Doctoral Thesis

Bioethanol Conversion of Cellulosic Biomass Using a Combination of Cellulase and Recombinant Yeast

ZHAO XUZHE

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Chapter 1
General Introduction

1.1 Bioethanol

Energy resource is the significantly important base of social and economic development. The unavoidable depletion of fossil fuels and negative impacts of fossil fuels on the environment, especially greenhouse gas emissions, have resulted in a worldwide interest in identifying and developing renewable fuel alternatives [1]. At present, biofuels are considered potential clean alternatives, which can be produced as liquid, gas and solid fuels from cellulosic biomass. Bioethanol is kind of biofuels and recently used as a

gasoline additive to improve burning of gasoline and reduce emission of greenhouse gas. Bioethanol has increased in popularity due to raise of oil price and the need for energy security [2].

Bioethanol is produced by saccharification and fermentation of cellulosic biomass. The study about bioethanol initially used sugar or starch crop such as corn and sugarcane as the raw materials. As food shortage and high cost could be caused by production from food materials, it is significantly important to use nonfood cellulosic biomass such as trees, corn stover and wheat straw as a feedstock for bioethanol production. Ethanol made from cellulosic biomass is attractive as renewable liquid fuel for transportation. Lignocelluloses materials are most promising feedstock as natural, abundant, and renewable resource and can potentially provide a long term sustainable fuel supply. In summary, bioethanol produced from nonfood cellulose is an attractive alternative because of its sustainability, lower-cost, efficient availability of feedstock reserves, renewable clean energy and reduction of greenhouse gas emission.

Ethanol production from lignocellulosic biomass comprises the following main steps: hydrolysis of cellulose and hemicellulose, sugar fermentation, separation of lignin residue and, finally, recovery and purifying the ethanol to meet fuel specifications. The production of bioethanol has been increased quickly in recent years.

1.2 Cellulosic biomass residues

Cellulosic biomass is a clean, renewable energy source that can help to significantly diversify transportation fuels. Biomass is biological material derived from living, or recently living organisms. In the context of biomass for energy this is often used to mean plant based material, but biomass can equally apply to both animal and vegetable derived material. Agricultural residues such as wheat straw and corn stover represent large renewable resources for cellulosic bioethanol conversion [3].

Biomass residue is mainly composed of cellulose, hemicellulose and lignin and they exist as cells form. Figure1.1 shows structure of cellulosic biomass with cellulose, hemicellulose, and lignin represented [4]. Hydrogen bonding made cellulose chains connected together and the long cellulose fiber is hold together with hemicellulose and lignin in order to protect plants against weather and attack by microorganisms and insects. Component of cellulose, hemicellulose and lignin are different according to variety of plants, region and development stages of plants. In addition, biomass residue also contains water, ash, mineral substance and other extractives.

Figure 1.1 Lignocellulose structure [4]

The content of cellulose, hemicellulose and lignin in biomass materials cover about 70%-90%. Table1.1 shows different cellulosic biomass composition of cellulose, hemicellulose and lignin [5-11].

Table 1.1 Cellulosic biomass composition of cellulose, hemicellulose and lignin [5-11].

Cellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Tree leaf	15-20	80-85	0
Wheat straw	30	50	15
Corn cob	45	35	15
Sugarcane bagasse	39	26	24
Hardwood sterm	40-55	24-40	18-25
Softwood srerm	45-50	25-35	25-35
Switchgrass	45	32	12
Bean stalk	35	25	20
Sorghum stalk	35	24	25
Rice hull	36	12	15
Nut shells	25-30	25-30	30-40
Rice straw	35	25	12

1.2.1 Cellulose

Cellulose is the major component in the rigid cell walls in plants and it has a long chain of linked sugar molecule of $(C_6H_{10}O_5)_n$ that could provide cell of plant firm structure and remarkable strength. Cellulose is a linear polysaccharide polymer with many glucose monosaccharide units by acetal linkage of ß-1, 4 glucosidic bond [11]. The ß-D-glucose units are linked when water is eliminated by combining the -OH group and –H bond. Linking just two of these sugars produces a disaccharide called cellobiose [12]. Figure1.2 shows the molecule structure of cellulose.

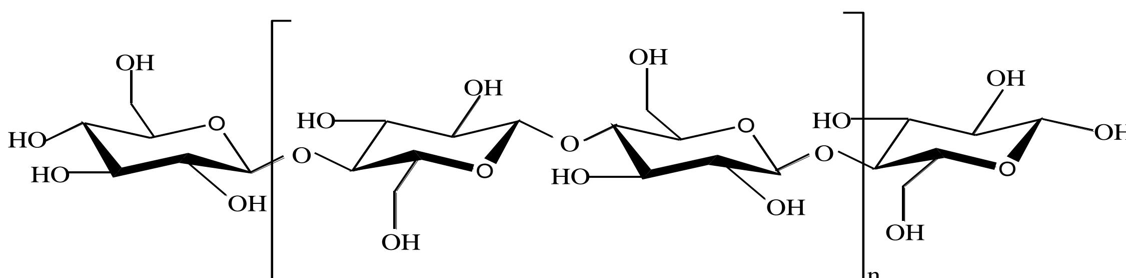


Figure 1.2 molecule structure of cellulose.

From figure 1.2 we could know that cellulose has plenty of hydroxyl groups, which can form strong hydrogen bonding network and lead to the formation of much crystalline cellulose [13]. The molecular weight of cellulose can reach to 50000-2500000 which is equivalent to 300-15000 glycosylic groups. Cellulose cannot be dissolved in water and general organic solvent

such as alcohol and acetone for the hydrogen bond of cellulose is very steady and cannot be destroyed at room temperature.

1.2.2 Hemicellulose

Hemicellulose is kind of heteropolymers that constituted of several monosaccharides include xylose, mannose, galactose, rhamnose, arabinose and the content of xylose achieves over 80%. For the low content of others monosaccharides the structure of hemicellulose is usually accredited to Figure 1.3. These monosaccharides connected each other with hydrogen bond, covalent bond, ether bond and ester bond. Hemicellulose has a random, amorphous structure with little strength and presents along with cellulose in almost all plant cell walls.

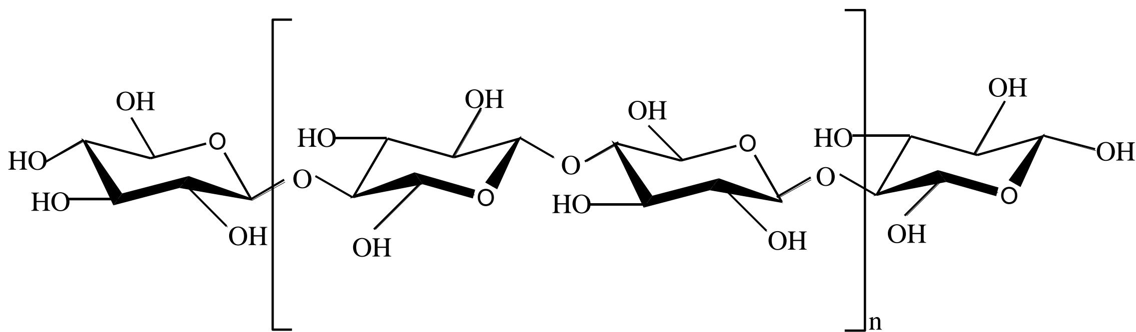


Figure 1.3 molecule structure of hemicellulose.

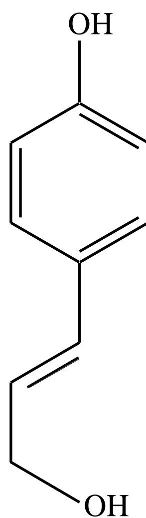
Hemicellulose can be hydrolyzed by dilute alkali and acid as well as hemicellulases easily. The degree of polymerization of hemicellulose can reach

to 500-3000 and it can be extracted though ethanol.

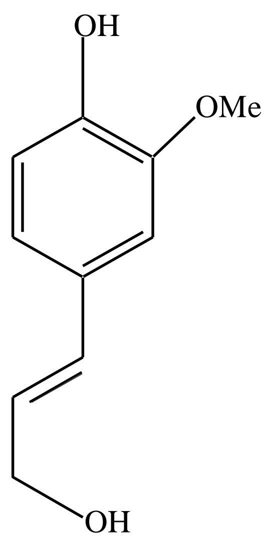
Hemicellose has any of various plant polysaccharides less complex than cellulose and easily hydrolyzable to simple sugars and other products. In addition, hemicellulose is a branched polymer, while cellulose is unbranched.

1.2.3 Lignin

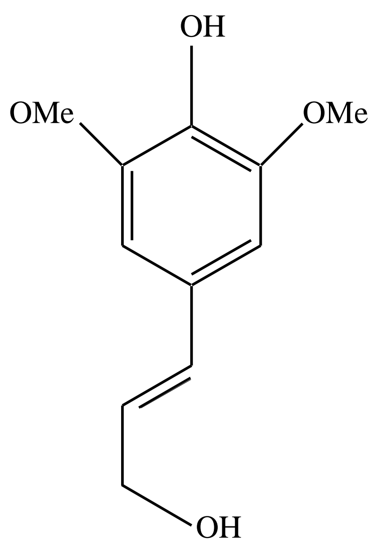
Lignin is a complex amorphous polymer related to cellulose that provides a structural function in plants and together with cellulose forms the woody cell walls of plants and the cementing material between them. Lignin composed of phenyl propane monomers and their derivatives as shown in figure 1.4.



ρ -coumaryl alcohol



Coniferyl alcohol



Sinapyl alcohol

Figure 1.4 Chemical structures of the phenyl propane monomers used to construct the lignin polymer.

It is very difficult to degrade lignin for its components being held together with strong chemical bonds and it also appears to have a lot of cross-linked internal H bonds. It is bonded in complex and various ways to hemicellulose

in wood. Lignin has no fixed structure and the degree of polymerization of lignin is very difficult to measure since it is fragmented during extraction.

1.3 Agricultural residues

Hokkaido is the second largest, northernmost, and most sparsely populated of the major islands of Japan. Hokkaido is the nation's largest agricultural producing region that boasts a food self-sufficiency ratio of 226 %, the highest in the country, and produces about a quarter of nation's agricultural products on a calorie basis. About 3500 thousand tons beets are produces every year in Hokkaido. Table 1.2 shows the production of beet and corn in Hokkaido in recent 5 years [63, 64].

Table 1.2 Production of beet and corn in Hokkaido in recent 5 years[63, 64].

Year	Beet(Kt)	Corn(Kt)
2013	3435	-
2012	3758	121
2011	3574	115
2010	3090	107
2009	3647	99
Average	3500	110

Beet pulp is the main by-product of sugar production after sugar juice extraction. The use of beet pulp has received considerable attention since beet

pulp includes high content of cellulose and hemicellulose and low lignin content.

1.4 Pretreatment

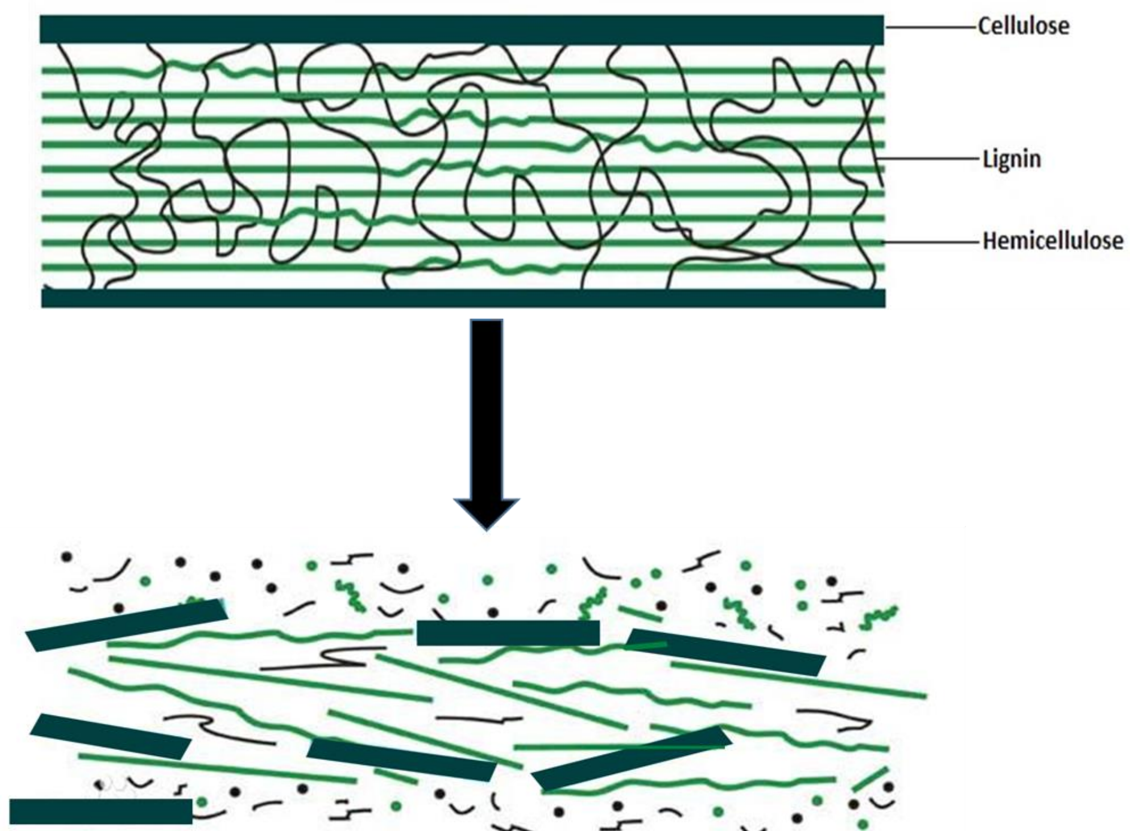


Figure 1.5 The image of structure changes before and after pretreatment on the lignocellulosic biomass [4].

As mentioned in section 1.2 cellulosic biomass we could know that cellulose is composed of a lot of hydroxyl groups, which can lead to the formation of stabilized hydrogen bonding network and supported to the foundation of these crystalline cellulose. Hemicellulose is determined as

branched polymer, which is mainly composed of a range of several different monosaccharides by the way of glycosidic bonds and connected with cellulose basic glucose units. Lignin is a complicated heterogeneous polymer with unfixable structure that constituted with phenyl propane units connected though ether and carbon bonds.

As the structure of lignin is high crystallized and stiff to be destroyed, the existence of lignin could significantly decrease the efficiency of enzymatic saccharification of cellulose and hemicellulose. Therefore, in order to improve the efficiency during the process of saccharification and fermentation of cellulosic biomass, pretreatment has become a crucial step. The fundamental purpose of the pretreatment is to decrease the crystallinity of cellulose and eliminate the resistance of lignin; hence, making them more accessible to the cellulase and yeast of fermentation. An efficient pretreatment method is the method that could improve the concentration of sugar saccharified, decrease the loss of cellulose and hemicellulose, and reduce the production of byproducts and so on. Most of the developed processes are cost intensive, excessive loss，require proper effluent treatment at the industrial scale and produce inhibitors like furfurals which further hinder the bioprocess at industrial scale making total process uneconomical. Current pretreatment research is focused on identifying, evaluating, developing and demonstrating promising approaches that primarily support the subsequent enzymatic hydrolysis of the treated biomass with lower enzyme dosages and shorter bioconversion times. A large number of pretreatment approaches have been investigated on a wide variety of feedstock types. Up to present，several

effective pretreatment methods have been suggested including physical, physicochemical, chemical, and biological pretreatments. Figure 1.5 shows the image of structure changes before and after pretreatment on the lignocellulosic biomass [4].

Physical pretreatment could increase the reaction surface area and decrease the size of lignocelluloses, crystallinity and polymerization degree [17]. Physical processes usually include different types such as milling, chipping, grinding and irradiation. These processes could improve the enzymatic digestibility of lignocellulosic biomass [18]. Physical pretreatment is considered to be one of the most effective methods for increasing the enzymatic accessibility to lignocellulosic biomass. Nevertheless, many of the physical pretreatment are not feasible for the reason that it needs high energy cost.

