Dissertation

Successive Conversion of Lignocellulose to Bio-ethanol Using Zwitterions

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Abstract

Utilization of lignocellulosic feedstock in bioethanol production is demanded. To convert biomass into ethanol, three main processes consist of biomass dissolution, hydrolysis, and fermentation are required. Recently, ionic liquids (ILs) have demonstrated to be promising pretreatment solvents or hydrolytic catalysts for lignocellulose. On the other hand, successive efficient process on conversion of lignocellulosic biomass into ethanol is needed from a viewpoint of efficiency of cost and energy. However, the application of ILs has been limited due to their inhibition effect on fermentable microorganisms, and this condition critically prevents the successive process on lignocellulosic biomass conversion. In this study, we realized the successive process avoiding the toxicity of ILs while exploiting ILs as cellulose solvents or hydrolytic catalysts.

ILs show toxicity to microorganisms by destruction of cell membranes. The cation part first approaches to anionic phospholipid in membranes, followed by insertion of alkyl chain of the cation into hydrophobic part of the membranes. We introduced the anion part of ILs to the end of the cation, namely zwitterions (ZIs), and the ZIs are expected to be effective to prevent the insertion of the alkyl chain of the cation to the membranes because ZIs no longer have the hydrophobic cation tail. As a result, the ZIs were not toxic to the growth of *E. coli*. We investigated the toxicity of ILs to fermentation, and the ILs which act as hydrolytic catalysts or cellulose solvents were highly toxic. On the other hand, ZIs were low-toxic regardless of the structure. By exploiting the low toxic of ZIs, we developed the two successive processes.

We developed a successive process with an IL as a hydrolysis catalyst. The catalytic IL, 1-(4-sulfobutyl)-3-methylimidazolium hydrogen sulfate ($[C_1imC_4SH]HSO_4$) is composed of sulfuric acid and a ZI, 1-(4-sulfobutyl)-3-methylimidazolium (C_1imC_4S). Sulfuric acid has pretreatment ability and $[C_1imC_4SH]HSO_4$ has high catalytic activity. Bagasse was pretreated by using sulfuric acid that included in IL, for pretreatment. Following the pretreatment, the ZI has added for i*n situ* synthesis of $[C_1imC_4SH]HSO_4$. This process improved the glucose yield at 77% for 40 minutes hydrolysis at 100 ºC. Subsequent to hydrolysis, the $[C_1imC_4SH]HSO_4$ then separated into C_1imC_4S and H_2SO_4 by electrodialysis to decrease the toxicity of IL because the remaining ZI no longer toxic. In addition, electrodialysis successfully separated H_2SO_4 from $[C_1imC_4SH]HSO_4$ by the recovery at 97%; the separation enabled the successive fermentation. This successive conversion of bagasse into ethanol has the ethanol yield at 52%.

We also developed a successive process with a ZI as a pretreatment solvent. In this strategy, we come up an idea to replace ILs with ZIs that have ability to dissolve cellulose as a pretreatment solvent; consequently one-pot conversion of bagasse into ethanol would be allowed. A novel carboxylate-type zwitterion (1-(3-carboxypropyl)-3-(methoxyethoxyethyl) imidazolium, OE_2imC_3C) was synthesized. OE_2imC_3C dissolved 6 wt% of cellulose at 100 ºC. Since OE_2imC_3C does not show toxicity to *E. coli*, the one-pot process using 0.5 M of OE_2imC_3C obtained ethanol yield at 28%, while when using ILs no ethanol was obtained due to the toxicity. The viscosity of OE_2imC_3C was noted as a critical inhibition factor in this one-pot process. Dimethyl sulfoxide (DMSO) was added as a co-solvent to improve the efficiency of conversion. The addition DMSO into OE_2imC_3C accelerated dissolution of cellulose. Furthermore, OE_2imC_3C/DMSO (8/2) at 150 gL^{-1} was chosen as a solvent to conduct the one-pot conversion and the ethanol yield was increasing at 51%.

This study successfully developed successive processes by exploiting ZIs to avoid the high toxicity of ILs while the superior ability of ILs maintained.

List of Contents

List of Figure

List of Table

List of Scheme

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Chapter 1. Introduction

1-1. Background

Scientifically, it is being undebatable that the evidence of emission of greenhouse gases (GHG), such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), coming up from fossil fuel combustion and reducing environment capacity as a result of human activities, has affected the climate change. The transport sector has the highest demand with oil as a primary source. Currently, the world demand is about 84 million barrels per days and is expected to rise up to about 116 million barrels a day by 2030[1]. However, concerns of supply this un-renewable fuel are becoming urgent and the availability of conventional fuels is going to geographically restricted. With that in mind, renewable technologies to replacing fossil fuel have grown rapidly. It is important to design an integrated biorefinery which develops renewable biomass feedstock processing it into bioenergy and chemicals.

A switch from fossil fuel to biofuel offers excellent advantage not only to overcome the problem of limited supply of fossil fuel, but also can contribute to pushing down GHG emission towards reducing the emission of CO_2. It is good application example when Brazilian sugar cane ethanol could save 80% of GHG emission compare to gasoline[2]. Nowadays, bioethanol production has been developed from edible biomass (sucrose and starch feedstock) that has directly competed with food production into inedible biomass such as lignocellulosic biomass. The major difference in the production process of the technology is the step how to obtain fermentable sugar. The first generation ethanol production is using sucrose that is extracted directly from sugarcane or glucose from enzymatic saccharification of starch, while the second generation one is using glucose and/or xylose from complex hydrolysis of lignocellulose. Utilization of lignocellulosic feedstock in bioethanol production also has promising more advantage for CO_2 emission saving than the previous technology[3].

Lignocellulose is abundant composite material that present in the cell wall of plants. It is composed of three major biopolymers (cellulose, hemicellulose, and lignin) which are organized into a complex structure and that structure has resistance to degradation. Breaking down the compact structure followed by hydrolysis process under mild condition without losing the natural functionality of valuable monomers is a key to lignocellulose conversion[4]. An achievement in lignocellulosic biorefinery was indicated by efficient pretreating the biomass in order to provide its components a smooth access to the future process[5].

Ionic liquids (ILs), which are defined as molten salts having melting point at below 100 ºC, have recently fascinated a lot of interest in the field of biorefinery, and have demonstrated to be promising pretreatment solvents[6,7]. In addition, IL-assisted pretreatment has been enforced toward several types of lignocellulosic material[8]. After Swatloski *et al.* (2002)[9] published their experiment result which 1-buthyl-3-methyl-imidazolium chloride ([C_4mim]Cl) could dissolve 10 wt% of cellulose at 100 ºC, it became an initial report used to develop some ILs which are more effective in cellulose dissolution.

However, application of ILs in biorefinery has been limited due to their inhibition effect on enzyme activity and toxicity to fermentable microorganisms. Overcoming these issues opens opportunity to develop not only specific biocompatible IL but also the strategy of integrated processes on biorefinery system. It is expected to synergism between upstream processing of biomass to produce fermentable sugar and downstream processing of bioconversion sugar into biofuels or the others chemicals.

1-2. Biorefinery Concept

1-2-1. Definition and Aspects of Biorefinery

A definition of biorefinery has been made by International Energy Agency (IEA) Bioenergy Task 42 as "*the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, and materials) and bioenergy (biofuels, power and/or heat)*". The main role of biorefinery is the sustainable aspect which should be considered for the entire value chain on their environmental, economics, and social sustainability.

There is an analogy between biorefinery concept and a petroleum refinery. In addition, in a petroleum refinery, the unrefined oil converts into fuels, chemical building blocks for petrochemistry, and product chemicals, such as lubricants and solvents. Meanwhile, in a biorefinery, biomass convert into biofuels, chemical building blocks for agro-biochemistry, and product chemicals, such as bio-lubricants and biosolvents[4].

Sugar and thermochemical are two primary platforms in biorefinery which can produce chemicals and fuels including methanol, ethanol, and polymers. The chemical and biological processes, which are based on the disruption of biomass into aqueous sugars, are the term for "sugar platform". The fermentable sugars were obtained from cellulose and hemicellulose in biomass can be further converted into ethanol, aromatic hydrocarbons or liquid alkanes by fermentation, the downstream processing, respectively. The residues (lignin) can be used for the others useful products. On the other hand, in the thermochemical platform, biomass is processed into synthesis gas via gasification, or into bio-oils by pyrolysis and hydrothermal conversion (HTC). Bio-oils can be further promoted to liquid fuels such as methanol, gasoline and diesel fuel, and other chemicals[14]. Figure 1-1 describes the platform of the biorefinery.

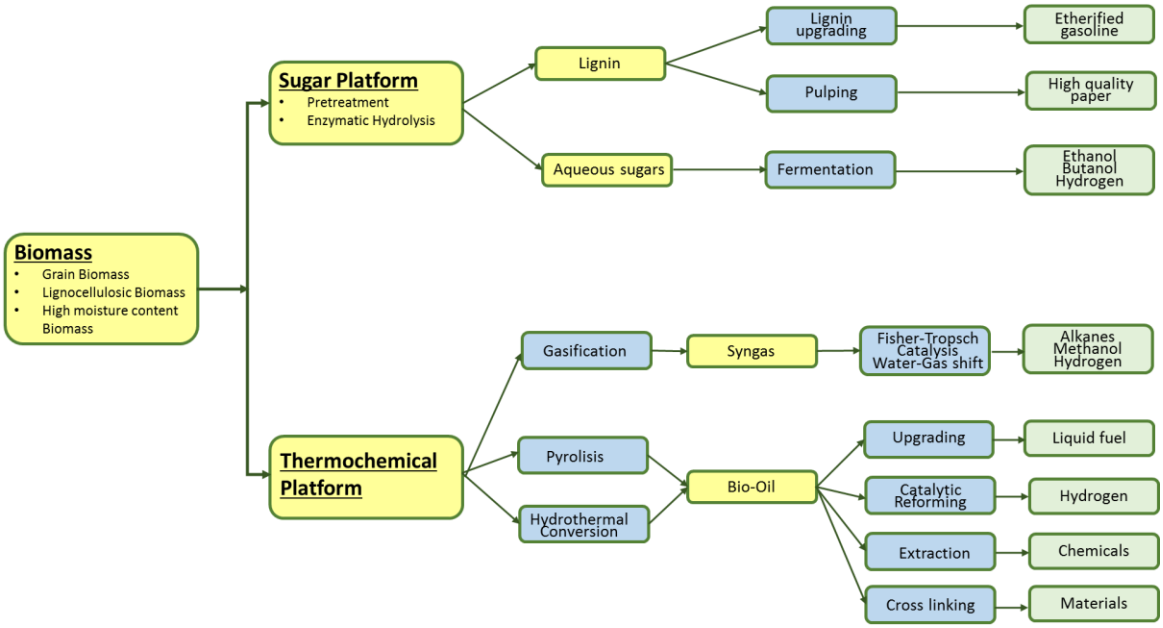


Figure 1- 1 Primary scheme for bioconversion of biomass into biofuel, chemicals, and material products (Adapted from reference 14, InTech, DOI: 10.5772/16417. Available from: https://www.intechopen.com/books/biofuel-s-engineering-process-technology/biorefinery-processes-for-biomass-conversion-to-liquid-fuel).

1-2-2. Biorefinery for Bioethanol

To date, bioethanol has already been mass produced in United State and Brazil. Sugarcane ethanol in Brazil has replaced 50% of the gasoline since the Brazilian ethanol program initially launched in 1970[15]. Increasing the global production of bioethanol was also significant which 48.0 billion liters ethanol was produced in 2007 to over 88.17 billion liters in 2013[16]. It takes 4% of the 1300 billion liters of gasoline consumed globally[17]. In addition, the consumption of bioethanol has been increasing rapidly in China, India, and some Asian countries. The demand for ethanol is a part of the decision of government programs in America, Asia, and Europe which is caused by total global fuel bioethanol and could grow to exceed 125 billion liters by 2020. Nowadays, ethanol was mixed with gasoline as a substituent for the lead, as a cleaner burning, and also octane enhancer[18].

Lignocellulosic ethanol, as a mark of the second generation, is a promising technology for at least two reasons: firstly, the lignocellulosic biomass is an abundant feedstock in the earth, the utilization does not compete with food crops, and less expensive than conventional agricultural feedstocks; secondly, by using plants

source of lignocellulose, it appears to be carbon-neutral when the carbons which emited to the atmosphere when biofuel burns are offset by carbons that plants absorb from the atmosphere while growing. However, increasing the complexity of the lignocellulosic biomass compared to sucrose and starch has created an obstacle to competitive lignocellulose utilization by economy and technology[19]. Main steps are required to produce ethanol from lignocellulose consists of pretreatment, hydrolysis of cellulose and hemicellulose, sugar fermentation, and finally recovery and purifying the ethanol to full fill a requirement specification[20]. Integrated biorefinery should address an efficient pretreatment to enhance hydrolysis physical-chemically or enzymatically and a high ethanol yield of ethanol. Enhancements in pre-treatment and advances in biotechnology, especially through process combinations can proceed the efficiency of production ethanol.

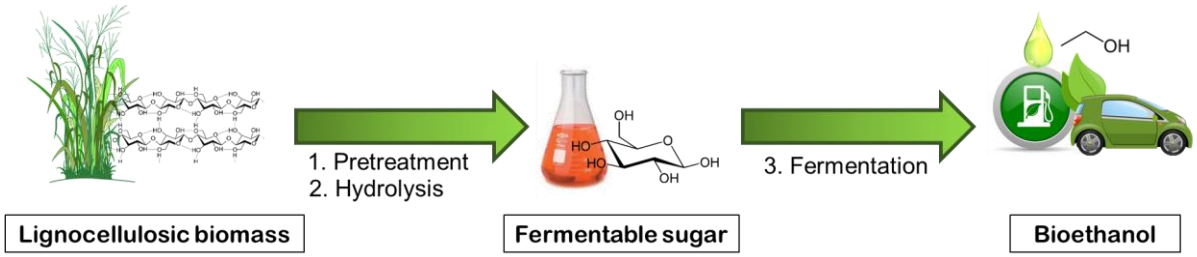


Figure 1- 2 General process of biorefinery lignocellulosic biomass into bioethanol

Efficient utilization lignocellulosic biomass to produce ethanol and value-added chemicals are still being researched until now. Research attentiveness has been focused extensively for over two decades to increase the digestibility of lignocellulosic biomass for the efficient conversion of cellulose to ethanol and another biofuel. The development of lignocellulose biomass conversion process covered five dominant steps are (1) choice of suitable biomass, (2) effective pretreatment, (3) production of a hydrolytic enzyme such as cellulose and hemicellulose along with the accessory enzyme, (4) fermentation of hexoses and pentoses, (5) downstream process[21].

The essential step in the production bioethanol from lignocellulosic biomass is pretreatment. Pretreatment should be able to dissolve or separate the valuable polymer components of biomass ie cellulose, hemicellulose and lignin and as a

result provides the easiness in polymers digesting in which are needed to achieve a high yield of fermentable sugar. For more details, the pretreatment described in the other part of this chapter (1-4). Furthermore, the researchers developed a multi-stage cellulosic ethanol production in which the treated biomass process using a variety of configuration processing such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and co-fermentation (SSCF), and consolidated biomass processing (CBP)[21] as described in Figure 1-3. SHF is a common two-steps process where the first step consists of hydrolysis of biomass to produce fermentable sugar, later in the second step the sugars are fermented to ethanol. Meanwhile, SSCF is a feasible option for ethanol production xylose-rich lignocellulosic materials. Finally, the CBP is a single process which configures of three biotransformations each production of saccharolytic enzymes (cellulase and hemicellulase), hydrolysis of cellulose and hemicellulose to monomeric sugars, and fermentation of hexose and pentose sugar.

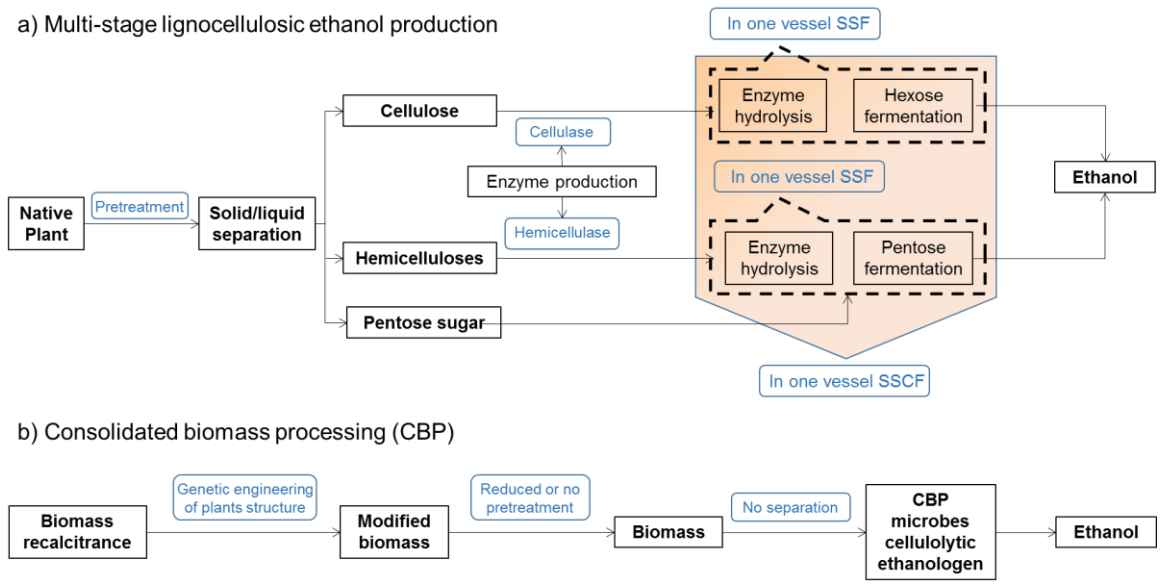


Figure 1- 3 Development of fermentation strategies and consolidated biomass processing in ethanol production (Adapted from reference 22 with permission from Elsevier).

1-3. Structure and Sources of Lignocellulosic Biomass

Lignocellulosic biomass is often referred to higher plants, softwood or hardwood. Three main polymers that compose this biomass are cellulose, hemicellulose, and lignin, also contains a few the other components such as acetyl groups, minerals, and phenolic substituents. Representative the structure of lignocellulose shows in Figure 1-4. These polymers are arranged into the complex non-homogenous three-dimensional structure to different degrees and varying relative composition. The interaction among molecule of the polymers produces the recalcitrance which comes from the crystallinity of cellulose, the hydrophobicity of lignin, and encapsulation of cellulose by the lignin-hemicellulose matrix[23].

Cellulose is the major component of lignocellulose which consists of glucose monomer. Cellulose is a linear polymer that is the *β*-1,4-polyacetal of the disaccharide cellobiose (4-O-*β*-D-glucopyranosyl-D-glucose)[24]. The number of glucose unit that makes up one polymer molecule of cellulose is known as the degree of polymerization (DP), as well as most of the cellulose properties depend on this value. Generally, DP of cellulose has a number of 1000-30000 glucose unit, even though the cellulose that is obtained from isolation usually has DP values range between 800-3000[25]. The polymerization of glucose on a long chain of cellulose via *β*-1,4-glicosidic bonding lead the hydroxides that distributed on both sides of the monomers to form extensive intra- and intermolecular hydrogen bonds[26].

The second abundant of polymer that contained in lignocellulose is hemicellulose. Unlike cellulose, hemicellulose polymer is not linear, it is a branched polymer chain. Hemicellulose is heteropolymer of pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose) and sugar acids (acetic). Hardwood hemicelluloses are xylans whereas softwood consists of glucomannans[23]. Xylan is the most common type of polymers which belongs to the hemicellulose family of polysaccharides, since arabinoxylans, glucomannans, galactans, and others are found in the plant cell wall. The molecule xylan includes 1->4 linkages of xylopyranosyl units with *α*-(4-O)-methyl-D-glucoronopyranosyl units attached to anhydroxylose units[24]. Consequently, it forms branching polymer chain which is primarily composed of five carbon sugar monomer, xylose, and rare consists of six

carbon sugar monomer such as glucose. Due to the highly branched structure, hemicellulose could not form the crystalline structure, however, it has an important role to contribute to strengthening the plant cell wall by interaction with cellulose and lignin[27].

The most complex natural polymer of lignocellulose is lignin. It is an aromatic polymer resulting from the oxidative combinatorial coupling of 4-hydroxyphenyl-propanoids. The three most abundant lignin monomers are p-hydroxyphenyl (H)/p-coumaryl (4-hydroxycinnamyl), guaiacyl (G)/conyferyl (3-methoxy 4-hydroxy-cinnamyl), and syringyl (S)/sinapyl (3,5-dimethoxy 4-hydroxycinnamyl)[24]. The function of lignin in the plant's cell is to act as the cellular glue which contributes compressive strength to the plant tissue and the individual fibers, hardness to the cell wall and protection counter to insects and pathogens[28].

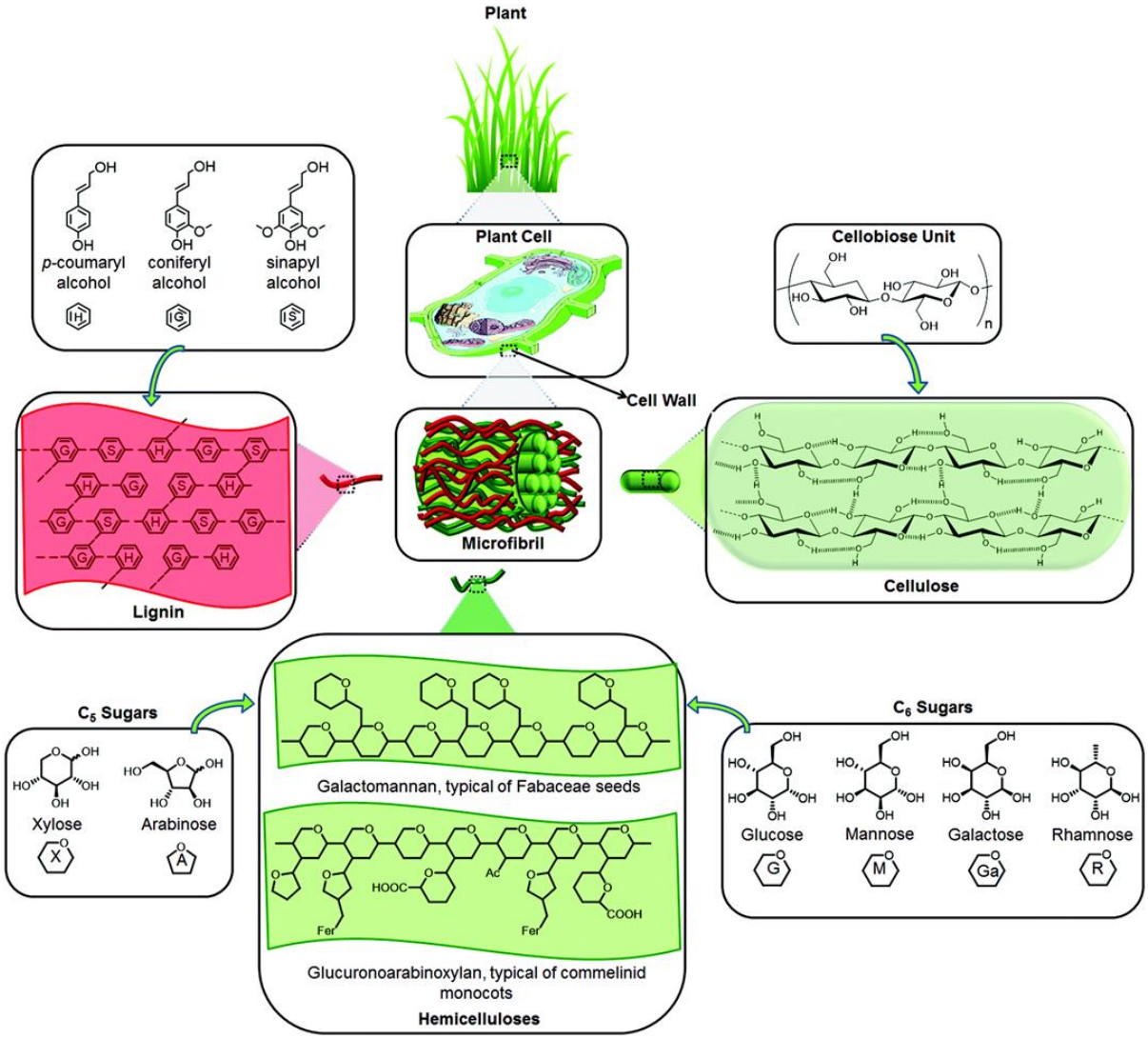


Figure 1- 4 The major components of lignocellulose. “Gl” represents glucuronic acid and “Fer” represents esterification with ferulic acid, which is characteristic of xylans in commelinid monocots (Adapted from reference 23, with permission of The Royal Society of Chemistry.)

Cellulose, hemicellulose, and lignin are not identically dispersed within the cells walls. The composition of lignocellulose highly relates to its biomass source and a number of components also depend on species, tissue, and age of plant cell wall. It is common that cellulose is composed of 35-50% of cellulose, 25-30% of hemicellulose, and 10-25% of lignin. The summary of the particular type of lignocellulosic biomass and their polymer composition is placed in Table 1-1.

Tabel 1- 1 Sources of lignocellulosic biomass and their polymers composition. (Adapted from reference 23, with permission of The Royal Society of Chemistry.)

Lignocellulose biomass		Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	Poplar	50.8-53.3	26.2-28.7	15.5-16.3
	Oak	40.4	35.9	24.1
	Eucalyptus	54.1	18.4	21.5
Softwood	Pine	42.0-50.0	24.0-27.0	20.0
	Douglas fir	44.0	11.0	27.0
	Spruce	45.5	22.9	27.9
Agricultural waste	Wheat Straw	35.0-39.0	23.0-30.0	12.0-16.0
	Barley Hull	34.0	36.0	13.8-19.0
	Barley Straw	36.0-43.0	24.0-33.0	6.3-9.8
	Rice Straw	29.2-34.7	23.0-25.9	17.0-19.0
	Rice Husks	28.7-35.6	12.0-29.3	15.4-20.0
	Oat Straw	31.0-35.0	20.0-26.0	10.0-15.0
	Ray Straw	36.2-47.0	19.0-24.5	9.9-240
	Corn Cobs	33.7-41.2	31.9-36.0	6.1-15.9
	Corn Stalk	35.0-39.6	16.8-35.0	7.0-18.4
	Sugarcane Bagasse	25.0-45.0	28.0-32.0	15.0-25.0
	Sorghum Straw	32.0-35.0	24.0-27.0	15.0-21.0
Grasses	Grasses	25.0-40.0	25.0-50.0	10.0-30.0
	Switchgrass	35.0-40.0	25.0-30.0	15.0-20.0

1-4. Pretreatment of Lignocellulosic Biomass

The first step to utilizing lignocellulosic biomass in ethanol producing is deconstruction the compact structure of polymers which are included inside of the biomass, namely pretreatment process. The pretreatment has a primary aim to eliminate the lignin seal and diminish the crystalline structure of cellulose for efficient hydrolysis of cellulose and hemicellulose chemically or biochemically by enhancing enzyme accessibility to the cellulose during hydrolysis step. In addition, the pretreatment is a critical step in the production bioethanol from lignocellulose because of it accommodates solubilization or separation the major components of biomass[22]. The primary of pretreating work on lignocellulosic ethanol is to prepare carbohydrate (especially cellulose) in a form that can be easily hydrolyzed and provide a high sugars concentration for ethanol production by fermenting microorganisms[29]. In the case of hydrolysis, it can be catalyzed by chemicals or enzymes. The illustration of the purpose of pretreatment process as mention above shows in Figure 1-5.

