University of Miyazaki

Doctoral Dissertation

Conversion of lignocellulose to biofuels using white-rot fungus and its co-culture with bacterium *Clostridium*

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Chapter 1

Overview

1.1. Biofuels

1.1.1 Promotion and advantage of using biofuels

Climate change and its negative impact on the biosphere is one of the most critical issues on the development process of living things on the Earth. Also, the demand for energy consumption to serve human socials is increasing yearly while traditional energy sources, like fossil fuel, natural gas and petroleum, are running out of stores. As a commitment to build sustainable societies, in the Paris Agreement 2015, the historic deal to pursuit the effort to limit the increase of global warming not over 1.5 $^{\circ}$C was mutually agreed upon by 195 countries. Contributing to that deal, the reduction of greenhouse gases (GHGs) emission is significant action (Puricelli et al., 2020).

To reduce GHGs emissions, the use of alternative biofuels, including ethanol, butanol and diesel, as an alternative to the traditional fossil fuels is a possible strategy. Not only giving benefit in the reduction of GHGs pollution, but the use of biofuels also increasing the heat efficiency and safety in the combustion engine (Manoj Babu et al., 2021).

Around the world, the USA, Brazil, Argentina, Germany and China are top countries in the level of production and consumption of biofuel for the combustion engine to alternative the use of traditional fossil fuels (Das, 2020). Globally, the use of biofuels in 2014 is equivalented to the use of 70.8 Megaton of oil (Mtoe). It is expected that this use of biofuel will be 720 Mtoe in 2050 (Hao et al., 2018).

1.1.2 Current status of biofuels production process

With the progress of science and technology, the development of biofuels production process could be classified to:

- The first generation: The current process in industrial biofuel production. Using starches, sugars, food and feed sources to produce bioethanol, biodiesel. In this generation, with well-known technologies, biofuel production could obtain a high yield and efficiency. However, it is raising the conflict between food security and fuels, what is the matter between human and machine, leading to the transition of first-generation to a newer generation.
- The second generation: Mostly knowledge is under laboratory research or small scale process. This production is using cellulose, lignocellulose, non-food and feed as raw materials. Unfortunately, with the recalcitrant structure of raw material, the second generation production requires a complex process and new advanced technology to apply to industrial scales.
- The third generation: Focusing on using algae as raw material to produce biofuels. However, up to date, the knowledge and technology about this generational production are still limited.

The study in this doctoral thesis is focused on the second generation of biofuels production from lignocellulosic materials.

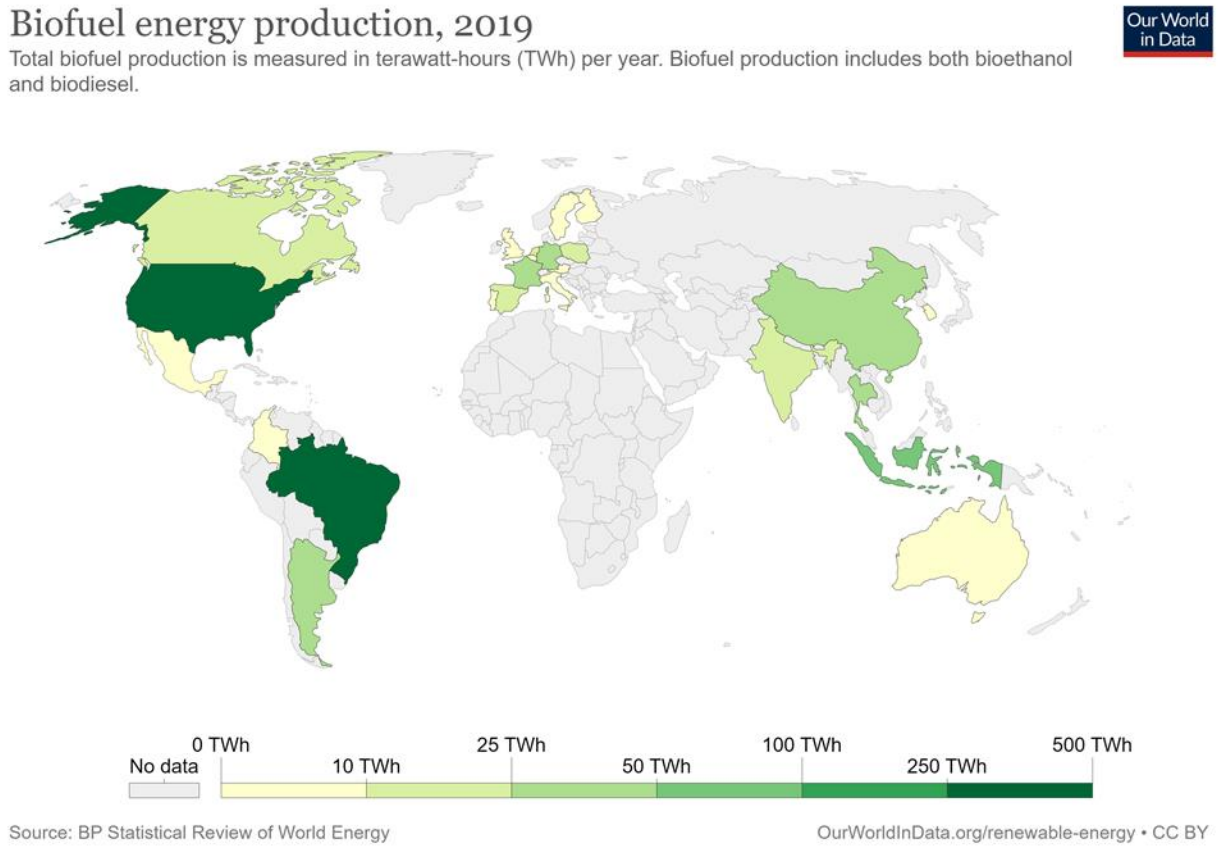


Fig 1-1. The production of biofuels by country in 2019. The figure is reproduced with permission from https://ourworldindata.org/

1.2. Lignocellulosic materials

1.2.1 Definitions

In 1813, lignin, one of the most complex natural phenolic compounds, was first mentioned after itself was isolated from wood by the Swiss botanist Augustin Pyramus de Candolle, (SjÖStrÖM, 1993). Since that discovery, the studies on lignin and its chemical structures have been becoming a focused topic in wood chemistry. It is said that the chemical composition and formula of lignin are varied from species to species as well as the growing conditions of individuals. Generally, lignins are cross-linked phenolic polymers with the incorporation of three monolignols: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) (Boerjan et al., 2003).

In 1834, cellulose, a polysaccharide with chemical formula as $(C_6H_{10}O_5)_n$ which consisting of a linear chain of several hundred to many thousands of D-glucose units, was first discovered by the French chemist Anselme Payen (Updegraff, 1969). It is indicated that cellulose is the most abundant organic polymer on Earth (Klemm et al., 2005). Nowadays, the main industrial application of cellulose is the production of paper and fiber, clothes, pharmaceuticals, building and construction materials.

Later, the term “lignocellulose” or “lignocellulosic material” was first scientifically defined since 1900 and documented in Webster's Dictionary (online at https://www.merriam-webster.com/) as any related substance constituting the essential part of woody cell walls of plants and consisting of cellulose intimately associated with lignin.

Modern studies have revealed that lignocellulose consists of not only cellulose and lignin but also hemicellulose – branched polysaccharides and mostly composed of pentoses. In the form of lignocellulose, the carbohydrate polymers (cellulose and hemicellulose) are tightly bound to the lignin (Brautaset & Ellingsen, 2011).

Currently, the number of research on the topic of lignocellulose is steadily increasing annually, showing that lignocellulose and related concerns are an important topic in scientific communities (Ferreira et al., 2018; Mehta et al., 2020; Wang et al., 2020).

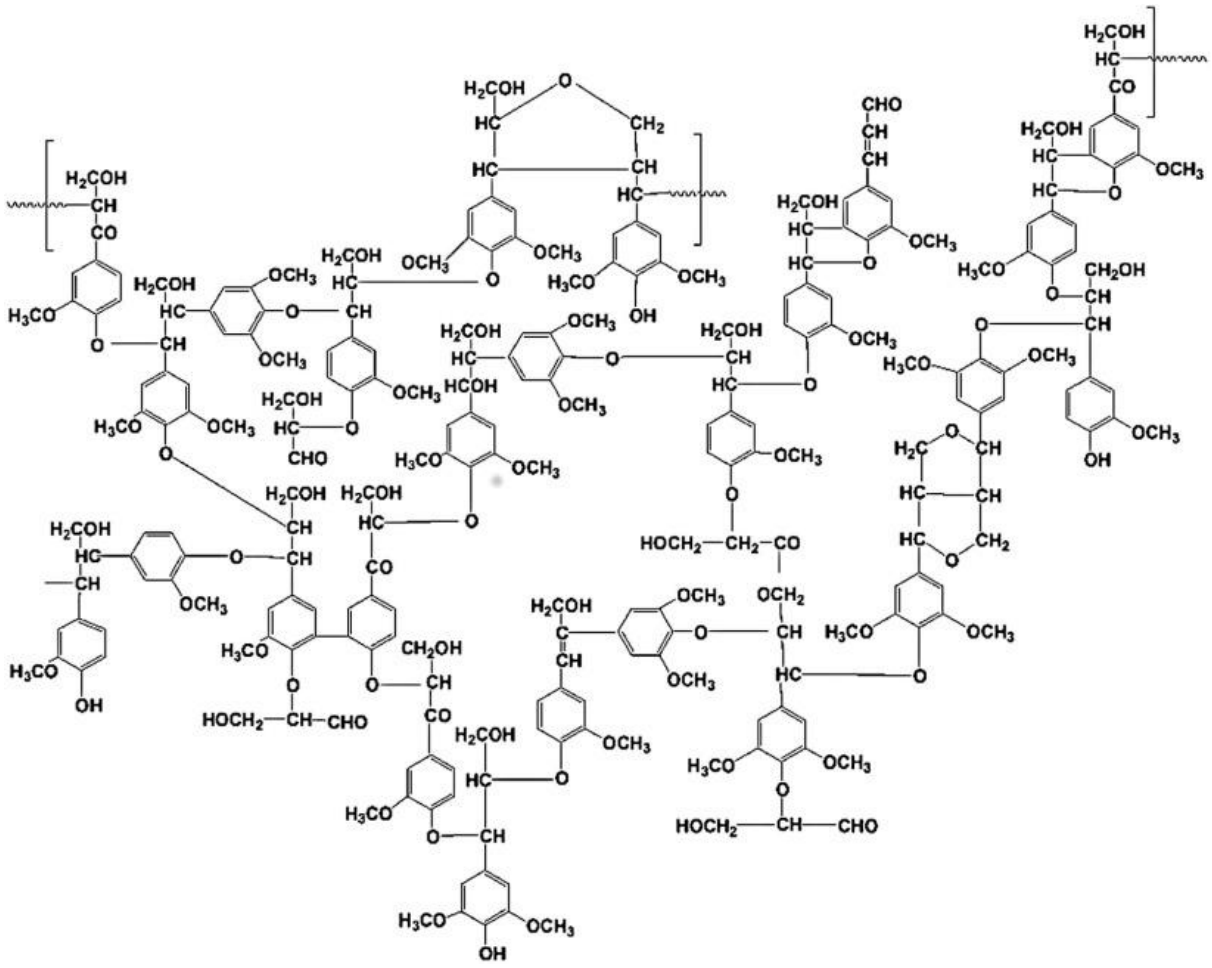


Fig. 1-2. Partial structure of a hardwood lignin molecule from European beech - *Fagus sylvatica* (Le Floch et al., 2015). The illustration is reproduced with the license (No. 4954230789983) from Elsevier.

1.2.2 Sources

Lignocellulose is the most abundant biomass resource on Earth and could be classified as virgin biomass (woody, grasses), waste biomass from agriculture and forestry (sugarcane bagasse, corn stover, wheat and straw, etc.), or energy crops. It is reported that the global yearly production yield of lignocellulose around 200×10^9 tons, comparing to synthetic polymers is 1.5×10^8 tons (Mohanty et al., 2000). Contributing to this quantitative, the annual production of lignocellulose from China, the United States, Canada and India are 1.0 billion tons, 1.3 billion tons, >200 million m^2 and 0.2 billion tons, respectively (Zhang, 2008). These huge yields are causes for several environmental pollutions, waste accumulation and reduction of agricultural efficiency (Green, 2019; Zou et al., 2020).

1.2.3 Characteristics

Lignocelluloses are mostly composed of three polymers: cellulose, hemicellulose and lignin, therefore, the characteristics of lignocelluloses are closely related to properties of their components in the independent form as well as in the composed form. Summary, the highlighted features of lignocellulose are pointed out as below (Chen, 2015)

(1) *Richness and Renewable*. Until the photosynthesis in the earth exists, the lignocelluloses are still produced as the main part of plant biomass. It is recognized that lignocelluloses are inexhaustible.

(2) *Degradable.* Physical, chemical, or biological treatments affect the degradation of lignocelluloses, therefore, the pollution caused by lignocelluloses possibly could be solved using environmentally friendly methods.

(3) *Transformable.* Lignocelluloses are polymers, hence, they have general characteristics of an organic polymer, such as flammability, molecular weight distribution inhomogeneity. Besides, according to their functional groups, the targeted chemical reactions could be designed to achieve modified lignocelluloses.

1.2.4 Conversion of lignocellulose to biofuels: issues and perspectives

In general, polysaccharides (cellulose and hemicellulose) in lignocelluloses are carbon sources to produce biofuels. Besides, lignin, the aromatic polymer in lignocellulose, is expected to utilize as an initial source for novel biomaterials or biochemical. Not only giving economic profit but lignocellulose utilization also might reduce the negative impacts of lignocellulose waste to environments, improves the efficiency of agricultural activities, etc.

Even the lignocellulose is showing the great potential as above, actually, the current utilization of lignocellulose still at low yield and almost under laboratory research. The main challenge on the way to improve this low yield is indicated: the recalcitrance in the chemical structure of lignocelluloses.

The main contribution that should be counted in the recalcitrant structure of lignocellulose is the formation of cellulose fibrils. These fibrils, containing both crystalline and amorphous regions, are made up of the β-(1→4) linked D-glucose and hydrogen bonding, making the high tensile strength property of lignocellulose. The mechanical role of cellulose fibers in the lignocellulose is significantly responsible for its strong structural resistance, which can be compared to that of the reinforcement bars in concrete.

In this concretely lignocellulose structure, the hemicellulose likely the connecting wires of cellulose. Lignin playing here the role of the hardened cement paste between the polysaccharides. This special complex structure protects lignocellulose from the attacks of microbial, enzymes,

environmental factors in nature (Fig. 1-2). But also, in the utilization process, it is recalcitrant to separate cellulose, hemicellulose and lignin before converting them to other valuable products.

The current strategy in the conversion of lignocellulose to biofuels is employing the serial of three steps: 1) pretreatment; 2) hydrolysis of polysaccharides and 3) fermentation of sugars

The first step, pretreatment, is the most important and expensive, which goals to modify initial substrates, break the "lignin cement paste" and disrupt the crystalline structure of cellulose (Mosier et al., 2005). After pretreatment, it is expected that lignocellulose will be fractionated to the high polysaccharide content substrate and high lignin content substrate. In the second step, the substrate with high polysaccharide content is more accessible to microorganism enzymes, which could convert polysaccharides to fermentable sugars (mono- or disaccharides). Simultaneously, microorganism metabolites these fermentable sugars to valuable products, such as biofuels (ethanol, butanol), biochemicals (xylitol, acetic acid, lactic acid, etc.). To complete the lignocellulose utilization, in the third step, high lignin content substrate is designed to produce novel biomaterial (biofilms, 3D inks, bio-paint), bio-chemicals (phenol, vanillins, phenolic components, etc.) through the various chemical, hydrothermal, or biological processes.

In the development of the lignocellulose utilization process, the sustainable integrated process, of the biological, physical, and chemical treatment as well as the thermal-equipment, to convert biomass to energy and other bio-based products (food, feed, chemicals, materials, etc.) could be defined as a biorefinery (The International Energy Agency Bioenergy Task 42).

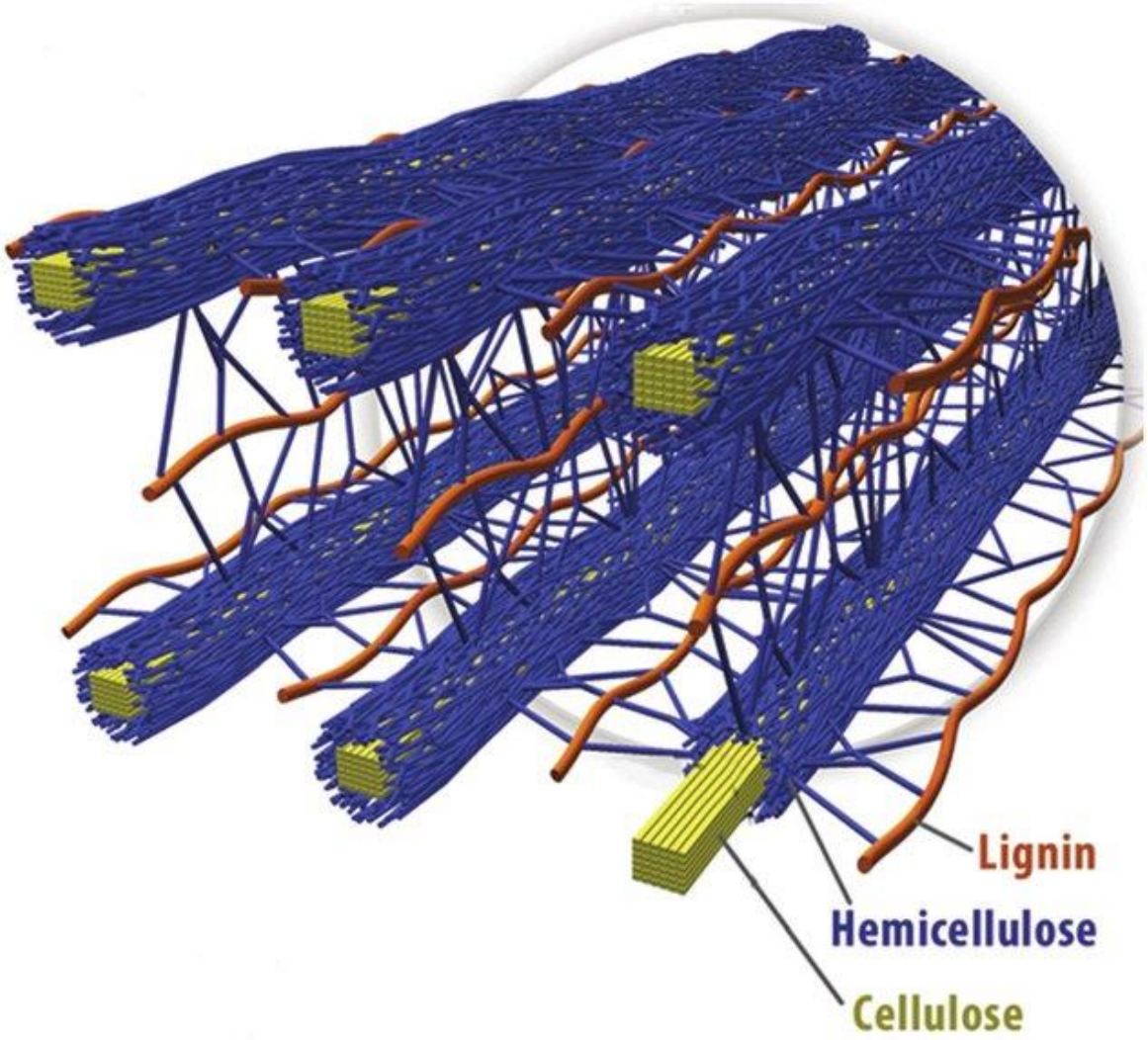


Fig. 1-3. The spatial arrangement of cellulose, hemicellulose and lignin in lignocellulose. This simulation is permitted to reproduce from the U.S Department of Energy Genome Programs, http://genomics.energy.gov (Brandt et al., 2013).

1.2.5 Improvement pathways in biofuel production from lignocellulose

Many studies have been focused on the pretreatment step as essential to improving the yield of biofuels production. The designed pretreatment aims to not only remove lignin but also retain the cellulose and hemicellulose for further fermentation steps (Roy et al., 2020). Among these conditions, using the mid-condition of temperature with chemical reagents, such as alkaline or diluted acids, are widely recognized.