Chemical pretreatment has the ability to remove lignin and hemicelluloses, decrease the degree of polymerization and crystallinity of the cellulose in lignocellulosic biomass [19]. It has been extensively investigated to be used for delignification of cellulosic material in the pulp and paper industry [20]. It has also been exploited to enhance biomass digestibility in an industrial pretreatment process [21]. Further, there are some chemicals which have been reported to have significant effect on the native structure of lignocellulosic biomass, do not produce toxic residues for the downstream processes, and where the reactions are carried out at room temperature and pressure [22].Chemicals ranging from oxidizing agents, alkali, acids and salts can be used to degrade lignin, hemicelluloses and cellulose from

lignocellulosic wastes. Organic acids such as oxalic acid, acetylsa licylic acid and salicylic acid can also be used as a catalyst, whereas an organic or aqueous organic solvent mixture with inorganic acids is also used to break the internal lignin and hemicellulose bonds [23]. However, concentrated acids are not preferred because they are corrosive and must be recovered to make the pretreatment economically feasible [24].

Physicochemical pretreatments that combine both the chemical and physical processes are of importance in dissolving lignin structure, which provide an improved accessibility of the cellulose for hydrolytic enzymes [25]. This category includes the vast majority of pretreatment methods such as steam explosion, liquid hot water, ammonia fiber/freeze explosion, wet oxidation, ammonia recycle percolation, and aqueous ammonia and CO_2 explosion [26]. Recently, ionic liquids and cellulose solvent-based lignocellulose fractionation processes have been proposed [27]. These types of pretreatments depend on process conditions and solvents used that affect the physical and chemical properties of the biomass.

Biological pretreatment is mostly associated with the action of fungi that are capable of producing enzymes to degrade lignin, hemicelluloses and polyphenols present in the biomass. It has attracted interest because of its potential advantages over physical/chemical pretreatments such as substrate and reaction specificity, low energy requirements, no generation of toxic compounds, and high yield of desired products [28]. However, its disadvantages are as apparent as its advantages, since biological pretreatment is a very slow process and requires careful control of growth

conditions and large space to perform [29]. In addition, most lignolytic microorganisms solubilize/consume not only lignin but also hemicellulose and cellulose [30]. Therefore, the biological pretreatment faces techno-economic challenges and is found less attractive commercially. Further, pretreatment of biomass is a global issue that demands an environment friendly process [31].

1.5 Successive saccharification and fermentation

Successive saccharification and fermentation is the process that saccharification and fermentation was carried out successively in each optimum conditions such as temperature, pH and so on. Saccharification is the critical step for bioethanol production where complex carbohydrates are converted to simple monomers. Compared to acid hydrolysis, enzymatic hydrolysis requires less energy and mild environment conditions [32]. The optimum conditions for cellulase have been reported as temperature of 40–50 °C and pH 4-5 [33]. Assay conditions for xylanase have also been reported to be 50 °C temperature and pH 4-5 [34]. Therefore, enzymatic hydrolysis is advantageous because of its low toxicity, low utility cost and low corrosion compared to acid or alkaline hydrolysis [35]. Here cellulase and hemicellulase enzymes cleave the bonds of cellulose and hemicellulose respectively. Figure 1.6 shows the image that cellulose is hydrolyzed into glucose and xylose.

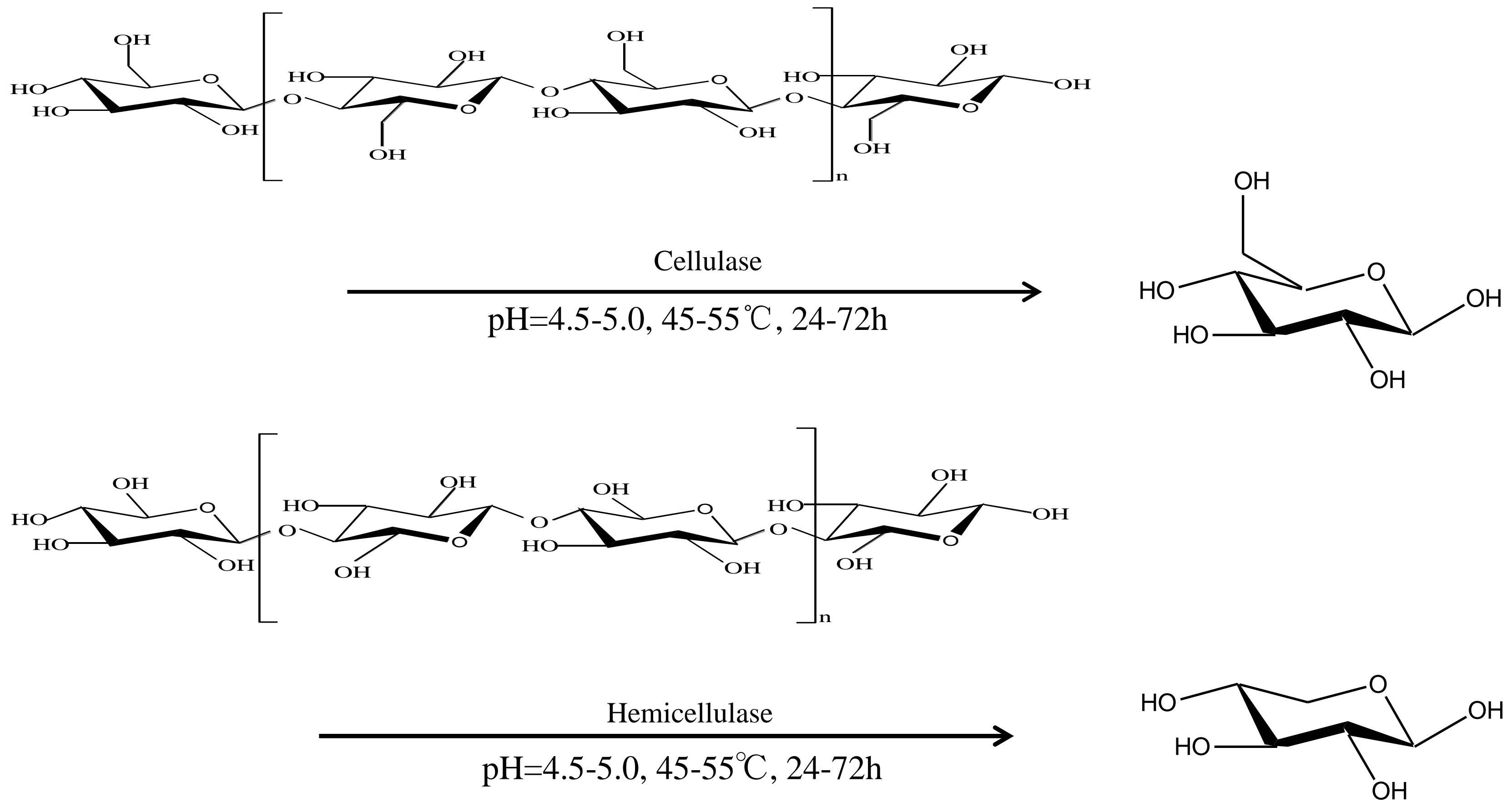


Figure 1.6 The image that cellulose is hydrolyzed into glucose and xylose.

Cellulase enzymes involve endo and exoglucanase and ß-glucosidases. Endoglucanase attacks the low crystallinity regions of the cellulose fiber, exoglucanase removes the cellobiase units from the free chain ends and finally cellobiose units are hydrolysed to glucose by ß-glucosidase [37]. Cellulose is hydrolysed to glucose whereas hemicellulose gives rise to several pentoses and hexoses. Several species of Clostridium, Cellulomonas, Thermonospora, Bacillus, Bacteriodes, Ruminococcus, Erwinia, Acetovibrio, Microbispora, and Streptomyces are able to produce cellulase enzyme. Many fungi such as Trichoderma, Penicillium, Fusarium, Phanerochaete, Humicola, Schizophillum sp. also have been reported for cellulase production [38]. Among the various cellulolytic microbial strains Trichoderma is one of the most well studied cellulase and hemicellulase producing fungal strains [39]. Trichoderma is able to produce at least two cellobiohydrolases and five endoglucanases and three endoxylanases [40]. However, Trichoderma lacks ß-glucosidase activity that plays an efficient role in polymer conversion [41]. On the other hand, Aspergillus is a very efficient ß-glucosidase producer [35]. Trichoderma cellulase supplemented with extra ß-glucosidase has been studied several times [42]. Combination of Trichoderma reesei ZU-02 cellulase and cellobiase from Aspergillus niger ZU-07 improved the hydrolysis yield to 81.2% with cellobiase activity enhanced to 10 CBU/g substrate [43].

Various factors influence yields of monomer sugars from lignocellulose. Temperature, pH and mixing rate are the main factors of enzymatic hydrolysis of lignocellulosic material [44]. Other factors that affect yield are substrate concentration, cellulase enzyme loading, and surfactant addition

[45]. High substrate concentration may lead to substrate inhibition. Cellulase contributes to the major cost of the lignocellulosic ethanol technology [36]. Therefore, an efficient pretreatment is to be selected to decrease cellulose crystallinity and to remove lignin to the maximum extent, so that hydrolysis time as well as cellulase loading will be minimized [46]. Surfactants modify the cellulose surface by adsorbing lignin onto surfactant and thus the surfactant prevents the enzyme from unproductive binding with lignin and lowers enzyme loading [47].

Several studies have been reported on the conversion of cellulosic biomass to sugars by enzymatic hydrolysis. Belkacemi and Hamoudi [48] studied enzymatic hydrolysis of corn stalk hemicellulose at 30 °C and pH 5 Saccharification was 90% and sugar was released after 10 h. Chen et al. [43] studied enzymatic hydrolysis of maize straw using cellulase from T. reesei ZU-02 and cellobiase from A. niger ZU-07. Addition of 5 g/L Tween 80 improved hydrolysis yield by 7.5%. Borjesson et al. [45] reported that PEG addition increased the enzymatic conversion of soft lignocellulose from 42% to 78% at 16 h where optimum hydrolysis temperature was 50 °C. Xu et al. [39] reported that T. reesei decomposed 68.21% of alkali pretreated rice straw whereas 73.96% conversion was obtained from alkali assisted photocatalysis of rice straw after enzymatic hydrolysis. Alkaline peroxide pretreated wheat straw showed 96.75% yield after enzymatic hydrolysis whereas atmospheric autocatalytic organosolv pretreated wet wheat straw gave above 75% yield [49].

The saccharified biomass is used for fermentation by several

microorganisms. But the industrial utilization of lignocelluloses for bioethanol production is hindered by the lack of ideal microorganisms which can efficiently ferment both pentose and hexose sugars [50]. For a commercially viable ethanol production method, an ideal microorganism should have broad substrate utilization, high ethanol yield and productivity, should have the ability to withstand high concentrations of ethanol and high temperature, should be tolerant to inhibitors present in hydrolysate and have cellulolytic activity. Genetically modified or engineered microorganisms are thus used to achieve complete utilization of the sugars in the hydrolysate and better production benefits.

1.6 Simultaneous saccharification and fermentation

To hydrolyze cellulose into soluble sugars, multiple enzymatic activities are required. These activities include endoglucanase, exoglucanases including cellobiohydrolase and cellodextrinase, and ß-glucosidase [51], whereas reaction products such as cellooligosaccharide and glucose inhibit cellulase activity [52]. Thus, to avoid the product inhibition, enzymatic hydrolysis is performed together with fermentation, a process referred to as simultaneous saccharification and fermentation (SSF). In the SSF process, glucose released by cellulase action is directly converted to ethanol by the fermenting microorganisms, which alleviates problems caused by the end product [53]. Also, the consumption of glucose and the presence of ethanol in the culture medium would reduce the risk of undesired contamination by

glucose-dependent organisms. Recently, consolidated bioprocessing which combines enzyme production, saccharification and fermentation in a single step, has gained recognition as a potential bioethanol production system because the costs of capital investment, substance and other raw materials, and utilities associated with enzyme production can be avoided [54].

However, one of the major drawbacks in the SSF process is the optimum temperature required for the saccharification and fermentation stages. Saccharification with cellulolytic enzymes is best done around 50 °C, while most fermenting microbes have an optimum temperature for ethanol fermentation between 28 °C and 37 °C [55]. In practice, it would be difficult to lower the optimum temperature of cellulases through protein engineering. Accordingly, high-temperature fermentation is in high demand for simultaneous saccharification and fermentation, and thermotolerant yeast strains have been screened for the ability to ferment ethanol [56]. This review will focus on the SSF process with thermotolerant yeast strains. In particular, development of yeast strains expressing heterologous cellulolytic enzymes for direct conversion of cellulosic materials to ethanol at elevated temperature is emphasized.

Ethanol production at elevated temperature has received much attention because of many advantages such as a reduction in the costs associated with cooling, continuous evaporation of ethanol from broth under reduced pressure, a reduced risk of contamination, suitability for application in tropical countries as well as the improvement of SSF efficiency [57]. Abdel-Banat et al. [56] calculated that a 5 °C increase in the fermentation temperature can

greatly reduce the cost of fuel ethanol production from starchy materials with a hyperthermostable α-amylase by reducing cooling energy. The generation of ethanol vapor during fermentation maintains the ethanol concentration at low levels that are not harmful to the fermenting yeast, which could also simplify subsequent distillation [56].

SSF is generally preferred because it can involve less equipment, which would reduce the investment cost. The operation is also simplified by the integration of saccharification and fermentation. One of the significant reasons for using SSF is the avoidance of cellulase inhibition by glucose and thereby increasing the saccharification rate and ethanol yield. However, the disadvantage of the SSF process is the lower efficiency of hydrolysis carried out at lower temperature to be compatible with yeast fermentation compared to separate hydrolysis and fermentation systems.

1.7 Recombinant yeast pYBGA1

A recombinant laboratory yeast pYBGA1 was applied for the fermentation of ethanol from the agricultural residues. Recombinant yeast pYBGA1 was produced by encoding both ß-glucosidase (*bglA*) gene of *Aspergillus kawachii* IFO4308 and uracil-encoding gene *Ura3* in *S. cerevisiae* YPH499 (*MATa ura3 lys2 ade2 trp1 his3 leu2*) [58]. IFO4308 encodes both extracellular and cell wall-bound ß-glucosidases [59, 60].It is found that glucose produced by cellobiose and cellotriose around cell wall could be absorbed efficiently by yeast strains. The pYBGA1 yeast could directly

ferment ethanol from cellobiose with a 1, 4-ß-glucopyranosidic linkage. Previously, it is reported a method for a two-step acid hydrolysis of cellulose materials such as tissue paper, cotton, and sawdust to give a mixture of cello-oligosaccharides containing glucose, cellobiose, and higher cello-oligosaccharides, a 40 g/L solution of which was fermented with pYBGA1 yeast to give ethanol as high as 70% conversion and 19 g/L concentration [60,61].

Recombinant yeast pYBGA1 was incubated in the agarose medium as a carbon source at 30℃ for a week. The compositions of the incubation medium was showed in Table 1.5.

Table 1.5 The compositions of pYBGA1 yeast incubation medium

Cellobiose	YNBw/oAA	-Ura Supplement
2%(w/v)	0.67%(w/v)	0.077%(w/v)

After preincubation recombinant yeast pYBGA1 was stored at the temperature of 4℃ and preincubated every 3 months. The amount of yeast cells was confirmed by a cell counter. It is reported that recombinant yeast pYBGA1 has ability to make cello-oligosaccharides saccharified and ferment to ethanol.

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Chapter 2

Successive Saccharification and Fermentation of Cellulosic Agricultural Residues using a Combination of Cellulase and Recombinant Yeast

2.1 Abstract

Bioethanol was produced from agricultural residues in Hokkaido Prefecture, Japan, by dilute alkali pretreatment, and then successive saccharification with cellulase and fermentation with recombinant yeast pYBGA1 that is a recombinant laboratory yeast expressing-glucosidase. The agricultural residues, beet pulp, beet leaf, corn stover, and weed were

pretreated with 2% NaOH aqueous solution for 1 h at 121°C to remove lignin.

The pretreated beet pulp (20g/L), for example, was then saccharified for 24 h by Cellic CTec (10 wt% to beet pulp) as the cellulase at 50°C to give glucose (11.8 g/L) at relatively good rates. After the temperature decreased to 30°C, the resulting glucose was continuously fermented for 36 h by addition of 1 x 108 cells/mL of pYBGA1 yeast to give 5.54 g/L of bioethanol in 60 % and 92% theoretical yields of cellulose in beet pulp and glucose saccharified, respectively.