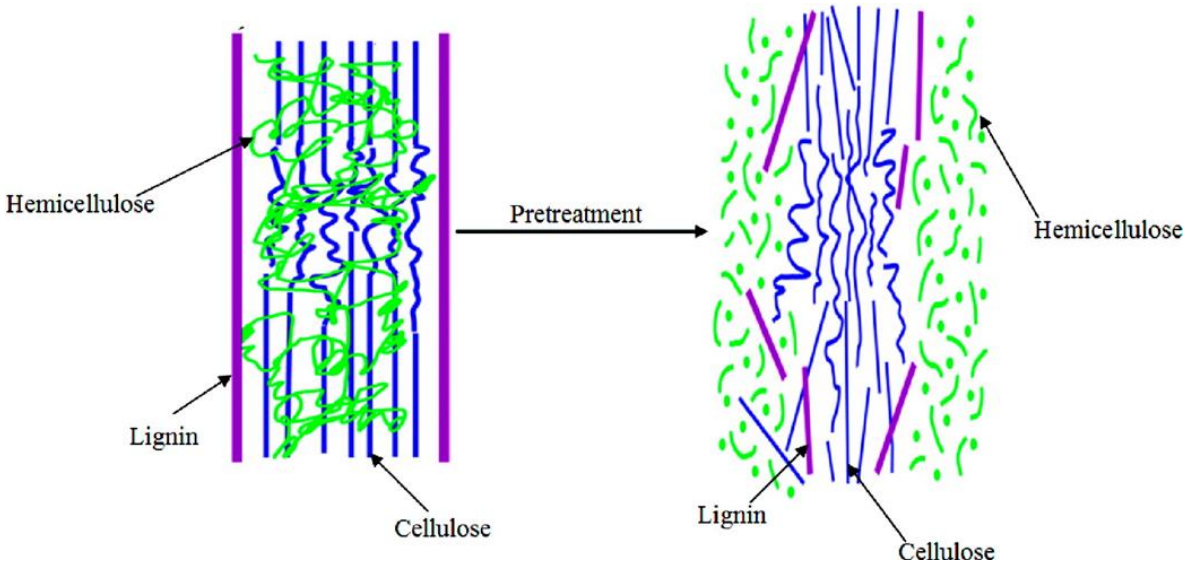


Figure 1- 5 Illustration of the lignocellulose's pretreatment process to remove the seal of lignin and hemicellulose on the natural lignocellulose composite, as well to decrystallization cellulose structure. (Adapted with permission from *Ind. Eng. Chem. Res.*, 2009, 48, 3713–3729 (reference 30). Copyright © 2009 American Chemical Society)

It has been also evaluated that pretreatment is the most second most expensive unit cost in the bioconversion of lignocellulose into ethanol via enzymatic saccharification[29,31,32]. Choosing effective and efficient methods, at the same time reducing the operational costs in pretreatment is a necessary step. Nowadays, pretreatment research is addressed to identifying, evaluating, developing, and demonstrating advantageous approaches that principally support the successive enzymatic hydrolysis of pretreated biomass with more diluted enzyme concentration and faster bioconversion rate[29]. Some criteria for the effectiveness and efficiency of the pretreatment process are being standard such as applicable for varying biomass, high digestibility of cellulose, facilitate high sugar concentration, avoid sugar degradation, produce less toxic compounds, available for lignin recovery, the operation minimize heat and power demand, suitable size of reactor in low-cost operation, and compatible with fermentation process[20]. However, it has not been decided which method is the best, because every pretreatment method belongs to specific advantages and disadvantages itself.

Generally, pretreatment classifies into physical pretreatment, psycho-chemical pretreatment, chemical pretreatment, and biological pretreatment. However, in the application of pretreatment, it is possible to combine one method and the others to meet the effectiveness and efficient processes including cost-effective[21]. Some of the advantages and disadvantages of pretreatment method described in Table 1-2.

Tabel 1- 2 Summary of the advantages and disadvantages of different methods for pretreating lignocellulose biomass (Adapted from reference 20 with permission from Elsevier)

Pretreatment method	Advantages	Disadvantages
Biological	- Degrades lignin and hemicellulose - Low Energy consumption	- Low rate of hydrolysis
Milling	- Reduces cellulose crystallinity	- High power and energy consumption
Steam explosion	- Causes lignin transformation and hemicellulose solubilization - Cost-effective - Higher yield of glucose and hemicellulose in the two-step method	- Generation of toxic compounds - Partial hemicellulose degradation
Ammonia fiber explosion (AFEX)	- Increases accessible surface area - Low formation of inhibitors	- Not effective for raw materials with high lignin content - High cost of large amount of ammonia
CO_2 explosion	- Increases accessible surface area - Cost-effective - Do not imply generation of toxic compounds	- Does not affect lignin and hemicelluloses - Very high-pressure requirements
Wet oxidation	- Efficient removal of lignin - Low formation of inhibitors - Minimize the energy demand (exothermic)	- High-cost of oxygen and alkaline catalyst
Ozonolysis	- Reduces lignin content - Does not imply generation of toxic compounds	- High-cost of large amount of ozone needed
Organosolv	- Does not imply generation of toxic compounds - Causes lignin and hemicellulose hydrolysis	- High-cost - Solvent need to be drained and recycled
Concentrated acid	- High glucose yield - Ambient temperatures	- High-cost of acid and need to be recovered - Reactor corrosion problems - Formation of inhibitors
Diluted acid	- Less corrosion problem than concentrated acid - Less formation of inhibitors	- Generation of degradation products - Low sugar concentration in exit stream

Lately, the most promising technology that researchers focus on, is the application of ILs on pretreatment lignocellulosic biomass. The achievement has already been satisfactory for biomass separation process and destruction of cellulose crystallinity. All this time, numerous data showing the great of ILs has been established using pure crystalline cellulose which it is not too complex structure such as in lignocellulose[20]. However, it was applied in the study on lignocellulosic feedstock pretreatments such as straw[33] or wood[34].

ILs are also eco-friendly while no toxic or explosive gasses are formed in biomass fractionation process. Nevertheless, ILs indicate have toxicity to enzymes and fermentative microorganisms. It is required to examine the effect of an IL to the cellulase/hemicellulase activity and growing up of fermentative microorganism before it can be selected as a catalyst in biomass pretreatment[20,35]. Base on the concentration of IL residues remaining, the significant inhibition effect on hydrolyzing enzyme activity and microorganism growth up may be investigated. So that, ILs residue removal would be demanded to avoid the low yield of sugars and it connects to concentration fermentation product automatically.

1-5. Ionic Liquid and Zwitterion

1-5-1. Ionic Liquid

ILs are defined as organic salts which have melting point or glass transition temperatures below 100 °C [36]. They are made from extensive organic cations, and either inorganic or organic anions. Since the first low melting salt, ethylammonium nitrate, with a melting point at 12 ºC was synthesized in 1914, to date, various structures of ILs have been synthesized and reported. Modern ILs are composed of an organic cation, frequently quaternised aromatic or aliphatic ammonium ions, alkylated phosphonium, and sulfonium. Meanwhile, the anions are either organic or inorganic compounds[37]. Figure 1-6 shows the structure of common cations (a) and anions (b) which usually compose an IL.

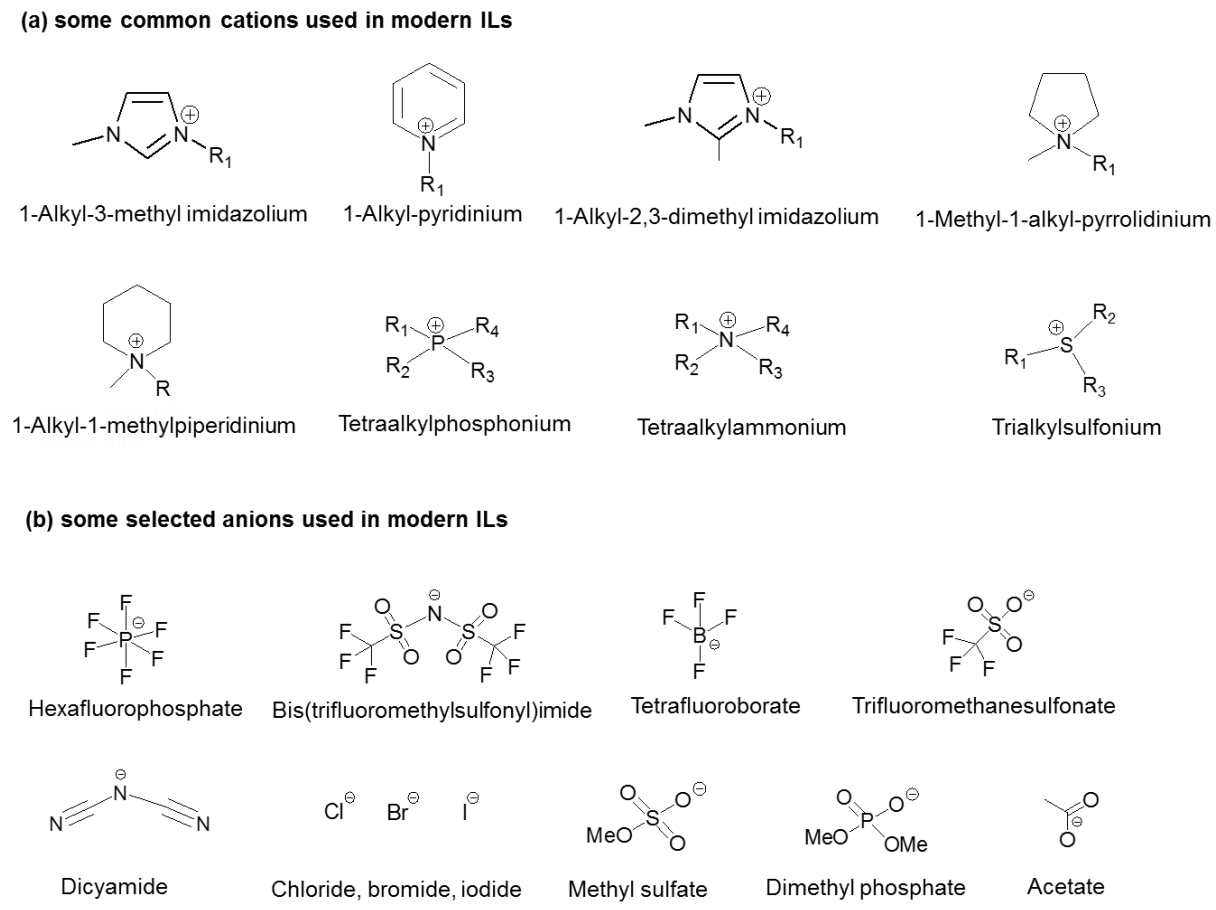


Figure 1- 6 Some common cations (a) and anion (b) structures which usually compose a modern IL (Adapted from reference 37, with permission of The Royal Society of Chemistry.)

Some properties of ILs are different from an ordinary solvents which have low melting point, high thermal stability, high density and viscosity, high conductivity, and vary in miscibility with water depend on their hydrophilicity or hydrophobicity property. In addition, Rogers and coworker have already published the properties of imidazolium cation-based ILs which a conclusion that the melting point goes down when the size and asymmetry of the cation increase. In conjunction with branching on alkyl chain, it indicates that higher the branching increases the melting-point[38]. Furthermore, ILs have a high thermal stability which commonly it will decompose at >400 ºC. On the other hands, ILs have high viscosity and this property allows to inhibit the rate of many organic reactions, also identically makes the diffusion rate of the redox species low. An increase in van der Waals forces over hydrogen bonding causes an increase in the viscosity of the various anion/cation combinations of ILs[39].

The properties of ILs are preferable for utilizing ILs as molecular solvents. ILs are able to dissolve a wide range of compounds in high concentrations, both of inorganics and biopolymers, also can lengthen the lifetime of unsteady reactive species, and kept the stabilization of catalyst [40]. The characters of ILs can be designed to adapt them to specific reaction condition by developing the substituents on the cation, e.g. by setting up the alkyl chain lengths, or by the specific of anion to regulate hydrophobicity properties. The opportunity to develop the structure of an IL that address to fill up a specific property is a reason to entitle ILs as “designer solvents”[41].

1-5-2. Application of ILs in Pretreatment and Hydrolysis Lignocellulosic Biomass

The observations of the ILs application in biomass pretreatment have been already exploited[42,43]. The efficient segregation of the main polymers of lignocellulose is essential as the first step of utilization of this biomass. Some researchers have observed capability of ILs in the scope of partial polymers in the dissolution of lignocellulose (cellulose, hemicellulose, and lignin), moreover, the inventions of the whole dissolution of lignocellulose have been achieved. Most of the research were using ILs with imidazolium cations, especially dialkylimidazolium cations. Rogers et al. (2007)[44] for the first time explained the dissolution of wood powder in 1-butyl-3-

methylimidazolium chloride ([C_4C_1im]Cl). Whereas Kilpeläinen et al. (2007)[45] made a progress in the dissolution of wood sawdust and wood pulp in 1-allyl-3-metylimidazolium chloride ([C=C_2)C_1im]Cl) and ([C_4C_1im]Cl). The next development comes from Sun et al. (2009)[46] which they reported the solubility of wood particles in [C_2C_1im][$MeCO_2$].

The capability of ILs in pretreatment lignocellulose is connected to its ability to dissolve the main polymer including in lignocellulosic biomass, it is also relevant to the fractionation of lignocellulose. Cellulose as a major polymer reported dissolve in some type of ILs. Some of the observation showed that an anion in an IL has an important function in describing its ability on the dissolution of cellulose. The anion is known from interacting with cellulose via a hydrogen bonding formation between the anion and an equatorial hydroxyl group on cellulose. With this mechanism, the anion breaks hydrogen-bonding inter- and intramolecular of cellulose[47]. In addition, a high value of hydrogen bond basicity of IL was mentioned as a factor that connected to ILs's ability an IL to dissolve cellulose[37]. Hydrogen bond basicity is examined by a solvatochromic measurement in the Kamlet-Taft system which the parameter characterizing the hydrogen-bond basicity (β), hydrogen-bond acidity (α), and dipolarity and polarisability (π^*)[48]. In spite of digestive lignin such as alkaline and organosolv lignin has been investigated for dissolution lignin procedure purpose, it could also assess that lignin dissolves in IL. The anion also has an important role in lignin dissolution, even thought the hydrogen-bond basicity does not require to be as high as for cellulose dissolution[37]. The representative of the action of an IL to disintegrate hydrogen-bonding in cellulose shows in Figure 1-7 using [Bmim]Cl as a model of IL.

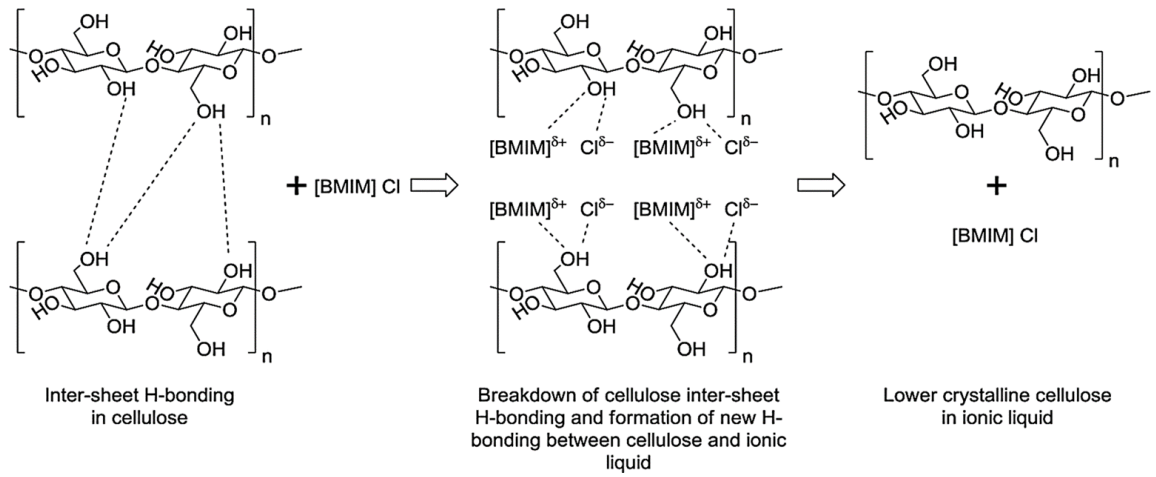


Figure 1- 7 Description of cellulose hydrogen-bond breaking by reaction of ILs (Adapted from reference 26)

Fermentable sugars are essential intermediate on the biological and chemical conversion of lignocellulose biomass, however the achievement of a high sugars from this conversion is inhibited by the recalcitrance of cellulose in biomass. Throughout it is known that the hydrolysis of cellulose on biomass can be usually catalyzed by acids and enzyme, the invention of ILs has to lead a mild conversion of lignocellulose via pretreatment and hydrolysis biologically or chemically. There are two principal of ILs application for biomass hydrolysis, first: importance on dissolution of the entire biomass, along with acid hydrolysis by acid or acidic IL[49], and second: importance on chemically disrupting the lignocellulose partially which fraction of cellulose remain largely and separate from hemicellulose and lignin, henceforth hydrolysis of the solid cellulose fraction catalyze by acid or enzyme[34]. Efficient pretreatment process by ILs is a key to enhancing the digestibility of cellulose on hydrolysis of cellulose. In the term of fractionation process, delignification and hemicellulose removal of lignocellulose are attract attention steps in pretreatment achievement which cellulose as a major component utilize to high sugar concentration producing. Table 1-3 summarizes pretreatment effect by using ILs for various biomass.

Tabel 1- 3 Effect of pretreatment by ILs to delignification and hemicellulose removal (Adapted from reference 37, with permission of The Royal Society of Chemistry)

Ionic Liquid	Biomass	Temperature (ºC)	Time (h)	Lignin removed (%)	Hemicellulose removed (%)
$[C_4C_1im][MeSO_4]$ (20% water)	*Miscanthus*	120	2	27	0
$[C_4C_1im][HSO_4]$ (20% water)			2	44	51
			22	93	82
$[C_4C_1im][MeSO_3]$ (20% water)				68	73
$[C_4C_1im][HSO_4]$ (20% water)			4	81	84
			20	80	92
$[C_4C_1im][HSO_4]$ (20% water)	Willow		22	85	79
	Pine			65	66
$[C_4C_1im]Cl$ (20% water)	*Miscanthus*			15	6
$[C_4C_1im]Cl$ (40% water)	Legume straw	150	2	30	9
$[C_4C_1im]Cl$	Triticale straw	90	24	15	11
$[C_4C_1im][MeSO_4]$ (7% water)	Sugarcane bagasse	125	2	26	88

1-5-3. Zwitterion

Zwitterion (ZI) is an ionic molecule in which both of cation and anion are bonded in the same molecule. The ZI material is identified by high dipole moments and is a family of materials that own moieties with both cationic and anionic groups. These materials are characterized by high dipole moments and notably charged compounds, on the other hands, its net charge is quietly neutral. The uniqueness of molecular structure spends much promising application of ZI material[51]. Nowadays, ZI has been present as a new family of biocompatible materials along with the ability of ZI to give some advances in biological activities[52].

ZI is also known as “inner salt” which by reason of a salt formed by molecules having both acid and basic properties. However, unlike ILs, most of ZIs have melting point (*T*m) at above 100 ºC. This character comes from the decreasing of movement freedom of cation and anion by fastening in a structure[53]. Yoshizawa et al (2004)[53] investigated the thermal properties of some series of zwitterion which are sulfonate and sulfonamide anion tied to various imidazolium cations, and they identified the ZIs have high melting point. Furthermore, the improvement to obtain a liquid phase of

zwitterion have been arising from their group. They published a mixture that comprises 1-(1-Butyl-3-imidazolio)propane-3-sulfonate (BIm_3S), a zwitterion, and bis(trifluoromethanesulfonyl)-imide (HTFSI) was a few viscous and transparent liquid at room temperature[54]. In 2011, with different research group, he also successfully designed a ZI containing two oxyethylene units on the imidazolium cation which has sulfonate cation, 3-(1-(2-(2-methoxyethoxy)ethyl)-1H-imidazol-3-ium-3-yl)propane-1-sulfonate (OE_2imps)[55]. They mentioned that ether groups were effective to decrease the crystallinity of ZI so that the ZI was a viscous liquid in room temperature.

Utilizing ZI directly in biomass processing is a novel field, so far there is no published paper that describes the application one. On the other hand, the investigation of enhancement of the saturated water content of hydrophobic IL, 1-butyl-3-methylimidazolium bis(trifluoromethanesulfonyl)imide ($[C_4mim][Tf_2N]$), by adding of designed zwitterion, 3-(1-butyl-3-imidazolio)propanesulfonate (C_4Im_3S) by Ohno group (2012)[56], has opened the window to apply this method in biomass processing. The discovery is useful for increasing the solubility of the enzyme in a hydrophobic IL. Since the enzyme dislikes to dissolving in the hydrophobic solvent, it would be convenient to be a medium enzymatic reaction when a hydrophobic IL is capable of dissolving and stabilizing of a protein enzyme. Furthermore, the hydrophobic IL-water biphasic system has been purposed for the extraction, separation, and condensation of numerous compounds. Hydrolysis of biomass in this system allows the separation of hydrolysis product which the fermentable sugars (glucose and xylose) are extracted in aqueous phase while the byproduct such as hydroxymethylfuran (HMF) and furfural, that are fermentation inhibitor, are dissolved in hydrophobic IL. Finally, because of ZI also could be a “designer material”, with the analogy of IL and also the invention of liquid zwitterion (ZIL) it might be an opportunity to utilize ZIL as biomass-solvent.

1-7. Toxicity Ionic liquid to Microorganism

The application of ILs in pretreatment lignoselulosic biomass was useful in bioconversion of the biopolymer into valuable chemical product including bioethanol. However, most of ILs are toxic to the microorganism and promote inactivation of the enzyme during the enzymatic saccharification process. A report mentioned that more than 20% (v/v) of IL presence inhibited enzyme activity significantly[57]. Furthermore, the investigation of ILs toxicity to the microbial fermentation indicated that the microbial was inhibited by imidazolium-type ILs by low concentration (0,1%)[58-59].

A mechanism of toxicity studied by Lim et al (2014)[60], and they explained how ILs destructed the membrane cells. First, cation part of ILs approach to anionic phospholipid of cell membrane by electrostatic interaction. After that, the hydrophobic part, which are mostly the alkyl chain part of cation, insert into the hydrophobic part of membrane, and finally the molecule of ILs accumulate in the membrane cell. Since the ILs accumulate, they will lead to deconstruction of membrane. The mechanism describes in Figure 1-8.

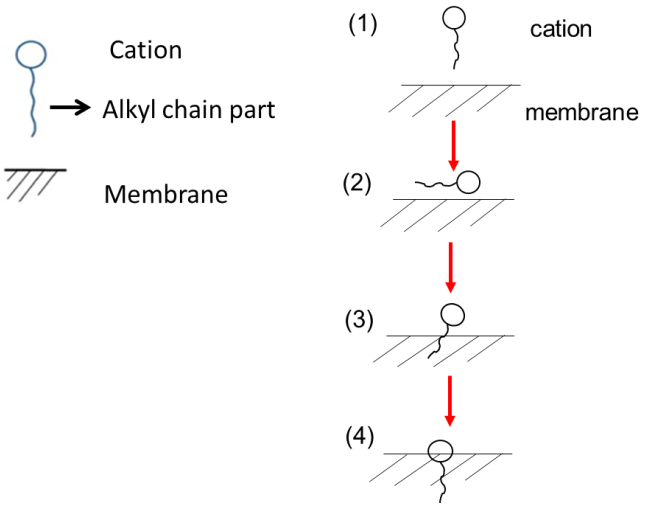


Figure 1- 8 The illustration of the cation insertion into membrane mechanisms, (1) and (2) cation approaches to anionic phospholipid by electrostatic interaction, (3) and (4) alkyl chain of the cation entered the hydrophobic part of membrane (Adapted with permission from *J. Phys. Chem. B*, 2014, 118, 10444−10459 (reference 60). Copyright © 2014 American Chemical Society)

1-8. Problem and Strategy

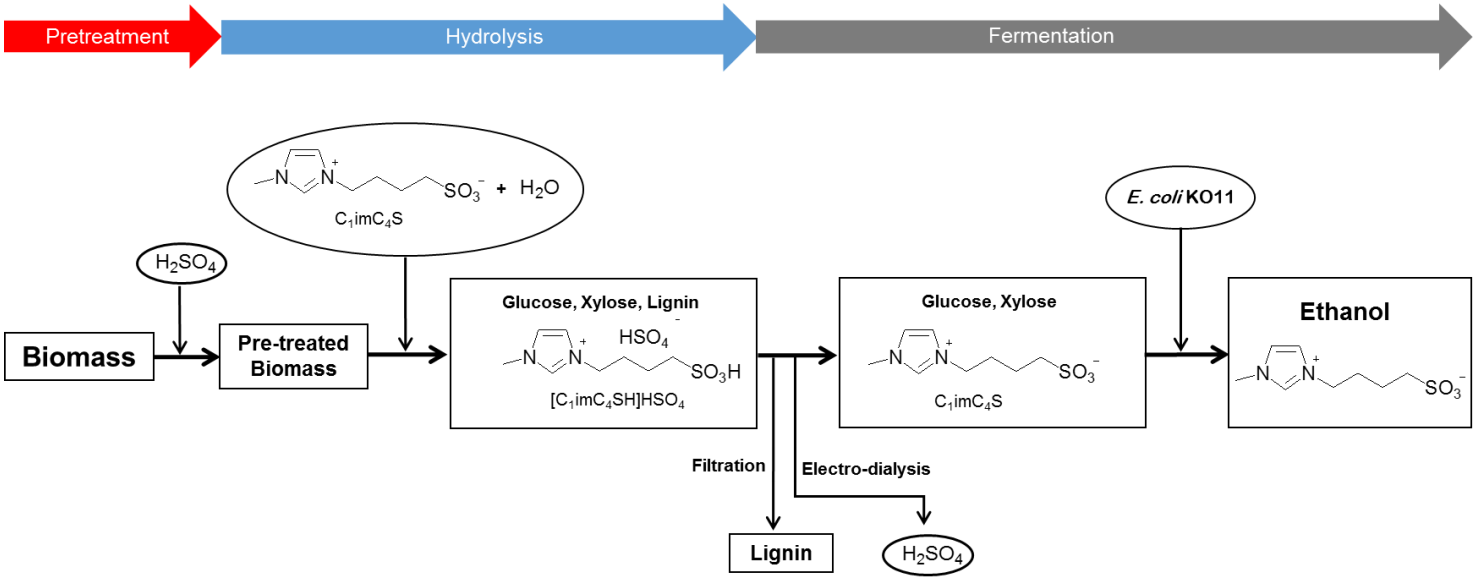
ILs have ability to fractionation lignocellulose biomass. Dual function of ILs, as a solvent and a catalyst in once time, also has beneficial to utilize ILs in pretreatment and hydrolysis carbohydrate polymer that including in lignocellulose at once. On the other hand, most of ILs are toxic for fermentable microorganism such as recombinant *Escherichia coli* (*E. coli*). The toxicity gives inhibition effect in bioconversion lignocellulose into ethanol. The contradictive fact between the function of ILs and its toxicity effect should be overcome using fine strategy in whole process bioconversion. Therefore, biocompatible ionic compound, namely zwitterion, has being a part structure and for many cases has similarity in structure and chemical properties with ILs.