The pretreated lignocellulose, containing both hemicellulose and cellulose, is required different enzymes to saccharify to fermentable sugars. The promising experimental design is expected to convert both cellulose and hemicelluloses to hexoses and pentoses. Besides, the by-products of saccharification need to show less negative impacts on the fermentation in promising design. With these expectations, the using of the microorganism, such as white-rot fungi, to saccharify hemicellulose and cellulose, is an advanced strategy (Liu et al., 2020). More importantly, white-rot fungi are capable of lignin degradation and co-fermentation of both pentoses and hexose to ethanol (Singh & Singh, 2014), leading to the efficient using white-rot fungi in the second generation of biofuel production.

1.3. Biofuel production from lignocelluloses by white-rot fungi

1.3.1 Taxonomy and biological characteristics of white-rot fungi

White-rot fungi (WRF) is the common name of a heterogeneous group of fungi that mostly belong to the basidiomycetes division. In nature, the common feature of these fungi, and only them, is that they can degrade lignin as well as cellulose and hemicelluloses, causing rotted wood to feel moist, soft, spongy, or stringy and appear white or yellow (Kamei, 2020; Schwarze, 2007). Many WRF is cultivating as the edible mushroom, providing nutrient food such as *Lentinula edodes* (Shiitake mushroom), *Pleurotus ostreatus* (oyster mushroom), *Armillaria mellea* (Honey mushroom), etc.

1.3.2 White-rot fungi and lignin degradation

It is strongly concluded that white-rot fungi could degrade lignin through activities of the oxidative and extracellular ligninolytic system, including lignin peroxidases (EC 1.11.1.14) (LiP), manganese peroxidases (EC 1.11.1.13) (MnP), laccases (EC 1.10.3.2) and versatile peroxidase (EC 1.11.1.16) (Sánchez, 2009). Under the aerobic solid-state, white-rot fungus *Phlebia* sp. MG-60 showed the selective degradation of lignin (40.7%) while the cellulose and hemicellulose contents were almost kept (Kamei et al., 2012a). Further study on white-rot fungus Phlebia sp. MG-60 revealed that the moisture content and inorganic nutrient also affect their biological delignification (Khuong et al., 2014b).

1.3.3 White-rot fungi and saccharification

The saccharification is an intermediate step in biological conversion from lignocellulose to biofuels, which produces fermentable sugars, mainly glucose and xylose, from cellulose and hemicellulose. The previous studies have confirmed the cellulase enzyme system, including endoglucanase, exoglucanase and β-glucosidase, that produced from WRF, is capable of complete hydrolysis of cellulose to glucose (Yoon et al., 2014). Besides, xylanase is the enzyme responsible for the hydrolysis of xylan (a main component of hemicellulose) to xylose, is also could be induced by WRF (Godoy et al., 2018b; Qinnghe et al., 2004). Many published studies showed the simultaneous saccharification of cellulose and hemicellulose in lignocellulose by cellulase and xylanase of WRF (Kobakhidze et al., 2016; Rastogi & Shrivastava, 2017).

1.3.4 Biofuel production from lignocellulosic materials by white-rot fungi

The conversion of pentose(s) and hexose(s) to ethanol (ethanol fermentation) is an ability of WRF (Okamoto et al., 2014). General, one molecule of hexose, such as glucose, produces two molecules of pyruvate in glycolysis. After that, under low oxygen conditions, two molecules of pyruvate are

converted to two ethanol molecules by enzymes alcohol dehydrogenase and pyruvate decarboxylase.

In the case of pentoses, such as xylose, the conversion is a little more complicated. At first, xylose is reduced to xylitol. Then, xylitol is oxidized to xylulose which is finally phosphorylated to xylulose-5-phosphate by the enzyme xylulokinase. The xylulose-5-phosphate then entering the pentose phosphate pathway in central carbon catabolism to produce ethanol (Kudahettige et al., 2012; Noor et al., 2010; Yano, 2015).

1.3.5 The possible pathways to advance biofuel production using white-rot fungi

Depend on substrates, microorganisms and experimental conditions, the modification or combination of delignification, saccharification, and fermentation could perform the biofuel production from lignocellulose. With the development of science and technology, there are several strategies have been using for biofuel production from lignocellulose: Separate hydrolysis and fermentation (SHF); Simultaneous saccharification and fermentation (SSF); Simultaneous Saccharification and Co-Fermentation (SSCF); Consolidated bioprocessing (CBP) (Amiri & Karimi, 2018; Khoo, 2015; Rastogi & Shrivastava, 2017).

In the aspect of an eco-friendly approach, the WRF was screened and isolated from the mangrove forest in Okinawa (Japan), named *Phlebia* sp. MG-60 was used in the integrated delignification and simultaneous saccharification and fermentation of hardwood (Kamei et al., 2012a), the results showed the possibility to produce ethanol directly from lignocellulose using only the biological process.

1.4 Japanese Moso bamboo - *Phyllostachys edulis*

Bamboo is a common name of woody-stemmed grass that belongs to the family Bambusoideae or Gramineae. In nature, there are approximately 1250–1500 species of bamboo, distributed across

approximately 31.5 million ha, the equivalent of 0.8% of the world's total "forested" area (Yuen et al., 2017).

Moso bamboo (*Phyllostachys edulis* (Carrière) J. Houz.) is one of the largest species of bamboo. In Japan, Moso-bamboo is a non-native invasive species that was introduced from China in the 1700s (Shinohara et al., 2019). In western Japan, Moso-bamboo forests have spread into and replaced surrounding broadleaved and coniferous forests, which was speculated that related to a decrease in ecosystem services, such as hydrology, soil erosion, and biodiversity. A previous review study also has indicated that Moso-bamboo (*Phyllostachys edulis*) forest accumulated at least 5.10 Mg Carbon per ha during a single year – a rate that is 33% higher than a tropical mountain rainforest (Yuen et al., 2017). With these huge carbon renewable sources, Moso bamboo was targeted as the raw material to produce many kinds of foods, functional foods, bio-materials, and bio-ethanol (He et al., 2014; Nirmala et al., 2018).

1.5 The aims of studies

Interesting in the bioenergy study, the serial experiments were conducted during the doctoral course aiming to produce bioethanol and biobutanol. At the beginning of the study, the characteristics of Japanese Moso bamboo (*Phyllostachys edulis*) after alkaline pretreatment and its use to produce ethanol in consolidated bioprocessing using white-rot fungus *Phlebia* sp. MG-60 fermentation was discussed (Chapter 2).

Among biofuels, butanol has a higher heating value than ethanol. However, butanol only could be produced from Acetone:Butanol:Ethanol (ABE) fermentation process of fermentable sugars by the bacterium *Clostridium.* Aiming to produce butanol directly from cellulosic material, in Chapter 3, the study has conducted the co-culture between white-rot fungus *Phlebia* sp. MG-60-P2 and bacterium *Clostridium,* aimed to produce butanol from cellulosic material Unbleached Hardwood

Kraft Pulp (UHKP). Also, the effects of the inhibition of fungal ethanol fermentation on characteristics of this fungus-bacterium co-culture were discussed.

An intensive study on the co-culture of white-rot fungi MG-60-P2 and *Clostridium* to utilize Moso bamboo is conducted in Chapter 4. The effects of co-culture on the utilization of lignocellulose substrates to produce butanol, acetone and ethanol, as well as the contribution of alkaline pretreatment to this process, are objects of this study.

Chapter 2.

The improvement of sodium hydroxide pretreatment in bioethanol production from Japanese bamboo *Phyllostachys edulis* using white-rot fungus *Phlebia* sp. MG-60

2.1 Introduction

Lignocellulosic biomass usually includes forest biomass and wastes, agricultural residues, and energy crops, which are mostly comprised of cellulose, hemicelluloses, and lignin components. These natural resources have been focused on as potential materials for the second generation of bioethanol production because of their beneficial characteristics, including high annual yield, abundance, and high concentrations of holocellulosic contents (Cai et al., 2017; Zabed et al., 2016). According to the previous researches, the second generation of bioethanol production is recognized as a key topic in energy science research because the results contribute not only to energy production but also to the reduction of environmental pollution. The primary research goal of second-generation bioethanol production is to increase the polysaccharide conversion and fermentation yield (Zabed et al., 2017).

The first step in bioethanol production is pretreatment. Successful methods were identified as the technologies able to alter or remove compositional impediments and resistant structures of biomass to improve enzymatic saccharification by using mechanical, biological, or chemical methods. Among these approaches, alkaline pretreatment was documented as one of the most effective methods to remove lignin and hemicelluloses, as well as increase surface contact area in various materials (Kim et al., 2016; Mosier et al., 2005; Saha et al., 2016; Suhara et al., 2012; Suhardi et al., 2013; Tomak et al., 2013; Yang et al., 2015; Zhang et al., 2017; Zhang et al., 2007). In bioethanol production, depending on pretreated sample features and experimental conditions,

various strategies have been applied such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and consolidated bio-processing (CBP). In general, modified SSF and CBP were identified as preferred processes with high economic feasibility (Chen & Fu, 2016; Mitchell et al., 2002).

Phlebia sp. MG-60 is a hypersaline-tolerant basidiomycete and is reported as a powerful bioethanol producer because its enzyme system can participate in lignin degradation, hydrolysis of polysaccharides, and fermentation of reducing sugars. Several studies have emphasized that *Phlebia* sp. MG-60 can produce ethanol from various types of biomass, including sugarcane bagasse, wood, and residues of mushroom production (Kamei et al., 2012a; Kamei et al., 2012b; Kamei et al., 2014; Khuong et al., 2014a) without addition of commercial hydrolytic enzymes.

Bamboo is the common name for more than 1,200 species groups of large woody grasses which have been used for food, construction materials, and traditional arts for approximately 2.5 billion people around the world. With such wide distribution, huge annual biomass yield, and high holocellulose content, bamboo has been recently recognized as a high-potential material for bioenergy production (Darabant et al., 2014; Fei et al., 2016; He et al., 2014; Scurlock et al., 2000).

To continue the focus on bioenergy performance from bamboo species, this study was conducted to 1) evaluate the effect of alkaline pretreatment on the chemical transformation of Japanese bamboo and 2) determine the efficiency of the integration of alkaline pretreatment and the white-rot fungus *Phlebia* sp. MG-60 fermentation in bioethanol production from Japanese bamboo, *Phyllostachys edulis*, with additional commercial hydrolytic enzyme (sSSF) or without (CBP).

2.2 Material and Methods

2.2.1 Bamboo samples

The stems of Japanese bamboo, *Phyllostachys edulis*, were kindly donated by Sanuki Kasei Co., Ltd. Samples were minced into 40 ~100 mesh size particles; four hundred grams of which were extracted with solution of benzene and ethanol (ratio 2:1 v/v in total 2.1 L) for 16 hours in a Soxhlet apparatus. Extracted bamboo – hereafter referred to as "initial" – was air-dried, then stored in plastic bags.

2.2.2 Fungal strain and cultures

White rot fungus *Phlebia* sp. MG-60 was used for this research. Phylogenetic analysis of internal transcribed spacers (ITSs) containing rRNA gene sequence (ITS-rDNA) clarified that this strain belongs to the genus *Phlebia* and is closely related to the fung*us Phlebia lindtneri.* This sequence was deposited to the database with the DDBJ accession number AB210077 (Kamei et al., 2005). The stock culture was maintained in Potato Dextrose Agar (PDA) slant at -80°C. The mycelial disk was then inoculated on PDA plates to recover at 28°C. The mycelia were cultured for 5 days in the PDA before being used in the fermentation steps.

2.2.3 Sodium hydroxide pretreatment

Three grams of initial bamboo were each added to 24 mL of sodium hydroxide (0.0, 0.5, 1.0, 3.0, 5.0 and 7.0%, w/v) aqueous solutions in individual 100 mL Erlenmeyer flasks. The mixtures were autoclaved at 120°C for 1 hour. After autoclaving, samples were filtered through glass filter type G3 and then washed with distilled water to achieve neutral pH. Pretreated samples were dried in the air for 72 hours and then kept in plastic bags to prevent environmental effects. The pretreatment step was done by three-time repeats.

2.2.4 Commercial enzymatic hydrolysis

Two hundred milligrams of initial and pretreated bamboo samples were placed in individual test tubes, each containing 20 mL sodium citrate buffer (50 mM, pH 4.8) and 200 mg of the commercial hydrolytic enzyme Meicelase (containing 0.437 g protein per 1 g of the enzyme, Meicelase-P; Meiji Seika Co. Ltd, Tokyo, Japan). The test tubes were then incubated at 60°C with 120 rpm for 72 hours. One mL of hydrolysate was collected after 24, 48, and 72 hours and used to determine the concentration of glucose and xylose.

The saccharification rate of the sample was determined by the following equation:

$$\text{SR (\%)} = \frac{\text{Total weight of glucose and xylose in hydrolysate}}{\text{Theoretical weight of glucose and xylose in 200 mg of sample}} \text{ x } 100$$

2.2.5 Consolidated bioprocessing using white-rot fungus Phlebia sp MG-60

Erlenmeyer flasks (100 mL type), each containing 0.8 g of sample and 40 mL of a basal liquid medium, which is the fundamental nutrients to fungal growth (10 g L^{-1} yeast extract, 10 g L^{-1} KH_2PO_4, 2 g L^{-1} $(NH_4)_2SO_4$ and 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$) with pH scale was 6.0, were autoclaved at 120°C for 15 min. To start fermentation, a 6 mm-diameter disc of *Phlebia* sp. MG-60 mycelium from PDA was transferred into each Erlenmeyer flask mentioned above. Flasks were sealed with silicone plugs and incubated at 28°C in the darkroom.

2.2.6 Semi-Simultaneous Saccharification and Fermentation

sSSF was done through a combination of pre-hydrolysis by commercial enzyme and fungal cultivation in the single bioreactor. In the pre-hydrolysis step, the bamboo substrate (0.8 g of each sample) was added into 100 mL Erlenmeyer flasks containing 40 mL sodium citrate buffer (50mM, pH 4.8) and 0.4 g Meicelase at 60°C with shaking (120 rpm) for 72 hours. After 72 hours, 1 mL of hydrolysate was taken to determine xylose and glucose contents. To start the fungal cultivation, a

6 mm-diameter disc of *Phlebia* sp. MG-60 mycelium (taken from PDA) was added into each flask, sealed with a silicone plug, and incubated at 28°C in the darkroom.

2.2.7 Chemical composition analysis of bamboo

Determination of structural carbohydrate and lignin contents in initial and pretreated samples was done by three-time repeats, following the Laboratory Analytical Procedure NREL/TP-510-42618 (Sluiter et al., 2008). The 300 mg of sample was placed in a 100 mL Erlenmeyer flask and hydrolyzed with 3 mL of 72% H_2SO_4 for 30 min at 60°C. Eighty-four mL distilled water was then added to the flask to dilute the solution, resulting in a 4% H_2SO_4 concentration. All samples were hydrolyzed a second time by autoclaving at 120°C for 1 hour. After being separated by glass filter G3, solid residues were dried at 100°C in an oven to determine lignin content. One mL of liquid was taken to determine glucose and xylose concentrations by high-performance liquid chromatography (ICSep ICE-coregel 87H3 column type; Refractive Index Detector RID-10A; Shimadzu, Kyoto, Japan) with a mobile phase consisting of 5.0 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}. The injection volume was 10.0 µL and the oven temperature was maintained at 70°C.

2.2.8 Ethanol production analysis

One mL of medium sample was taken from the incubated flask and then centrifuged at the 12,000 × *g* for 10 min at 4°C to separate supernatant and mycelium. The resulting supernatant was used to measure the concentration of produced ethanol by HPLC which is mentioned in section 2.2.7

The ethanol yield and conversion rate were determined by the following equations:

$$\text{Ethanol yield (\%)} = \frac{\text{Weight of produced ethanol (g)}}{\text{Weight of oven-dried sample (g)}} \text{ x } 100$$

$$\text{Conversion rate (\%)} = \frac{\text{Concentration of produced ethanol (g/mL)}}{\text{Theoretical concentration of ethanol (g/mL)}} \text{ x } 100$$

Ethanol production from xylose is described as $3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2$. In this equation, 3 moles of xylose (molecular mass 150) are required to produce 5 moles of ethanol (molecular mass 46). So, the coefficient of ethanol obtained from xylose equal to (5×46)/(3×150)

Ethanol production from glucose is $C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$. By that, 1 mole of glucose (molecular mass 180) produces 2 moles of ethanol, therefore the coefficient of ethanol obtained from glucose equal to (2×46)/180

Xylose and glucose are monosaccharides, which can be obtained from polysaccharides (xylan and glucan) after hydrolysis. To calculate the concentration of monosaccharides from the concentration of corresponding polysaccharides, an anhydro correction of 150/132 for xylan as well as the correction of 180/162 for glucan are needed.

Theoretical concentration of ethanol = [glucan concentration × (180/162) × (46×2/180) + xylan concentration × (150/132) × (46×5)/(150×3)] (Aita et al., 2011; Sluiter et al., 2008).

2.3 Results and Discussion

2.3.1 The effect of pretreatment on chemical composition of bamboo

The main components of initial and pretreated samples are shown in Table 1. Statistical analysis for data was examined by analysis of variance (ANOVA) in Microsoft Office 2016 with the probability value ($p \leq 0.05$) was considered as a statistically significant difference. The statistical analysis has revealed the significant effect of alkaline pretreatment on chemical composition changing of bamboo samples with all pretreatment modes (0.0-7.0% NaOH, w/v) have shown the significantly different ($p < 0.00001$). Following the data in Table 2-1, the initial bamboo was 43.0% glucan, 26.2% lignin, and 23.1% xylan. These high contents of carbohydrates show that Japanese bamboo is a potential material for bio-ethanol production, agreeing with other published research on bamboo species (He et al., 2014; Scurlock et al., 2000). In this study, the concentration

range of NaOH was prepared to examine the effect of alkaline pretreatment on the chemical transformation of the sample. Overall, the increase in NaOH concentration for pretreatment is closely related to the increase in glucan as well as to the reduction in xylan and lignin components in the samples. Following the 7.0% NaOH pretreatment, the glucan component of the sample reached to 67.7%; xylan and lignin contents were 13.2% and 16.7%, respectively (Table 2-1). In the case of lignin, when the NaOH concentration was increased from 0.0% to 0.5% and 1.0%, the decrease in lignin content from 26.6% to 22.1% and 16.6% were found. However, at higher concentrations of NaOH (3.0% to 7.0%), the reduction of lignin content was insignificant. On the other hand, the xylan content followed a reduction trend from 23.1% in the initial sample to 13.2% in the 7.0% NaOH pretreated sample. These results indicated that the removal of lignin in bamboo could be more efficient by using a low concentration of NaOH. The increase in sum contents of glucan, xylan, and lignin represents a composition more ideal for bioethanol production.

The mechanism of sodium hydroxide pretreatment can be explained by the hydroxide ion attacking the carbon of the ester bond, which is the linkage between lignin and hemicelluloses or between two hemicelluloses (Modenbach, 2013). NaOH has been documented as a successful pretreatment reagent for several other bamboo species (Kassaye et al., 2017; Kuttiraja et al., 2013; Li et al., 2016) by the removal of lignin and hemicelluloses, resulting in the reduction of solid residual weight. To evaluate the weight loss of Japanese bamboo during pretreatment using a concentration range of NaOH, the data were prepared in Table 2-2. Overall, a higher concentration of alkaline solution has a strong inverse relationship to the amount of residual solid. Following a 7.0% NaOH pretreatment, 56.7% of the initial sample remained, with glucan, xylan, and lignin residues of 88.7%, 32.7%, and 35.8%, respectively. This shows that weight loss during alkaline pretreatment was mainly attributed to the removal of hemicelluloses and lignin. In the concept of a bamboo

refinery, utilization of the waste materials in the liquid phase after pretreatment, including soluble lignin, hemicelluloses, and extractives, needs to be paid attention to (Gong et al., 2017; Peng & She, 2014; Wen et al., 2011).

Table 2-1. The chemical composition (%) of initial and sodium hydroxide-pretreated samples.