Other agricultural residues also afforded bioethanol by the successive saccharification and fermentation. It was found that the appropriate cellulase for saccharification differed with the kind of agricultural residue; beet pulp was effectively saccharified by Cellic CTec, beet leaf and corn stover by Sucrose C, and weed by Sumizyme C. These agricultural residues were found to be suitable for the production of bioethanol because Hokkaido Prefecture is the most abundant producer of beets and corn in Japan, and accordingly, agricultural residues are easily available. pYBGA1 was also found to be a good yeast for the fermentation of sugars saccharified from agricultural residues.

2.2 Introdution

Global energy inevitable depletion has generated strong interest in the development of more sustainable transportation fuels and renewable carbon-based raw materials, such as those produced from biomass. Biofuels derived

from lignocelluloses show promising future due to its sustainability, greenhouse gas emissions, lower-cost, and significantly greater availability of feedstock reserves as compared to starch-based ethanol.

Bioethanol produced from renewable plant resources is expected to provide an environment-friendly and clean energy to replace fossil fuels. The raw materials currently used for the industrial process are mainly starches of grains and corn, which are foods. However, cellulosic bioethanol from non-food plants is becoming increasingly attractive because of the great abundance of cellulose produced by plants on earth [1].

Although both starch and cellulose are composed of glucose, unlike starchy biomasses, native cellulose is highly resistant to enzymatic saccharification because it has crystalline regions in the molecule that are present as a lignin-cellulose complex. Lignin inhibits enzymatic saccharification and fermentation.

Therefore, pretreatment is needed to relax the highly crystalline structure and to remove lignin. Although the crystalline region was completely hydrolyzed to glucose by concentrated sulfuric acid [2], a large amount of toxic sulfuric acid is required and it is difficult to recover. Therefore, industrial saccharification of cellulosic raw materials to glucose is accomplished by use of dilute sulfuric acid, followed by fermentation with yeast [3, 4].

Several investigations of the production of bioethanol from cellulosic raw materials have been reported. Microorganisms appropriate for cellulase production and experimental conditions for saccharification were also

reported. Among them, Panicum virgatum, commonly known as switchgrass, was pretreated by soaking in ammonium hydroxide (30%) at 15° to 30°C in pilot scale bioreactors.

Simultaneous saccharification and fermentation of the pretreated switchgrass with Saccharomyces cerevisiae gave 73% of the theoretical yield bioethanol in the 50 L reactor and 52-74% in the 350 L reactor [5]. An alkali pretreated rice straw was saccharified by a microorganism to give glucose as a main component in the hydrolyzates, suggesting that Tricoderma ZM4-F3 is an effective and useful fungus for cellulase production [6].

Corn stover was used as a substrate for several investigations of the production of bioethanol from cellulosic raw materials have been reported. Microorganisms appropriate for cellulase production and experimental conditions for saccharification were also reported. Among them, Panicum virgatum, commonly known as switchgrass, was pretreated by soaking in ammonium hydroxide (30%) at 15° to 30°C in pilot scale bioreactors. Simultaneous saccharification and fermentation of the pretreated switchgrass with Saccharomyces cerevisiae gave 73% of the theoretical yield bioethanol in the 50 L reactor and 52-74% in the 350 L reactor [5].

An alkali pretreated rice straw was saccharified by a microorganism to give glucose as a main component in the hydrolyzates, suggesting that Tricoderma ZM4-F3 is an effective and useful fungus for cellulase production [6]. Corn stover was used as a substrate for enzymatic saccharification by a cellulase produced from Penicillium funiculosum.enzymatic saccharification by a cellulase produced from Penicillium funiculosum.

The conversion of hemicellulose and cellulose in corn stover pretreated with dilute NaOH was more than 80% after 24 h of saccharification when using a 5 g/L initial concentration with 80 units/mL of the enzyme, respectively [7, 8, 9]. Extracellular enzymes produced by Phanerochaete chrysosporium degraded lignin in corn fiber and saccharified it, and the subsequent simultaneous saccharification and fermentation of the hydrolysates using Saccharomyces cerevisiae enhanced bioethanol production [10]. The sugar composition and enzymatic saccharification of fractionated beet pulp, which is a residue after extraction of sugar from beets, were carried out to investigate microorganisms having cellulase, and indicated that Aspergillus niger enzyme produced much higher saccharification of beet pulp [11].

Previously, we reported a two-step acid hydrolysis of tissue paper, cotton, and sawdust, with the result that whole celluloses including the crystalline region were hydrolyzed to obtain a mixture of glucose and cello-oligosaccharides; that is, these cellulosic materials were partially hydrolyzed by concentrated sulfuric acid (80wt%) in the first step, and then the hydrolysis was continued in the second step with hot sulfuric acid that was diluted with water. The hydrolyzates from tissue paper, cotton, and sawdust were fermented with recombinant pYBGA1 yeast converting ß-glucosidase into ethanol in good yields [12, 13]. We also reported that ethanol was produced at good rates by direct saccharification and fermentation of cello-oligosaccharides with pYBGA1 yeast. For example, cellobiose in the concentration of 50 g/L was directly fermented for 60h with 1 x 10^8 cells/ml of

pYBGA1 yeast at 30°C to give ethanol at an 80% theoretical conversion rate and a concentration of more than 20g/L [14]. These results suggest that pYBGA1 yeast has some advantages as a simple procedure for effective fermentation of glucose and cello-oligosaccharides and for reducing time, cost, and energy consumptions [14].

Cellulosic agricultural residues are suitable raw materials for the production of bioethanol in local areas because they are wastes, not foods, and bioethanol, especially, is an ideal energy source for local production and local consumption. Hokkaido Prefecture is in the northern part of Japan, and agriculture is one of its important industries [15].

Among the locally available agricultural products, beets and corn are the most abundant in Hokkaido Prefecture. The recent 5-year average of the productions was 3663 kt and 111 kt for beets and corn, respectively [16, 17]. Therefore, these agricultural residues are promising raw materials for the production of bioethanol while protecting the environment, but effective application of agricultural residues has not been achieved yet.

In this paper, we report the saccharification of cellulosic agricultural residues by several cellulase enzymes, followed by fermentation of the resulting glucose by recombinant pYBGA1 yeast. A combination of cellulase and pYBGA1 yeast was also investigated with each agricultural residue.

2.3 Experimental

2.3.1 Materials

The plants used in the experiments include corn stalk, grass, beet and beet leaf. Beet pulp was supplied by a local company, Hokkaido Togyo Co. Ltd., Hokkaido, Japan. Beet leaf and corn stover were provided by a local farm in Kitami City, Hokkaido. Weeds were obtained from the lawn of our university. They were all washed buy tap water to remove soil and other particles, and then dried by oven at 40℃till constant weight . Thereafter, the plant was shredded and grinded to powder by a mill. The processed materials were stored in sealed glass bottles at low ambient temperature for further usage.

All the chemicals used were of analytical grade and mainly purchased from and used as such without further purification.

pYBGA1 yeast was kindly supplied by the National Research Institute of Brewing and was pre-incubated on agarose medium with cellobiose as a carbon source according to the procedure described in our previous paper [14]. The pYBGA1 yeast cells were counted using a cell counter.

Cellulases used in this work were obtained from the respective companies. Table 2.1 shows cellulases used in this work. Yeast extract and peptone were purchased from Merck Chemicals, Japan, and Kyokuto Pharmaceutical Industrial Co. Ltd., Japan, respectively.

Table 2.1 Cellulases complex used for saccharification of cellulosic agricultural residues .

No.	Enzyme	Co.	Declared Activity	Physical from	Optimal Temp. (°C)	Optimal pH
①	Cellic CTec	Novozymes	1000 EGU/g	Liquid 1.15 g/ml	45	5.0
②	Meicelase	Meiji Seika	400 FPU/g	Powder	45	4.5
③	"Onozuka" RS	Yakult	20,000 U/g	Powder	45	4.5
④	Sucrase C	Mitsubishi Kagaku	3,000 U/g	Powder	60	5.0
⑤	A [Amano]3	AMANO Enzymes	30,000 U/g	Powder	55	4.5
⑥	T [Amano]4		280 U/g	Powder	45	4.5
⑦	Sumizyme C	Shin Nihon Chem.	1,500 U/g	Powder	50	4.5
⑧	Sumizyme AC		2,000 U/g	Powder	60	4.0
⑨	GODO-TCL	Oenon	100 FPU/g	Liquid 1.1 g/ml	55	4.5
⑨	New Meicelase	Meiji Seika	-	Powder	45	4.5
⑪	Acremonium		-	Powder	45	4.5

2.3.2 Measurement

Thermogravimetric analysis (TGA) was conducted with a Shimadzu DTG-60 thermoanalyzer during heating at 10°C/min under a nitrogen atmosphere.

Saccharified sugars were measured quantitatively at 40 °C by a reverse-phase HPLC system with a Tosoh TSK-gel amide-80 column (5 μm, 4.6 mm x 250 mm) eluted with a 1:1 acetonitrile and water solution at a flow rate of 0.5 mL/min with a Tosoh RI detector using glucose as a standard in the calibration curve.

The concentration of ethanol produced was determined under a nitrogen atmosphere by a Shimadzu GC-8A gas chromatograph with a Shimadzu capillary column (SE-30, 3.2 mm x 30 m) and a hydrogen flame ionization detector. The column and injection temperatures were 60°C and 130°C, respectively.

2.3.3 Compositional analysis

Cellulose, hemilcellulose and ligin make up a major portion of biomass residue. These constituents must be determined as it is very important to the comprehensive analysis of biomass residue. The content of cellulose, hemilcellulose, ligin and ash present in the biomass was determined according to the procedures described by National Renewable Energy Laboratory (NREL).

Firstly, weighed 300.0±10.0 mg of the agricultural residue sample and then treated by the Soxhlet extraction method to determine ethanol extraction and oil extraction. Added 72% sulfuric acid to samples into pressure tube with cap and mixed with a stir until the samples totally mixed. The mixture was placed in a water bath at 30℃ for 1h. Keep stirring the residue sample without removing the sample from bath. Then the sulfuric was diluted to 4% by adding 84 mL deionized water and autoclaved at 121℃ for 1h.

After completion of the autoclave cycle, allowed the mixture to slowly cool to near room temperature before removing the caps. Thereafter, the reaction mixture was filtered. The residue was dried for lignin analysis and the solution was for cellulose and hemicellulose analysis by HPLC. Determination of ash and water was implemented by simultaneous DTA-TG apparatus (DTG-60, SHIMADZU).

2.3.4 Dilute alkali pretreatment of agricultural residues

The processed biomass materials were pretreated by dilute alkali before enzymatic saccharification. A typical procedure for the pretreatment of the agricultural residues with dilute alkali solution was as follows. The dried and powdered plant samples were soaked in 2% NaOH solutions with the ratio of solid to liquid as 1:5 for 1h. Then the alkali pretreatment was carried out in a autoclave at temperature of 121℃ and pressure of 115KPA in 0.5-2h.

Subsequently, the plant samples were washed with distilled water

several times until the used water was neutral. The resulting beet pulp was dried in the oven at 50 °C under vacuum to give 2.2 g of alkali treated beet pulp. Other agricultural residues (5.0 g each sample), beet leaf, corn stover, and weed, were also pretreated by the same procedure as above to give 2.1-2.3 g of pretreated residues, respectively.

2.3.5 Successive saccharification and fermentation of alkali-pretreated agricultural residues

A typical procedure for the successive saccharification and fermentation of alkalipretreated agricultural residue is as follows. In a 300 mL Erlenmeyer flask, 100 mL of deionized water was added to 2.0 g of the alkali-pretreated powdered beet pulp. The pH of the solution was adjusted to 4.5 by dilute aqueous HCl, and then Cellic CTec (10 wt% of beet pulp) was added. The flask was sealed with a sponge stopper and the mixture was incubated in an oven for 24 h at 45 °C using gentle stirring by a rotary shaker.

The samples were collected from saccharifacation solution at regular time intervals and was heated to 100℃ immediately for 20 minutes to denature the cellulase. Then the samples were centrifuged at 10000 rpm for 5 minutes and the supernatant was taken out for the sugar analysis. The saccharifacation residues were collected and weighted for further analysis. After saccharification the saccharifacation solution was denatured by autoclave for 20 minutes at 121℃ and then maintained at 4℃ for further fermentation.

The yeast SD-Ura pYBGA1 was provided by the National Research Institute of Brewing. The yeast pYBGA1 was maintained in the agar plates at 4℃ in the refrigetator and transferred periodically. The agar medium contained 6.7g/L of Difco Yeast Nitrogen Base w/o Amino Acide, 20g/L of cellobiose, 20g/L of agar and 0.77g/L -Ura DO suppropiate. The incubation of the yeast pYBGA1 was carried out for 150h at 30℃. A typical procedure for determination of the yeast cells quantity is as follows. Yeast pYBGA1 was added into deionized water and made sure that the sample was mixed thoroughly. The mixture was injected into cell counter and observed by microscope, and then the number of yeast pYBGA1 could be counted and calculated.

For the ethanol fermentation, 10 g/L of yeast extract and 20 g/L of peptone were added into saccharifacation solution. The pH of the solution was adjusted to 5.0 with dilute aqueous NaOH and then the mixture solution was autoclaved at 121℃ for 20 minutes. After lowering the temperature of the saccharified solution to 30 °C, pYBGA1 yeast with the quantity of 1×10^8 cells/mL was added to the saccharified solution. The fermentation was continued at 30°C for further 36 h in an oven with gentle stirring using a rotary shaker. The saccharified glucose and fermented ethanol was sampled using a syringe with a long needle every 6 h to measure the concentration of the products. The samples were withdrawed at regular time intervals and denatured immediately for sugar and ethanol analysis.

2.4 Results and Discussion

2.4.1 Alkali-pretreatment of agricultural residues

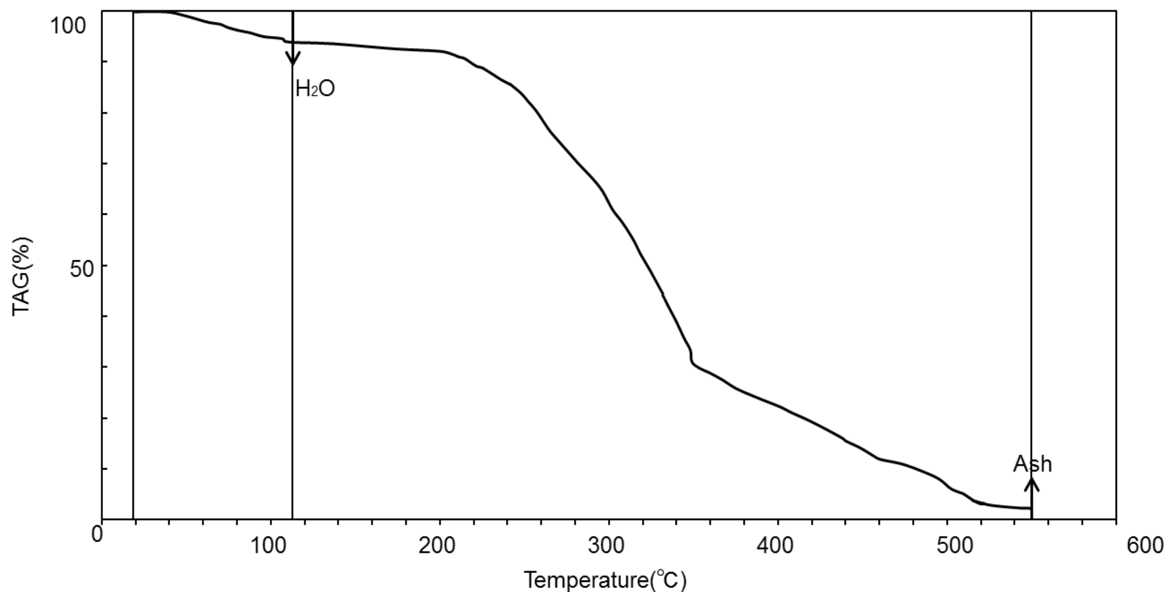

Figure2.1 Thermogravimetric analysis of beet pulp was carried out by DTG-60(SHIMADZU). Heated to 110℃ at 10℃/minute and held at 110℃ for 10minutes,the weight reduced is water content of 5.6%. And then heated to550℃ at 10℃/minute and held at 550℃ for 20minutes,the residue is ash content of 2.5%.