This working has a general aim to improve lignocellulose biomass conversion into ethanol by using zwitterion in a successive process. The first step is the investigation of ILs and zwitterion on toxic effect to the growth of *E. coli* and inhibition effect on ethanol fermentation by using recombinant *E. coli* KO11. This step will detail describe in Chapter 2.

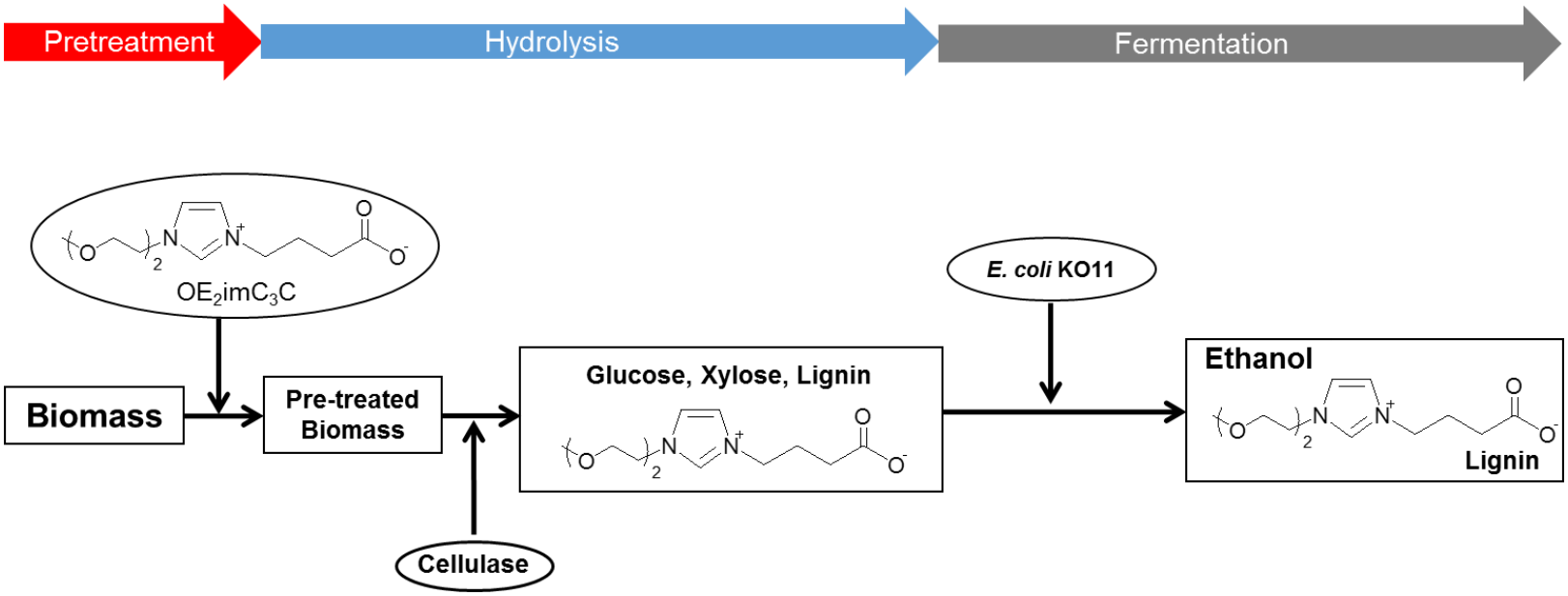
Furthermore, in Chapter 3 a new strategy on biorefinery of bagasse into ethanol is explained. The innate sulfuric acid included in an acidic ionic liquid, 1-(1-butylsufonic)-3-methyimidazolium hydrosulfate ($[C_1imC_4SH]HSO_4$), was using for the purpose of pretreatment, followed by addition of zwitterion, C_1imC_4S, for *in situ* synthesis of $[C_1imC_4SH]HSO_4$ successively for hydrolysis using microwave heating purpose. To overcome the toxicity of IL, the separation process via electrodialysis was applied to omit sulfuric acid part in IL, and finally, fermentation of sugar from hydrolysis was conducted to produce ethanol by using *E.coli* KO11. The strategy process in Chapter 3 shows in Scheme 1-1.

Moreover, the application of a novel synthesized carboxylate type zwitterion, OE_2imC_3C, which is liquid at room temperature has been applied on bioconversion of bagasse into ethanol. The biocompatibility property of this zwitterion allows the one-pot process of bioconversion of bagasse into ethanol by the sequential process consist of pretreatment, hydrolysis, and fermentation. The procedures and results of this research are mentioned in Chapter 4, and the strategy process are figured out in Scheme 1-2.

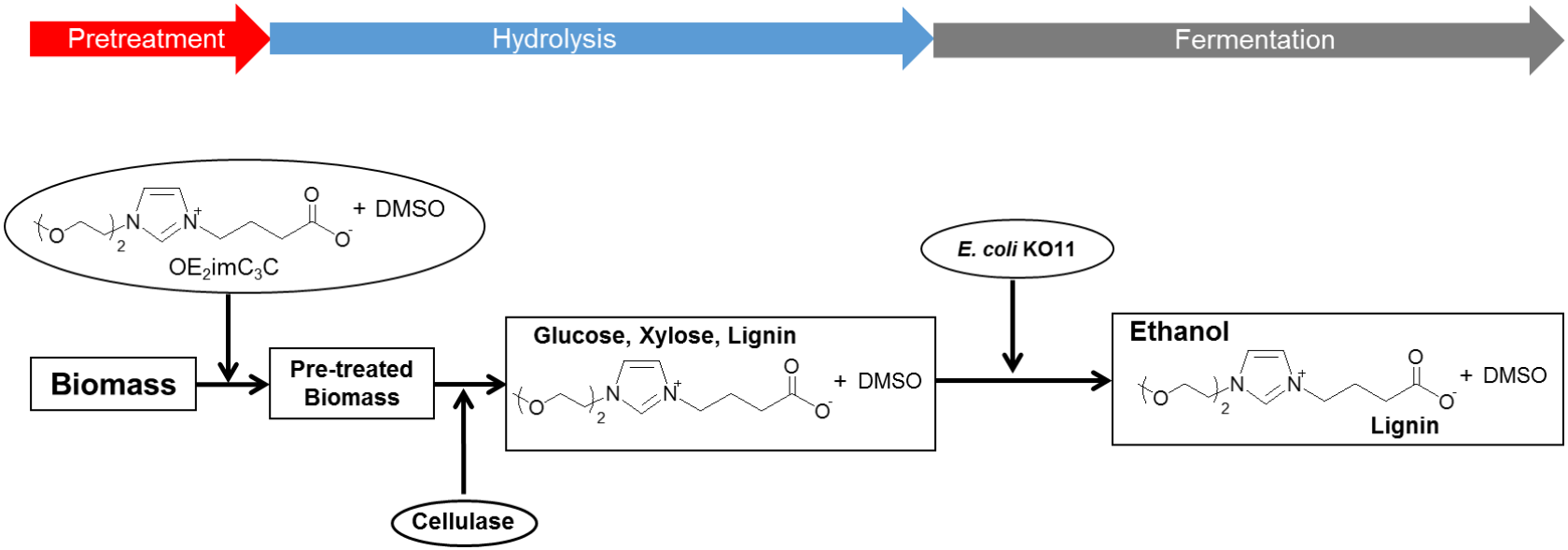
Finally, the improvement viscosity's property of OE_2imC_3C by adding dimethyl sulfoxide (DMSO) as co-solvent was employed on one-pot conversion of bagasse into ethanol. DMSO gave a synergy effect on the dissolution of cellulose, as a result pretreatment process was more effective. This research is presented in Chapter 5, and the design of working is displayed in Scheme 1-3.



Scheme 1- 1 Bioconversion of bagasse to ethanol strategy using zwitterion C_1imC_4S



Scheme 1- 2 One-pot bioconversion of bagasse to ethanol strategy using OE_2imC_3C



Scheme 1- 3 Bioconversion of bagasse to ethanol strategy using zwitterion OE_2imC_3C and DMSO co-solvent

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Chapter 2. Investigation of Toxicity and Inhibition of Ionic Liquid and Zwitterion in Ethanol Fermentation

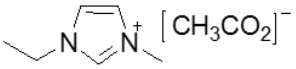
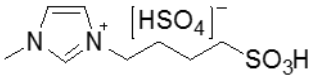
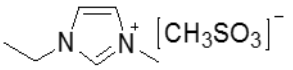
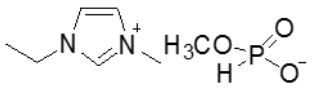
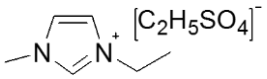
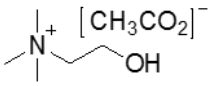
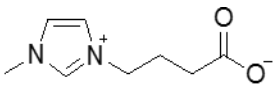
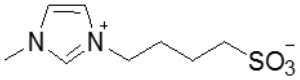
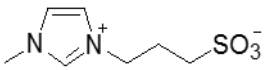
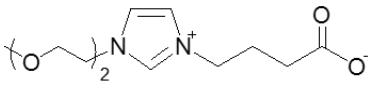
2-1. Introduction

Advance applications of Ionic liquid (ILs) in lignocellulose biorefinery have been published, including their utilization as solvents and catalyst for lignocellulose fractionation, cellulose dissolution and derivatization, and lignocellulose pretreatment. Brandt et al (2013)[1] made a resume of the application of 1-Ethyl-3-methylimidazolium-acetate ($[C_2C_1im][MeCO_2]$) for delignification and hemicellulose removal as an effectiveness parameter of pretreatment. Turning into detail, the pretreatments of various biomass at a range of temperature and time using $[C_2C_1im][MeCO_2]$ were effective to remove lignin from cellulose around 17-69% and also decreasing hemicellulose content up to 83%, since the glucose recovery achieve 97%. The other working reported that regenerated cellulose after pretreated by using $[C_4mim]Cl$ exhibited enzymatic hydrolysis kinetic acceleration[2].

However, the residue of ILs in pretreated cellulose promote inactivation of the enzyme during the hydrolysis process. There is strongly recommended to remove the residue of ILs after cellulose regeneration for the next process of cellulose[3]. It appeared that more than 20% (v/v) of IL presence inhibited enzyme activity significantly[4]. On the other hands, the downstream process after successfully obtain the fermented sugar by hydrolysis, the fermentation, is also prevented by low concentrations of ILs (0.1%)[5,6]. The unexpected condition is required to examine the effect of an IL on the growing up the microorganism as urgently as to investigate the effect of IL on the fermentation process.

In this chapter, the growth assay and fermentation assay by using several ILs and ZIs that presence in *E. coli* medium will be explained. The purpose of the study is to evaluate the toxicity effect on growing of *E.coli*, and the effect of ILs/ZIs to the fermentation glucose into ethanol by a recombinant *E. coli* KO11. The ILs and ZIs that used in the experiments describe in Table 2-1.

Tabel 2- 1 IL and ZI used in growth assay and fermentation assay

No	Abbreviation	Name	Structure	Molecular weight (mol g^{-1})
Ionic liquid				
1	$[C_2mim]OAc$	1-ethyl-3-methylimidazolium acetate		**170.21**
2	$[C_1im\ C_4SH]HSO_4$	1-(4-sulfobutyl)-3-methylimidazolium hydrogen sulfate		**316.25**
3	$[C_2mim][MeSO_3]$	1-ethyl-3-methylimidazolium methanesulfonate		**206.26**
4	$[C_2mim][(MeO)(H)PO_2]$	1-ethyl-3-methylimidazolium methylphosphonate		**209.11**
5	$[C_2mim][EtSO_4]$	1-ethyl-3-methylimidazolium ethyl sulfate		**236.29**
6	[Cho]OAc	Choline acetate		**163.21**
Zwitterion				
7	C_1imC_3C	1- (3-carboxypropyl)-3-methylimidazolium		**171.22**
8	C_1imC_4S	1- (4-sulfobutyl)-3-methylimidazolium		**218.27**
9	C_1imC_3S	1- (3-sulfopropyl)-3-methylimidazolium		**204.25**
10	OE_2imC_3C	1- (3-carboxypropyl)-3-(methoxyethoxyethyl)imidazolium		**256.30**

2-2. Growth Assay *E. coli*

2-2-1. Material and Methods

Materials and Instrumentations

LB medium is a sterile medium that composed by yeast extract (5 gL^{-1}), NaCl (5 gL^{-1}), tryptone (10 gL^{-1}) and ultrapure water up to 1.0 L. All of chemical was purchased from Nacalai Tesque, Kyoto, Japan). Sterilization medium and equipment was done by using autoclave (TOMY LSX-700), aseptically working was done in the clean bench (Bio-Labo clean bench NS-8A), incubation was done in shaker incubator (EYELA incubator FMS) and OD_{600} was measured by using spectrophotometer (Life Science UV/Vis spectrophotometer DU-730 Beckman Coulter).

Growth Assay Procedure

The assay medium contains LB medium and IL or ZI that the concentrations of IL or ZI have been set up each 0.00; 0.01; 0.05; 0.10; 0.50; 1.00 M, was prepared. Pre-culture of *E. coli* was prepared by inoculation 1 colony of *E. coli* into 5.0 mL LB aseptically and was incubated in a shaker incubator at 160 rpm, 37 ºC, for 18-24 hours. The density of pre-cultured cells was measured after 18-24 hours by using a spectrophotometer, and the absorbance at 600 nm then was defined as OD_{600}. The appropriate volume of pre-cultured *E. coli* was taken to set up initial OD_{600} at 0.1 in the 2.0 mL assay medium and was transferred into micro-tube and then was centrifuged at 15,000rpm, 4 ºC, for 5 minutes. The filtrate was then discharged, and the cells were re-suspended using the assay medium. Suspended of *E. coli* was inoculated into assay medium, then was incubated in a shaker incubator at 160 rpm, 37 ºC, for 24 hours. The measurement of OD_{600} was done at 6, 12, and 24 hours regularly. Relative OD_{600} 24h then was calculated using the following equation:

$$\text{Relative } OD_{600}\ 24h = \frac{OD_{600} \text{ of sample the IL or ZI at 24 hours}}{OD_{600} \text{ of 0.00 M at 24 hours}}$$

2-2-2. Result and Discussion of Growth Assay

Effect of ILs on *E. coli* growth has been examined, and the result presents in Figure 2-1. Firstly, imidazolium cation type of ILs show high inhibitor effect than cholinium one. The imidazolium cation-type of ILs inhibit *E. coli* growing up significantly at concentration 0.5 M, since the cholinium allows the *E. coli* to grow up, even though at this concentration substantially all of ILs appear to decrease the OD_{600} value. On the other hand, among of imidazolium-type of ILs, different type of anion have not given effect greatly. In addition, at concentration of ILs 1.0 M, none ILs were not inhibition the growth of *E. coli* in medium assay after 24 hours cultivation.

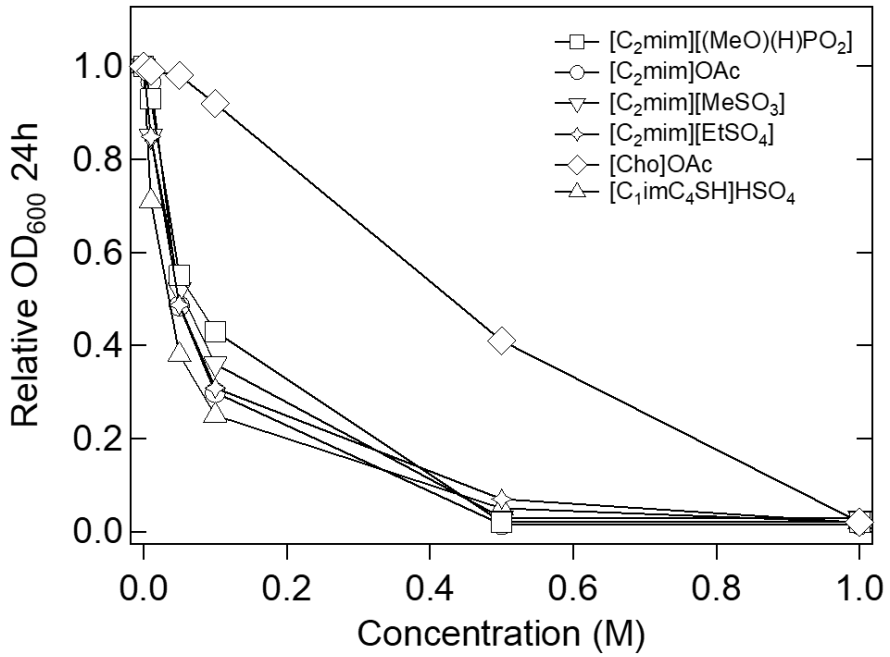


Figure 2- 1 The growth assay result that describes the effect of ILs on *E. coli* growth

Furthermore, there are significantly different effect of ZIs when it used on *E. coli* growth up. *E. coli* more tolerance at the high concentration of ZIs than ILs. As can be seen clearly in Figure 2-2, at concentration of ZI 1.0 M the ROD_{600} value of ZI is more than 0.1 that it indicate *E. coli* still allowed to grow up in this concentration. There also show that type of ZI, whereas we used carboxylate-type and sulfate-type of ZI, at concentration 0.5 M did not give significant inhibition effect to *E. coli*. Although, at concentration of ZI 1.0 M the distance of ROD_{600} appeared clearly. Turning into details, influence of alkyl length of spacer in sulfate type of ZI, when we compare between C_1imC_4S and C_1imC_3S, also gave slightly impact.

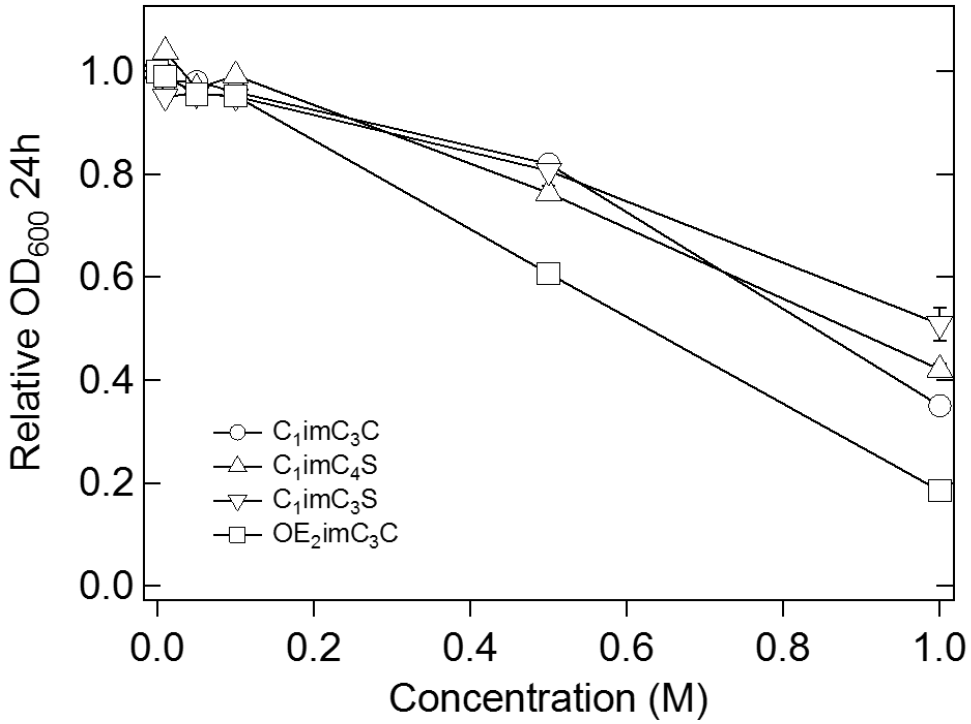


Figure 2- 2 The growth assay result that describes the effect of ZIs on *E. coli* growth

It was confirmed that *E. coli* could grow up better with the absence of ZI than IL. Figure 2-3 describes the difference structure between ILs and ZI. The toxicity of ILs or ZIs caused by the accumulation of the molecules on the cell. The molecule of ILs or ZIs could enter the cell with the mechanism of toxicity of ILs as was mentioned by Lim et al (2014)[7]. They explained that cation part firstly approaches to anionic phospholipid of the cell membrane by electrostatic interaction, followed by hydrophobic part (alkyl chain) of the cation insert into the hydrophobic part of the

membrane. Since ILs accumulate in cell membrane, it will lead to the destruction of membrane. When the anion part and cation part is tethered in the one molecule structure in ZI molecule structure, it showed more biocompatible than since they separate into each ion in IL. In the case of carboxylate type of ZI, structurally, a hydrophobic part in ZI has been loosen by attached the anion part into molecule. With this reason, the insertion of hydrophobic part into cell membrane could be avoided, as a result the accumulation would not happen. Moreover, strong acid anion in $[C_1imC_4SH]HSO_4$ is thought to be the cause of high toxicity of IL to *E. coli*. When the acid anion part was omitted in ZI molecule, acceptance *E. coli* to the molecule have increased.

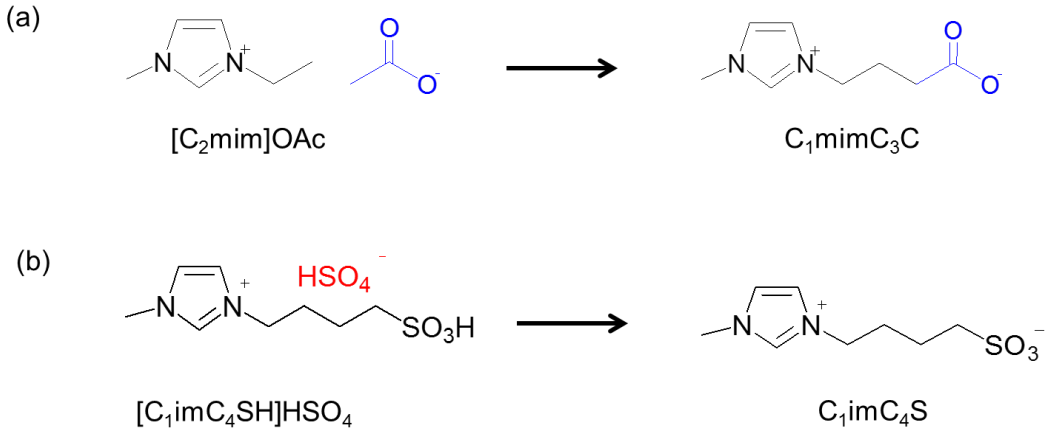


Figure 2- 3 The difference between IL and ZI structure, (a) omission of hydrophobic part with attachment of anion part into alkyl chain in cation part improved the tolerance of *E. coli* to C_1imC_3C (ZI) rather than $[C_2mim]OAc$ (IL) and (b) removal of acid anion part in $[C_1imC_4SH]HSO_4$ (IL) into C_1imC_4S (ZI) gave the positive effect to the growth of *E. coli*.

EC_{50} is defined as a critical concentration of chemical compounds for growth of microorganisms. We calculated EC_{50} value for all of ILs and ZIs that were examined, and it place in Table 2-2. The calculation result also show clearly that ILs more toxic than ZIs. Most of the EC_{50} value of ILs are under 20 gL^{-1} except for the [Cho]OAc (70 ± 0.0001 gL^{-1}), however, all of the EC_{50} value of ZIs are above 100 gL^{-1}.

Tabel 2- 2 EC_{50} value of ILs and ZIs that used in experiments.

No	Ionic liquid or Zwitterion	EC_{50} value (gL^{-1})
Ionic Liquid		
1	$[C_2min]OAc$	7 ± 0.00001
2	$[C_1imC_4SH]HSO_4$	11 ± 0.00001
3	$[C_2mim][MeSO_3]$	12 ± 0.00001
4	$[C_2mim][(MeO)(H)PO_2]$	19 ± 0.0001
5	$[C_2mim][EtSO_4]$	12 ± 0.00001
6	[Cho]OAc	70 ± 0.0001
Zwitterion		
7	C_1imC_3C	141 ± 0.0003
8	C_1imC_4S	> 200
9	C_1imC_3S	> 200
10	OE_2imC_3C	161 ± 0.00004

2-3. Fermentation Glucose into Ethanol Assay

2-3-1. Materials and Method

Materials and Instrumentations

Fermentation medium is a sterile medium that composed by yeast extract (5 gL^{-1}), NaCl (5 gL^{-1}), tryptone (10 gL^{-1}), glucose (50 gL^{-1}) and ultrapure water up to 1.0 L. All of chemical was purchased from Nacalai Tesque, Japan). *E. coli* KO11 ATCC®55124™ strain was used as fermented microorganism. Sterilization medium and equipment was done by using autoclave (TOMY LSX-700), and aseptically working was done in the clean bench (Bio-Labo clean bench NS-8A). OD_{600} was measured by using spectrophotometer (Life Science UV/Vis spectrophotometer DU-730 Beckman Coulter). Analysis of glucose and ethanol was determined by using a high-performance liquid chromatography equipped with a refractive index detector (Shimadzu Co., Kyoto, Japan), an ICSep ION-300 S1588 column (Tokyo Chemical Industry Co. Ltd., Japan) was used in tandem with an ICSep ICE-ION-300 S1589 guard column (Tokyo Chemical Industry Co. Ltd., Japan).

Fermentation Procedure

The fermentation assay medium, contains fermentation medium and IL or ZI that the concentrations of IL or ZI have been set up each 0.00; 0.01; 0.05; 0.10; 0.50; 1.00 M, was prepared. Pre-culture of *E. coli* was prepared by inoculation 1 colony of *E. coli* into 5.0 mL LB aseptically, and was incubated in shaker incubator at 160 rpm, 37 ºC, for 18-24 hours. The OD_{600} of pre-cultured was measured after 18-24 hours by using a spectrophotometer. The appropriate volume of pre-cultured *E. coli* was taken to set up initial OD_{600} at 1.0 in the 10.0 mL of fermentation assay medium and was transferred into micro-tube and then was centrifuged at 15,000rpm, 4 ºC, for 5 minutes. The filtrate was then discharged, and the cells were re-suspended using the fermentation assay medium. Suspended of *E. coli* was inoculated into assay medium, then was incubated in shaker incubator at 160 rpm, 37 ºC, for 72 hours. Sampling was done for 0; 6; 12; 24; 48; and 72 hours regularly by taken 500 µL of fermentation medium. The sample then was centrifuged at 15,000 rpm, 4 ºC, for 10 minutes, and the filtrate was collected and filtrated using 0.45 µm millipore filter. The analysis of glucose and ethanol was determined by HPLC to follow the analysis

condition: the volume of the injected sample was 20.0 µL, the column was operated at 70 ºC, and H_2SO_4 0.0085 N was used as the mobile phase with a flow rate was set up at 0.4 mL/min.