Sample	glucan	xylan	lignin	Sum of contents
Initial	43.0 ± 0.6	23.1 ± 0.3	26.2 ± 0.6	92.4
0.0% NaOH	43.8 ± 0.7	22.4 ± 0.3	26.6 ± 0.2	92.7
0.5% NaOH	50.1 ± 0.3	23.7 ± 0.2	22.1 ± 0.0	95.9
1.0% NaOH	55.7 ± 0.3	22.0 ± 0.7	16.6 ± 0.2	94.3
3.0% NaOH	61.0 ± 0.0	19.5 ± 0.2	16.1 ± 0.3	96.5
5.0% NaOH	64.7 ± 0.4	16.0 ± 0.1	16.9 ± 0.4	97.7
7.0% NaOH	67.7 ± 0.1	13.2 ± 0.0	16.7 ± 0.4	97.6

Table 2-2. The effect of sodium hydroxide pretreatment on the chemical composition (%) of samples.

Sample	Residual solid	Residual glucan	Residual xylan	Residual lignin
Initial	100	100	100	100
0.0% NaOH	96.0	97.7	92.9	97.3
0.5% NaOH	83.2	95.6	85.3	70.1
1.0% NaOH	71.1	92.1	67.7	46.8
3.0% NaOH	63.6	90.2	53.5	38.9
5.0% NaOH	60.5	91.1	41.9	39.0
7.0% NaOH	56.3	88.7	32.7	35.8

2.3.2 The effect of pretreatment on saccharification ability of bamboo

Reducing sugars are direct carbon sources for ethanol fermentation; therefore, a key point to improve bioethanol conversion from biomass is the enhancement of saccharification. In this study, a commercial enzyme was used to determine the ability of bamboo to be hydrolyzed before and after pretreatment, with the results shown in Fig. 2-1. The saccharification rates were accelerated in the first 24 hours and reached the highest conversion rate when 89.5% of the theoretical weight of polysaccharides (glucan and xylan) in the 7.0% NaOH pretreated sample was converted to fermentable sugars (glucose and xylose) after 72 hours. In the 5.0% NaOH pretreated sample, the saccharification rate after 72 hours was 82.0%, which is lower than that of the 3.0% NaOH pretreated samples (84.3%). This result could be interpreted as the higher lignin content samples showed a lower hydrolysis yield. A clearer relationship between lignin content and saccharification rate was seen in the initial, 0.0%, 0.5%, and 1.0% NaOH pretreated samples, where the significantly higher lignin content in the samples showed lower saccharification rates. As shown in Figure 2-1, the saccharification rate of bamboo was increased more than two-fold, from 42.5% in the initial to 89.5% in the 7.0% NaOH pretreated sample. Based on the concept of lignin removal in Japanese bamboo leading to the improvement of the saccharification rate, this study indicates that applying an alkaline compound is a successful pretreatment method. It is also comparable to the results of other studies that have used either chemical pretreatment (Kassaye et al., 2017; Li et al., 2016; Liu et al., 2017; Sun et al., 2014; Yamashita et al., 2010) or biological pretreatment (Suhara et al., 2012; Zhang et al., 2007) to enhance the hydrolysis in bamboo materials. Saccharification rates achieved in those studies varied from 60% to more than 90%, depending on the experimental conditions. Also, the saccharification of bamboo has shown a close relation to

the growth stages, species, and which part of the bamboo tree was used for the experiment (Cheng et al., 2015; Darabant et al., 2014; Shimokawa et al., 2009; Zhang et al., 2017).

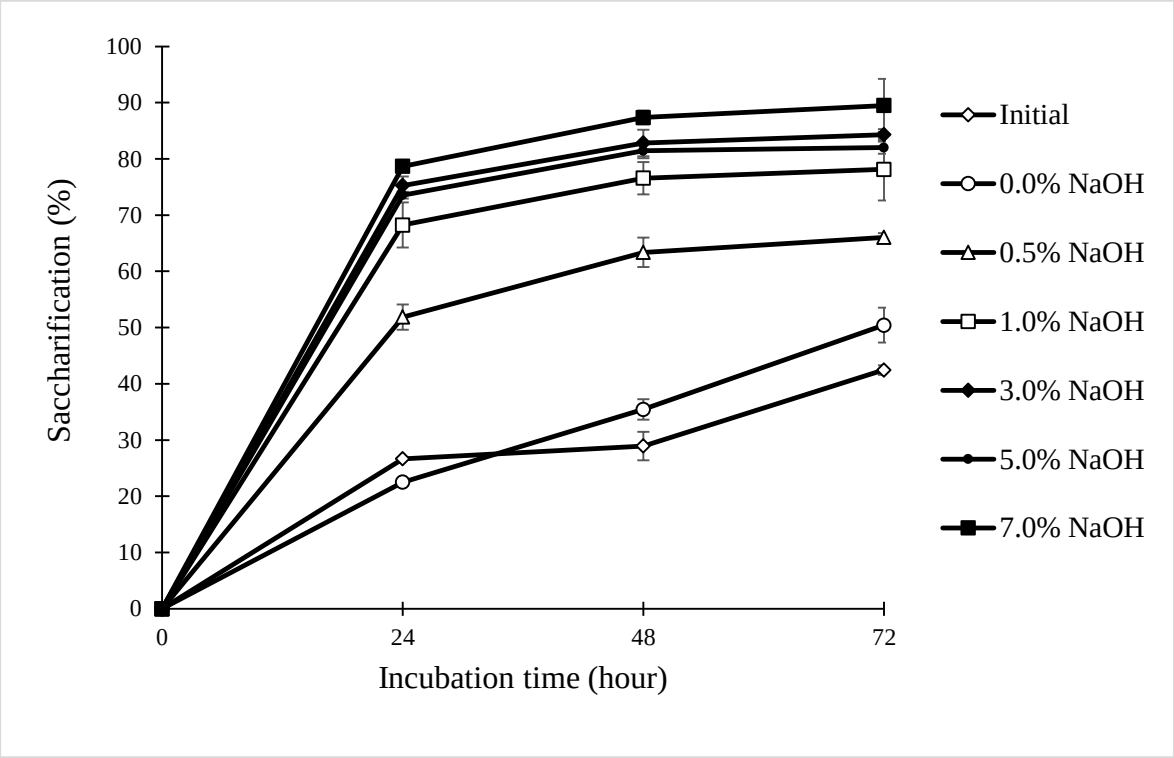


Fig. 2-1. The saccharification rates of initial and sodium hydroxide pretreated bamboo using a commercial hydrolytic enzyme. The error bars represent the standard deviations of three replicates.

2.3.3 Bioethanol production from bamboo by applying consolidated bioprocessing

After NaOH treatment, pretreated bamboo with a high content of polysaccharides became the suitable material for bioethanol production. To evaluate the feasibility of a combination of alkaline pretreatment and white-rot fungus fermentation to produce ethanol from Japanese bamboo, the incubation of *Phlebia* sp. MG-60 and pretreated bamboo was conducted without the addition of a commercial hydrolytic enzyme. White rot fungus *Phlebia* sp. MG-60 has been successfully applied to bioethanol fermentation of various biomaterials (Kamei et al., 2012a; Kamei et al., 2012b; Khuong et al., 2014a) through the activities of their generated enzymes, including lignin degradation, hydrolysis, and co-fermentation. This study reports the first published results on direct ethanol production from bamboo by consolidated bioprocessing (Fig. 2-2, 2-3). Processes quickly proceeded in the first 15 days and reached the highest ethanol yield after 20 days (12.8%, w/w), equivalent to 28.5% of polysaccharides (glucan and xylan) in the 7.0% NaOH pretreated sample being converted to ethanol. The initial and 0.0% NaOH pretreated samples, however, showed negligible ability to produce bioethanol. This phenomenon suggested that alkaline pretreatment is an important factor to improve the bioethanol production yield from bamboo by using *Phlebia* sp. MG-60 in CBP.

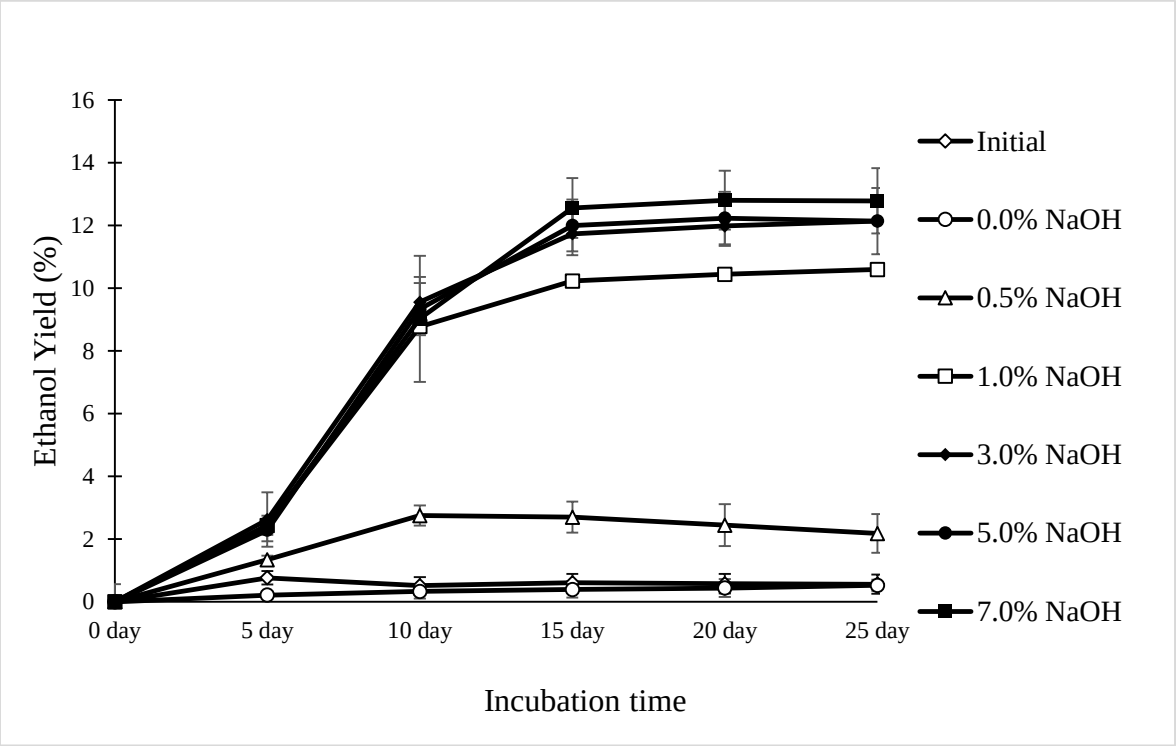


Fig. 2-2. The ethanol yield from Japanese bamboo by using the *Phlebia* sp. MG-60 in CBP. The calculation is based on the weight of produced ethanol and pretreated samples. The results and error bars show the average values and standard deviations, respectively, of three replicates.

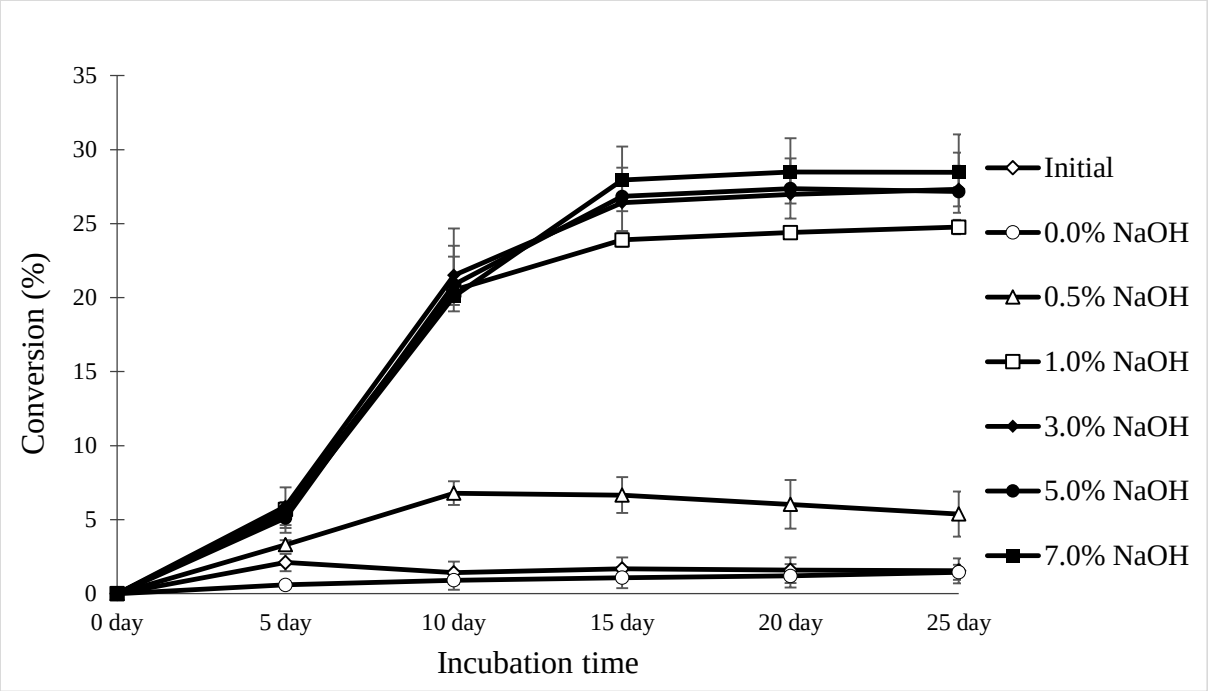


Fig. 2-3. The conversion of Japanese bamboo into ethanol by using the *Phlebia* sp. MG-60 in CBP. The theoretical concentration of ethanol is calculated with the concentration of polysaccharides in the pretreated samples. The results and error bars show the average values and standard deviations, respectively, of three replicates.

2.3.4 Bioethanol production from bamboo by using semi-simultaneous saccharification and fermentation

To evaluate the efficiency of a bioethanol production process, the ethanol yield, as well as incubation time of microorganisms and substrates, are important factors. To improve the efficiency in bioethanol production from Japanese bamboo by CBP, the sSSF strategy was established. In sSSF, the ethanol production by microorganism fermentation is supported by the hydrolysis of commercial enzyme and the result is shown in Fig. 2-4.

By the commercial enzyme hydrolytic assistance, the bioethanol production period was shortened, the bioethanol was rapidly produced during the first 6 days, then near-maximum conversion was achieved after 12 days, compare to incubation time in CBP. These results showed that not only fermentation time but also the ethanol production yield was improved in the sSSF. Figure 2-4A shows that, based on polysaccharide composition (glucan and xylan) in pretreated samples, the highest bioethanol conversion (58.9%) was in the 7.0% NaOH pretreated sample. The lowest bioethanol conversion was 15.9% in the 0.0% NaOH pretreated sample, which was due to negligible chemical transformation of Japanese bamboo during the alkaline-free hydrothermal pretreatment (autoclave with distilled water at 120°C for 1 hour).

During the alkaline pretreatment step, a part of lignin and hemicellulose structures in bamboo were selectively broken and removed, leading to weight decrease although glucan proportion increased. Therefore, to evaluate the efficiency of the whole bioethanol production process, from the initial sample through pretreatment and fermentation, the conversion rates were corrected by the weight loss of samples, and the results are shown in Fig. 2-4B. With this correction applied, the overall efficiency of the 1% NaOH pretreated sample achieved the highest conversion (38.1% after 15 days of sSSF), followed by the 5.0%, 3.0%, and 7.0% NaOH pretreated samples (34.4%, 33.9%,

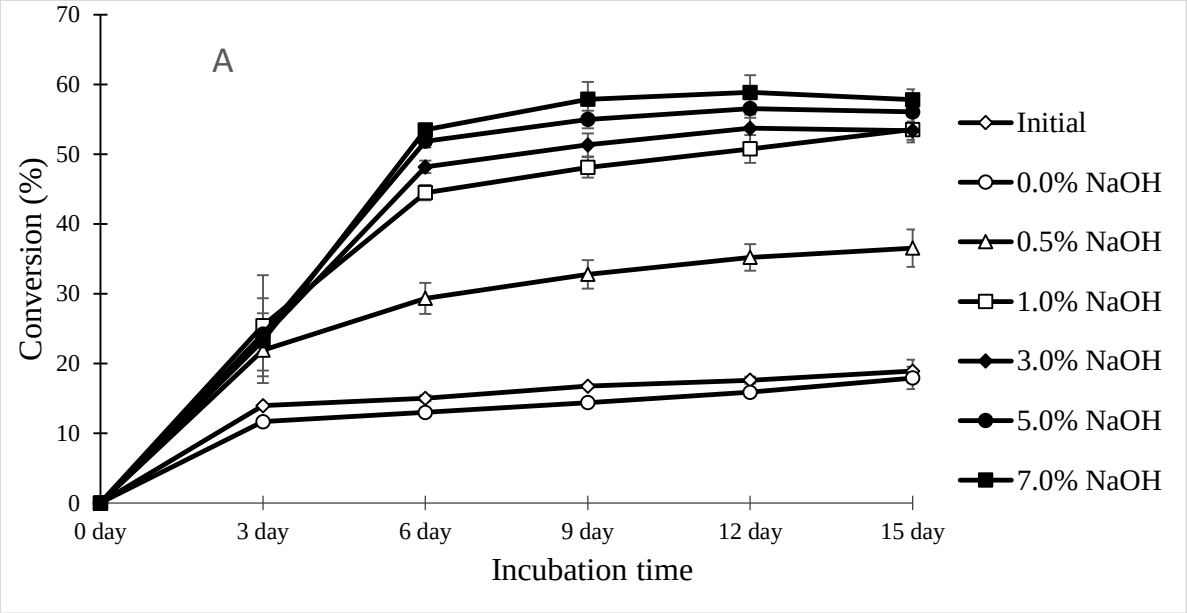
and 32.5%, respectively). These results suggest that using a diluted concentration of NaOH in pretreatment is a reasonable approach to bioethanol production from Japanese bamboo, based on the aspects of chemical consumption and production yield.

Many previous studies on bioethanol production from bamboo applied separate hydrolysis and fermentation, which were operated under optimal conditions. Mingxiong et al. (2013) reported that 55.8% of theoretical ethanol yield of pretreated bamboo shoot shell fiber was obtained by bacterium *Zymomonas mobilis* fermentation, which is smaller than the present result with the ethanol yield was 58.9% in the case of 7.0 % NaOH pretreated sample (Fig. 2-4A). Kuttiraja et al. (2013) reported that 42.8% of polysaccharides in their bamboo samples were used to produce ethanol by *Saccharomyces cerevisiae* fermentation. Compared to *Phlebia* sp. MG-60, the fungus *S. cerevisiae* performed negligible advantages under optimal conditions. Besides, using *S. cerevisiae*, Sindhu et al. (2014) indicated that the overall efficiency of bioethanol from processed bamboo was 41.7%, based on the theoretical yield of glucose content, which is not significantly different from the yield of the present study (Fig. 2-4B). These results showed a suggestion about *Phlebia* sp. MG-60 is a good bioethanol producer from lignocellulosic biomass.

2.4. Conclusions

The bioethanol production process from Japanese bamboo by the white-rot fungus *Phlebia* sp. MG-60 fermentation was examined with using of a sodium hydroxide pretreatment. The alkaline pretreatment showed the removal of lignin and hemicelluloses, leading to an increase in glucan composition as well as the saccharification rate of bamboo. The obtained results show that *Phlebia* sp. MG-60 is a powerful fungal candidate and is capable of bioethanol production from biomass, with or without a commercial hydrolytic enzyme. To achieve a promising strategy in bamboo

biorefinery, the utilization of the removed lignin and hemicelluloses is needed to be examined in further experiments.



