 115, 151, 134 and 2 g/L, respectively. Thus, the highest ethanol concentration was obtained at 35 ˚C. These temperatures are slightly higher than the optimum yeast growth temperature of 30 ˚C (Aldiguier et al., 2004) and lower than the optimum temperature for cellulase activity of 50-60 ºC (Zambare et al., 2011).

For the reason of optimum temperatures of enzymatic saccharification and

fermentation are different, the optimum temperature of FB-SSF was investigated at different temperatures in a 100-mL scale FB-SSF in the presence and absence of DOPEG-SCL, where pre-hydrolysis was conducted at 50 ºC. The highest ethanol concentration (g/L) during each FB-SSF condition for 144 h, ethanol conversion based on the theoretical maximum yield (%), and residual enzyme activity (%) are shown in **Table 4-2**. The maximum ethanol concentration (56.2 g/L) and ethanol conversion (62.8 %) were observed at 38 ˚C in the presence of DOPEG-SCL. Whereas, the highest recovery of enzyme activity (65.5 %) was observed at 35 ˚C in the presence of DOPEG-SCL. The results indicate that the observed maximum ethanol concentration at 38 ˚C might be driven by several different factors including optimum temperature for fermentation, optimum enzyme activity, temperature-dependent enzyme recovery rate and enzyme stability. As a conclusion of this section, the optimal FB-SSF temperature was decided to be 38 ºC from the viewpoint of production of high concentration ethanol, although the optimum fermentation temperature was 35 ºC.

Table 4-2. Optimum temperature test for the 100-mL scale FB-SSF with DOPEG-SCL after 6 days.

Temp. (°C)		Maximum Ethanol conc. (g/L)	Bioethanol conversion (%)	Residual enzyme activity (%)
25.0	Control	35.0	39.1	28.8
	DOPEG-SCL	40.1	44.8	63.8
35.0	Control	37.0	41.3	30.9
	DOPEG-SCL	49.6	55.4	65.5
36.5	Control	40.0	44.7	25.1
	DOPEG-SCL	51.7	57.8	55.6
38.0	Control	44.3	49.5	20.4
	DOPEG-SCL	56.2	62.8	42.7
40.0	Control	40.0	44.7	20.6
	DOPEG-SCL	43.3	48.4	38.2

4.3.2. Optimum loading of A-LDs in a 100-mL scale FB-SSF

In chapter 3, the loading of A-LDs for all FB-SSF was constant at 2.5 g/L. However, it was anticipated that A-LDs loadings could also affect the efficiency of FB-SSF. Optimization of A-LDs loading in 100-mL scale FB-SSF was investigated at A-LDs concentrations of 2.5, 3.0 and 5.0 g/L. The ethanol production curve based on days in the presence of either DOPEG-SCL or EPEG-SCL (2.5 g/L, 3.0 g/L, 5.0 g/L) for up to 144 h are shown in **Figure 4-2**. The highest ethanol concentration was observed in the

presence of 3.0 g/L DOPEG-SCL (**Figure 4-2-(B)**) or 3.0 g/L EPEG-SCL (**Figure 4-2-(E)**) at 144 h. However, at the 5.0 g/L A-LDs, slight reduction of ethanol production was observed at 144 h, and it might be due to the negative influence of ethanol fermentation by yeast in the presence of DOPEG-SCL (**Figure 4-2 A** to **C**) or EPEG-SCL (**Figure 4-2 D** to **F**) (Adeboye et al., 2014).

The residual enzyme activity results under those three A-LDs loadings shown in **Table 4-3** suggest that the higher amount of A-LDs have the better enzyme recovery. Apparently, the A-LDs greatly improved the enzyme activity compared to the residual activities measured in the absence of A-LDs (20.4%). These results strongly suggest that A-LDs suppress the reduction of cellulase activity. The maximum ethanol concentration (56.2 g/L) in this section at a DOPEG-SCL loading of 2.5 g/L is higher than in chapter 3 (49.4 g/L). The difference may be caused by difference in cellulose content between NUKP and unbleached cedar soda pulp.

As a conclusion of this section, the optimal A-LDs loading was decided to be 3 g/L from the viewpoint of production of high concentration ethanol. Existence of the optimum loading was my big surprise. Prior to this experiment, I anticipated that ethanol production would depend on A-LDs loadings only. Only residual activity, but not ethanol concentration, could, I thought, depend on the loading.