Yield of ethanol (Y_{EtOH}) 48h is defined as producing ethanol (mol L^{-1}) from initial glucose available (mol L^{-1}), and it is assumed that 1 mol of glucose is converted into 2 mol of ethanol. Relative Y_{EtOH} 48h then was calculated using the following equation:

$$\text{Relative } Y_{EtOH} \text{ 48h} = \frac{Y_{EtOH} \text{ of sample the IL or ZI at 48 hours}}{Y_{EtOH} \text{ of 0.00 M at 48 hours}}$$

2-3-2. Result and Discussion of Fermentation Assay

Ethanol productions from glucose including of ILs in the fermentation medium by using *E. coli* KO11 gave different response compared from growth assay. While 0.5 M of ILs mostly inhibit the growth of the *E.coli* in the growth assay, the ethanol still could be generated in the fermentation assay. The high yield of ethanol achieved in the fermentation medium that content of 0.5 M of $[C_2mim][EtSO_4]$, meanwhile at the similar concentration, the others IL in medium suppressed ethanol production by *E. coli* KO11. Moreover, the low yield of ethanol values are given in fermentation using $[C_2mim]OAc$ and $[C_2mim][MeSO_3]$, and ethanol totally could not produce under the influence of 0.5 M of $[C_1imC_4SH]HSO_4$ and neither of $[C_2mim][(MeO)(H)PO_2]$. In spite of [Cho]OAc more accepted in the growth assay of *E. coli*, however, it gave negative effect in ethanol fermentation. All of the data indicate that the cation-type did not influence a Y_{EtOH} achieved, on the other hand, various anion-type has a difference of the ethanol production level. Figure 11. shows the description of relative Y_{EtOH} 48 hours as a feedback of presence of each IL in the fermentation medium

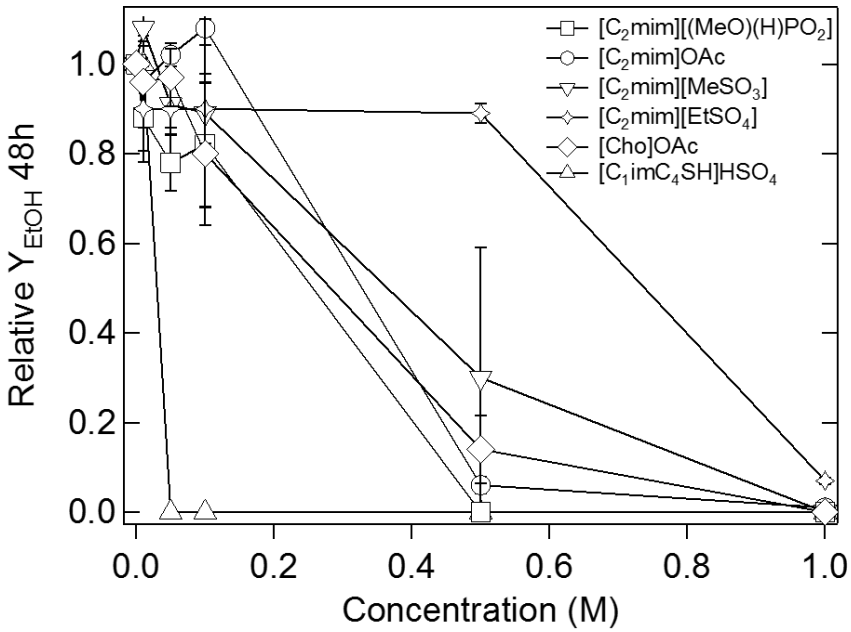


Figure 2- 4 The relative Y_{EtOH} 48 hours as a result fermentation of glucose (50 gL^{-1}) in presence of ILs in the medium.

In the presence of ZI, the ethanol fermentation at 0.5 M of ZI showed the positive impact. The Y_{EtOH} 48 hours in this condition reached around 80% for all of ZIs, and the trend shows a similarity with the using of ZIs in the low concentration. Both of sulfate and carboxylate type of ZIs up to 0.5 M have a difference Y_{EtOH} 48 value insignificantly. Furthermore, a sulfate type, C_1imC_3S, gave the highest Y_{EtOH} 48 value at 1.0 M, nevertheless, a carboxylate type, OE_2imC_3C, gave the lowest one. In the case of using liquid zwitterion, OE_2imC_3C, the viscosity is considered as a leading to the low yield. The result of fermentation assay is presented in Figure 2-5.

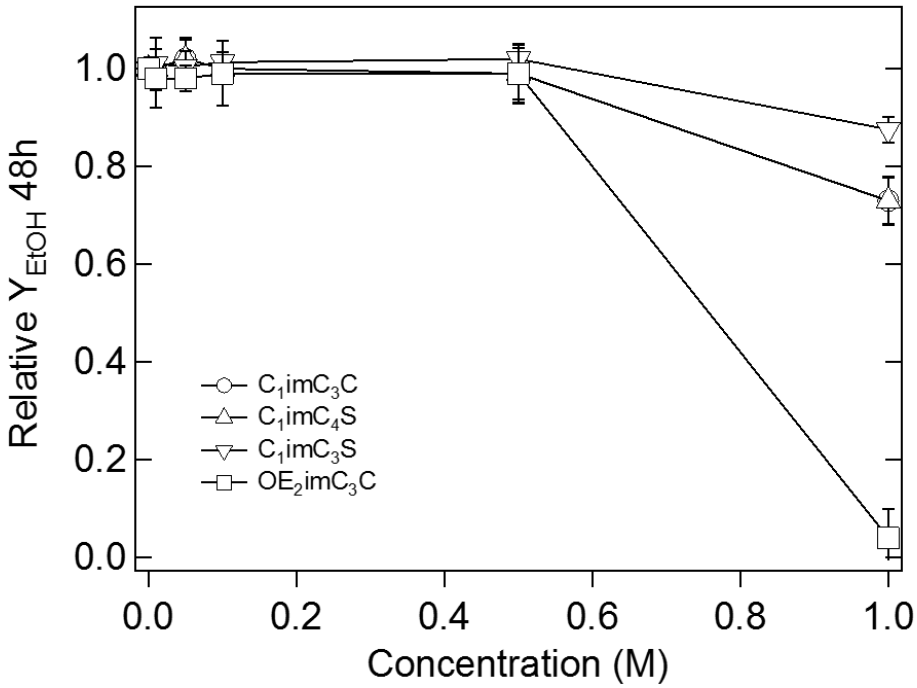


Figure 2- 5 The relative Y_{EtOH} 48 hours as a result fermentation of glucose (50 gL^{-1}) in presence of ILs in the medium.

To examine the production of ethanol in the presence of ILs and ZIs, the trend of glucose consumption during the fermentation assay is important to be evaluated. There can be seen that the low value of Y_{EtOH} 48 hours was caused by the poor of glucose consumption in the course of fermentation. The order of inhibition in ethanol production and glucose consumption for ILs is identic. Also in glucose consumption case, the cation-type did not express an impact to a Y_{EtOH} achievement, but the anion-type has strongly pointed out the ethanol production distinction. Even though this phenomenon did not occur during the application of ZIs in fermentation. Subsequently, we figured out the Kamlet-Taft β parameter that expresses a hydrogen basicity. This evaluation suggested that an increasing of inhibition effect of an IL to the ethanol production has given by a rise of β value. The information from Table 2-3 shows that imidazolium-type with higher β value inhibit fermentation vigorously so that the production of ethanol almost could not occur for β value over 0.8. The difficulty to consume glucose during fermentation also appears for the ILs that have high β value. Since the investigation of by-product results indicate almost no other metabolites detected, the glucose consumption declined with ascending IL β value. This condition may connect to denaturation protein associated with glucose

uptake or metabolism, that caused by disruption hydrogen bonds in protein. High *β* value IL acts as denaturant that destroys protein structure and function by formation hydrogen bonding with protein[4,8]. Consequently, glucose could not be consuming and the fermentation could not occur properly.

On the other hand, the presence of ZIs in the fermentation medium did not show inhibition effect. In contrast, high *β* value of OE_2imC_3C did not give inhibition effect both of in glucose consumption and ethanol fermentation. This condition indicates that high β value ZI is not denaturant, it is compatible for protein. The report from Ohno et al explained that hydrated ZIs from the specific structure related to hydrated biocompatible polymers, which is key to biocompatibility. Henceforth, hydrated ZIs did not give denaturation protein effect[9,10]. In order to none of ZIs indicated as an inhibitor of protein involved in glucose uptake, as a result fermentation of glucose into ethanol could be running appropriately.

Tabel 2- 3 The data of Kamlet-Taft *β* parameter (hydrogen basicity), glucose consumption and Y_{EtOH} 48 hours of ILs and ZIs

No	Ionic Liquid/Zwitterion	*β* value	Glucose Consumption (%)	Y_{EtOH} 48 hours (%)
Ionic liquid				
1	$[C_1imC_4SH]HSO_4$	-[a]	0.00	0.00
2	$[C_2mim][(MeO)(H)PO_2]$	1.00[b]	0.00	0.00
3	$[C_2min]OAc$	0.95[b]	7.41	3.88
4	$[C_2mim][MeSO_3]$	0.77	28.57	12.40
5	$[C_2mim][EtSO_4]$	0.71	95.15	76.41
6	[Cho]OAc	0.92	15.38	12.42
Zwitterion				
7	C_1imC_3C	-[a]	100.00	82.58
8	C_1imC_4S	-[a]	100.00	80.73
9	C_1imC_3S	-[a]	100.00	83.26
10	$OEimC_3C$	1.13	100.00	78.43

a: data did not measure, b: is referred to reference 11.

2-4. Summary

It is concluded that ILs were more toxic than ZIs. ILs have significant negative effect on the growth of *E.coli*, on the contrary, *E. coli* tolerance to ZIs. The cations strongly affected the growth of *E. coli*, whereas imidazolium-type cations have higher inhibition than cholinium-type one. Furthermore, it did not appear that anion-type has a similar impact to the *E. coli* growth. The omission of the hydrophobic part by attached anion part in one molecule to form carboxylate-type ZI, C_1imC_3C, adapted to IL, $[C_2min]OAc$, structure was effective to overcome toxicity effect. Meanwhile, the loosing of acid anion part in sulfate-type ZI (C_1imC_4S) as a derivative from IL ($[C_1imC_4SH]HSO_4$) also reduce the toxicity greatly.

Anion-type of ILs has a big impact to the fermentation of glucose into ethanol by *E.coli* KO11. It connected to the effect of ILs *β* value that the higher ILs *β* value decreased Y_{EtOH} 48 hours, either the glucose consumption. OE_2imC_3C that has a big *β* value and the other ZIs did not inhibit the fermentation.

2-5. Reference

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Chapter 3. Ethanol Production by Using Sulfate-type Zwitterion C_1imC_4S

3-1. Introduction

The conversion lignocellulosic biomass into bioenergy such as ethanol is one of purpose biorefinery. Lignocellulosic ethanol as a second generation in ethanol production technology still remain many opportunities to be developed. Generally, the main step to produce ethanol from lignocellulosic biomass is pretreatment followed by hydrolysis of cellulose and hemicellulose, sugar fermentation, separation of lignin residue, and finally purification and recovery of ethanol[1]. The cellulose is a major polymer in lignocellulose that gives a big barrier, with its crystallinity, to access the glucose, and it overcome with pretreatment. A wide number of pretreatment developments have been investigated and reviewed[2], and the choice of definite pretreatment has a large impact on all sequential process including digestibility of cellulose to serve a high fermentable sugar in ethanol production strategy.

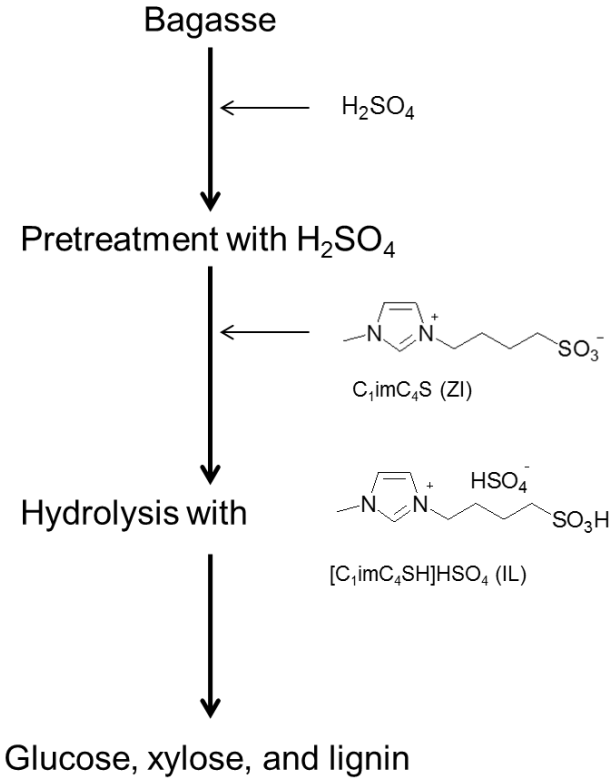
Acidic or enzymatic hydrolysis is a common process used to obtain glucose and xylose from cellulose and xylan respectively. In addition, application of dilute acid in hydrolysis has known as a modest and cost-effective method, although the major problem in this method is insufficient sugar yield[3]. There is strongly recommended to establish efficient catalysts, to upgrade this condition.

Since ILs reported dissolving cellulose[4], its application in cellulose treatment has developed rapidly. The capability of ILs to reduce crystallization of cellulose is one beneficial property to enhance the yield of sugar by enzymatic saccharification[5-7]. Meanwhile, acidic ILs, which have an acidic part in their structures, have been published as a catalyst in the chemical reaction[8-10]. Their capability for cellulose hydrolysis also has reported higher than sulfuric acid[3], even though the glucose yield achievement only 22%, after 3 hours treatment at 170 ºC. Hereinafter, the development hydrolysis cellulose has been done by a combination of acidic IL and microwave heating to upgrade yield of glucose and also shorten the reaction time[11]. With the assumption that synergy effect between catalytic activity of IL and

microwave heating, this experiment successfully achieve the increasing of glucose yield up to 40% for 12 min reaction at 160 ºC. Nevertheless, improvement to achieve the greater yield of glucose, more than 40%, important to realize as an efficient step to utilize biomass.

Although on average ILs have the ability to destruct crystalline structure of cellulose, our experiment using an acidic ionic liquid, $[C_1imC_4SH]HSO_4$, indicated this IL has no effectiveness to break down cellulose structure. The interesting one is when we are thinking about intrinsic sulfuric included in $[C_1imC_4SH]HSO_4$ for pretreatment. This IL is composed of sulfuric acid and a zwitterion, 1-(4-sulfobutyl)-3-methylimidazolium (C_1imC_4S), and be able to synthesize directly by mixing the two components.

In this study, the strategy of ethanol production from bagasse using ZI, C_1imC_4S, will be presented. The first strategy is an improvement of hydrolysis bagasse, whereas sulfuric acid (H_2SO_4) as an intrinsic part of IL, $[C_1imC_4SH]HSO_4$, structure used as a catalyst in pretreatment, then ZI, (C_1imC_4S) was added sequentially to *in situ* synthesized $[C_1imC_4SH]HSO_4$, and eventually hydrolysis of bagasse was done by $[C_1imC_4SH]HSO_4$ with microwave heating. The second strategy is reducing the toxic effect of $[C_1imC_4SH]HSO_4$ by electrodialysis. The hydrolyzate after hydrolysis contains IL, $[C_1imC_4SH]HSO_4$, glucose, xylose, and lignin. Insoluble lignin separated by filtration. The conversion fermentable sugar into ethanol with the presence of $[C_1imC_4SH]HSO_4$ by *E. coli* is already evaluated, and the IL has a negative effect on the fermentation (Chapter 1). It is considered to separation $[C_1imC_4SH]HSO_4$ into C_1imC_4S, ZI, and H_2SO_4, because ZI is known safe for fermentation. The separation has a purpose of removing innate H_2SO_4 in IL structure, so that glucose, xylose, and ZI remain in the hydrolysate. Finally, the hydrolyzate used as a medium for *E. coli* to produce ethanol. The strategy of pretreatment and hydrolysis improvements describes in Scheme 4.



Scheme 3- 1 The strategy of pretreatment and hydrolysis to increase glucose and xylose yield from bagasse

3-2. Material and Method

Materials and Instrumentations

Bagasse (approximately 3 mm in particle diameter) was purchased from Sanwa Ceruciron. The bagasse was ground by a mill and sieved to collect a powder, 250-500 μm in particle diameter. The bagasse (250-500 μm), Avicel PH-101 (Aldrich), Phosphoric acid-swollen cellulose (PASC) that prepared as a procedure in reference 13, were used as biomass. Microwave system StartSYNTH (Milestone S.r.l) was used for hydrolysis. Fermentation incubation was done in an incubator (Yamato Program Incubator IN600), and the incubator was equipped with multistirrer (AS-ONE slim stirrer). Analysis of glucose, xylose and ethanol were determined by using a high-performance liquid chromatography equipped with a refractive index detector (Shimadzu Co., Kyoto, Japan), a CARBOSep CHO-682 column (Tokyo Chemical Industry Co. Ltd., Japan) was used in tandem with a CARBOSep CHO-682 guard column (Tokyo Chemical Industry Co. Ltd., Japan). Electrodialysis was conducted using a Selemion electrodialyzer (DW-Lab, AGC Engineering Co. Ltd.) consist of a membrane stack, three compartments each dilute, concentrate and electrolyte compartment, and a DC power supply (PMC18-3A; Kikusui Electronic Co.).

Synthesis of C_1imC_4S

1-Methylimidazole (25 g) and 1,4-butane sultone (41.5 g) were mixed with acetone under a dry argon atmosphere at room temperature, and to complete the reaction, the mixture was refluxed for 4 days at 50°C. The insoluble zwitterion (the white sediment) was separated by filtration. The product was washed with acetone several times and dried under reduced pressure. The final product was obtained as a white powder.

^{1}H NMR δ_H (400 MHz; DMSO-d_6; Me_4Si); 1.48 (2H, quin, J= 6.8, $NCH_2CH_2CH_2CH_2SO_3$), 1.83 (2H, quin, J = 6.8, $NCH_2CH_2CH_2CH_2SO_3$) , 2.4 (2H , t , J = 6.8, $NCH_2CH_2CH_2CH_2SO_3$), 3.81 (3H, s, NCH_3), 4.13 (2H, t, J = 6.8, $NCH_2CH_2CH_2CH_2SO_3$), 7.66 (1H, t, J = 1.55 NCHCHN), 7.73 (1H, t, J = 1.55 NCHCHN), 9.09 (1H, s, NCHN). ^{13}C NMR δ_c (100 MHz; DMSO-d_6; Me_4Si); 22.27 ($NCH_2CH_2CH_2CH_2SO_3$), 29.13 ($NCH_2 CH_2CH_2CH_2SO_3$), 36.27 (NCH_3), 49.08 ($NCH_2CH_2CH_2CH_2SO_3$), 50.94 ($NCH_2CH_2CH_2CH_2SO_3$), 122.86 (NCHCHN), 124.13 (NCHCHN), 137.07(NCHN).

Elemental analysis: (Found: C, 43.9; H, 6.5; N, 12.8. Calc. for $C_8H_{14}N_2O_3S$: C, 44.0; H, 6.5; N, 12.8%).

Pretreatment of biomass with H_2SO_4, *in situ* Synthesis of $[C_1imC_4SH]HSO_4$, and Microwave-Assisted Hydrolysis

Avicel or bagasse was dipped in 72 wt% H_2SO_4 at room temperature for 60 min, with stirring in a 100 ml Teflon vessel. Water and an equimolar amount of C_1imC_4S were added to obtain the final concentration of $[C_1imC_4SH]HSO_4$ 1.0 M, and the final concentration of biomass 20 gL^{-1} in the 15 mL of solution. The pretreated biomass solution that consists of $[C_1imC_4SH]HSO_4$ and pretreated biomass was then heated in the microwave synthesizer. For collected sample, the vessel was pulled out from the microwave synthesizer and cooled down in an ice bath to stop the reaction. An aliquot of sample (500 µL) was centrifuged for 2 min at 15,000 rpm, and then the supernatant was filtered and was placed in HPLC vials for the analysis to determine glucose and xylose. The HPLC analysis follows the analysis condition: the volume of the injected sample was 20.0 µL, the column was operated at 85 ºC, and ultrapure water was used as the mobile phase with a flow rate was set up at 0.4 mL/min.

Hydrolysis by using H_2SO_4 was conducted as a control, without the addition of C_1imC_4S. Furthermore, for pretreatment Avicel using $[C_1imC_4SH]HSO_4$ that using as comparison hydrolysis, 72 wt% of $[C_1imC_4SH]HSO_4$ was used as an alternative to the 72 wt% H_2SO_4 solution.

The yield of glucose and xylose were calculated based on the concentration of glucose and xylose (primarily attributed to cellulose and xylan) involved in the original bagasse (lignocellulosic biomass). The amounts of glucose and xylose included in the original lignocellulosic biomass were determined by TAPPI method[13].

Electrodialysis

Electrodialysis was conducted using a Selemion electrodialyzer that contain a membrane stack, three compartments (dilute, concentrate, and electrolyte compartments), and a DC power supply. In the course of the electrodialysis, ions were transported from the dilute compartment to the concentrate compartment via cation and anion exchange membranes under a potential of 8 V. The membrane stack was arranged by five pairs of Selemion CMV cation exchange membranes and an AMV anion exchange membranes. The initial concentration of the $[C_1imC_4SH]HSO_4$ solution (250 g) in the dilute compartment was 0.05 M. The initial solution of the concentrate compartment was ultrapure water (250 g). All solutions were circulated at 4 L/min using pumps (RD-05 V24; Iwaki Co., Ltd.). The concentrations of H_2SO_4 or C_1imC_4S in dilute or concentrate compartments were analyzed by HPLC, follow the condition for the analysis of glucose yield described above.

Ethanol Production from Bagasse using C_1imC_4S

Hydrolyzate from the optimum condition of pretreatment and hydrolysis bagasse will be used for ethanol producing. The hydrolyzate was diluted up to 75 mL and was centrifuged for 10 min at 10,000 rpm, insoluble lignin was separated by filtration, the supernatant then was collected. The innate sulfuric acid that including in $[C_1imC_4SH]HSO_4$ was separated by electrodialysis, which the final solution in dilution compartment contains glucose, xylose, soluble lignin, and C_1imC_4S was collected, and filtered trough 0,45 µm millipore filter for medium fermentation. The fermentation was conducted anaerobically at 37 ºC. The concentration of glucose, xylose, and ethanol were determined using HPLC, follow the condition for the previous analysis condition.

3-3. Result and Discussion

The effectiveness of sulfuric acid on pretreatment as the first step of hydrolysis using *in situ* synthesis $[C_1imC_4SH]HSO_4$ evaluated by comparing unpretreated Avicel, pretreated Avicel with H_2SO_4, and pretreated Avicel with $[C_1imC_4SH]HSO_4$, that used as a substrate in hydrolysis. To compare the effect of crystallinity of cellulose PASC was used as the crystallinity index: 0.00. From the previous determination, it was known that crystallinity index of Avicel is 0.82. Figure 3-1 presents the result of pretreated cellulose when the substrate hydrolyze in a 1.0 M $[C_1imC_4SH]HSO_4$ solution under microwave heating at 100 ºC.

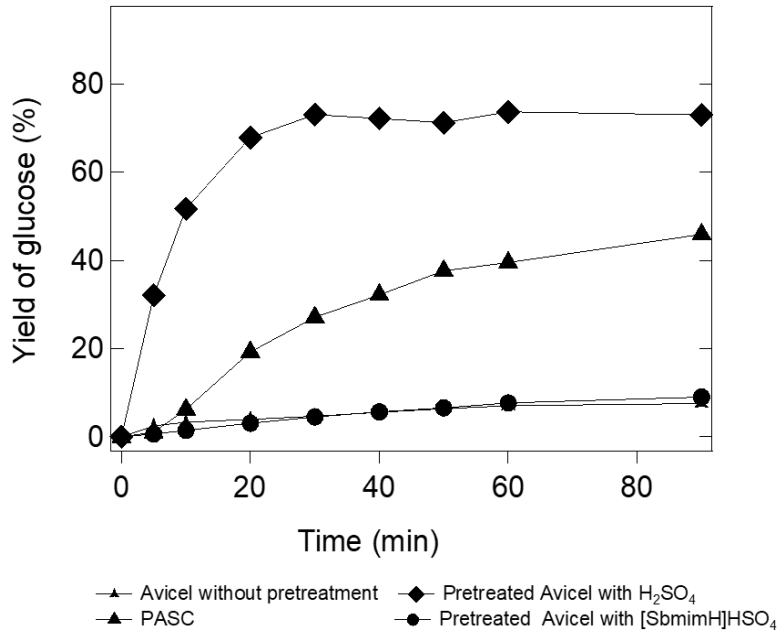


Figure 3- 1 Time course of the glucose yield during the hydrolysis of differently pretreated cellulose (Avicel and PASC) using 1.0 M of $[C_1imC_4SH]HSO_4$ solution under microwave heating at 100 ºC.

It was confirmed that successive pretreatment process followed by addition of ZI (C_1imC_4S) for *in situ* synthesis of $[C_1imC_4SH]HSO_4$ has a big impact to enhance the yield of glucose in hydrolysis. Since the pretreated Avicell was hydrolyzed with a high yield of 79.87% (for 90 minutes), the unpretreated one was only 8.05%. The pretreatment of Avicel with H_2SO_4 in the first step followed addition an equimolar of ZI, C_1imC_4S, and water in experiments, dissolved Avicel completely. It indicates that the crystallinity of Avicel already lost completely. In addition, the incapability of IL, $[C_1imC_4SH]HSO_4$, to break down the crystallinity of cellulose also appear clearly, which the yield of glucose was low at 10.24%. Turning into details, the efficacy of pretreatment using H_2SO_4 was proven by the higher yield of glucose from pretreated Avicell by using H_2SO_4 compare with PASC (48.98%). Given these points, the pretreatment using H_2SO_4, followed by hydrolysis using $[C_1imC_4SH]HSO_4$ is demanded to achieve the efficient hydrolysis.

In the next investigation, the comparison hydrolysis of bagasse using only H_2SO_4 and *in situ* synthesis $[C_1imC_4SH]HSO_4$ was performed, after pretreatment of bagasse was done previously using H_2SO_4. The result of this investigation presents in Figure 14. The increasing of glucose yield was significantly achieved when ZI was added after pretreatment to synthesis IL, $[C_1imC_4SH]HSO_4$, directly. Without this addition, the hydrolysis continued by H_2SO_4 that used in pretreatment, the glucose yield state under the ZI addition one (Figure 3-2 (a)). It indicates after the crystallinity of lignocellulose was decreased, acidic IL, $[C_1imC_4SH]HSO_4$, promoted the hydrolysis process. Xylose as a representative sugar from xylan, hemicellulose, also was investigated in the same experiment and expressed as xylose yield. The trend saw similar, whereas both of treatment could achieve the highest xylose yield, although the xylose yield from hydrolysis using in situ $[C_1imC_4SH]HSO_4$ less high than using H_2SO_4 (Figure 3-2 (b)). The destruction of hemicellulose was initiated at pretreatment process partially, because of H_2SO_4 easily access the hemicellulose in the surface of lignocellulose structure. Increasing of temperature under the influence of the catalyst was accelerated hydrolysis hemicellulose and there is no barrier such as crystallinity in hemicellulose structure[12], so that the hydrolysis more easy than cellulose. The xylose yield achieved more than 100% in this experiment, this is because of we compare the amount of xylose in xylan from bagasse by using NREL

method[13]. Since our method was using microwave heating to hydrolysis bagasse, the NREL one was using conventional heating and was generally reliable, but the method consists hydrolysis using H_2SO_4 at 121 ºC. On the other hand, the achievement of xylose yield that indicating in hydrolysis using $[C_1imC_4SH]HSO_4$ under microwave heating proved that the catalyst has a stronger effect to hydrolyze hemicellulose compare with H_2SO_4.