Thermogravimetric analysis (TGA) is a method of thermal analysis in which changes in physical and chemical properties of materials are measured as a function of increasing temperature (with constant heating rate), or as a function of time (with constant temperature and/or constant mass loss). Changes of water and ash was measured though the sample mass weight loss. Figure 2.1 show the mass loss of beet pulp with certain temperature increased though DTG-60(SHIMADZU). Heated the beet pulp from room temperature to 110℃ at rate of 10℃/min and then hold at 110℃ for 10 minutes. Because

of volatilization of water with temperature increased the weight percentage of resulting mass change was measured as water component. In the process of heating to 550℃ beet pulp sample lost weight continuously due to decomposition and oxidation of carbohydrates. The constant residue weight was measured as ash component. As shown in figure 2.1 water and ash component of beet pulp was 5.6% and 2.5%, respectively.

Table 2.2 shows the results of pretreatment of dried and powdered agricultural residues used in this work with 2% aqueous NaOH solution for 1 h at 121°C in the autoclave. Dilute acid pretreatment was also reported, but, toxic furfural derivatives were produced as a by-product [2, 3, 13]. Therefore, we used a dilute alkali for the pretreatment to remove lignin, which inhibits enzymatic saccharification, followed by fermentation. As shown in Table 2.2, the concentrations of cellulose and hemicellulose of beet pulp increased and decreased, respectively, to 74.0 wt% and 10.9 wt% from 58.3 wt% and 16.7 wt%. The lignin concentration decreased to below one third. The cellulose, hemicellulose, and lignin concentrations of other residues also showed the same tendency as beet pulp before and after pretreatment. The pretreatment caused a slight loss of polysaccharides but significantly reduced the lignin concentration of the agricultural raw materials, enhancing enzymatic saccharification.

Table2.2 Composition of unused plant resources in Hokkaido prefecture, Japan, before and after alkaline treatment.

	Weight g	Cellulose wt%	Hemicellulose wt%	Lignin wt%	Water wt%	Ash wt%	Others wt%
Beet pulp							
Before	5.0	58.3	16.7	4.2	5.6	2.5	12.7
After	2.2	74.0	10.9	1.2	6.5	2.2	5.2
Beet leaf							
Before	5.0	36.6	23.3	9.4	10.1	18.0	2.6
After	2.3	61.5	13.2	2.2	11.3	11.5	0.3
Corn stover							
Before	5.0	46.2	28.0	10.6	5.4	6.5	3.3
After	2.2	66.4	19.5	2.8	5.8	4.8	0.7
Weed							
Before	5.0	44.5	30.8	11.2	5.2	6.2	2.1
After	2.1	53.6	28.3	7.8	6.3	3.7	0.3

Figure 2.2 shows the remove of lignin of agricultural residues. It is very difficult to degrade lignin for its components being held together with strong chemical bonds and it also appears to have a lot of cross-linked internal H bonds. It is bonded in complex and various ways to hemicellulose in wood. After pretreatment by 2% aqueous NaOH solution for 1 h at 121°C in the autoclave, some lignin was removed and dissolved in the alkali solution.

The lignin of alkaline untreated beet pulp was 4.2% calculated by the method described by NREL, and it reduced to 1.2% by 70% of lignin removed after 2% aqueous NaOH solution treatment. It has been reported that amorphous cellulose with little presence of lignin and hemicellulose offers benefits in terms of the highest sugar yields, the fastest hydrolysis rates, the least use of enzyme, possible enzyme recycling, lower electricity consumption for hydrolysis mixing, and lower capital investment for smaller size hydrolysis reactors [33].Therefore, lignin residues in 2% NaOH-treated agricultural residues will be beneficial for enhancing enzymatic saccharification. The beet leaf, corn stover and weed lignin was removed by 75%, 75%, 30%, respectively. It has been reported that the delignification of lignin straw and wheat straw attained 77.3% and 72% reductions when pretreatment was carried out in an autoclave at 121°C by 2% NaOH for 90 min, respectively [34].The delignification of weed was low than other agricultural residues due to the composition was different and the untreated weed has high level lignin. The removal of lignin decreases the influence to the enzymatic saccharification and ethanol fermentation [35].

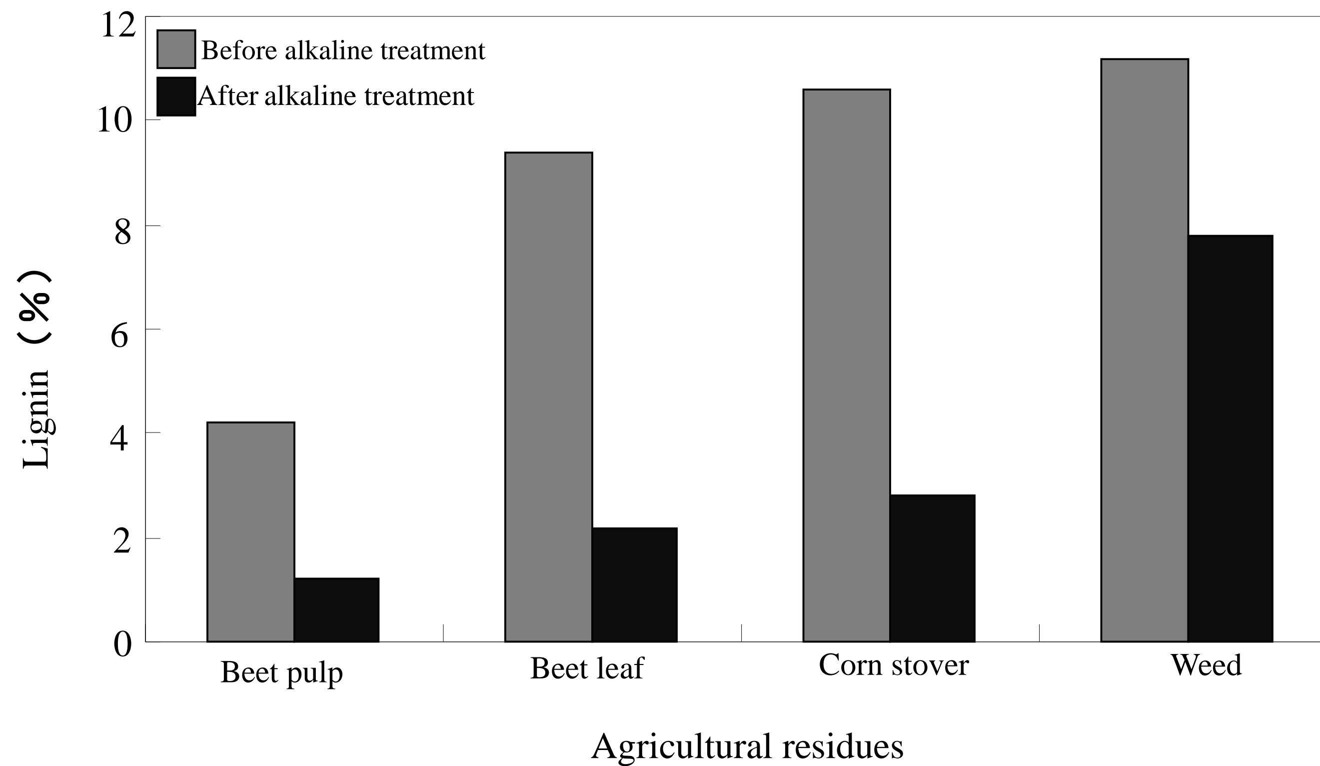

Figure 2.2 Lignin removal (weight %) from Hokkaido agricultural residues by dilute alkaline treatment. Dilute alkaline treatment was carried out in an autoclave at 121°C by 2% NaOH for 60 min.

2.4.2 XRD analysis of agricultural residues

The cellulose content of alkaline treated samples and natural agricultural residues are presented in Table 2.2. The slight decrease of cellulose recovery at 2% NaOH-treated sample was due to the strong alkaline effect of NaOH on agricultural residues fibres. The measurement of cellulose crystallinity provides a qualitative or semi-quantitative evaluation of the amount of amorphous and crystalline cellulosic compounds in the alkaline treated agricultural residues samples. The main effect of NaOH-treatment is the removal of hemicellulose and lignin, which are amorphous in structure. In the present work, the CrI values of agricultural residues samples were determined by measuring the relative amount of crystalline cellulose in the total solid. The amorphous part includes not only amorphous cellulose but also hemicellulose and lignin. The X-ray methods measure the crystallinity of the entire material, including the hemicellulose and lignin, in addition to amorphous cellulose [36].Therefore, the CrI is not only influenced by the composition of the biomass but also the crystallinity of cellulose itself. We performed XRD studies on 2% NaOH-treated agricultural residues samples, untreated agricultural residues samples, and natural cellulose powder. The XRD analysis of weed samples is graphically presented in Fig 2.3. The CrI of the NaOH-treated samples was calculated though the method described by Ant-Wuorinen and compared with the CrI of natural powder [37]. The CrI of the NaOH-treated weed and nature cellulose powder was 0.32 and 0.79, respectively. It is clear that weed treated with 2% NaOH led to significant

decrease of CrI compared to natural cellulose powder due to the alkaline treated changed the nature cellulose structure and loose the cellulose molecular chain. The nature weed XRD image shows that CrI cannot be calculated. That might because of the existence of hemicellulose and lignin which are amorphous in structure. After alkaline treated hemicellulose and lignin content was removed and the cellulose crystallinity was emerged. It has been reported that changes of CrI might be caused by the degradation and removal of amorphous components such as lignin and hemicelluloses [38]. The changed CrI of NaOH-treated weed suggests that cellulose became more exposed. Changed CrI after pretreatment has been observed in many previous investigations [39]. In the present work, the highest peak almost does not change in proportion to the cellulose content of the biomass. It indicates that the increased CrI upon alkaline NaOH-treatment is primarily due to removal of amorphous substances (lignin and hemicellulose), not due to changes in the basic crystalline structure of the cellulose. The decreased CrI value in 2% NaOH treated weed indicated that the crystalline cellulose was perhaps destructured.

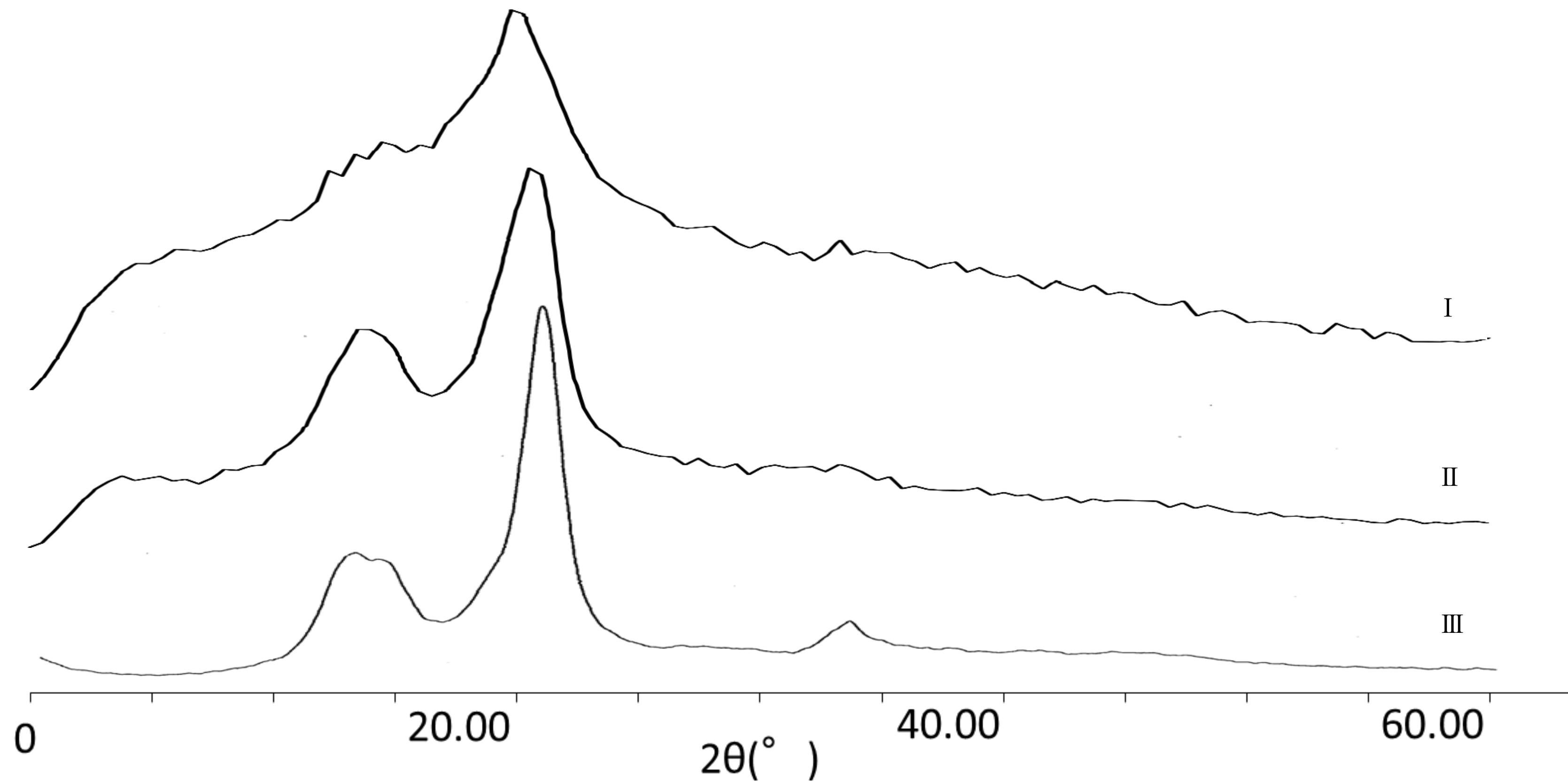

Figure 2.3 The XRD images of weed and cellulose powder

Ⅰ-Untreated weed, Ⅱ-Alkaline treated weed, Ⅲ-Cellulose powder.

All treatments were carried out at 10° /min. Here, xaxis represents diffraction angle (2θ) and y-axis represents intensity (cps) of Miscanthus. The graph has been recorded from the XRD-raw data.

2.4.3 Successive saccharification and fermentation of agricultural residues

Saccharifid sugar yield is the most important factor for evaluating the performance of sodium hydroxide in this pretreatment condition. Lignocellulose pretreatment conditions affect substrate characteristics, resulting in different enzymatic hydrolysis performances at different enzyme loadings [38].

In the current work, three different cellulases loadings have been tested for saccharification of natural and 2%NaOH-treated beet pulp to evaluate pretreatment effect properly. Saccharifid sugar yield of natural and 2%NaOH-treated beet pulp was showed in Table 2.3. The saccharifid sugar yield of untreated beet pulp was 0 and 2% NaOH-treated beet pulp saccharifid sugar yield was 11.8g/L when cellulase Cellic CTec was input. While Sucrase C was input saccharifid sugar yield increased from 0.7 g/L of untreated beet pulp to 9.6 g/L of 2% NaOH-treated beet pulp. In the case of cellulase Meicelase saccharifid sugar yield increased from 1.3 g/L of untreated beet pulp to 8.0 g/L of 2% NaOH-treated beet pulp.

Therefore, the data in Table 2.3 demonstrate that alkali treated significantly improved the enzymatic saccharification of beet pulp samples. The saccharifid sugar yield was different due to activity of cellulases was different. Cellulase Cellic CTec cannot saccharify untreated samples totally while Meicelase saccharifies untreated samples for a little of sugar.

Table2.3 Saccharifid sugar yield of natural and 2%NaOH-treated beet pulp.

Cellulase [1]	Sample [2]	pH	Saccharification Temp.	Time	Sugar
			℃	h	g/L
Cellic CTec	Beet pulp	5.0	45	24	0
	2%NaOH-treated beet pulp	5.0	45	24	11.8
Sucrase C	Beet pulp	5.0	60	24	0.7
	2%NaOH-treated beet pulp	5.0	60	24	9.6
Meicelase	Beet pulp	4.5	45	24	1.3
	2%NaOH-treated beet pulp	4.5	45	24	8.0

1) The addition of Cellic CTec was10 wt%.

2) The sample 20 g/L was carried out for the experiment.