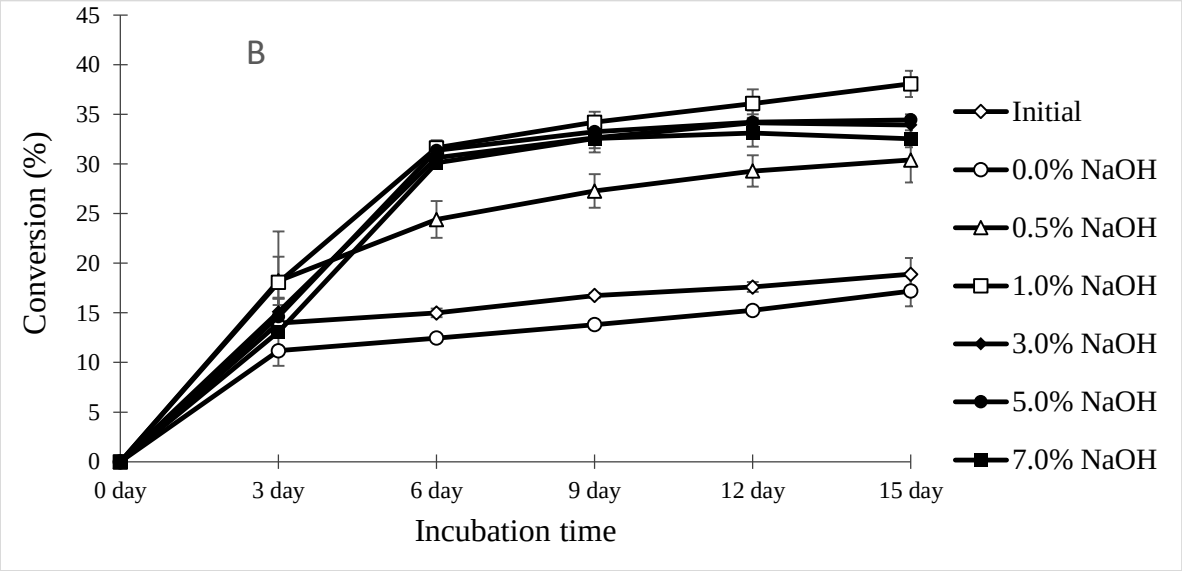


Fig. 2-4. The time course of bioethanol production from Japanese bamboo by using *Phlebia* sp. MG-60 in sSSF. The theoretical concentration of ethanol is calculated with the concentration of polysaccharides in the pretreated sample (A) and initial sample (B). The concentration of polysaccharides in the initial sample (B) was corrected by the weight loss of the sample during pretreatment step. The results and error bars show the average values and standard deviations, respectively, of three replicates.

Table 2-3. The concentration of fermentable sugars and produced ethanol during sSSF.

	glucose (g/L)		xylose (g/L)		ethanol (g/L)
Sample	0 day	15 day	0 day	15 day	15 day
Initial	1.3	0.0	0.9	0.0	1.3
0.0% NaOH	1.2	0.0	1.0	0.0	1.2
0.5% NaOH	3.3	0.1	2.4	0.8	2.7
1.0% NaOH	5.6	0.1	3.2	1.1	4.1
3.0% NaOH	6.7	0.1	2.8	0.9	4.3
5.0% NaOH	7.3	0.1	2.2	0.7	4.5
7.0% NaOH	7.9	0.1	1.8	0.6	4.7

Chapter 3.

Butanol production from cellulosic material by anaerobic co-culture of white-rot fungus *Phlebia* sp. MG-60 and bacterium *Clostridium* in consolidated bioprocessing

3.1 Introduction

Human society is facing a decrease in available fossil fuel resources while the demand for energy consumption is increasing continuously. This problem has promoted research into biofuel production and its application in internal combustion engines. Butanol is promising among biofuels, with high heating value while being safe to transport and handle (Li et al., 2019; Trindade & Santos, 2017).

Butanol production from fermentable sugars, such as glucose or cellobiose, through the acetone–butanol–ethanol (ABE) fermentation process, was previously documented. In this process, in anaerobic conditions, *Clostridium* metabolizes sugars to produce butanol (primarily), ethanol and acetone. For use of this process on the industrial scale, it is desirable to utilize raw, cheap and abundant materials such as lignocelluloses. However, it is difficult to produce butanol from these materials through biological processes only because the known wild-type strains of *Clostridium* that can take part in ABE fermentation are incapable of delignification and saccharification (Godoy et al., 2018a; Ibrahim et al., 2018; Jang et al., 2012; Lynd et al., 2002; Schwarz, 2001).

To overcome this problem, bacterial co-cultures were employed to perform butanol production from glucose, cellulose, or pretreated lignocelluloses (Kiyoshi et al., 2015; Li et al., 2013; Nakayama et al., 2011; Oliva-Rodríguez et al., 2019; Petitdemange et al., 1983; Tran et al., 2010; Wu et al., 2019). These studies confirm the possibility of establishing co-cultures of cellulolytic bacteria and ABE-fermenting bacteria under anaerobic conditions to produce butanol. However,

the role of each microbial component, as well as their growth in co-culture, was not clearly elucidated in previous studies.

White-rot basidiomycetous fungi have been recognized as advantageous for biofuel production from lignocellulose because of their ability to degrade the main components in the plant cell wall (cellulose, hemicellulose and lignin). Previous studies showed that the white-rot fungus *Phlebia* sp. MG-60 could directly produce ethanol from lignocelluloses via three specific processes: degradation of lignin in aerobic solid-state incubation; saccharification of polysaccharides and ethanol fermentation from monosaccharides in semi-aerobic conditions (Kamei et al., 2012b; Kamei et al., 2014; Khuong et al., 2014a; Motoda et al., 2019). Recently, the fungal transformant line KO77 was successfully established by knockout of the *pyruvate decarboxylase* (*pdc*) gene from strain *Phlebia* sp. MG-60-P2 (a protoplast-regenerated strain of wild-type *Phlebia* sp. MG-60 with uniform phenotypes) (Tsuyama et al., 2017). Interestingly, the transformant line KO77 accumulated glucose from cellulosic material in semi-aerobic consolidated bioprocessing (CBP), rather than fermenting ethanol as was observed for strain MG-60-P2 (Tsuyama et al., 2017). This finding indicates that it may be possible to co-culture KO77 with other microorganisms to produce valuable chemicals from cellulosic materials, based on the hypothesis that in co-culture, KO77 could supply glucose, while the other microorganism(s) would use that glucose as a carbon source to produce chemicals with a higher value than ethanol, such as butanol.

Several strategies can be considered for butanol production from lignocellulose, including separate saccharification and fermentation, simultaneous saccharification and fermentation, and CBP. Among these processes, the combination of cellulose saccharification and fermentation in CBP is recommended for biofuel production, showing numerous advantages, including reduction of contamination risk and increased overall production yield (Jang et al., 2012; Xin et al., 2019).

To advance knowledge about direct butanol production from cellulose, this study aimed to achieve butanol fermentation from unbleached hardwood kraft pulp (UHKP) using the anaerobic co-culture of the white-rot fungus *Phlebia* sp. MG-60-P2 and the bacterium *Clostridium saccharoperbutylacetonicum* strain NBRC109357 in CBP. The impact of the inhibition of ethanol fermentation in fungal line KO77 on the yield of butanol in co-culture with *C. saccharoperbutylacetonicum* was also determined.

3.2 Material and Methods

3.2.1 Fungal and bacterial strains and cultivation conditions

C. saccharoperbutylacetonicum NBRC 109357 stored at −80 °C was pre-cultured in 29.3 g/L of liquid thioglycollate medium (Nihon Pharmaceutical Co. Ltd.) for 36 h at 30 °C under anaerobic conditions. Before use in co-culture, the optical density at 600 nm (OD_{600}) of the 10-fold diluted *C. saccharoperbutylacetonicum* culture was 0.14.

Phlebia sp. MG-60-P2 (hereafter P2) was isolated from derived protoplasts of wild-type strain MG-60 to unify the protoplast phenotypes. The transformant line KO77 of P2 was constructed by knockout of the *pdc* gene (Tsuyama et al., 2017). The fungal strain P2 and the transformant line KO77 were inoculated into Petri dishes containing Difco™ potato-dextrose-agar (PDA; BD Biosciences, supplier number 213400) for 10 days at 28 °C.

3.2.2 Co-culture of *Clostridium* and *Phlebia*

UHKP (0.4 g) was loaded into 100-mL Erlenmeyer flasks containing 19 mL basal liquid medium [2.0 g/L yeast extract, 6.0 g/L tryptone, 2.57 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4.7H_2O$, 0.5 g/L KH_2PO_4, 10 mg/L $FeSO_4.7H_2O$, 3 g/L $CaCO_3$] (Zhao et al., 2019). This medium was autoclaved at 120 °C for 15 min.

Six millimeter-diameter discs of fungal mycelium grown for 10 days on PDA were added into flasks containing sterilized UHKP (Oji Paper Co., Ltd., Tokyo, Japan). The alkaline unbleached hardwood kraft pulp was washed with distilled water to neutral pH (approximately 6.5) and air-dried. Before adding to Erlenmeyer flasks, dried UHKP was defibrated by mixing blades rotating 20,000 rpm for 20 seconds. These flasks were kept at 28 °C for 5 days for fungal pre-cultivation. Then, 1 mL of bacterial culture (see section 2.1) was added to the flasks to begin co-culture. The whole experimental process (fungal pre-cultivation and co-culture) was kept anaerobic by supplying nitrogen gas flowing at 0.5 MPa to all flasks for 20 s then immediately sealing the flasks with silicone plugs.

3.2.3 Growth of *Clostridium* and *Phlebia* in consolidated bioprocessing

According to our previous study (Harry-asobara & Kamei, 2018), the co-culture was separated into liquid and solid components using a glass filter G2 with pore size 40–100 μm (PYREX IWAKI, ASAHI, AGC Techno Glass, Japan). The pH of the co-culture liquid was measured using a pH METER F-51 (HORIBA Ltd., Japan). OD_{600} of 10-fold diluted culture was determined using a V-630 Bio spectrophotometer (JASCO Deutschland GmbH) to measure bacterial growth.

The fungal mycelium was used as a sample for analysis of ergosterol content (Grant & West, 1986; Niemenmaa et al., 2008). First, the sample and 5 mL KOH (10% w/v in methanol) was added to a 10-mL glass vial and mixed in an ultrasonic bath for 10 min. Then, the mixture was kept at 80 °C for 1 h for saponification. After that, 1 mL of distilled water and 2 mL of *n*-hexane were added to the vial to extract ergosterol. The dissolved ergosterol in the *n*-hexane phase was collected and transferred to a new glass vial. These extraction and collection steps were repeated three times. The vial containing dissolved ergosterol in *n*-hexane was dried by nitrogen gas flow. The dried ergosterol was dissolved in 1 mL of methanol and filtered through a 0.22-μm filter (RF-Jet

Syringe). The dissolved ergosterol in methanol was analyzed by high-performance liquid chromatography (HPLC) using an LC-20AD system (SHIMADZU Corp., Japan) with a UV/VIS SPD-20A detector at 282 nm. Sample (10 µL) was injected into an Inertsil ODS-4 column (4.6 × 150 mm, particle size 5 µm; GL Sciences Inc.) at 40 °C with a flow rate of 1 mL/min of methanol mobile phase.

3.2.4 Analytical methods

After each interval of 48 h in the co-culture process, 1 mL of culture was pipetted from experimental flasks and centrifuged at 18,000 × *g* for 10 min to remove solids. The content of cellobiose, glucose, ethanol and butanol in the supernatant was quantified by HPLC using an LC-20AD system with refractive index detector RID-10A. Supernatant sample (10 µL) was injected into an ICSep ICE-core gel 87H3 column (7.8 × 300 mm; part number ICE-99-9861, Concise Separations, USA) at 70 °C with a flow rate of 0.6 mL/min of 5.0 mM H_2SO_4 mobile phase.

The conversion of cellulose in UHKP to ethanol and butanol was calculated referring to the laboratory protocol NREL/TP-510-42630 (Dowe, 2008) with the conversion of glucan to glucose (1) and glucose to ethanol (2) or butanol (3).

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \quad (1)$$

$$nC_6H_{12}O_6 \rightarrow 2nC_2H_5OH + 2nCO_2 \quad (2)$$

$$nC_6H_{12}O_6 \rightarrow nC_4H_9OH + nH_2O + 2nCO_2 \quad (3)$$

3.2.5 Filter paper activity (FPase) assay in consolidated bioprocessing

Co-culture broths were collected, solid material was removed by centrifugation, and the supernatant was used as a crude enzyme preparation to determine cellulase activity with filter paper as the substrate. Activity was determined by the production of reducing sugars in reaction mixture containing 125 µL crude enzyme and 20 mg filter paper Whatman No. 1 (15 × 10 mm) in

250 μL sodium acetate buffer (50 mM, pH 4.8) for 60 min at 50 °C. One filter paper unit (FPU) was defined as the amount of enzyme that released 1 μmol glucose per minute (Montenecourt et al., 1978). The release of reducing sugars during the assay and the glucose standard were determined by the 3,5-dinitrosalicylic acid method (Miller, 1959).

3.2.6 Determination of glucan content in cellulosic material

The glucan content in UHKP before and after fermentation was examined following NREL/TP-510-42618 (Sluiter et al., 2008). Briefly, the UHKP and medium in Erlenmeyer flasks were transferred into a centrifuge tube (50 mL). After centrifugation at 5,000 × *g*, the UHKP remaining as pellets were collected and dried in air for 24 h before hydrolysis by 3 mL of 72% (w/w) sulfuric acid for 1 h at 30 °C. Then, 84 mL of distilled water were added to the hydrolysate to adjust the concentration of acid in the solution to 4.0%. Hydrolysis of the remaining UHKP was completed by autoclaving the above solution at 120 °C for 1 h. A sample of the final hydrolysate (1 mL) was centrifuged at 18,000 × *g*, and the supernatant was collected to determine the glucose concentration by HPLC.

3.2.7 Statistical analysis

Experimental data were statistically analyzed using Microsoft Excel 2016 with the analysis of variance test for the production of biofuels. The $p \leq 0.05$ was considered as a statistically significant difference between variances (culture modes).

3.3 Results and Discussion

3.3.1 Production of biofuels

The production of ethanol and butanol from UHKP was examined over 11 days of CBP (fungal pre-cultivation for 5 days followed by co-culture of fungal strain P2 or KO77 with bacterium *C. saccharoperbutylacetonicum* for 6 days) (see Graphical abstract).

The production of ethanol and butanol are shown in Fig. 3-1. The culture of P2 alone produced 6.7 g/L ethanol from 20 g/L UHKP (Fig. 3-1A), which was similar to the result in a published study (Kamei et al., 2012b). However, when P2 was co-cultured with *C. saccharoperbutylacetonicum*, the ethanol production was reduced to around 2.2 g/L (Fig. 3-1A). No ethanol was produced by mono-cultures of KO77 or *C. saccharoperbutylacetonicum*, or by co-culture of *C. saccharoperbutylacetonicum* with KO77 (Fig. 3-1A).

Mono-culture of *C. saccharoperbutylacetonicum* or white-rot fungus (P2 or KO77) was unable to produce butanol from UHKP. However, when P2 was co-cultured with the bacterium, butanol production was observed (2.5 g/L) (Fig. 3-1B), and higher butanol production was achieved (3.2 g/L) in co-culture of KO77 and *C. saccharoperbutylacetonicum*. These findings indicated that co-culture of white-rot fungus P2 with bacterium *C. saccharoperbutylacetonicum* is a successful strategy to produce butanol from cellulosic material. Use of fungal strain KO77, from which the *pdc* gene was knocked out, led to the inhibition of ethanol fermentation, which could contribute to increased butanol production in co-culture with *C. saccharoperbutylacetonicum* by increasing the supply of glucose to the latter. As far as the authors know, this is the first report of the application of co-culture of aerobic white-rot fungus and anaerobic *Clostridium* to produce butanol from cellulose.

The chemical composition analysis was indicated that the main components of UHKP are cellulose, xylose and lignin (expressed in terms of glucan, xylan, lignin: 82.0%, 17.0% and 2.5% by weight, respectively (Kamei et al., 2012b). To further assess the effect of the co-culture on the production of ethanol or butanol, the conversion of cellulose in UHKP to ethanol or butanol is indicated in Fig. 3-2.

C. saccharoperbutylacetonicum mono-culture was unable to metabolize cellulose to biofuels. In mono-culture of fungal strain P2, 75.8% of the cellulose in UHKP was converted to ethanol. When P2 was co-cultured with *C. saccharoperbutylacetonicum*, the conversion of cellulose to ethanol was reduced to 23.5%, while 32.5% conversion to butanol was observed. Mono-culture of strain KO77 did not convert cellulose to ethanol or butanol. However, when KO77 was co-cultured with *C. saccharoperbutylacetonicum*, the conversion of cellulose to butanol was 45.7%. These results suggest that fungal-dependent conversion of cellulose to ethanol and bacterial-dependent conversion of fermentable sugars to butanol were co-occurring in the co-culture. Besides, the inhibition of fungal-dependent ethanol fermentation could increase the conversion of cellulose to butanol in the co-culture.

Recently, several studies have employed *Clostridium* with added commercial hydrolytic enzymes to produce butanol from cellulosic material (Xin et al., 2019; Zhao et al., 2019). In the present study, a CBP involving co-culture of *Clostridium* and a white-rot fungus without the addition of commercial enzymes was used and showed promising results in the production of biofuels from cellulosic material. Our previous studies have reported that *Phlebia* sp. MG-60 could produce ethanol from several lignocellulosic materials, including sugarcane bagasse (Khuong et al., 2014a), alkaline treated bamboo ((Tri et al., 2018), and hardwood (Kamei et al., 2012a). The present and previous results suggest the possibility of direct butanol production from lignocellulosic materials such as woody biomass, agricultural wastes, and so on, using co-culture of lignin-degrader P2 and their transformant line KO77.

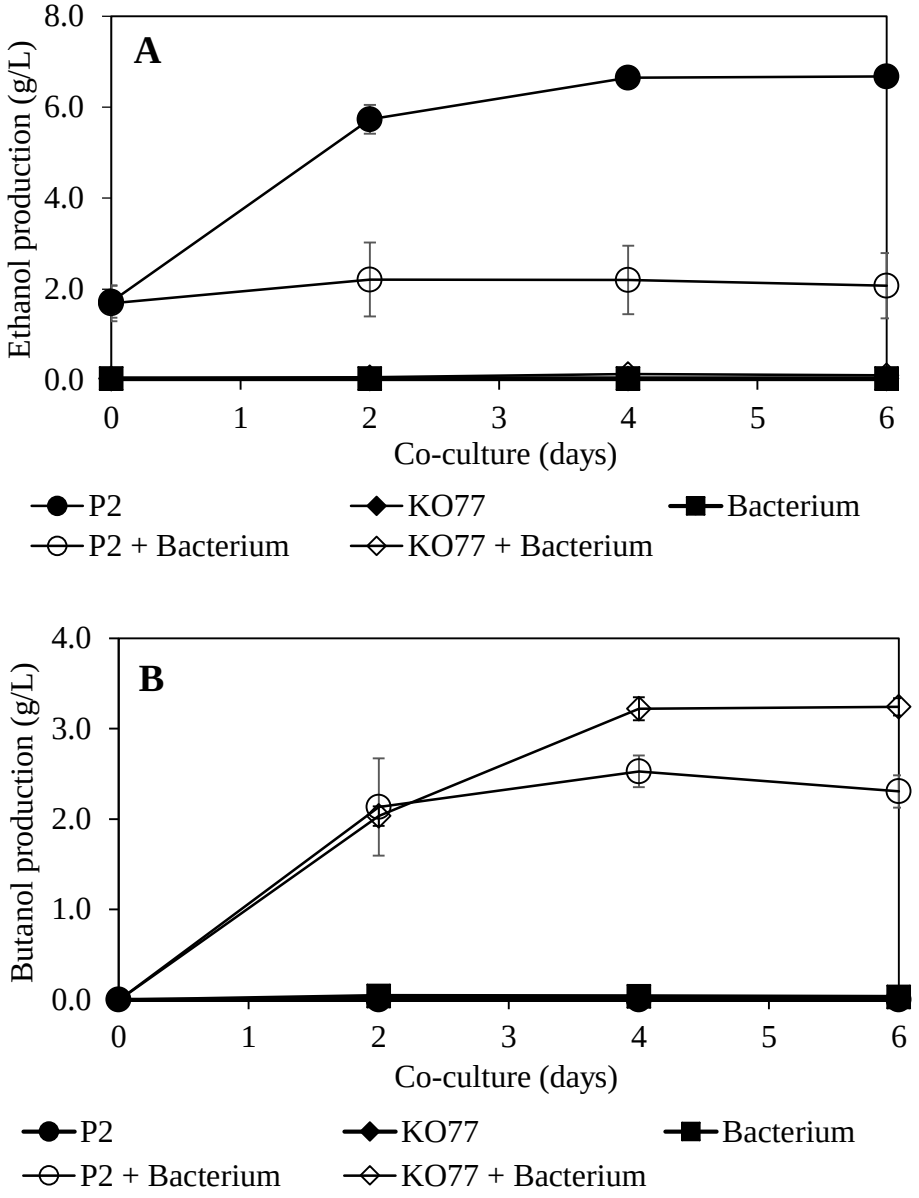


Fig. 3-1. Time course of ethanol (A) and butanol (B) production from unbleached hardwood kraft pulp in consolidated bioprocessing. The error bars represent standard deviations of mean values from three independent experiments (n = 3).