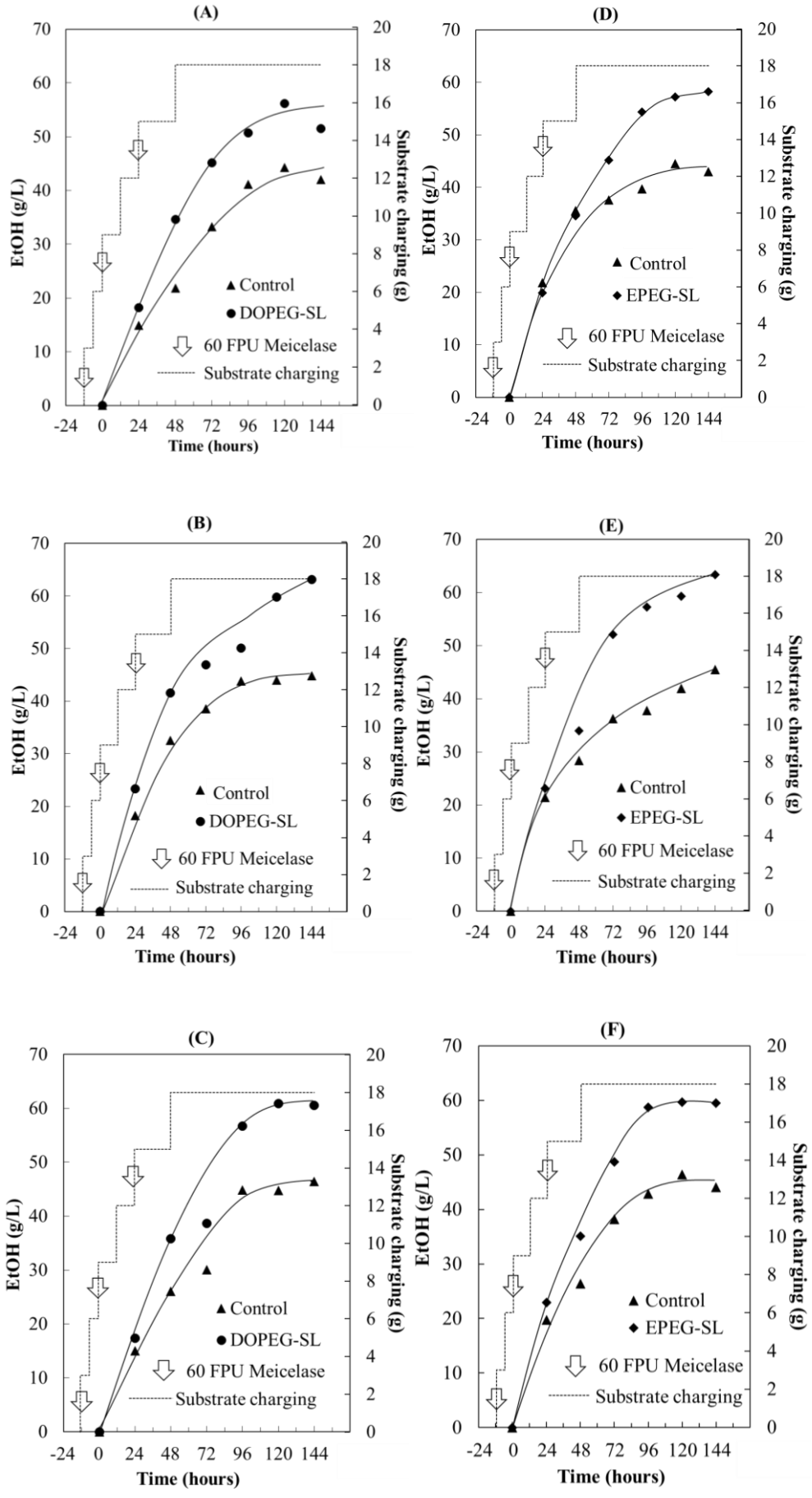

(A)
EtOH (g/L)
Substrate charging (g)
Time (hours)
Control
DOPEG-SL
60 FPU Meicelase
Substrate charging
(D)
EtOH (g/L)
Substrate charging (g)
Time (hours)
Control
EPEG-SL
60 FPU Meicelase
Substrate charging
(B)
EtOH (g/L)
Substrate charging (g)
Time (hours)
Control
DOPEG-SL
60 FPU Meicelase
Substrate charging
(E)
EtOH (g/L)
Substrate charging (g)
Time (hours)
Control
EPEG-SL
60 FPU Meicelase
Substrate charging
(C)
EtOH (g/L)
Substrate charging (g)
Time (hours)
Control
DOPEG-SL
60 FPU Meicelase
Substrate charging
(F)
EtOH (g/L)
Substrate charging (g)
Time (hours)
Control
EPEG-SL
60 FPU Meicelase
Substrate charging

Figure 4-2. Ethanol production (g/L) with a commercial cellulase (Meicelase) in a 100-mL scale FB-SSF at different DOPEG-SCL loadings: 2.5 g/L (A), 3.0 g/L (B) and 5.0 g/L (C); EPEG-SCL of 2.5 g/L (D), 3.0 g/L (E) and 5.0 g/L (F). Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow.

Table 4-3. Productivities and residual enzyme activities with varying A-LD loadings in 100-mL scale FB-SSF.

	A-LDs Loadings (g/L)	**Maximum Ethanol conc. (g/L)**	**Productivities based on theoretical maximum yield (%)**	**Residual enzyme activities (%)**
DOPEG-SL	2.5	56.2	62.8	42.7
	3.0	63.1	70.5	49.5
	5.0	60.9	68.0	91.6
EPEG-SL	2.5	58.3	65.1	45.2
	3.0	63.3	70.7	56.5
	5.0	59.7	66.7	88.7

4.3.3. Effect of stirring program on a 1.5-L scale FB-SSF bioethanol production

Agitation of the medium during SSF is one of the key conditions to produce bioethanol (Khongsay et al., 2012). Four types of stirring program were carried out in SSF experiments in the absence of A-LDs by using the large jar-fermenter equipped with a powerful stirrer, which enabled programmed stirring for high consistency of pulp

suspension. (**Table 4-1**). The reason for choosing those four strategies is explained below. Yeast fermentation favors anaerobic environment (van Maris et al., 2006). Vigorous agitation or stirring is a benefit for faster saccharification, because it enhances contact between enzyme and substrate. When stirring in SSF is carried out at high speed, the frequency of yeast exposing to air, resulting in the reduction of fermentation rate due to changing anaerobic condition to aerobic one. However, saccharification rate would be increased at a high-speed stirring. At a low speed stirring, opposite phenomena would occur. Programs 1 and 2 at a constant high speed and a constant low speed were carried out, respectively. In program 3, high speed was maintained during pre-hydrolysis, but the stirring speed was gradually decreased in FB-SSF. In program 4, the stirring rate was identical to that of program 3 until 72 h, but after all the substrate charge was finished, the stirring strategy was changed to intermittent mixing with 55 min rest in each hour. The reason why the stirring program changed after 72 h in program 4 was that a little stirring could help to keep the condition anaerobic, which was considered suitable for yeast fermentation, and that further saccharification was not needed.

Figure 4-3 shows ethanol production under 4 stirring programs in a 1.5-L scale FB-SSF. Programs 3 gave higher ethanol concentration than programs 1 and 2 did. This result of program 3 must be attributed to the high speed stirring at pre-hydrolysis and the low speed stirring at 72-144 h of SSF. In program 1, although a low speed stirring was carried out at 72-144 h, the maximum ethanol concentration was lowest, suggesting the importance of high speed stirring at pre-hydrolysis. Program 4 gave a slightly better

result than program 3. This result suggests that shorter stirring time in the fermentation stage at 72-144 h is more favorable.

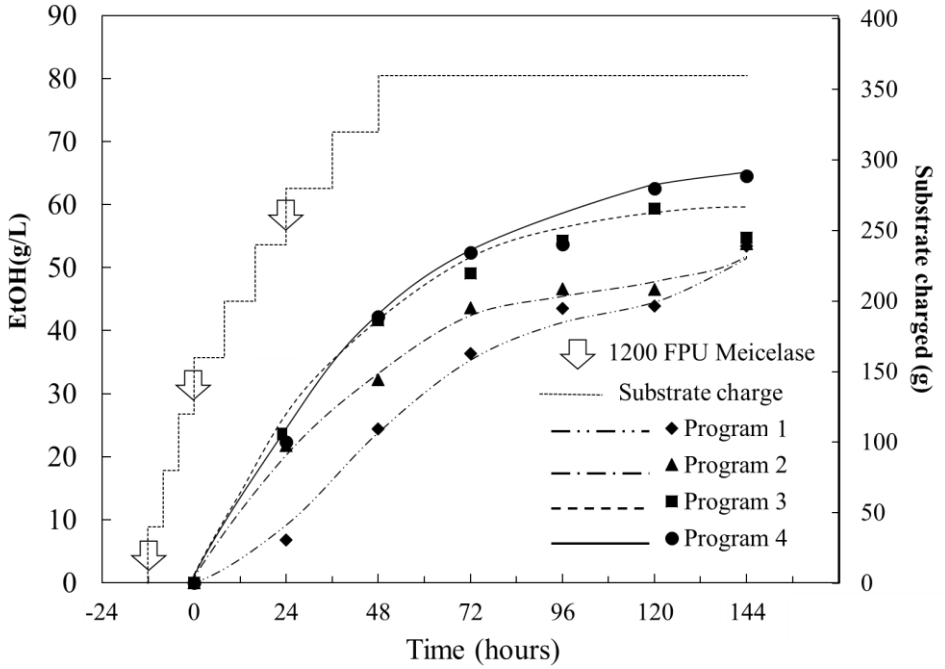
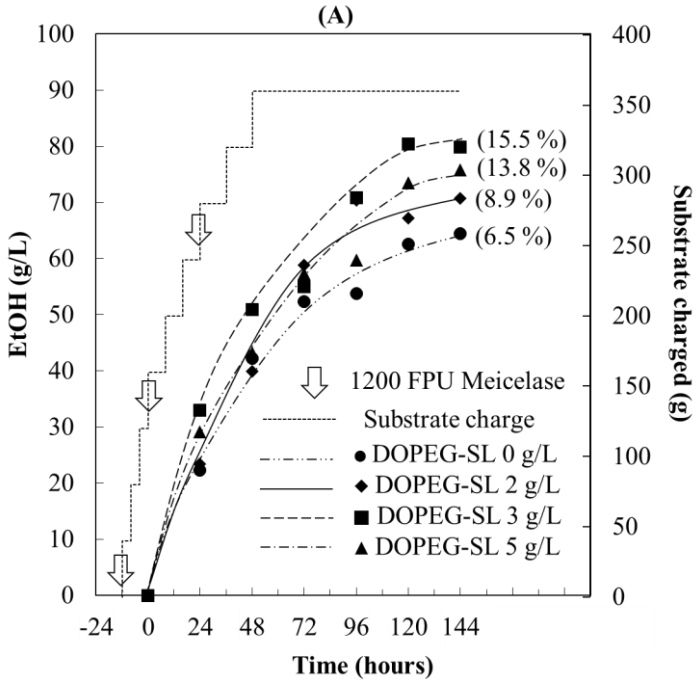