Different cellulases loadings showed in Table 2.1 have been tested for saccharification of 2%NaOH-treated beet pulp to evaluate cellulases saccharification effect properly. 20 g/L of alkaline-pretreated beet pulp was added with10 wt% of each cellulases and the saccharification was carried out at the optimal conditions of cellulases. As Table 2.2 showed, alkaline-pretreated beet pulp contains 74% cellulose and 1.2% lignin. When Cellic CTec was added and saccharified for 24h, 11.8 g/L sugar was reduced and the conversion 80% was obtained. The highest reducing sugar yield of alkaline-pretreated beet pulp was 65%, 60% and 58% when Sucrase C, Sumizyme C and Meicelase was used, respectively. Almost all of the cellulases saccharification at 24h obtained the highest sugar yield, which might because that sugar reduced was absorbed around the cellulased as the saccharification was going on. Sugar reduced could influence the saccharification and 24h was considered to be the optimal time for the saccharification when cellulases(showed in Table 2.1) was input with 10 wt%.Various factors influence yields of monomer sugars from lignocellulose. Temperature, pH and mixing rate are the main factors of enzymatic hydrolysis of lignocellulosic material [40]. Other factors that affect yield are substrate concentration, cellulase enzyme loading, and surfactant addition [41]. High substrate concentration may lead to substrate inhibition. Cellulase contributes to the major cost of the lignocellulosic ethanol technology [42]. Surfactants modify the cellulose surface by adsorbing lignin onto surfactant and thus the surfactant prevents the enzyme from unproductive binding with lignin and lowers enzyme loading [43].

Several studies have been reported on the conversion of cellulosic biomass to sugars by enzymatic hydrolysis. Belkacemi and Hamoudi [44] studied enzymatic hydrolysis of corn stalk hemicellulose at 30℃ and pH 5. Saccharificationwas 90% and sugar was released after 10 h. Chen et al. [45] studied enzymatic hydrolysis of maize strawusing cellulase from T. reesei ZU-02 and cellobiase from A. niger ZU-07. Addition of 5 g/L Tween 80 improved hydrolysis yield by 7.5%. Borjesson et al. [46] reported that PEG addition increased the enzymatic conversion of soft lignocellulose from 42% to 78% at 16 h where optimum hydrolysis temperature was 50℃. Xu et al. [47] reported that T. reesei decomposed 68.21% of alkali pretreated rice straw whereas 73.96% conversion was obtained from alkali assisted photocatalysis of rice straw after enzymatic hydrolysis. Alkaline peroxide pretreated wheat straw showed 96.75% yield after enzymatic hydrolysis whereas atmospheric autocatalytic organosolv pretreated wet wheat straw gave above 75% yield [48].

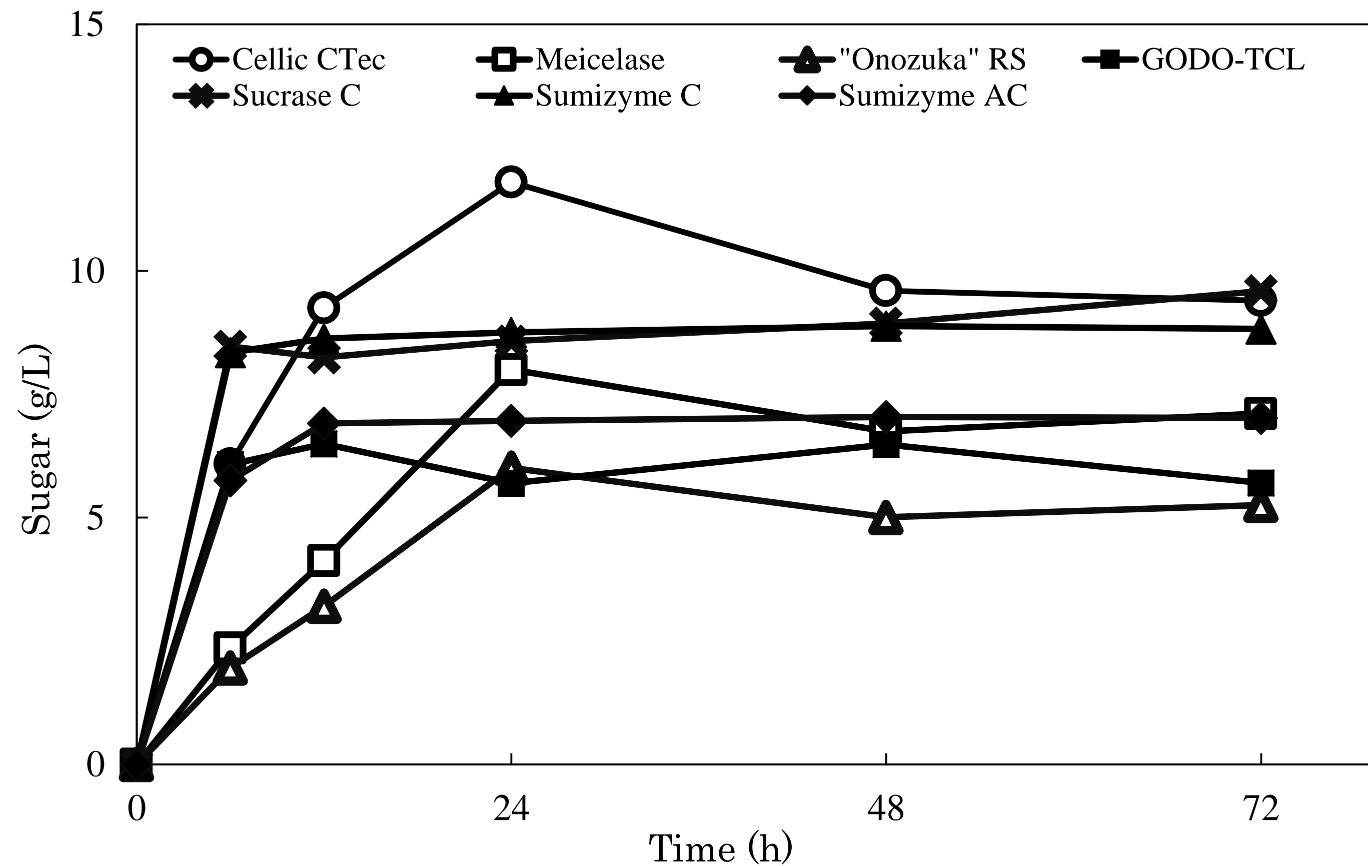

Figure 2.4 Saccharifid reducing sugar yield obtained from beet pulp by different cellulases.
20 g/L of alkaline-pretreated beet pulp was added with10 wt% of each cellulases and the saccharification was carried out at the optimal conditions of cellulases.

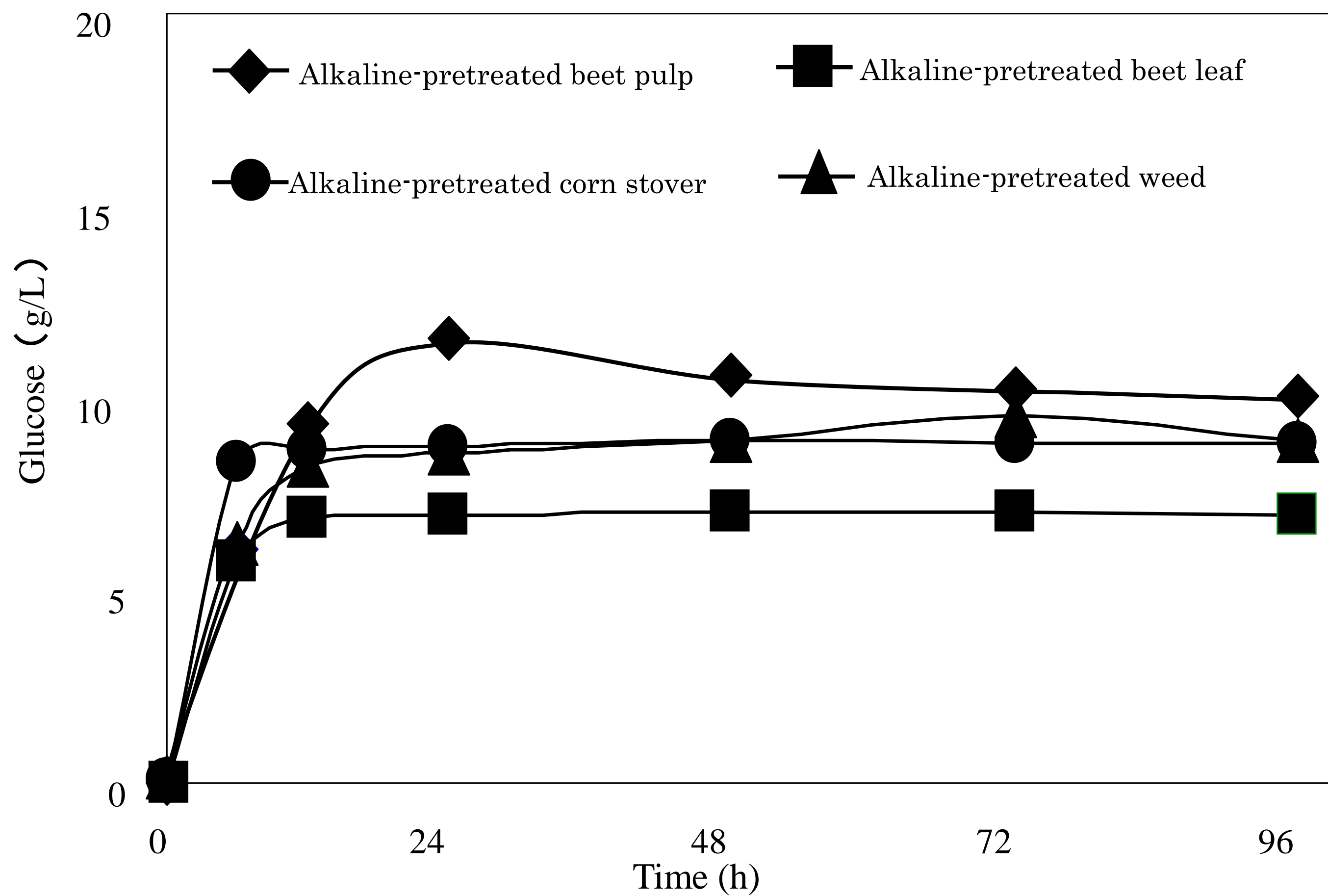

Figure 2.4 Saccharifid reducing sugar yield obtained from agricultural residues by cellulase Cellic CTec. 20 g/L of alkaline-pretreated agricultural residues was added with10 wt% of cellulase Cellic CTec and the saccharification was carried out at 45℃ and pH 5.0.

As cellulase Cellic CTec obtained highest sugar yield among these cellulases in the experiment of saccharification of alkaline treated beet pulp, its sugar conversion for other alkaline treated agricultural residues was tested. Figure 2.4 showed saccharification of alkaline treated agricultural residues by cellulase Cellic CTec.20 g/L of alkaline-pretreated agricultural residue samples was added with10 wt% of cellulase Cellic CTec and the saccharification was carried out at 45℃. As the saccharification proceeded, the concentration of glucose increased rapidly, and 8.6 g/L of glucose was obtained from alkaline-pretreated corn stover after 24 h of saccharification with the sugar conversion of 80%. 8.8g/L and 7.0 g/L glucose was obtained from alkaline-pretreated beet leaf and weed, respectively. Sugar concentration from alkaline-pretreated beet pulp was higher than other alkaline-pretreated agricultural residue samples due to the high cellulose content. The sugar convertion of alkaline-pretreated beet leaf and weed was 54% and 67%, respectively. Sucrase C, Sumizyme C and Meicelase was aslo carried out in saccharification experiment as they obtained relatively higher sugar conversion. Table 2.4 showed the optimum cellulase for each alkaline-pretreated agricultural residues. Alkaline-pretreated beet pulp and weed obtained 80% of sugar conversion when cellulase Cellic CTec was added. Alkaline-pretreated beet leaf and corn stover obtained 75% and 69% of sugar conversion when cellulase Sucrase C was added. Figure 2.5 and Figure 2.6 showed HPLC spectrum of saccharification of alkaline-pretreated beet pulp with cellulase Cellic CTec and Meicelase for 24h.

Table 2.4 The optimum cellulase for alkaline-pretreated agricultural residues.

Sample[1)]	Saccharification						
	Cellulase		pH	Temp.	Time	Sugar	
		Wt %		℃	h	g/L	%[2)]
Beet pulp	Cellic Ctec	10	4.5	45	24	11.8	80
Beet leaf	Sucrase C	10	5.0	45	24	9.2	75
Corn Stalk	Sucrase C	10	5.0	45	24	9.1	69
Weed	Cellic Ctec	10	4.5	55	24	8.6	80

1) The sample 20 g/L was carried out for the experiment.

2) Based on cellulose content.

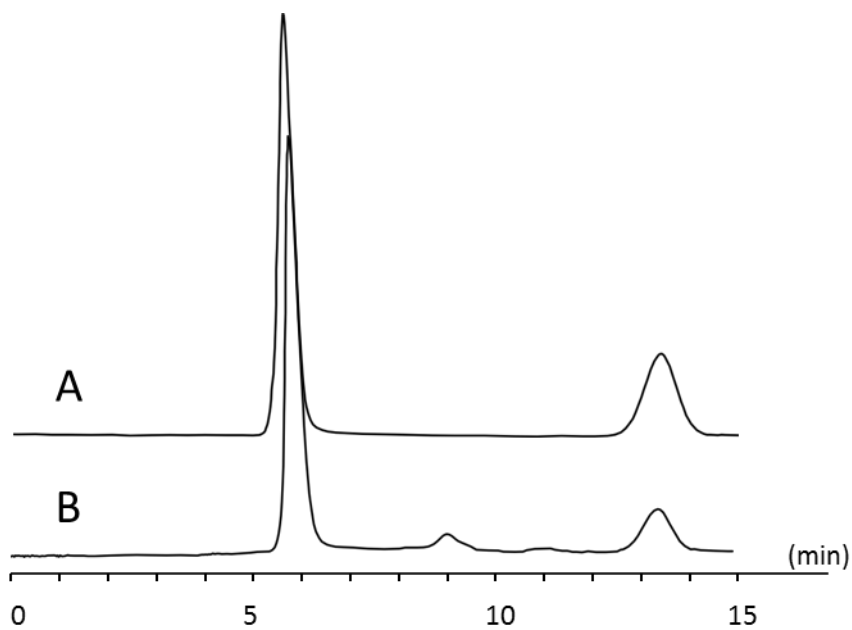

Figure 2.5 HPLC spectrum of saccharification of beet pulp with cellulase Cellic CTec for 24h. (A)Standard, (B) Saccharification with Cellic CTec. HPLC condition: column: amide-80(5μm, 4.6 mm x 250 mm);

Solvent: CH3CN: H2O = 7:3, speed: 0.5 mL/min.

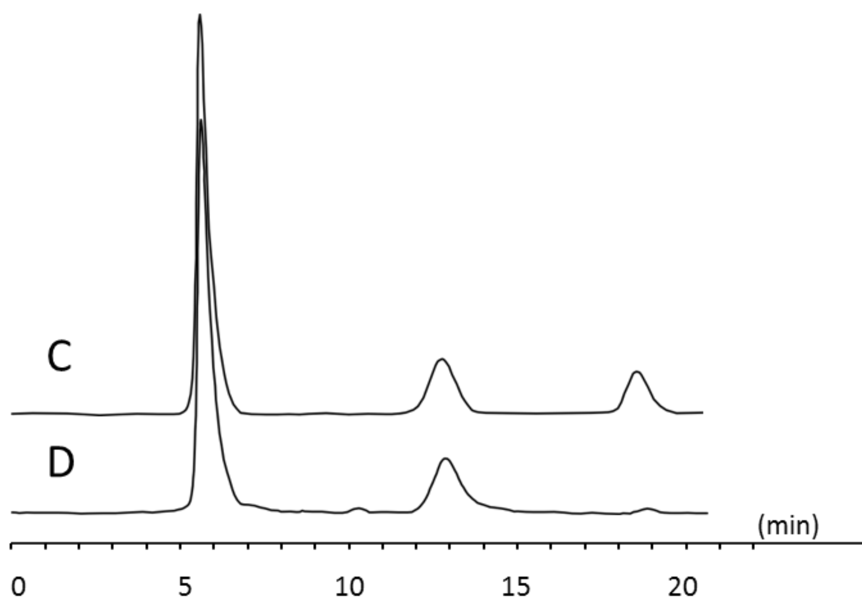

Figure 2.6 HPLC spectrum of saccharification of beet pulp with cellulases Meicelase for 24h. (C)Standard, (D) Saccharification with Meicelase. HPLC condition: column: amide-80(5μm, 4.6 mm x 250 mm);

Solvent: CH3CN: H2O = 7:3, speed: 0.5 mL/min.