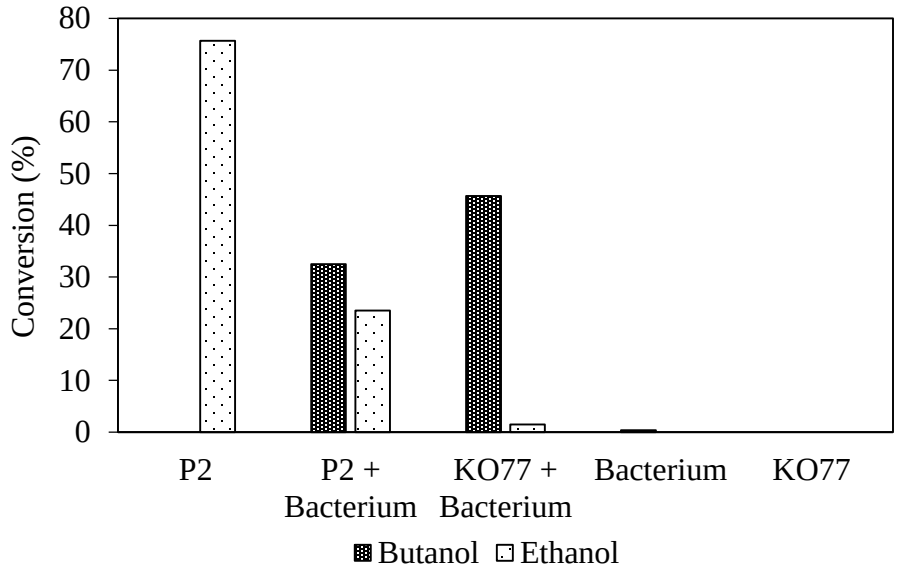


Fig. 3-2. Conversion of glucan content in unbleached hardwood kraft pulp to biofuels after consolidated bioprocessing. The error bars in figure represent standard deviations of mean values from three independent experiments (n = 3).

3.3.2 Enhancement of saccharification of cellulosic material by co-culture

The UHKP after fermentation was collected to determine the remaining cellulose content (expressed in terms of glucan) to evaluate the effectiveness of the saccharification and use of UHKP during CBP. In the mono-culture of *C. saccharoperbutylacetonicum*, almost all of the cellulose remained (Fig. 3-3), which corresponded with the trace level of biofuel production in these conditions (Fig. 3-1). This observation also suggested that the conversion of cellulose to saccharified fermentable sugars in the co-culture systems in the present study was sole because of the presence of the fungal strains.

Mono-culture of P2 successfully converted cellulose in UHKP to fermentation product; only 1.2% of the glucan remained after 6 days of co-culture. The value for mono-culture of KO77 was 22.3%. The higher use of UHKP in the monoculture of strain P2 compared with KO77 was similar to that in a previous study (Tsuyama et al., 2017).

Enhancement of saccharification was observed after 2 days of co-culture between P2 and *C. saccharoperbutylacetonicum* with remaining cellulose of 4.5% compared with 18.8% in mono-culture of P2. The saccharification was significantly improved in the co-culture involving KO77. After 6 days co-culture of KO77 with *C. saccharoperbutylacetonicum*, the remaining glucan was 2.3%, compared with 22.3% in the KO77 mono-culture (Fig. 3-3). These findings indicate that co-culture of P2 or its transformant line KO77 with *C. saccharoperbutylacetonicum* not only successfully produced butanol but also enhanced the saccharification of cellulosic material.

Previous studies have reported several factors that could affect hydrolysis of cellulose, including material features, enzyme activity, and inhibitors (Godoy et al., 2018a; Gregg & Saddler, 1996; Lynd et al., 2002; Schwarz, 2001; Yang et al., 2019). In the present study, *C. saccharoperbutylacetonicum* was unable to use cellulosic material itself, but the saccharification

of UHKP by white-rot fungus was significantly enhanced by co-culture with *C. saccharoperbutylacetonicum*. This suggests the existence of synergistic effects between *C. saccharoperbutylacetonicum* and the white-rot fungus *Phlebia* in the co-culture. Next, the growth characteristics of *C. saccharoperbutylacetonicum*, P2 and KO77 in CBP were examined.

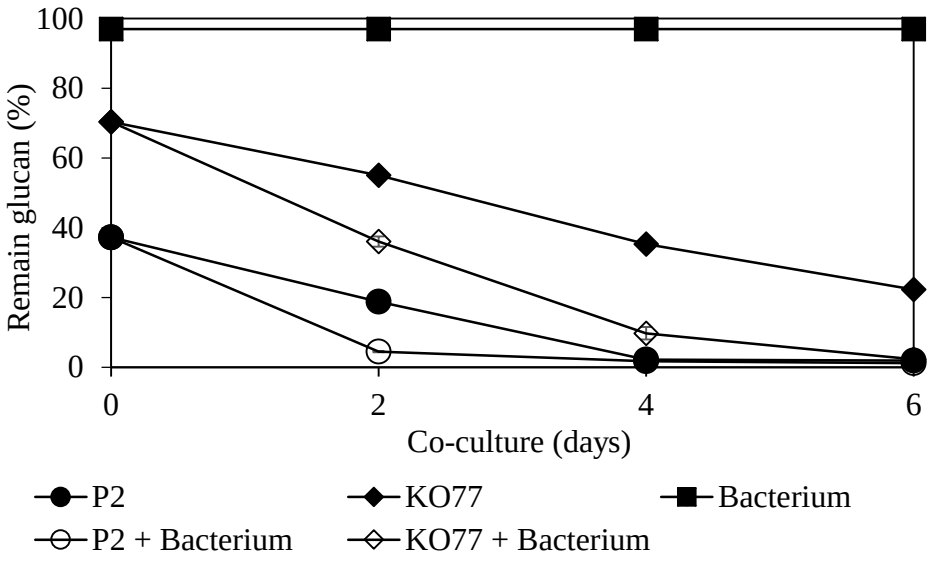


Fig. 3-3. Residual glucan content in unbleached hardwood kraft pulp (UHKP) during consolidated bioprocessing; 100% glucan indicates the amount of glucan in 0.4 g of UHKP at the beginning of fungal pre-cultivation. The error bars represent standard deviations of mean values from three independent experiments (n = 3).

3.3.3 Growth of *Clostridium* and *Phlebia*

To elucidate the growth of *C. saccharoperbutylacetonicum* in the CBP, the fermentation broth was collected and OD_{600} values were measured (Fig. 3-4A). There was no change in the OD_{600} value of the *Clostridium* mono-culture, i.e., *C. saccharoperbutylacetonicum* was unable to grow in mono-culture on the UHKP-containing medium, presumably because of its lack of cellulose saccharification ability. However, the OD_{600} increased significantly in the co-culture of *C. saccharoperbutylacetonicum* with fungal strain P2, and the highest value was observed in the co-culture of *C. saccharoperbutylacetonicum* with KO77.

To evaluate the growth of fungal strains P2 and KO77, the content of ergosterol, which is the main sterol in cell membranes of fungal mycelium, was used as an indicator (Grant & West, 1986; Montgomery et al., 2000; Niemenmaa et al., 2008). In the present study, the whole mycelium of strain P2 or KO77 was extracted to determine the ergosterol content (Fig. 3-4B). Less ergosterol was obtained from KO77 mono-culture than from P2 mono-culture; thus, in the liquid culture used here, the growth of fungal strain P2 was better than that of transformant line KO77. It was previously shown that the growth of P2 in liquid medium strongly corresponded with its ethanol fermentation metabolism (Motoda et al., 2019).

White-rot fungus is usually documented as aerobic or semi-aerobic (Cookson, 1995). In a previous study, the growth of strain P2 and their transformant line KO77 in semi-aerobic conditions was confirmed (Tsuyama et al., 2017). In the present experiments, the strain P2 also showed significant growth after 5 days of pre-cultivation under anaerobic conditions (see Fig. 3-4B; day 0 represents fungi after pre-cultivation in mono-culture for 5 days). These results clarify that P2 is a facultative anaerobic organism. However, the growth of transformant line KO77 was low after 5 days of pre-

incubation and then the growth of KO77 was almost resting in the consolidated bioprocessing. These results also indicated that KO77 lacked the ability to grow under severe anaerobic conditions. More ergosterol was observed in the mono-culture of strain P2 than in its co-culture with *C. saccharoperbutylacetonicum*. The ergosterol content in KO77 mono-culture was also slightly higher than that in its co-culture with *C. saccharoperbutylacetonicum* (Fig. 3-4B).

The finding that significant growth of *C. saccharoperbutylacetonicum* was observed only in co-cultures (Fig. 3-4A) supports the hypothesis that *C. saccharoperbutylacetonicum* could obtain fermentable sugars as carbon sources from the saccharification of UHKP by the white-rot fungus. These sugars could then be used by *C. saccharoperbutylacetonicum* to produce butanol.

3.3.4 FPase activity

FPase activity of crude fermentation broth produced during CBP was analyzed (Fig. 3-5). The broth from mono-culture of *C. saccharoperbutylacetonicum* did not saccharify filter paper (Fig. 3-5). The highest filter paper saccharification activity (0.53 FPU/mL of crude fermentation broth) was observed for the mono-culture of P2, followed by the co-culture of P2 and *C. saccharoperbutylacetonicum* (0.37 FPU/mL). The cellulase activities of the mono-culture of KO77 and the co-culture of KO77 with *C. saccharoperbutylacetonicum* were similar (0.12 FPU/mL) (Fig. 3-5). These results and the results of ergosterol accumulation experiments (Fig. 3-4B) indicate that co-culture with *C. saccharoperbutylacetonicum* did not induce an increase in white-rot fungal growth or fungal cellulase saccharification. These findings, therefore, suggest the indirect effects of *C. saccharoperbutylacetonicum* in co-culture on the enhancement of the saccharification of cellulose (Fig. 3-3).

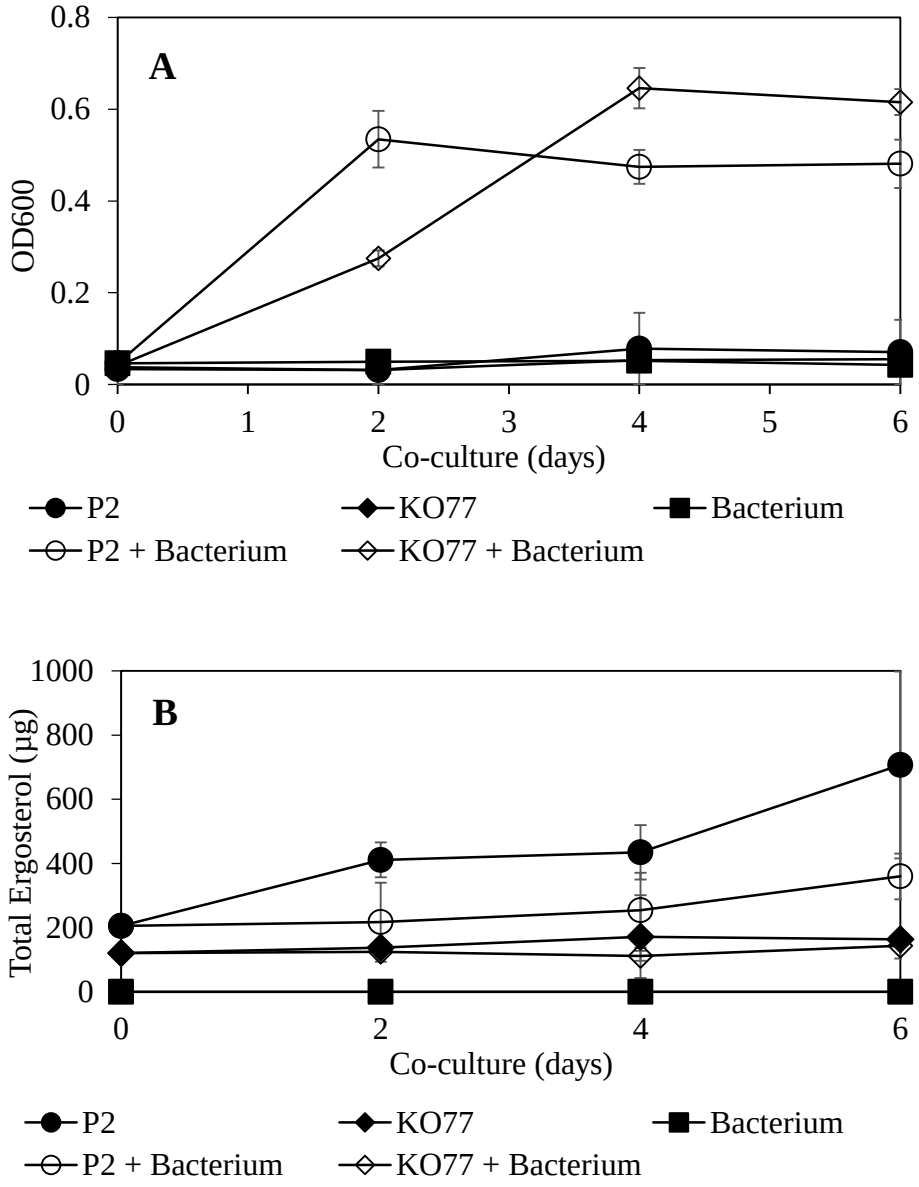


Fig. 3-4. The growth characteristics of *Clostridium saccharoperbutylacetonicum* and white-rot fungus *Phlebia* sp. MG-60-P2 or its transformant line KO77 in consolidated bioprocessing. (A) OD_{600} value of culture broth; (B) Accumulation of ergosterol (extracted from fungal mycelium). The error bars represent standard deviations of mean values from three independent experiments (n = 3).

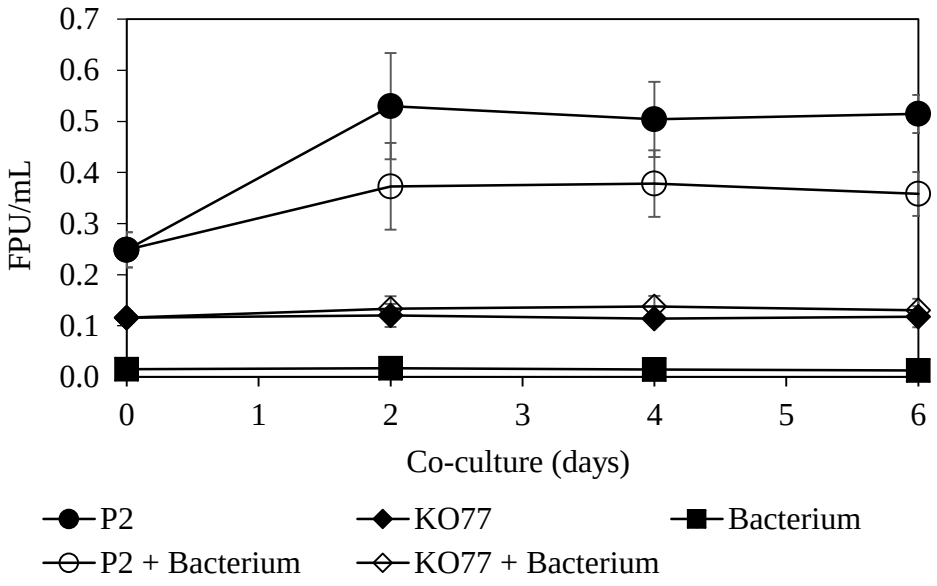


Fig. 3-5. Cellulase activity (measured by saccharification of filter paper) in fermentation medium during consolidated bioprocessing. One filter paper unit (FPU) was defined as the amount of enzyme that released 1 μmol glucose per minute. The error bars represent standard deviations of mean values from three independent experiments (n = 3).

3.3.5 Accumulation of saccharified glucose and cellobiose

To test the hypothesis that enhancement of UHKP saccharification in co-culture was caused indirectly by the activity of *C. saccharoperbutylacetonicum*, the content of two saccharified fermentable sugars (glucose and cellobiose) in the fermentation medium during co-culture was determined (Fig. 3-6). P2 simultaneously saccharifies cellulose and ferments glucose to ethanol under anaerobic conditions (Kamei et al., 2012b). Therefore, the concentration of accumulated glucose and cellobiose in the fermentation broth was low (Fig. 3-6). However, in mono-culture of KO77, conversion of glucose to ethanol is strictly inhibited; therefore, high-level accumulation of glucose was observed (Fig. 3-6A).

The saccharification of cellulose can occur via synergetic effects of three main types of cellulase: endoglucanases (EGs), cellobiohydrolases (CBHs), and β-glucosidases (BGLs). Among these enzymes, the EGs internally hydrolyze the cellulose polymer to expose reducing and non-reducing ends, while the CBHs primarily act to release cellobiose. The hydrolysis of cellulose is completed through the activity of BGLs, which cleave cellobiose, releasing glucose molecules (Godoy et al., 2018a; Lynd et al., 2002; Yang et al., 2019). Previous studies of cellobiose hydrolysis have concluded that glucose strongly inhibits BGL activity via negative feedback (Salgado et al., 2018; Singhania et al., 2013). Correspondingly, the data in this study showed that accumulated glucose might induce negative feedback to the BGLs activity, resulting in the high content of cellobiose that was obtained in mono-culture of KO77 (Fig. 3-6B).

C. saccharoperbutylacetonicum shows a highly efficient consumption of mixed fermentable sugars in liquid culture without carbon catabolite repression (Kihara et al., 2019; Zhao et al., 2019). In the present study, lower accumulation of glucose and cellobiose was observed in the co-culture of KO77 with *C. saccharoperbutylacetonicum* compared with that in KO77 mono-culture (Fig. 3-

6). This result suggests that glucose and cellobiose produced by KO77 were consumed by *C. saccharoperbutylacetonicum*. The success of the present study could be explained as follows: During the 5-day pre-cultivation, the fungus produces saccharified fermentable sugars. In the co-culture process, *C. saccharoperbutylacetonicum* metabolizes these saccharified fermentable sugars as carbon sources to produce butanol. Simultaneously, the consumption of saccharified fermentable sugars by *C. saccharoperbutylacetonicum* removes the negative feedback on cellulase hydrolysis, enabling further enhancement of UHKP saccharification.

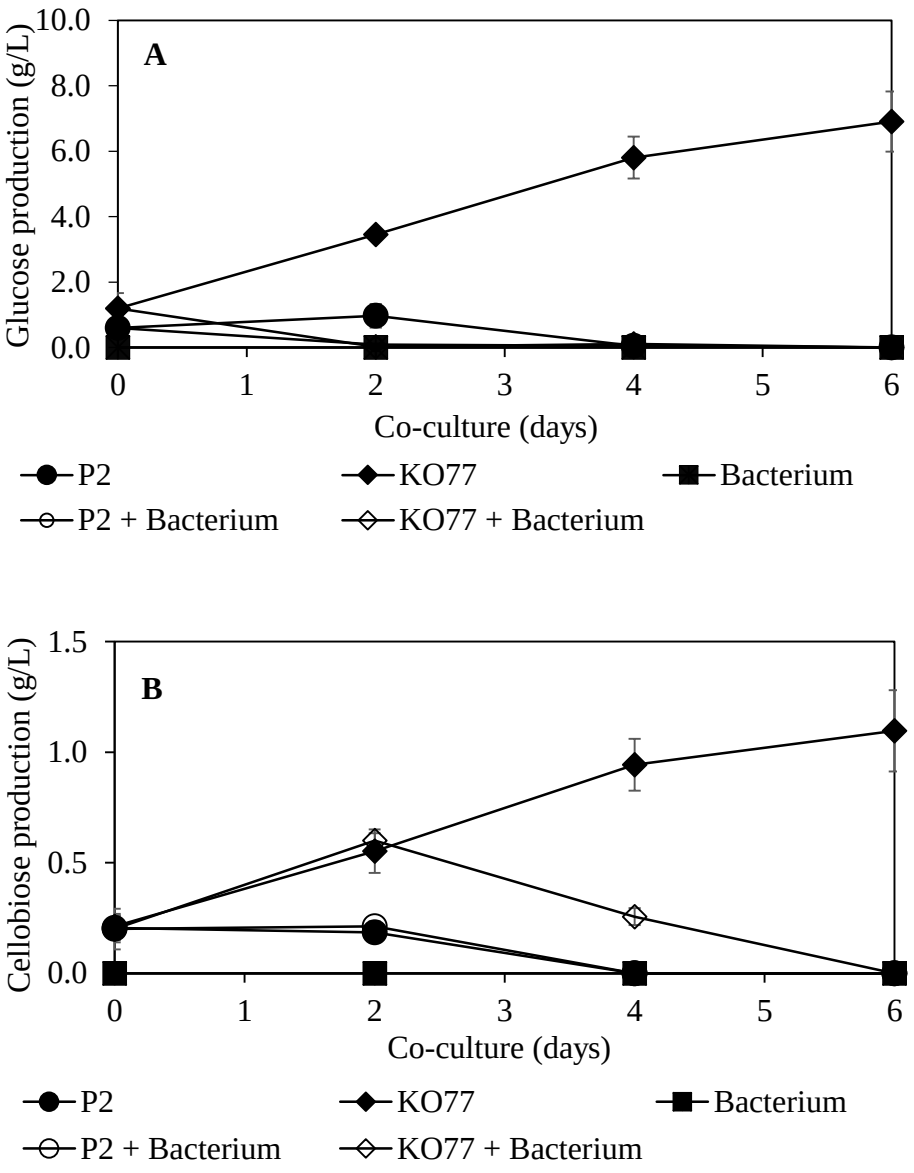


Fig. 3-6. The accumulation of glucose (A) and cellobiose (B) from unbleached hardwood kraft pulp saccharification during consolidated bioprocessing. The error bars represent standard deviations of mean values from three independent experiments (n = 3).