Figure 4-3. Ethanol production with a commercial cellulase (Meicelase) of the 1.5-L scale FB-SSF under 4 different stirring programs. Four different stirring programs are shown in Table 1. Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow.

4.3.4. Effect of A-LDs charging on 1.5-L scale FB-SSF on bioethanol production

As well as a 100-mL scale FB-SSF, effect of A-LDs loadings on a 1.5-L scale FB-SSF was investigated in the concentration range of 0-5 g/L. In these FB-SSF, total 360 g of NUKP and 3600 FPU of Meicelase were charged under the stirring program 4. The highest ethanol production of 80.5 g/L with ethanol conversion of 67.4 % was determined, as shown in **Figure 4-4-(A)**, when 3.0 g/L DOPEG-SCL was added to the

1.5-L scale FB-SSF. Similarly, the optimum amount of EPEG-SCL for the highest ethanol production of 81.0 g/L with ethanol conversion of 67.8 % was determined to be 3.0 g/L in the 1.5 L FB-SSF reaction [**Figure 4-4-(B)**]. It seems that EPEG-CSL is more useful than DOPEG-CSL for the ethanol production. Interestingly, the enzyme recovery rate during SSF was higher in the 3.0 g/L DOPEG-SCL (15.5 %) compared to the 5.0 g/L DOPEG-SCL (13.8 %), while the higher enzyme recovery was obtained in the 5.0 g/L EPEG-SCL (14.5 %) than the 3.0 g/L EPEG-SCL (13.1 %). This result means that the observed effect of A-LDs on ethanol production is less likely due to the enhanced enzyme recovery, but is more likely due to the enhanced fermentation by yeast. Perhaps, too much A-LDs negatively affects yeast cells to produce ethanol, and is consistent with the 100-mL scale FB-SSF in **Figure 4-2**. Among the tested A-LDs loadings in 1.5-L scale FB-SSF, the optimum concentration of A-LDs was decided to be 3.0 g/L, which was consistent with the optimum loading in 100-mL scale of SSF.



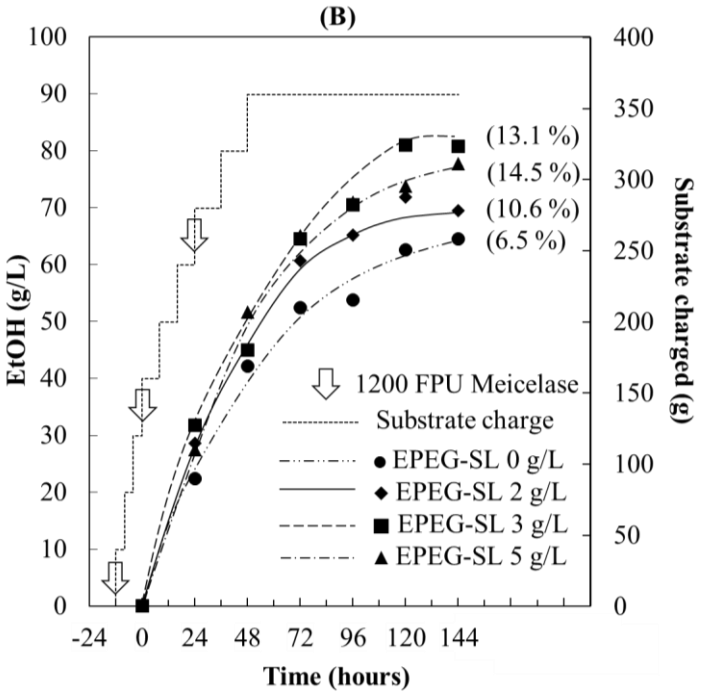


Figure 4-4. Ethanol production with a commercial cellulase (Meicelase) at different loadings of DOPEG-SCL (A) and EPEG-SCL (B) in the 1.5-L scale FB-SSF. Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow. Numbers in the parenthesis indicate the residual enzyme activity (%) after the completion of reaction.

4.3.5. Bioethanol production with high substrate loading under the best optimized conditions

In the above experiment with Meicelase, the ethanol conversion was 67.8 % (81.0 g/L) based on the theoretical maximum yield under the optimal temperature, A-LDs loading and suitable stirring program, suggesting that there were still 32.2 % of cellulose remaining that were not converted to ethanol. In this experiment, FB-SSF was attempted with GC220, which was a better enzyme than Meicelase, as described previously in chapter 2.3.2. GC220 in **Figure 4-5** gave higher ethanol concentration of

83.0 g/L with ethanol yield of 69.5 % than those with Meicelase. Additionally, the residual enzyme activity in GC220 (18.9 %) was higher than that of the Meicelase (14.5 %).

In order to achieve much higher ethanol concentration, an experiment at a high NUKP loading of 450 g/reaction (30 % w/w) was conducted in the optimized 1.5-L scale FB-SSF (**Figure 4-6**). The ethanol concentration of 87.9 g/L with theoretical ethanol conversion of 58.9 % was achieved in the presence of 3.0 g/L EPEG-SCL. In the absence of A-LD, ethanol concentration of 66.3 g/L and conversion of 44.5 % were very low.

In conclusion of this chapter, optimized parameters are as follows: SSF temperature is 38 ºC; A-LD loading is 3 g/L regardless of FB-SSF scale; the stirring program is at 40 rpm in pre-hydrolysis process, at 20 rpm for the initial 72 h of SSF process, and at 40 rpm for 5 min and no agitation for remaining 55 min in each hour for the latter half (72-144 h) of SSF process. By using a custom-made jar fermenter under the optimized conditions, the maximum ethanol concentration increased from 56.2 g/L (100-mL scale) to 87.9 g/L (1.5-L scale), which was 1.6-fold of ethanol concentration obtained without the jar fermenter.

Table 4-4. The residual enzyme activities based on the initial activity in the presence of A-LDs at 0, 2.5, 3.0 and 5.0 g/L of 100 mL FB-SSF after 144 h of incubation.