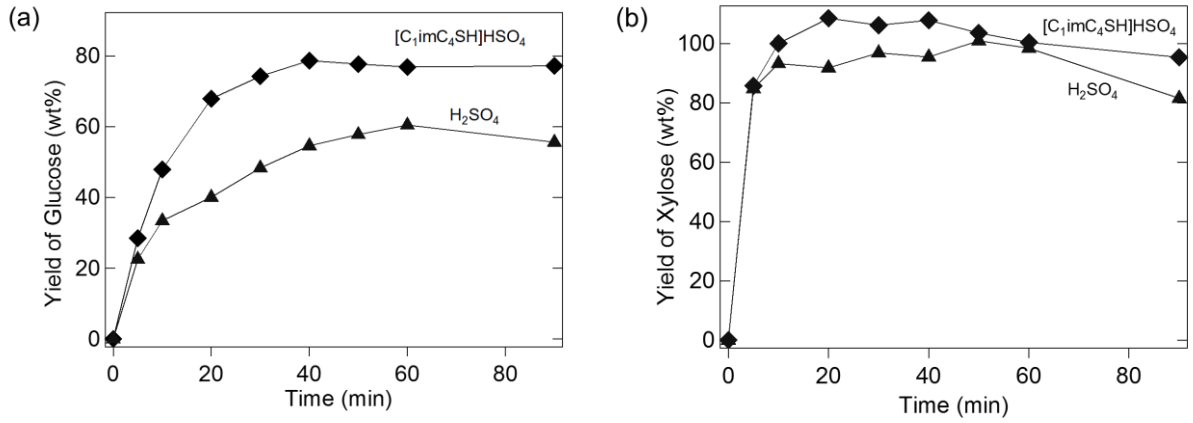


Figure 3- 2 Time courses of glucose yield (a) and xylose yield (b) during hydrolysis of bagasse in 1.0 M of $[C_1imC_4SH]HSO_4$ or H_2SO_4 solution at 100 ºC under microwave heating, after pretreatment using 72% of H_2SO_4 solution. $[C_1imC_4SH]HSO_4$ was synthesized in situ.

Furthermore, temperature effect investigation has conducted by hydrolysis with comparing the effect of pretreated bagasse with H_2SO_4 follow by in situ synthesis $[C_1imC_4SH]HSO_4$ and without pretreatment that hydrolysis was directly using $[C_1imC_4SH]HSO_4$. At high temperature, 160 ºC, hydrolysis pretreated bagasse achieved rapidly the peak, while the unpreteated bagasse has a peak after 15 minutes reaction. It also can be seen the degradation glucose occurred after glucose yield reaching the peak. Figure 3-3 shows the result of the investigation. From this point of view, hydrolysis at 160 ºC was not suggested, because of the high-temperature provide the harsh condition that allows decomposing the glucose. Generally, glucose generated by hydrolysis are immediately decomposed into a specific product such as 5-(hydroxylmetyl)furfural (HMF)[14]. The mild condition is required to prevent the decomposition glucose.

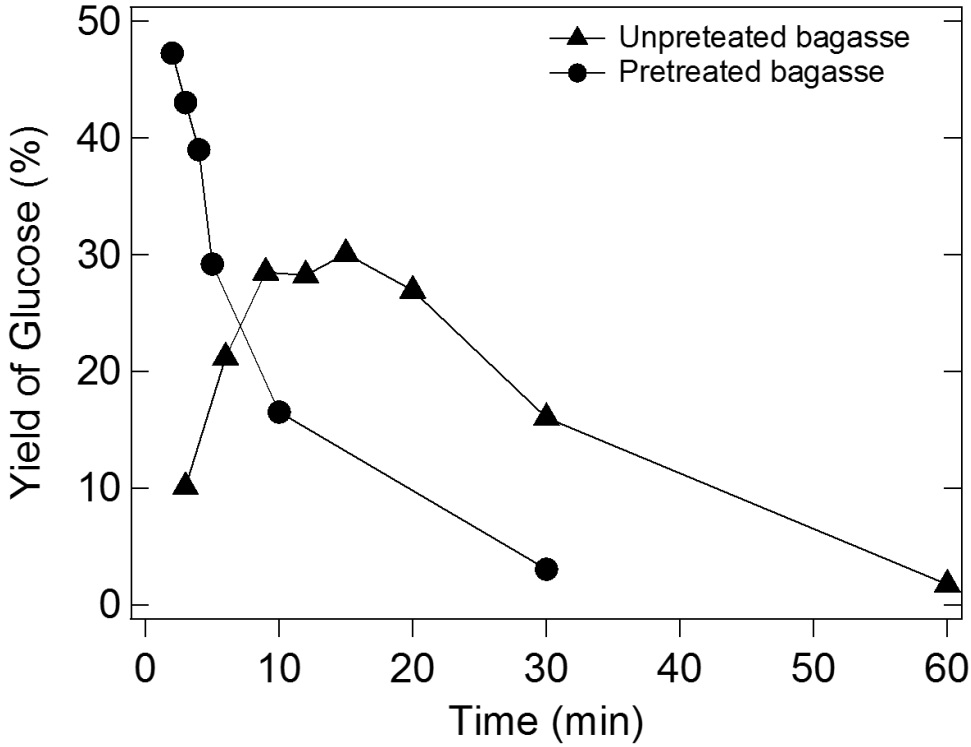


Figure 3- 3 Time courses of glucose yield during hydrolysis of bagasse in 1.0 M $[C_1imC_4SH]HSO_4$ solution at 160 ºC under microwave heating, pretreated bagasse was using 72% H_2SO_4, followed addition of C_1imC_4S for in situ synthesize $[C_1imC_4S]HSO_4$

Thereafter, optimum hydrolysis temperature was observed with a range of temperature 90-120 ºC. The result of observation was placed at Figure 3-4. Among of the examined temperature, the optimum condition was described by hydrolysis at 100 ºC. In this condition, the yield of glucose reached the peak at 40 minutes at gave glucose yield 77.44% and the glucose yield remains stable for up to 90 minutes. At the temperature below it, 90 ºC, the increase of glucose yield was slowly and the hydrolysis proceeded to achieve glucose yield of 58.30% at 90 minutes. Moreover, at temperature above it, 110 and 120 ºC, quite similar yields were generated (72.52% and 76.88%) at 30 and 10 minutes respectively, however a decrease in glucose yield appeared, caused by the relatively strong conditions. Figure 3-4 (a) describes the trend of hydrolysis as the mention above.

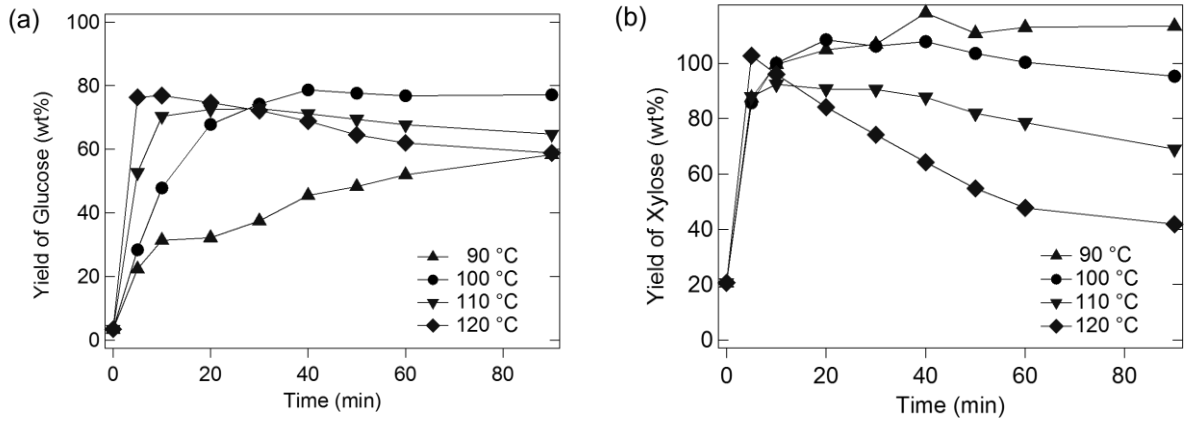


Figure 3- 4 Time courses of glucose yield (a) and xylose yield (b) during hydrolysis of bagasse pretreated with 72% H_2SO_4 in 1.0 M $[C_1imC_4SH]HSO_4$ solution under microwave heating at 90,100, 110, and 120 ºC

The xylose yield describes at Figure 3-4 (b), with the initial yield of xylose noted at 20.64%. This result indicates the xylan was partially hydrolyzed during pretreatment of bagasse. At all temperature, a high hydrolysis rate appeared, and nearly complete within 10 minutes at above 90 ºC. In addition at 90 and 100 ºC, xylose yield achieved 118.21% and 107.83% respectively, and significant decomposition of xylose was not observed. However, the decomposition of xylose caused by high temperature was confirmed at 110 and 120 ºC. Decomposition of xylose into furfural reached only 6.12% at 100 ºC since no HMF from the decomposition of glucose detected in this hydrolysis condition (Figure 3-5)

From the resulting trends of temperature optimization, it is noted that both high of yield and insignificant decomposition of main hydrolysis product, were simultaneously reached at 90 and 100 ºC. These conditions were suggested to perform hydrolysis of bagasse after pretreatment using H_2SO_4 and in situ synthesize $[C_1imC_4SH]HSO_4$ sequentially.

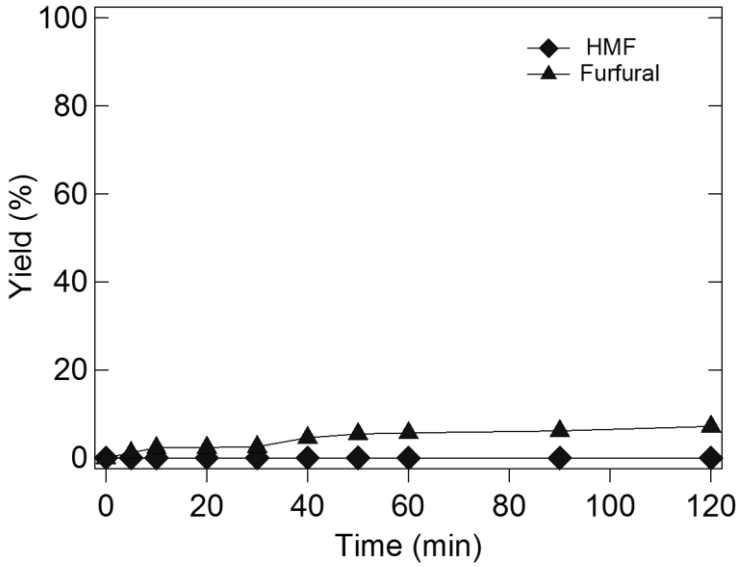


Figure 3- 5 Time courses of yield of HMF and furfural during hydrolysis of the bagasse, pretreated using H_2SO_4 in 1.0M $[C_1imC_4SH]HSO_4$ solution under microwave heating at 100 ºC.

Electrodialysis was used to separate $[C_1imC_4SH]HSO_4$ into its component, C_1imC_4S and H_2SO_4. Using the reverse analogy of in situ synthesize $[C_1imC_4SH]HSO_4$, the reversible reaction that considers hydrolyzing the IL into the ZI and H_2SO_4 would be repeatable, as shown in Figure 3-6.

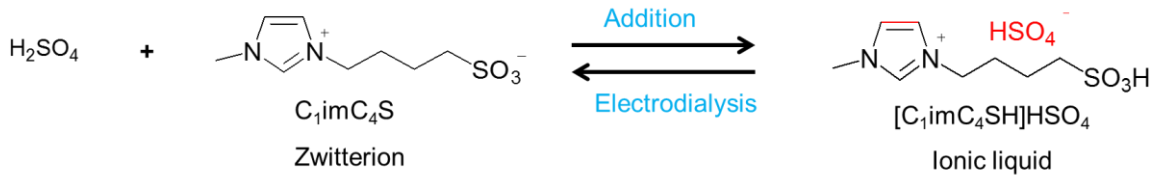


Figure 3- 6 Reaction of in situ synthesize $[(C_1imC_4SH]HSO_4$ from H_2SO_4 and C_1imC_4S and reversible separation of $[C_1imC_4SH]HSO_4$ into the components by electrodialysis

Electrodialysis is a technique for ion transport using ion-exchange membranes under an enforced potential gradient. It has been applied to separate neutral compounds from organic and inorganic salt solutions and reached recovery of ILs from mixtures of ILs and neutral compounds[15-17]. Acidic IL, $[C_1imC_4SH]HSO_4$, is comprised of the zwitterion and the acid moieties. While the acid is supposed to be transported as ions, it is assumed that the zwitterion would not move under an applied potential field because the net charge of zwitterion is neutral[18]. Since the separation of mixtures of

the acid and zwitterion has not been developed previously, in this experiment separation of $[C_1imC_4SH]HSO_4$ into C_1imC_4SH and H_2SO_4 using electrodialysis was applied. Figure 3-7 (a) presents the time courses for the concentration of H_2SO_4 in the dilute and concentrate compartments during electrodialysis of $[C_1imC_4SH]HSO_4$. The concentration of H_2SO_4 in the dilute compartment decreased with the passed time, and the desalination ratio was 99% at 60 min. On the other side, the concentration of H_2SO_4 in the concentrate compartment increased with time, and the recovery ratio was 97% at 60 min. Thereby, the recovery of the H_2SO_4 was almost complete. However, it is observed that there was a slight difference between the desalination ratio and the recovery ratio, and it was caused by remaining of the negatively charged species on the electrodialysis membrane[19].

Figure 3-7 (b) presents the time course for the concentration of C_1imC_4S in the dilute and concentrate compartments during electrodialysis of $[C_1imC_4SH]HSO_4$. On the contrary to the H_2SO_4 behavior, the concentration of C_1imC_4S remained constant in either compartment, whereas 99% of C_1imC_4S remained in the dilute compartment after 60 min. These results clearly indicate that most of the H_2SO_4 recovered in the concentrate compartment, and most of the C_1imC_4S remained in the dilute compartment. Thus, the application of electrodialysis to separate $[C_1imC_4SH]HSO_4$ into C_1imC_4S and H_2SO_4 components was conducted successfully.

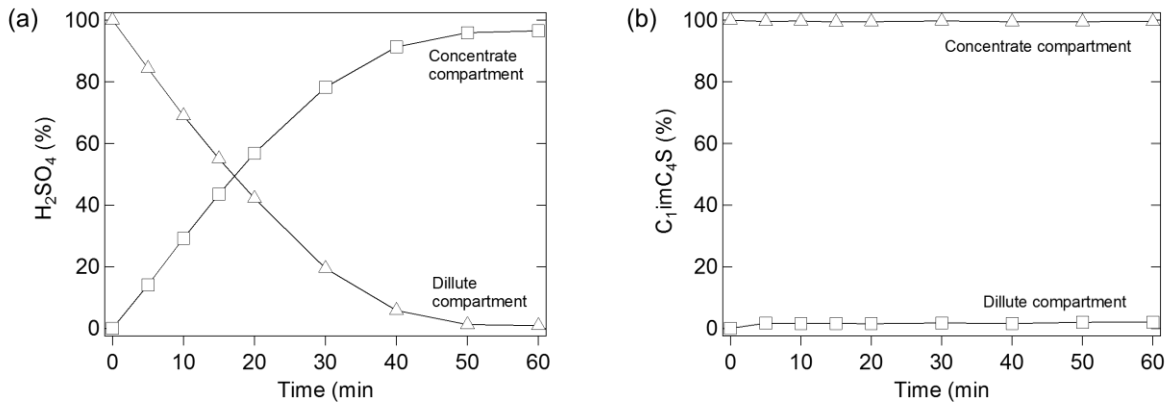


Figure 3- 7 Time courses of concentration of H_2SO_4 (a) and C_1imC_4S (b) in dilute and concentrate compartment during electrodialysis of $[C_1imC_4SH]HSO_4$

All part of whole procedures to convert bagasse into ethanol including the separation of IL, $[C_1imC_4SH]HSO_4$, into ZI, C_1imC_4S, and H_2SO_4 were already confirmed. To obtain the concrete description result of the process, successively conversion of bagasse into ethanol using C_1imC_4S was done. The mass balance as an indicator of successful process then was evaluated. Figure 3-8 presents the result of the mass balance calculation. Application of the whole system was able to convert 305 ± 1.8 mg of bagasse into 78 ± 14.2 mg of ethanol. The ethanol yield calculated from theoretical ethanol that could be produced from content of available fermentable sugar of glucan and xylan in bagasse, in this conversion, it was 52%. Meanwhile, the electrodialysis was successfully separation the H_2SO_4 including in $[C_1imC_4SH]HSO_4$, as a result improving pH of hydrolysate change from 1.0 to 6.0. It also showed that the glucose and xylose were kept in hydrolysate during the electrodialysis. This pH value allowed *E.coli* to growth up and automatically enable the fermentation of available fermentation sugar in this process.

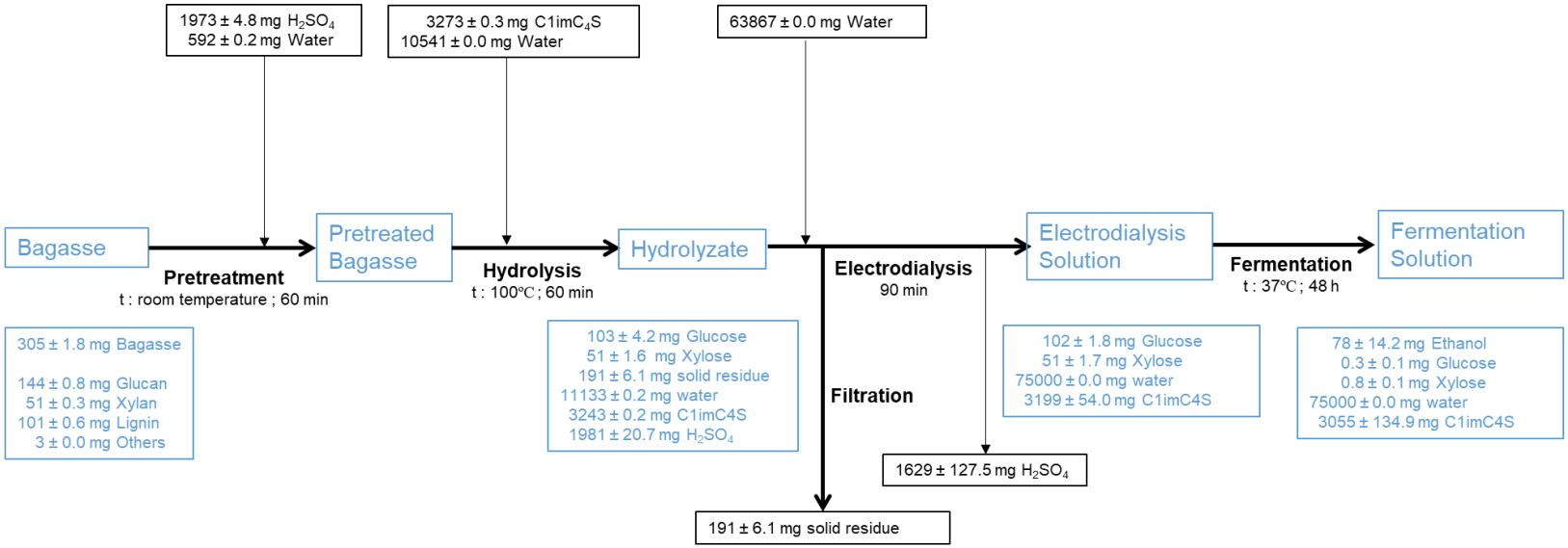


Figure 3- 8 Mass balance calculation of conversion bagasse into ethanol using C_1imC_4S

3-4. Summary

The effectiveness of sulfuric acid on pretreatment as the first step of hydrolysis using *in situ* synthesis $[C_1imC_4SH]HSO_4$ was successfully conducted. The successive process overcame the crystallinity problem and automatically has a big impact on enhancing sugar yield on biomass hydrolysis. It showed that the glucose yield from pretreated cellulose was increasing dramatically to 79.87%, since the unpretreated cellulose achieved only 8.05% and the other pretreated cellulose (PASC) was only 48.98%. The process also effective to apply on bagasse hydrolysis, whereas it showed the glucose yield was outperforming from acid hydrolysis using concentrated H_2SO_4.

The optimum temperature of hydrolysis was at 100 ºC. In this condition, the yield of glucose reached the peak at 77.44% for 40 minutes and the glucose yield remains stable for up to 90 minutes. At 90 ºC, the increase of glucose yield was slowly and the hydrolysis proceeded to achieve glucose yield of 58.30% at 90 minutes. Moreover, at the higher temperature, 110 and 120 ºC, quite similar yields were generated (72.52% and 76.88%) at 30 and 10 minutes respectively, however a decrease in glucose yield appeared in this condition. It was also noted that pretreatment bagasse followed by hydrolysis by *in situ* synthesis $[C_1imC_4SH]HSO_4$ sequentially, was able to hydrolysis all of the hemicellulose in bagasse.

Electrodialysis was used to separate $[C_1imC_4SH]HSO_4$ into its component, C_1imC_4S and H_2SO_4, the application of electrodialysis was conducted successfully. The recovery of the H_2SO_4 almost complete after 60 minutes, the concentration of H_2SO_4 changed in the both of compartment, which in dilute compartment H_2SO_4 decreased with the desalination ratio was 99%, and in the concentrate compartment increased with the recovery ratio was 97%. On the other hand, the concentration of C_1imC_4S remained constant, whereas 99% of C_1imC_4S kept in the dilute compartment after 60 minutes.

The successive conversion of bagasse into ethanol using H_2SO_4 pretreatment and in situ synthesis $[C_1imC_4SH]HSO_4$ by adding C_1imC_4S for hydrolysis by microwave heating was able to convert 305 ± 1.8 mg of bagasse into 78 ± 14.2 mg of ethanol and the ethanol yield at 70 ± 7.0%.

3-5. Reference

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Chapter 4. One-pot Ethanol Production by using Zwitterion OE_2imC_3C

4-1. Introduction

Application of room temperature ionic liquids (RTILs) in the pretreatment of lignocellulosic biomass has been developed rapidly. More recently, RTILs have been applied as solvents for lignocellulosic biomass processing with the purpose of developing preferences for lignocellulosic pretreatment[1-6]. However, most of ILs deactivated enzyme activity[7] and are also toxic for microorganism even by low concentration[8-9]. The ILs insert in the membrane by the mechanism was mentioned by Lim et al (2014)[10] that the cation part firstly approaches to anionic phospholipid of the cell membrane by electrostatic interaction, followed by hydrophobic part (alkyl chain) of the cation insert into the hydrophobic part of the membrane. The accumulation of ILs has triggered deconstruction of membrane cells, and consequently, the microorganism could not grow up. Since ILs have a toxic effect, microorganism resistance to ZIs. Investigation in Chapter-2 has proven that growing up of *E. coli* in the presence of ZIs tend to higher than ILs.

The ILs that have high polarity and high hydrogen basicity more than 0.8 commonly able to dissolve cellulose[11]. Carboxylic acid anions on imidazolium-type cation reported have high polarity and hydrogen basicity and have the ability to dissolve cellulose under mild condition[12]. It has noted that ILs composed of carboxylate anions are excellent candidates to be solvents for cellulose[13]. The strategy to keep the polarity of the molecule and reduce the hydrophobic is introducing the polar anion part onto the end of cation alkyl chain to convert IL structure into ZI. However, most of ZIs are reported solid below 100 ^oC[14], and only one report mention the utilization of ZI as solvent[15]. It also reported that attached oligoether (OE) chain onto 1-imidazol-3-ium-3yl)propane 1-sulfonate when they synthesized 3-(1-(2-(2-methoxyethoxy)ethyl)-1H-imidazol-3-ium-3yl)propane 1-sulfonate (OE_2imps) was decreasing its melting point[15]. Adapted from this working, 1-(3-carboxypropyl)-3-(methoxyethoxyethyl)imidazolium (OE_2imC_3C) was synthesized. This ZI is liquid at room temperature, whereas the melting point was not detected above -100 ºC and has the glass transition temperature at -62 ºC. The structure of OE_2imC_3C presents

at Figure 4-1 (b). The OE chain in the structure contribute to the liquid state of ZI, the other ZI, that has an analog structure in which the OE chain substituted by alkyl chain, C_1imC_3C (Figure 4-1 (a)), has melting point over 150 ºC.

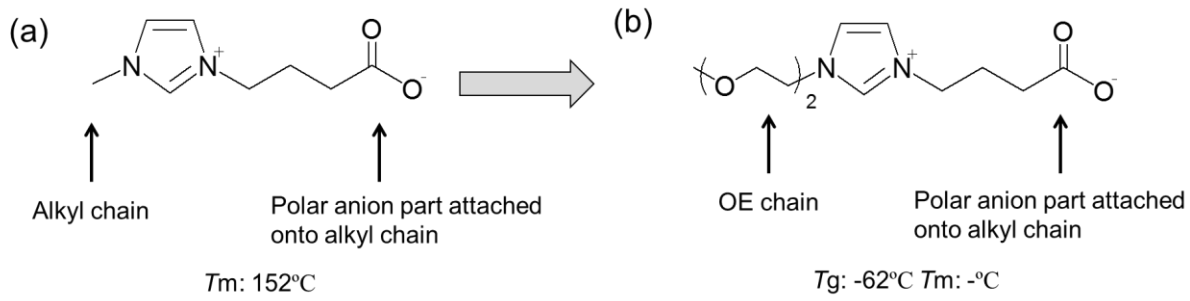

Figure 4- 1 Molecular structure of (a) C_1imC_3C and (b) OE_2imC_3C, substitute alkyl chain to OE chain change the state of ZI from solid into liquid

In this study, OE_2imC_3C used as an agent to convert bagasse into ethanol. At the first step, the capability of OE_2imC_3C to dissolve cellulose was evaluated to fill in the pretreatment purpose. The first step followed by enzymatic saccharification by cellulase to obtain fermentable sugar (glucose) from bagasse, and finally conversion of the fermentable sugar conducted by fermentation using *E. coli* KO11. All of the conversion arranged in a one-pot process, which means no material was discarded throughout the process.

4-2. Material and Method

Materials and Instrumentations

Oil base hot plate stirrer (AS-ONE Dry Stirring Bath HDBS-6) was used for control temperature in cellulose dissolution and pretreatment. Fermentation incubation was done in an incubator (Yamato Program Incubator IN600), and the incubator was equipped with multistirrer (AS-ONE slim stirrer). Analysis of glucose, xylose were determined by using a high-performance liquid chromatography equipped with a refractive index detector (Shimadzu Co., Kyoto, Japan), a CARBOSep CHO-682 column (Tokyo Chemical Industry Co. Ltd., Japan) was used in tandem with a CARBOSep CHO-682 guard column (Tokyo Chemical Industry Co. Ltd., Japan).

Tetrahydrofuran (THF), acetonitrile, and diethylether were purchased from Kanto Chemical, sodium hydride (NaH), 1-bromo-2-(2-methoxyethoxy)ethane, and ethyl 4-bromobutyrate were purchased from Tokyo Chemical Industry Co. Ltd.. Bagasse (approximately 3 mm in particle diameter) was purchased from Sanwa Ceruciron. The bagasse was ground by a mill and sieved to collect a powder, 250-500 μm in particle diameter. The bagasse (250-500 μm), Avicel PH-101 (Aldrich) was used as biomass. Cellulase Celic® CTec2 enzyme was purchased from Novozymes, Franklinton, NC, USA. Glucose C II kit test was purchased from Wako Pure Chemical Industries, Japan.

Synthesis of OE_2imC_3C

Under an argon atmosphere 15.7 g, 656 mmol as NaH in paraffin liquid was suspended in 50 mL THF and then 13.75 g, 202 mmol of imidazole, which was dissolved in 50 mL THF, was drop carefully added to the solution. The mixture was stirred at room temperature for 24 hours. Hereinafter, 37 g, 202 mmol of 1-bromo-2-(2-methoxyethoxy)ethane was added to the solution. After stirring at 70°C for 6 hours, the generated suspension was filtered under reduced pressure to eliminate white precipitation. The solvent was removed by evaporation under reduced pressure. The product was purified by distillation and a fraction was collected at 125°C under reduced pressure of 1 mmHg to obtain an imidazole derivative, 1-(2-(2-methoxyethyl)ethyl)-1*H*-imidazole (OE_2im). The obtained product, 43.7 g, 256 mmol of OE_2im then was washed with hexane several times to remove paraffin. After

evaporation of water, OE_2im was dissolved in 250 mL acetonitrile, and then 49.9 g, 256 mmol of ethyl 4-bromobutyrate was dropwise added to the solution under argon atmosphere. To complete the reaction, the mixture was refluxed at 70°C for 16 hours. After removing solvent under reduced pressure, the residue was washed three times with diethyl ether by decantation. Thereafter, anion exchange was carried out for 3 days using an anion exchange resin (Amberlite® IRN 78 hydroxide) in methanol and water solvent. The solvent was then removed by evaporation, and the obtained product was dried *in vacuo* at 80 °C for 5 hours to serve OE_2imC_3C as a viscous liquid.