3.4 Summary

The synergistic effect of the white-rot fungus *Phlebia* sp. MG-60-P2 and the bacterium *C. saccharoperbutylacetonicum* in anaerobic co-culture was successful in terms of both butanol production and enhancement of saccharification. Knockout of the *pdc* gene in fungal transformant line KO77 (derived from P2) led to the inhibition of ethanol fermentation and high accumulation of cellobiose and glucose in UHKP saccharification. In co-culture of KO77 with *C. saccharoperbutylacetonicum*, these saccharified sugars could be used effectively for butanol production by *C. saccharoperbutylacetonicum*.

Chapter 4.

Butanol production from sodium hydroxide pretreated bamboo (*Phyllostachys edulis*) using co-culture of white-rot fungus *Phlebia* sp. MG-60-P2 and bacterium *Clostridium*

4.1 Introduction

Bateria genus *Clostridium*, and the strain *C. saccharoperbutylacetonicum* particularly, is widely recognized as the strong biofuel producer using the Acetone – Butanol – Ethanol (ABE) fermentation process (Li et al., 2019; Noguchi et al., 2013). In this process, the wild type of *Clostridium* metabolize mono- or disaccharides to biofuels through two phases: acid fermentation and then solvent fermentation (Procentese et al., 2015; Yang et al., 2018). To improve the biofuel production yield, using metabolic engineering or pretreatment of biomass is recommended (Algayyim et al., 2018). Recently, a novel strategy to directly produce biofuels from cellulosic material using co-culture of white-rot fungus *Phlebia* sp. MG-60 and bacterium *C. saccharoperbutylacetonicum* was introduced (Tri & Kamei, 2020).

In the development of biofuel production, the utilization of abundant and renewable material likely lignocellulose should be considered (Jiang et al., 2017). Not only giving the economic benefits, but the biofuel production from lignocellulose is also reducing the environmental pollution issues, enhancing agricultural efficiency and leading the sustainable human society (Kim et al., 2019).

In Japan, the uncontrollable growth of the wild bamboo in “satoyama” and natural forests is a critical issue for agricultural activities and forest management (Indrawan et al., 2014; Katoh et al., 2009). In the concept of study on environmentally harmonized science and technology, the research on bioethanol production from Japanese bamboo (*Phyllostachys edulis*) was conducted (Tri et al., 2018). This previous study confirmed the possibility to produce ethanol from the stem

of Japanese bamboo with assistance from alkaline pretreatment. However, the conversion of xylan to ethanol by white-rot fungus *Phlebia* sp. MG-60 is weak with a significant amount of xylose was remained after the semi-simultaneous saccharification and co-fermentation process (Table 2-3). Interesting in the research on biofuel production, this intensive experiment was conducted to examine the production of biofuels from alkaline pretreated Japanese bamboo using the co-culture of *Phlebia* sp. MG-60-P2 and *C. saccharoperbutylacetonicum*

4.2 Material and Methods

4.2.1 Bamboo samples and alkaline pretreatment

The Japanese bamboo, *Phyllostachys edulis,* was donated by Sanuki Kasei Co., Ltd. Samples were minced into 40 ~100 mesh size particles and then extracted with a mixed solution of benzene and ethanol (ratio 2:1 v/v in total 2.1 L) for 16 hours in a Soxhlet apparatus. Extracted bamboo – hereafter referred to as "initial" – was air-dried.

Three grams of initial bamboo were added to 24.0 mL of sodium hydroxide (0.0-7.0%, w/v) aqueous solutions in individual 100 mL Erlenmeyer flasks. The mixtures were autoclaved at 120 °C for 1 hour. After autoclaving, remained bamboo, referred to as pretreated samples, were filtered through glass filter type G3 and then washed with distilled water to achieve neutral pH. Pretreated samples were dried in the air for 72 hours to final moisture content around 8.0 % and then kept in plastic bags for further experiments.

4.2.2 White-rot fungus *Phlebia* and bacterium *Clostridium*

Phlebia sp. MG-60-P2 (hereafter P2) was isolated from derived protoplasts of wild-type strain MG-60 to unify the protoplast phenotypes (Tsuyama et al., 2017). The fungal strain P2 was inoculated into Petri dishes containing Difco™ potato-dextrose-agar (PDA; BD Biosciences, supplier number 213400) for 5 days at 28 °C.

C. saccharoperbutylacetonicum NBRC 109357 stored at −80 °C was pre-cultured in 29.3 g/L of liquid thioglycollate medium (Nihon Pharmaceutical Co. Ltd.) for 36 h at 30 °C under anaerobic conditions. Before use in co-culture, the optical density at 600 nm (OD_{600}) of the 10-fold diluted *C. saccharoperbutylacetonicum* culture was 0.16-0.18

4.2.3 Co-culture of *Clostridium* and *Phlebia*

Beechwood xylan or 3.0% NaOH pretreated bamboo substrate (0.4 g) was loaded into 100-mL Erlenmeyer flasks containing 19 mL basal liquid medium [2.0 g/L yeast extract, 6.0 g/L tryptone, 2.57 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4.7H_2O$, 0.5 g/L KH_2PO_4, 10 mg/L $FeSO_4.7H_2O$, 3 g/L $CaCO_3$] (Zhao et al., 2019). This mixture of bamboo and medium was autoclaved at 120 °C for 15 min.

Six millimeter-diameter discs of fungal mycelium grown for 5 days on PDA were added into autoclaved flasks, then, were kept at 28 °C for 5 days for fungal pre-cultivation. In the next step, 1.0 mL of bacterial culture (see section 4.2.2) was added to the flasks to begin co-culture. The whole experimental process (fungal pre-cultivation and co-culture) was kept anaerobic by supplying nitrogen gas flowing at 0.5 MPa to all flasks for 20 s then immediately sealing the flasks with silicone plugs.

4.2.4 Determination of chemical composition in the bamboo substrate

After the fermentation process, the broth and solid in co-culture were separated by glass filter G2 with pore size 40–100 μm (PYREX IWAKI, ASAHI, AGC Techno Glass, Japan). The glucan and xylan content in bamboo substrates before and after co-culture was examined following NREL/TP-510-42618 (Sluiter et al., 2008). Briefly, separated bamboo was collected and dried in air for 24 h before hydrolysis by 4.0 mL of 72% (w/w) sulfuric acid for 1 h at 30 °C. Then, 111.0 mL of distilled water was added to the hydrolysate to adjust the concentration of acid approximately to 4.0%. Final hydrolysis of bamboo was completed by autoclaving the above solution at 120 °C for

1 h. A sample of the final hydrolysate (1.0 mL) was centrifuged at 18,000 × *g*, and the supernatant was collected to determine the concentration of glucose and xylose by HPLC.

4.2.5 Analytical methods

After each interval of 48 h in the co-culture process, 1.0 mL of cultural broth was pipetted from experimental flasks and centrifuged at 18,000 × *g* for 10 min to remove solids. The content of cellobiose, glucose, ethanol, acetone and butanol in the supernatant was quantified by HPLC using an LC-20AD system with refractive index detector RID-10A. Supernatant sample (10 µL) was injected into an ICSep ICE-core gel 87H3 column (7.8 × 300 mm; part number ICE-99-9861, Concise Separations, USA) at 70 °C with a flow rate of 0.6 mL/min of 5.0 mM H_2SO_4 mobile phase.

The representative to the balance of (C-mol/C-mol) in a chemical equation, the conversion of glucan $(C_6H_{10}O_5)_n$ and xylan $(C_5H_8O_4)_n$ in the bamboo substrate to acetone (C_3H_6O), ethanol (C_2H_5OH) and butanol (C_4H_9OH) in the ABE fermentation is calculated as below

$$\text{Acetone conversion (\%)} = (3 \times \text{Ca})/((6 \times \text{Cglu} + 5 \times \text{Cxyl})) \times 100$$

$$\text{Ethanol conversion (\%)} = (2 \times \text{Ce})/((6 \times \text{Cglu} + 5 \times \text{Cxyl})) \times 100$$

$$\text{Butanol conversion (\%)} = (4 \times \text{Cb})/((6 \times \text{Cglu} + 5 \times \text{Cxyl})) \times 100$$

Where Ca, Ce and Cb is the concentration (mole) of produced acetone, ethanol and butanol by ABE fermentation; Cglu and Cxyl is the concentration (mole) of glucan and xylan in the bamboo substrate.

4.2.6 Statistical analysis

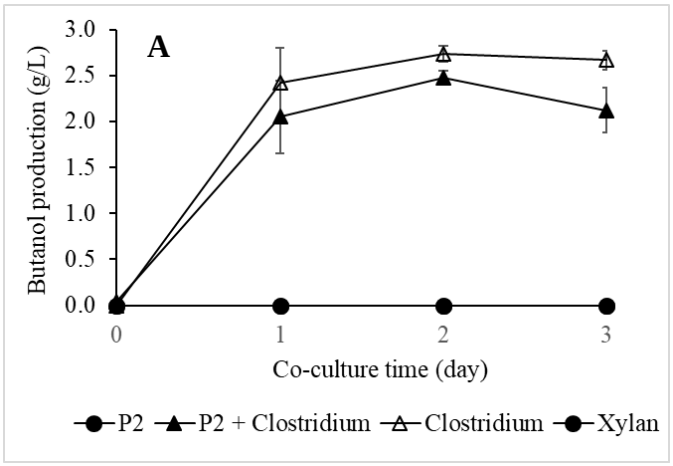
Experimental data were statistically analyzed using Microsoft Excel 2016 with the analysis of variance (ANOVA) test for the production of biofuels. The $p \leq 0.05$ was considered as a statistically significant difference between variances (culture modes).

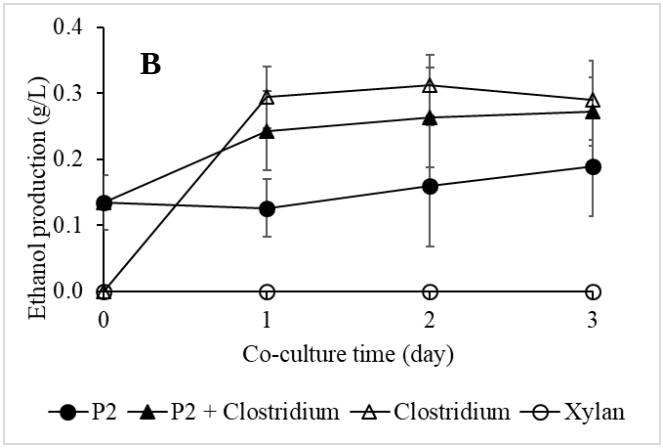
4.3 Results and Discussion

4.3.1 The utilization of xylan

Hemicellulose is the second most abundant polysaccharide in the chemical composition of lignocellulosic materials, therefore, to increase the efficient utilization of lignocellulose, the improvement of the hemicellulose conversion to valuable products is an important factor (Liang et al., 2020; Naidu et al., 2018). To provide more information about the hemicellulose consumption by *Clostridium*, the direct fermentation of beechwood xylan by *C. saccharoperbutylacetonicum* was conducted in both mono- or co-culture with white-rot fungi. The results are described in Fig. 4-1.

Similar to the previous study (Kamei et al., 2020), the partly utilization of 20.0 g/L beechwood xylan by MG-60-P2 mono-culture to produce a low ethanol concentration was confirmed (0.2 g/L, Fig.4-1B). Importantly, the experiment resulted that mono-culture of *C. saccharoperbutylacetonicum* could easily metabolize beechwood xylan to produce 2.5 g/L, 0.3 g/L and 1.7 g/L of butanol, ethanol and acetone, respectively (Fig.4-1). The production of butanol and ethanol was downed in the co-culture, which could be explained by the competition in the utilization of carbon sources between fungus and bacterium. It is noticed that a significant high in the production of acetone was found in both mono- or co-culture of *C. saccharoperbutylacetonicum.* Many studies have revealed that the ratio in butanol, acetone and ethanol production through ABE fermentation is affected by substrates, microorganisms and medium conditions (Ding et al., 2018; He & Chen, 2020; Mirfakhar et al., 2020; Qi et al., 2019). In our development of biofuel production, the optimum conditions to focus on the improving yield of butanol and ethanol production are considered in further studies (dos Santos Vieira et al., 2019).





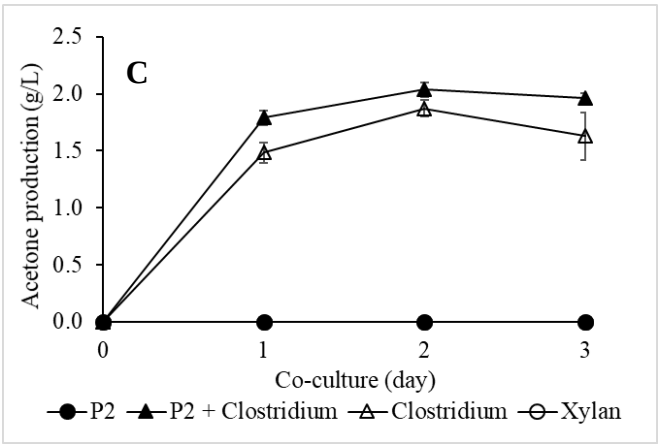


Fig. 4-1. The production of butanol (A), ethanol (B) and acetone (C) from 20.0 g/L beechwood xylan using co-culture of white-rot fungus Phlebia sp. MG-60-P2 and bacterium *C. saccharoperbutylacetonicum.*

4.3.2 The utilization of alkaline pretreated bamboo

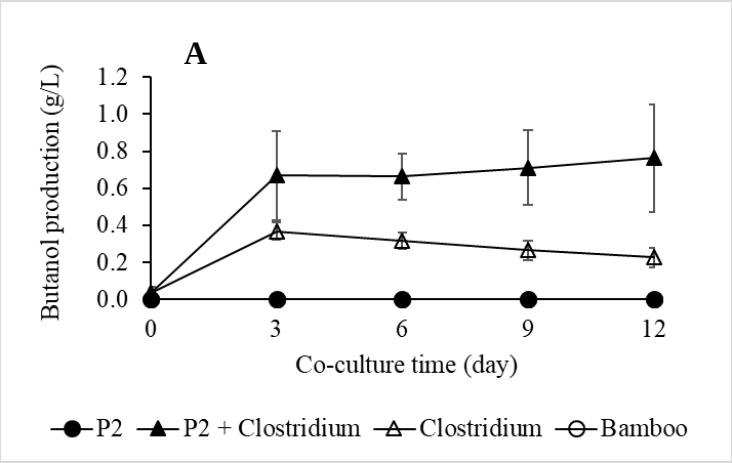
Hemicellulose contributes a significant content, 19.5% by weight, in the chemical composition of 3.0 % NaOH pretreated bamboo (Tri et al., 2018). However, the conversion of hemicellulose to ethanol production using MG-60-P2 is low (Fig. 4-1). Attempting to improve the yield of biofuel production from pretreated bamboo, the co-culture of MG-60-P2 and *C. saccharoperbutylacetonicum* was applied. The time courses of butanol, ethanol and acetone production using this co-culture are explained in Fig. 4-2.

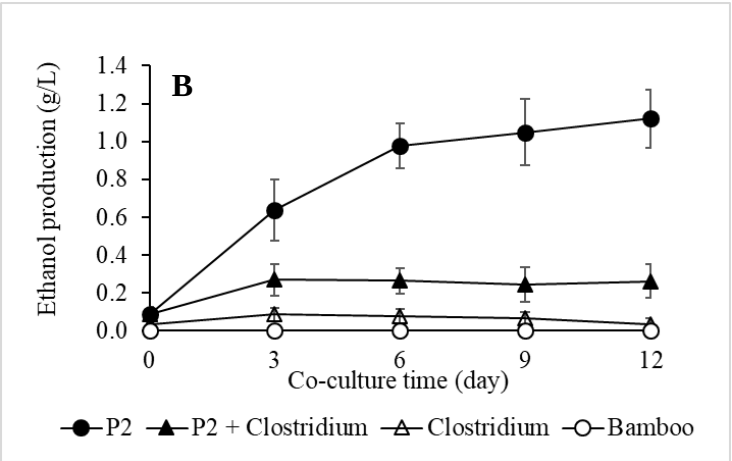
Even pure xylan was easily metabolized by mono-culture of *C. saccharoperbutylacetonicum*, however, this mono-culture showed the low utilization yield of pretreated bamboo to ABE fermentation. In the recalcitrant chemical structure of pretreated bamboo, while lignin is aromatics and cellulose is unable to nurture *C. saccharoperbutylacetonicum* (Tri & Kamei, 2020), only a part of bamboo xylan (24.0% by weight, Fig.4-3) was converted to 0.3 g/L, 0.1 g/L and 0.5 g/L of butanol, ethanol and acetone, respectively. Comparing, the co-culture between *C. saccharoperbutylacetonicum* and MG-60-P2 has successfully increased the yield of butanol, ethanol and acetone to 0.8 g/L, 0.2 g/L and 1.4 g/L, respectively. This increased production yield corresponds to the improvement of both cellulose and hemicellulose utilization, which is explained by the reduction of remaining glucan and xylan in bamboo substrates after 12 days of co-culture (Fig. 4-3).

It is noticed that the highest utilization of polysaccharides belonged to the mono-culture of MG-60-P2 with the lowest remaining of glucan and xylan was observed (Fig 4-3). However, this utilization by MG-60-P2 is less effective with only 17.5 % of the theoretical concentration of C-mol was converted to ethanol (Fig. 4-4). Comparing to *Clostridium* mono-culture, higher efficient conversion from polysaccharides to biofuels was achieved in co-culture with approximately

60.0 % of C-mole balance was converted to butanol (18.2%), acetone (36.1%) and ethanol (5.1 %) (Fig. 4-4). More importantly, discussing the remaining glucan and xylan in bamboo after co-culture (Fig. 4-3) and C-mole conversion (Fig. 4-4), it is indicated that this higher efficiency must be contributed from both cellulose and hemicellulose conversions. It also means that the production of biofuels from pretreated bamboo is synergistic effects of both bacterial co-fermentation of hexose and pentose and fungal saccharification of cellulose and hemicellulose.

The partly utilization of hemicellulose portion in bamboo by mono-culture of *C. saccharoperbutylacetonicum* showed the most effective C-mole conversion to ABE fermentation products, 88.9 % (Fig.4-4). Besides, both mono- and co-culture of *C. saccharoperbutylacetonicum* produced a significantly higher yield of acetone, comparing to ethanol and butanol. The results suggest the modification of the experimental design is needed, including medium conditions and micro-substrate interactions, to improve the ratio of butanol production in ABE fermentation.