	A-LDs loading (g/L)	Residual enzyme activity (%)
Control	0	20.4
DOPEG-SCL	2.5	42.7
	3.0	49.5
	5.0	91.6
EPEG-SCL	2.5	45.2
	3.0	56.5
	5.0	88.7

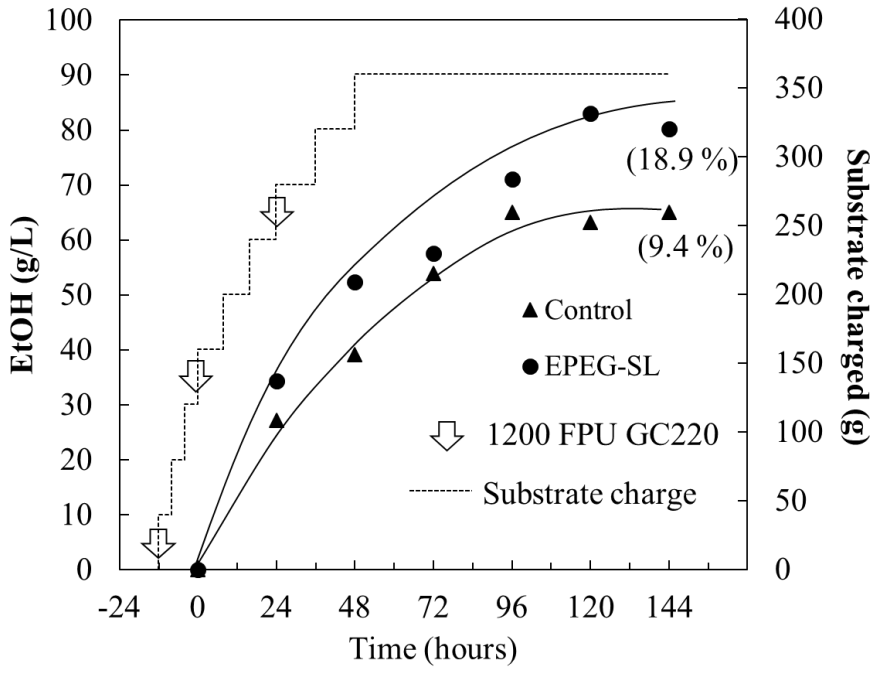


Figure 4-5. Ethanol production with a commercial cellulase (GC220) in the 1.5 L FB-SSF in the presence and absence of 3.0 g/L EPEG-SCL under the optimum condition. Total substrate loading was 360 g and total cellulase charge was 3600 FPU. Substrate

charge is shown as a dashed line, and enzyme charge is shown as an arrow. Numbers in the parenthesis indicate the residual enzyme activity (%) after the completion of reaction.

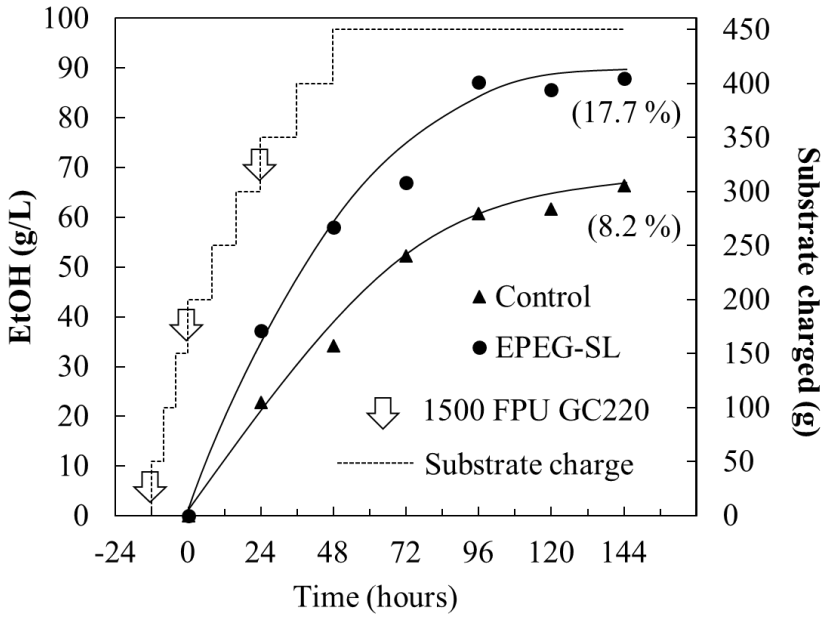


Figure 4-6. Ethanol production with a commercial cellulase (GC220) in the 1.5 L FB-SSF in the presence and absence of 3.0 g/L EPEG-SCL under the optimum condition. Total substrate loading was 450 g and total cellulase charge was 4500 FPU. Substrate charge is shown as a dashed line, and enzyme charge is shown as an arrow. Numbers in the parenthesis indicate the residual enzyme activity (%) after the completion of reaction.

Chapter 5 Conclusions

5.1. Concluding remarks

This study for my PhD thesis was conducted not only to produce high concentration bioethanol, but also to demonstrate the usefulness of A-LDs on FB-SSF. The obtained results in chapters 2, 3 and 4 are summarized as follows.

In Chapter 2,

1. Three kinds of A-LDs, PEGDE-CSL, EPEG-CSL and DOPEG-CSL, were prepared by the reaction of CSL with three kinds of epoxylated PEG analoges under alkaline conditions.
2. All the obtained A-LDs decreased water surface tension as well as previously prepared A-LDs from other isolated lignins. Among the A-LDs, DOPEG-SCL had the highest surface activity, which was comparable to commercial non-ionic surfactants.
3. Both A-LDs, EPEG-CSL and DOPEG-CSL, improved enzymatic saccharification efficiency and enzyme recovery of both cedar soda pulp and NUKP. Especially, EPEG-CSL was useful to improve the saccharification of unbleached cedar soda pulp with high lignin content (6.9%), and DOPEG-CSL was useful to improve the saccharification of NUKP with low lignin content (2.1%).
4. A commercial available cellulase cocktail, GC220, showed higher sugar yield and recovered enzyme activity than another commercial cellulase cocktail, Meicelase, in the saccharification of NUKP.

In Chapter 3,

1. LDs did not suppress the yeast fermentation, but rather accelerated the fermentation.
2. Both EPEG-SCL and DOPEG-SCL with any enzyme loadings remarkably increased ethanol production in 100-mL scale FB-SSF as compared to the control. The larger enzyme was loaded, the higher concentration of bioethanol was obtained.
3. In 100-mL scale FB-SSF, the highest ethanol concentration was 49.4 g/L by adding 2.5 g/L of A-LDs and 18 g of NUKP at the enzyme loading of 10 FPU/g, which was 1.3 times larger than the concentration without A-LD (37.8 g/L) as the control.

In Chapter 4,

1. The optimum temperature for Japanese Sake yeast fermentation of glucose was found to be 35 ^{o}C, whereas the optimum temperature for FB-SSF was 38 ^{o}C.
2. Both EPEG-CSL and DOPEG-CSL at a concentration of 3 g/L gave highest ethanol yield and concentration, independently of the FB-SSF scale.
3. A 3.5-L custom-made jar fermenter equipped with a powerful mechanical stirrer to achieve rigorous agitation for substrate slurry at high consistency was used. The optimum stirring program was that continuous stirring at 40 rpm in the pre-hydrolysis process, continuous stirring at 20 rpm for the initial 72 h of SSF, and then periodic stirring at 40 rpm for 5 min in every hour until 144 h.
4. The big jar fermenter enabled 30% (w/v) of substrate loading under the optimized conditions. As a result, finally, the highest ethanol concentration of 87.9 g/L could be achieved.