We found recently that pYBGA1 yeast has higher fermentation ability than that of commonly used Sake yeast Kyokai no. 7 (K7) for brewing [19]. For example, after saccharification of cellulose (20 g/L) with 1 wt% (to cellulose) of Acremonium cellulase (Meiji Seika Pharma Co. Ltd.), ethanol was obtained in 2.6 g/L and 6.8 g/L, respectively, by fermentation with 1 x 10^8 cells/mL of K7 yeast and with 1 x 10^8 cells/mL of pYBGA1 yeast [20]. Therefore, we used pYBGA1 yeast in this work.

Figure 2.7 shows the successive saccharification and fermentation 20 g/L of alkaline- pretreated beet pulp with addition of 10 wt% of Cellic CTec as the cellulase to the feed and with 1 x 10^8 cells/ml of pYBGA1 yeast. The weight of the beet pulp decreased rapidly during the first 24 h at 45°C and pH 4.5 after starting saccharification, and then the weight was almost constant at 0.2-0.15 g until 168 h. As the saccharification proceeded, the concentration of glucose increased rapidly, and 11.5 g/L of glucose was produced after 24 h of saccharification. After 24 h saccharification, the temperature and pH of the saccharification solution had decreased, and we increased it to 30°C and 5.0 to obtain the optimal condition of fermentation. Then pYBGA1 yeast was added to the saccharified solution, and the fermentation was continued for 144 h further after addition of yeast extract and peptone. After 36 h of fermentation, pYBGA1 produced the highest concentration of ethanol at 5.54 g/L in 60% and 92% theoretical yields based on cellulose content in alkaline-pretreated beet pulp and glucose saccharified, respectively. After fermentation for 60 h, the ethanol concentration decreased gradually,

suggesting that ethanol produced was being consumed by pYBGA1 yeast as a carbon source. Glucose had almost disappeared after 48 h of fermentation.

Figure 2.8shows the successive saccharification and fermentation 20 g/L of alkaline pretreated weed with addition of 10 wt% of Sumizyme C as the cellulase to the feed and with 1 x 10^8 cells/ml of pYBGA1 yeast. The weight of alkaline pretreated weed decreased rapidly during the first 24 h at 45°C and pH 4.5 after starting saccharification, and then the weight was almost constant at 0.2-0.15 g until 168 h. As cellulase Sumizyme C involves endo and exoglucanase and ß-glucosidases. Endoglucanase attacks the low crystallinity regions of the cellulose fiber, exoglucanase removes the cellobiase units from the free chain ends and finally cellobiose units are hydrolysed to glucose by ß-glucosidase [49]. As the saccharification proceeded, the concentration of glucose and cellobiose increased rapidly, and 0.7 g/L of cellobiose was produced when saccharification was carried out after 12 h. After 24 h almost all of the cellobiose was hydrolysed to glucose with the concentration of 7.6g/L and sugar conversion of 70%. After 36 h of fermentation, pYBGA1 produced the highest concentration of ethanol at 3.49 g/L in 58% and 95% theoretical yields based on cellulose content in alkaline-pretreated weed and glucose saccharified, respectively.

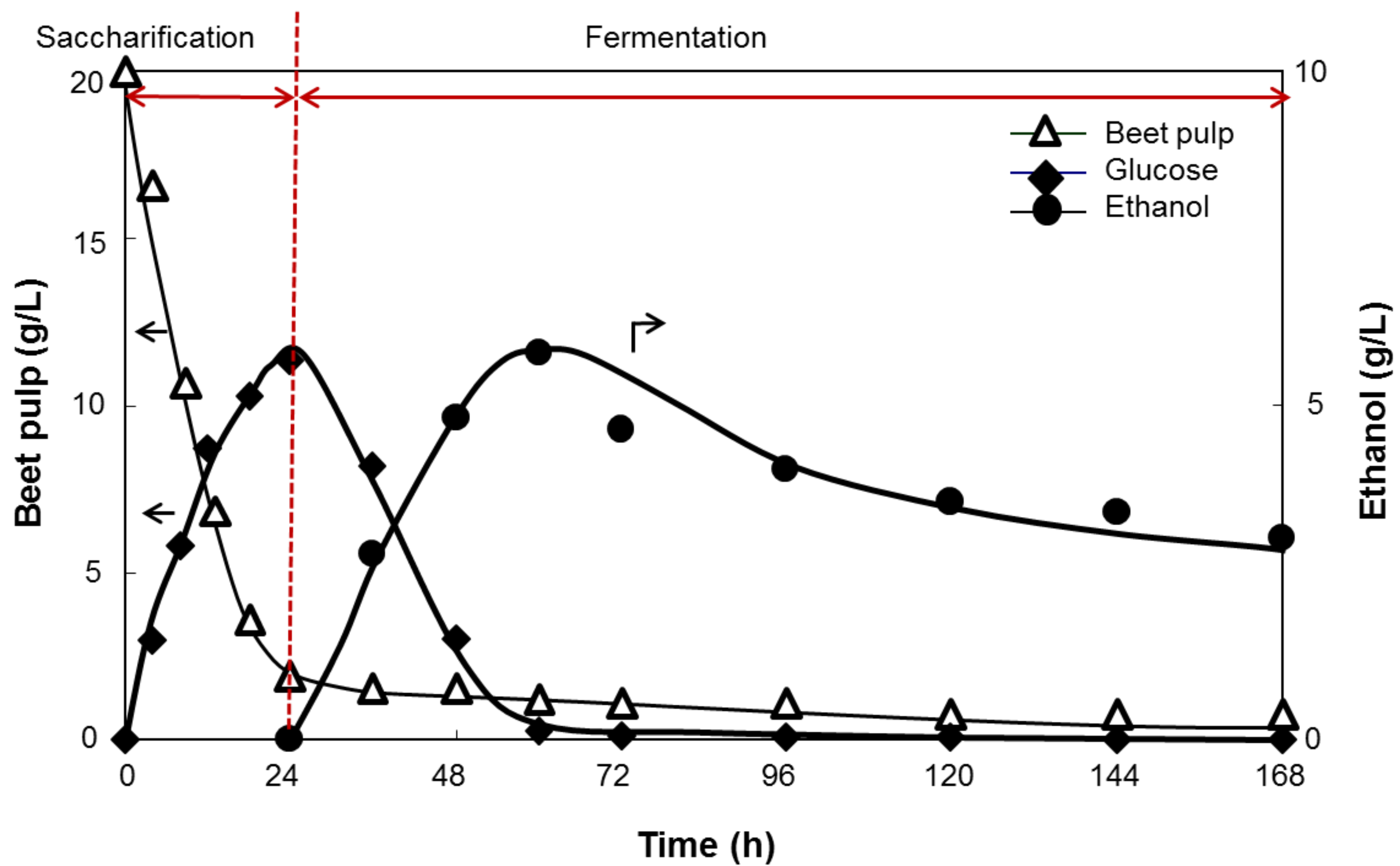

Figure 2.7 Time course of successive saccharification and fermentation of alkaline-pretreated beet pulp powder with Cellic CTec as a cellulase and pYBGA1 yeast. The saccharification was carried out for 24 h at 45℃and pH 4.5, and then fermentation for further 144 h at 30℃ and pH 5.0 after addition of yeast extract and peptone.

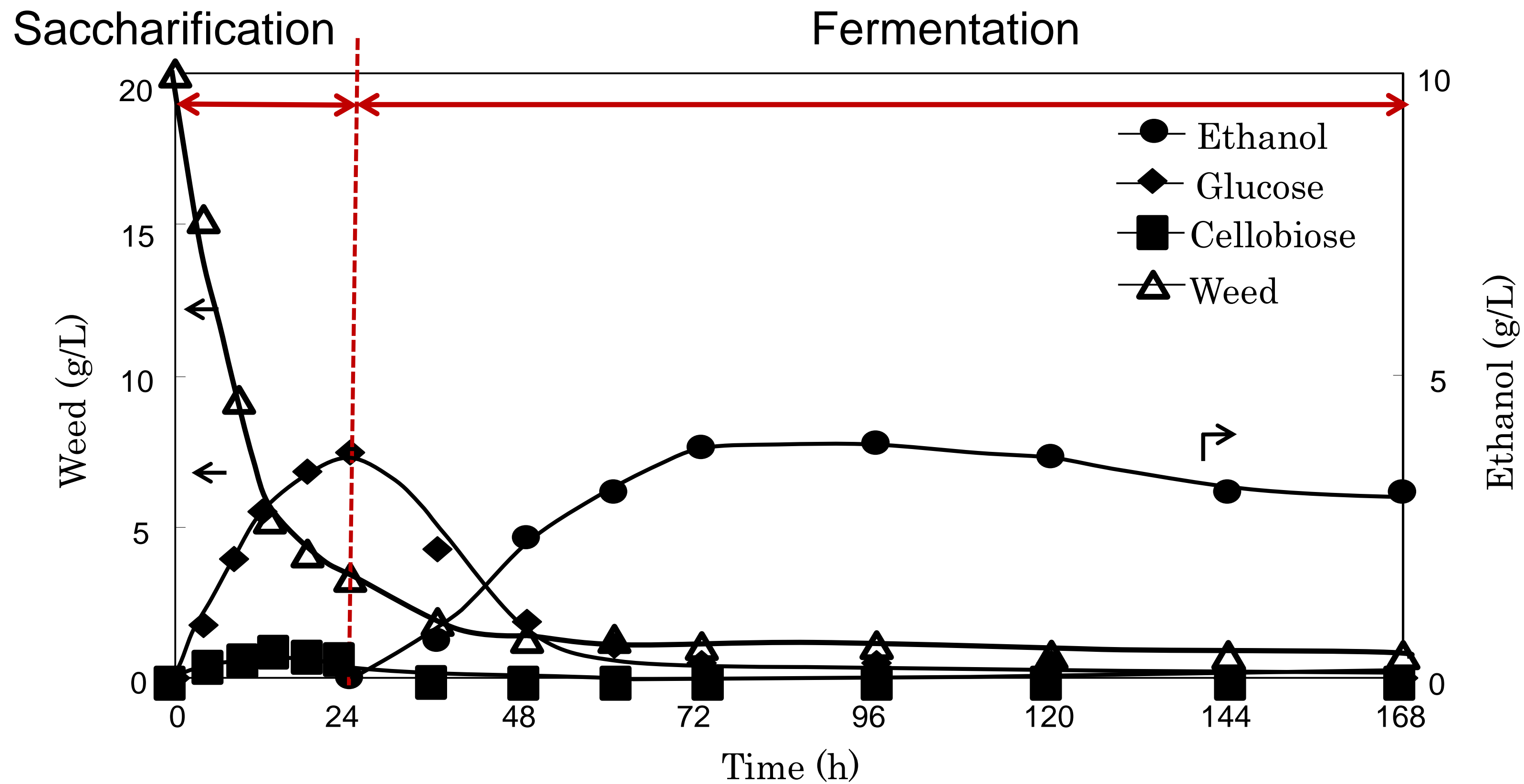

Figure 2.8 Time course of successive saccharification and fermentation of alkaline-pretreated weed powder with Sumizyme C as a cellulase and pYBGA1 yeast. The saccharification was carried out for 24 h at 55℃and pH 4.5, and then fermentation for further 144 h at 30℃ and pH 5.0 after addition of yeast extract and peptone.

Both glucose production and ethanol consumption with the cellulases used in this work and with pYBGA1 yeast for beet leaf, corn stover, and weed had almost the same tendency as those of the combination of Cellic CTec and pYBGA1 yeast shown in Figure 2.7. Table 2.5 shows saccharification and fermentation of alkaline-pretreated agricultural residue samples when the optimal cellulases were used. These results suggest that saccharification occurred for 24 h, and then the fermentation was continuously performed for a further 48 h.

Table2.5 Stepwise saccharification and fermentation of plant samples with cellulases and pYBGA1 yeast [1]

Sample[3]	Saccharification[2]						Fermentation [2]		
	Cellulase		pH	Temp.	Sugar		Ethanol		
	wt%			℃	g/L	%	g/L	%[4]	%[5]
Beet	Cellic Ctec	10	4.5	45	11.8	80	5.54	66	92
Beet leaf	Sucrase C	10	5.0	45	9.2	75	4.37	63	91
Corn Stalk	Sucrase C	10	5.0	45	9.1	69	4.23	57	91
Grass	Sumizyme C	10	4.5	55	7.6	70	3.49	58	95

1) cellulase was optimal for each plant samples.
2) The content of sugars and ethanol was determined by HPLC and GC, respectively.
3) Dry samples (2 g) in buffer (100 ml) was used.
4) Based on cellulose content.
5) Based on glucose saccharified.

Table2.6 Stepwise saccharification and fermentation of plant samples with cellulases Cellic CTec[1)]

	Saccharification					Fermentation			
Plant sample		Temp	pH	Sugar[2)]	Conversion	pYBGA1	Ethanol[5)]		
	wt%	°C		g/L	%	x10^8 cell/ml	g/L	%[3)]	%[4)]
Beet pulp	10	45	4.5	11.8	72	1	5.54	66	92
Beet leaf	10	45	4.5	5.3	39	1	2.35	34	87
Corn stover	10	45	4.5	5.1	35	1	2.26	30	87
Weed	10	45	4.5	4.1	34	1	1.81	30	86

1) Dry beet pulp (2 g) in buffer (100 ml) was used.
2) The content of sugars and ethanol was determined by HPLC and GC, respectively.
3) Based on cellulose content.
4) Based on glucose saccharifi

Table 2.7 represents the results of successive saccharification and fermentation of dilute alkali-pretreated beet pulp with several cellulases with saccharification for 24 h and then saccharified glucose fermented with pYBGA1 yeast for further 48 h. Cellulase played an important part in the saccharification of plant raw materials. The saccharification was carried out at the optimal pH and temperature for each cellulase. With Cellic CTec as a cellulase, glucose (11.8 g/L) was obtained in 72% conversion based on the concentration of cellulose. Sucrase C as a cellulase gave glucose (9.6 g/L) at 58%. When Meicelase was used, glucose was produced at 8.0 g/L at 49%. Glucose was also obtained from other cellulases, Sumizyme AC, GODO-TCL, and "Onozuka"RS, but the conversion rates were somewhat lower. Therefore, for the 24 h saccharification of beet pulp, Cellic CTec was found to be an effective cellulase.

For the fermentation, yeast extract and peptone were successively added to the saccharified solution, the temperature was decreased to 30°C, and the pH was adjusted at 5.0 for the optimal fermentation condition of pYBGA1 yeast. After adding pYBGA1, the fermentation was continued for a further 144 h. Saccharified glucose was converted to ethanol at the rates of around 90%. The pYBGA1 yeast also has a high ability to directly ferment cello-oligosaccharides to ethanol [12, 14]. pYBGA1 itself produced ß-glucosidase on the cell wall, and cello-oligosaccharides are hydrolyzed into glucose, followed by fermentation into ethanol. Therefore, ethanol was directly obtained from cello-oligosaccharides that were saccharified from cellulose by exo-type

cellulases. Among the cellulase complexes used in this work, cellulases, including the exo-type cellulases Sumizyme C and Meicelase, should saccharify beet pulp to glucose and cello-oligosaccharides, which were fermented with pYBGA1 to give ethanol. On the other hand, because Cellic CTec is also a complex type cellulase, glucose is the only sugar saccharified with ß-glucosidase. Therefore, pYBGA1 fermented the glucose to give ethanol.