^1H NMR (400 MHz; $CDCl_3$; Me_4Si) δ = 2.13-2.27 (4H, m, CH_2CO and CH_2CH_2CO), 3.37 (3H, s, CH_3O), 3.51-3.65 (4H, m, $CH_3OCH_2CH_2$), 3.86 (2H, t, J = 3.6 Hz, OCH_2CH_2N), 4.40 (2H, t, J = 6.7 Hz, NCH_2CH_2CH_2COO), 4.66 (2H, t, J = 3.7 Hz, OCH_2CH_2N), 7.29 and 7.49 (2H, t, J = both 1.6 Hz, NCHCHN), 11.00 (1H, s, NCHN).
^{13}C NMR (100 MHz; $CDCl_3$; Me_4Si) δ = 27.20 and 34.30 ($NCH_2CH_2CH_2COO$), 48.94 (OCH_2CH_2N), 49.47 ($NCH_2CH_2CH_2COO$), 58.65 (CH_3O), 69.19 (OCH_2CH_2N), 69.93 and 71.29 (OCH_2CH_2O), 121.22 and 122.58 (NCHCHN), 138.73 (NCHN), 176.63 (CH_2COO).
Elemental analysis: $OE_2imC_3C \cdot 2.5H_2O$ (Found: C, 48.0; H, 8.4; N, 9.3. Calc. for C12H25N2O6.5: C, 47.8; H, 8.4; N, 9.3%).

Dissolution of cellulose in OE_2imC_3C

Cellulose (1 wt%) was added into OE_2imC_3C and stirred at 100 ºC on oil base hot plate stirrer for 10 minutes. When it was confirmed that the cellulose soluble in OE_2imC_3C, the procedure was repeated until the maximum solubility of cellulose was achieved. The procedure of dissolution of cellulose mentions in Figure 4-2.

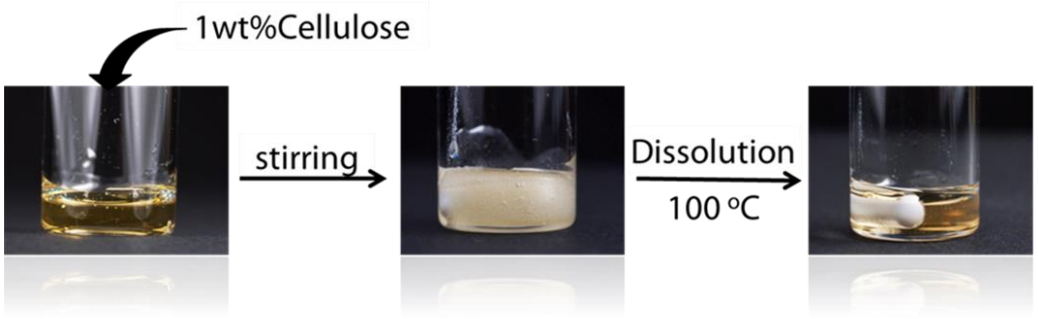

Figure 4- 2 Procedure of dissolution of cellulose in OE_2imC_3C

Optimation saccharification of cellulose using cellulase in OE_2imC_3C solution

Cellulase activity was evaluated by saccharification of avicel using Cellic® Ctec2 in the presence of OE_2imC_3C. The various concentration of OE_2imC_3C solvent (0.01-1.00 M) was prepared in acetate buffer. 12.8 mg of avicel was dissolved into 1 mL OE_2imC_3C solvent, and was stirred vigorously for a minute to performed a homogenous mixture. 0.64 µL of cellulase (100 $FPUmL^{-1}$) then was added into the mixture and was incubated at 50 ºC for 24 hours under stirring gently. After hydrolysis performed, 20 µL of the mixture was sampled and 3.0 mL of glucose CII Wako solution was added. This mixture then was incubated at 37 ºC for 15 minutes. The concentration of glucose then measured with UV-vis spectrophotometer and absorbance at 550 nm (main wavelength) and 600 nm (secondary wavelength) were recorded. A calibration curved was prepared for the range of glucose concentration from 50 $mgdL^{-1}$ to 500 $mgdL^{-1}$, and was used to calculate the concentration of glucose. The yield of glucose from saccharification was calculated with the following equation:

$$Yield(\%) = \frac{\frac{Mcell}{Mglu}\ x\ Cglu\ x\ V}{M\ x\ Rcell} \times 100$$

M_{cell} / M_{glu} : ratio of molecular weight of cellulose monomer to glucose
Cglu : concentration of glucose from saccharification (gL^{-1})
V : volume reaction (L)
M : weight of biomass
Rcell : percentage of cellulose in biomass

Optimation of pH reaction was conducted following the procedure that mentioned above to evaluated the optimum pH reaction of acetate buffer (50 and 200 mM), and $[C_2mim]OAc$ was used as a comparison.

Pretreatment of Bagasse using OE_2imC_3C and Enzymatic Saccharification

Pretreatment of bagasse by using OE_2imC_3C was conducted by mixing 0.128 g of bagasse that corresponding to 10 wt% in 1.28 g of OE_2imC_3C. The mixture then was stirred on oil base hot plate stirrer at 120 ºC for 8 hours. After pretreatment was complete, the volume of the mixer was adjusted to form 0.5 M of OE_2imC_3C by adding 200 mM acetate buffer. The mixture then was stirred vigorously for a minute, and 50 µL cellulase was added to conduct enzymatic saccharification at 50 ºC for 48 hours. The glucose after hydrolysis then was determined using glucose CII Wako solution as the mention above.

One-pot Ethanol Production from Bagasse using OE_2imC_3C

The procedure of pretreatment and enzymatic saccharification of bagasse using OE_2imC_3C was carried out to produce fermentable sugar. Successively after that process, fermentation was conducted by adding yeast (5.0 gL^{-1}), NaCl (5.0 gL^{-1}), and tryptone (10.0 gL^{-1}) into hydrolysate. The hydrolysate also was enriched with 100 mgL^{-1} chloramphenicol. The enriched hydrolyzate then was inoculated by *E. coli* KO11 as an initial OD_{600} at 1.0. The fermentation was done anaerobically at 37 ºC for 0-72 hours. The sample was collected periodically to determine glucose, xylose and ethanol by HPLC.

4-3. Result and Discussion

First of all, it was confirmed that cellulose was able to dissolve in OE_2imC_3C at 100 ºC. The solubility of cellulose at this condition so far achieved 6 wt%. The mixture of cellulose and OE_2imC_3C was too viscous so that after 6 wt% of cellulose soluble in OE_2imC_3C it could not be stirred anymore. Consequently, the solubility of cellulose in OE_2imC_3C above 6 wt% could not be evaluated.

Optimum activity of cellulase in the presence of OE_2imC_3C was investigated by using the buffer solvents was with a comparison of water, acetic buffer 50 and 200 mM. The Figure 4-3 describes the investigation result, it indicated that the highest activity of cellulase appeared in 200mM acetate buffer. The activity of cellulase was dropped in water, and in 50 mM acetate buffer was not higher than the optimum one.

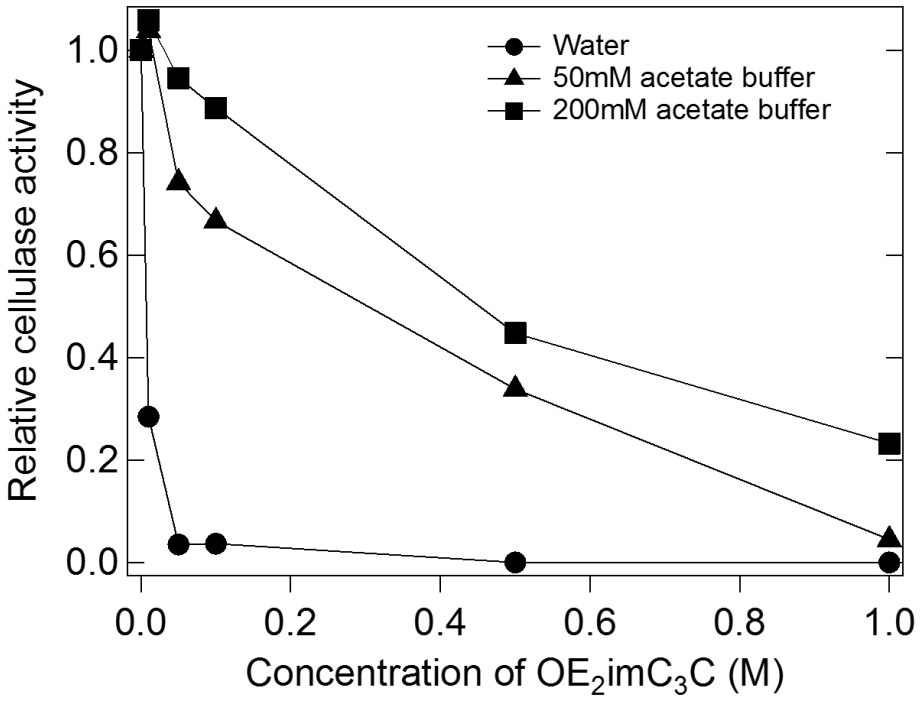

Figure 4- 3 Cellulase activity in the presence of OE_2imC_3C in water or acetate buffer

In addition, when the pH of each concentration of OE_2imC_3C determined, with the increasing of OE_2imC_3C concentration, the pH tended to rise up gradually. On the other hand, the enhancement of pH by influencing of increasing of OE_2imC_3C concentration made the activity of cellulase decreased. Consequently, the yield of glucose became lower at high concentration of OE_2imC_3C. The results indicated that pH around 5 gave the higher yield of glucose compare the other examined pH, and it a possibility to apply 200 mM acetate buffer pH 5 to maintenance the high activity of cellulase in the presence a high concentration of OE_2imC_3C. Figure 4-4 and 4-5 describe the difference of pH depending on the concentration of OE_2imC_3C and influence of pH on the glucose yield respectively.

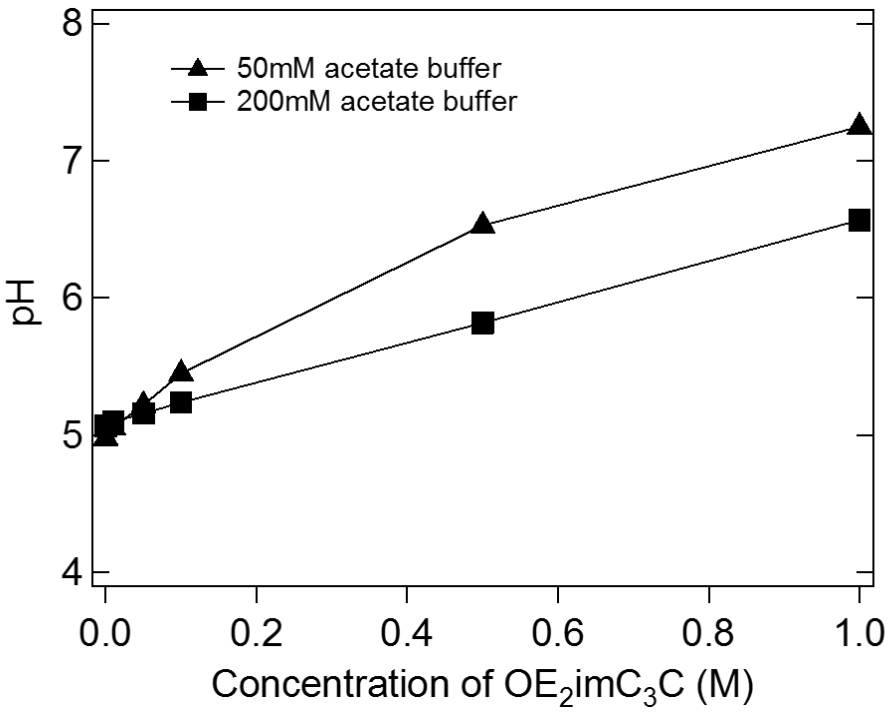

Figure 4- 4 pH determination result of the OE_2imC_3C solution in 50 and 200 mM acetate buffer

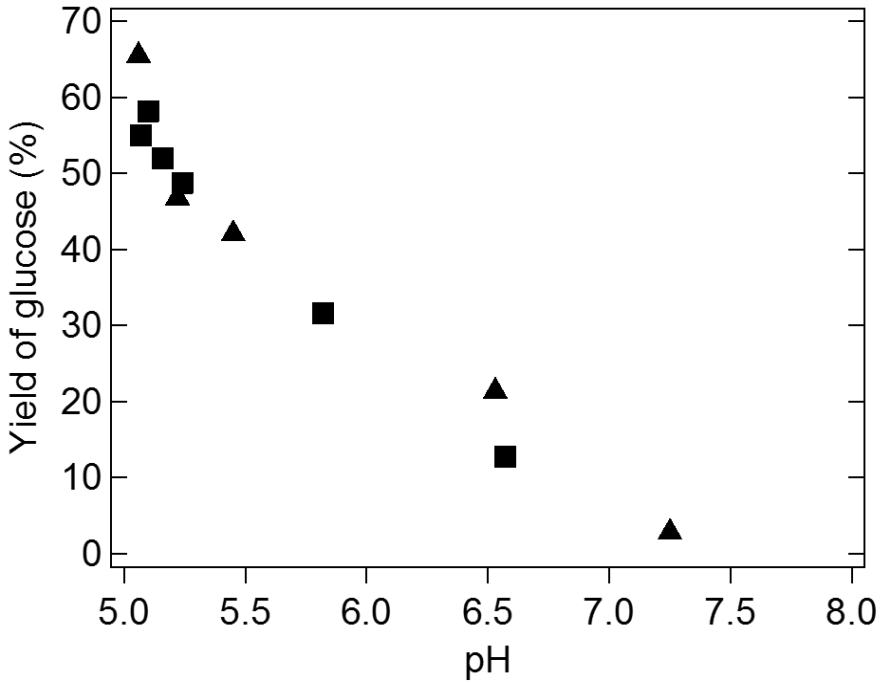

Figure 4- 5 pH dependency of glucose yield on cellulase activity determination.

The comparison activity of cellulose between in the presence of ZI, OE_2imC_3C, and IL, $[C_2mim]OAc$, in 200 mM acetate buffer then investigated. The purpose of comparison is to investigate the influence of carboxylic part on anion side in ZI and carboxylic acid anion in IL to the activity of cellulase. Figure 4-6 shows the trend of cellulase activity in presence of both of agents. From the result, it appeared that the activity of cellulase was not significantly different in presence of both ZI and IL carboxylate-type, whereas in 0.5 M the activity was kept in the middle high. This concentration considered to apply in the pretreatment of biomass followed by enzymatic saccharification in the part of one-port ethanol fermentation process.

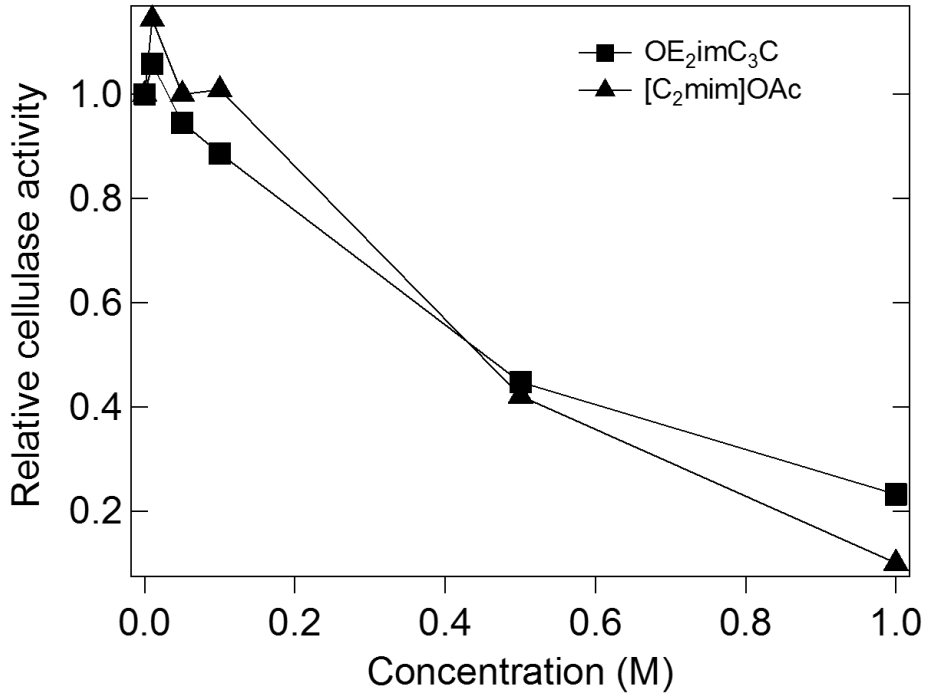

Figure 4- 6 Cellulase activity in the presence of OE_2imC_3C and $[C_2mim]OAc$ in 200 mM acetate buffer pH 5.0.

Furthermore, pretreatment bagasse was investigated by using 0.5 M of OE_2imC_3C and $[C_2mim]OAc$ respectively at 120 ºC for 8 hours, followed by enzymatic saccharification using cellulase in 200 mM acetate buffer pH 5.0 at 37 ºC for 48 hours successively. The result of those process was presented in Figure 4-7. It was confirmed clearly that both of pretreated bagasse using OE_2imC_3C and $[C_2mim]OAc$ gave increasing on the yield of glucose compared with unpretreated bagasse. After 48 hours hydrolysis by enzymatic saccharification, the pretreated bagasse using $[C_2mim]OAc$ achieve the highest yield of glucose at 64.15% since the pretreated bagasse using OE_2imC_3C reached to 49.61%. This hydrolyzate then was used as a medium for ethanol fermentation after enriched with nutrient and chloramphenicol as described in the method.

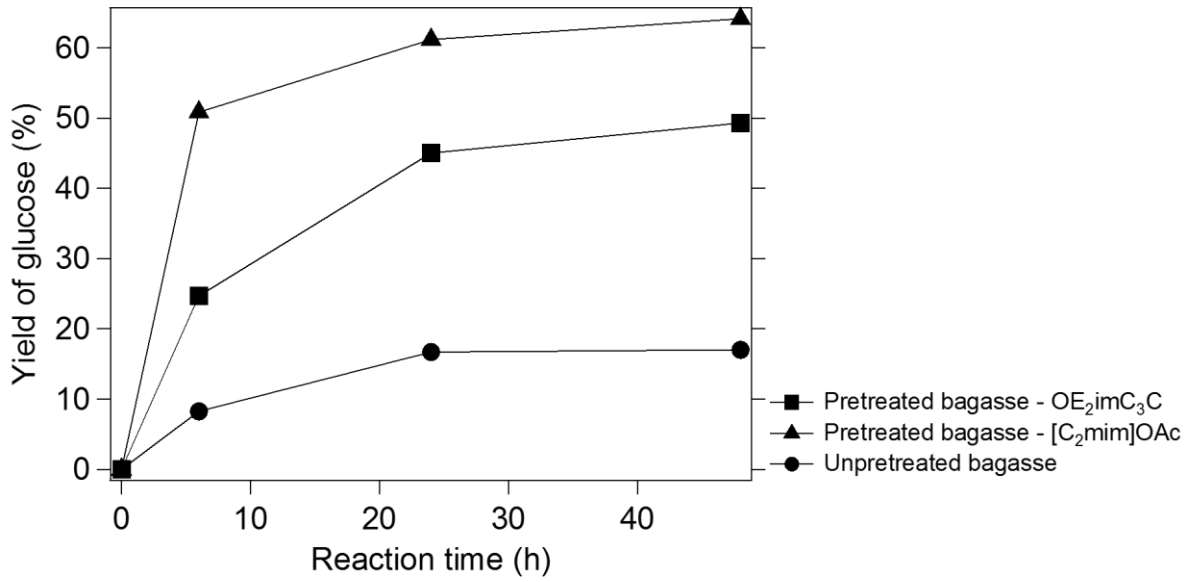

Figure 4- 7 Enzymatic saccharification of unpretreated bagasse and pretreated bagasse using OE_2imC_3C and $[C_2mim]OAc$

In one-pot ethanol fermentation using the saccharified bagasse from the previous process, the presence of IL can be seen clearly to have inhibited the production of ethanol. Since the ethanol yield in the using of OE_2imC_3C achieved 28% and in the unpretreaded bagasse hydrolyzate only reached 0.3%, in hydrolyzate containing $[C_2mim]OAc$, ethanol could not produce at all. It indicates that $[C_2mim]OAc$ has a high toxicity to the *E. coli* KO11, while the OE_2imC_3C has a lower one. It has been mentioned that hydrated ZIs from the specific structure related to hydrated biocompatible polymers, which is key to biocompatibility[16,17]. The time courses of glucose yield and concentration of produced ethanol in one-pot fermentation process present in Figure 4-8 and 4-9 respectively.

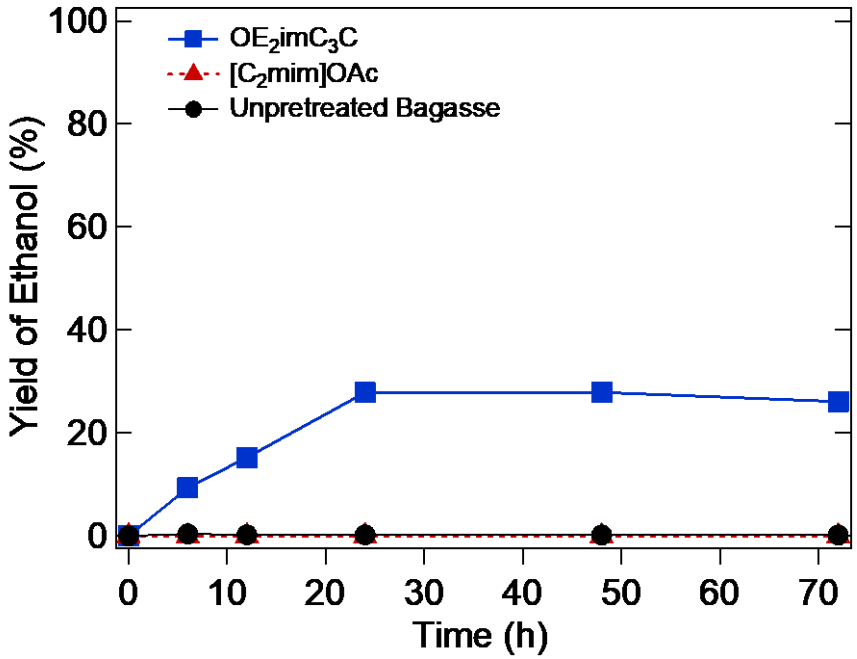

Figure 4- 8 Time courses of ethanol yield in one-pot fermentation in the presence of OE_2imC_3C and $[C_2mim]OAc$, the initial of OD_{600} *E. coli* KO11 was 1.0

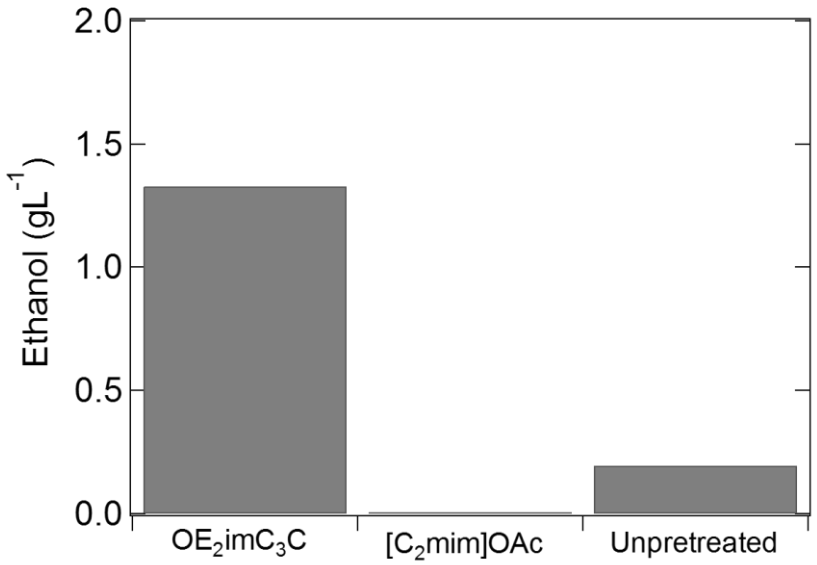

Figure 4- 9 The produced ethanol concentration in one-pot fermentation under the presence of OE_2imC_3C and $[C_2mim]OAc$, the initial of OD_{600} *E. coli* KO11 was 1.0

The confirmation of one-pot fermentation using higher density *E. coli* KO11 was investigated by applying the initial OD_{600} of inoculum at 10.0. When the initial OD_{600} was increasing to 10.0 the ethanol production was faster and the ethanol yield was increasing (38%). The peak of ethanol yield reached at 12 hours and in this case, ethanol could produce in the presence of $[C_2mim]OAc$ although the ethanol yield far bellows the OE_2imC_3C one. It indicates that high density of initial E.coli depressed the toxicity effect, because of the ratio between cells concentration to IL/ZI concentration increasing. Figure 4-10 shows the enhancement of ethanol yield when OD_{600} 10.0 was applied as an initial *E. coli* concentration.

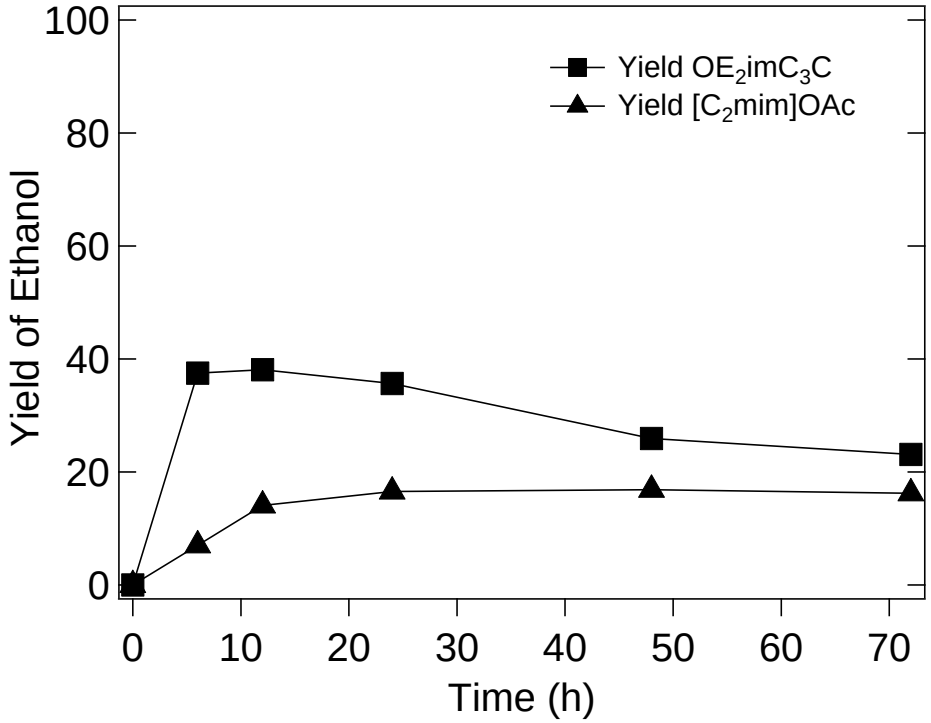

Figure 4- 10 Time courses of ethanol yield in one-pot fermentation in the presence of OE_2imC_3C and $[C_2mim]OAc$, the initial of OD_{600} *E. coli* KO11 was 10.0.