Thus, I successfully produced highly concentrated bioethanol, and elucidated the

usefulness of A-LDs on FB-SSF.

5.2. General discussion

In order to envision how the optimized FB-SSF performed compared to other studies in terms of produced ethanol concentration (g/L) and ethanol conversion (% on maximum theoretical yield), I looked closely at previously reported methods.

As far as I have investigated, Maeda et al. (2013) produced the highest ethanol concentration (100 g/L) with a relatively high conversion rate of 78% from pre-hydrolyzed sugar cane bagasse as the lignocellulosic substrate in SSF (Maeda et al., 2013) (**Table 5-1**). They used a special enzyme that they produced themselves, which may contribute to a higher ethanol concentration and conversion rate than my methods (87.9 g/L and 59%, respectively). Elliston et al. (2013) achieved an ethanol concentration of 11.6% (v/v), which was calculated to be 91.5 g/L (specific gravity of ethanol: 0.789 at 25 ºC), at an ethanol conversion rate of 54% (**Table 5-1**). These values are comparable to my results, although the enzyme loading (3.7 FPU/g substrate) is smaller than mine (10 FPU/g). The reason for their improved ethanol conversion is probably explained by the fact that they used special mixing equipment that enabled substrate loading at a very high consistency (65% w/v). In addition, the cellulase cocktail they used was supplemented with β-glucosidases.

A paper by Zhang et al. (2010) showed a higher ethanol conversion rate (79.6%) with a slightly lower ethanol concentration (84.7 g/L) at lower substrate loading (19% w/v) (Zhang et al., 2010) (**Table 5-1**). Their results were likely obtained by higher

enzyme loading (22.8 FPU/g substrate) than mine (10 FPU/g)

It is anticipated that much highly concentrated ethanol can be produced by a combination of three items, my A-LDs, the special enzyme produced by Maeda et al., (2013) and special mixing equipment (Elliston et al., 2013). Furthermore, ethanol production can be improved by searching yeast strain for FB-SSF. Thus, my results with A-LDs must contribute to ethanol production with a higher concentration than that reported previously.

The high concentration bioethanol leads to the reduction of distillation cost to produce absolute ethanol as liquid fuel. From this viewpoint, my findings in this thesis would also help to produce low-cost liquid fuel to establish sustainable society with effective energy recycle. Furthermore, this research will contribute to a novel, value-added utilization of isolated lignin and low quality lignocellulosics.

Table 5-1. The SSF parameters used in previously reported methods.

Substrate	**Lignin content (%)** [a)]	**Substrate loading**	**Enzyme**	**Enzyme loading (FPU/g substrate)**	**EtOH conc. (g/L)**	**Ethanol Conversion (%)**	**Refs**
Sugar cane bagasse	9.3 (19.4)	33 % w/v	Self-made	12.5	100.0	78.0	Maeda et al., 2013
Waste paper	1.0	65 % w/v	Accelerase 1500 (Genencor) and β-glucosidase (Novozyme)	3.7 FPU/g Accelerase 6.9 U/g β-glucosidase (Novozyme)	91.5 (11.6 % v/v)	54.0	Elliston et al., 2013
Corncob	4~5	25 % w/w	GC220	22.8	84.7	79.6	Zhang et al., 2010

a) The lignin content before pre-treatment is shown in the parentheses.

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REFERENCES

Adeboye, P.T., Bettiga, M., Olsson, L., 2014. The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in Saccharomyces cerevisiae in lignocellulose hydrolysates. *Amb Express*, **4**.

Aldiguier, A.S., Alfenore, S., Cameleyre, X., Goma, G., Uribelarrea, J.L., Guillouet, S.E., Molina-Jouve, C., 2004. Synergistic temperature and ethanol effect on Saccharomyces cerevisiae dynamic behaviour in ethanol bio-fuel production. *Bioprocess and Biosystems Engineering*, **26**(4), 217-222.

Alfaro, A., Rivera, A., Perez, A., Yanez, R., Garcia, J.C., Lopez, F., 2009. Integral valorization of two legumes by autohydrolysis and organosolv delignification. *Bioresour Technol*, **100**(1), 440-5.

Alkasrawi, M., Abu Jrai, A., Al-Muhtaseb, A.H., 2013. Simultaneous saccharification and fermentation process for ethanol production from steam-pretreated softwood: Recirculation of condensate streams. *Chemical Engineering Journal*, **225**, 574-579.

Alkasrawi, M., Eriksson, T., Borjesson, J., Wingren, A., Galbe, M., Tjerneld, F., Zacchi, G., 2003. The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol. *Enzyme and Microbial Technology*, **33**(1), 71-78.

Ask, M., Olofsson, K., Di Felice, T., Ruohonen, L., Penttila, M., Liden, G., Olsson,

L., 2012. Challenges in enzymatic hydrolysis and fermentation of pretreated Arundo donax revealed by a comparison between SHF and SSF. *Process Biochemistry*, **47**(10), 1452-1459.

Aso, T., Koda, K., Kubo, S., Yamada, T., Nakajima, I., Uraki, Y., 2013. Preparation of Novel Lignin-Based Cement Dispersants from Isolated Lignins. *Journal of Wood Chemistry and Technology*, **33**(4), 286-298.

Avci, A., Saha, B.C., Kennedy, G.J., Cotta, M.A., 2013. Dilute sulfuric acid pretreatment of corn stover for enzymatic hydrolysis and efficient ethanol production by recombinant Escherichia coli FBR5 without detoxification. *Bioresour Technol*, **142**, 312-9.

Aysu, T., Durak, H., 2015. Thermochemical conversion of Datura stramonium L. by supercritical liquefaction and pyrolysis processes. *The Journal of Supercritical Fluids*, **102**, 98-114.

Baeyens, J., Kang, Q., Appels, L., Dewil, R., Lv, Y.Q., Tan, T.W., 2015. Challenges and opportunities in improving the production of bio-ethanol. *Progress in Energy and Combustion Science*, **47**, 60-88.

Balat, M., Balat, H., Oz, C., 2008. Progress in bioethanol processing. *Progress in Energy and Combustion Science*, **34**(5), 551-573.

Ballesteros, M., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, I., 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with Kluyveromyces marxianus CECT 10875. *Process Biochemistry*, **39**(12), 1843-1848.

Bayrock, D., Ingledew, W.M., 2001. Changes in steady state on introduction of a Lactobacillus contaminant to a continuous culture ethanol fermentation. *Journal of Industrial Microbiology & Biotechnology*, **27**(1), 39-45.

Belbute, J.M., Massala, L.D., Delgado, J.A., 2016. Measuring Persistence in Inflation: Evidence For angola. *South African Journal of Economics*, **84**(4), 594-606.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. *Annu Rev Plant Biol*, **54**, 519-46.

Boonsawang, P., Subkaree, Y., Srinorakutara, T., 2012. Ethanol production from palm pressed fiber by prehydrolysis prior to simultaneous saccharification and fermentation (SSF). *Biomass & Bioenergy*, **40**, 127-132.

Borjesson, J., Engqvist, M., Sipos, B., Tjerneld, F., 2007a. Effect of poly(ethylene glycol) on enzymatic hydrolysis and adsorption of cellulase enzymes to pretreated lignocellulose. *Enzyme and Microbial Technology*, **41**(1-2), 186-195.