Table 2.6 summarizes the successive saccharification and fermentation of agricultural residues under the optimal conditions. Agricultural residues at 20 g/L and cellulase at 10 wt% or 15 wt% of the feed were used. As shown in Table 2.5 several cellulases were tested to determine the optimal saccharification condition. For beet leaf and corn stover, Sucrase C was the most effective cellulase for saccharification and produced glucose at 9.4 and 7.1 g/L, respectively, at 60% and 43% conversions based on the cellulose concentration for 24 h saccharification. Weed was saccharified with Sumizyme C to give 6.1 g/L of glucose at 51% conversion. Thus, the most appropriate cellulase for the saccharification depended on the kind of agricultural residue. As shown in Table 2.2, although the cellulose concentration in weed was lower than that of other agricultural residues, it was considered a convenient raw material because of the abundance of plant waste in general. The fermentation shown in Table 2.3 was also carried out successively with pYBGA1 yeast for 48 h at 30°C and pH 5.0. Ethanol was obtained at good conversion rates based on sugars in each saccharified agricultural residue.

Table2.7 Stepwise saccharification and fermentation of beet pulp with cellulases[1)]

Saccharification						Fermentation			
Cellulase		Temp	pH	Sugar[2)]	Conversion	pYBGA1	Ethanol[2)]		
	wt%	°C		g/L	%	$x10^8$ cell/ml	g/L	%[3)]	%[4)]
Cellic CTec	10	45	4.5	11.8	72	1	5.54	66	92
Sucrase C	10	45	5.0	9.6	58	1	4.56	54	93
Sumizyme C	15	55	4.5	8.9	54	1	4.04	48	89
Meicelase	10	45	4.5	8.0	49	1	3.68	44	90
Sumizyme AC	15	55	4.0	7.0	43	1	3.14	37	88
GODO-TCL	10	55	4.5	6.5	39				
"Onozuka" RS	10	45	4.5	6.0	37				

1) Dry beet pulp (2 g) in buffer (100 ml) was used.
2) The content of sugars and ethanol was determined by HPLC and GC, respectively.
3) Based on cellulose content.
4) Based on glucose saccharified.

Figure 2.9 and Figure 2.10 shows the successive saccharification and fermentation 20 g/L of alkaline pretreated agricultural residue samples with addition of 10 wt% cellulase to the feed and with 1 x 10^8 cells/ml of K7 yeast. Figure 2.9 show the combination of Cellic CTec and K7 yeast for alkaline pretreated beet pulp. As the saccharification proceeded, the concentration of glucose increased rapidly, and 11.8 g/L of glucose was produced after 24 h of saccharification. After 24 h saccharification, the temperature and pH of the saccharification solution had decreased, and we decreased it to 30°C and 5.0 to obtain the optimal condition of fermentation. Then K7 yeast was added to the saccharified solution, and the fermentation was continued for 144 h further after addition of yeast extract and peptone. After 36 h of fermentation, pYBGA1 produced the highest concentration of ethanol at 4.98 g/L in 60% and 82% theoretical yields based on cellulose content in alkaline-pretreated beet pulp and glucose saccharified, respectively. After fermentation for 60 h, the ethanol concentration decreased gradually, suggesting that ethanol produced was being consumed by K7 yeast as a carbon source. Glucose had almost disappeared after 48 h of fermentation. Figure 2.10 shows the successive saccharification and fermentation with combination of Sucrase C and K7 yeast for beet leaf. As cellulase Sucrase C involves endo and exoglucanase and ß-glucosidases, glucose and cellobiose was both produced during the process of saccharification. As the saccharification proceeded, the concentration of glucose and cellobiose increased rapidly, and 0.5 g/L of cellobiose was produced when saccharification was carried out after 12 h. After 24 h almost all of the cellobiose was hydrolysed to glucose with the concentration of 9.2

g/L and sugar conversion of 75%. After 36 h of fermentation, pYBGA1 produced the highest concentration of ethanol at 3.84 g/L in 56% and 80% theoretical yields based on cellulose content in alkaline-pretreated beet leaf and glucose saccharified, respectively.

Table2.8 showed stepwise saccharification and fermentation of plant samples with cellulases and K7 yeast. Ethanol was obtained at over 80% conversion rates based on sugars in each saccharified agricultural residue samples.

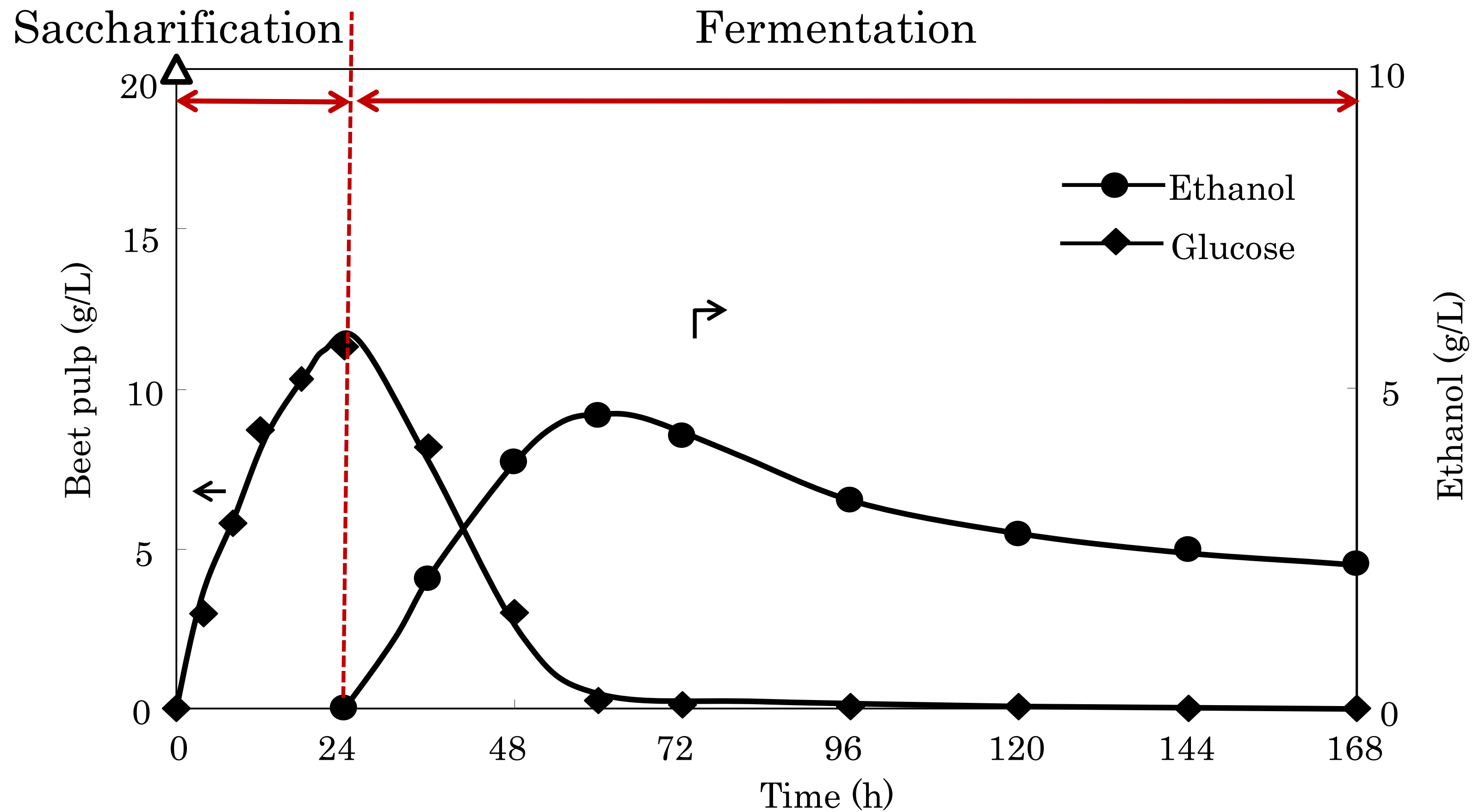

Figure 2.9 Time course of successive saccharification and fermentation of alkaline-pretreated beet pulp powder with Cellic CTec as a cellulase and K7 yeast. The saccharification was carried out for 24 h at 45℃and pH 5.0, and then fermentation for further 144 h at 30℃ and pH 5.0 after addition of yeast extract and peptone.

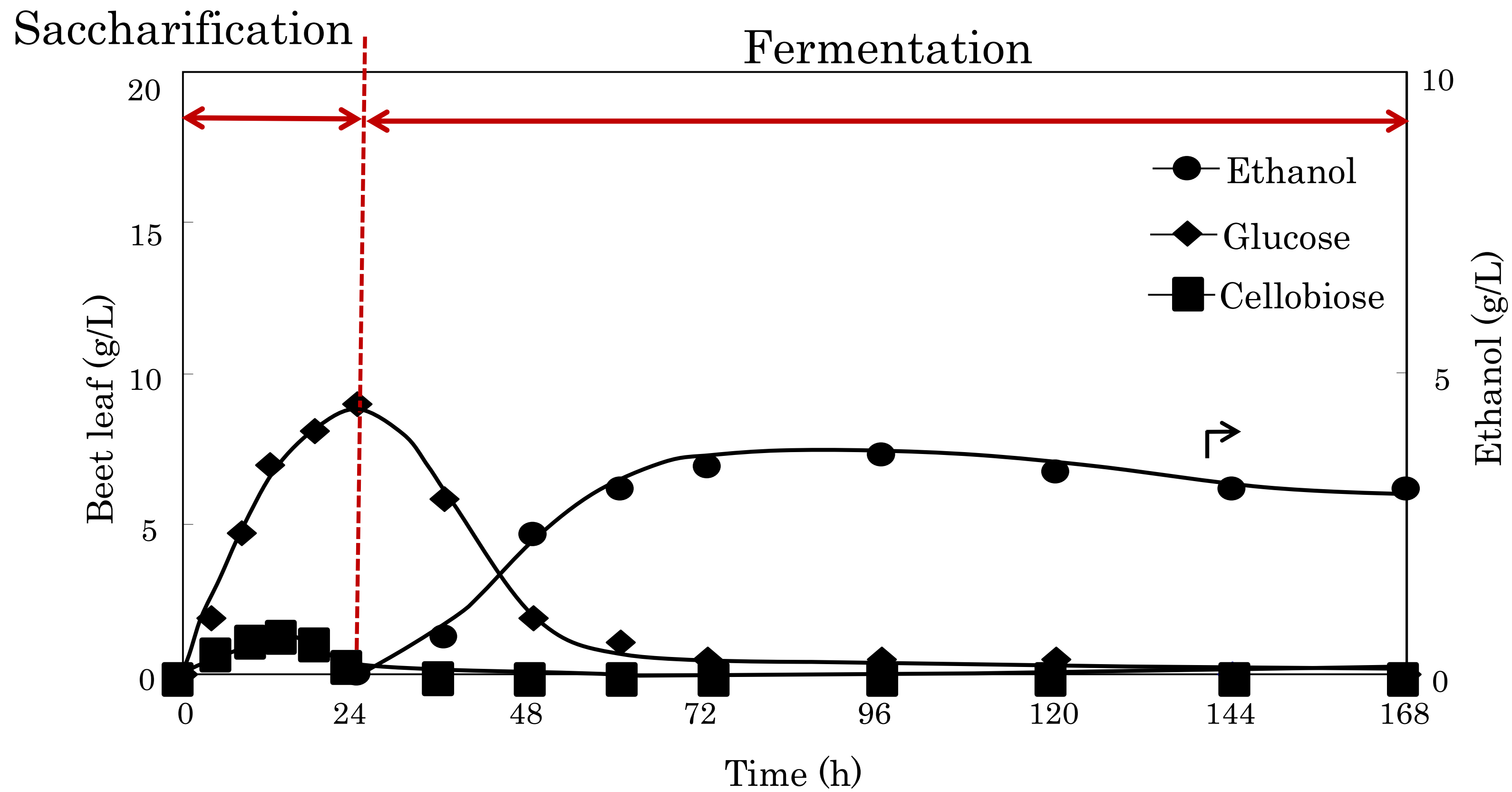

Figure 2.10 Time course of successive saccharification and fermentation of alkaline-pretreated beet leaf powder with Sucrase C as a cellulase and K7 yeast. The saccharification was carried out for 24 h at 55℃and pH4.5, and then fermentation for further 144 h at 30℃ and pH 5.0 after addition of yeast extract and peptone.

Table2.8 Stepwise saccharification and fermentation of plant samples with cellulases and K7 yeast [1)]

Sample[3)]	Saccharification[2)]						Fermentation [2)]		
	Cellulase		pH	Temp.	Sugar		Ethanol		
	wt%			℃	g/L	%	g/L	%[4)]	%[5)]
Beet	Cellic CTec	10	4.5	45	11.8	80	4.98	60	82
Beet leaf	Sucrase C	10	5.0	45	9.2	75	3.84	56	80
Corn Stalk	Sucrase C	10	5.0	45	9.1	69	3.77	51	81
Grass	Sumizyme C	10	4.5	55	7.6	70	3.01	50	82

1) cellulase was optimal for each plant samples.
2) The content of sugars and ethanol was determined by HPLC and GC, respectively.
3) Dry samples (2 g) in buffer (100 ml) was used.
4) Based on cellulose content.
5) Based on glucose saccharified.

2.4.4 Conclusion

As described above, cellulose in agricultural residues was mainly converted to ethanol; that is, the successive saccharification and fermentation of agricultural residues were carried out by a combination of cellulase for saccharification and pYBGA1 yeast for fermentation under the optimal conditions to give ethanol at good conversion rates. We tested several cellulases for saccharification of the agricultural residues. Among them, Cellic CTec was the most effective for beet pulp, Sucrase C for beet leaf and corn stover, and Sumizyme C for weed, respectively, for the 24 h saccharification. When beet pulp was saccharified with Cellic CTec for 24 h at pH 4.5 and 45°C, 11.8 g/L of glucose was obtained in 72% conversion based on the cellulose concentration of the alkali-pretreated beet pulp. The fermentation was successively performed by adding pYBGA1 yeast for further 36 h at 30°C and pH 5.0 to produce 5.54 g/L of ethanol at 60% and 92% conversions of cellulose content in beet pulp and of the glucose saccharified, respectively. Other agricultural residues, beet leaf, corn stover, and weed, gave ethanol at 91 and 96% conversions, respectively, in each optimal condition.

However, based on the cellulose concentration, the conversion to ethanol decreased slightly, probably because of incomplete saccharification of cellulose having crystalline moiety. In this work, we mainly investigated successive saccharification and fermentation of glucose saccharified from cellulose in the agricultural residues in Hokkaido Prefecture. Bioethanol

production fermented by cello-oligosaccharides and pentoses from cellulose and hemicellulose in agricultural residues will be further investigated to find the best combination of cellulase, hemicellulase, and yeast for good conversion rates.

2.5 References

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Chapter 3

Simultaneous Saccharification and Fermentation of Cellulosic Agricultural Residues using a Combination of Cellulase and Recombinant Yeast

3.1 Introdution

To hydrolyze cellulose into soluble sugars, multiple enzymatic activities are required. These activities include endoglucanase, exoglucanases including cellobiohydrolase and cellodextrinase, and ß-glucosidase [1], whereas reaction products such as cellooligosaccharide and glucose inhibit cellulase activity [2]. Thus, to avoid the product inhibition, enzymatic

hydrolysis is performed together with fermentation, a process referred to as simultaneous saccharification and fermentation (SSF). In the SSF process, glucose released by cellulase action is directly converted to ethanol by the fermenting microorganisms, which alleviates problems caused by the end product [3]. Also, the consumption of glucose and the presence of ethanol in the culture medium would reduce the risk of undesired contamination by glucose-dependent organisms. Recently, consolidated bioprocessing which combines enzyme production, saccharification and fermentation in a single step, has gained recognition as a potential bioethanol production system because the costs of capital investment, substance and other raw materials, and utilities associated with enzyme production can be avoided [4].

However, one of the major drawbacks in the SSF process is the optimum temperature required for the saccharification and fermentation stages. Saccharification with cellulolytic enzymes is best done around 50 °C, while most fermenting microbes have an optimum temperature for ethanol fermentation between 28 °C and 37 °C [5]. In practice, it would be difficult to lower the optimum temperature of cellulases through protein engineering. Accordingly, high-temperature fermentation is in high demand for simultaneous saccharification and fermentation, and thermotolerant yeast strains have been screened for the ability to ferment ethanol [6]. This review will focus on the SSF process with thermotolerant yeast strains. In particular, development of yeast strains expressing heterologous cellulolytic enzymes for direct conversion of cellulosic materials to ethanol at elevated temperature is emphasized.