4-4. Summary

The solubility of cellulose in OE_2imC_3C solvent reached 6 wt% at 100 ºC and the capability of OE_2imC_3C to dissolve cellulose was open the opportunity to apply the solvent in cellulose or lignocellulose pretreatment. Meanwhile, increasing of OE_2imC_3C concentration increased the pH of enzymatic saccharification medium, whereas the optimum cellulase activity was in 200 mM of acetate buffer pH 5.0.

Enzymatic saccharification of pretreated bagasse using both of 0.5 M of OE_2imC_3C, a ZI, and $[C_2mim]OAc$, an IL did not appear the inhibition effect. However, in the one-pot ethanol fermentation, 0.5 M of $[C_2mim]OAc$ has a high inhibition of ethanol production using initial OD_{600} of *E. coli* KO11 inoculum at 1.0. In this condition, OE_2imC_3C could convert glucose that produced in previous enzymatic saccharification into ethanol by the ethanol yield at 28%. Meantime, the increasing of initial OD_{600} of *E. coli* KO11 inoculum to 10.0 in one-pot fermentation has enhanced the ethanol yield to 38% in the presence of 0.5 M OE_2imC_3C and also accelerated the ethanol production time.

4-5. Reference

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Chapter 5. One-pot Ethanol Production by using Zwitterion OE_2imC_3C with DMSO Co-solvent

5-1. Introduction

The application of OE_2imC_3C in bioconversion of bagasse into ethanol was explained in Chapter 4. It has proven that *E. coli* KO11 produced ethanol via fermentation of sugar obtained from enzymatic saccharification of pretreated bagasse. OE_2imC_3C in this application showed the capability as cellulose solvent and also has low toxicity to facilitate one-pot process conversion of bagasse into ethanol. On the other hand, the high viscosity of OE_2imC_3C limited the dissolution of cellulose. To enhance the solubility of cellulose, in once time increase of the efficiency in pretreatment and reaction rate in the solvent, decreasing of OE_2imC_3C viscosity was considered.

Dimethyl sulfoxide (DMSO) is a polar aprotic co-solvent that frequently reported as co-solvent of ILs in the pretreatment of lignocellulosic biomass. It has published that DMSO has a wide of role in pretreatment process[1], including a function of co-solvent and viscosity reducer[1-3]. In fact of DMSO, its self, has no ability to dissolve cellulose, it considers that the solubility of cellulose was higher in low viscosity solvent (ILs) than the high one[4-5]. In other words, DMSO has a potency to apply in reducing of OE_2imC_3C viscosity.

Rinaldi et al (2011)[6] introduced a term of 'organic electrolyte solution' as a name of a solvent mixture of IL and polar organic solvent. In their investigation, the dissolution of cellulose was immediately faster at 100 ºC in 1-butyl-3-methylimidazolium chloride /1,3-dimethyl-2-imidazolidinone (DMI/BmimCl) mixture, in which the mixture had a low viscosity. It is reasonable that the expectation to enhance the dissolve of cellulose comes from improving the OE_2imC_3C viscosity.

Base on the previous investigation, there is an indication that toxicity property of IL strongly influenced on one-pot conversion bagasse into ethanol. In spite of DMSO has been widely applied in cryogenic cell protectant, the toxic property of DMSO to E.coli have not investigated so far. Meanwhile, it was confirmed that OE_2imC_3C has low toxicity to *E. coli* KO11 (Chapter 2).

In this study, the mixture solvent of OE_2imC_3C/DMSO applied as a solvent in the conversion of bagasse into ethanol. The first investigation was explored the capability of the mixture solvent to dissolve cellulose to mention their capacity in the pretreatment purpose. Then the examination of growth assay was conducted to describe the toxicity effect the mixture into *E.coli*. Successive enzymatic saccharification by cellulase then performed to obtain fermentable sugar (glucose) from bagasse, and finally, conversion of the fermentable sugar was carried out by fermentation using *E. coli* KO11. All of the conversion arranged in a one-pot process, which means no material was discarded throughout the process.

5-2. Material and Method

Materials and Instrumentations

Oil base hot plate stirrer (AS-ONE Dry Stirring Bath HDBS-6) was used for control temperature in cellulose dissolution and pretreatment. Growth assay was conducted in a shaker incubator (EYELA incubator FMS), and OD_{600} was measured by using spectrophotometer (Life Science UV/Vis spectrophotometer DU-730 Beckman Coulter). Fermentation incubation was done in an incubator (Yamato Program Incubator IN600), and the incubator was equipped with multistirrer (AS-ONE slim stirrer). The viscosity of solvent was determined using viscometer (Brookfied DV-II+ Pro). Analysis of glucose, xylose were determined by using a high-performance liquid chromatography equipped with a refractive index detector (Shimadzu Co., Kyoto, Japan), a CARBOSep CHO-682 column (Tokyo Chemical Industry Co. Ltd., Japan) was used in tandem with a CARBOSep CHO-682 guard column (Tokyo Chemical Industry Co. Ltd., Japan).

Mashed 250-500 µm bagasse (the approximately 3 mm in particle diameter was purchased from Sanwa Ceruciron) and Avicel PH-101 (Aldrich) was used as biomass. DMSO was purchased from Nacalai Tesque Inc. The solvatochromic dyes, (2,6-dichloro-4-(2,4,6-triphenyl-1-pyridinio)phenolate (Reichardt's dye #33) was purchased from Fluka, 4-nitroaniline was purchased from Tokyo Chemical Industries Co., Ltd, and *N*,*N*-diethyl-4-nitroaniline was purchased from Kanto Chemical Co., Inc.. Cellulase Celic® CTec2 enzyme was purchased from Novozymes, Franklinton, NC, USA. Glucose C II kit test was purchased from Wako Pure Chemical Industries, Japan.

Dissolution of cellulose in OE_2imC_3C/DMSO mixture

The various mixture of OE_2imC_3C/DMSO (0.3 g) were prepared with the comparison of OE_2imC_3C/DMSO were 0/10; 2/8; 4/6; 6/4; 8/2; and 10/0 respectively. Cellulose (1 wt%) was added into mixtures and stirred at 100 ºC on oil base hot plate stirrer for 10 minutes. When it was confirmed that the cellulose soluble in OE_2imC_3C, the procedure was repeated until the maximum solubility of cellulose was reached.

Measurement of Kamlet-Taft parameter

Kamlet-Taft parameters consist of three parameters: hydrogen bond acidity (α value), hydrogen bond basicity (β value), and bipolar parameter (π*). In order to examine the value, the solvatochromic dyes, 4-nitroaniline, N,N-diethyl-4-nitroaniline, and (2,6-triphenyl-1-pyridinio) phenolate (Reichardts' dye #33), were used. The stock solution of each dye was made by mix: 1.0 mg of 4-nitroaniline/1 ml methanol, 1.0 mg of N,N-diethyl-4-nitroaniline/1ml methanol, and 2 mg of Reichard's 'dye/1 ml methanol respectively. Severally, 30 µL stock solution of 4-nitroaniline and N,N-diethyl-4-nitroaniline and 100 µL stock solution of Reichard's 'dye were taken and placed in different vial. The methanol from each stock solution in vials then was carefully removed by vacuum drying. After that 200 µL of sample was mixed into each dried dye and was homogenized softly. These mix dye solutions then were placed into quartz cells with 0.1 mm light-path length, and the maximum absorption (λ_{max}) solutions then were examined. The α, β and π^* values were calculated by use of the following equations:

$$v(\text{dye}) = 1/(\lambda_{max(dye)}10^{-4})$$
$$E_T(30) = 0.9986\ (28\ 592/\lambda_{max\ (\text{Reichardt's dye \#33})}) - 8.6878$$
$$\pi^* = 0.314(27.52 - v_{(N,N\text{-diethyl-4-nitroaniline})})$$
$$\alpha = 0.0649E_T(30) - 2.03 - 0.72\pi^*$$
$$\beta = (1.035\ v_{(N,N\text{-diethyl-4-nitroaniline})} + 2.64 - v_{(4\text{-nitroaniline})})/2.80$$

Measurement of Viscosity

The viscosity of the mixture was determined using viscometer, with spindle spin rotation 1-100 rpm at temperature 25-80 ºC. The 500 µL of the mixture was used as a measurement volume, and viscosity was expressed in mPa.

Growth Assay Procedure

The assay medium contains LB medium and the OE_2imC_3C/DMSO mixtures (as described above) was prepared. The concentrations of the mixtures was set up 0.0; 50.0; 100.0; 150.0; 200.0; and 250.0 gL^{-1}, respectively. Pre-culture of *E. coli* was prepared by inoculation 1 colony of *E. coli* into 5.0 mL LB aseptically and was incubated in a shaker incubator at 160 rpm, 37 ºC, for 18-24 hours. The density of pre-cultured cells was measured after 18-24 hours by using a spectrophotometer. The appropriate volume of pre-cultured *E. coli* was taken to set up initial OD_{600} at 0.1

in the 2.0 mL assay medium and was transferred into micro-tube and then was centrifuged at 15,000 rpm, 4 ºC, for 5 minutes. The filtrate was then discharged, and the cells were re-suspended using the assay medium. Suspended of *E. coli* was inoculated into assay medium, then was incubated in a shaker incubator at 160 rpm, 37 ºC, for 24 hours. The measurement of OD_{600} was done at 6, 12, and 24 hours regularly. Relative OD_{600} 24h then was calculated using the equation in growth assay procedures at Chapter 2.

Fermentation Procedure

The fermentation assay medium contains fermentation medium and the OE_2imC_3C/DMSO mixtures (as described above) was prepared. The concentrations of the mixtures was set up 0.0; 50.0; 100.0; 150.0; 200.0; and 250.0 gL^{-1}, respectively. Pre-culture of *E. coli* was prepared by inoculation 1 colony of *E. coli* into 5.0 mL LB aseptically and was incubated in a shaker incubator at 160 rpm, 37 ºC, for 18-24 hours. The OD_{600} of pre-cultured was measured after 18-24 hours by using a spectrophotometer. The appropriate volume of pre-cultured *E. coli* was taken to set up initial OD_{600} at 1.0 in the 2.0 mL of fermentation assay medium and was transferred into micro-tube and then was centrifuged at 15,000rpm, 4 ºC, for 5 minutes. The filtrate was then discharged, and the cells were re-suspended using the fermentation assay medium. Suspended of *E. coli* was inoculated into assay medium, then was incubated in a shaker incubator at 160 rpm, 37 ºC, for 72 hours. Sampling was done for 0; 6; 12; 24; 48; and 72 hours regularly by taken 500 µL of fermentation medium. The sample then was centrifuged at 15,000 rpm, 4 ºC, for 10 minutes, and the filtrate was collected and filtrated using 0.45 µm millipore filter. The analysis of glucose and ethanol was determined by HPLC to follow the analysis condition: the volume of the injected sample was 20.0 µL, the column was operated at 70 ºC, and H_2SO_4 0.0085 N was used as the mobile phase with a flow rate was set up at 0.4 mL/min. Relative Y_{EtOH} 48h then was calculated using the equation in fermentation assay procedures in Chapter 2.

Saccharification of cellulose using cellulase in OE_2imC_3C/DMSO mixture

Cellulase activity was evaluated by saccharification of avicel using Cellic® Ctec2 in the presence of OE_2imC_3C/DMSO mixture. The various concentration of OE_2imC_3C/DMSO mixture was set up 0.0; 50.0; 100.0; 150.0; 200.0; and 250.0 gL^{-1}, respectively in 200mM acetate buffer pH 5. 12.8 mg of avicel was dissolved into 1 mL of OE_2imC_3C/DMSO mixture, and was stirred vigorously for a minutes to performed a homogenous mixture. 0.64 µL of cellulase (100 $FPUmL^{-1}$) then was added into the mixture and was incubated at 50 ºC for 24 hours under stirring gently. After hydrolysis performed, 20 µL of the mixture was sampled and 3.0 mL of glucose CII Wako solution was added. This mixture then was incubated at 37 ºC for 15 minutes. The concentration of glucose then measured with UV-vis spectrophotometer and absorbance at 550 nm (main wavelength) and 600 nm (secondary wavelength) were recorded. The yield of glucose from saccharification was calculated using the equation in saccharification using cellulase procedures in Chapter 4.

Pretreatment of Bagasse using OE_2imC_3C and Enzymatic Saccharification

Pretreatment of bagasse was conducted in a mixture solution containing OE_2imC_3C/DMSO (8/2) (later mention as an optimum solvent in previous procedure) by mixing 0.03 g of bagasse that corresponding to 10 wt% in the mixture of 0.24 g OE_2imC_3C/0.06 g of DMSO. The mixture then was stirred on oil base hot plate stirrer at 120 ºC for 8 hours. After pretreatment was complete, the volume of the mixer was adjusted by adding 1.945 mL 200 mM acetate buffer pH 5 (the final concentration of OE_2imC_3C/DMSO (8/2) was 150 gL^{-1}). The mixture then was stirred vigorously for a minute, and 10 µL cellulase (100 $FPUmL^{-1}$) was added to conduct enzymatic saccharification at 50 ºC for 48 hours. The glucose after hydrolysis then was determined using glucose CII Wako solution as the mention above.

One-pot Ethanol Production from Bagasse using OE_2imC_3C

The procedure of pretreatment and enzymatic saccharification of bagasse using OE_2imC_3C/DMSO (8/2) was conducted to produce fermentable sugar. Successively, after both of processes, fermentation was conducted by adding yeast (5.0 gL^{-1}), NaCl (5.0 gL^{-1}), and tryptone (10.0 gL^{-1}) into hydrolysate. The hydrolysate also was enriched with 100 mgL^{-1} chloramphenicol. The enriched hydrolyzate then was inoculated by *E. coli* KO11 as an initial OD_{600} at 1.0. The fermentation was done anaerobically at 37 ºC for 0-72 hours. The sample was collected periodically to determine glucose, xylose, and ethanol by HPLC.

5-3. Result and Discussion

As a co-solvent, DMSO was miscible well in OE_2imC_3C, for all various comparison was made (OE_2imC_3C/DMSO: 2/8; 4/6; 6/4; and 8/2). The first investigation purpose was for evaluated the capacity of mixture OE_2imC_3C/DMSO to dissolve cellulose. Figure 5-1 shows the result of the investigation. It was confirmed that pure OE_2imC_3C has ability to dissolve cellulose at 100 ºC, while pure DMSO has neither. Pure OE_2imC_3C dissolved cellulose up to 6 wt%, addition more 1% of cellulose after this condition made the mixture of OE_2imC_3-cellulose could not stirrer because of the mixture was too viscous. On the other hand, DMSO in the OE_2imC_3C/DMSO accelerated of cellulose solubility. Since the addition of 20% DMSO to OE_2imC_3C has a similar problem of viscosity as the pure OE_2imC_3C, but the dissolution of cellulose in this mixture appeared increasing up to 12 wt%. A higher of DMSO concentration (40%) in OE_2imC_3C/DMSO (6/4) showed a peak of cellulose solubility achievement, whereas 14 wt% of cellulose was soluble. In contrast, the addition of DMSO over 40% to OE_2imC_3C was decrease solubility of cellulose, although the dissolution of cellulose in the mixture containing 60% and 80% of DMSO still achieved 12 and 8 wt% respectively.

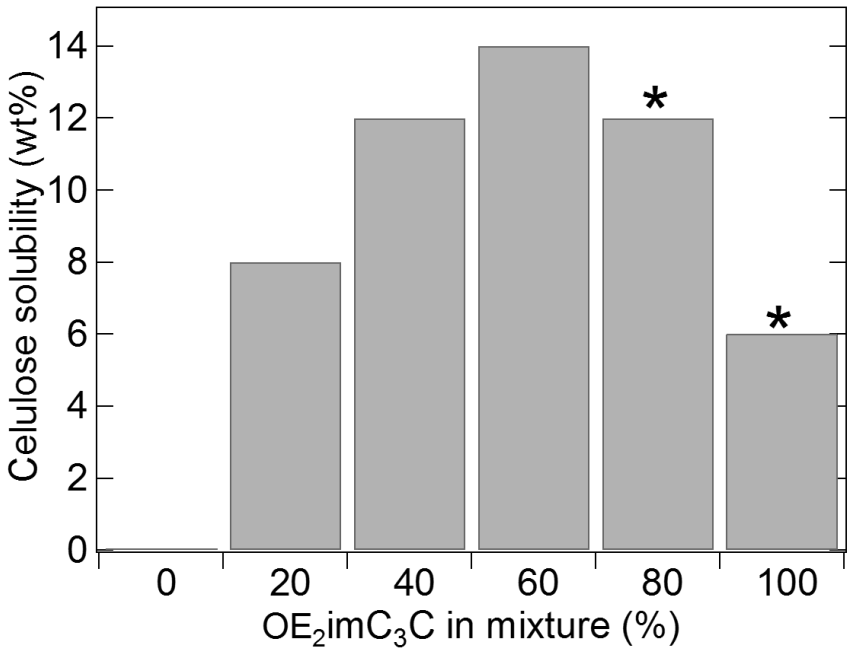

Figure 5- 1 Dissolution of cellulose in OE_2imC_3C/DMSO mixture.

The result of cellulose solubility in the mixture was interesting, and it requires an explanation in the various capability of mixture on dissolution of cellulose. Two parameters than were measured, the viscosity and hydrogen bonding basicity (β value of Kamlet-Taft parameter). The measurement of these parameter presents in Figure 5-2. It can see clearly that the addition DMSO to OE_2imC_3C made decreasing of mixtures viscosity almost exponentially. This reason considers explaining the highest dissolution of cellulose in OE_2imC_3C/DMSO (6/4). Meanwhile, base on Kamlet-Taft parameter measurement, the *β* value was considerably declined when the amount of DMSO was added into OE_2imC_3C. This reason was regarded to the low dissolution of cellulose in OE_2imC_3C/DMSO (2/8). High of *β* value is desired on the dissolution of cellulose[7], because of higher the *β* value a solvent connect to higher ability to destruct hydrogen bonding inside of cellulose molecules[6].

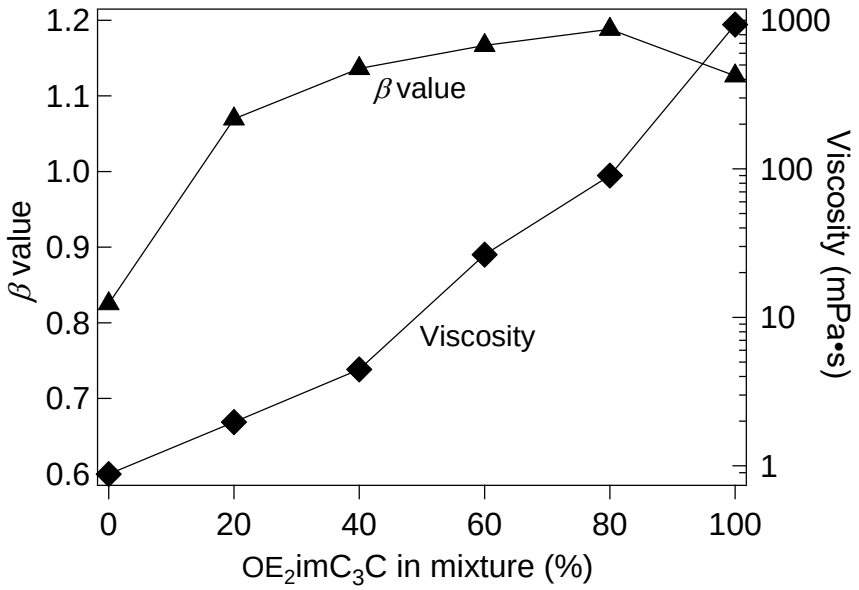

Figure 5- 2 The viscosity and the *β* value Kamlet-Taft parameter of OE_2imC_3C/DMSO mixture, viscosity was determined at 85 ºC.

Furthermore, the investigation of toxicity the mixture to *E. coli* was examined. Effective concentration 50 (EC_{50}) which is a critical concentration of chemical compounds to microorganisms was assumed as a level of toxicity. The description of EC_{50} for the all mixture put in Figure 5-3. It appears clearly that the toxicity of DMSO was higher than OE_2imC_3C one. EC_{50} of DMSO was almost half a fold than OE_2imC_3C one. The toxicity of IL or ZI in many cases was connected to the interaction of the agents into cells membrane. If the agents have ability to destruct the cell membrane, it will consider that the agent has toxicity effect to the microorganism cells[8]. The low toxicity of OE_2imC_3C due to omission the alkyl chain (hydrophobic part) in the structure as mentioned in Chapter 2. The structure could avoid insertion of alkyl chain, consequently, the accumulation of OE_2imC_3C in cell membrane could be prevented. This symptom followed the toxicity mechanism that mentioned by Lim at al (2014)[9].

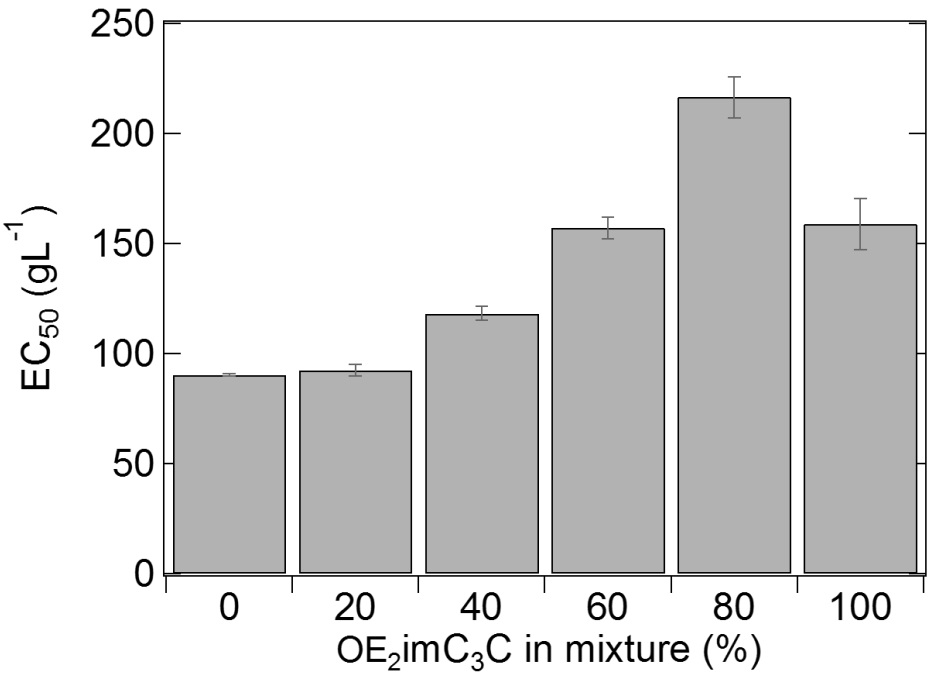

Figure 5- 3 EC_{50} value of the OE_2imC_3C/DMSO mixture in growth assay

Even though DMSO has a higher toxicity than OE_2imC_3C, in the examination, it was surprising that addition of 20% DMSO to OE_2imC_3C increased tolerance *E. coli* to the mixture. *E. coli* has the highest tolerance in this mixture, and in the addition of up to 40% DMSO, the toxicity level was similar with the pure OE_2imC_3C. The toxicity increased when DMSO was added above 40%, it indicated from the EC_{50} value was falling. The plotting the relation between the amount of OE_2imC_3C the mixture to the relative OD_{600} value indicated that the trend in the OE_2imC_3C/DMSO (80/20) and pure OE_2imC_3C was similar. Refer to the toxicity of OE_2imC_3C/DMSO (80/20), DMSO was not involved in the toxicity. It could be pre-assumed that OE_2imC_3C and DMSO have a different mechanism to inhibit the growth *E.coli*, consequently, the mixture has a low toxicity. Figure 5-4 presents the plotting the amount of OE_2imC_3C (a) or DMSO (b) in the mixture to the relative OD_{600} value.

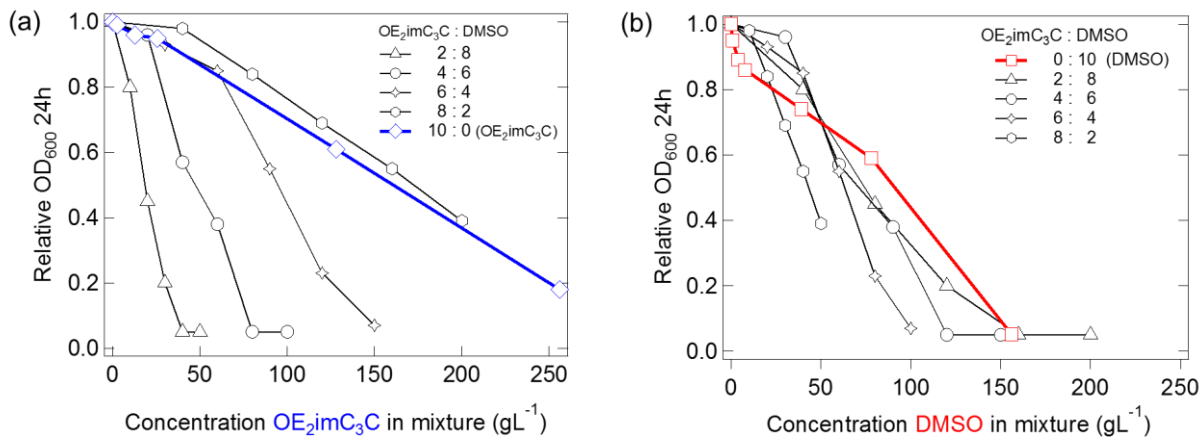

Figure 5- 4 Plotting of particular amount of OE_2imC_3C (a) or DMSO (b) in the mixture to the relative OD_{600} value

Despite the mechanism of DMSO toxicity and also the OE_2imC_3C have not been known clearly, an effect of DMSO on the membrane have ever been mentioned in a publication. Yamashita et al (2000)[10], in their discussion have underlined that the effect of DMSO on phospholipid membrane or biomembrane strongly dependent on the concentration of DMSO in water and temperature. It considered to the physical property of DMSO in water, whereas one molecule DMSO connected to two molecule water in low concentration around 20 ºC. Consequently, in a low concentration, DMSO stabilized water structure. In contrary, at higher concentration, the connected between DMSO and water break down, and DMSO has more hydrophobic property to interact with the membrane. In this assay, the lysogeny

broth (LB) medium, that has water as a solvent, was used. The low concentration of DMSO in OE_2imC_3C/DMSO (80/20) mixture that has the lowest toxicity connected to the DMSO character which has mentioned above might be a reasonable symptom.

In fermentation assay, the toxicity of the mixture became reduced when the calculation of EC_{50} was examined by the ability of *E. coli* to produce ethanol in the presence of mixture concentration (0-250 gL^{-1}). *E. coli* showed high tolerance in a mixture containing up to 40% DMSO whereas the EC_{50} value in this solution more than 250 gL^{-1}. The fermentation result indicated that the relative ethanol yield of OE_2imC_3C/DMSO (80/20) was almost similar with the pure OE_2imC_3C, while the OE_2imC_3C/DMSO (60/40) was a little bit lower although the relative ethanol yield kept remaining above 80%. Figure 5-5 and 5-6 present EC50 value for fermentation assay and relative ethanol yield of the OE_2imC_3C/DMSO mixtures respectively.