Borjesson, J., Peterson, R., Tjerneld, F., 2007b. Enhanced enzymatic conversion of softwood lignocellulose by poly(ethylene glycol) addition. *Enzyme and Microbial Technology*, **40**(4), 754-762.

Cha, Y.L., An, G.H., Park, Y., Yang, J., Ahn, J.W., Moon, Y.H., Yoon, Y.M., Yu, G.D., Choi, I.H., 2014. Partial Simultaneous Saccharification and Fermentation at High Solids Loadings of Alkaline-pretreated Miscanthus for Bioethanol Production. *Bioresources*, **9**(4), 6899-6913.

Claassen, P.A.M., van Lier, J.B., Contreras, A.M.L., van Niel, E.W.J., Sijtsma, L.,

Stams, A.J.M., de Vries, S.S., Weusthuis, R.A., 1999. Utilisation of biomass for the supply of energy carriers. *Applied Microbiology and Biotechnology*, **52**(6), 741-755.

Dias, M.O.S., Modesto, M., Ensinas, A.V., Nebra, S.A., Maciel, R., Rossell, C.E.V., 2011. Improving bioethanol production from sugarcane: evaluation of distillation, thermal integration and cogeneration systems. *Energy*, **36**(6), 3691-3703.

du Nouy, P.L., 1919. A new apparatus for measuring surface tension. *Journal of General Physiology*, **1**(5), 521-524.

Duff, S.J.B., Murray, W.D., 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. *Bioresource Technology*, **55**(1), 1-33.

Dwiarti, L., Boonchird, C., Harashima, S., Park, E.Y., 2012. Simultaneous saccharification and fermentation of paper sludge without pretreatment using cellulase from Acremonium cellulolyticus and thermotolerant Saccharomyces cerevisiae. *Biomass & Bioenergy*, **42**, 114-122.

Elliston, A., Collins, S.R.A., Wilson, D.R., Roberts, I.N., Waldron, K.W., 2013. High concentrations of cellulosic ethanol achieved by fed batch semi simultaneous saccharification and fermentation of waste-paper. *Bioresource Technology*, **134**, 117-126.

Eriksson, T., Borjesson, J., Tjerneld, F., 2002a. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme and Microbial Technology*, **31**(3), 353-364.

Eriksson, T., Karlsson, J., Tjerneld, F., 2002b. A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (cel7A) and endoglucanase I (cel7B) of Trichoderma reesei. *Appl Biochem Biotechnol*, **101**(1), 41-60.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M., Kammen, D.M., 2006. Ethanol can contribute to energy and environmental goals. *Science*, **311**(5760), 506-8.

Gaykawad, S.S., Zha, Y., Punt, P.J., van Groenestijn, J.W., van der Wielen, L.A.M., Straathof, A.J.J., 2013. Pervaporation of ethanol from lignocellulosic fermentation broth. *Bioresource Technology*, **129**, 469-476.

Ghose, A.T.K. 1987. Measurement of cellulase activities. Pure Appl. Chem. 59(2), 257-268.

Gladis, A., Bondesson, P.M., Galbe, M., Zacchi, G., 2015. Influence of different SSF conditions on ethanol production from corn stover at high solids loadings. *Energy Science & Engineering*, **3**(5), 481-489.

Goldstein, I.S., 1975. Potential for Converting Wood into Plastics: Chemicals from wood may regain importance as the cost of petroleum continues to rise. *Science*, **189**(4206), 847-52.

Gunjikar, T.P., Sawant, S.B., Joshi, J.B., 2001. Shear deactivation of cellulase, exoglucanase, endoglucanase, and beta-glucosidase in a mechanically agitated reactor. *Biotechnology Progress*, **17**(6), 1166-1168.

Guo, H., Su, R.X., Huang, R.L., Qi, W., He, Z.M., 2015. Co-optimization of sugar

yield and input energy by the stepwise reduction of agitation rate during lignocellulose hydrolysis. *Food and Bioproducts Processing*, **95**, 1-6.

Hameed, S., Dignon, J., 1988. Changes in the Geographical Distributions of Global Emissions of Nox and Sox from Fossil-Fuel Combustion between 1966 and 1980. *Atmospheric Environment*, **22**(3), 441-449.

Hayward, T., Combs, N., Schmidt, S., Philippidis, G., 1995. Chemical analysis and testing task: LAP-008 (SSF experimental protocols: lignocellulosic biomass hydrolysis and fermentation) National Renewable Energy Laboratory.

Himmel, M.E., Adney, W.S., Baker, J.O., Elander, R., McMillan, J.D., Nieves, R.A., Sheehan, J.J., Thomas, S.R., Vinzant, T.B., Zhang, M., 1997. Advanced bioethanol production technologies: A perspective. *Fuels and Chemicals from Biomass*, **666**, 2-45.

Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science*, **315**(5813), 804-7.

Hoel, M., Kverndokk, S., 1996. Depletion of fossil fuels and the impacts of global warming. *Resource and Energy Economics*, **18**(2), 115-136.

Homma, H., Kubo, S., Yamada, T., Koda, K., Matsushita, Y., Uraki, Y., 2010. Conversion of Technical Lignins to Amphiphilic Derivatives with High Surface Activity. *Journal of Wood Chemistry and Technology*, **30**(2), 164-174.

Homma, H., Kubo, S., Yamada, T., Matsushita, Y., Uraki, Y., 2008. Preparation and Characterization of Amphiphilic Lignin Derivatives as Surfactants. *Journal of*

Wood Chemistry and Technology, **28**(4), 270-282.

Hoyer, K., Galbe, M., Zacchi, G., 2013a. The effect of prehydrolysis and improved mixing on high-solids batch simultaneous saccharification and fermentation of spruce to ethanol. *Process Biochemistry*, **48**(2), 289-293.

Hoyer, K., Galbe, M., Zacchi, G., 2013b. Influence of fiber degradation and concentration of fermentable sugars on simultaneous saccharification and fermentation of high-solids spruce slurry to ethanol. *Biotechnol Biofuels*, **6**(1), 145.

Huang, Y., Baker, R.W., Vane, L.M., 2010. Low-Energy Distillation-Membrane Separation Process. *Industrial & Engineering Chemistry Research*, **49**(8), 3760-3768.

Ishola, M.M., Brandberg, T., Taherzadeh, M.J., 2015. Simultaneous glucose and xylose utilization for improved ethanol production from lignocellulosic biomass through SSFF with encapsulated yeast. *Biomass & Bioenergy*, **77**, 192-199.

Kaar, W.E., Holtzapple, M.T., 1998. Benefits from Tween during enzymic hydrolysis of corn stover. *Biotechnology and Bioengineering*, **59**(4), 419-427.

Kadar, Z., Szengyel, Z., Reczey, K., 2004. Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Industrial Crops and Products*, **20**(1), 103-110.

Kang, K.E., Chung, D.P., Kim, Y., Chung, B.W., Choi, G.W., 2015. High-titer ethanol production from simultaneous saccharification and fermentation using a

continuous feeding system. *Fuel*, **145**, 18-24.

Kanti, P., Srigowri, K., Madhuri, J., Smitha, B., Sridhar, S., 2004. Dehydration of ethanol through blend membranes of chitosan and sodium alginate by pervaporation. *Separation and Purification Technology*, **40**(3), 259-266.