Ethanol production at elevated temperature has received much attention because of many advantages such as a reduction in the costs associated with cooling, continuous evaporation of ethanol from broth under reduced pressure, a reduced risk of contamination, suitability for application in tropical countries as well as the improvement of SSF efficiency [7].

3.2 Experimental

3.2.1 Materials

The plants used in the experiments include corn stalk, grass, beet and beet leaf. Beet pulp was supplied by a local company, Hokkaido Togyo Co. Ltd., Hokkaido, Japan. Beet leaf and corn stover were provided by a local farm in Kitami City, Hokkaido. Weeds were obtained from the lawn of our university. They were all washed buy tap water to remove soil and other particles, and then dried by oven at 40℃till constant weight . Thereafter, the plant was shredded and grinded to powder by a mill. The processed materials were stored in sealed glass bottles at low ambient temperature for further usage.

All the chemicals used were of analytical grade and mainly purchased from and used as such without further purification.

pYBGA1 yeast was kindly supplied by the National Research Institute of Brewing and was pre-incubated on agarose medium with cellobiose as a carbon source according to the procedure described in our previous paper [14]. The pYBGA1 yeast cells were counted using a cell counter.

Cellulases used in this work were obtained from the respective companies. Cellic CTec (Novozymes Japan Ltd., 1000 EGU/g), Sucrase C (Mitsubishi-Kagaku Foods Corp., 3000 U/g), Sumizyme C (Shin Nihon Chemical Co.,Ltd., 1500 U/g), Meicelase (Meiji Seika Pharma-6 -Co.,Ltd., 400 FPU/g), Sumizyme AC (Shin Nihon Chemical Co.,Ltd., 2000 U/g), GODO-TCL (Oenon Holdings, Inc., 100 FPU/g), and "Onozuka"RS (Yakult Honsha Co.,Ltd., 20000 U/g) were obtained from the respective companies. Yeast extract and peptone were purchased from Merck Chemicals, Japan, and Kyokuto Pharmaceutical Industrial Co. Ltd., Japan, respectively.

3.2.2 Measurement

Thermogravimetric analysis (TGA) was conducted with a Shimadzu DTG-60 thermoanalyzer during heating at 10°C/min under a nitrogen atmosphere.

Saccharified sugars were measured quantitatively at 40 °C by a reverse-phase HPLC system with a Tosoh TSK-gel amide-80 column (5 μm, 4.6 mm x 250 mm) eluted with a 1:1 acetonitrile and water solution at a flow rate of 0.5 mL/min with a Tosoh RI detector using glucose as a standard in the calibration curve.

The concentration of ethanol produced was determined under a nitrogen atmosphere by a Shimadzu GC-8A gas chromatograph with a Shimadzu capillary column (SE-30, 3.2 mm x 30 m) and a hydrogen flame ionization detector. The column and injection temperatures were 60°C and 130°C,

respectively.

3.2.3 Simultaneous saccharification and fermentation of alkali-pretreated agricultural residues

A typical procedure for the successive saccharification and fermentation of alkalipretreated agricultural residue is as follows. In a 300 mL Erlenmeyer flask, 100 mL of deionized water was added to 2.0 g of the alkali-pretreated powdered beet pulp. The pH of the solution was adjusted to 5.0 by dilute aqueous HCl, and then Cellic CTec (10 wt% of beet pulp) and pYBGA1 yeast with the quantity of 1 x 10^8 cells/mL was added. The flask was sealed with a sponge stopper and the mixture was incubated in an oven for 96 h at 30 °C using gentle stirring by a rotary shaker. The samples were withdrawed at regular time intervals and denatured immediately for sugar and ethanol analysis.

3.3 Results and Discussion

Figure 3.1 shows time course of simultaneous saccharification and fermentation of alkaline-pretreated beet pulp powder with Cellic CTec as a cellulase and yeast. Ethanol 3.97 g/L was obtained with the conversion of 48% when pYBGA1 yeast was added in the process of simultaneous saccharification and fermentation from alkaline pretreated beet pulp. In the case of K7 yeast 3.13 g/L ethanol was produced with the conversion of 37%.

Ethanol 3.31 g/L was obtained with the conversion of 43% when pYBGA1 yeast was added in the process of simultaneous saccharification and fermentation from alkaline pretreated beet leaf compared with 4.73 g/L ethanol and conversion 63% in the process of successive saccharification and fermentation. Simultaneous saccharification and fermentation need low coats and could obtains high ethanol yields due to removal of end product inhibition of saccharification step, but it has the limitation of difference in optimum temperature conditions for saccharification and fermentation. Successive saccharification and fermentation has the features and advantages which include each step can be processed at its optimal operating conditions and separate steps minimize interaction between the steps. However, the limitations are obvious such as end product inhibition minimizes the yield of ethanol and possibility of contamination due to long period process.

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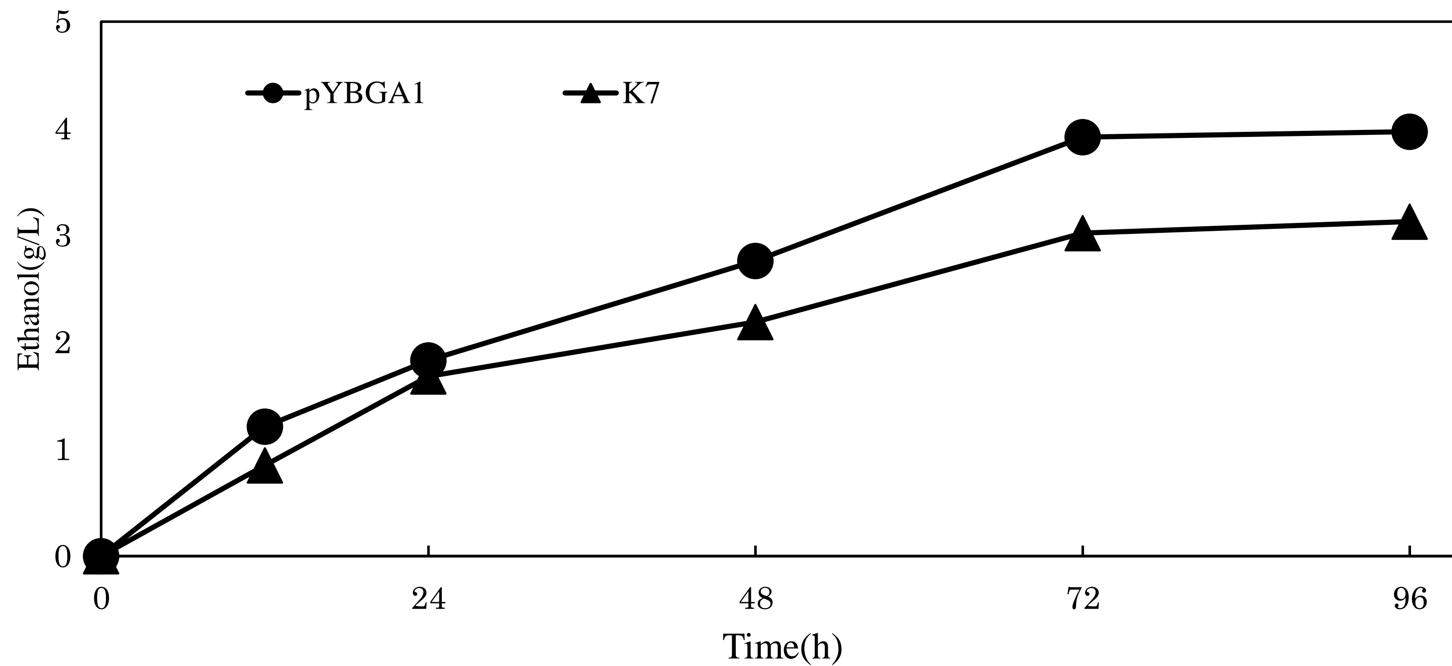

Figure 3.1 Time course of simultaneous saccharification and fermentation of alkaline-pretreated beet pulp powder with Cellic CTec as a cellulase and yeast. The experiment was carried out for 96 h at 30℃and pH 5.0.

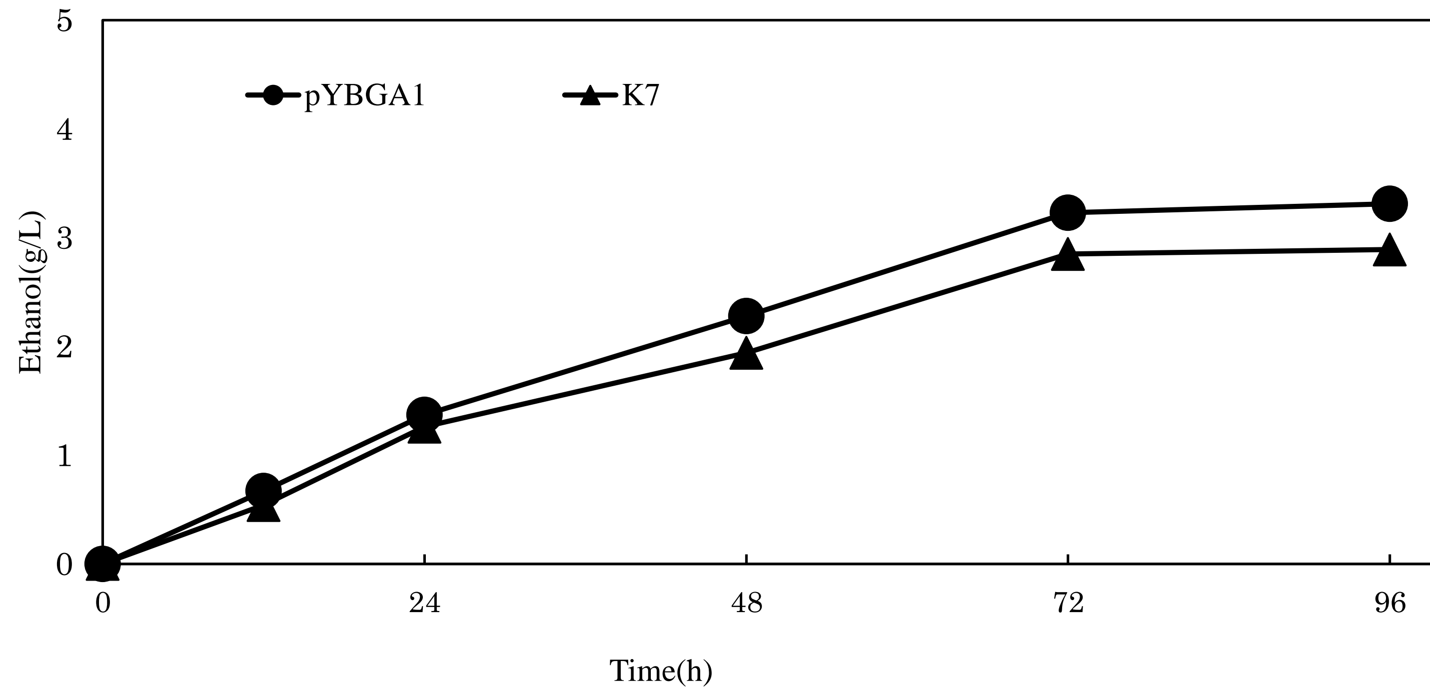

Figure 3.2 Time course of simultaneous saccharification and fermentation of alkaline-pretreated beet leaf powder with Sucrase C as a cellulase and yeast. The experiment was carried out for 96 h at 30℃and pH 5.0.

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Chapter 4
Conclusions

The unavoidable depletion of fossil fuels and negative impacts of fossil fuels on the environment especially greenhouse gas emissions, have resulted in a worldwide interest in identifying and developing renewable fuel alternatives. Bioethanol produced from renewable plant resources especially nonfood biomass residues is expected to provide an environment-friendly and clean energy becomes an attractive alternative recently. Due to Hokkaido was the largest base of agriculture production, this work used Hokkaido abundant agriculture resources such as corn stover, beet pulp, beet leaf and weed as raw material for the study of bioethanol production. Pretreatment is necessary for the reason that the existence of lignin could influence the saccharification of cellulose and hemiellulose.

The agriculture residue samples were pretreated with 2% NaOH aqueous solution for certain time at 121 ℃ by autoclave to remove major lignin and degrade the cellulosic crystal structure, and then successive saccharification with cellulase and fermentation was

carried out with yeast Sake yeast Kyokai no. 7 (K7) compared with recombinant yeast pYBGA1 which is a recombinant laboratory yeast expressing-glucosidase. The content of hemicellulose, cellulose and lignin was measured before and after alkali pretreatment according to the procedures described by National Renewable Energy Laboratory (NREL). The concentrations of cellulose and hemicellulose of samples increased and decreased, respectively, as part of hemicellulose was dissolved in the NaOH aqueous solution at the relative high temperature of 121℃. Take beet pulp for example, the concentrations of cellulose decreased from 53.8 wt% to 74.0 wt % while the hemicellulose changed from 16.7 wt% to 10.9 wt%. The lignin concentration decreased from 4.2 wt% to 1.2 wt%. Alkali pretreatment could remove a majority of lignin and improved the efficiency of saccharification significantly.

The saccharification was carried out at the optimal pH and temperature for each cellulase. Pretreatment in different time 30mins, 60mins, 90mins and 120mins was carried out and the results demonstrated that it is the optimal condition when agriculture residue samples pretreated in 60mins. The glucose reduced from beet pulp unpretreated was 1.3 g/L and the glucose concentration decreased to 9.6 g/L after the pretreatment by cellulase Meicelase (10 wt% to beet pulp).After alkali pretreated beet pulp (20 g/L), for example, was then saccharified for 24 h by Cellic CTec (10 wt% to beet pulp) as the cellulase at 45 ℃ to give glucose (11.8 g/L) at relatively good rates.

After the saccharification make temperature decrease to 30 ℃, the resulting glucose was continuously fermented for 36 h by addition of 1 x 10^8 cells/mL of pYBGA1 yeast to give 5.54 g/L of bioethanol in 60 % and 92% theoretical yields of cellulose in beet pulp and glucose saccharified, respectively. Other agricultural residues, beet leaf, corn stover, and weed, gave ethanol at 91% and 96% conversions, respectively, in each optimal

condition. In the case of yeast Sake yeast Kyokai K7 under the same condition saccharified glucose 9.4 g/L with cellulase Sucrase C (10 wt%) was fermented to 3.25 g/L of bioethanol in beet leaf, lower than bioethanol of 4.39 g/L fermented by yeast pYBGA1. Other agricultural residues also afforded bioethanol by the successive saccharification and fermentation.

It was found that the appropriate cellulase for saccharification differed with the kind of agricultural residue; beet pulp was effectively saccharified by Cellic CTec with glucose concentration of 11.8 g/L, beet leaf and corn stover by Sucrose C with glucose concentration of 9.4 g/L and 7.1 g/L, respectively, and weed by Sumizyme C with glucose concentration of 6.1 g/L. These agricultural residues were found to be suitable for the production of bioethanol because Hokkaido Prefecture is the most abundant producer of beets and corn in Japan, and accordingly, agricultural residues are easily available. Yeast pYBGA1 was also found to be good yeast for the fermentation of sugars saccharified from agricultural residues.

Simultaneous saccharification and fermentation was also carried out with cellulases and pYBGA1 yeast to compare with successive saccharification and fermentation. 3.97 g/L of bioethanol was fermented in 120h and 51% theoretical yields of cellulose in beet leaf with cellulase Sucrase C (10 wt%) while 4.39 g/L of bioethanol and 63% theoretical yields of cellulose. In the case of cellulase Meicelase and Sumizyme C it also could be demonstrated that the efficiency of successive saccharification and fermentation is higher than that of successive saccharification and fermentation.

Acknowledgment

I dedicated with best respect and particularly indebted to my supervior, Professor Dr. Takashi Yoshida of Kitami Institute of Technology, Japan. The works of my doctoral course in graduate school was achieved under his wonderful direction.

I am grateful to Dr. Xianxiang Liang of Kitami Institute of Technology for her guidance with experiment. I also thank all of the members of Yoshida laboratory for supports and assistance during my doctoral course.

I Thank MEXT Foundation for economical support, it is important to my stable and concentrated research.

Finally, I would like to thank my parents and my friends for supports during the doctoral researches and patience to fight together in doctoral course.