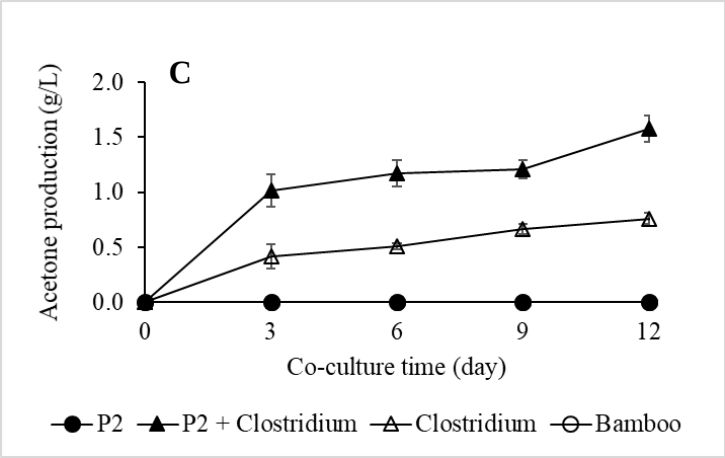


Fig. 4-2. The production of butanol (A), ethanol (B) and acetone (C) from 20.0 g/L of 3.0 NaOH pretreated bamboo using co-culture of white-rot fungus *Phlebia* sp. MG-60-P2 and bacterium *C. saccharoperbutylacetonicum*.

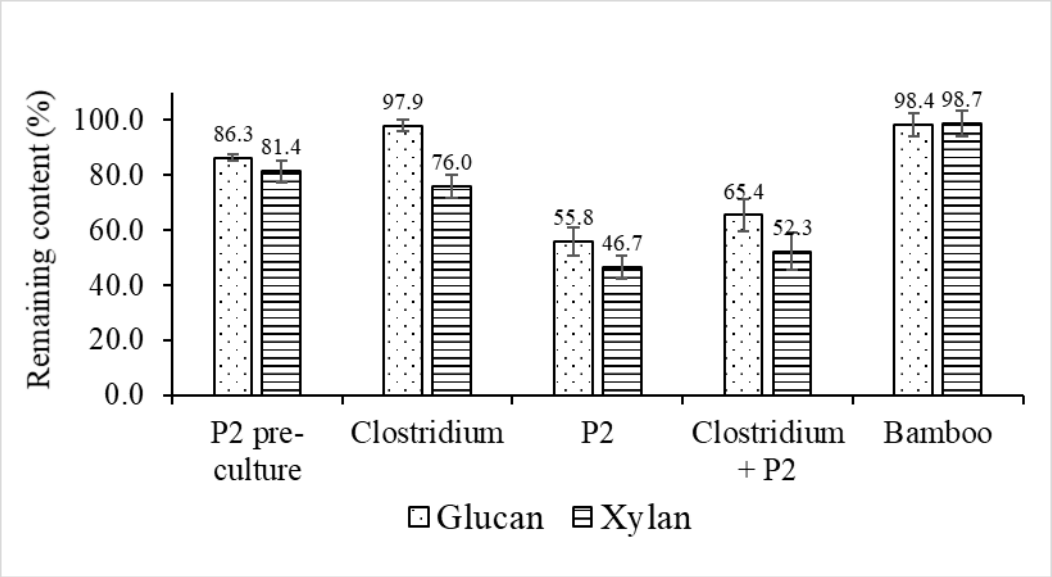


Fig. 4-3. The remaining content of glucan and xylan in 3.0% NaOH pretreated bamboo after co-culture of white-rot fungus *Phlebia* sp. MG-60-P2 and bacterium *C. saccharoperbutylacetonicum.*

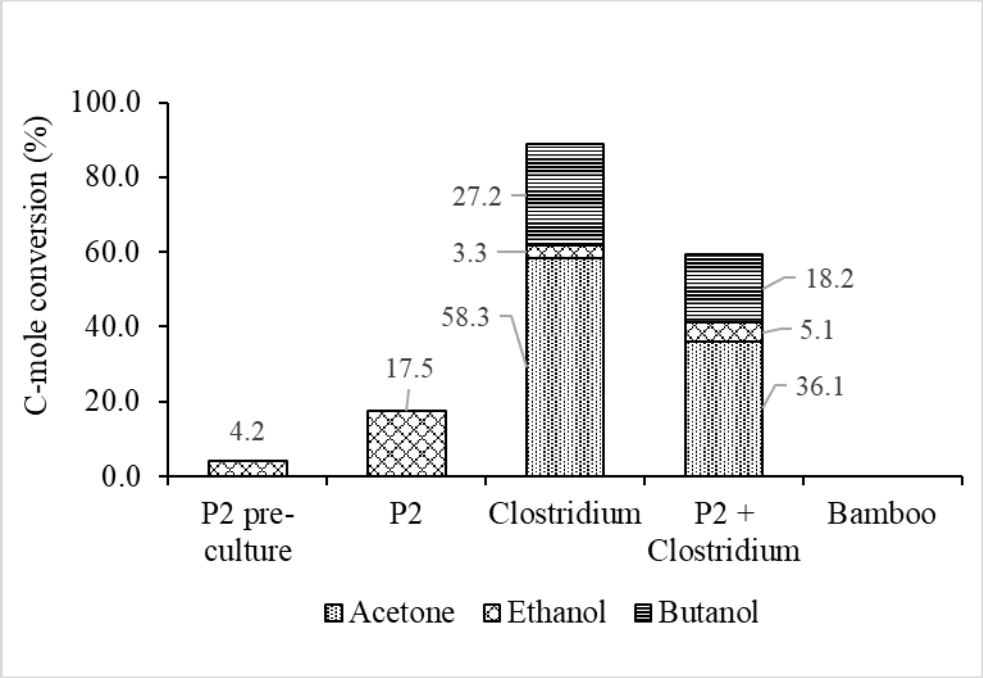


Fig. 4-4. The C-mole conversion of utilized glucan and xylan in 3.0% NaOH pretreated bamboo to butanol, ethanol and acetone using co-culture of white-rot fungus *Phlebia* sp. MG-60-P2 and bacterium *C. saccharoperbutylacetonicum.*

4.3 Summary

The study about ABE fermentation confirmed the direct production of butanol, acetone and ethanol from beechwood xylan by mono-culture of *C. saccharoperbutylacetonicum*. More importantly, the co-culture of white-rot fungus *Phlebia* sp. MG-60-P2 and *C. saccharoperbutylacetonicum* could produce biofuels from alkaline pretreated bamboo. In the co-culture, the synergistic effects of fungus and bacterium were achieved to utilize both cellulose and hemicellulose in bamboo to produce acetone, butanol and ethanol. In further experiments, the optimum condition of the experiment should be considered to increase the ratio of biofuel production in ABE fermentation using co-culture of fungus *Phlebia* sp. MG-60-P2 and *C. saccharoperbutylacetonicum.*

Chapter 5.

Conclusion of studies

With the high portion of polysaccharides (cellulose and hemicellulose) in chemical composition, lignocellulose is a potential renewable source for biofuel production. In this production, these polysaccharides are expected to be hydrolyzed to fermentable sugars (hexoses and pentoses). After that, the fermentable sugars are fermented to ethanol or butanol by the microorganism. This utilization is not only giving economic benefits but also reducing environmental pollutions, leading to more sustainable human socials. However, this conversion is facing a challenge caused by the presence of lignin in the recalcitrant structure of lignocellulose.

Lignin is one of the most complex aromatic compounds in nature. In native form, lignin is played the role likely hardened cement paste between the polysaccharides, which could avoid the attacks from microorganisms to polysaccharides. To breaking down this recalcitrance, the first step in biofuels production from lignocellulose, named pretreatment, is needed.

After pretreatment, the pretreated lignocellulose with a high content of polysaccharides is subjected to the next step in biofuel production: saccharification and fermentation. Among the microorganisms, it was reported that several white-rot fungi are powerful candidates in the simultaneous saccharification of both cellulose and hemicellulose to hexoses and pentoses, and co-fermentation of these sugars to ethanol. Recently, the development of a biological process that integrated both lignin degradation, saccharification, and fermentation using white-rot fungus *Phlebia* sp. MG-60 to produce ethanol from hardwood directly was reported.

In Chapter 2 of this thesis, the study about ethanol production from lignocellulose, the combination of alkaline pretreatment and white-rot fungal *Phlebia* sp.MG-60 saccharification &

fermentation was conducted to examine ethanol production from the stem of Japanese bamboo (*Phyllostachys edulis*). It was revealed that the sodium hydroxide pretreatment (0.0-7.0% w/v) showed the removal of lignin and increase of polysaccharides in the chemical composition of bamboo substrates. Not only that, but the alkaline pretreatment also improved the saccharification rate of bamboo substrate, leading to higher efficiency in utilization of pretreated bamboo to ethanol by fermentation in consolidated bioprocessing of white-rot fungus *Phlebia* sp. MG-60.

Among biofuels, butanol has a higher heating value than ethanol, however, butanol production is only obtained by the Acetone – Butanol – Ethanol fermentation (ABE fermentation) from mono- or di-saccharides by the bacterium *Clostridium.* Aiming to produce butanol from cheaper carbon sources, the co-culture of white-rot fungus *Phlebia* sp. MG-60-P2 (the re-generated strain from protoplast of *Phlebia* sp. MG-60, showing the unify phenotypes as *Phlebia* sp. MG-60) and bacterium *C. saccharoperbutylacetonicum* to produce butanol from cellulosic material - Unbleached Hardwood Kraft Pulp was focused on in Chapter 3. The fungus-bacterium co-culture showed success in direct butanol production from cellulose. The characteristics of co-culture proved that cellulose could be hydrolyzed to glucose and cellobiose by fungal cellulase enzymes. Simultaneously, the bacterium could metabolize these fermentable sugars to produce butanol. Also, this co-culture accelerated the saccharification rate of cellulose, reducing the time course of the conversion, which was explained by the removal of glucose and cellobiose by bacterium metabolism.

The success in the direct butanol production from cellulose promotes the establishment of an intensive study on biofuels production from pretreated lignocellulose. In Chapter 4, the utilization of sodium hydroxide pretreated Japanese bamboo (*Phyllostachys edulis*) using co-culture of white-rot fungus *Phlebia* sp. MG-60-P2 and *C. saccharoperbutylacetonicum* were examined. The data

elucidated that *C. saccharoperbutylacetonicum* can partly metabolize hemicellulose in NaOH pretreated bamboo to biofuels. More important, the co-culture with fungus *Phlebia* sp. MG-60-P2 has significantly improved the production yield of butanol, acetone, and ethanol. This improvement was explained by the synergistic effects of the bacterial fermentation of hexose and pentose, and the fungal saccharifications of cellulose and hemicellulose from bamboo substrates.

The outcome from studies on biofuel production from lignocellulose using microorganisms significantly contributes to the knowledge of the conversion of lignocellulose to biofuels. In further research, the conversion of lignin and other minor compounds, such as extractives, in lignocellulose into valuable chemicals is considered.

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References

Lignin. in: *Kirk-Othmer Encyclopedia of Chemical Technology*, pp. 1-26.

Aita, G.A., Salvi, D.A., Walker, M.S. 2011. Enzyme hydrolysis and ethanol fermentation of dilute ammonia pretreated energy cane. *Bioresource Technology*, **102**(6), 4444-4448.

Algayyim, S.J.M., Wandel, A.P., Yusaf, T., Hamawand, I. 2018. Production and application of ABE as a biofuel. *Renewable and Sustainable Energy Reviews*, **82**, 1195-1214.

Amiri, H., Karimi, K. 2018. Pretreatment and hydrolysis of lignocellulosic wastes for butanol production: Challenges and perspectives. *Bioresource Technology*, **270**, 702-721.

Boerjan, W., Ralph, J., Baucher, M. 2003. Lignin Biosynthesis. *Annual Review of Plant Biology*, **54**(1), 519-546.

Brandt, A., Gräsvik, J., Hallett, J.P., Welton, T. 2013. Deconstruction of lignocellulosic biomass with ionic liquids. *Green Chemistry*, **15**(3), 550-583.

Brautaset, T., Ellingsen, T.E. 2011. 3.47 - Lysine: Industrial Uses and Production. in: *Comprehensive Biotechnology (Second Edition)*, (Ed.) M. Moo-Young, Academic Press. Burlington, pp. 541-554.

Cai, J., He, Y., Yu, X., Banks, S.W., Yang, Y., Zhang, X., Yu, Y., Liu, R., Bridgwater, A.V. 2017. Review of physicochemical properties and analytical characterization of lignocellulosic biomass. *Renewable and Sustainable Energy Reviews*, **76**, 309-322.

Chen, H. 2015. 1 - Lignocellulose biorefinery engineering: an overview. in: *Lignocellulose Biorefinery Engineering*, (Ed.) H. Chen, Woodhead Publishing, pp. 1-17.

Chen, H., Fu, X. 2016. Industrial technologies for bioethanol production from lignocellulosic biomass. *Renewable and Sustainable Energy Reviews*, **57**, 468-478.

Cheng, L., Adhikari, S., Wang, Z., Ding, Y. 2015. Characterization of bamboo species at different ages and bio-oil production. *Journal of Analytical and Applied Pyrolysis*, **116**, 215-222.

Cookson, J.T., Jr. 1995. *Bioremediation engineering: design and application*. McGraw-Hill, Inc., New York.

Darabant, A., Haruthaithanasan, M., Atkla, W., Phudphong, T., Thanavat, E., Haruthaithanasan, K. 2014. Bamboo biomass yield and feedstock characteristics of energy plantations in Thailand. *Energy Procedia*, **59**, 134-141.

Das, S. 2020. The National Policy of biofuels of India – A perspective. *Energy Policy*, **143**, 111595.

Ding, J., Luo, H., Xie, F., Wang, H., Xu, M., Shi, Z. 2018. Electron receptor addition enhances butanol synthesis in ABE fermentation by Clostridium acetobutylicum. *Bioresource Technology*, **247**, 1201-1205.

dos Santos Vieira, C.F., Maugeri Filho, F., Maciel Filho, R., Pinto Mariano, A. 2019. Acetone-free biobutanol production: Past and recent advances in the Isopropanol-Butanol-Ethanol (IBE) fermentation. *Bioresource Technology*, **287**, 121425.

Dowe, N. 2008. SSF experimental protocols lignocellulosic biomass hydrolysis and fermentation : laboratory analytical procedure (LAP), (Ed.) J.D. McMillan, National Renewable Energy Laboratory. Golden, Colo.

Fei, B., Gao, Z., Wang, J., Liu, Z. 2016. Chapter 14 - Biological, Anatomical, and Chemical Characteristics of Bamboo A2 - Kim, Yoon Soo. in: *Secondary Xylem Biology*, (Eds.) R. Funada, A.P. Singh, Academic Press. Boston, pp. 283-306.

Ferreira, J.A., Brancoli, P., Agnihotri, S., Bolton, K., Taherzadeh, M.J. 2018. A review of integration strategies of lignocelluloses and other wastes in 1st generation bioethanol processes. *Process Biochemistry*, **75**, 173-186.

Godoy, M.G., Amorim, G.M., Barreto, M.S., Freire, D.M.G. 2018a. Agricultural residues as animal feed: protein enrichment and detoxification using solid-state fermentation. in: *Current Developments in Biotechnology and Bioengineering*, (Eds.) A. Pandey, C. Larroche, C.R. Soccol, Elsevier, pp. 235-256.

Godoy, M.G., Amorim, G.M., Barreto, M.S., Freire, D.M.G. 2018b. Chapter 12 - Agricultural Residues as Animal Feed: Protein Enrichment and Detoxification Using Solid-State Fermentation. in: *Current Developments in Biotechnology and Bioengineering*, (Eds.) A. Pandey, C. Larroche, C.R. Soccol, Elsevier, pp. 235-256.

Gong, W., Ran, Z., Ye, F., Zhao, G. 2017. Lignin from bamboo shoot shells as an activator and novel immobilizing support for α-amylase. *Food Chemistry*, **228**, 455-462.

Grant, W.D., West, A.W. 1986. Measurement of ergosterol, diaminopimelic acid and glucosamine in soil: evaluation as indicators of microbial biomass. *Journal of Microbiological Methods*, **6**(1), 47-53.

Green, A. 2019. Chapter 28 - Agricultural Waste and Pollution. in: *Waste (Second Edition)*, (Eds.) T.M. Letcher, D.A. Vallero, Academic Press, pp. 531-551.

Gregg, D.J., Saddler, J.N. 1996. Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process. *Biotechnology and Bioengineering*, **51**(4), 375-383.

Hao, H., Liu, Z., Zhao, F., Ren, J., Chang, S., Rong, K., Du, J. 2018. Biofuel for vehicle use in China: Current status, future potential and policy implications. *Renewable and Sustainable Energy Reviews*, **82**, 645-653.

Harry-asobara, J.L., Kamei, I. 2018. Bacterial strains isolated from cedar wood improve the mycelial growth and morphology of white rot fungus Phlebia brevispora on agar and liquid medium. *Journal of Wood Science*, **64**(4), 444-450.

He, M.-x., Wang, J.-l., Qin, H., Shui, Z.-x., Zhu, Q.-l., Wu, B., Tan, F.-r., Pan, K., Hu, Q.-c., Dai, L.-c., Wang, W.-g., Tang, X.-y., Hu, G.Q. 2014. Bamboo: A new source of carbohydrate for biorefinery. *Carbohydrate Polymers*, **111**, 645-654.

He, Q., Chen, H. 2020. Increased efficiency of butanol production from spent sulfite liquor by removal of fermentation inhibitors. *Journal of Cleaner Production*, **263**, 121356.

Ibrahim, M.F., Kim, S.W., Abd-Aziz, S. 2018. Advanced bioprocessing strategies for biobutanol production from biomass. *Renewable and Sustainable Energy Reviews*, **91**, 1192-1204.

Indrawan, M., Yabe, M., Nomura, H., Harrison, R. 2014. Deconstructing satoyama – The socio-ecological landscape in Japan. *Ecological Engineering*, **64**, 77-84.

Jang, Y.-S., Malaviya, A., Cho, C., Lee, J., Lee, S.Y. 2012. Butanol production from renewable biomass by clostridia. *Bioresource Technology*, **123**, 653-663.

Jiang, Y., Xin, F., Lu, J., Dong, W., Zhang, W., Zhang, M., Wu, H., Ma, J., Jiang, M. 2017. State of the art review of biofuels production from lignocellulose by thermophilic bacteria. *Bioresource Technology*, **245**, 1498-1506.

Kamei, I. 2020. Wood-Rotting Fungi for Biofuel Production. in: *Fungi in Fuel Biotechnology*, (Eds.) G. Salehi Jouzani, M. Tabatabaei, M. Aghbashlo, Springer International Publishing. Cham, pp. 123-147.

Kamei, I., Hirota, Y., Meguro, S. 2012a. Integrated delignification and simultaneous saccharification and fermentation of hard wood by a white-rot fungus, Phlebia sp. MG-60. *Bioresource Technology*, **126**, 137-141.

Kamei, I., Hirota, Y., Mori, T., Hirai, H., Meguro, S., Kondo, R. 2012b. Direct ethanol production from cellulosic materials by the hypersaline-tolerant white-rot fungus Phlebia sp. MG-60. *Bioresource Technology*, **112**, 137-142.

Kamei, I., Nitta, T., Nagano, Y., Yamaguchi, M., Yamasaki, Y., Meguro, S. 2014. Evaluation of spent mushroom waste from Lentinula edodes cultivation for consolidated bioprocessing fermentation by Phlebia sp. MG-60. *International Biodeterioration & Biodegradation*, **94**, 57-62.

Kamei, I., Suhara, H., Kondo, R. 2005. Phylogenetical approach to isolation of white-rot fungi capable of degrading polychlorinated dibenzo-p-dioxin. *Applied Microbiology and Biotechnology*, **69**, 358-366.

Kamei, I., Uchida, K., Ardianti, V. 2020. Conservation of Xylose Fermentability in Phlebia Species and Direct Fermentation of Xylan by Selected Fungi. *Applied Biochemistry and Biotechnology*, **192**(3), 895-909.

Kassaye, S., Pant, K.K., Jain, S. 2017. Hydrolysis of cellulosic bamboo biomass into reducing sugars via a combined alkaline solution and ionic liquid pretreament steps. *Renewable Energy*, **104**, 177-184.

Katoh, K., Sakai, S., Takahashi, T. 2009. Factors maintaining species diversity in satoyama, a traditional agricultural landscape of Japan. *Biological Conservation*, **142**(9), 1930-1936.

Khoo, H.H. 2015. Review of bio-conversion pathways of lignocellulose-to-ethanol: Sustainability assessment based on land footprint projections. *Renewable and Sustainable Energy Reviews*, **46**, 100-119.