Khongsay, N., Laopaiboon, L., Jaisil, P., Laopaiboon, P., 2012. Optimization of Agitation and Aeration for Very High Gravity Ethanol Fermentation from Sweet Sorghum Juice by Saccharomyces cerevisiae Using an Orthogonal Array Design. *Energies*, **5**(3), 561-576.

Kim, M.H., Lee, S.B., Ryu, D.D.Y., Reese, E.T., 1982. Surface Deactivation of Cellulase and Its Prevention. *Enzyme and Microbial Technology*, **4**(2), 99-103.

Kim, T.H., Choi, C.H., Oh, K.K., 2013. Bioconversion of sawdust into ethanol using dilute sulfuric acid-assisted continuous twin screw-driven reactor pretreatment and fed-batch simultaneous saccharification and fermentation. *Bioresour Technol*, **130**, 306-13.

King, A.T., Davey, M.R., Mellor, I.R., Mulligan, B.J., Lowe, K.C., 1991. Surfactant Effects on Yeast-Cells. *Enzyme and Microbial Technology*, **13**(2), 148-153.

Klemm, D., Heublein, B., Fink, H.P., Bohn, A., 2005. Cellulose: fascinating biopolymer and sustainable raw material. *Angew Chem Int Ed Engl*, **44**(22), 3358-93.

Kristensen, J.B., Borjesson, J., Bruun, M.H., Tjerneld, F., Jorgensen, H., 2007. Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose. *Enzyme and Microbial Technology*, **40**(4), 888-895.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P., 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. *Industrial & Engineering Chemistry Research*, **48**(8), 3713-3729.

Lai, C., Tu, M., Shi, Z., Zheng, K., Olmos, L.G., Yu, S., 2014. Contrasting effects of hardwood and softwood organosolv lignins on enzymatic hydrolysis of lignocellulose. *Bioresour Technol*, **163**, 320-7.

Lan, T.Q., Gleisner, R., Zhu, J.Y., Dien, B.S., Hector, R.E., 2013. High titer ethanol production from SPORL-pretreated lodgepole pine by simultaneous enzymatic saccharification and combined fermentation. *Bioresour Technol*, **127**, 291-7.

Lee, W.G., Lee, J.S., Lee, J.P., Shin, C.S., Kim, M.S., Park, S.C., 1996. Effect of surfactants on ethanol fermentation using glucose and cellulosic hydrolyzates. *Biotechnology Letters*, **18**(3), 299-304.

Lin, Y., Tanaka, S., 2006. Ethanol fermentation from biomass resources: current state and prospects. *Appl Microbiol Biotechnol*, **69**(6), 627-42.

Ling, W.S., Thian, T.C., Bhatia, S., 2010. Process optimization studies for the dehydration of alcohol-water system by inorganic membrane based pervaporation separation using design of experiments (DOE). *Separation and Purification Technology*, **71**(2), 192-199.

Liu, K., Lin, X., Yue, J., Li, X., Fang, X., Zhu, M., Lin, J., Qu, Y., Xiao, L., 2010. High concentration ethanol production from corncob residues by fed-batch strategy. *Bioresour Technol*, **101**(13), 4952-8.

Lou, H., Wang, M., Lai, H., Lin, X., Zhou, M., Yang, D., Qiu, X., 2013. Reducing

non-productive adsorption of cellulase and enhancing enzymatic hydrolysis of lignocelluloses by noncovalent modification of lignin with lignosulfonate. *Bioresour Technol*, **146**, 478-84.

Lynd, L.R., 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: Technology, economics, the environment, and policy. *Annual Review of Energy and the Environment*, **21**, 403-465.

Ma, A.Z., Hu, Q., Qu, Y.B., Bai, Z.H., Liu, W.F., Zhuang, G.Q., 2008. The enzymatic hydrolysis rate of cellulose decreases with irreversible adsorption of cellobiohydrolase I. *Enzyme and Microbial Technology*, **42**(7), 543-547.

Ma, Y., Wang, J.H., Tsuru, T., 2009. Pervaporation of water/ethanol mixtures through microporous silica membranes. *Separation and Purification Technology*, **66**(3), 479-485.

Maeda, R.N., Barcelos, C.A., Santa Anna, L.M., Pereira, N., Jr., 2013. Cellulase production by Penicillium funiculosum and its application in the hydrolysis of sugar cane bagasse for second generation ethanol production by fed batch operation. *J Biotechnol*, **163**(1), 38-44.

Monica, E.K., Gellerstedt, G.,, Henriksson, G., 2009. Pulp and Paper Chemistry and Technology Vol.1. Wood Chemistry and Wood Biotechnology (eds.). Stockholm, Sweden.

Nakagame, S., Chandra, R.P., Kadla, J.F., Saddler, J.N., 2011. Enhancing the Enzymatic Hydrolysis of Lignocellulosic Biomass by Increasing the Carboxylic Acid Content of the Associated Lignin. *Biotechnology and*

Bioengineering, **108**(3), 538-548.

Nakamura, Y., Sawada, T., Inoue, E., 2001. Enhanced ethanol production from enzymatically treated steam-exploded rice straw using extractive fermentation. *Journal of Chemical Technology and Biotechnology*, **76**(8), 879-884.

Ogawa, Y., Nitta, A., Uchiyama, H., Imamura, T., Shimoi, H., Ito, K., 2000. Tolerance mechanism of the ethanol-tolerant mutant of sake yeast. *Journal of Bioscience and Bioengineering*, **90**(3), 313-320.

Olsson, L., Soerensen, H.R., Dam, B.P., Christensen, H., Krogh, K.M., Meyer, A.S., 2006. Separate and simultaneous enzymatic hydrolysis and fermentation of wheat hemicellulose with recombinant xylose utilizing Saccharomyces cerevisiae. *Appl Biochem Biotechnol*, **129-132**, 117-29.

Pardo, A.G., 1996. Effect of surfactants on cellulase production by Nectria catalinensis. *Current Microbiology*, **33**(4), 275-278.

Park, J.M., Oh, B.R., Seo, J.W., Hong, W.K., Yu, A., Sohn, J.H., Kim, C.H., 2013. Efficient production of ethanol from empty palm fruit bunch fibers by fed-batch simultaneous saccharification and fermentation using Saccharomyces cerevisiae. *Appl Biochem Biotechnol*, **170**(8), 1807-14.

Park, J.W., Takahata, Y., Kajiuchi, T., Akehata, T., 1992. Effects of Nonionic Surfactant on Enzymatic-Hydrolysis of Used Newspaper. *Biotechnology and Bioengineering*, **39**(1), 117-120.

Qin, C.R., Clarke, K., Li, K.C., 2014. Interactive forces between lignin and cellulase as determined by atomic force microscopy. *Biotechnology for Biofuels*, **7**.

Saha, B.C., Nichols, N.N., Qureshi, N., Kennedy, G.J., Iten, L.B., Cotta, M.A., 2015. Pilot scale conversion of wheat straw to ethanol via simultaneous saccharification and fermentation. *Bioresour Technol*, **175**, 17-22.

Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: An overview. *Renewable Energy*, **37**(1), 19-27.

Seo, D.J., Fujita, H., Sakoda, A., 2011. Effects of a non-ionic surfactant, Tween 20, on adsorption/desorption of saccharification enzymes onto/from lignocelluloses and saccharification rate. *Adsorption-Journal of the International Adsorption Society*, **17**(5), 813-822.