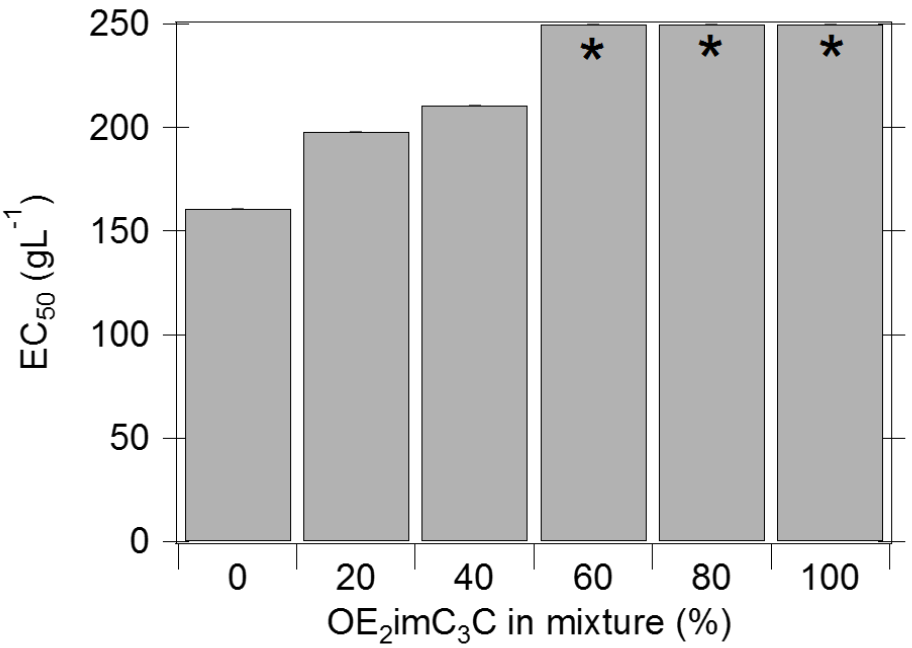

Figure 5- 5 EC_{50} value of the OE_2imC_3C/DMSO mixture in fermentation assay

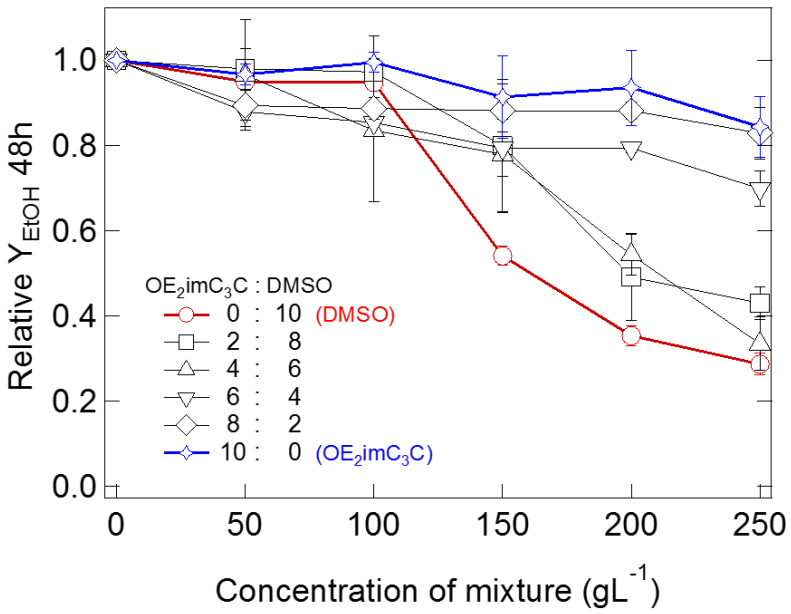

Figure 5- 6 Relative ethanol yield in fermentation assay under the presence of various concentration of OE_2imC_3C/DMSO mixture

Furthermore, the activity of cellulase in presence of the mixture was conducted in enzymatic saccharification assay. The result of examination presents in Figure 5-7. From that graph, it showed clearly that increasing of concentration of mixture gave inhibition effect to the cellulase activity. At 150 gL^{-1} of mixture, concentration DMSO gave higher inhibition than OE_2imC_3C, but at 250 gL^{-1} the opposite trend was appearing although for both of concentration the relative cellulase activity was not considerably different. Even though the inhibition concentration 50 (IC_{50}) of DMSO was lower than OE_2imC_3C, that describe the inhibition effect of DMSO higher than OE_2imC_3C, however the addition of DMSO the OE_2imC_3C showed that the IC_{50} value of mixture placed almost around the IC_{50} value of the pure OE_2imC_3C. Figure 5-8 presents the IC_{50} value of mixture in enzymatic saccharification assay.

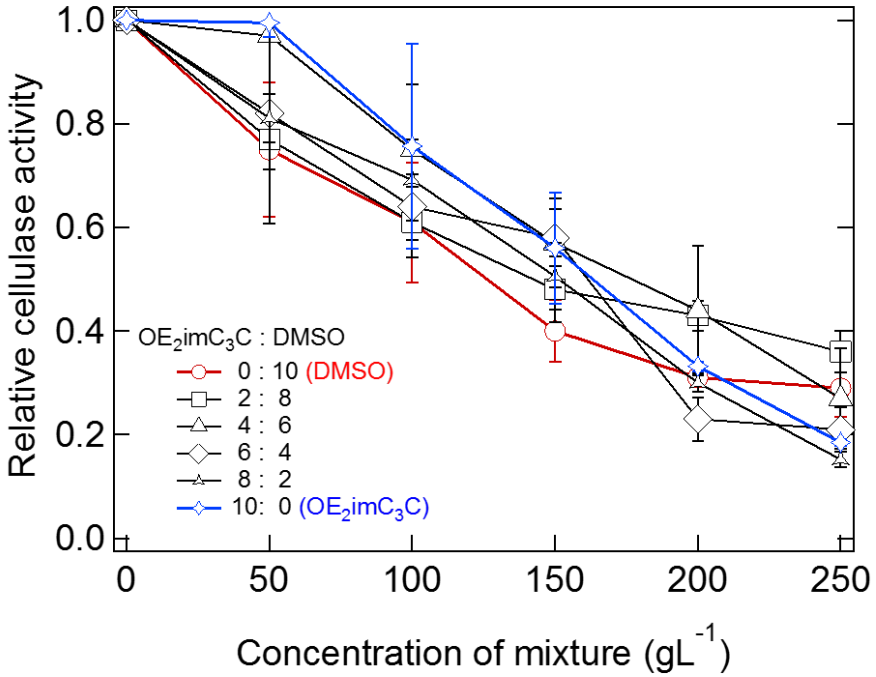

Figure 5- 7 Relative activity of cellulase in the presence of OE_2imC_3C/DMSO mixture

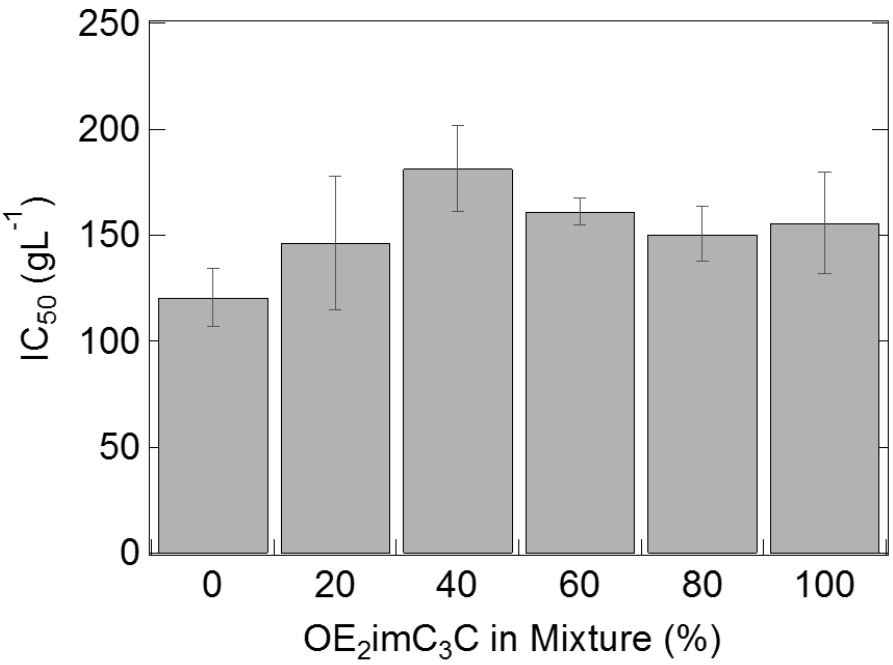

Figure 5- 8 IC_{50} value of OE_2imC_3C/DMSO mixture in enzymatic saccharification assay

Base on the investigation, further the OE_2imC_3C/DMSO (8/2) at 150 gL^{-1} was chosen as a solvent to conduct one-pot conversion of bagasse into ethanol. The treatment significantly has a big effect to the resultant of ethanol yield, when the comparison of yield ethanol was made. The unpretreated bagasse showed a low of ethanol yield because of the low fermentable sugar produced in enzymatic saccharification process. It indicated that pretreatment effect of OE_2imC_3C/DMSO (8/2) was effective to enhance the hydrolysis cellulose in bagasse by cellulase. The ethanol yield was achieved 51% for 12 hours fermentation. Figure 5-9 presents the ethanol yield in one-pot fermentation.

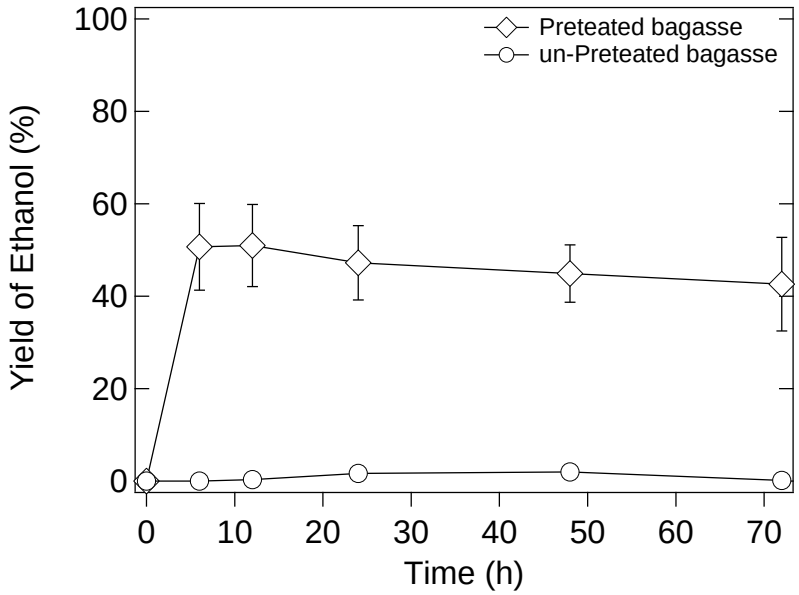

Figure 5- 9 Time courses of ethanol yield in one-pot conversion of bagasse into ethanol

The application of DMSO as co-solvent in mixture OE_2imC_3C/DMSO improved the amount of produced ethanol. Since using pure OE_2imC_3C in one-pot bioconversion of bagasse into ethanol process could produce 1.3 grL^{-1} of ethanol, using OE_2imC_3C/DMSO (8/2) was 3.1 grL^{-1} one (Figure 5-10.). The significant increasing of ethanol producing in the OE_2imC_3C/DMSO (8/2) caused by the improvement of glucose consuming in the fermentation. It was noted that OE_2imC_3C has high viscosity, consequently, the using of pure OE_2imC_3C compare with OE_2imC_3C/DMSO (8/2) mixture showed the different glucose consume rate. In higher viscosity, glucose consuming appeared slower in the beginning of fermentation, and the amount of glucose consuming was also lower during the fermentation (Figure 5-11). Co-solvent DMSO full filled the aim of reducing the

viscosity has a positive impact on the one-pot conversion of bagasse into ethanol generally.

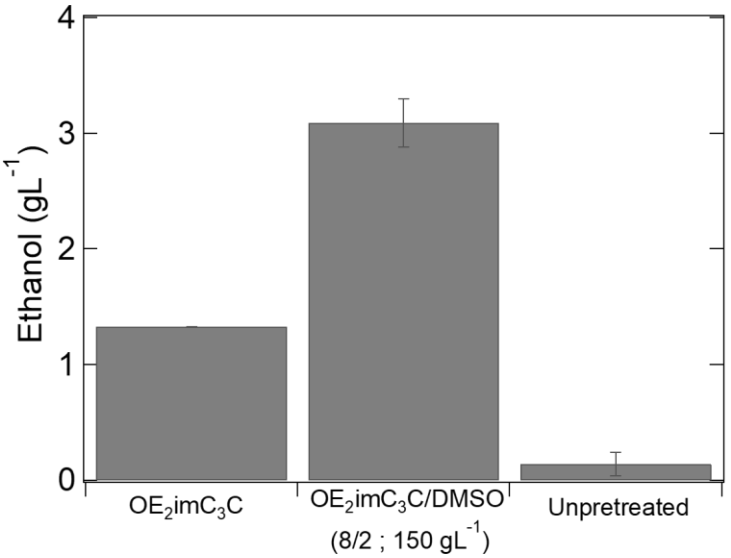

Figure 5- 10 Ethanol production by pretreated bagasse using OE_2imC_3C, OE_2imC_3C/DMSO (8/2) mixture, and unpretreated in one-pot process.

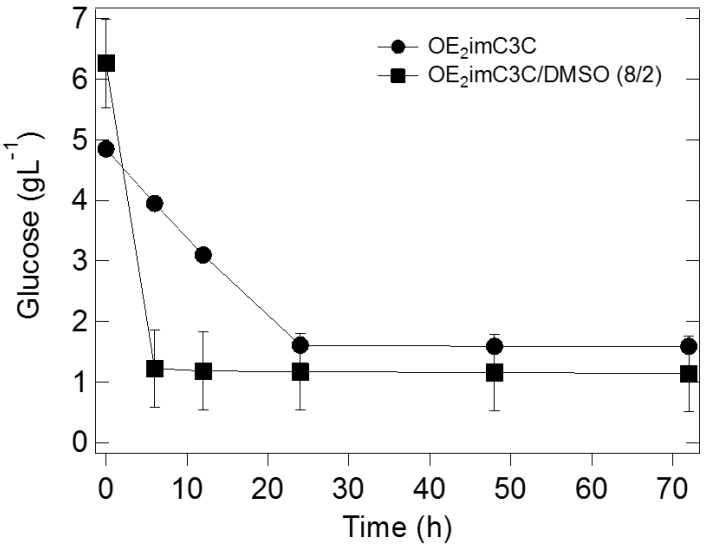

Figure 5- 11 Comparison of glucose consume in ethanol production by pretreated bagasse using OE_2imC_3C and OE_2imC_3C/DMSO (8/2) mixture in one-pot process

5-4. Summary

DMSO was miscible well in OE_2imC_3C, for all various comparison was made (OE_2imC_3C/DMSO: 2/8; 4/6; 6/4; and 8/2). Since 6 wt% of cellulose dissolved in pure OE_2imC_3C at 100 ºC, pure DMSO has no the ability to dissolve cellulose. DMSO in the OE_2imC_3C/DMSO mixture accelerated of cellulose solubility. The addition of 20% DMSO to OE_2imC_3C appeared increasing the cellulose solubility up to 12 wt%. The highest dissolution of cellulose was in OE_2imC_3C/DMSO (6/4) whereas 14 wt% of cellulose was soluble. In contrast, the addition of DMSO over 40% to OE_2imC_3C was decrease solubility of cellulose, although the dissolution of cellulose in the mixture containing 60% and 80% of DMSO still achieved 12 and 8 wt% respectively. The viscosity and β value of Kamlet-Taft parameter influenced the dissolution of cellulose in the OE_2imC_3C/DMSO mixture, the mixture that has low viscosity and high β value was preferable on high capability to dissolve cellulose.

It was confirmed well that toxicity of DMSO higher than OE_2imC_3C. The addition of 20% DMSO to OE_2imC_3C increased tolerance *E. coli* to the mixture, and in the addition of up to 40% DMSO, the toxicity level was similar with the pure OE_2imC_3C. The toxicity increased when DMSO was added above 40%. The low toxicity in OE_2imC_3C/DMSO (8/2) was contributed to the toxicity of OE_2imC_3C, it did not appear that toxicity of DMSO involved in the toxicity of mixture.

In fermentation assay, *E. coli* showed high tolerance in a mixture containing up to 40% DMSO whereas the EC_{50} value in this solution more than 250 gL^{-1}. The fermentation result indicated that the relative ethanol yield of OE_2imC_3C/DMSO (80/20) was almost similar with the pure OE_2imC_3C, while the OE_2imC_3C/DMSO (60/40) was a little bit lower although the relative ethanol yield kept remaining above 80%.

Furthermore, the activity of cellulase in presence of the OE_2imC_3C/DMSO mixture showed that the increasing of concentration of mixture increased inhibition effect to the cellulase activity. At 150 gL^{-1} of mixture, concentration DMSO gave higher inhibition than OE_2imC_3C, but at 250 gL^{-1} the opposite trend was appearing although for both of concentration the relative cellulase activity was not considerably different.

The addition of DMSO to the OE_2imC_3C did not dropped the IC_{50} value dramatically, the IC_{50} of mixture placed almost around the IC_{50} value of the pure OE_2imC_3C.

The OE_2imC_3C/DMSO (8/2) at 150 gL^{-1} was chosen as a solvent to conduct one-pot conversion of bagasse into ethanol. The pretreatment effect of OE_2imC_3C/DMSO (8/2) was effective to enhance the hydrolysis cellulose in bagasse by cellulase. In addition, the improvement in ethanol yield was achieved at 51%.

5-5. Refference

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Chapter 6. Conclusion and Future Prospects

6-1. Conclusion

This dissertation explained the novel application of zwitterion (ZI) on bioconversion of bagasse into ethanol. Using approach of ionic liquids (ILs) on biomass pretreatment, the novel process enabled sequentially process in biomass conversion consists of pretreatment, hydrolysis, and fermentation. The keys of successful conversion were how to overcome the crystallinity of biomass in pretreatment, efficient hydrolysis process, and reduce toxicity effect in down process that allowed fermentation using microorganism.

In *Chapter 2*, the effect of ILs and ZIs to the growth of *E. coli* and glucose fermentation into ethanol by *E. coli* KO11 have been described. It summarized that ILs were more toxic than ZIs. The cations have been signed as strongly affecting the growth of *E. coli*, whereas imidazolium-type cations have higher inhibition than cholinium-type one. Furthermore, it did not appear that anion-type has the different influence on the *E. coli* growth. The structure of ZI allowed to reduce the toxicity. When the hydrophobic part was omitted by attached anion part in one molecule to form carboxylate-type ZI, C_1imC_3C, adapted to IL, $[C_2min]OAc$, structure, it was effective to overcome toxicity effect. Meanwhile, the omission of acid anion part in sulfate-type ZI (C_1imC_4S) as a derivative from IL ($[C_1imC_4SH]HSO_4$) also was depressing the toxicity greatly. Anion-type of ILs has a big impact to the fermentation of glucose into ethanol by *E.coli* KO11. It connected to the influencing of ILs *β* value that the higher ILs β value decreased Y_{EtOH} 48 hours, either the glucose consumption. The ZI, OE_2imC_3C, that has a big *β* value and the other ZIs did not inhibit the fermentation.

In *Chapter 3*, the strategy of ethanol production from bagasse using ZI, C_1imC_4S, has been explained. The first strategy is using sulfuric acid (H_2SO_4) for pretreatment and followed by addition of C_1imC_4S sequentially to *in situ* synthesized $[C_1imC_4SH]HSO_4$ that has a function as a catalyst in hydrolysis of bagasse with microwave heating. The second strategy is reducing the toxic effect of $[C_1imC_4SH]HSO_4$ by electrodialysis. The effectiveness of sulfuric acid on pretreatment as the first step of hydrolysis using *in situ* synthesis $[C_1imC_4SH]HSO_4$

was appearing. The glucose yield from pretreated cellulose was increasing dramatically to 79.87%, since the unpretreated cellulose achieved only 8.05% and the other pretreated cellulose (PASC) was only 48.98%. The strategy also effective on bagasse hydrolysis, whereas the glucose yield has been higher than acid hydrolysis using concentrated H_2SO_4. The optimum temperature of hydrolysis was at 100 ºC, which has glucose yield at 77.44% and the glucose was not significantly degradation. It was also able to hydrolysis all of hemicellulose in bagasse. Meanwhile, the separation of $[C_1imC_4SH]HSO_4$ into C_1imC_4S and H_2SO_4 by electrodialysis was conducted successfully to recover 96% H_2SO_4 in concentrate compartment, and 99% C_1imC_4S in the dilute compartment after 60 min. The strategy of conversion was able to convert 305 mg of bagasse into 78 mg of ethanol and the ethanol yield at 52%.

In *Chapter 4*, an application of a novel zwitterion, OE_2imC_3C, as an agent to convert bagasse into ethanol has been described. The solubility of cellulose in OE_2imC_3C achieved 6 wt% at 100 ºC, and it was useful for pretreatment lignocellulosic biomass purpose. The optimum pH for enzymatic saccharification in presence of OE_2imC_3C was in 200 mM of acetate buffer pH 5.0. Using 0.5 M of OE_2imC_3C, it did not appear the inhibition effect of ZI to the cellulase. The one-pot ethanol fermentation was conducted as successive integrated process in biorefinery using 0.5 M of OE_2imC_3C. The bioconversion enabled to convert bagasse into ethanol using *E.coli* KO11. The initial OD_{600} of *E. coli* KO11 inoculum was applied at 1.0 with the ethanol yield were 28 %, when initial OD600 was increased the yield achieved 38%.

In *Chapter 5*, the explanation of addition of DMSO as a co-solvent to OE_2imC_3C has been presented. The purpose of addition was to reduce the viscosity, and the mixture was applied to the conversion of bagasse into ethanol. DMSO was miscible well in OE_2imC_3C, for all various comparison was made (OE_2imC_3C/DMSO: 2/8; 4/6; 6/4; and 8/2). DMSO in the OE_2imC_3C/DMSO mixture accelerated of cellulose solubility. The addition of 20% DMSO to OE_2imC_3C increased dissolution of cellulose up to 12 wt%, and maximum cellulose (14 wt%) could dissolve in OE_2imC_3C/DMSO (6/4). The viscosity and β value of Kamlet-Taft parameter influenced the cellulose solubility in the OE_2imC_3C/DMSO mixture, the mixture that has low viscosity and high β value was preferable on high capability to dissolve cellulose. Even though toxicity of DMSO was higher than OE_2imC_3C, the addition of

20% DMSO to OE_2imC_3C increased the tolerance of *E. coli* to the mixture. In addition, the mixtures containing up to 40% DMSO have low toxicity to *E. coli* in fermentation assay and it achieved high ethanol yield around 80%. Furthermore, the activity of cellulase in presence of the OE_2imC_3C/DMSO mixture was inhibited along with the increasing of concentration. However, the addition of DMSO to the OE_2imC_3C did not dropped the IC_{50} value dramatically, because the IC_{50} of mixture was almost around the IC_{50} value of the pure OE_2imC_3C. Eventually, the OE_2imC_3C/DMSO (8/2) at 150 gL^{-1} was selected as a solvent to conduct one-pot conversion of bagasse into ethanol. The pretreatment effect of OE_2imC_3C/DMSO (8/2) was effective to enhance the hydrolysis cellulose in bagasse by cellulase. The improvement in ethanol yield was achieved at 51%.

6-2. Future Prospects

The applications of zwitterion (ZI) for biomass processing have not investigated intensively in recently time. The prospect and the limitation of the ZI application will herein explain.

The strategy and method developed in using of ZI, C_1imC_4S, on biorefinery of bagasse into ethanol are useful to overcome some limitation of ILs application to biomass processing. The using of sulfuric acid (H_2SO_4) for pretreatment first and followed by addition of C_1imC_4S sequentially to *in situ* synthesized $[C_1imC_4SH]HSO_4$ for hydrolysis already answered the crystallinity problem that provides resistance in the hydrolysis of lignocellulosic biomass. It is open opportunity to apply to various biomass processing, especially to enhance the glucose yield of the process. According to the structure of $[C_1imC_4SH]HSO_4$ that can synthesize in situ from H_2SO_4 and C_1imC_4S, and also can separate following a reversible reaction of its synthesizing, it has great beneficial to overcome the toxicity of application acidic ionic for the downstream process of the biorefinery. $[C_1imC_4SH]HSO_4$ solution has low pH at 1.0 and it is undesirable for fermentation process using microorganism. Separation of H_2SO_4 from the structure increased pH up to 6.0 and it is preferable for almost of the fermentation process. In this part, electrodialysis has already well proven to separate it, and open opportunity to use recycled C_1imC_4S.

A small limitation for the upscale challenge the whole process of the process. In this investigated 1.0 M solution was used in hydrolysis and the biomass loading was 20 gL^{-1}. It considers in the upscale application that the biomass loading that conducted in this investigation suitable for the efficient process in a big scale. Therefore, in this study, the ethanol yield of 52% was already achieved.

The novel ZI, OE_2imC_3C, has a beneficial in biorefinery of lignocellulose into ethanol. Firstly, it is liquid in room temperature and the polarity properties allow for dissolution of cellulose process. This study was the invention study that investigated on application OE_2imC_3C for biomass process, and the achievement was open the opportunity to utilize this ZI in another biomass process. The disadvantage property of OE_2imC_3C, a high viscosity, has been overcome with the addition of DMSO as co-solvent to OE_2imC_3C, which it did not worsen the biocompatible property of this ZI.

The addition of DMSO also improved the dissolution ability and also enhance the enzymatic saccharification of pretreated biomass. The important thing is OE_2imC_3C and the mixture of OE_2imC_3C/DMSO enabled for one-pot process. It has a wide spectrum for beneficial application of this ZI to the biocompatible conversion process.

The complicated synthesis process of OE_2imC_3C might contribute to limitation of OE_2imC_3C utilization, and it also addressed to the application on a big scale of biomass process. Developing of simple synthesize of OE_2imC_3C would be preferable for providing this ZI in the large amount. It is a reasonable concern, because of OE_2imC_3C has an advantage property that would be needed in biomass processing.

List of Publications

1. **H. Satria**, K. Kuroda, T. Endo, K. Takada, K. Ninomiya, and K. Takahashi, Efficient hydrolysis of polysaccharides in bagasse by in situ synthesis of an acidic ionic liquid after pretreatment, *ACS Sustain. Chem. Eng.*, 2017, **5**, 708-713.

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3. T. Endo, M. Tatsumi, K. Kuroda, **H. Satria**, Y. Shimada, K. Ninomiya, and K. Takahashi, Efficient recovery of ionic liquid by electrodialysis in the acid hydrolysis process, *Sep. Sci. Tech.*, 2017, **52**, 1240-1245.

4. K. Kuroda, K. Inoue, K. Miyamura, **H. Satria**, K. Takada, K. Ninomiya, and K. Takahashi, Efficient Hydrolysis of lignocellulose by acidic ionic liquids under low-toxic condition to microorganisms, *Catalysts*, 2017, 7, 108; doi:10.3390/catal7040108

5. K. Kuroda, **H. Satria**, K. Miyamura, Y. Tsuge, K. Ninomiya, and K. Takahashi, Design of Wall-Destructive but Membrane-Compatible Solvents, *Sci. Adv.*, Under Revision

6. **H. Satria**, K. Kuroda, Y. Tsuge, K. Ninomiya, and K. Takahashi, Improvement of biocompatibility cellulose solvents by toxic co-solvent, Chem. Commun. In Preparation