Khuong, L.D., Kondo, R., De Leon, R., Kim Anh, T., Shimizu, K., Kamei, I. 2014a. Bioethanol production from alkaline-pretreated sugarcane bagasse by consolidated bioprocessing using Phlebia sp. MG-60. *International Biodeterioration & Biodegradation*, **88**, 62-68.

Khuong, L.D., Kondo, R., Leon, R.D., Anh, T.K., Meguro, S., Shimizu, K., Kamei, I. 2014b. Effect of chemical factors on integrated fungal fermentation of sugarcane bagasse for ethanol production by a white-rot fungus, Phlebia sp. MG-60. *Bioresource Technology*, **167**, 33-40.

Kihara, T., Noguchi, T., Tashiro, Y., Sakai, K., Sonomoto, K. 2019. Highly efficient continuous acetone–butanol–ethanol production from mixed sugars without carbon catabolite repression. *Bioresource Technology Reports*, **7**, 100185.

Kim, J.-Y., Lee, H.W., Lee, S.M., Jae, J., Park, Y.-K. 2019. Overview of the recent advances in lignocellulose liquefaction for producing biofuels, bio-based materials and chemicals. *Bioresource Technology*, **279**, 373-384.

Kim, J.S., Lee, Y.Y., Kim, T.H. 2016. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresource Technology*, **199**, 42-48.

Kiyoshi, K., Furukawa, M., Seyama, T., Kadokura, T., Nakazato, A., Nakayama, S. 2015. Butanol production from alkali-pretreated rice straw by co-culture of Clostridium thermocellum and Clostridium saccharoperbutylacetonicum. *Bioresource Technology*, **186**, 325-328.

Klemm, D., Heublein, B., Fink, H.-P., Bohn, A. 2005. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. *Angewandte Chemie International Edition*, **44**(22), 3358-3393.

Kobakhidze, A., Asatiani, M., Kachlishvili, E., Elisashvili, V. 2016. Induction and catabolite repression of cellulase and xylanase synthesis in the selected white-rot basidiomycetes. *Annals of Agrarian Science*, **14**(3), 169-176.

Kudahettige, R.L., Holmgren, M., Imerzeel, P., Sellstedt, A. 2012. Characterization of Bioethanol Production from Hexoses and Xylose by the White Rot Fungus Trametes versicolor. *BioEnergy Research*, **5**(2), 277-285.

Kuttiraja, M., Sindhu, R., Varghese, P.E., Sandhya, S.V., Binod, P., Vani, S., Pandey, A., Sukumaran, R.K. 2013. Bioethanol production from bamboo (Dendrocalamus sp.) process waste. *Biomass and Bioenergy*, **59**, 142-150.

Le Floch, A., Jourdes, M., Teissedre, P.-L. 2015. Polysaccharides and lignin from oak wood used in cooperage: Composition, interest, assays: A review. *Carbohydrate Research*, **417**, 94-102.

Li, K., Wan, J., Wang, X., Wang, J., Zhang, J. 2016. Comparison of dilute acid and alkali pretreatments in production of fermentable sugars from bamboo: Effect of Tween 80. *Industrial Crops and Products*, **83**, 414-422.

Li, L., Ai, H., Zhang, S., Li, S., Liang, Z., Wu, Z.-Q., Yang, S.-T., Wang, J.-F. 2013. Enhanced butanol production by coculture of Clostridium beijerinckii and Clostridium tyrobutyricum. *Bioresource Technology*, **143**, 397-404.

Li, Y., Tang, W., Chen, Y., Liu, J., Lee, C.-f.F. 2019. Potential of acetone-butanol-ethanol (ABE) as a biofuel. *Fuel*, **242**, 673-686.

Liang, J., Nabi, M., Zhang, P., Zhang, G., Cai, Y., Wang, Q., Zhou, Z., Ding, Y. 2020. Promising biological conversion of lignocellulosic biomass to renewable energy with rumen microorganisms: A comprehensive review. *Renewable and Sustainable Energy Reviews*, **134**, 110335.

Liu, J., Li, R., Shuai, L., You, J., Zhao, Y., Chen, L., Li, M., Chen, L., Huang, L., Luo, X. 2017. Comparison of liquid hot water (LHW) and high boiling alcohol/water (HBAW)

pretreatments for improving enzymatic saccharification of cellulose in bamboo. *Industrial Crops and Products*, **107**, 139-148.

Liu, Y.-J., Li, B., Feng, Y., Cui, Q. 2020. Consolidated bio-saccharification: Leading lignocellulose bioconversion into the real world. *Biotechnology Advances*, **40**, 107535.

Lynd, L.R., Weimer, P.J., Van Zyl, W.H., Pretorius, I.S. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews*, **66**(3), 506-577.

Manoj Babu, A., Saravanan, C.G., Vikneswaran, M., Edwin Geo, V., Sasikala, J., Femilda Josephin, J.S., Das, D. 2021. Analysis of performance, emission, combustion and endoscopic visualization of micro-arc oxidation piston coated SI engine fuelled with low carbon biofuel blends. *Fuel*, **285**, 119189.

Mehta, S., Jha, S., Liang, H. 2020. Lignocellulose materials for supercapacitor and battery electrodes: A review. *Renewable and Sustainable Energy Reviews*, **134**, 110345.

Miller, G. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugars, Ann, Chern.

Mingxiong, H., Li, Q., XY, L., Wu, J., GQ, H., Pan, K., Zhu QL, Wu, J. 2013. Bio-ethanol Production from bamboo residues with lignocellulose fractionation technology (LFT) and separate hydrolysis fermentation (SHF) by Zymomonas mobilis. *American Journal of Biomass and Bioenergy*, **Vol2. No1**(10.7726), 15-24.

Mirfakhar, M., Asadollahi, M.A., Amiri, H., Karimi, K. 2020. Co-fermentation of hemicellulosic hydrolysates and starch from sweet sorghum by Clostridium acetobutylicum: A synergistic effect for butanol production. *Industrial Crops and Products*, **151**, 112459.

Mitchell, D.A., Berovic, M., Krieger, N. 2002. Overview of solid state bioprocessing. *Biotechnology Annual Review*, **8**, 183-225.

Modenbach. 2013. Sodium hydroxide pretreatment of corn stover and subsequent enzymatic hydrolysis: An investigation of yields, kinetic modeling and glucose recovery. *Theses and Dissertations--Biosystems and Agricultural Engineering*, **17**.

Mohanty, A.K., Misra, M., Hinrichsen, G. 2000. Biofibres, biodegradable polymers and biocomposites: An overview. *Macromolecular Materials and Engineering*, **276-277**(1), 1-24.

Montenecourt, B.S., Eveleigh, D.E., Elmund, G.D., Parcells, J. 1978. Antibiotic disks—an improvement in the filter paper assay for cellulase. *Biotechnology and Bioengineering*, **20**(2), 297-300.

Montgomery, H.J., Monreal, C.M., Young, J.C., Seifert, K.A. 2000. Determinination of soil fungal biomass from soil ergosterol analyses. *Soil Biology and Biochemistry*, **32**(8), 1207-1217.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, **96**(6), 673-686.

Motoda, T., Yamaguchi, M., Tsuyama, T., Kamei, I. 2019. Down-regulation of pyruvate decarboxylase gene of white-rot fungus Phlebia sp. MG-60 modify the metabolism of sugars and productivity of extracellular peroxidase activity. *Journal of Bioscience and Bioengineering*, **127**(1), 66-72.

Naidu, D.S., Hlangothi, S.P., John, M.J. 2018. Bio-based products from xylan: A review. *Carbohydrate Polymers*, **179**, 28-41.

Nakayama, S., Kiyoshi, K., Kadokura, T., Nakazato, A. 2011. Butanol production from crystalline cellulose by cocultured clostridium thermocellum and clostridium saccharoperbutylacetonicum N1-4. *Applied and Environmental Microbiology*, **77**(18), 6470-6475.

Niemenmaa, O., Galkin, S., Hatakka, A. 2008. Ergosterol contents of some wood-rotting basidiomycete fungi grown in liquid and solid culture conditions. *International Biodeterioration & Biodegradation*, **62**(2), 125-134.

Nirmala, C., Bisht, M.S., Bajwa, H.K., Santosh, O. 2018. Bamboo: A rich source of natural antioxidants and its applications in the food and pharmaceutical industry. *Trends in Food Science & Technology*, **77**, 91-99.

Noguchi, T., Tashiro, Y., Yoshida, T., Zheng, J., Sakai, K., Sonomoto, K. 2013. Efficient butanol production without carbon catabolite repression from mixed sugars with Clostridium saccharoperbutylacetonicum N1-4. *Journal of Bioscience and Bioengineering*, **116**(6), 716-721.

Noor, E., Eden, E., Milo, R., Alon, U. 2010. Central Carbon Metabolism as a Minimal Biochemical Walk between Precursors for Biomass and Energy. *Molecular Cell*, **39**(5), 809-820.

Okamoto, K., Uchii, A., Kanawaku, R., Yanase, H. 2014. Bioconversion of xylose, hexoses and biomass to ethanol by a new isolate of the white rot basidiomycete Trametes versicolor. *SpringerPlus*, **3**(1), 121.

Oliva-Rodríguez, A.G., Quintero, J., Medina-Morales, M.A., Morales-Martínez, T.K., Rodríguez-De la Garza, J.A., Moreno-Dávila, M., Aroca, G., Rios González, L.J. 2019. Clostridium strain selection for co-culture with Bacillus subtilis for butanol production from agave hydrolysates. *Bioresource Technology*, **275**, 410-415.

Peng, P., She, D. 2014. Isolation, structural characterization, and potential applications of hemicelluloses from bamboo: A review. *Carbohydrate Polymers*, **112**, 701-720.

Petitdemange, E., Fond, O., Caillet, F., Petitdemange, H., Gay, R. 1983. A novel one step process for cellulose fermentation using mesophilic cellulolytic and glycolytic Clostridia. *Biotechnology Letters*, **5**(2), 119-124.

Procentese, A., Raganati, F., Olivieri, G., Russo, M.E., Salatino, P., Marzocchella, A. 2015. Continuous lactose fermentation by Clostridium acetobutylicum – Assessment of solventogenic kinetics. *Bioresource Technology*, **180**, 330-337.

Puricelli, S., Cardellini, G., Casadei, S., Faedo, D., van den Oever, A.E.M., Grosso, M. 2020. A review on biofuels for light-duty vehicles in Europe. *Renewable and Sustainable Energy Reviews*, 110398.

Qi, G., Huang, D., Wang, J., Shen, Y., Gao, X. 2019. Enhanced butanol production from ammonium sulfite pretreated wheat straw by separate hydrolysis and fermentation and simultaneous saccharification and fermentation. *Sustainable Energy Technologies and Assessments*, **36**, 100549.

Qinnghe, C., Xiaoyu, Y., Tiangui, N., Cheng, J., Qiugang, M. 2004. The screening of culture condition and properties of xylanase by white-rot fungus Pleurotus ostreatus. *Process Biochemistry*, **39**(11), 1561-1566.

Rastogi, M., Shrivastava, S. 2017. Recent advances in second generation bioethanol production: An insight to pretreatment, saccharification and fermentation processes. *Renewable and Sustainable Energy Reviews*, **80**, 330-340.

Roy, R., Rahman, M.S., Raynie, D.E. 2020. Recent advances of greener pretreatment technologies of lignocellulose. *Current Research in Green and Sustainable Chemistry*, **3**, 100035.

Saha, B.C., Qureshi, N., Kennedy, G.J., Cotta, M.A. 2016. Biological pretreatment of corn stover with white-rot fungus for improved enzymatic hydrolysis. *International Biodeterioration & Biodegradation*, **109**, 29-35.

Salgado, J.C.S., Meleiro, L.P., Carli, S., Ward, R.J. 2018. Glucose tolerant and glucose stimulated β-glucosidases – A review. *Bioresource Technology*, **267**, 704-713.

Sánchez, C. 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances*, **27**(2), 185-194.

Schwarz, W. 2001. The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology*, **56**(5), 634-649.

Schwarze, F.W.M.R. 2007. Wood decay under the microscope. *Fungal Biology Reviews*, **21**(4), 133-170.

Scurlock, J.M.O., Dayton, D.C., Hames, B. 2000. Bamboo: an overlooked biomass resource? *Biomass and Bioenergy*, **19**(4), 229-244.

Shimokawa, T., Ishida, M., Yoshida, S., Nojiri, M. 2009. Effects of growth stage on enzymatic saccharification and simultaneous saccharification and fermentation of bamboo shoots for bioethanol production. *Bioresource Technology*, **100**(24), 6651-6654.

Shinohara, Y., Misumi, Y., Kubota, T., Nanko, K. 2019. Characteristics of soil erosion in a moso-bamboo forest of western Japan: Comparison with a broadleaved forest and a coniferous forest. *CATENA*, **172**, 451-460.

Sindhu, R., Kuttiraja, M., Binod, P., Sukumaran, R.K., Pandey, A. 2014. Bioethanol production from dilute acid pretreated Indian bamboo variety (Dendrocalamus sp.) by separate hydrolysis and fermentation. *Industrial Crops and Products*, **52**, 169-176.

Singh, A.P., Singh, T. 2014. Biotechnological applications of wood-rotting fungi: A review. *Biomass and Bioenergy*, **62**, 198-206.

Singhania, R.R., Patel, A.K., Sukumaran, R.K., Larroche, C., Pandey, A. 2013. Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresource Technology*, **127**, 500-507.

SjÖStrÖM, E. 1993. Chapter 4 - LIGNIN. in: *Wood Chemistry (Second Edition)*, (Ed.) E. SjÖStrÖM, Academic Press. San Diego, pp. 71-89.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2008. Determination of structural carbohydrates and lignin in biomass.

Suhara, H., Kodama, S., Kamei, I., Maekawa, N., Meguro, S. 2012. Screening of selective lignin-degrading basidiomycetes and biological pretreatment for enzymatic hydrolysis of bamboo culms. *International Biodeterioration & Biodegradation*, **75**, 176-180.

Suhardi, V.S.H., Prasai, B., Samaha, D., Boopathy, R. 2013. Evaluation of pretreatment methods for lignocellulosic ethanol production from energy cane variety L 79-1002. *International Biodeterioration & Biodegradation*, **85**, 683-687.

Sun, S.-L., Wen, J.-L., Ma, M.-G., Sun, R.-C. 2014. Enhanced enzymatic digestibility of bamboo by a combined system of multiple steam explosion and alkaline treatments. *Applied Energy*, **136**, 519-526.

Tomak, E.D., Topaloglu, E., Gumuskaya, E., Yildiz, U.C., Ay, N. 2013. An FT-IR study of the changes in chemical composition of bamboo degraded by brown-rot fungi. *International Biodeterioration & Biodegradation*, **85**, 131-138.

Tran, H.T.M., Cheirsilp, B., Hodgson, B., Umsakul, K. 2010. Potential use of Bacillus subtilis in a co-culture with Clostridium butylicum for acetone–butanol–ethanol production from cassava starch. *Biochemical Engineering Journal*, **48**(2), 260-267.

Tri, C.L., Kamei, I. 2020. Butanol production from cellulosic material by anaerobic co-culture of white-rot fungus Phlebia and bacterium Clostridium in consolidated bioprocessing. *Bioresource Technology*, **305**, 123065.

Tri, C.L., Khuong, L.D., Kamei, I. 2018. The improvement of sodium hydroxide pretreatment in bioethanol production from Japanese bamboo Phyllostachys edulis using the white rot fungus Phlebia sp. MG-60. *International Biodeterioration & Biodegradation*, **133**, 86-92.

Trindade, W.R.d.S., Santos, R.G.d. 2017. Review on the characteristics of butanol, its production and use as fuel in internal combustion engines. *Renewable and Sustainable Energy Reviews*, **69**, 642-651.

Tsuyama, T., Yamaguchi, M., Kamei, I. 2017. Accumulation of sugar from pulp and xylitol from xylose by pyruvate decarboxylase-negative white-rot fungus Phlebia sp. MG-60. *Bioresource Technology*, **238**, 241-247.

Updegraff, D.M. 1969. Semimicro determination of cellulose inbiological materials. *Analytical Biochemistry*, **32**(3), 420-424.

Wang, C., Zhang, X., Liu, Q., Zhang, Q., Chen, L., Ma, L. 2020. A review of conversion of lignocellulose biomass to liquid transport fuels by integrated refining strategies. *Fuel Processing Technology*, **208**, 106485.

Wen, J.-L., Xiao, L.-P., Sun, Y.-C., Sun, S.-N., Xu, F., Sun, R.-C., Zhang, X.-L. 2011. Comparative study of alkali-soluble hemicelluloses isolated from bamboo (Bambusa rigida). *Carbohydrate Research*, **346**(1), 111-120.

Wu, J., Dong, L., Zhou, C., Liu, B., Feng, L., Wu, C., Qi, Z., Cao, G. 2019. Developing a coculture for enhanced butanol production by Clostridium beijerinckii and Saccharomyces cerevisiae. *Bioresource Technology Reports*, **6**, 223-228.

Xin, F., Dong, W., Zhang, W., Ma, J., Jiang, M. 2019. Biobutanol production from crystalline cellulose through consolidated bioprocessing. *Trends in Biotechnology*, **37**(2), 167-180.

Yamashita, Y., Shono, M., Sasaki, C., Nakamura, Y. 2010. Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo. *Carbohydrate Polymers*, **79**(4), 914-920.

Yang, S., Yang, B., Duan, C., Fuller, D.A., Wang, X., Chowdhury, S.P., Stavik, J., Zhang, H., Ni, Y. 2019. Applications of enzymatic technologies to the production of high-quality dissolving pulp: A review. *Bioresource Technology*, **281**, 440-448.

Yang, S., Yuan, T.-Q., Li, M.-F., Sun, R.-C. 2015. Hydrothermal degradation of lignin: Products analysis for phenol formaldehyde adhesive synthesis. *International Journal of Biological Macromolecules*, **72**, 54-62.

Yang, Y., Nie, X., Jiang, Y., Yang, C., Gu, Y., Jiang, W. 2018. Metabolic regulation in solventogenic clostridia: regulators, mechanisms and engineering. *Biotechnology Advances*, **36**(4), 905-914.

Yano, S. 2015. Enzymatic Saccharification and Fermentation Technology for Ethanol Production from Woody Biomass. *Journal of the Japan Petroleum Institute*, **58**(3), 128-134.

Yoon, L.W., Ang, T.N., Ngoh, G.C., Chua, A.S.M. 2014. Fungal solid-state fermentation and various methods of enhancement in cellulase production. *Biomass and Bioenergy*, **67**, 319-338.

Yuen, J.Q., Fung, T., Ziegler, A.D. 2017. Carbon stocks in bamboo ecosystems worldwide: Estimates and uncertainties. *Forest Ecology and Management*, **393**, 113-138.

Zabed, H., Sahu, J.N., Boyce, A.N., Faruq, G. 2016. Fuel ethanol production from lignocellulosic biomass: An overview on feedstocks and technological approaches. *Renewable and Sustainable Energy Reviews*, **66**, 751-774.

Zabed, H., Sahu, J.N., Suely, A., Boyce, A.N., Faruq, G. 2017. Bioethanol production from renewable sources: Current perspectives and technological progress. *Renewable and Sustainable Energy Reviews*, **71**, 475-501.

Zhang, J.-G., Li, Q.-L., Wei, Z.-J. 2017. Degradation of bamboo-shoot shell powder by a fungal consortium: Changes in chemical composition and physical structure. *International Biodeterioration & Biodegradation*, **116**, 205-210.

Zhang, X., Yu, H., Huang, H., Liu, Y. 2007. Evaluation of biological pretreatment with white rot fungi for the enzymatic hydrolysis of bamboo culms. *International Biodeterioration & Biodegradation*, **60**(3), 159-164.

Zhang, Y.H.P. 2008. Reviving the carbohydrate economy via multi-product lignocellulose biorefineries. *Journal of Industrial Microbiology & Biotechnology*, **35**(5), 367-375.

Zhao, T., Yasuda, K., Tashiro, Y., Darmayanti, R.F., Sakai, K., Sonomoto, K. 2019. Semi-hydrolysate of paper pulp without pretreatment enables a consolidated fermentation system with in situ product recovery for the production of butanol. *Bioresource Technology*, **278**, 57-65.

Zou, L., Liu, Y., Wang, Y., Hu, X. 2020. Assessment and analysis of agricultural non-point source pollution loads in China: 1978–2017. *Journal of Environmental Management*, **263**, 110400.