Siggia, S., Starke, A.C., Garis, J.J., Stahl, C.R., 1958. Determination of Oxyalkylene Groups in Glycols and Glycol and Polyglycol Ethers and Esters. *Analytical Chemistry*, **30**(1), 115-116.

Sipos, B., Szilagyi, M., Sebestyen, Z., Perazzini, R., Dienes, D., Jakab, E., Crestini, C., Reczey, K., 2011. Mechanism of the positive effect of poly(ethylene glycol) addition in enzymatic hydrolysis of steam pretreated lignocelluloses. *Comptes Rendus Biologies*, **334**(11), 812-823.

Sivers, M.V. and Zacchi, G., 1995. A techno-economical comparison of three processes for the production of ethanol from pine. *Bioresour. Technol.* **51**, 43–52.

Slavin, J.L., Marlett, J.A., 1983. Evaluation of High-Performance Liquid-Chromatography for Measurement of the Neutral Saccharides in Neutral Detergent Fiber. *Journal of Agricultural and Food Chemistry*, **31**(3), 467-471.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T., Vorwerk, S., Youngs, H., 2004. Toward a systems approach to understanding plant cell walls. *Science*, **306**(5705), 2206-11.

Stenberg, K., Bollok, M., Reczey, K., Galbe, M., Zacchi, G., 2000. Effect of substrate and cellulase concentration on simultaneous saccharification and fermentation of steam-pretreated softwood for ethanol production. *Biotechnology and Bioengineering*, **68**(2), 204-210.

Sun, S.L., Huang, Y., Sun, R.C., Tu, M.B., 2016. The strong association of condensed phenolic moieties in isolated lignins with their inhibition of enzymatic hydrolysis. *Green Chemistry*, **18**(15), 4276-4286.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour Technol*, **83**(1), 1-11.

Taneda, D., Ueno, Y., Ikeo, M., Okino, S., 2012. Characteristics of enzyme hydrolysis of cellulose under static condition. *Bioresource Technology*, **121**, 154-160.

Tomas-Pejo, E., Oliva, J.M., Ballesteros, M., Olsson, L., 2008. Comparison of SHF and SSF processes from steam-exploded wheat straw for ethanol production by xylose-fermenting and robust glucose-fermenting Saccharomyces cerevisiae strains. *Biotechnology and Bioengineering*, **100**(6), 1122-1131.

Torija, M.J., Rozes, N., Poblet, M., Guillamon, J.M., Mas, A., 2003. Effects of fermentation temperature on the strain population of Saccharomyces cerevisiae. *Int J Food Microbiol*, **80**(1), 47-53.

Uraki, Y., Ishikawa, N., Nishida, M., Sano, Y., 2001. Preparation of amphiphilic lignin derivative as a cellulase stabilizer. *Journal of Wood Science*, **47**(4), 301-307.

Valjamae, P., Kipper, K., Pettersson, G., Johansson, G., 2003. Synergistic cellulose hydrolysis can be described in terms of fractal-like kinetics. *Biotechnology and Bioengineering*, **84**(2), 254-257.

van Maris, A.J., Abbott, D.A., Bellissimi, E., van den Brink, J., Kuyper, M., Luttik, M.A., Wisselink, H.W., Scheffers, W.A., van Dijken, J.P., Pronk, J.T., 2006. Alcoholic fermentation of carbon sources in biomass hydrolysates by Saccharomyces cerevisiae: current status. *Antonie Van Leeuwenhoek*, **90**(4), 391-418.

Wang, Z., Lv, Z., Du, J., Mo, C., Yang, X., Tian, S., 2014. Combined process for ethanol fermentation at high-solids loading and biogas digestion from unwashed steam-exploded corn stover. *Bioresour Technol*, **166**, 282-7.

Wang, Z., Zhu, J., Fu, Y., Qin, M., Shao, Z., Jiang, J., Yang, F., 2013. Lignosulfonate-mediated cellulase adsorption: enhanced enzymatic saccharification of lignocellulose through weakening nonproductive binding to lignin. *Biotechnol Biofuels*, **6**(1), 156.

Winarni, I., Koda, K., Waluyo, T.K., Pari, G., Uraki, Y., 2014. Enzymatic Saccharification of Soda Pulp from Sago Starch Waste Using Sago Lignin-Based Amphipathic Derivatives. *Journal of Wood Chemistry and Technology*, **34**(3), 157-168.

Winarni, I., Oikawa, C., Yamada, T., Igarashi, K., Koda, K., Uraki, Y., 2013.

Improvement of Enzymatic Saccharification of Unbleached Cedar Pulp with Amphipathic Lignin Derivatives. *Bioresources*, **8**(2), 2195-2208.

Wu, J., Ju, L.K., 1998. Enhancing enzymatic saccharification of waste newsprint by surfactant addition. *Biotechnology Progress*, **14**(4), 649-652.

Xiao, Z.Z., Zhang, X., Gregg, D.J., Saddler, J.N., 2004. Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates. *Applied Biochemistry and Biotechnology*, **113**, 1115-1126.

Yamamoto, Y., Cheng, N.N., Koda, K., Igarashi, K., Tamai, Y., Uraki, Y., 2017. Association of amphipathic lignin derivatives with cellobiohydrolase groups improves enzymatic saccharification of lignocellulosics. *Cellulose*, **24**(4), 1849-1862.

Yamashita, Y., Sasaki, C., Nakamura, Y., 2010. Effective enzyme saccharification and ethanol production from Japanese cedar using various pretreatment methods. *J Biosci Bioeng*, **110**(1), 79-86.

Yoo, C.G., Kuo, M., Kim, T.H., 2012. Ethanol and furfural production from corn stover using a hybrid fractionation process with zinc chloride and simultaneous saccharification and fermentation (SSF). *Process Biochemistry*, **47**(2), 319-326.

Zambare, V.P., Zambare, A.V., Muthukumarappan, K., Christopher, L.P., 2011. Potential of Thermostable Cellulases in the Bioprocessing of Switchgrass to Ethanol. *Bioresources*, **6**(2), 2004-2020.

Zhang, M., Wang, F., Su, R., Qi, W., He, Z., 2010. Ethanol production from high dry

matter corncob using fed-batch simultaneous saccharification and fermentation after combined pretreatment. *Bioresour Technol*, **101**(13), 4959-64.

Zhang, T., Zhu, M.J., 2017. Enhanced bioethanol production by fed-batch simultaneous saccharification and co-fermentation at high solid loading of Fenton reaction and sodium hydroxide sequentially pretreated sugarcane bagasse. *Bioresour Technol*, **229**, 204-210.

Zhao, X.B., Dong, L., Chen, L., Liu, D.H., 2013. Batch and multi-step fed-batch enzymatic saccharification of Formiline-pretreated sugarcane bagasse at high solid loadings for high sugar and ethanol titers. *Bioresource Technology*, **135**, 350-356.

Zhou, H.F., Lou, H.M., Yang, D.J., Zhu, J.Y., Qiu, X.Q., 2013. Lignosulfonate To Enhance Enzymatic Saccharification of Lignocelluloses: Role of Molecular Weight and Substrate Lignin. *Industrial & Engineering Chemistry Research*, **52**(25), 8464-8470.